McCleary Lab Standard Techniques

W.R. McCleary (adapted from - J.S. Parkinson, Univ. of Utah)

Basic Methods in Microbial Genetics

Aseptic Techniques ……2
Recipes for Media and Solutions 3
Streaking for Colonies 4
Overnight Cultures and Strain Storage 5
Serial Dilutions and Pipetting 6
Pipetting …….....7
Use of the Pipetman ……8
Spreading Samples on Plates 9
Pour Plating and Agar Overlays.....10
Picking and Patching.....11-12
Preparing Competent Cells and Transformation ……13
Analysis of DNA on Agarose Gels.....14
Alkaline Phosphatase Assays ……15-17
Plasmid Minipreps ……18
P1 transduction ……...19
Colony PCR ……20

Five Lab Commandments

ALWAYS follow the best aseptic techniques

ALWAYS pay attention to the small details of an experiment

Never return sterile items to the common supply.

Don't covet your neighbor's supplies.

Never assume that your neighbor's supplies are sterile.

Principle:

Aseptic technique is a fundamental and important laboratory skill in the field of microbiology. Microbiologists use aseptic technique for a variety of procedures such as transferring cultures, inoculating media, isolation of pure cultures, and for performing microbiological tests. Proper aseptic technique prevents contamination of cultures from foreign bacteria inherent in the environment. For example, airborne microorganisms (including fungi), microbes picked up from the researcher's body, the lab bench-top or other surfaces, microbes found in dust, as well as microbes found on unsterilized glassware and equipment, etc. may potentially contaminate cultures, thus interfering with the lab results. Using proper aseptic technique can greatly minimize or even eliminate the risk of contamination. In addition, aseptic technique is of utmost importance to maintain pure stock cultures while transferring cultures to new media. Aseptic technique is also essential for isolation of a single species of microorganism from a mixed culture to obtain a pure culture. Furthermore, proper aseptic technique prevents microbes used in the laboratory from accidentally being released into the environment and/ or infecting people working in the laboratory. This is especially relevant when pathogens are being handled.

Overview

Aseptic technique involves developing both manual dexterity in safely handling the microorganisms and mental dexterity in thinking ahead about what you are doing with the microorganism. The main principle of good microbiological practice is a zero tolerance approach: it's good to be a little paranoid about maintaining sterile conditions. If in doubt whether something is sterile, assume that it is not. For example, tips that have touched anything, even a supposedly sterile part of the outside of a bottle, or have just been on the pipette for too long while you carry it around, should be discarded. Here are a few pointers for maintaining good sterile technique:

- o decontaminate your lab bench
- o safely organize your workspace
- o use sterile inoculating tools
- o aseptically transfer organisms from broth/plate cultures

General safety considerations

- o Access to the lab is limited.
- o Wear your lab coat.
- o Leave all food and drink in your backpack. Do not chew gum in lab.
- o The only thing on your lab bench should be the equipment you are working with and your lab book. Place your backpacks on the floor where you or someone else will not trip over them.
- o Discard contaminated material in the appropriate container. Anything that has been in contact with microorganisms must be disinfected with a disinfectant such as bleach or autoclaved.
- o Clean up all spills immediately!
- o Wash your hands before leaving the lab.

Media and Solution Recipes for Bacterial and Phage Experiments

Growth Media

LB broth [complex medium, contains all amino acids; generation time ~30' at 37°C]

Bacto-tryptone 10 g Yeast Extract 5 g NaCl 10 g H_2O 1000 ml

LB plates [used for colony isolations and plaque assays]

LB broth solidified with 1.0-1.5% Bacto-agar; ~30 ml. per 100 mm diameter Petri dish

MOPS LoPi [used to test phosphate response]

0.1 vol 10X sterile MOPS Modified buffer
Glucose to 0.4% from a 20% stock solution
K_2HPO_4 to 0.1 mM from a 132 mM stock solution

MOPS HiPi [used to test phosphate response]

0.1 vol 10X sterile MOPS Modified buffer
Glucose to 0.4 from a 20% stock solution
K_2HPO_4 to 2 mM from a 132 mM stock solution

Antibiotics	Working Concentration	Stock Concentration
Ampicillin	100 µg/ml	50 mg/ml
Kanamycin	50 µg /ml	20 mg/ml
Chloramphenicol	40 µg /ml	34 mg/ml
Streptomycin	100 µg /ml	100 mg/ml
Tetracycline	15 µg /ml	15 mg/ml

X-Phos can be added to a final concentration of 40 µg /ml from a 20 mg/ml stock solution made up in dimethylformamide

* recommended nutrient concentrations for minimal plates:

required amino acids ~1 mM
required vitamins 0.1-10 µg/ml
required nucleotides, etc. 10-100 µg/ml

Dilution Buffer

TN [all-purpose buffer for serial dilutions, etc.]

10 mM Tris HCl (pH 7.4) 150 mM NaCl

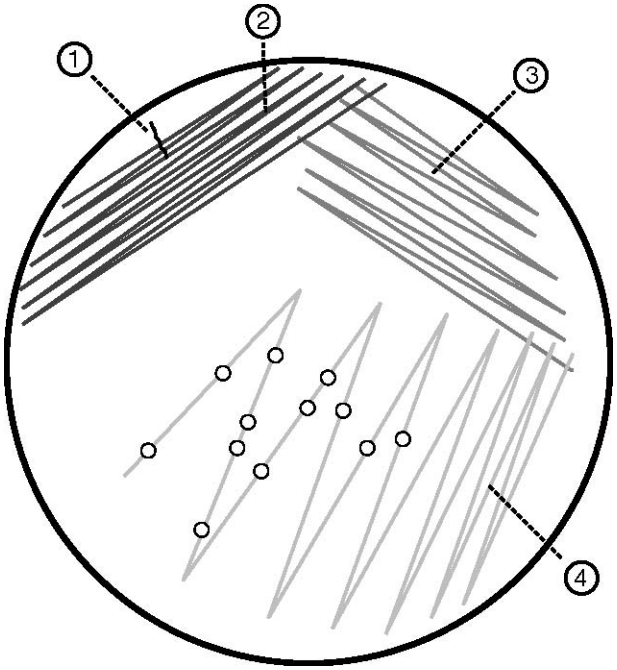
Streaking Plates for Clonal Isolates

Bacterial colonies and phage plaques represent clones of genetically identical organisms that are descended by vegetative reproduction from a single individual. Thus, bacterial and phage strains can be genetically "purified" of contaminants, mutants, revertants, etc. by inoculating cultures from single colonies or plaques produced in the manner shown below:

- Add sample to plate [1]. This is usually done by inserting a sterile toothpick into a colony, plaque, liquid culture of phage or bacteria, or a spot of phage or bacteria on a plate. The inoculum is placed in a short, straight line on the plate, near the outer edge.

