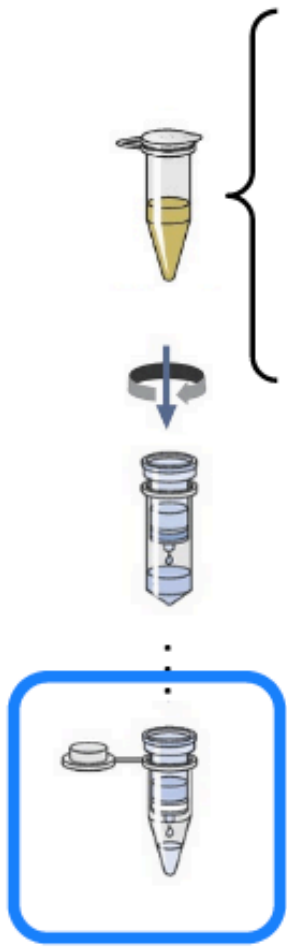


M1D5: Induce protein expression

02/24/2016

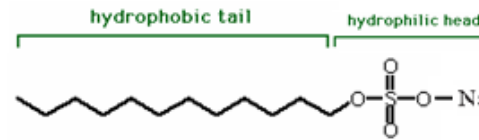
Mini-prep to isolate DNA using QIAprep kit by Qiagen



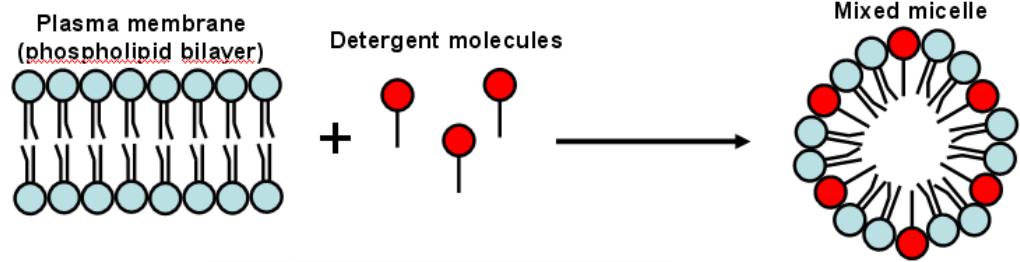
steps	contents	purpose
prepare P1	Tris/EDTA buffer RNase	resuspend NEB 5alpha cells weaken the cell walls
lyse P2	SDS surfactant NaOH alkaline lysis	solubilize proteins, denature DNA
neutralize N3	acetic acid, chaotropic salt, potassium acetate	short (plasmid) renature long DNA (rest of E. Coli) precipitate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

Review of mini-prep: Lysis with SDS/NaOH

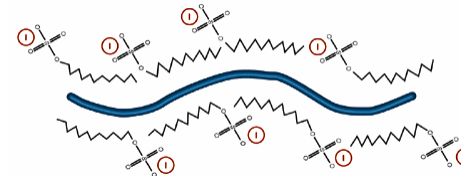
1. Sodium dodecyl sulfate (SDS)



- dissolves membranes

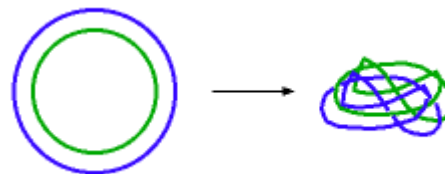


- binds to and denatures proteins



2. Sodium hydroxide (NaOH)

- denatures DNA

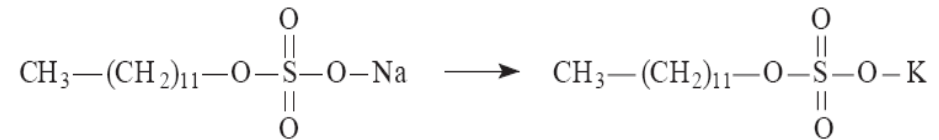


Because plasmids are supercoiled, both DNA strands remain entangled after denaturation

Review of mini-prep: Neutralization

1. Acetic acid / potassium acetate solution

- neutralizes NaOH (renatures plasmid DNA)
- converts soluble SDS into insoluble PDS (white fluff)



