



Profiling the Diversity of Microbial Communities with Single-Strand Conformation Polymorphism (SSCP).

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Title:

Profiling the diversity of microbial communities with single-strand conformation polymorphism (SSCP)

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20 Summary

21 Genetic fingerprinting techniques for microbial community analysis have evolved over the
22 last decade into standard applications for efficient and fast differentiation of microbial
23 communities based on their diversity. These techniques commonly analyse the diversity of
24 PCR products amplified from extracted environmental DNA usually utilizing primers
25 hybridizing to suspected conserved regions of the targeted genes. In comparison to the more
26 commonly applied terminal restriction fragment length polymorphism (TRFLP) or denaturing
27 gradient gel electrophoresis (DGGE) techniques, the here described single-strand
28 conformation polymorphism (SSCP) fingerprinting technique features some advantageous
29 key characteristics. *i*) Primers for the polymerase chain reaction (PCR) do only need minimal
30 5'-end alterations; *ii*) SSCP is adaptable to high throughput applications in automated
31 sequencers; *iii*) A second dimension in the SSCP gel electrophoresis can be implemented to
32 obtain high resolution 2D gels. One central key requirement for SSCP gel electrophoresis is a
33 tight temperature control. Gels that run at different temperatures will produce entirely
34 different fingerprints. This can be exploited for an improved analysis of highly diverse
35 communities by running the same template at different temperatures or by 2D-SSCP gel
36 electrophoresis.

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40 1. Introduction

41 Genetic fingerprinting techniques provide an important means to display the diversity of
42 microbial community members and thus allow the comparison of their composition from
43 different environmental samples. Currently applied methods in environmental microbiology
44 rely on the analyses of products amplified by the polymerase chain reaction (PCR) from
45 extracted DNA. Typically such amplifications are conducted with PCR primers which
46 hybridize to phylogenetically conserved regions of a target gene and thereby amplify a
47 mixture of PCR products, which require further processing in order to generate genetic
48 fingerprints by electrophoretic separation techniques. Electrophoretic separation can either be
49 achieved under denaturing conditions, if the DNA fragments under investigation have a
50 different size, or under native (non-denaturing) conditions. The native conditions may be
51 combined with increasingly denaturing conditions either by chemical or temperature
52 gradients. Under fully native conditions it is in fact possible to differentiate between DNA
53 fragments of the same length as the electrophoretic mobility is then affected by the secondary
54 structure of the DNA molecule which is strongly influenced by the nucleotide sequence of the
55 four bases adenine (A), thymine (T), guanine (G) and cytosine (C).

56 Among the genetic fingerprinting techniques, the terminal restriction fragment length
57 polymorphism (T-RFLP) technique is most commonly applied when DNA-fragments are
58 separated by size. Since amplification of community DNA typically generates DNA
59 fragments of a similar length, these products must first be digested with restriction
60 endonucleases to obtain fragments of different lengths. To detect terminal restriction
61 fragments (T-RF), one (or both) PCR primers need a fluorescent dye as a label (*I*).

Denaturing gradient gel electrophoresis (DGGE, (2)), temperature gradient gel electrophoresis (TGGE, (3)) and single strand conformation polymorphism (SSCP, (4, 5)) separate whole PCR products despite their lack of size differences. DNA separations in DGGE and TGGE are based on a denaturing or temperature gradient and a GC-clamp on one of the primers in use. Such a clamp which is typically composed of additional 30 nucleotides (2) allows the maintenance of part double stranded (ds) DNA even when the opposite DNA strands of the PCR-targeted gene are completely separated by denaturation. In contrast, SSCP does not require dsDNA by works with single stranded (ss) DNA. PCR products are denatured by heat into ssDNA prior to electrophoresis in non-denaturing gels. The impact of the base sequence on the electrophoretic mobility can in fact be very strong and substitutions of a single base in a 300 base long sequence can be picked up (4). In contrast to DGGE and TGGE, fingerprinting with SSCP only requires minimal modifications of the primers in order to generate antisense free ssDNA.

