Lab Protocols

Bhaskar Kumawat

2025

Table of contents

Introduction

Part I

Molecular Biology

P1 Transduction

Strains: Donor *E. coli* (eg. Keio knockout), Recipient *E. coli* (eg. K12), P1 phage stock (high titer)

Reagents: NZCYM media, 1M Sodium Citrate, 10mM CaCl2, antibiotic stocks, P1 salts solution (LB with 10mM CaCl2 and 5mM MgSO4), LB plates

Time required: 2-3 days (excluding day 0)

A. Donor lysate preparation

Day 0

1. Inoculate a 3ml overnight culture of donor strain in LB supplemented with antibiotics (eg. Kan for keio collection donors).

Day 0

2. Inoculate a 3ml overnight culture of recipient strain in LB. (For part B)

Day 1

3. Dilute saturated donor culture 1:100 into 3mL NZCYM supplemented with 10Mm CaCl2 and antibiotic if required (eg. Kan for keio collection donors). Incubate at 37C and start checking for cells after 40 minutes.
    - Make an additional such dilution to visually check for phage clearing later. No P1 phage will be added to this control culture.
    - Start a 42C water bath for incubation after addition of P1 phage.

Day 1

4. After around 45-60 minutes, if the culture has swirls when held up the light and shaken, add 50uL of the high-titer P1 phage stock to the culture. Incubate at 42C for 1 hour.

Day 1

5. After 60 minutes, check if the culture with P1 phage has started to clear (compared to control). If so, move the culture to 37C for 2 hours.

Day 1

6. Transfer lysed culture to a 15mL conical centrifuge tube and add 200uL $CHCl_3$. Vortex the tube vigorously to lyse any remaining cells and incubate at 37C for another 5 minutes.

    - At this point, start fast-cooling the centrifuge to 4C.

Day 1

7. After 5 minutes, centrifuge the 15 mL conical tubes at 9200g for 5 mins (or at max speed for 10 minutes).

    - Collect syringes and 0.45um filters for filtering the lysate.
    - Label new tubes that will be used to store the lysate.

Day 1

8. After centrifugation, carefully transfer the supernatant to the syringe and filter it through the 0.45um filter into its final tube. This P1:Donor lysate can now be stored at 4C.

    - Discard the chloroform contaminated tubes in biohazard bins.

B. Recipient transduction

Day 1 or 2

1. Pellet 1mL of saturated recipient culture for 2 minutes at maximum speed.

Day 1 or 2

2. Resuspend the cells in 500uL P1 salts solution. Transfer 100uL of these cells to 3 tubes (100uL each).

    - One of these tubes is a no-phage control and gets no P1:Donor lysate.
    - Add 10uL P1:Donor lysate to one of the other tubes.
    - Add 100uL P1:Donor lysate to the final tube.

Day 1 or 2

3. Incubate the tubes at 37C without shaking (desk top heater w/ water in the wells) for 30 minutes to allow the phage to adsorb to the cells.

Day 1 or 2

4. After 30 minutes, add 1mL of LB and 200uL of 1M Sodium Citrate to each tube and incubate for 1 hour with shaking.

- Spread plain LB plates with 5mM Sodium Citrate (125uL of 1M for a 25uL plate) and any antibiotics required for selection of the transduced strain. (eg. Kan for keio collection donors).

Day 1 or 2

5. After 1 hour, spin down the cells, discard the supernatant, and resuspend in 100uL LB media. Spread, the cells on LB plates containing 5mM Sodium Citrate and antibiotic. Incubate the plates at 37C

Day 2 or 3

6. Check the plates the next day, no-phage plates should have no colonies. Restreak colonies from the plates made with P1:Donor lysate transduced cultures onto selective plates. You can directly inoculate these colonies (after restreaking) for step 2 of FLP recombination.

FLP Recombination

Strains: Transformant strain

Reagents: TSS buffer, pcp20 plasmid, LB plates, antibiotics

Time required: 5 days (excluding day 0)

A. pcp20 Transformation

Day 0

1. Inoculate a 3ml overnight culture of transformant strain in LB supplemented with antibiotics (eg. Kan for keio collection transduced strains).

Day 0

2. Dilute the overnight culture 1:100 in 3mL LB supplemented with antibiotics and incubate at 37C for 2-3 hours.

Day 1

3. After 2-3 hours, once the culture is translucent but not opaque, swirl the culture in ice water bath for 5-10 minutes to stop cell growth.

