1.4. A PCR PRIMER

1.4.1. ORIGINS AND FUTURE

The importance of PCR

Considering the terse introductory words of *Kary B. Mullis* team in their 1985 article "*We have developed a procedure for the detection of the sickle cell mutation that is very rapid and at least two orders of magnitude more sensitive than standard Southern blotting*" [Saiki1985], it seems clear that not even the inventor of the PCR technique was able, at the time, to gauge the fundamental importance and repercussions of his discovery, which were to grant him both the Japan and the Nobel prizes in 1993. This should not come as a bolt from the blue, since, as we have already seen (see p. 38), the PCR methodology is quite intuitive in its overall scheme. It might certainly be that, precisely because of its inherent simplicity, the technique was not discovered earlier [Mullis1990]. Not without irony, PCR is often referred to as the "Why didn't I think of that?" technique [Powledge1996]. Nonetheless, the simplicity of PCR should not shed clouds on its importance, both as an analytical technique and as a milestone to life sciences. The invention of the PCR is of crucial importance because it introduces, for the first time, a reliable method for the amplification of genetic material and, thus, it paves the way for the myriad techniques that compose today's biotech armamentarium. In a way [Appenzeller1990], PCR democratized biotechnology, making experimentation with DNA much more affordable as the necessary laboratory setup scaled down. All in all, as Mark R. Hughes[6] is quoted saying, "*PCR is the most important new scientific technology to come along in the last hundred years*" and, certainly, there are not many niches in the life sciences that the introduction of PCR has not transformed utterly.

From baths to cyclers

Next to its conception in 1983 and its formal presentation in 1986 [Mullis1986], the single most remarkable technological development in PCR [Guyer1989] has been the discovery [Chien1976], purification and commercial distribution [Lawyer1989] of a heat-resistant polymerase enzyme from the thermophilic bacterium *Thermus aquaticus* (Taq) and its application to PCR

[Saiki1988]. Initial PCR procedures were carried on by switching the PCR mix vessel between three water baths set at denaturation, annealing and extension temperatures, and relied on the use of the *Escherichia coli* polymerase. Since this enteric bacterium thrives typically at 37 °C, its polymerase has not evolved to withstand high temperatures and, consequently, denatures at 95 °C (the temperature of the denaturation bath -typically a boiling water bath [Gibbs1990]- required to separate double-stranded DNA in PCR). Hence, prior to 1988, *E. coli* polymerase had to be replaced after each denaturation step. Apart from being tedious, this procedure resulted, recurrently, in the contamination of the PCR mixture, which led to all sorts of false positive and negative results, either by contamination from environmental DNA or from reaction inhibitors.

The introduction of Taq polymerase (avoiding polymerase reloading after each cycle), gave way to the automation of PCR in closed thermal-cycling systems. Thermocyclers [Haff1991], together with the development of disposable, efficient heat-transfer, plastic vessels and the discovery of low-cost methods for reliable automated synthesis of oligonucleotides [Caruthers1987] (which were to supply the endless demand for the specific primers that are necessary in each PCR), transformed PCR from a laborious and arduous workbench technique into a systematic analytical tool that could be repeatedly used for DNA selective amplification, and laid the grounds for the biotechnology revolution of the 1990's [Erlich1991].

Amplifying alternatives, chips and the future of PCR

As seen before (see p.37), PCR does no longer stand alone in the realm of amplification techniques. Many rival technologies, like strand displacement amplification (SDA), have emerged and continue to come out in a constant fight to depose PCR of the amplification throne. Nevertheless, although many of these alternatives offer interesting advantages (such as partial isothermal operation in SDA or single-base specificity in ligase chain reaction (LCR)), none is a direct rival to PCR in the global context of amplification. The reasons for this case are many. Firstly, only one of the alternative techniques, rolling circle amplification (RCA), is truly isothermal (a sensible advantage to PCR), but at the expense of requiring a lengthy circular probe into which primers for SDA must be carefully inserted to achieve exponential amplification (RAM). Plain SDA, on the other hand, is

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[6] Mark R. Hughes, Deputy Director of the National Center for Human Genome Research, National

isothermal in the amplification phase, but continues to call for an initial denaturation in the elaborate process of inserting the restriction enzyme recognizing primers. Lastly, both invader probe amplification (IPA) and LCR are powerful signal amplifiers, but they do not provide true fragment amplification capabilities. Moreover, although it lacks some of the advantages of its competitors, PCR has many rewards in its knapsack. For one, current PCR is a decade-old technique with a built-up set of well established and contrasted protocols, an item in which biologists, with due reason, are quite choosy. Besides, the load of clinical and research applications in which PCR has been introduced and benchmarked is enormous (see p.90), a fact that is complemented by the robustness and versatility of PCR as an analytical technique, even in such special setups as those of *in-situ* PCR. Finally, the main surge towards alternative amplification techniques has been brought about on economic grounds. While the PCR patent was held by Hoffman-LaRoche (which acquired it from Cetus, *Mullis'* company, for $300M in 1991), the invention of alternatives to PCR was a lucrative endeavor, since it avoided royalty payments. But now that the PCR patent rights are on the verge of expiration, the tide may reverse, some analysts say, making PCR more attractive than its competitors and conferring to it a second youth. These predictions may hold true, of course, in the standard analysis bench. Yet, if the trend towards miniaturization heralded by DNA-chips prevails in the following years, there is a compulsory requirement PCR must meet in order to stand its ground above contenders: it must face the challenge of scaling down and adapting to chip environments, gaining as much advantage as possible during the process.

