Introduction to Gene cloning Practicals

Master Development of Drugs and Health Products

November 2024

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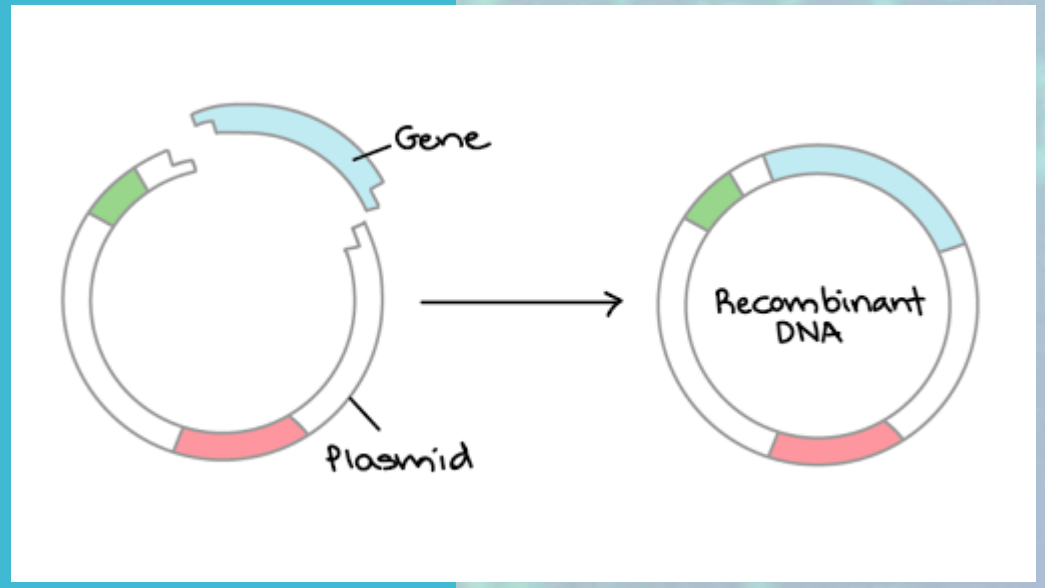
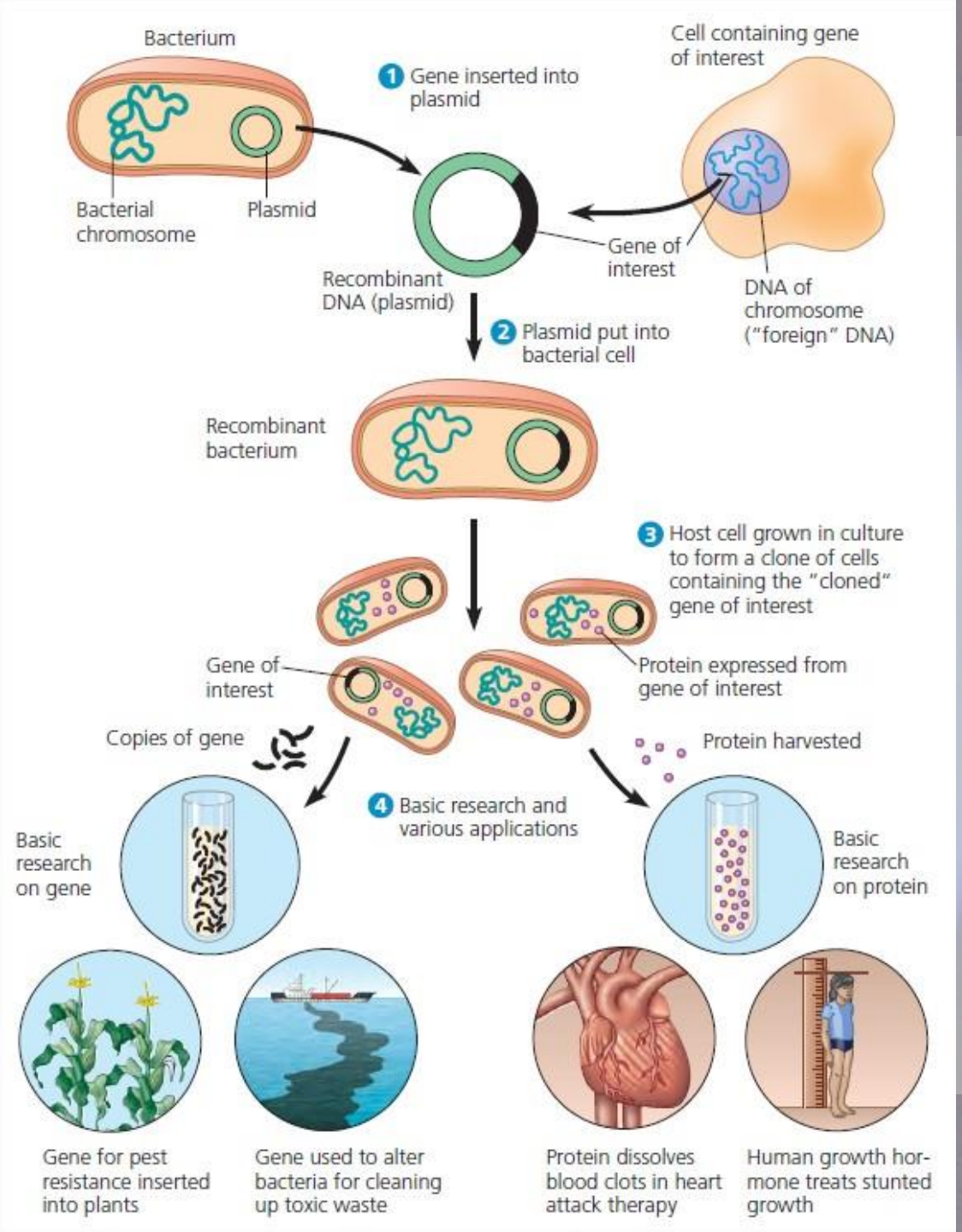
Context and applications





General objectives

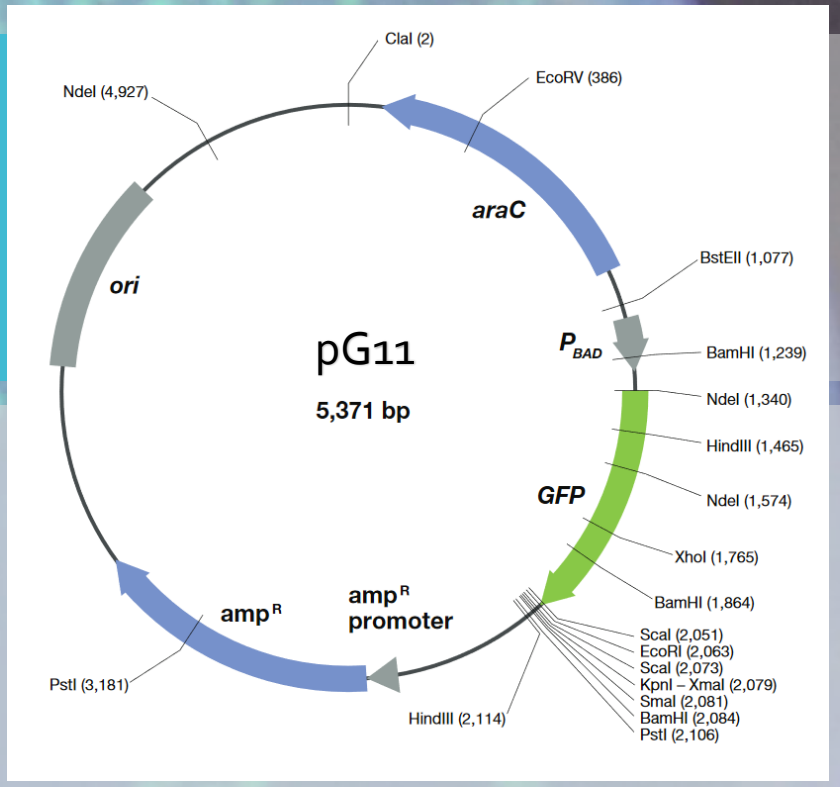
- Learn the lab good practices + Work safely in a lab
- Know how to perform each experiment:
- Think about organisation to optimise working time (retro-planning, running 2 experiments in parallel, anticipation from one experiment to the other,…)
- Perform a full experimental strategy
- Write-up a protocol

