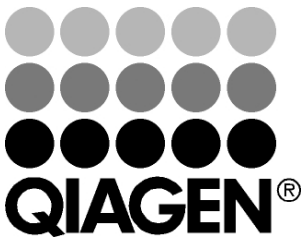
January 2010

SeqTarget LongRange PCR Handbook

For reliable and accurate amplification of long-range PCR fragments (>2 kb)



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- microRNA research and RNAi
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Contents

Kit Contents

SeqTarget LongRange PCR Kit	**(500)**
Catalog no.	**122117**
Number of 25 µl reactions	**500**
LongRange PCR Enzyme Mix	100 µl
LongRange PCR Buffer, 10x	1250 µl
dNTP Mix	3 x 200 µl
Q-Solution®, 5x	2 ml
RNase-Free Water	6 x 1.9 ml
$MgCl_2$	2 x 1.2 ml
Control Primer Pair (10 µM each)	40 µl
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Shipping and Storage

The SeqTarget LongRange PCR Kit is shipped on dry ice. It should be stored immediately upon receipt at –20°C in a constant-temperature freezer. When stored under these conditions and handled correctly, the product can be kept at least until the expiration date (see the inside of the kit lid) without showing any reduction in performance. The kit also retains full activity when stored at room temperature (15–25°C) for up to 2 weeks, but only until the kit expiration date.

Product Use Limitations

The SeqTarget LongRange PCR Kit is intended for research use. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Product Warranty and Satisfaction Guarantee

QIAGEN 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. Should any product fail to perform satisfactorily

due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department or distributor. We will credit your account or exchange the product — as you wish. Separate conditions apply to QIAGEN scientific instruments, service products, and to products shipped on dry ice. Please inquire for more information.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see back cover or visit www.qiagen.com).

Technical Assistance

At QIAGEN, we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in sample and assay technologies and the use of QIAGEN products. If you have any questions or experience any difficulties regarding the SeqTarget LongRange PCR Kit or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information, please see our Technical Support Center at www.qiagen.com/Support or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of SeqTarget LongRange PCR Kit is tested against predetermined specifications to ensure consistent product quality.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/Support/MSDS.aspx where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

Product Specifications

LongRange PCR Enzyme Mix

LongRange PCR Enzyme Mix is a mixture of highly pure recombinant thermostable DNA polymerases and processivity-enhancing factors, cloned in *E. coli*.

Concentration:	5 units/µl; refers to the amount of *Taq* DNA polymerase per microliter of LongRange PCR Enzyme Mix, although the specific activity (by nucleotide incorporation) of *Taq* DNA polymerase in combination with the other enzymes is higher than the specific activity of pure *Taq* DNA polymerase of the same concentration.
5'→3' exonuclease activity:	Yes
3'→5' exonuclease activity:	Yes
Extra A addition:	Yes

Buffers and reagents:

LongRange PCR Buffer, 10x:	10x concentrated, contains 25 mM Mg^{2+}
Q-Solution:	5x concentrated
dNTP mix:	10 mM each of: dATP, dCTP, dGTP, dTTP
RNase-free water:	Ultrapure quality, PCR-grade
Enzyme storage buffer:	LongRange PCR Enzyme Mix: Tris·HCl pH 8.0 (at 25°C), KCl, EDTA, DTT, 50% glycerol, Tween® 20, Nonidet® P-40
Control Primer Pair:	The premixed primer pair allows amplification of a 10 kb PCR fragment using human genomic DNA as template.

Introduction

Recent technological advances in sequencing systems have rapidly increased sequencing capacity and sequence output. However, these second generation sequencing systems currently do not have the throughput capacity to sequence the whole human genome cost-effectively. Therefore, a reduction in the complexity of genomic DNA samples to a manageable subset is required prior to sequencing.

The SeqTarget system has been developed to overcome this limitation and enable long-range PCR amplification of target genomic DNA regions for subsequent purification and normalization of PCR products in order to meet second generation resequencing throughput requirements.

