QIAGEN News

Innovation Working for You

1

February
2002

New

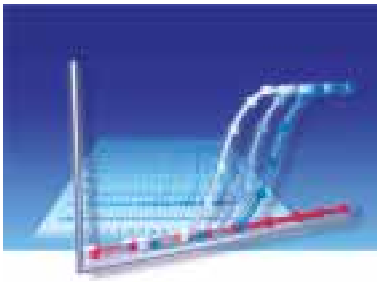
QuantiTect™ Probe PCR and RT-PCR Kits — minimize your PCR optimization for highly sensitive results!

QIAGEN introduces two new kits for quantitative, real-time PCR and RT-PCR using sequence-specific probes — the QuantiTect™ Probe PCR Kit (for PCR and two-step RT-PCR) and the QuantiTect Probe RT-PCR Kit (for one-step RT-PCR). Both kits are designed for use with all types of probes, including TaqMan® or QIAGEN® Operon® dual-labeled probes; LightCycler® hybridization probes or fluorescence resonance energy transfer (FRET) probes; and Molecular Beacons. The optimized system is designed for use on any real-time PCR cycler, such as the ABI™ (ABI PRISM® 7700 and GeneAmp® 5700), LightCycler (Roche), iCycler™ (Bio-Rad), and DNA Engine Opticon™ (MJ Research) systems.

QuantiTect Probe Kits offer:

- **Simple assay development** — no need for optimization
- **High sensitivity** —stringent built-in hot start with HotStarTaq™ DNA Polymerase
- **Easy handling** — ready-to-use master-mix format
- **Versatility** — optimized for use with all types of real-time PCR cyclers and sequence-specific probes

▶▶ New QuantiTect Probe Kits, page 10



New

FlexiGene technology — for efficient isolation of DNA

The FlexiGene DNA Kit provides a rapid and convenient method for purification of DNA from variable volumes of human whole blood, buffy coat, or cultured cells. The simple procedure requires less than 1 hour of hands-on time for purification of DNA from 12 samples.

Advantages of the FlexiGene DNA Kit include:

- **Easy handling** — purification in a single tube reduces waste and minimizes the risk of sample mix-up
- **Flexibility in amount of starting material** — purification of DNA from 0.1–20 ml whole blood
- **Direct purification from whole blood**
- **Speed** — protocol involves only 3 short centrifugation steps and 3 buffers
- **No organic extraction**
- **High yields of pure DNA**, suitable for downstream PCR analysis

▶▶ FlexiGene DNA Kit, page 24

What's New?

- The new ***QIAGEN® Operon® Product Guide 2002*** is included with this issue of *QIAGEN News.* QIAGEN Operon (formerly Operon) is a leading supplier of synthetic DNA, providing custom oligonucleotides, Array-Ready Oligo Sets™, and custom gene synthesis to life science researchers worldwide.

- Did you know that **German, French, or Japanese versions** are available of many of our handbooks? Some of these can be downloaded from our web site at **www.qiagen.com/literature/handbooks/**. Others are available on request; please contact QIAGEN Technical Services for further information.

- Don't miss the new **QIAamp® UltraSens™ Virus Kit**, described on page 29 of this issue! This kit uses new UltraSens technology to concentrate viral RNA and DNA from plasma and serum samples without ultracentrifugation. The resulting highly pure nucleic acids are suitable for use in a wide range of downstream applications.

- Are you looking for more sensitivity in ELISA procedures? A new Ni-NTA coating procedure used in the manufacture of **Ni-NTA HisSorb™ Strips and Plates** significantly increases sensitivity and signal-to-noise ratios, allowing you to use less protein per assay. Find out more on page 18.

- We welcome your feedback on *QIAGEN News*. Please send your comments or suggestions to **news.editor@qiagen.com**.

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High sensitivity in quantitative, competitive RT-PCR using QIAGEN® enzymes*

Frédéric Bonino, Julie Milanini, Jacques Pouysségur, and Gilles Pagès

Centre Antoine Lacassagne, Nice, France

This article describes a highly sensitive competitive RT-PCR assay to accurately quantify mRNA levels of a tumor marker. Use of Omniscript™ RT and the QIAGEN® Taq PCR Master Mix Kit provided high sensitivity and specificity for accurate quantification of low levels of a specific mRNA using small amounts of total RNA.

Competitive RT-PCR using internal standards

Competitive RT-PCR uses internal standards that are nearly identical to the target sequence and have the same primer binding sites. The standards are modified slightly from the target sequence so that amplification products can be differentiated, for example, by restriction digestion, gel electrophoresis, or HPLC. RT-PCR is then carried out using known amounts of the standard. The standards and the target compete for primers to amplify the sequence. When the amounts of standard and target RNA are equal, signals derived from the amplification of each are also equal. This allows precise quantification of the target. For competitive RT-PCR, it is important to measure values in the linear amplification phase. As the amplification rate reaches a plateau, quantification becomes less precise.

For absolute quantification of RNA, the copy number or concentration of the nucleic acids used as standards must be known. It is best to use RNA rather than DNA as standards for quantitative RT-PCR since this takes into account variability in the RT reaction due to differences in RNA quality, sequence, and structure.

Vascular endothelial growth factor (VEGF) is implicated in the progression of a number of human cancers. Its expression corresponds well with tumor growth and metastasis. High levels of VEGF correlate with the aggressiveness of the tumor and poor prognosis (2).

In this paper, we describe a new, competitive RT-PCR assay based on internal RNA standards for accurate quantification of *VEGF* mRNA (see box for details about quantification of mRNA using competitive RT-PCR). Use of Omniscript RT and the *Taq* PCR Master Mix Kit provided a highly sensitive and specific assay, capable of measuring low levels of *VEGF* mRNA in small amounts of total RNA.

Materials and methods

Two oligonucleotides were synthesized, a 53mer and 70mer with a 25-base overlap. The oligo sequences corresponded to bases 434 to 559 of the mouse *VEGF 164 A* mRNA, with a 26-base deletion of bases 457 to 482. The oligonucleotides were annealed and the non-annealed regions were filled in using QIAGEN *Taq* DNA Polymerase. The resulting 97 bp DNA fragment was cloned into a plasmid containing a T7 promoter, and RNA was transcribed from the plasmid in vitro using T7 RNA polymerase (Figure 1).

For RNA quantification, differing amounts of the in vitro transcribed RNA (1 pg to 10 ng) were added to a set amount of sample RNA (0.5 µg) as an internal competitive standard. Competitive, quantitative RT-PCR was then carried out using forward and reverse primers corresponding to sequences identical in the in vitro transcribed standard RNA and the sample RNA. These 23mer primers corresponded to the 5' and 3' ends of the ▶

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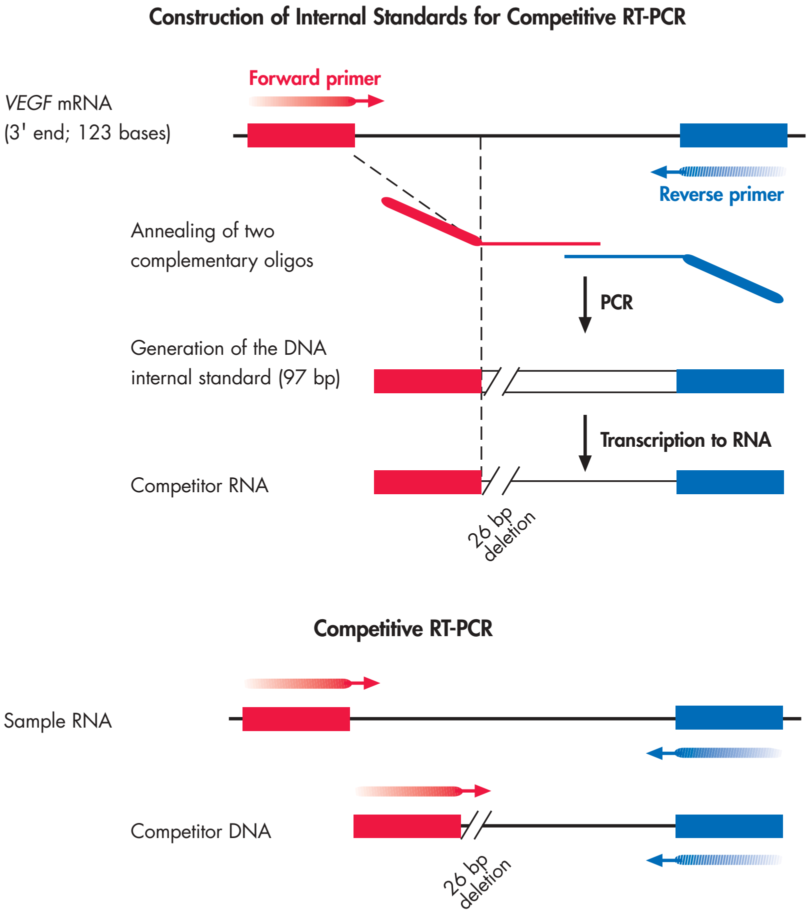
* *Excerpted from Bonino, F., Milanini, J., Pouysségur, J., and Pagès, G. (2001) RT-PCR method to quantify vascular endothelial growth factor expression. BioTechniques* ***30****, 1254 (reference 1) with permission from* BioTechniques.


Construction of Internal Standards for Competitive RT-PCR

Competitive RT-PCR

Figure 1 *A vector for competitive RNA for internal standards was constructed as described in "Materials and methods". The internal standard contains a 26 nt deletion, allowing competitor and sample RNAs to be distinguished by PAGE.*

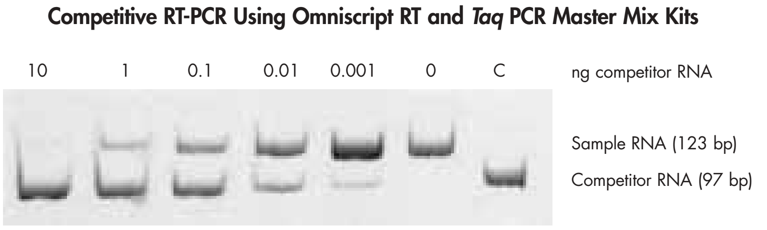

Competitive RT-PCR Using Omniscript RT and *Taq* PCR Master Mix Kits

Figure 2 *Autoradiogram of a representative set of competitive RT-PCR assays for quantification of* VEGF *mRNA. Known amounts of competitive RNA, as indicated, were added to 0.5 µg of sample RNA as internal standards. Two-step RT-PCR was carried out using Omniscript RT and the* Taq *PCR Master Mix Kit with primers specific for a 123 nt fragment of the* VEGF *mRNA. The competitive standard RNA contains a 26 nt deletion, resulting in a 97 bp amplification product using the same primers. When the standard and target RNA numbers are equal, signals derived from amplification of each are also equal.* ***C****: control with competitive standard RNA alone.*

competitive RNA construct (bases 559–537 and 434–456 of the mouse cDNA). Thus RT-PCR of the sample RNA and the standard RNA produces PCR products with lengths of 123 bp and 97 bp, respectively (Figure 1).

For competitive RT-PCR, the reverse-transcription step was carried out using the Omniscript RT Kit, priming with the gene-specific reverse primer described above. PCR was then performed using both gene-specific primers, *Taq* PCR Master Mix Kit (QIAGEN), and ^{33}P-labeled dATP in a T3 Thermocycler (Biometra) with the following cycling conditions: 95°C for 3 minutes; 28 cycles of 95°C for 30 seconds, 56.5°C for 30 seconds, 72°C for 30 seconds; and a final extension at 72°C for 7 minutes. The PCR products were resolved by polyacrylamide gel electrophoresis (PAGE) and quantified by phosphorimager analysis on a Fujix Bas 1000 Model IPR 1000 (Fuji Photo Film). The gel clearly resolved the 123 bp fragment, from the sample RNA, and the 97 bp fragment, from the internal standard RNA containing the 26 nt deletion.

