Back to basics: Fundamental concepts and special considerations in RNA isolation

Speaker: Phoebe Loh – Global Product Manager, RNeasy
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3-part Webinar Series

RNA

Part I: Basics of RNA isolation – Methods, Sample Handling, and Challenging Samples

Part II: RNA Integrity and Quality – Standardize RNA Quality Control

DNA

Part III: Challenges of FFPE Sample Materials – Where Does Variation in Quantity of Purified DNA Come From?
What challenges do you face in your RNA preparation?
RNA Basics: Agenda

- RNA Universe - What is RNA, types of RNA and role in cells
- Handling of RNA - General Remarks
- Stabilization of RNA in sample
- RNA Isolation and special considerations
  - Step 1: Sample disruption and homogenization
  - Step 2: Tips & Tricks of isolating RNA from different samples
  - Step 3: gDNA removal for sensitive applications
- miRNA purification & exosome RNA purification
- QIAGEN solutions for quality RNA
- Question and Answers
Did you know?
Thirty scientists have received Nobel Prizes for research on RNA.

Name: Ribonucleic acid

Abbreviation: RNA

Destination: Throughout the cell (nucleus, nucleolus, cytoplasm, ribosome, mitochondria, chloroplasts, and endoplasmic reticulum) / in exosomes for communication between cells

Types:
• Messenger RNA (mRNA), transcribed from DNA, serves as a template for synthesis of proteins
• ribosomal RNA (rRNA) present in ribosomes for protein synthesis
• transfer RNA (tRNA) Amino acids delivery for protein synthesis are delivered to the ribosome
• miRNAs noncoding RNAs that mediate post-transcriptional gene regulation
• Long non-coding RNAs (IncRNA) ranging from 200nt to100kb in length. Function in diverse areas including epigenetics, alternative splicing, and nuclear import.

To get the complete picture
  ▪ Download our RNA Universe App
RNA distribution in cells

RNA content of a typical human cell

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total RNA per cell</td>
<td>&lt;1–30 pg</td>
</tr>
<tr>
<td>Proportion of total RNA in nucleus</td>
<td>~14%</td>
</tr>
<tr>
<td>DNA:RNA in nucleus</td>
<td>~2:1</td>
</tr>
<tr>
<td>mRNA molecules</td>
<td>$2 \times 10^5 – 1 \times 10^6$</td>
</tr>
<tr>
<td>Typical mRNA size</td>
<td>1900 nt</td>
</tr>
</tbody>
</table>

RNA distribution in a typical mammalian cell

<table>
<thead>
<tr>
<th>RNA species</th>
<th>Relative amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>rRNA (28S, 18S, 5S)</td>
<td>80–85%</td>
</tr>
<tr>
<td>tRNAs, snRNAs, low MW species</td>
<td>15–20%</td>
</tr>
<tr>
<td>mRNAs</td>
<td>1–5%</td>
</tr>
</tbody>
</table>

mRNA classification based on abundance

<table>
<thead>
<tr>
<th>Abundance</th>
<th>Copies/cell</th>
<th>No. of different messages/cell</th>
<th>Abundance of each message</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>5–15</td>
<td>11,000</td>
<td>&lt;0.004%</td>
</tr>
<tr>
<td>Intermediate</td>
<td>200–400</td>
<td>500</td>
<td>&lt;0.1%</td>
</tr>
<tr>
<td>High</td>
<td>12,000</td>
<td>&lt;10</td>
<td>3%</td>
</tr>
</tbody>
</table>
Generic Gene Expression Workflow

Instruments

Sample collection → Disruption → Purification → cDNA synthesis → Analysis

Kits
Sample to Insight

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Handling RNA – The key factors

Working quickly but carefully is the key

General handling
- Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible.

Disposable plasticware
- The use of sterile, disposable polypropylene tubes is recommended when working with RNA. These tubes are generally RNase-free and do not require pretreatment to inactivate RNases.

Non-disposable plasticware
- Non-disposable plasticware should be treated before use to ensure that it is RNase-free. Rinse thoroughly with 0.1 M NaOH, 1 mM EDTA followed by RNase-free water. Alternatively, chloroform-resistant plasticware can be rinsed with chloroform to inactivate RNases.
Glassware

- Glassware used for RNA work should be cleaned with a detergent, thoroughly rinsed, and oven baked at 240°C for at least 4 hours before use. Autoclaving alone will not fully inactivate many RNases.

