Introduction to BIO 510

- Dr. John Rawls, 316 T.H. Morgan Building,
  257-4647 Office Hours Tuesday & Thursday 9:00-10:00 a.m. (or by appointment)

- Dr. Grace Jones, 304 T.H. Morgan Building, gjones@uky.edu 257-2105
  Office Hours Monday, Wednesday, and Friday 3:00-4:00p.m. or by appointment

Course Requirements

- **Course Content:**
  - Theory and application of recombinant DNA technology.
  - Emphasis is placed on learning through direct experimentation.
- **Prerequisites:**
  - This advanced course has firm requirements for **GENETICS** [Bio 304/404G] and for **BIOCHEMISTRY** [Bch 401G (or Bch 501 or Che 580].

Course Textbooks

- **Required Textbook:**
  - Molecular Biology: A Project Approach by Susan J, Karcher. 1995, Academic Press.
  - *Methods in Enzymology, Volume 152, Guide to Molecular Cloning Techniques* (eds., S.L. Berger and A. R. Kimmel; Academic Press, 1987)
  - *Molecular Cloning, A Laboratory Manual* (J. Sambrook, E. Fritsch, and T. Maniatis; Cold Spring Harbor Laboratory Press, 1989).

Additional Materials

- You will be given the lab instructions one class period before you will conduct the experiment (flexibility is needed in order to accommodate for some experiments taking longer than one session)
- **A lab coat must be worn at all times** in the laboratory.
- Free copy of New England Bio Labs product and resource catalog.

Grading Policy

- **Your grade will assigned based on your performance in:**

Midterm exam	30%
Final exam	30%
Weekly quizzes	30%
Notebook	10%

	100%

Grading Con't

- **Final Exam**
  - **Essay and short-answer questions**
  - **Main emphasis is on the second half of the course**
- **Weekly Quizzes**
  - Essay and short-answer questions
  - Weekly quizzes may occur during lab or lecture
- **Laboratory notebook**
  - Record and analyze laboratory experiences
  - Notebooks will be graded during "dead" week

Attendance Policy

- **Attendance is mandatory**
  - Laboratory exercises require group participation
  - Multiple (2 or more) unexcused absences will lower your final grade by one letter.

Grading Policy

Numerical Grade	Letter Grade
100-90	A
89-80	B
79-70	C
69-60	D
<60	E

- A “D” is not available for graduate students
- The +/- grading system will **NOT** be used

Additional Help

- I would like to encourage you to ask questions and the best way to communicate with me is via e-mail. I will respond within 24 hours upon receiving the e-mail.
  - My e-mail address is gjones@uky.edu

- Please feel free to stop by my office during office hours for additional assistance (Monday, Wednesday, Friday 3:00-4:00p.m.)

Overview

- Mini project
  - Analysis of promoter
    - Luciferase assay

Determine the sequence of DNA binding site

Anneal the single strand DNA to form a double strand DNA

Fuse the DNA binding site in front of the promotor

Assay of promotor activity by measuring the reporter expression

Assay the lucifcerase activity from reporter gene

Add hormone to induce promotor activity

Handling Oligonucleotides

To precipitate small oligonucleotides:

- Add one-tenth volume of 3M NaOAc, pH 6.5, and two volumes of cold absolute ethanol.
- Place at -70C for at least one hour.
- Spin the tube and save the pellet

Explanation of Handling Oligonucleotides

*the salt will neutralize the salt of the phosphate

*thus, the DNA does not dissolve in organic solvent because it does not have a charge

Determination of Small Fragments

- **We usually assume:**
  - 1 o.D.= 25 μg/mL oligo
  - 1 o.D= 40 μg/mL ds DNA
  - 1 o.D=20 μg/mL RNA
- **This is a rough estimate**
  - We won't worry about exact amounts and use coefficient
  - Accurate concentration requires the extinct coefficient for the nucleotide
    - e.g. GG CCC C
    - e.g. AAA TTT

Annealing

- GC pairing is tighter than AT pairing
- Conditions for annealing
  - Temperature (above 70C, it will melt the ds DNA)
  - Salt concentration (low salt causes DNA to separate; high salt promotes annealing)
  - Inorganic reagent (e.g., formamide weakens the H bonds of DNA- therefore, it lowers the annealing temperature)

Annealing

- Make sure that during your gel isolation of your vector you do not damage the DNA under the UV light

- Use long wavelength instead of short wavelength

Recovery of DNA from electrophoresis gels

- Kinds of DNA electrophoresis
  - Agarose gels
  - Acrylamide gels

Methods to Extract DNA from Agarose Gels

1. **Melt, dilute, phenol extract**
2. Agarose – enzyme digests agarase
3. Trough method
4. Slice out gel band, electroelution
5. Ionically charged paper
6. Freeze – squeeze
7. Glass beads
8. Isodyne

Methods to Extract DNA from Agarose Gels: Melt, dilute, phenol extraction method

- Use a gel made with low-melting point (LMP) agarose.
  (Compare LMP with regular agarose)
- After electrophoresis, use sterile razor blade to cut out the band **as small as possible**
- Melt LMP at 65 ^{o}C for 10 minutes
- Dilute the melted volume of the slide **to 0.4% agarose**
  - Ex. Initially the LMP is 1% and the volume of slice is 20 ul. Add H_2O to dilute the % to 0.4%. The volume of H_2O is 50 ul.
    Calculation: $\frac{20 \times 0.4}{1}$ Final volume = 100 ul
- Add phenol separately and vortex the mixture and briefly spin
- Take out the upper phase without contaminating the upper phase with the inner phase
- Add chloroform at an equal volume
- Vortex and spin the mixture again
- Remove the upper phase and proceed to precipitate the recovery DNA in the upper phase

Methods to Extract DNA from Agarose Gels

- Variation on LMP Agarose/Phenol method

-excise DNA band of interest with a sterile razor blade
-place in a microcentrifuge tube, freeze at –70 °C, and then melt
-add TE-saturated phenol
-**freeze mixture again** and thaw
-centrifuge the tube and remove the aqueous layer to a new tube.
-remove residual phenol with **two ether extractions**
-concentrate the DNA by ethanol precipitation

Variation on LMP Agarose/Phenol Method

Phenol-based Method for the Isolation of DNA Fragments from Low-Melting Temperature Agarose

Reference: Favre, D. 1992. Biotechniques vol. 13

1. After melting the slice at 65 °C for 5-10 min, add 1 volume Tris-buffered phenol at room temperature, mix by inversion.
2. Spin 3 min at 10-12k rpm and transfer aqueous phase to new tube.
3. Phenol extract again.
4. Spin as in (2) and transfer aqueous phase to new tube **containing 0.1 volume 4 M LiCl**.
5. Mix by inversion; a white precipitate forms immediately. Place tube on ice 2 min. Spin as above, 3 min.
6. Transfer to new tube, leaving transparent pellet behind.
7. **Add 1 ul carrier (glycogen)** and precipitate with 2.5 volumes cold ethanol.
8. Mix, leave at -70 C 5-10 min, and spin as above, 10 min.
9. Wash pellet with 1 ml 70% ethanol, dry under vacuum, and resuspend in 10-20 ul water or TE.

Extraction of DNA from Acrylamide Gel

- Usually for purification of short oligos
- The matrix is more dense than agarose gel
- The gel sieve of DNA yields a higher resolution than agarose gel
- Visualization of DNA in acrylamide gel
  - Examples:
    - Stain the gel with ETBr and place under UV
    - TLC shadowing
    - Methylene blue

Visualization of DNA in Acrylamide Gel: *ETBr*

Visualizing DNA in Acrylamide Gels: *TLC Shadowing Method*

- The method is based on UV interaction with DNA
- Molecules which have double bond and ring structure will absorb UV
- DNA has double bond and ring structure, therefore DNA will absorb UV, preventing the UV from reaching the fluorescent surface underneath

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- Place the gel on a piece of saran wrap on an X-ray cassette intensifying screen (or a thin-layer chromatography plate).
- Then illuminate with a hand-held UV light source
- Where there is no oligo, the screen fluoresces, so the DNA appears as a black band. This area appears as a shadow
- The shadow created will mark the position of the DNA

Visualizing DNA in Acrylamide Gels: *Methylene Blue Staining*

- Run the oligo into a mini PAGE
- When finished, soak the gel in a soln of 0.0002% Methylene Blue and 0.1X TBE.
- Find something to do for 4 hours like growing your other stuff and reading a journal.
- Come back and the stained bands will have appeared.
- Cut out the bands and do your usual elution procedure, desalt.

UV Damage to DNA

- UV-generated damage to DNA stained with ethidium bromide reduces the transformation efficiency (it often ruins the sticky ends on the vector)
- One minute of exposure to 312 nm UV can reduce the transformation efficiency by > 90%.
- Keeping the gel in the gel tray while setting it on the UV box stops the damaging effects of the UV.
- Guanosine is reported to protect DNA from damage by UV transillumination. 1 mM guanosine added to the gel and the running buffer "increased the yield of clones by a factor of about 400, compared with conventionally prepared, unprotected DNA" exposed to 312 nm UV.

Kinasing Reaction

- The only enzyme that does not work in gel containing solutions is T4 polynucleotide kinase.

Dephosphorylation

A common reason for failure to obtain colonies: used too much BAP or CIP

- BAP, CIP frequently contaminated with exonucleases and phosphodiesterases
- The contaminating exonucleases nibble away restriction overhangs.
- Use BAP and CIP at elevated temperatures because the contaminants are less active at high temperatures.
- BAP is more heat-resistant and is difficult to completely inactivate.
- Use exactly enough BAP or CIP for the number of ends. This usually requires dilution of the stock.
- SAP is thermally sensitive and must be used at 37°C, but lacks the contamination problems of BAP and CIP.
- The byproducts of the dephosphorylation inhibit the dephosphorylation reaction itself (dephosphorylation generally only proceeds to 95% completion)
- Don't try to overcome incomplete dephosphorylation by adding more enzyme: is better to do one reaction, clean it up, and repeat the dephosphorylation step

Ligation Reaction

- This protocol uses 0.5 pmols (866 ng) of pUC vector in a 20 µL ligation reaction for a final vector concentration of 43 ng/µL. While the quantity of vector can be scaled down to as little as 30 fmols (52 ng) of nucleic acid, the larger amount avoids problems that can arise from inherent errors in spectrophotometer measurements of nucleic acid concentrations or loss of material during purification.

- Since 66 µM dATP inhibits T4 DNA ligase by 60%[9], the PCR amplified insert should be purified before being used in the ligation reaction
- Do not use tRNA carrier, since it will inhibit the ligation

- A **5:1 ratio** is sufficient if the vector and insert are cut separately (allowing removal of the polylinker fragment via a 30K cartridge) and the concentrations are accurately determined. A 10:1 ratio is recommended if the polylinker is still present in the ligation reaction. If the insert concentration is in doubt, use both ratios or increase the ratio at the risk of double inserts. Higher ratios lead to double-inserts and should be avoided.

Second Lecture

Determine the sequence of DNA binding site

Anneal the single strand DNA to form a double strand DNA

Fuse the DNA binding site in front of the promotor

Transfect and add hormone to induce promotor activity

Assay of promotor activity by measuring the reporter expression

Assay the luciferase activity from reporter gene

Selection of Reporter Genes

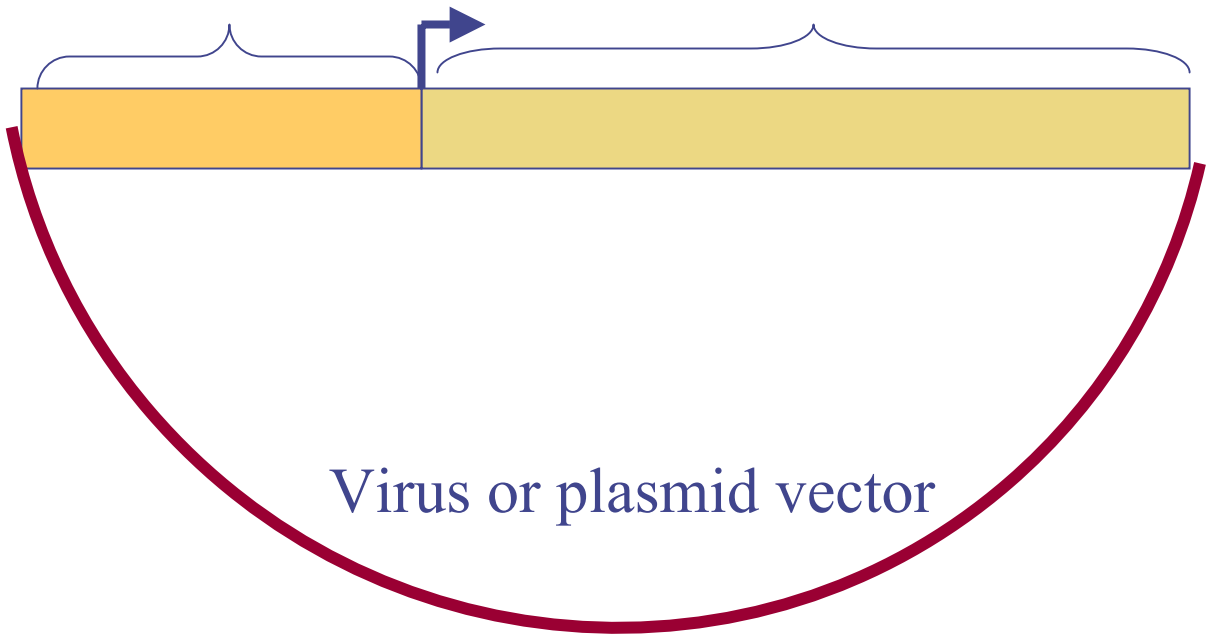
Reporter genes are chosen for a number of different purposes:

- As a Promoter Activity Reporter
- As a Biosensor of Metabolic or Environmental Surroundings
- As an Indicator of Gene Transfer (gene therapy)
- As an Indicator of Extent of Transgene Expression
- As a Detector of Cellular Biochemical Mechanisms

Structure of "Reporter Genes"

Promoter (constituitive vs inducible)

Coding Sequence (encodes transcript or protein to be detected/monitored)



Reporter Genes for Monitoring Test Promoter Activity

In vitro transcription analysis

G-Free cassette e.g. ATACTATCCTCATACCG

[ATP, CTP, TTP]

Transcription stops

Primer Extension

Cell transfection transcription analysis

Beta-galactosidase

CAT

luciferase

In vivo transfection transcription analysis

Bioreporters (Biosensors) and Reporter Genes in Microbial Ecology

Currently, physical and chemical analytical technologies exist for the detection of environmental pollutants

Urgently needed are bioassays which report not only on the presence of a chemical but also on its bioavailability and its biological effects.

"Biosensors" based on genetically engineered bacteria

Biosensor reporter constructs typically combine a promoter-operator, which acts as the sensing element, with reporter gene(s) coding for easily detectable proteins

Are designed to detect different groups of target chemicals

Additional improvements will make microbial biosensors an important tool for future environmental analysis

Bioreporters (Biosensors) and Reporter Genes in Microbial Ecology

Most have

(1) an environmentally or metabolically responsive promoter

(2) fused to a suitable reporter gene (e.g. genes for β-galactosidase, luciferase, GFP, etc.).

Reporter Genes Introduced into the Microbes

(1) either contained on plasmids maintained by antibiotic selection or

(2) transferred to the chromosome via transposable elements followed by antibiotic selection

- insertions occur at random positions in the chromosome, or
- can be integrated at a specific location (usually phage attachment sites) in the chromosome via integration proficient vectors

Bioreporters (Biosensors) and Reporter Genes in Microbial Ecology

Changes in abundance of the reporter protein indicates

(1) changes in the transcriptional activity of the promoter and, thus,
(2) changes in the stimulus to which that promoter is responsive.

