
SECTION II

**BACTERIA
AND
CLONING**

BACTERIAL PROPAGATION

INTRODUCTION:

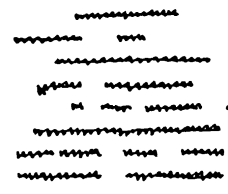
There is heavy utilization of microbiological methods in molecular biology, from molecular cloning of plasmid and phage DNA in *Escherichia coli* to transformation of plant cells with *Agrobacterium tumefaciens*. In the next several weeks we will be inserting foreign DNA (from plants, fungi, or whatever other source you want to use) into a plasmid (pBR325), that will then be introduced into and replicated by *E. coli* cells for later study.

The first step in this process is to select and grow the proper strain of bacteria. We will use *E. coli*, strain HB101, cells. These cells have been developed to be extremely disabled with respect to their ability to survive outside the lab. Next, you should choose the vector that is the best suited for the purpose at hand. For DNA pieces up to 15-20 kb in size, plasmids can be used (although pieces below 10 kb are much easier to clone than larger pieces). For fragments up to 25-30 kb, bacteriophage lambda can be used. For fragments up to 50 kb, cosmids can be used, although pieces of this size are often difficult to clone. For DNA sequencing, single stranded DNA bacteriophage M13 is generally used. In each of the above categories, there are many different commercially (and academically) available choices. The plasmids we will be using (based on pBR322, pBR325, and their derivatives) have genes that confer resistance to the drugs chloramphenicol, ampicillin and tetracycline to the host bacterium. [Chloramphenicol binds to the 50S ribosomal subunit and halts protein synthesis, ampicillin is a derivative of penicillin and inhibits cell wall formation, tetracycline binds to the 30S ribosomal subunit and stops protein synthesis.] There are some unique restriction enzyme sites in each of the resistance genes that make selection of recombinant molecules possible. For example, we will be cloning into the *EcoRI* site located in the gene that confers chloramphenicol resistance to the host. All of the bacteria that carry an uninterrupted gene are able to grow on media with chloramphenicol. However, the bacteria whose chloramphenicol resistance gene has been interrupted by the insertion of a foreign DNA fragment, will not be resistant to the drug. They will, however, be resistant to ampicillin and tetracycline. We will also use plasmids that carry the gene for β -galactosidase. The host bacteria have been engineered to lack this coding region, and only contain the α -galactosidase coding region. Since both parts are necessary to produce functional galactosidase enzyme, only bacteria that have been transformed with a functional β -galactosidase (on the plasmid) will exhibit galactosidase activity. Since the β -galactosidase complements the α -galactosidase, the process is known as " α -complementation." The activity of galactosidase can be made visible by the addition of a dye (5-bromo-4-chloro-3-indoyl- β -D-galactoside, also known as X-gal) that is colorless (to slightly yellow) until acted on by galactosidase, after which it forms a blue precipitate. Thus, a bacterial colony exhibiting galactosidase activity appears blue, and one that lacks functional galactosidase appears white (or creamy).

There are many different media for growing bacteria. We will use one of the most common media, known as LB (Luria-Bertani) medium. This first lab period will be spent preparing the liquid medium and agar plates for the growth of the bacteria. Each group should prepare three bottles each of liquid LB medium and LB medium

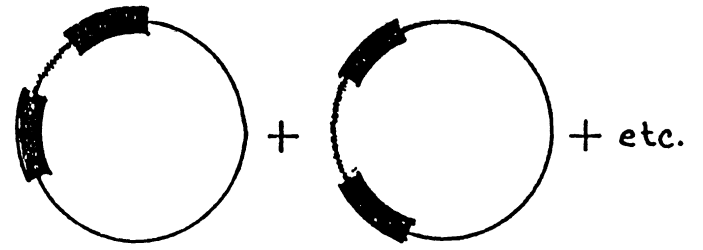


Isolate DNA

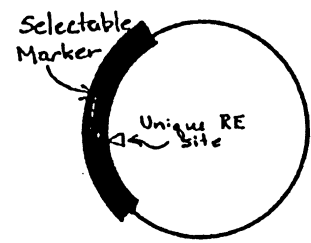


Digest with RE (EcoRI)

