



Breaking PCR: A Systematic Investigation of Intentional Violations of a Basic Polymerase Chain Reaction Amplification Protocol

Contents

Introduction	1
Our Basic PCR Protocol	2
PCR Buffer Dilution	3
MgCl ₂ Concentration.....	4
dNTP Amount.....	5
Target DNA Amount.....	5
Polymerase Amount	6
Annealing Temperature	6
Polymerase Extension Step Duration	7
Discussion.....	9
References	9

Introduction

The polymerase chain reaction (PCR) is the backbone of modern molecular biology. Our experience at Integrated DNA Technologies shows that there are myriad ways of setting up and running PCR amplifications. Within limits, there are no reagents or parameters that cannot be altered to fit the needs of the individual researcher. This also has shown us that PCR is a very forgiving technique. Indeed, it is precisely this unique combination of specificity and flexibility that led to the emergence of PCR as a dominant laboratory technology.

We are aware at the same time that, since it was first developed in the early 1980s, many “myths” have grown up around the PCR technique. Given that there are so many ways to do PCR, it occurs to us that much of what we recognize as codified PCR “truths” may be not be true at all or they may have been rendered obsolete as a consequence of two decades of improvements in equipment and reagents. For this reason we set out to take a basic PCR protocol used in our research labs and to systematically violate both the reaction set up and the amplification conditions.

Here, we report on the results of this systematic attempt to “break” PCR. Much of what we found will come as no surprise but there were a couple of unanticipated results. We hope that you will find this information useful with respect to your own PCR work.

Our Basic PCR Protocol

As noted above, there are dozens of ways to set up and run a PCR amplification. Since we could not hope to address each and every nuance, we settled on a single PCR protocol that has been successfully used in our own research labs for many years. The reaction assembly is as follows;

In a 0.2 ml PCR tube add:

- 37.5 μ L water
- 5.0 μ L 10X PCR reaction buffer w/o MgCl₂
- 3.0 μ L MgCl₂
- 1.0 μ L dNTP mix
- 1.0 μ L forward primer
- 1.0 μ L reverse primer
- 1.0 μ L genomic DNA
- 0.5 μ L polymerase

The polymerase we use is Sigma (D-4545) Taq polymerase along with the 10X reaction buffer and MgCl₂ supplied by the vendor. Our dNTP mix is a 10mM mix also from Sigma (DNTP-10). Water is nuclease-free supplied by IDT. Genomic DNA is human extracted from peripheral blood using a Qiamp DNA Blood Maxi Kit (Qiagen) and standardized to a working stock concentration of 100 ng/ μ l.

For these experiments we chose two sets of PCR primers that would represent two ends of normal PCR amplicon size ranges. Our short amplicon primers (SAP) yield a 225 bp product against human genomic DNA while our long amplicon primers (LAP) yield a 1729 bp product against human genomic DNA. It is important to note here that our designation “long” is not to be confused with the Long PCR technique but, rather, it is intended simply to refer to a longer than average PCR product using the basic technique. The primer sequences and their melting temperatures (T_m) are:

SAP FOR: 5'-TTTGGTTGGTCTTCGTGCTGGG-3' (60.2°C)
SAP REV: 5'-TTTCACAGATGGCCTTGATGCTGC-3' (60.2°C)

LAP FOR: 5'-TTGGTGTGGGCAGAACCTCACAGCCGAG-3' (70.9°C)
LAP REV: 5'-CATGCAAACATCAGTGCATGACACCTAATTTATCAGG-3' (64.8°C)

These primer sets have previously been used by one of us (EJD) in other experiments. Each primer was selected using PrimerQuest and analyzed for secondary structures using OligoAnalyzer 3.0, both available on-line as part of our SciTools suite of software (see <http://www.idtdna.com/SciTools/SciTools.aspx>). New syntheses were ordered on the 25 nmole scale with standard desalting as the only purification measure. Upon receipt each primer was standardized to a 25 nM working stock concentration. Our PCR protocol thus uses 25 pmole of each primer in each amplification.

