



# Benchtopics™ in Amplification

A forum for research applications

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## The Basics of PCR

The Polymerase Chain Reaction (PCR) process uses multiple cycles of template denaturation, primer annealing, and primer elongation to amplify DNA sequences (1). Since amplified products from the previous cycle serve as templates for the next cycle of amplification, PCR is an exponential process and a highly sensitive technique for nucleic acid detection. Typically, enough amplified product is generated after 20 to 30 cycles of PCR so that it can be visualized on an ethidium bromide-stained gel.

Several reaction components play a role in PCR (Table 1). The template can include purified genomic or plasmid DNA, RNA converted to cDNA, or unpurified, crude biological samples such as bacterial colonies or phage plaques. The primers determine the sequence and the length of the amplified product. The most frequently used thermostable polymerase is *Taq* DNA Polymerase<sup>1,2</sup>. This enzyme is appropriate for routine applications, but the use of other thermostable polymerases can enhance results. Amplification reactions also contain buffer, deoxynucleotide triphosphates, and magnesium. The magnesium ion concentration affects enzyme activity, primer

**Table 1** – Reaction components

| Component                   | Final Concentration                                     |
|-----------------------------|---|
| Template                    | 10 <sup>4</sup> –10 <sup>6</sup> copies of DNA template |
| Primer 1                    | 0.1–0.5 μM  |
| Primer 2                    | 0.1–0.5 μM  |
| 10X Reaction buffer         | 1X  |
| Magnesium                   | 1.0–3.0 mM  |
| dNTP mix                    | 200 μM each dNTP  |
| Thermostable DNA polymerase | 1–4 units/100 μl reaction                               |

annealing, melting temperature of the template and the PCR product, fidelity, and primer-dimer formation (2). This edition of *Benchtopics in Amplification* discusses how the interaction of reaction components, cycling parameters, as well as other factors contribute to successful PCR.

### References:

1. Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., and Arnheim, N. (1985) *Science* **230**: 1350.
2. Innis, M.A., Gelfand, D.H., Sinsky, J.J., and White, T.J. (1990) *PCR Protocols, A Guide to Methods and Applications*. Academic Press, San Diego, California.

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# Improving PCR specificity

## Primer Design

Careful primer design is one of the most important aspects of PCR. The ideal primer pair anneals to unique sequences that flank the target and not to other sequences in the sample. Poorly designed primers may amplify other, non-target sequences. The following guidelines describe the desirable characteristics of a primer sequence that increase specificity:

- Typical primers are 18 to 24 nucleotides in length. The primer needs to be long enough for the sequence to be unique and to reduce the probability of the sequence being found at non-target sites. However, primers greater than 24 nucleotides do not confer greater specificity. Longer sequences can hybridize with some mismatching, which decreases specificity, and hybridize more slowly than shorter sequences, which may decrease yield (1).
- Select primers that are 40% to 60% GC or mirror the GC content of the template.
- Design primers with G or C residues in the 5' and central regions. This increases the primer's stability and confers hybridization stability with the target sequence.
- Avoid complementary sequences at the 3' end of primer pairs. This prevents amplification from the primers themselves to form primer-dimers.
- Avoid a GC-rich 3' end. Design primers to contain 3 As or Ts within the last 5 nucleotides (2).

**Table 2** – Formulas to estimate  $T_m$

**Simple Formula (6)** (valid for primers <18 bases)  $T_m = 4^\circ\text{C} \times (\text{G} + \text{C}) + 2^\circ\text{C} \times (\text{A} + \text{T})$

**$T_m$  for Oligonucleotides (7)** (dependent on salt concentration)

$$T_m = 81.5 + 16.6 \times (\log_{10}[\text{Na}^+]) + 0.41 \times (\% \text{G} + \text{C}) - 675/n$$

Where  $n$  = number of bases and  $[\text{Na}^+]$  = monovalent ( $\text{Na}^+$  or  $\text{K}^+$ ) cations

- Avoid mismatches at the 3' end. The last 3' nucleotide needs to anneal to the template for the polymerase to catalyze extension (3).
- Avoid sequences with the potential to form internal secondary structure. This destabilizes primer annealing.

Additional sequences that are not present on the target, such as restriction sites and promoter sequences, can be added to the 5' end of a primer without affecting specificity. These sequences are not included when estimating the  $T_m$  of a primer. However, check these regions for complementarity and internal secondary structures.

### Degenerate Primers

Sometimes only limited sequence information is available for primer design. For example, if only the amino acid sequence is known, degenerate primers are designed. Degenerate primers are a mix of different sequences representing all possible bases coding for a single amino acid. To improve specificity, minimize the degeneracy by consulting codon usage tables for base preference in different organisms (1). In addition, deoxyinosine may be used in places where more than one base is possible. Inosine pairs with all bases and will lower the annealing temperature of the primer. Do not include degenerate bases at the 3' end of the primer, since annealing of the last three bases on the 3' end can be enough to ini-

tiate PCR at the wrong sites. Use higher primer concentrations (1  $\mu\text{M}$  to 3  $\mu\text{M}$ ), because many of the primers in the degenerate mixture are not specific to the target (4).

### Primer Annealing Temperature

Another important parameter for primers is the melting temperature ( $T_m$ ). This is the temperature at which 50% of the primer and its complementary sequence are present in a duplex DNA molecule. The  $T_m$  is necessary to establish an annealing temperature for PCR. Ideally, the annealing temperature is low enough to guarantee efficient annealing of the primer to the target, but high enough to minimize nonspecific binding. Reasonable annealing temperatures range from 55°C to 70°C. Annealing temperatures are generally set about 5°C below the  $T_m$  of the primers.

