Laboratory 3: Identification of NMDA receptor subunit genes – Part II

In last week’s lab, we extracted RNA from rat brain, reverse transcribed it into cDNA, and then amplified it by PCR using degenerate primers. We expect to have amplified approximately a 600 bp product, which we can visualize on an agarose gel. But this 600 bp band likely has several types of NMDA receptor subunits in it. This is because we used degenerate primers, which should amplify more than one type of NMDA receptor gene. If we were to sequence the PCR product we would find a mixture of sequences making the sequence unreadable. To circumvent this problem, we will subclone the PCR product into a plasmid.

How is this accomplished?
1) PCR product will be purified on a column so that we get rid of the primers, the Fail Safe enzyme, and buffer used in the PCR reaction.
2) Purified product will be digested with the restriction enzymes, BamHI and XbaI (to make ends sticky to facilitate subcloning).
3) A plasmid (Bluescript KS+) will also be digested with BamHI and XbaI (the plasmid will be used to subclone our PCR product).
4) Both the PCR product and the plasmid will be run on an agarose gel.
5) The DNAs will be purified from the gel and quantified.
6) The gel purified plasmid and PCR product will be ligated together.
7) This ligation will then be used in a bacterial transformation (before next lab).

Lab Procedure

Note: Please do not discard tips in the trash bins! Use the provided container on your benchtop.

Part A - Purifying the PCR product

1) Thaw your tubes containing PCR products. Then pipet 50 µl from each of the 4 identical samples into a single separate tube.

2) Add 1 ml of buffer PB to pooled PCR reactions, Mix.

3) Pipet 0.5 ml of this mixture into the top of a QIAquick PCR purification column.

4) Spin for 1 minute at 12,000g. Pour off flowthrough, and pipet an additional 0.5 ml of PCR/PB mixture into column. Spin again and then pour off flowthrough.

5) Add 0.75 ml of PE to column. Spin 1 minute at 12000g. Pour off flowthrough.
6) Spin AGAIN for 1 minute at 12000 to get rid of residual PE. Transfer column to a new clean tube.

7) Pipet 30 µl of heated EB solution into the column, let sit 1 minute. Spin 1min at 12,000g.

8) This time collect flowthrough and SAVE - This is your purified PCR product. Discard column.

**Part B - Digesting PCR product and Bluescript**

**Bluescript Digest:**
1) Take an aliquot of Bluescript. In a 1.5 ml tube pipet:
   a. 5 µl of Bluescript
   b. 20 µl of water
   c. 3 µl of 10X NEB #4 buffer, and mix.
2) Add 1 µl of XbaI and 1 µl of BamHI, mix gently, spin down contents.
3) Incubate for 45 minutes at 37°C.
4) After 45 minutes of incubation, add 1 µl of calf intestinal phosphatase (CIP), mix gently, spin down contents.
5) Return tube to 37°C for another 15 minutes.
6) Remove tube from heat block and add 4 µl of agarose loading buffer, mix and set aside.

**PCR Digest:**
1) Remove 25 µl of purified PCR product from tube and pipet into a fresh tube.
2) Add 3 µl of 10X NEB #4 buffer and mix.
3) Add 1 µl of Xba I. Then add 1 µl of BamHI, mix gently, spin down contents.
4) Incubate for 1 hour at 37°C.
5) Add 4 µl of loading buffer and set aside.

**Undigested/Controls:**
1) To remaining 5 µl of purified (but undigested PCR product) add 5 µl of loading buffer, mix.
2) For the control PCR reaction, remove 20 µl and pipet into a new tube, then add 5 µl of loading buffer and mix.

**Part C - Gel Purifying PCR product and Bluescript**

Two agarose gels (one gel is 1% agarose, the second gel is 2%) containing ethidium bromide will be supplied to you **(wear gloves! ethidium bromide is a known mutagen).**

1.) In Gel #1, load 10 µl of 1 kb ladder in lane 1. Then skip a lane and load your digested Bluescript.
2.) In Gel #2, load 10 µl of 100 bp ladder in lane 1. Skip a lane and load PCR product digest. In the next lane run your purified but undigested PCR product. In the next lane load 25 µl of the control PCR reaction.
3.) Run gels at 100V until the blue dye has run halfway.
4.) Document the gel on the Gel Doc System.
5.) While wearing UV protective goggles cut out Bluescript band with a clean razor blade (cut the smallest size you can but without losing the DNA). The Bluescript band should be around 3 kb. Place the gel fragment in a clean 1.5 ml tube. For the PCR product, cut out the band that was digested with BamHI/XbaI with a clean razor blade into a separate tube.
6.) To each gel slice (Bluescript and PCR product) add 1 ml of NaI solution. Incubate at 55°C for 10 minutes or until gel slice is completely dissolved.
7.) Take a tube containing glass milk and vortex vigorously until the glass is resuspended. Then pipet 5 µl of the glass milk into the tube with the melted slice.
8.) Invert tube to mix and do this several times over a 5 minute period. Spin tube for 15 seconds to pellet glass.
9.) Remove supernatant, add 200 µl of ethanol wash buffer, resuspend glass pellet, then spin. Remove supernatant, wash with ethanol wash buffer, resuspend and pellet again. Repeat this process one more time. Then remove all ethanol traces from pellet (you can re-spin the drops down and remove remaining drops with a P200).
10.) Add 10 µl of TE pH 8 to pellet. Resuspend glass in TE.
11.) Put at 42C for 3 minutes, then spin for 5 minutes.
12.) Transfer supernatant to new tube (the DNA is in the TE solution) and discard pellet.

**Part D - Ligation of PCR product with Bluescript**

1.) We will determine your yield of DNA from the gel with an ethidium spot test (this will be demonstrated to you).

2.) You will ligate approximately 100-200 ng of Bluescript to the PCR product. You should include approximately a 3 fold excess (molar–wise) of PCR product to plasmid. Since the plasmid is 3 kb and the insert is 600 bp, if you added 100 ng of bluescript and a 100ng of PCR product, your PCR product would be in 5 fold excess.

3.) The volume of the ligation should be 15 µl. You will perform two ligations. One will be a negative control with bluescript but no PCR product – the other ligation will contain bluescript and PCR product.
   a. To each tube add the same amount of bluescript (100-200 ng) then add PCR product to one tube and the equivalent volume of water to the negative control tube.
   b. Then add enough water so the volume is 11 µl.
   c. Add 3 µl of 5X ligation buffer. Mix.
   d. Then add 1 µl of T4 DNA ligase to each tube. Mix.
   e. Store at room temperature overnight.

**Part E - Plating Bacteria (done sometime before the following lab day)**
Transformation and plating:
1) Thaw aliquot of competent cells on ice.
2) Add 50 µl of bacteria to each of two pre-cooled 1.5 ml tubes.
3) Pipet 10 µl of your ligation of PCR product in one tube and 10 µl of your control ligation in the other tube. Leave on ice 30 minutes.
4) Then place in heat block at 37°C for 20 seconds. Remove and place on ice 2 minutes.
5) Add 0.9 ml of LB media to tube and shake at 37°C for 45 minutes.
6) Spin tube at 5000 RPM for 3 minutes. Shake out excess LB and resuspend in remaining liquid (about 50 µl).
7) Pipet bacteria on ampicillin plates containing X-gal, and spread. Grow overnight at 37°C.

Culture:
1.) Pick single colonies with a toothpick and drop into tubes containing LB with 100µg/ml ampicillin.
2.) Place tubes in shaker overnight at 37°C.