STS marker development protocol

Fragment isolation from acrylamide gel

Excise band from gel:

You need the following:

- **Clean scalpel**

1. Cut band from fresh gel.

2. Place band into 1.5 ml eppendorf with 50 µl of water.

3. Incubate overnight at 4ºC.

4. Run PCR using the original primers with 5 µl of the template (be sure to subtract the volume difference from the original reaction from the water component).

5. Check that the PCR worked by running a 4% NuSieve GTG agarose gel using TBE (70 volts) at 4ºC. Be sure to skip a lane between bands that you want to cut.
    a. Load the whole reaction into the well

6. Weigh 1 eppendorf tube for every band that you want to excise.

7. Put gel on UV box and cut out band using a sterile scalpel. Make sure that you don't touch any of the other bands surrounding the band of interest.

8. Trim the band on the UV box with the scalpel if necessary.

9. Weigh tube and subtract from previous weight to determine weight of gel. Use this weight to follow the protocol included in the kit.

10. Extract DNA from agarose: QIAquick™ Gel Extraction Kit (50) from QIAGEN (followed protocol exactly).

    a. Add 3 volumes of Buffer GQ to 1 volume of gel (100 mg ~100 µl). Note: for gel >2% agarose, add 6 volumes of buffer. The maximum amount of gel slice per QIAquick column is 400 mg; for gel slices >400 mg use more than one column.
    b. Incubate at 50°C for 10 minutes of until the slice has completely dissolved. To help dissolve the gel, mix by vortexing the tube every 2-3 minutes during the incubation. Note: for gel of >2% agarose, increase incubation time.
    c. After the gel slice has dissolved completely, check that the color of the mixture is yellow. If the color is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0 and mix. The mixture will turn to yellow. Note:

The adsorption of DNA to the membrane is efficient only at pH</= 7.5. The buffer contains a pH indicator which is yellow at pH </= 7.5 and orange or violet at higher pH.

d. Add 1 gel volume of isopropanol to the sample and mix.
e. Place a spin column in a provided 2 ml collection tube.
f. To bind the DNA, apply the sample to the column and centrifuge for 1 minute. The maximum volume of the column reservoir is 800 µl. For sample volumes of more than that, simply load and spin again.
g. Discard the flow through and place the column back in the same collection tube.
h. Add 0.5 ml of buffer QG to the column and centrifuge for 1 min at 13,000 rpm.
i. Discard the flow through and centrifuge the column for an additional 1 minute at 13,000 rpm.
j. Place the column into a clean 1.5 ml eppendorf tube.
k. To elute the DNA, add 50 µl of buffer EB to the center of the membrane and centrifuge the column for 1 minute at maximum speed. To concentrate the DNA more use 30 µl instead

Transformation/Cloning reaction:

You need the following:

- **TOPO TA Cloning® Kit for Sequencing Version E (Invitrogen, Carlsbad, CA 92008**
- **42ºC water bath**
- Approximately 10 LB plates (75 µg/ml ampicillin) recipe for 250 ml

1.0% Tryptone	2.50 g
0.5% Yeast extract	1.25 g
1.0 % NaCl	2.50 g
agar	15.00 g

(autoclave for 25 minutes, cool to about 60ºC then add the ampicillin)

- **Shaker at 37ºC**
- **Make sure you have enough vials of competent cells ("TOP10 one shot" cat.# C404010, Invitrogen Corporation, 1-800-828-6686)**
- **Warm SOC medium to room temperature**
- **X-gal (40 mg/ml)**
- **IPTG (100 mM)**
- **Sterilized toothpicks**
- **37°C incubator**

Note: Anything that comes into contact with antibiotics should be autoclaved.

1. Using the DNA from the extraction, run PCR with the original marker.

2. Remove 10 µl of the PCR product and use it to test your reaction by running it on an agarose gel.

3. If the band on the gel is clean and clear use the rest of the PCR product to do the transformation. NOTE: The PCR must be FRESH for the transformation to work well!

4. Used the TOPO TA Cloning® Kit for Sequencing Version E (Invitrogen, Carlsbad, CA 92008) but can use any PCR cloning kit. Followed the manufacturers manual exactly, refer to manual included in kit in case of differences between versions.

    a. Set up the TOPO cloning reaction into one tube: Add the following in THIS order.
        i. 3 µl of PCR product
        ii. 1 µl of salt solution (included in kit)
        iii. 1 µl of vector (included in kit)

    b. Mix gently by tapping and incubate at room temperature for 5 minutes. Then place on ice.

    c. Get vial of chemically competent cells and THAW ON ICE. Add 2 µl of the TOPO cloning reaction from above to vial.

    d. Incubate on ice for 30 min.

    e. Heat-shock the cells for 30 sec at 42ºC without shaking.