There are several formulas for estimating the  $T_m$  (5-7). Table 2 lists two of the commonly used formulas for determining a primer's  $T_m$ . The first formula was derived for hybridization in high salt (1 M) and is valid for primers <18 bases. The second formula estimates  $T_m$  based on GC content and salt concentration. The most reliable method for determining the  $T_m$  of a primer is nearest-neighbor analysis (5). This method predicts the hybridization stability of a primer from the primary sequence and the identity of the neighboring bases. Most software applications use nearest-neighbor analysis.

*continued on page 3*

## Improving PCR specificity *continued from page 2*

The  $T_m$  can vary significantly depending on the formula used and the primer sequence. Since most formulas provide an estimated  $T_m$  value, the annealing temperature is only a starting point. Analyzing several reactions with increasingly higher annealing temperatures can increase specificity. Begin at 5°C below the estimated  $T_m$  and increase the annealing temperature in 2°C increments. Higher annealing temperatures can reduce the formation of both primer-dimer and nonspecific products. For best results, both primers need to have a similar  $T_m$ . Primer pairs whose  $T_m$  varies by more than 5°C exhibit greater mispriming due to the use of lower annealing temperatures during cycling. If two primers have different  $T_m$  values, set the annealing temperature 5°C below the lowest  $T_m$ . To increase specificity, perform 5 cycles at the annealing temperature established by the primer with the higher  $T_m$  and then the remaining cycles at the annealing temperature established by lower  $T_m$ . This strategy allows some copies of the target to be generated under more stringent conditions.

### Touchdown PCR

Touchdown PCR increases sensitivity by using stringent annealing conditions during the early cycles of PCR (8). Cycling begins with annealing temperature approximately 5°C above the estimated  $T_m$ . It is incrementally decreased by 1°C to 2°C every cycle until the annealing temperature is about 5°C below the  $T_m$ . Only targets with the greatest homology are amplified. These products continue to be amplified and compete out nonspecific products amplified in later cycles. Touchdown PCR is useful in applications where the degree of homology between the target sequences and the primer are unknown, such as in DNA fingerprinting or 5' RACE.

### Primer Concentration

Primer concentration can affect specificity. Optimal primer concentration typically falls between 0.1 to 0.5 µM. Higher concentrations of primer may result in the amplification of nonspecific products.

To determine primer concentration, read the optical density at 260 nm ( $OD_{260}$ ). Then, using Beers Law (Formula 1) calculate the concentration by using the absorbance and the reciprocal of the millimolar extinction coefficient (nmol/OD). The millimolar extinction coefficient can be calculated using Formula 2 (9). Unlike for large double-stranded DNA molecules, where an

averaged extinction coefficient can be used, the extinction coefficient calculated for the primer is essential to accurately determine the concentration (9). This is because primers are short and the base composition can vary greatly. Both the extinction coefficient (OD/µmol) and the reciprocal of the extinction coefficient (µmol/OD) are provided on the Invitrogen Custom Primer certificate of analysis. In addition, do not estimate primer concentration on ethidium bromide-stained gels using oligonucleotides as standards, since the staining capability of the standard and the primer can vary greatly depending on their sequence (10). *continued on page 4*

#### Formula 1

**Concentration** =  $A_{260} \times \text{dilution factor} \times \text{the reciprocal of the extinction coefficient} \times \text{conversion factors}$

**Example:** To calculate the concentration of an oligonucleotide in 1 ml, measure the  $A_{260}$  of 10 µl of the oligonucleotide in 990 µl of water (1:100 dilution). If the  $A_{260}$  = 0.14 OD and the oligonucleotide has a reciprocal extinction coefficient of 4.9 nmol/OD, the concentration would be calculated as follows:

$$\begin{aligned} \text{Concentration} &= \frac{0.14 \text{ OD}}{\text{ml}} \times 100 \times \frac{4.9 \text{ nmol}}{\text{OD}} \times \frac{1 \text{ } \mu\text{mol}}{10^3 \text{ nmol}} \times \frac{10^3 \text{ ml}}{\text{L}} \\ &= 69 \text{ } \mu\text{M} \end{aligned}$$

#### Formula 2

**Millimolar Extinction Coefficient of Oligonucleotide =**

$$A(15.2) + C(7.05) + G(12.01) + T(8.4) \text{ at pH } 8.0$$

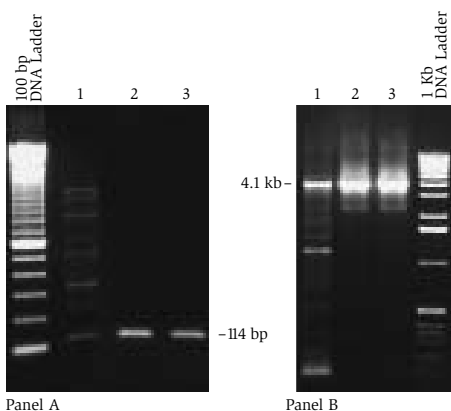
Where A, C, G, and T are the number of dAs, dCs, dGs, and dTs (2).

## Improving PCR specificity *continued from page 3*

### Hot Start

In addition to good primer design, hot-start PCR is one of the most important methods used to increase PCR specificity. Even though the optimal extension temperature for *Taq* DNA Polymerase is approximately 72°C, the polymerase has activity at room temperature (11). As a result, nonspecific products are often generated during PCR setup as well as at the start of thermal cycling, when reactions are briefly incubated at temperatures well below the annealing temperature (12,13). Once these nonspecific products are formed they can be efficiently amplified. Hot-start PCR is particularly effective when the sites available for primer design are limited due to the location of genetic elements, such as in site-directed mutagenesis, expression cloning, or the construction and manipulation of genetic elements for DNA engineering.

