GeneChip® Globin-Reduction Kit Handbook

AFFYMETRIX®

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Mat. No. 1029528

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Not for use in diagnostic procedures.

The PAXgene Blood RNA System and the PAXgene 96 Blood RNA System are for research use only and not for use in diagnostic procedures.

P/N 701828 Rev. 1

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Chapter 1

Kit Information

Chapter 1

Kit Contents

Table 1.1
GeneChip Globin-Reduction Kit

Description	Amount
GeneChip® Blood RNA Concentration Kit (P/N 900585) for 30 reactions	
• RNA Concentration Spin Columns	30 columns
• RNA Binding Buffer[a]	45 mL
• RNA Wash Buffer[b]	11 mL
• RNase-Free Water	10 mL
• Collection Tubes (2 mL)	60 tubes
• Collection Tubes (1.5 mL)	30 tubes
• Buffer BR5	5 mL
GeneChip® Globin-Reduction RNA Controls (P/N 900586) for 12 reactions	
• Jurkat RNA	1 vial[c]
• Jurkat + Globin RNA	1 vial[c]
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a: Contains a guanidine salt. Not compatible with disinfecting reagents containing bleach.

b: RNA Wash Buffer is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%), as indicated on the bottle, to obtain a working solution.

c: For 6 reactions.

Shipping and Storage

The GeneChip Globin-Reduction Kit is shipped as follows:

- The GeneChip Blood RNA Concentration Kit is shipped at room temperature.
- The Globin-Reduction RNA Controls Kit is shipped on dry ice.

The RNA Concentration Spin Columns should be stored at 4 to 8°C. The rest of the GeneChip Blood RNA Concentration Kit should be stored dry at room temperature. The Globin-Reduction RNA Controls should be stored at –20°C. All components are stable for at least 9 months under the recommended conditions.

Safety Information

General Safety Information

Please call Affymetrix at 1-888-DNA-CHIP within the United States, +44 (0) 1628 552 550 in Europe and +81 (0) 3 5730 8200 in Japan.

24-hour Emergency Information

Please call CHEMTREC at 1-800-424-9300 within the United States, Canada, Puerto Rico and the Virgin Islands. All other callers can use CHEMTREC's international number (emergency collect calls are accepted) at 1-703-527-3887.

Chapter 2

Getting Started

Chapter 2

Introduction

The GeneChip Globin-Reduction Kit is designed to enable sensitive gene expression analysis on Affymetrix GeneChip arrays using cRNA targets prepared from RNA purified from human whole blood samples. In contrast to RNA purified from fractionated blood samples, cellular RNA purified from whole blood samples contains high amounts of globin transcripts. Using RNA from whole blood for GeneChip target preparation results in cRNA containing a very large number of target transcripts generated from globin mRNAs leading to reduced sensitivity of gene expression analysis on GeneChip arrays (see the Technical Note *Globin-Reduction Protocol: A Method for Processing Whole Blood RNA Samples for Improved Array Results*, available at www.affymetrix.com).

The kit reduces the globin effect by blocking reverse transcription of globin mRNA during the target preparation procedure. The kit is intended for use with methods that isolate RNA from unfractionated human whole blood only, such as the PAXgene™ Blood RNA System from PreAnalytiX. The kit contains all the necessary reagents to concentrate purified cellular RNA to a volume suitable for target preparation. Globin-reduction controls are also available (Affymetrix P/N 900586) that provide verification that reverse transcription of globin mRNA was successfully blocked and to confirm the procedure affected only globin transcripts. The procedure for globin reduction fits seamlessly with the GeneChip Target Labeling Assay recommended by Affymetrix.

PRINCIPLE AND PROCEDURE

The GeneChip Globin-Reduction Kit improves assay sensitivity by reducing the amount of cDNA generated from globin mRNA during the first-strand cDNA synthesis step of the target preparation protocol for GeneChip arrays. To achieve this goal, a mixture of peptide nucleic acid (PNA) oligonucleotides is added to the cellular RNA sample together with the T7-Oligo (dT) Promoter Primer. These PNAs are complementary to human globin mRNA transcripts and anneal during the primer hybridization step to the globin mRNA transcripts. During the subsequent first strand cDNA synthesis reaction, the PNAs bound to the globin mRNA block reverse transcription of the corresponding transcripts. Therefore, the amount of cDNA and subsequently synthesized cRNA generated from globin mRNAs is reduced, enabling sensitive and unbiased gene expression analysis from other cells found in blood using GeneChip arrays.

The kit seamlessly integrates the globin reduction procedure into the GeneChip Target Labeling Assay described in the *GeneChip Expression Analysis Technical Manual* (see Figure 2.1), and requires minimal additional steps and time. In addition, the globin reduction procedure can be easily tested using control RNAs available in the GeneChip Globin-Reduction RNA Controls prior to or in parallel with the processing of human whole blood samples to check that the globin reduction procedure works successfully (see *Controls for the Globin Reduction Procedure on page 9*).

NOTE **The PNAs are not supplied with the kit and need to be purchased to perform the protocol. The sequences of the 4 PNAs and recommended supplier are listed on page 13**

