QIAGEN News
Innovation Working for You

4
August
2002

New

LabelStar™ Array Kit — efficient cDNA labeling and cleanup for high signal-to-noise ratios on arrays

- **High signal intensity and low background** — identification of true positives at low expression levels
- **Labeling using a wide range of RNA amounts** — from just 0.2 µg to 50 µg RNA
- **Flexibility in choice of label** — incorporation of any commonly used modified nucleotide
- **Optimized labeling and cleanup procedures** — reproducible, high-quality results
- **A fast and easy system** — reliable performance

▶▶ New LabelStar Array Kit, page 8

Coming soon!

PAXgene™ Blood DNA System — a standardized system for blood collection and genomic DNA isolation

- **Integrated, standardized system** — blood collection, transport, and storage, with DNA purification in one system
- **Convenient storage** — blood can be stored in PAXgene™ Blood DNA Tubes for up to 10 days at room temperature before DNA purification
- **Enhanced workflow efficiency**
- **Easy handling** — purification in a single tube minimizes the risks of sample mix-up and cross-contamination, and reduces plasticware consumption
- **Rapid** — only 1 hour of hands-on time for DNA purification from 12 samples
- **High yields of pure, high-molecular-weight DNA** — up to 500 µg of DNA from each sample

▶▶ PAXgene Blood DNA System, page 17

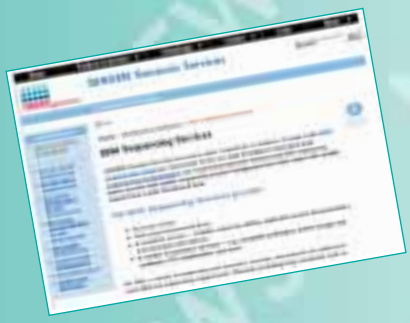
QIAGEN

What's New?

- **Xeragon**, a technology leader for products and services focusing on synthetic RNA, is now part of the QIAGEN Group of Companies. Xeragon focuses on **siRNA oligonucleotides**, an important tool for silencing target gene expression. QIAGEN now offers custom and pre-manufactured stock siRNA products. Further details and ordering information can be found at **www.xeragon.com**.

XERAGON
EXPERTS IN RNA SYNTHESIS
A QIAGEN Company

- QIAGEN provides integrated solutions for success in **molecular diagnostics**. Improve the sensitivity and specificity of your assays in molecular oncology, infectious disease research, and genetic testing. Find out more by visiting **www.qiagen.com/goto/MDx-news2002**.



- **The new QIAGEN Genomic Services website is now online!** Go to **www.qiagengenomics.com** to find out the latest about QIAGEN Genomic Services — your solution for fast and accurate results in genomics and molecular biology research. We offer SNP analysis, sequencing, DNA isolation services, and more. Plus you can order sequencing services online and try out our bioinformatics services.

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

Editor
Douglas J. McGarvey, Ph.D.

Assistant editor
Kate E. Bendall, Ph.D.

Editorial assistant
Joanne R. Bailey, B.Sc. (Hons.), M.Sc.

Writers
Sally E. Bee, Ph.D., Kate E. Bendall, Ph.D., Emma Duncan, Ph.D., Douglas J. McGarvey, Ph.D., Kevin J. Mobbs, Ph.D., Jason Smith, Ph.D.

Graphics and layout
Marion Urbschat

Production management
Roland Stelzer

Production
Katja Zündorf

news.editor@qiagen.com

New

Complete separation and detection of phosphorylated proteins from eukaryotic cell lysates

The new PhosphoProtein Purification System is used for purification of phosphorylated proteins from cell lysates, significantly reducing complexity in proteomic and cell signaling studies. The affinity chromatography-based procedure enables a complete separation of phosphorylated and unphosphorylated protein fractions. Both fractions retain biological activity and can be further purified if required. PhosphoProtein Antibodies are used for highly specific immunodetection of phosphoserine and phosphothreonine residues in blotting procedures.

The PhosphoProtein Purification Kit and PhosphoProtein Antibodies offer:

- Complete separation of phosphorylated and unphosphorylated proteins
- Cell-signaling studies without the need for radioactivity
- A complete system, including columns, buffers, and reagents
- Detection of phosphorylated serine or threonine residues, irrespective of surrounding amino acids

Complete separation on the basis of phosphorylation

Post-translational modifications of individual proteins add significantly to the complexity of the proteome. Phosphorylation of serine, threonine, and tyrosine residues — which plays a vital role in cell signaling, oncogenesis, apoptosis, and immune disorders — is one of the most common post-translational modifications.

Currently available methods for chromatographic separation of phosphorylated proteins from cell lysates offer at best an enrichment of a fraction containing phosphorylated proteins, with large quantities of acidic proteins often being copurified. Until now, studies of a cell's phosphorylation status have typically used radioactivity, with its attendant handling problems, and expense.

The PhosphoProtein Purification Kit, which is based on affinity chromatography, delivers a complete separation of phosphorylated and unphosphorylated proteins from a cell lysate and therefore greatly facilitates studies of the phosphorylation status of both entire cells and specific proteins (Figures 1 and 2). The complete separation allows the ratio of phosphorylated to unphosphorylated ▶

Highly Specific Separation of Phosphorylated Proteins

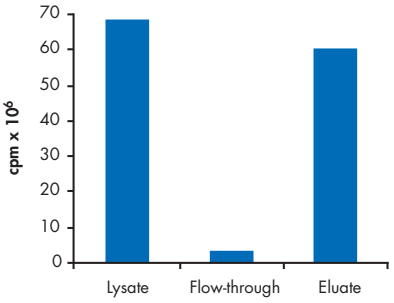


Figure 1 *Non-stimulated Jurkat cells were radioactively labeled in vivo using ^{32}P. Cell lysate was processed using the PhosphoProtein Purification Kit and the radioactivity in each fraction measured. (Data kindly provided by Gudrun Rehg and Sascha Dammeier, Byk Gulden, Konstanz, Germany.)*

Complete Separation of Unphosphorylated and Phosphorylated Proteins

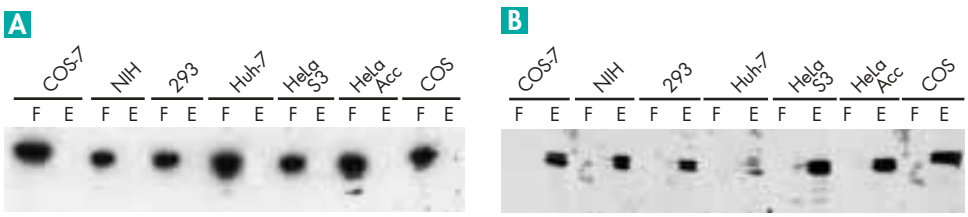


Figure 2 *Protein-specific immunodetection of A unphosphorylated HSP-60 protein, and B phosphorylated p44 and p42 mitogen-activated protein kinase (MAPK) proteins. **F**: flow-through; **E**: eluate fractions. The antibody used to detect MAPK recognizes an epitope containing phosphorylated residues at Thr202 and Tyr204 in the p44 **(upper band)** and p42 **(lower band)** MAPK amino acid sequences. The absence of unphosphorylated HSP-60 in the eluate fraction and the absence of phosphorylated MAPK in the flow-through fraction demonstrate the complete separation of phosphorylated proteins using the PhosphoProtein Purification Kit.*

SDS-PAGE Analysis of Flow-Through and Eluate Fractions

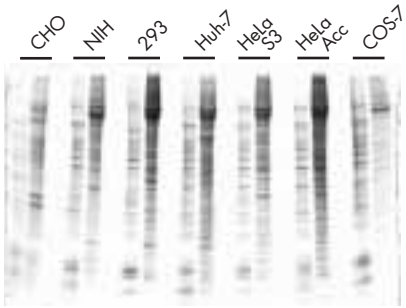


Figure 3 *Flow-through fractions* ***(left-hand lanes)*** *and eluates* ***(right-hand lanes)*** *from cell lysates processed using the PhosphoProtein Purification Kit. Both fractions were concentrated by a factor of 10 before loading onto the gel, and proteins were visualized by Coomassie® staining.*

forms of proteins to be easily determined. The drastic reduction of the complexity of each fraction is especially useful when studying proteins of low abundance.

The PhosphoProtein purification procedure

Each PhosphoProtein Purification Column can be used to purify phosphorylated proteins from 10^7 eukaryotic cells (equivalent to approximately 2.5 mg total protein). Cells are lysed in a detergent-containing buffer that provides gentle disruption of large protein complexes. Cleared lysates are loaded onto the column where phosphorylated proteins in the lysate bind to the affinity matrix, while unphosphorylated proteins are found in the flow-through fraction. After a wash step, phosphorylated proteins are eluted from the column. Depending on cell type and status, about 10% of protein loaded is recovered in the phosphorylated fraction (Table 1). The phosphorylated and unphosphorylated fractions retain full biological activity and can be further purified if desired. Nanosep® Ultrafiltration Columns are supplied with the kit to enable efficient concentration and desalting of protein fractions using a microcentrifuge.

Highly specific detection of phosphorylated proteins

Proteins containing phosphorylated serine or threonine residues can be detected after blotting using mouse monoclonal Phospho-Serine and PhosphoThreonine Antibodies. These highly specific antibodies recognize and bind to phosphorylated serine and threonine residues, irrespective of surrounding amino acids.

The complete separation of phosphorylated proteins offered by the PhosphoProtein Purification Kit makes it unique among currently available chromatography-based methods, and an invaluable tool for research in cell signaling and proteomics. ■

Table 1. Yields of phosphorylated proteins obtained using the PhosphoProtein Purification Kit

Cell type	No. of cells processed	Total protein in cell lysate	Protein loaded onto column	Protein in eluate	Phosphorylated proteins
CHO	1.5 x 10^7	3400 µg	2500 µg	300 µg	12%
NIH/3T3	n.d.	2750 µg	2500 µg	165 µg	7%
293	1.5 x 10^7	3650 µg	2500 µg	200 µg	8%
COS-7	4.5 x 10^6	1700 µg	1700 µg	120 µg	7%
Huh-7	8.5 x 10^6	2650 µg	2500 µg	235 µg	9%
HT 29	n.d.	n.d.	2500 µg	200 µg	8%
LT 23	n.d.	n.d.	2500 µg	275 µg	11%
HeLa S3	1.8 x 10^7	5950 µg	2500 µg	280 µg	11%
HeLa Acc57	6.6 x 10^6	2500 µg	2500 µg	235 µg	9%

n.d.: not determined

Ordering Information

Product	Contents	Cat. No.
PhosphoProtein Purification Kit (6)	6 PhosphoProtein Purification Columns; buffers; reagents; 6 Nanosep Ultrafiltration Columns	37101
PhosphoThreonine Antibody Q7 (100 µg)	100 µg anti-phosphothreonine antibody (isotype mouse IgG1, for 200 ml working solution)	37420
PhosphoSerine Antibody Q5 (100 µg)	100 µg mixture of anti-phosphoserine antibodies (isotypes mouse IgG1 and IgM, for 200 ml working solution)	37430

QIAGEN News

Highly efficient stable and transient transfection using Effectene® Reagent

Catherine Smith

Molecular Toxicology, Division of Biomedical Sciences, Imperial College, London, UK

A series of optimization experiments was performed in order to maximize efficiency of both stable and transient transfection of Chinese hamster V79-MZ cells. Using Effectene® Reagent, transient transfection efficiencies of up to 40% and stable transfection efficiencies of up to 0.8% were obtained.

Therapies based on gene transfer require reproducible and efficient transfection procedures and development of standardized protocols to enable comparison of the effects of transfected genes. Development and optimization of these procedures play a vital role in determining the potential effectiveness and safety of nucleic acid-based therapies. We aimed to develop an optimized transfection procedure for Chinese hamster V79-MZ cells grown in flasks, using an enhanced green fluorescent protein (EGFP) reporter gene.

