Module 1: Genetic Engineering

- Design and Construct Vectors for Gene Expression
- Learn to analyze fluorescence using flow cytometry.
What we are building and Why
Two-ended Double Strand Breaks

Double Strand Break Repair
Mice expressing *EGFP*

Normal light

UV light

Microscopy or Flow Cytometry

(5 x 10^6 cells)

Microscopy or Flow Cytometry
Recombinant yellow ear cell from a transgenic mouse viewed under a fluorescent microscope.
Green FL Intensity

Orange FL Intensity

Wild-Type Ear Cells

EGFP Ear Cells

Mouse #12 Ear Cells

%Fluorescent: 0

%Fluorescent: 99.98

Recombination Frequency:

$\sim 1 \times 10^{-5}$

Recombinant Yellow Cells
Create a Vector to Test for Recombination in Mammalian Cells

- Vector design (D1)
- Primer design (D1)
- Perform PCR and digest vector backbone (D1)
- Prepare PCR product and vector for ligation (D2)
- Perform Ligation and Electroporation (D3)
- Electroporation (D3)
- Colony screening by PCR (D4)
- Miniprep and Restriction Digestions (D5)

Flow Cytometry

- Prepare samples for flow cytometry (D6)
- Learn to analyze samples using a flow cytometer.
Ligations and PCR
Overview of Cloning

Parent Vector

Vector

Insert

Parent Insert Vector

Insert +
Overview of Cloning

Insert

Vector

Final Product
Mix DNA Vector that confers drug resistance (e.g., amp$^R$) with cells.
Add electric potential.

(Wait for cells to recover)
Spread cells onto selective plate.

Wait 24 hours for each drug resistant cell to form a colony.

Ampicillin Resistant Colonies Carry DNA
Overview of Cloning

Parent Vector

Insert

Parent Insert Vector

Insert +
Overview of Cloning

Vector

Insert

Final Product
DNA into Cells
(Transformation)

Ampicillin Resistant Colonies

Do all of these colonies carry the desired final product?

What other DNAs might be present?
Contaminants:

- Single-Cut Insert Vector
- Uncut Insert Vector
- Single-Cut Vector

Diagram:

- Uncut Vector
- Single Cut Vector
- Uncut Insert Vector
- Single-Cut Insert Vector
Overview of Cloning
Beware of Significant Contamination By Reclosed Vector Without Insert
PCR

Basic Principles:

5' ___________________________ 5'

5'  

5' ___________________________ 5'

5'  

5' ___________________________ 5'

5'  

5' ___________________________ 5'
PCR can be used to add sequences to the ends of a product.
PCR

Basic Principles:

5’

5’

5’

5’

5’

5’
Blunt vs Sticky Ends

-Make sure your sticky ends are sticky!

-Digest your PCR product

-Only a perfect match can be ligated

<table>
<thead>
<tr>
<th>EcoR1 Uncut</th>
<th>EcoR1 Cut</th>
</tr>
</thead>
<tbody>
<tr>
<td>......GAATTC.... .</td>
<td>...G AATTC.... .</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>......CTTAAG.... .</td>
<td>...CTTAA G.... .</td>
</tr>
</tbody>
</table>
Blunt vs Sticky Ends

-Blunt end ligations are more difficult
  -Avoid if possible
Methods for Matching Ends

- Fill in
  - Klenow
- Chew back
  - Beware of imperfect exonuclease processing
- Use different enzymes that share compatible sticky ends
- Adaptors
- Partial fill in
- Terminal transferase
Example of an Adaptor

NcoI

...C   CATGG...
...GGTAC   C...

Conversion of NcoI (CCATGG) into PvuI (CGATCG):

...C   CATGCGATCG   CATGG...
...GGTAC   GCTAGCGTAC   C...

...CCATGCGATCGCATGG...
...GGTACGCTAGCGTACC...
Destroying Sites

- Open, fill, close
- Open, chew, close
- Can get rid of one of two sites by partial digestion
- Beware: sometimes one of the two is favored.
Ways to Screen for Final Construct Using Restriction Enzymes

- Use same enzymes as you used to create the construct to observe drop out of the insert.
- Use sites in the MCS to drop out the insert.
- Cut with enzymes that would only cut the final product.
- Cut with more than one enzyme at a time to show correct fragment sizes.
- Cut with enzymes that should not cut the final product to show that the site was lost (remember to run control uncut DNA!). (Note that negative results are never definitive.)
Primer Design Considerations

- GC content
  - 50% is recommended
- Annealing to other primers
  - self annealing and hairpins
  - annealing to partner
    - primer dimers (match at 3’ end vs 5’ end)
  - avoid stems of 7-8 bps
Primer Design Considerations

- Need extra nucleotides (NT) beyond the restriction site at the end of a primer (ultimately at the end of the PCR product)
  - See NEB for number of NTs needed to cut near end
  - If in doubt, add 6 NT
- GC base pair at the end decreases fraying
- Avoid runs
  - Runs of GC or AT can form secondary structures
  - Secondary structures can interfere with PCR
About the Vector We Will Be Using…. pCX-NNX-EGFP
pCX-EGFP

