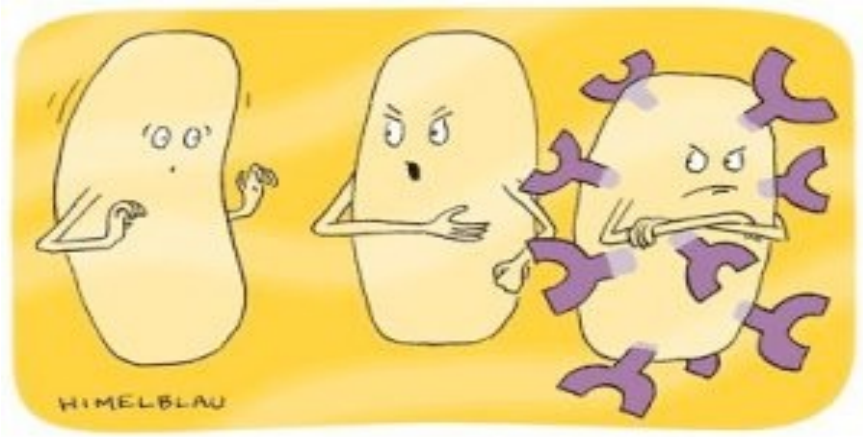
M1D2:
Perform protein purification protocol

1. Prelab discussion
2. Purify MAX-6xHis protein
3. Electrophorese confirmation digest

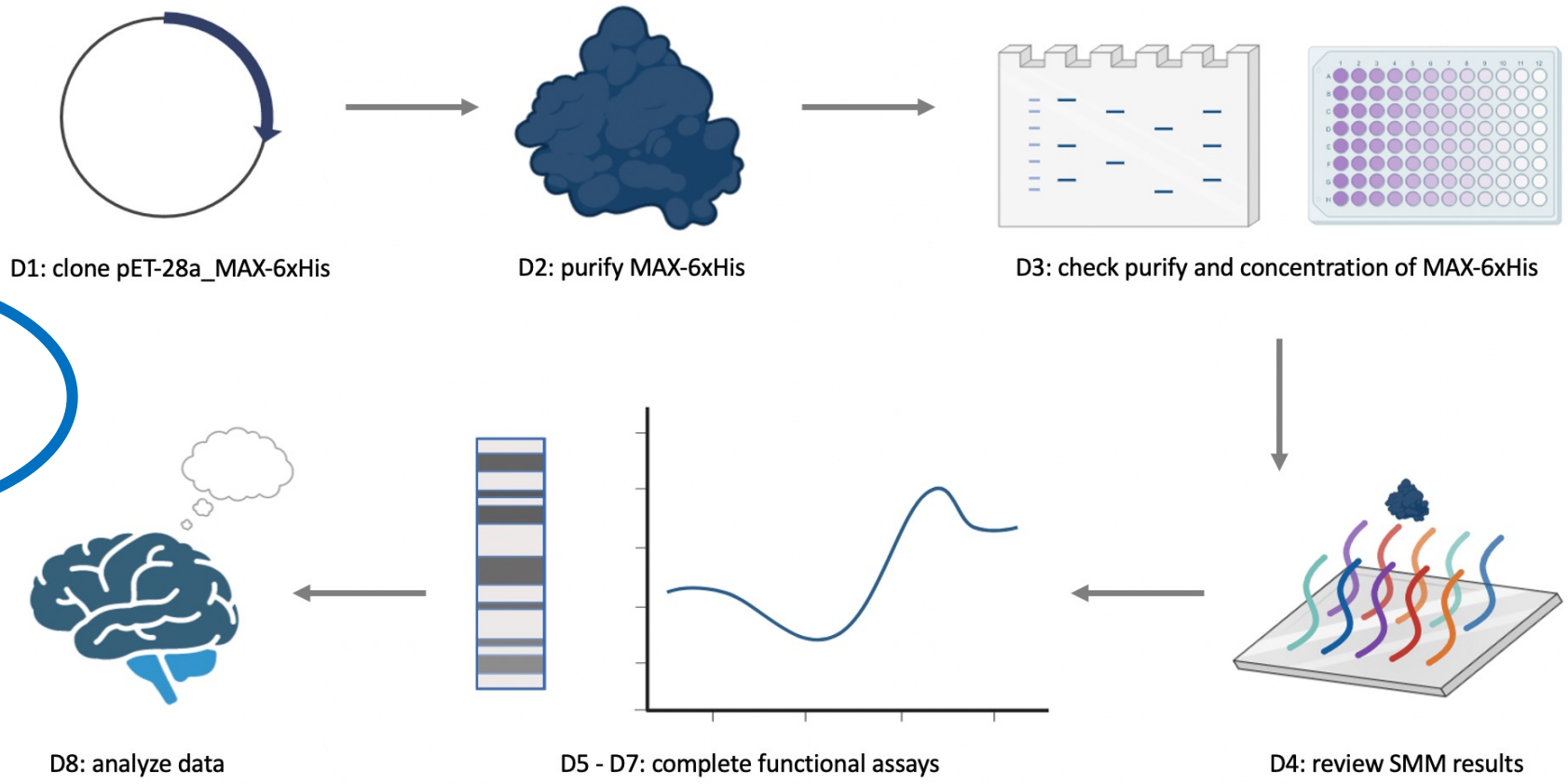


"Don't pick it up," I say, and he says, "It's just a *plasmid*, what harm could it do?" Well just look at him now…who knows *what* protein he's expressing!

