Tempus™ Blood RNA Tube and Large Volume Consumables

Stabilization and Isolation of Total RNA from Whole Blood

Protocol

Applied Biosystems

For Research Use Only. Not for use in diagnostic procedures.

NOTICE TO PURCHASER: LICENSE DISCLAIMER

TRADEMARKS:

Part Number 4345218 Rev. D
2/2005

Contents

Preface

Chapter 1 Introduction

Chapter 2 Collecting and Storing Blood Samples

Chapter 3 Assembling the Large Volume Format Consumables

Chapter 4 Extracting RNA From Whole Blood

Preface

This preface covers:

Safety

Safety Alert Words

Four safety alert words appear in Applied Biosystems user documentation. Each word implies a particular level of observation or action, as described below:

IMPORTANT! Indicates information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

CAUTION Indicates a potentially hazardous situation that, if not avoided, can result in minor or moderate injury. It can also alert against unsafe practices, damage to an instrument, or loss of data.

WARNING Indicates a potentially hazardous situation that, if not avoided, can result in serious injury or death.

DANGER Indicates an imminently hazardous situation that, if not avoided, will result in serious injury or death. This signal word is to be limited to the most extreme situations.

Chemical Hazard Warning

WARNING **CHEMICAL HAZARD**. Some of the chemicals used with Applied Biosystems instruments and protocols are potentially hazardous and can cause injury, illness, or death.

Chemical Safety Guidelines

To minimize the hazards of chemicals:

- Read and understand the MSDSs provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. See "About MSDSs."
- Minimize contact with chemicals. When handling chemicals, wear appropriate personal protective equipment such as safety glasses, gloves, and protective clothing. For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, a fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the cleanup procedures recommended in the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to *new* customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining MSDSs

You can obtain from Applied Biosystems the MSDS for any chemical supplied by Applied Biosystems. This service is free and available 24 hours a day.

To obtain MSDSs:

1. Go to **https://docs.appliedbiosystems.com/msdssearch.html**
2. In the Search field, type in the chemical name, part number, or other information that appears in the MSDS of interest. Select the language of your choice, then click **Search**.
3. Find the document of interest, right-click the document title, then select any of the following:
   - **Open** – To view the document
   - **Print Target** – To print the document

- **Save Target As** – To download a PDF version of the document to a destination that you choose

4. To have a copy of a document sent by fax or e-mail, select **Fax** or **Email** to the left of the document title in the Search Results page, then click **RETRIEVE DOCUMENTS** at the end of the document list.
5. After you enter the required information, click **View/Deliver Selected Documents Now**.

Chemical Waste Hazard

WARNING CHEMICAL WASTE HAZARD. Some wastes produced by the operation of the instrument or system are potentially hazardous and can cause injury, illness, or death.

Chemical Waste Guidelines

To minimize the hazards of chemical waste:

- Read and understand the MSDSs for the chemicals in a waste container before you store, handle, or dispose of chemical waste.
- Provide primary and secondary waste containers.
- Minimize contact with and inhalation of chemical waste. When handling chemicals, wear appropriate personal protective equipment such as safety glasses, gloves, and protective clothing.
- Handle chemical wastes in a fume hood.
- After you empty a chemical waste container, seal it with the cap provided.
- Dispose of the contents of a waste container in accordance with good laboratory practices and local, state/provincial, and/or national environmental and health regulations.

Site Preparation and Safety Guide

A site preparation and safety guide is a separate document sent to all customers who have purchased an Applied Biosystems instrument. Refer to the guide written for your instrument for information on site preparation, instrument safety, and chemical safety.

Waste Disposal

If potentially hazardous waste is generated when you operate the instrument, you must:

- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure the health and safety of all personnel in your laboratory.

- Ensure that the instrument waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.

Note: Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological Hazard Safety

BIOHAZARD. Biological samples such as tissues, body fluids, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective eyewear, clothing, and gloves. Read and follow the guidelines in these publications:

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (stock no. 017-040-00547-4, http://bmbl.od.nih.gov)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR §1910.1030, http://www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html).

Additional information about biohazard guidelines is available at: **http://www.cdc.gov**

How to Obtain Support

For the latest services and support information for all locations, go to **http://www.appliedbiosystems.com**, then click the link for **Support**.

At the Support page, you can:

- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents
- Download PDF documents
- Obtain information about customer training
- Download software updates and patches

In addition, the Support page provides access to worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.

Introduction 1

This chapter covers:

Product Overview

Purpose

Gene expression measurements in human whole blood are becoming an increasingly important research tool. However, isolating high quality RNA from human whole blood is complicated by the instability of gene expression profiles in blood stored at room temperature.

With the Tempus™ Blood RNA Tube, you can draw blood directly into a reagent that stabilizes RNA at room temperature for up to five days. A set of reagents and disposables, called large-volume format consumables, allow you to extract 2–8 µg of high quality RNA per milliliter of whole blood within 45–50 minutes from up to six samples.

About Tempus Blood RNA Tubes

The Tempus™ Blood RNA Tube (PN 4342792) contains 6 mL of Applied Biosystems Stabilizing Reagent, which effectively lyses blood cells. After the blood is drawn into the tube and mixes with the reagent, lysis occurs almost immediately. The stabilizing reagent inactivates cellular RNases and selectively precipitates RNA; genomic DNA (gDNA) and proteins remain in solution.

Following blood draw into the Tempus Blood RNA Tube, novel RNA isolation chemistry allows you to purify high quality RNA without sample pretreatments such as leukocyte isolation or selective red blood cell (RBC) lysis.

About Large Volume Format Consumables

Using large-volume format consumables, you can isolate RNA from 3 mL of whole blood. The following is a list of large-volume format consumables required for this protocol:

- RNA Purification Wash Solution 1 (PN 4305891)
- RNA Purification Wash Solution 2 (PN 4305890)
- Nucleic Acid Purification Elution Solution (PN 4305893)
- AbsoluteRNA Wash Solution (PN 4305545)
- Splash Guards (PN 4311758)
- Large Volume Upgrade Starter Kit (PN 4344440)
  (1 Large Volume Adapter Plate, 1 2-mL Tube Collection Plate, 6 Large Volume RNA Prep Filters, 6 20-mL Reservoirs, 18 5-mL Reservoirs, 5 Cap Plugs)

You can also purchase individual components of the Large Volume Upgrade Starter Kit separately, as listed in "Materials and Equipment" on page 1-5.

Benefits of Tempus Blood RNA Tube Chemistry

Tempus Blood RNA Tube chemistry is the combination of collecting blood in Tempus Blood RNA tubes and purifying RNA through Applied Biosystems Total RNA chemistry. It has the following benefits:

- Applied Biosystems Stabilizing Reagent in Tempus Blood RNA tubes lyses whole blood cells and stabilizes RNA in a single step. No pretreatment of blood is required before purification of RNA from the sample.
- The large-volume format consumables make it possible to isolate RNA from larger starting volumes of blood.
- Extracted RNA is pure ($A_{260/280}$ ratio > 1.9), with very low levels of protein and gDNA contamination.
- You can extract up to 6–25 µg of RNA (from 3 mL blood).
- The gene expression profile of important gene targets (see References on page 2-13) is immediately frozen and remains stable for up to five days at room temperature and at least one week at 4 °C.

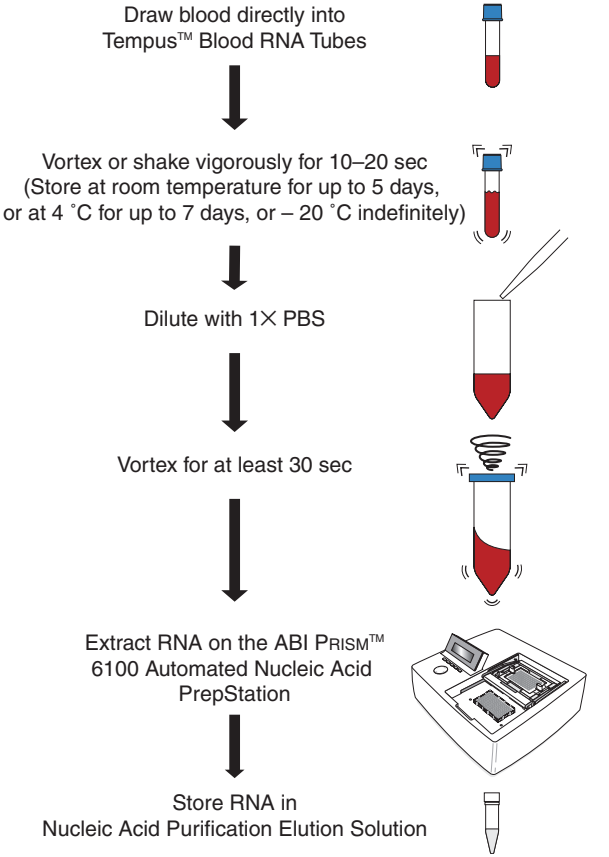
Protocol Overview

About This Protocol

This protocol describes the steps required to purify RNA from a 3-mL sample of whole blood collected in Tempus Blood RNA tubes. When the tubes are used with the large-volume format consumables, you can process six blood samples simultaneously on the ABI PRISM™ 6100 Automated Nucleic Acid PrepStation.

Procedure Flowchart

The following diagram provides an overview of the procedure for isolating RNA from human whole blood collected in Tempus tubes.