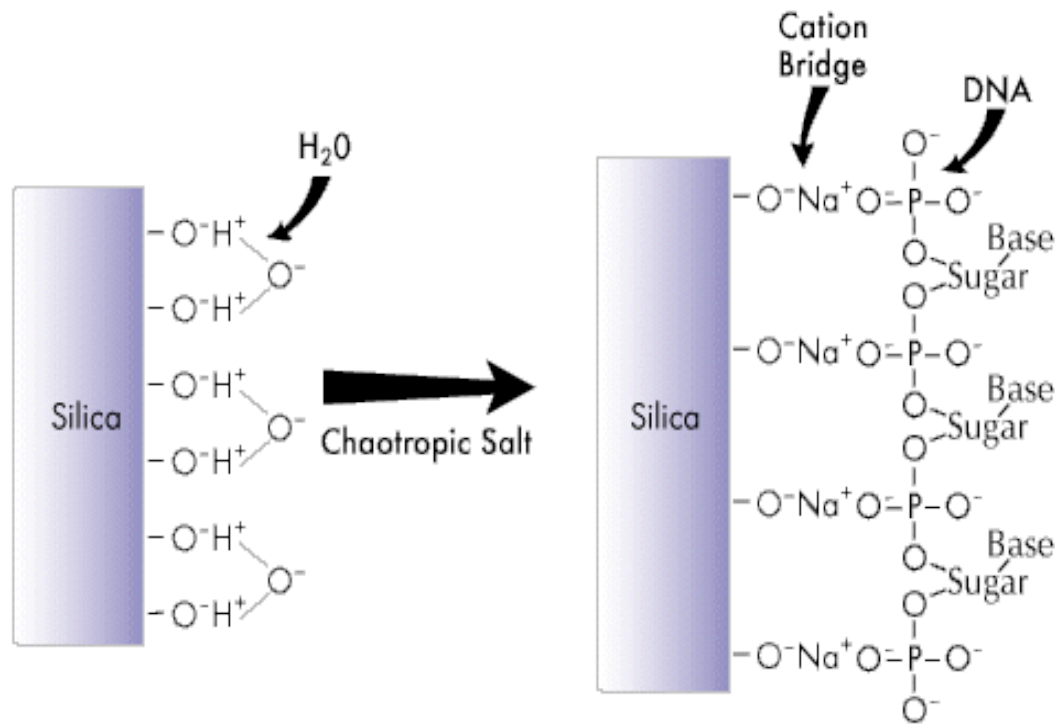
2. Chaotropic salt

- facilitates DNA binding to silica

➤ After centrifugation

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

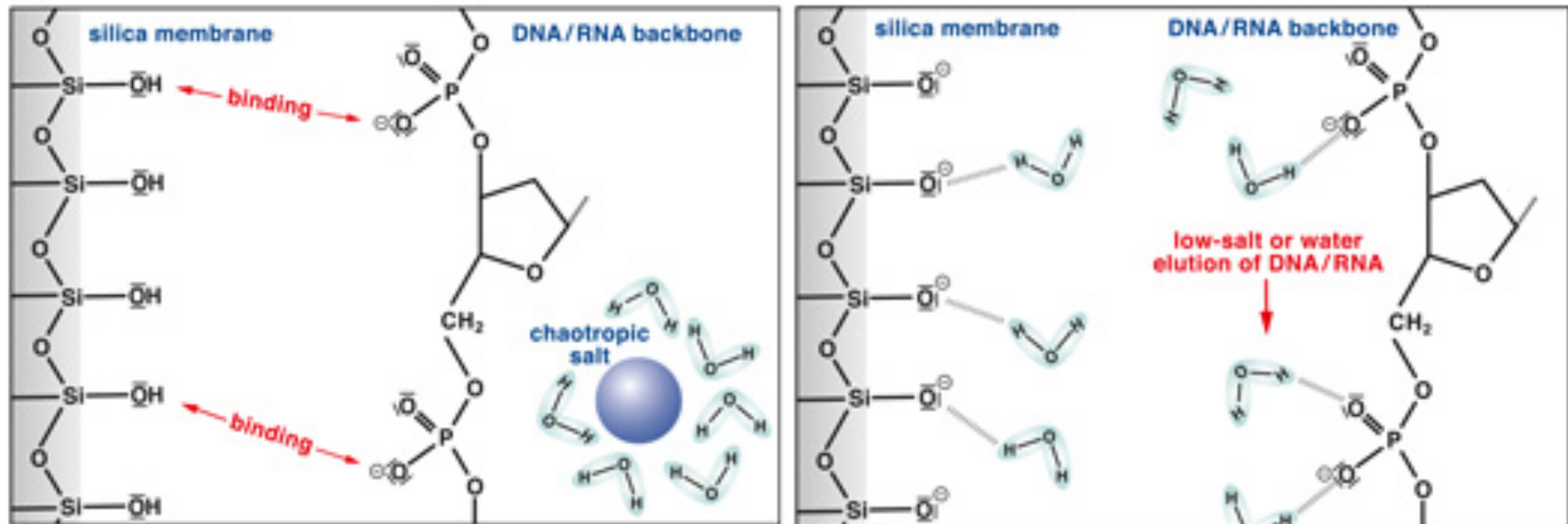
Review of mini-prep: Bind DNA to column



- 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

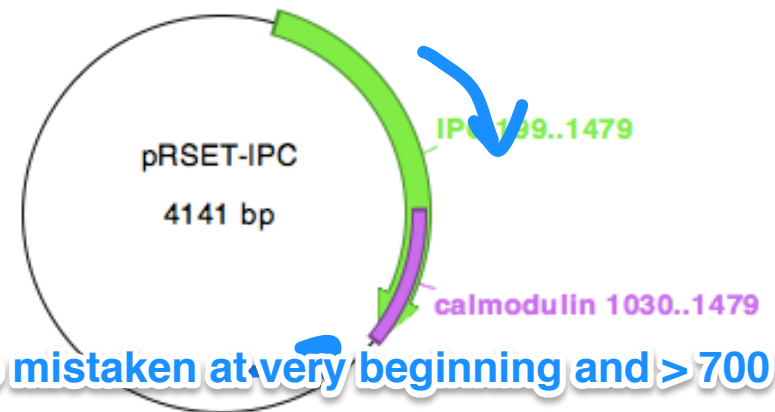


Do we have the intended mutant?