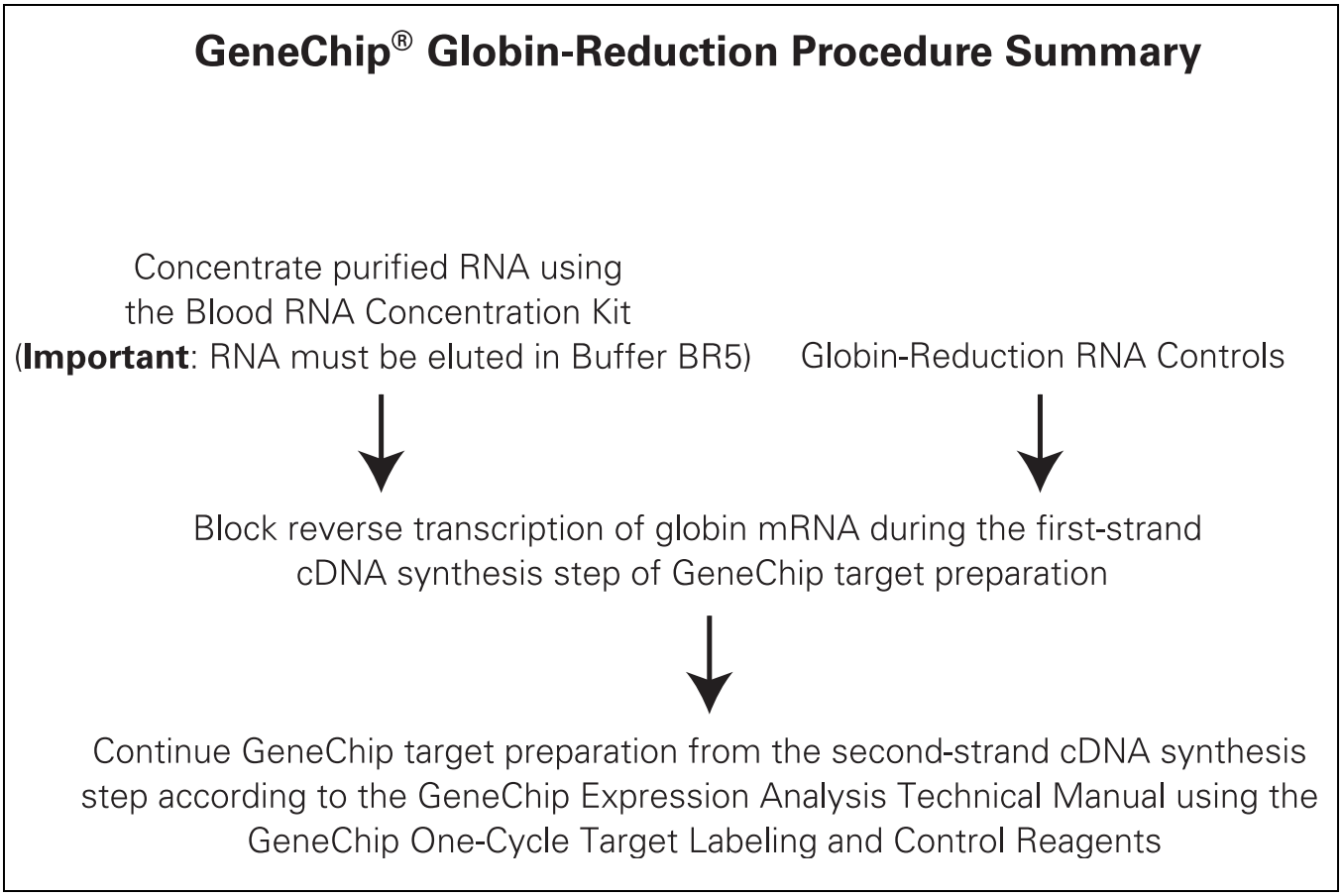

Figure 2.1
Overview of the GeneChip® Globin Reduction Procedure with RNA controls.

CONTROLS FOR THE GLOBIN REDUCTION PROCEDURE

The Globin-Reduction RNA Controls (P/N 900586) box contains 2 vials of control RNA that validate the globin reduction procedure:

- **Jurkat RNA**: This control RNA is purified from the Jurkat cell line that originated from human peripheral blood and contains negligible amounts of any Globin mRNAs. The Jurkat RNA control therefore represents RNA purified from the leukocyte fraction of whole blood (i.e., it mimics RNA purified from whole blood that had been subjected to erythrocyte lysis before RNA purification).
- **Jurkat + Globin RNA**: This globin-reduction control RNA is the Jurkat RNA described above but spiked with *in vitro* synthesized poly-adenylated globin transcripts. The Jurkat + Globin RNA control mimics RNA purified from unfractionated whole blood. The amount of globin *in vitro* transcripts in the Jurkat + Globin RNA control has been adjusted so that the derived cRNAs will provide GeneChip array results that are similar to those obtained using RNA purified from human whole blood.

The kit provides sufficient **Jurkat RNA** and **Jurkat + Globin RNA** for six reactions each, for a total of twelve reactions. The protocol recommends preparing (see Figure 2.2) both Jurkat RNA and Jurkat + Globin RNA with and without globin reduction treatment with sufficient material for 3 technical replicates each:

Table 2.1
Recommended globin-reduction controls and their expected results.

Sample	Rx	Globin Reduction	Total RNA Input (µg)	Replicates	Expected Result
Jurkat RNA	1	No	2	3	Optimal GeneChip array results are expected. This reaction is used as a reference to determine success of the GeneChip Target Labeling.
	2	Yes	2	3	Optimal GeneChip array results are expected. This reaction is used to verify that the globin reduction procedure does not affect non-globin transcripts.
Jurkat RNA + Globin RNA	3	No	2	3	Sub-optimal GeneChip array results are expected due to a loss in assay sensitivity. This reaction is used to demonstrate the effects of high amounts of globin mRNAs on array analysis.
	4	Yes	2	3	Optimal GeneChip array results similar to those obtained using the Jurkat RNA control are expected. This reaction is used to demonstrate the increase in assay sensitivity due to the globin reduction procedure.

The cRNA derived from the globin reduction control reactions can be analyzed using one of the following methods:

- The first method involves hybridizing the cRNAs to GeneChip arrays and then analyzing the gene expression pattern. The Jurkat + Globin RNA control without globin reduction is anticipated to result in a reduced percent Present call compared with that for the Jurkat RNA control. The Jurkat + Globin RNA control after globin reduction should provide a gene expression pattern comparable to that for the Jurkat RNA control.
- The second method for analyzing cRNA from the control reactions is less quantitative and involves using the Agilent® 2100 Bioanalyzer (see the example traces in Figure 2.2 on page 11). cRNA prepared from RNA samples containing high levels of globin mRNA typically shows a characteristic peak (see Figure 2.2). This peak is significantly reduced if the samples were subjected to the globin reduction procedure, and does not appear if the samples contains globin mRNA. Alternatively, agarose gel electrophoresis can be used to analyze cRNA fragment size distribution. Both methods give an indication of the success of globin reduction, but provide less detailed information than GeneChip array analysis.

