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Recombinant DNA technology

Lecture by:
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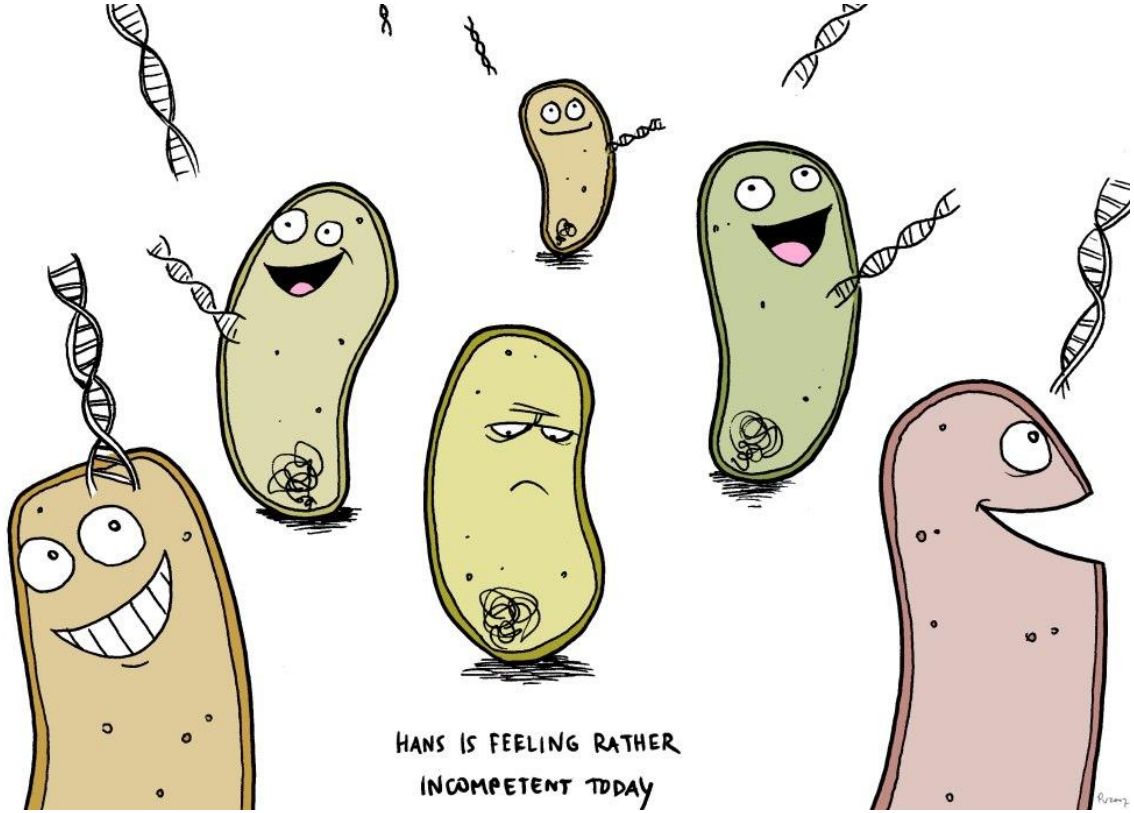

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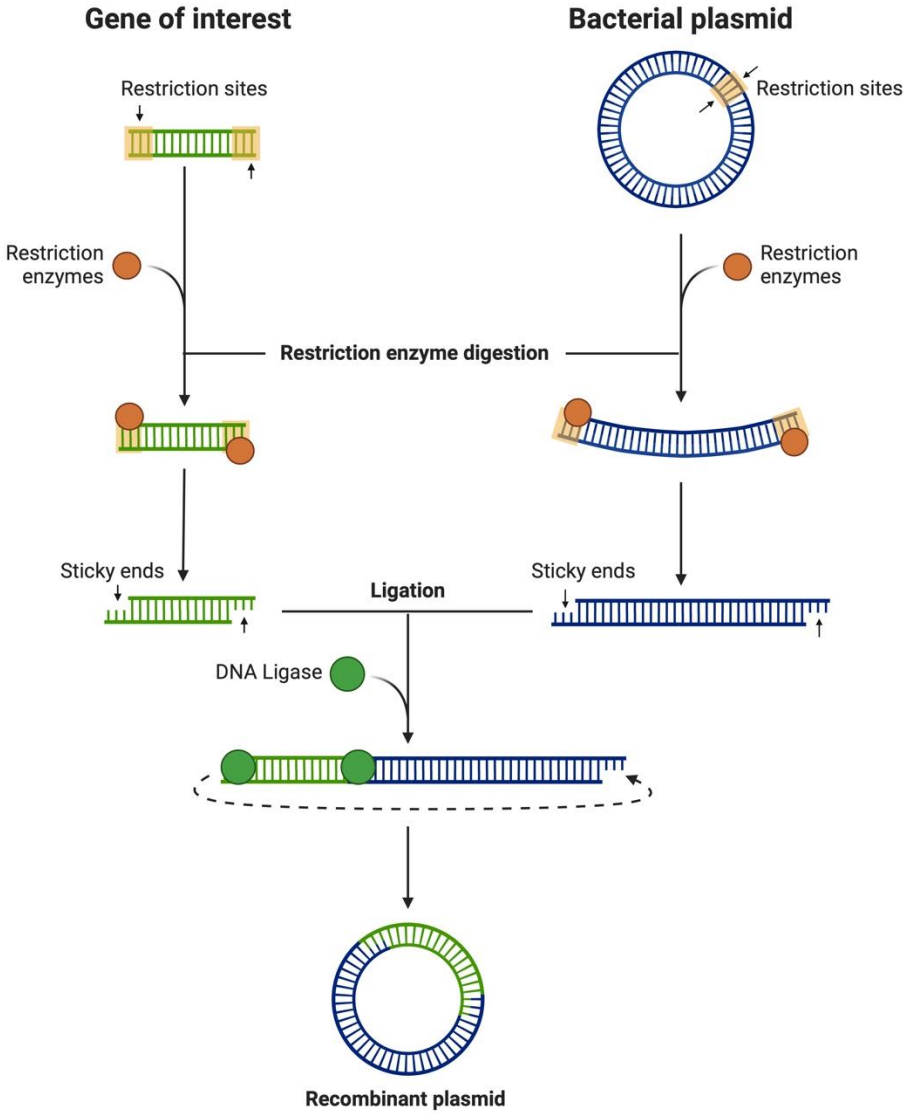
Molecular Cloning

