Fingerprinting aquatic virus communities using denaturing gradient gel electrophoresis (DGGE)

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Abstract

Using DGGE, similar-sized PCR products that differ in nucleotide composition (sequence) can be separated in denaturing gradient gels. Denaturing gradient gels are created using acrylamide (structural material) solutions that contain different amounts of denaturants (urea and formamide), such that the highest concentration of denaturants is at the bottom of the gel and the lowest concentration is at the top. As dsDNA fragments differing in sequence migrate into the gel during electrophoresis, they encounter increasing concentrations of denaturants, and each fragment partially melts (i.e., double-stranded regions dissociate into single-stranded) at a different place in the gel depending on its sequence. Because the electrophoretic mobility of partially melted DNA fragments is greatly reduced compared to dsDNA, same-sized DNA fragments with different sequences focus at different positions in these gels. Because PCR with universal primers can be used to amplify related, but different, DNA sequences, and different sequences focus at different positions in a denaturing gradient gel, DGGE can be used to produce a unique banding pattern, or fingerprint, for each PCR product amplified from different microbial communities.

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Guidelines

Equipment and reagents:

* DGGE apparatus, e.g., Hoefer Scientific SE600, Bio-Rad DCode system (includes gradient former), Ingeny, CBS Scientific;

* Peristaltic pump and gradient maker for casting gradient gels, e.g., SG Gradient Maker (GE Healthcare);

* Power supply for electrophoresis systems;

* Denaturing gel solutions: Solution A (for 250 mL of an 8% gel solution with 0% denaturant), 50 mL 40% acrylamide/bis stock solution (37:5:1 acrylamide:bis-acrylamide solution), 2.5 mL 50× TAE, adjust to 250 mL with sterile distilled water (sdH_2O); and Solution B (for 250 mL of an 8% gel solution with 100% denaturant; i.e., 7 M urea and 40% formamide), 50 mL 40% acrylamide/bis stock solution (37:5:1 acrylamide:bis-acrylamide solution), 2.5 mL 50× TAE, 105 g urea, 100 mL deionized formamide, adjust to 250 mL with sdH_2O;

* 10% wt/vol ammonium persulfate solution in sdH_2O (APS); this reagent should be prepared fresh

each time it is used;

* TEMED (N,N,N′,N′ -tetramethylenediamine);

* 1× TAE running buffer (from 50× buffer stock solution: 20 mM Tris-acetate (pH 7.4), 10 mM sodium acetate, 0.5 mM EDTA);

* 6× loading buffer (e.g., 60% glycerol, 10 mM Tris-HCl, pH 7.6, 60 mM EDTA, 0.03% bromophenol blue, 0.03% xylene cyanol FF);

* Fluorescent DNA strain (e.g., SYBR Gold or SYBR II gel stain; Molecular Probes);

* UV transilluminator and gel documentation system.

Figures and Tables:

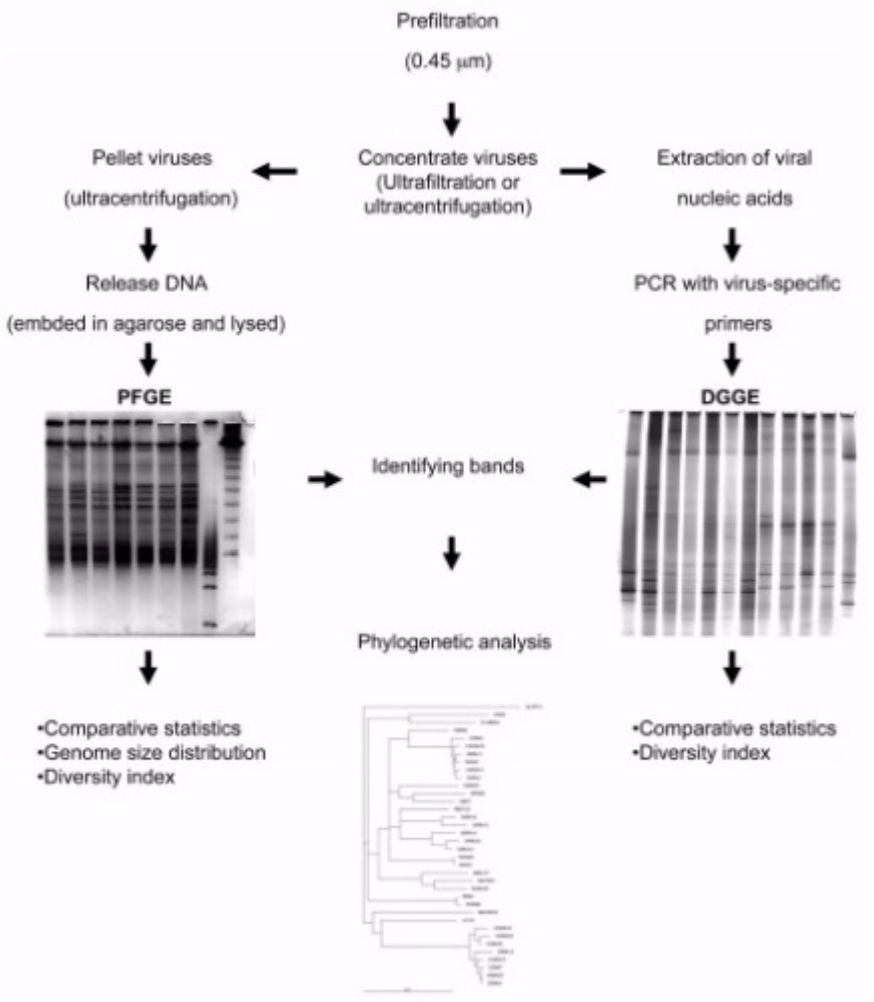

Fig. 1. Flowchart of methods involved in figerprinting aquatic virus communities.

Table 1. Formulation for DGGE gels using 0% and 100% denaturing solutions.

% denaturant of desired gel solution	Volume of solution A (0% denaturants)	Volume of solution B (100% denaturants)
0	14.50	0.00
15	12.32	2.18
20	11.60	2.90
25	10.87	3.63
30	10.15	4.35
40	8.70	5.80
45	7.97	6.53
50	7.25	7.25
55	6.54	7.96
60	5.80	8.70
65	5.07	9.43
70	4.35	10.15

Table 2. Denaturant amounts for various denaturing gel solutions.*

Denaturant concentration	**10%**	**20%**	**30%**	**40%**	**50%**	**60%**	**70%**	**80%**	**90%**
Formamide, mL	4	8	12	16	20	24	28	32	36
Urea, g	4.2	8.4	12.6	16.8	21.0	25.2	29.4	33.6	37.8

*For 8% acrylamide gels, add 20 mL of a 40% acrylamide/bis (37:5:1) solution, 2 mL 50x TAE, and bring the volue up to 100 mL using sdH_20

Analysis of PFGE and DGGE fingerprints:

DGGE and PFGE fingerprints can be analyzed using a variety of commercially available gel analysis software products (e.g., GelCompar II, Applied Maths; BioNumerics 5.1, Applied Maths; Quantity One, Bio-Rad). A common method to analyze DGGE/PFGE fingerprints involves creating a binary matrix representing the bands occurring in a set of DGGE/PFGE patterns. The presence or absence of bands in a sample is simply scored in a binary manner as 1 (present) or 0 (absent), relative to all of the bands detected in a set of DGGE/PFGE patterns. The binary data can then be presented in a dendrogram where the differences in fingerprint patters are represented in a graphical format or as a dendrogram using a distance-based cluster analysis techniques such as unweighted pairwise grouping with mathematical averages (UPGMA). Another possibility is to use multidimensional scaling (MDS) to reduce a complex fingerprint pattern to a point in a two-dimensional space (Van Hannen et al. 1999a). It is important to note that these types of analyses depend on consistency when detecting bands, and subjective determination of presence or absence should be avoided. Luckily, most commercially available gel analysis software programs allow researchers to use automated band detection parameters (e.g., band width and intensity), or even set their own thresholds for each parameter. As an alternative to comparisons based on presence or absence, the overall pattern of gel lanes can be compared directly using densitometry profiles (i.e., the pixel intensities at discrete positions in the gel). This type of analysis is based on pairwise correlations of profiles and can be used to avoid biases associated with band detection or loading unequal amounts of DNA. For densitometry profile analyses, each lane profile is compared to the others, and the resulting matrix of correlation

values correspond to lane similarities. The correlation values can then be transformed to dissimilarity values (1 – similarity) that can be used for cluster analysis via UPGMA.

Quantitative analysis of DGGE and PFGE gels is also possible. For DGGE, only semiquantitative analysis is possible, since individual band intensities cannot be used to infer target abundances in natural samples because differences in band intensity can arise from variable amplification efficiencies for different targets, and/or differences in background DNA. Thus fingerprint patterns can be compared, but individual band intensities should not be used to infer target abundance in natural samples. For PFGE, quantitative analysis involves measuring the relative fluorescence of each band. Based on this information, it is possible to obtain values for richness and abundance that can be used to calculate diversity indices. This type of analysis can be conducted using the commercial software noted above. Of course, it should be noted that both DGGE and PFGE are subject to a number of confounding errors. These sources of error should be carefully considered when deciding what types of analyses or comparisons should be conducted and not the least when the outcome of the analysis is interpreted.

