October 2010

QIAGEN® LongRange PCR Handbook

For reliable and accurate long-range PCR up to 40 kb

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Kit Contents

QIAGEN LongRange PCR Kit			
Catalog no.	**206401**	**206402**	**206403**
No. of units	**40**	**200**	**500**
No. of 50 µl reactions	**20**	**100**	**250**
LongRange PCR Enzyme Mix	8 µl	40 µl	100 µl
LongRange PCR Buffer, 10x	100 µl	500 µl	1250 µl
dNTP Mix	50 µl	250 µl (1 x 200 µl and 1 x 50 µl)	600 µl (3 x 200 µl)
Q-Solution®, 5x	400 µl	2 ml	2ml
RNase-Free Water	1.9 ml	5.7 ml (3 x 1.9 ml)	11.4 ml (6 x 1.9 ml)
$MgCl_2$	1.2 ml	1.2 ml	2.4 ml (2 x 1.2 ml)
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Shipping and Storage

The QIAGEN 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 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).

Quality Control

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

Product Use Limitations

The QIAGEN LongRange PCR Kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

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 molecular biology and the use of QIAGEN products. If you have any questions or experience any difficulties regarding the QIAGEN 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).

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/ts/msds.asp 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 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 (terminal transferase activity):	Yes

Buffers and reagents

LongRange PCR Buffer, 10x:	10x concentrated, contains 25 mM Mg^{2+}
Q-Solution:	5x concentrated
dNTP mix:	10 mM of each: dATP, dCTP, dGTP, dTTP
RNase-free water:	Ultrapure quality, PCR-grade
Enzyme storage buffer:	LongRange Enzyme Mix: Tris·HCl pH 8.0 (at 25°C), KCl, EDTA, DTT, 50% glycerol, Tween® 20, Igepal® CA-630

Introduction

The QIAGEN LongRange PCR Kit combines a powerful polymerase blend with an innovative buffer system designed for efficient amplification of long targets up to 40 kb. It also provides reliable amplification of smaller fragments of 0.1 to 10 kb with a simplified optimized protocol. Additionally, it facilitates amplification of GC-rich regions and other difficult templates.

The LongRange PCR Enzyme Mix is a blend of thermostable DNA polymerases with enhanced processivity. This provides both a very high extension rate and a proofreading ability, leading to increased fidelity. The LongRange PCR Buffer is designed to ensure long-range PCR with no optimization required, enabling reliable amplification of genomic targets longer than 20 kb and episomal targets up to 40 kb. It has a unique zwitterionic formulation that improves buffering of pH at high temperatures (72–94°C), thus minimizing pH-driven template degradation. In combination with Q-Solution (provided with the kit), it enables amplification of GC-rich templates.

Q-Solution

The QIAGEN LongRange PCR Kit includes Q-Solution, an innovative PCR additive that facilitates amplification of difficult templates by modifying the melting behavior of DNA. This unique reagent 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. For further information, refer to the protocol for long-range PCR using Q-Solution, page 14.

Applications

Applications of the QIAGEN LongRange PCR Kit include amplification of:

- Long targets (all targets in the range 0.1–10 kb)
- Very long targets (genomic DNA >10 kb; episomal DNA 10–40 kb)
- Long cDNA templates from reverse transcription reactions (e.g., those generated using the QIAGEN LongRange 2Step RT-PCR Kit [see Ordering Information])
- GC-rich and other difficult templates

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, please 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 H, page 36)
- Thermal cycler
- PCR tubes (use thin-walled 0.2 ml PCR tubes recommended by the manufacturer of your thermal cycler)
- Primers and template DNA
- For thermal cyclers without a heated lid: mineral oil
- For analysis of PCR products: agarose-gel electrophoresis system

Important Notes

Protocol selection

The LongRange PCR Kit can be used to amplify a range of targets, including human genomic DNA, cDNA, bacterial DNA, and plasmid DNA. The most suitable protocol for different templates is shown in Table 1.