Materials and methods

For optimization studies, Chinese hamster V79-MZ cells were transfected in six-well plates with the plasmid vector pL3112BSKS, which codes for EGFP and incorporates a *neo* resistance gene. DNA (0.8 µg) was transfected into cells using 10:1, 25:1, and 50:1 ratios of Effectene Transfection Reagent to DNA (µl:µg) according to the protocol in the *Effectene Transfection Reagent Handbook*. Plasmids were purified using the QIAGEN® Plasmid Maxi Kit. For scaled-up experiments, V79-MZ cells were seeded at a density of 1.5 x 10^6 cells per T75 cell culture flask (Nunc). Cells were cultured in DMEM supplemented with 5% fetal bovine serum, 2 mM L-glutamine, and penicillin/streptomycin. Cells were cultured for 24 hours before transfection using 3.47 µg plasmid DNA and a range of transfection reagent:vector ratios. After transfection, cells were cultured at 37°C and 5% CO_2 and medium was changed after 16 hours. After a further 24 hours growth, cells were harvested and an aliquot analyzed by FACS® to determine transient transfection efficiency. To determine stable transfection efficiency, 100 and 1000 cells were plated using medium containing 0.8 mg/ml G418 (replaced every 4 days) and cultured for 2–3 weeks until colonies could be counted. All transfections were carried out in triplicate. Mock-transfected V79-MZ cells were used as a control. ▶

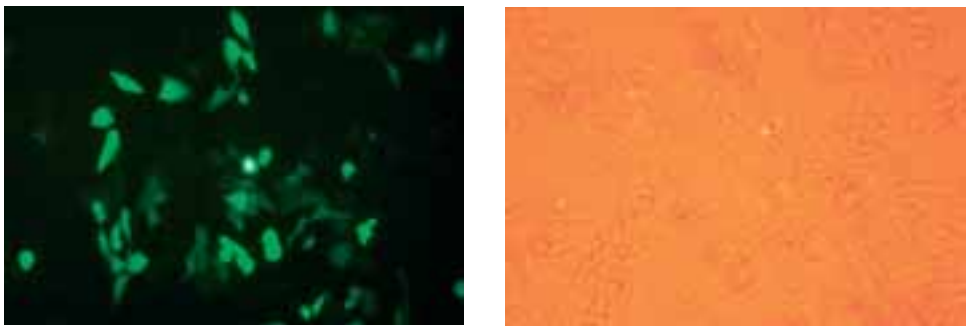


Figure 1 *Fluorescent and light micrograph images of transfected V79-MZ cells. Cells were transfected in T75 flasks with Effectene Reagent–DNA complexes for 16 hours and viewed 24 hours later.*

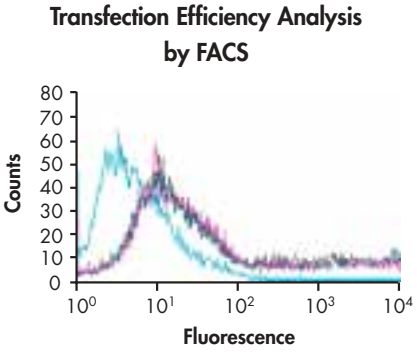


Figure 2 *FACS analysis of transfection efficiency. V79-MZ cells were treated with a 25:1 ratio (v/w) of Effectene Reagent:DNA. Experiments were carried out in triplicate. The cyan trace shows analysis of mock-transfected cells.*

Results

Four different ratios of Effectene Reagent:DNA were used. Figure 1 shows fluorescent and light microscope images of transfected cells. FACS traces from one set of experiments are shown in Figure 2. Figures 3 and 4 show the efficiencies of transient and stable transfection respectively. The most efficient transient and stable transfection was obtained using an Effectene Reagent to DNA (µl:µg) ratio of 25:1. Transient transfection efficiencies of up to 40% and stable transfection efficiencies of up to 0.8% were obtained.

Conclusions

By optimizing the ratio of Effectene Reagent to DNA used, high efficiencies were obtained in both transient and stable transfection experiments. By allowing transfection in the presence of serum, cytotoxicity is reduced. Transfection reactions can be easily scaled up using the simple procedure. The high efficiency and reproducibility of the procedure facilitates comparison of the effects of transfected genes and their gene-products on cells. ■

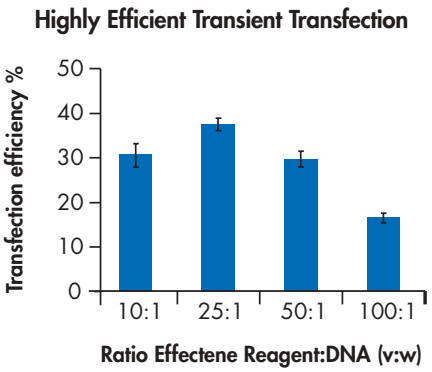


Figure 3 *Efficiency of transient transfection. V79-MZ cells were transfected in triplicate using the indicated ratios of Effectene Reagent to DNA. Cells were incubated for 16 h, the medium was changed, and the efficiency of transfection was analyzed by FACS after a further 24 hours of growth.*

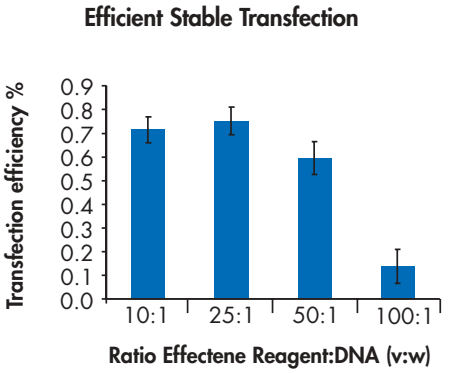


Figure 4 *Efficiency of stable transfection. V79-MZ cells were transfected in triplicate using the indicated ratios of Effectene Reagent to DNA. Efficiency of stable transfection was determined as described in "Materials and methods".*

Ordering Information

Product	Contents	Cat. No.
Effectene Transfection Reagent (1 ml)	1 ml Effectene Reagent, Enhancer, Buffer; for 40 transfections in 60 mm dishes or 160 transfections in 12-well plates	301425
Effectene Transfection Reagent (4 x 1 ml)	4 x 1 ml Effectene Reagent, Enhancer, Buffer; for 160 transfections in 60 mm dishes or 640 transfections in 12-well plates	301427

New

Clone blunt-ended PCR products easily and efficiently with the QIAGEN® A-Addition Kit

PCR products generated using proofreading DNA polymerases, such as ProofStart™ DNA Polymerase, are blunt-ended and cannot be used directly in UA- or TA-cloning procedures. These PCR products can be cloned using blunt-end cloning procedures. However, blunt-end cloning is inefficient and can cause a number of problems, including vector re-ligation, which results in a large proportion of colonies without the cloned insert. The QIAGEN® A-Addition Kit provides an easy and efficient method to modify blunt-ended PCR products for use in UA- or TA-cloning strategies.

The QIAGEN A-Addition Kit offers:

- **Ease of use** — simply add blunt-ended PCR products to the master mix and incubate for 30 minutes
- **Fast procedure** — no PCR product purification or precipitation required
- **High efficiency of UA-/TA-cloning** — large numbers of colonies contain desired insert

Fast and simple procedure

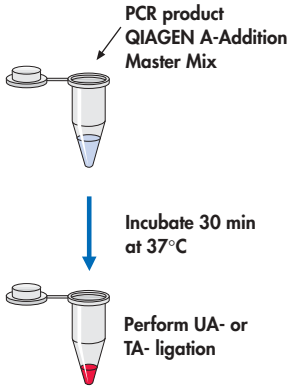
The QIAGEN A-Addition Kit is designed for use with practically any PCR fragment generated using a proofreading polymerase and allows insertion of the PCR product into any UA- or TA-cloning vector. The procedure is fast and simple (see flowchart). Simply add an aliquot of your PCR product to 2 µl of QIAGEN A-Addition Master Mix, incubate at 37°C for 30 minutes, and proceed directly with ligation. A distinct advantage is that there is no need for PCR purification or precipitation, as long as you start with a specific PCR product.

More colonies containing the desired insert

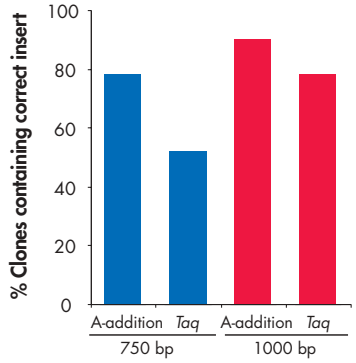
Blunt-ended PCR products processed with the QIAGEN A-Addition Kit are more efficiently cloned than PCR products that have an A-overhang added during amplification by *Taq* DNA polymerase. More colonies were transformed and a greater percentage of these contained the desired insert when using the QIAGEN A-Addition procedure and QIAGEN PCR Cloning Kits (Figure 1). ■

For ordering information, see page 10.

QIAGEN A-Addition Procedure



A

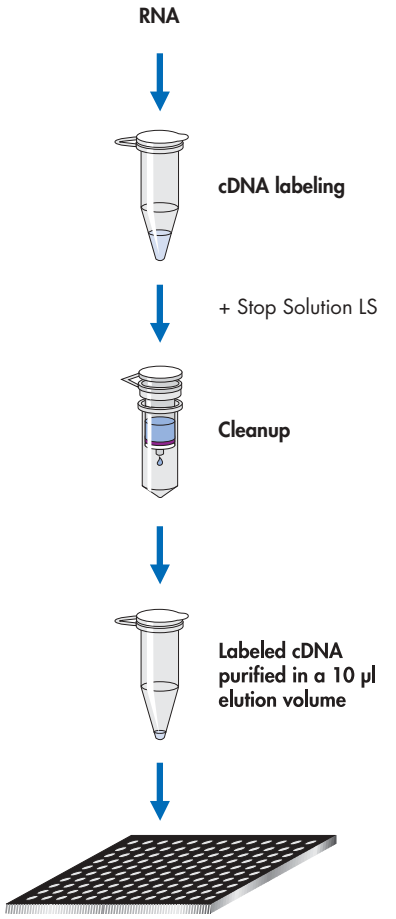


B

Method	Number of colonies	Number of colonies
Proofreading polymerase + QIAGEN A-Addition	421	4287
Taq DNA polymerase	150	441

Figure 1 *Two PCR products (**blue**: 750 bp; **red**: 1000 bp) were cloned into the pDrive UA-cloning vector using the QIAGEN PCR Cloning Kit. PCR products were amplified using either a proofreading DNA polymerase followed by the QIAGEN A-Addition procedure or using Taq DNA polymerase. All reactions were performed in parallel. A Cloning efficiency is given as a percentage of colonies containing the correct insert. B The total number of transformed colonies is shown.*

LabelStar Array Procedure



continued from page 1

Efficient cDNA labeling and cleanup

The new LabelStar™ Array Kit provides efficient labeling and cleanup of cDNA before array hybridization. Optimized reaction conditions ensure high signal intensity and low background. Reproducible and robust labeling over a range of RNA amounts and a wide choice of labels results in a flexible easy-to-use system. The LabelStar Array Kit allows highly sensitive array analysis through identification of true positives at low gene expression levels.

The rapidly expanding field of microarray analysis has led to many important advances in gene expression profiling. To meet the demands of this rapidly expanding field, more robust and sensitive technology is required. The most important factor for sensitivity is the efficiency of the labeling reaction, which can be compromised by inhibitors of reverse transcription that are copurified with the RNA and by inefficient incorporation of the modified nucleotide. Following the labeling reaction, removal of unincorporated nucleotides is necessary to reduce background and increase signal-to-noise ratios on the chip. Both labeling and cleanup are therefore required to maximize sensitivity of array analysis.

The LabelStar procedure

The LabelStar Array Kit combines efficient cDNA labeling, followed by cleanup, with high DNA recovery in a few simple steps (see flowchart). The LabelStar Array Kit is comprised of two modules. The cDNA Labeling Module contains LabelStar Reverse Transcriptase, dNTPs, RNase inhibitor, and all buffers and solutions required for labeling (excluding labeled nucleotides). The Cleanup Module contains MinElute™ spin columns and buffers optimized for cleanup of labeled cDNA used in array analysis.

During the LabelStar procedure, isolated RNA is denatured using Denaturation Solution Plus. This solution also neutralizes inhibitors of reverse transcription copurified with the RNA. During reverse transcription of the denatured RNA using LabelStar Reverse Transcriptase, a modified nucleotide of choice is incorporated (Table 1). For high signal intensity, degradation of remaining RNA is necessary after the labeling reaction. The exoribonuclease activity of LabelStar Reverse Transcriptase efficiently degrades RNA, eliminating the need for a separate degradation step. Stop Solution LS stops the labeling reaction and reduces nonspecific binding of components of the labeling reaction to the array, reducing background signal.

