Announcements

- Chapter 7 post-lab write-up will be due end of lab day starting week of Feb 20th (our "lab week" runs Thursday through Tuesday)
- Radioactivity training **MUST** be completed before the start of your Chapter 8AB lab this week.
- The next two weeks, NO personal items other than you notebook and a pen should be brought INTO the lab.
- METABOLISM PROJECT: New due date – midnight Wed. 2/26

Announce > Concepts > Procedure > Hazards > Tips > Clarification > End

Chapter 8: In vitro transcription & translation

Objectives

- To use an *in vitro* transcription and translation system to synthesize proteins from genes cloned into a plasmid.
- To learn about the use of radioisotopes in biochemistry
- To determine amount of ^{35}S incorporated into the protein
- Identify which of the two plasmids you prepared have the correct ORF for synthesis of the REL transcription factor

Procedures

- Use a **combined RNA polymerase transcription system** and a **wheat germ lysate-translation system** to ***express and synthesize proteins*** from the cloned gene in your plasmid preps
- Use **TCA (Trichloroacetic acid)** to ***precipitate all proteins***.
- Use **scintillation counting** to determine ***amount of ^{35}S incorporated*** into protein
- Use SDS-PAGE and autoradiography to determine if the protein you made is the correct size of REL.

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Transcription and Protein Synthesis

Week 1: Add DNA template and perform transcription/translation using TNT® Wheat Germ Extract.

TCA precipitation

Measure amount of radioactive methionine incorporated into proteins by Scintillation counting.

Week 2: Separate translation products by SDS-PAGE

Dry gel

Autoradiography

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Central dogma of molecular biology

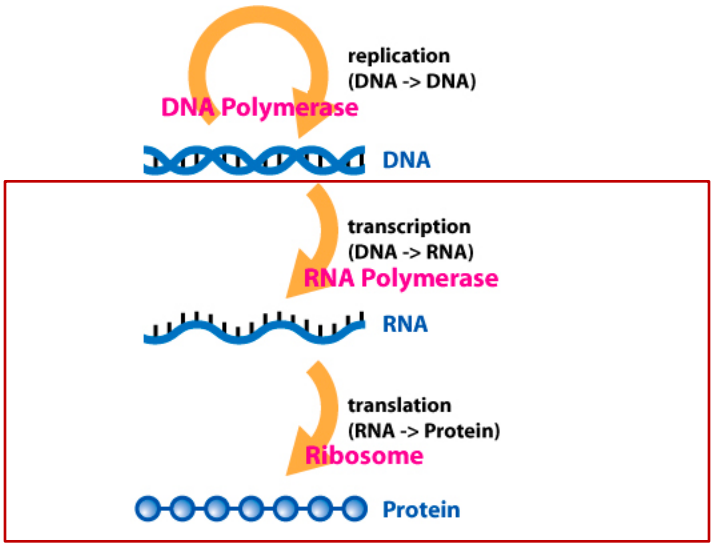

Photo: Wikipedia (2008).

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In vitro transcription/translation

- DNA systems: coupled transcription/translation
- RNA systems: translation only

- Chapter 8AB will use a ***DNA system*** (plasmid DNA > RNA > Protein)
  - Components in system include:
    - SP6 RNA polymerase
    - NTPs (ATP, GTP, CTP, UTP)
    - Wheat Germ Extract
      - Ribosomes and other translation factors
      - tRNAs and aminoacyl-tRNA synthetases
      - Amino-acid mixture (-Cys and/or -Met)
      - ATP and GTP as the source of translation energy
  - Students will supply the plasmid DNA as well as the ***radioactively labeled mixture of [^{35}S]Cys & Met***

