Protocols iGEM HR 2019

Digestion

x µL DNA
2 µL 10x restriction
buffer y µL Milli-Q water
Mix well and finally add: 1 µL restriction enzyme (1 U/µL)

Total volume: 20 µl

At https://www.neb.com/tools-and-resources/usage-guidelines/nebuffer-performance-chart-with-restriction-enzymes you can see which buffer you need as well as the incubation and heat inactivation temperatures.

Dephosphorylation and Ligation

Dephosphorylation

If necessary, dephosphorylate 1 of the 2 DNA fragments that need to be ligated, for example only the vector DNA according to the following scheme:

x µl (1 µg) DNA
2.5 µl Phosphatase buffer (10x)
y µl Milli-Q
Mix well and finally add: 1 µl Phosphatase (1 U / µl)

Total volume: 25 µl

Mix well and centrifuge briefly. Incubate for 10 minutes at 37 C. Then inactivate the enzyme by placing the mixture at 75 C for 2 minutes.

Ligation

x µl (100 ng) (dephosphorylated) vector DNA (pSB1C3 for example)(… ng/µl)
y µl (.. ng) fragment DNA (… ng/µl)
4 µl 5x T4 ligation buffer
z µl Milli-Q
Mix well and finally add: 1 µl T4 DNA ligase (1 U/ µl)

Total volume: 20 µl
For the dephosphorylation control, you will leave out the fragmented DNA and use more Milli-Q.

Mix well and centrifuge briefly. Incubate for 20 minutes at room temperature.

TEV Cleaving assay

The following protocol has been set-up after talking to Eddy van der Linden (Biochemist Lecturer University of Rotterdam) and Sari (analyst at Erasmus University).

From 100 mM stock DTT, the final concentration must be 1 mM DTT.

1 M Tris-HCl buffer with 10 mM DTT, pH 8.0 (20x buffer)
Make 100 ml of 20x concentrated buffer. Do this as follow:
10 mM DTT = 0.1542 grams.
Tris: 121.14 g/L, so for 100 mL you take 12.114 g. Dissolve this in 80 ml of dH_2O.
Add the 0.1542 g DTT.
Measure the pH and adjust it to 8.0 with 1 M HCl.
Add dH_2O till 100 mL

50 µl TEV protease is contained in a tube, donated by the Erasmus Medical Center (EMC) in Rotterdam.
The number of units per mg = 3000 U.
We want a final concentration of 10U in 150µl.
The concentration is 0.8 µg/µl = 3000/1000 * 0.8 = 2.4U/µl.
So 10U / 2.4 U/µl = 4.2µl TEV is added to the tubes.

Fusion protein (GST-TEVcleavingsite-BRCA2) is 20 µg (also donated by EMC), or 10 µl (if the concentration is not known) -> with known concentration this is also 10 µl.

Tube 1:
- 4.2 µl TEV,
- 7.5 µl 20x Buffer,
- 1.5 µl 100 mM DTT,
- 136.8 µl dH_2O

Tube 2:
- 7.5 µl 20x Buffer,
- 1.5 µl 100 mM DTT,
- 10 µl BRCA2,
- 131 µl dH_2O

Tube 3:
- 4.2 µl TEV,
- 7.5 µl 20x Buffer,
- 1.5 µl 100 mM DTT,
- 10 µl BRCA2,
- 126.8 µl dH_2O

- Incubate the mixture at 30 °C.
- Take 3 times a 50 µl sample after 1, 2 and 4 hours respectively.
- Load all this on an SDS-PAGE gel.

SDS-PAGE Electrophoresis

Wash the glass plates with a sponge and soap, then rinse it with a spray bottle with ethanol and dry the plates well.
Place the glass plates in the gel holder
Place the comb between the glass plates and mark up to ± 1.5 cm below the bottom of the comb.
Make the running gel (12% acrylamide/bisacrylamide gel) as follow:

Running gel	
- Demi water	4,2 mL
- 40% acryl/bisacrylamide solution	3,0 mL
- 1,5 M Tris-HCl (pH 8,8)	2,5 mL
- 5% SDS-solution	0,2 mL
- *10% APS	0,1 mL
- TEMED	15 µL

* 0,1501 grams + 1,5 mL demi water.

