Ligase chain reaction (LCR)

The ligase chain reaction, first described in 1988 [Landegren1988], is a two-step variation of the PCR technique in which a ligase enzyme, instead of a polymerase, is used to provide selective amplification of a previously known DNA sequence. The technique makes use of the discriminating ineffectiveness of DNA-ligase to ligate unbound, single-stranded, DNA in order to achieve detection of single-base mutations, a feat that in PCR can only be achieved by indirect methods (see p.92).

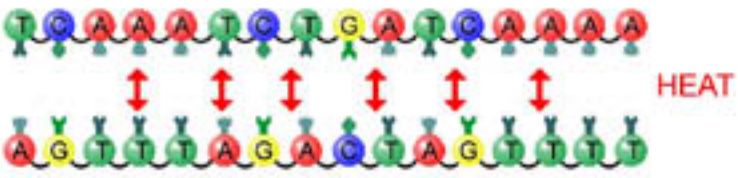

Figure 18 - LCR: Denaturing of the initial double-stranded DNA sample.

In a typical LCR assay, two sets of contiguous primers are designed to anneal at a specific region of the sample DNA. After the double-stranded sample DNA has been denatured (see Figure 18), DNA-ligase will only ligate primers that have perfectly annealed to the sample DNA. Otherwise, the primers will not be ligated (see Figure 19).

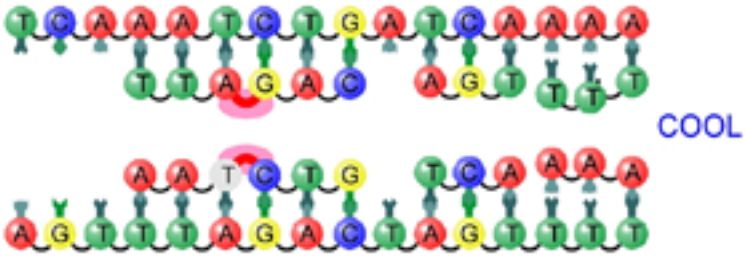

Figure 19 - LCR: Ligation of annealed primers. Single-base mutation impedes ligation.

After ligation, the newly ligated primers conform a template that can be used for primer annealing and, hence, if there is ligation, the ligation reaction becomes exponential, amplifying the ligated product until primers are exhausted. Although LCR cannot be used, as PCR, to amplify (generate copies) of long regions (since very long primers would have to be

synthesized), it is a very powerful technique for selectively detecting (by absence of amplification) single-base mutations at a known locus, and it is widely used for precise diagnostic applications and for ligase detection reactions in hybridization chips [Favis2000].

Strand displacement amplification (SDA)

A more direct rival to PCR, at least in certain kinds of analysis, was introduced in 1993. Strand displacement amplification [Walker1993] is a variation of the PCR that introduces a novel agent (a restriction enzyme) in order to achieve a mainly isothermal amplification.

SDA has two distinct phases, of which only the second (the exponential amplification) is truly isothermal. The first phase consists in the denaturation of the double-stranded sample DNA and its annealing with specific primers that contain recognition sites for a restriction enzyme (see Figure 20).

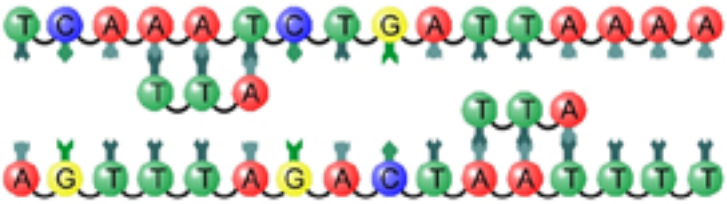

Figure 20 - SDA: annealing of restriction enzyme recognition site primers.

Once this altered sample DNA has been generated, SDA amplification begins at a fixed temperature (typically around 37 °C), with the annealing of the amplification primers. After the amplification primers are set in, a DNA polymerase bounds to them and starts replication.