Specific objective

- To produce a strain of *E. coli* expressing a His-tagged GFP protein

Background

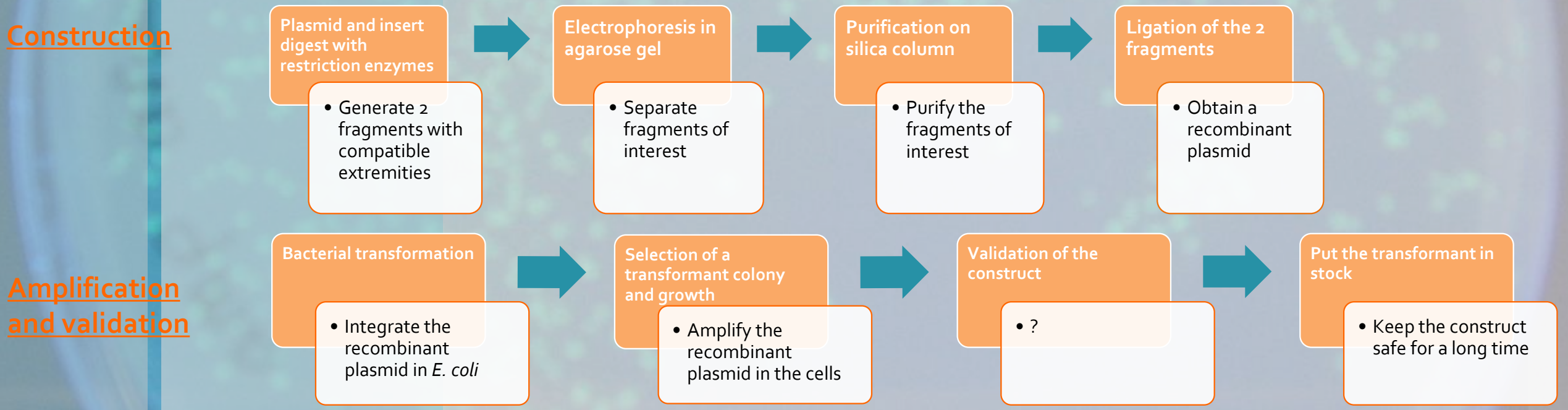
Aequorea victoria



- Why GFP?
- Bacterial strain: DH5-α (*endA recA*)
- Plasmid
- Cloning of a 6xHis encoding sequence fused to the GFP gene (! reading frame)

  => replace the WT GFP-encoding gene by the 6xHis-tagged GFP endoding gene
- Induction

Workflow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Validation of the construct
• ?
Put the transformant in stock
• Keep the construct safe for a long time

Workflow

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Preparation of required materials in advance

=> anticipate

Amplification and validation

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  - Integrate the recombinant plasmid in *E. coli*
- **Selection of a transformant colony and growth**
  - Amplify the recombinant plasmid in the cells
- **Validation of the construct**
  - ?
- **Put the transformant in stock**
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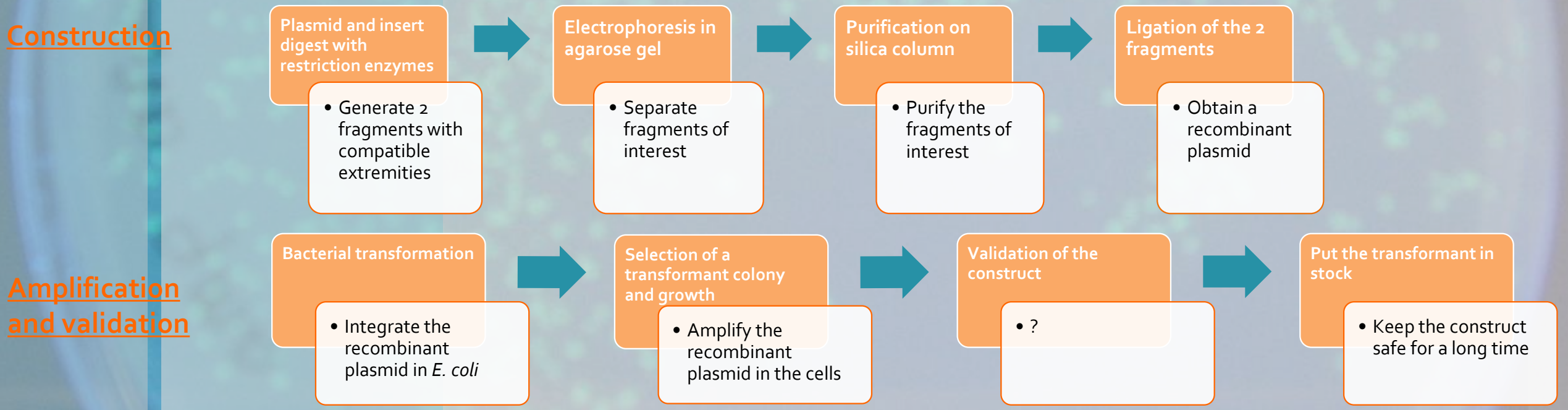
Preparation of your experimental work

To do before the lab course:

- Protocols: understand each step and order
  - Role of each reactive/component at each step
  - Search for and understand technical data (DNA sequence analysis, ligation conditions, agarose %, …)
- Controls
- Calculations + DNA sequence analysis to make
- Working conditions (sterility, safety equipment, temperature, …)
- Safety issues -> MSDS (Material Safety Data Sheet)
- Waste management
- Required materials and consumables (anticipation)
- See specific questions in your documents