Materials and Equipment

Required Materials

The following tables list the equipment, accessories, and chemicals required to perform this protocol. Unless otherwise noted, many items listed can be obtained from a major laboratory supplier (MLS).

Disposables and reagents

Item	Supplier	Part Number
Disposables		
Large Volume Upgrade Starter Kit Each starter kit contains: • 1 Large Volume Adapter Plate • 1 2-mL Tube Collection Plate • 6 Large Volume RNA Prep Filters • 6 20-mL Reservoirs • 18 5-mL Reservoirs • 6 2-mL Microcentrifuge Tubes • 6 Microelution vials • 5 Slip-fit Cap Plugs **Note:** These items are also available individually, as listed in "Alternatives" on page 1-6.	Applied Biosystems	4344440
Microcentrifuge tubes, 2-mL **Note:** The 2-mL Tube Collection Plate is designed specifically to hold Applied Biosystems 2-mL microcentrifuge tubes with screw caps.	Applied Biosystems	4305936
Splash Guard	Applied Biosystems	4311758
Tempus™ Blood RNA Tube	Applied Biosystems	4342792
Sterile tubes (BD Falcon), 50-mL	MLS	–
Pipette tips	MLS	–
Pipettes, 5-mL, 10-mL, 25-mL	MLS	–

Item	Supplier	Part Number
Reagents		
Absolute RNA Wash Solution	Applied Biosystems	4305545
Nucleic Acid Purification Elution Solution	Applied Biosystems	4305893
RNA Purification Wash Solution 1	Applied Biosystems	4305891
RNA Purification Wash Solution 2	Applied Biosystems	4305890
Ethanol, 100%	MLS	–
Glycogen, 5 mg/mL	MLS	–
NaCl, 5M	MLS	–
1× Phosphate buffered saline (PBS), calcium/magnesium-free	MLS	–
RNase-Free Water	MLS	–
Alternatives		
Large Volume Adapter Plate (20/pk) Includes • 120 2-mL Microcentrifuge Tubes with Screw Caps • 10 Slip-fit Cap Plugs	Applied Biosystems	4344438
Large Volume RNA Prep Filter (50/pk)	Applied Biosystems	4344439
5-mL Reservoir (150/pk)	Applied Biosystems	4344437
20-mL Reservoir (50/pk)	Applied Biosystems	4344435
2-mL Tube Collection Plate	Applied Biosystems	4344436

Equipment

Equipment	Supplier
ABI PRISM™ 6100 Automated Nucleic Acid PrepStation	See your Applied Biosystems sales representative
Vortexer	MLS

Optional Materials

Item	Supplier	Part Number
High Capacity cDNA Archive Kit	Applied Biosystems	4322171
Montage PCR Centrifugal Filter	Millipore	136461

Documentation

Document	Part Number
ABI PRISM™ 6100 Automated Nucleic Acid PrepStation User's Manual	4326242
High Capacity cDNA Archive Kit Protocol	4322169
Isolation of Total RNA from Whole Blood and from Cells Isolated from Whole Blood Protocol	4332809

Note: For additional protocols, see the Applied Biosystems Web site, **www.appliedbiosystems.com**, and refer to the tutorials section under the Support menu.

Collecting and Storing Blood Samples

2

This chapter covers:

Collecting and Storing Blood in Tempus™ Blood RNA Tubes

Standard Procedures for Drawing Blood

Follow your laboratory's normal procedures for drawing blood from individuals into blood collection tubes containing liquid reagents. Observe the appropriate safety practices when collecting blood.

Tempus Blood RNA tubes are used for the collection of venous whole blood specimens to stabilize RNA prior to purification for gene expression profiling. Refer to the product documentation of your blood collection set for specific instructions on venipuncture technique and blood collection. If you are using the Greiner Vacuette® Safety Blood Collection Set, refer to Blood Collection Tube Instructions on the Vacuette Web site (**www.vacuette.com**) for additional information.

IMPORTANT! Tempus Blood RNA tubes are designed to collect 3 mL of blood. Because Tempus Blood RNA Tube chemistry is optimized to extract RNA from a 2:1 ratio of Applied Biosystems Stabilizing Reagent and blood, it is critical that a full 3 mL of blood is drawn into the tube.

BIOHAZARD SAFETY. For additional safety information, follow the precaution, cautions, and prevention instructions listed below for specimen collection.

Precaution

Do not use Tempus tubes if foreign matter is present!

Cautions

- Handle all biological samples and blood collection "sharps" (lancets, needles, luer adapters, and blood collection sets) according to the policies and procedures of your facility.
- Obtain appropriate medical attention in the case of any exposure to biological samples (for example, through a puncture injury), since they may transmit HIV (AIDS), viral hepatitis, or other infectious disease.
- Discard all blood collection "sharps" in biohazard containers approved for their disposal.

- Transferring a sample from a syringe to a tube is not recommended. Additional manipulation of sharps increases the potential for needle stick injury. In addition, depressing the syringe plunger during transfer can create a positive pressure, forcefully displacing the stopper and sample and causing a potential blood exposure. Using a syringe for blood transfer may also cause over or under filling of tubes, resulting in an incorrect blood-to-additive ratio and potentially incorrect analysis results.
- If blood is collected through an intravenous (IV) line, ensure that the line has been cleared of IV solution before beginning to fill blood collection tubes. This is critical to avoid erroneous laboratory data from IV fluid contamination.
- All liquid preservatives and anticoagulants are clear and colorless. Do not use if they are discolored or contain precipitates.
- Do not use tubes after their expiration date.

Prevention of Backflow

Most evacuated blood collection tubes contain chemical additives. Therefore it is important to avoid possible backflow from the tube, due to the possibility of adverse reactions in the individual. To prevent backflow from the tube into the individual's arm, observe the following precautions:

1. Place the individual's arm in a downward position.
2. Hold the tube with the cap uppermost.
3. Release the tourniquet as soon as the blood starts to flow into the tube.
4. Make sure the tube contents do not touch the cap or the end of the needle during venipuncture.

Stabilizing RNA in Blood Samples

During collection, vigorously shake or vortex the tube for 10–20 seconds immediately after drawing blood into it. Shaking ensures that the Applied Biosystems Stabilizing Reagent makes uniform contact with the sample.

IMPORTANT! Failure to mix the stabilizing reagent with the blood leads to inadequate stabilization of the gene expression profile and the formation of clots that clog the purification filter.

Storage and Transportation of Blood in Tempus Blood RNA Tubes

Applied Biosystems recommends that you store or ship Tempus Blood RNA tubes containing stabilized samples in the following order of preference:

Storage / Shipping Options	Temperature Requirement
Store or ship on dry ice. (Recommended) **IMPORTANT!** Avoid direct contact of sample with dry ice!	–20 to –80 °C
Store or ship refrigerated within 7 days or less.	4 °C
Store or ship at room temperature within 5 days or less.	18–25 °C

Stability of RNA from Blood Collected in Tempus Tubes

Effects of Exposure to High Temperatures

Prolonged exposure to temperatures in excess of 37 °C (> 1 hr) leads to degradation and zero yield of RNA. Exposing the tube containing blood and the diluted blood lysate to high temperatures for short periods of time (37 °C for 1hr) causes RNA to dissolve in the stabilizing reagent. As a result, the filter membrane fails to capture RNA, leading to lower yields. It is possible to reverse this effect and reprecipitate RNA by freezing the sample and processing the blood lysate after thawing.

Altered Gene Expression in Unstabilized Blood

Prolonged storage of fresh, whole blood at room temperature may affect the expression levels of a number of important gene targets (Tanner et al., 2002).

In the experiment shown in Figures 2-1, 2-2, and 2-3 (on pages 2-5 to 2-7), fresh, whole, human blood was stored at three time points (1 hr, 4 hr, and overnight) either at room temperature (RT) or 4 °C.

Blood samples were then lysed using Applied Biosystems Total RNA chemistry and purified using the standard RNA protocol on the ABI PRISM™ 6100 Nucleic Acid PrepStation.

Relative gene expression across the different storage conditions was then analyzed by a two-step RT-PCR protocol using 85 different gene targets (derived from Applied Biosystems Pre-Developed Assay Reagents list). The list of 85 targets included 11 endogenous control or "housekeeping" genes. Figure 2-4 on page 2-8 and Figure 2-5 on page 2-9 compare gene expression stability for blood stabilized in Tempus Blood RNA tubes and EDTA collection tubes. (For more information, refer to the paper listed in "References" on page 2-13.)

For some gene targets, such as interleukin-8 [IL-8], c-myc, CCR-3, and MCP-2, there was significant up- or down-regulation (up to 50X) for samples stored at ambient conditions for more than one hour.

