Paradise®Plus Reagent System
User Guide

Part of the Arcturus Systems for Microgenomics

MDS Analytical Technologies

MDS Analytical Technologies
Paradise®Plus (WT-RT)
User Guide

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Warranty

MDS Analytical Technologies warrants that the products described in this manual meet the performance standards described in literature published by the company. If a product fails to meet these performance standards, MDS Analytical Technologies will replace the product or issue credit for the full purchase price, including delivery charges. MDS Analytical Technologies provides no other warranties of any kind, expressed or implied. MDS Analytical Technologies' warranty liability shall not exceed the purchase price of the product and shall not extend to direct, indirect, consequential or incidental damages arising from the use, results of use, or improper use of its products. The Paradise Plus Reagent System is intended for laboratory use.

Related Documents

When using the Paradise Plus Reagent System User Guide, the following user guides may be helpful references:

- → Arcturus^{XT}™, Veritas™, AutoPix®, or PixCell® LCM System User Guide
- → *Turbo* Labeling™ kit user guide
- → CapSure HS Caps User Guide

Quality Control

MDS Analytical Technologies performs functional testing on all components of the Paradise Plus Reagent System. The information sheet provided with the system highlights the tests performed.

Staining. MDS Analytical Technologies performs functional testing on the Paradise Plus Reagent System staining components to confirm the absence of nucleic acids and nuclease activity. The staining components are functionally tested using LCM to ensure proper dehydration and that good quality RNA is recoverable.

Extraction/Isolation. MDS Analytical Technologies performs functional testing on the Paradise Plus Reagent System RNA Extraction/Isolation using all components. MiraCol Purification Columns are tested by lot to confirm the absence of nucleic acids and nuclease activity. Column nucleic acid binding and recovery performance must meet quality standards.

Amplification. Functional Testing MDS Analytical Technologies performs functional testing on each lot of materials using the amplification protocol described in this manual.

Reagent Testing. MDS Analytical Technologies tests each lot of enzymes to confirm activity. Buffer components must perform correctly under reaction or nucleic acid purification conditions. Purification Column Testing Purification columns are tested by lot to confirm the absence of nucleic acids and nuclease activity. Column nucleic acid binding and recovery performance must meet quality standards.
Visual Inspection Finished kits are inspected for proper assembly.

Challenges of FFPE Tissue. MDS Analytical Technologies strongly recommends performing quality assessment of FFPE samples. Tissue that has degraded RNA prior to fixation will not yield good results, nor will samples that have been over-fixed. If the quality of the source tissue is unknown, then performing a quality assessment of the tissue block prior to spending the time and expense of Laser Capture and amplification is imperative.

The amplification process generates product from the 3' end of the mRNA. For best results use qRT-PCR primer sets designed within the first 300 bases from the poly A tail.

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

Technical Support

You may access services and support in the following ways:

- Online connection at www.moleculardevices.com,
- Phone at : +1-800-635-5577
  +1-408-747-1700
  Fax: +1-408-747-3603
- Web: www.moleculardevices.com/support
- e-mail to: support@moldev.com

For additional offices, please see the contact information on the back cover of this User Guide.

Comments and Suggestions

MDS Analytical Technologies welcomes your comments and suggestions for improving our documents. Send your comments to support@moldev.com.

MDS Analytical Technologies maintains an ongoing research program to test and validate the Paradise Plus Reagent System. Call MDS Analytical Technologies Technical Support at 1-800-635-5577 or +1-408-747-1700*, or send an email inquiry to support@moldev.com for an up-to-date list*

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A. Appendices

1. Introduction

1.1 BACKGROUND

The Paradise® Plus Reagent System provides an integrated system enabling gene expression studies using Formalin-Fixed Paraffin-Embedded tissue (FFPE).

Components provided include:

- → Sample preparation and staining reagents
- → RNA extraction and isolation reagents
- → RNA amplification reagents

Paradise Plus reagents are intended to be used together as a system. They are not as a Staining kit; Extraction and Isolation kit; or Amplification kit by itself. Alternate reagents have not been tested. The system is designed to be processed together. The issue is that the formalin fixation causes cross linking, and when the RNA is extracted, the cross-linking leaves artifacts on the RNA backbone. These artifacts need to be dealt with before successful amplification can be achieved. While our process is proprietary, the Paradise Plus system deals with these cross-linking artifacts during the amplification process. So using only the isolation components may not be effective with another company's downstream reagents.

This user guide is divided into sections describing the steps involved using staining, extraction/isolation and amplification separately. To get the most out of the Paradise Plus Reagent System, please examine the components and read each section of the user guide carefully. A principal application of this kit is use in conjunction with Laser Capture Microdissection (LCM). LCM experiments often involve the analysis of gene expression patterns in cells captured from specimens. Obtaining accurate results from gene expression analysis experiments, including microarray hybridization and quantitative PCR, depends on careful preservation of intact RNA molecules in captured cells.

Staining

The Paradise® Plus Reagent System Staining components are part of a series of LCM-certified LCM analysis products for preparing and staining tissues while preserving intact nucleic acid and protein species from captured cell populations. The staining components work with the additional modules provided in this reagent system. Paradise Plus extraction and isolation reagents and RNA amplification reagents provide a complete solution for studying RNA from cells isolated by LCM. The reagents and protocol have been optimized for use with Formalin-Fixed Paraffin-Embedded (FFPE) samples.

Extraction/Isolation

The Paradise® Plus Reagent System RNA Extraction/Isolation reagents enable researchers to recover total cellular RNA from formalin fixed paraffin embedded samples. They are optimized for use with cells acquired using Laser Capture Microdissection (LCM) on CapSure® LCM Caps. Total cellular RNA isolated using the Paradise Plus Reagent System RNA Extraction/ Isolation reagents produces RNA in a small volume of low ionic strength buffer, ready for use in linear amplification using

the Paradise Plus Reagent System RNA amplification reagents. The Paradise Plus Reagent System RNA Extraction/Isolation Kit contains RNA extraction and purification reagents and MiraCol™ Purification Columns.

Amplification

The Paradise Plus® Reagent System RNA Amplification reagents enable the production of large quantities of amplified antisense RNA (aRNA) from small quantities of total cellular RNA. This process for linear amplification provides efficient, reproducible results through protocols, reagents, and nucleic acid purification technology using MDS Analytical Technologies ' MiraCol™ Purification Columns. The Paradise Plus RNA Amplification reagents can amplify total cellular RNA to generate sufficient aRNA, ready for use in microarray, quantitative real-time PCR or other applications.

1.2. PERFORMANCE SPECIFICATIONS

The Paradise Plus kits should yield enough amplified RNA (aRNA) to complete multiple microarray experiments when starting with the recommended amount of starting material from a tissue block which contains good quality RNA.

1.3. MASTER-MIXES

The Paradise Plus kits are designed with the assumption that master-mixes will be made when using three or more samples, and will not be used for two or less samples. The kits have been designed with a 10% overage for 3 samples. Exceeding 10% overage for master-mixes may result in insufficient material to complete all reactions. A suggested master mix size for six samples is included where appropriate.

1.4. RNA INPUT REQUIREMENTS

The Paradise Plus kits are designed and optimized for use with Formalin-Fixed Paraffin Embedded tissue samples. The kit is designed to for use with an expected total RNA input amount of 5-40 ng. The amount of total RNA found in a cell varies by cell type, length of fixation, age of the sample block and quality of the material prior to fixation. Different sources of total RNA contain varying amounts of mRNA; consequently, the total RNA input needed to obtain microgram quantities of aRNA depends on the total RNA source. For example, RNA from rapidly dividing cells may be relatively mRNA-rich and thus may result in higher output of aRNA. In general one can expect anywhere from 1 - 10 pg of total RNA per cell based on factors mentioned above. We recommend brining in a minimum of 5 ng of total RNA into the Paradise Plus system amplification reaction.

1.5. STORAGE AND STABILITY

MDS Analytical Technologies makes recommendations for storage temperatures throughout this document. Realizing that not ever laboratory has a freezers set at these temperatures, we have defined the acceptable temperature ranges for our recommendations.

Acceptable ranges for storage:

- → $-70^{o}C = -65^{o}C$ to $-80^{o}C$
- → $-20^{o}C = -15^{o}C$ to $-30^{o}C$
- → $4^{o}C = 2^{o}C$ to $8^{o}C$
- → Room Temperature = $10^{o}C$ to $30^{o}C$

Staining

Inspect all kit components upon receipt. Ethanol and xylene are flammable and should be unpacked and stored at room temperature in a fireproof storage cabinet or fume hood with adequate ventilation. Cap bottles tightly between uses. Store remaining kit supplies at room temperature in a clean, dust-free environment.

Extraction and Isolation

Store the Paradise Plus Reagent System RNA Extraction/Isolation components at room temperature. Store the DNase I solution and DNase Buffer at –70ºC until use. Once the reagents are used, storage at –20ºC is recommended.

Amplification

The Paradise Plus amplification kits have both room temperature and frozen components. The room temperature components should be stored at normal room temperature. The frozen components are shipped on dry ice and should be stored at -70°C until initial use. After initial use -20°C is recommended to prevent unnecessary freeze-thaws of the enzymes. The control RNA and any RNA generated from Paradise Plus kits should always be stored at -70°C.

The Control RNA vial should be stored at –70°C or below immediately upon arrival to ensure maximum stability. For optimal results, using the reagents as soon as possible after receipt is recommended.

Expiration

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

1.6. MATERIAL SAFETY AND DATA SHEET (MSDS)

Material Safety and Data Sheets (MSDS) for kit chemical components are available from the MDS Analytical Technologies web site at www.moleculardevices.com. They may also be acquired by calling MDS Analytical Technologies Technical Services 1-800-635-5577 or +1-408-747-1700, or send an email inquiry to support@moldev.com

1.7. RELATED ARCTURUS PRODUCTS

Most common part numbers provided. Additional configurations available depending on individual need.

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

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

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

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 standard 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

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 1,000-3,000-fold in one round of amplification, and amplifications of up to 1,000,000-fold in two rounds. The kits 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 – 6 reactions
KIT0525 RiboAmp HS Plus – 6 reactions

Paradise® Plus Reagent System
The Paradise 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

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

1.8. ADDITIONAL EQUIPMENT AND MATERIALS REQUIRED

Ensure that you have ready access to the following laboratory equipment and materials before you begin. These items are not included with the Paradise Plus Reagent System

Staining

Equipment

- → Rotary Microtome
- → Fume hood
- → –70°C freezer
- → Tweezers
- → Cover glass forceps
- → Microslide box – plastic (VWR Cat. # 48444-004)
- → Tissue Flotation Water Bath
- → Oven
- → 20 – 200 µL pipettor

Materials

- → Disposable gloves
- → Detergent (Fisher Scientific, Cat. # 04-355
- → RNase AWAY (Life Technologies, Cat. # 10328-011)
- → 100% ethanol
- → Kimwipes or similar lint-free towels
- → Disposable microtome blades
- → Microslides
- → Pipette tips, nuclease free

Extraction/Isolation

Equipment

- → Microcentrifuge (Eppendorf 5415D or similar)
- → 2–20 µL pipettor
- → 20–200 µL pipettor
- → Incubation oven (50°C)

Materials

- → Nuclease-free pipette tips
- → 0.5 ml extraction tubes (Applied BioSystems #N8010611 or USA Scientific, Inc, #1605-0000)

Amplification

Equipment

- → Thermal cycler with heated lid
- → Microcentrifuge for 1.5 mL and 0.5 mL tubes (Eppendorf 5414D or similar)
- → 0.5 – 10 µL pipettor
- → 20 µL pipettor
- → 200 µL pipettor

- → 1000 µL pipettor
- → Ice bath or cold block (4*C)
- → Vortex mixer (optional)

Materials
- → 0.5 mL or 0.2 mL RNase-free microcentrifuge tubes
- → 2 mL lidless tube for centrifuge (PGC Scientific, Cat # 16-8101-06)
- → Nuclease-free pipette tips

Reagents
- → **SuperScript™ III Reverse Transcriptase, 200 U/µL** (Enzyme only) Invitrogen part number: 18080-093, 18080-044 or 18080-085

1.9. RECOMMENDATIONS FOR NUCLEASE-FREE TECHNIQUE

Staining

RNase contamination will cause experimental failure. Minimize RNase contamination by adhering to the following recommendations throughout your experiment:
- → Wear disposable gloves and change them frequently.
- → Use RNase-free solutions, glassware and plasticware.
- → Do not re-purify Paradise Plus Reagent System Section Staining Kit components. They are certified Nuclease Free.
- → Wash scalpels, tweezers and forceps with detergent and bake at 210°C for four hours before use.
- → Use RNase AWAY® (Life Technologies) according to the manufacturer's instructions on the horizontal staining rack and any other surfaces that may come in contact with the sample.
- → Use Kimwipe soaked in RNase Away to wipe down and clean the interior of tissue flotation water bath.

Extraction and Isolation

RNase contamination will cause experimental failure. Minimize RNase contamination by adhering to the following recommendations throughout your experiment:
- → Always handle RNA in a manner that avoids introduction of RNases.
- → Wear disposable gloves and change them frequently to prevent the introduction of RNases from skin surfaces.
- → After putting on gloves, avoid touching surfaces that may introduce RNases onto glove surfaces.
- → Do not use reagents not supplied with the Paradise Plus Reagent System. Substitution of reagents or Kit components may adversely affect yields or introduce RNases.
- → Use only new plasticware that is certified nucleic acid-free.
- → Use only new, sterile, RNase-free pipette tips and microcentrifuge tubes.
- → Clean work surfaces with commercially available RNase decontamination solutions prior to performing reactions.

Amplification

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

- → Wear disposable gloves and change them frequently.
- → After putting on gloves, avoid touching surfaces that may introduce RNases onto the glove surface.
- → Do not use reagents not supplied. Substitutions of reagents or components may adversely affect yields or introduce RNases.
- → Use only new, sterile RNase-free pipette tips and microcentrifuge tubes.
- → Work surfaces should cleaned with commercially available RNase decontamination solutions prior to performing reactions.

Amplified aRNA Contamination

Stray amplified aRNA and cDNA in work area can contaminate precious samples if the work area is routinely used for performing amplifications. To ensure a work area free of amplified aRNA, please do the following:

1. Irradiate the work area/hood with UV overnight every three to four days.
2. Clean surfaces and devices (pipettors, racks, centrifuge, etc.) with commercially available decontamination solutions everyday or more frequently depending on use.

2. Configurations

2.1. KIT COMPONENTS

Table 2.1: Paradise® Plus Kit Configuration with Catalog Numbers.

