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VCE Biology ¾
Recombinant Plasmids [1.7]
Workbook

Outline:

Study Design: CRISPR-Cas9 and Recombinant Plasmids

The use of recombinant plasmids as vectors to transform bacterial cells is demonstrated by the production of human insulin.

Learning Objectives:

- ☐ **BI34 [1.7.1] -** Describe the role of plasmids as a vector to transform bacteria & other cells.
- ☐ **BI34 [1.7.2] -** Explain how a gene of interest is isolated & inserted into a plasmid.
- ☐ **BI34 [1.7.3] -** Explain electroporation & heat shock as methods to transform bacterial cells.
- ☐ **BI34 [1.7.4] -** Describe the process by which bacterial cells can be used to produce human proteins, including the application of this to human insulin production.
- ☐ **BI34 [1.7.5] -** Explain the significance of beta-galactosidase, other reporter genes, & antibiotic resistance genes in the selection of transformed, recombinant bacterial cells.

Section A: Using Recombinant Plasmids for Protein Production

Sub-Section: Introducing Recombinant DNA

How can we get bacteria to produce human proteins?

Overview

- Recombinant DNA is when DNA is "combined" from multiple sources (human, bacteria).
  - We can utilise this to put our own DNA in other organisms to get them to produce our proteins and other gene products.
- This process of inserting recombinant DNA into a bacterium is called transformation, as is often undertaken by a plasmid or another vector.
  - A **vector** is a vehicle carrying foreign genetic material, allowing it to be expressed/replicated within a cell.
  - A plasmid is integrated with a target gene, making a recombinant plasmid, which is placed into bacteria, thereby transforming them.
  - This bacteria will then go and produce the desired protein, which can be collected and purified.

Active Recall: What characteristic of the genetic code allows for this?

UNIVERSALITY → all codons in ALL organisms code for the same amino acid

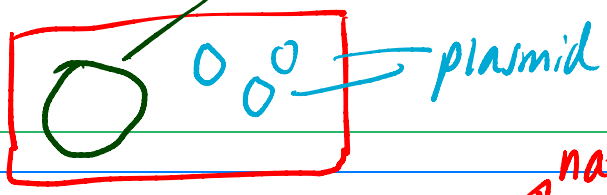
Active Recall: What could be other examples of vectors?

- VIRUS
- PLASMID
- Lipoprotein Particles → vesicle

Sub-Section: Structure of Plasmid

Active Recall: What is a plasmid?

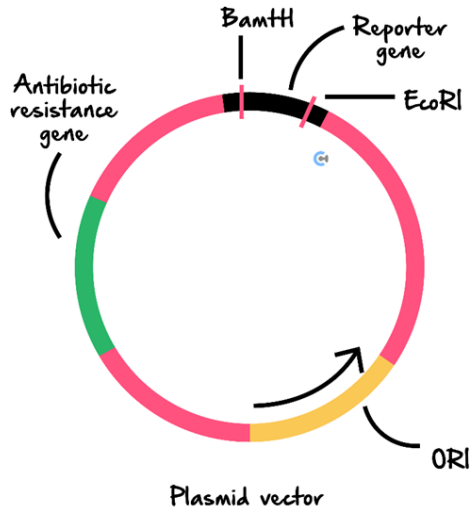
main circular chromosome



Plasmid Vector

naturally helps transfer characteristic

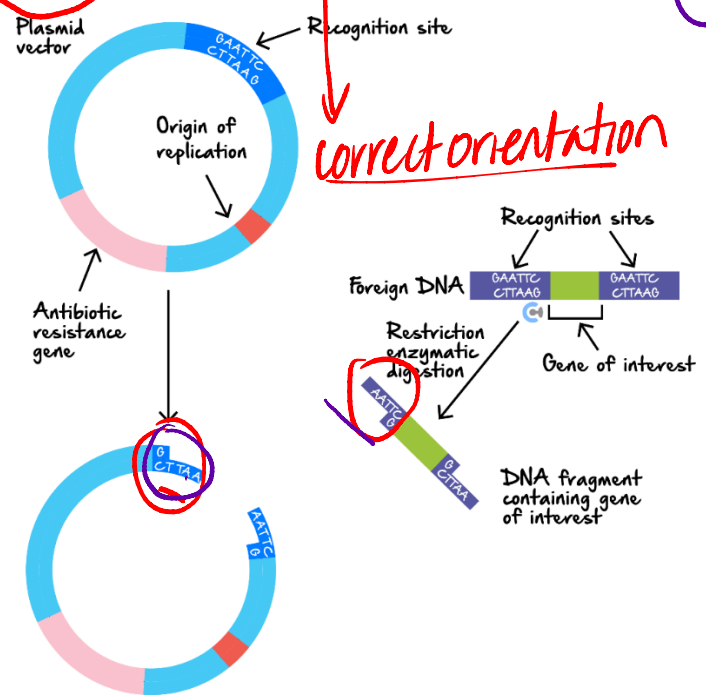
- A plasmid is a small circular piece of DNA found in bacteria, replicating independently from the main chromosome and transferable between bacteria.
- **Origin of replication**: Specific DNA sequence in which replication is initiated.
- **Antibiotic resistance gene**: Gene governing resistance to a specific antibiotic.
- **Multiple cloning site/recognition sites** (MCS): DNA region containing multiple different recognition sites.
  - ↳ endonuclease will cut the DNA
- **Selectable marker**: Genes in plasmid for certain traits that help to distinguish the transformant from the non-transformant cell (e.g. antibiotic-resistant gene).
- **Screening marker**: Sites in vector plasmid that help to confirm that a plasmid is recombinant (e.g. lacZ screening marker). → reporter gene — very distinct phenotype
- **Promoter region:** Upstream region of target gene where RNA polymerase binds to initiate transcription.