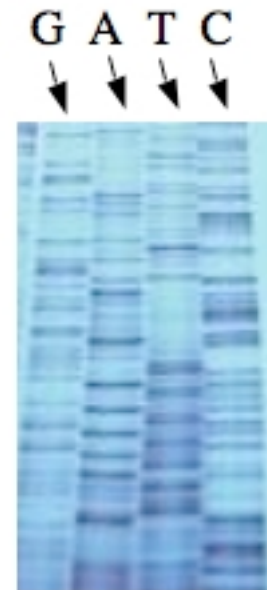
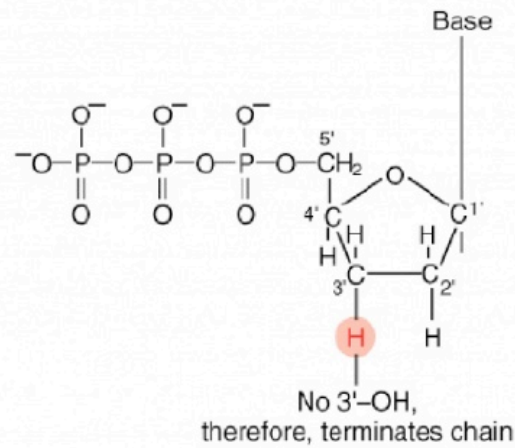
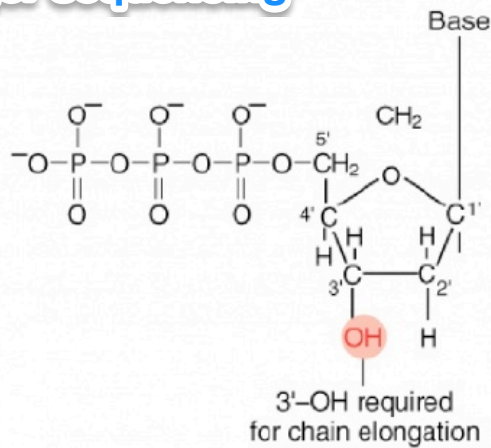
- Diagnostic digests
- Sequencing
 - good to have both F and R primers

coverage hence accuracy; polymerase mistaken at very beginning and > 700 bp double-check

- di-deoxynucleotides terminate elongation



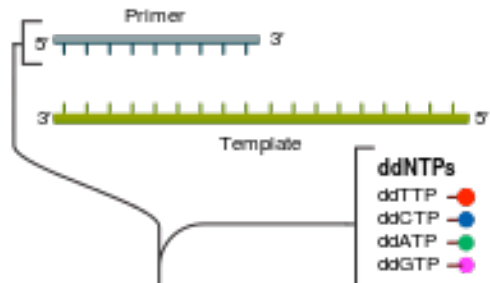
Sanger sequencing



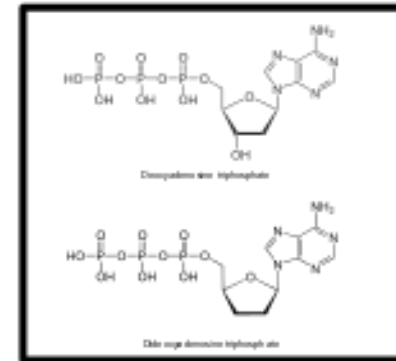
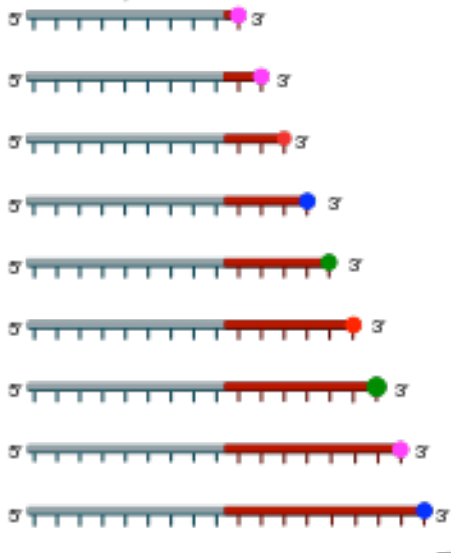
Sanger sequencing by Genewiz

① Reaction mixture

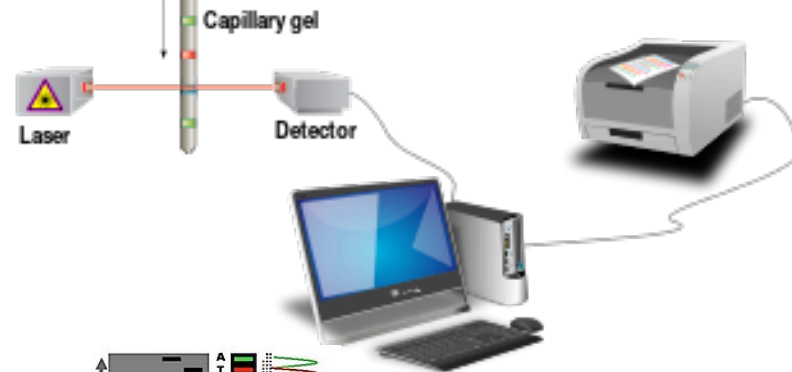
- ▶ Primer and DNA template
- ▶ DNA polymerase
- ▶ ddNTPs with flouorchromes
- ▶ dNTPs (dATP, dCTP, dGTP, and dTTP)