Mix well and pipette this solution until the mark is reached.
Carefully pipette 0.5 mL of butanol on top of the running gel solution. Ensure that no mixing occurs. This covering is necessary to obtain a nice straight surface of the gel and to seal off the gel for oxygen from the air.

Allow the gel to polymerize for approx. 15 minutes.
Remove the butanol from the polymerized running gel with a folded tissue and discard it in the acrylamide waste container.

Make the solution for the stacking gel:

Stacking gel	
- Demi water	2,9 mL
- 40% acryl/bisacrylamide solution	0,5 mL
- 0,5 M Tris-HCl (pH 6,8)	0,5 mL
- 5% SDS-solution	0,08 mL
- 10% APS	50 µL
- TEMED	5 µL

Pipette on the polymerized running gel so much stacking gel solution until the top of the glass plates is reached. Immediately insert the 10 slots comb carefully into the stacking gel and allow it to polymerize for approx. 20 minutes.

Grab the tubes with the fractions that you are going to put on gel.
Pipette 36 µL each time in a new tube.
Add 12 µL 4x sample buffer to these tubes.

Heat these samples (not the Blue protein standard) in a heat block for 5 minutes at 95 °C.
Then centrifuge the tubes shortly.

Connect the electrophoresis device to a voltage source. Use a voltage of 60 V until the samples have passed through the stacking gel. After this, use a voltage of 120 V until the blue band has almost reached the bottom of the gel.

Remove the gel from the glass plates. Coloring is done in a large Petri dish with Instant Blue. If the color is optimal, the gel can be analyzed by using the Gel Doc EZ imaging system.

DNA Electrophoresis

- Prepare 800 mL of 1 x TAE buffer.
- Prepare 100 ml of 0.8% agarose solution in the 1 x TAE buffer in a 250 ml Erlenmeyer flask.
- Bring the suspension (in the microwave) to let it boil until all agarose particles have dissolved. Make sure that it is hand warm.
- In the meantime, take all the parts that you need for electrophoresis.
- Tape the gel holder wall with masking tape.
- Place the comb in the gel holder in the recesses made for this.
- Add 100 µL Gelred solution to the hand warm agarose solution and mix well, remove any air bubbles (with a pasteurized pipette or pipette tip) and allow the gel to solidify. Do not move it during the solidification process. Immediately rinse the Erlenmeyer flask with plenty of warm water.
- If the gel has solidified (10 - 15 minutes), you can remove the tape and put the gel holder in the electrophoresis device.
- Pour so much 1 x TAE buffer into the electrophoresis tray that the buffer just flows over the gel. Then carefully remove the comb from the gel. Check whether the locks are filled with buffer. Test if a current is going to run and check the electrophoresis direction.

Competent cells

1) Remove a single colony from an LB plate and inoculate it in a tube with 5 ml of LB medium. Incubate overnight at 37 C in the shaker (200 rpm). This must be done the day before the experiment!

2) On the day of the experiment: Dilute the overnight (O/N) culture 1: 100 in LB in 20 ml LB with 20 mM $MgSO_4$ in 100 ml flask. Incubate at 37 C in a shaker. (200 rpm) until the culture has an A600 of approximately 0.5 (between 0.4 and 0.6) relative to the blank (LB medium with 20 mM $MgSO_4$). To measure absorbance in between, preferably use a plastic cuvette that is rinsed with alcohol after each measurement.

3) Centrifuge 10 ml of the culture for 5 minutes at 2500 rpm and 4 C in a sterile Greiner tube. Estimate the volume here. (= initial volume).

4) Absorb the cells gently at 0.4 times the initial volume in ice-cold Tfb1. For 10 ml this is therefore 4 ml. Resuspend the cells by tapping by hand or sucking up with a Pasteur pipette and pipetting again. The cells must remain cold!

5) Incubate the cells on ice for 15 minutes.

