Aim LISV I

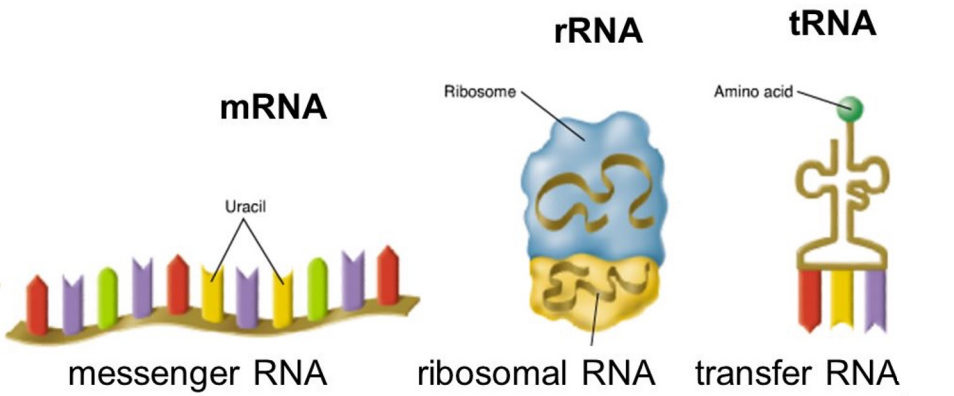
- Cloning mouse pancreatic α-amylase cDNA into a mammalian expression plasmid

cDNA cloning: Experimental steps

- Isolation of total RNA (Lab 2)
- Reverse transcription (Lab 2)
  - oligo-dT primer anneals to polyA tail of mRNA
  - Reverse transcriptase will synthesize first-strand cDNA

- PCR amplification using first-strand cDNA as a template (Lab 3)
- Ligation of PCR amplicon (Amy2 coding sequence) into expression plasmid / Bacterial Transformation to select recombinant plasmids (Lab 4)

Total RNA Composition



1-5%	80-85%	20-25%

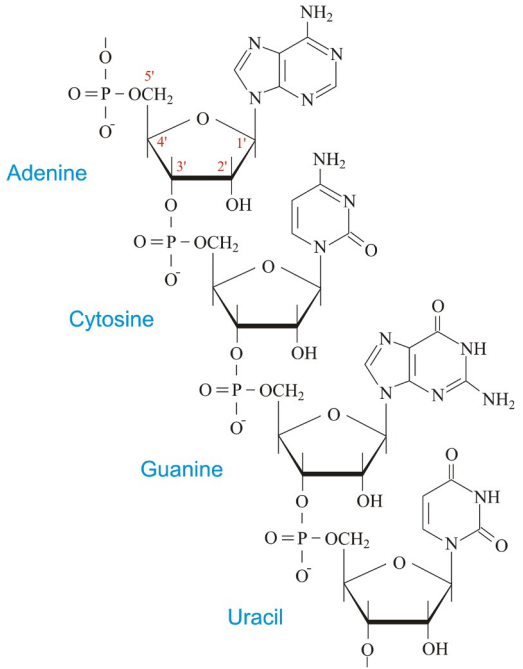
Experiments Lab2

1. RNA isolation
2. Quality control
3. Reverse transcription (RT)

Working RNAse Free

RNAases present on skin etc degrade RNA!