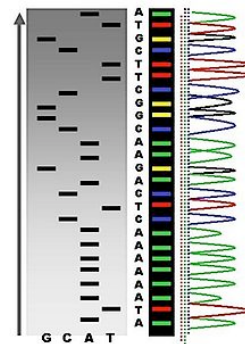
② Primer elongation and chain termination



③ Capillary gel electrophoresis separation of DNA fragments



④ Laser detection of flouorchromes and computational sequence analysis



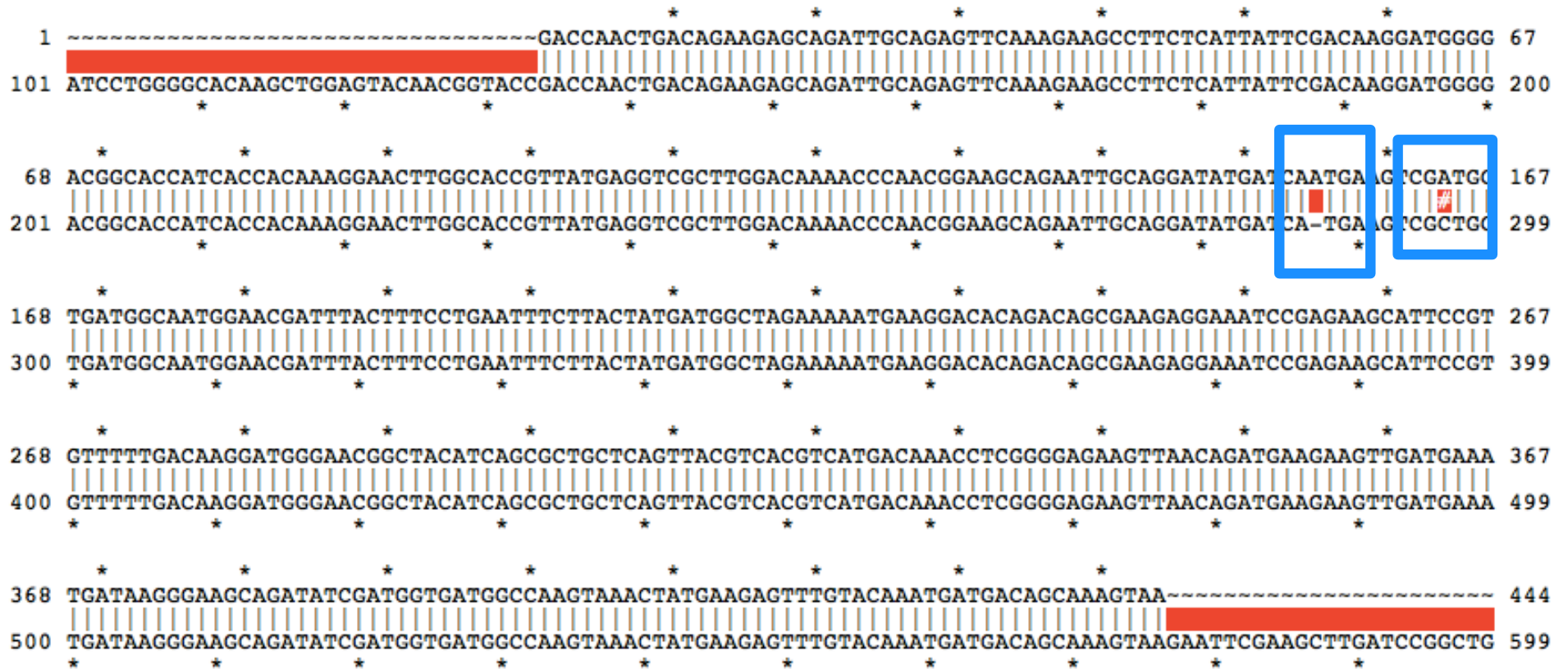
chromatogram

change of reading frame is catastrophic

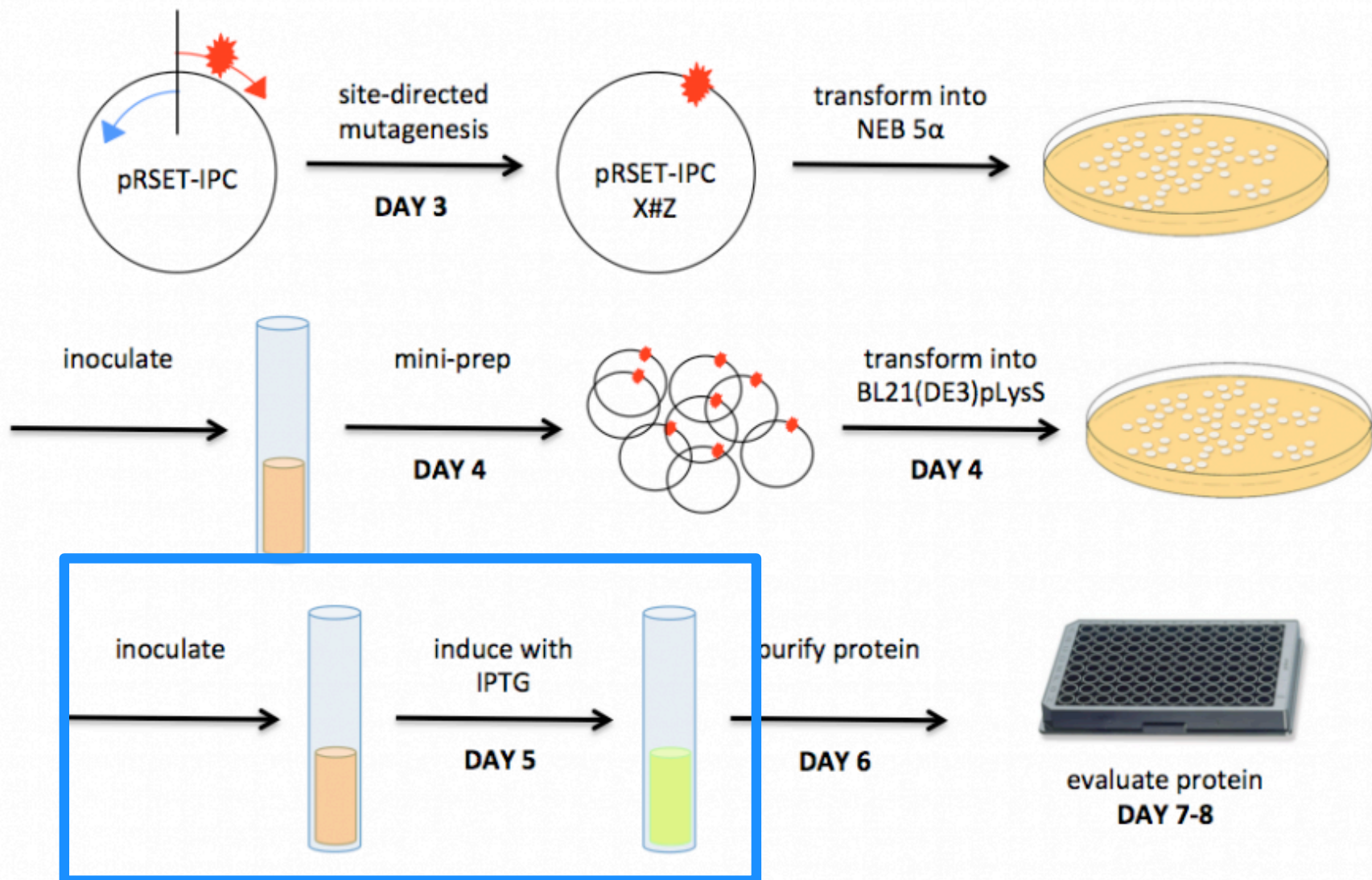
Good or bad?