Finally, our starting PCR amplification conditions are,

95°C^{5:00} [95°C^{0:30}; T_A^{0:30}; 72°C^y]₃₅ 72°C^{7:00}

where the annealing temperature, T_A, was set at 58°C for the SAP primer set and 62°C for the LAP primer set and y is set at 0:30 for SAP amplicons and 2:00 for LAP amplicons. All amplifications were performed on an MJ PTC-200 thermal cycler. Once we verified that the conditions set out above reliably yielded the expected amplicons, we systematically varied the reaction conditions;

PCR buffer dilution, MgCl₂ concentration, dNTP amount, PCR primer Concentration, Target DNA amount, Polymerase amount, Annealing temperature, and Extension step duration

The results of an amplification using the standard protocol and PCR conditions are shown in Figure 1. As can be seen, the two amplicons are easily visualized and there are no artifacts in the amplification.

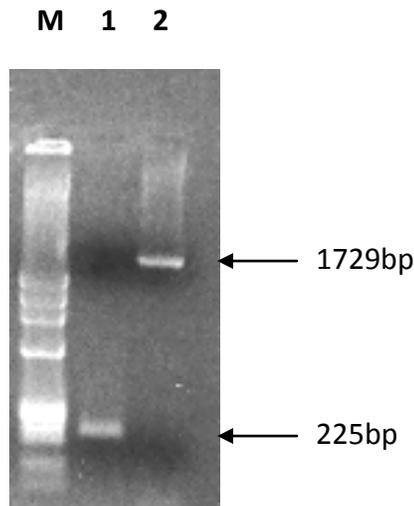


Fig. 1. PCR amplifications under default conditions.
Lane 1 = SAP, Lane 2 = LAP.

PCR Buffer Dilution

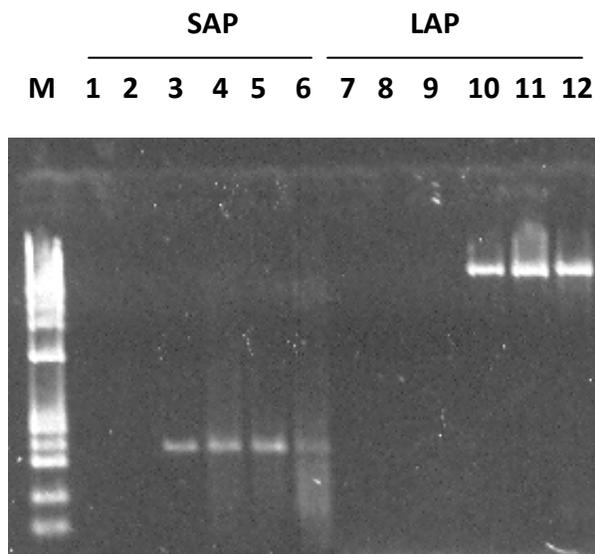
In all cases in which one of the reaction components is involved one default condition is the standard protocol and the other default condition is to leave that reagent out of the reaction completely. Not surprisingly, in no case does leaving out one of the reaction components result in any kind of amplification. Thus, discussions about reaction components use the two defaults as given starting points. Note as well that whenever a reaction component is altered, any required change in volume is adjusted by altering the amount of water to compensate.

PCR reaction buffer is supplied as a 10X concentrate. We varied the final buffer concentration over a range of 0.1X, 0.5X, 1X, 1.5X, and 2X. In both sets of primers the reduction of PCR

reaction buffer below 1X seriously compromised amplicon production. At 0.1X there were no amplicons and at 0.5X amplicons were present but at a very low level. On the other hand, increasing PCR reaction buffer to 1.5X and 2X had no apparent effect whatsoever.

MgCl₂ Concentration

Magnesium chloride (MgCl₂) is a necessary co-factor for all Type II enzymes including restriction endonucleases and polymerases. The divalent cation Mg²⁺ binds to the enzyme inducing a conformational shift that puts the subunits in proper configuration to function. Presumably, a significant reduction in MgCl₂ concentration prevents a sufficient number of enzyme molecules from being in the correct conformation for an efficient amplification to occur. However, it is also known that too much MgCl₂ is equally deleterious in most cases. In the case of excess Mg²⁺ the usual effect is a substantial increase in secondary products produced by non-specific priming. Thus, we varied the MgCl₂ concentration over a range from 0.5 mM to 3.0 mM with 1.5 mM being the normal default final concentration. Results of varying MgCl₂ concentration are shown for both primer sets in Figure 2.



