Applications Manual

Model 4300 DNA Analyzer

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This table of contents is an overview of the entire Model 4300 Applications Manual. The first three sections are manuals for DNA sequencing, microsatellite analysis, and AFLP® analysis. Each of these three sections corresponds to an application package available from LI-COR® Biosciences. You will notice some redundant instructions between the various manuals. Note, however, that each application has subtle variations to procedures that enhance operation, so read each manual carefully. The *Miscellaneous* section may contain pack inserts, data sheets, brochures, application notes and other documents of general interest.

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DNA Sequencing Manual

Model 4300 DNA Analyzer

LI-COR®

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Section 1 Introduction

This manual is a basic guide to DNA sequencing with the LI-COR® infrared DNA sequencers. All aspects of automated sequencing are discussed, including template preparation, primer design, reagents, labeled primer sequencing, bi-directional sequencing, gel preparation, and software. Each chapter contains a discussion of theory, critical points, and a set of protocols. Each protocol includes a list of reagents, step-by-step instructions, and tips highlighting important steps.

The System

The LI-COR System detects DNA using infrared (IR) fluorescence. In a DNA sequencing reaction, a DNA polymerase extends either an IRDye® infrared dye-labeled primer using unlabeled deoxy and dideoxy nucleotides or an unlabeled primer with unlabeled deoxy and labeled dideoxy nucleotides into a set of chain-terminated fragments. The IRDye infrared dye-labeled chain-terminated fragments separate according to size on an acrylamide gel. A solid-state diode laser excites the infrared dye on the DNA fragments as they migrate past the detector window. A focusing fluorescence microscope containing a solid-state silicon avalanche photodiode scans back and forth across the width of the gel collecting data in real time. The raw image data are a series of bands displayed on the computer monitor in a format similar to an autoradiogram. The image can be analyzed by the DNA sequencer or using e-Seq software. During analysis, lanes are found for each sample and bases are called. The sequence data are presented in text (ASCII) or curve format (standard chromatogram format (SCF)).

The Model 4300 is a dual laser system that detects IRDye 700 and IRDye 800 at the same time without spectral overlap between detection channels. The detection wavelengths are separated by 110 nm. Two independent image files are created from the same gel during electrophoresis. One file contains image information from the 700 channel and the other file contains image information from the 800 channel. The protocols in this manual employ either dye except for the simultaneous bi-directional sequencing (SBS™) method that requires both IRDye 700 and IRDye 800. The system requires borofloat glass plates, which are nearly colorless. Older LI-COR systems used soda lime glass plates, which are distinguished by their blue-green color.

IRDye® 800

IRDye 800 is a heptamethine cyanine dye absorbing and fluorescing in the near infrared region of the spectrum. The absorption maximum at 787 nm is well-matched to the 785 nm laser in the detection microscope. The extremely high absorptivity and good quantum efficiency of the dye provide excellent sensitivity. Bands containing less than 10 attomoles of IRDye 800 label have been detected.

IRDye 700

IRDye 700 is a pentamethine carbocyanine dye fluorescing in the near infrared region of the spectrum. The absorbtion maximum (681 nm) is just outside the visible region and matches the 685 nm laser of the detection microscope. While the absorptivity of IRDye 700 is slightly less than that of IRDye 800, higher fluorescence efficiency compensates for the absorption difference. Bands containing less than 10 attomoles of IRDye 700 label have been detected.

Dye Spectral Properties

DYE (Aqueous solution)	**IRDye 800**	**IRDye 700**
Absorbance max	787	681
Emission max	812	712
Molar Absorptivity	275000	170000
Stokes Shift	25	25

Automated vs. Manual Sequencing

IR fluorescent DNA sequencing differs from manual approaches in several ways. First, the LI-COR® system is extremely sensitive, detecting as little as 10 attomoles of label. Since the system is so sensitive, it is necessary to correctly estimate the amount of DNA in each sequencing reaction to avoid signal saturation. In other words, all reactions on a gel will have similar signal intensity if similar molar DNA concentrations are used in all sequencing reactions. Finally, the fluorescent signal is detected in real time as the labeled DNA fragments pass the detector. Therefore, each band must have sufficient signal for detection since it is not possible to stop each band in front of the detector for extended periods of time as is the case with x-ray film on a radioactive gel. For these reasons, it is very important to correctly estimate the DNA concentration of each template.

Section

2 Template

Introduction

Read length and sequence data quality are partially dependent upon the purity of the DNA template. This chapter reviews methods for the preparation and purification of plasmids, PCR products, and M13. In each case, several commercial DNA purification kits are recommended as well as a “do-it-yourself” protocol for the cost-conscious lab. The related topics of bacterial growth and PCR optimization, which can affect template quality, are also discussed.

In addition to the quality, it is important to use similar molar amounts of DNA template in each reaction for consistent data and similar band intensities. The advantages of DNA quantitation using a fluorometer, spectrophotometer, or gel estimation methods are discussed.

Plasmid DNA

High quality template is essential for long reads. Contaminating salt, RNA, protein, DNases, and polysaccharides from the host bacteria can inhibit sequencing reactions and produce low signal, high background, or spurious bands. To get consistently high quality DNA for sequencing on the Model 4300, several commercial DNA preparation kits can be used. Where cost is a concern, the mini-prep protocol given at the end of this chapter yields good quality template.

Bacterial Growth

Most commercial DNA purification kits discuss bacterial culture conditions. We recommend recA-, endA- *E.coli* strains such as DH5α or XL1-blue. Incubate cells at 37 °C until the optical density at 600 nm reaches 1.5 or grow overnight at 30 °C.

Template for Simultaneous Bi-directional Sequencing

Contamination of a plasmid preparation with plasmid that contains deletions in the multiple cloning site between the forward and reverse priming sites can

cause a second sequence to appear and obscure the beginning of the intended sequence during simultaneous bi-directional sequencing (SBS). The shortened area between the priming sites amplifies very efficiently during SBS to generate fairly dark bands. The resulting double sequence ladder (the deleted clone plus the template with the insert) obscures the first 50 bases of the intended sequence. At the end of the smaller insert, the expected, single sequence reappears. If this problem occurs, adjust the growth conditions to minimize deletion and contamination. Carefully select individual colonies. Harvest cultures shortly after reaching stationary phase, particularly in rich media (e.g., terrific broth), or grow cells at 30 °C overnight.

Commercial Kits

There are a number of kits available for plasmid purification. These kits are easy to use and provide high quality DNA. Template concentrations should be in the 0.5 - 1.0 µg /µl range for plasmid DNA. If any of the methods give lower yields, re-suspend the final pellet in a smaller volume of water or concentrate the DNA using a SpeedVac. Check the purity of the preparation on an agarose gel.

PCR Products

PCR products should give a single band on an agarose gel and be free of primers, nucleotides, and enzymes. Secondary PCR products can result in high backgrounds and bands in all four lanes (full stops). Some PCR conditions may require optimization to produce a more robust reaction that gives a single band.

PCR Optimization

The annealing temperature, the amounts of primer, template, magnesium, the nucleotides and enzyme used, any special additives, and the reaction pH are all factors that can influence the yield of the desired amplification product. The following comments may be useful in optimizing your PCR reaction.

Annealing Temperature

Calculate the optimal annealing temperature (T_a) from the melting temperature (T_m) with the following formula:

T_m = 69.3°C + 0.41x (%GC) - 650/Primer Length

T_a: = T_m - 3°C

This formula predicts the melting temperature (T_m), where approximately 50% of the available primer hybridizes to the target template. Early cycles contribute to most of the specificity. One can increase the specificity of PCR by using a higher annealing temperature in the first ten cycles (+2 °C) and lowering the T_a in the following 20 to 30 cycles (-5 °C). This touchdown method improves both specificity and yield.

Reaction Reagents
Yields from PCR reactions can be increased by using optimal reaction conditions. PCR optimization kits are helpful in determining the appropriate magnesium concentration and pH for the template, primer, and enzyme you are working with.

Nucleotides
When amplifying GC-rich DNA, the addition of 7-deaza-dGTP (1:2 dGTP: c7dGTP) to the reaction mix results in a PCR product that is easier to sequence.

Purification

After the PCR reaction, check the PCR product (2-5 µl) on an agarose gel to verify the presence of a single band of the expected size. Purify the product using a PCR purification kit or the PEG precipitation method given at the end of this section.

Following purification, check the purified product on an agarose gel and measure the concentration of the PCR product using the methods described in this manual.

Determining DNA Concentration

The best sequence data are obtained when the signal intensity for all the samples on a gel falls within the optimal range of the detector. Signal intensity is dependent, in part, on the number of molecules of template used in a sequencing reaction. Therefore, it is critical to accurately measure the concentration of every DNA sample. The most common methods for measuring DNA concentration are with a fluorometer or spectrophotometer, or by agarose gel estimation.

Fluorometer Measurement

Fluorometer measurements generally provide the most accurate measurement if instrument directions are followed carefully. Consult the instrument manual for protocol.

Spectrophotometric Measurement

Plasmids, cosmids, phage, PCR products, and primer concentrations can be estimated by measuring A_{260}. The sample is diluted so the A_{260} values lie between 0.01 and 0.10. For DNA preparations that generally yield concentrations in the range of 0.1 - 1 µg/µl, dilute the DNA solution 1:200 in water:

1.	Add 5 µl of DNA to 995 µl of distilled water in a microcentrifuge tube and mix well.

2.	Blank or zero the spectrophotometer with distilled water.
3.	Measure the optical density of the diluted DNA at 260 and 280 nm or scan from 200 to 400 nm.
4.	Calculate the A_{260}/A_{280} ratio. A value of approximately 1.8 indicates good quality DNA. Lower values indicate protein contamination and higher values are due to the presence of RNA.
5.	Assuming a 200-fold dilution, calculate the DNA concentration from the table below. Otherwise, change the dilution factor (200) in the equation.

DNA Type	OD of 1.0=	Concentration (in µg/µl)
Double stranded DNA	50 µg/ml	(A_{260}) x 50 x 200 / 1000µl
Single stranded DNA	40 µg/ml	(A_{260}) x 40 x 200 / 1000µl
Oligonucleotides	35 µg/ml	(A_{260}) x 35 x 200 / 1000µl

Calculate the molar concentration of a template with the following formulas:

Single-stranded DNA and primers

$$\text{pmoles/µl} = \frac{(\text{concentration of DNA in µg/µl})\,(10^6)}{(\#\text{ of bases})\,(325)}$$

Double-stranded DNA

$$\text{pmoles/µl} = \frac{(\text{concentration of DNA in µg/µl})\,(10^6)}{(\#\text{ of bases})\,(650)}$$

Agarose Gel

Agarose gel estimation is the least accurate yet simplest method because one commonly runs a gel to check the size and purity of a PCR product anyway. The intensity of a band (stained with ethidium bromide) on an agarose gel can be used to estimate the DNA concentration of a PCR product by running a molecular weight standard with known amounts of DNA adjacent to the unknown DNA sample. The intensity of the sample band is then compared to the intensity of a similarly sized band in the standard. A single dilution of a sample provides only a very rough estimate of the amount of DNA, and overloading causes band saturation that can also lead to errors in estimating the amount. For the best results, load several dilutions of a sample on the gel. This method is not recommended for plasmid DNA.

Plasmid Mini-prep Protocol

Introduction

This method provides very high quality DNA that works well with labeled primer protocols.

Reagents

Lysozyme	10 mg/ml solution of lysozyme.
RNase A	10 mg/ml solution of DNase-free RNase A
STET Buffer	100 mM NaCl, 50 mM Tris-Cl (pH 8.0), 1mM EDTA (pH 8.0) and 5% Triton X-100
PEG/NaCl	20% PEG8000 and 2.5M NaCl. Heating may be required to dissolve the PEG.
Chloroform	Reagent grade
70% Ethanol	
3M sodium acetate	
Isopropanol	
TE Buffer	10 mM Tris-Cl (pH 7.5), 1mM EDTA (pH 8.0)

Culture Isolation

1.	Prepare an overnight bacterial culture in the appropriate medium.
2.	Fill a 1.5 ml microcentrifuge tube with the overnight culture.
3.	Centrifuge at high speed for 1 minute to pellet the cells.
4.	Remove supernatant and repeat the previous two steps with the same tube.

