Screening Strategies for Cloning Results Verification

By

Ass.Prof. Dr. Hassan M. Al-Tameemi

University of Basrah

School of Veterinary Medicine

Department of Microbiology and Immunology

Email: hisan.jasim@uobasrah.edu.iq

Lecture topics

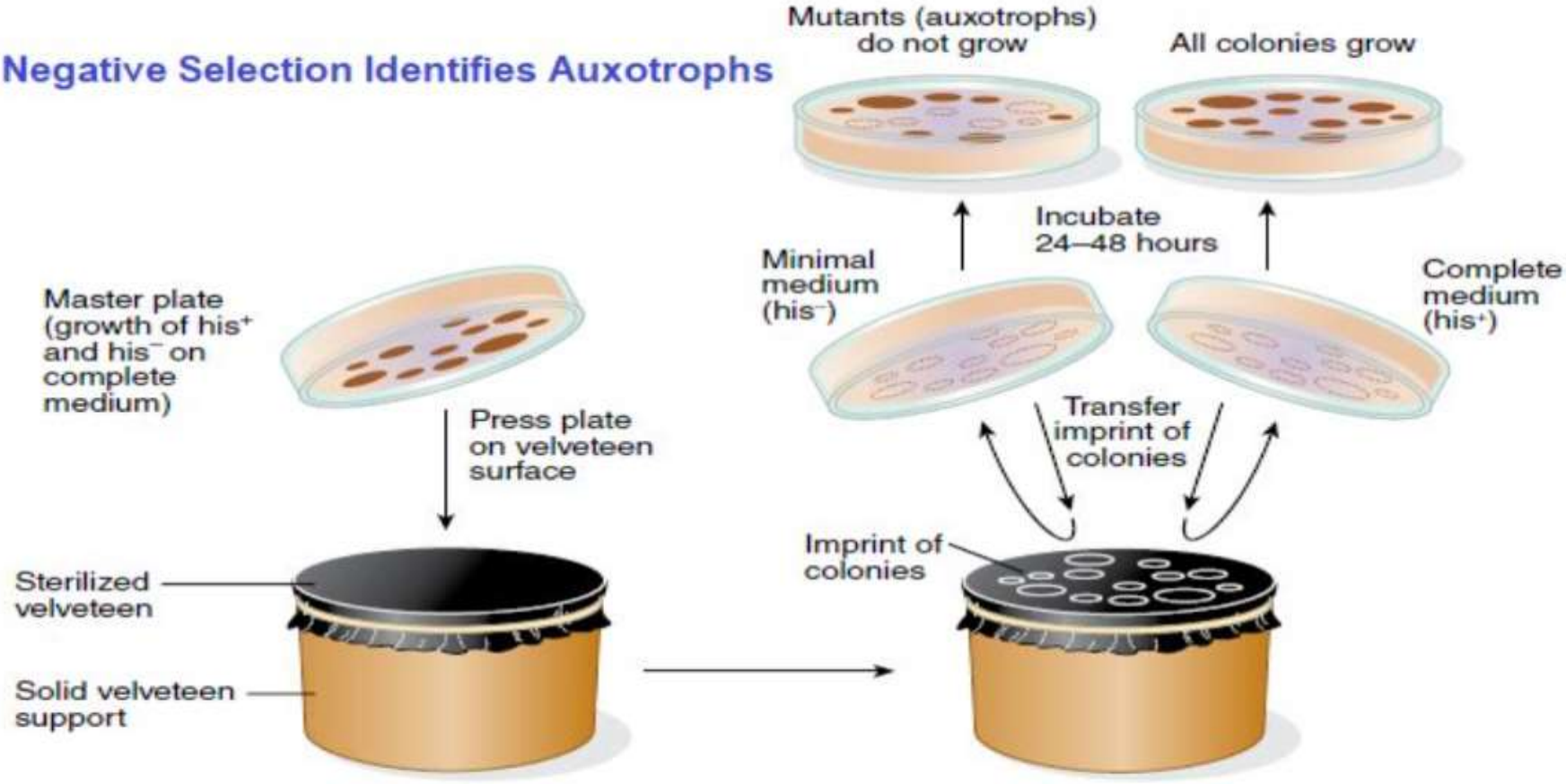
1. Replica Plating
2. Blue-White Screening
3. Colony PCR

Replica Plating

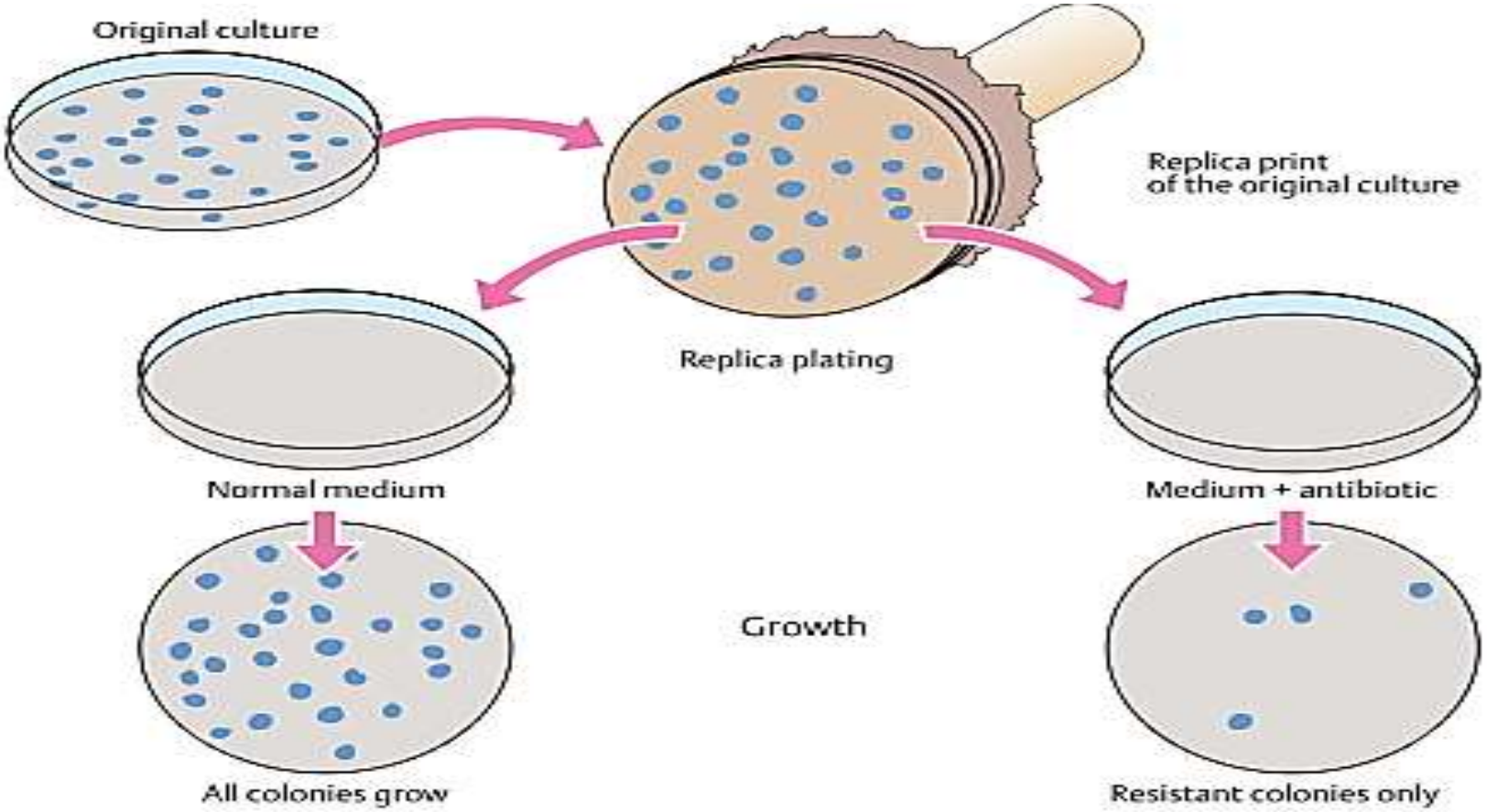
- **Replica plating to recognize mutants**
- In 1952, Joshua and Esther Lederberg developed replica plating of bacterial cultures.
- With this method, individual colonies on an agar plate can be taken up with a stamp covered with velvet and placed onto other culture dishes with media of different compositions.
- Some mutant bacteria differ from non mutants in their ability to grow on such plates.

