M1D5:
Induce protein expression

02/24/2016

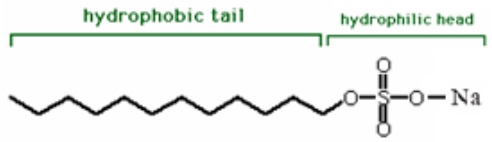
Mini-prep to isolate DNA

using QIAprep kit by Qiagen

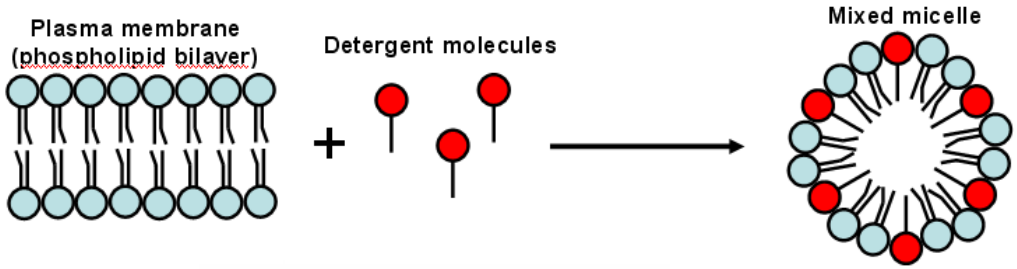
steps	contents	purpose
prepare P1	Tris/EDTA buffer RNase	resuspend NEB 5alpha cells weaken the cell walls
lyse P2	SDS surfactant NaOH alkaline lysis	solubilize proteins, denature DNA
neutralize N3	acetic acid, chaotropic salt, potassium acetate	short (plasmid) renature long DNA (rest of E. Coli) precipitate
		clear lysate
concentrate	spin: bind to silica column	pellet "garbage"
wash PB, PE	ethanol use 'Qiagen waste'	** get rid of *all* ethanol
elute	water, pH 8.0	high-purity DNA

Review of mini-prep: Lysis with SDS/NaOH

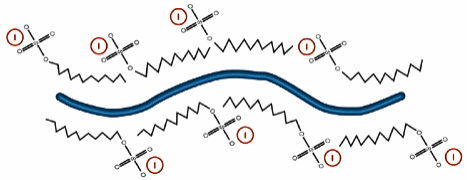
1. Sodium dodecyl sulfate (SDS)



   - dissolves membranes

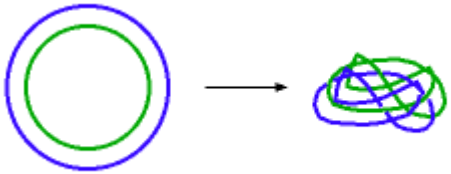


   - binds to and denatures proteins



2. Sodium hydroxide (NaOH)

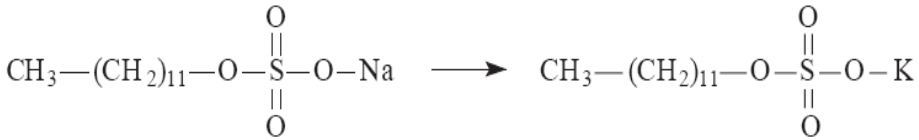
   - denatures DNA



Because plasmids are supercoiled, both DNA strands remain entangled after denaturation

Review of mini-prep: Neutralization

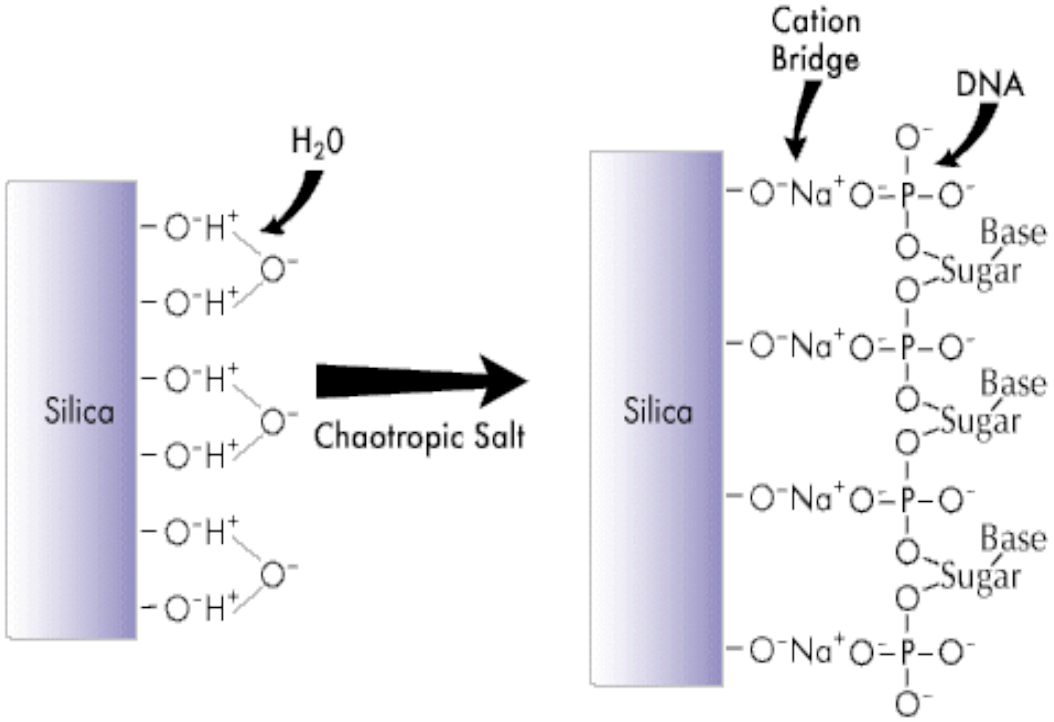
1. Acetic acid / potassium acetate solution
   - neutralizes NaOH (renatures plasmid DNA)
   - converts soluble SDS into insoluble PDS (white fluff)



