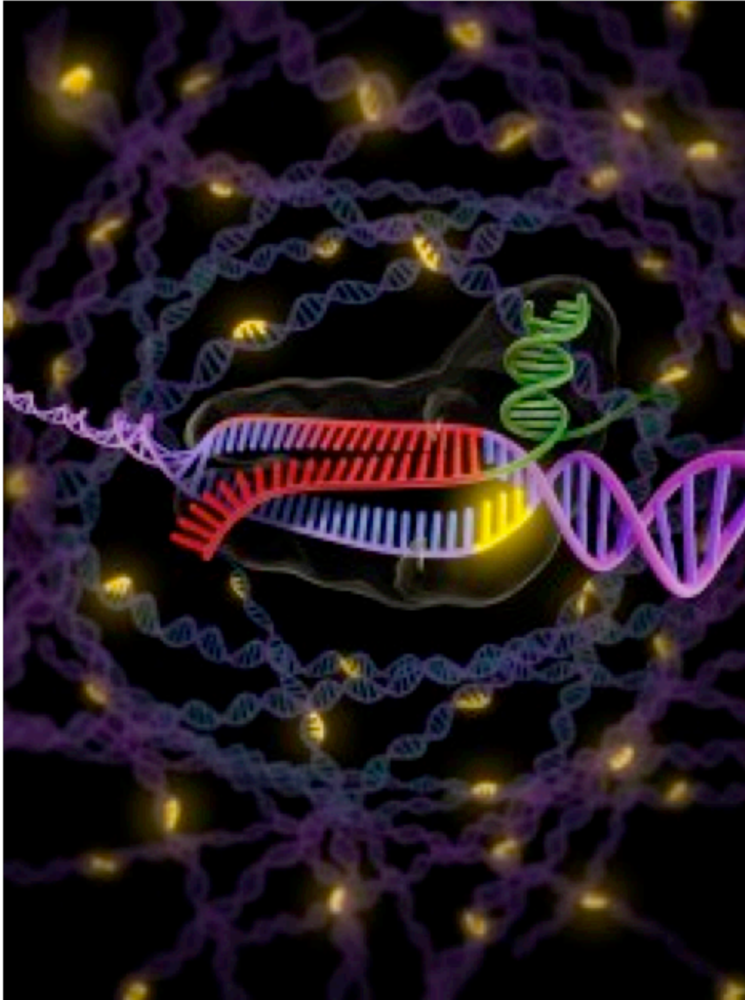
Seminar of great interest



Prof. Feng Zhang

Development and applications of CRISPR-Cas for genome editing

Wednesday, October 21

10:00 am

in 46-3002

M2D2:Prepare protein expression system

10/16/2015

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

Sign up for journal club

1. Pick 1 of 25 papers, or suggest your own
2. Sign up by adding your name next to paper[LMM/WF/Rainbow]
   - **first come first serve!**
   - **one T/R and one W/F per article**
3. Sign up for a time slot: M2D5 (Oct. 28) or M2D8 (Nov. 6)

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 LMM/WF/Rainbow
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 sy… modulated by glutamate at ATP.* (2015) J Neurosci 35:3016-3021. PMID:25698739

