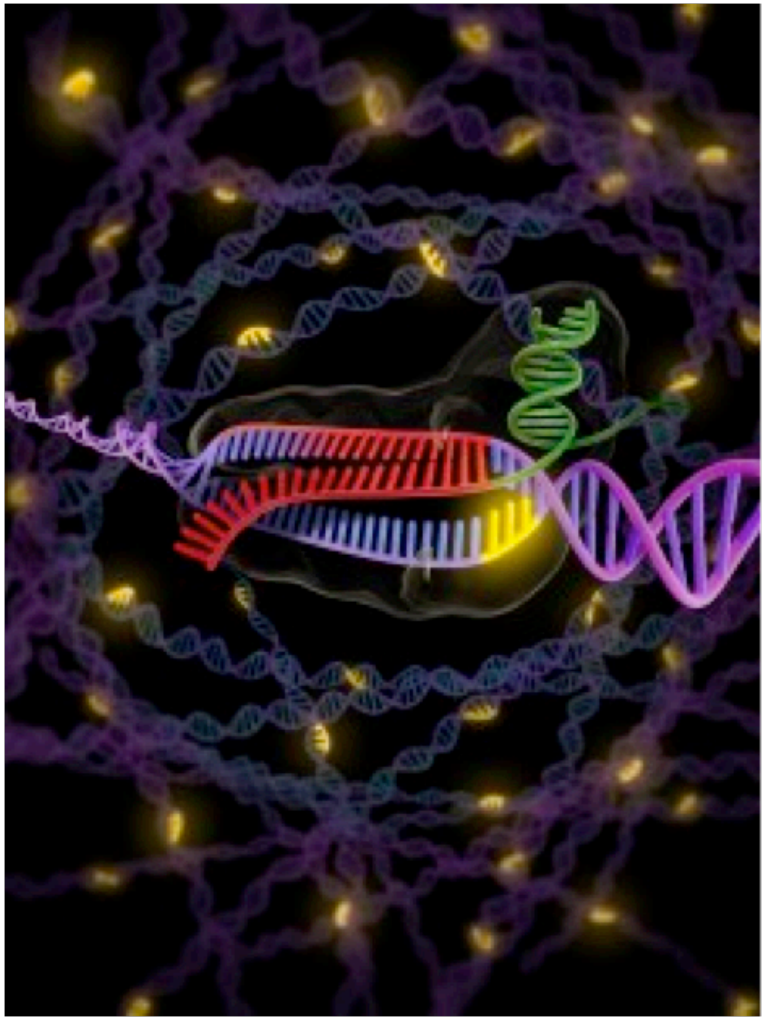
M2D2:
Prepare protein expression system

10/15/2015

The real end of M1 is in sight…
Let's celebrate this accomplishment!

Seminar of great interest



Prof. Feng Zhang

Development and applications of CRISPR-Cas for genome editing

Wednesday, October 21

10:00 am

in 46-3002

Sign up for journal club

Slot	Day 5 (T/R)	Day 8 (T/R)	Day 5 (W/F)	Day 8 (W/F)
1				
2				
3				
4				
5				
6				

- Pick 1 of 25 papers, or suggest your own
- Present M2D5 (Oct. 27) or M2D8 (Nov. 5)
- Sign up by adding your name next to listing [MJ/TR/Rainbow]
  - first come first serve!
  - one T/R and one W/F per article

Developing and examining calcium sensors [edit]

1. Robin, J. et al. *Differential nanosecond protein dynamics in homologous calcium sensors.* (2015) ACS Chem Biol epub ahead of print. PMID:26204433
2. Cai, B. et al. *A cell-based functional assay using a green fluorescent protein-based calcium indicator dCys-GCaMP.* (2014) Assay Drug Dev Tech 12:342-351. PMID:25105973
3. Wu, J. et al. *Red fluorescent genetically encoded Ca2+ indicators for use in mitochondria and endoplasmic reticulum.* (2014) Biochem J 464:13-22. PMID:25164254

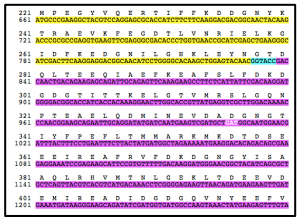
Using calcium sensors in biological systems [edit]

