Polymerase Chain Reaction (PCR)

Standard PCR Protocol

Molecular Biology Techniques Manual, 3rd ed. (2001) Edited by: Vernon E Coyne, M Diane James, Sharon J Reid and Edward P Rybicki.

Contents

Materials:

- Template DNA (genomic, plasmid, cosmid, bacterial/yeast colony, etc.)
- Primers (resuspended to a known concentration with sterile TE or water)
- Buffer (usually 10X, usually sold with Taq polymerase or you can make your own) **Note**: different buffer receipes follow at the end of this protocol
- $MgCl_2$ (25mM is convenient)
- Taq polymerase
- dNTPs (2mM stock) **Note**: a 2mM stock of dNTPs means that the final concentration of each dNTP (dATP, dCTP, dGTP, and dTTP) is 2mM -- **NOT** that all dNTPs together make 2mM. dNTPs come as 100mM stocks -- thaw and add 10µL of each dNTP to 460µL of (deionized distilled water) ddH_20 to make 2mM. Store at -20^OC.
- Sterile ddH_20
- Gloves
- PCR machine
- Aerosol tips, if desired
- Pipet

Recommended Reagent Concentrations:

- Primers: 0.2 - 1.0 uM
- Nucleotides: 50 - 200 uM each dNTP
- Dimethyl sulphoxide (DMSO): 0 - 10% (v/v)
- Taq polymerase: 0.5 - 1.0 Units/50ul reactions

Protocol for PCR cocktail

1. Determine the volume of reaction to be amplified. **Note**: The sample volume should not exceed 1/10th reaction volume.
2. Determine the working concentrations of your reagents as well as the final concentration of your PCR reaction and fill the table below.

Table 1. PCR cocktail

PCR components	Volume (___ul)	X no. of samples	[working]	[final]
DNA			20-50ng/ul	___ ng
Buffer			10x	1x
MgCl			25mM	2mM
Forward primer			5uM	200nM
Reverse primer			5uM	200nM
dNTPs			1mM	200uM
Taq. Polymerase			1U	0.5U
Distilled water	Up to desired volume			

3. Carefully thaw the reagents. Mix and quickly spin down the tubes to bring down the components then place on ice.
4. In a separate microtube, mix the reagents (based on the calculated volume) by first putting the water followed by buffer, MgCl, primers, dNTPs and Taq.
5. Carefully label the tubes or the reaction plate and dispense the PCR cocktail. The DNA can be placed in the tubes or plate before or after dispensing the cocktail.

6. Overlay the reaction with mineral oil to avoid evaporation. Close the tubes well or cover the plate with an aluminum foil tightly to avoid evaporation.

7. Spin down the reaction in order to remove bubbles and bring down all components.

8. Place the tube or plate in the thermal cycler programmed with the desired temperature and time of amplification.

9. Examine the product by agarose gel or acrylamide electrophoresis

Additional information in preparing PCR reactions

Target DNA may range from 1 ng - 1 ug (**Note**: higher concentration for total genomic DNA; lower for plasmid /purified DNA /virus DNA target). Nucleotide concentration need not be above 50uM each: long products may require more, however.

Buffer: use proprietary or home-made 10x reaction mix; eg: Cetus, Promega. This should contain: minimum of 1.5mM Mg2+, usually some detergent, perhaps some gelatin or BSA. Promega now supply 25mM MgCl2, to allow user-specified [Mg2+] for reaction optimization with different combinations of primers and targets.

Recommended buffers generally contain:

- 10-50mM Tris-HCl pH 8.3,
- up to 50mM KCl, 1.5mM or higher MgCl2,
- gelatin or BSA to 100ug/ml,
- and/or non-ionic detergents such as Tween-20 or Nonidet P-40 or Triton X-100 (0.05 - 0.10% v/v)

Higher than 50mM KCl or NaCl inhibits Taq, but some is necessary to facilitate primer annealing. [Mg2+] affects primer annealing; Tm of template, product and primer-template associations; product specificity; enzyme activity and fidelity. Taq requires *free* Mg2+, so allowances should be made for dNTPs, primers and template, all of which chelate and sequester the cation; of these, dNTPs are the most concentrated, so [Mg2+] should be 0.5 - 2.5mM *greater* than [dNTP]. A titration should be performed with varying [Mg2+] with all new template-primer combinations, as these can differ markedly in their requirements, even under the same conditions of concentrations and cycling times/temperatures.

