DGGE FOR *Symbiodinium*

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This protocol explains the step-by-step process for running a DGGE for PCR products of the *Symbiodinium* ITS2. DGGE, denaturing gradient gel electrophoresis, is a technique that uses electrophoresis and denaturing agents, such as urea and formamide, to denature DNA. Thus, the denaturing agents are in a gradient throughout the gel while temperature and voltage are kept constant. DGGE enables the researcher to separate PCR products based on sequences, as opposed to size when doing agarose or polyacrylamide gel electrophoresis. In the case of *Symbiodinium*, DGGE has been used to differentiate between the different strains of *Symbiodinium* (e.g. LaJeunesse, 2001; LaJeunesse, 2002; LaJeunesse et al., 2005).

PCR

- The primers for the amplification of the ITS2 from *Symbiodinium* are ITSintfor2 5'-GAA TTG CAG AAC TCC GTG-3' and ITS2CLAMP 5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GGG ATC CAT ATG CTT AAG TTC AGC GGG T-3' (LaJeunesse & Trench, 2000).

- Amplification is obtained from a touchdown PCR. The touchdown protocol consisted of an initial denaturing step at 92 °C for 3 min, 21 cycles at 92 °C for 30s, 62 °C for 40s, and 72 °C for 30s, decreasing each cycle 0.5 °C, followed by 15 cycles with a 52 °C annealing step, and a final extension at 72 °C for 10 min. Please, follow these steps.

- The conditions are: 1 µL of template at a concentration between 3-10 ng/µL, 10 µL of GoTaq Green Master Mix 1X (2X Green GoTaq reaction buffer, 400 µM each dNTP, 3.0 mM $MgCl_2$, GoTaq DNA polymerase, Promega), 0.25 µM forward primer, 0.75 µM reverse primer, and added Milli-Q water for a final volume of 20 µL per reaction. Although you may vary the final volume and concentration of primers, you should ALWAYS keep a 1:3 ratio because the reverse primer with clamp will tend to form primer dimmers. This is why you always add an excess of reverse primer.

- Concentration of template varies depending upon quality of DNA, source of DNA (from a host, free-living, or culture). Test different dilutions. Once you have good bands, you are ready to go.

THE GEL

Day 0 - SOLUTIONS

1. Prepare all your stock solutions or by making sure you have enough of them. The solutions are:

   Buffer TAE20X
   55g sodium acetate
   7.5g EDTA
   95g Tris base
   Mix with diH_2O and adjust to pH 7.4 with HCL (37%). Bring to a final volume of 1L.

   10% APS
   2ml APS
   18ml diH_2O

   40% Acrylamide
   40g Acrylamide
   1.09g Bis-acrylamide
   Bring to a final volume of 100ml.

   8% Acrylamide
   40% x Vi=8% x 100ml
   Vi=20ml acrylamide 40% and 80ml diH_2O

   Formamide
   Original

   Urea
   Original

   TEMED
   Original

USE NITRILE GLOVES AT ALL TIMES!

Day 1 – AM - CASTING THE GEL

(*I would recommend running a maximum of 2 gels at a time)

1. Start by preparing your 100% denaturing solution as follows:

Final volume	40% Acrylamide	Formamide	Urea	20X TAE	diH_2O
30mL (2 gels)	6mL	12mL	12.62g	1.5mL	4.5mL
15mL (1 gel)	3mL	6mL	6.31g	0.75mL	2.25mL

2. Prepare the glass plates and thaw APS while the urea dissolves.

3. Clean both surfaces of the glasses with 100% EtOH and let it air dry.

4. Check that the yellow gel wrap gasket is dry and has no holes. Place it around the thinner round-in-two-corners glass plate. Make sure it is tight against the glass and the corners match perfect with the "cuts" of the yellow gel wrap gasket. Pay attention to the raise on one side of the yellow gel wrap gasket. This will be your INNER part.

5. Place one spacer on each side of the glass plate, next to the raise of the yellow gel wrap gasket (INNER side). They have to be in a straight line.

