Using Terminal Restriction Fragment Length Polymorphism (T-RFLP) Analysis to Assess Microbial Community Structure in Compost Systems

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Abstract

Terminal restriction fragment length polymorphism (T-RFLP) analysis of PCR-amplified genes is a widely used fingerprinting technique in composting systems. This analysis is based on the restriction endonuclease digestion of fluorescently end-labeled PCR products. The digested product is mixed with a DNA size standard, itself labeled with a distinct fluorescent dye, and the fragments are then separated by capillary or gel electrophoresis using an automated sequencer. Upon analysis, only the terminal end-labeled restriction fragments are detected. An electropherogram is produced, which shows a profile of compost microbial community as a series of peaks of varying height. This technique has also been effectively used in the exploration of complex microbial environments and in the study of bacterial, archaeal, and eukaryal populations in natural habitats.

Key words: T-RFLP, fingerprinting technique, microbial composition, composting, PCR-based technique, culture-independent method.

1. Introduction

Composting is a biological conversion of solid organic wastes into stable materials such as fertilizers (1). The decomposition of organic matter during composting is mediated by a succession of microbial communities. The initial phase of composting process is characterized by the growth and activity of mesophilic microbes, which in turn leads to a rapid increase in temperature. At the next stage, the thermophilic microbes become responsible for the degradation process. The final stage, which includes

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cooling-down and maturing phases, is characterized by the development of a new mesophilic community (2–3). The optimization of compost quality is directly linked to the composition and succession of microbial communities in the composting process. Therefore, it is important to monitor the succession of microbial communities for effective management of the composting process.

Culture-independent methods have been recently employed to monitor the succession of microbial communities during the composting process. One of the most important methods for the survey of compost microorganisms is the analysis of a clone library (4) or, more and more promisingly, the analysis of a metagenomic library (5). However, due to the complexity of the compost communities and the effort required for these analyses, clone libraries have been restricted to the analysis of a single sample or a few samples. To circumvent the limitation of the clone library approach, several PCR-based methods now exist to allow rapid fingerprinting and monitoring of the composting process. These techniques include terminal restriction fragment length polymorphism (T-RFLP) (6), denaturing gradient length polymorphism (DGGE) (2), single-strand conformation polymorphism (SSCP) (7), and phospholipid fatty acid analysis (PLFA) (8). In recent years, T-RFLP has been widely used for the analysis of microbial communities during composting process (6, 9–12) due to its high throughput and phylogenetic resolution.

Typically, T-RFLP analysis involves amplification of target genes from whole-community DNA extracts by using specific primer pairs, one of which is fluorescently labeled. Subsequently, amplicons are digested with restriction enzymes (usually tetranucleotide recognizing) and fragments are size-separated by electrophoresis on automated sequencers, whereby only the labeled terminal fragments (T-RFs) are detected and quantified. Individual T-RFs can be assigned presumptively to operational taxonomic units, which ideally correspond to phylogenetically related microorganisms, based on in silico search for matching restriction sites in sequences from clone libraries established in parallel from the same sample. In general, the T-RFLP technique has been proven to be reproducible as an accurate tool for community fingerprinting (13–16) . However, since T-RFLP is based on PCR amplification, all biases related to this technique apply (17).

2. Materials

2.1. DNA Extraction and PCR Amplification

1. Kit for extracting environmental DNA. We routinely use the Power Soil DNA extraction kit (MO BIO Laboratories, Inc., Carlsbad, CA) for compost samples (*see* **Note 1**).

Alternatively, good results are obtained from Soil FASTDNA kit or from the extraction protocols developed by Yang et al. (18).

2. Double distilled water (ddH_2O) sterilized by autoclaving or filtering. Prepare 100 µL aliquots before sterilization and keep at −20°C. Discard the aliquot after use.
3. 10× PCR buffer: 500 mM KCl, 10 mM Tris−HCl, 0.1% Triton X-100, pH 9.0
4. 50 mM $MgCl_2$ stock solution (Invitrogen, Carlsbad, CA)
5. Stock solution of a mixture of dexoyribonucleotide triphosphates (dNTPs): 2 mM of each dNTP in ddH_2O. Prepare aliquots of 20 µL and store at −20°C.
6. *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) or other thermostable DNA polymerase
7. Primers for the amplification of the gene of interest: For instance on 16S rDNA, 8f primer (5′-AGAG TTTGATCCTTGGCTCAG-3) and 1492r primer (GCY-TACCTTGTTACGACTT) give good results (*see* **Note 2**). 8f primer is labeled at the 5′ end with 6-FAM (6-carboxyfluo-rescein) fluorescent dye (Applied Biosystems, Foster City, CA). Prepare stock solutions of primers at 20 µM and store at −20°C.
8. Bovine serum albumin (BSA): This protein eliminates PCR inhibitors in compost DNA samples.
9. Dimethyl sulfoxide (DMSO): Aliquot 500 µL and keep at −20°C.

