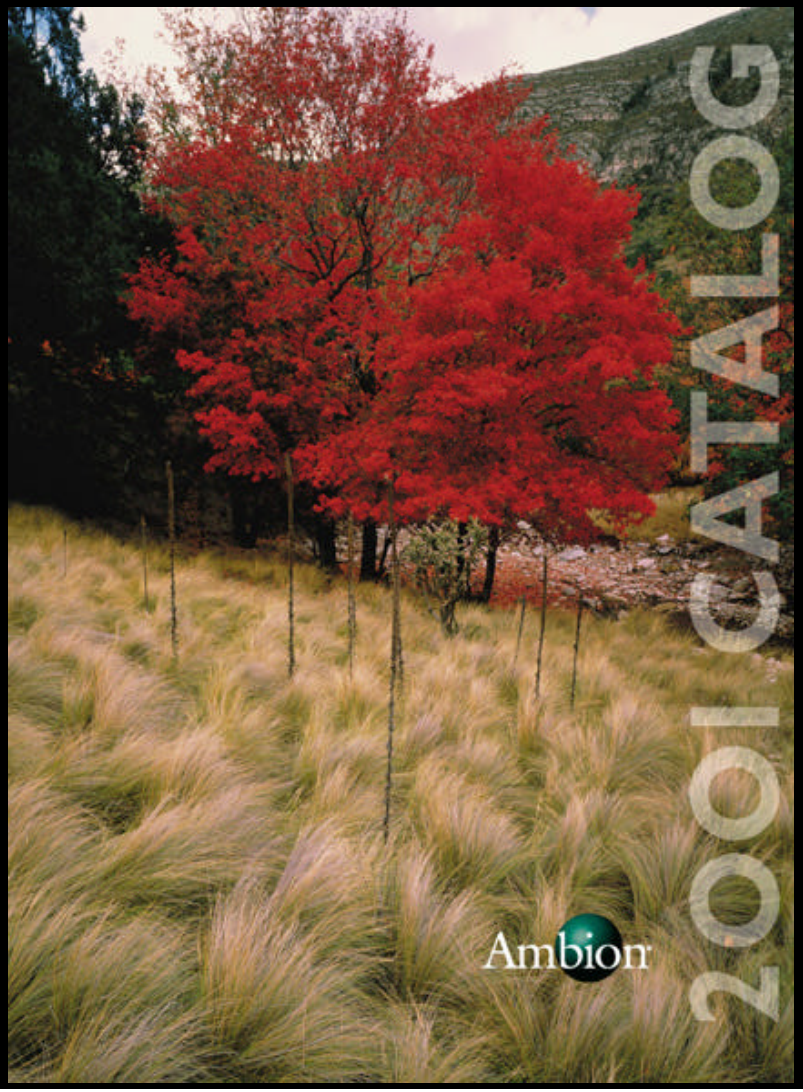
Sample Prep for Real-Time RT-PCR



Eric Lader, Ph.D
elader@ambion.com

Marketing Product Manager

Manager, Product Applications Group

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

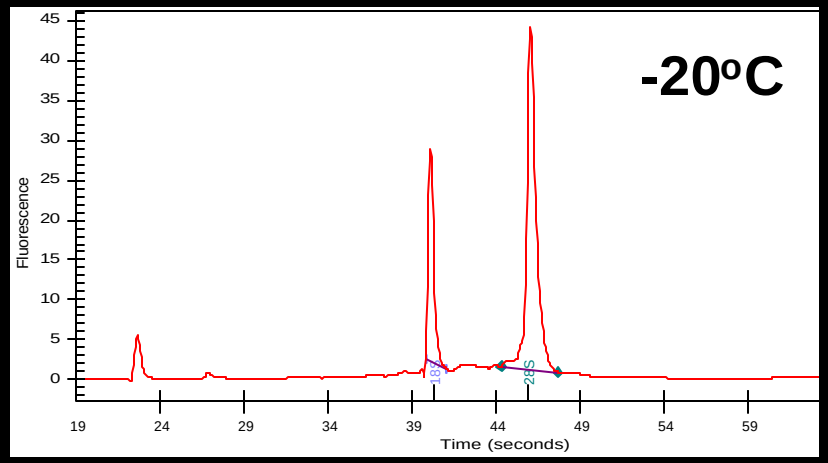
- rapid tissue preservation
- rapid and thorough sample disruption

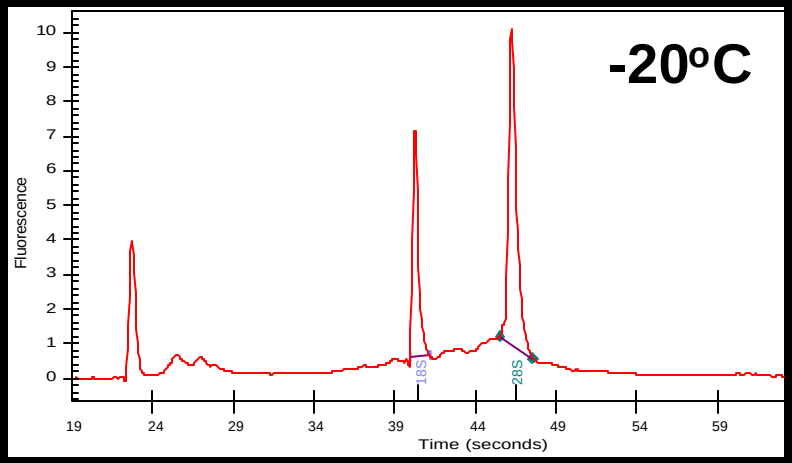
Quality

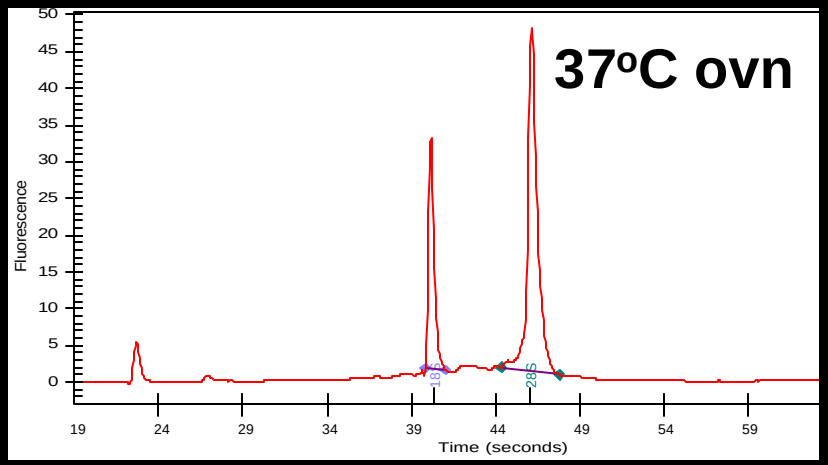
- intactness: rapid sample preservation and disruption
- purity: removal of contaminants and genomic DNA
- stability: complete inactivation of RNase

