BIOFOUNDRY

in collaboration with the

OPEN INSULIN

global initiative
presents...

Insulin Biosynthesis:
A practical course on the production of a miracle drug using bacteria

Name: _____

6 Digit PD #: _____

Date of Lesson: _____

Lab induction complete? _____
(Please Sign)

Safety Induction:

Welcome to Biofoundry. Biofoundry is a community bioscience laboratory. In order to make this work, every person who enters the lab must be responsible for their safety and for the safety of others.
We will start today with a 30 minute safety induction during which you will learn the common hazards and safety protocols specific to our lab.
Please pay attention and be responsible! Just because we have public liability insurance, doesn't make this a safe space!

Pro Pipetting Pointers

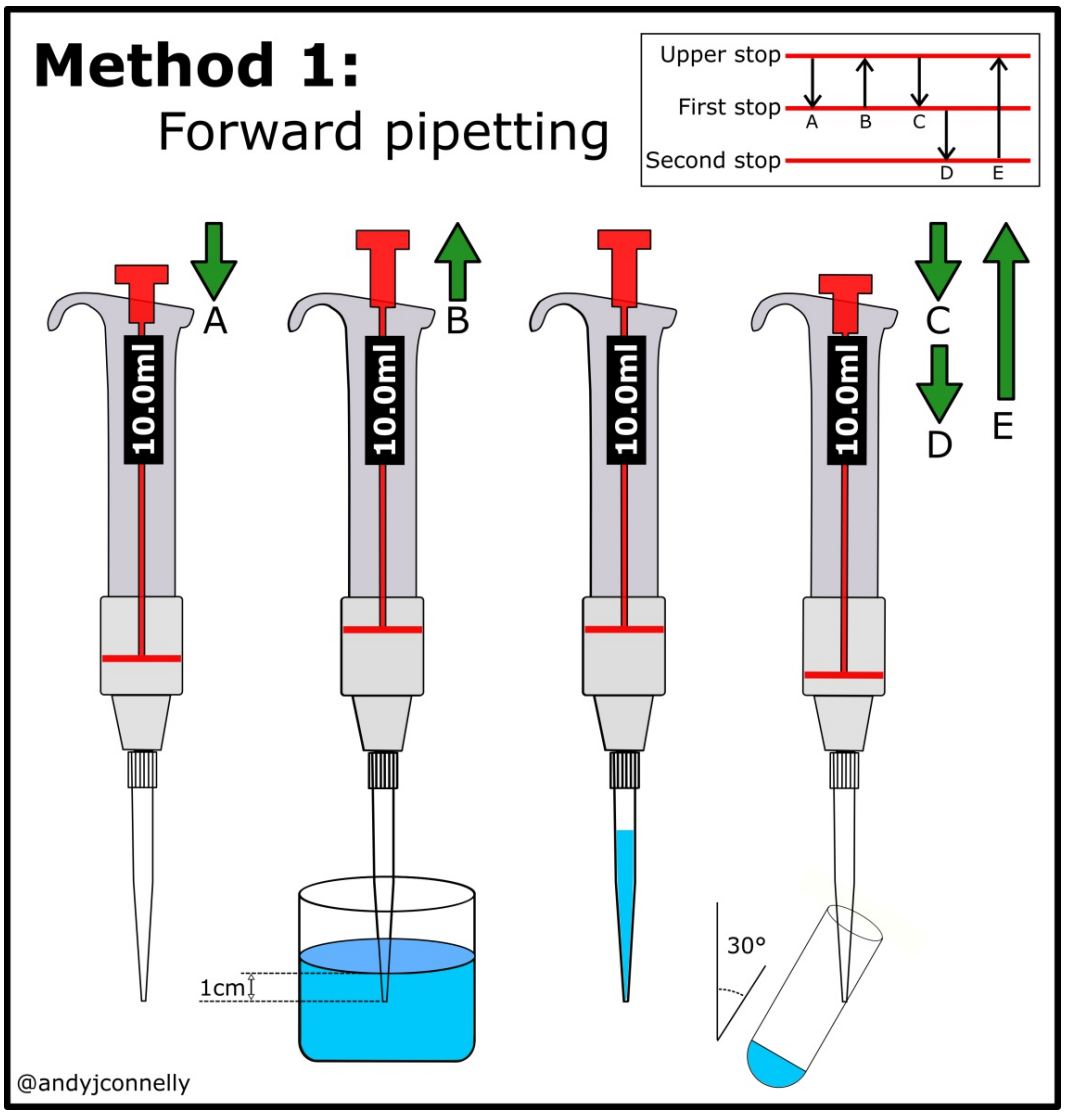
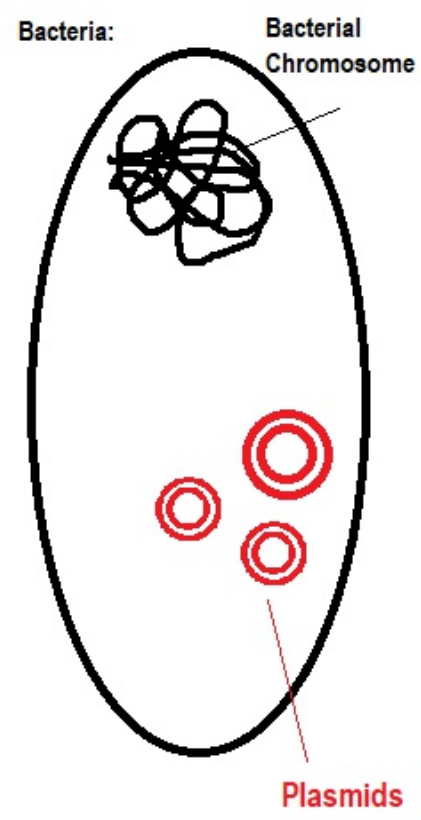


Image Credit: Andy Connelly Science and Laboratory Techniques Blog
https://andyjconnelly.wordpress.com/2017/02/12/practical-pipetting-a-guide/

Part 1: Restriction Digest of Plasmid and Gblock

Estimated time: 1 hour 30 minutes
Description: Welcome to DNA Arts + Crafts, where you will use molecular-sized scissors to precisely cut "a circle" and "a strand" of DNA, then power up a molecular-sized hot-glue gun to seal them together.

Background:

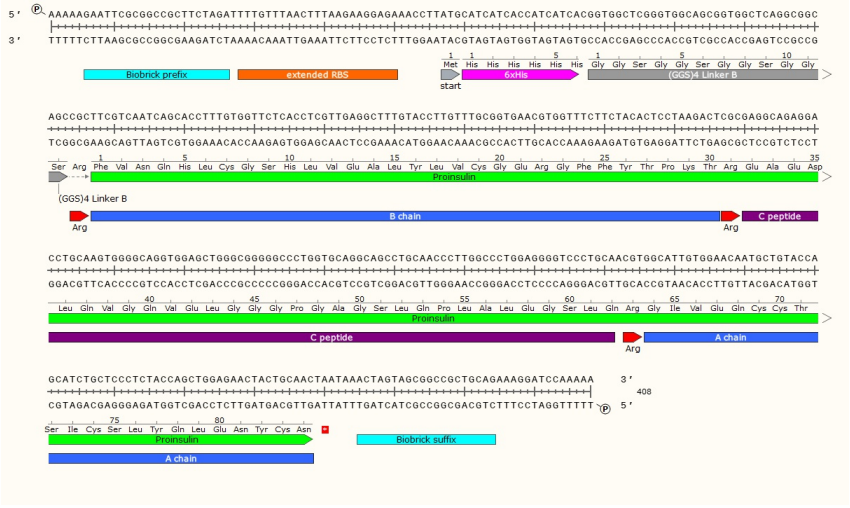


What is a Plasmid?