Paradise® Plus Kit Configurations with Catalog Numbers			Solvents / Stain		Extraction / Isolation		Amplification		IVT		Turbo Label
Description	Catalog Number	# of Samples	Room temp	Room temp	Frozen	Room temp	Frozen	Room temp	Frozen	Room temp	
Arcturus Paradise Plus 1.5 Round (12 reactions)	KIT0311	12	1x RA7013	1x RA7014	1x RA7007	1x RA7001	2x RA7018	2x RA7011	2x RA7008	x	x
Arcturus Paradise Plus 1.5 Round (12 ext/iso, 6 amp)	KIT0321	6	1x RA7013	1x RA7014	1x RA7007	1x RA7001	1x RA7018	1x RA7011	1x RA7008	x	x
Arcturus Paradise Plus 1.5 Round (6 Amplification only)	KIT0321-A	6	x	x	x	x	1x RA7018	1x RA7011	1x RA7008	x	x
Arcturus Paradise Plus 1.5 Round (12 extractions only)	KIT0312-I	12	x	x	1x RA7007	1x RA7001	x	x	x	x	x
Arcturus Paradise Plus staining components (24 samples)	KIT0312-S	12	1x RA7013	1x RA7014	x	x	x	x	x	x	x
Arcturus Paradise Plus stain, slide jars & slides (24 samples)	KIT0312-J	24	x	1x RA7014	x	x	x	x	x	x	x
Arcturus Paradise Plus 1.5 Round (12 reactions) - No solvents	KIT0311-NS	12	x	1x RA7014	1x RA7007	1x RA7001	2x RA7018	2x RA7011	2x RA7008	x	x
Arcturus Paradise Plus 1.5 Round (12 ext/iso, 6 amp) - No solvents	KIT0321-NS	6	x	1x RA7014	1x RA7007	1x RA7001	1x RA7018	1x RA7011	1x RA7008	x	x
Arcturus Paradise Plus 1.5 Round (Bulk, 48 reactions)	KIT0301	48	2x RA7013	4x RA7014	4x RA7007	4x RA7001	8x RA7018	8x RA7011	8x RA7008	x	x
Arcturus Paradise Plus 2 round (12 reactions)	KIT0312	12	1x RA7013	1x RA7014	1x RA7007	1x RA7001	2x RA7018	2x RA7011	2x RA7009	x	x
Arcturus Paradise Plus 2 Round with Biotin Labeling (12 reactions)	KIT0312B	12	1x RA7013	1x RA7014	1x RA7007	1x RA7001	2x RA7018	2x RA7011	2x RA7009	x	1x KIT0608
Arcturus Paradise Plus 2 Round with Cy3 Labeling (12 reactions)	KIT0312C	12	1x RA7013	1x RA7014	1x RA7007	1x RA7001	2x RA7018	2x RA7011	2x RA7009	x	1x KIT0609
Arcturus Paradise Plus 2 Round with Cy5 Labeling (12 reactions)	KIT0312D	12	1x RA7013	1x RA7014	1x RA7007	1x RA7001	2x RA7018	2x RA7011	2x RA7009	x	1x KIT0610
Arcturus Paradise Plus 2 Round (12 ext/iso, 6 amp)	KIT0322	6	1x RA7013	1x RA7014	1x RA7007	1x RA7001	1x RA7018	1x RA7011	1x RA7009	x	x
Arcturus Paradise Plus 2 Round (6 Amplification only)	KIT0322-A	6	x	x	x	x	1x RA7018	1x RA7011	1x RA7009	x	x
Arcturus Paradise Plus 2 Round (12 reactions) - No solvents	KIT0312-NS	12	x	1x RA7014	1x RA7007	1x RA7001	2x RA7018	2x RA7011	2x RA7009	x	x

Paradise® Plus Kit Configurations with Catalog Numbers (continued)			Solvents / Stain		Extraction / Isolation		Amplification		IVT		Turbo Label
Description	Catalog Number	# of Samples	Room temp	Room temp	Frozen	Room temp	Frozen	Room temp	Frozen	Room temp	
Arcturus Paradise Plus 2 Round (12 ext/iso, 6 amp) - No solvents	KIT0322-NS	6	x	1x RA7014	1x RA7007	1x RA7001	1x RA7018	1x RA7011	1x RA7009	x	x
Arcturus Paradise Plus 2 Round with Biotin Labeling (12 reactions) - No solvents	KIT0312B-NS	12	x	1x RA7014	1x RA7007	1x RA7001	2x RA7018	2x RA7011	2x RA7009	x	1x KIT0608
Arcturus Paradise Plus 2 Round with Cy3 Labeling (12 reactions) - No solvents	KIT0312C-NS	12	x	1x RA7014	1x RA7007	1x RA7001	2x RA7018	2x RA7011	2x RA7009	x	1x KIT0609
Arcturus Paradise Plus 2 Round with Cy5 Labeling (12 reactions) - No solvents	KIT0312D-NS	12	x	1x RA7014	1x RA7007	1x RA7001	2x RA7018	2x RA7011	2x RA7009	x	1x KIT0610
Arcturus Paradise Plus 2 round - Amino Allyl (12 reactions)	KIT0314	12	1x RA7013	1x RA7014	1x RA7007	1x RA7001	2x RA7018	2x RA7011	2x RA7010	2x RA7012	x
Arcturus Paradise Plus 2 Round - Amino Allyl (12 ext/iso, 6 amp)	KIT0324	6	1x RA7013	1x RA7014	1x RA7007	1x RA7001	1x RA7018	1x RA7011	1x RA7010	1x RA7012	x
Arcturus Paradise Plus 2 Round - Amino Allyl (6 Amplification only)	KIT0324-A	6	x	x	x	x	1x RA7018	1x RA7011	1x RA7010	1x RA7012	x
Arcturus Paradise Plus 2 Round - Amino Allyl (12 reactions) - No solvents	KIT0314-NS	12	x	1x RA7014	1x RA7007	1x RA7001	2x RA7018	2x RA7011	2x RA7010	2x RA7012	x
Arcturus Paradise Plus 2 Round - Amino Allyl (12 ext/iso, 6 amp) - No solvents	KIT0324-NS	6	x	1x RA7014	1x RA7007	1x RA7001	1x RA7018	1x RA7011	1x RA7010	1x RA7012	x
Arcturus Paradise Plus 2 round (Bulk, 48 reactions)	KIT0302	48	2x RA7013	2x RA7014	4x RA7007	4x RA7001	8x RA7018	8x RA7011	8x RA7009	x	x
Arcturus Paradise Plus 2 round (Bulk, 48 reactions)	KIT0304	48	2x RA7013	1x RA7014	4x RA7007	4x RA7001	8x RA7018	8x RA7011	8x RA7010	8x RA7012	x
Arcturus Paradise Plus qrtPCR kit (12 reactions)	KIT0310	12	1x RA7013	1x RA7014	1x RA7007	1x RA7001	2x RA7018	1x RA7011	2x RA7008	x	x
Arcturus Paradise Plus qrtPCR kit (12 reactions) - No Solvents	KIT0310-NS	12	x	1x RA7014	1x RA7007	1x RA7001	2x RA7018	1x RA7011	2x RA7008	x	x
Arcturus Paradise Plus qrtPCR kit (Bulk, 48 reactions)	KIT0300	48	2x RA7013	1x RA7014	4x RA7007	4x RA7001	8x RA7018	4x RA7011	8x RA7008	x	x
Arcturus Paradise Plus qrtPCR kit (Bulk, 48 reactions) - No solvents	KIT0300-NS	48	x	1x RA7014	4x RA7007	4x RA7001	8x RA7018	4x RA7011	8x RA7008	x	x
Arcturus Paradise Plus QC Kit (12 reactions)	KIT0313	12	x	x	1x RA7007	1x RA7001	1x RA7018	x	x	x	x

* Note KIT0313 should follow Section II in the Appendix

3. Sample Preparation and Staining

3.1. COMPONENTS

3.1.1. REAGENTS AND SUPPLIES

The Paradise Plus Reagent System Staining components include:

Table 3.1: Staining Solvents RA7013

Component	Size
100% Ethanol	0.5 L
95% Ethanol	0.5L
75% Ethanol	1 L
Nuclease-free Water	1 L
Xylene	0.5 L

Table 3.2: Staining Components RA7014

Component	Size
Paradise Plus Stain	6 ml
Slide jars	10x

3.2. PRELIMINARY STEPS

3.2.1. MATERIAL AND PROTOCOL REVIEW

To get the most from your staining reagents, take a few moments to examine the components of the kit and read the information in the following sections.

3.3. PROTOCOL

3.3.1. SLIDE PREPARATION

⚠ ***Precautions:*** *Wear clean disposable gloves throughout the Slide Preparation procedure. Use clean RNase-free instruments. Wear clean disposable gloves throughout the Slide Preparation procedure.*

Depending on humidity in the environment, drying may take longer for the sections to dry. The section must be dry before proceeding. Do not allow sections to air dry for longer than 3 hours.

1. Prior to starting slide preparation, minimize RNase contamination of the equipment by cleaning as follows:
   a. Rotary Microtome: Remove and discard old disposable microtome blade. Use a Kimwipe soaked with RNase Away to wipe down the knife holder. Dry holder with a clean Kimwipe. Install a new disposable microtome blade into holder.

b. Tissue Floatation Bath: Use a Kimwipe soaked with RNase Away to wipe down and clean the interior of the water bath. Rinse the interior with Milli-Q or RNase free water. Fill the water bath with Milli-Q or RNase free water. Heat water to appropriate temperature for the paraffin used in your laboratory, typically 41°C–43°C. Do not add any adhesives to the water bath.

2. Set cutting thickness to 7 µm on the microtome.
3. Place paraffin block into specimen holder. Trim off any excess paraffin from the block face. Cut and discard the first five sections after trimming.
4. From the fresh surface, cut 7 µm sections from your specimen. If you are cutting more than one specimen, move to a new section of the blade, use gauze soaked in RNase Away to clean blade, or use a new disposable blade for each one to avoid cross contamination.
5. Remove section(s) from microtome and float them onto heated water bath. Allow section(s) to flatten. Minimize time in water bath to no longer than 2 minutes. Mount each section on a room-temperature slide.
6. Prop slide on end in a vertical, not horizontal, position to allow water to drain away from section. Air-dry the slide for a minimum of 30 minutes at room temperature. Discard any slides that have wrinkles or folds in the section.
7. Proceed immediately to the Deparaffinization, Staining and Dehydration segment of the protocol or store slides at –70°C in a microslide box for up to two weeks.
8. After completion of the slide preparation process, remove any paraffin debris from the microtome. Clean surfaces with a Kimwipe soaked with RNase Away and dry all surfaces. Discard water from water bath and clean the interior with RNase Away and dry all surfaces.

3.3.2. DEPARAFFINIZATION, STAINING AND DEHYDRATION

1. Label 10 plastic slide jars as follows:
   a. Xylene
   b. Xylene
   c. 100% ethanol
   d. 95% ethanol
   e. 75% ethanol
   f. Nuclease free water
   g. 75% ethanol
   h. 95% ethanol
   i. 100% ethanol
   j. xylene
2. Using the LCM-certified solutions provided, fill the labeled plastic slide jars with 25 ml of the appropriate solution.
3. Remove up to four slides from the slide box or from the –70°C freezer, and place in a 50–60°C oven for 2 minutes.
4. Place the slides in plastic slide jar “a” containing xylene for 2 minutes. Invert jar gently.
5. Transfer the slides to plastic slide jar “b” containing xylene for 2 minutes. Invert jar gently.
6. Transfer the slides to plastic slide jar “c” containing 100% ethanol for 2 minutes. Invert jar gently.
7. Transfer the slides to plastic slide jar “d” containing 95% ethanol for 1 minute.

8. Transfer the slides to plastic slide jar "e" containing 75% ethanol for 1 minute.
9. Transfer the slides to plastic slide jar "f" containing nuclease free water for 30 seconds.
10. Using an RNase free pipette tip, apply 100 µL of the Paradise Plus Staining Solution so that it covers the entire section. Stain for 15-45 seconds at room temperature. Tap off excess stain before proceeding with the following steps.
11. Transfer the slides to plastic slide jar "g" containing 75% ethanol for 30 seconds.
12. Transfer the slides to plastic slide jar "h" containing 95% ethanol for 30 seconds.
13. Transfer the slides to plastic slide jar "i" containing 100% ethanol for 1 minute.
14. Transfer the slides to plastic slide jar "j" containing xylene. Hold slides in xylene until ready for microdissection. The minimum incubation in xylene should be 5 minutes, or up to a maximum of ~2 hours.
15. Place the slides on a Kimwipe to dry in the hood for five to ten minutes prior to LCM. LCM should be performed within 2 hours after removal from xylene.
16. Discard all used staining and dehydration solutions according to standard procedures.

⚠ ***Precautions:*** *Carry out the Staining and Dehydration segment of the protocol in a fume hood. Wear clean disposable gloves.*

Xylene jar "a" must be changed after processing up to a maximum of 4 slides.

75% Ethanol jar "e" must be changed after processing up to a maximum of 4 slides. Staining times may vary depending on tissue types.

Performing Laser Capture Microdissection (LCM)

⚠ *Please consult the User Guide for the instrument you will use for detailed instructions*.

4. RNA Extraction / Isolation

4.1. COMPONENTS

4.1.1. REAGENTS AND SUPPLIES

The Paradise Plus Reagent System RNA Extraction/Isolation components include the following items:

Table 4.1: Paradise Extraction - Room Temperature RA7001

Component	Vial Color	Vial Label
Extraction Conditioning 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
Extraction Binding Buffer	Blue	BB
Pro-k Reconstitution Buffer		Pro K
0.5 mL Microcentrifuge Tubes		
Purification columns		

Table 4.2: Paradise Extraction - Frozen RA7007

Component	Vial Color	Vial Label
DNase Buffer	Blue	DNB
DNase Mix	Blue	DNase

4.2. PRELIMINARY STEPS

4.2.1. MATERIAL AND PROTOCOL REVIEW

To get the most from your extraction reagents, take a few moments to examine the components of the kit and read the information in the following sections.

Overview

Separate protocols are provided for extraction/isolation of RNA from:

- → Microdissected samples using CapSure LCM Macro caps,
- → Microdissected samples using CapSure LCM HS caps or
- → Tissue scrapes (0.5 cm x 0.5 cm)

The flow chart illustrates the Paradise Plus Reagent System RNA Extraction/ Isolation procedure:

1. Extract RNA from a CapSure LCM Cap or tissue scrape.
2. Mix and load cell extract onto a preconditioned purification column.
3. Spin the extract through the column to capture RNA on the purification column membrane.

4. Wash.
5. DNase treat, and wash again.
6. Wash the column twice with wash buffer, and
7. Elute the RNA in low ionic strength buffer.

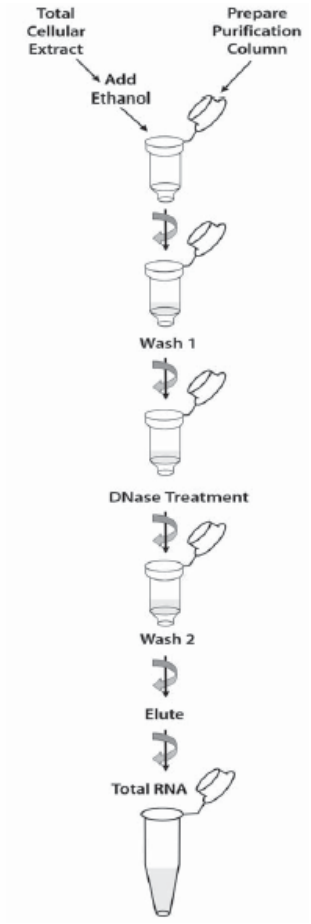


Figure 4.1: Paradise® Plus Reagent System RNA Extraction/Isolation procedure

The entire isolation process, including incubations, can be completed in less than an hour, and the isolated total cellular RNA is ready for use in downstream applications. The Paradise Plus Reagent System RNA Extraction/ Isolation reagents are capable of isolating small amounts of RNA. It is important not to introduce nucleic acid contamination.

4.3. PROTOCOL

4.3.1. PROTOCOL FOR USE WITH CAPSURE MACRO LCM CAPS

RNA Extraction

1. Dispense Pro K Mix and incubate as follows:
   a. Capture cells using the CapSure Macro Cap. Refer to the instrument User Guide for complete instructions.
   b. Add 300 µL of Reconstitution Buffer to vial of dried Pro K Mix (600 µg/tube). Dissolve completely by gently vortexing the tube to mix the reagents and place the tube on ice immediately. Excessive mixing may denature Proteinase K. One vial of Pro K Mix is adequate for 12 extractions. All mixed proteinase K solution should be used within one workday (up to 12 hours). *Discard any mixed Proteinase K solution that is not used within one day.*
2. Pipette 50µL of mixed Proteinase K Extraction Solution into a 0.5 ml extraction tube (not provided).

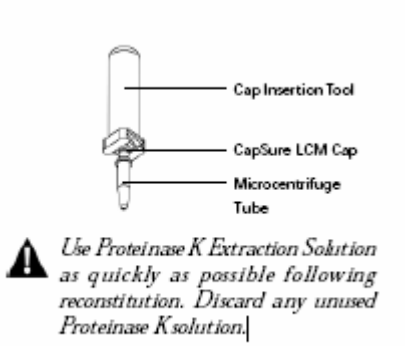


Figure 4.2: Proteinase K Extraction Solution

3. Insert the CapSure Macro LCM Cap with LCM captured cells into the microcentrifuge tube using the LCM Cap Insertion Tool.
4. Invert the extraction tube with the inserted CapSure Macro LCM Cap and shake down the 50 µL volume of Proteinase K Extraction solution until it completely covers the inside surface of the CapSure Macro LCM Cap.
5. Incubate at 37°C for the correct time period according to the following table:

Table 4.3: RNA Incubation Times

Samples Age	Incubation Time
Samples >3 years old	16 hours
Samples ≤3 years old	5 hours*

* For complete extraction this time can be increased to 16 hours.

Note: *If multiple LCM captures are performed, it is recommended that each cap be incubated in Pro K Mix immediately after collection. Caps may be incubated up to 24 hours.*

6. After incubation, remove the tubes from the incubator, place them in a microcentrifuge and centrifuge for one minute at 800x *g*.
7. Remove the CapSure Macro LCM Cap. Close the microcentrifuge tube containing the extract.
8. Proceed with RNA isolation protocol or freeze cell extract at –70°C.

   It is okay to stop at this point in the protocol.