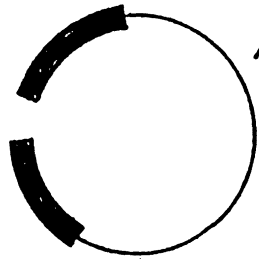
Mix and treat with DNA ligase



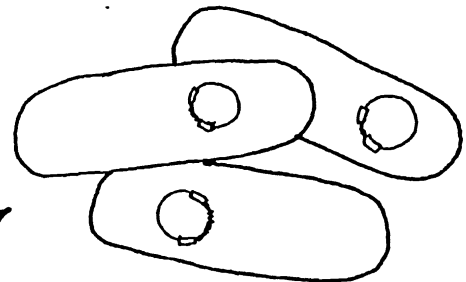
Mixture of recombinant molecules



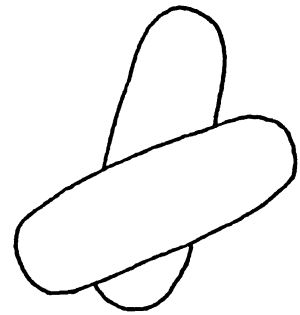
Isolate Vector-DNA (plasmid)



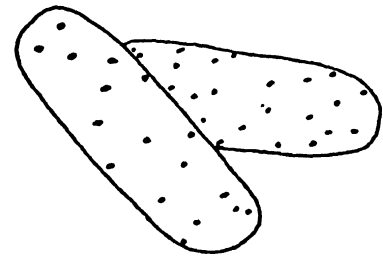
Digest with RE (EcoRI)



Transform bacteria



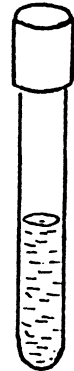
Grow bacteria



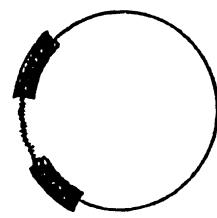
Treat with $CaCl_2$, etc. to make cells competent to take up DNA



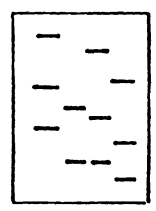
Select recombinant colonies



Grow single colonies in liquid culture



Isolate recombinant plasmid



Characterize Clones

with agar. After autoclaving, followed by about 5-15 minutes of cooling at room temperature, the drugs are added and the plates are poured. The addition of X-gal to the "X-gal/LBA" plates should be done an hour or so prior to spreading of the bacteria onto the plates. X-gal is not stable in solution at room temperature. The following are the amounts of liquid medium and the number of agar plates that you will need for the next few weeks:

Liquid medium,

- 150 ml LB
- 175 ml LBA (LB with ampicillin)
- 175 ml LBC (LB with chloramphenicol)

For plates (all with agar),

- 150 ml LB (for six plates)
- 350 ml LBA (for fourteen plates)
- 100 ml LBC (for four plates)

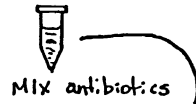
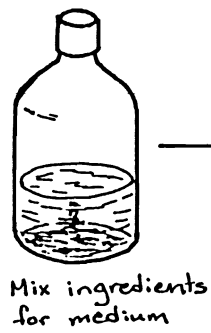
For recombinant DNA work you should be aware that there are restrictions on lab setting, containment, disposal and type of organisms that can be used. Here are some general guidelines for our work in this lab:

1. The doors and windows in the lab must be closed when using any recombinant molecules or bacteria containing the recombinant molecules.
2. All unused recombinant molecules must be securely stored (a freezer or refrigerator is fine).
3. Any recombinant molecules or bacteria must be autoclaved before disposal.
4. DNA from pathogens of plants or animals may not be used for these exercises unless specific permission is obtained.
5. Use of human DNA or human pathogen DNA is forbidden, unless you obtain special U.S. government authorization.