1.4.2. BASICS

The PCR overall scheme

The basic operation of the PCR method, briefly discussed in a previous section (see p.38), is sketchily depicted in Figure 40. Firstly, the original double-stranded DNA (dsDNA) is denatured at 94-96 ºC to produce two single-stranded DNA molecules (ssDNA). These ssDNA molecules are then left to anneal with provided *primers* that will mark the region of DNA within the sample to be amplified. Annealing is carried on at temperatures ranging from 40 to 70 ºC (depending on the length and composition of the primers)

Institutes of Health (NHGRI, NIH), 1998.

and it will tend to occur preferentially between primers and ssDNA (instead of between ssDNAs) due to the shorter nature of primers with respect to ssDNA. Once annealing has taken place, temperature is raised to 72 ºC to induce the activity of Taq polymerase, which attaches to the primer-ssDNA complex and starts replicating (extending) the molecule using the ssDNA portion as a complementary template. Once the first round is completed, the hybrid dsDNA is denatured again and the process carries on until enough DNA has been amplified.

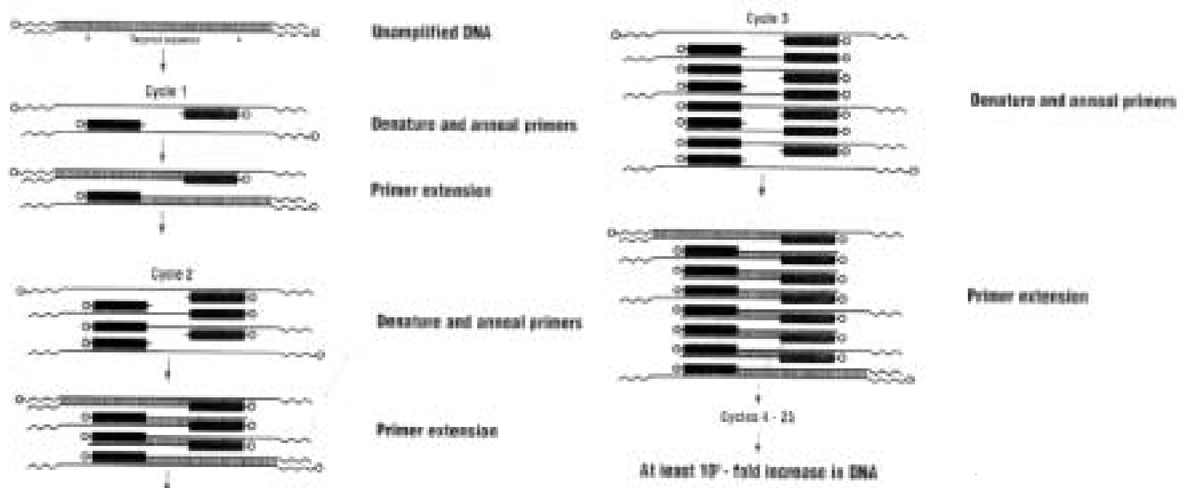

Figure 40 - Basic operation of the PCR method (first three initial rounds of the PCR cycle).

It must be noted [Gibbs1990] that the template molecules (the original dsDNAs) will produce at each cycle two hybrid copies of themselves; but this amplification is linear, generating $2 \cdot n \cdot x$ molecules at the end of the reaction, where n is the number of cycles and x the number of original ssDNA molecules. In contrast, after the third cycle, molecules comprising just the desired amplified region will start to be produced. Since each of these molecules will serve as a template for identical-length molecules in the following round, the amplification of these targeted molecules follows an exponential rule, producing as much as 2^{n-2} molecules at the end of the PCR. With cycle numbers ranging from 20 to 40, the exponential amplification of the targeted region dwarves the linear amplification of template molecules, which is usually not even appreciated in subsequent gel-electrophoresis separations.

The PCR mix

As seen above, the basic PCR mixture (often abbreviated as PCR mix) has four main ingredients: template dsDNA, a set of two primers to determine

the amplified region, abundant Taq polymerase enzyme and free oligonucleotides (dNTPs) of each type (ATP, GTP, TTP and CTP) to be used as building bricks by the polymerase; but, clearly, there is more to the picture than it shows. The following is a brief description of the standard PCR mix (i.e. the nature of its components and their usual concentrations and function):

- Template DNA
  Obviously, template DNA is a basic prerequisite for conducting any PCR amplification. The template can be either linear or circular, single-stranded or double-stranded DNA from previously amplified fragments, plasmid or genomic material. Although PCR can amplify from just a single copy of template ([Scharf1986], [Saiki1988]), above 10^4 copies of template are recommended for easy operation. Typical initial concentrations range between 0.01-0.1 ng/reaction for plasmid DNA and 10-500 ng/reaction for genomic DNA.

- Taq polymerase
  A heat-resistant polymerase is required to perform the extension process of PCR without excessive contamination (see p.62). Although Taq polymerase is the most widely used enzyme, there exist alternative polymerases that can improve yields in certain cases. The typical concentration for Taq polymerase is between 0.25-2 U, where U is the unit of enzymatic activity for Taq polymerase, defined as follows: 1 U = the amount of enzyme required to efficiently convert (in 30 min at 72 °C) 10 nmol dNTP into acid insoluble material (sc. incorporate 10 nmoles of dNTPs to the template in 30 min at 72 °C).

- Primers
  Primers also play an essential role in the PCR, since they provide selectivity to the amplification process and are required by Taq polymerase to initiate extension. Typical primers have lengths between 17 and 28 bp long and a %G+C content between 40 and 60%. Optimum primer concentrations are 0.1-1 μM.

- dNTPs
  Free oligonucleotides in solution are required by the Taq polymerase to replicate the template molecule during the extension step. It is

important to ensure an equal concentration of each type of oligonucleotide (A, T, G and C), which should range between 50-250 µM at a pH 7.5.

- Buffer

  The PCR buffer is a composite solution that adds indispensable elements to the PCR mix. Basically, the buffer consists of three separate items:

  - Potassium chloride (KCl)

    Salt is required to stabilize the DNA molecules (see p.30), facilitating primer annealing and polymerase activity. Typical concentration for KCl is about 30-50 mM.