Mutant bacteria identified through an auxotrophic medium

Negative Selection Identifies Auxotrophs

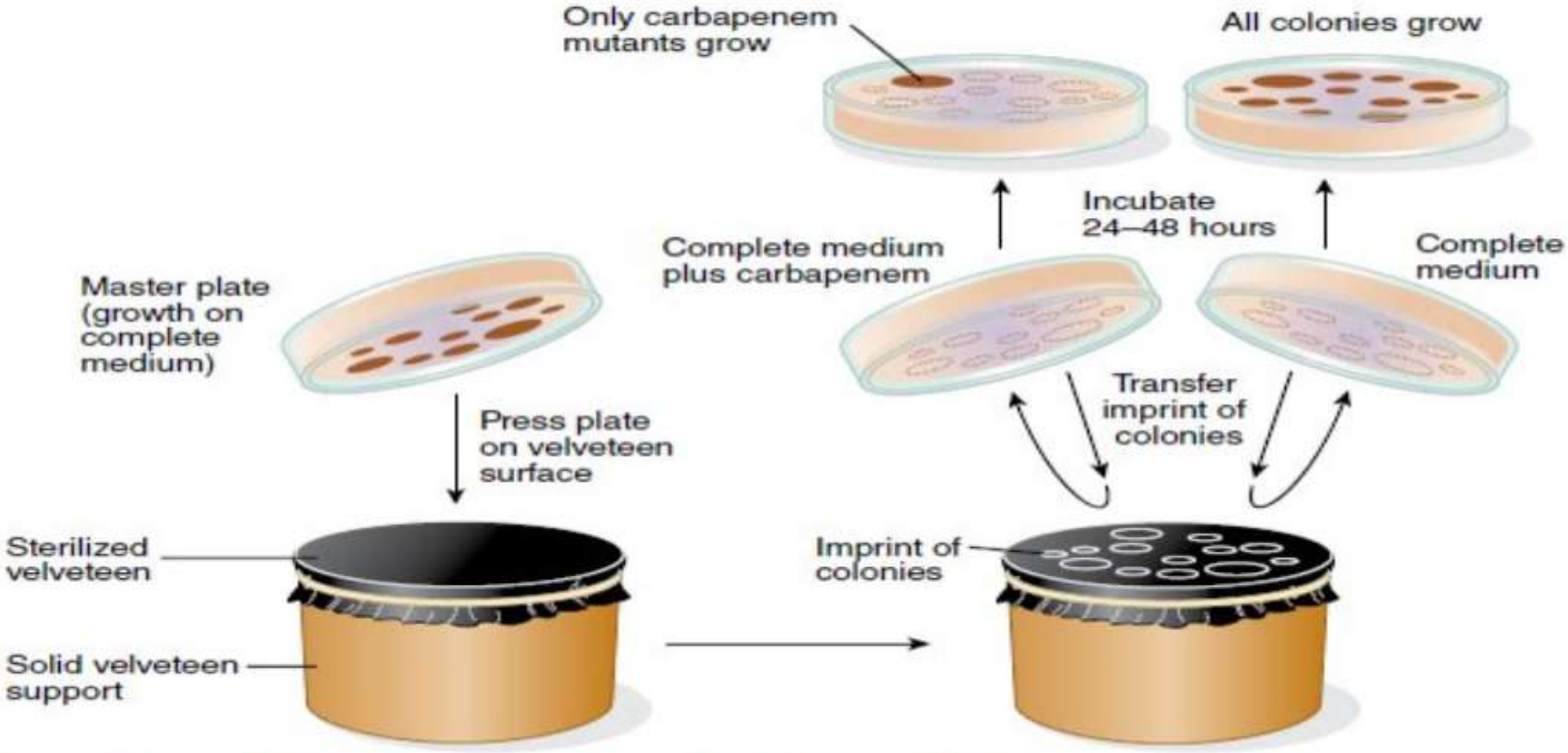


Negative selection plating techniques can be used to detect nutritional mutants (auxotrophs) that fail to grow when replica plated on minimal medium (in this example, a growth medium lacking histidine). Comparison to replica plating on complete medium visually identifies the auxotrophic mutants.



https://magazinescience.com/en/biology/isolation-of-mutant-bacteria/?print=print

Positive Selection of Mutants



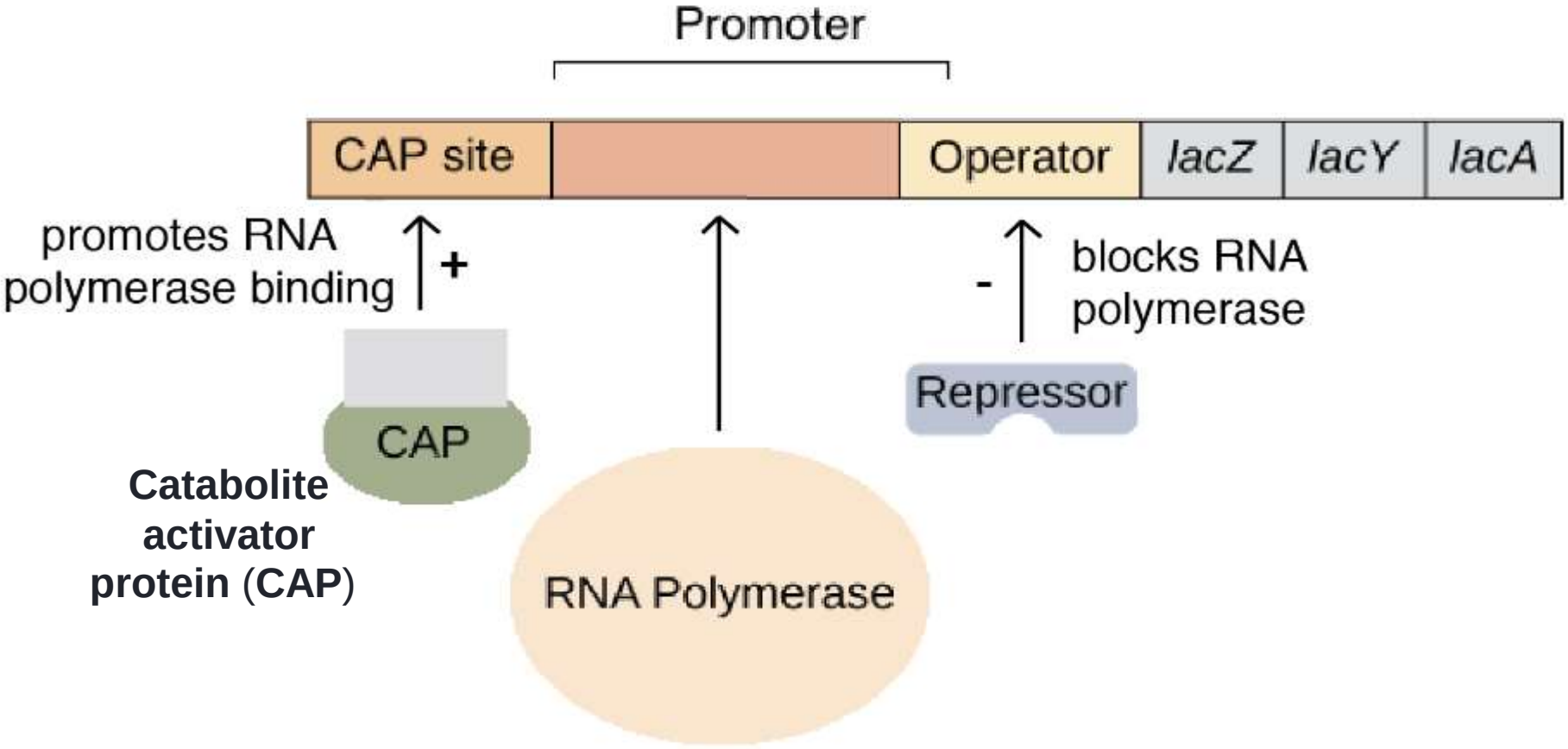
Positive selection plating techniques can be used to identify antibiotic-resistant mutants.

- https://youtu.be/uYb-hYSVYw?si=mcLdMZ_XIQ8bO_N9

Blue-White Screening

- Blue-white screening is a rapid and efficient technique for the identification of recombinant bacteria.
- It relies on the activity of β-galactosidase, an enzyme occurring in *E. coli*, which cleaves lactose into glucose and galactose.
- *E. coli* is a model bacterium used in the lab for molecular biology. And it has an enzyme called beta-galactosidase that helps *E. coli* break down sugars like lactose.
- The functional beta-galactosidase enzyme, capable of breaking down lactose and its analogs like **X-gal**, is formed by the *tetramerization* of four identical monomers of beta-galactosidase polypeptides.