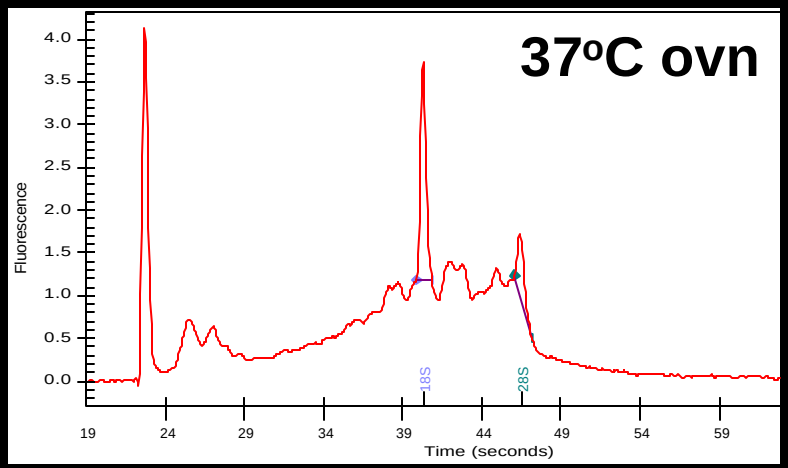
Isolating RNA: Stability?

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: can not pause until lysate obtained**

  or …

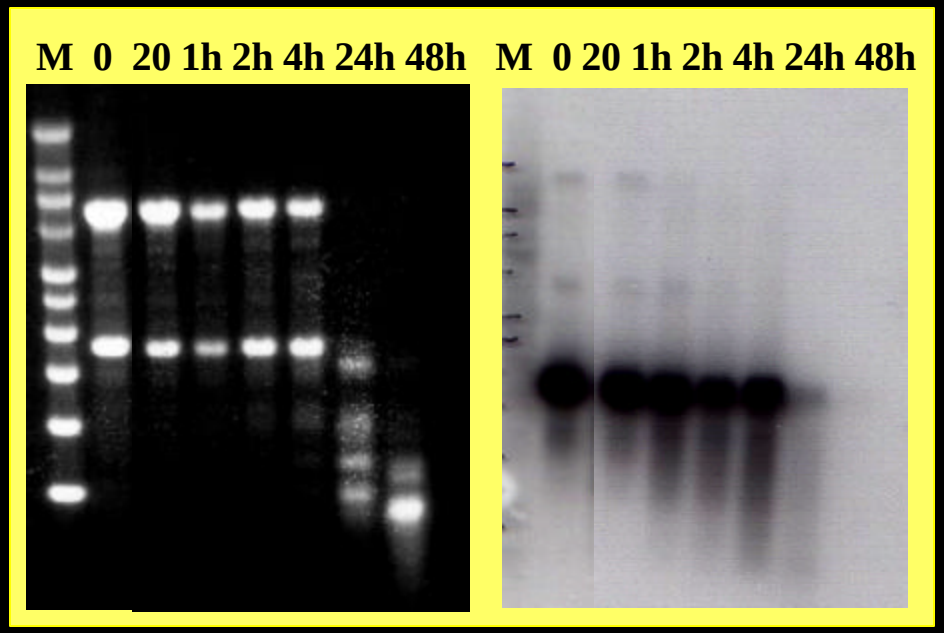
- **Snap Freeze with Liquid N_2:**
- **Not possible in many settings**
- **Cumbersome; must carry Dewar**
- **Grinding tissue is laborious and potentially hazardous**
- **Handling and transfer difficult (cross-contamination?)**
- **Processing large fragments is especially problematic**

Step 1 in RNA Isolation: sample handling

Obtain tissue sample

Fresh tissue: can not pause until lysate obtained

(well, not exactly…..)



bActin

Mouse Liver 25^{0}C, Processed at indicated time points

Step 1 in RNA Isolation: sample handling

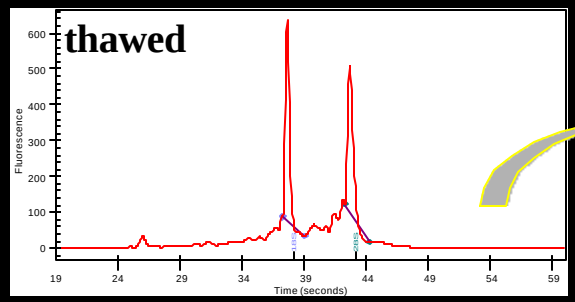
Obtain tissue sample

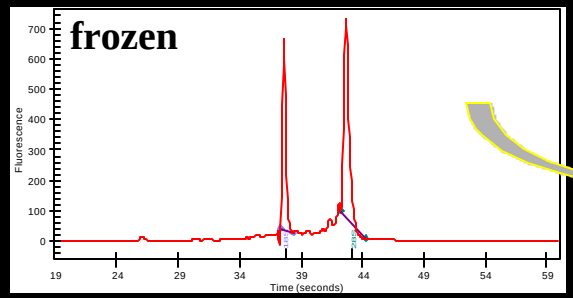
- **Fresh tissue: can not pause until lysate obtained**

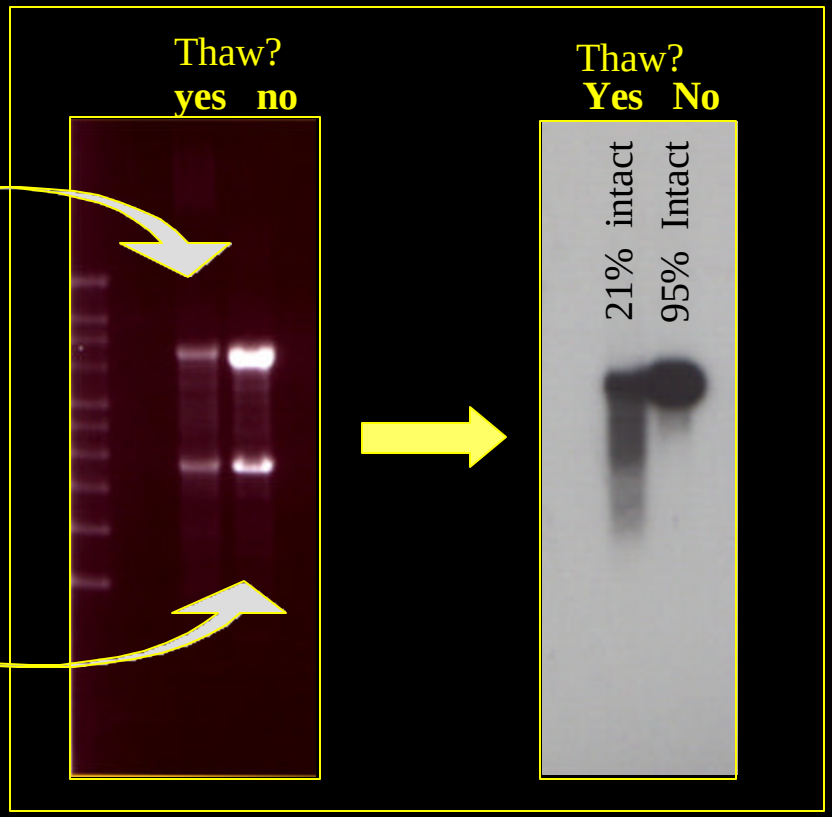
  or …

- **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)