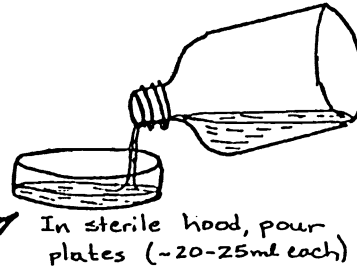
STEPS IN THE PROCEDURE:

PART I: MAKING MEDIA AND AGAR PLATES:

1. Measure out and dissolve the ingredients for the LB and LB with agar, as listed above. These should be in bottles that have caps. [NOTE: The agar will not go into solution until autoclaving.] Be sure that the bottles are less than half full or the liquid will boil out of them. Also, leave the caps loose on the bottles or they will explode in the autoclave.
2. Autoclave all solutions on a 20 minute liquid cycle.



AGAR
LIQUID

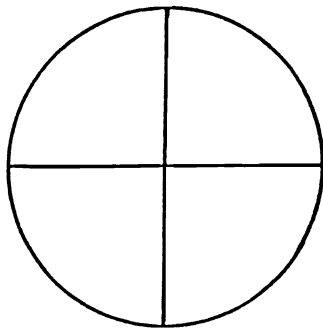
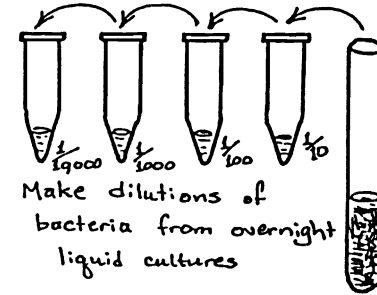


In sterile hood, pour plates (~20-25ml each)

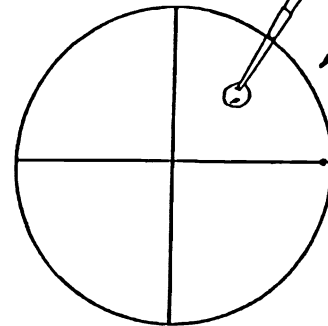
store at +4°C until needed



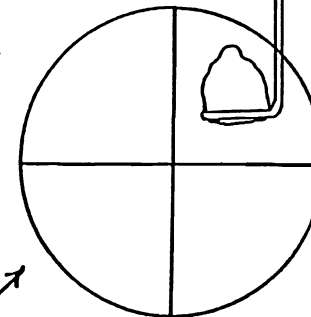
Stack and invert plates. Place at +4°C for storage



Draw lines on the outside of the plate to create quarters

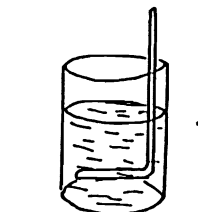


Place drop of bacteria onto appropriate quadrat of plate (This should be repeated for each plate type used)

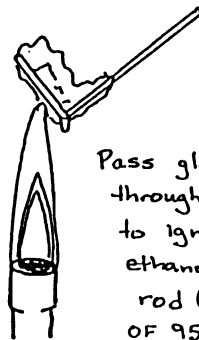


Spread drop of cells with sterilized (flamed) glass rod

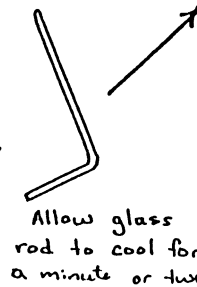
Incubate overnight at 37°C and record results



Place bent glass rod into 95% ETOH



Pass glass rod through flame to ignite the ethanol on the rod (KEEP CONTAINER OF 95% ETOH FAR FROM BURNER.)