RNA Isolation

1. Pre-condition the MiraCol Purification Column as follows:
   a. Pipette 200 µL Conditioning Buffer (**CB**) onto the purification column filter membrane.
   b. Incubate the purification column with Conditioning Buffer for 5 minutes at room temperature.
   c. Centrifuge the purification column in the provided collection tube at 16,000 *x g* for one minute.
2. Pipette 53 µL of Paradise Plus Reagent System Binding Buffer (**BB**) into the cell extract from Part 1 (RNA Extraction). Mix well by pipetting up and down. DO NOT CENTRIFUGE. Pipette 103 µL of Ethanol Solution (**EtOH**) into tube and mix well.
3. The cell extract mixture will have a combined volume of approximately 206 µL.
4. To bind RNA, centrifuge for 2 minutes at 100 x *g*, immediately followed by a centrifugation at 16,000 x *g* for 1 minute.
5. Pipette 100 µL Wash Buffer 1 (**W1**) into column and centrifuge for 1 minute at 8000 *x g*.
6. Mix 2 µL DNase Mix (**DNase**) with 18 µL of DNase buffer (**DNB**). Add 20 µL mixture to the column and incubate at room temperature for 20 minutes.
7. Pipette 40 µL Wash Buffer 1 (**W1**) into the purification column and centrifuge for one minute at 8000 *x g*.
8. Pipette 100 µL Wash Buffer 2 (**W2**) into the purification column and centrifuge for one minute at 8000 *x g*.
9. Pipette another 100 µL Wash Buffer 2 (**W2**) into the purification column and centrifuge for two minutes at 16,000 *x g*.

   Note: *Check the purification column for any residual wash buffer. If wash buffer remains, re-centrifuge at 16,000 x g for one minute.*
10. Transfer the purification column to a new 0.5 mL microcentrifuge tube provided.
11. Pipette12 µL Elution Buffer (**EB**) directly onto the membrane of the purification column (Gently touch the tip of the pipette to the surface of the membrane while dispensing the elution buffer to ensure maximum absorption of EB into the membrane).
12. Incubate the column for one minute at room temperature.
13. Place each column tube assembly into the 2 ml support tube in the rotor with the 0.5 ml tube cap trailing the tube.
14. Centrifuge the column for one minute at 1,000 *x g* to distribute EB in the column, and then spin for one minute at 16,000 *x g* to elute RNA. The entire sample may be used immediately or stored at –70°C.

    Note: *Flow through waste following centrifugation is usually present as only a small volume, and therefore it is not necessary to discard the flow through waste after every centrifugation step. Make sure that the accumulated flow through waste does not make contact with the purification column. Flow through waste should be*

discarded when the waste fluid level approaches the surface of the purification column.

⚠ *Prior to use, mix Binding Buffer (BB) thoroughly. Binding Buffer (BB) may form precipitate upon storage. Dissolve precipitate prior to use by mixing thoroughly. If necessary, warm the BB vial to re-dissolve Binding Buffer prior to use.*

⚠ *Remove all traces of wash buffer prior to transferring purification column to the new microcentrifuge tube. To remove wash buffer, discard flow through waste and re-centrifuge the column for one minute at 16,000 x g.*

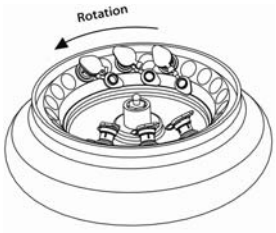


Figure 4.3: Centrifuge.

⚠ *To avoid potential breakage of the microcentrifuge tube cap during centrifugation, insert the purification column/ 0.5 mL tube assembly into a lidless 2.0 mL tube. Insert this assembly into adjacent rotor holes as illustrated. Rest the tube cap against the tube immediately clockwise to it. Place an empty, lidless 2.0 mL tube into the rotor hole adjacent in the clockwise direction to the last assembly.*

4.3.2. PROTOCOL FOR USE WITH CAPSURE HS LCM CAPS

RNA Extraction

1. Dispense Pro K Mix and incubate as follows:
   - **a.** Capture cells and assemble the CapSure HS Cap with the ExtracSure Extraction Device. Refer to the CapSure HS Caps User Guide for complete instructions.
   - **b.** Add 300 µL of Reconstitution Buffer to vial of dried Pro K Mix (600 µg/tube). Dissolve completely by gently vortexing the tube to mix the reagents and place the tube on ice immediately. Excessive mixing may denature Proteinase K. One vial of Pro K Mix is adequate for 60 extractions. All mixed proteinase K solution should be used within one work day (up to 12 hours). The remaining unmixed Reconstitution Buffer should be stored at -20°C. *Discard any mixed Proteinase K solution that is not used within one day.*
2. Place the CapSure–ExtracSure assembly in a CapSure HS Alignment Tray and pipette 10 µL Pro K Mix solution into the buffer well. Place pipette tip down to the film surface to avoid trapping a bubble.

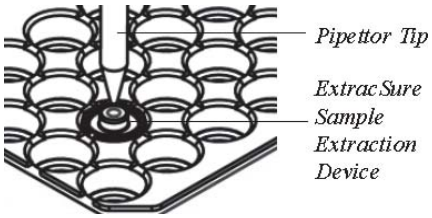


Figure 4.4: CapSure HS Alignment Tray

3. Place a new 0.5 ml extraction tube (not provided) onto the CapSure–ExtracSure assembly (see CapSure HS Caps User Guide for more details about assembly).
4. Cover with Incubation Block.

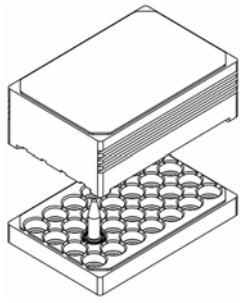


Figure 4.5: Incubation Block

5. Incubate at 37°C for the correct time period according to the following table:

Table 4.4: RNA Incubation Times

Samples Age	Incubation Time
Samples >3 years old	16 hours
Samples ≤3 years old	5 hours*

* For complete extraction this time can be increased to 16 hours.

⚠ *If multiple LCM captures are performed, it is recommended that each cap be incubated in Pro K Mix immediately after collection. Caps may be incubated up to 24 hours.*

6. Centrifuge the microcentrifuge tube with the CapSure–ExtracSure assembly at 800 *x g* for two minutes to collect cell extract into the tube.
7. After centrifugation, the microcentrifuge tube contains the cell extract required to complete the protocol. Remove the microcentrifuge tube from the CapSure–ExtracSure assembly and save the microcentrifuge tube with the cell extract in it.
8. Proceed with RNA isolation protocol or freeze cell extract at –70°C.

OK *It is okay to stop at this point in the protocol.*

RNA Isolation

1. Pre-condition the MiraCol Purification Column as follows:
   a. Pipette 200 µL Conditioning Buffer (**CB**) onto the purification column filter membrane.
   b. Incubate the purification column with Conditioning Buffer for 5 minutes at room temperature.
   c. Centrifuge the purification column in the provided collection tube at 16,000 *x g* for one minute.
2. Pipette 11 µL of Paradise Plus Reagent System binding buffer (**BB**) into the cell extract from Part 1 (RNA Extraction). Mix well by pipetting up and down. DO NOT CENTRIFUGE. Pipette 21 µL of Ethanol Solution (**EtOH**) into tube and mix well.
3. Pipette the cell extract mixture into the preconditioned purification column. The cell extract mixture will have a combined volume of approximately 110 µL.
4. To bind RNA, centrifuge for 2 minutes at 100 x *g*, immediately followed by a centrifugation at 16,000 x *g* for 1 minute.
5. Pipette 100 µL Wash Buffer 1 (**W1**) into column and centrifuge for 1 minute at 8000 *x g*.
6. Mix 2 µL DNase Mix (**DNase**) with 18 µL of DNase buffer (**DNB**). Add 20 µL mixture to the column and incubate at room temperature for 20 minutes.
7. Pipette 40 µL Wash Buffer 1 (**W1**) into the purification column and centrifuge for one minute at 8000 *x g*.
8. Pipette 100 µL Wash Buffer 2 (**W2**) into the purification column and centrifuge for one minute at 8000 *x g*.
9. Pipette another 100 µL Wash Buffer 2 (**W2**) into the purification column and centrifuge for two minutes at 16,000 *x g*. Check the purification column for any residual wash buffer. If wash buffer remains, at 16,000 *x g* for one minute.
10. Transfer the purification column to a new 0.5 mL microcentrifuge tube provided.
11. Pipette12 µL Elution Buffer (**EB**) directly onto the membrane of the purification column (Gently touch the tip of the pipette to the surface of the membrane while dispensing the elution buffer to ensure maximum absorption of EB into the membrane).
12. Incubate the column for one minute at room temperature.
13. Place each column tube assembly into the 2 ml support tube in the rotor with the 0.5 ml tube cap trailing the tube.
14. Centrifuge the column for one minute at 1,000 *x g* to distribute EB in the column, and then spin for one minute at 16,000 *x g* to elute RNA. The entire sample may be used immediately or stored at –70°C or below.

⚠ *NOTE: Flow through waste following centrifugation is usually present as only a small volume, and therefore it is not necessary to discard the flow through waste after every centrifugation step. Make sure that the accumulated flow through waste does not make contact with the purification column. Flow through waste should be discarded when the waste fluid level approaches the surface of the purification column.*

⚠ *Prior to use, mix Binding Buffer (BB) thoroughly. Binding Buffer (BB) may form precipitate upon storage. Dissolve precipitate prior to use by mixing thoroughly. If necessary, warm the BB vial to re-dissolve Binding Buffer prior to use.*

⚠ *Remove all traces of wash buffer prior to transferring purification column to the new microcentrifuge tube. To remove wash buffer, discard flow through waste and re-centrifuge the column for one minute at 16,000 x g.*

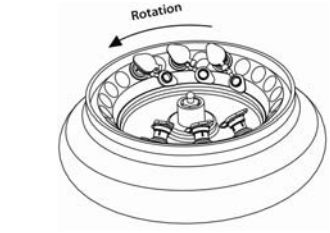


Figure 4.5: Centrifuge.

⚠ *To avoid potential breakage of the microcentrifuge tube cap during centrifugation, insert the purification column/ 0.5 mL tube assembly into a lidless 2.0 mL tube. Insert this assembly into adjacent rotor holes as illustrated. Rest the tube cap against the tube immediately clockwise to it. Place an empty, lidless 2.0 mL tube into the rotor hole adjacent in the clockwise direction to the last assembly.*

4.3.3. TISSUE SCRAPE PROTOCOL

⚠ *One vial of proteinase K is adequate for 3 tissue scrape samples*.

⚠ *Use a new scalpel blade for each sample to avoid cross-contamination*.

⚠ *Discard flow through waste when the waste fluid level approaches the bottom surface of the purification column*.

Slide preparation

Follow slide prep protocol, section 3.3.1.

Deparaffinization, Staining and Dehydration – no staining

⚠ *Important: If you are not staining your tissue, use the following protocol. Otherwise if you are staining you tissue use the deparaffinization, staining and dehydration protocol found in Chapter 3, section III, part b*.

1. Label three plastic slide jars as follows:
   a. Xylene
   b. Xylene
   c. Xylene
2. Fill each of the three jars with 25 mL of certified histology grade Xylene.
3. Retrieve up to four of the prepared slides
4. If the slides have been frozen, place them in a 50–60°C incubation oven for 2 minutes.

⚠ ***Note**: Do not perform this step if the slides have been at room temperature*.

5. Place the slides in jar **A. Xylene** for 3 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*.

6. Transfer the slides to jar **B. Xylene** for 3 minutes. Invert the jar gently three or four times.
7. Transfer the slides to jar **C. Xylene** for 3 minutes. Invert the jar gently three or four times.
8. Hold the slides in jar **C. Xylene** until ready to perform tissue scrape.

⚠ IMPORTANT: *The minimum incubation in xylene should be 3 minutes, up to a maximum of 2 hours*.

9. When ready to perform tissue scrape, remove the slides from the xylene, then dry in a fume hood for 5–10 minutes.

⚠ IMPORTANT: *Perform RNA extraction and isolation within 2 hours after removing the slides from the xylene*.

10. Repeat steps c through i for any remaining slides.
11. Discard the used xylene according to standard procedures, and then clean the jars following the procedure, "Cleaning the Plastic Slide Jars." found in the appendix of this document.
12. Proceed to RNA extraction and isolation.

Scrape and RNA Extraction

1. Add 300 µL of Reconstitution Buffer to vial of dried Pro K Mix (600 µg/tube). Dissolve completely by gently vortexing the tube to mix the reagents and place the tube on ice immediately. Excessive mixing may denature Proteinase K.
2. Pipette enough Pro K solution to cover entire tissue section (25 µL, 50 µL, 75 µL, 100 µL or 150 µL) into a 0.5 ml extraction tube (not provided).
3. Using a clean, sterile scalpel blade, take the dried slide and scrape off the tissue section and place the scrape into the microcentrifuge tube containing the Proteinase K solution.
4. Vortex slightly. Visually inspect to ensure that the tissue scrape is in the Pro K solution and not stuck to the side of the microcentrifuge tube.
5. Incubate at 37°C for the correct time period according to the following table:

Table 4.5: RNA Incubation Times

Samples Age	Incubation Time
Samples >3 years old	16 hours
Samples ≤3 years old	5 hours*

* For complete extraction this time can be increased to 16 hours.

6. Proceed to RNA isolation or store at –70oC or below.

RNA Isolation

1. Pre-condition the MiraCol Purification Column as follows:
   a. Pipette 200 µL Conditioning Buffer (**CB**) onto the purification column filter membrane.
   b. Incubate the purification column with Conditioning Buffer for 5 minutes at room temperature.

c. Centrifuge the purification column in the provided collection tube at 16,000 *x g* for one minute.

2. 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.

Table 4.6: Binding solutions chart

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

a. The volume of Binding Buffer is 1.06 × the volume of cell extract/PK solution.

b. The volume of Ethanol Solution is 2.06 × the volume of cell extract/PK solution, rounded up.

3. 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*.

4. Centrifuge the purification column for 2 minutes at 100 x *g* to bind the RNA on the column membrane.

5. Repeat steps c and d until all of the cell extract mixture has been loaded and bound to the purification column.

6. 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* to pellet the debris.

7. Repeat step d until all of sample mixture has been loaded and centrifuged.

8. Pipette 100 µL Wash Buffer 1 (**W1**) into column and centrifuge at *16,000 x g* for 1 minute.

9. Mix 2 µL DNase Mix (**DNase**) with 18 µL of DNase buffer (DNB). Add 20 µL mixture to the column and incubate at room temperature for 20 minutes.

10. Pipette 40 µL Wash Buffer 1 (**W1**) into the purification column and centrifuge for one minute at 8000 *x g*.

11. Pipette 100 µL Wash Buffer 2 (**W2**) into column and centrifuge at 16,000 *x g* for 1 minute.

12. Pipette 100 µL Wash Buffer 2 (**W2**) into column and centrifuge at 16,000 *x g* for 2 minutes.

13. Transfer column to a 0.5ml microcentrifuge tube provided in the Kit.

14. Pipette 12 µL of Elution Buffer (**EB**) direction onto the membrane of the purification column.

15. Incubate for 1 minute at room temperature.

16. Centrifuge at 1,000 *x g* for 1 minute and then at 16,000 *x g* for 1 minute.

The sample maybe used immediately or stored at –70°C or below.

