Laboratory 4:
Ligation / Transformation

Objectives

- To ligate the PCR- amplified *Amy2* coding region into a mammalian expression vector
- To transform *E. coli* with the ligation reaction, to determine transformation efficiency
- To learn the basics of sterile technique by working with bacteria

Ligated plasmids are **transformed** and amplified in an appropriate *Escherichia coli (E. coli)* bacterial strain and transformants are **selected** on media containing antibiotics.

1. DNA ligation

The aim of this experiment is to ligate the PCR fragment containing *Amy2* coding sequence into the digested pcDNA6/*myc*-His A plasmid. Insert and vector were both digested with *BamH*I and *Xho*I restriction enzymes. Remember that we added *BamH*I and *Xho*I restriction sites to *Amy2* specific primers. **T4 ligase** was originally isolated from the bacteriophage T4 and is used to ligate DNA fragments (cohesive or blunt ends) and to repair nicks in double-stranded DNA having 3'-hydroxyl and 5'-phosphate. During the ligation reaction, hydrogen bonds will form between overhangs of insert and vector DNA, which allows DNA ligase to catalyze the formation of phosphodiester bonds, creating a stable circular plasmid.

For practical reasons, we have prepared insert and vector DNAs in advance, as described below. This involved restriction enzyme digestion and purification of the corresponding DNA fragments.

Steps performed by us:

PCR products corresponding to *Amy2* coding sequence (sample A) were **digested** with *BamH*I and *Xho*I and **purified** using a silica spin column (QIAquick PCR purification kit) to remove primers, salts, enzymes and nucleotides. Similarly, the plasmid expression vector was first digested with *BamH*I and *Xho*I and then treated with **Antarctic Phosphatase** to remove of 5´ phosphate groups from the DNA ends. This reduces vector self- ligation, since ligase requires both 5´ phosphate and 3' OH ends for recircularization. Finally, to remove undigested plasmids, the reaction was separated on a 0.8% agarose gel and linearized plasmid (vector) was **cut out** from the gel and **purified** using the QIAquick gel extraction kit (Qiagen). Thereafter concentration of vector and insert DNA was quantified using the NanoDrop. For optimal ligation conditions we will use a **molar ratio of 1:3** vector to insert (e.g.: if your vector and insert are respectively 3 and 1 kb long you should use the same amount of DNA in your ligation reaction) and keep the total DNA concentration between 1-10 µg/ml.

Materials

- Insert (PCR fragment digested with *BamH*I and *Xho*I and purified)
- Plasmid pcDNA6/*myc*-His A (digested with *BamH*I and *Xho*I and purified)
- T4 DNA Ligase (1U/µl ; Invitrogen)
- 5X ligation buffer (Invitrogen)
- Sterile water

Procedure

In order to set up your ligation reaction you need to know the concentration and size of fragments to be ligated. Please fill in the following table:

	Concentration (ng/µl)	Size (bp)
Vector	40	
Insert	30	

For the ligation we will use 50 ng of vector DNA. Calculate the amount of insert corresponding to a 3-fold molar excess and complete the table below.

Help: https://nebiocalculator.neb.com/ - !/ligation

Add the following components (in the given order) into a sterile 1.5 ml tube:

tube	**A** **ligation** (vector + insert)	**B** **negative control** (vector only)
Volume	in µl	in µl
Digested **vector** DNA (pcDNA6/*myc*-His): 50 ng		
Digested **insert** DNA (PCR amplicon) : _____ ng (3-fold molar excess of insert)		**–**
5 X Ligation buffer	4	4
Water		
T4 ligase enzyme	1	1
Total volume	20	20
Total DNA concentration (ng/µl)		

Pipette up and down for gentle mixing (no vortexing!) followed by a quick spin, incubate for **15 min at room temperature**. Store on ice until you do the transformation.

2. Transformation of *E. coli* cells

The process by which foreign DNA is introduced into a bacterial cell is called **transformation**. *E. coli* cells have been chemically pretreated with divalent cations (e.g. Ca2+), which render the cell membrane permeable to allow incorporation of foreign plasmids (= they are **chemically competent**). Cells are incubated with the DNA on ice before they get heat-shocked whereby the DNA enters the cells (the bacterium is transformed). **Selection** of positive clones will be done on ampicillin-containing agar plates since the plasmid contains the ampicillin resistance

gene. Ampicillin acts as a competitive inhibitor of the enzyme transpeptidase, needed to make the bacterial cell wall.

Wild-type E.coli cells have a restriction-modification system, which protects them from foreign DNA (e.g. bacteriophage infection). Restriction enzymes cut foreign DNA (restriction), while the host DNA is methylated by methylases (modification) to protect it from the restriction enzymes. *E.coli* strains used for cloning, like DH5α have mutations in the system of methylation and restriction allowing efficient transformation of exogenous DNA. We will use *E. coli* **DH5α competent cells**. with a transformation efficiency > 1 x 10^6 colony forming units (cfu)/µg supercoiled plasmid DNA and allow stable replication of high-copy number plasmids.