1. Muto, A. et al. *Real-time visualization of neuronal activity during perception.* (2013) Curr Biol 23:307-311. PMID:23375894
2. Luongo, F. et al. *Putative microcircuit-level substrates for attention are disrupted in mouse models of autism.* (2015) Biol Psych epub ahead of print. PMID:26022075
3. Tang, W. et al. *Stimulation-evoked Ca2+ signals in astrocytic processes at hippocampal CA3-CA1 synapses of adult mice are modulated by glutamate at ATP.* (2015) J Neurosci 35:3016-3021. PMID:25698739

Assignments… you know ‘em already

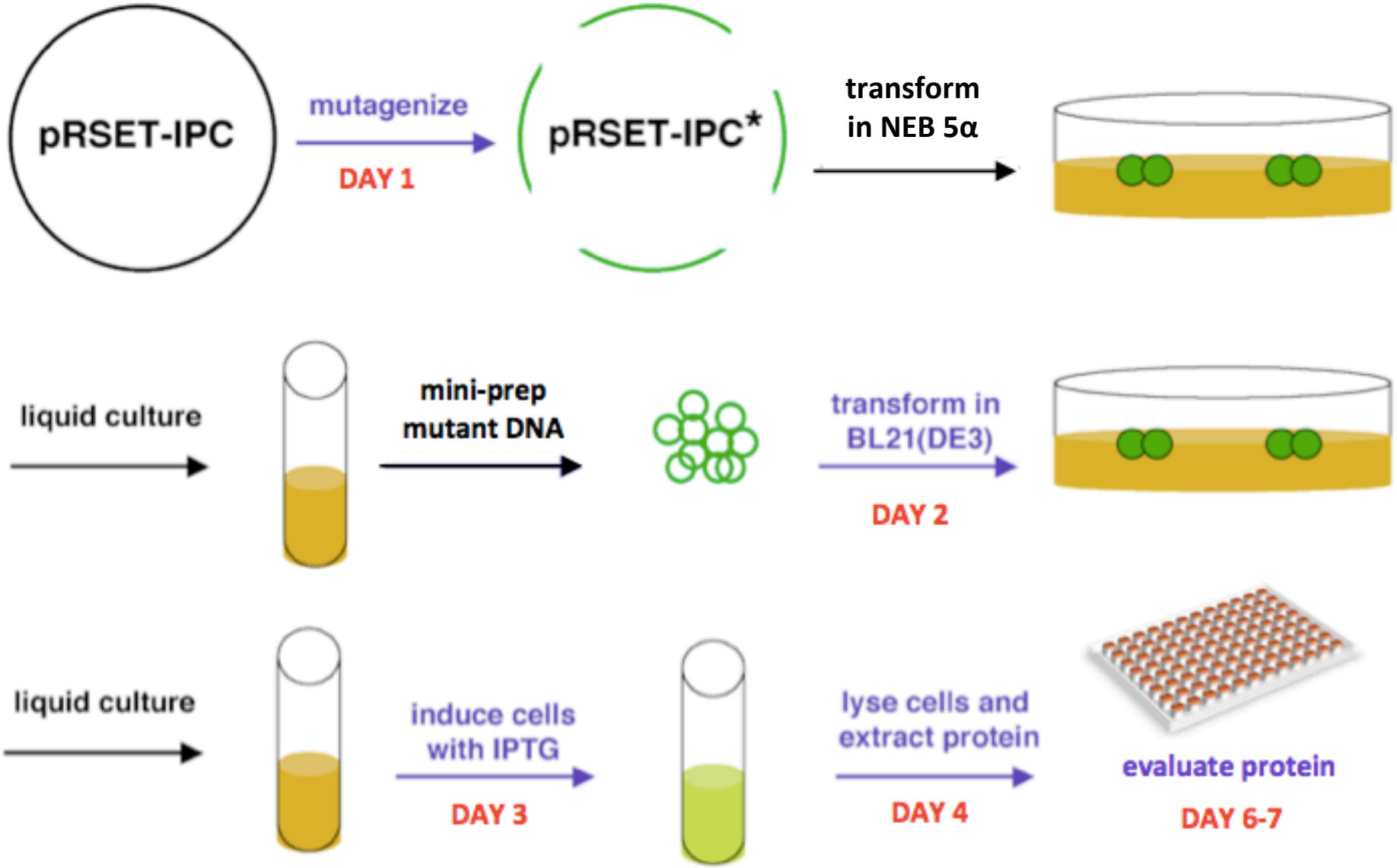
- DNA mini presentation
  - due by 5pm on Saturday, Oct. 17
- DNA engineering summary revision
  - due by 5pm on Saturday, Oct. 24
- Blog post for M1
  - due by 5pm on Sunday, Oct. 25