Results and discussion

We developed a competitive, quantitative RT-PCR assay for quantification of *VEGF* RNA (Figure 1) as described in "Materials and methods". Varying amounts of competitive standard RNA were added to a set amount of sample RNA. Since the standard RNAs contained a 26 nt deletion, the sample and competitive RNAs were easily separated by PAGE (Figure 2). The relative amounts of each RNA were measured using phosphorimager analysis. When the standard and target RNA amounts are equal, signals derived from each of them will also be equal. This amount can be determined graphically by plotting the standard RNA amounts against the ratio of the 2 RNA species on a logarithmic scale. The line crosses the abscissa when the ratio is 1:1 (log 1 = 0), giving the amount of sample RNA (see Figure 3A). This method allowed quantification of as little as 10–30 pg *VEGF* RNA, quantities that are undetectable by northern blot analysis (3).

To validate our RT-PCR method, we looked at the effect of estradiol stimulation on *VEGF* mRNA expression in nontransformed CCL39

Highly Sensitive Quantification by Competitive RT-PCR

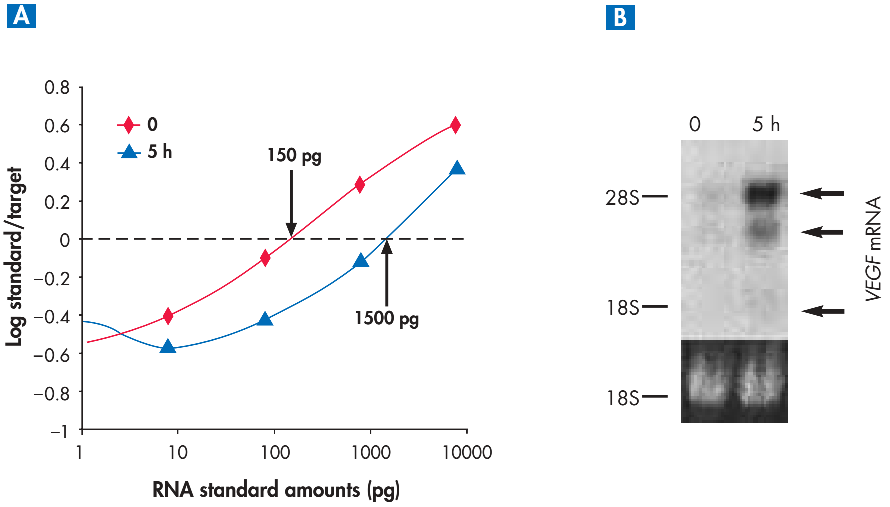


Figure 3 *Comparison of sensitivity using competitive, quantitative RT-PCR or northern blot analysis. Total RNA from quiescent CCL39 Raf-1:ER cells (**0**) and from cells stimulated with estradiol for 5 hours (**5 h**) was used as sample RNA in both analyses.* **A** *Competitive RT-PCR was carried out using Omniscript RT and the Taq PCR Master Mix Kit as described in "Materials and methods" with 0.5 µg RNA per reaction. The amount of internal standard RNA in each reaction was plotted against the ratio of the amplification products on a log scale. The amount of* VEGF *mRNA in the sample is determined from the value where the line crosses the abscissa (arrows).* **B** *Northern blot analysis using 20 µg total RNA per lane. The arrows indicated 3 differentially spliced forms of* VEGF *mRNA (all of which are amplified in the RT-PCR assay). The lower panel shows ethidium bromide staining of the 18S rRNA as a control.*

Chinese hamster lung fibroblasts stably expressing a Raf-1:ER chimeric protein. In this system, estradiol activates the Raf-1 signal-transduction domain of the chimera via the estrogen receptor (ER). *VEGF* mRNA is, in turn, induced by the Raf-1 domain, with maximal induction at 5 hours (3).

Using the competitive RT-PCR assay, *VEGF* mRNA levels were measured in quiescent and estradiol-activated CCL39 cells. Both basal and induced *VEGF* levels were quantifiable, showing that estradiol treatment caused a 10-fold induction of *VEGF* (Figure 3A). In contrast, northern blot analysis was not sensitive enough to measure basal levels, making it impossible to determine the magnitude of *VEGF* induction (Figure 3B).

As a further test of the assay, expression of *VEGF* was quantified in different cell lines with varying tumorigenic potential. Highly transformed cell lines, such as rat colon carcinoma PRO cells, human colon carcinoma HT-29 cells, and Ras-transformed CCL39 cells, express *VEGF* at 15- to 30-fold higher levels than quiescent cells (Figure 4). These results correlate with the capacity of these cells to induce highly vascular tumors. ▶

***VEGF* mRNA in Different Cell Types**

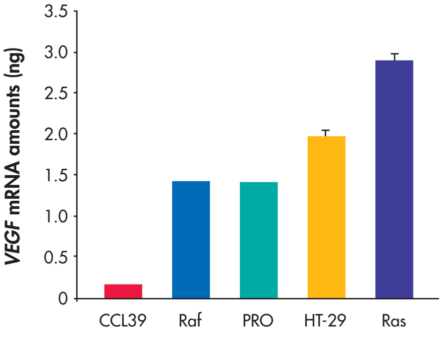


Figure 4 VEGF *mRNA levels were quantified in various cell types by competitive, quantitative RT-PCR using Omniscript RT and the Taq PCR Master Mix Kit as described in "Materials and methods".* ***CCL39****: quiescent CCL39 Raf-1:ER cells;* ***Raf****: CCL39 Raf-1:ER cells stimulated for 5 hours with estradiol;* ***PRO****: exponentially growing rat colon carcinoma PRO cells;* ***HT-29****: human colon carcinoma HT-29 cells;* ***Ras****: Ras-transformed CCL39 cells.*

References

1. *Bonino, F., Milanini, J.,Pouysségur, J., and Pagès, G. (2001) RT-PCR method to quantify vascular endothelial growth factor expression. BioTechniques **30**, 1254.*
2. *Lee, Y.H. et al. (1999) Cell-retained isoforms of vascular endothelial growth factor (VEGF) are correlated with poor prognosis in osteosarcoma. Eur. J. Cancer **35**, 1089.*
3. *Milanini, J., Vinals, F., Pouysségur, J., and Pagès, G. (1998) p42/p44 MAP kinase module plays a key role in the transcriptional regulation of vascular endothelial growth factor gene in fibroblasts. J. Biol. Chem. **273**, 18,165.*

Related articles in this issue

QuantiTect™ Probe PCR and RT-PCR Kits — minimize your PCR optimization for highly sensitive results! (page 1).

Conclusions

- A highly sensitive, competitive, quantitative RT-PCR assay was developed using Omniscript RT and the QIAGEN *Taq* PCR Master Mix Kit. Using this assay, as little as 10–30 pg *VEGF* mRNA could be accurately quantified. This allows quantification of basal levels that were impossible to measure using northern blot analysis.
- Using this assay, *VEGF* mRNA levels could be measured using as little as 3 μg total RNA, comparable to amounts obtained from biopsy samples.

  In contrast, a classical ELISA method to evaluate *VEGF* levels in human serum can only detect significant increases when the tumor has already reached a critical size.
- The assay provides a practical tool for researchers and clinicians to evaluate the potential of tumor cells to induce highly aggressive vascularized tumors. The high sensitivity and specificity of Omniscript RT and the *Taq* PCR Master Mix Kit improves the sensitivity and usefulness of the assay, allowing analysis of small amounts of sample.

Reader Inquiry No. 02103

Ordering Information

Product	Contents	Cat. No.
Omniscript RT Kit — for highly sensitive and specific reverse transcription using 50 ng – 2 μg RNA		
Omniscript RT Kit (10)[*]	For 10 reverse-transcription reactions: 40 units Omniscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix,[†] RNase-free water	205110
***Taq* DNA Polymerase — for standard PCR**		
Taq PCR Master Mix Kit (250 U)[*]	3 x 1.7 ml *Taq* PCR Master Mix[‡] containing 250 units *Taq* DNA Polymerase total, 3 x 1.7 ml distilled water	201443
Taq DNA Polymerase (250 U)[*]	250 units *Taq* DNA Polymerase, 10x PCR Buffer,[§] 5x Q-Solution, 25 mM $MgCl_2$	201203
Related products		
QuantiTect RT-PCR Kits — for quantitative, real-time RT-PCR		
QuantiTect SYBR® Green RT-PCR Kit (200)	For 200 x 50 μl reactions: 3 x 1.7 ml QuantiTect SYBR Green RT-PCR Master Mix;[¶] 1 x 100 μl QuantiTect RT Mix; 2 x 2.0 ml RNase-free water	204243
QuantiTect Probe RT-PCR Kit (200)	For 200 x 50 μl reactions: 3 x 1.7 ml QuantiTect Probe RT-PCR Master Mix;[**] 1 x 100 μl QuantiTect RT Mix; 2 x 2.0 ml RNase-free water	204443
Oligonucleotide Synthesis Service — high-quality oligos, modified oligos, and longmers		
Oligonucleotide Synthesis Service	Custom-made oligonucleotides up to 100 nt and a wide range of modified oligos, including Molecular Beacons, dual-labeled probes, and many more	Inquire

[*] *Larger kit sizes available: please inquire.*

[†] *Contains 5 mM each dNTP*

[‡] *Provides a final concentration of 1.5 mM $MgCl_2$ and 200 μM each*

[§] *Contains 15 mM $MgCl_2$*

[¶] *Contains 5 mM $MgCl_2$*

[**] *Contains 8 mM $MgCl_2$*

A new protocol for highly efficient amplification of long PCR products

Susan Kobsch, Katja Decker, and Dirk Löffert
QIAGEN GmbH, Hilden, Germany

Amplification of PCR products longer than 3–4 kb is often compromised by nonspecific primer annealing, suboptimal cycling conditions, and secondary structures in the DNA template. Lengthy optimization is often necessary, by varying factors such as cycling conditions, primer and dNTP concentrations, and special additives. Here, we describe a standardized, simple protocol for robust amplification of PCR fragments longer than 10 kb by the combined use of QIAGEN® *Taq* and ProofStart™ DNA Polymerases, corresponding PCR buffers, and Q-Solution.

Materials and methods

Human genomic DNA was isolated from blood using the QIAamp® DNA Blood Mini Kit. PCR was performed using QIAGEN *Taq* DNA Polymerase, PCR Buffer, Q-Solution, and ProofStart DNA Polymerase. Alternatively, reactions were carried out using two specialized long-range PCR kits from Suppliers R and A_{II}, following suppliers' protocols. One-tenth of each reaction was loaded onto a 0.8% TAE agarose gel. Cycling programs and reaction mixtures are detailed in Table 1 (page 9).

Effect of cycling conditions

While depurination is usually not a problem in standard PCR, it can significantly influence the amplification of longer PCR fragments. This is because longer templates are proportionally more depurinated than shorter ones. For this reason, very short denaturation steps of only 10 seconds give higher yields and no background smearing compared to denaturation steps of 30 seconds or 1 minute (which leads to PCR failure; Figure 1A). Extensive depurination is also observed during the final extension step. Therefore, using a lower extension temperature of 68°C instead of 72°C dramatically improves yield of longer amplification products (Figure 1B).

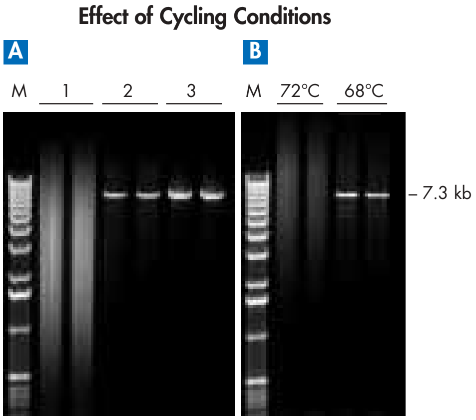


Figure 1 Cycling conditions were as listed in Table 2. **A** To illustrate the effects of prolonged heating of the DNA template or the DNA polymerase, three different PCR cycling programs were used to amplify a 7.3 kb fragment of the human interleukin 9 receptor gene. **1**: Each PCR cycle had a 60-second denaturation step at 94°C. **2**: A reaction mixture containing Taq DNA Polymerase but lacking the template DNA was incubated at 94°C for 30 minutes; the template DNA was then added and the PCR cycling program started, using 10-second 94°C denaturation steps. **3**: The PCR cycling program was comparable to 2, except that there was no additional prior incubation. **B** The 7.3 kb PCR product could only be amplified when the temperature of the extension step was lowered from 72°C to 68°C. **M**: markers. Results from duplicate PCR amplifications are shown.

