

PRI AFLP protocol for fingerprinting of *Phytophthora infestans*

AFLP (for 1 reaction)

DIGESTION EcoRI/MseI

Mix: 250 ng DNA
 10 Units EcoRI
 10 Units MseI
 5 µl 5x RL+ buffer (1/5 of the end volume)
 x µl MQ

Total: 50 µl

Overnight incubation (O/N) at 37°C (stove); 4 à 5 hrs is also sufficient (heating block)

10 µl on 1 % agarose gel (marker: 5 µl 100 bp ladder/10 ul lambda HindIII/EcoRI)

Store sample at 4°C

Incomplete digestion → add more enzyme and incubate for another 4 hours at 37°C

5xRL+ buffer: 50 mM Tris pH 7.5, 50 mM Mg-acetaat, 250 mM K-acetate,
 25 mM DTT, 250 ng / µl BSA

((50 mM Tris pH 7.5, 50 mM Mg-acetate, 250 mM K-acetate) = O.P.A. buffer)

LIGATION

Mix: 1 µl EcoRI-adapter (5 pMol/µl)
 1 µl MseI-adapter (50 pMol/µl)
 1 µl 10 mM ATP
 0.4 µl T4 DNA ligase (±6 WeissUnits/µl)
 2 µl 5x RL+ buffer (1/5 of the end volume)
 4.6 µl MQ

Total: 10 µl

Reaction: 40 µl digestion product + 10 µl mix

O/N incubation at 10-12°C (Over weekend (O/W) is also possible)

Dilute RL sample 10x (add 450 µl MQ). Store sample
 at 4°C

E0/M0 (E0/Mse0) AMPLIFICATION

Mix: 2.5 µl 10x PCR+Mg buffer
 2.5 µl 600 µM dNTPs (or 0.3 µl 5 mM dNTPs; adjust quantity MQ)
 2.5 µl E00 primer (50 ng/µl)
 2.5 µl M00 primer (50 ng/µl)
 0.2 µl Taq DNA polymerase (5 U/µl)
 9.8 µl MQ

Total: 20 μ l
 Reaction: 5 μ l 10x diluted RL sample + 20 μ l mix
 (take 1 blank as PCR control)

PCR: cycle 1: 2' at 94°C
 cycle 2-35: 30" at 94°C
 30" at 56°C
 90" at 72°C
 cycle 36: 10' at 72°C
 cycle 37: 4°C ∞

10 μ l on 1% agarose gel (marker: 5 μ l 1kb ladder)
 Remainder of PCR product (15 μ l) dilute 20x (add 285 μ l MQ)
 Store at -20°C

SECOND AMPLIFICATION

Mix: 0.80 μ l 5 mM dNTPs
 2 μ l 10x PCR+Mg buffer
 0.2 μ l 1mM spermidine
 0.1 μ l primer 1 (fluorescent; 50 ng/ μ l)
 0.6 μ l primer 2 (50 ng/ μ l)
 0.08 μ l Taq DNA polymerase (5 U/ μ l)
 11.22 μ l MQ

Total: 15 μ l

Reaction: 5 μ l 20x diluted PCR product + 15 μ l mix

PCR: cycle 1: 30" at 94°C -> denaturation
 30" at 65°C -> annealing
 60" at 72°C -> extension
 cycle 2-13: annealing temp. lower each cycle 0.7°C
 cycle 14-36: 30" at 94°C
 30" at 56°C
 60" at 72°C
 cycle 37: 4°C ∞

Add 20 μ l formamide mix per reaction
 Formamide mix: 1 ml formamide
 20 μ l 0.5 M EDTA (pH 8.0)
 spatula point Blue Dextran
 mix well and centrifuge

5' at 95°C

Store at -20°C

For use first put at 4°C and then in ice water

→ De-frost slowly and note that the samples don't have to be heated again.

Load $5\ \mu\text{l}$ on acrylamide gel.

POORING AFLP-GEL (on an ALFexpress sizer 50 – 500 from Pharmacia)

- Clean the 'gel'-site of both the glass plates with MQ (use Kimwipes Red tissue)
- The same with 96% EtOH
- Make sure to clean from bottom to top; this because of bits of Bind-Silane solution
- Make a fresh Bind-Silane solution:
0.5 ml 96% EtOH
1.25 μl Bind-Silane (toxic; work in hood)
mix
125 μl 10% acetic acid (HAc)
- Wrap the Bind-Silane solution with a tissue on the top 3 à 4 cm of both glass plates (where the comb is situated) (or by pipetting)
- Let it dry
- Wipe the rest off with a tissue
- Clean the glass plates again with MQ followed by 96% EtOH
Clean from bottom to top
- Clean the spacers (0.5 mm) with 96% EtOH
 - especially that part where the laserbeam is going through the spacers should be very clean
 - Manipulate the spacers only at the flat sites and not at the edges
- Place the spacers in the corners just above the lower corner of the back glass plate.
- Push the spacers at the white rubber bands at the sides of the glass plate
- Place the thin glass plate upon the spacers
- Place the clips, with the black rollers up, at both sides of the glass plates
 - one as high as possible, one as low as possible and two in between.
- Flush the comb with demi water and let dry.
- Put the comb between the glass plates so far as possible to the left.
- Take enough Sequagel solution (60 ml for the long cassette and 40 ml for the short one) in a plastic "flask". Be sure that the solution is homogenous. Let it come to room temperature and deaerate for 2-5 min.
- Make a fresh 10% APS solution (about 500 μl) and add 480 μl to 60 ml Sequagel or 320 μl to 40 ml Sequagel.
- Mix very carefully, avoid air bubbles !!!
- Pour the gel from the bottom up by squeezing the plastic bottle very carefully and by moving it from right to left and back.

Avoid air bubbles or remove them with the plastic strip.

The gel will be polymerised within 20-30 min.

Leave it for at least 2 hrs. and max. 5 hrs.

RUN THE AFLP-GEL

Make a fresh 0.6x TBE solution: 14.54 gram Tris
 0.45 gram EDTA
 6.16 gram Borate
 to 2L with MQ

The size marker (ALFexpress Sizer 50-500 from Pharmacia) is divided in small portions (8 μ l / vial) and is kept in the freezer.

Denaturate before use: 5min. at 95°C and then in ice water.

3 μ l size marker is used per lane.

Start computer program ALFwinControl (=ALFexpress)

Turn on the ALFexpress machine when prompted by the software program.

FILE

OPEN CASEBOOK

CONDITIONS

-> 1500 V, 60 mA, 35 W, 55°C en 500 min.

OK

Clean the back-side of the cassette with 96% EtOH; especially at the place of the laser.

Put the cassette on the apparatus.

-The cassette must be horizontal.

-the slots must be aligned with the numbers behind.

Connect the water circulation (control the water level).

Exam the laser beam for dust particles and for reflections and adjust if necessary the height with the wheels. Always keep the cassette in level, look for the black line in the thermoplate in relation to the white one at the filter front.

Realign the laser by carefully pulling the cassette.

The laser value has to be between 700 and 900.

Add buffer to the lower buffer tank.

Allow the gel to reach working temperature (55°C) → PRESET

Fill in the casebook:

EDIT

CASEBOOK

SAMPLE INFORMATION →fill in the names of the samples and primers you used.

NAME OF RESULT

NOTES

When the gel temp. is about 55°C, add buffer to the upper buffer tank.

THE SAMPLES MUST BE LOADED WITHIN 30 MIN.!!!!

Remove the comb and rinse the wells with buffer using a syringe.

Load the samples (5 μ l), adjacent at both sides the size marker, and loading buffer to the rest of the wells.

Put the electrodes in place, re-align the laser and start the run.

After 10 min., examine the background on the screen.

Look for high noise in any channel. High noise levels should be removed by opening the instrument and adjusting the cassette with the wheels.

THE LASER MUST BE RE-ALIGNED !!!

AT THE END OF THE RUN:

Close ALFWIN.

Turn off the power.

Get the cassette out off the instrument and pour away the buffer.

LOOK AT THE RESULTS:

start : ALFwin Evaluation

select FILE: OPEN and select the result file you want to evaluate.

Click OPEN: the result window is shown.

Select VIEW: CLONE

Click the maximise button in the upper right corner, with F7 or F8 you can zoom.

For other possibilities read the manual !!!!

CLEANING THE CASSETTE

In the lab:

- White mat in the sink
- Remove the clams
- Try to get air between the two glass plates so you can separate the two plates
- Carefully remove the spacers
- Remove the gel material with the pink plastic
- Clean the glass plates and the spacers very carefully with special soap (Alconox), hand warm water and the AFLP-brush.
- Rinse with demi water and let dry