Determining the Primers and Methods for T-RFLP Analysis of Yellowstone National Park Soil and Soil from Washington and Lee University's Campus to be Used with the LI-COR 4300

Honors Thesis

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Introduction

The relationship and interaction between aboveground and belowground biota is the driving force behind the development and maintenance of ecosystems (Wardle et al., 2004). Soil biota consists of both prokaryotes and eukaryotes with species grouped under all of the various kingdoms. Soil microbes are just one of the many parts of soil organisms that are responsive to plant community composition. Wardle et al., 2004, explain that plant species and the microbial diversity of soil exist in a cyclical relationship; the quantity and quality of resources returned to the soil depends on the plant species composition of the aboveground biota, and it is believed that the quantity and quality of plants that can be supported depends upon the diversity of the microbes. The distribution of soil organisms can differ depending on the coexisting plant species. Individual species of plants can have very important effects on the microbes in the soil and the processes that they regulate; however, it is uncertain as to the degree to which specific microbe species impact the species of plants that can exist (Wardle et al., 2004). There is an estimated 4600 distinct microbe genomes per gram of soil; the divisions of bacteria commonly found to dominate soil are known, however, the impact of most of these species on the ecosystem has yet to be determined (Kent and Triplett, 2002). Even in the bacterial phyla thought to be well known more diversity is continually discovered due to improved methods of sequencing and identifying microorganisms. According to Kent and Triplett, the fraction of cells that can at this time be cultured is a poor representation of the abundance and phylogenetic breadth of the microbe community present in the environment. Microbial ecologists are working on determining the diversity of bacterial communities in order to better analyze the various existing environments.

Such microbial analyses might help ecologists to gain a more clear understanding of the development and evolution of plant communities, and provide insight as to how environmental changes impact the ecosystem.

The Interaction Between Aboveground and Belowground Biota

In general, in the cyclical relationship between aboveground and belowground biota as described by Wardle et al., plants provide the organic carbon necessary for the functioning of the decomposers and the resources needed for such organisms as root herbivores, pathogens, and mutual symbiotes, like mycorrhizal fungi; decomposers unlock the nutrients in dead plant material and determine the supply of available soil elements, which thus indirectly impacts the plant growth. Those organisms that are more specifically connected with the roots have a more direct relationship with plants, influencing the quality, direction, and flow of energy and nutrients between aboveground and belowground biota (Wardle et al., 2004, see Figures 1 and 2). Plant species differ in the quality and amount of resources that they contribute to the soil, and individual species may have unforeseen effects on the soil biota and the processes that are regulated. However, the overall plant community has been shown to greatly impact the composition of root-associated organisms, and influence several of the aspects of the decomposer communities as well. Both of these pieces of the belowground biota are very important to plants not only for the nutrients that they make available directly, but also in their function in the nitrogen cycle.

Nitrogen is the mineral nutrient that is most often in short supply for plant communities, and yet it is the fourth most common element in plant make-up. Plants can only use nitrogen in two forms, the ammonium ion (NH_4^+) and the nitrate ion (NO_3^-), but

most obtain the nitrogen they need as inorganic nitrate. Most ecosystems store nitrogen in living and dead organic matter; therefore, in order for plants to use the nitrogen it must first be changed into an alternate form. Decomposers are the key elements in the process of modifying the nitrogen of organic matter. The nitrogen is changed from ammonia (NH_3) to ammonium salts (NH_4) by various bacteria, actinomycetes, and fungi through a process called mineralization. The ammonium can then be chemically altered through a process of nitrification into nitrite (NO_2^-) by autotrophic bacteria, and then further transformed into nitrate (NO_3^-) by different bacteria. (Paul, E.A., and F.E. Clark, 1996) Nitrogen and the process of nitrification are also strongly connected with belowground biota through the function of herbivory.

Foliar herbivory encourages the release of carbon into the rhizosphere, which in turn positively impacts microbial activity, leading to an increase in nitrogen availability for the plants. Herbivores can positively affect the quality and quantity of plant-derived resources over time through various mechanisms; herbivores return organic matter to the soil in the form of fecal matter, which induces an increase in the concentration of nutrients in the remaining plant material and impairs plant succession, thus preventing other plant species with poorer litter quality from taking over (Wardle et al., 2004). Herbivores also promote photosynthesis in grazed plants, and there is believed to be a large increase of rhizospheric microbial growth 24 hours after clipping, providing the plants with an increased availability of nutrients (Hamilton and Frank, 2001).

Culture-Independent Analyses of Microbial Diversity in Soils

Most of the technology used in microbial ecology today is centered on the molecular phylogeny of ribosomal RNA. rRNA molecules are very useful in ecological

research because they have been discovered to serve as molecular clocks. These molecules are also found in all organisms and they are very highly conserved both structurally and functionally over time. The large rRNA molecules have been noted to contain many domains that each has their own rate of sequence change (Kent and Triplett, 2002). Studying these changes over time can provide insight to phylogenetic changes, and thus to relationships among microbes and plants within ecosystems. Such culture-independent methods of microbial diversity analysis most often make use of polymerase chain reactions, PCR, to amplify the genetic markers of the extracted DNA (Kent and Triplett, 2002). 16SrDNA has been the sequence of choice in most of the PCR-based methods because of its relatively slow rate of change; it is therefore possible to more easily compare microbial DNA and look for differences and similarities that might determine the specific microbial species present. Slight variations in sequencing of the microbial DNA can occur even if the 16SrDNA is the same because of natural variation and chimeric genes; however, slight differences are often not enough to consider the DNA that of a new or different species of microbe because it often has not evolved enough to be considered separate (Rappe and Giovannoni, 2003).

The use of PCR in community analyses can be more complicated than realized at first glance. A number of steps of the process provide the possibility of introduced biases, most of which cannot be avoided (Kent and Triplett, 2002). Kent and Triplett explain that DNA extraction is one step that can be difficult because bacterial cell structure varies among taxonomic groups, making it more or less easy to disrupt the cells. Environmental factors can also play a role in sample collection and DNA extraction. Therefore, the process must optimize the sample collection and the lysis of structurally different cells.

According to Kent and Triplett, attention must be paid to detail because the extraction and purification methods are very important to the soil bacteria community profiles, considering that they can differ greatly according to the methods used. Despite these few challenges, PCR-based community analysis is very commonly used for its ease of studying many different samples, and the ability to alter the analysis to examine specific organisms or taxa through the use of universal or group-specific primers (Kent and Triplett, 2002).

The phylogenetic information about specific organisms or taxa is obtained through isolation and sequence analysis of the various PCR fragments. Terminal restriction fragment length polymorphism (T-RFLP) is the method commonly used to analyze these fragments. T-RFLP is the only fragment analysis method that provides phylogenetic information directly without any further sequencing (Kent and Triplett, 2002). T-RFLP uses a fluorescently labeled oligonucleotide primer in PCR amplification, and after the DNA is digested it is followed by gel electrophoresis to separate and detect the various PCR fragments. Band intensity of specific fragments in the gels is measured to compare community profiles. This measuring can be done with automated fluorescence detection methods in order to more accurately analyze the data. As Kent and Triplett explain, the information collected offers insight as to the relative abundance of individual fragments, and thus the abundance of different species. The fragment lengths obtained from T-RFLP analysis are then compared with the expected fragment length of known 16SrRNA gene sequences in order to differentiate the various community members present (Kent and Triplett, 2002).

Purpose

At the onset of our research, our goals consisted of determining the primers that should be used in the T-RFLP protocol with the LI-COR 4300, optimizing the conditions for soil DNA extraction, PCR, and T-RFLP, and ultimately testing the methods on soil samples in order to obtain results concerning which populations of bacteria are present and in what quantity. The importance of perfecting the methodology used with the LI-COR 4300 for Dr. Bill Hamilton's lab at Washington and Lee University is that it will provide a resource for comparing soil communities. Comparison of communities will provide information as to what species are present and ultimately how the communities change in response to surrounding environmental factors. The variations seen among different soil samples could provide more clear insight as to how the aboveground and belowground biotas interact. The comparisons may also help explain the exact impact that changes in nitrogen, carbon, oxygen, and mineral levels may have on the microbe community. The effects of herbivory can thus be examined as well considering that herbivory incorporates numerous interactions that are often studied separately.

The research proved to be very challenging because there is no set protocol for the many steps leading to T-RFLP or for T-RFLP itself that has been shown to work universally for different soil types. Multiple primers were tested including the forward primers 8F and Com1 and reverse primers Com2, 1492, 1406, and 518 in order to find the primer pair that would be most suitable for a diverse range of microbes found in various soils. 8F and Com2 had the most consistently strong results on the agarose gels, and thus it was the primer pair that was used for T-RFLP. Determining the primer pair to use was a challenge made more difficult because of the many elements of the methods for

DNA extraction, PCR, and T-RFLP that needed to be adjusted. Optimizing the conditions was a very large part of the research that consumed almost all of the DNA retrieved in the five soil DNA extractions. The soils used for the extractions included samples from the Dell and Colonnade on Washington and Lee University's campus, samples obtained by Dr. Bill Hamilton in Yellowstone, and samples prepared by Clint Oakley from Dr. Bill Hamilton's supply of Serengeti soil.

Materials and Methods

DNA Extraction and Sample Preparation

DNA extraction was done according to the *Epicentre SoilMaster DNA Extraction Kit* (see Appendix I). According to the protocol provided by *Epicentre*, the kit utilizes a hot detergent lysis process combined with a chromatography step that removes enzymatic inhibitors known to coextract with DNA from soil and sediment samples. The kit thus provides PCR ready DNA which can be used to obtain T-RFLP profiles of soil samples.

The soil DNA purification was done according to the protocol, with a few exceptions:

- step 3 of part B: the optional step was completed
- step 10 of part B: for the fourth and fifth DNA extractions 150ul of the supernatant was transferred instead of 100ul
- step 16 of part B: for the fifth DNA extraction the pellet was resuspended in 100ul of TE buffer instead of 300ul

Polymerase Chain Reaction (PCR)

The basic recipe for the PCR setup was provided by Dr. Paul Cabe of Washington and Lee University, using the materials from the Promega PCR kit. The recipe was based on a 20ul reaction as follows:

- 10x buffer	2ul
- $MgCl_2$	2.4ul
- dNTP	0.4ul
- Primer (forward)	0.8ul
- Primer (reverse)	0.8ul
- Taq	0.1ul
- dH_20	9.5ul
- template	4ul (or 2ul and 2 additional ul of dH_20)

The recipe was typically prepared in larger quantities as a master mix and then the template was added to the PCR tubes separately. Dilutions of the template were used in an attempt to optimize the conditions and results for PCR and gel electrophoresis. The

Qiagen buffer was substituted for the Promega buffer in one of the last PCR preparations in order to keep all of the conditions of the samples the same.