**Figure 1** – Improved specificity with Platinum® *Taq* DNA Polymerase



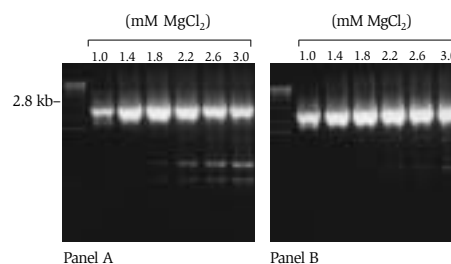
Panel A. Detection of cloned HIV DNA in human genomic DNA. 1,000 copies of plasmid DNA with the HIV gag region was mixed with 100 ng of genomic DNA and amplified with primers to the gag region. Lane 1. *Taq* DNA Polymerase with room temperature assembly. Lane 2. *Taq* DNA Polymerase with manual hot start by addition of enzyme at 94°C. Lane 3. Platinum® *Taq* DNA Polymerase with room temperature assembly. Panel B. Amplification of 4.1 kb of human  $\beta$ -globin from 100 ng of human genomic DNA. Lane 1. *Taq* DNA Polymerase with room temperature assembly. Lane 2. *Taq* DNA Polymerase with assembly on ice and placed in a preheated (80°C) thermal cycler. Lane 3. Platinum® *Taq* DNA Polymerase with room temperature assembly.

A popular method that limits *Taq* DNA Polymerase activity is to set up amplification reactions on ice and place them into a pre-heated thermal cycler. This method is simple and inexpensive, but does not completely inactivate enzyme activity and therefore does not completely eliminate the amplification of nonspecific products.

Hot start delays DNA synthesis by withholding one of the essential components until the thermal cycler reaches the denaturation temperature. Most manual hot-start methods involve the delayed addition of *Taq* DNA Polymerase, which can be cumbersome, especially for high-throughput applications (13). Other hot-start methods use wax barriers to encapsulate an essential component, such as magnesium or enzyme, or to physically separate reaction components, such as template and buffer, from each other. Melting of the wax during thermal cycling releases and mixes all of the components together. Like manual hot-start methods, wax barrier methods can be cumbersome, prone to contamination, and unreliable in high-throughput applications.

Platinum® DNA Polymerases are convenient and highly effective for automatic hot-start PCR (Figure 1). Platinum® *Taq* DNA Polymerase<sup>1,2,14,37</sup> is comprised of recombinant *Taq* DNA Polymerase complexed with monoclonal antibodies to *Taq* DNA Polymerase (14). The antibodies prevent enzyme activity during PCR setup and even during prolonged incubations at room temperature. *Taq* DNA Polymerase is released into the reaction after incubation at 94°C during the denaturation step, restoring full polymerase activity. In comparison to chemically modified *Taq* DNA Polymerase for hot-start PCR, Platinum® enzymes do not require prolonged incubation at 94°C (10 to 15 min)

**Figure 2** – Broader magnesium range with Platinum® *Taq* DNA Polymerase



A 2.8-kb region of the human  $\beta$ -globin gene was amplified from 100 ng of human genomic DNA. Panel A. *Taq* DNA Polymerase with assembly on ice and placed in a preheated (80°C) thermal cycler. Panel B. Platinum® *Taq* DNA Polymerase with room temperature assembly.

to activate the polymerase. More than 90% of *Taq* DNA Polymerase activity is restored in 2 min at 94°C with Platinum® *Taq* DNA Polymerase (15).

### Magnesium Concentration

Magnesium affects several aspects of PCR including DNA polymerase activity, which can affect yield, and primer annealing, which can affect specificity. The dNTPs and template bind magnesium and reduce the amount of free magnesium available for enzyme activity. The optimal magnesium concentration varies for each primer pair and template, but the starting concentration in typical PCR containing 200  $\mu$ M dNTPs is 1.5 mM (Note: for real-time quantitative PCR use 3 to 5 mM magnesium with fluorescent probes) (1). Higher concentrations of free magnesium can result in greater yield, but can also increase nonspecific amplification and reduce fidelity (16,17). To determine the best concentration, perform a magnesium titration using 1 mM to 3 mM in 0.5 mM increments. To reduce the need for magnesium optimization, use Platinum® *Taq* DNA Polymerase. Less optimization is required using Platinum® *Taq* DNA Polymerase because it functions over

*continued on page 5*

## Improving PCR specificity continued from page 4

a broader range of magnesium concentration than *Taq* DNA Polymerase (Figure 2) (14).

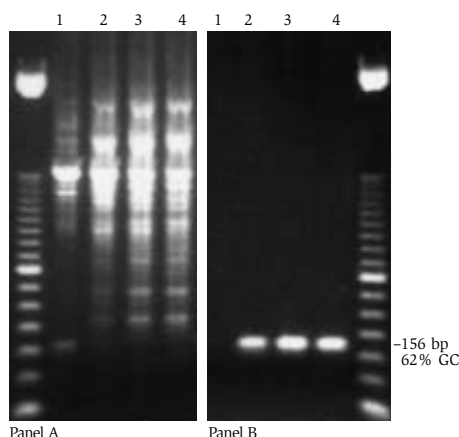
### Additives to Enhance PCR

Optimization of the annealing temperature, primer design, and magnesium concentrations is adequate to achieve high-specificity amplification of many targets. However, some targets, including those with high GC content, require additional measures. Additives that affect DNA melting temperature provide another method for improving product specificity and yield (Figure 3). Complete denaturation of the template is required to obtain the best results. In addition, secondary structure can prevent primer binding and enzymatic elongation. PCR additives, including formamide, DMSO, glycerol, betaine, and PCR<sub>x</sub> Enhancer Solution<sup>4,12</sup> can enhance amplification (18-20). Their proposed mechanism is to lower the melting temperature, thereby aiding primer annealing and helping the DNA polymerase extend through regions of secondary structure (20). PCR<sub>x</sub> Enhancer Solution has additional benefits. It requires less magnesium optimization and is compatible with Platinum<sup>®</sup> *Taq* DNA Polymerase (Figure 3) and Platinum<sup>®</sup> *Pfx* DNA Polymerase<sup>1,2,14,37</sup>. Combining Platinum<sup>®</sup> technology with additives enhances specificity while minimizing the need for the third approach, magnesium optimization. For best results, optimize the concentration of the additives (Figure 4), especially DMSO, formamide, and glycerol that inhibit *Taq* DNA Polymerase (13,19).