- Sterile wooden applicator sticks are used to streak the sample across the surface of the plate. A new stick is used for each of the three passes indicated in the figure [2, 3, 4].

- For single plaque isolations, streak a phage sample in the same manner, then overlay the plate with agar containing indicator cells.



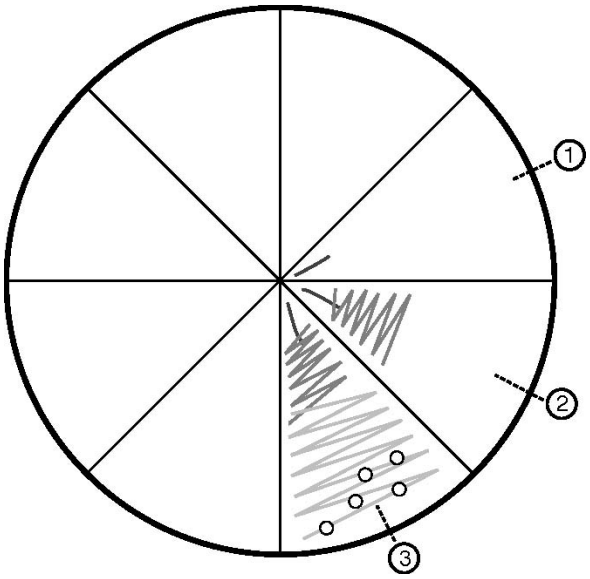
Several different samples can be streaked on the same plate:

- Divide and mark the plate into sectors (up to about eight)

- Inoculate each sample near the center of the plate [1].

- Streak half way with a sterile stick [2].

- Finish streaking with a new stick [3].



Overnight Cultures

Overnights are liquid cultures of bacteria that have reached the stationary growth phase ($\sim 10^9$/ml) They can be used as starting stocks for inoculating other liquid cultures and as indicator cells for making phage plaques.

Procedure:

1 Streak for colonies a day in advance. *It is inadvisable to start new overnight cultures from old ones (to avoid propagating any mutant variants that may have arisen during growth or storage of the original overnight).*

2 Determine the amount and type of liquid medium needed for the overnight culture. One ml overnights are typically prepared in plating tubes; larger volumes in dilution tubes or screw top flasks. Dispense medium with a sterile pipette. Be particularly careful to avoid touching the tip of the pipette to anything other than the medium itself. Any contaminants introduced at this stage will become part of the overnight culture and could cause considerable grief later on.

3 Pick a fresh, isolated bacterial colony with a sterile wooden applicator stick.

4 Immerse the end of the stick in the culture medium and agitate to dislodge some cells. In principle, *one cell is sufficient!* It is not necessary to use all of the cells in a colony to start an overnight culture.

5 Incubate the culture with shaking overnight (12-24 hours) at the desired temperature.

6 Overnight cultures should be stored at 4°C and can be used for periods up to two weeks.

Storing Bacterial Cultures

Overnight cultures

Good for a few days if left at room temperature; good for up to two weeks if stored at 4°C.

Colonies or patches on plates

Good for 1-2 weeks if left at room temperature; good for up to one month if stored at 4°C.

Stabs

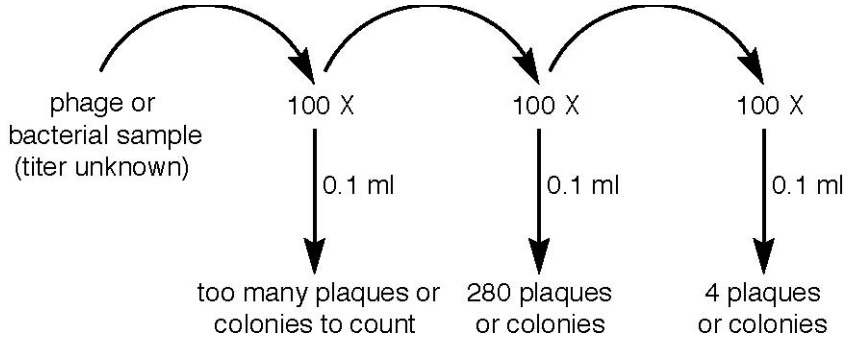
Good for many months when stored at either room temperature or 4°C. Stabs must be airtight to prevent desiccation.

Frozen

Good for years when frozen at -20°C or (preferably) -80°C, using glycerol (final concentration 10%) or (preferably) dimethyl sulfoxide (DMSO; final concentration 8-10%) as a cryoprotectant.