  - Magnesium dichloride ($MgCl_2$)

    Magnesium dichloride is necessary for PCR since, in solution, this divalent cation provides the free Mg^{++} ion required by the Taq polymerase to conduct its enzymatic activity. Optimal concentrations for $MgCl_2$ are 1-1.5 mM.

  - Tris·HCl [Tris(Hydroxymethyl) aminomethane· HCl] buffer

    The Tris·HCl buffer is required to set the global pH of the reaction, which affects both polymerase activity and specificity. The usual pH is 8.3 at 25 °C (which will become pH 7.2 at 72 °C), provided by a concentration of 10 mM Tris·HCl buffer.

- Water

  To achieve the desired proportions and concentrations of all reactants, they are typically dissolved in sterile 18.2 M Ω milliQ water. MilliQ water is rendered free of organic material by double-distillation and then de-ionized in ionic (cationic and anionic) exchange columns. To achieve further sterility, milliQ water can be sterilized in autoclave prior to de-ionization or under UV irradiation after de-ionization.

The PCR cycles

The PCR mix is a critical factor in successful amplification, but it is not the only one. As seen before, PCR is a three-step iterative cycling reaction. The nature of each step will be determined, mainly, by two parameters: the

temperature set and the interval of time spent at that temperature, and both factors will influence on the overall performance of the PCR assay.

- Denaturation
  Denaturation is typically carried out at 94-96 °C, with times varying from 5 s to 2 min or more for long PCR fragments [Cheng1994].

- Extension
  Taq polymerase activity reaches its optimum at 75 °C, but extension is usually carried out at 72 °C to avoid primer fall-off from the template. The time required for extension will vary depending on the length of the DNA region to be extended, but a common rule of thumb is to suppose that Taq polymerase will extend 1 kb of DNA per minute. It must be noted that (fortunately, because this partially avoids primer fall-off at the extension temperature) extension will have already begun at annealing temperatures, since Taq polymerase is partially active down to 50 °C.

- Annealing
  Annealing temperature (T_a) and time do strongly influence the efficiency and specificity of the PCR reaction. Hence, although there exist some rules for deriving annealing temperature, it is recommended that this parameter should be assessed empirically [von Ahsen2001]. Different formulae for deducing the annealing temperature (conversely, the melting temperature of the primers (T_m) and thus their upper limit for annealing → $T_a<T_m$) are shown in Equation 1. Annealing times are best experimentally determined too, although 30-90 s typically are recommended for primers between 16 and 24 bp.

(a) $$Tm = 81.5 + 16.6 \cdot \log_{10}\left(\left[Na^{+}\right]\right) + 0.41 \cdot (\%G + C) - \frac{675}{n}$$

(b) $$Tm = 2 \cdot \left[\#A + \#T\right] + 4 \cdot \left[\#G + \#C\right]$$

Equation 1 - *Meinkoth* [Meinkoth1984] (a) and (b) *Wallace* [Wallace1979] equations for primer T_m (typically, $T_a = T_m - 5$ °C), where (a) n is the primer length in bp, %G+C the global G+C percentage of the primer and (b) $\#X$ is the number of occurrences of base X in the primer.

Equipment

As already stated (see p.62), the typical setup for a current PCR experiment involves the use of a thermocycler to carry out the necessary temperature

cycles [Haff1991]. Thermocyclers consist mainly of an oil reservoir that is heated and cooled to attain the desired temperatures. The driving force behind the temperature cycling is typically a resistance (and air-liquid ventilation for cooling) or, more recently, a set of Peltier cells (see *Materials and Methods*, p.305). The oil reservoir temperature is constantly monitored by a Pt100 or thermocouple probe, and controlled to within 1 ºC accuracy by means of an ON/OFF or PID (proportional-integrative-derivative, see p.163) controller. A heat-dissipater block of either aluminum or oxygen-free copper is placed on top of the oil reservoir to convey heat to the PCR vessels (typically thin-walled, high heat-transfer *eppendorf* tubes) that are inserted into holes drilled onto the heat-dissipater. PCR vessels contain the PCR mix, with an overlaid layer of paraffin or mineral oil to avoid evaporation within the vessel. In more modern systems, the mineral-oil layer has been eliminated by means of a hot-plate cover (typically at 105 ºC) that establishes a differential temperature gradient across the vessel and prevents evaporation.

1.4.3. PCR OPTIMIZATION

The perfect beast

Judging on the above section, and taking into consideration the years PCR has been around, it might seem that PCR is quite a straightforward, even simple, technique. Indeed, and fortunately for the biotech world, once a particular PCR has been optimized, the method is quite robust and reproducible, yielding the extraordinary results that have enthroned it as the most important analytical procedure of modern genetics. However, optimizing the PCR is far from straightforward. Instead, it becomes a sort of tortuous path that often drives biologists mad due to its frustrating results. As some have noted [Swanson1999], "the PCR reaction can be a beast to optimize".

Two plus two do not yield four

The reasons for this difficult optimization, which invariably leads to many hours of empirical optimization in the lab, are many and multifaceted. At the core, nonetheless, there is the fact, often cited by many a life scientist, that in life sciences two plus two do not often add up to four [Erill2000a]. This is to mean that, in a reaction such as PCR, the same formula will almost never work twice, since the own subject of the experiment, the template

DNA and its primers, is a basic and interacting fraction of the reaction to optimize. If we take into account the fact that, as it will be seen, almost every element of the PCR (primers, polymerase, temperatures, times, template DNA, KCl and $MgCl_2$ concentration, etc.) is intermingled with almost every other member of the reaction in a number of different ways, PCR optimization becomes a very complex and almost intractable problem, in which theoretical methods are welcome to enlighten some aspects of the reaction ([Wetmur1968], [Schnell1997]), but are usually discarded in favor of simple rules of thumb.

