Northern BC iGEM

Experiments & Recipes

Experiments

PCR (NEB HF Phusion)

We use NEB HF Phusion (protocol from NEB PCR Protocol for Phusion® High-Fidelity DNA Polymerase (M0530)).

1. Prepare PCR reaction(s) on ice at the appropriate volume.

Component	20 µL reaction	50 µL reaction
5X Phusion HF buffer	4 µL	10 µL
10 mM dNTPs	0.4 µL	1 µL
10 µM forward primer	1 µL	2.5 µL
10 µM reverse primer	1 µL	2.5 µL
Template DNA	(variable)	
Phusion DNA Polymerase	0.2 µL	0.5 µL
Nuclease Free Water	to 20 µL	to 50 µL

   a. Add enzyme last, or second last (to pipette mix upon addition of nuclease-free water). Keep enzyme in the freezer until needed.

2. Run PCR reaction(s) in thermocycler (place tubes in machine once lid is heated to ~70 °C).

Step		Temperature	Time
Initial denaturation		98 °C	30 s
(30 cycles)	Denaturation	98 °C	5 - 10 s
	Annealing	45 - 72 °C	10 - 30 s
	Extension	72 °C	15 - 30 s per kb
Final extension		72 °C	5 min
Hold		4 °C	∞

   a. Determine the annealing temperature for the primers using the NEB Tm Calculator. Ensure that the proper polymerase/primer concentration are selected.
   b. It is best to overestimate extension time.

RE digest

1. Ensure that an excess of DNA is available to be digested (usually 1 µg of insert or 2 µg of vector; should be in excess of what is required for a ligation).
2. Check the information (buffer compatibility, time for digestion, enzyme concentration, issues with over-digestion/star activity, etc.) for the restriction enzymes to be used. This can be done using a tool such as NEBcloner.
    a. Ensure that the exact enzyme is chosen (for example, HF versions of enzymes may have different properties).
3. Set up the reaction as per enzymes' protocol.
    a. Ensure that the amount of enzyme used is proportional to the amount of DNA.
    b. For RE digestion of vectors, single-enzyme digests should also be performed to ensure proper cutting. For single-enzyme digests, set up reactions with 200 ng of vector.
4. Incubate in a 37 °C water bath for the appropriate amount of time.
    a. The digestion time is indicated in the protocol for the enzymes used. Even for Time-Saver qualified enzymes, incubation times of up to 30 minutes to 1 hour can be appropriate to ensure thorough digestion.
5. For RE digestion of vectors: check 200 ng of each product on a gel, and then gel purify the double-enzyme digest.
6. For RE digestion of inserts: purify using PCR clean-up system.
7. Quantify product(s) using a NanoDrop™, and store at -20 °C.

Ligation (NEB T4 DNA Ligase)

We use NEB T4 DNA Ligase (protocol from NEB Ligation Protocol with T4 DNA Ligase (M0202)).

1. Determine the desired ratio of vector:insert for your ligation.
    a. In general, a 1:3 ratio is desirable, though other ratios can work as well. We use 30 fmol vector and 90 fmol insert in our ligation reactions.

    To calculate how many nanograms of insert is required for a ligation:

$$ng = \#\,bp \times \frac{660\ g}{1\ mol\ bp} \times 90 \times 10^{-15} mol \times \frac{10^9\ ng}{1\ g}$$

    (to calculate for the vector, multiply by 30 fmol instead of 90).

2. Set up the ligation reaction.
    a.

Component	**20 µL reaction**
10X T4 DNA Ligase Buffer	2 µL
Vector DNA	(variable)
Insert DNA	(variable)
T4 DNA Ligase	1 µL
Nuclease-free water	to 20 µL

    b. For each set of ligation reactions, a control reaction should also be prepared. For the control, replace insert DNA with nuclease-free water. The control measures whether the vector effectively re-circularizes without the insert.
3. Gently pipette up and down, and spin down briefly if necessary.
4. We have found that overnight ligations at 16 °C work well. Prepare the ligation reactions in PCR tubes, and place in a PCR machine that is set to hold at 16 °C for ~16 hours. After this time, the reaction may be heat-inactivated at 65 °C for 10 minutes on a dry heating block, and stored at -20 °C until ready for transformation.

Transformation

This protocol can be used for DH5α and BL21(DE3) cells.

