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Experimental Protocols

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Cloning

PCR:

Purpose: amplify DNA segment of interest

Materials:
- DNA template
- dNTP
- Forward primer
- Reverse primer
- 5X Q5 Reaction buffer
- Q5 High-Fidelity DNA Polymerase
- DI water
- PCR tubes
- Pipettes and tips
- Thermocycler

Protocol:
1. Perform the following dilutions:

DNA template	Dilute until concentration is 10ng/μL
Forward and reverse primers	Dilute each primer 10X

2. Make a mix with the following in a PCR tube, making sure to add the DNA polymerase last. The total volume should be 50 μL.

DNA template	0.5 μL
Forward primer	2.5 μL
Reverse primer	2.5 μL
dNTP	1 μL
Q5 Reaction Buffer	10 μL
DI Water	33 μL
Q5 DNA Polymerase	0.5 μL

3. Thermocycler settings:
    - 98°C for 15s
    - 72°C for 15s
    - 72°C for 1 min (x 25)
    - 72°C for 2 min
    - Store at 4°C

Restriction Enzyme Digest:

Purpose: cleave DNA template plasmid at specific sites to isolate backbone

Materials:
- 10X Cutsmart Buffer
- Template plasmid DNA
- DI water
- Restriction enzymes (preferably high fidelity (HF) enzymes)
- PCR tubes
- Pipettes and tips

Protocol:
1. Make a mix with the following in a PCR tube, making sure to add the restriction enzymes last. The total volume should be 20 μL.

Cutsmart Buffer	2 μL
Plasmid DNA	5 μL (roughly 1 μg DNA)
DI water	2.5 μL
Restriction enzyme #1	0.5 μL
Restriction enzyme #2	0.5 μL
DI Water	Add until final volume is 20 μL

2. Place in 37 C incubator for 1 hour

Gel Electrophoresis:

Purpose: determine sizes of DNA fragments

Materials:

- PCR samples
- 1X TAE buffer
- Agarose
- SYBR Safe DNA Gel Stain
- DNA ladder
- Gel loading dye
- Gel tray
- Gel comb
- Gel electrophoresis machine
- Gel imaging system
- Pipettes and tips

Protocol:

Preparing the gel:

1. To prepare a 1% gel, add 40 mL 1X TAE buffer to 400 mg agarose in an Erlenmeyer flask
2. Microwave for 1-2 minutes until agarose is fully dissolved
3. Add 4 µL SYBR Safe to gel
4. Place gel comb in gel tray
5. Pour gel into gel tray and let it cool for 15-20 minutes

Running the gel:

1. Remove gel comb
1. To each PCR sample, add loading dye (1 part dye : 6 parts sample)
2. Mix with pipette
3. Slowly pipette sample containing dye into each well
4. In one well, pipette 4 µL of DNA ladder
5. Run gel for approximately 25 minutes at 140V and 3.00A

Imaging the gel:

1. Remove gel tray and place into gel imaging system
2. Select SYBR Safe
3. Select run protocol

Gel Extraction:

Purpose: isolate DNA from agarose gel

Materials:
- Razor blade
- UV tray
- 1.5 mL microcentrifuge tubes
- Zymo-Spin IIN columns
- Collection tubes
- Agarose Dissolving Buffer (ADB)
- DNA Buffer Wash
- DI water
- Microcentrifuge
- Pipettes and tips

Protocol:
1. Place gel on UV tray, making sure to use UV protection shield
2. Carefully cut out the desired DNA fragment with a razor blade, while minimizing the amount of excess gel surrounding the fragment. Place the gel slice in a 1.5 mL microcentrifuge tube.
3. Add 450 µL of Agarose Dissolving Buffer (ADB) into the tube. Incubate the tube at 55 C for 20 minutes. Invert the tube a few times to make the gel dissolve better.
4. Transfer liquid to a Zymo-Spin IIN column that is placed in a collection tube. Centrifuge at 13 xg for 15 seconds. Throw out the flow through.
5. Add 200 µL of DNA Buffer Wash to the Zymo-Spin IIN column. Centrifuge at 13 xg for 30 seconds. Repeat this step.
6. Throw away the collection tube and place the Zymo-Spin IIN column in a clean 1.5 mL microcentrifuge tube.
7. Add 12 µL of DI water to the column. Incubate for 1 minute. Centrifuge at 13 xg for 1 minute.
8. Throw away the column. The DNA is now in the 1.5 mL tube.

Nanodrop:

Purpose: measure concentration of plasmid DNA

Materials:
- Nanodrop Spectrophotometer
- DNA sample
- DI water
- Pipette and tips

Protocol:

1. Turn on the Nanodrop Spectrophotometer machine. Select 'Nucleic Acids', then select 'dsDNA' to measure a double stranded DNA sample.
2. Life the arm and wipe both pedestals clean.
3. Pipette 1 µL of DI water onto the pedestal and lower the arm. Select 'Blank' to blank.
4. Pipette 1 µL of the sample onto the pedestal and lower the arm. Select 'Measure' to measure the concentration.
5. Repeat if there are multiple samples, making sure to wipe the pedestals between measurements.
6. Record the concentrations of each sample.

