Isolation of Total RNA from Cultured Cells

ABI PRISM™ 6700 Automated Nucleic Acid Workstation or 6100 Nucleic Acid PrepStation

Protocol

Applied Biosystems

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Introduction 1

Overview

In This chapter

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RNA Chemistry Overview

Applied Biosystems has developed new techniques for producing highly purified total RNA from biological sources such as cultured cells. These new techniques respond to the demand for high-quality, high-throughput total RNA extraction methods posed by real-time quantitative PCR and other array-based assays.

By using Applied Biosystems total RNA chemistry and instrument systems, you can:

- Eliminate RNase degradation
- Purify high quality RNA

There are three techniques discussed in this protocol:

- Lysis of suspension cell cultures
- Lysis of adherent cell cultures
- Purification of RNA from cell lysates (the technique is the same for both types of cell cultures)

About Total RNA Chemistry from Cultured Cells

Extracting and purifying RNA from cultured cells is one of the most widely used applications. Traditionally this has been a highly repetitive and labor-intensive procedure requiring significant time investment, and often involving the use of toxic organic chemicals.

Applied Biosystems total RNA chemistry offers the following advantages:

- The procedure is significantly streamlined
- The highest quality total RNA is produced
- Cross contamination is controlled

Applied Biosystems total RNA chemistry uses a unique formulation to effectively lyse cultured cells. Lysis occurs almost immediately after cells are mixed with 1X lysis buffer – without any physical disruption or enzymatic digestion. The 1X lysis buffer inactivates cellular RNases, preventing degradation of the isolated nucleic acid while keeping genomic DNA (gDNA) and protein in solution. This allows a separation of total RNA from gDNA and other cellular debris.

The total RNA chemistry has been evaluated against several different cell lines to obtain highest purity and yield, while the 96-well format allows high throughput capabilities. The total RNA purification tray consists of:

- 96 wells (700 µL/well)
- A proprietary membrane to physically capture the RNA
- A low dead volume drip director
- An aerosol guard to prevent well-to-well cross-contamination

Use of AbsoluteRNA Wash Solution

The total RNA chemistry recovers a very low amount of gDNA – guaranteed to be less than 0.5% by weight from cell cultures. However, the need to look at genes expressed at a very low level is becoming significant as gene expression analysis is becoming a routine procedure in many areas of research. The lack of detailed sequence information on these targets may also limit the ability to design intron-spanning 5′-nuclease assay TaqMan® probes and primer sets, so primers may also amplify contaminating gDNA.

AbsoluteRNA Wash Solution can be incorporated directly into the RNA purification protocol and virtually eliminate contaminating gDNA from eluted RNA products.

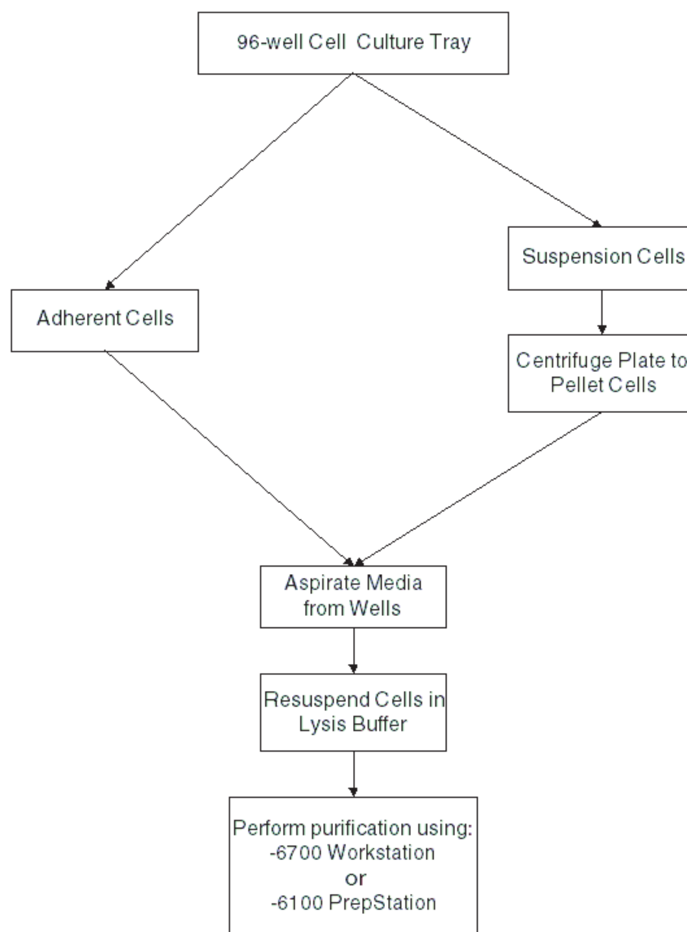
Instrument Systems

The lysis and purification techniques discussed in this protocol can be performed on the following Applied Biosystems instrument systems:

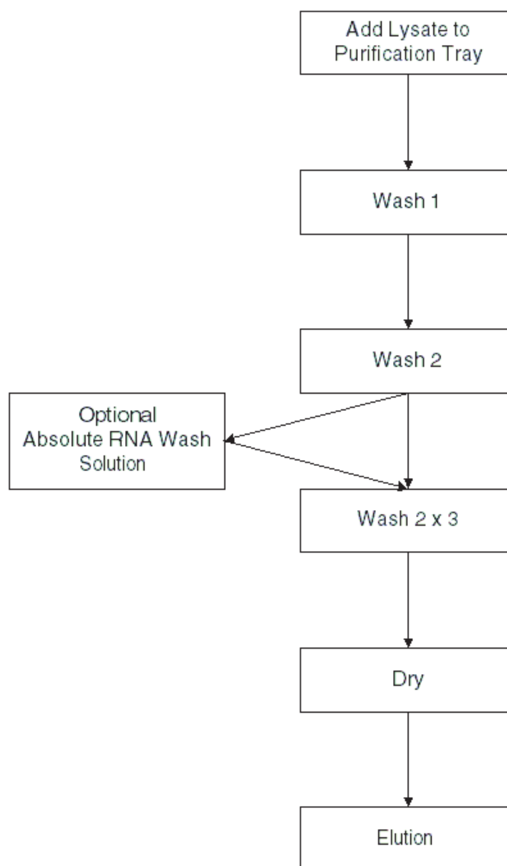
- ABI PRISM™ 6700 Automated Nucleic Acid Workstation
- ABI PRISM™ 6100 Nucleic Acid PrepStation

Summary Diagram

Cellular RNA isolation and purification is summarized in the following diagram.



Purification Process Diagram



Materials and Equipment

Equipment and Materials Required But Not Supplied

The following tables list the equipment, accessories, and chemicals required for RNA isolation from cultured cells.

Equipment	Source
ABI PRISM 6700 Automated Nucleic Acid Workstation or ABI PRISM 6100 Nucleic Acid PrepStation	See your Applied Biosystems sales representative
Centrifuge with 96-well plate adapters	Major Laboratory Supplier (MLS)
Pipettors	MLS
Vortexer	MLS

Accessories	Supplier	Part Number
96-Well Optical Reaction Plate with Barcode (also called “archive plate” and “PCR plate”)	Applied Biosystems	4306737
Splash Guard	Applied Biosystems	4311758
Total RNA Purification Tray	Applied Biosystems	4305673
15- and/or 50-mL sterile tubes (*e.g.*, BD Falcon)	MLS	—
300-µL, flat-bottom, 96-well cell culture plate, one of the following: • Costar® 3596 • BD Falcon • Nuncion Delta 1056	MLS	—

Accessories	Supplier	Part Number
Additional Materials for Purification on 6700 Workstation		
Archive Covers	Applied Biosystems	4306286
Conductive Pipette Tips, 1000-µL	Applied Biosystems	4306377
Conductive Pipette Tips, 200-µL	Applied Biosystems	4306375
Reagent Reservoirs, 120-mL **Note:** This product comes with a sheet of barcode labels for Applied Biosystems nucleic acid purification reagents.	Applied Biosystems	4304831
Deep-Well Plates	Applied Biosystems	4308641

Chemicals	Supplier	Part Number
AbsoluteRNA Wash Solution (optional)	Applied Biosystems	4305545
Nucleic Acid Purification Elution Solution	Applied Biosystems	4305893
2X Nucleic Acid Purification Lysis Solution **IMPORTANT!** 2X Nucleic Acid Purification Lysis Solution is supplied as a 2X formulation for sample storage. It should always be used at a final concentration of 1X (called "1X lysis buffer"). See "Buffer for Lysing Cultures" on page 2-2 or page 3-1.	Applied Biosystems	4305895
RNA Purification Wash Solution 1	Applied Biosystems	4305891
RNA Purification Wash Solution 2	Applied Biosystems	4305890
Phosphate buffered saline (PBS), calcium/magnesium-free	MLS	—

Safety

Documentation User Attention Words

Five user attention words appear in the text of all Applied Biosystems user documentation. Each word implies a particular level of observation or action as described below.

Note: Calls attention to useful information.

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 which, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

⚠ WARNING Indicates a potentially hazardous situation which, if not avoided, could result in death or serious injury.

⚠ DANGER Indicates an imminently hazardous situation which, if not avoided, will result in death or serious injury. 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.

- Read and understand the material safety data sheets (MSDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (*e.g.,* safety glasses, gloves, or 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 (*e.g.*, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer’s cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

General Biohazard Warning

⚠ WARNING **BIOHAZARD**. Biological samples such as tissues, body fluids, and blood of humans and other animals have the potential to transmit infectious diseases. Read and follow the guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (http://bmbl.od.nih.gov) and in the Occupational Safety and Health Standards, Bloodborne pathogens (http://www.access.gpo.gov/nara/cfr/ waisidx_01/ 29cfr1910a_01.htm). Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective eyewear, clothing, and gloves.

Chemical Waste Hazard Warning

⚠ WARNING **CHEMICAL WASTE HAZARD**. Wastes produced by Applied Biosystems instruments are potentially hazardous and can cause injury, illness, or death.

- Read and understand the material safety data sheets (MSDSs) provided by the manufacturers of the chemicals in the waste container before you store, handle, or dispose of chemical waste.
- Handle chemical wastes in a fume hood.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (*e.g.,* safety glasses, gloves, or 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 (*e.g.*, fume hood). For additional safety guidelines, consult the MSDS.
- After emptying the waste container, seal it with the cap provided.
- Dispose of the contents of the waste tray and waste bottle in accordance with good laboratory practices and local, state/provincial, 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, chemical safety, and waste profiles.