M=Marker, Lane 1,7: 0 MgCl₂, Lane 2, 8: 0.5mM MgCl₂, Lane 3, 9: 1.0mM MgCl₂, Lane 4,10: 1.5mM MgCl₂, Lane 5, 11: 2.0mM MgCl₂, Lane 6, 12: 3.0 mM MgCl₂.

Fig. 2. Effects of varying MgCl₂ concentration.

As can be seen, the amplifications were affected differently by changing the MgCl₂ concentration. The SAP primer set gave no result at 0.5mM MgCl₂ but did give satisfactory results between 1.0 mM and 2.0 mM concentrations. At 3.0 mM MgCl₂ the result shows the effect of non-specific priming. Conversely, the LAP amplification gave no results at both 0.5 mM and 1.0 mM concentrations but excellent results over the upper range of 1.5 mM to 3.0 mM MgCl₂.

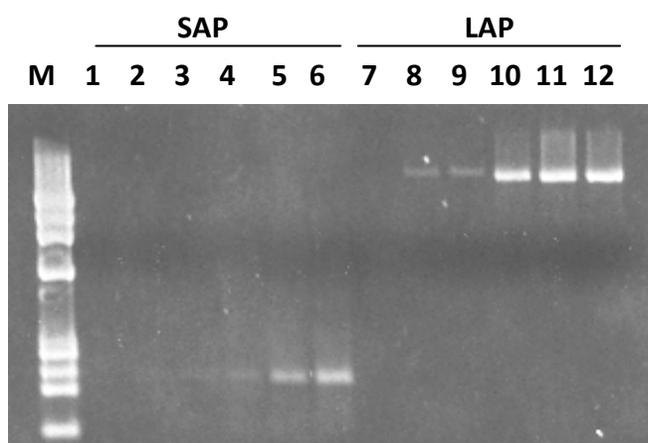
dNTP Amount

The standard concentration of our dNTP mix is 10 mM of each nucleotide triphosphate. Thus, in 1.0 μ L of mix we add 10 pmole of each dNTP. The range we chose to vary here was 0 pmole, 0.1 pmole, 1 pmole, 10 pmole, 100 pmole. Here, again, there was nothing surprising. The dearth of dNTPs at 0.1 pmole and at 1 pmole resulted in a proportional reduction in the amount of amplicon. The effect was more pronounced in the longer LAP amplicon as would be expected.

Another aspect of dNTP use that should be mentioned here is that, of all of the reagents used in a PCR reaction, dNTPs appear to be the most labile when subjected to repeated freezing and thawing. Frozen dNTPs are quite stable and have a fairly long shelf life but repeated freezing and thawing significantly shortens that shelf life. For this reason, we routinely aliquot dNTPs and only thaw what we need for any particular reaction set up. Obviously, this is good advice for all reagents but it is particularly important with dNTPs.

Target DNA Amount

Beginning with the 100 ng/ μ L stock concentration of genomic DNA we covered the range of 0 ng, 0.1 ng, 1 ng, 10 ng, 100 ng, 1000 ng (1 μ g). The obvious answer here is that more starting material yields more amplicon. Though we were unable to quantify this result, it appeared that there was an observable increase in amplicon density on the gel up to the 100 ng starting amount though the trend appeared to be diminishing returns above 10 ng (Figure 3). That is, there was not a proportionate advantage to increasing the starting material to 1 μ g over 100 ng and nowhere near a ten-fold advantage to 100 ng over 10 ng. This is consistent with the literature regarding the plateau phenomenon, observed in real-time PCR at the end of the log-linear amplification stage. The explanation for the plateau effect was recently demonstrated by Kainz who showed that it is, in fact, the production of the amplicons themselves that quench amplification efficiency by binding ever increasing amounts of polymerase [1].