6) Pellet the cells by spinning for 5 minutes at 2500 rpm and 4 C.

7) Gently absorb the cells in 1/25 of the initial volume in ice-cold Tfb2. 400 l for a 10 ml culture. You then have enough for 8 transformations.

If the cells are stored: 15-60 minutes on ice, then freeze quickly (“snap freeze” in liquid nitrogen or a mixture of dry ice and isopropanol).

Transformation

Protocol: Single Tube Transformation Protocol, iGEM

1. Resuspend DNA in selected wells in the Distribution Kit with 10µl dH_20. Pipet up and down several times, let sit for a few minutes. Resuspension will be red from the cresol red dye.

2. Label 1.5ml tubes with the part name or well location. Fill lab ice bucket with ice, and pre-chill 1.5ml tubes (one tube for each transformation, including your control) in a floating foam tube rack.

3. Thaw competent cells on ice: This may take 10-15min for a 260µl stock. Dispose of unused competent cells. Do not refreeze unused thawed cells, as it will drastically reduce transformation efficiency.

4. Pipette 50µl of competent cells into 1.5ml tube: 50µl in a 1.5ml tube per transformation. Tubes should be labeled, pre-chilled, and in a floating tube rack for support. Keep all tubes on ice. Don't forget a 1.5ml tube for your control.

5. Pipette 1µl of resuspended DNA into 1.5ml tube: Pipette from well into an appropriately labeled tube. Gently pipette up and down a few times. Keep all tubes on ice. Pipette 1µl of control DNA into 2ml tube: Pipette 1µl of 10pg/µl control into your control transformation. Gently pipette up and down a few times. Keep all tubes on ice.

6. Close 1.5ml tubes, incubate on ice for 30min: Tubes may be gently agitated/flicked to mix the solution, but return to ice immediately.

7. Heat shock tubes at 42°C for 45 sec: 1.5ml tubes should be in a floating foam tube rack. Place in a water bath to ensure the bottoms of the tubes are submerged. Timing is critical.

8. Incubate on ice for 5min: Return transformation tubes to an ice bucket.

9. Pipette 950µl SOC media to each transformation: SOC should be stored at 4°C, but can be warmed to room temperature before use. Check for contamination.

10. Incubate at 37°C for 1 hour, shaking at 100rpm.

11. Pipette 100µL of each transformation onto Petri plates. Spread with a sterilized spreader or glass beads immediately. This helps ensure that you will be able to pick out a single colony.

12. Spin down cells at 6800g for 3mins and discard 800µL of the supernatant. Resuspend the cells in the remaining 100µL, and pipette each transformation onto Petri plates ***with an antibiotic,*** Spread with a sterilized spreader or glass beads immediately. This increases the chance of getting colonies from lower concentration DNA samples.

13. Incubate transformations overnight (**24 hr**) at 37°C: Incubate the plates upside down (agar side up). If incubated for too long, colonies may overgrow and the antibiotics may start to break down; un-transformed cells will begin to grow.

DNA isolation

Protocol: Miniprep Zymo Research

1. Centrifuge 3 ml of bacterial culture in a clear 1.5 ml tube at full speed for 20 seconds in a microcentrifuge. Discard supernatant.

2. Add 250 µl of P1 Buffer (Red) to the tube and resuspend pellet completely (i.e., by vortexing or pipetting).

3. Add 250 µl of P2 Buffer (Green) and mix by inverting the tube 6 - 8 times. Do not vortex! Let sit at room temperature for 3 minutes. Cells are completely lysed when the solution appears clear, purple, and viscous.

4. Add 250 µl of ice-cold P3 Buffer (Yellow) and mix gently but thoroughly. Do not vortex. Invert the tube an additional 3-4 times after the sample turns completely yellow. The sample will turn yellow when the neutralization is complete.

5. Incubate the neutralized lysate on ice for 5 minutes.

6. Centrifuge the neutralized lysate for 5 minutes at 16,000 x g.

7. Transfer 600 µl of the supernatant from step 6 into a clean 1.5 mL microcentrifuge tube. *Be careful not to disturb the yellow pellet and avoid transferring any cellular debris to the new tube.*

8. Add 275 µl of ZymoPURE binding buffer to the cleared lysate from step 7 and mix thoroughly by inverting the capped tube 8 times.

