Protocol for
Cloning GFP into Mammalian cells

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Introduction

In your three days at Aarhus University you are going to conduct an experiment in order to make human cells emit green fluorescent light. This is done by using the gene of green fluorescent protein (GFP) from jellyfish. We have prepared the gene for you to work with, consisting of a double stranded piece of DNA. You will also receive a flask of living mammalian cells. In order to get the gene into the cells, you have to convert the gene into a circle of DNA, a so called plasmid. Plasmids are more easily taken up by cells, than linear pieces of DNA, and do not have to be inserted into the genome of the host, but will functions as an individual small genome alone.

To prepare the plasmid, you will receive an already existing ring of DNA, the expression plasmid, which we have cut open for you. The GFP gene is ligated into the open plasmid to recreate a circle. To ligate, an enzyme called T4 ligase is used. The outcome is a closed plasmid containing our GFP gene.

In the next step we want to sort and amplify our newly prepared GFP plasmid. Our reaction mixture from above contains both unclosed and closed circles of DNA, and some maybe with a wrong insert. To sort out the plasmids with a flawless GFP insert, we will begin with transforming all plasmids from the sample into E. coli bacteria cells. E. coli easily takes up plasmids, and once taken up, the cell will believe the DNA to be part of its genome, and will thus pass on a copy of the plasmid to future daughter cells. This means that one single E. coli cell, which has taken up the GFP plasmid, will give rise to a whole colony of bacteria cells all expressing the plasmid harboring our GFP gene. To get a sufficient amount of plasmid, we also have to rely on E. coli cell to mass produce our GFP containing plasmid.

To select the single E. coli cells which have taken up the GFP plasmid, and to transform this cell into a small colony, we seed the cells from above on media plates. Our GFP plasmid contains, together with GFP, a gene for the antibiotic kanamycin resistance. This means that E. coli cells which have taken up our plasmid, also are resistant to kanamycin. Cells which have not taken up any plasmid will die in the presents of kanamycin. So to sort out the cells, we grow them on plates containing kanamycin.

E. coli colonies surviving on the plates have the resistance gene, but we have to test whether they also contains the GFP gene. A plasmid closed without GFP insert, still has resistance towards kanamycin. This is done with PCR, where DNA can be amplified and visualized through gel
electrophoresis. We test for GFP and will in our gel see which *E. coli* colonies contains the real gene.

The next step we will perform for you, since we have no time for it together. The colonies positive for GFP will be transferred to a flask with media to grow. When a sufficient number of cells are reached, they are harvested and lysed (broken open) and the plasmids are purified.

Now you have your plasmid containing the GFP gene and you are ready to put it into mammalian cells. This is done by using calcium phosphate transfection. That transfection method is based on forming a calcium phosphate-DNA precipitate, which facilitates the binding of the DNA to the cell surface. DNA then enters the cell by endocytosis. This means that the cell membrane, which covers the cell, will fold around the DNA and drag it into the cell.

After transfection, the cells are allowed to grow for two days. On the second day you will have to change their media in order for them to maintain healthy. On the third day the GFP protein have been expressed in appropriate amounts to be visualized under UV light. This means that you can see your cells glowing in a green light if you look in a microscope.

The procedure above takes six days of work, and since you only have three, we will divide the experiment in two. This means that you on day one will begin by putting GFP into a plasmid as explained. At the same day, you will receive some already made GFP plasmid which you will transfect into mammalian cells. On your last day, you will thus hopefully have an *E. coli* colony expressing plasmid containing GFP, and mammalian cells green from GFP protein.
Day 1

Plasmid production: Ligation of the GFP gene into an expression plasmid
You are going to ligate GFP into an already cut open expression plasmid. By ligation the GFP becomes a covalent part of the plasmid which is at the same time circularized. It is only the circular form that can be replicated inside cells.

The GFP and the plasmid have both been cut with the restriction enzymes SalI and NotI, which recognizes specific DNA sequences. This means that the gene and the plasmid have ends matching each other. A map and sequence of the plasmid with GFP (called pEGFP-N1) inserted is in the appendix.