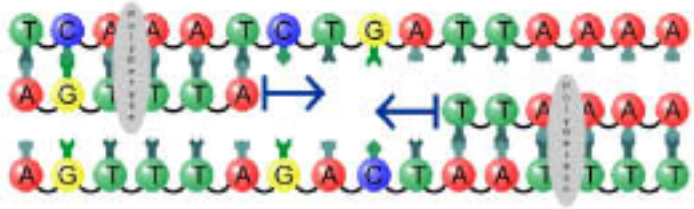

Figure 21 - SDA: annealing of the amplification primers and polymerase extension.

Upon completion, the DNA polymerase will have generated two full complementary strands, which both encode a restriction enzyme site. The restriction enzyme then binds to the double-stranded DNA segment at its recognition site and cleaves only one of the strands, forming a nick. After

nick formation, the DNA polymerase binds again to the amplification primer and starts replicating, displacing the previous complementary strand (hence the name strand displacement amplification).

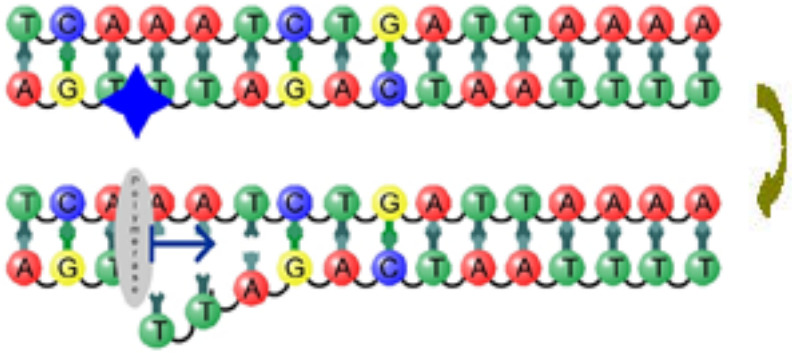

Figure 22 - SDA: *nicking* and polymerase extension/displacement.

The process does not only generate a full single-stranded segment for each sample that can be used as a new template, but it also can take place concurrently, with the same DNA strand being *nicked* and polymerized at the same time. This provides a highly exponential amplification and, since the exponential phase is isothermal, it can be readily adapted to hybridization chips ([Radtkey2000], [Westin2000]).

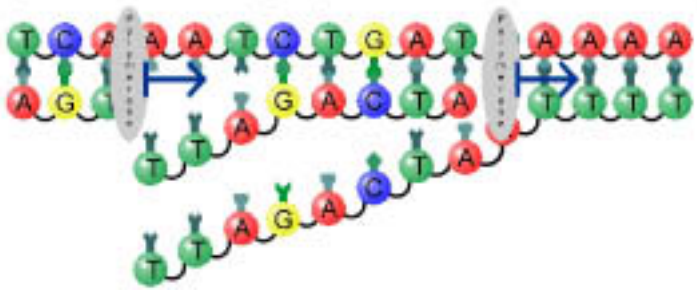

Figure 23 - SDA: concurrent *nicking* and extension.

Rolling circle amplification (RCA)

A more recent challenger to PCR exploits is rolling circle amplification. First described in 1995 [Fire1995], this technique makes use of processes similar to those carried out by viruses in order to replicate their genomes in a host cell. RCA combines the stringiness of ligase reaction with the exponential amplification power of modified strand displacement amplification.

Rolling circle amplification has two different phases. In the first phase, or ligation phase, a quasi-circular DNA probe containing two strand displacement primers and two ligation primers is left to anneal with the

target molecule at its ligase primer sites. The ligation (mismatch sensitive) closes the open circle molecule, in a process known as circularization.