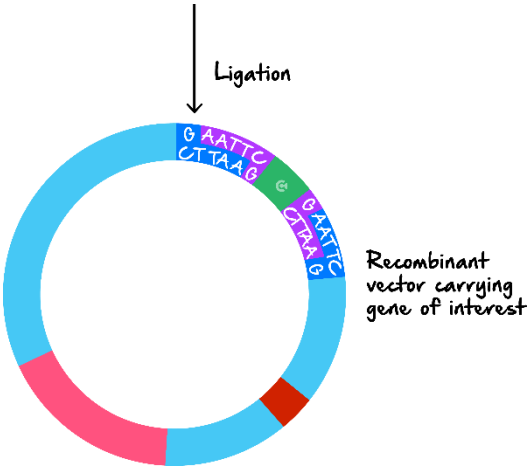
Sub-Section: Making a Recombinant Plasmid

- Adding the gene of interest to the plasmid!

- We must obtain a gene of interest and a plasmid of the structure described above.
  - Both the plasmid and gene of interest are cut with the same endonuclease to create sticky ends, which allow for specificity in the insertion of the gene of interest.



- DNA ligase
  - To join the gene of interest and the plasmid together.



Discussion: What are the possible outcomes at the end of this?

→ target gene is inserted successfully

(1) RECOMBINANT PLASMID

(2) NONRECOMBINANT PLASMID

↳ not inserted successfully

Space for Personal Notes

Sub-Section: Transforming Bacteria

- Putting the plasmid inside the bacteria!

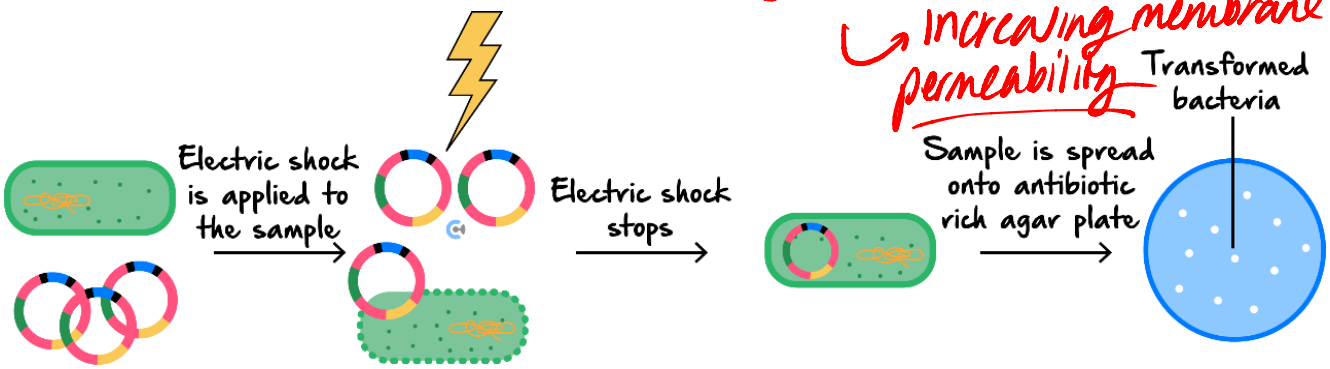
How do we put the plasmid inside the bacteria?

NO - they are large and negatively charged

Can plasmids move easily across the cell membrane?

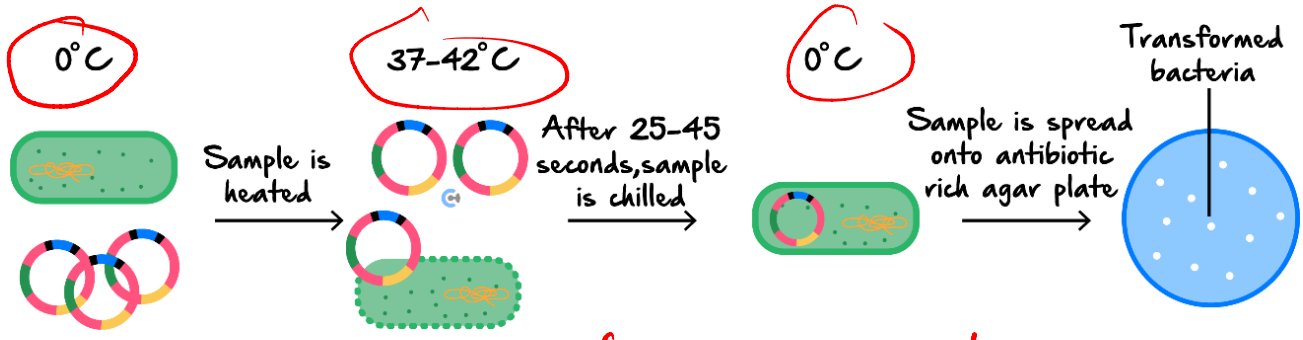
Transforming Bacteria

- We can do this via two methods: electroporation and heat shock.
  - Electroporation involves passing an electric current through the solution containing bacteria and the recombinant plasmids.
  - Creates pores in the membrane, allowing for the DNA to pass through.



