Optimizing RNA Preparation and Analysis

https://www.youtube.com/watch?v=dklyRYBZv4c

Step 1: Sample Collection & Protection

RNA is very fragile and can degrade easily. That's why protecting it immediately after collection is crucial.

- Challenges: Hard tissues (like bone), microbes with tough walls, delayed processing, or too many samples can make immediate RNA isolation hard.
- Common solution: Freeze samples using liquid nitrogen or dry ice and process them later — but this is time-consuming and complex.

A better way: Use RNAlater

- Simply place your tissue/cells in RNAlater solution at room temperature.
- It stabilizes RNA, allowing storage at 4°C (RNAlater) or –20°C (RNAlater-ICE).
- Samples can be stored for days to months without loss in RNA quality.
- Works with most RNA isolation methods.

Step 2: RNA Preparation

There are 4 main methods to extract RNA, each with pros and cons:

1. Organic Extraction (e.g., TRIzol)

- Pros: Great for difficult samples, denatures RNases fast, scalable.
- Cons: Uses toxic chemicals, labor-intensive, hard to automate.

2. Spin Column (Filter-based)

- Pros: Easy, suitable for high throughput and automation.
- Cons: Can clog, has fixed capacity, may retain DNA.

3. Magnetic Particles

- Pros: No filter clogging, good for automation, effective in solution.
- Cons: Particles may carry over, slow in thick solutions, manual steps can be tedious.

4. Direct Lysis

- Pros: Fastest, simplest, good for small samples, no solid-phase bias.
- Cons: No yield measurement, best for concentrated samples, not suitable for all downstream methods.

Thermo Fisher offers kits for all these methods, tailored to different sample types and needs.

Step 3: RNA Quantification

Before using RNA for analysis, you need to know how much you have.

Most common method: UV Spectroscopy

- Measures absorbance at 260/280 nm.
- Uses Beer-Lambert law to calculate RNA concentration.
- Tip: Always dilute samples and use clean equipment for accurate readings.

Sure! Here's a simplified version of the content, broken down for clarity and easier understanding:

Measuring RNA Concentration and Purity

1. UV Spectroscopy (A260/A280 Measurement)

- **A260 reading of 1.0** = ~40 µg/mL of single-stranded RNA.
- **A260/A280 ratio** shows RNA purity. A good ratio is **between 1.8 and 2.1**.
- **UV Spectroscopy** is commonly used because it’s simple and available in most labs.

Tips for Accurate Readings:

- **Remove DNA contamination** using RNase-free DNase. UV can't tell RNA from DNA.
- **Clean your sample:** Proteins and phenol can affect absorbance.
- **Use clean quartz cuvettes** and check for dust. Measure at 320 nm to correct background noise.
- **pH matters:** Water can lower the A260/A280 ratio. Use a slightly basic buffer like **TE (pH 8.0)**.
- **Stay in the right range:** Absorbance should be between **0.1 and 1.0** for accurate results.

RNA Storage Tips

After RNA isolation, store it carefully to avoid degradation.

Best Storage Practices:

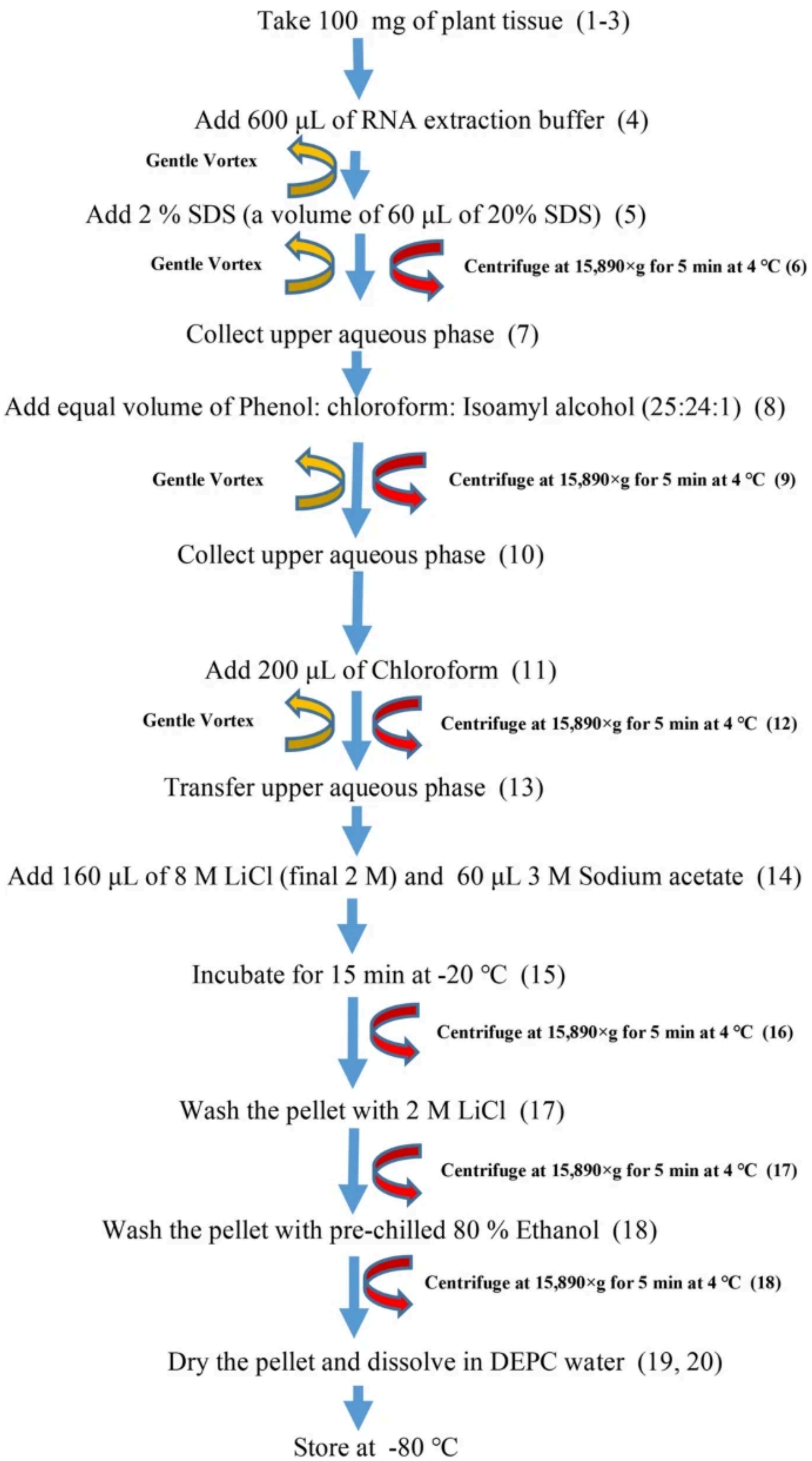
- Store at **–80°C** in small, single-use tubes.
- Use **RNase-free** buffers like:
    - THE RNA Storage Solution (more stable due to low pH and citrate)
      0.1 mM EDTA
      TE Buffer
      RNAsecure Resuspension Solution

RNAsecure:

- Inactivates RNases by heating to **60°C for 10 min**.
  Can be reheated to keep destroying new RNases.

Method	Pros	Cons
Organic Extraction	**High quality, denatures RNases quickly**	**Time-consuming, uses toxic chemicals**
Spin Basket	**Easy, good for many samples**	**Can clog, less effective for large RNAs**
Magnetic Beads	**Fast, automatable**	**May require special equipment**

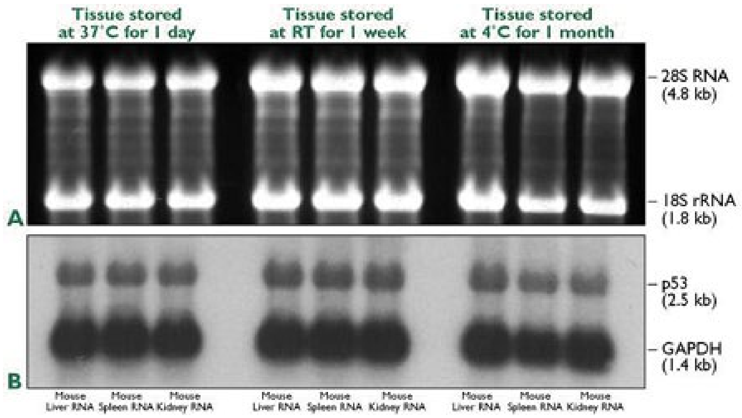
Direct Lysis	**Very quick, simple automation possible**	**Can't measure yield traditionally, RNase risk**



