# Laboratory 5:
# Plasmid isolation and digestion

## Objective

- To purify plasmid DNA from *E. coli* cells
- To analyze the plasmid DNA by restriction digest

## 1. Plasmid isolation

A widely used method for the purification of plasmid DNA from *E. coli* is **alkaline lysis** of the bacteria in the presence of the detergent sodium dodecyl sulfate (SDS). Lysis in **SDS** at high pH opens the cell wall and denatures the chromosomal DNA, the plasmid DNA and proteins. In the next step the pH is neutralized: the plasmid DNA re-natures in contrast to the chromosomal DNA. During this step the chromosomal DNA, the proteins, and the cell wall debris are precipitated and removed by centrifugation, the plasmid remains in solution. Finally, the plasmid DNA is bound to an affinity matrix (QIAprep column), washed and finally eluted.

As a follow up of the previous lab session you will isolate plasmid DNA from overnight (O/N) cultures from *E. coli* transformed with pcDNA6/myc-His ligation mix A containing the insert and with pcDNA6/*myc*-His control plasmid (negative control without insert, ligation mix B).

### Materials

- Bacterial overnight cultures (each group purifies one culture A or B):
  A = pAmy2-His clone (pcDNA6/*myc*-His + *Amy2* cDNA)
  B = pcDNA6/*myc*-His clone
- QIAprep Spin Miniprep Kit
  - P1 = resuspension buffer + RNase A and LyseBlue
  - P2 = lysis buffer
  - N3 = neutralizing buffer
  - PB = wash buffer
  - PE = wash buffer
  - EB = elution buffer
  - QIAprep spin column
  - Collection tube
  - 1.5 ml microfuge tube
- Microcentrifuge

**SAFETY REMINDER:**

You are working with an antibiotic-resistant bacterium. Continue to use the precautions that you learned in lab 4 (e.g. no spills, wear gloves and goggles, separate biohazard waste collection). Be careful not to contaminate your pipettes or electronic devices and wipe them with 70% ethanol after use.

## Procedure

Plasmid purification will be performed according to the QIAprep Spin Miniprep kit protocol (QIAGEN). All steps are performed at room temperature.

1. You receive 1.9 ml overnight culture (colonies were picked the day before). Centrifuge for **5 min at 13’000 rpm to pellet the bacteria**.
2. Remove the supernatant into a container specified for bacterial waste.
3. Resuspend each bacterial pellet in **250 µl buffer P1**. **Vortex** to obtain homogenous solution of bacteria (no clumps) .
4. Carefully re-open the tube (no spilling) and add **250 µl lysis buffer P2**. Mix by inverting the tubes 4-6 times. DON’T VORTEX at this or next step! Do not allow the lysis reaction to proceed for more than 5 minutes.
5. Carefully re-open the tube (no spilling) and add **350 µl neutralization buffer N3**. Mix immediately by **inverting the tube** 6 times. The solution should become cloudy and turns colourless.
6. Centrifuge **10 min at 13’000 rpm**. A compact white pellet will form.
7. Apply **supernatant to a QIAprep spin column** without carrying over material from the pellet.
8. Centrifuge **30 sec at 13’000 rpm**, discard the flow-through and put column back into collection tube.
9. Wash column by adding **500 µl PB buffer** and centrifuge **30 sec at 13’000 rpm**. Discard the flow-through and put column back into collection tube.
10. Wash column with **750 µl PE buffer** and centrifuge **30 sec at 13’000 rpm**. Discard the flow-through and put column back into collection tube.
11. Centrifuge for an additional **1 min at 13’000 rpm** to remove residual wash buffer. (EtOH in buffer can disturb downstream applications).
12. Place the QIAprep column in a **clean 1.5 ml microfuge tube**. Add **50 µl elution buffer EB onto the membrane**, let stand for **1 min (this will inmprove elution)**, and then centrifuge for **1 min at 13’000 rpm**.
13. Put tubes on ice.
14. Label tube with **sample name, group number, date, concentration** (see DNA quantification)
15. Analyse the samples for concentration and purity as described below and use the purified DNA for restriction digest.