2. Chaotropic salt
   - facilitates DNA binding to silica

➢ After centrifugation
   - supernatant: plasmid DNA (and soluble cellular constituents)
   - pellet: PDS, lipids, proteins, chromosomal DNA

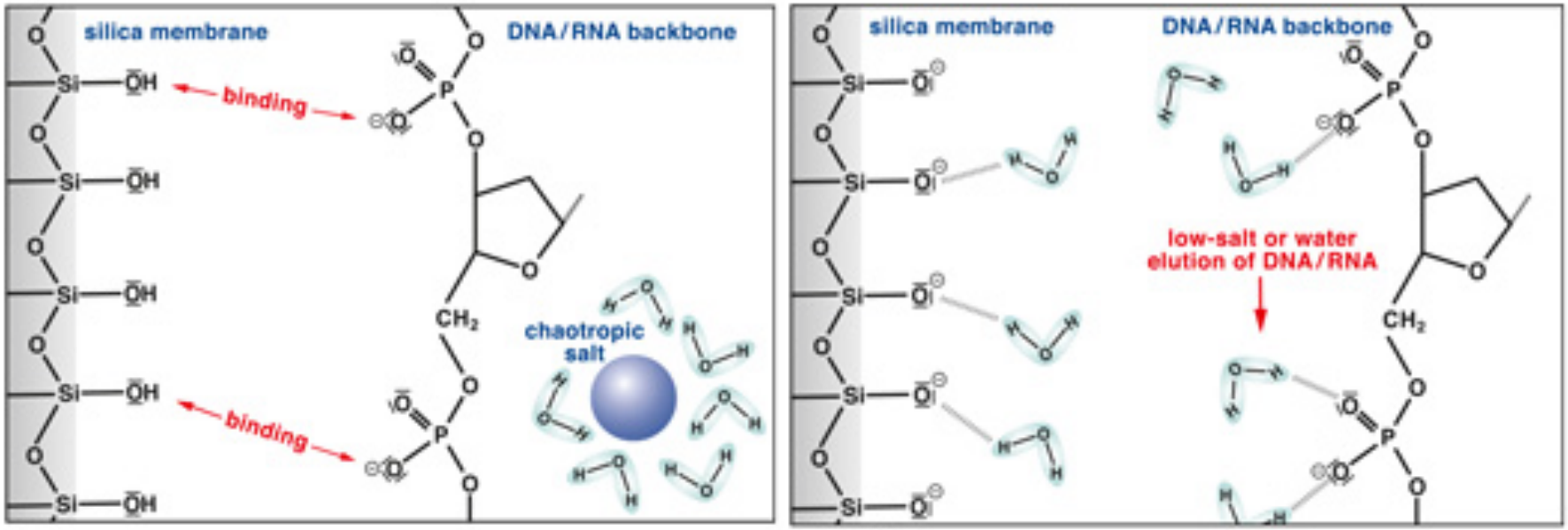
Review of mini-prep: Bind DNA to column



- Washes with PB and PE
  - remove residual contaminants (eluent)
  - maintain DNA onto column