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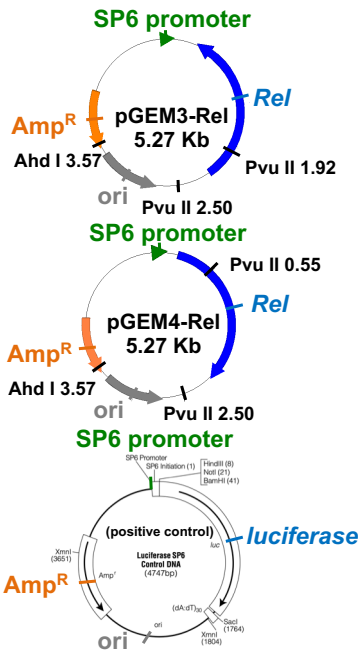
Recall from last lab

- Isolated pGEM3-Rel and pGEM4-Rel from *E. coli* using mini-prep
- Each plasmid contains:
  - **Ampicillin resistance gene**
  - **Origin of replication**
  - **Restriction enzyme recognition sites**
  - **SP6 promoter**
  - **Target gene - *Rel***

- Transcription only starts at sites with specific promoter sequences
- Incorporated just upstream of cloning sites of any opening reading frame



Plasmid maps Figure 6-5, p. 209

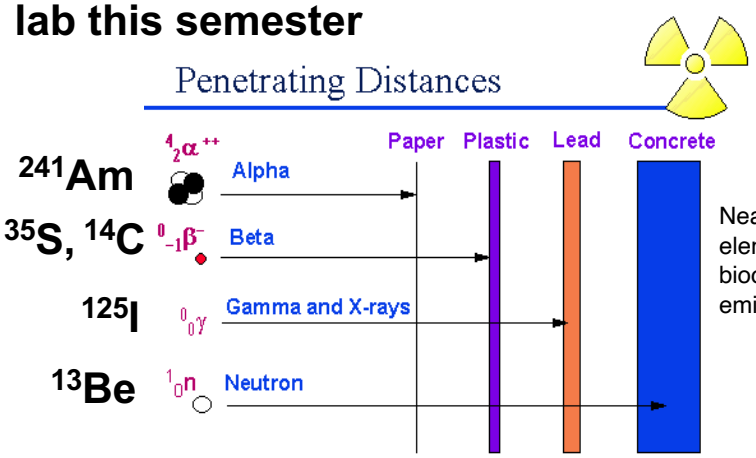
Announce | Concepts | Procedure | Hazards | Tips | Clarification | End

Properties of RNases

- RNA is easily degraded by ribonucleases, RNases
- These enzymes are on your clothes, skin, saliva
- Very important to work with gloves
- RNases are very stable. Autoclaving will not remove such contamination
- You can inactivate RNases by sterilizing solutions at high temperatures in the presence of DEPC, or by using chemical modifications
- You should have dissolved your plasmid preparations from last lab in nuclease-free water (likely DEPC treated).

We want to make RNA transcripts, be careful of RNase contamination!

You will be working with ^{35}S in Biochemistry lab this semester



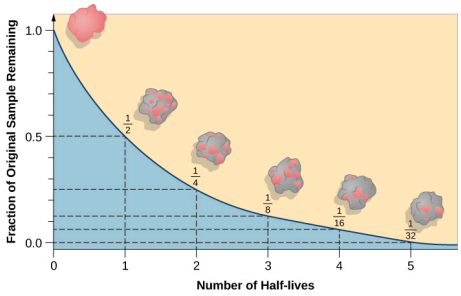
Nearly all radioactive elements used in biochemistry are β-emitters

Refer to the chart of radioisotopes on pages 233- 234 in lab manual for more physical constants

Half-lives of Isotopes

- The time required for any given radioisotope to decrease to one-half of its original activity by radioactive decay.
- This period of time is called the half-life.

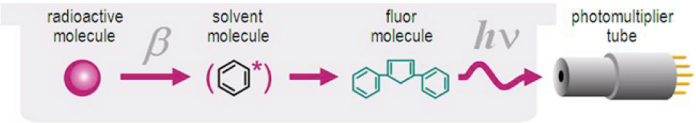
- **^{14}C – 5,730 years**
- ^{3}H – 12.3 years
- **^{35}S – 89.7 days**
- ^{125}I – 60 days
- ^{32}P – 14.3 days



Refer to the chart of radioisotopes on page 234 in lab manual for half-lives.