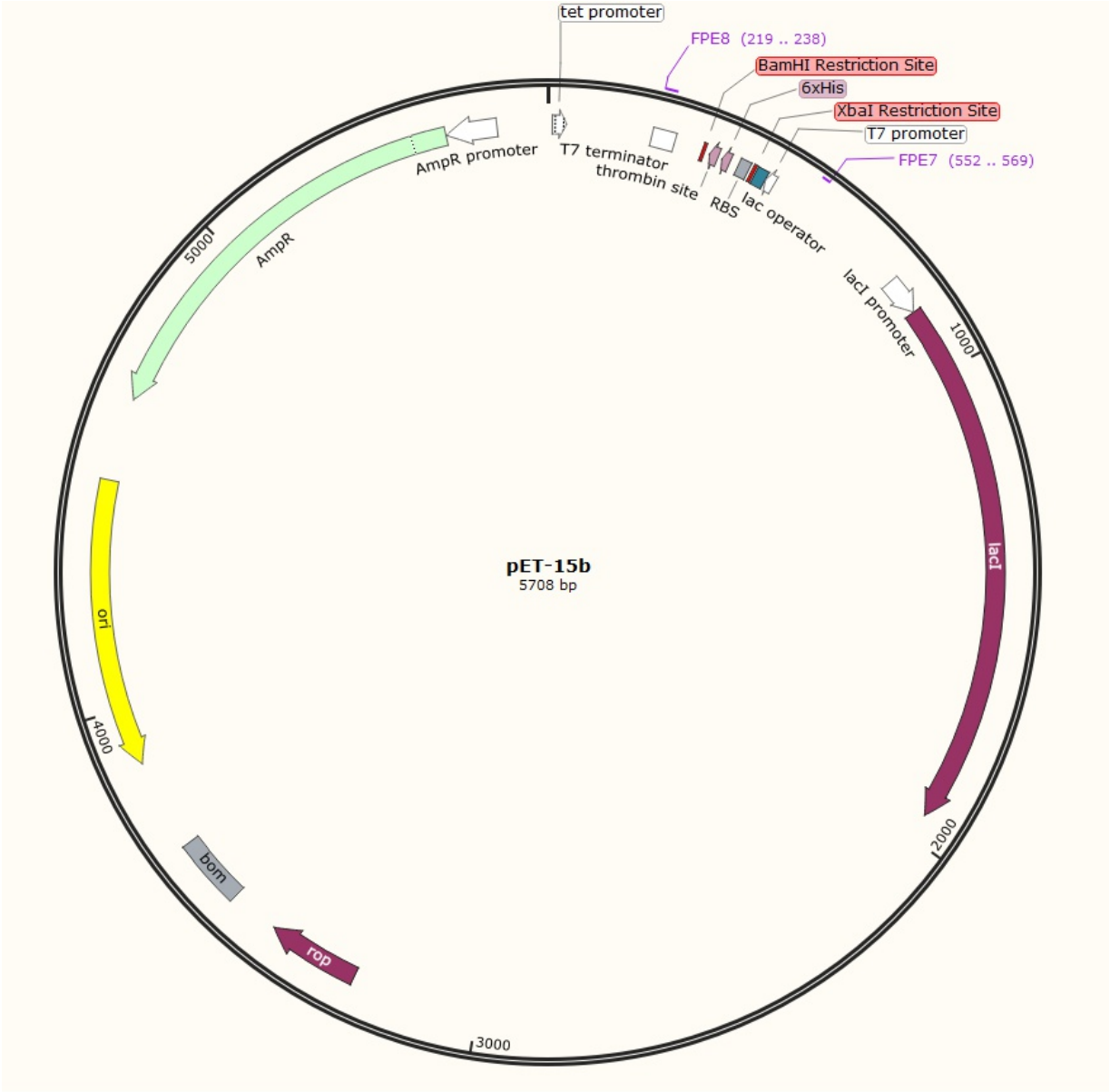
Plasmids are circular pieces of double-stranded DNA, generally between 2,000-20,000 base-pairs in length. They are a suprisingly common motif in nature, especially in bacteria. They are small enough to be transferred across the cellular membrane, but transient enough that a bacterial cell can kick out a plasmid that confers no benefit.
A plasmid acts in a similar manner to the genome while inside the bacteria, the lack of a nucleus allows easy access by transcription/translation proteins to the encoded genes.
That said, plasmids require specific code (ori) to instruct the bacteria to replicate them during binary fission if they are to remain through successive generations.

What is a Gblock?

A Gblock is a large segment of artificially synthesised DNA. The genes used today were originally synthesised by "phosphoramidite chemistry" by the company IDT, according to designs put together by the 2017 USYD iGEM Team.



Let's get specific: pET15b



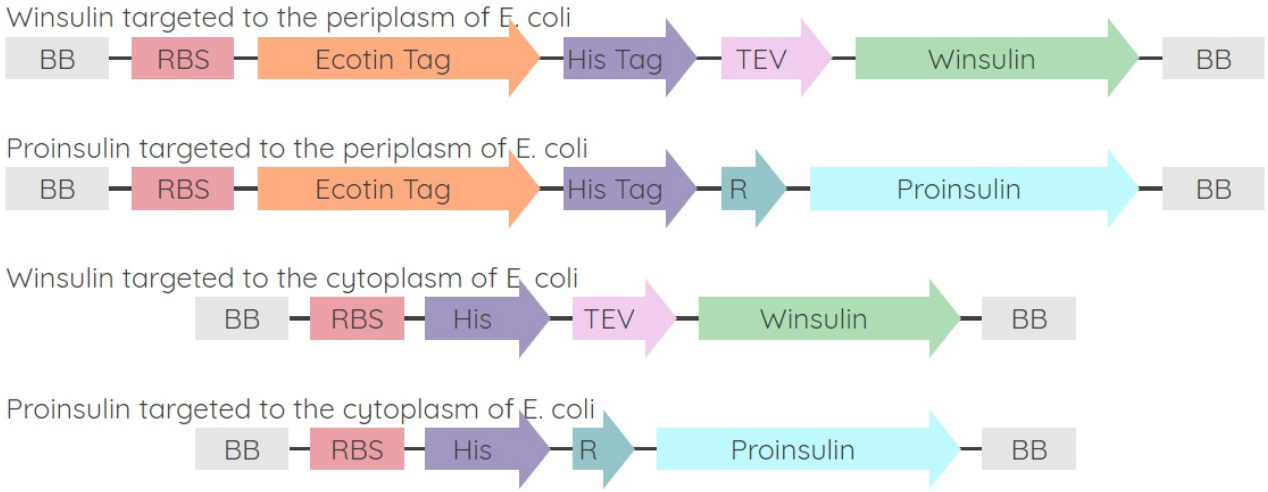
Say hello to today's plasmid, pET15b. This little beauty uses the "T7 Inducible Expression" system, a hijacked and improved cellular pathway that once provided lactase in the presence of lactose/IPTG. Instead we're going to use this system to instruct the cell to produce human insulin in the presence of lactose/IPTG.

Take note of the BamHI and XbaI sites! This is where our restriction enzymes will cut, so features in the small region between these sites will be cut out and replaced by our insulin gblock.

Knowing this, lets go through some of the relevant features of this plasmid;

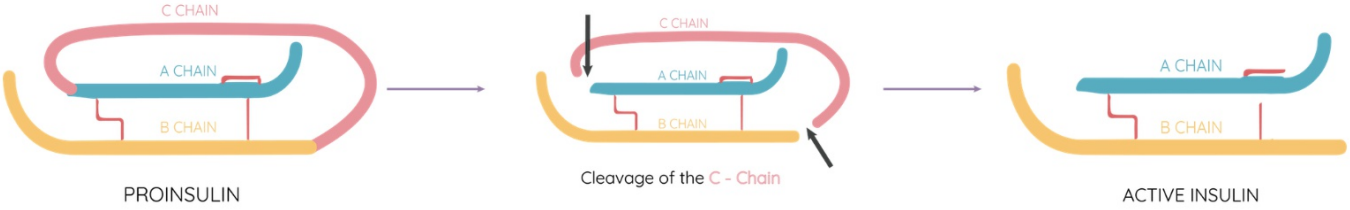
- AmpR/AmpR promoter: The Ampicillin resistance gene + promoter.
- ori: Origin of replication, tells the cell how much plasmid to make and how to split plasmids during binary fission.
- lacI promoter/lacI: Lac repressor, prevents expression of gene adjacent to lac operator unless lactose/IPTG is present.
- T7 Promoter: Very strong promoter, encourages binding of T7 polymerase.
- Lac Operator: Repressor binds here until lactose/IPTG is present.
- XbaI/BamHI Restriction Site: 6-base-pair recognition sequences
- RBS: Ribosome Binding Site (removed, but included in Gblock)
- T7 terminator: Forms a stem-loop structure that unbinds T7 polymerase.