be20109f15.blogspot.com
be20109.blogspot.com



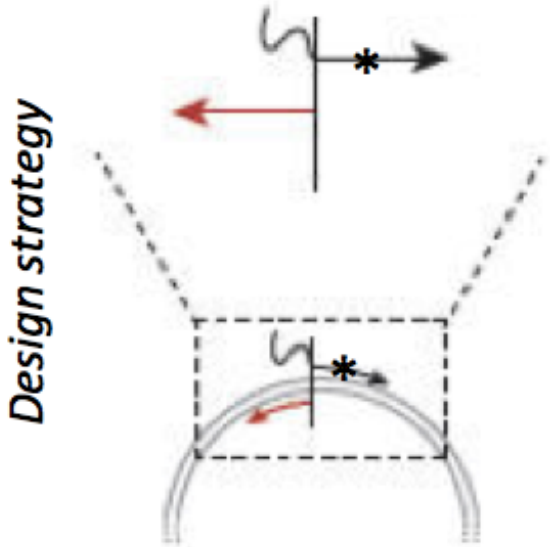
- For M2D3:
  - draft intro of your report, including references
  - schematic (with title and caption) of mutagenesis strategy

Since last time…



Well, lesson learned!

- Frame shift hiccup
  - primers auto-generated by NEB didn't start *in frame*
  - we carefully added a flap sequence (recognition site for endonuclease) of multiple-of-3-bp length…



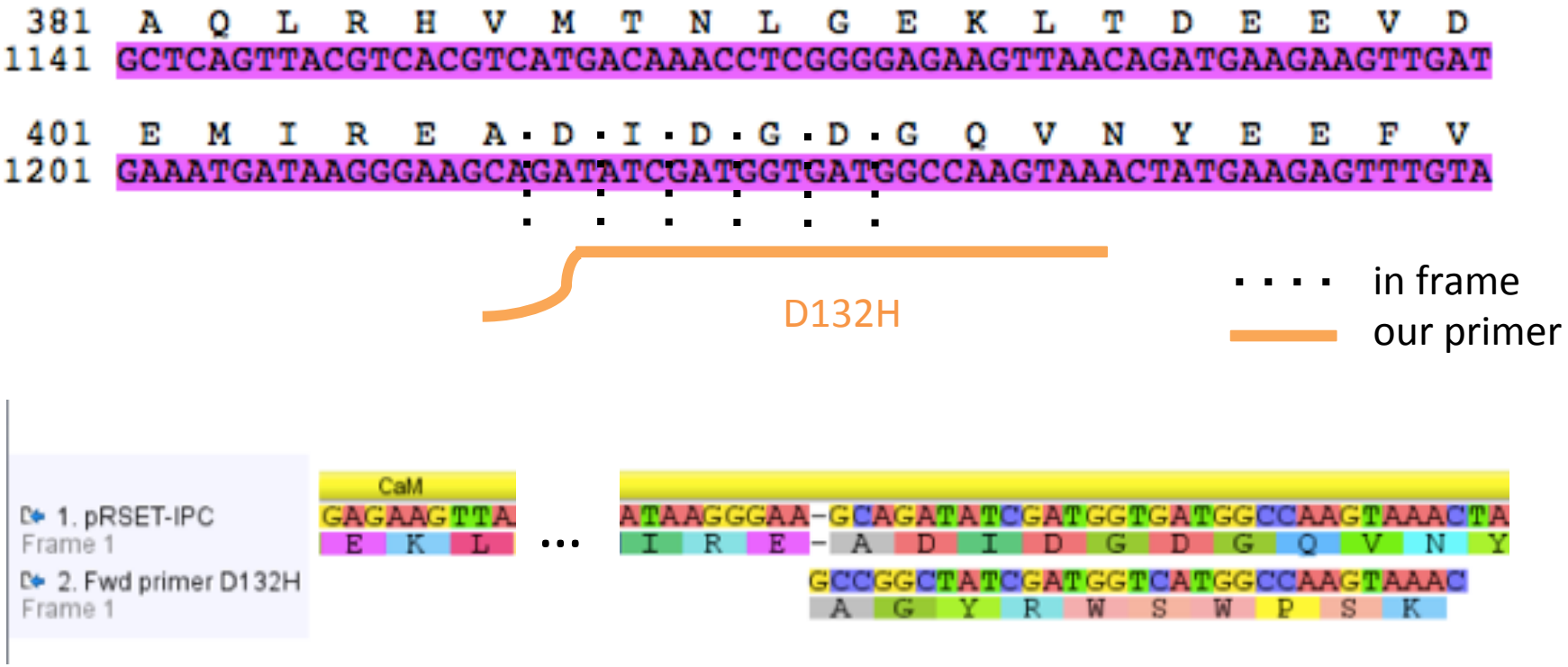

```
 381  A  Q  L  R  H  V  M  T  N  L  G  E  K  L  T  D  E  E  V  D
1141  GCTCAGTTACGTCACGTCATGACAAACCTCGGGGAGAAGTTAACAGATGAAGAAGTTGAT

 401  E  M  I  R  E  A  D  I  D  G  D  G  Q  V  N  Y  E  E  F  V
1201  GAAATGATAAGGGAAGCAGATATCGATGGTGATGGCCAAGTAAACTATGAAGAGTTTGTA
                       .  .  .  .  .  .
                       .  .  .  .  .  .
```