DNA Isolation

1.	Remove supernatant completely and resuspend pellet in 300 µl of STET Buffer and 50 µl of lysozyme.
2.	Place the tube in boiling water for 1 minute.
3.	Remove the tube and centrifuge at high speed for 10 minutes.
4.	Remove the cell debris pellet with a toothpick.
5.	Add 500 µl chloroform, vortex to mix well and centrifuge for 1 minute at high speed to separate the aqueous and organic phases.
6.	Transfer the upper aqueous phase (DNA) to a new 1.5 ml microcentrifuge tube.
7.	Add 1 µl of RNase A and incubate for 30 minutes at 37 °C.
8.	Add one volume of isopropanol, mix gently, and incubate at room temperature for 15 minutes.

9.	Centrifuge the tube at high speed for 15 minutes.
10.	Remove and discard supernatant.
11.	Add 1 ml of 70% ethanol (room temperature) to the tube, centrifuge for 1 minute at high speed, and remove supernatant. (The pellet may not be visible).
12.	Dry pellet (SpeedVac or air dry) and re-suspend in 200 µl of TE (pH 7.5).

PEG Precipitation

1.	Add 0.5 volumes of PEG/NaCl solution.
2.	Place on ice for at least one hour or refrigerate overnight.
3.	Centrifuge at high speed for 15 minutes.
4.	Remove the supernatant and wash pellet with 70% ethanol (room temperature).
5.	Dry pellet and re-suspend in 100 µl of TE buffer.
6.	Add one volume of chloroform, mix well, and centrifuge at high speed for 1 minute to separate the aqueous and organic phases.
7.	Remove the aqueous phase (DNA) to a new tube.
8.	Repeat the previous two steps two times.
9.	Add 10 µl of 3M Sodium Acetate and 300 µl of ice cold 100% ethanol. Mix well.
10.	Place the tube at -80 °C for 15 minutes.
11.	Centrifuge at high speed for 15 minutes.
12.	Remove and discard the supernatant.
13.	Add 1 ml of 70% ethanol (room temperature) slowly to wash the DNA pellet and remove with the same pipet tip. (The pellet may not be visible).
14.	Dry the pellet and re-suspend in 50 µl of distilled, deionized water.
15.	Proceed to the section "Determining DNA Concentration".

Plasmid DNA concentrations should be about 0.5 to 1.0 µg /µl for sequencing. If the method you choose to purify your DNA gives lower yields, make sure that you re-suspend the final DNA pellet in a smaller amount of water, or concentrate the DNA by using a vacuum concentrator.

PCR Product Purification With PEG

Modified procedure similar to Kusukawa et al, BioTechniques 9, 66-72 (1990).

Reagents Required

PEG/NaCl	20% PEG8000 and 2.5M sodium chloride. Heating may be required to dissolve the PEG.
Chloroform	Reagent grade
70% & 100% Ethanol	
3M Sodium acetate	

Protocol

1.	Transfer the PCR reaction to a 1.5 ml microcentrifuge tube.
2.	Add an equal volume of distilled water.
3.	Add 0.6 volumes of PEG/NaCl.
4.	Incubate at 37 °C for 15 minutes.
5.	Microcentrifuge at high speed for 10 minutes.
6.	Remove and discard the supernatant.
7.	Gently introduce 50 µl of 70% ethanol (at room temperature) along the side of the tube opposite the pellet. Immediately remove the ethanol with the same tip.
8.	Dry the pellet at room temperature or briefly in a SpeedVac (do not over-dry).
9.	Re-suspend pellet (pellet may not be visible) in 200 µl of distilled water.
10.	Add 600 µl of chloroform, mix well and centrifuge at high speed for 1 minute to separate the organic and aqueous phases.
11.	Transfer the upper aqueous phase (DNA) to a new 1.5 ml tube.
12.	Repeat the previous two steps two times.
13.	Add 10 µl of 3M sodium acetate and mix well.
14.	Add 500 µl of ice cold 100% ethanol and mix well.
15.	Place the tube at -80 °C for 15 minutes.
16.	Centrifuge at high speed for 15 minutes.
17.	Remove and discard the supernatant.
18.	Gently introduce 50 µl of room temperature, 70% ethanol along the side of the tube opposite the pellet. Immediately remove the ethanol with the same tip.
19.	Dry the pellet and re-suspend in 50 µl of distilled deionized water.
20.	Check the quality of the DNA on an agarose gel.

The DNA concentration of PCR products (up to about 600 bp long) should be about 0.05 to 0.10 µg /µl for sequencing.

Section 3 Primers

Introduction

The primer is critical to the success of sequencing reactions. Lack of specificity (annealing at more than one site), low melting temperatures, or hidden secondary structure can result in multiple bands, high background, or weak signals. Choosing a new primer sequence that binds several bases away from the current primer can dramatically affect its performance. This discussion offers rules for primer design as well as guidelines on nested primers, determining primer concentration, and primer purification.

Rules for Primer Design

While many parameters are important, there are three critical rules of primer design:

1. Primers should have a GC content of 40-50%.
2. Primers should have a G or a C at the 3'-end.
3. Primers should avoid single base repeats greater than 3 bases. For example, TTT or GGG is acceptable but not TTTT or GGGG.

If these rules are followed, other factors, such as annealing temperature, become much less critical. In situations where one of the rules is not followed, try to design a primer with an annealing temperature of at least 50 °C. Lower annealing temperatures tend to produce higher background, stops, and artifacts in cycle sequencing.

Primer Concentration

Accurate primer concentrations are important. Commercial primers arrive as a dried product in a tube. The product information identifies the amount of DNA in nanomoles, making it relatively easy to redissolve the primer to the desired concentration.

Primer Purity

Labeled primers should be purified by HPLC to assure the highest quality sequencing data. For unlabeled primers, commercial suppliers offer a number of different purification options. If unlabeled primers are synthesized in an individual laboratory or core facility, it is essential that they meet or exceed the quality of commercial sources. The main impurities that can affect sequencing are salts or organic groups that have not been removed following DNA synthesis. Crude primers that have not been purified should not be used for automated sequencing.

Handling Labeled Primers

All fluorescent dyes are sensitive to light. ***Minimize the light exposure of IRDye infrared dye-labeled primer solutions.*** The standard IRDye infrared dye-labeled sequencing primers are provided in several tubes in a light-tight plastic box. Wrap working tubes of primer with aluminum foil and return them to the freezer immediately after each use as a precaution against repeated light exposure. Sequencing reactions can be handled normally in ambient light after the labeled primer is added, as long as light exposure is kept to a minimum (i.e. keep ice bucket tightly covered). Completed reactions can be stored for at least 1 week in a dark freezer (-20 °C).

Common Sequencing Primers

Sequences of common sequencing primers:

Primer Name	Sequence (5' to 3')
M13 Forward (-29)	CACGACGTTGTAAAACGAC
M13 Reverse	GGATAACAATTTCACACAGG
T3 Promoter	AATTAACCCTCACTAAAG
T7 Promoter	TAATACGACTCACTATAGGG
SP6 Promoter	GATTTAGGTGACACTATAG

Please refer to www.licor.com/bio for availability of select common sequencing primers.

Custom Primers

Custom primer services are available from the following providers:

IDT http://www.idtdna.com

Biolegio	http://www.biolegio.com
Metabion	http://www.metabion.com

T_m Calculation

There are two different formulas presented below that can estimate the melting temperature (T_m) of a primer. The first is a very simple method (AT + GC) T_m that will give a rough estimate. This estimate is based on the following:

4 °C for every G or C, and 2 °C for every A or T.

Example: 5'-TAC CTG GTT GAT CCT GCC AGT AG-3'

This primer is a 23-mer containing 12 G and C's, plus 11 A and T's. Therefore,

$$(12 \times 4) + (11 \times 2) = 48 + 22 = 70\ °C.$$

The second method (based on thermodynamic T_m) results in a closer prediction to the actual melting temperature and is the preferred formula. This method is as follows:

69.3 °C + 0.41x - 650/Primer length

where x is the GC percentage.

Example:

Using the same primer shown above, the GC percentage is 52.2% and primer length is 23 bases, so

$$69.3\ °C + 0.41(52.2) - 650/23 = 69.3 + 21.4 - 28.26 = 62.4\ °C.$$

Section 4 Sequencing Protocols

Introduction

Labeled primer protocols for Thermo Sequenase® are designed for plasmids, PCR products, and M13-based templates. Larger templates like BACs, cosmids, and lambda phage can be sequenced with modifications in template and DNA polymerase concentrations and increased numbers of cycles. Additionally, IRDye 700 or IRDye 800 primers are interchangeable in the protocols.

General Considerations

Sequencing reactions can be run in either 0.2 ml strip tubes or microplates. Mineral oil can be used to seal the reactions during cycling. Alternatively, thermal cyclers with heated lids allow cycling reactions to be run without mineral oil as long as the tubes or plates are tightly sealed using caps or sealing mats. Test the brand of tubes or plates you will be using by running a standard cycling reaction using 6 µl of water instead of a sample. There should be no significant loss of volume after cycling if the tubes or mats are sealing properly.

Simultaneous Bi-Directional Sequencing

Simultaneous bi-directional sequencing (SBS™) employs two labeled primers on a single template in the same reaction. For example, a cDNA clone in pBluescript can be sequenced from each end using differently labeled T3-T7 or M13Forward - M13Reverse primer pairs. PCR products are ideal candidates for SBS, as mutations can be confirmed by obtaining the sequence of both strands in a single set of reactions.

Primers

An SBS reaction employs equal amounts of IRDye® 700 and IRDye 800 primers to obtain equal signal strength in both channels. In some cases, the relative amounts of each primer may require adjustment if annealing temperatures are significantly different or secondary structure complicates the binding site.

Template

The signal in both the 700 and 800 channels is dependent on the amount of template and primer in the reaction. The amount of template used in the reaction is based on the size of the insert between the two primers. The table below gives guidelines for an SBS™ reaction.

Size (bp)	Template (fmol*)
300-600	50 - 100
600-1200	125 - 200
1300-1800	225 - 300
>1800	300 - 500

* 1 femtomole = 10^{-15} mole; 1 picomole = 10^{-12} mole; 1 nanomole = 10^{-9} mole; 1 micromole = 10^{-6} mole; 1 millimole = 10^{-3} mole.

The template must also be uniform. Plasmids and PCR products can be contaminated with smaller DNA fragments that have the same priming sites as the desired template, but contain deletions or shorter inserts between the two priming sites. Since smaller inserts amplify very easily, a second sequencing pattern can appear in the lower portion of the gel, obscuring the data. A minor contaminant can cause significant background in this region of the gel (>60 bases). Carefully isolating single clones and limiting the time the miniprep culture is at stationary phase can eliminate this problem. See Section 2 for plasmid DNA isolation.

PCR Product Sequencing

PCR products can easily be sequenced with any of the labeled primer methods outlined above. The need for a labeled primer and its priming site on the PCR product can be met by one of the following options.

Labeled Primer

Make a labeled primer with the same sequence as the PCR primer. This may not be the best option for two reasons. First, in many cases PCR primers do not work as well as sequencing primers. The second reason is that the cost per sequencing reaction for a labeled primer could be considerable if used only a few times.

Nested Labeled Primers

If the sequence of the template is known for 10-20 bases in from the 3' side of the PCR primer site, you could design a labeled nested primer that would perform better. However, this would also be comparatively expensive if not used enough times.

M13 Tailed Primers

Use a PCR primer with the M13F primer sequence at the 5' end to generate a PCR product with a labeled primer-binding site. If you will be doing SBS reactions with your PCR product, the other PCR primer could have the M13REV sequence at its 5' end. By doing this you would only need to buy one or two labeled primers, M13F and/or M13REV (for SBS they should be labeled with different dyes) for all of your PCR product sequencing. The tail sequences that go on the 5' end of the PCR primers to generate binding sites for the labeled primers are M13F 5' CAC-GAC-GTT-GTA-AAA-CGA-C 3' and M13REV 5' GGA-TAA-CAA-TTT-CAC-ACA-GG 3'.

Difficult Templates

Compressions

Secondary structures at the end of chain-terminated fragments can cause shifts in band mobility. Bands differing in size by one or two nucleotides can appear to have the same mobility or be compressed. Compressions are characterized by very narrow band spacing (compression) followed by an area where the spacing expands (decompression). Including c7dGTP in the sequencing mixes and/or using high gel temperatures eliminates many compressions. Another approach is to sequence both strands using simultaneous bi-directional sequencing to resolve the sequence at a compression.

Thermo Sequenase® Labeled Primer Cycle Sequencing for USB® Kits

Template Recommendations

Template	Amount (fmol*)
Plasmid	200-500
PCR products	20-50
M13	100-200
Cosmids	50 (1.5 µg)

* fmol to µl conversion equations for DNA templates are listed in the Appendix. 1 fmol = 10^{-15} mol.