RNA*later*™

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 RNA*later* can be stored:

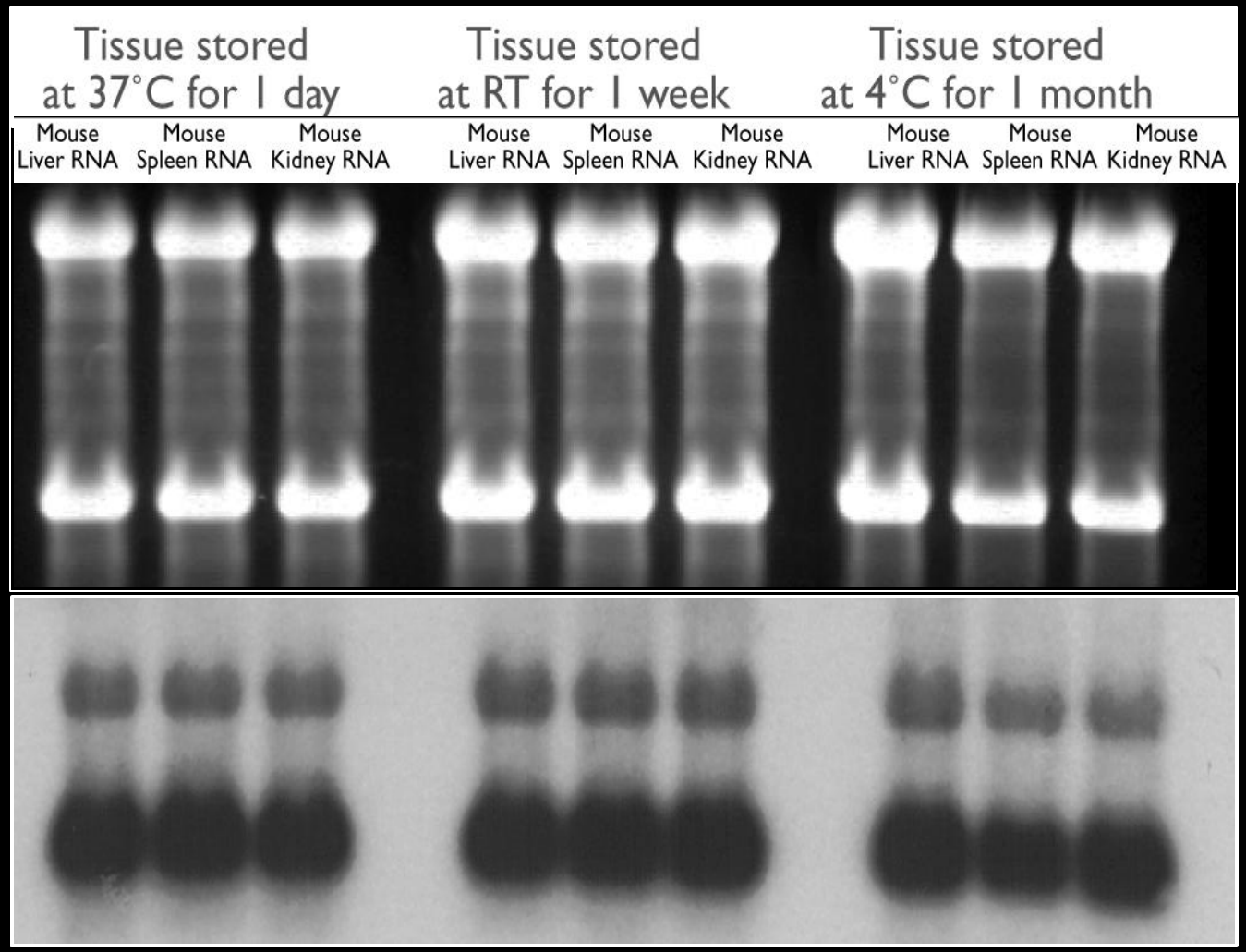
  at 37°C for one day

  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 RNA*later*-treated Tissue



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 redissovling 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**

