Terminal Fragment Length Polymorphism (T-RFLP) Analysis on Applied Biosystems Capillary Electrophoresis Systems

Introduction

T-RFLP analysis is a technique used to study complex microbial communities based on variation in the 16S rRNA gene (1). T-RFLP analysis can be used to examine microbial community structure and community dynamics in response to changes in different environmental parameters or to study bacterial populations in natural habitats. It has been applied to the study of complex microbial communities in diverse environments such as soil (2), marine and activated sludge systems (3) as well as in a study to characterize oral bacterial flora in saliva in healthy subjects versus patients with periodontitis (4). The T-RFLP technique is a culture-independent, rapid, sensitive and reproducible method of assessing diversity of complex communities without the need for any genomic sequence information. The technique provides information on a collection of microorganisms that may be present in a given sample. After the completion of a T-RFLP project, if a researcher is interested in a finer level of analysis at a species level, the Applied Biosystems MicroSeq® identification kit may be used.

Figure 1 shows an overview of the T-RFLP technique. Total DNA is first extracted from the microbial community and the16S rRNA gene is amplified from samples using fluorescently-labeled forward and reverse primers. Next, the PCR product is purified and subjected to restriction enzyme digestion with enzymes that have 4 base pair recognition sites. This step generates fluorescently-labeled terminal restriction fragments. The digested products are then separated and detected on an appropriate electrophoresis platform. For a given sample the terminal fragments will contain a fluorescent label at the 5' end and will therefore be detected. The output will be a series of peaks (fragments) of various sizes and heights that represents the profile of that sample.

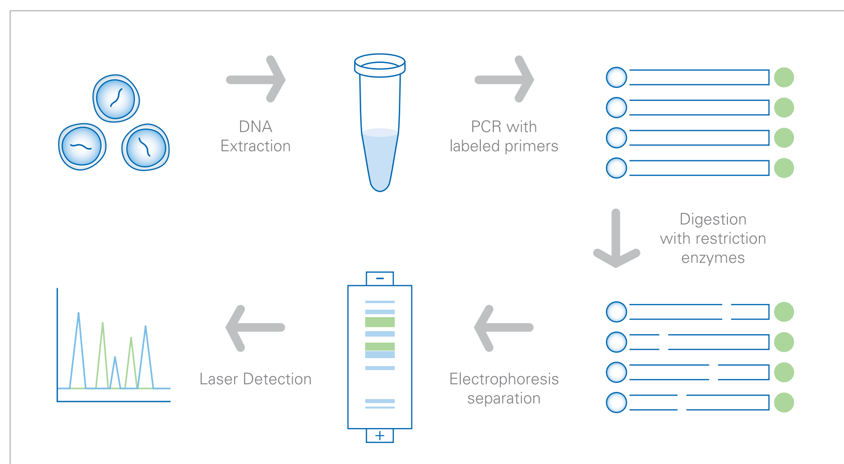

Figure 1. Flowchart of steps required for a T-RFLP assay.

Typical T-RFLP analysis comprises five major steps:

1. DNA isolation and purification
2. PCR amplification and restriction enzyme digestion
3. Separation and detection of the digested products via electrophoresis
4. Analysis of data to generate the fragment profile for each sample
5. Clustering analysis based on the profile of samples from step 4

In this application note, we will present data from T-RFLP assays that were performed on microbial communities present in enrichment cultures of Perchlorate Respiring Bacteria (PRB). PRB are used as part of the biological reduction of perchlorate to innocuous chloride, a process which is inexpensive and has been demonstrated in bench and full-scale reactors (5). Several PRB have been isolated and are ubiquitous in a variety of environments (6). Engineered biological reactors for the removal of perchlorate from waters may necessitate bioaugmentation with a PRB mixed culture that can be enriched from environmental samples. To maintain stable operation of

engineered bioreactors for perchlorate removal, it is important to assure that the PRB enrichment culture remain present in the bioaugmentation culture organism after long periods of operations. Since testing and identification of individual PRB would be laborious and costly, T-RFLP analysis was used as a potential tool to evaluate changes in the enrichment culture composition with time. Our results will demonstrate that the Applied Biosystems capillary electrophoresis platforms and GeneMapper® software v3.7 or higher provide an optimal system for performing T-RFLP projects.