- Alternatively, glassware can be treated with DEPC (diethyl pyrocarbonate). Fill glassware with 0.1% DEPC (0.1% in water), incubate overnight (12 hours) at 37°C, and then autoclave or heat to 100°C for 15 minutes to eliminate residual DEPC.

Electrophoresis tanks

- Electrophoresis tanks should be cleaned with detergent solution (e.g., 0.5% SDS), thoroughly rinsed with RNase-free water, and then rinsed with ethanol and allowed to dry.

- Important: Plastics used for some electrophoresis tanks are not resistant to ethanol. Take proper care and check the supplier’s instructions.

For complete tips: Download from RNA purification resource center
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Sample collection and stabilization

Changes in mRNA levels following sample harvesting
RNA later Reagent

Purification of RNA without degradation

Unstabilized

| 0 | 5 | 10 | 15 | 30 | 60 |

Stabilized

| 0 | 5 | 10 | 15 | 30 | 60 | min |

Northern blot: Expression of GAPDH
Immediate RNA stabilization at room temperature

Storage and transport of samples at ambient temperature

**RNAprotect Reagent**
- Cells
- Bacteria
- Saliva

**RNAlater RNA Stabilization Reagent**
- Freshly harvested tissue

<table>
<thead>
<tr>
<th>Sample</th>
<th>RNA stabilization</th>
<th>RNA stabilization and purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissues</td>
<td>RNAlater® RNA Stabilization Reagent</td>
<td>RNAeasy® Protect Kits</td>
</tr>
<tr>
<td></td>
<td>RNAlater TissueProtect Tubes</td>
<td></td>
</tr>
<tr>
<td>Mammalian cells</td>
<td>RNAprotect® Cell Reagent</td>
<td>RNAeasy Protect Cell Mini Kit</td>
</tr>
<tr>
<td>Human saliva</td>
<td>RNAprotect Saliva Reagent</td>
<td>RNAeasy Protect Saliva Mini Kit</td>
</tr>
<tr>
<td>Human blood</td>
<td>PAXgene™ Blood RNA Tubes</td>
<td>PAXgene Blood RNA Kit*</td>
</tr>
<tr>
<td>Bacteria</td>
<td>RNAprotect Bacteria Reagent</td>
<td>RNAeasy Protect Bacteria Kits</td>
</tr>
</tbody>
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- Sample collection
- Disruption
- Purification
- cDNA synthesis
- Analysis

Kits
Disruption and Homogenization: Two Distinct Steps

**Disruption:** Complete disruption of tissue structure, cell walls, and plasma membranes of cells is required to release all the RNA contained in the sample.
- Different samples require different methods to achieve complete disruption.
- Incomplete disruption results in significantly reduced yields.

**Homogenization:** Homogenization is necessary to reduce the viscosity of the cell lysates produced by disruption. Homogenization shears the high-molecular-weight genomic DNA and other high-molecular-weight cellular components to create a homogeneous lysate.
- Incomplete homogenization results in inefficient binding of RNA and therefore significantly reduced yields.

Efficient disruption and homogenization - an absolute requirement
Manual Disruption & Homogenization

**Classic Method**

Step 1: Liquid nitrogen to freeze the sample

Step 2: Mortar and pestle to grind the tissue to a powder
  - Approach works well but it is not consistent

Step 3: Powdered sample resuspended in a chaotropic lysis buffer
  - Genomic DNA is still high molecular weight and will add viscosity to the sample that can clog spin filters

Step 4: Shear with needle and syringe, improves efficiency of removing the genomic DNA from the columns
  - Care needed to prevent foaming but still be effective
Mechanical Disruption and Homogenization

Simultaneous Disruption and Homogenization

**TissueRuptor**
- 1 sample/run
- Disposable probes
- Rotor-stator

**TissueLyser LT**
- Up to 12 samples in parallel
- Bead mill

**TissueLyser II**
- Up to 48 or 192 samples in parallel
- Bead mill

Human/animal tissue
Plant tissue

Human/animal tissue
Plant tissue

Human/animal tissue
Plant tissue
Bacteria
Yeast

Human/animal tissue
Plant tissue
Bacteria
Yeast
Effective tissue disruption

Various rat tissues were disrupted using the TissueLyser LT or TissueLyser II

RNA was purified from 20 mg samples on the QIAcube using the RNeasy Fibrous Tissue Mini Kit (skin, heart, and lung) or RNeasy Lipid Tissue Mini Kit (brain).
Comparison of RNA yields with different homogenization methods

