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Arcturus® Paradise® PLUS Whole Transcript Reverse Transcription (WT-RT) Reagent System

Optimized RNA Extraction, Isolation, and Reverse Transcription from Archived FFPE Tissues

User Guide

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EXPIRATION

All reagents included with the system should be used within six (6) months of receipt.

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1. Introduction

The following topics are covered in this chapter:

→ Getting Started

→ About the Reagents

→ Module Components

→ User-Supplied Materials

→ Recommendations for Nuclease-Free Technique

1.1. GETTING STARTED

This user guide provides recommended applications and protocols for use of the Arcturus® Paradise® PLUS Whole Transcript Reverse Transcription (WT-RT) Reagent System.

Life Technologies recommends proceeding as follows:

1 See Section 1.2., *"About the Reagents"* to familiarize yourself with the Paradise PLUS WT-RT Reagent System.

2 Upon receipt, immediately inspect and store the module components as described in Chapter 1.

3 Obtain all required user-supplied materials (Chapter 1).

4 Review the nuclease-free techniques in Chapter 1.

5 Perform the protocols (Chapter 2 through Chapter 4). For each protocol, be sure to:

→ Read through the entire chapter before performing any of the procedures in the protocol.

→ Perform the procedures in the order given.

Note: Life Technologies recommends performing a sample assessment protocol. Refer to Appendix A.

1.2. ABOUT THE REAGENTS

1.2.1. CONTENTS

The Paradise PLUS WT-RT Reagent System includes reagents for extraction, isolation, and reverse transcription of total RNA from Formalin-Fixed Paraffin-Embedded (FFPE) tissues.

The reagents allow you to efficiently extract and isolate RNA, then perform reverse transcription to obtain whole transcripts of quality sufficient for quantitative real-time polymerase chain reaction (qRT-PCR) analysis.

1.2.2. AVAILABLE KITS

Reagents contained in the Paradise PLUS WT-RT Reagent System are packaged as convenience kits in two sample sizes. The kits include the modules listed below. Recommended protocols for each module are provided in this User Guide.

⚠ **IMPORTANT:** Recommended protocols are based on use of Paradise PLUS general purpose reagents when used together. Alternate reagents have not been tested; substitution of alternate reagents cannot be supported by Life Technologies Technical Support.

1.2.3. KIT PACK SIZE CATALOG NO.

Paradise PLUS WT-RT Reagent System, includes:

1. KIT0305 (48 Samples) Paradise PLUS WT-RT Reagent System, includes:
   - **a** Frozen Module (RA7003ARC)
   - **b** Room Temperature Module (RA7004a & RA7004b)
2. KIT0315 (12 Samples) Paradise PLUS WT-RT Reagent System, includes:
   - **a** Frozen Module (RA7001)
   - **b** Room Temperature Module (RA7002ARC)

1.2.4. STORAGE

The Paradise PLUS WT-RT kits have both room temperature and frozen components. The room temperature components should be stored at room temperature (10 °C to 30 °C). Proteinase K vials should be stored in a desiccator at room temperature.

The frozen components are shipped on dry ice and should be stored at -65 °C to -80 °C until initial use. After initial use -15 °C to -30 °C is recommended to prevent unnecessary freeze-thaws of the enzymes.

The control RNA and any RNA generated from Paradise PLUS WT-RT kits should always be stored at -65 °C to -80 °C.

1.3. SAFETY DATA SHEET (SDS)

1.3.1. ABOUT THE SDS

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

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

1.3.2. OBTAINING AN SDS

The SDS for any chemical supplied by Applied Biosystems is available to you free 24 hours a day. To obtain SDSs:

1. Go to **www.appliedbiosystems.com**, click **Support**, then select **SDS**.
2. In the Keyword Search field, enter the chemical name, product name, SDS part number, or other information that appears in the SDS 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

NOTE: For the SDSs of chemicals not distributed by Applied Biosystems, contact the chemical manufacturer.

1.4. MODULE COMPONENTS

Inspect all module components upon receipt and store as recommended. For optimal results, use the reagents as soon as possible after receipt.

RNA EXTRACTION AND ISOLATION REAGENTS

The components listed below are shipped in the RNA Extraction and Isolation Module.

KIT0315 WT-RT (12 Reactions)

Room Temperature Components

RA7001–12 Reactions

Component	Description	Vial Label
PK Mix (lyophilized)	6 x 600 µg/tube	–
PK Buffer	–	PKB
Extraction Conditioning Buffer	Blue	CB
Extraction Binding Buffer	Blue	BB
Extraction Ethanol Solution	Blue	EtOH
Extraction Wash Buffer 1	Blue	W1
Extraction Wash Buffer 2	blue	W2
Extraction elutionBuffer	Blue	EB
Purification columns w/ tubes	1 x 12 columns	–
Microcentrifuge tubes	1 x 12 tubes	–

Frozen Components

RA7002ARC–12 Reactions

Component	Description	Vial Label
Formalin-Fixed uRNA, 500 pg/µL	White	C
RT Enzyme Mix	Red	1
RT Master Mix	Red	2
Enhancer	Yellow	E
Nuclease Mix	Gold	–
Random Primer	Grey	–
sDNase Buffer	Green	1
sDNase Enzyme Mix	Green	2
sDNase Stop Solution	Green	3

KIT0305 WT-RT (48 Reactions)

Room Temperature Components

RA7004a–Bulk

Component	Description
Extraction Conditioning Buffer	4 x 12 columns
Microcentrifuge tubes	4 x 12 tubes

RA7004b–Bulk

Component	Description	Vial Label
PK Mix (lyophilized)	8 x 1800 µg/tube	–
PK Buffer	White	PKB
Extraction Conditioning Buffer	Blue	CB
Extraction Binding Buffer	Blue	CB
Extraction Ethanol Solution	Blue	EtOH
Extraction Wash Buffer 1	Blue	W1
Extraction Wash Buffer 2	Blue	W2
Extraction Elution Buffer	Blue	EB

Frozen Components

RA7003ARC–

Component	Vial Color	Vial Label
Formalin-Fixed uRNA, 500 pg/µL	White	C
RT Enzyme Mix	Red	1
RT Master Mix	Red	2
Enhancer	Yellow	E
Nuclease Mix	Gold	–
Random Primer	Grey	–
sDNase Buffer	Green	1
sDNase Enzyme Mix	Green	2
sDNase Stop Solution	Green	3

1.5. USER-SUPPLIED MATERIALS

The materials listed in this section are required, but not included with the Paradise PLUS WT-RT Reagent System. Be sure you have access to these materials before beginning the recommended protocols in this user guide.