Stimuli can be either

(1) chemical (e.g, nutrients, metals or antibiotics]),
(2) physical (e.g,ultraviolet light, temperature or water potential), or
(3) biological (e.g, n-acylhomoserine lactone, which bacteria use to monitor their population density)
(4) a microbe's growth rate or
(5) microbes deficiency in an essential resource such as carbon, oxygen, nitrogen or phosphorus.
(6) other indicators of metabolic stress (reflecting status of the environment)

Reporter Genes As a Detector of Cellular Biochemical Mechanisms

Example: Use of reporter genes to label selected neuronal populations in transgenic mice
(e.g., labelling neurons that contain gonadotropic releasing hormone [GnRH])

Kinds of Reporter Genes Used Include
(1) the β-galactosidase
(2) green fluorescent protein (GFP)
(3) luciferase
(4) β-lactamase

Target expression of reporter gene by
(1) fusing the corresponding to a cell-type or brain-region-specific promoter (I.e. GnRH promoter)
(2) may or may not also include a subcellular localization signal

The Reporter Gene Studies Revealed
(1) promoter elements required for cell type-specific expression of GnRH
(2) the full anatomical profile of the GnRH neuronal network, and
(3) the electrophysiological activity of that network

Prog Neurobiol 2001 Apr;63(6):673-86

Reporter Genes As a

Detector of Cellular Biochemical Mechanisms In Vivo

Vital step in transgenic animal study is the ability to assay the extent of transgene expression.

Desirable to noninvasively and repetitively determine the location, duration, and magnitude of transgene expression in living animals.

Classical Reporter Genes (b-galactosidase, alkaline phosphatase) would require invasive sampling or death of transgenic animal

Several Alternative Possible Approaches

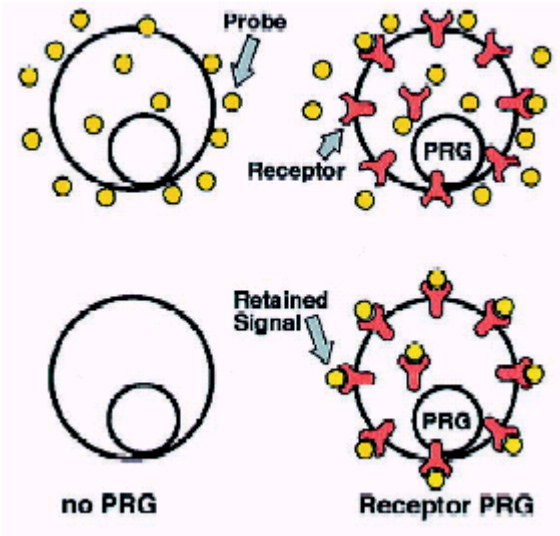
(1) radionuclide (single photon emission computed tomography [SPECT], positron emission tomography [PET])
(2) optical (green fluorescent protein, luciferase)
(3) magnetic (magnetic resonance imaging) approaches

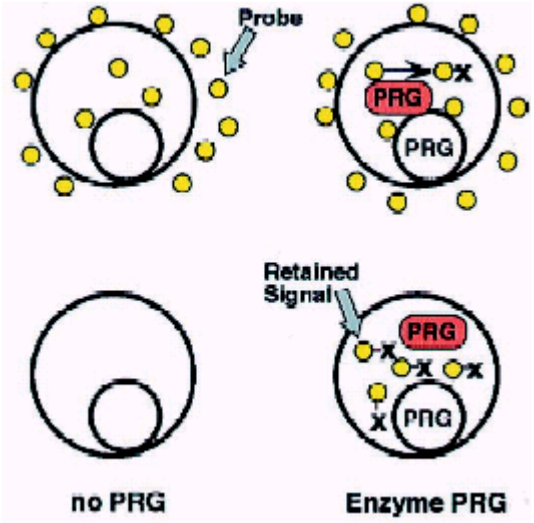
Radionuclide approach has
(1) highest sensitivity
(2) full quantitation [PET] to measure the expression of genes in vivo in large or nontransparent animals.
(3) can be extend small animal assays directly into clinical human applications.

For PET Scanning, the reporter gene must be capable of either
(1) binding positron-emitting ligand probes (e.g., dopamine type 2 receptor)
(2) be enzymes that modify the positron-emitting substrate probes to produce sequestered positron-emitting products (e.g. certain thymidine kinase substrates)

Reporter Genes As a

Detector of Cellular Biochemical Mechanisms In Vivo



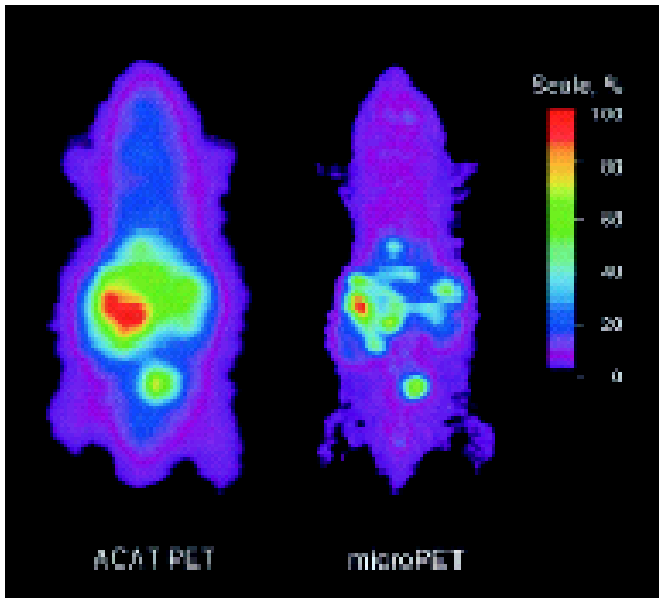


Expression of reporter gene produce that binds radionuclide that is infused to the animal

Expression of reporter gene product that binds enzymatically converts radionuclide substate into product that is ‘trapped’ inside the cell the animal

Reporter Genes As a

Detector of Cellular Biochemical Mechanisms In Vivo



Comparison of positron emission tomography (PET) imaging systems. A nude (NU/NU) mouse was imaged with a radiolabeled probe, 3-(2'-[^{18}F]fluoroethyl)spiperone (FESP

Third

Lecture

1. Review of Pop Quiz
2. Calculation for Conversion to Picomol
3. Counting Cells
4. Continuation of Reporter Genes and Subsection of Gene Therapy

a. How did you visualize the products and how is it that the method you used enables you to see the products? Be sure to describe the chemical details of the reason that the method works.

Visualization of DNA in Acrylamide Gel: *ETBr*

- ETBr is mutagen
- ETBr intercalates the ring structure of the DNA and absorb the UV
  - Under UV, DNA band can be seen glowing

b. What alternative method could you have used?

Visualizing DNA in Acrylamide Gels: *TLC Shadowing Method*

- The method is based on UV interaction with DNA
- Molecules which have double bond and ring structure will absorb UV
- DNA has double bond and ring structure, therefore DNA will absorb UV, preventing the UV from reaching the fluorescent surface underneath

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- Place the gel on a piece of saran wrap on an X-ray cassette intensifying screen (or a thin-layer chromatography plate).
- Then illuminate with a hand-held UV light source
- Where there is no oligo, the screen fluoresces, so the DNA appears as a black band. This area appears as a shadow
- The shadow created will mark the position of the DNA

c. What experimental problems might arise by the method you used, and what steps could you take to reduce those problems?

UV Damage to DNA

- UV-generated damage to DNA stained with ethidium bromide reduces the transformation efficiency (it often ruins the sticky ends on the vector)

- One minute of exposure to 312 nm UV can reduce the transformation efficiency by > 90%.

- Keeping the gel in the gel tray while setting it on the UV box stops the damaging effects of the UV.

- Guanosine is reported to protect DNA from damage by UV transillumination. 1 mM guanosine added to the gel and the running buffer "increased the yield of clones by a factor of about 400, compared with conventionally prepared, unprotected DNA" exposed to 312 nm UV.

d. By what procedure did you recover the concatamerized products from the gel?

The laboratory protocol handed out stated that the DNA would be eluted out of the gel slice over night into buffer.

*melting the gel slice was a protocol discussed for recovery of DNA from agarose gels; it was not the procedure stated to use in the lab for recovery of DNA from acrylamide gels

Conversion to Picomoles

Calculation of conversion of gram units to picomole units

Example: How many picomoles of 5000 base pair vector do you have, if you have 0.1 ug of that vector?

1. First, calculate the molecular weight of the vector

First, calculate the molecular weight of the vector

5000 bases

x 2 (for being double stranded)

x 320 g/mole/base

$$3.2 \times 10^6 \ \frac{g}{mole}$$

$3.2 \times 10^6 \ \frac{g}{mole}$ **Molecular weight of vector**

Inverting: $\frac{1}{3.2 \times 10^6} \ \frac{mole}{g}$

$3.2 \times 10^6 \ \frac{g}{mole}$ **Molecular weight of vector**

Inverting: $\frac{1}{3.2 \times 10^6} \ \frac{mole}{g}$

converting units: $\frac{1}{3.2 \times 10^6} \ \frac{umole}{ug}$

$3.2 \times 10^6 \ \frac{g}{mole}$ Molecular weight of vector

Inverting: $\frac{1}{3.2 \times 10^6} \ \frac{mole}{g}$

converting units: $\frac{1}{3.2 \times 10^6} \ \frac{umole}{ug}$

Multiplying by amount of vector available:

$$0.1 \text{ ug} \times \frac{1}{3.2 \times 10^6} \ \frac{\text{umole}}{\text{ug}} = 0.33 \times 10^{-7} \text{ umole}$$

$3.2 \times 10^6 \ \frac{g}{mole}$ Molecular weight of vector

Inverting: $\frac{1}{3.2 \times 10^6} \ \frac{mole}{g}$

converting units: $\frac{1}{3.2 \times 10^6} \ \frac{umole}{ug}$

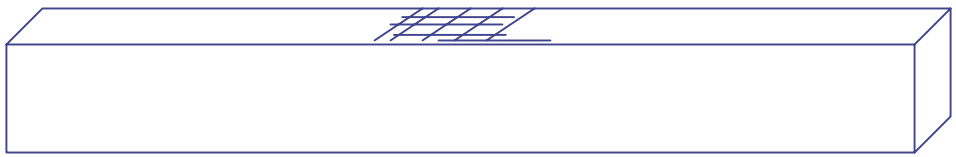
Multiplying by amount of vector available : $0.1 \ \cancel{ug} \times \frac{1}{3.2 \times 10^6} \ \frac{umole}{\cancel{ug}} = 0.33 \times 10^{-7}$ umole

