RNA EXTRACTION

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15th Anniversary biosciences eastern and central africa

NEPAD TRANSFORMING AFRICA

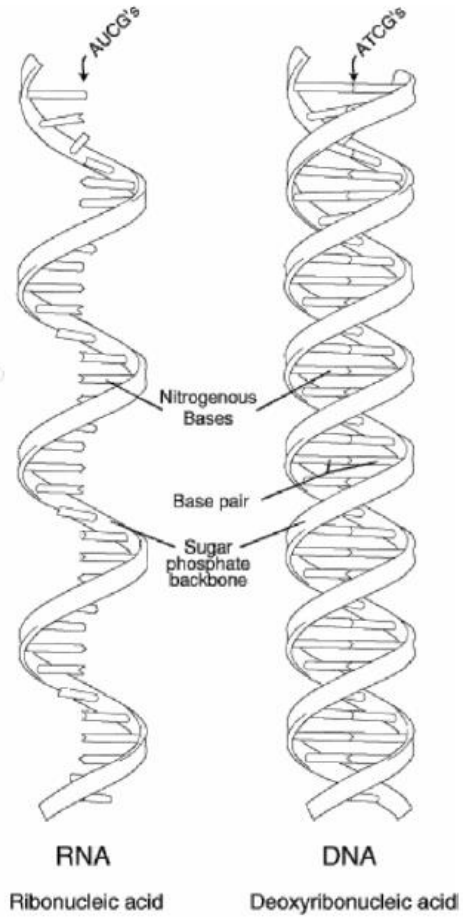
ILRI INTERNATIONAL LIVESTOCK RESEARCH INSTITUTE

Nucleic acid

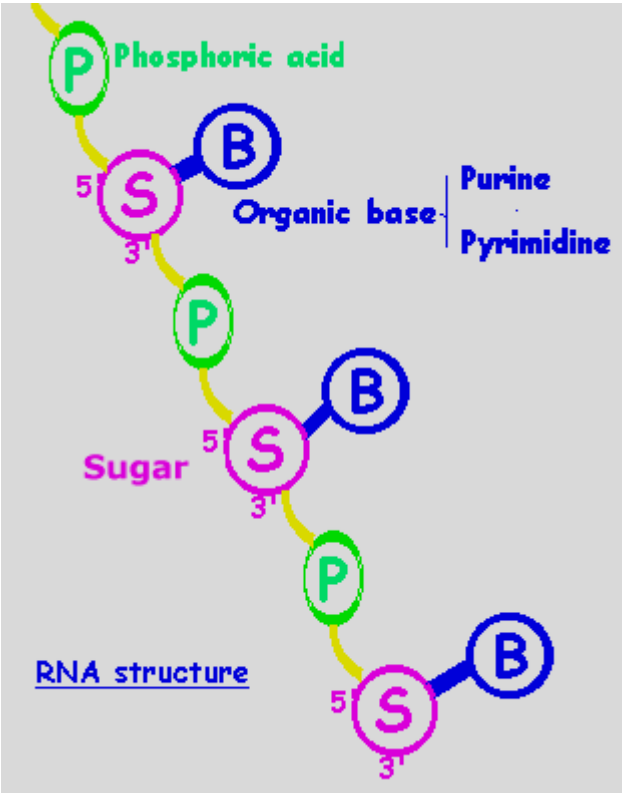
•DNA (deoxyribonucleic acid) and RNA (ribonucleic acid) store and transfer genetic information in living organisms.

- **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



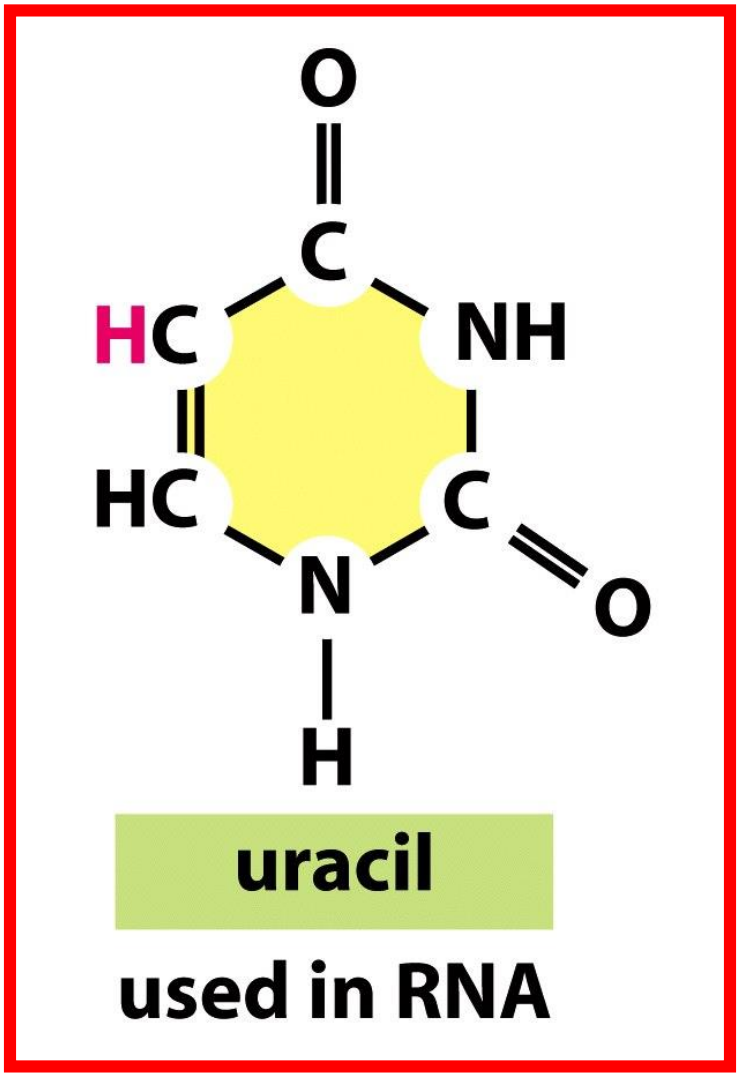
RNA

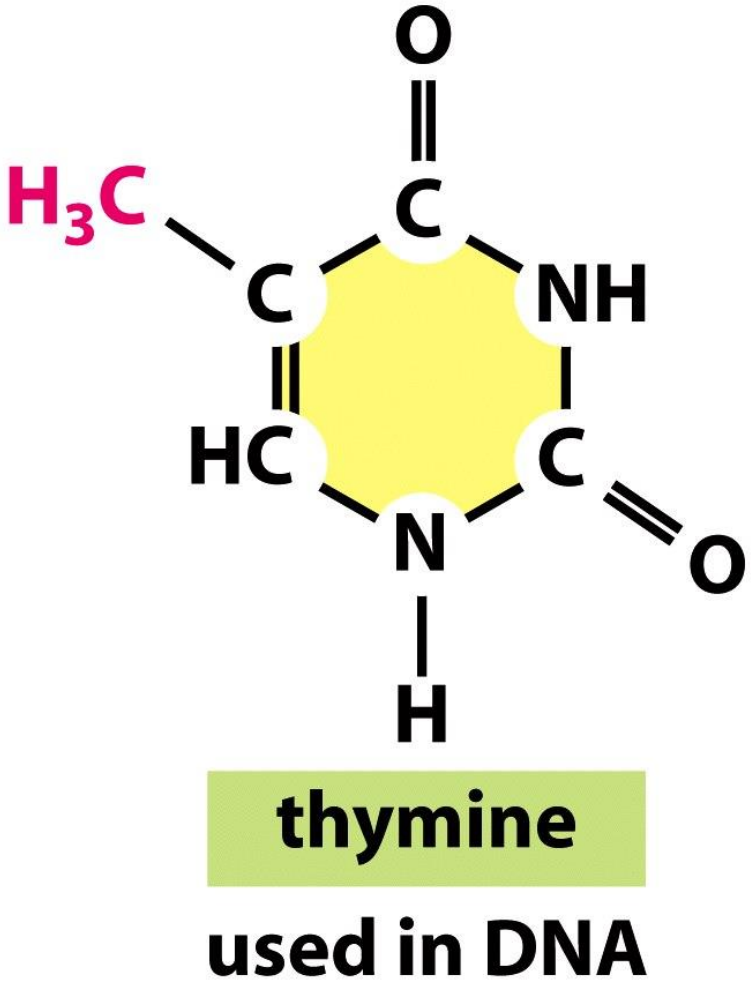


R	Ribo: the pentose has an oxygen in position 2.
N	Nucleic: these molecules were first found in the nucleus of the cell..
A	Acid: the acid groups of the phosphoric acid, used to form the RNA chain.