Materials
The purified restricted GFP
10 x T4 Ligase buffer
T4 DNA Ligase
Water
Restricted plasmid

Protocol
1. Prepare the following ligation mixes for your restricted GFP: (amounts in µL)

<table>
<thead>
<tr>
<th>Sample</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td>10 x T4 Ligase buffer</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Restricted GFP</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Restricted plasmid</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>T4 DNA Ligase</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

2. Incubate the ligation mixtures at 16°C in a heat block until next day.
Cell Transfection: The GFP plasmid is transfected into mammalian cells
To get the new GFP plasmid into the mammalian cells, the plasmid is mixed directly with a concentrated solution of CaCl$_2$. This mixture is then added drop wise to a phosphate buffer to form a fine precipitate. Aeration of the phosphate buffer while adding the DNA-CaCl$_2$ solution helps to ensure that the precipitate that forms is as fine as possible, which is important because clumped DNA will not adhere to or enter the cell as efficiently. This solution is then added drop wise to the cells.
The cells used in this experiment are a line of immortalized human cells. They grow on an active surface on the bottom of a plastic bottle. Be careful not to disturb the cells. They have to stay attached to the bottom of the bottle in order to remain healthy.

Materials
Human cells
2.5M CaCl$_2$
Plasmid
Hepes buffer

Protocol
1. Add 50 µl 2.5M CaCl$_2$ to your tube containing 450 µl DNA. Mix by taking the liquid up and down with your pipet. Avoid air bubbles.

2. Add the 500 µl DNA-CaCl$_2$-solution slowly one drop at a time to the tube containing 500 µl 2x Hepes buffer. While you do this, you continuously make bubbles in the solution with a bigger pipet.

3. Leave the mixture for 5 min at room temperature.

4. Add the mixture to your cells one drop at a time.

5. Leave the cells to grow in the incubator overnight.
**Day 2:**

**Plasmid production: Transformation of *E. coli* cells with ligation mixes**

The plasmids carrying insert (GFP) are to be transferred into *E. coli* in order to be sorted and replicated. This process is called transformation. The *E. coli* cells have been treated in such a manner that they are able to take up DNA. The cells are incubated with DNA plasmids and will, after heat shock at 42°C for 20 sec., take up the plasmids having accumulated on their cell surface. The transformed cells are plated on agar plates containing a selective antibiotic (here kanamycin). *E. coli* cells that have received intact plasmids will then be able to divide and grow into colonies on the agar plates, because the plasmids carry the gene for antibiotic resistance. Note, that the antibiotic resistance does not give any information whether the cells also have received the GFP gene.

**Materials**

- LB- medium
- 2 LB-agar plates containing kanamycin
- 2 Eppendorf tubes with *E. coli* cells.
- The ligation mixtures from yesterday
- Plastic Drigalsky spatulas for plating the bacteria
- 42°C heat block

**Protocol**

1. Transfer 5 µL of each ligation mix (A and B) into separate tubes with *E. coli*. Use a pipet tip to stir around.

2. Incubate the transformation tubes on ice for 30 min.

3. Heat-chok the cells in a 42°C heat block for 20 sec. and immediately thereafter incubate on ice for at least 2 min.

4. Add 950 µL LB-medium to each of the two transformation tubes.

5. Incubate at 37°C for 1 hour.
6. Plate 150 µL of each transformation mix on each of two LB-agar plates marked A and B. Write name and group on the plates.

7. The agar plates are incubated (bottom up!) in a 37°C incubator overnight.

**Cell transfection: Maintenance of cells**

Today the cells have to have their old media taken away, get washed and receive new media. The media contains among other the nutrients the cells need to grow.

**Materials**
- Waste tube
- PBS wash buffer
- Media

**Protocol**
1. Remove the old media from the cells, by transferring it to a waste tube.

2. Wash the cells with 5 mL wash buffer (PBS). Be careful not to disturb the cells. Add the buffer, let it flow around and empty the flask again by transferring the buffer to the waste tube.

3. Add 10 mL of new media.
Day 3

Plasmid production: Check colonies for inserted GFP by colony PCR

Today you are going to find out whether any of your *E. coli* colonies contain the GFP insert. You will be doing this by colony-PCR using a primer set where one primer anneals upstream of (before) the GFP gene and one anneals inside the gene itself. A PCR product of the right size tells you that the insert is most probably GFP and that it is oriented correctly in the plasmid.