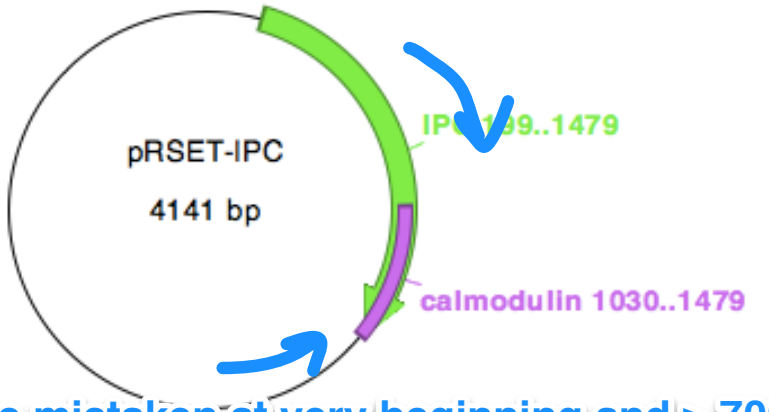
Review of mini-prep: Elution with water

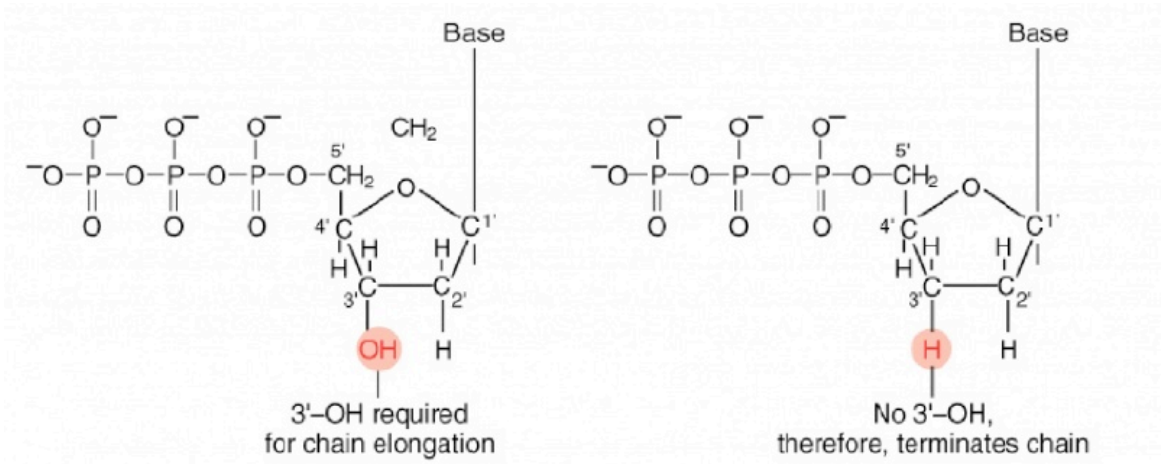
- Water competes DNA off of column



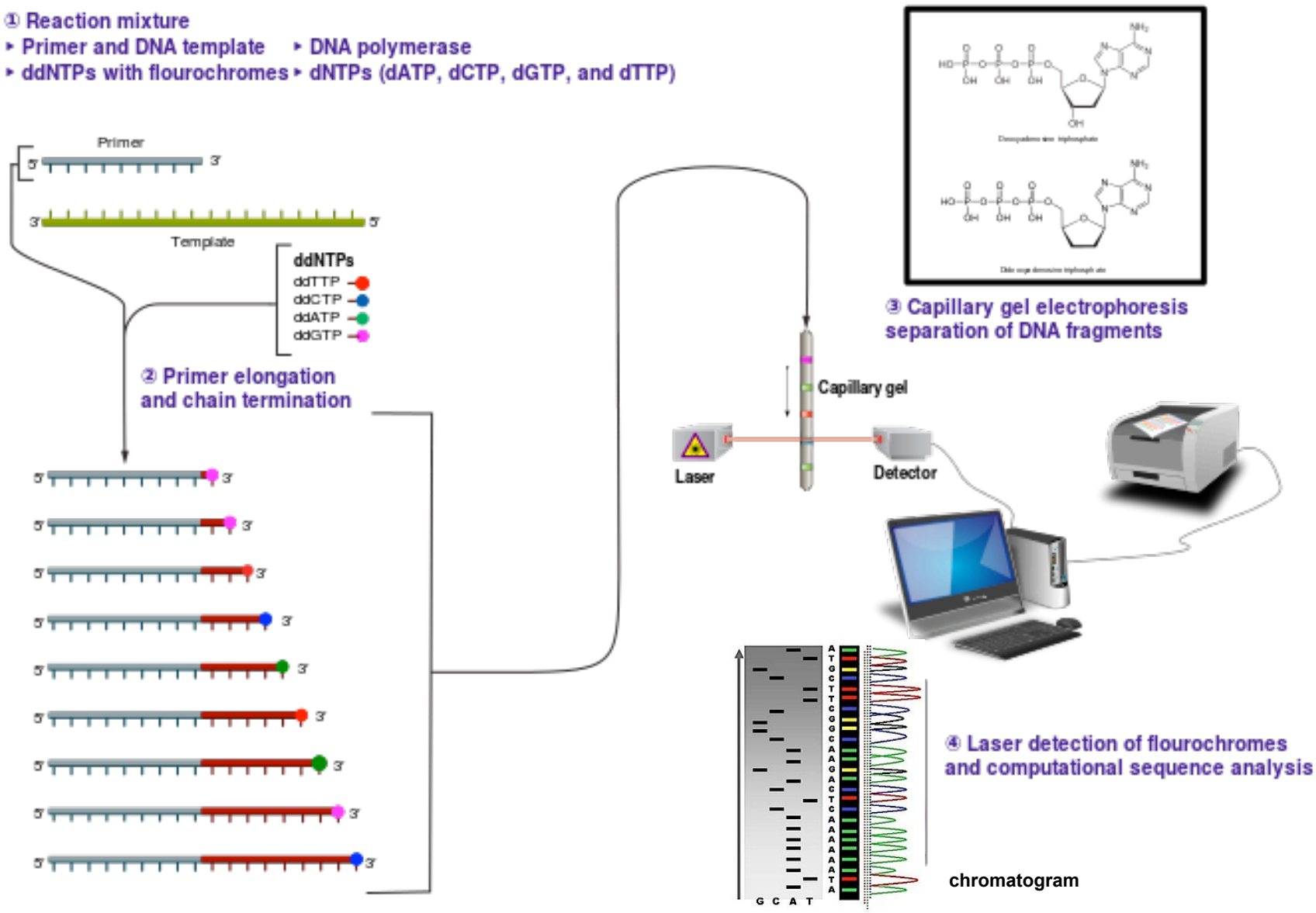
Do we have the intended mutant?

- Diagnostic digests
- Sequencing
  - good to have both F and R primers
    - double-check
    - coverage hence accuracy; polymerase mistaken at very beginning and > 700 bp
  - di-deoxynucleotides terminate elongation