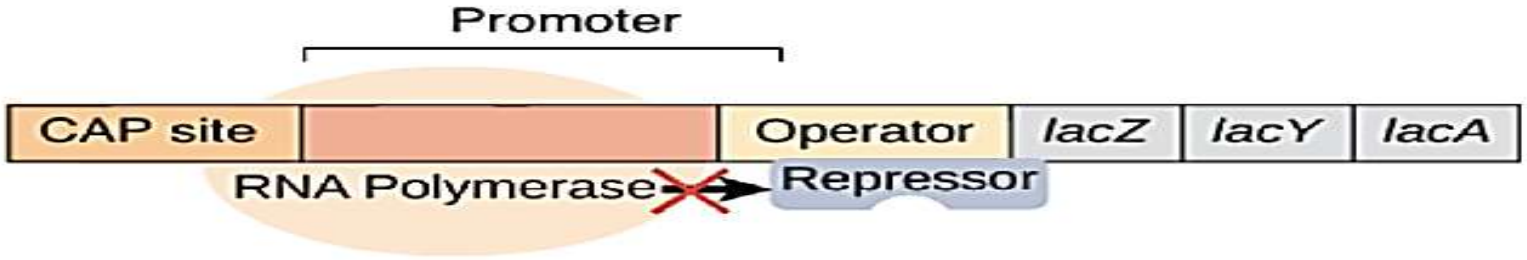
The *lac* operon:



Catabolite activator protein (CAP)

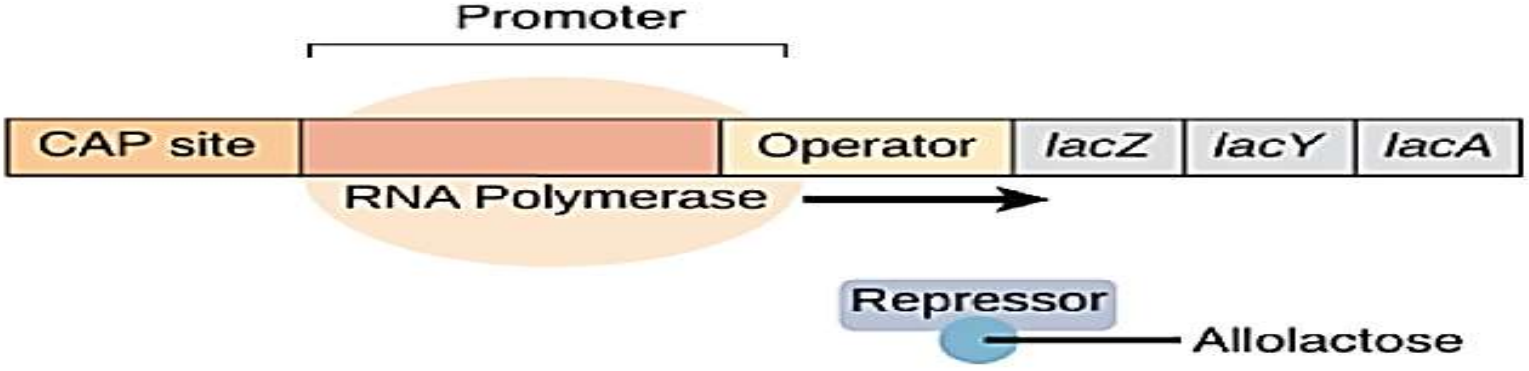
No lactose:

When lactose is absent, the *lac* repressor binds tightly to the operator. It gets in RNA polymerase's way, preventing transcription.



With lactose:

Allolactose (rearranged lactose) binds to the *lac* repressor and makes it let go of the operator. RNA polymerase can now transcribe the operon.



X-gal

(5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)

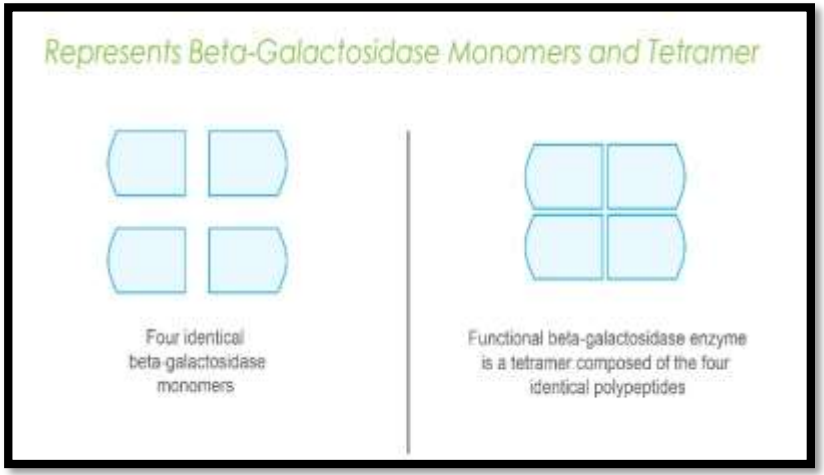
- X-gal is an analog of lactose, and therefore hydrolyzed by the β-galactosidase enzyme which cleaves the β-glycosidic bond in D-lactose.
- X-gal, when cleaved by β-galactosidase, yields **galactose** and **5-bromo-4-chloro-3-hydroxyindole**. The latter then spontaneously dimerizes and is oxidized into 5,5'-dibromo-4,4'-dichloro-indigo - 2, an intensely **blue** product which is **insoluble**.
- X-gal itself is colorless, so the presence of blue-colored product may therefore be used as a test for the presence of active β-galactosidase. This also allows for bacterial β-galactosidase (so called *lacZ*) to be used as a reporter in various applications

(IPTG)

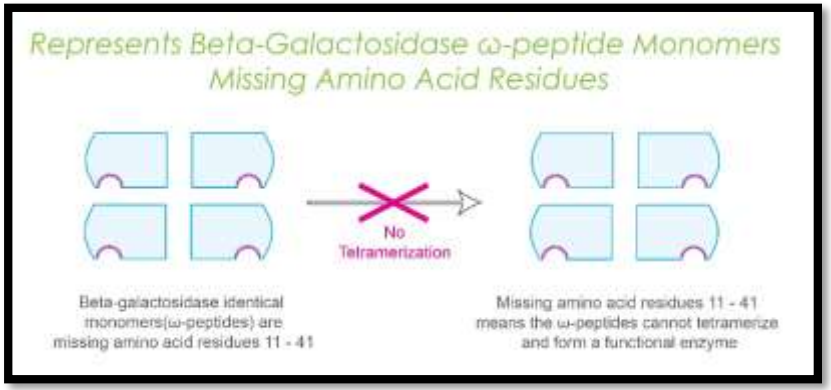
Isopropyl β-D-1-thiogalactopyranoside

- Isopropyl β-D-1-thiogalactopyranoside (IPTG) is used along with X-gal for blue-white screening.
- **It** mimics of allolactose, a lactose metabolite that triggers transcription of the *lac* operon, and it is therefore used to induce protein expression where the gene is under the control of the lac operator.
- It should be noted that IPTG is not a substrate for β-galactosidase but only an inducer. For visual screening purposes, chromogenic substrate like X-gal is required.

- *Tetramerization* –four monomers bond with each other to form a *tetramer* (a larger molecule made up of four small molecules, or *monomers*).
- Within each beta-galactosidase enzyme, there are sequences of amino acids. When amino acid residues 11-41 are removed from the amino-terminus, the monomers cannot form the tetramer.
- The peptide missing these residues is called the **omega** peptide (ω).
- Without the monomers being able to tetramerize, the complete enzyme cannot form, and therefore *E. coli* cannot break down x-gal.