### Nested PCR

Sequential rounds of amplification using nested primers can improve specificity and sensitivity (21). The first round is a standard amplification of

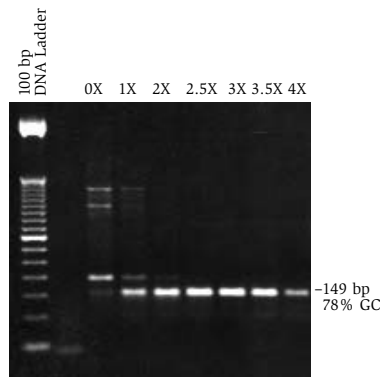
**Figure 3** – Improved specificity with PCR<sub>x</sub> Enhancer Solution



A 156-bp fragment (62% GC content), was amplified from 100 ng of human genomic DNA using Platinum<sup>®</sup> *Taq* DNA Polymerase. MgCl<sub>2</sub> was titrated (1.0, 1.5, 2.0, 2.5 mM, lanes 1 to 4 respectively) using standard PCR buffer (panel A) or PCR<sub>x</sub> Amplification Buffer with 1X PCR<sub>x</sub> Enhancer Solution (panel B). Cycling was 35 cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 1 min.

15 to 20 cycles. A small aliquot of the initial amplification is diluted 1:100 to 1:1,000 and added to the second round of amplification with 15 to 20 cycles. Alternatively, the initial amplification product can be size selected by gel purification. Two different primer sets are used for the two rounds of amplification. The second amplification uses a nested set of primers that binds to the target just inside the first set of primers. With nested PCR, the chance of amplifying multiple targets is reduced since fewer targets will be complementary to both sets of primers. In contrast, performing the same total number of cycles (30 to 40) with the same set of primers often amplifies nonspecific targets. Nested PCR can increase the sensitivity from limited amounts of target, such as amplification of a rare message, and increase the specificity of more challenging PCR applications such as 5' RACE.

**Figure 4** – Titration of PCR<sub>x</sub> Enhancer Solution



A 149-bp (78% GC) trinucleotide repeat-containing sequence was amplified from 100 ng of human genomic DNA with Platinum<sup>®</sup> *Taq* DNA Polymerase in 1X PCR<sub>x</sub> Amplification Buffer with varying amounts of PCR<sub>x</sub> Enhancer Solution (0 to 4X).

### References:

- Innis, M.A., Gelfand, D.H., Sinsky, J.J., and White, T.J. (1990) *PCR Protocols, A Guide to Methods and Applications*. Academic Press, San Diego, California.
- Lear, W., McDonel, M., Kashyap, S., and Boer, P. (1995) *BioTechniques* **18**: 84.
- Kwok, S., Kellogg, D.E., McKinney, N., Spasic, D., Goda, L., Levenson, C., and Sinsky, J. (1990) *Nucleic Acids Res.* **18**: 999.
- White, B.A. (1993) *PCR Protocols, Current Methods and Applications*. Humana Press, Totowa, New Jersey.
- Breslauer, K.J., Frank, R., Blocker, H., and Marky, L.A. (1986) *Proc. Natl. Acad. Sci. USA* **83**: 3746.
- Nelson, T. and Brutlag, D. (1979) *Methods Enzymol.* **68**: 41.
- Rychlik, W., Spencer, W.J., and Rhoads, R.E. (1990) *Nucleic Acids Res.* **18**: 6409.
- Don, R.H., Cox, P.T., Wainwright, B.J., Baker, K., and Mattick, J.S. (1991) *Nucleic Acids Res.* **19**: 4008.
- Fox, D.K. (1998) *Focus* **20**: 84.
- Sewall, A., Natarajan, P., and Fox, D.K. (1999) *Focus* **21**: 2.
- Li, H., Cui, X., and Arnheim, N. (1990) *Proc. Natl. Acad. Sci. USA* **87**: 4580.
- Chou, Q., Russell, M., Birch, D., Raymond, J., and Bloch, W. (1992) *Nucleic Acids Res.* **20**: 1717.
- Erllich, H.A. (1989) *PCR Technology, Principles and Applications for DNA Amplification*. Stockton Press, New York, New York.
- Westfall, B., Sitaraman, K., Solus, J., Hughes, J., and Rashtchian, A. (1997) *Focus* **19**: 46.
- Westfall, B., Darfler, M., Xu, R., and Rashtchian, A. (1998) *Focus* **20**: 17.
- Eckert, K.A. and Kunkel, T.A. (1990) *Nucleic Acids Res.* **18**: 3739.
- Williams, J.F. (1989) *BioTechniques* **7**: 762.
- Pomp, D. and Medrano, J.F. (1991) *BioTechniques* **10**: 58.
- Varadaraj, K. and Skinner, D.M. (1994) *Gene* **140**: 1.
- Baskaran, N., Kandpal, R., Bhargava, A., Glynn, M., Bale, A., and Weissman, S. (1996) *Genome Res.* **6**: 633.
- Dieffenbach, C.W. and Dveksler, G.S. (1995) *PCR Primer: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

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# Increasing PCR sensitivity and improving fidelity

## Template Concentration

The amount of starting template is an important factor to obtaining good product yields. For most amplifications,  $10^4$  to  $10^6$  starting target molecules allows sufficient amplification to visualize the product on an ethidium bromide-stained gel (1). The optimal amount of template required depends on the size of the genome (Table 3) (2). For example, 100 ng to 1 µg of human genomic DNA, correlating to  $3 \times 10^4$  to  $3 \times 10^5$  molecules, is sufficient to detect a PCR product from a single-copy gene. For plasmid DNA, which is much smaller, the amount of DNA added to PCR is in the picogram range.