## 2. DNA quantification

You will determine the concentration and purity of your DNA samples using the NanoDrop spectrophotometer. DNA concentration can be calculated by measuring the absorption at 260 nm, specific for nucleic acids (see Lab 2). Reminder:

- An A260 value of 1.0 corresponds to 50 µg/ml of double stranded (ds) DNA.
- Concentration (µg/ml) = (A260 reading – A320 reading) × 50 µg/ml × dilution factor. The NanoDrop software automatically corrects for background absorbance at 320 nm.
- The ratio of the absorbance at 260 and 280 nm is used to assess the purity of nucleic acids. Expected values for pure DNA samples: A260/A280 ~ 1.8

## Material

- miniprep samples from your group
- Elution buffer (EB)
- NanoDrop

## Procedure

1. Add 2 µl of elution buffer EB to the pedestal and close the arm. Click ***Blank*** on the screen.
2. Wipe the pedestal with a *Kimwipe*. Add 2 µl of your sample to the pedestal and close the arm. Click ***Measure*** on the screen.
3. Record the given concentration, the absorbance reading and the A260/A280 ratio of your sample.

**Calculate the total yield**

DNA yield (µg) = DNA concentration x total sample volume (µl)

Total yield plasmid A (pAmy2-His): ___________ µg

Total yield plasmid B (pcDNA6/*myc*-His): ___________ µg

Expected yield for a 1.5 ml overnight culture: 5 to 15 µg of plasmid DNA; Source: QIAprep Miniprep Handbook.

## 3. Restriction enzyme digestion and analysis

Restriction enzymes are important tools not only for cloning of DNA fragments (see *Recombinant cloning strategies*) but also for analyzing DNA. These commercially available enzymes recognize a specific sequence within DNA and cleave it. For example, the recognition site for *Bam*HI is GGATCC (Table1 ).

| Restriction enzyme | Recognition sequence | Bacterial strain |
|---|---|---|
| ***Eco*RI** | 5'...**G↓A A T T C**...3'<br>3'...**C T T A A↑G**...5' | ***Escherichia coli RY13*** |
| ***Bam*HI** | 5'...**G↓G A T C C**...3'<br>3'...**C C T A G↑G**...5' | ***Bacillus amyloliquefaciens*** |

Table 1. Restriction enzymes *Eco*RI and *Bam*HI. Both cleave after the 5' G on both strands.

**Task**

You will use restriction enzymes to analyze plasmid DNA preparations. You will confirm the presence of the *Amy2* insert in two ways:

1. By digesting with *Bam*HI and *Xho*I, the original enzymes used to clone the *Amy2* cDNA.
2. By digesting with *EcoR*I, an enzyme that cuts **within** the *Amy2* cDNA.

Digestion of the parental plasmid (pcDNA6/*myc*-His) will serve as a negative control.

Single *Bam*HI and *Xho*I digests will serve as a positive control for the restriction enzyme digest.

**Materials**

- pAmy2-His (pcDNA6/*myc*-His + *Amy2* insert)______ng/µl =______µg/µl
- pcDNA6/*myc*-His ______ng/µl =______µg/µl
- *Bam*H1 (FastDigest enzyme, Fermentas ; 1 FDU*/µl)
- *Xho*1 (FastDigest enzyme, Fermentas ; 1 FDU*/µl)
- *EcoR*1 (FastDigest enzyme, Fermentas; 1 FDU*/µl)
- 10X Fast digest green buffer (Fermentas)
- Molecular biology-grade water
- DNA ladder (see datasheet on Moodle)

*Definition of activity unit: 1 µL of FastDigest enzyme (1 FDU) cleaves 1 µg of substrate DNA in 5-15 minutes in 1X FastDigest Buffer

**Procedure**

We will set up the following sample reactions (see table below):

- Sample # 1 and 6: undigested plasmid DNA control
- Sample # 2 and7: *Bam*HI single digest
- Sample # 3 and 8: *Xho*I single digest
- Sample # 4 and 9: *Bam*HI / *Xho*I double digest
- Sample # 5 and 10: *Eco*RI single digest

Two groups share the purified DNA, but each group sets up their own digestions. For each reaction you will need **1 µg of plasmid DNA**. In case you have not enough DNA may use less DNA per reaction (min 0.5 ug). Calculate the amounts and fill in the table below before you start. Check your completed table with an assistant before preparing the reactions!