    f. Immediately transfer the tubes to ice.

    g. Add 250 µl of ROOM TEMPERATURE SOC medium to the vial.

    h. Cap the tube tightly and shake horizontally at 37ºC for 1 hour at 200 rpm then place on ice or at 4ºC until ready to plate. NOTE: Don't plate until after 3 pm. In the meanwhile prepare the plates:

        i. Warm LB plates in 37ºC for 30 min.
        ii. Place 40 µl of X-gal and 40 µl of IPTG in the center of a plate and spread evenly until completely dry. Keep plates covered while working in the hood to avoid photodegredation of X-gal.
        iii. Place in 37ºC incubator for at least one hour.

    i. For each vial, make 3 plates. Each plate will have a different volume of the bacterial culture (10, 50 and 100 µl). For each volume place the bacteria in the center of the plate and spread evenly until completely dry. For the 10 µl sample, add 20 µl of SOC medium to the center of the plate to facilitate spreading.

j. Incubate overnight at 37ºC.

k. Take plates out of the incubator in the morning and place in 4ºC until 3 pm.

Picking positive clones:

You need the following:

- Approximately 10 LB plates (75 µg/ml ampicillin) recipe for 250 ml

1.0% Tryptone	2.50 g
0.5% Yeast extract	1.25 g
1.0 % NaCl	2.50 g
agar	15.00 g

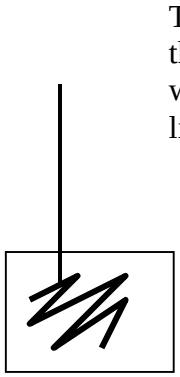
(autoclave for 25 minutes, cool to about 60ºC then add the ampicillin)

- **Sterilized toothpicks**
- **37°C incubator**

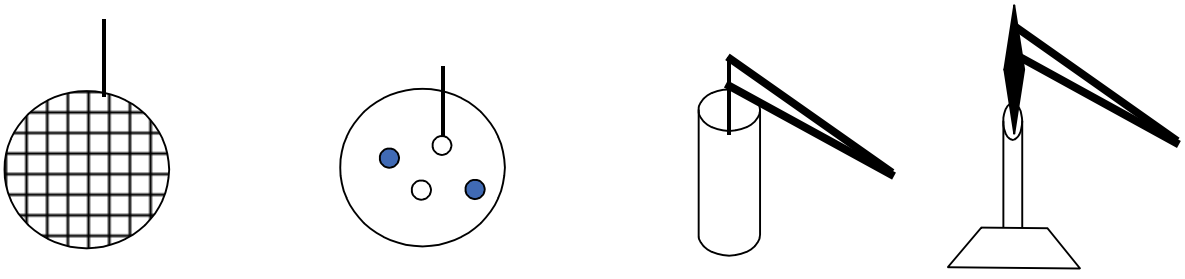
5. Before beginning this procedure you must prepare the plates.
   a. Warm LB plates in 37ºC for 1 hour.
   b. Draw a grid on the underside of the plate

6. Flame sterilize a tweezer and use it to pick a sterile toothpick. You can now transfer the toothpick to your hand. Using the end of the toothpick that has not touched your fingers, **TAP** the toothpick to the white colony you want to pick and streak the toothpick within the one of the squares of the grid. Repeat this procedure until you have either filled a plate or don't have any more white colonies, whichever comes first.

This is how the square will look like.



4 3 2 1



7. Place plate in the 37°C incubator overnight to grow.

Growing selected bacterial colonies in liquid media:

You need the following:

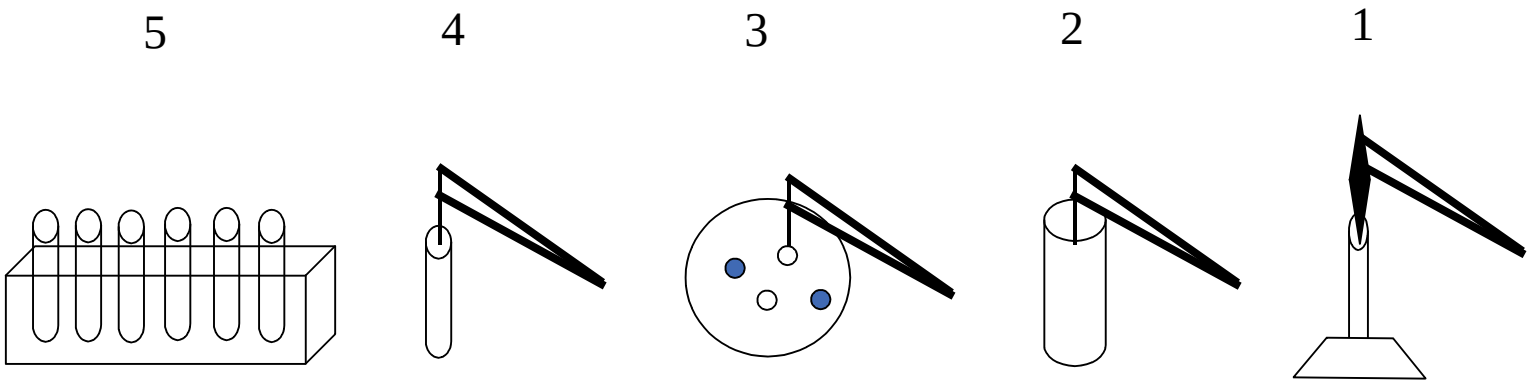
- **200 ml "Magnificent Broth" liquid media (MacConnell research, San Diego, CA 92121, 1-800-466-7949).**
- **Sterilized toothpicks**
- **Need to have sterile tubes with caps for bacterial cultures**