Overview of Mod 1 experiments:

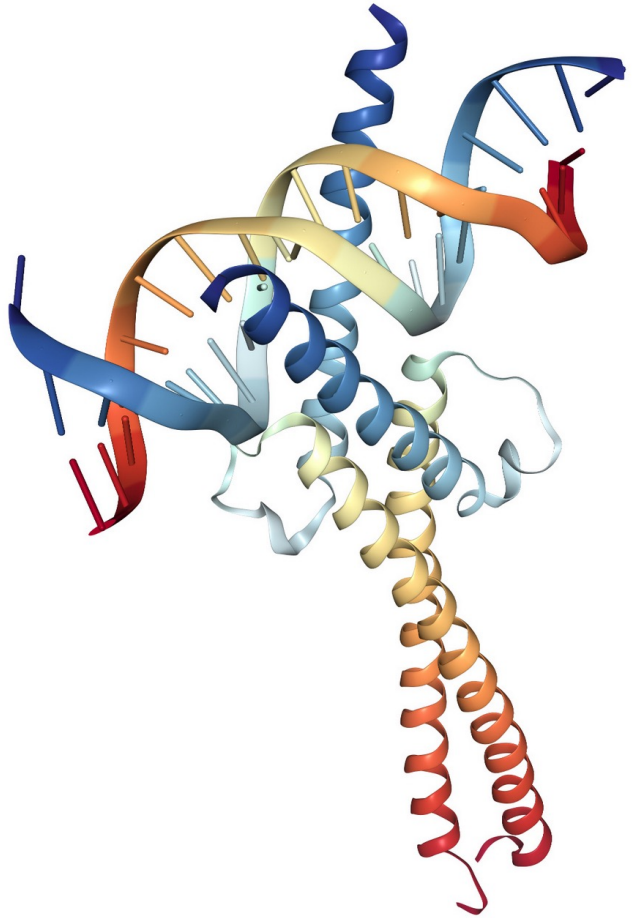
We will talk about this on M1D3 ☺

How is this biological engineering?

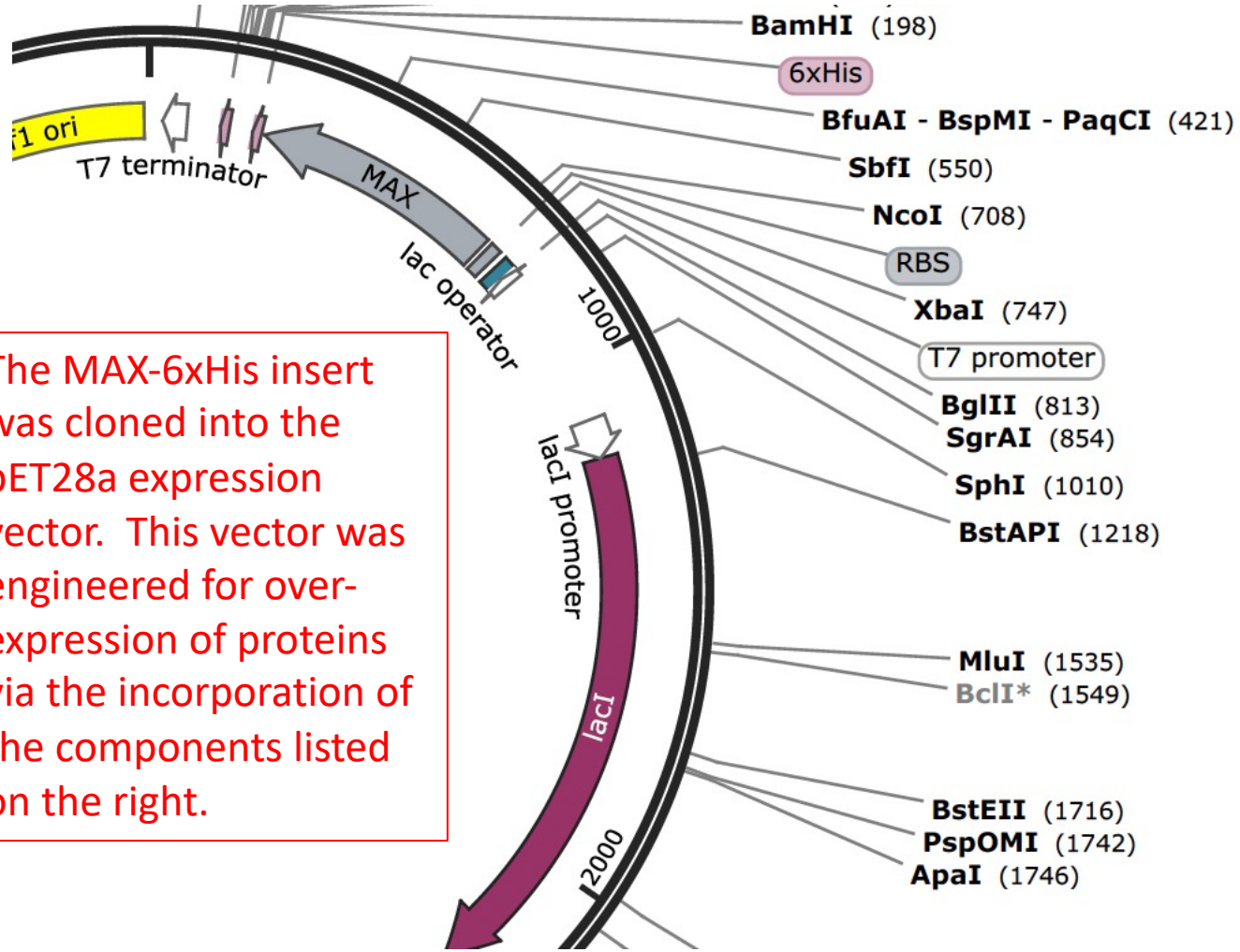


What is our protein of interest?

- MAX functions as a transcription factor
  - Forms homodimers and heterodimers
  - Dimerizes with Myc, which is an oncogenic transcription factor
  - Homodimers and heterodimers compete for binding at promoters to provide regulatory system of target genes