Table 1. Specifications

Amount of total RNA used in labeling reaction	**0.2–50 µg**
Amount of mRNA used in labeling reaction	**0.2–5 µg**
Modified nucleotides suitable for cDNA labeling using the LabelStar Array Kit:	
Cyanine-3/Cyanine-5-dCTP	✔
Cyanine-3/Cyanine-5-dUTP	✔
Biotin-dCTP or -dUTP	✔
5-(3-aminoallyl)-2'-dUTP	✔
^{32}P-dCTP	✔
^{33}P-dCTP	✔
Final elution volume	**10 µl**

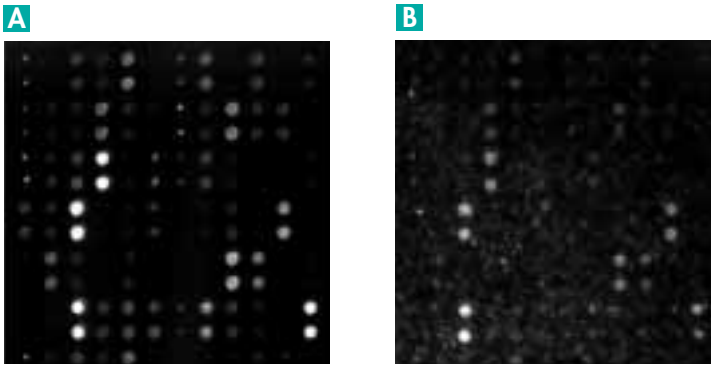
Optimized cleanup procedure

Cleanup of labeled cDNA is performed with silica-membrane–based MinElute spin columns using a simple bind–wash–elute procedure. All contaminants from the labeling procedure, such as unincorporated nucleotides, proteins, or salts are efficiently removed in the cleanup procedure. Optimized buffer sets and a novel spin-column design ensure high recovery and high purity of labeled cDNA in low elution volumes. Compared with other methods (e.g., ultrafiltration), use of the LabelStar Cleanup Module results in reproducibly higher signal-to-noise ratios (Figure 1). In addition, the very low elution volume of 10 µl often eliminates the need for further concentration of labeled cDNA before array hybridization. cDNA labeled and purified using the LabelStar Array Kit is ready for hybridization to arrays, independent of the type of probe (e.g., oligos, PCR fragments) or support material (e.g., glass slides, membrane arrays).

High signal intensities

The LabelStar Array Kit offers a labeling and cleanup system that can be adapted for direct and indirect cDNA labeling using a wide range of modified nucleotides and amounts of RNA (Table 1). Using the LabelStar Array Kit, higher signal intensities and lower background are obtained (Figure 2). The LabelStar procedure results in up to ninefold higher signal intensities, compared with other commercially available kits (Figure 3). The LabelStar Kit enables the detection of more true positive spots when using low amounts of RNA and at low levels of gene expression (Figure 1). ▶

Optimized LabelStar Cleanup Procedure



LabelStar Cleanup Module Supplier M_{III}

Figure 1 *cDNA labeling was performed using 20 µg of total RNA from mouse brain using the LabelStar cDNA Labeling Module. Purification of the labeled cDNA was performed using* **A** *the LabelStar Cleanup Module or* **B** *a commonly used ultracentrifugation-based method from Supplier M_{III}. Purified cDNAs (1/10 total volume) were hybridized to QIAGEN SensiChip™ DNA Arrays carrying a set of mouse genome oligonucleotide probes. Hybridization and washing procedures were identical in both cases.*

More Positives and Higher Signal-to-Noise Ratio with the LabelStar Array Kit

LabelStar Supplier L Supplier I Supplier A_{II} Supplier S_{III}

Figure 2 *cDNA labeling (with Cy®3) and purification was performed using 20 µg total RNA from HeLa cells using the LabelStar Array Kit* ***(LabelStar)*** *or kits from the 4 suppliers indicated, following the manufacturers' instructions. Each labeled and purified cDNA preparation was hybridized to a chip containing a set of human genome oligonucleotide probes. Hybridization and washing conditions were the same on all chips.*

Higher Signal Intensities Using the LabelStar Array Kit

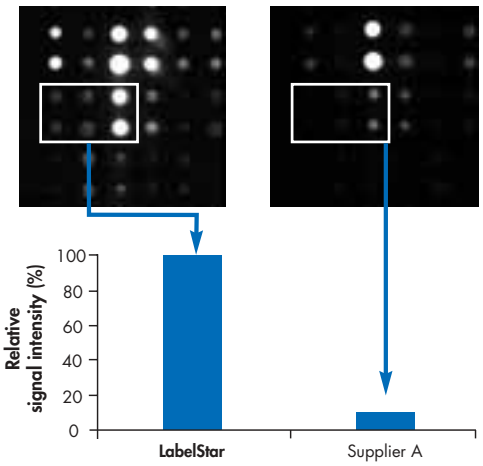


Figure 3 *cDNA labeling (with Cy5) and purification were performed using 2 µg total RNA from mouse brain using the LabelStar Array Kit and a kit from Supplier A, following the manufacturers' instructions. The signal intensities of six spots were compared. Mean values of relative signal intensities were calculated (see bar graph). On average, signal intensities were ninefold higher using the LabelStar Array Kit.*

Summary

The LabelStar Array Kit provides a complete solution for highly efficient labeling and purification of cDNA generated by reverse transcription using 0.2–50 µg of RNA. Compared with other methods, cDNA labeled using the LabelStar Array Kit gives higher signal intensities and lower backgrounds on chips, yielding more true positive spots. This is a result of optimized conditions in the labeling reaction and subsequent purification. ■

Ordering Information

Product	Contents	Cat. No.
LabelStar Array Kit (12)	For 12 labeling reactions: LabelStar Reverse Transcriptase, dNTPs,* RNase Inhibitor, Oligo-dT Primer, 12 MinElute Spin Columns, RNase-Free Reagents, Buffers	28902
LabelStar Array Kit (50)	For 50 labeling reactions: LabelStar Reverse Transcriptase, dNTPs,* RNase Inhibitor, Oligo-dT Primer, 50 MinElute Spin Columns, RNase-Free Reagents, Buffers	28904

** 20 mM solutions of each dNTP; labeled nucleotides to be supplied by user*

"Clone blunt-ended PCR products easily and efficiently with the QIAGEN A-Addition Kit", page 7

Product	Contents	Cat. No.
QIAGEN A-Addition Kit	For 40 A-addition reactions: 5x QIAGEN A-Addition Master Mix, Nuclease-free water	231994
Related products		
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
QIAGEN PCR Cloning^{plus} Kit (10)†	For 10 reactions: 2x Ligation Master Mix (50 µl), pDrive Cloning Vector (0.5 µg), distilled water (1.7 ml), QIAGEN EZ Competent Cells (10 tubes, 50 µl each), SOC medium (2 x 1.9 ml)	231222
ProofStart DNA Polymerase (500 U)	500 units ProofStart DNA Polymerase, 10x ProofStart PCR Buffer,‡ 5x Q-Solution, 25 mM $MgSO_4$	202205

† Larger kit sizes available; please inquire.
‡ Contains 15 mM $MgSO_4$.

Easy assay setup and high-quality data using the LiquiChip™ System

The LiquiChip™ Protein Suspension Array System is a bead-based platform that enables a wide range of protein interaction arrays to be carried out quickly and with high sensitivity (see *QIAGEN News* 2002 No 2, 1). Here we show how assays using 6xHis-tagged proteins can be quickly and easily developed and used to obtain high-quality assay data.

Easy assay setup

One of the major advantages of the LiquiChip System is that it enables QIA*express*® 6xHis technology to be used in *x*MAP™ bead-based assays. This means that a single chemistry can be used for all steps of the protein-handling process, from purification to immobilization and assay. Using a common immobilization protocol for 6xHis-tagged proteins increases standardization in assays and eliminates the need for lengthy and cost-intensive optimization trials (Figure 1). Proteins are bound to beads in a batch procedure and in most cases can be added directly to LiquiChip Assays. Table 1 shows a comparison of the steps involved in coupling 6xHis-tagged proteins to LiquiChip Ni-NTA and Penta·His™ Beads with those required for covalent protein immobilization.

In addition to being quicker, immobilization of 6xHis-tagged proteins on Ni-NTA and Penta·His Beads requires less protein per assay point. Proteins are bound in a directed manner, maximizing accessibility to interaction partners and increasing signal intensity.

High-quality data sets

Assay data quality is reflected in signal-to noise and signal-to-background ratios. The Z-factor (see Table 2 and reference 1) is a dimensionless, simple statistical characteristic and the most suitable parameter for assay quality assessment. The Z-factor reflects assay signal dynamic range and variation associated with signal measurements.

To determine the Z-factor of a typical LiquiChip assay, a hypothetical drug-screening assay using proteases was established (Figure 2). 6xHis-tagged Thioredoxin–Tag·100 was immobilized on LiquiChip Ni-NTA Beads, incubated with differing amounts of enterokinase, and residual noncleaved protein was quantified after 3 hours incubation using Tag·100 specific antibodies. Data were collected from three processed 96-well plates (n = 288). ▶

Table 1. Steps involved in coupling 6xHis-tagged proteins to LiquiChip Ni-NTA and Penta·His Beads in comparison with covalent protein immobilization

	Ni-NTA Bead coupling procedure	Penta·His Bead coupling procedure	Covalent protein coupling procedure
Volume of beads required for 500 assay points	250 µl	250 µl	1 ml
Washing step	Not required	Not required	2 centrifugation steps
Activation step	Not required	Not required	EDC/Sulfo-NHS (1 h)
Protein required for 500 assay points	<5 µg	<5 µg	25–250 µg (optimization recommended)
Incubation	1 h or overnight	1 h or overnight	0.5 h – overnight
Additional steps required	Ready to use	Ready to use	Further wash step Enumeration of beads

Reference

1. *Zhang, J.H., Chung, T.D., and Oldenburg, K.R. (1999) A simple statistical parameter for use in evaluation and validation of high-throughput screening assays. J. Biomol. Screen.* ***4****, 67.*

Table 2. The Z-factor

The Z-factor is based on assay signal-to-noise and signal-to-background ratios.

$$\text{Z-factor} = 1 - \frac{3\sigma \text{ positive control} + 3\sigma \text{ negative control}}{\text{mean positive control} - \text{mean negative control}}$$

For the experiment presented in Figure 2 the following values were calculated.

Background (negative control): Mean = 18, σ (standard deviation) = 2.5
Positive control: Mean = 2524, σ (standard deviation) = 66.5

These data give a Z-factor for the assay shown in Figure 2 of 0.92.

Sensitive Multiplex Assays

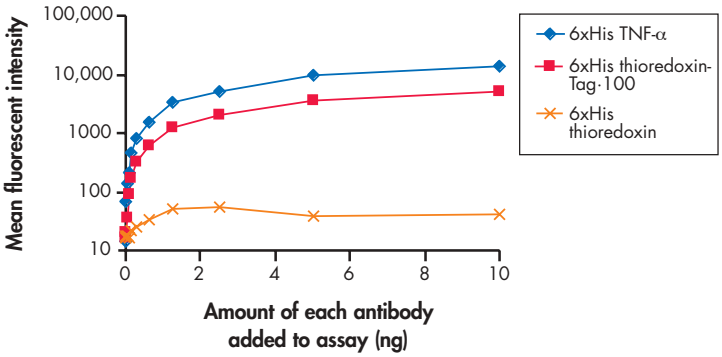


Figure 1 *Multiplex ELISA assay in which three different proteins were bound to LiquiChip Ni-NTA Beads using a common procedure, and used to quantify protein-specific antibodies.*

Highly Reproducible Assays

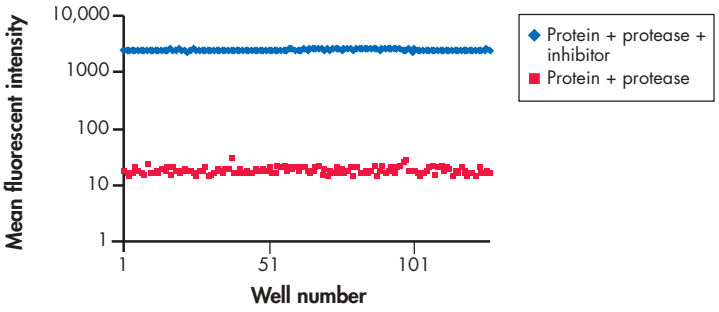


Figure 2 *High signal-to-background ratio and low background noise result in high Z-values. As a positive control, protease activity was inhibited with the serine-protease inhibitor aprotinin (10 ng/well). Data derived from 3 microtiter plates, processed on three successive days, are shown.*

By enabling fast assay development and setup and delivering high-quality data, the LiquiChip System is a valuable addition to any lab performing protein interaction assays. For more information on the LiquiChip System and how it can help you to streamline assay development visit **www.qiagen.com** or call QIAGEN Technical Services. ■

Optimizing probe hybridization in real-time PCR for quantification and SNP genotyping

Katja Decker, Thorsten Träger, Andreas Missel, Katja Heitz, Susan Kobsch, Katharina Machura, and Dirk Löffert

QIAGEN GmbH, Hilden, Germany

Real-time PCR is used for quantitative gene expression analysis as well as single nucleotide polymorphism (SNP) detection. A prerequisite for successful quantification and genotyping is high PCR specificity. Adapting PCR to increase specificity often also increases sensitivity of the assay, due to the lack of non-specific PCR products competing for limiting PCR reagents in the course of the reaction (1). Therefore, highly sensitive quantitative and genotyping assays that employ minimal amounts of DNA are more likely to work efficiently.