⚠ *Gently touch the tip of the pipette to the surface of the membrane while dispensing the elution buffer to ensure maximum absorption of EB to the membrane.*

5. RNA Amplification

5.1. COMPONENTS

5.1.1. REAGENTS AND SUPPLIES

Table 5.1: Paradise® Plus cDNA kit - RA7018

Component	Vial Color	Vial Label
1st Strand Master Mix	Red	1
1st Strand Enzyme Mix*	Red	2
Enhancer	Yellow	E
1st Strand Nuclease Mix	Gold	
2nd Strand Master Mix	White	1
2nd Strand Enzyme Mix	White	2
Primer 1	Grey	1
Primer 2	Grey	2
Primer 3	Grey	3
Control RNA	White	C

*Also requires SuperScript™ III enzyme, not included

In vitro Transcription (IVT)

Table 5.2: In vitro Transcription (IVT) – RA7008

Component	Vial Color	Vial Label
IVT Buffer	Blue	1
IVT Master Mix	Blue	2
IVT Enzyme Mix	Blue	3
DNase Mix	Blue	4

Table 5.3: In vitro Transcription (IVT) 2-round – RA7009

Component	Vial Color	Vial Label
IVT Buffer	Blue	1
IVT Master Mix	Blue	2
IVT Enzyme Mix	Blue	3
DNase Mix	Blue	4

Table 5.4: Amino-Allyl IVT – RA7010

Component	Vial Color	Vial Label
IVT Buffer	Blue	1
IVT Master Mix	Blue	2
IVT Enzyme Mix	Blue	3
DNase Mix	Blue	4
Amino-allyl IVT Master Mix	Light Blue	AA
Labeling Buffer	Light Blue	LB
DMSO	Light Blue	DMSO

Table 5.5: aRNA Purification – RA7011

Component	Vial Color	Vial Label
DNA Binding Buffer	Red	DB
DNA Wash Buffer	Red	DW
DNA Elution Buffer	Red	DE
RNA Binding Buffer	Blue	RB
RNA Wash Buffer	Blue	RW
RNA Elution Buffer	Blue	RE
0.5 mL Microcentrifuge Tubes		
Purification columns		

⚠ *Please read this entire protocol prior to performing amplifications.*

⚠ *MDS Analytical Technologies recommends using quantitative real-time PCR for the most accurate measurement of RNA quantity of FFPE samples.*

⚠ *For maximum stability, store the frozen reagents at –70ºC or below until used. After use, storage at -20ºC is recommended.*

5.2. PRELIMINARY STEPS

5.2.1. MATERIAL AND PROTOCOL REVIEW

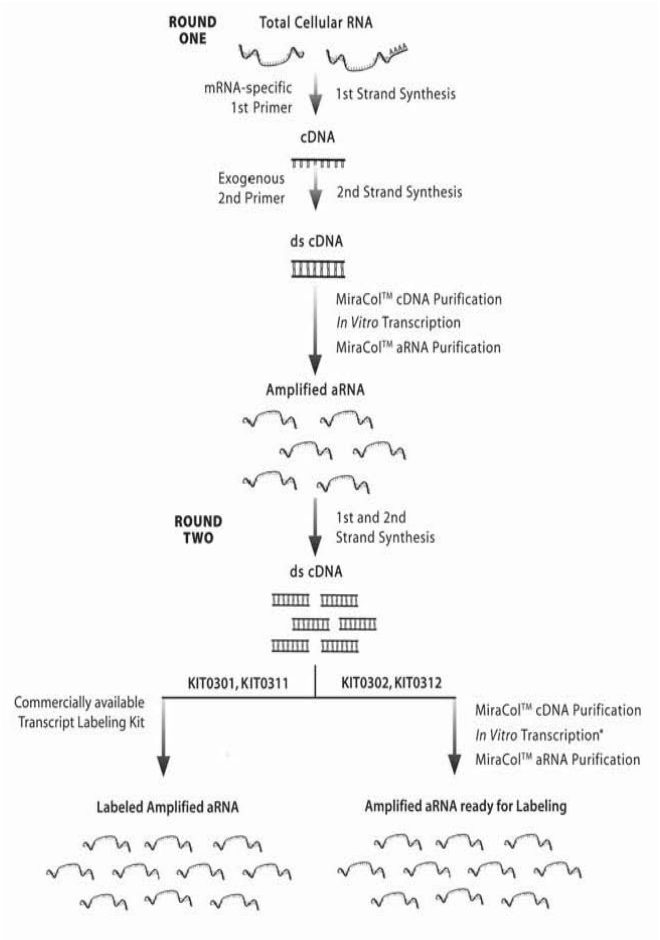
To get the most from your amplification reagents, take a few moments to examine the components of the kit and read the information in the following sections.

5.2.2. OVERVIEW

The Paradise Plus Reagent System RNA Amplification reagents are optimized to amplify formalin fixed RNA. The reagents utilize two rounds of a five-step process for linear amplification of the mRNA fraction of total cellular RNA:

a. first-strand synthesis reaction that yields cDNA incorporating a T7 promoter sequence;
b. second-strand synthesis reaction utilizing exogenous primers that yields double-stranded cDNA;
c. cDNA purification using specially designed MiraCol™ Purification Columns;
d. *in vitro* transcription (IVT) utilizing T7 RNA polymerase yields antisense RNA (aRNA); and
e. aRNA isolation with the MiraCol Purification Columns.

To save time, *in vitro* transcription may be performed overnight with the proper thermal cycler programming.



* *Optional reagents are supplied to enable the generation of amino allyl aRNA for hybridization on to sense oligo arrays. See Section V.A Application 3 for details.*

Figure 5.1: Paradise Amplification Schematic

⚠ *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 mixture concentrations.*

5.2.3. 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 for use appears on page 3-12. 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.

Table 5.6: Paradise® Plus Thermal Cycler Program – Round 1

	°C	Time
1st Strand Synthesis	70	1 hour
	4	hold
	42	1.5 hour
	4	hold
	37	30 minutes
	95	5 minutes
	4	hold
2nd Strand Synthesis	95	2 minutes
	4	hold
	25	10 minutes
	37	30 minutes
	70	5 minutes
	4	hold
IVT	42	8 hours
	4	hold (optional overnight hold)
	37	15 minutes
	4	hold

Table 5.7: Paradise® Plus Thermal Cycler Program – Round 2

	°C	Time
1st Strand Synthesis	70	5 minutes
	4	Hold
	25	10 minutes
	37	1.5 hour
	4	Hold
2nd Strand Synthesis	95	5 minutes
	4	Hold
	37	30 minutes
	70	5 minutes
	4	Hold
IVT	42	8 hours
	4	hold (optional overnight hold)
	37	15 minutes
	4	Hold

⚠ *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 mixture concentrations.*

5.2.4. TIME REQUIREMENTS

The table below presents typical time requirements for completion of the protocol. Times reflect total handling and reaction times of each step. Note that there are safe stopping points for pausing the amplification process, and the times presented reflect a continuous, uninterrupted process.

Table 5.7: Paradise® Plus Time Requirements

Paradise® Plus	1st Round	2nd Round
Steps	(hours)	(hours)
1st Strand Synthesis	3.5	2
2nd Strand Synthesis	1	1
cDNA Purification	0.5	0.5
Total (before IVT)	5	3.5
In Vitro Transcription	8	8
aRNA Purification	0.5	0.5
Total	13.5	12

⚠ *Samples requiring labeling for microarrays (biotin, Cy3, Cy5) labeling protocol will take an additional 30-45 minutes.*

⚠ *For samples processed using the optional IVT Master Mix, an additional 2.5 hours will be required for amino-allyl aRNA labeling.*

⚠ *Do not allow incubation times and temperatures to deviate from the protocol.*

⚠ *The 4°C steps in the thermal cycler program allow for buffer and reagent addition and mixing steps at certain points during the amplification process and are* ***not intended for indefinite hold unless noted.***

5.2.5. PROTOCOL NOTES

1. When adding reagent to samples or master mixes, pipette mixtures up and down several times to ensure complete transfer of reagent from the pipette tip.
2. Prior to the first use of an enzyme, gently mix (do not vortex) and briefly microcentrifuge the vial to ensure that all enzyme is mixed and collected at the bottom of the vial. Enzyme may collect on the vial wall or cap during shipment.
3. Keep thawed reagents and reaction tubes in cold blocks at 4°C while adding reagents to samples.
4. Prior to each incubation, mix samples thoroughly by flicking the reaction tube (unless noted otherwise in protocol) to ensure process performance. Spin down before proceeding. DO NOT VORTEX REACTION SAMPLES.
5. Use a microcentrifuge to spin down all components and samples following each mixing step.
6. Clean all amplification process equipment with an RNase eliminator such as RNase® AWAY (Life Technologies) to minimize the risk of RNase contamination.
7. **During enzyme and buffer dispensing, keep the reaction tube with sample on ice or chilled in a 4°C cold block**. Do not freeze samples unless it is indicated to be safe to do so in the protocol.

5.2.6. SAMPLE AND REAGENTS PREPARATION

1. Thaw frozen kit components, as needed, and mix with gentle vortexing or by inverting the tubes several times, spin down, and place on ice. When enzyme mixtures must be removed from –20°C storage for use, always keep them in a cold block or in an ice bucket at the lab bench.
2. Allow *In Vitro* Transcription (IVT) Buffer (Blue-labeled Vial 1), Master Mix (Blue-labeled Vial 2) and Enhancer (Yellow-labeled Vial) to assume room temperature (22–25°C), and mix by inverting or flicking the tube. Spin down if necessary. Dissolve all visible solids prior to use.
3. The Paradise Plus Reagent System RNA Amplification reagents are optimized for the input of formalin modified total cellular RNA.
4. Although excess enzyme and reagents are provided in all vials, there is insufficient volume to prepare extra reactions.
5. Two IVT Master Mix reagents are provided with kits designed for amino-allyl incorporation. The amino-allyl nucleotide mix should only be used in the second round IVT mix.

⚠ *When making master mixes, use only 10% overage per sample to avoid running out of reagent.*

5.2.7. NUCLEIC ACID ELUTION USING SPIN COLUMNS

Spin columns and 0.5 ml microcentrifuge tubes are provided for nucleic acid elution. Improper orientation of tubes during centrifugation may result in cap breakage or sample loss.

To correctly use the column-tube assembly, insert a spin column into the 0.5 ml tube, aligning the two cap hinges as illustrated. Load Elution Buffer onto the column and incubate as directed. Place the column-tube assembly into a 2 ml lidless support tube (PGC Scientific, Catalog #16-8101-06 or similar) in the centrifuge rotor; alternately, retain and reuse the 2 ml lidless collection tubes provided. (Some varieties of 2 ml tubes will not provide enough support. Contact MDS Analytical Technologies Technical Support for other alternatives). Skip one rotor position between assemblies and position assemblies with the 0.5 ml tube cap trailing the tube during centrifugation as shown. (Check for a mark on the centrifuge indicating rotation direction.) Centrifuge as directed in the protocol.

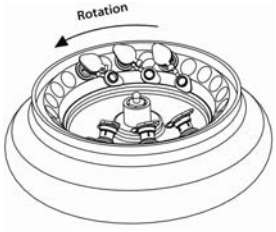


Figure 5.2: Centrifuge.

5.2.8. CONTROL AMPLIFICATIONS

A control RNA sample is provided along with each kit to be used as a control template to verify amplification efficacy. Use 10 µL of this RNA for control amplifications. 10 µL of this RNA contains 5 ng of formalin-fixed total RNA. Enough control RNA is provided for three control reactions per six-reaction kit. The control RNA provides a good positive control to assess amplification efficiency and success when run in parallel with samples following the procedures outline in the Appendix.

5.2.9. WORK SPACE RECOMMENDATIONS

Due to the high sensitivity of the reagents, it is very important to prevent RNA, DNA, and nuclease contamination. Work surfaces should be cleaned before and after each use. Perform all dispensing in a work hood that has been irradiated with UV to remove contaminants from previous amplification experiments.

5.2.10. IMPORTANT ADDITIONAL CONSIDERATIONS

MDS Analytical Technologies strongly recommends performing quality assessment of FFPE samples. In order to complete the Sample Assessment Protocol, a universal reference RNA (Stratagene) must also be run in parallel, in addition to the FFPE samples. Please see the Appendix for protocol details.

⚠ *MDS Analytical Technologies recommends using quantitative real-time PCR for the most accurate measurement of RNA quantity of FFPE samples.*

5.3. PROTOCOL

5.3.1. PARADISE® PLUS ROUND ONE: 1ST STRAND cDNA SYNTHESIS

⚠ *Read all Detailed Protocol notes on the previous pages prior to beginning.*

1. Prepare RNA sample in a total volume of 10 – 11 µL in a 0.5 mL or 0.2 mL RNase-free microcentrifuge tube and place on 4-8°C block.
2. Thaw Primer 1 (**Gray-1**), thoroughly mix, and spin down.

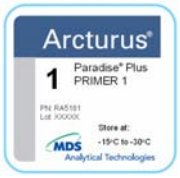


Figure 5.3: Gray 1

3. Add 1.0 µL of Primer 1, mix thoroughly by flicking the tube and spin down.
4. Incubate at 70°C for 1 hour then chill the samples to 4-8°C for at least one minute. Spin down the contents and place on 4-8°C block before proceeding to the next step.
5. Place 1st Strand Synthesis components (**Red**) at 4-8°C (cold block). 1st Strand Master Mix (**Red-1**) and Enhancer (**Yellow**) must be thawed, thoroughly mixed with all solids dissolved, and maintained at 4-8°C until used. 1st Strand Enzyme Mix (**Red-2**) and SuperScript III enzyme do not require thawing and can be placed directly at 4-8°C. Mix enzyme thoroughly by inverting several times. Spin briefly.

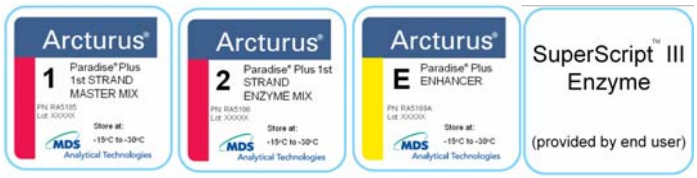


Figure 5.4: Red 1, Red 2, Yellow E and SuperScript III

6. Add 1st Strand Synthesis components in the order listed in the following table. If you are performing several amplifications, you may wish to prepare a Complete 1st Strand Synthesis Mix based on the following table, and add 9.0 µL Complete 1st Strand Synthesis Mix to each sample. Mix thoroughly by flicking the tube and spin down. **DO NOT VORTEX.**

Table 5.8: Complete 1st Strand Synthesis Mix

Component	Amount (µL)	Vial #	6 reaction Master Mix with 10% overage (µL)
Enhancer	2	Yellow E	13.2
1st Strand Master Mix	5	Red 1	33.0
1st Strand Enzyme Mix	1	Red 2	6.6
SuperScript™ III Enzyme*	1		6.6
Total per sample	**9**		59.4

* Not included in the kit

⚠ *Place components back onto the cold block or refreeze immediately after dispensing the reagent. Do not leave reagents at room temperature.*

7. Incubate at 42°C for 1.5 hours then chill the sample to 4-8°C for at least one minute. Do not hold samples at this step for a prolonged period of time. Keep samples at 4-8°C until next incubation.
8. (Optional) You may remove a 2.0 µL sample at this point in the protocol to assess the integrity of the starting mRNA by Quantitative Real-Time PCR (qRT-PCR.) ***Note:*** *This may reduce your final yield.*

⚠ IMPORTANT: **QC Kit Customers (KIT0313): STOP HERE and continue to appendix section II**

9. Thoroughly mix and spin down 1st Strand Nuclease Mix. Place on ice.
10. Add 2.0 µL of 1st Strand Nuclease Mix **(Gold)** to the sample, **mix thoroughly by flicking the tube and spin down**.

Figure 5.5: 1st Strand Nuclease Mix (Gold)

11. Incubate the sample at 37°C for 30 minutes followed by 95°C for five minutes.
12. Chill the sample to 4-8°C for at least one minute.

OK *It is okay to stop at this point in the protocol. Sample may be stored at –20°C overnight.*

⚠ *Removal for qrtPCR confirmation may not be suitable for low RNA inputs.*

5.3.2. PARADISE® PLUS ROUND ONE: 2ND STRAND cDNA SYNTHESIS

1. Place sample on 4-8°C block and allow to thaw if frozen (at 4-8°C).
2. Thaw Primer 2 (**Gray-2**), thoroughly mix, and spin down.

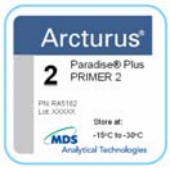


Figure 5.6: Gray 2

3. Add 1.0 µL of Primer 2. Mix thoroughly by flicking the tube and spin down.
4. Incubate sample at 95°C for 2 minutes, then chill and maintain the sample at 4-8°C for at least 2 minutes.
5. Thaw 2nd Strand Master Mix at 4-8°C (cold block) (**White-1**). Thoroughly mix and spin 2nd Strand Master Mix. 2nd Strand Enzyme Mix (**White-2**) does not require thawing. Mix enzyme thoroughly by inverting several times, spin briefly and place at 4-8°C.

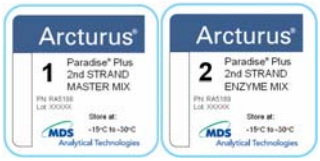


Figure 5.7: White 1 and White 2

6. Add 2nd Strand Synthesis components separately in the order listed in the following table. If you are performing several amplifications, you may wish to prepare a Complete 2nd Strand Synthesis Mix based on the following table, and add 30 µL Complete 2nd Strand Synthesis Mix to each sample. Mix thoroughly by flicking the tube and spin down.