D132H

. . . . in frame

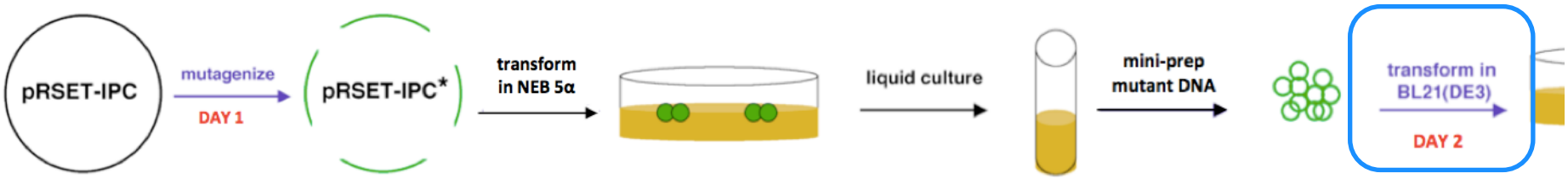
our primer

ApE does not reveal frame shift Geneious would have



Since last time…

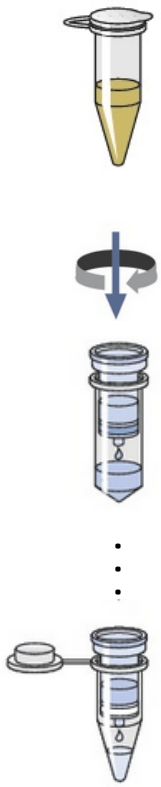
- Frame shift surprise
- We ordered new primers (no flap)
  - even though IPC protein expressed
  - effect on calcium binding not elucidated: due to mutation?
- We repeated SDM reactions for you



Transformation controls & outcomes

sample	expectation **/ what if?**	**role**
no DNA	no colony. What if many? **contamination** **wrong antibiotic (Amp)**	**negative control**
control	many. What if none/few? **wrong antibiotic killed cells (vortex)** **NEB says pUC19 vector is taken up efficiently**	**positive control reagents and methods good**
your X#Z or wt IPC	some. What if X#Z << control? **problem during KLD reaction** **transformation efficiency with pRSET is poor** **DNA degradation, low [DNA]**	**experimental**

