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Reverse Sample Genome Probing to Monitor Microbial Communities

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1. Introduction

Environmental microbial communities are often highly complex. To evaluate community activities, it is desirable to be able to accurately monitor community composition. A variety of methods to monitor environmental microbial diversity exist, e.g., the use of 16S rRNA probes, combined with fluorescence *in situ* hybridization (FISH), or fatty acid methylester analysis. However, no currently available method completely overcomes the difficulties presented by the complexity of environmental microbial communities.

Reverse sample genome probing (RSGP) monitors culturable members of microbial communities. Extracted and labeled total community DNA is used to probe a filter containing chromosomal DNAs from many pure strains. These are selected to have limited cross-hybridization and are referred to as standards. Information on the occurrence of multiple standards is thus obtained in a single hybridization assay. The information obtained is limited to the culturable component of the microbial community. RSGP thus measures microbial diversity in the selected target environment by following the fate of selected culturable community members. It can screen for the presence of specific microbial strains in the target environment under a variety of conditions. Its advantage: once a set of standard microbial strains has been selected, quantifying their relative abundance is straightforward. Its drawback is that environmental microbial diversity is huge. Thus a master filter containing a set of standards may be of limited use outside its intended target environment. Once a filter for a target environment has been obtained, RSGP is a straightforward

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method to evaluate changes in a microbial community over time, and during various treatments (e.g., bioremediation).

RSGP requires isolating and purifying total DNA from microbial community samples. This DNA is radiolabeled and used to probe a master filter that consists of chromosomal DNA from individual standard microorganisms of interest bonded to a nylon membrane **(Fig. 1)**.

2. Materials (see Note 1)

2.1. Designing and Preparing the Master Filter

2.1.1. Isolation of DNA From Pure Bacterial Strains

1. Lysis buffer (0.15 *M* NaCl, 0.1 *M* EDTA, pH 8.0).
2. Freshly made lysozyme (1 mg/mL in lysis buffer).
3. 25% SDS.
4. 5 *M* $NaClO_4$.
5. $CHCl_3$:isoamyl alcohol (24:1, v:v).
6. Tris-EDTA (TE) ***(1)***: 10 m*M* Tris-Cl, pH 7.4; 0.1 m*M* EDTA, pH 8.0.
7. 95% and 70% ethanol, ice cold.
8. 10 mg/mL DNase-free RNase ***(1)***.
9. 14 mg/mL Proteinase K.
10. TE-buffered phenol ***(1)***. Phenol is toxic and should be handled with gloves in a fume hood.

2.1.2. Quantification of DNA

Ethidium bromide is extremely toxic and should be handled with gloves at all times.

1. λ DNA (usually obtained as a 500 ng/µL stock solution).
2. Ethidium bromide plates.
   a. Combine 25 mL of melted 1% (w/v) agarose with 3 mL of 10 mg/mL ethidium bromide and pour the mixture into a 100 × 15 mm Petri plate.
   b. Allow plate to solidify. Plates can be stored in the dark at 4°C for several weeks.

2.1.3. Preparation of Filters

1. λ DNA (**Subheading 2.1.2.**).
2. Purified DNA from microorganisms of interest; at least 100–200 ng/filter.
3. Nylon membrane (e.g., Hybond™-N, Amersham).
4. Sterile-distilled water.
5. 0.5 *N* NaOH.
6. 6X SSC: 20X SSC ***(1)*** is 175.3 g NaCl, 88.2 g Na_3 citrate per liter, pH 7.

