

Product Information

PCR OPTIMIZATION KIT II

Product Number **OPT-II**

TECHNICAL BULLETIN

Product Description

Polymerase Chain Reaction (PCR)[†] has become one of the most powerful techniques used in molecular biology. It is the method of choice for selectively amplifying a specific region of DNA or RNA without extensive gene manipulations. Many parameters can influence the specificity, fidelity and yield of a specific PCR product. These include the concentration and pH of the various reaction buffer components (enzyme, dNTPs, KCl or MgCl₂). Also, the quality and quantity of the DNA primers and templates (purity, GC content, and secondary structure) can affect the outcome of a PCR. The magnesium concentration is believed to affect primer annealing and template denaturation as well as enzyme activity and fidelity. Excess magnesium can cause an increase in nonspecific product, while too little can cause reduced yield.¹ Accordingly, PCR reactions should contain between 0.5 and 2.5 mM magnesium over the total dNTP concentration.² The KCl concentration (up to 50 mM) is also believed to have an effect on the annealing and denaturing process. However, a KCl concentration above 50 mM can inhibit the polymerase.² As a result, any combination of primer-template sets run in standard assay conditions may result in either low yields of product or high yields of nonspecific product. Therefore, many primer-template sets will require optimization for PCR.

The PCR Optimization Kit is designed to offer a variety of buffers and adjuncts for optimizing the specificity, fidelity and yield of a PCR product. The kit is composed of twelve 10x PCR buffers, varying in pH, concentration of MgCl₂ and KCl. In addition, seven adjuncts are included. All reagents in this kit have been tested for the absence of DNase, nickase, and RNase and shown not to inhibit the PCR process.

Preliminary Considerations

1. Denaturing Temperature

The denaturing temperature is the temperature used to separate the two strands of template DNA. This temperature is usually 90-95 °C, but can be as high as 98 °C for primers with a high G-C content. The denaturing temperature must be high enough to completely disassociate complementary DNA strands and avoid "snap back" or quick reannealing.² However, the *Taq* polymerase is less stable at the higher temperatures, so choosing the optimal denaturing temperature for the primer-template combination is important in maximizing yield. If the initial denaturing temperature is >95 °C, it is recommended that the *Taq* polymerase be added after the initial denaturing. Most templates melt below 95 °C, allowing *Taq* polymerase to be added at the start of the cycle. If the initial template is genomic DNA, the denaturing temperature should be at 97 °C for the first few cycles. The denaturing temperature should then be reduced since the PCR product will be smaller and denature at a lower temperature.

2. Annealing Temperature

Annealing temperature is a vital parameter of the PCR. If annealing is allowed to proceed at too low a temperature, nonspecific DNA fragments may be amplified. Conversely, too high of an annealing temperature will result in low yield and purity. This temperature is usually determined by calculating the T_m of the primer and subtracting 5 °C.^{2,10} If two different primers are to be used, calculate the T_m for both and subtract 5 °C from the lower T_m . In general, a higher annealing temperature will increase primer-template specificity and result in fewer artifacts.

3. Extension Temperature

Typically, 72 °C is the temperature that is optimal for the PCR reaction to proceed using *Taq* DNA polymerase.

4. Cycling Time

Cycling time refers to the time for the complete denaturing, annealing and extension process. The time for denaturing the double-stranded DNA should be long enough to completely denature the DNA, but not long enough to threaten the integrity of the *Taq* DNA polymerase. The initial denaturing step should be at least 94-96 °C for one minute. The annealing time should be long enough for the primer to anneal to the template, but not long enough for nonspecific annealing to occur. Extension time is determined by the length of the template and *Taq* DNA polymerase activity. *Taq* DNA polymerase is reported to incorporate 2,000-4,000 bases per minute. As a general rule, one minute/kb of PCR product should be used as the extension time. If the PCR product is longer than 2 kb, it may be necessary to add approximately 15 seconds to the extension time. This is because the *Taq* polymerase becomes the limiting component in the reaction.