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

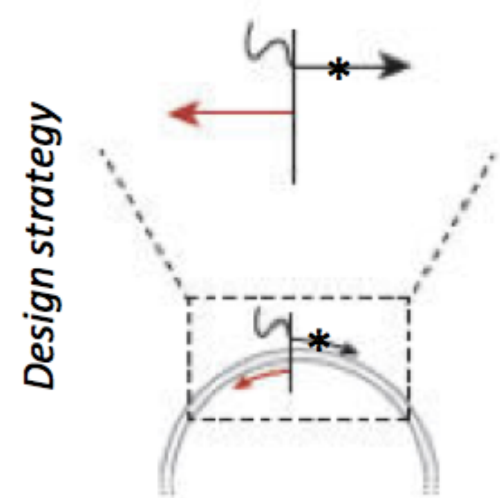
Assignments… you know 'em already

- 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

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

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
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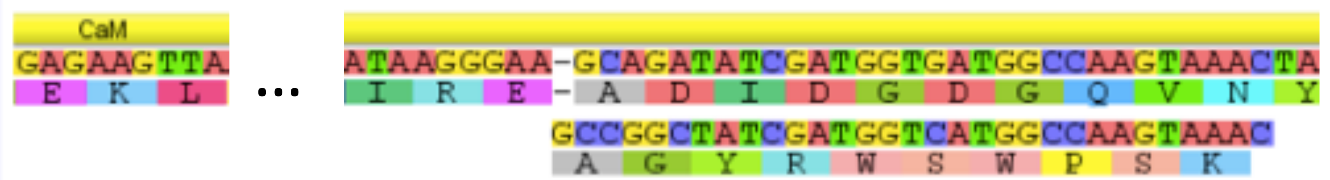
· · · · in frame

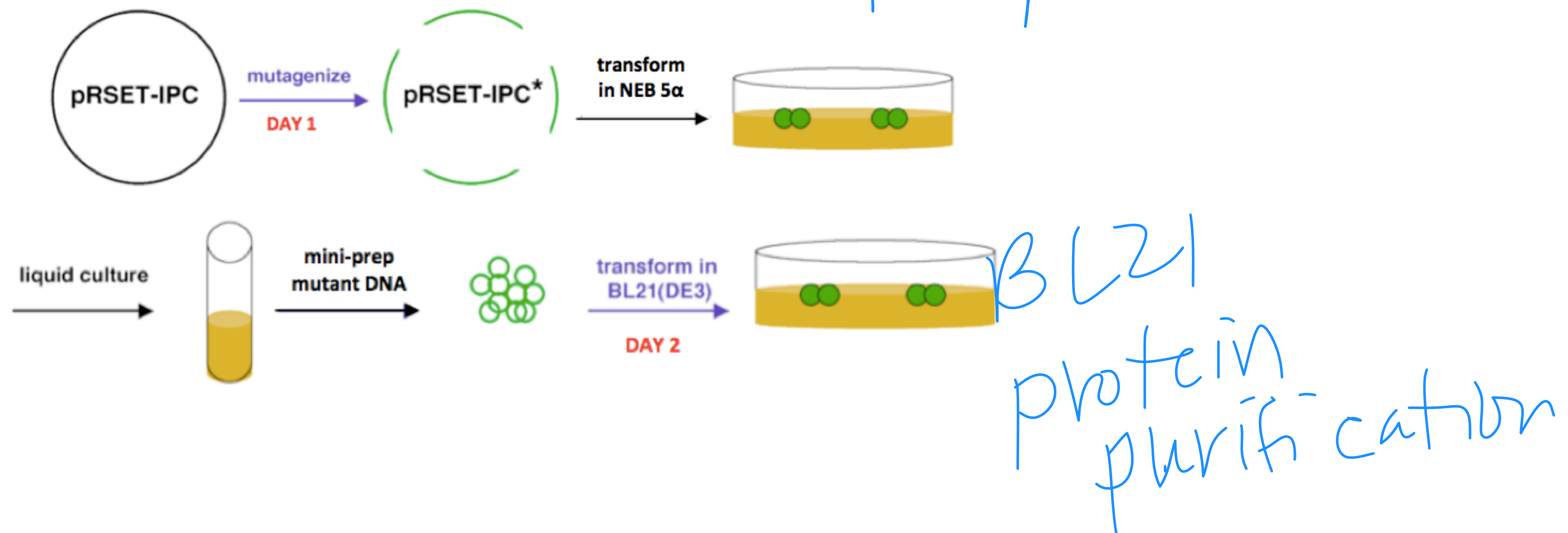
our primer



Since last time…

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

1. We repeated SDM reactions with new primers
2. Performed Kinase/ligase/DpnI (K/L/D)
3. Transformed into NEB α5 (E. coli)
4. Harvested – plasmid (mini prep)