Originally, SSCP was developed to detect gene polymorphism in human DNA (4) and mutations by comparing PCR-products (6). In these pioneering works, dsDNA was used for the gel electrophoresis. In order to obtain conformation specific information, dsDNA was denatured with formamide and heat to achieve strand separation and thus obtain ssDNA. However, during electrophoresis, re-annealing occurred among the complementary ssDNA with the result that typically for each DNA fragment analyzed, three bands occurred: two generated by the complementary ssDNA molecules and one caused by the reannealed dsDNA. The method was also applied to characterize microbial community by PCR, but due to the heterogeneity of the amplified PCR products from environmental DNA, the patterns of even simply structured communities were very complex. While the different mobility of opposite ssDNA strands is doubling the number of detectable signals, the formation of dsDNA molecule heteroduplexes built by reannealing of the complementary or almost

complementary ssDNA (7) introduces a multitude of additional signals in the community profile. In microbial ecology this classical approach has its merits to screen differences between pure culture isolates or cloned 16S rRNA gene libraries, as demonstrated by Tebbe et al. (8). However, for the analyses of diverse microbial communities the extensive level of heteroduplex formations makes the method unfeasible. Ideally, each community member should only generate one signal (band or peak, depending on the detection) and not an undefined amount of products and side products (5). The selective removal of one of the DNA strands from the dsDNA PCR product was the solution of the problem, as published by Schwieger and Tebbe in 1998 (5). This was achieved by the introduction of a 5'-end phosphorylation of one of the two PCR primers that allows the digestion of the phosphorylated DNA strand with Lambda exonuclease prior to the SSCP-gel electrophoresis. The feasibility of this modified approach for microbial community analysis was first demonstrated for analysing rhizosphere soils and composts or the gut contents of invertebrates (5, 9). Compared to the more commonly applied fingerprinting techniques, i.e., TRFLP and DGGE the introduction of the 5'-end phosphorylation has some significant advantages because it is much smaller than a fluorescent dye (TRFLP) and the above mentioned GC-clamp (DGGE).

Since its introduction to environmental microbiology, several novel applications have been added to the SSCP technique. Nested PCR applications have widened the spectrum of phylogenetic groups that can be displayed via SSCP and increase the sensitivity of detection (10) in direct comparison to the profiling of the dominant members of the bacterial community. The adaptation to automated sequencing machines as capillary electrophoresis (CE) SSCP has established a high throughput application comparable to TRFLP (11, 12). In addition to fingerprinting, DNA sequencing of the silver-stained bands from SSCP gels became feasible, allowing to characterize the contributors to the microbial community by

phylogenetic analyses of the targeted genes (5, 13). The transfer of single stranded DNA from the acrylamide gels onto nylon membranes is another option to identify specific DNA sequences from SSCP profiles by means of Southern hybridization using gene probes (14, 15). And more recently, the SSCP technique was extended to create two-dimensional gels by choosing different temperature settings for each dimension creating a separation based on potentially two different conformations for each molecule (16).

In the following section we describe the SSCP method for bacterial community analyses, as it is has evolved in different laboratories during 12 years of its use in environmental microbiology.

2. Materials

All solutions should be made up with ultra-pure (18 M Ω) or bi-distilled water with the exception of the 1 X electrophoresis buffer and the gel staining solutions where deionised water is sufficient.

2.1 PCR

- Thermocycler for PCR with wells for 0.2 ml micro reaction (PCR) tubes
- 0.2 ml PCR tubes, DNA free (pre-sterilized or autoclaved)
- Clean micropipettes and DNA free barrier tips
- PCR tube rack on ice
- HotMaster *Taq* polymerase (5 PRIME) with supplied buffer (see Note 1)
- MgCl₂ solution (5 PRIME)
- DNA free water (see Note 2)

- 0.5 μ M primers; standard universal 16S, COM1
(5'CAGCAGCCGCGGTAATAC3') and COM2-PH
(5'CCGTCAATTCCTTTGAGTTT3' with a phosphate group at the 5'-end) (see
Note 3) supplied by Invitrogen, Metabion and other companies
- Deoxynucleotides 200 μ M each
- Optional: enhancer solution (5 PRIME and other companies) or self-made (1M
betaine, final conc.)
- Approx. 1 ng template DNA or 0.1-1.0 μ l of template DNA (see note 4)

2.2 PCR product processing

- Lambda exonuclease (New England Biolabs or GE Life Sciences)
- PCR purification kit with a small elution volume of 10-12 μ l (e.g. Invitex,
Fermentas, Qiagen)