Workflow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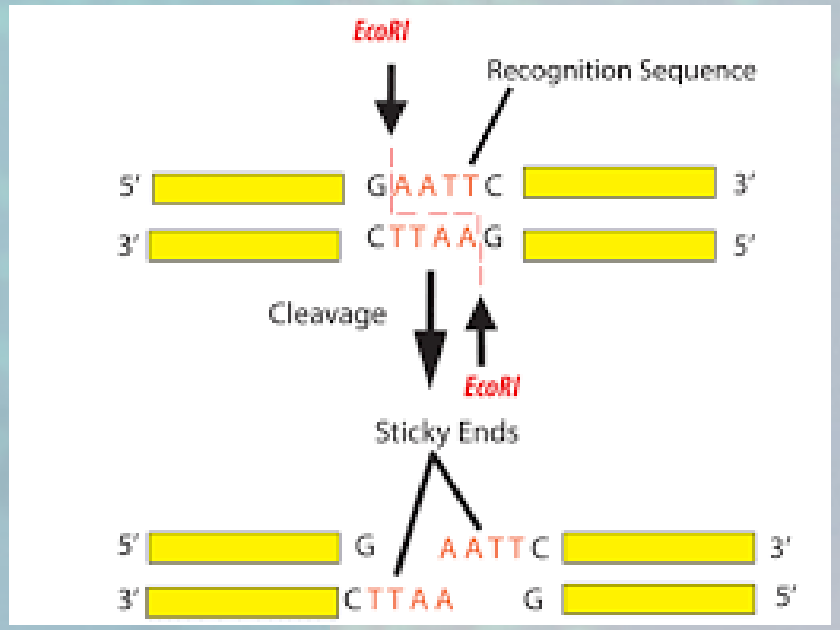
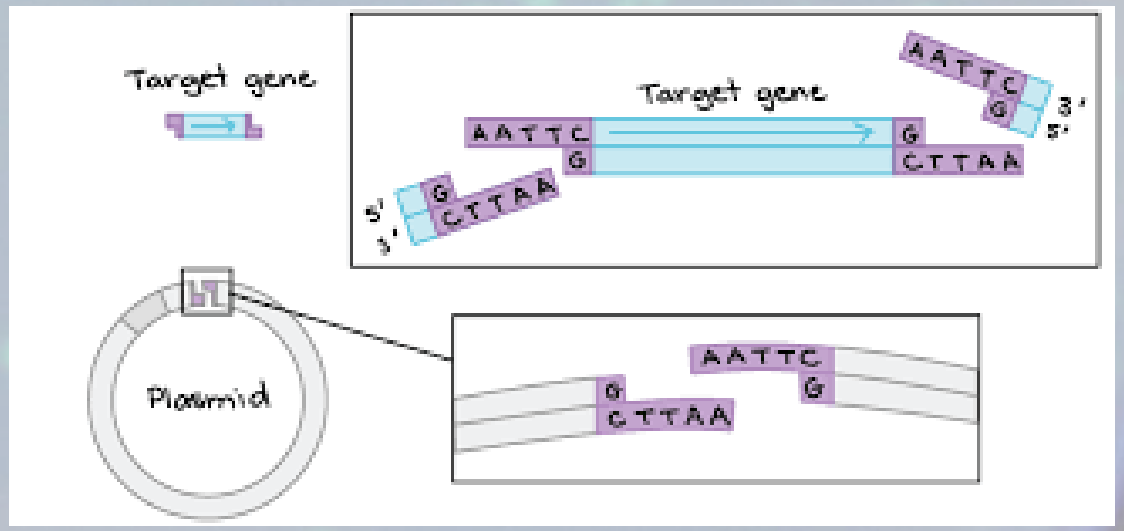
Plasmid and insert digest
Principle

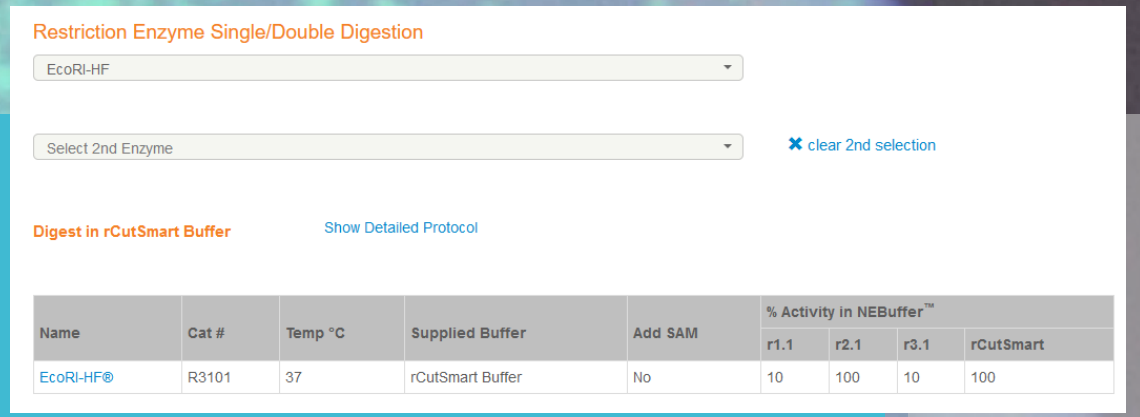
- Restriction enzymes





Plasmid and insert digest

Reaction



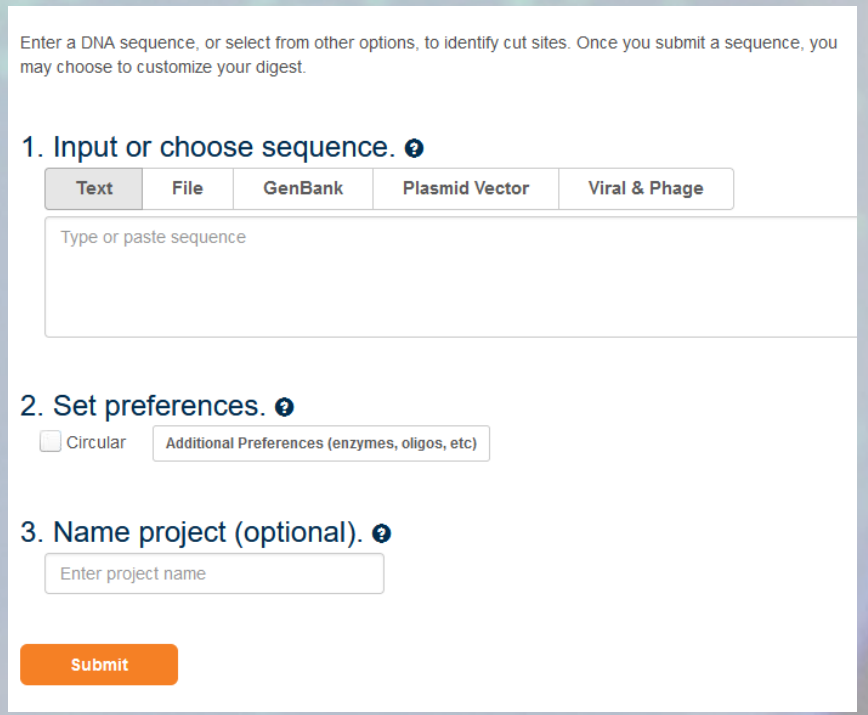
	pG11 plasmid	Insert
Ultra-pure H_2O	qsf 20 µl	qsf 20 µl
Restriction buffer (10 X): YOU MUST DETERMINE WHICH ONE IN ADVANCE	1 X	1 X
DNA (pG11 @250 ng/µL; Insert @25 ng/µL)	500 ng	150 ng
*Eco*RI-HF (5,000 U/ml)	5 U	5 U
*Nhe*I-HF (5,000 U/ml)	5 U	5 U

- ! Order of the reactives for the mix + temperature
- Which buffer? Sequence & length of produced fragments?
- Volume of each reactive ?