Reagents

Item	Supplier	Catalog Number
USB Thermo Sequenase Cycle Sequencing Kit	Affymetrix	78500
2.5 mM dNTP mix (with 7-deaza dGTP)*	Any Supplier	
IRDye labeled Primer (1 pmol/µl)	Any Supplier	
IR^2 Stop Solution	LI-COR	830-04997

* Prepare a solution of: 2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dTTP, 2.5 mM 7-deaza dGTP.

Reactions

1. Program the thermal cycler as follows:

Program:

Step	Temperature (°C)	Time	
1.	92	2 minutes	
2.	92	30 seconds	**30 cycles total** (steps 2–4)
3.	54 **	30 seconds	
4.	70	1 minute	
5.	4	hold	

** The annealing temperature may be adjusted depending on the T_m of the primer used. A good place to start is 5°C below the T_m of the primer.

2.	Combine these reagents in a 0.2 ml tube (add the largest volume first, then follow the order below): **Your Reaction** Template DNA — __ µl IR Dye labeled Primer (1.0 pmole/µl) — 2.0 µl Thermo Sequenase Reaction Buffer — 2.0 µl 2.5 mM dNTP nucleotide mix — 1.0 µl Thermo Sequenase DNA Polymerase — 2.0 µl ddH2O to bring final volume to 17.0 µl — __ µl TOTAL VOLUME — 17.0 µl
3.	Mix well. Mix the components well by pipetting. Simply tapping the tube to mix is not sufficient.
4.	Label a set of four 0.2 ml tubes A, T, G and C for each template/primer combination.
5.	Add 4.0 µl of each Thermo Sequenase® termination mix (purple capped tubes) to each tube labeled in step 4.
6.	Add 4.0 µl of the appropriate template/primer combination to each A, T, G, and C tube and mix well.
7.	For thermal cyclers without heated lids, add a drop of mineral oil to each tube. Place tubes in the thermal cycler and start the program.
8.	At the completion of the cycling program, add 4 µl of IR^2 stop solution to each tube.
9.	Remove oil (if used) from samples. Denature samples at 92°C for 3 minutes and place on ice.
10.	Load gel (volume depends on comb and gel thickness).

SBS™ Modifications

The amount of template used in SBS™ reactions is based on the size of the insert between the forward and reverse priming sites. The table below gives guidelines for an SBS™ reaction.

Insert Size (bp)	Template Amounts (fmol*)
300-600	50 - 100
600-1200	125-225
1300-1800	250 - 300
>1800	300 - 500

* 1 fmol = 10^{-15} mole

1.	Add the following components to a 0.2 ml tube to prepare the template/primer mix for each template (add the largest volume first, then follow the order below): Template DNA — __ µl IRDye™ 700 Fwd Primer (1.0 pmol/µl) — 1.5 µl IRDye™ 800 Rev Primer (1.0 pmol/µl) — 1.5 µl Thermo Sequenase Reaction Buffer — 2.0 µl 2.5 mM dNTP nucleotide mix — 1.0 µl Thermo Sequenase DNA Polymerase — 2.0 µl ddH2O to bring final volume to 17.0 µl — __ µl TOTAL VOLUME — 17.0 µl Mix well by pipetting.
2.	Label a set of four 0.2 ml tubes A, T, G, and C for each template/primer combination.
3.	Add 4.0 µl of the A reagent to the A tube(s), the T reagent to the T tube(s), etc.
4.	Proceed with the addition of 4 µl of the template/primer mix to each of the A, T, G, and C reagent tubes and continue with the protocol as given above.

Section 5

Gel Electrophoresis

Choosing Plates, Spacers and Combs

When using LI-COR® KB^{Plus} Gel Reagents, choose a gel size based on the following table.

	Fragment Length	Gel Length	Run Time	Spacer Thickness	Gel Comp.	e-Seq Electrophoresis Settings File Name
Medium Fragments	700-900 bp	41 cm	6 hr	0.20 mm	5.5%	4300 41cm (0.2mm)KB+
Long Fragments	900-1300 bp	66 cm	10 hr	0.20 mm	3.7%	4300 66cm (0.2mm) KB+

When using gel reagents other than KB^{Plus}, choose a gel size based on the following table.

	Fragment Length	Gel Length	Run Time	Spacer Thickness	Gel Comp.	e-Seq Electrophoresis Settings File Name
Medium Fragments	600-800 bp	41 cm	6-8 hr	0.20 mm	5.5%	4300 41cm (0.2mm)
	700-900 bp	41 cm	9-10 hr	0.25 mm	6%	4300 41cm (0.25mm)
Long Fragments	800-1300 bp	66 cm	9-11 hr	0.20 mm	3.75%	4300 66cm (0.2mm)
	900-1300 bp	66 cm	10-18 hr	0.25 mm	4.0%	4300 66cm (0.25mm)

The 64-well comb is best for most applications. A Hamilton 8-channel syringe loads the gel quickly and conveniently from a microplate or 0.2 ml strip tubes in a 96 well format. For fewer samples, a 48 well comb is an excellent alternative. Combs are available in sharkstooth or square well format.

Spacers and combs are available in two thicknesses: 0.25 mm and 0.2 mm. See the table above for run times. Generally, the thicker spacer is slightly easier to load and pour. Use the 0.2 mm Hamilton Syringe or flat tips to load 0.2 mm gels.

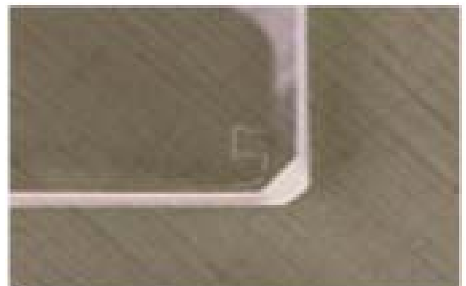
Plate Assembly

The following items are required to assemble the electrophoresis apparatus:

Gloves (non-powdered)
Safety glasses
Non-abrasive tissues (Kaydry or Kimwipes)
Front plate (notched)
Back plate (rectangular)
1 set of spacers
Comb
1 set of rail assemblies
Casting plate
Casting stand (optional)
Concentrated laboratory detergent solution (Micro-90®, International Products Corp., Burlington NJ; Liqui-Nox®, Alconox® Inc., New York, NY)
Deionized water (18.0 MOhm-cm)
3-(trimethoxysilyl)propyl methacrylate 98% (bind silane)
10% acetic acid
Test tube or centrifuge tube
Isopropanol (100%)

Caution: Always follow proper laboratory safety procedures. Always wear gloves and safety glasses when working with chemicals.

Caution: Newer Borofloat plates purchased from LI-COR are etched to show an ID number that reflects the level of fluorescence. Any two plates with the same ID number or whose numbers do not differ by more than a value of one may be used together as part of a set. Mismatched plates with ID numbers differing by more than one may result in focus errors during the focusing routine that the 4300 follows prior to every run.



Borofloat plate etched as Grade 5.

Cleaning the Plates

Materials needed:

- 2% laboratory detergent solution made from a concentrate such as Micro-90 (International Products Corp., Burlington NJ), or Liquinox (Alconox Inc., New York, NY).
- Spray bottle capable of spraying a fine mist, containing 70% ethanol.

1.	Pour a small amount (6 cm circle) of 2% detergent onto the side of the plate that will contact the gel.
2.	Work the solution into a lather with the bristle brush included, and thoroughly scrub the entire plate. Remove any dried-on polyacrylamide with a razor blade, if necessary. Rinse well with water. Buffer solution that has dried onto the plates can be removed with 0.1N NaOH.
3.	Pour distilled water onto the plate. Work the water around the plate with a gloved hand and watch for bubble formation from detergent residue. Repeat until no bubbles form.
4.	Repeat steps 1-3 above with the second plate.
5.	Rinse the plates with deionized distilled water. Use a pump spray bottle to spray a fine mist of 70% ethanol/ddH_2O for a final rinse. Stand plates in a rack to air dry.

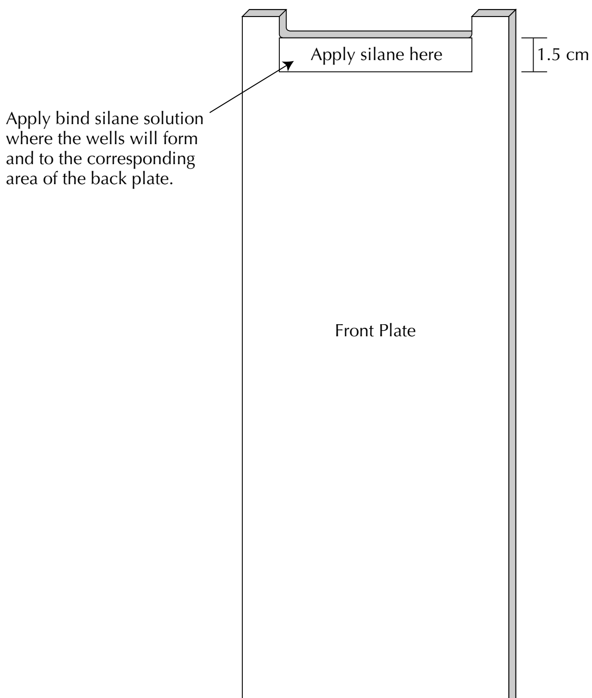
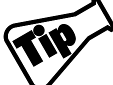
Preparing Bind Silane Solution

3-(trimethoxysilyl)propyl methacrylate 98% is a *binding* silane used to bind the gel to the glass in the area where the comb is inserted. This treatment helps maintain good well morphology for sample loading. Infrared fluorescence detection does not require repelling silane treatments.

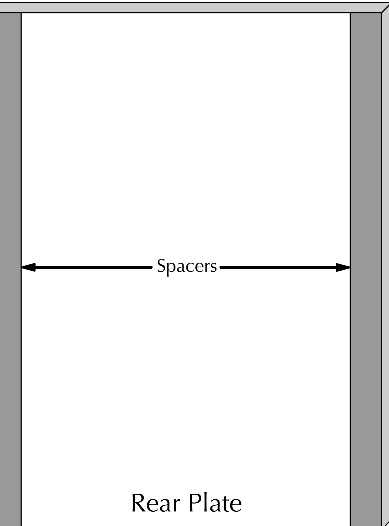
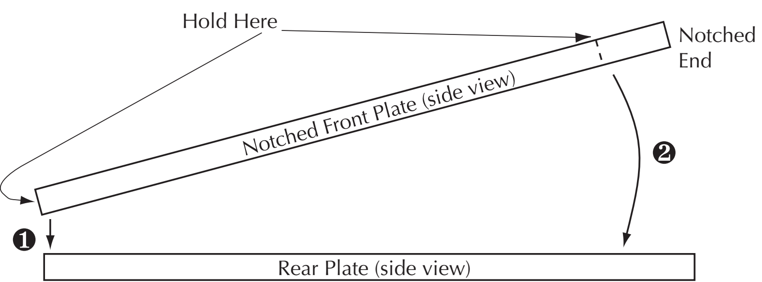
To prepare bind silane for use:

1.	Add 50 µl of bind silane to 10 ml of 100% ethanol.
2.	Mix well and store at 4 °C in an amber colored bottle, or wrap the bottle in aluminum foil.

Applying the Bind Silane Solution

1.	Wipe both plates (gel side) with 100% isopropanol.
2.	Combine 25 µl of stock bind silane solution and 25 µl of 10% acetic acid in a tube per gel. 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). Use the front plate as a guide to determine where to place the bind silane on the back plate. Allow the solution to dry before gel assembly.  ***Figure 5-1.*** *Apply bind silane to the plates as shown.*  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.

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.*

3. Make sure the rails are completely dry from prior runs before assembly. Align the spacers with the outside edges of the plates. Place the left and right rail assemblies over the plate edges (Figure 5-4).