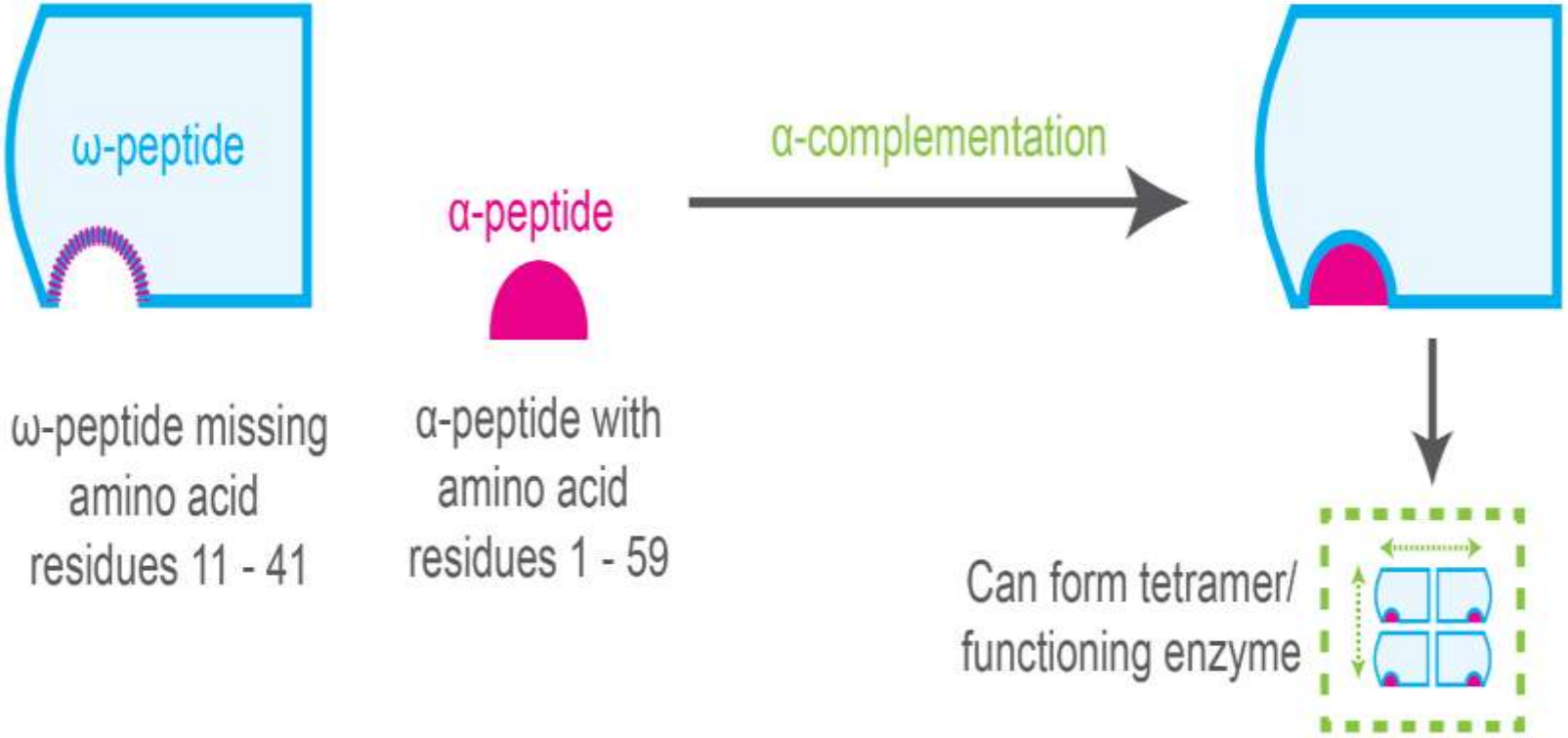
Monomers Tetramers



Monomers No Tetramers

https://goldbio.com/articles/article/explain-alpha-complementation-and-blue-white-screening-like-im-10#:~:text=The%20technical%20explanation%20of%20alpha,have%20the%20desired%20genetic%20construct.

Alpha-Complementation With ω-Peptide & α-Peptide



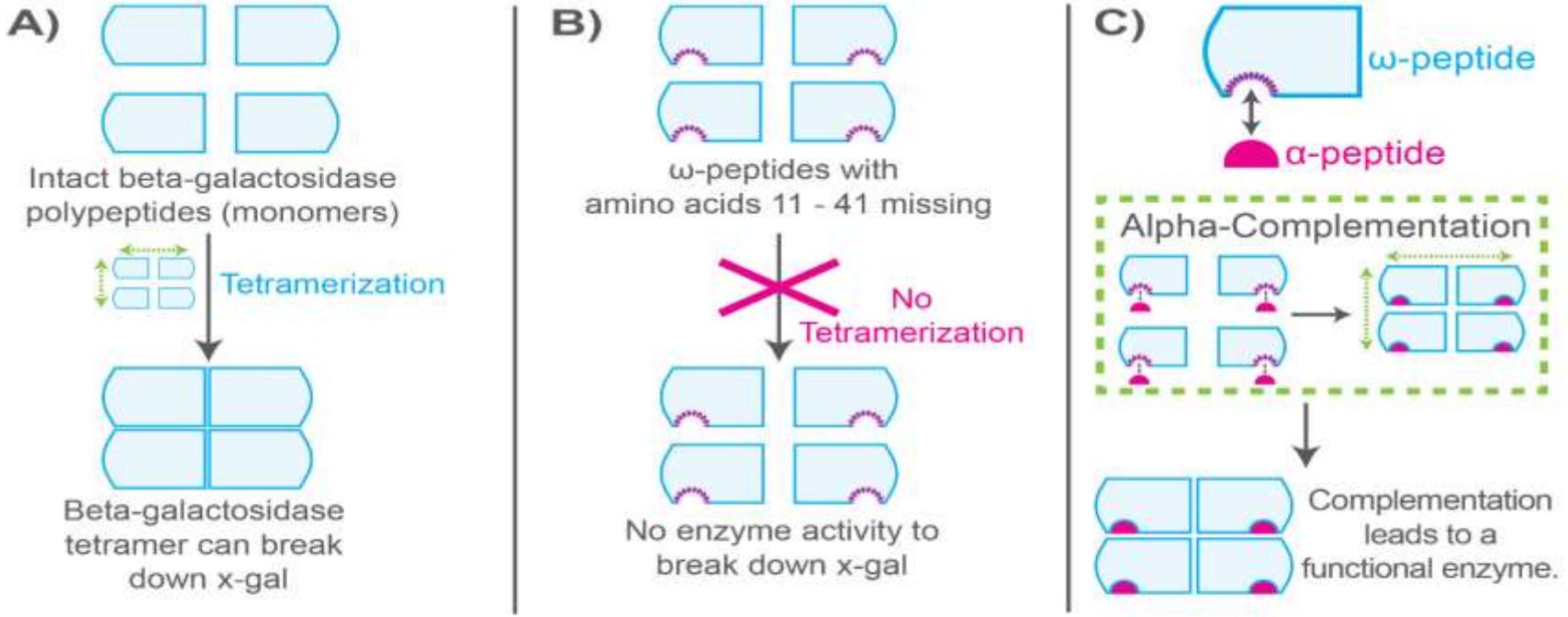


Figure 5. Summarizes beta-galactosidase monomers, the ω-peptide and the α-peptide along with how they interact. Panel A represents functional monomers that form a homotetramer. Panel B represents the ω-peptide missing amino acid residues 11-41. Panel C represents the ω-peptide and it's α-peptide complement.

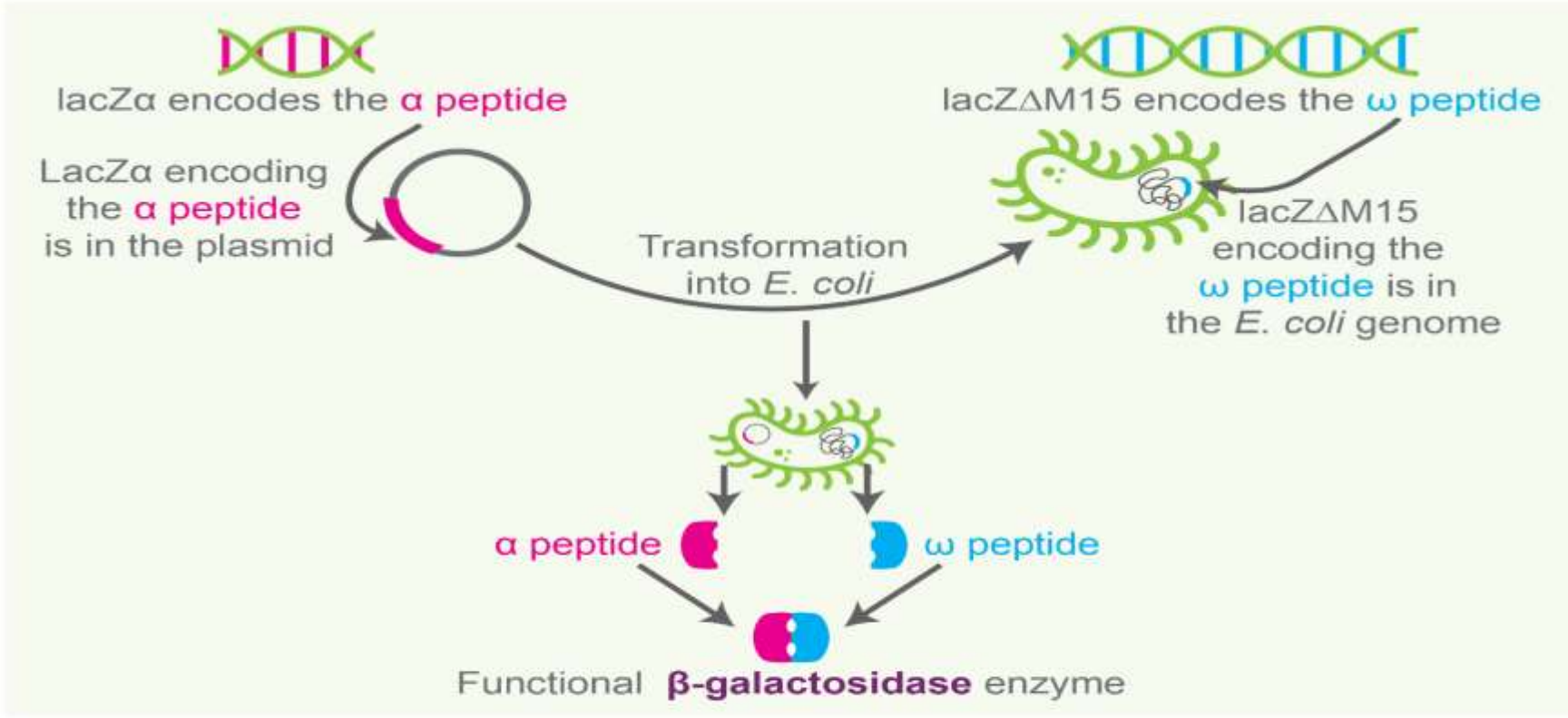


Figure 6. The *lacZα* sequence encoding the α-peptide is shown in the plasmid. The *lacZΔM15* sequence encoding the ω-peptide is within the *E. coli* genome. *E. coli* transformation leads to α-peptide and ω-peptide production and alpha-complementation.

