# Optimizing RNA Preparation and Analysis

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

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## 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.

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## 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.

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## 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.

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

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## 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.

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## 2. Fluorescent Dye Method (RiboGreen)

- **RiboGreen dye** binds RNA and increases fluorescence.
- Detects very low RNA levels (as little as **1 ng/mL**).
- Results are compared to a standard curve made from a known RNA sample.
- Accurate over a wide range (**1 ng/mL to 1 µg/mL**).

**Tips for Best Results:**

- **Remove DNA**, as the dye also binds DNA.
- Use **nonstick plastic tubes** to avoid dye sticking to sides.
- **Protect dye from light** and use it soon after preparing.
- Avoid **repeated freezing and thawing** of RNA samples.

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## 3. Agilent 2100 Bioanalyzer

- Combines **microfluidics and fluorescence** to measure both RNA **concentration and quality**.
- Uses a **chip system** to analyze RNA and compare it to a known ladder.

- Two versions:
    - **Nano Chip**: Measures 25–500 ng/µL
    - **Pico Chip**: Measures 50–5,000 pg/µL

**Key Feature:**

- Gives an **RNA Integrity Number (RIN)** from 1 to 10. The higher the number, the better the RNA quality.

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## 4. RNA Storage Tips

After RNA isolation, store it carefully to avoid degradation.