Sanger sequencing by Genewiz

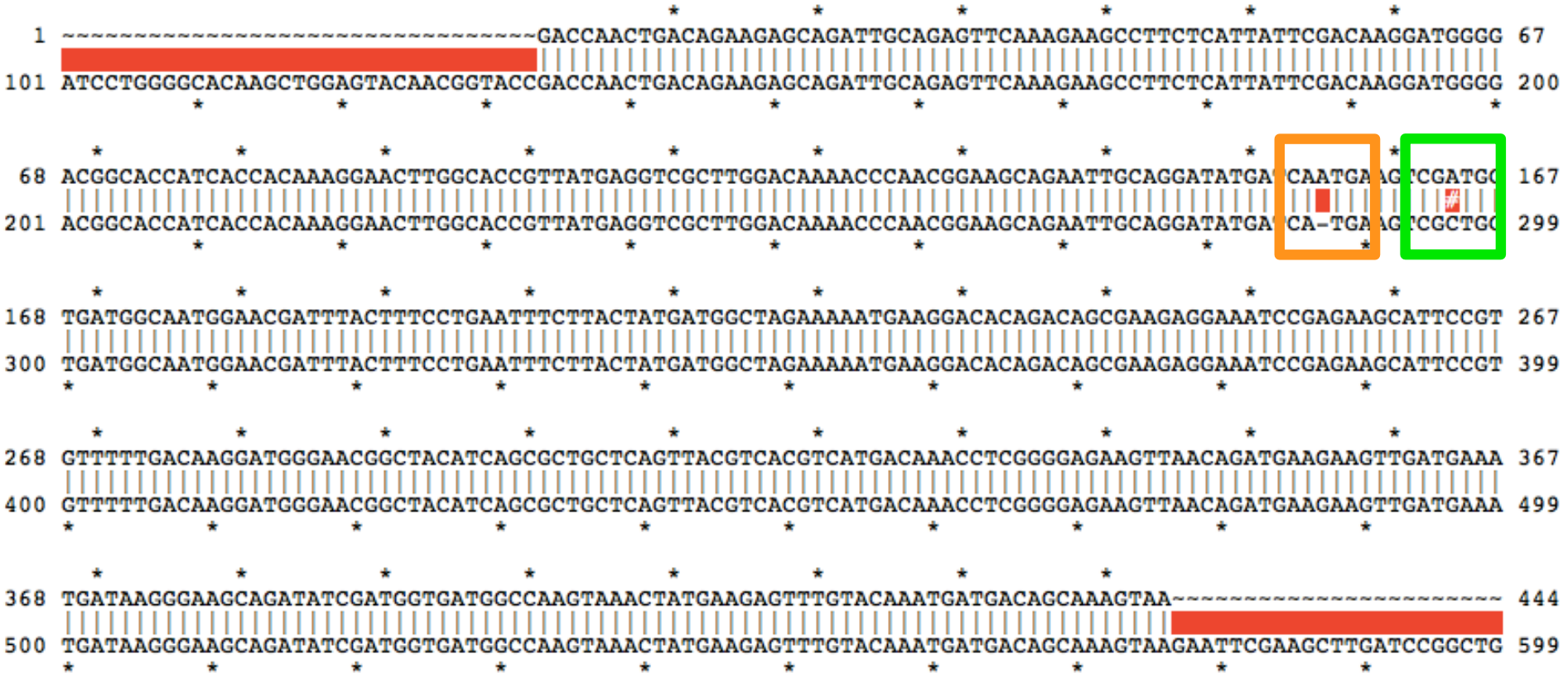


change of reading frame is usually catastrophic

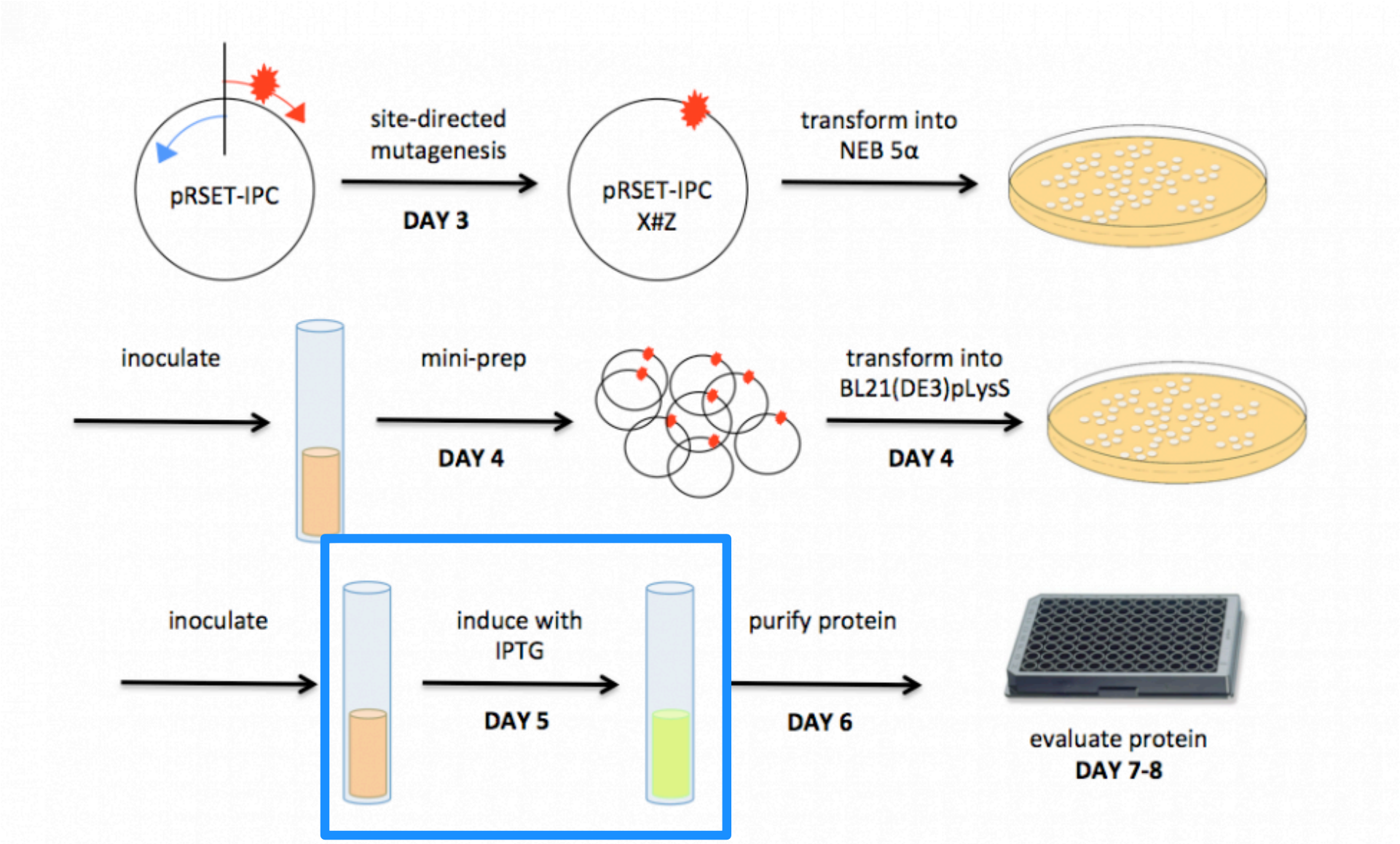
Good or bad?