Closer look at pET28a_MAX-6xHis

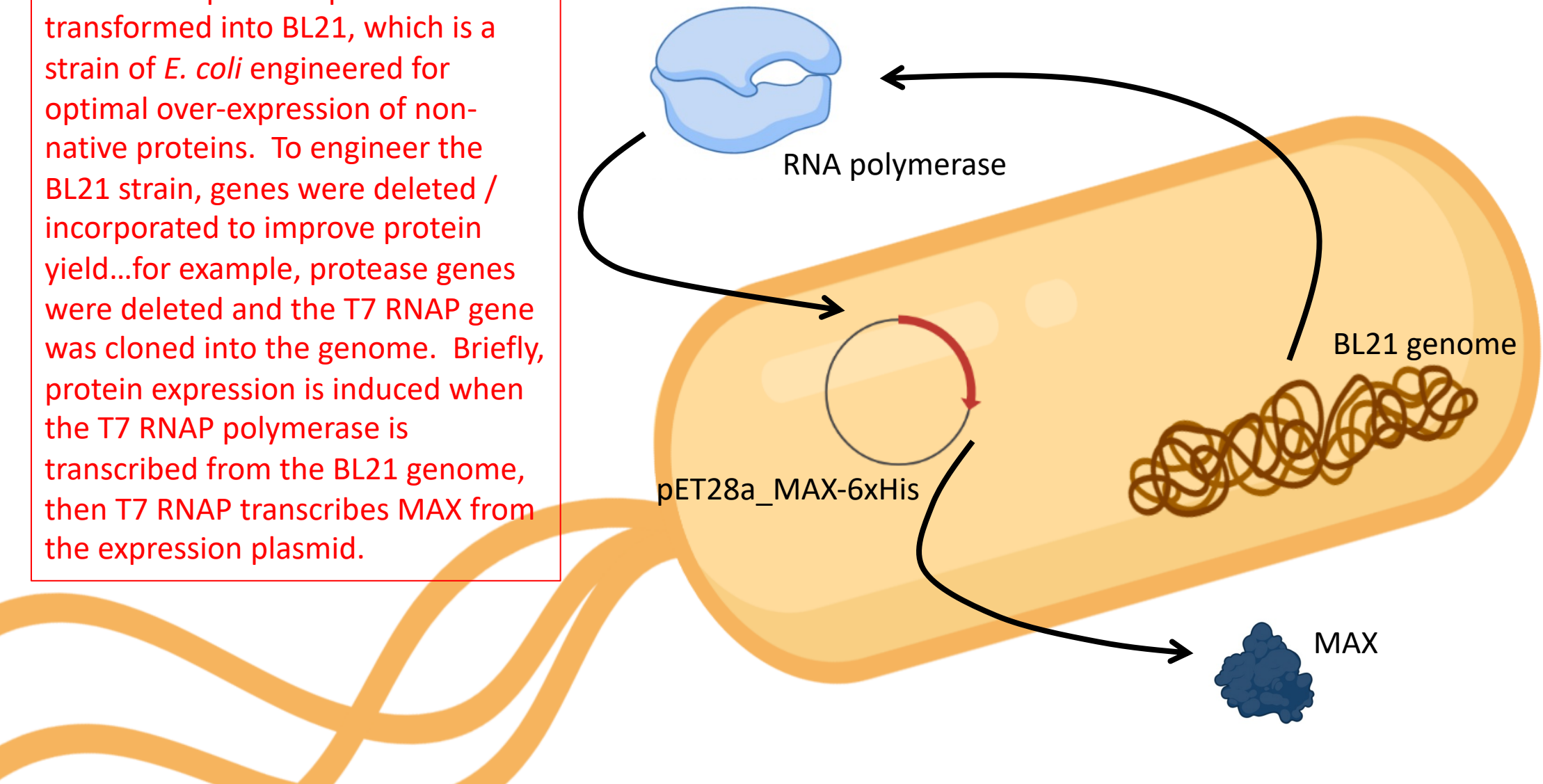


The MAX-6xHis insert was cloned into the pET28a expression vector. This vector was engineered for over-expression of proteins via the incorporation of the components listed on the right.

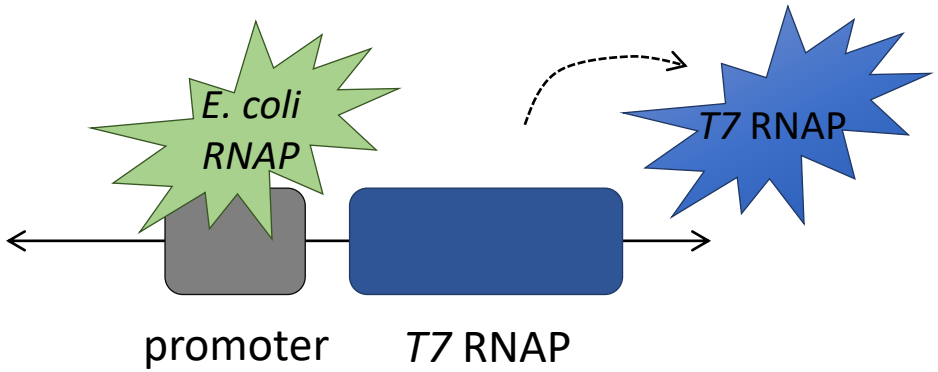
- 6xHis — 'Tag' that will allow us to purify the expressed MAX protein
- *lac* operator — Sequence found in bacterial genomes that is used to control transcription of certain genes
- *lac*I — Gene that encodes LacI, repressor protein that inhibits transcription of certain genes
- T7 promoter — Promoter found in phage that is used to control certain genes
- T7 terminator — Terminator found in phage that is used stop transcription
- RBS — Ribosome binding site sequence

Overview of protein expression system

The MAX expression plasmid was transformed into BL21, which is a strain of *E. coli* engineered for optimal over-expression of non-native proteins. To engineer the BL21 strain, genes were deleted / incorporated to improve protein yield…for example, protease genes were deleted and the T7 RNAP gene was cloned into the genome. Briefly, protein expression is induced when the T7 RNAP polymerase is transcribed from the BL21 genome, then T7 RNAP transcribes MAX from the expression plasmid.



