Lethbridge HS

iGEM

Laboratory Protocols 2017

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NOTE: Protocols found in this document are adapted from protocols developed by the Kothe and Wieden groups of the Alberta RNA Research and Training Institute (ARRTI) at the University of Lethbridge. We thank them for their advice and for sharing resources.

Cloning IDT g-blocks using the pJET Kit from Thermo Fisher Scientific

1. Combine the following reagents sequentially on ice:

Component	Volume (µL)
2x Reaction Buffer	10
DNA fragment or g-block (50ng/µL)	1
Water, nuclease free	Up to 17
DNA blunting enzyme	1
Total volume	18

2. Vortex briefly and centrifuge for 3-5 seconds
3. Incubate the mixture at 70°C for 5 minutes and chill on ice
4. Set up the ligation reaction on ice. Add the following to the blunting reaction mixture

Component	Volume (µL)
pJET1.2/blunt Cloning Vector (50ng/µL)	1
T4 DNA ligase	1
Total volume	20

5. Vortex briefly and centrifuge for 3-5 seconds. Then incubate at room temperature (approximately 22°C) for 5 minutes. (Note for DNA fragments in excess of 3 kb, ligation can be prolonged to 30 mins).
6. Transform ligation mixture into chemically competent cells.

Transformation of Competent Cells

Work sterile!!! Open flame!!!

1. Thaw 20 µL of pre- aliquoted cells (DH5α or BL21(DE3)) on ice. Competent cells are stored at -80°C. (Often cells are frozen as 50 µL aliquots – split under sterile conditions for 2 transformations.)
2. Gently pipet max. 2.0 µL (better 1.8µL) of DNA into the competent cells and pipet once up and down to rinse the tip.

   ATTENTION:

   Never use more DNA than 10% of the volume of the competent cells

   Otherwise the cells get destroyed by osmotic shock
3. Mix the DNA into the cells by swirling the tip in the solution.
4. Incubate the cells on ice for 30 minutes
5. Heat shock the cells in a water bath at 42 ^{0}C for exactly 45 seconds.
6. Incubate the cells on ice 1 minute.
7. Add 250 mL of sterile media to the cells and incubate at 37 ^{0}C for 1 hour with shaking (tape microcentrifuge tube in shaking incubator).
8. Label the LB plates on the outside perimeter:
   a. Your name
   b. Date
   c. Cell strain (e.g. DH5a, BL21DE3 etc.)
   d. Plasmid
   e. Volume plated
9. Plate 100 µL and 50 µL on pre-warmed LB plates containing the appropriate antibiotic.
   For ligations and mutagenesis: plate all 250 µL on (1 or) 2 plate
10. leave plate for 10-15 min to soak the cell suspension into the agar
11. Flip plate over (agar on top).
12. Incubate the plates in the 37^{0}C oven overnight.
13. Keep the remaining solution in the 4^{0}C fridge overnight until transformation has been confirmed.

Preparation of Competent Cells

1. Wear gloves at all times, work under sterile conditions with an open flame, and keep all samples on ice in closed tubes whenever possible to avoid contamination. KEEP CELLS ON ICE AS MUCH AS POSSIBLE!
2. Inoculate from glycerol stock and grow cell culture overnight in 2 x 50 mL LB flasks.
3. Centrifuge cells at 2700 x g for 7 minutes at 4°C.
4. Pour off supernatant being careful not to disturb the pellet. Tap gently on paper towel to remove any remaining supernatant.
5. Add 30 mL ice cold 80 mM $MgCl_2$ 20 mM $CaCl_2$ to each tube. Resuspend by pipetting gently up and down. DO NOT VORTEX.
6. Centrifuge for 5 minutes at 2700 x g.
7. Pour off supernatant.
8. Resuspend cell pellet in 2 mL ice cold 100 mM $CaCl_2$.
9. Combine both tubes for a final volume of 4 mL.
10. Add 1.2 mL sterile glycerol and mix gently.
11. Aliquot samples (20 – 100 µL each), flash freeze with liquid nitrogen, and store at -80°C.

Buffers

- 80 mM $MgCl_2$ 20 mM $CaCl_2$
- 100 mM $CaCl_2$

Sterilize prior to use.