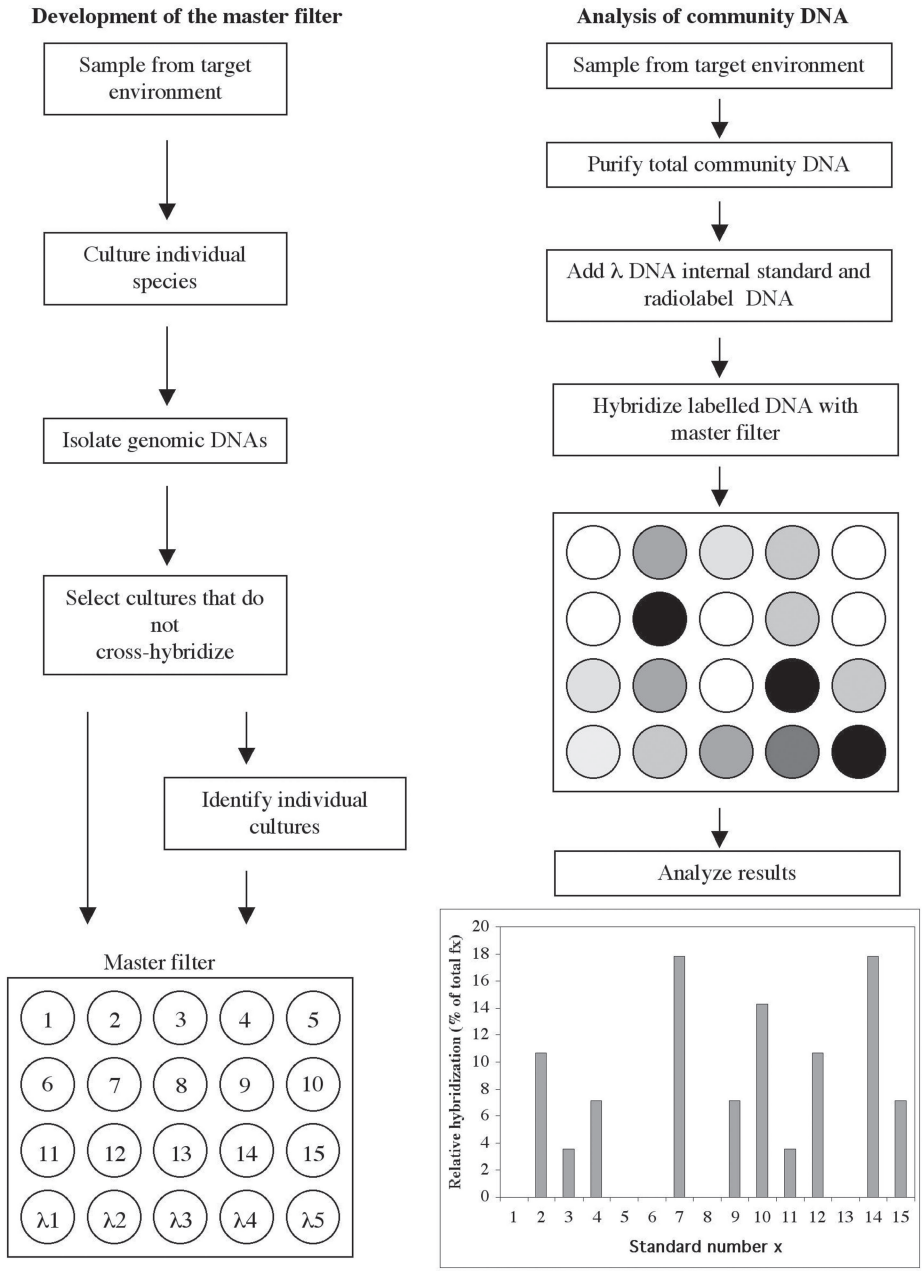

Fig. 1. Overview of the RSGP method. The λ spots represent five different concentrations of λ DNA, which are used to calculate the relative amount of hybridization of labeled community DNAs to various master filter standards.

2.1.4. Generation of Probes and Hybridization With Filters

The work described in this section involves the use of radioisotope (^{32}P); the appropriate precautions should be observed.

2.1.4.1. Generation of Probe

1. DNA (**Subheading 2.1.2.**).
2. DNA samples for generating probe (at least 100 ng of each).
3. Primer extension (PE) mix ***(2)***: 44 µL of 0.9 *M* HEPES, 0.1 *M* $MgCl_2$, pH 6.6; 25 µL of 1 *M* Tris-Cl pH 7.4; 10 µL of 0.1 *M* dithiothreitol; 4 µL each of 50 m*M* dATP, dGTP, and dTTP; 10 µL of 10 mg/mL randomly generated hexanucleotides. PE mix can be stored indefinitely at –20°C.
4. DNA polymerase I Klenow fragment, 1,000 U/µL.
5. [$\alpha^{32}P$]dCTP (10 mCi/mL, 3000 Ci/mmol).

