Ras2 is important for growth and pathogenicity in *Fusarium circinatum*

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GENOMIC DNA EXTRACTION

Adapted from Murray and Thompson (1980)

Notes:
i) Handle Phenol and chloroform with care and work in a fume hood
ii) Room temperature (RT) = 22-25 °C.

1. Grow fungal strains on half strength Potato Dextrose Agar (PDA; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at 25 °C for seven days.

2. Cut a small agar block from the culture and inoculate Difco Potato Dextrose Broth (PDB; Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

3. Incubate with shaking at approximately 200 rpm for seven days at RT.

4. Filter the culture through two layers of Miracloth and freeze dry.

5. Label and unscrew an empty sterile 1.5-ml screw cap tube and place a metal bead in it using sterile tweezers.

6. Using the tweezers, pinch and place about 50-100 mg of the freeze-dried mycelia into the tube.

7. Add 700 µl of TES buffer and homogenize until mycelia are completely ground and then incubate in a deep freezer (−70 to −80 °C) for 10 min, followed by incubation in a water bath at 60 °C for 1 hour.

   - TES buffer: 100 mM Tris-HCL [pH 8.0]; 10 mM EDTA (ethylenediaminetetraacetic acid) [pH 8.0]; and 2 % [w/v] SDS (sodium dodecyl sulfate)

8. Add 230 µl volume CTAB:NaCl mixture, vortex and incubate in a water bath at 65 °C for 10 min.

   - CTAB:NaCl mixture: 2.5 % [w/v] CTAB (cetyltrimethylammonium bromide) and 3.75 M NaCl. These two constituents are made up separately and then combined before use.

9. Add 500 µl of 1:1 [v/v] phenol-chloroform, mix by vortexing, and centrifuge at 11 000 x g for 45 min at RT.

10. Carefully transfer 600 µl of the aqueous (top) phase to a labeled sterile empty tube 1.5-ml Eppendorf tube.

11. Add RNase A 6 µl (Thermo Fisher Scientific, Vilnius, Lithuania) and incubate at 37 °C for 1 hour to eliminate contaminating RNAs.

12. Repeat steps 9-10, but centrifuge for 20 min at RT.

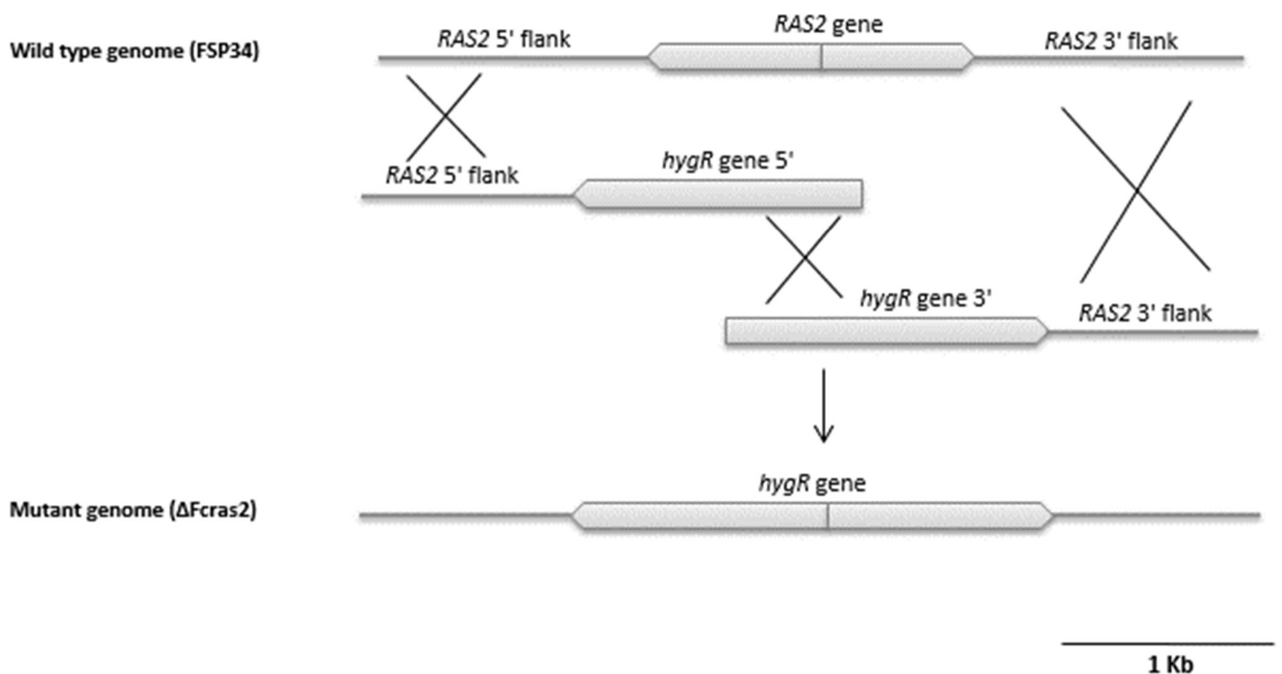
13. Add 300 µl of chloroform, mix by vortexing and centrifuge for 10 min at RT.