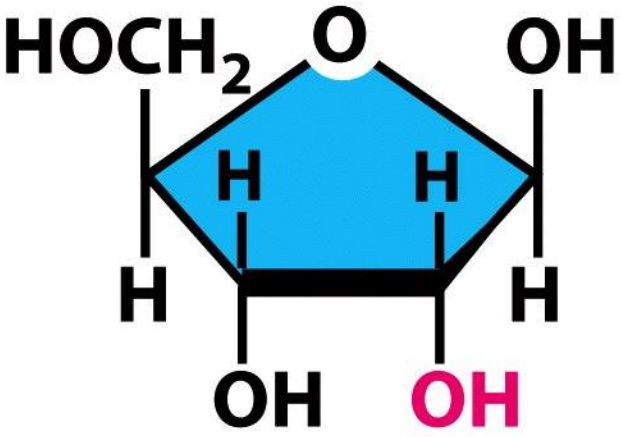
Major Differences Between DNA and RNA

RNA Has a Uracil Instead of Thymine



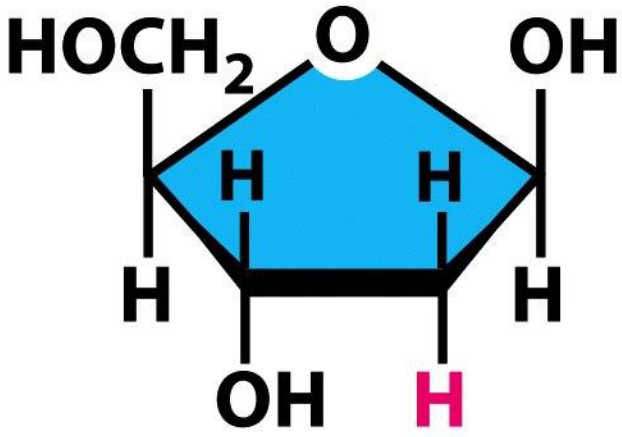


RNA Has Ribose Sugar in Nucleotide



ribose

used in ribonucleic acid (RNA)

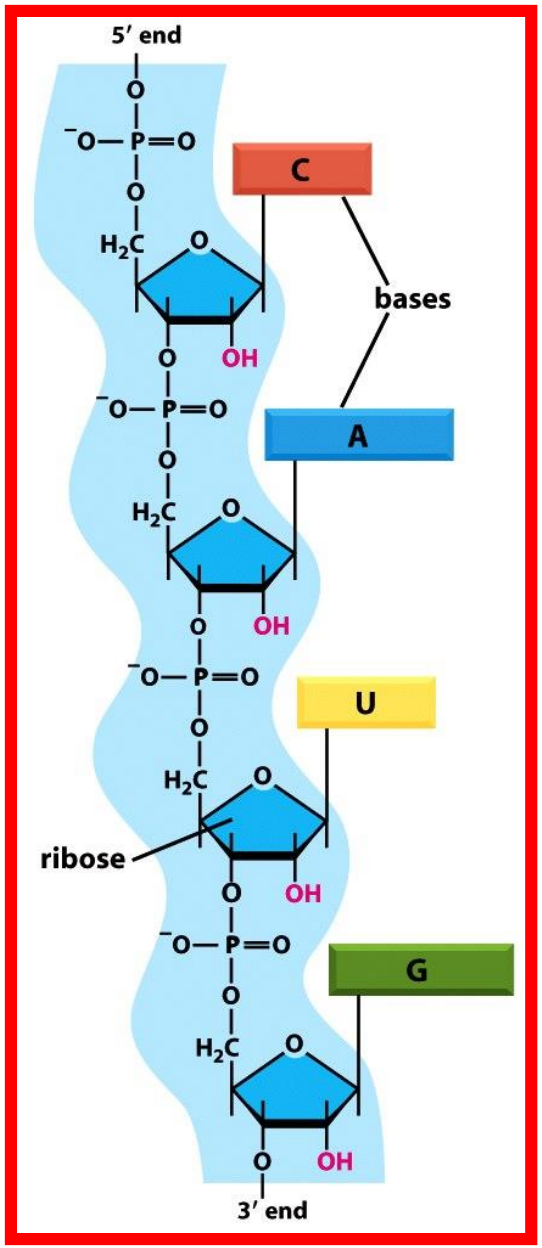


deoxyribose

used in deoxyribonucleic acid (DNA)

Eukaryotic RNAs Are Single-Stranded Polynucleotides

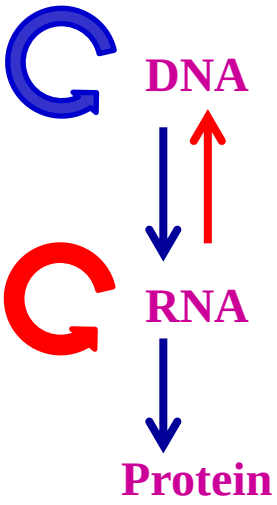
Nucleotides Joined By Phosphodiester Bonds Like All Nucleic Acids