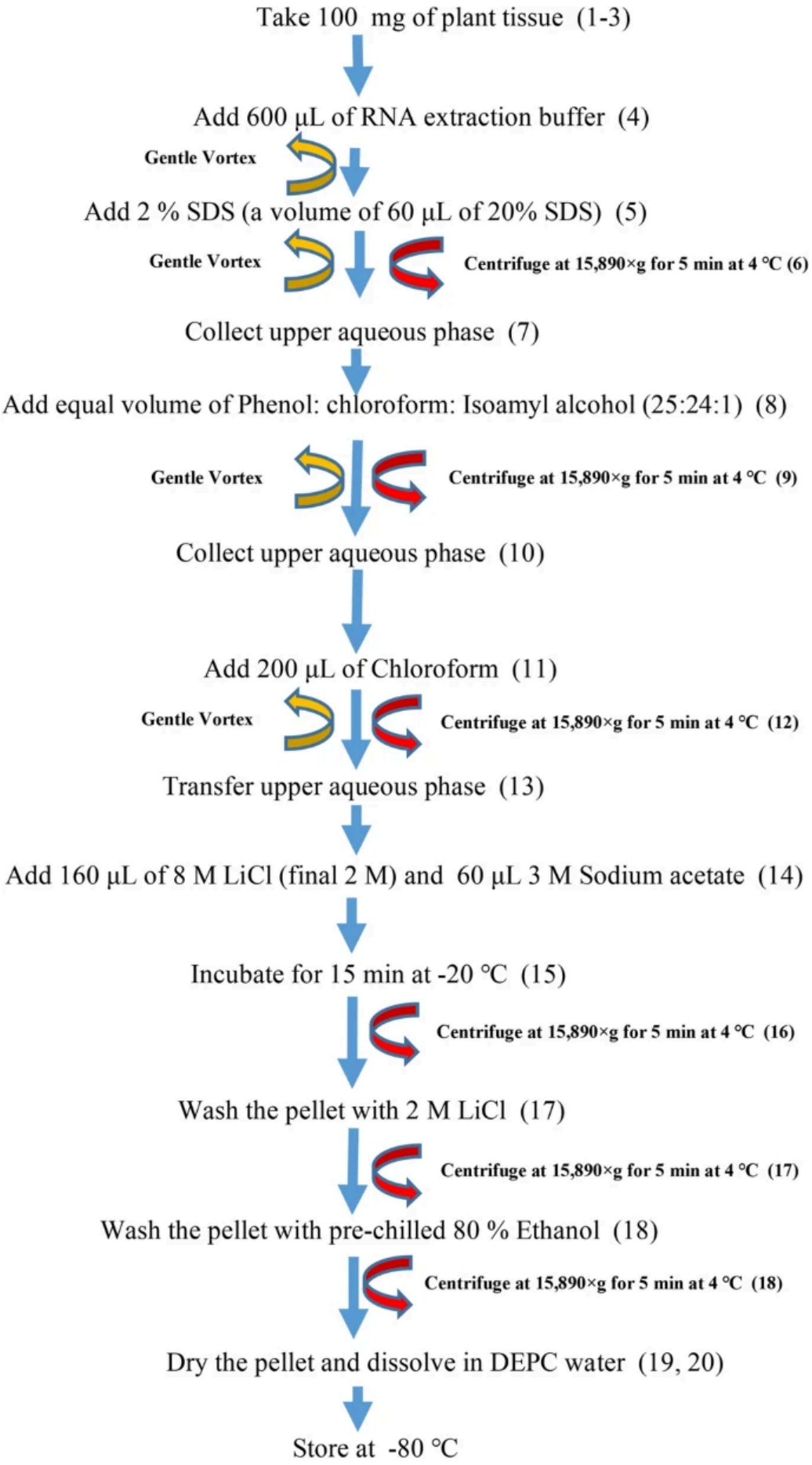
**Best Storage Practices:**

- 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** |


Take 100 mg of plant tissue (1-3)
Add 600 µL of RNA extraction buffer (4)
Gentle Vortex
Add 2 % SDS (a volume of 60 µL of 20% SDS) (5)
Gentle Vortex
Centrifuge at 15,890×g for 5 min at 4 °C (6)
Collect upper aqueous phase (7)
Add equal volume of Phenol: chloroform: Isoamyl alcohol (25:24:1) (8)
Gentle Vortex
Centrifuge at 15,890×g for 5 min at 4 °C (9)
Collect upper aqueous phase (10)
Add 200 µL of Chloroform (11)
Gentle Vortex
Centrifuge at 15,890×g for 5 min at 4 °C (12)
Transfer upper aqueous phase (13)
Add 160 µL of 8 M LiCl (final 2 M) and 60 µL 3 M Sodium acetate (14)
Incubate for 15 min at -20 °C (15)
Centrifuge at 15,890×g for 5 min at 4 °C (16)
Wash the pellet with 2 M LiCl (17)
Centrifuge at 15,890×g for 5 min at 4 °C (17)
Wash the pellet with pre-chilled 80 % Ethanol (18)
Centrifuge at 15,890×g for 5 min at 4 °C (18)
Dry the pellet and dissolve in DEPC water (19, 20)
Store at -80 °C

## Key Factors for Optimizing RNA Preparation:

1. **Sample Collection and Handling**
   It's important to choose the right method for cell or tissue disruption to maximize RNA yield and quality. Freezing samples immediately after collection can help preserve RNA, especially when working with hard tissues or when rapid processing isn't possible. RNAlater and RNAlater-ICE solutions stabilize RNA for days or even months without degradation, making RNA extraction more flexible. RNAlater also works well with many RNA extraction methods and doesn't affect RNA quality, yield, or analysis.

2. **RNA Preparation**
   Different RNA extraction methods include organic extraction, spin basket formats, magnetic particle methods, and direct lysis. Each method has pros and cons, and the right one depends on sample type, sample size, and throughput needs.

   - **Organic extraction** is considered the gold standard for RNA purification but is labor-intensive and difficult to automate.
   - **Spin baskets** are easy to use and suitable for higher throughput but may clog or retain large nucleic acids.
   - **Magnetic particles** offer fast and efficient RNA collection with minimal clogging and can be automated.
   - **Direct lysis** methods are quick and easy but may not allow for traditional yield measurements and can be prone to RNase contamination if not handled carefully.