Materials

You will get 55 µL of following stock mix made by the instructors:

- 5.5 µL 10 x SuperTaq buffer
- 0.55 µL SuperTaq DNA polymerase
- 2.2 µL CMV forward primer, 5 pmol/µL
- 2.2 µL eGFP – N reverse primer, 5 pmol/µL
- 1.1 µL 10 mM dNTP
- 43.45 µL water

Eppendorf tubes

1% agarose gel (incl. GelRed)

Gel apparatus

Power supply

6 x Ficoll DNA loading buffer (Ficoll pulls the dye down in the wells)

100 bp DNA size marker (see appendix for the size of each band in the marker)

Protocol

1. Number 5 PCR tubes (1-5).

2. Add 10 µL stock mix to each of the PCR tubes.

3. Mark the colonies that you want to test on the bottom of the plate with a pen.
4. Pick the 5 colonies with a small pipette tip (those for Pipetman P10) by dipping the pipette tip into a colony and then dipping it in one of the PCR tubes and stir a bit about, so that the cells gets into the liquid. Close the lids on the PCR tubes.

6. The 5 PCR tubes are placed in the PCR machine (remember team numbers!) and the PCR is started.

7. The PCR cycle program is:
   
   1. Initial denaturation of template DNA: 2 min at 95°C
   2. Amplification cycles (repeated 30 times):
      - 30 sec. at 95°C (melting)
      - 30 sec. at 60°C (annealing)
      - 1 min. at 72°C (elongation)

8. After ended PCR add 2 µL 6 x Ficoll DNA loading buffer to each PCR tube and to the size marker.

9. Load the agarose gel with the entire volumes of each sample. Write down where you load the samples. (See appendix for how to load a gel).

10. Run electrophoresis at 60 mA until the blue dye is around 2.5 cm from the bottom of the gel.

11. Visualize the agarose gel on a UV box and photograph your gel.

12. Write up sample numbers on your photograph.

- Do all the samples contain the plasmid with the insert?
Cell transformation: GFP-glowing cells
Today you will see whether the transformation has worked.

Materials
Fluorescence microscope

Protocol
1. Look at your cells in a microscope.
Appendix

Map of pEGFP-N1
The plasmid with GFP insert.

**pEGFP-N1 Vector Information**
GenBank Accession #U55762

![Map of pEGFP-N1](image)

Restriction Map and Multiple Cloning Site (MCS) of pEGFP-N1 Vector. (Unique restriction sites are in bold.) The NotI site follows the EGFP stop codon. The XbaI site (*) is methylated in the DNA provided by CLONTECH. If you wish to digest the vector with this enzyme, you will need to transform the vector into a strain and make fresh DNA.
The sequence of pEGFP-N1

1 tagttattaa tagtaatcga ttaaaggggtc attagttcat agcccatata tggagttccg
61 cgttacataa cttaagggta attgccgccg cccaaagaccc cccgcccaatt
121 gacgtcaata atgaatctag atcccatatt aaggcgaata gggacctccc attgacagtca
181 atggccagag taatctggttac aatgcgtggtg ggtacgctttg atcatatatgc
241 aagttarray cctttgcagc ttaaatgaccc gcctggcatt atgacgactta
301 ctagacatct tggacatttc ctacttggca gtacatctac gtattagtca tcgctattac
361 ctagctagc gcttttggca tgggacttttg tcggcagcgt tcggcagctg
421 atttgagecct ccaccccaatt ccccttaatt cttcatagt gcctggcatt atgcaggtac
481 ggacctttacc aaatggtgta aacaaactcgc cccacccggt ctgggtgctggtttgttgt
541 acgcttcttg gcttttggcc cggcctttgg ccgactttgg ttgggacttttg ttgtgcaccc
601 ccgaccttc gattccacttg tgggacttttg gcagcttcttg ccgacttttg ttgtgcaccc
661 gatcaggggg cctttggtga tgcattcgg ctcgctattac tcgctattac tcgctattac
721 atcttccttt agtttggacc cggacttttg cggcggccg cggcggccg cggcggccg
781 gaggatcgcg aacaaactcgc cccctatatt ctttgggacttttg gcagcttcttg ccgacttttg
841 cctgtgcc ccctggttg cgcgtctggt cgcgtctggt cgcgtctggt cgcgtctggt
901 taccagtctg ctcgctattac tcgctattac tcgctattac tcgctattac tcgctattac
961 ccaaggtgag ccttttggca tgggacttttg tcggcagcgt tcggcagctg
1021 tcggcagctg aacacactgt gacgcttcttg gcgcgttcag gccgcgttcag gccgcgttcag
1081 gccgaggtgc caaactcgcct ccccttggga cagctttctt gttgctattac tcgctattac
1141 gcgctttgctg ccggtgcttt gttgctattac tcgctattac tcgctattac tcgctattac
1201 gcgctttgctg ccggtgcttt gttgctattac tcgctattac tcgctattac tcgctattac
1261 ccgctttgctg ccggtgcttt gttgctattac tcgctattac tcgctattac tcgctattac
1321 gcgctttgctg ccggtgcttt gttgctattac tcgctattac tcgctattac tcgctattac
1381 gcgctttgctg ccggtgcttt gttgctattac tcgctattac tcgctattac tcgctattac
1441 taggttttga atcgtttttg ctcgctttg ccggtgcttt gttgctattac tcgctattac
1501 tcgctttgctg ccggtgcttt gttgctattac tcgctattac tcgctattac tcgctattac
1561 atcgtttttg ctcgctttg ccggtgcttt gttgctattac tcgctattac tcgctattac
1621 ccccttttttt ctcgctttg ccggtgcttt gttgctattac tcgctattac tcgctattac
Protocol for cloning PFP into mammalian cells