## Enzyme Choice

The choice of polymerase also affects yield (Table 4, page 10). Platinum® polymerases provide better yield than other polymerases because they prevent amplification of nonspecific product during PCR setup (Figure 5). For high-sensitivity PCR of long products (up to 12 kb), choose a Platinum® enzyme mix such as Platinum® *Taq* DNA Polymerase High Fidelity.<sup>1,2,14</sup> This enzyme combines the benefits of Platinum® technology with those of enzyme mixes (*Taq* DNA Polymerase mixed with a proofreading polymerase).

## Enzymes with Proofreading Capability

PCR applications involving cloning and sequence analysis, expression of PCR products, or site-directed mutagenesis require high-fidelity PCR. *Taq* DNA Polymerase is considered a low-fidelity polymerase since it lacks 3' → 5' exonuclease (proofreading) activity. The use of thermostable polymerases with 3' exonuclease activity improve fidelity (3,4). However, these polymerases can provide lower yields than *Taq* DNA Polymerase. Platinum® *Pfx* DNA

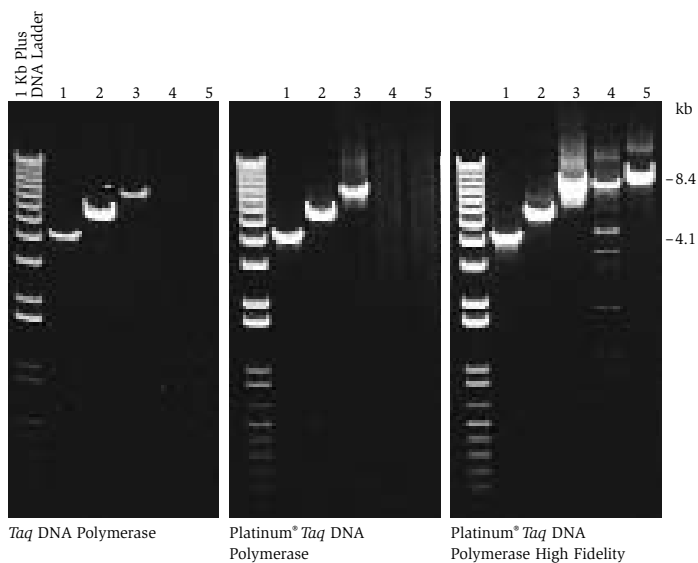
*continued on page 7*

**Table 3** – Correlation of genome size and number of molecules

| Genomic DNA                     | Size (bp)*           | Target Molecules/µg of Genomic DNA | Amount of DNA (µg) for ~10 <sup>5</sup> Molecules |
|---------------------------------|----------------------|------------------------------------|---|
| <i>E. coli</i>                  | $4.7 \times 10^6$    | $1.8 \times 10^8$                  | 0.001   |
| <i>Saccharomyces cerevisiae</i> | $2.0 \times 10^7$    | $4.5 \times 10^7$                  | 0.01  |
| <i>Arabidopsis thaliana</i>     | $7.0 \times 10^7$    | $1.3 \times 10^7$                  | 0.01  |
| <i>Drosophila melanogaster</i>  | $1.6 \times 10^8$    | $6.6 \times 10^5$                  | 0.5   |
| <i>Homo sapiens</i>             | $2.8 \times 10^9$    | $3.2 \times 10^5$                  | 1.0   |
| <i>Xenopus laevis</i>           | $2.9 \times 10^9$    | $3.1 \times 10^5$                  | 1.0   |
| <i>Mus musculus</i>             | $3.3 \times 10^9$    | $2.7 \times 10^5$                  | 1.0   |
| <i>Zea mays</i>                 | $1.5 \times 10^{10}$ | $6.0 \times 10^4$                  | 2.0   |
| pUC 18 plasmid DNA              | $2.69 \times 10^3$   | $3.4 \times 10^{11}$               | $1 \times 10^{-6}$                                |

\* Haploid genome size

**Figure 5** – Amplification with different thermostable polymerases



DNA was amplified directly in 50 µl using *Taq* DNA Polymerase (panel A), Platinum® *Taq* DNA Polymerase (panel B), and Platinum® *Taq* DNA Polymerase High Fidelity (panel C). Amplified products were 4.1, 5.2, 7.5, 8.0, and 8.4 kb in lanes 1 to 5, respectively.

## Increasing PCR sensitivity and improving fidelity *continued from page 6*

Polymerase has significantly better fidelity than *Taq* DNA Polymerase and offers the advantages of high yield and specificity featured in Platinum® products (Figure 6) (5).

### Enzyme Mixes

Mixing *Taq* DNA Polymerase with a second polymerase with 3' exonuclease activity provides greater fidelity than *Taq* DNA Polymerase alone and allows for higher yield and amplification of longer templates. The Invitrogen high fidelity enzyme mix, Platinum® *Taq* DNA Polymerase High Fidelity has 6 times greater fidelity than *Taq* DNA Polymerase alone and can amplify up to 12 kb.