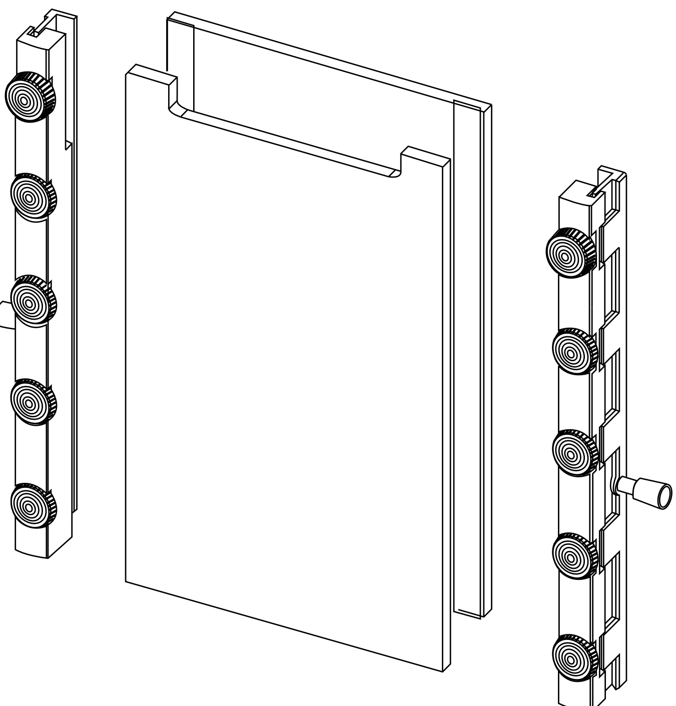


Figure 5-4. *Orientation of rails and plates before assembly.*

The top of a rail is identified by the groove where the upper buffer tank or casting plate is inserted.

Make sure the rails fit tightly against the edges of both glass plates (Figure 5-5). The spacer must also be tight against the rail. A leak will occur if there is a gap between the rail and either plate, or between the rail and the spacer.

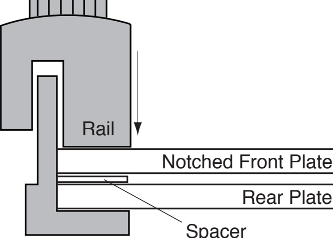


Figure 5-5. *Bottom view of assembled gel apparatus showing the proper fit of the plates and spacer in the rail.*

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.

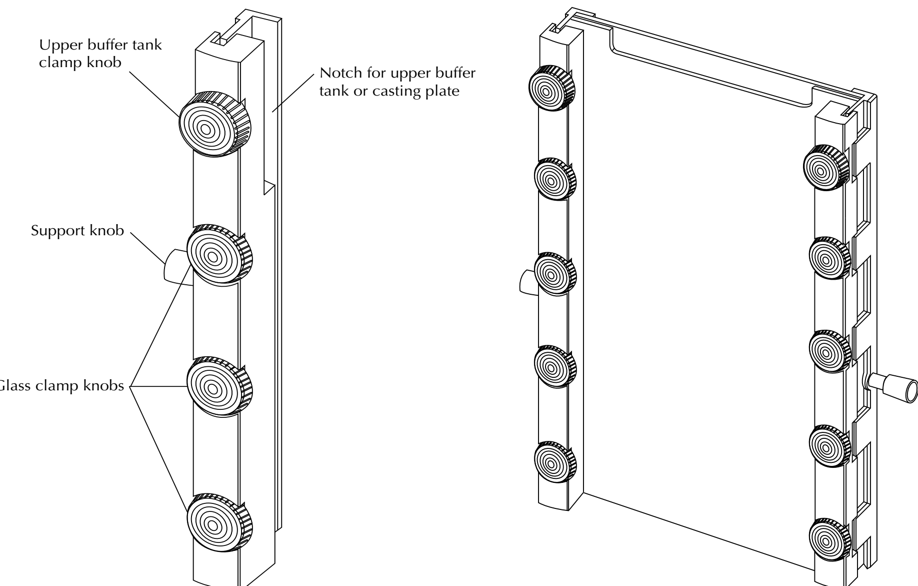
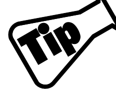


Figure 5-6. *Note orientation of rail assemblies (left rail shown).*

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.

KB^{Plus} Gel Preparation

Caution: Always follow proper laboratory safety procedures. Always wear gloves and safety glasses when working with chemicals.

Deionized Water Requirements

The conductivity of the water used in the gel and buffer should be 18 MOhm-cm or greater.

Preparing KB^{Plus} Buffer

Gel and running buffer solutions are prepared from a standard 10x TBE buffer.

10x TBE Buffer:

1.	Pour about 600 ml of distilled water (18MΩ) into a 1 l beaker.
2.	Add about one-third of the contents of the KB^{Plus} 10X TBE pouch to the water in the 1 liter beaker.
3.	Stir the solution well using a stir bar and stir plate or a stir rod until all of the solids have gone into solution and the solution is clear.
4.	Repeat steps 2 and 3 until finished.
5.	Add enough water to bring the final volume to 1000 ml.

Store at room temperature. Note that some precipitation may occur during prolonged storage.

0.8X Running Buffer: (Use this buffer for electrophoresis.)

1.	Add 80 ml of 10X TBE prepared as above to 920 ml of distilled water (18MΩ).
2.	Mix well.

Preparing Ammonium Persulfate Solution

APS provides a source of free radicals needed for polymerization of the gel (Sambrook, 1989). A 10% APS solution is made by adding 0.1 g APS to 1.0 ml deionized water in a small test tube. **Prepare APS daily to ensure a fresh working solution.** "Stale" APS will adversely affect band quality and give bands a “fuzzier” appearance with less distinct edges.

KB^{Plus} Protocol for 0.2mm, 66 cm Gels

1.	Bring 40 ml KB^{Plus} 3.7% Gel Matrix to room temperature (10-15 minutes) in a beaker. **Important:** Make sure KB^{Plus} is at room temperature before proceeding.
2.	Prepare glass plates for gel injection while KB^{Plus} warms.
3.	Add 230 µl 10% APS and 23 µl TEMED when ready to inject gel solution.
4.	Mix to homogenate and inject into gel cassette (see *Pouring The Gel* below).
5.	Allow at least 1.5 hour for polymerization before use.

KB^{Plus} Protocol for 0.2mm, 41 cm Gels

1.	Bring 30 ml KB^{Plus} 5.5% Gel Matrix to room temperature (10-15 minutes) in a beaker. **Important:** Make sure KB^{Plus} is at room temperature before proceeding.
2.	Prepare glass plates for gel injection while KB^{Plus} warms.
3.	Add 175 µl 10% APS and 17.5 µl TEMED when ready to inject gel solution.
4.	Mix to homogenate and inject into gel cassette (see *Pouring The Gel* below).
5.	Allow at least 1.5 hour for polymerization before use.

Estimating Gel Volumes Needed:

0.25 spacers: Multiply gel length by 0.625.

0.20 spacers: Multiply gel length by 0.50.

Example: A 66cm long, 0.2 mm thick gel needs 33 ml of gel solution (66 x 0.50 = 33 ml).

These volumes will just fill the gel. Mixing gel solutions in 30 or 60 ml batches will allow extra for gel run-off and shrinkage.

Preparing Gel Solutions From Other Manufacturers

Deionized Water Requirements

The conductivity of the water used in the gel and buffer should be 18 MOhm-cm or greater.

Preparing Buffer

Gel and running buffer solutions are prepared from a standard 10x TBE buffer.

Sequencing gels generally contain 1.0x-1.2x TBE while the running buffer is 0.8x-1.0x TBE.

Prepare 10x TBE as follows:

1.	Add the following to a 1000 ml beaker: **Component** / **Amount** / **Molarity** Tris Base / 107.8 g / 0.89 M Boric Acid / 55.0 g / 0.89 M EDTA (disodium salt) / 7.4 g / 0.02 M Distilled water / 950 ml TOTAL VOLUME / 1000 ml
2.	Stir to dissolve. Bring to a final volume of 1000 ml.
3.	Check the freshly prepared 10x TBE to make sure that it has a pH of 8.3 at 25 °C.
4.	Store at room temperature. Note that some precipitation may occur during prolonged storage.

Component	Amount	Molarity
Tris Base	107.8 g	0.89 M
Boric Acid	55.0 g	0.89 M
EDTA (disodium salt)	7.4 g	0.02 M
Distilled water	950 ml	
TOTAL VOLUME	1000 ml	

Preparing Ammonium Persulfate Solution

APS provides a source of free radicals needed for polymerization of the gel (Sambrook, 1989). A 10% APS solution is made by adding 0.1 g APS to 1.0 ml deionized water in a small test tube. **Prepare APS daily to ensure a fresh working solution.** "Stale" APS will adversely affect band quality and give bands a “fuzzier” appearance with less distinct edges. Do not store alliquots of APS solution.

Acrylamide

The tables below give examples for mixing gel solutions:

Long Ranger™ Gel Components

	41 cm		66 cm	
	0.25 mm, 6%	0.20mm, 5.5%	0.25 mm, 4.0%	0.20mm, 3.75%
Urea (7M)	12.6 g	12.6 g	25.2 g	25.2 g
50% Long Ranger™ acrylamide	3.6ml	3.3 ml	4.8 ml	4.5 ml
10X TBE buffer	3.0 ml	3.0 ml	7.2 ml	6.0 ml
dd water	to 30.0 ml	to 30.0 ml	to 60.0 ml	to 60.0 ml

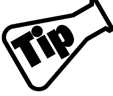
Adjust volume accordingly for 18 or 25 cm gels.

Gene-Page Plus™ Gel Components

	41 cm		66 cm	
	0.25 mm, 6%	0.20 mm, 5.5%	0.25 mm, 4.0%	0.20 mm, 3.75%
Urea (7M)	12.6 g	12.6 g	25.2 g	25.2 g
40% Page Plus™ acryla-mide	4.5 ml	4.125 ml	6.0 ml	5.6 ml
10X TBE buffer	3.0 ml	3.0 ml	6.0 ml	6.0 ml
dd water	to 30.0 ml	to 30.0 ml	to 60.0 ml	to 60.0 ml

Adjust volume accordingly for 18 or 25 cm gels.

A number of other commercial acrylamides can be used to cast gels for the LI-COR system including RapidGel-XL (Affymetrix) and Sequagel (National Diagnostics).



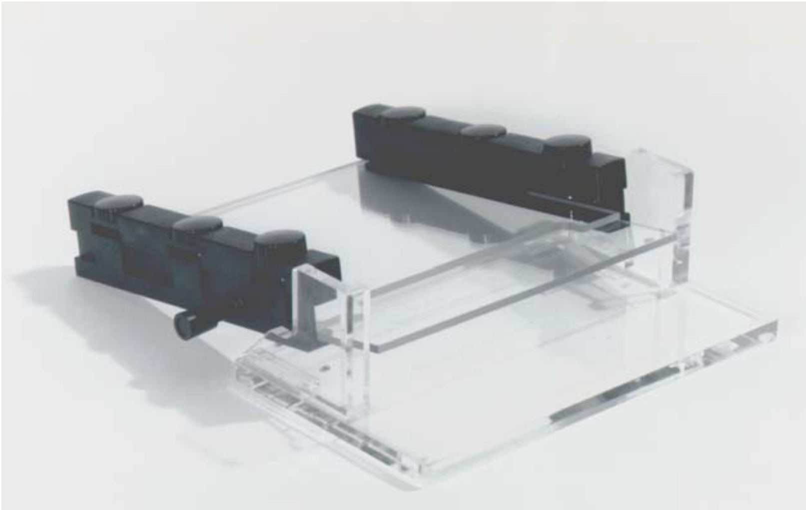
To mix 6.0 ml gel solution in one beaker: Place a 100 ml beaker with a stir bar on a balance and tare the balance. Measure 25.2 grams of urea into the beaker, then add the acrylamide solution and 10x TBE buffer. Add dd water to a target weight of 67.5 grams (= 60 ml gel solution).

*These quantities can be adjusted for smaller or larger amounts of gel.

Mixing the Gel Solution

1.	Add 350 μl of 10% APS per 60 ml of gel solution and swirl gently.
2.	Just before pouring, add 35 μl TEMED. You have approximately 3-5 minutes to pour the gel before it polymerizes.

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.

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

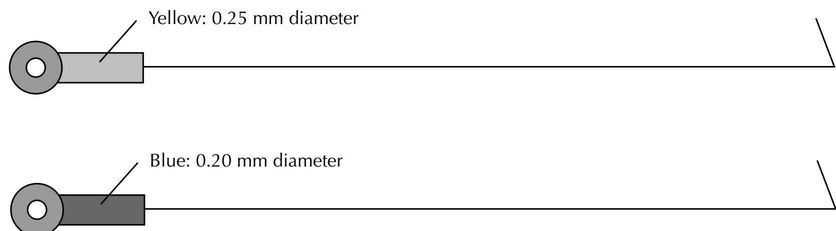

Figure 5-9. *Bubble Hooks.*
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<td>5.</td>
<td>
Insert the comb.

