RNA Isolation and Technology Applications

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Overview

• Brief overview of basic RNA/DNA chemistry.
• Overview of total and poly(A+) RNA isolation.
• Discuss the problem of RNase contamination.
• Discuss some major RNA analysis techniques.
Nucleic Acid Chemistry

- **DNA** (deoxyribonucleic acid) and **RNA** (ribonucleic acid) store and transfer genetic information in living organisms.

- **DNA:**
  - major constituent of the nucleus
  - stable representation of an organism’s complete genetic makeup

- **RNA:**
  - found in the nucleus and the cytoplasm
  - key to information flow within a cell
Nitrogenous Bases: Purines and Pyrimidines

- **Purines**: fused 5- and 6-member rings
  - Adenine
  - Guanine

- **Pyrimidines**: 6-member ring
  - Cytosine
  - Uracil
  - Thymine
Structure of RNA nucleotides

- **Nitrogen-containing Base** (pyrimidine or purine)
- **Sugar**
  - in RNA, the sugar is ribose
  - Hydroxyl group at position 2 of sugar ring

**phosphate**
Structure of DNA nucleotides

Nitrogen-containing Base (pyrimidine or purine)

Sugar
- in DNA, the sugar is deoxyribose
- Hydroxyl group at position 2 of sugar ring is absent
RNA is a Polymer of rNTPs

- Sugar-phosphate backbone
  - 5’ position of one sugar is connected to the 3’ position of the next sugar via the phosphate group
- Single-stranded
- Purines: Adenine, Guanine
- Pyrimidines: Cytosine, Uracil
DNA is a Polymer of dNTPs

- Sugar-phosphate backbone
- Double-stranded
- Rules govern base pairing
  - **Adenine** pairs with **Thymine**
  - **Cytosine** pairs with **Guanine**
- Antiparallel strands
  - 5’ end of coding strand aligns with 3’ end of anticoding strand
## RNA vs. DNA

### RNA:
- 2´ OH group (ribose)
- Uracil binds Adenine (A,U,C,G)
- Multiple types and roles
- Often permanently modified via splicing
- Usually single-stranded
- Intermolecular binding

### DNA:
- 2´ H (deoxyribose)
- Thymine binds Adenine (A,T,C,G)
- One biological form
- Permanently modifications are rare (mutation)
- Double stranded
- Double helix structure
The Central Dogma

DNA

RNA

Protein

Nucleus

Cytoplasm

Ribosome

mRNA

mRNA

Amino acid chain folds into a protein
Cellular “total” RNA

- **Messenger RNA (mRNA):** 1-5%
  Serves as a *template for protein synthesis*

- **Ribosomal RNA (rRNA):** >80%
  *Structural component of ribosomes*

- **Transfer RNA (tRNA):** 10-15%
  *Translates mRNA information into the appropriate amino acid*
RNA Isolation Techniques
Why Isolate RNA?

*Messenger RNA synthesis is a dynamic expression of the genome of an organism. As such, mRNA is central to information flow within a cell.*

- **Size** – examine differential splicing
- **Sequence** – predict protein product
- **Abundance** – measure expression levels
- **Dynamics of expression** – temporal, developmental, tissue specificity
RNA isolation

Tissue → Cells

Break open the cell → RNA Purification

Purity → Quality Parameters → Integrity
RNA Purification

- Total RNA from biological samples
  - Organic extraction
  - Affinity purification

- mRNA from total RNA
  - Oligo(dT) resins

- mRNA from biological samples
  - Oligo(dT) resins
Total RNA Purification

• Goal: Isolate RNA from other cellular components
  – Cells or tissue must be rapidly and efficiently disrupted
  – Inactivate RNases
  – Denature nucleic acid-protein complexes
  – RNA selectively partitioned from DNA and protein

• Isolation from different tissues/sources raises different issues
Ribonucleases (RNases)

- RNases are naturally occurring enzymes that degrade RNA
- Common laboratory contaminant (from bacterial and human sources)
- Also released from cellular compartments during isolation of RNA from biological samples
- Can be difficult to inactivate
Protecting against RNase

- Wear gloves at all times
- Use RNase-free tubes and pipet tips
- Use dedicated, RNase-free, chemicals
- Pre-treat materials with extended heat (180°C for several hours), wash with DEPC-treated water, NaOH or H₂O₂
- Supplement reactions with RNase inhibitors
- Include a chaotropic agent (guanidine) in the procedure
  - Chaotropic agents such as guanidine inactivate and precipitate RNases and other proteins
Organic Extraction of total RNA