2.2. Gel Electrophoresis and PCR Purification

1. TAE buffer: 40 mM Tris-acetate, 1 mM EDTA, pH 8. Prepare a 50× stock solution.
2. Agarose (Sigma-Aldrich St. Louis, MO).
3. Ethidium bromide stock solution: 10 mg/mL. Store in a dark bottle. Caution: Ethidium bromide is a mutagen, suspected carcinogen, and at high concentrations is irritating to the eyes, skin, mucous membranes, and upper respiratory tract. Preparation of stock solutions and any operations capable of generating ethidium bromide dust or aerosols should be conducted in a fume hood to prevent inhalation. Nitrile gloves should be worn at all times.
4. 10× DNA-loading buffer: 70% (w/v) glycerol, 0.5% bromophenol blue. Store at 4°C.
5. DNA size marker: 100-bp or 1-kbp molecular marker (Invitrogen, Carlsbad, CA) for agarose gel electrophoresis.

6. Kit for purification of PCR products from unincorporated primers and salts. Good purification results are obtained with Qiagen PCR purification kit (Qiagen Inc., Valencia, CA).
7. Ethanol (100%)

2.3. Restriction Digestion and T-RFLP

1. Restriction enzymes with four-base recognition sequence (i.e., *Hha* I, *Msp* I, *Rsa* I) and their specific buffers (Roche Applied Science, Indianapolis, IN). Four base pair cutter restriction enzymes are most appropriate as the probability of having a restriction site within the amplicon is high. Various restriction enzymes can be used in single-enzyme reactions in order to determine which one yields the highest number and most even distribution of terminal restriction fragments (*see* **Note 3**).
2. Double-distilled water (Prepared in Section **2.2.1**).
3. TAMRA 500 (Applied Biosystems, Foster City, CA) molecular size marker for capillary electrophoresis.

3. Methods

3.1. DNA Extraction

Numerous methods are available for extracting community DNA from composts. It is important that the extraction procedure works both for Gram-positive bacteria and for Gram-negative bacteria. We employ a bead-beating technique using the Power Soil DNA extraction kit following the manufacturer's instructions, which briefly are as follows:

1. Transfer 0.25 g of compost sample to the PowerBead tubes and gently vortex to mix.
2. Add 60 μL of Solution C1 (contains SDS and other disruption agents required for complete cell lysis) and invert several times.
3. Secure PowerBead tubes horizontally using the MO BIO Vortex Adapter tube holder for the vortex or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 min.
4. Centrifuge tubes at 10,000 × *g* for 30 s at room temperature.
5. Transfer the supernatant to a clean 2 mL collection tube.
6. Add 250 μL of Solution C2 (contains a reagent that precipitate non-DNA organic and inorganic materials) and vortex for 5 s. Incubate at 4°C for 5 min and then centrifuge the tubes at room temperature for 1 min at 10,000 × *g*.
7. Avoiding the pellet, transfer up to 600 μL of supernatant to a clean 2 mL collection tube 7. Add 200 μL of Solution

C3 (a second reagent to precipitate additional non-DNA organic and inorganic material) and vortex briefly. Incubate at 4°C for 5 min. Centrifuge the tubes at room temperature for 1 min at 10,000 × *g*.

8. Transfer up to 750 μL of supernatant to a clean 2 mL collection tube.
9. Add 1.2 mL of Solution C4 (a high concentration salt solution) to the supernatant and vortex for 5 s.
10. Load the supernatant spin filter and centrifuge at 10,000 × *g* for 1 min at room temperature. A total of three loads for each sample processed are required.
11. Add 500 μL of Solution C5 (an ethanol-based wash solution) and centrifuge at room temperature for 30 s at 10,000 × *g*. Discard the flow through from the 2 mL collection tube. Centrifuge again at room temperature for 1 min at 10,000 × *g*.
12. Carefully place spin filter in a clean 2 mL collection tube. Avoid splashing any Solution C5 onto the spin filter. Centrifuge at room temperature for 30 s at 10,000 × *g*. Discard the spin filter. The DNA in the tube is now ready for any downstream application.