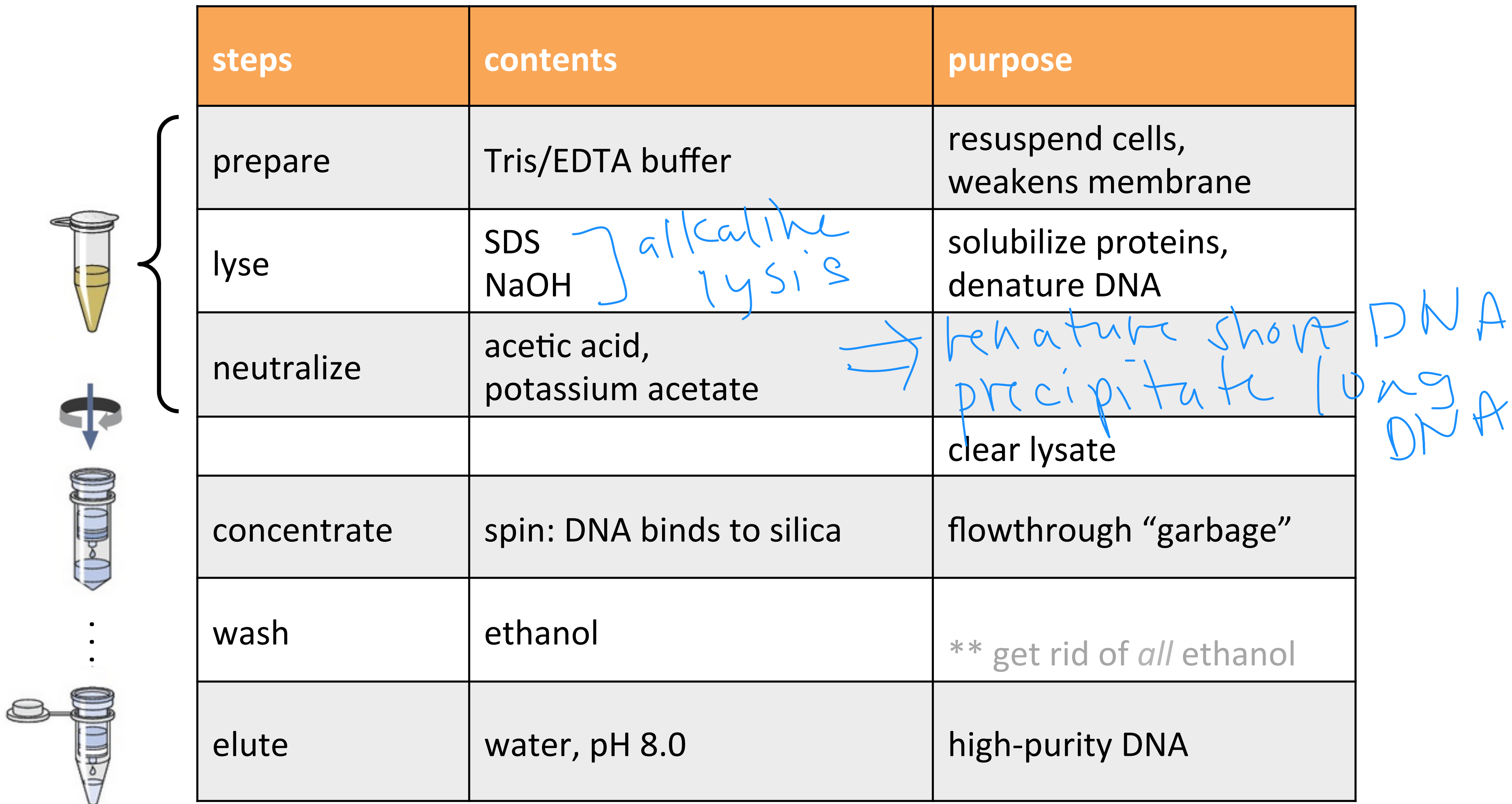


Transformation controls & outcomes

BL21 → appropriate antibiotics (CAM AMP)

sample	expectation / what if?	role
no DNA	no colony. What if many? contamination wrong antibiotics	negative
control DNA	many. What if none/few? · low transformation efficiency · killed · wrong antibiotic	positive
your X#Z or wt IPC	some. What if X#Z << control? low transformat'n eff. - SDM rxn - DNA degradation low [DNA]	experimental