14. Carefully transfer the aqueous phase to a new labeled tube 1.5-ml Eppendorf tube.

15. Precipitate DNA by adding 2 X aqueous phase volume of ice-cold 100% ethanol and incubate in a −20 °C freezer overnight.
16. Pellet DNA by centrifugation at 13 200 x g for 30 min at 4 °C.
17. After removing the supernatant, wash pellet by adding 500 µl of 70 % ethanol followed by centrifugation at 13 200 x g for 10 min at 4 °C.
18. Repeat steps 17.
19. Briefly centrifuge (for about 30 sec) to and remove the excess ethanol using a micropipette.
20. Open the tube and place it under a laminar flow until the pellet is dry.
21. Dissolve the pellet by adding 50 µl sterile distilled water and incubating the tube on the laboratory bench for about 30 min.
22. Check the integrity, quality and quantity of the DNA and then store it at −20 °C for later use or use immediately.

OVERVIEW OF THE SPLIT MARKER APPROACH EMPLOYED

Replacement of *RAS2* in the genome of *F. circinatum* wild type (FSP34) using the split marker approach (see the diagram below). The *hygR* gene is a hygromycin resistance gene from *Escherichia coli* plasmid pCB1004.



KNOCKOUT AND COMPLEMENT CONSTRUCTS GENERATION

Adapted from Catlett et al. (2003)

Notes:
i) All Polymerase Chain Reactions (PCRs) were performed following manufacturers' protocols (see Appendix A) together with variable melting temperatures corresponding to primers used.
ii) Primer sequences are provided in Table 1 of our research paper.
iii) Unless otherwise stated, primers were used at 10 mM concentration.
iv) About 100-200 ng of template DNA was used in PCR experiments.

Knockout constructs (split marker constructs)

1. In two independent PCRs, amplify 5' flanking region of the *RAS2* gene from the genomic DNA of *F. circinatum* wild type strain FSP34 using the Phusion High-Fidelity Master Mix (Thermo Fisher Scientific, Vilnius, Lithuania) and primer set *Ras2*U F1 + *Ras2*U R1, the 3' flanking region of the *RAS2* gene using primer set *Ras2*Dw F1 + *Ras2*Dw R1.

2. In two independent PCRs, produce two overlapping amplicons of the hygromycin resistance gene (*hygR*) using the Phusion High-Fidelity Master Mix and primer sets HygR + ygF and HygF + hyR directly on *Escherichia coli* colonies carrying plasmid pCB1004 (Carroll et al., 1994).

3. Fuse the 5' *RAS2* flank to the first *hygR* amplicon using the LongAmp® *Taq* 2X Master Mix (New England BioLabs, Ipswich, MA, USA) and primer set *Ras2*U F1 + ygF.

4. Fuse the 3' *RAS2* flank to the second *hygR* amplicon using the LongAmp® *Taq* 2X Master Mix and primer set *Ras2*Dw R1 and hyR.

Complementation constructs (random integration construct)

1. Amplify the *RAS2* allele from the genomic DNA of *F. circinatum* wild type strain FSP34 (the full gene sequence including the promoter and terminator regions) using Expand™ Long Range dNTPack (Sigma-Aldrich, Mannheim, Germany) with primer set *ras2*allele_F1 + *ras2*allele_R.