Table 5.9: Complete 2nd Strand Synthesis Mix

Component	Amount (µL)	Vial #	6 reaction Master Mix with 10% overage (µL)
2nd Strand Master Mix	29	White 1	191.4
2nd Strand Enzyme Mix	1	White 2	6.6
Total per sample	30		198.0

*Store at 4°C until use

7. Incubate the sample as follows:
→ 25°C 10 minutes
→ 37°C 30 minutes
→ 70°C 5 minutes
→ 4-8°C Hold until ready to proceed (up to a maximum of 30 minutes)

⚠ *Place components back onto the cold block or refreeze immediately after dispensing the reagent. Do not leave reagents at room temperature for any extended period of time.*

5.3.3. PARADISE® PLUS ROUND ONE: cDNA PURIFICATION

1. Add 250 µL of DNA Binding Buffer (**DB**) to a DNA / RNA Purification Column seated in the collection tube provided. Hold for five minutes at room temperature. Centrifuge at 16,000 x *g* for one minute.

Figure 5.8: Binding Buffer DB

Note: *DNA Binding Buffer (DB) must be at room temperature and thoroughly mixed by shaking before use. A precipitate may form during long term storage. Dissolve precipitate prior to use by mixing. If necessary, warm the DB vial to re-dissolve*.

2. Add 200 µL of DNA Binding Buffer (**DB**) to the 2nd Strand Synthesis sample tube, mix well, and pipette the entire volume into the purification column.
3. To bind cDNA to column, centrifuge at 100 x *g* for two minutes (or lowest speed setting available), immediately followed by a centrifugation at 10,000 x *g* for one minute to remove flow-through.
4. Add 250 µL of DNA Wash Buffer (**DW**) to the column and centrifuge at 16,000 x *g* for two minutes. Check the purification column for any residual wash buffer. If any wash buffer remains, re-centrifuge at 16,000 x *g* for one minute.

Figure 5.9: Wash Buffer DW

5. Discard the flow-through and collection tube.
6. Place the column into the provided 0.5 mL microcentrifuge tube and carefully add 11 µL of DNA Elution Buffer (**DE**) onto the center of the purification column membrane. (Gently touch the tip of the pipette to the surface of the membrane while dispensing the elution buffer to ensure maximum absorption of DE into the membrane). Gently tap the purification column to distribute the buffer, if necessary. Incubate for one minute at room temperature.

Figure 5.10: Elution Buffer DE

7. Place the assembly into the centrifuge as shown, and centrifuge at 1,000 x *g* for one minute and then at 16,000 x *g* for one minute Discard the column and retain the elution containing the cDNA in the microcentrifuge tube for further processing.

Note: *Avoid splashing flow-through in the collection tube onto the column. If flow-through waste liquid wets the outside of the purification column, re-centrifuge the column at16,000 x g to remove liquid.*

OK *It is safe to stop at this point in the protocol. You may store the sample overnight at –20°C.*

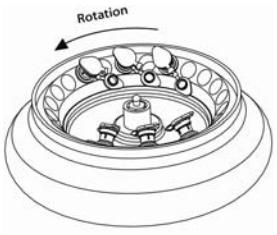


Figure 5.11: Centrifuge.

Note: *To avoid potential breakage of the microcentrifuge tube cap during centrifugation, insert the purification tube/0.5 mL tube assembly into a lidless 1.7/2.0 mL tube. Insert this assembly into adjacent rotor holes as illustrated. Rest the tube cap against the tube immediately clockwise to it. Place an empty, lidless 1.7/2.0 mL tube into the rotor hole adjacent in the clockwise direction to the last assembly.*

5.3.4. PARADISE® PLUS ROUND ONE: *IN VITRO* TRANSCRIPTION

1. Thaw IVT Buffer (**Blue-1**), Master Mix (**Blue-2**) and Enhancer (**Yellow**) to room temperature and thoroughly mix to dissolve all solids. IVT Enzyme Mix (**Blue-3**) does not require thawing and can be put directly 4-8°C. Mix enzyme thoroughly by inverting several times. Spin briefly.

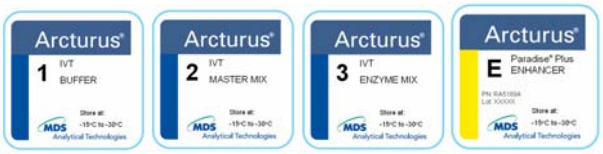


Figure 5.12: Blue 1, Blue 2, Blue 3 and Yellow E

Note: *IVT reaction components must be thawed thoroughly mixed with all solids dissolved, and brought to room temperature just before use.*

2. Add IVT components in the order listed in the following table. If you are performing several amplifications, you may wish to prepare a Complete IVT Reaction Mix according to the following table, and add 12 µL Complete IVT Reaction Mix to each sample. Mix thoroughly by flicking the tube and spin down.

Table 5.10: Complete IVT Reaction Mix

Component	Amount (µL)	Vial #	6 reaction Master Mix with 10% overage (µL)
IVT Buffer	2	Blue 1	13.2
IVT Master Mix	6	Blue 2	39.6
IVT Enzyme Mix	2	Blue 3	13.2
Enhancer	2	Yellow E	13.2
Total per sample	12		79.2

3. Incubate at 42°C for 8 hours. Chill the sample(s) to 4-8°C.

OK *At this point in the protocol, you may hold the reaction mixture at 4-8°C in the thermal cycler overnight.*

4. Move the samples directly to a 4-8°C block.
5. Add 1 µL DNase Mix **(Blue-4)**. Mix thoroughly and spin down. Incubate at 37°C for 15 minutes. Chill the sample(s) to 4-8°C. **Proceed immediately to aRNA purification.**

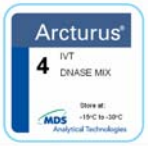


Figure 5.12: Blue 4

5.3.5. PARADISE® PLUS ROUND ONE: aRNA PURIFICATION

1. Add 250 µL of RNA Binding Buffer (**RB**) to a new purification column and incubate for five minutes at room temperature. Centrifuge at 16,000 x g for one minute.

Figure 5.13: Binding Buffer RB

⚠ *RNA Binding Buffer (RB) must be at room temperature and thoroughly mixed before use. A precipitate may form during long-term storage. Dissolve precipitate prior to use by mixing. If necessary, warm the RB vial to re-dissolve.*

2. Add 120 µL of **RB** to the IVT reaction sample and mix thoroughly. Pipette the entire volume into the purification column.
3. To bind aRNA, centrifuge at 100 x g (or lowest speed setting available) for two minutes, immediately followed by a centrifugation at 10,000 x g for one minute to remove flow-through.
4. Add 200 µL of RNA Wash Buffer (**RW**) to the purification column and centrifuge at 10,000 x g for one minute.

Figure 5.14: Wash Buffer RW

5. Add 200 µL of fresh **RW** to the purification column, and centrifuge at 16,000 x g for two minutes. Check the purification column for any residual wash buffer. If any wash buffer remains, re-centrifuge at 16,000 x g for one minute.

⚠ *Avoid splashing flow-through in the collection tube onto the purification column. If flow-through waste liquid wets the outside of the purification column, re-centrifuge the column at 16,000 x g to remove the liquid.*

6. Discard the collection tube and flow-through.
7. Place the purification column into a new 0.5 mL microcentrifuge tube provided in the kit and carefully add **12** µL of RNA Elution Buffer (**RE**) directly to the center of the purification column membrane. Gently touch the tip of the pipette to the surface of the membrane while dispensing **RE** to ensure maximum absorption of **RE** into the membrane. Gently tap the purification column to distribute the buffer, if necessary.

Figure 5.15: Elution Buffer RE

8. Incubate at room temperature for one minute.
9. Place each column-tube assembly into the centrifuge rotor with the 0.5 mL tube cap trailing the tube.
10. Centrifuge at 1,000 x g for one minute, immediately followed by 16,000 x g for one minute. Discard the purification column and retain the elution containing the aRNA.
11. Immediately proceed to Round Two or store the purified aRNA at –70°C overnight.

***Note**: Tubes must be properly oriented in the rotor during elution. See Section ?5.2.7?*

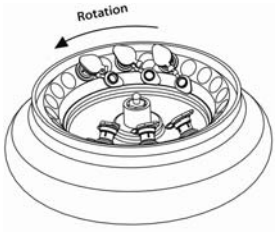


Figure 5.16: Centrifuge.

***Note:** To avoid potential breakage of the microcentrifuge tube cap during centrifugation, insert the purification tube/0.5 mL tube assembly into a lidless 1.7/2.0 mL tube. Insert this assembly into adjacent rotor holes as illustrated. Rest the tube cap against the tube immediately clockwise to it. Place an empty, lidless 1.7/2.0 mL tube into the rotor hole adjacent in the clockwise direction to the last assembly.*

End of Round 1.

5.3.6. PARADISE® PLUS ROUND TWO: 1ST STRAND cDNA SYNTHESIS

1. Thaw samples at 4-8°C if necessary. Place samples on a 4-8°C block.
2. Thaw Primer 2 (**Gray-2**), thoroughly mix, spin down and place on a 4-8°C block.

Figure 5.17: Gray 2

3. Into eluted aRNA product from Round One, add 1.0 µL of Primer 2, mix thoroughly by flicking the tube and spin down.
4. Incubate the microcentrifuge tube at 70°C for 5 minutes then chill the samples to 4-8°C for one minute. Spin down the contents and place on 4-8°C block before proceeding to next step.
5. Place 1st Strand Synthesis components (**Red**) at 4-8°C. 1st Strand Master Mix and Enhancer (**Yellow**) must be thawed, thoroughly mixed with all solids dissolved, and maintained at 4-8°C until used. 1st Strand Enzyme Mix does not require thawing and can be placed directly at 4-8°C. Mix enzyme thoroughly by inverting several times. Spin briefly.

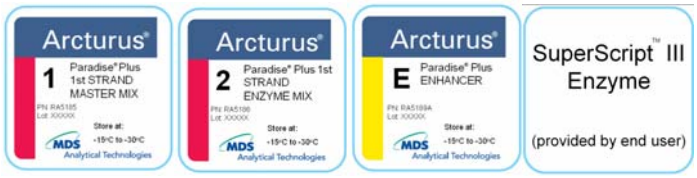


Figure 5.18: Red 1, Red 2, Yellow Enhancer and SuperScript III Enzyme

6. Add 1st Strand Synthesis components in the order listed in the following table. If you are performing several amplifications, you may wish to prepare a Complete 1st Strand Synthesis Mix based on the following table, and add 9.0 µL Complete 1st Strand Synthesis Mix to each sample. Mix thoroughly by flicking the tube and spin down. **DO NOT VORTEX**.

Table 5.11: Complete 1st Strand Synthesis Mix

Component	Amount (µL)	Vial #	6 reaction Master Mix with 10% overage (µL)
Enhancer	2	Yellow E	13.2
1st Strand Master Mix	5	Red 1	33.0
1st Strand Enzyme Mix	1	Red 2	6.6
SuperScriptTM III Enzyme*	1		6.6
Total per sample	9		59.4

* Not included in the kit

7. Incubate the sample(s) at 25°C for 10 minutes then at 37°C for 1.5 hours.
8. Chill the sample(s) to 4-8°C, for at least one minute.

OK *It is safe to stop at this point in the protocol. You may store the sample overnight at –20°C.*

⚠ *Place components back onto the cold block or refreeze immediately after dispensing the reagent. Do not leave reagents at room temperature for any extended period of time*.

⚠ IMPORTANT: **qRT-PCR Kit Customers (KIT0300 KIT0300-NS, KIT0310 & KIT0310-NS): STOP HERE and continue with qRT-PCR protocol as directed by instrument manufacturer.**

5.3.7. PARADISE® PLUS ROUND TWO: 2ND STRAND cDNA SYNTHESIS

1 Place sample on 4-8oC block and allow to thaw if frozen (at 4-8oC).

2 Thaw Primer 3 (Gray-3), thoroughly mix, spin down, and place on 4-8°C block.

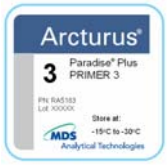


Figure 5.19: Gray 3

3 Add 1.0 µL of Primer 3 to the sample. Mix thoroughly by flicking the tube and spin down.

4 Incubate the sample at 95°C for five minutes then cool sample to 4-8°C for at least one minute. Hold the sample at 4-8°C until ready to proceed. Spin down the contents and place on 4-8°C block before proceeding to the next step.

5 Thaw 2nd Strand Master Mix at 4-8°C (cold block) (White-1). Thoroughly mix and spin 2nd Strand Master Mix. 2nd Strand Enzyme Mix (White-2) does not require thawing. Mix enzyme thoroughly by inverting several times, spin briefly and place at 4-8°C.

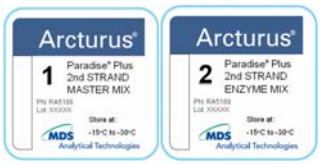


Figure 5.20: White 1 and White 2

6 Add 2nd Strand Synthesis components separately in the order listed in the following table. If you are performing several amplifications, you may wish to prepare a Complete 2nd Strand Synthesis Mix based on the following table, and add 30 µL Complete 2nd Strand Synthesis Mix to each sample. Mix thoroughly by flicking the tube and spin down.

Table 5.12: Complete 2nd Strand Synthesis Mix

Component	Amount (µL)	Vial #	6 reaction Master Mix with 10% overage (µL)
2nd Strand Master Mix	29	White 1	191.4
2nd Strand Enzyme Mix	1	White 2	6.6
Total per sample	30		198.0

*Store at 4°C until use

7 Incubate the sample(s) as follows:

→ 37°C 30 minutes

→ 70°C 5 minutes

→ 4-8°C Hold until ready to proceed (up to a maximum of 30 minutes).

Note: *Place components back onto the cold block or refreeze immediately after dispensing the reagent. Do not leave reagents at room temperature.*

5.3.8. PARADISE® PLUS ROUND TWO: cDNA PURIFICATION

1. Add 250 µL of DNA Binding Buffer (**DB**) to a new purification column seated in the collection tube provided. Incubate for five minutes at room temperature. Centrifuge at 16,000 x g for one minute.

Figure 5.21: Binding Buffer DB

Note: *DNA Binding Buffer (DB) must be at room temperature and thoroughly mixed before use. A precipitate may form during long-term storage. Dissolve precipitate by mixing. If necessary, warm the DB vial to re-dissolve*

2. Add 200 µL of **DB** to the 2nd Strand Synthesis sample tube, mix well, and pipette the entire volume into the purification column.
3. To bind cDNA, centrifuge at 100 x g (or lowest speed setting available) for two minutes, immediately followed by a centrifugation at 10,000 x g for 1 minute to remove flow-through.
4. Add 250 µL of DNA Wash Buffer (**DW**) to the column and centrifuge at 16,000 x g for two minutes. Check the purification column for any residual wash buffer. If any wash buffer remains, re-centrifuge at 16,000 x g for one minute.

Figure 5.22: Wash Buffer DW

Note: *Avoid splashing flow-through in the collection tube onto the column. If flow-through waste liquid wets the outside of the purification column, re-centrifuge the column at16,000 x g to remove liquid.*

5. Discard the collection tube and flow-through.
6. Place the column into the provided 0.5 mL microcentrifuge tube and carefully add **11** µL of DNA Elution Buffer (**DE**) onto the center of the purification column membrane. Gently touch the tip of the pipette to the surface of the membrane while dispensing **DE** to ensure maximum absorption of **DE** into the membrane. Gently tap the purification column to distribute the buffer, if necessary.

Figure 5.23: Elution Buffer DE

7. Incubate for one minute at room temperature.
8. Place each column-tube assembly into the 2 mL support tube in the rotor with the 0.5 mL tube cap trailing the tube.
9. Centrifuge at 1000 x g for one minute, followed immediately by 16,000 x g for one minute. Discard the column and retain the elution containing the cDNA.

OK *It is safe to stop at this point in the protocol. You may store the sample overnight at –20°C.*

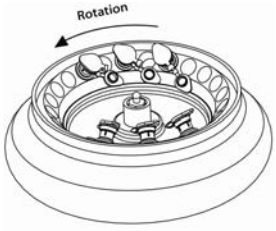


Figure 5.24: Centrifuge.

Note: *To avoid potential breakage of the microcentrifuge tube cap during centrifugation, insert the purification tube/0.5 mL tube assembly into a lidless 1.7/2.0 mL tube. Insert this assembly into adjacent rotor holes as illustrated. Rest the tube cap against the tube immediately clockwise to it. Place an empty, lidless 1.7/2.0 mL tube into the rotor hole adjacent in the clockwise direction to the last assembly.*

IMPORTANT: 1.5 Round Customer (KIT 0301, KIT0301-NS KIT0311, & KIT0311-NS): **STOP HERE** and proceed to Appendix IX.