Review of mini-prep steps



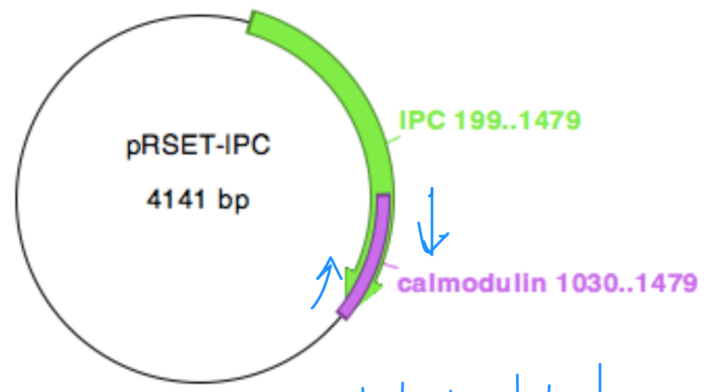
steps	contents	purpose
prepare	Tris/EDTA buffer	resuspend cells, weakens membrane
lyse	SDS NaOH] alkaline lysis	solubilize proteins, denature DNA
neutralize	acetic acid, potassium acetate ⇒	renature short DNA precipitate long DNA
		clear lysate
concentrate	spin: DNA binds to silica	flowthrough “garbage”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

good seq ~ 1000 bps

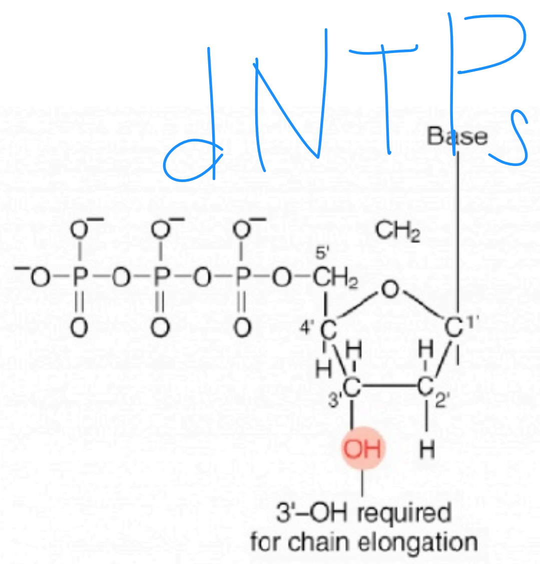
high / low — 1 seq 100

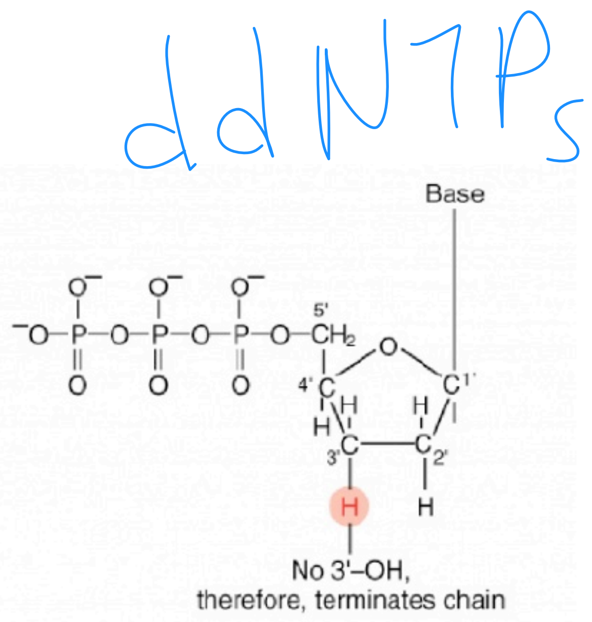
Do we have the intended mutant?

