Back To Basics I:
The good sample PREP for a successful gene expression quantitation

Objective of this talk

• Make sure your gene expression quantitation foundations are good by giving the right tools to get good starting material.

• Lots of problems are related to sample prep issues.

Characteristics of RNA

• Hydrophilic: It dissolves in water. It does not dissolve in phenol or alcohol or lipids
• pH: RNA is unstable in a basic environment (pH ~9 or more). It stable in mild acid (pH 4) but not strong acid (pH 1).
• Heat: Fairly stable up to ~65°C if divalent cation are not present
• Absorbance Spectra : Maximum UV absorbance at 260nm
RNA Degradation

The OH group attacks the nearby phosphate, breaking the RNA molecules phosphate backbone. This is catalyzed by high pH, divalent cations plus heat, RNases, etc...

RNases

- Found just about everywhere: the benchtop, your hands, in the air and more.
- Incredibly stable enzymes (can withstand extreme conditions such as heat and high salt).
- Some are sequence-specific, others are not. RNase A, for example, cuts 3’ to U and C residues.
- Are not destroyed by autoclaving. They must be destroyed with DEPC or RNaseZAP or inactivated with RNase inhibitors like SUPERase-In.

Sample Handling before Lysis

Example: Effect on Gene Expression in Blood Samples

Sample has to be processed ASAP or freeze in liquid nitrogen.
Main points to be careful when Isolating RNA

Problems are not at the extraction step but more before and after

• Treatment and Handling
  • How you collected
  • How you disrupted

• Storage
  • How will you store your isolated RNA

We will review TEN ways to improve your RNA Isolation

1. Immediately inactivate endogenous, intracellular RNases.
   • There are 3 effective methods to accomplish this:
     I. Homogenize samples immediately after harvesting in a chaotropic-based cell lysys solution (e.g. Guanidinium)
     II. Flash freeze in liquid nitrogen. Tissue pieces must be small
     III. Place sample in RNAlater™
RNAlater™

- Eliminates the need to immediately process or freeze samples
- Samples in RNAlater can be stored for extended periods
- Tissues can be stored indefinitely in RNAlater at –20°C or below
- Tissues stored in RNAlater are simply removed and processed by homogenization via a dounce, Polytron® (Brinkman), or other mechanical apparatus in the lysis buffer specified by your RNA isolation procedure.

2. Use proper Cell or Tissue storage conditions

- If Flash frozen, must be stored at -80°C and never allowed to thaw prior homogenization and isolation.

3. Thoroughly homogenize samples

- Essential step to prevent both loss and degradation
- Needs to be fast and thorough
- Method should be tailored to the cell type
- Usually Mechanical:
  - Dounce, polytron, vortex, sonication, French press, coffee grinder, bar shaker....
- or Enzymatic
  - Lysozyme, zymolase and lysostaphin etc....
4. Pretreat homogenate before RNA Isolation

- Needed for some samples after homogenization before isolation to increase RNA yield
  - High fat contain tissues, like Brain or Adipose tissue should be extracted with Chloroform to remove lipids
  - Plant tissues high in polyphenolics and polysaccharides should be pretreat with polyvinylpyrrolidone (PVP)

5. Choose the best RNA isolation method

- Sample type
  - Tissues, blood, cells, FFPE, LCM, Bacteria or yeast
- What is the downstream application
  - Microarray, qRT-PCR, Northern
- How many samples
- Other Isolation Needs
  - Viral RNA, microRNAs, mRNA isolation/enrichment
Methods in RNA Isolation

- Total RNA isolation
- Solid Phase/Glass Filter Methodology
- Magnetic Bead Methods
- Organic Extraction Phenol-based Method
- Combination of All the above

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6. DNase treatment

- Recommended when RNA is to be used in RT-PCR
- Good Idea when isolating RNA from DNA rich tissues like spleen

