

BioBrick DTU iGEM Workshop 2015

SOP1: PCR reaction

1. Primers

Add sterile MilliQ water (MQ) to primer vial, for the final concentration of 100 μM . The amount of water to be added is dependent on the nmol of primer. Add 10 μL of water per nmol of primer. Then dilute the primer in a new eppendorf tube to 10 μM (10x dilution), which is the one to be used for PCR reactions.

2. PCR mix

The following components are mixed together in an eppendorf tube. Since you are only setting up 1-3 PCR reactions, there is no need to make a master mix. Before adding each of the reaction components spin them down for a few seconds in the mini centrifuge on the lab benches.

Add the components in the order that they appear on the list.

The list of primer and templates will be written on the board in the lab.

For each reaction (50 μL) use:

Component	Total reaction volume = 50 μL
5x HF buffer	10 μL
2μM dNTPs	5 μL
Primer forward	2.5 μL
Primer reverse	2.5 μL
Phusion Polymerase	0.5 μL
DNA template	1 μL
MQ	to 50.0 μL

Try to avoid making too many bubbles when you pipet. Spin down the tubes in the table centrifuge if necessary.

Temperature (degC)	Time (min:sec)	Cycles
98	00:30	
98	0:10	
60	0:15	30
72	15 sec/kb plasmid DNA or 30 sec/kb for genomic DNA template	
72	5:00	
12	forever	

Annealing temp (red) can be changed according to the melting temperatures of the primers. Annealing temperature is set 3 degree above the T_m of the lowest primer. Please see the board for which annealing temperature you will be using.

Elongation time (blue) can be changed depending on the length of the fragments. Phusion polymerase synthesizes ~2000 bp per minute when the template is genomic DNA and double the amount when the template is plasmid DNA.

SOP2: Purification of PCR products

After your PCR is done add 0.5 μ L DpnI enzyme to the PCR mixture and incubate for 5 minutes at 37 degC before you purify the PCR product. DpnI degrades *dam* methylated DNA. The PCR product is not methylated, but the template is. This way we can remove the template and thereby reduce the amount of background after transformation.

This step is not necessary if you use a plasmid that has a gene on the plasmid that kills the cell. This step is also not necessary, if the plasmid you use as template has a different selection marker than what your constructed plasmid has.

1. Analytical gel

Each PCR product should be verified by gel electrophoresis.

- Cast a gel with 1% agarose, using the small molds. The gel should not be very thick and is ready after 10-20 mins.
- Mix 5 μ L of PCR product with 1 μ L of loading dye on a piece of parafilm
- The gel is transferred to the electrophoresis chamber with 1xTAE or 1xTBE, and the samples+dye are loaded.
- At least one well should be loaded with ladder.
- Set the voltage to 80-95V (Lower voltage=longer time=better resolution)
- Set the time depending on expected lengths of the fragments. 20-30 mins is usually appropriate.
- Take a picture and evaluate if the fragments have the expected lengths.

2. PCR Purification

While the gel is running start the PCR purification. Remember to share the centrifuge with other groups.

We will use Qiagen PCR Purification kit and the protocol that comes with it.

SOP3: Cloning and transformation

USER teams follow #1. Restriction/ligation teams follow #2. Both teams follow #3 (transformation protocol).

1. USER cloning

You need one eppendorf tube for the USER reaction. Normally you will also do a negative control, where you do not any USER enzyme, but as we do not expect any background due to the *ccdA/B* negative selection) we won't need a control.

The reaction volume is 10 μL . You will use 1 μL USER enzyme and 0.5 μL buffer and X μL of each of your three PCR reactions. Based on the amount of DNA you add calculate the amount of MQ water you need. Mix water, buffer, and DNA. Then add the enzyme. The USER enzyme is very robust and can work in MQ water if you forget to add the buffer.

Typical incubation: 37°C for 30 minutes, then 25°C for 30 minutes, then hold at 4°C.