Table 1.1: Tissue Preparation

Equipment	Supplier
Rotary microtome	Major laboratory suppliers (MLS)
Tissue flotation water bath	MLS
Forceps	MLS
Superfrost Micro Slides with Plastic Micro Slide Box	VWR (Cat # 48311-600)
Fume hood	MLS
Sterile PAP jars (plastic slide jars)	Evergreen Scientific (Cat # 222-5450-G8S)
If storing the slides: Non-frost-free freezer, -65 °C to -80 °C Desiccator	MLS MLS
If slides have been frozen: Incubation oven, 50 °C to 60 °C	MLS

Consumables	Supplier
Disposable gloves	MLS
RNase AWAY® or other nuclease decontamination solution	Invitrogen (Cat # 10328-011)
Kimwipes® or similar lint-free towels	MLS
Nuclease-free water	MLS
Disposable rotary microtome blades	MLS
Xylene, histological grade	Fisher Scientific (Cat # 1330-20-7)

Table 1.2: RNA Extraction and Isolation

Equipment	Supplier
Vortexor	MLS
Thermal cycler (heated lid required)	MLS
Incubation oven, 50 °C	MLS
Non-frost-free freezer, -65 °C to -80 °C	MLS
Scalpel and scalpel blades, sterile	MLS
Microcentrifuge	Eppendorf 5415D or similar
Picofuge	MLS
Pipettors: 0.5–10 µL 2–20 µL 20–200 µL 100–1,000 µL	MLS MLS

Equipment	Supplier
Optional: NanoDrop® ND-1000, or Agilent® 2100 Bioanalyzer	NanoDrop Technologies Agilent Technologies

Consumables	Supplier
Disposable gloves	MLS
RNase AWAY or other nuclease decontamination solution	Invitrogen (Cat # 10328-011)
Kimwipes or similar lint-free towels	MLS
Pipette tips, nuclease-free, aerosol resistant (assorted sizes)	MLS
0.5-mL microcentrifuge tubes	Bio-Rad Laboratories (Cat # TBI-0501)
2.0-mL microcentrifuge tubes, nuclease-free	MLS

Table 1.3: Reverse Transcription

Consumables	Supplier
Disposable gloves	MLS
RNase AWAY or other nuclease decontamination solution	Invitrogen (Cat # 10328-011)
Kimwipes or similar lint-free towels	MLS
0.5-mL Individual Flat-Cap Tubes	Bio-Rad Laboratories (Cat # TBI-0501, 1,000 tubes/package)
Poly (I)	Sigma (Cat # P4154)
Pipette tips, nuclease-free, aerosol resistant (assorted sizes)	MLS
SuperScript® III Reverse Transcriptase, 200 U/μL (Enzyme only)	Invitrogen (Cat # 18080-093, 18080-044 or 18080-085)

1.6. RECOMMENDATIONS FOR NUCLEASE-FREE TECHNIQUE

Nuclease contamination will cause experimental failure. Minimize nuclease contamination by adhering to the following recommendations throughout your experiment:

→ Use only the reagents/components that are recommended or included in the Paradise PLUS WT-RT Reagent System. Substitutions may adversely affect performance or introduce nucleases.

→ Wear disposable gloves and change them frequently to prevent the introduction of nucleases from skin surfaces. After putting on gloves, avoid touching surfaces that may introduce nucleases onto glove surfaces.

→ Use nuclease-free solutions.

→ Use only new plasticware that is certified nuclease-free.

→ Use only sterile, nuclease-free pipette tips and microcentrifuge tubes.

→ Wash scalpels, tweezers, and forceps with detergent, then rinse three times with nuclease-free water.

→ Clean work surfaces with commercially available nuclease decontamination solutions (for example, RNase AWAY solution) prior to performing the procedures in this User Guide.

2. Tissue Preparation

The following topics are covered in this chapter:

→ Before You Begin

→ Cleaning the Work Area and Equipment

→ Preparing the Tissue Scrapes

→ Performing Deparaffinization

2.1. BEFORE YOU BEGIN

2.1.1. MATERIALS REQUIRED

To perform the procedures in this protocol, you need all materials listed in "Tissue Preparation" in Chapter 1.

2.1.2. WORKFLOW

The workflow for performing tissue preparation is as follows:

1 Review the recommendations for nuclease-free technique in Section 1.6.

2 Obtain the materials required (see Section 1.5., *"User-Supplied Materials"*).

3 Read through this entire chapter before performing any of the procedures.

4 Perform the procedures in the order listed below.

5 Unless otherwise noted, proceed immediately from one procedure to the next.

6 Clean the work area and equipment (see Section 2.2., *"Cleaning the Work Area and Equipment"*).

7 Prepare the slides (see Section 2.3., *"Preparing the Tissue Sections"*).

8 Perform deparaffinization (see Section 2.4., *"Performing Deparaffinization"*).

2.2. CLEANING THE WORK AREA AND EQUIPMENT

To minimize nuclease contamination, clean the work area, rotary microtome, and tissue flotation water bath.

⚠ **PRECAUTION**: Wear clean, disposable gloves throughout this entire procedure.

2.2.1. WORK AREA

Use RNase AWAY® solution and follow the manufacturer's instructions to clean any other surfaces that may come in contact with the sample.