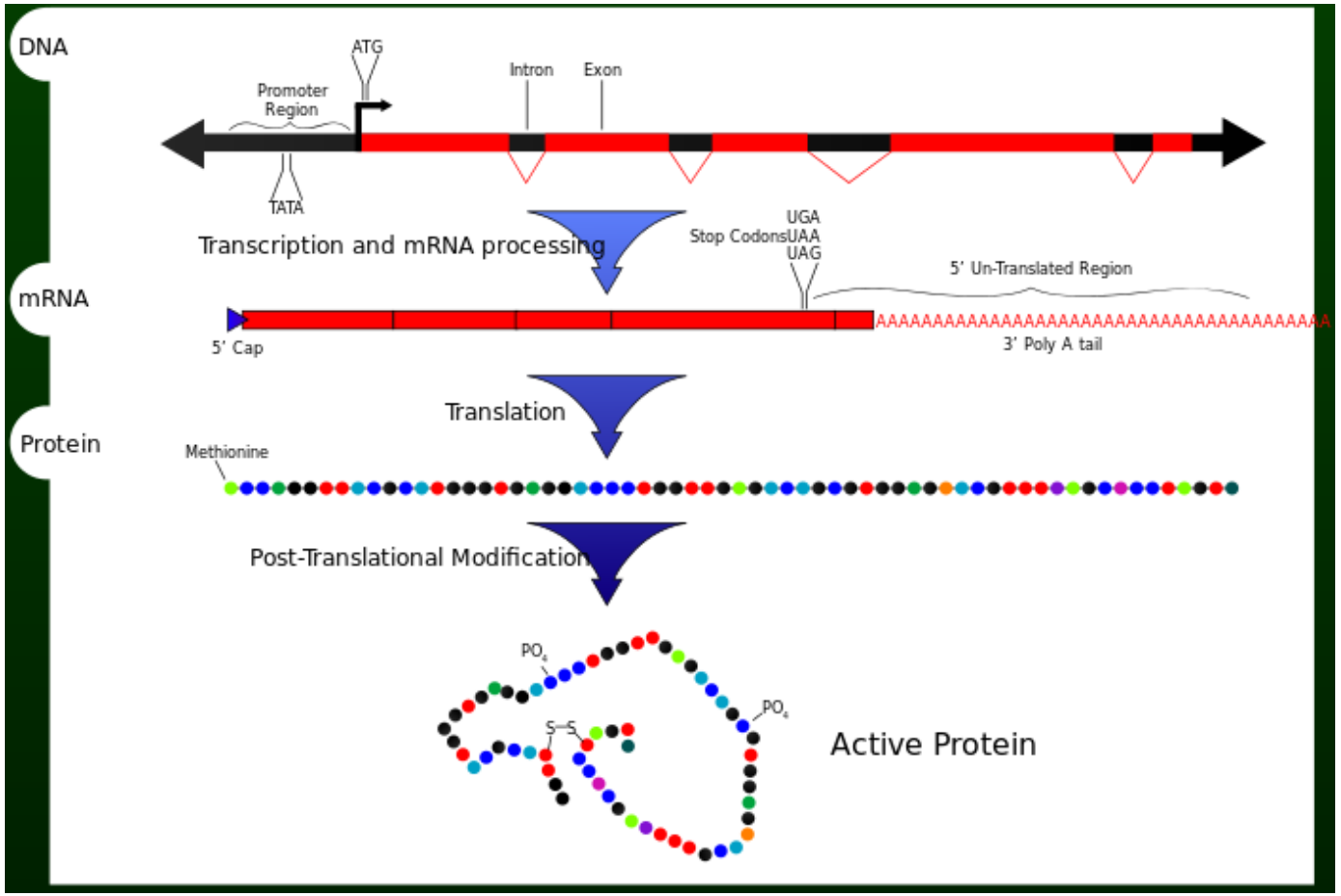


Eukaryotic model of central dogma of molecular biology

Flow of genetic information in a biological system

Crick, F.H.C. (1956)

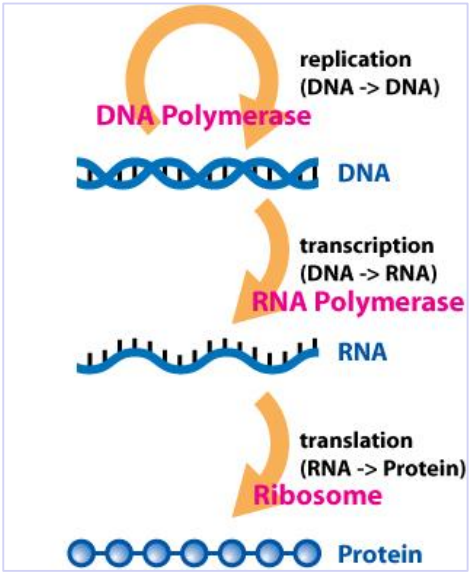




Some "primitive" organisms like retroviruses still use RNA as the carrier of the genetic information **(reverse central dogma.** e.g. HIV**)**.

Main Enzymes in the Flow of Genetic Information

A



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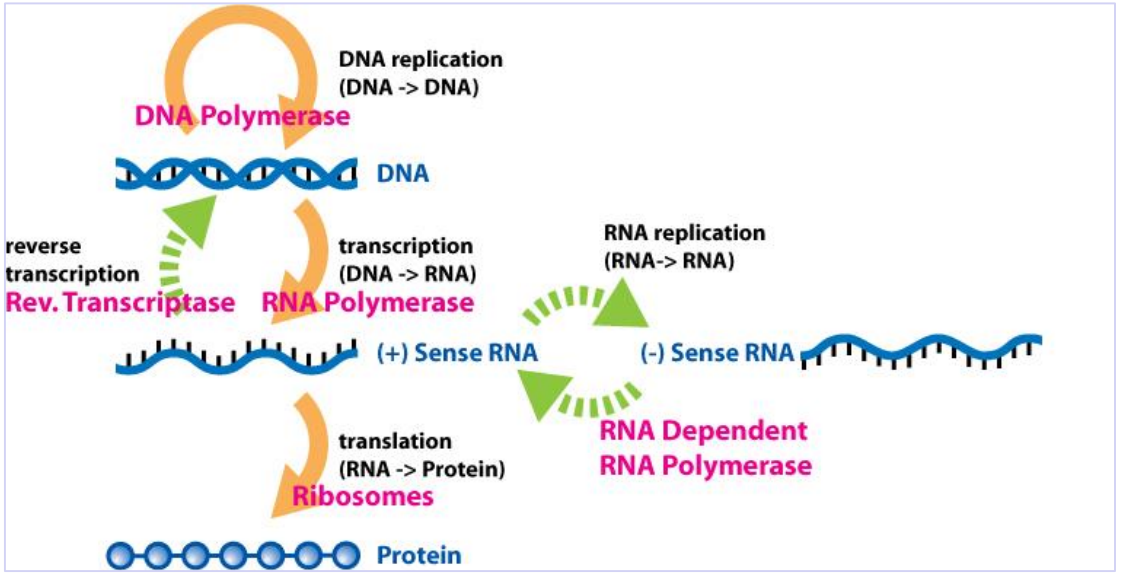