1. Thaw competent cells on ice. Avoid thawing larger volumes than is required, as multiple freeze-thaw cycles negatively impact cell competence. While cells are thawing, add the appropriate amount of DNA into microcentrifuge tubes on ice.
    a. For circularized plasmids (from miniprep), ranges between 100 pg - 50 ng of DNA have worked well for us.
    b. For ligation products, use between 1 - 10 µL of the reaction to transform.
2. Once cells are thawed, add 50 µL cells to each tube on ice. Slowly pipette mix twice. Incubate on ice for 30 minutes.
    a. To be extra gentle, wide bore tips may be used to pipette cells.
3. Heat shock cells in a 42 °C water bath for 45 seconds.
4. Immediately transfer cells to ice without shaking; let cool for 2 minutes.
5. Add 950 µL LB and incubate, shaking, at 37 °C, 150 rpm for 1 hour.
6. Spin down cells at 3 000 rpm at 4 °C. Remove ~850 µL supernatant, and carefully resuspend cells in remaining media.
7. Plate 100 - 150 µL of the transformation onto room temperature plates using aseptic techniques. Incubate at 37 °C for 16 - 20 hours.

Colony PCR (NEB *Taq*)

We perform colony PCR with NEB *Taq* polymerase (protocol from NEB PCR Protocol for *Taq* DNA Polymerase with Standard *Taq* Buffer (M0273)).

1. Pick and label colonies on plate.
2. Set up PCR master mix for 25 µL reactions. Assume no template DNA volume (dispense 25 µL master mix in each PCR tube).
    a. For n colonies, make a 1.5n volume of master mix.
3. Set up autoclaved test tubes in a rack, with lids labelled for each colony. Add 4 mL of LB + appropriate antibiotic to each tube.
    a. The amount of LB + antibiotic to grow up in is arbitrary; 4 mL allows for the preparation of a glycerol stock (requires 1 mL) with enough liquid culture left over to miniprep from.
4. For each colony:
    a. Using your smallest pipette, touch the colony with the tip.
    b. Swirl the tip into the appropriate PCR tube with master mix.
    c. Dispense the tip into the appropriate test tube. Place tubes with tips in broth in 4 °C fridge.
5. Run colony PCR in thermocycler (place tubes in machine once lid is heated to ~70 °C).

Step		Temperature	Time
Initial denaturation		95 °C	5 min
(30 cycles)	Denaturation	95 °C	15 s
	Annealing	45 °C	20 s
	Extension	68 °C	1 min per kb
Final extension		68 °C	5 min
Hold		4 °C	∞

6. Run each 25 µL reaction + 5 µL loading dye on an agarose gel alongside an appropriate ladder. Determine which colonies have your successful transformants.
7. Incubate appropriate tubes at 37 °C for 16 hours, shaking at 200 rpm.

Making a glycerol stock

1. Grow up a saturated culture (yeast or *E. coli*) containing plasmid of interest for 16 hours in LB + appropriate antibiotic.
2. Mix 1 mL culture with 0.5 mL 50% glycerol in 1.5 mL sterile cryovial. Label tube on side with the date and strain name.
3. Store in -80 °C freezer.

To grow up from a glycerol stock, scrape the bacteria with a sterile toothpick and quadrant streak a plate with appropriate antibiotic; incubate at 37 °C for 16 hours. Do not allow glycerol stock to thaw.

Overexpression of soluble protein

Expression

For our assays, we made induced and uninduced controls, as well as untransformed BL21 controls, by this protocol.

1. Grow BL21 cells in 2 mL LB with chloramphenicol at 37 °C, shaking at 200 rpm, overnight (16 hours).
2. Use the entire 2 mL culture to inoculate 100 mL LB with chloramphenicol. Grow at 37 °C, shaking at 200 rpm, until it reaches OD_{550} = 0.5 - 0.6.
3. Spin down 1 mL cells for negative control. Lyse.
4. Add 50 µL IPTG (0.5 mM end concentration) to appropriate cultures, and label as “induced”; label others as “uninduced”.
    a. Grow for at least 3 hours at 37 °C, shaking at 200 rpm. We grew our cultures overnight.
5. Spin down cultures at 3 270 rcf for 14 minutes at 4 °C in pre-weighed conical tubes. Decant supernatant and determine wet weight.

Cell lysis

1. Resuspend pellet in lysis buffer (2 - 5 mL/g of wet weight).
    a. Lysis buffer is 20 mM sodium phosphate, 300 mM NaCl, pH 7.4.
2. Sonicate using a microtip on ice, using 6×10 second bursts at low power. Ensure that there is at least a 10 second cooling period in between rounds.
3. Centrifuge 12 000 rcf for 15 minutes.
4. Store lysate at -20 °C.

Protein Purification

We used NEBExpress® Ni Spin Columns for purification of our His-tagged proteins (followed protocol from NEBExpress® Ni Spin Column Reaction Protocol (NEB #S1427)).