Table 1. Protocol Selection According to Template Size

Size and nature of template	Protocol
0.1–10 kb	Long-range PCR (page 11)
0.1–10 kb, GC-rich or other difficult templates	Long-range PCR using Q-Solution (page 14)
>10 kb	Very long-range PCR (page 18)

Template DNA

- Ensure that the template DNA or cDNA is of sufficiently high quality and is not degraded.
- Use high-molecular-weight DNA templates only, with average size >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 should have annealing temperatures above 60°C, as determined by the 4+2 rule (see Appendix B, page 29 for further information).

Amplification

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

Analysis of PCR products

- Analyze PCR products on an agarose gel under appropriate running conditions (see Appendix D, Table 17, page 32 for further information). Alternatively, fast and fully automated PCR analysis can be performed using the QIAxcel system (cat. no. 9001421). The QIAxcel utilizes reusable capillary electrophoresis cartridges to allow convenient high-resolution analysis of up to 96 samples per run. For more information, visit www.qiagen.com .

Protocol: Long-Range PCR

This protocol is designed for amplification of targets up to approximately 10 kb in size.

Important points before starting

- Read the Important Notes on page 10 before starting the protocol.
- 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.

Procedure

1. **Thaw 10x LongRange PCR Buffer, dNTP mix, primer solutions, and RNase-free water.**

   Mix the solutions thoroughly before use.

2. **Prepare a reaction mix as shown in Table 2.**

   We strongly recommend starting with an initial Mg^{2+} concentration of 2.5 mM as provided by the LongRange PCR buffer.

   Important: Set up all reactions on ice.

Table 2. Composition of Reaction Mix for Long-Range PCR (0.1–10 kb)

Component	Volume in each reaction	Final concentration
Reaction mix		
LongRange PCR Buffer with Mg^{2+}, 10x	5 µl	1x; 2.5 mM Mg^{2+}
dNTP mix (10 mM each)	2.5 µl	500 µM of each dNTP
Primer A	Variable	0.4 µM
Primer B	Variable	0.4 µM
RNase-free water	Variable	
LongRange PCR Enzyme Mix	0.4 µl	2 units per 50 µl reaction
Template DNA		
Added at step 4	Variable	See Table 3
Total volume	**50 µl**	

3. **Mix the reaction mix thoroughly and dispense appropriate volumes into PCR tubes.**
4. **Add template DNA to each tube containing reaction mix, following the amounts recommended in Table 3.**

   For two-step RT-PCR, the volume of cDNA added as template from the reverse transcription reaction should not exceed 10% of the final PCR volume. See Appendix E, page 33, for more information about RT-PCR.

Table 3. Amount of Template for Long-Range PCR (0.1–10 kb)

Type of template*	Optimal amount
Human genomic DNA	50–500 ng
cDNA†	50–500 ng
Bacterial DNA	100 pg–10 ng
Phage DNA	1–100 ng
Plasmid DNA	1–20 ng

* Refer to the section "Template DNA" in the Important Notes, page 10.

† We recommend that cDNA is generated using the QIAGEN LongRange 2Step RT-PCR Kit (catalog no. 205920) or an equivalent method.

5. **Program the thermal cycler according to the manufacturer's instructions, using the conditions outlined in Table 4.**

   When using a thermal cycler with a heated lid, do not use mineral oil.

Table 4. Cycling Protocol for Long-Range PCR (0.1–10 kb)

			Comments
Initial activation step:	3 min	93°C	Initial denaturation of template DNA.
3-step cycling:			
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 13, page 29).
Extension	1 min/kb	68°C	Use an extension time of 1 min per kilobase DNA for genomic DNA targets
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 32 for guidelines.
End of PCR cycling:	Indefinite	4°C	

6. **For a simplified hot start, place the tubes immediately into a thermal cycler that is preheated to 93°C and start the cycling program as outlined in Table 4.**

   Use the simplified hot start to ensure PCR specificity.

   After amplification, samples can be stored overnight at 2–8°C or for longer periods at –20°C.

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

   See Appendix D, Table 17, page 32, for choosing the optimal percentage of agarose.

   A TA/ UA cloning system can be used for direct cloning of amplified fragments (see Appendix I, page 37).