1 bp intended mutation + 1 bp insertion/deletion

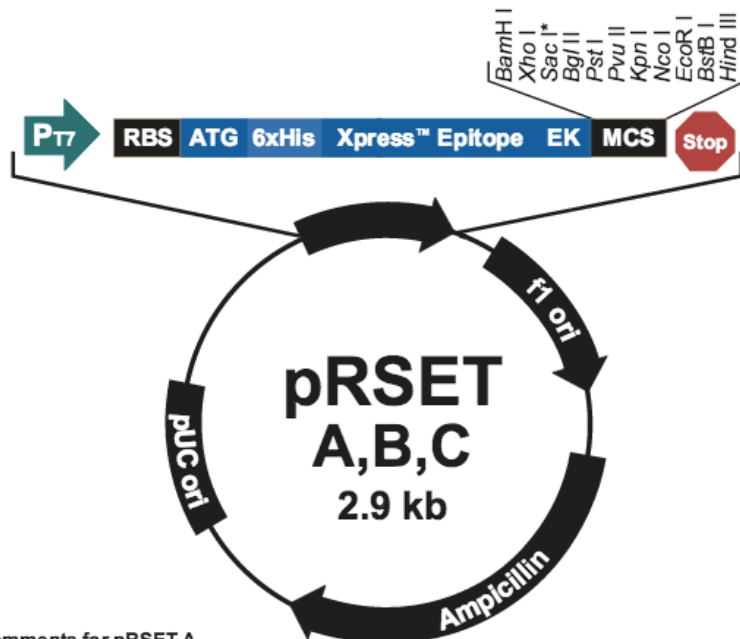
- Align and compare CaM with DNA sequenced by Genewiz (top) (bottom)



We're making progress... and proteins today!



BL21(DE3)pLysS competent cells



Comments for pRSET A
2897 nucleotides

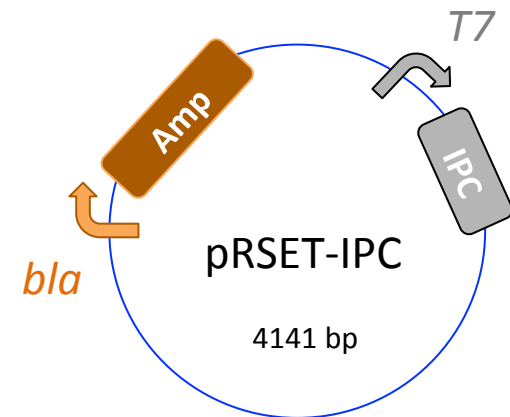
*Version C does not contain Sac I

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)