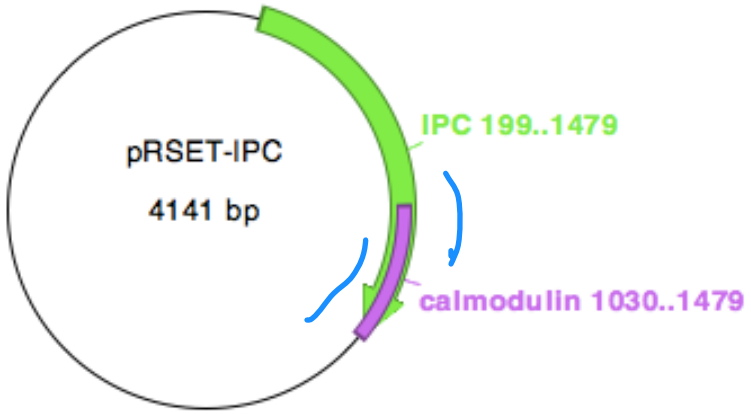
Review of mini-prep steps

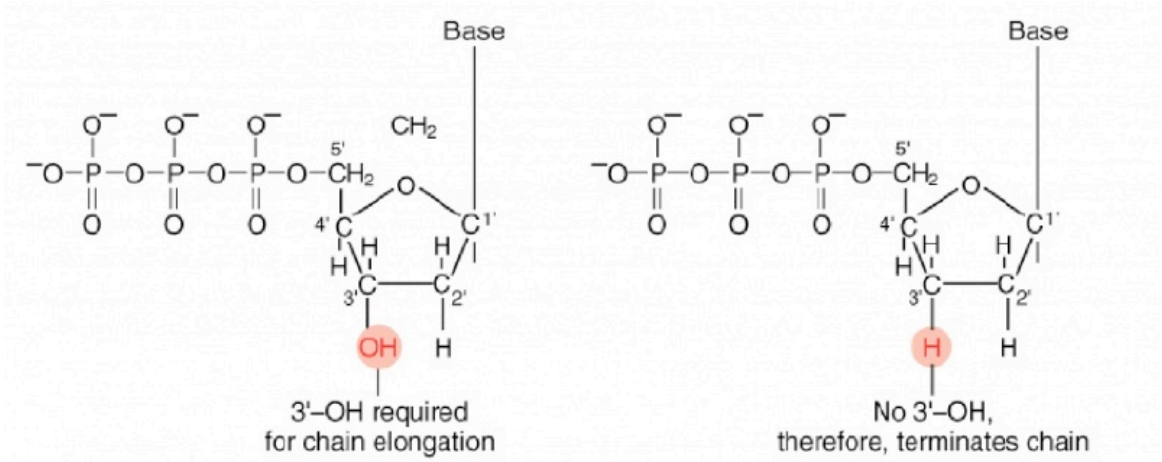


steps	contents	purpose
prepare	Tris/EDTA buffer RNAase	resuspend cells, weakens membrane
lyse	SDS NaOH alkaline lysis	solubilize proteins, denature DNA
neutralize	acetic acid, potassium acetate	renature short DNA (pRSET-IPC) precipitate long DNA
		clear lysate
concentrate	spin: bind to silica column	pellet “garbage”
wash	ethanol	** get rid of *all* ethanol
elute	water, pH 8.0	high-purity DNA