The aim of this experiment is to transfer the ligation reactions (= new recombinant plasmid pcDNA6-Amy2-His) into *E. coli* cells and select clones containing the plasmid using antibiotics. Once inside the bacterial cell the plasmid will be replicated due to the origin of replication and we can isolate large amounts of plasmid DNA in the next lab.

Be careful, competent cells are highly sensitive to temperature changes or mechanical lysis caused by pipetting. Always keep cells on ice and mix cells gently (no vortex).

Safety

At all times during the practical work be aware that you are working with a genetically modified organism (GMO). You are expected to adopt safe working practices to protect yourself and others. Please adopt the the following rules:

- Wear a lab coat, gloves and eye protection.
- Clean bench before and after use with 70% EtOH.
- At the end dispose of all waste collected on the bench (pipette tips, gloves, tubes) in the yellow biohazard bag. The waste will be treated to kill the bacteria.
- Clean any spillage with 70% EtOH.
- Don't touch your face, doorknobs, touchscreen etc with gloves while handling bacteria.
- After removal of gloves, wash your hands.

All steps will be carried out at the bench (non-sterile), antibiotics will minimize the risk of contamination. To prevent contamination, the same protocol can be carried out in sterile conditions by using a Bunsen burner or biosafety cabinet.

Materials

- DH5α chemically competent E. coli cells (stored at -80°C and thawed on ice)
- SOC medium (2% Tryptone, 0.5% Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose), prewarmed at 37°C
- Control plasmid (pcDNA6/myc-His undigested; 0.2 ng/µl)
- LB (Luria-Bertani) – Ampicillin plates (1.0% Tryptone 0.5% Yeast Extract 1.0% Sodium Chloride (NaCl) pH 7.0 ; 100 µg/ml ampicillin; 15 g/L agar)
- LB plates
- Heating block at 42°C (switch on before you start to let the temperature stabilize)
- Sterile spreader

Procedure

You will receive three tubes with each 50 µl competent *E. coli* cells. Label them A-C. Add the ligation mix or control according to the table below and mix by swirling or tapping the tube gently (not vortex).

Amounts in (µl)	**A** ligation	**B** negative control	**C** transformation control
Competent *E. coli* cells (already in tube to avoid pipetting!)	50	50	50
Ligation mix **tube A** from above	5	-	-
Ligation mix **tube B** from above	-	5	-
Control plasmid (total 1 ng)	-	-	5

1. Incubate on ice for **30 min**.
2. Heat-shock the cells for exactly **20 sec** at **42°C**. Immediately transfer the tube on **ice for 2 min**.
3. Carefully add **250 µl prewarmed SOC medium**.
4. Incubate the samples for **1 h at 37°C** with shaking (225 rpm) for recovery and expression of the antibiotic resistance gene. Note: due to logistics we will incubate at 37°C without shaking, this may reduce the transfection efficiency.
5. Label your plates on the bottom (since lids may get exchanged accidentally) close to the edge with date, group number, sample A-C and volume to spread. Storing plates upside down prevents contamination and evaporation.
6. Spread each **200 µl** on LB+Amp plates using a **sterile plastic spreader**. The correct technique for spreading is outlined below.
   a.Remove the lid of the plate. Use filter tips to pipette in the center of the plate.

   b. Remove sterile spreader from package

   c. Spread the liquid on the entire surface while turning the plate with the other hand, continue spreading until dry (minimum 1 minute). Do not make holes.

   d. Put back the lid, turn upside-down.
7. We will incubate your **plates overnight at 37°C** (upside down) to select for transformants (e.g. *E. coli* cells that took up the plasmid). After overnight incubation colonies are visible and will be imaged (with the next lab group).

Counting transformants (after overnight incubation)

To know whether the transformation was successful (this refers to both cells and procedure) we transformed the undigested plasmid of known amount. The commercial competent cells used have a transformation efficiency of > 1 x 10^6 colony forming units (CFU)/µg supercoiled plasmid DNA.

1. Count the number of colonies from each of your plates:

	Transformation	µl plated	Number of CFU
Plate A	Ligation (vector + insert)	200	
Plate B	Negative control (vector only)	200	
Plate C	Transformation control (undigested plasmid)	200	

2. Calculate the **transformation efficiency** of your competent cells (plate C). Take into account the amount of plasmid DNA used in your transformation control and the volume you plated onto LB-Amp plates.

   Transformation efficiency = Total number of colonies growing on the agar plate / Amount of DNA spread on the agar plate (in µg)

   Transformation efficiency: _____ transformants / µg DNA

3. Calculate the **% of background colonies** (vector ligation control, plate B). Remember that you added the same amount of linearized vector to the ligation mix A and B. Background colonies: _____%

Questions

Q1 What are background colonies, and how do they differ from recombinant colonies in a ligation and transformation experiment?

Q2 Did your transformation work as expected? Justify your answer.

Q3 How many colonies from plate A do you need to pick in order to find at least one positive clone? Take into account the % background colonies. (In practice you will usually pick 2-6 times more clones to increase the probability of obtaining the correct one).

