Day 5 Objectives:
- Extract DNA
- Analyze DNA by Restriction Mapping
- Start Recombination Assay (Lipofection)

Day 6 Objective:
Determine the frequency of Recombination between D3 and D5 by Flow Cytometry
Overview for Today:

1) Background and Significance: Recombination Assay

2) Review of Experimental Procedures
   Miniprep
   Restriction Analysis
   Lipofection

3) Experiments (Starting at 1:00)

Group I: **Toxic Avengers**
1-2  Miniprep
2-3  Restriction Digests
3-4  Lipofection
4-5  Load Gels

Group II: **Recombinators**
1-2  Lipofection
2-3  Miniprep
3-4  Restriction Digests
4-5  Load Gels

*Gel analysis during class is optional.*
Background and Significance: **Recombination Assay**

Adapted from Sandberg and from Lodish, *Molecular Cell Biology*
Sunlight

Pollution & Food

Cigarette Smoke

Oxidation

NO·
Base Damage

Chemical Changes

“Bulky” Lesions

Loss and Fragmentation
DNA Backbone Damage

- Sugar Damage
- Single Strand Breaks
- Double Strand Breaks
Loss of Heterozygosity

Mutant

Normal
Recombinational Repair
Translocations

Deletions

Mutant

LOH

Translocations
PROBLEM: LACK OF IN VIVO MODELS

OBJECTIVE: DETECT RECOMBINATION IN MAMMALS IN VIVO
II) New Tools: Recombomice!

Green Fluorescence Expression
Recombination Substrate

5′eyfp

532 bp

3′eyfp

△

532 bp

Pronuclear Injection

Candidate Founders

532 bp

Primers
Green FL Intensity
Orange FL Intensity

Wild-Type Ear Cells
EGFP Ear Cells
Mouse #8 Ear Cells
Mouse #12 Ear Cells

Flow Cytometry

(5 x 10^6 cells)

Recombination Frequency: 1 x 10^{-5}
Ear Cells from Mouse #12
Recombination Frequency = ~ 3/100,000

Non-Fluorescent Cells

Fluorescent Cells
DNA Purification the Fun and Easy Way!
pCXNNNX-EGFP

△

What You Created!

pCXNNNX-D5

Parent Vector
Experiment:

Purify DNA from Candidates and Controls

You will receive 4 cultures:

Positive control  
pCX-NNX-D5

Negative Control  
pCX-NNX

Two of your “PCR Hits”  
??
Miniprep in Short Form:

Start water boiling.
Spin overnight cultures for 30 seconds in an eppendorph centrifuge.
Remove supernatant.
Add 350 ul STET.
Poke toothpick into the lysozyme and twizzel to fully resuspend the pellet.
Boil for 40 seconds.
Spin 5 minutes.
Pull out yucky stuff.
Add 40 ul of 2.5 M NaAcetate (pH 5.2) and 420 ul of isopropanol.
Spin 5 minutes.
Pour off supernatant.
Squirt 70% Ethanol into the tube to fill it.
Pour off supernatant.
Spin for 5 seconds in the eppendorph centrifuge.
Remove the remaining small amount of ethanol.
Leave horizontal on the bench for 5 minutes to let it dry.
Resuspend in 40 ul of TE. Use 10 ul for each digest.
What enzymes did you choose?
Example Digestions:

Enzymes:  SalI w/ and w/out EcoRV

Best to use Buffer #3

2X Digestion Buffer:

Per Reaction:  For 5 Reactions:

0.25 ul SalI  1.25 ul SalI
2 ul Buffer #3 (stock)  10 ul Buffer #3 (10X stock)
7.75 ul ddH2O  38.75 ul ddH2O

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10 ul  50 ul
### For 5 Reactions (one extra):

<table>
<thead>
<tr>
<th></th>
<th>SalI Alone</th>
<th>EcoRV Alone</th>
<th>SalI/EcoRV</th>
<th>Neg. Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25 ul SalI</td>
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<td>10 ul Buffer #3 (10X)</td>
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<td>50 ul</td>
<td>50 ul</td>
<td>37.5 ul ddH2O</td>
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Add enzymes last to these master mixes! Label tubes carefully. Then combine 10 ul of purified DNA with 10 ul of master mix.

From each digest, load: 6 ul (be sure to add loading buffer!)