→ *NEB cutter tools :*

https://nebcloner.neb.com/#!/redigest

http://nc2.neb.com/NEBcutter2/



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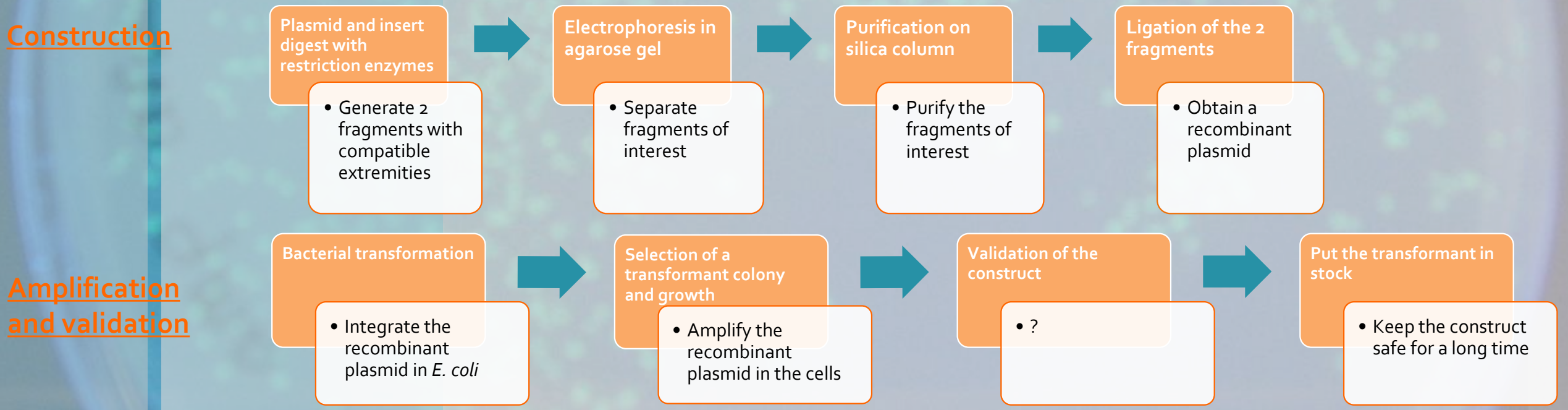
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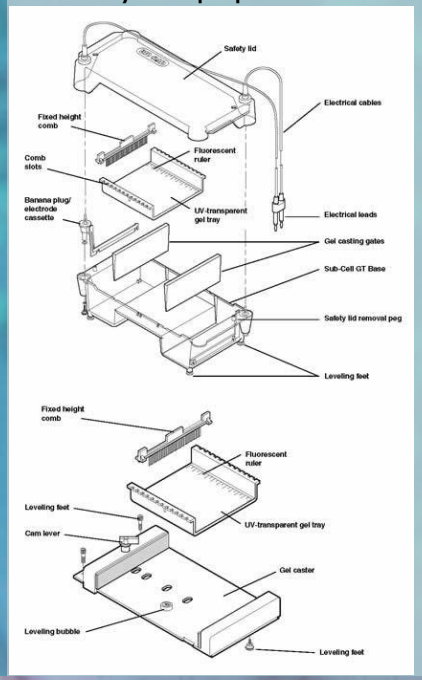
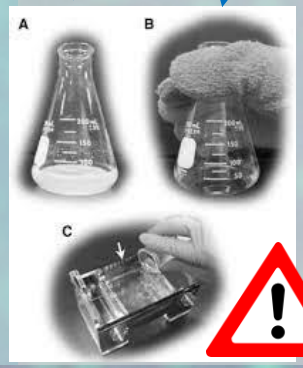
Electrophoresis in agarose gel

1. Gel casting system preparation

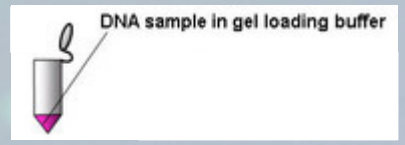


2. Agarose gel preparation



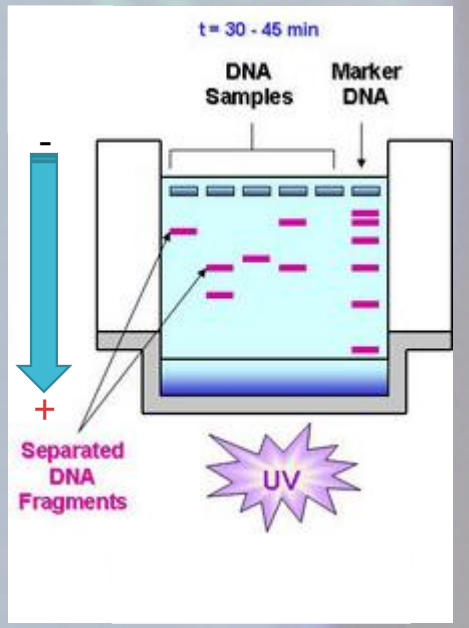
EtBr = CMR

3. Addition of loading buffer & sample loading



4. Setting up connection & start migration

5. Results observation



Electrophoresis in agarose gel

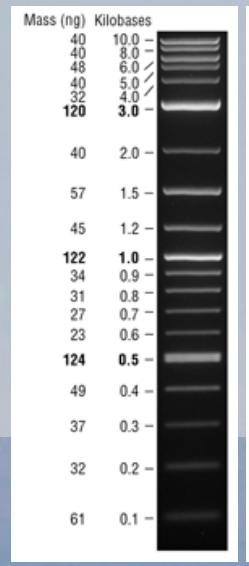
sample prepation for loading

Sample of digested plasmid or insert	500 ng pG11 or 150 ng insert
Loading buffer (6 X or 10 X)	1 X final
H_2O UP (only if necessary)	qsf 24 µl

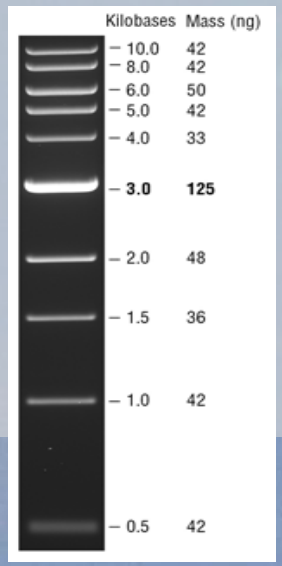
1. Which volume of loading buffer and H_2O?