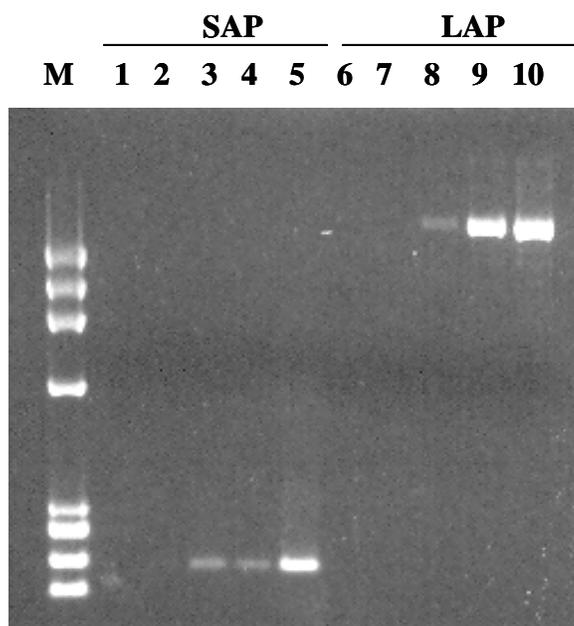


M=Marker, Lane 1,7: No Template, Lane 2, 8: 0.1ng., Lane 3, 9: 1.0ng, Lane 4,10: 10ng, Lane 5, 11: 100ng, Lane 6, 12: 1 μ g.

Fig. 3. Effect of varying template DNA amounts.

Polymerase Amount

Standard PCR protocols usually call for 2.5 units of polymerase per reaction. This is the standard we have used for some time. Here we covered a range of 0.1 U to 5.0 U. The results, shown below in Figure 4, indicate that there is a fairly proportionate effect on the LAP amplification and a more threshold-like effect on the SAP amplification. Using a very small amount of polymerase is not advised in the light of the Kainz results that suggest the polymerase is being bound by increasing numbers of amplicons [1]. The difference in the amplification patterns in the LAP and SAP amplifications are also consistent with those results if smaller amplicons are more efficient in binding polymerase molecules than are longer amplicons. However, to date, this has not been demonstrated to be the case.



**M=Marker, Lane 1,6: 0 polymerase,
Lane 2, 7: 0.1U polymerase., Lane 3, 8:
1.0U polymerase, Lane 4,9: 2.5U
polymerase, Lane 5, 10: 5U polymerase**

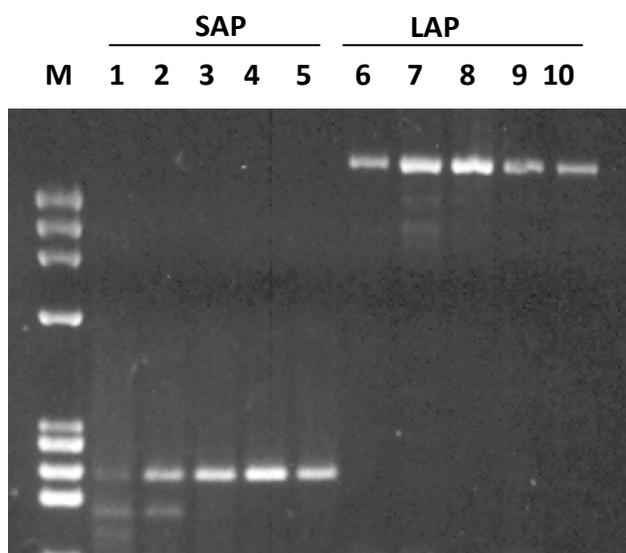
Fig. 4. Effect of varying polymerase amount.

Annealing Temperature

The temperature at which the primer annealing step is set is conventionally determined by the melting temperature (T_m) of the PCR primers. Since melting temperature is defined as the temperature at which half of the primers are bound to their complementary targets and half are dissociated, or melted off, it is the usual practice to set the annealing temperature of the PCR reaction (T_A) at a point a few degrees under the lower of the two melting temperatures of the primers in order to have as high a proportion of the primers bound to available targets as possible without sacrificing specificity. This task is greatly aided by ensuring that the two PCR

primers have equal or nearly equal T_m s and that there are no stable uni-molecular or bimolecular secondary primer structures (i.e., hairpins or dimers) at the desired T_A .