Photo: Key Stage Wiki (2020).

Scintillation Counting



- Method for detecting **β-particles** by reaction with fluorescent compounds (fluors)
- Allows for efficient accurate counting of isotopes
  - Low energy emitters (^{3}H), which ***cannot*** be detected with Geiger Counter, can be measured
- Detector is a photomultiplier tube (PMT) near radioactive sample in scintillation fluid
  - Scintillation fluid = solvent containing dissolved fluors
  - Put sample in to scintillation fluid to dissolve

Why use Scintillation Counting?

Advantages

- High efficiency with even low-energy particles (^3H)
- Ability to distinguish and count different radionucleotides in same sample simultaneously
- Easy sample preparation for preparation of fluid soluble compounds

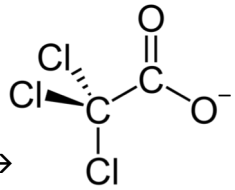
Disadvantages

- High cost of equipment
- Difficult sample preparation for some solids
- Difficulty in analyzing samples that quench and interfere with the signal intensity

Announce Concepts Procedure Hazards Tips Clarification End

Trichloroacetic acid precipitation

- TCA precipitation of proteins is commonly used to concentrate protein samples or remove contaminants
- Addition of TCA disrupts the salt-bridges and hydrogen-bonded water molecules (hydration sphere) surrounding a protein → protein no longer soluble
- Protein is quickly denatured



$pK_a = 0.7$

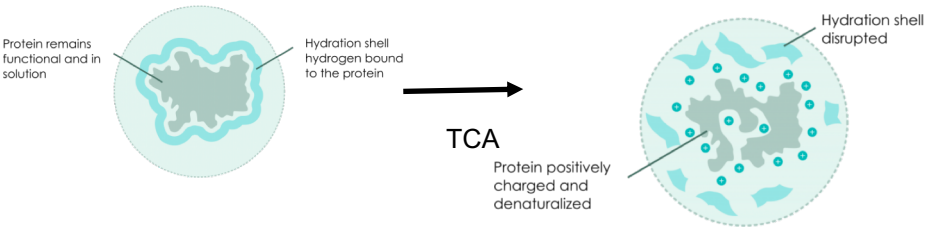


Photo: BQC Redox Technologies (2023).

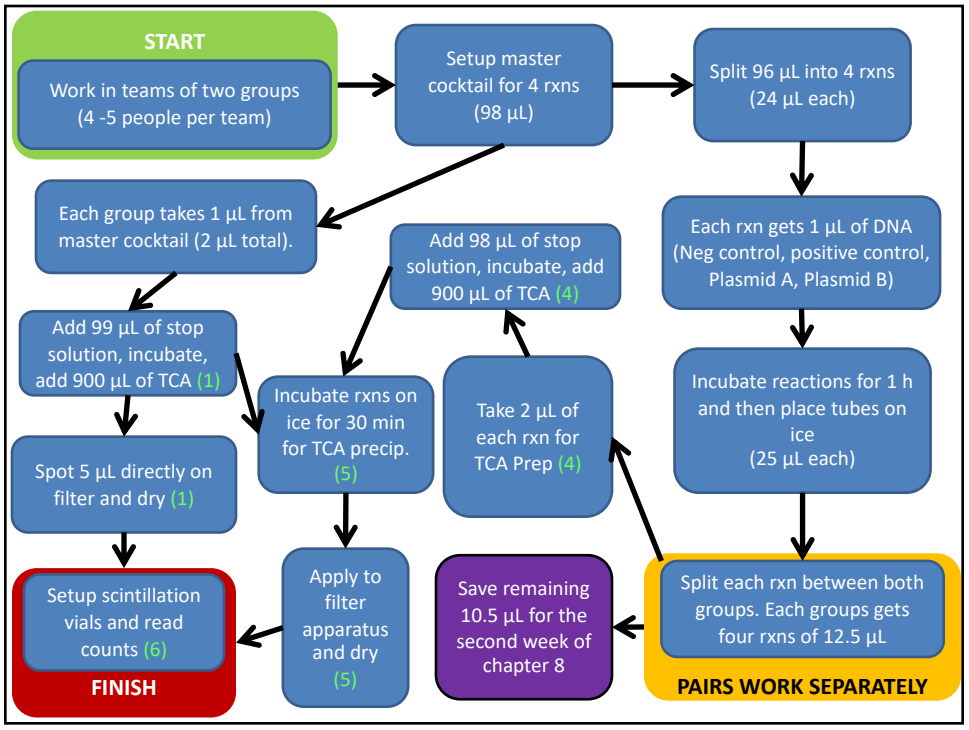
Announce Concepts Procedure Hazards Tips Clarification End