converting units: 0.33×10^{-7} umole $= 0.33 \times 10^{-1}$ pmole

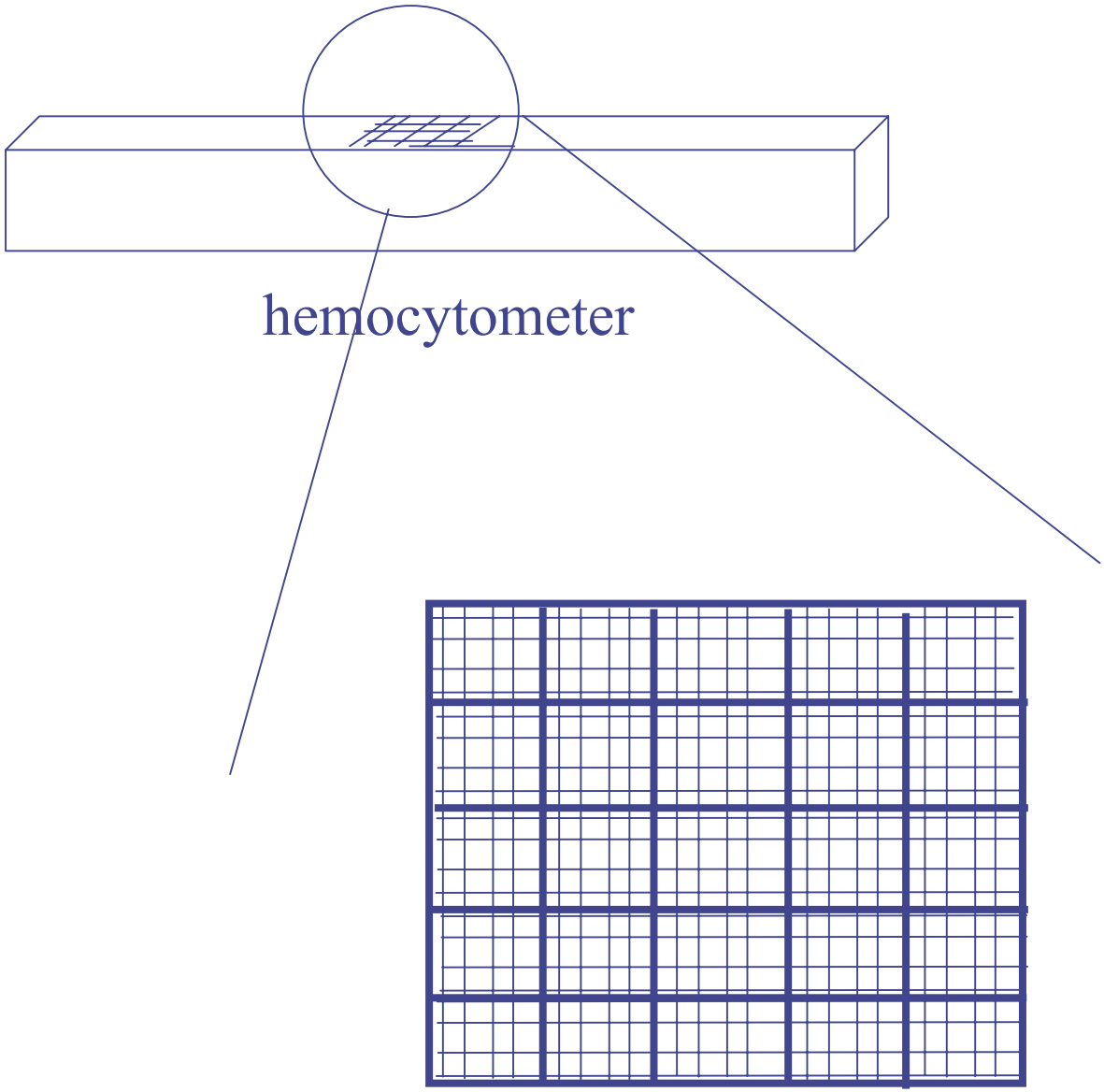
$= 0.033$ pmole

Calculating # of Cells

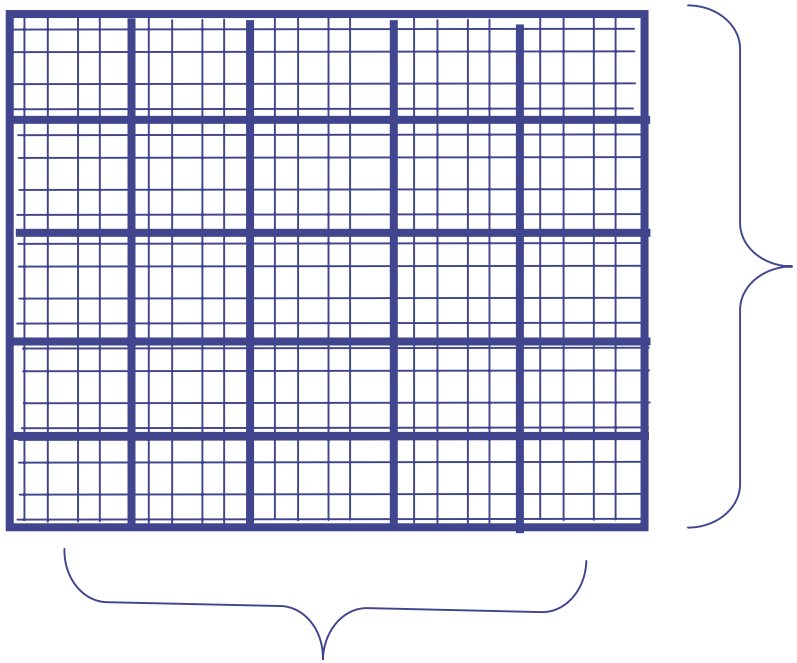
Calculating the number of cells/ml

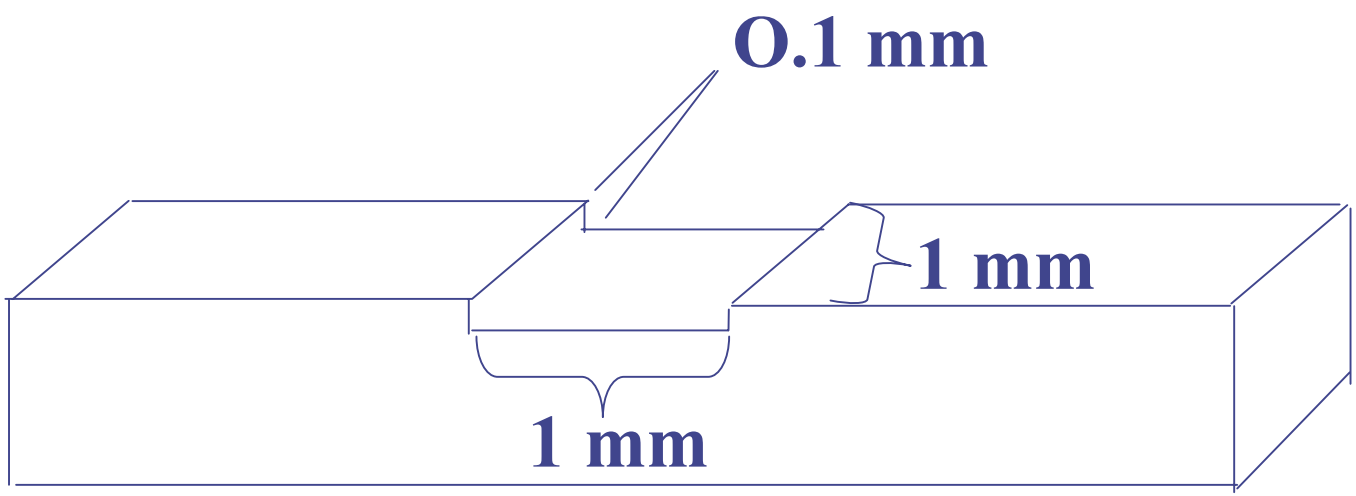


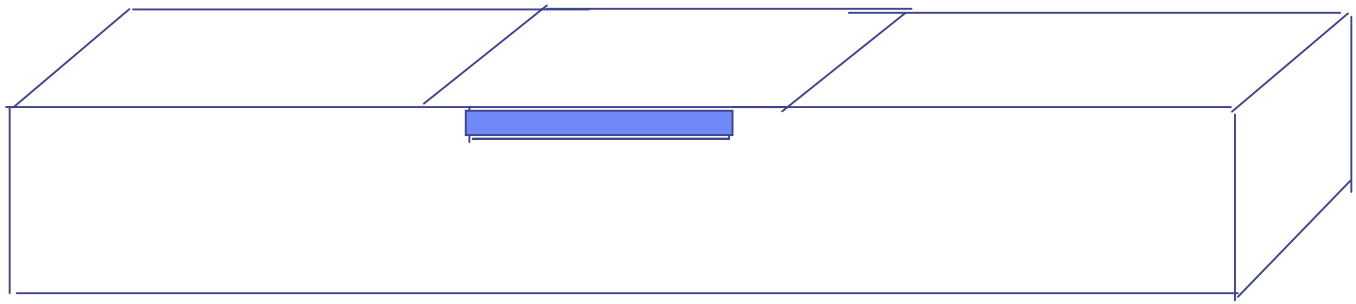
hemocytometer


hemocytometer

25 Medium Squares




O.1 mm
1 mm
1 mm



Volume underneath the cover slip = 1 mm x 1 mm x 0.1 mm

$= 0.1\ mm^3$

$= 0.1$ ul

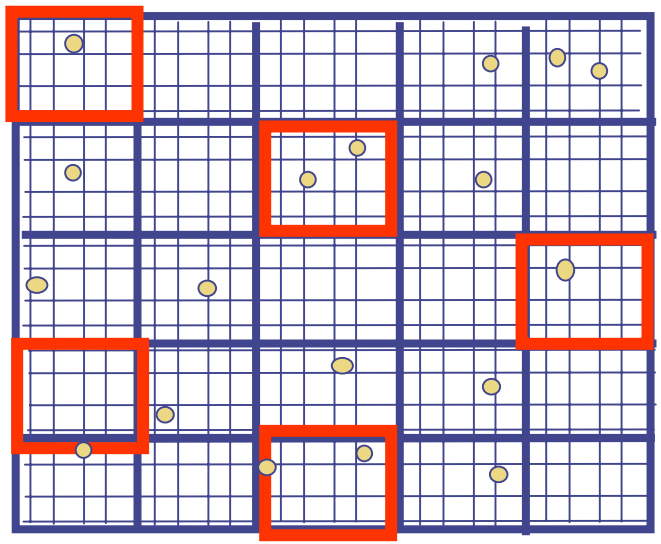
$=10^4$ ml

Add aliquot of cell solution to fill up the area under the cover slip.

Examine from above under microscope.

Count the number of live cells in 5 squares

25 large squares



5 large squares

5 large squares

Rule for cells on line

- **Left and top: count**
- **Right and bottom: disregard**

# of cells counted x 5 x 10^4 = # cells /ml

In our example:

6 cells x 5 x 10^4 = 3 x 10^5 cells/ml

Continuation of Reporter Genes and Subsection of Gene Therapy

D. As an Indicator of Gene Transfer (gene therapy)

- **Gene-marking studies** - were the first approved clinical protocols for introducing exogenous genetic material into human cells.
- **The reporter genes are coupled with a therapeutic gene of interest** to indirectly monitor the expression of the therapeutic gene.
- **Reporter genes are emerging as very powerful tools to monitor the**

(1) delivery,
(2) magnitude, and
(2) time variation of therapeutic gene transfer in vivo

D. As an Indicator of Gene Transfer (gene therapy)

Rapid advances in imaging technologies offer new opportunities to optimize clinical trials of human gene therapy

- **Several reporter genes are currently being in connection With imaging technology**

(1) herpes simplex virus type 1 thymidine kinase,
(2) dopamine type 2 receptor,
(2) somatostatin receptor type 2

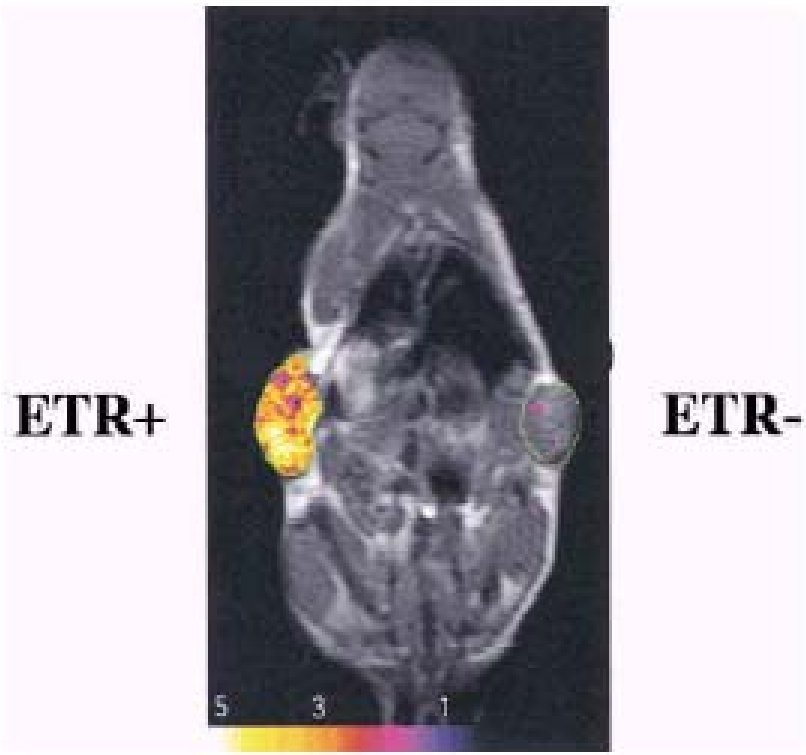
- **These reporter genes are currently being successfully used with several imaging approaches**

(1) gamma camera,
(2) single photon emission computed tomography
(3) positron emission tomography imaging (PET)

D. As an Indicator of Gene Transfer (gene therapy)

Nuclear imaging techniques

uniquely provide the necessary sensitivity required to:
(1) evaluate the success of the gene delivery and expression (transcription and translation)
(2) detect unwanted expression by non-target tissues.



- **In vivo magnetic resonance imaging of an engineered transferrin receptor (ETR) using a superparamagnetic transferrin probe (Tf) as a ligand.**

- **In this model, the expression of a magnetic resonance marker gene (ETR) is imaged with Tf-MION in a stably transduced tumor cell line implanted in vivo.**

D. As an Indicator of Gene Transfer (gene therapy)

Enzymes as reporters in gene therapy

Are attractive targets for diagnostic imaging in gene therapy because:
(1) they convert multiple molecular copies of the substrate (radiotracer) per molecule of enzyme,
(2) thereby greatly increasing the ultimate sensitivity (relative to the sensitivity offered by receptors that bind with 1:1 stoichiometry)

Enzymes have been the preferred molecular targets to date for scintigraphic imaging of gene therapy. (radiolabelled nucleosides and nucleoside bases for imaging in gene therapy)

There is interest in exploitation of enzyme systems for scintigraphic imaging of gene expression in gene therapy of cancer.

D. As an Indicator of Gene Transfer (gene therapy)

- **Gene-based therapies offer hope for treatment of a number of blood cell diseases**

(1) hereditary hemoglobinopathies,
(2) immunodeficiency syndromes,
(3) hemophilia
(4) AIDS.

- **Successful treatment with gene-modified HSCs requires**

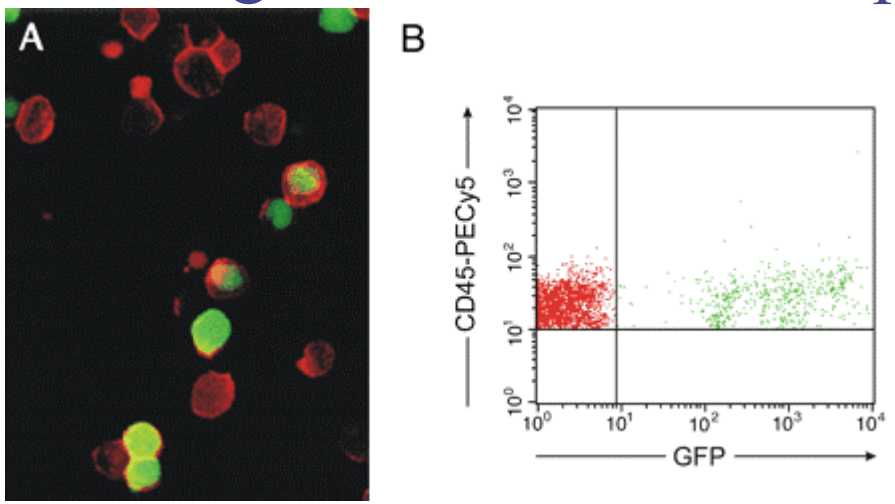
(1) high efficiency gene delivery to the target cell population
(2) persistence of transgene expression following differentiation.

- **Use of proper gene linked-reporters allows monitoring of gene delivery and persistence by noninvasive means**

- blue, cyan, and yellowish-green fluorescent reporters offer this possibility

D. As an Indicator of Gene Transfer (gene therapy)

Example of use of gene-linked fluorescent protein reporters to monitor gene transfer and the persistence of transferred gene



•Fluorescence microscopy and flow cytometric analysis of fluorescent protein (EGFP) expression in transduced human blood stem cells.

Panel A) CD34+ Cells were transduced with the MGIN oncoretroviral vector encoding (Fig. 2A), and analyzed for CD34 (red) and EGFP (green) expression by confocal fluorescence microscopy.

Panel B) Six weeks after receiving human CD34+ cord blood progenitors transduced with a lentiviral vector containing EGFP reporter, bone marrow was recovered, stained with an antihuman CD45 antibody conjugated to PE-Cy5, and analyzed for CD45 and EGFP expression by flow cytometry.

Additional Considerations in Use of Reporter Constructs

Artifactual Effects of One Cotranfected Plasmid on Another

- *Trans* effects between promoters on cotransfected plasmids can potentially affect reporter gene expression

•The **thymidine kinase promoter** is relatively weak; most useful in providing neutral constitutive expression

•The **SV40 and CMV promoter/enhancer regions** are strong, be less suitable for coreporter applications

• Cross-bleeding between channels in multiple-fluorescent protein applications

Fourth

Lecture

Agenda

- Transfection
- Transformation
- DNA Maxi Prep
- Clarify questions on reporter gene
- Pop Quiz

Transformation

- Usually transformation is referred to getting exogenous DNA inside the cell and that it is permanently maintained/replicated as the cell divides and replicates.
- If is a plasmid, must have 'origin of replication'
  - **bacteria**
  - **yeast**
- The transformation process essentially weakens the bacterial wall/cell membranes and shocks the DNA inside the cell
- The bacteria or yeast uptakes the DNA

Transformation

Common techniques for transformation

1. Heavy salts (calcium or rubidium)

The commercial competent cells are cells whose membranes are presensitized by the company with heavy salts making it easier for you to get the DNA to get inside the bacteria

The researcher uses the differential temperature to 'shock' the bacterial cell walls to allow foreign DNA to enter

2. Electrical shock (electroporation) to cells (yeast)

Transfection

***Transfection*—Process in which the gene of interest is introduced into eukaryotic cells by biochemical or physical methods.**

***Infection*—Viral-mediated process where target cells are infected with a virus carrying the cloned sequence of interest in its genome.**

Transfection Techniques

1. Calcium chloride coprecipitation
2. Electroporation
3. DEAE Dextran (polybrene)
4. Mechanical
5. Cationic lipids

Transfection

Calcium Phosphate Coprecipitation

The mixing of calcium chloride, DNA, and phosphate buffer precipitates extremely small, insoluble particles of calcium phosphate containing condensed DNA.

Complexes adhere to cell membrane

Enter cell by phagocytosis

Transfection

Electroporation

Exposure of a cell suspension to an electrical field

Induces a potential difference across the membrane of the cells.

Induces temporary pores in the cell membrane.

Transfection

DEAE-dextran and Polybrene

Positively charged polymers DEAEdextran or polybrene complex with negatively charged DNA molecules

Enables the DNA to bind to the cell surface.