1 bp intended mutation + 1 bp insertion/deletion

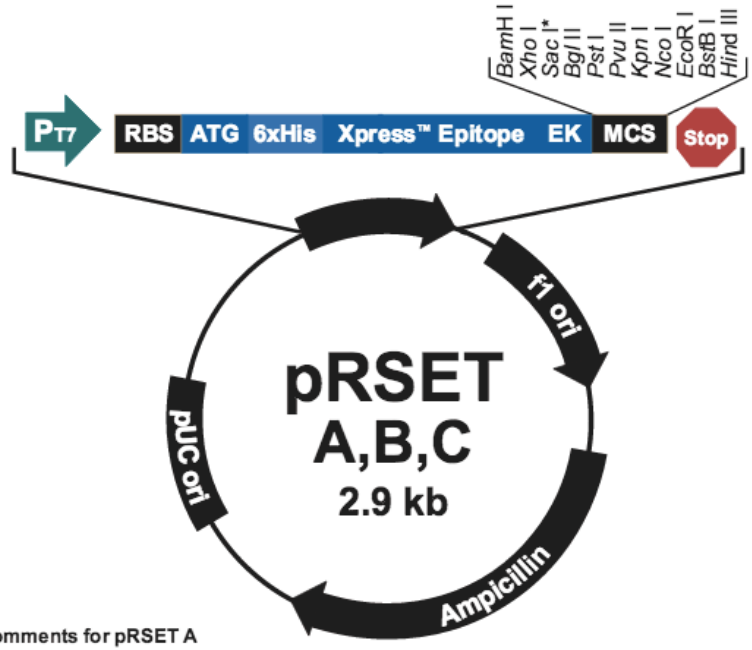
- Align and compare CaM with DNA sequenced by Genewiz
  (top) (bottom)



We're making progress… and proteins today!



BL21(DE3)pLysS competent cells



Comments for pRSET A
2897 nucleotides

*Version C does not contain *Sac* I

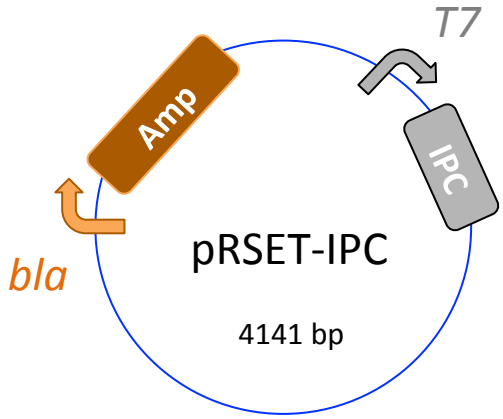
T7 promoter: bases 20-39
6xHis tag: bases 112-129
T7 gene 10 leader: bases 133-162
Xpress™ epitope: bases 169-192
Multiple cloning site: bases 202-248
T7 reverse priming site: bases 295-314
T7 transcription terminator: bases 256-385
f1 origin: bases 456-911
bla promoter: bases 943-1047
Ampicillin (*bla*) resistance gene (ORF): bases 1042-1902
pUC origin: bases 2047-2720 (C)

- BL21: E. coli bacterial strain
- can express IPC protein
  - induction by lactose or analog: isopropyl β-D-thiogalactoside
  - under T7 promoter control in pRSET vector
- DE3: bacteriophage (virus)
  - used to integrate the *lac*/T7RNAP construct into *E. coli*
- pLysS: protein that produces
  - lysosyme, which binds to T7RNAP, reducing basal "leaky" expression
  - retained by chloramphenicol (Cam) selection

Let's piece together this "protein induction" story

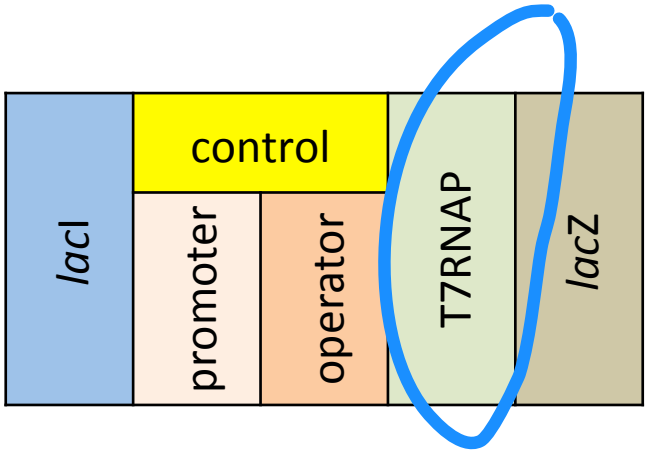
① in the pRSET plasmid

- bla promoter is constitutively *on*
- T7 promoter is turned *on* in the presence of T7 RNA polymerase