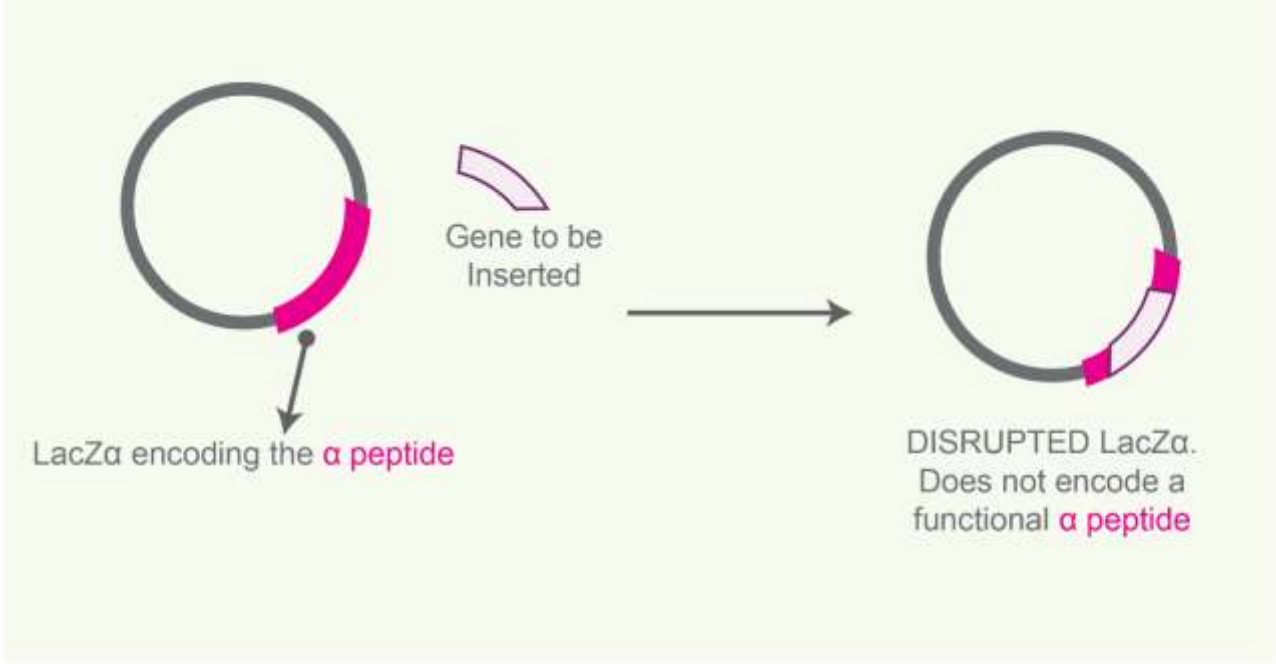


Figure 12. Gene of interest (purple outline) is inserted into a section of the plasmid and the insertion breaks up the *lacZα* gene that encodes the α -peptide. Because the *lacZα* gene is disrupted, the α -peptide cannot be produced

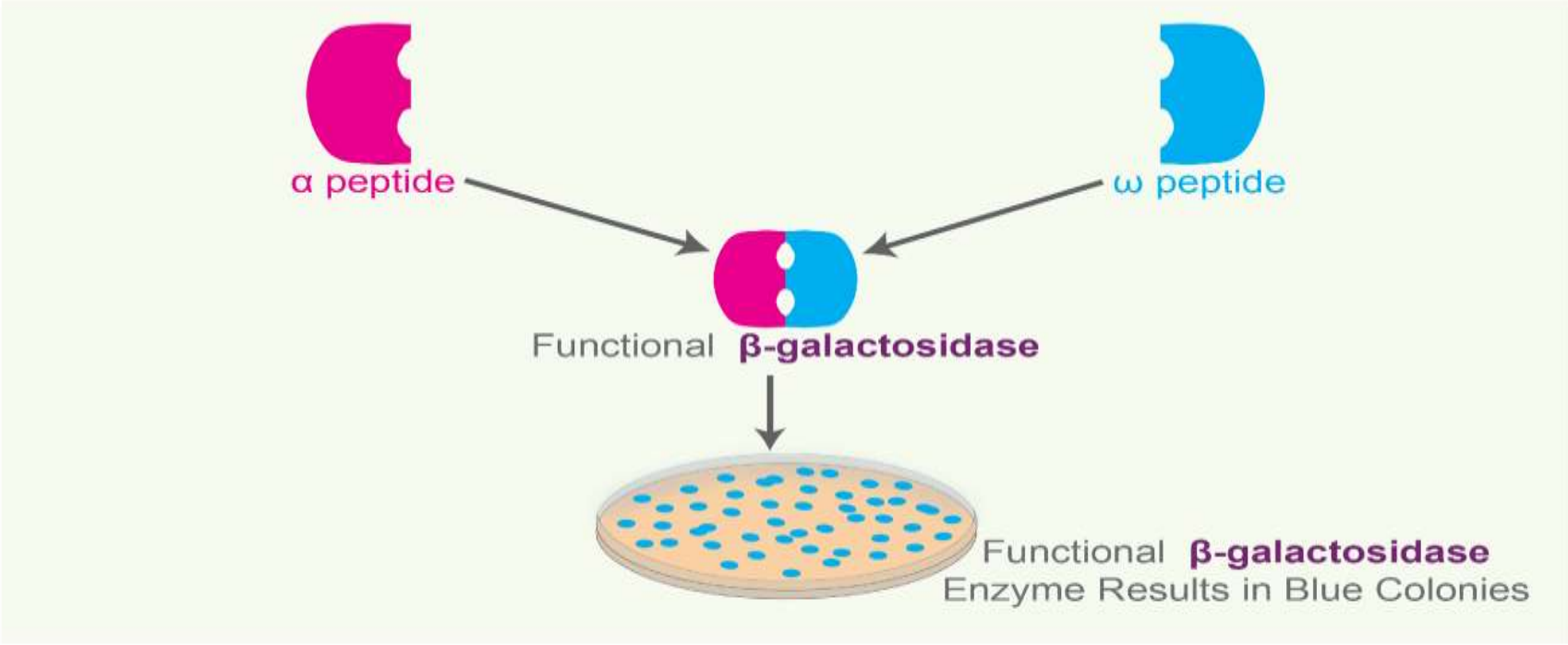


Figure 13. Both the α-peptide and ω-peptide combine, allowing peptides to form a tetramer that leads to beta-galactosidase. *E. coli* colonies with beta-galactosidase are blue due to a color change that occurs when the enzyme breaks down x-gal.

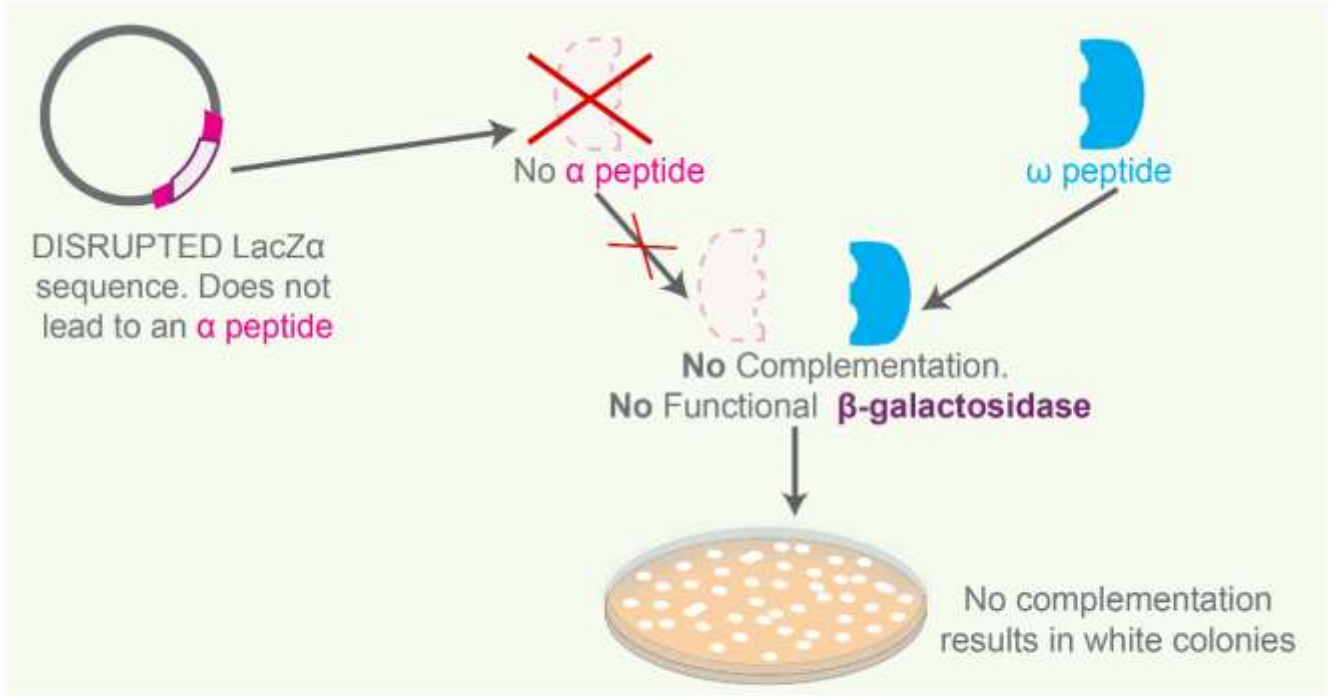


Figure 14. The plasmid with the target gene inserted disrupts the *lacZα* sequence. There is no α-peptide and no alpha-complementation that occurs. *E. coli* colonies with the recombinant plasmid appear white.