increasing membrane permeability

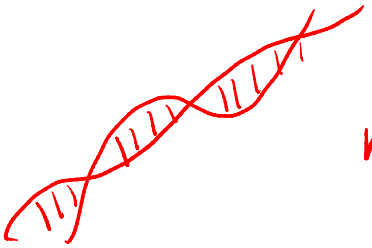
  - The heat shock method involves the solution being included with Ca^{2+} ions, placed on ice. It is then heated to 37-42°C and then placed back on the ice.



increase membrane permeability

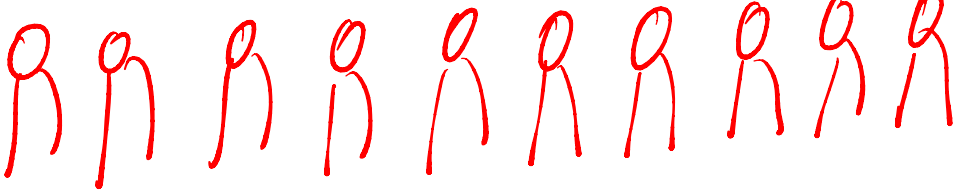
Exploration: Why would calcium ions help with the transformation of the bacteria?

Ca^{2+} ions



negatively charged

phosphate group



phosphate = negatively charged

Ca^{2+} ions are responsible for NEUTRALISING the charge of the membrane, increasing plasmid uptake!

Question 1 (3 marks)

Explain how electroporation and heat shock can be used to deliver plasmids to bacterial cells.

1. Electroporation
2. Heat shock
3. Both of them are responsible for increasing membrane permeability to plasmids, increasing uptake.

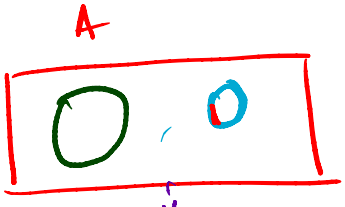
Is this process going to be 100% successful?

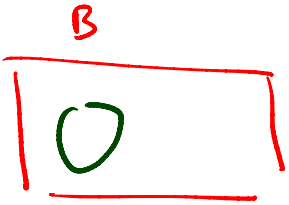
Sub-Section: Selecting Bacteria

- Determining which bacteria have got the recombinant plasmids!

Exploration: Selecting the Bacteria

- Throughout this whole process, we want to make sure we can separate the bacteria that have both RECEIVED a plasmid and also received a RECOMBINANT plasmid.
  - These are the ones that will ultimately be able to produce our desired protein.
- We will have 3 groups of bacteria – those with recombinant plasmid ✓, those with non-recombinant plasmid ✗ and those with no plasmid ✗.
- Antibiotic selection will be able to distinguish the transformed from non-transformed bacteria.
  - How?



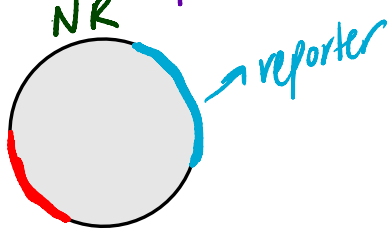


Bacteria with no plasmid will have no resistance gene ∴ will die when antibiotic is applied

those WITH plasmid will survive

- The bacteria that have taken up the recombinant plasmid would be determined using the reporter gene.
  - How?

→ gene that produces an obvious phenotype → turn bright blue



Ab resistance

- Once the selected colony is determined, they are grown with their products purified from clinical use.

Exploration: Why is there a difference in the function of the reporter gene in recombinant and non-recombinant plasmids?

The expression of the reporter gene will be impacted as it is interrupted by the gene of interest

Because the protein product is easily identifiable this can be used to distinguish between recombinant AND non recombinant plasmid

Key Takeaways

- **Recombinant DNA**:
  - Involves combining DNA from multiple sources to produce desired proteins.
  - Transformation is the insertion of recombinant DNA into a bacterium, often using a plasmid or vector.
  - A vector carries foreign genetic material into a host organism for replication or expression.
  - Plasmids, usually carrying the target gene, transform bacteria to produce the desired protein.
- **Structure of a Plasmid**:
  - **Plasmid**: Small circular DNA in bacteria replicates independently and is transferable.
  - **Origin of Replication**: Sequence where replication starts.
  - **Antibiotic Resistance Gene**: Provides resistance to specific antibiotics.
  - **Multiple Cloning Site (MCS)**: Contains recognition sites for inserting genes.
  - **Selectable Marker**: Distinguishes transformant from non-transformant cells.
  - **Screening Marker**: Confirms plasmid is recombinant (e.g., lacZ).
  - **Promoter Region**: Site where RNA polymerase binds to initiate transcription.
- **Making a Recombinant Plasmid**:
  - Obtain a gene of interest and plasmid, cut with the same endonuclease to create sticky ends.
  - Use DNA ligase to join the gene and plasmid.
  - Possible outcomes include recombinant and non-recombinant plasmids.
- **Transforming Bacteria:**
  - Electroporation and heat shock methods introduce plasmids into bacteria.
  - Electroporation: Electric current creates membrane pores for DNA entry.
  - Heat Shock: Uses Ca^{2+} ions, ice, and heat to facilitate DNA uptake.