The T-RFLP Assay

DNA isolation, purification and PCR amplification were performed in collaboration with Dr. Jaci Batista, University of Las Vegas, Nevada. For the PCR step, the forward primer was labeled with FAM™ (5'-CAGGC-CTAACACATGCAAGTC-3') dye-label and the reverse primer was labeled with PET® (5'-ACGGGCG-GTGTGTACAAG-3') dye label. The labeled PCR product was purified and subjected to restriction enzyme digestion with either Alu I or Hha I restriction enzymes. The T-RFLP assay was performed on duplicate samples that had been isolated from the bioreactor at 21 different time points over a span of about 4 months.

Capillary Electrophoresis

Having a robust and reliable electrophoresis system that can generate reproducible profiles is crucial to the success of the T-RFLP assay. The Applied Biosystems 3130 series Genetic Analyzers and 3730 series DNA Analyzers are fully automated, high-performance, fluorescence-based, multi-capillary systems that can run 4 (3130 Genetic Analyzer), 16 (3130*xl* Genetic Analyzer), 48 (3730 DNA Analyzer) or 96 (3730*xl* DNA Analyzer) samples. Sample analysis on these instruments is fully automated, from the moment each 96- or 384-well plate is placed on the instrument and the run is initiated. The systems provide continuous, unattended operation, from automated polymer loading and sample injection to separation, detection, and data generation.

When the Autoanalysis feature of GeneMapper® software is used for data analysis, the systems offers true one-button operation from sample loading to data analysis. The instruments also contain several features, such as:

- A detection cell heater designed for use with POP-7™ for better thermal control resulting in more reproducible T-RFLP profiles
- An automated polymer delivery system that significantly reduces set-up time and cost enabling the generation of increased amounts of data
- Easy to use wizards for instrument operation and maintenance

Electrophoresis of the T-RFLP products from the Perchlorate Respiring Bacteria

An aliquot of the digested PCR product was diluted 1:20 and a portion (1 µl) of the diluted product was mixed with the GeneScan™-500 LIZ® size standard and denatured. The samples were then subjected to electrophoresis on the 3130*xl* Genetic Analyzer using the FA_36_POP-7™ run module and G5 dye set. For the 3730 series DNA analyzer the GeneMapper_36_POP-7 run module and G5 dye set can be used for electrophoresis. Typical electropherograms obtained from these runs are shown in Figure 2.

Automated Data Analysis with GeneMapper® Software v3.7 or higher

Having analysis software that can accurately and reproducibly analyze the peak patterns from T-RFLP assays is crucial to the successful completion of a T-RFLP project. GeneMapper software contains an "AFLP®" analysis method that contains analysis parameters for pattern recognition of

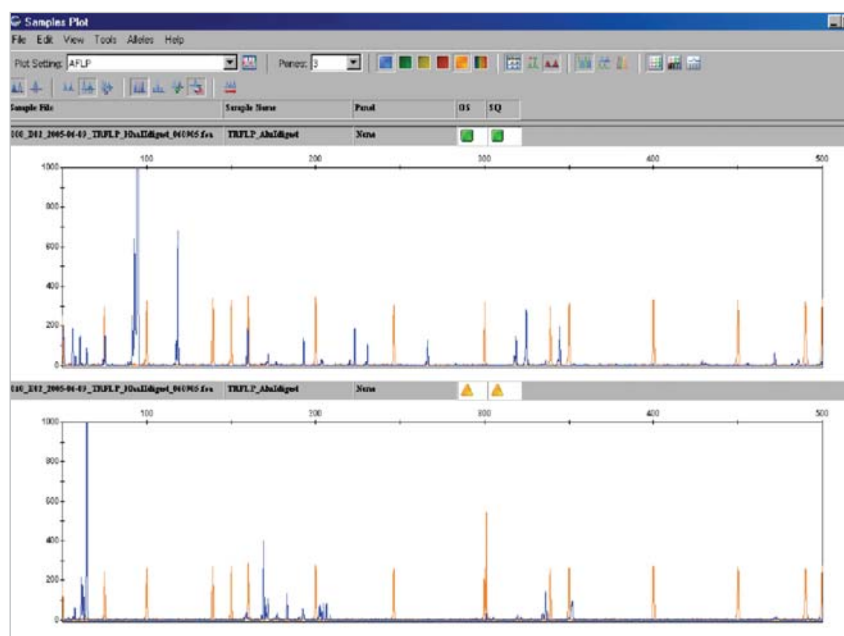

Figure 2. T-RFLP peaks isolated from the reactor at two different time points. The blue peaks represent the FAM-labeled terminal restriction fragments while the orange peaks represent the GS500 LIZ size standard.

fragments across samples to generate a "fingerprint" for every sample. Although labeled as AFLP this method can be used to analyze any type of data from fragment length polymorphism assays such as RFLP or T-RFLP. Features of the software useful for T-RFLP analysis include:

- Ability to generate a panel (the collection of markers) data from sample files that have been added to a project.
- Sizing Quality and Genotyping Quality values that flag poor quality samples enabling easy identification and decrease manual review.
- Automatic generation of final marker genotypes in a standard binary format where "1s" represent presence of a given fragment while "0s" represent absence of the correspon-ding fragment (see Table 1).