Serial Dilutions and Pipetting

Serial dilutions are used to reduce the astronomically large population sizes of microbial cultures to countable numbers. For example, to determine the titer of a bacterial or phage stock of unknown concentration, a series of dilutions are made and each of the diluted samples is assayed for colonies or plaques. After counting the number of colonies or plaques the next day, the concentration of organisms in the original culture is calculated by multiplying the number of organisms in the diluted sample by the dilution factor. This gives the *viable count* of organisms in the culture; dead bodies are not detectable as colonies or plaques. An example is shown below:



☞ Calculation of titer: = *280* (most accurate count) *X 100 X 100* (sample dilution) *X 10* (only 0.1 ml was plated) = *2.8 X* 10^7*/ml*

Typical dilutions:

Dilution factor Sample volume Buffer volume
10 X 0.5 ml 4.5 ml
20 X 0.1 ml 1.9 ml
50 X 0.1 ml 4.9 ml
100 X 0.1 ml 9.9 ml
200 X 0.05 ml 9.95 ml

Choosing a dilution series:

Titers are most accurately determined from counts of 100-300 colonies or plaques per plate. Thus, the dilution series should be designed to get this number of organisms on one of the assay plates. The amount of diluted sample to plate should usually be 0.1 ml (or occasionally 0.05). This volume is accurately measured in a 0.1 ml pipette and facilitates subsequent calculations. *Remember that titers are expressed as number of organisms per milliliter, so the volume of sample plated must be taken into account in determining the overall dilution factor.*

Sometimes the approximate titer of a culture can be guessed. For example, overnight bacterial cultures typically have titers of ~10^9/ml; a turbid bacterial stock will most likely have 10^7-10^{10} cells/ml; undiluted phage lysates typically have titers of ~10^{10}/ml When a sample is not visibly turbid, or when its history is not known, an extensive dilution series may be required to obtain samples with countable numbers of organisms. This can involve considerable labor, but when extreme accuracy is not required, dilutions can be sampled by spotting to save time and materials (see method #5).

Proper pipetting technique:

1 Store sterile pipettes in cans in a *horizontal* position. The lid of the can be left open for long periods (if the room is not drafty) because air-borne contaminants cannot drift sideways into the can to fall onto the pipette barrel or tip. (The end you put in your mouth sticks out of the can and is not sterile, but it's not the important part.)

2 *Do not flame pipettes before use!* This doesn't make them any more sterile, and probably contributes to contamination problems as it involves waving the pipette around in the air more. Just remove the pipette and use it, with as little wasted motion as possible.

3 *The end of the pipette should be sealed with your index finger — never your thumb or any other appendage!* Thumb pipetting is both awkward and inaccurate.

4 To withdraw a sample from a tube or flask, insert the pipette into the solution only as far as necessary. Do not immerse it all the way to the bottom of the container because this just deposits more fluid on the *outside* of the pipette. Fill the pipette in excess of the amount desired and withdraw it from the surface of the liquid, but do not remove it entirely from the sample container. Gradually reduce the pressure of your index finger, allowing the contents to slowly leak from the pipette until the correct liquid level is reached. Finally, remove as much as possible of the unwanted material on the outside of the pipette by rubbing the tip around the inside top edge of the sample container.

5 To introduce a sample into a tube or flask, insert the loaded pipette *all the way* into the container and blow vigorously enough to form bubbles as you withdraw the pipette, stopping only when the tip is above the surface of the liquid. Blowing ensures adequate mixing of the sample; no shaking, thumping, vortexing, etc. is needed. As long as you don't spit into the tube, you need not fear introducing contaminants; your breath is effectively sterile (except, perhaps, for cold and flu viruses, etc.)

6 Place the used pipette tip-up *(gently!)* in a pipette bucket for washing and re sterilization.

7 Use a clean pipette for each new operation; <u>never, ever reuse a pipette!</u>

INSTRUCTIONS FOR PIPETMAN USE (from Pipetman® manual) note: 1ml = 1000µl
SCALE MODEL VOLUME RANGE INCREMENTS

P10 0.5-10µl 0.02µl
P-20 2-20 µl 0.02 µl
P-200 50-200 µl 0.2 µl
P-1000 100-1000 µl 2.0 µl

Operation of pipetpman:

1) Set volume by turning the volume adjustment knob until correct volume shows on the digital indicator.
2) Always use disposable tips --Yellow tip for P-20 & P-200 Blue tip for P-1000
3) To withdraw SAMPLE, depress the plunger to the FIRST POSITIVE STOP. Hold the pipetman vertically, immerse the disposable tip into the sample liquid and allow the pushbutton to return SLOWLY. Never permit it to snap up.
4) Wait 1 to 2 seconds to insure that full volume of sample is drawn into tip before withdrawing tip from sample liquid. (This is particularly important for viscous fluids.)
5) To DISPENSE SAMPLE, depress plunger SLOWLY to the FIRST STOP. Then depress plunger to SECOND STOP to expel any residual liquid in tip.
6) Withdraw the pipetman from receiving container with plunger fully depressed. Allow plunger to return to TOP POSITION slowly.
7) DISCARD TIP by depressing tip ejector button.

Care/Caution:

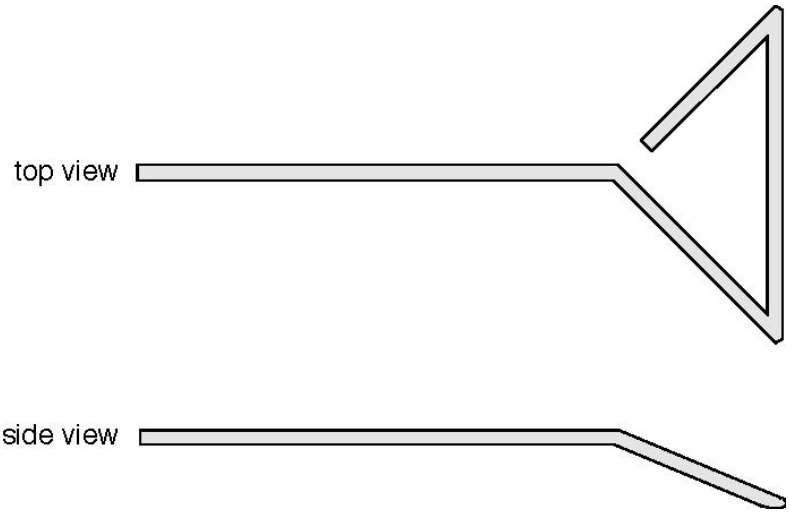
1) Pipet very SLOWLY to prevent liquid from being sucked into plastic shaft.
2) Never hold a pipetman at an angle greater than 20° from vertical axis when pipetting.
3) Never invert or lay a pipetman on its side with liquid in the tip.
4) Never eject tip in the direction of another person.

