Universitat de Girona

DEVELOPMENT OF MOLECULAR-BASED TECHNIQUES FOR THE DETECTION, IDENTIFICATION AND QUANTIFICATION OF FOOD-BORNE PATHOGENS

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Doctoral Thesis

Development of molecular-based techniques for the detection, identification and quantification of foodborne pathogens

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CERTIFICA:

Que el Licenciado en Veterinaria y en Ciencia y Tecnología de los Alimentos David Rodríguez Lázaro, ha realizado bajo su dirección, el trabajo titulado "*Development of molecular-based techniques for the detection, identification and quantification of foodborne pathogens*" que presenta en esta memoria la cual constituye su Tesis para optar al grado de Doctor europeo por la Universitat de Girona.

Y para que conste a los efectos oportunos, firma la presente en Girona, el día 19 de abril de 2004.

Dra. Maria Pla de Solà-Morales

"¡No os dejéis contagiar por un escepticismo estéril y negativo! ¡No desanimaros por la tristeza de ciertas horas que pasan sobre las naciones! Vivid en la paz serena de los laboratorios y de las bibliotecas. Preguntaos, ante todo: ¿Qué he hecho yo por instruirme?, y después, según vayáis avanzando gradualmente: ¿Qué he hecho yo por mi patria? Hasta que llegue un día en que alcancéis la inmensa felicidad de pensar que habéis contribuido de algún modo al progreso y a la dicha de la humanidad…"

Louis Pasteur

A mis padres,
a quien les debo todo lo que soy

A mi hermano
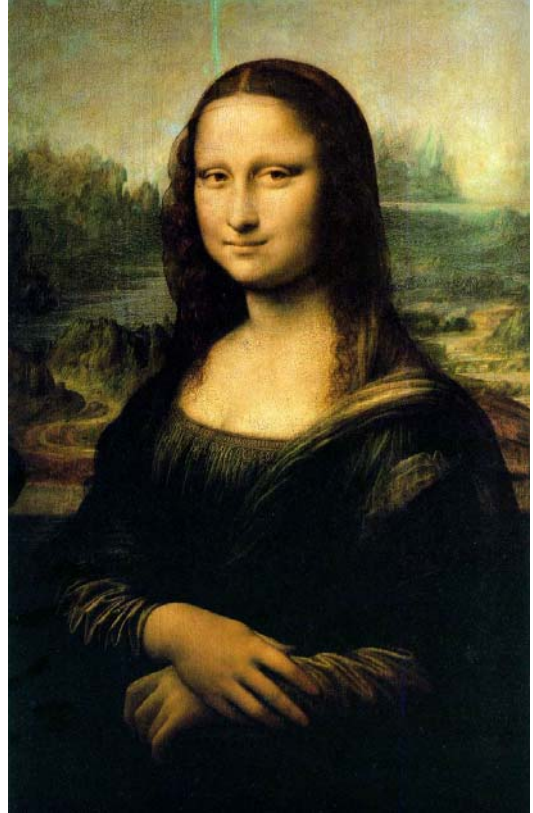
A Marta,

Aunque hable las lenguas de los hombres y de los ángeles, si no tengo amor, no soy más que una campana que toca o unos platillos que resuenan. Aunque tenga el don de la profecía y conozca todos los misterios y toda la ciencia y aunque tenga tanta fe que traslade las montañas, si no tengo amor, no soy nada

San Pablo, I Corintios 13:1-2

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SUMMARY

The presence of pathogens in foods is among the most serious public health concerns, and the diseases produced by them are a major cause of morbidity. Consequently, the application of microbiological control within the quality assessment programs in the food industry is a premise to minimize the risk of infection for the consumer. Classical microbiological methods involve, in general, the use of a non-selective pre-enrichment, selective enrichment, isolation on selective media, and subsequent confirmation using morphological, biochemical and/or serological tests. Thus, they are laborious, time consuming and not always reliable (e.g. in viable but non-culturable VBNC forms). A number of alternative, rapid and sensitive methods for the detection, identification and quantification of foodborne pathogens have been developed to overcome these drawbacks. PCR has become the most popular microbiological diagnostic method, and recently, the introduction of a development of this technique, RTi-PCR, has produced a second revolution in the molecular diagnostic methodology in microbiology. RTi-PCR is highly sensitive and specific. Moreover, it allows accurate quantification of the bacterial target DNA. Main advantages of RTi-PCR for its application in diagnostic laboratories include quickness, simplicity, the closed-tube format that avoids risks of carryover contaminations and the possibility of high throughput and automation.

In this work, specific, sensitive and reliable analytical methods based on molecular techniques (PCR and NASBA) were developed for the detection, identification and quantification of foodborne pathogens (*Listeria* spp., *Mycobacterium avium* subsp. *paratuberculosis* and *Salmonella* spp.). Real-time PCR based methods were designed and optimised for each one of these target bacteria: *L. monocytogenes*, *L. innocua*, *Listeria* spp. *M. avium* subsp. *paratuberculosis,* and also a real-time PCR based method previously described for *Salmonella* spp. was optimised and multicenter evaluated. In addition, an NASBA-based method was designed and optimised for the specific detection of *M. avium* subsp. *paratuberculosis*. The potential application of the NASBA technique for specific detection of viable *M. avium* subsp. *paratuberculosis* cells was also evaluated.

All the amplification-based methods were 100 % specific and the sensitivity achieved proved to be fully suitable for further application in real food samples. Furthermore, specific pre-amplification procedures were developed and evaluated on meat products, seafood products, milk and water samples. Thus, fully specific and highly sensitive real-time PCR-based methods were developed for quantitative detection of *L. monocytogenes* on meat and meat products and on salmon and cold smoked salmon products; and for quantitative detection of *M. avium* subsp. *paratuberculosis* on water and milk samples. The *M. avium* subsp. *paratuberculosis*-specific real-time PCR-based method was also applied to evaluate the presence of this bacterium in the bowel of Crohn's disease patients using colonic biopsy specimens form affected and unaffected volunteers. In addition, fully specific and highly sensitive real-time NASBA-based methods were developed for detection of *M. avium* subsp. *paratuberculosis* on water and milk samples.

In conclusion, this study reports selective and sensitive amplification-based assays for the quantitative detection of foodborne pathogens (*Listeria* spp., *Mycobacterium avium* subsp. *paratuberculosis* and) and for a quick and unambiguously identification of *Salmonella* spp. The assays had an excellent relative accuracy compared to microbiological reference methods and can be used for quantification of genomic DNA and also cell suspensions. Besides, in combination with sample pre-amplification treatments, they work with high efficiency for the quantitative analysis of the target bacteria. Thus, they could be a useful strategy for a quick and sensitive detection of foodborne pathogens in food products and which should be a useful addition to the range of diagnostic tools available for the study of these pathogens.

RESUMEN

La presencia de microorganismos patógenos en alimentos es uno de los problemas esenciales en salud pública, y las enfermedades producidas por los mismos es una de las causas más importantes de enfermedad. Por tanto, la aplicación de controles microbiológicos dentro de los programas de aseguramiento de la calidad es una premisa para minimizar el riesgo de infección de los consumidores. Los métodos microbiológicos clásicos requieren, en general, el uso de pre-enriquecimientos no-selectivos, enriquecimientos selectivos, aislamiento en medios selectivos y la confirmación posterior usando pruebas basadas en la morfología, bioquímica y serología propias de cada uno de los microorganismos objeto de estudio. Por lo tanto, estos métodos son laboriosos, requieren un largo proceso para obtener resultados definitivos y, además, no siempre pueden realizarse. Para solucionar estos inconvenientes se han desarrollado diversas metodologías alternativas para la detección identificación y cuantificación de microorganismos patógenos de origen alimentario, entre las que destacan los métodos inmunológicos y moleculares. En esta última categoría, la técnica basada en la reacción en cadena de la polimerasa (PCR) se ha convertido en la técnica diagnóstica más popular en microbiología, y recientemente, la introducción de una mejora de ésta, la PCR a tiempo real, ha producido una segunda revolución en la metodología diagnóstica molecular, como pude observarse por el número creciente de publicaciones científicas y la aparición continua de nuevos kits comerciales. La PCR a tiempo real es una técnica altamente sensible -detección de hasta una molécula- que permite la cuantificación exacta de secuencias de ADN específicas de microorganismos patógenos de origen alimentario. Además, otras ventajas que favorecen su implantación potencial en laboratorios de análisis de alimentos son su rapidez, sencillez y el formato en tubo cerrado que puede evitar contaminaciones post-PCR y favorece la automatización y un alto rendimiento.

En este estudio el proceso de implantación de la metodología de PCR a tiempo real como una herramienta diagnóstica alternativa a los métodos microbiológicos clásicos se desarrolló en una serie de fases sucesivas: (i) diseño de los sistemas de PCR a tiempo real, (ii) su optimización, y (iii) su aplicación a muestras alimentarias reales.

El diseño de sistemas de sistemas de PCR a tiempo real está basado en la selección de secuencias de ADN diana, lo que determinará la especificidad del sistema de detección. Por tanto, la selección de una región de ADN de la bacteria o grupo bacteriano sujeto al estudio es indispensable para asegurar que éste sea adecuado. En este estudio, se eligieron genes ribosomales, genes que codifican para proteínas y elementos repetitivos. De esta manera, para el sistema específico de detección de *Listeria* spp., se eligió una secuencia consenso dentro de las diferentes especies del género *Listeria* perteneciente al 23S rDNA. Interesantemente, una mutación puntual en una única base en la secuencia de *L. innocua*, varió la eficiencia de la reacción, siendo este hecho subsanado con la adición de un cebador extra totalmente específico para esta bacteria. Además, como las regiones ribosomales están presentes en el genoma en múltiples copias (p.ej. 6-8 copias en *L. monocytogenes* y *L. innocua*), éste sistema desarrollado para *Listeria* spp. presentó una excelente sensibilidad; detección consistente de una única copia. Por otro lado, en este estudio se utilizaron genes asociados a la virulencia pata la detección específica de *L. monocytogenes* (el gen de la Listeriolisina O -*hly*-, o el gen que codifica para proteína p60 -*iap*-) y de *Salmonella* spp. (el gen *invA*). Por ejemplo, el gen *hly* está presente únicamente en *L. monocytogenes* y permitió el diseño de 2 sistemas diferentes de PCR a tiempo real (en formato *uniplex* y en formato *duplex* para la detección simultánea de otros miembros del género *Listeria*). En el caso de *L. innocua,* al tratarse de una bacteria apatógena, se eligió el gen *lin02483* que codifica para una proteína potencialmente asociada al estrés, similar a proteínas asociadas a la resistencia al estrés osmótico y a variaciones en el equilibrio del pH. Asimismo, para el diseño de un sistema de PCR a tiempo real específico para *Mycobacterium avium* subsp. *paratuberculosis* se utilizó una secuencia de inserción propia de esta bacteria y presente en un número elevado de copias (15-18 copias); la secuencia de inserción 900 (*IS900*). Esto es especialmente relevante en el caso de este patógeno ya que se encuentra generalmente en pequeñas cantidades en los alimentos.

Tras la selección de los genes más adecuados para cada uno de los microorganismos estudiados, se realizó una búsqueda *in silico* de una región específica dentro de cada uno de los mismos usando herramientas bioinformáticas como Blast-N (NCBI, USA) o Fasta-3 (EMBL, UK). Se diseñaron sistemas de PCR a tiempo real en de las regiones específicas para cada uno de las organismos dentro de cada gen seleccionado utilizando herramientas bioinformáticas como Primer Express v2.0 (Applied Biosystems, USA), DNA*DNASTAR (Lasergene, DNASTAR Inc, USA) y primer 3 (http://www-genome.wi.mit.edu/genome_software/other/primer3.html). Posteriormente, la especificidad de cada sistema de PCR a tiempo real fue revisada *in silico* evaluando la especificidad global del amplicon así como la especificidad de cada uno de los oligonucleótidos diseñados. Por ejemplo, el gen *hly* de *L. monocytogenes*, contenía una región (posiciones 113 a 177) sin una homología significativa con ninguna otra región depositada en las bases de datos. Por lo tanto, se diseñaron un par de *cebadores* y una sonda TaqMan® dentro de la misma y se compararon con las secuencias publicadas, mostrando homología únicamente con secuencias depositadas de *L. monocytogenes*.

Posteriormente, se optimizaron los sistemas de PCR a tiempo real. Para ello, se evaluaron experimentalmente tres parámetros: la concentración de los oligonucleótidos, la concentración del ión Mg^{2+}, y la temperatura de hibridación. Las condiciones óptimas para las concentraciones de los *cebadores* y la sonda fueron las mínimas con el valor más bajo de ciclo umbral (C_T) y el más alto de incremento de fluorescencia normalizada (ΔR_n). En general, 50 nM-300nM de cada primer produjeron los mejores resultados. La concentración óptima del ión Mg^{2+} varia de 0.8 a 9.0 mM $MgCl_2$. La polimerasa que se utilizó generalmente en este trabajo fue la Ampli*Taq* Gold® DNA polymerase, la cual tiene una alta fidelidad independientemente de la concentración del ión Mg^{2+} utilizada, y por tanto, permitiendo la utilización de condiciones muy favorables a la amplificación sin afectar la especificidad de la misma en la mayoría de los sistemas de PCR a tiempo real desarrollados en este estudio. Finalmente, se seleccionó la temperatura de hibridación más alta que no produjera una afectación significativa de los valores de C_T. El escenario de consenso que fue adoptado en este estudio, especialmente para las condiciones de temperatura/tiempo, con el objeto de simplificar la aplicación rutinaria de los sistemas desarrollados y su aplicación potencial en una única reacción fue el siguiente: 2 min a 50 ºC, 10 min a 95 ºC, y 50 ciclos de 15 s a95 ºC y 1 min a 63 ºC.

Antes de la aplicación de un sistema de PCR a tiempo real a muestras reales es necesario establecer la especificidad, la sensibilidad, y la exactitud relativa del mimo. Un sistema de PCR a tiempo real debe ser totalmente capaz de detectar los microorganismos diana, y ser totalmente capaz de no detectar los organismos que no lo son. Para ello, en este estudio se siguieron las directrices marcadas por la norma de carácter internacional ISO 16140:2003: evaluar la especificidad empleando al menos 50 (25 para *Salmonella*) cepas bacterianas diana, y 30 cepas que no sean diana del sistema de detección. Todos los sistemas desarrollados fueron 100 % específicos. Además, aquellos que fueron diseñados con propósitos cuantitativos, tuvieron, para las cepas bacterianas positivas, una variación inferior a 1 unidad de C_T, excepto el sistema de PCR a tiempo real diseñado sobre el gen *iap*, por lo que éste no fue considerado adecuado para análisis cuantitativos de *L. monocytogenes*. Asimismo, se confirmó que esta divergencia en los valores de C_T era debida a variaciones en la secuencia dentro de los diferentes serotipos de esta especie. De esta manera, secuencias de cepas perteneciendo a la misma división filogenética de *L. monocytogenes* estaban altamente conservadas, y por tanto, produjeron valores de C_T más conservados que aquellos pertenecientes a divisiones diferentes. Esto permitió diseñar, por primera vez, un test diagnóstico para la clasificación de una capa de esta especie en la división II o I/III mediante el empleo de 2 sistemas de PCR a tiempo real (diseñados en las secuencias altamente conservadas del gen *hly* y en las más variables del gen *iap*).

Otro parámetro evaluado fue la sensibilidad. Ésta indica el nivel mínimo en el cual el analito puede ser detectado con una probabilidad dada. En este estudio, se estableció la sensibilidad o límite de detección mediante el análisis de de diluciones seriadas del organismo diana en diferentes experimentos (al menos 3) en lo cuales se empleaban varios replicados (al menos 3). Además, también se empleó el test diseñado por Knutsson y colaboradores para establecer la probabilidad de detección en el caso de los sistemas de PCR y NASBA a tiempo real específicos para *Mycobacterium avium* subsp.

paratuberculosis. Todos los sistemas tuvieron un límite de detección (con una probabilidad del 95 %) por debajo de 10 células. Como era esperado, los sistemas más sensibles fueron aquellos que usaron secuencias de genes multicopia: una única copia para el sistema específico de *Listeria* spp. (gen 23S rDNA) y por debajo de 3 copias para el sistema específico de *Mycobacterium avium* subsp. *paratuberculosis* (IS900). Interesantemente, el límite de detection fue similar usando tanto ADN bacteriano purificado o células, lo que tiene implicaciones prácticas importantes por la simplicidad de uso de éstas últimas como analito.

En los sistemas diseñados con propósitos cuantitativos, se analizaron 3 parámetros adicionales: la cuantificabilidad, el rango de cuantificación y el límite de cuantificación. El término cuantificabilidad no está definido formalmente, pero sin embargo, es asumido por los usuarios de la técnica de PCR a tiempo real como dependiente de la linealidad y la eficiencia de la PCR. En este estudio, la linealidad se estableció mediante el coeficiente de regresión (R^2) de la curva de regresión obtenida por concentraciones conocidas de ADN del microorganismo diana. Valores por encima de 0,90-0,95 son considerados adecuados. En este estudio, todos los sistemas presentaron valores por encima de 0,95. La eficiencia de la reacción (E) fue establecida mediante la siguiente ecuación: $E=10^{-1/s} - 1$; donde s es la pendiente la curva de regresión. Se puede considerar que el funcionamiento es adecuado para un rango de valores de E entre 0,78 ($s= -4,00$) y 1,15 ($s=-3,00$), y las eficiencias obtenidas para todos los sistemas de este estudio estuvieron dentro de este intervalo. El nivel más bajo donde el valor de C_T obtenido en dos diluciones consecutivas no se solapaban con un 95% de probabilidad, se consideró el límite de de cuantificación. En paralelo a la evaluación experimental, se calculó el límite de cuantificación teórico mediante el análisis de simulaciones de MonteCarlo y calculando un intervalo de confianza del 95% para cada dilución. En este estudio, los límites de cuantificación teórico y experimental coincidieron y se establecieron generalmente alrededor de 30 copias genómicas o células bacterianas. Por lo tanto, el rango de cuantificación era, al menos, de 5 órdenes de magnitud (generalmente desde 3×10^6 ó 3×10^5 a 3×10^1).

Finalmente, se analizó la exactitud relativa. Ésta define el grado de correspondencia entre la respuesta obtenida por el método de referencia y un método alternativo. En este estudio, la exactitud relativa se calculó mediante la comparación de los resultados obtenidos de los diferentes sistemas de PCR a tiempo real y los obtenidos mediante el método microbiológico de referencia, considerándose adecuados aquellos con valores comprendidos entre 70-130%. En este estudio, se calculó la exactitud relativa para cada sistema, obteniéndose valores dentro del intervalo previamente definido.

Además, se desarrolló un sistema de NASBA a tiempo real para la detección de específica de *Mycobacterium avium* subsp. *paratuberculosis*. Los criterios y parámetros empleados fueron los mismos que los resumidos en los párrafos anteriores, obteniéndose un sistema 100% específico con un límite de detección de 150-200 células por reacción. La principal característica de la técnica NASBA es que amplifica únicamente ARN, incluso en presencia de ADN. Por lo tanto mediante la aplicación de esta técnica se podría, en teoría, realizar estudios sobre la viabilidad celular ya que el ARN (especialmente el ARN mensajero) es mucho más lábil que el DNA. Sin embargo, aunque en un principio esta técnica debe teóricamente amplificar únicamente RNA, es necesario comprobar la naturaleza del origen de la señal producida en cada caso. Por ello, en este estudio se analizó el origen de señal producida mediante la técnica de NASBA en diferentes sistemas basados en la misma para la detección de *Mycobacterium avium* subsp. *paratuberculosis*, y se comprobó que podría amplificarse también DNA, lo que desaconseja estos sistemas como adecuados para la detección inequívoca de células viables de *Mycobacterium avium* subsp. *paratuberculosis*. Este descubrimiento puede servir a todos aquellos que pretendan utilizar sistemas basados en la técnica de NASBA como un ejemplo saludable para considerar si los sistemas amplifican de hecho, únicamente RNA, y por tanto son adecuados para el estudio de la viabilidad celular.

La eficiencia de los métodos moleculares puede estar influenciada negativamente por la presencia de sustancias inhibidoras en la muestra que debe ser analizada. Esto puede resultar en señales más débiles e incluso negativas y permitir la subestimación de la cantidad de ácidos nucleicos

en la muestra. Por tanto, un aspecto fundamental es un adecuado control de la eficiencia de la reacción de amplificación. Para ello, en este estudio se desarrollaron controles internos de amplificación (IAC). Un IAC es un ácido nucleico quimérico que es añadido para ser co-amplificado por los mismos cebadores que el ácido nucleico diana, y la amplificación de mismo indicará si la eficiencia de la reacción es adecuada. En este estudio, se construyeron y evaluaron IACs para los sistemas de PCR a tiempo real específicos de *L. monocytogenes* (*hly*) y de *Mycobacterium avium* subsp. *paratuberculosis* (IS900) y para el sistema de NASBA a tiempo real específico de *Mycobacterium avium* subsp. *paratuberculosis*. Estas secuencias quiméricas (DNA para RTi-PCR y RNA para RTi-NASBA) no mostraron ninguna homología con otra secuencia. Por tanto, el IAC pudo se amplificado con los *cebadores* específicos de cada sistema, aunque no pudo ser detectado con la sonda específica del microorganismo diana pero sí con la diseñada para cada uno de los IACs. Además, se evaluó el funcionamiento de la amplificación de diferente número de copias de IAC, así como la posible interferencia de distintas cantidades de IAC sobre la reacción principal y finalmente se eligieron 100 copias (para los sistemas de PCR a tiempo real) o 1 fg (para los sistemas de NASBA a tiempo real).

Finalmente, se evaluó la aplicación de los distintos sistemas a muestras reales para su futura implantación como herramientas diagnósticas en laboratorios de microbiología de los alimentos. Para ello, en este estudio se desarrollaron estrategias de preparación de la muestra antes de su aplicación a los sistemas de amplificación desarrollados, ya que este paso previo puede ser crucial para la robustez y funcionamiento de los mismos. La aproximación más racional para el procesamiento de la muestra es la combinación de diferentes procedimientos, generalmente procedimientos físicos y la extracción de los ácidos nucleicos, y por tanto fue la aproximación utilizada en este trabajo. De esta manera, se evaluó el sistema de PCR a tiempo real específico desarrollado para *L. monocytogenes* (*hly*) para productos cárnicos (carne de cerdo, productos cárnicos cocidos –jamón cocido y salchichas Frankfurt-, y un producto cárnico fermentado -fuet-) y para productos pesqueros (salmón, y salmón ahumado) al haber sido ambas categorías consideradas como fuentes importantes de transmisión de la listeriosis a los consumidores. Además, también se evaluaron los sistemas específicos para *Mycobacterium avium* subsp. *paratuberculosis* (tanto RTi-PCR como RTi-NASBA) para dos productos alimentarios: leche (leche pasterizada semi-desnatada) y agua, al haberse sugerido que se tratan de las dos fuentes potenciales más importantes de transmisión de *Mycobacterium avium* subsp. *paratuberculosis* al hombre. Para mejorar la sensibilidad de estos sistemas, se aplicaron diferentes procedimientos de procesado de cada uno de los tipos de muestras analizadas. Además, para reproducir las condiciones normales empleadas en los análisis rutinarios de los alimentos, se emplearon el volumen o peso que generalmente se emplean en éstos (20-25 ml o 25 g). Para *L. monocytogenes*, se desarrolló un procedimiento rápido y sencillo basado en la filtración de la muestra y en la posterior extracción de ADN. Así en los productos cárnicos se pudo detector hasta 100 CFU/g y cuantificar hasta 1000 CFU/g con una excelente exactitud comprada con el método oficial de referencia. Para productos pesqueros, el tramiento previo a la PCR también consistió en un filtración y posterior purificación del ADN, pudiéndose detectar en el caso del salmón ahumado hasta 10 CFU/g y cuantificar hasta 1000 CFU/g con una excelente exactitud comprada con el método oficial de referencia. Para *Mycobacterium avium* subsp. *paratuberculosis,* cuando se analizó muestras de agua, un procedimiento simple basado en la filtración fue suficiente para obtener los mismos resultados que cuando se analizaban ADN purificado. Para la leche semi-desnatada, el procedimiento consistió en un tratamiento enzimático asociado a uno detergente previo a la centrifugación y posterior extracción de los ácidos nucleicos, pudiéndose de esta manera detectar hasta 10^2 *Mycobacterium avium* subsp. *paratuberculosis* en 20 ml de muestras. Con los mismos pasos previos para las muestras de agua y leche, su pudo detectar consistentemente 10^3 células de *Mycobacterium avium* subsp. *paratuberculosis* en 20 ml de agua, y 10^4 células en 20 ml empleando el sistema de NASBA a tiempo real.

RESUM

La presència de microorganismes patògens en aliments és un dels problemes més importants en salut pública, i les malalties produïdes per ells és una de les causes més importants de malaltia, per tant, l'aplicació de controls microbiològics dins dels programes d'assegurament de la qualitat és una premisa per a minimitzar el risc d'infecció del consumidor. Els mètodes microbiològics clàssics requereixen, en general, l'ús de pre-enriquiments no selectius, enriquiments selectius, aïllament en medis selectius i la confirmació posterior utilitzant assajos basats en la morfologia, bioquímica i serologia pròpies de cadascun dels microorganismes objecte d'estudi. Per tant, aquests mètodes són laboriosos, requereixen un llarg procés per obtenir resultats definitius i, a més, no sempre poden realitzar-se. Per solucionar aquests inconvenients se han desenvolupat diverses metodologies alternatius per la detecció identificació i quantificació de microorganismes patògens d'origen alimentari, entre les que destaquen els mètodes immunològics i moleculars. En aquesta última categoria, la tècnica de PCR s'ha convertit en la tècnica diagnòstica més popular en microbiologia, i recentment, la introducció d'una millora d'aquesta, la PCR a temps real, ha produït una segona revolució en la metodologia diagnòstica molecular en microbiologia, com es pot observar pel nombre creixent de publicacions científiques i l'aparició continua de nous kits comercials. La PCR a temps real és una tècnica altament sensible i específica, que permet a més la quantificació exacta del DNA dels bacteris que s'analitzen. Els principals avantatges de la tècnica de PCR a temps real per la seva potencial en laboratoris d'anàlisis són la seva rapidesa, senzillesa, el format en tub tancat que pot evitar contaminacions post-PCR i la possibilitat d'elevat rendiment i automatització.

En aquest estudi, el procés d'implantació de la metodologia de PCR a temps real com una eina diagnòstica alternativa als mètodes microbiològics clàssics, es va desenvolupar en una sèrie de fases successives: (i) disseny dels sistemes de PCR a temps real, (ii) la seva optimització, i (iii) la seva aplicació a mostres alimentàries reals.

El disseny de sistemes de PCR a temps real està basat en la selecció de seqüències d'ADN diana, que determinaran l'especificitat del sistema de detecció. Per tant, la selecció d'una regió d'ADN específica del bacteri o grup bacterià en estudi és indispensable per assegurar que aquest sistema sigui adequat. En aquest estudi, es van escollir gens ribosomals, gens que codifiquen per proteïnes i elements repetitius. D'aquesta manera, pel sistema específic de detecció de *Listeria* spp., es va escollir una seqüència consens per les diferents espècies del gènere *Listeria* pertanyent al 23S rDNA. Una mutació puntual en una única base en la seqüència de *L. innocua*, va fer variar significativament l'eficiència de la reacció, i això es va poder solucionar metjançant l'addició d'un encebador més totalment específic per aquest bacteri. A més, com que les regions ribosomals estan presents en el genoma en múltiples copies (per exemple, 6-8 copies en *L. monocytogenes* i *L. innocua*), aquest sistema desenvolupat per *Listeria* spp. Va presentar una excel·lent sensibilitat: la detecció consistent d'una única copia. D'altra banda, en aquest estudi es van fer servir gens associats a la virulència per a la detecció específica de *L. monocytogenes* (el gen de la Listeriolisina O -*hly*-, o el gen que codifica per proteïna p60 -*iap*-) i de *Salmonella* spp. (el gen *invA*). Per exemple, el gen *hly* està present únicament en *L. monocytogenes* i va permetre el disseny de 2 sistemes diferentes de PCR a temps real (en format *uniplex* i en format *duplex* per la detecció simultània d'altres membres del gènere Listeria). En el cas de *L. innocua,* al tractar-se d'un bacteri apatògen, es va escollir el gen *lin02483* que codifica per una proteïna potencialment associada a l'estrès, similar a proteïnes associades a la resistència a l'estrès osmòtic i a variacions en l'equilibri del pH. Tanmateix, pel disseny d'un sistema de PCR a temps real específic per *Mycobacterium avium* subsp. *paratuberculosis* va utilitzar-se una seqüència d'inserció pròpia d'aquest bacteri i present en un nombre elevat de copies (15-18 copies); la seqüència d'inserció 900 (*IS900*). Això és especialment rellevant en el cas d'aquest patògen ja que generalment es troba en quantitats petites en els aliments.

Després de la selecció dels gens més adequats per cadascun dels microorganismes estudiats, es va realitzar una cerca *in silico* d'una regió específica dintre de cadascun d'aquests gens, utilitzant eines

bioinformàtiques com Blast-N (NCBI, USA) o Fasta-3 (EMBL, UK). Es dissenyaren sistemes específics de PCR a temps real en les regions seleccionades utilitzant eines bioinformàtiques com Primer Express v2.0 (Applied Biosystems, USA), DNA*DNASTAR (Lasergene, DNASTAR Inc, USA) i primer 3 (http://www-genome.wi.mit.edu/genome_software/other/primer3.html). Posteriorment, l'especificitat de cada sistema de PCR a temps real va revisar-se *in silico* avaluant l'especificitat global de l'amplicó i de cada oligonucleòtid dissenyat. Per exemple, el gen *hly* de *L. monocytogenes*, contenia una regió (posicions 113 a 177) sense homologia significativa amb cap altra regió dipositada en les bases de dades. Per tant, es dissenyaren un parell d'encebadors i una sonda TaqMan® dins d'aquesta regió i van comparar-se amb les seqüències publicades, observant-se homologia únicament amb seqüències dipositades de *L. monocytogenes*.

Posteriorment, s'optimitzaren els sistemes de PCR a temps real. Van avaluar-se experimentalment tres paràmetres: la concentració dels oligonucleòtids, la concentració de l'ió Mg^{2+}, i la temperatura d'hibridació. Les condicions òptimes per les concentracions dels encebadors i la sonda van ser les mínimes que donaren el valor més baix de cicle llindar (C_T) i el més alto d'increment de fluorescència normalitzada (ΔR_n). En general, 50 nM-300nM de cada encebador van produir els millors resultats. La concentració òptima d'ió Mg^{2+} varia de 0.8 a 9.0 mM $MgCl_2$. La polimerasa que es va utilitzar majoritàriament en aquest treball va ser Ampli*Taq* Gold® DNA polymerase, que té una elevada fidelitat independentment de la concentració de Mg^{2+}, i per tant, va permetre la utilització de condicions molt favorables a l'amplificació sense afectar-ne l'especificitat en la major part dels sistemes de PCR a temps real desenvolupats en aquest estudi. Finalment, va seleccionar-se la temperatura d'hibridació més alta que no produís una afectació significativa dels valors de C_T. L'escenari de consens adoptat en aquest estudi, especialment per les condicions de temperatura/temps, per tal de simplificar l'aplicació rutinària dels sistemes desenvolupats i la seva aplicació potencial en una única reacció va ser el següent: 2 min a 50 ºC, 10 min a 95 ºC, i 50 cicles de 15 s a95 ºC i 1 min a 63 ºC.

Abans de l'aplicació d'un sistema de PCR a temps real a mostres reals és necessari establir-ne l'especificitat, la sensibilitat, i l'exactitud relativa. Un sistema de PCR a temps real ha de ser totalment capaç de detectar els microorganismes diana, i ser totalment capaç de no detectar els organismes que no ho són. Per això, en aquest estudi van seguir-se les directrius marcades per la norma de caràcter internacional prEN ISO 16140:2002: avaluar l'especificitat utilitzant almenys 50 (25 per *Salmonella*) soques bacterianes diana, i 30 soques que no són diana del sistema de detecció. Tots els sistemes desenvolupats van ser 100 % específics. A més, els que van ser dissenyats amb finalitat de quantificació, van tenir, per les soques bacterianes positives, una variació inferior a 1 unitat de C_T, excepte el sistema de PCR a temps real dissenyat sobre el gen *iap*, per tant aquest sistema no va ser considerat adequat per anàlisis quantitatives de *L. monocytogenes*. Tanmateix, va confirmar-se que aquesta divergència en els valors de C_T era deguda a variacions en la seqüència dintre dels diferents serotips d'aquesta espècie. D'aquesta manera, seqüències de soques que pertanyen a la mateixa divisió filogenètica de *L. monocytogenes* estaven altament conservades, i van produir valors de C_T més conservats que aquelles soques de divisions diferents. Això ens va permetre dissenyar, per primera vegada, un test diagnòstic per la classificació d'una soca d'aquesta espècie en la divisió I o II/III utilitzant de 2 sistemes de PCR a temps real conjuntament (dissenyats en les seqüències altament conservades del gen *hly* i en les variables del gen *iap*).

Un altre paràmetre que va ser avaluat és la sensibilitat. Indica el nivell mínim en el qual l'analit pot ser detectat de manera reproduïble amb una probabilitat determinda. En aquest estudi, va establir-se la sensibilitat o límit de detecció per l'anàlisi de dilucions seriades de l'organisme diana en diferents experiments (almenys 3) en els quals s'utilitzaven diversos replicats (almenys 3). A més, també es va fer servir el test dissenyat per Knutsson i col·laboradors per establir la probabilitat de detecció del microorganisme diana en el caso dels sistemes de PCR i NASBA a temps real específics per *Mycobacterium avium* subsp. *paratuberculosis*. Tots els sistemes van presentar un límit de detecció (amb una probabilitat del 95 %) per sota de 10 cèl·lules. Com era d'esperar, els sistemes més sensibles van ser els que utilitzaven seqüències de gens multicopia: un únic microorganisme pel sistema

específic de *Listeria* spp. (gen 23S rDNA) i menys de 3 microorganismes pel sistema específic de *Mycobacterium avium* subsp. *paratuberculosis* (IS900). És interessant notar que el límit de detecció va ser similar quan s'utilitzava ADN bacterià purificat o cèl·lules, lo qual té implicacions pràctiques importants per la simplicitat d'ús d'aquestes darreres com a analit.

En els sistemes dissenyats amb propòsits quantitatius, van analitzar-se 3 paràmetres addicionals: la quantificabilitat, el rang de quantificació i el límit de quantificació. El terme quantificabilitat no està definit formalment, però els usuaris de la tècnica de PCR a temps real l'entenen en general com a depenent de la linealitat i l'eficiència de la reacció. En aquest estudi, la linealitat va establir-se mitjançant el coeficient de regressió (R^2) de la corba de regressió obtinguda per concentracions conegudes d'ADN del microorganisme diana. Valors per sobre de 0,90-0,95 són considerats adequats. En aquest estudi, tots els sistemes presentaren valors superiors a 0,95. L'eficiència de la reacció (E) va ser establerta mitjançant la següent equació: $E=10^{-1/s} - 1$; on s és el pendent de la corba de regressió. Pot considerar-se que el funcionament és adequat per un rang de valors de E entre 0,78 ($s= -4,00$) i 1,15 ($s=-3,00$), i les eficiències obtingudes per tots els sistemes d'aquest estudi van estar en aquest interval. El nivell més baix en el qual els valors de C_T obtinguts en dues dilucions consecutives no es solcaven amb una probabilitat del 95%, va considerar-se el límit de quantificació. En paral·lel a l'avaluació experimental, va calcular-se el límit de quantificació teòric a través de el anàlisi de simulacions de MonteCarlo i calculant un interval de confiança del 95% per cada dilució. En aquest estudi, els límits de quantificació teòric i experimental van coincidir i van establir-se en la major part dels casos entorn de 30 copies genòmiques o cèl·lules bacterianes. Per tant, el rang de quantificació estava per sobre de 5 ordres de magnitud (generalment des de 3×10^6 ó 3×10^5 a 3×10^1).

Finalment, va analitzar-se l'exactitud relativa. Defineix el grau de correspondència entre la resposta obtinguda a través del mètode de referència i d'un mètode alternatiu. En aquest estudi, l'exactitud relativa va calcular-se mitjançant la comparació dels resultats obtinguts amb els diferents sistemes de PCR a temps real i els obtinguts per mètodes microbiològics de referència (recompte en placa o hemocitometria), considerant-se adequats els que van presentar valors compresos entre 70 i 130%. En tots els casos van obtenir-se valors inclosos en l'interval definit.

A més, va desenvolupar-se un sistema de NASBA a temps real per a la detecció específica de *Mycobacterium avium* subsp. *paratuberculosis*. Els criteris i paràmetres utilitzats foren els mateixos que els resumits en els paràgrafs anteriors, i es va obtenir un sistema 100% específic amb un límit de detecció de 150-200 cèl·lules per reacció. La principal característica de la tècnica NASBA és que està descrit que amplifica únicament ARN, fins i tot en presència d'ADN. Per això, l'aplicació d'aquesta tècnica podria permetre, en teoria, realitzar estudis sobre la viabilitat cel·lular ja que l'ARN (especialment l'ARN missatger) és molt més làbil que el DNA. Però, encara en un principi aquesta tècnica amplifica en teoria només RNA, en un intent de corroborar-ho hem descobert que no sempre és així. En aquest estudi vam analitzar l'origen del senyal produït per NASBA en 3 sistemes NASBA diferents que permetien detectar específicament *Mycobacterium avium* subsp. *paratuberculosis* amplificant 3 seqüències diferents. Vam poder comprovar que en realitat s'amplificava DNA de *Mycobacterium avium* subsp. *paratuberculosis* en tots 3 sistemes, però que s'amplificava RNA en el sistema NASBA específic de *Salmonella* spp. que vam utilitzar com a control. Això indica que els sistemes NASBA desenvolupats per *Mycobacterium avium* subsp. *paratuberculosis* no són adequats per l'estudi de cèl·lules viables. Això és molt útil pels científics que es plantegin utilitzar sistemes basats en NASBA per l'estudi de la viabilitat cel·lular: sempre és necessari comprovar la naturalesa de la molècula origen del senyal.

L'eficiència dels mètodes moleculars pot estar influïda negativament per la presència de substàncies inhibidores en la mostra que ha de ser analitzada. Això pot donar lloc a senyals més dèbils o negatives i permetre la subestimació de la quantitat d'àcids nucleics en la mostra. Per tant, un aspecte fonamental és control de l'eficiència de la reacció d'amplificació adequat. En aquest estudi van desenvolupar-se controls interns d'amplificació (IAC). Un IAC és un àcid nucleic quimèric que

s'afegeix a la reacció per ser co-amplificat pels mateixos encebadors que l'àcid nucleic diana, i la seva amplificació indicarà si l'eficiència de la reacció és adequada. En aquest estudi, van construir-se i avaluar-se IACs pels sistemes de PCR a temps real específics de *L. monocytogenes* (*hly*) i de *Mycobacterium avium* subsp. *paratuberculosis* (IS900); i pel sistema de NASBA a temps real específic de *Mycobacterium avium* subsp. *paratuberculosis*. Aquestes seqüències quimèriques (DNA per RTi-PCR i RNA per RTi-NASBA) no mostraren cap homologia amb altres seqüències. Els nostres IACs van poder-se amplificar amb els encebadors específics de cada sistema; no van poder-se detectar amb la sonda específica del microorganisme diana; però sí que van poder-se detectar amb la sonda dissenyada específicament per cadascun d'ells s. A més, va avaluar-se el funcionament de l'amplificació de diferent nombre de copies de IAC, així com la possible interferència de diferents quantitats de IAC sobre la reacció principal; i finalment van triar-se 100 copies (per RTi-PCR) o 1 fg (per RTi-NASBA). Finalment, va avaluar-se l'aplicació dels diferents sistemes a mostres reals per la seva futura implantació com a eines diagnòstiques en laboratoris de microbiologia dels aliments. En aquest estudi van desenvolupar-se estratègies de prelació de la mostra abans de la seva aplicació als sistemes d'amplificació desenvolupats, ja que aquest pas previ pot ser crucial per la seva robustesa i bon funcionament. L'aproximació més racional pel processament de la mostra és la combinació de diferents procediments, generalment físics i d'extracció dels àcids nucleics; i per tant va ser l'aproximació utilitzada en aquest treball. D'aquesta manera, va avaluar-se el sistema de PCR a temps real específic desenvolupat per *L. monocytogenes* (*hly*) per productes càrnics (carn de porc, productes càrnics cuits –pernil dolç i salsitxes de Frankfurt-, i un producte càrnic fermentat -fuet-) i per productes de pesca (salmó, i salmó fumat) en haver estat ambdues categories considerades com a vies de transmissió de la listeriosi als consumidors. A més, també s'avaluaren els sistemes específics per *Mycobacterium avium* subsp. *paratuberculosis* (tant RTi-PCR com RTi-NASBA) per dos productes alimentaris: llet (llet pasteuritzada semi-desnatada) i aigua, en haver-se suggerit que són les dues fonts potencials més importants de transmissió de *Mycobacterium avium* subsp. *paratuberculosis* a l'home. Per millorar la sensibilitat d'aquests sistemes, s'aplicaren diferents procediments de processament de cada tipus de mostres analitzades. A més, per tal de reproduir les condicions normals utilitzades en les anàlisi rutinàries dels aliments, van utilitzar-se els volums o pesos que generalment s'empren en aquestes anàlisis (20-25 ml o 25 g). Per *L. monocytogenes*, va desenvolupar-se un procediment ràpid i senzill basat en la filtració de la mostra i en la posterior extracció d'ADN. Així, en els productes càrnics van poder-se detector fins a 100 CFU/g i quantificar fins a 1000 CFU/g amb excel·lent exactitud respecte al mètode oficial de referència. Per productes de pesca, el tractament previ a la PCR també va consistir en una filtració i posterior purificació de l'ADN, que va permetre detectar en el cas del salmó fumat fins a 10 CFU/g i quantificar fins a 1000 CFU/g amb excel·lent exactitud respecte al mètode oficial de referència. Per *Mycobacterium avium* subsp. *paratuberculosis,* quan van analitzar-se mostres d'aigua a través d'un procediment molt simple basat en una filtració van poder-se obtenir els mateixos resultats que quan s'analitzava ADN purificat. Per la llet semi-desnatada, el procediment òptim va consistir en un tractament enzimàtic associat a un detergent, centrifugació i posterior extracció dels àcids nucleics, i d'aquesta manera van poder-se detectar fins a 10^2 *Mycobacterium avium* subsp. *paratuberculosis* en 20 ml de mostra. Amb aquests mateixos tractaments pre-PCR, van poder-se detectar consistentment 10^3 cèl·lules de *Mycobacterium avium* subsp. *paratuberculosis* en 20 ml de aigua, i 10^4 cèl·lules en 20 ml de llet mitjançant el sistema de NASBA a temps real.

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ABREVIATIONS and SYMBOLS

∅:CHCl₃:AIA	phenol/chloroform/isoamyl alcohol
ΔRn	normalized fluorescence increment
AcNH₄	amonium acetate
AN	accession number
BSA	bovine seroalbumine, fraction V
CEN	European Committee for Normalization
CIP	Calf intestinal phospatase
C_T	threshold cycle
DABCYL	4-(dimetilamin)azo benzene sulfonic acid
dd	double-destilled
DMSO	dimetilsulfoxide
DNA	deoxyribonucleic acid
cDNA	complementary DNA
dsDNA	double-stranded DNA
gDNA	genomic DNA
ssDNA	single-stranded DNA
DNase	deoxyribonuclease
dNTP	deoxi-ribonucleotide-trifosfate
ds	double-stranded
EDTA	etilendiaminotetracetate disodium acid (Na₂EDTA-2 H₂O)
EtBr	ethidium bromide
EtOH	ethanol
FAM	6-carboxifluorescein
g	gram(s)
g	gravity (relative centrifugation units)
HAc	acetic acid
hsp	*heat-shock* protein
IAA	isoamyl alcohol
IAC	Internal Amplification Control
kb	kilobase(s)
kDa	kiloDalton(s)
kV	kilovolt(s)
l	litre(s)
LB	Luria Bertani medium
M	molar
m-, μ-, nm	mili-, micro-, nanometre(s)
m-, μ -, n-, pM	mili-, micro-, nano-, picomolar
m-, μ-, n-, p-, fg	mili-, micro-, nano-, pico-, fentogram(s)
m-, μl	mili-, microliter(s)
mA	miliampere
MDa	megaDalton(s)
min	minute(s)
NaAc	sodium acetate
NaCl	sodium chloride
NaOH	sodium hydroxide
NASBA	nucleic acid sequence-based amplification
nt	nucleotide(s)
°C	Celsius degrees
OD	optical density
w/v	weight/volume
bp	base pairs

PCR	polymerase chain reaction
cPCR	conventional PCR
PEG	polietilenglicol
pH	inverse of logarithm hydrogenous ions concentration
Rn	normalized fluorescence
rNTP	ribonucleotide-triphosphate
RNA	ribonucleic acid
mRNA	messager RNA
RNase	ribonuclease
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcriptase PCR
RTi-PCR	real-time PCR
s	second(s)
SD	standard deviation
SDS	sodium dodecilsulphate
T	temperature
Ta	annealing temperature
TAE	Tris-acetate (40 mM); EDTA (1 mM)
TAMRA	6-carboxi-tetrametilrhodamine
TE	Tris-HCl (10 mM); EDTA (1 mM)
T_m	dissociation or melting temperature
Tris	Tris-hidroximetil-aminometane
Tris-HCl	Tris-hidroximetil-aminometane choride hydroxide
U	unit(s)
UNG	Uracil N-Glicosilase
UV	ultraviolet light
V	volt
v/v	volume/volume
Vol.	Volume

I. INTRODUCTION

"It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic materials"

J.D. Watson and F.H.C. Crick

Nature 4356, 1953

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I.1   Foodborne pathogens and diseases: Importance and worldwide incidence

Foodborne diseases are among the most serious public health concerns all over the world and are a major cause of morbidity (Wallace *et al.*, 2000; Anonymous, 2001). This threat has been increasing with global trade and travel over the past decades, affecting both industrialised and developing countries (Käferstein *et al.*, 1997). More than 200 known diseases are transmitted trough food (Bryan, 1982), with symptoms ranging from mild gastroenteritis to life-threatening syndromes, with the possibility of chronic complications or disability (Mead *et al.*, 1999). The causes of foodborne illness include pathogens, toxins and metals. More than 40 different foodborne pathogens are known to cause human illness (CAST, 1994), among which over 90 % of confirmed foodborne human illness cases and deaths caused by foodborne pathogens reported to the Center for Disease Control and Prevention (CDC) have been attributed to bacteria, being the rest due to fungi, parasites and viruses (Bean *et al.*, 1990). In consequence, microbiological quality control programs are being increasingly applied throughout the food production chain in order to minimize the risk of infection for the consumer. Surveillance systems that include quantification of reported foodborne illnesses and identification of emerging pathogens are needed (Blackburn and McClure, 2002).

Salmonella spp., *Listeria monocytogenes*, *E. coli* and *Campylobacter* spp. can be considered the major foodborne pathogens. However, the impact of the foodborne pathogens has important geographical- and seasonal-dependent aspects. For instance, in USA, noroviruses cause the largest number of illnesses, followed by *Salmonella* spp., *Campylobacter* spp., *Giardia lambia,* staphylococci, *E. coli* and *Toxoplasma gondii,* respectively (Mead *et al.*, 1999). In developing countries, the principal causes of diarrhoea are enterotoxigenic *E. coli*, *Shigella* spp., enteropathogenic *E. coli*, *Campylobacter jejuni*, *Vibrio* spp., enteroinvasive E. *coli* and *Entamoeba enterocolytica* (Dupont, 1995).