Hi-Fi Assembly:

Purpose: joins DNA fragments

Materials:

- HiFi mix (2X)
- PCR product(s)
- Backbone DNA from restriction enzyme digest
- PCR tubes
- Pipettes and tips
- Thermocycler

Protocol:

1. Label one PCR tube with (+) and label another PCR tube with (-). The tube labeled (-) is the negative control.
2. Calculate how much of each insert and backbone to add based on the New England Biolabs protocol. If amounts are not pipettable, dilute the samples until they are.
3. In the tube labeled (+), add calculated amount of backbone and inserts.
4. In the tube labeled (-), add calculated amount of backbone but replace one of the inserts with DI water.
5. Get two new PCR tubes (tubes #3 and #4). In PCR tube #3, add 2 µL of the contents of the tube labeled (+). In PCR tube #4, add 2 µL of the contents of the tube labeled (-).
6. Add 2 µL of HiFi mix to each tube (tubes #3 and #4).
7. Place tubes #3 and #4 in Thermocycler and run the HiFi protocol for 30 minutes.

Bacterial Transformation:

Purpose: introduces plasmid to bacterial cells

Materials:
- Agar plate
- Bucket of ice
- KCM (stored at 4 C)
- Competent bacterial cells (stored at -80 C)
- PCR tubes containing DNA samples from HiFi assembly
- Heat bath
- Pipettes and tips

Protocol:
1. Add 10 µL of KCM solution to 50 µL of competent bacterial cells and keep on ice.
2. Add 25 µL of the mixture into each tube from the HiFi assembly. Keep the tubes on ice for 25 minutes.
3. While the tubes remain on ice, split the agar plate into sections, labeling one side as the negative control and the other side as the construct you want to clone. Put the plate in a 37 C incubator.
4. After 25 minutes, heat shock the tubes in a 42 C heat bath for 1 minute.
5. Place tubes on ice for 5 minutes.
6. Take the agar plate out of the incubator. Pipette out the bacteria from the tubes into the corresponding sections of the plate.
7. For each side of the plate: gently bend a pipette tip using the plate cover and streak the bacteria on the agar, making sure not to break the agar layer.
8. Place the plate in a 37 C incubator for 7-15 hours.

Bacterial Inoculation:

Purpose: grow bacterial colonies in media

Materials:
- Carbenicillin LB media
- Agar plate
- Pipettes and tips
- 15 mL glass tubes

Protocol:
1. Add 2 mL carbenicillin media to a 15 mL glass tube.

2. Pick a bacterial colony off of an agar plate using a pipette tip. Place the tip into the 15 mL glass tube.
3. Leave in a shaking 37 C incubator overnight.

Miniprep (DNA Purification):

Purpose: DNA purification

Materials:
- Inoculated bacteria (in 37 C shaking incubator)
- 7X Lysis Buffer
- Neutralization Buffer
- Endo Wash Buffer
- Zyppy Wash Buffer
- DI water
- Zymo-Spin IIN columns
- Collection tubes
- Microcentrifuge
- 1.5 mL microcentrifuge tubes

Protocol:
1. When the media appears cloudy/dense, take the glass tube containing the bacteria out of the 37 C incubator.
2. Transfer media into a 1.5 mL microcentrifuge tube and centrifuge at 13 xg for 30 seconds. Discard the supernatant.
3. Add 600 µL of DI water to the tube to resuspend the pellet. Vortex the tube.
4. Add 100 µL of 7X Lysis Buffer to the tube. Mix by inverting 4-6 times. Incubator at room temperature for 1-2 minutes. The solution should change from opaque blue to clear blue, indicating complete lysis.
5. Add 350 µL of cold Neutralization Buffer to the tube. Mix by inverting. The solution should turn yellow throughout, indicating complete neutralization.
6. Centrifuge the tube at 11 xg for 2-4 minutes. A pellet should form.
7. Place a Zymo-Spin IIN column in a collection tube. Transfer the supernatant from step 6 into the column.
8. Centrifuge the Zymo-Spin IIN column at 11 xg for 15 seconds. Discard the flow through. Return the column to the same collection tube.
9. Add 200 µL of Endo Wash Buffer to the column. Centrifuge at 11 xg for 30 seconds.
10. Add 400 µL of Zyppy Wash Buffer to the column. Centrifuge at 11 xg for 1 minute.

11. Transfer the column to a clean 1.5 mL microcentrifuge tube. Add 30 µL of DI water to the column. Incubate for 1 minute at room temperature.
12. Centrifuge at 11 xg for 30 seconds to elute the plasmid DNA. The purified DNA should be in the 1.5 mL microcentrifuge tube.

*Best practice after Miniprep is to Nanodrop the purified DNA sample to determine its concentration.

