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In gel ligation

I learned this in gel ligation from Dan Gestaut from the Frydman Lab.

Background

Gibson Assembly would not work for the tough cloning of putting a 4 kb insert into 6 kb vector. Therefore, I turned to more traditional approaches of restriction enzyme cut and paste. Performing an in gel ligation is more efficient than a normal ligation because the gel acts as a crowding agent to push the DNA into closer contact. The following is the basic information to perform an in gel ligation.

Digest

Considerations

- Needs to be performed on the same day as the in gel ligation
    - The sticky ends of the DNA will degrade and the ligation will be less efficient.
- There needs to be 1 ug of DNA per 20 uL of digest
    - This is plenty of DNA to see on the gel
- The glycerol concentration need to be less than 0.5%
    - Restriction enzymes are less efficient if there is more glycerol
    - If only 1 enzyme cuts there will be very low efficiency for the ligation because one side will ligate and the other side wont. This won't form a vector that can be transformed. Making sure both restriction enzyme cut is imparative
    - Make a master mix
- If possible cut the digest out of a vector to know for sure both restriction enzymes cut
    - TOPO coloning can be used for subcloning to cut the insert out of a vector
- Phosphatase treat the vector
    - CIP is harsh and stays bound to the DNA leading to a lower efficiency ligation
    - Arctic phosphatase is less harsh and leads to more transformants

2X Digest mastermix

This protocol assumes 100 ng/uL concentration of DNA. If your DNA is more concentrated adjust the water to get 1 ug. If the DNA is less concentrated don't add more, just hope you can see it on the gel. If the DNA concentration is really low do an ethanol precipitation.

Add reagents in the follow order

- 22.8 uL ultrapure water
- 6 uL 10X digest buffer
    - Don't need to add BSA because NEB has switch from dtt to BSA in its buffers
- 0.6 uL restriction enzyme 1
    - 20 U/uL or 10 U/uL concentration
- 0.6 uL restriction enzyme 2
    - 20 U/uL or 10 U/uL concentration

Digest Mix

- 12.5 uL DNA

- 12.5 uL 2X Digest mastermix

Digest Conditions

- 1 hour and 45 minutes at 37°C
- After 1 hour of incubation for the plasmid only add 2.5 uL arctic phosphatase buffer and 1 uL of arctic phosphatase
- Do not heat inactivate
- Place on ice until gel is ready

Gel

Considerations

- Electrophoresis grade low melt agarose must be used
- The gel will only solidify in the cold room at 4°C
- Don't use ethidium bromide as a stain, UV exposure is too harsh for the DNA. Use sybr gold or sybr safe and a blue light box
- A very thin comb must be used (1 mm thick) otherwise the gel slice will be very big and the DNA will not be concentrated enough. We have thick combs associated with our gel boxes so I use the gel box in the Frydman lab with thin combs.
- Don't pour buffer on top of the gel or into the wells in the gel box because the gel will rip
- Don't let the gel run too long or the bands smear and become bigger
- The goal is to have super concentrated DNA in the gel slice. It is better to cut off some of the DNA around the edge of the band than to have excess gel in the band and get every bit of DNA.
- Treat this procedure like RNA work rinsing everything in DI water to prevent single stranded DNA sticky ends from degrading

Mixing the gel

1. Add 60 mL TAE buffer to flask
2. Slowly swirl in 0.5 g low melt agarose powder
    a. Add agrose slowly to TAE or it will clump and your gel will smear
3. Microwave until gel dissolves
    a. Monitor so gel doesn't explode all over the microwave, the low melt agarose is prone to explode
4. Add 3 uL 10,000X syber gold
5. Rinse gel box stuff while gel cools a bit with DI water
6. Pour gel in cold room
    a. Use small ladder
    b. Place in ladder after gel is poured otherwise the gel will creep up the side of the comb and it will be impossible to take out
    c. Make sure comb is 1 mm from bottom, otherwise the wells rip
    d. Let solidify for 30 minutes

Running the gel

1. Mix the digest with 2.8 uL of 10X blue juice
2. Load 20 uL of digest into each well leaving 2 wells in between different pieces of DNA
3. Run the gel at 100 mV for 20 minutes