2.2.2. ROTARY MICROTOME

To clean the rotary microtome:

1. Remove and discard the old disposable microtome blade.

2. Using a Kimwipes® cleansing wipe soaked with RNase AWAY solution, clean the knife holder.

3. Dry all cleaned surfaces with a clean Kimwipes cleansing wipe.

4. Install a new disposable microtome blade into the knife holder.

5. Clean forceps, brushes or any other instruments used during sectioning with RNase AWAY solution.

2.2.3. TISSUE FLOTATION WATER BATH

To clean the tissue flotation water bath:

1. Using a Kimwipes cleansing wipe soaked with RNase AWAY solution, clean the interior of the water bath.

2. Rinse the interior of the water bath with autoclaved DEPC treated or nuclease-free water.

3. Fill the water bath with autoclaved DEPC treated or nuclease-free water.

4. Heat the water to an appropriate temperature for the paraffin used in your laboratory, typically 41 °C – 43 °C.

⚠ **IMPORTANT:** Do not add any adhesives to the water bath.

2.3. PREPARING THE TISSUE SECTIONS

⚠ **PRECAUTION**: Wear clean, disposable gloves throughout this entire procedure.

2.3.1. PREPARING THE SLIDES

⚠ **IMPORTANT:** Life Technologies recommends using tissue fixed in 10% Neutral Buffered Formalin for 14–24 hours. For complete tissue fixation guidelines, contact Technical Support at 1-800-831-6844 option 5.

To prepare the slides:

1 On the rotary microtome, set the cutting thickness to 7 µm.

2 Prepare a paraffin block:

a Place a paraffin block into the specimen holder.

b Trim any excess paraffin from the block face or realign face of the block such that it is parallel to knife edge.

c After trimming, cut and discard the first five sections.

3 From the fresh surface of the paraffin block, cut 7 µm section(s) from your specimen.

4 Remove the section(s) from the rotary microtome, then float them onto the heated tissue flotation water bath.

5 Allow the section(s) to flatten, but do not leave them in the water bath for more than 2 minutes.

6 Mount each section on a room-temperature Superfrost Micro Slide.

7 Dry the slides:

a Prop each slide on end to allow water to drain away from the section.

b Air-dry the slides for a minimum of 90 minutes at room temperature.

⚠ **IMPORTANT:** Do not dry the slides in an oven.

⚠ **IMPORTANT:** Depending on the humidity in the environment, it may take longer for the sections to dry. The sections must be dry before proceeding. However, do not allow sections to air-dry for longer than 3 hours.

c Discard any slides that have wrinkles or folds in the section.

8 If you are cutting more than one specimen, do one of the following to avoid cross contamination:

a Move to a new section of the blade, or

b Use a new disposable blade for each specimen.

9 When the slides are dry:

a Proceed immediately to Section 2.4., *"Performing Deparaffinization"*, or

b Store the slides in a micro slide box in a room temperature desiccator for up to 2 weeks, or

c Store the slides in a micro slide box at -65 °C to -80 °C for up to 3 months.

10 After completing the slide preparation process:

a Remove any paraffin debris from the rotary microtome, clean the surfaces with a Kimwipes cleansing wipe soaked with RNase AWAY solution, then dry all surfaces.

b Discard the water from the tissue flotation water bath, clean the interior with RNase AWAY solution, then dry all surfaces.

2.4. PERFORMING DEPARAFFINIZATION

Precautions: Wear clean, disposable gloves throughout this entire procedure. Xylene is a known toxin. Personal protective equipment should be worn at all times while performing the deparaffinization procedure. Perform all steps in a fume hood.

2.4.1. PREPARING THE SLIDE JARS

1. Label three plastic slide jars as follows:
   - **a** Xylene
   - **b** Xylene
   - **c** Xylene
2. Fill each jar with 25 mL of certified histological-grade Xylene.

2.4.2. REMOVING THE PARAFFIN

1. Retrieve up to four of the prepared slides (Section 2.3., *"Preparing the Tissue Sections"*).
2. Optional: If the slides have been frozen, place them in a 50 °C to 60 °C oven for 2 minutes.

   Note: Do not perform this step if the slides have been at room temperature.
3. Place the slides in jar A. Xylene for 5 minutes. Invert the jar gently three or four times.

   ⚠ **IMPORTANT:** Jar A. Xylene must be changed after processing up to a maximum of four slides. Discard the xylene appropriately and clean the jar following the procedure, "Cleaning the Plastic Slide Jars." in the appendix
4. Using forceps, transfer the slides to jar B. Xylene for 5 minutes. Invert the jar gently three or four times.
5. Using forceps, transfer the slides to jar C. Xylene for 5 minutes. Invert the jar gently three or four times.
6. Hold the slides in jar C. Xylene until ready to perform tissue scrape.

   ⚠ **IMPORTANT:** The minimum incubation in xylene should be 5 minutes, up to a maximum of 1–2 hours.
7. When ready to perform tissue scrape, remove the slides from the xylene, then dry in a fume hood for 5 minutes.

   ⚠ **IMPORTANT:** Perform RNA extraction and isolation within 2 hours after removing the slides from the xylene.
8. Repeat steps 1 through 7 for any remaining slides.
9. Discard the used xylene according to standard procedures.
10. Proceed to Chapter 3 to begin RNA extraction and isolation.

3. RNA Extraction and Isolation

The following topics are covered in this chapter:

→ Before You Begin

→ Extracting the RNA from Tissue Scrapes

→ Isolating the RNA

3.1. BEFORE YOU BEGIN

3.1.1. ABOUT THE MODULE

The RNA Extraction and Isolation module contains reagents to recover total cellular RNA from Formalin-Fixed Paraffin-Embedded (FFPE) tissues. The reagents were developed and optimized to work with 25–150 mm^2 of FFPE tissue. Reagents contained in the RNA Extraction and Isolation module produce RNA in a small volume of low-ionic strength buffer, ready for cDNA synthesis using the recommended Reverse Transcription module (Chapter 4).