3. **Quantifying RNA**
   It's important to measure RNA concentration before analysis. Common methods include:

   **Beer-Lambert Law for calculating UV absorbance by nucleic acid**

   $$A = \in C l$$

   Where,

   A = absorbance at a particular wavelength

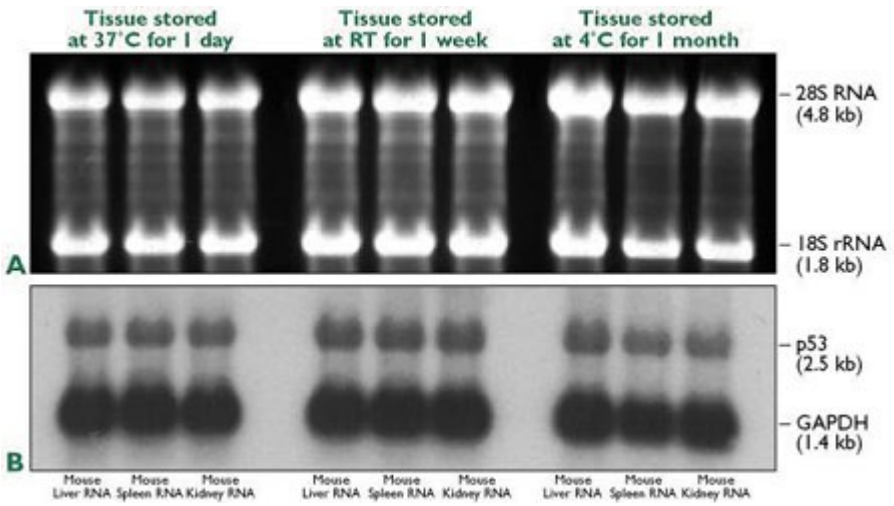
   C = concentration of nucleic acid

   l = path length of the spectrophotometer cuvette (typically 1 cm)

   $\in$ = the extinction coefficient ($\in$ for RNA is 0.025 $(mg/ml)^{-1}cm^{-1}$)

   **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.

## RNA Precipitation Methods

There are two common methods for precipitating RNA:

1. Alcohol Precipitation: RNA is precipitated by adding isopropanol or ethanol with a salt solution. Isopropanol works well in the presence of ammonium ions, and it helps separate free nucleotides.
2. Lithium Chloride Precipitation: Lithium chloride (LiCl) is used to precipitate RNA, particularly from solutions that contain inhibitors of translation. This method does not precipitate other biomolecules like proteins or DNA.

## RNA Yield and Integrity

1. Measuring RNA Yield: RNA concentration can be measured using a spectrophotometer at 260 nm. One unit of absorbance (A260) corresponds to 40 µg RNA/mL.
2. Checking RNA Integrity: RNA can be visualized using RNA size markers and electrophoresis. The ribosomal RNA bands (18S and 28S) are important markers for integrity.