Complexed-DNA is delivered by osmotic shock using DMSO or glycerol

Transfection

Mechanical Methods

Microinjection

Compressed air particle delivery (e.g. DNA on fine gold particles, pierces cell membrane and enters cell)

Transfection

Cationic Lipid Reagents

E.g., cellfectin

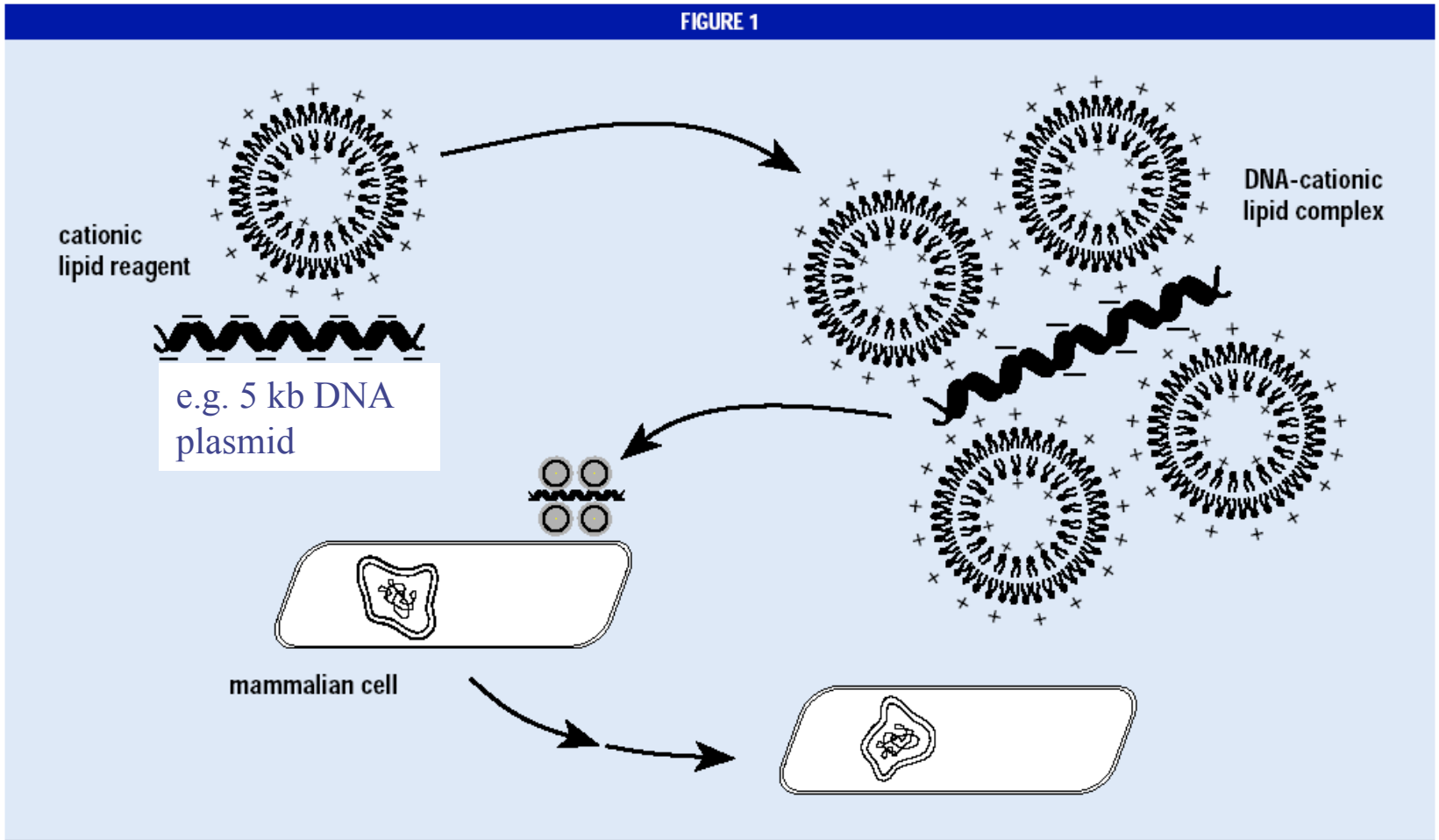
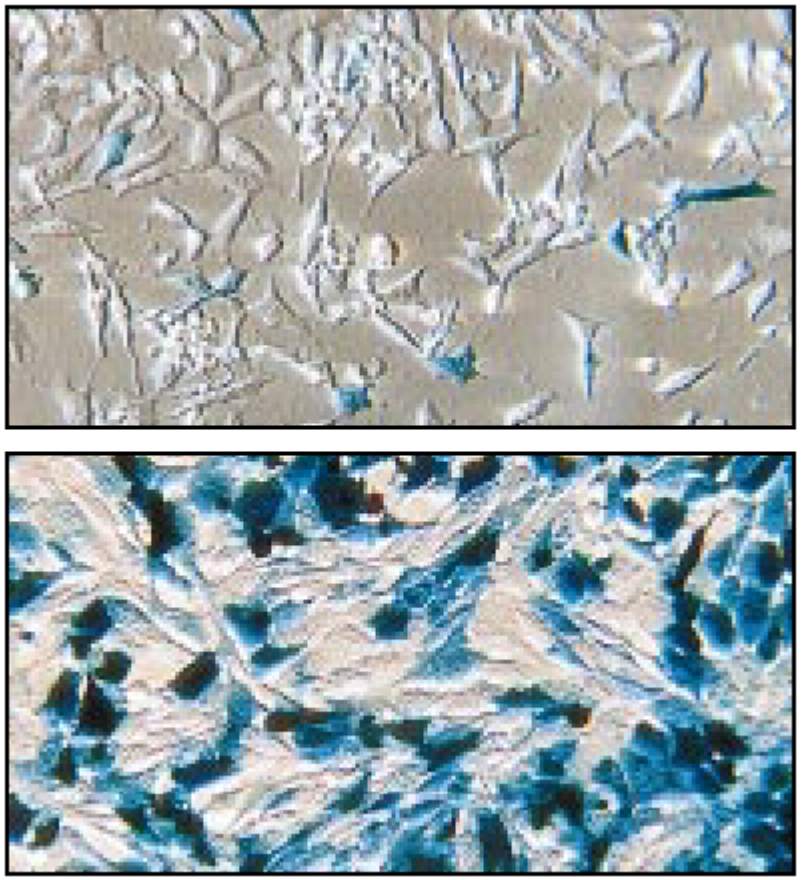


FIGURE 1. Cationic lipid reagent–mediated transfection

Transfection

of plasmid expressing beta-galactosidase



Calcium phosphate method

Cationic lipid method (lipofectamine)

Result of staining for beta-galactosidase reporter

Transfection

Considerations in transfection with cationic lipids

-Serum in cell culture media

-previously a problem

- now form DNA/cationic lipid complexes away from serum, then add to media containing serum

-Antibiotics in the Culture Medium

- cationic lipid increases toxicity of antibiotics to cells

-Health of cells

- passage

-Plating density

-DNA quality

"MaxiPrep"

- Harvest the DNA and essentially it's like minprep but on a larger scale
- The point you have to pay most attention to is the handling of the DNA
- **Common methods of 'maxiprep' of DNA:**
- Cesium Chloride salt/centrifugation
- 'Qiagen' or 'Wizard' using affinity resins

MaxiPrep (cont'd)

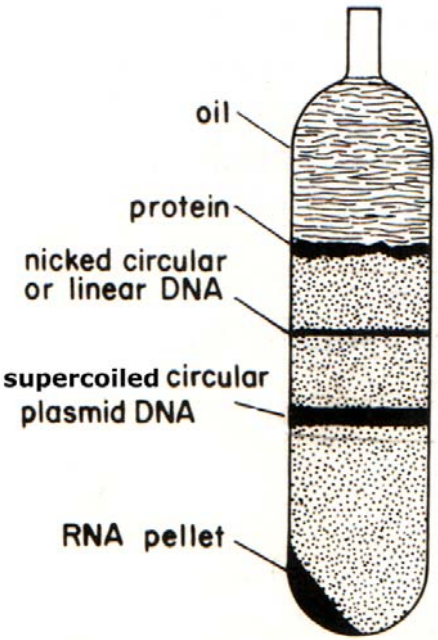
- The DNA, RNA, protein components of cell lysate separate on the cesium chloride gradient.
- When you add ethidium, the DNA will absorb the ethidium
- Depending on the form, the DNA will absorb the ethidium differently and have different density
- Superhelical circular DNA will absorb the most
- Linear DNA will absorb the least

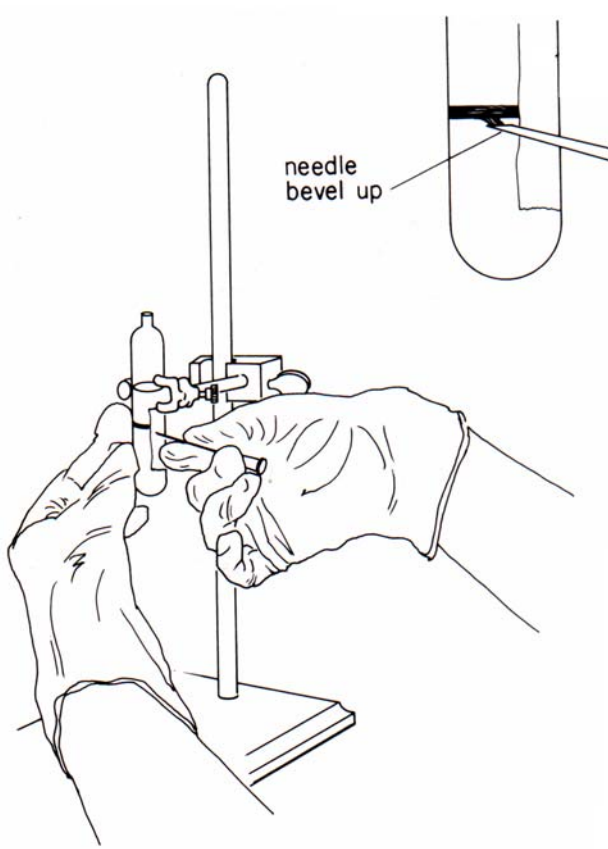
MaxiPrep (cont'd)

- The densities of the different DNA forms (and RNA and protein) thus each achieve a different density and floats during centrifugation to the point of the equilibrium on the cesium chloride density
- The RNA will form a pellet on the bottom of the gradient because it is single stranded and it doesn't absorb ethidium as well.
- Protein will float on the top of the CsCl density gradient

Cesium Chloride-Ethidium Bromide Gradient Method

- The density gradient method is a centrifugation method
- Cesium is a heavy salt. It interferes subsequent reactions:
- Be sure to later ge[illegible]ne
  - Dialysis
  - Changing buffer
- An example final gradient is illustrated at right:



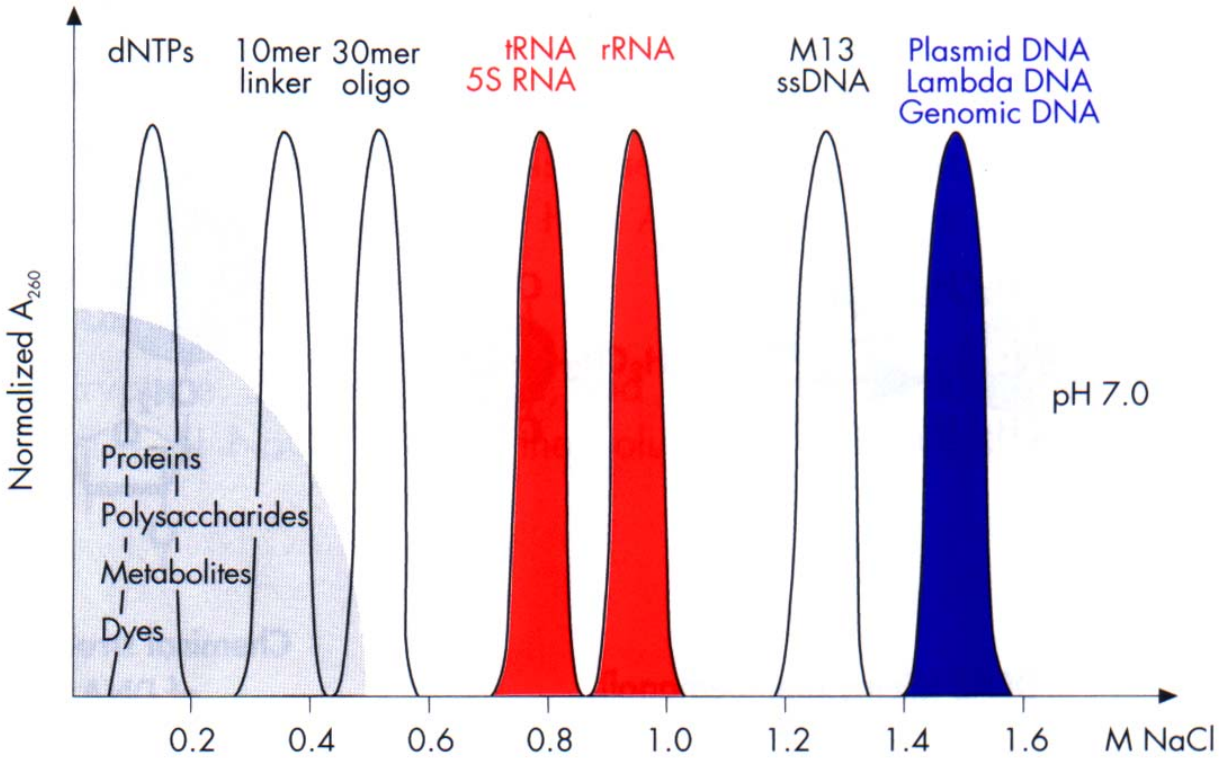


MaxiPrep (cont'd)

- Now you have the superhelical DNA separated from the other contaminates (RNA, protein, linear DNA)
- Draw the DNA band by syringe. Put on Qiagin column
- The Qiagin method is based on charge column (e.g. DEAE column) to separate the DNA and contaminating RNA
- Elute with high molar salt at different retention times and at different intervals

Qiagen Method

- Essentially uses an ion exchange column
- The DNA sticks to the column and is washed off by high salt NaCl
- Here is a visual representation below:



Separation of nucleic acids at neutral pH on QIAGEN Anion-Exchange Resin.

Reporter Genes

Gene product vs. detection method

Reporter gene products

-GFP

-beta galactosidase

-luciferase

Method to detect the reporter gene product

-fluorescence (GFP)

-enzymatic product color (b-gal)

-luminescence (luciferase)

So, NMR is NOT a reporter. It is a method to detect a reporter gene product.

Fifth

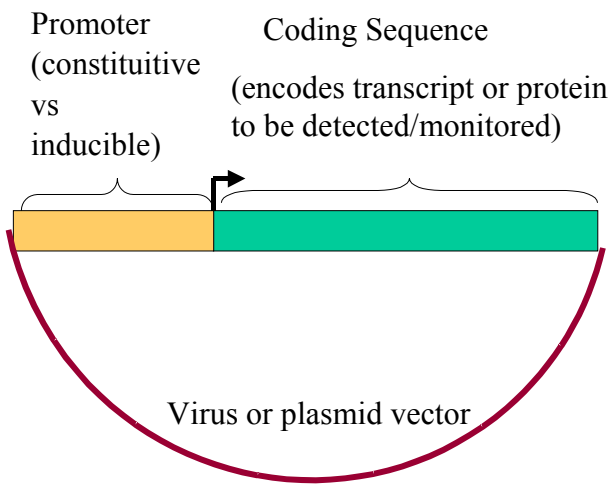
Lecture

Reporter Genes

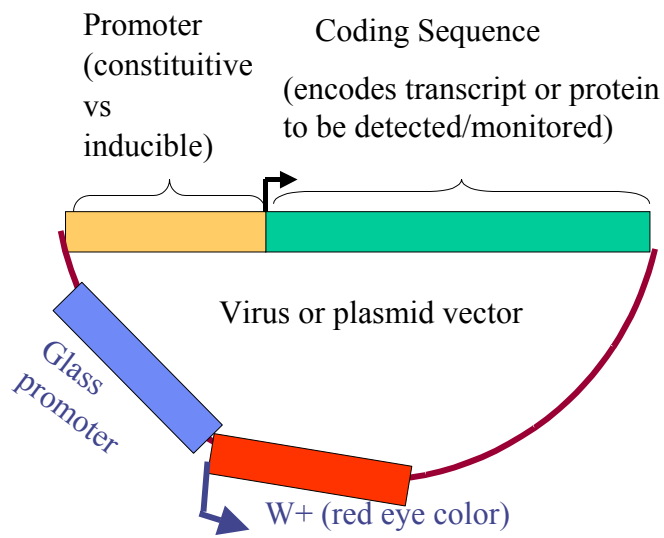
Points to remember

1. Reporter gene product vs. method to detect reporter gene product (e.g. in our project – "luciferase" vs. luminscence detection)
2. Reporter gene product of interest directly vs. indicating the presence of a gene of interest

Reporter gene is of direct interest

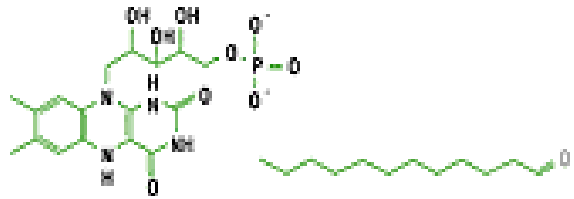
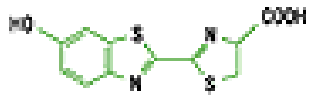
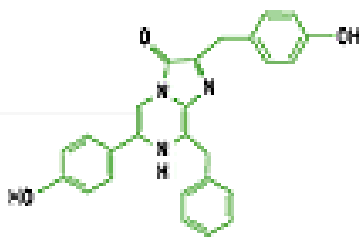