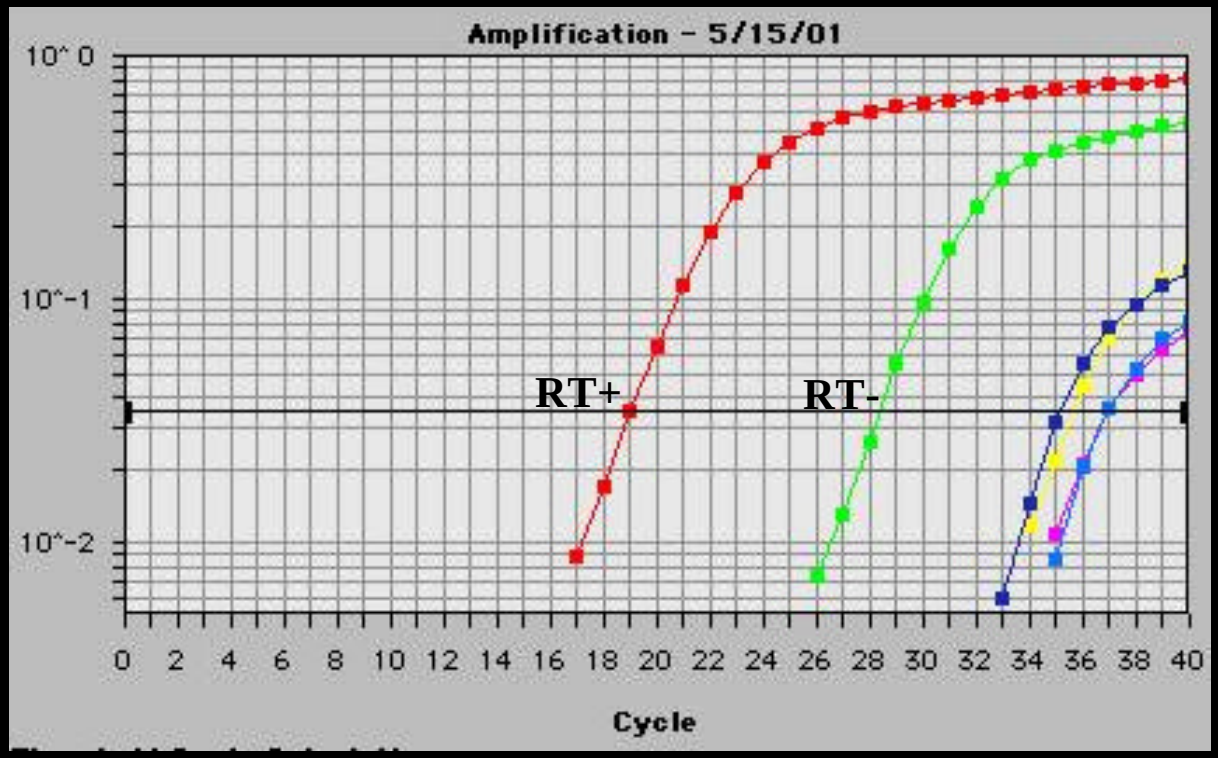
g DNA Contamination

- **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



G3PDH TaqMan assay

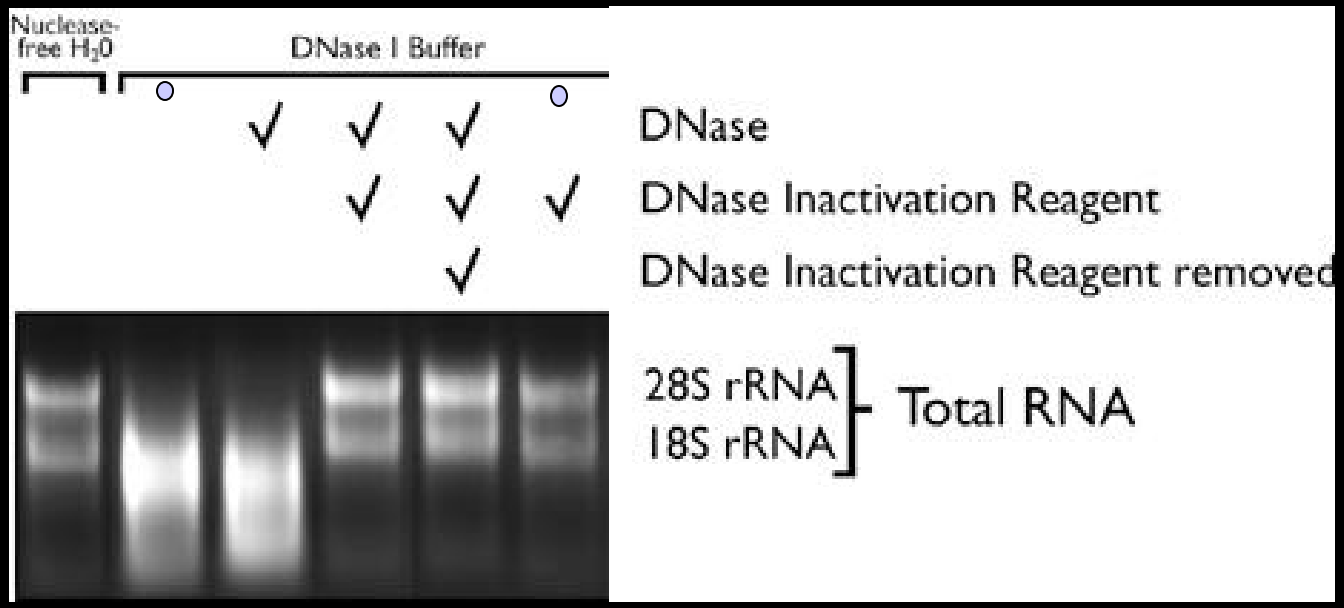
1X DNase = 0.02 units/ul

DNase I/time	fold reduction
RT+	-
0	0
1X/30’	~100
1X/60’	~250
2X/30’	~250
4X/15’	~100

99.6% removal (0.016% DNA)

Divalent Cations and Heat Degrade RNA

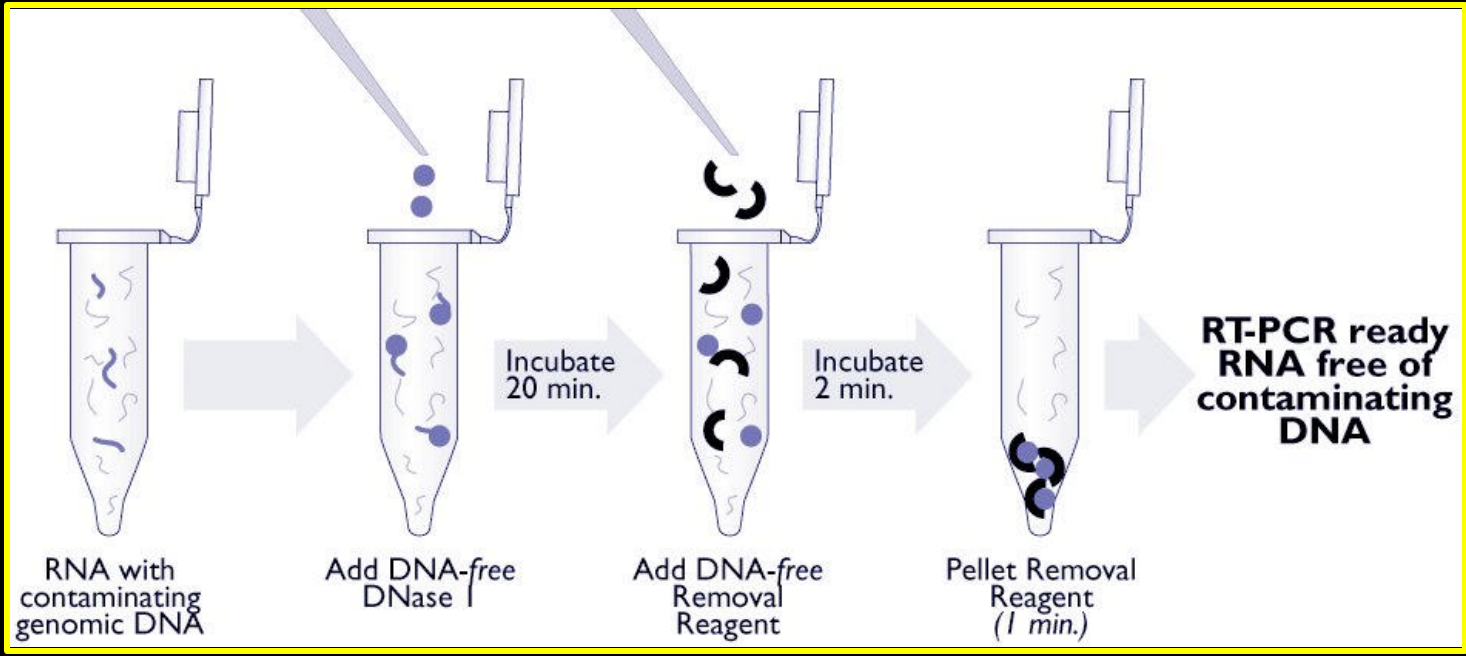
- **DNase I Buffer with 0.1 mM $CaCl_2$, 2.5 mM $MgCl_2$**
- **Heated 90C, 5 minutes**
- **Run on formaldehyde agarose gel**