Remember sample volume should not exceed 1/10th reaction volume, and sample DNA/NTP/primer concentrations should not be too high as otherwise all available Mg2+ is chelated out of solution and enzyme reactivity is adversely affected. Any increase in dNTPs over 200uM means [Mg2+] should be re-optimized. AVOID USING EDTA-CONTAINING BUFFERS AS EDTA CHELATES Mg^{2+}

Primer concentrations should not go above 1uM unless there is a high degree of degeneracy; 0.2uM is sufficient for homologous primers.

Use of detergents is recommended only for Taq from Promega (up to 0.1% v/v, Triton X-100 or Tween-20). DMSO apparently allows better denaturation of longer target sequences (>1kb) and more. Some enzymes do not need additional protein, others are dependent on it. Some enzymes work markedly better in the presence of detergent, probably because it prevents the natural tendency of the enzyme to aggregate.

Low primer, target, Taq, and nucleotide concentrations are to be favored as these generally ensure cleaner product and lower background, perhaps at the cost of detection sensitivity.

Pool MASTER MIX OF REAGENTS IN ABSENCE OF DNA using DNA-free pipette, then dispense to individual tubes (using DO NOT USE SAME PIPETTE FOR DISPENSING NUCLEIC ACIDS AS YOU USE FOR DISPENSING REAGENTS DNA-free pipette), and add DNA to individual reactions USING PLUGGED TIPS. OVERLAY REACTIONS WITH 50UL OF HIGH-QUALITY LIQUID PARAFFIN OR MINERAL OIL to ensure no evaporation occurs: this changes reactant concentrations. Vaseline was also found applicable especially for "HOT START" PCR.

PCR Profile:

Table 2. PCR thermal cycling.

Cycle No.	Step	Temperature	Time	Description
1x	1	94^{O}C	3 min.	Initial template denaturation
30x	2	94^{O}C	30 sec.	Template denaturation
	3	55^{O}C	30 sec.	Primer annealing
	4	72^{O}C	1 min.	Base extension
1x	5	72^{O}C	5 min.	Final elongation
1x	6	4^{O}C	α	