2.1.4.2. Preparation of Filters for Hybridization

1. Polypropylene bags and bag sealer.
2. Prehybridization solution ***(3)***: 300 mL 20X SSC (**Subheading 2.1.3.**); 50 mL 10% SDS; 100 mL 50X Denhardt's reagent (***[1]***: 5 g Ficoll Type 400), 5 g of polyvinylpyrrolidone, 5 g bovine serum albumin (Fraction V, Sigma, St. Louis, MO) made up to 500 mL in distilled water); 10 mL salmon sperm DNA ***(1)***. Prehybridization solution can be stored indefinitely at –20°C.

2.1.4.3. Probing Filters With Labeled DNA

1. 1X SSC (**Subheading 2.1.3.**).
2. 1X SSC, 0.2% (w/v) SDS, preheated to 68°C.

2.2. Isolation and Purification of DNA From Environmental Samples

2.2.1. Isolation of Bacterial Cells

1. Freshly made 0.1% (w/v) $Na_4P_2O_7 \bullet 10H_2O$.
2. Acid-washed polyvinylpolypyrrolidone (PVPP; ***[4]***).
3. Mix 300 g PVPP with 4 L of 3 *N* HCl, let stand overnight.
4. Filter with a Buchner funnel apparatus and Whatman no. 1 filter paper.
5. Resuspend PVPP in 20 m*M* KH_2PO_4, stir and filter. Repeat until pH is 7.0–8.0.
6. Air-dry PVPP. Acid-washed PVPP can be stored indefinitely at room temperature.

2.2.2. Cell Lysis and DNA Isolation

1. Lysis buffer (**Subheading 2.1.1.**).
2. Freshly made 300 mg/mL lysozyme in lysis buffer.
3. 25% SDS.
4. 5 *M* $NaClO_4$.
5 $CHCl_3$:isoamyl alcohol (24:1, v:v).

6. 95% Ethanol, ice cold.
7. 70% Ethanol, ice cold.
8. TE-buffered phenol (**Subheading 2.1.1.**).

2.2.3. Removal of Humic Acids From Soil DNA

1. TE (**Subheading 2.1.1.**).
2. Spin columns (based on the protocol of Jackson et al. ***[5]***).
3. Soak glass wool in 1 *N* HCl for at least 1 h, rinse with 0.1 *M* KH_2PO_4 until the pH is 7.0–8.0, then air dry overnight (*see* **Note 2**).
4. Pack the bottom 0.5 cm of a 1-mL syringe with acid-washed glass wool, then fill with Sepharose 4B. Centrifuge for 4.5 min at 1000*g*. Refill and pack the material by centrifugation until the syringe is filled to the 0.9-mL mark.
5. Wash with 200 µL of TE, centrifuge for 4.5 min at 1000*g*. Repeat for a total of four washes; after the final wash centrifuge the column again to remove excess TE. Packed columns can be stored up to 2 wk at 4°C with both ends sealed.
6. 0.7% Agarose gel and running buffer for analysis of spin column eluent.
7. Ice-cold 95% and 70% ethanol.

2.2.4. Final DNA Purification

1. 1 mg/mL DNase-free RNase (**Subheading 2.1.1.**).
2. 14 mg/mL Proteinase K.
3. TE-buffered phenol (**Subheading 2.1.1.**).
4. Ice-cold 95% and 70% ethanol.
5. 0.7% Agarose gel and running buffer for analysis of purified DNA.
6. Ethidium bromide plates for DNA quantification.

3. Methods

*3.1. Designing and Preparing the Master Filter (***see** *Note 3)*

3.1.1. Isolation of DNA From Pure Bacterial Strains

Cells should be grown to a high density in appropriate medium. The procedure described below is designed for approx 0.5 g (wet weight) of cells.