Transcription

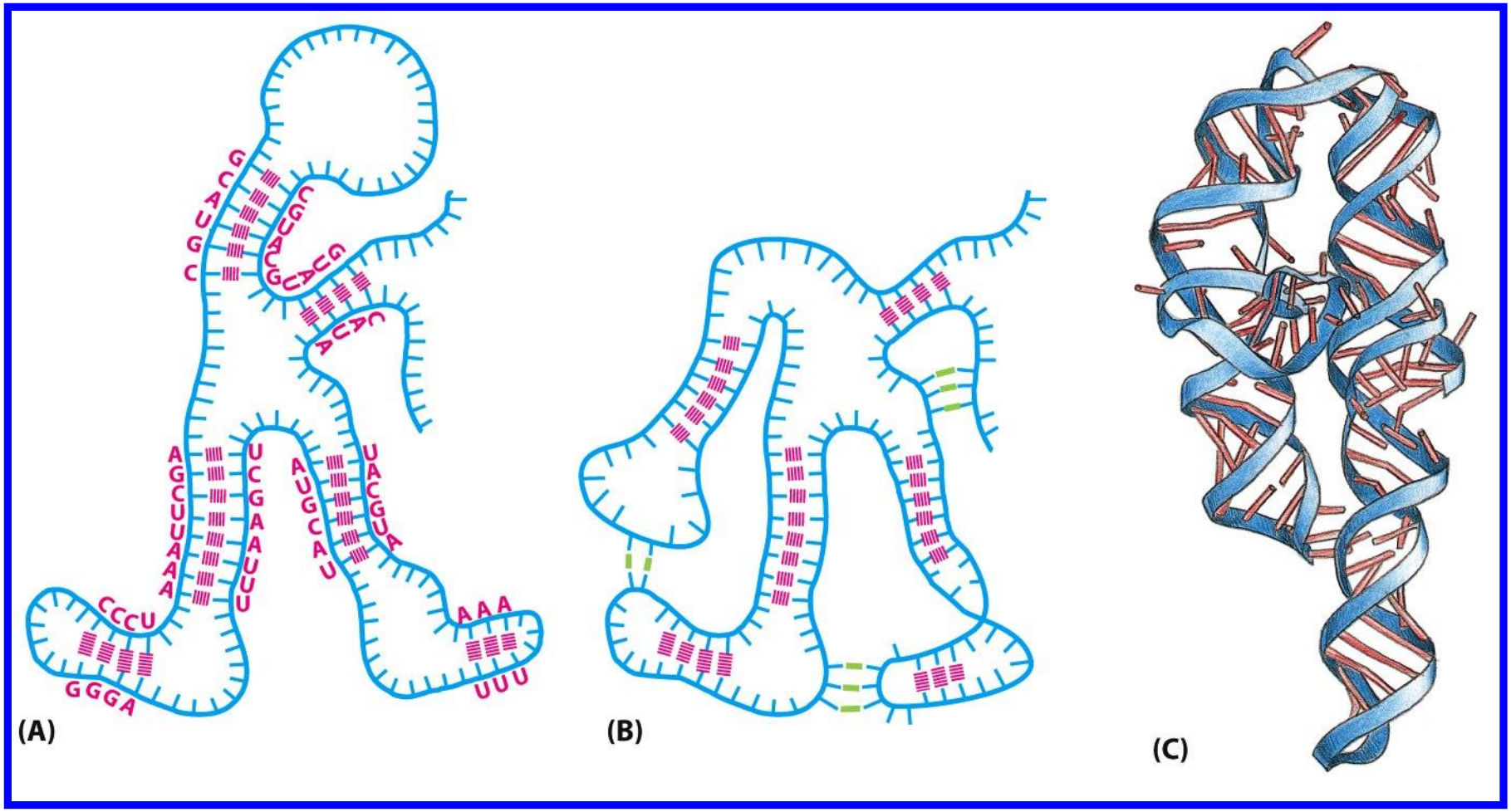
RNA pol. transcribes from 5' to 3' direction

```
5' ATG GCT GGC TTC ATG GCT GGC TTC  3'
3' TAC CGA CCG AAG TAC CGA CCG AAG  5'
```

- The strand from which RNA pol. copies is called the **antisense** or template strand, and the other strand, to which it is identical, the **sense** or **coding** strand.

- Since there is no nucleus in prokaryotes, ribosomes can begin making protein from an mRNA immediately upon its synthesis.

RNA Has Intra-Strand Double Helices or Secondary Structure



There is more than one RNA...

Some RNA are found in the nucleus, where they are synthesised, and in the cytoplasm, where they are transported; or in organelles.

1-Messenger RNA or **mRNA** (1-5%): carries the genetic **information** out of the nucleus for protein synthesis.

2-Transfer RNA or **tRNA** (10-15%): **decodes** the information.

3-Ribosomal RNA or **rRNA** (>80%): constitutes 50% of a ribosome, which is a molecular assembly involved in protein synthesis.

4-Catalytic RNA: involved in many reactions in the cytoplasm of the cell.

- **Guide RNA or gRNA**: A short 3'-uridylated RNA that can form a perfect duplex (except for the oligo[U] tail) with a stretch of mature edited mRNA.
- **The tmRNA** (earlier called "10S RNA") has properties of tRNA and mRNA combined in a single molecule.

More RNAs?

It was long thought that all enzymes were proteins.
RNA can in some cases act as an enzyme (ribozymes).

- **Ribozymes** are capable of cleaving other RNA molecules (substrates) in a sequence-specific manner.

- **siRNA** (small interfering RNA: 20-25nt) also mediates mRNA degradation and inhibition of gene expression.

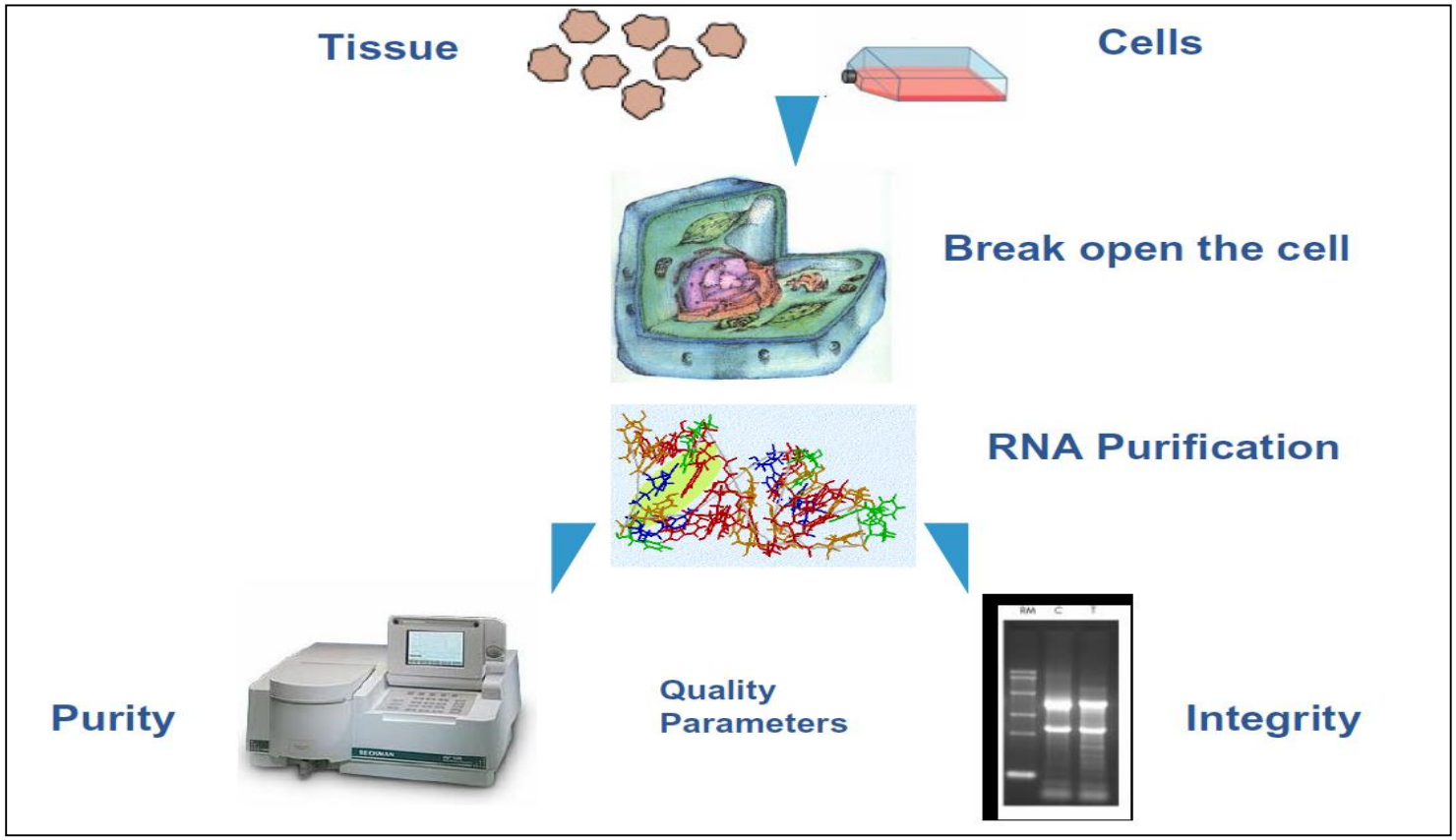
What RNA is needed for?