Protocol

Gel preparation

Step 1.

Using lint-free tissues, wash glass plates, spacers, and combs thoroughly with 70% ethanol.

NOTES

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Do not use soap or harsh abrasive cleaning materials to clean any of the equipment. If the materials are cleaned diligently, there is no need to use any detergents; a simple water rinsing followed by 70% ethanol will suffice.

Gel preparation

Step 2.

Assemble the gel sandwich by placing the small glass plate on top of the large plate, being sure to correctly place a 1-mm spacer along each edge of the plate assembly.

NOTES

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The following instructions will vary depending on the apparatus used; refer to the manufacturer for specific instruction pertaining to their system.

Gel preparation

Step 3.

To prevent current leakage and the resultant “smiles” in the bands near the edges of the gel, grease both sides of the spacers with as little as possible silicon grease to cover the full length of the spacer but only a quarter of the spacer width.

Gel preparation

Step 4.

Attach the plate clamps and place the entire assembly into the casting stand.

Gel preparation

Step 5.

Inspect the plate assembly to ensure that the two glass plates and the spacers form a flush surface

along the sides, and ensure that all gaskets adequately seal the plate assembly.

NOTES

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Breaches in the seal of the plate assembly with the bottom of the pouring stand will result in leakage during gel polymerization.

Gel preparation

Step 6.

Check the gradient maker and flush with sdH_2O.

Gel preparation

Step 7.

Empty pump tubing and attach pipette tip at the outlet tube to the top-middle of the gel chamber.

NOTES

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Although the reagents listed specify the formulation of an 8% gel, the percentage of acrylamide in the gel depends on the size of the PCR products to be resolved; i.e., 6% gel is recommended for 300–1000 bp, 8% for 200–400 bp, and 10% for 100–300 bp (BioRad manual, DCode Universal Detection System)

Gel preparation

Step 8.

To optimize the gradient conditions for a new DGGE experiment (new primer sets, new sample type/habitat, etc.), we usually start with a relatively broad gradient (20% to 80% denaturant). We then focus the gradient around the area of interest to include the highest and lowest bands in different samples.

Gel preparation

Step 9.

Table 1 (guidelines) can be used to determine the appropriate composition of the denaturing gradient gel (16 × 16 cm) that has a total volume of 29 mL.

NOTES

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The size and volume may vary between the different apparatuses.

Gel preparation

Step 10.

Make up two solutions of 14.5 mL each, a low denaturant concentration solution and a high denaturant concentration solution.

NOTES

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For example, if you wish to make a 30% to 55% gradient, then you would make a 30% (low) solution and a 55% (high) solution based on the reagent volumes in the table.

Gel preparation

Step 11.

Mix the solutions A and B (guidelines) to the desired percentage.

NOTES

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Alternatively, once desired gradient conditions are empirically determined, each denaturing solution can be made directly using the reagent volumes and amounts shown in Table 2

(guidelines).

Gel preparation

Step 12.

After preparing the denaturing gel solutions, degas for 15 min and filter through a 0.45-mm syringe filter.

DURATION

00:15:00

Gel preparation

Step 13.

Immediately before casting the gel, add 145 µL 10% APS and 7.25 µL TEMED into each solution and swirl gently to mix.

NOTES

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These reagents begin the polymerization of the acrylamide.

Gel preparation

Step 14.

At this point, you will have approximately 10 min to pour the gel.

DURATION

00:10:00

Gel preparation

Step 15.

Make sure the pump is off and the gradient maker-channel is closed (handle up).

NOTES

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We use the SG gradient maker attached to a peristaltic pump. In our hands, this system has produced more consistent gradients than other systems.

Gel preparation

Step 16.

Pour solution with the highest denaturant in the right leg of the gradient maker (at the pump side) and the solution with the lowest denaturant in the left leg.

Gel preparation

Step 17.

Turn the magnetic stirrer on, while simultaneously starting the pump (5 mL/min).

Gel preparation

Step 18.

Simultaneously, start the pump (5 mL/min) and move the handle of the gradient maker to horizontal position (channel open).

NOTES

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The gel chamber fills slowly.

Gel preparation

Step 19.

Use approximately 4–5 min to fill the gel.

DURATION

00:05:00

NOTES

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It is important to avoid bubbles in the gel, as this will stop the products from migration.

Gel preparation

Step 20.

Empty the tubing and flush thoroughly with sdH_2O.

Gel preparation

Step 21.

Insert the comb, flat or straight side down, making sure that there are no bubbles under the comb.

NOTES

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This is to ensure a smooth, even finish when you come to create your wells after your gel has set.