The programs used for the PCR reactions were run on the Peltier Thermal Cycler (PTC-200) and were adjusted according to the primer pair's necessary annealing temperature. The programs used were as follows:

8F-1406

Calculated
1) 94C, 2min
2) 94C, 30sec
3) 58C, 45sec
4) 72C, 1min 30sec
5) Go to step 2, 29 times
6) 72C, 5min
7) 4C for ever

8F-518

Calculated
1) 94C, 2min
2) 94C, 30sec
3) 55C, 45sec
4) 72C, 1min 30sec
5) Go to step 2, 29 times
6) 72C, 5min
7) 4C for ever

8F-Com2

Calculated
1) 94C, 2min
2) 94C, 30sec
3) 58C, 1min
4) 72C, 1min 30sec
5) Go to step 2, 29 times
6) 72C, 5min
7) 4C for ever

Com11492

Calculated
1) 94C, 2min
2) 94C, 30sec
3) 55C, 1min
4) 72C, 1min 30sec

5) Go to step 2, 29 times
6) 72C, 5min
7) 4C for ever

Com11406
Calculated
1) 94C, 2min
2) 94C, 30sec
3) 58C, 1min
4) 72C, 1min 30sec
5) Go to step 2, 29 times
6) 72C, 5min
7) 4C for ever

Com1-518
Calculated
1) 94C, 2min
2) 94C, 30sec
3) 55C, 1min
4) 72C, 1min 30sec
5) Go to step 2, 29 times
6) 72C, 5min
7) 4C for ever

In an attempt to optimize conditions and improve the results of PCR, a temperature gradient from 57-68C was run on primer pair 8F and Com2 to help determine the best annealing temperature. Epicentre's Failsafe PCR Premix Selection kit was also used in hopes of improving the results of PCR, with premixes A-L being used, and the following master mix and PCR program:

Based on a 25ul reaction-

- Enzyme	0.2ul
- dH_2O	9.5ul
- Template	2.0ul
- Primer (forward)	0.4ul
- Primer (reverse)	0.4ul
- Premix	12.5ul

PCR Program FAILSAFE
Calculated
1) 98C, 2min
2) 98C, 30sec

3) 52.2C, 30sec
4) 70C, 1min
5) Go to step 2, 29 times
6) 4C for ever

Gel Electrophoresis

Following PCR, 5ul of each sample was mixed with 2ul of stain and then loaded onto a 2% agarose gel along with a Promega 100bp ladder and run for 60 minutes at 100 volts in TBE. After the gel was finished running it was stained in ethidium bromide for 1 to 4 minutes and destained in deionized water for 10 to 20 minutes, and then a photo was taken.

T-RFLP and LI-COR4300

The basic preparation for T-RFLP begins with the PCR of the template with a tailed and untailed primer, which ended up being 8FT3 and Com2 respectively. The resulting amplicon was then tested alongside a ladder on a 2% agarose gel. The concentrations of the original extracted DNA samples were tested with NanoDrop Spectrophotometer before setting up a PCR reaction.

Next, using the amplicon obtained in the previous step, a 60ul reaction was set up in order to amplify the amplicon and attach the IR label. The thermal cycler program used for attaching the T3700IR label was AAT3:

Calculated
1) 94C, 2min
2) 94C, 30sec
3) 45C, 30sec
4) 72C, 1min 30sec
5) Go to step 2, 29 times
6) 72C, 5min
7) 4C for ever

After amplifying the amplicon, the amplicon must be purified / cleaned-up. This step was done following the Wizard PCR Preps DNA Purification System and using the direct purification method and vacuum manifold.

The concentration of the resulting purified DNA was again checked with the NanoDrop before moving on to the next step to make sure that there was between 15 and 40 ng/ul.

Next, a restriction enzyme digest was run. 100ng of the purified DNA sample was digested in a 20ul reaction with the desired enzymes, which for our research were HhaI and RsaI. Separate digestions were prepared for each enzyme with the following basic recipe:

- 10x Optimized Buffer C	2.0ul
- 10x BSA	0.2ul
- restriction enzyme	0.5ul
- Wizard prep'd amplicon	1-6ul (depending on DNA concentration)
- ddH_2O	varies for a total reaction volume of 20ul

These digestions were then run in the thermal cycler using the program CUT37/65.

Calculated
1) 37C, 6 hours
2) 65C, 20min
3) 4C for ever

Following the completion of the digestion, the results were verified on a 2% agarose gel using 10ul of each digestion and 2ul of the undigested Wizard Prep'd sample as a comparison.

At this point the 6.5% acrylamide LI-COR gel was prepared according to the Saga manual (see Appendix II).

Then the digested DNA was denatured and run on the LI-COR gel. The basic set-up for the denaturing step was as follows:

- In a PCR tube, add 3ul of the digested sample
- Add 3ul of LI-COR Stop Solution to each tube as well
- Mix well and then briefly centrifuge the sample
- Put in the thermal cycler and run the Denature program(95 C for 3 min); make sure to also denature the molecular weight standard at this point, too
- Chill the denatured sample on ice for 10min

1-1.4ul of each sample was then loaded on the gel, starting with a molecular weight standard and then all of the samples with the same enzyme. A molecular weight standard must separate each different restriction enzyme and must flank both sides of the loaded gel. The gel was then run according to the Saga manual at 1500V, 35mA, 35W, 45C, and slow scan speed for approximately 3 hours.

Fragment Analysis

The software programs FragSort (http://www.oardc.ohio-state.edu/trflpfragsort/default.htm) and MiCA3 Ribosomal Database Project III (http://mica.ibest.uidaho.edu/) were used to analyze the peak intensities collected with the LI-COR 4300. MiCA3 uses the Ribosomal Database to provide all of the sequences for 16sRNA. A file was created with the information provided by the MiCA3 website which was then uploaded into Excel. This information was then put into the FragSort program along with the peak value percentage for the band volume data provided by LI-COR.

Results

Throughout the research five different soil DNA extractions were performed. The resulting DNA from the first three extractions was used to help determine which primer pair would be best for T-RFLP. While working with all five extractions of DNA the methods were tested and altered in order to optimize results.

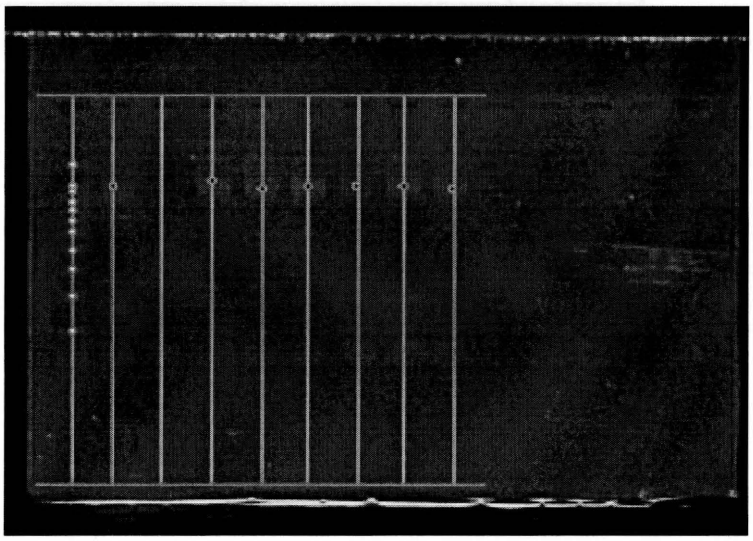
Extraction 1: Two samples of non-descript Yellowstone Soil

PCR was run on the DNA first with Com1/Com2, then 8F(tailed)/1492 two different times. Each run was set up with a combination of straight 4ul of template and a dilution of 2ul template and 2ul dH_2O in order to try and determine whether or not the template needed to be more dilute to improve the results. Following PCR the samples were run on a 2% agarose gel with the Promega 100 bp ladder for comparison. The results for Com1/Com2 were inconclusive, however, bands were seen in two of the lanes with the diluted template. The first run of 8F(tailed)/1492 was inconclusive as well, and there was a lack of a primer dimer for all of the experimental samples. The second run of 8F(tailed)/1492 again saw bands in two of the lanes with diluted template, but unlike the first run a primer dimer was present in all of the lanes.

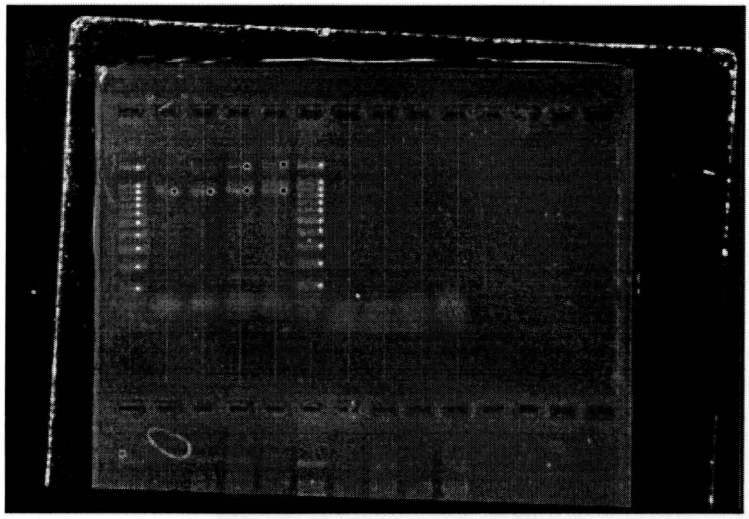
Extraction 2: Four samples of Soil from the Dell at Washington and Lee University

PCR was run on the DNA with 8F/1492, 8F/1406, 8F/518, 8F/Com2, Com1/1406, Com1/518, and Com1/1492 with straight 4ul template and diluted 2ul template. In an attempt to trouble shoot and improve the gel results, a new 8F was ordered and used from this point on that did not have a tail. The program on the thermal cycler that had been used was also altered by increasing the annealing temperature from 50C for 30sec to 55C for 1min, and by increasing the extension time from 1min to 1min 30sec. The gels of

8F/1492 and 8F/1406 had strong primer dimers, but no other bands were visible. The gel of 8F/518 showed some weak bands, which were overall inconclusive, and therefore another gel was run, but unfortunately only primer dimers were seen. 8F/Com2 was gelled as well showing some promising results for both the straight and diluted samples (see Gel 1 below). Com1/1406 and Com1/518 were run on a gel revealing bands with Com1/1406 that were strongest with the diluted sample, but only primer dimers with Com1/518 (see Gel 2). The gel of Com1/1492 showed bands for both the straight and diluted samples. 8F/Com2 was run again resulting in bands for both the straight template and the dilution (see Gel 3). Due to the lack of bands and large primer dimer seen in all of the gels with 518R, a new dilution of 518R was created and PCR'd with 8F and Com1. The thermal cycler programs were also altered for 8F/518 and Com1/518 by lowering the annealing temperature from 58C to 55C. Three gels were run for each primer pair, all of which were inconclusive due to a streaking of the samples on the gels.