Leave a post it note with lane labels before leaving if you don’t collect your image before you go!
Lipofection of Mammalian Cells
30 Seconds on Mammalian Cell Culture!
(More in your next module…)

Sterility

Media

Attached vs Nonadherent

Trypsinization
Methods of Transfection:

Chemical

Physical

Viral
pCXNNX-D3

Δ

pCXNNX-EGFP+

Δ

pCXNNX-EGFP+
Invitrogen’s Lipofectamine 2000

Contents and Storage
Lipofectamine 2000 is supplied in liquid form at a concentration of 1 mg/ml. Store at +4°C. **DO NOT FREEZE.** Product is guaranteed for 6 months from the date of shipment if stored properly.

Description
Lipofectamine 2000 is a proprietary formulation suitable for the transfection of nucleic acids into eukaryotic cells. Using Lipofectamine 2000 for transfection provides the following advantages:

- The highest transfection efficiency in many cell types and formats (e.g. 96-well).

- DNA-Lipofectamine 2000 complexes can be added directly to cells in culture medium (in the presence or absence of serum).

- It is not necessary to remove complexes or change or add medium following transfection, although complexes can be removed after 4-6 hours without loss of activity.
Invitrogen’s Lipofectamine 2000

Product Qualification

Lipofectamine 2000 is tested for the absence of microbial contamination using blood agar plates, Sabaraud dextrose agar plates, and fluid thioglycolate medium, and functionally by transfection of CHO-K1 cells with a luciferase reporter-containing plasmid.
Invitrogen’s Lipofectamine 2000

Important Guidelines

Follow these guidelines when performing transfections:

1. The ratio of DNA (in µg):Lipofectamine. 2000 (in µl) to use when preparing complexes should be 1:2 to 1:3 for most cell lines.

2. Transfect cells at high cell density. 90-95% confluence at the time of transfection is recommended to obtain high efficiency and expression levels, and to minimize decreased cell growth associated with high transfection activity. Lower cell densities are suitable with optimization of conditions. Take care to maintain a standard seeding protocol between experiments because transfection efficiency is sensitive to culture confluence.

3. Do not add antibiotics to media during transfection as this will cause cell death.
Invitrogen’s Lipofectamine 2000

For optimal results, we also recommend the following:

1. Use Opti-MEM® I Reduced Serum Medium (Catalog no. 31985-062) to dilute Lipofectamine. 2000 prior to complexing with DNA.
Invitrogen’s Lipofectamine 2000

Transfection Procedure

Use the following procedure to transfet mammalian cells in a 24-well format.

Up or Down Transfections.

1. **Adherent cells:** One day before transfection, plate $0.5-2 \times 10^5$ cells in 500 µl of growth medium without antibiotics per well so that they will be 90-95% confluent at the time of transfection.
Invitrogen’s Lipofectamine 2000

2. For each transfection sample, prepare DNA-Lipofectamine 2000 complexes as follows:

   a. Dilute DNA in 50 µl of Opti-MEM® I Reduced Serum Medium without serum (or other medium without serum). Mix gently.

   (BE109: You will be given DNA at 200 ng/ul. You should add 5 ul to get 1 ug.)

   b. Mix Lipofectamine 2000 gently before use, then dilute the appropriate amount in 50 µl of Opti-MEM® I Medium (or other medium without serum). Mix gently and incubate for 5 minutes at room temperature.

   c. After the 5 minute incubation, combine the diluted DNA with the diluted Lipofectamine 2000 (total volume is 100 µl). Mix gently and incubate for 20 minutes at room temperature to allow the DNA-Lipofectamine 2000 complexes to form. The solution may appear cloudy, but this will not inhibit the transfection.

3. Add the 100 µl of DNA-Lipofectamine 2000 complexes to each well containing cells and medium. Mix gently by rocking the plate back and forth.

4. Incubate the cells at 37°C in a CO2 incubator for 24-48 hours until they are ready to assay for transgene expression.
Materials:

One 24-well plate, with 7 wells of adherent Mouse Embryonic Stem cells

Opti-MEM

0.2 ug/ul Δ3 DNA
0.2 ug/ul Δ5 DNA
0.2 ug/ul pEGFP DNA
Lipofectamine

Procedure Under TC Hood:

1. Follow instructions as per protocol for a 24-well plate.

   Use the following DNA amounts/well:

   1 ug of Δ3
   1 ug of Δ5
   1 ug of Δ3, plus 1 ug of Δ5
   1 ug of pEGFP

   Two samples using conditions that you have chosen to test

   Leave one well of cells for the negative control.

   g. Note: The protocol says to use a DNA sample of 0.8 ug in 50 ul of Opti-MEM. We will use 1.0 ug in 50 ul of Opti-MEM for all samples except the Δ3/Δ5, which will contain 2 ug of total DNA in 50 ul of Opti-MEM.
If you decide to test Cut versus Uncut:

Use 1 ug Delta3
Use 0.025 ug Delta5 (cut or uncut)
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