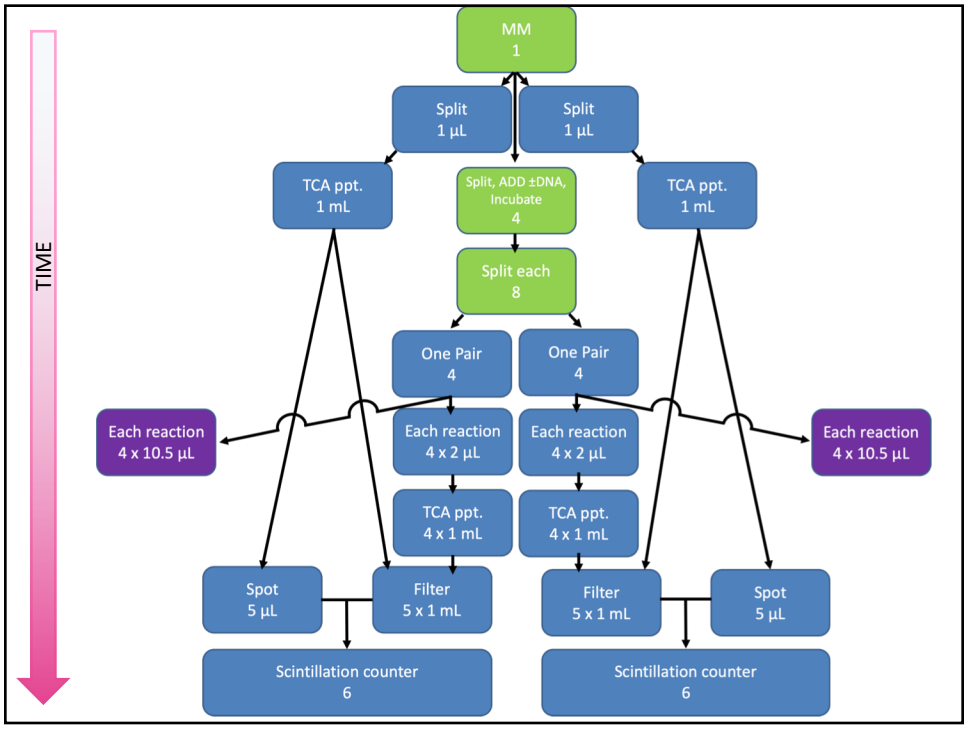
Chapter 8AB: Procedure

- Use gloves at **all times** to prevent RNase contamination
- We will provide sterile/RNase-free pipet tips, centrifuge tubes, etc. **Please keep them that way!!!**
- You will be pipetting **very small volumes!**
  - We should have some P-2 pipets available **(0.2 – 2 µL)**

Chapter 8AB: Procedure

- You will be working with radioactive materials:
  - You must have training to enter room and work with radioactive material
  - Wear **plastic aprons** and **double gloves**
  - Work on bench coat paper marked with radiation tape
  - Your TFs need to check your workspace for radioactivity **BEFORE** you can leave the lab (use of a Geiger Counter)
  - Special waste disposal for liquid and solid radioactive waste
  - Initially, you will be working in teams of 4 (or 5)


