# M1D2: Complete small molecule microarray analysis and induce protein expression

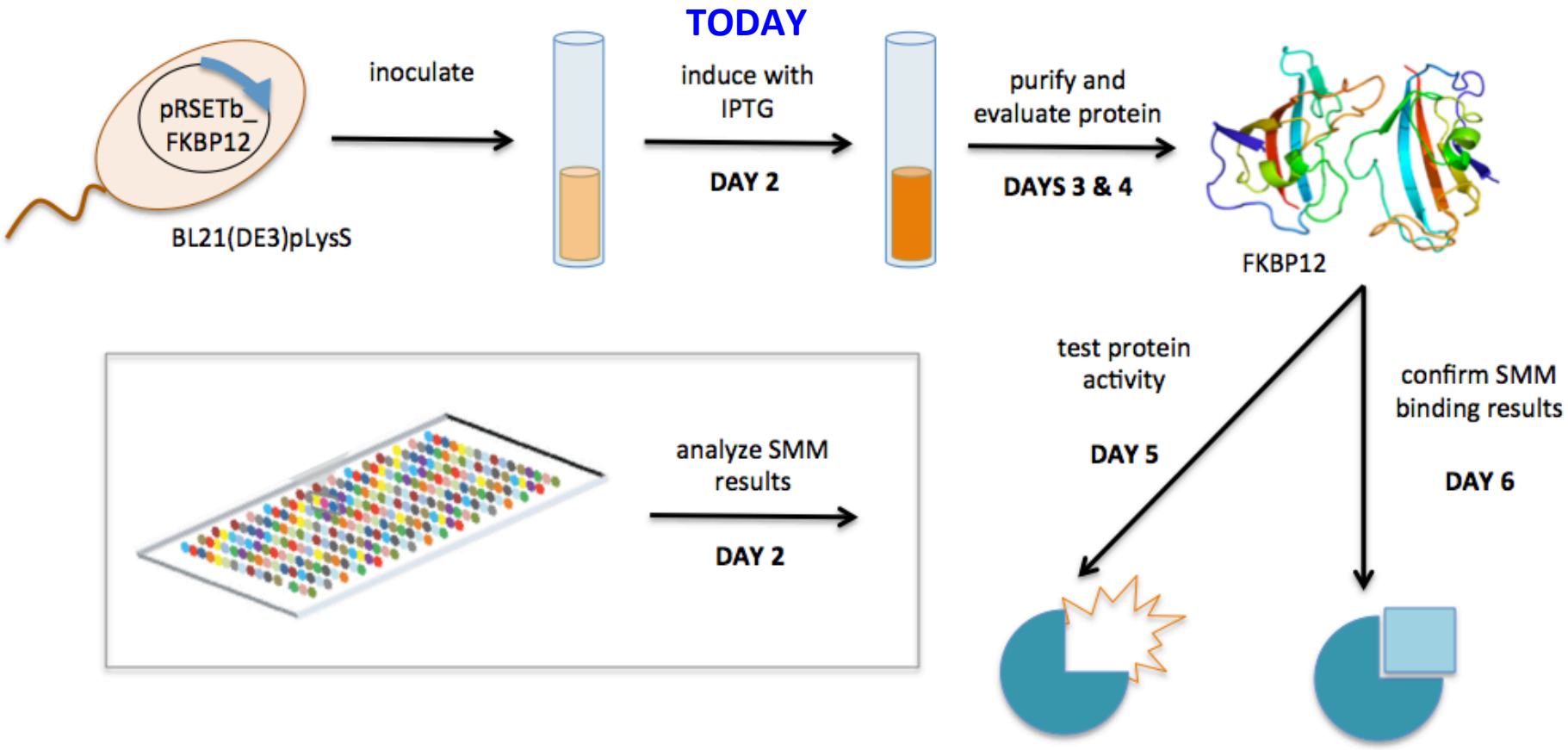
**Announcements**

Office hours:
**Mon 2-5pm** (Noreen, 16-317)
**Tu 4-5pm** (Josephine, 56-341c/322)
**Th 10-11am** (Josephine, 56-341c)
Email us for other times

1. Pre-lab discussion
2. Induce FKBP12 expression
3. Gel electrophorese confirmation digests
4. Complete SMM data analysis