3.1.2. MATERIALS REQUIRED

To perform the procedures in this protocol, you need:

1 The RNA Extraction and Isolation module

2 All materials listed in "RNA Extraction and Isolation" in Chapter 1.

3.1.3. WORKFLOW

The workflow for the RNA Extraction and Isolation module is as follows:

1 Be sure you have inspected and stored the module components (section 1.3.1).

2 Review the recommendations for nuclease-free technique (section 1.5).

3 Obtain the materials required for this module (section 1.4; table 1.3).

4 Read through this entire chapter before performing any procedures.

5 Perform the procedures in the order listed below.

6 Unless otherwise noted, proceed immediately from one procedure to the next.

a Collect tissue scrape and extract the RNA

b Isolating the RNA

3.2. TISSUE SCRAPE AND RNA EXTRACTION

⚠ **PRECAUTION:** Wear clean, disposable gloves throughout this entire procedure. Use a new, sterile scalpel blade for each tissue scrape to avoid cross-contamination.

3.2.1. PROTEINASE K DIGESTION

Preparation of Proteinase K Digestion Solution:

1. Remove the PK Mix (PK) and PK Buffer (PKB) from the RNA Extraction and Isolation module. Add the appropriate volume of PK Buffer to the PK Mix as per the table below:

Reagent	12-Sample Kit	48-Sample Kit
PK Mix (PK)	600 µg/tube	1800 µg/tube
PK Buffer (PKB)	300 µL/tube	900 µL/tube

2. Mix the PK Buffer and PK Mix thoroughly by gently vortexing. Be sure to completely dissolve the dried PK Mix.
3. Spin down briefly to collect the PK solution.
4. Pipette 25–150 µL of the PK solution into a nuclease-free, 0.5-mL microcentrifuge tube, adjusting the volume of PK solution according to the area of tissue to be scraped.

   Note: Use 1 µL of PK solution per 1 mm^2 of tissue scrape. Do not use more than 150 µL of PK solution per sample:

   a. Minimum of 25 µL of PK solution for 25 mm^2 of tissue area

   b. Maximum of 150 µL of PK solution for 150 mm^2 of tissue area

5. Using a new, sterile scalpel blade, take the dried slide and scrape off an area not exceeding 150 mm^2 from the section.

   Note: Life Technologies recommends scraping a minimum of 25 mm^2 of tissue area into 25 µL of PK solution to ensure adequate amounts of RNA for downstream applications.

6. Place the tissue scrape into the microcentrifuge tube containing the appropriate amount of PK solution.
7. Vortex gently, then spin briefly to collect the solution at the bottom of the tube. Make sure that the:

   a. Tissue scrape is completely submerged in the PK solution and is not adhering to the side of the microcentrifuge tube.

   b. The tissue is suspended in the PK solution and a pellet was not formed from the spin process.

8 Incubate the microcentrifuge tube for 16–20 hours at 37 °C in an oven.

9 After incubation, briefly spin down the cell extract. If there are large amounts of debris in the cell extract:

   a Centrifuge at 16,000 x g for 1 minute.

   b Transfer the supernatant to a new 0.5-mL microcentrifuge tube (do not use the tubes supplied in the kit), trying not to disturb the pellet.

10 At this point, you can:

   a Proceed immediately to Section 3.3., *"Isolating the RNA"*, or

   b Freeze the cell extract at -65 °C to -80 °C until you are ready to perform RNA isolation.

3.3. ISOLATING THE RNA

The RNA isolation process, including incubations, will take approximately 1 hour.

⚠ **PRECAUTION:** Wear clean, disposable gloves throughout this entire procedure. You will be centrifuging the nucleic acid purification column several times throughout this procedure. Handle the purification column and collection tube carefully so that the flowthrough waste does not splash back onto the column.

Note: Flow-through waste following centrifugation is usually present as only a small volume. With this protocol, you should not need to discard the flowthrough waste after every centrifugation step. However, if the level of flowthrough waste approaches the purification column, discard the flowthrough waste and repeat centrifugation. Small amounts of flowthrough waste can inhibit the RT reaction.

3.3.1. THERMAL CYCLER RECOMMENDATION

Life Technologies recommends performing incubations in a thermal cycler. Please see Appendix B.3., *"Thermal Cycler Programming"* for suggested times and temperatures.

3.3.2. PRE-CONDITIONING THE PURIFICATION COLUMN

1 Pipette 200 µL of Conditioning Buffer (CB) onto the purification column filter membrane.

2 Let the purification column sit at room temperature for at least 5 minutes.

   Note: At this point, it is okay to leave the purification column while performing steps 1 through 6 in "Mixing and Loading the Cell Extract" (below).

3 Centrifuge the purification column at 16,000 x g for 1 minute, using the collection tube provided with the purification column.

3.3.3. MIXING AND LOADING THE CELL EXTRACT

1 Retrieve the cell extract (from step 9 above).

2 If the cell extract has been frozen, thaw at room temperature.

⚠ **IMPORTANT:** Do not let the cell extract sit at room temperature for more than 15 minutes.

3 In a thermal cycler, heat the cell extract to 95 °C for 5 minutes.

4 Chill the cell extract at 4 °C in the thermocycler or ice bath for approximately 1 minute.

⚠ **IMPORTANT:** The Binding Buffer (BB) may precipitate during storage. If necessary, prepare the Binding Buffer as follows:

5 Mix the buffer thoroughly by inverting the vial. Be sure all solids are dissolved.

6 If necessary, warm the vial at 37 °C to dissolve the precipitate.

7 Using the amounts indicated in the table below:

a Pipette the Binding Buffer (BB) into the cell extract, then mix well by pipetting up and down.

b Pipette the Ethanol Solution (EtOH) into the cell extract, then mix well by pipetting up and down.

Solution	Volume (µL)				
Cell extract/PK solution	25	50	75	100	150
Binding Buffer (BB)[1]	27	53	80	106	159
Ethanol Solution (EtOH)[2]	52	103	155	206	309

1. The volume of Binding Buffer is 1.06× the volume of cell extract/PK solution.
2. The volume of Ethanol Solution is 2.06× the volume of cell extract/PK solution, rounded up.