Protocol: Long-Range PCR Using Q-Solution

This protocol is designed for using Q-Solution in PCR assays for amplification of targets of up to approximately 10 kb.

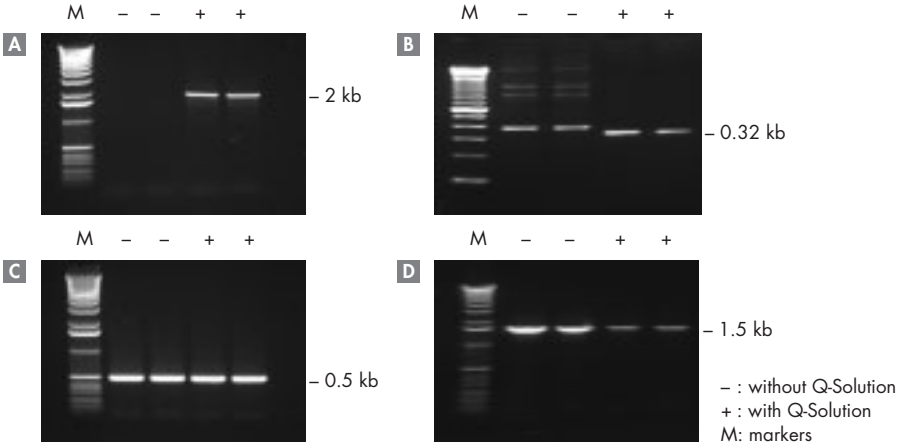
Q-Solution changes the melting behavior of DNA and can be used for PCR systems that do not work well under standard conditions. When using Q-Solution, the following effects may be observed, depending on the individual PCR assay:

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.



Important points before starting

- Read the Important Notes on page 10 before starting the protocol.
- 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.

Procedure

1. **Thaw 10x LongRange PCR Buffer, dNTP mix, primer solutions, and Q-Solution.**
   Mix the solutions thoroughly before use.
2. **Prepare a reaction mix according to Table 5.**
   We strongly recommend starting with an initial Mg^{2+} concentration of 2.5 mM as provided by the LongRange PCR buffer.
   Important: Set up all reactions on ice.

Table 5. Composition of Reaction Mix for Long-Range PCR Using Q-Solution (0.1–10 kb)

Component	Volume in each reaction	Final concentration
Reaction mix		
LongRange PCR Buffer with Mg^{2+}, 10x	5 µl	1x; 2.5 mM Mg^{2+}
dNTP mix (10 mM each)	2.5 µl	500 µM of each dNTP
5x Q-Solution	10 µl	1x
Primer A	Variable	0.4 µM
Primer B	Variable	0.4 µM
RNase-free water	Variable	
LongRange PCR Enzyme Mix	0.4 µl	2 units per 50 µl reaction
Template DNA		
Added at step 4	Variable	See Table 6
Total volume	**50 µl**	

3. **Mix the reaction mix thoroughly and dispense appropriate volumes into PCR tubes.**
4. **Add template DNA to each tube containing reaction mix, following the amounts recommended in Table 6.**
   For two-step RT-PCR, the volume of cDNA added as template from the reverse transcription reaction should not exceed 10% of the final PCR volume. See Appendix E, page 33, for more information about RT-PCR.

Table 6. Amount of Template for Long-Range PCR Using Q-Solution (0.1–10 kb)

Type of template*	Optimal amount
Human genomic DNA	50–500 ng
cDNA†	50–500 ng
Bacterial DNA	100 pg–10 ng
Phage DNA	1–100 ng
Plasmid DNA	1–20 ng

* Refer to the section "Template DNA" in the Important Notes, page 10.

† We recommend that cDNA is generated using the QIAGEN LongRange 2Step RT-PCR Kit (catalog no. 205920) or an equivalent method.

5. Program the thermal cycler according to the manufacturer's instructions, using the conditions outlined in Table 7

When using a thermal cycler with a heated lid, do not use mineral oil.