2. Which DNA ladder?

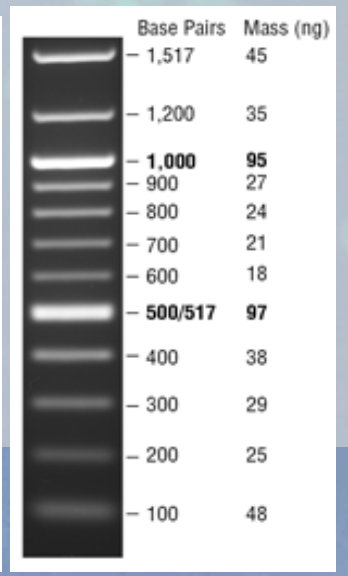
1 kb Plus DNA Ladder



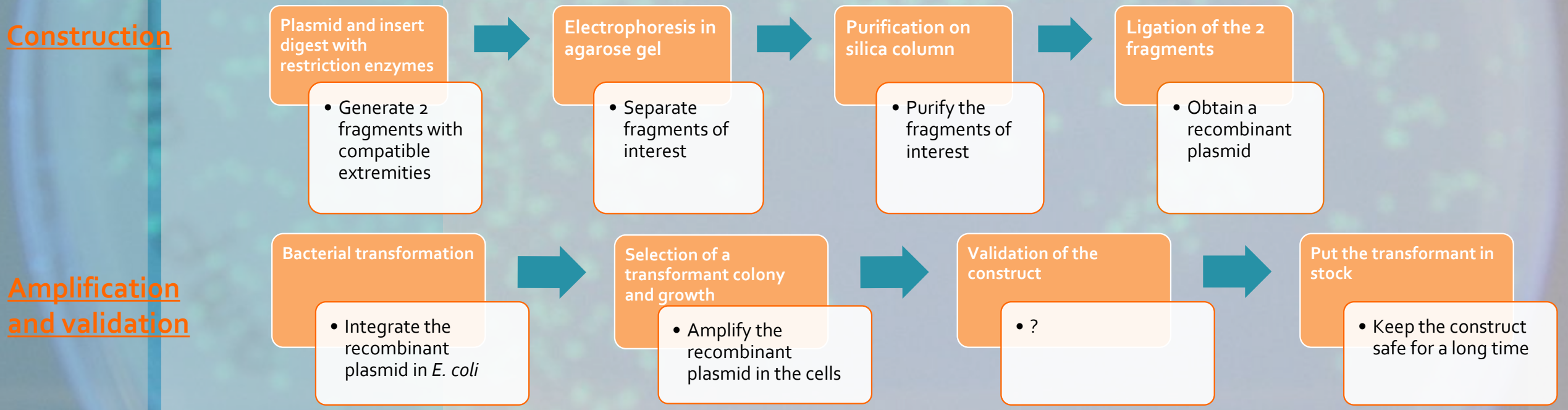
1 kb DNA Ladder



100 bp DNA Ladder



Workflow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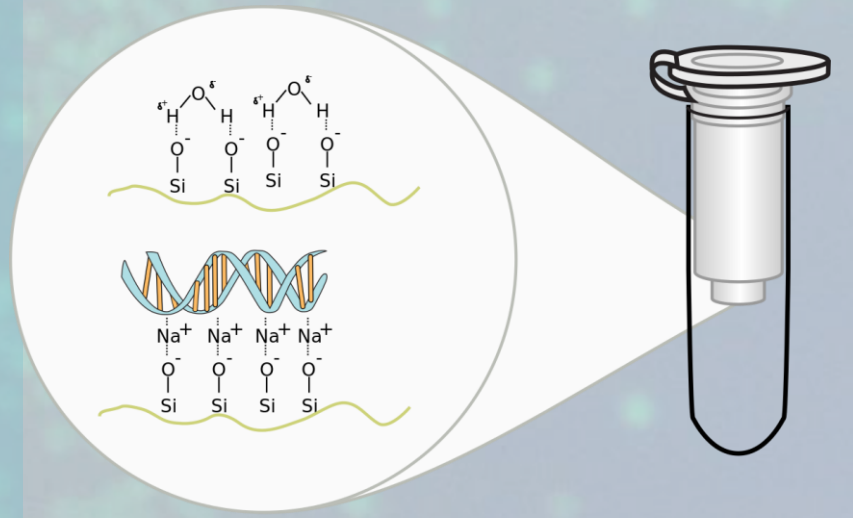
Purification on silica column

Materials

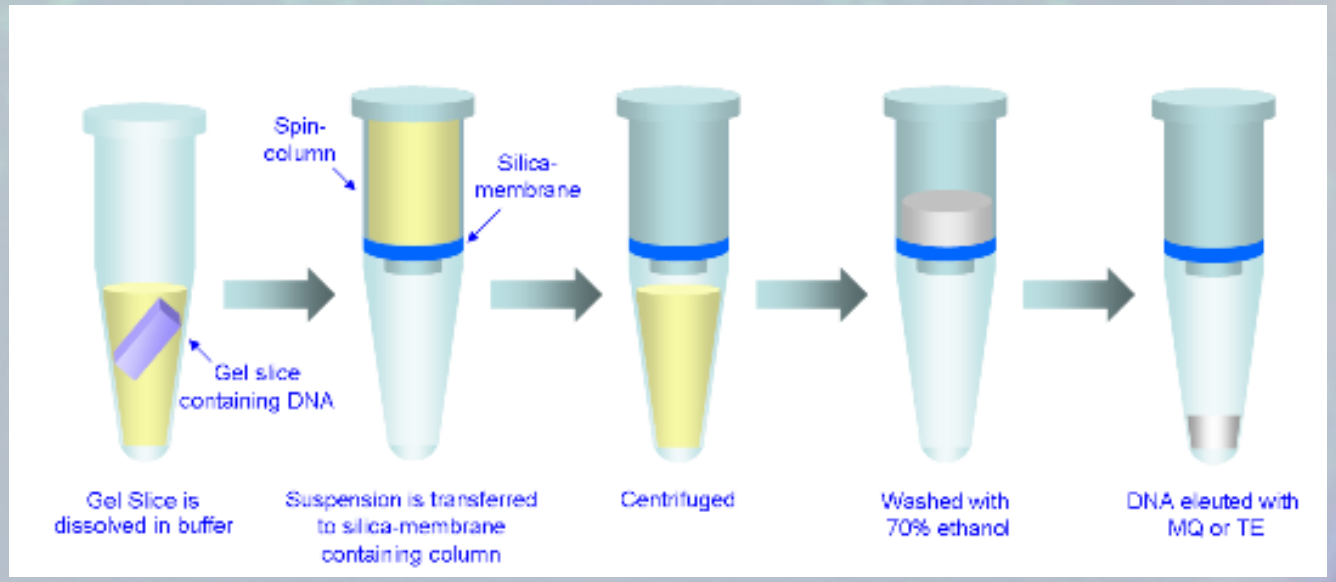


DNA adsorbs to silica matrices in high-salt buffer, at optimal pH.

Purification on silica column

Procedure overview

Eye protection against burn
= wearing an anti-UV mask



Purification on silica column

Anticipate expected results

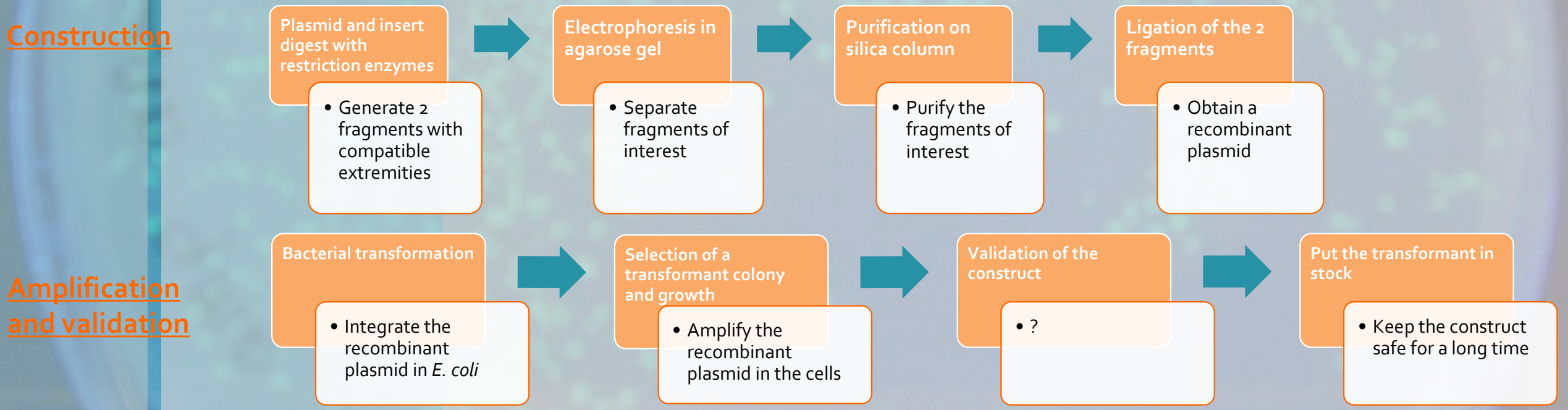
1. What is the expected size (length) and sequence of the 2 DNA fragments obtained after digest?