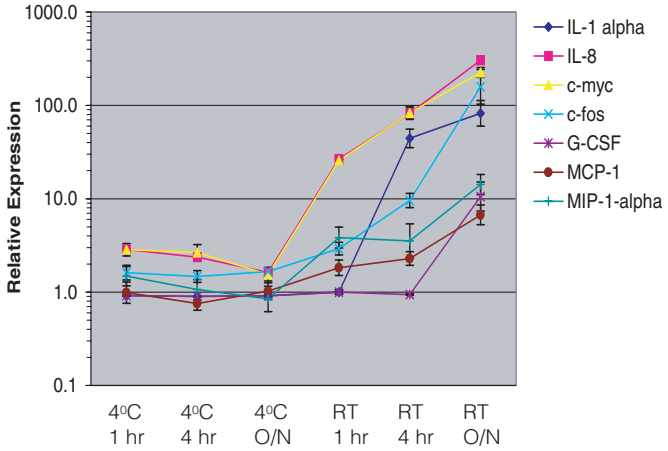


Figure 2-1 Genes up-regulated after prolonged storage at RT

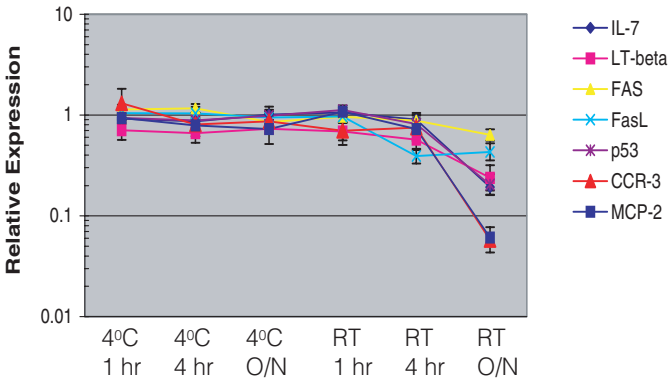


Figure 2-2 Genes down-regulated after prolonged storage at RT

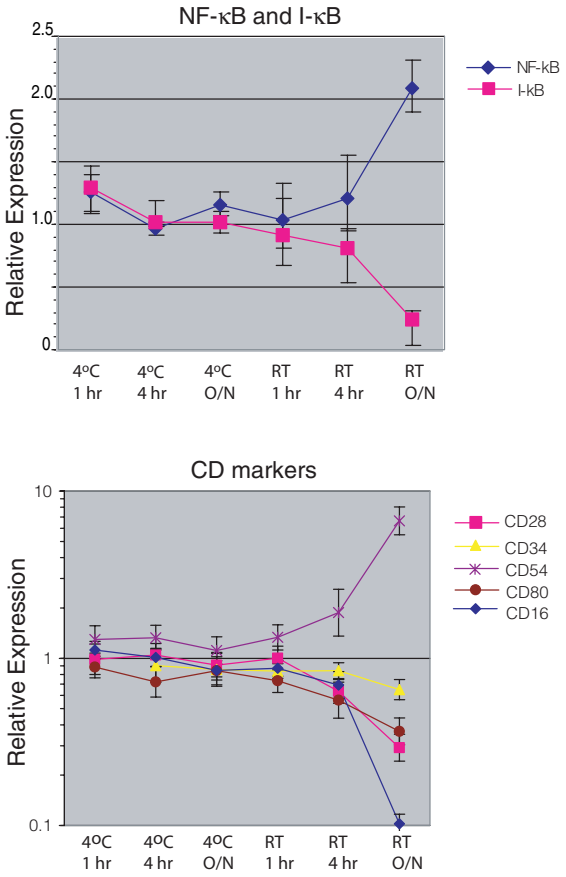


Figure 2-3 Changes in other important gene targets after prolonged storage at RT

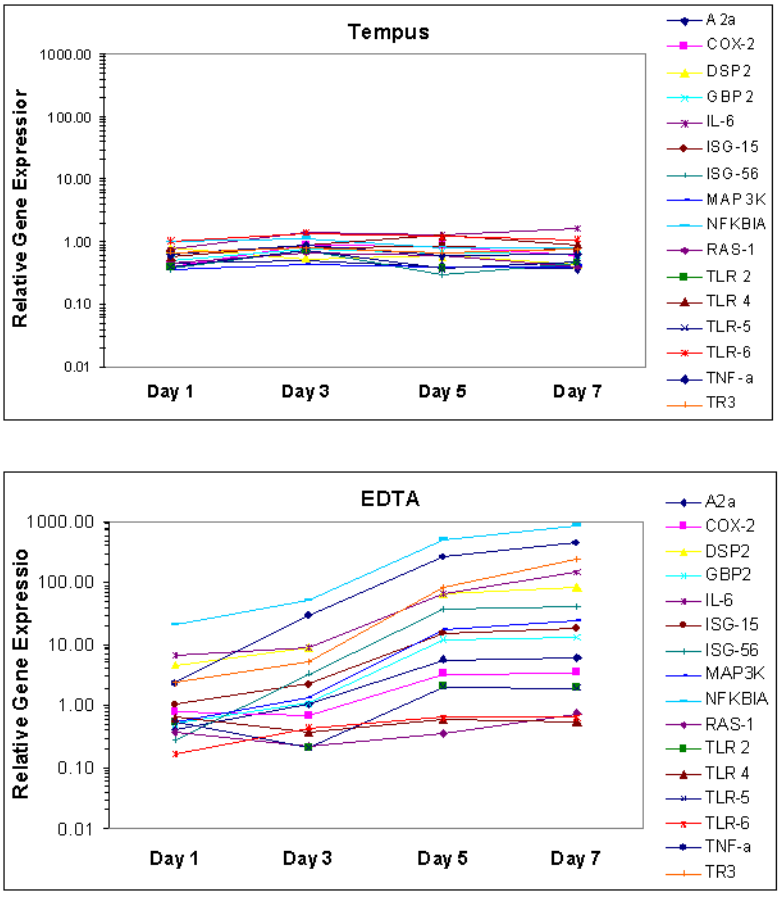


Figure 2-4 Comparison of Gene Expression Stability from blood stabilized in Tempus Blood RNA tubes and standard EDTA blood collection tubes

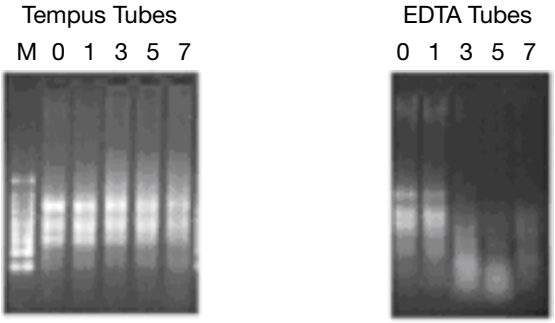


Figure 2-5 Agarose gel (1%) images of total RNA isolated after storage in Tempus and EDTA tubes at room temperature. Lane M has 2 µg RNA Millenium Markers; Lanes 0, 1, 3, 5, and 7 have 2 µg RNA from human whole blood after 0, 1, 3, 5, and 7 days storage at room temperature in Tempus or EDTA tubes.

Applied Biosystems Stabilizing Reagent Prevents RNA Degradation

The Applied Biosystems Stabilizing Reagent in Tempus Blood RNA tubes selectively precipitates RNA, effectively isolating it from gDNA and proteins that remain in solution. Additionally, the chaotropic properties of the stabilizing reagent inactivates RNases.

In the gel images shown in Figure 2-5, blood was collected and stored in Tempus tubes and tubes with standard EDTA anticoagulant for several days at room temperature. RNA was then extracted and run on a 1.2% agarose gel.

Stable RNA Transcripts in Stabilized Blood

You can also assess the stability of RNA transcripts by measuring the threshold cycle (C_T) of selected gene targets. The C_T of stable transcripts does not change over time. (explains the concept of threshold cycles.)

Figure 2-4 on page 2-8 shows the stability of gene targets measured by the TaqMan® assay on a real-time PCR instrument from blood stored in Tempus tubes versus blood stored in EDTA tubes. The results show that Tempus tubes stabilize RNA for at least five days in comparison to EDTA tubes.

Purity, Yield, and Concentration of RNA Purified From Whole Blood

Advantages of Total RNA Chemistry

Automated RNA purification from whole blood is difficult because blood contains high levels of RNases, multiple PCR inhibitors (such as heme, immunoglobulin G, and lactoferrin), protein, and gDNA. This protocol, which uses Applied Biosystems Total RNA Chemistry, generates total RNA that should be virtually free of proteins. Treatment with AbsoluteRNA Wash Solution (DNase reagent) brings contaminating gDNA down to levels of <0.002% by weight.

Additionally, Total RNA Chemistry makes sample pretreatments, such as red blood cell lysis, centrifugation, Ficoll™ buffer, or Proteinase K treatment, unecessary.

Extracted RNA is Virtually Protein– and gDNA–free

Finally, Total RNA Chemistry allows selective precipitation of RNA, retaining gDNA and proteins in solution. Precipitated RNA is trapped in a filter membrane and washed free of most contaminants. This process makes it possible to consistently isolate RNA, with $A_{260/280}$ ratios > 1.9. These ratios are typically close to the theoretically pure limit of absorbance ratio of $A_{260/280}$ = 2.1, indicating that the RNA is protein-free.

Expected RNA Yield

Normal human blood typically contains 2–8 µg of RNA per mL. You should expect to extract 6–25 µg of RNA (for 3 mL blood) in 500 µL of elution buffer (vacuum based elution), or 75–100 µL (centrifuge based elution).

The following experiment shows the yield and purity of RNA extracted from blood that has been stored at room temperature (RT) for several days after collection in Tempus tubes. In this experiment, three samples of blood were collected from each of two individuals and stored as shown in Table 2-1 on page 2-11. Figure 2-6 on page 2-11 shows the results of the experiment.

Table 2-1 Storage times and temperatures

Sample	Storage
1A	Immediately frozen and stored at –20 °C
1B	1 day at RT
1C	3 days at RT
2A	Immediately frozen and stored at –20 °C
2B	1 day at RT
2C	3 days at RT

Figure 2-6 shows the results of the experiment.