Prepared with help & slides from Rob Wilson

# Overview of Mod1 experiments

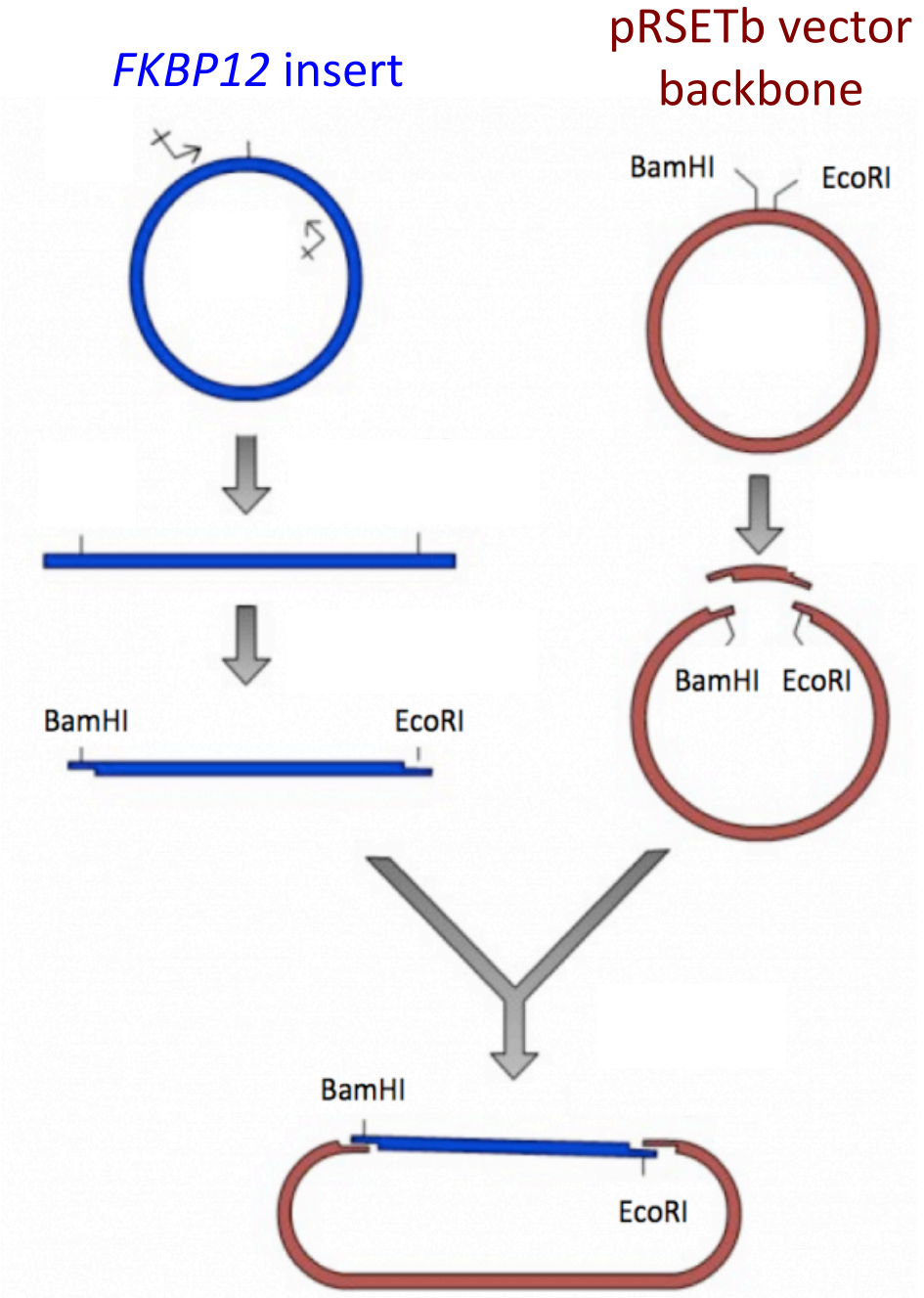


# Review of cloning: generate plasmid to make FKBP12 protein

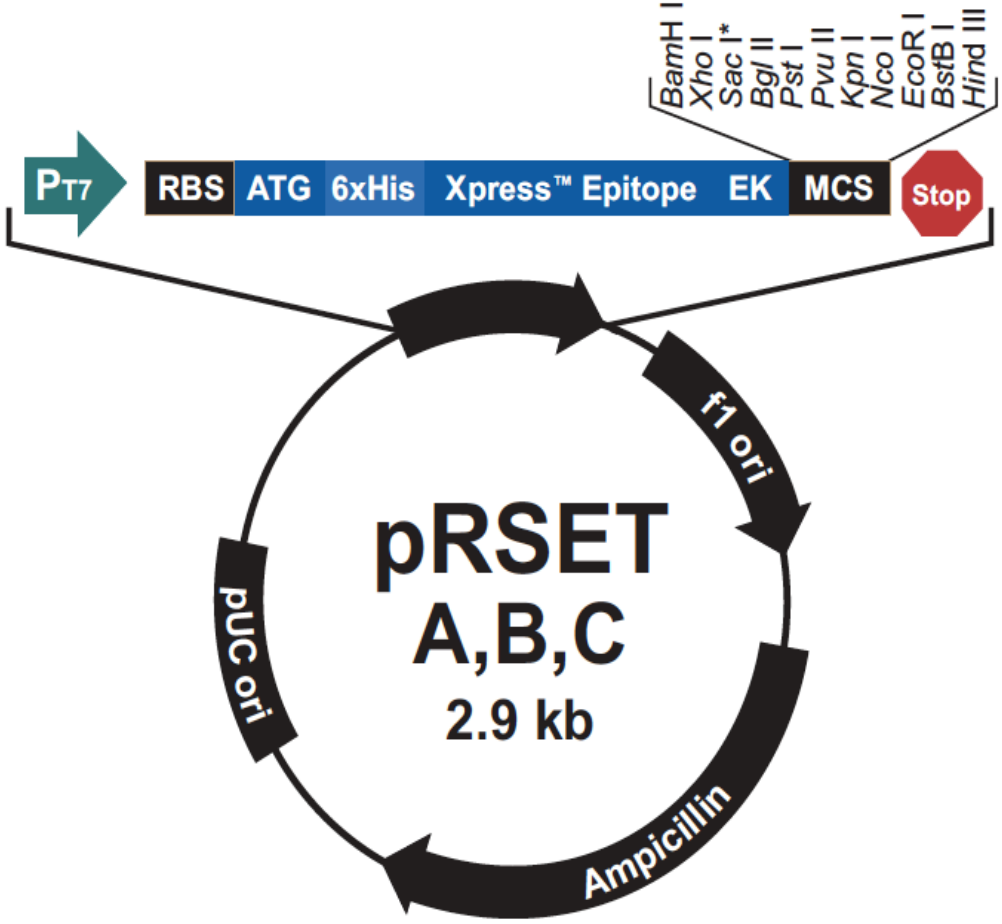
1) Amplification

2) Digestion

3) Ligation



# A closer look at the pRSETb expression vector (a.k.a. backbone)




- $P_{T7}$ promoter

  engineered to be IPTG-inducible

- 6xHis

  6 histidine tag
  binds to Nickel
  Helps with purification

- MCS

  Multiple cloning site

# M1D1 Part 2c: Calculate volumes of insert and backbone needed for ligation

Knowns:

- Need 50-100 ng backbone (specified by manufacturer)
- pRSET vector: 2853 bp
- FKBP12 Insert: 331 bp
- Molar mass ~660g/(mol*bp)
- Desired molar ratio of insert to backbone is 4:1
- Concentration of backbone (from recovery gel):
- Concentration of insert (from recovery gel):

# Calculate 4:1 (insert:backbone) *molar* amounts, final volumes for ligation

1. Calculate moles of backbone (50-100 ng, e.g. choose 60 ng)
   - 2853 bp * ( 660 g / (mol*bp) ) = $1.88 \times 10^6$ g/mol
   - so 60 ng / ($1.88 \times 10^6$ g/mol) = 3.19e-14 mol

2. Determine moles of insert needed (4x backbone)
   - 4 x 3.19e-14 mol ~ $1.26 \times 10^{-13}$ mol
   - with 331 bp * ( 660 g / (mol*bp) ) = $2.18 \times 10^5$ g/mol
   - so use $1.26 \times 10^{-13}$ mol * $2.18 \times 10^5$ g/mol ~ 27 ng

3. Calculate volume of backbone and insert needed
   - Backbone: 60 ng/( 20 ng/uL) = 3 uL
   - Insert: 27 ng / ( 40 ng/µL ) = 0.68 µL

# MIDI Part 2C: Ligation Calculation

Recovery gel:

backbone: 100ng/5μL = 20ng/μL

insert: 200ng/5μL = 40ng/μL

① Volume of backbone (50–100ng)

eg. let's choose 60ng → 3μL

② Calculate moles of backbone

backbone: $\frac{60 \times 10^{-9}\ g}{} \cdot \frac{mol \cdot bp}{660\ g} \cdot \frac{1}{2853\ bp} =$

$3.19 \times 10^{-14}$ moles

③ Determine moles of insert needed

(4:1 molar ratio of insert to backbone)

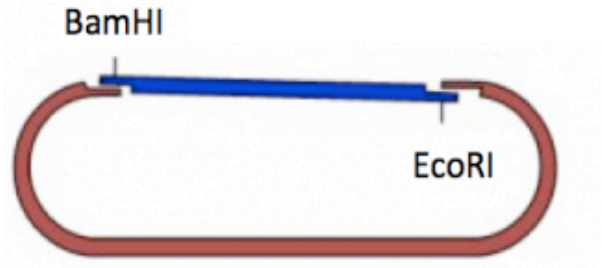
$4 \times 3.19 \times 10^{-14}\ moles = 1.26 \times 10^{-13}\ moles$

④ Calculate volume of insert needed

$1.26 \times 10^{-13}\ mol\ insert \left(\frac{660\ g}{mol \cdot bp}\right)\left(\frac{3316\ bp}{1}\right)\left(\frac{\mu L}{4 \times 10^{-9}\ g}\right)$

