



Perfect*Taq* Plus DNA Polymerase Manual

For specific hot-start PCR without optimization

Perfect*Taq* Plus DNA Polymerase Manual, January, 2010

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Product specifications

Product description

The easy-to-use, hot-start PerfectTaq Plus DNA Polymerase kits provide the components and procedures necessary for efficient and robust standard end-point PCR applications without optimization.

Product limitations

PerfectTaq Plus DNA Polymerase is developed, designed, and sold for research purposes only. It is not to be used for human diagnostic or drug purposes or to be administered to humans unless expressly cleared for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. All due care and attention should be exercised in the handling of the materials described in this text.

Materials supplied

Kit	Catalog no.	Size
PerfectTaq Plus DNA Polymerase	2200055	50 Units
PerfectTaq Plus DNA Polymerase	2200065	250 Units
PerfectTaq Plus DNA Polymerase	2200075	1000 Units
PerfectTaq Plus MasterMix	2200095	200 Rxn (250U)

PerfectTaq Plus DNA Polymerase

Kit	PerfectTaq Plus DNA Polymerase	PerfectTaq Plus DNA Polymerase	PerfectTaq Plus DNA Polymerase
Ordering no.	2200055	2200065	2200075
PerfectTaq Plus DNA Polymerase (5 U/ μ l)	1 x 10 μ l	1 x 50 μ l	4 x 50 μ l
PerfectTaq Plus PCR Buffer, 10x ¹	1 x 1.2 ml	1 x 1.2 ml	4 x 1.2 ml
PerfectLoad Dye Concentrate, 10x	1 x 1.2 ml	1 x 1.2 ml	4 x 1.2 ml
5P-Solution, 5x	1 x 400 μ l	1 x 2.0 ml	4 x 2.0 ml
25 mM MgCl ₂	1 x 1.2 ml	1 x 1.2 ml	4 x 1.2 ml

¹ Contains 15 mM MgCl₂.

PerfectTaq Plus MasterMix

Kit	PerfectTaq Plus MasterMix
Ordering no.	2200095
2x PerfectTaq Plus MasterMix ¹	3 x 1.7 ml
PerfectLoad Dye Concentrate, 10x	1 x 1.2 ml
RNase-free Water	3 x 1.8 ml

¹ Contains PerfectTaq Plus DNA Polymerase, PerfectTaq Plus PCR Buffer with 3 mM MgCl₂ and 400 μM each dNTPs.

Additional materials

To perform the protocols described in this manual, the following additional materials must be provided by the user:

- dNTPs (e.g. Deoxynucleotide Mix [10 mM], see ‘Ordering information’, page 30)
- RNase-free water
- Reaction tubes
- Pipets and pipet tips (aerosol resistant)
- Thermal cycler
- Mineral oil (if the thermal cycler does not have a heated lid)
- Primers

Primers should be purchased from an established oligonucleotide manufacturer. Lyophilized primers should be dissolved in TE buffer to provide a stock solution of 100 μM. Primer concentration should be checked using a spectrophotometer. Primer stock solutions should be stored in aliquots at -20°C

For convenience, additional materials to be supplied by the user are listed at the beginning of the protocol for which they are required.

Shipping and storage conditions

The PerfectTaq Plus DNA Polymerase Kit and PerfectTaq Plus MasterMix are shipped on dry ice, but retain full activity at room temperature (15-25 °C) for at least 2 weeks.

The PerfectTaq Plus DNA Polymerase Kit and PerfectTaq Plus MasterMix should be stored immediately upon receipt at 2-8 °C or at -20 °C in a constant-temperature freezer. If handled correctly and stored as recommended, the kits are stable until the expiration date printed on the label.

Safety information

All due care and attention should be exercised in the handling of this product. We recommend all users of 5 PRIME products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines. Specifically, always wear a suitable lab coat, disposable gloves, and protective goggles when working with chemicals.

Additional safety information is available from www.5PRIME.com in material safety data sheets (MSDSs) for 5 PRIME products and 5 PRIME product components.

Quality assurance

5 PRIME products are manufactured using quality chemicals and materials that meet our high standards. All product components are subjected to rigorous quality assurance testing process:

- **Component testing:** each component is tested to ensure the composition and quality meet stated specifications.
- **Performance testing:** each product is tested to ensure it meets the stated performance specification.

Additional quality information is available from www.5PRIME.com. Certificate of analysis sheets for 5 PRIME products and 5 PRIME product components can be obtained on request.

Product warranty

5 PRIME is committed to providing products that improve the speed, ease-of-use and quality of enabling technologies.

5 PRIME guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use.

This warranty is in place of any other warranty or guarantee, expressed or implied, instituted by law or otherwise. 5 PRIME provides no other warranties of any kind, expressed or implied, including warranties of merchantability and fitness for a particular purpose. Under no circumstance shall 5 PRIME be responsible for any direct, indirect, consequential or incidental damages or loss arising from the use, misuse, results of use or inability to use its products, even if the possibility of such loss, damage or expense was known by 5 PRIME.

