RNA Isolation and cDNA Synthesis

Complete Protocol from Cells to cDNA

Overview

This combined protocol guides you through the complete process of isolating RNA from cultured cells using TRIzol Reagent and converting it to cDNA using the High-Capacity cDNA Reverse Transcription Kit. The complete procedure takes approximately 4 hours (1 hour for RNA isolation + 3 hours for cDNA synthesis).

> **SAFETY**
> TRIzol contains phenol and guanidine isothiocyanate. Always wear gloves, lab coat, and safety glasses. Work in a fume hood when possible. Consult Safety Data Sheet before use.

Sample Requirements

Sample Type	Amount per 1 mL TRIzol
Cells in monolayer	1×10^5 to 1×10^7 cells (3.5 cm dish)
Cells in suspension	$5\text{-}10\times10^6$ cells

Required Materials

Equipment

- Centrifuge capable of 12,000 × g at 4°C
- Water bath or heat block (55-60°C)
- Polypropylene microcentrifuge tubes (RNase-free)

- Micropipettes and RNase-free pipette tips
- Vortex mixer
- PCR tubes or 96-well PCR plate
- Thermal cycler
- NanoDrop or spectrophotometer

Reagents for RNA Isolation

- TRIzol Reagent (store at 15-30°C)
- Chloroform (molecular biology grade)
- Isopropanol
- 75% ethanol in RNase-free water
- RNase-free water
- RNase-free glycogen (optional, for small samples)

Reagents for cDNA Synthesis

- 10X RT Buffer
- 10X RT Random Primers
- 25X dNTP Mix (100 mM)
- MultiScribe™ Reverse Transcriptase (enzyme)
- RNase Inhibitor (optional)
- Nuclease-free water

All stored at -20°C

Part 1: RNA Isolation

Part 1A: Cell Lysis and Phase Separation

Mark Done

For Cells Grown in Monolayer:

1. Remove growth media from culture dish
2. Add 0.3-0.4 mL TRIzol Reagent per 1×10^{5}-10^{7} cells directly to the dish
3. Pipet lysate up and down several times to homogenize
4. Transfer lysate to microcentrifuge tube

For Cells Grown in Suspension:

1. Pellet cells by centrifugation and discard supernatant
2. Add 0.75 mL TRIzol per 0.25 mL cell pellet (5-10×10^6 cells)
3. Pipet lysate up and down several times to homogenize

NOTE: Do NOT wash cells before adding TRIzol to avoid mRNA degradation

Continue for All Samples:

4. Incubate lysate **5 minutes at room temperature**
   → This allows complete dissociation of nucleoprotein complexes
5. Add **0.2 mL chloroform** per 1 mL TRIzol used (1/5 ratio)
6. Cap tube securely and shake vigorously by hand for **15 seconds**
7. Incubate **2-3 minutes at room temperature**
8. Centrifuge **15 minutes at 12,000 × g at 4°C**
   → Mixture separates into 3 phases: upper aqueous (RNA), interphase, lower organic (DNA/proteins)
9. Carefully transfer the **upper aqueous phase** to a new tube

CRITICAL: Angle tube at 45° and pipet carefully. AVOID transferring any interphase or organic layer!

Part 1B: RNA Precipitation

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1. (Optional) For small samples (<10^6 cells): Add 5-10 µg RNase-free glycogen as carrier
2. Add **0.5 mL isopropanol** per 1 mL TRIzol used (1/2 ratio)
3. Mix gently by inverting tube
4. Incubate **10 minutes at room temperature**
5. Centrifuge **10 minutes at 12,000 × g at 4°C**
   → RNA precipitates as white gel-like pellet at bottom of tube
6. Carefully discard supernatant with micropipette
   → Pellet may be loose, pipet carefully!