3.2. PCR Amplification, Gel Electrophoresis, and Purification of PCR Products

1. Perform PCR amplification in a total volume of 50 μL. The PCR set up is laid out on **Table 6.1**. Consider preparing a master mix solution. For instance, for 10 samples, prepare a master mix solution for 11 reactions. Prepare a master mix containing 394.9 μL of dH_2O, 55 μL of 10× PCR

Table 6.1
PCR reaction

Components	Volume	Final concentration
10× PCR buffer	5 μL	1×
25 mM $MgCl_2$	2.5 μL	2.5 mM
10 mM PCR nucleotide mix	1 μL	0.2 mM each
10 pmol forward primer	1 μL	0.2 pmol
10 pmol reverse primer	1 μL	0.2 pmol
5U/μL *Taq* DNA polymerase	0.50 μL	2.5 U
BSA	0.60 μL	1.2%
DMSO	1.50 μL	3%
Environmental DNA (50 ng/μL)	1 μL	
Sterile dH_2O	To 50 μL	

buffer, 25.5 μL of 25 mM $MgCl_2$, 11 μL of 10 mM PCR nucleotide mix, 11 μL of each primer solution, 5.5 μL of *Taq* DNA polymerase (5 U/μL), 6.6 μL BSA, and 16.5 μL DMSO. Mix and aliquot 49 μL of master mix solution in 0.2 mL PCR tubes. Add 1 μL of template DNA (from extraction previously prepared at 50 ng/μL, *see* **Section 3.1**) to each PCR tubes containing the 49 μL of master mix.

2. The above PCR protocol has been optimized in an iCycler Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA). The reaction mixture, after incubation at 94°C for 2 min, is cycled through the following temperature profiles: 94°C for 30 s, 58°C for 1 min, and 72°C for 1 min for 25 cycles. Finally, the reaction mixtures are incubated at 72°C for 7 min.
3. Prior to the PCR product being analyzed, check 5 μL of each amplification mixture by agarose gel (1.0% w/v) electrophoresis in TAE buffer containing 1 μg/mL (w/v) of ethidium bromide.
4. If nonspecific products are observed, then the desired product should be purified by excising the respective part of the gel using a gel purification system. Otherwise, the PCR product is purified using commercial kit such as QIAquick PCR purification kit to remove excess primers.
5. It might be necessary to pool several PCR reactions to obtain enough products for further steps (200–300 ng of DNA recommended per restriction digest). The amplification efficiency of labeled primers tends to be lower than that of unlabeled primers, frequently leading to lower yields. The concentrate DNA and the volume of the pooled PCR reactions can be reduced to half to a fifth of the original volume using a Speedvac or ethanol precipitation.

3.3. Restriction Digestion of the PCR Products

1. Once purified, the PCR products are digested with a restriction enzyme. Restriction enzyme digestion of PCR product will generate products of varying length with respect to sequence diversity. For each digestion, 200–300 ng of purified PCR product (assuming a 50% loss during purification) and 10–20 U of restriction enzyme should be used. The incubation period can vary from 4 to 12 h at 37°C to assure complete digestion (*see* **Note 3**).
2. Restriction enzymes are inactivated by heating to 65°C for 20–25 min.
3. Separate the digested products by capillary electrophoresis on an automated sequencer. Inject 5 μL of digestion product with 0.5 μL of molecular weight standard TAMRA-500.

4. Run the electrophoresis as indicated by the manufacturer of the instrument.

3.4. Desalting of Digested PCR Products

In capillary electrophoresis the injection of DNA samples can be achieved by two methods. First, hydrodynamic injection requires pressure difference over the capillary. Alternatively, electrokinetic injection uses a combination of electrophoresis and electroendosmosis to inject the sample. PRISM 310 and 3100 Genetic Analyzers use the latter. The presence of ions can interfere with the uptake of DNA using electrokinetic injection because of preferential injection of higher charge-to-mass molecules (e.g., Cl⁻ons). Therefore, it is essential to desalt the inactivated restriction digest with Microcon columns, Qiaquick Nucleotide Removal Kit, or conventional ethanol precipitation. In our case, the restriction products are diluted with water upto 500 μL, before concentration and desalinization on Microcon columns.

