

Results

The results depicted in Figure 110 suggested that 2.5 $\mu\text{g}/\mu\text{l}$ was a seemingly optimal BSA concentration, since 5 $\mu\text{g}/\mu\text{l}$ results proved negative twice. Furthermore, the results also indicated that the optimal annealing temperature probably lay in the 62-62.5 $^{\circ}\text{C}$ range, since the positive control for the 63.5 $^{\circ}\text{C}$ experiment displayed a somewhat lower efficiency than its 62 $^{\circ}\text{C}$ and 61 $^{\circ}\text{C}$ counterparts, while both these controls showed similar efficiencies. This hypothesis was further confirmed by the similarly low efficiency of 63.5 $^{\circ}\text{C}$ chip experiments. Even so, the 62 and 63.5 $^{\circ}\text{C}$ experiments on PCR-chips indicated that correct annealing was not taking place either at these corrected temperatures.

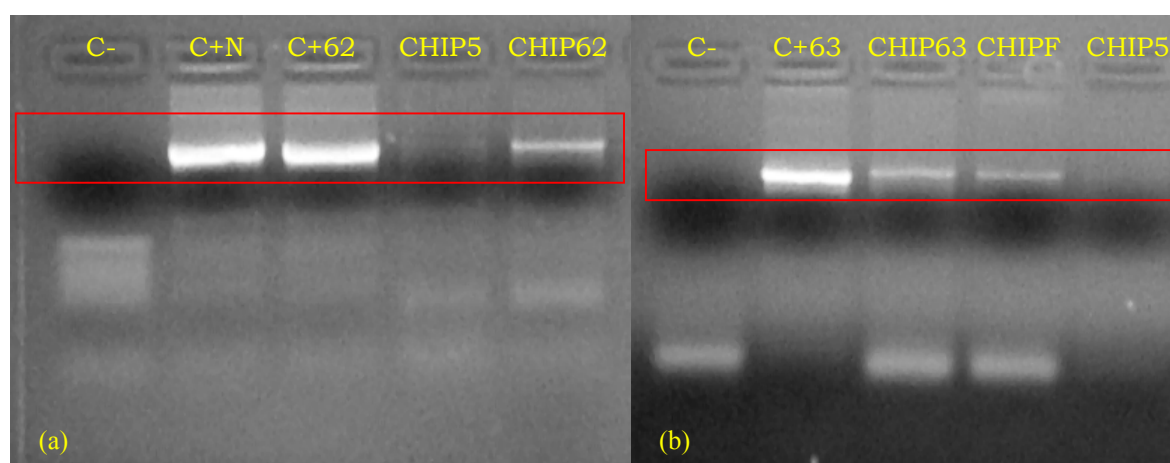


Figure 110 - Images of the 3% agarose gels (15 μl per well) results for annealing temperature titration using fast-cooling ramps. All chip experiments contained doubled template DNA and polymerase concentrations. CHIP5 lanes show results for 5 $\mu\text{g}/\mu\text{l}$ BSA concentrations, while the CHIPF lane (b) corresponds to the quick-PCR experiment at 62 $^{\circ}\text{C}$ annealing temperature. C+ and CHIP 62 and 63 lanes correspond, respectively, to positive control and chip experiments at 62 and 63.5 $^{\circ}\text{C}$, while C+N (a) is the positive control for the standard 61 $^{\circ}\text{C}$ annealing temperature. Total chip PCR analysis times were ~1:49 h for normal assays and 52:28 min for the quick-PCR assay.

On close inspection, it was seen that the PCR primers used had wildly different annealing temperatures (above 10 $^{\circ}\text{C}$, see *Materials and Methods*, p.325) and that they also presented a significant affinity to produce primer-dimer complexes, two facts that could well bring havoc on annealing temperature titration and PCR efficiency. Therefore, for future experiments, it was decided to switch to a new, carefully selected set of primers and template that would permit optimization of the chip PCR experiments. Nonetheless, fast cooling (and fast transition times) was just one of the

predicted advantages for PCR-chips. The other one should come from the fast temperature stabilization and distribution across the chip, which, theoretically, would allow shorter hold times at annealing, denaturation and even extension temperatures. Hence, prior to switching to the new primers and template, it was decided to test this hypothesis with the available setup, in a quick-PCR experiment using slow-cooling rates to avoid improper annealing.