The impact of foodborne pathogens in public health systems is very considerable. For example, in the USA, it has been estimated that foodborne diseases cause approximately 76 million illnesses, 325,000 hospitalisations and 5,000 deaths each year, with known pathogens accounting for 14 million illnesses, 60,000 hospitalisations and 1,800 deaths per year (Mead *et al.*, 1999). Only three pathogens, *Salmonella*, *Listeria*, and *Toxoplasma*, are responsible for more than 1,500 deaths each year (Mead *et al.*, 1999). Foodborne illnesses account for around 1 % USA hospitalisation cases and 0.2 % deaths (Buzby *et al.*, 2001); with annual medical and productivity losses due to the five major foodborne pathogens estimated at around 6,500 million of dollars (Crutchfield and Roberts, 2000). In England and Wales, foodborne pathogens produce 1.3 million illnesses, 20,759 hospitalisations and 480 deaths each year (Adak *et al.*, 2002).

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I.2   A brief description of the foodborne pathogens used in this PhD Thesis

Listeria monocytogenes

This bacterium was first described in 1924 by E.G.D. Murray, R.A. Webb, and M.B.R. Swann as a short, Gram-positive, non-sporing, rod-shaping bacterium, causing a septicemic disease in rabbits and guinea pigs (Murray *et al.*, 1926). It was named *Bacterium monocytogenes* because it infected the monocytes in the blood. In 1930, Pirie isolated a similar organism from livers of sick gerbils and called it *Listerella hepatolytica*, after the famous surgeon Joseph Lister. Since the name '*Listerella*' had previously been adopted for a group of slime moulds, the name *Listeria monocytogenes* was finally agreed (Pirie, 1940).

Taxonomy

Listeriae are catalase positive and facultatively anaerobic bacteria of low G-C content closely related to *Bacillus, Clostridium, Enterococcus, Streptococcus*, and *Staphylococcus* (Vázquez-Boland *et al.*, 2001). They occasionally give rise to coccoid forms or individual cells of 10 μm in length (Rocourt, 1999). They are motile at 10 to 25°C, showing a characteristic 'tumbling' motility, but not motile at 35 ºC (Collins *et al.*, 1991; Rocourt, 1999; Sallem *et al.*, 1996). The genus *Listeria* currently includes six species (Table I.1): *L. monocytogenes, L. ivanovii, L. seeligeri, L. innocua, L. welshimeri*, and *L. grayi*. A seventh species, previously named *Listeria denitrificans*, has been reclassified *Jonesia denitrificans* (Rocourt *et al.*, 1987). *L. innocua* and *L. grayi* are considered non-pathogenic, while *L. seeligeri, L. ivanovii* and *L. welshimeri* rarely cause human infection (Jones, 1991). *L. ivanovii* can cause infection in ruminants (González-Zorn *et al.*, 1999); and *L. monocytogenes* causes serious localized and generalized infections in humans and a variety of other vertebrates, including fish, birds and mammals (Roberts *et al.*, 1996). The infectious disease caused by these bacteria is known as listeriosis.

Table I.1. Principal characteristics of the genus *Listeria*.

(A)   Cultural   and   biochemical characteristics.

Characteristic	Reaction
Catalase activity	+
Oxygen requirement	F
Growth at 35 ºC	+
Motility at 22 ºC	+
Motility at 37 ºC	-
Methyl red reaction	+
Voges-Proskauer reaction	+
H_2S production	-
Acid from glucose	+
Indol Production	-
Citrate utilization	-
Urease activity	-
Mannitol	-
Nitrate	-
Gelatine	-

F, facultative

(B) Differences between *L. monocytogenes* and other *Listeria* spp.

Listeria species	Carbohydrate fermentation			CAMP Test[*]		β-haemolysis
	M	R	X	SA	RE	
monocytogenes	-	+	-	+	-	+
innocua	-	+/-	-	-	-	-
welshimeri	-	+/-	+	-	-	-
seeligeri	-	-	+	+	-	+
ivanovii	-	-	+	-	+	+
grayi	+	+/-	-	-	-	-

M, manitol; **R,** L-rhamnose; **X,** D-Xylose.
[*] Christie, Atkins, Munch-Petersen phenomenon, *i.e. L. monocytogenes* shows a typical haemolytic zone on blood plates when streaked together with β-haemolytic *Staphylococcus aureus* (SA) and/or *Rhodococcus equi* (RE).

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Distribution and Epidemiology

L. monocytogenes has been isolated from a wide variety of environmental sources, including soil, silage, water, sewage, food-processing environments, a large variety of foods, and the faeces of humans and animals (Farber and Peterkin, 1991; McCarthy, 1990; Watkins and Sleath, 1981; Weis and Seeliger, 1975; Welshimer and Donker-Voet, 1971). It has been hypothesised that its natural habitat can be soil, water and plant material, particularly that undergoing decay (Rocourt and Seeliger, 1985). Aerobically spoiled silage, capable of supporting a high load of *L. monocytogenes,* has been cited as a major source of infection of farm animals, and may be the origin of spreading the contamination along the food chain. Domesticated ruminants probably play a key role in its maintenance in the rural environment via a continuous faecal-oral enrichment cycle (Fenlon, 1999; Vázquez-Boland *et al.*, 2001).

As this organism is so widely distributed in the environment, animals and humans frequently are in contact with *L. monocytogenes*. The first cases of human listeriosis caused by *L. monocytogenes* were reported in 1929 in Denmark (Nyfelt, 1929). During the subsequent five decades listeriosis occurred sporadically, and the epidemiology of the disease was unresolved (Seeliger, 1972; Seeliger, 1988). However, during the late 1970's and early 1980's, the number of reports of *Listeria* isolations increased, with *L. monocytogenes* emerging as a serious foodborne pathogen (Ryser, 1999). From then on, several outbreaks associated with processed foods, such as coleslaw (Schlech *et al.*, 1983), soft cheese (Bille, 1990; Linnan *et al.*, 1988), paté (Gilbert *et al.*, 1993; McLauchlin *et al.*, 1991), milk (Fleming *et al.*, 1985), pork tongue (Jaquet *et al.*, 1995) and pork fillets (Jaquet *et al.*, 1995) have been reported. It is noteworthy that the strains implicated in several of these temporally and geographically unlinked outbreaks were of genetically related serotypes (Piffaretti *et al.*, 1989; Jacquet *et al.*, 1995). In contrast to their clinical prevalence, these serotypes are not frequently recovered in routine surveys of contaminated foods (Hayes *et al.*, 1991). This suggests that they may represent an epidemic clonal lineage (Kathariou, 2000).

Symptoms

Listeriosis is usually a very severe disease with a mean mortality rate in humans of at least 20-30 % despite early antibiotic treatment (Schuchat *et al.*, 1991). It has a bimodal distribution of severity with most cases being either mild or severe (CAST, 1994). Mild cases are characterized by a sudden onset of fever, severe headache, vomits, and other influenza-type symptoms. Severe manifestations of listeriosis correspond to disseminated infection or to local infection in the central nervous system, including septicaemia, meningitis (or meningoencephalitis), encephalitis, and intrauterine or cervical infections in pregnant women, which may result in spontaneous abortion (2nd/3rd trimester) or stillbirth (McLauchlin, 1990a; 1990b). The incubation period for listeriosis varies from 4 days to several weeks and the duration of the illness from a few days to several weeks (CAST, 1994).

Two basic forms of listeriosis can be distinguished: perinatal and in adult patients (Vázquez-Boland *et al.*, 2001). About one-third of human *L. monocytogenes* infections are perinatal (Benenson, 1990), involving pregnant women and their unborn or newly born infants, and the rest occurring in non-pregnant adults older than 40. This age distribution correlates with *Listeria* being more likely to cause severe illnesses and death in persons with compromised immune systems (*i.e.*, people with AIDS, cancer, diabetes, heart disease, or renal disease) or in those with immature immune systems (*e.g.*, foetuses) (Schwartz *et al.*, 1988).

The infection is usually asymptomatic in pregnant women or may be manifested as a mild flu-like syndrome with chills, fatigue, headache, and muscular and joint pain about 2 to 14 days before miscarriage. Infected pregnant women can transmit the disease to the foetus via the placenta and generate chorioamnionitis. Infected foetuses may be stillborn, develop meningitis (in the neonatal period), or are born with septicaemia and with a clinical syndrome, granulomatosis infantiseptica, characterized by the presence of pyogranulomatous microabscesses disseminated over the body and a

high mortality (Klatt *et al.*, 1986). Less frequently (10 to 15 % of perinatal cases), late neonatal listeriosis is observed (Vázquez-Boland *et al.*, 2001). The mortality of late-onset neonatal listeriosis is lower (10 to 20 %), but like early-onset neonatal listeriosis, it may have sequelae such as hydrocephalus or psychomotor retardation (Evans *et al.,* 1984). Non-pregnant adults with listeriosis typically develop septicaemia (15 to 50 % of cases) or meningitis (55 to 70 % of cases) syndromes. There are other atypical clinical forms (5 to 10 % of cases), such as endocarditis, myocarditis, arteritis, pneumonia, pleuritis, hepatitis, colecystitis, peritonitis, localized abscesses, arthritis, osteomyelitis, sinusitis, otitis, conjunctivitis and ophthalmitis (Blenden *et al.*, 1987; Farber and Peterkin, 1991; Gallaguer and Watakunakorn, 1988; Gauto *et al.*, 1991; Lorber, 1990).

Mycobacterium avium subsp. *paratuberculosis*

Mycobacterium avium subsp. *paratuberculosis* (*MAP*) is a facultative intracellular bacterium (Thorel *et al.*, 1990) first isolated and identified in 1895 from cattle with severe enteritis (Johne and Frothingham, 1895). *MAP* has been also isolated from domestic rabbit and wild animals (Ayele, 2001). This pathogen is the etiological agent of ruminant paratuberculosis (Johne's disease), and recently has been hypothesised that it could play a role on the etiopathogenesis of Crohn's disease (CD).

Taxonomy

MAP is a member of the family *Mycobacteriaceae* (Wayne and Kubica, 1986), which includes around 15 species known to be pathogenic for man, with *Mycobacterium tuberculosis* the best known. Analysis of the rRNA genes (rDNA) of mycobacteria has resulted in the division of this genus into two separate clusters. They correspond to the traditional fast-growing mycobacteria, represented by non-pathogenic environmental isolates; and the slow-growing mycobacteria, containing most of the pathogens (Rogall *et al.*, 1990; Stahl and Urbance, 1990; Wayne and Kubica, 1986). The mycobacterial species *M. avium* is currently subdivided into three subspecies, *M. avium* subsp. *avium (MAA), MAP*, and *M. avium* subsp. *silvaticum (MAS)*. The subspecies designation of *MAP* is based on DNA-DNA hybridization studies (Hurley *et al.*, 1988; Saxegaard *et al.*, 1988; Thorel *et al.*, 1990, Yoshimura and Graham, 1988) and numerical taxonomy analysis (Thorel *et al.*, 1990). At the subspecies level, *MAP* can be differentiated phenotypically from *MAA* and *MAS* by its dependence on mycobactin (Thorel *et al.*, 1990) and iron-binding hydroxamate compounds (Griffiths, 2002), and genotypically by the presence of multiple copies of an insertion element, IS*900* (Collins and de Lisle, 1986; Green *et al.*, 1989).

MAP is an aerobic, non-spore-forming, non-motile, acid-fast bacillus (Thorel *et al.*, 1990), although some non-acid fast, lightly staining acid-fast, and cell wall deficient types are encountered. It generally occurs in clumps linked together by a network of intercellular filaments. *MAP* has a complex cell wall, relatively impermeable and rich in lipids, which confers acid-fast properties and may enhance its survival in the environment. It grows in the temperature range 25-45 °C, at salt concentrations below 5 % and at a pH 5.5 or greater (Collins *et al.*, 2001). A major constituent of the cell wall is lipoarabinomannan (LAM), which may play a role in the formation of the granulomatous lesions found in infected patients. LAM differs immunogenically in bovine and ovine isolates, is highly immunogenic and is the target of the enzyme linked immunoassays (ELISA) developed to strain-type the species.

Paratuberculosis

Paratuberculosis (Johne's disease) is a chronic, granulomatous enteritis of ruminants (Stabel, 2000), currently regarded as one of the most serious chronic bacterial diseases of dairy cattle (Collins *et al.*, 2001; McNab *et al.*, 1991). Clinical manifestations are characterized by profuse, non-treatable diarrhoea, emaciation, and, ultimately, death. During the course of the disease, the intestine becomes thickened and corrugated, disallowing proper absorption of nutrients. Cattle are most susceptible to infection during the first 6 months of life, frequently being infected by ingestion of faeces and milk

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contaminated with *MAP* or by *in utero* transmission from infected dams (Morgan, 1987). There is a long and variable incubation period, and clinical manifestations seldom occur before the age of 2-5 years (Chiodini *et al.*, 1984a; Larsen *et al.*, 1975). Indeed, not all infected cattle will develop clinical disease.

Paratuberculosis is prevalent in domestic animals worldwide and has significant impact on the global economy (Sweeney, 1996). The incidence has been reported in 20 to 40 % of dairy herds in the United States (Wells *et al.*, 1998), causing an estimated loss of 1,500 million dollars per year (Stabel, 1998). Cows with subclinical paratuberculosis suffer an increased incidence of mastitis, decreased milk production, and increased calving intervals (Stabel, 1998), representing further losses of around 40 to 227 million of dollars per cow inventoried per year (National Animal Health Monitoring System, 1997).

Crohn's disease (CD)

The first clinical description of CD was drawn by T.K. Dalziel in 1913 (Dalziel, 1913). Several years later, B. Crohn and two colleagues reported a series of cases (Crohn *et al.*, 1932), and the disease got its name. CD is a chronic, debilitating, recurring, intestinal inflammatory disease. It most commonly affects the distal ileum and colon, but occasionally involves the entire gastrointestinal tract or any part of it (Hanauer, 1998). Extraintestinal clinical forms, such as ocular and cutaneous, have been also reported (Kapoor, 1997). Nowadays, there is no known cure for this life-long debilitating disease, which tends to pursue a variable course, characterised by periods of activity interspersed with remissions, when the disease is either absent or relatively quiescent, and up to 74 % of patients require surgery (Andres and Friedman, 1999; Greenstein *et al.*, 1975).

Pathologically, it is characterised by transmural inflammation with deep ulceration, and thickening of the bowel wall often leading to obstruction, discontinuous involvement, and fistula formation. Microscopically the ileum is infiltrated with mononuclear inflammatory cells, macrophages and lymphocytes, and often macrophages fuse together to form giant cells and a diffuse granulomatous inflammation results (Jewell, 1987). Pain is a common feature, particularly in those with small bowel involvement. Diarrhoea, often with bleeding, occurs in colonic CD. Fistulae are characteristic of CD.

CD can be considered a major human healthcare problem in developed societies (Rubery, 2001). The impact of CD was kept at a low level for many years; however it began to emerge in Western Europe and North America in the last few decades. At least 500,000 CD sufferers are estimated in the USA, rising by about 250,000 new cases each year; and the estimates for Western Europe are comparable (Rubery, 2001).

Reasons to link <u>Mycobacterium avium</u> subsp. <u>paratuberculosis</u> and CD

The relationship between *MAP* and CD was first proposed by Dalziel (Dalziel, 1913). Given the similarity of clinical signs (diarrhea and weight loss), pathology (transmural diffuse granulomatous inflammation), and epidemiology (rising incidence, long incubation period, and familial occurrence pattern) between ruminant paratuberculosis and CD, several authors have suggested that there is a strong probability that *MAP* plays a role in the aetiology of most CD (Chamberlein *et al.*, 2001; Chiodini and Rossiter, 1996; Chiodini *et al.*, 1984b; Chiodini *et al.*, 1986.; Dell'Isola *et al.*, 1994; El-Zaatari *et al.*, 2001; Greenstein, 2003; Hermon-Taylor, 1993; Hermon Taylor and Bull, 2002; Hermon-Taylor *et al.*, 2000; McFadden *et al.*, 1992; Murray *et al.*, 1995). It has been suggested that *MAP* could have fulfilled Koch's four postulates in Crohn's disease (Brown *et al.*, 1996). To verify this claim, individual publications should be combined (Greenstein, 2003). Viable *MAP* has been isolated and subsequently cultured by some laboratories from some patients with CD (Gitnick *et al.*, 1989; Chiodini *et al.*, 1984b; Coloe *et al.*, 1986; Thorel, 1989; Haagsma *et al.*, 1991). This finding, although not universally achievable, may be considered to satisfy the first and second postulates. Cultured human *MAP* has been administered orally to goats (Gitnick *et al.*, 1989; Chiodini *et al.*, 1984b; van

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Kruiningen *et al.*, 1986) and intestinal and mesenteric inflammation compatible with early Johne's disease was identified in a single animal (Chiodini *et al.*, 1984b). However, in other studies, no microorganisms were identified (Gitnick *et al.*, 1989). In more extensive studies, human (Chiodini *et al.*, 1984b) and bovine (Veazey *et al.*, 1995) *MAP* were inoculated in several species, resulting in liver and splenic granulomata in normal mice (Chiodini *et al.*, 1984b) and splenic isolates in immune-deficient mice (Veazey *et al.*, 1995). No disease was observed in rats, guinea-pigs, rabbits, or chickens (Chiodini *et al.*, 1984b) indicating interspecies variability in susceptibility to human *MAP*. These studies may be interpreted as confirming Koch's third postulate. *MAP* of human (Chiodini *et al.*, 1984b; van Kruiningen *et al.*, 1986) and bovine origin has been re-isolated and recultured (Veazey *et al.*, 1995; Hines *et al.*, 1995). These data may be interpreted as confirming Koch's fourth postulate. However, the hypothesis of the implication of *MAP* in CD continues to generate a big controversy, in part due to conflicting evidence and differences in laboratory techniques among studies (Chiodini, 1989; Thompson, 1994; Van Kruiningen, 1999). In addition, although there is considerable similarity between ruminant paratuberculosis and CD, there are also significant differences in the pattern of the disease (Rubery, 2001): (i) there is no fissuring ulceration, fistula formation or fibrosis in ruminant paratuberculosis, whereas these are common complications of CD; (ii) there is no caseation in the granulomata in CD; (iii) the course of the disease is progressive once established in ruminant paratuberculosis, but intermittent in CD; (iv) the organism is easy to recover in many cases of ruminant paratuberculosis but has rarely been recovered and then only with difficulty in CD; (v) there is no evidence of the organism on histochemical, immunostaining or *in situ* hybridisation to demonstrate the organism in CD; (vi) steroids exacerbate tuberculosis and other mycobacterial infections in AIDS patients, but produce remissions in CD.

Salmonella spp.

Salmonella was first described in 1885 by D.E. Salmon who characterised the hog cholera bacillus causing 'swine plague', naming it *Bacterium suipestifer* (now type species *Salmonella cholerasuis*). However, it was not until the 1960's when *Salmonella* became the widely accepted name for this genus.

Taxonomy

Salmonella spp. is a Gram-negative, usually motile, facultative anaerobic, flagellated rod-shaped bacterial group, and pathogen agents with major impact on public health (Garcia del Portillo, 2000; Roberts *et al.*, 1996). Table I.2 shows some key biochemical characteristics of *Salmonella* spp.

Table I.2. Biochemical characteristics of *Salmonella*.

Characteristic	Usual reaction
Catalase	+
Oxidase	-
Acid produced from lactose	-
Gas produced from glucose	+
Indol	-
Urease produced	-
Hydrogen sulphide produced from triple-sugar iron agar	+
Citrate utilised as sole carbon source	+
Methyl Red	+
Voges-Proskauer	-
Lysine decarboxylase	+
Ornithine decarboxylase	+

There has been a considerable debate over the classification and nomenclature of *Salmonella* (Old, 1992; Old and Threlfall, 1997; Thelfall *et al.*, 1999). *Salmonella* is a complex genus with more than 2000 serotypes that has been subdivided into 7 subspecies (I, II, III, IV, V, VI and VII). Molecular techniques have revealed that the genus *Salmonella* contains two lineages that diverged early in its evolution (Reeves *et al.*, 1989; Bäumler *et al.*, 1998). In accordance, they have been proposed to represent two distinct species, designated *S. enterica* and *S. bongori* (Boyd *et al.*, 1996; Le Minor and

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Popoff, 1987; Reeves *et al.*, 1989). Members of *S. enterica* species branche into several distinct phylogenic groups (subspecies I, II IIIa, IIIb, IV, VI and VII). The bacteria causing diseases in human and warm-blooded animal are included in Group I (Bäumler *et al.*, 1998). Group II to VII include *S. enterica* serovars frequently isolated from cold-blooded vertebrates (Bäumler *et al.*, 1998). *S. bongori* corresponds to subspecies V (Boyd *et al.*, 1996).

Distribution and Epidemiology

Salmonellosis is a major public health problem because of its large and varied animal reservoir, the existence of human and animal carrier states, and the lack of a concerted nationwide program to control salmonellae (Humphrey, 2000). Furthermore, *Salmonella* is the main cause of documented foodborne human illnesses in most developed countries (CAST, 1994; Tirado and Schmidt, 2001; Wallace *et al.*, 2000). Of the outbreaks of foodborne illness recorded in the WHO report for 1993 to 1998, salmonellae were most often reported as the causative agent (54.6 % of cases) (WHO, 2001). In the year 2000, 150,165 cases of human salmonellosis were reported from 17 regions in Europe, covering 14 EU Member States and Norway (EC, 2002). Throughout the European Union, the reported number of cases of human salmonellosis in the year 2000 is still high, ranging from 79,535 in Germany to 164 domestic cases in Norway (EC, 2002). The strains most frequently implicated in human gastroenteritis are *S. Typhimurium* and, especially in more recent years, *S. Enteritidis*, particularly Phage Type 4 (PT4) (ACMSF, 2001; EC, 2002; WHO, 2001). The other serotypes involved in human illness vary geographically but frequently include *S. Agona, S. Hadar, S. Heidelberg, S. Infantis, S. Newport, S. Panama, S. Saint-paul, S. Thompson*, and *S. Virchow* (WHO, 2001).

Salmonellae are ubiquitous bacteria due to their metabolism and physiology. The principal animal reservoir of *Salmonella* is the gastrointestinal tract of mammals, reptiles and birds, and salmonellae have been isolated from very different sources and can survive in the environment for prolonged periods (Roberts *et al.*, 1996). Most are widely adaptable and thus have the potential for transmission through human, animal and plant habitats, and the environment in general. Consequently, routes of infection to humans can vary, but, it has been estimated that consumption of contaminated food has been implicated in more than 90 % of the cases of human salmonellosis (Bennett *et al.*, 1987; Helmick *et al.*, 1994; Tauxe and Blake, 1992). Because of the ability of salmonellae to survive in meats and animal products that are not thoroughly cooked, animal products are the main vehicle of transmission. The foods most usually implicated in salmonellosis are eggs and egg products (35 %), cakes and ice-cream (28 %), meat and meat products (8 %), meat and eggs (7 %), poultry and poultry products (4 %), and salads, dressings and mayonnaise (4 %) (WHO, 2001). Other food sources of *Salmonella* may include raw milk or other dairy products and pork. *Salmonella* outbreaks also have been traced to contaminated vegetables and fruits (Helmick *et al.*, 1994). Thus, food categories possibly posing a greater hazard to public health include raw meat and some products intended to be eaten raw, raw or undercooked products of poultry meat, eggs and products containing raw eggs, unpasteurised milk and some products thereof (EC, 2002).

Symptoms

Human salmonellosis comprises several clinical syndromes including enteric (typhoid) fever, localised enterocolitis and systemic infections by non-typhoid microorganisms. Enteric fever and septicaemia due to salmonellae are life-threatening human illnesses but rarely occur in the developed countries (Mølbak *et al.*, 2002). Typically, clinical manifestations of salmonellosis are limited to an acute gastroenteritis. The symptoms generally appear 6 to 74 hours after ingestion of contaminated food or water with an average incubation period of 12 to 36 hours (Benenson, 1990), and include mild abdominal discomfort, with diarrhoea lasting less than a day. Other symptoms may include dehydration, fever, headache, nausea, stomach-ache, and sometimes vomiting (Benenson, 1990). In rare cases, blood may be present in the stools. This clinical condition is generally self-limiting, and remission of the characteristic non-bloody diarrhoeal stools and abdominal pain usually occurs within 5 days of onset of symptoms. Furthermore, human infections with non-typhoid strains can also

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progress to systemic infections and cause secondary-disease syndromes, some of which may be chronic illnesses such as reactive arthritis, Reiter's syndrome and ankylosing spondylitis (Archer, 1984; 1985; Mossel, 1988; D'Aoust, 1991; 1997; 2000). The patients most vulnerable to *Salmonella* infection and secondary complications are infants, the elderly, and the immunocompromised (Helmick *et al.*, 1994), and they face a higher risk of death (Benenson, 1990).

The infective dose for *Salmonella* can vary, depending on the bacterial strain ingested as well as on the immuno-competence of individuals. It has been considered that ingestion of at least 100 organisms is required for infection for serotypes not presenting particular adaptations to an animal host (McCullough and Eisele, 1951; Benenson, 1990). However, data from outbreaks of foodborne diseases indicate that infections can be caused by ingestion of as few as 10 cells or less (Archer and Young, 1988; CAST, 1994; D'Aoust *et al.*, 1985; Lehmacher *et al.*, 1995). It has repeatedly been reported that the infectious dose is lower when salmonellae are present in food with a high content of fat or protein, substances which protect bacterial cells against the low pH of gastric juices (D'Aoust *et al.*, 1975; Blaser and Newman, 1982).

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I.3 Molecular amplification-based methods for detection, identification and quantification of foodborne pathogens

I.3.1 Pre-amplification processing of the samples

It is well known that components of food samples, growth media, and nucleic acids extraction reagents can reduce or even block amplification reactions. They are generally known as amplification inhibitors, and they may cause a dramatic decrease in sensitivity of these reactions compared to pure solution of nucleic acids (Hill, 1996; Lantz *et al.*, 1994; Lantz *et al.*, 2000; Powell *et al.*, 1994; Rossen *et al.*, 1992; Scheu *et al.*, 1998). Consequently, sample preparation prior to the amplification reaction is crucial for the robustness and performance of amplification-based methods. Amplification inhibitors may interfere with the cell lysis, degrade or capture nucleic acids and/or inhibit the amplification reaction (Wilson, 1997). In PCR-based methods, the thermoestable DNA polymerase is probably the most important target site of PCR-inhibitory substances (Al Saud and Rådstöm, 1998). A wide range of inhibitors has been reported; however the identities and modes of action of many of them remain unclear. Common inhibitors include various components of body fluids and reagents encountered in clinical samples (*e.g.*, haemoglobin, bile salts, complex polysaccharides, urea and heparin); food constituents (*e.g.*, organic and phenolic compounds, milk proteinases, glycogen, fats, and Ca^{2+}); environmental compounds (*e.g.*, phenolic compounds, humic acids, and heavy metals); constituents of bacterial cells; non-target nucleic acids; and laboratory contaminants such as glove powder, plasticware and cellulose. Consequently, the characterization and removal of inhibitory substances that may affect an amplification reaction represents an important step in the development of efficient sample preparation procedures to allow correct performance and robustness of the amplification-based assay.

Sample preparation

The purposes of sample preparation are (i) to homogenise the sample for amplification; (ii) to increase the concentration of the target organism to the practical operating range of a given assay; and (iii) to reduce or exclude amplification-inhibitory substances. Hence, pre-amplification treatment aims to convert biological samples into amplifiable samples. As food samples vary in homogeneity, consistency, composition, and accompanying microbiota, pre-amplification procedures should be adapted to each food matrix. A large range of pre-amplification procedures have been developed, many of them being laborious, expensive, and time-consuming (Jaffe *et al.*, 2001). They can either be biochemical; immunological; physical; or physiological procedures (Rådstöm *et al.*, 2003) (Table I.3 and Table I.4); or a combination of them, *e.g.* a pre-step with a biochemical nucleic acid extraction protocol (Dahlenborg *et al.*, 2001; Chen *et al.*, 1997) or with a physical pre-amplification procedure (Lantz *et al.*, 1998).

Table I.3. Sample preparation procedures used for different types of samples. Adapted from Rådström *et al.*, 2003.

Category	Subcategory	Sample preparation procedure	Sample
Biochemical	Adsorption	Lectin-based separation	Beef meat
		Protein adsorption	Blood
	Nucleic acids extraction	Nucleic acid purification procedures	Diverse matrixes
		Lytic procedures	Diverse matrixes
Immunological	Adsorption	Immunomagnetic capture	Diverse matrixes
Physical		Aqueous two-phase systems	Soft cheese
		Buoyant density centrifugation	Minced meat
		Centrifugation	Diverse matrixes
		Dilution	Diverse matrixes
		Filtration	Diverse matrixes
		Boiling	Diverse matrixes
		Other heat treatments	Diverse matrixes
Physiological		Enrichment	Diverse matrixes

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Table I.4. Comparison of the performance of different pre-PCR sample preparation procedures. Adapted from Rådström *et al.*, 2003.

Category	Product of sample preparation			Removal of inhibitors	Time required	Cost
	Type	Homogeneity	Concentration			
Biochemical: nucleic acid extraction	Nucleic acids	Good	Average	Yes	3-6 h	High
Immunological: Immunomagnetic capture	Cell/ nucleic acids	Average	Average	Average	2-4 h	High
Physical: Buoyant-density centrifugation	Cell	Average	Good	Average	30 min	Average
Physiological: Enrichment	Cell	Low	Good	Low	6-24 h	Low

Biochemical procedures

Nucleic acids extraction (DNA and/or RNA) is the most widely employed biochemical procedure. Many different protocols are currently used (Ausubel *et al.*, 2002; Doyle and Doyle, 1991; Helbling *et al.*, 2001; Jeffrey *et al.*, 1994; Murray *et al.*, 1998; Sambrook *et al.*, 2001; Wang *et al.*, 1996). They typically rely on bacterial cell membrane lysis by enzymatic activity or/and mechanical disruption, mostly in the presence of detergents; cell solublization using reagents like guanidinium isothiocyanate; and cleanup steps using organic solvents and alcohol precipitation, suspended silica, affinity or ion/exchange purification columns, ultrafiltration or proprietary compounds. Binding and elution from silica has become the procedure of choice for most nucleic acid extraction procedures as it was developed to isolate all classes of nucleic acids from a wide range of biological samples, (Smith *et al.*, 2003). In addition, many different commercial kits are currently available, such as BAX (Qualicon, Inc.); PrepMan (Applied Biosystems); Purugene (Gentra Systems, Inc.); QIAmp, DNeasy and RNeasy (Qiagen); Nuclisens (BioMerieux); Wizard™ (Promega); Chelex®-100 (Bio Rad) and XTRAX (Gull Laboratories. Inc.). Several studies have compared and evaluated the quality and quantity of the extracted nucleic acids using commercial kits (*e.g.* Fahle and Fisher, 2000; Shafer *et al.*, 1997; Kramvis *et al.*, 1996; Zimmerman *et al.*, 1998a). In general, commercial kits including mini-columns with affinity resins produce low yields of high quality nucleic acids for amplification; whilst simpler, quicker and more economic procedures such as Chelex®-100 produces in general higher yields of nucleic acids of less quality. One advantage of purifying nucleic acids is that homogenous samples that have been mostly depleted of any inhibitors are obtained. However, for microbiological diagnostic purposes, pre-enrichment of the target microorganism is usually required prior to nucleic acids extraction (Rådström *et al.*, 2003). Furthermore, batch-to-batch variation after nucleic acids extraction may also exist with respect to purity and concentration of the template. Purification procedures using lectin-coated magnetic microspheres can be also used to specifically bind bacteria, but the type of food matrix strongly affects the efficiency of binding of bacteria to lectins (Skjerve and Ølsvik, 1991).

Immunological procedures

Immunomagnetic separation (IMS) is currently a widely used bacterial concentration procedure. It is based on the use of magnetic beads coated with antibodies (Blackburn and Patel, 1989; Skjerve *et al.,* 1990; Skjerve and Ølsvik, 1991), and thus the specificity will be dictated by the corresponding antigen present in the captured cells. After allowing the antibody to bind target bacterial antigens within a food matrix, target cells are separated from mixtures by exposing them to a magnetic field. The purpose of these procedures is to increase the concentration of the target organism, as well as to reduce the concentration of inhibitors. The homogeneity of the final sample may differ depending on the processing steps following the capture, but in general the quality of the nucleic acids is adequate for amplification purposes. The drawback of this kind of procedures is that the specificity of the capture is directly dependent on the formation of the complex antigen-antibody, and thus, false negative results can be obtained as a result of cross-reactions, as magnetic beads are usually not specific for the target bacteria (Rijpens and Hermans, 2002). In addition, these procedures

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can lose specificity and sensitivity when applied to matrices rich in fat compounds, such as whole milk, meat and fish products. Finally, this methodology is quite expensive and also very laborious and time consuming.

Physical procedures

Physical procedures are dependent on the physical properties of the target cells, for example cell density or size, and different strategies have been described such as aqueous two-phase systems (Lantz *et al.*, 1994), buoyant-density centrifugation (Lindqvist *et al.*, 1997), centrifugation (Gerritsen *et al.*, 1991), filtration (Starbuck *et al.*, 1992), dilution (Wang *et al.*, 1992) boiling (Wang *et al.*, 1996) and more stronger heat treatments (microwaves [Herman *et al.*, 1995] or autoclave [Simmon *et al.*, 2004]) or a combination of several of these procedures. Aqueous two-phase systems use two polymers with different chemical structures that can properly separate PCR inhibitors from target cells into two immiscible phases. However, in some cases, the sensitivity can be negatively affected by the presence of target bacteria in the interphase or in the same phase containing the inhibitors (Lantz *et al.*, 1998). Buoyant-density centrifugation is a very simple and promising alternative (Lindqvist, 1997) since the target microorganism is highly concentrated. However, the homogeneity of the sample will depend on the kind of matrix, and the components of the sample matrix with the same density as the cells will not be depleted and thus they may inhibit the amplification reaction. Centrifugation, filtration or a combination of both can be used to remove large food particles from the sample and to concentrate bacteria. Often, samples are centrifuged at low speeds to sediment food particles, leaving bacterial cells in the supernatant fluid. Alternatively, samples are centrifuged at higher speeds to sediment bacterial cells, although other particles of equal or greater density will sediment as well. Filtration through a filter such as cheese-cloth and filter paper can remove solid particles from samples, while bacteria are recovered in the filtrates. Alternatively, a food product can be homogenised and passed through an adequate filter to retain the target microorganisms. Filter type, pore shape and dimension, and physical and chemical properties contribute to recovery efficiencies. The principal disadvantages of centrifugation and filtration procedures are the high affinity for food components of some bacteria (Rijpens *et al.*, 1996) or the capacity of some food particles to entrap bacteria. In addition, the choice of the filter is important, as food particles tend to block certain types of filters.

Physiological procedures

These procedures are based on bacterial growth with the double purpose of concentrating target bacteria and diluting inhibitory substances. Cultures can be carried out both in enrichment broths or on agar plates, and selective or non-selective media. The selection of the appropriate medium will depend on the complexity and homogeneity of the matrices and the accompanying microbiota. This methodology is very simple and cost-effective. Furthermore, as only viable cells can grow, these procedures can be adapted to viability assays (Candrian, 1995; Cook, 2003). Indeed, Cook (2003) stated that the use of an enrichment step prior to NASBA detection may also be a safeguard against false positive results in a viability assay caused by residual RNA in dead cells. However, when enrichment is applied prior to PCR, the accuracy of this approach will depend on the background of DNA from dead cells in a sample.

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I.3.2 Amplification techniques

I.3.2.1 PCR

History

Kleppe *et al.* described the principles of polymerase chain reaction (PCR) for first time in 1971. But it was in 1985, with the introduction of a thermoestable DNA polymerase, that Saiki *et al.* published the first experimental data (Saiki *et al.*, 1985; 1988). The discovery of PCR is attributed to a collaborator of Saiki, Kary B. Mullis, laureate with the Nobel Prize in Chemistry in 1993. Since then, PCR has been used in more than 40,000 scientific publications (White, 1996). This technique has been applied in different areas due to its versatility, specificity and sensitivity. Accordingly, PCR has been successfully used for microorganism identification (Allmann *et al.*, 1995; Candrian, 1995; Olsen *et al.*, 1995), for the detection of constituents of food products *e.g.* cereals (Allmann *et al.*, 1993), vegetables (Jankiewicz *et al.*, 1997), and different animal (Zimmerman *et al.*, 1998b) and fish species (Hübner *et al.*, 1997).

The principle of PCR

PCR is a simple, versatile, sensitive, specific and reproducible assay (Saiki *et al.*, 1988; Cha *et al.*, 1993). PCR is an exponential amplification of a DNA fragment, and its principle is based on the mechanism of DNA replication *in vivo*: dsDNA is denatured to ssDNA, duplicated, and this process is repeated along the reaction according to the following formula (Higuchi *et al.*, 1993; Innis *et al.*, 1999).

$$C = C_0\ (1+E)^n$$

$$E = 10^{-1/s} - 1;\ \text{if } E = 1 \text{ then } s = -3.3219$$

where,
C: final amount of DNA
C_0: initial amount of DNA
E: efficiency
n: number of cycles
s: slope of the exponential phase.

During the denaturation step, the dsDNA melts opening up to ssDNA, and all enzymatic reactions stop (*i.e.* the extension from a previous cycle). To DNA denaturation, the temperature is usually raised to 93-96 ºC, breaking the H-bonds and thus increasing the number of non-paired bases. The temperature at which half of the dsDNA is single-stranded is known as the melting temperature, T_m. The type of solvent, the salt concentration and the pH influence the denaturation process. The concentration of G/C and T/A can also affect the T_m value. The DNA sequences rich in G/C have higher T_m values compared to those rich in T/A.

The second phase, *i.e.* annealing of primers to ssDNA, takes place at temperatures closer to their T_m (usually 55-65 ºC). The oligonucleotides typically consist of relatively short sequences (15-25 nt) and complementary to recognition sites flanking the segment of target DNA to be amplified. Once the temperature is reduced, the two complementary ssDNA chains tend to rehybridise into a dsDNA molecule. In this phase, ionic bonds are constantly formed and broken between the single-stranded primer and the single-stranded template. If primers adequately anneal to the template, the ionic bond is strong enough between the template and the primer to stabilise the nascent double stranded structure and allow the polymerase to attach and begin copying the template.

The extension phase is carried out across the target sequence by using a heat-stable DNA polymerase in the presence of dNTPs, resulting in a duplication of the starting target material. This enzyme has 5'→3' DNA polymerase activity, *i.e.* it adds dNTPs from 5´to 3', reading the template from 3' to 5'. When the primers have been extended a few bases, they posses a stronger ionic attraction to the template, which reduces the probability of unbinding. Primers that do not match exactly, come loose because of the high temperature and do not prime extension. The duration of the

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extension step can be increased if the region of DNA to be amplified is long (> 1000 bp); however, for the majority of PCR experiments less than 1 min is sufficient to get a complete extension.

After each cycle, the newly synthesised DNA strands can serve as template in the next cycle. As shown in Figure I.1, the major product of this exponential reaction is a segment of ds-DNA whose termini are defined by the 5' termini of the 2 primers and whose length is defined by the distance between the primers. The products of a successful first round of amplification are heterogeneously sized DNA molecules, whose lengths may exceed the distance between the binding sites of the two primers. In the second round, these molecules generate DNA strands of defined length that will accumulate in an exponential fashion in later rounds of amplification and will form the dominant products of the reaction.
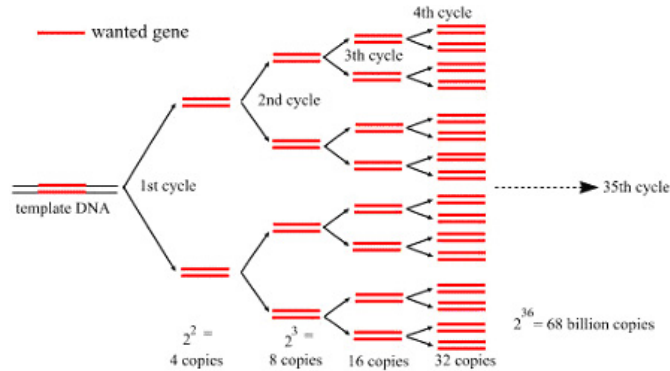
Figure I.1. The exponential amplification of DNA in PCR.

Thus, amplification, as a final number of copies of the target sequence, is expressed by the following equation:

$(2^n - 2n)$ x   where,   **n** is the number of cycles
2n is the first product obtained after the first cycle and second products obtained after the second cycle with undefined length.
x is the number of copies of the original template.

Potentially, after 20 cycles of PCR there will be around 2^{20}-fold amplification, assuming 100 % efficiency during each cycle. The efficiency of a PCR will vary from template to template and according to the degree of optimisation that has been carried out.

Multiplex PCR

Whereas standard PCR usually uses one pair of primers to amplify a specific sequence, multiplex PCR allows the simultaneous amplification of more than one target sequence in a single reaction (Elnifro *et al.*, 2000; Higuchi *et al.*, 1992; Wittwer *et al.*, 2001). This saves considerable time and effort, and decreases the number of reactions that need to be performed to detect the desired targets in the sample. The presence of many PCR primers in a single tube can cause some problems, such as the increased formation of misprimed PCR products, 'primer dimers', and the amplification discrimination of longer DNA fragments (Atlas and Bej, 1994; Higuchi *et al.*, 1992). For multiplex PCR, primers should be chosen with similar annealing temperatures. The lengths of amplified products should be similar; as large differences in the lengths of the target DNAs may favour the amplification of shorter targets over the longer ones, which results in differential yields of amplified products.

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I.3.2.1.1   Real-time PCR

The development of real-time (RTi-) PCR represents a significant advance in many molecular techniques involving nucleic acids analysis. RTi-PCR allows monitoring of the synthesis of new amplicon molecules during the PCR (*i.e.*, in real time). Data is therefore collected throughout the PCR process, not only at the end of the reaction (as occurs in conventional PCR). RTi-PCR is used for many different purposes, particularly for quantifying nucleic acids and for genotyping. Since the fist scientific work published in 1996 (Heid *et al.*, 1996), the number of publications where RTi-PCR is used has increased nearly exponentially (Figure I.2).
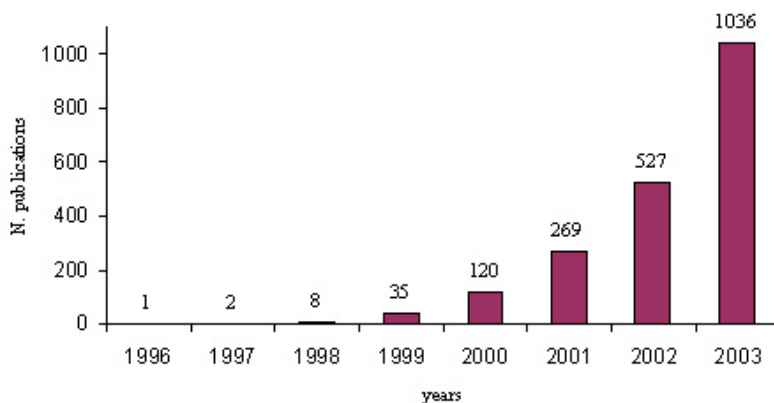
Figure I.2. Result of a PubMed search using 'real-time PCR', sorted by year of publication. Adapted from Wilhem and Pingoud, 2003.

The uniqueness of RTi-PCR is that the process of amplification is monitored in real time by using fluorescence (Heid *et al.*, 1996; Higuchi *et al.*, 1993; Nazarenko *et al.*, 1997). The results of RTi-PCR consist of amplification curves (Figure I.3), that can be used to quantify the initial amounts of template DNA molecules with high precision over a wide range of concentrations (Schmittgen *et al.*, 2000). Major advantages of RTi-PCR are the closed-tube format (that avoids risks of carryover contamination), fast and easy to perform analysis, the extremely wide dynamic range of quantification (more than eight orders of magnitude) (Heid *et al.*, 1996) and the significantly higher reliability of the results compared to conventional PCR.
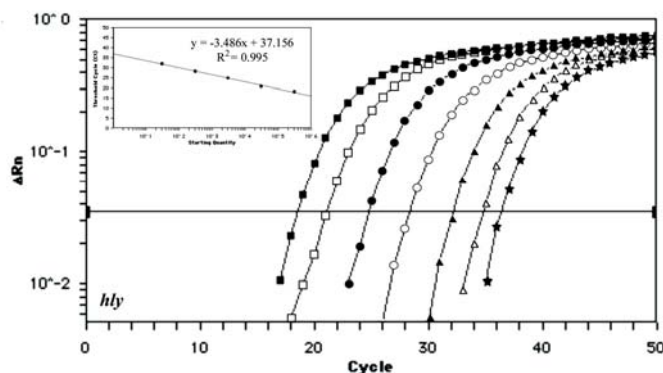
Figure I.3. Amplification curves in semi-logarithmic view obtained from serial dilutions of a target DNA. Inset, regression curve obtained from C_T values.

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Instrumentation

Commercial RTi-PCR instruments include GenAmp 5700, ABI Prism 7700, ABI Prism 7000, ABI Prism 7900 (Applied Biosystems), iCycler (BioRad), LightCycler (Roche Molecular BioChemicals), SmartCycler (Cepheid), MX3000, MX4000, (Stratagene), Rotor-Gene (Corbett Research), DNA Engine Opticon Continuous Fluorescence Detection System (MJ Research) and R.A.P.ID (Idaho Technology). The main characteristics of the real-time thermocyclers are showed in Table I.5.

Table I.5. Instrumentation for RTi-PCR*. Adapted from Cockerill and Uhl, 2002. Additional information was obtained from www.biocompare.com.