START
Work in teams of two groups (4 -5 people per team)
Setup master cocktail for 4 rxns (98 µL)
Split 96 µL into 4 rxns (24 µL each)
Each group takes 1 µL from master cocktail (2 µL total).
Each rxn gets 1 µL of DNA (Neg control, positive control, Plasmid A, Plasmid B)
Add 98 µL of stop solution, incubate, add 900 µL of TCA (4)
Add 99 µL of stop solution, incubate, add 900 µL of TCA (1)
Incubate rxns on ice for 30 min for TCA precip. (5)
Take 2 µL of each rxn for TCA Prep (4)
Incubate reactions for 1 h and then place tubes on ice (25 µL each)
Spot 5 µL directly on filter and dry (1)
Setup scintillation vials and read counts (6)
FINISH
Apply to filter apparatus and dry (5)
Save remaining 10.5 µL for the second week of chapter 8
Split each rxn between both groups. Each groups gets four rxns of 12.5 µL
PAIRS WORK SEPARATELY


TIME
MM
1
Split
1 µL
Split
1 µL
TCA ppt.
1 mL
Split, ADD ±DNA, Incubate
4
TCA ppt.
1 mL
Split each
8
One Pair
4
One Pair
4
Each reaction
4 x 10.5 µL
Each reaction
4 x 2 µL
Each reaction
4 x 2 µL
Each reaction
4 x 10.5 µL
TCA ppt.
4 x 1 mL
TCA ppt.
4 x 1 mL
Spot
5 µL
Filter
5 x 1 mL
Filter
5 x 1 mL
Spot
5 µL
Scintillation counter
6
Scintillation counter
6

Chapter 8AB: Reagent List

Reagent Name	Cap mark
Nuclease-free water	"NF H_2O"
TNT reaction buffer	BLUE
Amino Acid mix –Met	BLACK
RNasin RNase Inhib.	ORANGE
TNT SP6 RNA polymerase	GREEN
[S-35] methionine	RED ●
TNT Wheat Germ Extract	PURPLE
SP6-Luciferase (0.5 µg/µL)	"SP6 Luci"
Plasmid A (0.2-1.0 µg/µL)	"A"
Plasmid B (0.2-1.0 µg/µL)	" B"

← **Prepare mastermix in this tube!**

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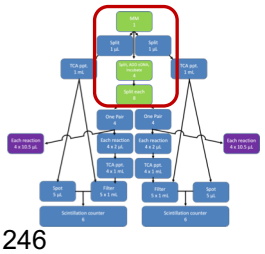
Chapter 8AB: Procedure



A. Transcription/Translation:

- Work together with a second group for the first part
- Thaw reagents
- Set up cocktail for 4 reactions according to Table IV p. 246

→ Prepare mastermix in tube pre-aliquoted with [^{35}S] Cys+Met (red dot)

- Remove 1 µL x 2 for zero-time point control reactions
  - ➢ Add 99 µL of NaOH stop solution to zero-time point
- Aliquot cocktail, 24 µL / reaction and add 1 µL DNA* to each
  - ➢ Plasmid A
  - ➢ Plasmid B
  - ➢ Positive control – DNA template encoding luciferase (SP6-luciferase)
  - ➢ Negative control – No DNA (nuclease-free water)

*Only if >500 ng/µL (from the gel)
If your [DNA] is <500 ng/µL, but >100 ng/µL use 2 µL; if it is less than 100 ng/µL, use TF samples

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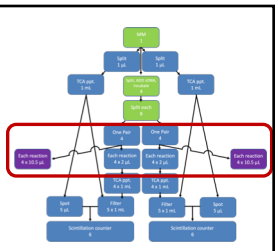
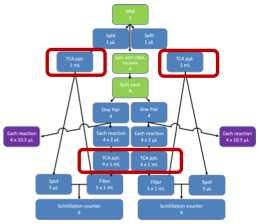
Chapter 8AB: Procedure