Key Factors for Optimizing RNA Preparation:

UV Spectroscopy measures absorbance at 260 nm and 280 nm. An **A260/A280 ratio between 1.8–2.1 indicates pure RNA.**

- **Fluorescent dyes**, like RiboGreen, allow for sensitive RNA quantification, even at low concentrations, and are less affected by contaminants.
- **Agilent 2100 Bioanalyzer** provides both RNA concentration and integrity, generating a gel-like image for quality assessment.

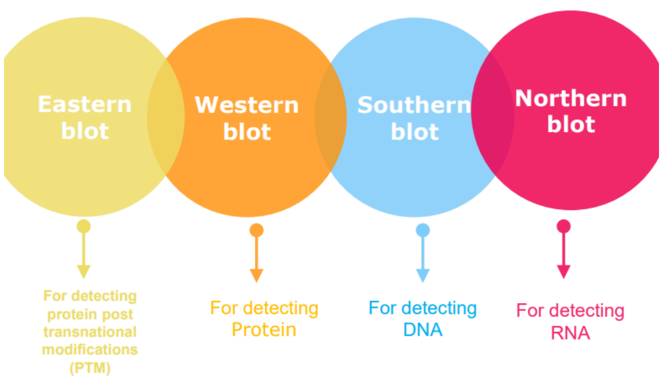


Quality of RNA isolated from tissue stored in RNA*later* reagent. Tissues were stored in RNA*later* reagent for the indicated times and RNA was purified from the tissues using Invitrogen TRIzol Reagent. Equivalent mass amounts of each RNA sample were analyzed using an Agilent 2100 Bioanalyzer instrument. The top panel shows 2100 Bioanalyzer traces of the purified RNA. The bottom panel indicates the yield based on A260 measurement.

Blotting

- **DNA Probe**: A short, labeled strand of DNA or RNA used to find its matching strand in a DNA sample.
- **Gel Electrophoresis**: A method for separating substances like DNA by how fast they move through a gel under an electric field.
- **Complementary DNA (cDNA)**: DNA made in the lab by converting mRNA into DNA using reverse transcriptase.
- **Hybridization**: The process where a single-stranded DNA probe pairs with its matching single-stranded DNA target to form double-stranded DNA.

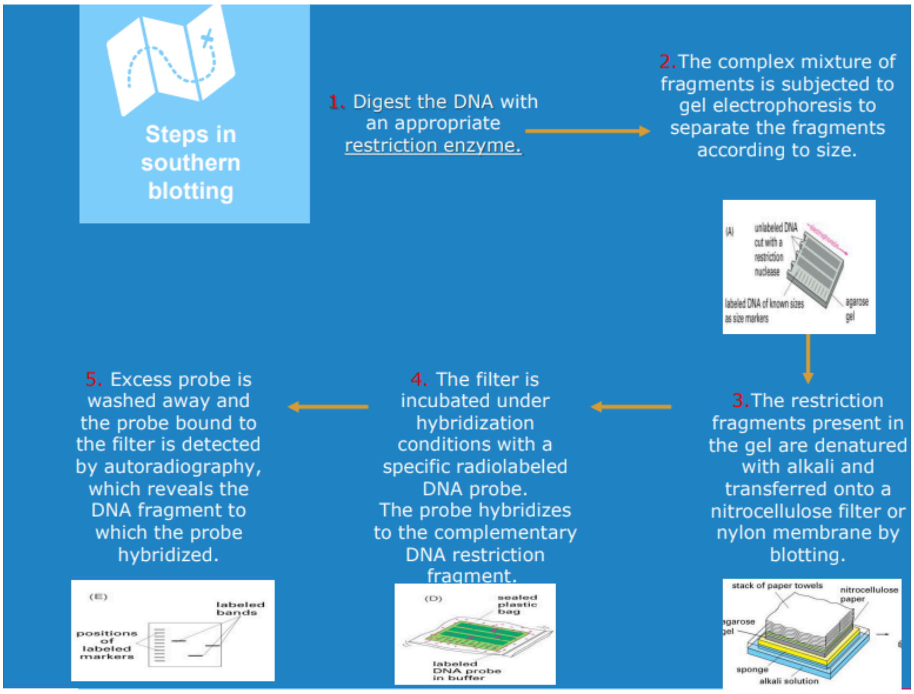
https://www.youtube.com/watch?v=AKGPrRtSu04