$= 0.68\ \mu L$ insert

# How do we confirm the plasmid product?




- Amplify plasmid
  - Transform into bacteria

- Purification
  - Separate plasmid from chromosomal DNA

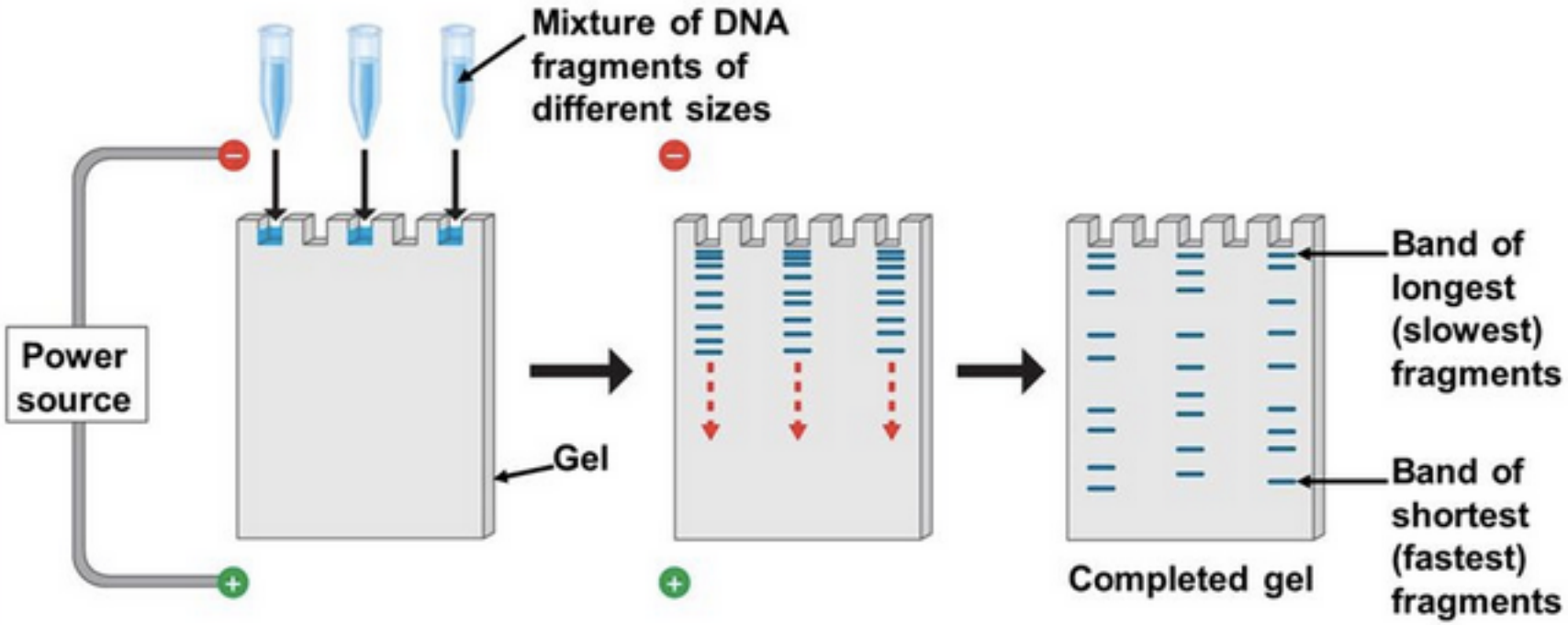
- Digestion This is what you did last time (M1D1)
  - Confirm the plasmid contains expected fragments