Methods for calculating primer T_m range from the simple $C=G=4^\circ\text{C} + A=T=2^\circ\text{C}$ paper and pencil method to the most accurate estimates available (see OligoAnalyzer 3.0 in SciTools at www.idtdna.com) based upon base stacking and nearest neighbor and next-nearest neighbor interactions determined in the context of both mono-valent and divalent ions [2, 3, 4, 5]. However, T_M estimates, no matter how accurate they are, are estimates of the behavior of a PCR primer in solution. Results of varying T_A in our PCR amplifications over a 20°C range starting at our defaults of 58°C for SAP and 62°C for LAP show that PCR primers can be extremely robust over a wide range of annealing temperatures (Figure 5).



M=Marker, Lane 1,6: $T_A-10^\circ\text{C}$, Lane 2, 7: $T_A-5^\circ\text{C}$.,
Lane 3, 8: T_A , Lane 4,9: $T_A+5^\circ\text{C}$, Lane 5,
10: $T_A+10^\circ\text{C}$

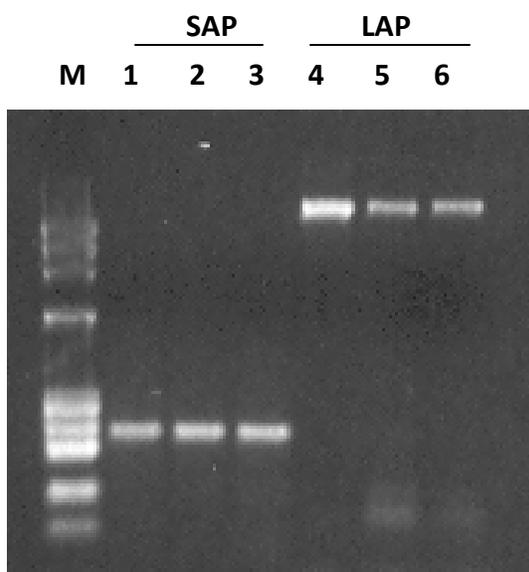
Fig. 5. Effect of varying primer annealing temperature.

Note that for both primer sets setting the primer annealing temperature too low does result in the presence of secondary bands formed by sequence mismatch tolerance (the secondary bands in Lane 6 are very faint as is the product band. This could easily be due to pipetting error in adding the template DNA). Note, too, that T_A appears to be the best choice for the LAP primer set but that $T_A + 5^\circ\text{C}$ seems to be better for the SAP primer set as judged by the intensity of the PCR products bands and the absence of any secondary bands.

Polymerase Extension Step Duration

This was the most surprising result we obtained in this study. Traditionally, polymerase extension times have been determined by estimates of enzyme processivity. That is, how fast does the polymerase make template copies. In nature, polymerase processivity is very fast, as

high as 1000 bases per second in *E. coli* [6]. The standard estimate for Taq polymerase *in vitro* (*Thermus aquaticus*) is 61 bases/ second and for Pfu polymerase (*Pyrococcus furiosus*) is 25 bases/second. From this, the extension step duration in PCR reactions using Taq polymerase is set at 30 seconds for each 500 bases in the amplicon. Thus, the default SAP extension step duration was fixed at 30 seconds (225 bp amplicon) and the default LAP extension step duration was fixed at 120 seconds (1729 bp amplicon). It is already well known that setting extension step times too long reduces reaction efficiency by depleting dNTPs in overly long anchored products. We were interested in observing the effects of extension step times that were too short. Thus, we set times of 30 sec, 20 sec, 10 sec, and 5 sec for SAP amplifications and 120 sec, 90 sec, 60 se, and 30 sec for LAP amplifications. The results we observed, particularly for the LAP amplifications were unexpected (Figure 6). In fact, the gel shown in Figure 5 is a second, independent LAP amplification because we frankly did not believe the first results.



M=Marker, Lane 1: 30 seconds, Lane 2: 20 seconds, Lane 3: 10 seconds, Lane 4: 120 seconds, Lane 5: 60 seconds, Lane 6: 30 seconds.

Fig. 6. Effect of varying polymerase extension step duration.