The SeqTarget system consists of 3 components:

- SeqTarget Primer Select (cat. no. 122001) and SeqTarget Primer Select 96 Plate (cat. no. 122004)
- SeqTarget LongRange PCR Kit (500) (cat. no. 122117)
- SeqTarget Normalization Kit (480) (cat. no. 122217)

SeqTarget Primer Select

SeqTarget Primer Select is a tool that enables primer design and convenient ordering at QIAGEN's GeneGlobe® Web portal (www.qiagen.com/GeneGlobe). This innovative tool ensures optimal design and ordering of bioinformatically evaluated, unique primers for overlapping long-range PCR fragments of human genes. Binding of primers to annotated repeat regions is avoided and special requirements for successful amplification of fragments of approximately 10 kb are taken into consideration. SeqTarget Primer Select is available in a convenient tube or 96-well plate format.

SeqTarget LongRange PCR Kit

The SeqTarget LongRange PCR Kit contains a powerful enzyme blend of thermostable DNA polymerases with enhanced processivity and proofreading ability. In addition, the enzyme mix facilitates amplification of GC-rich regions and other difficult templates. This ensures both a very high extension rate as well as increased fidelity, enabling reliable amplification of genomic targets longer than 20 kb. The unique PCR buffer included in the kit has a unique zwitterionic formulation which improves buffering of pH at high temperatures (72–94°C), minimizing pH-driven template degradation. The SeqTarget LongRange PCR Kit includes Q-Solution, an innovative PCR additive that facilitates amplification of difficult templates by modifying the melting behavior of DNA, and a control primer pair which allows checking of the suitability of

human genomic DNA for use as a template.

SeqTarget Normalization Kit

The SeqTarget Normalization Kit enables isolation of long-range PCR fragments by size-selective purification. The first step involves removal of unincorporated primers and nucleotides and reduction of PCR background (fragments ≤2 kb). Subsequently, the PCR fragments are normalized, (i.e., brought to comparable amounts by binding to beads with limited binding capacity). Excess PCR products are simply washed off and normalized amounts of PCR products are eluted with a mild buffer at a slightly alkaline pH. Normalized PCR products of similar size can be pooled together as they are equimolar. No further quantification and normalization steps are required for library preparation. Subsequent sequencing can be easily performed without introducing any bias. No expensive detection devices for A_{260}/A_{280} or fluorescence measurements are required. Further adjustment to obtain the desired concentration is also not needed.

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- Ice
- Pipets and pipet tips (use of pipet tips with hydrophobic filters is strongly recommended; see Appendix F, page 27)
- Thermal cycler with heated lid
- PCR tubes (use thin-walled 0.2 ml PCR tubes recommended by the manufacturer of your thermal cycler)
- Primers: use of SeqTarget Primer Select is recommended to achieve optimal yields.
- Template DNA
- For analysis of PCR products: agarose-gel electrophoresis system

Important Notes

Template DNA

- Ensure that the template DNA is of sufficiently high quality and is not degraded.
- Use high-molecular-weight DNA templates only, with an average size of >20–50 kb, depending on the size of the fragment to be amplified.
- Use of genomic DNA isolated using QIAamp®, DNeasy®, FlexiGene®, or EZ1® Kits is recommended.

Primers

- Primers designed using SeqTarget Primer Select and ordered via QIAGEN's GeneGlobe Web portal (see www.qiagen.com/GeneGlobe) have annealing temperatures above 60°C.
- The annealing temperature of manually designed primers can be calculated by the 4+2 rule (see Appendix B, page 23 for further information).

Amplification

- Use thin-walled 0.2 ml PCR tubes or PCR plates recommended by the manufacturer of your thermal cycler, to ensure that the tubes or plates fit correctly in the PCR block.

Analysis of PCR products

- Analyze PCR products on a standard 0.7–0.8% agarose gel, preferably in TAE buffer, using appropriate DNA markers for size determination and visual quantitation. Usually, the long-range PCR product of desired size (>2 kb) should be clearly visible on the agarose gel after loading a 1/50 volume of the PCR reaction (e.g., for dilution, add 18 µl TE to 2 µl PCR reaction and subsequently load 5 µl on the gel).