1. Collect cells by centrifugation for 20 min at 4°C, 15,000*g*. Some samples may require centrifugation for longer periods of time at higher speeds to collect cells.
2. Resuspend cells in 250 µL of lysis buffer and transfer to a 1.5-mL Eppendorf tube.
3. Add 10 µL of 1 mg/mL lysozyme and incubate at 37°C for 30 min.
4. Add 20 µL of 25% SDS and lyse cells using three freeze/thaw cycles (30 min at –80°C followed by 30 min at 65°C).
5. Add 60 µL of 5 *M* $NaClO_4$ and 300 µL of $CHCl_3$:isoamyl alcohol, then gently mix on a tube roller for 30 min.
6. Centrifuge in a microfuge at maximum speed, room temperature, for 5 min.

7. Transfer aqueous phase to a fresh 1.5-mL Eppendorf tube and add 1 mL of 95% ethanol; incubate at –20°C for at least 1 h. Centrifuge in a microfuge at maximum speed, 4°C, for 15 min. Discard supernatant, rinse pellet once with 500 µL of 70% ethanol, then air-dry pellet.
8. Resuspend in 300 µL TE, then add 15 µL of 1 mg/mL DNase-free RNase. Incubate 30–60 min at 37°C, add 5 µL of 14 mg/l proteinase K, and incubate at 37°C for 30–60 min.
9. Add 100 µL of TE-buffered phenol, mix, and centrifuge (maximum speed in a microfuge) for 5 min at room temperature.
10. Transfer aqueous phase to a fresh 1.5-mL Eppendorf tube and precipitate with 750 µL ethanol as in **step 7**.
11. Resuspend DNA pellet in TE at desired concentration.

*3.1.2. Quantification of DNA (*see ***Notes 4–6****)*

Two microliters of known concentrations of λ DNA (5, 10, 15, 20, 25, 30, 40, 50, 60, 80, and 100 ng/µL) are spotted on an ethidium bromide plate, followed by 2 µL of sample DNA. The plate is allowed to "develop" for 1 h, then DNA spots are visualized on a UV light box. Standards must be spotted at the same time as samples.

*3.1.3. Preparation of Filters (*see ***Note 7****)*

Filters can be prepared using various amounts of DNA; 20–1450 ng per spot have been used successfully ***(6,7)***. A useful concentration range is 100 to 200 ng of DNA per spot.

1. Mark 1 × 1 cm squares on a nylon membrane for each bacterial DNA standard, plus eight squares for λ DNA concentration standards.
2. Dilute λ DNA to 10, 20, 30, 50, 60, 80, and 100 ng/µL concentrations.
3. Dilute microbial standard DNA samples such that 2 µL of DNA solution provides the amount to be spotted on the master filter.
4. DNA is denatured by boiling for 3 min followed by placing on ice for 3 min.
5. Spot 2 µL of each DNA solution onto the nylon membrane.
6. Dry the nylon membrane for 15 min at 80°C in a drying oven.
7. Fix the DNA to the nylon membrane by exposing it to ultraviolet light (365 nm and 7000 $\mu W/cm^2$) for 3 min.
8. Wash filters in 6X SSC, and air-dry. Filters can be stored at –20°C indefinitely.

3.1.4. Generation of Probes and Hybridization with Filters

3.1.4.1. Generation of Probe

1. Add 100 ng sample DNA to a 1.5-mL Eppendorf tube; make up to 15 µL with sterile distilled water. Add 5 µL of freshly prepared 0.5 ng/µL λ DNA (*see* **Note 8**).
2. Boil DNA mixture for 3 min, then ice for 3 min. Briefly centrifuge to collect sample.

3. Add 6 µL of PE mix, 2 µL of DNA polymerase I Klenow fragment, and 2 µL of [α^{32}P]dCTP (*see* **Note 9**), then incubate at room temperature for approx 3 h (*see* **Note 10**).