Assays

We prepared our assays in black 384 well plates; our plates had a maximum well volume of 120 µL. We designed all our assays to have 100 µL of liquid in each well (dilutions to this volume were made with MilliQ).

For assays with vitamin D, lysate and water were first added to the wells; then, vitamin D was added row-by-row to the plate, with measurements taken immediately after each row.

Fluorescence Readings

We used a Synergy™ 2 Multi-Mode Microplate Reader to measure the fluorescence of the system. The following settings were used for reads:

Read Type	Fluorescence Endpoint
Optics Type	Filters
Mirror	Top 50%
Gain	110
Read Speed	Normal
Read Height	7 mm
Light Source	Xenon Flash

Excitation and emission wavelengths were set as follows:

To test mNeonGreen:

Excitation	485/20 nm
Emission	528/20 nm

To test mCherry:

Excitation	540/25 nm

Emission	620/40 nm

To test FRET:

Excitation	485/20 nm
Emission	620/40 nm

Recipes

LB broth

Mix 6.25 g LB broth powder (we use Difco™) with 250 mL MilliQ in a large beaker; stir to dissolve completely. Aliquot into 125 mL bottles and autoclave (15 minute liquid cycle). Do not re-autoclave.

LB antibiotic plates

This recipe makes 1 L of media, which pours approximately 40 plates.

1. Mix 25 g LB Broth Miller (we use Difco™) and 1 L MilliQ; stir to dissolve completely.
2. Add 20 g agar (we use Bacto™ Agar).
3. Autoclave with stir bar (15 minute liquid cycle). Prepare plates (stack right side up in groups of 4-5 on bench, and mark to indicate antibiotic). 1 L pours about 40 plates.
4. Stir slowly, so as to avoid creating bubbles; once the flask is ~60 °C (can hold bottom with palm uncomfortably), add 1 mL of the appropriate antibiotic and stir to mix.
    a. All antibiotic stocks are made in dH_2O except for chloramphenicol and tetracycline, which are made in EtOH. Stocks are filter sterilized through a 0.2 µm low protein binding filter before use.

Antibiotic	**1000X**
Ampicillin	100 mg/mL
Carbenicillin	50 mg/mL
Chloramphenicol	34 mg/mL
Kanamycin	25 mg/mL
Spectinomycin	100 mg/mL
Tetracycline	12.5 mg/mL

5. Pour plates from the bottom of each stack, lifting plates with one hand and pouring with the other; pour only enough LB mixture to just cover the bottom of each plate.
6. Allow plates to cool undisturbed on the benchtop overnight. Store upside down in a tape-labelled sleeve in 4 °C fridge.

10X TBE buffer (1 L)

Recipe from Cold Spring Harbor Protocols.

Weigh out the following into an autoclaved beaker:

- 121.1 g Tris
- 61.8 g Boric acid
- 7.4 g EDTA disodium salt

Dissolve in MilliQ (will need to stir vigorously); adjust volume to 1 L and autoclave. Autoclaving, though not strictly necessary, helps to keep the buffer in solution.

Agarose gel with SYBR Safe

For a 75 mL gel:

1. Mix 0.75 g agarose with 75 mL 1X TBE in a 250 mL Erlenmeyer flask.
2. Add 5 µL SYBR Safe; swirl gently to mix.
3. Microwave in 20 - 30 second intervals, stopping to swirl the flask, until agarose is thoroughly melted. Ensure that the mixture does not boil over.
    a. Use a paper towel to handle the hot flask. If preferred, a Kimwipe® may be stuffed into the mouth of the flask while microwaving.
4. Pour into gel cast and place comb. Allow to polymerize undisturbed.

SDS PAGE

Gel Preparation

1. Make resolving gel mixture.
2. Add 20 µL 20% APS and 6 µL TEMED; using 1 mL pipette, pour gel into cast.
3. Pipette about 200 µL methanol on top of the gel to flatten and allow to polymerize for ~ 20 - 30 min.
4. Pour off methanol and dry gel cast with Kimwipe®.

5. Make stacking gel mixture and pour; insert comb, and allow to polymerize.

Sample Preparation

1. Prepare 10% (v/v) β-mercaptoethanol in 2 X SDS Loading Dye.
2. To 500 ng protein per sample, add an equal volume 2 X SDS Loading Dye.
3. Boil for 5 minutes in a fume hood; rest on ice for 5 minutes.
4. Set up apparatus, and fill inner and outer chamber with running buffer (10X Tris/Glycine) to appropriate levels. Wash gel wells.
5. Load 20 µL of each sample into wells, along with a low molecular weight marker.