2. Amplify the geneticin resistance cassette (i.e., *nptII*; includes promoter and terminator) directly from *E. coli* colonies carrying plasmid pGEN-Not1 (Proctor et al., 2008) using Phusion High-Fidelity Master Mix and the primer set Not_1F_rp619 + Not_1R_rp620.

3. Fuse the *RAS2* allele amplicon to geneticin resistance cassette using LongAmp® *Taq* 2X Master Mix and primers *ras2*allele_F1 and Not_1R_rp620.

PROTOPLASTING AND TRANSFORMATION

Adapted from Hallen-Adams et al. (2011)

Notes:
i) All experiments should be performed in a Biosafety/sterile hood.
ii) Room temperature (RT) = 22 – 25 °C.

1. Inoculate 100 ml of CMC solution in a 250-ml Erlenmeyer flask with an agar block of mycelia or an equivalent amount grown from Potato Dextrose Broth. Incubate for 5 days on a rotary shaker table at 25 °C at 250 rpm. Prepare two CMC flasks per culture.

    - Carboxymethyl cellulose medium (CMC): dissolve 15 g of carboxymethyl cellulose sodium salt (Sigma-Aldrich, St. Louis, MO), 1 g NH_4NO_3, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, and 1 g yeast extract in 1 l water (carboxymethyl cellulose dissolves slowly, and will need to be heated and stirred). Aliquot 100 ml apiece into 250-ml flasks; autoclave for 20 min.

2. Filter the culture from the two CMC flasks through a sterile Miracloth in a glass funnel into a 50-ml Falcon tube. Rinse with sterile dH_2O. Spin at RT at 4 000 x g in an appropriate rotor for 10 min.

3. Discard all but 2-4 ml of the supernatant and resuspend conidia. Place conidia in 100 ml of YEPD broth in a 250-ml Erlenmeyer flask, and grow in a rotary shaker for 10-12 h at 25 °C at 175 rpm. Timing is critical here as older cultures do not digest well into protoplasts.

    - YEPD: 3 g yeast extract (Difco Laboratories, Detroit, MI); 10 g Bacto peptone (Difco Laboratories, Detroit, MI); 20 g dextrose (anhydrous; = d-glucose); dissolve in 1 ml water, aliquot 100 ml apiece into 250-ml flasks and autoclave.

4. Filter culture from each YEPD flask, through a sterile Miracloth, in a Büchner funnel under vacuum (can use regular glass funnels without vacuum) and collect the mycelial mat. Rinse the mat with sterile dH_2O and allow the water to drain. Place the mat back into the flask from where it was obtained or into a new sterile 250-ml Erlenmeyer flask if needed. Add 30 ml of Protoplasting buffer to each flask (should have already been prepared and filter sterilized).

    - Protoplasting buffer: To 20 ml of 1.2 M KCl, add 500 mg Driselase from Basidiomycetes (Sigma Chemical Co., St. Louis; D8037); 1 mg Chitinase from *Streptomyces griseus* (Sigma Chemical Co., St. Louis; C6137); and 100 mg lysing enzyme from *Trichoderma harzianum* (Sigma Chemical Co., St. Louis; L1412); stir for 30 min and filter sterilize through a 0.45-mm Millex-HA filter (Millipore, Bedford, MA).

5. Digest for 1.5 h on a rotary shaker table at 30 °C at 80 rpm. Check for protoplasts after the first 45 min and then after every 15-20 min.

   - Protoplasts are spherical, while intact *Fusarium* cells occur in a variety of shapes, but are not spherical. Under the microscope, many round protoplasts should be present in the field of view when observed with a 40× objective; if only a few protoplasts are present, continue the reaction. After 2½ h, further incubation will not be of benefit.

6. Filter the digestion mixture through two-three layers of sterile Miracloth into 50-ml Falcon tubes. The filtrate should be turbulent due to the presence of protoplasts. Centrifuge at RT at 3 000 x g for 5 min in an appropriate rotor. Protoplasts are very fragile. Treat them gently.

7. Discard the supernatant and gently resuspend protoplasts in 10 ml of STC Buffer using wide orifice glass pipettes. Spin the solution at 3 000 x g for 5 min.