What about those Gblocks?



These gene sequences vary in length between 300-600 basepairs and were designed using research papers and expired intellectual property:

- BB: Biobrick prefix and suffix. These each contain 3 different restriction enzyme sites, allowing for a dynamic choice when choosing where to insert these genes.
- RBS: Strong Ribosome Binding Site. Once the T7 RNA polymerase transcribes these genes from the plasmid, this sequence will encourage the Ribosome to bind the RNA strand and initiate translation.
- Ecotin Tag: Ecotin is a secondary protein that can be fused to insulin to encourage export to the Periplasmic Space (between the outer membranes of a gram-negative bacteria). This is important as the periplasm is an oxidizing environment, which will help insulin form the disulfide bridges necessary to fold correctly.
- His: 6x Polyhistidine Tag. This 18 base-pair sequence encodes 6 Histidine Amino Acids. This is useful for protein purification due to histidine's affinity for the Nickel ions used in column chromatography.
- TEV/R: Protease cleavage sites. Once bound on the affinity column and washed of contaminants, enzymes can be added that will cleave the insulin from the rest of the molecule at these sites.
- Proinsulin: The immediate precursor to Regular Human Insulin. Produced naturally in our pancreas, proinsulin is stabilised by the C-peptide - which is cleaved to activate the insulin.
- Winsulin: A single-chained analogue of human insulin, designed to prove a point. More on this later.



Sticky Ends: Using two restriction enzymes to ensure correct orientation of the gene!

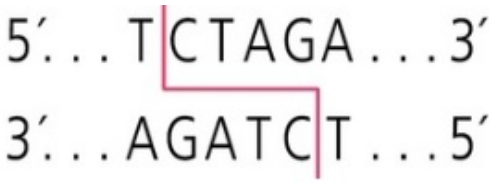
Meet our Enzymes, BamHI and XbaI

(the I is an i not a 1, roman numeral style!)

These precise proteins hunt down a specific 'recognition sequence" - bind the DNA, and the induce a cut (observe the red line).

BamHI

XbaI



These cuts each leave 4-base-pair single-stranded overhangs, known as "Sticky Ends". If we cut both the plasmid + gblock with the same two enzymes, the sticky ends will match - and we will be able to succesfully ligate them together!

Protocol i:

Be careful to minimise the amount of time that materials are taken off the ice! Many of the materials used in synthetic biology are very heat sensitive!

1) The following materials should be assembled in your ice box. Due to the expensive nature of the enzymes, you may need to take turns. Pass the group ice-box around to minimise heat-exposure to the enzymes.
    - pET15b plasmid
    - Gblock (Cpro, Ppro, Cwin or Pwin)
    - Cutsmart Restriction Buffer (group ice box)
    - BamHI (group ice box)
    - XbaI (group ice box)
    - Sterile RO water
2) Label a 1.5mL eppendorf tube as “Plasmid Digest (your initials)” and place on ice.
3) Add materials to the tube in the following order, changing tips EVERY TIME!
    - 10µl restriction buffer
    - 84µl Sterile RO water
    - 2µl pET15b plasmid
    - 2µl BamHI
    - 2µl XbaI
4) Mix by flicking, then quickly spin in centrifuge to get liquid to the bottom of the tube.
5) Label a second 1.5mL eppendorf tube as “Insert Digest (your initials)” and place on ice.
6) Add materials to the tube in the following order, changing tips EVERY TIME!
    - 10µl restriction buffer
    - 84µl Sterile RO water
    - 2µl PCR Product (Cpro, Ppro, Cwin OR Pwin)
    - 2µl BamHI
    - 2µl XbaI
7) Mix by flicking, then quickly spin in centrifuge to get liquid to the bottom of the tube.
8) Incubate both tubes in the 37°C incubator for one hour - set a timer!

Part 2: Confirmation of Digest - Agarose Gel Electrophoresis

Estimated time: 1 hour
Description: Okay you've just mixed 5 indistinguishable clear liquids together - are you getting suspicious that I might be playing sillybuggers, watching you mix water with water and chuckling to myself? Rest assured, in this section you will (hopefully!) get to visualise your DNA and confirm that it is the right size.

Background:

DNA has a negative charge that increases linearly with the length of the DNA strand.
By loading our DNA into a salty, agarose jelly and running an electrical current through it, we can seperate out DNA strands based upon size.
Smaller = faster!

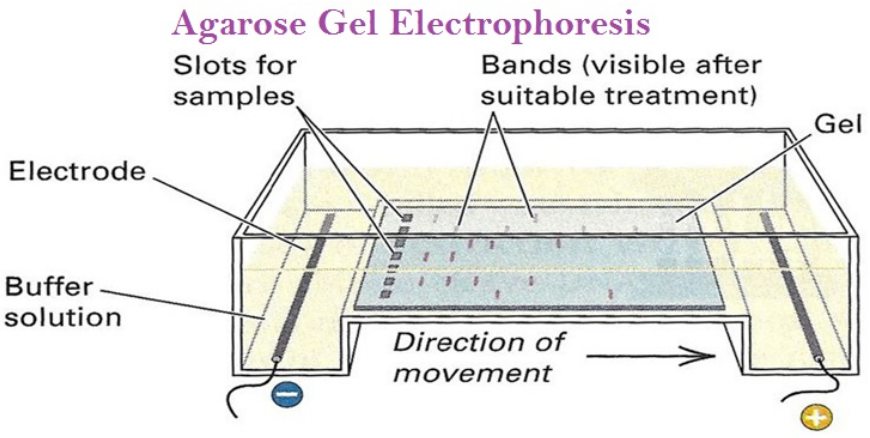
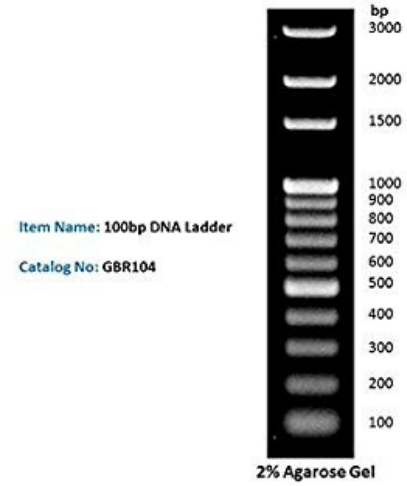


Image Credit: Microbiology Online Notes
https://microbiologyonlinenotes.com/agarose-gel-electrophoresis/

An Unclimbable Ladder

Context is key! Without anything to compare it to, a band on an agarose gel is pretty useless. A DNA ladder is a Size Standard, with a dozen quantified lengths of DNA mixed in an equimolar ratio. A correctly loaded DNA ladder is satisfying to observe, and even more satisfying to use.



A Tale of Two Dyes

Simply pulling DNA through the gel will be useless if it cannot be visualised. To facilitate this, we use two dyes for two distinct purposes:

GelGreen: An intercalating Nucleic Acid stain, this dye will be mixed into the agarose before it solidifies. When exposed to UV light, it will fluoresce - strongly increasing in intensity once bound within (intercalated) the DNA.

6x Loading Dye: A colour marker, this dye will be mixed directly with our DNA sample before it is loaded, helping the sample sink fully into the well. Clear bands in the visual spectrum allow for the progress of electrophoresis to be monitored, but this dye will not fluoresce when exposed to UV light.