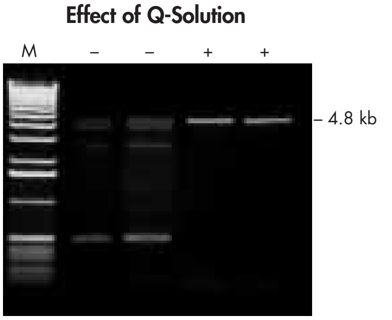


Figure 2 A 4.8 kb product from the human tissue plasminogen activator gene was amplified using QIAGEN Taq DNA Polymerase and QIAGEN PCR Buffer in the presence (**+**) or absence (**–**) of Q-Solution. The specific product was amplified only when Q-Solution was included in the reaction mixture. **M**: markers. Results from duplicate PCR amplifications are shown.

Effect of Q-Solution

Secondary structures such as hairpin loops, which are often caused by GC-rich template stretches, interfere with efficient amplification of long PCR products. This problem can be overcome by adding reagents that modify the melting behavior of DNA to help resolve secondary structures at lower temperatures. Figure 2 shows that the specificity of amplification of a 4.8 kb PCR fragment was dramatically improved by the addition of Q-Solution.

▶

Effect of 3'→5' Exonuclease Activity

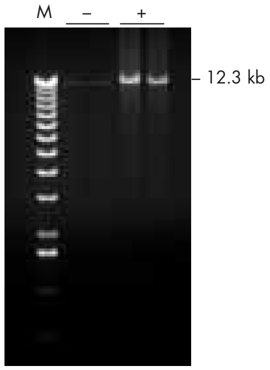


Figure 3 *A 12.3 kb fragment of the human interleukin 9 receptor gene was amplified from human genomic DNA using QIAGEN* Taq *DNA Polymerase, either with an exonuclease-deficient proofreading enzyme mutant from Supplier S_{III} (**–**) or with ProofStart DNA Polymerase (**+**) containing the exonuclease activity. Results from duplicate PCR amplifications are shown.* ***M****: markers.*

Effect of 3'→5' exonuclease activity

Taq DNA Polymerase introduces more errors into the PCR product while copying the template than do so-called proofreading DNA polymerases. Once a mismatch occurs during synthesis, *Taq* DNA polymerase will either extend the mismatched strand or fall off the template strand, leading to mutated or incomplete PCR products, respectively. Although this does not generally affect PCR efficiency when amplifying shorter PCR fragments, amplification of longer PCR products can be significantly impaired by mismatches introduced during DNA synthesis.

Proofreading DNA polymerases, such as ProofStart DNA Polymerase, usually contain an inherent 3'→5' exonuclease activity that removes base-pair mismatches. Adding a small amount of ProofStart DNA Polymerase to the PCR mixture therefore significantly improves the amplification efficiency of longer PCR products. This is shown by comparing PCR mixtures containing, in addition to *Taq* DNA Polymerase, either ProofStart DNA Polymerase or a proofreading polymerase with point mutations to inactivate the 3'→5' exonuclease activity (Supplier S_{III}). PCR mixtures containing ProofStart DNA Polymerase consistently enable amplification of long products with high yield (Figure 3). These results emphasize the importance of the 3'→5' exonuclease activity to correct misincorporated bases, increasing PCR efficiency, especially for longer PCR products.

Combined effect of 3'→5' exonuclease, hot start, PCR Buffer, and Q-Solution

The parameters described above are summarized in a final, optimized protocol (Tables 1 and 2) using QIAGEN *Taq* DNA Polymerase, PCR Buffer, Q-Solution, and ProofStart DNA Polymerase. This optimized protocol was then compared with commercially available kits for long-range PCR. Two different template–primer systems were tested, generating 9.3 kb and 15 kb PCR products. The optimized mixture of QIAGEN DNA polymerases and reagents gave superior amplification yields of the specific PCR product in all cases (Figure 4). This is due to the reduction of secondary structures in the template by Q-Solution and the efficient removal of misincorporated bases by ProofStart DNA Polymerase. Specificity of the reaction was further increased by the inactive state of ProofStart DNA Polymerase before the initial activation step (hot start). The lack of ProofStart exonuclease activity before this hot start prevents primer degradation during reaction setup and the initial heating phase of the thermal cycler.

Conclusions

- The new protocol presented here enables amplification of long PCR products with high yields and specificity, without the need for time-consuming optimization.
- The simple hot start provided by ProofStart DNA Polymerase eliminates the need for manual hot-start procedures or two-mix setup strategies that are often recommended in other protocols.
- The efficiency, specificity, and ease of handling offered by QIAGEN DNA polymerases, buffers, and reagents leads to genuine time savings in reaction setup and amplification procedures.

Optimized Reaction Conditions for Longer PCR Products

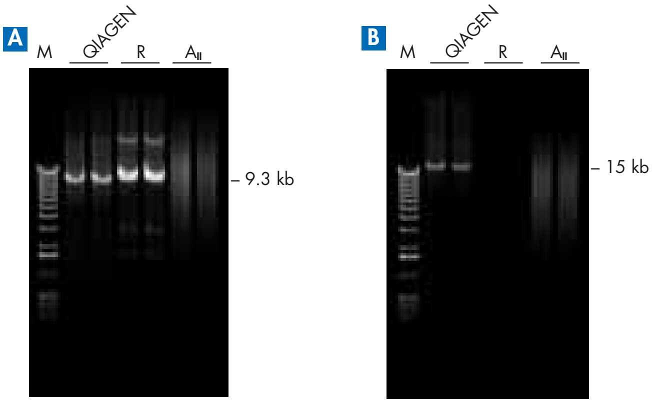


Figure 4 *Long PCR products were amplified from human genomic DNA using either the optimized QIAGEN reagent mixture (****QIAGEN****), a commercially available mixture for long-range PCR from Supplier R (****R****) or a commercially available mixture for long-range PCR from Supplier A_{II} (**A_{II}**). Amplification was performed according to suppliers' protocols. Equal volumes were analyzed. Primers were designed to amplify* **A** *a 9.3 kb fragment of the human tissue plasminogen activator gene or* **B** *a 15 kb fragment of the human coagulation factor IX gene.* ***M****: markers.*

Table 1. PCR components for long amplification products (master mix and template)

Component	Volume/reaction	Final concentration
PCR Buffer,* 10x (supplied with QIAGEN *Taq* DNA Polymerase)	5 µl	1x
Q-Solution, 5x	10 µl	1x
dNTP mix (10 mM of each)	1.5 µl	300 µM of each dNTP
Primer A	variable	0.5 µM
Primer B	variable	0.5 µM
Taq DNA Polymerase	1 µl	5 units
ProofStart DNA Polymerase (diluted)†	1 µl	0.2 units
Distilled water	variable	
Template DNA	variable	200 ng – 1 µg for genomic DNA or 5–50 ng for plasmid or λ DNA
Total volume	**50 µl**	

* *Contains 15 mM $MgCl_2$*

† *Dilute enzyme in 1x ProofStart PCR Buffer to a concentration of 0.2 units/µl.*

Table 2. Cycling conditions for amplifying longer PCR products

Initial activation step	**2 min**	**95°C**
3-step cycling:		
Denaturation	10 s	94°C
Annealing	1 min	50–68°C ‡
Extension	1 min/kb	68°C
Number of cycles	35–40	
End of PCR cycling	Indefinite	4°C

‡ *Approximately 5°C below T_m of primers*

■

Reader Inquiry No. 02104

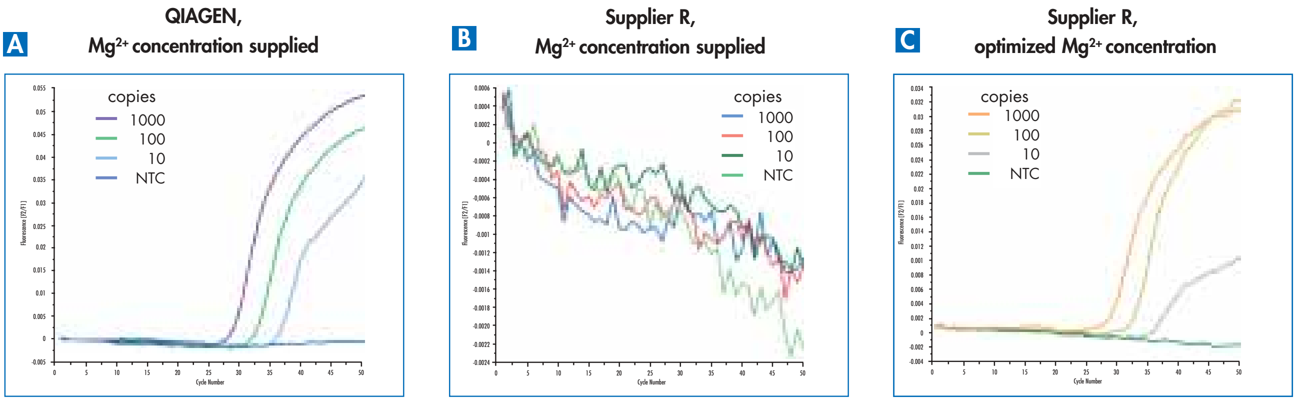
Ordering Information

Product	Contents	Cat. No.
Taq DNA Polymerase (250 U)	250 units *Taq* DNA Polymerase, 10x PCR Buffer,§ 5x Q-Solution, 25 mM $MgCl_2$	201203
Taq PCR Core Kit (250 U)	250 units *Taq* DNA Polymerase, 10x PCR Buffer,§ 5x Q-Solution, 25 mM $MgCl_2$, dNTP Mix¶	201223
ProofStart DNA Polymerase (100 U)	100 units ProofStart DNA Polymerase, 10x ProofStart PCR Buffer,** 5x Q-Solution, 25 mM $MgSO_4$	202203

§ *Contains 15 mM $MgCl_2$* ¶ *Contains 10 mM each dNTP* ** *Contains 15 mM $MgSO_4$*

continued from page 1

Successful Real-Time PCR without Mg^{2+} Optimization


A QIAGEN, Mg^{2+} concentration supplied

B Supplier R, Mg^{2+} concentration supplied

C Supplier R, optimized Mg^{2+} concentration

Figure 1 *Real-time PCR analysis of a fragment of the human protease inhibitor 1 gene was carried out using FRET probes and the QuantiTect Probe PCR Kit or a kit from Supplier R. Analyses were performed on the LightCycler system. Reactions contained human genomic DNA with the indicated copy numbers.* **A** *QIAGEN, with Mg^{2+} concentration supplied* **B** *Supplier R, with Mg^{2+} concentration supplied* **C** *Supplier R, with optimized Mg^{2+} concentration (2.5 mM).* **NTC***: no template control.*

Simple assay development with minimal optimization

Developing quantitative, real-time PCR and RT-PCR assays with fluorescent probes often requires extensive and time-consuming optimization procedures. This can involve complex, simultaneous adjustment of primer, probe, and magnesium concentrations in a coordinated matrix. QIAGEN QuantiTect Probe Kits minimize or even eliminate the need for optimization, making assay development both simple and fast.

Optimal results can be achieved the first time, using the recommended, pre-optimized primer and probe concentrations given in the QuantiTect Probe protocols. This greatly minimizes the need to adjust reaction parameters.

A balanced combination of KCl and $(NH_4)_2SO_4$ in the specially formulated buffer provides a greater tolerance to variable magnesium concentrations. The Mg^{2+} concentration in the buffer is already optimized so that, in most cases, the user does not need to experiment with different magnesium concentrations for each new target (Figure 1).