Gel 1. 8F/Com2; Lanes left to right: Ladder, Straight, Straight, Dilution, Dilution, Straight, Straight, Dilution, Dilution

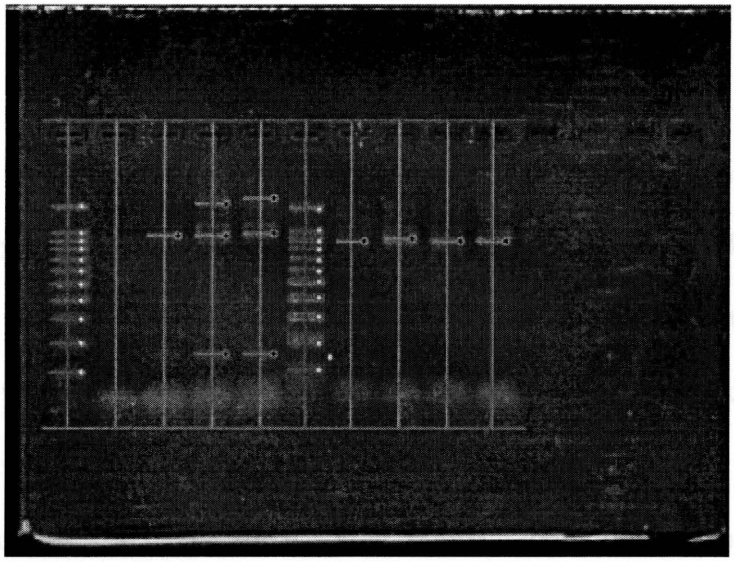


Project:
Date: 1/13/06 3:58:22 PM
Name: Com1/1406/518 KAG

1: 100 bp DNA Ladder (LTI)
2: Standard Com1 + 1406
3: standard Com1+1406
4: x5 dilution Com1+1406
5: x5 dilution Com1+1406
6: 100 bp DNA Ladder (LTI)
7: standard Com1+518
8: standard Com1+518
9: x5 dilution Com1+518
10: x5 dilution Com1+518

	MW (bp)					
	1:	2:	3:	4:	5:	6:
1	2072	1390	1390	2033	2132.7	2072
2	1500			1410	1410	1500
3	1400					1400
4	1300					1300
5	1200					1200
6	1100					1100
7	1000					1000
8	900					900
9	800					800
10	700					700
11	600					600

Gel 2. Com1/1406/518

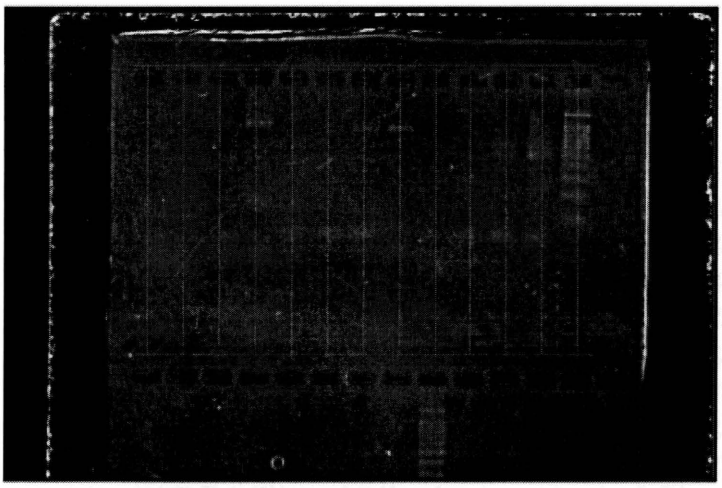


Gel 3. Com1/1492, 8F/Com2; Lanes from left to right: Ladder, Straight Com1/1492, Straight Com1/1492, Dilution Com1/1492, Dilution Com1/1492, Ladder, Straight 8F/Com2, Straight 8F/Com2, Dilution 8F/Com2, Dilution 8F/Com2

Extraction 3: One sample of Yellowstone Soil, Controlled, 72 hours

PCR was run with the new DNA and primer pairs 8F/Com2, Com1/1406, 8F/518, and Com1/518. The gels revealed results for almost every lane, however, the best results were seen with the diluted samples (see Gel 4). Based on the results throughout the research thus far, it was decided that 8F/Com2 would be the primer pair to use with T-RFLP, and thus a PCR of 8F/Com2 was set up adding the T3700 tail to 8F. After PCR was run with 8FT3/Com2, a gel was set up with 8F/Com2 and 8FT3/Com2 with DNA samples from the Dell and the most recent Yellowstone DNA extraction. The gel revealed bands for both primer pairs, both soil DNA types, and both the straight and diluted samples (see Gel 5). A second PCR was set up after the gel was run using the amplified amplicon of 8FT3/Com2 and Yellowstone Soil DNA (sample re-named YS8FT3/Com2), and the program AAT3. We forgot to run a gel to check the second PCR, and went on to purify the DNA with Wizard PCR Preps DNA Purification System. The

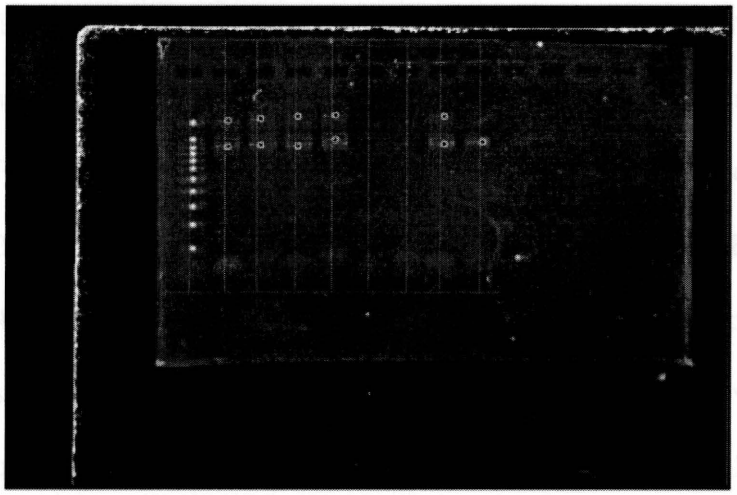
concentration of the purified sample was checked and found to be 20.68ng/ul. Next, a restriction enzyme digest was run with program CUT37/65 and enzymes Hha1 and Rsa1, as well as a control of E. coli and Hha1. The digestion was verified on a 2% agarose gel, but unfortunately no bands or primer dimers were seen, however, it was decided that we should go ahead with the LI-COR gel. The gel was set up with a straight template for each enzyme as well as a 1:3 and 1:6 dilution (diluted with ddH_2O). The dilutions were used to help determine the optimum conditions, and it was ultimately decided that dilutions were not necessary. The gel was seen to be running fast, and thus it was stopped after two hours. The gel had many problems including bad/old acrylamide and degraded molecular weight standards, which made the results inconclusive (see Appendix III, gel 1). Kelly Hemminger, lab technician for Dr. Paul Cabe's lab at Washington and Lee University, decided to try the samples on a thicker 13% acrylamide gel while new acrylamide was being ordered. The thicker gel appeared to improve the band separation, but it was decided that for the future T-RFLP runs a new KB^+ acrylamide would be used. In order to cut down on variables and steps in which an error may occur, labeled 8F primer was ordered as well.



Project:
Date: 2/10/06 2:37:43 AM
Name: yellowstone soil KAG 1

1:
2:
3:
4:
5:
6:
7:
8:
9:
10:
11:
12:
13:

Gel 4. Com1/1492, 8F/Com2, 8F/518; Lanes from left to right: Straight Com1/1492, Straight Com1/1492, Diluted Com1/1492, Diluted Com1/1492, Straight 8F/Com2, Straight 8F/Com2, Diluted 8F/Com2, Diluted 8F/Com2, Straight 8F/518, Straight 8F/518, Diluted 8F/518, Diluted 8F/518



Project:
Date: 2/24/06 12:19:15 PM
Name: 8F/8FT3+Com2

1: Promega 100bp ladder
2:
3:
4:
5:
6:
7:
8:
9:

	MW (bp)						
	1:	2:	3:	4:	5:	8:	9:
1	1500	1558.1	1616.3	1674.4	1709.3	1674.4	976.2
2	1000	914.3	938.1	923.8	1000	947.6	
3	900						
4	800						
5	700						
6	600						
7	500						
8	400						
9	300						
10	200						
11	100						

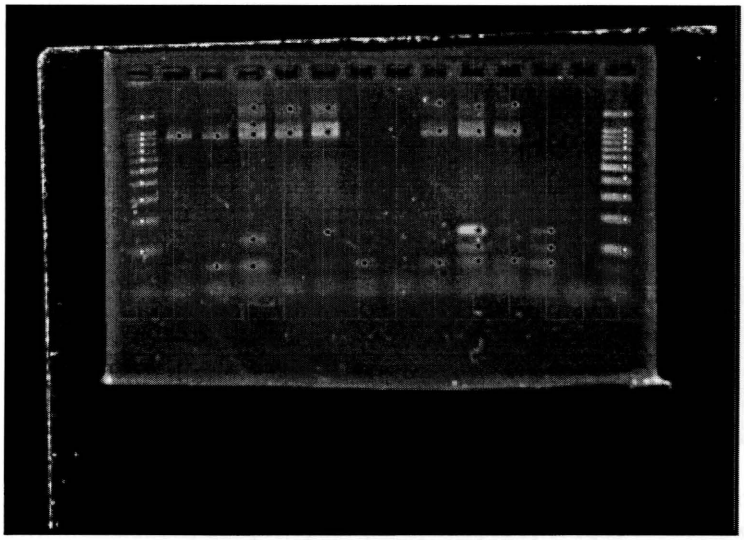
Gel 5. 8F,8FT3/Com2; Lanes from left to right: Ladder, Straight Dell 8F/Com2, Straight Dell 8FT3/Com2, Diluted Dell 8F/Com2, Diluted Dell 8FT3/Com2, Straight YS 8F/Com2, Straight YS 8FT3/Com2, Diluted YS 8F/Com2, Diluted YS 8FT3/Com2

Extraction 4: Twelve samples of Yellowstone Soil, Controlled/Clipped, 24/72 hours

The twelve samples of DNA and their concentrations were as follows:

1) R1 Control 24hr. 1	1.07ng/ul
2) R1 Clipped 24hr. 1	0.84ng/ul
3) R1 Control 72hr. 1	1.37ng/ul
4) R1 Clipped 72hr. 3	1.05ng/ul
5) R3 Control 24hr. 2	2.24ng/ul
6) R3 Clipped 24hr. 1	1.90ng/ul
7) R2 Control 72hr. 4	4.59ng/ul
8) R1 Clipped 72hr. 2	1.89ng/ul
9) R4 Control 24hr. 1	1.27ng/ul
10) R4 Clipped 24hr. 1	1.21ng/ul
11) R4 Control 72hr. 3	2.58ng/ul
12) R4 Clipped 72hr. 3	4.56ng/ul

The concentration of each sample was checked using the NanoDrop, and the results were shockingly lower than expected with concentrations ranging from 0.84ng/ul to 4.59ng/ul. PCR was run on the samples with the primer pair 8FT3/Com2 in 100ul reactions with 20ul template, as well as with the unlabeled primer pair 8F/Com2 in 20ul reactions with 4ul template. A gel was then run of the unlabeled primer pair reaction resulting in nice strong bands where they were expected (see Gel 6); however, there were also bands present lower on the gel above the primer dimer that should not have been there, and thus another PCR was run of these same samples and primer pair in order to try and clarify the result. The gel of the second PCR again had strong bands, but the unexpected bands at the bottom of the gel were still present (see Gel 7). Due to the presence of the unexpected bands, a PCR temperature gradient (program GRAD5768) was run on sample 9 (Control, 24 hr.) of the Yellowstone DNA with the unlabeled primer pair 8F/Com2 in order to try and rule out one of the variables. Unfortunately, the gel did not reveal anything, and therefore the annealing temperature was left the same (see Gel 9).