Reporter gene is indirectly indicating the presence of gene of interest

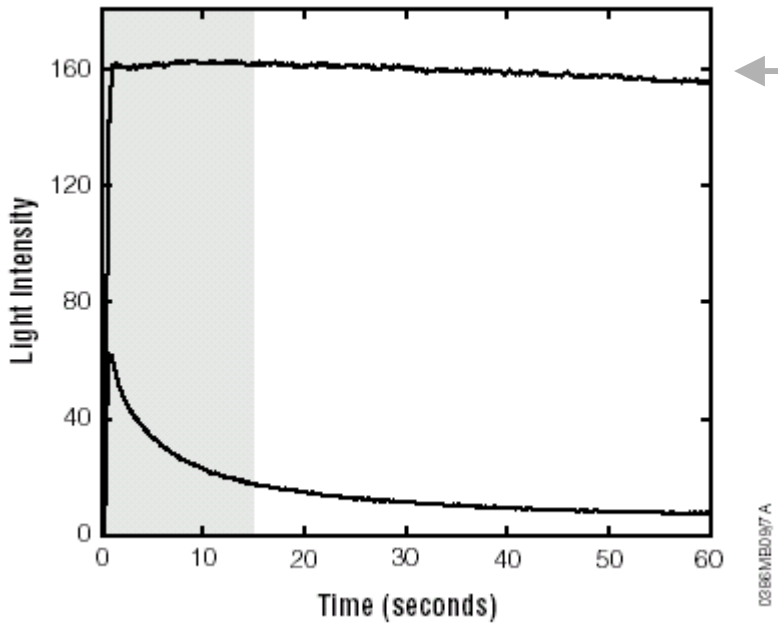


Bioluminescence as detection method of activity of reporter gene

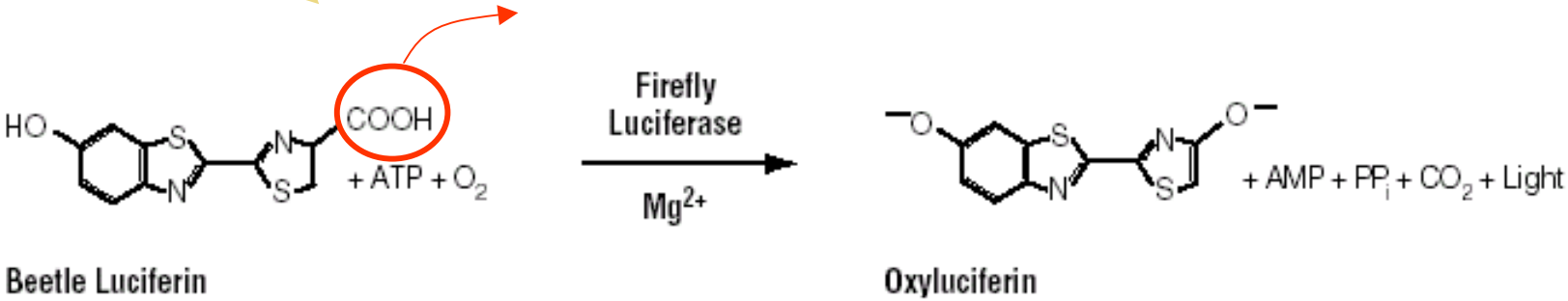
The assay is very sensitive because
--its light production has the highest quantum efficiency known for any chemiluminescent reaction and
--no background luminescence is found in the host cells or the assay chemistry.

Bioluminescence	Reaction		Luciferin Structure
Bacterial	Aldehyde + $FMNH_2$ + O_2	Luciferase → dimeric 80kDa	Carboxylate + FMN + Light	
Firefly	Luciferin + ATP + Mg^{2+} + O_2	Luciferase → monomeric 61kDa	Oxyluciferin + AMP + PPi + CO_2 + Light	
Renilla	Coelenterazine + O_2	Luciferase → monomeric 36kDa	Coelenteramide + CO_2 + Light	

Also include AcylCoA in reaction



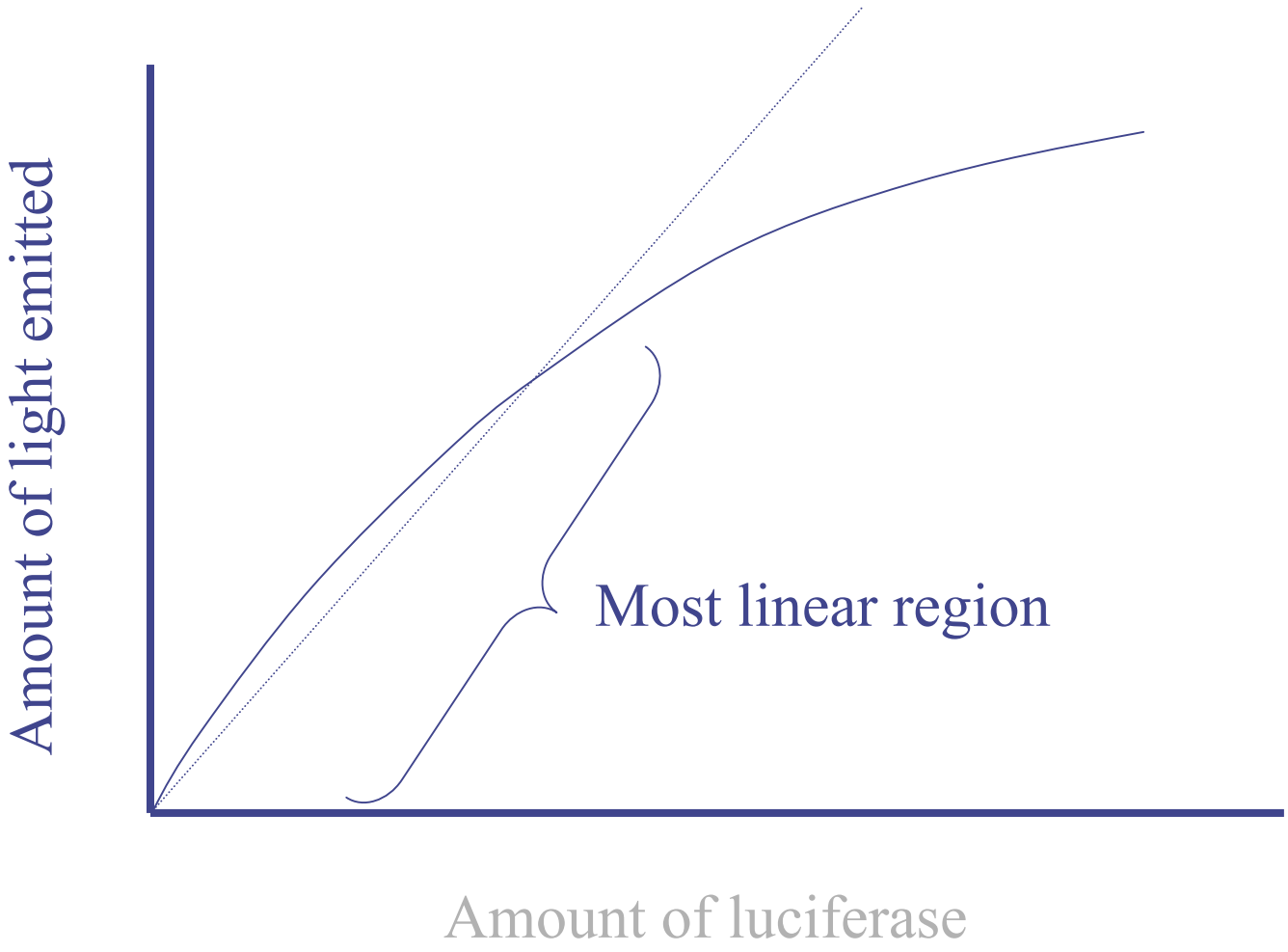
Natural reaction in insect



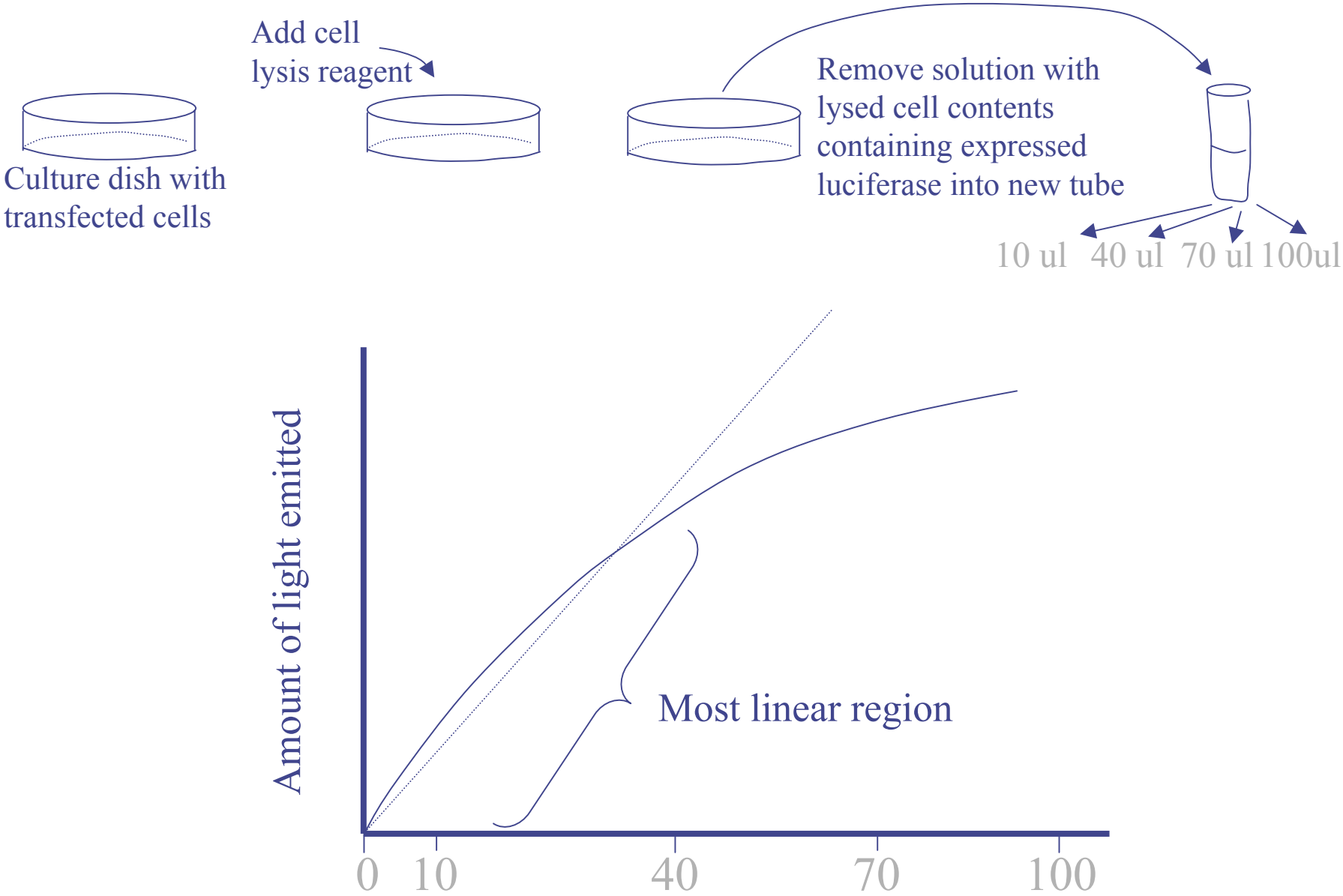
Beetle Luciferin

Oxyluciferin

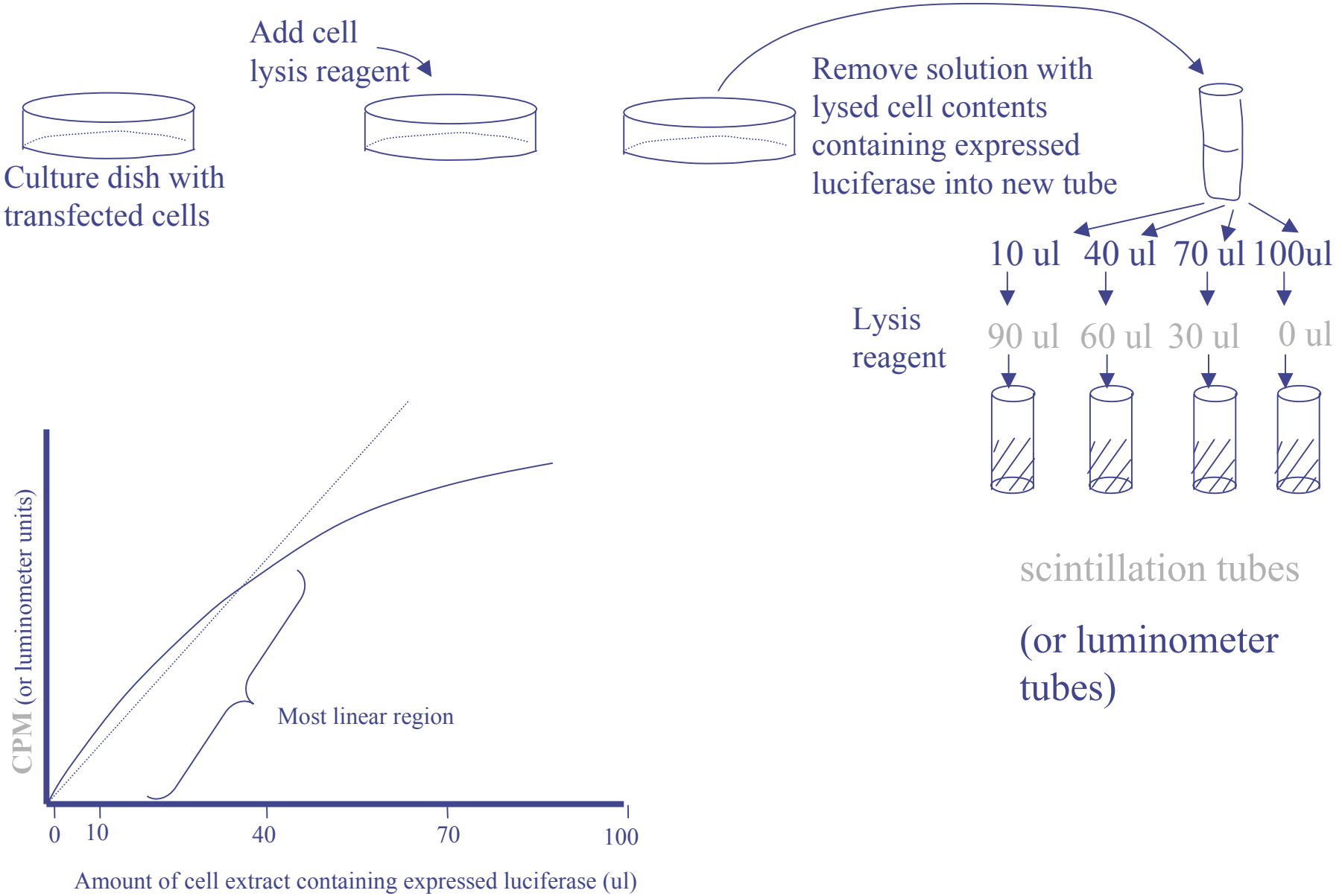
Determine linear region of the reaction kinetics



Determine linear region of the reaction kinetics



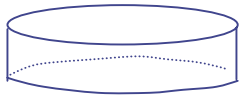
Determine linear region of the reaction kinetics



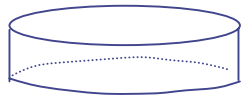
Determine linear region of the reaction kinetics

What to do if you have more than one treatment, e.g.:

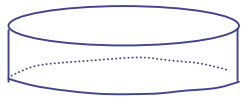
Culture dish with transfected cells



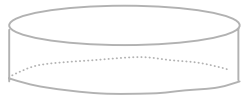
Transfected with empty vector



No hormone

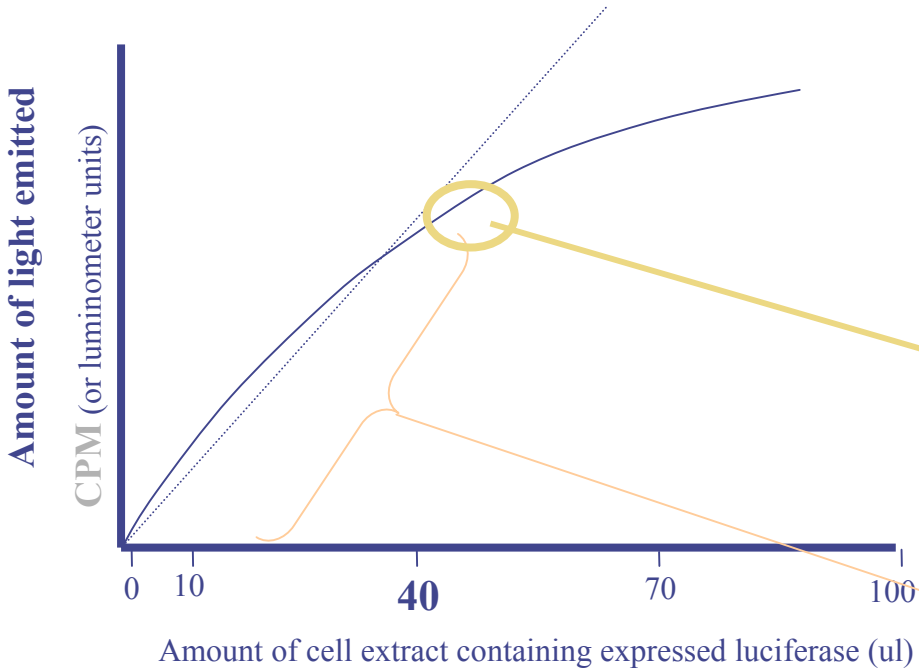


Low dose of hormone



High dose of hormone

Transfected with reporter vector



--Select the one anticipated to have the highest activity (here, the highest dose)

--perform preliminary test on it to find dilution (here, 40 ul) that is at the top edge of linear region

--all other treatments at that dilution or less (here, 40 ul or less) therefore must give lower cpm, that will also be down lower in the linear region

Considerations in setting up the assay conditions:

Determining the probable linear region

-for a given range of *aliquot volumes* of cell lysate (discussed on the previous slides)

-for a *fixed time*

-for a *fixed temperature*

For a fixed time

-typically *short time* (seconds up to minute)

-limited by maintenance of *saturating* amount of substrate

-limited by *product feedback inhibition*

For a fixed temperature

-*room temperature* is optimum (all reaction components at room temperature)

Considerations in setting up the detection method

Luminometer

-Most sensitive

Scintillation Counter (to be used in our course exercise)

Turn off the "**coincidence circuit** of the scintillation counter" (a feature used in isotope counting in which two signals are detected simultaneously) (we have a program for this in our Biology Dept. scintillation counter)

Because the enzymatic reaction produces light at all wavelengths, read the samples with **all channels open** (open window).

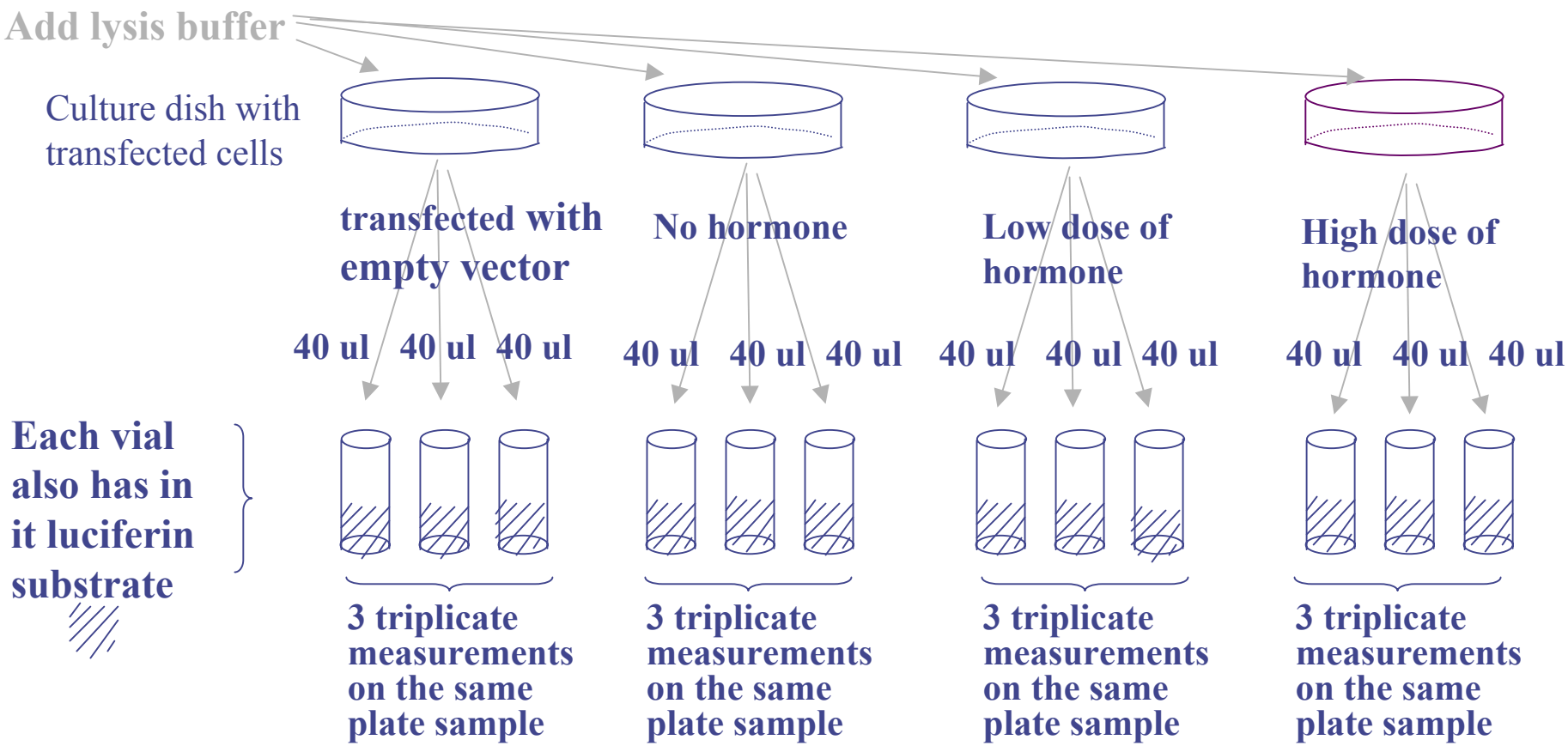
To measure **background cpm**, add Luciferase Assay Reagent to lysis buffer without cells or to a lysate of nontransfected cells.

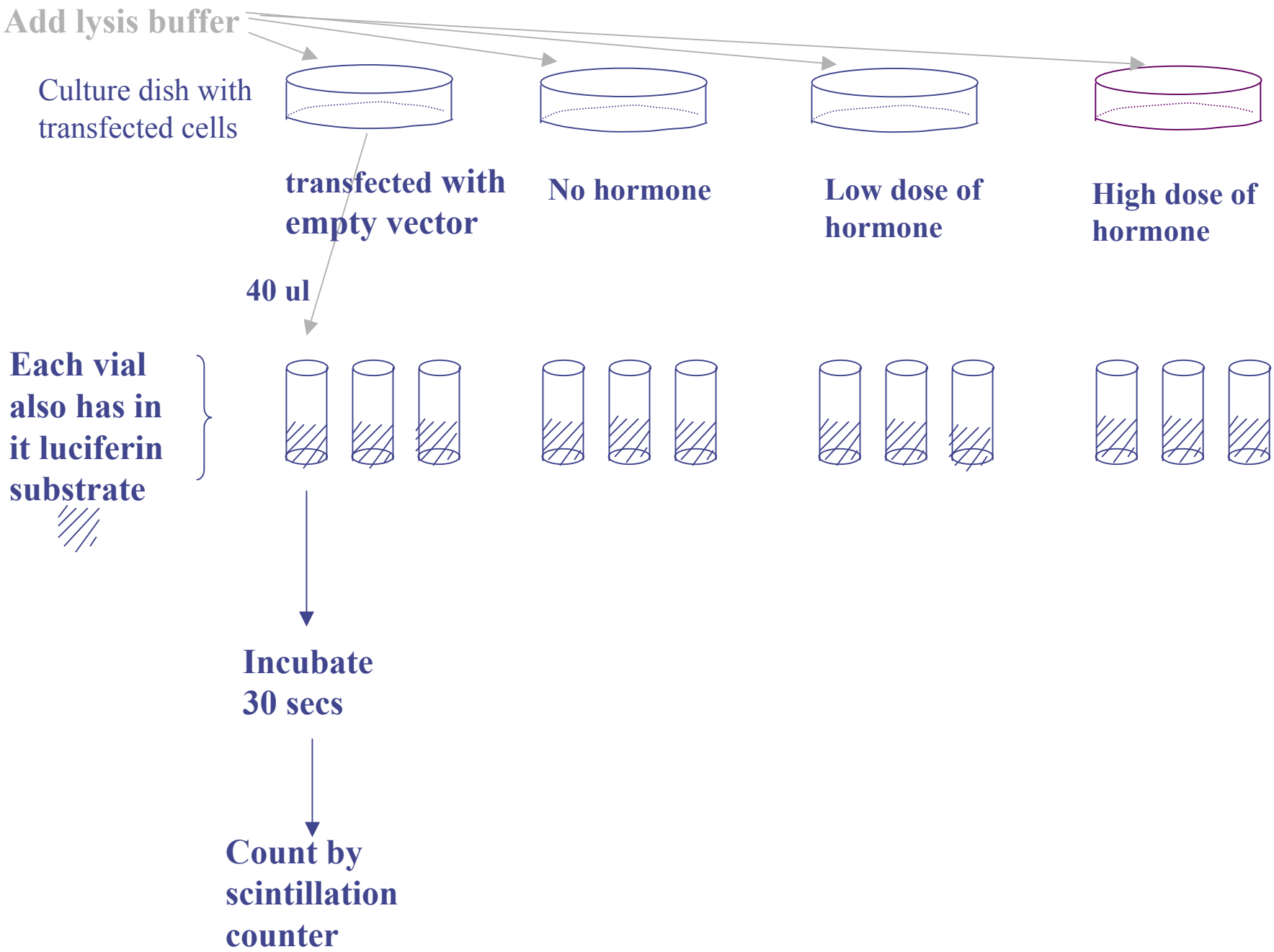
The sample may be placed **directly in the scintillation vial** if it completely covers the bottom of the vial (clear or translucent vials are acceptable) or put it into a microfuge tube and place the tube into a scintillation vial.

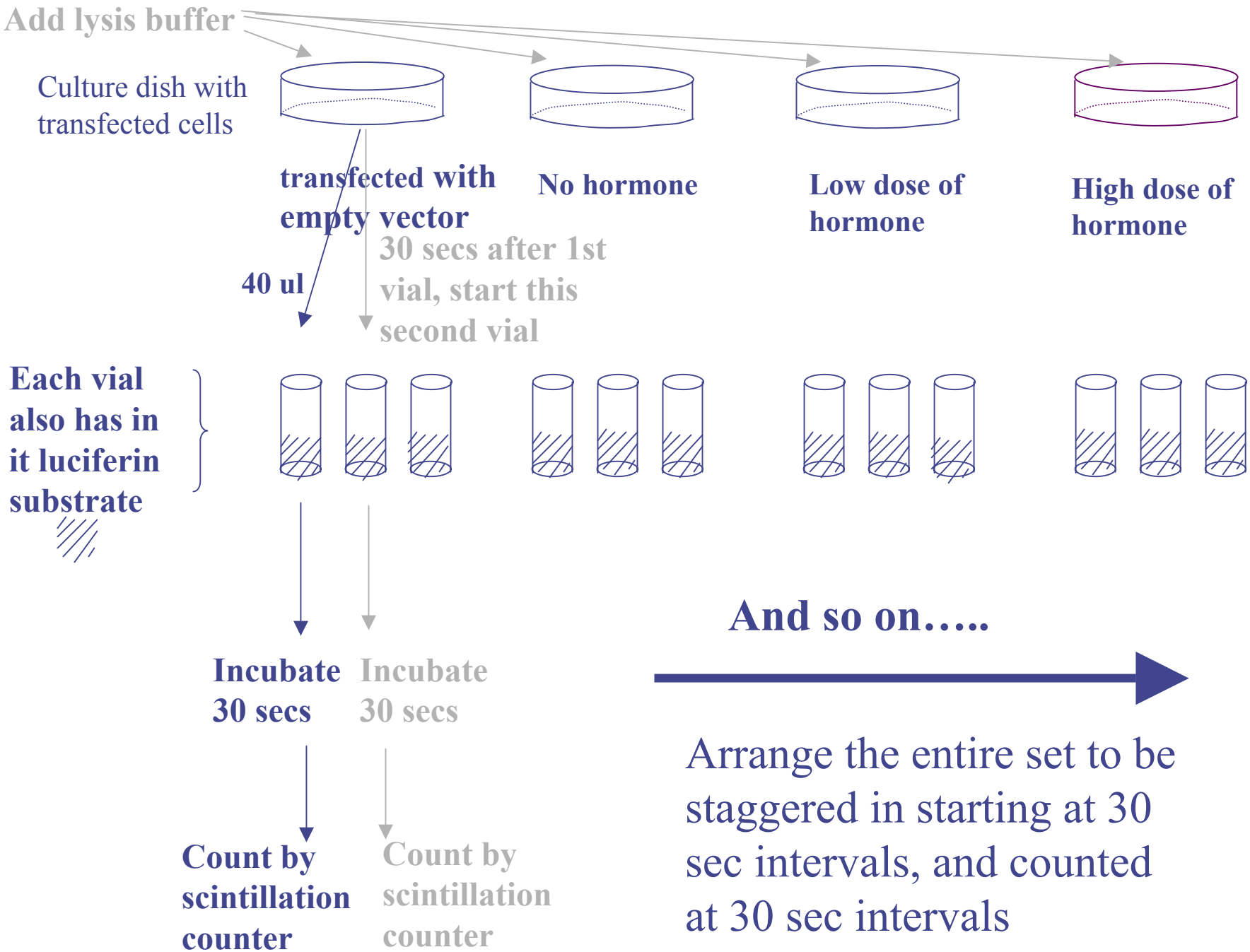
Do not add scintillant, because it will inactivate luciferase.