High sensitivity for reliable quantification

PCR sensitivity is directly related to the specificity of the assay. Extension of nonspecifically annealed primers and primer-dimers can occur at low temperatures during reaction setup and the first denaturation step. A particular problem with low-copy targets is the increased opportunities for these artifacts due to the high ratio of primers and probes to the small amount of target sequences. High background due to nonspecific products can block out specific signals, meaning that accurate detection is only possible after more cycles.

HotStarTaq DNA Polymerase, in both kits, provides a hot start to the PCR for highly specific and sensitive amplification. The hot start prevents nonspecific products, primer-dimer formation, and background, and increases the yields of specific PCR products. This leads to increased sensitivity, allowing detection of low-copy targets that could otherwise be obscured by high background levels.

In addition to HotStarTaq DNA Polymerase, the QuantiTect Probe RT-PCR Kit contains a unique blend of Sensiscript™ and Omniscript™

Reverse Transcriptases. The combination of both enzymes ensures highly efficient and sensitive reverse transcription over a wide range of template amounts (1 pg to 500 ng) without optimization. Both reverse transcriptases have high affinities for RNA, providing efficient synthesis of full-length cDNAs, even through regions with complex secondary structure.

Compared with other real-time RT-PCR kits that use sequence-specific probes, the QuantiTect Probe RT-PCR Kit provides significantly higher sensitivity for accurate real-time quantification (Figure 2).

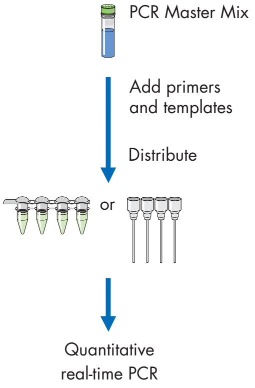
Easy handling with convenient master-mix format

Both QuantiTect Probe Kits contain a unique master mix, with HotStarTaq DNA Polymerase, an optimized PCR or RT-PCR buffer, and dNTPs. dUTP is included, enabling an optional UNG (uracil-N-glycosylase) treatment. In addition, both master mixes contain the internal reference dye ROX. This allows normalization on ABI sequence detection systems without interfering with reactions on other real-time cyclers that do not have this normalization option. Master mixes for both kits are stable at –20°C and 4°C for many months. Storage at 4°C allows even faster setup of amplification reactions by eliminating thawing time.

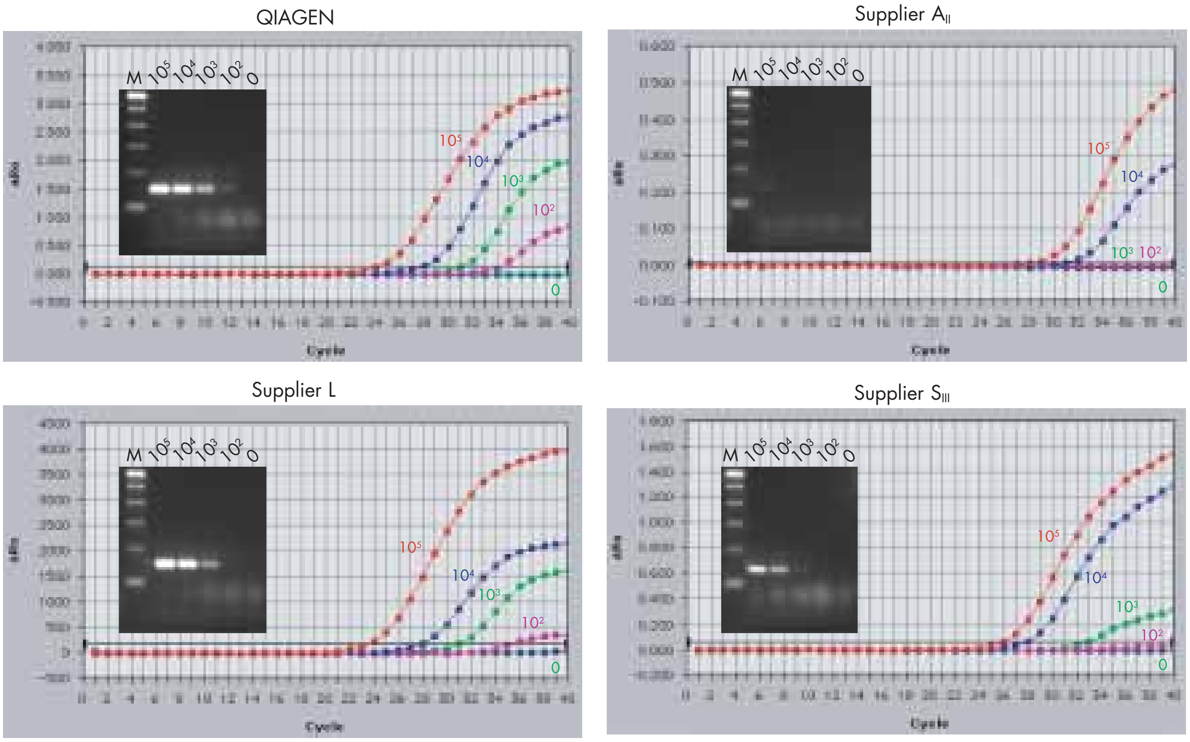
The QuantiTect Probe RT-PCR Kit also contains a blend of Omniscript and Sensiscript Reverse Transcriptases, provided as a separate solution to enable simple setup of control reactions without reverse transcriptases. RNase-free water for the addition of primers and templates is provided with both kits. This ready-to-use format minimizes pipetting steps and eliminates tedious calculations, maximizing both convenience and accuracy (see flowchart).

▶

PCR and two-step RT-PCR



Highly Sensitive Quantification Using the QuantiTect Probe RT-PCR Kit


QIAGEN

Supplier A_{II}

Supplier L

Supplier S_{III}

Figure 2 *Amplification plots of real-time RT-PCR analysis using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). One-step, quantitative RT-PCR was carried out using the QuantiTect Probe RT-PCR Kit (**QIAGEN**) or one-step, quantitative RT-PCR kits from suppliers A_{II}, L, or S_{III}, as indicated, according to suppliers' instructions. Reactions were performed with the indicated number of copies of an in vitro transcript of the TATA-box binding protein (TBP).*
***Inset**: Agarose-gel analyses of end-point PCR results. **M**: markers.*

Related articles in this issue

High sensitivity in quantitative, competitive RT-PCR using QIAGEN enzymes, page 3.

Versatile use with any real-time cycler and fluorescent probe

QuantiTect Probe Kits can be used with all types of fluorescent probes for real-time PCR and RT-PCR. Compatible probes include TaqMan or other dual-labeled probes that rely on the 5'→3' exonuclease activity of *Taq* DNA polymerase, as well as LightCycler hybridization probes or FRET probes, and Molecular Beacons. Both kits contain detailed, optimized protocols for using these probes with the ABI, LightCycler, iCycler, or DNA Engine Opticon systems (Table 1).

QIAGEN Operon offers a wide variety of these and other modified custom oligos. Please contact QIAGEN Technical Services for more details. ■

Table 1. Optimized protocols for QuantiTect Probe Kits

	Detection system			
Probes	**ABI Sequence Detection Systems**	**LightCycler (Roche)**	**iCycler (Bio-Rad)**	**DNA Engine Opticon (MJ Research)**
TaqMan or dual-labeled probes	✔	✔	✔	✔
Molecular Beacons	✔	✔	✔	✔
LightCycler hybridization or FRET probes	**n.t.**	✔	**n.t.**	**n.t.**

✔ : *Optimal for use with QuantiTect Probe Kits*
n.t.: *not tested*

Reader Inquiry No. 02101

Ordering Information

Product	Contents	Cat. No.
QuantiTect Probe Kits — for quantitative, real-time PCR and RT-PCR using sequence-specific probes		
QuantiTect Probe PCR Kit (200)	For 200 x 50 µl reactions: 3 x 1.7 ml QuantiTect Probe PCR Master Mix;* 2 x 2.0 ml RNase-free water	204343
QuantiTect Probe RT-PCR Kit (200)	For 200 x 50 µl reactions: 3 x 1.7 ml QuantiTect Probe RT-PCR Master Mix;* 1 x 100 µl QuantiTect RT Mix; 2 x 2.0 ml RNase-free water	204443

* *Contains 8 mM $MgCl_2$*

For related products, see page 6.

Important considerations for generating knockout mice

Following the article "Important considerations for generating transgenic mice" (*QIAGEN News* 2001 No. 5), this article discusses important factors when generating knockout mice, as well as an overview of the procedure.

Gene targeting technology uses homologous recombination to replace a specific region of the genome with a different DNA sequence. In knockout animals, a specific gene is replaced by vector sequences so that the gene no longer functions. Such animals are used in a variety of ways and allow the researcher to test the specific function of a gene by monitoring the effect of absence of its protein product. "Knock-in" animals can also be generated where the sequence of a given gene is changed. Mice are most commonly used in these studies since they are small and easy to handle, reproduce quickly, and have genes that are easy to manipulate. Mice provide valuable models of human disease since the genetic patterns of mice and humans are similar.

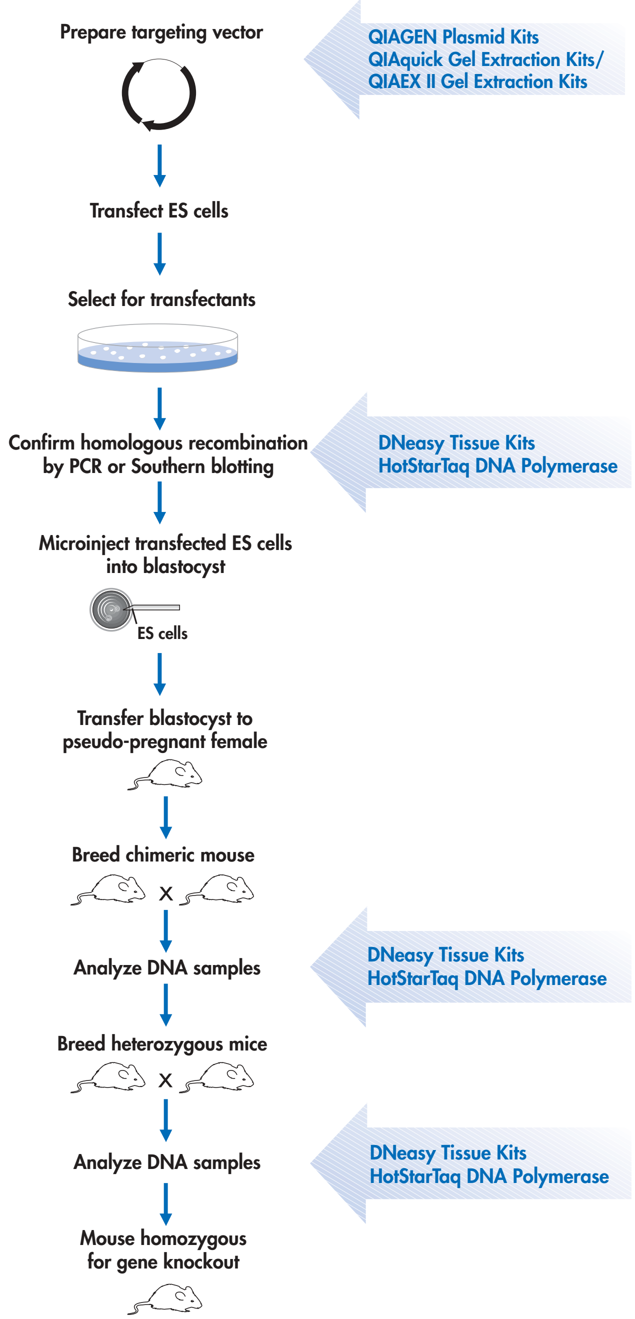
In contrast to the knockout technique, which alters the gene(s) of a host cell, generating transgenic mice involves adding genes to the chromosomes within the egg and can involve the transfer of genes from one species to another. Knockout mice take around 14 months to engineer, which is considerably longer than transgenic mice, which take around 6 months. This is because gene knockout technology is much more specific.

Generation of knockout mice

The process of creating a knockout mouse follows several basic steps (see flowchart). Firstly, a gene-targeting construct must be engineered, and then transfected into murine embryonic stem (ES) cells, usually via electroporation. ES cells are pluripotent and can be maintained indefinitely in an undifferentiated state. However, under the correct conditions ES cells are capable of differentiating into any cell type.

▶

Procedure for Generating Knockout Mice



Transfected ES cells are selected, and homologous recombinants are identified using PCR and Southern blotting. Successfully transfected stem cells are then microinjected into a blastocyst, and blastocysts are transferred to a pseudo-pregnant female. A proportion of the offspring have some tissues derived from the original blastocysts (with a wild-type genotype), and others derived from the manipulated ES cells (with the altered genotype). ES cell lines and donor blastocysts from mice with different natural coat colors are used so that in the chimeric mouse, skin cells derived from the ES cells will be one color and cells derived from the blastocyst will be another. These offspring are called chimeric, and are easily identified by having 2 coat colors.

Chimeric mice are then back- and self-crossed over several generations in order to generate homozygous knockout mice. The time taken for this process can be substantially shortened by using marker-assisted techniques, also known as "speed congenics". Using this technology the number of generations necessary to develop a congenic strain is considerably reduced (1, 2).

Important considerations

Vector preparation

The design of the vector is perhaps the most important step in the generation of knockout mice. The composition of the vector has a major impact on transfection efficiency and hence the ability to select and screen ES cell transformants, and also on whether the gene in question is actually knocked out.

A typical targeting vector comprises a plasmid DNA backbone, along with selection markers and DNA homologous to the regions flanking the gene to be knocked out. The plasmid vector usually contains an origin of replication and a selective cassette, which contains antibiotic selection markers that are used to discriminate between transfected and untransfected ES cells. The plasmid backbone provides the framework for DNA manipulation within the host. QIAGEN® EndoFree® Plasmid Kits can be used to isolate ultrapure, endotoxin-free plasmid DNA which is highly suited for vector preparation. QIAquick® and QIAEX® II Gel Extraction Kits can be used for purification of linear DNA fragments from agarose gels following restriction digestion reactions.

Another important factor in designing the target vector is the DNA flanking the selective cassette of the plasmid DNA. This flanking DNA should be derived from the genome of the ES cell line into which the targeting construct will be transfected, and homologous to the regions surrounding the gene to be knocked out. The longer the homologous regions, the greater the targeting efficiency. As a general guide it is recommended to include 3–8 kb of homologous DNA on either side of the selective cassette.

Screening for homologous recombinant clones

Once the vector has been transfected into ES cells and transfected clones have been isolated, clones that have undergone homologous recombination for the gene of interest must be selected, and differentiated from those that have undergone random (non-homologous) recombination. This is done by PCR or Southern blotting.

PCR is usually used as a preliminary method of screening, and Southern blotting is used to confirm the identity of positive clones. DNeasy® Tissue Kits are highly suited for isolation of genomic DNA, yielding DNA that performs well in both PCR reactions and Southern blotting analyses (3, 4). HotStarTaq™ DNA Polymerase is well suited for highly specific PCR.

Screening mice for gene knockout

Once chimeric mice are obtained they are bred with wild-type mice to produce heterozygous offspring. These heterozygous progeny are then back- and self-crossed a number of times in order to generate pups homozygous for the knockout gene. At all stages, progeny are screened for the knockout gene using Southern blotting or PCR analysis. DNeasy Tissue and DNeasy 96 Tissue Kits have been successfully used for isolation of DNA from mouse tails when screening knockout mice (3, 4, 5, 6).

References

1. *Markel, P. et al. (1997) Theoretical and empirical issues for marker-assisted breeding of congenic mouse strains. Nature Genet.* ***17****, 280.*
2. *Wakeland, E., Morel, L., Achey, K., Yui, M., Longmate, J. (1997) Speed congenics: a classical technique in the fast lane (relatively speaking). Immunol. Today* ***18****, 472.*
3. *Levy, J., Montross, L., Andrews, N. (2000) Genes that modify the hemochromatosis phenotype in mice. J. Clin. Invest.* ***105****, 1209.*
4. *Saari, J. et al. (2001) Visual cycle impairment in cellular retinaldehyde binding protein (CRALBP) knockout mice results in delayed dark adaptation. Neuron* ***29****, 739.*
5. *Doyonas, R. et al. (2001) Anuria, omphalocele, and perinatal lethality in mice lacking the CD34-related protein podocalyxin. J. Exp. Med.* ***194****, 13.*
6. *Iakoubova, O. et al. (2000) Microsatellite marker panels for use in high-throughput genotyping of mouse crosses. Physiol. Genomics* ***3****, 145.*

Summary

◆ Vector design is the most critical step in the generation of knockout mice. QIAGEN Plasmid Kits and DNA Cleanup Systems ensure high-quality DNA for use in vector construction.

◆ Accurate identification of recombinant clones and pups homozygous for gene knockout is crucial. DNeasy Tissue Kits provide a rapid and reliable method for isolating DNA from ES cells and mouse tails, while HotStarTaq DNA Polymerase facilitates high-specificity PCR. ■

> “As a leading supplier of knockout and transgenic mice and related services, efficient and reliable mouse genotyping tools are of paramount importance. The DNeasy 96 Tissue Kit and QIAGEN HotStarTaq DNA Polymerase consistently meet our high standards, and provide excellent results.”
>
> Jan L. Seymour
> Supervisor, Molecular Analysis
> Taconic Biotechnology
> **www.taconic.com**

Reader Inquiry No. 02105

Ordering Information

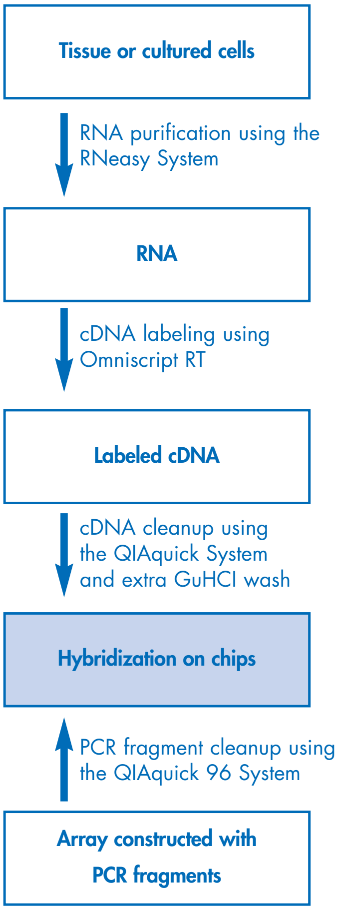
Product	Contents	Cat. No.
EndoFree Plasmid Kits — for purification of endotoxin-free ultrapure plasmid DNA		
EndoFree Plasmid Maxi Kit (10)[*]	10 QIAGEN-tip 500, 10 QIAfilter™ Maxi Cartridges, Buffers	12362
QIAquick Gel Extraction Kits — for purification of 70 bp – 10 kb DNA fragments		
QIAquick Gel Extraction Kit (50)[†]	50 QIAquick Spin Columns	28704
QIAEX II Gel Extraction Kits — for purification of 40 bp – 50 kb DNA fragments		
QIAEX II Gel Extraction Kit (150)[†]	For up to 150 extractions: QIAEX II Suspension, Buffers	20021
HotStarTaq DNA Polymerase — for highly specific, robust amplification		
HotStarTaq DNA Polymerase (250)[†]	250 units HotStarTaq DNA Polymerase, 10x PCR Buffer[‡], 5x Q-solution, 25 mM $MgCl_2$	203203
DNeasy Tissue Kits — for isolation of genomic DNA from animal tissues and cells, yeast, or bacteria		
DNeasy Tissue Kit (50)[†]	50 DNeasy Spin Columns, Reagents and Buffers, Collection Tubes (2 ml)	69504
DNeasy 96 Tissue Kits[§] — for high-throughput isolation of DNA from animal tissues and cells		
DNeasy 96 Tissue Kit (4)[†]	For 4 x 96 DNA minipreps: 4 x DNeasy 96 Plates, Reagents and Buffers, Collection Microtubes (1.2 ml), Collection Microtube Caps, 96-Well Plate Registers	69581

[*] *Different kit formats available; please inquire*
[†] *Larger kit sizes available; please inquire*
[‡] *Contains 15 mM $MgCl_2$*
[§] *Requires the use of the QIAGEN 96-Well-Plate Centrifuge System*

Efficient cDNA labeling and cleanup for successful microarray analysis

Christian Korfhage, Ivonne Schröder-Stumberger, and Evelyn Fisch
QIAGEN GmbH, Hilden, Germany

Production of Target DNA



The rapidly expanding field of microarray analysis has led to many important advances in gene expression profiling and mutation analysis. Improvements in array manufacture now allow simultaneous quantitative analysis of RNA transcription levels across an entire genome. As the technique has developed, microarray technology has become considerably more robust and sensitive. However, one of the most important factors for successful analysis remains the quality of the nucleic acids used in the procedure.

For reliable results, microarrays must be constructed using reproducible amounts of probe DNA (the DNA that is spotted onto chips), and the labeled target DNA used for hybridization must be of a high quality and purity.

Problems mainly occur during the production of target DNA. Nonspecific priming during the labeling step can misrepresent the gene expression profile by producing extraneous cDNAs from rRNA, multiple cDNAs from different regions of the same mRNA, or short cDNAs that hybridize nonspecifically (1). In addition, carryover of salts, unincorporated labeled nucleotides, and proteins during the subsequent cleanup step can further compromise the purity of target DNA. The presence of any one of these contaminants in the target DNA often leads to high background and artifactual signals.

We describe here a procedure using Omniscript™ Reverse Transcriptase for cyanine-5 (Cy®5) labeling of target DNA followed by cleanup using a modified QIAquick® PCR Purification Kit protocol (see flowchart). The QIAquick cleanup procedure delivered high yields of high-purity target cDNA that gave strong signal intensities and minimal background in microarray analysis.

Materials and methods

Total RNA was purified from mouse brain using the RNeasy® Mini Kit. Labeled target cDNA was generated by reverse transcription using Omniscript RT in a reaction containing Cy5-dCTP and 10 µg total RNA in a total volume of 50 µl. Cleanup of target cDNA was carried out using a modified QIAquick PCR Purification Kit protocol containing an additional wash step. This wash step, using 35% (w/v) guanidine hydrochloride, is carried out after cDNA binding and ensures maximum removal of unincorporated nucleotides. Hybridization was performed using target cDNA corresponding to 2 µg total RNA.

Semiquantitative PCR was used to estimate the yield of cDNA recovered after the QIAquick cleanup procedure. 1/2500th of the labeling reaction or the eluate following cleanup was added to a PCR containing a primer that amplified a 199 bp fragment. Half of each PCR was loaded onto an agarose gel and quantified by densitometry.

High Recoveries of Labeled cDNA

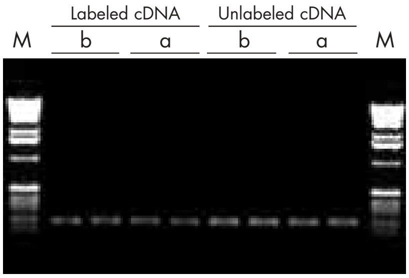


Figure 1 *Semiquantitative PCR analysis of cDNA before* ***(b)*** *and after* ***(a)*** *the modified QIAquick procedure. Calculated average recoveries were 89% (labeled cDNA) and 91% (unlabeled cDNA) of input.* ***M****: markers.*

Results

Figure 1 shows agarose gel analysis of the PCR products obtained in the semiquantitative PCR analysis. On average, 89% of the labeled input cDNA was recovered.