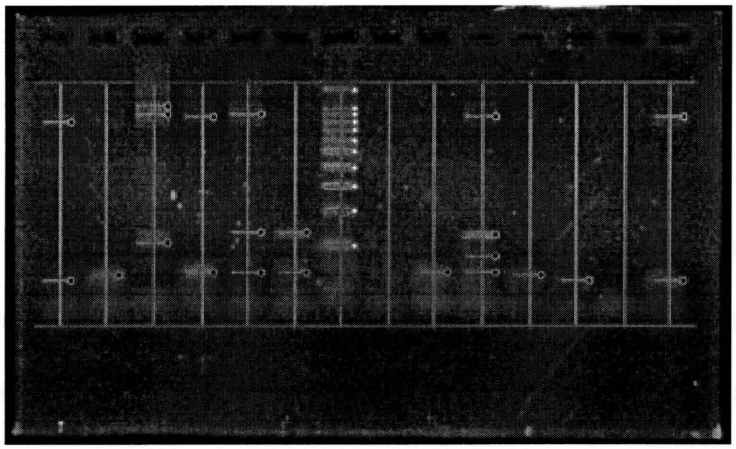
Project:
Date: 3/10/06 12:03:20 PM
Name: YS 8F-Com2 KAG

1: Promega 100bp ladder
2:
3:
4:
5:
6:
7:
8:
9:
10:
11:
12:
13:
14: Promega 100bp ladder

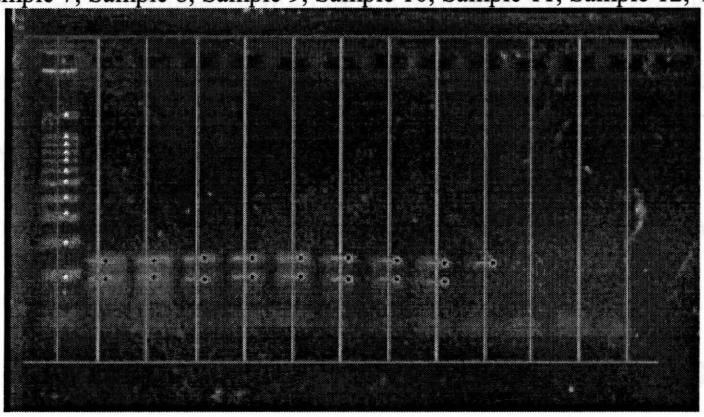
MW (bp)							
1:	2:	3:	4:	5:	6:	7:	9:
1500	924.1	924.1	1736.4	1698.9	1736.4	58.78	1899
1000		47.97	1223.6	991.4	1011.3		1041.1
900			961.5		161.5		61.49
800			137.2				
700			47.97				
600							
500							
400							
300							
200							
100							

MW (bp)			
10:	11:	12:	14:
1773.9	1836.5	168.2	1500
1041.1	1041.1	110.1	1000
168.2	68.24	61.49	900
116.9			800
68.24			700
			600
			500
			400
			300
			200
			100

Gel 6. 8F/Com2; Lanes from left to right: Ladder, Sample1 Control 24hr., Sample 2 Clipped 24hr., Sample 3 Control 72hr, Sample 4 Clipped 72hr., Sample 5 Control 24hr., Sample 6 Clipped 24hr., Sample 7 Control 72hr., Sample 8 Clipped 72hr., Sample 9 Control 24hr., Sample 10 Clipped 24hr., Sample 11 Control 72hr., Sample 12 Clipped 72hr., Ladder



Gel 7. 8F/Com2; Lanes from left to right: Sample 1, Sample 2, Sample3, Sample 4, Sample 5, Sample 6, Ladder, Sample 7, Sample 8, Sample 9, Sample 10, Sample 11, Sample 12, YS original



Gel 8. YS sample 9 Temperature Gradient; Lanes from left to right: 57C, 57.3C, 57.9C, 58.8C, 60.1C, 61.8C, 63.6C, 65.2C, 66.4C, 67.2C, 67.8C, 68C

Extraction 5: Sixteen new samples of Yellowstone Soil, Controlled/Clipped, 24/72 hours

The sixteen samples of DNA and their concentrations were as follows:

1) R2 Control 72hr. 4 9.79ng/ul
2) R1 Control 72hr. 2 1.21ng/ul
3) R2 Control 72hr. 1 5.28ng/ul
4) R4 Clipped 72hr. 3 5.61ng/ul
5) R1 Clipped 72hr. 4 4.78ng/ul
6) R2 Clipped 72hr. 3 17.18ng/ul
7) R3 Control 24hr. 2 7.57ng/ul
8) R2 Control 24hr. 1 9.32ng/ul
9) R1 Control 24hr. 2 7.57ng/ul
10) R3 Clipped 24hr. 1 7.18ng/ul
11) R3 Clipped 24hr. 2 7.86ng/ul
12) R3 Clipped 24hr. 3 11.95ng/ul

13) Control Pooled 72hr. 6.55ng/ul
14) Clipped Pooled 72hr. 3.71ng/ul
15) Control Pooled 24hr. 2.96ng/ul
16) Clipped Pooled 24hr. 17.54ng/ul

During the extraction process the samples were rehydrated in 100ul TE buffer instead of 300ul in order to try and concentrate the DNA more. PCR was then run on the samples with unlabeled primers 8F/Com2 in a 20ul reaction with 4ul of template, and the reactions were gelled, but the gels were inconclusive, lacking any other bands besides the primer dimers. The concentrations of the DNA samples were then checked with NanoDrop (seen above) revealing slightly higher concentrations than the 12 samples from the fourth extraction. The samples were PCR'd a second time with the unlabeled primer pair 8F/Com2 in a 20ul reaction with 2ul template. The gels of the reactions were again inconclusive with a lack of primer dimers or any other bands besides the ladder. Another 20ul PCR reaction was set up with unlabeled 8F/Com2 in which an additional 5ul of BSA was added to each of the PCR tubes to possibly help bind inhibitors. Before the master mix was added to the PCR tubes, the tubes with the BSA were heated up to 90C for 1min. 2ul template was used, but four more PCR tubes were set up with samples 1, 4, 7, and 10 in which 4ul template was used. Gels were run for these samples revealing bands for 2, 3, and 13, but otherwise the results were inconclusive. Kelly Hemminger set up a new PCR reaction with the 12 unbulked samples and a new 8FT3 primer and fresh Com2 primer. Before the samples were PCR'd they were concentrated by evaporating off approximately half of the liquid. The PCR did not look very good on the agarose gel, but Kelly decided to go ahead and check the concentrations (listed below) and clean up the DNA with Promega's Wizard PCR Prep.

1) YS1-WP	32.0ng/ul
2) YS2-WP	20.0ng/ul
3) YS3-WP	40.78ng/ul
4) YS4-WP	36.60ng/ul
5) YS5-WP	39.38ng/ul
6) YS6-WP	32.48ng/ul
7) YS7-WP	25.73ng/ul
8) YS8-WP	33.08ng/ul
9) YS9-WP	30.0ng/ul
10) YS10-WP	27.10ng/ul
11) YS11-WP	40.93ng/ul
12) YS12-WP	11.41ng/ul

The 12 samples were then set up to be digested with Hha1 and Rsa1; each template had its own recipe based on its concentration so that each digestion contained 100ng of DNA. An additional sample from the third extraction was used as well in order to compare the labeled direct PCR amplicon with the amplified amplicon. We were also interested in seeing what the results of the third extraction (sample 13 in this run) would be with fresh, new acrylamide. A LI-COR gel was prepared with lane 1 containing the molecular weight standard (MWS), lanes 2 through 13 containing the 12 Yellowstone samples with Hha1, lane 14 containing the old Yellowstone sample 13 with Hha1, lane 15 containing undigested Yellowstone sample 4 with no restriction enzyme, lane 16 containing the MWS, lanes 17 through 28 containing the 12 Yellowstone samples with Rsa1, lane 29 containing the old Yellowstone sample 13 with Rsa1, lane 30 containing the undigested Yellowstone sample 4 with no restriction enzyme, and lane 31 containing the final MWS. After loading and starting the gel, however, we realized that we forgot to load the final MWS. The gel also only ran for 1 hour and 30 minutes instead of 3 hours due to a programming problem that occurred after a power outage (see Appendix III, gel 2). Due to these mistakes the gel was unable to be analyzed, but despite the problems with the gel,

it was apparent that the PCR set up needed to be altered because the only sample that appeared to work was the double PCR'd sample 13 of the older Yellowstone extraction. In order to try and narrow down some of the possible problems, we used Epicentre's Failsafe PCR Premix Selection kit (premixes A through L) and ran a new PCR with 12 tubes of clipped sample 5 and 12 tubes of control sample 3. The master mix for the Failsafe program was as follows:

25ul reaction

- Premix (A-L)	12.5ul
- Enzyme	0.2ul
- dH_20	9.5ul
- Template	2.0ul
- Primer (Unlabeled 8F)	0.4ul
- Primer (Com2)	0.4ul

PCR program FAILSAFE was then run.

The PCR'd samples were then run on an agarose gel, but the results were disappointing because there were not even any primer dimers visible. Due to the results a new Failsafe kit was ordered to try and rule out any problems that may have arose due to the age of the kit. Kelly re-gelled the Yellowstone Failsafe samples as well as a sample of *Seratia marcescens* and observed similar results to the first attempt; the poor electrophoresis of two of the gels was attributed to a malfunctioning gel box. Once the new kit arrived another PCR was run with premixes A-L and Yellowstone sample 6 from the fourth extraction. The gel did show some results, suggesting that premixes D-L might work, but the bands were lower than expected.

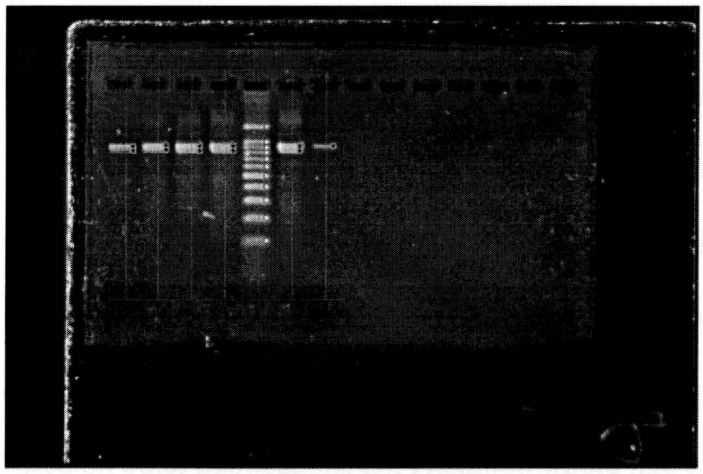
Pooled samples from extractions 4 and 5

We decided to pool the samples from extractions 4 and 5 due to a limited amount of DNA after having run various PCRs and gels. By pooling the samples into 6 tubes as

follows, we hoped to increase the concentration (listed below) of DNA in order to be able to run a LI-COR gel.

1) Extraction 4 Control 24hr. pooled 8.47ng/ul
2) Extraction 4 Clipped 24hr. pooled 12.93ng/ul
3) Extraction 4 Control 72hr. pooled 13.69ng/ul
4) Extraction 4 Clipped 72hr. pooled 10.96ng/ul
5) Extraction 5 Control pooled 16.33ng/ul
6) Extraction 5 Clipped pooled 20.79ng/ul

Once the samples were pooled they were cleaned up with Wizard PCR Preps DNA Purification System, and we made sure to elute with TE buffer heated to 65C. While the samples were being cleaned up, Kelly ran PCR with Failsafe premix E and primers 8FT3/Com2 and a gel of straight and serial dilutions of sample 7 from extraction 4. The PCR reactions were doped with *Seratia marcescens* in order to determine the effect of the soil template's inhibitors. The *Seratia marcescens* was not inhibited, suggesting that inhibitors were not the problem (see Gel 9). The pooled samples were then PCR'd in 100ul reactions with labeled primer 8FT3 and Com2. Instead of using the Promega buffer from the kit, the Qiagen buffer was used because four of Clint Oakley's optimized templates were going to be run on the same LI-COR gel, and therefore we wanted to make sure that all of the preparations of the samples were the same. A gel was run of the PCR'd samples in order to check the reactions; only Clint's samples showed results, but we decided to move forward with all of the samples. Hha1 and Rsa1 restriction enzyme digests were run on all six of my samples as well as all four of Clint's samples. A LI-COR gel was prepared and run for 3hours and 20minutes (see Appendix III, gel 3). The results looked much better than the previous sequencing gels, suggesting that there is a difference between control and clipped samples.



Project:
Date: 4/25/05 11:29:22
Name: S. marcescens doping experiment

1: Straight YS7-doped
2: 1:2 YS7 -doped
3: 1:4 YS7 -doped
4: 1:8 YS7-doped
5: Promega 100bp ladder
6: S. marcescens control
7: TE--Neg control??

	MW (bp)						
	1:	2:	3:	4:	5:	6:	7:
1	914.3	935.7	921.4	921.4	1500	928.6	921.4
2	842.9	857.1	842.9	842.9	1000	835.7	
3					900		
4					800		
5					700		
6					600		
7					500		
8					400		
9					300		
10					200		
11					100		

Gel 9. 8F/Com2

Further Testing

Since the completion of my research, which was limited by time, Kelly has continued to work with the samples to try and optimize the outcomes on gels. The most recent LI-COR gel was run with three restriction enzymes (Hha1, Rsa1, and Msp1) in order to try and gain more specificity in the results. The gel looked good, perhaps suggesting that there is a difference between the control and clipped samples at 24 hours.

Discussion

At the onset of our research we had hoped to determine and optimize the methods for examining soil microbial diversity using T-RFLP. Unfortunately there is no standard for the preparation leading up to T-RFLP, and as we discovered the preparation changes with each soil sample, making the task of designing the methods very difficult. We did determine that the primer pair 8F/Com2 could be useful with soil microbial DNA, but based on other publications Dr. Hamilton would like to test the primer pair 8F/1492 further because it would provide a larger piece of DNA. Besides narrowing down a useful primer pair, we tested and tried to optimize the annealing temperatures for different primer pairs, as well as the amount of template to use. Soil DNA seems to be capable of producing results at much lower DNA concentrations than other specimens. In an attempt to improve our results with the primer pair 8F/Com2 it has been discussed that lowering the annealing temperature might help, as well as using less of the template or a more dilute sample. Lowering the annealing temperature may lead to interference, though, because it may allow for other undesired specimens to amplify along with the desired DNA. Using a more diluted template may improve the results because the inhibitors, too, would be more diluted, and therefore less of a problem. In the article *Polyvinylpyrrolidone-Agarose Gel Electrophoresis Purification of Polymerase Chain Reaction-Amplifiable DNA from Soils* by Young et al., 1993, it is suggested that another way to try and improve the results of gel electrophoresis is by adding polyvinylpyrrolidone (PVP) to the agarose gel, which would retard the electrophoretic mobility of denaturing phenolic compounds, preventing the comigration with nucleic acids. Since the completion of my portion of the research, another LI-COR gel has been

run with three enzymes, which should increase the specificity of the T-RF length, thus revealing more clear results for more complex communities (Kent et al., 2003). Kelly Hemminger will continue to work with the research in hopes of finalizing a pre-T-RFLP and T-RFLP protocol that will work for multiple soil microbial DNA samples. T-RFLP is not a perfect nor conclusive solution for working with soil samples because it only provides an idea of what specimens might be present based on the bands that appear on the completed LI-COR gel; however, it does offer a rapid analysis of samples, which can easily be used to compare phylotype richness among multiple samples (Young et al., 1993). Our T-RFLP results suggested the presence of soil microbial DNA, which is what we had hoped for, but in order to obtain a more informative analysis the DNA samples would need to be cleaned of the inhibitors and re-run. T-RFLP will ultimately be useful for Dr. Bill Hamilton's research because it should provide insight as to the effects of grazing on soil communities, and thus insight into the interactions between aboveground and belowground biota as well as the role of environmental factors of soil diversity.

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Figures

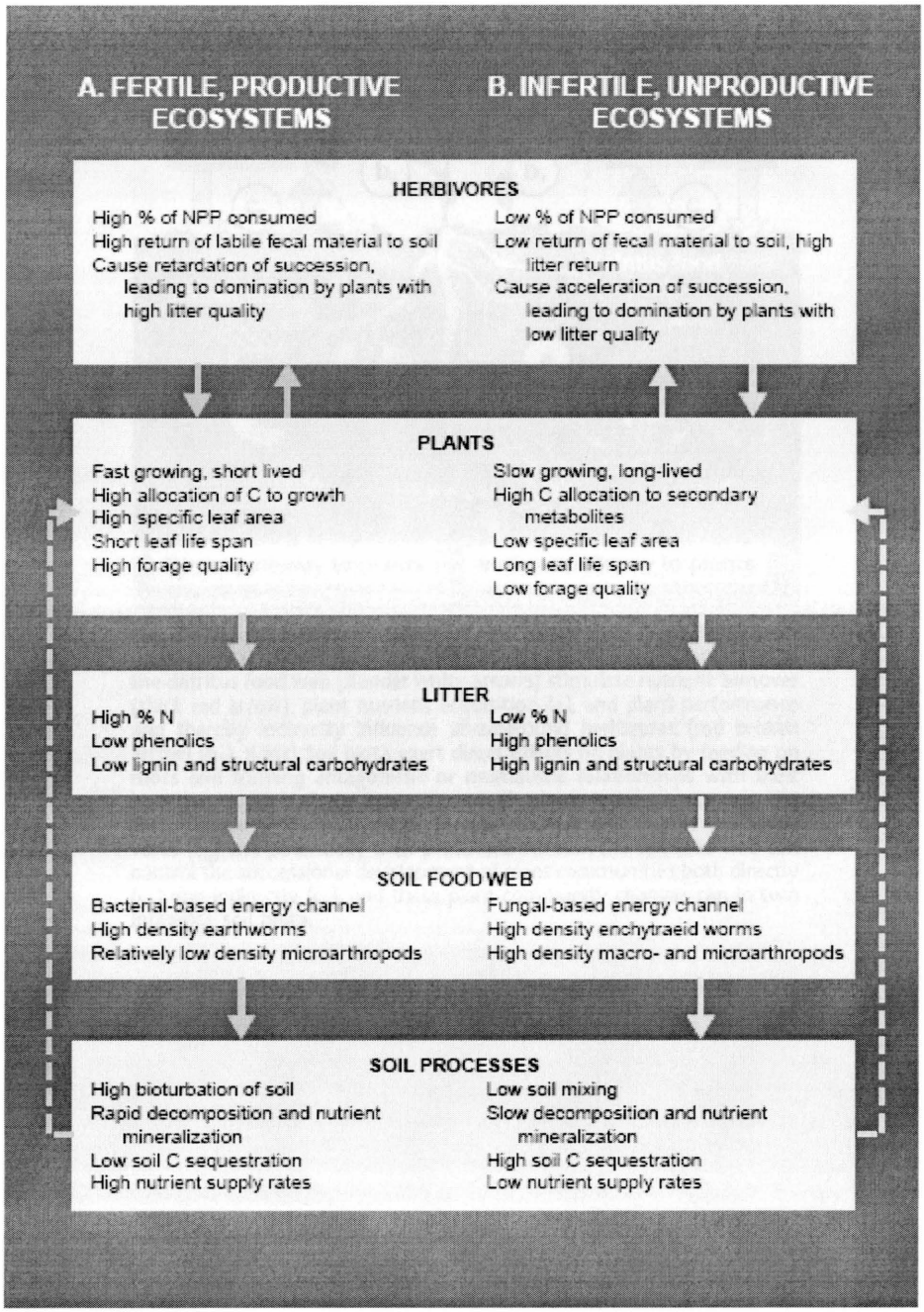

Fig. 1. One major ecological driver is the difference in fundamental plant traits between species that dominate (A) fertile systems that support high herbivory and (B) infertile habitats that support low herbivory. Plant traits serve as determinants of the quality and quantity of resources that enter the soil and the key ecological processes in the decomposer subsystem driven by the soil biota. These linkages between belowground and aboveground systems feed back (dotted line) to the plant community positively in fertile conditions (A) and negatively in infertile ecosystems (B).

Wardle et al., 2004

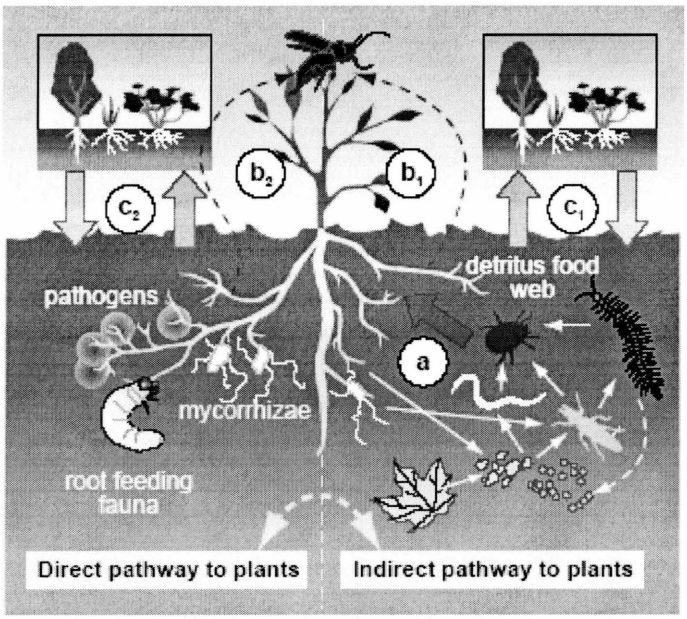

Fig. 2. Aboveground communities are affected by both direct and indirect consequences of soil food web organisms. (**Right**) Feeding activities in the detritus food web (slender white arrows) stimulate nutrient turnover (thick red arrow), plant nutrient acquisition (a), and plant performance and thereby indirectly influence aboveground herbivores (red broken arrow) (b_1). (**Left**) Soil biota exert direct effects on plants by feeding on roots and forming antagonistic or mutualistic relationships with their host plants. Such direct interactions with plants influence not just the performance of the host plants themselves, but also that of the herbivores (b_2) and potentially their predators. Further, the soil food web can control the successional development of plant communities both directly (c_2) and indirectly (c_1), and these plant community changes can in turn influence soil biota.

Wardle et al., 2004

Appendix I: SoilMaster DNA Extraction Kit

Protocol for SoilMasters' DNA Extraction Kit. Epicentre. Online. http://www.epibio.com/pdftechlit/178pl064.pdf 2005

The **SoilMaster™ DNA Extraction Kit** provides all of the reagents necessary to recover PCR-ready DNA from a variety of environmental samples. The kit utilizes a hot detergent lysis process[1,2] combined with a chromatography step, which removes enzymatic inhibitors known to co-extract with DNA from soil and sediment samples.[3,4] The extracted DNA is PCR-ready, and can be used with the FailSafe™ PCR System to amplify bacterial, plant or fungal templates.

Product Specifications

Storage: Store the Proteinase K and DNA Precipitation Solution at -20°C in a freezer without a defrost cycle. Store the remainder of the kit components at room temperature.

Storage Buffer: Proteinase K is supplied in a 50% glycerol solution containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 10 mM $CaCl_2$, 0.1% Triton® X-100 and 1mM dithiothreitol.

Quality Control: The SoilMaster DNA Extraction Kit is function-tested by assaying extracted soil sample DNA by PCR and agarose gel electrophoresis.

Related Products: The following products are also available:

- MasterPure™ Complete DNA and RNA Purification Kit
- MasterPure™ DNA Purification Kit
- MasterPure™ RNA Purification Kit
- MasterPure™ Plant Leaf DNA Purification Kit
- MasterPure™ Yeast DNA Purification Kit
- FailSafe™ PCR System
- MasterAmp™ PCR Optimization Kits
- MasterAmp™ *Taq*, *Tth*, *Tfl* and AmpliTherm™ DNA Polymerases
- BuccalAmp™ DNA Extraction Kit

SoilMaster™ DNA Extraction Kit Contents

The SoilMaster™ DNA Extraction Kit contains sufficient reagents to perform 50 extractions. The kit contains the following reagents: Cat. No. SM02050

Soil DNA Extraction Buffer	12.5 ml
Proteinase K (50 µg/µl)	100 µl
Soil Lysis Buffer	2.5 ml
Protein Precipitation Reagent	3 ml
Inhibitor Removal Resin	55 ml
Spin Columns	50
DNA Precipitation Solution	300 µl
Pellet Wash Solution*	15 ml
TE Buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA)	15 ml

Also available:

SoilMaster™ Spin Columns
Cat. No. SC04350 50

SoilMaster™ Inhibitor Removal Resin
Cat. No. SR04350 ..... 55 ml

* **Note**: Ethanol must be added to the Pellet Wash Solution before its first use (see Preparation of Pellet Wash Solution on page 3).

If a cap is desired for later spins of the column, the original cap can be cut off its tube and substituted for the cap of other 1.5 ml tubes.

References:

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-continued

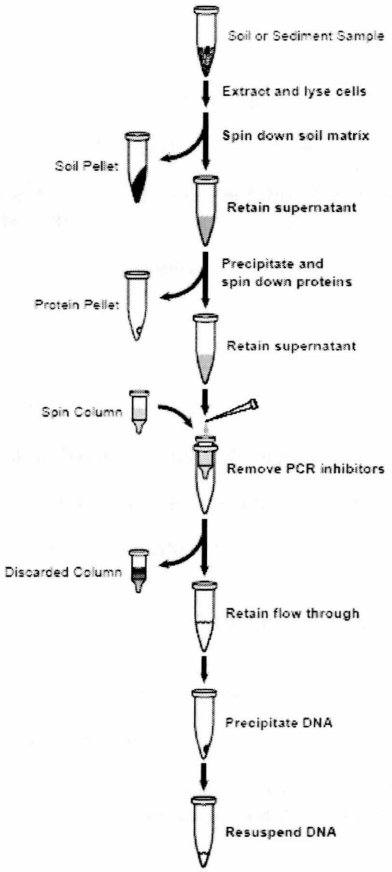
General Considerations

1. **Sample Sources**: We have used this kit to isolate PCR-ready DNA from a variety of soil and sediment sources including: forest soil, marsh soil, garden soil and cave sediment. Due to the wide variety of organic contaminants in different soil samples, some optimization of the initial sample size used and the amount of extract loaded onto the spin column may be required.

2. **Sample Size**: Generally 100 mg of soil will provide 300 µl of PCR-ready DNA. Users can extract DNA from larger sample sizes by using multiple spin columns. Do not load more than 150 µl of extract on a column.

3. **Quantitation of DNA:** If necessary, the concentration of PCR-ready DNA extracted by the SoilMaster DNA Extraction Kit should be measured by comparison to a standard amount of genomic DNA run side-by-side on an agarose gel. The remaining organic compounds co-extracted with the DNA may interfere with fluorimetry and spectrophotometry.

Figure 1. An overview of the process for extraction of DNA from soil samples.



Soil DNA Purification Protocol

A. Preparation:

Spin Columns

1. Add 550 µl of Inhibitor Removal Resin to each empty Spin Column to be used. Centrifuge for 1 minute at 2000 x g to pack the column.
2. Decant flow-through and place the column in the same collection tube.
3. Add another 550 µl of Inhibitor Removal Resin to each packed column. Centrifuge for 2 minutes at 2000 x g.
4. Move the column to a clean 1.5 ml collection tube.

Pellet Wash Solution

1. Add 45 ml of ethanol to the Pellet Wash Solution **before first use**.

B. Cell Lysis:

1. Weigh out 100 mg of the soil sample into a 1.5 ml tube.
2. Add 250 µl of Soil DNA Extraction Buffer and 2 µl of Proteinase K; vortex briefly.
3. (**Optional**) To increase the yield of DNA, shake the tube at 37°C for 10 min. or vortex for 2 min. Note: vortexing may shear the DNA.
4. Add 50 µl of Soil Lysis Buffer and vortex briefly.
5. Incubate at 65°C for 10 minutes.
6. Centrifuge for 2 minutes at 1000 x g.
7. Transfer 180 µl of the supernatant to a new tube.
8. Add 60 µl of Protein Precipitation Reagent, mix thoroughly by inverting the tube.
9. Incubate on ice for 8 minutes. Centrifuge the tube for 8 minutes at maximum speed.
10. Carefully transfer 100-150 µl of the supernatant directly onto the prepared Spin Column (from Section A).
11. Centrifuge for 2 minutes at 2000 x g into the 1.5 ml tube. Discard the column.
12. Add 6 µl of DNA Precipitation Solution, vortex briefly. Incubate the tube at room temperature for 5 minutes.
13. Centrifuge for 5 minutes at maximum speed. Carefully decant the supernatant.
14. Wash the pellet with 500 µl of Pellet Wash Solution (prepared in Section A). Invert to mix then spin for 3 minutes at maximum speed. Carefully decant the supernatant.
15. Repeat the wash and spin.
16. Resuspend the pellet in 300 µl of TE Buffer.

Troubleshooting DNA Extractions

DNA does not amplify by PCR

1) **Optimize cycling conditions.** Decrease the annealing temperature of the cycling profile by 2 degrees or more. Some primer pairs require a lower annealing temperature (less stringent conditions) when amplifying soil DNA.

2) **Use less starting material.** Some environmental samples contain significantly larger amounts of enzymatic inhibitors. When using these samples, begin the extraction with less starting material (50 mg).

3) **Load less extract onto the column.** If any color remains in the extract after the Inhibitor Removal Spin Column step, load less extract onto the column.

4) **Dilute the extracted DNA.** Dilute the extracted DNA 2-10 fold before amplification to decrease the effects of any remaining enzymatic inhibitors.

5) **Rewash the pellet with the Pellet Wash Solution.** This step is important in removing residual inhibitors of DNA amplification.

DNA is sheared

1) **Eliminate the vortex mixing step.** Eliminate the 2 minute vortex mixing step when extracting the DNA. Shake at 37°C instead or simply skip this step entirely. The yield of DNA will be decreased, but the integrity of the DNA will be significantly improved and hundreds of PCR amplifications can be performed on the resulting DNA.

Appendix II: Saga Procedure for Microsatellite Analysis and LI-COR Instructions

Saga Procedure
for Microsatellite Analysis using 25cm plates

1. Perform PCR reactions.

2. While the PCR reaction is running:
   a. Make agarose gel, buffers, PCR marker, etc. as needed.
   b. **Thoroughly** wash 25 cm plates with 2% micro-90 soap (do NOT use dish soap or Alconox!)
      Thoroughly rinse with dH2O.
      Spray with EtOH and dry with paper towels.
      Ensure that .25 mm spacers, .25 mm comb and the short rails are clean and dry.

3. Perform gel electrophoresis, stain, view and document in lab book.

4. Remove the 6.5% acrylamide, APS(ammonium persulfate),TEMED, stock bind saline and acetic acid reagents from the Li-Cor refrigerated box. Allow to warm to room temp.

5. Make the following working reagents fresh daily --10%APS & Bind Saline:
   10% APS: Add 0.1 gm ammonium persulfate pellets to 1.0 ml dH2O. Vortex.

   Bind Saline: 100 ul 10% acetic acid to 100 ul stock bind silane (wrapped in foil). Vortex.

6. Apply the bind saline to comb binding area of the inside of both glass plates and assemble the plates per the Li-Cor instructions on the following pages:

Applying the Bind Silane Solution

1.	Select a set of plates. Wipe both plates (gel side) with 100% isopropanol.
2.	Combine 100 µl of stock bind silane solution and 100 µl of 10% acetic acid in a test tube. (Or 1:1 as needed for the number of gels.) Mix thoroughly (pipette or vortex).
3.	Use a cotton swab to apply the solution to the area on the inside of the notched front plate where the wells will form (Figure 5-1). For 64-well squaretooth combs, the solution must be put on both front and back plates. Use the front plate as a guide to determine where to place the bind silane on the back plate.

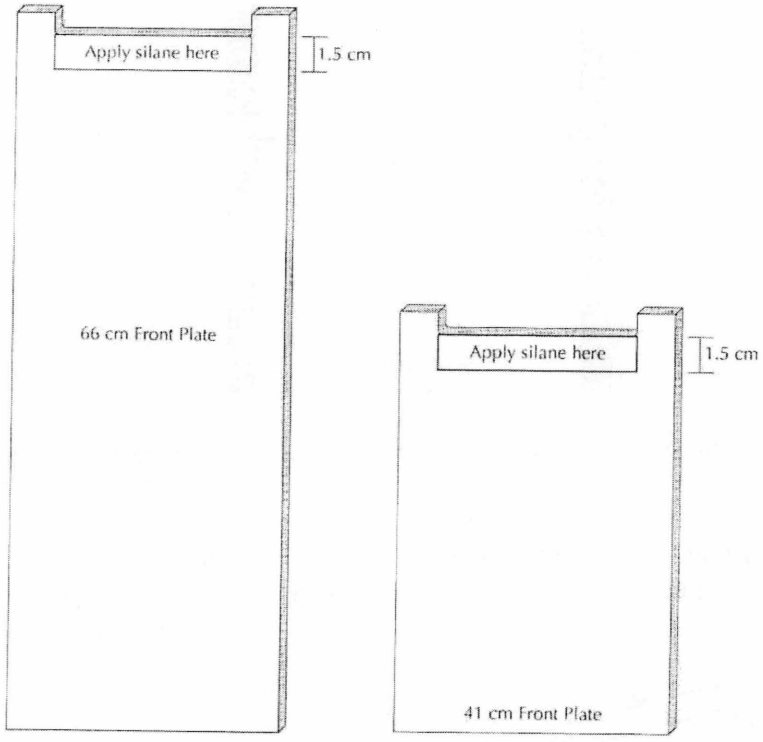


Figure 5-1. *Apply bind silane to the plates as shown. Note that only 64-well square tooth combs require back plate silane treatment.*

Always put the beveled side of the plate to the inside (gel side). The same side of the plates should always be on the inside because over time the upper buffer tank gasket leaves a permanent residue on the plate.