Protocols

Product principle

Introduction

PerfectTaq Plus DNA Polymerase provides highly efficient and robust PCR performance in a wide range of PCR applications. The time and effort required for the optimization of PCR conditions are reduced because of the combination of PerfectTaq Plus DNA Polymerase, the unique PCR Buffer, and the innovative Hot-Start/Cold-Stop® technology. The hot-start allows storage at 4°C and convenient reaction setup - without working on ice. In addition, an innovative loading dye permits direct gel electrophoresis.

PerfectTaq Plus DNA Polymerase

PerfectTaq Plus DNA Polymerase has an increased specificity and provides high PCR product yield with no or minimal optimization required.

The PerfectTaq Plus DNA Polymerase is a proprietary recombinant thermostable DNA Polymerase (94 kDa) with an enzyme concentration of 5 U/μl.

The PerfectTaq Plus DNA Polymerase possesses a 5'-3' exonuclease activity (but no 3'-5' exonuclease activity) and adds an additional A. The extension rate of the DNA polymerase activity at 72°C ranges from 2-4 kb per minutes, depending on the complexity of template DNA used in the reaction.

PerfectTaq Plus DNA Polymerase features the superior Hot-Start/Cold-Stop PCR technology: The Hot-Start/Cold-Stop technology prevents nonspecific product amplification throughout the reaction and facilitates room temperature reaction set-up. The proprietary inhibitor blocks the substrate binding site of DNA polymerases in a temperature-dependent manner. Inactive polymerase-inhibitor complexes are formed at temperatures below 40°C when the affinity of the inhibitor is higher than the binding affinity of the target DNA. As the temperature is elevated to the primer-specific annealing temperature, the binding equilibrium is shifted towards complex-formation only with the target-specific primed template DNA. Unlike other hot-start Taq polymerase formulations that block the enzyme activity only prior to the first high-temperature step, the inhibitor is not denatured or inactivated at higher temperatures. It provides sustained control of nonspecific binding and extension events throughout the PCR cycling program.

PerfectTaq Plus MasterMix

PerfectTaq Plus MasterMix is a 2x concentrated, ready-to-use reagent mix containing the PerfectTaq Plus DNA Polymerase, dNTPs, and PerfectTaq Plus PCR Buffer. 5 ml of PerfectTaq Plus MasterMix is sufficient for 200 amplification of 50 μl.

PerfectTaq Plus MasterMix provides all the benefits of the PerfectTaq Plus DNA Polymerase and offers high reproducibility when processing large numbers of samples. Only primers and template need to be added to the MasterMix reducing the number of pipetting steps and the risk of contamination. High PCR yields are achieved even after storage of the PerfectTaq Plus MasterMix for 4 months at 25°C.

PerfectTaq Plus PCR Buffer

During the annealing step of every PCR cycle, the buffer greatly increases the ratio of specific primer binding. The innovative PerfectTaq Plus PCR Buffer assures the amplification of specific PCR products over a wide range of temperatures and Mg²⁺ concentrations; dramatically reducing the time needed for PCR optimization.

Specificity and sensitivity

The combination of PerfectTaq Plus DNA Polymerase and the unique PCR Buffer promotes specific primer-template annealing and leads to maximum yields of specific products, even with low amounts of starting material.

PerfectLoad Dye Concentrate

The PerfectLoad Dye Concentrate supplied with the PerfectTaq Plus DNA Polymerase Kits and PerfectTaq Plus MasterMix allows the direct loading of the PCR products onto an agarose gel without prior addition of loading buffer. The PerfectLoad Dye Concentrate contains a gel loading reagent and two gel tracking dyes which facilitate estimation of DNA migration distance and optimization of agarose gel run time.

PerfectLoad Dye does not interfere with most downstream enzymatic applications. However, for reproducible results, purification of PCR products (e.g. PCR Extract Mini Kit; 5 PRIME) is recommended prior to enzymatic manipulation.

5P-Solution

The PerfectTaq Plus DNA Polymerase Kits include 5P-Solution, an innovative PCR additive that facilitates amplification of difficult templates by modifying the melting behavior of DNA. This unique reagent can enable or improve suboptimal PCR results caused by templates that have a high degree of secondary structure or that are GC-rich. Unlike other commonly used PCR additives, such as DMSO, 5P-Solution is used with only one working concentration; it is nontoxic, and PCR purity is guaranteed. Adding 5P-Solution to PCR does not compromise PCR fidelity. For further information, see Protocol 2, 'PCR using PerfectTaq Plus DNA Polymerase and 5P-Solution', page 12.

Standardized protocol and cycling program

The standardized protocol and PCR cycling program has been proven to work for a large variety of primer-template systems. The protocol includes standardized primer concentrations and annealing temperatures. It is, therefore, recommended to start with these optimized values. Alternatively, conditions for already established PCR assays can be used.