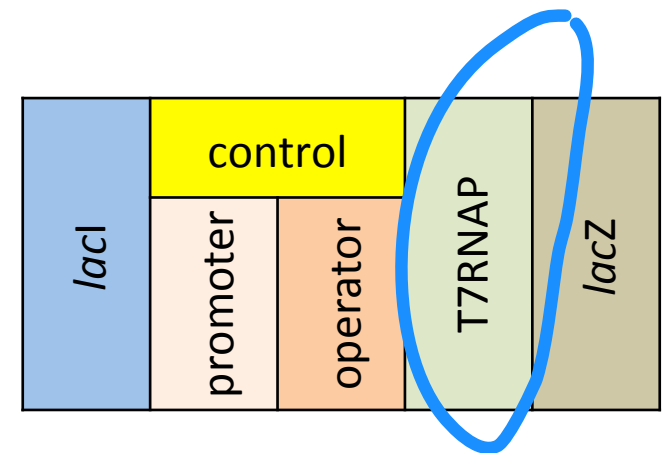
- BL21: **E. coli** bacterial strain
- can express IPC protein
 - induction by lactose or analog: isopropyl β-D-thiogalactoside
 - under **T7** promoter control in pRSET vector
- DE3: bacteriophage (**virus**)
 - used to integrate the *lac*/T7RNAP construct into *E. coli*
- pLysS: protein that produces
 - 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
 - *lacI* encodes a repressor that binds to control area, thereby turning it *off*
 - in addition, T7 lysosyme inactivates T7 promoter



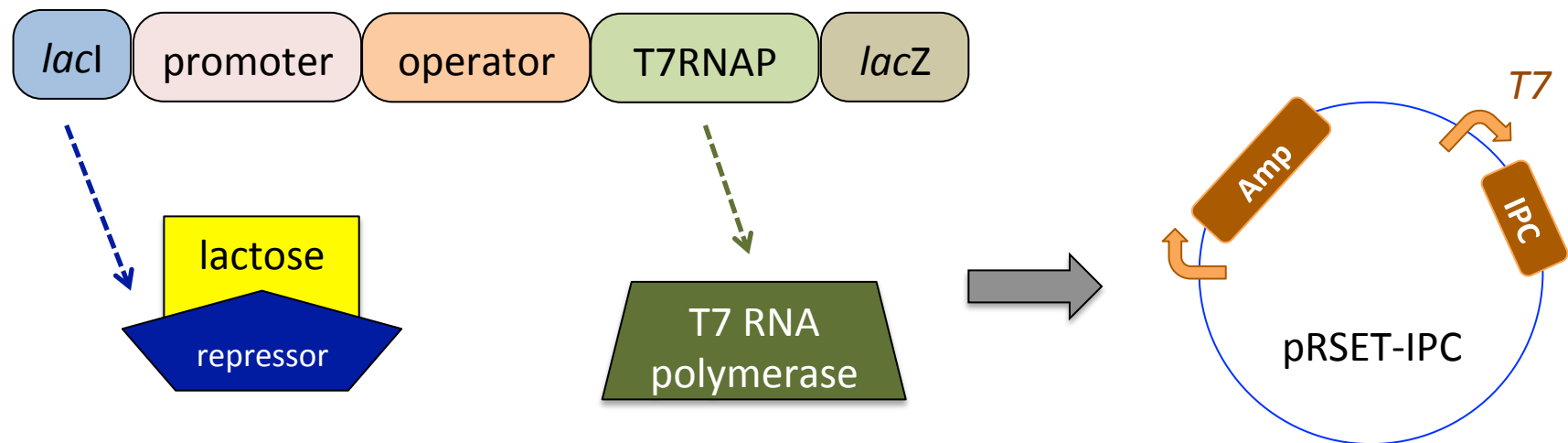
genes of the lac operon

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, *lacI* => repressor binds control area => T7RNAP turned *off*