→ Performed beforehand (not done during this lab)



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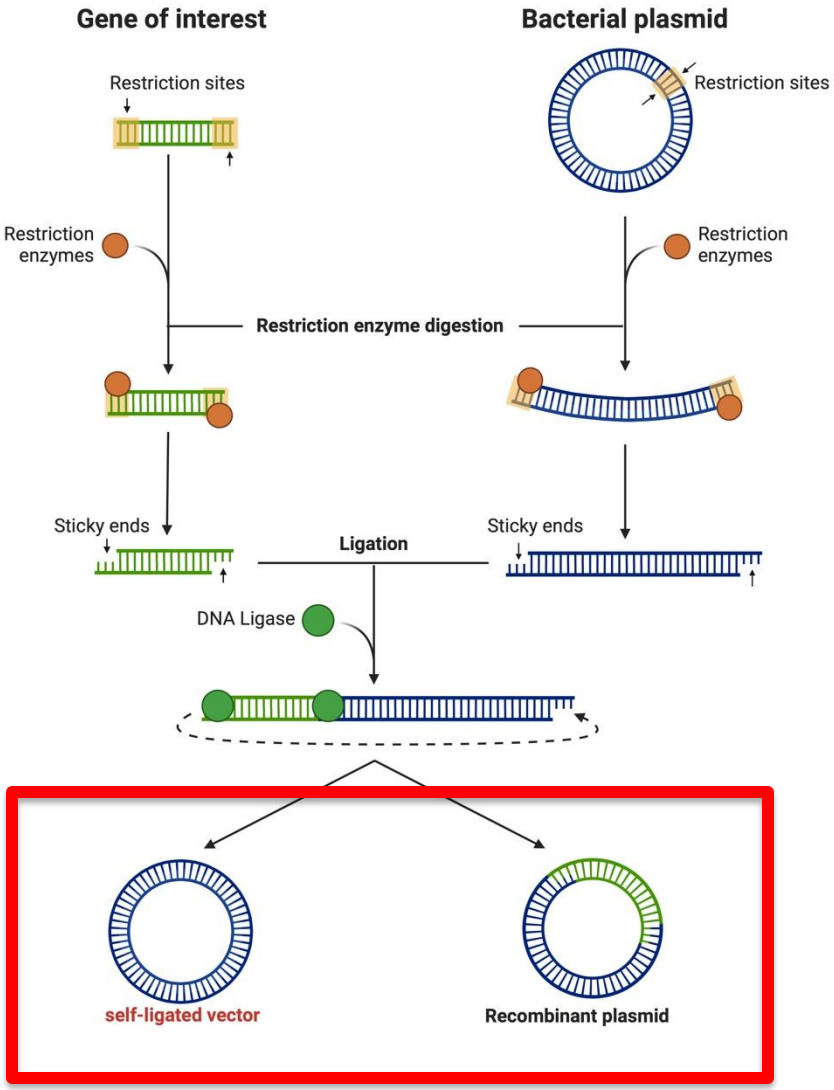
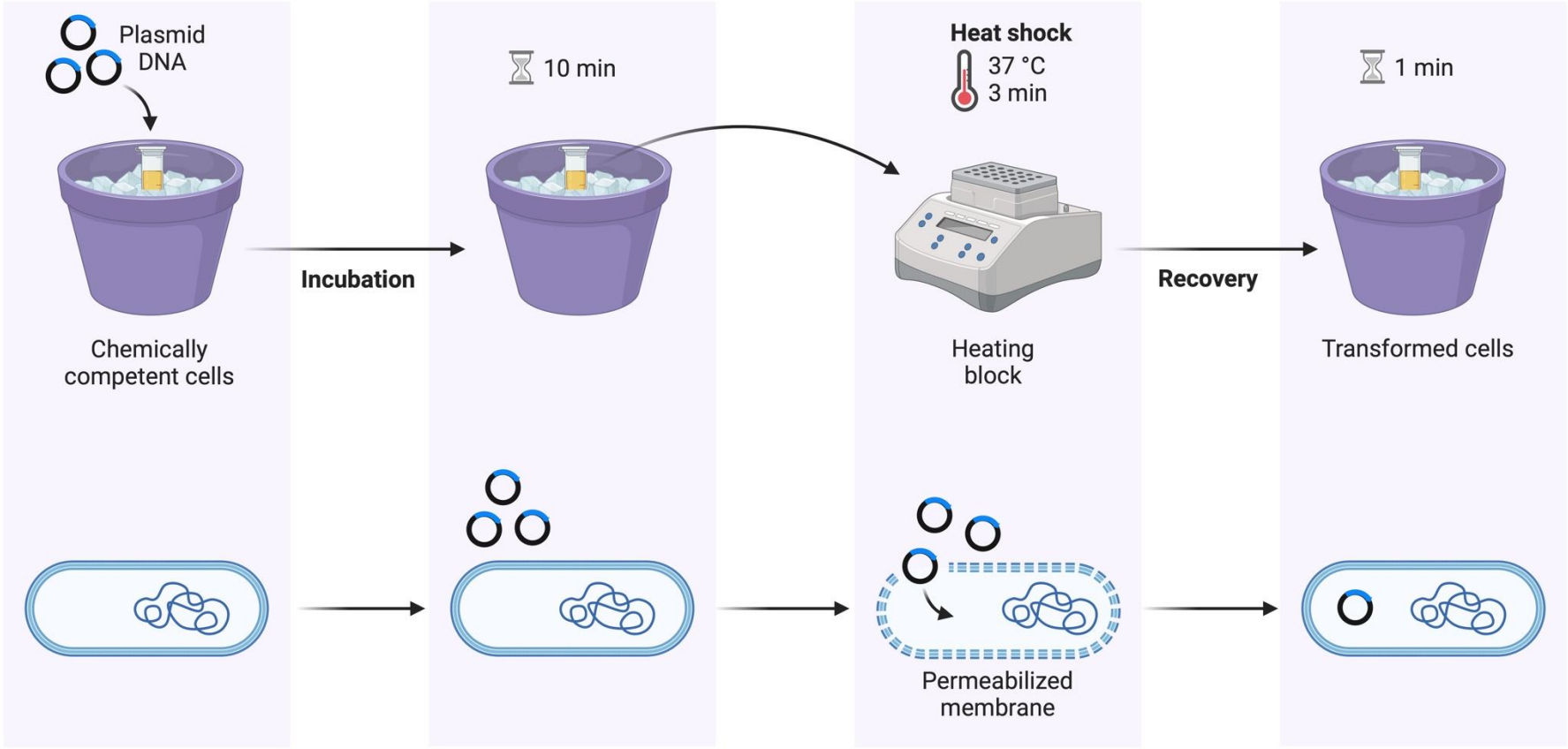