Company	Device	Sample formats	No. fluorophores	Excitation emission (nm)	Excitation source	Emission detection	Thermal cycling format
Applied Biosystems	GeneAmp 5700 SDS	Microplates, strips, tubes (96)	2	500-660	Tungsten lamp	CCD camera	Heating block
	ABI Prism 7700 SDS	Microplates, strips, tubes (96)	2	500-660	Laser	CCD camera	Heating block
	ABI Prism 7000 SDS	Microplates, strips, tubes (96)	2	500-660	Tungsten-halogen lamp	CCD camera	Peltier
	ABI Prism 7900 HT SDS	Microplates, strips, tubes, microfluidic cards (96-384)	2	500-660	Laser	CCD camera	Peltier
BioRad	iCycler	Microplates, strips, tubes (96)	4	400-700	Broad wave-length lamp with filters	CCD camera	Peltier and Joule
	MyiCycler	Microplates, strips, tubes (96)	1	400-585	Broad wave-length lamp with filters	CCD camera	Peltier and Joule
Roche Diagnosis	LightCycler	Capillaries (32/64)	2	530-710	blue LED light	Photodiode	Ambient air cooling
	LightCycler II	Capillaries (32)	6	530-710	blue LED light	Photodiode	Ambient air cooling
Cepheid	SmartCycler II	Single-use disposable tubes (16-96)	4	450-750	LED light	Pothodetector	Ceramic heating plate and forced air cooling
	TD SmartCycler	Single-use disposable tubes (16-96)	4	450-750	LED light	Pothodetector	Ceramic heating plate and forced air cooling
Stratagene	MX3000	Microplates, strips tubes (96)	4	350-750	Tungsten-halogen lamp	Scanning photomultiplier tube	solid-state heating and cooling
	MX4000	Microplates, strips tubes (96)	4	350-830	Tungsten-halogen lamp	Scanning photomultiplier tube	solid-state heating and cooling
Corbett Research	Rotor-Gene	Microtubes (36-72)	4	470-660	LED high power diodes with filters	Photodiode	Ambient air cooling
MJ Research	DNA Engine Opticon CFDS	Microplates, strips tubes (96)	2	450-545	blue LED Light	CCD camera	Peltier
	DNA Engine Opticon 2 CFDS	Microplates, strips tubes (96)	2	470-700	blue LED Light	CCD camera	Peltier
Idaho Technology	R.A.P.I.D.	glass/plastic reaction (32)	3	450-730	blue LED light r	Photodiode	fan & heating coil

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The ABI Prism 7700 Sequence Detection System (SDS) (Figure I.4a) was the first RTi-PCR instrument commercialized in 1997 and still is the best-selling. It is based on a conventional 96-well Kapton heat-block cycler with an additional fluoremeter device. The fluorescence induction source is an argon-laser light. Continuous fluorescence wavelength detection (Figure I.5) from 500 to 660 nm allows multiplex PCR by the use of multiple fluorophores in a single reaction. The instrument can be used for assays based on hydrolysis probes, hybridization probes, and dsDNA-binding dyes. However, although PCR

Figure I.5. Detection system of the ABI PRISM® 7700 SDS.

and fluorescence emission occur in real time, the data observation is not available to the user until the thermocycling program is completed. In contrast, for all the other systems (except GeneAmp 5700), real time observation of data is possible at each PCR cycle. The ABI GeneAmp 5700, also from Applied Biosystems, is a less expensive alternative but only allows single- wave-length detection. This system, like the 7700 SDS, can carry out RTi-PCR using hydrolysis probes, hybridization probes or the DNA-binding dye SYBR Green I. The 5700 SDS differs from the 7700 SDS in its use of a halogen lamp instead of a laser. Recently, Applied Biosystems has launched the ABI Prism 7900 HT SDS (Figure I.4b), which has the same specifications as the 7700 SDS but with a Peltier system to heat/cool, and is designed especially for very high throughput applications. The process is completely automated: it is able to prepare the plates that will load the sequence detector and it has the ability to handle microtiter plates ranging in size from 96 to 384 wells and microfluidic cards. The system also offers an optional automation accessory capable of loading up to eighty-four 384-well plates into the instrument for a 24-h, hands-free operation.

Only a little later than SDS 7700, Roche Diagnostics started distributing the LightCycler (Figure I.4c) based on an entirely different instrument design, which was originally developed by Idaho Technologies (Wittwer, *et al* 1989; Wittwer, *et al* 1997). The LightCycler system was the first RTi-PCR device applied for diagnosis of infectious agents (Cockerill and Uhl, 2001). Reactions are performed in borosilicate glass capillaries which can hold up to 20-µl of sample and a rapid heat exchange as compared with the reaction tubes used in other RTi-PCR instruments. A whole PCR run with 40 cycles can be performed within only 20-30 min (Wittwer, *et al* 1997). The capillaries are placed in a sample rotating carrousel, within a thermostatically controlled chamber. The combination of a small sample volume, the cylindrical shape of the capillary tubes, and temperature adjustment with air allows very steep temperature gradients. Thus, short cycle times can be used, which increases the specificity of the reaction. Additionally, in contrast to all other instruments available, the LightCycler instrument uses a robust blue light-emitting diode for excitation instead of a delicate laser and is read by three silicon photodiodes with different-wavelength filters, allowing detection of spectrally distinct fluorophores and thus multiplex RTi-PCR. This system can be used with hydrolysis probes, hybridization probes and dsDNA-binding dyes. Advantages of the LightCycler, compared with the 7700 SDS are its lower price, the ability to view the data while PCR amplification is still in progress, and the high throughput, since one run can be completed in 20–30 min. Disadvantages are, however, the use of capillaries as opposed to tubes, their use being less practical. Moreover, the small sample format, allowing only 32 wells to be analyzed simultaneously, is a disadvantage. This is especially important when performing quantification studies, where both standards and samples have to be compared within a single experiment.

BioRad offers the iCycler (Figure I.4d), which has a cost-effective fluoremeter device connected to the PCR thermocycler, measuring fluorescence emission during the PCR. The iCycler has a tungsten lamp as a fluorescence excitation source, and a set of filters. The fluorescence emission can be viewed during the course of PCR amplification and it is able to multiplex four different fluorophores per sample tube. Moreover, the 96 samples are tracked simultaneously, thereby providing a very fast assay.

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RTi-PCR systems are now available from many companies, such as Cepheid (SmartCycler), Stratagene (MX4000), Corbett Research (Rotor-Gene 3000), MJ Research (DNA Engine Opticon Continuous Fluorescence Detection System) and Idaho Technology (R.AP.I.D.). The Smart Cycler System (Cepheid, Sunnyvale, CA) (Figure I.4e) can be operated with hydrolysis probes, hybridization probes and dsDNA-binding dyes. An advantage of this system is its high flexibility, as it contains 16 different modules. Each module can be individually programmed and has its own optical subsystem, being able to detect four different fluorophores in one reaction. Different operators can define the parameters for each reaction and different runs can be carried out at the same time for individual experiments. With the software it is also possible to view the data as soon as they are collected. A disadvantage of this system, especially accounting for quantification analysis, is the small sample number. The MX4000 Multiplex (Stratagene, La Jolla, CA) (Figure I.4f) uses a quartz tungsten halogen lamp as light source that generates a broad excitation range of 350 to 750 nm, allowing the performance of multiplex PCR. This system can be used with hydrolysis probes and hybridization probes. The design of the sample block accommodates samples in a variety of formats (96-well plates, 8-strip tubes, or individual tubes). The system generates real-time amplification plots that can be viewed as the PCR run progresses. The Rotor-Gene (Corbett Research, Mortlake, Sydney, Australia) (Figure I.4g) is a 'centrifugal' thermal cycler comparable to the LightCycler. It uses two light sources, a blue one that emits fluorescence at 470 nm and a green one at 530 nm. A wide variety of fluorophores can be detected. Hydrolysis probes, hybridisation probes as well as intercalating dsDNA dye such as SYBR Green I can be used. Furthermore, the Rotor-Gene 3000 contains a 36- or 72-well rotor for 0.2-ml propylene PCR tubes that spin at 500 rpm, guaranteeing the temperature homogeneity in the samples carousel.

Figure I.4. Instrumentation for RTi-PCR. **A** ABI Prism 7700 SDS (Applied Biosystems) **B** ABI Prism 7900 HT SDS (Applied Biosystems) **C** LightCycler (Roche BioChemicals) **D** iCycler (BioRad) **E** SmartCycler (Cepheid) **F** Mx4000 (Stratagene) **G** Rotor-Gene (Corbett Research) **H** DNA Engine Opticon CFSD (MJ Research) **I** R.A.P.ID. (Idaho Technology).

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Detection Formats

Fluorescence signals that are proportional to the amount of PCR product can be generated by a **unspecific detection strategy** independent of the target sequence, *e.g.* through fluorescent dyes that have special fluorescent properties when bound to dsDNA, or by sequence-specific fluorescent oligonucleotide probes; *i.e.* a **sequence-specific strategy**.

Unspecific detection formats

Fluorescent dsDNA- specific dyes

The first dye used for this purpose was ethidium bromide (Higuchi *et al.*, 1993; Le Pecq and Paoletti, 1966; Wittwer *et al.*, 1997). Other intercalating dyes such as YO-PRO-1 have since been used (Ishiguro *et al.*, 1995; Tseng *et al.*, 1997).

SYBR Green I is the most frequently used dsDNA-specific dye in RTi-PCR (Figure I.6). The binding affinity is more than 100 times higher than that of ethidium bromide. It is an asymmetric cyanine dye, structurally related to the dsDNA-specific dyes YOYO-1 and TOTO-1 (Jin, *et al.*, 1994; Singer *et al.*, 1994). In contrast to ethidium bromide, intercalation of cyanine dyes is negligible under the assay conditions of RTi-PCR experiments. Instead, SYBR Green I largely binds sequence independently to the minor groove of ds-DNA. SYBR Green I can be excited with blue light with a wavelength of 480

Figure I.6. SYBR Green I bound to dsDNA.

nm, and its emission spectrum is comparable to that of fluorescein with a maximum at 520 nm and a quantum yield of 0.8 (Jin, *et al.*, 1994). The fluorescence of the bound dye is above 1000-fold higher than that of the free dye and, therefore, is well suited for monitoring the product accumulation during PCR (Wittwer *et al.*, 1997; Morrison *et al.*, 1998).

When monitored in real time, this results in an increase in the fluorescence signal that can be observed during the polymerisation step, and that falls off when the DNA is denatured. Consequently, fluorescence measurements have to be performed at the end of the elongation step of every PCR cycle. This method obviates the need for target-specific fluorescent probes, and hence it can be used with any pair of primers for any target, making its use less expensive (Giulietti *et al.*, 2001). However, its major disadvantage is that specificity is determined entirely by the primers and thus the risk of amplifying non-specific PCR products has to be considered during optimization (Simpson *et al.,* 2000). However, PCR product verification can be achieved at the end-point by plotting fluorescence as a function of temperature to generate a melting curve of the amplicon (Ririe *et al.*, 1997).

A new minor groove-binding asymmetric cyanine reporter dye (BEBO) is now available. BEBO and SYBR Green I are reported to be similar in all important aspects of their behaviour, such as specificity, PCR inhibition and quantum efficiency (Bengtsson *et al.*, 2003).

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Sunrise primers (AmpliFluor™ system)

The AmpliFluor™ system is an unspecific detection system developed by Intergen co. AmpliFluor™ technology uses a universal energy-transfer hairpin primer (UniPrimer™) which emits a fluorescent signal when unfolded during its incorporation into an amplification product. The UniPrimer™ contains a 18-nucleotides sequence (Z sequence: 5´-act gaa cct gac cgt aca-3´) at its 3' end, that is also present at the 5' end of one of the target-specific primers so that it anneals to the PCR product and acts as universal PCR primer. In the first step, the forward primer is extended. This extended product serves as template for the reverse primer in the second step. In the end, the polymerase opens the hairpin structure and a double-stranded PCR product is formed in which reporter and quencher are separated (Nazarenko *et al.*, 1997) (Figure I.7).
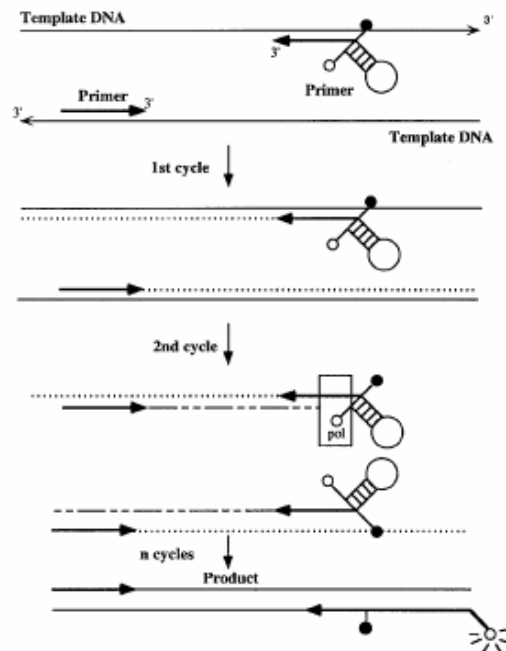
Figure I.7. Detection system based on AmpliFluor™. Adapted from Whitcombe *et al.* (1999).

Sequence-specific fluorescent oligonucleotide probes

There are different types of specific-sequence fluorescent probes, and they can be classified into two major groups: **hydrolysis probes** and **hybridization probes**, both types being homologous to the internal region amplified by the two primers. The fluorescence signal intensity can be related to the amount of PCR product (i) by a product-dependent decrease of the quench of a reporter fluorophore or (ii) by an increase of the fluorescence resonance energy transfer (FRET) from a donor to an acceptor fluorophore. FRET, also called Förster transfer, is the radiationless transfer of excitation energy by dipole-dipole interaction between fluorophores with overlapping emission and excitation spectra. The FRET and the quench efficiency are strongly dependent on the distance between the fluorophores (Förster, 1948; Clegg, 1992). Therefore, the PCR-product-dependent change in the distance between the fluorophores is used to generate the sequence-specific signals. Several different formats can be used. In principle, all of them could function by a decrease of quench or an increase of FRET; in practice, most formats are based on a decrease of quench. The most commonly used fluorescent reporter dyes are FAM, TET (tetrachloro-6-car-boxyfluorescein), JOE (2,7-dimethoxy-4,5-dichloro-6-carboxy-fluorescein), HEX (hexacholoro-6-carboxyfluorescein),

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or VIC®, and the most frequently used quenchers are TAMRA, DABCYL and the recently developed Black Hole Quencher (BHQ).

Sequence-specific probes allow multiplexing (Bernard *et al.*, 1998; Dupont *et al.*, 2002; Wittwer *et al.*, 2001) and easy identification of point mutations (Giesendorf *et al.*, 1998; Lay and Wittwer, 1997; Lyon, 2001). A common drawback of probe systems that use the decrease-of-quench mechanism is unwanted generation of a signal due to probe destruction (*e.g.* by unintentional hydrolysis of the probes by the *Taq* DNA polymerase) or by formation of secondary structures of the probes that lead to a decrease in quench (Wilhem and Pingoud, 2003).

Hydrolysis probes

The hydrolysis probes are cleaved by the 5'-3' exonuclease activity of several DNA polymerases (Holland *et al.*, 1991) during the elongation phase of primers, yielding a real time measurable fluorescence emission directly proportional to the concentration of the target sequence. The best known hydrolysis probes are TaqMan® probes and TaqMan® MGB (minor groove binder) probes, both developed by Applied Biosystems.

TaqMan® probes

A TaqMan® probe is an oligonucleotide double-labelled with a reporter fluorophore at the 5' end (reporter dye) and with a quencher internally or at the 3' end (quencher dye). In addition, the probes must be blocked at their 3'- end to prevent the extension during the annealing step. The TaqMan® chemistry exploits the 5'-3' exonuclease activity (Holland *et al.*, 1991) of particular DNA polymerases to produce a fluorescence signal as a result of the hydrolysis of a fluorescently labelled template-specific oligonucleotide included in the PCR (Heid *et al.*, 1996). It usually utilises either *Taq* or *Tth* polymerase, but any enzyme with equivalent 5'-3' exonuclease activity properties (*e.g. Tfl*) can be used (Gut *et al.* 1999).

The TaqMan® assay (Figure I.8) uses three oligonucleotides. Two template-specific primers (forward and reverse) allow amplification of the product, to which a third dual-labeled fluorogenic oligonucleotide, the TaqMan® probe, will anneal. The quencher dye absorbs the fluorescence of the reporter dye due to its proximity, which permits FRET. When the correct amplicon is amplified, the probe can hybridise to the target after the denaturation step. It remains hybridised while the polymerase extends the primers until it reaches the probe. Then, it displaces its 5' end to hold it in a forked structure. The enzyme continues to move from the now free end to the bifurcation of the duplex, where cleavage takes place (Lyamichev *et al.* 1993). The quencher is hence released from the fluorophore, which now fluoresces after excitation (Heid *et al.*, 1996; Holland *et al.*, 1991; Gibson *et al.*, 1996). The signal generated is proportional to the amount of specific amplicon produced during the PCR and is detected by a sensitive charge-coupled device (CCD), which allows the earliest possible differentiation between the baseline and the true signal.

Polymerisation and 5' Nuclease activity
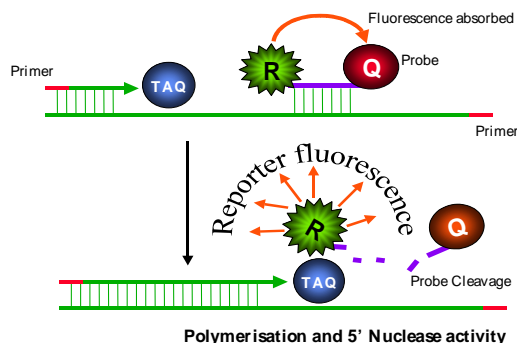
Figure I.8. 5'-3' polymerase and exonuclease activity of the *Taq* DNA polymerase (TAQ). R: reporter and Q: quencher.

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As the polymerase will cleave the probe only while it remains hybridised to its complementary strand, the temperature conditions of the polymerisation phase of the PCR must be adjusted to ensure probe binding. Most probes have a T_m of around 70 ºC; therefore, the TaqMan® system uses a combined annealing and polymerisation step at 60-63 ºC. This ensures that the probe remains bound to its target during the primer extension step. It also ensures maximum 5'–3' exonuclease activity of the *Taq* and *Tth* DNA polymerases (Tombline *et al.* 1996).

TaqMan® MGB probes

The TaqMan® MGB probes are similar to TaqMan® probes. They contain a <u>n</u>on-<u>f</u>luorescent <u>q</u>uencher (NFQ) and an oligopeptide at the 3' end. This oligopeptide is a DNA <u>min</u>or groove <u>b</u>inder (MGB), with very high affinity for the minor groove of A–T-rich double-stranded DNA (Afonina *et al.*, 1997). Addition of the MGB ligand significantly enhances duplex stability. The shorter the probe, the greater the MGB contribution to the overall duplex stability: with 12-18-bp oligonucleotides, it raises the T_m from 44-56ºC up to 66-70ºC (Kutyavin *et al.*, 2000). This allows designing suitable probes in sequences such as those rich in A-T, in which conventional TaqMan® probes require an excessive length.

Hybridization probes

In contrast to hydrolysis probes, hybridization probes are not hydrolyzed during PCR. The fluorescence is generated by a change in its secondary structure during the hybridization phase, which results in an increase of the distance separating the reporter and the quencher dyes. The most relevant hybridization probes are those containing hairpins (Molecular Beacons, Scorpion primers, etc), and FRET hybridization probes.

Molecular beacons

Molecular beacons form a stem-and-loop structure through complementary sequences on the 5' and 3' ends of the probe (Tyagi and Kramer 1996). The loop portion is complementary to the target nucleic acid (Tan *et al.*, 2000). A reporter and a quencher fluorophore are attached one at the end of each arm. The quencher is a non-fluorescent chromophore that dissipates the energy it receives from the fluorophore as heat. The fluorescence is quenched when the probe is in a hairpin-like structure (stem-and-loop structure) due to the proximity between quencher and fluorophore allowing FRET (Cardullo *et al.*, 1998; Stryer, 1978). In the presence of a complementary sequence, designed internal to the primer binding sites, the probe undergoes a conformational transition that forces the stem apart and results in the formation of a probe/target hybrid that is more stable than the former stem (Bonnet *et al.*, 1999). This conformational change separates the fluorophore from the quencher and consequently FRET no longer occurs, thus increasing reporter fluorescence emission (Kostrikis *et al.*, 1998; Tyagi *et al.*, 1998) (Figure I.9).
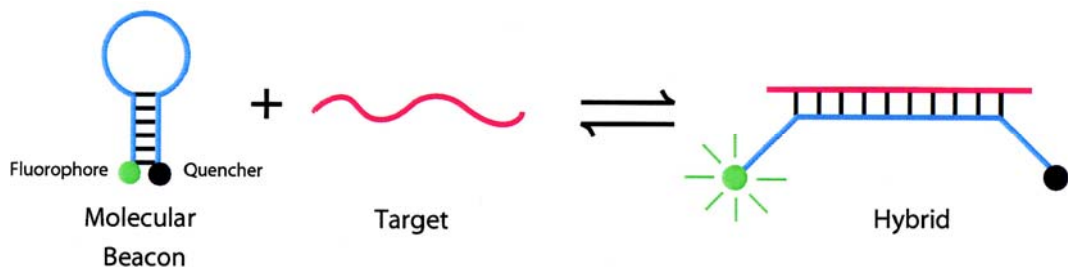
Figure I.9. Principle of Molecular Beacons. In the non-hybridized stage, the stem-loop structure of the probe brings fluorophore and quencher moieties into close proximity, such that the fluorophore is quenched and the probe does not fluoresce. When molecular beacons hybridize to target DNA sequences, the fluorophore and quencher components become spatially separated and the probe fluoresces (adapted from Tyagi and Kramer, 1996).

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Molecular Beacons have been reported to be significantly more specific than conventional oligonucleotide probes of equivalent length, due to the presence of a stem structure (Bonnet *et al.*, 1999; Kaboev *et al.*, 2000; Tyagi *et al.*, 1998). The main drawback of Molecular Beacons is associated with its design as the fluorescence yield is very sensitive to the hybridisation conditions. Thus, optimal design is crucial to avoid the Molecular Beacon folding into alternate conformations that would place the reporter and the quencher at different distances, resulting in large background signals. Alternatively, a too strong stem would interfere with the target hybridisation and affect the fluorescence yield.

Molecular Beacons have been used for many RTi-PCR based applications (Wilhelm and Pingoud, 2003). They can also be used in non-PCR amplification assays such as strand-displacement amplification (Walker *et al.*, 1992), nucleic acid sequence-based amplification (Leone *et al.*, 1998) or rolling-circle amplification (Lizardi *et al.*, 1998); and for the construction of self-reporting oligonucleotide arrays (Steemers *et al.*, 2000). Examples of RTi-PCR using Molecular Beacons include the detection and quantification of diverse species, in particular pathogens (*e.g.* Chen *et al.*, 2000; Fortin *et al.*, 2001; Piatek *et al.*, 1998; Vet *et al.*, 1999), the identification of single nucleotide variations in DNA (Durand *et al.*, 2000; Giesendorf *et al.*, 1998; Kostrikis *et al.*, 1998; Marras *et al.*, 1999; Smit *et al.*, 2001), or the detection of RNA molecules in living cells (Matsuo, 1998; Sokol *et al.*, 1998).

FRET Probes or Hybridization probes (Idaho Technology)

These use four oligonucleotides *i.e.* two primers and two sequence-specific probes to maximise specificity (Wittwer *et al.*, 1997). Each probe has a single label: either a donor fluorophore at the 3'-end or an acceptor fluorophore at the 5'-end. The emission spectrum of the donor fluorophore overlaps the excitation spectrum of the acceptor fluorophore. As TaqMan® probes, the FRET probes must be blocked at their 3'- end to prevent the extension during the annealing step. The two probes hybridize to the target sequences in a head-to-tail arrangement, thus bringing the two dyes close (typically 1–5 nucleotides distant), allowing FRET.

During PCR, only the donor fluorophore is excited. In solution, only background fluorescence is emitted by the donor. During annealing, the two probes hybridise adjacently to their target sequence and thus the excitation energy is transferred by FRET from the donor dye in one of the probes to the acceptor dye in the other probe (Figure I.10), allowing the acceptor dye to dissipate fluorescence at a different wavelength. This fluorescence emission is proportional to amplicon generation (Bernard and Wittwer, 2000). The use of two independent probes results in high specificity and flexibility for probe design. Furthermore, as the probes are not hydrolysed, fluorescence is reversible and allows the generation of melting curves.
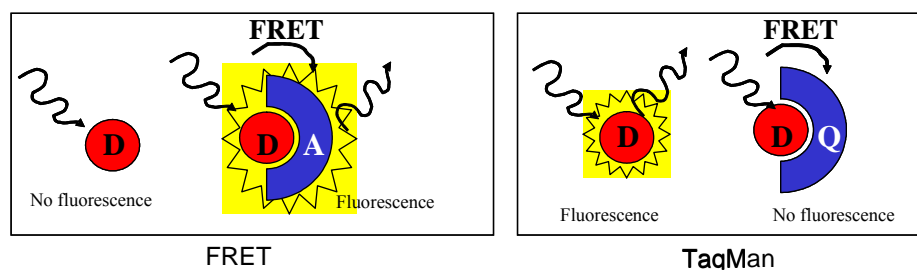
Figure I.10. A) Energy transfer in FRET probes (the e⁻ acceptor emits fluorescence when placed close to the e⁻ donor. B) Energy transfer in hydrolysis probes (TaqMan®) (the e⁻ donor emits fluorescence when the distance to the acceptor or quencher is higher than 10 nm. A: acceptor; D: donor and Q: quencher.

The FRET probe system is used mostly for LightCycler experiments. The LightCycler device has special filters for FRET use and Roche has developed fluorophores (RED-640 and RED-705) with corresponding emission characteristics (Wittwer *et al.*, 1997). Nevertheless, fluorophores with similar spectroscopic properties like Cy5 have been used (Connolly and Patel, 2002). Examples of its

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use are mutation detection (Bernard *et al.*, 1998; Cane *et al.*, 1999; Neoh *et al.*, 1999), pathogen detection (Koo and Jaykus, 2003), viral load quantification (Schalasta *et al.*, 2000), and detection of minimal disease after therapy (Emig *et al.*, 1999).

Scorpion primers

Scorpion primers are structurally and functionally related to molecular beacons, but serve as primers in the PCR reaction. They consist of a probe sequence linked to the 5' end of a primer via a non-amplifiable stopper moiety (Whitcombe *et al.*, 1999). The probe presents a fluorophore linked at the 5'-end and a quencher at the 3'-end, and is held in a hairpin loop structure by complementary sequences on its 5'- and 3'- ends. This configuration brings the fluorophore in close proximity with the quencher and avoids fluorescence (similar to Molecular Beacons). In addition, the probe sequence is complementary to an internal region of the sequence extended by the adjacent primer. The polarity is such that the 5'-end of the probe is complementary to the 3'-end of the target sequence. In the first step, the primer is extended, yielding a single-stranded template for the reverse primer in the second step. Upon hybridization, the hairpin is opened, producing a physical separation of the fluorophore and quencher such that increases in signal are observed (Figure I.11). In contrast to the sunrise primers, the reverse extension is blocked by a hexethylene glycol group. This ensures that the reporter of the scorpion primer remains quenched in unspecific products like primer dimers (Whitcombe *et al.*, 1998; Whitcombe *et al.*, 1999).
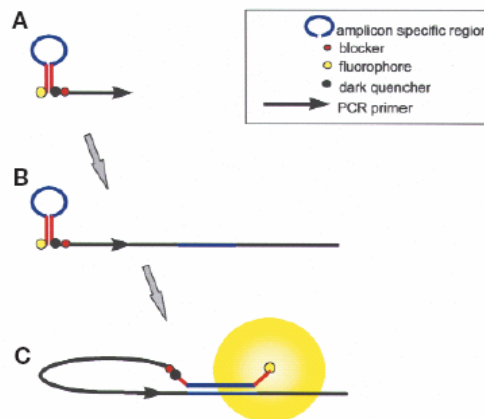
Figure I.11. Detection of PCR products with Scorpion primers. (A) A Scorpion primer carries a 5´ extension comprising a probe element, a pair of self-complementary stem sequences, and a fluorophore/quencher pair. The extension is 'protected' from copying by the inclusion of a blocking HEG monomer. (B) After a round of PCR extension from the primer, a newly synthesized target region is now attached to the same strand as the probe. (C) Following a second round of denaturation and annealing, the probe and target hybridize. Adapted from Whitcombe *et al.*, 1999.

Light-up probes

In contrast to the probe assays described above, the light-up technology utilizes a nucleic acid analogue instead of natural DNA as sequence recognizing element (Isacsson *et al.*, 2000). Light-up probes are peptide nucleic acids (PNAs) (Nielsen *et al.*, 1991) that use thiazole orange, a derivative of the asymmetric cyanine dye, as reporter fluorophore (Svanvik *et al.*, 2000a). PNA forms sequence-specific complexes with DNA and RNA which are more stable than double-stranded natural nucleic acids (Iyer *et al.*, 1995; Egholm *et al.*, 1993). These features are attributed mainly to the charge-neutral nature of PNA, which eliminates the electrostatic repulsion between the hybridizing strands. The probe has low fluorescence when free in solution (due to the intrinsic properties of the reporter dye), however they show increased fluorescence intensity upon hybridisation with DNA (Figure I.12).

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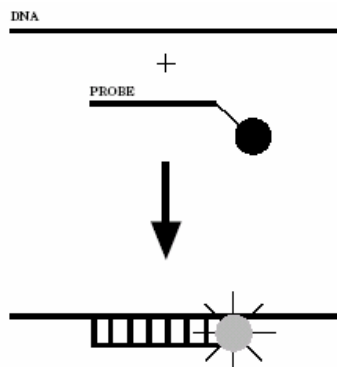
Figure I.12. Schematic diagram of the principle of detection using light-up probes.

Light-up is highly specific since a mismatch within the target sequence results in a significant decrease in thermal stability (*i.e.* greater than for dsDNA) (Egholm *et al.*, 1993). As a consequence, PNAs more efficiently discriminate mismatches than DNA oligonucleotides. An additional advantage of PNAs is that they are not a substrate for nucleases, proteases or peptidases. However, this technique is limited by the unspecific fluorescence that increases during PCR and therefore restricts the achievable sensitivity (Isacsson *et al.*, 2000; Svanvik *et al.*, 2000a; Svanvik *et al.*, 2000b).

RTi-PCR design

The following requisites (Table I.6) have been extensively studied on the TaqMan® system. However, most of them apply to other RTi-PCR systems.

Table I.6. Requisites for the design of oligonucleotides for RTi-PCR assays.

Parameter	Primers	Probes
G-C content (%)	30-80 %	30-80 %
Calculated T_m	50-60 ºC, always >55 ºC as UNG works at 50 ºC. T_m of the primers should not differ >2 ºC	68-70 ºC (5-10ºC above primer T_m)
Runs of identical nucleotides	Maximum 3 (no Gs!!)	Maximum 3 (no Gs!!)
Sequence length	Minimum 15 bp (15-30 bp)	Maximum. 30 bp
Amplicon length	The shorter the better. With TaqMan probes, 50-150 bp	
Distance forward primer to probe	Maximum 50 bp	
Primer dimers, hairpin loops	Avoid	Avoid
3'- instability (3'- rule)	Primers only. Maximum 2 Gs/Cs in the 5 last bp	
Autoquenching		No G at 5'-end
G-C ratio		Select the strand with C>G

When an RTi-PCR assay is designed, the most important parameters are the amplicon length and the T_m of the primers and probe. The optimal RTi-PCR amplicon length should be less than 150 bp, but it is advisable to reduce the length below 80 bp. However, amplicons up to 300 bp amplify efficiently. Shorter amplicons amplify more efficiently than longer ones and are more tolerant to suboptimal reaction conditions. This is because they are more likely to be denatured during the 92-95 ºC PCR step, allowing the probes and primers to compete more effectively for binding to their complementary targets. As the extension rate of *Taq* polymerase is between 30 and 70 bases per second (Jeffreys *et al.*, 1988), polymerisation times as short as 5 s are sufficient to replicate such amplicon, making amplification of artefacts less likely and reducing the time of the assay. Primers are generally used in the 50-300 nM range. Higher concentrations may promote mispriming and accumulation of non-specific products, and lower concentrations may lead to primer exhaustion, although target copy numbers will have been calculated well before. Non-specific priming can be minimised by selecting primers that have only one or two G/Cs within the 3' last five nucleotides. A

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relative instability at the 3' ends makes primers less likely to hybridise transiently causing non-specific extension.

The T_m of the probe is also a critical parameter. Since amplification primers are extended as soon as they bind to their targets, the hybridisation target sequence is rapidly masked with newly synthesised DNA. Therefore, the T_m of the probes must be significantly greater (approximately 10 °C) than that of the primers. The presence of G at the 5' end of the probe is to be avoided, because it slightly quenches the reporter signal, even after probe cleavage. Furthermore, the probe should contain more C's than G's; if this is not the case, the antisense probe should be used. The probe should never overlap with, or be complementary to either of the primers. The optimum concentration of fluorogenic probes will vary with the type of probe, as it depends on background fluorescence: quenching of hydrolysis probes is often below 100 %, and thus they produce background fluorescence levels higher than Molecular Beacons and FRET probes.

An additional requirement of the FRET system is that the target sequence should be located towards the 3' end of the amplicon, close to the reverse primer-binding site on the opposite strand. As fluorescence is measured during the annealing phase, this arrangement allows the RTi-PCR device to measure fluorescence before the polymerase displaces the probes. Paired probes used for quantification should have equal T_m, whereas for mutational analysis the T_m of the sensor probe should be lower than that of the anchor probe, thus ensuring that the sensor probe controls the generation of the fluorescent signal.

Quantification analysis

For hydrolysis and hybridization probes, the fluorescence emission is proportional to the synthesized DNA. Table I.7 and Figure I.13 show the most frequently used fluorophores and their excitation and emission wavelengths.

Table I.7. Excitation and emission spectre of different fluorophores.

Fluorophores	Excitation (nm)	Emission (nm)
DABCYL	453	-
6-FAM	492	515
SYBR® Green I	497	520
TET	521	536
JOE	527	548
HEX	535	556
TAMRA	555	580
ROX	575	602
VIC®	538	554
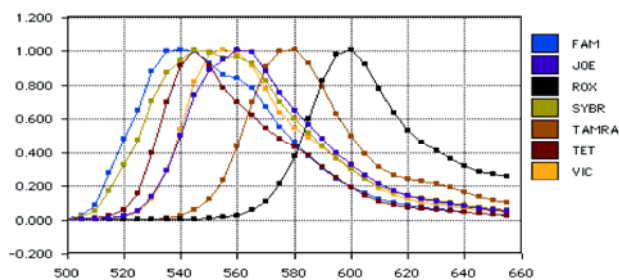
Figure I.13. Emission spectrum of fluorophores.

The fluorescence is measured by the RTi-PCR device and can be visualized as an amplification plot (Figure I.14). Typically, an amplification curve presents three different phases (Figure I.15). The first is called the *initiation phase*, it occurs during the first PCR cycles where the emitted fluorescence can not be distinguished from the baseline. During the *exponential* or *log phase* there is an exponential increase in fluorescence, before the plateau phase is reached. In this last phase, the reagents are exhausted, and no increase in fluorescence is observed. Only in the exponential phase, quantification is possible.

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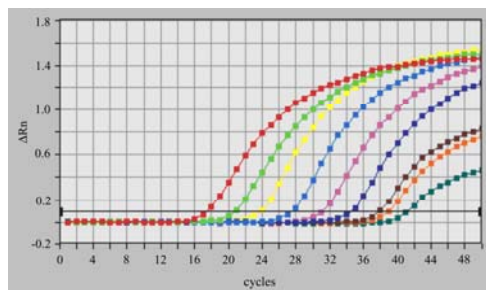
Figure I.14. RTi-PCR amplification curves (linear representation). *x-axis:* RTi-PCR cycles; *y-axis*: normalised fluorescence. Amplification curves correspond to 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10, 4 and 1 copies of a plasmid, respectively. Black line: the threshold.
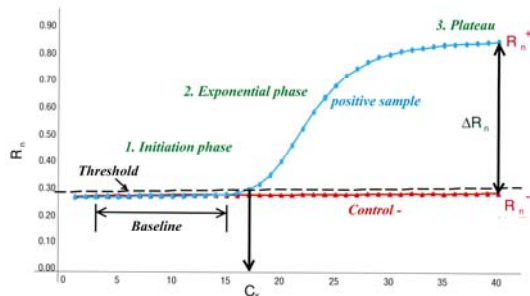
Figure I.15. Phases of a PCR amplification curve. Blue: amplification curve of a positive sample. Red: negative control. Rn, ΔRn and C$_T$; see the text.

A fluorophore (so-called passive reference) may be present in the PCR buffer in order to well-to-well normalize the fluorescence. This helps to overcome the effects of pipetting errors. This normalization is accomplished by dividing the emission intensity of the reporter dye by the emission intensity of the passive reference to obtain a ratio, *i.e.* **Rn** (normalized reporter) for any given reaction. At any given cycle, **Rn$^+$** is the Rn value of a reaction containing all components including the template and **Rn$^-$** is the Rn value of an unreacted sample obtained from the early cycles of the PCR, *i.e.*, those prior to a detectable increase in fluorescence, or from a reaction not containing template. The **ΔRn** is the difference between the Rn at the end point (Rn$^+$) and at the starting point (Rn$^-$). It reliably indicates the magnitude of the signal generated by the given set of PCR conditions, and is proportional to the DNA amount during the exponential phase. The fluorescence emitted in the first cycles is used to calculate the **baseline**. A threshold is established at the fluorescence value of the average standard deviation of Rn for the baseline cycles, multiplied by an adjustable factor (usually ten times). Alternatively, it can be established by the operator in order to compare different RTi-PCR experiments. The **threshold cycle** or **C$_T$** value is the cycle at which fluorescence achieves a defined threshold. It corresponds to the cycle at which a statistically significant increase in ΔRn is first detected. The concept of the threshold cycle is at the heart of accurate and reproducible quantification using RTi-PCR (Higuchi *et al.*, 1993). The higher the template copy number, the shorter the time to reach C$_T$ (Gibson *et al.*, 1996). Therefore, the number of cycles needed for the amplification-associated fluorescence to reach a specific threshold level of detection (the C$_T$ value) is inversely correlated to the amount of nucleic acid that was in the original sample (Walker, 2002). C$_T$ always is in the exponential phase of amplification, when amplification is most efficient, and therefore quantification is least affected by reaction-limiting conditions.

The quantity of DNA at the start of the PCR can then be determined by interpolation of the resulting C$_T$ value in a linear **standard curve** of C$_T$ values obtained from serially diluted known-amount standards (Figure I.16). This standard curve correlates the emitted fluorescence (C$_T$ value) with the initial concentration of the standards used and the final result is achieved by interpolation of the produced fluorescence (C$_T$ value) during the amplification of the sample in this standard curve. In practice, such curves are linear over more than five orders of magnitude.

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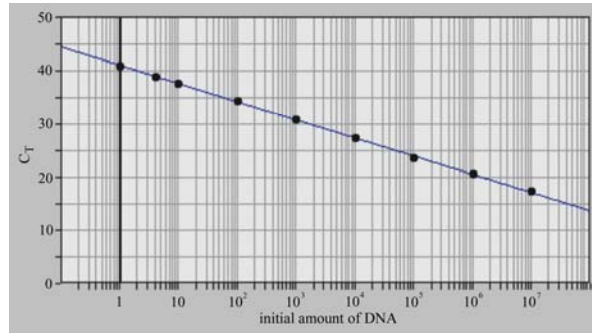
Figure I.16. Standard curve built with DNA standards of known concentration (Figure I.14). *x-axis*: initial amount of DNA (number of copies, ng, etc.); *y-axis*: C_T values. In this case, a dynamic range of 8 logarithmic units is shown. The slope of the curve is -3.41 and the regression coefficient or R^2 is 1.

I.3.2.2 Nucleic Acid Sequence based Amplification (NASBA)

The nucleic acid sequence-based amplification (NASBA) technique was first described by J. Compton in 1991, as a primer-dependent technology that can be used for the continuous amplification of nucleic acids, in a single mixture, at isothermal conditions (Compton, 1991). It is a sensitive transcription-based amplification (TAS) system specifically designed for the detection of RNA (Deiman *et al.*, 2002).

For the amplification process, NASBA employs three different enzymes: T7 RNA polymerase, RNase H and avian myeloblastosis virus (AMV) reverse transcriptase, which act in concert to amplify sequences from an original single-stranded RNA template. (Blais *et al.*, 1997; Compton, 1991; Heim *et al.*, 1998; Jean *et al.*, 2001; Kievits *et al.*, 1991; Mahony *et al.*, 2001; Simpkins *et al.*, 2000; Uyttendaele *et al.*, 1995a; Cook, 2003). The reaction also includes two oligonucleotide primers, complementary to the RNA region of interest, deoxyribunucleoside triphosphates for the activity of the AMV reverse transcriptase, and ribonucleoside triphosphates for the activity of the T7 RNA polymerase (van de Strijp and van Aarle, 1995). One of the primers, primer 1, also contains a promoter sequence that is recognized by T7 RNA polymerase at the 5'-end. The reaction is performed at a single temperature, normally 41 °C for 1 to 2 h in a self-sustained manner. At this temperature, the genomic DNA from the target remains double-stranded and does not become a substrate for amplification, and specificity values close to 100 % can be attained. The predominant product of the reaction is an RNA molecule of defined length limited by primer sequences complementary to the target.

Amplification process

Although NASBA is a continuous reaction, in order to simplify the description of the amplification process, it can be considered that the NASBA reaction is divided into two different phases (Compton, 1991; Rodríguez-Lázaro and Cook, 2003). In the first, so-called 'non-cyclic' phase, a double-stranded DNA product with a recognition sequence for T7 RNA polymerase is obtained. This product will be used as template in the second, 'cyclic' phase. In this second phase, RNA will be exponentially produced. In Figure I.17, a flow-chart that summarizes the reaction is shown.

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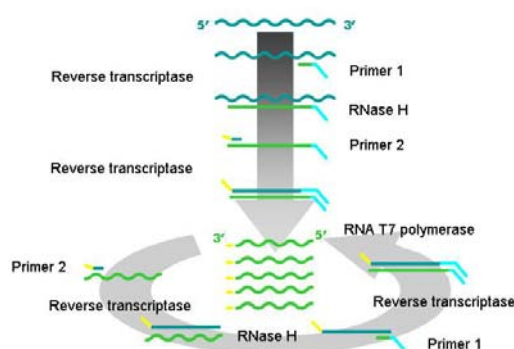
Figure I.17. NASBA reaction.

In the 'non-cyclic' phase, the primer containing the T7 RNA polymerase promoter sequence (primer 1) anneals to the RNA target sequence. AMV reverse transcriptase uses the deoxynucleoside triphosphates to extend the 3' end of primer 1, thereby forming a cDNA copy of the template and resulting in a RNA:DNA hybrid. Subsequently, RNase H hydrolyses RNA from the RNA:DNA hybrids. Consequently, the original RNA is destroyed, leaving a single strand of DNA to which primer 2 anneals. AMV reverse transcriptase synthesizes the second DNA strand, rendering the promoter region double stranded.

The third enzyme, T7 RNA polymerase, transcribes RNA copies from the now transcriptionally active promoter, generating as many as 100 copies from each template molecule. Each new RNA molecule is now available as template for reverse transcriptase in the 'cyclic' phase of the NASBA process, but with the primer annealing steps reversed. In this phase, primer 2 binds to the template first and the action of AMV reverse transcriptase elongates the complementary DNA strand, thus generating a RNA:DNA hybrid as before. Then RNase H hydrolyses the RNA strand, so primer 1 is able to bind the resulting single-stranded DNA. AMV reverse transcriptase synthesizes DNA again, yielding the transcriptionally active promoter. Thus, the final product of a NASBA reaction is mainly single-stranded RNA.

Characteristics of NASBA

The NASBA reaction requires fewer 'cycles' than conventional PCR to produce a desired amplification of 1×10^9 molecules from each initial template (Chan and Fox, 1999). When using conventional PCR the number of molecules doubles in each step, so it requires approximately 20 cycles to produce an amplification of one million fold (Compton, 1991). However, with NASBA, 10-100 copies of RNA are generated in each transcription step, which means that only four to five 'cycles' are required to achieve a similar amplification (Chan and Fox, 1999; Jean *et al.,* 2001). Errors that are inherent in some enzymatic activities (*e.g.* reverse transcriptase) are cumulative, so fewer 'cycles' should reduce the likelihood of these errors occurring. Compton (1991) evaluated the fidelity of the NASBA process by direct sequencing of the RNA product. From the sequences derived this way, 90 % were readable and 10 % were unreadable sequences which appeared to be due to either the inherent enzyme 'stops' in the sequence or strand degradation, which is to be expected in any amplification reaction. Furthermore, Chadwick *et al.* (1998) found that the NASBA error ratio by reverse transcription and sequencing of the NASBA product was 0.38 %.

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As the NASBA is an exponentially amplification-based technique, theoretically a detection of 1 copy could be achieved. Experimental detection limits below 10 copies have been reported (Baeumner *et al.*, 2001; Blais *et al.*, 1997; Compton, 1991; Cook *et al.*, 2002; Loeffler *et al.*, 2001; Uyttendaele *et al.*, 1994; 1995a; 1995b), and several NASBA methods have been reported to be more sensitive than RT-PCR on equivalent targets (Birch *et al.*, 2001; Jean *et al.*, 2001; Lanciotti and Kerst, 2001; Loeffler *et al.*, 2001).

NASBA is a potentially powerful technique for specific detection of viable cells. In contrast with RT-PCR (Klein and Kuneja, 1997; Szabo and Mackey, 1999), it does not required DNase treatment, since, as stated before, it runs at a single temperature in which dsDNA is not denatured and thus cannot became a substrate for NASBA.

The principal characteristics of NASBA are summarised in Table I.8.

Table I.8. Characteristics of NASBA. Modified from Deiman *et al.*, 2002.

- A single step isothermal amplification reaction at 41 °C.
- Especially suited for RNA analytes because of the integration of RT into the amplification process.
- The single-stranded RNA product is an ideal target for detection by various methods including real-time detection using molecular beacons.
- The fidelity of NASBA is comparable to other amplification processes that use DNA polymerases lacking the 3' exonuclease activity.
- The use of a single temperature eliminates the need of special thermocycling equipment.
- Efficient ongoing process results in exponential kinetics caused by production of multiple RNA copies by transcription from a given cDNA product.
- Unlike amplification processes such as PCR, in which the initial primer level limits the maximum yield of product, the amount of RNA obtained in NASBA exceeds the level of primers by at least one order of magnitude.
- NASBA RNA product can be sequenced directly with a dideoxy method using RT and a labelled oligonucleotide primer.
- The intermediate cDNA product can be made double-stranded, ligated into plasmids, and cloned.
- Three enzymes are required to be active at the same reaction conditions.
- Low temperature can increase the non-specific interactions of the primers. However, these interactions are minimized by the inclusion of DMSO.
- A single melting step is required to allow the annealing of the primers to the target.
- The NASBA enzymes are not thermoestable and thus can only be added after the melting temperature.
- The length of the target sequence to be amplified efficiently is limited to approximately 100 to 250 nucleotides.

Until recently, the RT-PCR technique had been the only molecular amplification approach put into practice to viability assays. However, the NASBA process offers numerous advantages over RT-PCR (Cook, 2003; Jean *et al.*, 2001; Keer and Birch, 2003; Simpkins *et al.*, 2000). Table I.9 shows a comparison of NASBA and RT-PCR technologies.

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Table I.9. Comparison of NASBA with RT-PCR.

Features	NASBA	RT-PCR
Reaction	One simultaneous reaction (isothermal at 41 °C)	Two consecutive reactions (non-isothermal)
Fragment size	Preference for production of short fragments	Can be optimised to produce long fragments
Enzymes	Three required; AMV reverse transcriptase, RNase H, T7 RNA polymerase	Two usually required, reverse transcriptase and thermoestable DNA polymerase (a single enzyme used in some protocols)
Specificity	Lower (enzymes currently are non-thermoestable; temperature cannot exceed 42 °C without compromising the reaction)	Control of time, temperature and enzyme mix to increase specificity
End amplified product	Single-stranded RNA	Double-stranded DNA complementary to original RNA
Amplification factor	Exponential increase in product/transcription reaction (1×10^9-fold amplification after five cycles).	Binary increase in amplified product/PCR cycle (1×10^6-fold amplification after 20 cycles)
Fidelity	2×10^4 (for both polymerases)	2×10^4 (for *Taq* DNA polymerases)
Other features	DNA background does not interfere with isothermal amplification of RNA	Intron-flanking primers/DNases required to prevent DNA amplification

NASBA primer and probe design

As indicated before, primer 1 must contain a T7-promoter sequence at the 5' end (Figure I.18). Primer 2 may include a 5' non-specific sequence that can be used for detection with a generic probe when electrochemiluminescence (ECL) detection is used (Figure I.18).
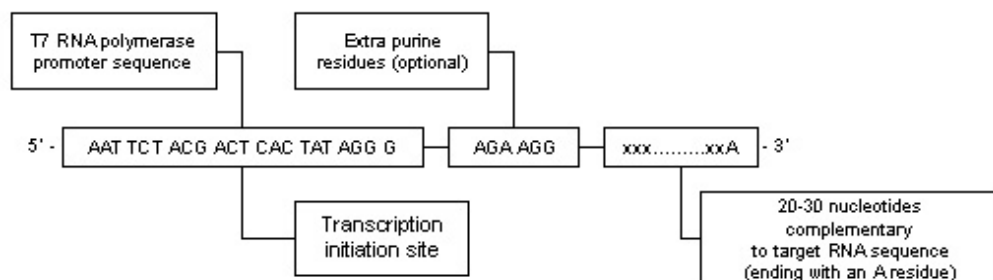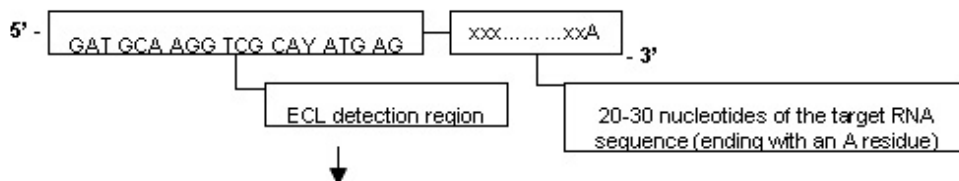
Figure I.18. Schematic representation of NASBA primers.