Protocol: SeqTarget Long-Range PCR

This protocol is optimized for amplification of targets of up to approximately 20 kb in size.

Important points before starting

- See “Important Notes" on page 10 before starting the protocol.
- For PCR fragments larger than 8 kb, use high-molecular-weight template DNA only and ensure that it is not degraded.
- High-molecular-weight template DNA should be stored at 2–8°C and ideally should not have been frozen.
- Primer pairs designed by SeqTarget Primer Select are provided as lyophilized oligonucleotides and are reconstituted by adding 840 µl buffer TE, pH 8.0. For a detailed protocol, see the product sheet provided with the primer pair.
- Set up all reactions on ice.
- Always use an elongation temperature of 68°C.
- Always perform denaturation at 93°C for 15 s. Do not exceed this temperature.
- Use a final dNTP concentration of 500 µM.
- When using Q-Solution for the first time with a particular primer–template pair, always perform parallel reactions with and without Q-Solution; this recommendation should also be followed if another PCR additive (such as DMSO) was previously used for a particular primer–template pair. Q-Solution often enables or improves a suboptimal PCR 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, Q-Solution is used at just one working concentration, is nontoxic, and PCR purity is guaranteed. Adding Q-Solution to the PCR does not compromise PCR fidelity.

Q-Solution changes the melting behavior of DNA and can be used for PCR systems that do not work well under standard conditions. When using Q-Solution, the following effects may be observed, depending on the individual PCR assay:

Case A: Q-Solution enables amplification of a reaction that previously failed.

Case B: Q-Solution increases PCR specificity in certain primer–template systems.

Case C: Q-Solution has no effect on PCR performance.

Case D: Q-Solution causes reduced efficiency or failure of a previously successful amplification reaction. In this case, addition of Q-Solution disturbs the previously optimal primer–template annealing. Therefore, when using Q-Solution for the first time for a particular primer–template system, always perform reactions in parallel with and without Q-Solution.

Procedure

1. **Thaw 10x LongRange PCR Buffer, dNTP mix, primer solutions, and Q-Solution, if required.**

   Mix the solutions thoroughly before use.

2. **Prepare a reaction mix according to Table 1.**

   Note: We strongly recommend starting with an initial Mg^{2+} concentration of 2.5 mM as provided by 10x LongRange PCR Buffer.

   IMPORTANT: Set up all reactions on ice.

Table 1. Reaction composition for SeqTarget long-range PCR

Component	Volume per reaction	Final concentration
Reaction mix		
10x LongRange PCR Buffer with Mg^{2+}	2.5 µl	1x (2.5 mM Mg^{2+})
dNTP mix (10 mM each)	1.25 µl	500 µM of each dNTP
Optional: 5x Q-Solution	5 µl	1x (see pages 11 and 12)
RNase-free water	Variable	
Template DNA	Variable*	
LongRange PCR Enzyme Mix	0.2 µl	1 unit per 25 µl reaction
SeqTarget Primer Select primer pair (added at step 4)	2 µl†	0.8 µM each
Alternative: Primer A and B (added at step 4)	Variable	0.8 µM each
Total volume per reaction	**25 µl**	–

* Human genomic DNA (50–500 ng); bacterial DNA (100 pg–10 ng). For evaluation of the quality of prepared human genomic DNA, control primers supplied with the kit can be used. Add 2 µl of the control primer mix to set up a 25 µl reaction. The expected PCR product is 10 kb in size.

† SeqTarget primer pairs designed using GeneGlobe are delivered premixed and have a concentration of 10 µM each when reconstituted as recommended.

3. **Mix the reaction mix thoroughly and dispense appropriate volumes into PCR tubes or into wells of a PCR plate precooled on ice to 2–4°C.**
4. **Add the primer pair to each tube or well containing reaction mix.**
5. **Mix the reaction by vigorous shaking for 3 s and centrifuge briefly.**
6. **Program the thermal cycler according to the manufacturer's instructions, using the conditions outlined in Table 2 for PCR fragments 2–8 kb in size and Table 3 for PCR fragments larger than 8 kb.**

Note: When using a thermal cycler with a heated lid, do not use mineral oil. Purification and normalization with the SeqTarget Normalization Kit does not work with samples containing mineral oil.