About MSDSs

Some of the chemicals used with this instrument may be listed as hazardous by their manufacturer. When hazards exist, warnings are prominently displayed on the labels of all chemicals.

Chemical manufacturers supply a current material safety data sheet (MSDS) before or with shipments of hazardous chemicals to new customers and with the first shipment of a hazardous chemical after an MSDS update. MSDSs provide you with the safety information you need to store, handle, transport and dispose of the chemicals safely.

We strongly recommend that you replace the appropriate MSDS in your files each time you receive a new MSDS packaged with a hazardous chemical.

⚠ WARNING **CHEMICAL HAZARD**. Be sure to familiarize yourself with the MSDSs before using reagents or solvents.

Ordering MSDSs

You can order free additional copies of MSDSs for chemicals manufactured or distributed by Applied Biosystems using the contact information below.

To order documents by automated telephone service:

1.	From the U.S. or Canada, dial **1.800.487.6809**.
2.	Follow the voice instructions to order documents (for delivery by fax). **Note:** There is a limit of five documents per fax request.

In the U.S.	Dial **1.800.345.5224**, and press **1**.
In Canada	Dial **1.800.668.6913**, and press **1** for English or **2** for French.

3.	Go to **http://www.appliedbiosystems.com**
4.	In the **SEARCH** field at the top of the page, type in the chemical of interest.
5.	Select **MSDS** from the **IN** drop-down list to the right, then click **GO**.
6.	When the **Search Results** page opens, find the document you want in the language of your choice and click on it to open a PDF of the document.

For chemicals not manufactured or distributed by Applied Biosystems, call the chemical manufacturer.

Services and Support

Applied Biosystems Web Site

A services and support page is available on the Applied Biosystems Web site. To access this, go to:

http://www.appliedbiosystems.com

and click the link for services and support.

At the services and 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 services and support page provides worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.

Purification Using the ABI PRISM™ 6700 Automated Nucleic Acid Workstation

2

Introduction

In This Chapter

This chapter includes the following topics:

Buffer for Lysing Cultures 2-2
Lysing Suspension Cell Cultures. 2-3
Lysing Adherent Cell Cultures 2-6
Purifying RNA Automatically 2-8

About the 6700 Workstation

The 6700 Workstation is a flexible, fully automated system. It is designed to dramatically increase the throughput of nucleic acid purification and setup. For example:

- In just over 1 hour, the assay produces 96 purified samples of total RNA.
- In less than 2.5 additional hours, the 6700 Workstation prepares up to four fully assembled, 96-well reaction plates complete with standards, and controls for 5′ nuclease assay analysis or other nucleic acid-based assays.

Recommended Parameters

The following parameters are recommended for lysing cell cultures on the 6700 Workstation with Applied Biosystems total RNA chemistry:

Table 2-1 Suspension Cell Seeding Parameters

Parameter	Value
Input range	10 to 10^6 cells per well
Recommended range	10^4 to 10^6 cells per well
Maximum lysate volume	600 µL of lysed cells per well in a deep-well plate

Cell Culture Plates

You may use one of two types of plates.

- A 300-µL, flat-bottom, 96-well cell culture plate. We recommend one of the following:
  - Costar® 3596
  - BD Falcon
  - Nuncion Delta 1056
- A deep-well plate.

Buffer for Lysing Cultures

The total RNA 1X lysis buffer is a 1:1 mixture of 2X Nucleic Acid Purification Lysis Solution and calcium/magnesium-free phosphate-buffered saline (PBS).

IMPORTANT! The 2X Nucleic Acid Purification Lysis Solution is supplied as a 2X formulation. The 2X Nucleic Acid Purification Lysis Solution should always be used at a final concentration of 1X for maximum RNA recovery.

Chemical Hazard Warnings

⚠ WARNING **CHEMICAL HAZARD**. **2X Nucleic Acid Purification Lysis Solution** causes eye, skin, and respiratory tract irritation. It is harmful if swallowed. Contact with acids or bleach liberates toxic gases. DO NOT ADD acids or bleach to any liquid waste containing Nucleic Acid Purification Lysis Solution. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

⚠ WARNING **CHEMICAL HAZARD**. **1X Lysis Buffer (1:1 2X Nucleic Acid Purification Lysis Solution:PBS)** causes eye, skin, and respiratory tract irritation. It is harmful if swallowed. Read the MSDS for 2X Nucleic Acid Purification Lysis Solution, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Making 1X Lysis Buffer

To make 1X lysis buffer:

1.	Make 1X lysis buffer by diluting 1 volume of 2X Nucleic Acid Purification Lysis Solution with an equal volume of calcium/magnesium-free PBS.
2.	Cap the container and invert to mix.

Lysing Suspension Cell Cultures

Two Methods

For the 6700 Workstation, two methods can be used to lyse the suspension cell cultures:

- Method 1, which uses automated reagent addition by the 6700 Workstation
- Method 2, which uses manual reagent addition

Method 1: Automated Lysis Reagent Addition

Cultured Cell Biohazard Warning

⚠ WARNING **BIOHAZARD**. Biological samples such as cultured cells have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective eyewear, clothing, and gloves.

Preparing the Suspension Cell Cultures

To prepare the suspension cell cultures:

1.	Pellet the suspension cell cultures by centrifugation.
2.	Aspirate the growth media from all wells, making sure not to disturb the cell pellet.
3.	Resuspend the cell pellet in 125 µL of calcium/magnesium-free PBS.
4.	Place the cell culture plate in the primary input position of the 6700 Workstation. **Note:** Refer to the *ABI PRISM™ 6700 Automated Nucleic Acid Workstation User Guide* (PN 4304309) for the automated lysis procedure.