5.3.9. PARADISE® PLUS ROUND TWO: *IN VITRO* TRANSCRIPTION

1 Thaw IVT Buffer (**Blue-1**), Master Mix (**Blue-2**) and Enhancer (**Yellow**) to room temperature and thoroughly mix to dissolve all solids. IVT Enzyme Mix does not require thawing and can be put directly 4-8°C. Mix enzyme thoroughly by inverting several times. Spin briefly.

Note: *IVT reaction components must be thawed thoroughly mixed with all solids dissolved, and brought to room temperature just before use.*

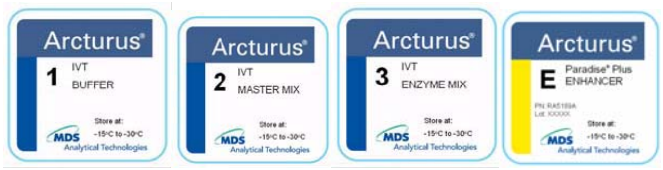


Figure 5.24: Normal IVT Blue-1, Blue-2, Blue-3, and Yellow E.

or

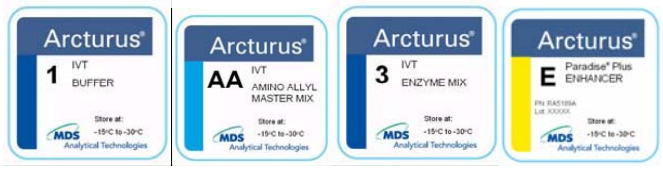


Figure 5.25: Amino-Allyl incorporation IVT: Blue-1, Light Blue AA Blue-3, and Yellow E.

2 Add IVT components in the order listed in the following table. If you are performing several amplifications, you may wish to prepare a Complete IVT Reaction Mix according to the following table, and add 12 µL Complete IVT Reaction Mix to each sample. Mix thoroughly by flicking the tube and spin down.

Note: *IVT reaction components must be thawed thoroughly mixed with all solids dissolved, and brought to room temperature just before use.*

Table 5.13: Complete IVT Reaction Mix

Component	Amount (µL)	Vial #	6 reaction Master Mix with 10% overage (µL)
IVT Buffer	2	Blue 1	13.2
IVT Master Mix*	6	Blue 2*	39.6
IVT Enzyme Mix	2	Blue 3	13.2
Enhancer	2	Yellow E	13.2
Total per sample	**12**		79.2

*If doing Amino-Allyl incorporation, substitute Amino-Allyl IVT Master Mix (light blue AA) here

3 Incubate at 42°C for 8 hours. Chill the sample(s) to 4-8°C.

OK *At this point in the protocol, you may hold the reaction mixture at 4-8°C in the thermal cycler overnight*.

4 Move the samples directly to a 4-8°C block.

Figure 5.26: Blue 4

5 Add 1 µL DNase Mix **(Blue-4)**. Mix thoroughly and spin down. Incubate at 37°C for 15 minutes. Chill the sample(s) to 4-8°C. **Proceed immediately to aRNA purification.**

5.3.10. PARADISE® PLUS ROUND TWO: aRNA PURIFICATION

1 Add 250 µL of RNA Binding Buffer (**RB**) to a new purification column seated in the collection tube provided. Incubate for five minutes at room temperature. Centrifuge at 16,000 x g for one minute.

Figure 5.27: RNA Binding Buffer

⚠ *RNA Binding Buffer (RB) must be at room temperature and thoroughly mixed before use. A precipitate may form during long-term storage. Dissolve precipitate prior to use by mixing. If necessary, warm the RB vial to re-dissolve.*

2 Add 120 µL of **RB** to the IVT reaction sample and mix thoroughly. Pipette the entire volume into the purification column.
3 To bind aRNA, centrifuge at 100 x g (or lowest speed setting available) for two minutes, immediately followed by a centrifugation at 10,000 x g for 1 minute.
4 Add 200 µL of RNA Wash Buffer (**RW**) to the purification column and centrifuge at 10,000 x g for one minute.

Figure 5.28: RNA Wash Buffer

⚠ *Avoid splashing flow-through in the collection tube onto the purification column. If flow-through waste liquid wets the outside of the purification column, re-centrifuge the column at 16,000 x g to remove the liquid.*

5 Add 200 µL of fresh **RW** to the purification column, and centrifuge at 16,000 x g for two minutes. Check the column for any residual wash buffer. If any wash buffer remains, re-centrifuge at 16,000 x g for one minute.
6 Discard the collection tube and flow-through.
7 Place the purification column into a new 0.5 mL microcentrifuge tube provided in the Kit and carefully add 30 µL of RNA Elution Buffer (**RE**) directly to the center of the purification column membrane. Gently touch the tip of the pipette to the surface of the membrane while dispensing **RE** to ensure maximum absorption of **RE** into the membrane. Gently tap the purification column to distribute the buffer, if necessary.

Figure 5.29: RNA Elution Buffer

8 Incubate for one minute at room temperature.
9 Place each column-tube assembly into the 2 mL support tube in the rotor with the 0.5 mL tube cap trailing the tube.
10 Centrifuge at 1000 x g for one minute, followed immediately by 16,000 x g for one minute. Discard the column and retain the elution containing the aRNA.
11 Measure the O.D. of the product at A_{260} and A_{280}
12 Analyze the aRNA using the Agilent Bioanalyzer or by gel electrophoresis.
13 The purified aRNA is ready for use in a labeling reaction with the TURBO microarray labeling kit (see Appendix Appendix 1, Application 1) or in a reverse transcription application with the Paradise cDNA kit (see "related Arcturus reagents" or www.molecualrdevices.com for more information)

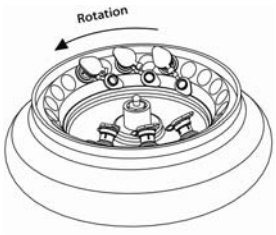


Figure 5.30: Centrifuge.

Note: *To avoid potential breakage of the microcentrifuge tube cap during centrifugation, insert the purification tube/0.5 mL tube assembly into a lidless 1.7/2.0 mL tube. Insert this assembly into adjacent rotor holes as illustrated. Rest the tube cap against the tube immediately clockwise to it. Place an empty, lidless 1.7/2.0 mL tube into the rotor hole adjacent in the clockwise direction to the last assembly.*

End of Round 2.

A. Appendices

A.1. APPLICATIONS OF aRNA

The Paradise Plus Reagent System can be used to yield a suitable labeled RNA sample for hybridization to nucleic acid in a variety of formats. The RNA sample may be labeled in a number of different ways, including those listed below.

A.1.1. DIRECT aRNA LABELING WITH TURBO LABELING KIT

After analysis of the aRNA with the Agilent Bioanalyzer (Appendix E) or by gel electrophoresis (Appendix F) as described in Round Two: Antisense RNA (aRNA) Purification, aRNA may be directly labeled with a biotin or a fluorescent marker. Direct mRNA labeling can be accomplished using the Turbo Labeling kits:

KIT0608 Turbo Labeling – Biotin, 12 reactions
KIT0609 Turbo Labeling – Cy3, 12 reactions
KIT0610 Turbo Labeling – Cy5, 12 reactions

For more information go to www.moleculardevices.com/turbo

A.1.2. DIRECT cDNA FLUORESCENT LABELING

For use with complete 2 round kits

The protocol described here may be used to prepare Cy3- or Cy5-labeled cDNA from aRNA generated using the Paradise Plus Reagent System RNA Amplification Kit for hybridization to cDNA microarrays. This protocol provides labeled **probe of sense orientation from 5-10 micrograms** of aRNA, a sufficient quantity for replicate hybridizations on cDNA microarrays. However, such probes are typically not used for oligonucleotide arrays, since the targets on such arrays are also generally in the sense orientation.

Table A.1: Suppliers

Reagents Used	Maker	Catalog#
RNase AWAY®	Invitrogen	10328-011
Cy3 labeled dUTP	Amersham	PA53022
Cy5 labeled dUTP	Amersham	PA55022
RNAsin® Ribonuclease Inhibitor	Promega	N2515
SuperScript™ III RT and Buffer	Invitrogen	18080-044
Nuclease Free Water	Invitrogen	10977-023
Rnase H	Invitrogen	18021-071
Random Hexamer	Operon	custom-made
QiaQuick® PCR Purification Kit	Qiagen®	28106

Protocol

1 Take 5-10 µg of amplified aRNA and adjust the volume to 22 µL with nuclease-free water.

⚠ *Adjust to 22 µL using a vacuum concentrator, paying attention to not completely dry down the aRNA sample.*

2 Add 2 µL of 5 mg/ml random hexamer.

3 Mix well by flicking, then briefly spin down by centrifugation.

4 Heat the tube to 70°C for 10 minutes, then 4°C for 2 minutes in the thermal cycler.

5 During incubation, prepare the first-strand master mix as described below.

Table A.2: First Strand Master Mix - 1x

First Strand Buffer	10 µL
0.1 M DTT	5 µL
25 mM dNTP	1 µL
1 mM dUTP-Cy3 or Cy5	2 µL
1 mM dTTP	2 µL
RNAsin®	2 µL
Superscript™ II RT	4 µL
Total	**26** µL

6 When incubation is complete, mix the tube well by flicking, and then briefly spin down by centrifugation.

7 Add 26 µL of the above first stand master mix to each reaction tube.

8 Mix well by flicking, and then briefly spin down by centrifugation.

9 Incubate at 27°C for 10 minutes, followed by 37°C for 2 hours in the thermal cycler.

10 Treat with 2 units of RNase H for 20 minutes at 37°C in the thermal cycler.

11 Immediately proceed to PCR product purification using QiaQuick® PCR Purification Kit. Pre-treat the columns placed in collection tube by incubating 100 µL of QiaQuick PB buffer for 5 minutes, and then centrifuge at 13200 rpm (or full speed on a 5415C Eppendorf Centrifuge) for 1 minute.

12 Add 260 µL of QiaQuick® PB buffer to the sample tube.

13 Mix well by flicking, and then briefly spin down by centrifugation.

14 Load the sample onto the pre-treated columns. Centrifuge at 6000 rpm for 1 minute.

15 Discard flow through. Place the column into the same collection tube.

16 Wash with 750 µL of QiaQuick PE buffer. Centrifuge at 13200 rpm for 1 minute.

17 Discard flow through. Place the column back into the same collection tube.

18 Centrifuge at 13200 rpm for an additional 2 minutes to remove residual wash solution.

19 Place the column into a clean 2 ml microcentrifuge tube.

20 Add 50 µL of nuclease-free water, pH 8.5, directly onto the column membrane. Incubate for 3–5 minutes, and then centrifuge at maximum speed for 1 minute.

21 If the column still shows residual probe, add another 30 µL of nuclease free water, pH 8.5, directly onto the column membrane, incubate for 1minute, and centrifuge at maximum speed for 1 minute.

⚠ *After centrifuging, make sure that the entire sample has passed through the column, and that the column is completely dry. If not, centrifuge for an additional 1 minute at 6000 rpm. When dry, the column will be visibly pink (Cy3) or blue (Cy5) if the reaction was successful.*

⚠ *If the labeling reaction was successful, the eluate should be visibly pink (Cy3) or blue (Cy5) in color.*

A.1.3. GENERATION OF TEMPLATE FOR QRT-PCR

The aRNA generated using the Paradise Plus Reagent System RNA Amplification reagents can be used in reactions measuring relative gene expression using quantitative PCR methods.

The amplification process generates product from the 3' end of the mRNA. For best results use qRT-PCR primer sets designed within the first 300 bases from the poly A tail.

The following protocol may serve as a guide for qRT-PCR experiments using aRNA converted to cDNA as a template.

Protocol

Reverse Transcription

1 MDS Analytical Technologies recommends an aliquot of 100 ng of amplified RNA from each sample in a volume of 10 µL.
2 Add 1 µL of 5 mg/ml random hexamer.
3 Mix well by flicking, and then briefly spin down by centrifugation for 2 minutes in thermal cycler.
4 Incubate samples at 70ºC for 10 minutes. Chill sample to 4ºC.
5 Assemble a master mix with the following components: (components listed are for one reaction)

Table A.3: Master Mix Components

Item	Volume (µL)	Vendor	Catalog #
First Strand Buffer	4	Invitrogen	18080-044
0.1M DTT	2	Invitrogen	18064-044
10 mM dNTP	1	Amersham	US77212-500µL
Rnasin	1	Promega	N2511
Superscript III	1	Invitrogen	18064-044
Total	9		

6 When incubation (Step 4) is complete, mix the tube well by flicking, and then briefly spin down by centrifugation.
7 Add 9 µL of the First Strand Master Mix to each reaction tube.

8 Incubate at 27°C for 10 minutes, followed by 37°C for 1.5 hours in the thermal cycler.
9 The sample is now ready for Q-PCR.

qRT-PCR
Please consult the protocol from the system manufacturer.

A.2. SAMPLE ASSESSMENT PROTOCOL

MDS Analytical Technologies recommends performing this simple, in process, protocol to assess the quality of RNA in FFPE tissue blocks. This protocol will enable estimation of RNA quantity and quality using a quantitative real-time PCR assay with primers designed to β-actin.

The assumption is that the β-actin mRNA in the sample represents the average status of other RNA molecules in the same sample. The total estimated RNA amount in a given sample is expressed as an equivalent of universal RNA that contains the same amount of β-actin mRNA.

The protocol measures the average β-actin cDNA length by quantification of the PCR product yield from the 3' end (primer 1650-1717) and another relative 5' sequence (primer 1355-1472). If all cDNA contains both the 3' and 5' sequence target, the ratio of the PCR product for 3'/5' would be 1. As the RNA from FFPE samples tends to exhibit some degradation, the 3'/5' ratio is usually greater than one. Depending on the ratio, an estimation of the quality of the RNA can be made.

It is recommended to perform the reverse transcription steps with 100-200 ng of total RNA. Be sure to determine the yield of the total RNA following isolation and make the appropriate dilutions to ensure your samples are within this concentration range. Perform necessary dilutions of total RNA in 10 ng/µL Poly-I.

In parallel to the testing sample, a control of 10 ng/ µL uRNA (100 ng in 10 µL) (Stratagene 740000) needs to be carried through the complete analysis as the quantitation standard for **EVERY** experiment. Dilution factor may vary for aRNA generated after one round of amplification.

⚠ *MDS Analytical Technologies recommends this protocol to customers who are starting a new set of FFPE samples or new type of FFPE samples. Experiments should be done on scraped sections*.

The RNA quantity derived from the 3' primer set (1650-1717) should be used as the quantity measurement of the RNA in the FFPE sample. The ratio of the RNA yield obtained from both sets of PCR primers is the 3'/5' used as an indication of RNA quality.

Materials Needed

Table A.4: Materials needed

Component	Vendor	Catalog #
RNase free water	Invitrogen	18054-015
Universal Human Reference RNA	Stratagene	740000
Poly-I	Sigma	P4154
Uracil-DNA Glycosylase	Roche	1444646
Thermal cycler	MLS	--

Table A.5: Primer sequences:

HBAC1650	TCCCCCAACTTGAGATGTATGAAG	50 µM*
HBAC1717	AACTGGTCTCAAGTCAGTGTACAGG	50 µM*
HBAC1355	ATCCCCCAAAGTTCACAATG	50 µM*
HBAC1472	GTGGCTTTTAGGATGGCAAG	50 µM*

* Stock concentration

Table A.6: Materials needed for qrtPCR

Component	Vendor	Catalog #
For ABI PRISM 7900HT:		
QuantiTect SYBR Green PCR Master Mix	Qiagen	204143
ABI optical Reaction Plate	ABI	4314320
Optical Adhesive covers	ABI	4311971
Splash Free Support Base for 96well plate	ABI	4312063
Microseal F foil	MJ Research	MSF 1001
For Roche Light Cycler:		
LightCycler DNA SYBR Green kit	Roche	2158817
BD Taqstart Antibody	BD-Clontech	639251

⚠ IMPORTANT: *Depending on the exact model of the real-time PCR instrument, the protocol may vary. Provided are protocols for the ABI PRISM 7900 HT Sequence Detection System and the Roche LightCycler*.

Protocol

Extraction

Follow the protocol detailed in Section 4.3.3.: Tissue Scrape Protocol of this user guide.

Quantitate the concentration of the RNA extracted (see Appendix A.6)

Reverse Transcription

Your experimental design should include:

→ 100-200 ng of each sample

→ 100 ng of universal reference RNA (Stratagene cat # 740000) to be used later as a standard curve for qRT-PCR

⚠ *Note: it is not necessary to run the control that comes with the kit. That control RNA is an amplification control and is not of high enough concentration for the standard curve.*

⚠ *Note: This is the same procedure as Section 5.3.1.: Paradise® Plus Round one: 1st strand cDNA synthesis of this user guide.*

⚠ *Read all Detailed Protocol notes in Chapter 5 prior to beginning this procedure.*

1 Prepare each 100-200 ng RNA sample in a total volume of 10 – 11 µL in a 0.5 mL or 0.2 mL RNase-free microcentrifuge tube and place on 4-8°C block.