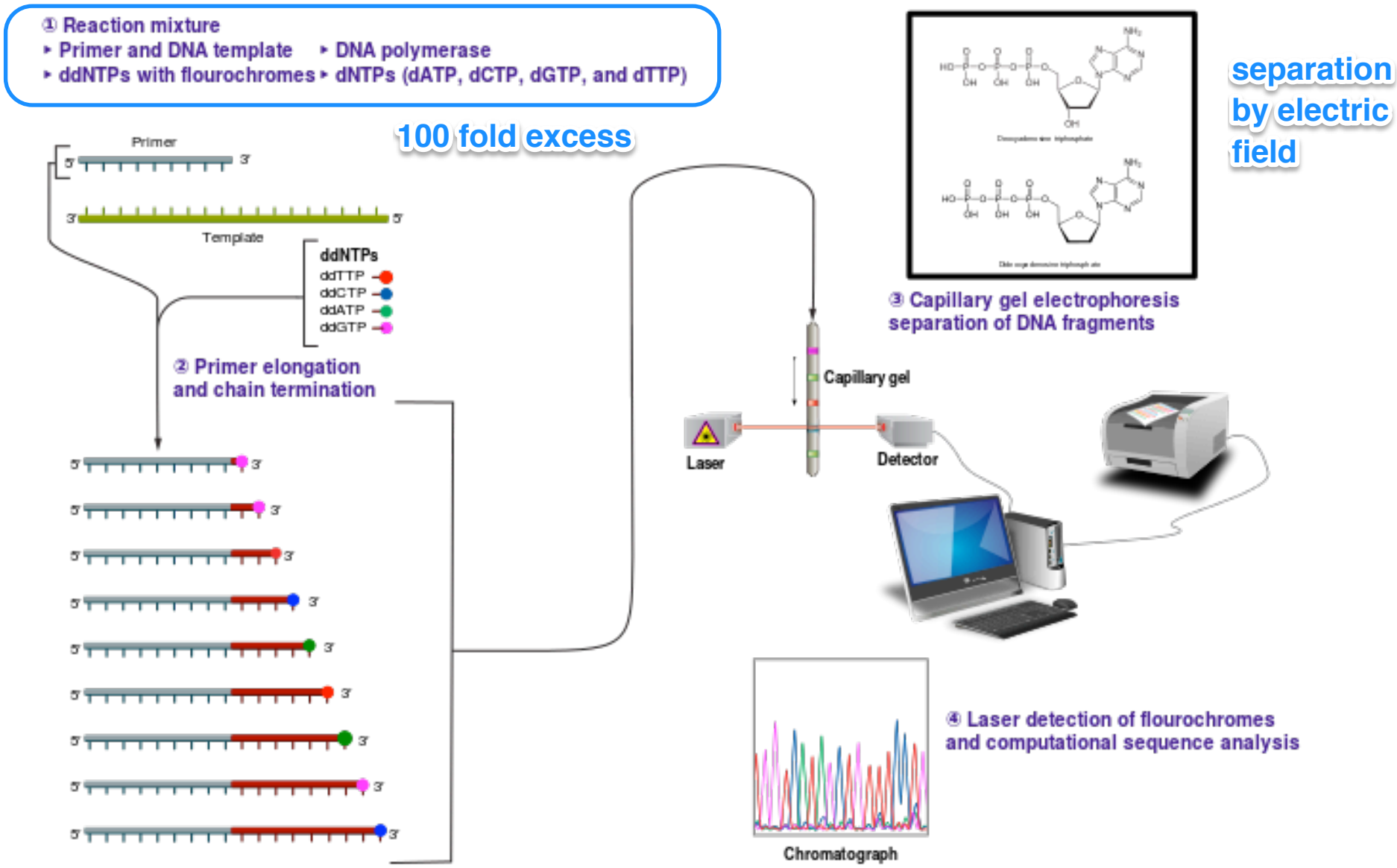
Do we have the intended mutant?

- Diagnostic digests
- Sequencing
  - good to have both F and R primers
    - **double-checking sequence**
    - **coverage of > 500 bp**
  - di-deoxynucleotides terminate elongation