A. Transcription/Translation:

- Incubate all samples at **30 ℃ for 60 min**
- Stop reactions on ice
- Split reactions – 12.5 µL/group
- Each group works separately from now on
- Remove 2 µL aliquot of experimental reactions
  - ➢ Add 98 µL of NaOH stop solution
- Store rest of samples at -20 ℃ until next lab

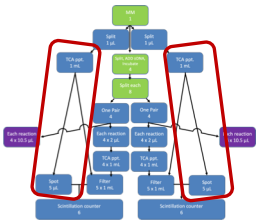
Chapter 8AB: Procedure



B. Scintillation counting: TCA ppt

- Should have 1 "zero-time" reaction and 4 "60-min" reactions
- From the "Zero-time" Reaction = measure TOTAL counts (1) and BACKGROUND counts (1)
- "60-min" Reactions = Level of incorporation of radioactive tag
- Vortex the 100 µL stopped reactions, incubate tubes at 37 ℃, 10 min
  - ➢ NaOH will hydrolyze the amino-acyl tRNAs, and unincorporated AA's will not precipitate when adding TCA.
- Add ice-cold 25% TCA (0.9 mL), incubate 30 min on ice
  - ➢ Precipitate translation product

Chapter 8AB: Procedure



B. Scintillation counting: Control for input

- Prepare the control for number of counts in the reactions
- This is a **"Direct Spot"** filter
  - ➢ Spot 5 µL of the TCA reaction from "zero-time point" reaction **DIRECTLY** on filter
  - ➢ Dry the filter 10 min, and put in scintillation vial
- TFs will give you vials with pre-numbered caps: **record which sample is in which number!!**

The tops of caps are labeled, not on the sides of the vial
Why?

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Chapter 8AB: Procedure

B. Scintillation counting: Filtered samples

- Set up a filter apparatus
- Filter **all** FIVE samples
  - ➢ *Everything except direct spot*
- Wash filter with ice cold 5% TCA 3x
- Wash filter with 95% ethanol 1x
- Allow filters to dry and put in scintillation vials

Your TFs will demonstrate techniques for these TCA steps

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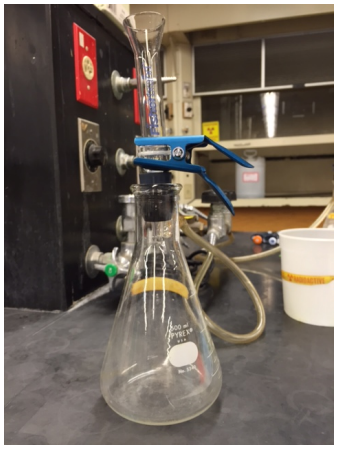
Chapter 8AB: filter apparatus



Video missing the 5% TCA rinse of the tube, as described for the glass tubes in the lab manual

