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# PAXgene® Blood RNA Kit Handbook

Version 2

50 (catalog no. 762174)

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IVD

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PreAnalytiX GmbH
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Produced by QIAGEN GmbH for PreAnalytiX

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**Conditional Sale**

The present product comes with a license under certain claims of US-7,270,953, and US-7,682,790, as well as EP-1820793 B1 and foreign equivalents of these patent claims to use the product to process the nucleic acid complex formed in the course of sample collection in a PAXgene Blood RNA Tube.



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## PreAnalytiX Distributors

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Please see the last page for contact information for your local PreAnalytiX distributor.

# Contents

# Kit Contents

| **PAXgene Blood RNA Kit** | | | **(50)** |
|---|---|---|---|
| **Catalog no.** | | | **762174** |
| **Number of preps** | | | **50** |
| BR1 | Resuspension Buffer | RES BUF | 20 ml |
| BR2 | Binding Buffer* | BIND BUF | 18 ml |
| BR3 | Wash Buffer 1* | WASH BUF 1 | 45 ml |
| BR4 | Wash Buffer 2 (concentrate)† | WASH BUF 2 CONC | 11 ml |
| BR5 | Elution Buffer | ELU BUF | 6 ml |
| RNFW | RNase-Free Water (bottle) | PEL WASH | 2 × 125 ml |
| PK | Proteinase K (green lid) | PROTK | 2 × 1.4 ml |
| PRC | PAXgene RNA Spin Columns (red) | PAXgene RNA COL | 5 × 10 |
| PT | Processing Tubes (2 ml) | PROC TUBE | 6 × 50 |
| Hemogard | Secondary BD Hemogard™ Closures | SEC CLOS | 50 |
| MCT | Microcentrifuge Tubes (1.5 ml) | MIC TUBE | 3 × 50, 1 × 10 |
| RNFD | DNase I, RNase-free (lyophilized) | DNA REM | 1500 Kunitz units‡ |
| RDD | DNA Digestion Buffer (white lid) | DNA DIG BUF | 2 × 2 ml |
| DRB | DNase Resuspension Buffer (tube, lilac lid) | DNase RES BUF | 2 ml |
| PSC | PAXgene Shredder Spin Columns (lilac) | PAXgene SHRED COL | 5 × 10 |
| Handbook | PAXgene Blood RNA Kit Handbook (Version 2) |  | 1 |

*Not compatible with disinfecting reagents containing bleach. Contains a guanidine salt. See page 9 for safety information.

† Wash Buffer 2 (BR4) is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%, purity grade p.a.) as indicated on the bottle to obtain a working solution.

# Symbols

| | |
|---|---|
|  | Contains reagents sufficient for <N> tests |
|  | Consult instructions for use |
|  | Use by |
| IVD | In vitro diagnostic medical device |
| REF | Catalog number |
| LOT | Lot number |
| MAT | Material number |
| COMP | Components |
| NUM | Number |
| STERILE R | Method of sterilization using irradiation |
| KU | Kunitz units |
| ADD | Adding |
| CONT | Contains |
| RCNS | Reconstituted |
| DNase | Deoxyribonuclease I |

‡ Kunitz units are the commonly used units for measuring DNase I, defined as the amount of DNase I that causes an increase in $A_{260}$ of 0.001 per minute per milliliter at 25°C, pH 5.0, with highly polymerized DNA as the substrate (Kunitz, M. (1950) J. Gen. Physiol. **33**, 349 and 363).

| | |
|---|---|
| EtOH | Ethanol |
| GITC | Guanidine isothiocyanate |
| RNase-Free DNase Set | RNase-Free DNase Set |
| GTIN | Global Trade Item Number |
|  | Do not reuse |
|  | Temperature limitation |
|  | Upper limit of temperature |
|  | Manufacturer |
|  | Important note |

# Storage Conditions

PAXgene RNA spin columns (PRC), PAXgene Shredder spin columns (PSC), proteinase K (PK), and buffers (BR1, BR2, BR3, BR4, and BR5) should be stored dry at the temperature indicated on the kit label.

The RNase-Free DNase Set, which contains DNase I (RNFD), DNA digestion buffer (RDD), and DNase resuspension buffer (DRB), is shipped at ambient temperature. Store all components of the RNase-Free DNase Set immediately upon receipt at the temperature indicated on the label. When stored properly, the kit is stable until the expiration date on the kit box.

# Intended Use

The PAXgene Blood RNA System consists of a blood collection tube (PAXgene Blood RNA Tube, BRT) and nucleic acid purification kit (PAXgene Blood RNA Kit). It is intended for the collection, storage, and transport of blood and stabilization of intracellular RNA in a closed tube and subsequent isolation and purification of host RNA from whole blood for RT-PCR used in molecular diagnostic testing.

**Performance characteristics for the PAXgene Blood RNA System have only been established with FOS and IL1B gene transcripts. The user is responsible for establishing appropriate PAXgene Blood RNA System performance characteristics for other target transcripts.**

## Indications for use

The PAXgene Blood RNA Kit is for the purification of intracellular RNA from whole blood collected in the PAXgene Blood RNA Tube (BRT). When the kit is used in conjunction with the PAXgene Blood RNA Tube (BRT), the system provides purified intracellular RNA from whole blood for RT-PCR used in molecular diagnostic testing.

# Product Use Limitations

The PAXgene Blood RNA Kit is intended for purification of intracellular RNA from human whole blood ($4.8 \times 10^6$ – $1.1 \times 10^7$ leukocytes/ml) for in vitro diagnostics applications. It is not for the purification of genomic DNA or viral nucleic acids from human whole blood. Due to the limited number of transcripts validated for stabilization specifications (FOS and IL1B gene transcripts), the performance characteristics have not been established for all transcripts. Users

should review the manufacturer's data and their own data to determine whether validation is necessary for other transcripts.

The product is intended to be used by professional users, e.g. technicians and physicians who are trained in in vitro diagnostic procedures.

See the *PAXgene Blood RNA Tube Handbook* for information about the use of PAXgene Blood RNA Tubes (BRT).

# Quality Control

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

# Technical Assistance

At QIAGEN we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in molecular biology and the use of PreAnalytiX products. If you have any questions regarding the PAXgene Blood RNA Kit, please do not hesitate to contact us.

For technical assistance and more information please call QIAGEN Technical Services.

# Safety Information

EU - Users should report any serious incident related to the device to the manufacturer and National Competent Authority. Outside EU - Contact your local QIAGEN representative for any incident or inquiry related to the device.

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles.

To avoid the risk of infection (e.g., from HIV or hepatitis B viruses) or injury when working with biological and chemical materials, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at **www.preanalytix.com** where you can find, view and print the SDSs for this kit.

**CAUTION**

DO NOT add bleach or acidic solutions directly to the sample-preparation waste.

Binding buffer (BR2) and wash buffer 1 (BR3) contain guanidine thiocyanate, which can form highly reactive compounds when combined with bleach. If Binding buffer (BR2) or wash buffer 1 (BR3) are spilt, clean with suitable laboratory detergent and water. If liquid containing potentially infectious agents is spilt, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite (bleach).

The RNA stabilizing solution and blood mixture from the PAXgene Blood RNA Tube (BRT) can be disinfected using 1 volume of commercial bleach solution (5% sodium hypochlorite) per 9 volumes of the RNA stabilizing solution and blood mixture.

Sample-preparation waste, such as supernatants from centrifugation steps in the RNA purification procedure, is to be considered potentially infectious. Before disposal, the waste must be autoclaved or incinerated to destroy any infectious material. Disposal must be made according to official regulations.

The following hazard and precautionary statements apply to components of the PAXgene Blood RNA Kit. See the *PAXgene Blood RNA Tube Handbook* for safety information about PAXgene Blood RNA Tubes (BRT).

**Buffer BR2**

Contains: guanidine thiocyanate. Danger! Harmful if swallowed. May be harmful in contact with skin or if inhaled. Causes serious eye damage. Harmful to aquatic life with long lasting effects. Contact with acids liberates very toxic gas. Wear protective gloves/ protective clothing/ eye protection/ face protection. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Immediately call a POISON CENTER or doctor/ physician.

**Buffer BR3**

Contains: ethanol; guanidine thiocyanate. Danger! Flammable liquid and vapor. Causes serious eye damage. Contact with acids liberates very toxic gas. Keep away from heat/sparks/open flames/hot surfaces. No smoking. Wear protective gloves/ protective clothing/ eye protection/ face protection. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Immediately call a POISON CENTER or doctor/ physician.

**DNase I**

Contains: DNase. Danger! May cause an allergic skin reaction. May cause allergy or asthma symptoms or breathing difficulties if inhaled. Avoid breathing dust/ fume/ gas/ mist/ vapors/ spray. Wear protective gloves/ protective clothing/ eye protection/ face protection. Wear respiratory protection. IF exposed or concerned: Call a POISON CENTER or doctor/ physician. Remove victim to fresh air and keep at rest in a position comfortable for breathing.

**Proteinase K**

Contains: proteinase K. Danger! Causes mild skin irritation. May cause allergy or asthma symptoms or breathing difficulties if inhaled. Avoid breathing dust/ fume/ gas/ mist/ vapors/ spray. Wear protective gloves/ protective clothing/ eye protection/ face protection. Wear respiratory protection. IF exposed or concerned: Call a POISON CENTER or doctor/ physician. Remove victim to fresh air and keep at rest in a position comfortable for breathing.

# Introduction

Collection of whole blood is the first step in many molecular assays used to study cellular RNA. However, a major problem in such experiments is the instability of the cellular RNA profile in vitro. Studies at PreAnalytiX have shown that the copy numbers of individual mRNA species in whole blood can change more than 1000-fold during storage or transport at room temperature.* This is caused both by rapid RNA degradation and by induced expression of certain genes after the blood is drawn. Such changes in the RNA expression profile make reliable studies of gene expression impossible. A method that preserves the RNA expression profile during and after phlebotomy is therefore essential for accurate analysis of gene expression in human whole blood.

## Principle and procedure

PreAnalytiX has developed a system that enables the collection, stabilization, storage and transportation of human whole blood specimens, together with a rapid and efficient protocol for purification of intracellular RNA. The system requires the use of PAXgene Blood RNA Tubes (BRT; US Patents 6,602,718 and 6,617,170) for blood collection and RNA stabilization, followed by manual or automated RNA purification using the PAXgene Blood RNA Kit. Both manual and automated protocols provide substantially equivalent performance with regards to RNA quality and yield. Performance data for the manual protocol (pages 22–29) and the automated protocol (pages 31–35) are included in this handbook.

The QIAGEN QIAcube Connect MDx is not available in all countries. For further details please contact QIAGEN Technical Service.

* Rainen, L. et al. (2002) Stabilization of mRNA expression in whole blood samples. Clin. Chem. **48**, 1883.

## Sample collection and stabilization

PAXgene Blood RNA Tubes (BRT) contain a proprietary reagent composition based on a patented RNA stabilization technology. This reagent composition protects RNA molecules from degradation by RNases and minimizes ex vivo changes in gene expression. PAXgene Blood RNA Tubes (BRT) are intended for the collection of human whole blood and stabilization of cellular RNA for up to 3 days at 18–25°C (Figures 1 and 2, pages 15 and 16) or up to 5 days at 2–8°C (Figures 3 and 4, pages 17 and 18). Currently available data shows stabilization of cellular RNA for at least 11 years at –20°C or –70°C*. For more information from ongoing studies evaluating stability for longer time periods, please contact QIAGEN Technical Services.

The actual duration of RNA stabilization may vary depending upon the species of cellular RNA and the downstream application used. Due to the limited number of transcripts validated for stabilization specifications (FOS and IL1B gene transcripts), the performance characteristics have not been established for all transcripts. Users should review the manufacturer's data and their own data to determine whether validation is necessary for other transcripts.

* A long-term study of blood storage in PAXgene Blood RNA Tubes is ongoing.

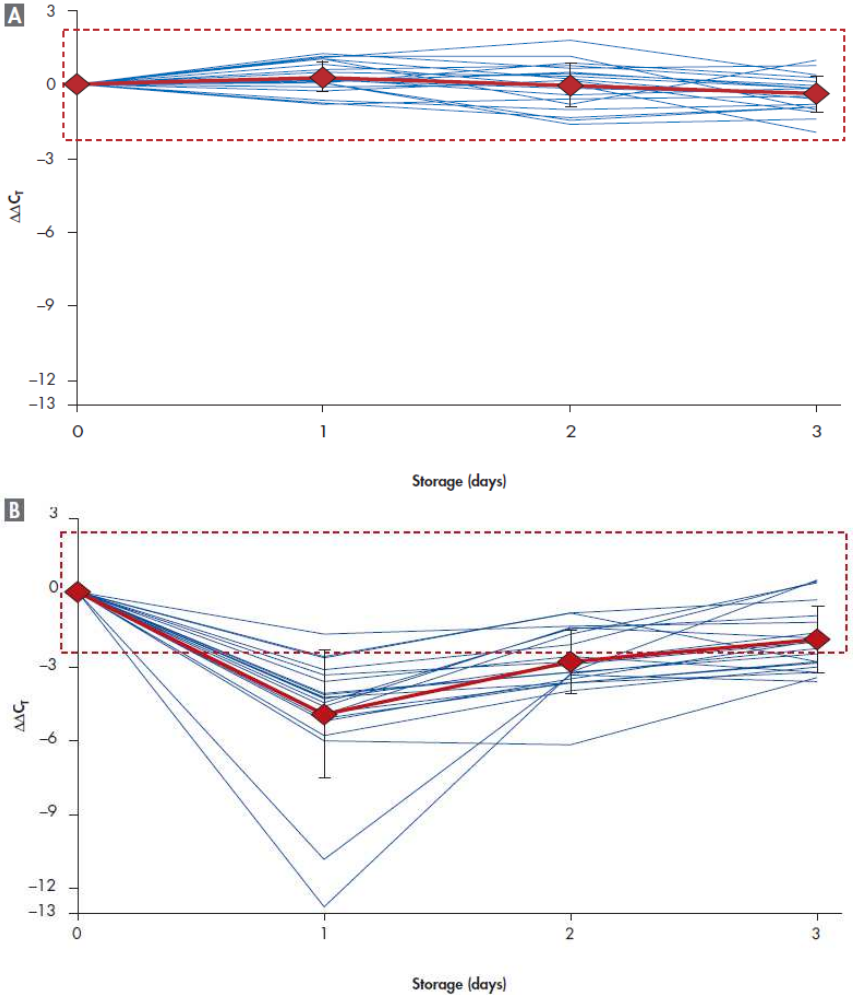



**Figure 1. RNA stability in blood samples at 18–25°C: FOS.** Blood was drawn from 10 donors, with duplicate samples and stored at 18–25°C for the indicated number of days, followed by total RNA purification. [**A**] Blood was collected and stored in PAXgene Blood RNA Tubes (BRT), and total RNA was purified using the PAXgene Blood RNA Kit. [**B**] Blood was collected and stored in standard blood collection tubes with EDTA as an anticoagulant, and total RNA was purified using a standard organic-extraction method with silica-membrane-based RNA cleanup. Relative transcript levels of FOS were determined by real-time, duplex RT-PCR, using 18S rRNA as an internal standard. The values for all samples are plotted, with means and standard deviations of all samples shown. The dashed lines indicate the ±3x total precision of the assay (2.34 $C_T$).