# Gel electrophoresis to visualize confirmation digest results

Driving force: electric field, charge

DNA is + / — charged

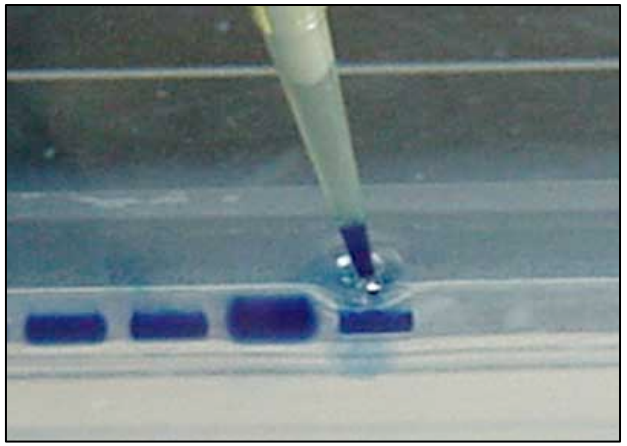
Separates DNA by size

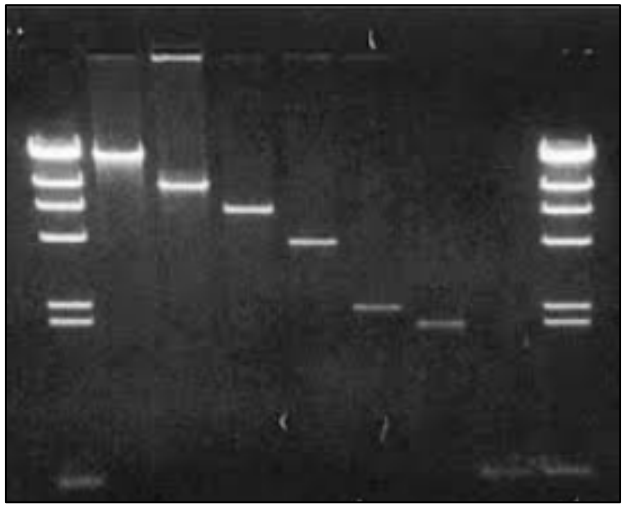



1% agarose gel

# Visualize DNA + save a picture!

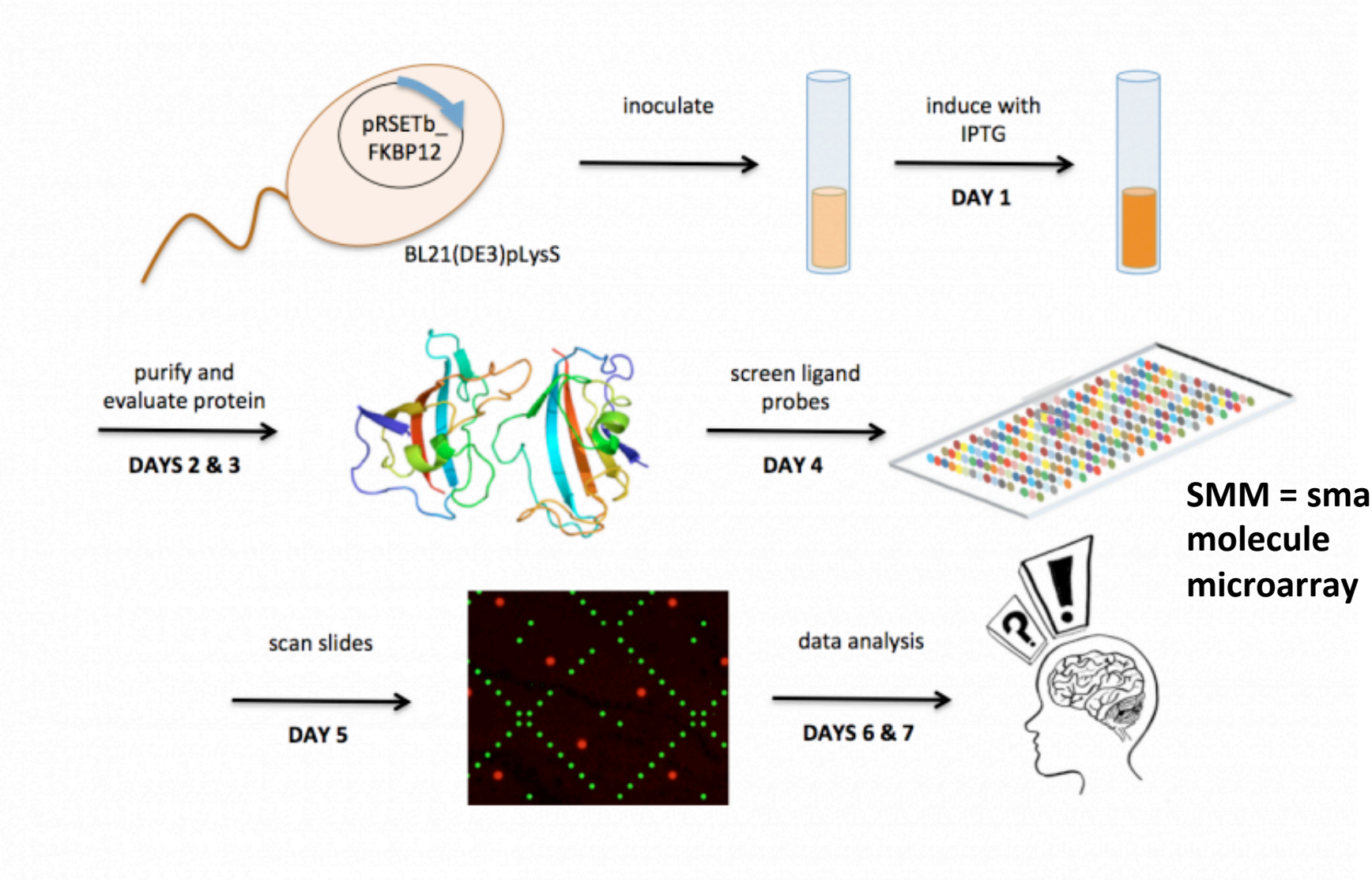
- DNA Loading dye (6X):
  - bromophenol blue
    - -runs as ~500 bp
    - -visualize progress
  - glycerol
    - -viscous, dense
    - -DNA sinks in well
- Sybr-Safe DNA stain:
  - -DNA intercalator
  - -Fluorescent
  - -Visualize with UV or blue light