Figures 5-10 and 5-11 show how to insert the mylar sharkstooth and rectangular tooth combs after pouring the gel. 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.
</td>
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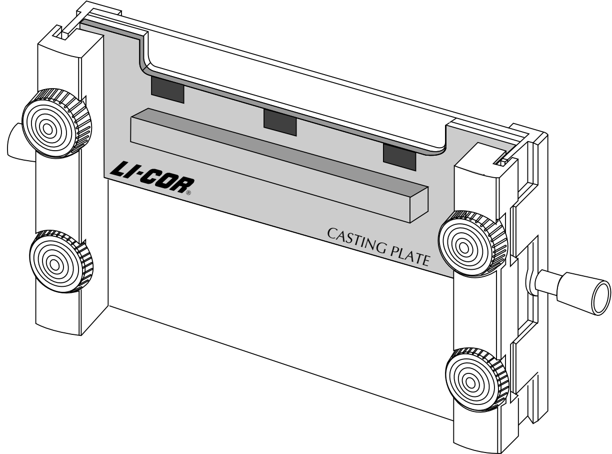
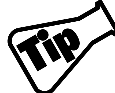
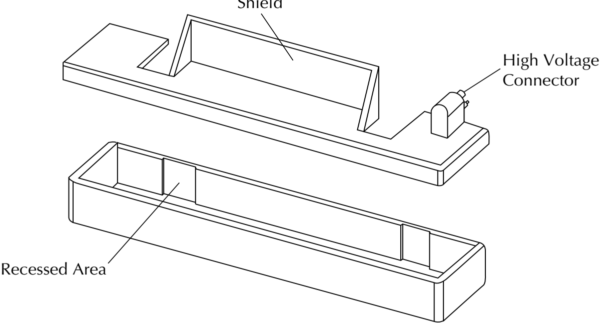
6.	Place the casting plate in the grooved area in the rails normally occupied by the upper buffer tank. Tighten the two upper clamp knobs until finger tight to secure the comb in place.  ***Figure 5-12***. *Insert the casting plate and tighten the knobs.*
7.	Allow the gel to polymerize for 1 to 1 1/2 hours. Check the tightness of the clamp knobs after polymerization to make sure they are still tight.

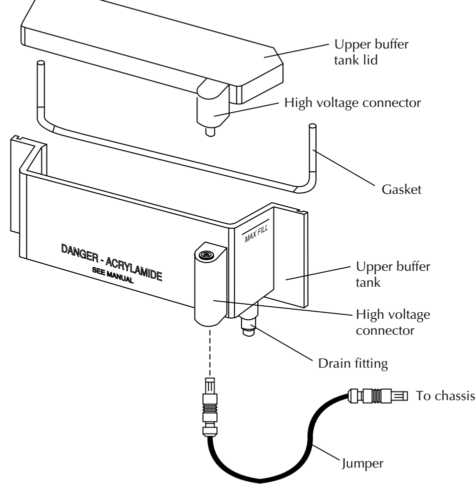
Pre-Electrophoresis Preparation

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 TBE buffer 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 TBE buffer 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.	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.)  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.
7.	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).
8.	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.
9.	Tighten the upper clamp knobs "finger tight". The electrophoresis apparatus is now fully assembled.
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 they are free of smears, dust, or spots that may interfere with detection.

Filling the Buffer Tanks

13.	For KB^{Plus} gels, use 0.8X KB^{Plus} running buffer (see Gel Preparation). For other gel formulations use 10x TBE buffer stock solution to make 1000 ml of TBE running buffer. For example: To make 0.8x running buffer for 0.2mm gels, measure 80 ml of 10x TBE buffer into a one liter graduated cylinder and add dd water to 1000 ml. Seal the top of the cylinder with Parafilm and invert the cylinder several times (carefully!) to mix completely before filling the tanks.
14.	Fill the upper buffer tank to the Max Fill line. Do not fill past the Max Fill line. Pour the remainder of the buffer into the lower buffer tank. (Pour to the left of the left rail.) The level of buffer solution must be high enough to immerse the platinum electrodes (attached to the tank lids), so power can be applied to the gel.
15.	Fill a 20cc syringe with buffer from the upper tank, add a 22 gauge needle and flush the wells with buffer to remove crystallized urea and air bubbles. Be careful not to dislodge the teeth when flushing wells around the sharkstooth comb.
16.	Place the upper and lower buffer tank lids onto the tanks. Insert the power cable on the upper buffer tank and connect it to the high voltage connector on the instrument chassis, as shown in Figure 5-14. Make sure that both connectors are fully inserted.  ***Figure 5-14**. Upper buffer tank connections.*

Starting a New Run With e-Seq Software

e-Seq Software, version 3.1 or higher, can be used to start runs and sequence data from the NEN® Model 4300 DNA Analyzer. e-Seq software automates nearly the entire sequencing process by controlling pre-electrophoresis, electrophoresis, base calling, and more. The main requirement of the user is to load the samples when e-Seq automatically pauses operation after pre-electrophoresis.

Starting a new run with e-Seq is described in the e-Seq Tutorial and the User Guide. Only a general description of e-Seq operation is given below. Runs can also be started using an Internet browser as described in the Model 4300 Operator's Manual.

Settings

Perhaps the most important step to getting successful runs with e-Seq is choosing electrophoresis settings and sequencing settings. The default electrophoresis settings include current, voltage, temperature, etc., and are designed for a specific gel height and thickness. The gel height and thickness are given in the default settings file names to distinguish the settings (listed at the beginning of this section).

The sequencing settings are also important since they contain information like the comb specification and lane loading order.

Starting the Pre-Run

Runs are started by selecting **New Run** from the e-Seq **File** menu. At the start of each run, e-Seq initializes the DNA sequencer, focuses the laser/microscope and starts the electrophoresis. The pre-run is an important process during which heat and voltage are applied to the gel.

The time interval for pre-run is set in the electrophoresis settings. At the end of the pre-run, e-Seq pauses operation automatically (gel temperature is maintained) and waits for samples to be loaded. A Scanner Console window updates run status. For example, during the pre-run it will display "Pre-run in Progress" and then "Waiting to Load Samples" at the end of the pre-run.

Sample Loading

Materials required: 20 cc syringe with 25 gauge tip.

Denature the samples as recommended in the sequencing protocol.

1.	Open the instrument door and remove the upper buffer tank lid (Figure 5-14). *Flush* the wells completely with buffer using a 20 cc syringe to remove urea or other particulate matter that may have settled into the wells during pre-electrophoresis.

<table>
<tr><td>2.</td><td>Load using either the 8-channel 0.2 mm Hamilton syringe or a pipette with flat 0.2 mm micropipette tips (Midwest Scientific MFF-2). Load volume will depend on comb and gel thickness. Carefully position the tip(s) between the glass plates and slowly release the mixture into the wells.

Loading Tips:
• Do not load wells that are deformed or contain air bubbles that cannot be removed.
• Do not overload the wells – samples could flow into adjoining wells.
• Avoid injecting an air bubble into the well after the sample is loaded, as this can force the load into adjoining wells.
• When loading rectangular tooth combs with a single pipette, flush four or five wells, load them, and repeat. If you wait too long after flushing the wells, urea will leach into the wells and make them even more transparent.
• One of the accessories for the Model 4300 is a well visualization aid that can help you see rectangular wells. This aid has a mylar sheet that slides in behind the rear plate and can make the wells more visible. Don't forget to remove the visualization aid before electrophoresis.
• One way to visualize rectangular wells is to look for the reflection of the wells in the upper surface of the notched front plate. Load by putting the tip in so it touches the back plate, then down slightly.</td></tr>
<tr><td>3.</td><td>After sample loading, replace the upper buffer tank lid, and close the instrument door.</td></tr>
</table>

Starting the Run

After sample loading, replace the upper buffer tank lid, close the door, and click the **Start Run** button in the Scanner Console window. The Scanner Console window in e-Seq displays "Run in Progress" during the run, and "Run Complete" after electrophoresis is complete. The elapsed run time is also shown in the Scanner Console window. The run is completed in one of three ways:

- The full run time elapses (run time is specified in the electrophoresis settings).
- The user clicks **End Run** button to finish the run early and save all related files.
- The user clicks **Cancel Run** because the run is bad and no files should be saved.

Base Calling and Editing

Base calling, editing and data output in reports are described in the e-Seq User Guide.

Starting a New Run With the 4300 Browser Software

Starting runs using an Internet browser or the front panel is fully described in the 4300 Operator's manual. Sample loading at the end of the pre-run is as described above.

Disassembly

1.	Remove the buffer tank lids.
2.	Disconnect the power cable.
3.	***For 18 cm gels***, remove the entire gel apparatus from the sequencer and carefully dispose of the buffer solution.
4.	***For 25 cm or larger gels***, the upper buffer tank has a fitting for draining the buffer solution, while the apparatus is still secured to the sequencer. A coupling attached to a length of rubber tubing is provided to drain the tank (Figure 5-15) Make sure the tubing is placed in a proper receptacle, as buffer will begin to drain immediately upon connection of the coupling. Insert the coupling until it snaps into place. Press here to release Rubber tubing ***Figure 5-15.*** *Buffer tank drain fitting.* Depress the metal ring on the coupling and pull straight down to remove the drain hose when finished.

Cleanup

1.	After removing the upper and lower buffer tank lids and disconnecting the power cable, take the gel assembly off the sequencer and remove the upper buffer tank and rails.
2.	Remove the lower buffer tank and dispose of the buffer solution.
3.	Rinse the rails, spacers, and comb, and allow to air dry.
4.	Rinse and air dry the upper and lower buffer tanks and lids.
5.	Clean any spills on the heater plate, chassis, or front panel.

Cleaning the Plates

Materials needed:

- 2% laboratory detergent solution made from a concentrate such as Micro-90® (International Products Corp., Burlington NJ), or Liqui-Nox® (Alconox® Inc., New York, NY).
- Spray bottle capable of spraying a fine mist, containing 70% ethanol.

1.	A small black plastic wedge is included in the spare parts kit to aid in separating the gel plates. Insert the wedge between the plates to pry them apart for cleaning. **Caution:** Do not pry on the left and right sides of the front plate where the glass is narrow. *Never use metal tools to pry plates apart.* Lab spatulas, razor blades, or similar tools can chip plates.
2.	Dispose of the acrylamide in compliance with local regulations. Try to clean gels within 1-2 hours after the run is complete. If the gel has recently cooled to room temperature, acrylamide will adhere to paper towels, which can be used to lift the acrylamide off the plates. If gels have been left for more than 4 hours, use a razor blade to scrape the acrylamide from the plates.
3.	Pour a small amount (6 cm circle) of 2% detergent onto the side of the plate that will contact the gel.
4.	Work the solution into a lather with a nylon bristle brush and thoroughly scrub the entire plate. Remove any dried-on polyacrylamide with a razor blade, if necessary. Rinse well with water.

5.	Pour distilled water onto the plate. Work the water around the plate with a gloved hand and watch for bubble formation from detergent residue. Repeat until no bubbles form.
6.	Repeat steps 3-5 above with the second plate.
7.	Rinse the plate with deionized distilled water. Use a pump spray bottle to spray a fine mist of 70% ethanol/ddH_2O for a final rinse. Stand plates in a rack to air dry.

Section 6 Troubleshooting

Electrophoresis Apparatus Troubleshooting

This section is intended to provide some tips and techniques for assembling and using the electrophoresis apparatus. In addition, some of the common problems and possible solutions are outlined in the following troubleshooting sections; Apparatus Assembly and Mounting, Electrophoresis, and Apparatus Disassembly. Only problems associated with the electrophoresis apparatus are addressed; help for software problems can be found in the software User Guides or on-line help systems.