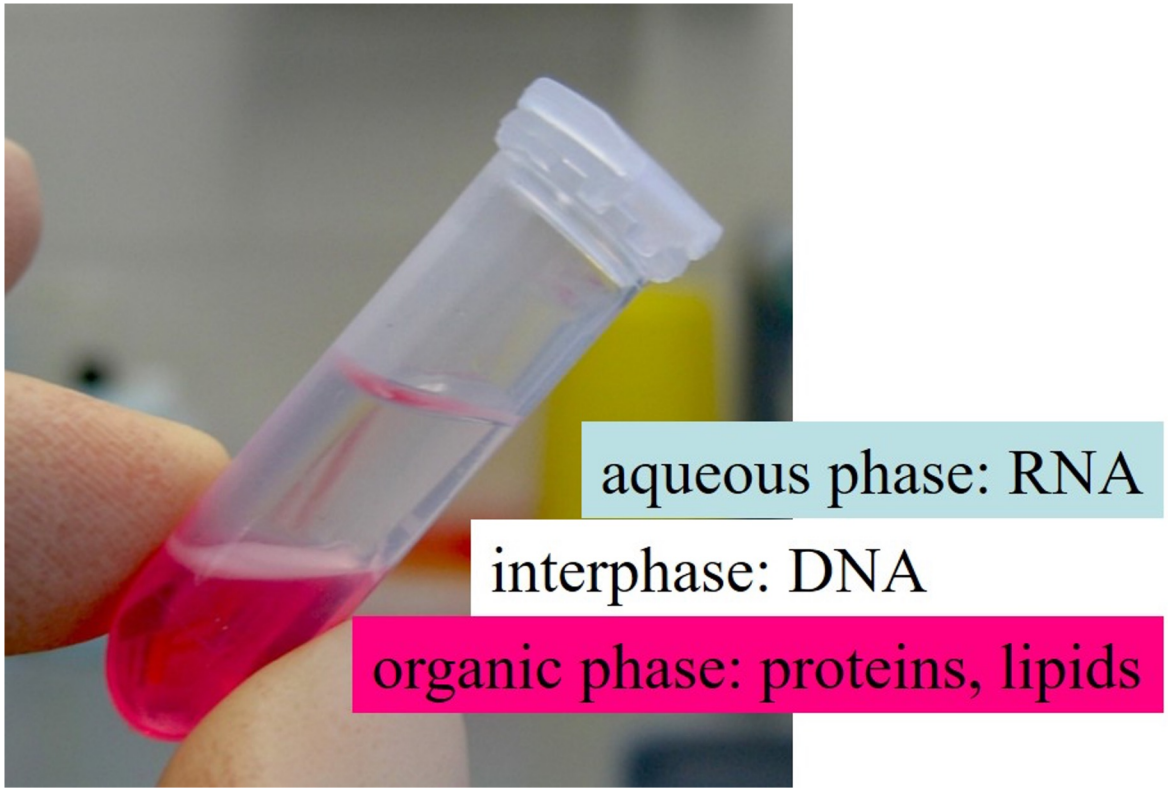
- Clean benches with 70% ETOH
- Wear gloves
- Use filter tips
- Keep RNA on ice



Total RNA Isolation

- **Trizol extraction** (prepared by us)
  - Guanidinium thiocyanate => Denatures proteins, including RNases, and preserves RNA integrity.
  - Phenol-chloroform extraction => RNA separates into the aqueous phase.
  - Video
- Purification on **RNeasy spin column** (prepared by you)
- You will isolate RNA from mouse tissue

Trizol Extraction



RNA isolation: RNeasy spin column

Use aqueous phase Trizol extract
Selective binding of the RNA to a silica-based membrane.

Purification of RNAs > 200 nucleotides.

Safety: wear goggles

RNA Purity

NanoDrop Spectrophotometer

Assess purity of total RNA

Compare to control RNA

RNA Quality Control

- Concentration: how much?
- Purity: how clean?
- Integrity: how intact/ how much degraded?

Determination of RNA concentration

- Nucleic acids have an absorption maximum at at **260 nm**
- The Beer-Lambert law **A=Ecl**
- Linear change in **absorbance** with **concentration**
- Spectrophotometric conversion for RNA:
  1.0 A260 unit = 40 µg/ml (1 cm path)

Chemical Purity of Nucleic Acids is Assessed by Absorbance Ratios

Absorption at 280 nm may be caused by proteins (aromatic amino acids) or phenol

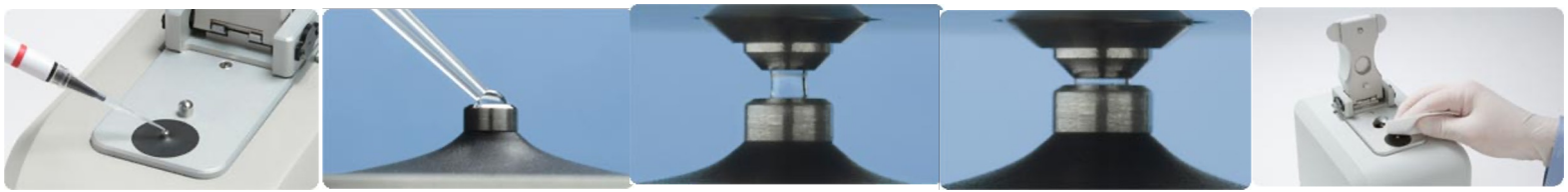
▪ A260/A280	DNA	1.7 - 1.8
▪ A260/A280	RNA	1.9 - 2.2

Absorption at 230 nm can be caused by guanidinium thiocyanate, other organic compounds or proteins.