The published rules for designing NASBA primers and probes (Deiman *et al.*, 2002; Sooknanan *et al.*, 1995, Soumitra *et al.*, 1995) are summarized in Table I.10. Briefly, the primer binding site should be specific for the target nucleic acid, the hybridizing part of the primer should be preferably 20 bases long although up to 30 can be used and the G/C contents should be 40-60 %. The ability to form intramolecular and intermolecular structures should be avoided. The distance between the two primer binding sites should be preferably 80 to 200 nucleotides.

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Table I.10. Primer and probe design rules. Modified from Deiman *et al.*, 2002.

Primer Design Rules

- The distance between the binding site of primer 1 (complementary to the target sequence) and primer 2 (identical to the target sequence) should be about 80-200 nucleotides resulting in amplicons with a length of 120-250 nucleotides.
- If the target is obtained from different sources a sequence alignment should be performed. The primers should be directed against the conserved regions.
- Primer 1 contains a 5'-T7 promoter sequence consisting of 25 nucleotides:
  5'- AAT TCT AAT ACG ACT CAC TAT AGG G 3'
- A purine-rich region of 6 to 10 nucleotides directly downstream of the 5'-promoter sequence and upstream of the 3'-hybridizing sequence of primer 1 could improve amplification. If the 5'-part of the hybridizing sequence is purine rich, an additional purine stretch is not required.
- If the Nuclisens® Basic Kit is used, a defined non-hybridizing sequence consisting of 20 nucleotides:
  5'- GAT GCA AGG TCG CAT ATG AG -3' can be added to the 5'-end of primer 2 for generic ECL detection.
- The hybridizing parts of both primers should be 20-30 nucleotides.
- The G/C content of the hybridizing part should be 40-60 %
- The final nucleotide at the 3'-end of the hybridizing sequence is preferably an A-residue.
- A G/C-rich region at the 5'-end of the hybridizing part and A-T rich at the 3'-end of the hybridizing part is preferred.
- Full-length primers should be used. Primers purified by polyacrylamide gel electrophoresis or HPLC are recommended.
- Tracks of four or more of the same nucleotides in the primer sequence should be avoided.
- Both primer sequences should be screened for undesired matches with other nucleic acid sequences by using a DNA/RNA sequence data bank.
- Both primer sequences should be checked for internal secondary structures by using a DNA folding program.
- The secondary structure of the target sequence and amplicon sequence could be predicted with an RNA folding program.

Probe Design Rules

- The hybridizing sequence of the probe is identical to the target sequence and thus complementary to the amplicon sequence.
- The hybridizing sequence should be 20-25 nucleotides in length.
- The G/C content should be 40-60 %.
- The hybridizing sequence should not overlap with the primer 1 or primer 2 binding site.
- The melting temperature of the hybridizing sequence should be at least 7-10 ºC higher than the NASBA annealing temperature (41 ºC).
- Internal sequences in the hybridizing sequence and dimerization or interactions with the primers should be avoided.

ECL-detection:
- Biotin is attached to the 5'end of the capture probe to immobilize it on streptavidin-coated paramagnetic beads.
- In the Basic Kit, the hybridizing sequence of the generic detection probe is identical to the 5'-tail of primer 2.

Molecular beacons:
- Two complementary stems should be added, one at each end of the hybridizing sequence (loop).
- A G-residue may act as a quencher. A C-residue is therefore recommended directly adjacent to the fluorophore.
- The stem region should be 6-7 bp in length, dependent on the length of the loop: a 6 bp stem is recommended for a loop of 20 nucleotides and a 7-bp stem for a loop of 25 nucleotides.
- The G/C content of the stem region should be 70-80 %.
- The melting temperature of a 6 bp stem should be approx 60-65 ºC and of a 7-bp stem approx. 67-70 ºC.
- A DNA folding program could be used to predict the structure and melting temperature of the beacon, though the latter should preferably be determined by means of a melting curve analysis.
- Beacons with multiple predicted structures should be avoided.
- A free energy (ΔG) of -3 ± 0.5 Kcal/mole is recommended.

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Detection of NASBA products

There are several approaches for the detection of NASBA products. The simplest one is the use of standard agarose-gel electrophoresis and ethidium bromide staining (Malek *et al.*, 1994; Uyttendaele *et al.*, 1995c).

The most used detection method is the ECL-based technique (Baeumner *et al.*, 2001, Cook *et al.*, 2002; Min and Baeumner, 2002; Rodríguez-Lázaro *et al.*, 2003; Simpkins *et al.*, 2000; van Gemen *et al.*, 1994). The RNA product is detected by means of a one-step sandwich hybridization assay using 2 oligonucleotide probes that are complementary to a two different regions of the NASBA product. The probes are the capture probe (biotynilated), and the detection probe (labelled at the 5' end with electrochemiluminescent Tris (2,2' - bipyridine) ruthenium (Ru) [TAG]). The two probes are incubated together with the NASBA product and paramagnetic beads coated with streptavidin. Then the solution is analysed by an ECL reader (Figure I.19). The beads are drawn to the instrument's electrode with a magnet. The TAG label is therefore brought to the electrode surface in an amount that is proportional to the amount of specific nucleic acid in the NASBA product. A low voltage is then applied to the electrode, which triggers a cyclical oxidation-reduction reaction involving a ruthenium metal ion that produces photons (Blackburn *et al.*, 1991; Gudibande *et al.*, 1992; Kenten *et al.*, 1992). Tripolamine (TPA), which is present in excess, is consumed in the oxidation- reduction reaction and

Figure I.19. Nuclisens® ECL reader.

the ruthenium ion is recycled. The light signal is amplified during the cycling of these two components and is proportional to the amount of RNA target anchored to the electrode surface. Light is detected by the ECL reader at 620 nm using a photomultiplier tube. Figure I.20 illustrates the NASBA-ECL process.
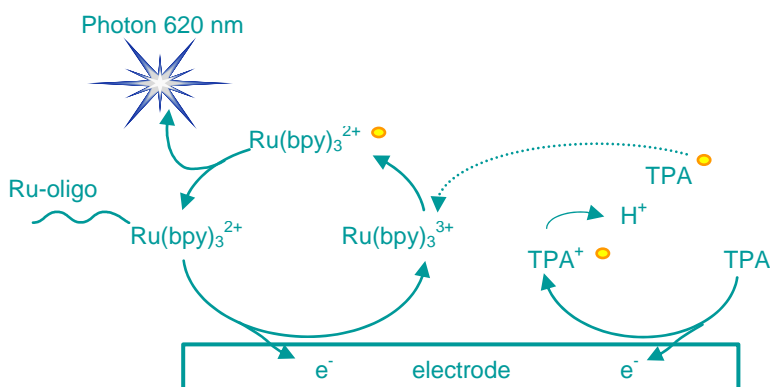
Figure I.20. Process of detection of NASBA products by electrochemiluminescence (ECL).

Alternatively, the enzyme-linked gel assay (ELGA) (Uyttendaele *et al.*, 1994; 1995a; 1995b; 1995c; 1996; 1997; 1999; van der Vliet *et al.*, 1993) can be used. NASBA products are identified with a specific oligonucleotide probe (ELGA probe) 5'-labelled with horseradish peroxidase (HRP). After hybridization, the RNA probe complex is separated from the non-hybridized probe by acrylamide gel electrophoresis, and subsequently stained and visualised.

The use of molecular beacons has recently been developed to allow real time detection of NASBA products (De Baar, *et al.*, 2001a; 2001b; De Ronde *et al.*, 2001; Greijer *et al.*, 2002; Klerks *et al.*, 2001; Lanciotti and Kerst, 2001; Leone *et al.*, 1998; Polstra *et al.*, 2002; Szemes *et al.*, 2002; Van Beckhoven *et al.*, 2001; Van Beuningen *et al.*, 2001; Weusten *et al.*, 2002; Yates *et al.*, 2001).

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This procedure facilitates the analysis of the kinetics of the reaction, and consequently the establishment of strategies for quantification (*e.g.* similar to that used in RTi-PCR assays). The use of diverse molecular beacons labelled with different fluorophores also allows multiplex detection in a single reaction (Tyagi *et al.*, 1998). In addition, no post-NASBA step is needed, which reduces risks of cross contamination.

Blais *et al.* (1997) detected NASBA amplimers by hybridization with immobilized capture probes complementary to the minus-strand RNA, followed by colorimetric visualization of conjugates. Denaturing agarose gel electrophoresis, Northern and Dot blotting (Jean *et al.*, 2001; 2002a; 2002b), or solid phase hybridization have also been reported for detection of NASBA products.

I.3.2.3 Analytical controls

The molecular-based detection techniques are instrumental techniques, and thus can produce false negative and false positive results (Knowk and Higuchi, 1989). Contamination remains an issue for diagnostic laboratories (Pellet *et al.*, 1999; Scherczinger *et al.*, 1999). The main causes of production of false positive results are the accidental contamination of the samples or the reagents with positive samples (cross-contamination) or with amplification products and plasmid clones (carry over contamination). To minimize these risks, several practices should be applied such as the following (Dieffenbach *et al.*, 1995; Hernández, 2003; Mifflin, 2003):

1. Space and time separation of pre- and post-amplification activities (Heinrich, 1991; Yap *et al.*, 1994). In particular, separation of pre-PCR activities from recombinant DNA activities.
2. Define 2 different pre-PCR areas: (i) for the preparation of the master mix, (ii) for the addition the nucleic acid template to the reaction mixture.
3. Each working area must have its own separate set of equipment, including pipettes, reagents, pipette tips, racks, centrifuges, vortex, lab coats, etc. Lab coats and gloves should be worn at all times, and gloves should be changed frequently.
4. All reagents should be prepared free from contaminating nucleic acids and/or nucleases. Water should be of highest quality, freshly distilled/deionised, filtered using a 0.22-µm filter, and autoclaved. All reagents should be prepared in large volumes and aliquoted into single-use volumes for storage.
5. The use of UV light in the sample preparation lab and in the cabinet where the amplification mixture is prepared, is recommended before carrying out the experiments. Be aware that instruments exposed to UV light may become discoloured.
6. DNA samples should be manipulated with specialized barrier or positive-displacement pipettes, which prevent the carryover of aerosols created during pipetting. Aerosols should also be minimized by briefly centrifuging the tubes prior to opening and avoiding popping the tubes open.
7. With PCR Uracil-DNA-glycosylase (UDG; also called uracil-N-glycosylase or UNG) can be used for elimination of contamination arising from PCR products (Longo *et al.*, 1990; Thornton *et al.*, 1992). During amplification, dTTP is substituted with dUTP, so the DNA product will contain dU instead of dT. Subsequent PCR runs are programmed to include a 50 ºC incubation step with UDG, which will mediate cleavage of any contaminating DNA strands containing dU.

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In order to correctly interpret the results of an amplification reaction , the following controls are recommended (Table I.11).

Table I.11. Analytical controls for molecular-based techniques. Adapted from Dieffenbach *et al.*, 1995; Hernández, 2003; Hoorfar and Cook, 2003; and Stirling, 2003.

- **<u>P</u>rocessing <u>P</u>ositive <u>C</u>ontrol (PPC):** A negative sample spiked with sufficient amount of target (*e.g.* pathogen, species, etc), and processed throughout the entire protocol. A positive signal should be obtained indicating that the entire process (from nucleic acids extraction to amplification reaction) was correctly performed.

- **<u>P</u>rocessing <u>N</u>egative <u>C</u>ontrol (PNC):** A negative sample spiked with sufficient amount of non-target or water, and processed throughout the entire protocol. A negative signal should be obtained indicating the lack of contamination along the entire process (from nucleic acids extraction to amplification reaction).

- **Premise Control or Environmental Control:** A tube containing the master mixture or water left open in the PCR setup room to detect possible contaminating nucleic acids in the environment.

- **Amplification Positive control:** A template known to contain the target sequence. A positive amplification indicates that amplification was performed correctly.

- **<u>No</u> <u>T</u>emplate <u>C</u>ontrol (NTC) or Reagent Control (Blank):** Including all reagents used in the amplification except the template nucleic acids. Usually, water is added in stead of the template. A negative signal indicates the absence of contamination in the amplification assay.

- **<u>I</u>nternal <u>A</u>mplification <u>C</u>ontrol (IAC):** Chimerical non-target nucleic acid added to the master mixture in order to be co-amplified by the same primer set as the target nucleic acid but with an amplicon size visually distinguishable or different internal sequence region from the target amplicon. The amplification of IAC both in presence and absence of target indicates that the amplification conditions are adequate.

Internal Amplification controls (IAC)

The efficiency of amplification-based techniques can be negatively influenced by several conditions including malfunction of equipment, incorrect reaction mixture, poor enzyme activity, or the presence of inhibitory substances in the original sample matrix (Al Soud and Rådström, 2000, Al Soud and Rådström, 2001; Rossen *et al.*, 1992). This can result in weak or negative signals and lead to underestimation of the amount of target nucleic acid in the sample. Many components of food products and culture media are amplification inhibitors. Their potential presence in the reaction is a serious problem that can compromise the applicability of the amplification-based techniques for analyses of these matrixes. Therefore, adequate control of the efficiency of the reaction is a fundamental aspect in such assays (Hoorfar and Cook, 2003, Hoorfar *et al.*, 2004; Hoorfar *et al.*, in press). An internal amplification control or IAC is a non-target nucleic acid sequence which is co-amplified simultaneously with the target sequence (Cone *et al.*, 1992; Hoorfar and Cook, 2003; Hoorfar *et al.*, 2004; Hoorfar *et al.*, in press). In a reaction without an IAC, a negative response (no signal) can mean that there was no target sequence present in the reaction. But, it could also mean that the reaction was inhibited. In a reaction with an IAC, a control signal will always be produced when there is no target sequence present. When no control signal is observed, this means that the reaction has failed, and the sample must be reanalysed.

In an amplification-based assay, an IAC should be based on flanking nucleic acid sequences (DNA for PCR assays, and RNA for RT-PCR and NASBA assays) with the same primer recognition sites as the target, with non-target internal sequences (Ballagi-Pordány and Belák, 1996; Cook, 2003; Hoorfar *et al.*, in press). An optimal IAC should fulfil the criteria summarized in Table I.12.

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Table I.12. Requirements of an optimal internal amplification control (IAC) for use in diagnostic assays. Modified from Hoorfar *et al.*, in press.

- Target nucleic acid and IAC should share the same primer binding sites. This avoids two primer pairs to be present in a multiplex reaction.
- IAC amplicons should be easily distinguishable from the target nucleic acid amplicons. Depending on the detection method of the assay, an IAC should contain either different internal sequences to bind to IAC-specific probes, or produce an amplicon of a different size which is easily detected by gel electrophoresis.
- Only highly purified templates should be used.
- IAC concentration should be determined by titration of the IAC according the method described in Rosenstrauss *et al.* (1998) or by fluorescence methods.
- For dilution use polyallomer tubes and aerosol-resistant, sterile pipette tips. IAC nucleic acid can be stabilized, and storage should be at high concentrations of stabilized IAC nucleic acid (10^3 copies in aliquots at $-20°C$).
- The optimal amount of IAC in the assay shall be as low as possible, while still eliciting a signal through amplification. This will maximise the potential to identify false negatives by detecting amplification inhibition, and will ensure the reliable detection of low target concentrations.
- For PCR assays, the source of IAC should be plasmid DNA carrying the cloned IAC sequence, or purified PCR products. For RT-PCR and NASBA assays, the source of IAC should be an *in vitro*-synthesized chimerical single-stranded RNA.
- Add the IAC in the master mixture to ensure equal amount in each reaction tube.
- It is not strictly necessary that the amplification efficiency of target and IAC nucleic acid is identical. An experimental approach is needed to show the amplification efficiency of each nucleic acids during the amplification reaction.

There are two main strategies for use of an IAC in diagnostic assays: competitive IAC and non-competitive IAC (Hoorfar *et al.*, in press). Both strategies are useful, although the first is recommended to avoid the risk of undesired interactions of multiple primers, and, especially, to get comparable amplification efficiency of the IAC and the target sequence.

1st. Competitive IAC strategy. The target and the IAC are co-amplified in a single reaction tube with the same primer set, and, in consequence, there is always some competition between target nucleic acid and IAC. Thus, the most critical parameter to consider is the optimal initial number of IAC copies in the diagnostic assay as it directly affects the target detection limit (Abdulmawjood *et al.*, 2002; Brightwell *et al.*, 1998; Sachadyn and Kur, 1998). If used at high concentration, the IAC might not allow detection of weak inhibition which could cause false-negative results if the target is present in low concentrations (Rosenstraus *et al.*, 1998). However, small amounts of initial IAC lead to substantial variations in IAC amplification, indicating poor reproducibility. The initial IAC copy number in the reaction must be determined in a compromise level that allows reproducible IAC detection and avoids inhibition of the target-specific reaction. In this sense, Ballagi-Pordány and Belák (1996) recommend a range of 50-500 copies.

2nd. Non-competitive IAC strategy. The target and the IAC are amplified by two different primer sets. Thus, the amplification harbours at the same time two reactions with different kinetics. The IAC primer set can target a synthetic nucleic acid (DNA for PCR assays or RNA for RT-PCR or NASBA assays) or a gene different from the target. In this approach, amplification of the IAC must be limited by a controlled concentration of the IAC-specific primers (Figure I.21).

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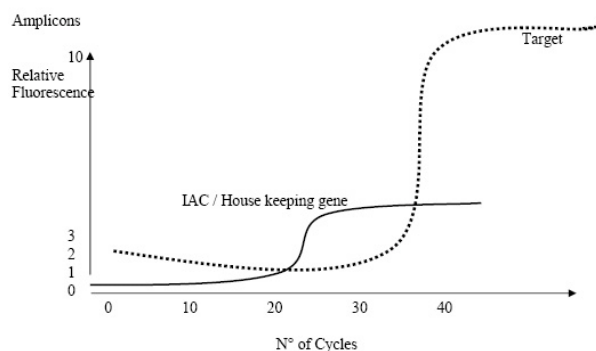
Figure I.21. Amplification plots of an RTi-PCR containing an IAC using a non-competitive strategy. Amplification of the IAC is limited by a controlled concentration of the IAC-specific primers.

Theoretically, the quantity of IAC introduced in the reaction and the potential contamination with amplicons derived from the IAC should not be relevant to the detection limit of the target nucleic acid. However, non-competitive sequences may not accurately reflect the amplification of the primary target due to differences in the primer sequences and size of the amplified product.

Construction of an IAC

As a general rule, the IAC should be chosen as the likelihood of its presence in the sample is low. The IAC and the target sequences usually have similar base-pair nucleotide composition, but not sequence. This facilitates reaching comparable amplification efficiencies for IAC and target. At the same time, formation of heteroduplex products must be avoided; which should be empirically tested for each amplification system.

The simplest approach is to produce amplification products, which differ in size or internal region and hence can be easily distinguished by visualizing separately from the native product in agarose gel electrophoresis, or with multiplex detection by colour using different fluorescent probes. The most common approach is the composite primer technique (Siebert *et al.*, 1992).

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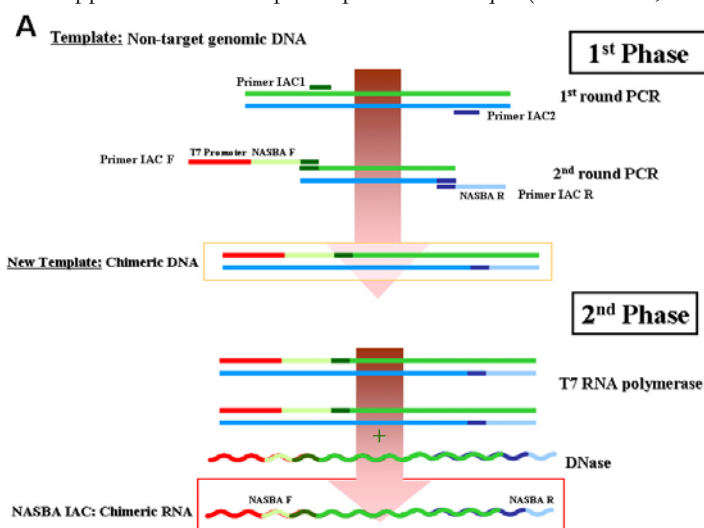
B <u>Template:</u> Non-target genomic DNA
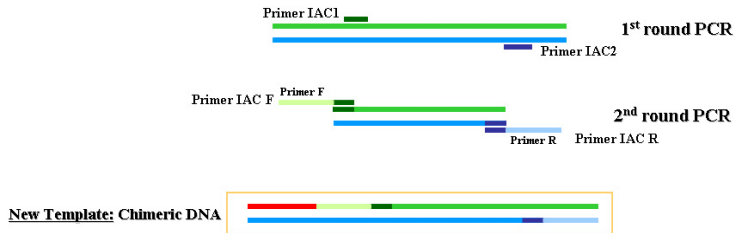
Figure I.22. Illustration of the composite primer technique where the same primer set is used to amplify both the target and the non-target chimerical nucleic acid spiked in the reaction mixture. **A.** For diagnostic NASBA or RT-PCR assays. **B.** For diagnostic PCR assays.

For a size-dependent discrimination (by gel electrophoresis) of target and IAC nucleic acids, chimerical IACs may be produced by deleting, or inserting sequences between the recognition primer sites (Abdulmawjood *et al.*, 2002; Cubero *et al.*, 2002; Müller *et al.*, 1998; Sachadyn and Kur, 1998). A smaller size IAC can be constructed by deleting an internal sequence fragment. In this approach, the original amplicon is digested at 2 sites with restriction enzymes. After purification of the two outer fragments, they are re-ligated to create an amplicon which is smaller than the target gene. In order to generate an IAC larger than the target amplicon, it is cloned and digested at a unique site. Then, a new sequence of defined size is ligated in the linearized and dephosphorylated recombinant plasmid to create a new final recombinant plasmid containing the IAC (Cubero *et al.*, 2002). In order to produce similar biophysical amplification conditions between target and IAC, both sequences should not be too heterologous. For sequence discrimination (*e.g.* multiplex by-colour) of target and IAC nucleic acids, modification of various nucleotides from the amplicon may be performed using mutagenesis technology (Courtney *et al.*, 1999). Alternatively, an IAC can be constructed by linking the two target specific primer sequences with a non-relevant sequence (Figure I.22) *e.g.* from a fish virus (Hoorfar *et al.*, 2000; Lübeck *et al.*, 2003) or a plant (Jofré *et al.*, in press; Rodríguez-Lázaro *et al.*, submitted for publication) for the detection of veterinary or foodborne pathogens.

Finally, another IAC construction methodology allows the creation of an IAC with a completely designed nucleotide sequence (Malorny *et al.*, 2003a; Rosenstraus *et al.* 1998). Several pairs of partially overlapping oligonucleotides that contain the entire IAC sequence are annealed and extended using an *E. coli* DNA polymerase. The product is finally reamplified with the terminal primers. DNA IACs may be used for PCR assays or as template for reverse transcription to produce IACs for RT-PCR or NASBA assays.

I.3.2.4 Diagnostic accuracy parameters

An international standard guideline (ISO 16140:2003) has been recently prepared by CEN/TC275 in collaboration with the Technical Committee ISO/TC34 to establish the general principle and technical procedure for the validation of alternative methods in the field of microbiological analysis of food, animal feeding stuffs, and environmental and veterinary samples. Validation of a method is defined as the process of determining the suitability of a measurement system for providing useful analytical data. In that standard guide, several terms and definitions with a critical importance in the implementation of alternative methods are described (Table I.13).

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Table I.13. Definitions of validation concepts for alternative methods in Microbiology (ISO 16140:2003).

1. Analyte: The analyte is the component demonstrated or measured by the method of analysis. It may be the microorganism, its components or products.
2. Qualitative method: A method of analysis whose response is either the presence or absence of the analyte in a certain amount of sample.
3. Quantitative method: A method of analysis whose response is the amount of the analyte measured either directly (enumeration in a mass or a volume), or indirectly (colour absorbance, impedance, etc.) in a certain amount of sample.
4. Reference method: A reference method is a method that is internationally recognised and accepted (*e.g.* NMKL, ISO, CEN and AOAC methods, and certain national standards of equivalent standing).
5. Alternative method: An alternative method is a method of analysis that demonstrates or estimates, for a given category of products, the same analyte as is measured using the corresponding reference method. The method can be proprietary or non-commercial, and does not need to cover an entire analysis procedure, that is from the preparation of samples to the test report. The alternative method exhibits attributes appropriate to the users' needs, for example:
     i. speed of analysis and/or response;
     ii. ease of execution and/or automation;
     iii. analytical properties (precision, accuracy, limit of detection, etc.);
     iv. miniaturisation;
     v. reduction of cost.
6. Validation of an alternative method: The validation of an alternative method is the procedure to demonstrate if the results obtained by the alternative method are comparable to those obtained using the reference methods. The validation comprises two phases:
     A. A method comparison study of the alternative method against a reference method (performed by an expert laboratory).
     B. An interlaboratory study of the two methods (organised by an expert laboratory).
7. Organising laboratory: Laboratory having the qualified staff and skills to perform the method comparison study and organise the interlaboratory study. The availability of an experienced statistician is essential for the analysis of the results.
8. Methods comparison study: Study, performed by the organising laboratory of the alternative method against the reference method.
9. Inter-laboratory study: Study of the alternative method's performance using common samples in several laboratories and under the control of the organising laboratory.

Many of the performance criteria are well recognized and accepted by numerous internationally recognized organizations (ISO, EMEA, Codex Alimentarius, etc.). There are a series of concepts that define an analytical method and that are applied to molecular-based detection techniques. They can be considered in the context of validation and implementation of a particular method (Bertheau *et al.*, 2002).

A. *Linearity, range and accuracy.*

a. Linearity: Ability of the method, when used with a given matrix, to give results that are in proportion to the amount of analyte present in the sample; that is, an increase in analyte corresponds to a proportional increase in the result (Anonymous, 2003). In the same manner, linearity has been defined by Paoletti and Weighardt (2002) as the proportionality of the signal to the amount of reference material, demonstrated by the calculation of a regression line with an adequate statistical method.

A **response curve** or a signal function is obtained when measuring the relationship between the **signal** or the method's response and the **analyte concentration** (doses) in different samples of reference materials having known values. In microbiology, where practically no stable reference material is available, these 'known values' can be obtained after replicate measurements using the reference method.

The European Commission, in the particular case of genetically modified organisms (GMO), has established that the linearity within the range of applicability of the method should be not less than 98 % (Paoletti and Weighardt, 2002)

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b. <u>Range</u>: Interval of analyte concentration over which the method is considered to perform in a linear manner (Paoletti and Weighardt, 2002) or interval of analyte concentration within which the method can be regarded as validated (Thompson *et al.*, 2002).

c. <u>Accuracy</u>: The accuracy describes the veracity of the test results (Skog and Leary, 1992), and can be defined as closeness of agreement between a test result and the accepted reference value (Anonymous, 1993; Paoletti and Weighardt, 2002).

d. <u>Bias or total systematic error</u>: Difference between the expectation of the results and an accepted reference value (Anonymous, 1993).

e. <u>Trueness</u>: Closeness of agreement between the average value obtained from a large series of test results and an accepted reference value (Anonymous, 1994; Thompson *et al.*, 2002).

f. <u>Relative accuracy</u>: Degree of correspondence between the response obtained by the reference method and the response obtained by the alternative method on identical samples (Anonymous, 2003; Hoorfar and Cook, 2003). The term 'relative accuracy' is complementary to the 'accuracy' and 'trueness'.

B. *Detection and quantification limits*

a. <u>Critical level</u>: The smallest amount of analyte which can be detected (not null), but not quantified as an exact value. Below this value, a negative result does not mean a null (Anonymous, 2003). At the critical level, the false negatives probability (β) is 50 %.

b. <u>Detection limit (LOD)</u>: The smallest critical level detected with a probability (1- β), which has to be well over 50 %, for example 95 % (Anonymous, 2003) or 99 % (Hoorfar and Cook, 2003; Knutsson *et al.*, 2002). Detection limit has been also described as the smallest amount or concentration of analyte in the test that can be distinguished from zero (Anonymous, 1997; IUPAC, 1995; Thompson *et al.*, 2002); the smallest number of culturable target microorganisms necessary to create a positive test response (Hoorfar and Cook, 2003), or the minimum level at which the analyte can reliably be detected with a probability of 95 % (Paoletti and Weighardt, 2002).

c. <u>Quantification limit (LOQ)</u>: The smallest amount of analyte which can be measured and quantified with defined precision and accuracy by the method under validation (Anonymous, 2003). The Association of Analytical Communities (AOAC) defines the quantification limit for quantitative methods as: $LOQ = 10 \ SD_0$ (Coleman *et al.*, 1997).

C. *Relative sensitivity:*

The relative sensitivity is the ability of the alternative method to distinguish two different amounts of analyte (as measured by the reference method) within a given matrix, at a specified average value, or over the whole measurement range; that is, the minimal quantity variation which gives a significant variation of the measurement signal (Anonymous, 2003). The sensitivity differs from detection limit because it is calculated for each value of the measurement range. From a qualitative approach (*i.e.* for qualitative methods), the relative sensitivity can also be defined as the ability of the alternative method to detect the analyte when it is detected by the reference method (Anonymous, 2003; Hoorfar and Cook, 2003).

D. *Specificity and selectivity:*

a. <u>Specificity</u>: Specificity is the degree to which a method is affected by the other components present in a multi-component sample (Anonymous, 2003; Malorny *et al.*, 2003b). In other words, the ability of a method to measure a given analyte within the sample without interference from non-target components or background noise (Anonymous, 2003). For qualitative methods, the term used is **relative specificity**, and is defined as the ability of the alternative method not to detect the analyte when it is not detected by the reference method (Anonymous, 2003; Hoorfar and Cook, 2003).

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b. <u>Selectivity:</u> Selectivity is defined as a measure of the degree of non-interference in the presence of non-target analytes (Anonymous, 2003). A method is selective if it can be used to detect the analyte and that a guarantee can be provided that the detected signal can only be a product by the specific analyte. In qualitative methods, there is another term; *relative selectivity,* which is defined as a measure of the inclusivity, and the exclusivity (Hoorfar and Cook, 2003; Malorny *et al.*, 2003b; NordVal, 2001).

c. <u>Inclusivity:</u> Inclusivity is the ability of an alternative method to detect the target analyte from a wide range of strains.

d. <u>Exclusivity:</u> Exclusivity is the lack of interference from a relevant range of non-target strains of the alternative method.

E. *Precision:*

a. <u>Precision:</u> The precision describes the reproducibility of the test results (Skog and Leary, 1992), and can be defined as the closeness of agreement between independent test results obtained under stipulated conditions of repeatability and reproducibility (Anonymous, 1993; Thompson *et al.*, 2002).

b. <u>Repeatability:</u> Closeness of agreement between successive and independent results obtained by the same method on identical material, under the same repeatability conditions (apparatus, operator, laboratory and short intervals of time) (Anonymous, 2003).

c. <u>Repeatability limit (r):</u> value less than or equal to which the absolute difference between two test results obtained under repeatability conditions is expected to be with a probability of 95 % (Anonymous, 2003).

d. <u>Reproducibility:</u> Closeness of agreement between single test results on identical test material using the same method and obtained by operators in different laboratories using different equipment (that is reproducibility conditions) (Anonymous, 2003).

e. <u>Robustness:</u> Reproducibility by other laboratories using different batches and brands of reagents and validated equipment (Hoorfar and Cook, 2003).The method should be tolerant of a range of physical and chemical parameters. The most critical parameters usually are quality of template DNA, batch differences in purity of the reagents, pipetting errors, accuracy of temperatures reached during PCR, adequacy of time duration of each PCR step, and ramping rates between the different temperatures required during amplification (Malorny *et al.*, 2003b).

f. <u>Ruggedness:</u> Resistance to change in the results produced by an analytical method when minor deviations are made from the experimental conditions described in the procedure (Thompson *et al.*, 2002).

g. <u>Outlier:</u> extreme value which normally appears randomly in less than 1 % of tests, but more frequently if abnormal situations occur (Anonymous, 1993).

I.3.2.5 Application of amplification techniques for the detection of foodborne pathogens

Bacterial detection and control are two key aspects in food microbiology (Stewart, 1997). Microbiological quality control programs are increasingly applied throughout food chain production in order to minimize the risk of infection for the consumer. Thus, the availability of reliable, rapid and accepted test systems to detect the presence or absence, or even the degree of contamination of pathogens, becomes increasingly important for the agricultural and food industry. Such systems would also find a place within the framework of legislative control measures. During the last decade, molecular approaches have significantly contributed to this field.

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Traditional and standardized analysis of food for the presence of microorganisms relies on the enrichment and isolation of presumptive colonies of bacteria on agar media, using approved diagnostic artificial media. This is generally followed by biochemical and/or serological identification. Traditional methods of detection, although reliable and efficient, require several days to weeks before results are produced. Furthermore, phenotypic properties by which the bacteria are identified may not be always expressed; and when expressed, they may be difficult to interpret and classify. Another disadvantage of traditional methods is that cells which are viable but otherwise non-culturable cannot be detected, *e.g.* some stressed *Campylobacter* spp. (Rollins and Colwell, 1986; Tholozan *et al.*, 1999). Thus, introduction of amplification techniques in microbial diagnostics has been established in research laboratories as a valuable alternative to traditional detection methods. The most extensively used amplification method is PCR, but other methods have also been developed such as NASBA, the Q-beta replicase amplification system and the ligase chain reaction (LCR), although until now, they have had limited practical relevance for food monitoring and control. Speed, excellent detection limit, selectivity, specificity, sensitivity and potential for automation are among the most important advantages of amplification techniques. These advantages compared to traditional detection methods might well encourage end-users to adopt amplification techniques in routine testing for foodborne pathogens.

PCR-based methods

The more general aspects of PCR as a diagnostic tool in food microbiology and its application to identify foodborne bacterial pathogens have been reviewed elsewhere (Hill, 1996; Lantz *et al.*, 2000; Olsen *et al.*, 1995; Scheu *et al.*, 1998). PCR-based methods are predicted to be established as routine reference methods alongside traditional detection techniques within the next ten years (Hoorfar and Cook, 2003). However, further developments are needed to universalise its application in food microbiology, including pre-PCR treatment of the samples (section 1.3.1); distinguishing viable from dead cells, direct quantitative RTi-PCR and automation of the whole PCR process. In this sense, the implementation of PCR is currently hampered by its lack of ability to distinguish between viable and dead cells, since establishing bacterial viability is an essential issue for appropriate risk management. PCR-based methods detect DNA, which survives cell death and, thus a positive PCR signal is not a cell viability indicator. An approach to detect only viable bacterial cells by using PCR-based methodology is the use of mRNA as template for amplification (Klein and Juneja, 1997; Sheridan *et al.*, 1998; Szabo and Mackey, 1999). However, these require removing any trace of bacterial DNA in the reaction in order to avoid false-positive results in viability assays (Nogva *et al.*, 1999; Cook, 2003).

Quantitative analysis of foodborne pathogens is a main aspect of molecular microbiological diagnostics. Although several approaches have been described based on conventional PCR (Rijpens and Hermann, 2002; Scheu *et al.*, 1998), the most promising method for quantitation is RTi-PCR. Several RTi-PCR methods for food microbiology diagnostics have been published (Table I.14).

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Table I.14. RTi-PCR-based methods published for detection of the five most representative foodborne pathogenic bacteria.

Target	Sequence	System	Matrix	Detection limit	Reference
Campylobacter jejuni		TaqMan	chicken faecal and cecal samples	2-25 cfu/reaction	Rudi *et al.*, 2004
C. jejuni	ORF-C sequence.	TaqMan	raw chicken, offal, shellfish, raw meat, and milk	12 cells/reaction	Sails *et al.*, 2003
C. jejuni	VS1 sequence	TaqMan	poultry, milk and water	1 cfu/reaction	Yang *et al.*, 2003
C. jejuni		SYBR Green	chicken rinse water	10 cfu/ml*	Cheng and Griffiths, 2003
C. jejuni		SYBR Green	stool	10^5 cfu/ml	Fukushima *et al.*, 2003
C. jejuni	*gryA*	TaqMan	chicken cloacal and carcass swabs	- - -	Padungtod *et al.*, 2002
C. jejuni	381121-381206	TaqMan	culture	1 cfu/reaction	Nogva *et al.*, 2000
C. jejuni, C. coli	*mapA* and *ceuE*	TaqMan	agar plate	- - -	Best *et al.*, 2003
C. jejuni, C. coli		BAX			Manfreda *et al.*, 2003
Campylobacter spp.	*16S rDNA*	SYBR Green	faecal samples	- - -	Logan *et al.*, 2001
Escherichia coli 0157		BAX	faecal samples	1cfu/g*	Bono *et al.*, 2004
E. coli 0157		BAX	alfalfa sprouts and mushrooms	10 cfu/ml	Strapp *et al.*, 2003
E. coli 0157	*uidA*	TaqMan MGB	culture	- - -	Yoshitomi *et al.,* 2003
E. coli 0157	*uidA*	TaqMan	culture	- - -	Jinneman *et al.*, 2003
E. coli 0157		BAX	fresh vegetables	1-10 cfu/ml*	Bhagwat, 2003
E. coli 0157	Intimin, stx1, stx2	TaqMan	faecal samples	1 cfu/g*	Sharma and Nystrom, 2003
E. coli 0157	stx1, stx2	TaqMan	bread slices, ground beef, commercially bagged salad greens, and salad dressing	5340 cfu/g	Heller *et al.*, 2003
E. coli 0157	*stx1* and *eae*	TaqMan	faeces	10 cfu/g*	Ibekwe and Grieve, 2003
E. coli 0157		BAX	ground beef	1 cfu/g*	Lionberg *et al.*, 2003
E. coli 0157	*stx1,stx2* and *eae*	TaqMan	water	10 cfu/g*	Ibekwe *et al.*, 2002
E. coli 0157	*stx1,stx2* and *eae*	TaqMan	beef and bovine faeces	1-10 cfu/g*	Sharma, 2002
E. coli 0157	*stx1* and *stx2*	SYBR Green	culture broth	10^3 cfu/ml	Jothikumar and Griffiths, 2002
E. coli 0157	*rfbE*	molecular beacons	raw milk and apple juice	1 cfu/ml*	Fortin *et al.,* 2001
E. coli 0157		TaqMan E. coli O157:H7 Kit	water	- - -	Fratamico and Bagi, 2001
E. coli 0157		BAX	beef chuck shoulder	0.5 cfu/c1m^2*	Bhaduri and Cottrell,2001
E. coli 0157		BAX	fresh fruits and vegetables	1 cfu/ 25g*	Shearer *et al.*, 2001
E. coli 0157		BAX	bovine carcasses	- - -	Kang *et al.*, 2001

(Cont)

Organism	Gene	Method	Sample	Detection limit	Reference
E. coli 0157	*sltII*	molecular beacons	milk	10^3 cfu/ml	McKillip and Drake, 2000
E. coli 0157	*stx1,stx2* and *eae*	TaqMan	beef and faece	1 cfu/g[*]	Sharma *et al.*, 1999
E. coli 0157	*eaeA*	TaqMan	culture medium	10^2 cfu/ml[*]	Oberst *et al.*, 1998
E. coli 0157		BAX	ground beef	- - -	Johnson *et al.*, 1998
Listeria monocytogenes	*hly, iap*	TaqMan, Amplyfluor	culture broth	1 cell/reaction	Rodríguez-Lázaro *et al.,* 2004
Listeria monocytogenes	*hly*	SYBR Green	raw sausage meat	3 cell/g[*]	Wang *et al.*, 2004
L. monocytogenes	*hly, iap*	FRET	non-fat dry milk	10^3-10^4 cfu/25 ml	Koo and Jaykus, 2003
L. monocytogenes		BAX	fresh vegetables	1-10 cfu/ml[*]	Bhagwat, 2003
L. monocytogenes	*hly*	SYBR Green	culture broth	2.5 cells /reaction	Jothikumar *et al.*, 2003
L. monocytogenes	*hly*	TaqMan	cabbage	9 cfu/reaction	Hough *et al.*, 2002
L. monocytogenes		BAX	chicken nugget processing plant	- - -	Rodrigues *et al.*, 2002
L. monocytogenes		BAX	beef chuck shoulder	0.5 cfu/c1m²[*]	Bhaduri and Cottrell, 2001
L. monocytogenes	*iap*	TaqMan	milk	6 cfu/reaction	Hein *et al.*, 2001
L. monocytogenes		BAX	environmental and raw fish samples	- - -	Hoffman and Wiedmann, 2001
L. monocytogenes		BAX	several	- - -	Hochberg *et al.*, 2001
L. monocytogenes	*Hly*	TaqMan	pure cultures, water, skim milk, and whole milk	6 cfu/reaction	Nogva *et al.*, 2000
L. monocytogenes		BAX	cold-smoked fish	- - -	Norton *et al.*, 2000
L. monocytogenes		BAX	culture	10^5-10^6 cfu/ml	Stewart and Gendel, 1998
Salmonella spp.	*invA*	SYBR Green	raw sausage meat	3 cfu/g[*]	Wang *et al.*, 2004
Salmonella spp.		iQ-Check PCR kit	poultry and pork carcasses	50–500 cfu/ml[*]	Uyttendaele *et al.*, 2003
Salmonella spp.	*fimI*	SYBR Green	culture broth	2.5 cells /reaction	Jothikumar *et al.*, 2003
Salmonella spp.		BAX	frankfurters, raw ground beef, mozzarella, fish and orange juice	- - -	Fukushima *et al.*, 2003
Salmonella spp.		SYBR Green	stool	10^5 cfu/ml	Fukushima *et al.*, 2003
Salmonella spp.		RAPID System *Salmonella* Detection Kit	milk	- - -	Van Kessel *et al.*, 2003
Salmonella spp.	*invA*	FRET	chicken	3 cfu/ml[*]	Eyigor and Carli, 2003
Salmonella spp.	*invA*	TaqMan	agar plate	- - -	Rodríguez-Lázaro *et al.*, 2003
Salmonella spp.		BAX	fresh vegetables	1-10 cfu/ml[*]	Bhagwat, 2003
Salmonella spp.		BAX	chicken rinses and chicken hot dogs	1-250 cfu/g[*]	Bailey and Cosby, 2003

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(Cont)

Salmonella spp.		BAX	alfalfa sprouts and mushrooms	10 cfu/ml	Strapp *et al.*, 2003
Salmonella spp.	*invA*	SYBR Green	poultry	6 cfu/ml[*]	Eyigor *et al.*, 2003
Salmonella spp.	*invA*	TaqMan	food outbreak	- - -	Daum *et al.*, 2002
Salmonella spp.		BAX	beef chuck shoulder	0.5 cfu/c1m^2[*]	Bhaduri and Cottrell, 2001
Salmonella spp.		BAX	fresh fruits and vegetables	1 cfu/ 25g[*]	Shearer *et al.*, 2001
Salmonella spp.		Applied Biosystems (TaqMan) Kit	meat	- - -	Kawasaki *et al.*, 2001
Salmonella spp.		BAX	animal feeds	40 cfu/10 g[*]	Maciorowski *et al.*, 2000
Salmonella spp.	*himA*	Molecular beacons	culture broth	2 cfu/reaction	Chen *et al.*, 2000
Salmonella spp.	Applied Biosystems (TaqMan) Kit		culture broth	25 cfu/reaction	Nogva and Lillehaug, 1999
Salmonella spp.	Applied Biosystems (TaqMan) Kit		raw meat and shrimp	3 cfu/reaction	Kimura *et al.*, 1999
Salmonella spp.		BAX			Bennett *et al.*, 1998
Salmonella spp.		BAX	poultry	10^4 cfu/ml[*]	Bailey, 1998
Salmonella spp.	*invA*	TaqMan	chicken carcass	2 cfu/reaction	Chen *et al.*, 1997
Salmonella enterica	*invA*	TaqMan	culture broth	- - -	Knutsson *et al.*, 2002
Salmonella enterica	*invA*	TaqMan	agar plate	- - -	Hoorfar *et al.*, 2000
S. Enteritidis	*sefA*	SYBR Green	poultry	10^3 cfu/ml*	De Medici *et al.*, 2003
Yersinia enterocolytica	16S rDNA	SYBR Green	meat	4.2 10^3 cfu/ml	Wolffs *et al.*, 2004
Y. enteroolytica		TaqMan	ground pork	1-10 cfu/ml[*]	Wu *et al.*, 2004
Y. enteroolytica		SYBR Green	stool	10^5 cfu/ml	Fukushima *et al.*, 2003
Y. enteroolytica	16S rDNA	SYBR Green	culture	10^1 cfu/ml[*]	Knutsson *et al.*, 2002b
Y. enteroolytica	ERIC fingerprint fragment	SYBR Green	culture	- - -	Aarts *et al.*, 2001
Y. enteroolytica	16S rDNA	light-up			Wolffs *et al.*, 2001
Y. enteroolytica	*ail*	TaqMan	pork products	0.4 cfu/g[*]	Boyapalle *et al.*, 2001
Y. enteroolytica	*yst*	TaqMan	raw meat and tofu	10^3 cfu/g	Vishnubhatla *et al.*, 2001
Y. enteroolytica	*yst*	TaqMan	ground pork	10^3 cfu/g	Vishnubhatla *et al.*, 2000
Y. enteroolytica	*ail*	TaqMan	ground pork	1 cfu/g[*]	Jourdan *et al.*, 2000

[*] After pre-enrichment step.

NASBA-based methods

NASBA is a promising diagnostic tool for the analysis of viable microorganisms, since it is based on amplification of RNA rather than DNA. A critical review of the application of this technique to food and environmental microbiology has recently been published (Cook *et al.*, 2003). In that review, Cook states that NASBA for detection of foodborne pathogens is at around the same stage as PCR was a decade or so ago, with a few methods being sporadically published in the scientific press. Hence, considerable further development is required before NASBA can follow in PCR's footsteps to realize its potential for routine use. However, since NASBA can equal the rapidity and accuracy of PCR and has the additional potential advantage of unambiguous detection of viable pathogens, NASBA is a very promising diagnostic tool for food and clinical microbiology, and even

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can become a reference in future decades. In Table I.15, published NASBA-based methods applied to pathogenic microorganisms in food and environmental samples are summarised.

T**able I.15.** NASBA-based methods reported for detection of pathogenic microorganisms in food and environmental samples. Adapted from Cook, 2003.