3.5. Analysis of T-RFLP Profiles

The profiles generated by T-RFLP can vary in two ways. First, there can be variation in the number and size (base pairs) of T-RFs present in a profile. For example, profiles from day 0 (D0) compost and day 28 (D28) compost are clearly different (**Fig. 6.1**). Secondly, differences can be found in the height (and consequently the area) of any particular peak. This variation can have a major influence in estimates of the biodiversity represented in the numerically rarer members of the community. Such a variation is clearly seen when comparing the heights of peaks in T-RFLP profiles from D0, D5, D12, D21, and D28 manure composts. The height of each peak can provide a measure of relative proportion of each component of a population, although biases caused by preferential annealing of the primer of templates (19) means that the absolute values should be treated with caution.

The second output generated from the analysis program is numerical and consists of a table, which includes the size in base pairs of each of the peaks (calculated by reference to the internal standard) and the height of each peak (relative to the amount of fluorescence detected) (**Table 6.2**). When analyzing any particular profile, a minimum threshold of fluorescence is first defined to exclude background noise. The minimum peak height is normally set at 50–100 fluorescent units (6, 11, 13, 16) (*see* **Note 4**). The calculation of T-RF size after comparison with internal standard can be estimated using the Local Southern size calling algorithm method on the GENESCAN software.

3.6. Computer Programs Used for Exploratory Analysis T-RFLP Microbial Community Fingerprints

The output generated from automatic sequencers is rarely suitable for statistical analysis and for assigning detected T-RFs and their relative abundances to appropriate ribotypes. The process of manually sorting and manipulating profiles into the desired format is tedious, time-consuming, and often influenced human error. The

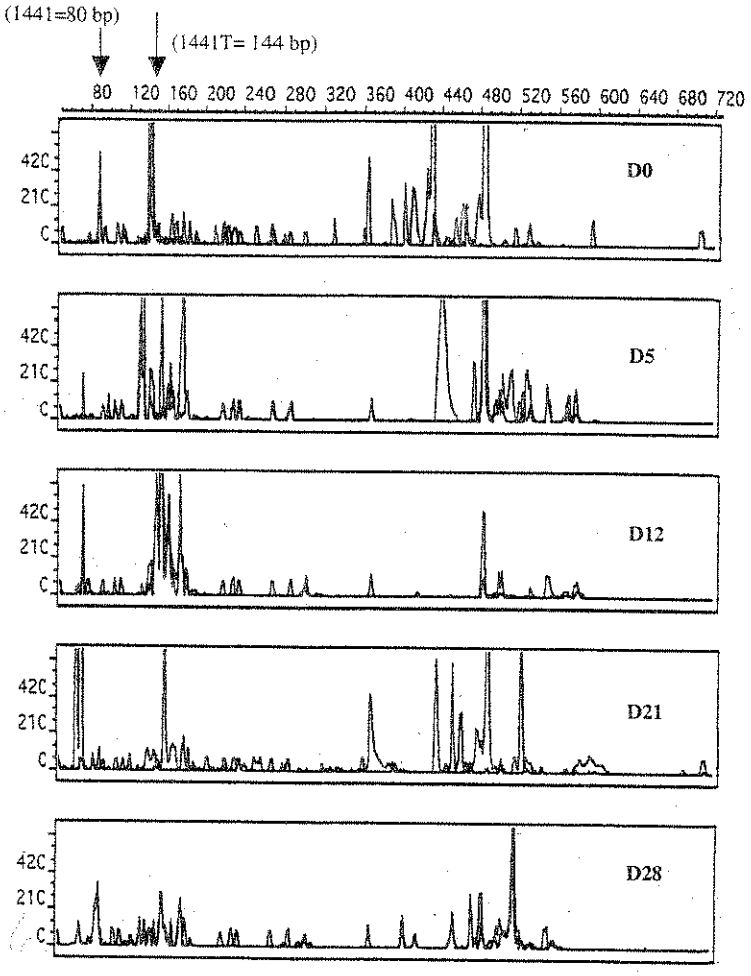

Fig. 6.1. T-RFLP profiles from *Hha* I analysis of 16S rRNA gene PCR products from DNA isolated from manure compost at different stages of composting (D0, day 0; D5, day 5; D12, day 12; D21, day 21; D28, day 28).

following are computer programs that are available for various analyses of T-RFLP data:

1. RIBOSORT is a computer package used for editing of automates T-RFLP data (20). It is designed to eliminate the laborious task of manually classifying community fingerprints in microbial ecology studies. The program automatically assigns detected fragments and their respective relative abundance to appropriate ribotypes.
2. T-RFLP FRAGSORT correlates multiple 16S rRNA gene T-RFLP profiles with corresponding in silico *a* mplification and Digestions of Ribosomal Database Project II Alignments (http://www.oardc.ohio-state.edu/trflpfragsort).