2. Why and how are they able to ligate to each other?

→ Tool recommended for sequence analyses: APE free software

→ ! You must consider all the possibilities of ligation that can happen

Workflow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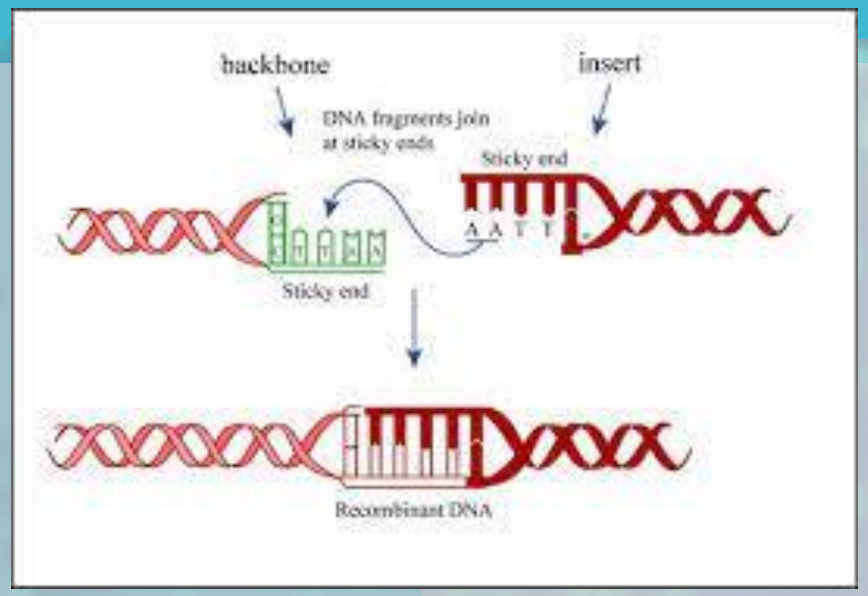
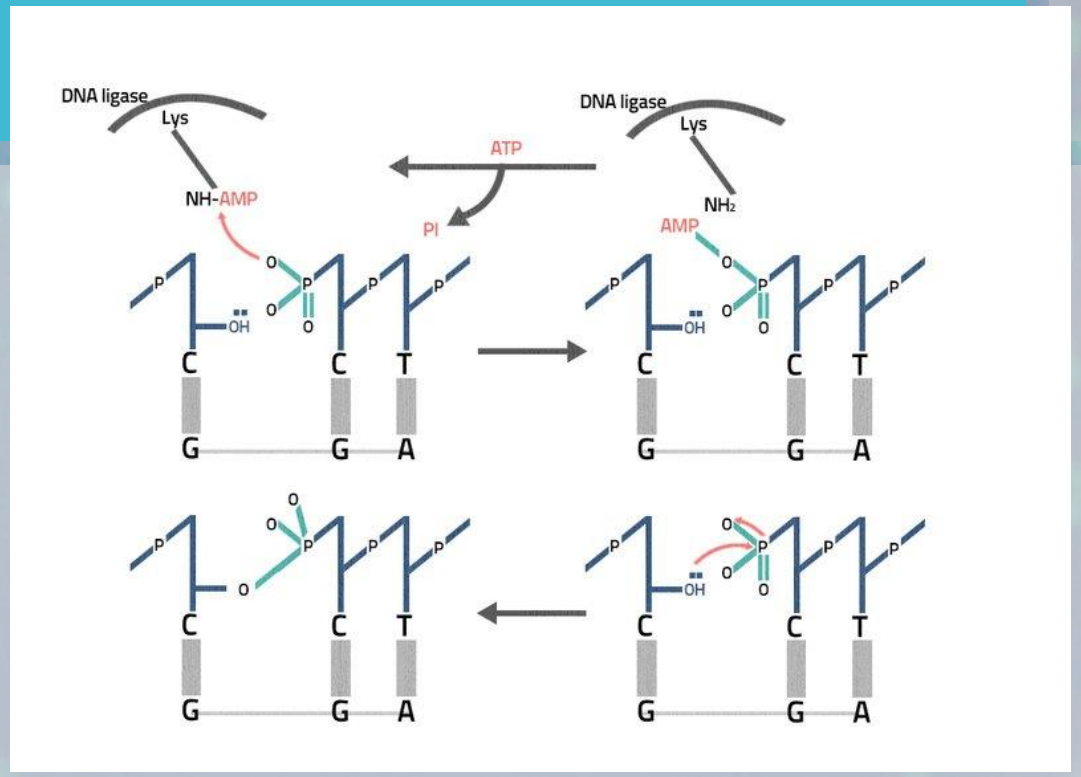
Ligation
Principle





- ! Molar ratio and not quantity ratio

Ligation
Reaction

	Tube Lig A (ratio 1:2)	Tube Lig B (ratio 1:3)	Tube Lig C (ratio idem A or B)	Tube Lig D
H_2O	qsf 20 µl	qsf 20 µl	qsf 20 µl	qsf 20 µl
Ligase buffer (10 X) **! Make sure you vortex it before pipetting it**	1 X	1 X	1 X	1 X
Cloning vector (previously digested and purified)	50 ng	50 ng	50 ng	50 ng
Insert (previously digested and purified)	x ng **(YOU MUST CALCULATE*)**	x ng **(YOU MUST CALCULATE*)**	x ng **(YOU MUST CALCULATE*)**	-
Ligase	1 µl	1 µl	-	1 µl

You need to calculate the required amount of insert

! Molar ratio (and not mass ratio)

→ *Tool recommended for calculation:* http://nebiocalculator.neb.com

Or simply apply the following calculation:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

Ligation

Ligation Calculator

This tool will calculate the mass of insert required at several molar insert:vector ratios in the range needed for typical ligation reactions.

Ligation | Tutorials

Insert DNA length

kb

Vector DNA length

kb

Vector DNA mass

ng

Required insert DNA mass

--- (1:1)

--- (2:1)

--- (3:1)

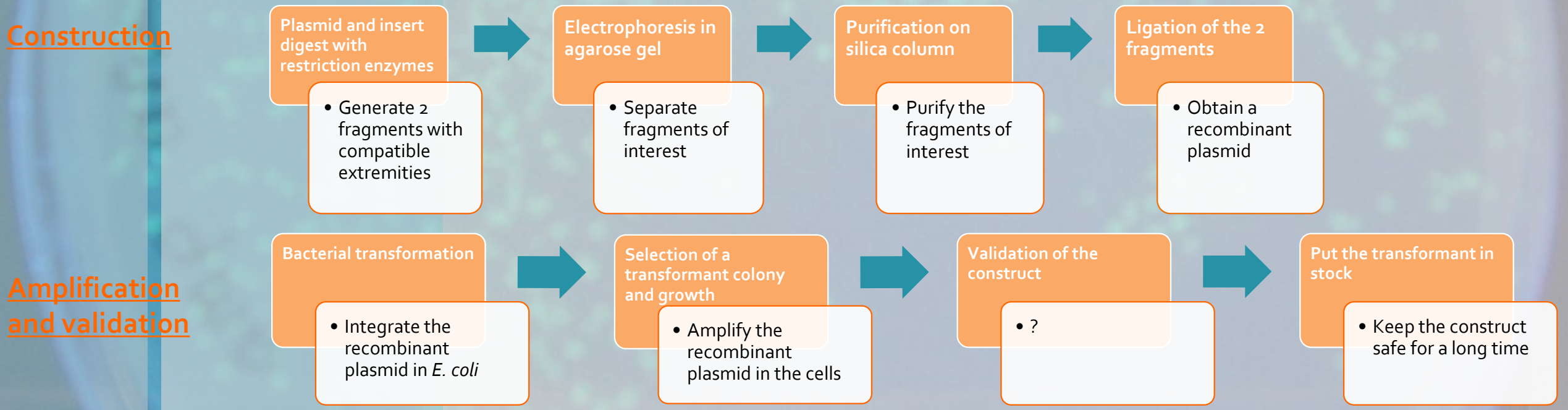
--- (5:1)

--- (7:1)