We aim to isolate/separate these two plasmids to select the recombinant plasmid and to verify the presence of the gene of interest

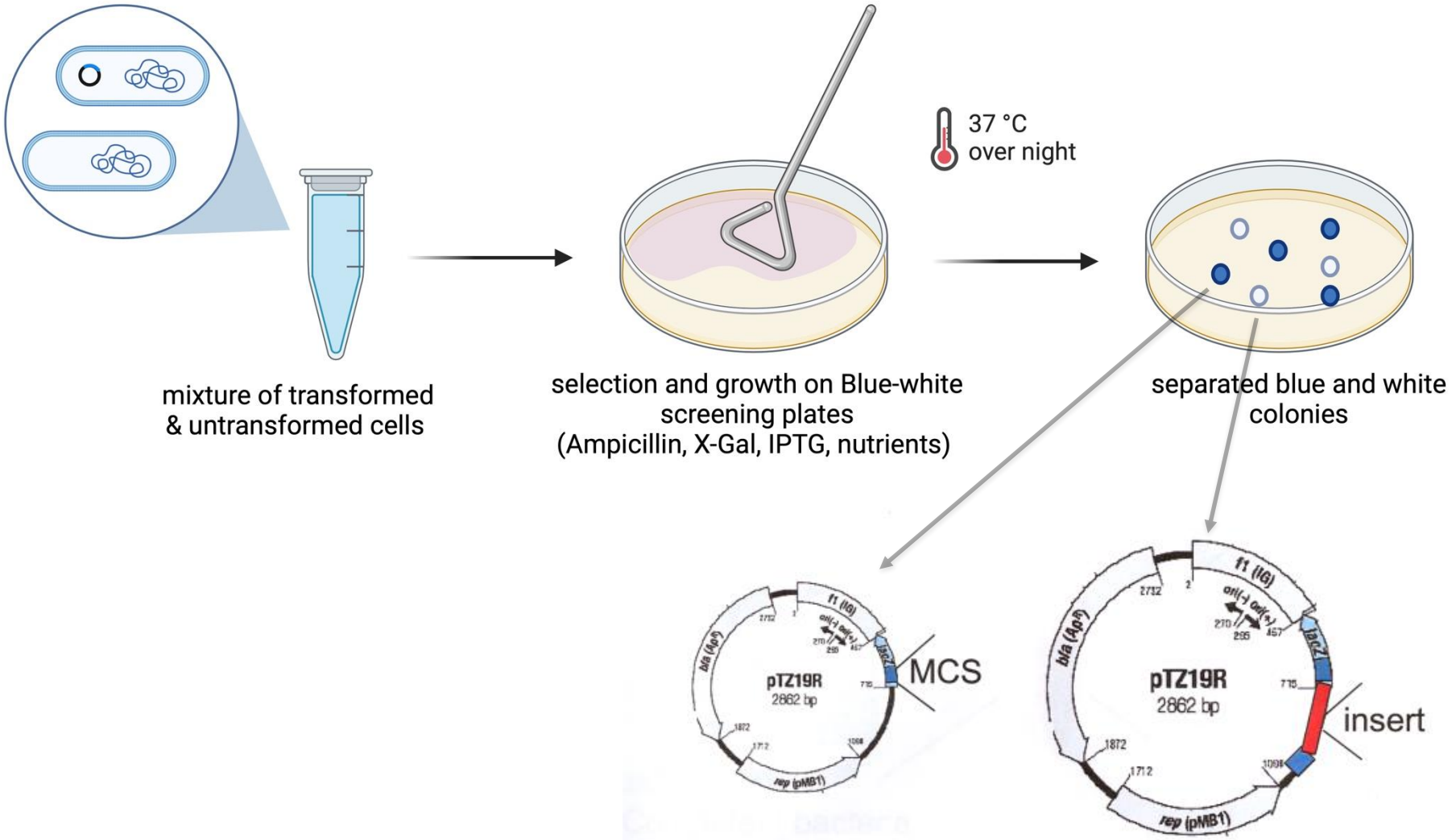
Day 1: Transformation of competent cells

→ performed during this lab (day 1)