Table 6.2
Example of numerical T-RFLP output of 3′ fragments generated by *Hha* I digestion of 16S rRNA from PCR products directly amplified from D0 (day 0) manure compost

Dye/sample peak[a]	Minutes[b]	Size[c]	Peak height[d]	Peak area[e]	Data point[f]
G, 17	91.68	199.68	167	1659	3438
G, 18	93.23	203.68	127	1658	3496
G, 19	93.60	204.65	431	4517	3510
G, 20	93.97	205.62	335	3747	3524
G, 21	94.29	206.45	198	2390	3536
G, 22	94.69	207.50	108	1259	3551
G, 23	95.17	208.75	162	3086	3569
G, 24	97.47	214.80	156	1584	3655
G, 25	97.81	215.72	553	6019	3668

[a]G indicates green, i.e., 3′ fragments. 17–25 represents the number of each fragment in the profile.
[b]Time in minute of migration of the fragment during electrophoresis before it reaches the detector.
[c]Size of fragments in base pairs.
[d]Peak height is given as fluorescent units.
[e]Peak area is a function of the peak height and the relative spread of each peak.
[f]Each peak is assigned a data point.

3. TAP-TRFLP uses virtual digests of sequence databases to determine predicted fragment lengths to ribosomal DNA (21).
4. TRAMPR matches a database of eukaryotic T-RFLP profiles from multiple restriction digests to environmental samples (22).
5. T-RFLP PROFILE calculates the similarity between profiles (https://rdp8.cme.msu.edu/cgis/trflp.cgi).
6. T-RFLP STATS has been developed for clustering T-RFLP profiles from multiple communities (23).
7. T-ALIGN is a program that compares multiple T-RFLP profiles to identify shared and unique components of microbial communities and also constructs consensus profiles from multiple T-RFLP profiles (24); http://inismor.ucd.ie/~talign/index.html).
8. FRAGMATCH matches a database of eukaryotic T-RFLP profiles from multiple restriction digests to environmental samples (25).

9. TRFCUT uses virtual digests of sequence databases to determine predicted fragment lengths to functional marker genes (26).
10. MiCA T-RFLP Analysis (APLAUS+) compares the data from one or more T-RFLP profiles to the outcomes of in silico analyses of sequences in the database done using the same primers and enzymes (http://mica.ibest.uidaho.edu/pat.php).
11. MiCA: T-RFLP Analysis (PAT+) a phylogenetic assignment tool that enables investigators to quickly find possible phylogenetic assignments based on data from series of restriction enzyme digests (http://mica.ibest.uidaho.edu/pat.php).
12. T-RFLP PROFILE MATRIX has been developed for clustering T-RFLP profiles from multiple communities (https://rdp8.cme.msu.edu/cgis/trflp.cgi).

3.7. Application of Diversity Statistics to Describe Diversity Patterns of T-RFLP Profiles

Diversity of T-RFLP patterns, such as the Shannon index ($\hat{H}$), equitability index (J), richness (d), and evenness (e), can be used as means of evaluating microbial diversity of compost samples using the formula suggested by Atlas and Bartha (27). The T-RFLP-based diversity statistics can be calculated as follows:

$$\text{Shannon index}\,(H) = \frac{C}{N} N \log_{10} N - \sum ni \log_{10} ni,$$

where $C = 2.3$; N = sum of peak areas in a given T-RFLP; n_i = area of T-RF i; and i = number of T-RFs of each T-RFLP pattern. This calculation was derived on Shannon and Weaver's formula based on the

$$\text{Equilitability index}\,(J) = \frac{H}{H_{\max}},$$

where $\hat{H}$ = Shannon-diversity index and $H_{\max}$ theoretical maximal Shannon index for the T-RFLP examined, assuming that each peak represents only one member.

$$\text{Richness}\,(d) = \frac{S-1}{log N},$$

where S = number of T-RFs, N = sum of all peak areas in a given T-RFLP pattern.

$$\text{Evenness}\,(e) = \frac{H}{log S},$$

where $\hat{H}$ = Shannon index, S = total number of T-RFs.

