# 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)

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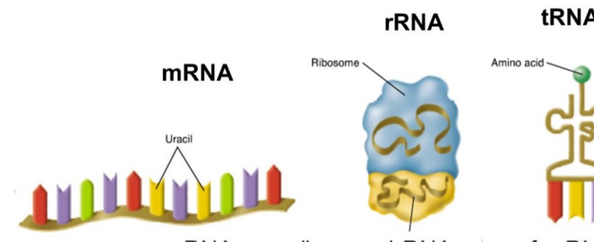
# cDNA cloning: Experiments Lab 2

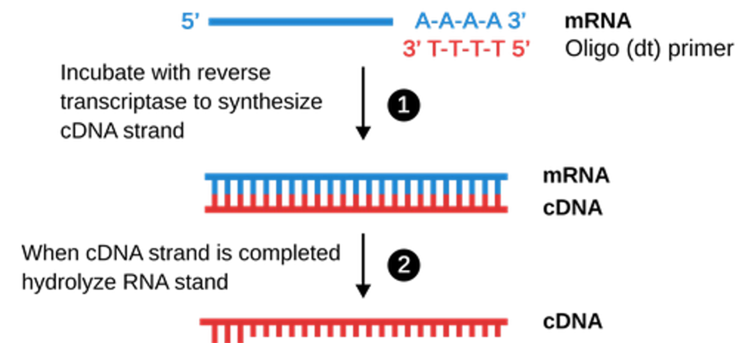


Isolation of total RNA



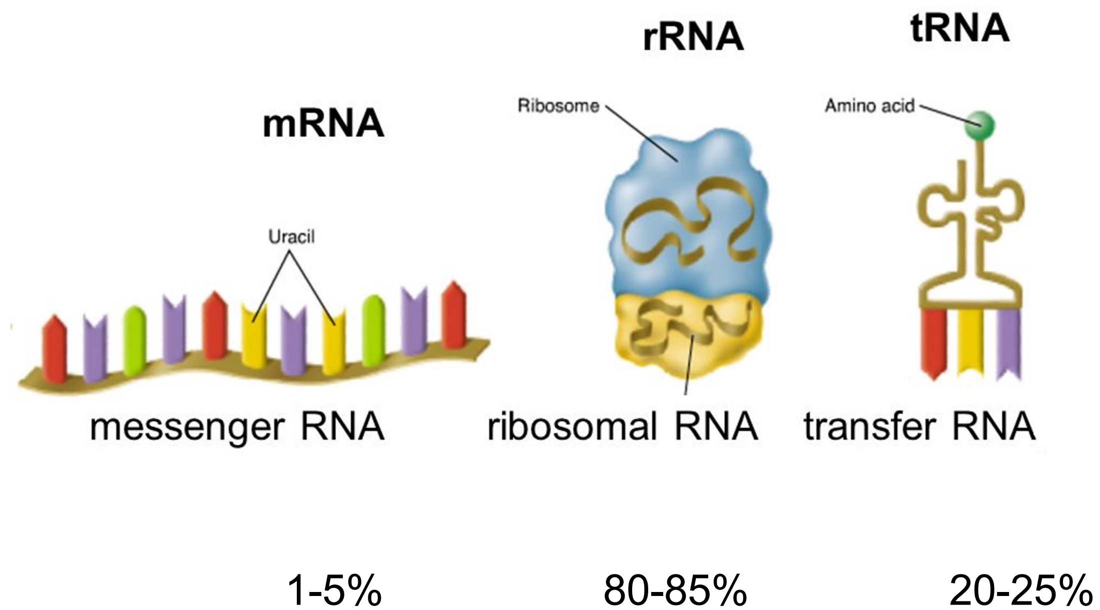

Quality control

Reverse transcription



# Total RNA Composition






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

# Working RNAse Free

**RNAases present on skin etc degrade RNA!**

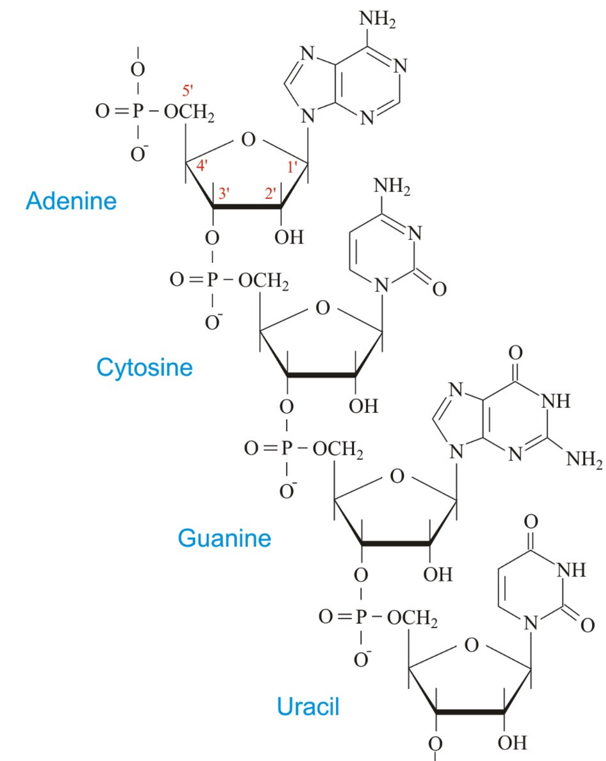
Clean benches with 70% ETOH