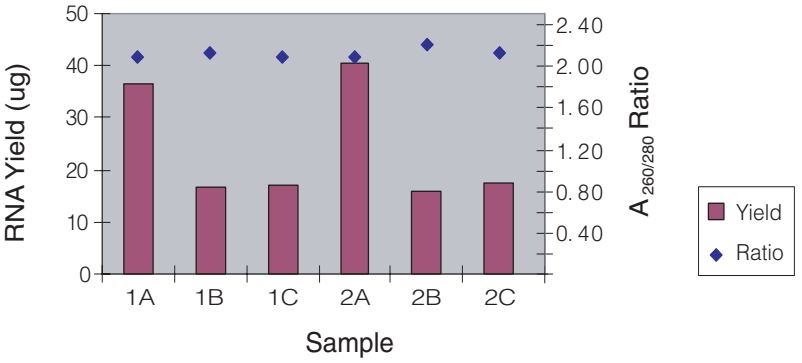


Figure 2-6 Purity and yield of RNA after storage in Applied Biosystems Stabilizing Reagent

The vacuum based elution procedure, using 2-mL microcentrifuge tubes, has an expected yield of 6–25 µg of RNA and an expected concentration of approximately 20–40 ng/µL as seen below in Figure 2-7. (See "To elute RNA from whole blood collected in Tempus tubes using the vacuum based elution (low concentration RNA):" on page 4-10.)

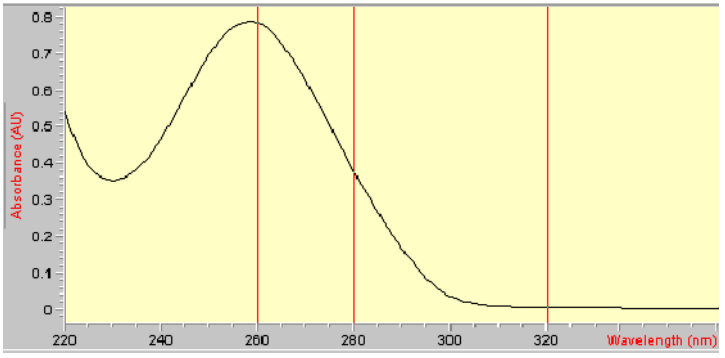
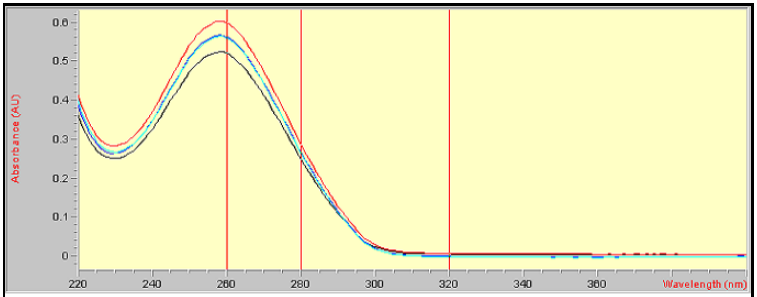


Figure 2-7 Using the vacuum elution procedure, Tempus tubes with an elution volume of 400 µL yield 12.6 µg of RNA at a concentration of 31 ng/µL

The centrifugation based elution procedure yields 6–25 µg of RNA from 3 mL of blood in 100 µL volume with an expected concentration of 80–120 ng/µL as seen below in Figure 2-8. (See "To elute RNA from whole blood collected in Tempus tubes using centrifugation based elution (high concentration RNA):" on page 4-15.)



#	Name	Dilut. Factor	Ratio	Abs<260nm>	Abs<280nm>	Abs<320nm>	Abs<410nm>
1	conc1	5.00000	2.09020	0.51963	0.24860	4.9186E-3	2.8362E-3
2	conc2	5.00000	2.08590	0.59770	0.28654	7.3562E-3	3.5515E-3
3	conc3	5.00000	2.11260	0.56232	0.26617	-6.9475E-4	-9.7561E-4
4	conc4	5.00000	2.12160	0.55910	0.26353	-1.6870E-3	-2.4033E-3

Figure 2-8 Using the centrifugation elution procedure, Tempus tubes with an elution volume of 100 µL yield 11 µg of RNA at a concentration of 110 ng/µL

Concentration of Extracted RNA

As mentioned earlier, you should expect to extract 6–25 µg of RNA (for 3 mL blood) in 500 µL of elution buffer. This elution volume, which is larger than typical elution volumes, helps maximize RNA yield.

References

Tanner, M.A., Berk, L.S., Felten, D.L., Blidy, A.D., Bit, S.L., and D.W. Ruff. 2002. Substantial changes in gene expression level due to the storage temperature and storage duration of human whole blood. *Clin. Lab. Haematol.* 24:337–341.

Assembling the Large Volume Format Consumables

3

This chapter covers:

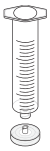
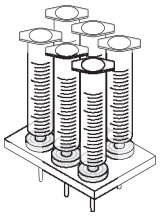
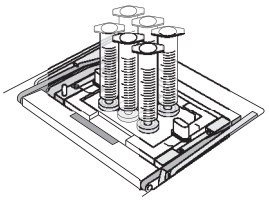
Assembly for Six Samples

You can process a maximum of six samples at one time on the ABI PRISM™ 6100 Nucleic Acid PrepStation.

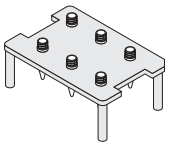
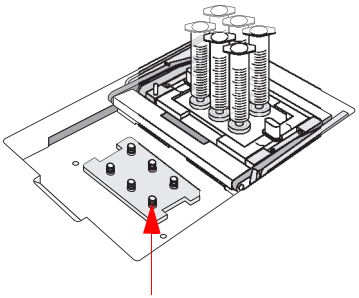
Filter assemblies and reservoirs have luer-fitting connections. The following instructions explain how to assemble the components of the purification consumable for extracting RNA from six samples.

Note: If you are purifying fewer than six samples at one time, follow the instructions for "Assembly for Fewer Than Six Samples" on page 3-4.

To assemble the purification consumable for six samples:

1.		Insert a 20-mL Reservoir (PN 4344435) into the top of each of six Large Volume RNA Prep Filters (PN 4344439).
2.		Insert the filters with attached reservoirs onto each fitting on the top of the Large Volume Adapter Plate (PN 4344438).
3.		Place the assembled Large Volume Adapter Plate into the Carriage of the 6100 PrepStation.

To assemble the purification consumable for six samples: ***(continued)***

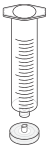
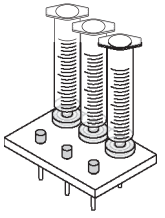
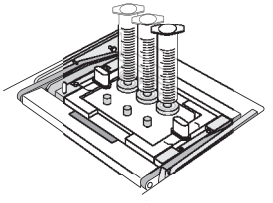
4.		Put 6 2-mL microcentrifuge tubes (PN 4305936) without the caps into the 2-mL Tube Collection Plate. **Note:** Label the tubes before placing them in the tube collection plate. **Note:** The 2-mL Tube Collection Plate is designed to hold Applied Biosystems 2-mL microcentrifuge tubes with screw caps.
5.	 Make sure all tubes are capped until the elution step	When you are ready to collect the eluate, place the 2-mL Tube Collection Plate into the Collection position on the ABI PRISM™ 6100 PrepStation.

Assembly for Fewer Than Six Samples

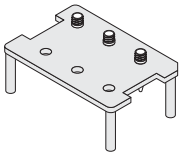
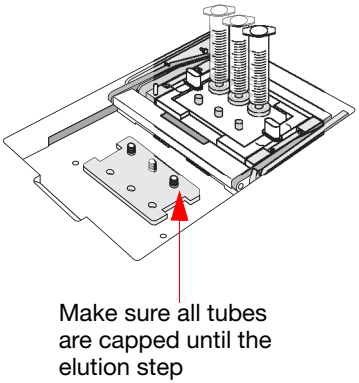
If you are purifying fewer than six samples at the same time, plug unused positions on the adapter plate to ensure that the vacuum reaches the correct set-point. Take care to position the 2mL-microcentrifuge tubes under the appropriate adapter positions (that is, positions that have an RNA Prep Filter Assembly and Reservoir for purification).

Filter assemblies and reservoirs have luer-fitting connections. The following instructions explain how to assemble the components of the purification consumable for extracting RNA from six samples.

To assemble the purification consumable for fewer than six samples:

1.		Insert a 20-mL Reservoir (PN 4344435) into the top of each Large Volume RNA Prep Filter (PN 4344439).
2.		Insert the appropriate number of filters with attached reservoirs onto the Large Volume Adapter Plate (PN 4344438).
3.	(See illustration for step 2.)	Cover unused openings with cap plugs by pushing them down onto the adapter plate fittings. **Note:** Cap plugs are included in the Large Volume Adapter Plate Kit, PN 4344438.
4.		Place the assembled Large Volume Adapter Plate into the Carriage of the ABI PRISM™ 6100 Nucleic Acid PrepStation.

To assemble the purification consumable for fewer than six samples: *(continued)*

5.		Put the appropriate number of 2-mL microcentrifuge tubes (PN 4305936) into the 2-mL Tube Collection Plate. **IMPORTANT!** Make sure the microcentrifuge tubes are under positions on the adapter plate that have RNA Prep Filters and reservoirs. **Note:** Label the tubes before placing them in the tube collection plate. **Note:** The 2-mL Tube Collection Plate is designed specifically to hold Applied Biosystems 2-mL microcentrifuge tubes with screw caps.
6.		When you are ready to collect the eluate, place the 2-mL Tube Collection Plate into the collection position on the ABI PRISM™ 6100 Nucleic Acid PrepStation.