Cutting the gel

1. Place a piece of saran wrap over the blue light box
2. When the gel is done running drain as much buffer from the gel as possible
3. Slide the gel onto the bottom of the light box. Push the gel to the top of the light box to drain off more buffer
4. Wear the orange glasses and turn on the blue light. Estimate the intensities of each band. For example the insert is 10 times more bright than the vector.
5. Cut the gel slices. They should have a 15-30 uL volume. Trim of any unnecessary gel that does not contain DNA
6. Place the gel slices into 1.5 mL Eppendorf tubes
7. Spin down gel slice to the bottom of the tube
8. Calculate the ratio to mix the insert to vector in the tubes. This will be used to help calculate the amount of insert and vector to mix into the 10 uL ligation reaction. The insert should be in a 2-10 molar excess (m) to vector

$$\frac{\text{moles insert}}{\text{moles vector}} = \frac{\text{length bp insert}}{\text{length bp vector}} \frac{\text{volume gel slice vector}}{\text{volume gel slice insert}} \frac{\text{band brightness insert}}{\text{band brightness vector}} = \text{r}$$

$$10\ \text{uL} = \text{r} * \text{m} * \ \text{x uL vector} + \text{x uL vector}$$

$$\text{x} = \text{uL vector}$$

$$10 - \text{x} = \text{uL insert}$$

Ligation

Considerations

- Don't let the 2X ligase solution sit out before using
- Make sure all of the white flakes (dtt) are dissolved after thawing the 5X ligase buffer. Dtt is necessary for the ligase to function properly.
- Ligases are finicky enzymes. Only add ligase directly before mixing into the DNA. Within 5 minutes of adding ligase to the ligase is much less active
- Must perform a vector only control to know the background level of colonies forming from the vector closing on itself. This should be small to none due to the phosphatase treatment.

Melting Gel slices

1. Label two 1.5 mL Eppendorf tubes for the ligation reaction. One for vector and insert and another for vector only.
2. Pipet ultrapure water in place of insert into the vector only control
3. Place the gel slices and empty ligation reaction tubes in a heat block set to 67°C for 30 seconds or until the gel is melted.
4. Pipet the melted gel slices up and down to mix to make sure the DNA is evenly distributed.
5. Add the amounts of vector and insert calculated above to each ligation tube.
6. Once all of the DNA has been added, hang the tubes off the side of the heat block to cool
    a. Don't let the tube cool below 37°C

Ligation

1. While waiting for the gel slices to cool mix the 2X ligase solution (recipe for 3 reactions)
    a. 21 uL ultra pure water
    b. 6 uL 5X ligase buffer
    c. 3 uL T4 ligase
        i. Only add directly before adding to DNA
        ii. Pipet gently up and down to make sure the ligase is well mixed into the rest of the buffer
2. When the DNA has cooled add 10 uL of the 2X ligase solution to the DNA and gently pipet up and down to mix well
3. Let the ligation sit on the bench top for 1 hour at room temperature
4. After an hour add the ligation mix back to the heat block at 67°C for 1 minute.
5. Add 80 uL of ultrapure water and pipet up and down to mix
    a. Skip this step if performing chemical transformation
    b. Don't place ligation on ice even before adding to transformation

Transformation

Considerations

- I use electroporation because it is more efficient
- The salts from the ligation mix must be diluted out before performing the electroporation
    - Water was added at the end of the ligation

Protocol

1. Take out electrocompetent cells from freezer (20 uL per transformation) and place on ice along with the electroporation cuvettes with 1 mm gap
2. For each transformation fill a 1.5 mL tube with 1 mL of SOC medium
3. When electrocompent cells have thawed aliquot 20 uL into 1.5 mL eppendorff tubes.
4. Add 1 uL of diluted ligation mix to each transformation. Pipet up and down 3 times to mix and transfer to chilled electroporation cuvette
5. Tap cuvette on table to remove and bubbles, wipe contacts with chem wipe to remove water and electroporate at 1.25 kV.
6. Immediately add 1 mL of SOC to save cells
7. Tape to rotator for 1 hour a 37°C
8. Spin down at 4500 x g for 1 minute
9. Remove supernatant and resuspend pellet in 100 uL of LB
10. Plate entire 100 uL on appropriate antibiotic resistance plate.
11. Screen with colony PCR