- **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++)**

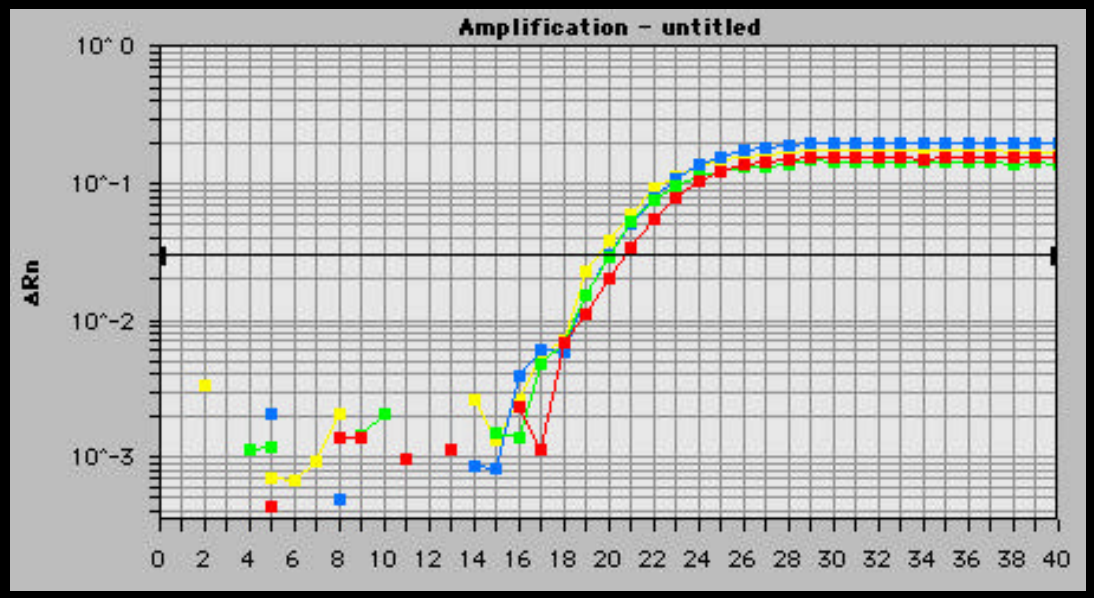


Priming 1st strand synthesis

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)

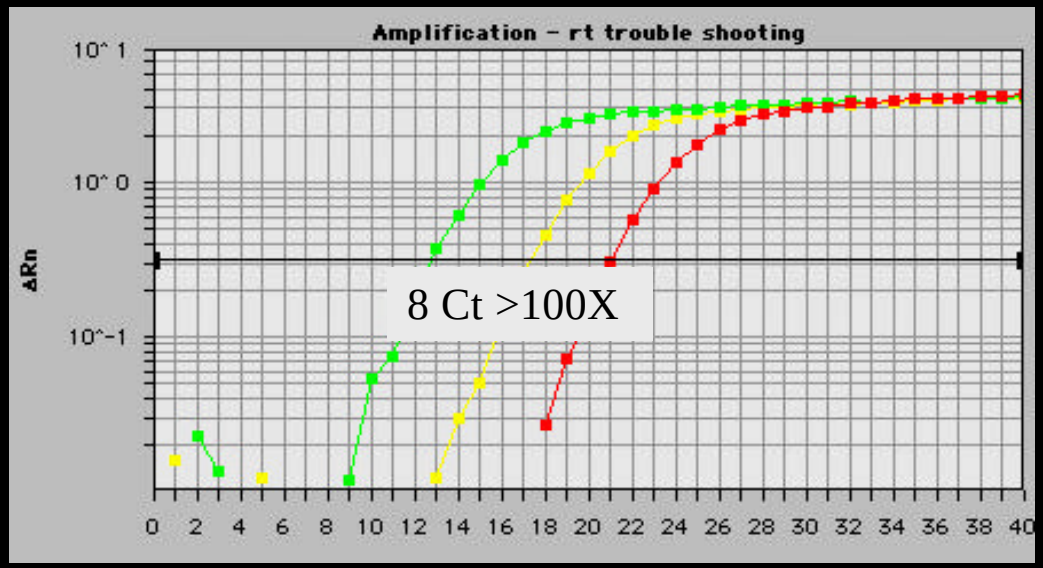
Efficiency of different 1st strand primers on standard RT reactions



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

42°C MMLV RT, G3PDH Taqman PCR

Minimizing endogenous priming in RT Reactions



- MMLV 42ºC
- SSII 48ºC
- AMV 52ºC

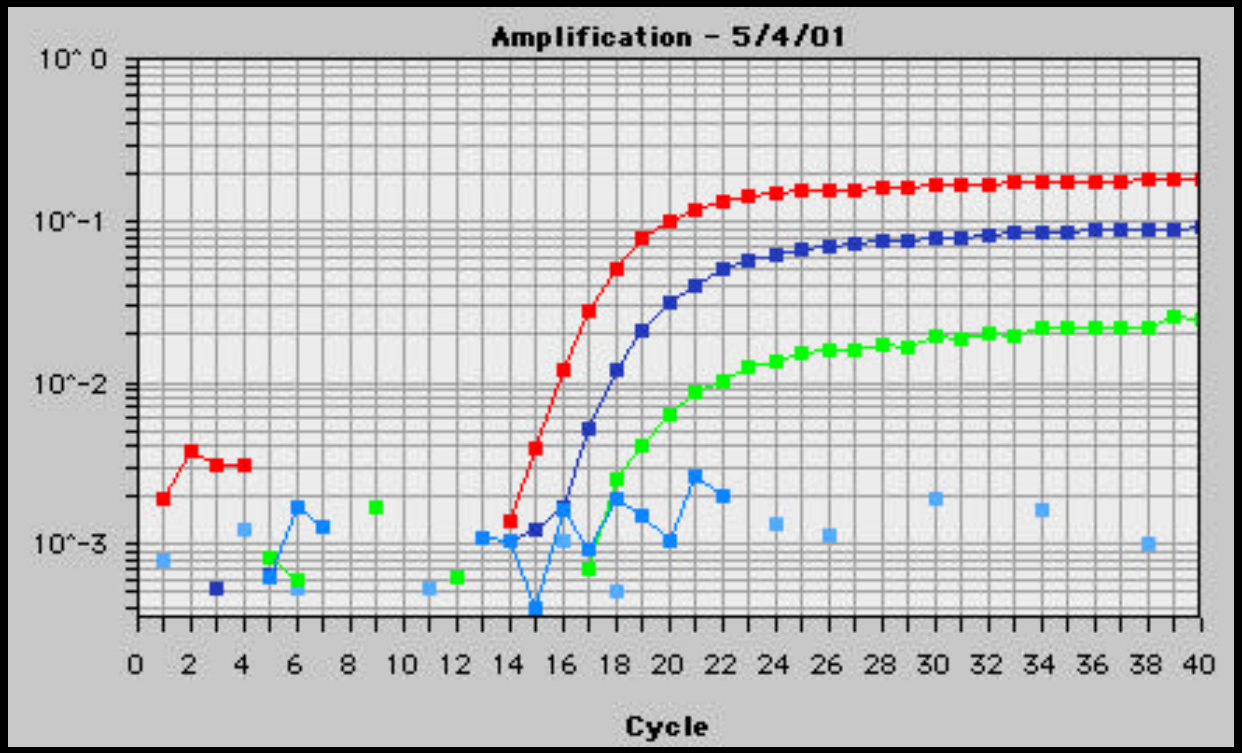
For standard qRT-PCR, random priming at 42ºC is fine.