Clearly, decreasing the duration of the polymerase extension step has very little effect under the conditions of these reactions. The explanation for this finding is that a DNA polymerase will bind to a double-stranded primer::template duplex as soon as it forms and will begin to extend. The 72°C polymerase extension step is meant to ensure that the polymerase has adequate time to finish its task. It is not the case that the polymerase will only begin to extend at 72°C. At the T_A , the enzyme may be a bit slower but it is not inert! While a DNA polymerase *in vitro* does not have the benefit of the clamp and clamp-loader proteins that are present *in vivo*, these enzymes are quite efficient at carrying out their assigned task [6]. That seems to be particularly true for the very short stretches of DNA that are the usual subject of PCR amplifications.

Discussion

Overall, we have to conclude that the polymerase chain reaction is an even more forgiving technique than we first assumed. The nature of some of the robust results we obtained, particularly for MgCl₂ concentration, primer annealing temperatures, and polymerase extension step duration, suggest that an investment in systematic optimization of PCR reactions can save a great deal of trouble in the long run. The four aspects of the PCR amplification that should be the first line candidates for optimization are; 1. primer annealing temperature, 2. MgCl₂ concentration, 3. polymerase extension time, and 4. target DNA amount.

One additional experimental practice that should be adopted by any lab carrying out PCR reactions is the inclusion of both a negative (no template) control and a positive control. The choice of a positive control should incorporate a pair of very robust primers in which the investigator has great confidence. A negative control guards against reagent contamination. However, if a PCR amplification fails completely to produce a product, a negative control tells you nothing since it was not supposed to work in the first place. On the other hand, a positive control can alert the investigator to whether the failure is design-related or reagent-related. A design-related failure will not affect the positive control whereas a reagent-related failure will.

Of course, one issue that we have yet to raise is the most obvious of all. The proper design of your primers is critical to the success of your experiment. If the primers are poorly designed, all of the optimization in the world will have no effect on outcome. Second only to this is the quality of the primer synthesis. A standard IDT 24-mer PCR primer will be greater than 90% full-length using our phosphoramidite synthesis chemistries. This means that the vast majority of primer annealing by mass will be with the full-length complementary target sequence. Moreover, the process of capping base-coupling failures, which removes that sequence from participating in any further couplings, effectively “kills” base drop outs. Studies of chemical synthesis of PCR primers have shown that more than one-half of the coupling failures occur within the first six bases of the 3' end of the sequence and that almost none occur near the 5' end [7, 8]. Capped sequences that are less than half full length will never be able to anneal under normal PCR conditions because they are too short and, consequently, have T_Ms that are too low to form a stable duplex. Thus, most of the non-full-length mass of a primer synthesis will be “invisible” to the PCR process. Since PCR primer synthesis proceeds in the 3' to 5' direction, this makes the “effective full-length” proportionate mass greater than 97%.

At IDT we have made state-of-the-art primer design and analysis software available on-line to aid in primer selection. IDT also provides the highest quality primer synthesis available anywhere.

References

1. Kainz P. (2000) The PCR plateau phase- towards an understanding of its limitations. *Biochim Biophys Acta*, 1494: 23–27.

2. Moreira BG, You Y, et al. (2005) Effects of fluorescent dyes, quenchers, and dangling ends on DNA duplex stability. *Biochem Biophys Res Comm*, 327: 473–484.
3. Owczarzy R, Vallone PM, et al. (1997) Predicting sequence-dependent melting stability of short duplex DNA oligomers. *Biopolymers*, 44: 217–239.
4. Owczarzy R, Dunietz I, et al. (2003) Thermodynamic treatment of oligonucleotide duplex-simplex equilibria. *Proceedings of the National Academy of Science USA*, 100: 14840–14845.
5. Owczarzy R, You Y, et al. (2004) Effects of Sodium Ions on DNA Duplex Oligomers: Improved Predictions of Melting Temperatures. *Biochemistry*, 43: 3537–3554.
6. Bowman GD, O'Donnell M, and Kuriyan J. (2004) Structural analysis of a eukaryotic sliding DNA clamp-clamp loader complex. *Nature*, 429: 724–730.
7. Hecker KH, and Rill RL. (1998) Error analysis of chemically synthesized polynucleotides. *BioTechniques*, 24: 256–260.
8. Temsamani J, Kubert M, and Agarwal S. (1995) Sequence identity of the n-1 product of a synthetic oligonucleotide. *Nucleic Acids Res*, 23: 1841–1844.