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

Purification of RNA

– Cells or tissue must be rapidly and efficiently disrupted
– Inactivate RNases
– Denature nucleic acid-protein complexes
– RNA selectively partitioned from DNA and protein
– Different tissues/sources raises different issues



Purification of RNA

- Improved methods use potent denaturing agents such as **guanidine HCl** and **guanidinium thiocyanate** in combination with reducing agents such as β-**mercaptoethanol** and **dithiothreitol**.

- Chaotropic agents such as guanidine inactivate and precipitate RNases and other proteins

- **RNases** are inactivacted by 4 M guanidinium thiocyanate and 1% β-mercaptoethanol.

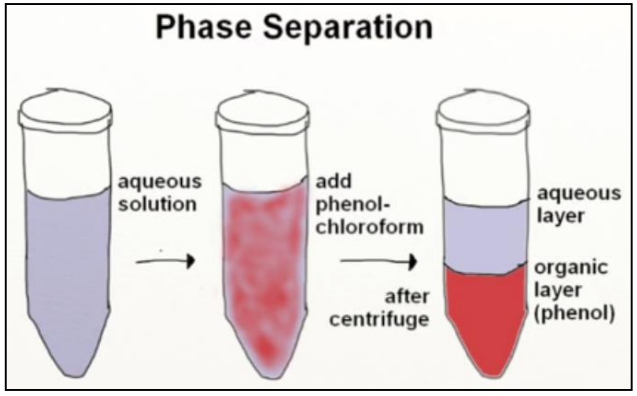
- Proteins are removed by **phenol/chloroform** extraction.

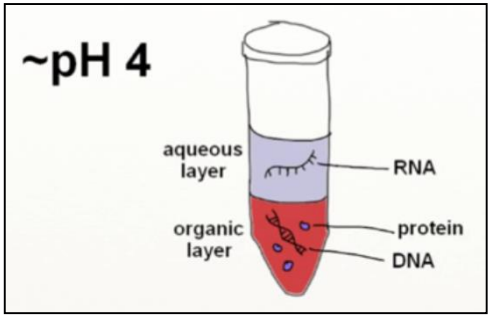
Purification of RNA

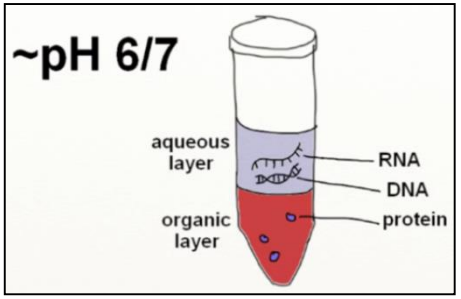
Total RNA and mRNA (from biological samples or total RNA)

- Organic extraction (e.g.: Trizol reagent)
- Affinity purification (e.g.: Oligo(dT) resins)

Principle of RNA Extraction with Trizol

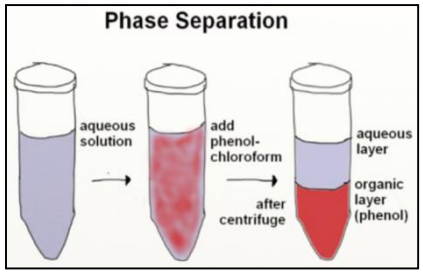


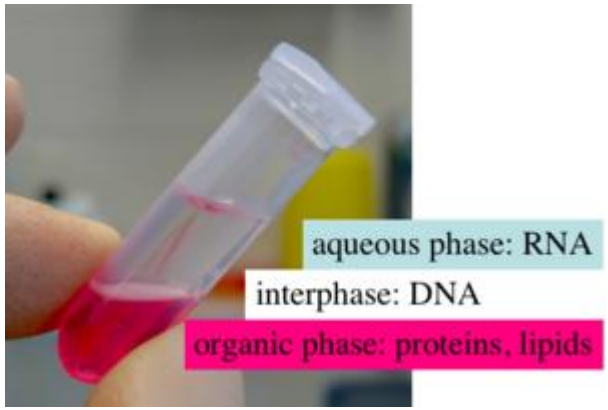


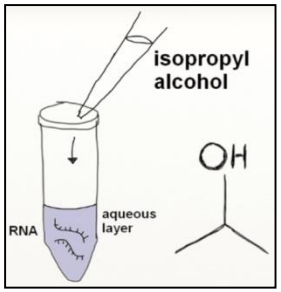


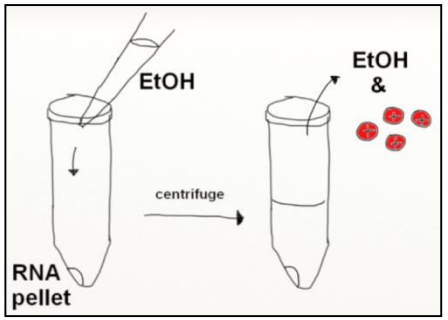
RNA Extraction Using Trizol

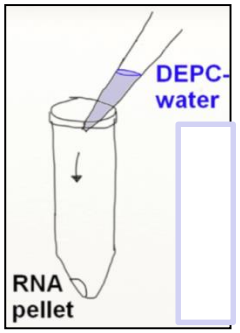
- Lyse cells/tissue in the lysis buffer and centrifuge to remove particles
- Transfer the supernatant in a fresh tube then proceed to phase separation by adding and mixing with phenol-CHCl3
- Centrifuge to separate phases











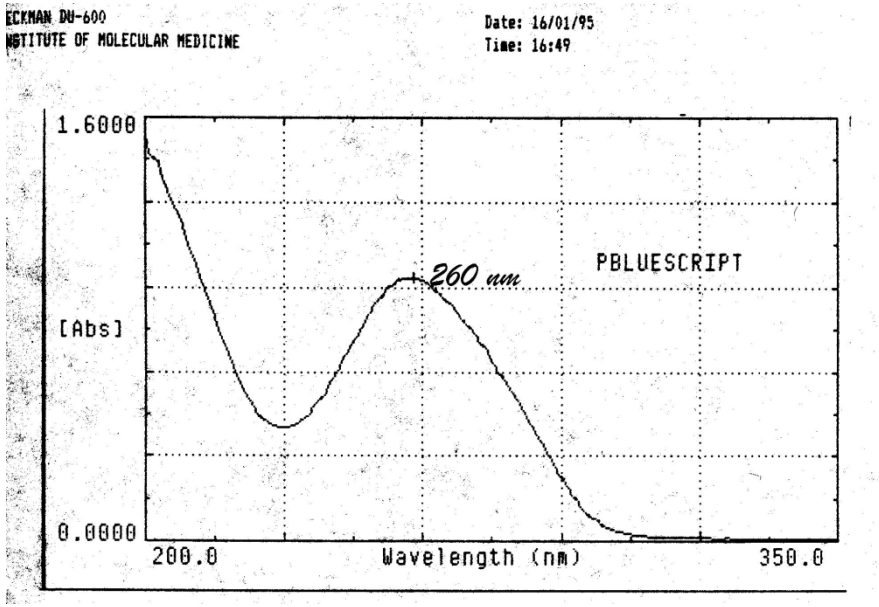
Analysis of RNA: Spectrophotometer and gel electrophoresis

1-UV absorbance: Photometric measurement of RNA concentration

-The pick of absorption for RNA is 260 nm and a solution whose absorbance at 260 (A_{260}) = 1 contains ~ 40µg RNA/ml. => Conc=40xOD_{260}

-Proteins have 2 picks (at 230 and 280 nm). The value of these picks increase with the amount of protein in the RNA preparation.

-For a good RNA solution, the ratios of A_{260}/A_{230} and A_{260}/A_{280} must be about 2.2 and 2.0, respectively.