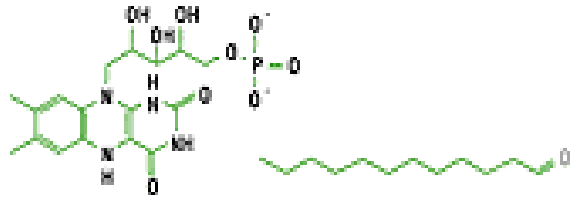
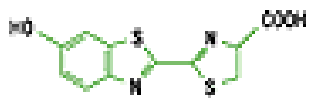
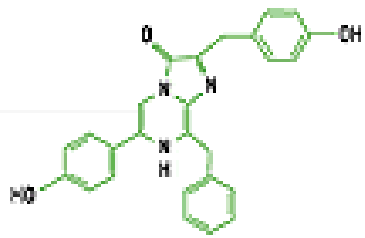
Setting up the reaction

(after preliminary determination of linear region conditions, e.g., 40 ul lysate)




Add lysis buffer
Culture dish with transfected cells
transfected with empty vector
No hormone
Low dose of hormone
High dose of hormone
40 ul
Each vial also has in it luciferin substrate
Incubate 30 secs
Count by scintillation counter


Add lysis buffer
Culture dish with transfected cells
transfected with empty vector
No hormone
Low dose of hormone
High dose of hormone
40 ul
30 secs after 1st vial, start this second vial
Each vial also has in it luciferin substrate
Incubate 30 secs
Incubate 30 secs
Count by scintillation counter
Count by scintillation counter
And so on…..
Arrange the entire set to be staggered in starting at 30 sec intervals, and counted at 30 sec intervals

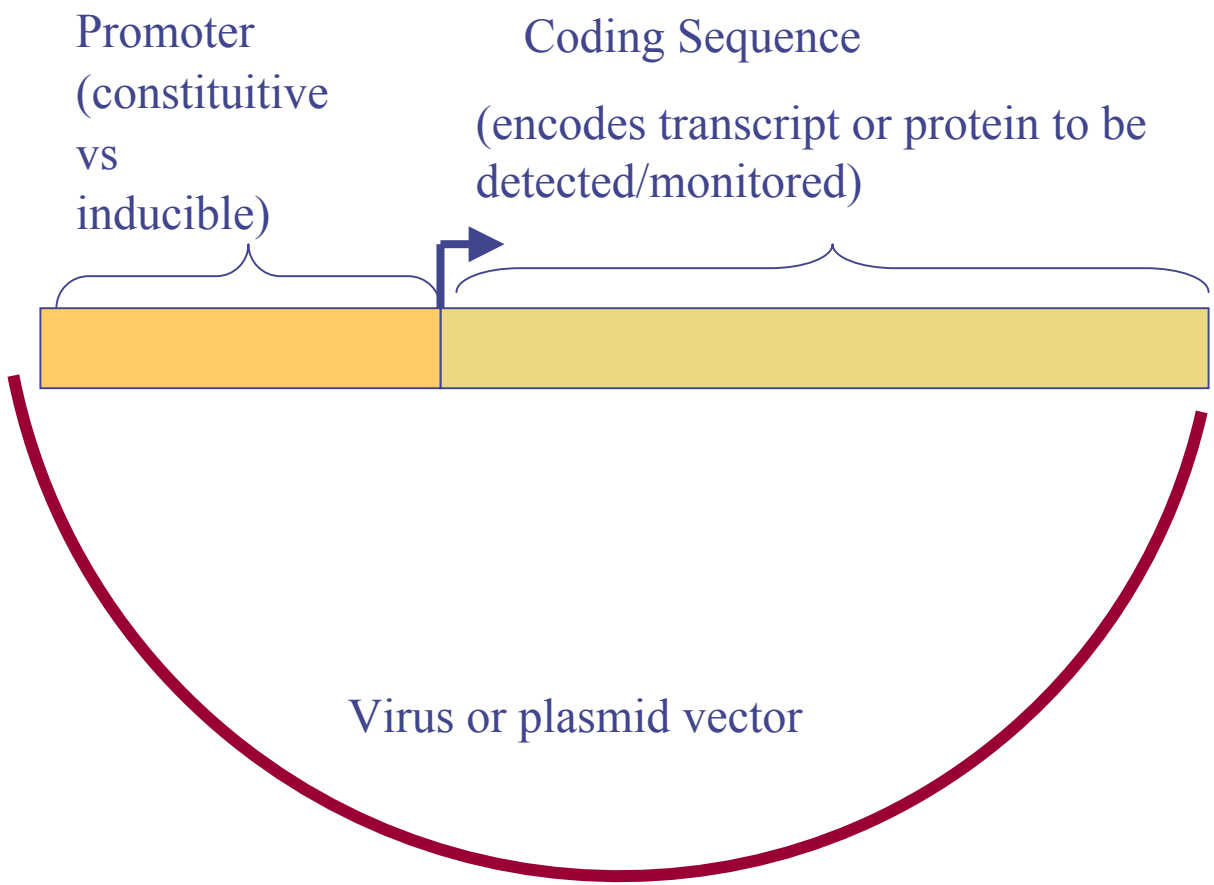
Bioluminescence	Reaction		Luciferin Structure
Bacterial	Aldehyde + $FMNH_2$ + O_2	Luciferase → dimeric 80kDa	Carboxylate + FMN + Light	
Firefly	Luciferin + ATP + Mg^{2+} + O_2	Luciferase → monomeric 61kDa	Oxyluciferin + AMP + PPi + CO_2 + Light	
Renilla	Coelenterazine + O_2	Luciferase → monomeric 36kDa	Coelenteramide + CO_2 + Light	

Sixth

Lecture

BIO 510 Second POP QUIZ
Nov. 15, 2002

1. Diagram the general structure of a reporter gene:



2. List different functional uses of reporter genes.

Selection of Reporter Genes

Reporter genes are chosen for a number of different purposes:

- As a Promoter Activity Reporter
- As a Biosensor of Metabolic or Environmental Surroundings
- As an Indicator of Gene Transfer (gene therapy)
- As an Indicator of Extent of Transgene Expression
- As a Detector of Cellular Biochemical Mechanisms

in the mini-project of our class. (2 pts).

Luciferase gene

4. We observed in the lab this week that on some cases, the plates of 'vector plus insert' had many more colonies than the 'vector only'. In other cases, there were very few colonies in both the 'vector plus insert' and 'vector only' and in still other cases there could have been many colonies on both the 'vector plus insert' and 'vector only.' Discuss reasons why

A) you might get back few colonies on both. (2 pts)

Possible reasonable answers:

- the transformation of DNA into the bacteria failed for both

- the vector only should have only a few if dephosphorylation worked; the problem is that the ligation of the insert into the vector failed.

B) you might get back many colonies on both.(2 pts)

Possible reasonable answers:

- the dephosphorylation failed so in the vector only reaction the vector ligated back its two ends (it recircularized), giving a high number of colonies in vector only. The vector plus insert would be expected to have a high number regardless of whether everything worked well, or whether the vector in the reaction improperly recircularized.

Third Pop Quiz
Bio 510
Dr. Grace Jones Section
Nov. 22, 2002

1. By what specific method did you remove the ethidium bromide from the DNA purified by the CsCl procedure? (2 pts)

Isopropanol extraction

2. On the CsCl gradients, there were at least <u>two bands</u> of DNA visible. Why did these two bands migrate to different places in the CsCl gradient? (2 pts)

Circular, supercoiled binds more EtBR, becoming denser, migrate farther down into the denser CsCl

Circular nicked, or linear, bind less EtBR, are less dense

3. What is the purpose of the dialysis step that is now going on? (2 pts)

To remove the CsCl and other solutes from the DNA, by the CsCl and other dissolved components diffusing out of the dialysis bag through the small pores in the bag. The DNA is too large to diffuse out through the pores.

4. In the sequencing of the constructs you made in which you inserted DR1 elements in front of a reporter gene, the sequencing of the various constructs showed that some constructs had different numbers of DR1 elements inserted, and that not all the DR1 elements were in the same orientation.

a. Would you have expected the constructs to have the same number of DR1 elements? Why or why not? (2 pts)

No, because

1. the ligation process of the DR fragments to each other is controlled by random encounter,
2. as seen on the purification gel, there was a smear generally in the background of the bands of larger and larger sizes, so that the multiple-sized DR-DR bands were not completely purified from each other.
3. The ligation process of the 'semipurified DR-DR fragments to the opened vector is controlled by random encounter

4.b. Would you have expected the DR1 elements to be in the same orientation? Why or why not? (2 pts)

No, because each end of the DR fragments had the same restriction site, and the vector was opened in such a way that each free end was also that same restriction site, so there was no control over which direction the DR fragments ligated to each other or to the vector.

"Promoter Analysis"

What are the important regulatory DNA sequences in a promoter of interest?

-Basic research interest. How are promoters controlled so that genes are regulated to express at the proper developmental time?

-Clinical interest. What diseases are caused by mutations in the regulatory promoter sequences?

-Biotechnological interest. Use of appropriate or designed promoter to "biosense" environment or to drive expression of a reporter gene.

Experimental systems for promoter analysis

1. "Cell free" in vitro, test tube biochemical reactions
2. **Cell transfection assay (in living cells)**
3. **In vivo (transformation of organisms, e.g., flies, mice, humans, etc.)**

In vitro analysis promoter regulation

1. Electrophoretic mobility shift assay

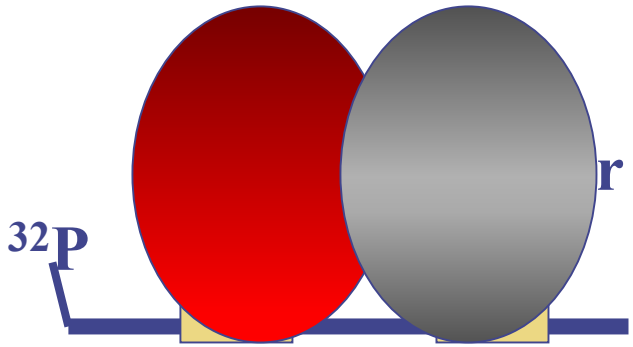
"gel shift"

Direct physical detection of protein binding to DNA

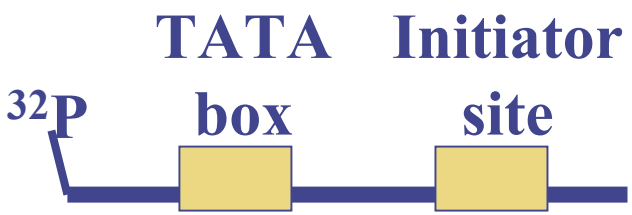
2. In vitro transcription assay

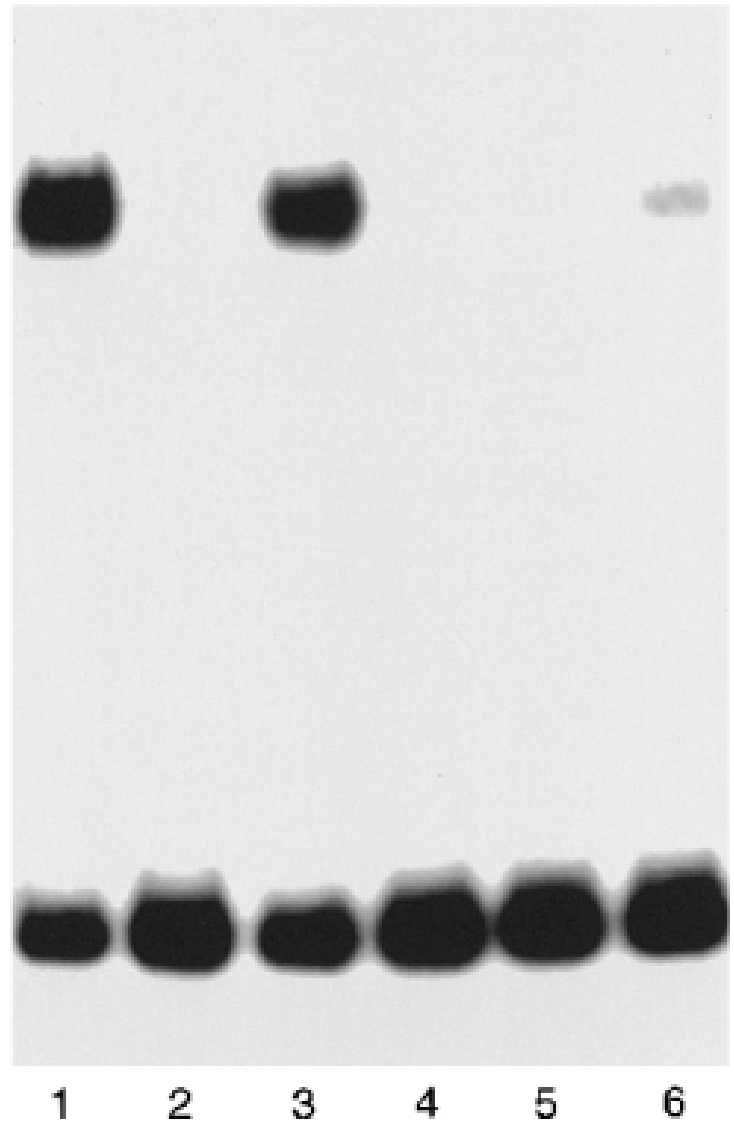
Functional test of effect of protein binding to DNA (i.e., does it have an effect on the function of the DNA to support transcription)

Electrophoretic mobility shift assay



Hot probe





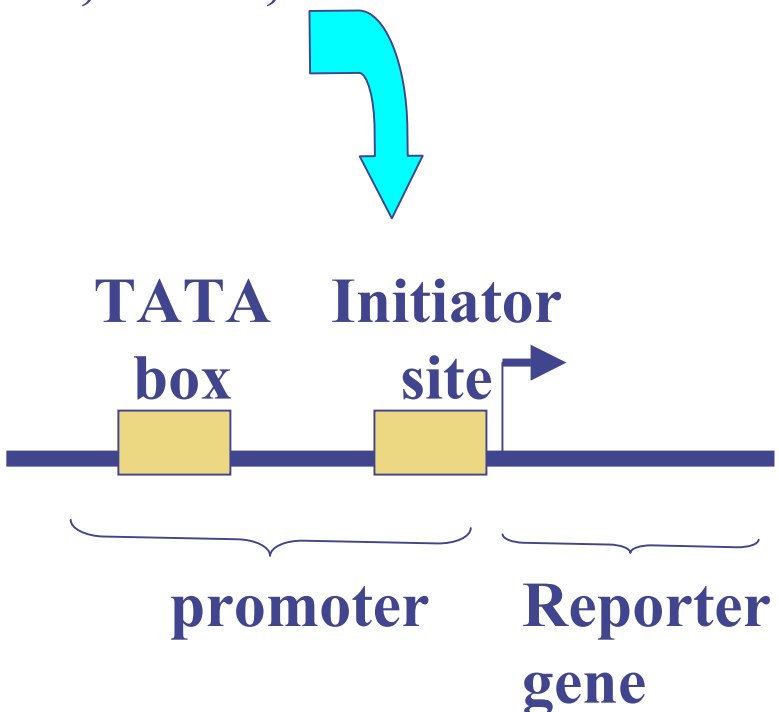
In vitro transcription assay

Detection of reporter:

- **G-free cassette**
- **Primer extension assay**

Primer extension example:

If extract nuclear proteins from cells, NTPs, add to

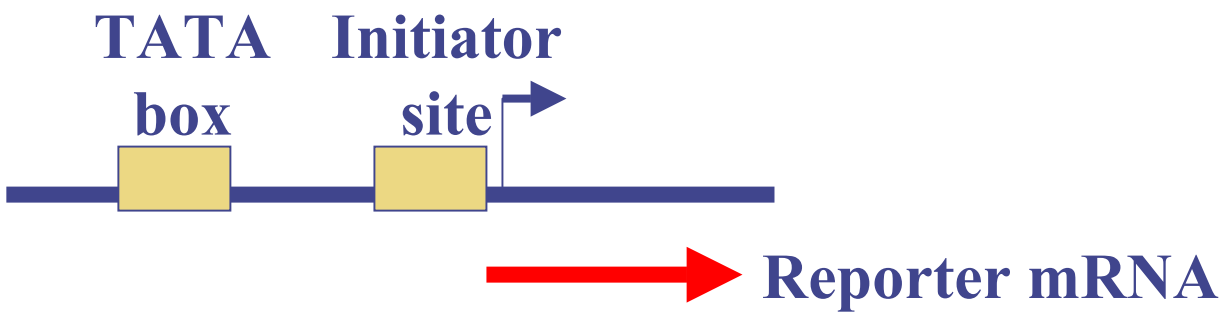


Then it will initiate transcription of reporter gene to make reporter mRNA, if all necessary components are present and promoter is of correct sequence

In vitro transcription assay

Primer extension assay

Extract nuclear proteins from cells, add to



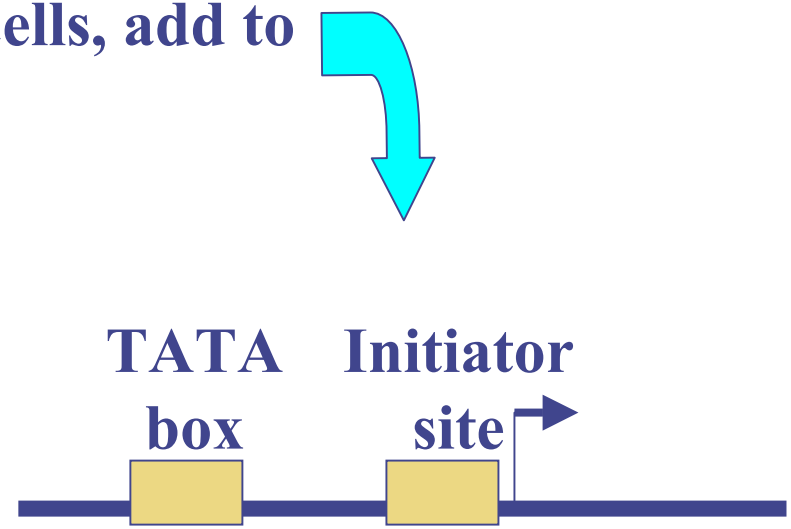
But how to detect the reporter mRNA has been made?