5. Fidelity

Fidelity refers to the accuracy of incorporation. Several factors will influence the fidelity of the final PCR product.³ An equimolar concentration of each dNTP is recommended to prevent misincorporation and early chain termination.² A low concentration of deoxynucleotides (10-50 µM) in the amplification reaction decreases the chance of incorporating the wrong dNTP. Low magnesium concentration has been reported to increase fidelity of the finished product.² Finally, limiting the number of cycles will decrease the chance for misincorporation of deoxynucleotides.

Reagents

The reagents in this kit are sufficient for up to 1200 individual amplification reactions

- 10x PCR Buffer #1, Product No. P 1956 500 µl
100 mM Tris-HCl, pH 8.3, 15 mM MgCl₂, 250 mM KCl
- 10x PCR Buffer #2, Product No. P 2081 500 µl
100 mM Tris-HCl, pH 8.3, 15 mM MgCl₂, 750 mM KCl
- 10x PCR Buffer #3, Product No. P 2206 500 µl
100 mM Tris-HCl, pH 8.3, 35 mM MgCl₂, 250 mM KCl
- 10x PCR Buffer #4, Product No. P 2331
500 µl 100 mM Tris-HCl, pH 8.3, 35 mM MgCl₂,
750 mM KCl
- 10x PCR Buffer #5, Product No. P 2456
500 µl 100 mM Tris-HCl, pH 8.8, 15 mM MgCl₂,
250 mM KCl
- 10x PCR Buffer #6, Product No. P 2581
500 µl 100 mM Tris-HCl, pH 8.8, 15 mM MgCl₂,
750 mM KCl
- 10x PCR Buffer #7, Product No. P 2706
500 µl 100 mM Tris-HCl, pH 8.8, 35 mM MgCl₂,
250 mM KCl
- 10x PCR Buffer #8, Product No. P 2831
500 µl 100 mM Tris-HCl, pH 8.8, 35 mM MgCl₂,
750 mM KCl
- 10x PCR Buffer #9, Product No. P 2956
500 µl 100 mM Tris-HCl, pH 9.2, 15 mM MgCl₂,
250 mM KCl
- 10x PCR Buffer #10, Product No. P 3081
500 µl 100 mM Tris-HCl, pH 9.2, 15 mM MgCl₂,
750 mM KCl
- 10x PCR Buffer #11, Product No. P 3206
500 µl 100 mM Tris-HCl, pH 9.2, 35 mM MgCl₂,
250 mM KCl
- 10x PCR Buffer #12, Product No. P 8081
500 µl 100 mM Tris-HCl, pH 9.2, 35 mM MgCl₂,
750 mM KCl
- 50x Universal Buffer, Product No. U 3506
1250 µl 20 mM Tris-HCl, pH 8.0, 250 nM EDTA
- Single Strand Binding Protein, 25 µg
Product No. S 3917, from *E. coli*
- 50% Formamide, 500 µl
Product No. F 8912
- 750 mM Ammonium Sulfate, 1 ml
Product No. A 1079
- Bovine Serum Albumin, Non-acetylated 1 vial
Product No. B 8667, 20 mg/ml
Dilute to 1.5 mg/ml using water, PCR reagent
- Glycerol, Product No. G 8778 1 vial
1.5 ml

- Dimethyl sulfoxide (DMSO), Product No. D 9170 1 ml
- 5 M Betaine, Product No. B 0300 1.5 ml

Reagents Required but Not Provided

Product numbers are given where appropriate

- Water, PCR reagent, Product No. W 1754
- *Taq* DNA Polymerase, Product No. D 1806 or D 4545
- Primers
- DNA template
- Deoxynucleotide mixture, containing each of the four nucleotides at 10 mM, Product No. D 7295
- Mineral oil, Product No. M 8662

Precautions and Disclaimer

Sigma's PCR Optimization Kit is for R&D use only. Not for drug, household or other uses. Kit contains components that are hazardous. Consult the MSDS for information regarding hazards and safe handling practices. When the control template DNA is radioactively labeled, standard procedures for safely handling radioactive materials should be followed.