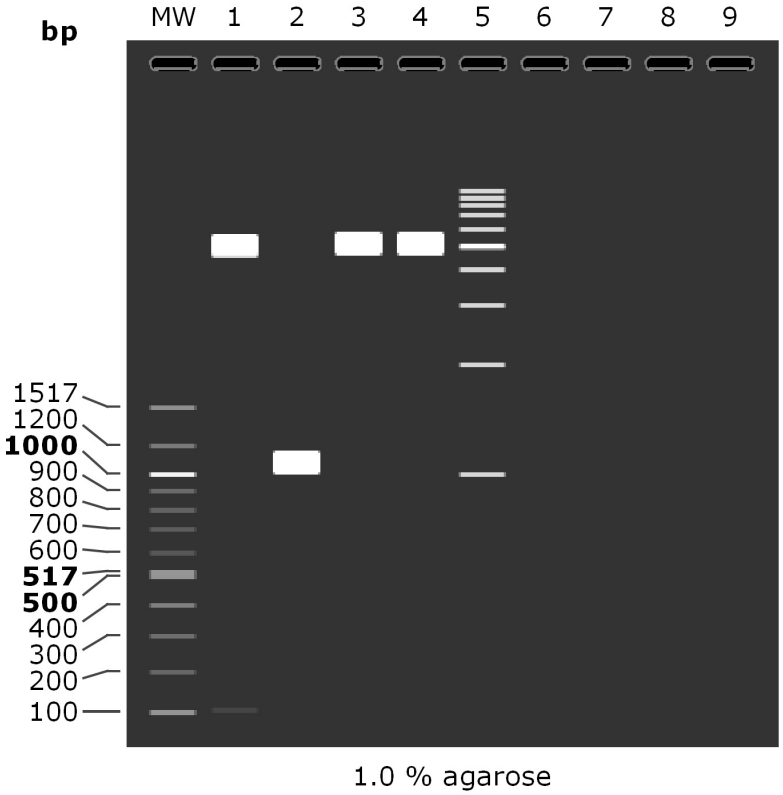
Protocol ii:

Caution: Because of the properties of agarose, the solution can SUPERBOIL when in the microwave. Always use the heatglove, and take care!

As a group:

1) Weigh out 0.7g of Agarose into a weigh boat.
2) Measure out 70mL of 1xTAE buffer into the measuring cylinder
3) Add Agarose and TAE to a conical flask and microwave for 90 seconds on high heat.
   CAUTION: Agarose can “superboil” - always use a heat glove when removing from the microwave, stir cautiously at first to expel heat.
4) Assemble the gel-tray and seal the edges with sticky-tape.
5) Add 7µl of GelGreen or ETBR to the molten agarose solution, mixing well.
   CAUTION: ETBR is a carcinogen, wear PPE.
6) Line the edges of the gel tray with a thin line of molten agarose solution to create a strong seal. Let it set over 2 minutes.
7) Pour the remaining molten agarose solution into the gel tray, watch out for leakage!
8) Add the comb to the gel and set gel box aside to harden.
9) Once the gel is solid, remove the tape, side-panels and comb.
10) Add the gel and tray to the gel electrophoresis bath with the wells furthest away from the positive electrode (RUN towards RED).
11) Take turns loading Plasmid + Insert Digests: Mix 5ul of sample with 2ul of 6x Loading dye (on parafilm) and add to well. Ladder will be loaded by demonstrator.
12) Attach the powerpack (once again, red lead furthest away from the wells) and run at 100V for 1 hour.
13) At the end of one hour, turn off the powerpack, remove the gel and place on the UV transilluminator. Gear up in PPE before turning on the transilluminator.

Expected Result:



1.0 % agarose

MW: 100 bp DNA Ladder
1: pET-15b
XbaI + BamHI
1. 5599 bp
2. 109 bp
2: pET15b-PPro PCR
1. 1147 bp
3: pET-15b
XbaI
1. 5708 bp
4: pET-15b
BamHI
1. 5708 bp
5: 1 kb DNA Ladder

Simulation of gel courtesy of SnapGene's excellent software. They have a free trial dontchaknow?

Part 3: Ligation

Estimated time: 2 hours
Description: DNA Arts + Crafts Part Deux, this time featuring an extremely selective molecular stapler (or welder, hot-glue-gun, allegory-of-your-choice) - requiring complementary sticky ends in order to repair the 'breaks' in the backbone.

Background:

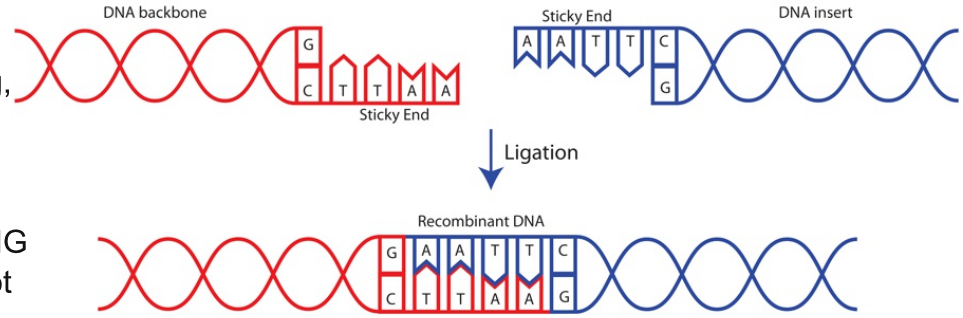
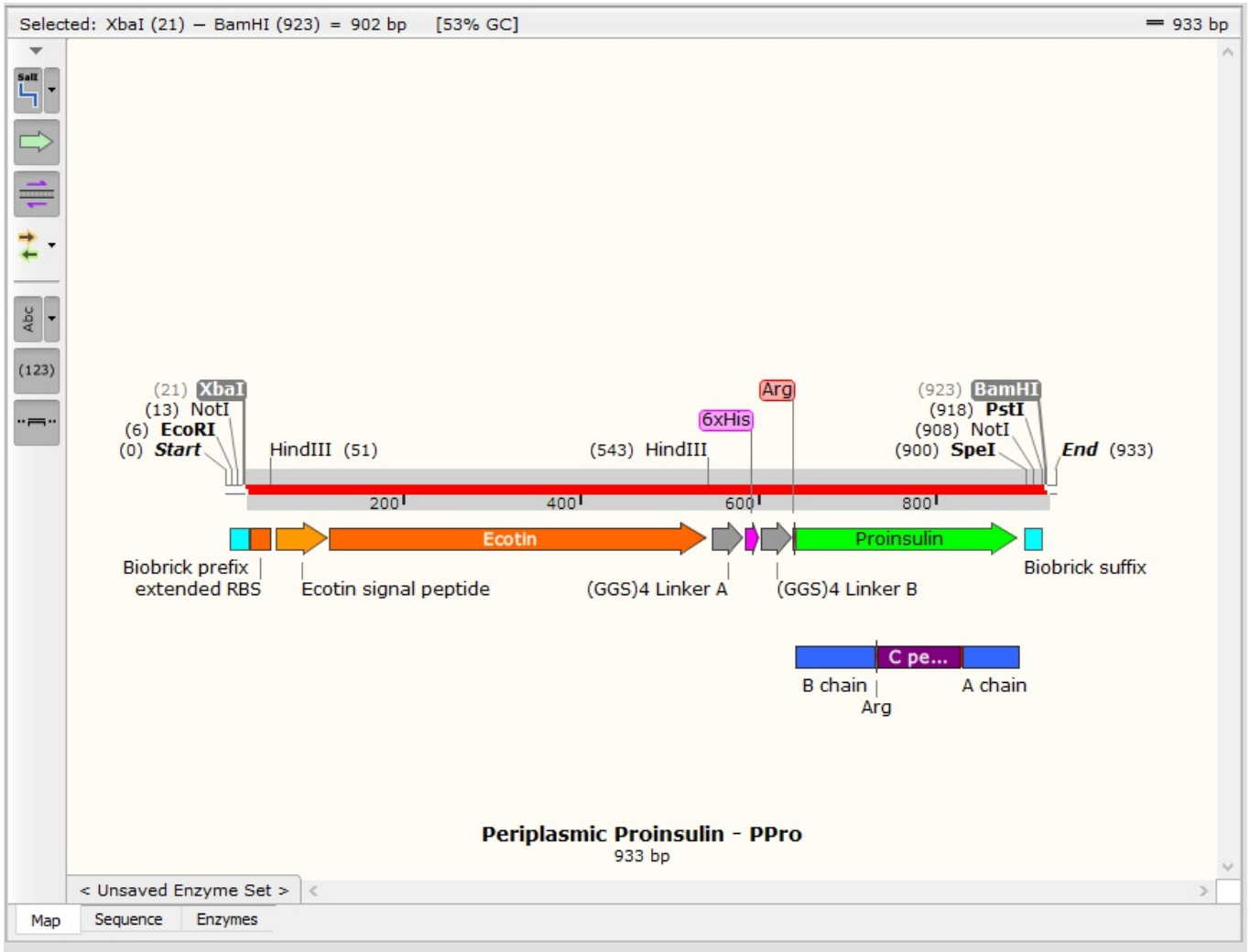
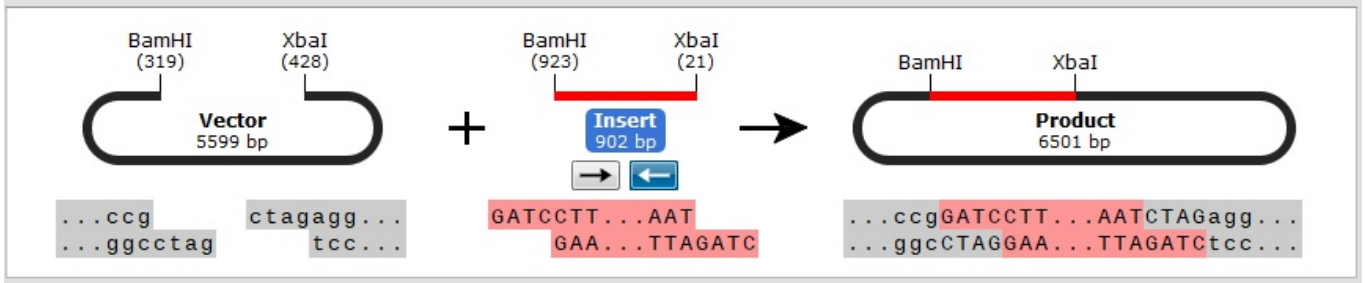