   - STC buffer: 1.2 M sorbitol; 10 mM Tris–HCl, pH 8.0; 50 mM CaCl2; autoclave. Use 4.1 ml per transformation reaction.

8. Discard the supernatant and gently resuspend protoplasts in 1 ml of STC Buffer using wide orifice pipette tips. Transfer to a 2-ml tube. Spin in a microcentrifuge at RT at 3 500 x g for 5 min. Repeat once.

9. Resuspend protoplasts in a final volume of 200-300 µl. Quantify using a hemocytometer; a good preparation can be expected to yield 106-108 protoplasts/ml. This is your protoplast suspension. Make the following mixture in a 50-ml Falcon tube: 100 µl – protoplast suspension, 100 µl – STC Buffer, 50 µl of 30 % PEG Solution, and PCR product (0.25-0.5 µg for each split-marker constructs). Extra protoplasts can be frozen for later use: add DMSO to 7% volume, aliquot, and freeze at − 80°C. When using frozen stocks, spin to collect and resuspend in STC at least twice before use. Start at step 9.

   - 30% PEG solution: 30% polyethylene glycol (PEG) 8000 (Sigma-Aldrich, St. Louis, MO; P2139); 10 mM Tris–HCl, pH 8.0; 50 mM CaCl2; filter sterilize using a 0.45-µm Millex-HA filter (Millipore, Bedford, MA). Always prepare fresh on the day of use. 2.05 ml is used per reaction.

10. Add 2 ml of 30 % PEG solution and incubate for 5 min.

11. Add 4 ml of STC Buffer and gently mix by inversion.

12. Pour reactions into cooled Regeneration Medium (RM): 9 ml RM per transformation reaction. Incubate at RT overnight (about 18 hours) with agitation at 75 rpm to regenerate protoplasts.

- Regeneration medium (RM): 135.5 g sucrose in 500 ml water; heat to dissolve, then add 0.5 g yeast extract; 0.5 g N-Z-Amine AS (Sigma-Aldrich, St. Louis, MO), autoclave.

13. Pour 1 ml reactions into 90 mm petri dishes.

14. Pour 10 ml cooled RM agar amended with 150 µg/ml hygromycin B into each petri dish and mix by gently swirling. *RM must be cool enough to touch and hold to the inside of your arm else protoplasts will be killed!* If the medium feels hot, allow it to cool more. The agar should be close to solidifying.

    - RM agar: 135.5 g sucrose in 500 ml water; heat to dissolve, then add 0.5 g yeast extract; 0.5 g N-Z-Amine AS (Sigma-Aldrich, St. Louis, MO; N4517); and 3.72 g agar; autoclave. Keep regeneration medium warm (55-65 °C) and liquid until use.

    - For complementation experiments, amend RM agar with 120 ug/ml of geneticin (G-418 sulfate; Thermo Fisher Scientific, Rockford, IL, USA).

15. Incubate at RT in the dark until protoplasts emerge (usually for 10-14 days).

16. Screen for putative transformants by transferring emerged protoplasts to half strength PDA containing 150 µg/ml hygromycin and incubate at RT in the dark until protoplasts grow (usually for 5-7 days).

    - Half strength PDA: 20 g PDA (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and 5 g agar in 1 l water; dissolve and autoclave.

    - For complementation experiments, augment PDA with 120 ug/ml of geneticin.

SCREENING FOR TRANSFORMANTS

Screening of knockout transformants

1. In two independent PCRs, use LongAmp® *Taq* 2X Master Mix and primer sets *ras*2F + *ras*2R and HygF + HygR to confirm the presence of the *RAS2* and *hygR* genes, respectively directly on the mycelia of putative transformants.
2. Perform a PCR using LongAmp® *Taq* 2X Master Mix and *ras*2left_F1 and *ras*2right_R1 to test for the replacement of *RAS2* by the *hygR* genes in mutants.
3. Screen for copy number of the *hygR* construct in genomes of knockout mutants using Southern Blot capillary analysis. See Appendix B of the protocol.

Screening of complementation transformants

1. Perform colony PCR using LongAmp® *Taq* 2X Master Mix and primer set *Ras2*_probeF1 + *Ras2*_probeR1 to test for the presence of the *RAS2* gene in complementation transformants,
2. Perform the Southern Blot analysis described above to test for copy number of the *ras* gene in genomes of the complementation mutants.

APPENDIX A

CYCLING CONDITIONS FOR THE VARIOUS DNA POLYMERASES AND/OR MASTER MIXES

Phusion High Fidelity Master Mix (Thermo Fisher Scientific, Vilnius, Lithuania)

Step	Temperature	Time	Cycles
Initial Denaturation	98 °C	30 seconds	1
Denaturation	98 °C	5-10 seconds	
Annealing	variable *	10-30 seconds	25-35
Extension	72 °C	15-30 seconds per kb	
Final Extension	72 °C	5-10 minutes	1
Hold	4 °C	∞	

* *Ras2*U F1 + *Ras2*U R1 = 65 °C; *Ras2*Dw F1 + *Ras2*Dw R1 = 65 °C; HygR + ygF = 60 °C and HygF + hyR = 60 °C; Not_1F_rp619 + Not_1R_rp620 = 60 °C; HygF + HygR = 55 °C.

LongAmp® *Taq* 2X Master Mix (New England BioLabs, Ipswich, MA, USA)

Step	Temperature	Time	Cycles
Initial Denaturation	94 °C	30 seconds	1
Denaturation	94 °C	10-30 seconds	
Annealing	variable *	15-60 seconds	30
Extension	65 °C	50 seconds per kb	
Final Extension	65 °C	10 minutes	1
Hold	4-10 °C	hold	

* *Ras2*U F1 + ygF = 65 °C; *Ras2*Dw R1 and hyR = 65 °C; *ras2*allele_F1 and Not_1R_rp620 = 60 °C; *ras2*F + *ras2*R = 53 °C; *Ras2*_probeF1 + *Ras2*_probeR1 = 53 °C; *ras2*left_F1 and *ras2*right_R1 = 50 °C.

Expand™ Long Range dNTPack (Sigma-Aldrich, Mannheim, Germany)

Step	Temperature	Time	Cycles
Initial Denaturation	92 °C	2 minutes	1
Denaturation Annealing Extension	94 °C 60 °C 68 °C	10 seconds 15 seconds 60 seconds per kb	10
Denaturation Annealing Extension	94 °C 60 °C 68 °C	10 seconds 15 seconds 60 seconds per kb + 20 seconds cycle for each successive cycle	15-25
Final Extension	68 °C	Up to 7 minutes	1
Hold	4-10 °C	∞	

Taq DNA polymerase and reaction buffer (Roche Diagnostics, Mannheim Germany) *

Step	Temperature	Time	Cycles
Initial Denaturation	98 °C	30 seconds	1
Denaturation Annealing Extension	98 °C 60 °C 72 °C	30 seconds 30 seconds 30 seconds	30
Final Extension	72 °C	10 minutes	1
Hold	4 °C	∞	

* This polymerase was used using primers CIRC1A and CIRC4A, and the reaction mixture contained 2.5 mM each dNTP, 25 mM, $MgCl_2$, 10 mM each primer, 50 ng/mL template DNA, 0. 03 U/ mL *Taq* DNA polymerase and reaction buffer.

APPENDIX B

SOUTHERN BLOT ANALYSIS PROTOCOL

Adapted from Sambrook and Green (2012), and Eisel et al. (2008)

1. Synthesize DIG-labeled probe (about 300 bp in this study)

Component	Full volume (50 µl reaction)	Half volume (25 µl reaction)*
H_20	34.25 µl	17.125 µl
Buffer (with $MgCl_2$)	5 µl	2.5 µl
DIG Mix	5 µl	2.5 µl
Primer 1 (10 mM)	1.5 µl	0.75 µl
Primer 2 (10 mM)	1.5 µl	0.75 µl
Enzyme (High fidelity)	0.75 µl	0.375 µl
Template DNA (10 ng/µl)	2 µl	1 µl

*Half volumes were used during the current study, and the LongAmp® *Taq* 2X Master Mix was used together with primer set HygR + Hyg-int-R with an annealing temperature of 66 °C.

- Include non-labeled control (replace DIG mix with normal dNTPs mix).
- Use no more than 100 pg of plasmid DNA or 1-50 ng (10 ng used in this study) of genomic DNA as template for PCR (optimal amount of template is 10 pg for plasmid DNA and 10 ng for genomic DNA). It is better to use plasmid as a template for probe synthesis if available.
- Perform PCR for 35 to 40 cycles. Check for specificity and efficiency of PCR product by loading 2 µl on Agarose gel. DIG-labeled PCR product should run a bit slower when compared with the non-labeled product.
- There is no need to purify the DIG-labeled PCR product. DIG-labeled PCR product can be used directly during hybridization. Store the DIG-labeled PCR product at – 20 °C for later use.

2. Preparation of genomic DNA

Isolate genomic DNA using a method suitable for isolates being examined (the CTAB extraction protocol described on page 1 was used in this study).

- DNA Genomic DNA used for Southern blot needs to be of high quality (high molecular weight, and free of inhibitor for restriction enzymes). Any commercially available DNA isolation kit should be suitable.

- A total of 5 µg of genomic DNA (up to 10 µl for bacteria and fungi) will be used for each lane/restriction enzyme used. Prepare enough genomic DNA for the relevant number of digestion setups.

3. **Digest genomic DNA using restriction enzymes relevant to the probe being used for screening** (Fast Digest HindIII and BamHI were used in this study - Thermo Fisher Scientific).

Setup digestion restriction(s) following the protocol relevant to the restriction enzymes being used.

- Digest 5 µg of genomic DNA per reaction. If using 10 µg per isolate, use double the volume of all reagents or perform the reaction in 2 tubes. In this study, the following Thermo Fisher Scientific FastDigest protocol was used:

Component	**Reagent volume (50 µl total volume reaction)**	**Double volume (100 µl reaction)***
Nuclease-free water	30 µl	60 µl
10X FastDigest® buffer	5 µl	10 µl
DNA	10 µl (up to 5 µg)	20 µl (up to 10 µg)
FastDigest® enzyme	5 µl	10 µl

*Double volumes were used during the current study.

- Use a PCR machine for incubation if doing 100 µl reactions, and a water bath if doing higher volumes.
- Incubate at appropriate temperature (37 °C in this study) for up to 16 hours. Check digestion progress by running a small volume (~ 0.1 µg of DNA) of the digestion mixture on a 0.75 % agarose gel using after 1 hour of incubation to decide if additional incubation time is required.

After desired incubation time has reached, check for completed digestion by loading a small amount of digestion mixture (~ 0.25 µg of DNA) on a 0.75 % agarose gel. Run and visualize under the UV light.

- Completed digestion can be used immediately or kept in a freezer until use.

Precipitate the digested DNA using the following Ethanol/Sodium Acetate (EtOH/NaOAc) precipitation method (combine reactions if they were divided):

1. Add one tenth of NaOAc to the total digestion reaction (*i.e.*, if the reaction is 100 µl, add 10 µl of NaOAc).
2. Add 2.5 volume of 100% EtOH to the above reaction.

3. Put reaction on ice for 10 min.
4. Spin at 4 °C for 30 min at maximum speed.
5. Discard supernatant.
6. Wash pellet by adding 500 µl 70 % EtOH.
7. Spin at 4 °C for 10 min at maximum speed.
8. Discard supernatant.
9. Repeat steps 6-8.
10. Spin tubes again for 5 min to remove excess EtOH. Use a P20 pipette to remove the excess EtOH.
11. Dry pellet under lamina flow for 5-10 min (assess progress after 4 min to avoid over-drying).
12. Resuspend the pellet in no more than 20 µl (25 µl used in this study) of 1x loading buffer with Gel-red or an equivalent DNA straining dye. Keep on ice until ready to load on Agarose gel for transfer.
13. Pulse spin the above mixture.
14. Incubate at 60 °C for 5-10 min.
15. Keep reaction at 4 °C until loading time.

4. Preparing and running the gel

Prepare 0.75 % agarose gel in 1x TAE (Tris Acetate EDTA) buffer. The thickness of the gel should be just enough (about 5 mm thick) to accommodate the samples' volumes.

Load DIG-labeled marker on the first lane (use 5 µl, mix with loading dye with Gel-red), followed by samples and positive control.

- ➢ Use PCR product or linearized plasmid DNA as a positive control, and load no more than 1 ng of it.
- ➢ Mix 98 µl of dH_2O with 2 µl of the control PCR product to make 100 µl total volume.
- ➢ Only load 2 µl of the above on the Agarose gel (remember to add Gel Red for the loading).

Run the gel at 80 V until the yellow dye gets close to the bottom of the gel (usually about 90 min).

Stop the gel, check and take a picture under UV light.

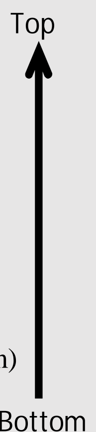
- ➢ Handle gel carefully as it is fragile, and avoid damage to the DNA by longtime UV exposure.

5. Transfer DNA to a membrane.

Note:
Recipes of all solutions used are detailed in the last section of this protocol.

1. Depurinate the DNA by submerging gel in 250 mM HCl with shaking (30 rpm) at RT until the bromophenol blue marker changes from blue to yellow. DO NOT incubate for more than 20 minutes.
2. Rinse the gel briefly with sterile, double distilled water.
3. Submerge the gel in Denaturation Solution (0.5 M NaOH, 1.5 M NaCl) 15 min at RT, with gentle shaking. Repeat once.
4. Rinse the gel briefly with sterile, double distilled water.
5. Submerge the gel in Neutralization Solution (0.5 M Tris-HCL, pH 7.5, 1.5 M NaCl) for 15 min at RT, with gentle shaking. Repeat once.
6. Rinse the gel briefly with sterile, double distilled water.
7. Equilibrate the gel for at least 10 min in 20X SSC (Saline Sodium Citrate).
8. Setup the blot transfer as follows (container, base, paper, membrane and tissue used must be same width and length as gel unless otherwise stated, and avoid the formation of air bubbles):

1 Kg weight/two or three thick stable books
Glass/thick hard-cover book
Stack of tissue paper (15-20 cm thick)
Pour 2 ml 5X SSC on to Whatman filter paper
Whatman filter paper 1 layer (5X SSC)
Whatman filter paper 1 layer
Nylon membrane
Pour 2 ml 20X SSC on gel center
1 cm Parafilm coat at all four gel edges
Gel (inverted)
Pour 20X SSC to drench paper and fill container up to 5 cm
2 layers Whatman filter paper as a bridge (long enough for edges to touch container bottom)
Gel tray inverted as a base (width and length must fit gel)
Container



9. Allow the blot to transfer overnight.
10. Unpacking the blot.
11. Remove ge1 + Whatman filter paper together then make a small cut at the top left edge to mark membrane orientation and cut off edges not containing any DNA.
12. Place the membrane (DNA side facing up) on Whatman 3MM paper that has been soaked with 2x SSC. Expose the membrane to UV light to permanently fix DNA on to the membrane using a Stratalinker UV crosslinker at 1200 mJ.
13. The membrane can now be used directly for hybridization or dried and stored at 4 °C for later use.

6. Pre-hybridize the blotted membrane with DIG Easy Hyb

1. Pre-heat an appropriate volume of DIG Easy Hyb (10 ml/100 cm2 of membrane; a total of 15 ml was used in this study) to hybridization temperature (usually at 42 °C; 68 °C was used) for 20 min.
2. Pre-hybridize the membrane for 30 min in a roller bottle with rotation at 42° C together with
3. Denature DIG-labeled probe by heating at 99 °C for 5 min in a PCR machine and rapidly cool submerged in or using ice-cold water. It is good to adjust the volume of probe to 50 µl with distilled water before denaturing by diluting 15 µl of probe with 35 µl water.
4. Add denatured DIG-labeled probe (15 µl) to the 15 ml pre-heated DIG Easy Hyb above and mix well. Avoid forming bubbles.
5. Pour out the pre-hybridization solution and add the above mixture into the roller with the membrane.
6. Close the cap tightly and incubate overnight with rotation.

7. Wash the membrane

1. Open the hybridization bottle, pour out the hybridization into a falcon tube (this hybridization can be stored at -15 to -20 °C and be reused several times. Freshly denature stored probe by incubating the solution at 68 °C for 10 min before reuse).
2. Wash membrane with 2X washing solution (2X SSC, 0.1 % SDS) at RT with gentle agitation for 5 min. Repeat once.
3. Wash in 0.1X washing solution (0.1X SSC, 0.1% SDS) at 65-68 °C under constant agitation for 5 min, and repeat once.
    - Pre-warm 0.1X washing solution to washing temperature before use.

8. Immunological detection

Notes:

i) Do not let the membrane dry out during the following steps of the detection protocol.

ii) Use the appropriate amount of solutions; do not use more than the necessary amount considering the price of solutions.

1. After washing, rinse the membrane briefly (1-5 min) in 100 ml washing buffer (Maleic acid buffer with 0.3 % Tween 20).
2. Incubate for 30 min (up to 3 hours) in blocking solution with shaking.
3. Incubate for 30 min in 10 ml antibody solution with shaking.

4. Wash min in 100 ml washing buffer (Maleic acid buffer with 0.3 % Tween 20) for 15 min. Repeat once.
5. Equilibrate 1-5 min in detection buffer enough to cover the membrane.
6. Incubate the membrane in 10 ml freshly prepared color substrate solution in an appropriate container in the dark. DO NOT shake during color development.
   - The color precipitate starts to form within a few minutes and the reaction is usually complete after 16 hours. Stop the reaction, when desired spot or band intensities are achieved, by washing the membrane for 5 min with sterile double distilled water or low TE buffer. Immediately photocopy, scan or take picture of the wet membrane.

SOLUTIONS FOR SOUTHERN BLOT ANALYSIS

Depurination solution (200 ml)	250 mM HCL (dilute 3M HCl twelve times in distilled water).
Denaturized solution (500 ml)	NaCl 43.83 g, NaOH 10 g, and distilled water (DW) until 500 ml.
Neutralized solution (500 ml)	NaCl 87.6645 g, Trisma-base 30.275 g, pH to 7.5 with HCl and DW until 500 ml (pH 7.5 with HCl ~ 23 ml).
DIG Easy Hyb	Add 64 ml of sterile double distilled water in two portions to the DIG Easy Hyb Granules; dissolve by stirring immediately for 5 min at 37 °C.
2X Washing solution (1,000 ml)	20X SSC 100 ml: 3M NaCl + 300 mM Sodium Citrate, 10 % SDS 10 ml, and DW 890 ml.
0.1X Washing solution (1,000 ml)	20X SSC 25 ml (5 ml), 10 % SDS 10 ml, and DW 965 ml.
20X SSC (1,000 ml)	NaCl 350.658 g (175.329 g), Sodium citrate 176.460 g (88.23 g), and DW 1,800 ml (900 ml) (pH to 7.0 with HCl) +/- 20 µl of 37% HCL.
5X SSC (1,000 ml)	20X SSC 250 ml and DW 750 ml.
10% SDS (100 ml)	SDS 10 g in 100 DW (heat at 65 ºC for 20 min).
Maleic acid buffer (1,000 ml)	11.61 g Maleic Acid, 8.766 g NaCl. Fill with dH2O. Check pH 7.5 with NaOH pellets (~7 g). Autoclave.
10X Blocking solution	Freshly prepare an appropriate amount of 1x working solution by diluting 10X blocking solution 1:10 with Maleic acid buffer *i.e.*, 10 ml Blocking solution: 90 ml Maleic acid buffer.
Antibody solution	Centrifuge Anti-Digoxigenin-AP for 5 min, 4 °C at 10 000 rpm and pipet the necessary amount from the surface. Dilute Anti-Digoxigenin-AP 1:5000 in blocking solution. Add 10 µl to 50 ml of 10X blocking solution.
Color-substrate solution	Add 1 ml of NBT/BCIP stock solution to 50 ml of detection buffer.
Washing buffer	Add 0.3 % (0.9 ml) Tween 20 to maleic acid buffer using a 1,000 µl tip that is cut at the bottom.
Detection buffer	0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5. Add $MgCl_2.6H_2O$ to a final concentration of 5 mM.
TE buffer	10 mM Tris-HCl, 1 mM EDTA, pH 8.0
Sterile double distilled water	

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