Wear gloves

Wear goggles

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

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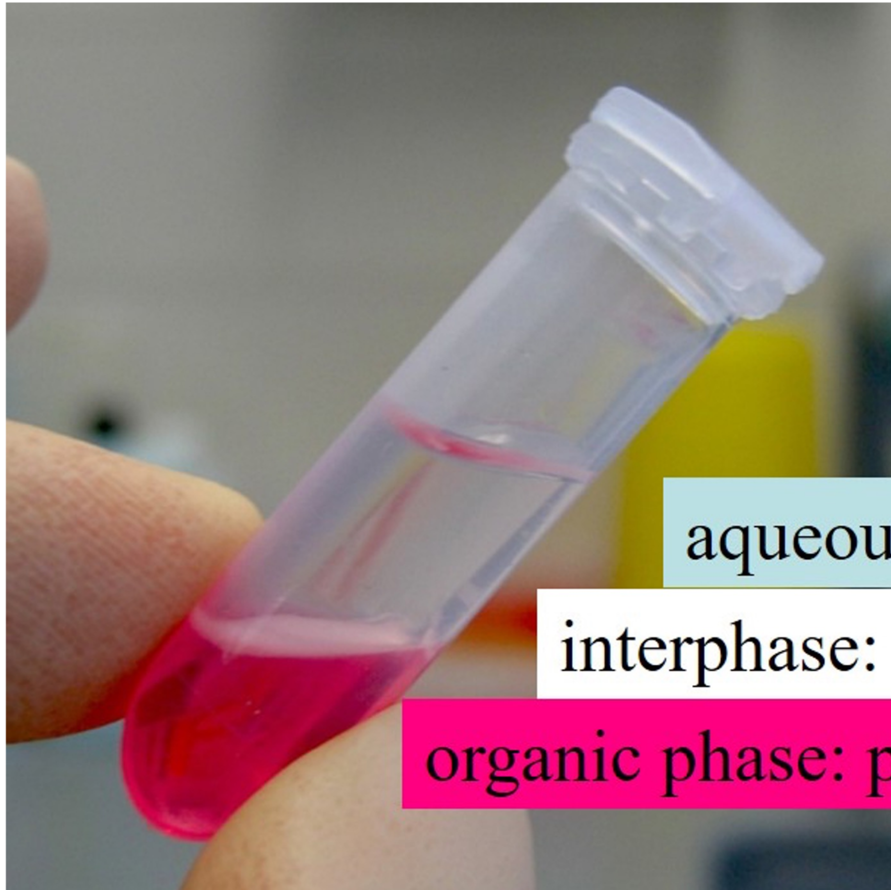
# Trizol Extraction (prepared)



# 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

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# 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 |

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# NanoDrop Spectrophometer



Small samples: 0.5 µl - 2.0 µl

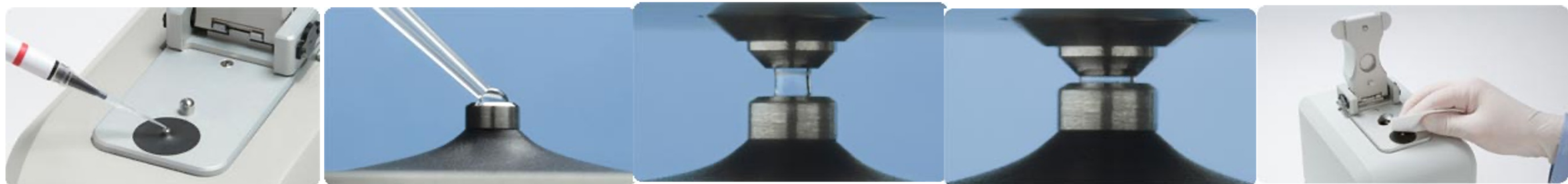
No need for cuvettes or capillaries.

