Microbial DGGE

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Techniques and primers described here originate from Dr. Andrei Chistoserdov.

Microbial DGGE is a culture-independent technique for profiling bacterial communities. Template DNA starting material can originate from environmental or organismal samples.

Primers used for this protocol are as follows:

AC341F	CCTACGGGDGGCWGCAG
AC907R	CCGYCWATTCMTTTGAGTTT
AC341F-Clamp	CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGCCTACGGGDGGCWGCAG

Reagents:
GoTaq Green Master Mix (Promega, Madison, WI)
25 mM $MgCl_2$
PCR-grade water
Milli-Q water
Deionized water
40% Acrylimide, 1.06 % Bis-Acrylimide
7 M urea, 40% formamide
50x TAE
Ammonium persulfate
Tetramethylethylenediamine (TEMED)
Loading dye
100% molecular biology grade ethanol

***This protocol uses the CBS Scientific (Del Mar, CA) DGGEK-2401-110 DGGE apparatus and associated CBS Scientific gradient maker and gel casting equipment.**

Day 1

A word of advice: perform the PCR amplification either in the morning of day 1, or the day before. Began the acrylimide gel casting in the afternoon and allow it to run overnight.

PCR Amplification of 16s fragments

PCR products for DGGE are created by amplifying the template with degenerate universal primers for a fragment of 16s.

16s PCR mix, for each reaction:
25 uL Promega Green Mastermix
7.5 uL AC341F-Clamp (10 uM)
2.5 uL AC907R (10 uM)
1 uL $MgCl_2$ (25 mM)
13 uL water
1 uL template*

*Amount and concentration of template may require optimization.

PCR Program:
Following an initial denaturation at 95°C for 3 minutes, a touchdown program began, with 21 cycles consisting of a 1 minute denaturation at 95°C, 1 minute annealing beginning at 65°C and ending at 55°C (decreasing 0.5°C per cycle), and a 1 minute extension at 72°C. An additional fifteen cycles (55°C annealing) followed, with a 5 minute final extension at 72°C.

Confirm efficient and consistent amplification by running 3 uL of the reaction product on an agarose gel. The desired PCR product is 594 b.p. (including the GC clamp). However, when amplifying from cDNA, a fragment of approximately 800 b.p. from the host transcriptome is also amplified. This fragment does not appear in the final DGGE gel.

Gel Casting

1. Clean the glass gel casting plates thoroughly, either with a commercial solution for this purpose, or with detergent, DI water, and ethanol. Dry with a lab wipe, not a paper towel. One side of each plate, the side which will be on the inside, must be critically clean.

2. Affix the blue gasket* to the glass plate with curved corners The gasket seal is the shorter side of the gasket, with a raised ridge; this will be on the inside of the casting rig. Insert the dark grey separators on the inside of the gasket-covered glass., according to shape. Place the glass with the square corners atop the glass with the curved corners. Secure the plate together (with gasket and spacers in place) with the casting clamps (closed, tighter, stronger clamps), with 2 on each side and 2 on the bottom. Inspect to ensure that a seal is made by the gasket.

***The blue gasket and dark grey separators are used for casting gels of ~40 mL.**

3. Check that the gradient maker mixing chamber and pump are working by running DI water through it. Ensure that it is clean and not clogged, and run it dry. Close the valve on the mixing chamber (put it in the “up” position) and the inline valve on the attached tubing.

4. Either thaw a frozen aliquot of 10% APS, or make a fresh stock. Prepare by mixing 1000 ul DI water with 0.1 g ammonium persulfate (molecular biology grade). Ready the following reagents:

Reagents	**Left Chamber**	**Right Chamber**
Milli-Q Water	10.832 mL	3.102 mL
Loading dye	---	500 uL
40% Acrylimide, 1.06 % Bis-Acrylimide	3.075 mL	3.075 mL
7 M urea, 40% formamide	6.183 mL	13.413 mL
50x TAE	410 uL	410 uL
APS	100 uL	120 uL
TEMED	6 uL	7.5 uL

*The addition of APS and TEMED cause the polymerization. Add these reagents last, and do so quickly. Immediately after the adding APS, stir the appropriate side of the missing chamber. Immediately after the adding TEMED, stir the appropriate side of the missing chamber. Both sides need to be stirred with a stirring rod; the magnetic stir bar (which should be in the left chamber and turned on) may not be sufficient, and clogging may occur.