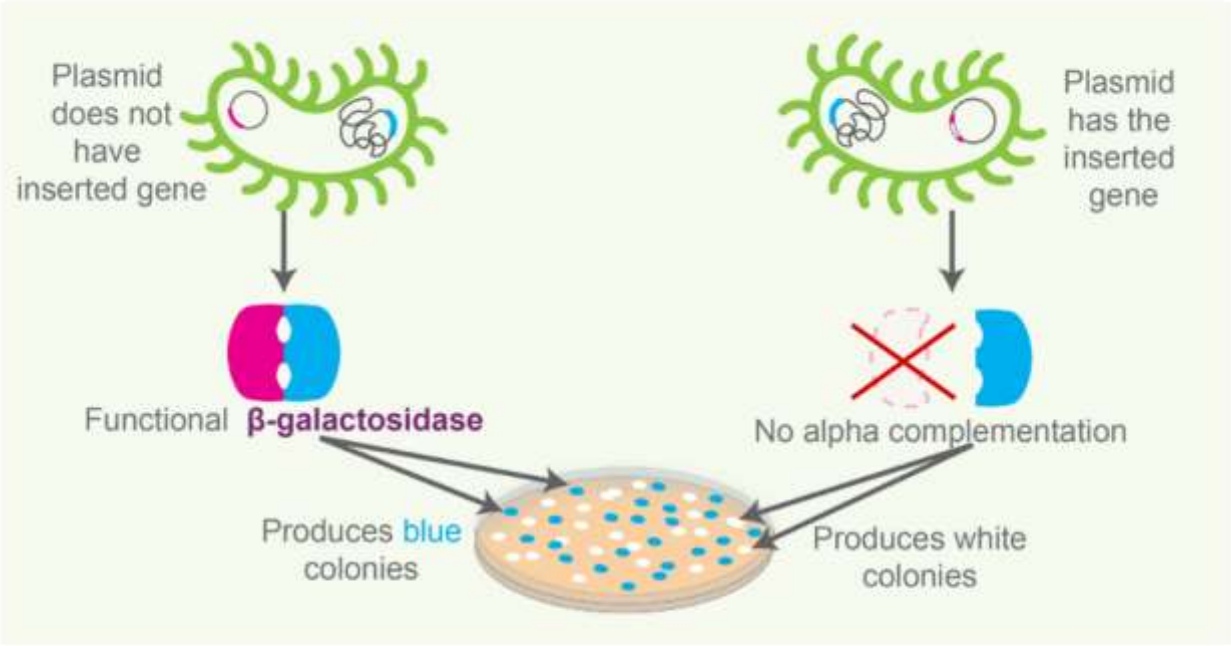


Figure 15. Summarizes blue-white screening and colony selection. Colonies with the recombinant plasmid grow white on the petri dish. Colonies that do not have the recombinant plasmid with the desired gene inserted will appear blue.

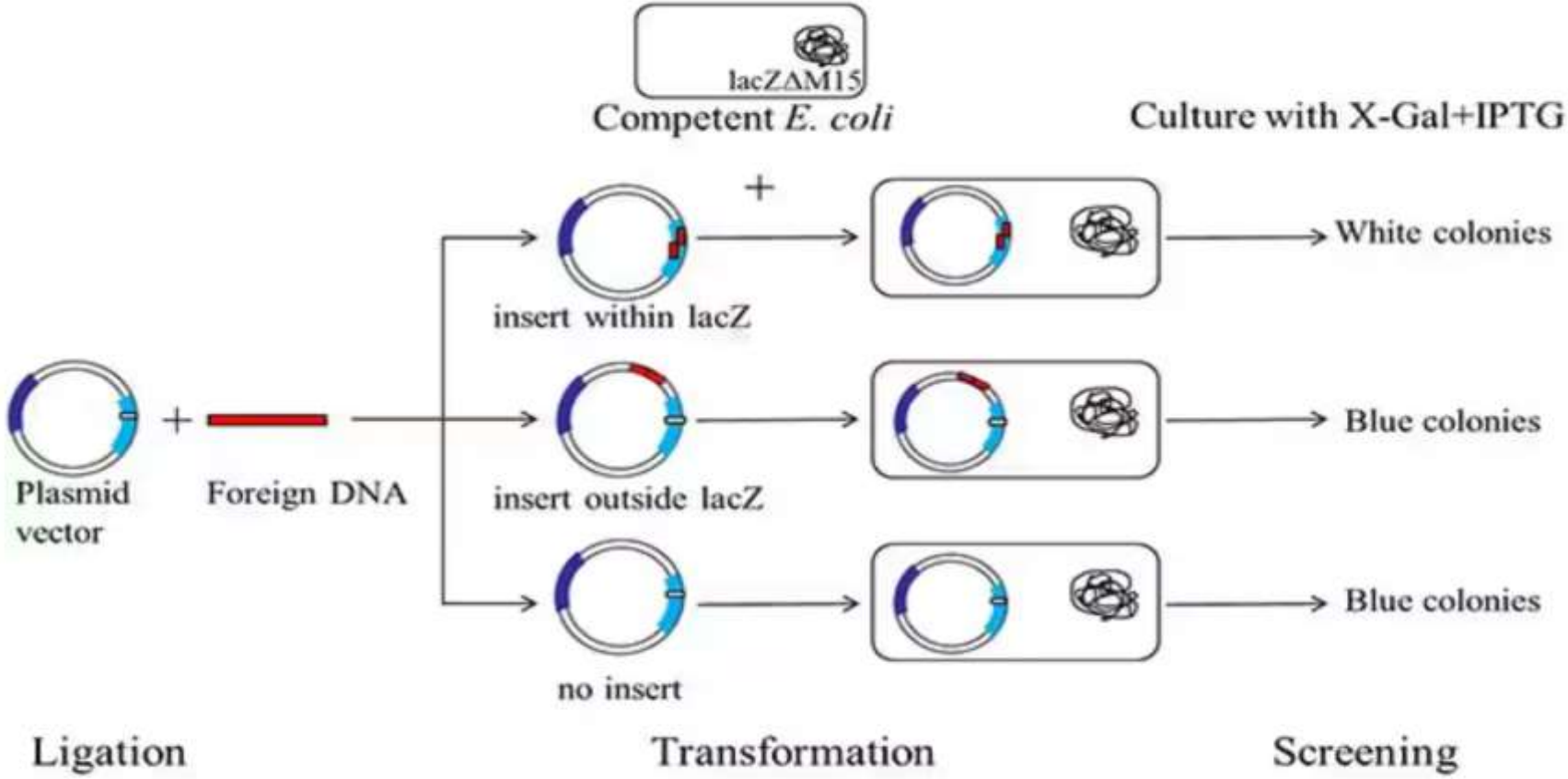
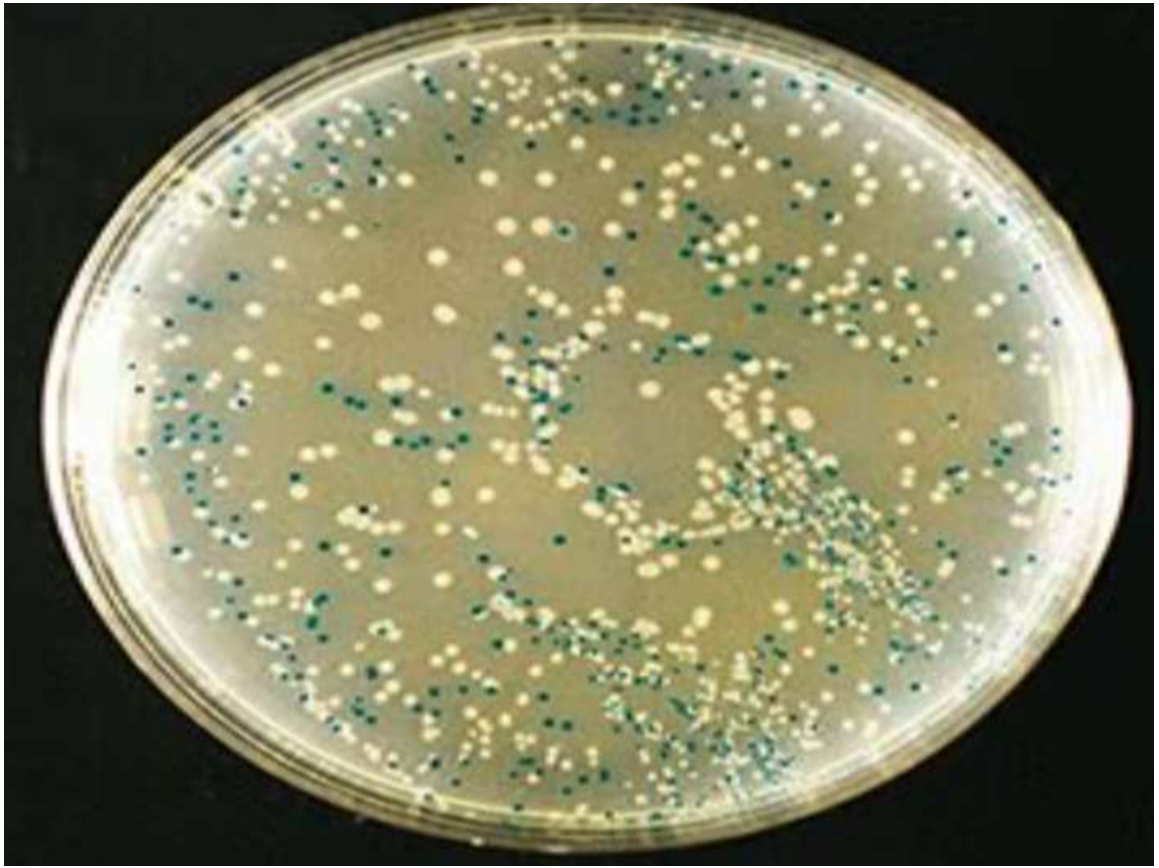