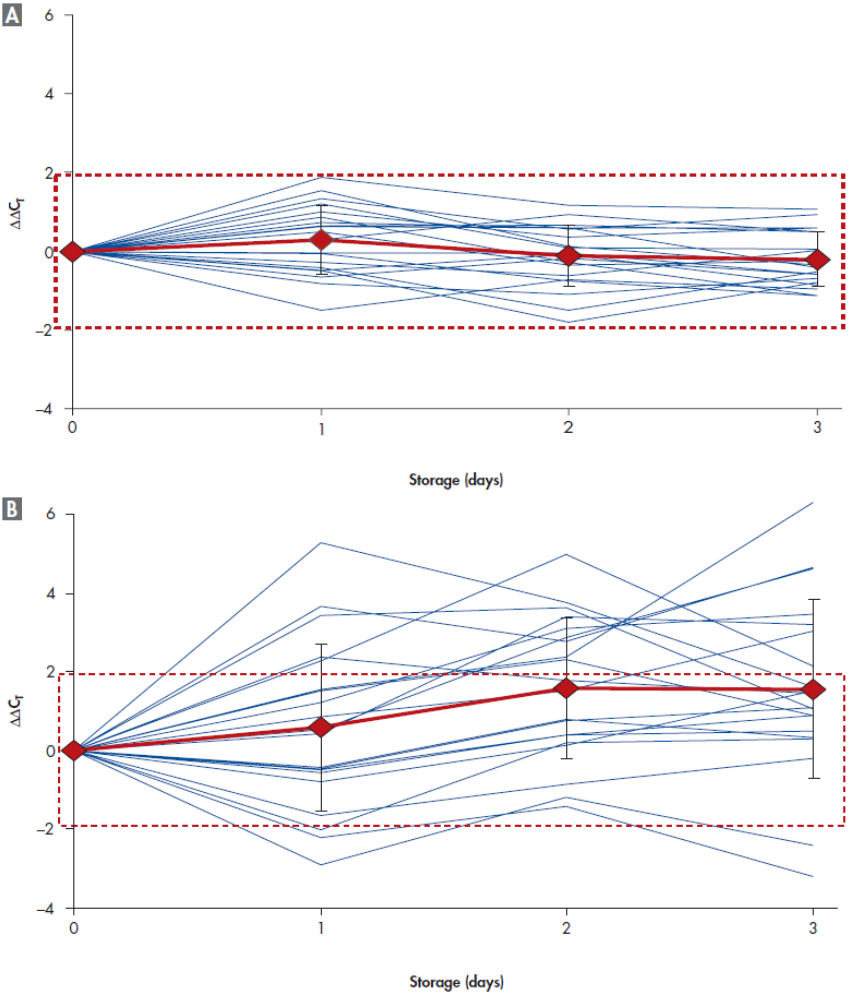



**Figure 2. RNA stability in blood samples at 18–25°C: IL1B.** Blood was drawn and total RNA purified, after storage at 18–25°C, as described in Figure 1. Relative transcript levels of IL1B were determined by real-time, duplex RT-PCR, using 18S rRNA as an internal standard. The values for all samples are plotted, with means and standard deviations of all samples shown. The dashed lines indicate the ±3x total precision of the assay (1.93 $C_T$).

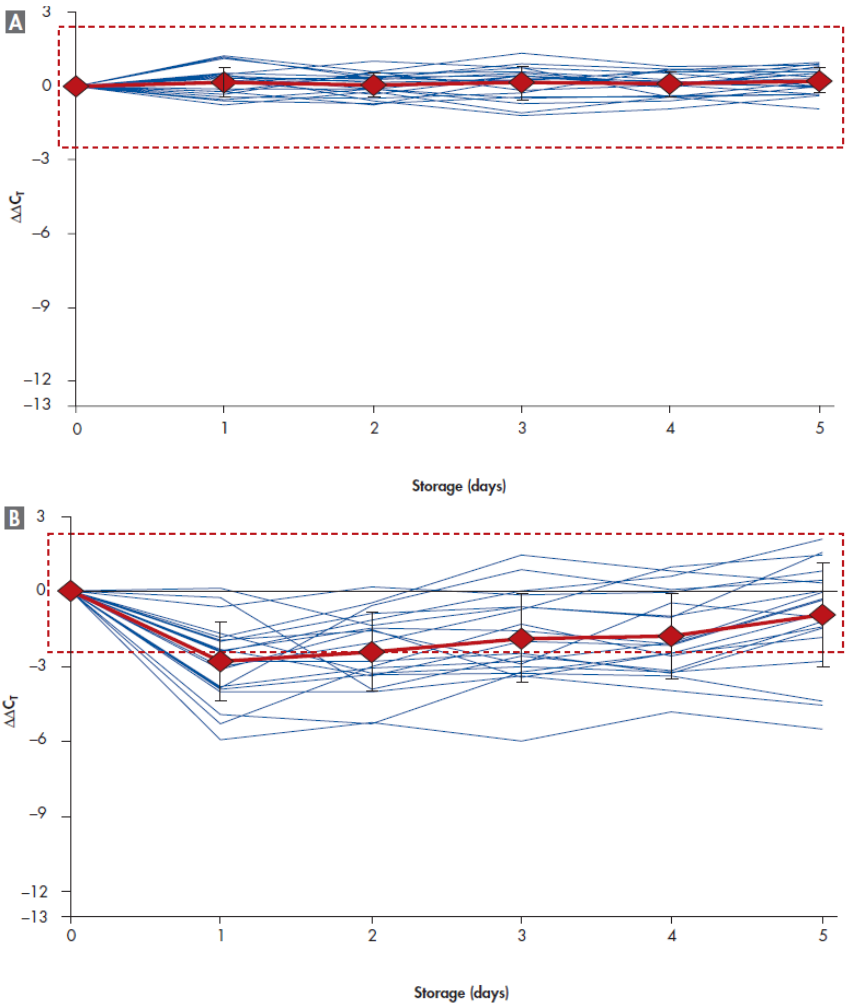



**Figure 3. RNA stability in blood samples at 2–8°C: FOS.** Blood was drawn from 10 donors, with duplicate samples and stored at 2–8°C for the indicated number of days, followed by total RNA purification. [**A**] Blood was collected and stored in PAXgene Blood RNA Tubes (BRT), and total RNA was purified using the PAXgene Blood RNA Kit. [**B**] Blood was collected and stored in standard blood collection tubes with EDTA as an anticoagulant, and total RNA was purified using a standard organic-extraction method with silica-membrane–based RNA cleanup. Relative transcript levels of FOS were determined by real-time, duplex RT-PCR, using 18S rRNA as an internal standard. The values for all samples are plotted, with means and standard deviations of all samples shown. The dashed lines indicate the ±3x total precision of the assay (2.34 $C_T$).

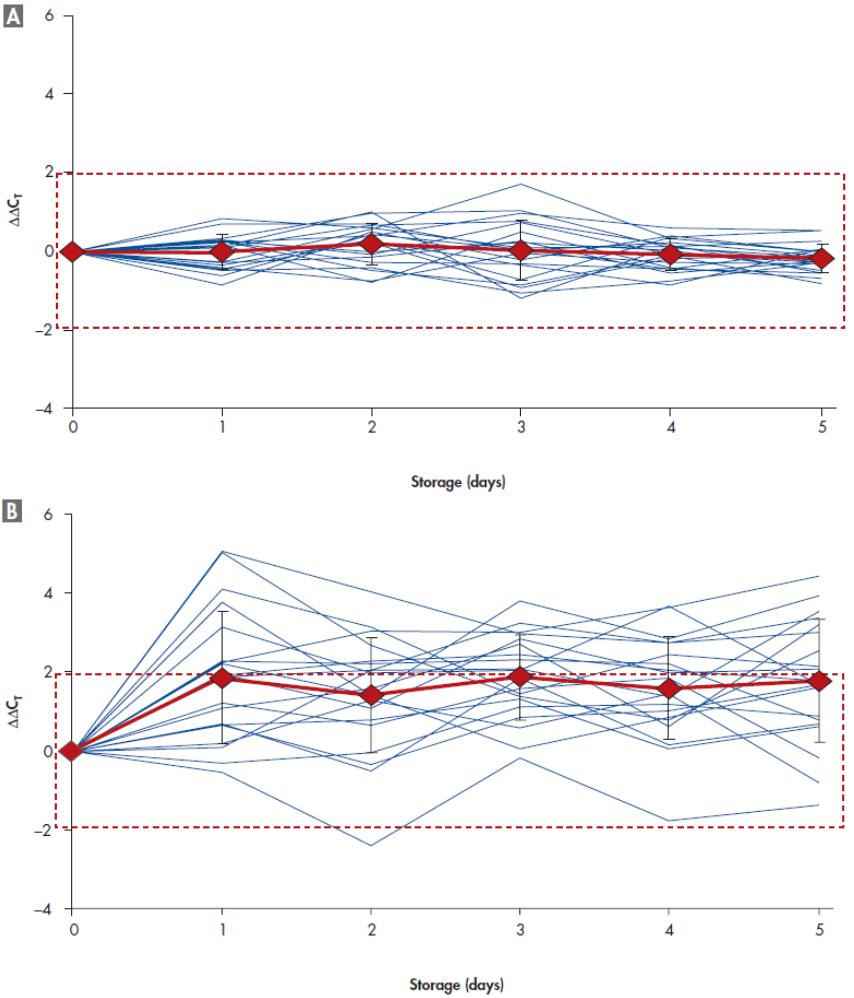



**Figure 4. RNA stability in blood samples at 2–8°C: IL1B.** Blood was drawn and total RNA purified, after storage at 2–8°C, as described in Figure 3. Relative transcript levels of IL1B were determined by real-time, duplex RT-PCR, using 18S rRNA as an internal standard. The values for all samples are plotted, with means and standard deviations of all samples shown. The dashed lines indicate the ±3x total precision of the assay (1.93 $C_T$).

## RNA concentration and purification

The PAXgene Blood RNA Kit is for the purification of total RNA from 2.5 ml human whole blood collected in a PAXgene Blood RNA Tube (BRT). The procedure is simple and can be performed using manual or automated procedures (see Figures 5 and 10, pages 20 and 30). In both protocols, purification begins with a centrifugation step to pellet nucleic acids in the PAXgene Blood RNA Tube (BRT). The pellet is washed and resuspended, followed by manual or automated RNA purification. In principle, both protocols follow the same protocol steps with the same kit components.

## Manual RNA purification

In detail, the resuspended pellet is incubated in optimized buffers together with proteinase K (PK) to bring about protein digestion. An additional centrifugation through the PAXgene Shredder spin column (PSC) is carried out to homogenize the cell lysate and remove residual cell debris, and the supernatant of the flow-through fraction is transferred to a fresh microcentrifuge tube. Ethanol is added to adjust binding conditions, and the lysate is applied to a PAXgene RNA spin column (PRC). During a brief centrifugation, RNA is selectively bound to the PAXgene silica membrane as contaminants pass through. Remaining contaminants are removed in several efficient wash steps. Between the first and second wash steps, the membrane is treated with DNase I (RNFD) to remove trace amounts of bound DNA. After the wash steps, RNA is eluted in elution buffer (BR5) and heat-denatured.

Total RNA isolated using the PAXgene Blood RNA System is pure. Using the manual protocol, $A_{260}/A_{280}$ values are between 1.8 and 2.2, and ≤1% (w/w) genomic DNA is present in ≥95% of all samples, as measured by quantitative, real-time PCR of a sequence of the beta-actin gene. At least 95% of samples show no inhibition in RT-PCR, when using up to 30% of the eluate.

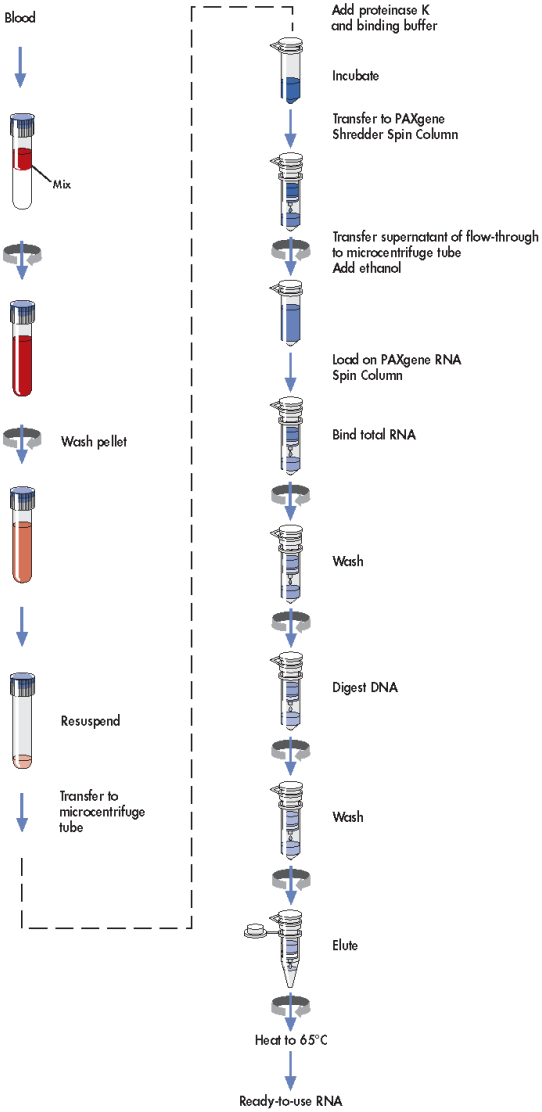



**Figure 5. The manual PAXgene Blood RNA procedure.**

Using the manual protocol, average sample preparation time (based on data from 12 sample preparation runs) is approximately 90 minutes*, with only 40 minutes of hands-on time. RNA yields from 2.5 ml healthy human whole blood are ≥3 µg for ≥95% of the samples processed. Since yields are highly donor-dependent, individual yields may vary. For individual donors, the PAXgene Blood RNA system provides highly reproducible and repeatable yields (Figures 6 and 7, pages 22 and 23) and reproducible and repeatable RT-PCR (Figures 8 and 9, pages 27 and 28), making it highly robust for clinical diagnostic tests.

Figure 6 (page 22) indicates the overall repeatability and reproducibility of the PAXgene Blood RNA System. Additional studies were conducted to show the influence of different PAXgene Blood RNA kit lots and different operators on the reproducibility of RNA yield and real time RT-PCR performance. As pooled blood samples instead of individual PAXgene Blood RNA Tubes (BRT) were used for these studies, the results do not reflect the system repeatability, including fluctuation between individual blood draws, but only the repeatability of the sample preparation (see Figure 7, page 23).

* Total protocol runtime, including upfront handling of PAXgene Blood RNA Tubes (centrifugations, pellet wash and pellet resuspension).

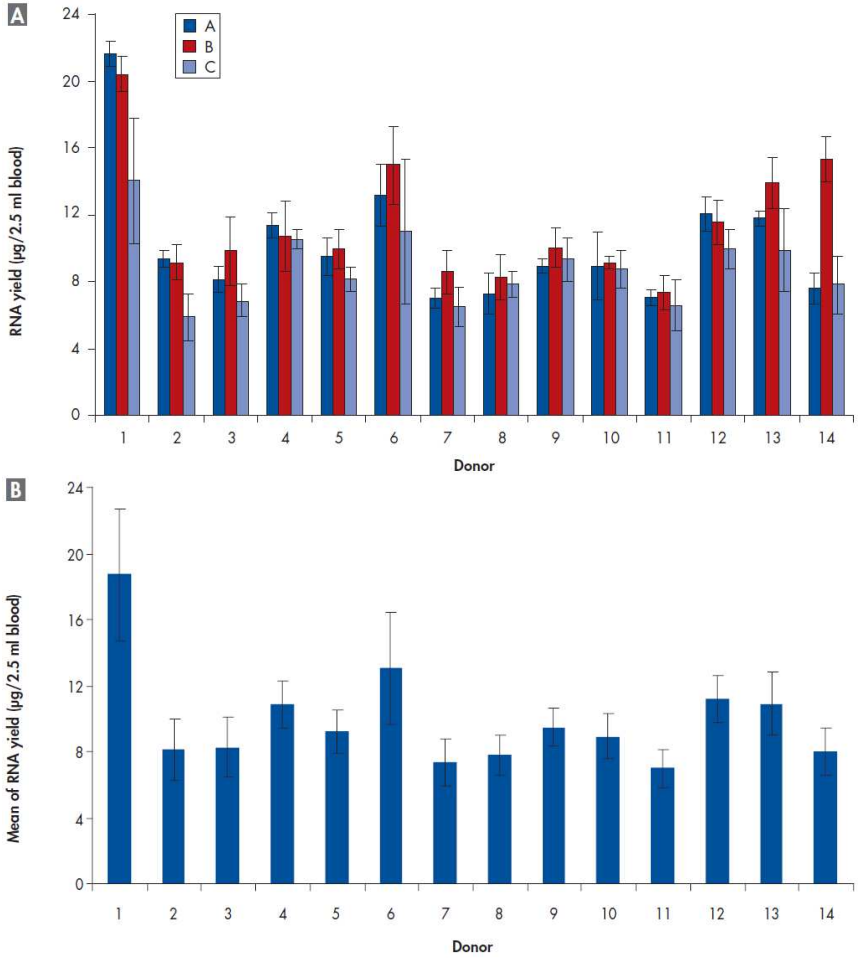



**Figure 6. Reproducible and repeatable RNA purification.** Quadruplicate blood samples from 14 donors were manually processed by each of 3 technicians (A, B, C). Three sets of equipment were used, and all samples prepared by a single technician were processed using the same equipment. [**A**] Means and standard deviations of RNA yield per replicate samples from the same donors and different technicians are shown. [**B**] Twelve replicate blood samples from each of 14 donors were processed by the 3 different technicians. Means and standard deviations of RNA yield per samples from the same donors and all technicians are presented. For all RNA samples, $A_{260}/A_{280}$ ratios ranged from 1.8 to 2.2.