3.1.4.2. Preparation of Filters for Hybridization

1. Place each filter in a polypropylene bag (*see* **Notes 11** and **12**). Add prehybridization mixture to the bag (approx 125 µL per DNA spot), then remove all bubbles and seal.
2. Place sealed bags in a container of water and heat to 68°C in a hybridization oven; incubate rocking for at least 1 h before adding probe.

3.1.4.3. Probe Filter

1. After the probe has incubated for 3 h, boil for 3 min, then place on ice for 3 min.
2. Cut off the corner of the polypropylene bag, add probe to the prehybridized filter, then reseal the bag, removing all bubbles. This step should be done quickly so that the solution does not cool excessively.
3. Return to the 68°C oven and incubate overnight, rocking.
4. Remove filters from bags, wash in 100 mL of 1X SSC for 15 min at room temperature, rocking; then wash a second time in 100 mL of 1X SSC plus 0.2% SDS for 1 h at 68°C, rocking (*see* **Note 13**). Remove filters from wash and air-dry.
5. Expose a phosphoimager plate to the filters for 1 to 3 h, read the results (e.g., using a Fuji Bas1000 Bio-Imaging Analyzer), then quantify the relative intensity of each spot (e.g., using the MacBAS program) (*see* **Notes 14** and **15**).

3.2. Isolation and Purification of DNA From Environmental Samples

*3.2.1. Isolation of Bacterial Cells From Soil (*see ***Note 16****)*

1. This protocol is designed for approx 5 g soil. Dry soil or soil cultures can be used.
2. Place 5 g soil, 20 mL 0.1% $Na_4P_2O_7 \cdot 10H_2O$ (or culture medium, made up to 20 mL total liquid volume with 0.1% $Na_4P_2O_7 \cdot 10H_2O$), and 1 g acid-washed PVPP (*see* **Note 17**) in a 50-mL beaker, and mix on a magnetic stirrer for 20 min.
3. Centrifuge for 10 min at 4°C, 1000*g*; collect supernatant.
4. Wash soil twice more with 10 mL 0.1% $Na_4P_2O_7 \cdot 10H_2O$. Pool collected supernatants.

3.2.2. Cell Lysis and DNA Isolation

1. Centrifuge liquid sample or cells washed from solid support (**Subheading 3.2.1.**) for 20 min at 4°C, 15,000*g*.
2. Resuspend the cell pellet in 5 mL of lysis buffer.
3. Add 250 µL of 300 mg/mL lysozyme then incubate at 37°C for 30–60 min.
4. Add 2.5 mL of 25% SDS per tube, mix by inversion. Lyse cells using three freeze/thaw cycles (30 min at –80°C followed by 30 min at 65°C).
5. Add 0.6 mL of 5 *M* $NaClO_4$ and 3 mL of $CHCl_3$:isoamyl alcohol, then mix on a tube roller for approx 30 min.

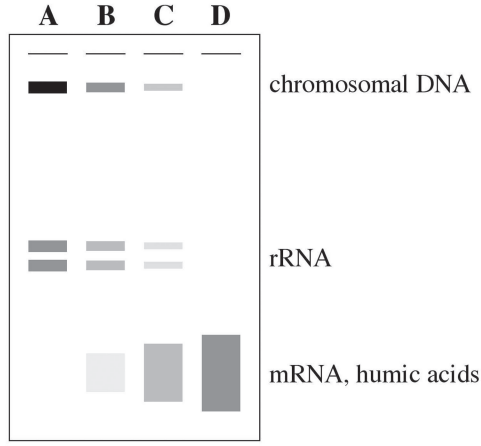

Fig. 2. Schematic of a typical agarose gel showing fractions eluted from spin columns. (A) Fraction 1; (B) fraction 2; (C) fraction 3; (D) fraction 4.

6. Centrifuge at 3500*g* for 10 min at room temperature, then transfer the aqueous phase into 50-mL glass centrifuge tubes.
7. Add 2.5 volumes of 95% ethanol; incubate at –20°C for at least 1 h. Centrifuge at 15,000*g,* 4°C for 20 min. Wash pellet with 1 mL of 70% ethanol and air-dry.
8. Resuspend DNA pellet in TE (*see* **Note 18**).