6. Put the squared-corner glass plate on top of the curved one. It has to match perfectly in the bottom and sides to avoid leaking.

7. Use the casting clamps (closed clamps) to join both glass plates. (* I suggest starting from the bottom part of the glass plates and then place the ones on the side).

8. Check that there are no leaks with Milli-Q H_2O. If there are leaks, dissemble everything, dry and start again until you have no leaks.

9. We are now going to prepare the gradient for the DGGE. In the case of ITS2 of *Symbiodinium* we use 45-80%, using 8% acrylamide and 100% acrylamide, which consisted of 7 M urea and 40 % deionized formamide (which should be dissolved by now).

10. Marked are two falcon tubes with 45 and 80 (if not, go ahead and mark them).

11. In the 45-tube, add 6.3mL of 8% (0%) acrylamide and 5.2mL of 100% denaturing acrylamide solution (the one that you just dissolved).

12. In the 80 tube, add 2.3mL of 8% (0%) acrylamide and 9.2mL of 100% acrylamide (the one that you just dissolved).

13. BEFORE adding the TEMED and APS, prepare the gradient maker, the pump (if not doing by gravity) and make sure the glass-assemble is empty. The silicone tubing from the gradient maker should connect to the glass-assemble. Place a small magnet on the left hole of the gradient maker and put the gradient maker on a shaker. Check that both valves of the gradient maker are closed! (The one that goes to the tubing and the one that connects both chambers).

14. Once you have everything to pour your gel, add 5µL of TEMED and 95µL of APS to EACH gradient solution, 45% and 80%.

15. Immediately place the mix in the gradient maker. It is important that you put the 80% gradient on the LEFT chamber of the gradient maker. This is because you greater percentage of denaturing solution will be in the lower part of the gel.

16. Pour the gel by opening the valve that goes to the tubing. This will make the 80% denaturing solution to be poured FIRST.

17. When the level of the 80% denaturing solution reaches the silver sticker on the gradient maker, open the middle valve, allowing the two denaturing solutions to mix.

18. Place the combs. No bubbles should be seen next to the combs. In case that you have bubbles, remove the combs and place them again slowly.

19. Overflow the volume of gel. This way, you will ensure that, upon polymerization, the volume of the gel will be enough to still have wells.

20. When you finish pouring, immediately place the gradient maker in running water to avoid polymerization in the tubing.

21. Repeat from steps **11-20** for the second gel.

DAY 1 – PM - LOADING AND RUNNING THE DGGE

22. ONE HOUR BEFORE LOADING, Fill the tank with 19L of diH_2O and 1L of 20X TAE for a running buffer of 1X TAE. Heat to 60°C.

    Note: to know the time to start running, keep in mind that it will run for 14 hours. * My advice will be to start early on day 2 for staining, visualization and excising bands. Thus, I suggest start running at 6 p.m. to the latest. In this case, start warming at 4:00 p.m., pre-run from 5:00-5:30p.m., and load from 5:30-6:00p.m.

23. Remove the casting clamps, the combs and the gel wrap gasket only in the lower part of the gel.

24. Label the gels e.g. "Gel 1" and put an arrow. This will help you the next day to remember which gel you loaded first and the direction in which you loaded (see **steps 33** and **39**).

25. Place gel in the gel cassette and attach with the running clamps (opened clamps). If you are running only one gel, make sure you put a set of glass plates in the other side of the gel cassette. This will create a "pool" of buffer constantly pump by a tubing. The "pool" will be formed in between the glass plates, which is important for keeping the electrode wet during the run.

26. Connect the cable and the hose to the gel cassette. Make sure, buffer is running through the hose and the pool, mentioned above, is formed.

27. Pre-run at 100V for 30 min. Clean the wells using a Pasteur pipette with the same buffer of the tank.

28. Load your samples. Clean well by well with buffer as you load. This will ensure that the entire sample will go to the bottom of the well and you will have a better run of the sample in the gel.