Data Analysis of the PRB T-RFLP profiles

Since both the forward and reverse PCR primers were fluorescently labeled, two profiles were generated for each sample. In addition since two restriction enzymes were used, a total of four profiles as listed below were expected for each sample:

1. AluI-Blue (FAM)
2. AluI-Red (PET)
3. HhaI-Blue (FAM)
4. HhaI-Red (PET)

Table 1. Generation of genotypes in standard binary format where 1 represents the presence of an allele/peak and 0 represents the absence of the corresponding allele.

Samples | Genotypes

	Sample File	Sample Name	Dye	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Allele 6	Allele 7	Allele 8	Allele 9
1	002_A04_2005-0	TRFLP_Aluldigest	B	0	0	0	1	1	0	1	1	1
2	004_B04_2005-0	TRFLP_Aluldigest	B	0	0	0	0	1	0	1	1	0
3	006_C04_2005-0	TRFLP_Aluldigest	B	0	0	0	0	0	1	1	0	0
4	008_D04_2005-0	TRFLP_Aluldigest	B	0	0	0	0	0	1	1	1	0
5	010_E04_2005-0	TRFLP_Aluldigest	B	0	0	1	0	0	1	0	0	0
6	012_F04_2005-0	TRFLP_Aluldigest	B	0	0	1	0	0	1	0	0	0
7	014_G04_2005-0	TRFLP_Aluldigest	B	0	0	1	0	0	1	0	1	0
8	016_H04_2005-0	TRFLP_Aluldigest	B	0	0	1	1	0	1	1	1	0

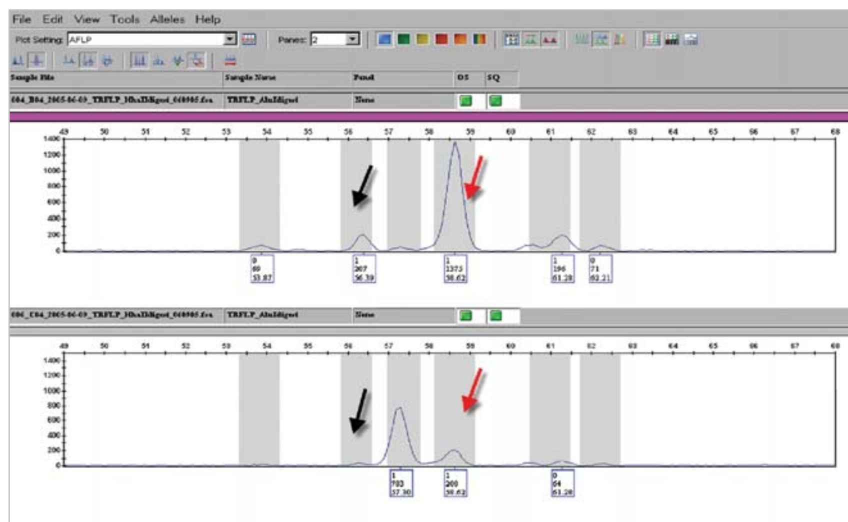

Figure 3. Comparison of a section of the T-RFLP profile of samples from two different time points. The grey bars represent the bins and squares below each peak represent allele labels that contain information on the allele calls, size and height of each peak. The black arrow indicates a peak present at one time point (top panel) but absent in another time point (lower panel). The red arrow points to the reduction in peak height of the same peak across the two time points. It should be noted that the arrows were added manually to the figure to indicate the appropriate peaks.

Data analysis was performed with GeneMapper® software using the AFLP analysis method and panels and bins were generated using the fragments across all samples. For each of the 4 (four) profile groups analysis was done on the duplicate samples that had been isolated at the 21 different time points from the reactor. An example of typical bins and the corresponding labels containing the allele calls are shown in Figure 3. As can be seen in the sample data shown there is a change in the fragment profile between the two time points as indicated by the absence of a peak (black arrow) as well as a reduction in the height of a peak (red arrow) upon comparison of the two sample plots. The peak patterns are automatically converted to a table of binary marker genotypes as shown in Table 1, which was then exported and analyzed for similarity and generation of dendrograms using the statistics software package Minitab (data not shown). The flexible binary format of the GeneMapper genotypes data should enable other downstream analysis software to be used for this type of clustering analysis.