8 Pipette up to 210 µL of the cell extract mixture onto the preconditioned purification column.

⚠ **IMPORTANT:** Do not load more than 210 µL of the cell extract mixture onto the purification column at one time.

9 Centrifuge the purification column for 2 minutes at 100 x g to bind the RNA onto the column membrane.

10 Repeat steps 6 and 7 until all of the cell extract mixture has been loaded and bound to the purification column.

11 Once all of the cell extract mixture has been bound onto the purification column, centrifuge the column for 1 minute at 16,000 x g.

3.3.4. WASHING AND ELUTING THE RNA

1 Pipette 100 µL of Wash Buffer 1 (W1) onto the purification column, then centrifuge for 1 minute at 8,000 x g.

2. Pipette 100 µL of Wash Buffer 2 (W2) onto the purification column, then centrifuge for 1 minute at 8,000 x g.
3. Pipette another 100 µL of Wash Buffer 2 (W2) onto the purification column, then centrifuge for 2 minutes at 16,000 x g.
4. Check the purification column for any residual Wash Buffer.
   a. If Wash Buffer remains:
   b. Discard the flowthrough waste.
   c. Centrifuge the purification column for 1 minute at 16,000 x g.
   d. d. Repeat this step as needed.

   ⚠ **IMPORTANT:** *All of the Wash Buffer must be removed prior to the next step (step 5). Residual Wash Buffer can inhibit the RT reaction.*
5. Transfer the purification column to a new 0.5-mL microcentrifuge tube (supplied with kit).
6. Pipette 12 µL of Elution Buffer (EB) directly onto the purification column membrane.

   Note: To ensure maximum absorption of the Elution Buffer into the membrane, gently touch the pipette tip to the surface of the membrane while dispensing the Elution Buffer, taking care not to disturb the membrane.
7. Incubate the purification column for 1 minute at room temperature.
8. To avoid potential breakage of the 0.5-mL microcentrifuge tube cap during centrifugation:
   a. Insert the purification column/0.5-mL microcentrifuge tube assembly into a lidless, 2.0-mL tube.
   b. Insert each assembly into adjacent rotor holes in the centrifuge, with the 0.5-mL tube cap trailing the 2.0-mL tube.
   c. Rest the 0.5-mL tube cap against the tube immediately clockwise to it.
   d. Place an empty, lidless, 2.0-mL tube into the rotor hole adjacent in the counter-clockwise direction to the last assembly.
9. Centrifuge the purification column for 1 minute at 1,000 x g to distribute Elution Buffer in the column.
10. Centrifuge the purification column for 1 minute at 16,000 x g to elute the RNA.
11. Remove the purification column from the 0.5-mL microcentrifuge tube, then discard the column. The 0.5-mL microcentrifuge tube should contain approximately 11 µL of isolated RNA.
12. At this point, you can:
    a. Proceed immediately to “Performing the DNase Treatment” below, or

b Store the isolated RNA eluate at -65 to -80 °C until ready for downstream analysis.

3.3.5. PERFORMING THE DNASE TREATMENT

1 Prepare the sDNase solution:

a Thaw the sDNase Buffer (Green 1) and sDNase Enzyme (Green 2).

b Thoroughly mix each tube by flicking, then spin quickly to collect each solution at the bottom of its tube.

2 For each sample, prepare an sDNase reaction mix by combining 1 µL of sDNase Buffer (Green 1) and 1 µL of sDNase Enzyme (Green 2) in a sterile, 0.5-mL microcentrifuge tube (do not use tubes supplied in the kit).

3 Mix by flicking the tube, then spin quickly to collect the solution at the bottom of the tube.

4 Add 2 µL of the sDNase reaction mix created above to the isolated RNA.

5 Mix by flicking the tube, then spin quickly to collect the sample at the bottom of the tube.

6 In a thermal cycler, incubate the tube at 37 °C for 15 minutes to digest the genomic DNA.

7 Chill the tube to 4 °C for at least 1 minute.

8 Add 1 µL of sDNase Stop Solution (Green 3) to each tube.

9 Mix by flicking the tube, then spin quickly to collect the sample at the bottom of the tube.

⚠ **IMPORTANT:** To ensure complete inactivation of DNase, make sure the entire sample is collected at the bottom of the tube.

10 Incubate the tube at 70 °C for 10 minutes to inactivate the DNase.

11 Chill the tube to 4 °C for at least 1 minute.

12 Spin quickly to collect the sample at the bottom of the tube.

13 *Optional.* At this point, you may remove 1–2 µL of sample for measuring the yield of total RNA and for RNA profiling. To measure the RNA concentration, use the NanoDrop® ND-1000 or Agilent® 2100 Bioanalyzer instrument. Centrifuge the sample at 16,000 x g for 1 minute, then draw the sample from the top for OD measurement. You may also perform a QC of the RNA using qRT-PCR (refer to Appendix B).

14 The sample (isolated total cellular RNA) is immediately ready for use in downstream applications. At this point, you can:

a Store the sample at -65 to -80 °C until ready for downstream analysis, or

b Use the sample to make first strand cDNA (proceed to Chapter 4, *"Reverse Transcription"*).

Note: For tissue scrapes of large areas, you may quantify the isolated RNA and reverse transcribe 100–2,000 ng of total RNA per the procedures in Chapter 4.

4. Reverse Transcription

The following topics are covered in this chapter:

→ Before You Begin

→ Getting Ready

→ Performing First Strand cDNA Synthesis

4.1. BEFORE YOU BEGIN

4.1.1. ABOUT THE MODULE

The Reverse Transcription module contains reagents developed and optimized to reverse transcribe total cellular RNA to generate high quality cDNA. The cDNA is then ready for use in quantitative real-time polymerase chain reaction (qRT-PCR) gene expression studies using quantitative PCR instrumentation (e.g., the Applied Biosystems 7900HT Fast Real-Time PCR System). Following the recommended protocol, each Reverse Transcription reaction will convert 10–2,000 ng total RNA to cDNA.