2.3 Gel electrophoresis

- Electrophoresis power supply with the capacity of at least 500 V (e.g. Biorad
Universal Power Supply)
- LKB 2010 Macrophor system (LKB, Amersham, discontinued) (see Note 5)
- 20 cm notched glass plates for Macrophor (Amersham, now GE Life Sciences)
- 0.4 mm spacer strips, 40 cm
- 0.4 mm comb
- 8 large bulldog clamps
- Horizontal gel pouring stand with levelling feet (Amersham, discontinued) or
level laboratory bench
- Vacuum chamber or vacuum flask with vacuum pump (e.g. water pump)

- 160 • Multitemp II or III recirculating chiller (Amersham, now GE Life Sciences)
- 161 • Magnetic stirrer
- 162 • TBE buffer 10X (TBE 10X per litre: 108 g tris base, 55 g boric acid, 40 ml 0.5 M
- 163 EDTA pH 8)
- 164 • MDE acrylamide gel (Lonza, double strength stock solution) (see Note 6)
- 165 • N, N, N', N', -tetramethylethylenediamine (TEMED)
- 166 • 40% ammoniumpersulfate (APS, wt/vol)
- 167 • 100 ml beaker, vacuum flask and magnetic flea
- 168 • Bind silane solution (17 µl Plus One Bind Silane (Amersham, now GE life
- 169 Sciences), 170 µl acetic acid 10 % (vol/vol), 10 ml ethanol)
- 170 • Plus One Repel Silane (Amersham, now GE life Sciences)
- 171 • Gel loading tips 0.2 mm (e.g. Starlab)

172

173 Preparing the ssDNA

- 174 • Heating block for 1.5 ml microtubes (e.g. Eppendorf Thermomixer)
- 175 • Denaturing dye solution (95% formamide vol/vol, 10 mM NaOH, 0.002%
- 176 bromophenol blue and xylene cyanol wt/vol)
- 177 • Ice bucket with crushed ice

178

179 2.4 Band visualization type A

- 180 • Trays made of stainless steel or plastic approx. 25X25 cm in pristine condition
- 181 • 10% acetic acid (vol/vol)
- 182 • Deionised or distilled water

- Silver staining solution (per litre: 1 g silver nitrate, 1.5 ml 37% formaldehyde)
- Developing solution (per litre: 56.3 g sodium bicarbonate decahydrate, 2 ml 37% formaldehyde, 1 ml sodium thiosulfate (0.2% wt/vol))

2.5 Band visualization type B

- SybrGold (Invitrogen)
- Visiblue transilluminator (UVP) or Darkreader (Clare Chemical) or Storm scanner or Typhoon imager (both Amersham, now GE Life Sciences)

2.6 Extraction of ssDNA from bands in SSCP gels

- Tray approx. 25X25 cm, deionised water
- Scalpel or razor blade, 1.5 ml micro reaction tubes
- DNA elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA pH 8, 0.1% sodium dodecyl sulfate (SDS, wt/vol))
- Thermomixer (for 1.5 ml tubes) or mixer for 1.5 ml tubes in 37°C incubator
- Microtube centrifuge, refrigerated, 16,000 rcf minimum
- Ethanol (96%)
- Freezer compartment
- 10 mM Tris buffer (pH 8)

3. Methods

3.1 PCR and PCR product preparation

For SSCP, a 5'-end phosphorylated primer is needed. Several primers have been published for PCR-SSCP, most of which target the 16S rRNA gene (**8, 10, 17**). This

protocol refers to one of the so called universal primer pairs namely COM1 and COM2-PH (the latter with a phosphate group at the 5'-end) covering the variable regions 4 and 5 of the 16S. PCR was performed with an initial denaturation at 94°C for 3 min, followed by cycles of 1 min at 94°C, 50°C for 1 min, 72°C for 1 min and a final extension at 72°C for 10min. The numbers of cycles may vary according to primer and template choice. The COM primers have been found to be very efficient at an annealing time of 50°C and produce sufficient yields within 28-30 cycles. Amplification reaction components for 50 µl reaction volumes were are follows:

- 1-1.5 U of hot start polymerase with supplied buffer
- 1.5 – 2.5 mM MgCl₂ (5 PRIME supplies buffer with 2.5mM MgCl₂)
- 0.5 µM primers
- Deoxynucleotides, 200 µM each
- Optional: enhancer solution
- Approx. 1 ng template DNA or 0.1-1.0 µl of template DNA

PCR products need to be further processed before they can be used for gel electrophoresis. The dsDNA PCR products are digested with Lambda exonuclease (New England Biolabs, 5-10 U per 50 µl PCR) at 37°C for 45 min in a total volume of 0.1 ml. In order to provide an optimal performance of the exonuclease, PCR products should be purified upfront using a PCR purification kit. ssDNA products can be applied directly to the gel or can be purified again (PCR purification kit) to improve the fingerprint quality. The applications of PCR purification kits with a small elution volume of 10-12 µl are particularly recommended to maximise the amount of ssDNA that can be loaded onto the gel (see Note 7).