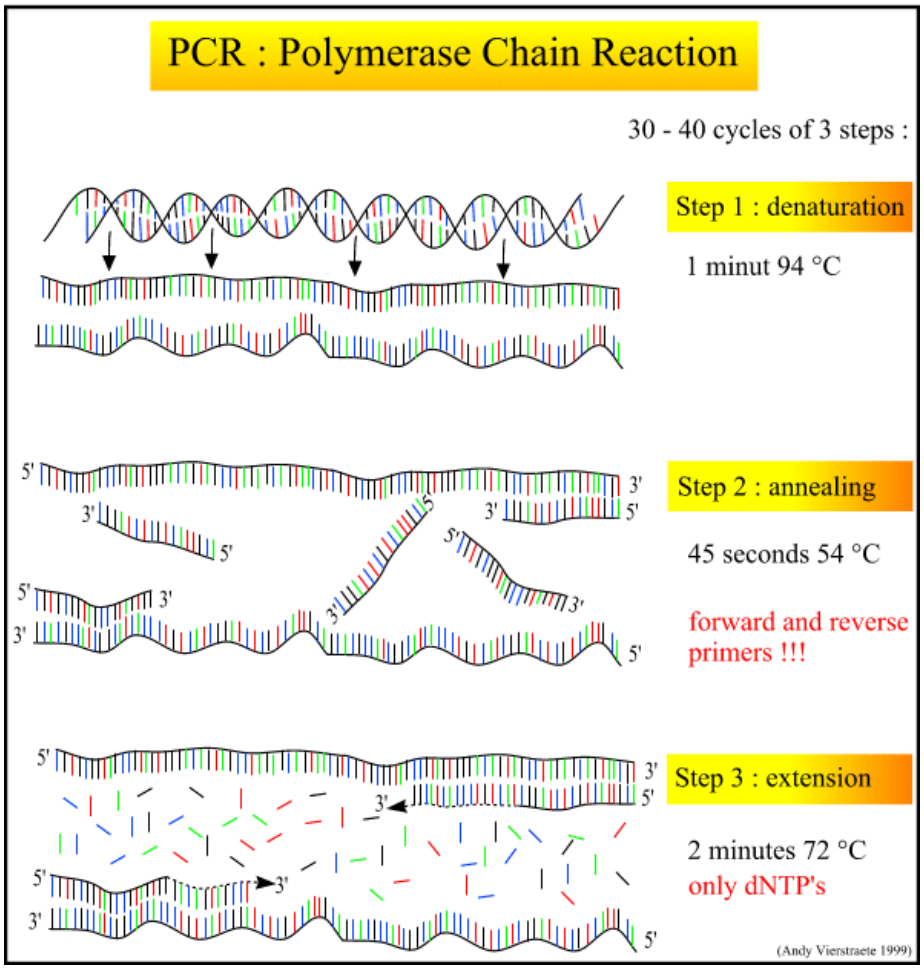


Figure 1. Difference in PCR profile

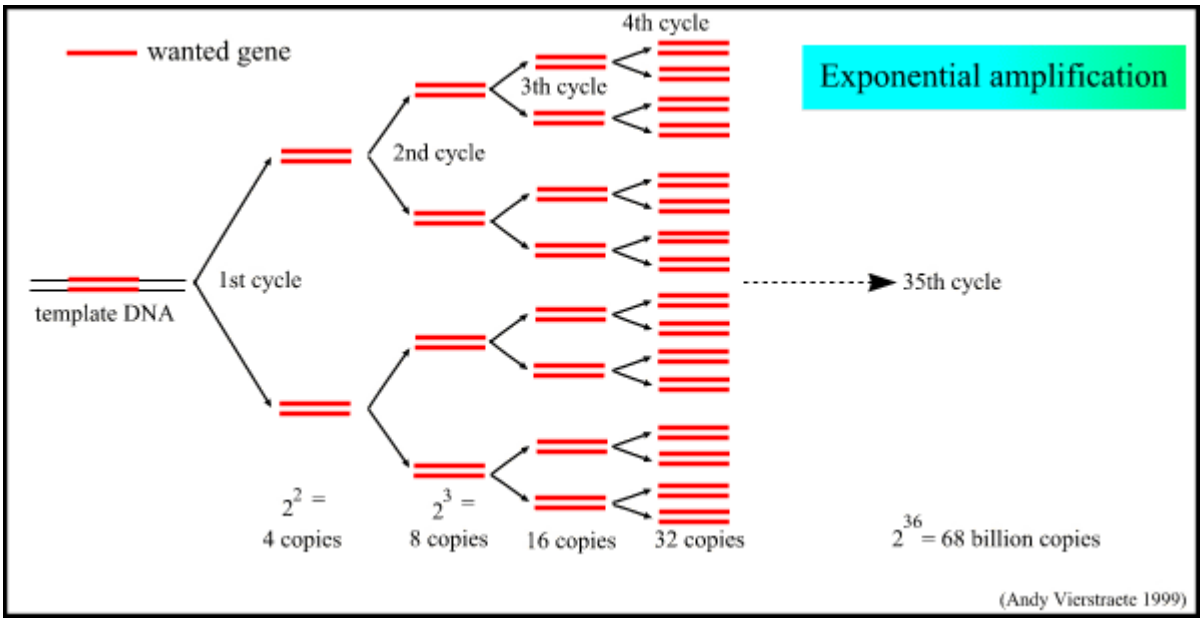


Figure 2. Show exponential increment of the number of copied product

Additional information for PCR profile

Initial Conditions

Initial denaturation can start at 92 – 97^{O}C for 3 - 5 min. If you denature at 97^{O}C, denature sample only with high GC content.

Temperature Cycling:

- 92 – 94^{O}C for 30 - 60 sec (Denature)
- 37 – 72^{O}C for 30 - 60 sec (Anneal)
- 72^{O}C for 30 - 60 sec (Elongate) (60 sec per kb target sequence length)

Note: 25 - 35 cycles only (otherwise enzyme decay causes artifacts)

Complete Elongation:

- 72^{o}C for 5 min at end to allow complete elongation of all product DNA

YOU CAN USE GLYCEROL IN THERMAL CYCLER REACTION TUBE HOLES TO ENSURE GOOD THERMAL CONTACTS

DON'T RUN TOO MANY CYCLES: if you don't see a band with 30 cycles you probably won't after 40; rather take an aliquot from the reaction mix and re-PCR with fresh reagents.