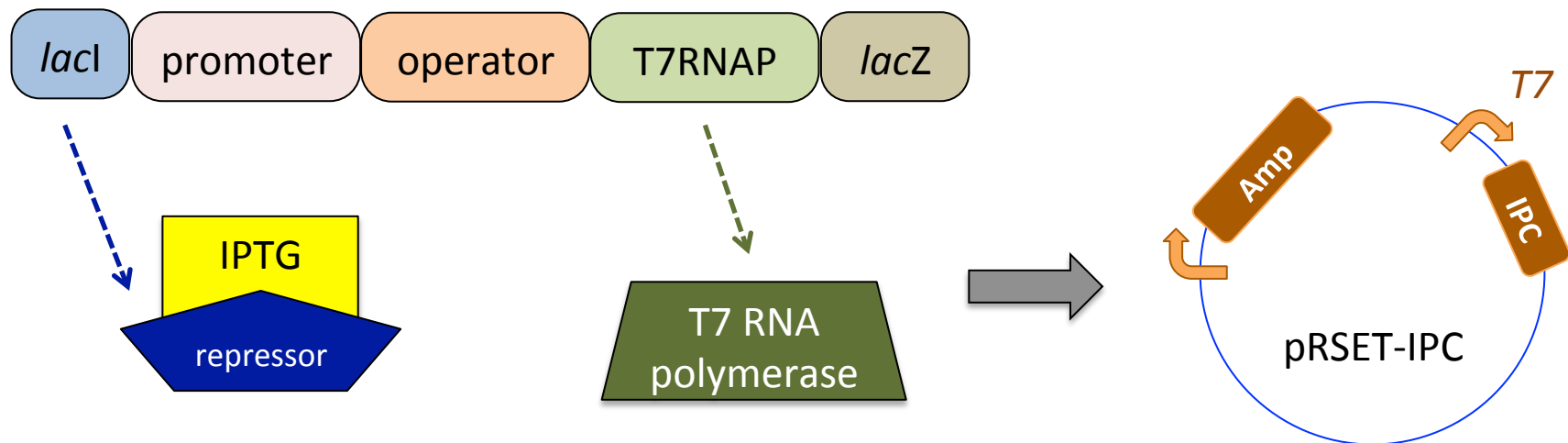
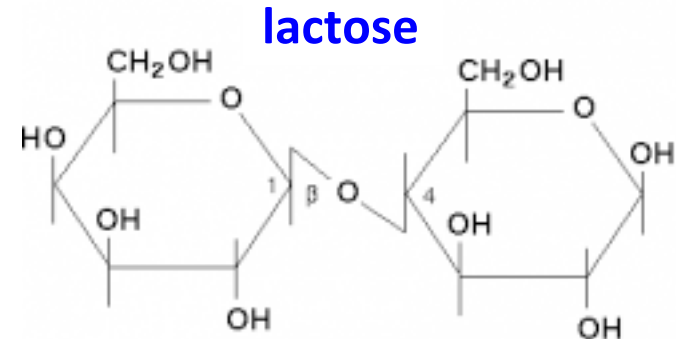
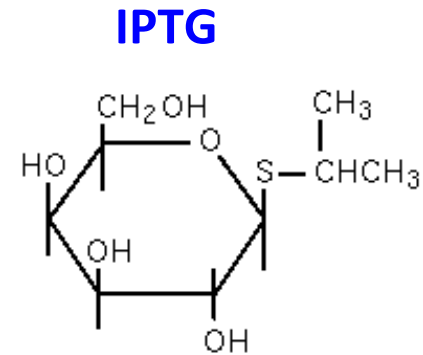
③ if lactose is present

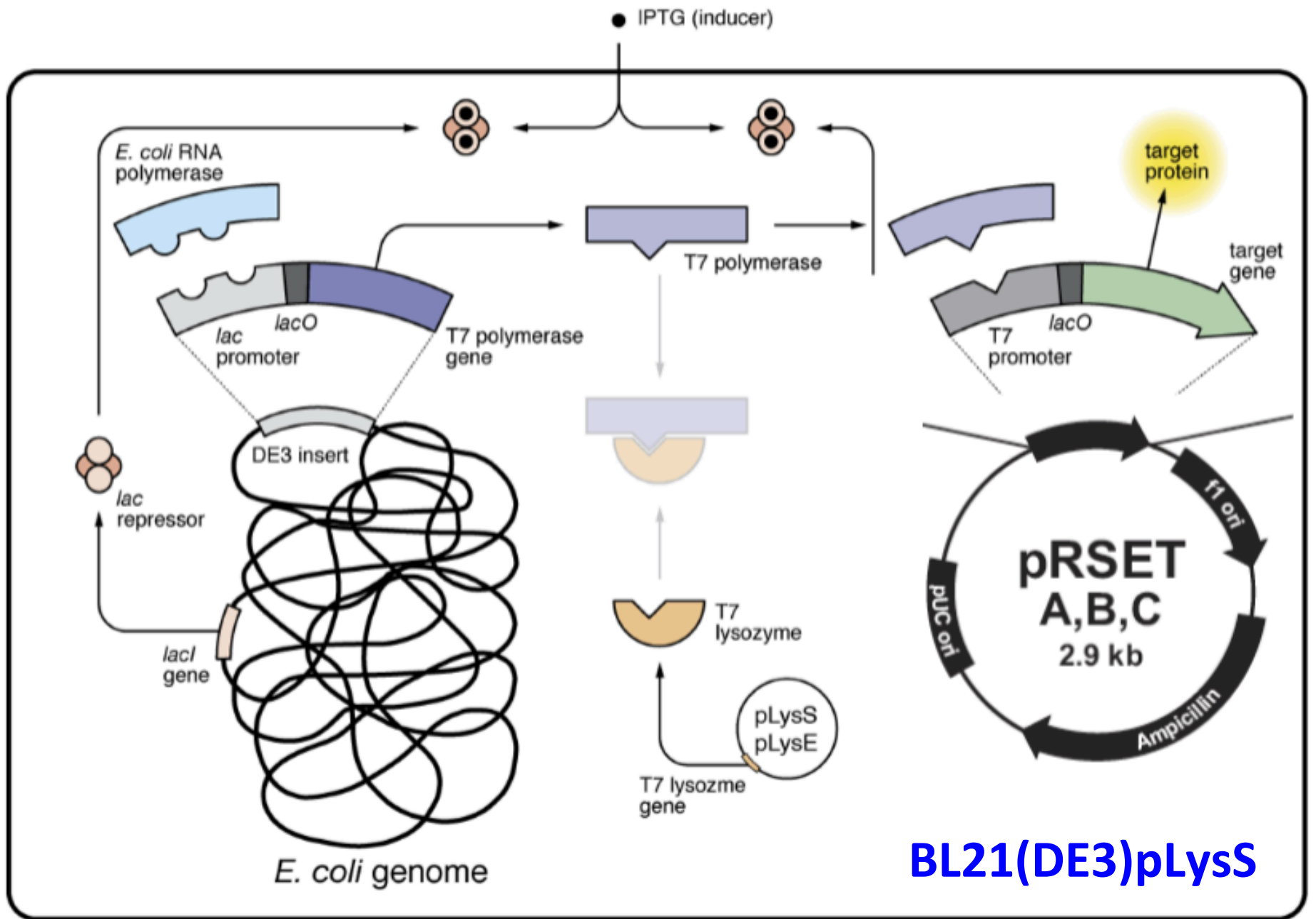
- lactose binds to repressor and makes it inactive, thus turning on expression of T7RNAP
- with T7RNAP present, the T7 promoter is on, and IPC expressed