3.2 Gel electrophoresis

For the SSCP gel electrophoresis keeping a constant temperature is a key factor. Many acrylamide electrophoresis systems have only limited capability to maintain a constant temperature in the range of 0.1°C. The Macrophor system is very efficient in controlling the gel temperature since gels are casted directly on a thermostatic plate connected to a cryostat/chiller.

A high voltage is necessary to migrate the ssDNA through the gel. Casting thin gels of approx. 0.4 mm is therefore recommended (see Note 8). The use of 0.6-0.65 times MDE acrylamide gel (Lonza) has been found to deliver high quality gel profiles. The following acrylamide gel concentrations apply for a 25 ml 20X20 cm gel, 0.4 mm thick:

- 2.5 ml TBE stock solution (10X)
- 7.8 ml MDE stock solution (2X)
- 14.7 ml water
- 10 µl TEMED
- 25 µl 40% APS

Pouring the gel on a Macrophor system with 20 cm notched glass plates:

1. Clean glass and thermostatic plate with ethanol, treat the thermostatic plate with repel silane, the glass plate (optional) with binding silane, (0.5 ml each) (see Note 9).
2. Position the thermostatic plate horizontally on the gel casting stand, use levelling feet and a spirit leveller to ensure the system is level.
3. Fix 0.4 mm thick and 40 cm long spacers on both sides of the thermostatic plate with clamps and place the glass plate with the notches facing forward onto the lower part of the spacer (bind silane solution side facing downwards).

4. To degas the gel, pour the gel matrix into a vacuum flask, add a magnetic stirrer and apply a vacuum (water pump or membrane pump) for approx. 3 min to the solution so that a few air bubbles accumulate on the floor whilst stirring the matrix slowly on a magnetic stirrer.
5. Add APS and TEMED to the matrix solution and after a brief stir pour the matrix slowly onto the thermostatic plate and at the same time slowly slide the glass plate with the notches facing forward towards the upper location of the thermostatic plate.
6. Fix the glass plate to the thermostatic plate with clamps holding the spacers and insert the comb between the notched side of the glass plate and the thermostatic plate. Ensure that the gel casting system is level.
7. The acrylamide needs to polymerise for approx. 2 h at room temperature.
8. Fit the gel sandwiched between the glass plate and the thermostatic plate into the electrophoresis unit (glass plate facing the buffer chambers) and fill upper and lower buffer chamber with 1 litre of 1X TBE buffer each.
9. Connect the thermostatic plate to the cryostat and set the temperature to 20 or 30°C (see Note 10).

Preparing the ssDNA

1. Mix 1 volume of purified ssDNA (5 µl or half of the PCR) with 1 volume (5 µl) of denaturing loading dye in a 1.5 ml reaction tube .
2. Heat the ssDNA sample to 95°C to denature the single strands for 2 min in a heating block (see Note 11).
3. Cool the ssDNA on ice for 3 min before loading the gel.

279

280 Loading and running the gel

- 281 1. Pull the comb and rinse the wells with a syringe and needle. For visualization
282 purposes, load small volumes of denaturing dye into the wells and apply the
283 voltage to the system for about 1 min, then rinse the wells again
- 284 2. Load 10 μ l of denatured sample into the well using a gel loading tip.
- 285 3. Apply 350-400 V and 8-10 mA for 16 hours (6,000 Vh).
- 286 4. After removing the thermostatic plate with the gel and glass plate attached,
287 carefully lift the glass plate from the thermostatic plate without breaking the
288 notches. The gel should stick to the glass plate. If you intend to use the gel for a
289 subsequent DNA transfer, avoid using bind silane solution on the glass plate (use
290 a dedicated untreated glass plate for this type of experiment).