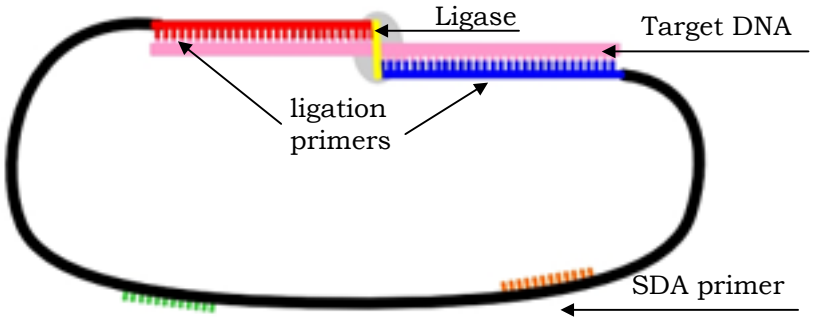

Figure 24 - RCA: ligation and circularization

After circularizing the probe, DNA polymerase, primers and oligonucleotides are inserted into the solution. The polymerase attaches to the first strand-displacement primer and starts copying the circular probe. After a complete revolution, the copied strand is displaced by the same polymerase, which continues to replicate the circular template. This leads to a continuous polymerization of the circular template and the unending creation of a concatamer (the linear repetition of the circular probe). Hence, with only one extension primer, RCA provides linear amplification.

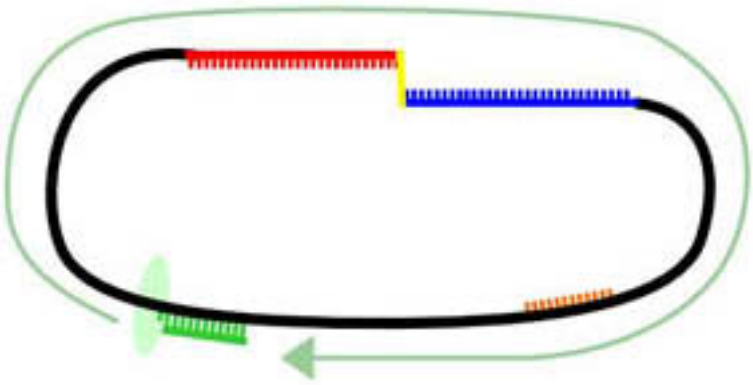

Figure 25 - RCA: Linear extension of the circularized probe.

However, if a second strand displacement primer has been encoded in the circular probe (see Figure 26), then strand displacement amplification occurs by means of the polymerase, which acts both as a copying and displacement enzyme. In this way, the amplification process grows exponentially in what is commonly known as ramification amplifying method (RAM).

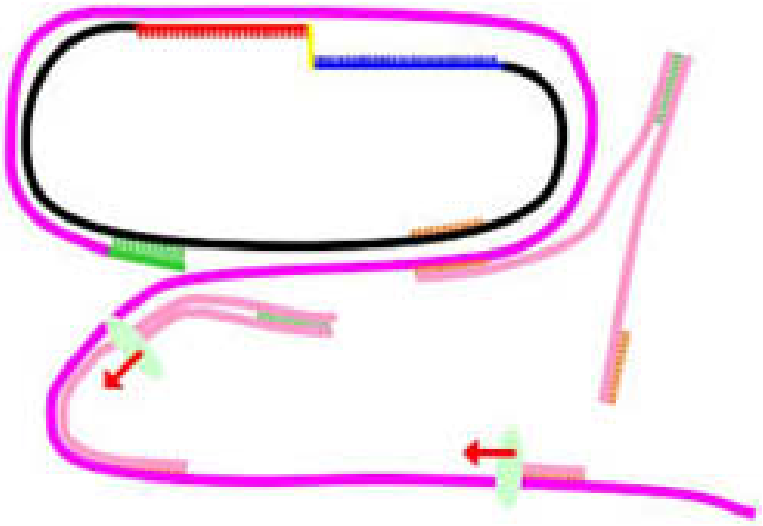

Figure 26 - RCA: multiple *ramified* strand displacement amplification.