Image Credit: AddGene Blog, DNA Ligation.
I HIGHLY RECOMMEND THIS RESOURCE FOR DEEPER SYNBIO LEARNING
https://www.addgene.org/protocols/dna-ligation/

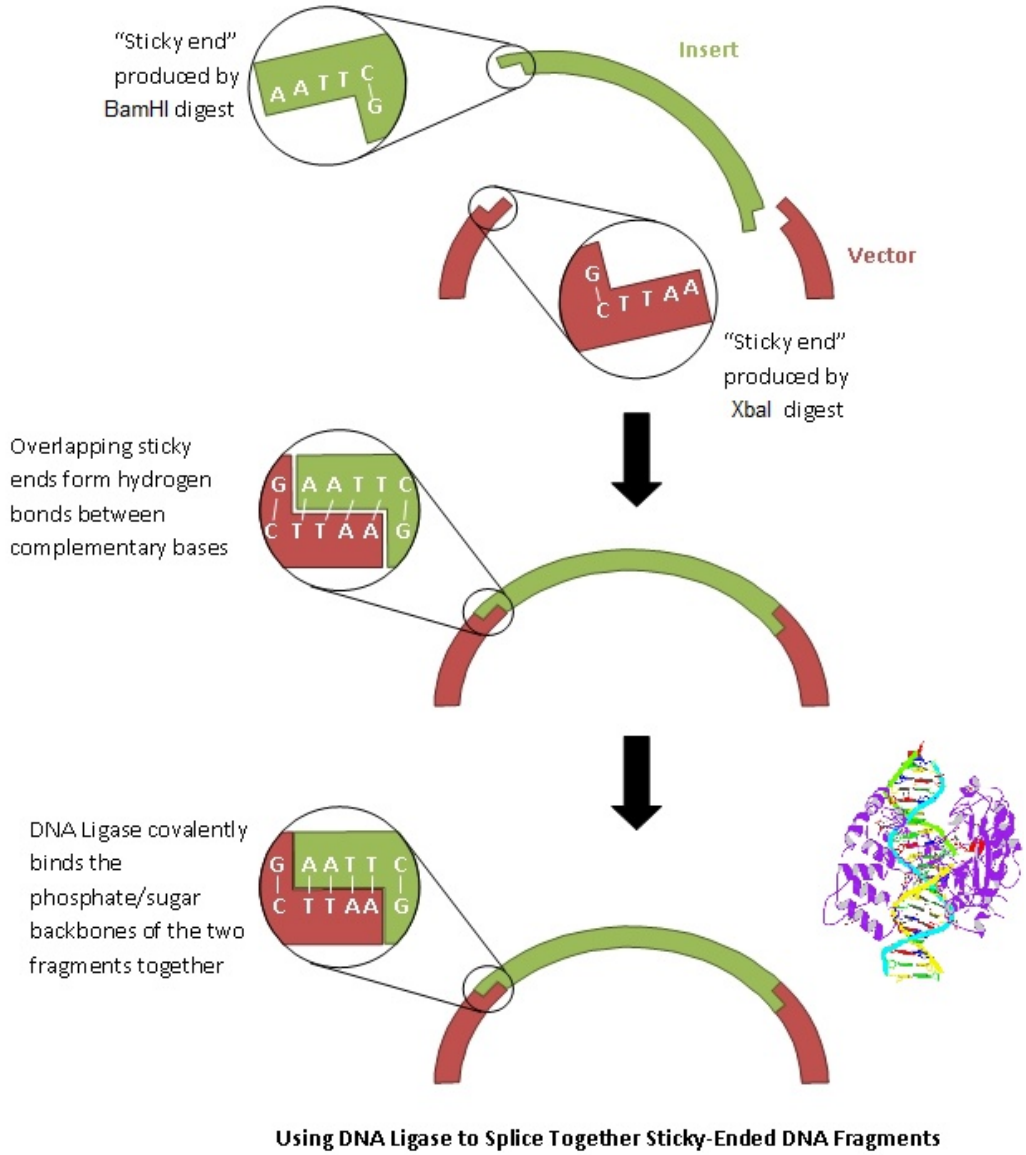




Protocol iii:

Caution: T4 DNA Ligase requires ATP to function, which is contained in the T4 Ligase Buffer. It is extremely heat-sensitive, keep submerged in ice at all times!

1) The following materials should be assembled in the ice boxes, MINIMISE any warming! These materials are considered extremely heat-sensitive in an industry where everything is heat-sensitive;
   - 10µl 10x Ligase buffer aliquot (group ice-box)
   - T4 Ligase Enzyme (group ice-box)
   - Your Plasmid digest
   - Your Gblock digest
2) Label a 1.5mL Eppendorf tube with “Ligation (Your Initials)” and set up on ice (Be careful to not get ice in the tube - this is not sterile!)
3) Add reagents to the Ligation tube in the following order, changing tips every time!
   - 2µl of 10 x ligase buffer
   - 8µl of insert DNA
   - 8µl of plasmid DNA
   - 2µl of T4 DNA Ligase Enzyme
4) Mix by briefly flicking, then spin down in the centrifuge and incubate at room temperature for 1 hour.
5) Return the ligase enzyme immediately to -20°C freezer. Throw out any unused thawed ligase buffer.