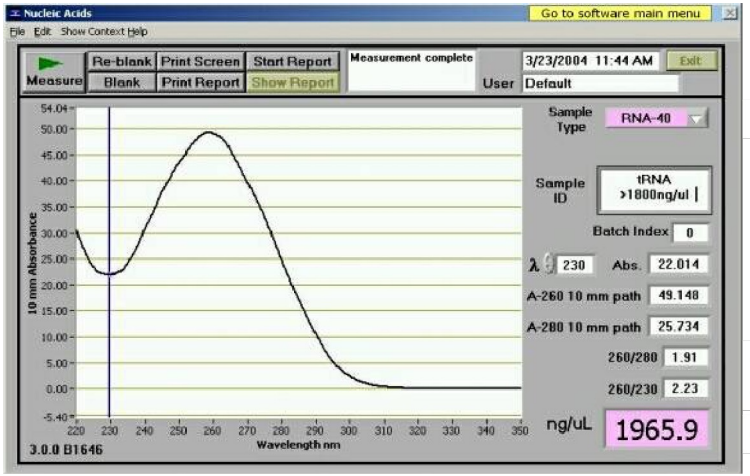
▪ A260/A230	RNA and DNA	> 1.9

NanoDrop Spectrophometer

- Small samples: 0.5 µl - 2.0 µl
- No need for cuvettes or capillaries.
- With the arm open, a sample is pipetted directly onto the pedestal.
- After the arm is closed, a sample column is formed.
- The pedestal then moves to automatically adjust for an optimal path length (1 mm).
- When the measurement is complete, the surfaces are simply wiped with a lint-free lab wipe before going on to the next sample.



Typical NanoDrop Spectral Profile



RNA sample

A260/A230

A280/A230

Concentration

RNA integrity

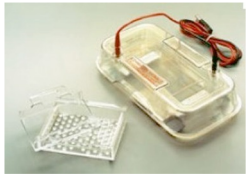
Agarose gel electrophoresis

Assess integrity of ribosomal RNA bands of purified RNA

Compare to control RNA

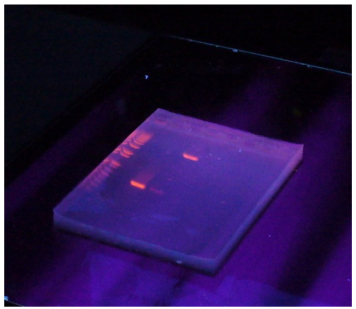
Agarose Gel Electrophoresis

- How to run an agarose gel:
  - Wear gloves (GelRed dye)
  - Plug cables correctly (+/-)
  - Turn power ON after loading your sample
  - Watch samples migrate in the correct direction
  - Turn power OFF before taking out the gel

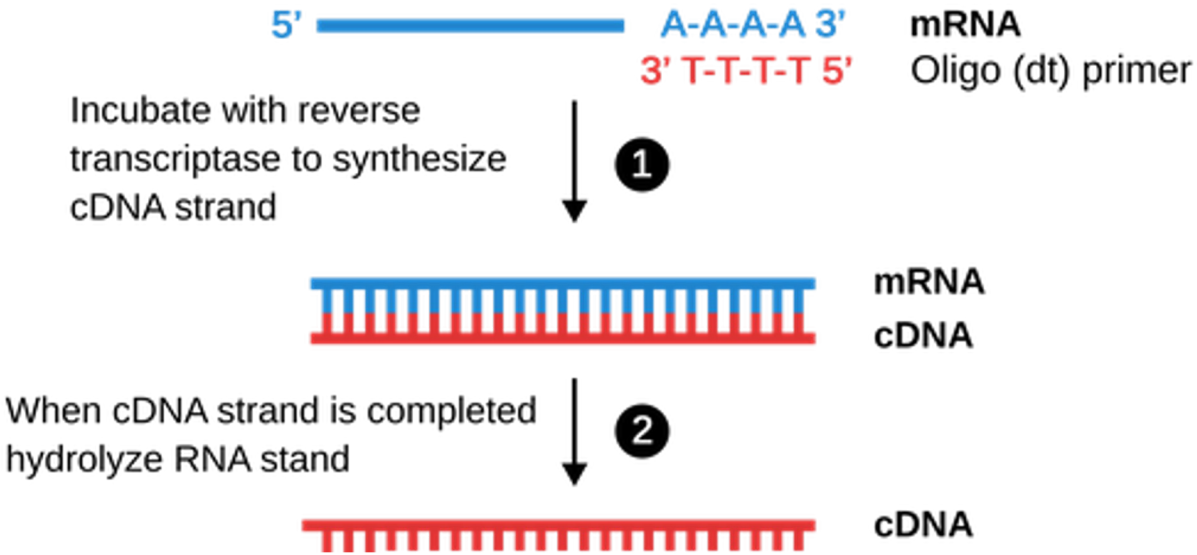


UV transilluminator

- The gel contains a fluorescent nucleic acid dye (GelRed)
- When illuminated with UV light the nucleic acids are visible
- Save the image with group number



Reverse Transcription (RT) Reaction



RT Key Reaction Components

- Template: total RNA from **mouse pancreas** (provided by us)
- Oligo-dT primer
- Reverse transcriptase: derived from Moloney Murine Leukemia Virus
- Deoxyribonucleoside triphosphates (dNTPs)

Control Reaction for RT

- **Sample without reverse transcriptase (-RT)**

  This control assesses the amount of DNA contamination present in an RNA preparation. Important control during PCR amplification (Lab 3)