### Other Parameters

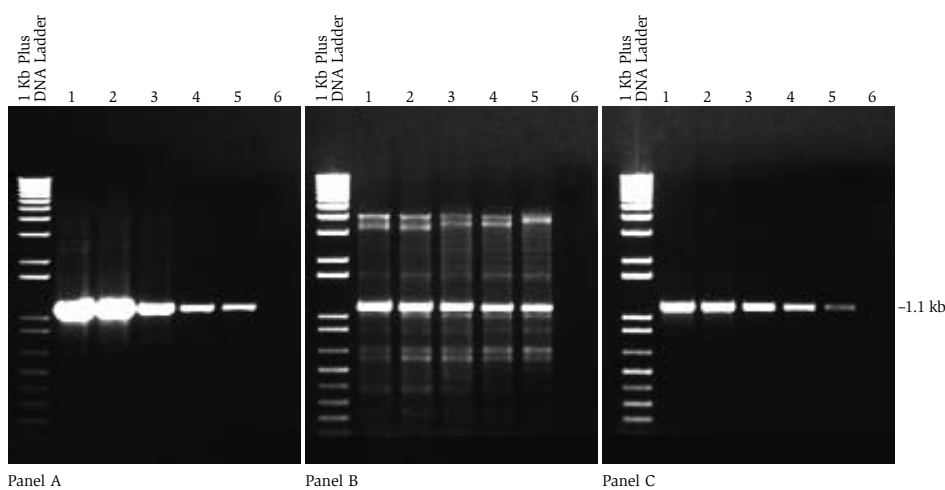
Besides enzyme, high dNTP or magnesium concentrations can reduce fidelity. Decreasing the concentration of dNTPs from 200 µM to 25-50 µM can increase accuracy. If the concentration is not the same for all four nucleotides, the fidelity will be affected. Performing fewer cycles of PCR also can help increase fidelity, since the probability of a mutation rises with increasing cycle number and product length.

### References:

1. Innis, M.A., Gelfand, D.H., Sinsky, J.J., and White, T.J. (1990) *PCR Protocols, A Guide to Methods and Applications*. Academic Press, San Diego, California.
2. Brown, T.A. (1991) *Molecular Biology Labfax*. Academic Press, Inc., San Diego, California.
3. Cline, J., Braman, J.C., and Hogrefe, H.H. (1996) *Nucleic Acids Res.* **24**: 3546.
4. Tindall, K.R. and Kunkel, T.A. (1988) *Biochemistry* **27**: 6008.
5. Westfall, B., Sitaraman, K., Lee, J.E., Borman, J., and Rashtchian, A. (1999) *Focus*® **21**: 46.

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**Figure 6** – High sensitivity and specificity with Platinum® *Pfx* DNA Polymerase



Genomic DNA (100, 50, 10, 5, 1, and 0 ng, lanes 1 to 6) was amplified with primers for a 1.1-kb fragment of human thrombospondin for 35 cycles. Panel A. 1 unit of Platinum® *Pfx* DNA Polymerase with room temperature setup. Panel B. 2.5 units of *PfuTurbo*® DNA Polymerase with setup on ice. Panel C. 1 unit of *Taq* DNA Polymerase with setup on ice.

# Long targets and multiplex PCR

## Amplifying Long Targets

When amplifying targets > 5 kb, use an enzyme or enzyme mix for long PCR to obtain the best yield (Table 4, page 10 and Figure 5, page 6). *Taq* DNA Polymerase does not efficiently amplify longer targets (> 5 kb), presumably because it lacks 3' → 5' exonuclease activity and cannot correct dNTP misincorporations (1). A mismatch greatly reduces the elongation rate, which decreases the yield of longer products. Mixing *Taq* DNA Polymerase with a thermostable DNA polymerase containing 3' → 5' exonuclease activity can allow amplification of targets up to 30 kb (2). Platinum® *Pfx* DNA Polymerase, which has 3' → 5' exonuclease activity, can also amplify longer products (< 12 kb).

In addition to choosing the correct thermostable polymerase, amplification of longer products requires changes to the extension times, denaturation times, and buffer pH of the standard protocol. Increase extension times to 1 min/kb to allow the polymerase to complete synthesis. Typically the extension temperature

is lowered to 68°C for more effective long PCR (1,2). Since the elongation times are long – up to 20 min for a 20-kb target – a buffer with a higher initial pH is used. If the pH falls below pH 7.0, the DNA can depurinate. To protect the template against damage, minimize the denaturation time at 94°C to 30 s or less during each cycle and limit the preamplification denaturation time to < 1 min at 94°C. Primers are designed in the same manner as those used in a standard protocol, with primers of 18 to 24 bases giving good product yields (1,2).

## Multiplex PCR

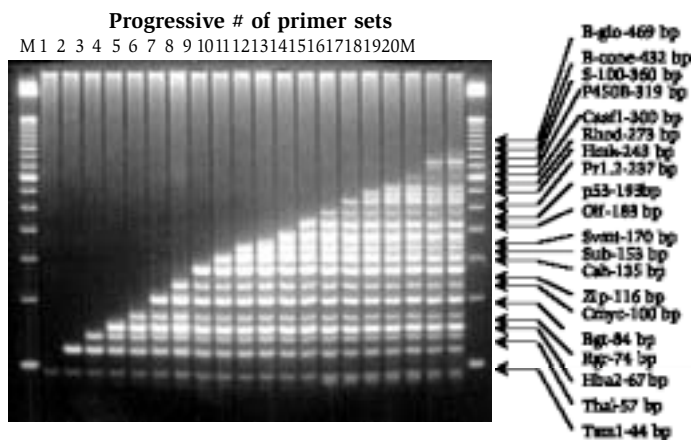
In multiplex PCR, multiple primer sets are used simultaneously to amplify several different loci. This complex PCR technique often results in low product yield and requires high concentrations of magnesium (3). A false negative result may be obtained if reactions are not optimized correctly. In multiplex PCR, AccuPrime™ *Taq* DNA Polymerase<sup>2,12,14,33</sup> delivers a single band for as many as 20 targets per reaction (Figure 7).