Method 2: Manual Lysis Reagent Addition

Chemical Hazard Warnings

⚠ WARNING **CHEMICAL HAZARD**. **2X Nucleic Acid Purification Lysis Solution** causes eye, skin, and respiratory tract irritation. It is harmful if swallowed. Contact with acids or bleach liberates toxic gases. DO NOT ADD acids or bleach to any liquid waste containing Nucleic Acid Purification Lysis Solution. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

⚠ WARNING **CHEMICAL HAZARD**. **1X Lysis Buffer (1:1 2X Nucleic Acid Purification Lysis Solution:PBS)** causes eye, skin, and respiratory tract irritation. It is harmful if swallowed. Read the MSDS for 2X Nucleic Acid Purification Lysis Solution, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Cultured Cell Biohazard Warning

⚠ WARNING **BIOHAZARD**. Biological samples such as cultured cells have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective eyewear, clothing, and gloves.

Lysing the Suspension Cell Cultures Manually

To lyse the suspension cell cultures manually:

Step	Action
1.	Pellet the suspension cells by centrifugation.
2.	Aspirate the growth media from all wells, making sure not to disturb the cell pellet. **Note:** If the cell culture growth media is calcium/magnesium-free, you may skip step 3 and lyse the cells directly in the growth media by adding an equal volume of 2X Nucleic Acid Purification Lysis Solution.
3.	Resuspend each cell pellet in 250 µL of 1X lysis buffer. **Note:** Make 1X lysis buffer by diluting 1 volume of 2X Nucleic Acid Purification Lysis Solution with an equal volume of calcium/magnesium-free PBS.
4.	Mix each cell pellet thoroughly into the 1X lysis buffer by pipetting the lysate up and down in the pipet tip at least three times, changing tips for each well.

To lyse the suspension cell cultures manually: ***(continued)***

5.	Place the cell culture plate in the primary input position of the 6700 Workstation.

Lysing Adherent Cell Cultures

Two Methods

For the 6700 Workstation, two methods can be used to lyse the adherent cell cultures:

- Method 1, which uses automated reagent addition by the 6700 Workstation
- Method 2, which uses manual reagent addition

Method 1: Automated Lysis Reagent Addition

Preparing the Adherent Cell Cultures

To prepare adherent cell cultures for lysis:

1.	Aspirate the growth media from all wells, making sure not to disturb the cell pellet.
2.	Resuspend the cell pellet in 125 µL of calcium/magnesium-free PBS.
3.	Place the cell culture plate in the primary input position of the 6700 Workstation. **Note:** Please refer to the *ABI PRISM™ 6700 Automated Nucleic Acid Workstation User Guide* (PN 4304309) for the automated lysis procedure.

Method 2: Manual Lysis Reagent Addition

Chemical Hazard Warnings

WARNING **CHEMICAL HAZARD**. **2X Nucleic Acid Purification Lysis Solution** causes eye, skin, and respiratory tract irritation. It is harmful if swallowed. Contact with acids or bleach liberates toxic gases. DO NOT ADD acids or bleach to any liquid waste containing Nucleic Acid Purification Lysis Solution. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

WARNING **CHEMICAL HAZARD**. **1X Lysis Buffer (1:1 2X Nucleic Acid Purification Lysis Solution:PBS)** causes eye, skin, and respiratory tract irritation. It is harmful if swallowed. Read the MSDS for 2X Nucleic Acid Purification Lysis Solution, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Cultured Cell Biohazard Warning

WARNING **BIOHAZARD**. Biological samples such as cultured cells have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective eyewear, clothing, and gloves.

Lysing the Adherent Cell Cultures Manually

To lyse the adherent cell cultures manually:

1.	Aspirate the growth media from all wells, making sure not to disturb the adherent cells. **Note:** If the cell culture growth media is calcium/magnesium-free, you may skip step 3 and lyse the cells directly in the growth media by adding an equal volume of 2X Nucleic Acid Purification Lysis Solution.
2.	Resuspend each cell pellet in 250 µL of 1X lysis buffer. **Note:** Make 1X lysis buffer by diluting 1 volume of 2X Nucleic Acid Purification Lysis Solution with an equal volume of calcium/magnesium-free PBS.
3.	Mix each cell pellet thoroughly into the 1X lysis buffer by pipetting the lysate up and down in the pipet tip at least three times, changing tips for each well.
4.	Place the cell culture plate in the primary input position of the 6700 Workstation.

Purifying RNA Automatically

Purification Overview

Use the 6700 Workstation to purify the RNA automatically. The procedure is the same for both suspension and adherent cell lysates. The purification of RNA on the 6700 Workstation consists of the following procedures:

Purifying RNA Automatically 2-8
Purifying RNA Automatically 2-9

Creating a New RNA Archive Protocol

Create a new Archive protocol using the following illustration and table. For further information on creating protocols, please refer to the *ABI PRISM™ 6700 Automated Nucleic Acid Workstation User Guide* (PN 4304309).