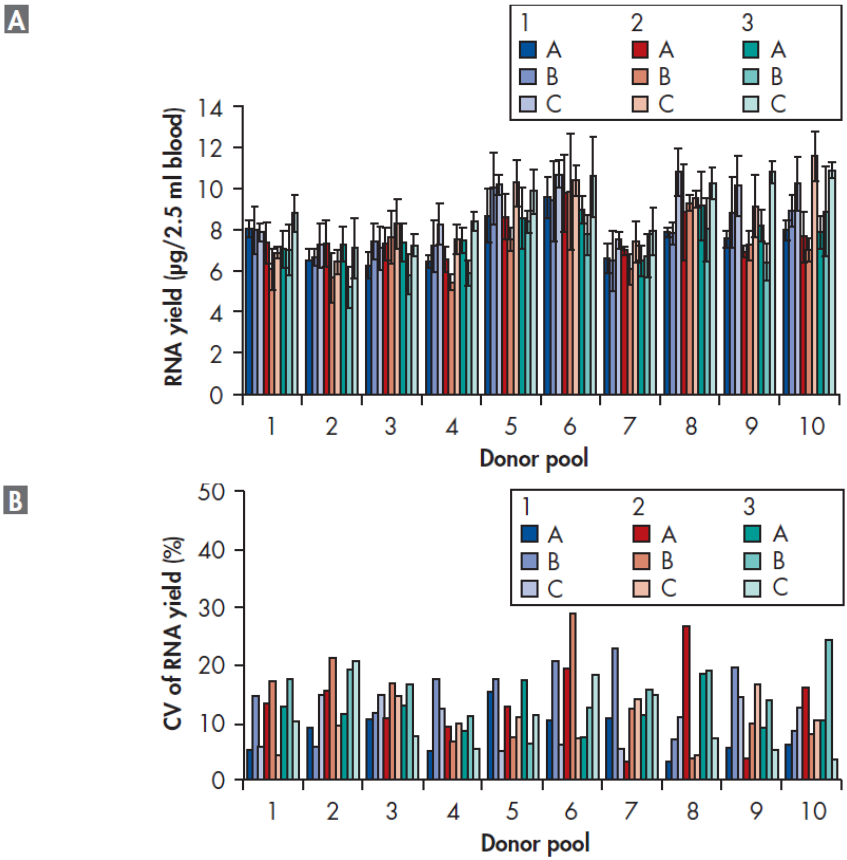



**Figure 7. Repeatability and reproducibility of RNA yield for different operators and PAXgene Blood RNA Kit lots using pooled blood samples.** Blood samples from 30 different donors were collected in PAXgene Blood RNA Tubes (BRT; 12 tubes per donor, 360 tubes in total). The contents of the tubes from 3 donors were pooled and subsequently realiquoted into 36 samples. These 36 samples per 3-donor-pool were manually processed by 3 different operators. Each operator used 3 different PAXgene Blood RNA Kit lots for the extraction and processed quadruplicate samples from each of the 10 donor pools. [**A**] RNA yield and standard deviation for every operator–lot combination. Quadruplicate blood samples from 10 donor pools were processed by 3 different operators (A, B, C) with each of 3 kit lots (1, 2, 3). The mean yields (columns) and standard deviations (error bars) per quadruplicate sample from the same donor pool for different operator and different kit lot are presented. [**B**] CV of RNA yield per donor pool for all operator–lot combinations (A, B, C; 1, 2, 3) as calculated from the mean yield and standard deviation of the yield shown in Figure 7A.

**Table 1A. Reproducibility within each lot and within each user for selected donor pools (1, 6, 9, 10)**

| | Donor pool 1<br>5.1 x $10^6$ cells/ml | | | Donor pool 6<br>6.5 x $10^6$ cells/ml | | |
|---|---|---|---|---|---|---|
| **Combination of data** | **Mean yield (µg)** | **SD (µg)** | **CV (%)** | **Mean yield (µg)** | **SD (µg)** | **CV (%)** |
| Lot 1, user A | 8.03 | 0.42 | 5 | 9.55 | 0.99 | 10 |
| Lot 1, user B | 7.98 | 1.17 | 15 | 9.38 | 1.94 | 21 |
| Lot 1, user C | 7.87 | 0.45 | 6 | 10.71 | 0.65 | 6 |
| Lot 2, user A | 7.32 | 0.98 | 13 | 9.78 | 1.89 | 19 |
| Lot 2, user B | 6.09 | 1.04 | 17 | 9.82 | 2.83 | 29 |
| Lot 2, user C | 6.87 | 0.31 | 4 | 10.37 | 0.74 | 7 |
| Lot 3, user A | 7.04 | 0.90 | 13 | 8.96 | 0.68 | 8 |
| Lot 3, user B | 6.98 | 1.22 | 17 | 7.73 | 0.97 | 13 |
| Lot 3, user C | 8.78 | 0.89 | 10 | 10.59 | 1.94 | 18 |
| | **Donor pool 9**<br>**8.4 x $10^6$ cells/ml** | | | **Donor pool 10**<br>**10.2 x $10^6$ cells/ml** | | |
| **Combination of data** | **Mean yield (µg)** | **SD (µg)** | **CV (%)** | **Mean yield (µg)** | **SD (µg)** | **CV (%)** |
| Lot 1, user A | 7.52 | 0.41 | 6 | 7.96 | 0.49 | 6 |
| Lot 1, user B | 8.82 | 1.72 | 19 | 8.90 | 0.76 | 9 |
| Lot 1, user C | 10.14 | 1.46 | 14 | 10.22 | 1.29 | 13 |
| Lot 2, user A | 6.92 | 0.27 | 4 | 7.63 | 1.23 | 16 |
| Lot 2, user B | 7.20 | 0.71 | 10 | 7.00 | 0.56 | 8 |
| Lot 2, user C | 9.14 | 1.52 | 17 | 11.56 | 1.21 | 10 |
| Lot 3, user A | 8.18 | 0.76 | 9 | 7.85 | 0.82 | 10 |
| Lot 3, user B | 6.41 | 0.88 | 14 | 8.88 | 2.17 | 24 |
| Lot 3, user C | 10.78 | 0.56 | 5 | 10.88 | 0.37 | 3 |

**Table 1B. Reproducibility within each user and between all lots for selected donor pools (1, 6, 9, 10)**

| | Donor pool 1 5.1 x $10^6$ cells/ml | | | Donor pool 6 6.5 x $10^6$ cells/ml | | |
|---|---|---|---|---|---|---|
| **Combination of data** | **Mean yield (µg)** | **SD (µg)** | **CV (%)** | **Mean yield (µg)** | **SD (µg)** | **CV (%)** |
| User A, all lots | 7.46 | 0.85 | 11 | 9.43 | 1.22 | 13 |
| User B, all lots | 7.02 | 1.31 | 19 | 8.98 | 2.09 | 23 |
| User C, all lots | 7.84 | 0.98 | 13 | 10.56 | 1.15 | 11 |
| | **Donor pool 9 8.4 x $10^6$ cells/ml** | | | **Donor pool 10 10.2 x $10^6$ cells/ml** | | |
| **Combination of data** | **Mean yield (µg)** | **SD (µg)** | **CV (%)** | **Mean yield (µg)** | **SD (µg)** | **CV (%)** |
| User A, all lots | 7.54 | 0.72 | 10 | 7.81 | 0.82 | 11 |
| User B, all lots | 7.48 | 1.50 | 20 | 8.26 | 1.54 | 19 |
| User C, all lots | 10.02 | 1.34 | 13 | 10.89 | 1.10 | 10 |

**Table 1C. Reproducibility within each lot and between all users for selected donor pools (1, 6, 9, 10)**

| | Donor pool 1 5.1 x $10^6$ cells/ml | | | Donor pool 6 6.5 x $10^6$ cells/ml | | |
|---|---|---|---|---|---|---|
| **Combination of data** | **Mean yield (µg)** | **SD (µg)** | **CV (%)** | **Mean yield (µg)** | **SD (µg)** | **CV (%)** |
| Lot 1, all users | 7.96 | 0.69 | 9 | 9.88 | 1.34 | 14 |
| Lot 2, all users | 6.76 | 0.93 | 14 | 9.99 | 1.84 | 18 |
| Lot 3, all users | 7.60 | 1.27 | 17 | 9.09 | 1.71 | 19 |
| | **Donor pool 9 8.4 x $10^6$ cells/ml** | | | **Donor pool 10 10.2 x $10^6$ cells/ml** | | |
| **Combination of data** | **Mean yield (µg)** | **SD (µg)** | **CV (%)** | **Mean yield (µg)** | **SD (µg)** | **CV (%)** |
| Lot 1, all users | 8.83 | 1.63 | 19 | 9.02 | 1.27 | 14 |
| Lot 2, all users | 7.75 | 1.36 | 18 | 8.73 | 2.31 | 26 |
| Lot 3, all users | 8.46 | 1.99 | 24 | 9.20 | 1.80 | 20 |

**Table 1D. Reproducibility between all lots and all users for selected donor pools (1, 6, 9, 10)**

| | Donor pool 1<br>5.1 x $10^6$ cells/ml | | | Donor pool 6<br>6.5 x $10^6$ cells/ml | | |
|---|---|---|---|---|---|---|
| **Combination of data** | **Mean yield (µg)** | **SD (µg)** | **CV (%)** | **Mean yield (µg)** | **SD (µg)** | **CV (%)** |
| Lot 1, all users | 7.44 | 1.09 | 15 | 9.66 | 1.65 | 17 |

| | Donor pool 9<br>8.4 x $10^6$ cells/ml | | | Donor pool 10<br>10.2 x $10^6$ cells/ml | | |
|---|---|---|---|---|---|---|
| **Combination of data** | **Mean yield (µg)** | **SD (µg)** | **CV (%)** | **Mean yield (µg)** | **SD (µg)** | **CV (%)** |
| Lot 1, all users | 8.35 | 1.70 | 20 | 8.99 | 1.80 | 20 |

Detailed analysis of 4 representative donor pools. The pools were selected according to the white blood cell count and reflect the upper, medium, and the lower values of the normal range of white blood cell counts (4.8 x $10^6$ – 1.1 x $10^7$ leukocytes/ml). The white blood cell count represents the mean value of the 3 white blood cell counts from the 3 donors per donor pool.

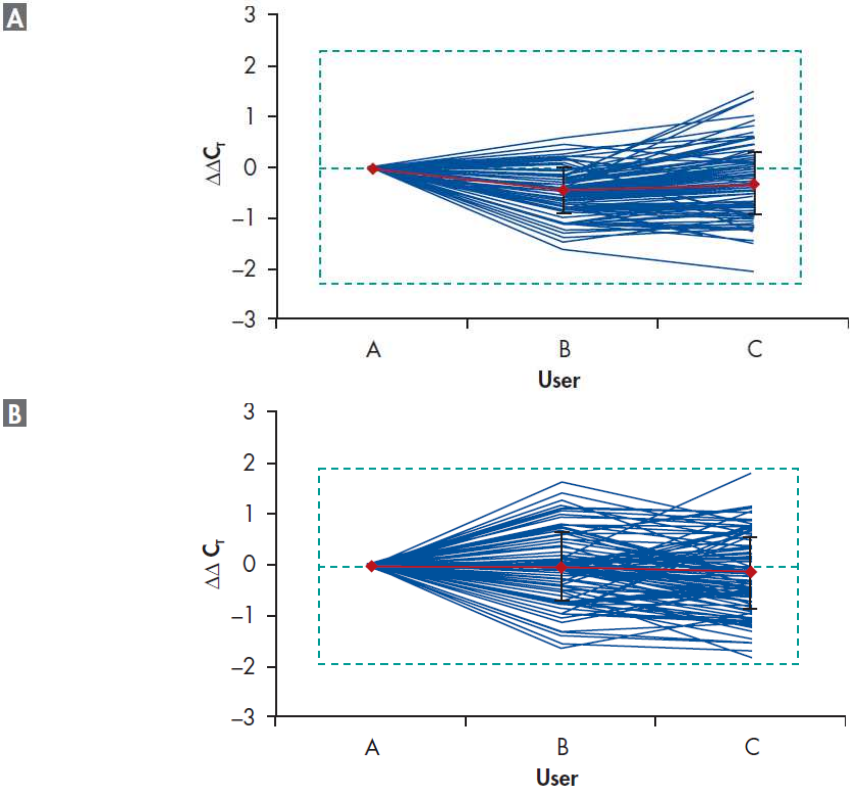



**Figure 8. Reproducibility of RT-PCR — between users.** RNA purified in the experiment described in Figure 7 was used for real-time RT-PCR. Relative transcript levels of [**A**] FOS and [**B**] IL1B were determined by real-time, duplex RT-PCR using 18S rRNA as an internal standard. The values for all samples are plotted, relative to the values for user A (10 donor pools x 3 kit lots x 4 replicates = 120 data sets for each gene), with means (red lines) and standard deviations (black bars) for all samples shown. The dashed lines indicate the ±3x total precision of the assays (FOS: 2.34 $C_T$; IL1B: 1.93 $C_T$).

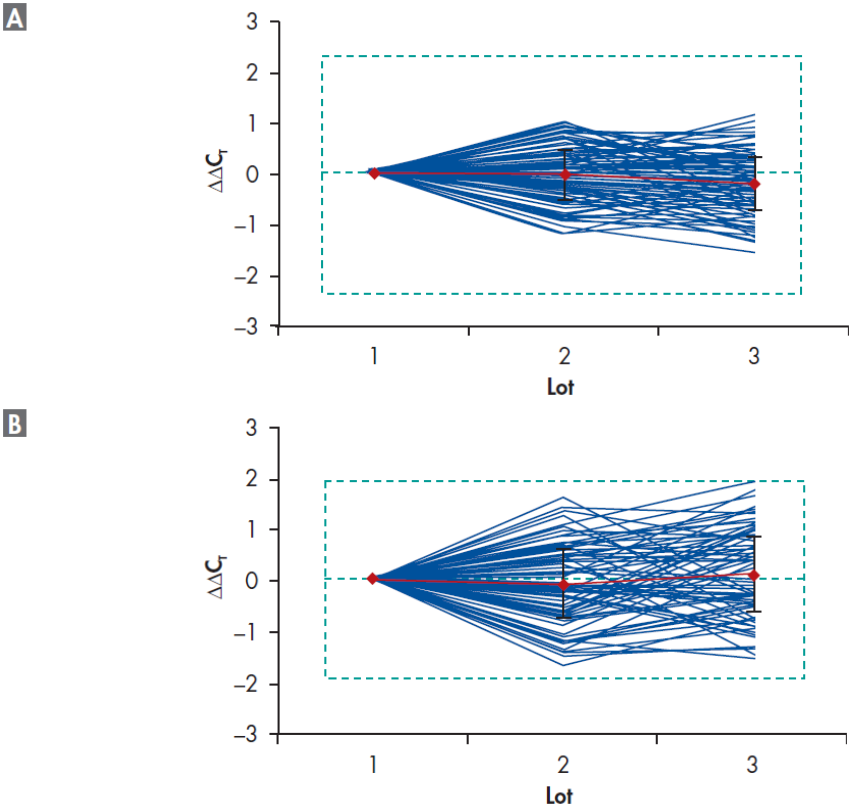



**Figure 9. Reproducibility of RT-PCR — between kit lots.** RNA purified in the experiment described in Figure 7 was used for real-time RT-PCR. Relative transcript levels of [**A**] FOS and [**B**] IL1B were determined by real-time, duplex RT-PCR using 18S rRNA as an internal standard. The values for all samples are plotted, relative to the values for kit lot 1 (10 donor pools x 3 users x 4 replicates = 120 data sets for each gene), with means (red lines) and standard deviations (black bars) for all samples shown. The dashed lines indicate the ±3x total precision of the assays (FOS: 2.34 $C_T$; IL1B: 1.93 $C_T$).

**Table 2. Summary of RT-PCR data from Figures 8 and 9**

| Test system | FOS/18S rRNA assay | | IL1B/18S rRNA assay | |
|---|---|---|---|---|
| Comparison of data | Mean ($\Delta\Delta C_T$) | ± SD ($\Delta\Delta C_T$) | Mean ($\Delta\Delta C_T$) | ± SD ($\Delta\Delta C_T$) |
| **Reproducibility within each user and between all lots** | | | | |
| All users, lot 1–lot 1 | 0.00 | 0.00 | 0.00 | 0.00 |
| All users, lot 1–lot 2 | –0.03 | 0.48 | –0.07 | 0.66 |
| All users, lot 1–lot 3 | –0.21 | 0.52 | 0.11 | 0.71 |
| **Reproducibility within each user and between all lots** | | | | |
| All lots, user A–user A | 0.00 | 0.00 | 0.00 | 0.00 |
| All lots, user A–user B | –0.46 | 0.44 | –0.06 | 0.69 |
| All lots, user A–user C | –0.31 | 0.60 | –0.15 | 0.71 |

User: Technician, performed the study.
Lot: Number of kit lot used in this study.
SD: Standard deviation.
Mean $\Delta\Delta C_T$ values (N = 120) and standard deviations are shown for the data presented in Figures 8 and 9.