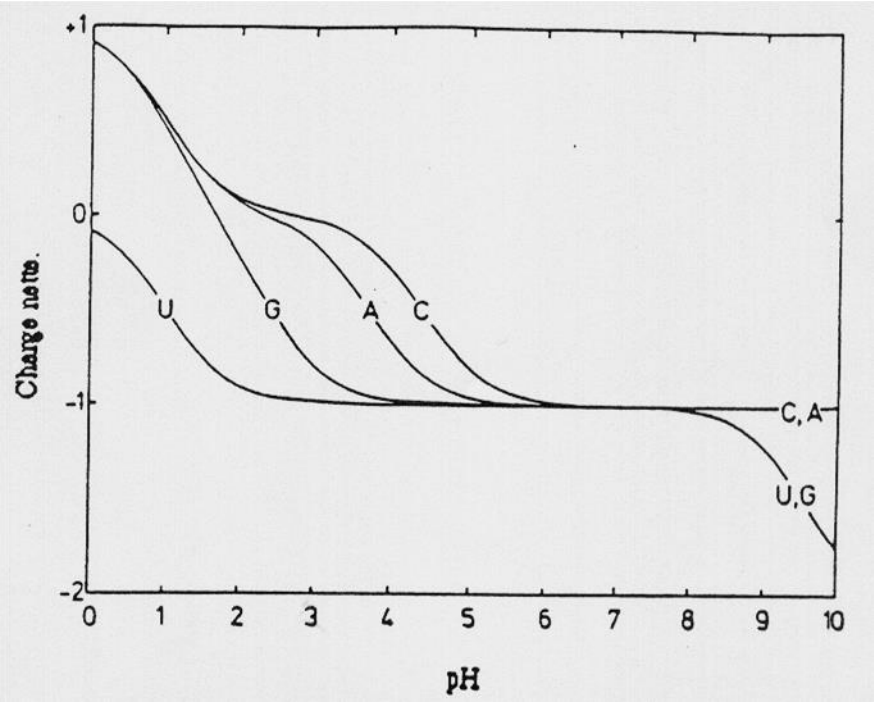
Analysis of RNA: Spectrophotometer and gel electrophoresis

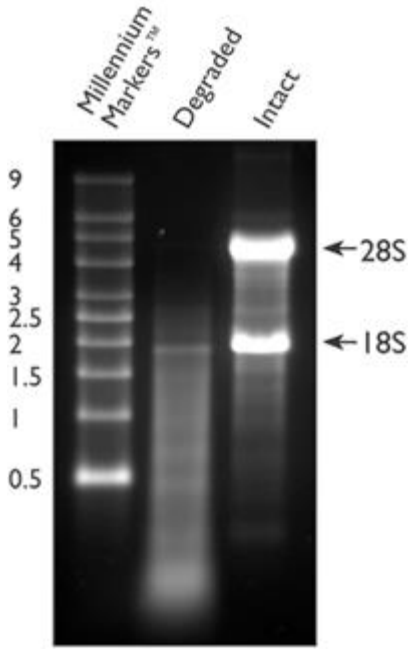
2-Gel electrophoresis

-The ionisation of the RNA molecule is highly affected by the pH of the medium.

-The speed of migration of the RNA depends on its size, form and net charge.

-The net charge dictates the direction of migration.





Contribution of a nucleotide to the net charge of an RNA molecule

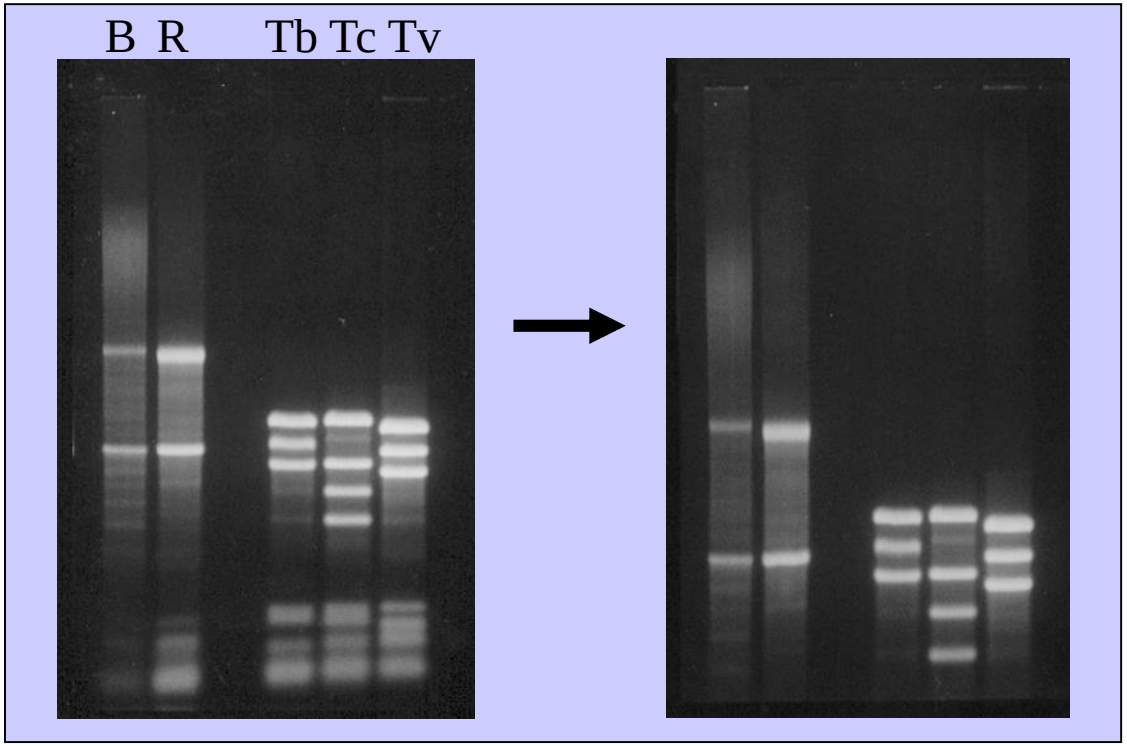
Agarose Gel Electrophoresis

- Standard agarose gel electrophoresis separate RNA in the presence of chemicals such as:
  - -Methylmercury hydroxyde;
  - -Formaldehyde/ formamide;
  - -Glyoxal/DMSO.

- Some of these chemicals are extremely toxic.

- Gels need to be treated then stained with EtBr after electrophoresis to visualize RNA bands.

New method is simple, faster and practical:
Ref: **Pelle R, Murphy NB.** Nucleic Acids Res. 1993 Jun 11;21(11):2783-2784.