Special:

"Hot Start" PCR:

In certain circumstances one wishes to avoid mixing primers and target DNA at low temperatures in the presence of Taq polymerase: Taq pol is almost as efficient as Klenow pol at 37^{o}C; consequently, if primers mis-anneal at low temperature prior to initial template denaturation, "non-specific" amplification may occur. This may be avoided by only adding enzyme after the initial denaturation, before the reaction cools to the chosen annealing temperature. This is most conveniently done by putting wax "gems"^{TM} into the reaction tube after addition of everything except enzyme, then putting enzyme on top of the gem: the wax melts when the temperature reaches +/-80^{o}C, and the enzyme mixes with the rest of the reaction mix while the molten wax floats on top and seals the mix, taking the place of mineral oil. "Gems" may be substituted by Vaseline^{TM}.

Asymmetric PCR for ssDNA Production:

Simply use a 100:1 molar ratio of the two primers (eg: primer 1 at 0.5uM, primer 2 at 0.005uM). This allows production of mainly ssDNA of the sense of the more abundant primer, which is useful for sequencing purposes or making ssDNA probes.

Factors Affecting the PCR:

Denaturing Temperature and time

The specific complementary association due to hydrogen bonding of single-stranded nucleic acids is referred to as "annealing": two complementary sequences will form hydrogen bonds between their complementary bases (G to C, and A to T or U) and form a stable double-stranded, anti-parallel "hybrid" molecule. One may make nucleic acid (NA) single-stranded for the purpose of annealing by heating it to a point above the "melting temperature" of the double- or partially-double-stranded form, and then flash-cooling it: this ensures the "denatured" or separated strands not re-anneal. Additionally, if the NA is heated in buffers of ionic strength lower than 150mM NaCl, the melting temperature is generally less than 100^{O}C - which allows PCR to work with denaturing temperatures of 91-97^{O}C.

Taq polymerase is given as having a half-life of 30 min at 95^{o}C, which is partly why one should not do more than about 30 amplification cycles: however, it is possible to reduce the denaturation temperature after about 10 rounds of amplification, as the mean length of target DNA is decreased: for templates of 300bp or less, denaturation temperature may be reduced to as low as 88^{O}C for 50% (G+C) templates (Yap and McGee, 1991), which means one may do as many as 40 cycles without much decrease in enzyme efficiency.

"Time at temperature" is the main reason for denaturation/loss of activity of Taq. Thus, if one reduces this, one will increase the number of cycles that are possible, whether the temperature is reduced or not. Normally the denaturation time is 1 min at 94^{o}C: it is possible, for short template sequences, to reduce this to 30 sec or less. Increase in denaturation temperature and decrease in time may also work: Innis and Gelfand (1990) recommend 96^{O}C for 15 sec.

Annealing Temperature and Primer Design

Primer length and sequence are of critical importance in designing the parameters of a successful amplification. The melting temperature of a NA duplex increases both with its length, and with increasing (G+C) content. A simple formula for calculation of the Tm is

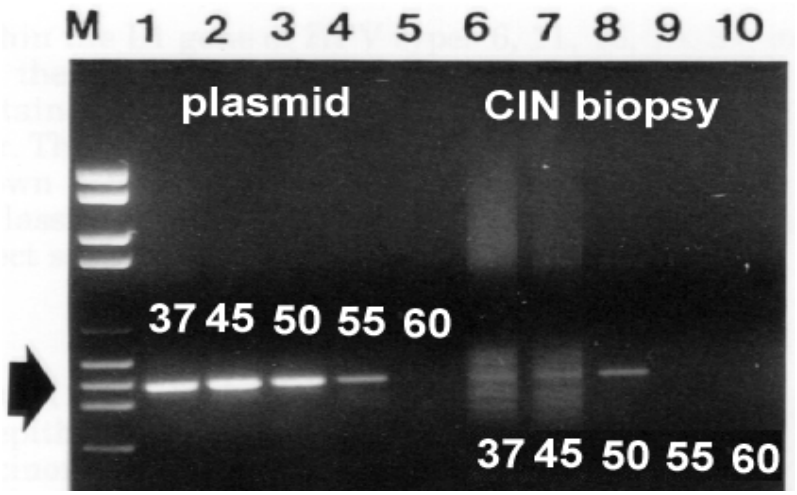
Tm = 4(G + C) + 2(A + T)^{o}C.