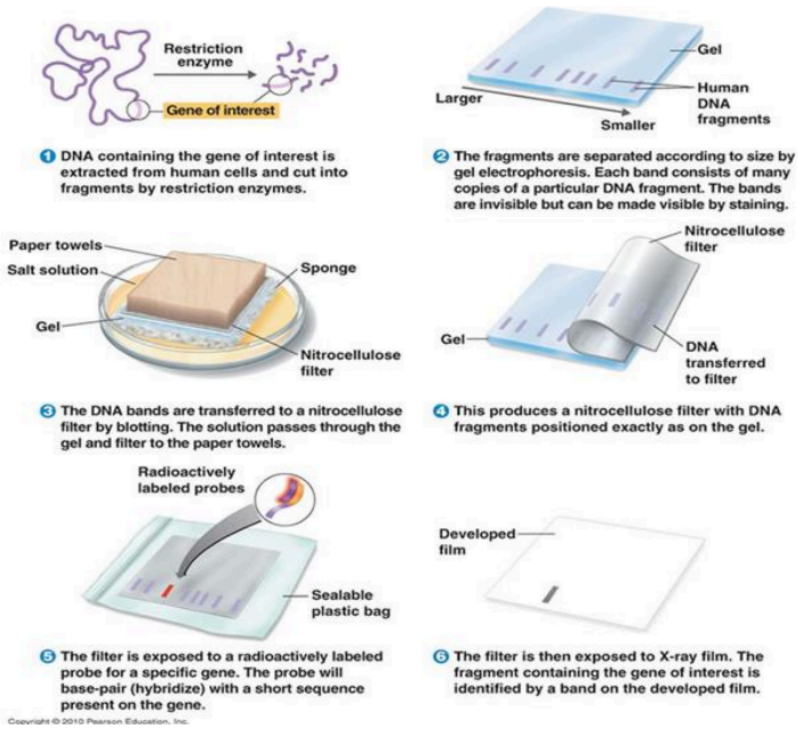


Blotting techniques are used to transfer DNA, RNA, or proteins onto a carrier for separation, often following gel electrophoresis. The **Southern blot** is for DNA, **Northern blot** for RNA, and **Western blot** for proteins. Developed by Professor Sir Edwin Southern in 1975, the Southern blot is a method for detecting specific DNA sequences.

- **Southern Blot**: A technique to detect specific DNA sequences by transferring DNA from a gel onto a filter, then using a probe for identification.
- **Applications**: Gene discovery, mapping, diagnostics, forensics, identifying transferred genes in transgenic organisms, and analyzing genetic patterns. It helps determine the molecular weight of DNA fragments and measures DNA amounts across samples.
- **Alternatives to a Southern blot include**: microarrays, next-generation sequencing (NGS), and real-time quantitative PCR (qPCR), which are generally considered more efficient and sensitive for detecting specific DNA sequences, especially when analyzing large datasets or requiring high throughput analysis.
- https://www.youtube.com/watch?v=CSrUm-EgTK4

Southern blotting is not widely used today because it is a relatively labor-intensive, time-consuming technique that requires a large amount of starting DNA, making it less efficient compared to newer methods like PCR which can amplify specific DNA sequences much faster and with smaller sample sizes; therefore, for most applications, PCR has largely replaced Southern blotting as the preferred method for detecting specific DNA sequences.