Parameters to optimize

Whichever set of rules of thumb is chosen, it will used as the means to optimize some aspect of the PCR reaction. Disregarding specific applications, optimization will cover mainly four basic topics: functionality, efficiency, sensitivity and specificity.

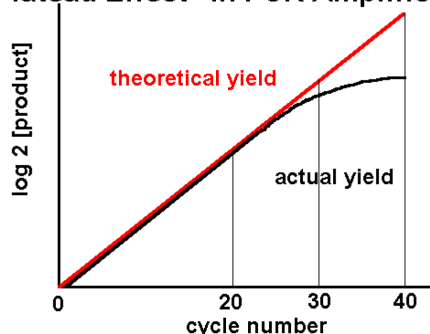

Figure 41 - The plateau effect: theoretical vs. actual yield as a function of cycle number.

- Functionality
  Functionality in PCR should be interpreted as a Boolean topic, defining whether the reaction works or does not work at all. Although almost every element of the reaction can play a role in defining functionality (it is evident that a PCR without polymerase will not be functional), key issues are typically related to contamination, be it by external DNA that masks or confounds results [Schmidt1995] or by inhibitors and chelators present either in the medium or the template solution [Wilson1997].

- Efficiency
  Once a PCR has become functional, its efficiency can be assessed as the number of amplicate copies it produces at the end of the reaction. The

practical efficiency of the PCR is quite far from its theoretical efficiency (2^n), reaching what is known as a *plateau* (see Figure 41) after approximately 25-40 cycles. This is mainly due to the heat-driven functional degradation of the polymerase and other actors in the final stages of PCR. Additionally, a multi-causal low efficiency at the initial stages of PCR (which has a cascade-like effect on overall efficiency) can also be a factor of drastic reduction in PCR efficiency. Both factors can be readily addressed (as by adding more polymerase to the mix at the final PCR stages to overcome enzyme decay), but care must be taken in order not to override other optimization factors (contamination, specificity and sensitivity).

- Sensitivity
  Sensitivity [Gibbs1990] should not be confused with the lower limit of detection in PCR. The minimal amount of material that can be identified by PCR, as it has been repeatedly demonstrated ([Saiki1988], [Li1988]), is a single template molecule. Conversely, sensitivity, the capacity to discern target from background signal in a particular reaction, is affected by many parameters (such as primers, purity of template, contamination, etc.) and, thus, it is a parameter that must be optimized.

- Specificity
  Specificity is both a paramount requisite and an inherent advantage of the PCR method, since it provides PCR with combined amplification and built-in filtering capabilities. In fact, the own methodology of PCR makes it a specificity booster when compared with conventional hybridization. Evidently, as in direct hybridization techniques, primers may anneal incorrectly at positions in the template that do not match them exactly. But, since PCR will provide the exponential amplification factor by using previously amplified segments, the probability of such false anneals occurring at proper distances to undergo subsequent extension is very low and, consequently, mismatched primer segments bear a similar fate to the initial template segments (see p.64), achieving, at their best, a linear amplification rate that is engulfed by the exponential amplification of correct-priming segments. Nonetheless, there exist many problems precluding optimal PCR specificity. For one, primers may anneal between themselves, producing primer-dimers and spoiling efficiency. And, obviously, an extremely low specificity of primer annealing can also lead to decreased efficiency and to confusing results.

Moreover, although specificity in PCR is often sought just to improve efficiency, experiments in which the size of the amplicate band is not known a priori require stringent specificity conditions if conclusive results are to be inferred.

Rules of thumb

In short, and albeit it would prove enormously important to obtain a set of equations to predict PCR behavior, biologists currently resort onto a variable set of rules of thumb, general principles and much common sense to optimize the PCR reaction. The following is a detailed, although not exhaustive, account of the general rules of thumb that are commonly used, sorted by the nature of each component, and of some extra hints (like the use of adjuvants) that can become important when dealing with PCR in atypical circumstances (such as those posed by the use of PCR-chips).

PCR mix

Template

Although PCR can work on very different templates (single-stranded or double stranded, cloned, plasmidic or genomic DNA [or even RNA, as in RT-PCR, see p.91]), not all templates work equally well.

- Basic template rules of thumb
  In general, it is recommended to use linear templates (instead of circular) and to cleave very large genomic DNA into smaller fragments (using a restriction enzyme, like Not-I). This will tend to avoid the presence of tertiary DNA configurations that may interfere with polymerase activity, and will also reduce initial denaturing and extension times and the number of oligonucleotides needed to carry on the reaction. The concentration of template should be enough to conduct PCR easily (10^5-10^6 copies ~ an extraction from a 10,000 cell culture), but not too large, since it may then reduce the specificity of the reaction by competing with primers at the annealing step. Template concentration will vary depending on the length of the template and the amplicate, since the same amount (weight) of a short template will proportionally contain (all other things being equal) many more amplicate regions than a longer one.

- Extraction and purification
  Template extraction and purification procedures should also be regarded in PCR. DNA is typically extracted (separated from the rest of cell material) by tissue digestion, which is followed by phenol-chloroform extraction and alcohol precipitation (see *Materials and Methods*, p.321). Salts may also be used to facilitate precipitation. As it happens, residual proteins from phenol-chloroform extraction, salts, the same phenol and other reagents can inhibit polymerase activity. Optional methods for extraction, like the use of guanine detergents also pose inhibition problems due to the own traces of detergent. A common solution to these problems is to conduct a last filtering with diatomite ionic-exchange or silica [Böddinghaus2001] columns and/or to re-precipitate in ethanol before PCR.