New RNA/DNA Archive Protocol

Protocol Name: New RNA Archive Protocol ☑ In Use

Conditions for Transferring Samples to the Purification Tray

Lysis/DNA Precipitation Input: Falcon 96-Well Plate

	Add Soln. (µL)	Transfer (µL)	Mix (#)	Starting (µL)
First Transfer:	None	200	3	250
Second Transfer:	0	0	0	

☐ High Viscosity Sample

Filtration Conditions

☐ Create Deep-Well Filtrate Plate

Incubation Time: 0 (min.)

Vacuum Time: 120 (sec.)

Vacuum Pressure: 20 %

Wash Conditions

Step	Add	Volume (µL)	Temp. (°C)	Incubation (min)	Vacuum (sec)	Repeat (count)	Vacuum (%)
1. ☑	Wash Solution 1	400		0	120	1	20
2. ☑	Wash Solution 2	500		0	120	1	20
3. ☑	Wash Solution 2	300		0	120	2	20
4. ☐		300		0	120	1	20
5. ☐		300		0	120	1	20
6. ☐		300		0	120	1	20
7. ☐		300		0	120	1	20
	Pre-Elution Vacuum				300		90
	Elution Solution	150	-	0	120	1	20

Cancel OK

Table 2-2 New RNA Archive Protocol

Field or Submenu	Action
Protocol Name	Type in a name.
Lysis/DNA Precipitation Input	Select a plate.
Default parameters	Leave unchanged.

Loading the Samples

To load the samples, place the input plate into the primary input position on the 6700 Workstation.

Purification Using the ABI PRISM™ 6100 Nucleic Acid PrepStation

3

Introduction

In This Chapter

This chapter includes the following topics:

Buffer for Lysing Cultures 3-1
Lysing Cell Cultures 3-2
Using the 6100 PrepStation. 3-5

About the 6100 PrepStation

The 6100 PrepStation is a semi-automated nucleic acid purification platform. The firmware and an integrated vacuum system automatically perform all vacuum operations. Users perform sample and reagent additions.

Purification Overview

The purification of RNA on the 6100 PrepStation consists of:

- Preparing the 1X lysis buffer
- Lysing the suspension or adherent cell cultures
- Using the 6100 PrepStation

Buffer for Lysing Cultures

The total RNA buffer is a 1:1 mixture of 2X Nucleic Acid Purification Lysis Solution and calcium/magnesium-free PBS.

IMPORTANT! The 2X Nucleic Acid Purification Lysis Solution is supplied as a 2X formulation. The 2X Nucleic Acid Purification Lysis Solution should always be used at a final concentration of 1X for maximum RNA recovery.

Chemical Hazard Warnings

⚠ WARNING **CHEMICAL HAZARD**. **2X Nucleic Acid Purification Lysis Solution** causes eye, skin, and respiratory tract irritation. It is harmful if swallowed. Contact with acids or bleach liberates toxic gases. DO NOT ADD acids or bleach to any liquid waste containing Nucleic Acid Purification Lysis Solution. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

⚠ WARNING **CHEMICAL HAZARD**. **1X Lysis Buffer (1:1 2X Nucleic Acid Purification Lysis Solution:PBS)** causes eye, skin, and respiratory tract irritation. It is harmful if swallowed. Read the MSDS for 2X Nucleic Acid Purification Lysis Solution, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Making 1X Lysis Buffer

To make 1X lysis buffer:

1.	Make 1X lysis buffer by diluting 1 volume of 2X Nucleic Acid Purification Lysis Solution with an equal volume of calcium/magnesium-free PBS.
2.	Cap the container and invert to mix.

Lysing Cell Cultures

Chemical Hazard Warnings

⚠ WARNING **CHEMICAL HAZARD**. **2X Nucleic Acid Purification Lysis Solution** causes eye, skin, and respiratory tract irritation. It is harmful if swallowed. Contact with acids or bleach liberates toxic gases. DO NOT ADD acids or bleach to any liquid waste containing Nucleic Acid Purification Lysis Solution. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves

⚠ WARNING **CHEMICAL HAZARD**. **1X Lysis Buffer (1:1 2X Nucleic Acid Purification Lysis Solution:PBS)** causes eye, skin, and respiratory tract irritation. It is harmful if swallowed. Read the MSDS for 2X Nucleic Acid Purification Lysis Solution, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Cultured Cell Biohazard Warning

WARNING **BIOHAZARD**. Biological samples such as cultured cells have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective eyewear, clothing, and gloves.

Lysing Suspension Cell Cultures

To lyse suspension cell cultures:

1.	Pellet the suspension cells by centrifugation.
2.	Aspirate the growth media from all wells, making sure not to disturb the cell pellet. **Note:** If the cell culture growth media is calcium/magnesium-free, you may skip step 3 and lyse the cells directly in the growth media by adding an equal volume of 2X Nucleic Acid Purification Lysis Solution.
3.	Resuspend each cell pellet in 250 µL of 1X lysis buffer. **Note:** Make 1X lysis buffer by diluting 1 volume of 2X Nucleic Acid Purification Lysis Solution with an equal volume of calcium/magnesium-free PBS.
4.	Mix each cell pellet thoroughly into the 1X lysis buffer by pipetting the lysate up and down in the pipet tip at least three times, changing tips for each well.

