Protocols iGEM Team 2017 UPMC-Paris

Bacterial competentiation :

We started from frozen *E. coli* DH5α which we thaw on ice and then streak on Lysogeny Broth (LB) agar plates (autoclaved 20 g sterile agar for 1L distilled water) that we incubated overnight at 37°C. The next day we inoculated one colony on 4 mL LB (autoclaved 25g LB in distilled water), overnight at 37°C and 220 rpm. The following day, we inoculated 500µL of these pre-culture in 50 mL of LB and incubated them at 37°C until OD600: 0,5. At this stage, bacterial cells are centrifuged (10mn, 4°C, 4200 rpm), and pellets were gently resuspended in 2 mL of T1 solution (30mM acétate K, 100mm KCl, 10mM CacI2, 15% glycerol and 50mM MnCl2 added extemporary) and we completed to 15mL. We incubated cells in this solution for one hour and centrifuge them 10mn at 4°C/ 2300 rpm. We resuspended in 2 mL of solution T2 (MOPS pH7 10mM, KCl 10mM, CaCl2 75mM, Glycérol 15%) and was aliquoted into Eppendorf carefully frozen on dry ice with ethanol.

Transformation :

DNA was resuspended with 10 µL of water and 1 µL of DNA incubated with 50 µL of the competent cells DH5α on ice for 30 minutes. We realized heat-shock at 42°C for 45s and transferred quickly tubes on ice for 5mn. We added 950 µL of SOC media per tube, and incubated them at 37°C for 1 hour shaking at 200-300rpm. We collected 100 µL from each tubes and spread in triplicate the mixture evenly across the LB agar plates with chloramphenycol (25 µg/mL). We incubated them with the agar side at the top at 37°C overnight for approximately 16 hours, because growing with chloramphenycol is slower than with other antibiotics. The next day we evaluated the number of colonies on a light field based.

Plasmid DNA extraction:

We used the Monarch Plasmid Miniprep kit from NEB.
We realized all steps of centrifugation at 16000g. 5 mL of culture was pelleted by centrifugation during 30 seconds and the supernatant was discarded. Cells was totally resuspended in 200µl of Plasmid Resuspension Buffer (B1) and lysed with 200 µl Plasmid Lysis Buffer (B2) and 5-6 time inversion tubes. After one minute of incubation and in order to avoid plasmid irreversible denaturation, we added to neutralize lysate 400 µl of Plasmid Neutralization Buffer (B3). After 2 minutes, we centrifuge and carefully transfer supernatant to the spin column and repeat centrifugation for 1mn. We removed RNA, protein and endotoxin with 200 µl of Plasmid Wash Buffer 1 and then after centrifugation with 400 µl of Plasmid Wash Buffer2. To finish, we made elution with 30µl of DNA elution buffer. Plasmid DNA concentration was measured with nanodrop.

Polymerisation Chain Reaction :

We realized DNA amplification with Q5® High-Fidelity 2X Master Mix (NEB #M0492S). To make 25µL reaction, we added 12.5µL of Q5 High-Fidelity 2X Master Mix, 1.25µL of each forward/ reverse primers (10µM), 250 ng of DNA and we completed with nuclease free water. We

gently mixed reaction and transferred PCR tubes from the ice to a PCR machine with the block preheated to 98°C and began thermocycling.
We have designed our primers to amplify the desired DNA sequence, adding restriction recognition sites of the enzymes that will be used in cloning at 5' ends of the primers, with a few extra nucleotides prior to the restriction site to increase restriction efficiency. To do so, we used a Tm calculator tool from NEB https://tmcalculator.neb.com/#!/ to design primer pair with no more difference of temperature than 5°C.
The thermocycling program was:

- 98°C/ 30s for initial denaturation
- 98°C/ 10s for denaturation, TM°C/ 20s for hybridation, 72°C/ 20s for elongation (X30)
- 72°C/ 2mn for final extension
- hold 4°C

Purification products PCR :

We used the Monarch DNA Cleanup extraction Kit from NEB. We realized all steps of centrifugation at 13K RPM. First, we resuspended PCR products with 80µl of DNA Cleanup Binding Buffer and transfer mix into spin column. After one minute of spinning we discarded flow-through. We added 200 µl of DNA Wash Buffer was added before 1 minute of spinning (these steps were repeated twice time). We transferred columns into a clean 1.5 ml microfuge tube before to add 20µl of DNA Elution Buffer to the center of the matrix. After 1 minute, we eluted DNA by one minute of centrifugation. Concentration and purity of DNA was assessed with nanodrop.