Selecting Bacteria:

- Identify bacteria with recombinant plasmids using antibiotic selection and reporter genes.
- Three groups: Without plasmid, with non-recombinant plasmid, and with recombinant plasmid.
- Antibiotic resistance indicates transformed bacteria.
- Reporter gene function changes indicate recombinant plasmids, often through colour change.
- Selected bacteria colonies produce the desired protein for purification.

Space for Personal Notes

(1) MAKE RECOMBINANT PLASMID

(2) TRANSFORMATION

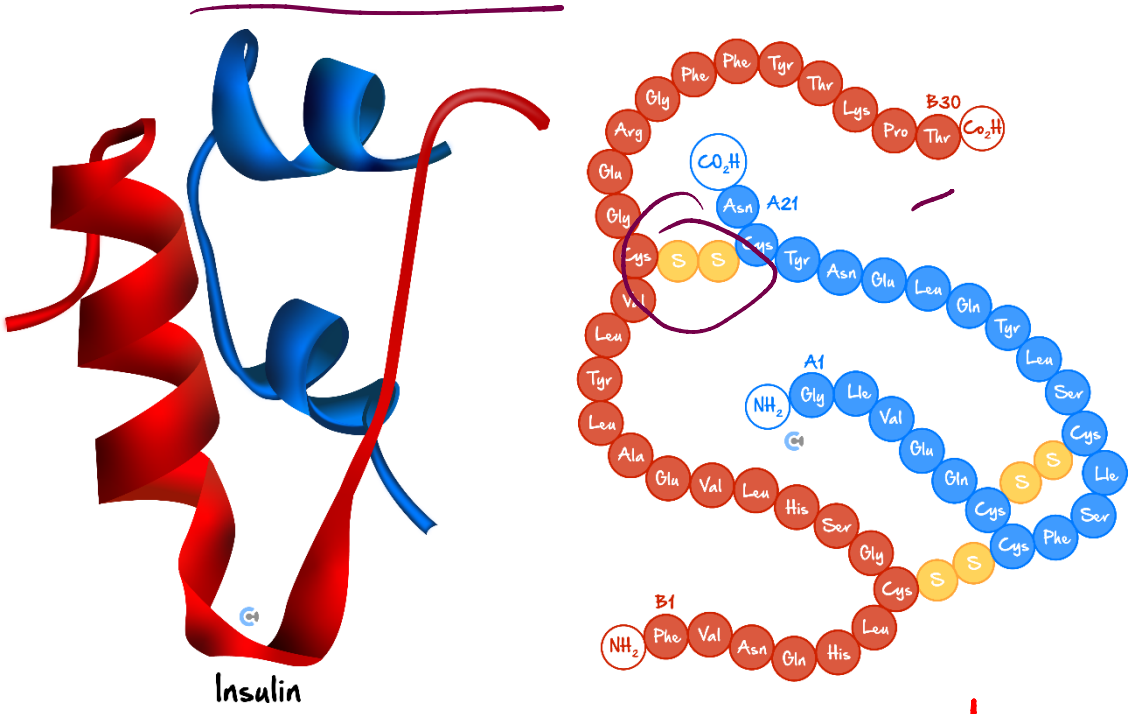
(3) SELECTION

Section B: Recombinant DNA and Insulin Production

Sub-Section: Introducing Insulin

Overview of Insulin

- Insulin is a quarternary structure protein composed of two peptide chains, an alpha chain, and a beta chain.
- It is the response to lowering blood glucose levels as the main anabolic hormone in the body.