Protocol 1: PCR using PerfectTaq Plus DNA Polymerase

Before starting

- It is recommended to start with the standardized protocol, which provides standardized primer concentrations and an annealing temperature suitable for most primer-template systems. Alternatively, PCR conditions for previously established PCR assays can be used
- To ensure optimal PCR performance, it is essential to use the provided 10x PerfectTaq Plus PCR Buffer
- To minimize cross-contamination, use disposable tips containing hydrophobic filters
- Addition of PerfectLoad Dye Concentrate is not recommended when downstream applications require fluorescence or absorbance measurement
- If required, prepare a dNTP mix containing 10 mM of each dNTP. Store the mix in aliquots at -20°C. Ready-to-use, high-quality dNTP mix is available from 5 PRIME (see 'Ordering information', page 30)

Procedure

1. Thaw dNTP mix and primer solution at room temperature or on ice. Remove PerfectTaq Plus DNA Polymerase, PerfectTaq Plus PCR Buffer, and PerfectLoad Dye Concentrate from storage at 4°C, or, if stored at -20°C, thaw on ice.

Mix the solutions thoroughly before use to avoid localized concentrations of salts.

2. Set up the master mix according to Table 1 (page 10).

The Hot-Start/Cold-Stop technology means that it is not necessary to keep reaction vessels on ice, since the polymerase is inactive at room temperature.

The master mix contains all components needed for PCR, other than the template DNA. Prepare a volume of master mix 10% greater than that required for the total number of PCR assays to be performed. A negative control (without template DNA) should always be included.

Table 1. Recommended reaction composition using PerfectTaq Plus DNA Polymerase

Component	Volume/reaction	Final concentration
Master mix 10x PerfectTaq Plus PCR Buffer*	5 μ l	1x
dNTP mix (10 mM of each)	1 μ l	200 μ M of each dNTP
Optional: 10x PerfectLoad Dye Concentrate	5 μ l	1x
Primer A	Variable	0.2 μ M [†]
Primer B	Variable	0.2 μ M [†]
PerfectTaq Plus DNA Polymerase	0.25 μ l	1.25 unit/reaction
RNase-free water	Variable	
Template DNA (added in step 4)	Variable	\leq 1 μ g/reaction
Total volume	50 μ l	

Note: For lower reaction volumes, the amount of each component should be scaled accordingly.

* Contains 15 mM MgCl₂.

† 0.2 μ M is suitable for most PCR systems. If necessary optimize the primer concentration in a range of 0.1-0.5 μ M.

- Mix the master mix thoroughly and dispense appropriate volumes into PCR tubes.
- Add template DNA (\leq 1 μ g/reaction) to the individual tubes containing the master mix.

For RT-PCR, add an aliquot from the reverse transcriptase reaction. This should not exceed 10% of the final PCR volume (see 'RT-PCR', page 22)

- If using a thermal cycler with a heated lid, proceed directly to step 6. If the thermal cycler does not have a heated lid, first overlay each reaction with approximately 50 μ l mineral oil.
- Program the thermal cycler according to the manufacturer's instructions. The PCR cycling program outlined in Table 2 (page 11) has been standardized for most primer-template systems. Should optimization of the annealing temperature be required, see Supporting information, page 20.

Table 2. Standardized cycling protocol

Step	Time/ cycles	Temperature	Comments
Initial denaturation	3 min	94°C	
3-step cycling:			For most PCR systems, an annealing temperature of 60°C is suitable. If optimization of annealing temperature is required, see 'Primers', page 20.
Denaturation	30 s	94°C	
Annealing	30 s	60°C	
Extension	1 min/kb	72°C	
Number of cycles	25-35		
Final extension	10 min	72°C	

7. Place the PCR tubes in the thermal cycler and start the cycling program.
PCR products can be stored overnight at 2-8°C or at -20°C for longer storage.
8. If PerfectLoad Dye Concentrate is used, the PCR reaction can be directly loaded onto an agarose gel without prior addition of a PCR loading buffer and gel tracking dye.
A gel loading reagent and two gel tracking dyes are contained in the PerfectLoad Dye Concentrate. See Table 3 for DNA migration distances with different agarose gels.

Table 3. Migration distances of gel tracking dyes

% TAE (TBE) agarose gel	Red dye	Orange dye
0.8	500 bp (270 bp)	~80 bp (<10 bp)
1.0	300 bp (220 bp)	~40 bp (<10 bp)
1.5	250 bp (120 bp)	~20 bp (<10 bp)
2.0	100 bp (110 bp)	<10 bp (<10 bp)
3.0	50 bp (100 bp)	<10 bp (<10 bp)

Protocol 2: Protocol: PCR using PerfectTaq Plus DNA Polymerase and 5P-Solution

5P-Solution changes the melting behavior of DNA and can be used for PCR systems that do not work well under standard conditions. When using 5P-Solution for the first time with a particular primer-template pair, always perform parallel reactions with and without 5P-Solution. This recommendation should also be followed if another PCR additive (such as DMSO) was previously used for a particular primer-template pair. Optimal reaction conditions, such as incubation times, temperatures, and amount of template DNA, may vary and must be individually determined for primer-template pairs. When using 5P-Solution, the following effects may be observed, depending on the individual PCR assay:

- 5P-Solution enables amplification of a reaction which failed previously
- 5P-Solution increases PCR specificity in certain primer-template systems
- 5P-Solution has no effect on PCR performance
- 5P-Solution causes reduced efficiency or failure of a previously successful PCR. In such cases, addition of 5P-Solution disturbs the previously optimal primer-template annealing.