Due to its isothermal properties and its specific targeting capabilities, RCA can also be readily adapted to hybridization chips [Nallur2001]. When used in hybridization chips, the immobilized target is not amplified, but serves instead as an anchoring site for a specific target to which an already circular probe attaches (see Figure 27). The probe incorporates a binding site for a fluorescent dye and the subsequent amplification product can be easily detected and, more important to hybridization assays, spatially resolved.

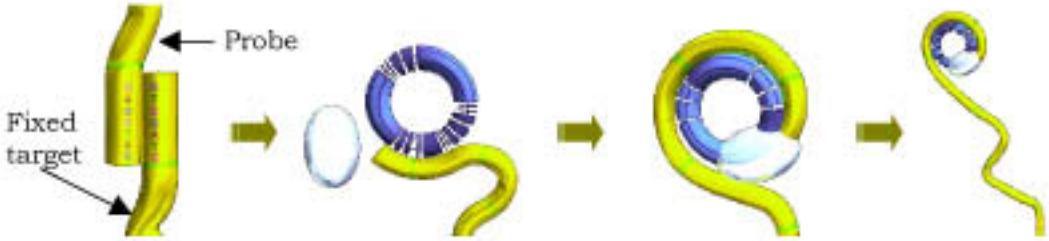

Figure 27 - RCA: linear amplification of hybridized probe. Source: *Molecular Staging, Inc.*

Invader probe amplification (IPA)

The last contestant to the crown of amplification techniques is another method for amplifying the probe signal, rather than the target DNA. Invader probe amplification [Lyamichev1999] makes use of the specificity of DNA cleavases to attain mismatch-discriminating amplification. In the Invader assay, two target-specific probes are used as a binding site for a cleavase enzyme. One of the probes, which completely anneals upstream to the

target DNA, is called the invader probe. The other probe, the signal probe, anneals downstream and contains a signaling short sequence that does not bind to the target DNA. In order to work properly, the two probes must anneal correctly and become overlapped in at least one base. This is the necessary condition for the DNA cleavase to bind, cleave and free the signaling sequence. If there is a mismatch and the two probes do not overlap correctly, the cleavase will not act.

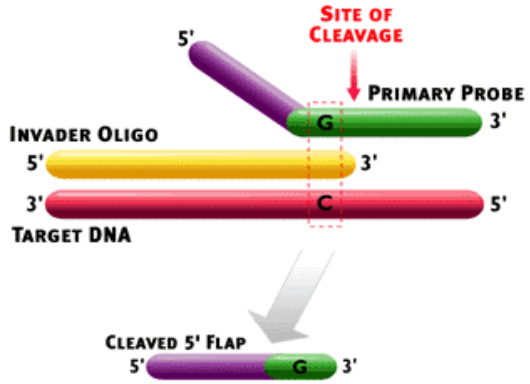

Figure 28 - IPA: cleavage of the signaling probe. Source: *Third Wave Technologies, Inc.*

In principle, the assay could finish straightforward, but the amplification signal provided would be linear. In order to exponentially increase the magnitude of the invader probe amplification signal, a semi-palindrome fluorescence resonance energy transfer (FRET) probe is also inserted into the solution. The previously released signaling sequence acts as an invader probe for the FRET probes, a signaling fraction of which the cleavase frees. Provided that enough FRET probes are available, the process can proceed undeterred until enough signal yield has been achieved.

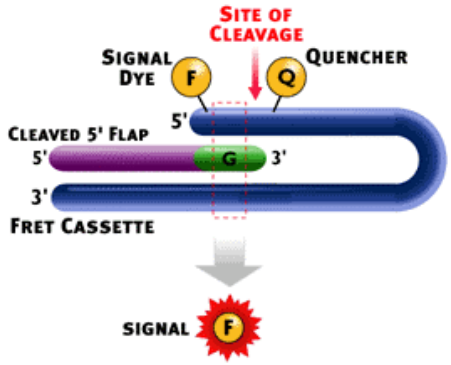

Figure 29 - IPA: signal amplification by FRET probe. Source: *Third Wave Technologies, Inc.*