Table 7. Cycling Protocol for Long-Range PCR Using Q-Solution (0.1–10 kb)

			Comments
Initial activation step:	3 min	93°C	Initial denaturation of template DNA.
3-step cycling:			
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 13, page 29).
Extension	1 min/kb	68°C	Use an extension time of 1 min per kilobase DNA for genomic DNA targets For targets of low complexity such as phage or plasmid DNA, use 45 s per kilobase 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 32 for guidelines.
End of PCR cycling:	Indefinite	4°C	

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

   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.

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

   See Appendix D, Table 17, page 32, for choosing the optimal percentage of agarose.

   A TA/ UA cloning system can be used for direct cloning of amplified fragments (see Appendix I, page 37).

Protocol: Very Long-Range PCR

This protocol is optimized for amplification of PCR templates longer than 10 kb.

Important points before starting

- Read the Important Notes on page 10 before starting the protocol.
- Use high-molecular-weight template DNA only and ensure that it is not degraded.
- Template DNA should be stored at 2–8°C and ideally should not have been frozen.
- Set up all reactions on ice.
- Always use an elongation temperature of 68°C.
- Always use denaturation conditions of 93°C for 15 s. Do not exceed this temperature.
- Use a final dNTP concentration of 500 µM.
- For difficult targets, the use of 1x Q-Solution may improve results.

Procedure

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

   Mix the solutions thoroughly before use.

2. **Prepare a reaction mix according to Table 8, page 19.**

   We strongly recommend starting with an initial Mg^{2+} concentration of 2.5 mM as provided by the LongRange PCR buffer.

   Important: Set up all reactions on ice.

Table 8. Composition of Reaction Mix for Very Long-Range PCR (>10 kb)

Component	Volume in each reaction	Final concentration
LongRange PCR Buffer with Mg^{2+}, 10x	5 µl	1x; 2.5 mM Mg^{2+}
dNTP mix (10 mM each)	2.5 µl	500 µM of each dNTP
Optional: 5x Q-Solution	10 µl	1x
Primer A	Variable	0.4 µM
Primer B	Variable	0.4 µM
RNase-free water	Variable	
LongRange PCR Enzyme Mix	0.4 µl	2 units per 50 µl reaction
Template DNA		
Added at step 4	Variable	See Table 9
Total volume	**50 µl**	

3. **Mix the reaction mix thoroughly and dispense appropriate volumes into PCR tubes.**
4. **Add template DNA to each tube containing reaction mix, following the amounts recommended in Table 9.**

   For two-step RT-PCR, the volume of cDNA added as template from the RT reaction should not exceed 10% of the final PCR volume. See Appendix E, page 33, for more information about RT-PCR.

Table 9. Amount of Template DNA for Very Long-Range PCR (>10 kb)

Type of template*	Optimal amount
Human genomic DNA	100–500 ng
cDNA†	100–500 ng
Bacterial DNA	100 pg–10 ng
Phage DNA	1–100 ng
Plasmid DNA	1–20 ng

* Refer to the section "Template DNA" in the Important Notes, page 10.

† We recommend that cDNA is generated using the QIAGEN LongRange 2Step RT-PCR Kit (catalog no. 205920) or an equivalent method.

5. **Program the thermal cycler according to the manufacturer's instructions, using the conditions outlined in Table 10.**

   When using a thermal cycler with a heated lid, do not use mineral oil.

Table 10. Cycling Protocol for Very Long-Range PCR (>10 kb)

			Comments
Initial activation step:	3 min	93°C	Initial denaturation of template DNA.
3-step cycling: 38 cycles total*			
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 13, page 29).
Extension	1 min/kb	68°C	Use an extension time of approximately 1 min per kilobase 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 13, page 29).
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 32 for guidelines.

† Program an extended extension time, referred to as "Time Increment", in which the extension time is increased by increments 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.

6. **For a simplified hot start, place the tubes immediately into a thermal cycler that is pre-heated to 93°C and start the cycling program as outlined in Table 10.**

   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.

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

   See Appendix D, Table 17, page 32, for choosing the optimal percentage of agarose.