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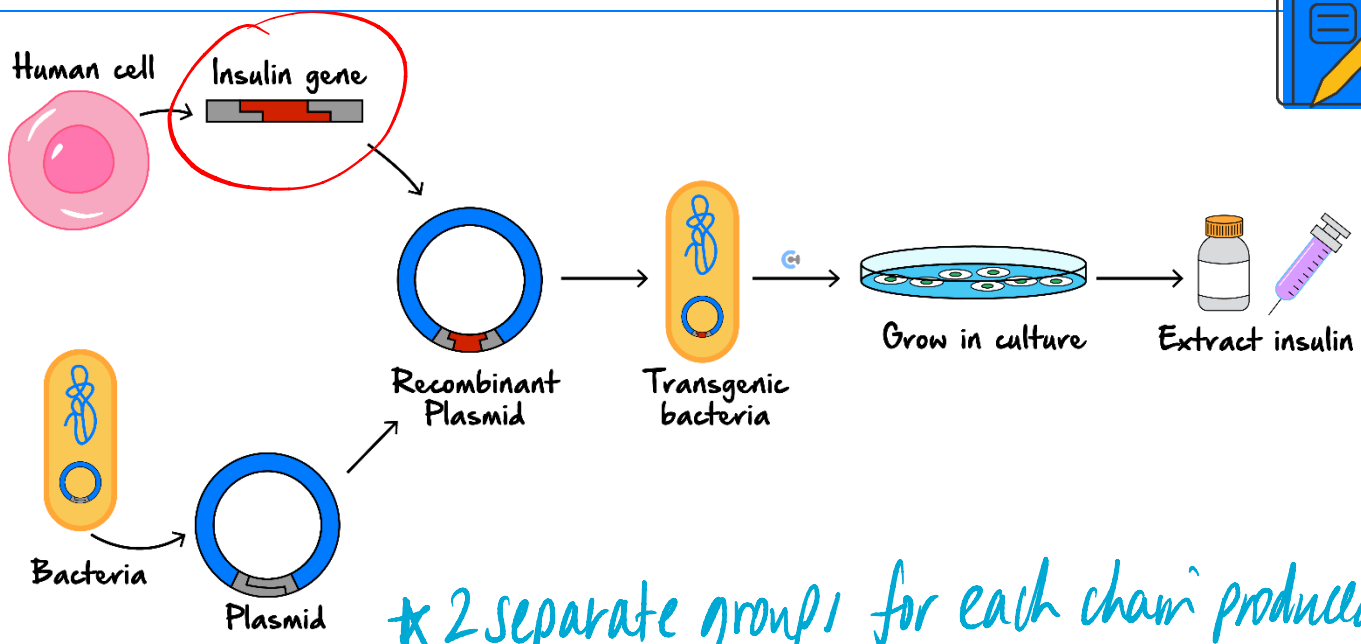
Diabetics require insulin → type 1 → autoimmune
CANNOT PRODUCE
*require insulin
previously obtained from animals
→ effective?/allergies
→ type 2
*final stage – need insulin
Bacterial Production began in 1980's

Sub-Section: Using Recombinant Plasmids for Insulin Production



* 2 separate groups for each chain produced

Bacteria lack the organelles to adequately process insulin

Exploration: Applying to Insulin Production

- A plasmid with an antibiotic resistance gene, reporter gene and endonuclease restriction site is prepared.
- In the case of insulin, as it has a quaternary structure with 2 chains, 2 genes of interest are cut and ligated to form recombinant plasmids.
- Bacteria are transformed through heat shock or electroporation.
- Transformed bacteria are selected using antibiotics, and recombinant plasmids are selected using the reporter gene.
- Insulin subunits are expressed, and the separate components are isolated and purified and then joined together to form insulin.

A + B

Exploration: How is the insulin gene obtained?

difference between delivered gene & human gene

Introns must be removed – Bacteria cannot remove them as they do not undergo mRNA processing