Allow glass rod to cool for a minute or two

3. While the LB is autoclaving make the stock solutions of ampicillin and chloramphenicol. Make only enough to add to the liquid medium and to the agar plates. Keep these in the freezer until they are added to the medium.
4. Prepare the sterile hood (in room 432 Illick) for use. If they are not already on, turn on the fan and the fluorescent lamp (not the UV lamp). If the Bunsen burner is on, turn it OFF! Squirt some 80% ethanol onto the hood table and use a tissue to wipe the entire surface of the hood table with the ethanol. Leave the light and the fan on until you are ready to use the hood.
5. When the LB is out of the autoclave swirl the containers to assure complete mixing, especially for those containing the agar. [If someone else is using the hood, or for some other reason you cannot pour the agar-containing medium, keep it warm in a 65 °C water bath until ready. However, if you plan to pour the plates on a different day, place the container into a refrigerator and melt the agar-containing medium in a microwave oven or a hot water bath when ready to pour the plates.]
6. Move to the sterile hood with all of the containers of medium, the stock solutions of the drugs, 20 sterile plastic petri plates, Pipetman and sterile tips (for addition of the drugs).
7. After cooling the medium at room temperature for 5-15 minutes, add the drugs to the appropriate containers.
8. Pour 20-25 ml of the agar media into each plate. Be sure to mark the ones containing the drugs accordingly. Place the tops of the plates so that they are tipped to the side. This allows some of the water vapor to escape so that the tops of the plates will not be coated with condensation (which can cause problems later).
9. Allow the plates to cool for at least 30 minutes to one hour before trying to move them. When the agar has hardened, replace the lids and store the plates upside-down in the refrigerator. Also store the liquid media in the refrigerator.
10. When finished in the hood, always turn off the Bunsen burner, then wipe the table down with 80% ethanol and turn off the light and fan (unless you know that other people are planning on using the hood immediately after you).

PART II: TO TEST THE PLATES:

1. Make four dilutions of the *E. coli* cells containing no plasmid and four dilutions of the *E. coli* cells that contain the plasmid pBR322 (which confers resistance to ampicillin and tetracycline). Remove 2 µl of each of the *E. coli* cell suspensions and separately dilute them with 18 µl of LB (for the *E. coli* HB101 or RR1 cells alone) or LBA (for the *E. coli* HB101 or RR1 cells with the pBR-based plasmids) or X-gal/LBA (for the *E. coli* DH5α cells with the plasmids conveying galactosidase activity). These will be a one-tenth dilution of the cells. [NOTE: Each time you take any suspension or medium from a glass bottle or test tube, you should sterilize the opening of that container by passing the top opening of the container through a Bunsen burner (or other) flame. This will

cut down on contamination of your solutions and bacterial cultures.] Remove 2 μl of these and dilute in 18 μl of LB (or LBA, as before). These will be a one-one hundredth dilution of the original cell suspension. Again, remove 2 μl of these and add 18 μl of LB (or LBA), for the 1/1000 dilution. Finally, make one more 1/10 dilution of the last suspension (a 1/10000, or 10^{-4} , dilution of the original suspension).

2. Draw lines on the plastic part of the plates (the side containing the agar) to divide the plate into four parts (quadrants).
3. Place the lower portion of the bent glass rod ("hockey stick") into 95% ethanol, then pass it through a flame. [NOTE: Be sure that the container of ethanol is at least a few feet from the flame or you will start a fire.] Allow this to cool for a minute or so.
4. Place the 18-20 μl of one of the bacterial suspensions onto the agar plate in one of the quadrants (remember to add and quickly spread 40 μl of the X-gal solution onto the appropriate LBA plates about 30 - 60 min prior to use). Using the sterilized bent glass rod spread the liquid suspension in that quadrant. Do this for each dilution for the two types of bacteria (i.e., with and without the plasmid) and on each of the three types of plates (i.e., LB, LBA and LBC). Therefore, each group will have four different dilutions of each of the two bacteria spread on each of the three types of plates, for a total of six plates.
5. Place all of the inoculated plates into the 37 °C incubator until the next day.
6. Examine the plates, count the colonies in each quadrant (if possible) and make a determination of whether the dilutions were accurate and whether the selection on the ampicillin and chloramphenicol plates is working.

SOLUTIONS:

LB (Luria-Bertani) medium (per liter)

10 g tryptone
5 g yeast extract
10 g NaCl
(Adjust pH to 7.5 with NaOH)

LB with agar

Make up LB (as above), then add 15 g of powdered agar per liter of medium

Ampicillin stock solution

25 mg/ml dissolved in sterile water

Chloramphenicol stock solution

34 mg/ml in 100% ethanol

LBA (LB with ampicillin)

add ampicillin to a final concentration of 50 µg/ml
in LB

LBC (LB with chloramphenicol)

add chloramphenicol to a final concentration of
30 µg/ml in LB

X-gal

20 mg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactoside dissolved in
dimethylformamide

REFERENCES:

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular Cloning, A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY