10/15/2016 – Quynh Nguyen

DNA Cloning Protocol

Aim : To get the desired gene of interest into a vector & introduce into proliferating bacteria to increase the quantity of the gene of interest.

Gene Isolation- Restriction

Generally, we want 3ug DNA/ 40uL rxn volume

Always thaw materials on ice ahead *(buffer has large volume and* ***take forever*** *to thaw)*

Prepare sample on ice

Also start heating block ahead *(take ~40mins to reach desired temperature)*

Heating block: low setting: #6 = 55C , #8 = 65C - Always add water to wells

1. In each tube, add in the below order:
   Buffer - 4 uL
   Water – top up all materials to 40uL
   Restricting enzymes (BamH1/NHe1/Not1…)
   *** Double check enzyme type and requirement buffer, inactivation.*
   Vector – volume determined based on concentration **(we want 3ug)**
   ***Choose buffer according to the enzyme(s) used. Check in product catalog or online.*
2. Incubate at 37C and wait for 1hr to 1.5hr for restriction enzymes to cut gene sequences.
   ***Protocol suggests apprx 30', but longer time may result in higher yield.*
   ***No longer than 1.5hr. Enzymes may chop up everything.*
3. Inactivate restriction enzyme at 65^{o}C for 20 mins
4. Prepare agarose gel and stain it with ethidium bromide.

1% agarose gel for DNA

----For 100mL ---

Mix 1 gr Agarose + 100mL of **1X TAE buffer** (leave 1-2 mL for volume of agarose powder)

Microwave for ~ 2' (leave the cap untighten in the microwave. Heat until solution dissolved)

----> For thick gel when loading 40uL rxn vol:

Measure 35 - 40mL/gel into conical tube - Wait for gel to cool slightly - Add 8 uL of EtBr **(toxic chemicals) ****Thicker gel may interfere with imaging*

Cap and mix gently by rocking back n forth

Insert tray into mold - Insert comb - Pour gel from the back of the comb to prevent bubbles

Wait ~30 mins for gel to set (gel sets faster in cold room ~15min) - Remove comb

4. Dilute dye to 1X and add to sample
   ***Dye stock is 6X ->For 1X, 1 dye:6 sample ||**For 40uL rxn, 1 part dye = 6.6uL*
5. Make running buffer (1X TAE)
   ***Stock is 50X ->For 1X, 1 Stock: 49 water||**500mL work buffer=10mL stock:490mL water*
6. Make ladder sample *1uL ladder : 4uL water : 1uL dye*
   Make dyed buffer for empty wells (if any) ***~30uL/ well -> 30uL buffer : 5 uL dye*
   Plan order of sample in gel. ***Load alternatingly (1 empty well in btw samples), if possible*
7. Place gel with wells toward "Fill line" side -Load buffer up to the "Fill line" - *Do not add more*
   Load samples with 10uL pipette tips (extended tips preferred)

***Load 40uL of sample in each well (splitting to 2 rounds to avoid overflow)*

***Wipe tip of pipette before loading to avoid contamination.*

8. Put lid on. Connect black wire to "Fill line" side + Black to black- Red to red.
   Use tape to keep wires in place, if needed - Run the gel for 1 to 1.5 hours at 100V

** Gel at this stage can be kept in the 4°C (cold room) for a day.

DNA Extraction from Gel

****Adjust volume ahead according to mg of gel:** *100 mg of gel ~ 100microL ~ x amount*

**Write down all numbers and calculations before proceeding.

** Soak gel in EtBr sltn before image for better visualization. (*10uL EtBr + 100mL 1X TAE)*

1. Prepare tubes to store gel fragments w DNA of interest - carefully remove gel from chamber
2. Image gel under UV light on the 5th floor (opposite to 5-269B Autoclave)
3. Use clean blade to cut out wanted gel fragments and put pieces of gel into appropriate tubes.
4. Follow the protocol as stated in the DNA Gel Extraction Kit.
   ***When applying to column, pipette max 700uL liquid to avoid overflow.*
   ***If use for ligation, elute in 30uL water. Wait 4', then centrifuge 2' for higher yield.*
5. Check DNA concentration and quality using the Nanodrop machine on the 5th floor.
   ***Besides concentration, also check purity (ratio 260/280 should be 1.6 - 2.0)*

Ligation <u> Or follow ligation protocol of kit</u>**

***Calculate concentration of vector and insert before proceeding*

1. Prepare **each** ligation rxn mixture as following:
   10X T4 DNA Ligase buffer – 2uL **(Buffer is temperature sensitive !!)**
   120 ng Vector : **240 ng** Insert
   T4 DNA ligase – 1uL
   Water - top up to 20uL
2. Incubate 15-20mins @ RT

Transformation

(For new DNA: use 50uL cell +3uL DNA +250uL SOC, plate all, or plate x2 (50uL &150uL))

1. Get ice, chill culture tube, thaw host cells **(XL1 BLUE**, stored in -80°C**)** & DNA (if frozen)
   Must thaw cells on ice (not hand temperature).* ***Cells are heat sensitive!!!!
2. Warm SOC media/ LB Broth to 37°C – Warm plates containing **appropriate antibiotics.**
3. Slightly mix cells in stock tube to ensure same concentration
   Put 25µL of cells and 3µL of DNA from ligation step into culture tube - Ice the tube for 30'
4. Heat shock bacteria for 50 seconds with 42°C water then immediately put into ice for 2'.
5. Add 125µL S.O.C media into tube – Incubate in shaker for 1 hour at 37°C and 225 rpm.
7. Start fire and prepare plate spinner, alcohol, & spreading loop.
8. Plate 25µL of bacteria culture
9. Store plates in incubator (37 C) for 14 to 18 hours.
10. Check for colonies next day.