Crystal structure source: https://www.rcsb.org/structure/6DT1

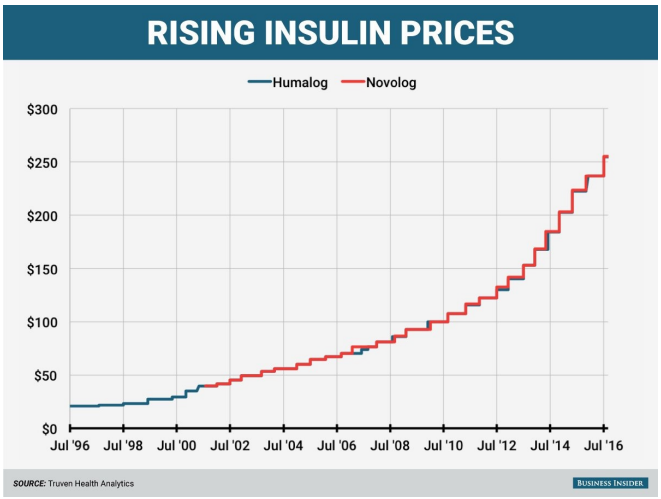
Using DNA Ligase to Splice Together Sticky-Ended DNA Fragments

Image credit: https://di.uq.edu.au/community-and-alumni/sparq-ed/sparq-ed-services/dna-ligation

Part 4: Lunchtime Economics

Estimated time: 1 hour
Description: While our DNA glues together, we'll head down to the atrium for lunch at Funkychino. While you eat, we'll have a roundtable discussion about the market issues at play and brainstorm solutions to the insulin accessibility problem.

A Collusive Oligopoly?

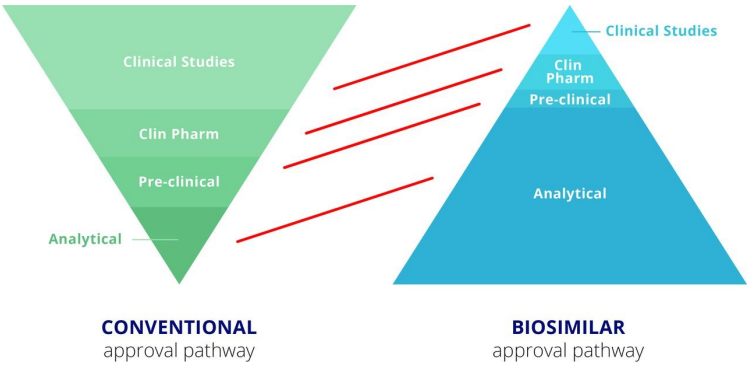


92% of the Global insulin market is dominated by three major players; Eli Lilly, Novo Nordisk and Sanofi.
The remaining 8% of the market includes a number of generic manufacturers, some of which are owned by the above pharma giants. Indian generic manufacturers 'Biocon' and 'Wockhardt' manage to keep their insulin price under US$2, albeit this is subsidised nationally by the Indian government.
Some questions to prime discussion:
Why might the price be increasing globally?
Why do the prices increase in lockstep?
Does a "free market" exist here?
Why are there so few generics?

Same drug, Same action... Different bacteria

Some definitions for you:
Biosimilar - is a biologic medical product that is almost an identical copy of an original product that is manufactured by a different company.
Clinical Trials - Clinical trials are research investigations in which people volunteer to test new treatments, interventions or tests as a means to prevent, detect, treat or manage various diseases or medical conditions.
Stage 0: 10 People - Pharmacokinetics; particularly, oral bioavailability and half-life of the drug.
Stage 1: 20-100 People - Testing of multiple drug doses on healthy volunteers for safety.
Stage 2: 100-300 People - Testing on patients to assess efficacy and side effects.
Stage 3: 300-3000 People - Testing on patients to assess efficacy/effectiveness/safety.
Stage 4: Market Surveillance, assessing ongoing efficacy/effectiveness/safety.
Biosimilar Clinical Trial: ~800 People - Testing of drug on patients after structure is confirmed.
Good Manufacturing Practice (GMP) - is a system for ensuring that products are consistently produced and controlled according to quality standards. It is designed to minimize the risks involved in any pharmaceutical production that cannot be eliminated through testing the final product.

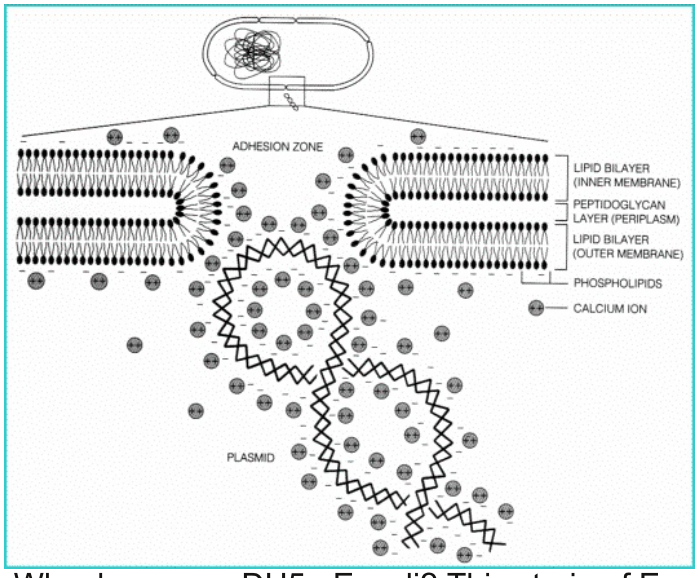
Image Credit:
Insulin Prices:
https://www.businessinsider.com.au/insulin-prices-increased-in-2017-2017-5?r=US&IR=T
Biosimilar Approval Process:
https://considerations.bmj.com/content/1/1/3



Part 5: Heat-Shock of Ligation Mixture into CC DH5α E. coli

Estimated time: 90 minutes
Description: We've now constructed our synthetic circuit, containing all the information necessary for the inducible production of insulin. We will now attempt to insert this plasmid into a strain of E. coli specially bred for the production of high-quality DNA.

Background:



Chemically competent (CC) cells are specially treated with calcium/rubidium chloride to facilite attachment of plasmid DNA to the cell membrane. The bacterial cell membrane is permeable to chloride ions, but is non-permeable to calcium ions. As the chloride ions enter the cell, water molecules accompany the charged particle. This influx of water causes the cells to swell and is necessary for the uptake of DNA. The exact mechanism of this uptake is unknown, but it has been suggested that the wider pores in the cell membrane may cause the DNA to be "sucked in" when the temperature is changed rapidly. The calcium ions MAY facilitate this by neutralising the DNA phosphate groups and inner core of membrane phospholipids.

Why do we use DH5α E. coli? This strain of E. coli has been engineered to be more efficient at recieving and reproducing DNA. The recA1 mutation disables specific recombinases that cause homologous recombination. The endA1 mutation disables the production of specific enzymes that degrade plasmid DNA when purified.

Will this strain produce insulin? No, not yet. Before producing insulin using the plasmid you've just made, it must be grown and repurified, then heat-shocked into a new strain: BL21(DE3). This strain of E. coli has been engineered to use the T7 Inducible expression system. In addition, this strain is deficient in Protease B - allowing each bacterial cell to accumulate large (even lethal) quantities of protein without degrading it.

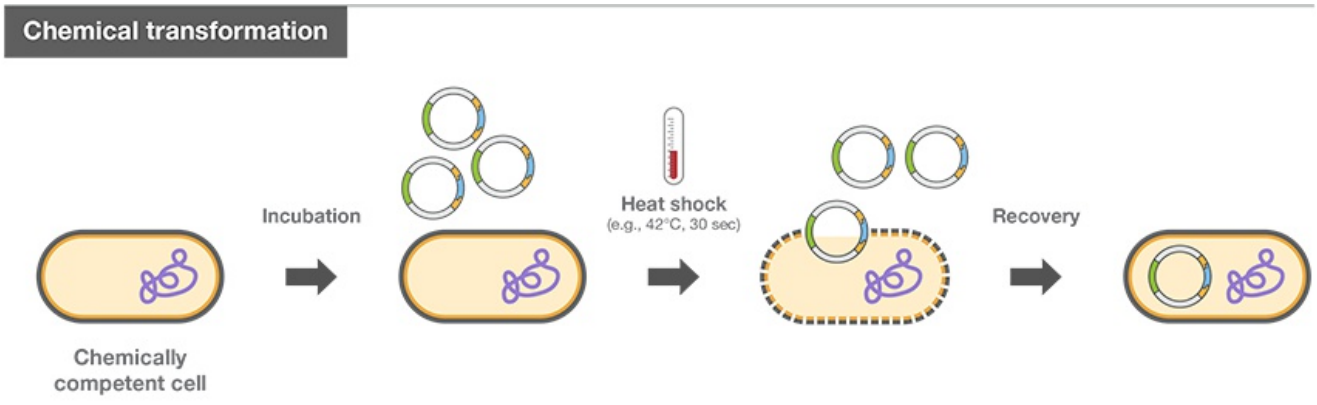


Image credit: https://www.thermofisher.com/au/en/home/life-science/cloning/
https://www.sciencedirect.com/science/article/pii/S0022283683802848?via%3Dihub

Protocol iv:

Caution: The -80°C freezer is cold...very cold! Let the demonstrator take the cells out for you and before of freezer burn while thawing them!

1) Turn on Heat-block to 42°C
2) Remove one aliquot of chemically competent DH5α E. Coli cells from the -80°C freezer. Thaw the cells by rubbing them in your hands, but don't let them stay warm! As soon as they are thawed, put them on ice.
3) Divide the cells into 50 µl aliquots (1 per group!) in separate Eppi tubes on ice. Label the tube with "HS (Your Initials)"
4) Add 10 µl of ligation mixture to the chemically competent cells.
5) Ensure at least the bottom half of the tube (approx 2 cm) is embedded in the ice, don't just rest them on top of the ice. Allow the cell/DNA mixtures to incubate on ice for 30 minutes.
6) The demonstrator will include both a positive control and a negative control in the transformation experiment. The positive control should be 1 µl of a plasmid with the correct antibiotic resistance (same resistance as the plasmid used for the ligation), and should also be a plasmid stock that you know is in good condition (based on agarose gel).The negative control is simply no DNA added.
7) Take your esky of ice over to the 42°C heat block. Put your tubes into the block. Allow 45 seconds for heat shock. (Plus or minus 10 seconds, this needs to be exact!). Then transfer the cells straight back onto ice (embed into ice, as above, don't just rest on top)

Comparison of Chemical Transformation (Heat-Shock) protocol with Electroporation, a commonly used alternative procedure for convincing E. coli to take up plasmid DNA:

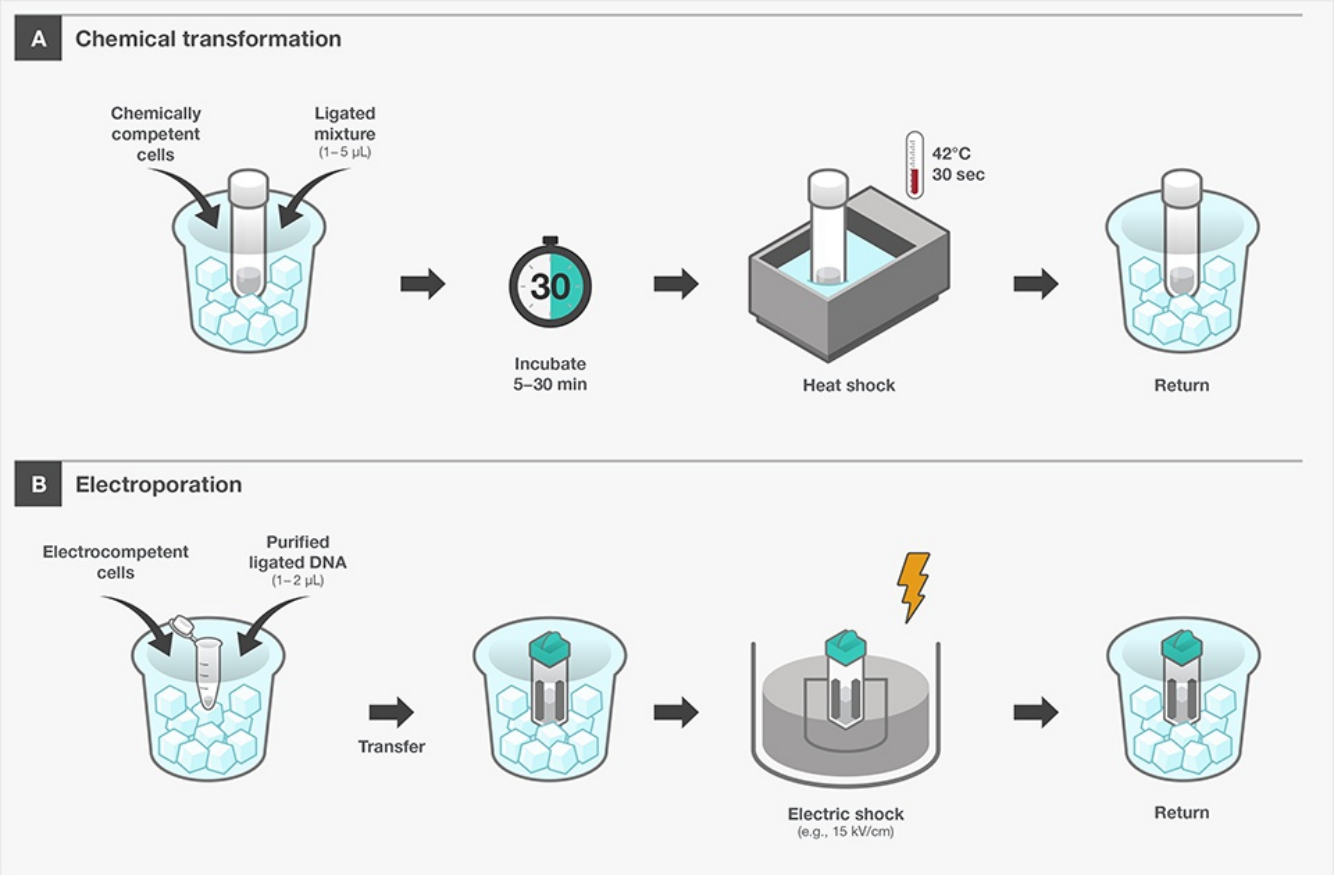


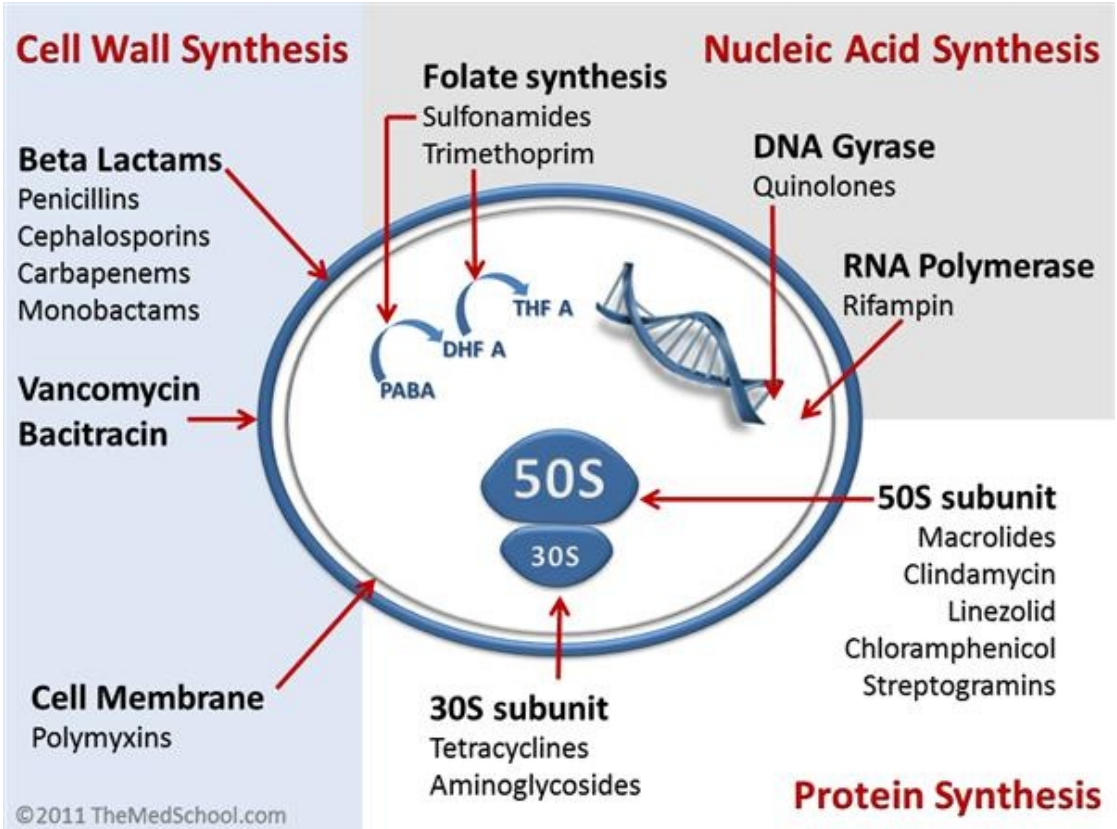
Image credit: https://www.thermofisher.com/au/en/home/life-science/cloning/cloning-learning-center/invitrogen-school-of-molecular-biology/molecular-cloning/transformation/bacterial-transformation-workflow.html

Part 6: Recovery and Plating

Estimated time: 1 hour 15 minutes
Description: Phew! That was tough - moving those cells back and from the waterbath, time for a break right? Okay it wasn't so difficult for you, but your bacteria have been through a lot. Time to give them a feed and an hour to grow and replicate. But most importantly... to get to work building the antibiotic resistance protein we just gave them.

Background:

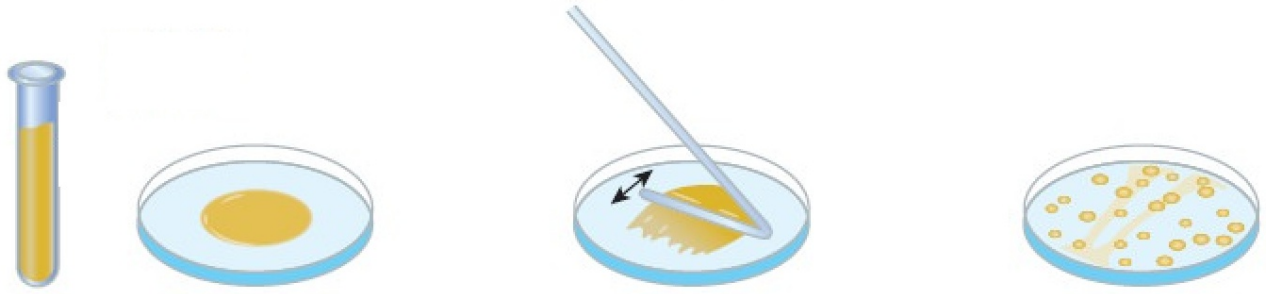
As mentioned previously, these bacteria won't be producing insulin themselves - they lack the T7 RNA Polymerase gene to make use of our insulin expression system, but that is not true for other genes encoded on our plasmid. Most importantly, the Ampicillin-resistance gene is under constitutive (constant, opposite of inducible) expression, i.e. it is always on.
We include antibiotic resistance in our plasmids for two very important reasons;
1) Positive Selection - Tomorrow when I examine your agar plates to look for successfully transformed bacteria, I will need a method to 'tell at a glance' whether you succeeded or not. By plating onto an agar plate that contains Ampicillin, I'll know that 100% of colonies on your plate have taken up a plasmid.
2) Ensuring our gene is necessary for survival - E. coli are efficient organisms, and a plasmid that conveys no evolutionary benefit will quickly be 'spat out' into the surrounding medium. Since we will be plating our E. coli onto agar plates that contain Ampicillin, these bacteria will have no choice but to keep the plasmid - as well as the insulin-producing genes it contains! However, if we were to immediately plate your bacteria onto the antibiotic plate, it would be carnage - none would survive. They've only just recieved the DNA and have had no time to produce the protein encoded protein, we must give them an hour to recover!



How Antibiotics Kill; Image credit: https://upload.orthobullets.com/topic/9059/images/antibiotics_mechanisms_of_action.jpg

Protocol v:

1) Add 1 ml sterile LB broth to each heat-shock mixture.
2) Incubate in the 37°C incubator for 1 hour. Occasional shaking of the cell mixture during this time will increase the growth rate.
3) Label two LB-Ampicillin plates before starting the next bit;
- (Your Name), (Date), Insert-Plasmid-Cell, 100µl
- (Your Name), (Date), Insert-Plasmid-Cell, Pellet
e.g. Alex Kelly, 11/06/19, Ppro-pET15b-DH5α, Pellet
4) Pipette 100 µl of the first cell suspension onto the 100µl LB-antibiotic plate. Sterilise the glass spreader with ethanol and flame and spread the cells around the plate with the spreader. Do this by pushing the spreader with a back-and-forth motion, while turning the plate around in a circular motion. Be careful not to touch the spreader on your fingers! Keep spreading for approx 10 seconds. If the plates are properly dried, you should feel the spreader start to ‘stick’ to the agar, this means the liquid has been drawn into the agar.



5) Centrifuge all the tubes at max speed for 1 minute in a micro-centrifuge. Pour off most of the supernatant into culture waste (being careful not to touch the tubes on the edge of the culture waste bottle). Leave a little bit of liquid behind (surface tension will hold a bit in the bottom of the Eppi)
6) Pipette the suspension onto the plate labelled “Pellet” - spread with the glass spreader using the described method.
7) Incubate all plates at 37°C overnight.
8) Photographs of your plates will be sent to your email. Transfer of your colonies, or the standard Open Insulin strains can also be organised. If you ask really nicely, I can even repurify your plasmid DNA/heat-shock it into BL21(DE3) for you. Rules for transfer of GMOs apply!

If you forget to ask for my contact information, just message the Biofoundry Facebook page: https://www.facebook.com/thebiofoundry/

Image credit: https://www.brainkart.com/article/Pure-Culture_35236/

Conclusion: Building an Open Insulin Lesson Plan:

Estimated time: To be dicussed throughout
Description: We're scientists, not high-school teachers! We have all this wonderful knowledge, but you are the ones whom we entrust the responsibility of teaching the youth about this world of opportunity!

Relevant Syllabus Points for Year 11-12 Biology Teachers: ACSBL076, ACSBL077, ACSBL086, ACSBL087

After the discsussion: Describe a series of lessons that could be used to teach the Open Insulin project to high school students.

Also: Describe the online platforms with which you could communicate with other teachers and the International Open Insulin Collaboration:

Open Insulin Collaboration Form

If you'd like to join our global network of scientists and educators to help make life-saving medicine affordable for every human-being on this earth, fill out this page and hand it to your demonstrator by the end of the lesson.

Full Name:

Preferred Contact Method:

Phone: Email: Facebook URL:

School you will represent:

(or) I wish to join as an individual researcher!

Area of research that most interests you (tick all that apply):

1) Creating new insulin-producing plasmids for expression (DNA work)

2) Attempting to induce protein expression using existing plasmids/strains (protein work)

3) Seeking to improve methods of protein purification (protein work)

4) Building an open-source insulin pump (engineering work)

5) Coding open-source software to recode existing insulin pumps (software work)

6) Other, please specify: _____

Do you wish to recieve regular updates about progress on Open Insulin? Yes No

Do you consent to the release of this personal information (strictly for the purpose of collaboration) in the knowledge that we will never release it to an annoying spam company?

YES! No, I just like filling out forms

Signed: _____ Date: _____