## Automated RNA purification

Purification of blood RNA is automated on the QIAGEN QIAcube Connect MDx or the classic QIAGEN QIAcube (hereafter called QIAcube). The innovative QIAcube instruments use advanced technology to process QIAGEN spin columns, enabling seamless integration of automated, low-throughput sample prep into your laboratory workflow. Sample preparation using QIAcube instruments follows the same steps as the manual procedure (i.e., lyse, bind, wash and elute), enabling you to continue using the PAXgene Blood RNA Kit for purification of high-quality RNA.

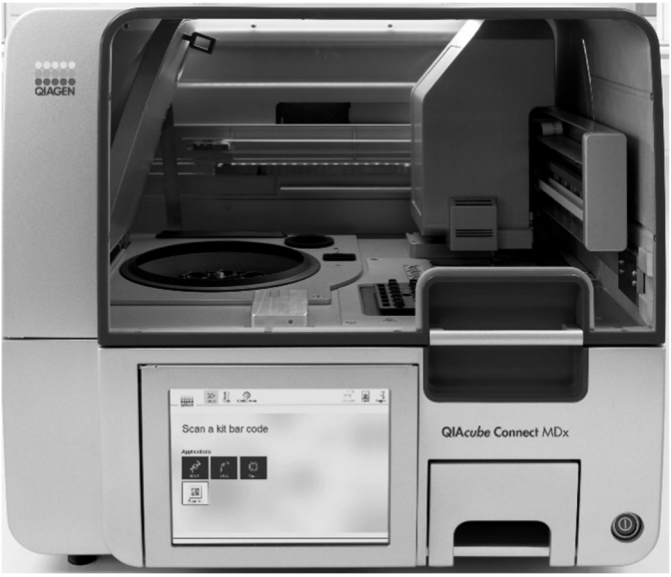



**Figure 10. QIAcube Connect MDx.**

The automated RNA purification protocol consists of 2 parts (or protocols), "PAXgene Blood RNA Part A" and "PAXgene Blood RNA Part B", with a brief manual intervention between the 2 parts (see Figure 11, page 31).

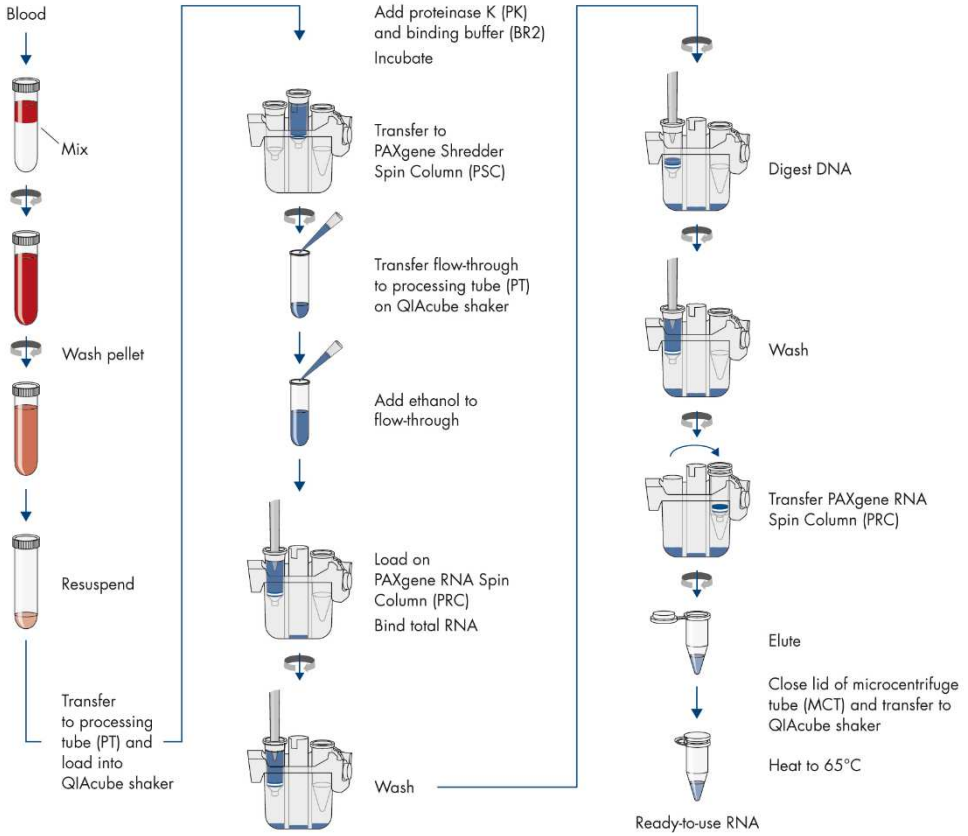



**Figure 11. The automated PAXgene Blood RNA procedure.**

The centrifuged, washed and resuspended nucleic acid pellet (see "RNA concentration and purification", page 19) is transferred from the PAXgene Blood RNA Tube (BRT) into processing tubes (PT), which are placed into the thermoshaker unit on the QIAcube instruments' worktable. The operator selects and starts the "PAXgene Blood RNA Part A" protocol from the menu. The QIAcube instruments perform the steps of the protocol through to elution of RNA in elution

buffer (BR5). The operator transfers the microcentrifuge tubes (MCT) containing the purified RNA into the thermoshaker unit of the QIAcube instruments. The operator selects and starts the "PAXgene Blood RNA Part B" protocol from the menu and heat denaturation is performed by the QIAcube instruments.

Average sample preparation time (based on data from 12 sample preparation runs) is 151 minutes*, with significantly less hands-on time compared to the manual protocol.

RNA yields from 2.5 ml healthy human whole blood are ≥3 µg for ≥95% of the samples processed. Figure 12 (page 33) indicates the RNA yields from a total of 216 samples prepared using the automated protocol with 3 kit lots by 3 operators. As pooled blood samples instead of individual PAXgene Blood RNA Tubes (BRT) were used for these studies, the results do not reflect the RNA yield expected from single samples of individual blood draws. Since yields are highly donor-dependent, individual yields may vary (Figure 12, page 33).

At least 95% of samples show no inhibition in RT-PCR, when using up to 30% of the eluate. Using the automated protocol, cross contamination between samples is undetectable, as measured by quantitative, real-time RT-PCR of sequences of the ABL1 and FOS transcripts in RNA-negative samples (water) paired with RNA-positive samples (human whole blood) in the same run.

RNA isolated with the PAXgene Blood RNA System and the automated protocol is pure, as shown by lack of RT-PCR inhibition and $A_{260}/A_{280}$ values between 1.8 and 2.2. Genomic DNA is present at ≤1% (w/w) in ≥95% of all samples, as measured by quantitative, real-time PCR of a sequence of the beta-actin gene. Figures 13 and 14 (pages 34 and 35) show the $A_{260}/A_{280}$ values and relative genomic DNA of a total of 216 samples prepared using the automated protocol with 3 kit lots by 3 operators.

* Total protocol runtime, including upfront handling of PAXgene Blood RNA Tubes (centrifugations, pellet wash and pellet resuspension).

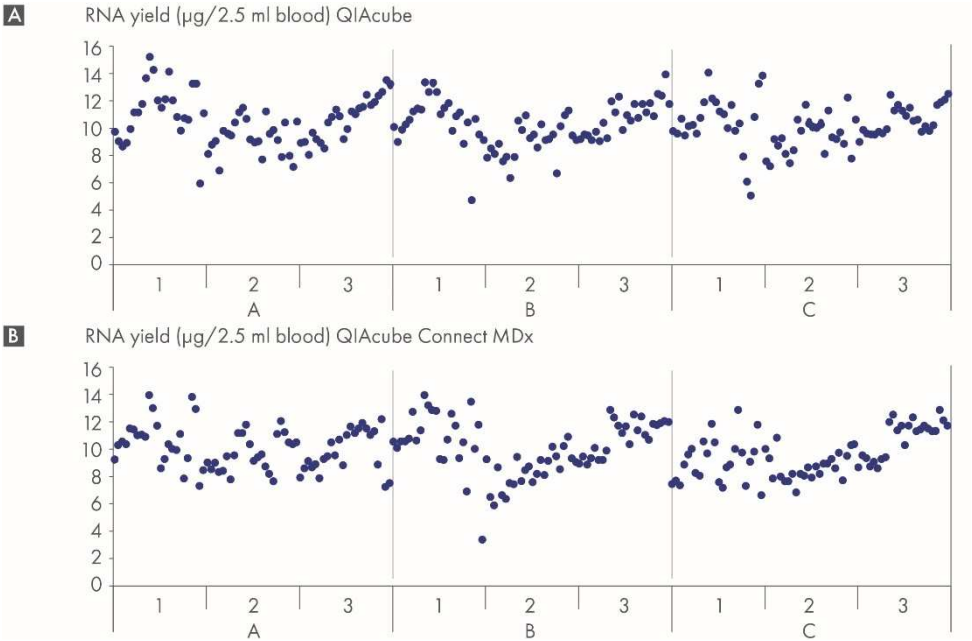



**Figure 12. RNA yield — automated processing A: QIAcube, B: QIAcube Connect MDx**. Blood samples from individual donors were collected in PAXgene Blood RNA Tubes (BRT). The contents of the tubes were pooled into 6 donor pools and subsequently realiquoted. A total of 216 tubes (i.e. 36 per pool) were processed by 3 different operators (A, B, C). Each operator used 3 different lots (1, 2, 3) of the PAXgene Blood RNA Kit for automated extraction with multiple QIAcube and QIAcube Connect MDx instruments and processed quadruplicate samples from each of the 6 donor pools. RNA yields of all individual samples are shown for every operator–lot combination.

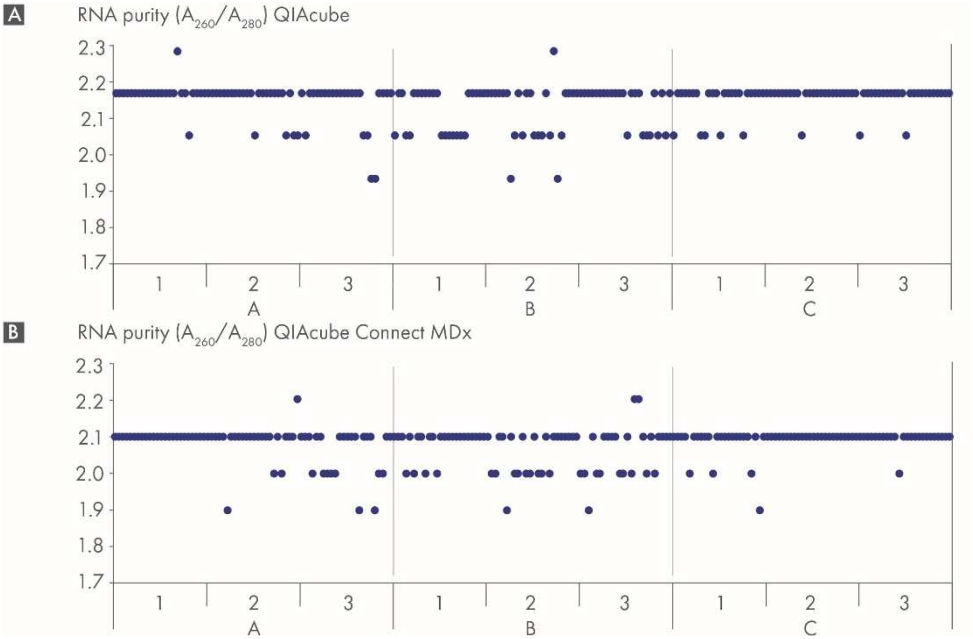



**Figure 13. RNA purity ($A_{260}/A_{280}$ values) — automated processing. A: QIAcube, B: QIAcube Connect MDx** RNA was purified by 3 different operators (A, B, C) using 3 different lots (1, 2, 3) of the PAXgene Blood RNA Kit with multiple QIAcube and QIAcube Connect MDx instruments in the experiment described in Figure 12. $A_{260}/A_{280}$ values of all individual samples are shown for every operator–lot combination.

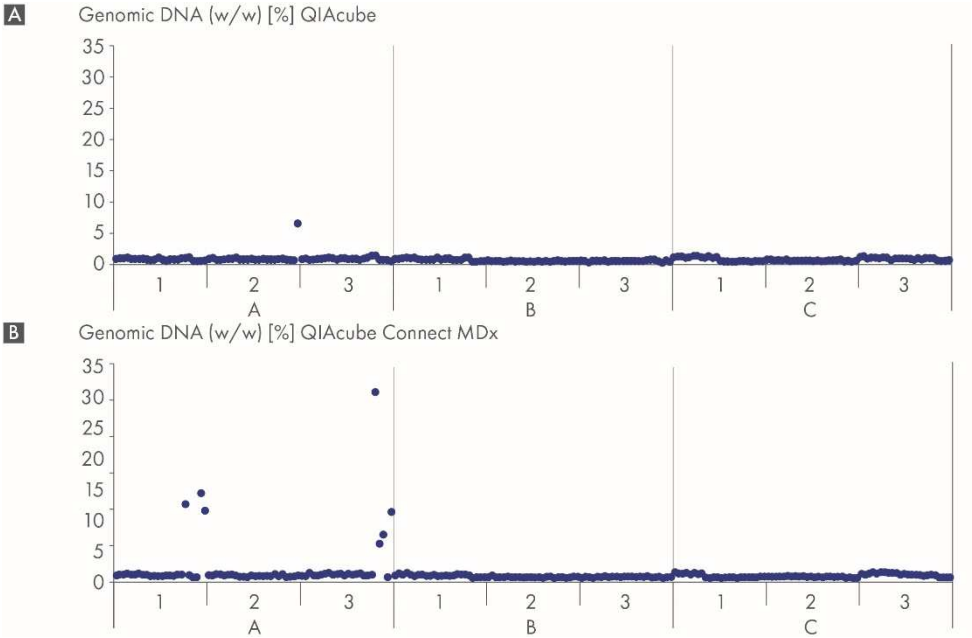



**Figure 14. RNA purity (% genomic DNA contamination) — automated processing, A: QIAcube, B: QIAcube Connect MDx.** RNA was purified by 3 different operators (A, B, C) using 3 different lots (1, 2, 3) of the PAXgene Blood RNA Kit with multiple QIAcube and QIAcube Connect MDx instruments in the experiment described in Figure 12. Genomic DNA amounts (w/w) in all individual samples are shown for every operator–lot combination.

The automated protocol of RNA purification using the PAXgene Blood RNA System provides highly reproducible and repeatable RT-PCR results, as shown in Figure 15 and Figure 16 (page 36 and 37), making it highly robust for clinical diagnostic tests.

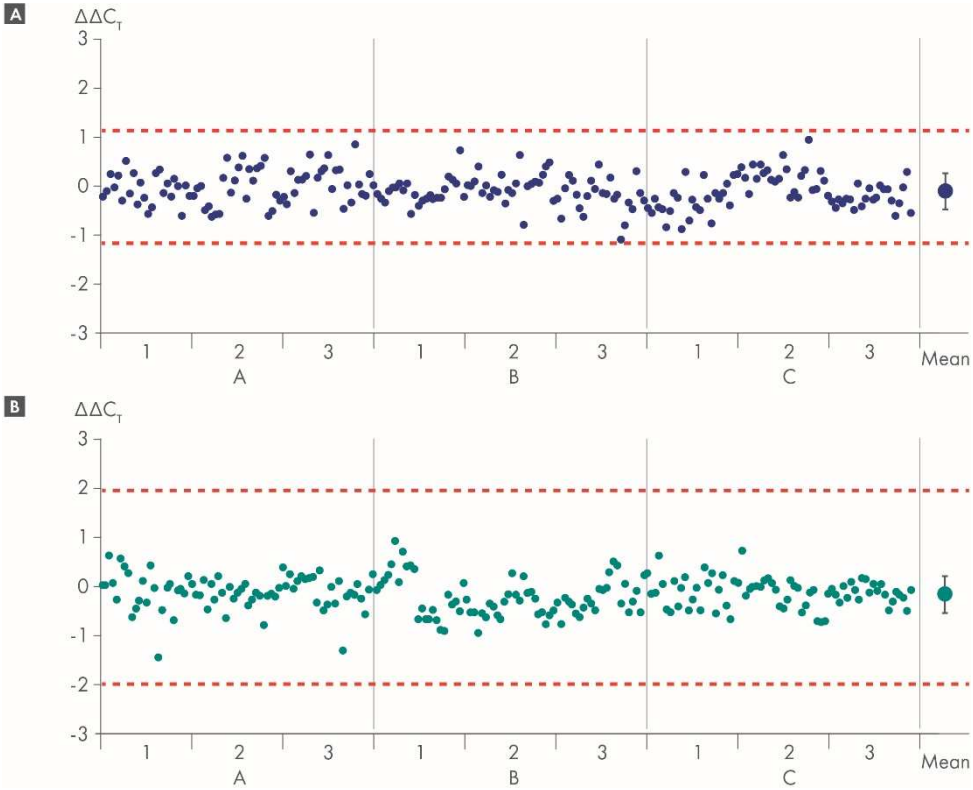



**Figure 15. Reproducibility of RT-PCR — between automated (QIAcube) and manual protocols.** RNA was purified by 3 different operators (A, B, C) using 3 different lots (1, 2, 3) of the PAXgene Blood RNA Kit with multiple QIAcube and QIAcube Connect MDx instruments using the automated protocol in the experiment described in Figure 12. In parallel, RNA was purified from the corresponding replicate tubes using the manual protocol. Relative transcript levels of [**A**] FOS and [**B**] IL1B were determined by real-time, duplex RT-PCR using 18S rRNA as an internal standard. Possible differences of transcript levels between RNA prepared from paired blood samples using both extraction protocols (automated and manual protocol) were calculated by the $\Delta\Delta C_T$ method. Individual $\Delta\Delta C_T$ values for all sample pairs (4 replicates x 6 donor pools x 3 kit lots x 3 operators = 216 pairs for each gene) are plotted as single dots with means (larger dots) and standard deviations (black bars) for all samples shown. The dashed lines indicate the ±3x total precision of the assays (FOS: 1.16 $C_T$; IL1B: 1.98 $C_T$; different assay precisions as compared to Figures 1–4, 8, and 9 due to different assay versions).