Table 2. Optimized cycling protocol for long-range PCR (2–8 kb)

Step	Time	Temp.	Comments
Initial PCR activation step	**3 min**	**93°C**	Initial denaturation of template DNA.
3-step cycling:			**Important: Optimal performance is only assured using these cycling conditions**
Denaturation	**15 s**	**93°C**	Do not exceed this temperature.
Annealing	**30 s**	**62°C**	Approximately 5°C below T_m of primers (see Appendix B, Table 6, page 23).
Extension	**1 min/kb**	**68°C**	Use an extension time of 1 min/kb DNA for genomic DNA targets. For targets of low complexity such as phage or plasmid DNA, use 45 s/kb DNA.
Number of cycles	35		Amplification for 35 cycles will produce satisfactory results in most cases. However, the optimum number of cycles depends on the amount of template DNA. See Appendix C, page 26 for guidelines.
End of PCR cycling	Indefinite	4°C	

Table 3. Optimized cycling protocol for very long-range PCR (>8 kb)

Step	Time	Temp.	Comments
Initial PCR activation step	**3 min**	**93°C**	Initial denaturation of template DNA.
3-step cycling: 38 cycles in total*			**Important: Optimal performance is only assured using these cycling conditions**
First 10 cycles:			
Denaturation	**15 s**	**93°C**	Do not exceed this temperature.
Annealing	**30 s**	**62°C**	Approximately 5°C below T_m of primers (see Appendix B, Table 6, page 23).
Extension	**1 min/kb**	**68°C**	Use an extension time of 1 min/kb DNA.
Next 28 cycles:			
Denaturation	15 s	93°C	Do not exceed this temperature.
Annealing	30 s	62°C	Approximately 5°C below T_m of primers (see Appendix B, Table 6, page 23).
Extension	1 min/kb + 20 s in each additional cycle†	68°C	Use an extension time of approximately 1 min per kb DNA.

* Amplification for 38 cycles will produce satisfactory results in most cases. However, the optimum number of cycles depends on the amount of template DNA. See Appendix C, page 26 for guidelines.

† Program an extended extension time, referred to as "time increment", in which the extension time is increased by an increment of 20 s in each cycle. For example, for a 10 kb fragment, program an extension time of 10 min 20 s in the 11th cycle, 10 min 40 s in the 12th, 11 min in the 13th, etc.

7. For a simplified hot start, place the tubes or plate immediately into a thermal cycler that is preheated to 93°C and start the cycling program as outlined in Table 2 or 3.

Note: Use the simplified hot start to ensure specificity in the PCR. After amplification, samples can be stored overnight at 2–8°C or for longer periods at –20°C.

8. **Analyze samples using an appropriate detection system such as agarose gel electrophoresis.**

   For further processing of the PCR fragment, see Appendix E, page 27.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocol in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

	Comments and suggestions
Little or no product	
a) Pipetting error or missing reagent	Repeat the PCR. Check the concentrations and storage conditions of reagents, including primers and template.
b) Insufficient template	Increase the amount of template used in PCR. Use the values given in Table 1 (page 13) as a starting point. See Appendix A, page 22, for information on template amounts and corresponding cycle numbers and enzyme concentrations. High-quality templates are essential for amplification of long targets.
c) PCR conditions not optimal	Using the same cycling conditions, repeat the PCR using Q-Solution. Vary the concentration of Q-Solution in steps of 0.25x to between 0.5x and 1.25x. Start with 1x. If Q-Solution has no effect, try adding 2.5–10% DMSO in steps of 2.5%.
d) Primer concentration not optimal	Use a concentration of 0.8 µM for each primer. For calculation of primer concentration, see Appendix B, page 23.
e) Enzyme concentration too low	If necessary, increase the amount of LongRange PCR Enzyme Mix in steps of 0.5 U.
f) Insufficient number of cycles	Increase the number of cycles in steps of 5 cycles (see Appendix C, page 26).