Figure 2 shows the results of two independent microarray analyses using target cDNA cleaned up using the modified QIAquick protocol. The absence of significant background and high reproducibility in the hybridized arrays demonstrates the superior results obtainable with QIAquick purified target cDNA.

Conclusions

Generation of target cDNA using Omniscript RT for the reverse transcription labeling reaction, followed by DNA cleanup using a modified QIAquick PCR purification protocol, gives high signal intensities with minimal background and reliable, reproducible results in microarray analysis. ■

References

1. Fisch, E., Brinker-Krieger, N., Schwarz, H., Schäfer, A., and Korfhage, C. (2001) Improved signal-to-noise ratios using Omniscript RT for microarray and LightCycler® analyses. QIAGEN News 2001 No. 3, 13.

Reproducible Microarray Analysis with High Signal Intensities and Low Background

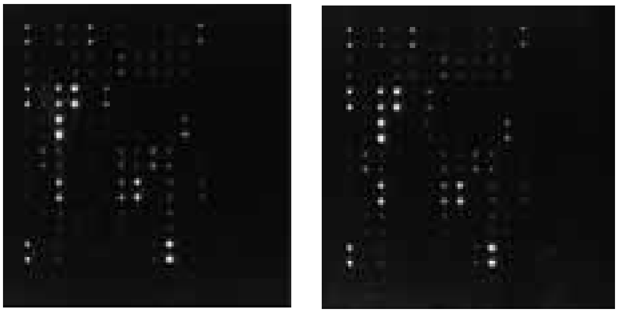


Figure 2 *Two independent microarray analyses using the same QIAquick 96 purified probe DNA derived from mouse brain. Cy5-labeled target cDNA was cleaned up using a modified QIAquick procedure as detailed in "Materials and methods".*

Reader Inquiry No. 02106

Ordering Information

Product	Contents	Cat. No.
QIAquick 96 PCR Purification Kit (4)*	For purification of 1 x 96 PCR reactions: 1 QIAquick 96 Plate, Buffers, Collection Microtubes (1.2 ml), Caps	28181
QIAquick PCR Purification Kit (50)*	For purification of 50 PCR reactions: 50 QIAquick Spin Columns, Buffers, Collection Tubes (2 ml)	28104
Omniscript RT Kit (50)*	For 10 reverse-transcription reactions: 40 units Omniscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix,[†] RNase-free water	205111
RNeasy Mini Kit (50)*	50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	74104

** Other kit sizes available; please inquire.*

[†] Contains 15 mM each dNTP

New

Increased sensitivity in bioassays using improved Ni-NTA HisSorb™ Plates and Strips

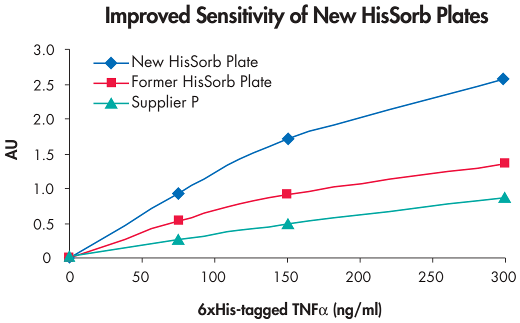


Figure 1 *Dilutions (150 µl) of 6xHis-tagged tumor necrosis factor α (TNFα) were bound to a new HisSorb Plate (◆), a former HisSorb Plate (■), or a metal chelate-coated plate from Supplier P (▲) for 30 minutes at room temperature, and washed 4 times. A peroxidase-conjugated anti-TNFα antibody (150 µl at 50 mU/ml) was added and allowed to bind for 30 minutes at room temperature. After four washes, color was developed using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS).*

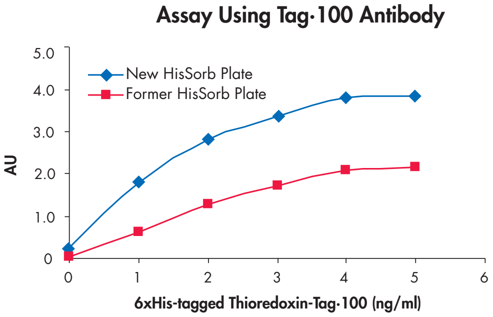
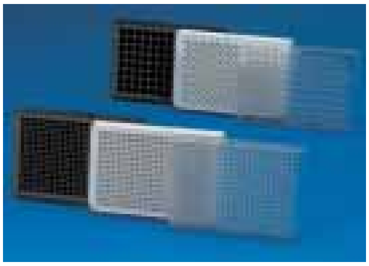


Figure 2 *Dilutions (200 µl) of thioredoxin tagged with 6xHis at the N-terminus and with Tag·100 at the C-terminus were bound to a new HisSorb Plate (◆) and a former HisSorb Plate (■) for 60 minutes at room temperature. The plate wells were washed four times and 200 µl of Tag·100 Antibody was added and allowed to bind for 60 minutes at room temperature. After 4 washes, 200 µl of a peroxidase-conjugated anti-mouse IgG secondary antibody was added and allowed to bind for 60 minutes at room temperature. After an additional 4 washes, color was developed using ABTS.*



Ni-NTA HisSorb plates in 96- and 384-well formats

Ni-NTA HisSorb™ Plates and Strips allow efficient, directed binding of 6xHis-tagged proteins — even from crude lysates — and subsequent assay using standard ELISA techniques and equipment. A new Ni-NTA coating procedure used in the manufacture of HisSorb Strips and Plates delivers significantly increased sensitivity in ELISA procedures.

The Ni-NTA–6xHis-tag interaction

Ni-NTA HisSorb Plates and Strips utilize the remarkable selectivity of Ni-NTA (nickel-nitrilotriacetic acid) for an affinity tag of six consecutive histidine residues (the 6xHis tag). Proteins, immobilized via an N- or C-terminal 6xHis tag, are optimally presented to antibodies or other interaction partners in a convenient and reproducible procedure.

The directed binding enhances assay sensitivity, minimizes nonspecific binding, and increases reproducibility and signal-to-noise ratios. By using the Ni-NTA–6xHis tag interaction for immobilization, time-consuming protein-specific optimization trials are eliminated. Now, due to a new coating procedure used in their manufacture, assays using HisSorb Plates and Strips are even more sensitive.

Increased sensitivity in assays

The improved HisSorb Strips and Plates offer enhanced 6xHis-tag binding efficiency and minimized nonspecific binding, significantly increasing the sensitivity of assays compared to former HisSorb products and other commercially available plates (Figures 1 and 2). This increase in sensitivity means that less material is required for each assay, which is especially important when assaying precious or rare samples.

Wide choice of formats

Ni-NTA HisSorb Strips are supplied framed and sealed in sets of twelve 8-well strips for maximum convenience when testing or optimizing assays using a limited number of samples. For higher throughput requirements, 96-well Ni-NTA HisSorb Plates are available. QIAGEN also offers a wide range of customized assay products to suit your individual requirements.* Ni-NTA HisSorb Plates in 96- and 384-well formats are available in transparent format for use with colorimetric assays and a white or black opaque format for luminescence- or fluorescence-based assays. All plates are compatible with standard multichannel pipets and automated plate washers and readers.

Reliable and reproducible assay results

All HisSorb products are charged with nickel and preblocked with BSA for immediate use. Vigorous quality control measures ensure that HisSorb products provide uniform binding, washing, and optical properties for reliable and reproducible measurements, both on a well-to-well and a plate-to-plate basis.

The Tag·100 Antibody

The Tag·100 Antibody is used to detect proteins, expressed using the pQE-100 DoubleTag™ vector, that carry a 6xHis tag at their N-terminus and the Tag·100 epitope at their C-terminus (Figure 3). Directed immobilization using the Ni-NTA–6xHis tag interaction, and detection of the optimally presented Tag·100 epitope using the Tag·100 Antibody, increases standardization in ELISA procedures.

Tag·100 Antibodies

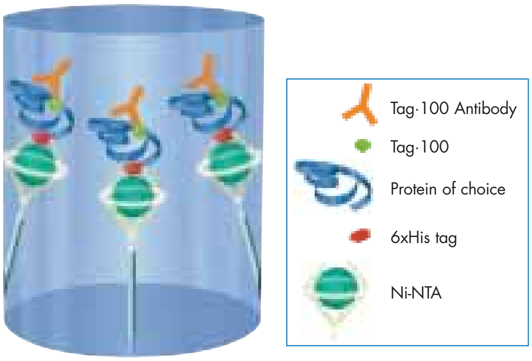


Figure 3 *Detection of proteins expressed by the pQE-100 Double-Tag vector using Tag·100 Antibodies.*

The high levels of sensitivity, convenience, and flexibility offered by HisSorb Plates and Strips make them the method of choice for assay of 6xHis-tagged proteins. To find out more about the QIA*express*® Assay System visit us at **www.qiagen.com** or contact QIAGEN Technical Services or your local distributor. ■

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* *Please inquire about customized assay products available in minimum orders of 100 plates (allow 6 weeks for delivery). Customized products available include Ni-NTA HisSorb Plates (transparent, white, or black) in 96- and 384-well format, as well as plates coated with any of the QIAexpress Anti·His Antibodies in 96- or 384-well format.*

Reader Inquiry No. 02107

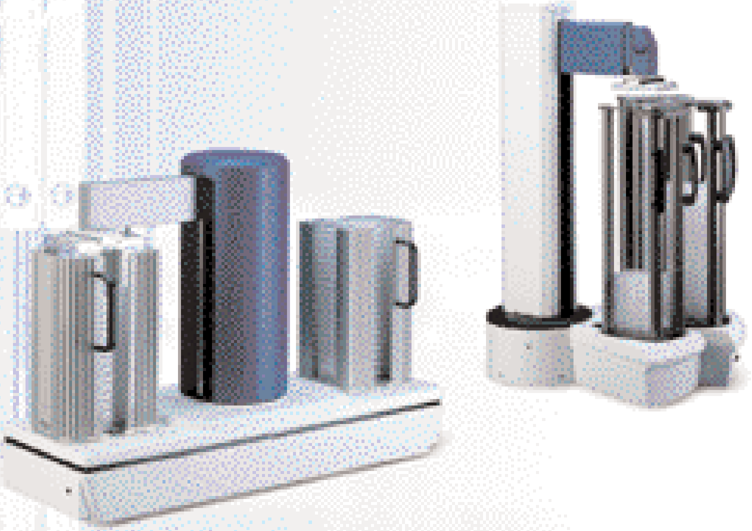
Ordering Information

Product	Contents	Cat. No.
Ni-NTA HisSorb Strips (24)	2 racks of 12 x Ni-NTA–coated 8-well strips in 96-well format	35023
Ni-NTA HisSorb Plates (5)	5 Ni-NTA–coated, transparent 96-well plates	35061
Ni-NTA HisSorb Plates, white (5)	5 Ni-NTA-coated, opaque, white 96-well plates	35081
Related products		
Tag·100 Antibody, BSA-free (100 µg)	100 µg mouse anti-Tag·100 (lyophilized, BSA-free, for 1000–2500 ml working solution for ELISA)	34680
pQE-100 DoubleTag Vector DNA	25 µg pQE-100 (lyophilized)	33003

New BioRobot® Twister™ robotic arms — expand your integration options

BioRobot® Twister™ robotic arms are versatile systems for the transfer and storage of microplates and other labware. Twister robotic arms are used with BioRobot 3000 and BioRobot 8000 workstations to allow integration of external instruments for walkaway automation of sequential tasks. BioRobot Twister I and Twister II robotic arms are compatible with most laboratory instruments, ensuring seamless integration into your workflow.

Using BioRobot Twister robotic arms with BioRobot workstations allows integration of instruments such as thermal cyclers, spectrophotometers, plate sealers, and storage incubators. Walkaway automation of sequential tasks such as sample purification, normalization of sample concentration, reaction setup and cycling, and reaction cleanup is now simple.