Conclusion

The Applied Biosystems capillary electrophoresis platforms, the 3130 Series and 3730 series Analyzers, in conjunction with GeneMapper® Software v3.7 or higher, provide an optimal solution for performing T-RFLP assays. Using this system we were successfully able to perform analysis of microbial samples that had been isolated from PRB enrichment bioreactors. System features include high resolution, excellent sizing precision, low turnaround times, and ease of use. The integration of GeneMapper software with these instruments provides one-button operation for sample loading, data

collection, fragment size-calling, and accurate marker scoring in both graphical and standard binary formats. All of these features enable the generation of ample high-quality data with minimal hands-on time required and little need for manual data review.

Acknowledgements

We would like to acknowledge Dr. Jaci Batista at the University of Las Vegas, Nevada for her generous contribution of samples used to generate the data and her technical input for this application note.

References

1. Osborn, A. M., Moore, R.B. and Timmis, K.N. (2000). An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. Environmental Microbiology. 2(1): 39–50.
2. Derakshani, M., Lukow, T. and Liesack, W. (2001). Novel bacterial lineages at the (sub)division level as detected signature nucleotide-targeted recovery of 16s rRNA genes from bulk soil and rice roots of flooded rice microcosms. Applied Environmental Microbiology. 67(2):623–631.
3. Eschenhagen, M., Schuppler, M. and Roske, I. (2003). Molecular characterization of the microbial community structure in two activated sludge systems for the advanced treatment of domestic effluents. Water Res. 37(13):3224–32.
4. Sakamoto, M., Takeuchi, Y., Umeda, M., Ishikawa, I. and Benno, Y. (2003). Application of terminal RFLP analysis to characterize oral bacterial flora in saliva of healthy subjects and patients with periodontitis. Journal of Medical Microbiology 52:79–89.
5. Batista, J. R. and J. Liu, Biological Perchlorate Removal from Drinking Waters Incorporating Microporous Membranes, *In:Bioremediation of Inorganic Compounds*, Editors: Andrea Leeson, B. Peyton, J. Means, and V. Magar, Volume 6(9), page 265–272, Battelle Press, Columbus.
6. Coates, J.D., Michaelidou, U., Bruce, R.A., O'Connor, S.M., Crespi, J.N. and Achenbach, L.A (1990). Ubiquity and diversity of dissimilatory (per)chlorate-reducing bacteria. Appl Environ Microbiol. 65(12): 5234-5241.

Ordering Information

Product	P/N
3130*xl* and 3100 Capillary Array (36 cm)	4315931
3130 and 3100–*Avant* Capillary Array (36 cm)	4333464
3730 Capillary Array (36 cm)	4331247
3730*xl* Capillary Array (36 cm)	4331244
3130 POP-7™ Polymer	4352759
3730/3730*xl* POP-7™ Polymer	4363929
10X 31XX Genetic Analyzer Buffer with EDTA	402824
10X 3730/3730*xl* Running Buffer with EDTA	4335613
Hi-Di™ Formamide	4311320
Matrix Standard Set DS-33	4345833
GeneScan™-500 LIZ® Size Standard	4322682

For Research Use Only. Not for use in diagnostic procedures.

© 2005 Applied Biosystems. Applied Biosystems, GeneMapper are registered trademarks and AB (Design), Applera, FAM, Hi-Di,POP-7, LIZ and PET are trademarks of Applera Corporation and/or its subsidiaries in the US and certain other countries.

NOTICE TO PURCHASER: PLEASE REFER TO THE AFLP KIT, APPLIED BIOSYSTEMS 3130 GENETIC ANALYZER, APPLIED BIOSYSTEMS 3730 DNA ANALYZER AND GENEMAPPER SOFTWARE USER'S MANUAL FOR LIMITED LABEL LICENSE OR DISCLAIMER INFORMATION.

Printed in the USA, 12/2005, Publication 106AP22-01

Headquarters
850 Lincoln Centre Drive | Foster City, CA 94404 USA
Phone 650.638.5800 | Toll Free 800.345.5224
www.appliedbiosystems.com

International Sales
For our office locations please call the division headquarters or refer to our Web site at
www.appliedbiosystems.com/about/offices.cfm