Obtain mature mRNA → no introns, do reverse transcription to get cDNA

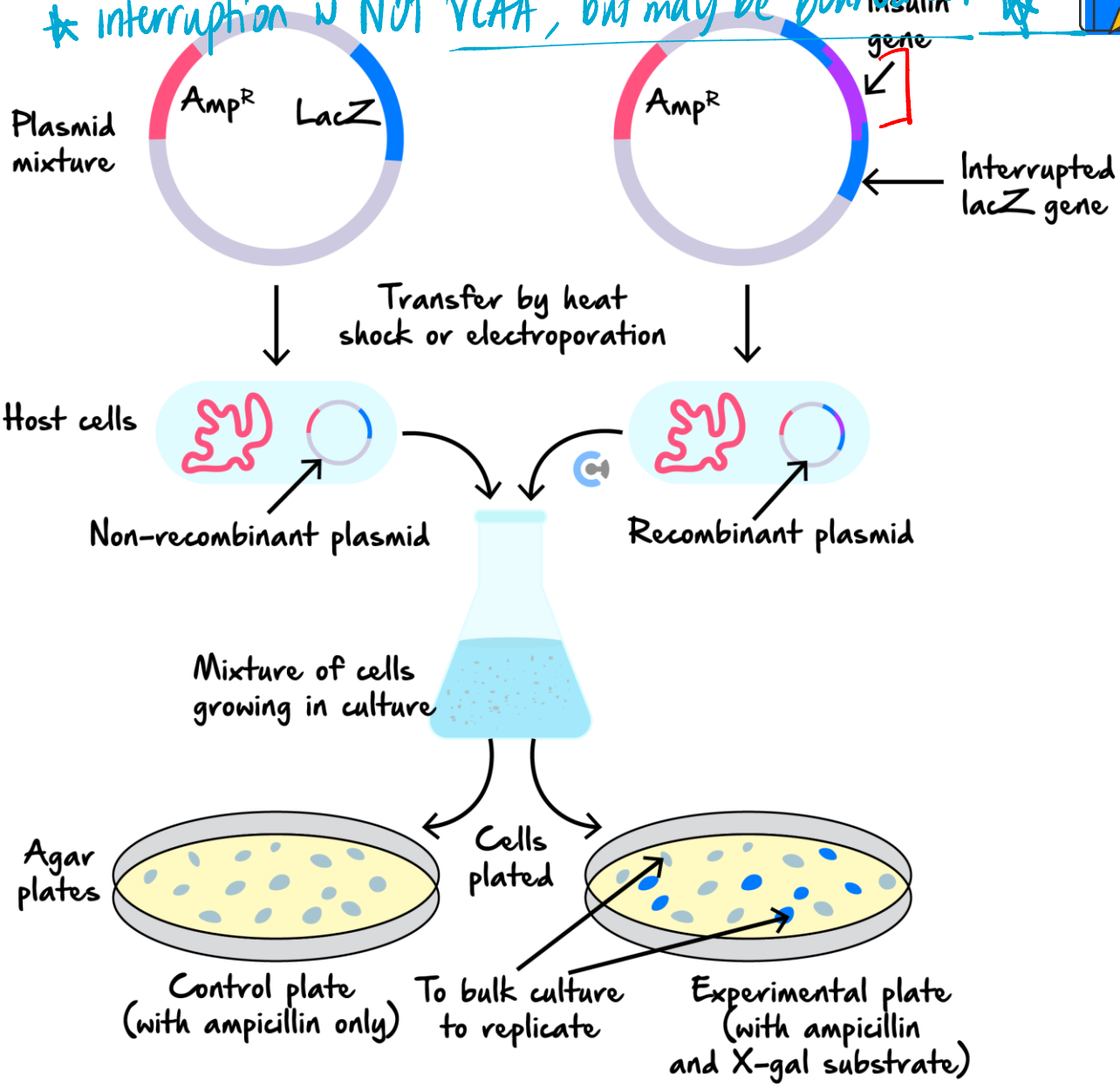
codes for a B-galactosidase protein

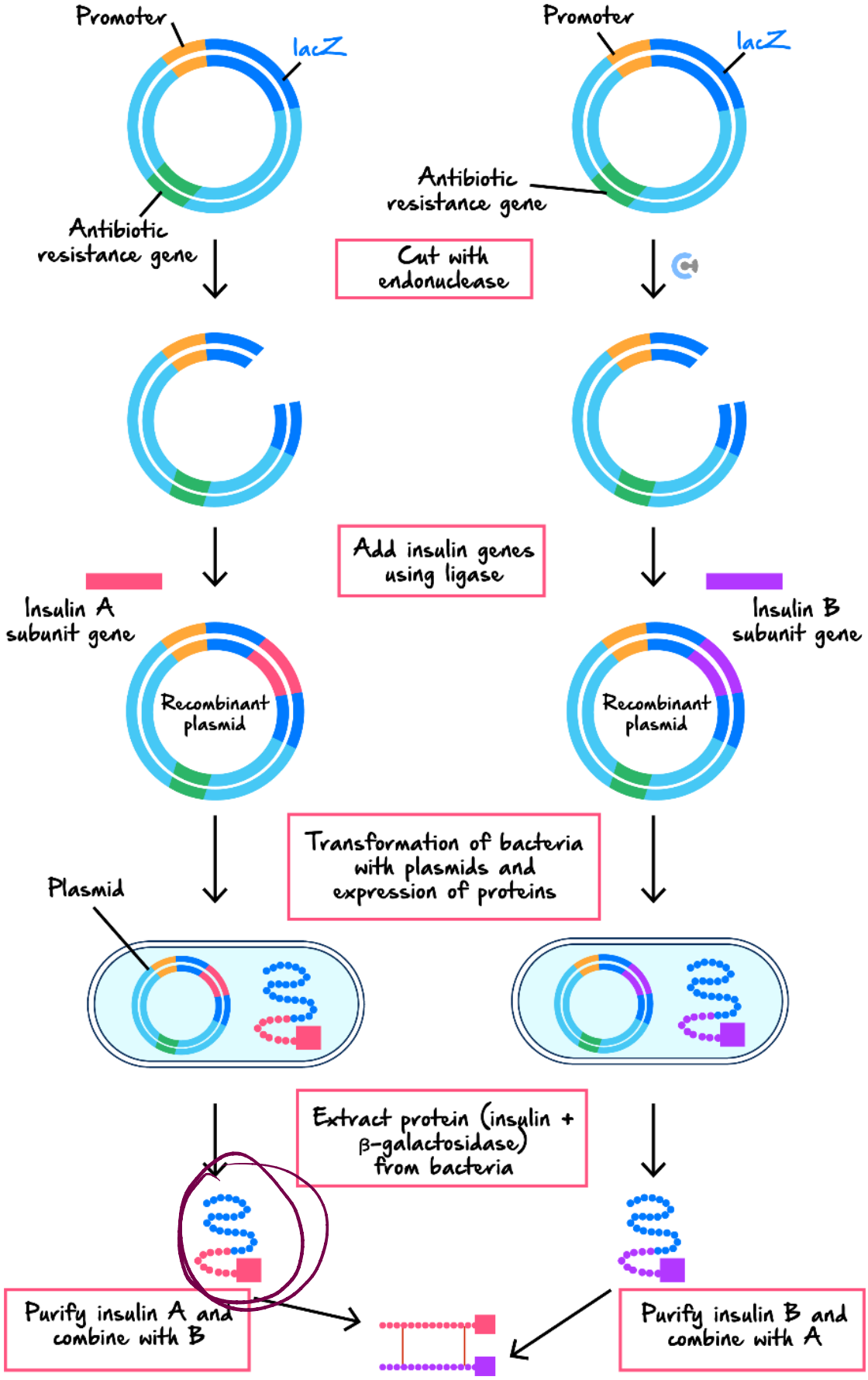
Exploration: The LacZ Gene — reporter gene