## Prevention of Carry-Over Contamination

PCR is susceptible to contamination problems because it is a sensitive amplification technique. Small amounts of contaminating DNA from an exogenous source can be amplified along with the desired template. A common source of contamination occurs when previously amplified products are introduced into new amplification reactions. This is called carry-over contamination. Purified DNA from other samples and cloned DNA can also be sources of contamination. Carry-over contamination can be minimized by using good laboratory procedures during PCR. Use aerosol-resistant tips to prevent aerosols from reaching the pipette barrel. Designate separate areas for PCR sample set-up and post-amplification analysis and change gloves before preparing new reactions. Always perform a negative control, without template, to check for contamination. Use premixed reaction components instead of adding each reagent to individual reactions.

One method to prevent carry-over contamination uses uracil DNA glycosylase (UDG) (4). This enzyme (also known as uracil-N-glycosylase or UNG) removes uracil residues from DNA. Substituting deoxyuracil for thymine in amplification reactions allows previously amplified products to be distinguished from template DNA. Since the previously amplified products are susceptible to UDG, newly assembled reactions are treated with UDG before PCR to destroy carry-over products.

**Figure 7** – Resolution of up to 20 specific PCR products from a single-tube multiplex reaction



Each lane from left to right represents the progressive number of primer sets (1-20) included in a single-tube, 50 µl PCR reaction. PCR reactions were assembled on ice, using 200 ng K562 human genomic DNA, and five units AccuPrime™ *Taq* DNA Polymerase, and amplified for 35 cycles (94°C for 15 seconds, 60°C for 30 seconds, 68°C for one minute).

## References:

1. Westfall, B., Sitaraman, K., Berninger, M., and Mertz, L. (1993) *Focus* 17: 62.
2. Cheng, S., Fockler, C., Barnes, W.M., and Higuchi, R. (1994) *Proc. Natl. Acad. Sci. USA* 91: 5695.
3. Chamberlain, J.S., Gibbs, R.A., Ranier, J.E., Nguyen, P.N., and Caskey, C.T. (1988) *Nucleic Acids Res.* 16: 11141.
4. Longo, M.C., Berninger, M.S., and Hartley, J.L. (1990) *Gene* 93: 125.

<sup>2,12,14,33</sup> Products mentioned above are subject to the Limited Use Label Licenses indicated by the superscript numbers. Please refer to the Invitrogen web site or catalog for the Limited Use Label Licenses corresponding to the numbers indicated.

## Troubleshooting guide

### PCR Sensitivity: Little or no PCR product visible after agarose gel analysis.

|                                    |   |
|------------------------------------|---|
| Poor PCR primer design             | Avoid complementary sequences at the 3' end of primers. Avoid sequences that can form internal hairpin structures. Design primers with similar $T_m$ s.   |
| DNA contains inhibitors            | Reagents such as DMSO, SDS, and formamide can inhibit <i>Taq</i> DNA Polymerase. If inhibitor contamination is suspected, ethanol precipitate the DNA sample.   |
| GC-rich template                   | For templates > 50% GC content, use PCR <sub>x</sub> Enhancer Solution.   |
| Template concentration is too low  | Start with $10^4$ copies of the target sequence to obtain a signal in 25 to 30 cycles.  |
| Magnesium concentration is too low | Determine the optimal magnesium concentration for each template and primer pair by performing a reaction series from 1 mM to 3 mM in 0.5 mM increments.<br><br>Note: Use 3 mM to 5 mM magnesium for real-time quantitative PCR. |

|                                   |   |
|-----------------------------------|---|
| Annealing temperature is too high | Use the equations listed in Table 2 (page 2) to estimate the $T_m$ and set the annealing temperature 5°C below the $T_m$ . Since these equations estimate $T_m$ values, the true annealing temperature may actually be higher or lower.   |
| Primer concentration is too low   | Optimal primer concentration is between 0.1 $\mu$ M to 0.5 $\mu$ M. To accurately determine primer concentration, read the optical density at 260 nm ( $OD_{260}$ ). Then, calculate the concentration using the absorbance and the extinction coefficient (See Primer Concentration Design, page 3). |

### PCR Specificity: Unexpected bands after agarose gel analysis

|  |  |
|--|--|
| Primer-dimer formation   | Design primers without complementary sequences at the 3' ends.   |
| Nonspecific annealing of primers to template                     | Increase the annealing temperature in 2°C to 5°C increments and minimize the annealing time.<br><br>Use higher annealing temperatures for the first few cycles, followed by lower annealing temperatures.<br><br>Use Platinum® <i>Taq</i> DNA Polymerase for automatic hot-start PCR.<br><br>Avoid 2 or 3 dGs or dCs at the 3' end of primers. |
| Magnesium concentration is too high                              | Optimize magnesium concentration for each template and primer combination.   |
| Primer mispriming due to amplification from complex templates.   | Use nested primers or touchdown PCR.   |
| Contaminating DNA from an exogenous source                       | Use aerosol-resistant tips and UDG (See Prevention of Carry-Over Contamination, page 8).   |
| Primer binding sites are inaccessible due to secondary structure | For templates > 50% GC content use (1X-3X) PCR <sub>x</sub> Enhancer Solution.   |

### PCR Fidelity: PCR induced errors found in the product sequence

|   |   |
|---|---|
| Polymerase has low fidelity                                 | Use a proofreading thermostable polymerase such as Platinum® <i>Pfx</i> DNA Polymerase.                                     |
| Too many cycles   | Reduce cycle number.  |
| The concentration of all four deoxynucleotides is not equal | Prepare a new deoxynucleotide mix and ensure that the concentration of all four nucleotides is equal or use a prepared mix. |