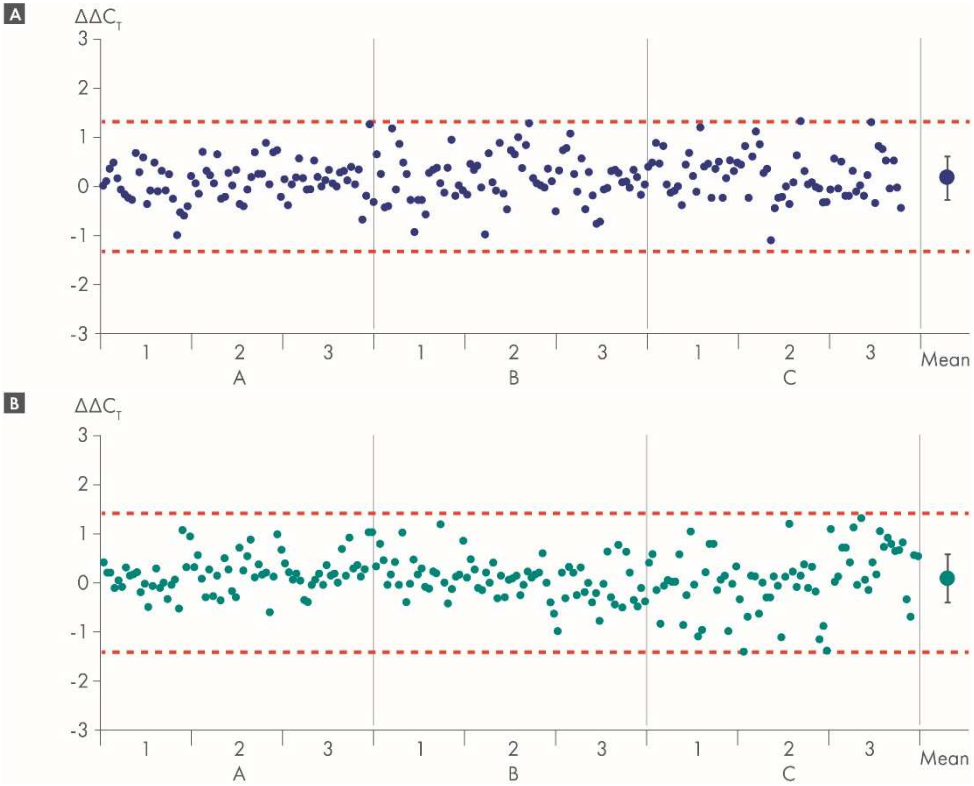



**Figure 16. Reproducibility of RT-PCR — between QIAcube and QIAcube Connect MDx using the automated protocol.** RNA was purified by 3 different operators (A, B, C) using 3 different lots (1, 2, 3) of the PAXgene Blood RNA Kit using the automated protocol on multiple QIAcube and QIAcube Connect MDx instruments in the experiment described in Figure 12. Relative transcript levels of **[A]** FOS and **[B]** IL1B were determined by real-time, duplex RT-PCR using 18S rRNA as an internal standard. Possible differences of transcript levels between RNA prepared from paired blood samples using both instruments were calculated by the $\Delta\Delta C_T$ method. Individual $\Delta\Delta C_T$ values for all sample pairs (4 replicates x 6 donor pools x 3 kit lots x 3 operators = 216 pairs for each gene) are plotted as single dots with means (larger dots) and standard deviations (black bars) for all samples shown. The dashed lines indicate the ±3x total precision of the assays (FOS: 1.30 $C_T$; IL1B: 1.42 $C_T$; different assay precisions as compared to Figures 1–4, 8, 9, and 15 due to different assay versions).

# Equipment and Reagents to Be Supplied by User

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

**For all protocols**

- PAXgene Blood RNA Tubes (BRT, PreAnalytiX; cat. no. 762165)
- Ethanol (96–100%, purity grade p.a.)
- Pipets* (10 µl – 4 ml)
- Sterile, aerosol-barrier, RNase-free pipet tips†
- Graduated cylinder‡
- Centrifuge* capable of attaining 3000–5000 x *g*, and equipped with a swing- out rotor and buckets to hold PAXgene Blood RNA Tubes (BRT)
- Vortex mixer*
- Crushed ice
- Permanent pen for labeling

**For the manual protocol**

- Variable-speed microcentrifuge* capable of attaining a range of at least 1000–8000 x *g*, though lower and higher g-forces are applicable (see protocol steps for details), and equipped with a rotor for 2 ml microcentrifuge tubes

* Make sure that the devices and instruments have been checked, maintained and calibrated regularly according to the manufacturer's recommendations.

† Make sure that you are familiar with the guidelines on handling RNA (Appendix A, page 71).

‡ For the addition of ethanol to Buffer BR4 concentrate.

- Shaker–incubator* capable of incubating at 55°C and 65°C and shaking at ≥400 rpm, not exceeding 1400 rpm (e.g., Eppendorf® Thermomixer Compact, or equivalent)

**For the automated protocol (using QIAcube or QIAcube Connect MDx)**

- Scissors

QIAcube instruments consumables:

- Filter-Tips, 1000 µl (1024) (QIAGEN, cat. no. 990352)†
- Reagent Bottles, 30 ml (6) (QIAGEN, cat. no. 990393)†
- Rotor Adapters (10 x 24) (QIAGEN, cat. no. 990394)†

QIAcube instruments accessories:

- Rotor Adapter Holder (QIAGEN, cat. no. 990392)†

**For the automated protocol using QIAcube Connect MDx**

- QIAcube Connect MDx* (QIAGEN, cat. no. 9003070)

QIAcube Connect MDx service bundles:

- QIAcube Connect MDx System FUL-2 (QIAGEN, cat. no. 9003071)
- QIAcube Connect MDx System FUL-3 (QIAGEN, cat. no. 9003072)
- QIAcube Connect MDx System PRV-1 (QIAGEN, cat. no. 9003073)
- QIAcube Connect MDx Device PRV-1 (QIAGEN, cat. no. 9003074)
- QIAcube Connect MDx System PRM-1 (QIAGEN, cat. no. 9003075)

* Make sure that the devices and instruments have been checked, maintained and calibrated regularly according to the manufacturer's recommendations.

† Also included in the Starter Pack, QIAcube (QIAGEN, cat. no. 990395).

**For the automated protocol using QIAcube**

- QIAcube* (QIAGEN, cat. no. 9001882 [110 V])

* Make sure that devices and instruments have been checked, maintained and calibrated regularly according to the manufacturer's recommendations.

# Important Notes

## Using QIAcube instruments

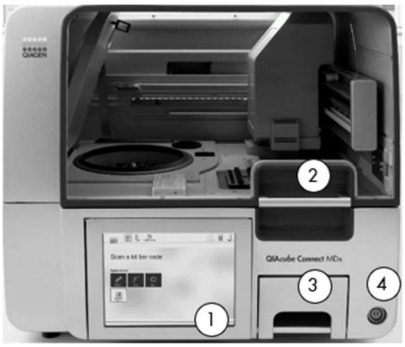
Ensure that you are familiar with operating the QIAcube instrument. Please read the appropriate QIAcube instrument User Manual and any additional information supplied with the QIAcube instrument paying careful attention to the safety information, before beginning the automated PAXgene Blood RNA protocols.

Instructions in this section apply to the QIAcube Connect MDx as well as the QIAcube where not specified separately.

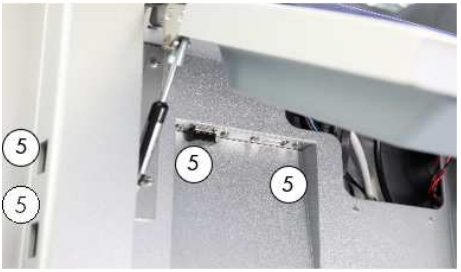
### Starting the QIAcube instruments

Close the QIAcube instrument hood, and switch on the QIAcube instrument with the power switch (QIAcube Connect MDx: see Figure 17, page 42; QIAcube: Figure 18, page 43).

A beeper sounds and the startup screen appears. The instrument automatically performs initialization tests.




**Front view of the QIAcube Connect MDx**




**Pulled-out touchscreen**


**Rear view of QIAcube Connect MDx**


**Rear view of QIAcube Connect MDx**

**Figure 17. External features of the QIAcube Connect MDx.**

1. Touchscreen
2. Hood
3. Waste drawer
4. Power switch
5. 2 USB ports on the left side of the touchscreen; 2 USB ports behind the touchscreen (Wi-Fi module plugged into 1 USB port)
6. RJ-45 Ethernet port
7. Power cord socket
8. Cooling air outlet

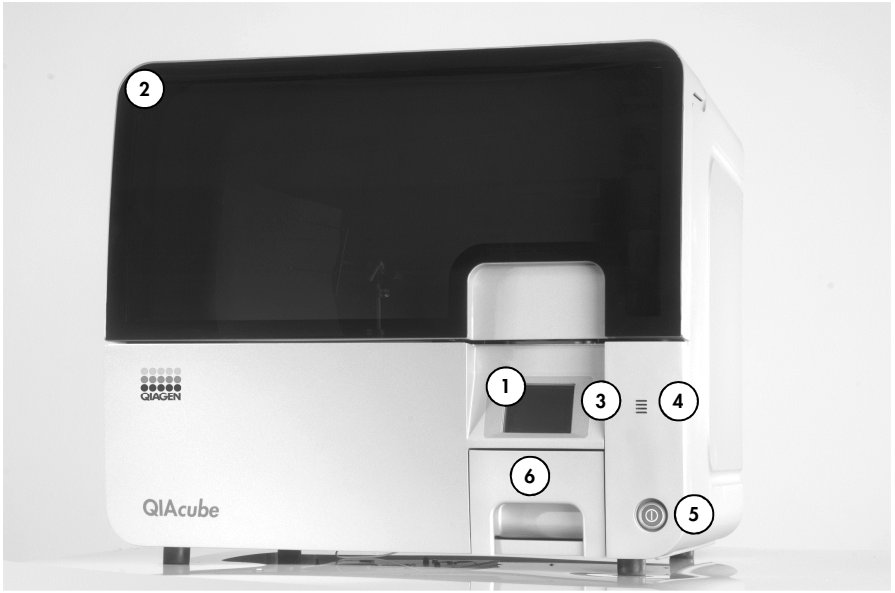



**Figure 18. Front view of the QIAcube.**

| | | | |
|---|---|---|---|
| 1 | Touchscreen | 4 | USB port behind protective panel |
| 2 | Hood | 5 | Power switch |
| 3 | RS232 serial port behind protective panel (for use by QIAGEN Instrument Service Specialists only) | 6 | Waste drawer |

### Touchscreen

QIAcube instruments are controlled using a touchscreen. The touchscreen allows the user to operate the instrument and guide the users through worktable setup. During sample processing, the touchscreen shows the protocol status and remaining time.

**Figure 19. Pulled-out touchscreen of QIAcube Connect MDx**

## Installing protocols on the QIAcube instruments

An initial protocol installation may be required before the first RNA preparation run on the QIAcube instruments can be performed. Install both "PAXgene Blood RNA Part A" and "PAXgene Blood RNA Part B" protocols.

Protocols for the QIAcube Connect MDx are provided at **www.qiagen.com/products/diagnostics-and-clinical-research/solutions-for-laboratory-developed-tests/qiacube-connect-mdx/#resources** (www.qiagen.com/MyQIAcube for QIAcube) and need to be downloaded to the USB stick supplied with the QIAcube instruments. These protocols will be transferred to the instrument via the USB port.

The USB port (QIAcube Connect MDx: located on the side of the touchscreen see Figure 17, page 42; QIAcube: behind the protective panel, see Figure 18, page 43) allows connection of the QIAcube instruments to the USB stick supplied with the QIAcube instruments. Data files,

such as log files or report files can also be transferred via the USB port from the QIAcube instruments to the USB stick.

The USB port is only for use with the USB stick provided by QIAGEN. Do not connect other devices to this port.

Do not remove the USB stick while downloading protocols or transferring data files or during a protocol run.

For further details on the process of uploading protocols to QIAcube instruments, please see the related handbook for the instrument used.

## Loading the QIAcube instruments

To save time, loading can be performed during one or both of the 10-minute centrifugation steps (steps 3 and 5) in "Protocol: Automated Purification of Total RNA from Human Whole Blood Collected into PAXgene Blood RNA Tubes (BRT)", page 62.

### Reagent bottles

Before every run on the QIAcube instrument, carefully fill the 4 reagent bottles with the reagents listed in Table 3 (page 46) up to the maximum indicator level or, if that is not possible, to the level allowed by the buffer volumes supplied in the PAXgene Blood RNA Kit. Label the bottles and lids clearly with buffer names and place the filled reagent bottles into the appropriate positions on the reagent bottle rack. Load the rack onto the QIAcube instrument worktable as shown (Figures 20 - 22, pages 46 - 48).

The supplied volume of Buffer BR2 will not fill a reagent bottle to the indicator level. Buffers BR3 and BR4 may not fill the bottle to the indicator level after processing multiple samples in previous runs.

Be sure to remove lids from the bottles before placing onto the worktable.

Buffer volumes provided in the PAXgene Blood RNA Kit (50) are sufficient for a maximum of 7 RNA preparation runs on the QIAcube instrument with sample numbers of 2 to 12 per run. In general, runs with lower sample numbers should be avoided in order to process a total of 50 samples per kit with a maximum of 7 RNA preparation runs. More than 7 RNA preparation runs can lead to insufficient buffer volumes for processing the last samples.

**Table 3. Positions in the reagent bottle rack**

| Position | Reagent |
|---|---|
| 1 | Binding buffer (BR2) |
| 2 | 96-100% ethanol |
| 3 | Wash buffer 1 (BR3) |
| 4 | Wash buffer 2 (BR4)* |
| 5 | – (leave empty) |
| 6 | – (leave empty) |

* Wash buffer 2 (BR4) is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%, purity grade p.a.) as indicated on the bottle to obtain a working solution.

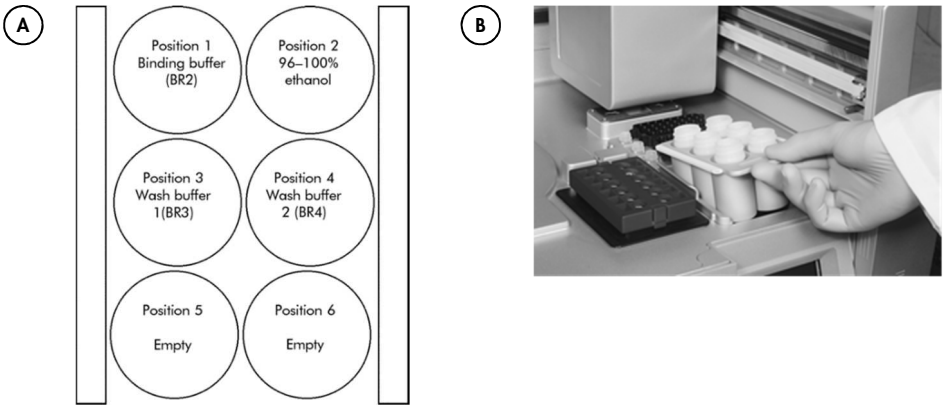



**Figure 20. Loading the reagent bottle rack.** [**A**] Schematic of positions and contents of bottles in the reagent bottle rack. [**B**] Loading the rack onto the QIAcube instrument (QIAcube shown as example).