↳ turn its substrate, X-gal, blue

The gene for human insulin is inserted next to a gene coding for beta galactosidase protein. This creates a functional fusion protein, which allows for detection of successful gene insertion. Also ensures the survival of the insulin peptide when expressed by bacteria

* interruption is NOT VCAA, but may be your school *




Promoter
lacZ
Antibiotic resistance gene
Promoter
lacZ
Antibiotic resistance gene
Cut with endonuclease
Add insulin genes using ligase
Insulin A subunit gene
Insulin B subunit gene
Recombinant plasmid
Recombinant plasmid
Transformation of bacteria with plasmids and expression of proteins
Plasmid
Extract protein (insulin + β-galactosidase) from bacteria
Purify insulin A and combine with B
Purify insulin B and combine with A

What does VCAA say?

- Students should understand that one of the main methods for the commercial production of human insulin involves the insertion of genes for two different insulin polypeptides into two different plasmids in two separate bacteria. The insulin genes are inserted next to a gene for the β-galactosidase protein, which allows for the detection of successful gene insertion. Expression of each gene from the two bacteria allows a functional fusion protein to be produced. Students should understand that introns need to be removed prior to inserting the relevant insulin gene. Once the genes are expressed and the fusion proteins are produced by each bacterium, these fusion proteins are then purified, and the insulin polypeptides are removed and then combined to produce functional insulin.
- VCAA Response 2023. Q – Describe production of human insulin 5 mark
- Students were required to identify that human insulin genes for chains A and B are isolated, the insulin A gene is placed in one plasmid, and the insulin B gene is placed in a different plasmid.
- Other key steps students may have identified include:
  - Restriction endonucleases are used to cut, e.g. the plasmid or human DNA.
  - DNA ligase joins the DNA sequences into the plasmid.
  - The plasmid is inserted by heat shock into the bacteria which is transformed OR each plasmid is inserted into separate bacteria.
  - Antibiotic selection or another suitable method (e.g. insulin genes inserted next to a gene coding for beta-galactosidase protein) is used to determine success.
  - Processing of the protein, such as joining insulin polypeptide chains A and B, occurs to create functional insulin.

Space for Personal Notes

Sample Response: Insulin Production

- **Isolation of Insulin Genes:**
  - mRNA strands coding for the A and B chains of insulin are isolated and used as templates to synthesise complementary DNA (cDNA) via **reverse transcription**. This ensures that introns are not present in the insulin genes.
- **Preparation of Plasmids and DNA:**
  - The plasmid (vector) and insulin DNA sequences are cut with the same **restriction endonuclease**, creating complementary **sticky ends**. This ensures the insulin DNA can be inserted into the plasmid.
- **Formation of Recombinant Plasmids:**
  - The insulin DNA sequences (A and B) are separately inserted into plasmids containing a **beta-galactosidase gene** for detection.
  - **DNA ligase** is used to join the insulin DNA and plasmid, creating **recombinant plasmids**.
- **Introduction to Bacteria:**
  - The recombinant plasmids are inserted into **E. coli** bacterial cells through **heat shock** or electroporation transforming the bacteria.
  - Each plasmid is introduced into separate bacterial strains to produce the A and B chains independently.
- **Selection of Transformed Bacteria:**
  - Bacteria are grown on **antibiotic agar plates**, allowing only those with recombinant plasmids (containing antibiotic resistance genes) to survive.
  - Successful incorporation of the insulin genes is confirmed using **beta-galactosidase activity**.
- **Expression and Protein Extraction:**
  - Transformed bacteria are cultured, producing **fusion proteins** consisting of beta-galactosidase and insulin chains.
  - Fusion proteins are extracted, and enzymes are used to **cleave** the insulin chains (A and B) from the beta-galactosidase portion.

- **Purification and Assembly:**
  - The insulin A and B chains are purified and then chemically combined.
  - **Disulfide bonds** are formed between the chains, resulting in functional human insulin ready for medical use.

NOTE: This is super detailed, as you can see, the VCAA response is more succinct, but this should cover most schools, create your own variation of this to memorise. Also, do not include the subheadings in your answer.