Quick-PCR

This new quick-PCR assay was mainly a repetition of the previous quick-PCR experiment, with some minor differences introduced in the PCR protocol. The denaturation temperature was increased in 0.5 °C, up to 95.5 °C, in an attempt to minimize the effects of primer-dimer formation. Accordingly, denaturation times were nearly reduced to spikes (5 s), and extension and annealing times were halved (30 s) for quick-PCR operation.

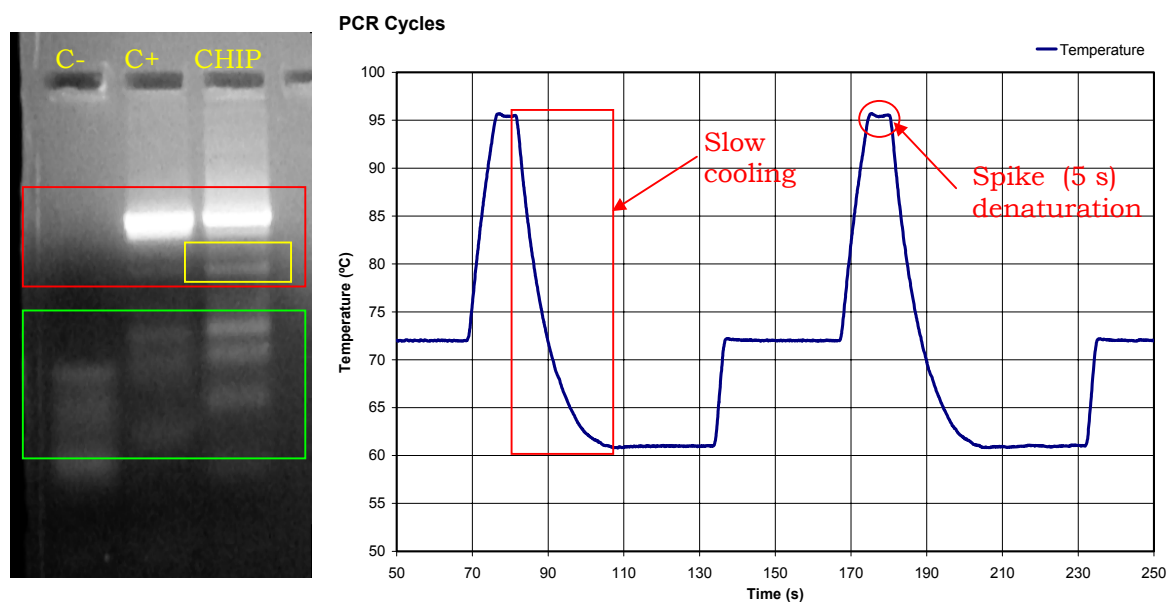


Figure 111 - Image of the 3% agarose gel (20 μ l per well) results and the PCR-cycles used in the slow-cooling quick-PCR experiment. All tubes contain the same BSA concentration: 2.5 μ g/ μ l. Chip contains doubled template DNA and polymerase concentrations. Total chip PCR analysis time: 1:04:39h. Due to shorter hold times, the slow-cooling effect can be observed here more clearly than in previous graphs.

The results (see Figure 111) confirmed that quick-PCR was possible using the developed system, even if with a somewhat smaller efficiency than positive controls. Moreover, the tradeoff in efficiency was handsomely paid

for in analysis time, which had been reduced two-fold, from the approximately 2 hours of the standard experiment down to one hour in quick-PCR. If such experiments could be further improved with a new set of primers and making full use of fast cooling, they prompted the way to a greater reduction in analysis time that could well lie within the 40 min range already described in the literature ([Northrup1993], [Shoffner1996], [Taylor1997]). Furthermore, the quick-PCR experiment also confirmed the necessity to switch to a new primer and template set, since primer-dimer occurrences were self-evident in the slab gel results (green box) and secondary amplicate DNA structures could also be observed below the main amplicate band (yellow box).