Spreading Samples on Plates

There are occasions when it is convenient or desirable to *spread* bacteria or phage on a plate (rather than streaking, spotting, or pouring). When bacteria are pour plated, many of the resulting colonies are embedded in the soft agar and grow slowly until they break through to the surface where there is abundant oxygen. In contrast, spreading distributes the cells only on the surface of the plate, leading to faster and more uniform colony growth. Spread plates are commonly used in conjunction with replica printing methods, which cannot be done with cells in agar overlays. Phage can also be spread on plates; for example, to ensure a uniform selective surface for spot testing bacterial samples for phage resistant mutants. Nutrients, such as essential amino acids or vitamins, and antibiotics can also be added to plates by spreading.

☞ Liquid samples are spread on plates with a glass spreader similar to the one pictured below. Spreaders are usually made from soft glass rod, but Pasteur pipettes will also do in a pinch.

Procedure:



1 Sample volumes are typically 0.05-0.2 ml. If less liquid is used, the sample soaks into the plate before it is uniformly spread; more material, and the sample takes an inconveniently long time to soak into the plate.

2 After adding the sample to the center of the plate, the spreader is sterilized by dipping it into 70% ethanol and burning off the alcohol by igniting it with the flame of a Bunsen burner. *Do not allow the spreader to linger in the flame—it may get so hot that it melts, or melts the plate, or vaporizes the agar, or kills part of the sample.*

3 To spread the sample, work the solution outward from the center, rotating the plate slowly to ensure even distribution. *It is important to continue spreading until the liquid has soaked into the plate.*

Pour Plating and Agar Overlays

Pour plating is a technique used to embed cells or cells plus phage in a thin layer of agar on the surface of a plate. It is the most convenient way to spread samples uniformly, but requires a bit of practice. In particular, one must work quickly because the overlay agar solidifies rapidly when it contacts the plate. (If the overlay is lumpy, colony and plaque morphology are grossly altered, essentially rendering the plate unusable.)

Procedure:

1. Determine the type and amount of top agar needed and melt it in an autoclave or microwave. *It is important that the agar be completely melted! Check by swirling it vigorously until unmelted clumps and Schlieren lines can no longer be seen.* Store melted agar in a water bath (45-55°C).
2. Add samples to plating tubes (13 X 100 mm). In general, sample volume should not exceed 0.5 ml to avoid excessive dilution of the overlay agar. For phage assays, the indicator cells (0.1-0.2 ml) should be added to the plating tube first, and the phage should be allowed to adsorb to the cells for 10-20 minutes at room temperature before plating.
3. Prepare the plates. Inspect them for contaminants, etc., and label them. *Plates should always be labeled on the bottom, not the lids!*

4. Remove melted agar from water bath just prior to use. Pipette about 2.5 ml of agar into each sample tube. It is permissible to dispense agar to several tubes at once with the same pipette, but be extremely careful not to touch the insides of the tubes with the tip of the pipette as this could transmit cells or phage from one sample to another. With practice, four samples can be plated at a time, using a 10 ml pipette to dispense the top agar. *Novices should do plates one at a time until proficient.*

5. Pour the contents of the plating tube onto the plate, swirling gently to get an even distribution. Touch the mouth of the tube to the plate to dispense the last drop of sample. If any bubbles have formed, use the tube to push them to the edge of the plate. *It is not necessary to shake, stroke, vortex, or otherwise attempt to mix the contents of the tube! Everything will be uniformly distributed on the plate with no special effort on your part.*

6. Wait several minutes to allow the overlay agar to harden thoroughly before attempting to move the plates or to put them into an incubator. *The plates should be incubated in inverted position* to minimize drying and to prevent condensation from dropping onto their surface.

Picking and Patching Cells and Phage
Bacterial colonies and phage plaques can be picked and transferred to other plates with sterile, round, wooden toothpicks. Because colonies and plaques contain millions of genetically identical organisms, it is not necessary to transfer all of them, just a few will suffice. So, don't use the toothpick like a spoon to scoop or scrap up every possible organism. A quick jab will serve to pick up plenty of organisms; a second jab in the fresh plate will transfer enough of them to start a new colony or plaque. It isn't necessary to jab all the way to the bottom of the agar; a shallow poke is adequate. Many organisms will remain on the toothpick, so it can be used to inoculate a series of plates, if desired.