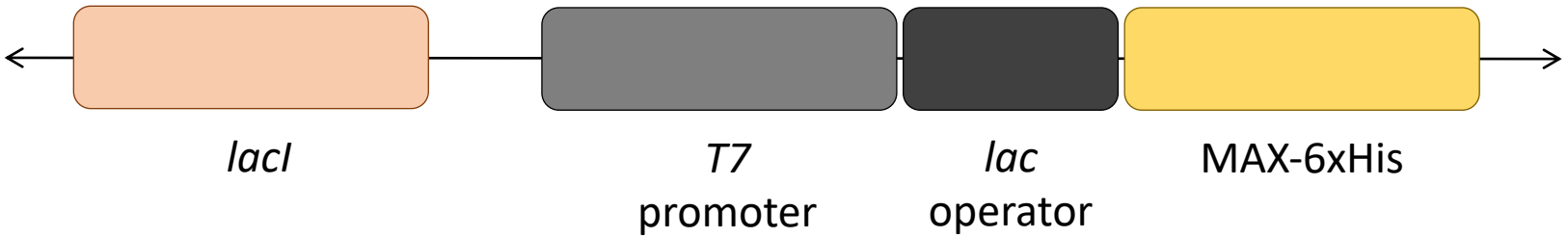
T7 RNA polymerase transcribes MAX-6xHis



E. coli BL21

There are several genetic components needed for the induction system used to express the MAX protein:

- The T7 RNAP is transcribed by the *E. coli* RNAP via a promoter native to the BL21 *E. coli* strain. From this promoter, the T7 RNAP is constitutively expressed.
- Components of the *lac* operon were cloned into the pET28a expression vector to control expression of a protein-of-interest, in our case this is MAX-6xHis.



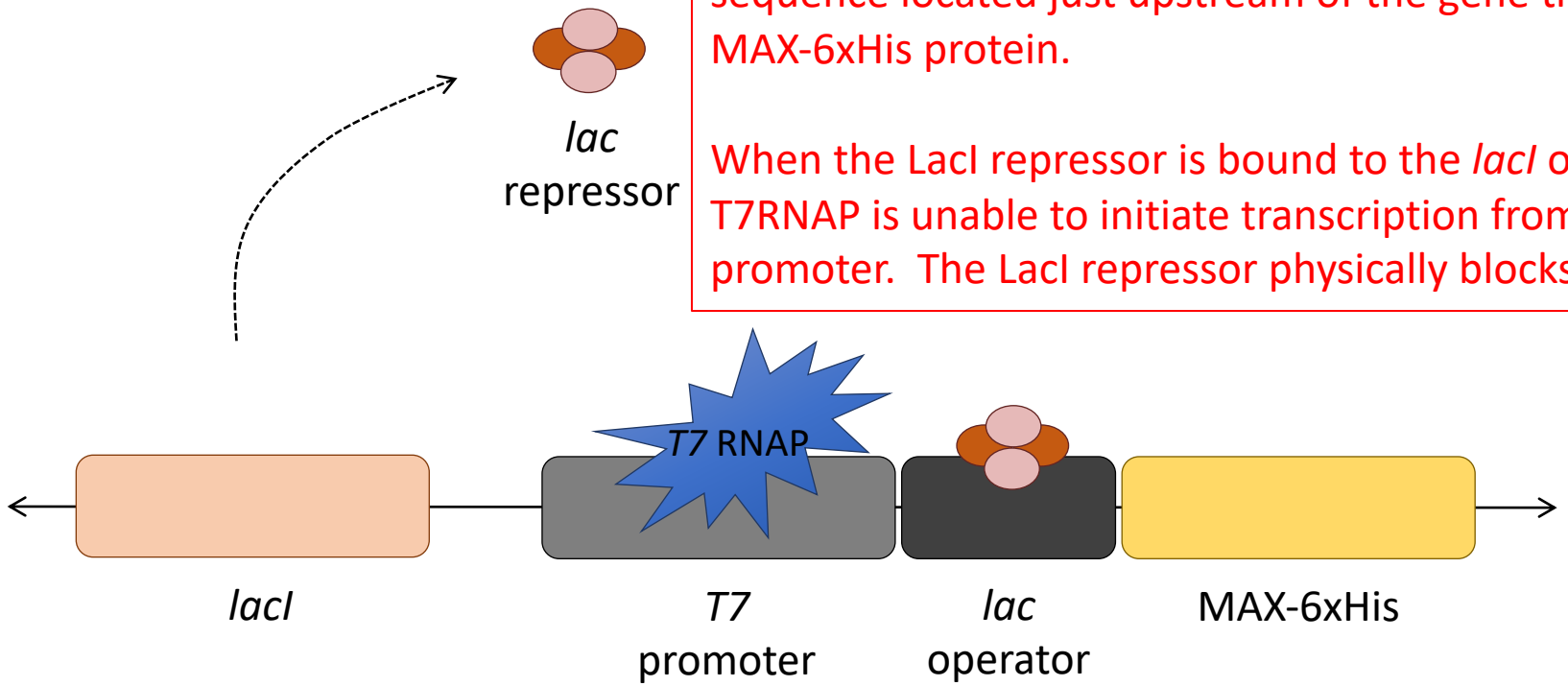
pET28a_MAX-6xHis

LacI repressor blocks transcription at *lac* operator

There are a few 'layers' involved in the biology that controls the expression of MAX-6xHis in our system.

The *lacI* gene located in the pET-28a expression vector is constitutively active. This means that the LacI repressor protein is always present in the BL21 cells and when not impeded, the LacI repressor binds to the lac operator sequence located just upstream of the gene that encodes our MAX-6xHis protein.

When the LacI repressor is bound to the *lacI* operator, the T7RNAP is unable to initiate transcription from the T7 promoter. The LacI repressor physically blocks elongation.



pET28a_MAX-6xHis

Some background information for context:

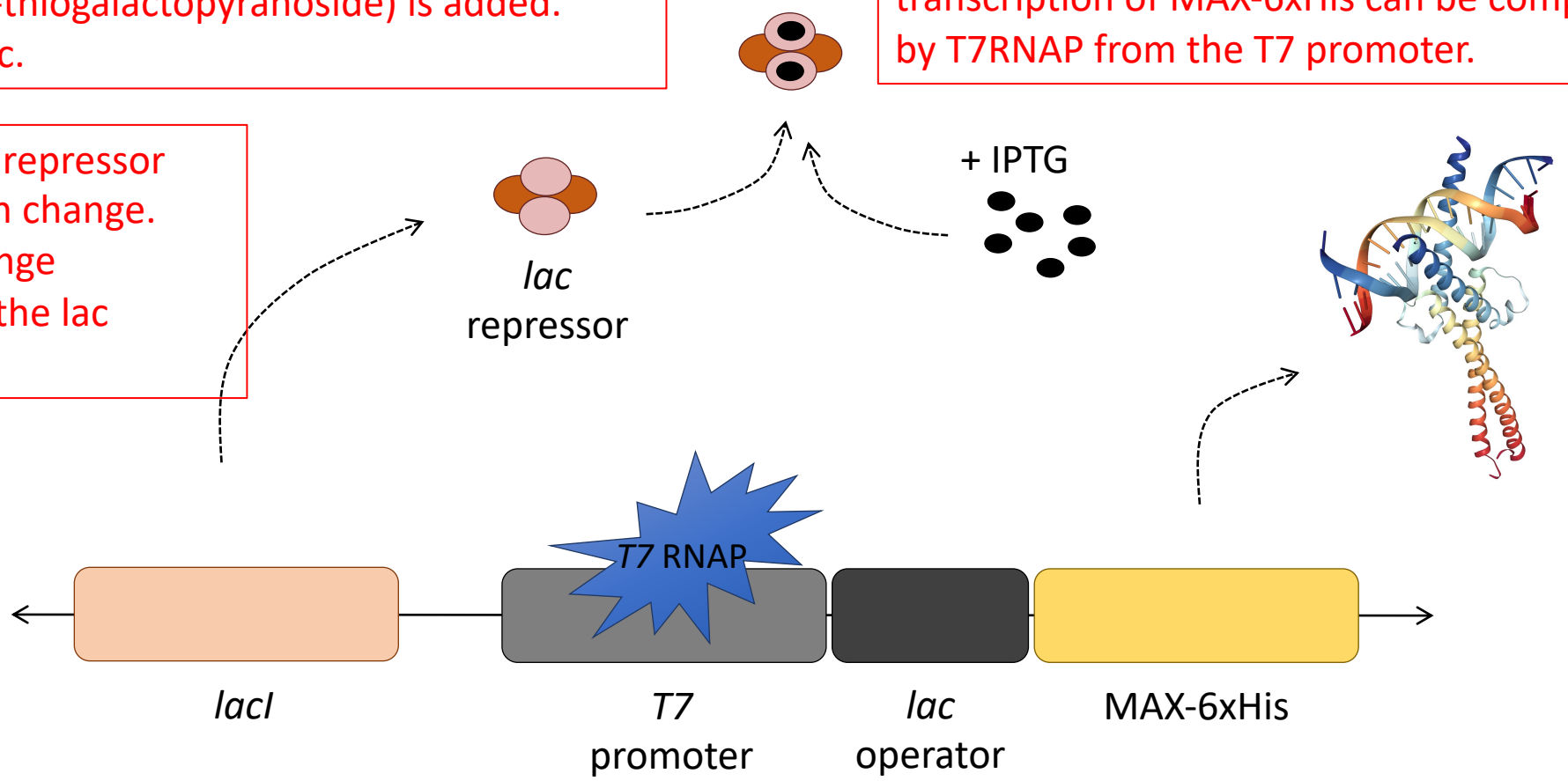
In native bacterial systems, the lac operon is only expressed when lactose is present as a means of conserving energy and taking advantage of available food sources. In this, the LacI repressor inhibits the expression of genes needed for lactose catabolism (via the the *lac* operator) sequence when lactose is absent. When lactose is present it binds to the LacI repressor causing a confirmational change that prohibits LacI from binding the lac operator.

IPTG ‘induces’ MAX-6xHis expression

To ‘induce’ expression of MAX-6xHis, a molecule known as IPTG (Isopropyl β-D-1-thiogalactopyranoside) is added. IPTG is a lactose mimic.

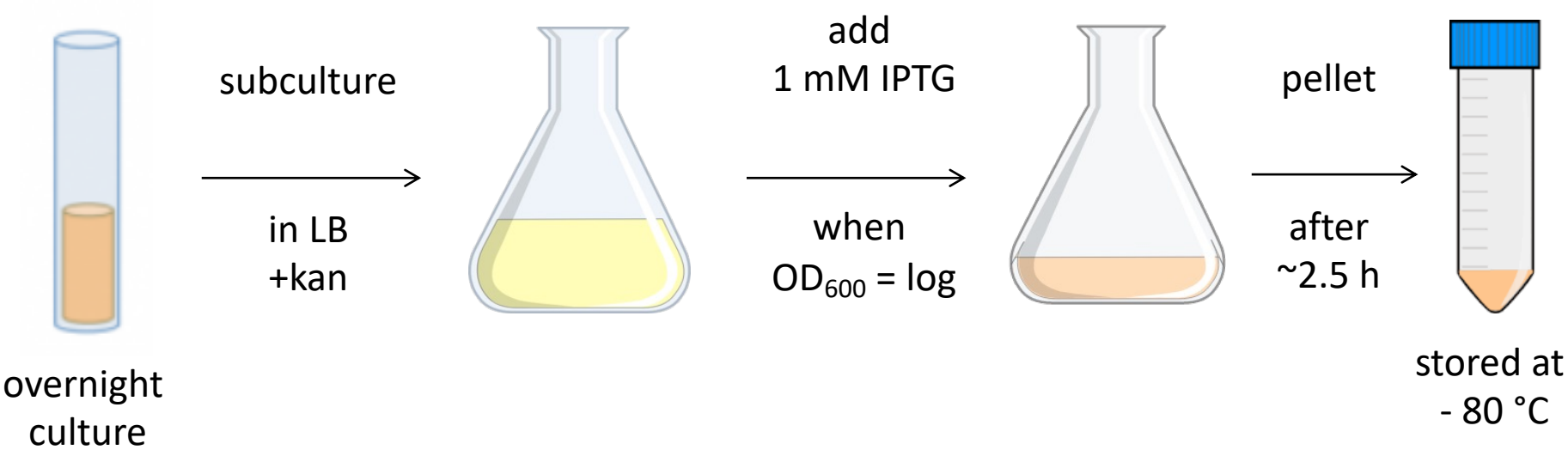
IPTG binds to the LacI repressor causing a confirmation change. This confirmation change eliminates binding to the lac operator sequence.

Without the physical block at the lac operator, transcription of MAX-6xHis can be completed by T7RNAP from the T7 promoter.



pET28a_MAX-6xHis

How did we induce protein expression?



These steps were completed by the instructors for timing reasons. Please note that this information should still be included in your methods homework!

How will you purify MAX-6xHis?