Part 1C: Wash RNA Pellet

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1. Add **1 mL of 75% ethanol** per 1 mL TRIzol used
   → RNA can be stored in 75% ethanol for 1 year at -20°C
2. Vortex sample briefly to resuspend pellet
3. Centrifuge **5 minutes at 7,500 × g at 4°C**
4. Carefully discard supernatant with micropipette
5. Air dry RNA pellet for **5-10 minutes**
   → Do NOT over-dry! Pellet should still look slightly wet

> **IMPORTANT: Do NOT dry by vacuum centrifuge. Do NOT let pellet dry completely - this makes RNA hard to dissolve!**

Part 1D: Dissolve RNA

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1. Add **20-50 µL RNase-free water**
   → Volume depends on expected RNA amount
2. Pipet up and down gently to dissolve
3. Incubate in water bath at **55-60°C for 10-15 minutes**
4. Mix by pipetting and check that pellet is fully dissolved
5. Proceed to quantification

Part 1E: RNA Quantification

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Method 1: Absorbance (NanoDrop or Spectrophotometer)

1. Dilute sample in RNase-free water if needed
2. Measure absorbance at 260 nm and 280 nm
3. Calculate concentration: **A_{260} × dilution × 40 = µg RNA/mL**
4. Check A_{260}/A_{280} ratio: **~2.0 indicates pure RNA**

Method 2: Fluorescence (Qubit or Quant-iT)

Fluorescence methods selectively measure intact RNA and are more accurate than absorbance. Follow manufacturer's instructions for Qubit RNA assay.

Expected RNA Yields:

Cell Type	Quantity	Typical Yield
Epithelial cells	1×10^6 cells	8-15 µg

Cell Type	Quantity	Typical Yield
Fibroblasts	1×10^6 cells	5-7 µg

> **STOP POINT: RNA can be stored at -70°C until ready to proceed with cDNA synthesis. Alternatively, continue immediately to Part 2.**

Part 2: cDNA Synthesis

Part 2A: Preparation

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1. Plan Your Experiment

- Calculate how many reactions you need
- Include at least 1 extra reaction (10% overage) in your master mix calculations
- Example: If running 8 samples, make master mix for 9 reactions

2. Thaw Kit Components

- Remove kit components from -20°C freezer
- Thaw on ice (takes ~15-20 minutes)
- Once thawed, keep on ice at all times
- DO NOT thaw the enzyme at room temperature

3. Prepare Your RNA

- You can use up to 2 µg of total RNA per reaction
- Common amounts: 500 ng - 1 µg per reaction
- Make sure RNA is in nuclease-free water or TE buffer

Part 2B: Make the Master Mix (work on ice!)

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You'll be making a 2X Master Mix that contains all the enzymes and reagents needed for reverse transcription.

For ONE 20 µL reaction:

Component	Volume	What it does
10X RT Buffer	2.0 µL	Provides optimal pH and salts
25X dNTP Mix	0.8 µL	Building blocks for cDNA
10X RT Random Primers	2.0 µL	Initiates cDNA synthesis
MultiScribe™ RT Enzyme	1.0 µL	The enzyme that makes cDNA
Nuclease-free H_2O	4.2 µL	Brings to correct volume
Total Master Mix	**10.0 µL**	
+ Your RNA sample	10.0 µL	
= Final volume	**20.0 µL**	

Master Mix Calculation Example (8 samples + 1 extra = 9 reactions):

Component	1 reaction	× 9 reactions
10X RT Buffer	2.0 µL	18.0 µL
25X dNTP Mix	0.8 µL	7.2 µL
10X RT Random Primers	2.0 µL	18.0 µL
MultiScribe™ RT Enzyme	1.0 µL	9.0 µL
Nuclease-free H_2O	4.2 µL	37.8 µL
TOTAL	**10.0 µL**	**90.0 µL**

> **Step-by-Step Master Mix Preparation:**
>
> 1. Get a clean tube for your master mix (1.5 mL microcentrifuge tube works well)
> 2. Label it: "RT Master Mix, [Date], [Your initials]"
> 3. Add components IN ORDER (on ice):
>   - First: Water (largest volume)
>   - Then: Buffer, dNTPs, Primers
>   - **LAST: Add enzyme** (it's the most sensitive!)
> 4. Mix gently:

- Close tube cap
- Flick tube gently with finger 5-6 times
- Quick spin in microcentrifuge (3 seconds)