Lyse/homogenize cells

Add phenol:chloroform:isoamyl alcohol to lysed sample, and centrifuge

Organic phase separates from aqueous phase
- Organic solvents on bottom
- Aqueous phase on top (contains total RNA)
- Cellular debris and genomic DNA appears as a “film” of debris at the interface of the two solutions

Remove RNA solution to a clean tube; precipitate RNA and wash with ethanol, then resuspend RNA in water
Organic Extraction of total RNA

• Advantages
  – Versatile
    • Compatible with a variety of sample types
  – Scalable
    • Can process small and large samples
  – Established and proven technology
  – Inexpensive

• Disadvantages
  – Organic solvents
  – Not high-throughput
  – RNA may contain contaminating genomic DNA
Affinity purification of total RNA

Lyse cells, and spin to remove large particulates/cell debris

Apply lysate (containing nucleic acids and cellular contaminants) to column with glass membrane

Wash with alcohol to remove contaminants; nucleic acids stick to glass membrane while contaminants wash through. Treat with DNase enzyme to remove contaminating DNA.

Apply water to the column; purified RNA washes off the glass and is collected
Affinity purification of total RNA

- Advantages
  - Eliminates need for organic solvents
  - Compatible with a variety of sample types (tissue, tissue culture cells, white blood cells, plant cells, bacteria, yeast, etc.)
  - DNase treatment eliminates contaminating genomic DNA
  - Excellent RNA purity and integrity
Messenger RNA Isolation

- mRNA molecules have a tail of A’s at the 3’ end (polyA tail)

- Oligo(dT) probes can be used to purify mRNA from other RNAs

- mRNA can be eluted from oligo(dT) matrix using water or low-salt buffer
Messenger RNA Isolation

Combine cytoplasmic RNAs and oligo(dT) matrix under hybridization conditions.

Poly-A tail of mRNA binds to oligo(dT) matrix.

rRNA and tRNA is washed away.

Purified mRNA is eluted from the oligo(dT) matrix in water or low-salt buffer.
Messenger RNA Isolation

- **Isolating mRNA from total RNA**
  - Purifying total RNA first enables larger sample sizes to be processed; this results in higher mRNA yield
  - Two purifications; takes longer than isolating mRNA directly

- **Isolating mRNA directly from a biological sample**
  - Quicker than doing an initial total RNA isolation followed by mRNA selection
  - However, sample size is limited
RNA sample absorbances are determined on the spectrophotometer at 260nm, 280nm, and 230nm.

- 260nm: nucleic acid (DNA, RNA, nucleotides)
- 280nm: protein
- 230nm: guanidine
Quantitation of RNA

• Nucleic acids absorb UV light maximally at 260nm

• For RNA:
  \[ 1 \text{ OD}_{260} \text{ Unit} = 40 \mu\text{g/ml of ssRNA} \]

• The concentration in the sample is calculated by using the formula:
  \[ A_{260} \times \text{dilution} \times 40 = [\text{RNA}] \mu\text{g/ml} \]
Estimating RNA purity by spectrophotometry

- $A_{260}/A_{280}$
  - pure RNA will exhibit an $A_{260}/A_{280}$ ratio within the range of 1.8 - 2.0
  - If the RNA exhibits a ratio lower than 1.7, this indicates protein contamination in your sample

- $A_{260}/A_{230}$
  - properly purified RNA should exhibit an $A_{260}/A_{230}$ ratio within the range of 1.8 - 2.0.
  - If the RNA exhibits a ratio lower than 1.7, this indicates guanidine contamination in your sample

- While spec readings do give an estimation of purity, they do NOT indicate RNA integrity
Two major ribosomal subunits: 28S and 18S

Approximately 80% of total RNA is associated with these two subunits

Integrity of the total RNA sample can be determined by evaluating the 28S and 18S rRNA
  - 28S and 18S rRNA should appear as distinct bands
  - Ratio of 28S:18S should be approximately 2:1
Evaluating RNA integrity

- High quality total RNA imaged on an Agilent 2100 Bioanalyzer
- 28S rRNA and 18S rRNA appear as distinct, intact bands
- Ratio of 28S : 18S approximately 2 : 1
Poor quality RNA

- Poor quality total RNA imaged on an Agilent 2100 Bioanalyzer
- Degradation of 28S rRNA and 18S rRNA is apparent
- Ratio of 28S : 18S falls below 1.5 : 1
mRNA isolated from Total RNA
RNA Gels

- Denaturing gels
- Denaturing loading dye
- RNase-free
- Check for integrity of ribosomal RNAs
Applications for RNA Analysis
Techniques for RNA analysis: Northern Blotting

• Once a gene has been identified, it is useful to determine the size and quantity of the mRNA message produced and whether alternative splice variants of different sizes exist.