4.1.2. CONTROL RNA

The frozen components module includes Formalin-Fixed Universal RNA, 500 pg/µL. Use 10 µL of this RNA to verify reverse transcription (RT) efficacy.

4.1.3. MATERIALS REQUIRED

To perform the procedures in this protocol, you need:

1 The frozen components module (RA7002 or RA7003)

2 All materials listed in "Reverse Transcription" in Chapter 1.

3 SuperScript® III Reverse Transcriptase, 200 U/µL (Invitrogen, cat # 18080-044)

4.1.4. WORKFLOW

1 Be sure you have inspected and stored the module components as described in Chapter 1.

2 Review the recommendations for nuclease-free technique on in Chapter 1.

3 Obtain the materials required for this module in Chapter 1.

4 Read through this entire chapter before performing any procedures.

5 Perform the procedures in the order listed below.

6 Unless otherwise noted, proceed immediately from one procedure to the next.

a Get ready (below).

b Perform first strand cDNA synthesis (below).

4.2. GETTING READY

4.2.1. PREPARING THE CONTROL RNA

Use 10 µL of the Formalin-Fixed Universal RNA, 500 pg/µL, for reverse transcription. Run the control RNA sample in parallel with your samples, following the procedures in this protocol.

Table 4.1: Input Recommendations Table

Total Number of qRT-PCR Reactions	Input of Total Cellular RNA
20–200	10–100 ng
200 or more	100–2,000 ng

4.3. PERFORMING FIRST STRAND cDNA SYNTHESIS

Note: A 20 µL RT reaction efficiently converts a maximum of 2 µg of total RNA to cDNA. Perform multiple RT reactions in multiple wells if you are using more than 2 µg of total RNA.

4.3.1. THERMAL CYCLER RECOMMENDATION

Precautions: Life Technologies recommends performing incubations in a thermal cycler. Please see Appendix B.3., *"Thermal Cycler Programming"* in the appendix for suggested times and temperatures. Wear clean, disposable gloves throughout this entire procedure.

4.3.2. PREPARING THE RNA SAMPLE

1 Transfer the isolated RNA sample (total volume of 12–14 µL) to a nuclease-free, 0.5-mL microcentrifuge tube. For recommended sample input quantities, see Table 4.1.

Note: If making a dilution from the RNA prepared per the protocol in Chapter 3, dilute the sample in 10 ng/µL of Poly (I) to a final volume of 12 µL.

2 Prepare the Random Primer (Gray):

a Thaw tube.

b Mix thoroughly by flicking the tube, then spin quickly to collect the solution at the bottom of the tube.

Note: The Random Primer may be substituted by gene-specific primers of your choice. Use gene-specific primer concentrations of 500 nM to 1 µM.

3 Add 1.0 µL of Random Primer to each 12–14 µL of RNA sample.

4 Mix thoroughly by flicking the tube, then spin quickly to collect the sample at the bottom of the tube.

5 Incubate at 70 °C for 5 minutes.

6 Chill the sample to 4 °C for at least 1 minute.

Note: Hold the sample at 4 °C until you are ready to use it.

7 Proceed to "Performing the RT Reactions" below.

4.3.3. PERFORMING THE RT REACTIONS

1 1. Prepare the First Strand Synthesis Mix components:

- **a** Thaw the RT Master Mix (Red 1), RT Enzyme Mix (Red 2), Enhancer (Yellow) and SuperScript III Reverse Transcriptase Enzyme, 200 U/µL
- **b** Mix each component thoroughly by flicking the tube until all solids are dissolved.
- **c** Place each tube on ice, maintaining at -2 °C until you are ready to use it.

2 Quickly spin the RNA sample (from above) to collect the sample at the bottom of the tube.

3 Prepare the First Strand cDNA Synthesis Mix per the table below.

Note: Kit volumes will support only a 10% overage for each sample.

Note: If you are processing multiple samples, prepare a master mix of the First Strand cDNA Synthesis Mix components, mix thoroughly, then aliquot 9 µL to each reaction tube.

Component	Volume (µL)	Vial
Enhancer	2	Yellow
RT Master Mix	5	Red 1
RT Enzyme Mix	1	Red 2
SuperScript III Enzyme[1]	1	---
Total per sample	9	---

1. Not included in the kit.

4 Pipette 9 µL of the First Strand cDNA Synthesis Mix into each reaction tube.

5 Mix thoroughly by flicking the tube, then spin quickly to collect the sample at the bottom of the tube.

⚠ **IMPORTANT:** Do not vortex.

6 Incubate at 25 °C for 10 minutes.

Note: If you are using gene-specific primers, then skip this step and proceed directly to step 7 below.

7 Incubate at 42 °C for 1.5 hours.

8 Chill the sample to 4 °C for at least 1 minute.

⚠ **IMPORTANT:** Do not hold the sample at 4 °C for a prolonged period. This is not a stopping point, but holding at 4 °C for a few minutes while getting ready to proceed is fine.

9 Prepare the Nuclease Mix (Gold):

a Mix thoroughly by inverting several times, then spin quickly to collect the solution at the bottom of the tube.

b Place the Nuclease Mix on ice, maintaining at 2 °C to 8 °C until you are ready to use it.

10 Add 2.0 µL of the Nuclease Mix to each reaction tube.

11 Mix thoroughly by flicking the tube, then spin quickly to collect the sample at the bottom of the tube.

12 Incubate at 37 °C for 30 minutes.

13 Incubate at 95 °C for 5 minutes.

14 Chill the sample to 4 °C for at least 1 minute.

15 At this point, you can:

a Hold the sample at 4 °C, if proceeding immediately to step 16 below, or

b Store the sample at -15 to -30 °C until ready for qRT-PCR.