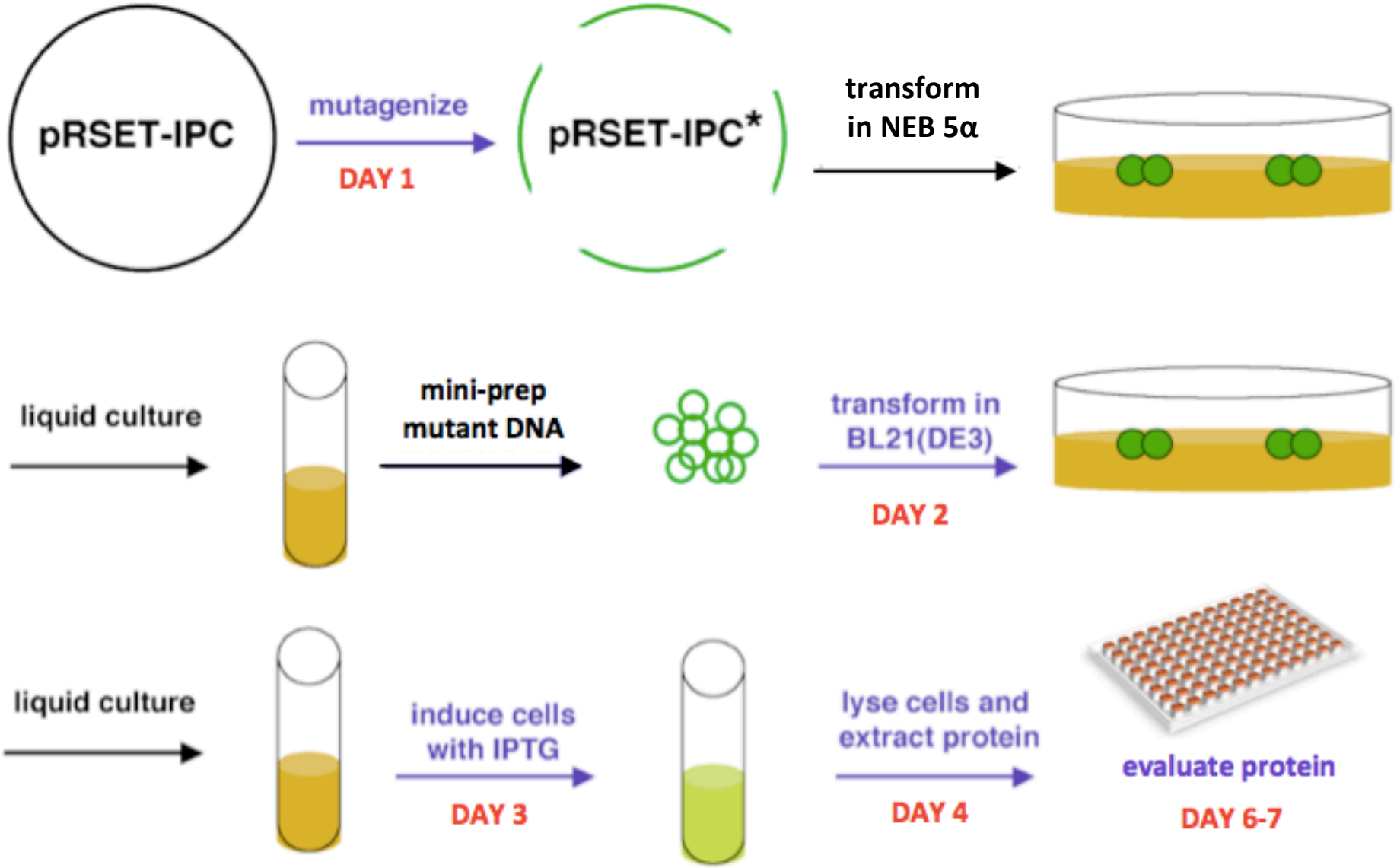




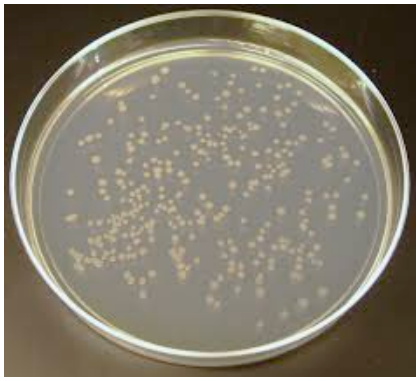
Sanger sequencing by Genewiz

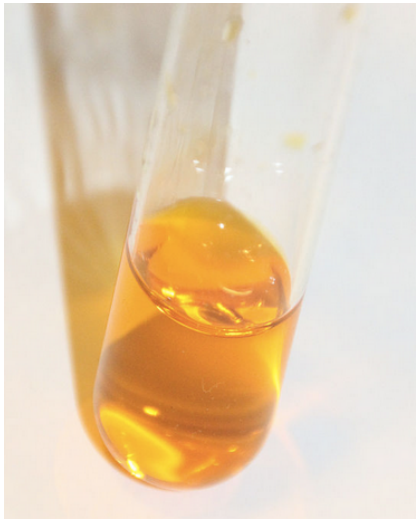


DNA vs. protein amplification in NEB 5α vs. BL21



Transforming BL21(DE3)pLysS competent cells



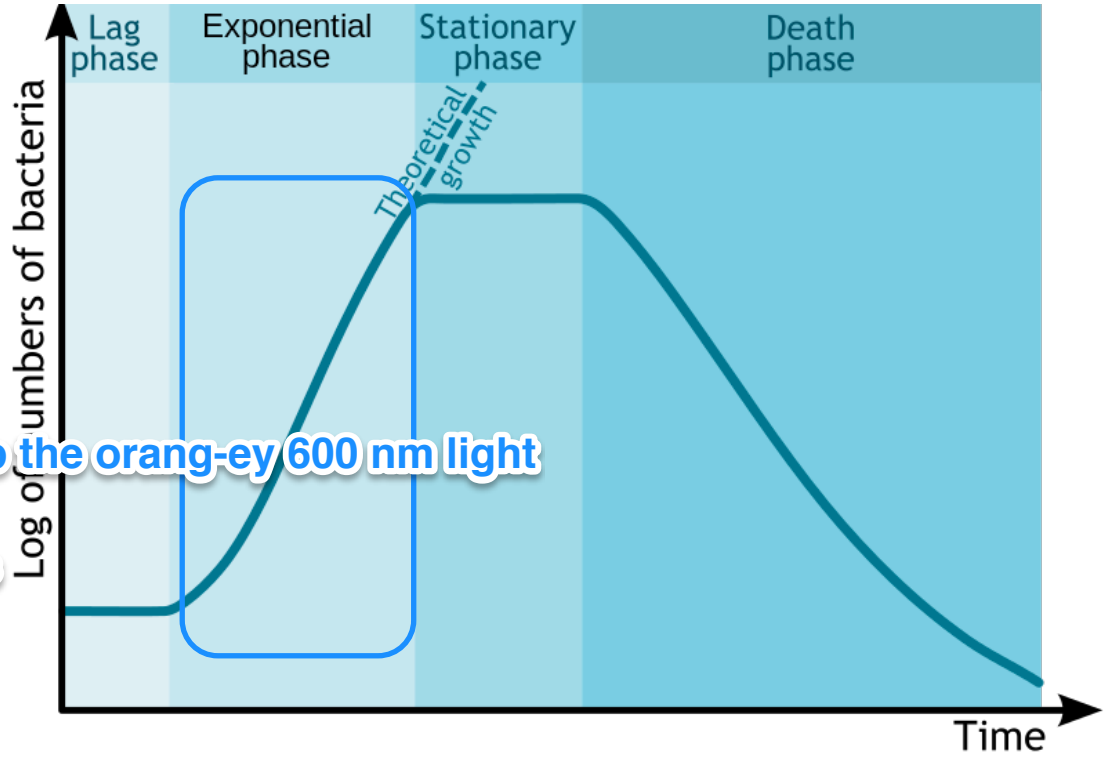


- can express IPC protein
  - when induced by lactose analog…
  - …details on M2D3!
- made competent by $CaCl_2$
  - Ca^{2+} ions attract both DNA and liposaccharides
  - heat shock
- in exponential growth phase, OD_{600} = 0.4-0.8
- handle very gently, or will lyse
  - *on ice* all the time, and with chilled solutions
  - not vortexed
- Cam (chloramphenicol) resistant *E. coli* strain
  - Amp (ampicillin) resistant if IPC insert uptaken

A few brief notes on *E. coli* growth curve

- exponential phase
  - binary fission
  - OD600 ~ 0.4 - 0.8
  - machinery ready

- OD ≠ absorbance
  - yellow cells don't absorb the orang-ey 600 nm light
  - turbidity, cell count
  - UV would be killing cells



Today in lab

- Obtain BL21(DE3)pLysS in mid-log phase, make them competent, and transform with X#Z #1, X#Z #2, wt IPC, or no DNA
- Prepare X#Z #1 and X#Z #2 for sequencing
- Count colonies from X#Z plate
- Discuss Nagai *et al.* paper