7. Reduce exposure to environmental RNases

- Essential that any items that could contact the purified RNA is RNames free
- Benchtop, Pipettors, Glassware should be decontaminated with RNaseZap™
- RNase free tips, tubes and solution should always be used and gloves should be changed frequently
8. Proper precipitation

- If purified RNA need to be concentrated by precipitation for downstream application
  - Ammonium Acetate (NH$_4$OAc) precipitation
    (0.1 volume s of 5M NH$_4$OAc, and 2-2.5 volumes 100% EtOH at -20°C for > 25min)
  - For low concentrations of RNA (ng/ml) use glycogen, yeast RNA or linear acrylamide

9. Resuspension

- Three ideal qualities of a resuspension solution
  - RNase-free
  - Low pH (pH 6-7)
  - Incorporate a Chelating agent (sodium citrate or EDTA) to protect RNA against degradation by introduced RNases

10. Storage

- Short term:
  - Stored at -20°C
- Long term:
  - Stored at -80°C in buffer
  - More stable in NH$_4$OAc/EtOH precipitation mixture at -80°C
  - Aliquot your RNA into several Tube
  - Avoid Freeze-thaw event
  - Prevent RNase contamination

Caution: MgCl$_2$ and other ions catalyze unspecific RNA breaks => use EDTA
Tips to make sure your RNA is of good quality

Method to assess RNA Quantity

- Fluorescent Dye (Ribo Green)
  - More sensitive than Spectrophotometry
  - Less sensitive to protein and free nucleotide
  - DNA contamination can cause inaccurate quantitation

- Spectrophotometry (A260)
  - Simple, widely used and uses common equipment
  - Considerably less sensitive than other methods

- Agilent 2100 bioanalyser
  - Requires only a small amount of RNA (25ng)
  - Can quantitate RNA and assess RNA integrity
  - Instrument not available everywhere

Assessing the RNA integrity

- Denaturing Agarose Gel electrophoresis

  A 2:1 ratio of 28S to 18S rRNA indicates intact RNA (requires at least 100ng)

  Smeared 28S and 18S rRNA bands indicate degradation. The ratio of 28S to 18S rRNA is significantly less than 2.0
Assessing the total RNA integrity

- Agilent 2100 Bioanalyzer

A 2:1 ratio of 28S to 18S rRNA indicates intact RNA (requires at least 1ng). Degradation is indicated by less pronounced peaks for 28S and 18S rRNA ratio is less than 2.0.

Identifying Genomic DNA contamination

- Denaturing agarose Gel
  - Genomic DNA contamination shows up as high molecular weight band or a smear

Assessing RNA purity

- Spectrophotometry

Pure RNA should have an A260/A280 ratio of ~2.0. RNA with significantly lower ratio should be further purified by phenol-chloroform extraction, LiCl precipitation or washing to remove residual salt.
Creating Primer and Probes Stock Solutions

- TAMRA™ dye-labeled probes
  - arrive lyophilized, elute in 1 mM Tris HCl (pH 8), 0.01 mM EDTA
  - Stock concentration e.g. 10 µM

- MGB Probes
  - arrive eluted (50 µM), at room temperature (stable for days)
  - dilute in 1 mM Tris HCl (pH 8), 0.01 mM EDTA
  - Stock concentrations e.g. 10 µM

Creating Primer and Probes Stock Solutions

- Primers
  - arrive lyophilized
  - dilute in 1 mM Tris HCl (pH 8), 0.01 mM EDTA
  - Stock concentrations e.g. 10 µM in TE buffer

- TaqMan® Genomic Assays
  - primer-probe mixtures
  - 20 x concentrated, can directly be used as stock solution
Primer and Probes Storage

- Aliquot stock solutions
  - Reduced number of freezing and thawing processes
  - Potential contamination would affect only one aliquot and not entire stock solution
  - Aliquot volume based on throughput
- Store aliquots at -20°C

You will always reap what you sow

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