• Northern Blotting: method to determine the abundance and size of a RNA molecule in a sample, by hybridization with a gene-specific probe.
Techniques for RNA analysis: Northern Blotting

- Total RNA or mRNA samples are run on denaturing agarose gels
- After electrophoresis, the RNA is transferred from gel to membrane
Techniques for RNA analysis: Northern Blotting

- A labeled probe that is specific to the gene of interest is applied to the RNA samples on the filter.

- Labeling methods:
  - Radioactive
  - Chemiluminescent
  - Fluorescent

- Analysis reveals the size and quantity of the mRNA message in each sample.
Techniques for RNA analysis: Northern Blotting

A. Gel analysis of RNA

<table>
<thead>
<tr>
<th>µg total RNA</th>
<th>µg mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
</tr>
<tr>
<td>1.5</td>
<td>M</td>
</tr>
</tbody>
</table>

B. Northern blot

<table>
<thead>
<tr>
<th>µg total RNA</th>
<th>µg mRNA</th>
</tr>
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<tr>
<td>12</td>
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<td>0.5</td>
</tr>
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<td>1.5</td>
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</table>

Probe used is specific to the mRNA sequence of interest

- Size and quantity of message can be determined
Techniques for RNA analysis: RNase Protection Assay

- RNase Protection Assay: sensitive technique for the quantitation of specific RNA transcripts in solution
- Can be performed using total RNA or mRNA as the target
- A probe that is complementary to the sequence of interest is used
  - defined length
  - radioactively labeled
- The RNA target and RNA probe are hybridized in solution
- Following hybridization, the RNA is digested with RNases specific for single-stranded nucleic acids
  - Any remaining unhybridized single-stranded RNA target and probe is degraded
  - Target:probe hybridized sequences are left intact
- Following RNase inactivation, RNA samples are run on denaturing polyacrylamide gels
- The amount of intact probe observed on the gel is proportional to the amount of target RNA in the original sample
Techniques for RNA analysis: RNase Protection Assay

- RNase Protection Assay is more sensitive than Northern blots for the detection and quantitation of low abundance RNAs
Techniques for RNA analysis: RT-PCR

- Reverse Transcription-Polymerase Chain Reaction
- Amplification of a specific RNA molecule into many copies of a cDNA molecule
- Potential applications:
  - Determine the presence or absence of a transcript
  - Estimate mRNA expression levels
  - Convert RNA message to cDNA for cloning
Techniques for RNA analysis: RT-PCR

- Initial step: first-strand synthesis
- mRNA template is copied into cDNA via reverse transcription
- The synthesized cDNA strand is *complementary* to the mRNA template
Techniques for RNA analysis: RT-PCR

- Subsequent step: thermal cycling for second strand cDNA synthesis and amplification
- Gene-specific primers and thermostable DNA polymerase are added to the reaction
- Thermal cycling results in amplification of sequence of interest

<table>
<thead>
<tr>
<th>bp</th>
<th>M</th>
<th>Sample #1</th>
<th>Sample #2</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,845</td>
<td>676</td>
<td>350</td>
<td>222</td>
</tr>
<tr>
<td>179</td>
<td>126</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Lanes 1 and 3: housekeeping gene
- Lanes 2 and 4: gene of interest

- Difference in intensity of amplified fragment reflects difference in amount of mRNA transcript in different samples
- Powerful tool for detecting low-copy messages
Techniques for RNA analysis: qRT-PCR

- In traditional PCR assays, too much amplification makes it impossible to quantitate the amount of starting nucleic acid material.

- Real-Time PCR (or, quantitative PCR) addresses this problem of end-point analysis

- Real-Time PCR monitors the product accumulation as the PCR amplification proceeds, allowing for higher sensitivity.
Techniques for RNA analysis: qRT-PCR

- Cells from the eye were evaluated for expression of IL1-beta
- Experimental group (“vit”): treated with vitreous humor
- Control group (“con”): no treatment
- IL1-beta shows dramatic difference in mRNA levels in treated vs control
Techniques for RNA analysis: Microarrays for Gene Expression Profiling