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



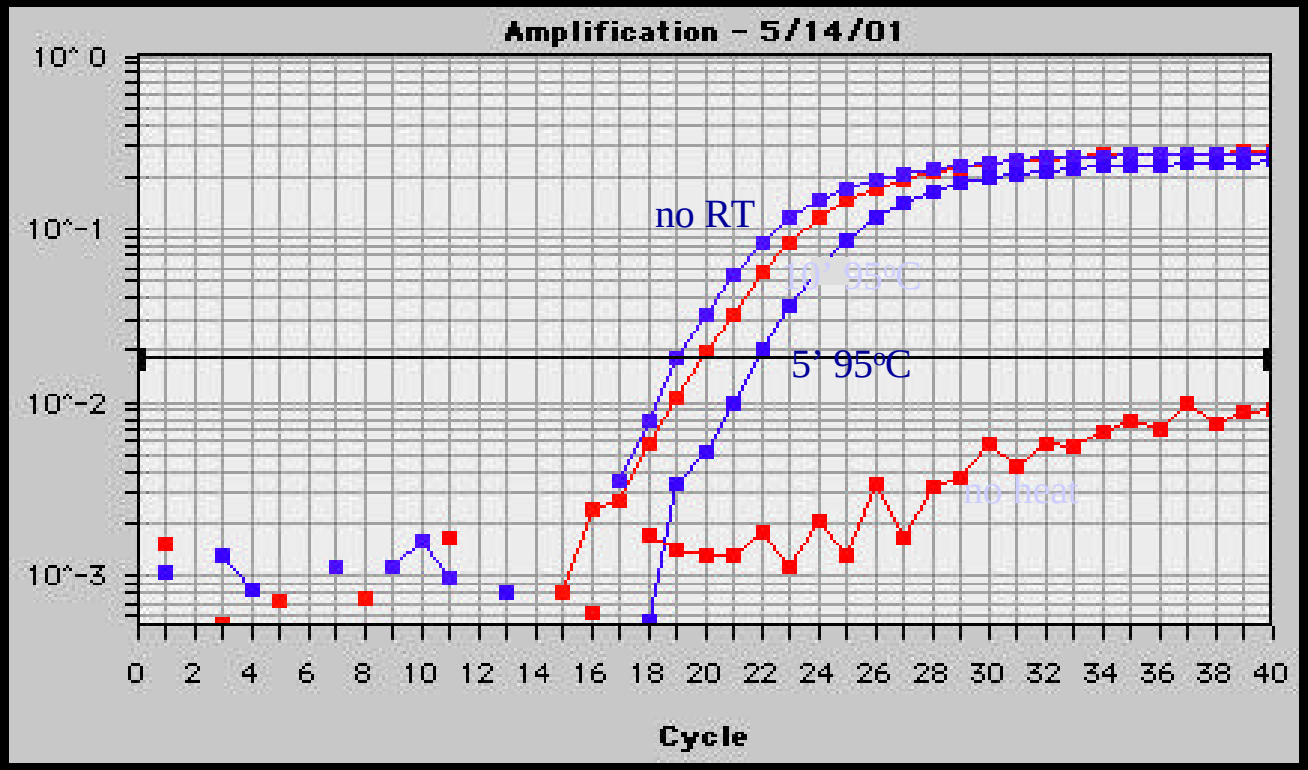
- No RT
- AMV (2 U)
- MMLV (20 U)

- SSII (20 U)

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

RT inhibition of real-time PCR:

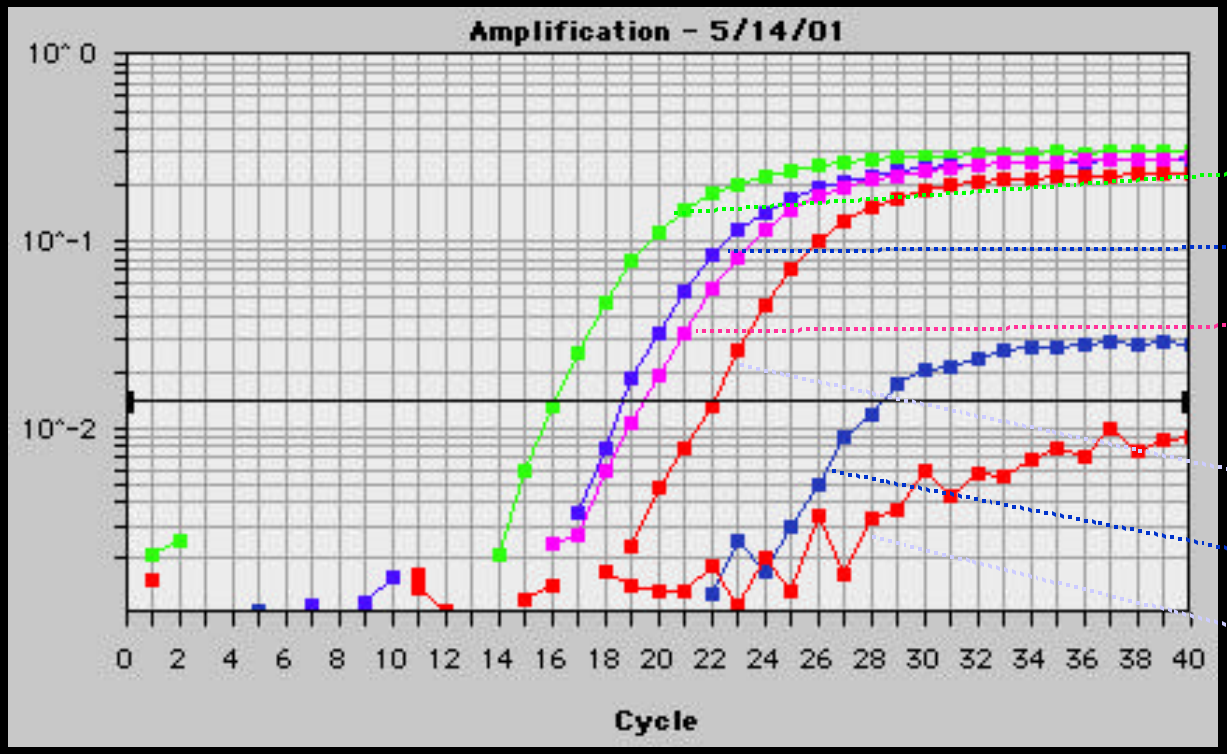
can be relieved by heat-denaturation



MMLV (20 U)