## RNA Storage

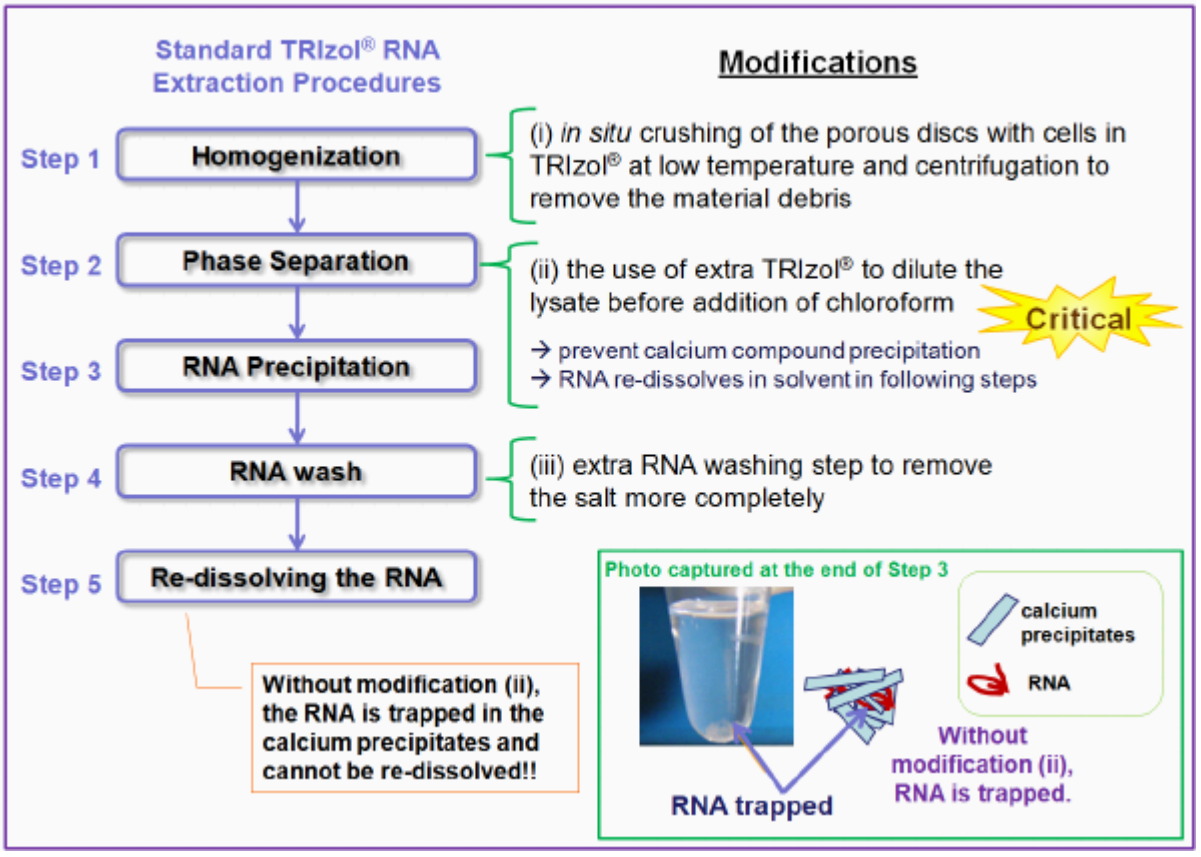
1. Short-Term Storage: Store RNA at -80°C with RNase-free water or TE buffer (Tris and EDTA).
2. Long-Term Storage: For long-term storage, RNA stabilizers help maintain RNA integrity. EDTA should be used to chelate metal ions that can cause RNA degradation.

**Table 1. Ribosomal subunit sizes in representative eukaryotes**

| | Average number of bases | |
|---|---|---|
| **Organism** | **18S** | **28S** |
| ***Drosophila*** | 1,976 | 3,898 |
| **Rat** | 1,874 | 3,898 |
| **Human** | 1,868 | 5,025 |