Northern Blotting

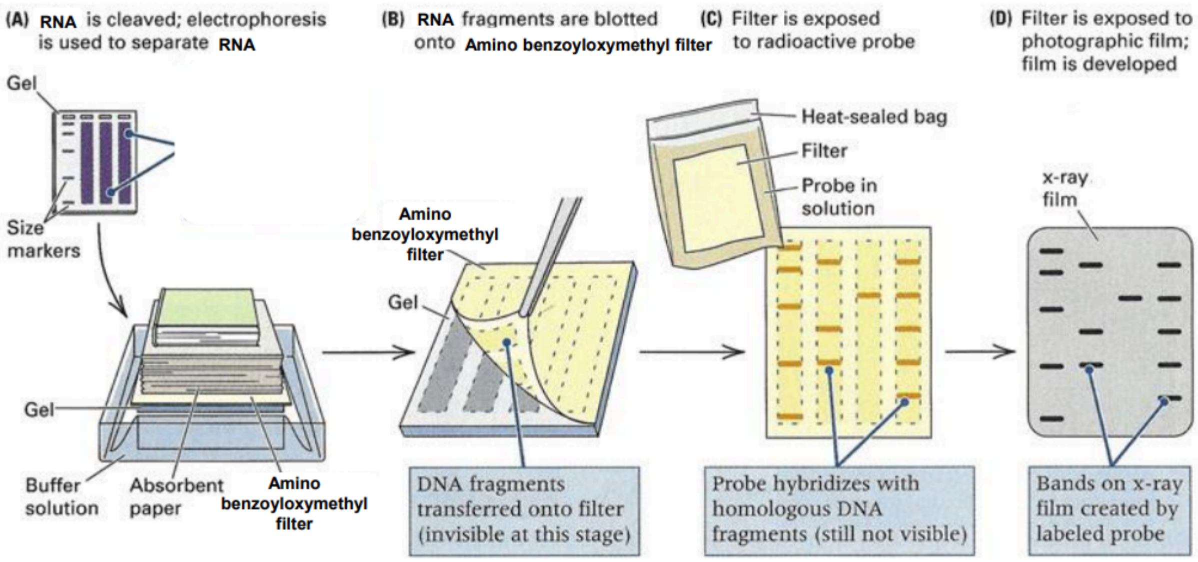
- **Definition**: Northern blotting is a technique used to detect specific RNA sequences.
- **Development**: It was developed by James Alwine and George Stark at Stanford University in 1979 and was named by analogy to Southern blotting.
- **Purpose**:
  - Detects the expression level (mRNA) and transcript size of a specific gene in a particular tissue or at a specific time.
  - Helps in identifying mutations that affect transcriptional regulatory sequences (e.g., promoter, splice sites, copy number, and transcript stability) rather than the coding regions.

Advantages:

- Can assess gene expression and transcript size.

Disadvantages:

1. **Sensitivity**: Less sensitive compared to other methods, such as nuclease protection assays and RT-PCR.
2. **Multiple Probes**: Detecting with multiple probes can be challenging.
3. **RNA Degradation**: Degraded RNA samples can severely affect data quality and the quantitation of expression.
4. **Radioactivity**: The traditional method uses radioactivity (though non-radioactive alternatives exist).
5. **Labor-Intensive**: The method is laborious, especially when testing many genes.
6. **Time-Consuming**: The assay is generally time-consuming.



https://www.youtube.com/watch?v=HoGBG2ebOzU

Northern blotting has fallen out of favor because of the perceived difficulty of working with RNA and because most people don't like working with radioactivity. In situ hybridization, quantitative real-time PCR (qRT-PCR), and membrane hybridization are some alternatives to northern blotting.

Comparison of Southern, Northern, and Western blotting techniques

	Southern blotting	Northern blotting	Western blotting
Molecule detected	DNA (ds)	mRNA (ss)	Protein
Gel electrophoresis	Agarose gel	Formaldehyde agarose gel	Polyacrylamide gel
Gel pretreatment	Depurination, denaturation, and neutralization	-	-
Blotting method	Capillary transfer	Capillary transfer	Electric transfer
Probes	DNA Radioactive or nonradioactive	cDNA, cRNA Radioactive or nonradioactive	primary antibody
Detection system	Autoradiography Chemiluminescent Colorimetric	Autoradiography Chemiluminescent Colorimetric	Chemiluminescent Colorimetric