Target	Sequence	Matrix	Detection system	Detection limit	Reference
Bacillus spp.	*hblC*	Milk	Real-time fluorescence	10^5 cfu/ml	Gore *et al.*, 2003
Campylobacter jejuni, C. coli, C. lari	16S rRNA	poultry products	ELGA	< 10 cfu/10 g	Uyttendaele *et al.*, 1995a
C. jejuni	16S rRNA	poultry products	ELGA	naturally contaminated (10 g)	Uyttendaele *et al.*, 1995b
C. jejuni	16S rRNA	poultry products	ELGA	naturally contaminated (10 g)	Uyttendaele *et al.*, 1996
C. jejuni	16S rRNA	poultry products	ELGA	< 10^5 cfu/10 g	Uyttendaele *et al.*, 1997
C. jejuni	16S rRNA	poultry products, meat products, dairy products	ELGA	5×10^2–5×10^4 cfu/20 g	Uyttendaele *et al.*, 1999
Cryptosporidium parvum	hsp70	water	ECL	50 oocysts/100 l	Baeumner *et al.*, 2001
Escherichia coli	*clpB*	water	ECL	40 cfu/ml	Min and Baeumner, 2002
E. coli	*clpB*	water	RNA biosensor	40 cfu/ml	Baeumner *et al.*, 2003
Hepatitis A virus	VP2	Blueberries, lettuce, wastewater, sewage	Probe hybridization / dot-blot		Jean *et al.*, 2001
Hepatitis A virus	VP2	- - -	Microtiter plate hybridization	400 PDU/ml	Jean *et al.*, 2002a
Listeria monocytogenes	16S rRNA	poultry products, meat products, seafood, vegetables, dairy products	ELGA	< 10 cfu/25 g	Uyttendaele *et al.*, 1995c
L. monocytogenes	*hly*	egg products, dairy products	colorimetric	< 10 cfu/60 g	Blais *et al.* 1997
Norwalk virus	RNA polymerase	stools	ECL	10^4 PDU/ml	Greene *et al.*, 2003
Rotavirus	gene 9	sewage	ELISA	10^4 PDU/ml	Jean *et al.*, 2002b
Rotavirus	gene 9	- - -	microtiter plate hybridization	40 PDU/ml	Jean *et al.*, 2002
Salmonella Enteritidis	*dna*K	liquid egg	ECL	< 10 cfu/25 g	Cook *et al.*, 2002
Salmonella Enteritidis	*dna*K	fresh meats, poultry, fish, ready-to-eat salads and bakery products	ECL	< 10^1-10^2 cfu/25 g	D'Souza and Jaykus, 2003

Finally, although RNA has been considered a good indicator of cell viability, not all RNA species (*i.e.* ribosomal RNA -rRNA-, transfer RNA -tRNA- and messenger RNA -mRNA-) are suitable for unambiguous detection of viable bacteria. Bacterial rRNA and tRNA have strong secondary structures and are much more stable than bacterial mRNA, which in general has a short half-life within viable bacterial cells, and is rapidly degraded by specific enzymes (RNases) (Nierlich and Murakawa, 1996; Rauhut and Klug, 1999; Sela *et al.*, 1957). Thus, the detection of mRNA has been proposed as an indicator of cell viability (Bej *et al.*, 1991, 1996; del Mar Lleo, *et al.*, 2000; Klein and Kuneja, 1997), and NASBA could be a promising alternative tool for its study.

II. OBJECTIVES

"*Si uno no tiene cualidades para ser un artista,*
¿qué otra cosa puede ser sino investigador?"

Max Dellbrück

OBJECTIVES

The main objective of this Thesis work has been to develop molecular tools for the detection, identification and quantification of food-borne pathogens. Nowadays, a huge number of bacterial pathogens has been reported of interest for the Food Industry; which show a very different biology, ecology and physiology.

Thus, a study that intends to develop diagnostic molecular tools for this wide class of microorganisms is unrealistic, and thus a serious, rational objective should be circumscribed to a limited number of foodborne pathogens. The selection of candidate microorganisms for this study is based on two premises: its impact produced on the economy and public health; and its emergent character.

Hence, three of the most relevant food-borne pathogens were selected for this study: *Salmonella* spp., *Listeria monocytogenes* and *Mycobacterium avium* subsp. *paratuberculosis*. *Salmonella* spp. is one the major concern in Public Health, both for its enormous incidence and for economic repercussion. The significance of *L. monocytogenes* has been increased since the notable food-related outbreaks happened in 1980's. *Mycobacterium avium* subsp. *paratuberculosis* is a relevant veterinary pathogen causing of the endemic disease called paratuberculosis. It has been recently hypothesised it could play a role on the etiopathogenesis of Crohn's disease, being the potential vehicles of transmission to humans food products and water. Thus, it can be considered as an emergent foodborne pathogen.

In order to achieve the main objective of this Thesis work, (1) procedures for purification of bacterial nucleic acids from different food matrixes will be developed as well as (2) protocols for amplifying target genes. Furthermore, (3) internal amplification controls (IAC) will be developed to assist the interpretation of the diagnostic results.

1.   **Nucleic acid purification procedures (optimisation and use).**

     a.   **Comparison of different protocols for genomic DNA isolation from a food model product.** Two procedures to extract bacterial genomic DNA from a model food product, a first one based on an available commercial kit and a second one based on an open formula, will be evaluated. The quality and quantity of the bacterial genomic DNA will be compared using fluorescence-based techniques and RTi-PCR.

     b.   **Comparison of different protocols for total RNA isolation from bacterial samples.** Different available protocols for total RNA isolation from bacteria will be evaluated. The quality and quantity of total RNA will be compared using fluorescence-based techniques. The protocol producing the best results will be optimised with the incorporation of a RNA stabilization reagent.

2.   **Amplification methods**

     a.   *Listeria* **spp.**

          i.   **Detection, identification and quantification of *Listeria* spp., *L. innocua* and *L. monocytogenes* in uniplex and duplex formats using RTi-PCR.** RTi-PCR assays, both based on universal and sequence-specific systems, will be developed to detect, identify and quantify *Listeria* spp., *L. innocua* and *L. monocytogenes*. In addition, RTi-PCR assays will be adapted to duplex format for simultaneous quantitative detection of *Listeria* spp. and *L. monocytogenes*.

          ii. **Quantitative detection of *L. monocytogenes* in different food samples using RTi-PCR.** Different pre-PCR procedures for meat products and fish products will be evaluated. The effect of different extraction buffers and the incubation with

different enzymes will be tested. Subsequently, the most suitable procedure for each food category will be combined to RTi-PCR assay for quantitative detection of *L. monocytogenes* in each food sample.

b. *Mycobacterium avium* **subsp.** *paratuberculosis* **(*MAP*)**

   i. Quantitative detection of *MAP* in food and clinical samples using RTi-PCR. Suitable *MAP* sequences for RTi-PCR will be identified, and a *MAP*-specific RTi-PCR assay will be developed and optimised. Specific pre-amplification procedures for the extraction of *MAP* nucleic acids from milk and water will be developed and combined with the RTi-PCR assay in order to be applied to water and milk samples. Finally, the RTi-PCR based method will be applied to clinical samples of Crohn's disease patients.

   ii. Detection of *MAP* in food samples using RTi-NASBA. Suitable *MAP* sequences for NASBA will be identified, and a *MAP*-specific RTi-NASBA assay will be developed and optimised. In addition, the capacity of this technique to detect living *MAP* will be also evaluated. Specific pre-amplification procedures for the extraction of *MAP* nucleic acids from milk and water will be developed and combined with the NASBA assay in order to be applied to water and milk samples.

c. *Salmonella*

   i. Optimisation of an RTi-PCR method for the identification of *Salmonella* spp. An RTi-PCR assay for the identification of *Salmonella* spp. to be used directly on an agar-grown colony will be optimised. It will be evaluated through multi-center ring trial.

3. Tools to assist the interpretation of the diagnostic results: Internal Amplification Control.

a. IAC for RTi-PCR. An IAC for RTi-PCR will be developed and optimised taking into account the most important RTi-PCR parameters.

b. IAC for RTi-NASBA. A construction strategy will be drawn to produce adequate IACs for RTi-NASBA. A practical example of IAC for a *MAP*-specific NASBA will be designed and built; and its use to test the inhibition of nucleic acid amplification (and thus to asses the reliability of the NASBA results) will be evaluated.

III. MATERIALS AND METHODS

"PCR has transformed molecular biology through vastly extending the capacity to identify, manipulate and reproduce DNA. It makes abundant what was once scarce – the genetic material required for experimentations."

Paul Rabinow

Making PCR, A Story of Biotechnology, University of Chicago Press, 1996

III.1 MATERIALS

III.1.1 Bacterial strains

Table III.1. *Listeria* spp. strains.

Species	Strain	Other designations	Serotype	Source
L. monocytogenes	ATCC[a] 5577*		1/2c	Collection
"	CECT[b] 911		1/2c	Collection
"	CECT 932		1/2a	Collection
"	CECT 933		3a	Collection
"	CECT 934		4a	Collection
"	CECT 935		4b	Collection
"	CECT 936		1/2b	Collection
"	CECT 937		3b	Collection
"	CECT 938		3c	Collection
"	CECT 940		4d	Collection
"	CECT 4031[T]	ATCC 15313[T]	1/2a	Collection
"	CECT 4032		4b	Collection
"	CECT 5725		6	Collection
"	CECT 5366		4b	Collection
"	UdG[c] 1010	CTC[h] 1010	1/2c	Food plant, meat
"	UdG 1011	CTC 1011	1/2c	Food plant, meat
"	UdG 1034	CTC 1034	4b	Food plant, meat
"	NC[d] Mi-3		3b	Cheese
"	NC Fe-2			Chicken
"	NC Fe-4			Paté
"	NC Fi-2		1/2b	Trout
"	NC Fi-4			Smoked salmon
"	NC U-3			Environment
"	NC U-4			Environment
"	NC Me-3			Clinical, human
"	NC Me-4			Clinical, human
"	NC Ve-1			Clinical, human
"	NC Ve-3			Milk
"	NC Re-9	NCTC[i] 11994	4b	Collection
"	NC Re-10	ATCC 5579	4c	Collection
"	PAM[e] 35	NCTC 7973, SLCC[j] 2371	1/2a	Collection
"	PAM 484	SLCC 2755	1/2b	Collection
"	PAM 485	NCTC 5348, SLCC 2373	1/2c	Collection
"	PAM 486	ATCC 19113, SLCC 2373	3a	Collection
"	PAM 487	SLCC 2540	3b	Collection
"	PAM 489	NCTC 5214, SLCC 2374	4a	Collection
"	PAM 491	NCTC 10527, SLCC 2375	4b	Collection
"	PAM 493	ATCC 19116, SLCC 2376	4c	Collection
"	PAM 494	NCTC 10888, SLCC 2377	4d	Collection
"	PAM 495	SLCC 2482	7	Collection
"	PAM 358	EGD	1/2a	Collection
"	PAM 61		1/2a	Cheese
"	PAM 62		1/2b	Cheese
"	PAM 70		4b	Cheese
"	PAM 75		3b	Cheese
"	PAM 68		1/2c	Environment
"	PAM 80		3c	Environment
"	PAM 9		4b	Clinical, ovine
"	PAM 51		1/2c	Clinical, human
"	PAM 348		1/2b	Clinical, human
"	PAM 349		4b	Clinical, human
"	PAM 602		1/2a	
L. innocua	DSMZ[f] 20649*		6a	Collection
"	CECT 910		6a	Collection
"	CECT 4030			Collection
"	CECT 5376			Collection
"	CECT 5377			Collection
"	CECT 5378			Collection
"	UdG 1012	CTC 1012		Food plant, meat

(cont.)

Species	Strain	Designation	Serotype	Source
L. innocua	UdG 1014	CTC 1014		Food plant, meat
"	NC IN-1			Shrimps
"	NC IN-2			Ham
"	NC IN-12	ATCC 5578		Collection
"	NC IN-17			Cheese
"	PAM 152	ATCC 33091, SLCC 3423	6b	Collection
"	PAM 153	ATCC 33090	6a	Collection
"	PAM 154	SLCC 3379	6a	Collection
"	PAM 443			
"	PAM 490	NCTC 10528, SLCC 4951	4ab	Collection
"	PAM 550		6b	
"	PAM 569		6b	Meat
"	PAM 583		6b	Milk
L. grayi	CECT 931[*]			Collection
"	CECT 942			Collection
"	CECT 4181			Collection
"	NC GR-1			Milk
"	NC GR-3	DSM 20601		Collection
"	PAM 450	SLCC 3322	-	Collection
"	PAM 466	SLCC 4425	-	Collection
L. seeligeri	CECT 917[T]			Collection
"	CECT 939[g]		1/2b	Collection
"	CECT 941[g]			Collection
"	CECT 5339		6b	Collection
"	CECT 5340			Collection
"	CECT 5341			Collection
	CECT 5342			Collection
"	NC SE-1			Collection
"	NC SE-3			Salad
"	PAM 600	DSM 20751	1/2b	Collection
"	PAM 498	SLCC 5921	1/2b	Collection
"	PAM 499	SLCC 3954, CIP[k] 100100	1/2b	Collection
"	PAM 606		1/2b	
"	UdG 1024	CTC 1024		Food plant, meat
L. welshimeri	PAM 497[T]	SLCC 5334, CIP 8149[T]	6a	Collection
"	CECT 919[T]		6a	Collection
"	CECT 5370		1/2b	Collection
"	CECT 5371		6a	Collection
"	CECT 5372			Collection
"	CECT 5380			Collection
"	UdG 1013	CTC 1013		Food plant, meat
"	NC We-1	DSM 20650		Collection
"	NC We-3			Salami
L. ivanovii	PAM 424	ATCC 19119	5	Collection
"	PAM 55		5	Clinical, ovine
"	CECT 913		5	Collection
"	CECT 5368		5	Collection
"	CECT 5369			Collection
"	CECT 5373		5	Collection
"	CECT 5374			Collection
"	CECT 5375			Collection
"	CECT 5379			Collection
"	UdG 2001		5	Clinical, caprine
"	UdG 2002		5	Clinical, caprine
"	UdG 2003		5	Clinical, caprine
"	UdG 2004		5	Clinical, ovine
"	UdG 2005		5	Clinical, ovine

(cont.)				
L. ivanovii	UdG 2006		5	Clinical, ovine
"	UdG 2007		5	Clinical, ovine
"	NC Iv-1		5	Milk
"	NC Iv-3	DSM 20750	5	Collection

ᵃ American Type Culture Collection.
ᵇ Spanish Type Culture Collection, Valencia, Spain.
ᶜ Collection of Food Microbiology, Department of the University of Girona, Spain.
ᵈ Kindly provided by Dr. Nigel Cook. Central Science Laboratory, Sand Hutton, York, UK.
ᵉ Collection of the Microbial Pathogenesis Group, Veterinary Molecular Microbiology Section, University of Bristol, UK.
ᶠ German Collection of Microorganisms.
ᵍ Recently assigned to the *L. seeligeri* species.
ʰ Collection of Food Microbiology Unit, Meat Technology Centre, Monells (Girona), Spain.
ⁱ National Type Culture Collection, UK.
ʲ H.P.R. Seeliger's Special *Listeria* Culture Collection.
ᵏ Institut Pasteur Collection, France.
ᵀ Type strain.

Table III.2. *Salmonella* spp. strains.

Species	Strain no	Subgenus[a]	Serogroup
Salmonella enterica Abony	CECT[b]545	I	O:4 (B)
Salmonella enterica Abortusbovis	CECT 995	I	O:4 (B)
Salmonella enterica Abortusequi	CECT 952	I	(B)
Salmonella enterica Agona	UdG[c] 686	I	O:4 (B)
Salmonella enterica Alburquerque	UdG 4321	I	O:6,14 (H)
Salmonella enterica Amsterdam	UdG 677	I	O:3,10 (E_1)
Salmonella enterica Arizonae	CECT 4395	III	
"	UdG 1048	III	
"	UdG 1952	III	
Salmonella enterica Blockley	CECT 4370	I	O:6,8 (C_2)
Salmonella bongori	UdG 1058	---	
Salmonella enterica Braenderup	CECT 921	I	O:6,7 (C_1)
Salmonella enterica cholerasuis	CECT 915[T]	I	O:6,7 (C_1)
Salmonella enterica Derby	UdG 688	I	O:4 (B)
Salmonella enterica Dublin	UdG 826	I	O:9,12 (D_1)
"	UdG 828	I	O:9,12 (D_1)
Salmonella enterica Enteritidis	CSL[d] 1015	I	O:9,12 (D_1)
"	CECT 4300	I	O:9,12 (D_1)
"	UdG 784	I	O:9,12 (D_1)
"	UdG 822	I	O:9,12 (D_1)
"	CSL E2187		O:9,12 (D_1)
Salmonella enterica Falkensee	UdG 942	I	O:3,10 (E_1)
Salmonella enterica Gallinorum	CECT 4182	I	(D_1)
Salmonella enterica Goldcoast	CECT 4373	I	O:6,8 (C_2)
Salmonella enterica Heidelberg	UdG 755	I	O:4 (B)
Salmonella enterica Hontenae	UdG 1054	IV	
Salmonella enterica Indica	UdG 1062	---	
Salmonella enterica Infantis	UdG 685	I	O:6,7 (C_1)
"	UdG 793	I	O:6,7 (C_1)
"	UdG 936	I	O:6,7 (C_1)
Salmonella enterica London	CECT 4376	I	O:3,10 (E_1)
Salmonella enterica Mbandaka	UdG 985	I	O:6,7 (C_1)
Salmonella enterica Minnesota	CECT 456	I	O:21 (L)
Salmonella enterica Muenchen	CECT 4378	I	O:6,8 (C_2)
Salmonella enterica Ohio	CECT 4379	I	O:6,7 (C_1)
Salmonella enterica Panama	CECT 702	I	O:9,12 (D_1)

(cont.)

Salmonella enterica Paratyphi B	CECT 554	I	O:4 (B)
Salmonella enterica Postdam	CECT 4382	I	O:6,7 (C_1)
Salmonella enterica Saint-paul	UdG 909	I	O:4 (B)
Salmonella enterica Salamae	CECT 4000[T]	II	
"	UdG 1042	II	
Salmonella enterica Stanley	CECT 4141	I	O:4 (B)
Salmonella enterica Senftenberg	CECT 4565	I	O:1,3,19 (E_4)
Salmonella enterica Taksony	CECT 4385	I	O:1,3,19 (E_4)
Salmonella enterica Typhi	CECT 409	I	O:9,12 (D_1)
Salmonella enterica Typhimurium	CECT 4594	I	O:4 (B)
"	UdG 756	I	O:4 (B)
"	UdG 728	I	O:4 (B)
"	UdG 888	I	O:4 (B)
"	UdG 914	I	O:4 (B)
"	CSL FMG2	I	O:4 (B)
Salmonella enterica Urbana	CECT 4151	I	O:30 (N)
Salmonella enterica Virchow	UdG 781	I	O:6,7 (C_1)

[a] According to Bergey's Manual of Systematic Bacteriology (Le Minor, 1984).
[b] Spanish Type Culture Collection (CECT), Valencia, Spain.
[c] Collection of Food Microbiology Department of the University of Girona, Spain.
[d] Collection of Food Microbiology Group, Central Science Laboratory, UK.
[T] Type strain.

Table III.3. *Mycobacterium* spp. strains.

Species	Strain
M. avium subsp. *paratuberculosis*	RIVM[a] 97-1863
"	RIVM 97-1855
"	RIVM 97-1090
"	RIVM 97-1779
"	RIVM 97-1516
"	RIVM 97-2186
"	RIVM 97-1796
"	RIVM 99-0016
"	RIVM 99-1876
"	RIVM 99-1574
"	RIVM 99-1938
"	RIVM 01-0029
"	RIVM 99-2210
"	RIVM 01-0035
"	RIVM 99-2212
"	RIVM 01-02231
"	RIVM 01-02226
"	ATCC[b] 19698[T]
M. avium complex	RIVM 96-01246
"	RIVM 96-01238
"	RIVM 97-00615
"	RIVM 97-00622
"	RIVM 97-01643
"	RIVM 98-1017
"	RIVM 96-01127
M. bovis	RIVM BCG P3
"	RIVM 97-2262
M. intracellulare	RIVM 97-01388
M. kansasii	CSL[c] 10001
"	RIVM 97-02355
M. malmoense	RIVM 98-01168
M. marinum	RIVM 98-1853
M. silvaticum	RIVM 98-0851
"	RIVM 98-01574
M. tuberculosis	RIVM 96-01604

[a] Collection of National Institute of Public Health and the Environment (RIVM), The Netherlands.

Table III.4. Other bacterial strains.

Species	Strain	Other designations	Source
Aeromonas hydrophila subsp. *hydrophila*	NCTC[a] 8049		Collection
Acinetobacter calcoaceticus	NCTC 7844		Collection
Bacillus cereus	PAM[b] 871	NCTC 7464	Collection
"	NCTC 11145		Collection
Bacillus subtilis	PAM 870	NCTC 10400	Collection
Brochothrix thermosphacta	UdG[c] 1510		Food plant, meat
"	PAM 873		Collection
Citrobacter freundii	PAM 878	ATCC[d] 8090	Collection
"	CSL[e] 1516		
Corynebacterium bovis	NCTC 3224		
Enterobacter aerogenes	PAM 863	NCTC 10006	Collection
Enterococcus faecalis	UdG 2708		Food plant, meat
"	PAM 872	NCTC 775	Collection
Enterococcus faecium	UdG 492		Food plant, meat
Enterococcus malodoratus	UdG 7007		Food plant, meat
"	UdG 7008		Food plant, meat
"	UdG 7009		Food plant, meat
Escherichia coli	CSL 25922		
Klebsiella aerogenes	PAM 862	NCTC 9528	Collection
Kleibsella pneumoniae	CSL 10T		
Kurthia gibsonii	NCIMB[e] 9758		
Kurthia gibsonii	PAM 876		Collection
Kurthia zopfii	PAM 875	ATCC 6900	Collection
Lactobacillus curvatus	UdG 742		Food plant, meta
"	UdG 759		Food plant, meat
"	UdG 1174		Food plant, meat
Lactococcus lactis subsp. *lactis*	NCTC 6681		Collection
Lactobacillus murinus	UdG 7004		Food plant, meat
"	UdG 7005		Food plant, meat
"	UdG 7006		Food plant, meat
Lactobacillus plantarum	UdG 305		Food plant, meat
Lactobacillus reuteri	UdG 7010		Food plant, meat
"	UdG 7011		Food plant, meat
"	UdG 7012		Food plant, meat
"	UdG 7013		Food plant, meat
Lactobacillus sakei	UdG 746		Food plant, meat
"	UdG 748		Food plant, meat
"	UdG 756		Food plant, meat
"	UdG 757		Food plant, meat
Lactococcus garvieae	UdG 7001		Food plant, meat
"	UdG 7002		Food plant, meat
"	UdG 7003		Food plant, meat
Lactococcus lactis	UdG 122		Food plant, meat
Leuconostoc carnosum	UdG 747		Food plant, meat
Micrococcus luteus	NCTC 8340		Collection
Nocardia spp.	NCTC 434		Collection
Pediococcus pentosaceus	UdG 745		Food plant, meat
Pediococcus acidolactici	UdG 771		Food plant, meat
Propionobacterium acnes	NCTC 737		Collection
Proteus vulgaris	CSL 4175		Collection
Pseudomonas aeruginosa	PAM 860		Collection
Pseudomonas fluorescens	NCTC 10038		Collection
Rhodococcus equi	CECT[g] 555[T]		Collection
Rhodococcus equi	NCTC 1621		Collection
Staphylococcus aureus	CECT 4520[T]		Collection
Staphylococcus aureus	ATCC 25923		Collection
"	PAM 868		Collection
Staphylococcus epidermidis	PAM 869		Collection
Streptococcus faecalis	PAM 879		Collection
Streptococcus pyogenes	PAM 880		Collection

c Collection of Food Microbiology Department of the University of Girona, Spain.
d American Type Culture Collection (ATCC), USA.
e Collection of Food Microbiology Group, Central Science Laboratory, UK.
f National Collections of Industrial, Food and Marine Bacteria (NCIMB), UK.
g Spanish Type Culture Collection (CECT), Valencia, Spain.
T Type strain.

III.1.2   Bacterial culture media

+ Brain Heart Infusion (BHI) Broth

Formula	g/l
Calf brain infusion solids	12.5
Beef heart infusion solids	5.0
Proteose peptone	10.0
Glucose	2.0
ClNa	5.0
Disodium phosphate	2.5
pH 7.4 ± 0.2	

Directions: Dissolve 37 g in 1 l of distilled water. Mix well and distribute into final containers. Sterilise by autoclaving at 121 °C for 15 min. For solid medium, add 1.5 % (w/v) agar.

+ MRS (De Man, Rogosa, Sharpe) Broth

Formula	g/l
Peptone	10.0
`Lab-Lemco' powder	8.0
Yeast extract	4.0
Glucose	20.0
Sorbitan mono-oleate	1 ml
Dipotassium hydrogen phosphate	2.0
NaAc 3H$_2$O	5.0
Triammonium citrate	2.0
Magnesium sulphate 7H$_2$O	0.2
Manganese sulphate 4H$_2$O	0.05
pH 6.2 ± 0.2	

Directions: Add 52 g to 1 l of distilled water at approximately 60 °C. Mix until completely dissolved. Dispense into final containers and sterilise by autoclaving at 121 °C for 15 min. For solid medium, add 1.5 % (w/v) agar.

+ Blood Agar

Formula	g/l
`Lab-Lemco' powder	10.0
Peptone Neutralised	10.0
ClNa	5.0
Agar	15.0
pH 7.3 ± 0.2	

Directions: Suspend 40 g in 1 l of distilled water. Bring to the boil to dissolve completely. Sterilise by autoclaving at 121 °C for 15 min. Cool the Base to 50 °C and add 7 % of defibrinated Horse Blood. Mix with gentle rotation and pour into Petri dishes.

+ FRASER Broth

Fraser medium

Formula	g/l
Proteose peptone	5.0
Tryptone	5.0
`Lab-Lemco' powder	5.0
Yeast extract	5.0
ClNa	20.0
Di-sodium hydrogen phosphate	12.0
Potassium dihydrogen phosphate	1.35
Aesculin	1.0
Lithium chloride	3.0
pH 7.2 ± 0.2	

Fraser Selective Supplement

Ferric ammonium citrate	0.25 g
Nalidixic acid	10.0 mg
Acriflavine hydrochloride	12.5 mg
to supplement 500 ml of Fraser medium	

Directions: Suspend 28.7 g of Fraser Medium in 500 ml of distilled water. Sterilise by autoclaving at 121 °C for 15 min, bring to the boil to dissolve completely. Cool to 50°C and aseptically add the contents of one vial of Fraser Selective Supplement reconstituted with 5ml of EtOH/sterile water (1:1). Mix well and distribute into sterile containers.

+ Half Fraser Broth

Fraser medium

Formula	g/l
Proteose peptone	5.0
Tryptone	5.0
`Lab-Lemco' powder	5.0
Yeast extract	5.0
ClNa	20.0
Di-sodium hydrogen phosphate	12.0
Potassium dihydrogen phosphate	1.35
Aesculin	1.0
Lithium chloride	3.0
pH 7.2 ± 0.2	

Half Fraser Selective Supplement

Ferric ammonium citrate	112.50 mg
Nalidixic acid	2.25 mg
Acriflavine hydrochloride	2.8125 mg
To supplement 225 ml of Fraser medium	

Directions: Suspend 14.35 g of Fraser Broth in 225 ml of distilled water. Sterilise by autoclaving at 121 °C for 15 min. Cool to 50 °C and aseptically add the contents of one vial of Half Fraser Selective Supplement reconstituted with 4 ml of (1:1) EtOH/sterile distilled water and mixed gently to dissolve. Mix well and distribute into final containers.

+ PALCAM Agar

PALCAM Agar Base

Formula	g/l
Columbia Blood Agar Base	39.0
Yeast extract	3.0
Glucose	0.5
Aesculin	0.8
Ferric ammonium citrate	0.5
Mannitol	10.0
Phenol red	0.08
Lithium chloride	15.0
pH 7.2 ± 0.2	

PALCAM Selective supplement

Polymyxin B	5.0 mg
Acriflavine hydrochloride	2.5 mg
Ceftazidime	10.0 mg
to supplement 500 ml of Palcam Agar Base	

Directions: Suspend 34.5 g of Palcam Agar Base in 500 ml of distilled water. Bring gently to the boil to dissolve completely. Sterilise by autoclaving at 121° C for 15 min. Cool to 50° C and aseptically add the PALCAM Selective Supplement, reconstituted with 2 ml of sterile distilled water. Mix well and pour into sterile Petri dishes.

+ Listeria Selective Agar (Oxford Formulation)

Listeria Selective Agar Base (Oxford Formulation)

Formula	g/l
Columbia Blood Agar Base	39.0
Aesculin	1.0
Ferric ammonium citrate	0.5
Lithium chloride	15.0
pH 7.0 ± 0.2	

Listeria Selective Supplement (Oxford Formulation)

Cycloheximide	200.0 mg
Colistin sulphate	10.0 mg
Acriflavine	2.5 mg
Cefotetan	1.0 mg
Fosfomycin	5.0 mg
to supplement 500 ml of Listeria Selective Agar Base	

Directions: Suspend 27.75 g of the Listeria Selective Agar Base (Oxford Formulation) in 500 ml of distilled water. Bring gently to the boil to dissolve. Sterilise by autoclaving at 121°C for 15 min. Cool to 50°C and aseptically add the contents of one vial of Listeria Selective Supplement (Oxford Formulation) reconstituted with 5 ml of 70 % EtOH. Mix well and pour into sterile Petri dishes.

+ LB Medium (Luria Bertani Medium)

Formula	g/l
Tryptone	10.0
Yeast extract	5.0
ClNa	10.0
pH 7.0 ± 0.2	

Direction: Suspend in 1 l of distilled water. Shake until the solutes have dissolved. Adjust the pH to 7.0 with 5 N NaOH (≈ 0.2 ml). Sterilise by autoclaving at 121 °C for 15 min. For solid medium, add 1.5 % (w/v) agar.

+ ID Medium

Formula	g/l
Peptone	1.0
ClNa	10.0
pH 7.0 ± 0.2	

Direction: Suspend in 1 l of distilled water. Bring gently to the boil to dissolve. Sterilise by autoclaving at 121 °C for 15 min.

+ Herrold's Egg Yolk Agar Slant with Mycobactin J and ANV (Becton, Dickinson and Co.)

Formula	g/l
Pancreatic Digest of Casein	9.0
Beef Extract	2.7
ClNa	4.0
Sodium pyruvate	4.1
Malachite green	0.1
Amphotericin B	0.05
Mycobactin J	0.002
Agar	15.3
Nalidixic acid	0.05
Vancomycin	0.05
Egg yolk	100.0 ml
Glycerol	27.0 ml

III.1.3   Enzymes, reagents and materials

III.1.3.1   Enzymes

- α-amylase (Sigma-Aldrich co., Saint Louis, USA).
- Alkaline phosphatase (Boehringer Mannheim, Mannheim, Germany)
- Calf intestinal phospatase (Sigma-Aldrich co., Saint Louis, USA).
- DNA Polymerase Ampli*Taq* Gold™ (Applied Biosystems-Roche Molecular Systems Inc., Branchburg, Germany).
- DNA Polymerase "BioTaq™ DNA polymerase" (Bioline, Luckenwalde, Germany).
- DNA Polymerase Jumpstart™ *Taq* (Sigma-Aldrich co., Saint Louis, USA).
- DNA Polymerase r*Tth* (Applied Biosystems-Roche Molecular Systems Inc., Branchburg, Germany).
- DNA Polymerase TaKaRa Ex Taq™ (TaKaRa Shuzo, Japan).
- DNA Polymerase TaKaRa LA Taq™ (TaKaRa Shuzo, Japan).
- DNA Polymerase (Amersham Pharmacia Biotech, Freiburg, Germany).
- DNA Polymerase (BioRad, Munich, Germany).
- Lysostaphin (Sigma-Aldrich co., Saint Louis, USA).
- Lysozyme (Sigma-Aldrich co., Saint Louis, USA).
- Proteinase K (Sigma-Aldrich co., Saint Louis, USA).
- Restriction endonucleases (Boehringer Mannheim, Manheim, Germany).
- Ribonuclease A from bovine pancreas (Sigma-Aldrich co., Saint Louis, USA).
- T4-DNA-ligase (Boehringer Mannheim, Mannheim, Germany).
- Trypsin (Sigma-Aldrich co., Saint Louis, USA).
- Uracil N-glycosylase (UNG) *AmpErase* (Applied Biosystems-Roche Molecular Systems Inc., Branchburg, Germany).

III.1.3.2   Reagents and materials

- Agarose Molecular Biology grade (Roche Molecular Systems Inc., Branchburg, Germany).
- Acetic acid (Sigma-Aldrich co., Saint Louis, USA).
- Agar (Oxoid, Hampshire, UK).
- Ammonium acetate (Merck, Darmstadt, Germany).
- β-Mercaptoethanol (Sigma-Aldrich co., Saint Louis, USA).
- Bromophenol blue (Sigma-Aldrich co., Saint Louis, USA).
- Bovine Serum Albumin (Promega, Madison, USA).
- Chelex® 100 (BioRad, Munich, Germany).
- Chloroform (Sigma-Aldrich co., Saint Louis, USA).
- CTAB (cetiltrimetilamonium bromide) (Sigma-Aldrich co., Saint Louis, USA).
- DNA molecular weight markers (BioRad, Munich, Germany).
- DNA molecular weight markers (Roche Molecular Systems Inc., Branchburg, Germany).
- DNA molecular weight markers (Invitrogen, Carlsbad, CA, USA).
- dATP, dCTP, dGTP, dTTP and dUTP (Sigma-Aldrich co., Saint Louis, USA).

- DTT (Promega, Madison, USA).
- EDTA (Sigma-Aldrich co., Saint Louis, USA).
- Glycine (Sigma-Aldrich co., Saint Louis, USA).
- EB Buffer (Qiagen, Hilden Germany).
- Ethanol (Merck, Darmstadt, Germany).
- Ethidium bromide (Sigma-Aldrich co., Saint Louis, USA).
- *Escherichia coli* DH5α.
- *Escherichia coli* XL1-Blue.
- Ficoll 400 (Merck, Darmstadt, Germany).
- Glass beads 22.222.0001 (Retsch, Germany).
- Glycerol (Merck, Darmstadt, Germany)
- Guanidinium thiocyanate (Sigma-Aldrich co., Saint Louis, USA).
- Isoamylic alcohol (Merck, Darmstadt, Germany).
- Isopropanol (Merck, Darmstadt, Germany).
- Latex Gloves (Satelec).
- Miracloth (Calbiochem Coroporation).
- 11-μm Nylon membrane (Millipore).
- Oligonucleotides (Applied Biosystems, Warrington, UK; MWG-Biotech AG, Ebensburg, Germany; Sigma-Genosys Ltd., Pampisford, UK).
- Peptone (Oxoid, Hampshire, UK).
- Phenol (Sigma-Aldrich co., Saint Louis, USA).
- Phosphate-buffered Saline (PBS Dulbecco's, Gibco, Paisley, Scotland)
- PicoGreen® dsDNA quantification reagent (Molecular Probes, Inc., Eugene, USA).
- Pioxietilensorbitan monolaurate (Tween 20) (Sigma-Aldrich co., Saint Louis, USA).
- Pipette filter tips (Genotek, Inmunogenetics and Labclinics).
- Pipettes Research (3 ml, 1 ml, 200 μl, 100 μl, 20 μl, 10 μl, 2.5 μl) (Eppendorf).
- Polyethylenglycol 6000 (Merck, Darmstadt, Germany).
- Potassium acetate (Merck, Darmstadt, Germany).
- RiboGreen™ RNA quantification Reagent (Molecular Probes, Inc., Eugene, USA)
- RNAprotect Bacteria Reagent (Qiagen, Hilden, Germany).
- RNase/DNase-free water (Sigma-Aldrich co., Saint Louis, USA).
- rNTP mix (Promega, Madison, USA).
- Sarcosyl (N-Lauril Sarcosine) (Sigma-Aldrich co., Saint Louis, USA).
- SDS (Sigma-Aldrich co., Saint Louis, USA).
- Sodium acetate (Merck, Darmstadt, Germany).
- Sodium chloride (Sigma-Aldrich co., Saint Louis, USA).
- Sodium citrate (Merck, Darmstadt, Germany).
- Sodium hydroxide (Sigma-Aldrich co., Saint Louis, USA).
- Sodium hypochlorite (Sigma-Aldrich co., Saint Louis, USA).
- Stomacher bags (Biochek).
- Streptavidin paramagnetic beads (Promega, Madison, USA).
- Surgical edges (Swann-Morton, UK).
- SYBR® Green I (Sigma-Aldrich co., Saint Louis, USA).
- SYBR® Green II (Sigma-Aldrich co., Saint Louis, USA).
- 50× TAE (Sigma-Aldrich co., Saint Louis, USA).
- 100× TE (Sigma-Aldrich co., Saint Louis, USA).
- Tris (Sigma-Aldrich co., Saint Louis, USA).
- Triton X-100 (Sigma-Aldrich co., Saint Louis, USA).
- Trypsin (Sigma-Aldrich co., Saint Louis, USA).
- Tryptone (Oxoid, Hampshire, UK).
- 0.5-ml ABI 310 tubes (Applied Biosystems).
- 30-ml and 15-ml tubes (Falcon).
- 2-ml, 1.5-ml and 0.5-ml micro-centrifuge tubes (Eppendorf).

- UniPrimer™ (Intergen Co., Purchase, USA).
- Xylen cianol FF (Sigma-Aldrich co., Saint Louis, USA).
- Yeast- Extract (Difco-Laboratories, Detroit, USA).

III.1.4 Commercial kits

- Access RTPCR system (Promega, Madison, USA).
- ABI PRISM® Big Dye™ Terminator Cycle Sequencing kit (Applied Biosystems; Foster city, USA).
- GENECLEAN® II kit (BIO101, Q-BIOgene, Carlsbad, USA).
- pGEM®-T Easy Vector System I (Promega, Madison, USA).
- QIAEX II DNA extraction from agarose gels (Qiagen, Hilden Germany).
- QIAGEN® Plasmid Midi Kit (Qiagen, Hilden Germany).
- QIAquick™ PCR and Gel Purification kit (Qiagen, Hilden Germany).
- NucliSens® Basic Kit Isolation Module (bioMérieux bv, The Netherlands).
- NucliSens® Basic Kit Amplification Module (bioMérieux bv, The Netherlands).
- NucliSens® Basic Kit Detection Module (bioMérieux bv, The Netherlands).
- RNase ONE™ Ribonuclease (Promega, Madison, USA).
- RNase-Free DNase Kit (Qiagen, Hilden, Germany).
- RNeasy® Mini Kit (Qiagen, Hilden, Germany).
- RNeasy MiniElute Cleanup Kit (Qiagen, Hilden, Germany).
- RQ1 RNase-free DNase kit (Promega, Madison, USA).
- S1 nuclease kit (Promega, Madison, USA).
- TaqMan™ PCR Core Reagent Kit (Applied Biosystems-Roche Molecular Systems Inc., Branchburg, Germany).
- T7 RNA polymerase kit (Promega, Madison, USA).
- Universal Master Mix (Applied Biosystems-Roche Molecular Systems Inc., Branchburg, Germany).
- Wizard® Genomic DNA Purification kit (Promega, Madison, USA).

III.1.5 Apparatus and equipment

- ABI PRISM® 310 DNA Sequencer (Applied Biosystems).
- ABI PRISM® 377 DNA Sequencer (Applied Biosystems).
- Cabinet CFL Telstar BV 30/70 (Telstar).
- Cabinet Clean Bench (Sanyo).
- Centrifuge Megafuge 1.0 (Haeraus).
- Centrifuge biofuge Pico (Haeraus).
- Centrifuges mod. J2-21, J2-HS (Beckman Coulter Inc.).
- Centrifuge 5415 D (Eppendorf).
- Centrifuge 5810 R (Eppendorf).
- Centrifuge rotors JA-14, JS-13, JLA, NVTi65 (Beckman Coulter Inc.).
- Delta Dilutor (IUL Instruments).
- Electrophoresis tanks (BioRad).
- Electroporation apparatus (BioRad).
- Freezer -20 °C (Zanussi).
- Fridge (Zanussi).
- Haemocytometer BS 748 Improved Neubauer.Weber, England. Depth 0.1mm, 1/400mm² (Improved Neubauer).
- Image Analyser Automatic Counter (Protos, Synoptics).
- Luminescence Spectrometer LS50B (Perkin-Elmer Corp.).
- Magnetic stirrer mod. Agimatic-N (Selecta).
- Magnetic stirrer mod. Magnetic S (Selecta).

- Micro-centrifuge mod. Biofuge pico (Haeraus).
- Microscopy Olympus BX50 (Olympus).
- Microwave Genius (Panasonic).
- Minicentrifuge mod. C1200 (National labnet C.O.).
- Mixer Mill MM 300 (Qiagen).
- Multiblock P Selecta (Grant Boekel).
- NucliSens® ECL reader (bioMérieux bv).
- Orbital shaker mod. ABT 4/50 y ABT 4/10 (SBS).
- Orbital shaker mod. IKA K5501 (IKA).
- Parafilm M (Sigma-Aldrich co., Saint Louis, USA).
- Pasteur pipettes (Sarstedt).
- Petri dishes (Bibby Steriline Ltd).
- 96-well PCR plates (Applied Biosystems).
- 0.2-ml PCR tubes and strips (Applied Biosystems).
- PCR optical caps PCR (Applied Biosystems).
- pHmeter GLP21 (Crison).
- PowePack 300 (BioRad).
- Refrigerated incubator haker innoca 4335 (New Brunswick scientific).
- Robot coupé (Blixter 3).
- Spectrophotometer UV 160A (Shimadzu).
- Spectrophotometer 2000 series (Cecil).
- Spectrophotometer UV GeneQuant RNA/DNA Calculator (Amersham Pharmacia Biotech).
- Stomacher masticator (INL Instruments).
- Stratalinker UV (Stratagene).
- Swinnex (Millipore).
- TD-360 Mini-Fluorometer (Turner DesignsSunnyvale, USA).
- Thermocycler ABI PRISM® 7700 Sequence Device Systems (Applied Biosystems).
- Thermocycler ABI PRISM® 7900 Sequence Device Systems (Applied Biosystems).
- Thermocycler Eppendorf Master-Cycler gradient (Eppendorf).
- Thermocycler GenAmp® PCR System 9600 (Applied Biosystems).
- Thermocycler 96-Well GenAmp® PCR System 9700 (Applied BiosystemsUSA).
- Thermo-mixer mod.compact (Eppendorf).
- Thermosealer (ABgene).
- Ultra-Centrifuge mod. Optima LE-80K (Beckman Coulter Inc.).
- Ultra-low temperature Freezer MDF ar1186S (Sanyo).
- Ultra-low temperature Freezer VX 430 series 2(Jouan).
- Vortex TK3S (Tecnokartell).
- Vacuum pump XF54 13050 (Millipore).
- Water bath mod. Atom 131 (Biotron).
- Weight (Mettler AC88).
- Weight mod. FBL-410S (Heraeus).
- Weight mod. AB204-S (Mettler-Toledo).
- Weight mod. ARD GF-300 (A&D Comp).
- Whitley Automatic Spiral Plater (WASP) (dw Scientific).

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III.1.6   Sequencing and bioinformatics software tools

- *Blast-n 2.2.6 software*; http://www.ncbi.nlm.nih.gov/BLAST/ (National Center for Biotechnology Laboratory –NCBI–, USA).
- *Chromas v 1.45 software* (Griffith University, Queensland, Australia).
- *Clustal-W software*; http://www.ebi.ac.uk/cultalw/index.html (European Molecular Biology Laboratory –EMBL–, Hinxton, UK).
- *DNA*DNASTAR* software package software including EditSeq 4.03, PrimerSelect 4.03 MapDraw 4.03 and SeqMan 4.03 (Lasergene, DNASTAR Inc, Madison, USA).
- *Entrez-Pubmed search software* http://www.ncbi.nlm.nih.gov/entrez/query.fcgi (National Center for Biotechnology Laboratory –NCBI–, USA).
- *Fasta-3 software;* http://www.ebi.ac.uk/fasta33 (European Molecular Biology Laboratory –EMBL–, Hinxton, UK).
- *GCG Software*, Wisconsin Packet (Genetic Computer Group, Sequence Analysis Software Package).
- *Dissociation Curve Analysis* software v1.0 (Applied Biosystems division of Perkin Elmer Corp., Foster City, USA).
- *Kodak Digital Science 1d* (Eastman Kodak Company, Rochester, NY, USA).
- *Primer Express™ v2.0* software (Applied Biosystems division of Perkin Elmer Corp., Foster City, USA).
- *Quanti One™* software (Bio-Rad Laboratories Inc., Hercules, USA).
- *RNAstrucutre 3.71* (Isis Pharmaceuticals, Inc.).
- Science Direct search tool http://www.sciencedirect.com/
- *Sequence Detection System* software v1.7 (Applied Biosystems division of Perkin Elmer Corp., Foster City, CA, USA).
- SAS software system for Windows v8.0 (SAS Institute Inc., Cary, NC, USA).
- SPSS v11.0.1 (SPSS INC., Chicago, USA).
- *SRS6 software*; http://srss6.ebi.ac.uk/ (European Molecular Biology Laboratory –EMBL–, Hinxton, UK).
- FL WinLab software (Applied Biosystems division of Perkin Elmer Corp., Foster City, USA).

III.1.7 Oligonucleotides

Table III.5. Oligonucleotides used in the PhD Thesis.