High PCR specificity is not only required for specific primer annealing and efficient detection of the PCR product but is also a critical factor in SNP analysis using real-time PCR probes, which are used to detect single nucleotide differences in the template. The optimized formulation of QuantiTect™ Probe PCR Master Mix, which selectively destabilizes nonspecific base pairing, provides highly specific and efficient probe hybridization conditions, enabling accurate allele calling in SNP analysis. Real-time PCR-based quantitative gene expression analysis and SNP genotyping are now possible without lengthy assay optimization, even when only minute amounts of template are available.

Materials and methods

Quantitative real-time PCR gene expression assays were performed on the DNA Engine Opticon™ Instrument (MJ Research), which enables users to program a temperature gradient. SNP analysis was performed using an ABI PRISM® 7700 Sequence Detection System. The performance of QuantiTect Probe PCR Master Mix was compared with reagents from supplier A_{II} in reactions containing Molecular Beacons or TaqMan® dual-labeled probes. Setup and cycling conditions followed provided standard protocols. The final volume of SNP analysis reactions was 25 µl. Reactions were used to detect the cytochrome P450 SNP, CYP2D6*4 G1934A, using TaqMan MGB probes.

LightCycler® Software Version 3.5 was used for analysis on the LightCycler System with LightCycler Hybridization (FRET) probes. ▶

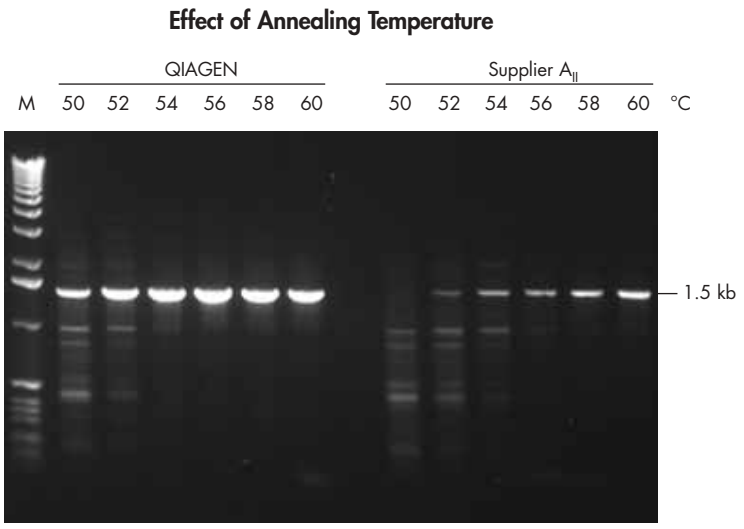


Figure 1 *Amplification of the human single-copy cystic fibrosis gene at the indicated annealing temperatures using QIAGEN® PCR Buffer and QIAGEN Taq DNA Polymerase. The same PCR was performed in parallel using PCR buffer and Taq DNA polymerase from another supplier (**Supplier A_{II}**). **M:** markers.*

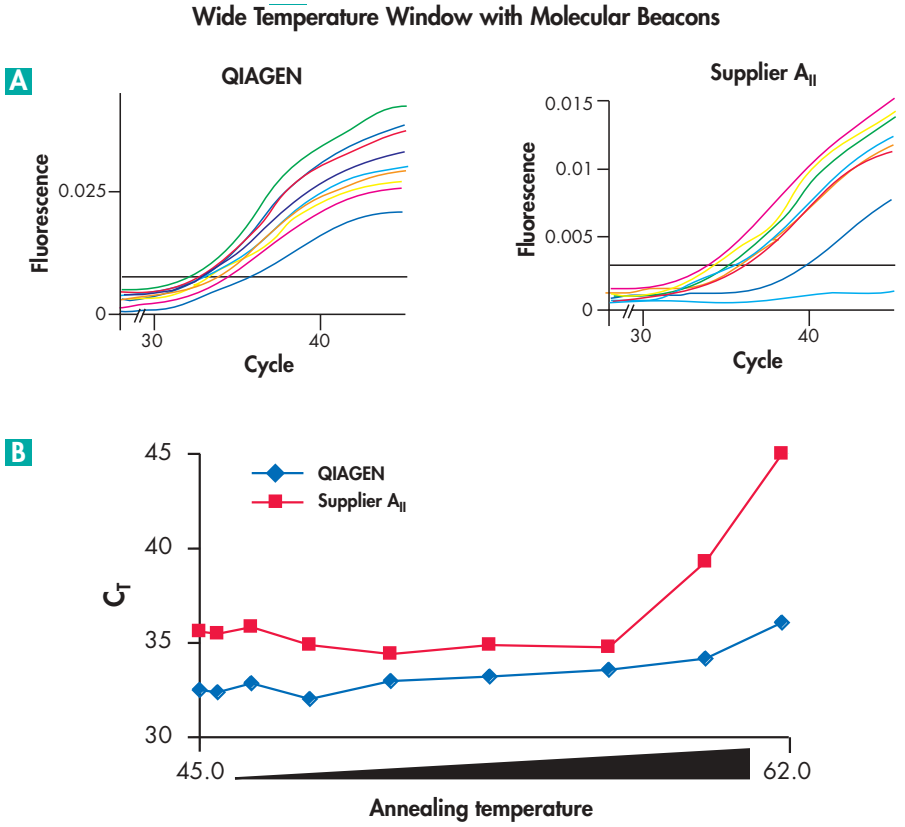


Figure 2 *One-step, quantitative RT-PCR was carried out using the QuantiTect Probe RT-PCR Kit* ***(QIAGEN)*** *or a one-step, quantitative RT-PCR kit from Supplier A, as indicated, using 10 pg HeLa total RNA. Reactions were performed on the DNA Engine Opticon Instrument according to suppliers' instructions, except that a temperature gradient from 45 to 62°C was used during the annealing/detection step of the 3-step cycling program. Primers and probe were specific for a fragment of the human GAPDH transcript.* **A** *Amplification plots. The horizontal line indicates the threshold limit.* **B** *Threshold-cycle (C_T) values with different annealing temperatures.*

Effect of buffer formulation on specificity and temperature of hybridization

PCR specificity depends on maintaining a high ratio of specific to nonspecific primer annealing. This ratio can be modulated by PCR buffer formulation. Cations such as K^+ or Mg^{2+} neutralize negatively charged phosphate groups on the DNA backbone and consequently weaken electrorepulsive forces between the two DNA strands. Reduction of these repulsive forces facilitates hybridization of primer or probe to template DNA or cDNA. NH_4^+, which is also contained in the buffer, can interact with hydrogen bonds between bases to destabilize weak hydrogen bonds at mismatched bases (2). The combined effect of K^+ and NH_4^+ maintains a high ratio of specific to nonspecific primer–template binding. This results in a wider temperature window for efficient and specific primer binding, providing more robust PCR conditions without lengthy PCR optimization (Figure 1).

The same principle holds true for fluorescent probe binding in quantitative or SNP real-time PCR analysis. To demonstrate the effect of a broader temperature tolerance for specific and efficient probe hybridization, real-time, quantitative, one-step RT-PCR was performed using the temperature gradient function of the DNA Engine Opticon Instrument. Assays were performed using either TaqMan dual-labeled probes or Molecular Beacons. Figure 2 shows that QuantiTect Probe RT-PCR Master Mix provides a wide temperature window for binding and detection of the Molecular Beacon. Binding of the probe becomes inefficient only at temperatures above 62°C.

The assay performed using the QuantiTect Probe RT-PCR Kit also shows high sensitivity, as indicated by the low C_T value. In contrast, when using reagents from Supplier A, hybridization of the probe is significantly weakened at temperatures as low as 57°C, limiting the range of temperatures suitable for analysis.

A similar phenomenon is observed when a TaqMan probe is used (not shown). Using QuantiTect reaction chemistry, probe binding and hydrolysis are uniform over a broad temperature range, eliminating the need for optimization, such as by adjusting the concentration of magnesium. In contrast, when using reagents from Supplier A, which do not contain the optimized ion composition used in QuantiTect buffers, probe hybridization and generation of fluorescent signal vary with temperature.

Reliable SNP genotyping, even from small amounts of DNA

In SNP genotyping, a single mismatch between probe and target sequences needs to be discriminated during PCR. Therefore, a PCR environment needs to be created that supports specific and strong oligonucleotide binding, as long as the probe perfectly matches its target sequence. In contrast,

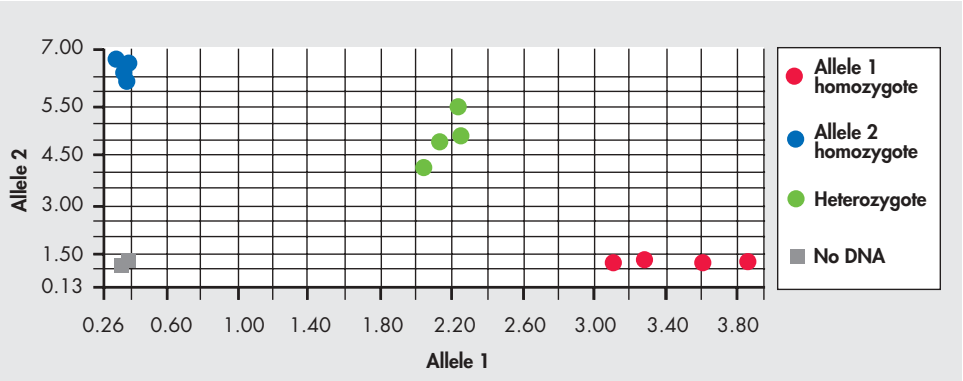


Figure 3 *PCR was carried out using the QuantiTect Probe PCR Kit and dual-labeled (TaqMan) probes for the CPY2D6*4 SNP, with 1 ng and 5 ng DNA template. End-point reads were performed on the ABI PRISM Sequence Detection System using SDS software version 1.7 (Applied Biosystems) for SNP genotyping. The allelic discrimination plots clearly indicate the homozygotes for each allele and the heterozygotes.*

probes that are mismatched in a single nucleotide position should not bind to the target. Binding of mismatched primers is prevented by a buffer composition that destabilizes weak hydrogen bonds at mismatched bases (see above). For genotyping, it is important that buffer composition offers such discrimination in a robust PCR environment using small amounts of DNA, since samples are often precious and cannot be routinely collected from an individual. We have tested the robustness of QuantiTect Probe PCR chemistry in SNP genotyping by reducing the amount of input DNA. Usually, use of 10 to 100 ng genomic DNA is recommended for real-time PCR SNP genotyping assays. Figure 3 shows results of assays where as little as 1 ng genomic DNA was used per reaction. DNA from individuals who were homozygous wildtype (allele 1), heterozygous (allele 1/2), or homozygous mutant (allele 2) for the CYP2D6*4, G1934A SNP was examined. Figure 3 shows that as little as 1 ng genomic DNA used in a 25 µl PCR provides accurate SNP genotyping with alleles clearly clustered in distinct regions of the scatter plot.