- First, need to lyse cells to release proteins
  - B-PER (Bacterial Protein Extraction Reagent):

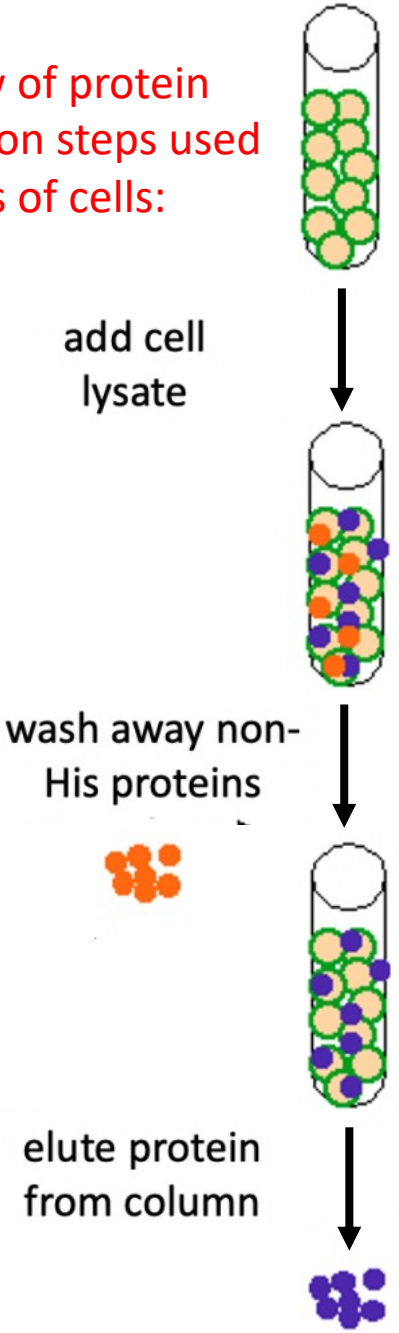
    Detergent in sodium phosphate buffer. Aids in lysis by solubilizing lipids and proteins in the membrane, thereby creating pores.
  - Lysonase:

    Enzyme solution that contains lysozyme and benzonase. Lysozyme cleaves glycosidic bonds in peptidoglycan and benzonase cleaves nucleic acids.
  - Proteinase inhibitor:

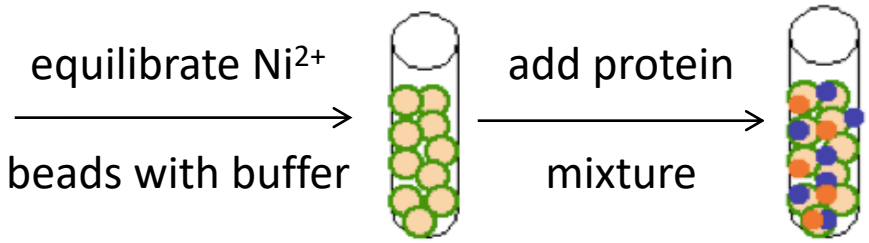
    Molecules that bind the proteolytic (active) sites of proteinase enzymes.
  - Dnase:

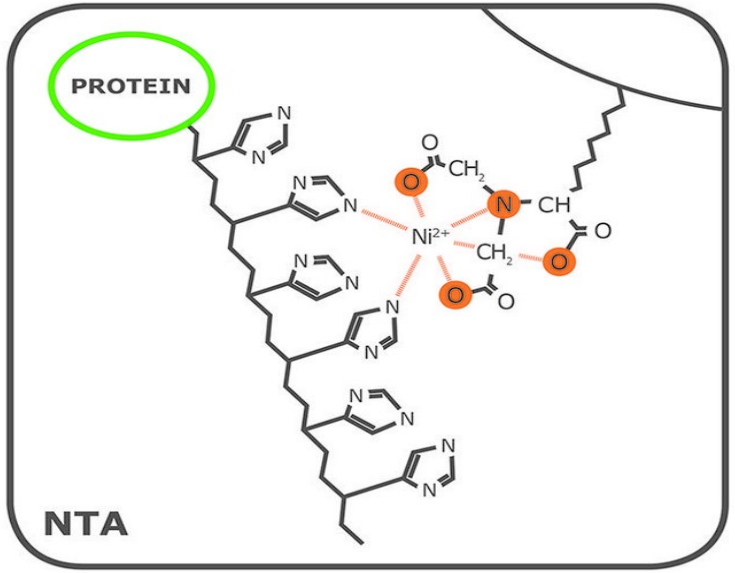
    Cleaves DNA. Added in addition to the benzonase as pilot experiments demonstrated that the DNA was not completely cleaved. Uncleaved DNA is quite viscous and can clog the column during protein purification.

Overview of protein purification steps used after lysis of cells:



6xHis tag binds to Ni^{2+} resin / column

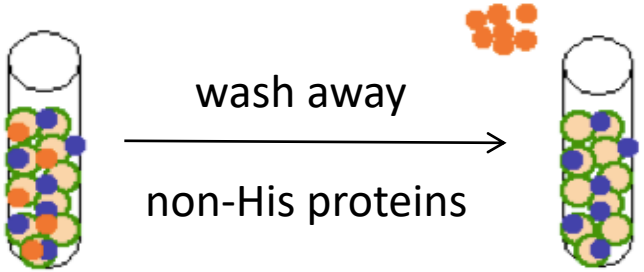


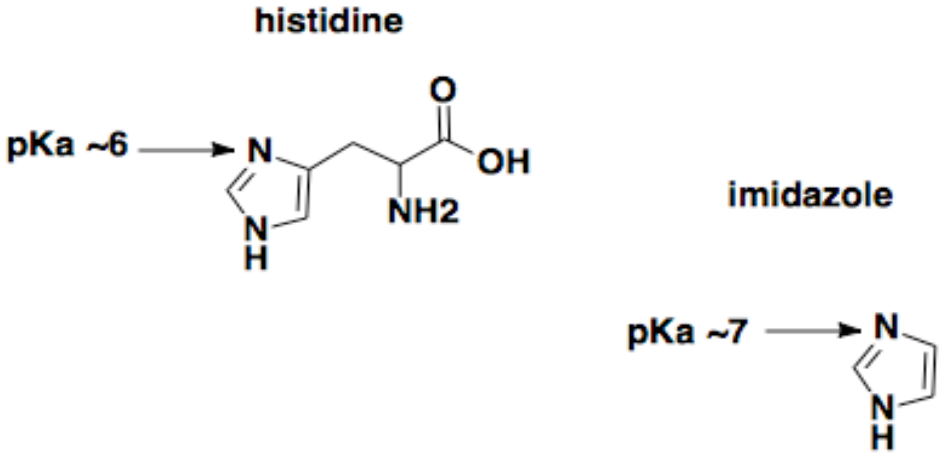


- Ni^{2+} chelated onto agarose resin via nitrilotriacetic acid (NTA) ligand
- His tag chelates to Ni^{2+} causing protein to ‘stick’ to resin / column

The basic imidazole ring of histidine binds to the Ni^{2+} ions causing tagged MAX protein to bind to the agarose resin while other proteins pass through the column.