*3.2.3. Remove Humic Acids From Soil DNA (*see ***Note 19****)*

1. Load up to 200 µL of DNA sample onto a spin column (*see* **Notes 20** and **21**).
2. Centrifuge for 4.5 min at 4°C, 1000*g*. Collect eluent in a 1.5-mL Eppendorf tube.
3. Wash the columns 2–3 times, each time by loading 100 µL of TE on the top of the column and centrifuging, collecting each eluent in a fresh Eppendorf tube.
4. Analyze the column eluents by gel electrophoresis. Pool all samples that contain DNA but no humic acids (**Fig. 2**).
5. Add 2.5 vol of ethanol to combined samples, incubate at –20°C for at least 1 h, then centrifuge for 15 min at 4°C, maximum speed in a microfuge. Rinse pellet with 70% ethanol, then air-dry pellet. Resuspend samples in 300 µL TE (*see* **Note 22**).

*3.2.4. Final DNA Purification (*see ***Note 23****)*

1. Add 15 µL of 1 mg/mL DNase-free RNase, incubate for 30–60 min at 37°C, then add 5 µL of 14 mg/mL proteinase K and incubate for 30–60 min at 37°C.
2. Add 100 µL of TE-buffered phenol, mix, then centrifuge at maximum speed in a microfuge for 5 min at room temperature.
3. Collect aqueous phase, add approx 750 µL of ice-cold 95% ethanol and precipitate at –20°C for at least 1 h.

4. Centrifuge at maximum speed in a microfuge at 4°C for 15 min, discard supernatant, rinse pellet with 500 µL of 70% ethanol, and air-dry. Resuspend pellet in an appropriate amount of TE (typically around 30 µL for samples isolated from soil; for samples containing more DNA, a larger volume may be required).
5. Quantify DNA (**Subheading 3.1.2.**). Dilute DNA with TE to a concentration that is useful for probe generation (e.g., 10 ng/µL).

3.3. Calculation of f_x

To quantify hybridization of radiolabeled sample DNA to various microbial standards, the hybridization intensity is evaluated using the following equation:

$$f_x = (k_\lambda/k_x)(I_x/c_x)(f_\lambda)(I_\lambda/c_\lambda)^{-1} \quad (1)$$

- f_x is the weight fraction of genome x present in the DNA mixture used for probing.
- k_λ/k_x is a constant that represents the relative genome complexity, which is unknown for the genomes of various bacterial standard organisms. This constant must be measured for each standard.

1. Mix λ DNA and DNA from a specific standard together such that $f_x = 0.976$ and $f_\lambda = 0.024$.
2. Label DNA and hybridize to the master filter as described in **Subheading 3.1.4.**
3. Calculate k_λ/k_x. This must be done for each standard on the master filter; this constant can be used to calculate the relative f_x for each standard when probed with unknown community DNA samples. The constant k_λ/k_x can be calculated using the following equation:

$$k_\lambda/k_x = (f_x/f_\lambda)(I_\lambda/c_\lambda)(I_x/c_x)^{-1} \quad (2)$$

4. Repeat this experiment in duplicate; use the average result from the duplicate samples as the value for k_λ/k_x.

- I_x is the relative intensity of standard x on a given master filter, as measured by densitometry or by cpm in a scintillation counter. Background radioactive signal should be subtracted from the total intensity.
- c_x is the amount of standard x DNA spotted on the master filter, in ng.
- f_λ is the fraction of λ DNA present in each labelled probe mixture.
- c_λ is the concentration of λ DNA spotted on the master filter.
- I_λ is the relative intensity of λ DNA on the master filter for each hybridization experiment, as measured by densitometry or by cpm in a scintillation counter. The presence of several concentrations of λ DNA on each master filter allows accurate measurement of this value.