RT inhibition of real-time PCR

Heat inactivation must be prior to addition of Taq

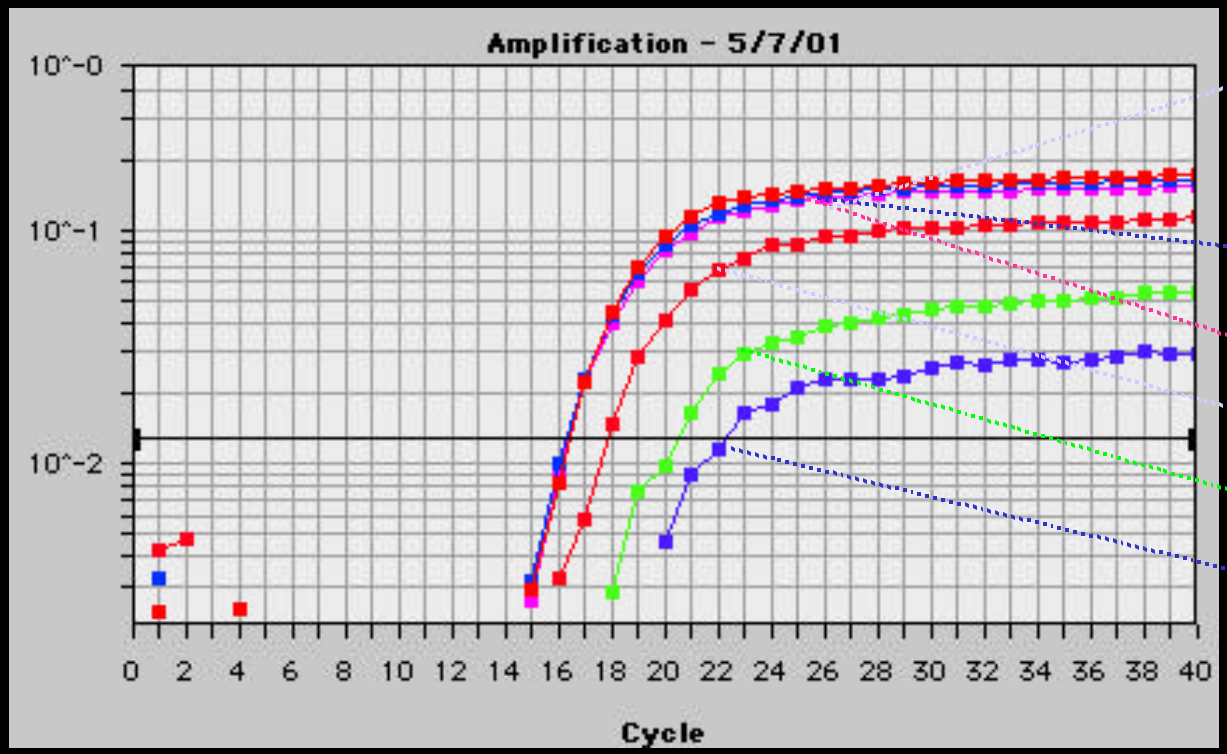


MMLV (20 U)

- 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
- ---

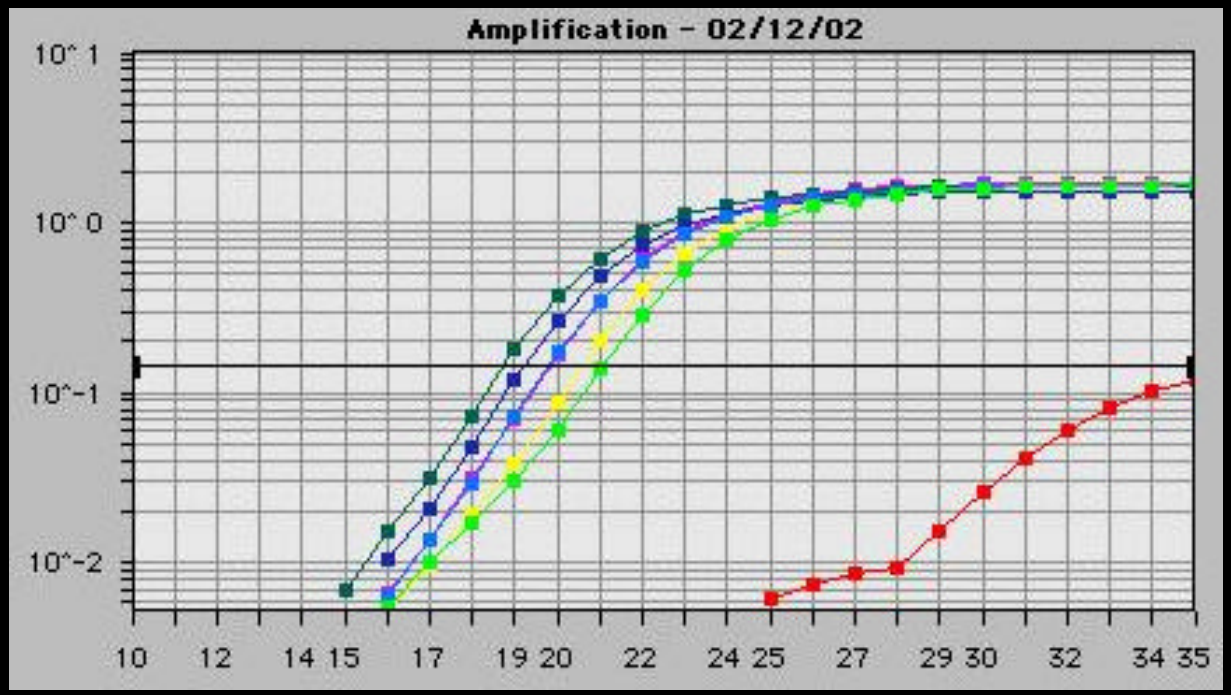
MMLV (20 U)