291

292 3.3 Band visualization

- 293 A) Silver staining. For permanent visualization and cutting out of bands, silver staining
294 of the gel has been proven to be most effective (Figure 1A). Silver staining procedure:
- 295 1. Use 2 clean trays of stainless steel or plastic, incubate the gel on the glass plate in
296 10% acetic acid for 30 min.
 - 297 2. Remove the acetic acid and wash the gel twice in deionised water for 5 min.
 - 298 3. Remove the water and add silver staining solution and incubate in darkness for 30
299 min. The gel can be gently stirred but must not fall dry.
 - 300 4. Remove the staining solution and rinse the gel briefly with deionised water (10 s).
 - 301 5. Wash the tray with the gel in it in a small volume of cold (approx. 8°C)
302 developing solution for approx. 20 s.

6. Stain the gel in cold developing solution in the second tray until the bands are clearly visible.
7. Stop the reaction by placing the gel into 10% acetic solution for at least 10 min.
8. Rinse the gel in deionised water and dry the gel.

B) SybrGold staining. Different stains can be employed to visualise ssDNA bands. A convenient form is to use SybrGold by overlaying the horizontal positioned gel for 30 min with 40 ml of 10,000 times diluted SybrGold (in 1 times TBE) in darkness. After submerging the gel in deionised water for 5 min visualise the bands of the fingerprint (ssDNA) on a blue light or UV transilluminator (Figure 1B) or a laser scanner/imager (Storm or Typhoon).

3.4 Extraction of ssDNA from bands in SSCP gels

Single bands can be cut out of dried and silver stained SSCP gels and re-amplified for sequence identification (see Note 12).

1. Incubate dried gels in tray with deionised water for 5 min.
2. Cut out selected bands with a scalpel or razor blade and deposit gel slice in a 1.5 ml micro reaction tube.
3. Add 50 µl of DNA elution buffer and crush gel slice with a micropipette tip on the tube wall.
4. Incubate for 3 h at 37 °C in a shaker at 700-1400 rpm.
5. Pellet gel fragments by centrifuging for 1 min 6,000 rcf and transfer 40 µl gel slice free solution to a new 1.5 ml tube.
6. Precipitate DNA with 2 volumes of ethanol (96%) for 2 h at -20°C.

7. Pellet DNA by centrifuging at 16,000 rcf for 15 min at 4-8°C.
8. Discard supernatant, air dry DNA pellet for 5-10 min and dissolve DNA in 10 µl of 10 mM Tris buffer (pH 8), store at 8°C or freeze for long time storage.
9. DNA sample is ready for PCR .

3.5 Comparative SSCP fingerprint analysis

Data analysis of SSCP gels is very similar to image analysis of fingerprints obtained through other techniques such as DGGE. However, if silver stained gels are generated then a scanner with an integrated top light is highly recommended to obtain digitalised fingerprints. We have used GelCompar (Applied Maths) and Phoretix (Nonlinear Dynamics) software to analyse SSCP gels but other software packages may also be suitable. These software packages were used to normalise the fingerprints and to carry out the cluster analysis embedded in the software e.g. Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Alternatively, normalised fingerprints can be exported to carry out Principal Component Analysis (PCA) or Correspondence Analysis (CA) with software packages such as Canoco (Microcomputer Power).

4. Notes

1. Although a variety of polymerases can be employed to obtain a PCR product for SSCP analysis the use of hot start polymerases such as HotMaster (5 PRIME), Platinum (Invitrogen) or Robust HotStart (Kappa) Taq improved the yield and reduced the amount of unspecific products.
2. Self-made PCR water: Filter ultra-pure (18MΩ) water through a 0.2 µm membrane filter into sterile 1.5 ml safe lock micro tubes and autoclave tubes locked, filled with 1 ml water each.

