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Identifying an active promoter in B. saltans using Luciferase assay

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Protocol status: Working
We use this protocol and it's working

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Abstract

Before starting prepare the following:

- Measure the Luciferase plasmid concentration using NanoDrop and Qubit.
- Design the PCR primers for the gene of interest with the RE (restriction enzyme) sequence at the 5′.
- Add 3 to 4 nucleotides at the end of the RE sequence (These bps will be removed during the fragment digestion)
- Order TOPO 10 chemically competent *coli* cells and the other kits for digestion, ligation, gel purification.

Protocol:

Day 1:

- **PCR amplification**: (Use 10 -100 ng/ul of DNA of *saltans*) and the following forward and reverse primers with RE sequence to amplify gene or region of interest. Here we are using two forward primers, and one reverse primer to 0.6 and 3 kb respectively. One of these regions include the putative ribosomal promoter

Forward 8683-8713 CTGAAGCTTTGAAGGAAAGTCGAAACGCCGTGTGTGTG Forward 6305-6332 CTGAAGCTTCGGTGTGTCGAGTGGAAGAAAGGACGAT Reverse 9603-9634 ATCAAGCTTAGATTCCTGCAATGCAGTGATTCTGAGG The PCR master mix (Master Mix 2 x NEB (Taq 2x)) PCR amplification profile is 1) 95 C -30 SEC 2) 95 C -30 SEC 3) 61 C – 30 SEC 4) 68C- 60 SEC GO TO 2 FOR 29 CYLCE 5) 68 C-5 MIN 6) HOLD 10 C Make sure that you have more than 75 ul per reaction (3×25ul), we need a lot of PCR product for the fragment of interest

- Gel electrophoresis:

Check the PCR product using gel electrophoresis to make sure that you amplified the right fragment.

- PCR purification:

This is a crucial step; you have to purify the PCR product using the column purification or beads method. Also make sure that you elute in a little volume around 15ul for total 75 ul of the purified PCR product.

- Restriction enzyme digest:

This step should be done for both the plasmid and the PCR product in parallel
Make sure that the digestion for both the PCR product and the plasmid does not exceed 3 hours, use the rapid digestion protocol.
Saw the plasmid on ice
Take only 750 ng of the luciferase plasmid (note: too much plasmid may not digest)

X ul of vector or plasmid with insert
1.7 ul of 10 Reaction Buffer
1.5 ul of 10 Units/ul restriction enzyme (Hind III)
Y ul of water such that the final volume is about= 17 ul
Digest at least 3 or 4 hours.
After digestion treat it with Calf Alkaline Phosphotase , CAP, for an hour at 37 C to prevent the vector from ligating back on itself.
Use add 1/10 volume of CAP 10X Buffer + 1 ul of the enzyme, then heat inactivate this at 65 C for 10 minutes.
Don't recommend this step: You can store this in a -20 C freezer before running it on the gel if you don't have time to run the gel that day.
Add gel loading buffer containing bromophenol blue and glycerol and run out digested material on a 1% agarose gel.
Make sure that you run the plasmid without digestion (control) as well to compare the sizes of the fragments.
If you did not want to run the PCR product on gel that should be fine as long as it will be purified after RE digestion (I recommend to NOT run it, just go a head and purify it again with column or beads)
Cut out the bands of interest (the vector and insert) with a separate sterile scalpel blade.
Place the gel fragments in a 1.5 ml centrifuge tube and freeze at -20 for at least 10 minutes (longer is fine).

- Clean up of gel fragment using Sigma Genelute kit: (I will send this kit to you)

Remove tubes containing gel fragments from -20 C freezer and place in 56 C heat block for 5 or 10 minutes to melt.
Break up the gel fragment into pieces as small as possible with either a small pipette tip or small sterile spatula.
Add 100 ul of TE Buffer to the columns and centrifuge at maximum micro-centrifuge speed for 10 seconds.
Transfer column to a new collection tube (discard old tube with TE buffer in it) and transfer broken up gel fragment to the column, being sure the fragments are at the bottom of the column.
Centrifuge at maximum micro-centrifuge speed for 10 minutes.
Measure volume of recovered gel fragment and add 1/10 volume of sterile 3 M Sodium Acetate solution (for salt precipitation).
Then add 2 volumes of 100% Ethanol and 1 micro-liter of 20 mg/ml glycogen solution (Qiagen #158930) to help precipitate the DNA.
Place this in a -20 C freezer for overnight.
Day 2:
Centrifuge the ethanol precipitation for 10-20 minutes at maximum micro-centrifuge speed and pour or pipette off the ethanol without losing the DNA pellet (pellet will be a small lightly colored "clear" pellet).
Add 200 ul of 70% Ethanol to the tube carefully avoiding disturbing theDNA pellet and centrifuge for 3 minutes at maximum micro-centrifuge speed and pour or pipette off the ethanol without losing the DNA pellet.
Briefly centrifuge again for 10 seconds and remove as much of the ethanol as possible with a pipette avoiding the DNA pellet then allow the pellet to air dry for about 10 or 15 minutes.
Add a small volume of TE buffer (15 ul) carefully to the pellet and place at room temperature for 30 minutes to suspend it.
After 30 minutes pipette up and down a few times to ensure pellet is suspended then run 1 ul of the vector and 1 ul of insert out on a gel for visualization and qubit for quantitation.