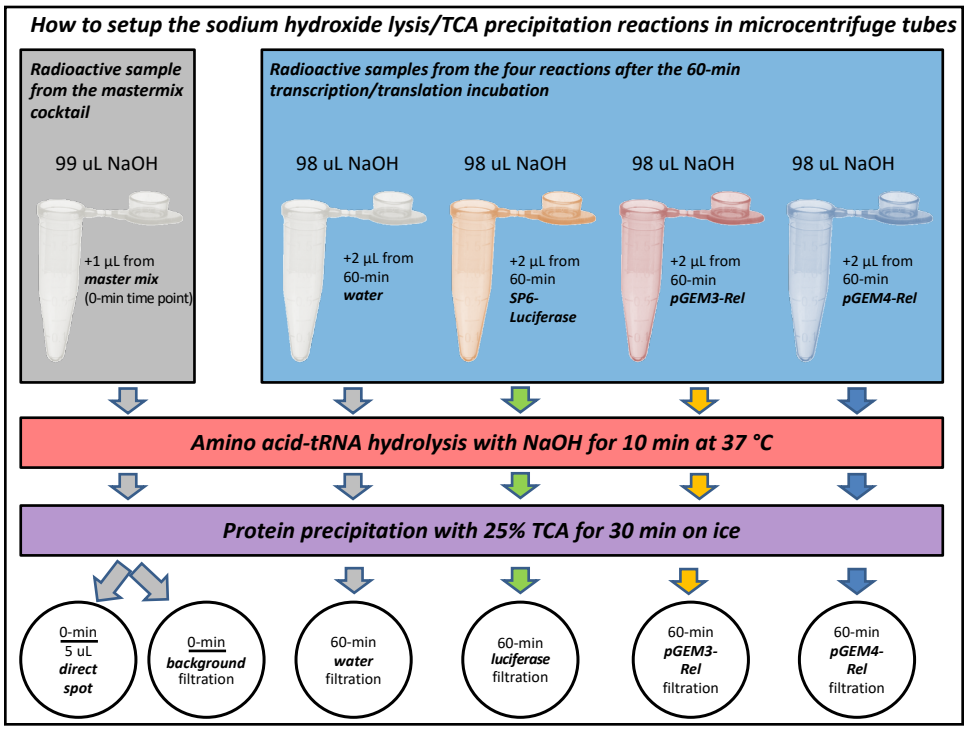
Check out the online videos for visual demonstrations!
(QR-codes and written directions are on pages 250-251 of Lab Manual)

Filtration apparatus: https://www.youtube.com/watch?v=W0HEwZnmHho
Direct spots: https://www.youtube.com/watch?v=SKOofiC2GOA
Prepare scintillation vials: https://www.youtube.com/watch?v=OK-6x14b4fI

Chapter 8AB: Procedure

At end of lab:

- Turn in 6 scintillation vials to TF for counting (record your vial numbers!):
  - 1 Direct Spot of "Zero-Time" reaction
  - 1 Precipitated Filter of "Zero-Time" reaction
  - 4 Precipitated Filters of "60-min" reactions
- **Dispose of TCA/Ethanol washes in DESIGNATED radiation-disposal sink as directed by TF**
- Clean up your bench space and dispose all solid radioactive waste
- Your TF must check your workspace and materials (esp. P-1000) for radioactivity before leaving lab!



Chapter 8AB: Procedure

B. Scintillation counting:

- TFs will run scintillation counter after lab
- You will get reading of counts the following week
- **Percentage incorporation:**

$$\text{percent incorporation} = \left(\frac{\text{"60-min precipitated filter CPM"} \times 12.5}{\text{"0-min direct spot CPM"} \times 200 \times 24.5}\right) \times 100\%$$

- **Fold stimulation over background:**

$$\text{fold stimulation} = \left(\frac{\text{"60-min } \textit{DNA} \text{ precipitated filter CPM"} - \text{"0-min precipitated filter CPM"}}{\text{"60-min } \textit{water} \text{ precipitated filter CPM"} - \text{"0-min precipitated filter CPM"}}\right) \text{"-fold"}$$

SAVE YOUR SAMPLES AT THE END OF LAB!!!

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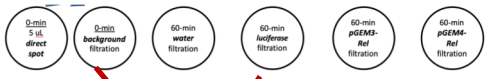
Chapter 8AB: Procedure



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SAVE YOUR SAMPLES AT THE END OF LAB!!!

Refer to p. 266 to see how equation is derived.

Chapter 8AB: Procedure

- You will be supplied with the positive control plasmid "SP6-Luciferase" containing the ***luciferase*** gene
  - ➢ This plasmid has the optimal coding sequence for the SP6 promoter
  - ➢ Lots of RNA > lots of protein > a lot of ^{35}S-Met incorporation
- You WILL use your own pGEM3-Rel & pGEM4-Rel plasmids from last lab (check concentration and determine how much to use)*