   For separation of products >25 kb in a standard electrophoresis chamber, a 12-hour run at 40 V is recommended.

   Generally, efficient separation of very large DNA fragments is achieved only by field inversion or by pulsed field gel electrophoresis.

   A TA/ UA cloning system can be used for direct cloning of amplified fragments (see Appendix I, page 37).

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 protocols 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 Tables 3, 6, or 9 in the relevant protocol as a starting point. See Appendix A, page 27, 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, following the protocol on page 14.
d)	Primer concentration not optimal	Use a concentration of 0.4 µM of each primer. For calculation of primer concentration, see Appendix B, page 29.
e)	Enzyme concentration too low	If necessary, increase the amount of LongRange PCR Enzyme Mix in steps of 0.5 U. See Appendix C, Table 16, page 32 for further suggestions.
f)	Insufficient number of cycles	Increase the number of cycles in steps of 5 cycles (see Appendix C, page 32). Refer to the protocols and to Appendix A, page 27, to optimize numbers of cycles and enzyme concentrations for different amounts of template.

		Comments and suggestions
g)	Problems with template	Check the concentration, storage conditions, and quality of template (see Appendix A, page 27). 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 29, for how 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 F, page 35). 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 5 of the PCR protocols (pages 12, 16, and 20)
j)	Primer design not optimal	Review primer design (see Appendix B, page 29).
k)	Reverse transcription reaction error	Refer to the *QIAGEN LongRange 2step RT-PCR Handbook*. For RT-PCR, consider the efficiency of the reverse transcription reaction, which is usually between 10 and 30%. Oligo-dT primers rather than random primers in the reverse transcription reaction are strongly recommended for amplification of long cDNA species. The volume of reverse transcription reaction added should not exceed 10% of the final PCR volume (see Appendix E, page 33).
l)	Problems with the thermal cycler	Check the power to the thermal cycler and that the thermal cycler has been correctly programmed.

		Comments and suggestions
m)	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.
n)	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.
o)	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.
Product is multibanded		
a)	PCR cycling conditions not optimal	Using the same cycling conditions, repeat the PCR using Q-Solution, following the protocol on page 14.
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 29, for how 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 F, page 35). 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 4, 7, or 10).

		Comments and suggestions
d)	Primer concentration not optimal or primers degraded	Use a concentration of 0.4 µM of each primer. For calculation of primer concentration, refer to Appendix B, page 29. 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 29). Design new or longer primers.
f)	Q-Solution not used	Repeat the experiment, performing parallel reactions with and without Q-Solution (see Protocol: Long-Range PCR Using Q-Solution, page 14).
g)	Template concentration too high	Reduce the amount of template DNA.
h)	Too many cycles	Reduce the number of cycles in steps of 2.
Product is smeared		
a)	Insufficient starting template	Increase the amount of template. Use the values given in Tables 3, 6, or 9 in the relevant protocol as a starting point. Also see Appendix A, page 27, for information on template amounts and corresponding cycle numbers and enzyme concentrations.
b)	Poor analysis by agarose gel electrophoresis	Prepare gels using a suitable concentration of agarose and run gels under appropriate conditions (see Table 17, page 32). Generally, efficient separation of very large DNA fragments (>approximately 30 kb) is achieved only by field inversion or by pulsed field gel electrophoresis.
c)	Incorrect enzyme concentration	Reduce the amount of enzyme in decrements of 0.5 U.

* 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
d)	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.
e)	Suboptimal ratio of Mg^{2+} ions:dNTP	Check the ratio of Mg^{2+} ions:dNTPs. A final dNTP concentration of 500 µM requires 2.5 mM Mg^{2+} ions, as recommended in the protocols.
f)	Primer design not optimal	Review primer design (see Appendix B, page 29).
g)	Too many cycles	Reduce the number of cycles in steps of 4.
h)	Elongation step too short	Increase the length of the elongation step. Use a minimum of 1min/kb. For very long targets see protocol on page 18.
Poor PCR fidelity		
a)	Insufficient starting template	Increase the amount of template used. Use the values given in Tables 3, 6, or 9 in the relevant protocol as a starting point. Also see Appendix A, page 27, 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.
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 of each dNTP. Increasing the dNTP concentration may negatively affect PCR results.