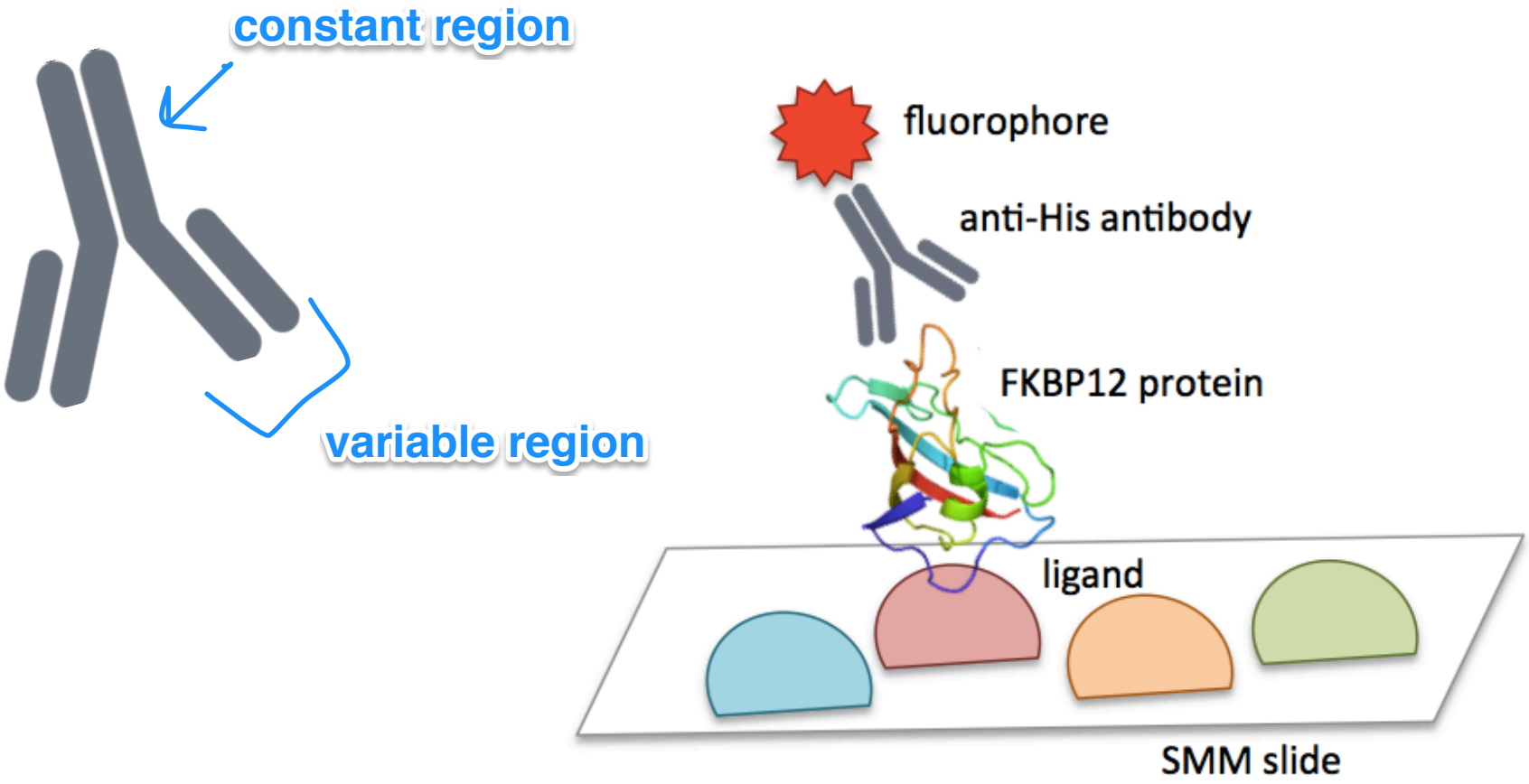


➢ Safety : wear nitrile gloves

# Your colleagues in Spring 2017…



# Using immunofluorescence to detect ligand binding

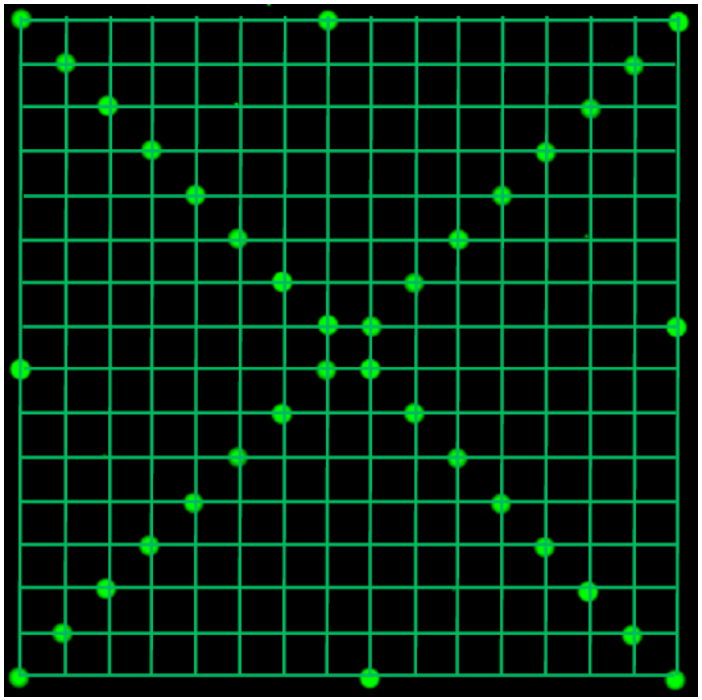


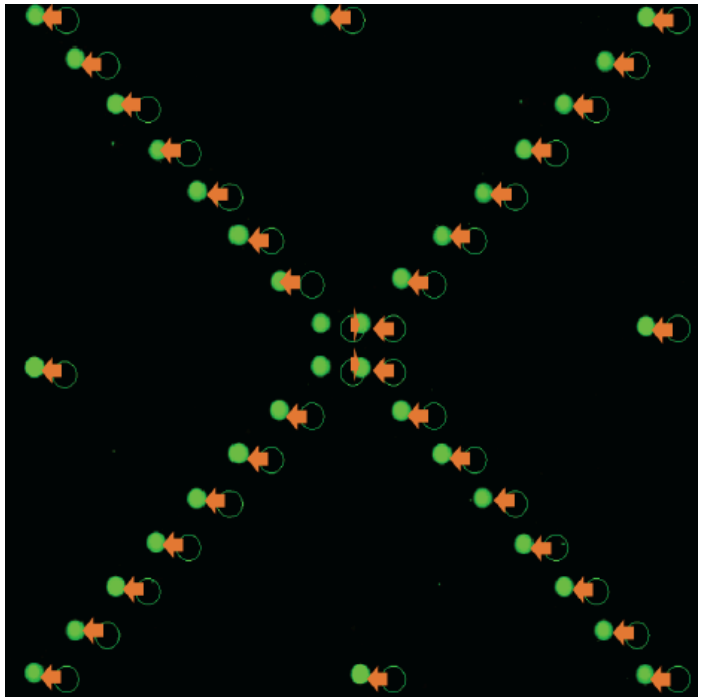
# SMM quantification steps

1. Align GAL file to fluorescence on 532 nm channel (sentinel spots)
2. Quantify fluorescence on 635 nm channel
3. Identify ‘hits’ with improbably high fluorescence
4. Identify compounds that hit repeatedly
5. Compare top hits to common binders list