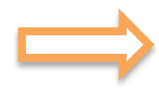
- Diagnostic digests
- Sequencing
  - good to have both F and R primers
    - 2x coverage
    - coverage of >500 bps
  - di-deoxynucleotides terminate elongation

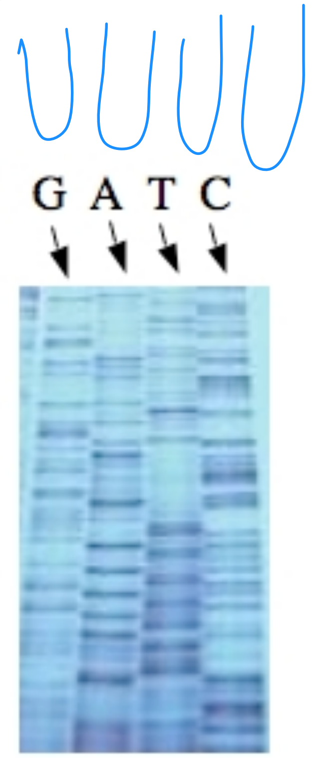


dNTPs ddNTPs







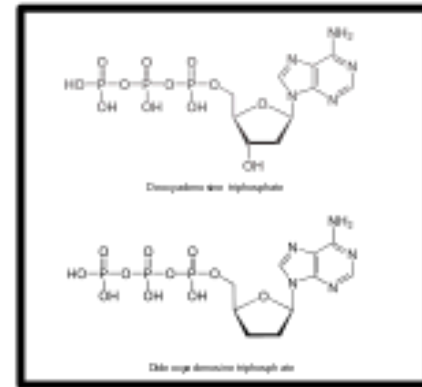


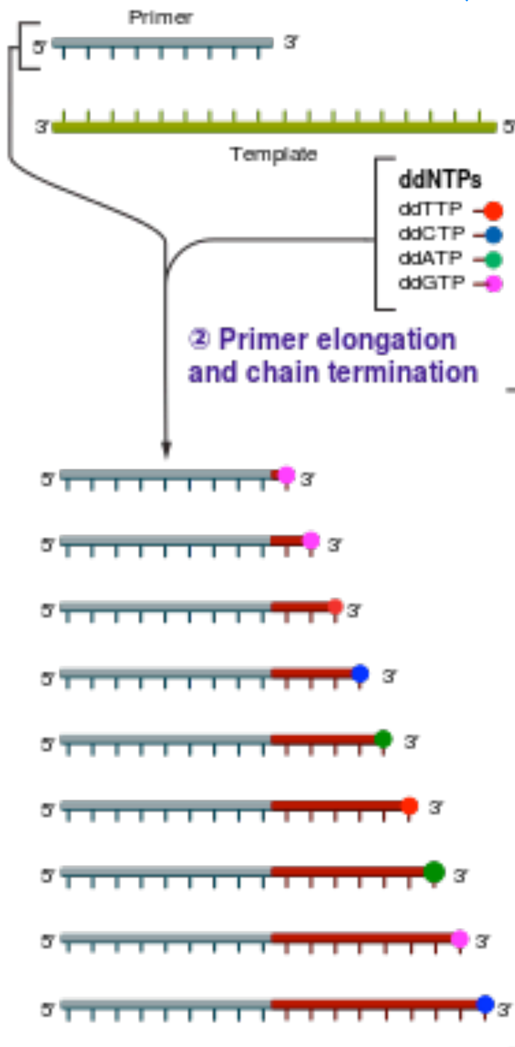
Sanger sequencing by Genewiz

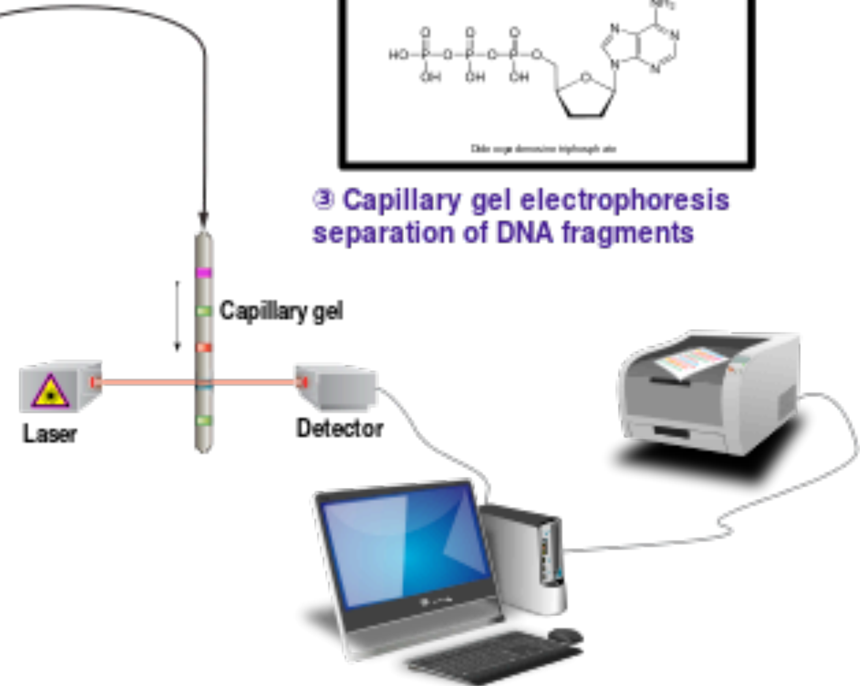
① Reaction mixture

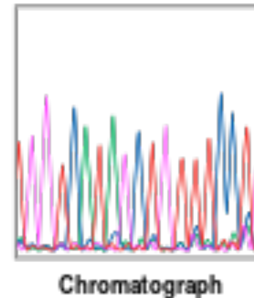
- Primer and DNA template
- DNA polymerase
- ddNTPs with flourochromes
- dNTPs (dATP, dCTP, dGTP, and dTTP)