9. Place a Zymo-Spin II-P column in a Collection Tube and transfer the entire mixture from step 8 into the Zymo-Spin II-P Column.

10. Incubate the Zymo-SPin II-P/Collection Tube assembly at room temperature for 2 minutes and then centrifuge at 5000 x g for 1 minute. Discard the flow-through.

11. Add 800 µl of ZymoPURE Wash 1 to the Zymo-Spin II-P Column and centrifuge at 5000 x g for 1 min. Discard the flow-through.

12. Add 800 µl of ZymoPURE Wash 2 to the Zymo-spin II-P Column and centrifuge at 5,000 x g for 1 min. Discard the flow-through. Repeat this wash step with 200 µl of ZymoPURE Wash 2.

13. Place the Zymo-Spin II-P Column in a Collection Tube and transfer it to a microcentrifuge. Centrifuge at > 10,000 x g for 1 minute in order to remove any residual wash buffer.

14. Transfer the Zymo-Spin II-P Column into a clean 1.5 mL tube and add 25 µl of ZymoPURE Elution Buffer directly to the column matrix. Incubate at room temperature for 2 minutes, and then centrifuge at > 10,000 x g for 1 minute in a microcentrifuge.

Nitrocefin

Protocol from: https://microbeonline.com/nitrocefin-test-principle-procedure-uses-limitations/

Prepare a 0.5 mM Nitrocefin solution. Do this by weighing 2.58 mg on an analytical balance and dissolving this in 500 µl DMSO (Do this is in a fume hood!). Then complete this with 9.5 ml of 0.1 M phosphate buffer (pH 7.0).

Take a clean object glass and pipette 20 µl of 0.1 M phosphate buffer (pH 7.0).

Scrape a colony off the plate with an öse or sterile Pasteur pipette and suspend it in the 20 µl on the object-glass.

Now add 20 µl of the Nitrocefin solution. The color should change to red within 1-2 minutes (maximum 10).

Remarks:
- The solution is stable for 10 days at 4 ° C, wrapped in aluminum foil.
- Glass must be used because DMSO breaks down plastic.
- A weak activity of β-lactamase can prolong the reaction time, anything that lasts longer than 10 minutes must be critically examined.

Annealing

Resuspend the gBLOCK DNA (3000 ng) with 30µl dH_20 to get an end concentration of 100 ng/µL.

Protocol from: https://www.sigmaaldrich.com/technical-documents/protocols/biology/annealing-oligos.html

Annealing buffer 10x:
- 1 mL 1M Tris pH 8.0
- 5 mL of 1 M NaCl
- 0.2 mL of 0.5 M EDTA
- 3.8 mL of demi water

Sterilize Annealing buffer with a 0,22 µm filter.

Pipette scheme

gBLOCK 1	gBLOCK 2	Control
2,5 µL gBLOCK 1 sense (250 ng)	2,5 µL gBLOCK 2 sense (250 ng)	-
2,5 µL gBLOCK 1 antisense (250 ng)	2,5 µL gBLOCK 2 antisense (250 ng)	-
13 µL DNA free water	13 µL DNA free water	18 µL DNA free water
2 µL Annealingbuffer 10x	2 µL Annealingbuffer 10x	2 µL Annealingbuffer 10x

Thermocycler settings:

1. Mix equal volumes of the oligonucleotides in a PCR tube.
2. Use the following thermal profile:
   a. Heat* to 95 °C and maintain the temperature for 2 min.
   b. Cool to 25 °C for over 45 min.
   c. Cool to 4 °C for temporary storage.
3. Centrifuge the PCR tube briefly to draw all moisture away from the lid.

