Protocol for bean SSR screening

This protocol combines the use of the C.B.C SCIENTIFIC Mega-Gel High Throughput electrophoresis system with the use of a silver staining procedure for fast and cheap SSR genotyping.

The gel system apparatus preparation and electrophoresis is according to the instructional manual of the Mega-Gel High-throughput Electrophoresis system C-DASG-400-50 from C.B.S .SCIENTIFIC.INC.

The silver Staining protocol has been optimized by modifying and combining various polyacrylamide gel silver staining protocols.

Glass plates preparation

1. Select a pair of glass plates to be used. One notched plate and one unnotched gel plate
2. Clean the back plate using 70% ethanol, wiping it with a lint –free wipe across and up and down three times
3. Apply the Bind Silane to the gel plate. For this, Mix 1.1 ml 95% Ethanol: 0.5 % Acetic Acid solution with 2.0 µl Bind Silane in an eppendorf tube, apply the Bind Silane solution to the surface of the plate and very quickly, wipe the glass plate as evenly as possible. Let the glass dry for 5 minutes and lightly clean it one more time with 70% ethanol and dry it with a lint free wipe.
4. Change gloves and clean the notched plate with 70% ethanol, wiping it with a lint –free wipe across and up and down three times

5. Treat the notched glass plate sigmacoate. For this, saturate a lint-free wipe with sigmacote and wipe the glass both vertically and horizontally. Wait 5 minutes to dry. Again clean it lightly with 70% ethanol and dry it with a lint- free wipe.
6. Assemble the glass plate unit: lay the back glass plate on the bench with treated face up. Fit blue gasket around the sides and bottom of the gel plate. Be sure the grooved surface of the gasket faces up. Leave approximately 2.5cm overhang on the top on each side. Slide a finger along the gasket to ensure a tight fit to the glass. Lay spacers on each side of the glass plate placing the curved end toward the bottom with the curved portion fitting against the blue gasket.
7. Carefully lay the notched plate, treated side down facing the gel plate on top of the bottom assembly. Working from the bottom corner, place clamps on both sides of the corner to firmly hold the glass plates together assuring a tight seal. Continue placing clamps on bottom and both sides of the glass unit.
8. Stand glass plate unit upright using the clamps as base.

    a. Note: If needed, the seal can be tested by pouring a small amount of distilled water between the glass plates. If water leaks from the edges, repeat the fitting of the glass plates by taking them apart and readjusting the gasket, spacer bars, and clamps. Test with water again.

9. Slowly and carefully pour the Acryl amide gel mixture between the glass plates up to the top.
    a. Note: Avoid to trap bubbles in the gel as much as possible since bubbles can cause the gel cracking as it dries up; a plastic ribbon can be used to release any bubbles trapped between the glass plates.

10. Insert the comb and continue to fill the gel space with acrylamide solution if the level has lowered. A pipette may be used to top up the gel if needed. The gel should fill the spaces between the teeth on the comb with no bubbles trapped. If bubbles are trapped, gently lift the comb and push down again allowing the bubbles to escape. Wait 1 hour for the gel to polymerize.

11. After the gel has completely polymerized, lay the apparatus on the bench, remove the clamps, and gently remove the blue gasket by pulling gently from one end until it is freed from the glass plates.

12. Holding the gel unit from both sides, place the unit carefully in the bottom reservoir which has been filled with approximately 300 ml 0.5 X TBE solution notched plate facing inward. Check for bubbles trapped on the bottom edge of the gel and if there is any, use the Pasteur pipette to push bubbles out towards the edge. Gently lift the glass unit up and rest it on the footing in the lower reservoir carefully without trapping in bubbles. Attach the lower reservoir safety covers to the lower reservoir.

13. Slide the upper reservoir to the glass plate sandwich and clamp using the bar clamp. Tighten the clamp by turning the black knobs until they are firm but not tight to avoid any breakage of the glass.

14. Verify that the upper buffer reservoir is closed and fill the top reservoir with 0.5XTBE solution to cover the top of the gel. Make sure that there are no buffer leaks from the upper reservoir. Using both hands, remove the comb by pulling up on the backside with your fingers and down on the gel plate with your thumb. Work slowly and carefully avoid damaging the wells.

15. After the gel is in place and all reservoirs filled, place covers and wires in their proper location. Turn the power pack on and verify if the power is being conducted through the gel

Loading the Sample

- After the PCR has been done, add 8 µl of the loading dye to each tube
- Load the DNA ladder and then 14 ml of your sample in each well

Electrophoresis

- After loading the sample, attach the upper safety cover. Connect the unit to the power supply with proper polarity: black leads connected to the black cathode and the red leads connected to the red anode. Turn on the power pack, set the voltage to 250V, and allow the gel to run for 2.5 hours. Monitor the progress of electrophoresis following the migration of the dye.

While waiting for the gel to run make the Fix Stop Solution, the Stain Solution, and the Developing Solution.

- When the electrophoresis is complete, turn of the power pack and disconnect both power cords from the power supply.