Appendix A: Starting Template

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, such as the QIAprep® system for rapid plasmid purification, the QIAamp, DNeasy, FlexiGene, and EZ1 systems for rapid purification of genomic DNA and viral nucleic acids, and the RNeasy® system for RNA preparation from a variety of sources. For more information about other QIAGEN products, contact one of our Technical Service Departments (see back cover).

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: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 11 and 12 respectively. For optimal template amounts to be used with the LongRange PCR System, see Table 16 on page 32 and Tables 3, 6, and 9 on pages 12, 16, and 20 respectively.

* 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).

† 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.

Table 11. Spectrophotometric Conversions for Nucleic Acid Templates

1 A_{260} unit*	Concentration (µg/ml)
Double-stranded DNA	50
Single-stranded DNA	33
Single-stranded RNA	40

* Absorbance at 260 nm = 1

Table 12. Molar Conversions for Nucleic Acid Templates

Nucleic acid	Size	pmol/µg	Molecules/µg
1 kb DNA	1000 bp	1.52	9.1×10^{11}
2 kb DNA	2000 bp	0.76	4.6×10^{11}
pUC19 DNA	2686 bp	0.57	3.4×10^{11}
pBluescript® II DNA	2961 bp	0.52	3.1×10^{11}
pBR322	4361 bp	0.35	2.1×10^{11}
Lambda DNA	48,502 bp	0.03	1.8×10^{10}
Average mRNA	1930 nt	1.67	1.0×10^{12}
Genomic DNA			
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 in Table 13.

Table 13. General Guidelines for Standard PCR Primers

Length:	20–35 nucleotides
G/C content:	40–60%
T_m :	Simplified formula for estimating melting temperature (T_m): T_m = 2°C x (A+T) + 4°C x (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., OLIGO 6, Rychlik, 1999) or web-based tools such as Primer3, Steve Rosen and Helen Skaletsky, 2000 (www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi), can be used for primer design.
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

- For most applications, use 1 µM of each primer (50 pmol of each primer per 50 µl reaction).

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. Table 14 gives recommendations for further optimizing PCR using degenerate primers. Table 15 shows the codon redundancy of each amino acid.

Table 14. Guidelines for Design and Use of Degenerate Primers

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 0.4 µM. ■ If PCR efficiency is poor, increase primer concentrations in increments of 0.1 µM until satisfactory results are obtained.

Table 15. 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 LongRange PCR System, a typical cycling program consists of 35–38 cycles, depending on the number of copies of the starting template. Table 16 provides general guidelines to determine the number of copies.

Table 16. General Guidelines for Choosing the Number of Copies of Template

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 12 to calculate the number of molecules for template DNA different than the examples given. When starting with cDNA templates, it is important to take into account the efficiency of reverse transcription in cDNA synthesis, which is on average 10–30%. Use oligo dT primers for cDNA synthesis for all PCR fragments >1 kb. Use the higher number of PCR cycles for maximum PCR product yield.

† Refers to single-copy genes.

‡ The recommended upper limit for human genomic DNA is approximately 500 ng.
N.R.: Not recommended to use such high amounts of starting template.

Appendix D: Gel Analysis of PCR products

Analyze large amplification products on a suitable agarose gel (see Table 17), preferably in TAE-buffer, using appropriate DNA markers. For separation of products longer than 25 kb in a standard electrophoresis chamber, a 12-hour run at approximately 40 V is recommended. Generally, efficient separation of very large DNA fragments is achieved only by field inversion or pulsed-field gel electrophoresis.