	Comments and suggestions
g) Problems with template	Check the concentration, storage conditions, and quality of template (see Appendix A, page 22). If necessary, make new serial dilutions of template nucleic acid from stock solutions and repeat the PCR using the new dilutions. Degraded template nucleic acid is not suitable for amplification of long target sequences.
h) Incorrect annealing temperature or time	Use an annealing temperature 5°C below the T_m of your primers. See Appendix B (page 23) for information on determining the annealing temperature of your primers. Difficulties in determining the optimal annealing temperature can often be overcome by performing a gradient PCR (see Appendix D, page 27). Use the optimized annealing time of 30 s.
i) Incorrect denaturation temperature or time	Denaturation should be at 93°C for 15 s. Ensure that the initial incubation for 3 min at 93°C was performed as described in step 6 of the protocol (page 13).
j) Primer design not optimal	Review primer design (see Appendix B, page 23).
k) Problems with the thermal cycler	Check the power to the thermal cycler and that the thermal cycler has been correctly programmed.
l) Use of incorrect consumables	Check the fit of the PCR tube caps in the thermal cycler. Poor thermal contact prevents effective temperature transfer. Use only consumables recommended by the manufacturer of your thermal cycler.
m) Air bubbles in PCR tube	Do not allow air bubbles to become trapped after mixing the reaction master mixes. Air bubbles prevent homogenous temperature distribution throughout the reaction volume.
n) Evaporation during thermal cycling	Check the fit of PCR tube caps or sealing foils on PCR plates. Long-range PCR is especially sensitive to evaporation.

	Comments and suggestions
Product is multibanded	
a) PCR cycling conditions not optimal	Using the same cycling conditions, repeat the PCR using Q-Solution.
b) Annealing temperature too low or annealing time incorrect	Use an annealing temperature 5°C below the T_m of your primers. See Appendix B, page 23, to determine the annealing temperature of your primers. Difficulties in determining the optimal annealing temperature can often be overcome by performing a gradient PCR (see Appendix D, page 27). First use the optimized annealing time of 30 s. If this is unsuccessful, reduce the annealing time in decrements of 10 s to a minimum of 10 s.
c) Incorrect extension time	Adjust the length of the extension step according to the size of the expected PCR product (see Tables 2 and 3, pages 14 and 15, respectively).
d) Primer concentration not optimal or primers degraded	Use a concentration of 0.8 µM for each primer. For calculation of primer concentration, refer to Appendix B (page 23). Check that primers have not degraded by analysis on a denaturing polyacrylamide gel,* particularly when performing highly sensitive PCR.
e) Primer design not optimal	Review primer design (see Appendix B, page 23). Design new or longer primers.
f) Q-Solution not used	Repeat the experiment, performing parallel reactions with and without Q-Solution.
g) Template concentration too high	Reduce the amount of template DNA.
h) Too many cycles	Reduce the number of cycles in steps of 2.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

	Comments and suggestions
Product is smeared	
a) Insufficient starting template	Increase the amount of template. Use the values given in Table 1 (page 13) in the protocol as a starting point. Also, see Appendix A, page 22, for information on template amounts and corresponding cycle numbers and enzyme concentrations.
b) Incorrect enzyme concentration	Reduce the amount of enzyme in decrements of 0.5 U.
c) Mg^{2+} concentration not optimal	Use an initial Mg^{2+} concentration of 2.5 mM as provided by the LongRange PCR Buffer. In very rare cases, an increased Mg^{2+} concentration may improve PCR performance. Increase the concentration of Mg^{2+} ions in increments of 0.25 mM.
d) Suboptimal ratio of Mg^{2+} ions to dNTP	Check the ratio of Mg^{2+} ions to dNTPs. A final dNTP concentration of 500 µM requires 2.5 mM Mg^{2+} ions, as recommended in the protocol.
e) Primer design not optimal	Review primer design (see Appendix B, page 23).
f) Too many cycles	Reduce the number of cycles in steps of 4.
g) Elongation step too short	Increase the length of the elongation step. Use a minimum of 1 min/kb. For very long targets, see cycling conditions in Table 3, page 15).
Poor PCR fidelity	
a) Insufficient starting template	Increase the amount of template used. Use the values given in Table 1 (page 13) in the protocol as a starting point. Also, see Appendix A, page 22 for information on template amounts and corresponding cycle numbers and enzyme concentrations.
b) Mg^{2+} concentration too high	Optimal PCR fidelity using LongRange PCR Enzyme Mix is achieved using Mg^{2+} concentrations of 2.5 mM (as supplied). Higher Mg^{2+} concentrations will lead to lowered fidelity.