Figure 2. A schematic representation of a typical blue-white screening procedure.

Blue-white color selection of recombinant bacteria using X-gal.



Colony PCR

By

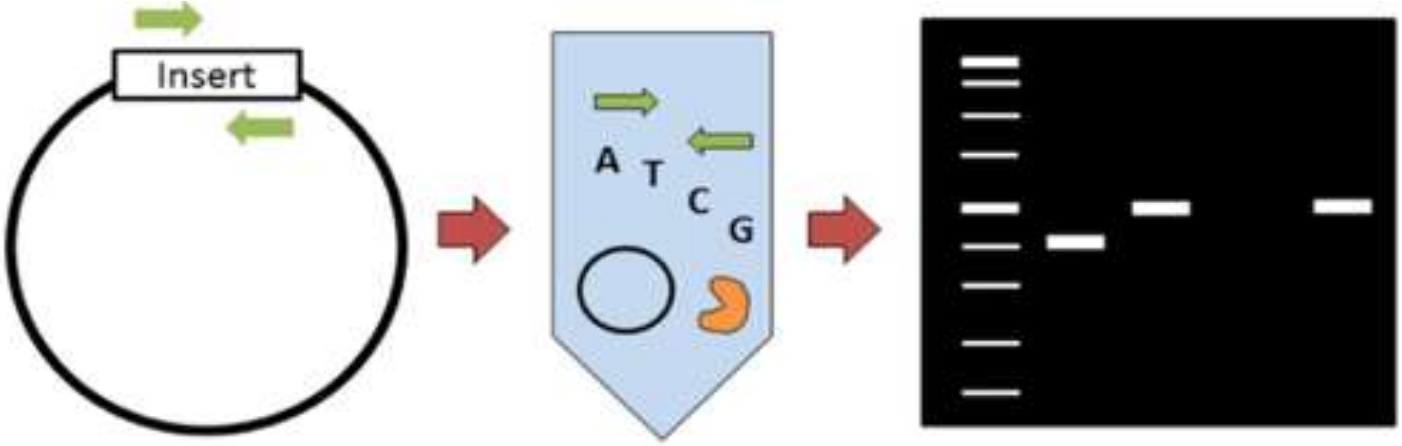
Prof. Dr. Rasha Munther Othman

What is colony PCR?

- Colony PCR is a method to quickly check bacterial colonies generated after the transformation step of a molecular cloning protocol .
- It is used to verify that the vector inserted during transformation contains the full desired genetic insert.
- Other methods used to screen clones may require plasmid DNA extraction and some level of purification, followed by a digestion reaction with a restriction enzyme, and verification of a specific DNA banding pattern on an agarose or acrylamide gel.

The key steps to colony PCR are:

1) design primers to detect the presence of your insert;
2) set up a standard PCR reaction (primers, dNTPs, polymerase) using the supernatant of lysed bacteria as template;
3) run your PCR product on a gel to analyze product size.

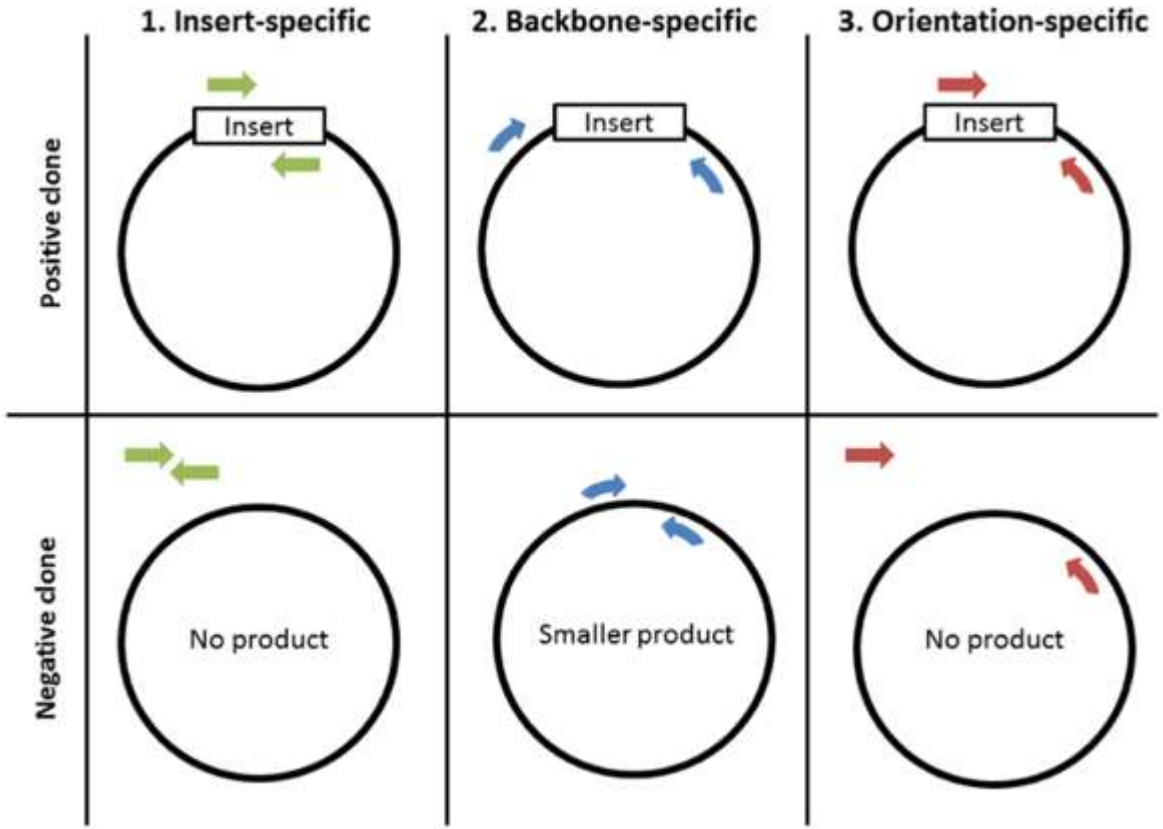

Insert
A
T
C
G
1. Design primers
2. Set-up PCR
3. Analyze PCR product

Designing colony PCR primers

The first and perhaps most important step to colony PCR is designing primers.

There are 3 strategies for primer design:

1) insert-specific primers,
2) backbone-specific primers,
3) orientation-specific primers.


1. Insert-specific
2. Backbone-specific
3. Orientation-specific
Positive clone
Negative clone
Insert
Insert
Insert
No product
Smaller product
No product

Insert-specific primers:

- Insert-specific primers are designed to anneal to an insert-specific sequence.
- This is a “yes or no” kind of test, with a positive clone amplifying a product and a negative clone resulting in no product.
- Additionally, this type of primer only tells you if your specific insert is present but not if it’s in the correct orientation or even if it’s in your plasmid backbone.