Table 17. Guidelines for Agarose Gel Analysis of Long PCR Products

Size of PCR product	Percentage of agarose	Run conditions
Up to 1 kb	1.7%	5–6 V/cm* for 0.5–1 h
1–3 kb	1.5%	4–4.5 V/cm* for 1–2 h
3–7 kb	1%	3–3.5 V/cm* for 2 h
7–15 kb	0.7%	2.5 V/cm* for 5–6 h
15–40 kb	0.5%	1.2 V/cm* for 10–12 h

* Voltage per cm between electrodes

Appendix E: RT-PCR

To perform PCR using RNA as a starting template, the RNA must first be reverse transcribed into cDNA in a reverse transcription reaction (RT reaction). Failure of the subsequent PCR is often a result of the limitations of the RT reaction. On average, only 10–30% of the original RNA molecules is reverse transcribed into cDNA. Use of random primers may result in relatively short cDNA molecules, which may limit the amplification of long fragments. To amplify long cDNA species, use of the QIAGEN LongRange 2Step RT-PCR Kit is recommended. This kit includes a new reverse transcriptase suitable for production of cDNA up to 12.5 kb in length, as well as the components needed for subsequent long-range PCR.

The expression level of the target RNA molecules and the relatively low efficiency of the reverse transcription reaction must be considered when calculating the appropriate amount of starting template for subsequent PCR. The volume of the RT reaction transferred should not exceed 10–15% of the total PCR volume. General guidelines as well as guidelines on the amount of cDNA required for amplification of high-, medium-, or low-abundance transcripts are shown in Table 18.

Table 18. General Guidelines for Amplification of cDNA Molecules in RT-PCR

RNA purification:	Intact RNA is a prerequisite for amplification of cDNA molecules in RT-PCR. The integrity of the RNA is especially important when you plan to analyze large fragments (e.g., amplification of complete cDNAs for protein expression). QIAGEN offers the RNeasy system for total RNA isolation and Oligotex® Kits for messenger RNA isolation.
Reverse transcription:	The QIAGEN LongRange 2Step RT-PCR Kit is recommended for amplification of cDNA species up to 12.5 kb in length. Oligo dT primers are recommended for reverse transcription of templates of 1–5 kb. Follow the detailed protocol in the *QIAGEN LongRange 2Step RT-PCR Handbook*, or follow the manufacturer's instructions when using an enzyme from another supplier. The following guidelines may be helpful. ■ 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 2.5 µl oligo dT primer, 12–18mer (0.2 µg/µl) reverse transcriptase† Add RNase-free water to a final volume of 20 µl. ■ Incubate following the manufacturer's instructions. ■ Heat the reaction mix to 93°C for 5 min to inactivate the reverse transcriptase.
PCR:	■ Prepare a PCR mixture following steps 1–3 in protocols. ■ Add 2–5 µl from the RT reaction to each PCR tube containing the reaction mix. Typically, 10 to 100 ng cDNA will give satisfactory results. For low-abundance transcripts, it is recommended to start with approximately 100 ng cDNA, whereas for highly abundant transcripts, low amounts (down to 1 ng–100 pg) may be sufficient. ■ Continue with step 5 in the PCR protocols.

* For further information about RNeasy and Oligotex, contact your local QIAGEN Technical Services or distributor (see back cover). Oligotex is not available in Japan.

† Refer to the manufacturer's instructions for the amount of enzyme required.

Appendix F: 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 29, 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 G: 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, mineral oil, and probably nonspecific amplification products. Before the specific PCR product can be used in subsequent experiments it is often necessary to remove these contaminants. 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 kb – 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 H: 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 reaction 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 reaction 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 RNase-free 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 RNase-free 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, 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.

Appendix I: Cloning of PCR Products

PCR products contain an A overhang at the 3' end and can therefore be directly cloned into any TA- or UA-cloning vector. QIAGEN PCR Cloning Kits provide highly efficient cloning of PCR products through UA hybridization. Ligation of the PCR product, transformation, and plating of QIAGEN EZ Competent Cells takes place in just 40 minutes.

References

QIAGEN maintains a large, up-to-date online database of scientific publications utilizing QIAGEN products. Comprehensive search options allow you to find the articles you need, either by a simple keyword search or by specifying the application, research area, title, etc. For a complete list of references, visit the QIAGEN Reference Database online at www.qiagen.com/RefDB/search.asp or contact QIAGEN Technical Services or your local distributor.