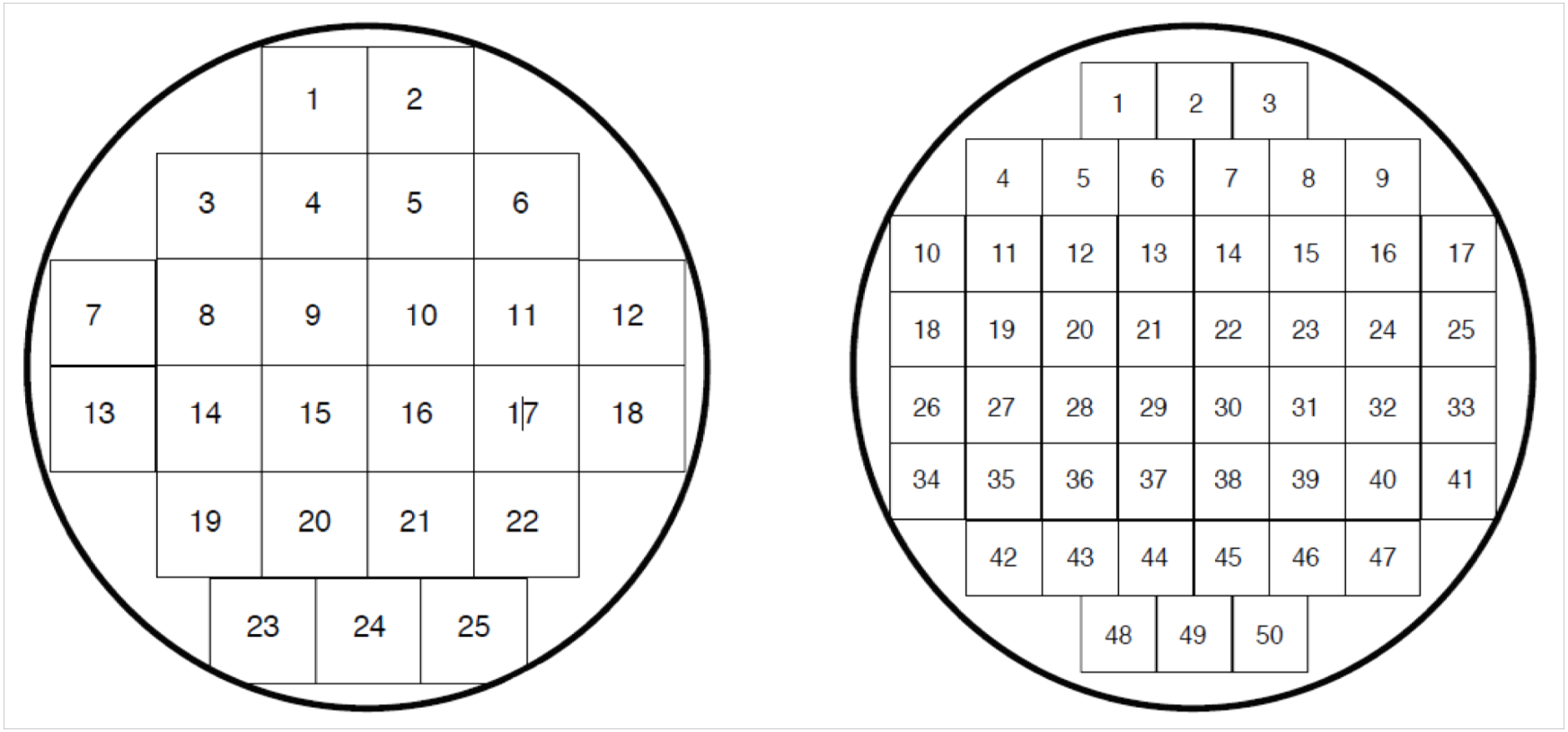
Obviously, each toothpick should only be used to pick up one colony or phage. *Never re-use a toothpick until it has been re-sterilized!*

Grid patterns like those below should be used to keep track of the pickates. The plates are placed over the grid patterns so that each pickate can be deposited at the same position on each plate in the series.

Hint: Mark the 12 o'clock position on the bottom (not the lid!) of each plate in order to align them for subsequent comparisons.

Hint: When testing for growth under various conditions, the *last* plate in the series should be one on which all of the pickates will grow (e.g., permissive temperature or host, complete medium, etc.). This accomplishes two purposes: (i) It serves as a control to validate the instances in which growth does not occur. (If growth occurs on the last plate in the series, then all previous plates must have received the organisms as well.) (ii) It also serves as a "master" plate for subsequently recovering the bacteria or phage that prove to have the desired properties.

Hint: When making master plates of bacterial colonies, it's often convenient to "patch" them in a grid pattern for storage or subsequent tests. The patches are made by scratching the surface of the agar with the transfer toothpicks, usually in a single, slanted line, or two intersecting lines that form an "X".


1 2
3 4 5 6
7 8 9 10 11 12
13 14 15 16 17 18
19 20 21 22
23 24 25
1 2 3
4 5 6 7 8 9
10 11 12 13 14 15 16 17
18 19 20 21 22 23 24 25
26 27 28 29 30 31 32 33
34 35 36 37 38 39 40 41
42 43 44 45 46 47
48 49 50

Introduction of Plasmids into Recipient Strains by Transformation

Materials:

0.1 M CaCl2
0.1 M CaCl2 / 15% glycerol Eppendorf tubes microcentrifuge 37°C and 42°C water baths

Preparation of competent cells:

1. Grow an *E. coli* strain in LB at 37°C with aeration until the OD_{600} is between ~ 0.4 – 0.5 (A common volume for this procedure is 25 ml, which will provide the researcher with ten 100 µl transformations or five 200 µl transformations).
    a. This is most frequently done by diluting a fresh overnight culture 1:100 into fresh LB medium and incubating it ~1.5 – 2 hr.
2. Pellet the cells in a centrifuge at 4°C by spinning for 10 minutes at 2000 X g. (*You can divide cells into two sterile 15 ml screw capped tubes for this*)
3. Discard the supernatant and then resuspend the pellet in 1/5 vol. of ice cold 0.1 M $CaCl_2$. (*So for a 25 ml culture, resuspend cells in 5.0 ml $CaCl_2$*)
4. Let the resuspended cells sit on ice for 30 minutes
5. Pellet the cells again as above.
6. Carefully resuspend the cells in 1/25 of the original volume of ice cold 0.1 M $CaCl_2$.
    1. Alternatively, resuspend the cells in ice cold 0.1 M $CaCl_2$/15% glycerol and store at -70°C for future use.

***Note**: Competent cells are very fragile. Store them on ice as much as possible!*

Transformation procedure:

Use the competent cells prepared above within several hours or thaw tubes of competent cells on ice.