- Microarrays enable researchers to examine the expression levels of thousands of genes in a single experiment
- As many as 50,000 or more unique DNA sequences are spotted on to a glass slide
  - Each spot represents one unique, known gene sequence
- RNA is isolated from two different cell or tissue samples
  - healthy vs. cancerous tissue, for example
- Fluorescently-labeled cDNA is generated from each RNA sample
  - samples are differentially labeled
- Gene expression levels are compared based on hybridization to the known gene sequences spotted on the slide
  - Fluorescent intensity of each spot corresponds to that gene’s expression level in the cells from which the sample was obtained
Techniques for RNA analysis: Microarrays for Gene Expression Profiling

Normal

→ Isolate Normal Lung RNA

→ Generate fluorescent cDNA from RNA; label with **GREEN** dye

Tumor

→ Isolate Lung Tumor RNA

→ Generate fluorescent cDNA from RNA; label with **RED** dye
Techniques for RNA analysis: Microarrays for Gene Expression Profiling

- Mix normal lung cDNA (labeled with green fluorescent dye) with lung tumor cDNA (labeled with red fluorescent dye)
- Apply both fluorescent cDNAs to spotted slide
Techniques for RNA analysis: Microarrays for Gene Expression Profiling

- Normal lung sample was labeled with green dye.
- The green fluorescent intensity of each spot indicates the level of expression of that gene in the normal sample:
  - Bright spots correspond to strongly expressed genes.
  - Dim spots indicate weak expression.
Techniques for RNA analysis: Microarrays for Gene Expression Profiling

- Lung tumor sample was labeled with red dye

- The red fluorescent intensity of each spot indicates the level of expression of that gene in the tumor sample
  - bright spots correspond to strongly expressed genes
  - dim spots indicate weak expression
Techniques for RNA analysis:
Microarrays for Gene Expression Profiling

By overlaying red and green data from the same slide, you can view the *differential expression* of various genes.

**Green** = gene expressed in normal sample

**Red** = gene expressed in tumor sample

**Yellow** = gene expressed in BOTH normal and tumor samples
Techniques for RNA analysis: Microarrays for Gene Expression Profiling

• Gene expression data generated in microarray experiments is often confirmed using qRT-PCR technology.

• Microarrays: most useful when screening many genes of interest.

• Real-Time PCR: most useful when many treatment conditions on a handful of genes.

• Example:
  – Screen a tumor sample vs a normal sample for expression differences in 10,000 genes: MICROARRAY
  – Screen the effect of 10 different drug treatments on the expression of one or two gene: REAL-TIME PCR
Techniques for RNA analysis: Microarrays for Gene Expression Profiling

- Some Examples of Potential Applications
  - Gene expression studies in disease
  - Drug discovery and development
  - Detection of bacteria, viruses in environmental samples
    - water quality management
  - Use as a diagnostic tool in the field of healthcare
    - Disease detection
    - Newborn screening
  - Detection of bioagents
    - Anthrax
    - biological weapons
Techniques for RNA analysis: 
*In vitro* Translation

- *In vitro* Translation: Expression of a protein product encoded by an RNA molecule in a cell-free system
  - Commonly used cell-free translation systems consist of extracts from rabbit reticulocytes, wheat germ and *Escherichia coli*
  - All are prepared as crude extracts containing all the macromolecular components required for translation of exogenous RNA
  - Standard translation systems, such as reticulocyte lysates and wheat germ extracts, use RNA as a template
Techniques for RNA analysis: *In vitro* Translation

- Making proteins in vitro is an important tool for:
  - studying protein mutations
  - protein-DNA binding studies
  - protein activity assays
  - protein-protein interaction studies.
Techniques for RNA analysis: RNA Interference (RNAi)

- RNAi: using dsRNA to inhibit expression of genes
  - Dual PCR fragments (complementary to the RNA message of interest) are transfected into cells
    - One fragment expresses the sense strand of the RNA
    - One fragment expresses the antisense strand of the RNA
  - Once inside cells, the DNA PCR fragments are transcribed into short RNA fragments (short interfering RNA, or siRNA)
  - The resulting complementary siRNAs anneal, forming siRNA duplexes (dsRNA)
  - These duplexes trigger the specific degradation of the target mRNA inside the transfected cells
Techniques for RNA analysis: RNA Interference (RNAi)

The siRNA associates with a nuclease complex, forming the RNA-induced silencing complex (RISC)

The siRNA-RISC complex locates the mRNA with the target sequence and degrades it, effectively silencing the gene.
Techniques for RNA analysis: RNA Interference (RNAi)

- Gene knockdown is a powerful tool for analyzing the function of a single gene.

- Selectively downregulating the expression of a particular gene allows researchers to determine its function in many cellular processes.

- Particularly useful in studies such as:
  - Mapping cellular pathways
  - Developing gene therapies
Questions?