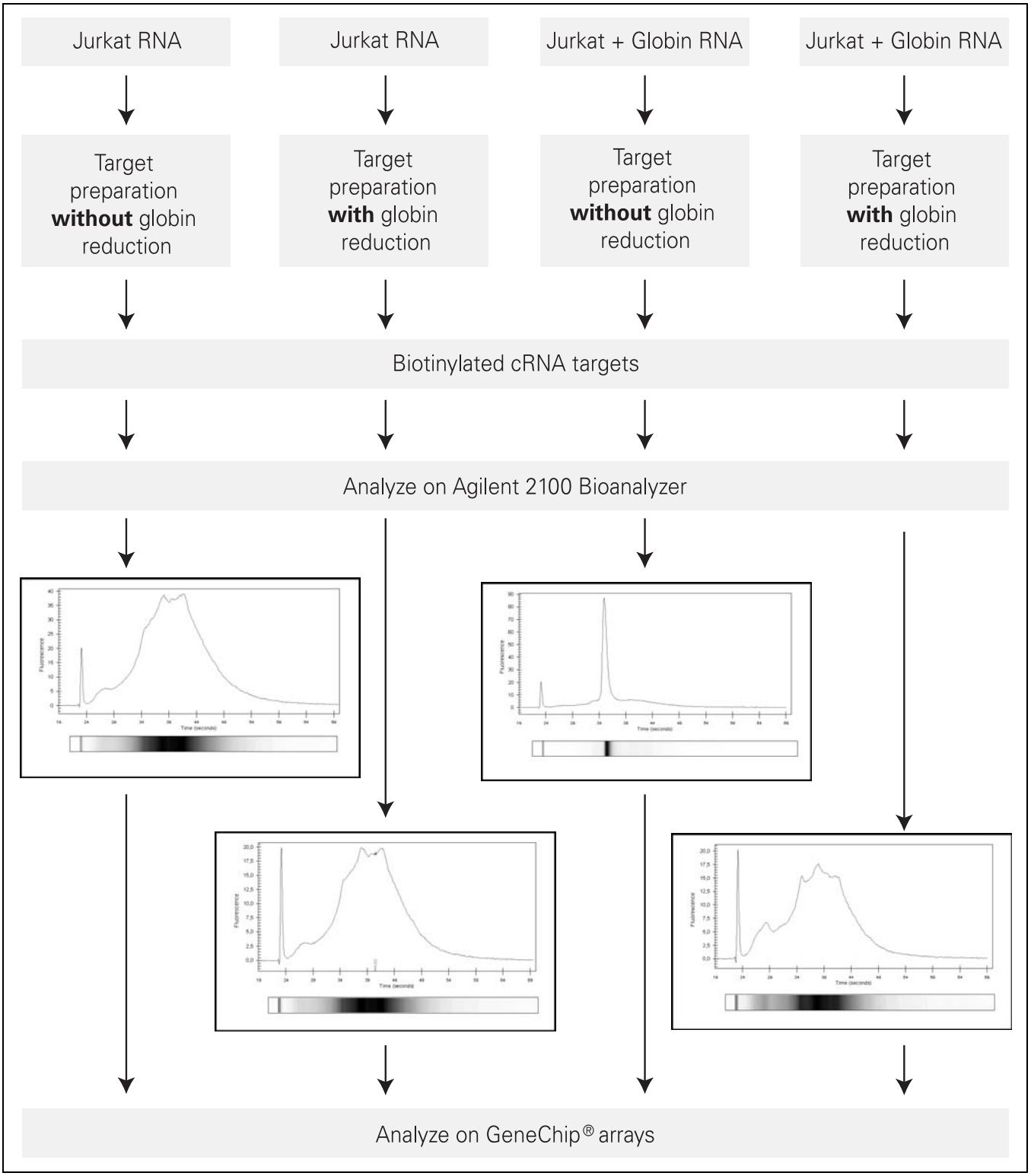

Figure 2.2
Summary of the expected results using an Agilent 2100 Bioanalyzer for the four RNA control reactions. The Jurkat + Globin RNA sample without globin reduction shows a characteristic narrow peak corresponding to the large amount of amplified globin transcript in the sample.

DESCRIPTION OF PROTOCOLS

This handbook contains the following protocols:

- *Protocol 1: Concentrating Purified Cellular RNA* on page 17
- *Protocol 2: Preparing cRNA Targets with Integrated Globin Reduction* on page 21

Equipment and Reagents to Be Supplied by User

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

For all protocols:

- 96–100% ethanol[1]
- Heating block or water bath
- RNase-free tubes (0.2 mL, 1.5 mL, 2 mL, and 25 mL)
- NanoDrop® spectrophotometer (recommended)
- Agilent 2100 Bioanalyzer
- Thermal cycler
- Vortexer
- Microcentrifuge
- Sterile, RNase-free pipet tips
- Disposable gloves

FOR PROTOCOL 1

- RNase-Free DNase Set (only required if RNA samples were not treated with DNase during the RNA purification procedure)

[1] *Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone.*

FOR PROTOCOL 2

- Globin-Reduction PNAs
  It is strongly recommended to purchase PNA oligomers from Applied Biosystems (for ordering information, refer to Applied Biosystems home page, available at www.appliedbiosystems.com). The sequences below are written from the N-terminal (5' end) to the C-terminal (3' end). The 5' end is acetylated (Ac).
  - Globin-Reduction PNA 1: Ac – TAA CGG TAT TTG GAG
  - Globin-Reduction PNA 2: Ac – GTA GTT GGA CTT AGG
  - Globin-Reduction PNA 3: Ac – GCC CTT CAT AAT ATC
  - Globin-Reduction PNA 4: Ac – ATC CAG ATG CTC AAG

NOTE **These sequences are specific to human adult globin mRNA transcripts.**

- One-Cycle Target Labeling and Control Reagents (Affymetrix P/N 900493)
  This is a convenient package containing all required labeling and control reagents to perform 30 one-cycle labeling reactions. Contains 1 IVT labeling Kit, 1 One-Cycle cDNA Synthesis Kit, 1 Sample Cleanup Module, 1 Poly-A RNA Control Kit, and 1 Hybridization Control Kit. Each of these components may be ordered individually as well as in this complete kit (for ordering information, refer to the *GeneChip Expression Analysis Technical Manual,* (P/N 900223 or 900365 for CD-ROM version) available online at www.affymetrix.com).

Chapter 3

Protocols

Chapter 3

Protocol 1: Concentrating Purified Cellular RNA

STARTING MATERIAL

The following protocol is meant as a guide to concentrate the RNA obtained using the PAXgene Blood RNA Kit (see Appendix B on page 31 or Appendix C on page 35). The purified RNA isolated by these methods may be too dilute for the GeneChip Target Labeling Assay and, therefore, may require concentration prior to starting the Globin- Reduction Protocol (see Protocol 2 on page 21).

Taking sample recovery into account, at least 8 µg cellular RNA should be used as starting material per donor. To achieve this, RNA eluates from several PAXgene Blood RNA Tubes must be pooled taking care not to exceed the binding capacity of 45 µg of RNA per RNA Concentration Spin Column.

If several different RNA samples are to be compared, similar amounts should be used in the procedures for target preparation and GeneChip array analysis. For whole blood total RNA samples purified by methods other than the PAXgene Blood RNA kit, the RNA can also be concentrated with this protocol.

NOTE

To assure the accurate estimation of RNA amount, RNA isolated from whole blood should be digested with DNase prior to this procedure if DNase digestion was not already performed. Follow the procedure in Appendix D (on page 39) to DNase-digest the RNA. The DNase is then removed during the RNA concentration procedure.

The RNA concentration procedure allows processing of a maximum of 200 µL RNA eluate. If 8 µg RNA is present in a volume greater than 200 µL, increase the amount of RNA Binding Buffer and ethanol in the procedure proportionally (see steps 3 and 4 of the procedure). Using 8 µg RNA in the concentration procedure the expected yield is at least 5.6 µg of concentrated RNA. Up to 9 µL of the 10 µL concentrated RNA is used for subsequent target preparation (Protocol 2).

Table 3.1
Concentration of RNA Using the GeneChip® Blood RNA Concentration Kit

Sample	Volume	Total RNA
Pooled RNA eluates from 3 PAXgene Blood RNA Tubes	Approximately 200 µL (using the PAXgene Blood RNA System)	At least 8 µg[a]
Sample after RNA concentration procedure	10 µL	At least 5.6 µg
RNA used for target preparation	Up to 9 µL	5 µg

a: In >95% of cases, RNA purified from 3 PAXgene Blood RNA Tubes provides sufficient RNA. For details, visit www.preanalytix.com/pdf/TN_YieldsOfRNA_PAX_1203_HR.pdf.

IMPORTANT POINTS BEFORE STARTING

- RNA Binding Buffer may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature (15–25°C).
- RNA Binding Buffer contains a guanidine salt and is therefore not compatible with disinfecting reagents containing bleach.
- Unless otherwise indicated, all steps of the procedure, including centrifugation, should be performed at room temperature (15–25°C). During the procedure, work without interruption.
- If the RNA samples were not treated with DNase during the purification procedure, we strongly recommend that you follow the protocol in Appendix D (on page 39) for DNase digestion before starting the RNA concentration procedure.

THINGS TO DO BEFORE STARTING

- RNA Wash Buffer is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%), as indicated on the bottle, to obtain a working solution.
- Prepare a stock solution of 80% ethanol. For example, pipet 4 mL RNase-free water (supplied) and 16 mL of 96–100% ethanol into an appropriately sized RNase-free tube and mix to give 20 mL of 80% ethanol, which should be sufficient for 30 samples.

PROCEDURE

1. Set a heating block or water bath to 65°C for use in step 10.

   If the entire concentrated RNA resulting from this protocol will be used for GeneChip target preparation, this step can be skipped. In this case, denaturation (step 10) is carried out in step 3 of Protocol 2. It is not necessary to denature the eluate more than once: it remains denatured after freezing and thawing.

2. Pipet the pooled RNA eluate from one donor (8–45 µg RNA) into a 2 mL tube (not supplied).

3. Adjust the volume of the eluate to 200 µL using Buffer BR5 (supplied). Add 700 µL RNA Binding Buffer, and mix thoroughly by pipetting up and down several times.

NOTE **If the eluate volume exceeds 200 µL, increase the volume of RNA Binding Buffer proportionally (e.g., if the eluate volume is 220 µL, add 770 µL RNA Binding Buffer).**

4. Add 500 µL of 96–100% ethanol to the diluted eluate, and mix thoroughly by pipetting up and down several times. Do not centrifuge. Continue immediately with step 5.

NOTE **If the eluate volume (see step 3) exceeds 200 µL, increase the volume of 96–100% ethanol proportionally (e.g., if the eluate volume is 220 µL, add 550 µL of 96–100% ethanol).**

5. Pipet 700 µL of the sample into an RNA Concentration Spin Column placed in a 2 mL collection tube (supplied). Close the lid of the spin column gently, and centrifuge for 15 seconds at 8000 x g (10,000 rpm). Discard the flow-through. Pipet the remaining sample (up to 700 µL) into the spin column and repeat the centrifugation. Discard the flow-through and collection tube.
6. Place the spin column in a new 2 mL collection tube (supplied). Pipet 500 µL RNA Wash Buffer into the spin column. Close the lid of the spin column gently, and centrifuge for 15 seconds at 8000 x g (10,000 rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step 7.

NOTE **RNA Wash Buffer is supplied as a concentrate. Ensure that ethanol is added to RNA Wash Buffer before use (see *Things to do before starting on page 18*).**

7. Pipet 500 µL of 80% ethanol into the spin column. Close the lid of the spin column gently, and centrifuge for 2 minutes at 8000 x g (10,000 rpm). Discard the flow-through and collection tube.

NOTE **After centrifugation, take care to avoid ethanol carryover: carefully remove the spin column from the collection tube so that the spin column does not come into contact with the flow-through.**

8. Place the spin column in a new 2 mL collection tube (supplied). Open the lid of the spin column, and centrifuge in a microcentrifuge at full speed for 5 minutes to dry the spin column membrane. Discard the flow-through and collection tube.

   To avoid damage to the lids, place the spin columns into the centrifuge so that at least one empty position is between each spin column. Position the lids so that they point in the opposite direction to the rotation of the rotor (i.e., if the rotor rotates clockwise, orient the lids in a counter-clockwise direction).

   It is important to dry the spin column membrane, since residual ethanol may interfere with downstream reactions. Centrifugation with opened lids ensures that no ethanol is carried over during elution in step 9.

9. Place the spin column in a 1.5 mL collection tube (supplied). Pipet 12 µL of Buffer BR5 directly onto the center of the spin column membrane. Close the lid of the spin column gently, and centrifuge for 1 minutes at maximum speed to elute the RNA. The dead volume of the RNA Concentration Spin Column is 2 µL (i.e., elution with 12 µL Buffer BR5 gives 10 µL eluate).

NOTE **Buffer BR5 may not be substituted by other solutions, e.g. water. GeneChip results will be significantly worse if Buffer BR5 is not used.**

10. Incubate the eluate for 5 minutes at 65°C in a heating block or water bath, and then chill immediately on ice. Denaturation of the eluate is essential for maximum efficiency in subsequent enzymatic reactions.

NOTE **If the entire eluate will be used for GeneChip target preparation, this step can be skipped. In this case, denaturation is carried out in step 3 of Protocol 4. It is not necessary to denature the eluate more than once: it remains denatured after freezing and thawing.**

11. Briefly centrifuge the tube to collect the entire eluate.

12. Remove 1 µL of the eluate for RNA quantification. The expected RNA concentration is approximately 0.5µg/µL. For convenience and due to the limited amount of RNA, access to a NanoDrop spectrophotometer is advantageous but not essential. Use up to 9 µL of the eluate (5 µg of concentrated RNA) in Protocol 2.

Protocol 2: Preparing cRNA Targets with Integrated Globin Reduction

IMPORTANT POINTS BEFORE STARTING

- This protocol is optimized for use with 5 µg of starting blood total RNA. If less material is available the protocol may not yield enough cRNA for a single array hybridization.
- We strongly recommend using a thermal cycler for incubation of the reaction mixtures.
- If using RNA that has been purified using a different method than the PAXgene Blood RNA System, concentrate it according to Protocol 1.
- The volume of the spike-in GeneChip® poly-A RNA controls (obtained from the One-Cycle Target Labeling and Control Reagents) must not exceed 1 µL. Therefore, the concentration of the spike-in GeneChip® poly-A RNA controls must be twice what is recommended in the standard One-cycle Target Labeling Assay, since in the standard protocol the volume of each spiked poly-A RNA control is 2 µL.
- To check that the globin reduction procedure works successfully, use the Globin-reduction control RNAs (on page 9).
- The procedure for GeneChip target preparation with globin reduction is slightly different to the standard GeneChip Target Labeling Assay (see Table 3.7 on page 26). To ensure successful globin reduction, be sure to follow Protocol 2 exactly.
- Dilutions of Globin-Reduction PNA may not be stored. Prepare fresh dilutions for each experiment, as described in the section *Things to do before starting on page 22*.
- Read the *GeneChip® Expression Analysis Technical Manual* carefully before starting the protocol.

THINGS TO DO BEFORE STARTING

1. Prepare the PNA Working Solution.

PNAs purchased from Applied Biosystems are supplied lyophilized and need to be reconstituted as Globin-Reduction PNA stock solutions (see Appendix A on page 27 for details). We strongly recommend following the instructions in Appendix A for directions on handling and storing the reconstituted PNAs.

A. Before diluting Globin-Reduction PNA stock solutions, incubate the stock solutions at room temperature (15–25°C) for 10 minutes; during incubation, pulse-vortex the PNAs 3 times. Then incubate at 50°C for 10 minutes, vortex, and centrifuge briefly. Leave the PNAs at room temperature until use.

NOTE **To ensure successful globin reduction, be sure to follow the recommendations for reconstituting, quantifying, handling and storing of PNAs exactly (see Appendix A on page 27).**

B. Prepare fresh dilutions of the four different PNA stock solutions before each experiment according to Table 3.2.

Table 3.2
Concentrations of Individual PNA Working Stocks

PNA	Concentration (µM)
Globin-Reduction PNA 1	28.8
Globin-Reduction PNA 2	48.0
Globin-Reduction PNA 3	48.0
Globin-Reduction PNA 4	48.0

To prepare a diluted PNA solution, use the formula:

$$V_{Diluted} = V_{Stock} \times C_{Stock} / C_{Diluted}$$

where:

$V_{Diluted}$ = Final volume of diluted PNA solution

V_{Stock} = Volume of (approximately 100 µM) PNA stock solution to use

C_{Stock} = Measured concentration of (approximately 100 µM) PNA stock solution (e.g., 108 µM); to determine PNA stock concentrations, see Appendix A on page 27

$C_{Diluted}$ = Desired concentration of diluted PNA solution (e.g., 48 µM)

For example, when preparing a diluted PNA solution with a concentration of 48 µM, the calculation is as follows:

$$V_{Diluted} = 2\ \mu L \times 108\ \mu M / 48\ \mu M$$

$$V_{Diluted} = 4.5\ \mu L$$

With these values, the user needs to add 2 µL of 108 µM PNA stock solution to a tube, and add RNase-free water to a final volume of 4.5 µL. The final concentration of PNA would then be 48 µM.

C. Prepare Globin-Reduction PNA master mix by adding equal amounts of diluted Globin-Reduction PNAs 1–4 to a tube, and mix by vortexing. For each globin- reduction reaction, 1 µL of the master mix is required. After vortexing, briefly centrifuge the tube to collect all liquid at the bottom of the tube. Keep the master mix at room temperature.

NOTE

To avoid inaccuracies, we do not recommend pipetting volumes less than 2 µL. We recommend pipetting, for example, 2 µL of each PNA to give a master mix of 8 µL. The master mix must be prepared fresh. Do not store the master mix or the individual PNA dilutions.

2. Prepare dilutions of poly-A RNA controls.
(Poly-A RNA controls are provided with the One-Cycle Target Labeling and Control Reagents)

Make dilutions with Dil. Buffer (supplied with the poly-A controls) according to Table 3.3, below.

Table 3.3
Preparation of Poly-A RNA Controls for both blood RNA and the globin-reduction RNA control reactions.

	Serial dilutions		
Starting amount of total RNA	**First**	**Second**	**Third[a]**
5 µg (blood RNA)	1/20	1/50	1/5
2 µg (globin-reduction control reactions)	1/20	1/50	1/12.5

a: When preparing cRNA targets with integrated globin reduction and globin-reduction control reactions, the volume of poly-A RNA control per reaction must be 1 µL. Therefore, the final dilution of the poly-A RNA controls should result in a solution that is twice as concentrated as the final dilution used in standard target preparation (without globin reduction). For details, Important Points Before Starting on page 21 and Table 3.7 on page 26.

PROCEDURE

1. Thaw the reagents needed for this protocol. These include RNA, globin-reduction control RNAs (optional) and reagents for first-strand cDNA synthesis. Keep the PNAs at room temperature (15–25°C). Keep the other reagents on ice until use.
2. Prepare sample tube(s) by adding following components in a 0.2 mL PCR tube, then mix by vortexing.

Table 3.4
Addition of diluted Poly-A Control RNA to blood RNA sample (tube 1) prior to denaturation

Component	Control	Samples
RNA	9 µL (2 µg)	up to 9 µL (5 µg)
Diluted Poly-A controls*	1 µL	1 µL
Buffer BR5	-	Adjust to 10 µL

* Do not exceed 1µL. For details see *Important points before starting on page 18* and Table 3.2 on page 22

3. Place the sample tubes in a thermal cycler. Incubate at 65°C for 5 minutes, and then chill immediately on ice.
4. Place sample tube(s) at room temperature, then add the following components and mix by vortexing.

NOTE **Do not prepare a master mix containing the T7-(dT) primer and Globin-Reduction PNA working solution. Pipet components in the order listed in the table.**

Table 3.5
Sample Annealing Reaction con

Component	Reaction with Globin Reduction	Reaction without Globin Reduction
T7-(dT) primer (50 µM)	1 µL	1 µL
Globin -Reduction PNA working solution	1 µL	--
RNase-free Water	--	1 µL

NOTE **Prepare these reaction mixes at room temperature. Do not put them on ice.**

5. Place the sample tube(s) in a thermal cycler preheated to 70°C, and incubate at 70°C for 10 min.

6. During incubation, prepare on ice the first strand synthesis reaction mixture in a 0.2 mL tube containing the following components.

Table 3.6
First Strand cDNA Synthesis Components (for each reaction)

Component	Volume	Final Concentration or Amount in Reaction
5x First-strand cDNA Buffer[a]	4 µL	1x
0.1 M DTT[a]	2 µL	10 mM
10 mM dNTP Mix[a]	1 µL	500 µM each dNTP
SuperScript II RT[a] (200 U/µL)	1 µL	200 U
Total Volume	8 µL	

a: Components of the One-Cycle Target Labeling and Control Reagents.

7. Mix the reaction mixture by vortexing, and place on ice until use in step 8.
8. Cool down the thermal cycler with sample tube(s) to 45°C. Place tube containing the reaction mixture into the same thermal cycler. Incubate all tubes at 45°C for 5 min. Pipet 8 µL of the preheated reagent mixture into each sample tube (12 µL) while both are incubating at 45°C. Mix well by flicking the tube several times. Centrifuge briefly (approximately 3 seconds) and immediately return the tube to 45°C.
9. Cool down the thermal cycler to 42°C, and incubate for 60 minutes to carry out the first-strand cDNA synthesis.
10. Carry out second-strand cDNA synthesis, cDNA cleanup, cRNA synthesis, and cRNA cleanup according to the *GeneChip Expression Analysis Technical Manual*. Use entire purified cDNA in the *in vitro* transcription (IVT) reaction.

COMPARISON OF THE GLOBIN-REDUCTION PROTOCOL TO THE STANDARD GENECHIP TARGET LABELING ASSAY

The following tables summarize the differences between the target preparation procedure of the Globin-Reduction Protocol and that of the standard procedure (without Globin-Reduction):

Table 3.7
Comparison of GeneChip® Target Preparation with Integrated Globin Reduction and the Target Preparation Procedure According to the Standard Affymetrix Protocol

	Globin-Reduction Protocol	GeneChip Target Labeling Assay[a]
Solution for dissolving input RNA	Buffer BR5	Variable (e.g., water)
Amount of input RNA	5 µg RNA in Buffer BR5	1–15 µg cellular RNA
Maximum volume of input RNA	Up to 9 µL	Up to 8 µL
Dilution steps of GeneChip Poly-A RNA controls		
• Starting with 5 µg blood RNA	1/20; 1/50; 1/5	1/20; 1/50; 1/10
• Globin-reduction control reactions (2 µg RNA)	1/20; 1/50; 1/12.5	1/20; 1/50; 1/25
Input volume of diluted poly A controls	1 µL (2-fold concentration)	2 µL
Incubation of RNA with poly-A RNA controls	Incubation for 5 minutes at 65°C, then chill on ice	None
Input volume of T7-(dT) primer (50 µM)	1 µL (final amount of 50 pmol)	2 µL (final amount of 100 pmol)
Use of globin-reduction PNA master mix	Yes	No
Preparation of first-strand master mix	With SuperScript® II	Without SuperScript II
Incubation steps for first-stand cDNA synthesis	10 minutes at 70°C; 5 minutes at 45°C; Add prewarmed (to 45°C) first-strand master mix with SuperScript II; 1 h at 42°C; Hold at 4°C	10 minutes at 70°C; 2 minutes at 4°C; Add first-strand master mix; 2 minutes at 42°C; Add SuperScript II; 1 h at 42°C; Hold at 4°C

a: According to standard Affymetrix protocol.

Appendix A

Reconstituting, Quantifying, Handling, and Storing Lyophilized PNAs

Appendix A

Introduction

PNAs from Applied Biosystems are supplied lyophilized and need to be reconstituted. Follow the instructions in this appendix before starting the globin-reduction procedure in Protocol 2 on page 21.

REAGENTS TO BE SUPPLIED BY USER

- PNAs from Applied Biosystems (see page 13 for sequences).

IMPORTANT POINT BEFORE STARTING

- Read the documentation supplied with the PNAs.

PROCEDURE

1. Centrifuge the tubes before opening them in order to collect lyophilized PNA at the bottom of the tubes.
2. Add RNase-free water (supplied with this kit) to each tube to obtain an approximately 100 µM solution of each PNA. The volume of RNase-free water to add is specified in the documentation supplied with each PNA.
3. Incubate the PNAs at room temperature (15–25°C) for 10 min. During incubation, pulse-vortex the PNAs 3 times.
4. Incubate the PNAs at 50°C for 10 min. Then vortex for 5 seconds and briefly centrifuge. For immediate use, keep at room temperature.
5. Since PNAs can be difficult to dissolve, make sure that the lyophilized PNAs are completely dissolved in the RNase-free water. To check that the PNAs are completely dissolved, measure their concentrations by spectrophotometry.
   A. Add 5 µL of reconstituted PNA solution to 495 µL deionized water.
   B. Measure absorbance at 260 nm (A_{260}).
   C. Calculate PNA concentration according to the following formula:

      Concentration (µM) = (A_{260} / Molar extinction coefficient) x Dilution factor

      Thus, for each PNA, the calculation is:

      Globin-Reduction PNA 1: Concentration (µM) = (A_{260} / 0.1629) x 100

      Globin-Reduction PNA 2: Concentration (µM) = (A_{260} / 0.1609) x 100

      Globin-Reduction PNA 3: Concentration (µM) = (A_{260} / 0.1425) x 100

      Globin-Reduction PNA 4: Concentration (µM) = (A_{260} / 0.1558) x 100
6. Prepare aliquots of the Globin-Reduction PNA stock solutions in polypropylene tubes. If using the stock solutions within 14 days, store at 4°C. For longer storage, store at –20°C.

   Before use, the Globin-Reduction PNA stock solutions must be heated and

diluted as described in Protocol 2 on page 21. Fresh working dilutions need to be prepared before each use.

Aliquots of PNA stock solutions can be heated up to 10 times without affecting performance.

Appendix B

Collecting Human Whole Blood and Purifying Cellular RNA Using the PAXgene Blood RNA Kit

Appendix B

Introduction

To guarantee sufficient yields of RNA for GeneChip array analysis, at least 8 µg of cellular RNA per donor is required. Internal evaluation of typical RNA yields from healthy donors have shown that 8 µg of cellular RNA can usually be achieved by collecting blood from each donor into 3 PAXgene Blood RNA Tubes. In >95% of cases, this will ensure that there will be sufficient RNA. For detailed study information on RNA yields from PAXgene Blood RNA Tubes, visit www.preanalytix.com/pdf/TN_YieldsOfRNA_PAX_1203_HR.pdf.

THINGS TO DO BEFORE STARTING

IMPORTANT !

BEFORE STARTING: Read the PAXgene Blood RNA Tube Product Circular and the PAXgene Blood RNA Kit Handbook (February 2004), paying careful attention to the safety information. These items of literature are included with the corresponding PAXgene products and can also be downloaded from www.preanalytix.com and www.qiagen.com.

See the *PAXgene Blood RNA Kit Handbook* for things to do before starting.

Prepare DNase I for the on-column DNase digestion according to the protocol in Appendix A of the *PAXgene Blood RNA Kit Handbook*.

PROCEDURE

1. Collect blood from a donor in 3 PAXgene Blood RNA Tubes according to the *PAXgene Blood RNA Tube Product Circular*. If storing the collected blood for longer than 24 hours, we strongly recommend storage at 2–8°C. If storing blood for longer than 3 days, we strongly recommend storage at –20°C.
2. Purify RNA from the 3 tubes by following steps 1 to 9 of the protocol in the *PAXgene Blood RNA Kit Handbook* (February 2004).
3. After performing step 9, carry out the on-column DNase digestion using the RNase-Free DNase Set according to Appendix A of the *PAXgene Blood RNA Kit Handbook*.
4. Continue the protocol in the *PAXgene Blood RNA Kit Handbook* from step 11 up to step 14. When performing step 13, apply 35 µL Buffer BR5 to the spin column membrane instead of the recommended 40 µL. When performing step 14, apply a second 35 µL of Buffer BR5.

NOTE

This modification of steps 13 and 14 allows concentration in Protocol 1 of the pooled RNA eluates obtained from the 3 PAXgene Blood RNA Tubes. It is not necessary to perform step 15 of the protocol in the PAXgene Blood RNA Kit Handbook (i.e., the RNA denaturation step), since the RNA will be denatured in Protocol 1.

5. Pool the 3 RNA eluates from the same donor. Use the pooled eluates as the starting material in Protocol 1 (on page 17).

NOTE

Since RNA is eluted from each spin column using 2 x 35 µL Buffer BR5, the combined eluate volume will be less than 70 µL due to the dead volume of the spin column. Therefore, when combining the eluates from 3 spin columns, the combined eluate volume does not exceed 200 µL.

Appendix C

Collecting Human Whole Blood and Purifying Cellular RNA Using the PAXgene 96 Blood RNA Kit

Appendix C

Introduction

To guarantee sufficient yields of RNA for GeneChip array analysis, at least 8 µg of cellular RNA per donor is required. Internal evaluation of typical RNA yields from healthy donors have shown, that 8 µg of cellular RNA can usually be achieved by collecting blood from each donor into 3 PAXgene Blood RNA Tubes. In >95% of cases, this will ensure that there will be sufficient RNA. For detailed study information on RNA yields from PAXgene Blood RNA Tubes, visit www.preanalytix.com/pdf/TN_YieldsOfRNA_PAX_1203_HR.pdf.

THINGS TO DO BEFORE STARTING

IMPORTANT !

BEFORE STARTING: Read the PAXgene Blood RNA Tube Product Circular and the PAXgene 96 Blood RNA Kit Handbook (August 2003), paying careful attention to the safety information. These items of literature are included with the corresponding PAXgene products and can also be downloaded from www.preanalytix.com and www.qiagen.com.

See the *PAXgene 96 Blood RNA Kit Handbook* for things to do before starting.

PROCEDURE

1. Collect blood from each donor in 3 PAXgene Blood RNA Tubes according to the *PAXgene Blood RNA Tube Product Circular*.

   If storing the collected blood for longer than 24 hours, we strongly recommend storage at 2–8°C. If storing blood for longer than 3 days, we strongly recommend storage at –20°C.

2. Purify RNA from each tube by following steps 1 to 24 of the protocol in the *PAXgene 96 Blood RNA Kit Handbook* (August 2003). When performing step 22, apply 40 µL Buffer BR5 to each well instead of the recommended 45–60 µL. When performing step 23, apply a second 40 µL of Buffer BR5 to each well.

NOTE

This modification of steps 22 and 23 allows concentration in Protocol 3 of the pooled RNA eluates obtained from the 3 PAXgene Blood RNA Tubes. It is not necessary to perform step 25 of the protocol in the PAXgene 96 Blood RNA Kit Handbook (i.e., the RNA denaturation step), since the RNA will be denatured in Protocol 1.

3. Pool the 3 RNA eluates from each donor. Use the pooled eluates (total volume of approximately 200 µL) as the starting material in Protocol 1 (on page 17).

NOTE

Since RNA is eluted from each well using 2 x 40 µL Buffer BR5, the combined eluate volume will be less than 80 µL due to the dead volume of the spin column. Therefore, when combining the eluates from 3 wells, the combined eluate volume does not exceed 200 µL.

Appendix D

DNase Digestion of RNA Before RNA Concentration

Appendix D

Introduction

This optional procedure is for DNase digestion of RNA that was purified using a procedure without integrated DNase digestion (see Protocol 1, on page 17). It is recommended to perform the DNase digestion prior to the pooling of RNA samples from the same donor.

REAGENTS TO BE SUPPLIED BY USER

- RNase-Free DNase Set[1]

IMPORTANT POINTS BEFORE STARTING

- Read the product sheet supplied with the RNase-Free DNase Set.
- Buffer RDD supplied with the RNase-Free DNase Set is optimized for on-column DNase digestion. Buffer RDD is also well-suited for efficient DNase digestion in solution.
- Do not vortex reconstituted DNase I, since it is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube.
- For long-term storage of reconstituted DNase I, divide it into single-use 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.

THINGS TO DO BEFORE STARTING

- Prepare DNase I stock solution when using the RNase-Free DNase Set for the first time. Dissolve the solid DNase I (1500 Kunitz units) in 550 µL of the RNase-free water provided. Take care that no DNase I is lost when opening the vial. Mix gently by inverting the tube. Do not vortex.

[1] *See Ordering Information on page 47.*

PROCEDURE

1. Into a microcentrifuge tube, add 87.5 µL RNA eluate, 10 µL Buffer RDD, and 2.5 µL DNase I stock solution. Adjust the total volume to 100 µL using RNase-free water.