In vitro transcription assay

Reporters:

Primer extension assay

Extract nuclear proteins from cells, add to



Detect the existenceof the reporter mRNA, by using the reporter mRNA as a template to make a second strand, made of DNA

Prime the reaction with a hot primer ← that is of complimentary sequence

In vitro transcription assay

Reporters:

Primer extension assay

Extract nuclear proteins from cells, add to

The reverse transcriptase synthesizes a new DNA fragment — onto the primer that is complimentary to the reporter mRNA

Reporter mRNA

^{32}P

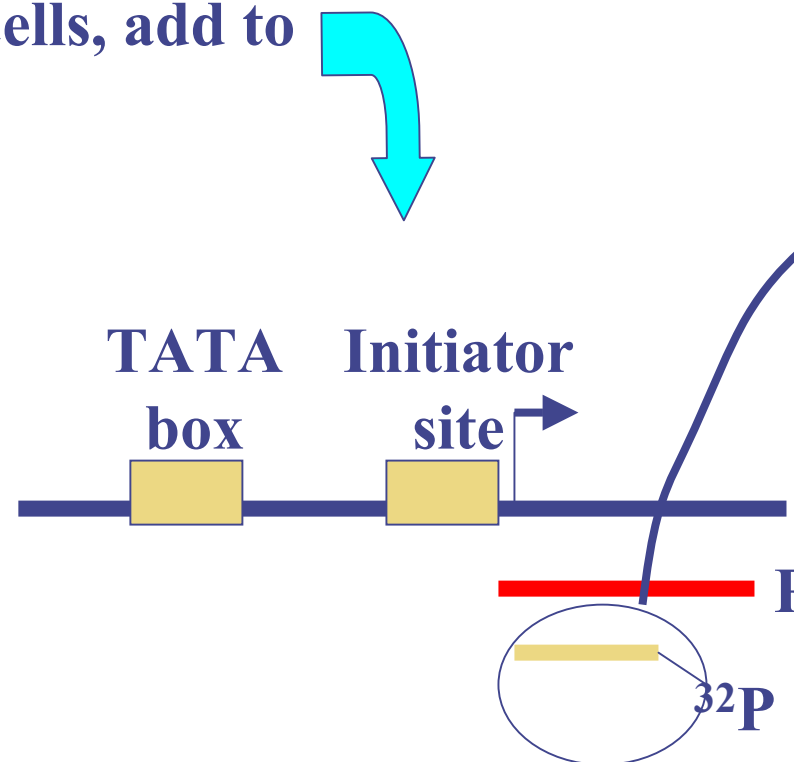
Hot primer + reverse transcriptase

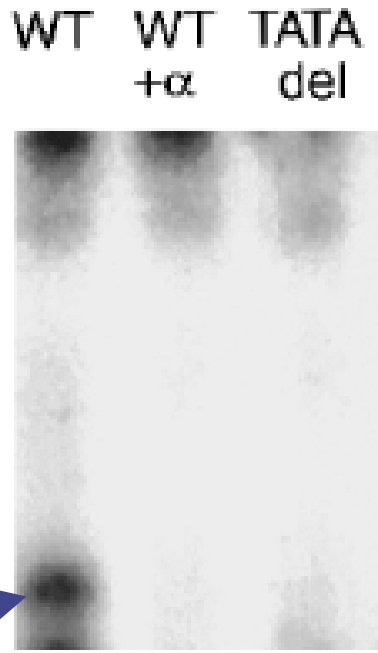
In vitro transcription assay

Reporters:

Primer extension assay

Extract nuclear proteins from cells, add to

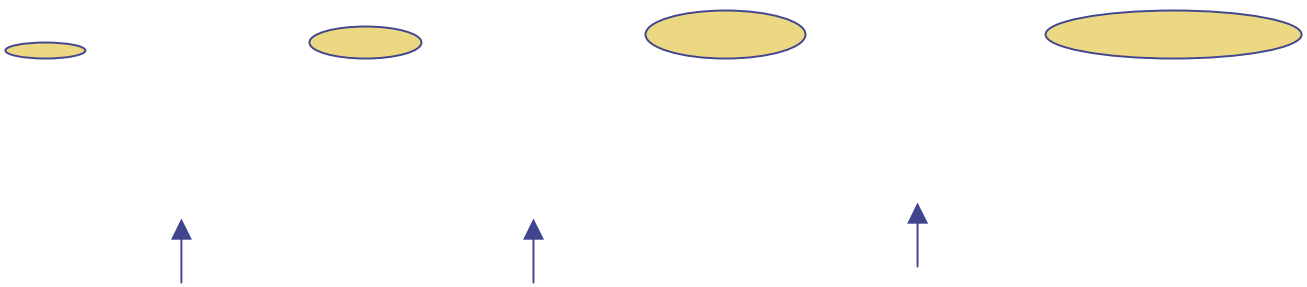




Detect the made DNA fragment by acrylamide gel electrophoresis; autoradiography

Ecdysone:

A steroid hormone that controls the timing of molting in insects



Seventh

Lecture

Bio 510 2002

Dr. Grace Jones Section

Outline of final class meeting

Review of Lab Exercises

- **-Global view**
- **-Technical view**

Review of Class Lectures

Review of Pop Quizzes

Discussion of Final Exam Organization

Discussion of Course Grading

Review of Lab Exercises

‘Global View’ of the lab

How would you answer the following questions, if they were on the final exam?

-What was the overall *scientific* objective of the lab project?

Example incorrect answers:

(1) The objective was to clone DR elements into a reporter gene

(2) The objective was to perform cell transfection assay

(3) The objective was to use make the most effective vector construct

Correct answer:?

Review of Lab Exercises

‘Global View’ of the lab

How would you answer the following questions, if they were on the final exam?

What were the series of technical steps used toward accomplishing the overall scientific objective?

Incorrect answers include:
(1) we measured the amount of DNA recovered from the acrylamide gel

(2) we dialyzed the DNA

(3) we transformed DNA into bacteria

Correct answer:?

Correct answer:

Toward the scientific objective of testing the effect of different sequences, numbers and orientations of DR enhancer elements to enhance the response of a core promoter to treatment with hormone,

we annealed upper and lower strand of specific DR sequences,

we ligated them into concatamers of varying multiples and varying orientations,

we selected the range of multiple DR numbers of the desired range in numbers,

we prepared and ligated the DRs into an appropriate vector that contained a core promoter and a luciferase reporter gene,

we finally transfected the different reporter constructs into a cell line and treated the transfected cells with either hormone or EtOH

Review of Lab Exercises

Technical View

What was the specific technical purpose of particular experimental activities performed at each lab meeting?

-Why was the technical procedure done?

-What would have happened if the procedure was not done or had failed?

-How would you have detected that the procedure failed?

Review of Lab Exercises

Technical View

First Lab Session

"It is not necessary to kinase these oligonucleotides because they have already been synthesized to contain a 5' phosphate group."

Why was the technical procedure of having a 5' phosphate necessary?

-What would have happened if the 5' phosphate was not on the oligos?

-How would you have detected that the procedure failed?

Review of Lab Exercises

Technical View

Second Lab Session

“Add 1 ul of CIP enzyme to dephosphorylate the vector.”

Why was the technical procedure of dephosphorylating the vector necessary?

-What would have happened if the procedure was not successful?

-How would you have detected that the procedure failed?

Review of Lab Exercises

Technical View

Third Lab Session

"1. For each of your four samples, set up two ligation reactions with the following ratios of DR to vector:

a. 3:1 DR to vector, where the picomoles of vector is 0.03

b. 10:1 DR to vector, where the picomoles of vector is 0.03."

-Why is it important to consider the ratio of DR to vector in the ligation?

-What would have happened if the ratio of DR to vector had been too high in favor of the DR?

-How would you have detected that ration was (a) too high in favor of DR or (b) too low in favor of DR?

Review of Lab Exercises

Technical View

4th Lab Session

"Obtain 20 m sterile LB media without ampicillin…Add 950 ul of LB without amplicillin to each of the 18 tubes and incubate at 37C for 1 hr...."

-Why was the technical procedure of leaving out the amplicillin necessary?

-What would have happened if the ampicillin had not been omitted the oligos?

-How would you have detected that the ampicillin had been omitted?

Review of Lab Exercises

Technical View

6th and 7th Lab Sessions

"Purify the DNA by CsCl ethidium bromide gradient centrifugation…the band that corresponds to the DNA sample must be removed with a syringe needle."

-Why was the technical procedure of subjecting the DNA to CsCl gradient centrifugation necessary?

-What would have happened to the remainder of your lab project the gradient had not separated the different forms of DNA?

-How could you have detected that the CsCl had failed separate the different DNA forms from each other?

Review of Lab Exercises

Technical View

8th Lab Session

"Each group prepare the following constructs for transfection."

a. pGL3 vector only

b. JHECore pGL3

c. DR1(two)JHECore pGL3

d. DR1 (five -F)JHECore pGL3

e. DR1 (five -C)JHECore pGL3

Why was it necessary to include the pGL3 vector only and the JHECore pGL3 constructs?

What does each test?

Review of Lab Exercises

Technical View

9th Lab Session

"Make a range of lysate [to be added to luciferase substrate] to be added as 0 ul, 5 ul, 10 ul and 20 ul. ."

-Why was the technical procedure of making this range of lysate volumes necessary?

-What could have happened had just arbitrarily selected one of the lysate volumes to use for all of your experimental samples?

-How could you have detected that the CsCl had failed separate the different DNA forms from each other?

Review of Lab Exercises

Technical View

10th Lab Session

Analyze the results to determine whether increasing the number of DR elements changed the activity (a) in the absence of hormone or (b) in the presence of hormone. That is, compare the activity of the different DR number constructs for the case where each received only ethanol. Then, compare the ratio of the ecdysone treatment/ethanol treatment for each DR construct. Is the ratio significantly different for the situation of two DR elements vs. five DR elements?

Review of Lab Exercises

Technical View

10th Lab Session

Perform the same kind of analysis as above in item 11, except this time the question you are considering is whether the differences in orientations, when the DR number is the same (we have two constructs each with five DRs), has any effect on (a) the level of activity in the absence of hormone or (b) on the ratio of activities of the ecdysone treatment/ethanol treatment.

Review of Pop Quizzes

First Pop Quiz - Most often missed question

In the past week's lab, you wanted to visualize after electrophoresis the products of the concatamerization reaction.

> How did you the products and how is it that the method you used enables you to see the products. Be sure to describe the chemical details of the reason that the method works.

Answer: TLC shadowing method. The double bonds in DNA bases absorb UV. So, place the gel onto a fluorescent plate, and illuminate it with UV. The plate will glow every place except where the gel has a DNA band that intercepts the UV. That spot will appear dark against the fluorescent plate.

Review of Pop Quizzes

Second Pop Quiz - Most often missed question

1. We observed in the lab this week that on some cases, the plates of 'vector plus insert' had many more colonies than the 'vector only'. In other cases, there were very few colonies in both the 'vector plus insert' and 'vector only' and in still other cases there could have been many colonies on both the 'vector plus insert' and 'vector only.' Discuss reasons why

A) you might get back few colonies on both

One possibility - the bacterial transformation failed (e.g., bacteria were not efficiently made "competent" to uptake DNA

Second possibility - the ligation of the DR insert into the vector failed, giving the vector an unexpected low number, along with the expection vector-only low number

B) you might get back many colonies on both

One possibility - the dephosphorylation of the vector failed, so that many vectors self ligated back into a circle, in both the vector only and vector plus insert, giving many colonies back in both.

Review of Pop Quizzes

Third Pop Quiz - Most often missed question

In the sequencing of the constructs you made in which you inserted DR1 elements in front of a reporter gene, the sequencing of the various constructs showed that some constructs had different numbers of DR1 elements inserted, and that not all the DR1 elements were in the same orientation.

Would you have expected the constructs to have the same number of DR1 elements? Why or why not? (2 pts)

No, for several reasons. First, we did not control during the ligation reaction the number of random encounter between DR oligos and the ligase enzyme. Second, on the acrylamide gel, there was smearing from one band to another, making each band (that was a particular number of DRs ligated together) contaminated with the DNA of adjacent bands.

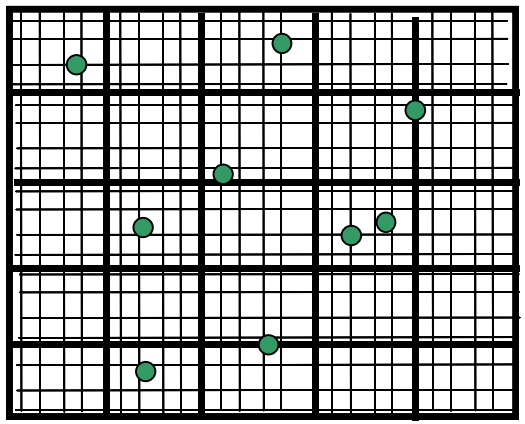
Would you have expected the DR1 elements to be in the same orientation? Why or why not? (2 pts)

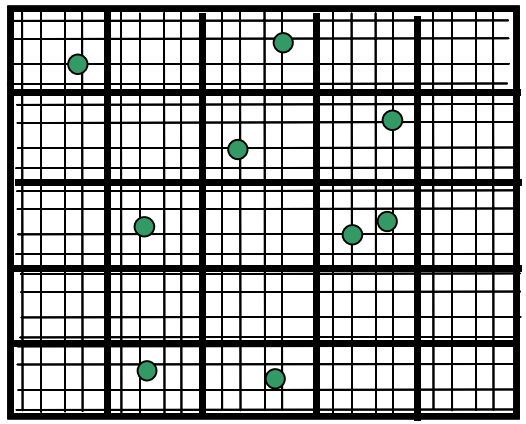
No, because both ends of the opened vector and both ends of the DR oligos were all the same restriction site. Thus, as the DR oligos randomly encounter each other and encounter the vector, there was no control on which orientation they were in as they ligated into the vector

Review of Pop Quizzes

Fourth Pop Quiz - Most often missed questions

1. **Assume that you have done two experiments in which you needed to know the density of cells/ml in you cell culture medium. For each of the two hemocytometer representations below (green circles are cells), provide the estimated number of cells per ml using the calculation method we went over in class. (2.5 points each)**





Either count ALL cells in ALL 25 squares and multiply by 10^4

OR,

Count a proportion of the squares, such as 5, and then multiply the number of cells by 25/5 x 10^4

2. **What is the functional (not methodological) difference between transformation and transfection?**

Transformation is permanent placement of the DNA into the cell (chromosomal integration or on a plasmid with its own origin of rep.)

Transfection temporarily places the DNA into the cell

Review of Pop Quizzes

Fifth Pop Quiz - Most often missed question

In the lab, you performed steps to determine the 'region of linearity' in the kinetics of the luciferase reaction. Describe two independent reasons why the kinetics might depart from linearity. Be specific as to the biochemical basis for the nonlinearity

1. Too much luciferase (in the lysate) for the limited amount of luciferin in the reaction. The luciferase will become not saturated in all enzymatic sites with luciferin substrate. This will cause the rate of the reaction to become slower as the luciferase has to 'look around harder' to find substrate.

2. Incubation time went to long. The amount of luciferase (in the lysate) was small enough that it was saturated with substrate at the beginning of the reaction, but by the end of the reaction enough luciferin substrate had been used up so that the luciferase was no longer saturated.

3. Product feedback inhibition

Additional Points to Especially Consider

During Studying of the Lectures

1st lecture

1. Conditions to consider when annealing oligos

2. Methods to recover DNA from gels

2nd lecture

1. What are ‘reporter genes’ and in what situations are the different kings of reporter genes most useful?

2. Distinguish between reporter genes and detection methods

3rd lecture

1. Conversion of ug or ng amounts of DNA to picomoles or nanomoles

2. Use of hemocytometer to estimate density of #cells/ml

Additional Points to Especially Consider During Studying of the Lectures

4th lecture

1. How does an CsCl gradient in the presence of EtBR separate DNA forms?

5th lecture

1. What is bioluminescence and how can it be measured?

6th lecture

1. In the 'primer extension assay' for promoter activity, what is the reporter and what is the detection method?

2. In the method, exactly and precisely what is being detected?