② in BL21(DE3)pLysS

- T7RNAP gene engineered in DE3 cells under a modified *lac* operon control
- *lac*I encodes a ___repressor___ that binds to ___control area___ , thereby turning it *off*
- in addition, T7 lysosyme inactivates T7 promoter



genes of the lac operon

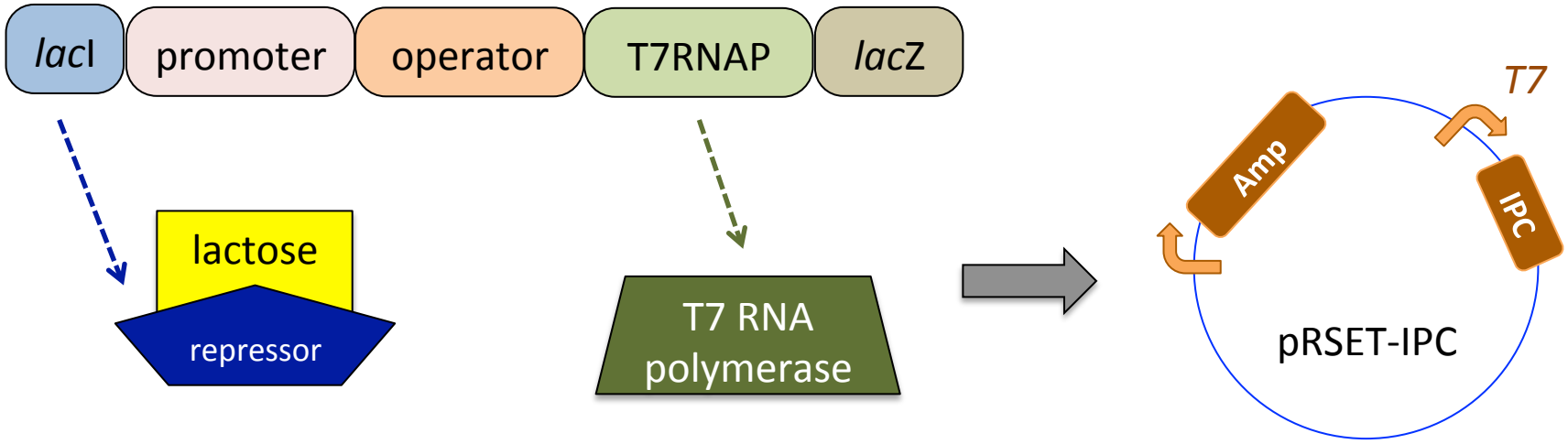
Let's piece together this "protein induction" story

① in the pRSET plasmid, T7 promoter *on* only if T7RNAP present

② in BL21(DE3)pLysS, *lac*I => repressor binds control area => T7RNAP turned *off*

③ if lactose is present

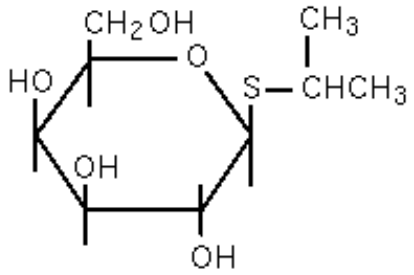
- lactose binds to repressor and makes it inactive , thus turning on expression of T7RNAP
- with T7RNAP present, the T7 promoter is on , and IPC expressed



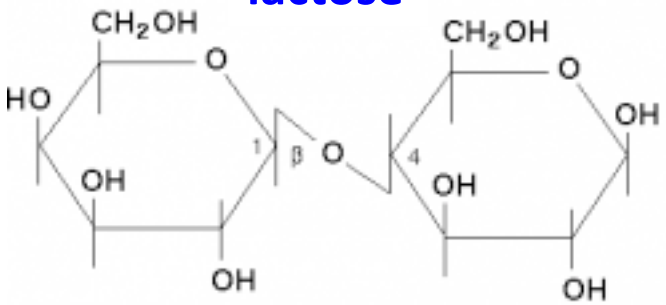
IPTG is a lactose analogue

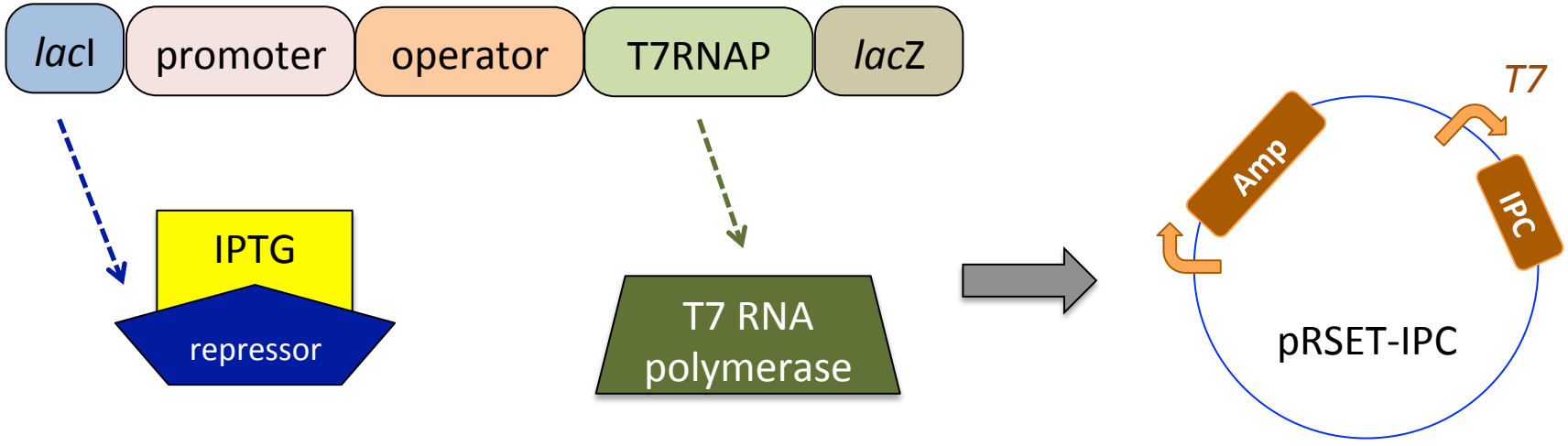
- isopropyl β-D-1-thiogalactoside
- structural mimic of lactose
- unlike lactose, IPTG is not cleaved by β-galactosidase and so will not used by the cell
  ➔ [IPTG] constant