The f_x values calculated with **Eqs. 1** and **2** cannot be corrected for contributions due to cross-hybridization. Cross-hybridization will cause every reported f_x value to be overestimated. This will cause the sum Σf_x of all f_x values to exceed 1. One can report data by setting $\Sigma f_x = 1$. The resulting relative f_x values

are reported as %, not as fraction between 0 and 1. A drawback of this is that relative f_x values (%) represent the fractions of standards in the portion of the community represented on the master filter.

4. Notes

1. Solutions can be prepared in advance, autoclaved, and stored at room temperature unless otherwise noted. Solutions stored at 4°C or –20°C are typically not autoclaved.
2. Glass wool used for the spin columns does not have to be acid-washed; however this treatment appears to give higher DNA yields.
3. Before preparing the master filter, it is important to determine which microbial strains of interest do not show significant cross-hybridization. This can be done using DNA filters to assess cross-hybridization between various potential standard genomes. Once standard bacterial strains have been selected, a master filter can be developed. It is important to select bacterial strains that are appropriate to the experimental design and are represented in the community of interest.
4. DNA can be quantified by fluorimetry, A_{260} or visual comparison of fluorescence with ethidium bromide staining and UV light after gel electrophoresis, or after spotting samples onto agarose containing ethidium bromide.
5. Condensation interferes with the accuracy of ethidium bromide plate determinations.
6. Quantification by A_{260} or ethidium bromide plates will work only for pure DNA samples. Humic acids from soil are also detected by these methods ***(5)***; when the presence of humic acids is suspected, gel electrophoresis and staining will be a more accurate method of DNA quantification.
7. Used filters can be reused when radioactivity can no longer be detected, or after washing. Washing procedure:
   a. Heat 1X SSC plus 0.2% SDS to boiling.
   b. Pour heated solution over filters. Incubate for 30–60 min at 80°C, rocking.
   c. Allow to cool to room temperature, remove filters, and air-dry.
   d. Repeat procedure until radioactivity is no longer detected.
8. Some RSGP users ***(8)*** digest DNA with a restriction enzyme (*Sau*3A) before labeling.
9. Other researchers have used ^{35}S ***(8)*** or a nonisotope labeling kit (DIG DNA Labeling and Detection ***[9]***) rather than ^{32}P.
10. While probe is being incubated, master filters can be prepared for hybridization (**Subheading 3.1.4.2.**).
11. Hybridization bags are less cumbersome if they are cut to fit the filter fairly precisely on three sides; there should be extra room on the fourth side as the bag needs to be cut and resealed during the probing process.
12. Rather than using the hybridization bag method, a tube roller and individual hybridization tubes can be used. However, if a large number of samples are being screened, polypropylene bags allow more samples to be processed simultaneously.

13. More than one filter can be washed in the same container; however, some background radioactivity may appear if several filters are present in a single container.
14. If no phosphoimager system is available, DNA hybridization to the master filter can be detected using X-ray film and quantified by densitometry.
15. An alternate method for quantifying sample DNA hybridization to the master filter is to collect each DNA spot and measure the total radioactivity by scintillation counting *(7,8)*; however, this method precludes reuse of the master filters.
16. This protocol is also useful for removing bacterial cells from solid support materials.
17. Acid-washed PVPP is used to remove humic acids; therefore, this compound may be omitted for extracting cells from solids that do not contain these substances.
18. The volume of TE used to resuspend DNA depends on the sample. Typically 300 µL is suitable for soil DNA; larger volumes may be required if the sample still contains substantial amounts of material from soil or if the DNA concentration is high.
19. This step is required only for samples that may contain humic acids.
20. DNA can also be isolated by gel electrophoresis and electroelution, or other means of gel purification. Spin columns are good for processing many samples at once.
21. If the DNA preparation contains suspended solids, a large amount of humic acids, or a large amount of DNA, only 50 to 100 µL should be loaded on the column; the remaining volume should be made up with TE. The column can become plugged, or removal of humic acids can be poor, if it is overloaded.
22. If a sample appears brown after purification on a spin column, it still contains humic acids and must be re-cleaned because they will interfere with the labeling procedure.
23. This step is not necessary if the sample was gel purified, because RNA and protein will have been removed during that process.

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