Lysing Adherent Cell Cultures

To lyse adherent cell cultures:

1.	Aspirate the growth media from all wells, making sure not to disturb the adherent cells. **Note:** If the cell culture growth media is calcium/magnesium-free, you may skip step 3 and lyse the cells directly in the growth media by adding an equal volume of 2X Nucleic Acid Purification Lysis Solution.
2.	Resuspend the adherent cells in each well in 250 µL of 1X lysis buffer.
3.	Mix the cells thoroughly in each well into the 1X lysis buffer by pipetting the lysate up and down in the pipet tip at least three times, for each well.

Using the 6100 PrepStation

Selecting the Predefined Method

You must select a predefined protocol for purifying RNA on the 6100 PrepStation before proceeding with the purification, or use Quick Method. For further information on purifying RNA, please refer to the *ABI PRISM™ 6100 Nucleic Acid PrepStation User Guide* (PN 4326242).

Table 3-1 gives the steps for isolating total RNA from cultured cells without a DNase wash.

Table 3-1 RNA Cell Method without DNase Wash

Step	Description	Volume (μL)	Position	Time (sec)	Vacuum (%)
—	Pre-Wet All Wells with Wash Solution 1	40	Waste	—	—
1.	Load Samples	10–650	Waste	120	20
2.	Add Wash Solution 1	500	Waste	120	20
3.	Add Wash Solution 2	400	Waste	120	20
4.	Add Wash Solution 2	400	Waste	120	20
5.	Add Wash Solution 2	300	Waste	120	20
6.	Pre-Elution Vacuum	—	Waste	300	90
7.	Touch Off at Waste	—	Touch Off	—	—
8.	Add Elution Solution	150	Collection	120	20
9.	Touch Off at Collection	—	Touch Off	—	—

Table 3-2 gives the steps for isolating total RNA from cultured cells using a DNase wash.

Table 3-2 RNA Cell Method with DNase Wash

Step	Description	Volume (µL)	Position	Time (sec)	Vacuum (%)
—	Pre-Wet all Wells with Wash Solution 1	40	Waste	—	—
1.	Load Samples	10–650	Waste	120	20
2.	Add Wash Solution 1	500	Waste	120	20
3.	Add Wash Solution 2	650	Waste	120	20
4.	Add AbsoluteRNA Wash Solution and Incubate	50	Waste	900	0
5.	Add Wash Solution 2 and Incubate	400	Waste	300	0
6.	Wash Solution 2 Removal	—	Waste	120	20
7.	Add Wash Solution 2	300	Waste	120	20
8.	Add Wash Solution 2	300	Waste	120	20
9.	Pre-Elution Vacuum	—	Waste	300	90
10.	Touch Off at Waste		Touch Off	—	—

Table 3-2 RNA Cell Method with DNase Wash *(continued)*

Step	Description	Volume (μL)	Position	Time (sec)	Vacuum (%)
11.	Add Elution Solution	50–150	Collection	120	20
12.	Touch Off at Collection	—	Touch Off	—	—

Loading Disposables

For complete instructions for the following procedure and for performing a run, refer to the *ABI PRISM™ 6100 Nucleic Acid PrepStation User Guide* (PN 4326242).

To load disposables onto the 6100 PrepStation:

1.	Place a 96-well archive plate in the collection compartment.
2.	Place a splash guard in the waste compartment.
3.	Place a Total RNA Purification tray in the carriage, and turn the two knobs to secure the tray in place.
4.	Slide the carriage to the waste position, and push the carriage handle down until the carriage locks into position and seals.

Use of AbsoluteRNA Wash Solution 4

Introduction

In This Chapter

This chapter includes the following topics:

Requirements for Highly Purified RNA 4-1
Use of AbsoluteRNA Wash Solution 4-1

Requirements for Highly Purified RNA

Some RNA Analyses

Highly purified RNA is required for:

- Real-time PCR
- Microarray analysis of gene expression levels
- Identification of pathogenic materials
- Analysis of cell population numbers

For these procedures, the RNA must be free of contaminating gDNA and reverse transcription or PCR inhibitors in order to obtain accurate and reliable results.

The current Total RNA Chemistry produces RNA that contains less than 0.5% gDNA contamination by weight. However, for samples where limited sequence knowledge is available, or for targets where exon-exon spanning primer-probe sets are impossible to design, even very low levels of gDNA contamination can bias results.

Use of AbsoluteRNA Wash Solution

About AbsoluteRNA Wash Solution

AbsoluteRNA Wash Solution was developed to meet the need for highly purified RNA. The use of AbsoluteRNA Wash Solution can reduce gDNA levels to virtually 0% by weight (<0.002%).

Addition to the Isolation Procedure

The use of AbsoluteRNA Wash Solution requires only a small modification to the existing Total RNA Chemistry procedure. The addition of a 15 minute on-column treatment provides an economical and high-throughput RNA preparation of very high purity.

Using AbsoluteRNA Wash Solution

The RNA isolation procedure incorporating the AbsoluteRNA Wash Solution requires the addition of 50 µL of AbsoluteRNA Wash Solution after the first addition of Wash Solution 2. It is incubated for 15 minutes with zero vacuum time. The following Wash Solution 2 step has an incubation time of 10 minutes and should have a volume of 600 µL.

Changing the Protocol

Make the equivalent changes to all protocols to include:

- 50-µL addition of the AbsoluteRNA Wash Solution with a 15 minute incubation
- 10 minute incubation with the following Wash Solution 2

All other steps remain the same as in the original protocols given in the *ABI PRISM™ 6700 Automated Nucleic Acid Workstation User Guide* (PN 4304309) and the *ABI PRISM™ 6100 Nucleic Acid PrepStation User Guide* (PN 4326242).