Before starting

- When using 5P-Solution for the first time in a particular primer-template system, it is important to perform parallel amplification reactions with and without 5P-Solution
- It is recommended to start with the standardized protocol, which provides standardized primer concentrations and an annealing temperature suitable for most primer-template systems. Alternatively, PCR conditions for previously established PCR assays can be used
- To ensure optimal PCR performance, it is essential to use the provided 10x PerfectTaq Plus PCR Buffer
- To minimize cross-contamination, use disposable tips containing hydrophobic filters
- Addition of PerfectLoad Dye Concentrate is not recommended when downstream applications require fluorescence or absorbance measurement
- If required, prepare a dNTP mix containing 10 mM of each dNTP. Store the mix in aliquots at -20°C. Ready-to-use, high-quality dNTP mix is available from 5 PRIME (see 'Ordering information', page 30)

Procedure

1. Thaw dNTP mix and primer solution at room temperature or on ice. Remove PerfectTaq Plus DNA Polymerase, PerfectTaq Plus PCR Buffer, 5P-Solution and PerfectLoad Dye Concentrate from storage at 4°C, or, if stored at -20°C, thaw on ice.

Mix the solutions thoroughly before use to avoid localized concentrations of salts.

2. Set up the master mix according to Table 4.

The Hot-Start/Cold-Stop technology means that it is not necessary to keep reaction vessels on ice, since the polymerase is inactive at room temperature.

The master mix contains all components needed for PCR, other than the template DNA. Prepare a volume of master mix 10% greater than that required for the total number of PCR assays to be performed. A negative control (without template DNA) should always be included.

Table 4. Recommended reaction composition using PerfectTaq Plus DNA Polymerase and 5P-Solution

Component	Volume/reaction	Final concentration
Master mix: 10x PerfectTaq Plus PCR Buffer*	5 µl	1x
Optional: 10x PerfectLoad Dye Concentrate	5 µl	1x
5 x 5P-Solution	10 µl	1x
dNTP mix (10 mM each)	1 µl	200 µM each dNTP
Primer A	Variable	0.2 µM [†]
Primer B	Variable	0.2 µM [†]
PerfectTaq Plus DNA Polymerase	0.25 µl	1.25 units/reaction
RNase-free water	Variable	
Template DNA (added at step 4)	Variable	≤1 µg/reaction
Total volume	50 µl	

Note: If smaller reaction volumes are used, reduce the amount of each component accordingly.

* Contains 15 mM MgCl₂.

† 0.2 µM is suitable for most PCR systems. If necessary optimize the primer concentration in a range of 0.1-0.5 µM.

3. Mix the master mix thoroughly and dispense appropriate volumes into PCR tubes.

4. Add template DNA (≤1 µg/reaction) to the individual tubes containing the master mix.

For RT-PCR, add an aliquot from the reverse transcriptase reaction. This should not exceed 10% of the final PCR volume (see 'RT-PCR', page 22)

5. If using a thermal cycler with a heated lid, proceed directly to step 6. If the thermal cycler does not have a heated lid, first overlay each reaction with approximately 50 µl mineral oil.

6. Program the thermal cycler according to the manufacturer's instructions.

The PCR cycling program outlined in Table 5 has been standardized for most primer-template systems. Should optimization of the annealing temperature be required, see 'Primers', page 20.

Table 5. Pre-optimized cycling protocol

Step	Time/ cycles	Temperature	Comments
Initial denaturation	3 min	94°C	
3-step cycling:			For most PCR systems, an annealing temperature of 60°C is suitable. If optimization of annealing temperature is required, see 'Primers', page 20.
Denaturation	30 s	94°C	
Annealing	30 s	60°C	
Extension	1 min/kb	72°C	
Number of cycles	25-35		See 'Number of PCR cycles', page 21.
Final extension	10 min	72°C	

7. Place the PCR tubes in the thermal cycler and start the cycling program.

PCR products can be stored overnight at 2-8°C or at -20°C for longer storage.

8. If PerfectLoad Dye Concentrate is used, the PCR reaction can be directly loaded onto an agarose gel without prior addition of a PCR loading buffer and gel tracking dye.

A gel loading reagent and two gel tracking dyes are contained in the PerfectLoad Dye Concentrate. See Table 6 for DNA migration distances with different agarose gels.

Table 6. Migration distances of gel tracking dyes

% TAE (TBE) agarose gel	Red dye	Orange dye
0.8	500 bp (270 bp)	~80 bp (<10 bp)
1.0	300 bp (220 bp)	~40 bp (<10 bp)
1.5	250 bp (120 bp)	~20 bp (<10 bp)
2.0	100 bp (110 bp)	<10 bp (<10 bp)
3.0	50 bp (100 bp)	<10 bp (<10 bp)

Protocol 3: PCR using PerfectTaq Plus MasterMix

Before starting

- It is recommended to start with the standardized protocol, which provides standardized primer concentrations and an annealing temperature suitable for most primer-template systems. Alternatively, PCR conditions for already established PCR assays can be used
- To minimize cross-contamination, use disposable tips containing hydrophobic filters
- Addition of PerfectLoad Dye Concentrate is not recommended when downstream applications require fluorescence or absorbance measurement

Procedure

1. Thaw primer solutions and template DNA.

Mix well before use.