4.	Allow to dry for 1 minute. Wipe both plates (gel side) with 70% isopropanol as needed to remove dust particles. *Do not rub the isopropanol over the area treated with silane*. The treated area may be wiped gently with a tissue that is lightly moistened with water.

Assembling the Gel Sandwich

1.	Lay the back plate down (gel side up) and place the spacers along the edges, as shown below.

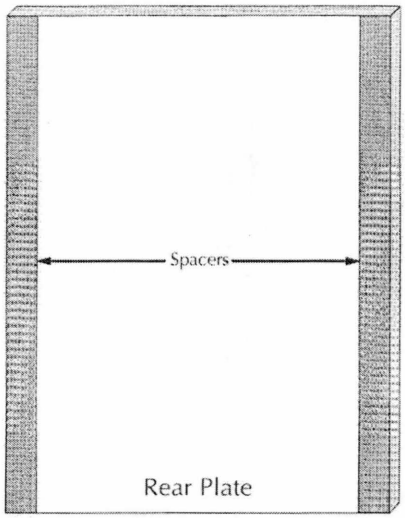


Figure 5-2. *Place spacers on the long edges of the plate.*

2.	Place the front plate (gel side down) on top of the rear plate by rotating the top plate down onto the bottom plate. Make sure that the plates are aligned at the bottom.

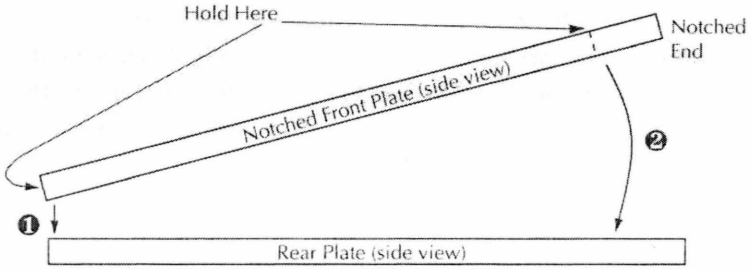


Figure 5-3. *Side view of front plate placement procedure.*

4. Tighten the glass clamp knobs (Figure 5-6) on each rail. ***Tighten only until finger tight*** (just past the point of resistance). Over tightening can break or distort the glass plates. Over tightening is also one of the primary causes of "smiles" on gel images because distorted plates cause uneven band migration across the gel.

   The assembled apparatus is shown in Figure 5-7.

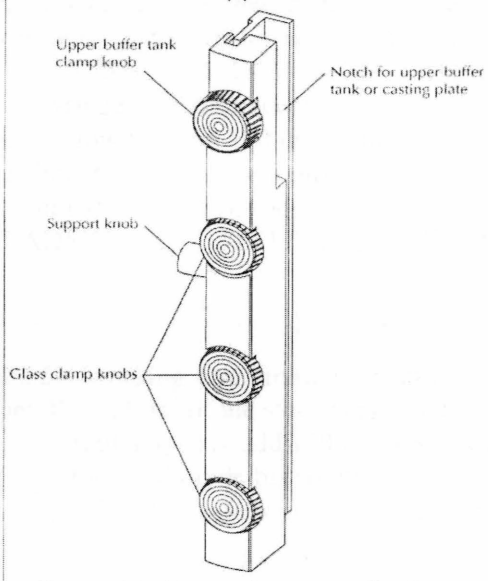


Figure 5-6. *Note orientation of rail assemblies (left rail shown).*

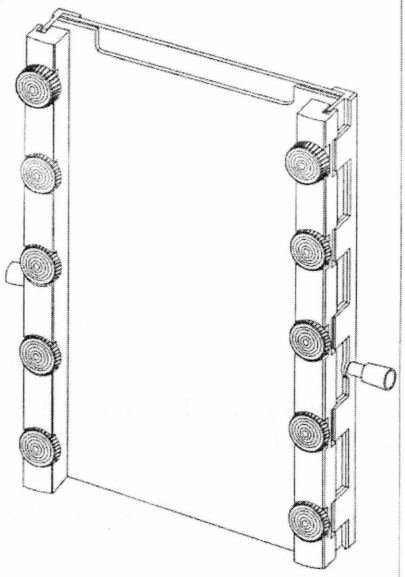


Figure 5-7. *Assembled apparatus.*

Recheck after tightening all knobs to make sure each knob is evenly tightened. Also, try to be consistent from day to day when tightening the knobs.

5. Select a comb (sharkstooth or rectangular tooth comb) with a thickness that matches the spacers. Clean the comb with water and/or isopropanol if necessary. Make sure that the comb fits between the two plates at the top of the gel. If it doesn't fit or is very loose, try another comb or adjust the gel assembly.

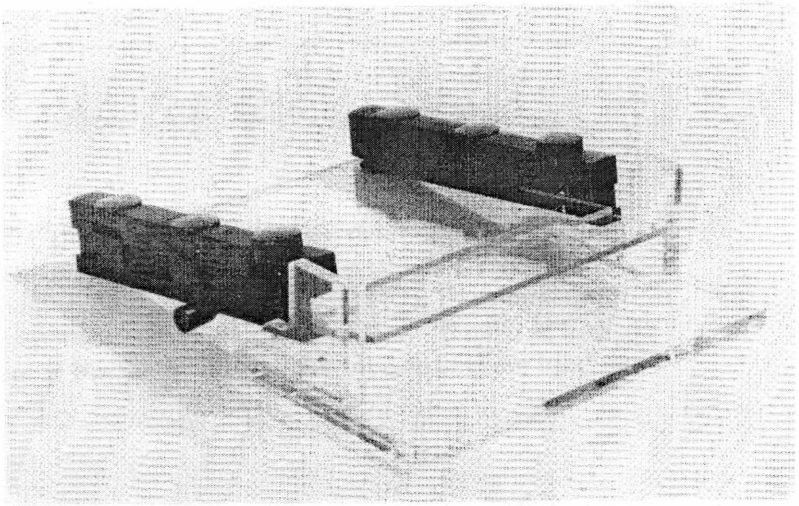
7. Once you have the plates ready and the rails attached, place the entire plate/rail assembly into the gel casting stand to ensure that rails and plates are assembled correctly.

8. The next step is *critical* and requires that the following are on/ready/within arms reach:
   - gloves, gown, goggles
   - clean, assembled 25 cm plates with .25 mm spacers
   - dark plexiglass positioned under the plates
   - Styrofoam lids to prop up the glass plates
   - yellow (0.25mm)bubble hooks
   - 60 cc syringe with blunt needle
   - small yellow tips and 100 ul pipette
   - large blue tips and 1000 ul pipette
   - 0.25 mm 48-well sharkstooth comb
   - STEADY HAND and NO INTERRUPTIONS

9. Make a 6.5% working acrylamide:bis solution:
   Recipe: Pour 20 ml of the stock acrylamide:bis solution into a plastic beaker.
   Simultaneously add 150 ul APS (prepared in step 5) and 15 ul TEMED to the acrylamide:bis solution.
   Quickly, but gently mix with the blue pipette tip from the 1000 ul pipette.

10. Wasting no time, pull about 18-19ml of the solution into the 60cc syringe and load plates per the following:

Pouring The Gel

1.	Draw the gel solution into a 60 cc syringe with a 14 gauge needle and begin pouring the gel.
2.	Hold or prop the apparatus at a slight incline when pouring the gel (Figure 5-8).  ***Figure 5-8**. Rest the apparatus on the casting stand (25 cm or larger plates) while pouring the gel.*
3.	Start a little above the bottom of the notch at the left or right side of the notch in the front plate. Inject the gel evenly at a steady rate while moving downward to the bottom of the notch and then side to side across the notch. Tap the front of the plates firmly to prevent the formation of air bubbles. If the gel is being injected correctly, you should get a smooth half moon shaped gel front advancing downward between the gel plates. If plates are dirty, the advancing primer front will be jagged. Never pull up the syringe after you start injecting. Any time you stop you are likely to create an air bubble. When the gel solution reaches the bottom of the plates and a small pool of gel overflows onto the notch in the front plate, quickly lay the plate assembly flat on the bench to prevent the gel solution from running out the bottom.

4. Remove any bubbles that form during gel pouring using a bubble hook (Figure 5-9).

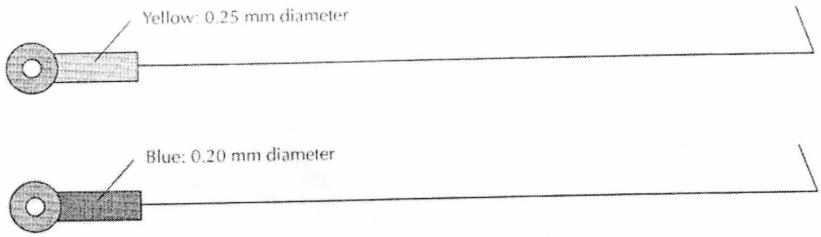


***Figure 5-9**. Bubble Hooks.*

5. Insert the comb.

Figures 5-10 and 5-11 show how to insert the mylar sharkstooth and rectangular tooth combs after pouring the gel. Instructions for inserting paper combs are given in the Appendices (Section 7). The sharkstooth comb is inserted upside down during polymerization to make a trough which forms the base of the wells, and is then inverted before loading the samples.

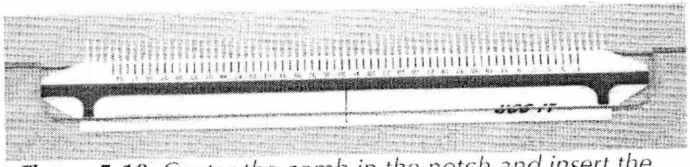


***Figure 5-10**. Center the comb in the notch and insert the sharkstooth comb upside down until the plastic depth gauge rests on top of the notch.*

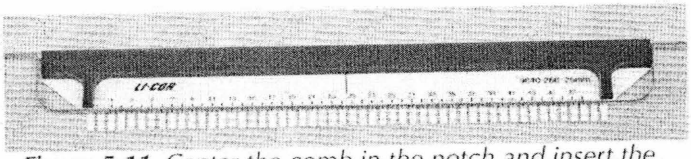


***Figure 5-11**. Center the comb in the notch and insert the rectangular tooth comb with the teeth downward, until the plastic depth gauge rests on the notch.*

Insert the comb slowly to avoid air bubbles forming around the comb. Air bubbles can destroy or deform the wells. Add a small amount of the gel solution over the comb (near the notch) to compensate for gel shrinkage as it polymerizes.