# Align SMM results to using sentinel spots

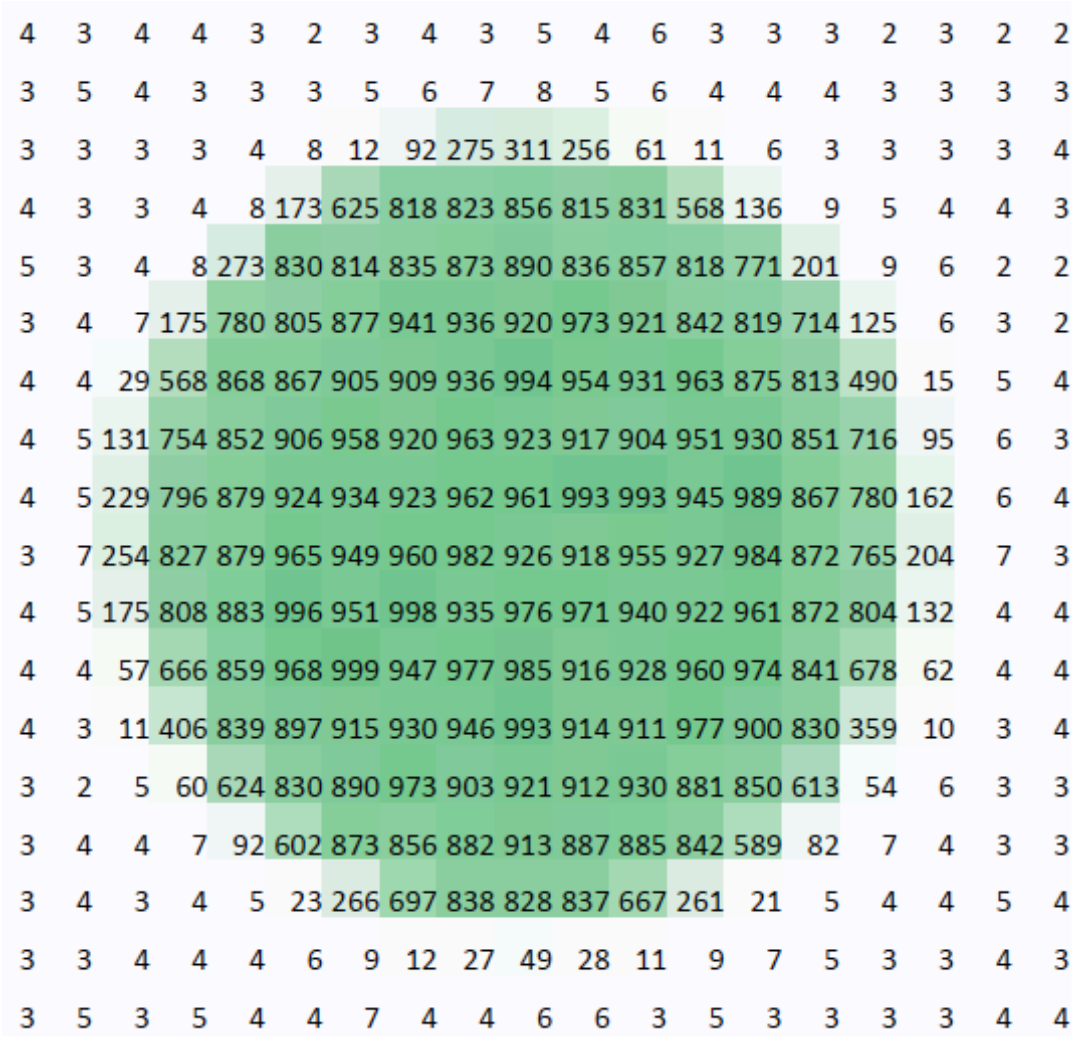
- Every spot can be located using intersecting lines between sentinels





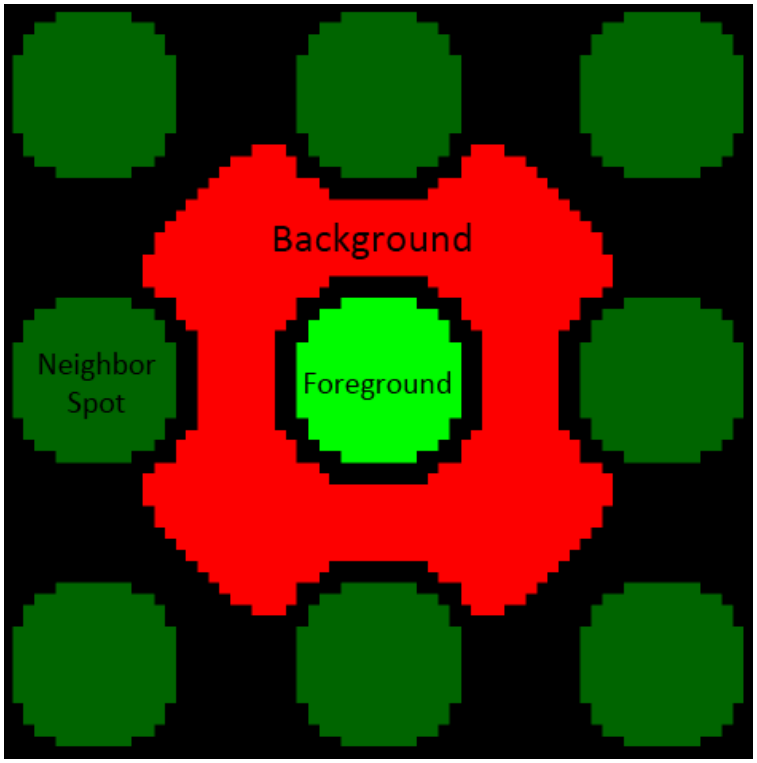
# Images represent arrays of numbers

- Each pixel is a 16-bit number that represents intensity
- Computational analysis used to define ‘hits’