Single colony streaks (each student streaks one plate)

Streaking is a technique to isolate on a plate a pure strain (colony) from a microorganism. By cell division a single cell will form a colony with genetically identical cells. In this exercise you will streak a mixed culture of genetically engineered *E. coli* cells expressing different fluorescent proteins from a liquid culture onto a LB + kanamycin plate. The (random) dilution on the surface of an agar plate is achieved by **streaking multiple times** and **using a new tip for each streak.** The same technique can be applied with a metal **inoculation loop** that is sterilized in the flame between the streaks (Figure 1). The appropriate technique will be demonstrated by the assistant.

Procedure

1. Label the plate at the bottom: write on the edge.
2. Dip (do not pipette!) a 20 µl tip on a micropipette into the cell suspension (vortex before opening). Close the tube.
3. Streak cell suspension back and forth (either 4-5 zig-zag or parallel lines) in one quadrant of the plate as shown below.
4. Take a new sterile tip (on micropipette or in hand). For the first dilution streak, move several times across the end of the first streak while streaking back and forth (either 4-5 zig-zag or parallel lines) in the next quadrant of the plate.
5. Repeat step 4 two more times with a new sterile tip to obtain four dilution streaks in the four quadrants (Figure 1). For the final streak, be careful not to touch the original inoculum across the top of the plate.
6. Plates will be incubated at 37°C overnight.

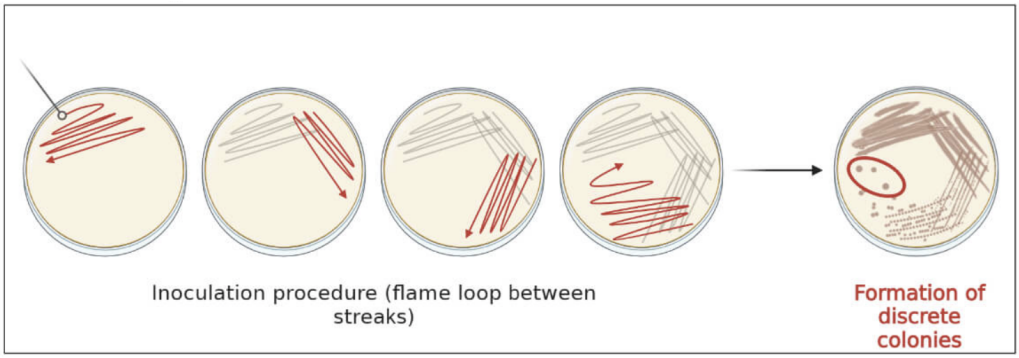

Figure 1: Isolation of single colonies by streaking. Four consecutive “dilution streaks” (arrows) with loop sterilization in between streaks were made from the original inoculum. After overnight incubation at 37°C, the fourth streak has generated well-separated single colonies that can be picked for further analysis. Image source: Microbenotes.

3. Inoculating cultures

You will inoculate liquid cultures starting from single colonies (after transformation normally you have single colonies the next day). In the next lab session plasmid DNA will be recovered from such overnight cultures.

Note: Satellite colonies may appear on LB/Amp plates because the beta-lactamase enzyme, which degrades ampicillin, is secreted and transformants will create an ampicillin-free area around them. Be careful not to pick satellite colonies, which are small surrounding colonies and correspond to non-transformed bacteria.

Materials

- LB ampicillin plates from the ligation + transformation (plate A)
- LB ampicillin medium
- 14 ml sterile round-bottom tubes with dual position snap cap (loose or tight)
- Pipette boy and disposable pipettes
- Sterile tips
- Shaker at 37°C

Procedure (each student prepares one tube)

1. Prepare a 14 ml tube with 2 ml LB/Amp medium.
2. Pick a single colony using a sterile tip.
3. Drop the tip into a bacterial culture tube containing 2 ml LB/Amp medium.
4. Close tubes (loose position for sterile aerobic culturing).
5. Put your tubes onto a shaker at 37°C and incubate overnight with agitation at 225 rpm.

Exercises

Exercise 1: You purchased some supercompetent bacteria that are provided at a transformation efficiency of 10^9 colony forming units/µg of DNA. You transform the cells with 1 ng of plasmid DNA and plate 1/1000th of the cells. How many colonies do you expect? Next you transform another aliquot of cells, also at 10^9 colony forming units/µg of DNA, with 2 µl of plasmid DNA. You spread 1/100th of the cells and find 50 colonies growing on the plate after 24 hours at 37°C. What is the concentration of plasmid?

Exercise 2: To illustrate your understanding and the importance of the controls you performed today, please write a one-sentence interpretation for each of the following transformation outcomes. There may be more than one valid interpretation for some of the data (one answer for each is required).

- Outcome 1: no colonies on any plate.
- Outcome 2: thousands of colonies on all the plates.
- Outcome 3: approximately the same number of colonies (around 100) on the plate with ligation (A) and vector control (B).