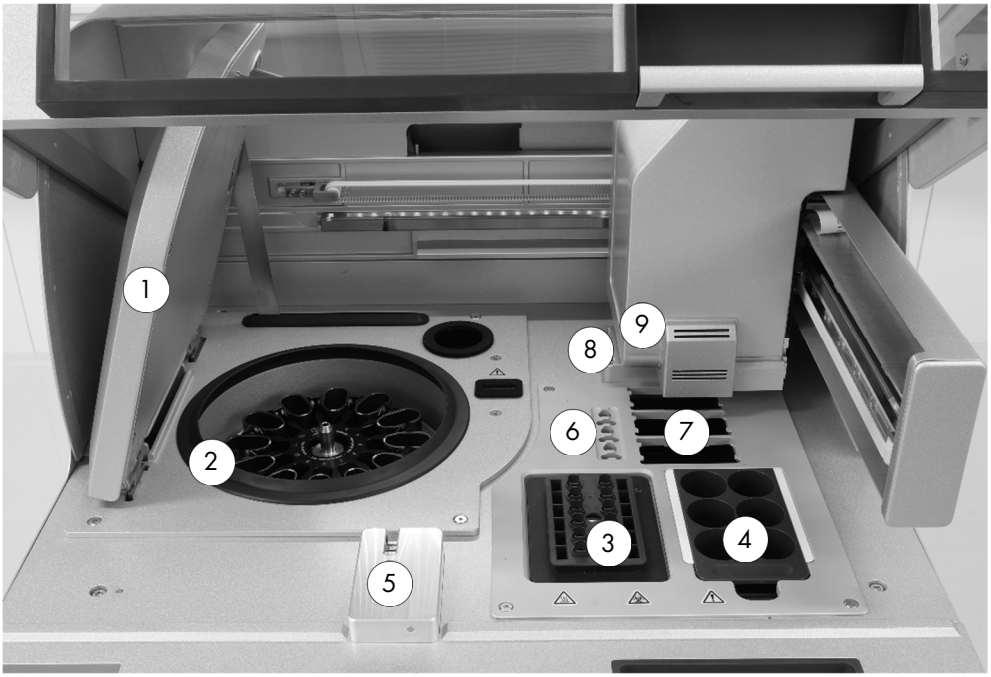



**Figure 21. Internal view of the QIAcube Connect MDx**.

1. Centrifuge lid
2. Centrifuge
3. Shaker
4. Reagent bottle rack
5. Tip sensor and hood lock
6. Microcentrifuge tube slots
7. 3 slots for tip racks
8. Disposal slots for tips and columns
9. Robotic arm (includes 1 channel pipettor, gripper, ultrasonic and optical sensor and UV LED)

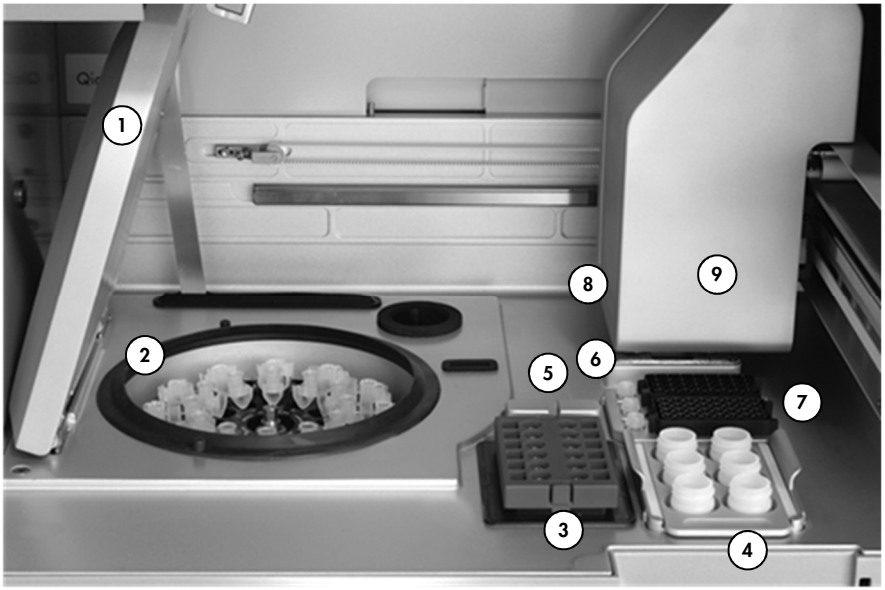



**Figure 22. Internal view of the QIAcube.**

1. Centrifuge lid
2. Centrifuge
3. Shaker
4. Reagent bottle rack
5. Tip sensor
6. Microcentrifuge tube slots
7. Tip racks
8. Disposal slots for tips and columns
9. Robotic arm

### Spin columns (PRC, PSC), microcentrifuge tubes (MCT), and QIAcube instruments plasticware

Place 2 tip racks filled with Filter-Tips 1000 µl onto the QIAcube instrument (see Figures 21 and 22, pages 47 and 48). Refill racks with tips when necessary.

Only use 1000 µl filter-tips designed for use with the QIAcube instruments.

Label rotor adapters and microcentrifuge tubes (MCT) for each sample using a permanent pen. Open the PAXgene Shredder spin columns (PSC) to be used, and cut the lids off completely using scissors (see Figure 23, page 49).

For proper operation of the QIAcube instruments robotic gripper, completely remove (cut off) the lids and all plastic parts connecting the lid to the PAXgene Shredder spin columns (PSC; see Figure 23). Otherwise, the robotic gripper cannot grip the spin columns (PSC, PRC) properly.

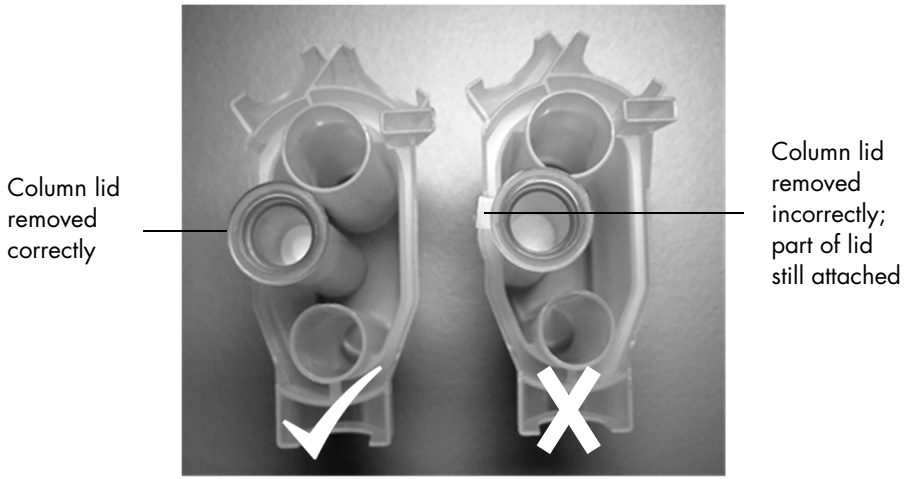



**Figure 23. Loading the PAXgene Shredder spin column (PSC). The PAXgene Shredder spin column (PSC) is loaded into the middle position of the rotor adapter. Cut off the lid before loading the column.**

Load the PAXgene RNA spin column (PRC), PAXgene Shredder spin column (PSC; without lid, see Figure 23, page 49), and labeled microcentrifuge tube into the appropriate positions in each labeled rotor adapter as shown in Table 4 and Figure 24.

Make sure that the spin column (PRC) and microcentrifuge tube (MCT) lids are pushed all the way down to the bottom of the slots at the edge of the rotor adapter, otherwise the lids will break off during centrifugation.

**Table 4. Labware in the rotor adapter**

| Position | Reagent | Lid position |
|---|---|---|
| 1 | PAXgene RNA spin column (red, PRC) | L1 |
| 2 | PAXgene Shredder spin column (lilac, PSC) (cut off lid before placing in rotor adapter) | – |
| 3 | Microcentrifuge tube (MCT)* | L3 |

* Use the microcentrifuge tubes (MCT; 1.5 ml) included in the PAXgene Blood RNA Kit.

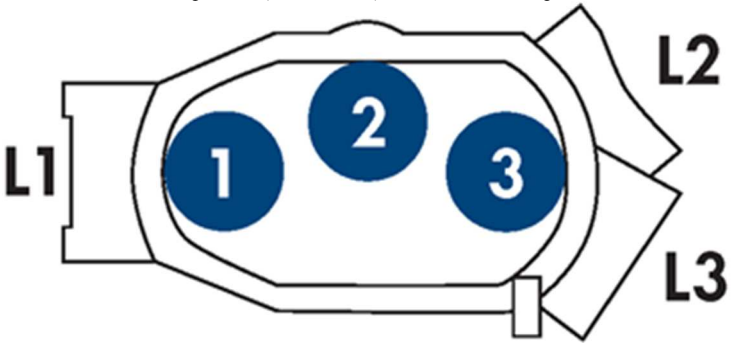



**Figure 24. Positions in the rotor adapter.** The rotor adapter has three tube positions (1–3) and three lid positions (L1–L3).

## Loading the centrifuge

Load the assembled rotor adapters into the centrifuge buckets as shown in Figure 25 below.

If processing fewer than 12 samples, make sure to load the centrifuge rotor balanced radially (see Figure 26, page 52). All centrifuge buckets must be mounted before starting a protocol run, even if fewer than 12 samples are to be processed. A single (one) sample or 11 samples cannot be processed.

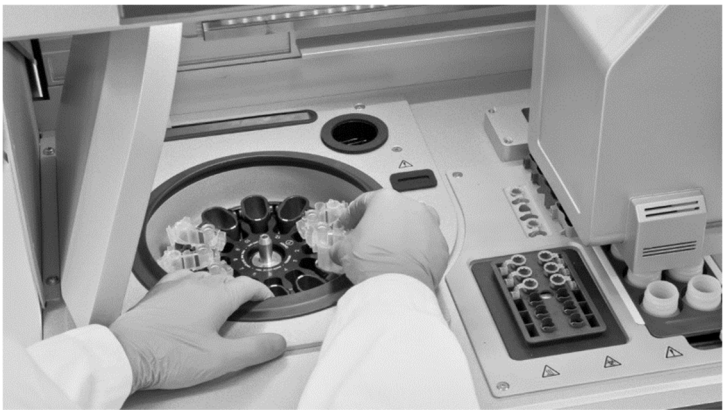


**Figure 25. Loading the centrifuge on the QIAcube instruments.** Load the assembled rotor adapters into the centrifuge buckets (QIAcube Connect MDx shown as an example).

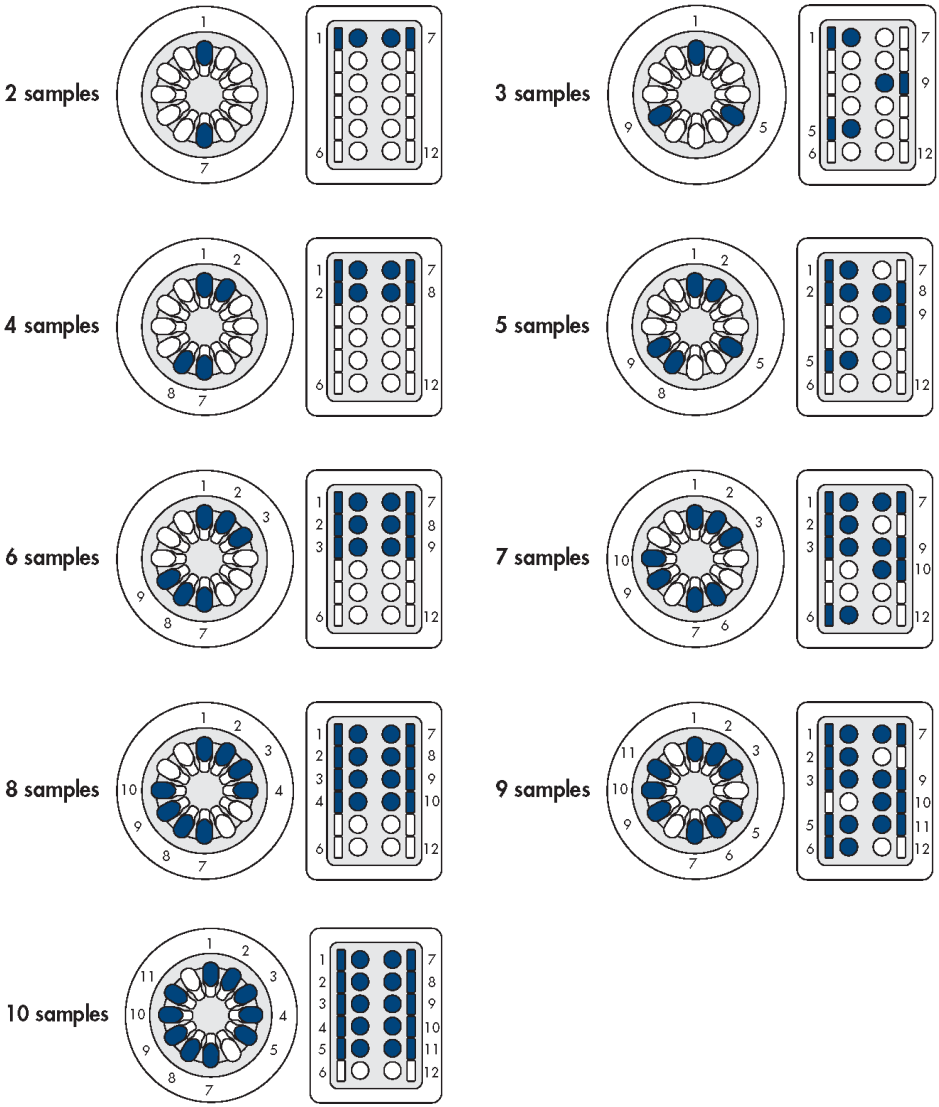



**Figure 26. Loading the centrifuge and shaker.** Centrifuge and shaker positions are shown for processing from two (2) to ten (10) samples. One (1) or 11 samples cannot be processed. For processing 12 samples, all centrifuge and shaker positions are loaded (image not shown).

## Processing tubes (PT)

Remove any processing tubes (PT) left in the microcentrifuge tube slots from previous runs (QIAcube Connect MDx: see Figure 21, page 47, QIAcube: see Figure 22, page 48). Fill 3 processing tubes (PT) with the amount of reagents given in Table 5 according to the number of samples in the run.

For DNase I incubation mix, pipet the indicated volume of DNA digestion buffer (RDD) into a processing tube (PT) and add the indicated volume of DNase I (RNFD) stock solution. Mix by gently pipetting the complete mixture up and down 3 times using a 1000 µl pipet tip.

Use the 2 ml processing tubes (PT) included in the PAXgene Blood RNA Kit. Label the tubes clearly with reagent names and place them into the appropriate position in the microcentrifuge tube slots, as indicated in Table 6 (page 54).

DNase I (RNFD) is especially sensitive to physical denaturation. Mix only by pipetting, using wide-bore pipet tips to reduce shearing. Do not vortex.

Be sure to only pipet the required volume as indicated in Table 5 below.

**Table 5. Volume of reagents required in processing tubes for the microcentrifuge tube slots.**

| Number of samples | Reagent volume for indicated number of samples (µl) | | |
|---|---|---|---|
| | Proteinase K (PK) | DNase I incubation mix | Elution buffer (BR5) |
| 2 | 126 | 187 (23 DNase I + 164 Buffer RDD) | 313 |
| 3 | 170 | 261 (33 DNase I + 228 Buffer RDD) | 399 |
| 4 | 213 | 334 (42 DNase I + 292 Buffer RDD) | 486 |
| 5 | 256 | 407 (51 DNase I + 356 Buffer RDD) | 572 |
| 6 | 299 | 481 (60 DNase I + 421 Buffer RDD) | 658 |
| 7 | 342 | 554 (69 DNase I + 485 Buffer RDD) | 745 |
| 8 | 386 | 627 (78 DNase I + 549 Buffer RDD) | 831 |
| 9 | 429 | 701 (88 DNase I + 613 Buffer RDD) | 918 |
| 10 | 472 | 775 (97 DNase I + 678 Buffer RDD) | 1004 |
| 12 | 558 | 921 (115 DNase I + 806 Buffer RDD) | 1177 |

**Table 6. Microcentrifuge tube slots**

| | Position | | |
|---|---|---|---|
| | **A** | **B** | **C** |
| **Content** | Proteinase K | DNase I incubation mix | Elution buffer (BR5) |
| **Vessel** | Processing tube (PT)* | Processing tube (PT)* | Processing tube (PT)* |

* Use the 2 ml processing tubes included in the PAXgene Blood RNA Kit.