2. Mix the PerfectTaq Plus MasterMix briefly by vortexing and dispense 25 µl into each PCR tube according to Table 7 (page 16).

To avoid localized concentrations of salts, mix PerfectTaq Plus MasterMix thoroughly. Due to the Hot-Start/Cold-Stop technology, it is not necessary to keep reaction vessels on ice, since the polymerase is inactive at room temperature.

3. Add diluted primer mix into the PCR tube containing the PerfectTaq Plus MasterMix.
4. Add template DNA (1 µg/reaction) to the individual PCR tubes.

For RT-PCR, add an aliquot from the reverse transcriptase reaction. This should not exceed 10% of the final PCR volume (see 'RT-PCR', page 22)

Table 7. Recommended reaction composition using PerfectTaq Plus MasterMix

Component	Volume/ reaction	Final concentration
2 x PerfectTaq Plus MasterMix	25 μ l	1.25 units PerfectTaq Plus DNA Polymerase 1x PerfectTaq Plus PCR Buffer 200 μ M of each dNTP
Optional: 10x PerfectLoad Dye Concentrate	5 μ l	1x PerfectLoad Dye Concentrate
Primer A	Variable	0.2 μ M*
Primer B	Variable	0.2 μ M*
RNase-free water	Variable	
Template DNA (added in step 4)	Variable	\leq 1 μ g/reaction
Total volume	50 μ l	

Note: For lower reaction volumes, the amount of each component should be scaled accordingly.

* 0.2 μ M is suitable for most PCR systems. If necessary optimize the primer concentration in a range of 0.1-0.5 μ M.

- If using a thermal cycler with a heated lid, proceed directly to step 6. If the thermal cycler does not have a heated lid, first overlay each reaction with approximately 50 μ l mineral oil.
- Program the thermal cycler according to the manufacturer's instructions.
The PCR cycling program outlined in Table 8 has been standardized for most primer-template systems. Should optimization of the annealing temperature be required, see 'Primers', page 20.

Table 8. Standardized cycling protocol

Step	Time/ cycles	Temperature	Comments
Initial denaturation	3 min	94°C	
3-step cycling:			For most PCR systems, an annealing temperature of 60°C is suitable. If optimization of annealing temperature is required, see 'Primers', page 20.
Denaturation	30 s	94°C	
Annealing	30 s	60°C	
Extension	1 min/kb	72°C	
Number of cycles	25-35		
Final extension	10 min	72°C	

7. Place the PCR tubes in the thermal cycler and start the cycling program.
PCR products can be stored overnight at 2-8°C or at -20°C for longer storage.
8. If PerfectLoad Dye Concentrate is used, the PCR reaction can be directly loaded onto an agarose gel without prior addition of a PCR loading buffer and gel tracking dye.

A gel loading reagent and two gel tracking dyes are contained in the PerfectLoad Dye Concentrate. See Table 9 for DNA migration distances with different agarose gels.

Table 9. Migration distances of gel tracking dyes

% TAE (TBE) agarose gel	Red dye	Orange dye
0.8	500 bp (270 bp)	~80 bp (<10 bp)
1.0	300 bp (220 bp)	~40 bp (<10 bp)
1.5	250 bp (120 bp)	~20 bp (<10 bp)
2.0	100 bp (110 bp)	<10 bp (<10 bp)
3.0	50 bp (100 bp)	<10 bp (<10 bp)

Supporting information

Starting template

The success of PCR can be affected by both the quality and quantity of nucleic acid starting template, especially the sensitivity and efficiency of amplification.

Quality and quantity of starting template

Since PCR consists of multiple rounds of enzymatic reactions, it is more sensitive to impurities (e.g. proteins, phenol/chloroform, salts, ethanol, EDTA) than single-step enzyme-catalyzed processes. 5 PRIME offers a complete range of nucleic acid preparation systems, to ensure high-quality templates for PCR. These include the PerfectPrep™ system for rapid plasmid purification and the ArchivePure DNA and PerfectPure™ DNA systems for rapid purification of genomic DNA and RNA from various sources. For more information, contact your local 5 PRIME distributor. A complete list of 5 PRIME distributors is available at www.5PRIME.com.

An important factor in PCR is the annealing efficiency of primer to template. Owing to the thermodynamic nature of the reaction, the primer:template ratio strongly influences the specificity and efficiency of PCR and should be optimized empirically. If too little template is used, primers may not be able to find their complementary sequences. Too much template may lead to an increase in mispriming events. As a guide, spectrophotometric and molar conversion values for different nucleic acid templates are listed in Tables 10 and 11, respectively.