- **37°C shaker**

8. The next day, in the morning, put the plates in the 4°C fridge until 3 pm.

9. After 3 pm, in the hood, pour 3 ml of magnificent broth into each sterile tube for liquid culture. Prepare 10 tubes for each transformation.

10. Select 10 bacterial colonies, that is, bacteria from 10 different squares from the gridded plates: Flame sterilize a tweezer and use it to pick a sterile toothpick. **Holding the toothpick with the tweezer**, TAP the toothpick to the white colony you want to pick and drop it into the tube with the magnificent broth. Cap the tube right away.



11. Put the tubes vertically into the 37ºC shaker and shake for 22-23 hours, but no longer than 24 hours!

Extract Plasmid DNA:

You need the following:

- **Wizard® Plus SV Minipreps DNA purification system (Promega Madison, Wi, 1-800-356-9526**
- **Bacteria grown in liquid culture for 23 hours**

12. The next day, extract the plasmid DNA using the Wizard® Plus SV Minipreps DNA purification system (Promega Madison, Wi, 1-800-356-9526). Followed their protocol exactly. Refer to manual included in kit in case of differences between versions.
13.
    a. Using a pipettor, transfer half of the volume (750 µl) to a 1.5 ml eppendorf tube and centrifuge for 5 minutes at 10,000 rpm.
    b. Pour off the supernatant into a designated container and add the remaining volume (750 µl) and centrifuge again.
    c. Add 250 µl of the Cell Re-suspension solution and completely re-suspend the cell pellet by vortexing well.

d. Add 250 µl of the Cell Lysis solution and mix by nverting the tube 4 times. DO NOT VORTEX. Should see some streaks of clear liquid but mostly still clouded.
e. Add 350 µl of Neutralization solution and mix by invering the tubes 4 times. DO NOT VORTEX.
f. Centrifuge the bacterial lysate at 14,000 rpms for 10 minutes at room temperature.
g. Transfer the cleared lysate, approximately 850 µl, by decanting into the spin column inserted into a 2 ml collection tube. These are provided in the kit.
h. Add 750 µl of column wash solution to the spin column.
i. Centrifuge at 14,000 rpm for 1 minute at room temperature. Pull the spin column out from the tube and discard the flow through from the collection tube. Place spin column back into collection tube.
j. Add 250 µl of column wash solution to the spin column.
k. Centrifuge at 14,000 rpm for 2 minutes at room temperature.
l. Transfer the spin column to a clean sterile 1.5 ml eppendorf tube.
m. Elute the plasmid DNA from the column by adding 100 µl of Nuclease-free water to the spin column.
n. Centrifuge for 1 minute at 14,000 rpm at room temperature.

Digesting plasmid:

You need the following:

- **EcoRI restriction enzyme**
- **EcoRI buffer**
- **37°C water bath**
- **0.8% agarose gel**

14. Select size of insert by digesting the plasmid DNA and running on an agarose gel.
    a. Add into a 0.5 ml eppendorf tube:
        i. 5 µl of plasmid DNA
        ii. 3 µl of EcoRI buffer
        iii. 1 µl of EcoRI enzyme
        iv. 21 µl of water
    b. Incubate at 37°C in a water bath for 1 hour.
    c. Place in 4°C until gel is ready to run.
    d. Pour a 0.8% agarose gel.
    e. Add 4µl of loading buffer to digest and load 28µl into each well.
    f. Run at 80 volts.

15. Quantify plasmid DNA

16. Select which colonies to sequence.
    a. For sequencing need to send them 1000 ng in 6 µl.

17. Send purified plasmid DNA for sequencing.

Note: You do not have to use the TOPO TA cloning kits (which are only sold with competent cells and therefore, very expensive. A cheaper alternative is to use Qiagen PDrive PCR cloning kit and to make your own competent cells.