# Protocol: Manual Purification of Total RNA from Human Whole Blood Collected into PAXgene Blood RNA Tubes (BRT)

## Important points before starting

- Make sure that the kit box is intact and undamaged, and that buffers have not leaked. Do not use a kit that has been damaged.
- When using a pipet, ensure that it is set to the correct volume and that liquid is carefully and completely aspirated and dispensed.
- To avoid transferring samples to the wrong tube or spin column, ensure that all tubes and spin columns are properly labeled using a permanent pen. Label the lid and the body of each tube (PT, MCT). For spin columns, label the body of its processing tube (PT). Close each tube or spin column after liquid is transferred to it.
- Spillages of samples and buffers during the procedure may reduce the yield and purity of RNA.
- Unless otherwise indicated, all steps of this protocol, including centrifugation steps, should be carried out at room temperature (15–25°C).

Because of the sensitivity of nucleic acid amplification technologies, the following precautions are necessary when handling samples to avoid cross-contamination:

- Carefully pipet the sample into the spin column (PRC, PSC) without moistening the rim of the column.
- Always change pipet tips between liquid transfers. Use aerosol-barrier pipet tips.
- Avoid touching the spin column (PRC, PSC) membrane with the pipet tip.

- After vortexing or heating a microcentrifuge tube (MCT), briefly centrifuge it to remove drops from the inside of the lid.
- Wear gloves throughout the entire procedure. In case of contact between gloves and sample, change gloves immediately.
- Close the spin column (PRC, PSC) before placing it in the microcentrifuge. Centrifuge as described in the procedure.
- Open only one spin column (PRC, PSC) at a time and take care to avoid generating aerosols.
- For efficient parallel processing of multiple samples, fill a rack with processing tubes (PT) to which the spin columns (PRC, PSC) can be transferred after centrifugation. Discard the used processing tubes (PT) containing flow-through, and place the new processing tubes (PT) containing spin columns (PRC, PSC) directly in the microcentrifuge.

### Things to do before starting

- Blood must be collected in PAXgene Blood RNA Tubes (BRT) according to the instructions in the *PAXgene Blood RNA Tube Handbook*. If necessary, see Appendix C (page 74) for recommendations on handling PAXgene Blood RNA Tubes (BRT).
- Ensure that the PAXgene Blood RNA Tubes (BRT) are incubated for at least 2 hours at room temperature after blood collection to ensure complete lysis of blood cells. Incubation of the PAXgene Blood RNA Tube (BRT) overnight may increase yields. If the PAXgene Blood RNA Tube (BRT) was stored at 2–8°C, –20°C or –70°C after blood collection, first equilibrate it to room temperature and then store it at room temperature for 2 hours before starting the procedure.
- Read the safety information on page 10.
- Read the guidelines on handling RNA (Appendix A, page 71).
- Ensure that instruments, such as pipets and the shaker–incubator, have been checked and calibrated regularly according to the manufacturer's recommendations.
- A shaker–incubator is required in steps 5 and 20. Set the temperature of the shaker–incubator to 55°C.

- Binding buffer (BR2) may form a precipitate upon storage. If necessary, warm to 37°C to dissolve.
- Wash buffer 2 (BR4) is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%, purity grade p.a.) as indicated on the bottle to obtain a working solution.
- If using the RNase-Free DNase Set for the first time, prepare DNase I stock solution. Dissolve the solid DNase I (RNFD; 1500 Kunitz units)* in 550 µl of the DNase resuspension buffer (DRB) provided with the set. Take care that no DNase I (RNFD) is lost when opening the vial. Do not vortex the reconstituted DNase I (RNFD). DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the vial.
- Current data shows that reconstituted DNase I (RNFD) can be stored at 2–8°C for up to 6 weeks. For long-term storage of DNase I (RNFD), remove the stock solution from the glass vial, divide it into single-use aliquots (use the 1.5 ml microcentrifuge tubes [MCT] supplied with the kit; there are enough for 5 aliquots) and store at –20°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.
- When reconstituting and aliquoting DNase I (RNFD), ensure that you follow the guidelines for handling RNA (Appendix A, page 71).

* Kunitz units are the commonly used units for measuring DNase I, defined as the amount of DNase I that causes an increase in $A_{260}$ of 0.001 per minute per milliliter at 25°C, pH 5.0, with highly polymerized DNA as the substrate (Kunitz, M. (1950) J. Gen. Physiol. **33**, 349 and 363).

## Procedure

1. Centrifuge the PAXgene Blood RNA Tube (BRT) for 10 minutes at 3000–5000 x *g* using a swing-out rotor.

   Ensure that the blood sample has been incubated in the PAXgene Blood RNA Tube (BRT) for a minimum of 2 hours at room temperature (15–25°C) in order to achieve complete lysis of blood cells.

   The rotor must contain tube adapters for round-bottom tubes. If other types of tube adapter are used, the tubes may break during centrifugation.

2. Remove the supernatant by decanting or pipetting. Add 4 ml RNase-Free Water (RNFW) to the pellet and close the tube using a fresh secondary BD Hemogard closure (supplied with the kit).

   If the supernatant is decanted, take care not to disturb the pellet and dry the rim of the tube with a clean paper towel.

3. Vortex until the pellet is visibly dissolved and centrifuge for 10 minutes at 3000–5000 x *g* using a swing-out rotor. Remove and discard the entire supernatant.

   Small debris remaining in the supernatant after vortexing but before centrifugation will not affect the procedure.

   Incomplete removal of the supernatant will inhibit lysis and dilute the lysate, and therefore affect the conditions for binding RNA to the PAXgene membrane.

4. Add 350 µl resuspension buffer (BR1) and vortex until the pellet is visibly dissolved.

5. Pipet the sample into a 1.5 ml microcentrifuge tube (MCT). Add 300 µl binding buffer (BR2) and 40 µl proteinase K (PK). Mix by vortexing for 5 seconds and incubate for 10 minutes at 55°C using a shaker–incubator at 400–1400 rpm. After incubation, set the temperature of the shaker–incubator to 65°C (for step 20).

   Do not mix binding buffer (BR2) and proteinase K (PK) together before adding them to the sample.

6. Pipet the lysate directly into a PAXgene Shredder spin column (PSC; lilac) placed in a 2 ml processing tube (PT) and centrifuge for 3 minutes at maximum speed (but not to exceed 20,000 x *g*).

   Carefully pipet the lysate into the spin column (PSC) and visually check that the lysate is completely transferred to the spin column (PSC).

   To prevent damage to columns (PSC) and tubes (PT), do not exceed 20,000 x *g*.

   Some samples may flow through the PAXgene Shredder spin column (PSC) without centrifugation. This is due to low viscosity of some samples and should not be taken as an indication of product failure.

7. Carefully transfer the entire supernatant of the flow-through fraction to a fresh 1.5 ml microcentrifuge tube (MCT) without disturbing the pellet in the processing tube.
8. Add 350 µl ethanol (96–100%, purity grade p.a.). Mix by vortexing and centrifuge briefly (1–2 seconds at 500–1000 x *g*) to remove drops from the inside of the tube lid.

   The length of the centrifugation must not exceed 1–2 seconds, as this may result in pelleting of nucleic acids and reduced yields of total RNA.

9. Pipet 700 µl sample into the PAXgene RNA spin column (PRC; red) placed in a 2 ml processing tube (PT) and centrifuge for 1 minutes at 8000–20,000 x *g*. Place the spin column (PRC) in a new 2 ml processing tube (PT) and discard the old processing tube (PT) containing flow-through.
10. Pipet the remaining sample into the PAXgene RNA spin column (PRC) and centrifuge for 1 minutes at 8000–20,000 x *g*. Place the spin column (PRC) in a new 2 ml processing tube (PT) and discard the old processing tube (PT) containing flow-through.

    Carefully pipet the sample into the spin column (PRC) and visually check that the sample is completely transferred to the spin column (PRC).

11. Pipet 350 µl wash buffer 1 (BR3) into the PAXgene RNA spin column (PRC). Centrifuge for 1 minute at 8000–20,000 x *g*. Place the spin column (PRC) in a new 2 ml processing tube (PT) and discard the old processing tube (PT) containing flow-through.

12. Add 10 µl DNase I (RNFD) stock solution to 70 µl DNA digestion buffer (RDD) in a 1.5 ml microcentrifuge tube (MCT). Mix by gently flicking the tube and centrifuge briefly to collect residual liquid from the sides of the tube.

    If processing, for example, 10 samples, add 100 µl DNase I (RNFD) stock solution to 700 µl DNA digestion buffer (RDD). Use the 1.5 ml microcentrifuge tubes (MCT) supplied with the kit.

    DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently flicking the tube. Do not vortex.

13. Pipet the DNase I (RNFD) incubation mix (80 µl) directly onto the PAXgene RNA spin column (PRC) membrane and place on the benchtop (20–30°C) for 15 minutes.

    Ensure that the DNase I (RNFD) incubation mix is placed directly onto the membrane. DNase digestion will be incomplete if part of the mix is applied to and remains on the walls or the O-ring of the spin column (PRC).

14. Pipet 350 µl wash buffer 1 (BR3) into the PAXgene RNA spin column (PRC) and centrifuge for 1 minute at 8000–20,000 x *g*. Place the spin column (PRC) in a new 2 ml processing tube (PT) and discard the old processing tube (PT) containing flow- through.

15. Pipet 500 µl wash buffer 2 (BR4) into the PAXgene RNA spin column (PRC) and centrifuge for 1 minute at 8000–20,000 x *g*. Place the spin column (PRC) in a new 2 ml processing tube (PT) and discard the old processing tube (PT) containing flow-through.

    Wash buffer 2 (BR4) is supplied as a concentrate. Ensure that ethanol is added to wash buffer 2 (BR4) before use (see “Things to do before starting”, page 56).

16. Add another 500 µl wash buffer 2 (BR4) to the PAXgene RNA spin column (PRC). Centrifuge for 3 minutes at 8000–20,000 x *g*.

17. Discard the processing tube (PT) containing the flow-through and place the PAXgene RNA spin column (PRC) in a new 2 ml processing tube (PT). Centrifuge for 1 minute at 8000–20,000 x *g*.

18. Discard the processing tube (PT) containing the flow-through. Place the PAXgene RNA spin column (PRC) in a 1.5 ml microcentrifuge tube (MCT) and pipet 40 µl elution buffer (BR5) directly onto the PAXgene RNA spin column (PRC) membrane. Centrifuge for 1 minute at 8000–20,000 x *g* to elute the RNA.

    It is important to wet the entire membrane with elution buffer (BR5) in order to achieve maximum elution efficiency.

19. Repeat the elution step (step 18) as described, using 40 µl elution buffer (BR5) and the same microcentrifuge tube (MCT).

20. Incubate the eluate for 5 minutes at 65°C in the shaker–incubator (from step 5) without shaking. After incubation, chill immediately on ice.

    This incubation at 65°C denatures the RNA for downstream applications. Do not exceed the incubation time or temperature.

21. If the RNA samples will not be used immediately, store at –20°C or –70°C.

    Since the RNA remains denatured after repeated freezing and thawing, it is not necessary to repeat the incubation at 65°C. If using the RNA samples in a diagnostic assay, follow the instructions supplied by the manufacturer.

    For accurate quantification of RNA by absorbance at 260 nm, we recommend diluting samples with 10 mM Tris-HCl, pH 7.5.* Diluting the sample in RNase-Free Water may lead to inaccurately low values.

    Zero the spectrophotometer using a blank consisting of the same proportion elution buffer (BR5) and Tris-HCl buffer as in the samples to be measured. Elution buffer (BR5) has high absorbance at 220 nm, which can lead to high background absorbance levels if the spectrophotometer is not properly zeroed.

For quantification in Tris HCl buffer, use the relationship $A_{260}$ = 1 ⇒ 44 µg/ml. See Appendix B, page 72.

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

# Protocol: Automated Purification of Total RNA from Human Whole Blood Collected into PAXgene Blood RNA Tubes (BRT)

## Important points before starting

- Make sure that the kit box is intact and undamaged, and that buffers have not leaked. Do not use a kit that has been damaged.
- When using a pipet, ensure that it is set to the correct volume, and that liquid is carefully and completely aspirated and dispensed.
- To avoid transferring samples to the wrong tubes and plastic consumables, ensure that all processing tubes (PT), microcentrifuge tubes (MCT) and rotor adapters are properly labeled using a permanent pen. Label the lid and the body of each microcentrifuge tube (MCT), the body of each processing tube (PT) and the outer wall of each rotor adapter.
- Spillages of samples and buffers during the procedure may reduce the yield and purity of RNA.
- Unless otherwise indicated, all steps of this protocol, including centrifugation steps, should be carried out at room temperature (15–25°C).

Because of the sensitivity of nucleic acid amplification technologies, the following precautions are necessary when handling samples to avoid cross-contamination:

- Carefully pipet the sample into the processing tube (PT), into the bottom of the tube without moistening the rim of the tube.
- Always change pipet tips between liquid transfers. Use aerosol-barrier pipet tips.
- Avoid touching the spin column (PRC, PSC) membrane with the pipet tip.

- After vortexing or heating a microcentrifuge tube (MCT), briefly centrifuge it to remove drops from the inside of the lid.
- Wear gloves throughout the entire procedure. In case of contact between gloves and sample, change gloves immediately.

### Things to do before starting

- Blood must be collected in PAXgene Blood RNA Tubes (BRT) according to the instructions in the *PAXgene Blood RNA Tube Handbook*. If necessary, see Appendix C (page 74) for recommendations on handling PAXgene Blood RNA Tubes (BRT).
- Ensure that the PAXgene Blood RNA Tubes (BRT) are incubated for at least 2 hours at room temperature after blood collection to ensure complete lysis of blood cells. Incubation of the PAXgene Blood RNA Tube (BRT) overnight may increase yields. If the PAXgene Blood RNA Tube (BRT) was stored at 2–8°C, –20°C or –70°C after blood collection, first equilibrate it to room temperature and then store it at room temperature for 2 hours before starting the procedure.
- Read the safety information on page 10.
- Read "Important Notes", page 41.
- Read the guidelines on handling RNA (Appendix A, page 71).
- Read the appropriate QIAcube instrument User Manual and any additional information supplied with the QIAcube instrument, paying careful attention to the safety information.
- Ensure that devices and instruments, such as pipets and the QIAcube instrument, have been checked and calibrated regularly according to the manufacturer's recommendations.
- Binding buffer (BR2) may form a precipitate upon storage. If necessary, warm to 37°C to dissolve.
- Wash buffer 2 (BR4) is supplied as a concentrate. Before using for the first time, add the appropriate volume of ethanol (96–100%, purity grade p.a.) as indicated on the bottle to obtain a working solution.

- If using the RNase-Free DNase Set for the first time, prepare DNase I stock solution. Dissolve the solid DNase I (RNFD; 1500 Kunitz units)* in 550 µl of the DNase resuspension buffer (DRB) provided with the set. Take care that no DNase I (RNFD) is lost when opening the vial. Do not vortex the reconstituted DNase I (RNFD). DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the vial.
- Current data shows that reconstituted DNase I (RNFD) can be stored at 2–8°C for up to 6 weeks. For long-term storage of DNase I (RNFD), remove the stock solution from the glass vial, divide it into single-use aliquots (use the 1.5 ml microcentrifuge tubes [MCT] supplied with the kit; there are enough for 5 aliquots), and store at –20°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.
- When reconstituting and aliquoting DNase I (RNFD), ensure that you follow the guidelines for handling RNA (Appendix A, page 71).
- Install the correct shaker adapter (included with the QIAcube instruments; use the adapter for 2 ml safe-lock tubes, marked with a "2"), and place the shaker rack on top of the adapter.
- Check the waste drawer and empty it if necessary.
- Install any related protocols if not already done for previous runs. The QIAcube Connect MDx requires all protocols found in the related zip file to be downloaded. For the classic QIAcube, install both "PAXgene Blood RNA Part A" and "PAXgene Blood RNA Part B" protocols. See "Installing protocols on the QIAcube instruments", page 44.

* Kunitz units are the commonly used units for measuring DNase I, defined as the amount of DNase I that causes an increase in $A_{260}$ of 0.001 per minute per milliliter at 25°C, pH 5.0, with highly polymerized DNA as the substrate (Kunitz, M. (1950) J. Gen. Physiol. **33**, 349 and 363).

## Procedure

1. Close the QIAcube instrument hood, and switch on the QIAcube instrument with the power switch (QIAcube Connect MDx: see Figure 17, page 42; QIAcube: see Figure 18, page 43**Figure 15**).

   A beeper sounds and the startup screen appears. The instruments automatically perform initialization tests.

2. Open the QIAcube instrument hood, and load the necessary reagents and plasticware into the QIAcube instrument. See "Loading the QIAcube instruments", page 45.

   To save time, loading can be performed during one or both of the following 10-minute centrifugation steps (steps 3 and 5).

3. Centrifuge the PAXgene Blood RNA Tube (BRT) for 10 minutes at 3000–5000 x *g* using a swing-out rotor.

   Ensure that the blood sample has been incubated in the PAXgene Blood RNA Tube (BRT) for a minimum of 2 hours at room temperature (15–25°C), in order to achieve complete lysis of blood cells.

   The rotor must contain tube adapters for round-bottom tubes. If other types of tube adapter are used, the tubes may break during centrifugation.

4. Remove the supernatant by decanting or pipetting. Add 4 ml RNase-Free Water (RNFW) to the pellet and close the tube using a fresh secondary BD Hemogard closure (supplied with the kit).

   If the supernatant is decanted, take care not to disturb the pellet and dry the rim of the tube with a clean paper towel.

5. Vortex until the pellet is visibly dissolved and centrifuge for 10 minutes at 3000–5000 x *g* using a swing-out rotor. Remove and discard the entire supernatant.

   Small debris remaining in the supernatant after vortexing but before centrifugation will not affect the procedure.

   Incomplete removal of the supernatant will inhibit lysis and dilute the lysate, and therefore affect the conditions for binding RNA to the PAXgene membrane.

6. Add 350 µl resuspension buffer (BR1) and vortex until the pellet is visibly dissolved.
7. Pipet the sample into a 2 ml processing tube (PT).

   Use the 2 ml processing tubes (PT) included in the PAXgene Blood RNA Kit.

8. Load the open processing tubes (PT) containing sample into the QIAcube instrument shaker (QIAcube Connect MDx: see Figure 21, page 47; QIAcube: see Figure 22, page 48**Figure 15**). The sample positions are numbered for ease of loading. Insert shaker rack plugs (included with the QIAcube instruments) into the slots at the edge of the shaker rack next to each processing tube. This enables detection of samples during the load check.

   Make sure that the correct shaker adapter (Shaker Adapter, 2 ml, safe-lock tubes, marked with a "2", included with the QIAcube instruments) is installed.

   If processing fewer than 12 samples, make sure to load the shaker rack as shown in Figure 26, page 52. One (1) or 11 samples cannot be processed. The position numbers in the shaker rack correspond to the position numbers in the centrifuge.

9. Close the QIAcube instrument hood (QIAcube Connect MDx: see Figure 17, page 42; QIAcube: see Figure 18, page 43).
10. Select the "PAXgene Blood RNA Part A" protocol and start the protocol.

    Follow the instructions given on the touchscreen of the QIAcube instrument.

    Make sure that both program parts (part A and part B) are installed on the QIAcube instrument (see "Installing protocols on the QIAcube instruments", page 44).

    The QIAcube instruments will perform load checks for samples, tips, rotor adapters and reagent bottles.

11. After the "PAXgene Blood RNA Part A" protocol is finished, open the QIAcube instrument hood (QIAcube Connect MDx: see Figure 17, page 42; QIAcube: see Figure 18, page 43). Remove and discard the PAXgene RNA spin columns (PRC) from the rotor adapters and the empty processing tubes (PT) from the shaker.

During the run, spin columns are transferred from the rotor adapter position 1 (lid position L1) to rotor adapter position 3 (lid position L2) by the instrument (see Figure 24, page **Error! Bookmark not defined.**).

12. Close the lids of all 1.5 ml microcentrifuge tubes (MCT) containing the purified RNA in the rotor adapters (position 3, lid position L3, see Figure 24, page **Error! Bookmark not defined.**). Transfer the 1.5 ml microcentrifuge tubes (MCT) onto the QIAcube instrument shaker adapter (QIAcube Connect MDx: see Figure 21, page 47; QIAcube: see Figure 22, page 48).
13. Close the QIAcube instrument hood (QIAcube Connect MDx: see Figure 17, page 42; QIAcube: see Figure 18, page 43).
14. Select the "PAXgene Blood RNA Part B" protocol and start the protocol.

    Follow the instructions given on the QIAcube instrument touchscreen.

This program incubates the samples at 65°C and denatures the RNA for downstream applications. Even if the downstream application includes a heat denaturation step, do not omit this step. Sufficient RNA denaturation is essential for maximum efficiency in downstream applications.

15. After the "PAXgene Blood RNA Part B" program is finished, open the QIAcube instrument hood (QIAcube Connect MDx: see Figure 17, page 42; QIAcube: see Figure 18, page 43). Immediately place the microcentrifuge tubes (MCT) containing the purified RNA on ice.

WARNING: Hot surface. The shaker can reach temperatures of up to 70°C (158°F). Avoid touching it when it is hot.

Do not let the purified RNA remain in the QIAcube instrument. Since the samples are not cooled, the purified RNA can be degraded. Unattended overnight sample preparation runs are therefore not recommended.

16. If the RNA samples will not be used immediately, store at –20°C or –70°C.

Since the RNA remains denatured after repeated freezing and thawing, it is not necessary to repeat the heat incubation protocol ("PAXgene Blood RNA Part B"). If using the RNA samples in a diagnostic assay, follow the instructions supplied by the manufacturer.

For accurate quantification of RNA by absorbance at 260 nm, we recommend diluting samples in 10 mM Tris-HCl, pH 7.5.* Diluting the sample in RNase-Free Water may lead to inaccurately low values.

Zero the spectrophotometer using a blank consisting of the same proportion elution buffer (BR5) and Tris-HCl buffer as in the samples to be measured. Elution buffer (BR5) has high absorbance at 220 nm, which can lead to high background absorbance levels if the spectrophotometer is not properly zeroed.

For quantification in Tris-HCl buffer, use the relationship

$A_{260}$ = 1 => 44 µg/ml. See Appendix B, page 72.

17. Remove the reagent bottle rack from QIAcube instrument worktable (QIAcube Connect MDx: see Figure 21, page 47; QIAcube: see Figure 22, page 48), and close all bottles with the appropriately labeled lids. Buffer in bottles can be stored at room temperature (15–25°C) for up to 3 months. Remove and discard remaining reagents in the processing tubes (PT) in the QIAcube instrument microcentrifuge tube slots. Remove and discard rotor adapters from the centrifuge. Empty the QIAcube Connect MDx waste drawer (QIAcube Connect MDx: see Figure 17, page 42; QIAcube: see Figure 18, page 43). Close the QIAcube instrument hood and switch off the instrument with the power switch.

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

# Troubleshooting Guide

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

| | Comments and suggestions |
|---|---|
| **RNA degraded** | |
| RNase contamination | Be careful not to introduce any RNases into the reagents during the procedure or later handling (see Appendix A, page 71). |
| **Low RNA yield** | |
| a) Less than 2.5 ml blood collected in PAXgene Blood RNA Tube (BRT) | Ensure that 2.5 ml blood is collected in the PAXgene Blood RNA Tube (BRT; see *PAXgene Blood RNA Tube Handbook*). |
| b) RNA concentration measured in water | RNA must be diluted in 10 mM Tris-HCl, pH 7.5* for accurate quantification (see Appendix B, page 72). |
| c) Cell debris transferred to PAXgene RNA spin column (PRC) in steps 9 and 10 of the manual protocol | Avoid transferring large particles when pipetting the supernatant in step 7 of the manual protocol (transfer of small debris will not affect the procedure). |

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

| | | Comments and suggestions |
|---|---|---|
| d) | Supernatant not completely removed in step 3 | Ensure the entire supernatant is removed. If the supernatant is decanted, remove drops from the rim of the PAXgene Blood RNA Tube (BRT) by dabbing onto a paper towel. Take appropriate precautions to prevent cross-contamination. |
| e) | After collection into the PAXgene Blood RNA Tube (BRT), blood is incubated for less than 2 hours | Incubate blood in the PAXgene Blood RNA Tube (BRT) for at least 2 hours after collection. |
| **Low $A_{260}/A_{280}$ value** | | |
| a) | Water used to dilute RNA for $A_{260}/A_{280}$ measurement | Use 10 mM Tris-HCl, pH 7.5 to dilute RNA before measuring purity* (see Appendix B, page 72). |
| b) | Spectrophotometer not properly zeroed | Zero the spectrophotometer using a blank consisting of the same proportion elution buffer (BR5) and 10 mM Tris-HCl, pH 7.5, as in the samples to be measured. Elution buffer (BR5) has high absorbance at 220 nm, which can lead to high background absorbance levels if the spectrophotometer is not properly zeroed. |
| **Instrument malfunction** | | |
| | QIAcube instruments did not operate properly | Read the appropriate QIAcube user manual, paying careful attention to the Troubleshooting section. Make sure that the QIAcube instrument is properly maintained, as described in the user manual. |

* Wilfinger, W.W., Mackey, M., and Chomczynski, P. (1997) Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. BioTechniques **22**, 474.

# Appendix A: General Remarks on Handling RNA

## Handling RNA

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. Since RNases are difficult to inactivate and even minute amounts are sufficient to degrade RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. Great care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the purification procedure. In order to create and maintain an RNase-free environment, precautions must be taken during pretreatment and use of disposable and non-disposable vessels and solutions while working with RNA.

## General handling

Proper microbiological, aseptic technique should always be used when working with RNA. Hands and dust particles carry bacteria and molds and are the most common sources of RNase contamination. Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible. Keep purified RNA on ice when aliquots are pipetted for downstream applications.

Protocols for removing RNase-contamination from glassware and solutions can be found in general molecular biology guides, such as Sambrook, J. and Russell, D. W. (2001) Molecular Cloning: A Laboratory Manual, 3rd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

# Appendix B: Quantification and Determination of Quality of Total RNA

## Quantification of RNA

The concentration of RNA should be determined by measuring the absorbance at 260 nm ($A_{260}$) in a spectrophotometer. To ensure significance, readings should be in the linear range of the spectrophotometer. An absorbance of 1 unit at 260 nm corresponds to 44 µg of RNA per ml ($A_{260}$ = 1 ⇒ 44 µg/ml). This relation is valid only for measurements in 10 mM Tris-HCl, pH 7.5,*. Therefore, if it is necessary to dilute the RNA sample and this should be done in 10 mM Tris-HCl. As discussed below (see "Purity of RNA", page 73), the ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity. When measuring RNA samples, be certain that cuvettes are RNase-free. Zero the spectrophotometer using a blank consisting of the same proportion elution buffer( BR5) and Tris-HCl buffer as in the samples to be measured. Elution buffer (BR5) has high absorbance at 220 nm, which can lead to high background absorbance levels if the spectrophotometer is not properly zeroed. An example of the calculation involved in RNA quantification is shown below.

| | | |
|---|---|---|
| Volume of RNA sample | = | 80 µl |
| Dilution (1/15) | = | 10 µl of RNA sample + 140 µl 10 mM Tris-HCl, pH 7.5 |
| Measure absorbance of diluted sample in a cuvette (RNase-free). | | |
| $A_{260}$ | = | 0.3 |
| Concentration of sample | = | 44 x $A_{260}$ x dilution factor |
| | = | 44 x 0.3 x 15 |
| | = | 198 µg/ml |
| Total yield | = | concentration x volume of sample in milliliters |
| | = | 198 µg/ml x 0.08 ml |
| | = | 15.8 µg RNA |

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

## Purity of RNA

The ratio of the readings at 260 nm and 280 nm ($A_{260}/A_{280}$) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV, such as protein. However, the $A_{260}/A_{280}$ ratio is influenced considerably by pH. Lower pH results in a lower $A_{260}/A_{280}$ ratio and reduced sensitivity to protein contamination.* For accurate values, we recommend measuring absorbance in 10 mM Tris-HCl, pH 7.5. Pure RNA has an $A_{260}/A_{280}$ ratio of 1.8–2.2 in 10 mM Tris-HCl, pH 7.5. Zero the spectrophotometer using a blank consisting of the same proportion elution buffer (BR5) and Tris-HCl buffer as in the samples to be measured. Elution buffer (BR5) has high absorbance at 220 nm, which can lead to high background absorbance levels if the spectrophotometer is not properly zeroed.

* Wilfinger, W.W., Mackey, M., and Chomczynski, P. (1997) Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. BioTechniques **22**, 474.

# Appendix C: Handling PAXgene Blood RNA Tubes (BRT)

The following recommendations from BD may be helpful when handling PAXgene Blood RNA Tubes (BRT). See the *PAXgene Blood RNA Tube Handbook* for more information about PAXgene Blood RNA Tubes (BRT).

**Instructions for removal of BD Hemogard Closure**

1. Grasp the PAXgene Blood RNA Tube (BRT) with one hand, placing the thumb under the BD Hemogard closure. (For added stability, place arm on solid surface.) With the other hand, twist the BD Hemogard closure while simultaneously pushing up with the thumb of the other hand ONLY UNTIL THE TUBE STOPPER IS LOOSENED.
2. Move thumb away before lifting closure. DO NOT use thumb to push closure off the PAXgene Blood RNA Tube (BRT). Caution: If the PAXgene Blood RNA Tube (BRT) contains blood, an exposure hazard exists. To help prevent injury during closure removal, it is important that the thumb used to push upward on the closure be removed from contact with the PAXgene Blood RNA Tube (BRT) as soon as the BD Hemogard closure is loosened.
3. Lift closure off the PAXgene Blood RNA Tube (BRT). In the unlikely event of the plastic shield separating from the rubber stopper, DO NOT REASSEMBLE CLOSURE. Carefully remove rubber stopper from the PAXgene Blood RNA Tube (BRT).

**Instructions for insertion of Secondary BD Hemogard Closure**

1. Replace closure over the PAXgene Blood RNA Tube (BRT).
2. Twist and push down firmly until stopper is fully reseated. Complete reinsertion of the stopper is necessary for the closure to remain securely on the PAXgene Blood RNA Tube (BRT) during handling.

# Ordering Information

| Product | Contents | Cat. no. |
|---|---|---|
| PAXgene Blood RNA Kit (50) | 50 PAXgene Spin Columns, 50 Shredder Spin Columns, Processing Tubes, RNase-Free DNase I, RNase-Free Reagents and Buffers. To be used in conjunction with the PAXgene Blood RNA Tubes | 762174 |
| PAXgene Blood RNA Tubes (100) | 100 blood collection tubes | 762165 |
| **Related Products that can be ordered from QIAGEN** | | |
| Starter Pack, QIAcube | Pack includes: reagent bottle racks (3); rack labeling strips (8); 200 µl filter-tips (1024); 1000 µl filter-tips (1024); 1000 µl filter-tips, wide-bore (1024); 30 ml reagent bottles (18); rotor adapters (240); rotor adapter holder | 990395 |
| Filter-Tips, 1000 µl (1024) | Sterile, Disposable Filter-Tips, racked | 990352 |
| Reagent Bottles, 30 ml (6) | Reagent Bottles (30 ml) with lids; pack of 6; for use with the QIAcube instrument reagent bottle rack | 990393 |
| Rotor Adapters (10 x 24) | For 240 preps: 240 Disposable Rotor Adapters; for use with QIAcube instruments | 990394 |
| Reagent Bottle Rack | Rack for accommodating 6 x 30 ml reagent bottles on the QIAcube instrument worktable | 990390 |

| | | |
|---|---|---|
| Rotor Adapter Holder | Holder for 12 disposable rotor adapters; for use with QIAcube instruments | 990392 |
| **Related products that can be ordered from BD*** | | |
| Blood Collection Set | BD Vacutainer® Safety-Lok™ 6 Blood Collection Set: 21G, 0.75 inch (0.8 × 19 mm) needle, 12 inch (305 mm) tubing with luer adapter; 50 per box, 200 per case | 367286 |
| BD Vacutainer One-Use Holder | Case only for 13 mm and 16 mm diameter; 1000/case | 364815 |
| BD Vacutainer Plus Serum Tubes | 13 x 75 mm 4.0 ml draw with red BD Hemogard closure and paper label; 100/box, 1000/case | 368975 |

* These blood collection accessories represent typical products that can be used with PAXgene Blood RNA Tubes (BRT). To find out more about these accessories, including how to order, visit **www.preanalytix.com**.

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

# Handbook Revision History

| Document and revision | Changes | Date |
|---|---|---|
| HB-0101-004, R2 | Changes to comply with GHS regulations throughout document | June 2015 |
| HB-0101-005, R3 | New template; revisions to automated protocol and performance data; update of Safety Information to comply with GHS regulations; changes to instrument details and Product Use Limitations statement. | February 2019 |
| HB-0101-006, R3 | Correction of kit name in kit contents table p. 5. | January 2020 |
| HB-0101-007, R4 | Added QIAcube Connect MDx to automated protocol; updated language throughout to include references to QIAcube Connect MDx; updated table, page and figure numbers throughout. | December 2020 |

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