BioRobot Twister robotic arms provide:

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- Unattended overnight processing capability
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Reader Inquiry No. 02108

New

UltraSens™ technology for highly efficient purification of viral nucleic acids

The QIAamp® UltraSens™ Virus Kit uses new UltraSens technology for increased viral nucleic acid yield from plasma and serum samples. Now your downstream assays can be even more sensitive!

In the QIAamp UltraSens Virus procedure, UltraSens technology is first used to capture viral nucleic acids from a 1 ml sample, allowing them to be highly concentrated. No ultracentrifugation or specialized laboratory equipment is required, and an internal control can be added at the start of the procedure. The concentrated nucleic acids are then purified using a fast silica-gel–based spin-column procedure, yielding highly pure, ready-to-use nucleic acids suitable for use in amplification-based assays.

The QIAamp UltraSens Virus Kit is suitable for purifying nucleic acids from viruses such as HIV-1, HAV, HBV, HCV, and B19V (see Table 1), as well as BHV-1, SIV, PCV, and PCMV. It is also well suited for efficient isolation of genomic DNA from serum and plasma. ■

Table 1. Highly sensitive detection of viruses using UltraSens technology

Virus	Detection limit	Detection method*
HCV	1.8 IU/ml	AMPLICOR®
HIV	10 IU/ml	AMPLICOR
HBV	15 genome equivalents/ml	TaqMan®
HAV	4 genome equivalents/ml	TaqMan
B19V	16 genome equivalents/ml	TaqMan

* *Viral nucleic acids were amplified and detected using either the Cobas® AMPLICOR or TaqMan systems from Roche Molecular Systems, Inc.*

Reader Inquiry No. 02109

Ordering Information

Product	Contents	Cat. No.
QIAamp UltraSens Virus Kit (50)	50 viral nucleic acid preps: 50 QIAamp Spin Columns, QIAGEN Proteinase K, Carrier RNA, Collection Tubes (2 ml), Buffers	53704
QIAamp UltraSens Virus Kit (250)	250 viral nucleic acid preps: 250 QIAamp Spin Columns, QIAGEN Proteinase K, Carrier RNA, Collection Tubes (2 ml), Buffers	53706

Rapid isolation of anthrax DNA from large-volume soil samples using QIAamp® Kits

Gudrun Zoll, Gudrun Grote, Roland Dierstein, and Stefan Köhne

Armed Forces Scientific Institute for Protection Technologies, Munster, Germany

QIAamp® Kits were used to develop a rapid method for the isolation of Bacillus anthracis *DNA from large-volume soil samples. The method provided good yields of high-quality DNA that performed well in downstream PCR.*

Anthrax is a potentially fatal infectious disease caused by a spore-forming bacterium, *Bacillus anthracis*. Approximately 95% of anthrax cases in humans result from exposure to infected soil or animals, through either ingestion or skin lesions. Rapid diagnosis is important for treating the disease, as antiobiotics are highly effective if administered to an infected individual before symptoms occur. A reliable and fast method for detection of *B. anthracis* in soil samples is therefore important for disease control. PCR-based assays should provide more rapid methods for detection of anthrax than protocols requiring bacterial cultivation.

An important factor in the success of PCR is the use of high-quality DNA templates. Preparation of high-quality DNA from soil samples is challenging for two reasons. Firstly, soil contains high levels of enzymatic inhibitors that will interfere with downstream PCR, and secondly, samples are often large in volume. Furthermore, DNA purity, and hence PCR detection limits, depend on soil type. For example, sandy soils yield the highest quality DNA, while it is very difficult to isolate amplifiable DNA from rich loamy soils containing high levels of humic acid. This is because the loam binds DNA efficiently, and humic acid is a potent PCR inhibitor.

We describe here the development of a rapid method for the isolation of *B. anthracis* DNA from loamy soil samples. Soil samples were inoculated with inactivated *B. anthracis* cells and DNA was isolated using the QIAamp DNA Stool Mini Kit according to a modified protocol. The resulting DNA was of a high quality and performed well in downstream PCR.

Materials and methods

Soil samples (5 g samples of sandy loam) were inoculated with 1 ml solutions containing either 10^5 or 10^7 inactivated *B. anthracis* cells. *B. anthracis* cells were inactivated using H_2O_2, resulting in bacterial inactivation without cell lysis. Samples were left for 5 minutes at room temperature, then 1 ml water was added to each tube to form soil suspensions. Samples were incubated in a water bath at 100°C for 10 minutes, and centrifuged for 5 minutes at 3000 rpm. The supernatant was removed and 7 volumes of Buffer ASL was added.

For DNA isolation, 2 InhibitEX™ tablets from the QIAamp DNA Stool Mini Kit were added to each tube, tubes were incubated for 1 minute, and the contents were mixed. Tubes were centrifuged for 4 minutes at 10,000 x *g* and supernatants were transferred to new tubes. One volume of Buffer AL was added to each sample, tubes were mixed by inversion, and one volume of 96–100% ethanol was added.

Washing and elution were carried out using the QIAvac 24. Samples were applied to columns from the QIAamp DNA Blood Midi Kit and washed with 1 ml volumes of Buffers AW1 and AW2. The columns were transferred to 15 ml tubes and centrifuged for 15 minutes at 4500 rpm. For elution, 300 µl water was pipetted onto each column, columns were incubated for 5 minutes at room temperature, and centrifuged for 5 minutes at 4500 rpm. The eluate containing DNA was applied again to the column and a second elution step was performed, using identical conditions.

The resulting DNA was used as a template in PCR using QIAGEN® *Taq* DNA Polymerase. Each reaction contained 2 µl DNA template and 16 pmol each primer. Soil supernatants spiked with *B. anthracis*, and DNA purified from these supernatants were both used as templates in quantitative, real-time PCR using the LightCycler®. Each reaction contained 2 µl DNA template, 16 pmol primer, and QIAGEN *Taq* DNA Polymerase.

Results and discussion

Successful detection of *B. anthracis* DNA from soil samples was achieved using quantitative, real-time PCR with the LightCycler (see Figure 1). Figure 2 shows the improved PCR sensitivity achieved by removing the inhibitors present in loam. Using supernatants from spiked soil as PCR templates, *B. anthracis* could be detected in samples inoculated with 10^7 *B. anthracis* cells/5 g soil. Following DNA purification, this sensitivity improved so that 10^5 *B. anthracis* cells /5 g soil were detected. The same sensitivity was found in positive control samples. The purification procedure therefore efficiently removed the many PCR inhibitors present in loamy soil.

Real-Time PCR Analysis

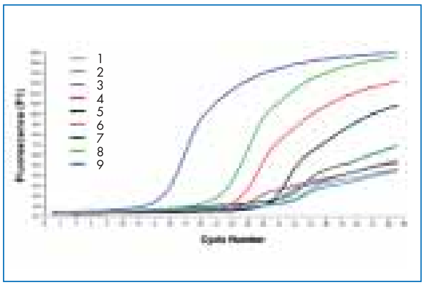


Figure 1 *Quantitative, real-time PCR using the LightCycler. Samples 1–9 are the products shown in Figure 2, lanes 1–9.*

High Sensitivity Following DNA Purification

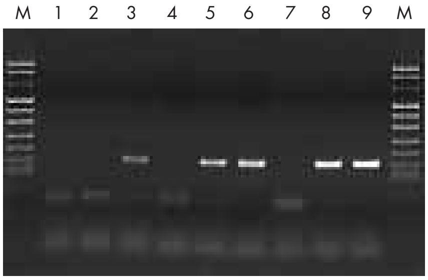


Figure 2 *Real-time PCR products: negative controls (**lanes 1, 4, and 7**); products from soil supernatants spiked with 10^5 (**lane 2**) and 10^7 (**lane 3**)* B. anthracis *cells/5 g soil; products from DNA purified from soil supernatants spiked with 10^5 (**lane 5**) and 10^7 (**lane 6**)* B. anthracis *cells/5 g soil; positive controls (**lanes 8 and 9**); markers (**M**).*

Conclusions

QIAamp Kits were used to develop a method for the rapid isolation of bacterial DNA from large volumes of soil. This method reduces the time required for DNA isolation by 1 hour in comparison with previously published methods. The QIAamp method provided efficient removal of PCR inhibitors, and *B. anthracis* could be detected down to a sensitivity of 10^5 cells/5g soil. ■

Reader Inquiry No. 02110

Ordering Information

Product	Contents	Cat. No.
QIAamp DNA Stool Mini Kit (50)	For 50 DNA preps: 50 QIAamp Spin Columns, QIAGEN Proteinase K, InhibitEX tablets, Buffers, Collection Tubes (2 ml)	51504
QIAamp DNA Blood Midi Kit (20)*	For 20 DNA midipreps: 20 QIAamp Midi Spin Columns, QIAGEN Protease, Buffers, Collection Tubes (15 ml)	51183
QIAvac 24	Vacuum manifold for processing 1–24 spin columns: includes QIAvac 24 Base, Lid, Luer Caps	19403

* *Larger kit sizes avaiable; please inquire*

continued from page 1

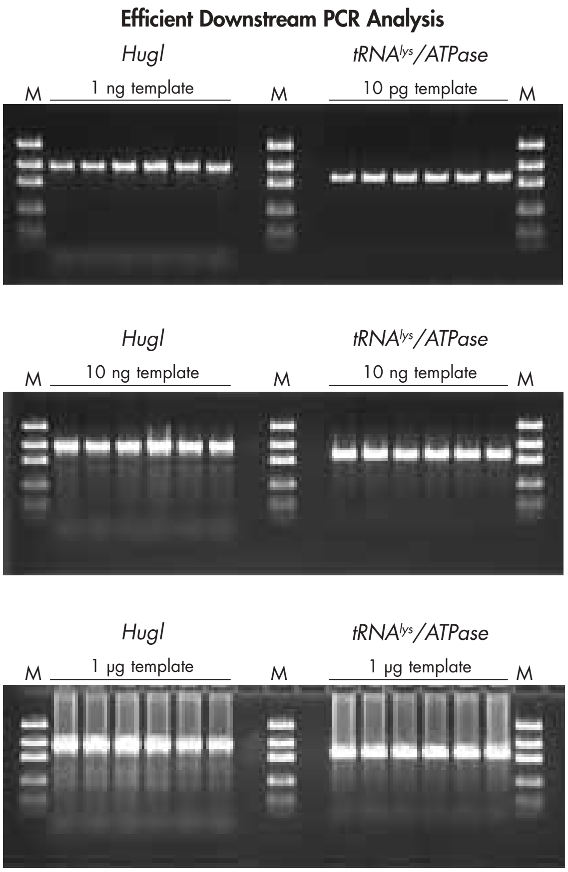


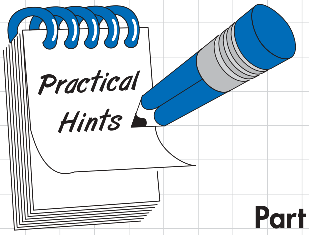
Figure 1 *Variable amounts of DNA template were used to amplify the single-copy* Hugl *gene and a mitochondrial target (tRNA^{lys}/ATPase). Each sample was analyzed 6 times, and reproducible results were achieved.* ***M****: markers.*

The FlexiGene protocol begins with addition of lysis buffer to samples. Cell nuclei and mitochondria are pelleted by centrifugation and resuspended in denaturation buffer containing QIAGEN Protease. Following protein digestion, DNA is precipitated by addition of isopropanol, recovered by centrifugation, washed in 70% ethanol, and dried. DNA is resuspended in hydration buffer and is ready for direct use in downstream assays or storage at –20°C. The efficient performance of the DNA in PCR assays is shown in Figure 1. ■

Reader Inquiry No. 02102

Ordering Information

Product	Contents	Cat. No.
FlexiGene DNA Kit (50)	For purification of DNA from 50 ml whole blood: Buffers, QIAGEN Protease	51204
FlexiGene DNA Kit (250)	For purification of DNA from 250 ml whole blood: Buffers, QIAGEN Protease	51206



The QIAGEN Guide to Animal Cell Culture

Part II: Safety and handling considerations for animal cell culture

Welcome to the second of a new series of articles aimed at providing useful hints for culturing animal cells. This article describes considerations for working with animal cell cultures as well as information on cell culture contamination. The series will continue in future issues of *QIAGEN News* with information on cell culture growth conditions, followed by cell culture protocols.