With the arm open, a sample is pipetted directly onto the pedestal.

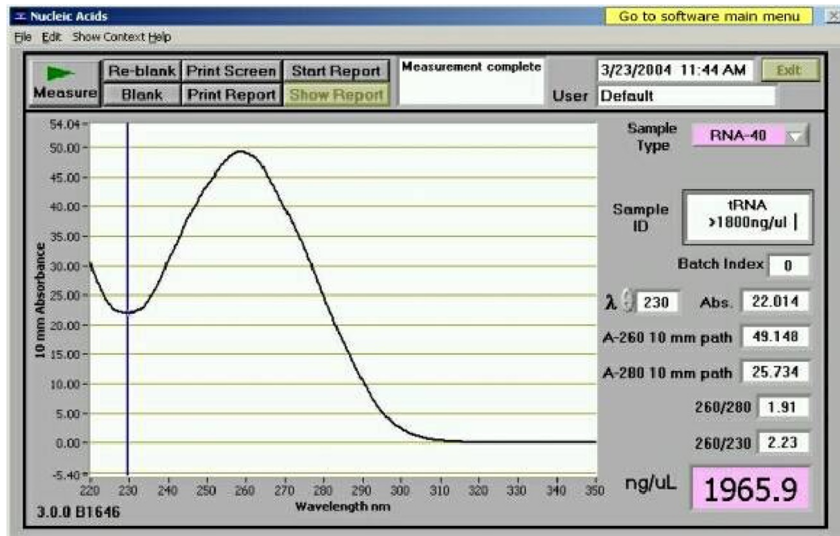
After the arm is closed, a sample column is formed.