Day 1

4. Centrifuge the culture in two 1.5mL centrifuge tubes at maximum speed for 5 minutes to get a cell pellet. Resuspend the cell pellet in 100uL TSS buffer.
    - One of these tubes can be used as a control and will get no plasmid DNA.

Day 1

5. Add 1uL pcp20 plasmid DNA to the tube and incubate on ice for 30 minutes.
    - Set a desk-top heater to 42C for the heat-shock step.

Day 1

6. Heat shock the cells twice by doing two 45 second heat exposures with a two minute gap on ice (45 sec shock → 120 second on ice → 45 sec shock). After these heat-shocks, let the tubes incubate on ice for 2 minutes and add 500uL SOC to each. Incubate the cells at 30C for 1 hour.

    - Make LB + Carbenicillin plates for plating transformants.

Day 1

7. Plate 100uL of the culture on LB + Carbenicillin plates. Grow the plates overnight at 30C.

B. Recombination, curing, and screening

Day 2

1. After checking for the absence of colonies on the control, inoculate 3 transformed colonies in 3mL LB and grow at 42C overnight for curing of the plasmid.

Day 3

2. Dilute the overnight culture by a factor of 106 (3x dilution of 10uL in 990uL LB) and plate 125uL of this dilution on LB plates. Incubate the plates at 30C.

Day 4

3. Pick three colonies from each plate (total 9 colonies) and restreak them on LB+Kan → LB+Carb → LB plates (in that order) followed by resuspension in 10uL nuclease free water in a PCR tube. Incubate the LB and LB+Kan plate at 37C and the LB+Carb plate at 30C overnight.

    - The colonies in nuclease free water can be used directly for a Colony PCR to check for deletion of the Kanamycin casette.

Day 5

4. The next day, isolate colonies that grew on LB but not on LB+Kan and LB+Carb plates. These are the recombined clones. Verify deletions with PCR for binding sites flanking the gene of interest.

TSS Transformation

Strains: Transformant strain

Reagents: TSS buffer, plasmid, LB plates, antibiotics

Time required: 4-5 hours (excluding Day 0)

Day 0

1. Inoculate a 3ml overnight culture of transformant strain in LB supplemented with any antibiotics required for plasmid/insert maintenance.

Day 1

2. Dilute the overnight culture 1:100 in 3mL LB supplemented with antibiotics and incubate at 37C for 1-2 hours.

Day 1

3. After 2-3 hours, once the culture is translucent but not opaque, swirl the culture in ice water bath for 5-10 minutes to stop cell growth.

Day 1

4. Centrifuge the culture in two 1.5mL centrifuge tubes at 10,000rcf for 4 minutes to get a cell pellet. Resuspend the cell pellet in 100uL TSS buffer.
    - One of these tubes can be used as a control and will get no plasmid DNA.

Day 1

5. Add 1uL plasmid DNA to the tube and incubate on ice for 30 minutes.
    - Set a desk-top heater to 42C for the heat-shock step.

Day 1

6. Heat shock the cells twice by doing two 45 second heat exposures with a two minute gap on ice (45 sec shock → 120 second on ice → 45 sec shock). After these heat-shocks, let the tubes incubate on ice for 2 minutes and add 500uL SOC to each. Incubate the cells at 37C (or another temperature required for plasmid stability) for 45 minutes.

- During this time, make selection plates for plating the transformants (with previous and new antibiotics)

Day 1

7. Plate 100uL of the culture on selective plates (You can also spin down the cells at 10000rcf for 3 mins and resuspend in a smaller amount of LB to plate all the cells). Grow the plates overnight at 37C (or alternative temperature for plasmid).

Colony PCR

Strains: Plate with any colonies to be tested (Plate of interest)

Reagents: Appropriate primers for verification, restreak plate with appropriate antibiotic

Time required: <1 day.