Ordering Information

Product	Contents	Cat. no.
QIAGEN LongRange PCR Kit (20)	For 20 x 50 µl reactions: LongRange PCR Enzyme Mix (40 U), LongRange PCR Buffer, 10x Q-Solution, RNase-Free Water, 10 mM dNTPs	206401
QIAGEN LongRange PCR Kit (100)	For 100 x 50 µl reactions: LongRange PCR Enzyme Mix (200 U), LongRange PCR Buffer, 10x Q-Solution, RNase-Free Water, 10 mM dNTPs	206402
QIAGEN LongRange PCR Kit (250)	For 250 x 50 µl reactions: LongRange PCR Enzyme Mix (500 U), LongRange PCR Buffer, 10x Q-Solution, RNase-Free Water, 10 mM dNTPs	206403
Related products		
QIAGEN LongRange 2Step RT-PCR Kit (20)	For 20 x 50 µl PCRs: Reverse transcription step (10 x 20 µl reactions) — LongRange Reverse Transcription Enzyme, Buffer, dNTPs, Oligo-dT, RNase-Free Water; PCR step — QIAGEN LongRange PCR Kit (20), see above	205920
QIAGEN LongRange 2Step RT-PCR Kit (100)	For 100 x 50 µl PCRs: Reverse transcription step (50 x 20 µl reactions) — LongRange Reverse Transcription Enzyme, Buffer, dNTPs, Oligo-dT, RNase-Free Water; PCR step — QIAGEN LongRange PCR Kit (100), see above	205922
HotStar HiFidelity Polymerase Kit (100 U)*	100 units HotStar HiFidelity DNA Polymerase, 5x HotStar HiFidelity PCR Buffer (inc. dNTPs),† 5x Q-Solution, 25 mM $MgSO_4$, RNase-Free Water	202602

* Larger kit sizes available; please inquire.

† Contains Factor SB, dNTPs, and optimized concentration of $MgSO_4$.

Ordering Information

Product	Contents	Cat. no.
HotStarTaq *Plus* DNA Polymerase (250 U)*	250 units HotStarTaq Plus DNA Polymerase, 10x PCR Buffer,† 10x CoralLoad PCR Buffer,† 5x Q-Solution, 25 mM $MgCl_2$	203603
HotStarTaq DNA Polymerase (250 U)*	250 units HotStarTaq DNA Polymerase, 10x PCR Buffer,† 5x Q-Solution, 25 mM $MgCl_2$	203203
QIAGEN Fast Cycling PCR Kit (200)*	For 200 x 20 µl reactions: 2x Fast Cycling PCR Master Mix, 10x Fast Cycling Dye, 5x Q-Solution, RNase-Free Water	203743
TopTaq™ DNA Polymerase (250)*	250 units TopTaq DNA Polymerase, 10x PCR Buffer,† 10x CoralLoad Concentrate, 5x Q-Solution, 25 mM $MgCl_2$	200203
Taq DNA Polymerase (250 U)*	250 units Taq DNA Polymerase, 10x PCR Buffer,† 10x CoralLoad PCR Buffer,† 5x Q-Solution, 25 mM $MgCl_2$	201203
dNTP Mix, PCR Grade (200 µl)*	Mix containing 10 mM each of dATP, dCTP, dGTP, and dTTP (1 x 200 µl)	201900
QIAGEN PCR Cloning Kit (10)*	For 10 reactions: 2x Ligation Master Mix (50 µl), pDrive Cloning Vector (0.5 µg), distilled water (1.7 ml)	231122
QIAamp DNA Mini Kit (50)*	For 50 DNA preps: 50 QIAamp Mini Spin Columns, QIAGEN Proteinase K, Buffers, Collection Tubes (2 ml)	51304

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Services or your local distributor.

* Larger kit sizes available; please inquire.

† Contains 15 mM $MgCl_2$.

Notes

Notes

Limited License Agreement

Use of this product signifies the agreement of any purchaser or user of the LongRange PCR Enzyme Mix and the QIAGEN LongRange PCR Kit to the following terms:

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