2. Backbone-specific primers:

- These primers are designed to anneal to sites that flank the insert site.
- A positive clone will produce a larger size product then a negative clone without the insert.
- This type of primer pair can tell you if the insert is the correct size and whether it's within your backbone.
- This type of primer pair is also great for screening clones created with the same backbone but that contain different inserts.
- When you design primers to anneal outside the cloning site, it doesn't matter what the sequence of the insert is, allowing you to use the same primer pair to screen for the presence of many different inserts.

3. Orientation-specific primers:

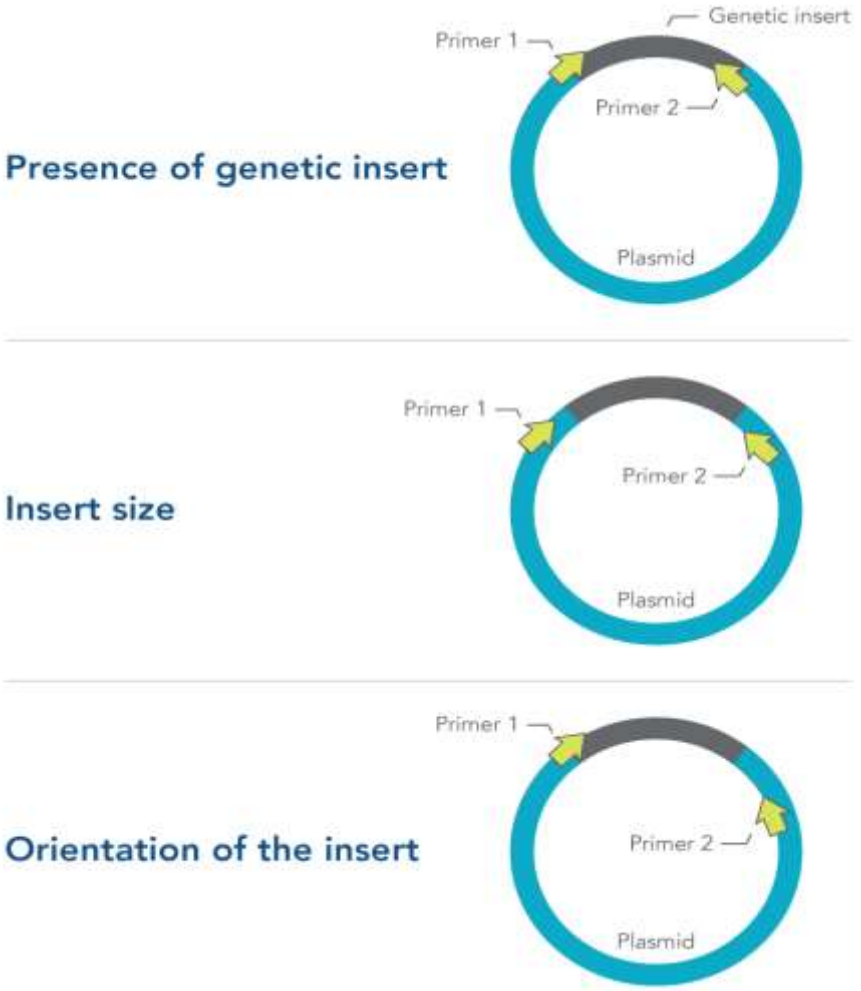
- If you need information about insert orientation, then you might consider designing orientation-specific primers.
- Blunt end cloning is an example of when you might want to know the orientation of the insert.
- One member of this type of primer pair anneals to a sequence flanking the insert and one primer anneals to the insert.
- A simple way to create this type of primer pair is to mix-and-match insert-specific and back-bone specific primers.

How is colony PCR done?

Primer design is especially important in colony PCR. Depending on what they are designed to target, primers selected for colony PCR can provide information about:

- Presence of genetic insert—primers are designed to target the insert and not the plasmid
- Insert size—primers are designed to flank the insert
- Orientation of the insert—one primer is designed to target the insert, while the other primer targets the plasmid

Figure 1. Different primer design strategies for colony PCR.



- **Procedure**
  - Setting up colony PCR reactions is nearly identical to preparing a standard PCR reaction: combine template, primers, polymerase, and dNTPs and then incubate with a standard PCR thermocycling program.
  - One key difference is the plasmid DNA must be released from the bacteria in order to serve as PCR template. Dealing with this and a few other colony PCR tips are highlighted below.

1. Preparing template: Pick a single colony with a sterile flat toothpick or pipette tip and swirl in a small amount of sterile water. Pick 3-10 colonies in total to test, depending on the number of background colonies on your no ligation control plate. The more background, the more colonies you will need to screen.

- **Procedure**

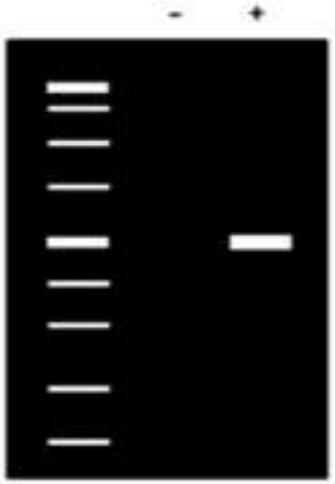
2. Saving clones for later culture: At this point, you will want to hang onto your clones for later use. There are a few ways you can do this. If you are going to complete your colony PCR analysis in the same day, you can save the leftover bacteria-water suspension and use them to start cultures of your positive clones. If you want to store your clones longer term, just streak the colonies on an LB plate. You can use this plate to start liquid cultures. Lastly, you can start small overnight liquid cultures with the clones you pick and only mini-prep the positive ones. Regardless of which method you choose, make sure to use the appropriate antibiotic for selection.

- **Procedure**

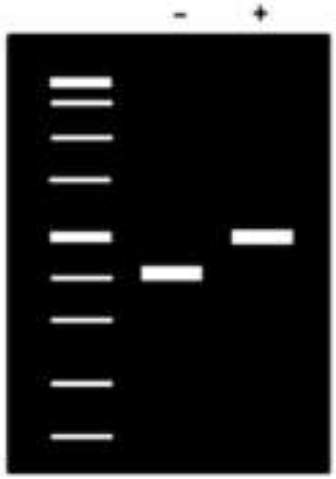
3.Lysing bacteria and setting up PCR reactions: The remaining bacteria-water suspension will serve as the template for your PCR reaction. You just need to lyse the bacteria to release the plasmid DNA by either briefly boiling the sample before use or by directly adding a small volume of the sample to the PCR reaction. The bacteria will be lysed during the initial heating step of the PCR reaction. A standard Taq polymerase is sufficient.

4. Controls: Controls can make or break an experiment. The best controls for a colony PCR are the same ones used to verify if the colony PCR primers work in the first place: the backbone vector with and without an insert. These controls are quick references you can use when you run your PCR products out on a gel to determine if the colonies contain an insert. They also serve as controls for your PCR reaction. Running a no template control PCR reaction for detecting contamination is also a good idea.

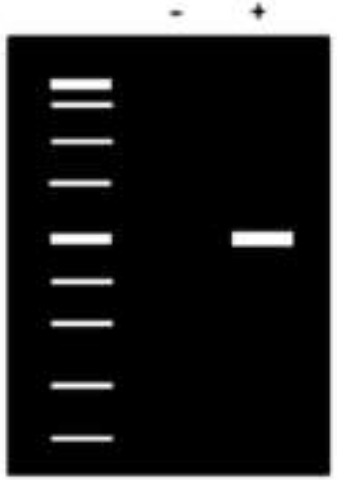
Analyzing PCR product size on a gel



1. Insert-specific primers



2. Backbone-specific primers



3. Orientation-specific primers

Analyzing PCR product size on a gel

- The figure above summarizes generalized expected results for the three primers previously described.
- When using insert-specific primers (1), positive clones (+) will give a band, while a negative clone (-) will not. Backbone-specific primers (2) give larger sized products for positive clones (+) compared to negative clones.
- Finally, orientation-specific primers (3) give the same band (+) or no band (-) result as insert-specific primers but also tell you whether the insert has the correct directionality.

Verifying the insert sequence with Sanger Sequencing

Tips for colony PCR

1. Don't pick too large of a colony. Too many bacteria can inhibit your PCR reaction or cause non-specific products to show up on your gel.

2. Beware of false positives. Just because you get the expected sized PCR product doesn’t mean there aren’t mutations in your insert. Make sure to submit multiple positive clones for sequencing to verify the insert sequence before proceeding with your experiment.

3. Shorter amplicons tend to be better. Shorter amplicons make for shorter PCR programs and are more likely to work in a PCR reaction that has bacterial debris.

Tips for colony PCR

4. Use a positive control. A good positive control is bacteria transformed with the same backbone plasmid. If this control doesn't amplify a product, then you know there could be something wrong with the PCR setup and/or the primer design.

5. Use a negative control strain. A good negative control strain is an untransformed culture of the same strain of bacteria you used for cloning. This type of control is especially important for insert-specific primers. If your negative control amplifies a product of the expected size, you know the genome of your bacteria already contains your target sequence.