1. Create/use LB Agar plates that contain the appropriate antibiotic that was used for selection of the colonies that are to be verified.
2. Divide the plate area into any number of subsections for restreaking the colonies. (I usually divide a plate into 8 sectors).
3. For 10uL reactions and N colonies, create a batch PCR mix that is 10 times (N+1) uL in volume. For example, for 8 samples, I will create a PCR master mix that is $10 \times (8+1) = 90$ uL in volume. This master mix will contain,
    - Water
    - Forward Primer
    - Reverse Primer, and
    - Polymerase w/ Buffer (i.e., PCR Master Mix)
4. Decide how much water, primers, and polymerase MM goes into this batch mix using the appropriate enzyme documentation and based on the total volume calculated above. For colony PCR, I suggest using a non-high fidelity enzyme like Promega GoTaq Green (but NEB Q5 can be used if the first try with GoTaq fails.)
5. Mix the batch using vortexing and spin it down. Divide the batch mix into individual PCR tubes, with 10ul volume per tube. Label one of them the negative control (-) and the rest as C1 to CN, where N is the number of colonies you wish to PCR.
6. Now, number N colonies on your plate of interest with a marker (from 1 to N). Number the subdivisions on the new plate the same way.
7. For each colony, pick it using a sterile pipette tip from the plate of interest and streak it in the correct section on the new plate. Then dip the same pipette tip into the prepared PCR reaction tube with the correct colony number. The negative control tube (-) gets no colonies.

8. Perform the PCR reactions with the appropriate settings for the polymerase and run the samples on a 1% Agarose gel for checking the results.

HMW Phage gDNA Extraction (NEB Monarch)

Reagents: High concentration phage lysate (ideally 10mL, 4mL might also work), Phage precipitation solution (30% PEG-8000, 3M NaCl, filter-sterilized), Resuspension buffer (5mM MgSO4), 10x DNAse I buffer, DNAse I enzyme (2000 U/mL), RNAse A enzyme (20mg/mL), NEB Monarch Kit

Time required: 1 day (excluding overnight phage precipitation)

A. Phage Precipitation

Day 0

1. Incubate 10mL of phage lysate mixed with 5mL precipitation solution at 4C overnight in a 50mL falcon tube (no shaking). This step precipitates the phage out of the lysate. (4mL lysate with 2mL precipitation solution may also work)

Day 1

2. Pre-chill a centrifuge to 4C. Centrifuge the precipitated phage lysate at 12,000 rcf for 12 minutes at 4C.

Day 1

3. Precipitated phage should form a mostly transparent pellet on the bottom of the tube. Carefully pour off supernatant or remove it gently using a pipette. Resuspend the pellet well in 300uL 1x DNAse I Buffer (made by diluting 30uL 10x buffer in 270uL NF Water). Move the final resuspension to a 1.5mL tube.
4. Add 10uL DNAse I + 1uL RNAse A to the resuspended phage, mix by inverting a few times. Incubate at 37C for 30 minutes without shaking. Incubate at 75C for 10 mins to deactivate enzymes.
5. Spin down the tube for 10 seconds in a benchtop centrifuge and transfer supernatant to a 2mL DNA LoBind tube.
6. Add 300uL HMW gDNA Tissue Lysis buffer and 20uL Proteinase K solution to the tube. Incubate samples in a thermal mixer at 56C for 1hr with agitation at 500rpm.

7. Add 300uL Protein Separation Solution and mix by inverting for 1 minute (each complete inversion should take 3-4 seconds).
8. Centrifuge the tube at 16,000 rcf for 10 minutes and carefully move it to the bench, trying not to agitate the phase boundary. The sample will separate into a large, clear DNA phase and a smaller protein phase at the bottom. Both phases are transparent but the boundary can be seen by holding the tube against a dark background.
9. Transfer ~800uL of the upper phase containing DNA to another labelled 2mL DNA LoBind Tube (use wide bore 1000uL or 200uL tips for this step). Avoid transferring material from the bottom protein layer.
10. Use clean, ethanol and flame sterilized forceps to add 2 DNA capture beads to the sample in the 2mL tube.
11. Add 550uL isopropanol to the tube, close the cap. Bind the DNA to the beads by inverting slowly and gently by hand for 25-30 times. A full inversion should take 5-6 seconds, with a full inversion completing when tube returns to the upright position.
    - You should be able to see the DNA precipitate as white filaments after a few inversion. Make sure these filaments are attached to the beads before you conclude this step. If not, invert gently a few more times.
12. After DNA is bound to the beads, discard liquid by pipetting. Avoid removing any of the DNA wrapped on the beads. Make sure to not let the beads dry too much between this and the next step (i.e., add the next buffer as soon as you're done removing the liquid). The process of removing liquid can be carried out in two ways,
    - Keep the tube upright, insert pipette tip, gently push the beads aside until they are up the wall of the tube with liquid at the bottom. Then aspirate the liquid out using the pipette making sure you don't get any DNA into the pipette.
    - Angle the tube so that beads remain at the bottom but liquid reaches the opening of the tube. Pipette liquid from the surface and continue to angle the tube as liquid is removed (tube will be almost horizontal at the end).
13. Add 500uL gDNA wash buffer, close the cap, and mix by inverting tube gently 2-3 times. The gDNA will condense around the beads more tightly. Remove the wash buffer as described in the previous step.
14. Repeat the wash with the gDNA wash buffer as in step 13. After the wash, remove the wash buffer as described in step 12.
15. Place a labelled bead retainer into a monarch collection tube. Pour the beads into the bead retainer and close the cap. Discard the used 2mL tube.