- Amplicate nature
  The region of template to amplify is also an important factor in PCR. Apart from the aspects of primer design it involves, which will be discussed later (see p.76), the length and nature of the amplicate region can widely affect the overall performance of the PCR. Typical amplicate lengths range from 100 to 1000 bp, and may rise up to 5 kb without excessive problems. Nonetheless, to achieve amplification of larger fragments, special setups and mixes need to be elaborated (see p.91). Amplicate %G+C content is also important, since it will influence its interaction with primers. For instance, amplicates with a high %G+C content will tend to produce less specific priming and can induce local tertiary structures that may impede or partially hinder polymerase activity. In this sense, amplicates with at least one breathing (A+T rich) region are often recommended.

- Special templates
  Finally, the nature of special templates (such as ancient [Powledge1996] or forensic DNA [Blake1992]) may require additional optimizations. For example, when using short amounts of template DNA (which is often the case in forensic analysis and the general rule in archeological analysis), high-fidelity polymerases, instead of Taq polymerase (see p.74), are recommended. The degraded nature of ancient DNA (aDNA) can pose by itself many problems. For instance, contamination by modern DNA (which is in a better shape than ancient DNA) can mask results and thus become a major problem [Schmidt1995]. The extraction and

purification methods must be also specially addressed in aDNA, since there may be many inhibitors in the medium or they may come from specific extraction procedures (such as EDTA [Ethylene-diamine tetra-acetic acid] decalcification of bones and teeth [Goodyear1994]). DNA stabilizer or Mg^{++}-chelator competing adjuvants (such as DMSO [Dimethyl-sulfoxide] and BSA [Bovine Albumin Serum], see p.83) may be required, together with an increase of $MgCl_2$ concentration to compensate for the presence of contaminant Mg^{++}-chelators. Additionally, since aDNA tends to be composed of very short fragments (below 300 bp on average), ligation of overlapped regions prior to PCR may be necessary to obtain comprehensive results ([Pääbo1990], [Pusch1998]).

Polymerase

As mentioned before (see p.62), the introduction of Taq polymerase has probably been the most important improvement in PCR methodology since its invention. Nowadays, two forms of the enzyme are available. The native enzyme may be isolated from cultures of the thermophilic bacterium *Thermus aquaticus*, or it may be obtained in a genetically engineered form, coming from recombinant strains of *E. coli*. Anyway, and although Taq polymerase works fairly well and is still widely used, *T. aquaticus* enzyme is no longer the only option available in heat-resistant polymerase enzymes.

- Error rates, proofreading capabilities and additional functionalities

  One of the main problems of Taq polymerase is that, even if it has 5'-3' polymerization dependent exonuclease activity, it lacks 3'-5' nuclease activity. This means that Taq polymerase is unable to correct mis-incorporation errors (i.e. it lacks *proofreading* capabilities). Since all polymerases (under a specified set of conditions) have an inherent error rate of base mis-incorporation (about 10^{-4} errors per nucleotide per extension in Taq polymerase, ([Tindall1988], [Keohavong1989])), the lack of proofreading capabilities implies that mis-incorporation errors will remain unbridled, a fact that can lead to many misleading conclusions in sequencing or SNP detection reactions. Most new polymerases overcome this problem. *Pyrococcus furiosus* (Pfu), *Thermogota maritima* (Ult), *Thermococcus litoralis* (Tli), *Thermus brockianus* (Tbr), *Thermococcus fumicolans* (Tfu) and *Pyrococcys woesei* (Pwo) polymerases all exhibit 3'-5' exonuclease activity and, thus, display lower error rates than Taq polymerase (down to 10^{-6} errors per base per extension in Pfu

polymerase ([Lundberg1991], [Hengen1995])). Other polymerases, such as *Thermus flavis* (Tfl) polymerase, offer a much greater thermal stability than Taq, while still other polymerases (like *Thermus termophilus* (Tth) polymerase, which can either reverse-transcribe RNA in presence of Mn^{++} or amplify DNA in presence of Mg^{++}) and polymerase cocktails are used in specific applications such as RT-PCR (see p.91) or the amplification of large fragments (see p.91). Many more parameters, like the rate of extension, are involved in the selection of the adequate polymerase. In general, the choosing of the best-fitted polymerase depends on the PCR application. For instance, Taq polymerase can amplify DNA much faster than Pfu polymerase, a fact that has a strong impact on global reaction times. Moreover, the proofreading exonuclease activity of Pfu (and others) polymerase can induce unwanted degradation of ssDNA during amplification, which may become critical if the template is very short (as in aDNA amplification). On the other hand, the modified N-terminal deletion mutant of Taq polymerase (*KlenTaq*) has a much broader range of optimal of Mg^{++} concentration than competing polymerases, making reactions easier to optimize. Still, other polymerases are better at withstanding the presence of contaminants, adjuvants and inhibitors, producing optimal results in certain applications. Finally, the manner in which different polymerases terminate the extension, leaving *blunt* (as Ult polymerase) or *sharp* ends can be of importance in subsequent analytical techniques, such as cloning or gene expression.

- Polymerase concentration

  As in the case of template concentration, polymerase concentration must rest in a delicate balance. Excessively low concentrations of polymerase will hamper the reaction, reducing the number of cycles at which the plateau effect will appear since, statistically, a homogenous amount of polymerase will be degraded by repeated denaturation regardless of its concentration. The obvious solution, and a partial solution to completely eradicate the plateau effect, would be a linear increase in polymerase concentration. However, an excessive concentration of polymerase will tend to lower the specificity of the reaction (forcing erroneous anneals, [Saiki1989] and, consequently, it will also indirectly affect the efficiency of PCR (see p.70). Additionally, the high price of polymerases (when compared to all the other reagents present in a PCR mix) makes the use of large concentrations

unwelcome, in spite of any beneficial effects. In general, and since most excessive enzyme levels are caused by pipetting errors, biologists often resort to the creation of a master mix (a general mix which is then homogenously distributed among mixes prior to the addition of template) to overcome the problem of inaccurate dispensing of sub-microliter volumes of enzyme solution (see *Materials and Methods*, p.322).