Formula

required mass insert (g) = desired insert/vector molar ratio x mass of vector (g) x ratio of insert to vector lengths

Workflow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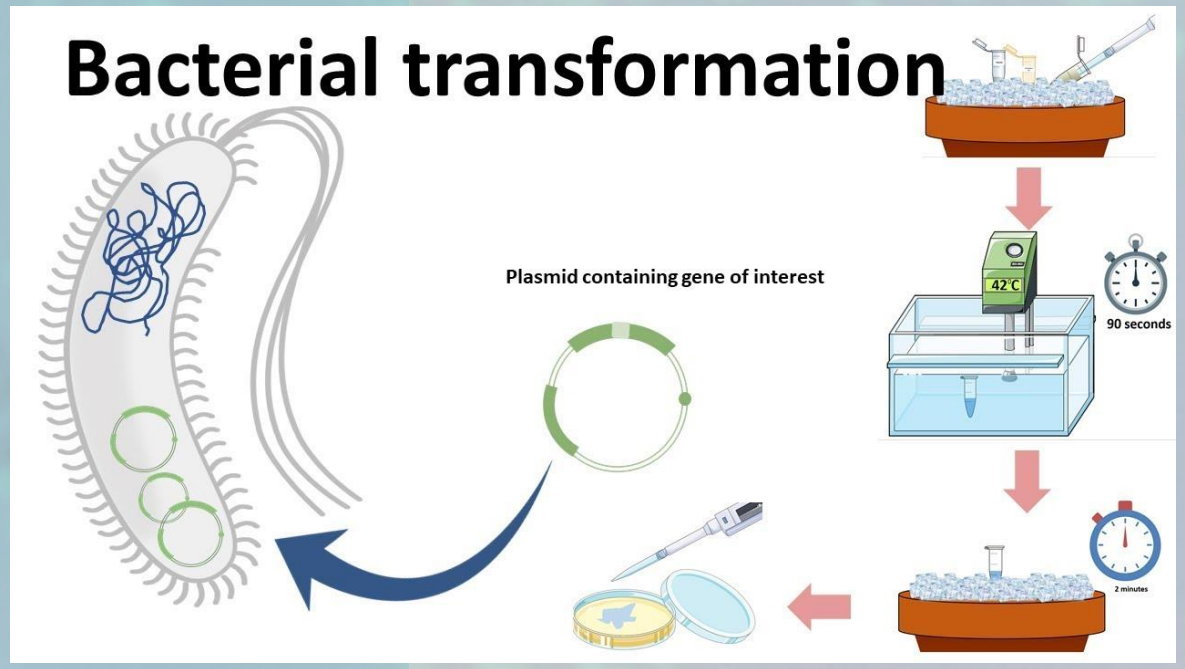
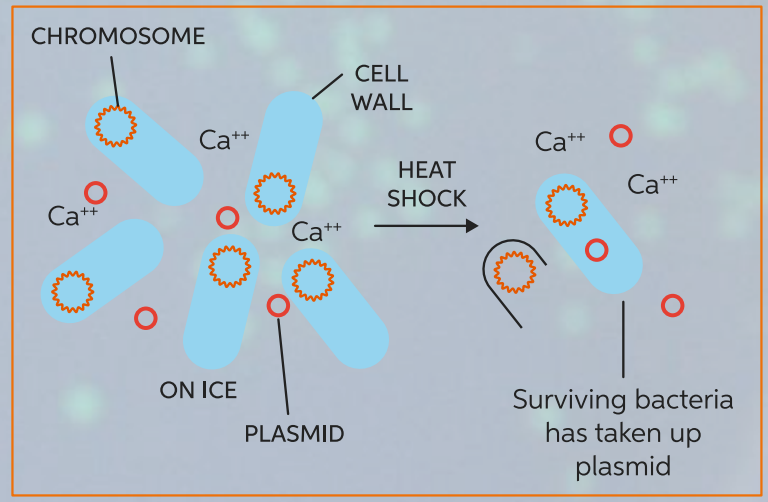
Bacterial transformation

Principle





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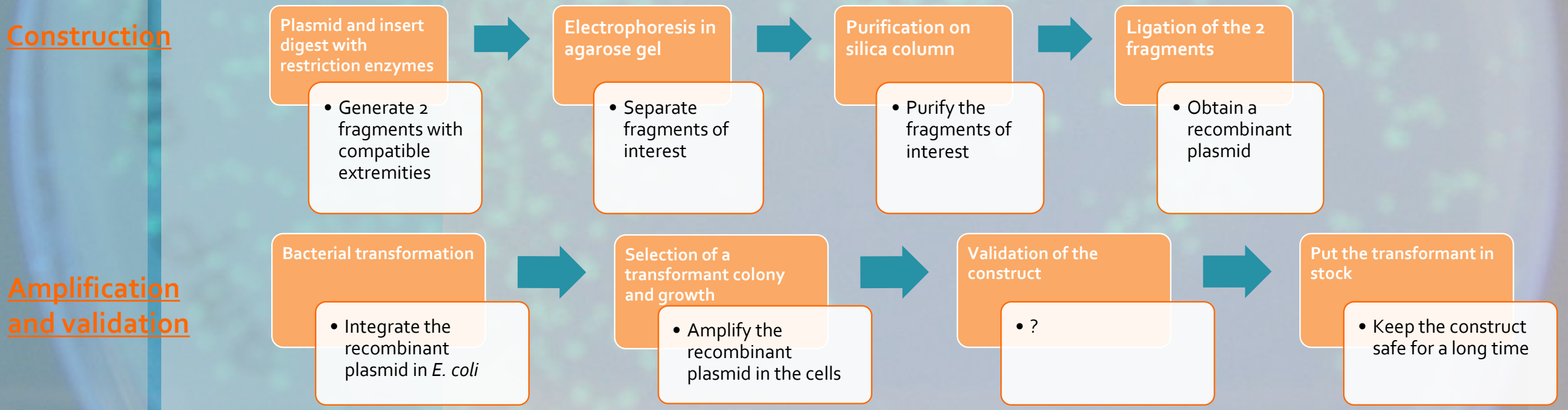
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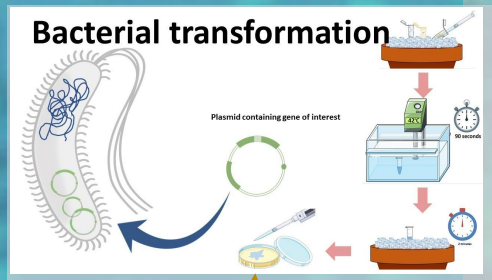
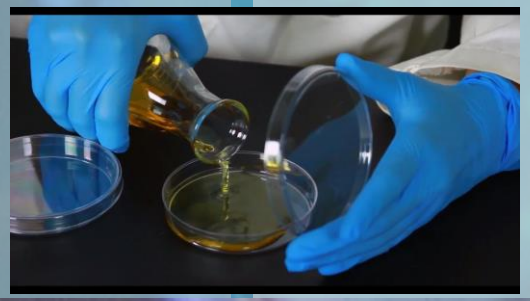
Selection of transformant colonies and growth in liquid nutritive medium



Medium = ?
Controls = ?