Non-specific binders washed from Ni^{2+} resin / column using imidazole

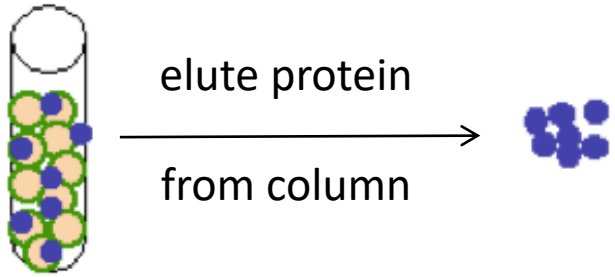


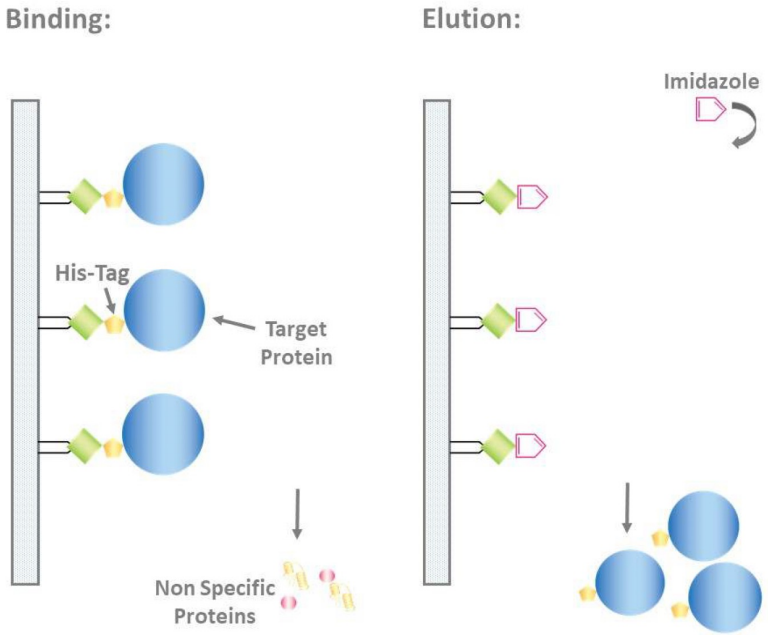


- Low concentration of imidazole included in wash buffer
- Imidazole competes for binding to Ni^{2+} resin
  - Low affinity binders / non-specific binders are outcompeted and released from the resin

Because other proteins in the cell have histidine residues, it is possible to have non-specific binding to the column. A low concentration imidazole solution is used to wash weakly bound proteins from the column.

Imidazole used to elute protein from column



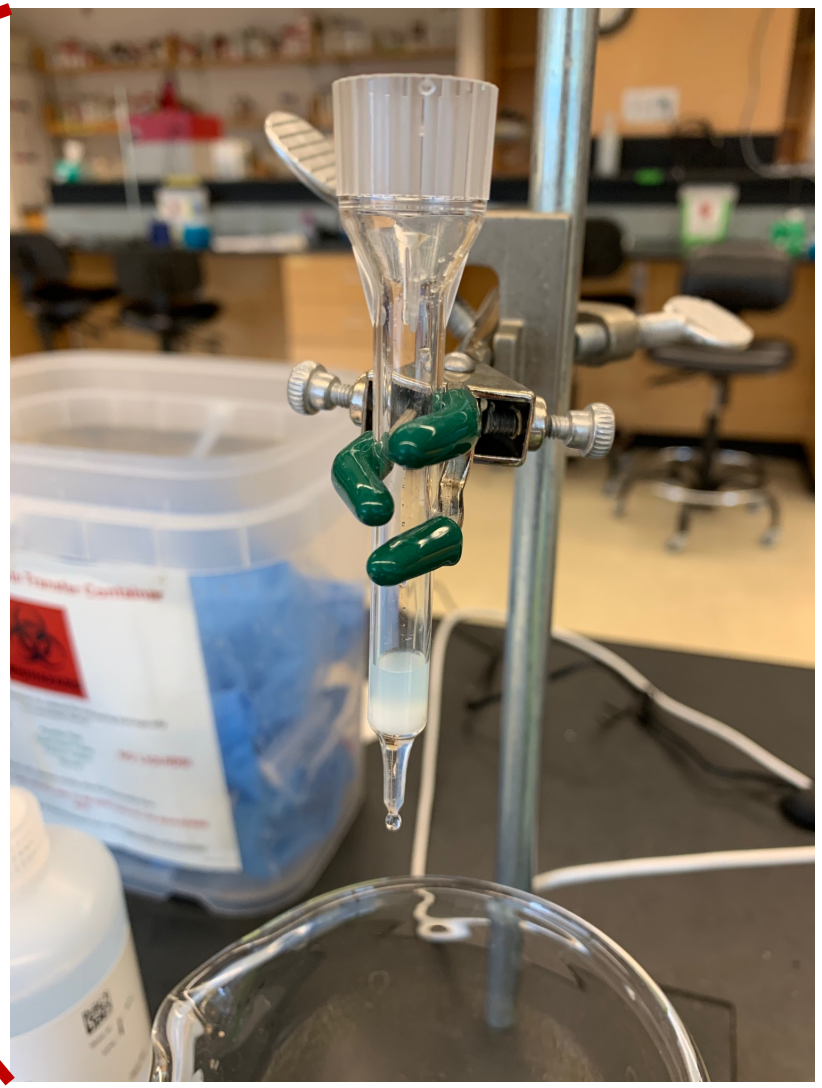


- Elution buffer contains higher concentration of imidazole compared to wash buffer
- Increased concentration allows imidazole to out-compete 6xHis for binding to Ni^{2+} resin