Table 10. Spectrophotometric conversions for nucleic acid templates

1 A_{260} unit*	Concentration ($\mu\text{g}/\text{ml}$)
Double-stranded DNA	50
Single-stranded DNA	33
Single-stranded RNA	40

* Absorbance at 260 nm = 1

Table 11. Molar conversions for nucleic acid templates

Nucleic acid	Size	pmol/ μ g	Molecules/ μ g
Genomic DNA			
<i>Escherichia coli</i>	4.7×10^6 *	3.0×10^{-4}	$1.8 \times 10^{8\dagger}$
<i>Drosophila melanogaster</i>	1.4×10^8 *	1.1×10^{-5}	$6.6 \times 10^{8\dagger}$
<i>Mus musculus</i> (mouse)	2.7×10^9 *	5.7×10^{-7}	$3.4 \times 10^{5\dagger}$
<i>Homo sapiens</i> (human)	3.3×10^9 *	4.7×10^{-7}	$2.8 \times 10^{5\dagger}$
1 kb DNA	1000 bp	1.52	9.1×10^{11}
pUC19 DNA	2686 bp	0.57	3.4×10^{11}
pTZ18R DNA	2870 bp	0.54	3.2×10^{11}
pBluescript® II DNA	2961 bp	0.52	3.1×10^{11}
Lambda DNA	48,502 bp	0.03	1.8×10^{10}
Average mRNA	1930 nt	1.67	1.0×10^{12}

* Base pairs in haploid genome

† For single-copy genes

Primers

The following factors are critical for the success of PCR:

- Design of optimal primer pairs
- Use of appropriate primer concentrations
- Correct storage of primer solutions

General guidelines for standard PCR primers

- The optimal primer length is 18-30 nucleotides with a G/C content of 40-60%
- The optimal annealing temperatures may be above or below the estimated T_m . For most PCR systems, an annealing temperature of 60°C is suitable. If optimization of annealing temperature is required, use an annealing temperature 5°C below T_m as a starting point, or carry out a temperature-gradient PCR using annealing temperatures from 50-68°C. Whenever possible, design primer pairs with similar T_m values.
- Following formula can be used to calculate the melting temperature:

$$T_m = 2^\circ\text{C} \times (\text{A}+\text{T}) + 4^\circ\text{C} \times (\text{G}+\text{C})$$
- To prevent primer dimers it is necessary to avoid complementarity of two or three bases at the 3' ends of primer pairs. Furthermore, avoid mismatches between the 3' end of the primer and the target-template sequence, a 3'-end T, runs of 3 or more Gs or Cs at the end and complementary sequences within a primer sequence and between the primer pair
- A primer concentration of 0.2 μM is suitable for most PCR systems. If necessary, optimize the primer concentration in a range of 0.1-0.5 μM
- To calculate the concentration of the primer, use following formula and spectrophotometric conversion for primers:

$$1 A_{260} \text{ unit} = 20\text{-}30 \mu\text{g/ml}$$

Table 12. Molar concentrations of primers

Primer length	pmol/ μg	20 pmol
18mer	168	119 ng
20mer	152	132 ng
25mer	121	165 ng
30mer	101	198 ng

- Store the primer in a concentrated stock solution; dissolve lyophilized in a small volume of distilled water or TE buffer. Store working solutions (10 pmol/ μl) in aliquots at -20°C. Primer quality can be checked on a denaturing polyacrylamide gel; resulting in a single band.

Number of PCR cycles

Depending on the number of copies of the starting template, a cycling program is usually between 25 and 35 cycles. More cycles do not necessarily lead to a higher yield of PCR product, instead they may increase nonspecific background and decrease the yield of specific PCR products. Table 13 provides a general guideline for choosing the appropriate number of cycles.

Table 13. General guidelines for choosing number of PCR cycles

No. of copies of starting template*	1 kb DNA	<i>E. coli</i> DNA [†]	Human genomic DNA	Number of cycles
10-100	0.01-0.11 fg	0.05-0.56 pg	36-360 pg	40-45
100-1,000	0.11-1.1 fg	0.56-5.56 pg	0.36-3.6 ng	35-40
$1 \times 10^3 - 5 \times 10^4$	1.1-55 fg	5.56-278 pg	3.6-179 ng	30-35
$>5 \times 10^4$	>55 fg	>278 pg	>179 ng	25-35

* Use Table 11 (page 21) to calculate the number of starting molecules. When starting with cDNA templates, consider the efficiency of reverse transcription in cDNA synthesis (on average 10-30%)

[†] Refers to single-copy genes.

RT-PCR

When using RNA as starting template for PCR, the RNA must first be reverse transcribed into cDNA in a reverse transcriptase (RT) reaction. It is usually a limitation of the RT reaction which causes a subsequent PCR to fail.

RT reactions are known to have a low efficiency, with only 10-30% of the original RNA molecules, on average, reverse transcribed into cDNA. It is important to consider the expression level of the target RNA molecules and the relatively low efficiency of the RT reaction when calculating the appropriate amount of starting template for subsequent PCR. The volume of the RT reaction transferred should not exceed 10% of the total PCR volume.

General guidelines for performing RT-PCR

For total RNA purification and reverse transcription, 5 PRIME offers the PerfectPure system and Masterscript™ system, respectively. Follow the detailed protocol in the Masterscript Kit and Masterscript RT-PCR System Manual or, when using an enzyme from another supplier, follow the manufacturer's instructions. The following guidelines may be useful.