1. For each transformation, place 100 – 200 µl of competent cells into a cold, sterile microcentrifuge tube. Keep cells on ice.
   Note: *When setting of a transformation, it is often useful to include a "No DNA" control tube to verify that the selective plates are working.*

2. Add 1-4 µl of your DNA {1 µl for purified plasmid, 1 -4 µl for a ligation reaction (0.05 to 0.5 µg of DNA) to each tube, mix gently and place on ice.
3. After 30 minutes on ice, transfer the tubes to a 42°C water bath for 60 seconds and then return them to ice following the heat shock.
4. After another 2 minutes on ice, add 1 ml of LB broth to each tube and then incubate the tubes at 37°C for 45 – 60 minutes to allow the cells to recover and to begin expressing resistance functions (*e.g.*,ß-lactamase).
5. Spread the cell samples on LB plates containing the appropriate selective antibiotic. ***Note**: It is often useful to spread several plates with different volumes of cells to make sure that individual colonies are obtained (i.e., spread one plate with 50µl, one with 200 µl, and one where the remainder of the cells were pelleted for 1 min in a microcentrifuge, resuspended with 100 µl LB and plated). Use 3 plates for the two ligations and one plate each for the no DNA (200 µl) and supercoiled controls (50 µl).*
6. Incubate the plates overnight at 37°C.

Analysis of DNA Samples on Agarose Gels

Materials:

10X restriction buffer Eppendorf tubes microcentrifuge TAE buffer 0.7% agarose in TAE dye loading buffer ethidium bromide horizontal gel boxes and combs ultraviolet light box

Restriction digests:

Transfer 0.5-1 µg of plasmid DNA into an Eppendorf tube. Add 1 µl restriction buffer (10X buffer). Add about 10 units of *restriction enzyme* to the tube. Mix the solutions, spin the tube briefly and incubate for at least one hour at 37°C. After the restriction digestion is complete add 2 µl of glycerol loading buffer, mix, and spin the tubes briefly. Samples can be stored at -20°C.

Agarose gel electrophoresis:

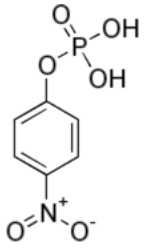
Take the 0.7% agarose in TAE and melt it in the microwave until the solution is clear. Place the agarose in a 50° water bath and until it has cooled, add 3 µl of ethidium bromide and then pour into the gel tray (don't forget the comb) and allow it to harden. Place the gel in the gel box and add 1X TAE buffer until the gel is just covered. Load 10 µl of DNA markers (1kb ladder) into the first lane and then load 10 µl of your restriction digests (mixed with 2 µl of loading buffer) into the other lanes. Apply ~75 volts to the gel (DNA moves toward the positive pole) and let the gel run until the first dye front nears the bottom of the gel. Examine the gel under ultraviolet light (make sure you wear a face shield when examining the gel).

Warning: Ethidium bromide is a carcinogen and a mutagen and will intercalate into your DNA! It should be handled with care. Wear gloves at all times when you work with it. Make sure you clean up any spills and drips.
for posterity.

AP assay

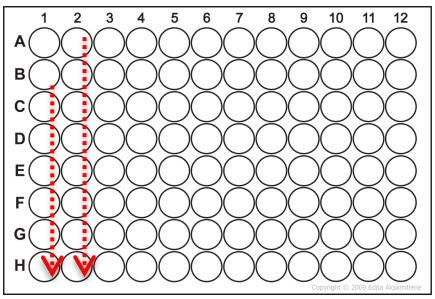
McCleary Lab 96-well plate AP assays

This assay measures the periplasmic enzyme, alkaline phosphatase (AP). AP is most active at high pH and hydrolyzes organophosphates to produce free phosphate. Cells are treated with a dilute detergent and chloroform to release AP from the periplasm. The substrate for these reactions is p-nitrophenol phosphate (PNPP), which when hydrolyzed forms the yellow compound p-nitrophenol that can be detected with a spectrophotometer at 420 nm. The AP levels in a culture are proportional to the amount of p-nitrophenol produced in these assays. The rate of p-nitrophenol production is normalized for the amount of cells in the reaction.



<u>Cultures grown in MOPS media</u>

1. materials
    a. Assays will require one 96-well plates, and lock-top microcentrifuge tubes for each replicate and blank
    b. Freshly prepare enough 20 mM PNPP in 1 M Tris HCl, pH 8.2 for all assays (40 µl for each well plus ~500 µl extra; 4.3 ml for an entire plate)
2. For each strain to be tested, pick three separate colonies from a freshly streaked MOPS plate and grow 2.5 – 3 ml cultures overnight on the RollerDrum in MOPS HiPi or MOPS LoPi + antibiotics, +/- inducer (IPTG).
3. Remove 600 µl of a freshly vortexed culture into a lock-top microcentrifuge tube
4. Spin the cells for 2 min at maximum speed
5. Carefully remove the supernatant liquid using a pipet tip attached to the vacuum pump and then resuspend the cells with 600 µl of 1 M Tris-HCl pH 8.2 by vortexing for 15-20 sec.
6. Remove 100 µl of the cell suspension into the wells of a 96-well plate containing 100 µl of 1 M Tris-HCl. This will be a 1:2 dilution. Add the 100 µl of Tris with the multichannel pipettor. Remember that cells A1 and B1 are blanks and will contain 200 µl of the 1 M Tris solution. The wells should be filled from 1C to 1D to 1E and so on.



7. Determine the OD_{594} for these diluted samples by running the Cell Density program on the plate reader. Export the data to a USB drive.
8. Add 10 µl of 0.1% SDS and 1 drop of chloroform to each microcentrifuge tube and vortex for 5 sec, rest 20 sec, vortex again for 5 sec. Let tubes sit for 1 – 5 min to allow the $CHCl_3$ to settle to the bottom of the tubes.
9. While tubes are resting and you are preparing the 96-well plate, Run the pre-warm program on the plate reader to get the Plate reader/Incubater to 37°C.
10. Starting in Column 7, add 50 µl of each cell suspension to 150 µl of 1 M Tris HCl, pH 8.2, which has been placed into each well using a multichannel pipettor. Remember that cells A7 and B7 are blanks and to include the blank (500 µl Tris, 10 µl SDS, 1 drop $CHCl_3$) in your preparations. The total volume in each well should be 200 µl. Place the plate in the 37°C incubator for 10 min.