Thus, the annealing temperature chosen for a PCR depends directly on length and composition of the primer(s). One should aim at using an annealing temperature (Ta) about 5^{o}C below the lowest Tm of the pair of primers to be used (Innis and Gelfand, 1990). A more rigorous treatment of Ta is given by Rychlik *et al*. (1990). It was emphasized that if the Ta is increased by 1^{O}C every other cycle, specificity of amplification and yield of products <1kb in length are both increased. One consequence of having too low a Ta is that one or both primers will anneal to sequences other than the true target, as internal single-base mismatches or partial annealing may be tolerated: this is fine if one wishes to amplify similar or related targets; however, it can lead to "non-specific" amplification and consequent reduction in yield of the desired product, if the 3'-most base is paired with a target.

A consequence of too high Ta is that too little product will be made, as the likelihood of primer annealing is reduced; another and important consideration is that a pair of primers with very different Tas may never give appreciable yields of a unique product, and may also result in inadvertent "asymmetric" or single-strand amplification of the most efficiently primed product strand.

Annealing does not take long: most primers will anneal efficiently in 30 sec or less, unless the Ta is too close to the Tm, or unless they are unusually long.

An illustration of the effect of annealing temperature on the specificity and on the yield of amplification of *Human papillomavirus type 16* (HPV-16) is given below (Williamson and Rybicki, 1991: J Med Virol 33: 165-171).



Plasmid and biopsy sample DNA templates were amplified at different annealing temperatures as shown: note that while plasmid is amplified from 37 to 55^{o}C, HPV DNA is only specifically amplified at 50^{O}C.

Primer Length

The optimum length of a primer depends upon its (A+T) content, and the Tm of its partner if one runs the risk of having problems such as described above. Apart from the Tm, a prime consideration is that the primers should be complex enough so that the likelihood of annealing to sequences other than the chosen target is very low. (See hybridn.doc).

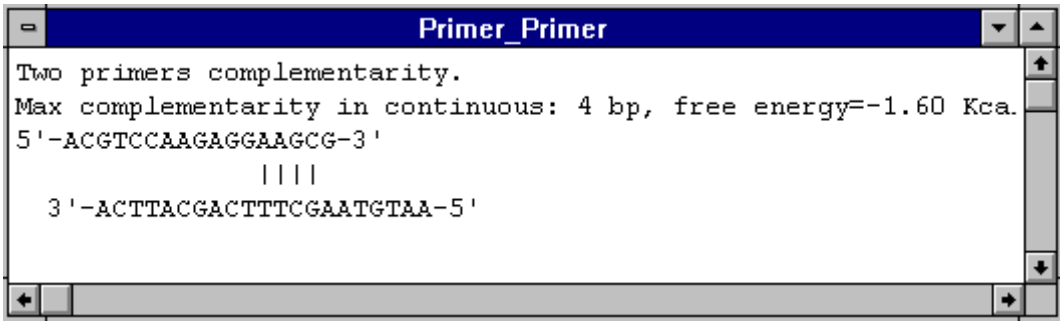
For example, there is a ¼ chance (4^{-1}) of finding an A, G, C or T in any given DNA sequence; there is a 1/16 chance (4^{-2}) of finding any dinucleotide sequence (eg. AG); a 1/256 chance of finding a given 4-base sequence. Thus, a sixteen base sequence will statistically be present only once in every 4^{16} bases (=4 294 967 296, or 4 billion): this is about the size of the human or maize genome, and 1000x greater than the genome size of *E. coli*. Thus, the association of a greater-than-17-base oligonucleotide with its target sequence is an extremely sequence-specific process, far more so than the specificity of monoclonal antibodies in binding to specific antigenic determinants. Consequently, 17-mer or longer primers are routinely used for amplification from genomic DNA of animals and plants. Too long a primer length may mean that even high annealing temperatures are not enough to prevent mismatch pairing and non-specific priming.

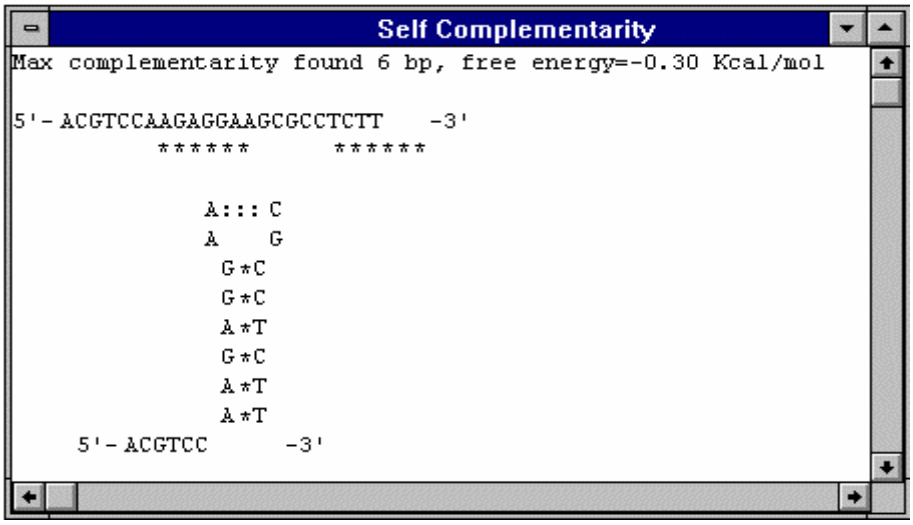
A simple set of rules for primer sequence design is as follows (adapted from Innis and Gelfand, 1991):

1. primers should be 17-28 bases in length;
2. base composition should be 50-60% (G+C);
3. primers should end (3') in a G or C, or CG or GC: this prevents "breathing" of ends and increases efficiency of priming;
4. Tms between 55-80^{o}C are preferred;
5. runs of three or more Cs or Gs at the 3'-ends of primers may promote mispriming at G or C-rich sequences (because of stability of annealing), and should be avoided;
6. 3'-ends of primers should not be complementary (ie. base pair), as otherwise primer dimers will be synthesised preferentially to any other product;

7. primer self-complementarity (ability to form 2^{o} structures such as hairpins) should be avoided.

Examples of inter- and intra-primer complementarity which would result in problems:





Screen shots taken from analyses done using DNAMAN (Lynnon Biosoft, Quebec, Canada; http://www.lynnon.com/).