6700 Workstation Archive Protocol Illustration

In the example below, the Protocol Name is RNA Cell + DNase and the entries reflect the required archive protocol changes.

New RNA/DNA Archive Protocol

Protocol Name: RNA Cell + DNase ☑ In Use

Conditions for Transferring Samples to the Purification Tray

Lysis/DNA Precipitation Input: Falcon 96-Well Plate

	Add Soln. (µL)	Transfer (µL)	Mix (#)	Starting (µL)
First Transfer:	None	200	3	250
Second Transfer:	0	0	0	

☐ High Viscosity Sample

Filtration Conditions

☐ Create Deep-Well Filtrate Plate

Incubation Time: 0 (min.)

Vacuum Time: 120 (sec.)

Vacuum Pressure: 20 %

Wash Conditions

Step	Add	Volume (µL)	Temp. (°C)	Incubation (min)	Vacuum (sec)	Repeat (count)	Vacuum (%)
1. ☑	Wash Solution 1	400		0	120	1	20
2. ☑	Wash Solution 2	500		0	120	1	20
3. ☑	DNase Wash	50		15	0	2	0
4. ☑	Wash Solution 2	600		10	120	1	20
5. ☑	Wash Solution 2	300		0	120	2	20
6. ☐		300		0	120	1	20
7. ☐		300		0	120	1	20
	Pre-Elution Vacuum				300		90
	Elution Solution	150	-	0	120	1	20

Cancel OK

Outline for the 6100 PrepStation

An outline of the steps incorporating the AbsoluteRNA Wash Solution using the 6100 PrepStation is in Chapter 3, Table 3-2 on page 3-6.

Yields and Purity 5

Introduction

In This Chapter

This chapter includes the following topics:

Total RNA Chemistry Yield 5-1
About Inhibition 5-5

Total RNA Chemistry Yield

When used as recommended, the Applied Biosystems Total RNA chemistry and instrument systems provide high yields and high purity for cell-derived total RNA. Average yields and examples are presented in this section.

Yield of Total RNA

For seeding densities of 10^4 to 10^6 cells per well, the yield of total RNA is specified to be greater than 50%, and typically is close to theoretical. Table 5-1 shows typical results of total RNA purified from a variety of cultured cells using the ABI PRISM™ 6700 Workstation and the 6100 PrepStation.

The maximum yield per well of RNA is >200 µg, but is highly dependant on the sample type.

Note: The gDNA contamination was determined using the One Step 18S Ribosomal 5′ Nuclease Assay performed on the ABI PRISM® 7700 Sequence Detection System

Table 5-1 Analysis of Total RNA from Cultured Cells; Quantitation by UV Spectroscopy

Cell Type	Cells per Well	Typical Yield per Well (μg)	% DNA by Weight	$A_{260/280}$ Ratio	Standard Deviation	% CV
Raji	10^6	25 to 100	0.10	2.10	0.60	3.35
Raji	10^4	1 to 10	0.10	~2	0.47	1.78
PC-3	10^6	32.5	0.01	2.08	0.20	1.36
HepG2	10^6	28.6	0.00	2.07	0.22	1.51
3T3	10^6	34.8	0.01	2.09	0.24	1.64
HeLa	3.5×10^4	30.7	0.01	2.05	0.26	1.81

Table 5-2 shows typical results of total RNA purified from PC-3 cells. In this assay, 60 wells of a PC-3 adherent cell culture were purified at a seeding density of approximately 10^5 cells per well.

Table 5-2 Analysis of Total RNA from PC-# Cell Culture; Quantitation by Beta-Actin RT-PCR

Cells per Well	Typical Yield per Well (μg)	$A_{260/280}$ Ratio	% CV
~10^5	7 (71 pg/cell)	2.06	13

Figure 5-1 shows the amplification plot for C_T vs. well position for the total RNA purified from PC-3 cells. The gDNA contamination was determined by the 18S Ribosomal 5′ Nuclease Assay performed on the 7700 Sequence Detection System.

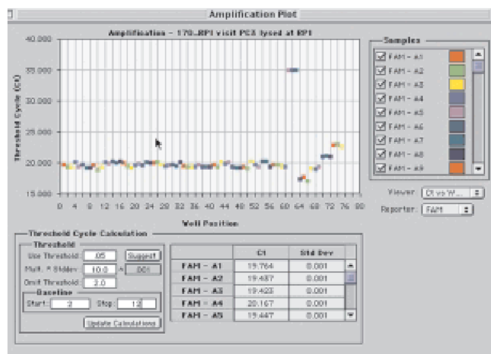


Figure 5-1   Total RNA Purified from PC-3 Cells

Precision and Reproducibility of Recovery

The total experimental coefficient of variation (CV) is specified to be less than 30%. This includes:

- Purification on the 6700 Workstation or 6100 PrepStation
- All pipetting steps
- Analysis on the ABI PRISM 7000, 7700, or 7900HT Sequence Detection Systems

Figure 5-2 shows the results for 16 samples of human Raji cells seeded at 10^5 cells per well and purified on the 6700 Workstation.The estimated recovery of 17 µg of total RNA per well was measured by UV absorbance where 1 ODU = 40 µg of RNA.