2 Thaw Primer 1 (**Gray-1**), thoroughly mix, and spin down.

3 Add 1.0 µL of Primer 1, mix thoroughly by flicking the tube and spin down.

4 Incubate at 70°C for 1 hour then chill the samples to 4-8°C for at least one minute. Spin down the contents and place on 4-8°C block before proceeding to the next step.

5 Place 1st Strand Synthesis components at 4-8°C (cold block). 1st Strand Master Mix (**Red-1**) and Enhancer (**Yellow**) must be thawed, thoroughly mixed with all solids dissolved, and maintained at 4-8°C until used. 1st Strand Enzyme Mix (**Red-2**) and SuperScript enzyme do not require thawing and can be placed directly at 4-8°C. Mix enzyme thoroughly by inverting several times. Spin briefly.

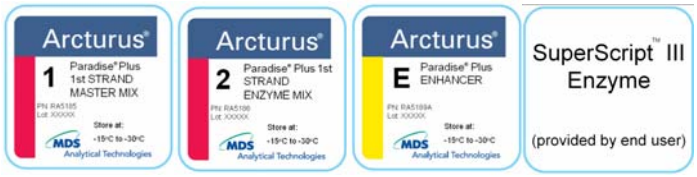


Figure A.1: Red 1, Red 2), Yellow Enhancer and SuperScript III Enzyme

6 Add 1st Strand Synthesis components in the order listed in the following table. If you are performing several amplifications, you may wish to prepare a Complete 1st Strand Synthesis Mix based on the following table, and add 9.0 µL Complete 1st Strand Synthesis Mix to each sample. Mix thoroughly by flicking the tube and spin down. **DO NOT VORTEX.**

Table A.7: Complete 1st Strand Synthesis Mix

Component	Amount (µL)	Vial #	6 reaction Master Mix with 10% overage (µL)
Enhancer	2	Yellow E	13.2
1st Strand Master Mix	5	Red 1	33.0
1st Strand Enzyme Mix	1	Red 2	6.6
SuperScript™ III Enzyme*	1		6.6
Total per sample	**9**		59.4

* Not included in the kit

7 Incubate at 42°C for 1.5 hours then chill the sample to 4-8°C for at least one minute. Do not hold samples at this step for a prolonged period of time. Keep samples at 4-8°C while creating the standard curve.

Creating the Standard Curve

Using the cDNA generated from the 100 ng of Universal Human Reference RNA, create other standard curve points from subsequent serial dilutions. Use the following guidelines:

- → The standard curve should consist of four standard points:
  - → 100 ng, 10 ng, 1 ng, and 0.1 ng (per reverse transcription input of total RNA).
  - → Use 10 ng/µL Poly-I as the diluent.
  - → Perform the serial dilutions as illustrated below.

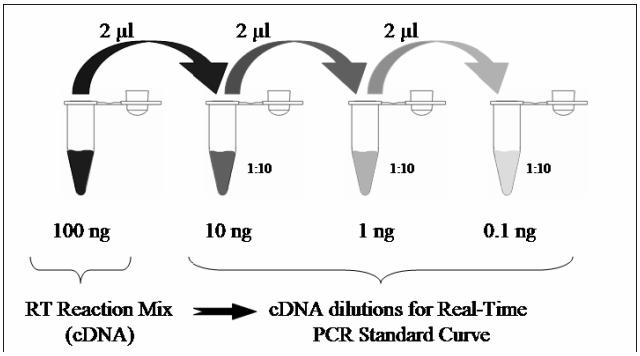


Figure A.2: cDNA dilutions for Real-Time PCR Standard Curve

  - → Store the unused volume of 100-ng cDNA at –65 to –80°C for use in creating subsequent standard curves.

⚠ *Set up one RT reaction for each testing samples, one for the blank, and one for the uRNA standard.*

⚠ *Use 10.0 µL of testing samples, 10.0 ul of water for a blank tube and 10.0 µL of the uRNA standard.*

⚠ *Many real-time PCR instrument systems utilize similar technology. We provide protocols for the ABI PRISM 7900HT and the Light Cycler. Modification to these*

protocols for other real-time platforms can be made according to the manufacturers' recommendations.

Protocol

1. Following the RT reaction serially dilute the uRNA control for use as a standard curve. The cDNA generated from the 100 ng of Universal Human Reference RNA (Stratagene, PN 740000; see above) is used as the highest template mass point for the standard curve. Therefore, the 100-ng sample represents 100% of the reverse transcription product.
2. Set up the PCR reactions following the steps below for the instrument you are using:

→ ABI PRISM 7900HT
→ Roche Light Cycler

For ABI PRISM 7900HT:

1. Prepare the PCR reactions per the table below, combine the PCR master mix components in a clean, nuclease-free microcentrifuge tube. To determine the total amount of PCR master mix required, multiply the amount of each component by the number of reactions you are performing (*n*), plus one: ***n* + 1**

Table A.8: For the 3′ β-actin Primers

	Amount for Each Reaction	
Component	**Volume (µL)[a]**	**Final Conc.**
SYBR® Green PCR Master Mix	10	—
Uracil-DNA Glycosylase	0.5	—
3' β-actin Forward Primer, 50 µM	0.25	625 nM
3' β-actin Reverse Primer, 50 µM	0.25	625 nM
Water, nuclease-free	1	—

Table A.9: For the 5′ β-actin Primers

	Amount for Each Reaction	
Component	**Volume (µL)[a]**	**Final Conc.**
SYBR® Green PCR Master Mix	10	—
Uracil-DNA Glycosylase	0.5	—
5' β-actin Forward Primer, 50 µM	0.25	625 nM
5' β-actin Reverse Primer, 50 µM	0.25	625 nM
Water, nuclease-free	1	—

a. The volumes listed above are specific to the Applied Biosystems 7900HT Fast Real-Time PCR System. Other real-time PCR instruments may require adjustments to these volumes. Please consult your instrument's user manual.

2. Mix thoroughly by inverting, then spin quickly to collect the master mix at the bottom of the tube.

3. Proceed to "Running the ABI PCR Reactions".

Preparing the reaction plate

1. Assemble the reactions in an optical tube or optical reaction plate:
   a. Dispense 12 µL of the 3′ Primer Set into the appropriate wells or tubes.
   b. Dispense 12 µL of the 5′ Primer Set into the appropriate wells or tubes.
   c. Add 8 µL of the diluted cDNA into the appropriate wells or tubes.
   d. Add 8 µL of the nuclease-free water into the appropriate NTC wells or tubes.

Note: For both the 3′ and 5′ Primer Sets, include the RNA samples, positive control, diluted control for a standard curve, and the blank (NTC) in the sample layout.

2. Seal the tube or reaction plate with an optical cap or optical adhesive cover.
3. Vortex the tube or reaction plate for 20 seconds, then spin quickly to collect the reactions at the bottom of the tube or wells.
4. Proceed to "Running the ABI PCR Reactions".

Running the ABI PCR Reactions

1. Place the tube or reaction plate in your real-time PCR instrument.
2. Program the thermal cycling conditions as follows:

Table A.10: Thermal Cycling Conditions

Step		Temperature (°C)	Time
1	Hold	50	2 minutes
2	Hold	95	15 minutes
3	PCR (40 cycles)	95	15 seconds
		58	30 seconds
		72	32 seconds

⚠ IMPORTANT: *The thermal cycling conditions listed above have been optimized for use on the Applied Biosystems 7900HT Fast Real-Time PCR System.*

3. *Optional.* If your instrument has the ability, include a dissociation curve.
4. When the run is completed, export the results into Microsoft® Excel software.
5. Proceed to "Interpreting the Results"

For Roche Light Cycler

Preparing the PCR reactions

1. Prepare the PCR reaction mix according to the table below:

Table A.11: For the 3′ β-actin Primers

	Amount for Each Reaction	
Component	**Volume (µL)[a]**	**Final Conc.**
SYBR® Green PCR Master Mix	2	—
BD Taqstart Antibody	0.16	
25 mM $MgCl^2$	2.4	—
Uracil-DNA Glycosylase	1.0	—
3’ β-actin Forward Primer, 50 µM	0.25	625 nM
3’ β-actin Reverse Primer, 50 µM	0.25	625 nM
Water, nuclease-free	11.94	—

Table A.12: For the 5′ β-actin Primers

	Amount for Each Reaction	
Component	**Volume (µL)[a]**	**Final Conc.**
SYBR® Green PCR Master Mix	2	—
BD Taqstart Antibody	0.16	
25 mM $MgCl^2$	2.4	—
Uracil-DNA Glycosylase	1.0	—
5’ β-actin Forward Primer, 50 µM	0.25	625 nM
5’ β-actin Reverse Primer, 50 µM	0.25	625 nM
Water, nuclease-free	11.94	—

2. For each reaction, add 18 µL of the PCR mix into a LightCycler capillary. Add 2 µL of the cDNA.

3. Spin the capillaries at 500g for 5 second in their adaptor. Load the capillaries into LightCycler.

4. Run the LightCycler following programs as listed below (set temperature transition rate to 20):

Step		Target temp	Time	Acquisition
1	Denaturation	95	1 min	none
2	Amplification (35 cycles)	95	0 sec	none
		58	5 sec	none
		72	10 sec	single
3	Melting	95	0 sec	none
		65	10 sec	none
		99	0 sec	cont
4	Cooling	40	1 min	none

5. Obtain Ct1650, Ct 1355 for the testing sample and the uRNA dilutions.
6. Quantify the input RNA:
   a. Plot the standard curve of log uRNA amount vs. Ct. For each pair of primers, one standard curve is generated.
   b. Obtain the uRNA equivalent of the testing sample from the corresponding standard curve (Standard curve $_{1650}$, Standard curve $_{1355}$).
   c. Use the uRNA equivalent from 1650 primer set to estimate the RNA quantity, use the ratio of RNA $_{1650}$/RNA $_{1355}$ to estimate the RNA quality.

Interpreting the Results

The RNA concentration using the 3′ Primer Set is the quantity of the sample RNA. The ratio of 3′/5′ is obtained for each sample using the corresponding RNA concentration.

Table A.13: Example of results

Primer	Ct	Quantity	Primer	Ct	Quantity	3'/5' Ratio
1355/1472	28.70	0.09	1650/1717	23.80	2.56	30.12
1355/1472	27.80	0.16	1650/1717	23.90	2.32	14.50
1355/1472	23.60	3.50	1650/1717	20.10	35.37	10.11
1355/1472	21.00	22.50	1650/1717	19.50	57.70	2.56

a. The 3′/5′ ratio is the ratio of the quantities of each Primer Set. For example, in Sample 1 the ratio is equal to 30.12; this is derived from 2.56/0.085.

Explanation of results

The 3′/5′ ratio evaluates the abundance of the average β-actin cDNA from the 3′ end (primer 1650-1717) compared to the abundance of a relatively 5′ sequence (primer 1355-1472) using the quantified PCR yields of each amplicon. If most of the cDNA contains both the 3′ and 5′ sequence target, the ratio of the PCR product for 3′/5′ is close to 1. As the RNA from FFPE samples starts exhibiting some level of degradation, the 3′/5′ ratio tends to become greater than 1. Depending on the ratio, an estimation of the RNA quality can be made.

For studies in which a known set of genes is being evaluated, ratios that are in a higher range (20 to 40) can be tolerated. In cases where the FFPE samples are being used to discover gene sets, to maximize the success and enable discovery of the maximum number of genes, samples with lower ratios (≤20) are optimal.

⚠ *Note: MDS Analytical Technologies does not have a strict cut-off for ratios from which no data will be obtained. As individual studies will have different tolerance levels, MDS Analytical Technologies recommends running a few samples with a variety of ratios to determine where the cut-off should be for a specific study.*

It is also important to remember that in addition to the ratio, the overall quantity reported for the 3′ Primer Set can be helpful in determining the quality of the sample. If a ratio of <10 is reported, but the quantity for that sample is <5 pg, there is likely not enough RNA in the sample to produce a quality result in a subsequent assay. The quantity reported can serve as a guide for determining how much of the original sample should be used to meet the input requirements for further sample processing.

A.3 TROUBLESHOOTING

A.3.1. STAINING

Staining

Symptom	Cause	Suggestion
Targeted cells do not lift from the slide during LCM	The sample may contain residual water.	Ensure that the ethanol solutions are fresh. Ethanol is hygroscopic. Keep the ethanol bottles tightly capped, and do not pour ethanol solutions until you are ready to use them. If you suspect that the 100% ethanol solution has absorbed water, discard and
	The sample may have dried in between protocol steps.	Carry out the Staining and Dehydration segment of the protocol at a steady pace.
RNA cannot be recovered from the sample	The sample starting material may contain poor quality RNA.	Run a quality control assesmnet (detailed in this protocol) on the tissue block to ensure it contains useable quality RNA.
	RNA may become degraded during RNA isolation.	Wear gloves; use RNase-free technique and RNase-free instruments and reagents.
	RNA may not be fully extracted and isolated from cells on the LCM cap.	Perform RNA extraction immediately after LCM to ensure complete extraction and optimum recovery of RNA.
	Amount of starting material may be insufficient.	Capture more cells. Amount of RNA in each cell may vary depending on cell type, RNA quality and length of fixation. For troubleshooting purposes, try starting with ~40,000 cells.

A.3.2. EXTRACTION AND ISOLATION

Extraction and Isolation

Symptom	Cause	Suggestion
Isolated RNA is of Poor Quality	Source tissue is of compromised quality	Verify quality of source tissue of LCM cells. The greatest factor affecting the quality of isolated RNA is the integrity of the RNA in the original tissue sample. RNA degradation due to RNase activity occurs rapidly, especially upon tissue removal such as
	RNA degridation during staining process	Use the Paradise Plus Reagent System Staining components to prepare slides for LCM. Specialized staining protocols and reagents are required for optimal RNA preservation in LCM samples. MDS Analytical Technologies has developed and validated the Paradise
	RNA degridation during LCM	Perform LCM immediately after preparing LCM slides. LCM sample slides are dehydrated in the final step of preparation so RNase activity is minimized. However, the risk of moisture and RNases entering the sample following preparation increases with the amo
	RNA quality compromised during slide storage	Use FFPE sections that are within 2 weeks of cutting. Extended storage of sections after being cut from blocks may result in RNA degradation.
RNA Yield is Low	RNA integrity has been compromised.	Verify quality of initial tissue sample or LCM slide (see A.1). Poor quality RNA may not bind effectively to the purification column membrane, decreasing overall RNA yield.

A.3.3. AMPLIFICATION

Amplification

Symptom	Cause	Suggestion
Amplification yield is Poor	Starting RNA sample quality varies	If you observe low yields with different RNA samples, run an amplification control using the Control RNA provided in the Paradise Plus Kit to verify kit functionality.
	Starting RNA sample quality has been compromised.	The greatest factor affecting amplification efficiency is the integrity of the RNA used in the Paradise Plus amplification process. Suspend RNA in nuclease-free water prior to amplification. Avoid using organic solvents such as phenol in RNA isolation pro
	There is no RNA in the input sample.	Run a control RNA sample with a known quantity of RNA to ensure that amplification is successful.
	Reagent concentrations in reaction mixtures are incorrect due to inadequate thawing or mixing.	Ensure all reagents are completely thawed, mixed, and all solids dissolved prior to use.
	Reagent concentrations in the reaction mixtures are incorrect due to inadequate reaction volume collection in the reaction tube.	Thoroughly thaw and mix all reagents prior to dispensing. Ensure all reagents are dispensed at proper volumes. Briefly spin down the reaction mix prior to incubation to ensure all reagents are collected in the reaction volume and the reaction mix has the
	Reagent concentrations in reaction mixtures are incorrect due to evaporative condensation onto the wall of the reaction tube during incubation.	Briefly spin down the sample following incubation steps to maintain proper volumes and concentrations of reagents and ensure that all nucleic acid templates are mixed with reaction components. Use a thermal cycler with a heated lid.
	Incubation temperatures are incorrect.	Verify the accuracy of all incubation temperatures. If you are using a thermal cycler, make sure that the programmed temperatures read correctly and the instrument has been calibrated to establish and maintain accurate temperature settings.
	RNA yield is diminished during column purification.	Verify centrifugal force used during nucleic acid purification. Improper binding, washing, and elution centrifugal forces can decrease the recovery of nucleic acid from the purification column. Microcentrifuges should be calibrated to deliver the correct
	Message content is low within the total RNA being used in your study.	Check amplification efficiency using control RNA. Use higher RNA inputs to compensate for lower message content.
Low Molecular Weight Product Appears on a Gel	Occasionally, a predominant band below the expected aRNA smear will appear on a gel. This band will lead to	
	Quality of the starting RNA is inadequate.	Poor RNA quality can lead to the formation of the reaction artifact, visible as a low molecular weight band. Check the quality of your input RNA. One approach is to utilize the Agilent Lab-on-a-Chip System with an RNA LabChip® Kit. For additional recommen
	Concentrations of Primer 1, Primer 2, Primer 3, or 1st Strand Nuclease Mix are incorrect due to inadequate thawing or dispensing.	Thaw and thoroughly mix each reagent vial prior to dispensing. If incompletely thawed and mixed, the concentrations of these reagents may not be dispensed at optimal concentrations for the reaction. Ensure that all pipettes are properly calibrated to disp
	Concentrations of Primer 1, Primer 2, Primer 3, or 1st Strand Nuclease Mix are incorrect due to inadequate mixing or reaction volume collection inside the reaction tube.	Thoroughly mix and spin down the sample after adding the primers or 1st Strand Nuclease Mix into the reaction mix and prior to incubation. This ensures the correct concentration of primers or nuclease in each respective reaction mix.
	Input RNA was not isolated using the PicoPure RNA Isolation Kit and no nucleic acid carrier was added.	Low molecular weight material may result from lack of RNA and carrier. Using the PicoPure RNA Isolation Kit is recommended to prepare samples that contain carrier.