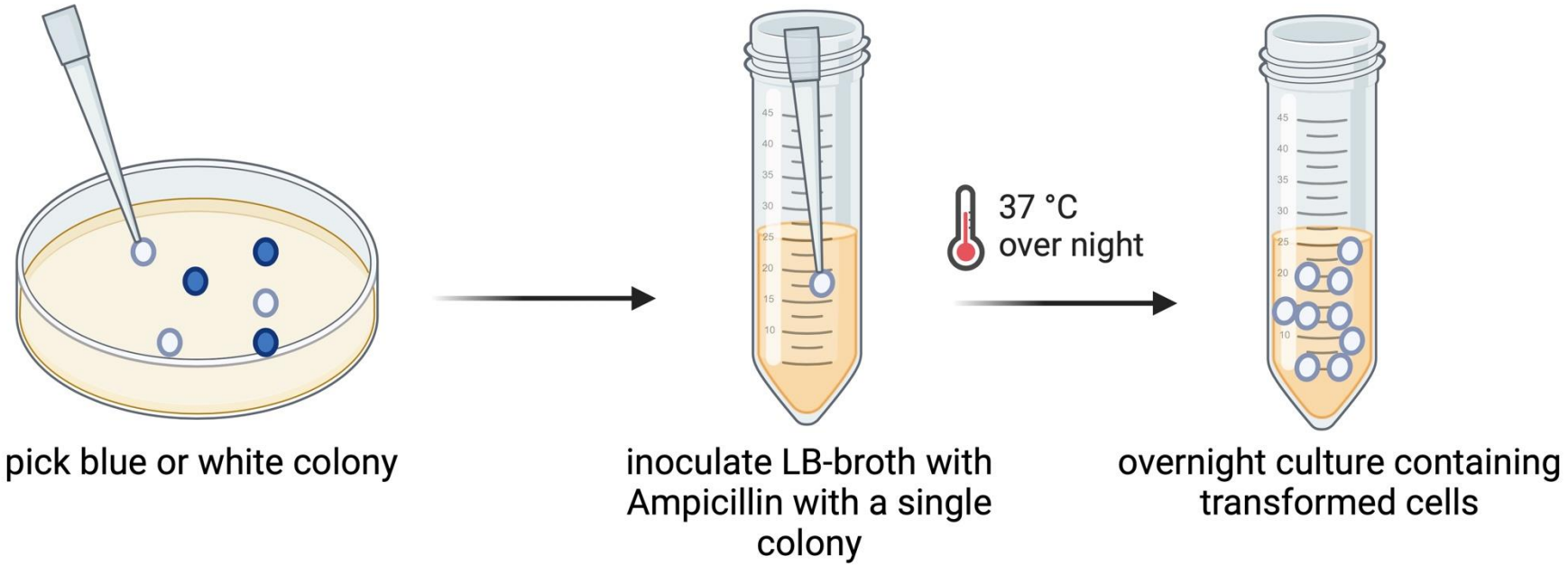
Day 1: Selection of transformed cells

→ performed during this lab (day 1 + day 2)



Day 2: Picking & expansion of blue and white colonies

→ performed during this lab (day 2)



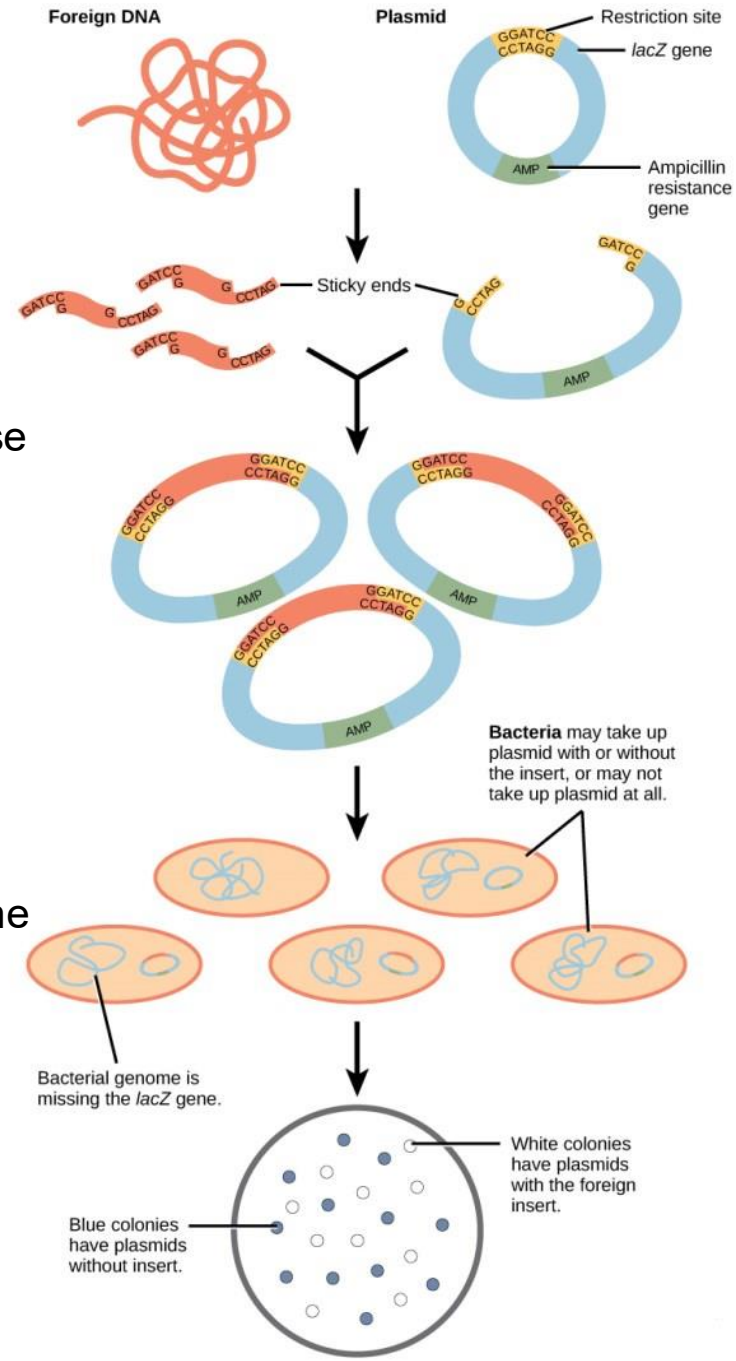
Revision
Blue-white screening

Plasmid:

- AmpR: Ampicillin resistance (β-lactamase)
- LacZ: α-peptide for functional β-galactosidase enzyme
- BamHI restriction site in LacZ gene for insertion of DNA

Growth medium:

- Ampicillin: Only successfully transformed bacteria carrying plasmids can survive in the presence of ampicillin
- IPTG : activates transcription of the LacZ gene by binding its repressor
- X-gal: β-galactosidase degrades X-gal. The product has a **blue colour**!



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Day 3 - work overview

- ✓ Purify plasmids
- ✓ Restriction enzyme digestion
- ✓ Run on agarose gel
- ✓ Interpret results

Day 3: Material

Plasmid preparation kit

Tubes labeled with
A1; A2; A3; A4; H_2O

Spin column

Collection tube &
spin column (blue)

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Day 3: Plasmid purification

→ performed during this lab (day 3)

<u>Buffer A1</u> (Cell Suspension)

Tris/HCl (pH 8.0), EDTA, RNase A

<u>Buffer A2</u> (Cell Lysis)

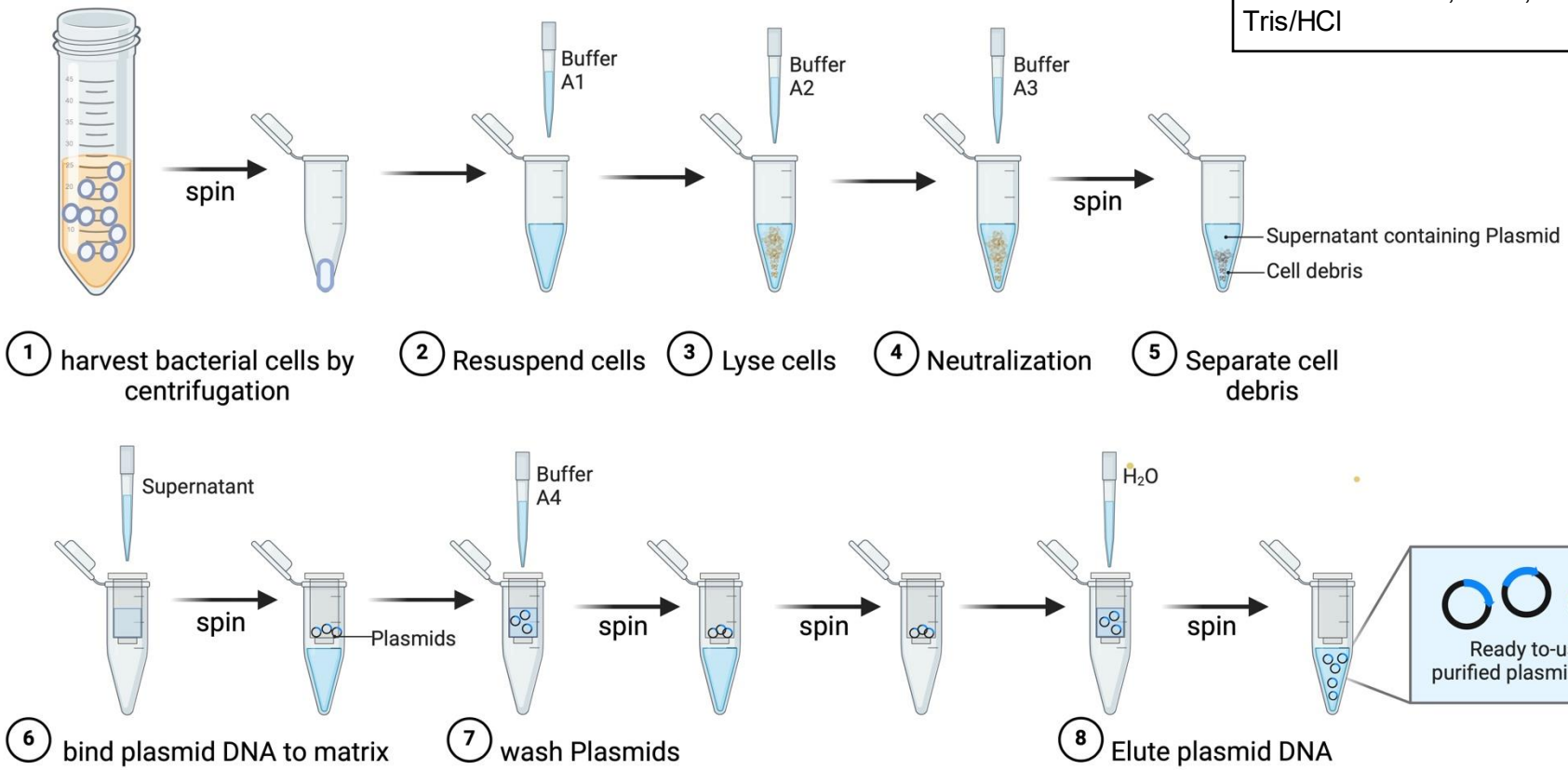
NaOH; SDS

<u>Buffer A3</u> (Neutralization/Binding)

Contains acetate and guanidine hydrochloride

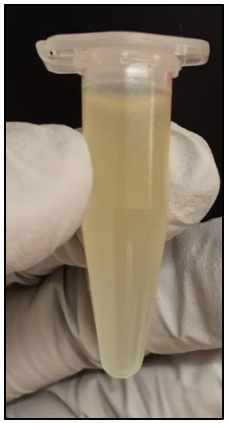
<u>Buffer A4</u> (Wash, reconstituted)