Target	Gene	Use	Name	Type	Sequence
L. monocytogenes	*hly*	RTi-PCR	*hlyQF*	Forward primer	5'- CAT GGC ACC ACC AGC ATC T -3'
			hlyQF	Reverse primer	5'- ATC CGC GTG TTT CTT TTC GA -3'
			hlyQP	TaqMan® probe	5'- *FAM* - CGC CTG CAA GTC CTA AGA CGC CA - *TAMRA* -3'
		AmpliFluor	*hlyZ*	Primer Z	5' - act gaa cct gac cgt aca CAT GGC ACC ACC AGC ATC T - 3'
		Sequencing	*hly-F*	Forward primer	5'- TAA CGA CGA TAA AGG GAC AGC AGG ACT A -3'
			hly-R	Reverse primer	5'- AAT GAA TCA CGT TTT ACA GGG AGA A -3'
	iap	RTi-PCR	*iapQF*	Forward primer	5'- AAT CTG TTA GCG CAA CTT GGT TAA -3'
			iapQR	Reverse primer	5'- CAC CTT TGA TGG ACG TAA TAA TAC TGT T -3'
			iapQP	TaqMan® probe	5'- *FAM* - CAA CAC CAG CGC CAC TAC GGA CG - *TAMRA* -3'
		Sequencing	*P60-F*	Forward primer	5'- TAA AGG GAC TAC TGT TGA CG -3'
			P60-R	Reverse primer	5'- GCT TCT GTT GGT GCT TTA GGT GCT GTT TG -3'
L. innocua	*Lin02483*	RTi-PCR	*lipHQF*	Forward primer	5'- AAC CGG GCC GCT TAT GA -3'
			lipHQR	Reverse primer	5'- CGA ACG CAA TTG GTC ACG -3'
			lipHQP	TaqMan® probe	5'- *FAM* - TTC GAA TTG CTA GCG GCA CAC CAG T – *TAMRA* -3'
	23S rDNA	RTi-PCR	*lin23S-r*	Reverse primer	5'- TTC GCA GAA AGC GGA TTT G -3'
Listeria spp.	23S rDNA	RTi-PCR	*l23SQ-f*	Forward primer	5'- AGG ATA GGG AAT CGC ACG AA -3'
			l23SQ-r	Reverse primer	5'- TTC GCG AGA AGC GGA TTT -3'
			l23SQ-p	TaqMan® probe	5'- *VIC* - TCT CAC ACT CAC TGC TTG GAC GC - *TAMRA* -3'
M. avium subsp. *paratuberculosis*	IS900	RTi-PCR	*IS900QF*	Forward primer	5'- CCG GTA AGG CCG ACC ATT A -3'
			IS900QR	Reverse primer	5'- ACC CGC TGC GAG AGC A -3'
			IS900QP	TaqMan® probe	5'- *FAM* - CAT GGT TAT TAA CGA CGA CGC GCA GC – *TAMRA* -3'
	clone uni7 Sequence	RTi-NASBA	MAP57F	Forward primer	5'- CAA CGA CGA CCA AGA CGA -3'
			MAP57R	Reverse primer	5'- aat tct aat acg act cac tat agg gag aag gAG CAA ACC GAT CAC GAC A -3'
			MAP57MB	Molecular beacon	5'- *FAM* - <u>CGA TCG</u> CTG ATG AAA CCG AGC TCG <u>TCG ATC G</u> - *DABCYL* -3'
	Hed	NASBA	*MptbNAS1*	Forward primer	5'- <u>aat tct aat acg act cac tat agg g</u> *aga* GCG CCT TCG ACT ACA ACA AGA –3'
			MptbNAS2	Reverse primer	5'- **gat gca agg tcg cat atg ag** ATG CTG TGT TGG GCG TTA –3'
			Mptb-CP	Capture Probe	5'- biotin- TAT CGG CGT TGC GGG CCT TGC GTT T –3'
	Tuf	NASBA	*Tuf-1*	Forward primer	5'- **gat gca agg tcg cat atg a** TCA GGT CGG TGT GCC CTA –3'
			Tuf-2	Reverse primer	5'- <u>aat tct aat acg act cac tat agg g</u> *aga agg* CAT CAG CTG CTC GAC GGA –3'
			Tuf-CP	Capture Probe	5'- biotin- TGG TCG CGC TGA ACA AGG CCG ACA T –3'
	Hsp70	NASBA	*MapHSP-F1*	Forward primer	5'- **gat gca agg tcg cat atg ag** GGA ATC GCT TGT CTA CCA GA -3'
			MapHSP-R2	Reverse primer	5'- <u>aat tct aat acg act cac tat agg g</u> *aga agg* GAG CTT CTC CAT CGC CGA CTT GA -3'
			MapHSP-CP	Capture probe	5'- biotin- TCC CGA GGA GAC GCT GTC-3'

Target	Gene	Use	Name	Type	Sequence
Salmonella spp.	*invA*	*RTi-PCR*	StyinvAJHO2-left	Forward primer	5'- TCG TCA TTC CAT TAC CTA CC -3'
			StyinvAJHO2-right	Reverse primer	5'- AAA CGT TGA AAA ACT GAG GA -3'
			Target probe	TaqMan® probe	5'- *FAM* - TCT GGT TGA TTT CCT GAT CGC A - *TAMRA* -3'
	dnaK	*NASBA*	SDnaK1	Forward primer	5'- <u>aat tct aat acg act cac tat agg g</u> *aga* GGC AGT CGG TTC GTT GAT GAT A-3'
			SDnaK2	Reverse primer	5'- **gat gca agg tcg cat atg ag** CTT GAT GTG AAA GGT CAG A -3'
			SBIO74	Capture probe	5'- biotin- TGA AGA TTA TCT GGG CGA ACC G –3'
Brassica napus	*BnACCg8*	IAC construction	*hlyccAF*	Forward primer	5'- CAT GGC ACC ACC AGC ATC TGG TGA GCT GTA TAA TC -3'
			hlyccAR	Reverse primer	5'- ATC CGC GTG TTT CTT TTC GAG GCG CAG CAT C -3'
Chimerical DNA	IAC	IAC for RTi-PCR	*accAP*	TaqMan® probe	5'- VIC- AAC ACC TAT TAG ACA TTC GTT CCA TTG GTC GA –TAMRA -3'
viral hemorrhagic septicaemia virus		IAC construction	Flank-1-left	Forward primer	5'- AAA CGT TGA AAA ACT GAG GAT ATA CAC TCA TCC CTC CAA C -3'
			Flank-1-right	Reverse primer	5'- TCG TCA TTC CAT TAC CTA CCT TCA TGA GGA CAC CTG AGT T -3'
Chimerical DNA	IAC	IAC for RTi-PCR	Control Probe	TaqMan® probe	5'- *TET* - TTC ATG AGG ACA CCT GAG TTG A - *TAMRA* -3'
L. monocytogenes	*hly*	IAC construction	hly-F	Forward primer	5'- CCG GTA AGG CCG ACC ATT ACA TGG CAC CAC CAG CAT CT –3'
			hly-R	Reverse primer	5'- ACC CGC TGC GAG AGC AAT CCG CGT GTT TCT TTT CGA -3'
Chimerical DNA	IAC	IAC for RTi-PCR	*IACQP*	TaqMan® probe	5'- *VIC* - CGC CTG CAA GTC CTA AGA CGC CA - *TAMRA* -3'
Clavibacter michiganensis subsp. *sepedonicus*	Cms 16S rRNA	NASBA IAC construction	Af2	Forward primer	5'- CGA TGC AAC GCG AAG AAC -3'
			Ar	Reverse primer	5'- GGT TGG CCC CGG CAG TCT -3'
			IACF	Forward primer	5' aat tct aat acg act cac tat agg gag aag gCA CGC ACG ACC AAG ACG ACG ATG CAA CGC G 3'
			IACR	Reverse primer	5' AGC AAA CCG ATC ACG ACA GGT TGG CCC CGG CAG TCT 3'
Chimerical RNA	IAC	IAC for RTi-NASBA	MB1	Molecular beacon	5' HEX <u>CGC AGG</u> AAC GTG CAG AGA TGT GCG CCC <u>CTG CG</u> DABCYL 3'

Underlined lowercase: T7 promoter sequence.
Italic lowercase: GA-rich sequence following to the T7 promoter sequence.
bold lowercase: 20 bp-sequence that is specific for the detection probe (5'- ruthenium- GAT GCA AGG TCG CA-3')

III.2   METHODS

III.2.1   Storage of bacterial cultures

1. Add 0.5 ml of sterile 60 % glycerol to 1.5 ml of bacterial culture. Sterilise by autoclaving at 121 °C for 15 min.

2. Mix with a Vortex to ensure that the glycerol is evenly dispersed.

3. Transfer to a labelled storage tube equipped with a screw cap and an air-tight gasket.

4. Freeze in liquid nitrogen, and then transfer the tube to -70 ºC fro long-term storage.

5. To recover the bacteria, scrape the frozen surface of the culture with a sterile inoculating loop, and then immediately streak the bacteria that adhere to the needle onto the surface of an LB agar plate. Return the frozen culture to storage at -70 ºC. Incubate the plate overnight at 37 ºC.

III.2.2   Revival of freeze-dried cultures

1. Particularly in culture collections, bacterial strains are usually kept for long-term storage freeze-dried in special ampoules (Figure III.1). Check each culture thoroughly upon receipt. Ampoules containing freeze-dried cultures should be either revived he week after receipt or stored in a dark place at 5° C or lower (*e.g.* a refrigerator). They should not be stored exposed to light, particularly direct sunlight.

2. Determinate the most suitable medium and incubation conditions when first reviving strains to ensure optimal conditions for recovery.

3. Make a file cut on the ampoule at the mid-point of the cotton wool plug (Figure III.1) and crack the glass by applying a red hot wire or glass rod to the file cut. Allow air to enter slowly before gently removing the pointed part. If the ampoule proves difficult to crack, use the ampoule snappers (place one at either side of the file cut) and physically snap the ampoule using thick wadding or gloves to protect the operator. Discard the upper part of the ampoule and the cotton plug into a disinfectant solution.
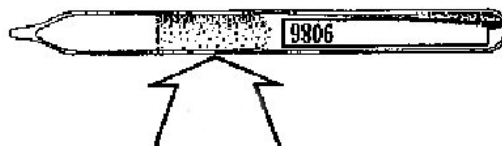
Figure III.1. Glass ampoule containing freeze-dried bacterial strains

4. Flame the open end of the ampoule, aseptically add 0.3 to 0.4 ml of a suitable liquid medium to the ampoule containing the freeze-dried material with a Pasteur pipette and mix well, avoiding frothing.

5. Transfer the total mixture into a test tube of the suitable broth medium (5 to 6 ml). Prepare more than one subculture and transfer the last few drops to an agar slant as a precaution against accidents.

6. Sub-cultures should be incubated at the optimum temperature for the organism under appropriate gaseous conditions. Given proper treatment and conditions, most freeze-dried cultures will grow out in a few days. However, some may exhibit a prolonged lag period and should be given twice the normal incubation time before discarding as nonviable.

7. Organisms should be sub-cultured at least twice before they can be optimally used in experiments.

III.2.3   Hemocytometry counting

1. Sample the cell suspension to be counted with a Pasteur pipette. Deliver each sample of cell suspension into chamber of the haemocytometer by capillary action. Avoid overflowing. Repeat sampling to lad the second chamber. Do not allow the cell suspension to dry on the haemocytometer.

2. Count the total number of cells in five of the nine large squares in each haemocytometer camber for a total of ten squares. The microscope field using a 10× objective and a 10× ocular should encompass the majority of one of the nine squares of the chamber and is a convenient magnification to use for counting. Cells overlapping the border on two sides of the square should be included in the cell count and not counted on the other two sides. If the initial dilutions results more than 50-100 cells/square, make further dilution to improve counting accuracy and speed the process of determining cell numbers.

3. Add the number of cells in a total of ten chambers (five corresponding each chamber) to give the number of cells per μl (1×10^{-4} ml/square × 10 squares = a volume of 10^{-3} ml). Multiply the total number of cells by 1000 to give the number of cells/ml in the sample.

4. Immediately after use, clean the haemocytometer and coverslip by rising in distilled H_2O followed by 70 % EtOH. Dry with lens paper.

III.2.4   Pre-treatment of food samples for bacterial genomic DNA extraction

a.   Filtration-based protocol

1. Weigh the food sample, usually 25 g for solid matrixes or 25 ml for liquid matrixes, and place it into a stomacher bag with 200-μm filter.

2. Dilute 1:10 with the appropriate culture media.

3. Apply the stomacher bag containing the bacterial solution into a stomacher for 1.5 min.

4. Place the bacterial solution in a sterile 10-ml tube.

5. Filter 5-10 ml of the bacterial solution trough a 22-25-μm Miracloth filter or 11-μm nylon filter.

6. If used 22-25-μm Miracloth filter, repeat the filtration with a new 11-μm nylon filter

7. Continue with a protocol for extraction of genomic DNA (III.2.5).

b.   Extraction of bacteria from milk

1. Place 25 ml of milk sample into a sterile 50-ml tube.

2. Add 3 ml Triton X-100 and mix vigorously using a vortex.

3. Add powdered trypsin to 1 % (w/v), and incubate at 37 °C for 30 min.

4. Pellet the cells by centrifugation at 2,500 × *g* for 30 min. Decant the supernatant carefully.

5. Resuspend the pellet in 1 ml of nuclease free-H_2O and mix gently.

6. Transfer the suspension to a sterile 1.5-ml micro-centrifuge tube.

7. Centrifuge at 13,000 × *g* for 5 min to compact the pellet.

8. Continue with the extraction of DNA from bacteria using the Wizard® Genomic DNA Purification protocol (III.2.5.d).

III.2.5   Extraction of genomic DNA from bacteria

a.   Chelex® 100 resin-based DNA extraction

<u>Preparation of 6 % Chelex® 100:</u> Immediately prior to use, resuspend 0.3 g Chelex® 100 in 5 ml double-destilled H₂O (not TE buffer). The Chelex® 100 suspension should be mixed at moderate speed on a magnetic stirrer to maintain the matrix in suspension. Always pipette Chelex® 100 suspension using large bore pipette tips, such as 1,000 µl pipette tip. Stirr the suspension during pipetting the suspension. Prepare always a fresh resuspension.

<u>Protocol:</u>

1. Transfer 1 ml bacterial culture to a clean 1.5-ml micro-centrifuge tube. Spin in a micro-centrifuge for 5 min at 10,000 × *g* and 4°C.

2. Carefully discard the supernatant using a pipette.

3. Repeat the steps 1 and 2.

4. Resuspend the pellet in 100 µl 6 % Chelex 100 suspension by vortexing and incubate at 56°C for 20 min. Vortex at high speed for 10 s.

5. Incubate at 100°C for 8 min. Vortex at high speed for 10 s and immediately chill on ice.

6. Centrifuge for 5 min at 14,000 × *g* and 4°C. Carefully transfer 50 µl of the supernatant into a new micro-centrifuge tube.

7. Centrifuge for 5 min at 14,000 × *g* and 4°C. Carefully transfer 20 of µl the supernatant into a new micro-centrifuge tube.

8. Store the supernatant at –20°C.

b.   DNA extraction with CTAB (protocol 1)

1. Transfer 1 ml of bacterial culture to a clean 1.5 ml micro-centrifuge tube. Spin tube in a micro-centrifuge for 5 min at 10,000 × *g* and 4°C.

2. Carefully discard the supernatant using a pipette.

3. Resuspend the pellet in 100 µl of 50 mM EDTA and 100 µl lysozyme 10 mg/ml.

4. Incubate at 37 °C for 60 min.

5. Add 200 µl of TE-SDS-K buffer (10 mM Tris HCl pH 8.00; 1 mM EDTA pH 8.00; 1 % SDS w/v; 100 µg/ml Proteinase K) and incubate at 37 °C for 30 min.

6. Add 1 ml of CTAB buffer and homogenize inverting the tube several times. Incubate at 65 °C for 60 min.

7. During the incubation period prepare 2 ml of PB Buffer with 4 mg/ml RNase A.

8. Centrifuge at maximum speed for 10 min.

9. Place the supernatant into a 2-ml micro-centrifuge tube with 700 µl of chloroform. Shake gently the tube until emulsion formation and centrifuge at high speed for 5 min.

10. Place 400 µl of the aqueous phase into a 15-ml tube containing the buffer prepared in step 7. Shake gently by inversion and load the sample into the QIAquick column by decanting or pipetting, and apply vacuum. After the sample has passed through the column, switch off the vacuum source.

11. To wash, add 0.75 ml of Buffer PE (Wash Buffer) to the QIAquick column and apply vacuum.

12. Repeat step 11.

13. Transfer each QIAquick column to a 2-ml collection tubes. Centrifuge for 2 min at 10,000 × *g* to remove residual EtOH (Buffer PE).

14. Place each QIAquick column into a clean 1.5 ml micro-centrifuge tube.

15. To elute DNA, add 50 µl of Buffer EB (10 mM Tris-Cl, pH 8.5) to the centre of each QIAquick membrane, and incubate for 2 min.

16.   Centrifuge the columns for 1 min at 10,000 × *g*.

17.   Store the DNA solution at –20°C.

c.   DNA extraction with CTAB (protocol 2 - adapted from Vázquez-Boland's group protocol).

1.   Inoculate a 5 ml of liquid culture with the bacteria strain of interest. Grow overnight in the appropriate conditions.

2.   Take 2 ml of overnight culture and add 2 ml of preheated 0.1 g glycine BHI. Incubate for 40 min at 37 ºC.

3.   Centrifuge at 6,000 rpm for 10 min at 4 ºC.

4.   Resuspend the pellet in 200 μl of 1× TE pH 8.00.

5.   Centrifuge at 6,000 rpm for 10 min at 4 ºC.

6.   Resuspend the pellet in 140 μl of TE-sucrose, 100 μl of 20 mg/ml lysozyme. Incubate 1 h at 37 ºC.

7.   Add 24 μl of 0.5 M EDTA, 24 μl of double-distilled H_2O, 16 μl of 20 % SDS and 4 μl of 5 mg/ml Proteinase K (-20 ºC). Mix thoroughly and incubate for 45 min at 37 ºC.

8.   Preheat the CTAB at 65 ºC.

9.   Add 36 μl of NaCl 5 M and mix thoroughly. Add 30 μl of CTAB, mix again and incubate 15 min at 65 ºC (don't close the tube).

10.   Add an approximately equal volume of chloroform pH 8.00, mix thoroughly, and centrifuge at 14,000 rpm for 5 min.

11.   Remove aqueous, viscous supernatant to a fresh micro-centrifuge tube, leaving the interface.

12.   Add 1.25 μl of 4 mg/ml RNase solution. Incubate 15 min at 37 ºC.

13.   Add an equal volume of ∅:CHCl$_3$:AIA. Mix thoroughly and centrifuge at 14,000 rpm for 5 min.

14.   Place aqueous phase into a fresh micro-centrifuge tube. Add 0.6 vol isopropanol. Shake the tube back and forth until a stringy white DNA precipitate becomes clearly visible.

15.   Centrifuge at 14,000 rpm for 5 min. Remove the supernatant.

16.   Wash the DNA with 70 % EtOH and centrifuge at 14,000 rpm for 5 min.

17.   Carefully remove the supernatant and dry the pellet at 37 ºC during 15-20 min.

18.   Add 50 μl of double distilled water to the tube and rehydrate the DNA for 1 hour.

d.   DNA extraction using the Wizard® Genomic DNA Purification kit

1.   Add 1 ml of an overnight culture to a 1.5 ml micro-centrifuge tube.

2.   Centrifuge at 13,000 × *g* for 2 min to pellet the cells. Remove the supernatant.

3.   Resuspend the cells thoroughly in 480 μl of 50 mM EDTA.

4.   Add 60 μl of 10 mg/ml lysozyme and 60μl of 10mg/ml lysostaphin and gently pipette to mix.

5.   Incubate the sample at 37°C for 60 min. Centrifuge for 2 min at maximum speed and remove the supernatant.

6.   Add 600 μl of Nuclei Lysis Solution. Gently pipette until the cells are resuspended.

7.   Incubate at 80 °C for 5 min to lyse the cells; then cool to room temperature.

8.   Add 3 μl of 4 mg/ml RNase A solution to the cell lysate. Invert the tube 5 times to mix.

9.   Incubate at 37 °C for 15min. Cool the sample to room temperature.

10.   Add 200 μl of Protein Precipitation Solution to the RNase-treated cell lysate. Vortex vigorously at high speed for 20 s. to mix the Protein Precipitation Solution with the cell lysate.

11.   Incubate the sample on ice for 5 min.

12. Centrifuge at maximum speed for 3 min.

13. Transfer the supernatant containing the DNA to a clean 1.5-ml micro-centrifuge tube containing 600 µl of room temperature isopropanol.

14. Gently mix by inversion until the thread-like strands of DNA form a visible mass.

15. Centrifuge at maximum speed for 2 min. Carefully pour off the supernatant and drain the tube on clean absorbent paper.

16. Add 600 µl of room temperature 70 % EtOH and gently invert the tube several times to wash the DNA pellet.

17. Centrifuge at maximum speed for 2 min. Carefully aspirate the EtOH.

18. Drain the tube on clean absorbent paper and allow the pellet to air-dry for 10–15 min.

19. Add 50 µl of DNA Rehydration Solution to the tube and rehydrate the DNA by incubating at 65 °C for 1 hour.

20. Store the DNA at -20 °C.

e. DNA extraction with Guanidinium thiocyanate

Lysis buffer: 5M Guanidinium thiocyanate; 0.1M EDTA; Sarcosyl (N-Lauril Sarcosine) 0.5 % (p/v).

1. Add 1 ml of an overnight culture to a 1.5 ml micro-centrifuge tube.

2. Centrifuge at $4,000 \times g$ for 10 min to pellet the cells. Remove the supernatant.

3. Repeat steps 1 and 2.

4. Add 550 µl of Lysis buffer and incubate for 30 min to lyse the pellet.

5. Add 250 µl of AcNH$_4$, and incubate 15 min.

6. Add 250 µl of chloroform:IAA (24:1) and mix the contents on the tube until an emulsion forms.

7. Centrifuge at $10,000 \times g$ for 5 min and collect the supernatant into a fresh 1.5 ml micro-centrifuge tube.

8. Repeat steps 6 and 7.

9. Add 0.6 V of isopropanol.

10. Centrifuge at maximum speed for 2 min and remove the supernatant.

11. Add 600 µl of room temperature 70 % EtOH and gently invert the tube several times to wash the DNA pellet.

12. Centrifuge at maximum speed for 2 min. Carefully aspirate the EtOH.

13. Repeat steps 11 and 12 twice.

14. Drain the tube on clean absorbent paper and allow the pellet to air-dry for 10–15 min.

15. Add 50 µl of water to the tube and rehydrate the DNA by incubating at 65°C for 1 hour.

16. Store the DNA at -20°C.

III.2.6   Extraction of plasmid DNA (minipreperation) by Alkaline Lysis with SDS

Alkaline Lysis Solution I:	50 mM glucose	
	25 mM Tris-Cl (pH 8.0)	
	10 mM EDTA (pH 8.0)	
	Autoclave for 15 min on liquid cycle, and store at 4 ºC.	
Alkaline Lysis Solution II:	0.2 N NaOH (freshly diluted from a 10 N stock)	
	1 % (w/v) SDS	
	Prepare Solution II fresh and use at room temperature.	
Alkaline Lysis Solution III:	5 M potassium acetate	60.0 ml
	Glacial HAc	11.5 ml
	H$_2$O	28.5 ml
	Store the Solution III at 4 ºC and transfer it to an ice bucket just before use.	

Preparation of cells

1. Inoculate 5 ml of LB medium containing 50 μg/ml ampicillin with a single bacterial colony containing the desired plasmid. Incubate the culture overnight at 37 °C with vigorous shaking.
2. Pour 1.5 ml of the culture into a 1.5 micro-centrifuge tube the next day. Centrifuge at 8,000 × *g* for 3 min in a micro-centrifuge. Discard the supernatant.
3. Repeat step 1.

Lysis of cells

4. Resuspend the pellet in 200 μl Alkaline Lysis Solution I by vigorous vortexing.
5. Add 400 μl of a freshly prepared Alkaline Lysis Solution II. Close the tube tightly, and mix the contents by inverting the tube rapidly 5 times. Store the tube on ice.
6. Add 300 μl of an ice-cold Alkaline Lysis Solution III. Mix gently. Place on ice for 10 min.
7. Centrifuge at maximum speed for 7 min at 4 °C. Transfer the supernatant (800 μl) to a clean tube on ice.
8. Add 500 μl of ∅:CHCl$_3$:AIA to the supernatant and mix. Centrifuge at maximum speed for 5 min.
9. Transfer the clear supernatant (700 μl) to a clean tube.

Recovery of plasmid DNA

10. Precipitate nucleic acids from the supernatant by adding 0.6 V of isopropanol at room temperature. Mix the solution by vortexing and incubate at room temperature for 2 min.
11. Centrifuge at maximum speed for 10 min. Discard immediately the supernatant by inversion.
12. Wash the pellet with 1 ml 70 % EtOH. Centrifuge at maximum speed for 5 min and discard the supernatant.
13. Repeat step 12.
14. Air dry the pellet. Resuspend in 10 μl of TE pH 8.0 containing 20 μg/ml RNase A.
15. Assay the DNA on a mini-gel with appropriate concentration standards before restricting the DNA. The expected yield is 1-4 μg plasmid DNA.
16. Store the DNA solution at -20 °C.

III.2.7   Phenol:chloroform extraction

1. Transfer the sample to a polypropylene tube and add an equal volume of ∅:CHCl$_3$:AIA pH 7.8-8.0.
2. Mix the contents on the tube until an emulsion forms.
3. Centrifuge the mixture at 13,000 rpm for 10 min in a micro-centrifuge.
4. Use a pipette to transfer the aqueous phase containing DNA. Discard the interface and organic phase.
5. Repeat Steps 1-4 until no protein is visible at the interface.
6. Add an equal volume of chloroform and repeat Steps 2-4.
7. Recover the nucleic acid by standard precipitation with EtOH or isopropanol.

III.2.8   Precipitation of DNA with ethanol

1. Estimate the volume of the DNA solution.
2. Add exactly 2 volumes of ice-cold 100 % EtOH and 0.1 volumes of 3 M NaAc (pH 5.2) and mix the solution well. Store the ethanolic solution on ice for 30 min to allow the precipitate of DNA to form.
3. Recover the DNA by centrifugation at 0 °C.

4. Carefully remove the supernatant. Take care not to disturb the pellet of nucleic acid (which may be invisible). Use the pipette tip to remove any drops of fluid that adhere to the walls of the tube. It is best to save the supernatant from valuable DNA samples until recovery of the precipitated DNA has been verified.

5. Fill the tube half way with 70 % EtOH and recentrifuge at maximum speed for 2 min at 4 ºC in a micro-centrifuge.

6. Carefully remove the supernatant.

7. Store the open tube on the bench at room temperature until the last traces of fluid have evaporated.

8. Dissolve the DNA pellet in the desired volume of buffer (usually TE pH 8.0). Rinse the walls of the tube well with the buffer.

III.2.9 Extraction of total RNA from bacteria

a. Enzymatic Lysis Protocol

1. Place 0.5 ml of an exponentially growing culture of the strain of interest into a 1.5-ml micro-centrifuge tube.

2. Add 1 ml of RNAprotect bacteria Reagent. Mix immediately by vortexing for 5 s.

3. Incubate for 5 min at room temperature.

4. Centrifuge at $5{,}000 \times g$ for 10 min at 4 °C.

5. Decant the supernatant. Remove residual supernatant by gently dabbing the inverted tube once onto a paper towel. For subsequent isolation of RNA use the RNeasy Mini Kit.

6. Loosen cell pellet by flicking the bottom of the tube. Resuspend cells in 100 μl of 1× TE buffer containing 1 mg/ml (Gram-negative bacteria) or 15 mg/ml (Gram-positive bacteria) lysozyme by vortexing for 10 s.

7. Incubate at room temperature for 5 min (Gram-negative bacteria) or 10 min (Gram-positive bacteria). Incubate with vortex for 10 s at least every 2 min during incubation. Incubate on a shaker–incubator, or vortex for 10 s at least every 2 min during incubation.

8. Add 350 μl of Buffer RLT containing 1:100 β-mercaptoethanol to the sample and vortex vigorously. If particulate material is visible, it should be pelleted by centrifugation, and only the supernatant should be used in subsequent steps.

9. Add 250 μl of 96 % EtOH to the lysate, and mix well by pipetting.

10. Apply sample, 700 μl, to a RNeasy mini spin column sitting in a 2 ml collection tube. Centrifuge at $13{,}000 \times g$ for 15 s. Discard flow-through.

11. Pipette 700 μl of Buffer RW1 onto the RNeasy column, and centrifuge at $13{,}000 \times g$ for 15 s to wash. Discard the flow-through.

12. Transfer RNeasy column into a new 2 ml collection tube. Pipette 500 μl of Buffer RPE onto the RNeasy column, and centrifuge at $13{,}000 \times g$ for 15 s to wash.

13. Pipette 500 μl of Buffer RPE onto RNeasy column, and centrifuge at $13{,}000 \times g$ for 2 min to dry the RNeasy membrane.

14. Place the RNeasy spin column in a new 2-ml collection tube, and discard the old collection tube with the filtrate. Centrifuge at $13{,}000 \times g$ for 1 min.

15. Transfer RNeasy column into a new 1.5-ml collection tube and pipette 50 μl of RNase-free water directly onto the RNeasy membrane. Centrifuge $13{,}000 \times g$ for 1 min to elute.

16. Store the RNA solution at -80 ºC.

If <u>DNase treatment </u>is performed, substitute step 11 by the following steps:

a. Pipette 350 μl of Buffer RW1 onto the RNeasy column, and centrifuge at $13{,}000 \times g$ for 15 s to wash. Discard the flow-through.

b.   Add 10 μl of DNase I stock solution to 70 μl Buffer RDD. Mix by gently inverting the tube.

c.   Pipette the DNase I incubation mix (80 μl) directly onto the spin-column membrane, and place on the bench top (20 ° to 30 ºC) for 15 min.

d.   Pipette 350 μl of Buffer RW1 into the spin column, and centrifuge at $13,000 \times g$ for 15 s. Discard the flow-through.

b.   Mechanical Disruption Protocol

1.   Place 0.5 ml of an exponentially growing culture of the strain of interest into a 1.5-ml micro-centrifuge tube.

2.   Add 1 ml of RNAprotect bacteria Reagent. Mix immediately by vortexing for 5 s.

3.   Incubate for 5 min at room temperature.

4.   Centrifuge at $5,000 \times g$ for 10 min at 4 °C.

5.   Decant the supernatant. Remove residual supernatant by gently dabbing the inverted tube once onto a paper towel. For subsequent isolation of RNA use the RNeasy Mini Kit.

6.   Add 350 of μl Buffer RLT containing 1:100 β-mercaptoethanol to the sample and vortex vigorously. If particulate material is visible, it should be pelleted by centrifugation, and only the supernatant should be used in subsequent steps.

7.   Transfer the lysate into a 2 ml reaction tube containing 50 mg acid-washed glass beads (22.222.0001, Retsch, Germany). Disrupt the cells in the Mixer Mill MM 300 at maximum speed for 5 min.

8.   Centrifuge tubes at $13,000 \times g$ for 5 s. Transfer 400 μl of supernatant into a new 1.5 ml reaction tube.

9.   Measure the volume of the solution. Add an equal volume of 70 % EtOH, and mix well by pipetting.

10.   Apply the sample to an RNeasy mini spin column sitting in a 2 ml collection tube. Centrifuge at $13,000 \times g$ for 15 s.

11.   Pipette 700 μl of Buffer RW1 onto the RNeasy column, and centrifuge at $13,000 \times g$ for 15 s to wash. Discard the flow-through.

12.   Transfer the RNeasy column into a new 2 ml collection tube. Pipette 500 μl of Buffer RPE onto the RNeasy column, and centrifuge at $13,000 \times g$ for 15 s to wash.

13.   Pipette 500 μl of Buffer RPE onto the RNeasy column, and centrifuge at $13,000 \times g$ for 2 min to dry the RNeasy membrane.

14.   Place the RNeasy spin column in a new 2-ml collection tube, and discard the old collection tube with the filtrate. Centrifuge at $13,000 \times g$ for 1 min.

15.   Transfer the RNeasy column into a new 1.5-ml collection tube and pipette 50 μl of RNase-free water directly onto the RNeasy membrane. Centrifuge at $13,000 \times g$ for 1 min to elute.

16.   Store the RNA solution at -80 ºC.

If <u>DNase treatment</u> is performed, substitute step 10 by the following steps:

a.   Pipette 350 μl of Buffer RW1 onto the RNeasy column, and centrifuge at $13,000 \times g$ for 15 s to wash. Discard the flow-through.

b.   Add 10 μl of DNase I stock solution to 70 μl Buffer RDD. Mix by gently inverting the tube.

c.   Pipette the DNase I incubation mix (80 μl) directly onto the spin-column membrane, and place on the bench top (20 ° to 30 ºC) for 15 min.

d.   Pipette 350 μl of Buffer RW1 into the spin column, and centrifuge at $13,000 \times g$ for 15 s. Discard the flow-through.

c. Enzymatic Lysis with Mechanical Disruption Protocol

1. Place 0.5 ml of an exponentially growing culture of the strain of interest into a 1.5-ml micro-centrifuge tube.

2. Add 1 ml of RNAprotect bacteria Reagent. Mix immediately by vortexing for 5 s.

3. Incubate for 5 min at room temperature.

4. Centrifuge at 5,000 × *g* for 10 min at 4 °C.

5. Decant the supernatant. Remove residual supernatant by gently dabbing the inverted tube once onto a paper towel. For subsequent isolation of RNA use the RNeasy Mini Kit.

6. Loosen cell pellet by flicking the bottom of the tube. Resuspend cells in 100 μl of 1× TE buffer containing 1 mg/ml (Gram-negative bacteria) or 15 mg/ml (Gram-positive bacteria) lysozyme by vortexing for 10 s.

7. Incubate at room temperature for 5 min (Gram-negative bacteria) or 10 min (Gram-positive bacteria). Incubate with vortex for 10 s at least every 2 min during incubation. Incubate on a shaker–incubator, or vortex for 10 s at least every 2 min during incubation.

8. Add 350 μl of Buffer RLT containing 1:100 β-mercaptoethanol to the sample and vortex vigorously. If particulate material is visible, it should be pelleted by centrifugation, and only the supernatant should be used in subsequent steps.

9. Transfer the lysate into a 2 ml reaction tube containing 50 mg acid-washed glass beads (22.222.0001, Retsch, Germany). Disrupt the cells in the Mixer Mill MM 300 at maximum speed for 5 min.

10. Centrifuge tubes at 13,000 × *g* for 5 s. Transfer 400 μl of supernatant into a new 1.5 ml reaction tube.

11. Add 220 μl 96 % EtOH to the supernatant, and mix well by pipetting.

12. Apply the sample to an RNeasy mini spin column sitting in a 2 ml collection tube. Centrifuge at 13,000 × *g* for 15 s.

13. Pipette 700 μl of Buffer RW1 onto the RNeasy column, and centrifuge at 13,000 × *g* for 15 s to wash. Discard the flow-through.

14. Transfer the RNeasy column into a new 2 ml collection tube. Pipette 500 μl of Buffer RPE onto the RNeasy column, and centrifuge at 13,000 × *g* for 15 s to wash.

15. Pipette 500 μl of Buffer RPE onto the RNeasy column, and centrifuge at 13,000 × *g* for 2 min to dry the RNeasy membrane.

16. Place the RNeasy spin column in a new 2-ml collection tube, and discard the old collection tube with the filtrate. Centrifuge at 13,000 × *g* for 1 min.

17. Transfer the RNeasy column into a new 1.5-ml collection tube and pipette 50 μl of RNase-free water directly onto the RNeasy membrane. Centrifuge at 13,000 × *g* for 1 min to elute.

18. Store the RNA solution at -80 ºC.

If DNase treatment is performed, substitute step 13 by the following steps:

a. Pipette 350 μl of Buffer RW1 onto the RNeasy column, and centrifuge at 13,000 × *g* for 15 s to wash. Discard the flow-through.

b. Add 10 μl of DNase I stock solution to 70 μl Buffer RDD. Mix by gently inverting the tube.

c. Pipette the DNase I incubation mix (80 μl) directly onto the spin-column membrane, and place on the bench top (20 º to 30 ºC) for 15 min.

d. Pipette 350 μl of Buffer RW1 into the spin column, and centrifuge at 13,000 × *g* for 15 s. Discard the flow-through.

d. Perfect RNA™ protocol

1. Place 0.5 ml of an exponentially growing culture of the strain of interest into a 1.5 ml micro-centrifuge tube.

2. Centrifuge at 5,000 × *g* for 10 min at 4 °C.

3. Decant the supernatant. Remove any residual supernatant by gently dabbing the inverted tube once onto a paper towel.

4. Add 350 μl of Lysis Solution (containing 1:100 β-Mercaptoethanol). Mix well by vigorous vortexing and pipetting up and down several times.

5. Transfer the homogenized cell sample to a 1.5 ml micro-centrifuge tube and centrifuge at 13,000 × *g* for 5 min.

6. Transfer the supernatant to a fresh 1.5-ml tube, taking care not to transfer any solid material.

7. Add 350 μl of 70 % EtOH to the lysate and completely mix by very gentle, repeated inversion.

8. Thoroughly mix Perfect RNA Binding Matrix Solution so that Binding Matrix is completely in suspension before use. Add 200 μl Perfect RNA Binding Matrix Solution to the lysate/ EtOH mixture from step 7, and completely mix by gentle inversion.

9. Place a Perfect RNA Mini Spin Column into fresh 1.5-ml micro-centrifuge tube. Carefully and evenly pipette lysate/Binding Matrix mixture into the Spin Column.

10. Centrifuge at 13,000 × *g* for 1 min. Discard the filtrate from the collection tube and return Spin Column to same micro-centrifuge tube.

11. Wash the RNA by adding 700 μl of Wash Solution 1 and centrifuge at 13,000 × *g* for 30 s. Discard the filtrate and return the Spin Column to the same collection tube.

12. Centrifuge the assembly for 30 s to remove any residual Wash Solution 1 from the Spin Column. Place the Spin Column into a fresh collection tube.

13. Wash the RNA twice by adding 500 μl of diluted Wash Solution 2 to the Spin Column, centrifuge at 13,000 × *g* for 15 s. Discard the filtrate and return the Spin Column to same collection tube.

14. Centrifuge at 13,000 × *g* for an additional 30 s to remove any residual Wash Solution 2 from the Spin Column.

15. Transfer the Spin Column to an RNase-free collection tube and elute the RNA by adding 50 μl of Molecular Biology Grade Water directly to the Binding Matrix. Vortex the Spin Column Assembly for 1 s to completely moisten the entire dried binding matrix.

16. Incubate the Spin Column Assembly at 50°C for 5 min.

17. Vortex the Spin Column assembly for 5 s and immediately centrifuge at 13,000 × *g* for 2 min.

18. Store the RNA at -80°C.

e. RNAWiZ™ protocol

1. Place 0.5 ml of an exponentially growing culture of the strain of interest into a 1.5-ml micro-centrifuge tube.

2. Centrifuge at 5,000 × *g* for 10 min at 4 °C.

3. Decant the supernatant. Remove any residual supernatant by gently dabbing the inverted tube once onto a paper towel.

4. Resuspend the cells in 100 μl of 1× TE buffer containing 1 mg/ml (Gram-negative bacteria) or 15 mg/ml (Gram-positive bacteria) lysozyme by vortexing for 10 s.

5. Incubate at room temperature for 5 min (Gram-negative bacteria) or 10 min (Gram-positive bacteria). Incubate with agitation with vortex for 10 s at least every 2 min during incubation.

6. Homogenize in 1 ml of RNAWIZ by simply pipetting vigorously several times.

7.   Incubate the homogenate at room temperature for 5 min to dissociate the nucleoproteins from the nucleic acids.

8.   Add 200 µl of chloroform to the homogenate. The chloroform should not contain IAA or other additives.

9.   Cover the sample, shake vigorously for ~20 s, and incubate at room temperature for 10 min.

10.   Centrifuge the mixture at 13,000 × *g* for 15 min at 4°C. The mixture separates into 3 phases, the colourless upper aqueous phase (containing the RNA), the semi-solid interphase (containing most of the DNA), and the lower organic phase.

11.   Without disturbing the interphase, carefully transfer the aqueous phase into a clean RNase-free tube. Add 500 µl of RNase-free water, and mix well.

12.   Add 1 ml of isopropanol, mix well, and incubate at RT for 10 min. Centrifuge at 13,000 × *g* for 15 min at 4°C to pellet the RNA.

13.   Decant the supernatant and wash the pellet with 1 ml cold 75 % EtOH by vortexing. Centrifuge at 13,000 × *g* for 5 min at 4°C. Discard the supernatant.

14.   Air dry the pellet for about 10 min; do not let the RNA dry completely, as this will make it difficult to resuspend.

15.   Resuspend the RNA in an appropriate amount of RNase-free water or 1× TE.

16.   Briefly vortex or repeatedly pipette to aid in resuspension, and if necessary, heat to ~60°C.

17.   Store RNA at -80°C.

f.   High Pure RNA protocol

1.   Place 0.5 ml of an exponentially growing culture of the strain of interest into a 1.5-ml micro-centrifuge tube.

2.   Centrifuge at 5,000 × *g* for 10 min at 4°C.

3.   Decant the supernatant. Remove any residual supernatant by gently dabbing the inverted tube once onto a paper towel.

4.   Resuspend the pellet in 200 µl of 10 mM Tris (pH 8.0), and add 4 µl of 50 mg/ml lysozyme Incubate for 10 min at 37°C.

5.   Add 400 µl of lysis/-binding buffer and mix well.

6.   Combine the High Pure filter tube and the collection tube and pipette the sample in the upper reservoir.

7.   Centrifuge at 8,000 × *g* for 15 s. Discard the flowthrough and again combine the filter tube and the used collection tube.

8.   Pipette 90 µl of DNase incubation buffer into a sterile reaction tube, add 10 µl DNase I, mix and pipette the solution in the upper reservoir of the filter tube. Incubate at room temperature for 60 min.

9.   Add 500 µl of wash buffer I to the upper reservoir, centrifuge at 8,000 × *g* for 15 s, discard the flowthrough and combine the filter tube and the used collection tube.

10.   Add 500 µl of wash buffer II to the upper reservoir and centrifuge at 8,000 × *g* for 15 s, discard the flowthrough and again combine the filter tube and the used collection tube.

11.   Add 200 µl of wash buffer II (blue cap) to the upper reservoir, centrifuge at 13,000 × *g* for 2 min to remove residual washing buffer. Discard the collection tube and insert the filter tube in a sterile 1.5-ml reaction tube.

12.   Add 50–100 µl of elution buffer into the filter tube and centrifuge at 8,000 × g for 1 min.

13.   Store the RNA at -80°C.

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III.2.10   Precipitation of RNA with ethanol

1.   Estimate the volume of the RNA solution.

2.   Add 2.5-3.0 volumes of ice-cold 100 % EtOH and 0.1 volumes of 3 M NaAc (pH 5.2) and mix the solution well. Store the ethanolic solution on ice for 30 min to allow the precipitate of RNA to form.

3.   Recover the RNA by centrifugation at 0 ºC.

4.   Carefully remove the supernatant. Take care not to disturb the pellet of nucleic acid (which may be invisible). Use the pipette tip to remove any drops of fluid that adhere to the walls of the tube. It is best to save the supernatant from valuable RNA samples until recovery of the precipitated RNA has been verified.

5.   Fill the tube half way with 70 % EtOH and recentrifuge at maximum speed for 2 min at 4 ºC in a micro-centrifuge .

6.   Carefully remove the supernatant.

7.   Store the open tube on the bench at room temperature until the last traces of fluid have evaporated.

8.   Dissolve the RNA pellet in the desired volume of buffer (usually TE pH 8.0). Rinse the walls well of the tube with the buffer.

III.2.11   Enzymatic manipulation of nucleic acids

a.   RNase treatment

+ <u>Preparation of DNase free-RNase:</u>

1.   Dissolve pancreatic RNase (RNase A) at a concentration of 10 mg/ml in 0.01 M NaAc (pH 5.2).

2.   Heat at 100 ºC for 15 min.

3.   Allow it to cool slowly to room temperature.

4.   Adjust the pH by adding 0.1 volume of 1 M Tris-Cl (pH 7.4).

5.   Dispense into aliquots and store at -20 ºC.

+ <u>Protocol:</u>

1.   Set up the RNase digestion reaction as follows:

i.	+ Nucleic acids sample in water or 1× TE	20-30 μl.
ii.	+ RNase A	1 U/2 μg RNA.
iii.	+ Nuclease-free water	to a final volume of 100 μl.

2.   Incubate at 37°C for 15 min.

3.   Add 5 μl of 0.5 mM EDTA to stop the reaction.

4.   Perform a phenol:chloroform extraction step as indicated in III.2.7.

5.   Perform the precipitation of DNA with EtOH as indicated in III.2.8.

6.   Store the resulting DNA solution at -20 ºC.

b.   DNase treatment.

- **DNase I protocol:**

+ <u>Preparation on of RNase free-DNase:</u>

1.   Dissolve 10 mg of pancreatic DNase I in 10 ml of 0.1 M iodoacetic acid, 0.15 M NaAc (pH 5.2).

2.   Heat at solution to 55 ºC for 45 min.

3.   Cool the solution to 0 ºC, and add 1 M $CaCl_2$ to a final concentration of 5 mM.

4. Dispense the DNase I into small aliquots and store at -20 °C.

    + Protocol:

1. Set up the DNase I digestion reaction as follows:
    i. + Nucleic acids sample in water or 1 × TE buffer   20-30 µl.
    ii. + DNase I   1 U/µg DNA.
    iii. + Nuclease-free water   to a final volume of 100 µl.

2. Incubate at 37°C for 30 min.

3. Add 5 µl of 0.5 mM EDTA to stop the reaction.

4. Perform a phenol:chloroform extraction step as indicated in III.2.7.

5. Perform the precipitation of RNA with EtOH as indicated in III.2.10.

6. Store the resulting RNA solution at -80 °C.

- **RQ1 RNase-Free DNase kit protocol (Promega):**

1. Set up the DNase digestion reaction as follows:
    + Nucleic acids sample in water or 1 × TE   20-30 µl.
    + RQ1 RNase-Free DNase 10× Reaction Buffer   10 µl.
    + RQ1 RNase-Free DNase   1 U/µg DNA.
    + Nuclease-free water   to a final volume of 100 µl.

2. Incubate at 37°C for 30 min.

3. Add 5 µl of RQ1 DNase Stop Solution to terminate the reaction.

4. Incubate at 65°C for 10 min to inactivate the DNase.

5. Perform a phenol:chloroform extraction step as indicated in III.2.7.

6. Perform the purification of RNA with EtOH as indicated in III.2.10.

7. Store the resulting RNA solution at -80 °C.

c. S1 nuclease treatment

1. Set up the S1 nuclease digestion reaction as follows:
    + Nucleic acids sample in water or 1 × TE   20-30 µl.
    + 10 × S1 nuclease Buffer   10 µl.
    + S1 nuclease   10 U/2 µg nucleic acids.
    + Nuclease-free water   to a final volume of 100 µl.

2. Incubate at 37°C for 30 min.

3. Add 5 µl of 0.5 mM EDTA to stop the reaction.

4. Perform a phenol:chloroform extraction step as indicated in III.2.7.

5. Perform the precipitation of DNA with EtOH as indicated in III.2.8.

6. Store the resulting dsDNA solution at -20 °C.

d. T7 RNA polymerase-based *in vitro* transcription

T7 RNA Polymerase is a DNA-dependent RNA polymerase that exhibits extremely high specificity for its cognate promoter sequences.

Protocol:

1. In a micro-centrifuge tube, add the following reagents at room temperature in the order listed:
    + 5 × Transcription Optimized Buffer (Promega)   20 µl
    + 100 mM DTT   10 µl
    + rNTP mix   20 µl
    + DNA template (2-5 µg)   2 µl
    + T7 RNA Polymerase   40 units
    + Nuclease-Free Water   to final volume of 100 µl

2. Incubate for 2 hour at 37°C.

3. Stop the reaction with 2 µl of 0.5 M EDTA.

III.2.12   Quantification of nucleic acids

a.   Spectrophotometry of DNA or RNA

For quantitating the amount of DNA or RNA, readings are taken at wavelengths of 260 nm and 280 nm. To ensure significance, readings should be greater than 0.15. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. An OD of 1 corresponds to ≈ 50 μg/ml for double-stranded DNA, 40 μg/ml for single-stranded DNA and RNA, and ≈ 33 μg/ml for single-stranded oligonucleotides. The ratio between the readings at 260 nm and 280 nm (OD_{260}:OD_{280}) provides an estimate of the purity of the nucleic acids. Pure preparations of DNA and RNA have OD_{260}:OD_{280} values of 1.8 and 2.0, respectively.

b.   Fluorometric quantification of DNA or RNA

- **DNA quantification using PicoGreen® dsDNA reagent and Luminescence Spectrometer LS50B**

PicoGreen® dsDNA quantification reagent is an ultrasensitive fluorescent nucleic acid stain for quantitating dsDNA in solution. The PicoGreen® dsDNA quantification reagent enables to quantitate as little as 25 pg/ml of dsDNA with a standard spectrofluorometer and fluorescein excitation and emission wavelengths. Using a fluorescence microplate reader, it is possible to detect as little as 250 pg/ml dsDNA.