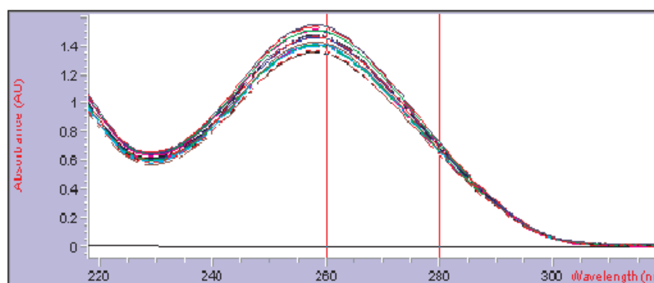


Figure 5-2   Results for 16 Samples of Total RNA from Raji Cells.

Range of Recovery

Figure 5-3 shows that for Raji cells, the total RNA recovery versus cell input is linear for the range 10 pg–100 mg (101 to 106 cells). The samples were analyzed in quadruplicate.

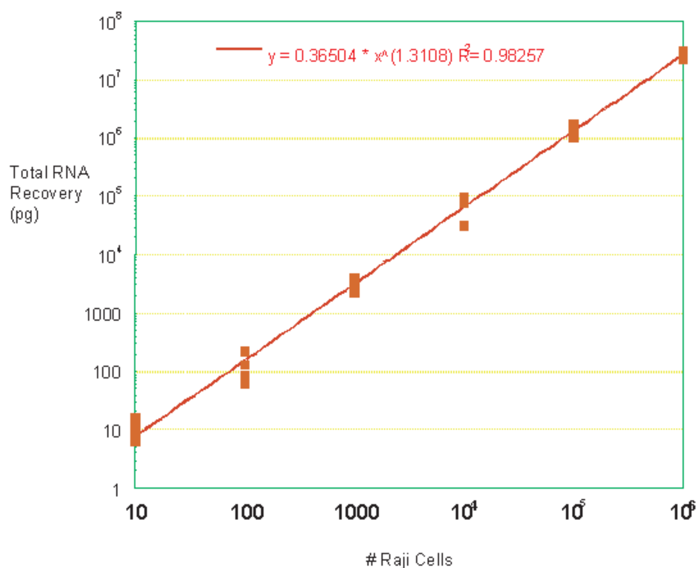


Figure 5-3 Total RNA Recovery from Raji Cells.

IMPORTANT! Input ranges greater than 10^6 cells can cause blockages in the Total RNA Purification tray, which may cause the wash solution to overflow. This can result in well-to-well contamination and a failed purification run. See Figure 3 for an example.

About Inhibition

The presence of inhibitors after purification of total RNA samples is a widespread problem with many total RNA extraction methods. Applied Biosystems total RNA chemistry and purification procedures readily remove inhibitors.

The threshold cycle (C_T) suppression of the neat samples is less than or equal to 0.5.

Detecting Inhibitors

The combination of the 7000, 7700, or 7900HT Sequence Detection Systems and the RT+GAPDH-based 5′ Nuclease assay easily detects the presence of inhibitors. Follow the procedure for a very sensitive assessment of PCR performance.

To assess PCR performance:

1.	Measure C_T values on four-fold serial dilutions of an individual sample.
2.	Measure inhibition by plotting a standard curve of the three most dilute samples.
3.	Extrapolate to compare the measure versus the calculated C_T values for 1:4 and undiluted samples.
4.	Determine performance as follows:

If the measured C_T value for the undiluted sample is ...	Then PCR inhibitor is ...
suppressed by >0.5 cycles from the calculated C_T value	present.
not suppressed by >0.5 cycles from the calculated C_T value	not present. See Figure 5 for an example.

Figure 5-4 gives the results of an inhibition assay of total RNA purified from Raji cell lysate. The 1:4 dilution series is from a single purified nucleic acid sample ($R^2 = 0.995$). The straight line relationship between the log dilution and C_T value for the six points of the 1:4 dilution series indicates that the sample contains no PCR inhibitors. If PCR inhibitors were present, there would be an obvious nonlinear relationship at the no dilution and 1:4 dilution points.

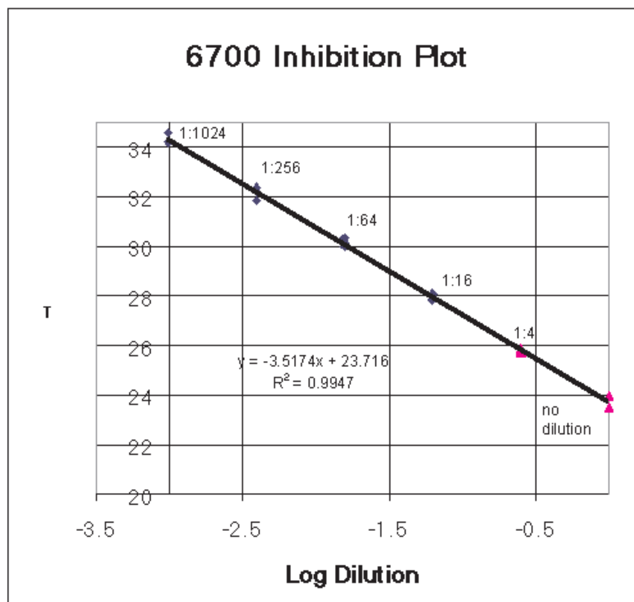


Figure 5-4 A 6700 Workstation Inhibition Plot.

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