# Quantify fluorescence to identify hits

- Foreground
- Background




$$\text{Signal-to-noise ratio (SNR)} = \frac{\mu_{foreground} - \mu_{background}}{\sigma_{background}}$$

signal

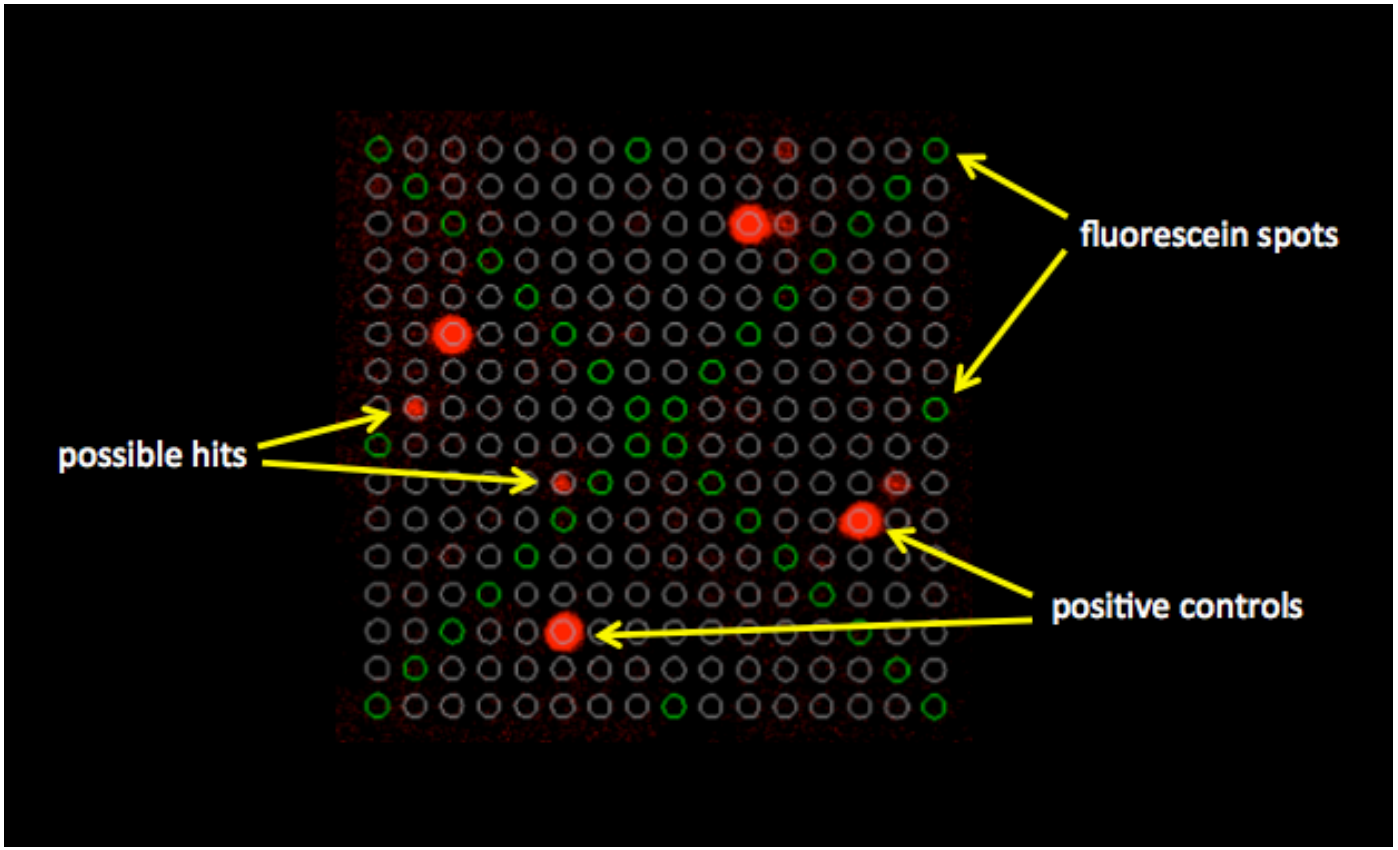
noise

Identify hits based on z-score (typically is a measure of how many standard deviations away from the mean the signal is). We will use a robust z-score, which is the same idea but based on the median instensity instead of the mean.

# How to evaluate the SMM results

- Is the background noisy?
- Are the positive controls easily recognized?
- Do any areas appear strange? Damaged?
  - Manufacturer or handling defects
- Are the hits aligned with printing spots?
- Do you trust the data?

# What do we expect to see?



# Factors that influence hit identification

- How many false positives are expected?
  - More hits needed if confidence is low
- How many chemical ‘patterns’ are evident?
  - Repeated patterns between compounds may increase confidence
- Are the hits unique to the screen?
  - Promiscuous binders may decrease confidence

# Today in lab…

- Reminder: wipe down bench with 70% EtOH before and after wetlab work
- Reminder: empty benchtop waste bucket into biowaste bin at end of day
- Measure OD of bacterial culture—when OD is >0.5, add IPTG to induce expression of FKBP12 protein
- Perform electrophoresis on confirmation digest reactions
- SMM data analysis

# For next time…

- Draft a figure with your confirmation digest results for your Data Summary
  - Include a title and caption
- Schedule appointment at BE Communication Lab before M1D5

# Notes on figure making:

- Image should not be the entire page
  - Only needs to be large enough to be clear
- Title should be conclusive
  - Don't include what you did, rather include what you found
- Caption should not detail the methods
  - Define abbreviations, symbols, etc.