Inoculating Overnight Culture of Bacteria for Plasmid Purification

Overnight culture

1. Using proper aseptic technique, sterilely transfer 5 mL of LB medium into a sterile culture tube or falcon tube. (or use prepared, autoclaved tube containing 5 mL LB medium)
2. Thaw the appropriate antibiotic and pipet in the amount needed for 1:1000 dilution.
3. Use an autoclaved toothpick (or yellow pipette tip) to pick one colony from a plate or scrape some media from a glycerol stock and transfer the cells to the media.
4. Secure the lids of the vessel and incubate overnight (about 15h) in shaking incubator at appropriate temp (usually 37 ^{0}C)

Harvesting of cells and plasmid purification

1. Prepare a glycerol stock if not yet available (see extra protocol)
2. Transfer 1 mL bacteria into a microcentrifuge tube and centrifuge at max speed for 2 minutes.
3. Remove the supernatant and dispose of in the bacteria waste vessel.
4. Add another 1mL of bacteria and repeat the centrifugation (steps 2 & 3).
5. Freeze cells at -20C or proceed to the miniprep protocol found in the miniprep kits.

Miniprep of Plasmids

1. Transfer 1500 µL of overnight culture into a microcentrifuge tube. Centrifuge for 2 min. Pour off the supernatant.
2. Resuspend: Add 100 µL of Solution I to the pellet, flick and shake the tube to mix, let sit for 1 min.
3. Lyse Cells: Add 200 µL of Solution II to the mixture, mix by very gently inverting 4-6 times, sit for 1 min.
4. Precipitate cell material: Add 350 µL of Solution III, mix gently by inverting, let sit 1 min.
5. Clear cell lysate: Centrifuge for 5 min at high speed.
6. Bind the DNA to column: Transfer the supernatant to an EZ-10 column. Let sit 2 min. Centrifuge 2 min.
7. Clean DNA: Discard the flow-through. Add 750 µL of Wash Solution to the column, centrifuge for 2 min.
8. Repeat wash step 7 for a total of two washes.
9. Discard flow through, spin again for 1 min. Remove all liquid.
10. Elute the DNA: Transfer column to a clean labeled 1.5 mL tube. Add 30 µL of Elution Buffer into the center part of the column and let sit 2 min, centrifuge for 2 min.
11. Determine the concentration of the plasmid DNA using the Biodrop, record.
12. Store purified plasmid at -20°C.

PCR of Gene Fragments for Cloning

Program the PCR machine:

Cycle Step	Temp.	Time	Number of Cycles
Initial Denaturation	95 ^{0}C	5 min	1
Denaturation	95 ^{0}C	30 s	
Annealing	Tm	30 s	25-35
Extension	72 ^{0}C	30 s – 120s/Kb	
Final Extension	72 ^{0}C	5-10 min	1
	4 ^{0}C	hold	

Set up the PCR reaction *Work on ice!*

1. If not yet done, prepare the primer solution: dissolve the purchased primers in appropriate amount of MilliQ H_2O to a final concentration of 100 µM (amount of primer in nmole given on tube or primer sheet). Prepare a 1:10 dilution, i.e. to 10 µM in MilliQ H_2O for your own use.
2. Pipet the reagents into an appropriate PCR tube (0.2 mL) in the order given. If you are setting up multiple reactions, then prepare a master mix containing all of the common reagents (e.g. Buffers, dNTPs, polymerase etc.) which is then aliquoted into separate tubes.
3. Add DNA last unless it is included in the master mix.
4. Place tubes in the thermocycler and start.
5. Run 5-10 µL of the PCR reaction after cycling on an agarose gel to verify products.

Component	Volume 50 µL	Volume 20 µL	Final conc.
MilliQ ddH_2O	add to 50 µL	Add to 20 µL	
10x Pfu buffer with $MgSO_4$	5 µL	2 µL	1X
10mM dNTPs	1 µL	0.4 µL	200 µM each
Forward Primer (10 mM)	2.5 µL	1 µL	0.5 mM
Reverse Primer (10 mM)	2.5 µL	1 µL	0.5 mM
Pfu DNA Polymerase	0.5 µL	0.2 µL	0.02 U/µL
Template DNA*	X µL	X µL	1-100 ng/µL

* Template amounts: minipreps - 2 µL for 20 µL PCR

Restriction Digestion

1. Pipet in this order into a 1.5mL microcentrifuge tube:

MilliQ H_2O	final total volume:	20 µL
10x Buffer (corresponding to enzyme)		2 µL
Plasmid DNA (Vol according to conc.)		10 µL
Restriction enzyme (1-2 U / microg DNA)		2 U, typically 1 µL

   Attention with handling restriction enzymes:

   > Take them only immediately before use out of -20°C fridge, store on ice, put back into fridge as soon as possible

2. incubate at optimal temperature for 1 h (incubation at 37°C in water bath)
3. take sample for agarose gel or store for short-term on ice or for long-term at -20°C

Volumes and concentrations may be adjusted to for preparative or analytic restriction digestions. Notes should be made in the lab book accordingly.