29. Run for 14 h, 100 V, 60°C.

DAY 2 – VISUALIZATION AND BAND EXCISING

30. Make EtBr staining bath: 1L of diH_2O and 120µl of concentrated EtBr.

31. Turn off power supply, unplug everything from the gel cassette, empty "pool" in between glass plates, and pull out everything from tank.

32. Remove gel wrap gasket and spacers.

33. Carefully separate the glass plates. Make sure you know the DIRECTION in which you loaded; this is critical in this step and in **step 39**.

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    Note: An easy way to separate both glass plates is by using a spatula or something similar that you can slide in between the glass plates. Once you separate one side, the rest will come out easily.

34. Place in EtBr for staining for 20 min.

35. Label 0.5mL tubes and cover each tube with aluminum foil. You may have up to 40 bands per gel.

36. Prepare another container with diH_2O only.

37. Place gel in de-staining bath for 20 min.

38. In the second 20-minute wait, finish labeling your tubes and covering them with aluminum foil. You may also want to prepare your material for excising bands: tips next to the transiluminator, TBE 1X (to wet the gel, see **step 42**), a small beaker with 30mL of 100% EtOH, and tweezers.

39. Place gel in transiluminator.

    Note: In this step it is also critical to know the DIRECTION in which you loaded. To remove the gel from the glass plate, make sure the gel is wet from the bath. Put the entire gel on the transiluminator and move it a little bit, so that water comes in between the gel and the glass plate. As with **step 33**, once one part of the gel detaches from the glass plate, the rest will peel off easily.

40. Take a picture of your gel and save it immediately!

41. Label the bands in your image. This will guide you through all the excising process.

42. Before excising bands, wet your gel. This will make band excising much easier and faster!

43. Use the tips to cut the bands. Use a new tip for each band. An easy way to cut the bands is by "drawing a rectangle" around the band, but start by cutting the longer sides of the rectangle ("up and down"). Then cut the sides ("left and right"). Start excising you bands from the faintest ones to the brightest ones. With the help of the tip, put the excised band in a different part of the gel. Use the tweezers to put the bands in the tubes. To clean the tweezers, submerge them in 100% EtOH. Dry with Kimwipe before picking the bands. Reduce, as much as possible, the amount of time you have the UV light on.

    Note: Tips are good to use because you can put as much pressure as you want without scratching the transiluminator. Do it accurately and fast.

44. Repeat **step 32-43** for a second gel.

45. After you finish cutting all the bands, add 30μL of nuclease-free H_2O to each band. Using a tip, squeeze the band in the water and make sure the band is in the water.

46. Leave shaking overnight covered with foil at a medium speed.

DAY 3-4 – PRECIPITATION

47. Transfer the liquid (NOT THE GEL, since it will inhibit the PCR) to a corresponding labeled 1.5mL tube.

48. Add 800µL of cold 100% molecular grade EtOH.

49. Put at -20°C overnight.

50. The next day, centrifuge at 12,000rpm for 30 min at 4°C.

51. Discard the EtOH.

52. Add 300µL of cold 70% EtOH.

53. Centrifuge at 12,000 rpm for 10 min at 4°C.

54. Discard the EtOH.

55. Dry completely (avoid over-drying).

56. When completely dry, add 30µL of nuclease-free H_2O or TE pH 8.0.

DAY 4 (or later) – REAMPLIFICATION

57. Reamplify the bands by adding only 1µL of template. The primers used are ITSintfor2 (see **"PCR"**) and the ITSRev 5'-GGGATCCATATGCTTAAGTTCAGCGGGT-3' (Coleman et al., 1994).

58. The conditions are: 1 µL of template, 10 µL of GoTaq Green Master Mix 1X (2X Green GoTaq reaction buffer, 400 µM each dNTP, 3.0 mM $MgCl_2$, GoTaq DNA polymerase, Promega), 0.25 µM ITSintfor2 primer, 0.25 µM ITSRev primer, and added Milli-Q water for a final volume of 20 µL per reaction.

59. The protocol consisted of 92 °C for 3 min, 35 cycles at 92 °C for 30s, 52 °C for 40s, and 72 °C for 30s, and a final extension at 72 °C for 10 min.