Extracting RNA From Whole Blood

4

This chapter covers:

Overview

RNA extraction involves passing diluted, stabilized blood across an RNA filter and eluting trapped RNA into 2-mL microcentrifuge tubes.

About the Purification Procedures

The purification procedure for diluted, stabilized blood involves the following procedures:

1. Loading the purification consumables (see page 4-2)
2. Creating a new run method (see page 4-4)
3. Diluting stabilized blood before purification (see page 4-5)
4. Performing the purification run (see page 4-7)

The parameters and reagents specific to this protocol are provided in the procedures.

For More Information

The procedures that follow provide a broad overview of the steps required to perform a purification run on the 6100 PrepStation. If you need more detailed instructions for operating the 6100 PrepStation, refer to the *ABI PRISM™ 6100 Nucleic Acid PrepStation User Guide* (PN 4326242).

Loading the Purification Consumables

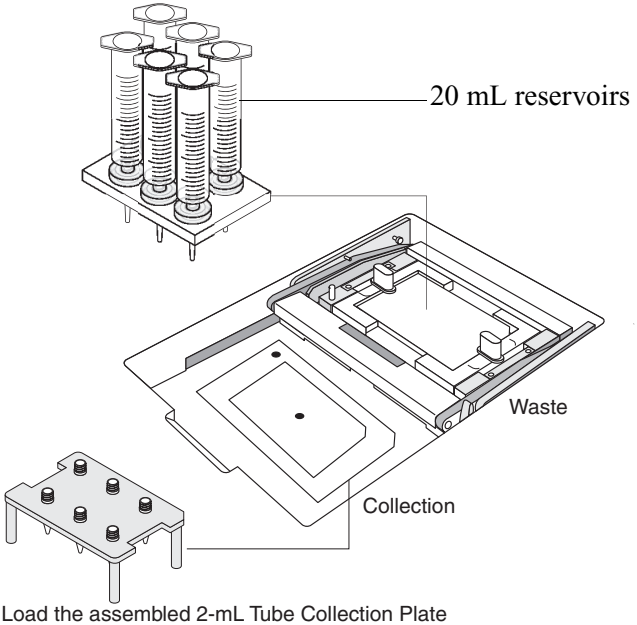
Load the assembled purification consumables into the ABI PRISM™ 6100 Nucleic Acid PrepStation before starting the purification run. Chapter 3, "Assembling the Large Volume Format Consumables," contains assembly instructions.

To load the purification consumables:

1.	Insert a new splash guard (PN 4311758) into the Waste position.
2.	Slide the carriage over the Waste position and depress the carriage handle to lock the carriage in the sealed position.

To load the purification consumables:

3.	Insert the assembled Large Volume Adapter Plate onto the purification tray carriage. **Note:** Check that all empty positions on the plate are covered with cap plugs.
4.	Press down on the edges of the plate to ensure that it is fully sealed.
5.	Lock the plate in position by rotating the locking knobs.
6.	When you are ready to collect the eluate, place the 2-mL Tube Collection Plate into the Collection position on the ABI PRISM™ 6100 Nucleic Acid PrepStation. Cap all unused positions.
7.	Create a new method named *Tempus RNA* (see "Creating a New Run Method" on page 4-4).



Load the assembled 2-mL Tube Collection Plate right before moving the carriage over to the collection position.

Figure 4-1 Properly loaded purification consumables

Creating a New Run Method

Method Definition

A *method* is a list of steps you perform on the ABI PRISM™ 6100 Nucleic Acid PrepStation for a purification protocol.

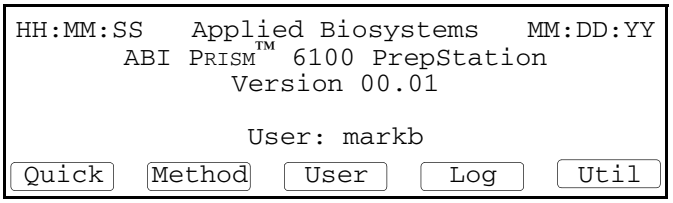
Tempus™ RNA Method

For the Tempus™ Blood RNA Tube protocol, you must program the method for the purification run.

To create a new method:

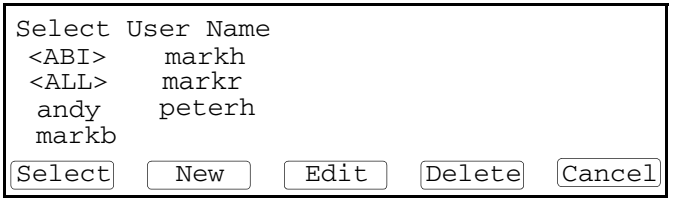
1. In the main menu, press **F3 (User)**.

   The Select User Name screen appears.

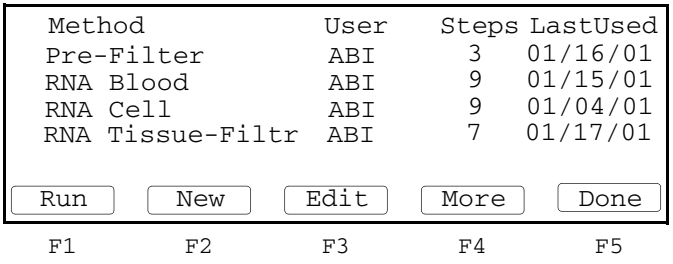


2. Use the arrow keys to highlight the user and press **F1 (Select)**.

   The main menu displays the user.



To create a new method: ***(continued)***

3.	Press **F2 (Method)**. The Method Select 1 screen appears. 
4.	Press **F2 (New)** and enter parameters for the *Tempus RNA* method. The parameters are provided in the procedure table "To elute RNA from whole blood collected in Tempus tubes using the vacuum based elution (low concentration RNA):" on page 4-10.
5.	Save the new method as *Tempus RNA*, then continue with "Diluting Stabilized Blood Before Purification" on page 4-5.

Diluting Stabilized Blood Before Purification

Safety Warnings

WARNING CHEMICAL HAZARD. Tempus Blood RNA tube. Exposure to the contents causes eye, skin, and respiratory tract irritation. Contents are harmful if swallowed. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

WARNING BLOODBORNE/INFECTIOUS WASTE HAZARD. Discard the supernatants following recognized disinfection procedures and in accordance with all local, state, and national bloodborne/infection regulations.

Diluting Stabilized Blood

During the blood collection process, stabilize blood by mixing it with the Applied Biosystems Stabilizing Reagent contained in the Tempus Blood RNA tube. To adjust the concentration of the stabilizing reagent for purification, you must dilute stabilized blood with calcium and magnesium-free phosphate-buffered saline (PBS) before extracting RNA. Failure to do so results in significantly lower RNA yields.

To dilute stabilized blood:

1.	Bring the blood collection tube to room temperature (18–25 °C).
2.	Open the collection tube and pour the contents into a 50-mL container (such as a 50-mL Falcon tube).
3.	Add 3 mL of 1✕ PBS (Ca^{2+}/Mg^{2+}–free), bringing the total volume of the diluted blood to 12 mL (3 mL blood, 6 mL Applied Biosystems Stabilizing Reagent, 3 mL 1✕ PBS). Cap the tube. **IMPORTANT!** Applied Biosystems Stabilizing Reagent must have a final concentration of 1✕. If the initial blood sample was less than 3 mL, make up the difference by adding 1✕ PBS to bring the total volume to 12 mL. Otherwise, RNA yields decrease significantly.
4.	Vortex vigorously for at least 30 sec. **IMPORTANT!** You must vortex the diluted sample for at least 30 sec; vortexing for less than 30 sec may cause clogging of the purification consumable.
5.	Proceed to "Performing the Purification Run" on page 4-7.

Performing the Purification Run

Purifying six samples on the ABI PRISM™ 6100 Nucleic Acid PrepStation takes about 45–50 minutes. For further information on running protocols on the 6100 PrepStation, refer to the *ABI PRISM™ 6100 Nucleic Acid PrepStation User's Manual* (PN 4326242).

To perform the purification run:

1.	Prewet the filters thoroughly with 350 µL of RNA Purification Wash Solution 1 before loading the samples. Make sure that no air bubbles are trapped inside the filter. Ensure that the highlighter is at step 1 of the Tempus Blood RNA method.
2.	Perform run steps 1 to 18, as described in the procedure "To elute RNA from whole blood collected in Tempus tubes using the vacuum based elution (low concentration RNA):" on page 4-10 and steps 1 to 19 in the procedure "To elute RNA from whole blood collected in Tempus tubes using centrifugation based elution (high concentration RNA):" on page 4-15.

Note: The vacuum time for every step may differ from what is presented in the table. When the sample wash solution is completely evacuated, proceed to the next step. However, it is important to perform the pre-elution drying step using the recommended time.

Eluting RNA Extracted From Tempus Blood RNA Tubes

Recommended Methods

Applied Biosystems recommends two methods for the elution of RNA extracted from Tempus Blood RNA tubes. Both methods and suggested sample volumes are briefly described below:

- Use the vacuum method on the ABI PRISM™ 6100 Nucleic Acid PrepStation for obtaining samples with lower concentrations of RNA. See "Elution Using Vacuum on the ABI PRISM™ 6100 Nucleic Acid PrepStation" on page 4-10.

- Use the centrifugation method for obtaining samples with higher concentrations of RNA. See "Elution Using Centrifugation" on page 4-15.