*if needed your TFs can supply you with pGEM3-Rel & pGEM4-Rel plasmids

CH 8AB Hazards and Radioactive waste

- In addition to safety eyewear & lab coats, you must double glove and wear plastic aprons
- Everything you dispose of is considered radioactive (please pay attention when disposing items)
  - Radioactive liquid waste from flasks goes down into the only one radioactive sink in the lab
  - All other radioactive solids go into solid waste that we will bring to the front of the lab
  - If apron and gloves are **NOT** hot they should go into the regular trash
- TCA is very corrosive; but will go down radioactive sink

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Chapter 8AB Lab Tips

- Always work on radioactive mats on the bench space
- No laptops, phones, calculators, or other unnecessary items should be brought to the bench space. If they are hot, we will confiscate them and seal them away for 10 half-lives [~2.4 years for ^{35}S]
- For scintillation vials, TFs will give you vials with pre-numbered caps. It's your responsibility to write down which sample corresponds to which number
  - Printout of numbered scintillation results will be available during the Chapter 8C lab, we will upload results on web site or through Slack

Announce > Concepts > Procedure > Hazards > Tips > Clarification > End

Horror stories from previous semesters

A student refused to work on the radioactivity mats and contaminated his new pair of Nike's Air Max 90 SE Shoes. He was forced to return to his dorm barefoot in the snow

"…why do cold things feel so cold when you step on them?"

- Jacob T.

One student had her Sponge Bob Squarepants pencil case on the radioactivity mat, when she spilled the ^{35}S on the fabric of the case

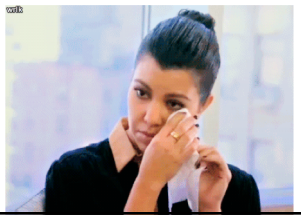
"…I can't even right now…so not gucci…"

- Ashley P.

Another student had their phone out on the bench when their collection flask was knocked over and contaminated their Blackberry

"…ugh, whatever…I'll just have my parents get me the iPhone 16 Pro Max"

- Gianna D.



Minor change to TCA precipitation procedure

GOAL:

Get **Complete** transfer of contents from reaction onto GF/C filter

- **We decided to pipet radioactive solutions would be safer that pouring them from a small culture tube. So, we will use 1.5 mL plastic microcentrifuge tubes** instead of glass culture tubes.
  - This is because we can't fit pipettes in the glass tubes to retrieve our samples
    - *Have your 99-98 µL of NaOH in your plastic tube **before adding** your 1 µL of 0' timepoint **OR** 2 µL of 60' timepoints*

      You want to minimized the amount of radioactivity sticking to the tubes by putting in the NaOH first and then the radioactive aliquots

- ***Use your p1000 to retrieve the TCA samples** and place onto the filter membrane as close as possible without touching/damaging the filter disc*

Video missing the 5% TCA rinse of the tube, as described for the glass tubes in the lab manual

Video suggests keeping vacuum on all the time, but this risks concentrating it on one spot, and not using the whole surface area of the disk.

Announce > Concepts > Procedure > Hazards > Tips > Clarification > End

Chapter 8AB requirements

- Please make sure to submit your Pre-Lab to GradeScope *before* your lab section
- Include in your procedures:
  - a flowchart for the transcription/translation protocol
  - a flowchart for the TCA precipitation step
- These two flowcharts can be combined into one

- Please submit your in-lab data collection assignment to Gradescope *by the end of the day after* your lab section

Chapter 8AB

Before the lab period, you should have:

- ✓ Completed your Pre-lab Write-up and submit on Gradescope
  - ✓ Title, purpose and procedures
  - ✓ Remember to include:
    - ✓ 2 flowcharts
    - ✓ For data collection pages, record the scintillation vial numbers you have been assigned, and the filter samples you have placed in each one

At the end of lab, you should have:

- ✓ Performed transcription/translation on 4 samples
- ✓ Saved 4 x 10.5 µL samples for next week (the bulk of the radioactivity!). Be sure to label well.
- ✓ Turned in 6 scintillation vials to TFs
- ✓ Before leaving, have TFs help you survey bench, pipettes, tools, notebook, your shoes

Questions?

In-class activity
&
Discussion Quiz