Protocol:

1.   Pipette 1-100 μl (usually 3 μl) of DNA solution in a 1.5-ml micro-centrifuge tube.

2.   Add 1 × TE (pH 8.0) up to a final volume of 900 μl. Mix well.

3.   Add 100 μl 1:40 PicoGreen® solution (diluted in 1 × TE). Mix well. Wait for 10 min in darkness.

4.   Switch on the Luminescence Spectrometer LS50B (Perkin-Elmer Corp., Norwalk, USA). Wait for 10 min.

5.   Select FL WinLab software.

6.   Select the window called "Applications", and then choose "Status".

7.   Select the correct parameter of measuring:
      + λ excitation: 460 nm
      + λ emission: 540 nm
      + slit: 2.5 nm
      + integration time: 2 s.
      + emission filter: open

8.   Close this software's window, and choose Read.mth.

9.   Determine the fluorescence background using destilled water.

10.   Check the calibration of the device using 10 μl of DNA solution form Herring's salmon sperm of known concentration (*e.g.* 10 μg/ml).

11.   Place the 1 ml of DNA solution in the device and read the fluorescence values produced.

12.   The Formula for the calculation of DNA concentration is $y = 0.012x + 0.083$ ($R^2 = 0.9999$), where y is the relative fluorescence and x the DNA concentration (ng/ml)

- **RNA quantification using RiboGreen™ and TD-360 Mini-Fluorometer.**

The Turner Designs TD-360 Mini-Fluorometer, in combination with Molecular Probe's RiboGreen™ RNA quantification reagent, provides a method for ultrasensitive quantification of RNA in solution. The RiboGreen™ RNA quantification assay, implemented with the TD-360 Mini-Fluorometer can detect as little as 5 ng/ml RNA. The linear quantification range extends over three orders of magnitude of RNA concentration. Two different dye concentrations are required to achieve the full linear dynamic range of the RiboGreen™ RNA quantification assay. Different working solutions of RiboGreen™ reagent are prepared for the high- range assay (20 ng/ml to 1 mg/ml RNA) and the low-range assay (5 ng/ml to 50 ng/ml RNA).

Protocol:

1. **Reagent preparation:** On the day of the experiment, prepare an aqueous working solution of the RiboGreen™ reagent by diluting an aliquot of the concentrated RiboGreen™ stock solution into 1 × TE. If performing the high-range assay, dilute 200-fold. If performing the low-range assay, dilute 2,000-fold. Prepare these solutions in sterile, disposable, polypropylene plasticware rather than glassware, as the reagent may adsorb to glass surfaces. Protect the working solutions from light by covering them with foil or placing them in the dark, as the RiboGreen™ reagent is susceptible to photodegradation.

2. **RNA Standard Curves:**

    a. Prepare a 2 µg/ml solution of RNA in 1 × TE using nuclease-free plasticware. Determine the RNA concentration on the basis of absorbance at 260 nm (A260) in a cuvette with a 1 cm pathlength; an A_{260} of 0.05 corresponds to 2 µg/ml RNA.

    b. For the high-range standard curve, dilute the 2 µg/ml RNA solution into nuclease-free plastic test tubes for transfer to quartz cuvettes as shown in Table III.6a. For the low-range standard curve, first dilute the 2 µg/ml RNA solution 20-fold with TE buffer to make a 100 ng/ml RNA stock solution and use this to prepare the dilutions shown in Table III.6b.

Table III.6a. Protocol for preparing high-range standard curve.

Vol (µl) of 1× TE	Vol (µl) of 2 µg/ml of RNA stock	Vol (µl) of 1:200 diluted RiboGreen™ reagent	Final RNA Concent. RiboGreen™ Assay
0	50	50	1000 ng/ml
25	25	50	500 ng/ml
45	5	50	100 ng/ml
49	1	50	20 ng/ml
50	0	50	Blank

Table III.6b. Protocol for preparing low-range standard curve.

Vol (µl) of 1× TE	Vol (µl) of 2µg/ml of RNA stock	Vol (µl) of 1:2000 diluted RiboGreen™ reagent	Final RNA Concent. RiboGreen™ Assay
0	50	50	50 ng/ml
25	25	50	25 ng/ml
40	10	50	10 ng/ml
45	5	50	5 ng/ml
50	0	50	Blank

    c. Add 50 µl of the appropriate aqueous working solution of RiboGreen™ reagent to each plastic test tube. The high-range working solution (200-fold dilution of stock) should only be used to perform the high-range assay and the low-range working solution (2,000-fold dilution of stock) should only be used to perform the low- range assay. Mix well and incubate for 2 to 5 min at room temperature, protected from light.

    d. For the high-range calibration, blank the instrument using the blank (Table III.6a). Calibrate the fluorometer, according to the instructions from the TD-360 manual, using the sample containing the highest concentration of RNA. Measure the fluorescence of the remaining samples. To equalize any photobleaching effects, insert the samples into the fluorometer for approximately equal time periods.

    e. For the low-range calibration, blank the instrument using the blank (Table III.6b). Calibrate the fluorometer, according to the instructions from the TD-360 manual, using the sample containing the highest concentration of RNA. For the calibration value, enter the value that is 100× concentration of the standard. Measure the fluorescence of the remaining samples. After all measurements are made, divide all results by 100 to obtain the real concentrations. To equalize any photobleaching effects, insert the samples into the fluorometer for approximately equal time periods.

3. **Sample Analysis:**

    a. Dilute 5 μl of RNA solution in $1 \times$ TE to a final volume of 50 μl in disposable test tubes.

    b. Add 50 μl of the aqueous working solution of the RiboGreen reagent to each sample. Incubate for 2 to 5 min at room temperature, protected from light.

    c. Measure the fluorescence of each sample using the same instrument calibration conditions as used to generate the standard curve. To equalize any photobleaching effects, insert samples into the fluorometer for approximately equal time periods.

    d. Determine the RNA concentration of each sample from the standard curve generated in step 2.

   c. **Minigel method**

1. Mix 2 μl of the DNA sample with 0.4 μl of Gel-loading buffer IV (bromophenol blue only) and load the solution into a slot in a 0.8 % agarose mini-gel containing EtBr (0.5 μg/ml).

2. Mix 2 μl of each of a series of standard DNA solutions (0.2, 5, 10, 20, 30, 40 and 50 μg/ml) with 0.4 μl of Gel-loading buffer IV. The standard DNA solutions should contain a single species of DNA approximately the same size of the unknown DNA. Loads the samples into the wells of the gel.

3. Carry out electrophoresis until the bromophenol blue has migrated ≈ 1-2 cm.

4. Destain the gel immersing it for 5 min in electrophoresis buffer containing 0.01 M $MgCl_2$.

5. Photograph the gel using short-wavelength UV irradiation. Compare the intensity of fluorescence of the unknown DNA with that of the DNA standards and estimate the quantity of DNA in the sample.

III.2.13   Separation of DNA by agarose gel electrophoresis

   DNA fragments can be separated by electrophoresis using horizontal agarose gels. The agarose concentration depends on the size of the fragment to be resolved, most usually 1 % was used to resolve fragments from 0.5 to 8 kb and 2 % for fragments smaller than 0.5 kb. DNA samples are mixed 4:1 with loading buffer and electrophoresis was generally performed at 5-10 V/cm in TAE buffer. As DNA size marker to estimate the size of unknown restriction fragments size, commercial DNA marker or λ bacteriophage DNA digested with *PstI* or *HindIII* were used. After electrophoresis the gel was stained for, at least, 15 min with the intercalating dye EtBr (0.5 μg/ml) and was photographed under UV light (254 nm).

Protocol

1. Seal the edges of a clean, dry glass plate with tape to form a mould. Set the mould on a horizontal section of the bench.

2. Prepare sufficient electrophoresis buffer (usually 1× TAE) to fill the electrophoresis tank and to cast the gel.

3. Prepare a solution of agarose in electrophoresis buffer at the appropriate concentration (Table III.7) for separating the particular size fragments expected in the DNA sample: Add the correct amount of powdered agarose to a measured quantity of electrophoresis buffer in an Erlenmeyer flask or a glass bottle.

Table III.7. Correspondence between % agarose and PCR products size.

% agarose	DNA fragments size (Kb)
0.7	1-30
1.0	0.5-10
1.2	0.4-7
1.5	0.2-3
2.0	0.05-2

4. Loosely plug the neck of the Erlenmeyer flask with Kim wipes. Heat the slurry in a microwave oven until the agarose dissolves.

5. Transfer the flask into a water bath at 55 ºC. When the molten gel has cooled, add EtBr to a final concentration of 0.5 µg/ml. Mix the gel solution thoroughly by gentle swirling.

6. While the agarose solution is cooling, choose an appropriate comb for forming the sample slots in the gel. Position the comb 0.5-1.0 mm above the plate so that a complete well is formed when the agarose is added to the gel.

7. Pour the warm agarose solution into the mould.

8. Allow the gel to set completely (30-45 min at room temperature), then pour a small amount of electrophoresis buffer on the top of the gel, and carefully remove the comb. Pour off the electrophoresis buffer and carefully remove the tape. Mount the gel in the electrophoresis tank.

9. Add just enough electrophoresis buffer to cover the gel to a depth of $\approx$ 1 mm.

10. Mix the samples of DNA with 0.20 volume of the desired 6× gel-loading buffer.

11. Slowly load the sample mixture into the slots of the submerged gel using a micropipette. Load size standards into slots on both the right and left sides of the gel.

12. Close the lid of the gel tank and attach the electrical leads so that the DNA will migrate toward the positive anode. Apply a voltage of 1-5 V/cm. If the leads have been attached correctly, bubbles should be generated at the anode and cathode (due to electrophoresis), and within a few min, the bromophenol blue should migrate from the wells into the body of the gel. Run the gel until the bromophenol blue and/or xylene cyanol FF have migrated an appropriate distance thorough the gel.

13. When the DNA samples or dyes have migrated a sufficient distance through the gel, turn off the electric current and remove the leads and lid the gel tank. If EtBr is present in the gel and electrophoresis buffer, examine the gel by UV light and photograph the gel. Otherwise, stain the gel by immersion it in electrophoresis buffer or H_2O containing EtBr (0.5 µg/ml) for 30-45 min at room temperature or by soaking in a 1:10,000-fold dilution of SYBR Gold stock solution in electrophoresis buffer.

III.2.14   DNA extraction from agarose gel

a.   QIAEX II protocol (Qiagen)

1. Excise the DNA band from the agarose gel with a clean, sharp scalpel.

2. Weigh the gel slice in a colourless tube. Add 3 volumes of Buffer QX1 to 1 volume of gel.

3. Resuspend QIAEX II buffer by vortexing for 30 s. Add 10 µl of QIAEX II buffer to the sample.

4. Incubate at 50°C for 10 min to solubilize the agarose and bind the DNA. Mix by vortexing every 2 min to keep QIAEX II in suspension. Check that the colour of the mixture is yellow (indicating an appropriate pH).

5. Centrifuge the sample for 30 s and carefully remove supernatant with a pipette.

6. Wash the pellet with 500 µl of Buffer QX1. Resuspend the pellet by vortexing. Centrifuge the sample for 30 sec and remove all traces of supernatant with a pipette.

7. Wash the pellet twice with 500 µl of Buffer PE. Resuspend the pellet by vortexing. Centrifuge the sample for 30 sec and carefully remove all traces of supernatant with a pipette.

8. Air-dry the pellet until the pellet becomes white.

9. To elute the DNA, add 20 µl of 10 mM Tris-Cl, pH 8.0 and resuspend the pellet by vortexing. Incubate at room temperature for 5 min.

10. Centrifuge for 30 sec. Carefully pipette the supernatant into a clean tube.

b. GENECLEAN® II Protocol (Q-BIOgene)

1. Excise the DNA band from an EtBr-stained agarose gel with a razor blade using long wave UV light for as short a time as practical.

2. Determine the weight of the gel slice in micrograms. Transfer gel slice to a 1.5-ml micro-centrifuge tube.

3. Add 3 volumes of NaI solution. This keeps the final concentration of NaI above 4M.

4. Place the tube in a 45-55 °C water bath for 1 min.

5. Mix the contents of the tube by tapping the side with a finger or gently pipetting. Continue the incubation until all the agarose has dissolved (approximately 5 min).

6. Resuspend GLASSMILK® by vortexing for 1 min.

7. Add 10 µl of GLASSMILK® to NaI/DNA solution and mix with vortex gently or stir with a pipette tip.

8. Incubate at room temperature for 5 min. Mix every 1-2 min to ensure that GLASSMILK® stays in suspension.

9. Centrifuge at $14,000 \times g$ for 5 s. Discard NaI wash supernatant.

10. Add 500 µl of prepared NEW Wash and resuspend. Carefully resuspend the pellet by pipetting up and down while stirring the pellet with the pipette tip.

11. Centrifuge at $14,000 \times g$ for 5 s and discard the supernatant.

12. Repeat the wash procedure (Steps 10-11).

13. After the supernatant from the final wash has been removed, centrifuge the tube again at $14,000 \times g$ for 5 s and remove the last bit of liquid with a small bore pipette tip to avoid diluting the elute with prepared NEW Wash.

14. Leave the cap open for 5-10 min at room temperature in order to dry the pellet to remove residual EtOH.

15. Add a volume of $1 \times$ TE (around 10-20 µl) equal to that of GLASSMILK®. Carefully resuspend the pellet by gently pipetting up and down with wide-bore pipette tip or by tapping the side of the tube with a finger to mix and elute the DNA.

16. Centrifuge at $14,000 \times g$ for 30 s and carefully remove the supernatant containing the eluted DNA and place in a new tube.

III.2.15 PCR amplification

III.2.15.1 Conventional PCR

Reagents:
+ 1× PCR Buffer II (Applied Biosystems).
+ 1.5-6 mM $MgCl_2$.
+ 0.2 mM dNTP mix
+ 50-900 nM each primer.
+ 0.2 U AmpErase® uracil N-glycosylase
+ 1 U Ampli*Taq* Gold™ DNA polymerase.
+ 3 fg - 100 ng DNA
  Final volume of 20 µl.

Thermocycler conditions:
1. 50 °C 2 min (activate the uracil *N*-glycosylase in order to digest any sequences containing dUTP).
2. 95 °C 10 min (initial denaturation step, activate the *Taq*Gold DNA polymerase with Hot Start activity).
3. 40-50 cycles:
   + 95 °C 15 s (denaturation)
   + 60-63 °C 1 min (annealing and extension)
   or
   + 95 °C 15 s (denaturation)
   + 55 °C (annealing)
4. 72 °C 5 min (final extension)

Reactions were run on an 96-Well GenAmp® PCR System 9700 (Applied Biosystems division of Perkin Elmer Corp., Foster City, USA). PCR products were detected by EtBr straining after electrophoresis in agarose gels as described in III.2.13

III.2.15.2   Reverse transcriptase PCR (RT-PCR)

In the RT-PCR, RNA is copied by using a retroviral reverse transcriptase to produce complementary DNA (cDNA). The cDNA can be amplified by a thermal stable DNA polymerase in a subsequent PCR.

Reagents:
+   1 × AMV/T*fl* reaction buffer
+   1 mM MgSO₄
+   200 μM of each dNTP
+   1 μM of each primer
+   5 U of AMV reverse transcriptase
+   5 U T*fl* DNA polymerase
+   5 μl containing RNA dilution
     Final volume of 50 μl.

Thermocycler conditions:
1.   48 ºC for 45 min (reverse transcription step).
2.   94 ºC for 2 min (initial denaturation step).
3.   45 cycles:
      +   94 ºC for 1 min (denaturation)
      +   55 ºC for 1 min (annealing)
      +   68 ºC for 1 min (extension)
4.   68 ºC 5 min (final extension)

Reactions were run on a 96-Well GenAmp® PCR System 9700 (Applied Biosystems division of Perkin Elmer Corp., Foster City, USA). The resulting RT-PCR products were detected by EtBr straining after electrophoresis in agarose gel as described in III.2.13.

III.2.15.3   Real-time PCR (RTi-PCR)

a.   TaqMan®-based RTi-PCR

The main TaqMan®-based RTi-PCR feature is the use of three oligonucleotides in the PCR reaction. Two of the primers (forward and reverse) allow amplification of the product to which a third dual-labelled fluorogenic oligonucleotide, the TaqMan® probe, will anneal. Upon polymerase amplification, the 5'-3' exonuclease activity of the *Taq* polymerase releases a 5' fluorescent tag from the annealed TaqMan® probe, yielding a real-time measurable fluorescence emission directly proportional to the concentration of the target sequence.

Reagents:
+   1 × PCR Buffer A (including ROX a passive reference).
+   3-6 mM MgCl₂.
+   0.2 mM dATP, dCTP, dGTP and 0.4 mM dUTP.
+   50-900 nM each primer.
+   100-300 nM TaqMan® probe.
+   0,2 U AmpErase® uracil *N*-glycosylase (UNG).
+   1 U Ampli*Taq* Gold™ DNA polymerase.
+   3 fg - 100 ng DNA
     Final volume of 20 μl.

Thermocycler conditions:
1.   50 ºC 2 min (activate the uracil N-glycosylase in order to remove any sequences with dUTP).
2.   95 ºC 10 min (initial denaturation step, activate *Taq* DNA polymerase with Hot Start activity and 5'-3' exonuclease).
3.   40-50 cycles:
      +   95 ºC 15 s (denaturation)
      +   60-63 ºC 1 min (annealing and extension)

Reactions were run on an ABI PRISM® 7700 Sequence Detection System device (Applied Biosystems division of Perkin Elmer Corp., Foster City, USA). Fluorescence was quantified on-line and at the end point with the sequence detection system software (version 1.7, Applied Biosystems). Quantification was performed by interpolation in a standard regression curve of C_T values generated from samples at known concentrations. Negative values or lack of amplification was considered for RTi-PCRs with threshold C_T value of >40-50.

b.   SYBR® Green I-based RTi-PCR

SYBR®Green I is an ultrasensitive stain for dsDNA. SYBR®Green I is maximally excited at 497 nm, but also has a secondary excitation peak near 254 nm. The fluorescence emission of SYBR®Green I-strained DNA is centred at 520 nm.

Reagents:
+ 1 × PCR Buffer II.
+ 3-6 mM $MgCl_2$.
+ 1:10,000 – 1:100,000 SYBR® Green I
+ 0.2 mM dATP, dCTP, dGTP and 0.4 mM dUTP.
+ 50-900 nM each primer.
+ 0,2 U AmpErase® uracil *N*-glycosylase (UNG).
+ 1 U Ampli*Taq* Gold™ DNA polymerase.
+ 3 fg - 100 ng DNA.
  Final volume of 20 µl.

Thermocycler conditions:
1. 50 ℃ 2 min (activate the uracil N-glycosylase in order to remove any sequences with dUTP).
2. 95 ℃ 10 min (initial denaturation step, activate *Taq* DNA polymerase with Hot Start activity).
3. 40-50 cycles:
   + 95 ℃ 15 s (denaturation)
   + 60-63 ℃ 1 min (annealing and extension)

Reactions were run on an ABI PRISM® 7700 Sequence Detection System device (Applied Biosystems division of Perkin Elmer Corp., Foster City, USA). Fluorescence was quantified on-line and at the end point with the sequence detection system software (version 1.7, Applied Biosystems). After PCR amplification, T_m curve analysis was performed using the *Dissociation Curve Analysis* software v1.0 (Applied Biosystems). The PCR products were heated to 95 ℃ during 15 s, cooled at 60 ℃ 20 s and then slowly heated back to 95 ℃ at a rate of 0.2 ℃/s. Obtained fluorescence signals were continuously monitored during the slow warming up gradient and showed a decreasing curve with a sharp fluorescence drop near to denaturation temperature. Plotting the negative derivate of the fluorescence over temperature versus the temperature (-dT/dT versus T) generated peaks from which the T_m of the products were calculated.

c. AmpliFluor™-based RTi-PCR

The AmpliFluor™ RTi-PCR is based on a universal energy-transfer hairpin primer (UniPrimer™) which emits a fluorescent signal when unfolded during its incorporation into an amplification product. The UniPrimer™ contains a 3'-Z tail sequence that is also present at the 5' end of one of the target-specific primers so that it anneals to the PCR product and acts as universal PCR primer.

Reagents:
+ 1 × Ex Taq™ buffer
+ 1.5 mM $MgCl_2$.
+ 0.25 mM dNTP mix
+ 50 nM *Z* primer
+ 500 nM primer
+ 500 nM UniPrimer™
+ 1 U TaKaRa Ex Taq™ polymerase
+ 3 fg - 100 ng DNA
  Final volume of 20 µl.

Thermocycler conditions:
1. 95 ℃ 4 min (initial denaturation step).
2. 45 cycles:
   + 95 ℃ 15 s (denaturation)
   + 55 ℃ 20 s (annealing)
   + 72 ℃ 40 s (extension)

Reactions were run on an ABI PRISM® 7700 Sequence Detection System device (Applied Biosystems division of Perkin Elmer Corp., Foster City, USA). Fluorescence was only measured at the melting point with the sequence detection system software (version 1.7, Applied Biosystems). Quantification was performed by interpolation in a standard regression curve of C_T values generated from samples at known concentrations. Negative values or lack of amplification was considered for real-time PCRs with threshold C_T value of >45.

III.2.16 NASBA technique

NASBA is specifically designed to detect RNA, and employs three enzymes, a reverse transcriptase, RNaseH and T7 RNA polymerase, which act in concert to amplify sequences from an original single-stranded RNA template. Oligonucleotide primers, complementary to sequences in the target RNA, are incorporated in the reaction. One primer also contains a recognition sequence for T7 RNA polymerase. The reaction contains both dNTPs and NTPs. The first primer binds to the RNA, allowing the reverse transcriptase to form a complementary DNA strand. Then the RNase digests away the RNA and the second primer binds to the cDNA, allowing the reverse transcriptase to form a double-stranded cDNA copy of the original sequence. This double-stranded DNA then acts as a kind of mini "gene", which is transcribed by the T7 RNA polymerase to produce thousands of RNA transcripts which then cycle through the reaction.

a. Conventional NASBA

Reagents:

+ 40 mM Tris-HCl, pH 8.5
+ 12 mM $MgCl_2$
+ 70 mM KCl
+ 5 mM dithiothreitol
+ 15 % v/v DMSO
+ 1 mM of each dNTP
+ 2 mM of each rNTP
+ 0.5 µM of each primer
+ 5 µl enzymatic mix containing 0.08 U of RNase H, 32 U of T7 RNA polymerase and 6.4 U of AMV reverse transcriptase.
+ 5 µl containing RNA solution
  Final volume of 20 µl.

Thermocycler conditions:

1. 65 ºC for 5 min (destabilize the secondary structure of RNA).
2. 41 °C for 5 min (annealing of primers)

addition of enzymatic mix

3. 41 ºC for 90 min

NASBA reactions were performed on a GenAmp® PCR System 9700 (Applied Biosystems division of Perkin Elmer Corp., Foster City, USA).

<u>NASBA products detection</u>

• Electrophoresis agarose gel detection

The resulting NASBA products were detected by EtBr straining after electrophoresis in agarose gel as described in III.2.13.

• Electrochemiluminescence (ECL) detection

The NASBA amplicons were detected using a NucliSens® ECL Reader (bioMérieux bv, The Netherlands) as described previously (Simpkins et al., 2000). After the amplification reaction, a 5 µl aliquot of NASBA amplification products was added to 100 µl of NucliSens® detection diluent (1:20 dilution). Each diluted NASBA amplification product was combined with its gene-specific capture probe bound to magnetic beads and the generic ECL detection probe, and the mixture was incubated at 41°C for 30 min with intermittent vortexing. Following hybridization, each reaction was diluted with 300 µl of assay buffer containing tripropylamine (TPA), and then read in a NucliSens® ECL reader. The NucliSens® ECL reader detects the amplified capture probe complex through the electrochemiluminescence emitted by the bound generic ECL probe, and calculates positive results on the basis of the values for the positive and negative controls included in the assay.

b. Molecular beacon-based real-time NASBA

Reagents:

+ 40 mM Tris-HCl, pH 8.5
+ 12 mM $MgCl_2$
+ 70 mM KCl
+ 5 mM dithiothreitol
+ 15 % v/v DMSO
+ 1 mM of each dNTP
+ 2 mM of each rNTP
+ 500 nM of each primer
+ 50-400 nM of molecular beacons
+ 5 µl enzymatic mix containing 0.08 U of RNase H, 32 U of T7 RNA polymerase and 6.4 U of AMV reverse transcriptase.
+ 5 µl containing RNA solution
  Final volume of 20 µl.

Thermocycler conditions:

1. 65 ºC for 5 min (remove the secondary RNA structure).
2. 41 °C for 5 min (annealing of primers)

   addition of enzymatic mix

3. 42 ºC for 100 min

NASBA reactions were performed on a ABIPrism® 7700 Sequence Detection System (Applied Biosystems, Foster City, USA) which allowed real-time monitoring of fluorescence as molecular beacon probe was bound to newly generated NASBA amplicons. Fluorescence was quantified on-line and at the end point with the sequence detection system software (version 1.7, Applied Biosystems).

III.2.17   DNA cloning

III.2.17.1   Dephosphorylation of Plasmid DNA

1. Digest ≈10 μg of closed circular plasmid DNA with a two- to threefold excess of the desired restriction enzyme for 1 hour.

2. Remove one 0.1 μg-aliquot, and analyze the extend of digestion by electrophoresis through a 0.7 % agarose gel containing EtBr, using undigested plasmid DNA as a marker. If digestion is not complete, add more restriction enzyme and continue the incubation.

3. When digestion is complete, extract the sample once with phenol:chloroform and recover the DNA by standard precipitation with EtOH. Store the ethanolic solution on ice for 15 min.

4. Recover the DNA by centrifugation at maximum speed for 10 min at 4 °C in a micro-centrifuge , and dissolve the DNA in 110 μl of 10 mM Tris-Cl (pH 8.3).

5. Reserve 20 μl of the DNA preparation for later use as a control. To the remaining 90 μl of the linearized plasmid DNA, add 10 μl of 10 × CIP buffer and the appropriate amount of CIP and incubated as described in Table III.8.

Table III.8. Conditions for Dephosphorylation of 5'-phospatase residues from DNA.

Type of terminus	Enzyme/Amount per Mole DNA ends	Incubation Temperature/Time
5'- Protunding	0.01 unit CIP[a]	37 °C/30 min
3'- Protunding	0.1-0.5 unit CIP[b]	37 °C/15 min
		then 55 °C/45 min
Blunt	0.1-0.5 unit CIP[b]	37 °C/15 min
		then 55 °C/45 min

[a] After the initial 30-min incubation, add a second aliquot of CIP enzyme and continue incubation for another 30 min at 37 °C
[b] Add a second aliquot of CIP just before beginning the incubation at 55 °C.

6. Inactivate the phosphatase activity of CIP at the end of the incubation period with add SDS and EDTA (pH 8.0) to final concentration of 0.5 % and 5 mM, respectively. Mix well, and add Proteinase K to a final concentration of 100 μg/ml. Incubate for 30 min at 55 °C.

7. Cool the reaction mixture to room temperature, and then extract it once with phenol and once with phenol: chloroform.

8. Recover the DNA by standard precipitation with EtOH. Mix the solution again and store it for 15 min at 0 °C.

9. Recover the DNA by centrifugation at maximum speed for 10 min at 4 °C in a micro-centrifuge . Wash the pellet with 70 % EtOH at 4 °C and centrifuge again.

10. Carefully remove the supernatant and leave the open tube on the bench to allow the EtOH to evaporate.

11. Dissolve the precipitated DNA in 1 × TE (pH 8.0) at a concentration of 100 μg/ml. Store the DNA in aliquots at -20 °C.

III.2.17.2   Blunt-ended Cloning into Plasmid Vectors.

1. In separate reaction, digest 1-10 μg of the plasmid DNA and foreign DNA with the appropriate restriction enzyme(s) that generate blunt ends.

2. Purify the digested foreign DNA and vector DNA by extraction with phenol:chloroform and standard EtOH precipitation.

3. Reconstitute the precipitated DNAs separately in 1 × TE (pH 8.0) at a concentration of ≈ 100 μg/ml. Calculate the concentration of the DNA (in pmol/ml) assuming that 1 bp has a mass of 660 daltons. Confirm the approximate concentration of the DNAs by analyzing aliquots by agarose gel electrophoresis.

4. Dephosphorylate the plasmid vector DNA as described in III.2.17.1.

5. Transfer appropriate amounts of the DNAs to sterile 0.5-ml micro-centrifuge tubes as follows

Tube	DNA
A and E	vector[1] (60 fmol [≈ 100 ng])
B	Foreign[2] (60 fmol [≈ 100 ng])
C and F	vector[1] (60 fmol) plus foreign (60 fmol)[3]
D	linearized vector (contains 5´- terminal phosphates9 (60 fmol)
G	Superhelical vector (6 fmol [≈ 10 ng])

[1] Vector DNA is desphosphorylated as described in III.2.17.1
[2] Linkers may be ligated to foreign target DNA.
[3] The molar ratio of plasmid vector to insert DNA fragment should be ≈ 1:1 in the ligation reaction.
The total DNA concentration in the ligation reaction should be ≈ 10 ng/μl

a. To tubes A, B, C add:

$10 \times$ Ligation buffer	1.0 μl
Bacteriophage T4 ligase	0.5 Weiss unit
5 mM ATP	1.0 μl
H_2O	to 8.5 μl
30 % PEG 8000	1-1.5 μl

b. To tubes D, E, F add:

$10 \times$ Ligation buffer	1.0 μl
5 mM ATP	1.0 μl
H_2O	to 8.5 μl
30 % PEG 8000	1-1.5 μl
no DNA ligase	

6. Incubate the reaction mixtures overnight at 16 °C or for 4 hours at 20 °C.

7. Transform competent *E. coli* with dilutions of each of the dilution reactions. As controls include known amounts of a standard preparation of superhelical plasmid DNA to check the efficiency of the transformation.

III.2.17.3   Ligation of DNA fragments

1. Prepare the ligation mixture in a final volume of 10 μl containing:
   + The purified DNA fragment.
   + digested plasmid.

2. Usually, a molar ratio of 3:1 between insert and vector was present in the ligation mixtures.

3. Incubate the mixture at 65 °C for 5 min.

4. Place the mixture on ice, and centrifuge at maximum speed for 10 s.

5. Add 1 μl of $10 \times$ T4 DNA ligase buffer and 1 μl 5 U/μl bacteriophage T4 DNA ligase.

6. Mix well and incubate at 16 °C for 2 hours to overnight.

When blunt ends were ligated, 6 % PEG (final concentration) was added to the ligation mixture to increase ligation efficiency.

40 % PEG: 40 % (w/v) polyethylenglycol 6000 in water. Autoclave at 120 °C fro 20 min and store at 4 °C.

III.2.17.4   Bacterial transformation

III.2.17.4.1   Bacterial transformation by thermal shock

This protocol is a modification of that described by D. Hanahan (1983). The resulting yield must be 10^6-10^7 transformed colonies/ μg DNA.

- **Preparation of competent cells**

1. Inoculate a well-isolated *E. coli* strain DH5α colony grown in a LB agar plate into a tube containing 5 ml of LB broth. Incubate at 37 °C overnight with moderate shaking.

2. Harvest *E. coli* strain DH5α into a LB agar plate and incubate at 37 °C for 16-18 h.

3. Use an inoculating loop from this LB agar plate to inoculate 50 ml of LB broth. Incubate at 37 °C overnight with moderate shaking.

4. Use 100 μl to inoculate 1,000 ml-LB broth. Incubate at 37 °C for 2.5-3 h with moderate shaking, monitoring the growth of the culture. For efficient transformation, it is essential that the number of viable cells not exceed 10^8 cells/ml, which for most strains of *E. coli* is equivalent OD_{600} of ≈ 0.4.

5. Cool the culture on ice for 15-30 min.

6. Transfer the cells to sterile, disposable, ice-cold 50-m polypropylene tubes.

7. Recover the cells by centrifugation at 2,700 × *g* (rotor Beckman JA-14) for 10 min at 4 °C.

8. Decant the medium from the cell pellets. Stand the tubes in an inverted position for 1 min to allow the last traces of medium to drain away.

9. Resuspend the pellets by swirling or gentle vortexing in ≈ 20 ml (per 50-ml tube) of ice-cold TFB. Store the resuspend cells on ice for 10 min.

10. Recover the cells by centrifugation at 2,700 × *g* for 10 min at 4 °C.

11. Decant the buffer from the cell pellets. Stand the tubes in an inverted position for 1 min to allow the last traces of buffer to drain away.

12. Resuspend the pellets by swirling in 10 ml of TFB2 (4 °C).

13. Aliquot in 100-µl in cold sterile tubes and snap-freeze the competent cells by immersing the tightly closed tubes in a bath of liquid nitrogen.

14. Store the tubes -80 °C until needed.

- **Transformation**

15. When needed remove a tube of competent cells from the -80 °C freezer. Thaw the cells by holding the tube in the palm of the hand. Just as the cells thaw, transfer the tube to an ice batch. Store the cells on ice for 10 min.

16. Add 10 µl of the transforming DNA (up to 25 ng per 50 µl of competent cells). Swirl the tubes gently several times to mix their contents.

17. Incubate the tubes on ice for 30 min.

18. Transfer the tubes to a rack paced in a preheated 42 °C water bath. Store the tubes in the rack for 3 min.

19. Transfer the tubes to an ice bath. Incubate for 2-5 min.

20. Add 800 µl of preheated-LB broth and incubate at 37 °C for 1 h.

21. Centrifuge at maximum speed, and remove 800 µl of supernatant. Resuspend the rest (≈ 100 µl).

22. Transfer the transformed competent cells onto the agar plate containing the appropriate antibiotic.

23. Store the plates in room temperature until the liquid has been absorbed.

24. Invert the plates and incubate them at 37 °C.

25. The white coloured-colonies obtained will be used to perform minipreps.

III.2.17.4.2   Bacterial transformation by electroporation

The electroporation is one of the most efficient techniques for bacterial transformation, with a resulting yield up to 10^9 transformed colonies/ µg DNA. The following protocol is a modification of that described by Sheen (1997).

- **Preparation of competent cells**

1. Inoculate a well-isolated *E. coli* strain DH5α colony grown in a LB agar plate into a tube containing 5 ml of LB broth. Incubate at 37 °C overnight with moderate shaking.

2. Inoculate 2.5 ml of this culture into 2-liter Erlenmeyer flasks containing 500 ml of prewarmed LB medium.

3. Incubate the flasks at 37 °C with agitation (300 rpm). Measure the OD_{600} of the growing bacterial cultures every 20 min.

4. When the OD_{600} of the cultures reaches 0.4, transfer the cultures to ice-cold 1-l propylene bottles.

5. Incubate on ice for 1 h.

6. Harvest the cell by centrifugation at 4,000 rpm (rotor Beckman JA-10) for 20 min at 2 °C.

7. Decant the supernatant and resuspend gently the cell pellet in 5 ml cold sterile double destilled water. Add 500 ml of double destilled water.

8. Repeat steps 6 and 7.

9. Repeat step 6.

10. Decant the supernatant immediately and resuspend gently the cell pellet in 40 ml of ice-cold 10 % glycerol.

11. Transfer the bacterial solution into two separate 50-ml propylene bottles.

12. Centrifuge at 4,000 rpm (rotor Beckman JA-20) for 10 min at 2 ºC. Decant the supernatant immediately. Calculate the sediment volume and add a equal volume of ice-cold 10 % glycerol. Resuspend gently the bacterial pellet.

13. Transfer 40-µl aliquots to -20 ºC cold sterile tubes and snap-freeze the elctrocompetent cells by immersing the tightly closed tubes in a bath of liquid nitrogen.

14. Store the tubes -80 ºC until needed.

- **Electroporation**

15. Pipette 40 µl of thawed elctrocompetent cells into ice-cold sterile 0.5-ml micro-centrifuge tubes.

16. Add 10-20 ng of the transforming DNA in a volume of 1-2 µl. Swirl the tubes gently several times to mix their contents.

17. Incubate the tubes on ice for 20 min. Place also an appropriate number of bacterial electroporation cuvettes on ice.

18. Set the electroporation apparatus (Bio-Rad) to deliver an electrical pulse of 25 µF capacitance, 2.5 kV, and 200 ohm resistance.

19. Pipette the DNA/cell mixture into a cold electroporation cuvette. Tap the solution to ensure the suspension of bacteria and DNA sits at the bottom of the cuvette. Dry condensation and moisture from the outside of the cuvette. Place the cuvette in the electroporation device.

20. Deliver a pulse of electricity to the cells.

21. As quickly as possible after the pulse, remove the electroporation cuvette and add 460 µl of LB medium, mix well by pipetting and transfer the cells to a 1.5-ml micro-centrifuge tube.

22. Incubate at 37 ºC for 1 h in moderate agitation (250 rpm).

23. Plate different volumes (up to 200 µl) of the electroporated cells onto the appropriate agar plate.

24. Store the plates in room temperature until the liquid has been absorbed.

25. Invert the plates and incubate them at 37 ºC.

26. The white coloured-colonies obtained to perform minipreps.

III.2.18 DNA Sequencing

a. Cycle sequencing reaction

Reaction Mix (final volume 10 µl)		Thermocycling conditions:	
+ DNA template (0.2 µg/ml)	1 µl	94 ºC 3 min.	
+ BigDye Terminator Mix	3 µl	25 cycles:	96 ºC 10 s
+ Primer 3.2 µM 1 µl			50 ºC 5 s
+ dd-H$_2$O 5 µl			60 ºC 4 min

b. Preparation of the Cycle sequencing reaction product

1. Add the Cycle sequencing reaction product into a 1.5-ml micro-centrifuge tube containing 62.5 µl of 95 % EtOH, 3.0 µl of 3M NaAc pH 4.6 and 14.5 µl of dd-H$_2$O, and vortex to mix.

2. Incubate at room temperature for 15 min.

3. Centrifuge at 12,000 rpm for 20 min.

4. Remove the supernatant, and add 250 µl of 70 % EtOH, and vortex to mix.

5. Centrifuge at 12,000 rpm for 5 min.

6.   Remove the supernatant, and store the open tube on the bench at room temperature until the last traces of fluid have evaporated.

7.   Add 30 μl of TSR (Template Suppression Reagent)

8.   Incubate the sample at room temperature for 20 min.

9.   Vortex gently and centrifuge briefly.

10. Denaturize the sample at 94 ºC for 3 min.

11. Cool on ice for 3 min.

12. Vortex gently and centrifuge briefly.

13. Place the sample into the 0.5-ml ABI 310 tubes, and close the tubes.

14. Place the tube into ABI PRISM® 310 DNA Sequencer.

IV. RESULTS AND DISCUSSION

"DNA testing is to justice what the telescope is for the stars"

Barry Scheck, Peter Neufeld and Jim Dwyer

IV.1 Pre-amplification procedures

IV.1.1 Extraction of bacterial genomic DNA from food samples

Comparison of two protocols for bacterial genomic DNA isolation from a model meat product

OBJECTIVE

The aim of this study was to compare two different protocols for extraction of bacterial genomic DNA present in a model meat product in order to be applied in combination with an RTi-PCR assay. A model meat product (*i.e.* cooked ham) was artificially contaminated with a fixed amount of *Listeria monocytogenes*. The selected extraction protocols were either based on an open formula (Protocol 1) or a commercial kit (Protocol 2). The reliability of each protocol was evaluated by performing three independent experiments using 8 replicate samples. The quality and quantity of the bacterial genomic DNA was compared using both fluorescence-based techniques and a *L. monocytogenes*-specific quantitative RTi-PCR assay.

MATERIALS AND METHODS

Artificial contamination of a model food product with *L. monocytogenes*. Cooked ham slices containing 2% fat (Anonymous, 1979) were artificially contaminated with an overnight culture of *L. monocytogenes* strain UdG 1010 as reported (Aymerich *et al.*, 2000). Briefly, slices were vacuum packed, diluted (1:10) with 0.1% peptone, 0.85% NaCl and homogenised for 1 min in stomacher bags (125-µm pore size, Biochek). *L. monocytogenes* was enumerated by the standard microbiological method (ISO 11290-2, Anonymous, 1998). The *L. monocytogenes* load added to the food samples was calculated to be 3×10^9 cfu/g.

Protocols for bacterial genomic DNA extraction. For each protocol three replicate experiments were performed, each on eight replicate samples.

(a) <u>Protocol 1: Chelex® 100 resin-based DNA extraction.</u> One ml of sample was transferred to a clean 1.5-ml micro-centrifuge tube, centrifuged for 5 minutes at $10,000 \times g$ and the supernatant was discarded. Subsequently, 100 µl 6% Chelex® 100 suspension was added to the pellet and the mixture was incubated at 56°C for 20 min. Then, the sample was mixed using a vortex and incubated at 100°C for 8 min. After vortexing, the

mixture was immediately chilled on ice. Finally, the sample was centrifuged for 5 minutes at $14,000 \times g$, and the supernatant was transferred to a fresh tube and stored at –20°C until use.

(b) <u>Protocol 2: Wizard® Genomic DNA Purification kit.</u> One ml of sample was transferred to a 1.5 ml microcentrifuge tube, centrifuged at $10,000 \times g$ for 5 min and the supernatant was removed. Subsequently, the pellet was resuspended thoroughly in 480 µl of 50 mM EDTA, and 120 µl of 10 mg/ml lysozyme was added. The sample was incubated at 37°C for 60 min, centrifuged for 2 min at maximum speed and the supernatant was removed. 600µl of Nuclei Lysis Solution was added to the pellet and the mixture was incubated at 80°C for 5 min and then allowed to cool down to room temperature. Then, 3 µl of 4 mg/ml RNase A solution was added to the cell lysate and the sample was incubated at 37°C for 15 min. 200µl Protein Precipitation Solution was added and well mixed to the RNase-treated cell lysate, and the sample was incubated for 5 min on ice. The sample was centrifuged at maximum speed for 3 min and the supernatant was transferred into a clean 1.5 ml tube containing 600 µl of isopropanol, gently mixed by inversion until the thread-like strands

of DNA became visible. After centrifugation at maximum speed for 5 min, the supernatant was carefully poured off. The DNA pellet was washed by adding 600μl of 70% ethanol, centrifuging at maximum speed for 5 min, carefully removing the ethanol and allowing the pellet to air-dry for 10–15 min. Finally, pellet was resuspended in 100 μl of DNA Rehydration Solution and stored at –20°C until use.

DNA quantification.

(a) Fluorescence-based procedure: DNA concentration was determined using PicoGreen® (Molecular Probes, Inc., Eugene, OR, USA) in a Luminescence Spectrometer LS50B (Perkin-Elmer Corp., Norwalk, CT, USA) -see III.2.12 of Materials and Methods section-.

(b) *L. monocytogenes*-specific RTi-PCR assay. RTi-PCR assay was performed using TaqMan® PCR core reagents (Applied Biosystems-Roche Molecular Systems Inc., Branchburg, NJ, Germany) in a 20 μl reaction volume containing 1× PCR TaqMan® buffer A (including ROX as a passive reference dye), 6 mM $MgCl_2$, 200 μM dATP, dCTP and dGTP, 400 μM dUTP, 50 nM *hly*-specific primers (*hly*Q-F and *hly*Q-R), 100 nM

*hly*Q-P probe, 1 unit of Ampli*Taq* Gold® DNA polymerase, 0.2 units of AmpErase® uracil *N*-glycosylase (UNG), and 1 μl of the target DNA solution. Reactions were run on an ABI PRISM® 7700 apparatus (Applied Biosystems division of Perkin Elmer Corp., Foster City, CA, USA) using the following program: 2 min at 50°C, 10 min at 95°C and 50 cycles of 15 s at 95°C and 1 min at 63°C. RTi-PCR assays were analyzed using Sequence Detection System software v1.7 (Applied Biosystems division of Perkin Elmer Corp., Foster City, CA, USA). Quantification was performed by interpolation in a standard regression curve of C_T values generated from *L. monocytogenes* DNA samples at known concentrations. Negative values or lack of amplification was considered for RTi-PCRs with threshold C_T value of >50. All reactions were performed in triplicate.

Statistical analysis. Data was analysed using SPSS v11.0.1 (SPSS INC., Chicago, Il, USA). Analyses of variance (ANOVA) were applied to the values of different extractions.

RESULTS AND DISCUSSION

Tables IV.1 and IV.2 show DNA yield values obtained using the two DNA extraction protocols here evaluated; calculated using two different techniques *i.e.* based on fluorescence and RTi-PCR. The former allows quantification of DNA in an unspecific manner (*e.g.* including possible swine DNA from the food matrix) and the latter allows specific quantification of *L.*

monocytogenes DNA. Moreover, if bacterial DNA is to be used for amplification based analyses, the latter approach should be of choice since it allows determining the amount of specific DNA suitable for amplification (*e.g.* taking into account the possible presence of PCR inhibitors in the DNA solution).

Table IV.1. DNA yields determined by PicoGreen®. Mean values (± standard deviation) obtained using each DNA extraction protocol in each replicate experiment and overall.

	DNA quantification by Picogreen (ng/μl)			
	1st	2nd	3rd	Overall
Protocol 1	20.5 ± 1.3	16.0 ± 2.0	10.1 ± 1.0	**15.2 ± 3.4**
Protocol 2	18.2 ± 5.9	13.3 ± 7.0	10.3 ± 3.0	**14.0 ± 5.0**

Table IV.2. *L. monocytogenes* DNA yields determined by RTi-PCR. Mean values (± standard deviation) obtained using each DNA extraction protocol in each replicate experiment and overall.

	C_T values			
	1st	2nd	3rd	Overall
Protocol 1	17.37 ± 0.31	19.33 ± 0.23	19.82 ± 0.43	**18.82 ± 1.13***
Protocol 2	15.25 ± 0.34	15.25 ± 0.34	15.18 ± 0.40	**15.23 ± 0.35***

*Results statistically different (p<0.05)

The two protocols produced statistically similar DNA yields (p>0.05) (Table IV.1). However, the presence of DNA other than *L. monocytogenes* DNA can not be excluded at this stage.

In contrast, the two protocols produced statistically different yields of *L. monocytogenes* DNA suitable for amplification (Table IV.2). In RTi-PCR reactions with optimal efficiency, the

C_T difference ($\Delta C_T = 3.59$) obtained among the two protocols signifies one order of magnitude (i.e $12.04=2^{3.59}$) less amplifiable *L. monocytogenes* DNA obtained by Protocol 1 compared to Protocol 2. This is a very significant parameter as it reveals the performance of each DNA extraction protocol for use in combination with the RTi-PCR assay; and it directly affects the detection limit of the method, and thus it could be predicted that the Wizard® based protocol would be around one order of magnitude more sensitive than the Chelex® based protocol.

It is noticeable that the RTi-PCR standard deviation among replicate samples processed in parallel is significantly smaller using DNA extractions obtained by the Wizard® based protocol compared to those obtained by the Chelex® based protocol. In addition, only using the Wizard® based protocol, equivalent samples processed on different days produced non-statistically different results ($p>0.05$). Thus, the reproducibility of the Wizard® based protocol is very convenient.

Comparison among DNA concentration values obtained by fluorescence and RTi-PCR for the two protocols suggested two possible interpretations, either (i) non- *L. monocytogenes* DNA amount was higher in extractions using the Chelex® based protocol; or (ii) the Wizard® based protocol produces a DNA solution less rich in other components that may negatively influence subsequent amplification reactions. Both, the presence of high amounts of non-target DNA and amplification inhibitors have been described to negatively affect the performance and the detection limit of subsequent PCR or RTi-PCR assays.

In conclusion, the Wizard® based protocol used in combination with RTi-PCR assay proved to be a very reproducible for the detection of *L. monocytogenes* in a model meat sample. However, the Chelex® based protocol is shorter and more cost-effective, thus its combination with an RTi-PCR assay may be desirable for a number of routine analytical purposes.

IV.1.2 Isolation of total RNA from bacteria (scientific article I)

Isolation of total RNA from bacteria

Scientific article I:

David Rodríguez-Lázaro, Maria Pla, Annette N. Jensen and Jeffrey Hoorfar

Usefulness of RNAprotect Bacteria Reagent (cat. no. 76506, Qiagen) for total RNA isolation of *Salmonella* spp.