Resuspention of synthetized DNA by IDT

DNA was centrifuge 5s/ 300g and TE 1X (1M Tris-HCL pH 7.5-8; 0.5M EDTA pH8; sterile H20) was added in order to reach a final concentration of 10 ng/uL. Resuspended DNA was vortexed and incubated 20mn at 50°C before centrifugation 3/5s (300g mini).

Cloning and part assembly :

We used the Biobrick assembly kit from NEB.
First, we digested purified PCR products with prefix and suffix restriction enzymes. 500 ng of upstream parts were individually digested with EcoRI and SpeI in a digestion mix containing 5µL of 10X digestion Buffer 2.1, 1 µL of each enzyme and sterile water to complete the mix until a final volume of 50µL. 500 ng of downstream parts were individually digested by XbaI and PstI and 100 ng of pSB1C3 plasmid by EcoRI and PstI enzymes in the same conditions that upstream parts. Digestion mix were incubated at 37°C for 10 minutes and then heat inactivated at 80°C for 20 minutes. Then, we gather our devices by using the T4 DNA ligase from NEB. 2 µl of EcoRI/SpeI digested upstream parts were mixed with 2 µl of XbaI/PstI digested downstream part, 2µL of EcoRI/PstI digested plasmid, 2 µl of 10X T4 DNA ligase Buffer, 1 µl of ligase and 11 µL of sterile water. Then, ligation mix was incubated at room temperature for 10 minutes and heat inactivated at 80°C for 20 minutes. The assembly construction was used for the transformation of *E. coli* DH5α. After transformation, cells were spreaded in LB culture overnight in LB+ Chloramphenycol.

GFP and RFP quantification:

After transformation, we selected recombinant clones directly by visualization of GFP and RFP fluorescence through epifluorescent microscope Zeiss Axiovision. We inoculated 4 mL of LB + Chloramphenycol with colonies emitting GFP and RFP fluorescence and incubated then overnight at 37°C. The next day, bacterial cells were all diluted in LB+ Chloramphenycol at a final OD600 of 0,2. We quantified GFP and RFP production of bacteria in 96-well microwell plate during their exponential stage (OD600 of 0.5). by using the spectrophotometer Molecular Device SpectraMax M4 with the following set-up.

Transformed cells were synchronized by dilution on LB+ Chloramphenycol at OD600: 0,2. Cultures were aliquoted (150 µl) into a 96-well plate (Greiner Bio-One) in which OD (600 nm) and fluorescence for GFP (BBa_I13500) and RFP (BBa_I13507) were read using a SpectraMax M4 fluorescence plate reader (Molecular Devices) along exponential stage.

Finaly, we made ratio between protein synthesis (fluorescence) and growth rate (optical density) of our different transformed strains and measured each sample in triplicates.

GFP (BBa_I13500) : Excitation wavelength : 485nm

Emission wavelenght : 530nm

Integration time : 500 ms

RFP (BBa_I13507) Excitation wavelength : 584 nm

Emission wavelenght : 607nm

Integration time : 500 ms

SKP cytoplasmic genomic integration:

The DH5a competent strain was transformed with 1uL of the construction and select on LB+ampicillin (100ug/ml) at 32°C in triplicate. After two days we count few colonies (approximately 10) resistant to ampicillin. We picked 3 colonies and streaked out once overnight in LB+ampicillin, 32°C. We started cultures overnight of 3 colonies in LB without ampicillin at 32°C (this step allows for some loss of plasmid) and grow overnight. We diluted cultures appropriately to recover single cells (I dilute 10-7 and plate 50 uL of culture) and plate on LB plates. Grow at 42°C to block replication of the plasmid. Streak colonies once on LB at 42°C to ensure the complete loss of the plasmid.

After this step of integration and before characterization of the effect of SKP in protein synthesis we made verifications:
A simple checking was to pick the next day 3 colonies to put them in culture in LB+/- ampicillin.

Electrophoresis :

We realized an agarose gel electrophoresis (1.5%) using agarose powder with 100ml of 1x TAE buffer, heated until all agarose is dissolved Agar Low Melting… dissolved in TAE 1X before to add 6µl of Bet. Mix 1µl of 1kbp DNA ladder with 6µl of loading dye (bromophenol blue) and 4µl of 1x TAE buffer (total 6ul) and load onto first well. We Mixed 10µl of PCR products with 2µl of loading dye 6X before loading into wells. Running gel electrophoresis was made at 10V for 30 minutes approximately and photograph gels under UV light.