Storage

Store all components at -20°C

Procedure

Optimization using 10x PCR Buffers

1. For a 50 μl reaction, label 12 individual tubes and add to each tube:

5 μl	each 10x PCR buffer
x μl	Water, PCR reagent, QS to a final volume of 50 μl
1 μl	50x Universal Buffer
1 μl	10 mM dNTP Mix
200 ng	each of the 2 primers
200 ng	genomic DNA template
or	
100 ng	plasmid DNA template
2.5 units	<i>Taq</i> DNA polymerase

NOTE: Special handling instructions for genomic DNA to prevent shearing:

- a) The genomic DNA should be added last
- b) Mix gently using a wide pore pipet tip
- c) DO NOT VORTEX!

For accuracy and reliability from sample to sample, prepare a master mix when using all twelve 10x PCR buffers for evaluation.

2. Layer 25 μl of mineral oil over the reaction mix in every tube if using a thermocycler without a heated lid.
3. Place the tubes in the thermocycler and proceed using the calculated parameters.
4. At the end of the cycling, load 10 μl from each reaction onto an agarose gel to evaluate the product. Observe the yield and size of the product as well as any nonspecific amplified product.

Based on the electrophoresis results, select the best 10x PCR buffer for the specific primer-template set.

Optimization by Addition of Adjuncts

Adjuncts have been shown, under specific conditions, to enhance PCR. The kit includes seven adjuncts reported in the literature to optimize PCR. Their reported effects on PCR are as follows:

- BSA (10-100 $\mu\text{g/ml}$) has been shown to stabilize the *Taq* DNA polymerase.⁴
- Formamide (1.25-10%) has been shown to increase specificity in regions of high GC content.⁵
- Dimethyl sulfoxide (1-10%) has been shown to accelerate strand renaturation and is believed to give the nucleic acid thermal stability against depurination.^{6,7}
- Glycerol (15-20%) is thought to increase thermal stability of the polymerase and lower the temperature necessary for strand separation.⁶

- Ammonium sulfate (15-30 mM) affects the denaturing and annealing temperatures of the DNA.⁸
- Single strand binding protein (0.7-1.5 µg) is thought to accelerate annealing of complementary primer to DNA template while inhibiting extension of mismatched primers.⁹
- Betaine (0.8-1.6 M) has been reported to reduce base pair composition dependence of DNA melting.¹⁰

1. Set up reactions as in step one of the optimization using 10x PCR buffers procedure, but use the 10x PCR buffer yielding the best results.
2. To each of eight tubes, add the appropriate amount of one of the adjuncts listed in the table below

a. Single strand binding protein	1.0 µg
b. 50% formamide	2.5 µl
c. 750 mM ammonium sulfate	1.0 µl
d. 1.5 mg/ml bovine serum albumin	3.0 µl
e. Glycerol	7.5 µl
f. Dimethyl sulfoxide	2.5 µl
g. Betaine	8-17 µl
h. Water, PCR reagent (For comparison use)	4.0 µl

Note: It is not necessary for final volume to be exactly 50 µl. The difference in volume from reaction to reaction is not significant.

3. Layer 25 µl of mineral oil over the reaction mix in every tube if using a thermocycler without a heated lid.
4. Place the tubes in a thermocycler and proceed using the calculated parameters.

5. At the end of the cycling, load 10 µl from each reaction onto an agarose gel to evaluate the product. Observe the yield and size of the product and also any nonspecific amplified product.

Based on these two optimization procedures, determine the optimal 10x PCR buffer and appropriate adjunct for the specific primer-template set. If desired, running a series of concentrations of the best adjunct in the ranges suggested will identify the optimum concentration of adjunct. This will result in the best yield/fidelity for the specific primer/template set.

References

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