3.8. Application of Multivariate Statistical Analyses to Interpret T-RFLP Data

T-RFLP experiments generate large data sets, and a major challenge in T-RFLP experiments is to extract meaningful information out of the data. Too often, noise and bias from large data sets cloud data analysis and thus hinder our ability to learn about what basic principles control microbial community diversity and composition, microbial processes, and interspecies interactions (28). The management of microbial communities for practical applications such as bioremediation and waste treatment is also impeded by our inability to predict community dynamics and function under different environmental conditions. The most simplistic T-RFLP community analysis approach is to compare the presence or absence of different peaks. Such an approach is valid; however, it lacks the benefits of quantitative analysis. Many different statistical methods have been used for analyzing T-RFLP data, such as principal components analysis (6, 29–30), cluster analysis (6, 9–10, 13), self-organizing maps (28), and multidimensional scaling (31). The combination of these analyses offers a balance between noise elimination and information retention, yielding a powerful and yet easily interpreted method to examine community patterns based on T-RFLP data.

4. Notes

To be useful as a biomarker in composting systems, T-RFLP data need to be highly reproducible and must reflect compost microbial community composition. Like any other method, T-RFLP suffers from its own inherent pitfalls that need to be taken account with. Below are the crucial steps in T-RFLP that require attention.

1. Sample preparation and DNA extraction have the potential to influence T-RFLP fingerprint of microbial community, thus the representative sample size and DNA extraction protocol should be selected and verified carefully to minimize the possibility of later misinterpretation of results. In order to minimize the inherent random bias in compost sample composition, numerous replicate samples should be analyzed or even replicate extractions pooled. Subsequent steps in T-RFLP analysis are highly dependent on the starting DNA purity and extent of shearing. It would be worthwhile to consider a DNA extraction protocol with significantly remove contaminants that co-elute with DNA and reduce the degree of shearing. It is needless to say that these factors should be kept at minimum or at least constant across various samples and replicates.
2. Primer selection and PCR conditions can be improved to increase specificity and reduce bias during PCR. The numbers of ribosomal, housekeeping, and other functional

gene sequences in databases have increased exponentially, thus enabling constant improvement in primer quality through their evaluation using freely available tools such as Amplicon (http://sourceforge.net/projects/amplicon), BLAST http://www.ncbi.nlm.nih.gov/BLAST/ and FunGene (http://flyingcloud.cme.msu.edu/fungene//). Classical PCR optimization of PCR composition and thermal cycling parameters should also not be neglected.

3. The choice of restriction enzyme to be employed should be based on those that cut frequently (i.e., four-base cutters) and produce unique patterns between operational taxonomic units. PCR product digestion with endonucleases should be explored to determine which enzyme results in the highest number of peaks detected, therefore yielding best enzyme for each primer and PCR condition set. The use of multiple enzymes is also suggested in order to obtain better resolution between different communities. By choosing the appropriate number and types of restriction endonucleases, the probability that the resulting T-RF size distributions more accurately reflect the natural diversity of microbial populations within a sampled community is increased. In some instances, enzymatic digestions of PCR products can yield incomplete digests due to various reasons such as template purity, complexity, PCR salt interference, or traces of PCR enhancers and additives. Longer restriction times and higher enzyme concentration in restriction reactions of PCR products from environmental samples (i.e., composts, soils, sludge) are therefore suggested.
4. For the analysis of chromatogram, peaks below 50–100 units of fluorescence are excluded because of their low level of reproducibility. However, differences in DNA loading can also generate slightly different profiles. For this reason, it could be useful to standardize the DNA quantities loaded into the capillary. There is yet, no agreed-upon method for normalizing samples with different amounts of DNA, which would allow easy comparison of profiles with different total amounts of fluorescent label (32). Kaplan et al. (33) present a method for standardizing T-RFLP patterns based on T-RF peak area. The amount of DNA loaded onto a gel or a capillary is estimated as the sum of all T-RF peak areas in a pattern (total peak area). Dunbar et al. (34) propose a method for standardizing T-RF patterns based on peak height. The sum of peak height values is then standardized between samples by proportionally decreasing the height of each peak in the profiles until the sum of peak heights (total fluorescence) for each profile equals the lowest value represented among the samples.

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