	Comments and suggestions
Poor performance in very long-range PCR	
a) Poor quality of DNA template	Perform denaturation at 93°C for 15 s. Prolonged denaturation may damage template nucleic acid.
b) Suboptimal concentration of dNTPs	Use a concentration of 500 µM for each dNTP. Increasing the dNTP concentration may negatively affect PCR results.

Appendix A: Starting Template

Both the quality and quantity of nucleic acid starting template affect PCR, in particular the sensitivity and efficiency of amplification.* These factors are especially important for amplification of long targets.

Quality of starting template

Since PCR consists of multiple rounds of enzymatic reactions, it is more sensitive to impurities such as proteins, phenol/chloroform, salts, ethanol, EDTA, and other chemical solvents than single-step enzyme-catalyzed processes. QIAGEN offers a complete range of nucleic acid preparation systems, ensuring the highest-quality templates for PCR. These include the QIAprep®, QIAamp, DNeasy, FlexiGene, and EZ1 systems for rapid purification of genomic DNA and viral nucleic acids. For more information about these products, contact one of our Technical Service Departments (see back cover) or visit www.qiagen.com/productfinder.

Quantity of starting template

The annealing efficiency of primer to template is an important factor in PCR. Owing to the thermodynamic nature of the reaction, the primer to 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 an initial guide, spectrophotometric and molar conversion values for different nucleic acid templates are listed in Tables 4 and 5, respectively. For optimal template amounts to be used with the SeqTarget LongRange PCR system, see Table 1 on page 13. Human genomic DNA can be evaluated for its suitability as template for SeqTarget LongRange PCR by a control PCR using the primer pair supplied with the kit.

Table 4. Spectrophotometric conversions for DNA templates

1 A_{260} unit†	Concentration (µg/ml)
Double-stranded DNA	50
Single-stranded DNA	33

† Absorbance at 260 nm = 1

* For further information see our guide *Critical Factors for Successful PCR*. To obtain a copy, visit the QIAGEN Web site at www.qiagen.com or call one of the QIAGEN Technical Service Departments or local distributors (see back cover).

Table 5. Molar conversions for nucleic acid templates

Organism	Size	pmol/µg	Molecules/µg
Escherichia coli	4.7×10^{6}*	3.0×10^{-4}	1.8×10^{8}†
Drosophila melanogaster	1.4×10^{8}*	1.1×10^{-5}	6.6×10^{5}†
Mus musculus (mouse)	2.7×10^{9}*	5.7×10^{-7}	3.4×10^{5}†
Homo sapiens (human)	3.3×10^{9}*	4.7×10^{-7}	2.8×10^{5}†

* Base pairs in haploid genome.

† For single-copy genes.

Appendix B: Primer Design, Concentration, and Storage

Standard PCR primers

Prerequisites for successful PCR include the design of optimal primer pairs, the use of appropriate primer concentrations, and the correct storage of primer solutions. Some general guidelines are given below

Length: 18–30 nucleotides

G/C content: 40–60%

T_m: Simplified formula for estimating melting temperature (T_m): $T_m = 2°C \times (A+T) + 4°C \times (G+C)$

Whenever possible, design primer pairs with similar T_m values.

Optimal annealing temperatures may be above or below the estimated T_m. As a starting point, use an annealing temperature 5°C below T_m.

Sequence:

- Avoid complementarity of two or three bases at the 3' ends of primer pairs to reduce primer–dimer formation.
- Avoid mismatches between the 3' end of the primer and the target-template sequence.

