Sample Prep for Real-Time RT-PCR

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Agenda

Sample prep considerations
• Tissue handling: do’s and don’ts
• RNA isolation: what’s important
• My favorite RNA isolation method
• Genomic DNA contamination

RT considerations you’ve never considered
• Endogenous priming
• RT inhibition of PCR
• RT reaction times
Isolating RNA: Issues and Concerns

Performance

suitability for downstream applications

Yield

rapid tissue preservation

rapid and thorough sample disruption

Quality

intactness: rapid sample preservation and disruption

purity: removal of contaminants and genomic DNA

stability: complete inactivation of RNase
Isolating RNA: Stability?

Variable Stability of Prepared RNA
Steps in RNA Isolation

1. Obtain Sample
   • process immediately
   • preserve it

2. Disrupt sample and release RNA
   • use of physical force to break cells (e.g. polytron, grinding)
   • homogenization in chaotrope (GuSCN, LiCl) or detergent (SDS)

3. Extract and purify RNA
   • acid phenol/chloroform (RNAwiz, TRIzol, etc.)
   • glass filter (RNAqueous, RNeasy, etc.)
   • oligo (dT) chromatography (PolyAPurist, etc.)

4. Store RNA (0.1X TE, 0.1mM EDTA, or 1 mM Citrate pH6.4)
Step 1 in RNA Isolation: sample handling

Obtain tissue sample

- Fresh tissue: cannot pause until lysate obtained
  - or …
- Snap Freeze with Liquid N₂:
  - Not possible in many settings
- Cumbersome; must carry Dewar
- Grinding tissue is laborious and potentially hazardous
- Handling and transfer difficult (cross-contamination?)
- Processing large fragments is especially problematic
Step 1 in RNA Isolation: sample handling

Obtain tissue sample

Fresh tissue: can not pause until lysate obtained

(well, not exactly.....)

Mouse Liver 25°C, Processed at indicated time points
Step 1 in RNA Isolation: sample handling

Obtain tissue sample

Fresh tissue: cannot pause until lysate obtained

or ...

Snap Freeze with Liquid N$_2$:
Not possible in many settings

Cumbersome; must carry Dewar

Grinding tissue is laborious and potentially hazardous

Handling and transfer difficult (cross-contamination?)

Processing large fragments is especially problematic
Effects of tissue freeze/thaw on RNA quality

Agilent 2100 Bioanalyzer

EtBr Agarose  β-actin Northern (equal mass loading on gel)

thawed

frozen

95% intact 21% intact

Thaw? Yes No

Yes No

Fluorescence

Time (seconds)

0 100 200 300 400 500 600

19 24 29 34 39 44 49 54 59
**RNAlater™**
Tissue preservation and RNA Stabilization Solution

- Aqueous, non-toxic tissue preservation
- Standardizes tissue preservation & nucleic acid isolation
- Provides spatial and temporal separation of collection and processing with no penalties in quality or throughput
- Samples in RNAlater can be stored:
  - at 37°C for one day
  - at 25°C for 1-2 weeks: ship samples at ambient temperature
  - at 4°C for months
  - frozen indefinitely: archival storage of samples
Quality of RNA from RNAlater-treated Tissue

<table>
<thead>
<tr>
<th>Tissue stored at 37°C for 1 day</th>
<th>Tissue stored at RT for 1 week</th>
<th>Tissue stored at 4°C for 1 month</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse Liver RNA</td>
<td>Mouse Liver RNA</td>
<td>Mouse Liver RNA</td>
</tr>
<tr>
<td>Mouse Spleen RNA</td>
<td>Mouse Spleen RNA</td>
<td>Mouse Spleen RNA</td>
</tr>
<tr>
<td>Mouse Kidney RNA</td>
<td>Mouse Kidney RNA</td>
<td>Mouse Kidney RNA</td>
</tr>
</tbody>
</table>

28S

18S

p53 (2.5 kb)

GAPDH (1.4 kb)
RNA Isolation: Sample Handling Summary

Process fresh samples as quickly as possible...
(some tissues may be more stable than others!)

.. or preserve in RNAlater and treat as fresh.
Still must be processed quickly into RNAlater, but can then be handled safely.

.. or snap-freeze and process frozen.
Must never thaw: however some small samples can be directly homogenized with a polytron.
Step 2 in RNA Isolation: Sample Disruption

Choice of disruption method critical for yield and quality

Dear Dr. Lader:

I am working at Johns Hopkins School of Medicine. Now we want to isolated RNA from mouse brain cortex for microarray. I have some problems for that and need your troubleshooting and some suggestion. I isolated total RNA following TRIZOL protocol, homogenizing tissue with Sonicator. After redissoving the RNA pellet with DEPC water, RNA concentration was round 2ug/ul. O.D. A260/280 was round 2.0. But when running the RNA at formaldehyde agarose gel (Northernax 10x MOPS gel running buffer, and Northernax formaldehyde load dye were from ambion a half year ago), the smear bands or nothing showed on the gel. I don't know what was wrong during isolation. Before I had worked another lab, my RNA quality was very good for RT-PCR, Northern, and RPA. Only different thing was using Polytron for homogenizing.