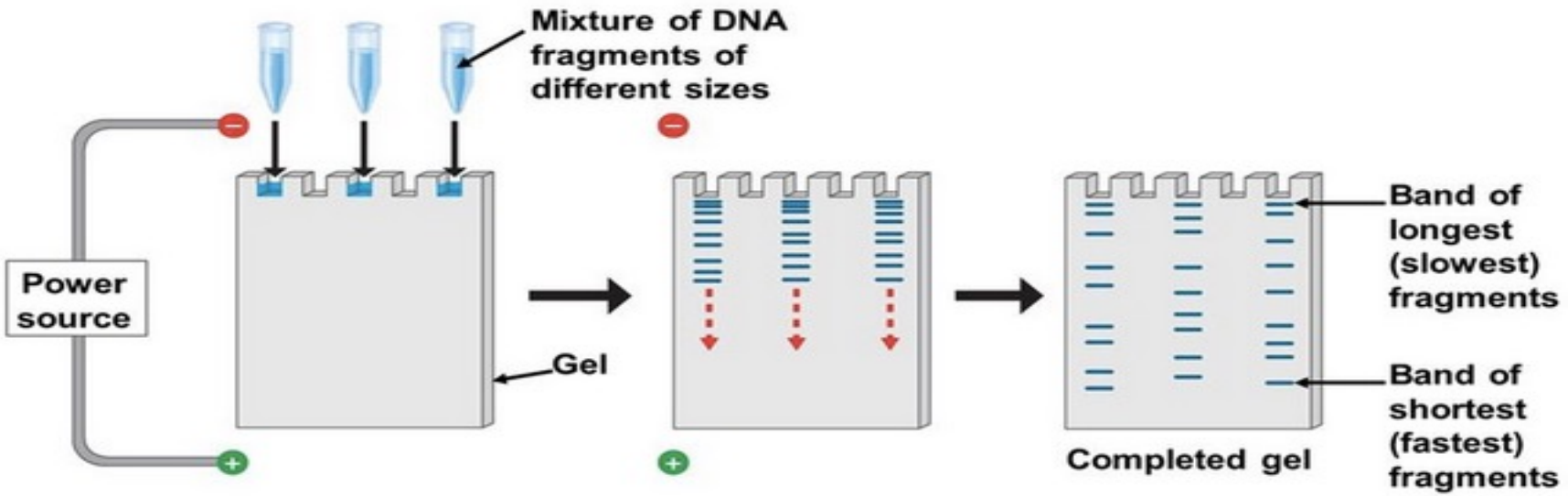
This takes advantage of the chemistry that enables the His tag to bind to the Ni2+ ions. The imidazole ring of His binds MAX-6xHis to the column. This interaction can be out-competed by adding a high concentration imidazole solution to the column.

Demonstration of protein purification

Please watch the video!



Wrap-up of confirmation digest



- How do you visualize migration through the gel?

  Loading dye is used to visualize migration during electrophoresis. The components are bromophenol blue (dye) and glycerol. The glycerol 'weights' the sample down into the well so it doesn't 'float' into the buffer.

- How do you visualize DNA bands in the gel?

  SYBER safe is used to visualize the DNA bands after electrophoresis. This is a fluorescent dye that binds to DNA. The DNA-dye complex absorbs blue light and emits green light.

For today…

- Start protein purification protocol
- Complete agarose gel electrophoresis during lysis incubation
- Be sure to clearly label all tubes containing protein purification aliquots!

For M1D3…

- Draft methods section for confirmation digest (M1D1) and protein purification (M1D2) protocols.

Please carefully review the information provided on how to craft a methods section!! And reach to me to schedule a time to meet if you would like to go over the assignment.

Notes on methods section…

Include enough information to replicate the experiment

- Cite manufacturer for supplies / equipment (Company)
- Be **concise and clear** in your description

Use subsections with descriptive titles

- Put in **logical order**, rather than chronological order
- Begin with topic sentence to introduce purpose / goal of each experimental procedure

Use clear and concise full sentences

- NO tables or lists, all information should be provided in **full sentences** and paragraphs
- Write in passive voice and use past tense

Use the most flexible units

- Write **concentrations** (when known) rather than volumes

Eliminate 20.109 specific language and obvious details

- Example “labeled Row A, Row B…”
- Do not include details about tubes and water!
- Assume reader has some biology experience
- Include parts of the protocol that the teaching faculty completed, but do not say “completed by teaching faculty.”

How can you improve this example?

DNA was cut to check insert. Enzymes were used for single and double

digest then run on gel made by adding 1 g of agar to 100 mL of water.

Gel was imaged on a gel box.

How can you improve this example?

What DNA? How much?

Consider more formal language.

What insert?

Which enzymes? From where were the enzymes acquired?

DNA was cut to check insert. Enzymes were used to cut DNA for

Specifically, why was this done?

Redundant

Provide details on how this was done.

Colloquial…use more scientific language. Also, include details.

single and double digests then run on a gel made by adding

What does this mean?

Be mindful of the order of information and of confusing sentence structure.

1 g of agar to 100 mL of water. Gel was imaged on the gel box.

Use the most flexible units / concise description.

What else was needed for imaging?

The?

What would be more informative?

Edited example…

Confirmation digest of pET28a_MAX-6xHis

To confirm that MAX-6xHis was cloned into pET28a expression vector, a digest was completed. Restriction enzymes AbcI and DefI were used to digest W ng of pET28a-MAX-6xHis in single digests (only one enzyme added) and in a double digest (both enzymes added) using X U / uL of each enzyme and Y buffer (NEB). Digests were incubated at 37C for Z hrs then stored at -20C. [Add gel electrophoresis details].

You can use this as part of your homework assignment! Be sure to include the details for placeholders and the information for the gel electrophoresis protocol.