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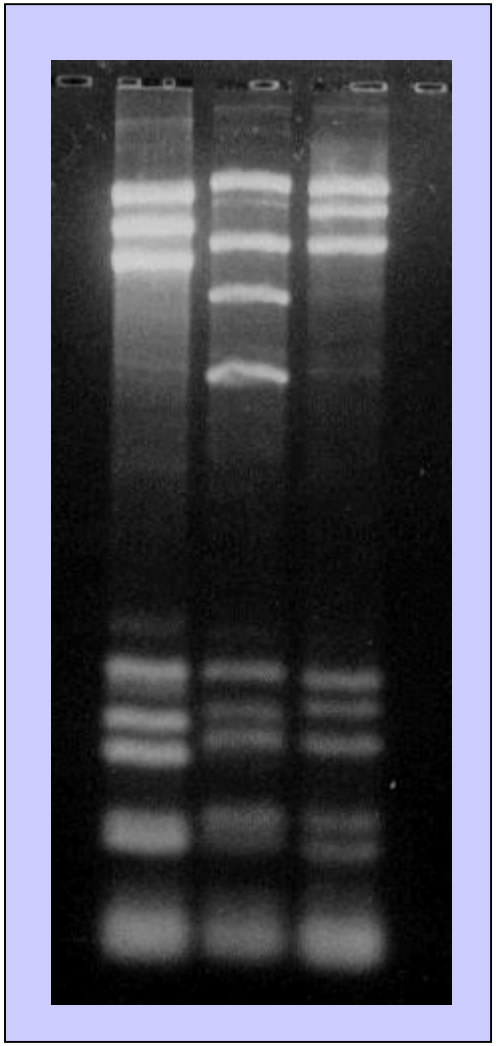

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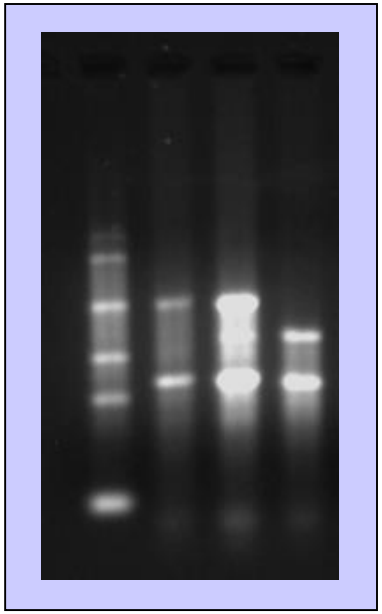
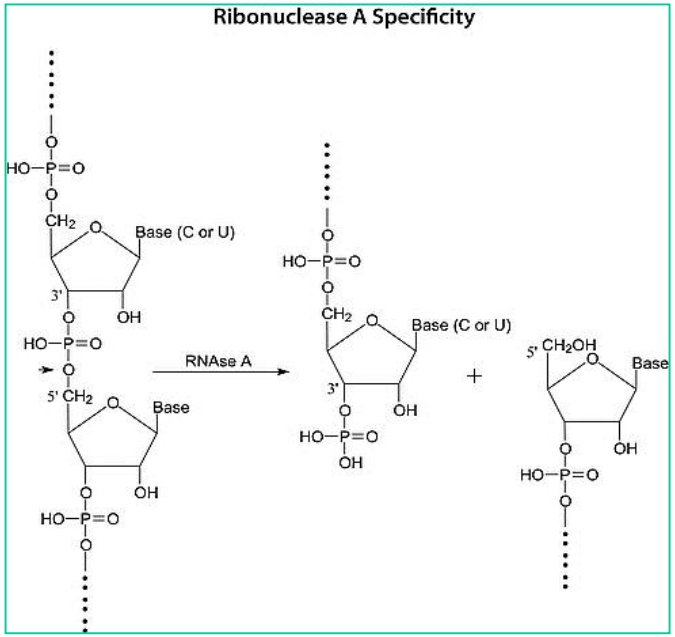


C

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

- RNase A is an endoribonuclease that attacks at the 3'OHphosphate of a pyrimidine nucleotide (U, T, C).



RNAse A solution preparation

The protocol below allows to obtain RNAse A (13.7 kDa) that is free of DNAse.

1. Dissolve RNAse A at a concentration of 10 mg/ml in 0.01M sodium acetate (pH 5.2);

2. Heat to 100°C for 15 minutes;

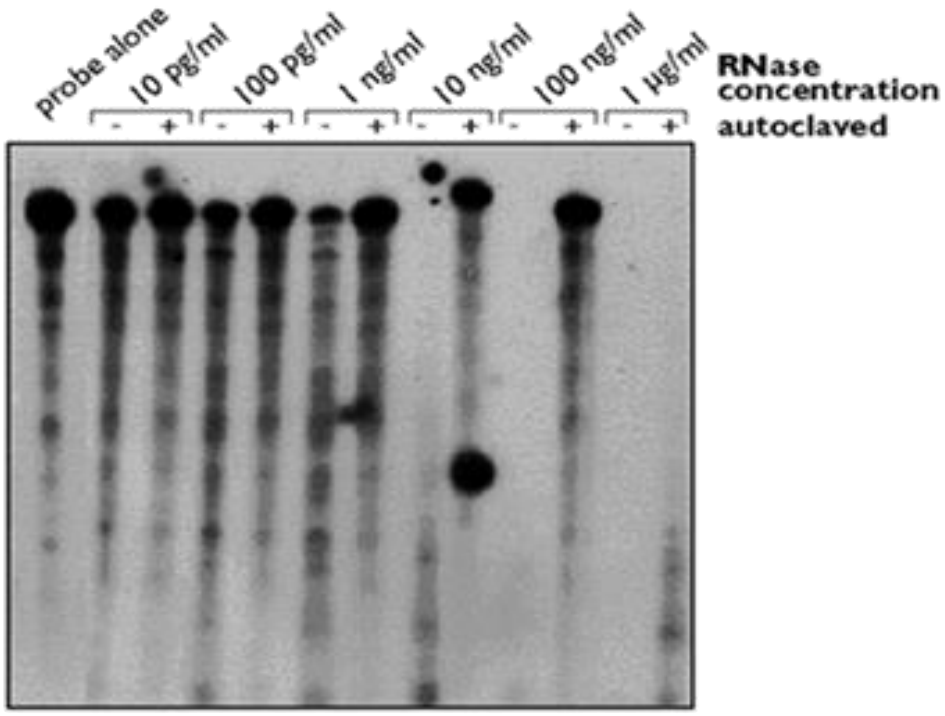
3. Allow to cool slowly to room temperature;

4. Adjust the pH by adding 0.1 volumes of 1M TrisHCl (pH 7.4);

Controlling Ribonuclease activity by autoclaving and DEPC treatment

1

- Autoclaving alone does inactivate a substantial amount of RNase A.
- Denatured RNases tend to regain their native structure and partial function after being cooled to room temperature in the absence of a denaturant.

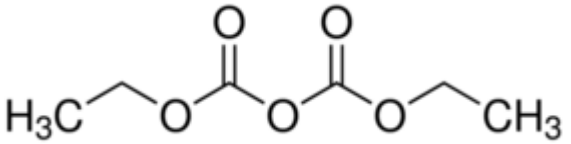


Effect of Autoclaving on RNase Activity

Controlling Ribonuclease activity by autoclaving and DEPC treatment

2

- Diethylpyrocarbonate (DEPC) destroys enzymatic activity by modifying -NH, -SH and -OH groups in RNases and other proteins.
- Autoclaving inactivate DEPC by causing hydrolysis of diethylpyrocarbonate. CO_2 and EtOH are released as reaction by-products.
- DEPC has a half-life of approximately 30 minutes in water, and at a DEPC concentration of 0.1%, solutions autoclaved for 15 minutes/litre can be assumed to be DEPC-free.
- Reagents containing primary amine groups (e.g., Tris) and some reagents containing secondary or tertiary amines (e.g., HEPES) cannot be DEPC-treated. The amine groups react with the DEPC, making it unavailable for inactivating RNases.
- Solutions that cannot withstand autoclaving, such as MOPS, also cannot be DEPC-treated since autoclaving is essential for inactivating DEPC.