Key Takeaways

- **Isolation of Insulin Genes:**
  - mRNA strands coding for the A and B chains of insulin are isolated and used as templates to synthesise complementary DNA (cDNA) via **reverse transcription**. This ensures that introns are not present in the insulin genes.
- **Preparation of Plasmids and DNA:**
  - The plasmid (vector) and insulin DNA sequences are cut with the same **restriction endonuclease,** creating complementary **sticky ends**. This ensures the insulin DNA can be inserted into the plasmid.
- **Formation of Recombinant Plasmids:**
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  - Transformed bacteria are cultured, producing **fusion proteins** consisting of beta-galactosidase and insulin chains.
  - Fusion proteins are extracted, and enzymes are used to **cleave** the insulin chains (A and B) from the beta-galactosidase portion.
- **Purification and Assembly:**
  - The insulin A and B chains are purified and then chemically combined.
  - **Disulfide bonds** are formed between the chains, resulting in functional human insulin ready for medical use.
- **Function of b-gal:**
  - The gene for human insulin will be inserted next to a gene coding for the production of beta-galactosidase protein. This will create a fusion protein, which allows for detection of successful gene insertion and ensures the survival of the insulin peptide when expressed by the bacteria. Detection of gene insertion is done when the fusion protein allows the plated X-gal substrate to turn blue.
- The insulin gene should not contain any introns and is created using reverse transcriptase.

Space for Personal Notes

Contour Check

Learning Objective: [1.7.1] – Describe the role of plasmids as a vector to transform bacteria & other cells

Key Takeaways

- **Recombinant DNA:**
  - Involves combining DNA from multiple sources to produce desired proteins.
  - Transformation is the insertion of recombinant DNA into a bacterium, often using a plasmid or vector.
  - A vector carries foreign genetic material into a host organism for replication or expression.
  - Plasmids, usually carrying the target gene, transform bacteria to produce the desired protein.
- **Structure of a Plasmid:**
  - **Plasmid:** Small circular DNA in bacteria, replicates independently, transferable.
  - **Origin of Replication:** Sequence where replication starts.
  - **Antibiotic Resistance Gene:** Provides resistance to specific antibiotics.
  - **Multiple Cloning Site (MCS):** Contains recognition sites for inserting genes.
  - **Selectable Marker:** Distinguishes transformant from non-transformant cells.
  - **Screening Marker:** Confirms plasmid is recombinant (e.g., lacZ).
  - **Promoter Region:** Site where RNA polymerase binds to initiate transcription.

Learning Objective: [1.7.2] – Explain how a gene of interest is isolated & inserted into a plasmid

Key Takeaways

- **Making a Recombinant Plasmid:**
  - Obtain a gene of interest and plasmid, cut with the same endonuclease to create sticky ends.
  - Complementary tick ends will attract via hydrogen bonding.
  - Use DNA ligase to join the gene and plasmid.
  - Possible outcomes include recombinant and non-recombinant plasmids.

Learning Objective: [1.7.3] – Explain electroporation & heat shock as methods to transform bacterial cells

Key Takeaways

- **Transforming Bacteria:**
  - Electroporation and heat shock methods introduce plasmids into bacteria.
  - **Electroporation**: Electric current creates membrane pores for DNA entry, increasing permeability.
  - **Heat Shock**: Uses Ca^(2+) ions, ice, and heat to facilitate DNA uptake, increasing permeability.

Learning Objective: [1.7.4] – Describe the process by which bacterial cells can be used to produce human proteins, including the application of this to human insulin production

Key Takeaways

- **Isolation of Insulin Genes:**
  - mRNA strands coding for the A and B chains of insulin are isolated and used as templates to synthesise __complementary__ DNA (cDNA) via __reverse transcription__. This ensures that introns are not present in the insulin genes.
- **Preparation of Plasmids and DNA:**
  - The plasmid (vector) and insulin DNA sequences are cut with the __same restriction endonuclease__, creating __complementary sticky ends__. This ensures the insulin DNA can be inserted into the plasmid.
- **Formation of Recombinant Plasmids:**
  - The insulin DNA sequences (A and B) are __separately inserted__ into plasmids containing a __beta-galactosidase gene__ for detection.
  - __DNA ligase__ is used to join the insulin DNA and plasmid, creating __recombinant__ plasmids.
- **Introduction to Bacteria:**
  - The recombinant plasmids are inserted into **E. coli** bacterial cells through __heat shock__ or __electroporation__ transforming the bacteria.
  - Each plasmid is introduced into separate bacterial strains to produce the A and B chains independently.
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  - Fusion proteins are extracted, and enzymes are used to **cleave** the insulin chains (A and B) from the beta-galactosidase portion.
- **Purification and Assembly:**
  - The insulin A and B chains are purified and then chemically combined.
  - Disulfide bonds are formed between the chains, resulting in functional human insulin ready for medical use.

Learning Objective: [1.7.5] – Explain the significance of beta-galactosidase, other reporter genes, & antibiotic resistance genes in the selection of transformed, recombinant bacterial cells

Key Takeaways

- **Selecting Bacteria:**
  - Identify bacteria with recombinant plasmids using antibiotic selection and reporter genes.
  - Three groups: Without plasmid, with non-recombinant plasmid, and with recombinant plasmid.
  - Antibiotic resistance indicates transformed bacteria.
  - Reporter gene function changes indicate recombinant plasmids, often through colour change.
  - Selected bacteria colonies produce the desired protein for purification.
- **Function of *b*-gal:**

The gene for human insulin will be inserted next to a gene coding for the production of beta-galactosidase protein. This will create a fusion protein, which allows for the detection of successful gene insertion and ensures the survival of the insulin peptide when expressed by the bacteria. Detection of gene insertion is done when the fusion protein allows the plated X-gal substrate to turn blue.

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