4.7.2. QUICK-PCR OPTIMIZATION

Template validation

For the new batch of chip PCR optimization assays, a new DNA template was used and primers carefully selected for optimal annealing conditions and minimization of primer-dimer structures. The chosen template was the IVI-2 segment of *Pasteurella multocida* PM25 high virulence strain, which contains, among other, *hfq* and *hflX* genes. The IVI-2 segment was cloned into a pGY2 6.3 kb plasmid and transferred and cultivated in an *E. coli* strain. The target amplicate (see Figure 112) was an intragenic region of the *hfq* gene, framed by two ultra-pure 21-mer primers with similar %G+C content (38.1%) and very close melting temperatures (56.7 °C, see *Materials and Methods*, p.325 for details).

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TACGGTTCAGTGATGACAATAAATTAATTTAATGTTATATTGAGATTTGGAGGAAATACAG
AAWTCTCTGTGTTAGCTAGTTGTATCATTTTATTTAGATTTAAAAGAAGGAAAGAAAAAT
GGCAAAGGACAATCTTTA CAAGATCCTTATTTAAACGCGTTACGTCGTGAACGTATCCCT
GTTCCATTTACCTCGTAAATGGGATTAACACTACAAGGTCAGATTGAATCATTGATCAAT
TGTTATTTTATTAATAAATAACAGTTAATCAAATGGTGTACAAACACGCAATTTCAACTGTT
TTCTGCGCGCTCAGTGTCTCATCACAATAATAGCAATAATTCCAATCAACAGAATTATCA
ACAAGAGCAACAACTGACAGCAACGTAGAAAAAGCAGAATAGTTTCAAGTTGAACACA1
TTATTGCAAGTGCGGTAGATCTTCAGAATGAATCGACCGCACTTTCTTCATTACCT
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Figure 112 - The *hfq* gene (red code, flanked by translation start (ATG) and end (TAG) codons), the 21-mer upstream CAAGATCCTTATTTAAACGCG and downstream GCAACGTAGAAAAAGCAGAAT primers and the amplified region (yellow background).

Prior to PCR-chip experimentation, amplification of the template was tested in a conventional thermocycler and annealing temperature was

experimentally titrated. The final parameters for *hfq* PCR amplification can be seen in Table 12 and its results in Figure 113.

Quantity	Reagent	Cycling protocol: 95 °C - 2 min 95 °C - 1 min \\ 53 °C - 1 min x30 72 °C - 1 min / 72 °C - 7 min 4 °C - ∞
17 µl	milliQ H ₂ O	
2.5 µl	10x MgCl ₂ Buffer	
2.5 µl	10 nM dNTPs	
1.25 µl	10 µM sense primer	
1.25 µl	10 µM antisense primer	
0.2 µl	3.5 U/µl Expand™ High Fidelity System (Boehringer Mannheim Corp.)	
1 µl	70 ng/µl sample DNA	

Table 12 - PCR mix and amplification protocol for the *hfq* gene.

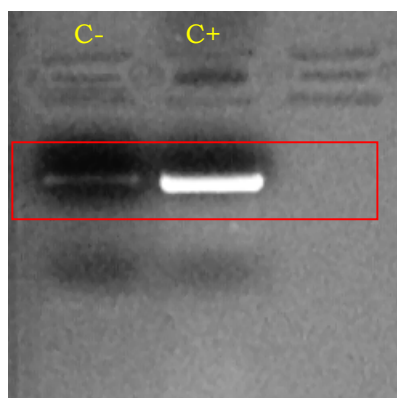


Figure 113 - Image of the 3% agarose gel (25 µl per well) PCR results for preliminary amplification of the *hfq* gene in a conventional thermocycler.

Chip PCR validation

After assessing the optimal preservation of the template DNA and the adequate performance of the chosen primers by conventional amplification, a second assay was carried out to determine the feasibility of chip amplification and the incidence (if any) of BSA addition into the PCR mix.

Cycling protocol:

95 °C - 2 min
95 °C - 5 s \\
53 °C - 10 s x40
72 °C - 20 s /
72 °C - 3 min
4 °C - ∞

Table 13 - PCR protocols for fast thermocycling.