X[th] International Congress of Bacteriology and Applied Microbiology, Paris, 2002

The aim of this study was to evaluate the new RNAprotect Bacteria Reagent for the stabilisation of *Salmonella* RNA prior to its extraction using the RNeasy kit. It was performed in collaboration with Qiagen (Hilden, Germany), manufacturer of the new reagent and the kit, in the context of an end-user field trial previous to the commercialisation of the product. Briefly, the RNA extraction protocol was as follows: RNA stabilisation, enzymatic digestion of bacterial cell walls, and RNA isolation with the RNeasy kit. The inclusion of a mechanical cell wall disruption step prior to its enzymatic digestion was also tested. As a control, the former protocol was used without any RNA stabilisation. From the results obtained, the application of RNAprotect Bacteria Reagent to the isolation of total RNA improves both the yield and reproducibility of the RNA obtained. Furthermore, its combination with cell mechanical disruption further improves RNA yields.

IV.2 Detection and enumeration of *Listeria* spp.

IV.2.1 Detection, identification and quantification of *L. innocua* and *L. monocytogenes* using RTi-PCR

(scientific article II)

IV.2.1 Detection, identification and quantification of *L. innocua* and *L. monocytogenes* using RTi-PCR.

Scientific article II:

David Rodríguez-Lázaro, Marta Hernández, Mariela Scortti, Teresa Esteve, José Antonio Vázquez-Boland and Maria Pla

The first aim of this study was the development of conventional and real-time PCR methods for qualitative and quantitative detection of *Listeria monocytogenes* and *Listeria innocua*. This was achieved by amplifying either a part of the listeriolysin O (*hly*) gene or a part of the extracellular invasion-associated protein (*iap*) gene (*L. monocytogenes*); or a part of the *lin2483* gene (*L. innocua*). The methods proved to be highly selective for identification of *L. monocytogenes* or *L. innocua*, with a detection limit of 1 target molecule in a percentage of the replicates. Moreover, quantification by RTi-PCR was possible along a wide dynamic range and down to 15 target molecules with R^2 values of 0.999.

A major goal of this study was to analyse the suitability of different target sequences for use in PCR experiments with quantitative purposes. For *L. monocytogenes*, only the *hly* based real-time PCR system proved to be suitable for accurate quantification irrespective of the particular bacterial strain. The sequence differences among *L. monocytogenes* strains belonging to different serotypes and/or divisions allowed placement of a particular bacterial strain into division I/III or II through the combined use of both, *hly* and *iap* directed RTi-PCR assays.

Finally, a novel quantification chemistry based on the universal AmpliFluor™ system was compared to the widely used TaqMan® system that is based on a sequence specific probe; targeting the same *hly* sequence. The specificity and suitability for quantification of the two chemistries are discussed. In both cases, not only *L. monocytogenes* or *L. innocua* (only for TaqMan probes) purified genomic DNA but also intact cells were suitable for use as external calibration standards for the elaboration of comparative amplification profiles. Detection could be also performed using broth culture or agar grown colonies.

IV.2.2 Simultaneous quantitative detection of *Listeria* spp. and *Listeria monocytogenes* using duplex RTi-PCR

(scientific article III)

IV.2.2 Simultaneous quantitative detection of *Listeria* spp. and *Listeria monocytogenes* using duplex RTi-PCR

Scientific article III:

David Rodríguez-Lázaro, Marta Hernández and Maria Pla

The aim of this study was the development, for the first time, of a duplex RTi-PCR based assay for the simultaneous quantitative detection of *Listeria* spp. and the food-borne pathogenic species *Listeria monocytogenes*. This single-tube system targets the 23S rDNA and *hly* genes of *Listeria* spp. and *L. monocytogenes*, respectively; using two compatible sets of primers and two probes labelled with different fluorophores (FAM for *hly* and VIC for 23S rDNA). The assay was efficient, 100% selective (*i.e.* it allowed accurate simultaneous identification of 52 *L. monocytogenes* and 120 *Listeria* spp. strains); and had a detection limit of one target molecule in 100% (23S rDNA) and 56% (*hly*) of the reactions. The RTi-PCR assay was highly linear, with R^2 values above 0.995 along a 5-log dynamic range, making the simultaneous quantification of *Listeria* spp. and *Listeria monocytogenes* possible (limit of quantification, 30 target molecules in both cases). Thus, it is a promising alternative to classical methods used for routine analysis in microbiology laboratories. In addition, its simplicity and rapidity makes this assay a good choice for use in studies aiming at the evaluation of the incidence of *Listeria* spp. and *L. monocytogenes* in food processing environments and raw and processed food products.

IV.2.3 Application of RTi-PCR for quantitative detection of *L. monocytogenes* in food samples

(scientific articles IV and V)

IV.2.3 Application of RTi-PCR for quantitative detection of *L. monocytogenes* in food samples

IV.2.3.1 Meat and meat products

Scientific article IV:

David Rodríguez-Lázaro, Anna Jofré, Teresa Aymerich, Marta Hugas and Maria Pla

Rapid quantitative detection of *Listeria monocytogenes* in meat products by real-time PCR.

The aim of this study was evaluate three different protocols for food sample preparation in order to be applied in combination with a *Listeria monocytogenes*-specific RTi-PCR. These three pre-PCR procedures were base on a simple filtration step ([i] filtration through the filter included in the stomacher bags (125-µm pore size, Biochek); [ii] through a 22 to 25 µm pore size filter; and [iii] through a nylon membrane with 11 µm pore size) in combination with a rapid nucleic acid extraction procedure based on Chelex resin. These procedures were evaluated in a cooked meat sample artificially contaminated with decreasing amount of *L. monocytogenes*. The pre-PCR procedure with better results was the combination of filtration through a nylon membrane with 11 µm pore size and an Chelex-based DNA purification. Subsequently, other three meat products (raw pork, Frankfurter sausages and fermented sausages) were analyzed using this pre-PCR method in combination with the previously described RTi-PCR (scientific article II). This method could detect as few as 100 CFU/g and quantify as few as 1000 CFU/g with excellent accuracy compared to the plate count method. Therefore, the PCR-based method (including this pre-PCR procedure and the RTi-PCR assay) is a promising alternative for the detection of *L. monocytogenes* in meat products.

IV.2.3.2 Fish and fish products

Scientific article V:

David Rodríguez-Lázaro, Anna Jofré, Teresa Aymerich, Margarita Garriga and Maria Pla

Rapid quantitative detection of *Listeria monocytogenes* in salmon products: evaluation of pre-real-time PCR strategies

The aim of this study was evaluate different protocols for food sample preparation in order to be applied in combination with a *Listeria monocytogenes*-specific RTi-PCR. These pre-PCR procedures were base on a simple filtration step with enzymatic and detergent step in combination with a rapid nucleic acid extraction procedure. These procedures were evaluated in a smoked salmon sample artificially contaminated with decreasing amount of *L. monocytogenes*. The pre-PCR procedure with better results was the combination of filtration through a nylon membrane with 22-25 μm pore size and an Wizard-based DNA purification. Its combination with the previously described RTi-PCR assay previously described could detect as few as 10 CFU/g and quantify as few as 1,000 CFU/g with excellent accuracy compared to the plate count method. In addition, raw salmon was analyzed. Therefore, the PCR-based method (including this pre-PCR procedure and the RTi-PCR assay) is a promising alternative for the detection of *L. monocytogenes* in seafood products.

IV.3 Detection of *M. avium* subsp. *paratuberculosis*
(scientific articles VI-IX)

IV.3.1 Quantitative detection of *M. avium* subsp. *paratuberculosis* using real-time PCR

(scientific articles VI and VII)

IV.3.1.1 Application of real-time PCR for quantitative detection of *M. avium* subsp. *paratuberculosis* in food samples

Scientific article VI:

David Rodríguez-Lázaro, Martin D'Agostino, Arnold Herrewegh, Maria Pla, Nigel Cook, and John Ikonomopoulos

Real-time PCR-based methods for quantitative detection of *Mycobacterium avium* subsp. *paratuberculosis* in water and milk

The aim of this study was the development and application of an RTi-PCR assay for quantitative detection of *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) in food samples. The RTi-PCR assay targeted the IS900 insertion element which had been previously reported to be specific for this bacterium. Furthermore, an adequate internal amplification control (IAC) was developed to be co-amplified with the target bacterium using the same primer pair; but differentially detected through an IAC-specific probe labelled with a different fluorophore as compared to that of the *MAP*-specific probe. The specificity of the assay was analysed using 18 isolates of *MAP*, 17 other mycobacterial strains and 25 non-mycobacterial strains, and it was fully selective. The sensitivity (with 99 % probability) of the assay was determined to be 3 genomic DNA copies or 12 cells added directly to the reaction. Being this assay developed with analytical purposes, its suitability for use on 2 different food matrixes (*i.e.* milk and water) was evaluated. A pre-PCR treatment specific for each food matrix was developed. A single centrifugation step prior to PCR allowed the consistent quantitative detection of 10^2 *MAP* in 20 ml of artificially contaminated drinking water. A simple detergent and enzymatic pre-treatment followed by centrifugation and nucleic acid extraction allowed the consistent detection of 10^2 *MAP* in 20 ml of artificially contaminated semi-skimmed milk. Hence, the assay could be a promising diagnostic tool to be implemented in routine diagnostic laboratories; and a useful addition to the range of diagnostic tools available for the study of *MAP*.

IV.3.1.2 Application of the real-time PCR for quantitative detection of *M. avium* subsp. *paratuberculosis* in clinical samples

Scientific article VII:

David Rodríguez-Lázaro, Xavier Fuentes, Xavier Aldeguer, Ferrán González-Huix, Doroteo Acero, Maria Pla and L. Jesús Garcia-Gil

Mycobacterium avium subsp. *paratuberculosis* not found in fresh ileocolonic mucosal biopsy specimens from patients with Crohn's Disease using real-time PCR

The aim of this study was to provide additional data regarding the controversial relation between Crohn's disease and *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*): whereas some authors have reported aetiological relationship of *MAP* respect to Crohn's disease (Hermon-Taylor, 2001; Chiodini *et al.*, 1989), others have failed to detect this bacterium in Crohn's patients (Al-Shamali *et al.*, 1997; Riggio et al, 1997). Here we report on the *MAP* analysis of 27 Crohn's patients selected with non-acute symptoms, included in the Crohn's Disease A Index (CDAI) and not under drug treatment. Innovative aspects of this work include: (i) the use of fresh biopsies from both, affected and non-affected bowel portions of Crohn's patients; and (ii) the recently developed RTi-PCR assay in combination with a new pre-PCR nucleic acids extraction protocol. Advantageous features of using this RTi-PCR assay for such purpose include the multi-copy nature of the target sequence (*IS 900* insertion sequence, allowing high sensitivity), the short PCR amplicon (67 bp, allowing the detection of highly degraded DNA molecules) and the inclusion of an internal amplification control (IAC, allowing the control of the performance of the reaction). The new pre-PCR protocol was developed aiming at the sole recovery of bacteria intimately related to bowel human cells, thus discarding the gastrointestinal tract transient microbiota, which largely depends upon food uptake and other personal conditions.

We could not detect *MAP* DNA in any of the samples analysed. Inhibition of the reactions was ruled out the IAC was properly amplified in all RTi-PCRs; and *MAP* positive controls were also detected correctly. Consequently, considering the sensitivity of the assay, it can not be considered an absolute absence of *MAP* in bowel from Crohn's patients. These results provide additional data for the controversy on aetiology of Crohn's disease.

IV.3.2 Detection of *M. avium* subsp. *paratuberculosis* in food samples using Nucleic Acid Sequence-Based Amplification (NASBA) technique

(scientific articles VIII and IX)

IV.3.2.1 Application of real-time NASBA for detection of *M. avium* subsp. *paratuberculosis* in food samples

Scientific article VIII:

David Rodríguez-Lázaro, Joy Lloyd, Arnold Herreweg, John Ikonomopoulos, Martin D'Agostino, Maria Pla and Nigel Cook

A molecular beacon-based real time NASBA assay for detection of *Mycobacterium avium* subsp. *paratuberculosis* in water and milk

The aim of this study was to develop and apply an RTi-NASBA assay for detection and identification of *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) in food samples. The molecular beacons chemistry was selected as real-time detection system. The assay targeted a nucleotide sequence specific for this bacterium, and includes an adequate internal amplification control. The specificity of the assay was analysed using 18 isolates of *MAP*, 17 other mycobacterial strains and 25 non-mycobacterial strains; and it was fully selective. The detection limit with 99 % probability was established at 150-200 cells per reaction. Being this assay developed with analytical purposes, its suitability for use on 2 different food matrixes (*i.e.* milk and water) was evaluated. A pre-amplification treatment specific for each food matrix was developed. A single centrifugation step prior to nucleic acids extraction allowed the consistent detection of 10^3 *MAP* in 20 ml of artificially contaminated drinking water. A simple detergent and enzymatic pre-treatment followed by centrifugation and nucleic acid extraction allowed the consistent detection of 10^4 *MAP* in 20 ml of artificially contaminated semi-skimmed milk. Hence, the assay could be a useful additional diagnostic tool for the study of *MAP*, and could be used to confirm and complement results of analyses performed with PCR- and culture-based methods.

IV.3.2.2 Evaluation of NASBA for detection of viable *Mycobacterium avium* subsp. *paratuberculosis*

Scientific article IX:

David Rodríguez Lázaro, Joy Lloyd, John Ikonomopoulos, Maria Pla and Nigel Cook

Unexpected detection of DNA by Nucleic Acid Sequence-Based Amplification technique

In this study we tested the capacity of Nucleic Acid Sequence-Based Amplification (NASBA) technique for the detection of viable *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*). NASBA is a technique previously shown to selectively mediate the detection of specific RNA molecules in microbial cells. In a series of parallel experiments performed in the same conditions, nucleic acids were extracted from *Salmonella enterica* serotype Typhimurium and *MAP*, and subjected to four enzymatic treatments (*i.e.* DNase, RNase, S1 nuclease, and RNase / S1 nuclease) prior to NASBA. We obtained different results for the two bacteria. As expected, with *S. enterica* serotype Typhimurium, only RNase and RNase / S1 nuclease treatments abolished the NASBA signal. In contrast, with *MAP* nucleic acids, RNase, S1 nuclease, and RNase / S1 nuclease treatments had no effect on the NASBA signal, although DNase treatment abolished it. In conclusion, unlike for *S. enterica*, *MAP* RNA was not the substrate of NASBA. Strikingly our results show for the first time, NASBA amplification of bacterial genomic DNA. This study is a salutary example to workers who are contemplating the use of NASBA for selective detection of viable microorganisms, and demonstrates the necessity of verifying that the nucleic acid origin of a NASBA signal has indeed been generated only by RNA (*i.e.* potential viable cells) and not DNA.

IV.4 Identification of *Salmonella* spp.
(Scientific article X)

IV.4 Identification of *Salmonella* spp.

Scientific article X:

David Rodríguez Lázaro, Marta Hernández, Teresa Esteve, Jeffrey Hoorfar and Maria Pla

In this study the optimization and validation of a rapid real-time (RTi-) PCR assay for the unequivocal identification of *Salmonella* spp. colonies is presented. This study was developed in laboratories of two different countries (Denmark: Danish Veterinary Institute and Spain: Molecular Biology Institute -IBMB-CSIC, Barcelona- and Food Molecular Microbiology Unit (University of Girona -UdG-, Girona). A previous RTi-PCR assay (Hoorfar *et al.*, 2000) was further optimized and validated through a four times repeated blind experiment performed in two different laboratories including 50 *Salmonella* spp. with representative strains from each of the 5 different *Salmonella* subgenus and 30 non-*Salmonella* strains.

Two RTi-PCR parameters *i.e.* ΔR_n and C_T were analyzed. Overall mean values for *Salmonella* strains were statistically different from values for non-*Salmonella* strains, allowing the establishment of cut-off values based on 95% confidence intervals that allowed the correct identification of all strains tested in each independent experiment. The RTi-PCR assay was 100% selective. Moreover, the PCR system was especially convenient since the pre-mix containing all PCR reagents except for the bacterial cells could be kept at –20ºC for at least one month before use. In conclusion, the optimized RTi-PCR assay is a useful, simple and rapid method for routine identification of *Salmonella* spp., irrespective of the particular subgenus.

IV.5 Development and optimisation of Internal Amplification Controls (IAC)

IV.5.1 Development and application of an IAC for real-time PCR
(Scientific paper XI)

IV.5.1 Development and application of an IAC for real-time PCR

Scientific article XI:

David Rodríguez Lázaro, Mariela Scortti, Jose Antonio Vázquez-Boland and Maria Pla

Assessment of quantitative detection of *Listeria monocytogenes* by real-time PCR: Development and application of an Internal Amplification Control

The aim of this study was to describe and illustrate the construction and optimization strategy of an internal amplification control (IAC) for a RTi-PCR assay. The IAC should be co-amplified with the target bacterium, in this case *Listeria monocytogenes*, as they should share the same primer binding sites. The target bacterium (*e.g. L. monocytogenes*) and the IAC should be detected through two different probes labelled with different fluorophores (*e.g.* FAM and VIC for *L. monocytogenes* and IAC, respectively). The IAC should be used in duplex format with the RTi-PCR assay for the quantitative detection of the target bacterium. It should assist the interpretation of the results of the *L. monocytogenes* reaction by assessing the performance of the RTi-PCR and thus allowing the identification of false-negative results arising from low efficiencies of the reaction due to the presence of inhibitors in the sample or to user/reagents/instrumental errors.

The selectivity of the duplex RTi-PCR was evaluated using 49 *L. monocytogenes* strains of different serotypes and sources; and 104 strains of other species; and the assay was fully selective. The limit of detection of duplex RTi-PCR was the same as the corresponding uniplex RTi-PCR. In addition, the limit of quantification was established at 30 CFU with excellent linearity ($R^2 = 0.997$) and efficiency (0.8), also similar to uniplex reactions, indicating that duplexing did not significantly affect the basic RTi-PCR parameters. Reliable quantification was possible even in the presence of broths such as Brain Heart Infusion or buffered peptone water. As it should be expected, the assay was capable of detecting underestimation of *L. monocytogenes* in reactions containing inhibitory culture broths or foodstuffs. As it occurred with the corresponding uniplex reaction, specific *L. monocytogenes* detection by duplex RTi-PCR could also be performed using agar-grown colonies and in conventional PCR format. In conclusion, the results obtained indicate that the optimized duplex RTi-PCR assay is a useful, simple and rapid method for quantitative detection of *L. monocytogenes*.

IV.5.2 Development and application of an IAC for real-time NASBA

(Scientific paper XII)

IV.5.2 Development and application of an IAC for real-time NASBA

Scientific article XII:

David Rodríguez Lázaro, Martin D'Agostino, Maria Pla and Nigel Cook

A construction strategy for an internal amplification control (IAC) for real time NASBA-based diagnostic assays

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The aim of this study was to present a general strategy for the construction of an IAC for real time (RTi-) NASBA assays. The IAC should be co-amplified simultaneously with the target sequence, and detected using a specific molecular beacon labelled with a fluorophore (HEX) different to the one used for the target (FAM). The construction process is divided into two different phases. In the first phase, a chimerical double-stranded DNA molecule is produced, which contains (i) non-target sequences flanked by (ii) sequences complementary to the NASBA primers and (iii) a T7 RNA polymerase binding sequence at the 5'-end. In the second phase, RNA transcripts are *in vitro* produced from the chimerical DNA by T7 RNA polymerase; subsequently the DNA template is degraded and the RNA (*i.e.* IAC) is purified. Such IAC is amplified along the NASBA reaction by the target NASBA primers; and the IAC NASBA product is fluorescently detected by a molecular beacon probe complementary to the internal non-target sequences. As a practical example, an IAC for use in a NASBA assay to detect *Mycobacterium avium* subsp. *paratuberculosis* is described, and its incorporation and optimisation in the assay in duplex format is detailed.

IV.6 General discussion and concluding remarks

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The presence of pathogens in foods is among the most serious public health concerns, and the diseases produced by them are a major cause of morbidity (Wallace *et al.*, 2000; Anonymous, 2001). Consequently, the application of microbiological control within the quality assessment programs in the food industry is a premise to minimize the risk of infection for the consumer. Classical microbiological methods involve, in general, the use of a non-selective pre-enrichment, selective enrichment, isolation on selective media, and subsequent confirmation using morphological, biochemical and/or serological test (Lübeck and Hoorfar, 2003). Thus, they are laborious, time consuming and not always reliable (*e.g.* viable but non-culturable VBNC forms). A number of alternative, rapid and sensitive methods for the detection, identification and quantification of foodborne pathogens have been developed to overcome these drawbacks, including bioluminescence-based, immunological and molecular methods (Fung, 2002; Giese, 1995; Olsen *et al.,* 1995; Scheu *et al.*, 1998; Swaminathan and Feng, 1994). Among the latter category, PCR has become the most popular microbiological diagnostic method, and recently, the introduction of a development of this technique, RTi-PCR, has produced a second revolution in the molecular diagnostic methodology in microbiology as stated by the increasing number of scientific publications and novel commercial kits available. RTi-PCR is highly sensitive and specific. Moreover, it allows accurate quantification of the bacterial target DNA. Main advantages of RTi-PCR for its application in diagnostic laboratories include quickness, simplicity, the closed-tube format that avoids risks of carryover contaminations and the possibility of high throughput and automation (reviewed in Klein, 2002). Variations of the RTi-PCR are described in the Introduction section.

The implementation process of RTi-PCR methodology as an alternative tool to classical microbiological approach, involves a series of successive phases, each with different key factors that must be carefully considered: (i) the design of the RTi-PCR assay; (ii) its optimization and (iii) its application to real food samples.

The design of the RTi-PCR assay is based on the selection of a bacterial target DNA sequence, which will determine the specificity of the detection system. A specific DNA region of the target bacterium or bacterial group is indispensable to assess its suitability. Among the possible types of candidate sequences, ribosomal RNA (rDNA) genes, protein coding genes and repetitive elements were used in this study. rDNA regions represent a versatile combination of highly conserved and moderately to highly variable segments. Thus, it allows the design of both species-specific and genus- or family-specific detection systems. A consensus sequence of 23S rDNA was used as target for the *Listeria* spp.-specific RTi-PCR assay. Interestingly, note that the problem of a mismatch in the target sequence of the *L. innocua* species could be overcome with the addition of an extra primer fully specific for this bacterium. In addition, rDNA regions are present in several copies in bacterial genomes (*e.g.* 6-8 copies in *L. monocytogenes* and *L. innocua*), which provides an excellent approach to increase the sensitivity of the RTi-PCR assays (*e.g.* the *Listeria* spp.-specific RTi-PCR consistently detected 1 target bacteria). Many RTi-PCR assays targeting protein coding genes have been currently developed (see table I.14). In general, they are specially designed for a particular bacterium or a small group of related microorganisms. The most used genes are those associated to virulence (*e.g. ail* for *Yesinia enterocolytica*). They are unique markers associated to a particular pathogenesis pathway of the target bacterium. Furthermore, such RTi-PCR assays can provide information on the virulence of the detected bacterium. In this study, several genes associated to virulence were used as target for the specific detection of *L. monocytogenes* (*i.e.* the Listeriolysin O gene -*hly*- [Mengaud *et al.*, 1988] and the protein p60 coding gene -*iap*- [Köhler *et al.*, 1990]) and *Salmonella* spp. (*i.e. Salmonella* invasion gene -*invA*- [Galán *et al.*, 1992]). For example, the *hly* gene is only present in the pathogenic species of the *Listeria* genus, and allows the design of RTi-PCR highly specific assays based on both, TaqMan and AmpliFluor chemistries (Rodríguez-Lázaro *et al.*, 2004a; Rodríguez-Lázaro *et al.*, 2004b). For the apathogenic bacterium *L. innocua*, it was selected the *lin02483* gene, coding for a protein potentially associated to

stress, similar to proteins involved in the resistance to cholate and to Na$^+$ and in pH homeostasis. Some microorganisms possess repetitive elements or insertion sequences present in multiple copies, which is a favourable prerequisite for the development of highly sensitive detection methods. Obtaining a highly sensitive assay was especially relevant for the slow-growing *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*), which usually is present in small numbers in food samples. The Insertion Sequence 900 (IS900) (Bull *et al.*, 2000), specific for this bacterium, was selected as target for the *MAP*-specific RTi-PCR assays.

Then, an *in silico* search for a specific region within the selected genes using bioinformatics software tools such as Blast-N (NCBI, USA) and Fasta-3 (EMBL, UK) is recommended. The more important rules for the design of a RTi-PCR system are described in Table I.6 and discussed in the Introduction section. Software tools such as for example Primer Express v2.0 (Applied Biosystems, USA), DNA*DNASTAR (Lasergene, DNASTAR Inc, USA) and primer 3 (http://www-ge-nome.wi.mit.edu/genome_software/other/primer3.html) are useful to assist the design. Subsequently, the specificity of the RTi-PCR system must be revised *in silico* by testing the overall specificity of the amplicon as well as the specificity of each individual oligonucleotide. As a example, the *hly* gene from L. monocytogenes, contained a region (positions 113 to 177) showing no significant homology to any other sequence deposited in public databases. A pair of primers and a TaqMan® probe were designed within this region and compared to other public sequences, showing only similarities to *L. monocytogenes* sequences. Thus, the system was considered specific and further optimised.

Next, RTi-PCR systems must be optimised. Three parameters must be empirically tested: oligonucleotides concentrations, Mg^{2+} concentration, and the annealing temperature. Optimal conditions for primers and probe concentrations are the minimum concentrations giving the lowest threshold cycle (C_T) and the highest fluorescence intensity by a normalized reporter value (ΔR_n). In general, 50 nM-300 nM of each primer produced the best RTi-PCR performance. However, in certain reactions the oligonucleotide in the same strand as the probe required higher concentration. The magnesium ion concentration should range from 0.8 to 9.0 mM MgCl$_2$. Low concentrations reduce the amplification efficiency, and high concentrations result in non-specific amplification. The most used DNA polymerase in this work, Ampli*Taq* Gold® DNA polymerase, has a high fidelity irrespective to the MgCl$_2$ concentration, thus generating specific products when used in Mg^{2+} conditions aiming to high PCR performance. If the samples are expected to contain EDTA, citrate, or other chelators, the MgCl$_2$ concentration in the reaction mix must be raised proportionately. In this work, most of optimised RTi-PCR assays used 6 mM MgCl$_2$ concentration. Finally, by increasing the annealing temperature, a higher astringency is obtained, and thus the higher temperature without a significant affection in the C_T must be selected for diagnostic purposes. A consensus scenario was adopted along the present work, most importantly including the RTi-PCR run conditions, with the aim of simplifying the routine application of the developed assays, and the potential combination in single runs. The consensus conditions were as follows: 2 min at 50ºC, 10 min at 95ºC and 50 cycles of 15 s at 95ºC and 1 min at 63ºC.

The specificity, sensitivity and relative accuracy must be established prior to implementation of the RTi-PCR systems to real food samples. Specificity is defined as the degree to which a method is affected by the other components present in a multi-component sample (Anonymous, 2003; Malorny *et al.*, 2003), in other words, the ability of a method to measure a given analyte within the sample without any interference from non-target components or background noise. Recently, a new concept has been introduced for microbiological assays *i.e.* selectivity. It can be defined as a measure of the degree of non-interference in the presence of non-target analytes (Anonymous, 2003), *i.e.* a method can be considered selective if it can be used to detect the analyte and that a guarantee is provided that the detected signal can only be a product by the specific analyte. In addition, the selectivity includes 2 complementary concepts: inclusivity and exclusivity. The first one is defined as the ability to detect the target analyte from a wide range of strains, and the second one as the lack of interference from a relevant range of non-target strains. Thus, specificity and selectivity share the same central conceptual core. However, they are used indistinctly, which creates confusion to authors and

readers of scientific publications. Besides, the concept of specificity in epidemiological studies is slightly different from one defined above. The most rational scenario should be the use of a unique concept totally accepted, born from the consensus of the microbiology community. However, the subconcepts of inclusivity and exclusivity are very direct and could play an explanatory role. In any case, the RTi-PCR must be fully capable to detect the target bacteria, and fully capable not to detect the non-target bacteria. In order to demonstrate this, the international standard guideline ISO 16140:2003 has established that at least 50 target (25 for *Salmonella*) bacterial strains and 30 non-target bacterial strains must be analysed. In this work, the specificity of RTi-PCR assays for the detection of *L. monocytogenes*, *L. innocua*, *Listeria* spp., *MAP* and *Salmonella* spp. was evaluated using the strains shown in tables III.1-III.4. All RTi-PCR assays were 100% specific. All RTi-PCR systems developed with quantitative purposes had a variation among equal amounts of target strains below 1 C_T unit, except for the *L. monocytogenes iap*-specific RTi-PCR assay, which was not considered adequate for quantitative analyses. It could be further confirmed that such divergent C_T values were due to sequence differences. Moreover, sequences of strains belonging to the same *L. monocytogenes* division were highly conserved, and thus produced more conserved C_T values than those belonging to different divisions. This allowed designing for the first time a test to classify each *L. monocytogenes* strain into either division II or I/III by using 2 RTi-PCRs targeting to a highly conserved (*hly*) and a more variable (*iap*) sequences. A flaw of current RTi-PCR studies was detected during this work, especially for RTi-PCR assays designed with quantitative purposes. It is extremely important to verify the conservation of the target sequence among target strains. Even small variations in such sequences may strongly affect the efficiency of RTi-PCR, and, in consequence, lead to underestimation of the number of target molecules and reduction of the limit of detection. Thus, the conservation of the target sequences in terms of C_T values was evaluated in this study for the first time on RTi-PCR assays for microbiological diagnostics. In optimal conditions, the DNA concentration is duplicated at each PCR cycle, thus, theoretically a variation above 3 C_T units implicates a variation of 1 order of magnitude of the initial DNA concentration.

Another diagnostic accuracy parameter is the sensitivity. As for specificity, different terms are currently associated to this concept: sensitivity, relative sensitivity, detection limit and detection probability (see Introduction section, p.43). They indicate the minimum level at which the target analyte can reliably be detected, with a given probability. The limit of detection establishes a threshold for such probability that varies from 95% (ISO 16140:2003; Paoletti and Weighardt, 2002) to 99% (Knutsson *et al.*, 2002). Thus, the detection limit must not be considered as that smallest level in which the amplification signal is achieved, but where the target is reliably detected with a high probability. The limit of detection of the RTi-PCR assays developed in this study was established by performing different experiments (at least 3) including several replicates (at least 3) of serial dilutions of target. In addition, the test designed by Knutsson *et al.*, (2002) was employed to establish the detection probability of the *MAP*-specific RTi-PCR (and *MAP*- specific RTi-NASBA). All RTi-PCRs developed had limit of detection (with 95% probability) below 10 targets cells. As expected, the most sensitive RTi-PCR assays were those targeting a multicopy gene: limit of detection of a single genomic DNA copy for *Listeria* spp. 23S rDNA-specific system and below 3 genomic DNA copies for *MAP IS900*-specific system. Interestingly, the limit of detection was similar when using purified bacterial DNA or cells as target, which has important practical implications for the simplicity of the use of the latter as template.

In the systems designed with quantitative purposes, 3 additional parameters must be evaluated: the quantificability, the quantification range and the limit of quantification. Approved definitions are available for the two last concepts: quantification range, as the interval of analyte concentration over which the method is considered to perform in a linear manner (Paoletti and Weighardt, 2002); and limit of quantification, as the smallest amount of analyte which can be measured and quantified with defined precision and accuracy by the method (Anonymous, 2003). Quantificability is not formally defined, but it is assumed for the RTi-PCR user's community as the capacity of the method to accurately establish the number of targets present in the sample, and depends upon the linearity and the PCR performance. Linearity is the ability of the method to give results that are in

proportion to the amount of analyte present in the sample; (ISO 16140:2003), and can be determined by the calculation of a regression line with an adequate statistical method. In this study, this regression line was built with known-concentration DNA standards, and the regression coefficient (R^2) was the value used to measure the linearity of the system. Values above 0.90-0.95 can be considered adequate, and all RTi-PCR developed presented R^2 values above 0.95. The PCR efficiency (E) can be determined using the same standard curve, according to the following equation $E=10^{-1/s} - 1$ (Klein *et al.*, 1999); where *s* is the slope of the standard curve. The optimal PCR performance is obtained when E equals 1 (*s*-3.3219). For diagnostic purposes, it is needed to describe the practical operating range of the assay, and generally is considered between E values of 0.78 (*s*= 4.00) and 1.15 (*s*=3.00). To determine the quantification range and the limit of quantification, the experimental design is crucial. In this study, ten-fold dilutions were performed from the highest concentration (usually 3×10^5 target molecules per reaction) to a dilution above the expected limit of quantification (usually 6×10^2 or 3×10^2). From this level, two-fold dilutions were performed to approximately a single DNA genomic copy per reaction. The smallest level at which the C_T obtained in two consecutive serial dilutions did not overlap with a 95% probability was considered the limit of quantification. In parallel to experimental evaluation, the theoretical limit of quantification was calculated using the MonteCarlo simulation analysis (considering the error associated to the particular experimental design such as volumes, pipetting, etc) and calculating a 95% confidence interval for every serial dilution. The theoretical and experimental limits of quantification matched each other, and were generally established around 30 DNA genomic copies or bacterial cells. The quantification range was dynamic and was above 5 orders of magnitude (usually from 3×10^6 or 3×10^5 to 3×10^1).

Finally the relative accuracy must be evaluated. This concept can be defined as degree of correspondence between the response obtained by the reference method and the response obtained by the alternative method on identical samples (ISO 16140:2003; Hoorfar and Cook, 2003). This parameter is considered fundamental for the international guideline for the validation of a microbiological alternative method (Anonymous, 2003), and unfortunately it has not been widely applied in RTi-PCR methods published to date. The accuracy of the results can be considered critical for the assumption of the results, especially when those are originated from an indirect signal (this is the case of RTi amplification based methods: indirect signal –fluorescence- associated to presence or growing of bacteria). Thus, it would be a salutary measure to consider determining the relative accuracy as a mandatory prerequisite for publication in scientific journals. The relative accuracy can be calculated by means of comparison of the results obtained from the RTi-PCR method and those obtained from the international reference method (usually established by ISO). Relative accuracy values between 70 % and 130 % can be considered adequate. In this study, the relative accuracy was calculated for each assay, obtaining values within the interval defined above.

In addition to RTi-PCR methods, a real-time NASBA method was designed and optimised for the specific detection of *MAP*. NASBA is a sensitive transcription-based amplification system specifically designed for the detection of RNA molecules (Compton, 1991). Due to the labile nature of RNA compared to DNA, NASBA is especially suitable for viability assays, and offers numerous advantages over reverse-transcription-(RT)-PCR (Cook, 2003; Deiman *et al.*, 2002): it does not require DNase treatment of the samples since, in theory, dsDNA cannot be substrate for NASBA. Thus, NASBA has the potential to be a powerful diagnostic tool for the detection of viable microorganisms. Although RNA is considered a good indicator of cell viability, not all RNA species are suitable for unambiguous detection of viable bacteria. Bacterial rRNA and tRNA molecules have strong secondary structures and are more stable than bacterial mRNAs, which in general have short half-lifes (Rauhut and Klug, 1999). This could be a drawback of the NASBA methods developed to date that target rRNAs; and one of the reasons why the developed *MAP*-specific NASBA systems target mRNA molecules. Our NASBA system was designed and optimised using the same parameters as described for RTi-PCR assays, with the only exception that molecular beacons were used for the real-time detection of the NASBA amplicons, since TaqMan probes cannot be used in this technique. As an additional control during the development of a NASBA system, we recommend verifying the nucleic acid origin of a NASBA signal if detection of RNA is the objective. This is due to the unex-

pected results obtained by treating the NASBA sample with enzymatic treatments with DNase, RNase, S1 nuclease, and RNase / S1 nuclease; which indicated that in *MAP* (but not in *Salmonella*) *hsp70* targeted NASBA can amplify and detect DNA.

The ultimate goal of the development of new analytical techniques is their adoption and establishment as routine diagnostic tools. Using such tools routinely necessitates employing reliable controls to verify the accuracy of the results obtained. A very important control for molecular amplification-based methods is the use of internal amplification controls (IAC). An IAC is a non-target nucleic acid present in every reaction, which can be co-amplified simultaneously with the target sequence. In a reaction with an IAC, a control signal should always be produced when there is no target sequence present; indicating the correct performance of the reaction *i.e.* lack of inhibition due to malfunction of equipment, operator error, incorrect reaction mixture, poor enzyme activity, or the presence of inhibitory substances. There are two different strategies for the application of an IAC: the competitive, *i.e.* the target and the IAC are co-amplified in a single reaction tube with the same primer set, and the non-competitive, *i.e.* the target and the IAC are amplified by two different primer sets. In this study, chimerical IACs were designed, constructed and optimised for the *L. monocytogenes hly*-specific and the *MAP IS900*-specific RTi-PCR assays and for the *MAP*-specific RTi-NASBA assay. For RTi-PCR, the IACs were constructed by linking the two target specific primer sequences with a non-relevant sequence (Figure I.22a; pp. 41). For RTi-NASBA, the IACs were constructed in two phases. In the first phase, a double-stranded DNA molecule is produced, which contains non-target sequences, flanked by target sequences complementary to the NASBA primers. At the 5' end of this DNA molecule is a T7 RNA polymerase binding sequence. In the second phase of the construction, RNA transcripts are produced from the DNA, by T7 RNA polymerase. This RNA is the IAC (Figure I.22b; pp. 42). Thus, the IAC include the sequences corresponding to the target specific primers and lack the internal sequences. Instead, they flank a different sequence against which a probe is directed. This chimerical nucleic acid sequence (DNA for RTi-PCR and RNA for RTi-NASBA) does not also exhibit homology to any other nucleic acid sequence to avoid any cross-reaction. Thus, the IAC can be amplified using the target-specific primers, although the IAC amplicon is not detected with the target-specific probe (*e.g.* FAM-labelled) but with the IAC-specific probe (*e.g.* VIC- or HEX-labelled). This makes multiplexing by colour possible by detection of target and IAC through *e.g.* FAM and VIC or HEX fluorescence, respectively. In the competitive IAC strategy, the most critical parameter to consider is the optimal initial number of IAC copies. It must be determined as a compromise level that allows reproducible IAC detection and at the same time it avoids inhibition of the target-specific reaction. In this study, the performance of the amplification reactions in the presence of different IAC initial copy numbers was evaluated, and 100 copies (for RTi-PCR assays) or 1 fg (for the RTi-NASBA assay) were selected as adequate.

In this study it has been shown that RTi-amplification based methods can be used with diagnostic purposes as complementary tools to the classical microbiological methods. However, such techniques should potentially allow going a step further in the food microbiology diagnostics: its use on food products, avoiding any enrichment and culturing steps, and thus reducing the time required for obtaining final confirmatory results, and even providing data about VBNC.

The application of the amplification-based assay to real food samples for a future implementation as a diagnostic tool in food microbiology laboratories requires special pre-amplification steps. It is well known that components of food samples, growth media, and nucleic acids extraction reagents can reduce or even block amplification reactions. Consequently, sample preparation prior to the amplification reaction is crucial for the robustness and performance of amplification-based methods, and thus, the purpose of the pre-amplification treatment aims to convert food samples into amplifiable samples, by means of: (i) homogenising the sample for amplification; (ii) increasing the concentration of the target organism to the practical operating range of a given assay; and (iii) reducing amplification-inhibitory substances. As food samples vary in homogeneity, consistency, composition, and accompanying microbiota, pre-amplification procedures should be adapted to each food matrix. In the Introduction section has been discussed the different approaches employed (see pp.

13-15). If only pre-enrichment is applied two advantages are achieved (i) increase the number of target bacteria and decrease the concentration of amplification inhibitors; and (ii) detect only the bacteria capable to grow, *i.e.* viable bacteria (but it must be considered the background non-viable bias). Furthermore, this procedure is simple and cost-effective. However, several cultures contain amplification-inhibitors, and, besides, after the growing the quantification is not achievable. In contrast, the most rational approach to obtain consistent and quantitative RTi-PCR or NASBA results is the combination of several pre-amplification procedures adapted to different food matrices, usually physical procedures coupled with nucleic acid extraction. The advantages are that the results are obtained more rapidly than using the enrichment of the food sample and the quantification is still achievable. However, it is more difficult and more expensive. In this study, the strategy used was to use procedures that reproduced the ISO analyses conditions (initial sample volume or weight, 20-25 ml or 25 g, respectively; diluted in 10-volumes of the recommended broth) with the aim of developing molecular tools fully compatible with the ISO methods; which would allow quick quantitative detection of high loads of bacteria and at the same time would not interfere with the accepted microbiological methods.

The implementation of a method can be horizontal, *i.e.* fully applicable for all categories of food matrixes, or vertical, *i.e.* only for a food product or a category of food matrixes. The international guideline for the validation of a microbiological alternative method (ISO 16140:2003) establishes that if it is sought for implantation in all foods, at least five categories must be studied. In addition, this international standard guideline defines in the annex B each food category and the products within each one that must be studied for each pathogenic or non-pathogenic bacterium. In this study, the *L. monocytogenes hly*-specific RTi-PCR assay was evaluated for the meat products category (raw, heat processed –cooked ham and frankfurter sausage and fermented -fuet-) and for fish and seafood products (raw -salmon- and smoked -smoked salmon) as these both categories have been considered as major vehicles of transmission of listeriosis to humans. The *MAP IS900*-specific RTi-PCR assay and *MAP*-RTi-NASBA assay were also evaluated for two food products, pasteurised semi-skimmed milk and drinking water, as they are hypothesised as major routes of potential transmission of *MAP* to humans. The use of pre-amplification procedures adapted to each category of food product allowed the detection of 10^2 *L. monocytogenes* CFU/g and quantification of 10^3 CFU/g of meat products, detection of 10 CFU/g and quantification of 10^3 CFU/g of smoked salmon, all with excellent accuracy compared to the plate count method. When the *MAP*-specific RTi-PCR was applied to water samples, one simple precipitation procedure was enough for obtain equivalent results to those when genomic DNA or cells from *MAP* were used, and 10^2 *MAP* were consistently detected in 20 ml pasteurised semi-skimmed milk using the RTi-PCR assay after a simple detergent and enzymatic sample pretreatment prior to centrifugation and nucleic acid extraction. *MAP*-specific RTi-NASBA produced slightly less sensitive results, with consistent detection down to 10^3 and 10^4 *MAP* cells in 20 ml water and milk, respectively. In consequence, the methods developed in this study can be considered less sensitive compared to the traditional microbiological methods or to molecular methods with pre-enrichment steps, but provide the advantage of allowing a direct, quick (around 2 hours) and accurate detection (and quantification in some cases) of medium or high loads of pathogenic bacteria in food samples, being in the same time, fully compatible with international standard methods.

V. CONCLUSIONS

"Las ciencias tienen las raíces amargas, pero muy dulces los frutos"

Aristóteles

V. CONCLUSIONS

1. Two different protocols for extraction of bacterial gDNA from food were compared for subsequent amplification by RTi-PCR. The Wizard®-based protocol was very reproducible for the detection of *L. monocytogenes* in meat samples. However, the Chelex®-based protocol was less time consuming and more cost-effective, and, thus, it could be desirable for a quick and simple routine analysis in combination with a specific RTi-PCR assay.

2. The use of RNAprotect Bacteria Reagent for the stabilisation of RNA prior to isolation of total RNA improved both, the yield and the reproducibility of the total RNA extraction. Furthermore, its combination with cell mechanical disruption further improved the total RNA isolation yield.

3. Three independent RTi-PCR methods were developed for detection, identification and quantification of *L. monocytogenes*, *L. innocua* and *Listeria* spp, respectively. These methods were fully specific, showing limits of detection and quantification adequate for microbiology diagnostics in food samples.

4. The combination of the *L. monocytogenes hly-* and *iap*-specific RTi-PCR assays enabled to classify *L. monocytogenes* isolates in one of the two major phylogenetic divisions of the species, I/III and II.

5. A duplex RTi-PCR method for simultaneous quantitative detection of *L. monocytogenes* and *Listeria* spp. was developed. This method was fully specific, showing adequate limits of detection and quantification, similar to those obtained in uniplex formats.

6. Protocols for DNA extraction to be applied in combination with RTi-PCR assay were developed for the following food matrixes: pork raw meat, pork cooked ham, frankfurter sausages, fermented meat product -*fuet*-, smoked salmon and raw salmon. Such methods allowed the detection down to 1×10^2 *L. monocytogenes* cfu per g of meat products and the quantification down to 1×10^3 cfu per g, with excellent accuracy compared to the plate count method. In addition, the selected method allowed the detection down to 10 *L. monocytogenes* cfu per g and the quantification down to 1×10^2 cfu per g of smoked salmon, with excellent accuracy compared to the plate count method. For raw salmon, this method could reliably detect as few as 1×10^4 cfu per g.

7. An RTi-PCR method was developed for detection, identification and quantification of *Mycobacterium avium* subsp. *paratuberculosis*. This method was fully specific, showing limits of detection and quantification adequate for the further application in microbiology diagnostics.

8. An RTi-NASBA method was developed for detection and identification of *Mycobacterium avium* subsp. *paratuberculosis*. The molecular beacons chemistry was selected as real-time detection system. This method proved to be fully specific, with a limit of detection (with 99 % probability) at of 150-200 cells per reaction.

9. Protocols for extraction of nucleic acids to be applied in combination with RTi-PCR or RTi-NASBA were developed for drinking water and pasteurised semi-skimmed milk. Such RTi-PCR-based methods allowed the quantitative detection down to 10^2 *Mycobacterium avium* subsp. *paratuberculosis* cells in 20 ml artificially contaminated drinking water, and the consistent detection of 10^2 *Mycobacterium avium* subsp. *paratuberculosis* cells in 20 ml artificially contaminated semi-skimmed milk. The RTi-NASBA-based methods allowed the consistent

detection down to $1{\times}10^3$ *Mycobacterium avium* subsp. *paratuberculosis* in 20 ml of artificially contaminated drinking water, and down to $1{\times}10^4$ *Mycobacterium avium* subsp. *paratuberculosis* in 20 ml of artificially contaminated semi-skimmed milk.

10. It was observed absence of *Mycobacterium avium* subsp. *paratuberculosis* DNA in fresh bowel mucosal biopsy specimens from patients with Crohn's disease using the *Mycobacterium avium* subsp. *paratuberculosis*-specific RTi-PCR. This result cannot support the aetiological significance of *Mycobacterium avium* subsp. *paratuberculosis* in Crohn's disease.

11. The *hsp70*-specific NASBA assay amplified *Mycobacterium avium* subsp. *paratuberculosis* double-stranded DNA. Thus, the use of NASBA-based methods for viability studies must be carefully evaluated prior to its application.

12. An RTi-PCR assay for the unequivocal identification of *Salmonella* spp. colonies was optimised and evaluated in a multi-center trial. This method was fully specific for identification of *Salmonella* spp., irrespective of the particular subgenus.

13. Internal amplification controls for the *Listeria monocytogenes*- and *Mycobacterium avium* subsp. *paratuberculosis*-specific RTi-PCR assays were designed, constructed and evaluated. Each internal amplification control was optimised for use in duplex format, and the specificity and the limits of detection and quantification was the same to those obtained in uniplex formats.

14. A general strategy for the construction of an IAC for RTi-NASBA assays was presented. The method is simple and easy to apply without the need of sophisticated molecular manipulation.

VI. REFERENCES

"Uno no es lo que es por lo que escribe, sino por lo que ha leído"

Jorge Luis Borges

VI. REFERENCES

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