* The lid will be set on 100 °C

TEV cleaving site – β-Lactamase

		bp	initial bp	initial Tm (°C)
XbaBlaPF	GCCGCTTCTAGAGCGCGGAACCCCTATTTGTTTATT	36	24	62
BlaPSSTEVR	TCCCTGAAAATACAGATTTTCcgagacgTGAGCAAAAACAGGAAGGCAAA	50	23	57
TEVBlaF	GAAAATCTGTATTTTCAGGGACCAGAAACGCTGGTGAAAG	40	19	57
SpeBlaTEVR	CCGCTACTAGTAcgtctcTTATCCCTGAAAATACAGATTTTCCCAATGCTTAATCAGTGAGGc	63	21	56
BlaSpeR	CGCTACTAGTATTACCAATGCTTAATCAGTGAGGC	35	24	57

C-terminal: Use XbaBlaPF primer in combination with SpeBlaTEVR with pUC18 as a template (see *table 1*). Digest the PCR product with XbaI and SpeI and clone in pSB1C3 between the XbaI and SpeI sites.

N-terminal: We need two fragments for this. Use the primers XbaBlaPF and BlaPSSTEVR for fragment 1 and TEVBlaF and BlaSpeR for fragment 2 and pUC18 as a template (see *table 1*). Purify both fragments and merge them in a "fusion PCR" where you now only use the outer primers, XbaBlaPF and BlaSpeR *(see table 2).* The outer primers contain the RFC10 prefix or suffix site. The long product thus obtained is purified and digested again with XbaI and SpeI and cloned in pSB1C3.

The PCR master mix contains:

	Conc.	For 1 reaction	For 5 reactions
Q5 polymerase buffer, NEB	10X	2,5 µl	12,5 µl
dNTPs, NEB	10 mM	0,5 µl	2,5 µl
Fw primer	10 µM	1 µl	5 µl
Rv primer	10 µM	1 µl	5 µl
Milli-Q		17,8 µl	89 µl
Q5 polymerase, NEB	5 U/µl	0,2 µl	1 µl
Total		**23 µl**	**115 µl**
Sample DNA	1-5 ng	**2 µl**	**10 µl**

PCR clean-up protocol from Macherey-Nagel:
https://www.mn-net.com/Portals/8/attachments/Redakteure_Bio/Protocols/DNA%20clean-up/UM_PCRcleanup_Gelex_NSGelPCR.pdf

Table 1: Thermocycler program for normal PCR.

Phase	Temperature	Time
1. Denaturation	95 °C	5 min
2. Denaturation	95 °C	30 sec
3. Annealing	58 °C	30 sec
4. Elongation	72 °C	1 min
5. Repeat step 2-4 for 29 times	-	-
6. Elongation	72 °C	5 min
7. Hold	4 °C	∞

Table 2: Thermocycler program for fusion PCR.

Phase	Temperature	Time
1. Denaturation	95 °C	5 min
2. Denaturation	95 °C	30 sec
3. Annealing	58 °C	30 sec
4. Elongation	72 °C	1 min
5. Repeat step 2-4 for 14 times	-	-
Now add 2 µL of primer mix	-	-
6. Denaturation	95 °C	30 sec
7. Annealing	58 °C	30 sec
8. Elongation	72 °C	1 min
9. Repeat step 6-8 for 29 times	-	-
10. Elongation	72 °C	5 min
11. Hold	4 °C	∞

Chromoprotein characterization

- Transform the BioBricks into DH5α-cells.
- The next day an O/N culture (300 mL LB medium with chloramphenicol) will be prepared, incubate this at 37 C in a shaker (200 rpm) for 24 hours.
- Centrifuge 15 mL of culture for 10 min at 10.000 rpm. Resuspend the pellet with 15 mL saline.
- Sonicate (amplitude of 20) the resuspended pellet 4x for 15 seconds.
- Save the supernatant on ice for the experiments.
- Make different pH samples (pH 0,3/4,3/7,3/10,3/14) by combining different concentrations of 1 M NaOH and 1 M HCL.
- Take 100 µl of the chromoprotein + µl 100 of the different pH samples.
- Mix well.
- As negative control, saline will be used.
- Measure the absorbance with a microtiter plate reader at full spectrum (300-1000 nm).