Method	Suggested Elution Volume
Vacuum elution	500–1000µL
Centrifugation elution	100–300µL

Safety Warnings

CHEMICAL HAZARD. **RNA purification wash solution 1** may cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

CHEMICAL HAZARD. **RNA purification wash solution 2** is a flammable liquid and vapor. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

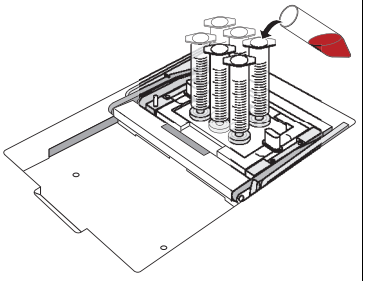
CHEMICAL HAZARD. **Tempus Blood RNA Tube.** Exposure to the contents causes eye, skin, and respiratory tract irritation. Contents are harmful if swallowed. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

BLOODBORNE/INFECTIOUS WASTE HAZARD. Discard the supernatants following recognized disinfection procedures and in accordance with all local, state, and national bloodborne/infection regulations.

Elution Using Vacuum on the ABI PRISM™ 6100 Nucleic Acid PrepStation

The ABI PRISM™ 6100 Nucleic Acid PrepStation, a 2 mL collection plate, and 2 mL microcentrifuge tubes are used to elute RNA from Large Volume RNA Prep Filters (see "Materials and Equipment" on page 1-5). A single pass with 500 µL of elution buffer is required to obtain the greatest yield. A second elution does not generally result in an improved yield.

To elute RNA from whole blood collected in Tempus tubes using the vacuum based elution (low concentration RNA):

Step	Description	Vol (mL)	Position	Incubation (sec)	Vacuum (%)	Vacuum (sec)
1	Thoroughly prewet the filters with RNA Purification Wash Solution 1. **Note:** Ensure the highlighter is at step 1 of the Tempus RNA method and that no air bubbles are trapped in the filter. Release any air bubbles by pipetting the solution up and down.	0.35	Waste	0	0	0
2	Load samples by pouring diluted stabilized blood from 50-mL tubes into the 20-mL reservoir, then turn the vacuum on. **Note:** If air is trapped underneath the blood lysate, pipet the lysate up and down to release the trapped air. 	12	Waste	0	80	300[a,b]
3	Add RNA Purification Wash Solution 1	4.5	Waste	0	80	300

To elute RNA from whole blood collected in Tempus tubes using the vacuum based elution (low concentration RNA): ***(continued)***

Step	Description	Vol (mL)	Position	Incubation (sec)	Vacuum (%)	Vacuum (sec)
4	Leaving the filter assemblies in place, remove the 20-mL reservoirs, turn vacuum on, and wash the neck and filter with 1–3 mL of RNA Purification Wash Solution 1 using a P1000 pipet. **Note:** Wash until the blood lysate is completely washed off and no color remains.	1–3	Waste	0	80	300
5	Attach new 5-mL reservoirs and add RNA Purification Wash Solution 1.	4.5	Waste	0	80	300
6	Add RNA Purification Wash Solution 2. Repeat steps 5–6 until the filter membrane is free of all red or brown heme pigment.[c] Detach the filter and verify that the bottom of the filter is completely clean. **IMPORTANT!** Make sure filter is completely dry (whitish in appearance) before proceeding.[d]	4.5	Waste	0	80	180
7	Remove 5-mL reservoir and replace with a new 5-mL reservoir. Add AbsoluteRNA Wash Solution and incubate. **Note:** Use a smaller pipet tip when adding AbsoluteRNA Wash Solution to ensure that the solution sufficiently wets the membrane. **IMPORTANT!** Do not turn on the vacuum until step 9.	0.35	Waste	900	0	0
8	Add RNA Purification Wash Solution 2 and incubate.	3	Waste	300	0	0

To elute RNA from whole blood collected in Tempus tubes using the vacuum based elution (low concentration RNA): *(continued)*

Step	Description	Vol (mL)	Position	Incubation (sec)	Vacuum (%)	Vacuum (sec)
9	Turn on the vacuum to remove RNA Purification Wash Solution 2.	–	Waste	0	80	120
10	Add RNA Purification Wash Solution 2.	3	Waste	0	80	120
11	Add RNA Purification Wash Solution 2 and then dry filters. **Note:** Make sure the adapter tips and splash guard are clean and free of blood lysate before proceeding. Replace the splash guard with a new one if necessary.	3	Waste	0	80	120
12	Remove the 5-mL reservoirs and adapter plate from the ABI PRISM™ 6100 PrepStation and wash the tips of the adapter plate and the splash guard with 70% ethanol before performing the pre-elution vacuum.	–	–	–	–	–
13	Perform pre-elution vacuum.	–	Waste	–	90	400
14	Touch off at Waste, and load the assembled 2-mL Tube Collection Plate in the Collection position.	–	Waste	–	–	–
15	Move the carriage to the Collection position, and attach a new 5-mL reservoir. **IMPORTANT!** Ensure that the filters are completely dry (free from RNA Purification Wash Solution 2) before adding Nucleic Acid Purification Elution Solution.[e]	–	Collection	–	–	–

To elute RNA from whole blood collected in Tempus tubes using the vacuum based elution (low concentration RNA): ***(continued)***

Step	Description	Vol (mL)	Position	Incubation (sec)	Vacuum (%)	Vacuum (sec)
16	Add Nucleic Acid Purification Elution Solution and incubate. **Note:** The minimum volume is 0.5 mL. If higher yields are desired, use a larger volume (for example 2× elution volume).	0.5	Collection	120	0	0

To elute RNA from whole blood collected in Tempus tubes using the vacuum based elution (low concentration RNA): *(continued)*

Step	Description	Vol (mL)	Position	Incubation (sec)	Vacuum (%)	Vacuum (sec)
17	Turn on the vacuum to elute the RNA. The eluate volume should be approximately 400 µL. If volume is significantly less, check the adapter plate tips to see if any liquid remains. Repeat this step at a higher vacuum if necessary.	–	Collection	0	60	120
18	Touch off at Collection.	–	Collection	–	–	–

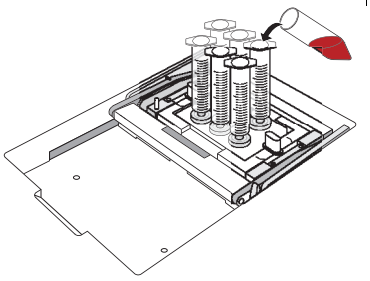
a. 300 seconds is the average time. Blood from different patients might require more time due to variations in blood composition. If necessary, repeat this step. On the other hand, if blood passes across the filter in less than 300 seconds, stop the vacuum when all reservoirs are empty. Blood samples from patients with very high leukocyte counts require more time. These donors have high RNA yields (>10µg/mL).
b. If the sample appears to be clogged, air is probably trapped underneath the blood lysate at the membrane surface. Place the tip of a 1.0 mL pipet in the bottom of the reservoir and pipet the lysate up and down to release the trapped air.
c. Failure to remove all pigment and protein can lead to degraded RNA due to residual RNase activity in the eluted RNA sample. Contaminating heme can also inhibit PCR.
d. RNA Purification Wash Solution 2 interferes with the ability of AbsoluteRNA Wash Solution (DNase reagent) to remove gDNA.
e. Unless the membrane is completely dry, ethanol (contained in RNA Purification Wash Solution 2) remains on the membrane. Ethanol inhibits solubilization of RNA in the elution solution, resulting in lower RNA yields.

Elution Using Centrifugation

For applications requiring a high concentration of RNA, such as microarray analysis, you can use centrifugation to elute the extracted RNA. This procedure typically gives concentrations of RNA in the range of ~100 ng/µL.

Note: Steps 1–11 for Elution Using Centrifugation are the same as steps 1–11 in the "Elution Using Vacuum on the ABI PRISM™ 6100 Nucleic Acid PrepStation" on page 10. At step 12 the two procedures diverge.

To elute RNA from whole blood collected in Tempus tubes using centrifugation based elution (high concentration RNA):

Step	Description	Vol (mL)	Position	Incubation (sec)	Vacuum (%)	Vacuum (sec)
1	Thoroughly prewet the filters with RNA Purification Wash Solution 1. **Note:** Ensure the highlighter is at step 1 of the Tempus RNA method and that no air bubbles are trapped in the filter. Release any air bubbles by pipetting the solution up and down.	0.35	Waste	0	0	0
2	Load samples by pouring diluted stabilized blood from 50-mL tubes into the 20-mL reservoir. Use a different reservoir for each sample. **Note:** If air is trapped underneath the blood lysate, pipet the lysate up and down to release the trapped air. 	12	Waste	0	80	300[a,b]

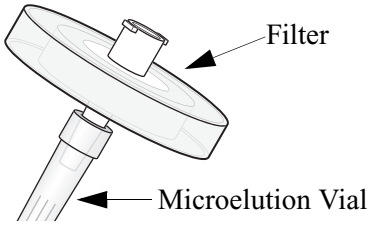
To elute RNA from whole blood collected in Tempus tubes using centrifugation based elution (high concentration RNA): *(continued)*