16. Pulse spin the retainer + collection tube for ≤ 1 second in a benchtop centrifuge to remove residual wash buffer from the beads.

17. Separate the bead retainer from the collection tube, pour the beads into a new, labelled 2mL DNA lobind tube. Insert the now empty retainer into a 1.5mL DNA lobind tube for later use. Discard the collection tube.

18. Immediately add 100uL Nuclease free water (prewarmed to 60C) to the glass beads and incubate for 5-10 minutes at 56C in a thermal mixer with agitation at 300rpm. Halfway through this step, ensure the beads are not stuck by tilting the tube almost horizontally and gently shaking.

19. Pour the eluate and glass beads from the 2mL tube into the bead retainer that was inserted into a 1.5mL DNA LoBind tube. Transfer any remaining liquid using a wide bore pipette. Close the cap of the bead retainer.

20. Centrifuge the bead retainer + 1.5mL tube for 30 seconds at 12,000 rcf to separate the eluted DNA from the glass beads. Discard the beads and the retainer.

21. Make sure the DNA is homogeneously dissolved by either incubating at 37C for 30 minutes or for > 24 hours at 4C. The DNA is now ready to be measured using a nanodrop or sent for sequencing.

Part II

Microbiology

3-Color Infection Assay

This protocol is used to check for phage *E. coli* receptor usage using three one-receptor strains containing only the LamB, OmpF, or OmpC receptor each. The phage are plated on a plate containing 1:1:1 ratio of the three strains, each containing a different fluoroscent reporter, and then imaged using a fluoroscent microscope setup.

Strains:

- 3-color strains
  - *E. coli* ΔLamB ΔOmpF w/ mClover3 (eOC)
  - *E. coli* ΔOmpF ΔOmpC w/ mTurquoise2 (eLB)
  - *E. coli* ΔOmpC ΔLamB w/ mScarlet-I (eOF)

Reagents: LB Media, 0.3% NZCYM Agar, 1000x Kanamycin stock, phage sample.

Time required: 2 days (excluding day 0)

Virocell Protocol

This protocol is used to make virocells (cells containing phage DNA/RNA) for stable storage at -80C and is derived from Golec, Piotr, et al. “A reliable method for storage of tailed phages.” Journal of Microbiological Methods 84.3 (2011): 486-489.

Strains:

- Permissive host strain (eg. E. coli BW25113)
- Clonal phage lysate for phage required to be stored (eg. any of the Basel phages)

Reagents: LB Media supplemented with 10mM MgSO4 and 10mM CaCl2, 80% Glycerol

Time required: 1 days (excluding day 0)

Day 0

1. Make an overnight culture of the permissive strain with any required antibiotics.

Day 1

2. On the next day, dilute the culture 1:100 in 3mL LB supplemented with 10mM MgSO4 and 10mM CaCl2 (30uL of 1M stocks for each). Let the cells grow to late exponential phase (2-3hrs) until they culture is almost near saturation (text outline is visible but not readable).

Day 1

3. Take the culture out of the incubator, let it sit at room temp for 10 minutes, add phage lysates to the cultures aiming for around 10^6 to 10^7 PFUs total and let the tubes sit at room temperature for 15 mins (Time-sensitive).

Day 1

4. Aliquot 800uL of the culture into cryotubes containing 200uL 80% Glycerol, mix and flash freeze in liquid nitrogen or dry-ice and ethanol. The virocells can now be stored at -80.

Part III

Bespoke

Receptor Switching Assay

This assay is used to test the receptor switching rate of phages. It requires an initial stock of the phage to be tested, and host knockouts with only specific receptors in them.

Strains:

- Permissive host strain (eg. E. coli BW25113)
- Alternate receptor knockouts (eg. eLamB, eOmpC, eOmpF containing only one of the three receptors each)
- Clonal phage lysate (eg. any of the Basel phages)

Reagents: LB Liquid Media, 0.5% LB Agar, 1M CaCl2, 1M MgSO4, Chloroform

Time required: 3 days (excluding day 0)

A. Replicate Lysate Preparation

Day 0

1. Make overnight cultures of the cognate host for the phage, the alternative receptor hosts, and a permissive host in 3mL LB.

Day 1

2. Dilute overnight culture of cognate host 1:100 (eg. eLamB for B24 phage) in 3mL LB supplemented with 10mM CaCl2 (30 uL of 1M) and 10mM MgSO4 (30uL of 1M). Let the culture grow for ~90 minutes. These are the 2' bacterial cultures (make 3x/10x tubes for replication + 1 control with no phage added).

Day 1

3. Add clonal phage lysate to the 2' cultures, aiming for a total of 10^6 PFUs of the phage. (eg 0.1uL for 10^{10} lysate). Incubate the cultures at 37C and check for clearing after 2 hours compared to the control with no phage. (I usually start seeing clearing 2 hrs after adding the phage). Let the tubes incubate for a total of 3hrs after addition of phage.

Day 1

4. Aliquot 1mL of the cultures in a 1.5mL tube, add 50uL chloroform, and vortex vigorously (~30 seconds). Incubate the cultures at 37C with shaking for ~10 minutes.

Day 1

5. Centrifuge the 1.5mL chloformed tubes at 20,000 rcf for 5 minutes and transfer ~900uL of the supernatant carefully to another 1.5mL tube. The replicate phage lysates are now ready for switching tests.

B. Low concentration plating

Day 2

1. Melt 0.5% LB Agar in the microwave, aliquot 8mL of it into a test tube or falcon and supplement with 10mM MgSO4 + 10mM CaCl2 (80uL each of 1M). Do this for each host you wish to test the phage lysates on. Let the agar cool down to ~45-50C in the hot bath.

Day 2

2. Add 300uL of the saturated overnight culture of each host to each of these agar aliquots, vortex well and pour into a 150mm petri plate with LB Agar. Swirl well to make sure the agar covers the entire surface of the plate.

Day 2

3. Prepare phage lysate dilutions by mixing 20uL of lysate into 180uL LB in a 96 well plate 8 consecutive times to get dilutions from 10^{-1} to 10^{-8}. Using a 8/12-well pipette, spot 2uL of these dilutions on the prepared host agar plates.

C. High concentration plating

Day 2

1. For a high-concentration test (to find and isolate rare switching mutants), mix 100uL of the undiluted phage lysates with 50uL saturated alternate host culture in a 1.5mL tube. Add 500uL of 0.5% LB Agar (supp. with 10mM MgSO4 + 10mM CaCl2) to the tube, mix well, and move all the liquid in the tube to the wells of a 12-well plate taking care to avoid bubbles.

Day 2

2. Cover the 12-well plate with breathe-easier strip, parafilm it, and incubate upright at 37C (with a thermal mass on top to prevent condensation).

D. Analysis

Day 3

1. Check the low concentration and high concentration plates the next day. Use the low concentration plates to calculate lysate concentrations, and efficiency of plating on alternate receptor hosts if any plaques are seen. Use the high concentration plates to isolate switching mutants and calculate the rate of switching.

Part IV

Recipes

TSS Buffer

Based on the Bennett lab TSS protocol

Pre-sterilized components

Makes **50mL** TSS Buffer

Component	Stock Concentration	Sterilization method	Amount
LB Media	1x	Autoclaved	42.5mL
PEG-3350	1g/mL	Filter-sterilized	5mL
MgCl2	1M	Autoclaved	1mL
DMSO	100%	–	2.5mL

Store at 4°C.

Post-sterilized components

Makes **50mL** TSS Buffer

Component	Stock Concentration	Amount
PEG-3350	pure	5g
MgCl2	1M	1mL
DMSO	100%	2.5mL
LB media	1x	make up to 50mL (~46.5mL)

Filter sterilize and store at 4°C.