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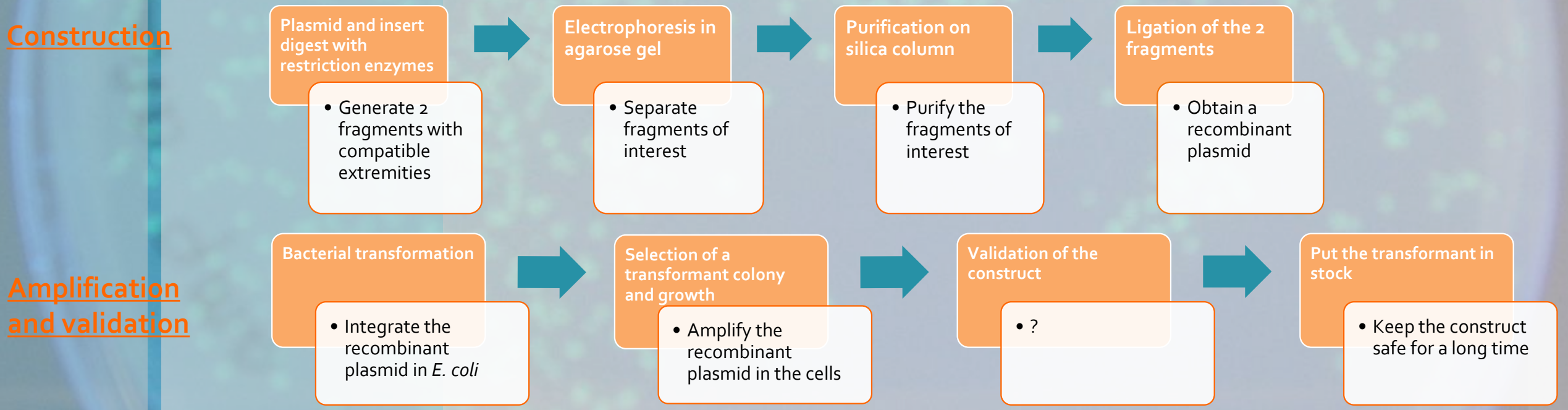
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Validation of the construct

- Has the insert been integrated in the plasmid? In the correct orientation?
- → You must plan the experimental steps you want to perform to prove it
- → To do so, you must make a scheme of all the possibilities of ligation reactions that can take place (some of them are more likely to happen than others)

Time schedule

- 4 lab sessions:
  - Day 1 = plasmid and insert digest, agarose gel preparation, electrophoresis and purification, ligation
  - Day 2 = bacterial transformation and discussion about validation protocol
  - Day 3 = analysis of transformation results and culture of transformants
  - Day 4 = cloning validation, finishing report writing-up

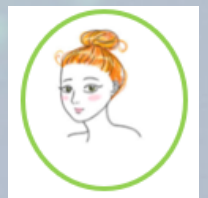
Requirements for lab work

- Basic hygiene and security rules in the lab:
  - !!! Work with alkaline/acidic/CMR reactives
  - Wear a lab coat in cotton
  - Be careful when and how to use lab gloves
  - No eating and drinking
  - Not use your mobile phone
  - Keep long hair attached

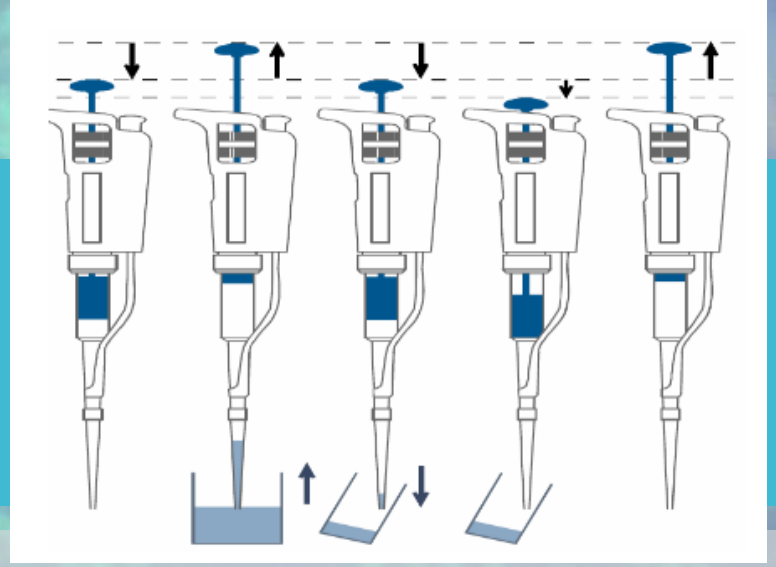








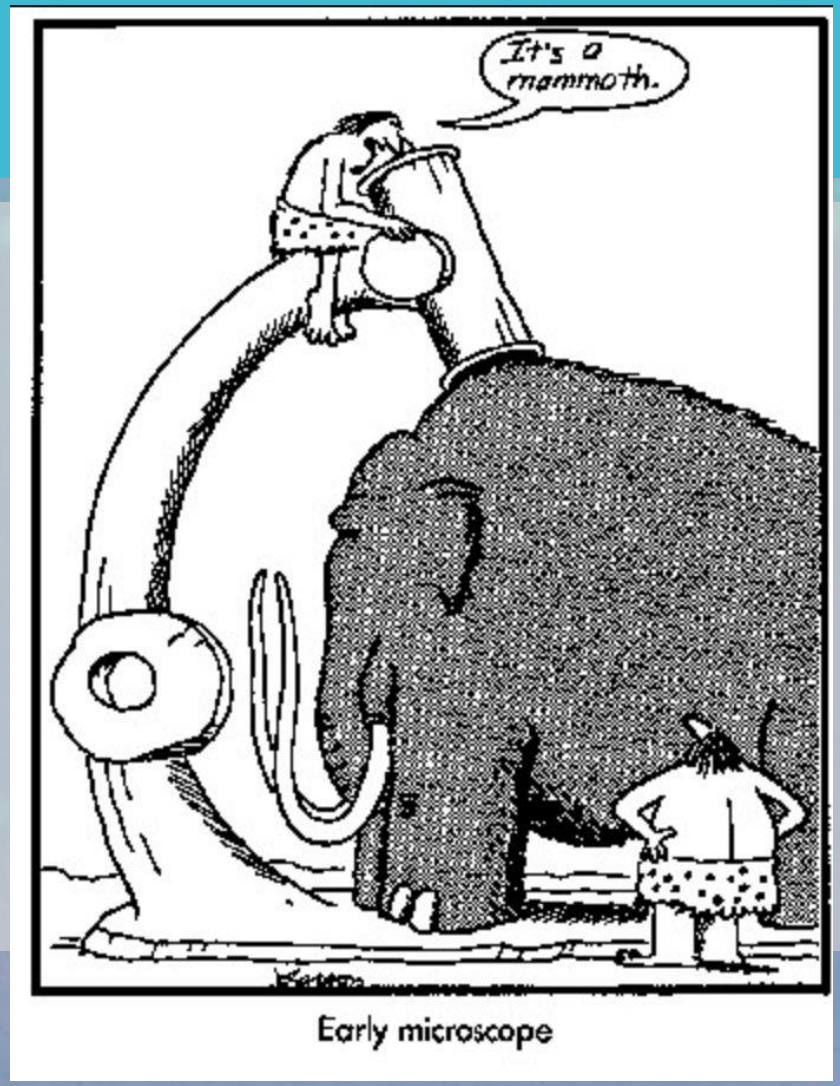
Pipeting technique



- Pipeting small volumes of reactives → use of micropiettes = precision tools
  - Handle with care and precision
  - Effect of different parameters on pipeting:
    temperature of sample, viscosity…
  - Most simple technique: direct pipeting
  - Always use pipette tips
  - Keep the end of the pipette tip just below the level of the taken liquid
  - Always look at the volume you take up and transfer

Thank you for you attention!

Hope you enjoy the lab class



Early microscope

Appendix - Purification of plas

Procedure overview