Glycerol Stock Preparation:

Purpose: Store plasmid in glycerol stock for future use

Materials:
- Plasmid DNA
- Agar plate
- 50% glycerol
- Glass tubes
- 1.5 mL microcentrifuge tube
- Pipettes and tips

Protocol:
1. Transform plasmid into bacterial cells using the transformation protocol, except this time, do not split the agar plate into sections. You only need to add 1 µL of the KCM+DNA+cell mixture to the agar plate (instead of 25 µL). Incubate in a 37 C incubator overnight.
2. Pick a bacterial colony with a pipette tip and add it to 1 mL LB media in a glass tube. Grow up the colony overnight in a shaking 37 C incubator.
3. Add 600 µL of bacterial media to 400 µL of 50% glycerol in a clean 1.5 mL microcentrifuge tube.
4. Label the tube and freeze it at -80 C.

Cell/Tissue Culture:

Passaging Cells:

Purpose: Helps keep cells alive and growing.

Materials:
- DBPS or PBS
- DMEM (media)

- Trypsin
- HEK 293T cells (provided in a flask)
- Centrifuge
- Aspirator
- 15 mL tubes
- 1.5 mL microcentrifuge tube
- Pipettes and tips
- Serological pipettes and serological pipette controller

Protocol: *make sure to work in a hood

5. Incubator DBPS, DMEM, and trypsin in a 37 C incubator for 5-10 minutes until solutions are warm.
6. Aspirate the media from the culture flask.
7. Add 10 mL of DPBS to the flask.
8. Aspirate.
9. Add 1 mL of trypsin. Let the flask sit for 1-2 minutes then rock the flask back and forth gently until cells lift off of the plate.
10. Incubate at 37 C for a few minutes.
11. Add 5-7 mL of DMEM (media) into the flask to inhibit trypsin activity.
12. Pipette cells into a 15 mL tube. Pipette up and down to mix.
13. Centrifuge for 2 minutes. A pellet should be visible.
14. Aspirate out the supernatant. Add 5 mL of DMEM to the tube to suspend the pellet and mix gently with a pipette.
15. Transfer 1 mL of cells to a new flask. Add 9 mL DMEM to the new flask and label the flask with the passage number and date.
16. Keep the remaining ~4 mL of cells to plate for an experiment. Transfer 500 µL of these cells to a 1.5 mL centrifuge tube for cell counting.

Counting and Seeding Cells:

Purpose: Determine the concentration of cells and add the desired amount of cells to plate for an experiment

Materials:

- Hemocytometer
- 96-well plate
- Fibronectin
- Cell media from passaging
- DMEM (media)
- DPBS or PBS

- Pipettes and tips

Protocol:

1. Add 15-20 µL of cells between the hemocytometer and coverslip/glass cover.
2. Using a computer, visualize the cells and take an image of a section of cells.
3. Record the concentration of cells.
4. Determine the desired number of cells per well, the desired volume (in µL) per well, and the total number of wells for the experiment. For our experiments in a 96-well plate, we wanted 30,000 cells and a volume of 100 µL per well.
5. Dilute fibronectin 100X into PBS/DPBS. Add 50 µL to each well in the 96-well plate that you plan to use. Place the plate in a 37 C incubator for 10 minutes.
6. Quickly "whip" the fibronectin into the sink.
7. *(These next steps should be done in a hood.)* Make a master mix of cell media and DMEM media to achieve the desired concentration described in the step 4. For our experiments, we added 100 µL of the mix to each well.
8. Place the plate in a 37 C incubator and leave overnight.

Testing:

Transfection:

Purpose: Introduce plasmids into HEK cells to run experiments

Materials:

- 96-well plate with cells seeded
- Plasmid DNA for constructs you wish to test
- HEBES
- $CaCl_2$
- DI water
- PCR tubes
- Pipettes and tips

Protocol:

1. For each construct you wish to transfect, make the following transfection mix in a PCR tube. If using many wells/replicates, you can make a master transfection mix by multiplying each volume in the table by the number of wells you wish to allocate for the construct. The total volume of transfection mix (per well) should be 10 µL. Make sure to add the $CaCl_2$ *LAST* for an efficient transfection.

Plasmid DNA	100 ng
DI water	Add to plasmid DNA until volume is 4.4 µL
HEBES	5 µL
$CaCl_2$	0.6 µL

2. For each construct you wish to transfect, add 10 µL of the transfection mix into each well.
3. Place the plate in a 37 C incubator overnight. *Since our constructs are light-activatable, we covered the plate in tinfoil when moving it to the incubator.

Luciferase Assay:

Purpose: Measure luminescence in the media of cells to determine if there was secretion.

Materials:
- Luciferase Assay System kit
- 96-well plate containing tested constructs
- Plate reader
- Pipettes and tips

Protocol:
1. Transfer 25 µL media from each well in the black 96-well plate to white 96-well plate.
2. Add 100 µL of Luciferase Assay Reagent to each well.
3. Luminescence measurement:
    a. Measure the light produced by each well using a plate reader.