Note on Leak Detection Circuitry

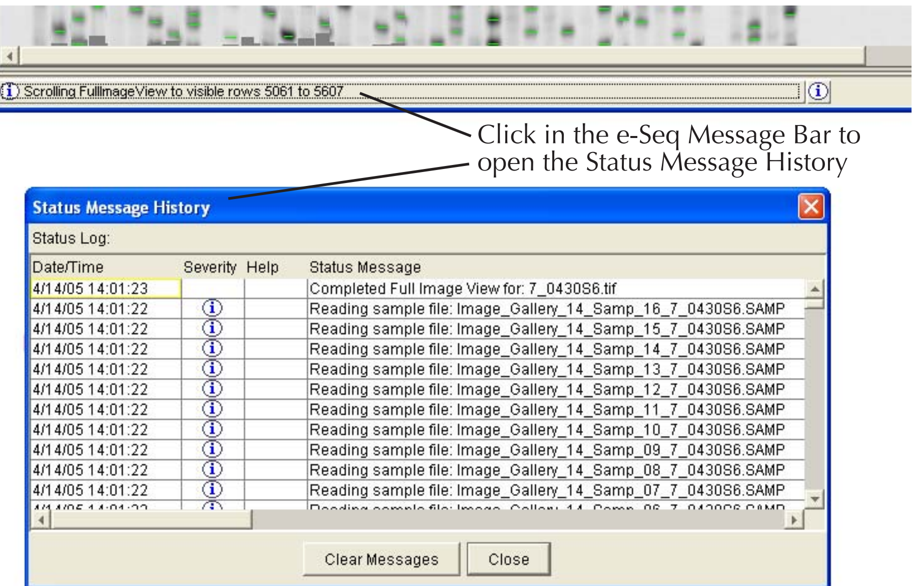
As with any electrophoresis apparatus, cleanliness is very important when preparing the system. With LI-COR systems, this is particularly important because the electrical leak detection circuitry is designed to monitor electrical leakage that may occur between the buffer solution and the heater plate (chassis ground). If the top surface of the plates is wet or is caked with acrylamide, a potential path exists whereby buffer solution in the upper tank can "wick" over the top of the plates and make contact with the heater plate, shutting down the system. This type of problem is usually indicated by a message that says "*Probable Electrical Leakage*".

A second type of electrical problem can occur if the connection between the upper and lower buffer tanks (via the buffer solution) is broken. This can happen when the level of the buffer solution in the upper tank drops below the electrode, or fails to make contact with the gel. In this instance, you may see a message that says "*Open Circuit Condition*". In an extreme situation, buffer may run down between the glass and the rails into the lower buffer tank. This can affect the electrical field in the gel, but may not be detected as an electrical "leak", since the electrical path is not being grounded to the heater plate.

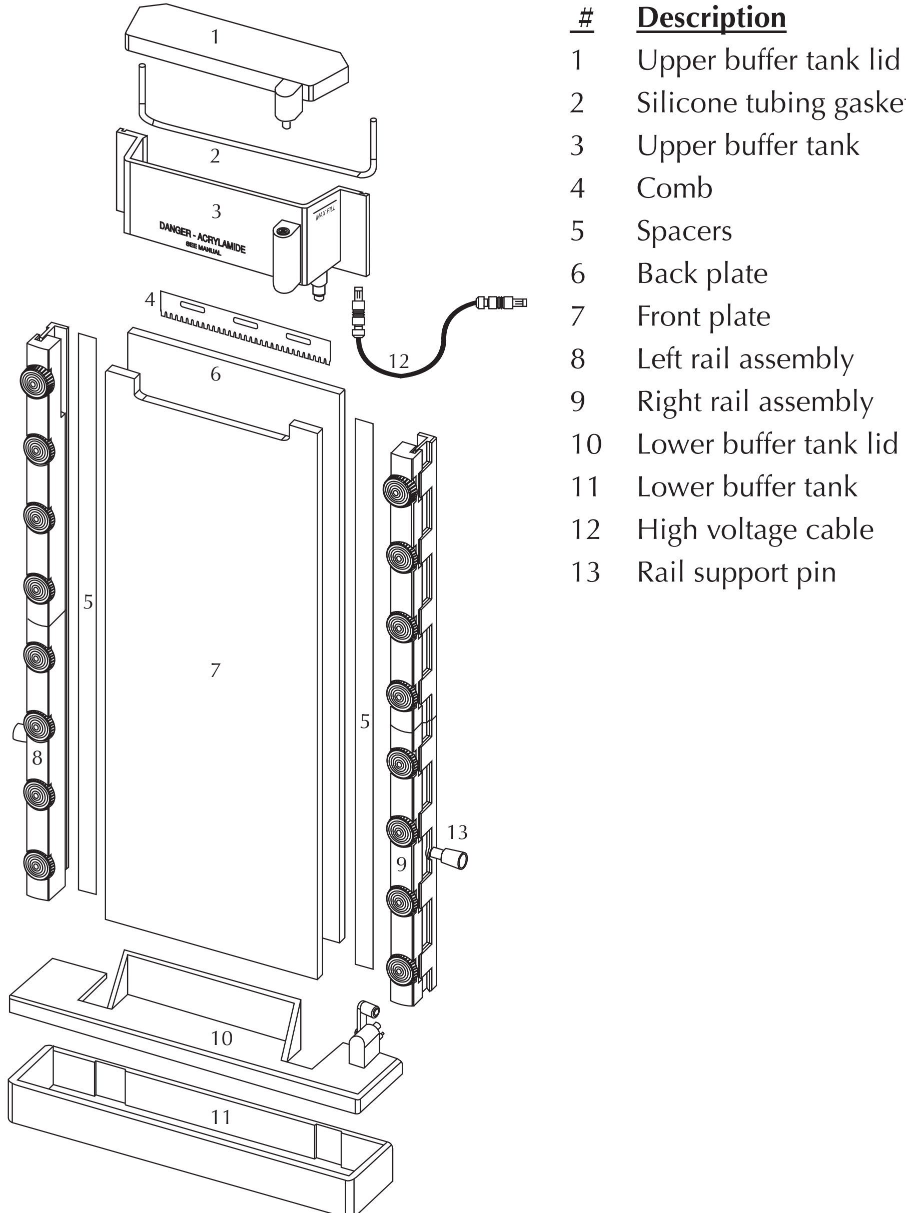
To avoid these types of problems, we recommend that you routinely inspect the apparatus parts to make sure that the buffer tanks aren't cracked or crazed, and that the joints are tightly sealed. We also recommend that you periodically wipe the instrument enclosure (heater plate, front door, stainless steel platform) with a damp cloth. Use "powder free" latex gloves when handling the glass plates or assembled apparatus.

When attempting to diagnose problems, the Message History window in e-Seq will often provide valuable information regarding the events that may have preceded the problem. A log file of messages for the run can also be accessed through the browser software (see Model 4300 Operator's Manual). LI-COR® service technicians may refer you to one of these diagnostic sources when troubleshooting problems.

To open the Message History window in e-Seq, click in the message bar at the bottom of the e-Seq window as shown below.



The diagram below shows the parts of the electrophoresis apparatus. The Troubleshooting sections to follow may periodically refer to these parts.



#	Description
1	Upper buffer tank lid
2	Silicone tubing gasket
3	Upper buffer tank
4	Comb
5	Spacers
6	Back plate
7	Front plate
8	Left rail assembly
9	Right rail assembly
10	Lower buffer tank lid
11	Lower buffer tank
12	High voltage cable
13	Rail support pin

Figure 6-1. *Exploded view and parts list for 66 cm gel apparatus.*

Gel Apparatus Assembly and Mounting

Problem	Possible Cause	Solution / Prevention
Trouble clamping gel.	Rail clamp cracked.	Replace rail clamp.
Bubble in gel.	Dirty plates.	Use a bubble hook to remove bubble.
Many bubbles in gel.	Interrupted flow during pouring.	Maintain a smooth continuous stream while pouring the gel.
	Oil from hands on plates.	Wear gloves when handling plates.
	Soap residue on plates.	Clean plates thoroughly.
	Dirty plates.	Reclean the plates thoroughly.
Gel polymerizes during pouring	Added too much APS and/or TEMED.	Add proper amount of APS/ TEMED
	Took too long to pour.	Pour gel with in 3-5 minutes of adding APS and TEMED.
	Gel solution was heated to dissolve components.	Leave gel solution at room temperature when preparing.
No polymerization of gel	No APS and/or TEMED added.	Add proper amount of APS/ TEMED
	Incorrect ratio of acrylamide:bis acrylamide used.	Use correct amounts of each, or use premixed gel solutions.
Poor well morphology	Removed comb before gel was completely polymerized.	Allow more time for polymerization.
	Didn't coat plates with bind silane at comb location.	Coat notched area with bind silane.
	Didn't use enough gel. Thick gels may shrink somewhat during polymerization.	Use excess gel, so that gel pools at notched area of plates.
	Gel not mixed thoroughly.	Mix completely after addition of APS and TEMED.
	Gel sandwich not clamped at top.	Use casting plate to hold comb in place during polymerization.
Comb is too tight or too loose between the plates.	Comb and spacers are different thickness.	Select comb and spacers of matching thickness.
	Dirty comb and/or spacers.	Clean comb and/or spacers.

Electrophoresis

Problem	Possible Cause	Solution / Prevention
Apparatus hangs crooked	Lower tank is not pushed completely to the rear or is in backwards.	Check orientation and push completely toward the rear of the platform.
	One rail support pin knob in and the other pulled out.	Hang again with both rail support knobs in or both out.
	Rail clamp cracked.	Replace rail clamp.
	Rail support pin broke.	Replace rail clamp.
"Open Circuit Condition" error message displayed in e-Seq or on 4300 display, run quits early, or arcing occurs.	Upper or lower lid not making good electrical connection.	Check for proper insertion of high voltage cables and connectors.
		Make sure lid is positioned properly on the tank.
	Upper tank leaks around the gasket.	Check that gasket is of proper length, is not stretched during installation, and that the gasket and groove are clean.
	Rails not tight against sides of glass.	Re-seat rails against the glass so that bottom surface of both glass plates, spacers, and rails are flush.
	Buffer level below electrode position.	Fill tanks to proper level.
		Check for cracked tanks or tank seams that may have failed.
	Buffer wicking from upper tank.	Remove gel residue from top edge of plates.
	Broken electrode.	Replace the tank lid.

"Probable Electrical Leakage" message. Run quits early, or arcing occurs.	Running buffer or gel shorting to heater plate or chassis "ground".	Check for cracked tanks or tank seams that have failed.
		Clean and dry the top surface of the back glass plate and heater plate to prevent wicking.
		The area along the rail edges (next to heater plate) should be clean and dry.
	Moisture on top surface of back glass plate.	Make sure the tank was not overfilled or buffer spilled during filling.
		Reclean; Be careful filling of upper buffer tank and/or flushing of the wells.
	Moisture in cable. Moisture in lower buffer tank lid connector due to rinsing without protective cap or submersion in water.	Remove moisture with clean compressed air, or dry with desiccant overnight in 37 °C incubator. Use retaining cap when rinsing lower buffer tank lid.
Streaks on the image.	Dirt or bubble in the gel at the precise position where the laser scans the gel.	If dirt is on the outer surface of the front plate, wipe it clean. Otherwise, use the "drop down" support pins on the rails.
		May require draining the upper tank, removing the apparatus, and cleaning the plates again.
	Detergent buildup on plates.	Scrub plates and rinse thoroughly; soak plates in 1N NaOH.
	Incorrect detergent used.	Use Liquinox or other detergent designed for laboratory glassware, not dish or liquid hand soaps.

Some sample lanes out of view on image.	Comb not properly centered in 6-inch scan zone.	On future runs, center the comb in the 6-inch scan zone.
	Excess salts in the sample cause lateral movement of the lanes.	Decrease the salts or load fewer lanes. Load stop buffer in empty lanes.
	Lanes outside scan zone loaded.	Skip the outside two lanes on both ends of the comb.
Run terminates early.	Power supply interruption.	Add a UPS (Uninterruptable Power Supply) to the system.

Gel Apparatus Disassembly

Problem	Possible Cause	Solution / Prevention
Upper buffer won't drain.	Drain fitting full of polymerized acrylamide.	Clean out drain fitting.
Gel plates hard to separate.	Gel dried out over time.	Run plastic wedge along edge of gel. Do not pry on notched glass plate "ears"!
		Soak gel/glass plate assembly in ddH_2O.
		Disassemble within eight hours of the end of the run.

Electrophoresis Apparatus "Open Circuit" Troubleshooting

This section provides tips and techniques for assembling and using the electrophoresis apparatus, particularly if you encounter the "*Open Circuit Condition*" error message.

The "Open Circuit Condition" Error Message

An electrical problem can occur if the connection between the upper and lower buffer tanks (via the buffer solution) is broken. This can happen when the level of the buffer solution in the upper tank drops below the electrode, or fails to make contact with the gel. In this instance, you may see a message that says "*EMBERR 258 Open Circuit Condition...Shutting Down!*" in e-Seq or "*Gel Open Circuit Condition*" on the 4300 display. If the top surface of the plates is wet or is caked with acrylamide, a potential path exists whereby buffer solution in the upper tank can "wick" over the top "ears" of the front plate, and if the spacer is not properly installed, a capillary can form. These two actions act like a pump that drains the upper buffer tank (Figures 6-2 and 6-3). This is especially true during long runs with 66 cm gels. As a result, buffer may run down between the glass and the rails into the lower buffer tank. This can affect the electrical field in the gel, but may not be detected as an electrical "leak", since the electrical path is not being grounded to the heater plate.