6.	Place the casting plate in the grooved area in the rails normally occupied by the upper buffer tank. Tighten the two upper clamp knobs until finger tight to secure the comb in place.

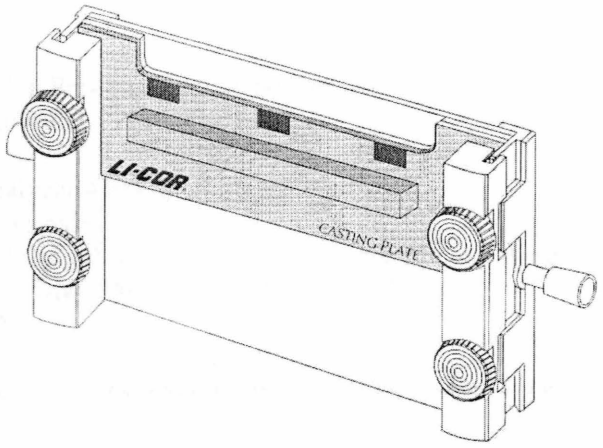


***Figure 5-12**. Insert the casting plate and tighten the knobs.*

7.	Allow the gel to polymerize for 1 to 1 1/2 hours. Check the tightness of the clamp knobs after polymerization to make sure they are still tight.

11. Once you have poured a successful gel, return the Li-Cor Sequencing reagents to the refrigerator.

12. Obtain 1 liter of 1x TBE buffer.

13. After the gel has polymerized for 1-2 hours, refer to the Li-Cor instructions on the following pages:

Starting a Pre-Run, Constructing a Gel, and Running a Microsatellite Gel in SAGA

Starting the Pre-Run

1. Prior to running a new gel, a Pre-Run must be performed.
2. Start the Pre-run from the Web
   - Internet Explorer
   - Favorites: DNA Analyzer 4300
   - Load Conditions: Microsatellites.
   - Use current date and the gel name in the both the pre-run and the gel manager.
     (Example: 25May2005_MpAAT96_Catbird2_kah)
   - Ensure that Group Name is correct
   - Start Pre-run…will take about 30 minutes.

Note: if a gel is being re-used, you would choose "Skip Focus/Pre-run" and go right to loading samples.

During the Pre-Run "Construct" your Gel and Perform Final Sample Preparations

While the Pre-run is going, perform the following tasks:

1. Use Gel Manager to construct your molecular weight standards, loci, and sample list.

Thoughts on Gel Management…

For each new species make a new project. In each new project, make a generic template for molecular weight standards and (loci).

Include spaces for your molecular weight standards at the first and last positions and every 8-10 samples in-between.

When constructing a gel, DO NOT insert loci for the markers (MWS) and DO NOT insert MWS for DNA samples. Saga will not find/call alleles if you ignore this advice!

When entering templates, enter them into Saga in the reverse order you want to load/run them (the first sample entered, goes to the bottom of Saga's list.)

Don't forget to include the appropriate # of bases in your loci range if using tailed primers.

"Copy" the template gel from the "Ready to Run" tab or the "Gel" tab to create a new "working gel".

If you are copying a previous gel, make changes as necessary for the new gel in the Gel Constructor window.

Print the gel constructor using expanded margins and 6 font in a "portrait"format. Retrieve the copy from the Biology office. Use this as a map to help with sample loading.

2. Obtain the approriate 700 or 800 MWS (molecular weight standards) from the freezer, and wrap the mws in foil to protect from the light as they thaw.
3. Label new PCR 8-strip tubes in a logical manner to match your gel constructor.
4. If needed, make template dilutions.
5. Mix 1 part AFLP Stop Solution (blue dye) to 2 parts template for each template (straight or diluted). We use 1ul dye: 2ul template as a "straight" sample.
6. Mix tubes, centrifuge, and denature all the templates *plus* the molecular weight standards using the DENAT program available on both thermocyclers.

Verify the Pre-Run

1. Review the Pre-Run from the Web's Li-Cor site:
   Utilities
   Diagnostic Utilities
   View User Focus Profiles
   Enter Group Name and Use drop-down menu to choose pre-run to view.
2. Look at position numbers and verify that numbers are close.
   (example Left=119 and Right=126.)
   If the numbers are too far apart, the Pre-Run may fail and in this case you will need to re-clean the exterior of the plates and/or re-position the plate and rail assembly.

Loading and Starting a SAGA Gel

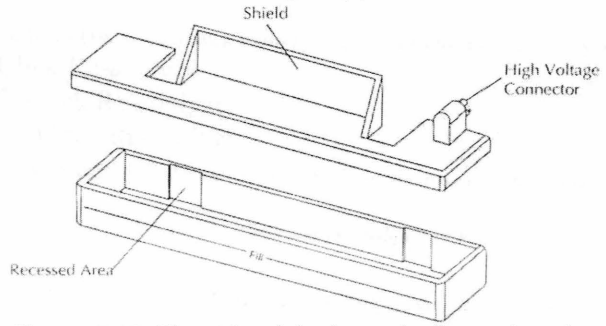
1. Remove the denatured samples from the thermocycler and place the rack of tubes in an ice bucket. Cover the racks with foil to protect the IR label from light.
2. Remove the upper buffer tank lid and the electrical cable.
3. Using a 20 ul pipette and the flat-tipped pipette tips, load 1ul of the MWS in the first well.
4. Rinse the tip x2 in buffer and wipe the pipette tip with a Kimwipe.
5. Load 1ul of the sample/dye mixture in the second position.
6. Rinse and wipe the pipette tip.
7. Continue loading, only removing one strip of samples at a time from the refrigerator.

Remember to load a MWS at least every 8-10th position per your gel constructor and make sure your last sample loaded is also a MWS.

8. Attach the upper tank lid and attach the electrical cable.
9. Choose Start Run from the Li-Cor Website.
10. A 25cm gel will take about 1.5 hours to complete.

Pre-Electrophoresis Preparation

1.	After the gel has polymerized, loosen the upper knob on each rail and remove the casting plate.
2.	Add a small volume of water to the notched area on the front plate where the comb is inserted. When the comb is removed water will be drawn into the wells, which helps to maintain good well morphology.
3.	Remove the comb: ***Rectangular tooth comb:*** Carefully remove the comb by slowly pulling it straight out. This is a critical step, in that the well morphology must be maintained for sample loading. If the comb does not slide out easily, it may help to use a razor blade to score along the edge between the top of the comb and the back plate to break the gel seal. Rinse the wells with water using a 20cc syringe fitted with a 22 gauge needle. ***Sharkstooth comb:*** Hold a razor blade at a 45° angle relative to the comb and lightly score the acrylamide along the interface between the glass and the plastic comb. This will prevent acrylamide from cracking off and dropping into the well. Carefully remove the comb from the gel and rinse the single well with water using a 20cc syringe fitted with a 22 gauge needle. Be sure to remove any small acrylamide fragments in well. Proceed with gel clean-up before re-inserting the comb.

4.	After removing the comb, use a razor blade to remove excess gel from the inside of the back plate above the notched area where the comb was previously inserted. Similarly, remove any gel from the outside of the plates, at the bottom and top of the gel sandwich, and next to the rails on the back plate.
5.	Use wipes and deionized water to clean the back and front plates, then 100% isopropanol (optional). The area on the plates (between the two bottom knobs) corresponding to the position of the sequencer's scanning window is the most important and should be carefully cleaned.
6.	Press the white rubber gasket into the recessed groove on the back of the upper buffer tank. Do not stretch the gasket while pressing it into place. (Note: Do not use alcohol to clean this gasket - use only water).
7.	Loosen the upper clamp knob on each rail and slide the upper buffer tank into place. Be careful not to let the gasket touch or drag against the plates while installing the tank, as this may pull the gasket from its position in the groove. If the gasket is displaced from the groove, buffer will leak from the upper tank during electrophoresis. For new gaskets, you may need to carefully wet plate near the gasket and rub water over the gasket with your fingers (only necessary the first 4-5 times a gasket is used). Don't let water contact the rails or run down the front plate.
8.	Tighten the upper clamp knobs "finger tight". The electrophoresis apparatus is now fully assembled.
9.	If using a sharkstooth comb, re-insert the comb until the teeth just touch the gel. Hold the gel upright against a good light source in order to see the bottom of the well. (A casting stand is useful for this.) Tip Lightly coat the teeth of the sharkstooth comb with Cello-Seal (Fisher #C-601) to help seal the wells and hold the comb in place.
10.	Open the instrument door and place the lower buffer tank into position at the base of the heater plate. The tank has two recessed areas where the rails rest when the assembled gel apparatus is installed.  ***Figure 5-13**. The side of the lower buffer tank with the recessed areas is placed against the heater plate.*

11.	Mount the gel apparatus on the instrument against the heater plate, with the bottom of the gel sandwich inside the lower buffer tank. Check to see that the support arms holding the gel assembly on the instrument are seated evenly on the bracket.
12.	Inspect the plates at the location of the scanning window to make sure that they are free of any smears, dust, or spots that may interfere with detection.

Viewing a Completed SAGA Gel

1. From the Li-Cor Website, view the gel (enter group and gel name)
2. Zoom out to 25%.
3. Can also just look at 1 channel (layer button)
4. Scroll up and down ¼ to 1 full page at a time using button, not bar.
5. If the gels look good(MWS are in ***correct*** positions, looks like the number of sample lanes matches the # of samples loaded), click on save icon and save the tif 700 image and then 800 image (to the desk top).
   If not using a primer labeled with IR800, there will only be a IR700 tif image to save.

   OR

   If you know or can see that MWS were loaded ***incorrectly***, edit the gel constructor accordingly prior to saving the tif. To edit the gel constructor: open SAGA, open gel manager, doubled click on gel to edit, say OK, gel constructor opens. Correct, print and save.
6. **On the desktop or where ever you save the images, look at the tif image properties and make sure to *unblock* the image! ..if you don't SAGA cannot import the image**
7. Then go back to gel manager Ready to Run tab, choose gel to have images imported to and click import tif images.
8. Highlight 700 and/or 800.
9. A window opens showing tifs from which to choose.
10. Click on 700, click open.
11. Window again opens showing tifs to choose from.
12. Click on 800, click open (if you've also used an 800 labeled dye).
13. The name of the gel disappears from "Ready to Run" tab and can now be seen on the "Gels" tab. The status will change from "Getting Image" to "Lane Analysis" to "Calibrated" to "Genotyping" to eventually "Genotyped" (takes about 5 minutes).
14. Click Show.
15. If necessary, edit Lane Lines.(start by editing a control point near a locus that is centrally located in the image rather that at the top or bottom.) Click Re-analyze.
16. If necessary, edit MWS. Click Re-analyze.
17. If necessary, edit Alleles. Click Re-analyze.
18. Refresh and Confirm. (sometimes Refresh doesn't seem to work, so you may need to exit and close the gel and then re-open it)
19. Print the tif image (*only the tif image will print, the allele analysis will not print!*).
20. Print desired reports.
21. On the printed tif indicate primers used, dilutions (if any), date/initials and name of gel.

Appendix III: LI-COR Gels

1) Gel from Extraction 3
2) Gel from Extraction 5
3) Gel from Pooled samples