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

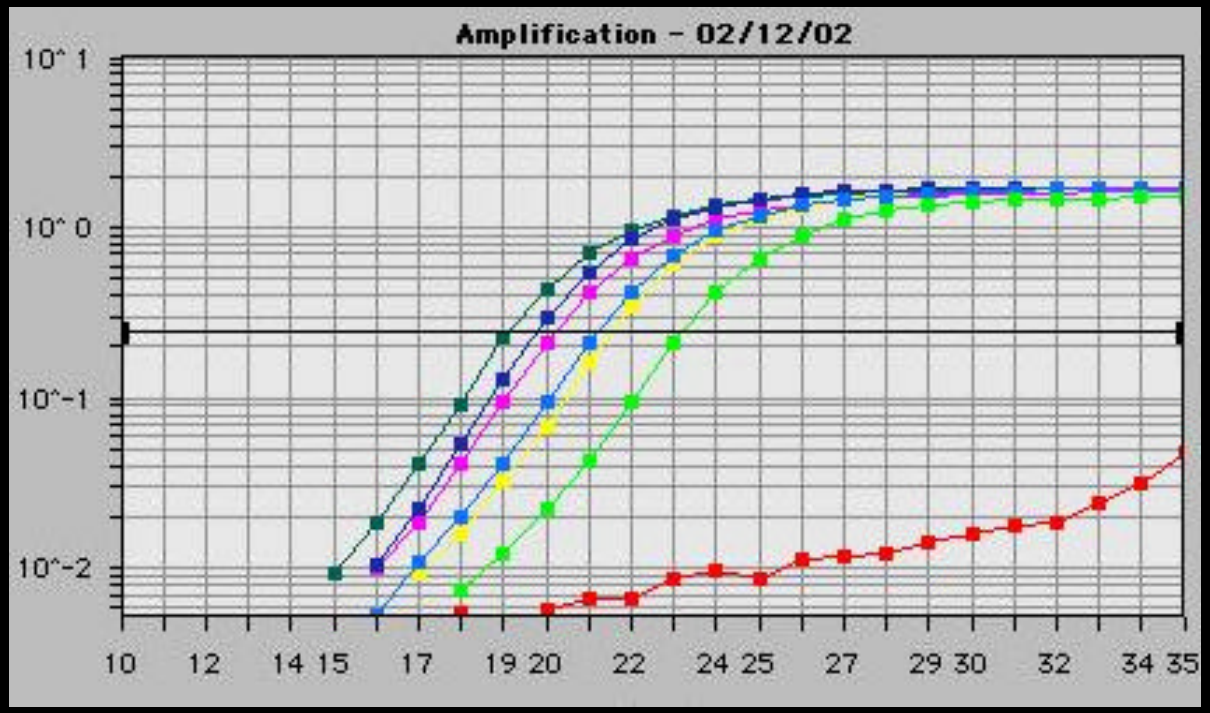
cDNA synthesis: yield vs. time 42^0C



Reaction time (min)	Relative cDNA
0	0
7.5	30
15	33
30	42
60	51
120	68
240	100

1 ug RNA, 200 units MMLV, random primed
β Actin TaqMan assay

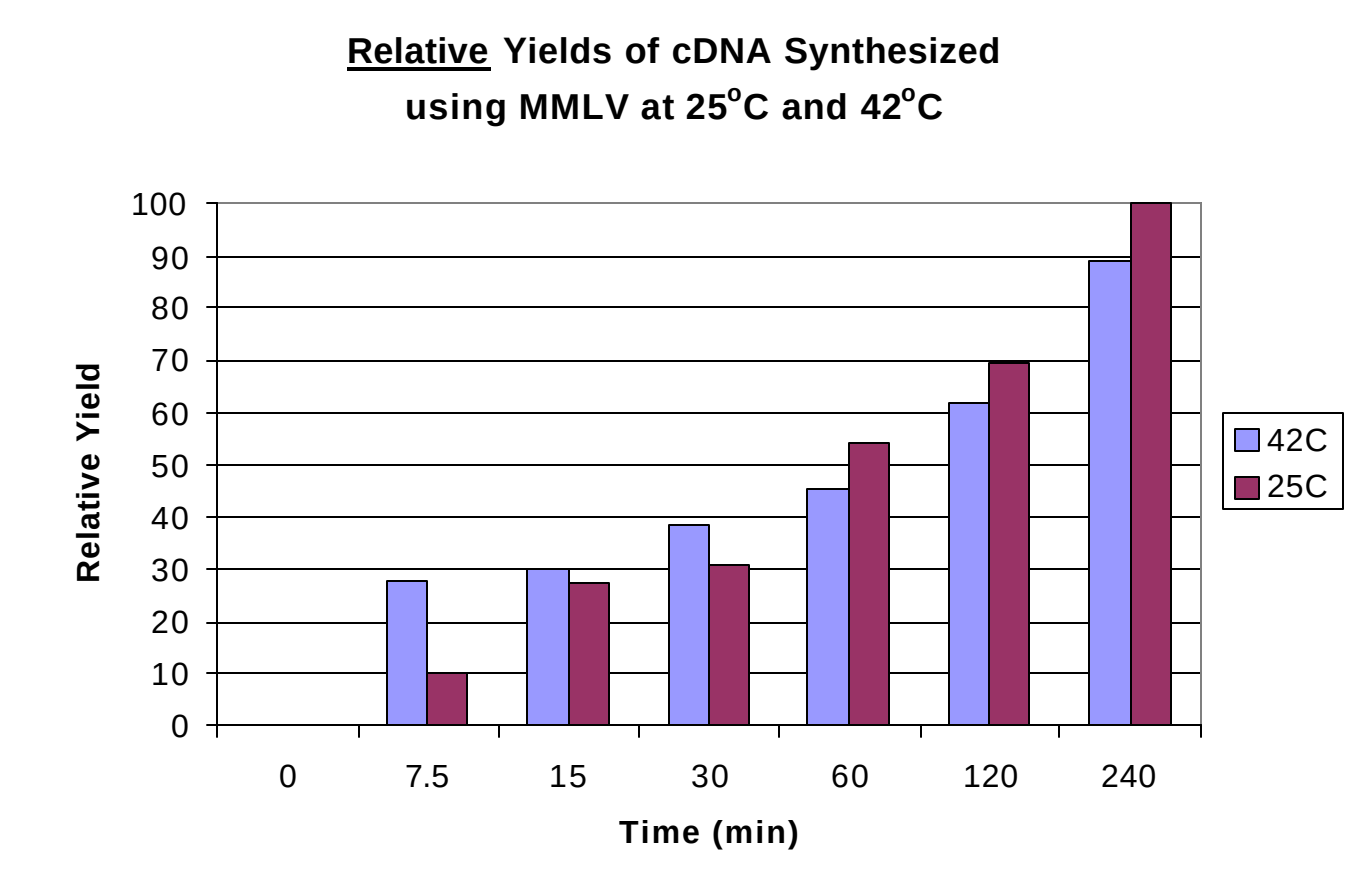
cDNA synthesis: yield vs. time 25^0C



Reaction time (min)	Relative cDNA
0	0
7.5	10
15	27
30	31
60	54
120	70
240	100

25^0C Time course, MMLV, β Actin TaqMan assay

cDNA synthesis: 25^0C vs. 42^0C



elader@ambion.com