QIAshredder - fast and simple homogenization of cell lysates
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Special consideration: Difficult-to-Lyse Tissues

Some tissues require stronger lysis conditions

### Easy-to-Lyse
- **Cells**
- **Yeast**
- **Some tissues**
  - Liver
  - Spleen
  - Kidney

Lysis: Phenol-free lysis buffer

### Difficult-to-Lyse
- **Fatty Tissues**
  - Brain
  - Skin
  - Adipose tissues – e.g. breast

Lysis: Phenol/Guanidine Reagent

- **Fibrous Tissues**
  - Muscle
  - Heart
  - Trachea
  - Lung

Lysis: Proteinase K or Phenol/Guanidine Reagent
Special consideration: Fibrous Tissue

Isolating RNA from heart, muscle, and other fibrous tissue

- Contractile proteins, connective tissue, and collagen, which can all interfere with the isolation process.
- Sample needs to be treated with a protease or phenol containing lysis reagents.
- Proper conditions that do not degrade RNA, such as with an RNase-free proteinase K digest (RNeasy Fibrous Tissue Kit).
## Precipitation vs. Spin Columns

<table>
<thead>
<tr>
<th>Precipitation</th>
<th>Spin Columns</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cheap, no kits required, scalable</td>
<td>Higher material cost</td>
</tr>
<tr>
<td>More handling, longer incubation times</td>
<td>Fast, easy to use</td>
</tr>
<tr>
<td>Often several rounds of precipitation required for decent purity</td>
<td>High purity</td>
</tr>
<tr>
<td>Risk to lose RNA pellet, esp. with small samples</td>
<td>Special formats for different sample types and sizes, incl. very small samples</td>
</tr>
<tr>
<td>More room for error; inaccurate pipeting between phases can cause phenol carryover</td>
<td>Consistent, no phenol carry-over</td>
</tr>
</tbody>
</table>
Special consideration: FFPE Samples

Minimize the effects of FFPE storage on RNA transcripts

- Remove and fix tissue as quickly as possible
- Use tissue samples no more than 5 mm thick and do not over-fix (max. 24 hours)
- Use high-quality reagents for paraffin embedding, without additives
- Avoid sample staining, if possible
- Store FFPE samples appropriately

Note: RNA remains intact for up to 1 year when stored at 4°C
- Use an appropriate deparaffinization step
- Have a crosslink-reversal step during RNA isolation

Complete FFPE guide: Critical factors for molecular analysis of FFPE samples
miRNeasy FFPE Kit

Successful detection of both miRNAs as well as for the mRNA from the same eluate

- Rat liver tissue was formalin fixed for 24 hours or 60 hours, followed by purification of total RNA including miRNA.
- Purified RNA was used as a template in quantitative, real-time RT-PCR using the miScript PCR System.
Special consideration: RNA from Human Blood Samples

- Very small amounts of RNA
- RNA integrity - Presence of RNases
- Cellular RNA or exosomal RNA
- Contaminants must be removed - such as the anticoagulants heparin and EDTA, and naturally occurring enzyme inhibitors - can all interfere with downstream RNA analysis.

PaxGene webinar and exosomes webinar available
Special consideration: Plant Material

- Plant metabolites are difficult to remove

- Healthy young tissues recommended
  - RNA yields often higher since young tissue generally contains more cells and fewer metabolites than the same amount of older tissue

- Many “home-made” protocols RNA isolation recommend growing plants in darkness for 1 to 2 days before harvesting to prevent high levels of plant metabolite accumulation
Dedicated Kits could solve problem...
Special consideration: RNA from bacteria and virus

Bacterial RNA
- Bacterial mRNA has no 5’ cap and only rarely has a poly-A tail
- mRNA isolation by hybrid capture is impossible
- The RNeasy Protect Bacteria Kit is makes bacterial gene expression studies possible

Viral RNA
- When purifying viral RNA and DNA from plasma and serum, a major challenge is to concentrate the nucleic acids, as they may be extremely diluted in a large sample volume
- QIAamp Kits allow purification of viral nucleic acids from starting volumes as high as 5 ml

Amplification of RNA from plasma. RT-PCR products of a 1026 nt RNA fragment purified from plasma. Serial tenfold dilutions (as indicated) were added to plasma and purified using the QIAamp® Viral RNA Mini Kit. M: markers; C: negative control.
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Removal of genomic DNA contamination

Why is the gDNA removal important?