Agarose Gel Electrophoresis

Prepare the agarose gel:

1. weight appropriate amount of agarose into a small Erlenmeyer flask (0.3 g for 30 ml small 1% gel)
2. add desired volume of 1x TAE buffer (or 1x TBE in special cases) (30 mL for small gel chamber)
3. weight the flask and write down its total weight
4. boil it to resolve the agarose:
   either by microwaving (without magnetic stir bar)

   or on hot plate (with magnetic stir bar)
5. let it cool down while stirring to about 60°C (hand-warm). Add Gel Red 1:10000, swirl to mix.
6. weight again and replace lost water
7. cast gel into gel plate and put in the comb, wait until solid

Prepare samples:

8. transfer about 0.3 µg DNA into a microcentrifuge tube
9. add 1x TAE or water to 5 µL
10. add 1 µL of 6x DNA loading buffer (NEB)

Run electrophoresis:

11. place gel into the big chamber in correct orientation (DNA migrates to the positive pole!)
12. fill chamber with 1x TAE buffer, about 0.5 cm above gel
13. carefully remove comb from solid gel
14. Load the 6 µL samples into the gel slots, and load 5 µL of DNA ladder (typically 1kb ladder) in at least one gel slot
15. close chamber with lid in the correct orientation (black/red!!!)
16. connect cables to power supply

17. run the gel with 100V, check for dye fronts should be at about 1/3 and 2/3 of gels (about 0.5-1h, depending on size of gel)

Documenting:

18. Gel Red already incorporated in the gel, can observe under UV directly without further staining. observe bands on UV illuminator and take a digital photo
    Careful: strong UV light, wear glasses and lab coat to avoid “sun burn”
19. clean UV illuminator

Buffers:

<u>50x TAE</u>

242 g Tris

57.1 mL acetic acid

100 mL 0.5 M EDTA pH 8.0

H_2O to final volume of 1 L

Dilution to 4 L of 1x TAE Buffer:

80 ml 50x TAE buffer

fill up to 4 L with MilliQ H_2O

Test Expression of MelA in *E. coli* BL21 (DE3)

1. streak a plate from a glycerol stock of melA_pJET or use a fresh transformation colony to inoculate a 5mL overnight culture in LB media. Incubate at 37°C shaking at 200 rpm.
2. Use 200µL of the overnight culture to inoculate another 5mL culture (OD600 ~0.1). Add ampicillin to a final concentration of 0.1 mg/mL.
3. Let grow at 37°C for 3-4h to allow the OD600 to reach 0.7-1. Take a 1OD sample, spin down cells, remove supernatant. Resuspend cells in 80µL 100mM Tris-HCl pH 8, 5M urea. Store at -20°C.
4. Add $CuSO_4$ to a final concentration of XXX. Add tyrosine to a final concentration of XXX. Induce melA expression by adding IPTG to a final concentration of 1mM.
5. Incubate at **30°C** overnight shaking at 200rpm.
6. Take a 1 OD sample, treat as in step 3.
7. Analyze samples on an SDS PAGE.

SDS PAGE of Proteins

Effective Range of Separation of SDS-Polyacrylamide Gels

Acrylamide Concentration (%)	*Linear Range of Separation (kDa)*
15	10 – 43
12	12 – 60
10	20 – 80
7.5	36 – 94
5.0	57 - 212

Pouring SDS-polyacrylamide Gels

1. Clean the glass plates and comb with H_2O and ethanol.
2. Assemble glass plates in casting unit.
3. In an old 15mL centrifuge tube, mix the components for the resolving gel in the order shown in the table. Polymerization will start as soon as the TEMED has been added!
   Be careful handling the acrylamide solution! Wear gloves! Don't spill it!
4. Vortex the mixture rapidly.
5. Using a Pasteur pipette, pour the acrylamide solution into the gap between the glass plates. Leave space for the stacking gel (teeth of comb plus 1 cm).
6. Overlay the gel with water (or technical isopropanol if available).
7. Let the gel stand at room temperature for polymerization (~ 30 min). – Keep remaining acrylamide solution in centrifuge tube to observe polymerization.
8. Pour the overlay off and remove remaining H_2O with edge of a paper towel.
9. Prepare the stacking gel in an old 15 mL centrifuge tube by mixing the components shown in the table, vortex rapidly, and pour on top of the resolving gel using a Pasteur pipette.
10. Immediately insert the clean Teflon comb – avoid air bubbles! If necessary, add more stacking gel solution to fill the spaces.
11. Let gel polymerize at room temperature (~ 30 min). Observe polymerization of stacking gel in the centrifuge tube.