Contains ethanol, NaCl, EDTA, and Tris/HCl



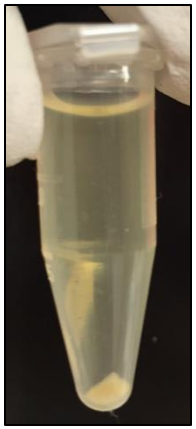
Day 3: harvest cells & purify plasmids

cells grown in LB-media overnight

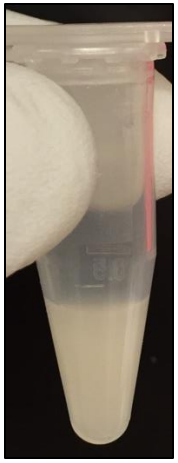


Transfer 2x 750 µl into microcentrifuge tube

Balance the centrifuge

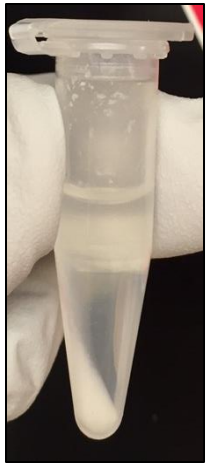


Bacterial pellet = cells + plasmid
→ Discard superatant



A1 - resuspension buffer
A2 – cell lysis buffer

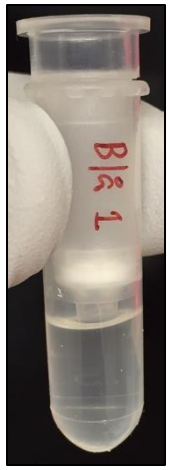
A3 – neutralization/ binding buffer



Plasmid in supernatant cell debris as pellet

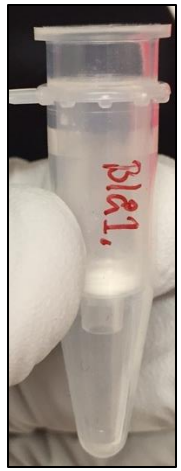
transfer supernatant to column

Plasmid binding



Discard flow-through

Wash with A4



Transfer column to new tube
add H_2O to elute plasmid

Plasmid purification

Buffer A1:
Bacterial cells are resuspended in a buffer containing Rnase A.

Buffer A2:
Bacteria are lysed under alkaline conditions (NaOH). SDS solubilizes the phospholipid and protein components of the cell membrane
Lysis and release of cells contents. Alkaline conditions: denaturation of chromosomal and plasmid DNA as well as proteins.

Buffer A3:
The lysate is neutralized and adjusted to high-salt-binding conditions. The high salt concentration causes denatured proteins, chromosomal DNA, cellular debris, and SDS to precipitate, while the smaller plasmid DNA renatures correctly and stays in solution. DNA is bound to silica membrane of spin columns in high-salt buffer. RNA, cellular proteins and metabolites are not retained on the membrane.

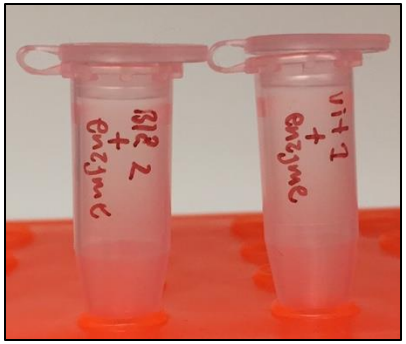
Buffer A4:
Washing and reconstitution of DNA. Salts are efficiently removed by this wash step.

H_2O:
The purified plasmid DNA is eluted from silica membrane by addition of water. The elution is pe is dependent on a low salt concentration and a stable pH (pH 7-8.5).

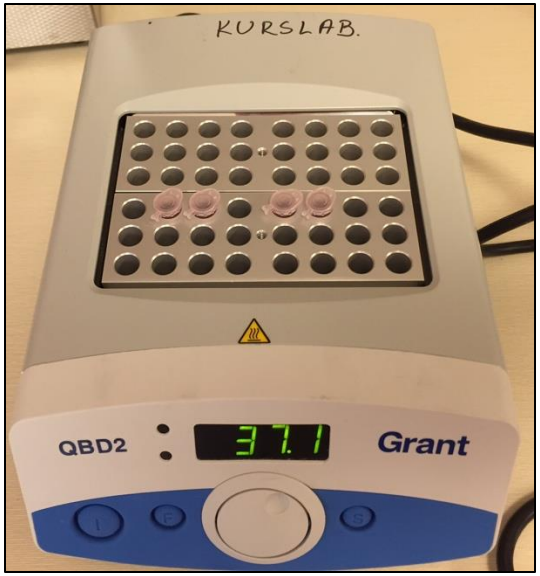
QIAprep Miniprep Handbook, Appendix A: Background Information, Preparation of cell lysates, p 43

Day 3: Restriction enzyme digestion

<u>Restriction enzyme working solution</u>:
Restriction enzyme buffer
H_2O
Restriction enzyme (keep it cold!)