Primers

Primer design is a matter of crucial interest in the outline of a PCR method. By themselves, primers can wildly affect the specificity, sensitivity, efficiency and even functionality of the reaction, and will impose many restrictions in the defined temperatures and times, thus limiting (or optimizing) the overall performance (both in terms of yield and speed) of the PCR.

- Primer length

  A basic rule of thumb in primer design is to use primers between 17 and 28 bp long. This is meant to ensure that primer sequence will not be easily found by chance in the template (statistically, a 16 bp primer should occur only once every 4^{16} [~4 billion] bases). Hence, shorter primers will lower specificity, since they will produce mismatching anneals more easily, and will tend to lower efficiency, as they may anneal to other regions of the template. On the other hand, longer primers will require longer annealing times and a higher annealing temperature (T_a). When the primer is too long, non-specific priming can occur if T_a is set too low and, if not, the efficiency of the reaction may be hindered due to a shorter amount of priming and to an excessively low advantage of primers in front of template during annealing. In general, primer length will be a compromise between specificity and primer composition (see next section).

- Primer composition

  Primer composition is a very important factor in PCR and often, as in the case of multiplex PCR (see p.90), a critical one. There are many aspects the analyst should take into account when designing a set of primers; the first is their %G+C content. Excessive %G+C content should be avoided on the basis that it will increase T_a and will promote the occurrence of secondary configurations in primers. This last issue

holds also true when %G+C content is excessively low. As a general rule, primer %G+C content should lie in the whereabouts of 40-60%G+C. Since the %G+C content will determine the primer melting temperature (and, conversely, its annealing temperature), primer pairs should be designed to have close T_m. If the T_m between primers differs excessively, the set T_a will either cause that one of the primers (the one with the highest T_a) anneals nonspecifically or, on the contrary, that the other primer (the one with the lowest T_a) primes poorly, precluding efficiency. If the difference in T_a is too great, PCR can even become asymmetric (meaning that only one of the primers will anneal and PCR will produce single stranded sequences only delimited at one end; an effect that is sometimes intended, but obviously detrimental when not sought).

Primer sequence is also a key issue in primer design. The presence of excessive Gs and Cs at the 3' end of the primer may induce mis-priming, manifestly in %G+C rich template sequences. In addition, complementary or palindromic sequences and inverted repeats should be avoided at all costs in primers, given that they will invariably result in the formation of secondary structures within or between primers (see Figure 7, p.24). The creation of primer-dimers (or even primer-trimers, the linking between primers at complementary positions) or self-dimers (hairpin structures between complementary sequences in a single primer) will reduce the number of available useful primers from the onset, leading to a decrease in efficiency, and may confound results if the amplicate length is short (primer-trimers of 28 bp primers may produce products of up to 70 bp long).

- Primer concentration

  Finally, the concentration of primers should also be carefully balanced. As in the case of template and polymerase, an exceedingly low concentration of primers will reduce the efficiency of the reaction, since they won't be available in sufficient numbers for priming. Conversely, an excessive concentration of primers will promote mis-priming and the formation of primer-dimers.

dNTPs

As stated before, dNTPs should be provided in equal concentrations to avoid mis-incorporation errors by the polymerase (if a dNTP is excessively present, it will tend to be more available to the polymerase and, thus, can

be mis-incorporated more easily in place of the corresponding dNTP). In addition, the concentration of dNTPs must also be carefully balanced. In this case, the balance must be sought between low concentrations (which will promote the plateau effect but enhance specificity) and too high concentrations, which will reduce specificity and will influence polymerase activity by sequestering free Mg^{++} ions (see next section). Finally, dNTP solutions should be neutralized to pH 7.0 in order to minimally disrupt pH conditions during PCR.

Buffer

The three components of buffer (salt, magnesium and Tris·HCl) must be optimized for each PCR reaction, since their concentrations and influence will vary depending on the polymerase, template and primers used. In this respect, Tris·HCl is perhaps the most stable component of the PCR buffer, although not many studies have been conducted to assess its optimality [Innis1990]. The main contribution of Tris·HCl is to provide the adequate pH for PCR (which influences polymerase activity and annealing efficiency, see p.65). Salt (KCl or, sometimes, NaCl) can be added to the reaction to facilitate priming, but its concentration should be reduced when mis-priming is a major effect. Additionally, salt concentration should never exceed 50 mM, since it has been shown that it may then inhibit polymerase activity ([Innis1988], [Gelfand1989]).

- Magnesium dichloride ($MgCl_2$)
  Magnesium dichloride is a critical factor in PCR optimization. Not only does it have an effect on primer annealing, strand dissociation temperatures, formation of primer-dimers and enzyme activity and fidelity, but it is, in turn, influenced by the concentrations of primers, template, contaminants and dNTPs. Hence, $MgCl_2$ concentration is a very difficult parameter to optimize and iterative titration with varying Mg^{++} concentrations is recommended to empirically optimize the reaction. Most crucially, the polymerase requires free Mg^{++} ions to achieve enzymatic activity. Since all DNA molecules have a net negative charge (due to their phosphate groups, see p.24), they will tend to form chelates with Mg^{++} ions. In addition, other chelators (like proteins or EDTA) may be present in the mix due to extraction techniques (see p. 72). This fact can be partially circumvented by the addition of salt, which generates K^+ or Na^+ ions that compete for chelation with Mg^{++}, while helping to stabilize the DNA molecules (due to the own process of

chelation), thus facilitating annealing and extension. Nevertheless, since the addition of excessive amounts of salt inhibits polymerase activity, Mg^{++} sequestering cannot be completely avoided and care must be taken to ensure that free Mg^{++} ions will remain available to the polymerase. Given that dNTPs are, by far, the most concentrated nucleotides (compared to template and primers) in the PCR mix, the concentration of Mg^{++} must always be greater than that of dNTPs. It must be noted here that the chelation of Mg^{++} ions by dNTPs is not an undesirable secondary effect, since Mg^{++} conforms a soluble complex with dNTPs that is essential for dNTP incorporation. Unfortunately, Mg^{++} concentration cannot be increased indefinitely. An excess of [Mg^{++}] gives nonspecific amplification products because it promotes imperfect priming and reduces enzyme fidelity.

PCR cycles

Optimization of the PCR mix is a cumbersome task, but is only halfway to optimizing the amplification reaction. As already stated, many parameters of the PCR mix (as primers or Mg^{++} concentration) exert some influence on the physical properties of the reaction, inducing changes in the physical parameters of PCR cycles (namely, temperatures and times). This influence is crisscrossed, since modulation of the physical parameters upon a prefixed mix will also provide means for optimization of the reaction.

Denaturation

Denaturation is a basic step in PCR. It is important because it separates the initial dsDNA for primer annealing and, thereafter, it denatures the amplified double-strands for further priming and extension.

- Denaturation temperatures

  Although heat-resistant polymerases partly circumvent the problem posed by denaturing temperatures, denaturation continues to impose limits on the efficiency of PCR, and is one of the main cofactors triggering off the plateau effect (see Figure 41). Taq polymerase, for instance, has an estimated half-life of 30 min at 95 ºC (5 min at 97.5 ºC, [Innis1990]), which is one of the main reasons why PCR should not exceed the 30 cycles barrier. In addition, the denaturation temperature is influenced by salt concentration. In buffers of ionic strength lower than 150 mM NaCl, DNA will denature at 91-97 ºC; this provides the means

for carrying out amplification at temperatures below the water boiling point, but it also places upper and lower limits on salt concentration. Excessively low salt concentrations will decrease performance because of poor annealing, while too high concentrations will require higher denaturation temperatures and longer denaturation times, contributing to enzyme degradation (and thus also limiting PCR yield). In general, denaturation temperatures are quite fixed by the nature of the buffer and should not be tampered with much. For instance, working with substantially lower denaturing temperatures can induce false negative results, since a low melting temperature (like 92 ºC) will be enough to denature short strands of DNA (200-300 bp), which are commonly used as PCR positive controls, while it will be unable to denature template DNA. Hence, the experimenter will perceive a false negative result due to an exceedingly low denaturation temperature.

- Denaturation times

  On the contrary, denaturation times are not as stringent as temperatures. An initial, long (1-3 min) denaturation step is highly recommended to achieve maximum disengaging of the original template and of the possibly conformed primer-dimer structures, maximizing the amount of reagents available in the first cycles of PCR and improving sensitivity. After this initial denaturation, denaturation times should be as short as possible, a factor that will depend on amplicate length. An additional strategy is to somewhat increase the denaturing temperature in order to lower the denaturation times, since the latter seems to be a closer factor in enzyme degradation than the former [Innis1990].

Annealing

Annealing temperatures (and to a lesser degree, times) are probably the most difficult physical parameters to optimize in PCR, since they are strongly linked to primer design and, thus, to specificity and efficiency.

- Annealing temperature

  Annealing temperature is usually derived from formulae as those seen in Equation 1 (p. 68). Typically, T_a is approximated to T_m-5 ºC to start empirical optimization of the T_a, which is usually raised to increase specificity. The upper limit on annealing temperature is the apparent loss of PCR efficiency, when primer annealing becomes difficult and scarce. Conversely, low annealing temperatures will induce nonspecific

amplification due to mis-pairing and, consequently (see p.70), they will also degrade PCR efficiency ([Rychlik1990], [Wu1991]). Since the polymerase enzyme is already active (even if little, some two orders of magnitude less) at annealing temperatures, the mis-priming induced by a low T_a will be aggravated by the fact that low-temperature extension will preclude primer fall-off, contributing to an increase in the enzyme mis-incorporation rates. To avoid specificity loss, some authors have suggested the use of maximally long primers to carry annealing at extension (~72 °C) temperatures, in what is known as two-step PCR ([Kim1988], [Rasmussen1994a]). In case of different melting temperatures for each primer, a compromise must be reached in T_a to allow enough annealing of one primer while maintaining the necessary stringency conditions for the other. If this compromise is unattainable, the general rule is to find a different set of primers for the desired region, rather than start tinkering with the reaction.

- Annealing times

  Annealing times depend on primer composition, length and concentration, but are generally low. Typical annealing times range from 5 s to 30 s for normal (17-28 bp long, 40-60 %G+C content, 0.2 µM) primers. The general rule of thumb is to extend annealing times when long, low %G+C content or highly concentrated primers are used, but at the cost of producing less specific results.

Extension

Extension times and temperatures basically depend on the nature of the polymerase enzyme used and on both the concentration of template and the length of the amplicate sequence, but, since they are held tight by fixed polymerase parameters, they are not usually optimized to a great extent. Even if it is sometimes raised to 75 °C to increase both speed and specificity, the optimal extension temperature is considered to be 72 °C if the polymerase provider does not state otherwise. Regarding extension times, the general rule of thumb is to assume that Taq polymerase will extend about 100 bases per second at 72 °C, which should provide reliable amplification of up to 2 kb samples in 1 min [Innis1990]. Longer products may require longer times, among other things, as it will be later discussed (see p.91). Longer times are also useful when the concentration of polymerase is lower than that of the product (namely, at the final stages of PCR). It is for this reason that standard PCR protocols incorporate a final 7-

15 min extension step to ensure that the ultimate amplification step (which, if effective, amounts to a huge increase in product) is conveniently carried out.

Preparation methods

Extraction and purification methods (see p.72) can introduce many contaminants that may pose problems due to masking of results (in case of contaminant DNA) or inhibition of the PCR.

- Avoiding inhibitors
  The problem of reaction inhibitors is mainly caused by the presence of these agents in the tissues or cells from which the DNA has been extracted. Reports on inhibitors coming from clinical, food and environmental samples [Wilson1997] have appeared since the first days of PCR, covering such effects as inhibition by hemoglobin, milk proteins and Ca^{++} ions [Rossen1992], pollen [St. Pierre1994] or humic compounds. Therefore, inhibition problems are mainly dealt with by using strict and stringent extraction protocols (see p.72). Nevertheless, strong inhibitors may also lurk in the backstage of laboratory benches. For instance, glove powder has been reported as a putative PCR inhibitor in many studies ([De Lomas1992], [St. Pierre1994]) and UV irradiated pipette tips [Linquist1998] and mineral oil [Dohner1995] have also been pinned down as potential PCR inhibitors, facts that should hint at the degree of suspiciousness an experimenter must wield when facing apparent PCR failure.

- The paranoia chain reaction
  Back in the early nineties, a jocose biologist re-dubbed PCR as the Paranoia Chain Reaction. In fact, to a profane in the field, the behavior of biologists when conducting PCR might seem as paranoiac as the guise in which technology engineers amble in clean room facilities. Actually, the reason is the same in both cases: contamination. But, whereas in a microelectronics clean room contamination is assessed by the presence of relatively small polluting particles, the black beast of PCR is much smaller and harder to smash, since its nature is the same as that of the amplification target: DNA. It is precisely the extraordinary capability of PCR to amplify from a single strand of DNA that makes it so sensitive to DNA contamination. With sufficiently high contamination levels, the

contaminant DNA may compete with template DNA in such a way as to completely *erase* it, while lower contamination levels may mask and/or confound results. Contrary to common intuitiveness, DNA is much harder to eradicate than it might seem and, once a workbench has been contaminated, the price of decontaminating it is much higher than the costs of avoiding contamination in the first place. For this reason, many procedures have accumulated since the invention of PCR to avoid contamination by *foreign* or carryover DNA, leading to the complex methodology of PCR that lead to the above cited rebaptism of the reaction. Basically, all PCR material tends to be one-use-only disposable material that has been manufactured under sterile conditions. The material that must be reused is sterilized in an autoclave (although some authors indicate that this is not enough to guarantee decontamination [Dwyer1992]). In the case of some degradable materials, like pipette tip dispensers, each disposable pipette tip contains a filter that impedes contamination of the dispenser. As an overall measure, the PCR workbench is physically isolated from other laboratory areas in which DNA work is conducted and the PCR-mix is prepared in a laminar airflow chamber inside an over-pressurized room to avoid the entrance of contaminants. Experimenters will wear gloves and lab-coats, and all the non-autoclavable material (including gloves, fountain-pens and all other material the experimenter carries with him) will be UV-irradiated in the laminar flow chamber for at least 20 min previous to the PCR mix elaboration ([Sarkar1990a], [Sarkar1990b], [Ou1991], [Sarkar1993]).

Adjuvants

Over the years, many reports concerning the use of adjuvants (additives or co-solvents) in PCR have appeared. Although there seem to be strong indicators favoring the use of some, the very nature of PCR often makes results irreproducible and can even lead to contradictory results [Gibbs1990]. The following is a short list of adjuvants, their main positive and negative properties and their applications.

Gelatin

The first adjuvant to be ever described [Saiki1988], gelatin clearly illustrates the controversial aspects in the use of adjuvants. Although it was supposed to facilitate PCR by thickening the PCR mix and helping stabilize the DNA

molecules, the role of gelatin has been subsequently disproved [Blanchard1993]. Nonetheless, it is still routinely used in many laboratories.

Dimethyl-sulfoxide (DMSO)

DMSO has been repeatedly reported ([Chamberlain1988], [Bookstein1990], [Smith1990], [Filichkin1992]) as a strong facilitator of PCR, both in terms of boosting specificity and efficiency, particularly with high %G+C or unusually long templates. The main role of DMSO is to disrupt base pairing and to prevent mismatched anneals. It also helps to break down secondary DNA structures that might hinder polymerase activity and, to some extent, it lowers the effective T_m of the primers. Thus, DMSO enhances PCR specificity (and consequently efficiency), and can be useful as a functionality booster when dealing with long or high %G+C DNA strands, which will tend to display secondary structures and facilitate mis-pairing. Standard concentrations for DMSO are 1-10% (v/v). Nevertheless, the use of DMSO is not gratuitous. Although the mechanisms are not clearly understood, it seems clear that DMSO decreases polymerase activity by up to 50% in concentrations of 10% and higher [Lawyer1989]. Thus, polymerase concentration has to be increased whenever DMSO is used, and titration of dNTPs and other reagents to optimize the reaction becomes mandatory. Moreover, since DMSO alters the melting temperature of primers (a key issue in PCR optimization, p.80), empirical optimization of this parameter following the addition of DMSO is also strongly recommended. Finally, although DMSO is mainly supposed to boost specificity, there may be cases in which this is not so [Frackman1998], as illustrated by Figure 42.

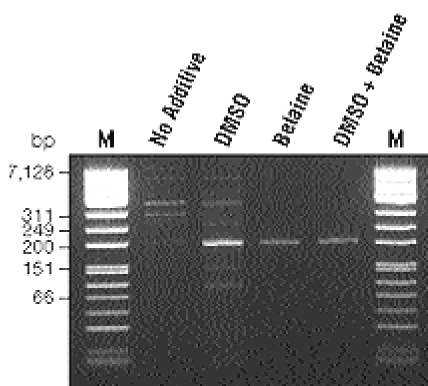

Figure 42 - Amplification a 75%G+C region of the human retinoblastoma gene following *Bookstein* assay [Bookstein1990]. No amplification is seen without additives, but the addition of DMSO, although it boosts efficiency, generates extraneous bands. Betaine, in turn, produces amplicate without nonspecific bands, but with a lower yield. Finally, combination of the two does not improve results. Source: [Frackman1998].