Preparation of Samples and Running the Gel

12. Mix sample in SDS gel-loading dye: add 1/5 of final Vol. 6x SDS gel-loading dye and 1/10 Vol. 1 M DTT
13. Heat sample for 3 min to 100°C by boiling in a beaker on a hot plate (denatures the proteins).
14. Mount the gel in the electrophoresis apparatus – the comb facing inward.

15. Add Tris-Glycine electrophoresis buffer to the top and bottom reservoirs – bottom reservoir should be filled at least 5 mm above lower edge of the glass plate. Make sure that there are no air bubbles trapped at the bottom of the gel.
16. Carefully remove the Teflon comb and wash the wells with electrophoresis buffer using a 200 µL pipette.
17. Load about 15 µL of each sample into the bottom of the wells. Record the order of your samples! Load 5 µL of the Protein Molecular Weight Marker.
18. Close electrophoresis chamber with the lid and connect to an electric power supply: the positive electrode should be connected to the bottom of the buffer reservoir!
19. Apply a voltage of 80 V to the gel.
20. After the dye front has moved into the resolving gel, increase the voltage to 180 V and run the gel until the dye reaches the bottom of the resolving gel. Then turn of the power supply.
    If time is limiting and the gel NOT important: run completely at 200 V.
21. Remove the glass plates from electrophoresis apparatus and discard old running buffer (can not be re-used!).
22. Place glass plates on a paper towel.
23. Using the green wedge, carefully pry the plates apart.
24. Mark the orientation of the gel by cutting with a disposable scalpel a corner from bottom of the gel that is closest to the leftmost well (slot 1).

Staining of SDS-PAGE with Coomassie Brilliant Blue

25. Place the gel into the staining solution and incubate on a slowly rotating platform for at least 30 min.
26. Remove the stain, pour it back into original bottle for future use.
27. Destain the gel by adding destaining solution and further incubate on rocking platform for several hours.
    More rapid destaining is achieved by heating the gel in the destaining solution in the microwave to about 45°C and by adding a piece of paper towel to the destaining solution.
28. Change the destaining solution 3 – 4 times.
29. Recycle staining solution by cleaning it from the dye using the active charcoal column.
30. Check the gel for bands on a white light transilluminator.

6x SDS gel-loading dye (5 mL)

100 mM Tris-HCl (pH 6.8)	0.5 mL 1.0 M Tris pH 6.8
20 mM 2-Mercaptoethanol	7 µL of stock (14.2M)
15 mM EDTA	
6 % SDS	0.3 g (weight in under hood!)
0.2 % Bromophenol Blue	10 mg

Add H_2O ad 1.5 mL, dissolve, then add glycerol:

60 % glycerol	3 mL

Add H_2O ad 5 mL

Store 1x SDS gel-loading buffer at room temperature.

10x Tris-glycine electrophoresis buffer	***1x Buffer***
30.2 g Tris base	25 mM Tris
188 g glycine (pH 8.4)	250 mM glycine (pH 8.3)

Add MilliQ H_2O to a final volume of 800 mL and dissolve chemicals by stirring

Add 100 mL 10% SDS	0.1 % (w/v) SDS

Adjust to 1000 mL with MilliQ H_2O

Coomassie Staining solution *(recipe from Wieden lab)*

800 mL H_2O

100 mL Isopropanol

100 mL glacial acetic acid

1 g Coomassie Brilliant Blue R-250

Destaining solution *(recipe from Wieden lab)*

800 mL H_2O

100 mL Isopropanol

100 mL glacial acetic acid

Extraction of Pigment from Supplement Tablets

1. Crush or open appx 3g of tablets, pour into 50mL falcon tube.
2. Add 5-10mL water or buffer, boil 10 min, allow to mix overnight
3. Centrifuge or strain out remaining material from tablets.
4. Adjust pH of solution if required (for anthocyanin to be red, pH must be appx 5)
5. Add acetone to appx 50% vol/vol
6. Add guar gum to the desired consistency. NOTE – not much is required! Optimal amount still TBD.