# PCR enzyme selection guide

**Table 4** – PCR enzyme selection guide

| Invitrogen PCR Enzyme                                | Product Size | Yield | Specificity | Fidelity | Convenience | GC Rich |
|--|--------------|-------|-------------|----------|-------------|---------|
| <i>Taq</i> DNA Polymerase                            | < 5 kb       | •     | •           | •        | •           | •       |
| PCR SuperMix   | < 5 kb       | •     | •           | •        | •••         | •       |
| <i>Taq</i> PCR <sub>x</sub> DNA Polymerase           | < 5 kb       | •     | •           | •        | ••          | •••     |
| Platinum® <i>Taq</i> DNA Polymerase                  | < 5 kb       | •••   | ••          | •        | ••          | •       |
| Platinum® PCR SuperMix                               | < 5 kb       | •••   | ••          | •        | ••••        | •       |
| Platinum® <i>Taq</i> PCR <sub>x</sub> DNA Polymerase | < 5 kb       | •••   | ••          | •        | •••         | •••     |
| AccuPrime™ <i>Taq</i> DNA Polymerase                 | < 5 kb       | ••••  | ••••        | ••       | •••         | •       |
| AccuPrime™ SuperMix I and II                         | < 5 kb       | ••••  | ••••        | ••       | ••••        | •       |
| Platinum® <i>Pfx</i> DNA Polymerase                  | < 12 kb      | ••    | ••          | ••••     | •••         | •       |
| Platinum® <i>Taq</i> DNA Polymerase High Fidelity    | < 12 kb      | ••••  | ••          | •••      | ••          | •       |
| Platinum® PCR SuperMix High Fidelity                 | < 12 kb      | ••••  | ••          | •••      | ••••        | •       |
| PCR SuperMix High Fidelity                           | < 12 kb      | •••   | •           | •••      | •••         | •       |
| Elongase® Enzyme Mix                                 | < 30 kb      | •••   | •           | •••      | •           | •       |
| ThermalAce™ DNA Polymerase                           | < 25 kb      | •••   | •           | ••       | •           | •••     |
| Platinum® Quantitative PCR SuperMix-UDG              | < 5 kb       | •••   | ••          | •        | ••••        | •       |

## Ordering information

| Product   | Quantity†                           | Cat. no.  |
|---|-------------------------------------|-----------|
| <b>Taq with Antibody Hot Start</b>                            |                                     |           |
| *Platinum® Taq DNA Polymerase <sup>1,2,14,37</sup>            | 100 rxns                            | 10966-018 |
| *Platinum® PCR SuperMix <sup>1,2,12,14</sup>                  | 100 rxns                            | 11306-016 |
| <b>UDG Products</b>   |                                     |           |
| *Platinum® Quantitative PCR SuperMix-UDG                      | 100 reactions                       | 11730-017 |
| *Uracil DNA Glycosylase                                       | 100 units                           | 18054-015 |
| <b>Highest Specificity Taq with Antibody Hot Start</b>        |                                     |           |
| *AccuPrime™ Taq DNA Polymerase <sup>2,12,14,33</sup>          | 200 rxns                            | 12339-016 |
| *AccuPrime™ SuperMix I <sup>2,12,14,33</sup> (≥ 200 bp)       | 200 rxns                            | 12342-010 |
| *AccuPrime™ SuperMix II <sup>2,12,14,33</sup> (0.2-4 kb)      | 200 rxns                            | 12341-012 |
| <b>Taq/Proofreading Blend with Antibody Hot Start</b>         |                                     |           |
| *Platinum® Taq DNA Polymerase High Fidelity <sup>1,2,14</sup> | 100 rxns                            | 11304-011 |
| *Platinum® PCR SuperMix High Fidelity <sup>1,2,12,14</sup>    | 100 rxns                            | 12532-016 |
| <b>High Fidelity PCR with Antibody Hot Start</b>              |                                     |           |
| *Platinum® Pfx DNA Polymerase <sup>1,2,14,37</sup>            | 100 rxns                            | 11708-013 |
| <b>Long PCR</b>   |                                     |           |
| *Elongase® Amplification System <sup>1,2</sup>                | 100 rxns                            | 10480-010 |
| <b>Taq</b>  |                                     |           |
| *Taq DNA Polymerase <sup>1,2</sup> , native                   | 100 units                           | 18038-018 |
| *Taq DNA Polymerase <sup>1,2</sup> , cloned                   | 100 units                           | 10342-053 |
| *PCR SuperMix <sup>1,2,12,14</sup>                            | 100 rxns                            | 10572-014 |
| <b>PCR Optimization &amp; Accessories</b>                     |                                     |           |
| PCR <sub>x</sub> Enhancer System <sup>4,37</sup>              | 250 rxns                            | 11495-017 |
| Custom Primers <sup>100</sup>                                 | <i>Please inquire: 800-955-6288</i> |           |
| PCR Optimizer™ Kit <sup>4</sup>                               | 100 rxns                            | K1220-01  |
| 10 mM dNTP Mix <sup>4</sup>                                   | 100 ul                              | 18427-013 |
| 100 mM dNTP Set <sup>4</sup>                                  | 4 x 25 umol                         | 10297-018 |
| E-Gels® <sup>61</sup> Pre-Cast Agarose Gels 0.8% Starter Pak  | 9 gels & 1 base                     | G5000-08  |
| Ready-Load™ 100 bp DNA Ladder                                 | 100 apps                            | 10380-012 |
| Ready-Load™ 1 kb Plus DNA Ladder                              | 100 apps                            | 12308-011 |

†Larger sizes available. See web site or catalog.

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