- Avoid runs of 3 or more G or C at the 3' end.
- Avoid a 3'-end T. Primers with a T at the 3' end have a greater tolerance of mismatch.
- Avoid complementary sequences within a primer sequence and between the primer pair.
- Commercially available computer software (e.g., Primer Designer 1.0, Scientific Software, 1990; Oligo, Rychlik, and Rhoads, 1989) can be used for primer design.
- Visit www.qiagen.com/GeneGlobe for more information.

Concentration:

- Spectrophotometric conversion for primers: 1 A_{260} unit = 20–30 µg/ml
- Molar conversions:

Primer length	pmol/µg	50 pmol
20mer	152	329 ng
25mer	121	413 ng
30mer	101	495 ng
35mer	92	543 ng

Storage:

Lyophilized primers should be dissolved in a small volume of TE* (10 mM Tris·Cl, 1mM EDTA, pH 8.0) to obtain a 50 or 100 µM stock solution.
Prepare small aliquots of working solutions containing 10 µM (10 pmol/µl) to avoid repeated thawing and freezing. Store all primer solutions at –20°C. Primer quality can be checked on a denaturing polyacrylamide gel;* please call one of the QIAGEN Technical Service departments or local distributor for a protocol (see back cover).

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

Degenerate PCR primers

Occasionally, the exact nucleotide sequence of the target-template DNA will not be known, for instance when it has been deduced from an amino acid sequence. To enable such templates to be amplified by PCR, degenerate primers can be used. These are actually mixtures of several primers whose sequences differ at the position that correspond to the uncertainties in the template sequence. See below for recommendations for further optimizing PCR using degenerate primers. Table 6 shows the codon redundancy of each amino acid.

Sequence:

- Avoid degeneracy in the 3 nucleotides at the 3' end.
- If degeneracy cannot be avoided at the 3'-terminal bases, the oligonucleotide supplier can synthesize primers with one phosphorothioate bond between the two 3'-terminal nucleotides.
- If possible, use Met- or Trp-encoding triplets at the 3' end.
- To increase primer–template binding efficiency, reduce degeneracy by allowing mismatches between the primer and template, especially towards the 5' end (but not at the 3' end).
- Try to design primers with less than 4-fold degeneracy at any given position.

Concentration:

- Begin PCR with a primer concentration of 0.8 μM.
- If PCR efficiency is poor, increase primer concentrations in increments of 0.1 μM until satisfactory results are obtained.

Table 6. Codon redundancy

Amino acid	Number of codons
Met, Trp	1
Cys, Asp, Glu, Phe, His, Lys, Asn, Gln, Tyr	2
Ile	3
Ala, Gly, Pro, Thr, Val	4
Leu, Arg, Ser	6

Appendix C: Number of PCR Cycles

When using the SeqTarget LongRange PCR Kit, a typical cycling program consists of 35–38 cycles, depending on the number of copies of the starting template. Table 7 provides general guidelines for choosing the number of cycles.

Table 7. General guidelines for choosing the number of PCR cycles

Number of copies of starting template*	*E. coli* DNA†	Human genomic DNA†	Number of cycles
$1 \times 10^4 – 5 \times 10^4$	5.56–278 pg	3.6–179 ng	35–40
$5 \times 10^4 – 2 \times 10^5$	278 pg – 2.78 ng	179 ng – 537 ng‡	35–40
$2 \times 10^5 – 2 \times 10^6$	2.8 ng – 28 ng	N.R§.	30–35
$2 \times 10^6 – 5 \times 10^6$	28–70 ng	N.R§.	25–30

* Refer to Table 5 (page 23) to calculate the number of molecules for template DNA different than the examples given.

† Refers to single-copy genes.

‡ The recommended upper limit for human genomic DNA is approx. 500 ng.

§ N.R.: Not recommended to use such high amounts of starting template. Use the higher number of PCR cycles for maximum PCR product yield.

Appendix D: Gradient PCR

Many thermal cyclers have a temperature-gradient function. Using this function, it is possible to easily determine optimal annealing temperatures by generating a temperature gradient across the heating block for the annealing step. If your primers conform to the criteria specified in Appendix B on page 23, we recommend using a gradient program that includes a temperature range from 50–70°C. In order to determine optimal annealing conditions, prepare 3 identical reactions and place in the block positions that most closely correspond to the temperatures 1°C, 5°C, and 8°C below the calculated T_m of your primers.