Add restriction enzyme to a portion of eluted plasmid
KEEP THE REST OF UNDIGESTED PLASMID AS CONTROLS FOR LATER USE



Incubate at 37°C for 60 min

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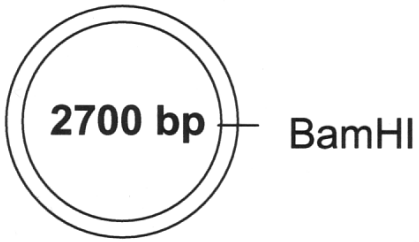
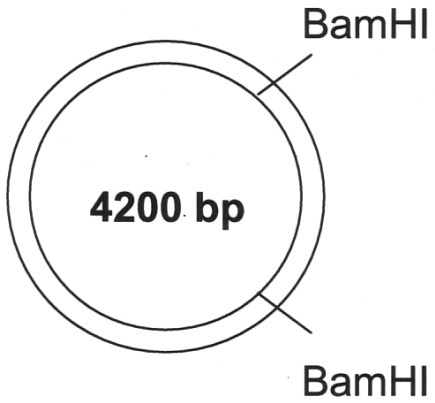
Day 3: Restriction enzyme digestion

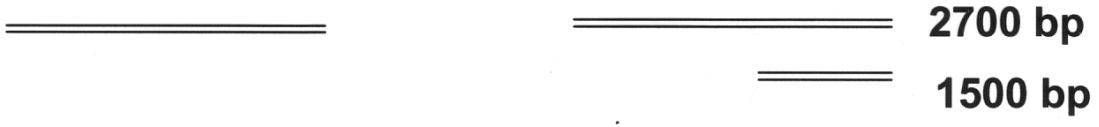
Plasmid without insert



Plasmid with insert (in BamHI site)