11. Add 40 µl of 20 mM PNPP to each well with the multichannel pipettor and start the AlkPhos1 program on the plate reader. If you expect high AP values, the wells can start turning yellow VERY quickly – so be prepared. The program will measure the A_{420} at one min intervals for 20 min with a 5 sec shake before each measurement. It will then determine the maximum kinetic rate (ΔA_{420}/min) using 6 time points and report that value from each well.
12. Export the data onto a USB drive and import it into Excel.
13. Calculate the AP units (1000*(rate)/(corrected cell density) for each well. Calculate the averages and standard deviations for each strain.

Tube Assay for AP

1. Prepare enough 20 mM PNPP in 1M Tris-HCl (pH 8.2) for all of your assays (100 µl / sample and blanks)
2. spin down 1 ml of cells for two min in a labeled lockable microcentrifuge tube. Save the remainder of the cells on ice for A_{600} determination.
3. carefully aspirate the supernatant being careful not to disturb the cell pellet
4. add 900 µl of 1 M Tris-HCl pH 8.2 to the cell pellet
5. add 2 drops chloroform and 1 drop 0.1% SDS to each tube. Lock each tube and vortex vigorously for 10 sec.
6. Prepare a blank tube w/ 900 µl of Tris, the SDS and the $CHCl_3$.
7. add 100 µl of 20 mM PNPP and mark the time.
8. incubate at 37°C until a yellow color is clearly visible and then add 400 µl of 1M K H PO_4 to stop the reaction. Note the time, mix well and keep the tube on ice until all samples are completed.
9. spin the tubes in a microcentrifuge for ~2 min.
10. Determine the Absorbance of each sample in a spectrophotometer at 420 nm using the Blank to zero the instrument.
11. Determine the Absorbance of the cells at 600 nm. Use an appropriate Blank to zero the instrument (water for MOPS media, LB for LB).
    If you are using an overnight culture grown in LB, then dilute your sample 1:5 in water (your blank should be diluted LB)
12. Calculate the arbitrary AP units with the formula

$$\text{AP Units} = \frac{1000 \times A_{420}}{\text{time(min)} \times A_{600}}$$

Sample	Time (min)	A_{420}	A_{600} (corrected for dilution)	Units

Plasmid Preparation.

Background: Plasmids are extra-chromosomal DNA. They often confer selective advantages upon an organism under certain environmental conditions.

In this procedure the researcher resuspends bacterial cells in a buffer that contains RNAase A. This enzyme eliminates RNA from the plasmid preparations. The cells are lysed with a detergent (SDS: sodium dodecyl sulfate) under alkaline conditions (Buffer P2). The chromosome and many cellular proteins will then be precipitated by the addition of potassium acetate (Buffer N3), leaving the smaller plasmid DNA in solution. The DNA in this solution will then be adsorbed onto a special matrix in a spin column and purified by washing the matrix with buffers that retain the DNA but let contaminating proteins pass through.

PROTOCOL

1. Grow bacterial strains overnight in 5 ml LB + antibiotic with shaking at the appropriate temperature (mostly 37°C). Label your microcentrifuge tube containing your cells with your initials.
2. Collect cells by centrifugation (10 min @ 1750 rpm)
3. Resuspend pelleted bacterial cells with 250 µl Buffer P1.
4. Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4-6 times. (The solution should turn blue)
5. Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times (the solution should turn colorless)
6. Centrifuge for 10 min at 13,000 rpm in a table-top microcentrifuge.
7. Apply the supernatant (from step 4) to the QIAprep spin column by pipetting.
8. Centrifuge for 30-60 s. Discard the flow-through into a waste beaker using a pipetman.
9. Wash the QIAprep spin column by adding 0.5 ml Buffer BP and centrifuging for 30-60 s. Discard the flow-through.
10. Wash the QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30-60 sec.
11. Discard the flow-through, and then centrifuge for an additional 1 min to remove residual wash buffer.
12. To elute DNA (get it off the column), place the QIAprep column in a clean 1.5 ml microcentrifuge tube. Add 50 µl Buffer EB to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.
13. Discard the column
14. Transfer the purified DNA to another clean, labeled microcentrifuge tube.

Save at -20°C

P1 Transduction

1. Spin down a fresh overnight culture of the strain to be transduced (1 ml in a microcentrifuge tube, spun at 12,000 X g for 2 min will be great). Remove the supernatant with a pipet and resuspend the cells in the same volume of MC buffer (0.1 M $MgSO_4$, 0.005 M $CaCl_2$).
2. Add 0.1 ml of the cells to each of three labeled microcentrifuge tubes. To the first tube, add 0.1 ml of the P1 lysate; to the second tube, add 0.01 ml (10 µl) of the lysate. Do not add lysate to the third tube, which will serve as a control.
3. To a fourth tube, add 0.1` ml of the lysate with no bacteria present. This serves as a control to ensure that the lysate is not contaminated with bacteria.

Tube	Bacteria	P1 lysate
1	0.1 ml	0.1 ml
2	0.1 ml	0.01 ml
3	0.1 ml	0
4	-	0.1 ml

4. Incubate in a 37°C water bath for 20 minutes.
5. Add 0.2 ml of 1 M sodium citrate to each tube. The citrate chelates the Ca^{2+} ions that are needed for further P1 infections.
6. Add 1 ml of LB + citrate medium (LB containing 10 mM Citrate) and incubate tubes with shaking at 37°C for 90 min while cells start to express their antibiotic resistance genes.
7. Spin down the cells in the microcentrifuge (12,000 X g for 2 min). This step will remove much of the remaining P1 phage. Remove the supernatant.
8. Resuspend the cell pellet with 0.2 ml of LB+citrate (10 mM Citrate) and plate on LB plates containing the correct antibiotic (usually kanamycin) by spreading with a sterile glass rod.
9. Incubate the plates at 37°C overnight.

Colony PCR

Typical colony PCR reaction

For Colony PCR, our lab typically uses the Apex Taq RED DNA Polymerase Master Mix from Genesee Scientific. This is a ready to use 2.0X reaction mix. Simply add primers, template, and water to carry out PCR.