3. Universal primers are published for SSCP applications targeting the variable regions V2-3, V4-5 and V6-8 of the 16S (13, 17). In addition, Peters et al. (9) demonstrated the diversity analysis of fungi by SSCP. Bacterial groups were also targeted more specifically employing a nested PCR approach (10).
4. DNA obtained from DNA extraction kits such as FastDNA spin kit for soil (MP Biomedicals) or Ultra Clean Soil DNA kit (MO BIO Laboratories) can be used directly or 5 times diluted as template DNA for the PCR.
5. Several other electrophoresis systems can be used for running SSCP gels. *i)* the Protean XL from Biorad (comes with a cooling core that can be connected to a chiller); *ii)* the Scie-Plas 20X20 cm protein chamber with an integrated temperature exchange chamber (now replaced by the TV400 series) has been used successfully by connecting the latter to a chiller and inserting magnetic stirrers; *iii)* the TGGE maxi system (Biometra) has also been used successfully to run SSCP gels. The system allows keeping a constant temperature. Unfortunately, horizontal gels have to be run in this chamber which can cause problems when running thin gels for 16 h; *iv)* The DCode system (Biorad) can also be used with an optional cooling coil but the standard coil size is too small to obtain sufficient constant temperatures; *v)* in some cases where systems can only heat but not cool the placing of the electrophoresis unit in a 4°C walk in room can be a solution and this has been done successfully with a Mutation Detection Chamber from CBS Scientific (Figure 1B).
6. Standard acrylamide is sufficient to cast an SSCP gel but the MDE gel solution has been used most successfully to generate high quality fingerprints and is therefore recommended.

7. The Lambda exonuclease digest can be carried out with raw PCR since the HotMaster buffer (5 PRIME) shows similar features to the lambda exonuclease buffer. However, optimal results will be obtained by purifying the PCR product upfront and using the buffer supplied with the exonuclease. The digested product can be applied to the gel directly but then the buffer solution in the digest will create smiling effects in the gel. For optimal presentation use a PCR purification kit with small elution volume such as the Apache from Invitex, the PCR purification kit from Fermentas or the Qiagen PCR mini elute. By eluting into 10-12 µl one can apply 50% of a PCR in a single well.
8. Running SSCP gels that are 1mm thick can cause problems since the voltage will be significantly lower at 10 mA and new settings have to be tried. With the TGGE maxi it is impossible to reach the 400V and the electrophoresis has to be carried out at a lower voltage setting or the strength of TBE has to be reduced. Many small standard power packs only allow a voltage of 200 or 300 V. Users have to source power packs that can run at a higher voltage.
9. Using bind silane solution and repel silane was found to very useful when using the macrophor system to run SSCP gels. Bind silane solution is beneficial when SSCP gels are stained with silver nitrate as many wash steps are included in the staining procedure. However, bind silane solution has to be avoided if the ssDNA in the gel needs to be transferred to a membrane. The use of gelbond films with the hydrophobic side facing the gel side is recommended although gels can also be transferred from the untreated glass plate to a sheet of blotting paper by rubbing the blotting paper over the gel and then peeling it off carefully. For the application of electro blotting and southern blot hybridization please refer to Schmalenberger and Tebbe (14).

401 10. SSCP running conditions have been optimised to 1X TBE buffer at 20°C but can
402 also be used to run gels at 10-30°C. We have used different temperatures for
403 different primers to obtain sharp bands in the fingerprints and users who try new
404 primers are advised to optimise for the best temperature. Liu and Sommer (18)
405 patented the SSCP5 method to detect mutations using multiple temperature
406 settings. However, if an apparatus is used that cannot cool actively it is advised to
407 run the gels at the highest possible temperature without losing a ssDNA
408 conformation effect.

409 11. Different additives have been tried for the loading/denaturing buffer and the SSCP
410 gel but the gel recipes above showed the best results. As the Lambda exonuclease
411 digests dsDNA to single stranded DNA, SSCP gels can be loaded without
412 denaturation but in practice the denaturation step improved the quality of the
413 fingerprint.

414 12. Retrieving ssDNA from SSCP bands was most successful by using silver stained
415 gels. DNA in the gels was found to be stable for several years. It is also possible
416 to retrieve ssDNA from SybrGold stained gels but the exposure to UV light is
417 usually disintegrating the DNA fragments quickly and we recommend the use of a
418 blue light transilluminator. However, faint bands can be easily overlooked by this
419 method due to low signal intensity.

422

423 5. References

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486

487 Figure legend

488 Figure 1

489 SSCP fingerprints of various bacterial soil communities flanked by species standards

490 (*Bacillus licheniformis*, *Rhizobium trifolii*, *Flavobacterium johnsoniae*, *Rhizobium*

491 *radiobacter*) using a A) MacroPhor chamber (Amersham) B) Mutation Detection chamber

492 (CBS Scientific) in a walk in cold room, 20 cm gel length at 20°C.