A.3.4. QUALITY ASSESSMENT PROTOCOL

Quality Assesment Protocol

Symptom	Cause	Suggestion
Tissue homogenate is viscous and difficult to pipet, resulting in low RNA yield	There was insufficient disruption or lysis of cells.	Limit the extraction to 1 mm2 of tissue scrape per 1 µL of PK solution:
		• Use a minimum of 25 mm2 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.
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 CT 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.4. AMINO-ALLYL aRNA LABELING

This protocol is intended for use with amino-allyl modified aRNA which was generated using the optional Amino-Allyl IVT components of RA7010 and RA7012.

Table A.14: Amino-allyl aRNA Labeling Purification - RA7012

Component	Vial Color	Vial Label
RNA Binding Buffer	Blue	RB
RNA Wash Buffer	Blue	RW
RNA Elution Buffer	Blue	RE
0.5 mL Microcentrifuge Tubes		
Purification columns		

Table A.15: Fluorescent dyes (not supplied with the kit)

Reagent	Maker	Catalog #
Cy3 mono reactive dye	Amersham	PA23001
Cy5 mono reactive dye	Amersham	PA25001
Alexa Fluor 647 reactive dye decapacks for microarrays	Molecular Probes	A-32756
Alexa Fluor555 reactive dye decapacks for microarrays	Molecular Probes	A-32757

Protocol

Labeling Reaction:

Re-suspend 1mg monoreactive dye in 51 µL of **DMSO**. Save unused vials in the dark at 2–6ºC.

Figure A.3: DMSO

1. Take 15 µg of amino-allyl aRNA in 7.5 µL of nuclease free water.
   a. Sample should be maintained on a cold block.
2. Add 2.5 µL of Labeling Buffer (**LB**) to the sample.

Figure A.4: Labeling Buffer

3. Add 10 µL of the re-suspended dye into 10 µL of the sample.
4. Mix thoroughly by flicking the tube. Spin down briefly.
5. Incubate at room temperature in the dark for 1 hour.
6. Proceed directly to purification of labeled aRNA.

aRNA Purification:

1. Pre-treat column by adding 250 µL of RNA Binding Buffer (**RB**) to a new purification column. Incubate the column at room temperature for 5 minutes. Centrifuge at 16,000 *x g* for one minute.
2. Add 225 µL of **RB** to the transcript labeling reaction sample and mix thoroughly. Pipette the entire sample volume into the purification column.

Figure A.5: RNA Binding Buffer

3. Centrifuge at 100 *x g* (or lowest speed setting available) for 2 minutes, immediately followed by a centrifugation at 10,000 *x g* for 1 minute.

⚠ *Do not use re-suspended dye that is over 2 days old. DMSO is hygroscopic. Store tightly capped*.

⚠ *To obtain 15 µg of aRNA in 7.5µL, you may dry down 15µg of aRNA and re-suspend in 7.5 µL of nuclease free water, or concentrate the aRNA to 2 µg /µL and use 7.5 µL of the sample*.

⚠ *Do not allow the samples to incubate longer than 1 hour. Use reagents supplied in the Labeling Purification Reagents box*.

4. Discard flow-through. Place the column into the same collection tube.
5. Add 250 µL of RNA Wash Buffer (**RW**) to the purification column and centrifuge at 10,000 *x g* for 1 minute.

Figure A.6: RNA Wash Buffer

6. Repeat Step 5.
7. Add 250 µL of fresh **RW** to the column and centrifuge at 16,000 *x g* (full speed) for 2 minutes. Check the purification column for any residual wash buffer. If any wash buffer remains, re-centrifuge at 16,000 *x g* for one minute.
8. Discard the collection tube and flow-through.
9. Place the purification column into a new 0.5 mL microcentrifuge tube provided in the kit and carefully add 50 µL of RNA Elution Buffer (**RE**) directly onto the center of the purification column membrane. Gently touch the tip of the pipette to the surface of the membrane while dispensing **RE** to ensure maximum absorption of **RE** into the membrane. Gently tap the purification column to distribute the buffer if necessary.

Figure A.7: RNA Elution Buffer

10. Incubate at room temperature for one minute.
11. Place the assembly into the 2 mL support tube in the rotor with the 0.5 mL tube cap trailing the tube.

12. Centrifuge at 1000 *x g* for one minute, immediately followed by 16,000 *x g* for one minute. Discard the purification column and retain the elution containing the labeled aRNA.
13. Measure the O.D. of the product at A_{260}, A_{280}, and A_{550}/A_{650} to determine the yield and frequency of incorporation (FOI) by making a dilution of 1:10 (5 µL sample + 45 µL nuclease free water).
14. Store any remaining samples at –70ºC until ready for hybridization.

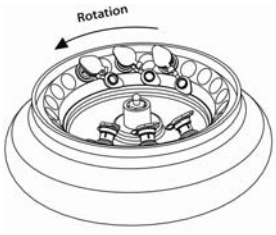


Figure A.8: Centrifuge.

A.5. RNA QUANTITATION USING SPECTRAMAX® MICROPLATE READERS WITH ABSORBANCE MODE AND PATHCHECK™ SENSOR

Introduction

This protocol describes how to measure RNA concentration using a 96-well, UV-clear microplate (e.g., Corning P/N 3635) and SpectraMax® microplate reader featuring the PathCheck™ sensor. The PathCheck sensor measures the pathlength in each well of the microplate and normalizes absorbance values to a 1 cm pathlength so that they are the same as values obtained in a 1 cm cuvette. Please note that some SpectraMax microplate readers are equipped with a cuvette port. See the user guide for your instrument for details on use of the cuvette port.

Method

Dilute RNA in RNase-free water. A total of 200 µL/well of diluted RNA will be used for quantitation, and diluting the sample more than 1:100 is not recommended. Prepare additional diluted sample if multiple wells are desired for analysis. Reading samples in triplicate is recommended. Pipette samples into a 96-well UV-transparent microplate; include a set of wells containing 200 µL of RNase-free water as blanks. Assuming that all well volumes are identical, and that all samples are blanked with the same solution (water), use the following method to subtract background OD due to the microplate itself:

1. In the template editor, select three or more wells in the microplate to contain the blanking solution (typically water). Assign the appropriate wells as 'Blank'. Designate the appropriate wells as 'Samples', and enter the dilution factor.
2. In the instrument settings dialogue box, make sure that the box for PathCheck is checked, the Water Constant button is on, and the box for Plate Background Constant is not checked.
3. Pipette blanks and RNA samples into the appropriate wells of the microplate.
4. Read the plate.

When the plate is read as indicated, SoftMax Pro automatically applies PathCheck to all samples and blanks and also subtracts the average of the blanks from each well of the microplate. Provided that all sample and blank path lengths are identical, potential error from applying path length normalization to $OD_{microplate}$ is cancelled out.

Table A.16: SpectraMax microplate reader settings for RNA quantitation. Other instrument settings not listed here should be left on the default values.

Read Mode	Absorbance
Wavelengths	Lm1: 260 nm Lm2: 280 nm
PathCheck	'PathCheck' box is checked 'Water Constant' button is checked 'Plate background constants' box is not checked
Assay Plate Type	96 Well Standard clrbtm
Wells To Read (or Strips)	Highlight wells to be read

A.6. aRNA YIELD AND PURITY DETERMINATION

aRNA quantitation by ultraviolet light absorbance is the simplest approach to determining amplification yield. An absorbance reading at 260 nm (A_{260}) using a spectrophotometer is taken on a diluted aliquot of aRNA. Typically, a 1:25 to 1:50 dilution of aRNA in nuclease free water is sufficient.

For single-stranded RNA, a measurement of A_{260} = 1.0 corresponds to 40 µg/mL. The yield can by calculated by:

$$(A_{260}) \text{ (dilution factor) } (40) = \mu g/mL \text{ RNA}$$

Measuring A_{280} and calculating the A_{260}/A_{280} ratio indicates the purity of the RNA sample. An $A_{260/}$ A_{280} ratio between 2.0 – 2.6 indicates very pure aRNA.

A.7. ASSESSMENT OF RNA QUALITY USING THE AGILENT BIOANALYZER

The Agilent Lab-on-a-Chip system provides a fast and effective approach to assessing the integrity of an aRNA sample. The system requires very small quantity of sample. Refer to the Agilent 2100 bioanalyzer and RNA LabChip™ Kit Instruction Manuals for details.

Equipment and Materials Required

Agilent 2100 bioanalyzer System (Agilent) RNA 6000 Nano Assay Kit (Agilent) Ice or cold block (4-8°C) Spectrophotometer Before you begin, refer to the instruction manual for the RNA 6000 Nano Assay Kit. Prepare necessary reagents and supplies as required by the kit.
Use RNase-free technique. Wipe all surfaces and equipment with RNase decontamination solution, use RNase-free solutions and plastic ware, and wear disposable gloves.

Protocol

1. Determine the concentration of the aRNA generated through Paradise® Plus by UV spectrophotometry.
2. Based on the optical density reading, prepare a dilution of the sample to a concentration of 200 – 300 ng/µL.
3. Store the sample on ice or in a cold block until ready to load on to the RNA chip.
4. Follow the RNA 6000 Nano Assay Kit protocol, loading 1 µL of the prepared sample dilution (from step 2).

For details of data interpretation refer to the bioanalyzer instruction manual. The aRNA appears on the bioanalyzer as a single, broad peak. The size of the aRNA ranges in length from 200 to 2000 bases.

A.8. ANALYSIS OF aRNA BY AGAROSE GEL ELECTROPHORESIS

Analysis of aRNA using agarose gel electrophoresis is one method to visualize the RNA profile and relative quantity after amplification. Standard protocols for agarose gel electrophoresis can be used. The following is a suggested protocol using commercially available reagents.

Materials

- → 1.25% Agarose Portrait Gel or 1.25 Agarose Medium Gel
- → (EmbiTec cat. # GE-6010 or GE-6030)
- → 10X RNA MOPS Running Buffer
- → (EmbiTec cat. # EC-1020) 2X Gel Loading Buffer (various) RNA Ladder

  (various) SYBR® Gold Nucleic Acid Gel Stain (Molecular Probes cat. # S-11494) or Ethidium Bromide Stain

  Nuclease-free Water

Protocol:

1. Determine the concentration of the aRNA by UV absorbance with a spectrophotometer. (Refer to Appendix A.)
2. Dilute the aRNA sample(s) with nuclease-free water. Each gel well can be loaded with 1 – 3 µg of aRNA.
3. Prepare aRNA gel sample by mixing 6 µL of diluted aRNA with 6 µL of 2X Gel Loading Buffer.
4. Incubate for 3 – 5 minutes at 65°C. Cool on ice.
5. Prepare 1X RNA MOPS Running Buffer and fill gel electrophoresis unit. Place agarose gel into the unit.
6. Load 12 µL of sample per well of the agarose gel. Include RNA Ladder in one or more lanes.
7. Electrophorese at 5 – 7 volts per centimeter for 30 minutes.
8. Stain the gel with SYBR® Gold Nucleic Acid Gel Stain for 30 minutes or according to the protocol supplied with the reagent. Alternatively, stain with Ethidium Bromide (0.5 – 1.0 µg/mL).
9. Visualize the gel on a UV transilluminator. The size of the aRNA ranges from 200 to 2000 bases in length.

A.9. GENERATION OF LABELED aRNA USING ALTERNATIVE IVT KITS

The Paradise® Plus Kits can be used with alternative IVT labeling (such as Affymetrix labeling kit 900449) to yield suitable RNA sample for hybridizing to GeneChip Probe Arrays as described below. These kit reagents and protocol are substituted during the second IVT reaction of the Paradise® Plus Kit protocol. Labeled aRNA is subsequently purified with the Paradise® Plus Kit and MiraCol Purification Columns as described below.

1. Perform Round One of amplification according to the Paradise® Plus Amplification Kit protocol starting from the recommended input for the kit. It is not recommended to use the minimum input amounts when using an alternative labeling kit due to IVT efficiency.
2. Perform Round Two of amplification through cDNA Purification according to the Kit protocol. (Stop at the end of Chapter 5, Section 3, Step H – "Round Two: cDNA Purification)
3. Perform RNA transcript labeling according to the protocol of the IVT labeling kit using the sample (from step #2 above) as the cDNA template. Adjust the final volume of the cDNA sample, as necessary.

Antisense RNA Purification:

⚠ ***Note:*** *Use the remaining components from RA7011 used during the amplification process*.

1. Add 250 µL of RNA Binding Buffer (**RB**) to a new purification column and incubate for five minutes at room temperature. Centrifuge at 16,000 x g for one minute.

Figure A.9: RNA Binding Buffer

2. Add 200 µL of **RB** to the Transcript Labeling Reaction sample and mix thoroughly. Pipette the entire sample volume into the purification column.
3. Centrifuge at 100 x g (or lowest speed setting available) for two minutes, immediately followed by a centrifugation at 10,000 x g for 1 minute.
4. Add 200 µL of RNA Wash Buffer (**RW**) to the purification column and centrifuge at 10,000 x g for one minute.

Figure A.10: RNA Wash Buffer

⚠ *RNA Binding Buffer (RB) must be at room temperature and thoroughly mixed before use. A precipitate may form during long-term storage. Dissolve precipitate by mixing. If necessary, warm the RB vial to re-dissolve*.

5. Add 200 µL of fresh **RW** to the column and centrifuge at 16,000 x g for two minutes. Check the purification column for any residual wash buffer. If any wash buffer remains, re-centrifuge at 16,000 x g for one minute.
6. Discard the collection tube and flow-through.
7. Place the purification column into a new 0.5 mL microcentrifuge tube provided in the kit and carefully add **30** µL of RNA Elution Buffer (**RE**) directly onto the center of the purification column membrane. Gently touch the tip of the pipette to the surface of the membrane while dispensing **RE** to ensure maximum absorption of **RE** into the membrane. Gently tap the purification column to distribute the buffer, if necessary.

Figure A.11: RNA Elution Buffer

8. Incubate at room temperature for one minute.
9. Place the assembly into the 2 mL support tube in the rotor with the 0.5 mL tube cap trailing the tube.
10. Centrifuge at 1,000 x g for one minute, immediately followed by 16,000 x g for one minute. Discard the purification column and retain the elution containing the labeled aRNA.
11. Measure the O.D. of the product at A_{260} and A_{280} to determine the yield of labeled aRNA. Perform electrophoretic analysis, if necessary.
12. Proceed to protocols for microarray hybridization.

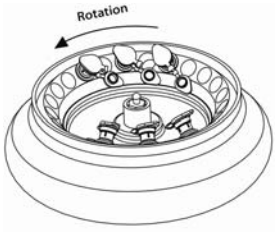


Figure A.8: Centrifuge.

A.10. CLEANING THE STAINING JARS

The staining jars can be reused, but must be cleaned. Rinse jars with 100% ethanol, followed by distilled water, then treat with RNase AWAY according to the manufacturer's protocol. Rinse jars thoroughly with nuclease-free water and allow to dry completely in the hood. Do not use reservoirs to store solutions.

A.11. CENTRIFUGE INFORMATION

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

Table A.17: Centrigual Forces (g)

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