Appendix E: Purification of PCR Products

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, and probably nonspecific amplification products. Before the specific PCR product can be used in subsequent experiments, it is often necessary to remove these contaminants. For next-generation sequencing library preparation, the samples can be directly used for normalization with the SeqTarget Normalization Kit. PCR cleanup is not required because the SeqTarget Normalization Kit includes a purification step to remove small, nonspecific PCR fragments (<2000 bp). For more information on the SeqTarget Normalization Kit, please call QIAGEN Technical Services or your local distributor (see back cover). For other downstream applications, the QIAquick® system offers a quick and easy method for purifying the final PCR product (70 bp–10 kb). Using the MinElute® system, PCR products (70 bp–4 kb) can be purified in higher concentrations due to the low elution volumes needed in this system. In addition, the QIAEX® II Gel Extraction Kit can be used for purification of PCR products 40 bp–50 kb in length. Gel loading reagent and tracking dyes are effectively removed with the QIAquick and MinElute system. For more information about QIAquick and MinElute products, please call QIAGEN Technical Services or your local distributor (see back cover).

Appendix F: Control of Contamination

It is extremely important to include at least one negative control that lacks the template nucleic acid in every PCR setup 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. Ideally, use separate rooms.
- Use a separate set of pipets for the PCR master mix. Use of pipet tips with hydrophobic filters 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 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 laborious, however, and its efficiency is difficult to control and cannot be guaranteed. We recommend storing solutions in small aliquots and using fresh aliquots 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.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

† Most commercial bleach solutions are approximately 5.25% sodium hypochlorate. Sodium hypochlorate is an irritant and should be handled with caution. When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

Ordering Information

Product	Contents	Cat. no.
SeqTarget LongRange PCR Kit (500)	For 500 x 25 µl reactions: LongRange PCR Enzyme Mix (500 U), LongRange PCR Buffer, 5x Q-Solution, RNase-Free Water, 10 mM dNTPs, Control Primer Pair	122117
Related products		
SeqTarget Primer Select — for primer design and convenient ordering at GeneGlobe (www.qiagen.com/GeneGlobe)		
SeqTarget Primer Select	Tube format: 8.4 nmol SeqTarget Primer Select (containing a mix of lyophilized forward and reverse primers for a specific target); for 400 x 25 µl reactions	122001
SeqTarget Primer Select 96 Plate	96-well plate format: 8.4 nmol SeqTarget Primer Select (containing a mix of lyophilized forward and reverse primers for a specific target); for 400 x 25 µl reactions	122004
SeqTarget Normalization Kit – for purification and normalization of long range PCR products		
SeqTarget Normalization Kit (480)	For 480 preps: QIAquick 96 Plates, Bead Filter 96 Plates, Normalization Beads, Buffer PBN1, Buffer PBN2, Buffer EB, Buffer EB2, Mineral Oil	122217
QIAGEN LongRange PCR Kit — for sensitive and accurate long-range PCR		
QIAGEN LongRange PCR Kit (20)*	For 20 x 50 µl reactions: LongRange PCR Enzyme Mix (40 U), LongRange PCR Buffer, 5x Q-Solution, RNase-Free Water, 10 mM dNTPs	206401
dNTP Mix — For sensitive and reproducible PCR and RT-PCR		
dNTP Mix, PCR Grade (200 µl)*	Mix containing 10 mM each of dATP, dCTP, dGTP, and dTTP (1 x 200 µl)	201900

* Larger kit sizes available; see www.qiagen.com.

Product	Contents	Cat. no.
QIAamp DNA Mini Kit — For isolation of genomic, mitochondrial, bacterial, parasite, or viral DNA		
QIAamp DNA Mini Kit (50)*	For 50 DNA preps: 50 QIAamp Mini Spin Columns, QIAGEN Proteinase K, Buffers, Collection Tubes (2 ml)	51304

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* Larger kit sizes available; see www.qiagen.com.

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