5. Immediately after the addition of APS and TEMED and stirring, open the valve on the mixing chamber, as well as the in-line valve on the tubing. Turn on the pump and place the outlet needle on one side of the gel casting rig. When the gel is 3-4 cm from the top of the casting plates, turn off the pump.
Run DI water through the mixing chamber and pump to clean it. Add a bit of water to the top of the gel. Allow the gel to polymerize (~2 hours, but gels can be cast in the morning and run at the end of the day, if necessary).

6. Fill the tank with TAE buffer: 200 mL 50x TAE and 20 L DI water. Heat to 60°C.

7. After the gel has polymerized, pour off the water on top of the gel, and prepare the stacking gel in a 15 mL conical tube.

Reagents	**Volume**
Milli-Q Water	8.3 mL
40% Acrylimide, 1.06 % Bis-Acrylimide	3.075 mL
50x TAE	200 uL
APS	70 uL
TEMED	10 uL

8. Mix immediately after the addition of APS and TEMED. With a disposable transfer pipette, load the stacking gel mix on top of the gradient gel till the casting rig is

nearly full and carefully insert the desired comb. Allow the stacking gel to polymerize, approximately 15 minutes.

9. Carefully remove the clamps and gasket, and clamp the gel/glass plates to the plastic support with the running clamps (not-so-tight/open clamps). Place the whole system inside of the DGGE tank and connect a buffer circulation hose such that buffer flows over the top of the gel. Carefully withdraw the gel comb and rinse wells.

10. Load the appropriate sameples (PCR products), up to 50 uL per lane. Promega Green Mastermix has an integral loading dye, so additional loading dye is unnecessary. Carefully note the order of samples.

11. Connect the power leads, close the lid of the DGGE system, and engage the power supply. Run at 97 volts for 14 hours.

Day 2

Staining and Visualization

Disengage the power supply, disconnect power lead, remove the gel system from the tanks, and remove clips. With extreme caution, separate the two plates using a thin plastic spatula as a wedge if necessary. The aim is to end up with the gel on one of the glass plates.

Transfer the plate with the gel attached gel-side up into an ethidium bromide bath (120 uL of 10 mg/mL EtBr in 1 L of DI water). Stain for 20 minutes. Destain in DI water for 20 minutes.

Carefully transfer the gel from the plate to the UV transilluminator by inverting the plate with one end lower and closer to the transilluminator and letting the gel slowly fall from the plate to the transilluminator, from one end to the next.

Capture the gel image and ensure a good exposure.

Band excision and reamplification in preparation for downstream sequencing

Identify bands to be excised. Bands can be cut with either a 10 uL pipette tip, or with a blade. If using a pipette tip, wet the get first.

Place each excised band in a 0.5 mL tube cover in aluminum foil. Once all bands are excised, add 30 uL of nuclease-free water to each tube. Just after adding the water, use the pipette tip to gently squeeze each gel slice against the tube wall, and confirm that the gel slice is immersed in water.

Cover all tubes with foil and shake overnight at room temperature.

Day 3

Label a set of 1.5 mL microcentrifuge tubes, one corresponding to each gel slice. Transfer the liquid from the incubating gel slice (NOT the gel itself, as this will inhibit PCR). Add 800 uL -20°C molecular biology grade ethanol. Incubate overnight at -20°C.

Day 4

Centrifuge at 12,000 g in a tabletop centrifuge for 30 minutes. Decant and discard ethanol. Add 300 uL -20°C 70% ethanol. Centrifuge at 12,000 g for 10 minutes. Carefully remove and discard ethanol by pipetting; you will not be able to see a pellet. Air dry at room temperature. Resuspend in 30 uL nuclease-free water.

Reamplification

Reamplifiy the DNA from the excised gel bands with the following reaction mix:

Band Reamplification PCR mix, for each reaction:
12.5 uL Promega Green Mastermix
1.25 uL AC341F (10 uM)
1.25 uL AC907R (10 uM)
8.5 uL water
1.5 uL template

Use the same touchdown program as for the original amplification. Notice that the reamplification reaction mix uses a forward primer lacking the GC clamp, and that no additional $MgCl_2$ is added to the mix. The PCR product should be 556 b.p. in length. Prepare for direct sequencing.