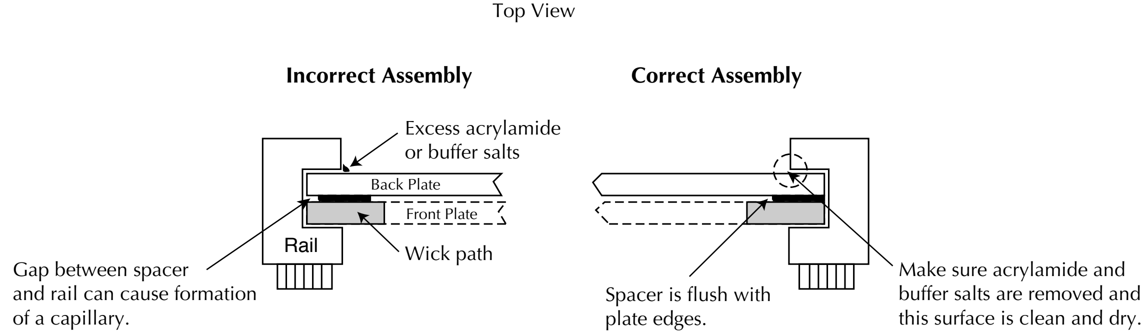


Figure 6-2. *Align the spacers with the edge of the glass plates.*

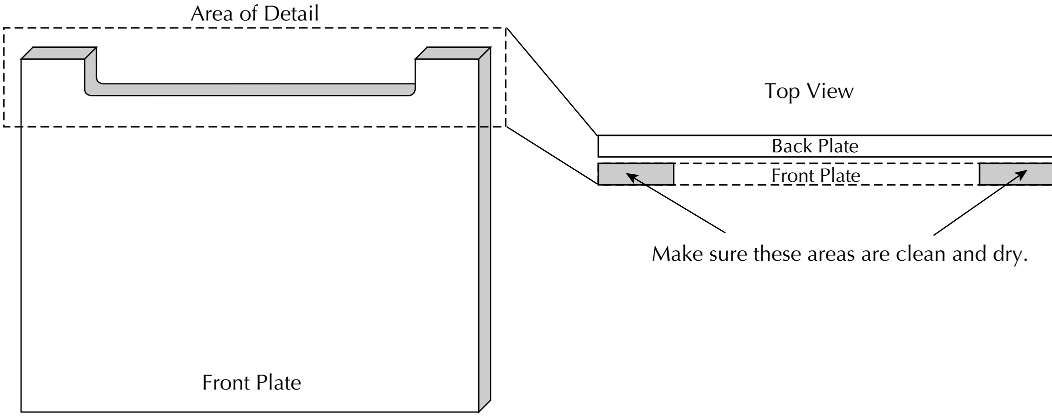


Figure 6-3. *Clean and dry the front plate "ears" thoroughly.*

Note on Leak Detection Circuitry

As with any electrophoresis apparatus, cleanliness is very important when preparing the system. With LI-COR systems, this is particularly important because the electrical leak detection circuitry is designed to monitor electrical leakage that may occur between the buffer solution and the heater plate (chassis ground). If the back plate is not clean and free of caked acrylamide or buffer salts (Figure 6-4), wicking from the bottom buffer tank can occur. Once the wicking action causes moisture to contact the heater plate, an error message that says "*EMBERR 284 Probable Gel Leak*" will be displayed in e-Seq and the 4300 display will show “*Probable Gel Leak*”. Make sure that the glass plates are clean and dry in the areas indicated in Figures 6-3 and 6-4.

To avoid these types of problems, we also recommend that you routinely inspect the apparatus parts to make sure that the buffer tanks aren't cracked or crazed, and that the joints are tightly sealed. Periodically wipe the instrument enclosure (heater plate, front door, stainless steel platform) with a damp cloth. Do not use alcohol on the buffer tank as this will craze the tank. Use "powder free" latex gloves when handling the glass plates or assembled apparatus.

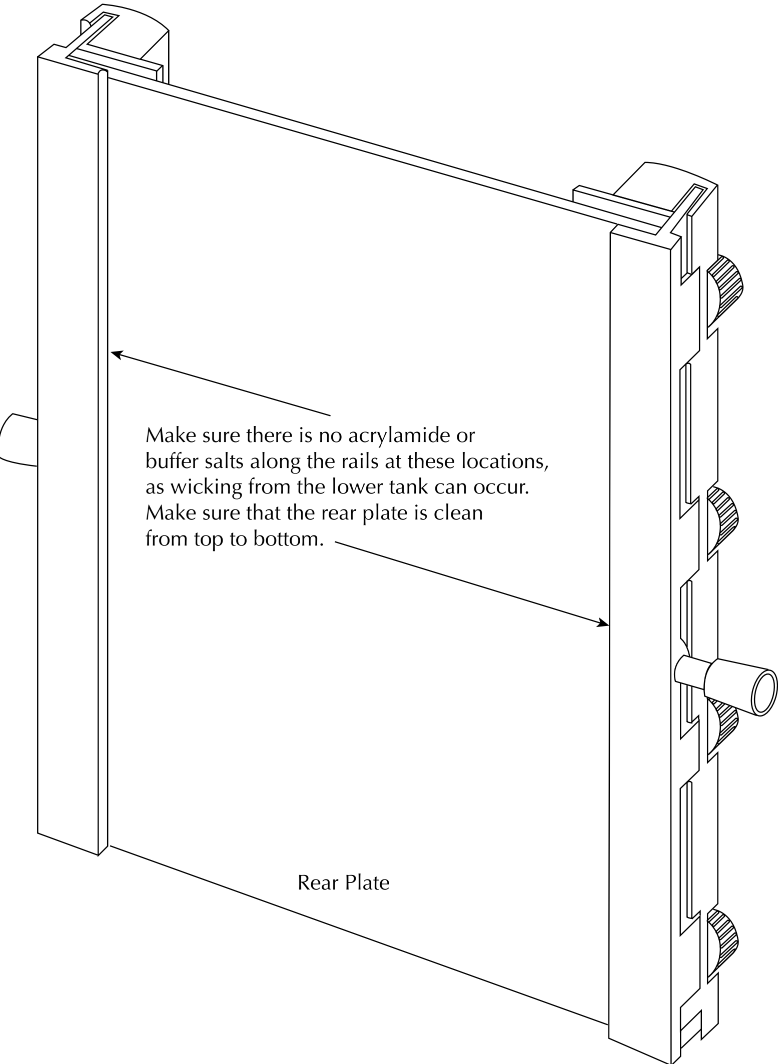


Figure 6-4. *Make sure there is no acrylamide or buffer salts along the edge of the rails.*

Section 7 Appendices

Conversion From fmol to µl for DNA Templates

General Equation for dsDNA Templates:

$$\frac{[\textit{fmol needed in reactions}]\ /\ 1000}{\left[\frac{\textit{Conc.}\ (\mu g/\mu l)}{\#\ \textit{Bases}}\right] \times\ 1538.46} = \mu l \text{ volume needed in reactior}$$

General Equation for ssDNA Templates:

$$\frac{[\textit{fmol needed in reactions}]\ /\ 1000}{\left[\frac{\textit{Conc.}\ (\mu g/\mu l)}{\#\ \textit{Bases}}\right] \times\ 3076.92} = \mu l \text{ volume needed in reactior}$$

These equations use 650 as the molecular weight of a base pair.

Typical Template Sizes:

Template	Size
M13mp18 & 19	7300 each
pUC 19	2686
pBluescript	2961
pGEM-3z	2743

Example with pUC:

Suppose we want to sequence the pUC19 template using the SequiTherm Excel cycle sequencing protocol.

pUC19 Template

Total Size = 2686 bp

Concentration = 1 µg/µl

SequiTherm requires 0.3 pmols of dsDNA. Therefore the volume (µl) needed for this reaction is

$$\frac{[300\textit{fmol}]\ /\ 1000}{\left[\frac{1\ \mu g/\mu l}{2686}\right] \times\ 1538.46} = 0.52\mu l$$

Microsatellite Analysis Manual

Model 4300 DNA Analyzer

LI-COR®

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Section 1 Plate Organization

Introduction

A system for the arrangement of samples in storage and reaction plates should be established at the beginning of a microsatellite analysis project. Samples should be placed in a specific arrangement in the plate to achieve the desired sample order on the gel. These arrangements can be used for reaction processing plates as well as stock plates that hold up to a milliliter of the DNA diluted to a standard concentration.

A consistent volume for each DNA sample can be conveniently transferred to the 96-well reaction plate with a multichannel pipette or a 96 needle Hydra (Robbins Scientific). Ideally, samples from an entire family are stored on one plate and aliquoted to 96-well plates at 20 to 50 ng/µl concentration. Plates are stored at -20 °C until needed.

The Hamilton 8-Channel Syringe

The Hamilton 8-channel 0.2 mm syringe (LI-COR, P/N 870-05848) has syringe needles that are spaced 9 mm apart. Since the wells formed by LI-COR® 64-well combs are on 2.25 mm centers, the 8-channel syringe can be used to load every fourth well. For 48-well combs, every third lane can be loaded. LI-COR Biosciences' ClickIR Assembly for the Hamilton 8-channel syringe assures consistent volume delivery through tactile feedback. The ClickIR assembly attaches to the 8-channel syringe and allows the user to feel clicks that correspond to 0.3 µl, 0.4 µl, or 0.5 µl (selectable) as sample is dispensed. Assembly instructions for the ClickIR Assembly can be found in the Miscellaneous tab of this applications manual.

Microplate Configurations

The arrangement of samples and standards in microplates should be predefined in order to achieve the desired order on the gel. Two arrangement examples for 64-well combs are shown in Figures 1-1 and 1-2. The wells in each microplate

are numbered according to the lane number that will be loaded. Both configurations satisfy Saga's requirement of at least 5 size standard lanes for di-nucleotide repeats (7 lanes or more are better), as should any plate configurations you design. The first example shows how to load half of a full microplate on one gel and the other half on a second gel. The second example shows how to configure a microplate for loading 60 samples on a 64-well comb.

Loading 48 Samples on a 64-well Comb

In this configuration, size standards are loaded into every fourth lane. Size standards (designated "S") are loaded from a second plate or a set of strip tubes. An entire microplate can therefore be loaded onto two gels.

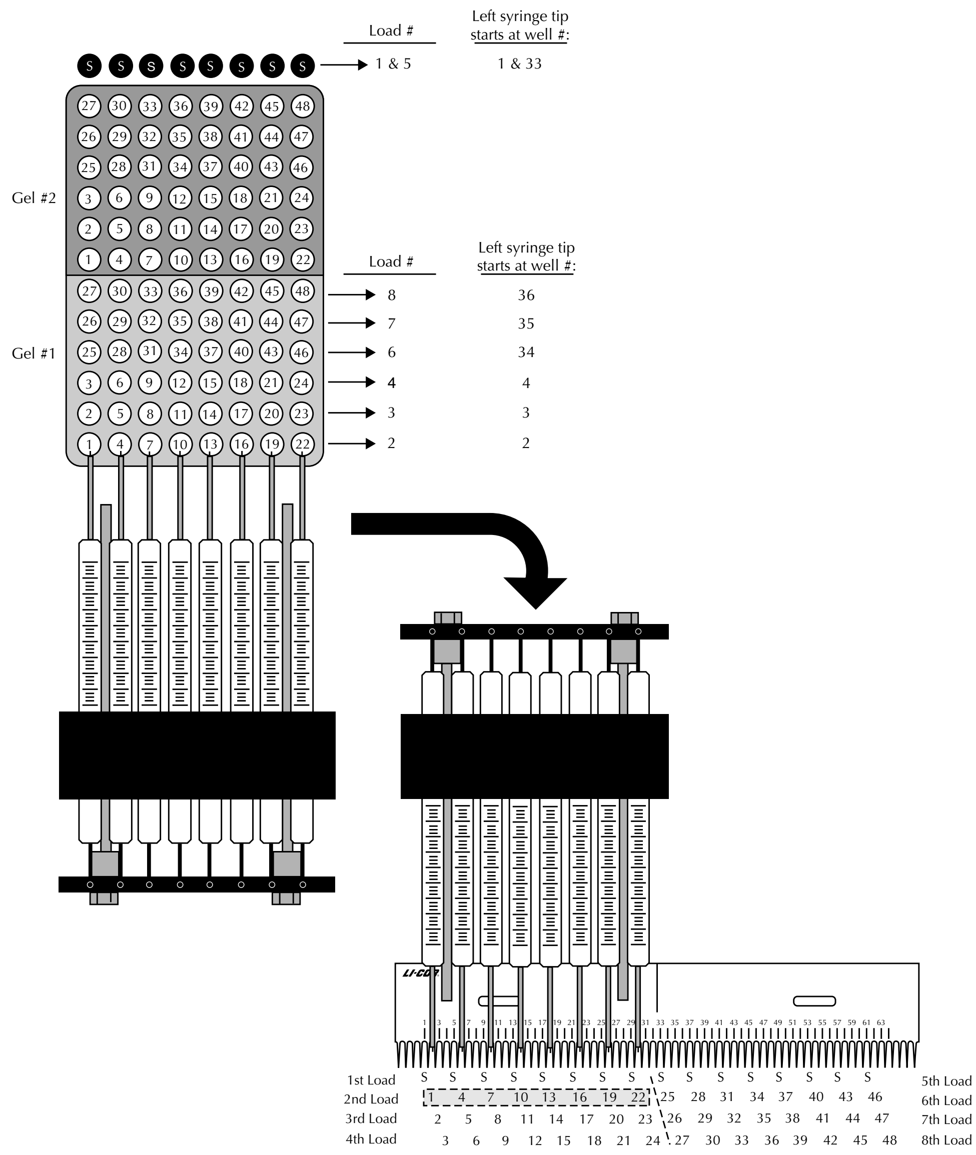


Figure 1-1. *Microplate showing 48 sample loading format. Size standards are loaded from a second plate or set of strip tubes.*