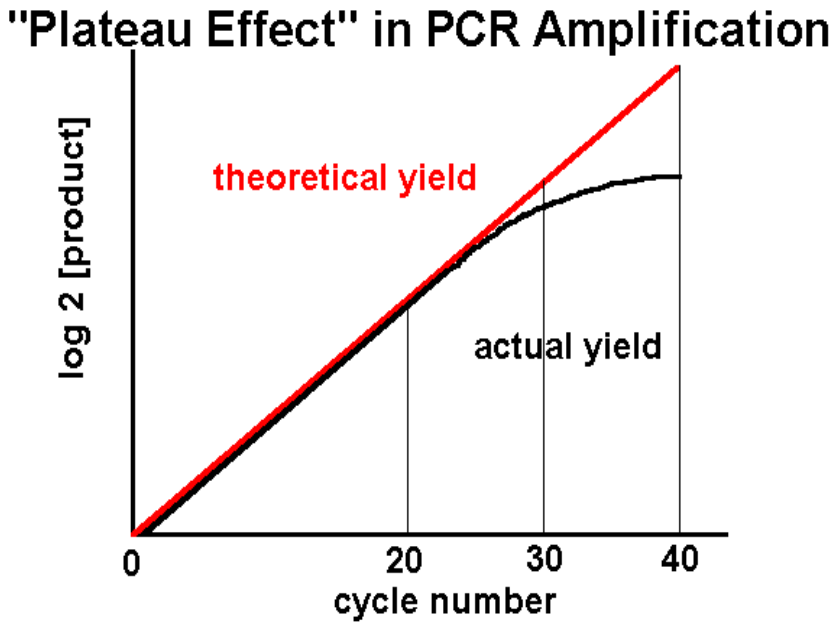
Elongation Temperature and Time

This is normally 70 – 72^{O}C, for 0.5 - 3 min. Taq actually has a specific activity at 37^{O}C which is very close to that of the Klenow fragment of *E coli* DNA polymerase I, which accounts for the apparent paradox which results when one tries to understand how primers which anneal at an optimum temperature can then be elongated at a considerably higher temperature - the answer is that elongation occurs from the moment of annealing, even if this is transient, which results in considerably greater stability. At around 70^{O}C the activity is optimal, and primer extension occurs at up to 100 bases/sec. About 1 min is sufficient for reliable amplification of 2kb sequences (Innis and Gelfand, 1990). Longer products require longer times: 3 min is a good bet for 3kb and longer products. Longer times may also be

helpful in later cycles when product concentration exceeds enzyme concentration (>1nM), and when dNTP and/or primer depletion may become limiting.

Cycle Number

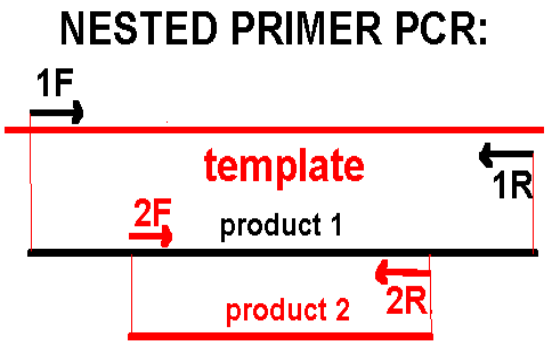
The number of amplification cycles necessary to produce a band visible on a gel depends largely on the starting concentration of the target DNA: Innis and Gelfand (1990) recommend from 40 - 45 cycles to amplify 50 target molecules, and 25 - 30 to amplify 3x105 molecules to the same concentration. This non-proportionality is due to a so-called *plateau effect*, which is the attenuation in the exponential rate of product accumulation in late stages of a PCR, when product reaches 0.3 - 1.0 nM. This may be caused by degradation of reactants (dNTPs, enzyme); reactant depletion (primers, dNTPs - former a problem with short products, latter for long products); end-product inhibition (pyrophosphate formation); competition for reactants by non-specific products; competition for primer binding by re-annealing of concentrated (10nM) product (Innis and Gelfand, 1990).

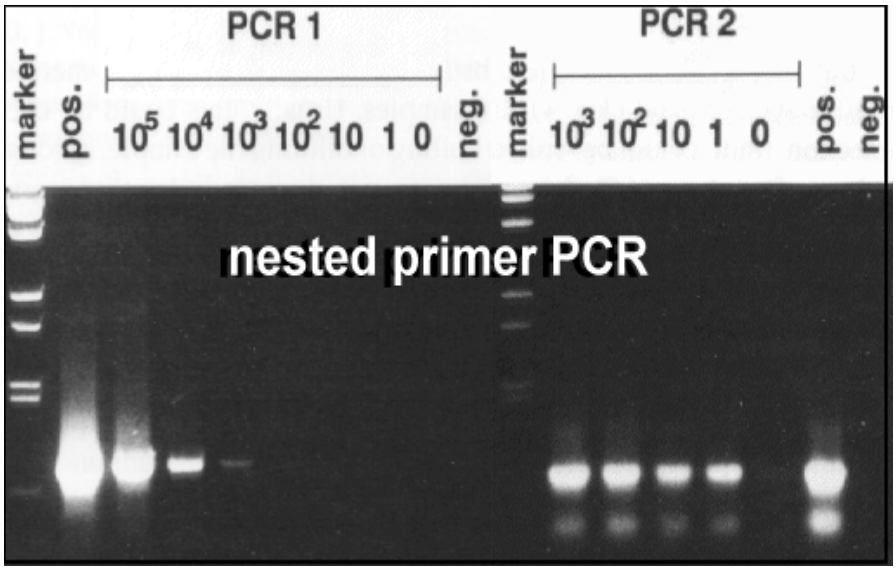


If desired product is not made in 30 cycles, take a small sample (1ul) of the amplified mix and re-amplify 20-30x in a new reaction mix rather than extending the run to more cycles: in some cases where template concentration is limiting, this can give good product where extension of cycling to 40x or more does not.