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

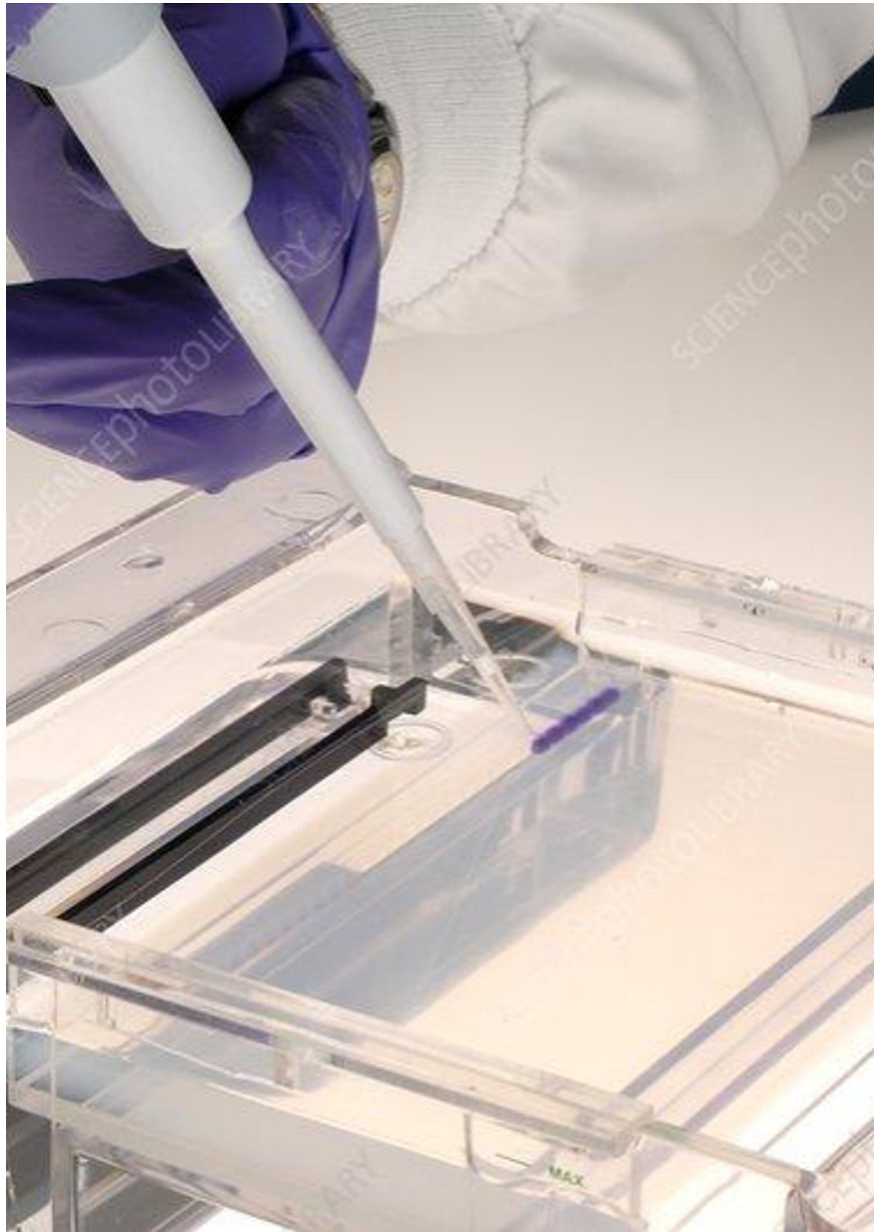



RNA sample

A260/A230

A280/A230

Concentration



# RNA integrity

Agarose gel electrophoresis

Assess integrity of ribosomal RNA bands of purified RNA

Compare to control RNA

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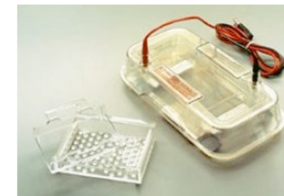
# Agarose Gel Electrophoresis



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



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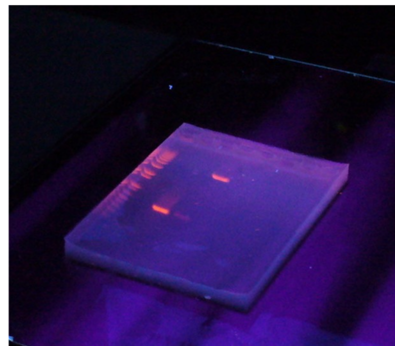
# UV transillluminator



The gel contains a fluorescent nucleic acid dye (GelRed)

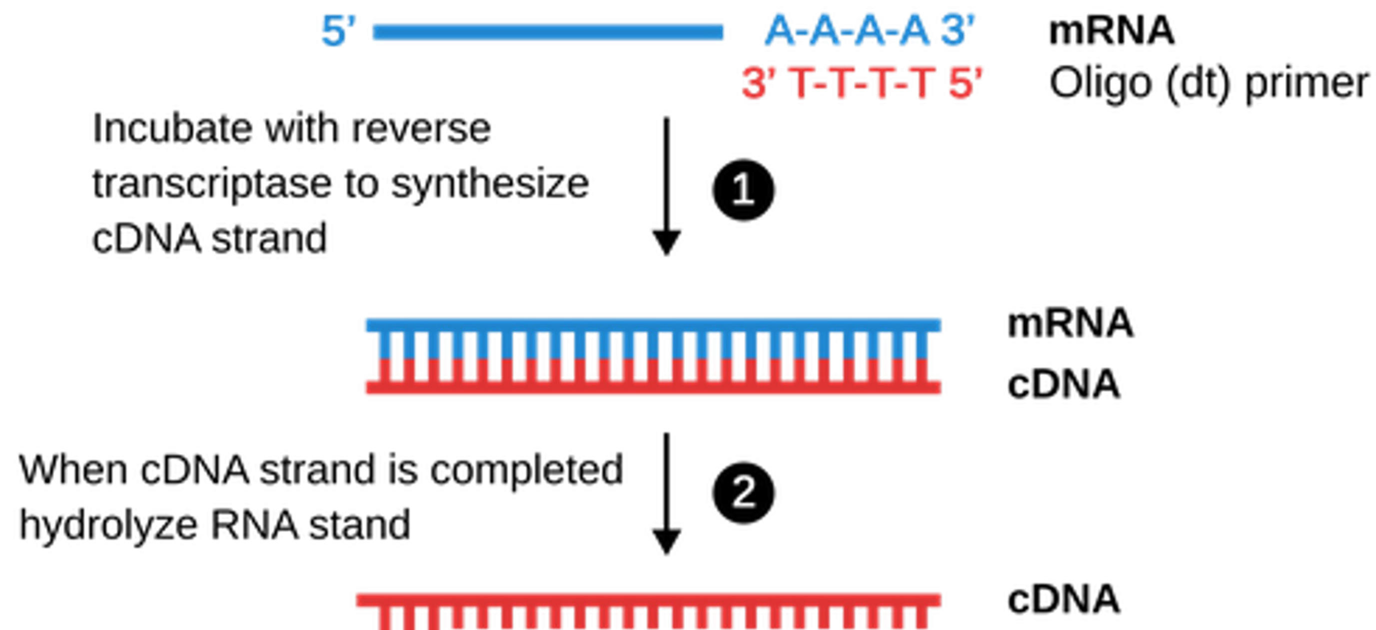
When illuminated with UV light the nucleic acids are visible

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)

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# 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)