Choose marker accordingly

https://pmc.ncbi.nlm.nih.gov/articles/PMC2789530/

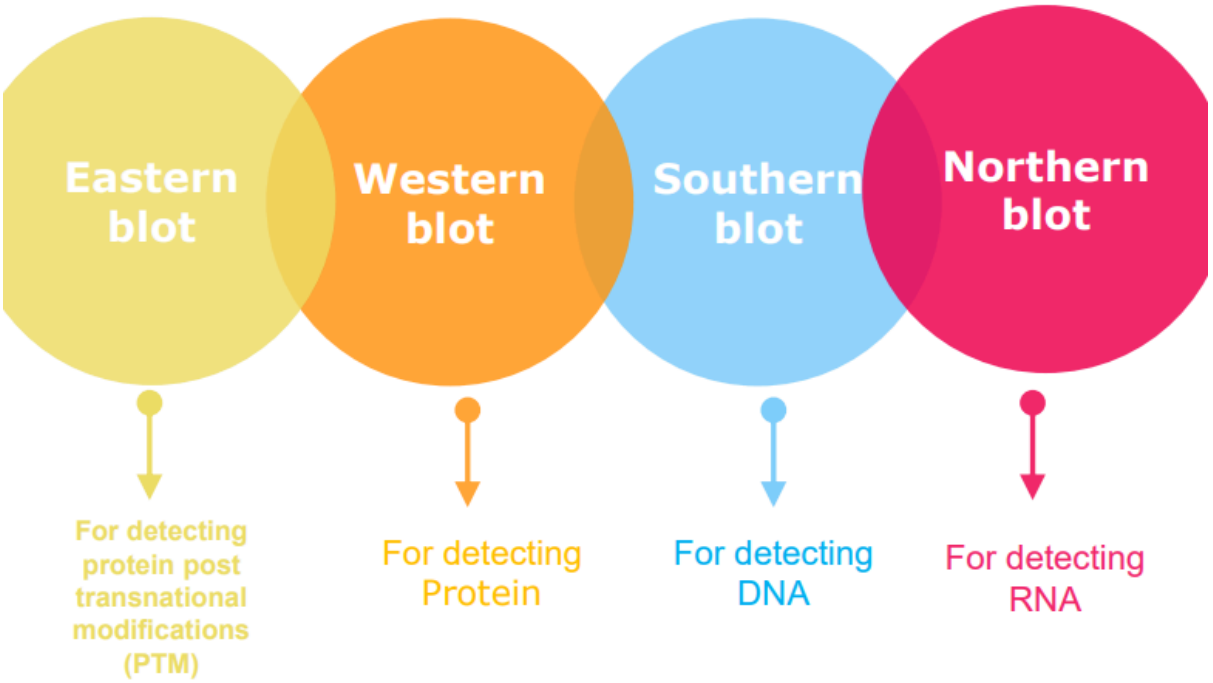



https://blog.quartzy.com/how-to-extract-rna-from-trizol

# 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

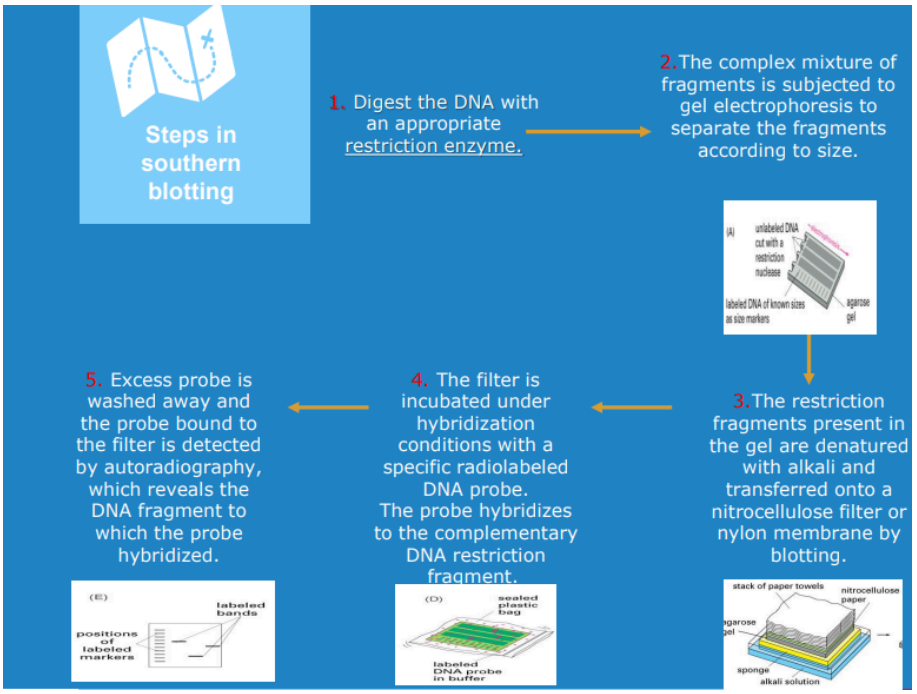


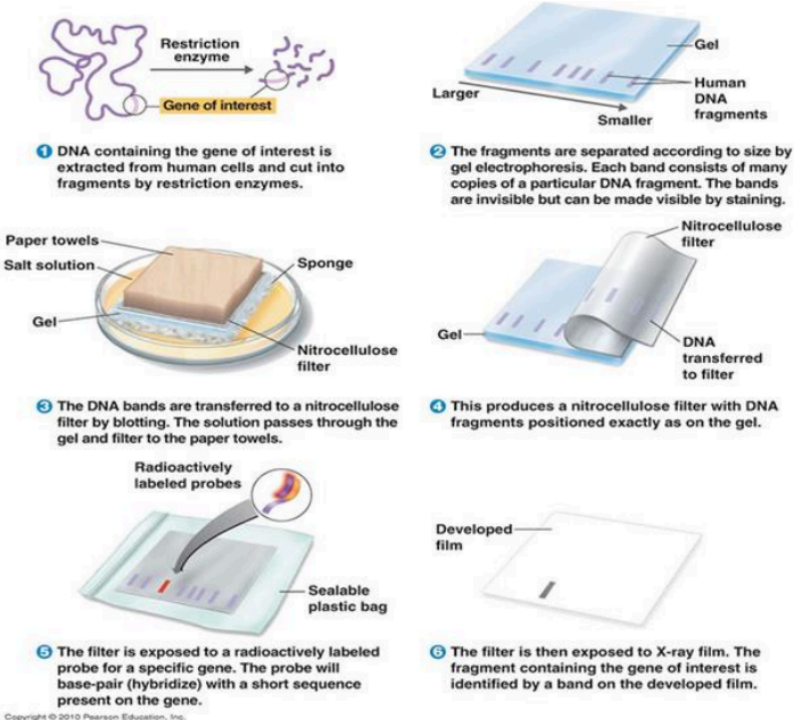

Blotting techniques are used to transfer DNA, RNA, or proteins onto a carrier for separation, often following gel electrophoresis. The **Southern blot** is for DNA, **Northern blot** for RNA, and **Western blot** for