A variant of this is nested primer PCR: PCR amplification is performed with one set of primers, then some product is taken - with or without removal of reagents - for re-

amplification with an internally-situated, "nested" set of primers. This process adds another level of specificity, meaning that all products non-specifically amplified in the first round will not be amplified in the second. This is illustrated below:





This gel photo shows the effect of nested PCR amplification on the detectability of *Chicken anaemia virus* (CAV) DNA in a dilution series: the PCR1 just detects 1000 template molecules; PCR2 amplifies 1 template molecule (Soiné C, Watson SK, Rybicki EP, Lucio B, Nordgren RM, Parrish CR, Schat KA (1993) Avian Dis 37: 467-476).

Helix Destabilisers / Additives

With NAs of high (G+C) content, it may be necessary to use harsher denaturation conditions. For example, one may incorporate up to 10% (w or v/v) in the reaction mix

- dimethyl sulphoxide (DMSO),
- dimethyl formamide (DMF),
- urea

- or formamide

these additives are presumed to lower the Tm of the target NA, although DMSO at 10% and higher is known to decrease the activity of Taq by up to 50% (Innis and Gelfand, 1990; Gelfand and White, 1990).

Additives may also be necessary in the amplification of long target sequences: DMSO often helps in amplifying products of >1kb. Formamide can apparently dramatically improve the specificity of PCR (Sarkar *et al*., 1990), while glycerol improves the amplification of high (G+C) templates (Smith *et al*., 1990).

Polyethylene glycol (PEG) may be a useful additive when DNA template concentration is very low because it promotes macromolecular association by solvent exclusion, meaning the polymerase can find the DNA.

The complementary association of two strands of polynucleotides is the basis for replication of all organisms; the complexity inherent in the sequence of the molecules renders the association extremely specific for any molecule longer than sixteen nucleotides. This is easily understood if one considers the combinatorial possibilities of given lengths of "probe" sequence: there is a chance (4-1) of finding an A, G, C or T (U for RNA) in any given DNA sequence; there is a 1/16 chance (4-2) of finding any dinucleotide sequence (eg. AG); a 1/256 chance of finding a given 4-base sequence. Thus, a sixteen base sequence will statistically be present only once in every

416 bases (=4 294 967 296, or 4 billion):

this is about the size of the human genome, and 1000x greater than the genome size of *E. coli*.

Thus, the association of two nucleic acid molecules - presumed to be at least a few hundred bases long - is an extremely sequence-specific process, far more so than the widely-used specificity of monoclonal antibodies in binding to specific antigenic determinants. The correct annealing of two sequences to each other does, however, depend on the physical and chemical solution conditions under which the reaction takes place.

Melting Temperatures

For example, all double-stranded nucleic acids - whether dsDNA, dsRNA or RNA:DNA hybrids - have specific "melting temperatures", which depend mainly upon their specific guanine+cytosine content, but also upon whether they are DNA, RNA, or a mixture

(RNA:RNA hybrids have the highest melting temperatures, followed by DNA:RNA hybrids, then dsDNA), and upon the ionic strength of solution.

The melting temperature is also dependent upon the length of the sequences to be annealed: the shorter the probe sequence, the lower the melting temperature. The degree of sequence mismatch also determines the effective melting temperature of a hybrid: Tm decreases by about 1oC for every 1% of mismatched base pairs. It therefore makes sense to maximise probe length in order to minimise Tm reduction due both to length and degree of sequence mismatch. Under standard conditions of annealing (0.8M NaCl, neutral pH) one may calculate the melting temperature ™ of any given DNA hybrid as shown:

Tm = 81.5oC + 0.41(%G + %C) - 550/n

where n=probe length (no. nucleotides).

One can see that the reduction in Tm becomes negligible for probes of length 200 nt or greater. Thus, one may vary the specificity of association of a specific single-stranded "probe" and a target by varying the incubation temperature of the annealing reaction: the higher the temperature, the higher the specificity of the reaction - and the lower the likelihood of annealing taking place.

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