Step	Description	Vol (mL)	Position	Incubation (sec)	Vacuum (%)	Vacuum (sec)
3	Add RNA Purification Wash Solution 1	4.5	Waste	0	80	300
4	Leaving filter assemblies in place, remove the 20-mL reservoirs, turn vacuum on, and wash the neck and filter with 1–3 mL of RNA Purification Wash Solution 1 using a P1000 pipet. **Note:** Wash until the blood lysate is completely washed off and no coloration remains.	1–3	Waste	0	80	300
5	Attach new 5-mL reservoirs and add RNA Purification Wash Solution 1.	4.5	Waste	0	80	300
6	Add RNA Purification Wash Solution 2. Repeat steps 5–6 until the filter membrane is free of all red or brown heme pigment.[c] Detach the filter and verify that the bottom of the filter is completely clean. **IMPORTANT!** Make sure filter is completely dry (whitish in appearance) before proceeding.[d]	4.5	Waste	0	80	180
7	Remove 5-mL reservoirs and replace with new 5-mL reservoirs. Add AbsoluteRNA Wash Solution and incubate. **Note:** Use a smaller pipet tip when adding AbsoluteRNA Wash Solution to ensure that the solution sufficiently wets the membrane. **IMPORTANT!** Do not turn on the vacuum until step 9.	0.35	Waste	900	0	0

To elute RNA from whole blood collected in Tempus tubes using centrifugation based elution (high concentration RNA): *(continued)*

Step	Description	Vol (mL)	Position	Incubation (sec)	Vacuum (%)	Vacuum (sec)
8	Add RNA Purification Wash Solution 2 and incubate.	3	Waste	300	0	0
9	Turn on the vacuum to remove RNA Purification Wash Solution 2.	–	Waste	0	80	120
10	Add RNA Purification Wash Solution 2.	3	Waste	0	80	120
11	Add RNA Purification Wash Solution 2 and then dry filters.	3	Waste	0	80	120
12	Perform pre-elution vacuum.	–	Waste	–	90	400
13	Remove 5-mL reservoirs.	–	Waste	0	–	–
14	Touch off at Waste position.	–	Waste	–	–	–
15	Remove the RNA Prep Filters and ensure the tips of the filter are dry and free from Wash Solution 2[e].	–	–	–	–	–
16	Fit the microelution vials to the RNA Prep Filters (see Figure 4-2 on page 4-20), and place assemblies in an Applied Biosystems 96-well tray retainer with a splash-free base. See Figure 4-3 on page 4-20.)	–	–	–	–	–

To elute RNA from whole blood collected in Tempus tubes using centrifugation based elution (high concentration RNA): *(continued)*

Step	Description	Vol (mL)	Position	Incubation (sec)	Vacuum (%)	Vacuum (sec)
17	Add 1 µL of RNase Inhibitor (PN N8080119) to 100 µL of RNA elution solution; then add this solution inside the RNA filter.  **Note:** The RNase Inhibitor concentration is 20 U/µL. **IMPORTANT!** Allow the membrane to get wet, but do not introduce air bubbles. **Note:** Adding an incubation time of 5 min prior to centrifugation, or increasing the volume of the elution solution to 130 µL may improve yields of RNA.	0.1 – 0.3	–	20 – 300	–	–

To elute RNA from whole blood collected in Tempus tubes using centrifugation based elution (high concentration RNA): *(continued)*

Step	Description	Vol (mL)	Position	Incubation (sec)	Vacuum (%)	Vacuum (sec)
18	Centrifuge the filter-vial assembly at 2100g (~3500–4000 rpm) in a splash-free base or a tray retainer set in a base for 3 min. **CAUTION** The filter-vial assembly should only be placed in a centrifuge that is qualified to spin 96-well plates.	–	–	–	–	–
19	When the spin is finished, repeat steps 17–18 with the same eluate to ensure that all RNA is removed from the RNA prep filter. **Note:** A 40% improvement in recovery is typically gained by repeating the elution. No further improvement is gained by recycling the eluate a third time.	–	–	–	–	–

a. 300 seconds is the average time. Blood from different patients might require more time due to variations in blood composition. If necessary, repeat this step. On the other hand, if blood passes across the filter in less than 300 seconds, stop the vacuum when all reservoirs are empty. Blood samples from patients with very high leukocyte counts require more time. These donors have high RNA yields (>10µg/mL).
b. If the sample appears to be clogged, air is probably trapped underneath the blood lysate at the membrane surface. Place the tip of a 1.0 mL pipet in the bottom of the reservoir and pipet the lysate up and down to release the trapped air.
c. Failure to remove all pigment and protein can lead to degraded RNA due to residual RNase activity in the eluted RNA sample. Contaminating heme can also inhibit PCR.
d. RNA Purification Wash Solution 2 interferes with the ability of AbsoluteRNA Wash Solution (DNase reagent) to remove gDNA.
e. Unless the membrane is completely dry, ethanol (contained in RNA Purification Wash Solution 2) remains on the membrane. Ethanol inhibits solubilization of RNA in the elution solution, resulting in lower RNA yields.

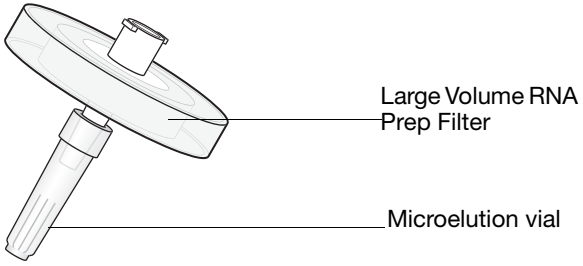


Figure 4-2 Filter-vial assembly

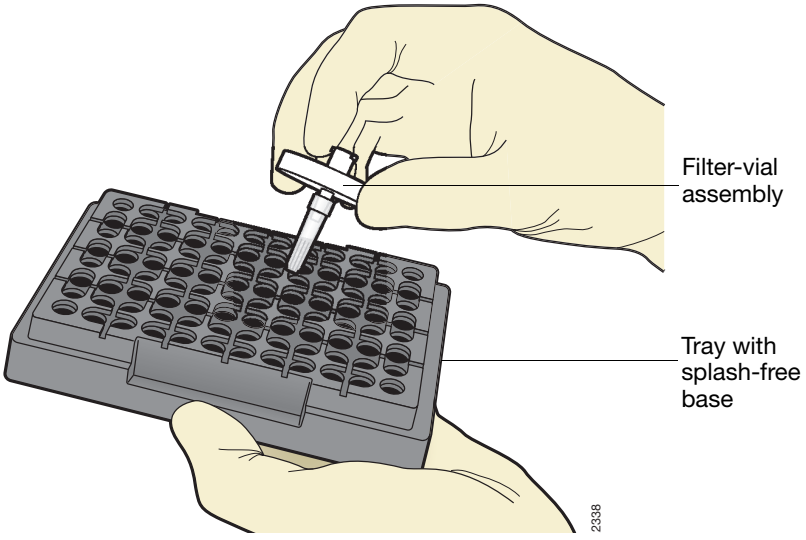


Figure 4-3 Filter-vial-base assembly

Concentrating Eluted RNA

Ethanol Precipitation of Extracted RNA

Applied Biosystems recommends performing a secondary ethanol precipitation to concentrate RNA from large sample volumes for use on microarray or other types of analysis. Using ethanol precipitation increases stability, purity, and recovery of RNA. Use RNase-free reagents and techniques, and follow the procedure below to precipitate the extracted RNA.

Note: The volumes specified in the procedure below are for 300 µL samples. Adjust the reagent volumes (except for Glycogen) accordingly for other sample volumes.

WARNING **CHEMICAL HAZARD. Ethanol** is a flammable liquid and vapor. Exposure causes eye, skin, and respiratory tract irritation and may cause central nervous system depression and liver damage. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Ethanol precipitation procedure:

1.	Pipet 300 µL of each sample into 1.5 µL microcentrifuge tubes.
2.	Pipet 18 µL of 5M NaCl into each sample microcentrifuge tube.
3.	Add 10 µL of a 5 mg/mL Glycogen stock solution to each tube, and pipet up and down a few times to mix. **Note:** Add 10 µL of this 5 mg/mL Glycogen solution regardless of the sample volume.
4.	Add 900 µL of Ethanol to each tube. Mix by inverting the tubes at least 12 times. **IMPORTANT!** Do not mix by vortexing or pipetting up and down.
5.	Place the tubes in a freezer and cool for at least 1 hr at –20 °C.

Ethanol precipitation procedure: ***(continued)***

6.	Remove tubes from freezer and place in a refrigerated centrifuge. **IMPORTANT!** Note the orientation of each tube in the centrifuge, and if needed, mark the outer radial side. The location of the outer radial side of the tube will help you locate the pellet after centrifugation. **Note:** It is also possible to obtain glycogen containing dyes which allow easier location of the pellet (Ambion Glyco Blue™, CN 9510).
7.	Centrifuge the tubes at full speed for 15 min at 4 °C.
8.	Carefully remove each tube from the centrifuge, and hold each tube to the light to visually locate the pellet.
9.	Carefully remove the supernatent with a pipet. **Note:** Pipet supernatent from the side opposite the pellet (the side opposite the marked outer radial side of the tube).
10.	Resuspend the pellet with 60 µL of RNase-free water or the appropriate volume for your application.

Reverse Transcription of Total RNA to cDNA

cDNA Archive Kit

To reverse transcribe 0.1 to 10 µg of total RNA to single-stranded cDNA, use the High Capacity cDNA Archive Kit (PN 4322171). The cDNA produced is suitable for quantitative PCR applications and short- or long-term storage.

Manual Method for Generating cDNA

In the *High Capacity cDNA Archive Kit Protocol* (PN 4322169), follow the procedure for the manual method to generate cDNA from total RNA.