Workshop Protocol: 37°C for 15 minutes, then 25°C for 15 minutes, then hold at 4°C in a PCR machine. Continue with step 3, Transformation.

2. Traditional cloning with restriction enzymes

You will need to prepare one eppendorf tube with your miniprep DNA, purified PCR product (which includes GFP), restriction enzymes, and the buffer to allow the restriction enzymes to cut out the GFP DNA.

Mix miniprep and PCR product in 1:3 ratio (maximum amount of DNA should be 10 μg total in max 16 μL volume), with 2 μL Cutsmart buffer, 1 μL of EcoRI restriction enzyme, and 1 μL PstI restriction enzyme in 20 μL total volume. Remember that your miniprep has fewer molecules per ng of DNA than your PCR product. You need to take that into consideration, when you calculate how much of the miniprep and how much of the PCR product you want to add. *{Example: GFP template PCR product is ~900 bp and pOSIP backbone is ~6800 bp. For an equivalent amount in ng of miniprep and PCR product there is 7.6X more PCR GFP fragments than backbone template plasmids from the miniprep ($6.8/0.9 = 7.6$). Therefore if you want a 1:3 ratio of miniprep:PCR product ($7.6/3 = 2.5$) you need to add 2.5 times more ng of miniprep than ng of PCR product. This number is not a hard and exact number so use your best estimate for ng of DNA material}*

Incubate reaction for 15 minutes at 37 degC.

Heat-inactivate restriction enzymes by incubating the eppendorf tube at 80 degC for 20 minutes. After 20 minutes transfer eppendorf tube to ice to cool the reaction.

When reaction is at room temperature, add 1 μL T4 DNA ligase and 2 μL 10 mM ATP to the reaction mixture. Incubate at room temperature for at least 10 minutes (longer ligation times = better yield). Then proceed with the transformation. (You can let the ligation stand as long as you want, so if it is longer than 10 minutes this is fine).

3. Transformation

- Thaw competent *E. coli* cells on ice.
- USER teams: Carefully add 50 μL of cells to 5 μL of your USER reaction (maximum 10% extra volume for your chemically competent cells). The cells are fragile, so pipet slowly. Add 50 μL cells to an empty eppendorf tube and 5 μL MQ water. This is your negative control.
- Restriction Enzyme/Ligase teams add 90 μL of cells to your ligation tube and in a separate tube make a negative control with 45 μL of cells 10 μL MQ water. The cells are fragile, so pipet slowly.
- Incubate on ice for 30 minutes.
- Incubate in 42 degrees water bath for 30 seconds.
- Cool immediately on ice for 2-5 minutes.
- Add 500 μL SOC media (or LB media if we don't have any SOC) and let the cells recover for 60 minutes at 37 degC.
- Plate bacteria on plates using a sterilised Drigalsky spatula or sterile glass beads. You will need 6 plates:
 - 10 μL (plate 1) and 100 μL (plate 2) of your transformation mix with DNA onto two **LB + kanamycin (kana)**
 - 10 μL (pt 3) and 100 μL (pt 4) of your transformation mix with DNA onto two **LB + IPTG (Inducer of GFP exp) + kana**
 - 2 μL (pt 5) of your negative control plus 1000 μL MQ water. Only plate 50 μL of this transformation mix without DNA onto an **LB** plate. This will be our positive control because there is no selection marker (*Kanamycin*). We will compare the colonies that grow on this plate to our successfully-transformed colonies. These cells won't fluorescence Sunday morning.
 - 100 μL (pt 6) of the negative control to an **LB + kana plate**. This plate should not grow any colonies, as only transformed cells will have the kanamycin resistance.
- Incubate at 37 degrees overnight.

4. Colony PCR to confirm Successful Genomic Integration of GFP into *E. coli*

1. Select 3 colonies and transfer each to a separate PCR tube with 50 μL MQ water. Resuspend the colony by vortexing for a few seconds.
2. Boil samples at 99°C with heated lid for 15 min in a PCR machine.
3. Use 1 μL of this mix in the PCR reaction SOP1 starting at step 2.