Mix the following reagents in a microcentrifuge tube:

- 4.0 µl 5x RT buffer, 1.0 µl RNase inhibitor (5 units/µl), 2.0 µl DTT (0.1 M), 1.0 µl each dNTP (10 mM), ~1 µg RNA and 2.5 µl primer (0.2 µg/µl) reverse transcriptase
- Add RNase-free water to a final volume of 20 µl
- Incubate following manufacturer's instructions
- Heat the reaction mix to 95°C for 5 min to inactivate the reverse transcriptase

For the PCR, prepare a PCR mixture by following steps 1-3 in respective protocol (pages 9 and 12). Add 2-5 µl from the RT reaction to each PCR tube containing the master mix and continue with step 5 in the protocol.

Contamination control

It is vital to include one or more negative controls that lack any template nucleic acid in every PCR setup in order to detect possible contamination.

General physical precautions

- Separate the working areas for setting up the PCR master mix and DNA handling, including the addition of starting template, PCR product analysis, or plasmid preparation. If possible, separate rooms should be used.
- Use of pipet tips with hydrophobic filters and use of a separate set of pipets for the PCR master mix is strongly recommended.
- Prepare and freeze small aliquots of primer solutions and dNTP mix. Use of fresh distilled water is strongly recommended.
- In case of contamination, laboratory benches, apparatus, and pipets can be decontaminated by cleaning them with RNaseKiller (5 PRIME) or a 1/10 dilution of a commercial bleach solution. Afterwards, the benches and pipets should be rinsed with distilled water.

General chemical precautions

- PCR stock solutions can also be decontaminated using UV light. This method is problematic, however, and its efficiency is difficult to control and cannot be guaranteed. Solutions should ideally be stored in small aliquots and a fresh aliquot should be used for each PCR.
- Another approach to preventing amplification of contaminating DNA is to treat individual reaction mixtures with DNase I or restriction enzymes that cut between the binding sites of the amplification primers used, before adding the template DNA sample.

Purified sample guide

After amplification, the PCR sample contains a complex mixture of specific PCR product and residual reaction components such as primers, unincorporated nucleotides, enzyme(s), salts, mineral oil, nonspecific amplification products, and, if used, PerfectLoad Dye Concentrate. Before the specific PCR product can be used in subsequent experiments, it is often necessary to remove these contaminants.

The PCRExtract Mini Kit offers a quick and easy method for purifying the final PCR product (see Ordering information, page 30). For more information, contact your local 5 PRIME distributor. A complete list of 5 PRIME distributors is available at www.5PRIME.com.

Troubleshooting guide

The following troubleshooting recommendations are designed to address unexpected or undesired results. To ensure optimal use, follow the guidelines and recommendations in the manual.

Observation	Little or no product
Possible cause	Pipetting error or missing reagent
Avoiding	Repeat the PCR. Check the concentrations and storage conditions of reagents, including the primers and dNTP mix.
Possible cause	Wrong PCR buffer
Avoiding	The 10x PerfectTaq Plus PCR Buffer provided is required for optimal performance.
Possible cause	Primer concentration not optimal or primers degraded
Avoiding	For most primer-template systems, a primer concentration of 0.2 μM is suitable. To optimize primer concentration, repeat the PCR with different primer concentrations from 0.1-0.5 μM of each primer (in 0.1 μM steps). Check possible degradation of the primers on a denaturing polyacrylamide gel or use fresh primers.
Possible cause	Problems with starting template
Avoiding	Check the concentration, storage conditions, and quality of the starting template ('Starting template', page 18). If necessary, prepare and repeat the PCR using new serial dilutions of template nucleic acid from stock solutions.
Possible cause	Suboptimal PCR cycling conditions
Avoiding	Repeat the PCR using the same cycling conditions, but use 5P-Solution (Protocol 2, page 12).

Possible cause Mg²⁺ concentration not optimal

Avoiding Perform PCR with different final concentrations of Mg²⁺ from 1.5-5.0 mM (in 0.5 mM increments) using a 25 mM MgCl₂ solution.

Possible cause Enzyme concentration too low

Avoiding When using PerfectTaq DNA Polymerase, use 1.25 units per 50 µl reaction. If necessary, increase the amount of PerfectTaq Plus DNA Polymerase (in 0.5 unit increments).

Possible cause Insufficient number of cycles

Avoiding Increase the number of cycles in increments of 5 cycles (see 'Number of PCR cycles', page 21).

Possible cause Incorrect annealing temperature or time

Avoiding Decrease annealing temperature in 2°C increments. Annealing time should be between 30 and 60 s. Optimal annealing temperature can be determined by performing touchdown PCR.

Possible cause Incorrect denaturation temperature or time

Avoiding Denaturation should be at 94°C for 30-60 s. Ensure that the initial 3 minute 94°C incubation step is performed as described in step 6 of the PCR protocols (pages 10, 14, and 16).

Possible cause Extension time too short

Avoiding Increase the extension time in increments of 1 minute.

Possible cause Primer design not optimal

Avoiding Review primer design (see 'Primers', page 20).