- Trace amounts of genomic DNA in an RNA sample can compromise the accuracy of sensitive applications such as real-time RT-PCR
- Both RNA and DNA targets may be amplified, leading to unreliable quantification of the intended RNA target
Removal of genomic DNA contamination

Eliminate genomic DNA contamination either during RNA purification or just prior to cDNA synthesis.

- DNase Digestion
  - on-column during isolation (DNA already bound to column)
  - after RNA isolation, prior to cDNA synthesis

- Non-enzymatic removal (columns or reagents)

- Primer Design to avoid coamplification of DNA targets

QIAGEN solutions:

- DNase digestion (with or without DNase Booster)
- gDNA Eliminator Column – RNeasy Plus Kits
- gDNA Eliminator Solution – RNeasy Plus Universal Kit

  - The gDNA eliminator solution is a novel non-enzymatic solution, which reduces gDNA contamination of the aqueous phase. It does not contain DNase.
Quick and Easy gDNA removal: RNeasy Plus

RNeasy Plus Procedure

Cells or tissue

Lyse and homogenize

Genomic DNA

Total RNA

gDNA Eliminator spin column:
Remove genomic DNA

Add ethanol

Total RNA

RNeasy spin column:
Bind total RNA

Wash

Eluted RNA

Elute

Total time: <25 minutes
gDNA removal for difficult-to-lyse tissue: RNeasy Plus Universal
The -RT curves demonstrate that RNA purified using the **RNeasy Plus Mini Kit** was virtually free of genomic DNA.

- Total RNA was purified from Jurkat cell samples
- (1 x 106 cells per sample)
- The **RNeasy Plus Mini Kit** is an RNA purification kit with integrated genomic DNA removal from Supplier AV.
- Duplicate real-time RT-PCR assays for β-actin were performed with (+RT) or without (-RT) reverse transcriptase.
Consistent RNA yields and effective gDNA removal

- Compared to the tested alternative suppliers, the RNeasy Plus Mini Kit provides the most reliable RNA purification.

- Isolates from PBMC had higher and more consistent yields of total RNA and elimination of gDNA contamination (Figure 1B) was approximately 10-fold better.

Figure 1. Consistently high yields and efficient gDNA removal with the RNeasy Plus Mini Kit. A Mean yield of RNA purified from $5 \times 10^6$ PBMC using the RNeasy Plus Mini Kit and solutions from two alternative suppliers. Yields were determined using the QIAxpert and exhibited decreased variability for the RNeasy Plus Mini Kit compared to Suppliers A and B. B A real-time PCR assay for HOXD9 was performed on the extracted RNA to determine the amount of residual gDNA. Contamination was significantly lower in the RNA purified with the RNeasy Plus Mini Kit.
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miRNA Isolation Technologies

miRNeasy - Efficient purification of miRNA from different starting materials

- Cells & Tissue - miRNeasy Mini
- FNB - miRNeasy Micro
- FFPE - miRNeasy FFPE
- Blood - miRNeasy Serum/Plasma
- Serum/Plasma - miRNeasy Serum/Plasma

Highly Pure RNA without Phenol Carryover
Exosome Isolation Technologies

Lower the risk of missing your biomarker!

exoRNeasy
Exosomal (Evs) RNA isolation
Serum / Plasma / CSF / Cell Culture
*Saliva has been tested by customer but we do not provide a protocol for this purpose.

exoEasy
Exosome (Evs) isolation
Serum / Plasma / Cell Culture

Special exosomes webinar available
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- **QIAGEN solutions for quality RNA**
- **Question and Answers**
# QIAGEN RNA Purification Kits