- Add 1/10$^{th}$ of the final volume (20 µl) of 10-fold concentrated (10X) reaction buffer (Fast digest buffer containing green gel loading dye).
- Add 1 µl of each FastDigest restriction enzyme (= 1 FDU*).
- Add water to reach a final total volume of 20 µl.

| **Sample reaction #** | **amounts in µl** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** |
| pAmy2CDS-HIS | | | | | | - | - | - | - | - |
| pcDNA6 | - | - | - | - | - | | | | | |
| 10X Fast buffer | | | | | | | | | | |
| *Bam*HI | | | | | | | | | | |
| *Xho*I | | | | | | | | | | |
| *EcoR*I | | | | | | | | | | |
| Water | | | | | | | | | | |
| **Total volume** | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 |

- 1. Pipette reagents into 1.5 ml microfuge tubes, **enzymes should be added last**.
- 2. Mix carefully by tipping the tube (no vortexing) followed by a quick spin.
- 3. Incubate the reactions at 37°C for 15 min, then put samples on ice.

**Agarose gel electrophoresis of restriction fragments**

You will run your samples on a 1% TAE agarose gel to check that your recombinant plasmid has the expected insert size by comparison to a DNA ladder. DNA migrates differently depending on its form: circular supercoiled DNA migrates faster than linear DNA of the same mass. To follow how far the DNA has migrated through the agarose gel, a colored loading dye is added to the DNA samples. Note that the DNA ladder contains bromophenol blue, a tracking dye that migrates with 300-400 bp DNA fragments. Fast digest green buffer contains blue and yellow tracking dyes and are ready for gel loading. In a 1% agarose gel the blue dye migrates with 3-5 kb DNA fragments and the yellow dye migrates faster than 10 bp DNA fragments.

Each group will prepare one agarose gel.

1. Prepare 50 ml of 1% agarose in 1X TAE buffer (Tris-acetate-EDTA) buffer in a 200 ml Erlenmeyer flask.
2. Melt the agarose in a microwave oven (no aluminium foil or Parafilm!).
3. Let cool down for approximately 5 minutes.
4. Add 1:10’000 parts GelRed dye (nucleic acid stain). Mix properly.
5. Set up the gel-casting mold into the frame. Place the comb onto the frame at the top of the mold.
6. Pour the agarose solution into the gel-cast (don’t overfill) and wait until the gel solidifies before loading your samples (1 hour).
7. Cover the gel with 1X TAE buffer and **carefully** remove the comb.
8. Load your samples in the following order:

| | | **sample #** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **ladder** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **ladder** |
| µl | 3 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 3 |

9. Run the gel at 120 Volt for about 30 minutes.
10. Remove the gel from the chamber and take a photograph at the UV transilluminator.
11. Paste the gel photograph into your notebook and label it with an adequate description.

**Analysis of restriction digest**

You will compare the expected pattern of digested DNA bands (based on Benchling virtual digestion) with the experimental results.

Use Benchling 'Virtual Digest' to determine the expected size of restriction digestion fragments of recombinant plasmid pcDNA6-Amy2-myc-His (created previously) and the parental plasmid pcDNA6/*myc*-His A (as a reminder how to digest https://help.benchling.com/hc/en-us/articles/9684268012557-Simulate-a-digest). Use the same order of lanes to facilitate comparison between the virtual and the experimental gel digest (omit the undigested samples - the software can only visualise migration of linear fragments). You can change the order of lanes on the Virtual Digest tab by rearranging the digest tabs with drag and drop. Note that the ladder in Benchling may differ from the ladder used in the lab.

1. Paste the circular plasmid map (from lab 3) with the restriction sites *Bam*HI, *Xho*I and *Eco*RI into SLIMS.
2. Run 'Virtual Digest' of all enzyme combinations as in the protocol.
3. Paste the virtual gel image into SLIMS and indicate the fragment length on the right side of the gel.
4. Paste the experimental gel image into SLIMS and describe the pattern of the bands that you observe. Indicate DNA fragment sizes of the 1 kb DNA ladder and fragments and estimate the sizes of the bands on your agarose gel.

**Address in your analysis:**

- Does the observed fragment size correspond to the expected size? Can you identify all expected fragments; if not explain.
- Can you distinguish between linear and circular plasmid DNA molecules? If yes, indicate their respective migration on your gel. Which of the supercoiled plasmid migrates faster? Justify your answer.
- Did the experiment work? Are the predicted and experimental digestion patterns the same? If not, try to explain why (discuss).
- Based on the result, can you be sure that your plasmid contains the expected *Amy2* insert? Justify your answer.

**Question:** Which other restriction enzyme could you use to check if your recombinant plasmid (pAmy2-His) contains the expected insert using Benchling. Explain your strategy (choose between cut sites within plasmid backbone, insert or in combination with sites used for cloning) and indicate the expected fragment sizes of the recombinant plasmid using 'Virtual Digest'. There are many solutions.