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 be used by the cell
→ [IPTG] constant

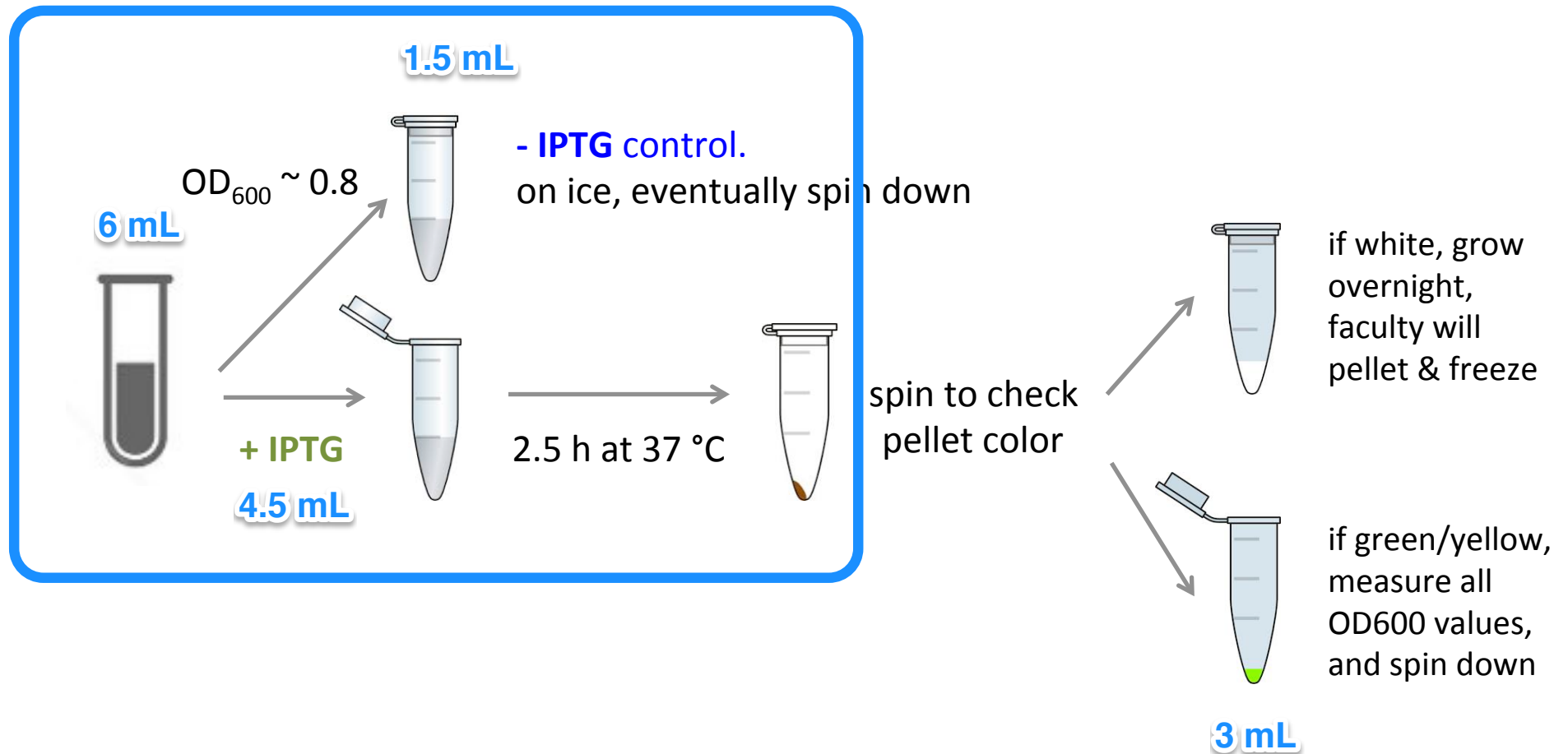




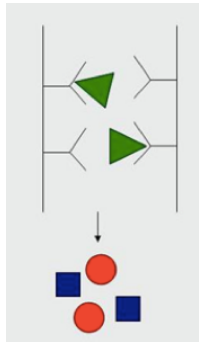
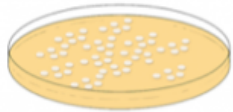
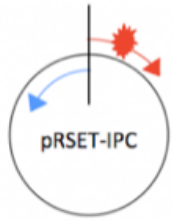
Start with Part 1 of the lab right away

Induce IPC protein expression

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



Tips to write Methods (due M1D6)



- 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 μ L) and primers were mixed with 20 μ L of
(final concentration of template)

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

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

~~A tube without template was prepared and labeled control.~~

A no-template control was performed.

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)

In lab today

- IPTG induction of IPC protein expression
- Prelab
- Analysis of DNA sequences
- Count mutant colonies (in BL21)
- Measure OD₆₀₀ 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