5. Keep on ice until use

Part 2C: Set Up Your Reactions

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1. **Label your PCR tubes or plate:**
   - Write sample names clearly
   - Include date
2. **Add Master Mix first:**
   - Pipette **10 µL of master mix** into each tube/well
   - Use a new tip each time to avoid contamination
3. **Add your RNA samples:**
   - Pipette **10 µL of RNA sample** into corresponding tube
   - Pipette up and down 2 times to mix
   - Use a new tip for each sample
4. **Seal tubes/plate:**
   - Cap tubes tightly OR
   - Seal plate with adhesive film
5. **Quick spin:**
   - Spin in microcentrifuge for 3-5 seconds
   - This removes air bubbles and brings liquid to bottom
6. Keep on ice until you're ready to load thermal cycler

Part 2D: Run the Thermal Cycler

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Program Settings:

Step	Temperature	Time	What's happening
1	25°C	10 min	Primers bind to RNA
2	37°C	120 min	Enzyme makes cDNA
3	85°C	5 min	Stops the reaction
4	4°C	Hold	Keeps samples cool

To run:

1. Turn on thermal cycler
2. Select or program the above settings
3. Set reaction volume: 20 µL
4. Load your samples
5. Close lid and START

The run takes about 2 hours and 15 minutes. You can leave while it runs, but don't forget about your samples!

Part 2E: After the Run - cDNA Storage

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Option 1: Use immediately

- Proceed directly to qPCR setup
- Keep cDNA on ice while setting up

Option 2: Short-term storage

- Store at 4°C (regular fridge)
- Good for up to 1 week

Option 3: Long-term storage

- Store at -20°C (freezer)
- Good for several months
- Make aliquots to avoid freeze-thaw cycles

Important Storage Tips:

- Label tubes with: "cDNA, sample name, date, your initials"
- Record in lab notebook or spreadsheet
- Avoid repeated freeze-thaw cycles (breaks down cDNA)
- Consider making duplicate aliquots for backup

Troubleshooting

RNA Isolation Issues

Problem	Possible Cause	Solution
Low RNA yield	Incomplete homogenization Pellet not fully dissolved	Use less starting material Pipet thoroughly Heat sample to 55-60°C
Degraded RNA	RNase contamination Sample not processed quickly	Use RNase-free materials Process samples immediately Clean work area
Low A260/A280 ratio (<1.8)	Phenol contamination Insufficient washing	Avoid transferring organic phase Add extra ethanol wash step
RNA won't dissolve	Pellet too dry	Heat to 55-60°C longer Add more water Pipet repeatedly

cDNA Synthesis Issues

Problem	Possible Cause	Solution
No amplification in qPCR	RNA was degraded Not enough RNA input RT enzyme was dead RNase contamination	Check RNA quality (260/280 ~2.0) Keep everything cold Add enzyme to master mix Use RNase-free materials
Master mix calculations confusing	Math errors	Write down number of samples Add 1 extra Multiply each volume Double-check with supervisor

Problem	Possible Cause	Solution
Forgot if component added	Uncertainty during pipetting	Make fresh master mix Work systematically Check off components as added

Quick Reference

RNA Isolation Key Ratios

Chloroform: 0.2 mL per 1 mL TRIzol

Isopropanol: 0.5 mL per 1 mL TRIzol

75% Ethanol: 1 mL per 1 mL TRIzol

cDNA Master Mix (per reaction)

2.0 µL → 10X RT Buffer
0.8 µL → 25X dNTP Mix
2.0 µL → 10X RT Random Primers
1.0 µL → MultiScribe RT Enzyme
4.2 µL → Nuclease-free Water
10 µL Total Master Mix + 10 µL RNA = 20 µL Final

Thermal Cycler Program

1. 25°C for 10 min
2. 37°C for 120 min
3. 85°C for 5 min
4. 4°C hold

Important Reminders

✓ Work in RNase-free environment
✓ Keep everything on ice

✓ Add enzyme LAST to master mix
✓ Don't let RNA pellet dry completely
✓ Label everything clearly
✓ Avoid freeze-thaw cycles

Storage Conditions

Material	Temperature	Duration
Lysed samples in TRIzol	4°C / -20°C	Overnight / Up to 1 year
RNA in 75% ethanol	-20°C / 4°C	1 year / 1 week
Dissolved RNA	-70°C	Long-term
cDNA	4°C / -20°C	1 week / Several months

Protocol based on TRIzol Reagent User Guide (Thermo Fisher Scientific) and Applied Biosystems High-Capacity cDNA Reverse Transcription Kit

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