16 For qRT-PCR, dilute the reverse-transcribed sample with water as required, then perform qRT-PCR per your instrument recommendations.

5. Troubleshooting

Table 5.1: Slide Preparation

Observation	Possible Cause	Recommended Action
RNA cannot be recovered from the sample	RNA may have degraded during RNA isolation.	Wear gloves; use nuclease-free technique and nuclease-free instruments and reagents.
	RNA may not be fully extracted and isolated from the cells.	Extract RNA immediately after performing the tissue scrape to ensure complete extraction and optimum recovery of RNA.
	The tissue quantity is insufficient.	Refer to the recommended input amounts in this User Guide.
	RNA in tissue is degraded due to extended storage times.	Use tissues that are more recent for the RNA isolation procedures.
	RNA in tissue is degraded due to improper fixation.	Procure samples that are properly fixed.

Table 5.2: RNA Isolation

Observation	Possible Cause	Recommended Action
Isolated RNA is of poor quality	The greatest factor affecting the quality of isolated RNA is the integrity of the RNA in the original tissue scrape.	Verify the quality of source tissue. For suggestions on verifying quality, see Appendix B.

Observation	Possible Cause	Recommended Action
Low RNA yield or purity	Poor quality RNA may not bind effectively to the purification column membrane, decreasing overall RNA yield.	Verify quality of initial tissue scrape. For suggestions on verifying quality, see Appendix B.
	RNA degradation due to RNase activity occurs rapidly.	• Use blocks that are < 5 years old. • Use nuclease-free techniques and reagents. • For optimum results, use FFPE sections that are within 2 weeks of cutting. Extended storage of sections after being cut from blocks may result in RNA degradation.
	Buffer concentrations in extraction mixtures are incorrect due to inadequate mixing with Ethanol Solution.	Ensure all buffers are completely mixed and all solids are dissolved prior to use.
	Forgot to perform centrifugation at 100 x g during the binding step.	Perform centrifugation at 100 x g during the binding step.
	Residual Wash Buffer 2 (W2) on the purification column filter membrane will alter the concentration of Elution Buffer (EB), resulting in inhibition of the RT reaction.	Ensure that all Wash Buffer 2 has been removed by centrifugation before proceeding to add Elution Buffer.
	The extraction step incubation was too short.	Incubate the tissue scrapes in PK solution for 16 hours at 37 °C (section 3.2). The incubation time may be increased to 20 hours, if necessary.
	The kit module components were not stored under optimal conditions.	Upon arrival, store the module components as specified in section 1.3.1.
	There is a high level of nuclease activity.	• Work in a nuclease-free environment. • Use nuclease-free tubes and pipet tips.

Observation	Possible Cause	Recommended Action
Tissue homogenate is viscous and difficult to pipette, resulting in low RNA yield	The RNA Extraction and Isolation module reagents were not added as specified.	Repeat the extraction and isolation procedures.
	There was insufficient disruption or lysis of cells.	Limit the extraction to 1 mm^2 of tissue scrape per 1 μL of PK solution: • Use a minimum of 25 mm^2 of tissue area in 25 μL of PK solution per extraction. • Do not use over 150 μL of PK solution per sample for isolation. • For >150 μL of PK solution, perform multiple isolations.

Table 5.3: Reverse Transcription

Observation	Possible Cause	Recommended Action
ΔRn ≤ No template Control Rn, and there is no amplification plot	Inappropriate reaction conditions.	Optimize qRT-PCR with positive controls to monitor the qRT-PCR performance.
	Poor quality PCR mastermix.	Contact the vendor/supplier of the PCR master mix.
	Incorrect dye components were chosen.	Check dye component prior to data analysis.
	The reaction component was omitted.	Check that all the correct reagents were added.
	Incorrect primer or probe sequence.	Verify primer and probe sequences. If necessary, re-synthesize with the appropriate sequence.
	PCR is not optimized.	Optimize PCR with cDNA standard curves to obtain a slope of -3.1 to -3.7 when plotting C_T against the concentration of cDNA.
	Degraded template or no template added.	Repeat the extraction and RT reaction with fresh template.
	Reaction inhibitor present.	Repeat with purified template.
ΔRn ≤ "No Template" Control Rn, and both reactions show an amplification plot.	Contamination of reagents or work area.	Check technique and equipment to confine contamination. Repeat the reaction with fresh reagents. Run negative controls along with the samples to monitor template contamination.

A. Sample Assessment Protocol

⚠ **IMPORTANT:** Life Technologies recommends performing a sample assessment protocol to customers who are starting a new set of Formalin-Fixed Paraffin-Embedded (FFPE) samples or a new type of FFPE samples. Experiments should be performed on tissue scrapes. You can perform one of the following sample assessment protocols:

1. Use the Paradise® PLUS Sample Quality Assessment Kit (cat # KIT0313) and follow the procedures in its User Guide (Paradise PLUS Reagent System User Guide (PN 12872-00) Sample Quality Assessment protocol).
2. Use the Paradise PLUS WT-RT Reagent System (cat # KIT0305 or KIT0315) for RNA extraction, isolation, and reverse transcription using an Oligo-dT primer (not supplied with the WT-RT Reagent System), followed by qRT-PCR according to the Paradise PLUS Reagent System User Guide (PN 12872-00) Sample Quality Assessment protocol.

   Note: The Paradise PLUS Reagent System User Guide is provided on the Life Technologies Web site: www.appliedbiosystems.com.

B. Appendix

B.1. RELATED PROTOCOLS

The following topics are covered in this appendix:

→ Cleaning the Plastic Slide Jars

→ Thermal Cycler Programming

→ Centrifuge Information

B.2. CLEANING THE PLASTIC SLIDE JARS

The plastic slide jars used for deparaffinization of the tissue scrapes (Chapter 2) can be reused, but must be cleaned first to reduce the potential for carryover contamination.