Controlling Ribonuclease activity by autoclaving and DEPC treatment

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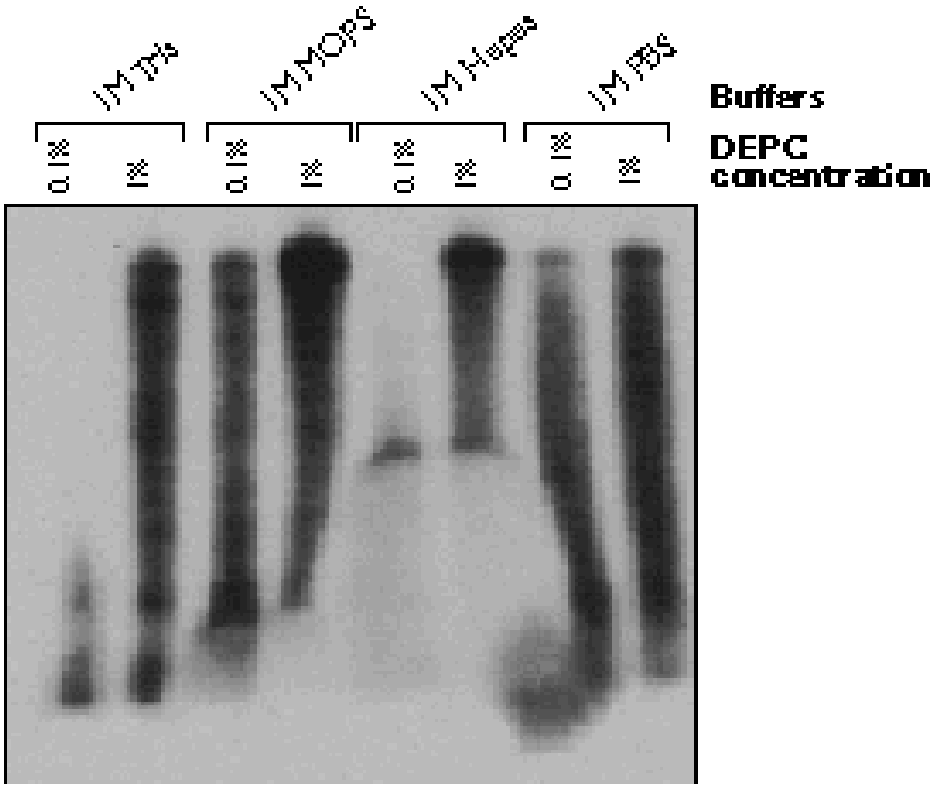
- Solutions containing Tris cannot be treated with DEPC.
- (Tris contains an amino group that "sops up" DEPC and makes it unavailable to inactivate Rnase)

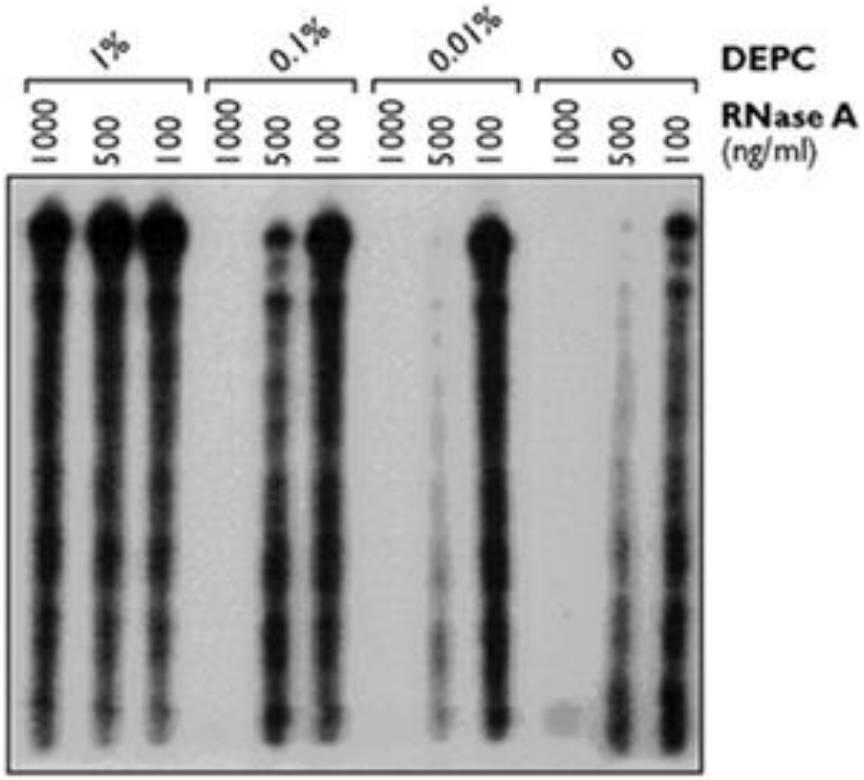


Effects of DEPC Treatment of Various Buffers

Controlling Ribonuclease activity by autoclaving and DEPC treatment

4

- The amount of DEPC required to inactivate RNase increases as the amount of contaminating RNase in a solution increases.
- High levels of residual DEPC or DEPC by-products in a solution can inhibit some enzymatic reactions or chemically alter (carboxymethylate) RNA.



Effects of Varying Percentages of DEPC on Increasing Concentrations of RNase

Sources of contaminating RNases

1-Even you are a source of RNase. Human skin and body fluids such as saliva, tears, perspiration and mucus, can contain RNases.

2-Tips and tubes can be an easily overlooked source of RNase contamination.

3-Water and buffers used in molecular biology applications.

4-Laboratory surfaces: benchtops, glassware, plasticware, and other surfaces and equipments.

5-Endogenous RNases: present in all tissue samples.

6-RNA samples: small amounts of RNases may co-purify with isolated RNA.

7-Plasmid preps: degradation of RNA in plasmid preps by RNase treatment.

8-RNA storage: trace amounts of RNase can compromise RNA integrity even if the samples are stored frozen in an aqueous environment.

9-Chemical nucleases: RNA molecules can also undergo strand scission when heated in the presence of divalent cations such as Mg^{2+} or Ca^{2+} at >80°C for 5 minutes or more.

10-Enzymes: Both commercially purchased and laboratory prepared enzymes.

Preventing RNases Contamination by:

1. Wearing gloves throughout experiments.
2. Changing gloves after touching skin (e.g., your face), door knobs, and common surfaces.
3. Having a dedicated set of pipettes that are used solely for RNA work.
4. Using tips and tubes that are tested and guaranteed to be RNase-free.
5. Using RNase-free chemicals and reagents, glassware and metalware [re-treat materials with extended heat (180°C for several hours), wash with DEPC-treated water, NaOH or H_2O_2]
6. Designating a "low-traffic" area of the lab that is away or shielded from air vents or open windows as an "RNase-free Zone".
7. Decontaminating shared laboratory surfaces and equipments with RNaseZap (or NaOH).
8. Supplement reactions with RNase inhibitors
9. Include a chaotropic agent (guanidine) in the procedure

Other ribonucleolytic proteins

- The terminator-gene **barnase** is a universal poison that breaks down RNA, an intermediate in the expression of all genes.

- Barnase is lethal to all cells in which it is expressed, unless its specific inhibitor, **barstar**, is also present. Both barnase and barstar are produced by a soil bacterium, Bacillus amyloliquefaciens.

Storing RNA

Short-term Storage

- For short-term storage, RNA samples can be resuspended in nuclease-free water or buffer and stored at -80°C.

- Using a buffer solution that contains a chelating agent is a better way to store RNA. It prevents heat-induced strand scission. Chelation of divalent cations such as Mg^{+2} and Ca^{+2} will prevent heat-induced strand scission.

Storing RNA

Long-term Storage

- For long-term storage (more than a few weeks), RNA samples are best stored at -20 to -80°C as a salt/ethanol slurry. The combination of low pH, low temperature and high alcohol content will stabilize the RNA and inhibit all enzymatic activity.

- Other alternatives: RNA in formamide, RNA in frozen aliquots at -20°C or below.

Thank you