## Sample to Insight

Back to Basics: RNA Isolation

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Kit for RNA purification</th>
<th>Capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells†</td>
<td>RNeasy Mini, Midi, and Maxi Kits</td>
<td>Micro: (&lt;5 \times 10^5) cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mini: (10^{10}) cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Midi: (5 \times 10^6) – (1 \times 10^8) cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Maxi: (5 \times 10^7) – (5 \times 10^8) cells</td>
</tr>
<tr>
<td>Easy-to-lyse tissues [e.g., kidney, liver, spleen]†</td>
<td>RNeasy Mini, Midi, and Maxi Kits</td>
<td>Micro: (&lt;5) mg tissue</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mini: (0.5–30) mg tissue</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Midi: (20–250) mg tissue</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Maxi: (150) mg – (1) g tissue</td>
</tr>
<tr>
<td>Fiber rich tissues [e.g., heart, muscle, skin]†</td>
<td>RNeasy Fibrous Tissue Mini and Midi Kits</td>
<td>Mini: (0.5–30) mg tissue</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Midi: (20–250) mg tissue</td>
</tr>
<tr>
<td>All types of tissue†</td>
<td>RNeasy Plus Universal Mini and Midi Kits</td>
<td>Multi: Up to (50) mg tissue</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Midi: Up to (250) mg tissue</td>
</tr>
<tr>
<td>All types of tissue (for microarray applications)†</td>
<td>RNeasy Microarray Tissue Mini Kit</td>
<td>(10–100) mg tissue</td>
</tr>
<tr>
<td>FFPE tissue sections</td>
<td>RNeasy FFPE Kit</td>
<td>Up to (8 \times 10) μm sections, with surface area of up to (250) mm²</td>
</tr>
<tr>
<td>Microsamples [e.g., fine needle aspirates, laser-microdissected cryosections]†</td>
<td>RNeasy Plus Micro Kit</td>
<td>(&lt;5 \times 10^5) cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(&lt;5) mg tissue</td>
</tr>
<tr>
<td>Animal blood [stabilized]†</td>
<td>RNeasy Protect Animal Blood Kit</td>
<td>(&lt;100) μl or (&lt;500) μl blood collected in RNAProtect Animal Blood Tubes</td>
</tr>
<tr>
<td>Human blood [stabilized]†</td>
<td>PAXgene Blood RNA Kit</td>
<td>(2.5) ml blood collected in PAXgene Blood RNA Tubes</td>
</tr>
<tr>
<td>Human blood [fresh]</td>
<td>QIAamp RNA Blood Mini Kit</td>
<td>(&lt;1.5) ml blood</td>
</tr>
<tr>
<td>Plasma and serum</td>
<td>miRNeasy Serum/Plasma Kit</td>
<td>Up to (200) μl</td>
</tr>
<tr>
<td>Yeast</td>
<td>RNeasy Mini, Midi, and Maxi Kits</td>
<td>Mini: (&lt;5 \times 10^7) cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mini: (2 \times 10^7) – (5 \times 10^8) cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Maxi: (2.5 \times 10^8) – (2.5 \times 10^9) cells</td>
</tr>
<tr>
<td>Plants and fungi</td>
<td>RNeasy Plant Mini Kit</td>
<td>(10^7–10^8) cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(10–100) mg tissue</td>
</tr>
<tr>
<td>RNA cleanup and concentration</td>
<td>RNeasy MinElute® Cleanup Kit</td>
<td>(&lt;45) μg RNA</td>
</tr>
<tr>
<td>RNA cleanup</td>
<td>RNeasy Mini, Midi, and Maxi Kits</td>
<td>Mini: (&lt;100) μg RNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Midi: (&lt;1) mg RNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Maxi: (&lt;6) mg RNA</td>
</tr>
</tbody>
</table>
RNA Isolation Kit Selection Wheel

RNA Sample Selection Wheel

- Column capacity/tissue amounts (estimated actual size):
  - 25-35 mg: Mini column
  - 150-175 mg: Midi column
  - 500-700 mg: Maxi column

*Automatable on the Gilacube®; visit www.qiagen.com/Qiagen for details.

Sample to Insight

Back to Basics: RNA Isolation
Your tools for success in RNA purification

Your experiments require high-quality RNA. Ensure your success with great resources for precise sample collection and robust RNA purification.

Get knowledge and special tips for dealing with challenging sample sources and overcoming five common issues in RNA preparation. See the best lots for your RNA purification needs.

The QIAGEN Resource Center is here for you!

- Enter the microRNA World
  - Download the brochure
  - Solutions for miRNA research, from bioguises to miRNAse profiling.

- New RNA-focused WebApp
  - Check it!
  - Grab your space suit, it's time to explore the RNA Universe!

- Video troubleshooting guide
  - Watch now
  - Easy solutions to 5 common problems in RNA isolation

https://www.qiagen.com/qdm/rna/resources/
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Questions?

Contact QIAGEN
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+49-2103-29-12400 (EU)
Email: BRC.US@qiagen.com

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Qiawebinars@qiagen.com