- Ligation reaction**:**

Make sure that the ligation does not exceed 2 hours, use the rapid ligation protocol
You need about a 1: 5 ratio of vector to insert (so more insert than vector) you can use the following calculator to calculate insert to vector ratio. (http://www.promega.com/a/apps/biomath/index.html?calc=ratio)
To a 1.5 ml tube in order, add:
X ul restriction enzyme digested CAP treated vector
Y ul insert
2 ul 5X T4 Ligase buffer
Z ul of water to bring final volume to 10 ul
0.4 ul of T4 HC ligase enzyme (Invitrogen #15224-041... this has both the ligase enzyme and ligase buffer in it)
Incubate tube at room temperature for 1-3 hour without disturbing it.
It is recommended to run PCR of the ligation mix to make sure that your insert has been integrated into the plasmid. Use the forward primer of the plasmid and the reverse primer of your insert.

- **Bacterial Transformation:**

Make sure that you transform positive and negative control as well along with your plasmids.
Take 1 ul of ligation and dilute to 5 ul and then take 2.5 ul of this mix and transform the bacteria.
Also you can use only 1 ul of the concentrated ligation and transform. I prefer the first option; it yielded more bacterial colonies.
thawed-on-ice TOP-10 cells [Be sure to keep the cells cold on ice! I usually don't take the tube of cells out of the -80 C freezer until 5 or 10 minutes before I'm going to use them] (One Shot TOP10 Chemically Competent E. coli) and hold for 10 minutes on ice.
Place rest of ligase reaction in -20 C freezer in case you need to repeat this.
Heat shock tube of TOP-10 cells for 35 seconds in a precise 42 C water bath.
Place tubes back on ice for 2 minutes and then add 210 ul of SOCmedia (this is included in the TOP-10 cells kit) to the tubes and incubate with shaking for 1 hour at 37 C.
Spread out on a Miller's LB + Ampicillin (50 or 100 ug/ml) plate using a sterile plate spreader
Divide the cells for three plates, use all of the cells, and grow up overnight at 37 C.
Don't let it go for more than 16 hours or you will get small background bacterial colonies growing up too.
Save some of the bacteria in glycerol stock for future replication

Day 3

- Screening

To screen for the right colonies prepare the following first
Depend on the number of the PCR reactions that you plan to run that day prepare equal number of eppendorf tubes that include 100 ul of LB broth with the ampicillin
Pick up the white colony for the LB plate using the tooth pick, and insert the tooth pick in the PCR master mix and swirl a little bit, then put the same tooth pick in the LB broth in the 1.5 eppendorf
Do that for each white colony you will amplify. To end up saving all the tooth picks in eppendofr that includes the LB broth.
Amplify the white bacterial colonies using plasmid's forward primer and your gene of interest reverse primer, this is to make sure that you have the right insertion size at the right orientation .

Gel electrophoresis image will confirm whether the insert with the expected size was introduced to the plasmid or not.
Go back to your eppendorf tubes and mark all the tubes that gave a positive PCR amplification at the expected size.
Transfer the LB from this tubes to flasks or glass tubes that include 100 ml of LB broth + ampcilin and grow these into culture over night, I usually make 4 different culture, each with 100 ml of LB broth.
Day 4
Next day at the morning start by saving some of the LB+ bacteria into glycerol stock (10-15 tubes), store them in -70C. For the rest of the culture start the plasmid prep following the protocol of Zymo Midi prep kit.

- Quantification:

Qubit all the eluted plasmids to document the plasmid concentration, I elute in 200 ul and usually I get between 200 to 300 ng/ul.

- PCR confirmation and sequencing: Using the plasmid forward primer and the insert reverse primer

Attachments

DOCX

protocol for lucifer...

24KB