Storage

The generated cDNA can be stored in 96-well plates at –20 °C under an attached archive cover (PN 5306286).

Cleaning the ABI PRISM™ 6100 Nucleic Acid PrepStation

Because large volumes of blood lysate pass through the ABI PRISM™ 6100 Nucleic Acid PrepStation, you need to clean the Waste position after every run.

IMPORTANT! Do not use bleach to clean the ABI PRISM™ 6100 Nucleic Acid PrepStation. Use a non bleach virocide, such as Lysol® or Vesphene®, to clean the equipment.

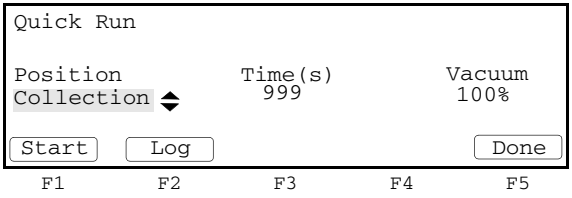
To clean the ABI PRISM™ 6100 Nucleic Acid PrepStation:

1.	Remove and discard the large volume adapter plate.
2.	Move the carriage to the Collection position.
3.	Remove and discard the splash guard from the Waste position.

To clean the ABI PRISM™ 6100 Nucleic Acid PrepStation:

4. Use the Quick Run feature to flush the Waste position thoroughly with water and cleaning agent, if necessary.

   a. From the main menu, press **F1 (Quick)**.
   The **Quick Run** screen appears.



   b. Enter the values shown below, then press **F1 (Start)**.

Position	Time	Vacuum
Waste	3 min	50%

Note: Take care that water does not spill over the capture area and the waste container does not overfill.

   c. Flush the waste position with the chosen cleaning agent.

5. If the white plastic piece that holds the splash guard in place is heavily contaminated, remove and clean it as follows:

   a. Unscrew the two Allen screws that hold the piece in place.

   b. Lift the plastic piece out.

   c. Wash the piece and the area underneath it thoroughly with Lysol®, Vesphene, or 70% alcohol, detergent, and water.

   d. Allow the piece and the area to dry before replacing the plastic piece.

6. Clean the area around the vacuum carriage and gasket with 70% alcohol.

Threshold Cycle

A

About Real-Time PCR Assays

Real-time PCR is the ability to monitor the progress of PCR amplification as it occurs. Data is collected throughout the PCR process, rather than at the end of the PCR process. In real-time PCR, reactions are characterized by the point in time during cycling when amplification of a target is first detected rather than the amount of target accumulated after a fixed number of cycles.

Real-time PCR can be used to quantify RNA, and can be performed as a one-step or two-step procedure.

- The one-step real-time PCR performs reverse transcription (RT) as well as PCR in a single buffer system. The reaction proceeds without the addition of reagents between the RT and PCR steps to provide the convenience of a single-tube preparation for RT and PCR amplification.
- The two-step real-time PCR is performed in two separate reactions. This two-step procedure is useful when detecting multiple transcripts from a single cDNA reaction, or when storing a portion of the cDNA for later use.

How Real-Time PCR Quantification Assays Work

Real-time PCR allows reactions to be characterized by the point in time during cycling when amplification of a PCR product achieves a fixed level of fluorescence, rather than by amount of PCR product accumulated after a fixed number of cycles. An amplification plot graphically displays the fluorescence detected over the number of cycles that were performed.

As shown in Figure A-1, in the initial cycles of PCR there is no significant change in fluorescence signal. This predefined range of PCR cycles is called the “baseline.” First, the software generates a baseline subtracted amplification plot by calculating a mathematical trend using Rn values corresponding to the baseline cycles. Then, an algorithm searches for the point on the amplification plot at which the delta Rn value crosses the threshold. The fractional cycle at which this search occurs is defined as the C_T.

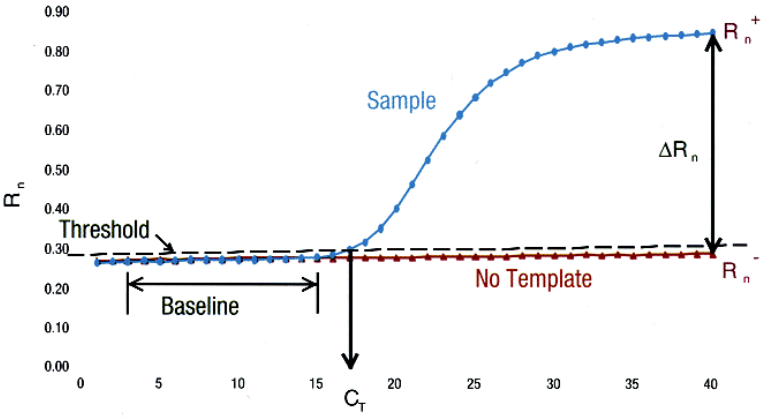


Figure A-1 Model of a single amplification plot, showing terms commonly used in quantitative analysis

Note: Refer to your sequence detection system instrument user guide for more information.

Troubleshooting Tips

B

The following table lists some problems that you might encounter when running the Tempus Blood RNA method.

Problem	Cause	Solution
Sample appears to be clogged	Air is probably trapped underneath the blood lysate at the membrane surface	Stop the vacuum step and restart the vacuum at 100%. Alternatively, place the tip of a 1.0 mL pipet in the bottom of the reservoir and gently pipet the lysate up and down to release the trapped air.
	Insufficient mixing immediately after blood draw	Vortex sample for 10–20 seconds immediately after drawing the blood into each tube.
	Insufficient mixing during sample dilution with PBS	Remove the sample from the reservoir. Vortex and replace Large Volume RNA Prep Filter. Restart purification procedure.
	Proteins and other blood components have clogged filter pores	Remove remaining blood lysate from the reagent reservoir. Attach a new filter reservoir and process a fraction of the remaining blood lysate (for example, 5 mL).
	Sample has too much RNA	Blood from human donors (or other animal species such as rats and mice) with very high leukocyte counts yields large amounts of RNA. Remove remaining blood lysate from the reagent reservoir. Attach a new reservoir and process a fraction of the remaining blood lysate (for example, 5 mL).
	Vacuum leak	Check seals and instrument lines.
Some samples have completely evacuated.	Other samples are clogged or evacuating more slowly.	Remove the filter/reservoir assemblies of samples that have completely evacuated and cover the openings with the cap plugs supplied. Continue to pull a vacuum for the rest of the samples. Replace filter/reservoir assemblies when all samples have evacuated.

Problem	Cause	Solution
RNA is degraded	• Residual protein (RNase activity)	• Increase number of wash steps with RNA Purification Solution Wash 1 and 2 in next run. • Ensure adapter plate tips are clean and free of blood lysate before performing final elution. • Perform ethanol precipitation immediately after isolation.
	• Blood lysate exposed to >37 °C for short period	• RNA has gone back into solution. Freeze any remaining lysate. Thaw and repurify.
	• Insufficient mixing after blood draw and during dilution	• Vortex sample.
gDNA contamination	• Filter was not completely dry when AbsoluteRNA Wash Solution was added • Vacuum was turned on too early after adding AbsoluteRNA Wash Solution	• Ensure filter is completely dry before proceeding to DNase treatment.
	• Air is trapped underneath AbsoluteRNA Wash Solution	• Ensure membrane is wetted by AbsoluteRNA Wash Solution.

Problem	Cause	Solution
Low RNA yield	• Less than 3 mL blood sample	• Add 1× PBS to bring the total sample volume to 3 mL.
	• Applied Biosystems Stabilizing Reagent did not reach 1× final concentration	• Add enough 1× PBS to bring the total volume of the diluted blood lysate to 12 mL.
	• Filters not completely dry before Nucleic Acid Purification Elution Solution was added	• RNA remains on the membrane (in residual RNA Purification Wash Solution 2). Re-elute RNA with another aliquot of Nucleic Acid Purification Elution Solution.
	• Insufficient mixing during sample dilution with PBS	• Remove the sample from the reservoir. Vortex and replace Large Volume RNA Prep Filter. Restart purification procedure.
	• Blood lysate exposed to >37 °C for short period	• RNA has gone back into solution. Freeze any remaining lysate. Thaw and repurify.
	• The filter membrane is not completey wet with the Elution Solution.	• Ensure that no air bubbles are trapped in the filter.
Vacuum does not reach set point and vacuum leak is detected	Misplaced adapter plate	Reposition adapter plate.
	Uncovered unused positions	Cover the unused positions with caps.
	Setting instrument to Collection instead of Waste	Set the vacuum to Waste.

Index

V

Y

iScience. To better understand the complex interaction of biological systems, life scientists are developing revolutionary approaches to discovery that unite technology, informatics, and traditional laboratory research. In partnership with our customers, Applied Biosystems provides the innovative products, services, and knowledge resources that make this new, **Integrated Science** possible.

Worldwide Sales and Support

Applied Biosystems vast distribution and service network, composed of highly trained support and applications personnel, reaches 150 countries on six continents. For sales office locations and technical support, please call our local office or refer to our Web site at **www.appliedbiosystems.com**.

Applera is committed to providing the world's leading technology and information for life scientists. Applera Corporation consists of the Applied Biosystems and Celera Genomics businesses.

Headquarters
850 Lincoln Centre Drive
Foster City, CA 94404 USA
Phone: +1 650.638.5800
Toll Free (In North America): +1 800.345.5224
Fax: +1 650.638.5884

02/2005

Applied Biosystems

Part Number 4345218 Rev. D