Probe hybridization on the LightCycler System

Stabilizing the probe–template complex is particularly important for rapid cycling using the LightCycler system. In this instrument, the detection of fluorescence with LightCycler Hybridization (FRET) probes takes place during the annealing step of the PCR cycle. ▶

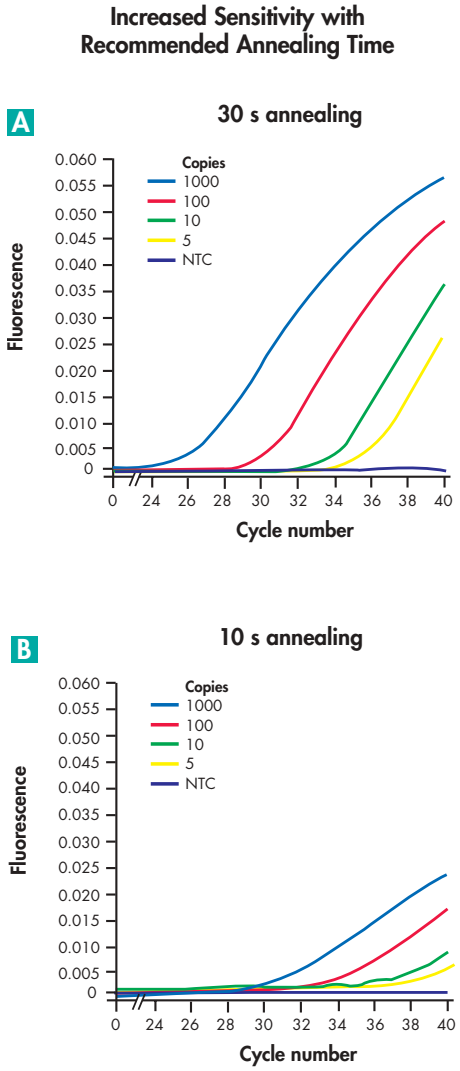


Figure 4 *Real-time, quantitative PCR was carried out using the QuantiTect Probe PCR Kit using primers and LightCycler Hybridization Probes specific for a 125 bp fragment of the human insulin-like growth factor 1. Reactions were performed on the LightCycler System with human genomic DNA corresponding to the indicated number of gene copies.* **A** *PCR was carried out using the 30-second annealing time recommended in the QuantiTect Probe protocol.* **B** *PCR using a shorter, 10-second annealing time.* ***NTC****: no template control.*

References

1. *QuantiTect Probe PCR and RT-PCR Kits — minimize your PCR optimization for highly sensitive results. QIAGEN News 2002 No.1, 1.*
2. *Missel, A., Decker, K., Arentzen, S., Kobsch, S., and Löffert, D. (2002) Reducing PCR optimization and costs with QuantiTect SYBR® Green PCR Kits on the LightCycler system. QIAGEN News 2002 No. 2, 3.*

Buffers used for this instrument often require optimization of salt concentration (e.g., Mg^{2+}) to sufficiently stabilize the probe–template complex. Salt concentrations (including Mg^{2+}) in QuantiTect Probe PCR Master Mix (or QuantiTect Probe RT-PCR Master Mix for one-step RT-PCR) are optimized for specific annealing of primers and probe. NH_4^+ ions in the Master Mix destabilize nonspecific primer and probe binding, and hybridization kinetics are slightly slowed down. Therefore, a moderate increase of the annealing time to 30 seconds is recommended for optimal sensitivity (Figure 4). Using pre-optimized QuantiTect Probe Master Mix allows more stringent primer and probe hybridization in real-time PCR, eliminating the need for lengthy optimization procedures.

Conclusions

Kits were developed for optimal performance with all types of fluorescent probes on all available real-time PCR instruments. The optimized formulation of QuantiTect Probe PCR and RT-PCR reagents increases PCR specificity, sensitivity, and efficiency. Using QuantiTect Probe Master Mix, probes bind to their target sequence with high specificity over a wider range of PCR conditions, such as temperature or Mg^{2+} concentrations, eliminating extensive assay optimization. QuantiTect Probe PCR and RT-PCR Kits enable highly sensitive and precise quantitative real-time analysis and accurate allele calling in SNP genotyping, even when minute amounts of genomic DNA are used. ■

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,* 100 µl QuantiTect RT Mix, 2 x 2.0 ml RNase-free water	204443
QIAGEN Operon® Oligonucleotide Synthesis Service — high-quality oligos, modified oligos, and longmers		
Oligonucleotide Synthesis Service	Custom-made oligonucleotides and a wide range of modified oligos, including Molecular Beacons, dual-labeled probes, and many more	Inquire

** Contains 8 mM $MgCl_2$*

continued from page 1

PreAnalytiX introduces the PAXgene Blood DNA System, an integrated and standardized system for collection of whole blood specimens and isolation of their genomic DNA. Blood is collected into PAXgene Blood DNA Tubes, which contain a proprietary blend of reagents that both prevents blood coagulation and stabilizes white blood cells. DNA is isolated from the tubes using the PAXgene Blood DNA Kit, which includes all buffers and reagents required for purification of high-quality genomic DNA.

High Quality and High Molecular Weight of Genomic DNA

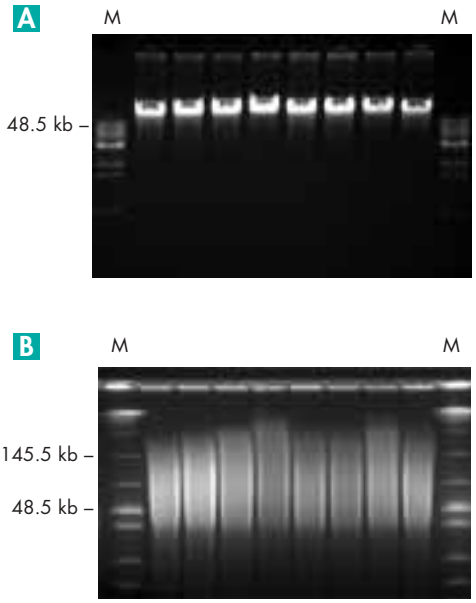


Figure 1 *Genomic DNA isolated from 8 blood donors using the PAXgene Blood DNA System.* **A** *Agarose gel analysis;* **B** *pulsed-field gel electrophoresis for enhanced separation of high-molecular-weight genomic DNA.* ***M****: markers.*

Simple, standardized procedure

Blood samples (8.5 ml) are collected into PAXgene Blood DNA Tubes. Tubes can be processed immediately, transported, or stored for up to 10 days at 18–22°C. For DNA isolation, the blood is transferred to processing tubes (supplied already filled with cell lysis buffer), and the solution is mixed to lyse red and white blood cells. Cell nuclei and mitochondria are pelleted by centrifugation, washed, and resuspended in digestion buffer. Protein contaminants are removed by incubation with a protease. DNA is precipitated in isopropanol, washed in 70% ethanol, dried, and resuspended in resuspension buffer.

High quality and yield of genomic DNA

DNA isolated using the PAXgene Blood DNA System has an A_{260}/A_{280} ratio of 1.7–1.9. It ranges from 20 to 200 kb in size, with an average length of 50–150 kb (Figure 1). Average DNA yields are 150–500 µg, depending on the number of nucleated cells present in the blood sample (Figure 2).

Efficient performance in downstream applications

Use of the PAXgene Blood DNA System provides DNA that can be used directly in sensitive downstream applications. For example, using only 5 ng of DNA template provided successful amplification of a single-copy gene fragment (Figure 3). Use of the QuantiTect™ Probe PCR Kit with the ABI PRISM® 7700 led to successful SNP analysis (Figure 4). Using the QIAGEN® Multiplex PCR Kit (available soon), 3 mitochondrial genes were successfully amplified in one assay (Figure 5).

Efficient Amplification of a Single-Copy Gene

Consistent Yields

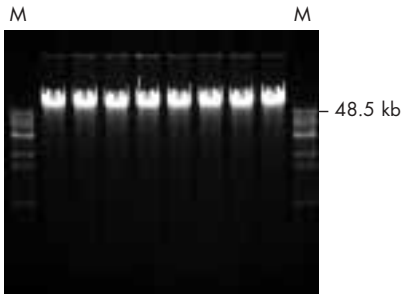


Figure 2 *Eight replicates of DNA isolated from one blood donor. 400 ng of DNA was loaded in each lane. Mean yield was 251.4 µg (SD 12.7 µg) DNA.* ***M****: markers.*

Figure 3 *Amplification of a 1.1 kb fragment from the single-copy gene Hugl, using 5 ng DNA from the donors 1–8 in Figure 1 as starting material.*

SNP Genotyping in the Cytochrome P450 Gene CYP2C19

Donor	Genotype
1	AL 1
2	AL 1
3	AL1 + AL 2
4	AL1 + AL 2
5	AL1
6	AL1
7	AL1 + AL 2
8	AL1

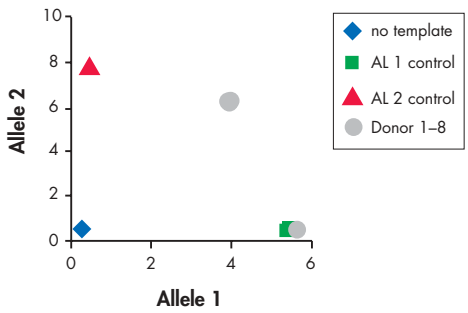


Figure 4 *Allelic discrimination assay using the QuantiTect Probe PCR Kit and a TaqMan® assay.* ***Allele 1****: CYP2C19*1;* ***allele 2****: CYP2C19*2.*

Efficient Multiplex PCR of 3 Mitochondrial Genes

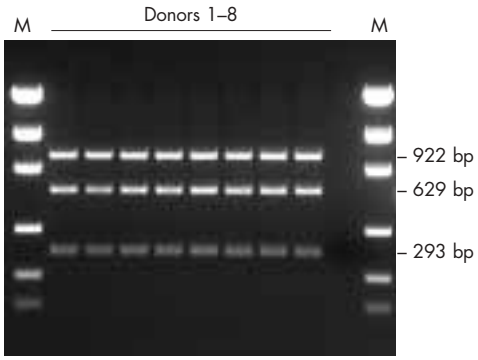


Figure 5 *Multiplex PCR of fragments from the mitochondrial genes tRNAlys/ATPase (0.92 kb), tRNAleu(UUR) (0.63 kb), and ND4 (0.29 kb), using 250 ng DNA from the donors 1–8 in Figure 1 as starting material.*

Discussion

Clinical applications, including pharmacogenomic studies, gene expression studies, and SNP genotyping, yield clinically important data that may directly affect patients' treatment regimes. Consequently, use of standardized methods for sample collection and nucleic acid isolation are of increasing importance. The PAXgene Blood DNA System provides an efficient method for standardized blood collection, transport, storage, and isolation of genomic DNA. High yields of pure DNA are obtained that perform well in sensitive downstream applications. ■

Ordering Information

Product	Contents	Cat. No.
PAXgene Blood DNA Kit (25)*	Processing tubes and buffers for 25 preparations	761133
PAXgene Blood DNA Validation Kit (10)*	Processing tubes and buffers for 10 preparations plus 10 PAXgene Blood DNA Tubes	761132
PAXgene Blood DNA Tubes (100)*	100 PAXgene Blood DNA Tubes	761115,† 761125‡

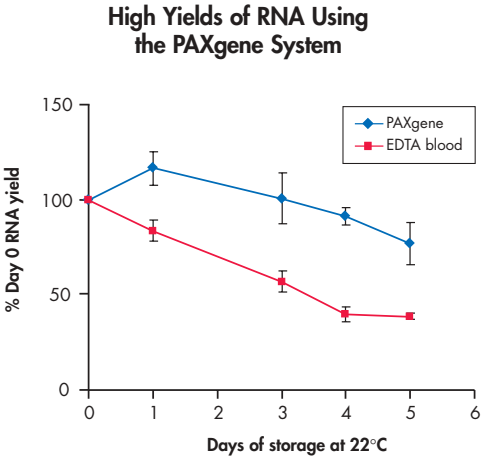
** For research use only. Not for use in diagnostic procedures.*
† USA and Canada
‡ All other countries

Stabilization of cellular RNA in whole blood samples using the PAXgene™ Blood RNA System

L. Rainen, C. Ballas, U. Oelmueller*, S. Jurgensen†, R. Wyrich*, J. Schram†, M. Walenciak, C. Herdman†, M. Paumen*

PreAnalytix (CH), c/o BD, Franklin Lakes, NJ, USA.
* PreAnalytix (CH), c/o QIAGEN GmbH, Hilden, Germany
† BD Technologies, RTP, NC, USA

A major difficulty in studying gene expression patterns in whole blood is that extensive changes in transcript levels occur during phlebotomy and during subsequent storage or transport of samples. In order to address this problem, PreAnalytiX developed the PAXgene™ Blood RNA System, an integrated and standardized system for the collection and stabilization of whole blood samples and isolation of cellular RNA. The system consists of PAXgene Blood RNA Tubes and the PAXgene Blood RNA Kit. PAXgene Blood RNA Tubes are used for blood collection, and contain a proprietary blend of reagents that provides immediate stabilization of RNA, allowing blood samples to be stored for days at 18–22°C (exact period depends on the downstream application). This blend also prevents the drastic changes in cellular RNA expression profiles that normally take place after blood collection. RNA is then isolated using proven silica-gel–membrane technology supplied in the PAXgene Blood RNA Kit.



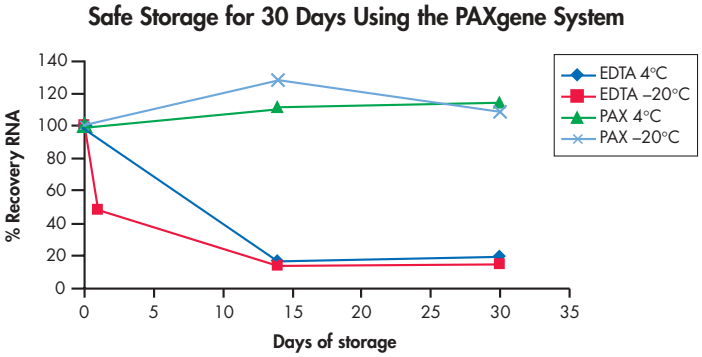


Figure 2 *Blood was collected in PAXgene Blood RNA Tubes and RNA isolated using the PAXgene Blood RNA Kit, or collected in EDTA tubes and RNA isolated without stabilization, as described in "Materials and methods". Samples were stored for up to 30 days at 4°C or –20°C. Samples were taken from one donor and processed in triplicate (mean values are shown).*

We compared RNA yields obtained using the PAXgene Blood RNA System with yields obtained using a method that did not include an RNA stabilization step. The performance of RNA isolated using the PAXgene System in downstream PCR was assessed.