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1861 gatgccccac tacgtgaacc atcaccctaa tcaagttttt tggggtcgag gtgccgtaaa
1921 gcacta aatc ggaaccctaa agggagcccc cgatttagt gaaagttgtg cagtttaggt
1981 aacgtgceca gaagaagacc gaagaagacc gaagaagacc gggctagggc gctggcaagt
2041 gtagegctca ccttcggtg ctaacaccgc gcctgacgctt ctaacaccgc gcctgacgctt
2101 gctgcaagt gcctgacgctt ctaacaccgc gcctgacgctt ctaacaccgc gcctgacgctt
2161 atacattca atatgtatcc gcctgacgctt ctaacaccgc gcctgacgctt ctaacaccgc
2221 tgaacaccgc gcctgacgctt ctaacaccgc gcctgacgctt ctaacaccgc gcctgacgctt
2281 ggtgaaagtc cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc
2341 cagcaacacc gcctgacgctt ctaacaccgc gcctgacgctt ctaacaccgc gcctgacgctt
2401 atacattca atatgtatcc gcctgacgctt ctaacaccgc gcctgacgctt ctaacaccgc
2461 gcctgacgctt ctaacaccgc gcctgacgctt ctaacaccgc gcctgacgctt ctaacaccgc
2521 cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc
2581 taggttttgg cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc
2641 gatgatgttc cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc
2701 gcctgacgctt ctaacaccgc gcctgacgctt ctaacaccgc gcctgacgctt ctaacaccgc
2761 cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc
2821 gcctgacgctt ctaacaccgc gcctgacgctt ctaacaccgc gcctgacgctt ctaacaccgc
2881 actaggaggc gcctgacgctt ctaacaccgc gcctgacgctt ctaacaccgc gcctgacgctt
2941 cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc
3001 cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc
3061 cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc
3121 cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc
3181 cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc
3241 cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc
3301 cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc
3361 cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc
3421 cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc
3481 cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc
3541 cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc
3601 cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc
3661 cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc
3721 cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc cccagcctcc
Underlined sequences are the sequences where the primers bind

Bold sequence is the GFP

Italic and red sequences are where the restriction enzymes cleaves
100 bp DNA size marker
How to load an agarose gel

The picture below shows a gel, which is ready to be loaded. There are 15 wells, which mean it can be used for 15 samples including a DNA size marker. Power will be added so that there will be a flow of negative charges towards the end of the gel, the positive pole. DNA is negatively charged.

![Charges](image)

The two pictures below show you how to hold your hands while loading the gel. It is important to have very steady hands to avoid you piercing the gel with the pipette or adding your sample in a wrong well.

Hold the pipette with one hand and use the other hand as support. This will make your hands more steady.

![Hands](image)
The picture below shows how to add a sample to a well. It is important not to touch the bottom of the well with the pipette tip. Add the sample very slowly by not pushing the pipette too hard.