Possible cause	Reverse transcription reaction error
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Avoiding	Consider the efficiency of the RT reaction, which averages 10-30%. The added volume of RT reaction should not exceed 10% of the final PCR volume (see 'RT-PCR', page 22).
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Possible cause	PCR overlaid with mineral oil when using a thermal cycler with a heated lid
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Avoiding	Do not overlay the PCR samples with mineral oil if using a thermal cycler with a heated lid, as this may decrease the yield of PCR product.
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Possible cause	Problems with the thermal cycler
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Avoiding	Check the power supply and program of the thermal cycler.
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Observation	Product is multi-banded
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Possible cause	Annealing temperature too low
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Avoiding	Increase annealing temperature in 2°C increments. Annealing time should be between 30 and 60 s. Difficulties in determining the optimal annealing temperature can be overcome in many cases by performing touchdown PCR.
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Possible cause	Primer concentration not optimal or primers degraded
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Avoiding	For most primer-template systems, a primer concentration of 0.2 µM is suitable. To optimize primer concentration, repeat the PCR with different primer concentrations from 0.1-0.5 µM of each primer (in 0.1 µM steps).
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Check possible degradation of the primers on a denaturing polyacrylamide gel or use fresh primers.

Possible cause	Suboptimal PCR cycling conditions
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Avoiding	Repeat the PCR using the same cycling conditions, but use 5P-Solution (Protocol 2, page 12).
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Possible cause Primer design not optimal

Avoiding Review primer design (see 'Primers', page 20).

Observation Product is smeared

Possible cause Too much starting template

Avoiding Check the concentration and storage conditions of the starting template (see 'Starting template', page 18). Make serial dilutions of template nucleic acid from stock solutions.

Perform PCR using the serial dilutions. When reamplifying a PCR product, start the reamplification by using 1 µl of a 1/10 10^3 – 10^4 dilution of the previous PCR.

Possible cause Carryover contamination

Avoiding If the negative-control PCR shows a PCR product or smear, change all reagents. Use disposable pipet tips containing hydrophobic filters to minimize cross-contamination.

Set up all reaction mixtures in an area separate from that used for DNA preparation or PCR product analysis.

Possible cause Enzyme concentration too high

Avoiding Use 1.25 units PerfectTaq Plus DNA Polymerase per 50 µl rxn.

Possible cause Too many cycles

Avoiding Reduce the number of cycles in increments of 3 cycles.

Possible cause Mg^{2+} concentration not optimal

Avoiding Perform PCR with different final concentrations of Mg^{2+} from 1.5–5.0 mM (in 0.5 mM increments) using the 25 mM $MgCl_2$ solution provided.

Possible cause Primer concentration not optimal or primers degraded

Avoiding The primer concentration of 0.2 μM is suitable for most primer-template systems with Perfect*Taq* DNA Polymerase. To optimize primer concentration, repeat the PCR with different primer concentrations from 0.1-0.5 μM of each primer (in 0.1 μM steps).
When performing highly sensitive PCR, check for possible degradation of the primers on a denaturing polyacrylamide gel.

Possible cause Primer design not optimal

Avoiding Review primer design (see 'Primers', page 20).

Ordering information

Product	Size	Order/ref. no.
PerfectTaq Plus DNA Polymerase	50 Units	2200055
PerfectTaq Plus DNA Polymerase	250 Units	2200065
PerfectTaq Plus DNA Polymerase	1000 Units	2200075
PerfectTaq Plus MasterMix	250 U (200 Rxn of 50 µl)	2200095
Taq DNA Polymerase	100 U (40-100 Rxn of 50 µl)	2200000
Taq DNA Polymerase	250 U (100-250 Rxn of 50 µl)	2200010
Taq DNA Polymerase	1000 U (400-1000 Rxn of 50 µl)	2200020
Taq DNA Polymerase	5000 U (5x 1000U; 2000-5000 Rxn of 50 µl)	2200030
HotMaster Taq DNA Polymerase	100 U (40-80 Rxn of 50 µl)	2200300
HotMaster Taq DNA Polymerase	250 U (100-200 Rxn of 50 µl)	2200310
HotMaster Taq DNA Polymerase	1000 U (400-800 Rxn of 50 µl)	2200320
HotMaster Taq DNA Polymerase	5000 U (2000-4000 Rxn of 50 µl)	2200330
MasterTaq™ Kit	100 U (40-100 Rxn of 50 µl)	2200200
MasterTaq™ Kit	250 U (100–250 Rxn of 50 µl)	2200210
MasterTaq™ Kit	500 U (200-500 Rxn of 50 µl)	2200220
MasterTaq™ Kit	1000 U (400-1000 Rxn of 50 µl)	2200230
5 PRIME Master Mix	100 Rxns	2200100
5 PRIME Master Mix	1000 Rxns	2200110
Deoxynucleotide Mix 10 mM	200 µl	2201200
Deoxynucleotide Mix 10 mM	1000 µl	2201210

Deoxynucleotide Set	4 x 100 µl	2201220
Deoxynucleotide Set	4 x 250 µl	2201230
Water, Mol Bio grade	1 liter	2500000
Water, Mol Bio grade	10 x 50 ml	2500010
Water, Mol Bio grade	5 liters	2500020
PCRExtract Mini Kit	50 preps	2300600
PCRExtract Mini Kit	250 preps	2300610
RNaseKiller	250 ml	2500080

5 PRIME distributors

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