Legislation and regulatory guidelines

Before undertaking any work with human or animal tissue (e.g., to establish a primary cell culture), it is necessary to ensure that the nature of the work conforms to the appropriate medical-ethical and animal-experiment legislation and guidelines. It may be necessary to seek approval from the relevant regulatory authorities and/or individuals.

Safety considerations and biohazards

When working with potentially hazardous material, it is important to be aware of the possible risks associated with both the material and the experimental protocol. All cell cultures are considered a biohazard because of their potential to harbor an infectious agent (e.g., a virus). The degree of hazard depends on the cells being used and the experimental protocol. Primary cell cultures in particular should be handled carefully as these cultures have a high risk of containing undetected viruses. Although commonly used cell lines are generally assumed to be free of infectious agents, care should still be exercised when working with these cell lines as it is possible that they contain infectious agents, such as latent viruses. Cell cultures used to study specific viruses should be assumed to have the same degree of hazard as the virus under study.

We recommend handling all material as potentially infectious to ensure the safest possible working environment. Work should be performed in an approved laminar flow hood using aseptic technique, and the creation of aerosols should be avoided (see below). After the work is complete, all waste media and equipment (i.e., used flasks, pipets, etc.) should be disinfected by autoclaving or immersion in a suitable disinfectant according to institutional and regional guidelines.

Handling cell cultures

Adherence to good laboratory practice when working with cell cultures is essential for two reasons: first, to reduce the risk of exposure of the worker to any potentially infectious ▶

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References

1. *Freshney, R.I. (1993)* Culture of Animal Cells, a Manual of Basic Technique. *3rd ed. New York: Wiley-Liss.*
2. *Spector, D., Goldman, R.R., and Leinwand, L.A., eds. (1998)* Cells: a Laboratory Manual. *Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.*
3. *Drexler, H.G. et al., eds. (1997)* DSMZ Catalog of Human and Animal Cell Lines. *6th ed.*

agent(s) in the cell culture, and second, to prevent contamination of the cell culture with microbial or other animal cells (see below).

Aseptic technique and minimization of aerosols

Aseptic technique and the proper use of laboratory equipment are essential when working with cell cultures. Always use sterile equipment and reagents, and wash hands, reagent bottles, and work surfaces with a biocide or 70% ethanol before beginning work.

Creation of aerosols should be avoided — aerosols represent an inhalation hazard, and can potentially lead to cross-contamination between cultures. To avoid aerosols, use TD (to deliver) pipets, and not TC (to contain) pipets; use pipets plugged with cotton; do not mix liquids by rapidly pipetting up and down; do not use excessive force to expel material from pipets; and do not bubble air through liquids with a pipet. Avoid releasing the contents of a pipet from a height into the receiving vessel. Expel liquids as close as possible to the level of liquid of the receiving vessel, or allow the liquid to run down the sides of the vessel.

Proper use of equipment can also help minimize the risk of aerosols. For example, when using a centrifuge, ensure the vessel to be centrifuged is properly sealed, avoid drops of liquid near the top of the vessel, and use centrifuge buckets with caps and sealed centrifuge heads to prevent contamination by aerosols.

Laminar flow hoods

For the most efficient operation, laminar flow hoods should be located in an area of the laboratory where there is minimal disturbance to air currents. Avoid placing laminar flow hoods near doorways, air vents, or locations where there is high activity. Hoods are often placed in dedicated cell culture rooms.

Tip Keep laminar flow hoods clean, and avoid storing equipment inside the hood.

Tip Before starting work, disinfect the work surface of the hood as well as the outside of any bottles (e.g., by wiping with 70% ethanol), and then place everything needed for the cell culture procedure in the hood.

Tip Arrange equipment, pipets, waste containers, and reagent bottles so that used items are not placed near clean items, and avoid passing used items over clean items.

Tip Place used items (e.g., pipets) in a container inside the hood, and disinfect or seal before removing from the hood.

Contamination

The presence of microorganisms can inhibit cell growth, kill cells, and lead to inconsistent results. Contamination of cell cultures can occur with both cell culture novices and experts. Potential contamination routes are numerous. For example, cultures can be infected through poor handling, from contaminated media, reagents, and equipment (e.g., pipets), and from microorganisms present in incubators, refrigerators, and laminar flow hoods, as well as on the skin of the worker and in cultures coming from other laboratories.

Bacteria, yeasts, fungi, molds, mycoplasmas, and other cell cultures are common contaminants in animal cell culture. To safeguard against accidental cell culture loss by contamination, we recommend freezing aliquots of cultured cells to re-establish the culture if necessary. A protocol for freezing cell cultures will be provided in a future issue of *QIAGEN News*.

Microbial contamination

The characteristic features of microbial contamination are presented in Table 1. The presence of an infectious agent sometimes can be detected by turbidity and a sharp change in the pH of the medium (usually indicated by a change in the color of the medium), and/or cell culture death. However, for some infections, no turbidity is observed and adverse affects on the cells are not easily observed.

Table 1. Characteristic features of microbial contamination

Characteristic	Bacteria	Yeast	Fungi
Change in pH	pH drop with most infections	pH change with heavy infections	pH sometimes increases
Cloudy medium: Under microscope (100–400x)	Shimmering in spaces between cells; rods or cocci may be observed	Round or ovoid particles that bud off smaller particles	Thin filamentous mycelia; sometimes clumps of spores

Cell cultures should be routinely evaluated for contamination. Mycoplasmal infections are one of the more common and difficult-to-detect infections; their detection and eradication are described in further detail below.

Mycoplasmal infection — detection

Mycoplasmas are small, slow-growing prokaryotes that lack a cell wall and commonly infect cell cultures. They are generally unaffected by the antibiotics commonly used against bacteria and fungi. Furthermore, as mycoplasma do not overgrow cell cultures and typically do not cause turbidity, they can go undetected for long periods of time and can easily spread to other cell cultures. The negative effects of mycoplasmal contamination include inhibition of metabolism and growth, as well as interference with nucleic acid synthesis and cell antigenicity. Acute infection causes total deterioration of the cell culture, sometimes with a few apparently resistant colonies that may, in fact, also be chronically infected. There are two main approaches to ▶

detect mycoplasma — Hoechst 33258 staining (1, 2) and mycoplasma-specific DNA probes (Fisher Scientific). Alternatively, a PCR-based, mycoplasma-testing service is offered by the ATCC (**www.atcc.org**) or BioReliance (**www.biomeva.com**) on a fee-for-service basis.

Mycoplasmal infection — eradication

The best action to take with a culture containing chronic mycoplasmal infection is to discard it by either autoclaving or incineration. Only if the cell culture is absolutely irreplaceable should eradication be attempted. This process should be performed by experienced personnel in an isolated hood that is not used for cell culture, preferably in a separate room. Elimination of mycoplasma is commonly achieved by treatment with various commercially available antibiotics such as a quinolone derivative (Mycoplasma Removal Agent), ciprofolxacin (Ciprobay®), enrofloxacin (Baytril®), and a combination of tiamulin and minocycline (BM-Cyclin). Treatment procedures and appropriate antibiotic concentrations can be found in the suppliers' instructions and in references 1 and 3.

Cross-contamination of cell lines

Cross-contamination of one cell culture with fast-growing cells from another culture (such as HeLa) presents a serious risk. To avoid cross-contamination, only use cell lines from a reputable cell bank; only work with one cell line at a time in the hood; use different pipets, bottles of reagents, and bottles of media for different cell lines; and check cells regularly for the correct morphological and growth characteristics.

The QIAGEN Guide to Animal Cell Culture will continue in future issues of *QIAGEN News* with information on cell culture growth conditions and cell culture protocols. If there is any other information you would like to see on these pages of *QIAGEN News*, please let us know by calling QIAGEN Technical Services or your local distributor. ■

QIA-Hints

QIAGEN Technical Services are always available to answer your questions!

TRANSFECTION

Q Can I use PolyFect® Transfection Reagent for other cell lines, besides the 5 that it's been optimized for?

A PolyFect Transfection Reagent is designed to provide optimal transfection results with COS-7, NIH/3T3, HeLa, 293, and CHO cells. We expect that other cell lines may also be successfully transfected using PolyFect Transfection Reagent, but we do not have detailed information. Therefore, it may be necessary to optimize transfection conditions for your specific cell type and DNA vector.

To choose the best reagent, a searchable list of cell lines and primary cells that have been successfully transfected using QIAGEN® Transfection Reagents is available at the QIAGEN Transfection Tools web site (**www.qiagen.com/transfectiontools/**). If your cell type of interest is not listed, or if you want to test different reagents to optimize transfection, QIAGEN offers the Transfection Reagent Selection Kit. This kit contains both Effectene® and SuperFect® Transfection Reagents and a comprehensive handbook to guide you through optimization and determine the best reagent and transfection conditions.

DNA

Q Our laboratory has developed its own set of optimized buffers and conditions for lysing specific bacteria. I've already lysed the bacteria using our lab's protocol. Can I still use the DNeasy® Tissue Kit or the QIAamp® DNA Mini Kit to isolate genomic DNA from them?

A If you have already optimized lysis conditions for a specific sample source and don't want to alter them, you can still use the DNeasy or QIAamp Kits for isolation of genomic DNA. Simply lyse the sample in your lysis buffer, and follow the protocol for isolation of genomic DNA from crude lysates. For cultured bacteria, see page 28 of the *DNeasy Tissue Kit Handbook* (April 1999), or, if you are working with human clinical samples containing bacteria, see page 52 of the *QIAamp DNA Mini Kit* and *QIAamp DNA Blood Mini Kit Handbook* (September 2001).

Please do not hesitate to call your local QIAGEN Technical Service Department if you have any questions or require further information regarding any QIAGEN products.

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QIAGEN exhibits and presents at many venues throughout the year. During the next few months, you can visit us at any of the meetings listed below. Come and see what's new, or talk to a company representative.

In North America

American Academy of Forensic Science (AAFS)	11–16 February, Atlanta, GA
CHI — Genome Tri-Conference 2002	23 February – 1 March, Santa Clara, CA
Association for Biomolecular Research (ABRF) Resource Facilities	9–12 March, San Diego, CA
Pittcon 2002	17–22 March, New Orleans, LA
American Association for Cancer Research (AACR)	6–10 April, San Francisco, CA
Microarrays Through Macroresults 3	19 April – 1 May, Boston, MA
Experimental Biology 2002: Translating the Genome	20–24 April, New Orleans, LA
Clinical Virology Symposium (CVS)	28 April – 1 May, Clearwater Beach, FL

In Europe

Swiss Society for Microbiology (SGM/SSM)	20–21 February, Lucerne, Switzerland
Union of the Swiss Societies of Experimental Biology (USGEB/USSBE)	7–8 March, Lugano, Switzerland
Forum Labo Biotech	26–29 March, Paris, France
DNA Vaccines 2002	10–12 April, Edinburgh, UK
ANALYTICA	23–26 April, Munich, Germany

In Asia and Australasia

27th Annual Conference on Protein Structure and Function	10–14 February, Lorne, Australia
14th Lorne Cancer Conference	14–17 February, Lorne, Australia
23rd Lorne Genome Conference	17–21 February, Lorne, Australia

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Hoffmann-La Roche owns patents and patent applications pertaining to the application of Ni-NTA resin (Patent series: RAN 4100/63: USP 4.877.830, USP 5.047.513, EP 253 303 B1), and to 6xHis-coding vectors and His-labeled proteins (Patent series: USP 5.284.933, USP 5.130.663, EP 282 042 B1). All purification of recombinant proteins by Ni-NTA chromatography for commercial purposes, and the commercial use of proteins so purified, require a license from Hoffmann-La Roche.

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DNA isolation from clinical samples

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