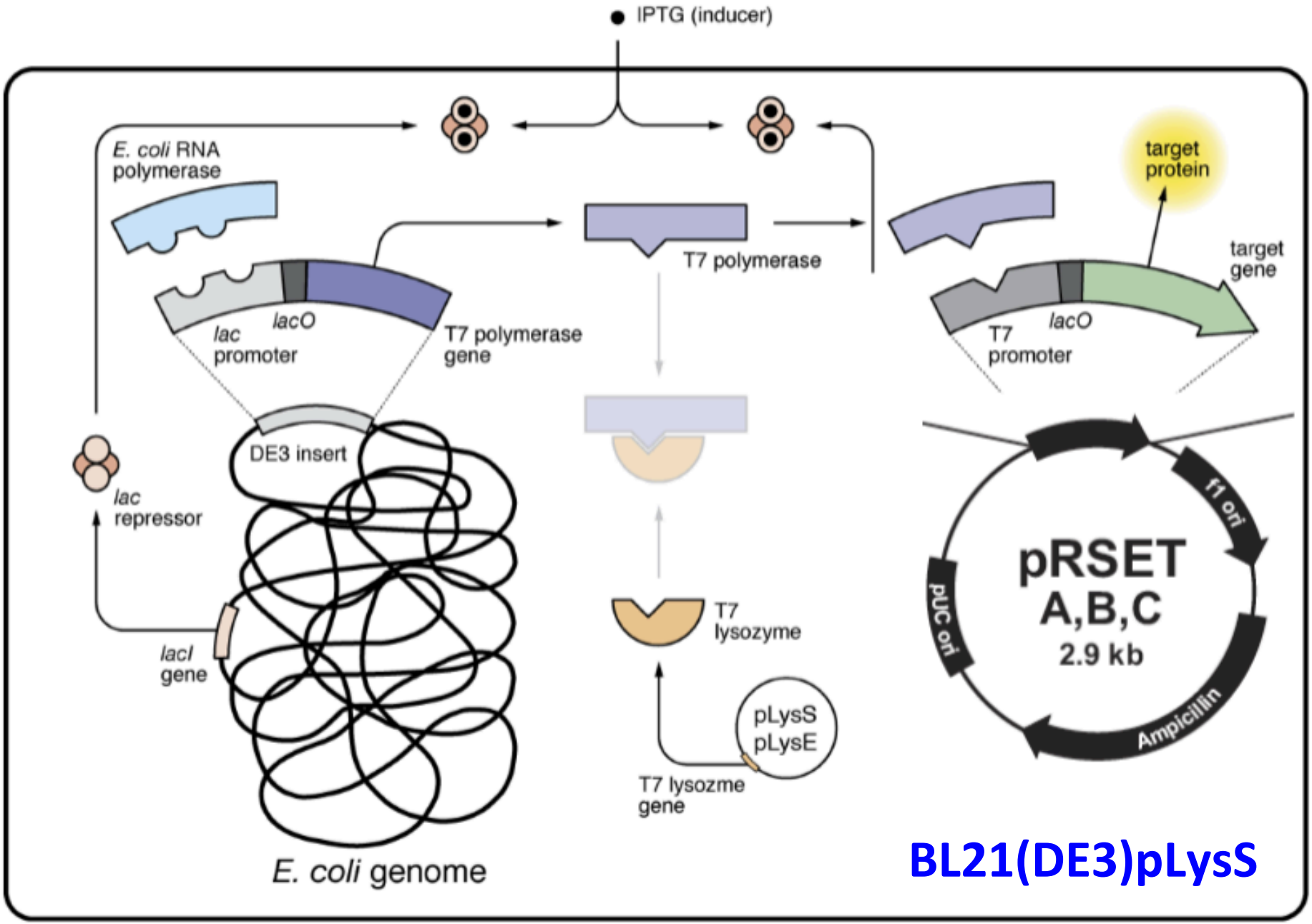
IPTG



lactose



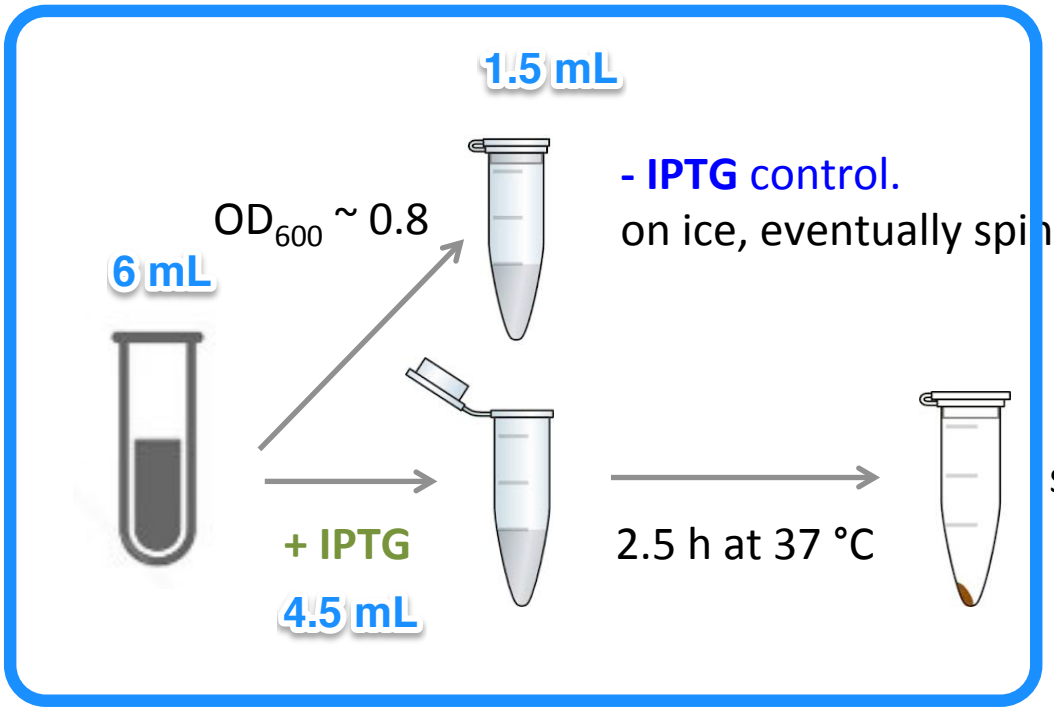



IPTG (inducer)
E. coli RNA polymerase
lac promoter
lacO
T7 polymerase gene
T7 polymerase
target protein
target gene
T7 promoter
lacO
DE3 insert
lac repressor
lacI gene
E. coli genome
T7 lysozyme
pLysS pLysE
T7 lysozme gene
pRSET A,B,C
2.9 kb
f1 ori
pUC ori
Ampicillin
BL21(DE3)pLysS

Start with Part 1 of the lab right away

Induce IPC protein expression

- for three samples: X#Z #1, X#Z #2, and wt inverse pericam

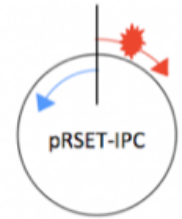


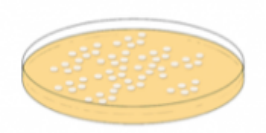
spin to check pellet color

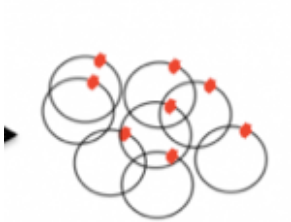
if white, grow overnight, faculty will pellet & freeze

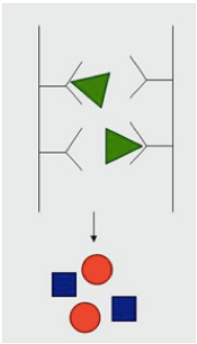
if green/yellow, measure all OD600 values, and spin down

3 mL









Tips to write Methods (due M1**D6**)

- Use subsections with descriptive titles
  - Put in logical order
  - Begin with topic sentence to introduce purpose
- Use clear and concise full sentences
  - Eliminate tables and lists
  - Passive voice expected
- Use the most flexible units
  - Write concentrations (when known) rather than volumes
- Eliminate ‘109 specific details
  - Do not include details about tubes and water!
  - Assume reader has some microbiology experience

Improving your Methods [1]

Inverse pericam (IPC) template

primers (name, 5' NNN 3', concentration)

Template DNA (5 µL) and primers were mixed with 20 µL of

(final concentration of template)

2.5X Master Mix in a PCR tube. Water was added to 50 µL.

using 1X Master Mix (manufacturer's name, city, state/country)