- Work on ice at all times!
- Thaw Taq @x Master Mix RED and primers on ice. It is important to thaw the solutions completely and mix thoroughly before use to avoid localized concentrations of salts.
- Template DNA preparation

  When picking colonies, use a sterile pipet tip. Angle the tip down towards a colony and just barely touch the colony. DO NOT scoop up LB agar since this inhibits the PCR reaction. After touching the colony, place the tip in 20 - 50 uL of Sterile PCR-grade water (in a clean PCR tube) and pipet up and down several times to resuspend the cells.
  - Run the DNA prep program on the Thermocycler. After the program is complete, place the PCR tube on ice until use. This program heats the cells to 98°C for 10 min and then cools the extract to 4°C. This procedure lyses the bacteria, releases the DNA and inactivates nucleases that could degrade the DNA.
- Prepare the reaction mix. We typically use a 20 µl reaction volume.

Component	Vol./reaction	Final concentration
Taq 2x Master Mix	10 µl	1X
25 mM MgCl2	0 µl	1.5 mM (variable, as needed)
Primer A*	0.5 µl	1 µM
Primer B*	0.5 µl	1 µM
PCR-grade H_2O	7 µl	
Template DNA	2 µl	
Total volume	20 µl	

*Sometimes the primers are supplied as a mixture of both forward and reverse primers. In that case use 1 µl of the mix.

PCR conditions

What happens?

1 cycle	1 min at 95°C	DNA denaturation
30 cycles	1 min at 95°C	DNA denatures into single strands
	1 min at 54°C	primers anneal to ssDNA template (temp depends on primers)
	1 min at 72°C	primers are extended from the 3'-end by Taq (1 min/kb)
1 cycle	5 min at 72°C	final extension to make sure all products are full length (72°C is optimal for Taq polymerase)

Preparation of P1 Liquid Lysate

1. Inoculate a 5ml overnight culture of donor strain in LB.
    - If you'd like to make a lysate and do a transduction on the same day, inoculate the recipient strain along with the donor strain.
2. Dilute saturated culture 1:100 into 5ml LB + 50ul of 20% glucose (final concentration = 0.2%) and 25 ul of 1M $CaCl_2$ (final concentration = 5mM).
3. Incubate 30 to 45 minutes at 37°C with shaking. Before proceeding to the next step, visually inspect the culture for cells. The culture should be slightly cloudy and you should see swirls when it is held up to the light.
4. Add 100 µl of P1 phage stock.
    - Some notes on phage titer: A good titer for a phage stock is 10^9 to 10^{10} pfu/ml. If the transduction fails, consider determining the titer of your phage stock.
5. Continue to incubate the culture, shaking at 37°C, until the culture lyses. This typically takes ~3 hours.
    - The culture should be clear and may contain clumps of floating debris. An unlysed culture will look smooth and silky.
6. Add 200µl of $CHCl_3$ and continue shaking for 5 minutes.
    - The $CHCl_3$ ensures complete cell lyses and kills the bacterial donor strain. $CHCl_3$ will not harm the phage, however it should be removed before storing at 4°C because it can lead to decreased viability of the stock.
7. Transfer the supernatant to a 15ml conical tube and centrifuge for 5 mins at ~9200 x g, 4°C.
8. Transfer ~4-5mL of the supernatant (your P1 lysate) into new 15mL conicals.
    - Take care to not disturb the $CHCl_3$ or any debris which may have pelleted.
    - Phase lysates can be stored at 4°C for several years.
    - Additionally, the lysate can be further purified using a 0.45µm filter to remove any residual bacterial cells. This can also be done at any time later. In particular consider doing this if plating 100µl of lysate not exposed to cells yields colonies.

Perform P1 Transduction

1. Inoculate a 5ml overnight culture of the recipient strain in LB.
    - Again, this step can be started at the same time donor cultures are started if you wish to do transduction on same day lysate generated.
2. Pellet 1.5ml of saturated culture, 2 min at maximum speed.
3. Resuspend the cells in 0.75ml in sterile P1 salts solution [P1 salt (10 mM CaCl, 5 mM MgSO4)]
4. Mix 100ul of cells + P1 salts mixture with 1-10ul of P1 lysate.
    - As a negative control, include a tube containing 100ul of cells without lysate.
    - If using the lysate for the first time or troubleshooting a transduction, try varying the amount of lysate that is added to the cells - ex: up to 200µl.
        - To date, 1-5µl has proven sufficient for P1 Transductions using the deconvoluter donor strains.
5. Allow the phage to adsorb to the cells for 30 min at 37°C.
6. Add 1ml of LB + 200ul of 1M sodium citrate.

    - The sodium citrate will minimize secondary infection by chelating the calcium, which is necessary for phage to adsorb to bacteria.
7. Incubate for 1 hour at 37°C with shaking.
8. Harvest the cells by centrifuging for 2 mins at maximum speed. Remove and discard the supernatant.
9. Resuspend the cells in 100ul of LB and spread on selective transduction plates containing 5mM sodium citrate.
    - Spread 100ul of P1 phage on another selective plate as a negative control if the lysate has not been tested previously, or there is concern it has become contaminated with bacteria.
    - It is good practice to supplement selective plates with 5mM sodium citrate to prevent reinfection of any residual phage into the recipient strain. Additionally, you may have to adjust the antibiotic concentration.
10. Incubate plates overnight.
11. Pick colonies from plates and re-streak on selective plates, incubate overnight.
    - Cell-only plates should have no colonies.
    - If using multiple concentrations of P1, pick colonies from the plate with the least amount of phage.
    - Usually plates with the lowest concentration of phage will have the fewest colonies.
    - If colonies are present on the phage-only plates, either the selection did not work or the phage lysate is contaminated with bacteria.
12. Confirm the genotype by PCR. It is also a good idea to sequence upstream/downstream of the insertion site, especially when the donor and the recipient are different *E.coli* strains.