- Drain the top reservoir by unclamping the binder clip on the clear plastic tube allowing the TBE solution to drain into a beaker.

- When all of the solution has been drained, remove the top cover by holding the cover with your index fingers and pushing up on the white post with your thumbs. Set the cover aside.

- Remove the gel clamp by loosening the black knobs and lifting the clamp off. Be sure the gel does not fall toward you.

- Lift the glass plates unit from the bottom reservoir.

- Place the notched glass plate up on a glass rack to allow easier handling of the gel.

- Separate the glass plates by inserting your fingers between the glass plates. Work slowly and gently to avoid damaging the gel. Clean the notched glass plate by rinsing with water and wiping with Ethanol.

- Place the gel, glass side down, into the large tray contains the "Stop/Fix" solution, and shake for 5 minutes.

- Carefully lift the gel out and drain the "Fix/Stop" solution into its beaker, reserve.

- Rinse the tray with water.

- Place the tray on the orbital shaker and add the staining solution. Carefully lay the gel in the staining solution and agitate for 5 minutes.

- Remove gel from the staining solution and prop up on the work surface.
- Drain the staining solution into its container and save it, it can be used up to 4 times.
- Rinse the tray with water.

- Place 2L distilled water in the tray; lay the gel into the tray, lifting a corner to gently agitate for 10 to 15 seconds. Remove the gel and prop up on the work surface.

- Place the tray on the shaker, add the developing solution. Place the gel in the tray and agitate until bands are visible. This takes approximately 5 minutes.
- When the desired darkness of bands has been reached, stop the reaction by adding the saved "Stop/Fix" solution to the developing solution and continue to agitate for about 2 minutes.

- Remove the gel, rinse it, and stand it upright to allow any solution to drain off. If the gel contains some bubble, it is advisable to cover it with plastic wrap to protect it from cracking when drying out.
- Use the light box to score the gel.

Glass plates clean up.

- Soak the gel plate in 3% Sodium Hydroxide solution.
- Use a razor blade to scrape the gel from the gel plate
- Use a small amount of “Sequestrip” and sprinkle over the gel side of the gel plate. Using your glove, rub firmly the surface of the glass.
- Rinse with water.
- Using a sponge wash the plate with “Sequesoap” and rinse.
- Hold the glass to the light to see any remaining acrylamide and repeat if necessary.
- Hold the plate upright and spray 70% ethanol and wipe it with Kim wipes to dry the glass plate
- Mark the back of the glass so that the next user knows which side has been treated.

Note: The short glass plate should not be soaked unless it has been contaminated with bind silane. If it has been contaminated, soak it in 3% Sodium hydroxide but never soak the two plates (Back plate and notched plate) in the same tray because this causes a cross contamination of both glasses.

References

Wang, D., J. Shi, S. R. Carlson, P. B. Cregan, R. W. Ward, and B. W. Diers. 2003. A low- cost, High-Throughput polyacrylamide Gel Electrophoresis System for Genotyping with Microsatelite DNA markers. Crop Sci. 43: 1828-1832.

Benbouza, H., J.M. Jacquemin, J.P. Baudoin, and G. Mergeai 2006. Optimization of a reliable, fast, cheap and sensitive silver staining method to detect SSR markers in polyacrylamide gels. Biotech. Agron. Soc. Environ*., 10 (2): 77 - 81.*

Solutions preparation

40% Acrylamide Solution (19:1)
190 g Acrylamide (powder)
10 g Bis Acrylamide (powder)
500 ml distilled water
Mix above.
Store at 4°C.

Or
Use readymade 40% acryl amide: bis-acrylamide (19:1) solution

6% Acrylamide Solution 19:1)
24.4 ml of 40% acrylamide: bis acrylamide solution
136.2 ml of 0.5931 TBE solution
1.13ml 10% APS solution
113 µl TEMED

10% Ammonium per sulfate (APS)
0.1g APS
1 ml distilled water

0.5931X TBE Solution
59.31 ml 10X TBE solution
940 ml distilled water
Mix above.
Store at 4°C.

0.5X TBE solution
50 ml of 10X TBE solution
950 ml of distilled water
Mix and store at 4° C

Loading Buffer
0.05 g Xylene Cyanol
13 ml 80% Glycerol
Volume to 20ml with distilled water.
Store at 4°C.

DNA Ladder Mix
137 µl distilled water
58 µl loading buffer
10 µl DNA ladder

Mix above.
Store at 4°C.

Fix/Stop Solution

210 ml 95% Ethanol or 200 ml of 100% Ethanol
10 ml Acetic Acid
1780 ml distilled water.
Mix above, cover with aluminum foil and place in freezer to chill (10 to 12 ° C) prior to using.

Stain Solution

3 g of Silver Nitrate (0.15%)
3ml of 37% Formaldehyde
2000 ml distilled water
Mix above and cover with aluminum foil and leave at room temperature.

Developing Solution

30 g Sodium Hydroxide pellets
3 ml of 37% Formaldehyde
2000 ml distilled water
Mix above and cover with aluminum foil and leave at room temperature.