A tube without template was prepared and labeled control.

A no-template control was prepared.

Improving your Methods [2]

1.5 mL

genotype, add OD600

A liquid bacteria culture was pelleted and the DNA was purified

volume

using a Qiagen kit.

QIAquick mini-prep kit (Qiagen, Hilden, Germany)

Improved Methods

[1] ***PCR amplification of inverse pericam (IPC)***

Inverse pericam (IPC) was amplified from a pcDNA3-IPC template (5 ng/uL) with 2 pmol/uL IPC-forward (5' NNN 3') and IPC-reverse (5' NNN 3') primers, using 1X MasterMix (company, city, state/country) and the following thermocycler conditions: initial denaturation at 98°C for 30 s, 25 cycles of amplification (melt at 98°C for 10 s, anneal at 55°C for 30 s, extend at 72°C for 2 min), final extension at 72°C for 2min.

[2] ***Site-directed mutagenesis and amplification of the pRSET-IPC plasmid***

Introductory topic sentence. SDM (including KLD, transformation in NEB5α)

The DNA of a 1.5 mL of NEB 5alpha (genotype: *fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*) overnight** culture was collected using a QIAquick mini-prep kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol with a final elution in 30 µL of distilled water pH 8.0.

Confirmation of intended mutation by sequencing (Genewiz, Cambridge, MA)

**grown to saturation (as opposed to exponential growth phase for transformation or induction of protein)

In lab today

- IPTG induction of IPC protein expression
- Prelab
- Analysis of DNA sequences
- Count mutant colonies (in BL21)
- Measure OD_{600} of, and spin down six samples
  - wt IPC 1.5 mL – IPTG 3 mL + IPTG
  - X#Z #1 1.5 mL – IPTG 3 mL + IPTG
  - X#Z #2 1.5 mL – IPTG 3 mL + IPTG