Technical University of Denmark

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 Tm 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 µL 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 cams. The gel should not be very thick and is ready after 10-20 mins.
- Mix 1-2 µL of PCR product with 5 µL 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 95V
- 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.

Incubate at 37 degrees for 30 minutes then at room temperature for 30 minutes. Then 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 uL PstI µL 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 to the reaction mixture. Incubate at room temperature for 10 minutes. 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 your 10 µL USER reaction. The cells are fragile, so pipet slowly. Add 50 µL cells to an empty eppendorf tube and 10 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 30 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**
    - 10 µL (pt 5) of your transformation mix onto **LB**. This will be our positive control (and the cells that won't fluorescence Sunday morning)
    - 100 µL (pt 6) of the negative control (MQ water + cells) to a **LB + kana plate**. This is your negative control.
- 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 uL 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 1uL of this mix in the PCR reaction SOP1 starting at step 2.

Run the PCR with T_p1 + T_p2 forward and reverse primers. Use 59C annealing for 30s with a 20s extension step. Use 1uL 50mM MgCl2 + 1uL 100% DMSO for enhancing this PCR rxn. Visualize results with an analytical gel that you cast while waiting for the PCR to finish.