Materials and methods

Blood samples were taken from adults with informed consent. Blood was collected either in BD Vacutainer™ PLUS K_2EDTA Tubes, or in PAXgene Blood RNA Tubes, and stored at –20°C, 4°C, and room temperature for up to 30 days. RNA was isolated from samples collected in EDTA tubes using a commercial ▶

Figure 1 *Blood was collected in PAXgene Blood RNA Tubes and RNA isolated using the PAXgene Blood RNA Kit, or collected in EDTA tubes and RNA isolated without stabilization, as described in "Materials and methods". Samples were stored for up to 5 days at room temperature. Samples were taken from 4 blood donors and processed in triplicate (mean values are shown)*

The PAXgene Blood RNA System Provides Highly Reproducible Results

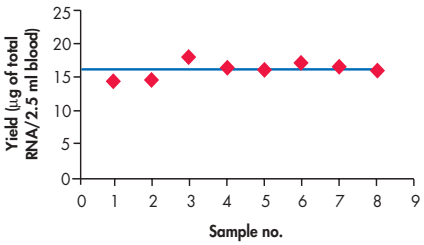


Figure 3 *Eight blood samples were taken from each donor and stored at room temperature for 24 hours before RNA purification. Yields are shown for a single donor.*

phenol-based reagent (Supplier I) according to the manufacturer's instructions, followed by cleanup using a silica-based method (no RNA stabilization step). Residual DNA was removed using an optional on-column DNase treatment, and RNA was eluted in RNase-free water. RNA was isolated from samples collected in PAXgene Blood RNA Tubes using the PAXgene Blood RNA Kit, including the optional on-column DNase treatment. Quantitative, real-time RT-PCR was performed using the TaqMan® system and primers designed to amplify the GAPDH gene, using 2 µl of eluate from the PAXgene System as a template.

Excellent Results in Quantitative, Real-Time RT-PCR

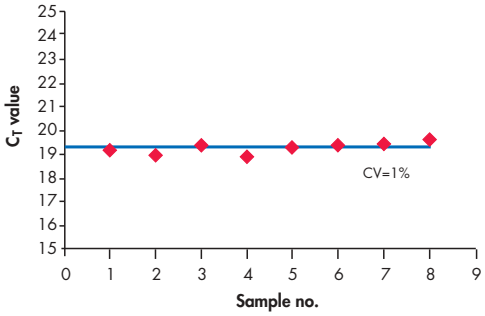


Figure 4 *The RNA samples analyzed in Figure 3 were amplified in quantitative, real-time RT-PCR using the TaqMan system with 2 µl of each eluate.*

Results and discussion

RNA yields were higher during 5 days of blood storage at 18–22°C when using the PAXgene System than when using a collection system that did not include an RNA stabilization step (Figure 1). The loss in RNA yield from blood stored in EDTA tubes indicates RNA degradation. When blood was stored at 4°C or –20°C, the PAXgene System provided constant RNA yields for a minimum of 30 days. In contrast, RNA yields decreased significantly when blood was collected and stored in conventional EDTA tubes (Figure 2). The PAXgene System yielded highly reproducible results (Figure 3), and the resulting RNA performed well in quantitative, real-time RT-PCR (Figure 4).

Conclusions

Use of the PAXgene Blood RNA System provides efficient stabilization of cellular RNA in whole blood during and after phlebotomy. Excellent and reproducible yields of RNA are achieved. ■

Ordering Information

Product	Contents	Cat. No.
PAXgene Blood RNA Tubes (100)*	100 blood collection tubes. To be used in conjunction with the PAXgene Blood RNA Kit (50).	762115,† 762125‡
PAXgene Blood RNA Kit (50)*	50 PAXgene Spin Columns, Processing Tubes, RNase-free Reagents and Buffers. To be used in conjunction with PAXgene Blood RNA Tubes.	762134
PAXgene Blood RNA Validation Kit (10)*	10 blood collection tubes, 10 PAXgene Spin Columns, Processing Tubes, RNase-free Reagents and Buffers	762132

** For research use only. Not for use in diagnostic procedures.*
† USA and Canada
‡ All other countries

QIAGEN News

Rapid analysis of the extracellular matrix protein decorin using the Penta·His™ Alexa Fluor® 488 Conjugate

Cordula Büttner and Annett Skupin

Institute of Immunology, Medical Faculty, Technical University Dresden, Dresden, Germany

Decorin is found in connective tissue where, among other roles, it helps to regulate cell growth by interacting with growth factors and collagen. After cloning of full-length human decorin cDNA into the pQE-TriSystem vector, human fibrosarcoma cells (HT-1080) were transfected, and the expression of decorin was analyzed by direct immunofluorescence using the Penta·His™ Alexa Fluor® 488 Conjugate.

Extracellular matrix (ECM) proteins play an essential role in regulation of differentiation, adhesion, and migration of cells and influence complex processes such as development, and tissue remodeling. Decorin is a member of the small leucine-rich family of proteoglycans, which are prominent constituents of both the ECM and the cell surface. Decorin is post-translationally modified by polymerization of a single glycosaminoglycan (GAG) chain onto a site near the N-terminus of the core protein and by N-glycosylation at three potential sites (1). It may modify vascular smooth muscle cell function by altering the response to growth factors, such as TGF-β1, and the accumulation of ECM proteins during vascular injury (2–4). Decorin has been shown to influence the proliferative capacity of cells (5).

The goal of this study was to produce a biologically active decorin protein, consisting of both the core protein and the GAG chain. To achieve this, the protein's cDNA was cloned into the pQE-TriSystem vector and overexpressed as a His-tagged fusion protein in a mammalian expression system (human fibrosarcoma cells). Expression of the protein was analyzed in situ by direct immunofluorescence using the Penta·His Alexa Fluor 488 Conjugate.

Materials and methods

Total RNA was isolated from human fibroblasts and the full-length decorin cDNA, containing the endogenous signal sequence, was amplified by RT-PCR using decorin-sequence–specific primers containing *Bam*HI and *Eco*RI restriction sites. PCR products were ligated into a TA-ligation vector and subjected to *Bam*HI and *Eco*RI digestion. Decorin cDNA was inserted into the *Bam*HI – *Eco*RI site of pQE-TriSystem, and the vector was transiently transfected into human fibrosarcoma cells (HT-1080).

The Penta·His Alexa Fluor 488 Conjugate was used to analyze transfection efficiency of HT-1080 cells. HT-1080 transfectants were fixed 24 hours after transfection (2% paraformaldehyde in PBS, 15 minutes at room temperature) and incubated with a 1:200 dilution of Penta·His Alexa Fluor 488 Conjugate for 1 hour at 4°C. His-tagged decorin was visualized by fluorescence microscopy.

Results and discussion

Human fibrosarcoma cells (HT-1080) were transfected with pQE-TriSystem vector containing the full-length cDNA of human decorin. Expressed protein could be detected using Penta·His Alexa Fluor 488 Conjugate as early as 24 hours post-transfection. Figures 1 and 2 show the intracellular localization of His-tagged decorin. Decorin is distributed in the nuclear envelope, endoplasmic reticulum, or confined to cisternae in the Golgi body. In Figure 2, nuclei were counterstained with blue-fluorescent DAPI. Some nuclear staining was observed in nontransfected cells (data not shown). ▶

References

1. Krusius, T., and Ruoslahti, E. (1986) Primary structure of an extracellular matrix proteoglycan core protein deduced from cloned cDNA. Proc. Natl. Acad. Sci. U.S.A. **83**, 7683.
2. Yamaguchi, Y., Mann, D.M., and Ruoslahti, E. (1990) Negative regulation of transforming growth factor-beta by the proteoglycan decorin. Nature **346**, 281.
3. Giri, S.N., Hyde, D.M., Braun, R.K., Gaarde, W., Harper, J.R., and Pierschbacher, M.D. (1997) Antifibrotic effect of decorin in a bleomycin hamster model of lung fibrosis. Biochem. Pharmacol. **54**, 1205.
4. Yamakawa, T., et al. (2000) Differential expression of proteoglycans biglycan and decorin during neointimaformation after stent implantation in normal and atherosclerotic rabbit aortas. Atherosclerosis **152**, 287.
5. Santra, M., Eichstetter, I., and Iozzo, R.V. (2000) An anti-oncogenic role for decorin. Down-regulation of ErbB2 leads to growth suppression and cyto-differentiation of mammary carcinoma cells. J. Biol. Chem. **275**, 35,153.

Direct Immunofluorescent Detection of His-Tagged Proteins

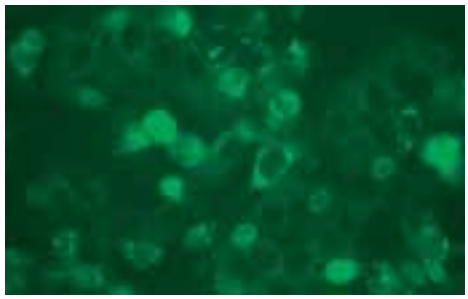


Figure 1 *Fluorescence micrograph (400x) of human fibrosarcoma cells transfected with a pQE-TriSystem construct encoding His-tagged decorin. Cells were fixed with 2% paraformaldehyde 24 hours post-transfection and stained using Penta·His Alexa Fluor 488 Conjugate.*

Sensitive and Specific Protein Localization

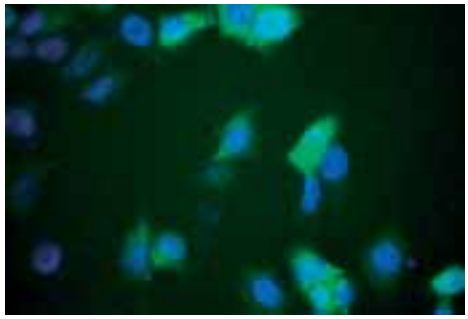


Figure 2 *Fixed cells were stained using Penta·His Alexa Fluor 488 Conjugate and nuclei were counterstained with DAPI (400x).*

Conclusions

- Expression in mammalian systems allows post-translational modification of over-expressed proteins. Post-translational modifications (e.g., signal sequence processing), may be essential for correct protein compartmentalization or function. The pQE-TriSystem vector contains 3 promoters allowing expression of His-tagged proteins in *E. coli*, insect, and mammalian cells using a single construct.
- The Penta·His antibody conjugated to green-fluorescent Alexa Fluor 488 is a sensitive and specific tool for localizing His-tagged proteins expressed in mammalian cells by direct immuno-fluorescence. Transfection efficiency, which is known to depend on several parameters such as cell type, size of transfected DNA, and time of transfection, can be easily checked. Such analyses can also be used to aid determination of protein expression levels before performing western blotting. ■

Ordering Information

Product	Contents	Cat. No.
For efficient expression of His-tagged proteins in *E. coli*, insect, or mammalian cells		
pQE-TriSystem Vector	25 µg pQE-TriSystem Vector DNA	33903
For direct detection of 6xHis-tagged proteins in immunofluorescence procedures		
Penta·His Alexa Fluor 488 Conjugate	125 µl Penta·His Alexa Fluor 488 Conjugate, 200 µg/ml	35310
Penta·His Alexa Fluor 532 Conjugate	125 µl Penta·His Alexa Fluor 532 Conjugate, 200 µg/ml	35330
Penta·His Alexa Fluor 555 Conjugate	125 µl Penta·His Alexa Fluor 555 Conjugate, 200 µg/ml	35350
Penta·His Alexa Fluor 647 Conjugate	125 µl Penta·His Alexa Fluor 647 Conjugate, 200 µg/ml	35370
For indirect detection of 6xHis-tagged proteins in immunofluorescence procedures		
Penta·His Biotin Conjugate	125 µl Penta·His Biotin Conjugate, 200 µg/ml	34440
Streptavidin-R-PE	250 µl Streptavidin–R-phycoerythrin Conjugate, 1mg/ml	922721

QIAGEN News

Automate front-end tasks using the BioRobot® 3000 for microarray fabrication and streamlined workflow

Katrine Verdun, Richard Rouse, and Gary Hardiman

BIOGEM, Division of Biology, University of California, San Diego, La Jolla, CA, USA
E-mail: ghardiman@ucsd.edu

A BioRobot® 3000 workstation was used to process 4 x 96 cDNA clones in parallel for a high-throughput microarray project to analyze gene expression profiles in mice. Bacterial cDNA clones were propagated in 96-well format and insert sequences were amplified to provide DNA for microarray spotting. Automation of clone propagation, PCR setup and cleanup, cherry picking, and sample rearray results in high yields of pure PCR products, ready to use in microarray spotting.