Your kind and help are greatly appreciated.

Best regards,
Step 3 in RNA Isolation: RNA recovery

Total RNA Isolation Methods

• Rapid
  - one-step phenol-based - scalable
  - glass-binding - higher potential throughput

• Phenol-free
  - glass-binding

• Difficult tissues, specific challenges
  - multi-step phenol based
The Hybrid RNA Isolation Method
(for people who ❤ RNA isolation)

Requires: RNAqueous or equivalent
Acid Phenol:Chloroform

Protocol
• Disrupt sample in GiTC lysis buffer with Polytron
• Extract sample with 1:1 acid phenol:chloroform, spin and collect aqueous phase
• Continue with filter-based protocol

• High yields
• Low residual protein (260:280 of ~2.0)
• Vacuum manifold adaptable
• Scalable 1mg - 1g tissue !!!
• Handles difficult tissues well
• No filter clogging problems
g DNA Contamination

- Filter-based purification methods yield RNA that is typically 1-10% DNA (based on real-time data).

- There is no RNA isolation method that generates RNA completely free of DNA contamination. Therefore you must DNase I treat your RNA samples.

- A follow-up problem is how to get rid of the DNase I and divalent cations so they won’t be present during subsequent cDNA synthesis.
DNase I treatment of RNA
increasing DNase I and time

This sample starts out at 4% DNA

<table>
<thead>
<tr>
<th>DNase I/time</th>
<th>fold reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT+</td>
<td>-</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1X/30’</td>
<td>~100</td>
</tr>
<tr>
<td>1X/60’</td>
<td>~250</td>
</tr>
<tr>
<td>2X/30’</td>
<td>~250</td>
</tr>
<tr>
<td>4X/15’</td>
<td>~100</td>
</tr>
</tbody>
</table>

99.6% removal
(0.016% DNA)

G3PDH TaqMan assay

1X DNase = 0.02 units/ul
Divalent Cations and Heat Degrade RNA

- DNase I Buffer with 0.1 mM CaCl$_2$, 2.5 mM MgCl$_2$
- Heated 90°C, 5 minutes
- Run on formaldehyde agarose gel

- Don’t heat kill DNase I
- Unless you remove the divalents, don’t heat your RNA in the RT reaction
DNA-free™ DNase Treatment and Removal Reagents

- Optimized DNA digestion reagents
- Inactivates DNase without heating, phenol extraction or precipitation
- DNase Removal Reagent is added directly after DNase digestion: inactivates DNase and removes divalent cations (Ca++, Mg++)
Priming 1st strand synthesis

General:
Oligo dT, anchored dT (3’ bias, library construction, 3’ RACE)
Random Primers (non biased distribution)
Gene Specific Primers (more sensitivity?)

Specialized:
Allele specific primers
Functionalized primers (e.g. T7 Promoter + dT, aRNA)
Primers with restriction sites on the ends (cloning)
Efficiency of different 1st strand primers on standard RT reactions

- random decamers
- oligo dT
- G3PDH primers
- no primers !!

42°C MMLV RT, G3PDH Taqman PCR
Minimizing endogenous priming in RT Reactions

For standard qRT-PCR, random priming at 42°C is fine.
If there is any reason that you need specific priming:
  • a specific site (gene specific or allele specific priming)
  • the 3’ end (for 3’ RACE or other ‘anchored’ applications)
  • using a chimeric or bifunctional primer (T7, restriction site)

...a high stringency RT reaction may be critical.
RT inhibition of real-time PCR

Reverse transcriptase (1/10th the amount in a standard RT reaction) added directly into a 25 ul G3PDH PCR

- No RT
- AMV (2 U)
- MMLV (20 U)
- SSII (20 U)
RT inhibition of real-time PCR: can be relieved by heat-denaturation
RT inhibition of real-time PCR

Heat inactivation must be prior to addition of Taq

MMLV (20 U)

- No RT
- heated 10'
- heated 5'
- heated 10’w/Taq
- heated 5’ w/ Taq
- no heat
RT inhibition of real-time PCR: can be relieved by addition of carrier protein

- Control
- RNase inhibitor
- α crystallin
- DNAk
- BSA
- MMLV (20 U)
RT inhibition of real-time PCR

Take home message –

• 1 tube RT-PCR must use carrier protein (RNase inhibitor, αCry)

• 2 tube RT-PCR can either heat kill or use carrier protein
cDNA synthesis: yield vs. time 42°C

1 ug RNA, 200 units MMLV, random primed
β Actin TaqMan assay
cDNA synthesis: yield vs. time 25°C

<table>
<thead>
<tr>
<th>Reaction time (min)</th>
<th>Relative cDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7.5</td>
<td>10</td>
</tr>
<tr>
<td>15</td>
<td>27</td>
</tr>
<tr>
<td>30</td>
<td>31</td>
</tr>
<tr>
<td>60</td>
<td>54</td>
</tr>
<tr>
<td>120</td>
<td>70</td>
</tr>
<tr>
<td>240</td>
<td>100</td>
</tr>
</tbody>
</table>

25°C Time course, MMLV, β Actin TaqMan assay
cDNA synthesis: 25°C vs. 42°C