100X excess





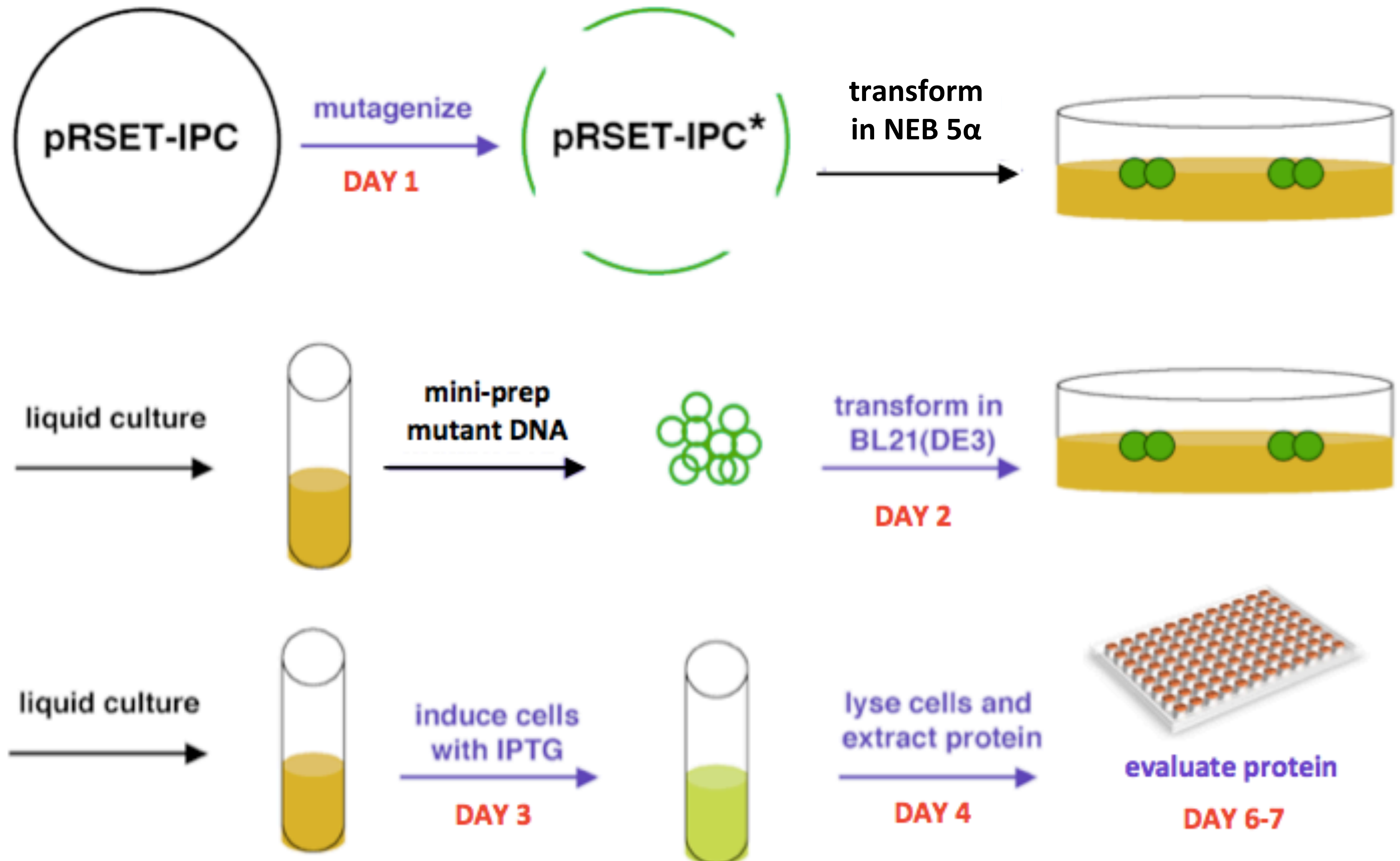




Chromatograph

④ Laser detection of flourochromes and computational sequence analysis

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 lipposacharide (cell wall)
  - 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

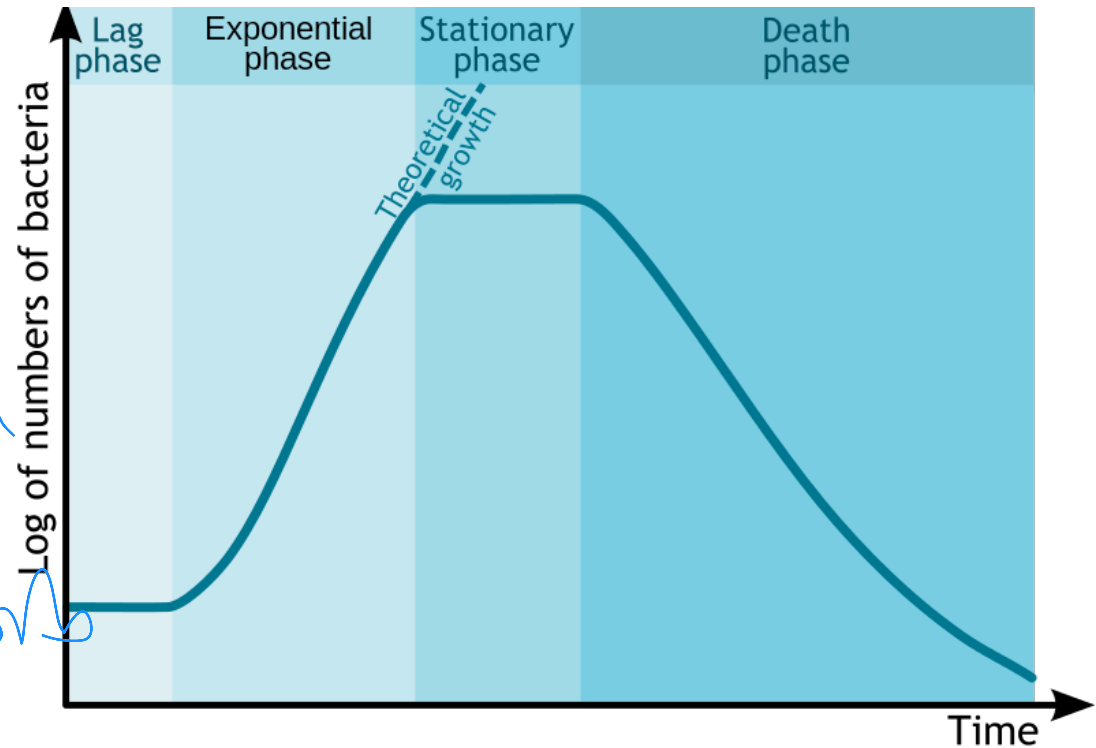
A few brief notes on *E. coli* growth curve

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

- OD ≠ absorbance
  - low wavelength
  - yellow =
  -

doesn't absorb @600nm

- turbidity



Today in lab

- Pick up BL21 liquid culture in 37C incubator
- Primers and mini-preps of potential mutants in ice bucket
- Set up sequencing reactions at front bench