proteins. Developed by Professor Sir Edwin Southern in 1975, the Southern blot is a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

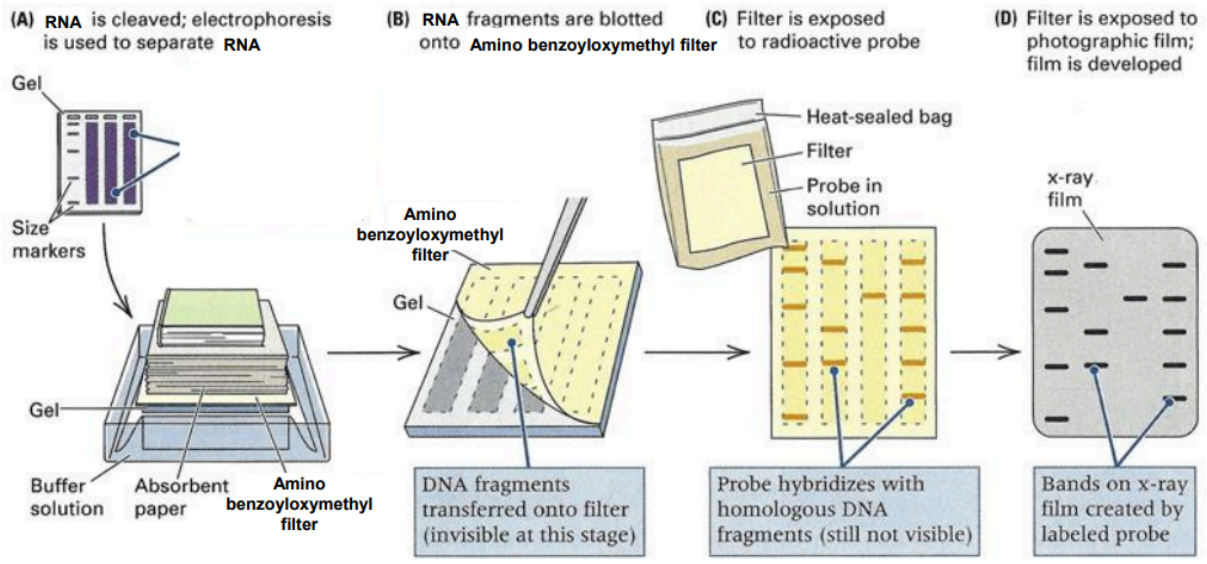
- **Definition**: Northern blotting is a technique used to detect specific RNA sequences.
- **Development**: It was developed by James Alwine and George Stark at Stanford University in 1979 and was named by analogy to Southern blotting.
- **Purpose**:
    - Detects the expression level (mRNA) and transcript 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 |