Gel preparation

Step 22.

Different combs (16 or 20 wells) are available, depending on the number of samples that you want to run.

Gel preparation

Step 23.

Cover gels with cling film and allow 2 h for the gel to polymerize.

DURATION

02:00:00

Gel preparation

Step 24.

The gel can be kept at 4°C until the next day.

Electrophoresis

Step 25.

Prepare approximately 7 L of 1× TAE and fill the buffer chamber. Put about 0.5 L aside for later use.

NOTES

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Different DGGE systems require different volumes of running buffer. The following procedure is based on the DCode system from Bio-Rad.

Electrophoresis

Step 26.

To enhance the circulation of the running buffer, place the tank on a magnet stirrer and add a magnetic stirrer bar in the bottom of the tank.

Electrophoresis

Step 27.

Preheat the buffer in the DCode apparatus to 60°C; this will take about 2 h.

DURATION

02:00:00

Electrophoresis

Step 28.

Attach the gel plates to the core assembly.

Electrophoresis

Step 29.

Loosen the clamps a quarter-turn counterclockwise to prevent breaking of the sandwich clamps (due

to heat expansion).

Electrophoresis

Step 30.

Then place the core assembly into the heated buffer in the tank.

NOTES

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Note: the following procedures can be carried out either while the gel is standing at the bench or when the core assembly is loaded in the tank.

Electrophoresis

Step 31.

Switch off the magnetic stirrer (if loading in tank).

Electrophoresis

Step 32.

Flush each well with buffer using syringe with needle to remove any unpolymerized acrylamide and excess urea.

NOTES

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Failure to do this might result in uneven well floors and unresolved bands.

Electrophoresis

Step 33.

Flush each well with buffer again before loading approximately 10–50 µL of PCR products mixed with loading dye into each well.

NOTES

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The volume loaded depends on yield and the expected diversity of the PCR products. For quantitative comparisons among samples, equal quantities of DNA must be loaded in each lane. It is also recommended to use standard markers on the gels to allow gel-to-gel comparisons. The standard marker should be composed of fragments covering a range of denaturant concentrations.

Electrophoresis

Step 34.

To quantify PCR products, we recommend gel quantification using a DNA mass standard (e.g., Low DNA Mass ladder; Invitrogen) and commercially available gel quantification software such as Quantity One (Bio-Rad) or free software such as Image J (available for download at http://rsbweb.nih.gov/ij/download.html).

Electrophoresis

Step 35.

In the DGGE gel, load a marker on each side of the gel adjacent to the samples (markers can be custom made for each DGGE application using known PCR products, or common molecular weight markers can be used) for determination of band positions, or comparisons of different gels.

Electrophoresis

Step 36.

Apply a loading voltage of 200 V for 5 min before starting the pump to circulate the buffer, then turn on the magnetic stirrer.

DURATION

00:05:00

Electrophoresis

Step 37.

The length of the run and the running voltage depend on the size of the PCR products and the percentage of acrylamide/bis in the gel. A good starting point is to run the gel at 60 V (about 20 mA for one gel) for 19 h.

DURATION

19:00:00

NOTES

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Optimal run times and conditions, however, should be empirically determined for each type of fragment.

Electrophoresis

Step 38.

When the electrophoresis is complete, take apart the apparatus and remove the glass plates from the gel clamps.

Electrophoresis

Step 39.

Carefully separate the plates, leaving the gel exposed on the large plate.

Electrophoresis

Step 40.

Use the edge of the small plate to trim the well walls, but be sure to leave the leftmost wall slightly higher than the others for use as a gel orientation reference.

Electrophoresis

Step 41.

For easy manipulation, the gel can either be stained on the large plate or transferred to, stained on, and transported on a plastic sheet.

Electrophoresis

Step 42.

Stain the gel for 30 min in 50–500 mL fluorescent gel stain.

DURATION

00:30:00

NOTES

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Depending on the container, and according to manufacturer’s instructions.

Electrophoresis

Step 43.

Destain the gel for 30 min in 1 × TAE.

DURATION

00:30:00

NOTES

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Not always necessary.

Electrophoresis

Step 44.

Remember, the fluorescent dye binds to nucleic acids; therefore, it is important to minimize contact with skin, so gloves (powder-free) should be worn.

Electrophoresis

Step 45.

If staining in a container, use plastic and not glass, as the fluorescent dyes accumulate over time on glass surfaces.

Electrophoresis

Step 46.

Slide the gel off of the plastic sheet or large plate onto a UV transilluminator and view the gel.

NOTES

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As an alternative to staining gels with fluorescent dyes, some researchers have used fluorescently labeled PCR primers for DGGE analyses (Neufeld and Mohn 2005).