NOTE **If necessary, the total volumes can be doubled to 200 µL.**

2. Incubate on the benchtop (20–25°C) for 10 min.
3. Proceed to Protocol 1 on page 17 to concentrate the RNA.

NOTE **Do not heat-inactivate the DNase, as this may cause RNA degradation.**

Appendix **E**

Troubleshooting Guide

Appendix E

Troubleshooting Guide

This troubleshooting guide may be helpful in problems that may arise with the globin reduction process. For additional troubleshooting guidance associated with the standard GeneChip Target Labeling Assay please refer to Section 5, Appendix B of the *GeneChip Expression Analysis Technical Manual.*

Table E.1
Table Title

Problem/Possible Cause	Comments and Suggestions
Low cRNA Yield	
Insufficient amount of starting material	At least 8 µg of total whole blood RNA is recommended before proceeding with the concentration protocol (Protocol 1). Approximately 5.6ug of total RNA will be available for labeling following sample concentration. Starting with a minimum of 3 PAXgene collection tubes will ensure sufficient material for the assay in >95% of healthy donors.
Excess amount of starting material	In subsequent preparations, reduce amount of starting material. A maximum of 45 µg total RNA can be purified using Protocol 1. Use 5 µg total RNA in the Globin-Reduction Protocol (Protocol 2).
Inefficient cDNA synthesis	Make sure to elute the concentrated RNA in Buffer BR5 (Protocol 1). Do not use water or any other buffer (e.g., Tris). Use Globin-Reduction RNA controls and/or Poly-A Spike Controls to ensure the cDNA and cRNA synthesis steps are processed properly.
RNA not pure	Concentrate and clean-up the RNA following Protocol 1.
Inadequate concentration procedure	If methods other than the GeneChip® Blood RNA Concentration Kit have been used for RNA concentration, the resulting cRNA yield may be low and may lead to poor array results. Follow Protocol 1 for sample concentration.
Insufficient yield after sample concentration	
Ethanol carryover in concentration protocol	After the 80% ethanol wash step, be sure to dry the silica-gel membrane by centrifugation at full speed for 5 minutes, as described in the protocol. Following centrifugation, remove the Spin Column from the centrifuge tube carefully so the column does not contact the flow-through as this will result in carryover of the ethanol.
Centrifugation temperature too low in concentration protocol	Centrifugation at low temperatures (e.g., under refrigeration) can cause precipitates to form that can clog the Spin Column. All steps of the protocol, including centrifugation, should be performed at room temperature.

Table E.1
Table Title

Problem/Possible Cause	Comments and Suggestions
Inefficient globin reduction	
Use of fetal blood	The PNA oligomer sequences are specific to the alpha and beta globin genes that are predominantly expressed in adult reticulocytes. In fetal blood, a fetal globin form is preferentially expressed. There is sufficient sequence divergence between the adult and fetal genes that the current PNA oligomer sequences are not expected to target fetal globin.
Low quality of PNAs	For best results use high quality PNAs. We have observed optimal results using PNAs ordered from Applied Biosystems, Inc.
Insufficient PNA amount in globin reduction procedure	Make sure to dissolve the PNA oligomers carefully before use. Therefore, incubate the solutions for 10 minutes at room temperature and afterwards for 10 minutes at 50°C while vortexing several times. Keep at room temperature until use. After resuspending lyophilized PNAs, incubate as described above and determine the concentration spectrophotometrically. A detailed description of how to do this is given in Appendix A.
Wrong determination of PNA concentration	Measure the PNA concentration according to Appendix A of the handbook using the extinction coefficients provided.
PNA working solutions too old	Prepare fresh dilutions of the four different PNA stock solutions before each experiment according to Table 3.6. Do not store PNA working solutions.
Too much input RNA	Use 5 µg total RNA in the Globin-Reduction Protocol
Use of non-human starting material	Make sure to use human blood or blood containing biological material for RNA isolation. PNA sequences used for the Globin-Reduction Protocol were not tested with animal globin mRNA.
Poor 3´/5´ Ratios in array analysis	
RNA degraded	Although all buffers have been tested and guaranteed RNase-free, RNases can be introduced during use. Be certain not to introduce any RNases during the procedure or later handling Do not put RNA samples into a vacuum dryer or microcentrifuge that has been used in DNA preparation where RNases may have been used. To determine starting RNA quality perform quality control (gel, Bioanalyzer analysis) of the RNA before starting the Globin-Reduction Protocol.

Appendix **F**

Ordering Information

Appendix F

Supplies from QIAGEN® Companies and QIAGEN

Table F.1
Supplies from QIAGEN Companies

Product	Contents	P/N
PAXgene Blood RNA System — for blood collection, and RNA stabilization and purification		
PAXgene Blood RNA Tubes (100)	100 PAXgene Blood RNA Tubes; to be used with the PAXgene Blood RNA Kit or PAXgene 96 Blood RNA Kit	762115[a] 762105[b] 762125[c]
PAXgene Blood RNA Kit (50)	For 50 RNA preps: 50 PAXgene RNA Spin Columns, Buffers, Proteinase K, and Processing Tubes; to be used with PAXgene Blood RNA Tubes	762134
PAXgene Blood RNA Validation Kit (10)	For 10 RNA preps: 10 PAXgene Blood RNA Tubes, 10 PAXgene RNA Spin Columns, Buffers, Proteinase K, and Processing Tubes	762132
PAXgene 96 Blood RNA Kit (4)	For 4 x 96 RNA preps: 4 PAXgene 96 RNA Plates, 4 PAXgene 96 Filter Plates, Buffers, Proteinase K, RNase-Free DNase Sets, AirPore Tape Sheets, Collection Vessels; to be used with PAXgene Blood RNA Tubes	762331
RNase-Free DNase Set — for DNase digestion during RNA purification		
RNase-Free DNase Set (50)[d]	1500 Units RNase-Free DNase I, RNase-Free Buffer RDD, and RNase-Free Water for 50 RNA minipreps	79254

a: Canada and USA.
b: Japan.
c: All other countries.
d: For use with the PAXgene Blood RNA Kit (50) and the PAXgene Blood RNA Validation Kit (10)

Supplies from Affymetrix

Table F.2
Supplies from Affymetrix

Product	Contents	P/N
One-Cycle Target Labeling and Control Reagents — for GeneChip target preparation		
One-Cycle Target Labeling and Control Reagents	Sufficient for 30 reactions	900493