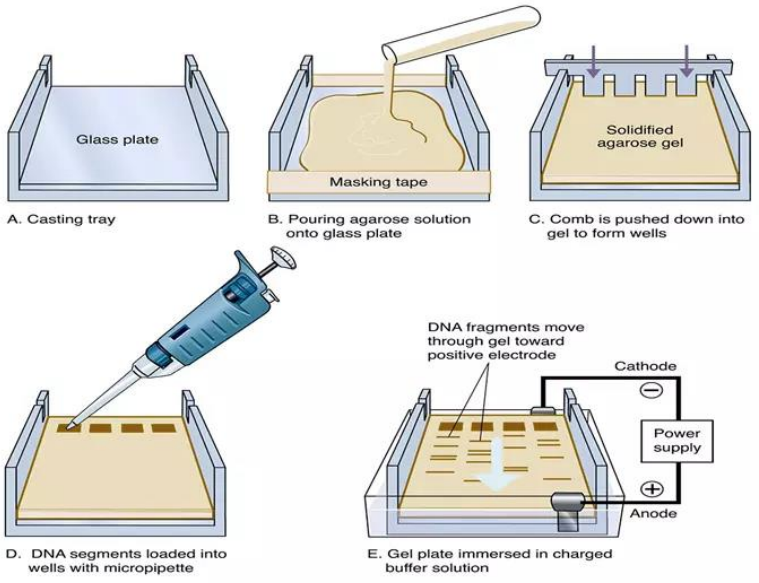


Cut with BamHI

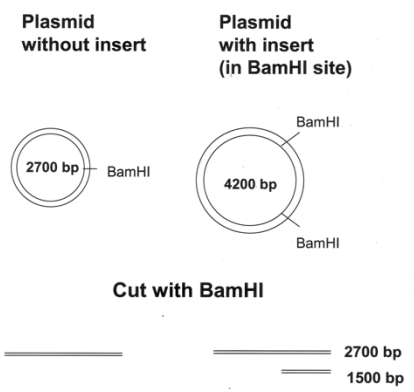


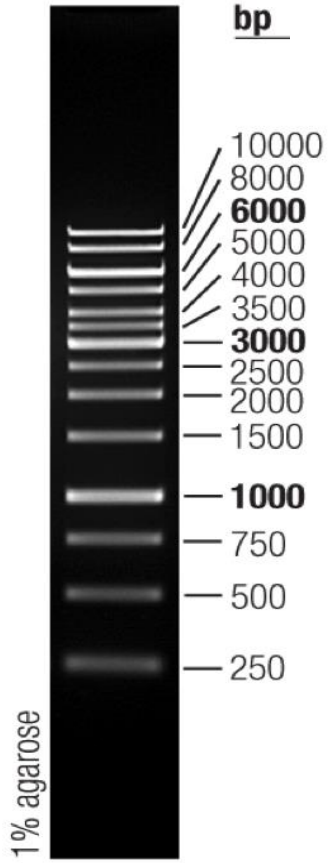
Day 3: Agarose Gel Electrophoresis

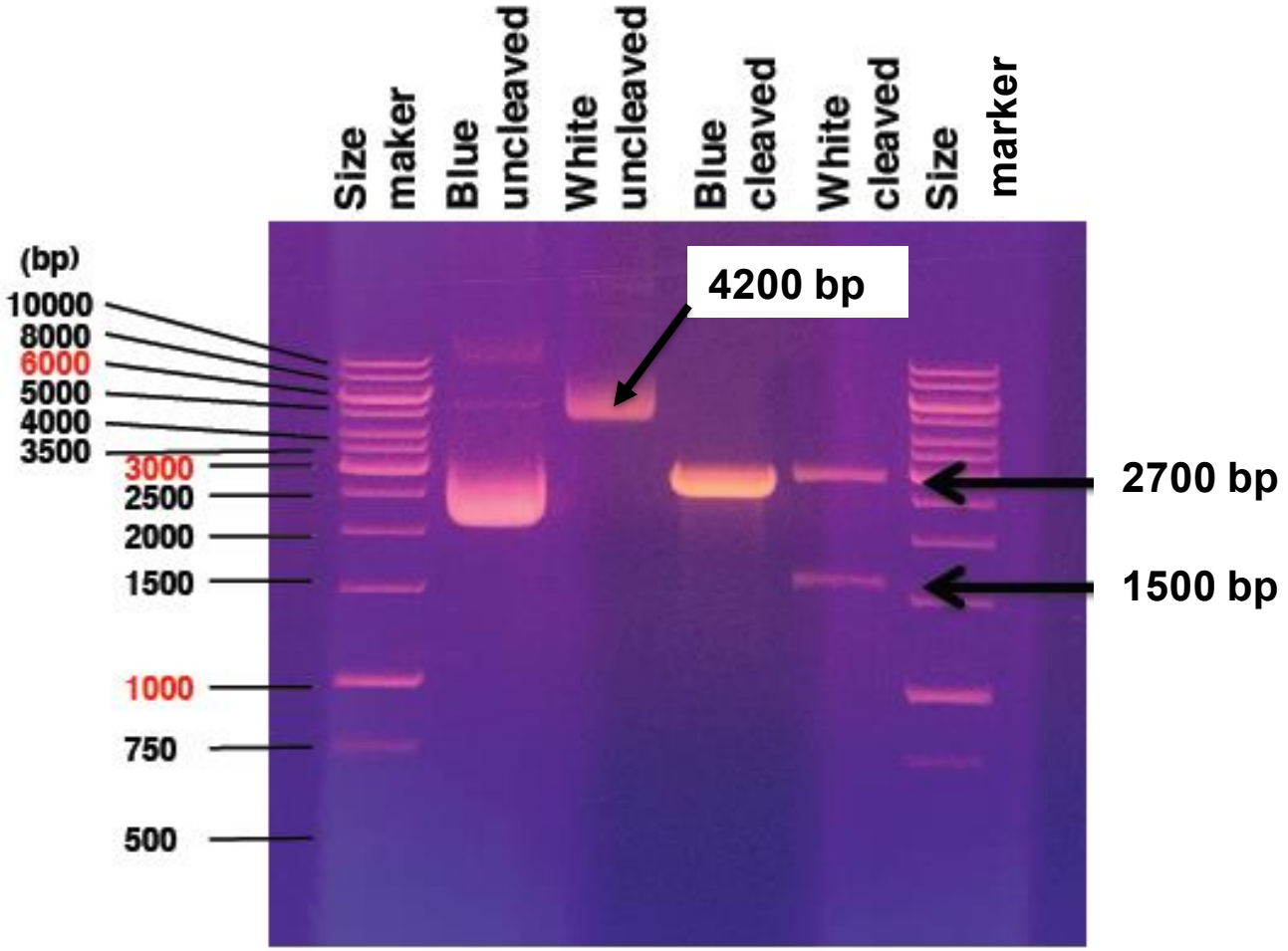
- Samples are mixed with 6x loading dye to make them ”heavier” to stay in wells
- Separation of DNA molecules based on their size
- DNA **negatively** charged
- Agarose gel for separation
- Shorter molecules move faster and migrate farther than longer ones
- Visualization of DNA with SYBR safe DNA stain



Day 3: Expected Results

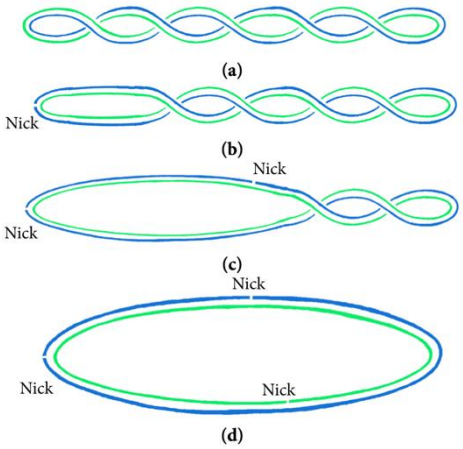


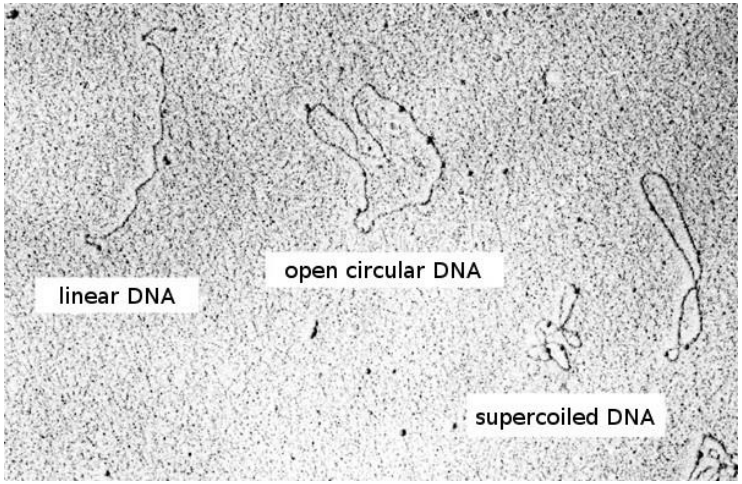




GeneRuler 1kb DNA ladder= size marker

Plasmid conformation affects migration





- Relaxed/linear: intact circle but “nick” in one strand
- Linear: both strands are cut (at the same location)
- Supercoiled: fully intact with both strands uncut, appears in a compact form

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Lab schedule

- ✓ Purify plasmids

- ✓ Restriction enzyme digestion
  1-2h incubation time → lunchbreak and everyone will be back at the same time

- ✓ Run on agarose gel
  approximately 1h → go through the expected results to be able to ask appropriate questions

- ✓ Interpret results
  make sure to ask a lot of questions while you have the chance!!!

Lab reports

- ✓ Write according to the guidelines on the handout on Canvas
- ✓ **One lab report per group** (Names and group number on the cover page)
- ✓ **In English**
- ✓ Upload your lab reports on CANVAS, deadline 07/12/2025

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Lecture Questions

1. What are the sites the plasmid contains that allow for this experiment?
2. What are the 3 compounds in the LB plates that allow for selection of bacteria with plasmid and the distinction of plasmids with and without the inserted gene?
3. What are the six steps in plasmid preparation and purification?
4. What is a restriction enzyme and why was it used in this experiment?
5. Explain the principle of Gel Electrophoresis, what is it for?
6. What´s the compound that allows for the visualization of the DNA in the gel?
7. How many times does BamHI cut the plasmid without the insert? And the plasmid with the insert?
8. Explain the different plasmid conformations that exist.