Microarray technology has revolutionized genomic research, allowing scientists to monitor the expression profiles of thousands of genes in parallel. A microarray consists of a nylon membrane or glass-derivative slide, onto which DNA sequences are spotted (1).

DNA probes immobilized as spots on microarray slides are cDNAs, gene fragments (ESTs), or oligonucleotides corresponding to known genes or putative open reading frames (ORFs). These arrays are usually hybridized with fluorescently labeled targets synthesized from cellular RNA. The widespread use of microarray technology means that university core facilities such as BIOGEM now require cost-efficient automated solutions to increase sample throughput and reduce handling variability (Figure 1).

The most basic type of experiment compares mRNA abundance in two target samples, a control mRNA and a test mRNA. During hybridization, both targets bind to complementary sequences on the microarray. The microarray is washed to remove nonspecific hybridization and then dried. Hybridization is detected by laser excitation of the fluorescently labeled target sequences. Comparing the intensities of the signals from individual spots shows the relative expression levels of genes in both test and control populations (2). ▶

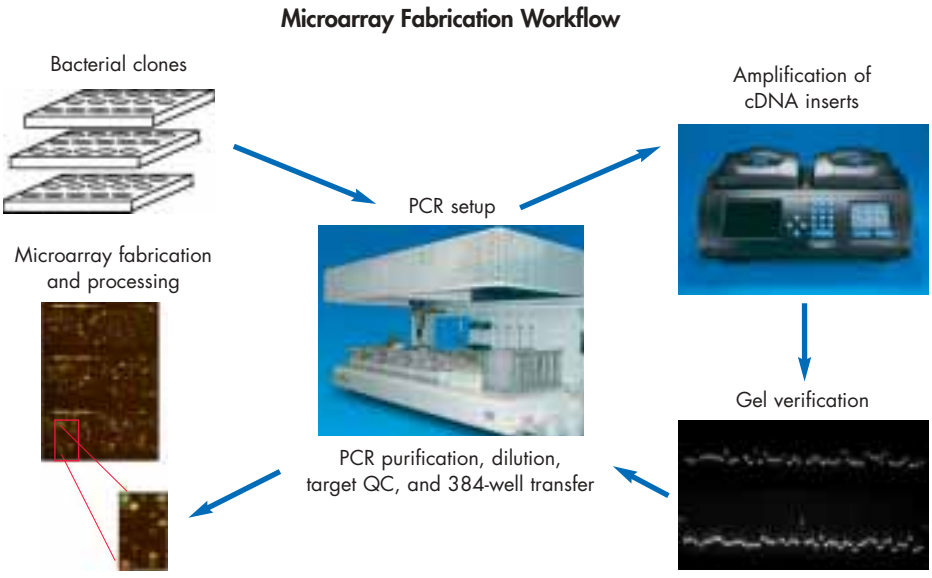


Figure 1 *A typical microarray fabrication procedure involves replication of bacterial clones, amplification of cDNA inserts, verification, purification, and dessication of the PCR amplicons. DNA is then resuspended at a concentration appropriate for microarray spotting.*

The cDNAs spotted onto microarrays are typically PCR products amplified from plasmid DNA purified from cDNA clones. The recommended concentration for spotting on aminosilane and poly-L-lysine slides is 200–400 fmol/µl. Lower concentrations result in weak signals while higher concentrations can cause smearing and create hybridization artifacts. Microarray hybridizations with unpurified PCR products give poor results due to carryover of salts, primers, and partial PCR products. Consequently, PCR amplification and cleanup strategies for array fabrication are optimized to maximize DNA yield and purity.

The precise liquid handling and robotic handling of the BioRobot 3000 workstation are ideally suited for automated purification of plasmid DNA, subsequent PCR setup, and reaction cleanup. High-throughput PCR projects such as microarray analysis rely on highly standardized purification and reaction setup procedures. Errors in front-end processes, such as PCR setup, will be amplified in downstream applications, such as amplification and hybridization, and may produce meaningless data. Microarray spotting is performed by highly specialized instruments that usually require purified DNA in 384-well plates. The robotic handling system of the BioRobot 3000 and the disposable tips of the pipetting system provide the precision and protection from carryover required to accurately rearray samples from 96- to 384-well format.

Materials and methods

A commercially available mouse library containing 8734 clones was used to generate a clone set. Bacterial clones containing cDNA inserts for growth and replication were inoculated into 96-well plates containing selective media and grown overnight with agitation at 37°C. PCR (150 µl each) was set up on the BioRobot 3000 workstation in 96-well microplates using a customized protocol within QIAsoft™, the BioRobot Operating System software. PCR cycling was performed using MJ Research thermal cyclers.

A 10 µl aliquot of each reaction was analyzed on a 0.8% agarose gel and stained with ethidium bromide (Figure 2). The yield of PCR product was assayed using a commercially available dsDNA quantification kit and a microplate fluorescence reader. Bacterial clones that gave no amplification product were cherry-picked using the BioRobot 3000 and inoculated into fresh media in 96-well plates for repeated growth and PCR amplification. Amplicons were purified using the QIAquick® 96 PCR

Evaluation of PCR Products

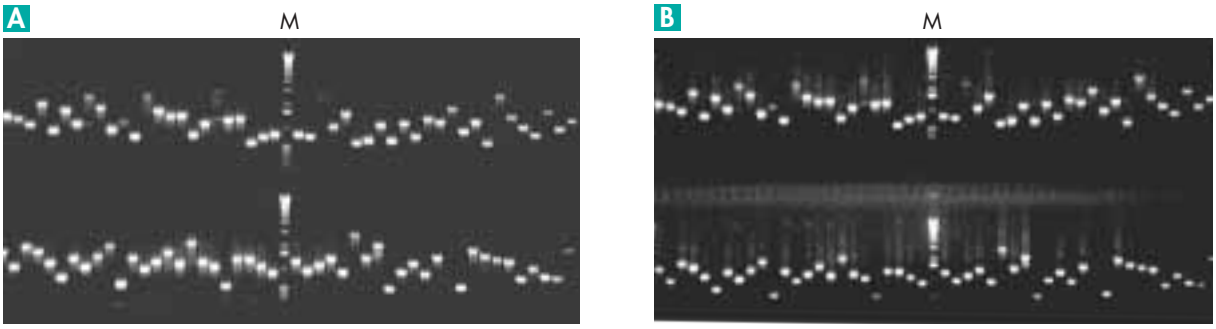


Figure 2 PCR products from a 96-well plate were separated by agarose gel electrophoresis. A Before purification using the QIAquick 96 PCR Purification Kit. B After purification using the QIAquick 96 PCR Purification Kit. M: Molecular weight markers.

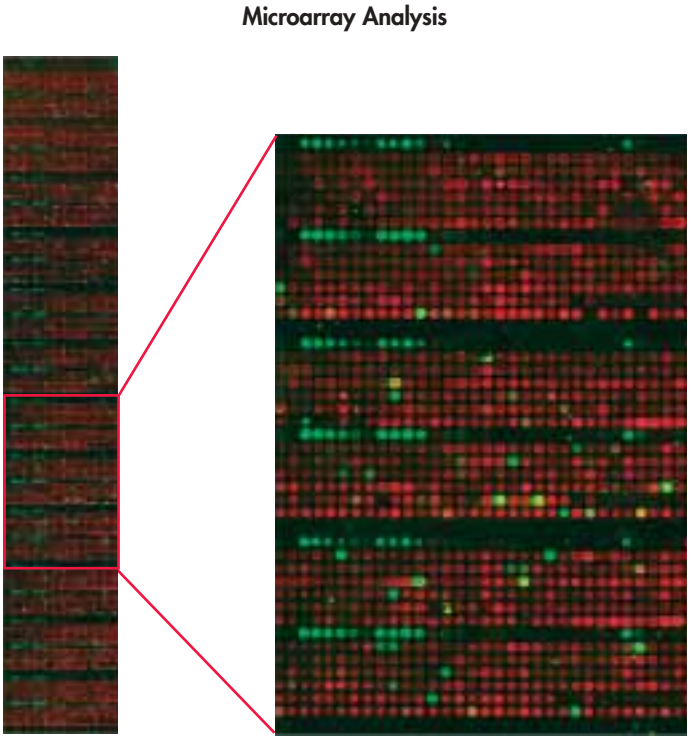


Figure 3 *Approximately 10,000 cDNA clones derived from the mouse clone set and controls were printed on Type 7 reflective slides. Fluorescent targets were synthesized from mouse spleen and control vector sequence RNAs and used to screen the microarray. The enlarged area shows signal color range when using cyanine targets, Cy5 amplicon and Cy3 spleen. Green, red, and yellow spots indicate positive hybridization with the labeled target. Green (Cy3) and red (Cy5) spots indicate that the genes are expressed differentially.*

Purification Kit and an automated protocol on the BioRobot 3000 workstation. QIAquick purified DNA was then rearrayed from 96-well plates to 384-well plates on the BioRobot 3000 workstation prior to spotting (Figure 1).

One microgram each of mouse spleen poly A+ RNA and a synthetic RNA target amplicon created from plasmid vector sequence were reverse transcribed and fluorescently labeled with Cy®3-dCTP and Cy5-dCTP as previously described (1). Remaining RNA was hydrolyzed by addition of 1 µl of 10 M NaOH and subsequent incubation at 37°C for 15 minutes, followed by neutralization. cDNA targets were purified using the QIAquick PCR Purification Kit. Typically, 20% of each reaction was used per hybridization.

Approximately 10,000 PCR products were amplified from the mouse clone set and control cDNA clones. QIAquick purified products were printed on Type 7 reflective slides using a commercially available microarray spotter (Molecular Dynamics). cDNA targets were lyophilized and redissolved in 32 µl hybridization buffer before hybridization for 14–18 hours at 42°C. The microarrays were washed at room temperature, briefly rinsed with water, and dried by centrifugation. Microarrays were scanned using an automated scanner (Molecular Dynamics).

Results and discussion

Amplicon yield and quality

Representative amplifications performed in 96-well format are shown in Figure 2. Most samples gave a single amplicon at high concentration. The quality of QIAquick purified DNA was high and DNA was recovered efficiently.

Of approximately 10,000 samples tested, 8356 samples gave single amplicons. Of these, 7997 samples gave high concentrations of PCR products. 293 samples gave multiple bands and 85 samples failed to give any PCR product. The cDNA clones that generated multiple bands were rearrayed and inoculated for growth using the BioRobot 3000 and amplified with a nested primer set (data not shown). Most samples still gave multiple bands, indicating that these cDNAs represent mixed clones. This indicates a very low rate of cross-contamination for amplification of a large clone set. This is due largely to automation of the array fabrication process using a BioRobot 3000 workstation. ▶

References

1. *Schena, M., Shalon, D., Davis, R.W. and Brown, P.O. (1995) Quantitative monitoring of gene expression patterns with complementary DNA microarray. Science* ***270****, 467.*
2. *Hegde, P. et al. (2000) A concise guide to cDNA microarray analysis. BioTechniques* ***29****, 548.*
3. *Verdun et al. (2002) submitted.*

Representative DNA samples from the entire set of amplifications were quantified. The mean yield per clone was 4955 ng dsDNA. DNAs used for printing were resuspended at a concentration of approximately 200 µg/ml in 25 µl 50% DMSO. Using 200 nl of this solution per spot enables printing of approximately 3000 microarray slides, even considering sample evaporation and other printing variables.

Microarray performance

PCR products amplified using this fabrication scheme generated strong specific signals in a microarray experiment (Figure 3). We printed this set of amplicons representing approximately 10^4 mouse cDNA clones and appropriate controls on a reflective slide. Fluorescent probes synthesized from mouse spleen poly A^+ and control amplicon RNAs were used to screen the microarray by hybridization (Figure 3). Positive hybridization of the labeled targets to the printed PCR products is indicated by green (Cy3), red (Cy5), and yellow fluorescent spots. The enlarged area shows the signal color range observed when using cyanine targets, Cy5 control RNA sequence amplicon and Cy3 spleen. Green and red spots indicate differential hybridization of the targets. The control amplicon hybridization represents quality control of the array as every probe is expected to hybridize to the target. Lack of hybridization indicated a poor probe and data from this spot were not counted. Generally, strong hybridization signals were observed indicating that the quality of the probes was good and that use of the BioRobot 3000 workstation was successful.