To clean the plastic slide jars:

1. Rinse jars with 100% ethanol (ACS grade or better), followed by distilled water.
2. Treat with RNase AWAY® solution according to the manufacturer's protocol.
3. Rinse jars thoroughly with nuclease-free water.
4. Allow to dry completely in a fume hood.

   Note: Do not use the plastic slide jars to store solutions.

B.3. THERMAL CYCLER PROGRAMMING

Thermal cyclers provide a convenient and reproducible method of incubating reactions according to specified temperatures and times in the protocol.

A thermal cycler program is provided below. The program is not intended for automatic progression from one time and temperature set to another. The program lists a 4 °C hold after each incubation or incubation cycle when it is necessary to remove the reactions from the thermal cycler to add reagents. After the addition of reagents, place the sample back into the thermal cycler and resume the program.

⚠ **PRECAUTIONS:** Using a thermal cycler with a heated lid is important. The heated lid ensures proper temperature distribution within the reaction tube and prevents evaporative condensation that alters the reaction mix concentrations.

Table B.1: Program

Protocol	Temperature	Time
RNA Extraction and Isolation (Chapter 3)	95 °C	5 minutes
	4 °C	Hold
	37 °C	15 minutes
	4 °C	Hold
	70 °C	10 minutes
	4 °C	Hold

Protocol	Temperature	Time
Reverse Transcription (Chapter 4)	70 °C	5 minutes
	4 °C	Hold
	25 °Ca	10 minutes
	42 °C	90 minutes
	4 °C	Hold
	37 °C	30 minutes
	95 °C	5 minutes
	4 °C	Hold

B.4. CENTRIFUGE INFORMATION

B.4.1. CENTRIFUGATION

The table below shows corresponding centrifugal forces (g) for selected rotations per minute (rpm) when working with the tabletop microcentrifuge Eppendorf 5415D.

Rotations Per Minute (rpm)	Centrifugal Force (g)
14,000	13,000
12,000	10,000
10,000	7,000
8,000	4,500
5,500	2,200
5,000	2,000

C. Appendix

C.1. RELATED ARCTURUS® PRODUCTS

Life Technologies offers additional research and general purpose laboratory kits in its Arcturus® product line for multiple applications. Most common part numbers provided. Additional configurations available depending on individual need.

C.1.1. HISTOGENE® LCM FROZEN SECTION STAINING KIT

The HistoGene LCM Frozen Section Staining Kit is used to process tissue sections for LCM that maximizes the quality and yield of RNA from LCM cells. The Kit comes with all dehydration and staining reagents, disposable staining jars, specially treated slides, and detailed protocol and troubleshooting guide.

KIT0401 – 72 slides

C.1.2. HISTOGENE® LCM IMMUNOFLUORESCENCE STAINING KIT

The HistoGene LCM Immunofluorescence Staining Kit is the only kit designed to enable retrieval of high-quality RNA from immunofluorescently stained frozen tissue. It enables convenient and reliable staining, dehydration and LCM of tissue sections with protocols streamlined and optimized both for optimal LCM captures and maintaining RNA quality for downstream applications that require intact RNA, like microarray analysis and RT-PCR.

KIT0420 – 32 slides

C.1.3. PICOPURE® RNA ISOLATION KIT

For extraction and isolation of total RNA from small samples particularly Laser Capture Microdissected (LCM) cells. The PicoPure RNA Kit comes with optimized buffers, MiraCol™ Purification Columns and an easy-to-use protocol to maximize recovery of high-quality total cellular RNA ready for amplification with the RiboAmp® PLUS RNA Amplification Kits.

KIT0204 – 40 isolations

C.1.4. PICOPURE® DNA EXTRACTION KIT

The PicoPure DNA Extraction Kit is optimized to maximize the recovery of genomic DNA from 10 or more cells captured by LCM. The kit comes with reagents and protocol tested to ensure complete extraction of DNA from LCM samples prepared with any stan-

dard tissue preparation procedure. DNA prepared using the kit is PCR-ready and needs no additional purification to perform amplification.

KIT0103 – 150 HS cap or 30 Macro cap extractions

C.1.5. RIBOAMP® PLUS RNA AMPLIFICATION KIT

The RiboAmp PLUS RNA Amplification Kit enables the production of microgram quantities of antisense RNA (aRNA) from as little as picogram quantities of total cellular RNA. Amplified RNA produced using the kit is suitable for labeling and use for probing expression microarrays. The kit achieves amplifications of up to 1000-fold in one round of amplification, and amplifications of up to 1,000,000-fold in two rounds. Kits options include microarray labeling options such as biotin, fluorescent dyes and amino allyl. Kits are available in two sensitivity options, RiboAmp PLUS (5-40 ng) and a high sensitivity version RiboAmp HS PLUS (0.1-5 ng).

KIT0521 RiboAmp PLUS – (12) 1-round amplifications or (6) 2-round amplifications

KIT0525 RiboAmp HS PLUS – (6) 2-round amplifications

C.1.6. PARADISE® PLUS REAGENT SYSTEM

The Paradise PLUS Reagent System is the only reagent system designed to enable gene expression studies using formalin-fixed paraffin-embedded (FFPE) tissue samples. Components include sample preparation and staining reagents, RNA extraction and isolation reagents, RNA amplification reagents and a comprehensive user guide.

KIT0312 – 12 samples

KIT0312B – 12 samples with biotin labeling

KIT0312C – 12 samples with Cy®3 labeling

KIT0312D – 12 samples with Cy5 labeling

C.1.7. TURBO LABELING™ KITS

The Turbo Labeling Kits provide a proprietary, non-enzymatic technology for labeling of unmodified aRNA for Gene Expression profiling. The unmodified aRNA is labeled post-amplification, thereby avoiding the need to incorporate modified nucleotides. The use of natural nucleotides in the amplification step results in unmodified aRNA with higher yields and longer aRNA fragments, thus providing better representation of the mRNA transcript for downstream analysis.

KIT0608 – Biotin – 12 samples

KIT0609 – Cy3 – 12 samples

KIT0610 – Cy5 – 12 samples

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