

Single-stranded Conformation Polymorphism (SSCP) Analysis

The PCR reactions were performed with Advantage® PCR Kits & Polymerase Mixes (Clontech, Catalog #639101), in the presence of S35dATP (MP Biomedicals, catalog # 01562000 in a total volume of 10 μ L.

Step 1: A) SSCP Labeling Mixture: Add the following amounts of each reagent per sample:

1.0 μ L 10X PCR Buffer 0.2 μ L dNTPs, 2.5 mM each of dGTP, dATP, dTTP, dCTP 7.0 μ L dH₂O 0.2 μ L Advantage® Taq polymerase,
0.2 μ L **S³⁵dATP**, 1mCi stock*
0.2 μ L (Gene specific Forward and reverse primer of 5pmol/ μ L concentration)
0.2 μ L (Actin Forward and reverse primer of 5pmol/ μ L concentration)
1.0 μ L of cDNA

**Note: Double this amount if the isotope is > 1 half-life old and change the water volume accordingly.*

(B) Run the following PCR cycle:

2 minutes @ 94°C
3 cycles of 30 sec @ 94°C
2min @ 60°C
1 min @ 72°C;
33 cycles of 30 sec @ 94°C
30 sec @ 57°C
1 min @ 72°C
one cycle of 10 minutes @ 72°C
Hold @ 4°C

Step 2: Running SSCP gels to resolve duplicated,

homoeologous genes in allohexaploid wheat.

This section details how to analyze radioactively-labeled SSCP products using manual sequencing gels. Gels can be run at room temperature or at 4° C (SSCP). Some genes resolve better at one temperature than the other.

For cleaning the sequencing gel plates use 50gm KOH dissolved in 1 litre of methanol and liquid detergent (both plastic and glass plate). Use KOH for cleaning only the glass plate and not the plastic plate followed by 100% ethanol. Use RAIN-X to clean the flat surface of plastic plate. Then use air supply for removing water droplets from the surface of plastic plate. Keep the glass plate in standing position for some time to air dry it completely.

(B) Gel preparation:

Assemble the glass plates and use the clamps as describe in *Molecular Cloning manual*.

Take a 200/500 mL beaker/flask.

Add 100mL of 8% polyacrylamide gel solution.

Once the gel plates are set up, add 168 µL of **TEMED** and 408 µL of 10% ammonium persulfate to the acrylamide/buffer solution. Continue stirring for ~ 30 seconds to mix properly. Check for bubbles..

Carefully pour the acrylamide solution into the glass plates using syringe following the procedure described in *Molecular Cloning manual*.

Once the gel solution fills the glass plates, slide in the comb with the flat side facing the surface of the gel solution (sharks' teeth facing up). Examine the gel for leaks.

Let the solution polymerize for at least 1 hour before use. If gel is going to sit overnight, wrap the upper surface of the gel by the comb with plastic wrap to reduce evaporation (for more precaution- Wet a towel in the 1x TBE solution and place it

over the comb and then cover the upper surface with plastic wrap).

(C) Running Gels

Materials needed: 1.5L of 0.5X running buffer per gel (TBE), chilled to 4°C Thermocycler Denaturation/Loading buffer : 95% formamide, 20mM EDTA, 10mM NaOH, 0.05% bromophenol blue and 0.05% xylene cyanol)

1. Before you begin, prepare 1 liter of 0.5X gel buffer (TBE) for each gel.

Put all electrophoresis materials into the walk-in cold room or 4°C glass door refrigerator.

Set sequencing gel plates into the gel rig (See *Molecular Cloning manual* for details).

Tighten down clamps, then add ~ 750 to 1000 mL of 0.5X running buffer to both the lower and upper chambers.

For SSCP analysis, the gel should be pre-chilled to 4°C for at least 5-6 hrs (or overnight). Do not pre-chill the gel in case of normal run.

Gently remove shark's tooth comb from gel.

Denature the SSCP samples by mixing 10 µL of PCR product with 10 µL of denaturation/ Loading buffer.

Heat to 95°C for 5 minutes, then immediately cool on ice. Store on ice until samples are loaded.

Prior to loading samples, rinse the upper surface of the gel with 0.5X buffer, and then put the shark's tooth comb into place.

Push the comb down until the teeth just enter the surface of the gel.

In standard run, pre-run the gel at 33mA constant current for 30min.

Using a sharpie, label 1st and last lane on the glass prior to loading samples! (optional step- but do remember in which direction was the first and last sample loaded).

Load up to 3-5 µL of each sample into each gel, and load as

rapidly as possible.

Once loaded, run the gel using between 10W Constant Power (not constant voltage!) for 16hrs (estimate the “optimal” run time for your gene of interest) at 4°C.

For standard run, run the gel at 70W constant power for 4 hours at room temperature.

When run is complete, shut off power and drain the upper tank. Remove the gel from the rig and lay the sandwich down with the buffer tank side facing up (See *Molecular Cloning manual* for details).

Gently pry apart the glass plates, leaving the gel on the glass plate.

To remove the gel from the glass plate, gently lay a piece of Whatman 3MM paper onto the surface of the acrylamide gel (note – once the gel touches the paper they can’t be separated again, so be sure that you have the paper aligned properly with the gel!).

Gently lift the Whatman paper, and the gel will stick to the paper. Wrap the gel with saran wrap.

Dry the gel on the gel dryer for 1 hour at 80°C.

Make sure to remove the saran wrap from the dried gel and then place an X-ray film on the dried gel in the dark room and expose it for three to seven days.

Develop the X-ray film to visualize the results.

SOLUTIONS:

(A) Mixing the Acrylamide Solution

Please refer to the Molecular Cloning manual for details:

Materials needed: Water 10X gel buffer, TBE 100 mL beaker with

stir bar 10% ammonium persulfate, fresh Urea solid **TEMED** (RT)

Acrylamide:

40% Acrylamide solution (make in dark bottle)

Acrylamide	= 190gm
N,N'-methylenebisacrylamide	= 10gm
Water	= 250 mL

Dissolve the chemicals and then make the volume to 500mL.

5x TBE, pH=8.3

Tris base	= 216 gm
Boric acid	= 110gm
0.5M EDTA, pH=8.0	= 80mL

Add water to make the final volume 4L and autoclave.

8% Polacryamide gel / 8M Urea

Urea	= 240.24gm
40% Acrylamide	= 100mL
5x TBE	= 50mL

Add water to make the final volume 500mL

To speed the dissolution of urea, microwave water until warm to the touch.

10% Ammonium persulfate (make fresh)

Dissolve 0.1gm in 1000 μ L water

TEMED, comes as liquid and stored at RT.