Conclusions

- Amplifying large clone collections for the production of microarrays requires minimal cross-contamination and reduction of sources of error due to manual intervention.
- Standardized processing using the BioRobot 3000 workstation together with QIAquick chemistry results in high yields of pure PCR products at concentrations ideal for microarray spotting.
- High yields of PCR product result in a greater number of arrays per PCR and a resultant reduction in time and cost per project. ■

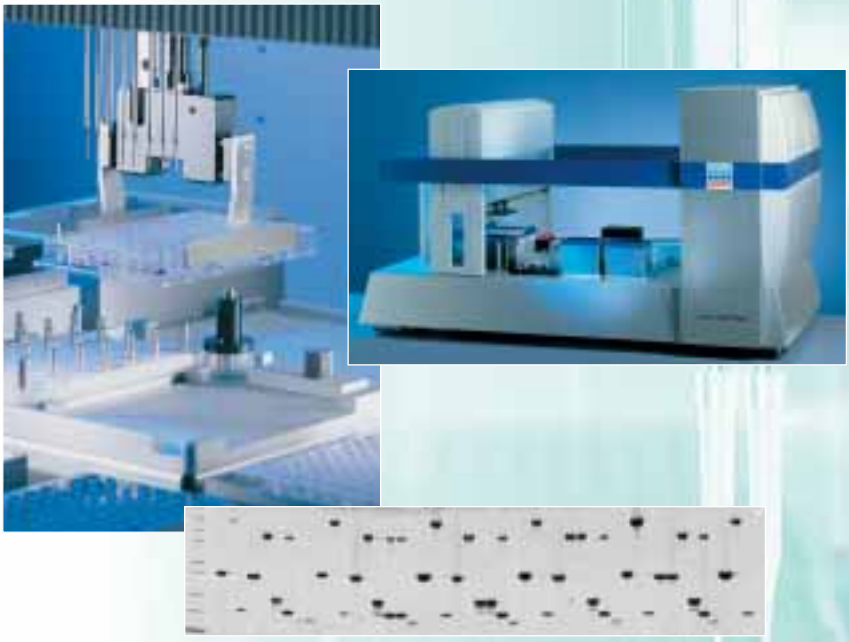
Ordering Information

Product	Contents	Cat. No.
BioRobot 3000	System includes: custom-designed robotic workstation comprised of 2–4 dilutor units, computer controlled shaker and vacuum pump, vacuum manifold, QIAsoft 4.0 Operating System , computer, 1 year warranty on parts and labor. Workstations of various sizes are available.	900400
QIAquick 96 PCR Purification Kit	For purification of 24 x 96 PCR products: 24 QIAquick 96 Plates, Buffers, Collection Microtubes (1.2 ml), Caps	28183
Related product for faster liquid-handling and rearray		
BioRobot RapidPlate	System includes: robotic system, controller unit, software, pre-programmed protocols for reaction setup, 1 year warranty on parts and labor	9000490

Automate high-throughput purification and assay of recombinant proteins

Walkaway protein purification and assay setup on the BioRobot® 8000 workstation includes:

- Cost-efficient Ni-NTA chemistry for medium-scale purification of up to 2 mg 6xHis-tagged protein per well
- Purification of 96 samples in 150 minutes for high sample throughput and streamlined workflow
- Highly pure protein suitable for applications such as solubility screening, functional studies, and for rapid definition of crystallization conditions



Contact QIAGEN today and discover automated solutions for your recombinant protein applications

001

The QIAGEN Guide to Animal Cell Culture

Part V: Counting cells

Welcome to the fifth of a series of articles providing useful hints for culturing animal cells. This article contains considerations for counting cells.

This protocol has been adapted from references 1–3. Protocols in this series are examples of methods for general cell culture and have not been rigorously validated and optimized by QIAGEN. There are many alternative protocols in current use.

Cell counting using a hemocytometer

It is often necessary to count cells, for example, when plating cells for transfection experiments. One method for counting cells is to use a hemocytometer. A hemocytometer contains 2 chambers (Figure 1). Each chamber is ruled into 9 major squares (volume of 0.1 mm^3 or 1 x 10^{-4} ml each). Cell concentration is determined by counting the number of cells within a defined area of known depth (volume).

1. Clean the surface of the hemocytometer with 70% ethanol or another suitable disinfectant, taking care not to scratch the surface of the central area. Dry with lens paper.

2. Clean the coverslip, wet the edges very slightly, lay it over the grooves and central area of the hemocytometer and gently press down.

Tip

It is important that the coverslip is properly attached to obtain the correct chamber depth. The appearance of Newton's rings (bright and dark rings caused by interference in the air between the coverslip and the glass surface of the hemocytometer) will confirm that the coverslip is attached properly.

3. Harvest the cells, either by trypsinization (adherent cell cultures; see *QIAGEN News* 2002 No. 3, 20) or by centrifugation at 200 x *g* for 5 min (suspension cell cultures). Resuspend the cells in an appropriate volume of prewarmed growth medium. At least 10^6 cells/ml are required for accurate counting.

Tip

It may be necessary to centrifuge cells and resuspend in a smaller volume to obtain the desired cell concentration for counting. For adherent cells, it is important to produce a single-cell suspension after trypsinizing. Cell clumping will make counting difficult and inaccurate.

4. Mix the cell suspension sample thoroughly. Using a pipet, immediately transfer 20 µl to the edge of one side of the coverslip to fill one chamber of the hemocytometer. Repeat for the second chamber.

Tip The cell distribution should be homogeneous in both chambers.

Tip The cell suspension is drawn under the coverslip and into the chamber by capillary action. The cell suspension should just fill the chamber. Blot off any surplus fluid without disturbing the sample underneath the coverslip.

5. Transfer the slide to the microscope, and view a large square ruled by 3 lines using a 10x objective and 10x ocular. Count the total number of cells in 5 of the 9 major squares.

Count cells that overlap the top and left border of squares but not those overlapping bottom and right borders (Figure 1). This prevents counting overlapping cells twice. If the cell density is too high, the cell suspension should be diluted, noting the dilution factor.

6. Repeat the counting for the second chamber to give a total of 10 squares.

7. Add the number of cells counted in all 10 squares together to give the number of cells in 1 x 10^{-3} ml. Multiply by 1000 to give the number of cells/ml.

IMPORTANT: If the original cell suspension was diluted for counting, multiply by the dilution factor to obtain the number of cells/ml.

8. Clean the hemocytometer and coverslip by rinsing with 70% ethanol and then with distilled water. Dry with lens paper.

Counting Cells Using a Hemocytometer

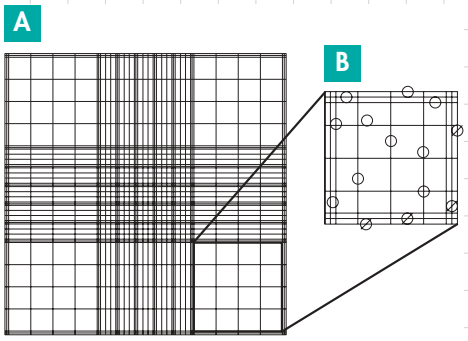


Figure 1 **A** *One chamber of a hemocytometer slide under 10x objective and 10x ocular. The chamber is divided into 9 major squares.* **B** *Detailed view of one of the 9 major squares. Only cells that overlap the top and left borders of squares should be counted to avoid overestimating the cell concentration.* **O***: cells that should be counted;* **Ø***: cells that should be ignored.*

The QIAGEN Guide to Animal Cell Culture will continue in future issues of *QIAGEN News*. 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.

References

1. *Freshney, R.I. (1993)* Culture of Animal Cells: a Manual of Basic Technique. *3rd ed. New York: Wiley-Liss.*

2. *Ausubel, F.M. et al., eds. (1991)* Current Protocols in Molecular Biology. *New York: Wiley Interscience.*

3. *Spector, D., Goldman, R.R., and Leinwand, L.A., eds. (1998)* Cells: a Laboratory Manual. *Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.*

QIA-Hints

QIAGEN Technical Services are always available to answer your questions!

DNA cleanup

Q What kit can I use to purify short PCR products?

A QIAGEN has developed a range of kits designed for specific DNA cleanup applications. For PCR products from 100 bp to 10 kb, the QIAquick® PCR Purification Kit provides typical recoveries of >90%. The MinElute™ PCR Purification Kit is well suited for cleanup of smaller fragments, from 70 bp to 4 kb, in only 6 minutes. PCR products purified using this kit are concentrated due to the small elution volumes used in the MinElute procedure. Alternatively, the QIAEX® II Gel Extraction Kit can be used for batch purification of PCR products as small as 40 bp, from either gels or solutions.

DNA isolation

Q I'm using tissue samples that are very tough and difficult to digest. How can I improve genomic DNA yields from these tissues using the DNeasy® Tissue Kit or the QIAamp® DNA Mini Kit?

A The efficiency of DNA isolation requires thorough disruption of the sample, generally using proteinase K. To improve digestion of tough tissue samples, lengthen the proteinase K incubation time at 55°C to 1–3 hours or even overnight. You can also improve DNA yields by increasing the amount of proteinase K in the reaction, or, alternatively, adding extra proteinase K after several hours of digestion.

Although the DNeasy and QIAamp DNA procedures require no mechanical disruption of the tissue sample, proteinase K digestion may be more effective following mechanical homogenization. Particularly tough tissues can be disrupted using the QIAGEN® Mixer Mill MM 300. Please contact QIAGEN Technical Services for a user-developed protocol.

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.

Come and See Us

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

13th International Symposium on Human Identification — 2002	7–10 October, Phoenix, AZ

In Europe

Ribozymes and RNA Catalysis — International Workshop	23–27 August, Dundee, UK
Public Health Laboratory Service Annual Scientific Conference	9–11 September, Warwick, UK
BSHI — 13th Conference & AGM	16–18 September, Glasgow, UK
BioTech Forum	8–10 October, Malmö, Sweden
10th Annual Meeting European Society of Gene Therapy	13–16 October, Antibes, France
European Biotech Crossroads (Carrefour Européen des Biotechnologies)	15–16 October, Lille, France
r+d in Life Sciences	15–18 October, Basel, Switzerland
British Society for Immunology and British Society for Allergy and Clinical Immunology Joint Meeting	3–6 December, Harrogate, UK

QIAGEN News

IN THIS ISSUE

www.qiagen.com

QIAGEN:

Australia Tel. 03-9489-3666 Fax 03-9489-3888
Canada Tel. 800-572-9613 Fax 800-713-5951
France Tel. 01-60-920-930 Fax 01-60-920-925
Germany Tel. 02103-29-12400 Fax 02103-29-22000
Italy Tel. 02-33430411 Fax 02-33430426
Japan Tel. 03-5547-0811 Fax 03-5547-0818
Switzerland Tel. 061-319-30-31 Fax 061-319-30-33
UK and Ireland Tel. 01293-422-999 Fax 01293-422-922
USA Tel. 800-426-8157 Fax 800-718-2056

Distributors:

Argentina Tecnolab S.A. (011) 4555 0010 **Austria** VWR International GmbH (01) 576 00 0 **Belgium/Luxemburg** Westburg b.v. 0800-1-9815 **Brazil** Uniscience do Brasil 011 3622 2320 **China** Gene Company Limited (852)2896-6283 **Cyprus** Scientronics Ltd (02) 357 22 765416 **Czech Republic** BIO-CONSULT spol. s.r.o. (420) 2 417 29 792 **Denmark** VWR International A/S 43 86 87 88 **Egypt** Clinilab 52 57 212 **Finland** VWR International Oy (09) 804 551 **Greece** BioAnalytica S.A. (10)-640 03 18 **India** Genetix (011)-542 1714 or (011)-515 9346 **Israel** Westburg (Israel) Ltd. 08 6650813/4 or 1-800 20 22 20 **Korea** LRS Laboratories, Inc. (02) 924-86 97 **Malaysia** RESEARCH BIOLABS SDN. BHD. (603)-8070 3101 **Mexico** Quimica Valaner S.A. de C.V. (55) 55 25 57 25 **The Netherlands** Westburg b.v. (033)-4950094 **New Zealand** Biolab Scientific Ltd. (09) 980 6700 or 0800 933 966 **Norway** VWR International AS 22 90 00 00 **Poland** Syngen Biotech Sp.z.o.o. (071) 351 41 06 or 0601 70 60 07 **Portugal** IZASA PORTUGAL, LDA (21) 424 7312 **Singapore** Research Biolabs Pte Ltd 2731066 **Slovak Republic** BIO-CONSULT Slovakia spol. s.r.o. (02) 5022 1336 **South Africa** Southern Cross Biotechnology (Pty) Ltd (021) 671 5166 **Spain** IZASA, S.A. (93) 902.20.30.90 **Sweden** VWR International AB (08) 621 34 00 **Taiwan** TAIGEN Bioscience Corporation (02) 2880 2913 **Thailand** Theera Trading Co. Ltd. (02) 412-5672 **In other countries contact:** QIAGEN, Germany

1021015 06/2002