5510 bp

EGFP Coding

Chicken Beta-Actin Promoter
CMV-IE Enhancer

Bovine Globin pA Signal

Bam HI 3517

Bam HI 3180

Hind III 2995

Pst I 2990

Bsg I 1480

Bsg I 1935

Bsg I 1622

Bsg I 2259

Amp

Intron

P
pCX-EGFP
5510 bp

Chicken Beta-Actin Promoter
CMV-IE Enhancer

Bovine Globin pA Signal

EcoR1

EGFP Coding
Cloning Strategy:

**pCX-EGFP**

- **EcoR1**

**Backbone**

- **pCX**
- **pA**

**Gel Purify Vector Backbone**

**pEYFP**

- **Start**
- **Stop**

- **A** = ApoI
- **B** = BamH1
- **S** = SacII
EcoR1 Uncut                      EcoR1 Cut

......GAATTC... .                        ...G                    AATTC... .
       | | | | |                             |                     |
......CTTAAG... .                        ...CTTAA                G.... .

Apo1 Uncut                      Apo1 Cut

......AAATTT... .                        ...A                    AATTT... .
       | | | | |                             |                     |
......TTTTAA... .                        ...TTTAA               A.... .

FUSION SITE:

......GAATTT... .
       | | | | |       
......CTTTAA... .

Loss of EcoR1 site.
PCR Conditions:

Your final reaction should contain the following:

**X ng of template** (theory vs reality; plasmid vs genomic; use 2.5 ul of the template provided)

0.1-3 uM Primers

200 uM dNTPs (stocks are 10 mM)

1X polymerase buffer (containing Mg++; diluted from the 10X buffer)

2.5 units of polymerase (stock is 2.5 units/ul)

**Mineral oil on top** (or heated lid) (use 50 ul for a 100 ul rxn)

Final Volume: 100 ul.

Note that the MgCl2 should be 100-2000 uM. It is usually but not always in the 10X buffer.
PCR: Melt – Anneal – Extend

Melting:

Generally set melting temp to 95C
Usually start with a longer melting temp (5 min)
In subsequent cycles, just need 1 min (30 sec is OK)

Annealing:

Annealing temp should be 5 degrees below the melting temp.
Usually anneal for 1 min (30 sec is OK)

Extending:

Make sure you use the right temperature for your enzyme.
Make sure you use 1 minute for every kb!
Standard Conditions:

1) 95C  5 min
2) 95C  1 min
3) 55C  1 min
4) 72C  1 min

5) Repeat 2-4 30X
Choosing the Annealing Conditions:

• Determine the annealing temperature for both primers.

• Go to 5 degrees below the melting temperature of the primer with the lowest melting temperature.

• Does the annealing temperature change during PCR?

  Yes! If the primer has a “landing site” and a “tail”. Make sure the program goes low enough for the “landing site”. If desired, raise the temperature during later cycles.
Touch-Down PCR:

Note that calculated values don’t always work!
Touch-Down can save you a lot of work!

95C 5 min
95C 1 min
60C 1 min (setting this 5 degrees higher than you think is right)
72C 1 min (repeat this cluster once or twice)
95C 1 min
58C 1 min (move the annealing temp down 1 or 2 degrees)
72C 1 min (repeat this cluster once or twice)
95C 1 min
56C 1 min (move the annealing temp down 1 or 2 degrees)
72C 1 min (repeat this cluster once or twice)
95C 1 min
54C 1 min (move the annealing temp down expected annealing temp)
72C 1 min (repeat this cluster 20X)
Setting Up PCR Reactions:

USE FILTER TIPS!

Contamination is a BIG problem! Filter tips are essential whenever you take from a “stock”.

Temperature Control

Keep everything cold until samples are put into the PCR machine.
Be sure to have good ice contact once polymerase is added.

Write out the list of all ingredients
Check them off as you add them.
Restriction Enzyme Conditions - General Guidelines

Aim for a final reaction volume of 20 ul (for analytical digests).

Make sure DNA is reasonably clean (phenol + EtOH kill enzymes!)

Use about 10 units for each ug (this is very conservative)

(you can use less if you cut longer; theoretically 1 unit cuts 1 ug in 1 hr)

Keep enzymes cold!!! Always use a clean tip!

Always add enzyme last.

Make sure your rxn is well mixed first since some enzymes die if they are put straight into water.

Dilute enzyme at least 1:10 (glycerol inhibits rxn).

Mix gently (do not vortex!). Touch spin to clean sides of tube.
Restriction Enzyme Conditions For Backbone Preparation:

Your final reaction should contain the following:

3 ug of vector
1X concentration of restriction enzyme buffer
Restriction enzyme diluted at least 1:10
BSA (sometimes)
Final Volume: 20-100 ul

For analytical digests, use as little as 200 ng DNA.