Loading 60 Samples on a 64-well Comb

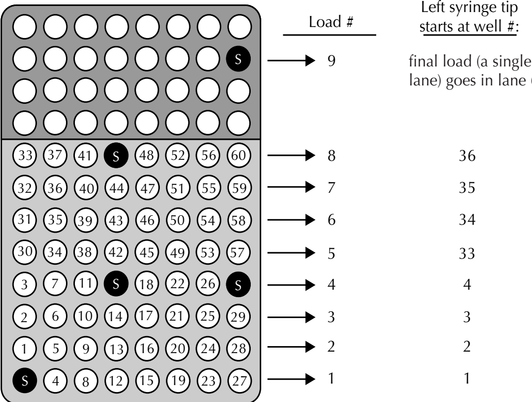


Figure 1-2. *Microplate showing 60 sample loading format.*

Section 2 Gel Preparation and Electrophoresis

Choosing Plates and Spacers

18 and 25 cm gel plates are both suitable for microsatellite analysis applications. Tri- and tetranucleotide repeats, as well as dinucleotide loci are clearly resolved on 18 cm gels, providing accurate data. However, for complex dinucleotide repeats, 25 cm gels provide increased resolution. 18 cm gels typically resolve fragments of 350 base pairs in 1 hour, while 25 cm gels require 1.5 hours. 0.25 mm spacers should be used with either gel height.

Choosing a Gel

Use 25 cm gels for standard runs since they provide optimum resolution and have adequate run speeds. Each gel can be loaded up to three times. Throughput can be increased by using 18 cm gels since they require less time for electrophoresis between successive loads. 18 cm gels give good performance with increased speed for many applications.

Table 2-1. Parameters for Standard and Fast Run gels.

Parameter	Standard Run	Fast Run
Plate Length	25 cm	18 cm
Spacer Thickness	0.25 mm	0.25 mm
Gel Composition	6.5% LI-COR® KB^{Plus}	6.5% LI-COR KB^{Plus}
Run time (for 350 bases)	1.25 hours	45 minutes
Reload gel after	1.5 hours	1 hour

Plate Assembly

The following items are required to assemble the electrophoresis apparatus:

- Gloves (non-powdered)
- Safety glasses
- Non-abrasive tissues (Kaydry and Kimwipes)
- Front plate (notched)
- Back plate (rectangular)
- 1 set of spacers
- Comb
- 1 set of rail assemblies
- Casting plate
- Casting stand (optional)
- Concentrated laboratory detergent solution (Micro-90®, Liqui-Nox®, etc. detergent and tap water)
- Deionized water (≈18.0 MOhm)
- 3-(trimethoxysilyl)propyl methacrylate 98% (bind silane)
- 10% acetic acid
- Test tube or centrifuge tube (must hold 170 µl of liquid)
- Isopropanol (70 - 100%)

Caution: Always follow proper laboratory safety procedures. Always wear gloves and safety glasses when working with chemicals.

Caution: Newer Borofloat plates purchased from LI-COR are etched to show an ID number that reflects the level of fluorescence. Any two plates with the same ID number or whose numbers do not differ by more than a value of one may be used together as part of a set. Mismatched plates with ID numbers differing by more than one may result in focus errors during the focusing routine that the 4300 follows prior to every run.

Borofloat plate etched as Grade 5.

Cleaning the Plates

Materials needed:

- 2% laboratory detergent solution made from a concentrate such as Micro-90 (International Products Corp., Burlington NJ), or Liquinox (Alconox Inc., New York, NY).
- Spray bottle capable of spraying a fine mist, containing 70% ethanol.

1.	Pour a small amount (6 cm circle) of 2% detergent onto the side of the plate that will contact the gel.
2.	Work the solution into a lather with the bristle brush included, and thoroughly scrub the entire plate. Remove any dried-on poly-acrylamide with a razor blade, if necessary. Rinse well with water. Buffer solution that has dried onto the plates can be removed with 0.1N NaOH.
3.	Pour distilled water onto the plate. Work the water around the plate with a gloved hand and watch for bubble formation from detergent residue. Repeat until no bubbles form.
4.	Repeat steps 1-3 above with the second plate.
5.	Rinse the plates with deionized distilled water. Use a pump spray bottle to spray a fine mist of 70% ethanol/ddH_2O for a final rinse. Stand plates in a rack to air dry.

Preparing Stock Bind Silane Solution

1.	Add 50 µl of bind silane to 10 ml of 100% ethanol.
2.	Mix well and store at 4 °C in an amber colored bottle, or wrap the bottle in aluminum foil.

Silane treatments are used in autoradiography to facilitate removal of the gel from the plates for exposure to film after electrophoresis. Infrared fluorescence detection does not require repelling silane treatments because DNA bands are detected in the gel, in real time, during electrophoresis.

3-(trimethoxysilyl)propyl methacrylate (98%), however, is a binding silane used to covalently bind the gel to the glass in the area where the comb is inserted. This treatment helps maintain good well morphology when loading gels multi-ple times.

Applying the Bind Silane Solution

1.	Combine 25 µl of stock bind silane solution and 25 µl of 10% acetic acid in a 1.5 ml microcentrifuge tube. Mix thoroughly (pipette or vortex).

2.	Use a cotton swab to apply the solution on the inside of the short plate over the area below the edge of the notch where the wells will form (Figure 2-1). Apply solution to the rear plate using the front plate as a guide to determine where to place the bind silane onto the rear plate . Allow the solution to dry before gel assembly.

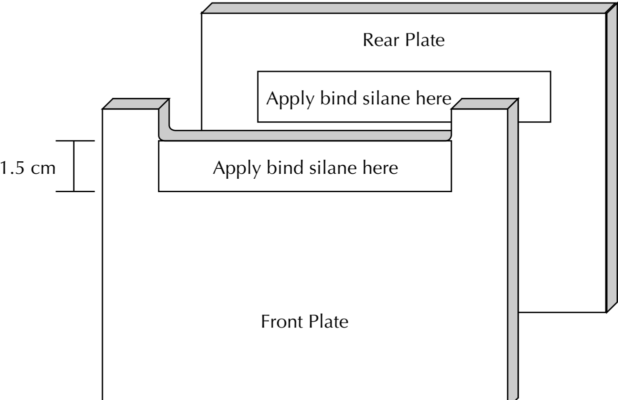
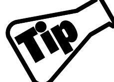


Figure 2-1. *Apply bind silane to the plates as shown.*



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.

Assembling the Gel Sandwich

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

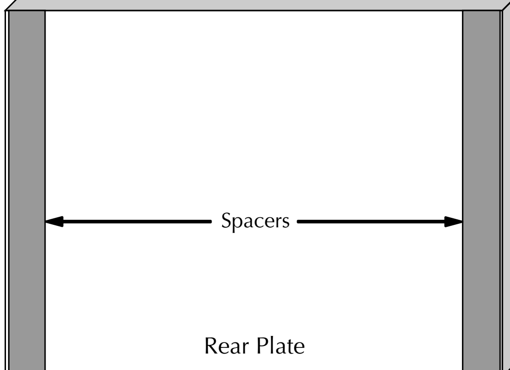


Figure 2-2. *Place a spacer on the long edges of the plate.*

2.	Place the front plate on top of the rear plate (gel side down) and align the spacers with the outside edges of the plates. Make sure that the plates are aligned evenly at the bottom.

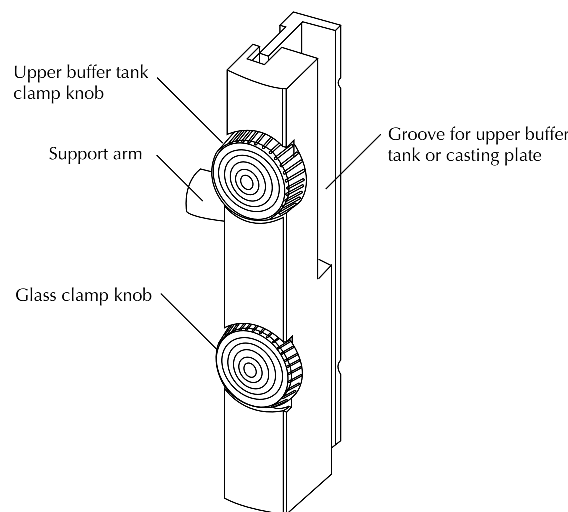
<table>
<tr><td>3.</td><td>Make sure the rails are completely dry from prior runs before assembly. Place the left and right rail assemblies over the plate edges. Note that the top portion of each rail is notched for insertion of the upper buffer tank or casting plate (Figure 2-3). The uppermost clamp knob on each rail is larger than the other one, as well.


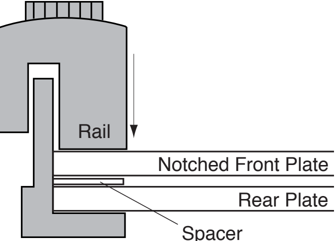
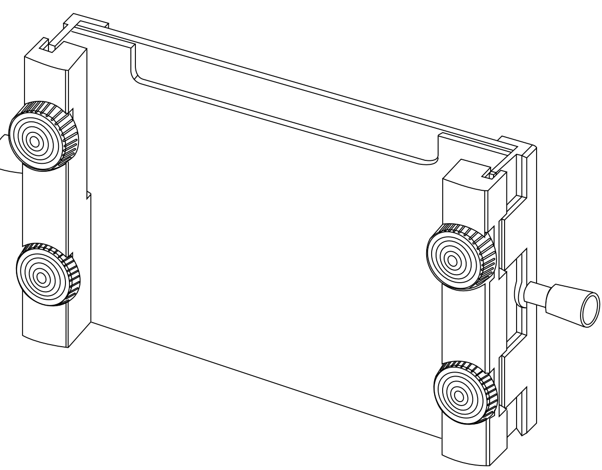
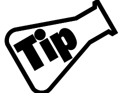
Figure 2-3. *Note orientation of rail assemblies (left rail shown).*</td></tr>
<tr><td>4.</td><td>Check to make sure the rails fit tightly against the edges of both glass plates (Figure 2-4). The spacer must also be tight against the rail. A leak will occur if there is a gap between the rail and either plate, or between the rail and the spacer.


Figure 2-4. *Bottom view of assembled gel apparatus showing the proper fit of the plates and spacer in the rail*</td></tr>
</table>

5.	Tighten the glass clamp knob 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. Assemble the apparatus as shown in Figure 2-5.  ***Figure 2-5**. 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.
6.	Select a comb (sharkstooth or rectangular tooth comb) with thickness that matches the spacers. Clean plastic combs with water and/or ethanol if necessary. Make sure that the comb fits between the two plates at the top of the gel, behind the notch in the front plate. If it doesn't fit or is very loose, try another comb.

KB^{Plus} Gel Preparation

Caution: Always follow proper laboratory safety procedures. Always wear gloves and safety glasses when working with chemicals.

Deionized Water Requirements

The conductivity of the water used in the gel and buffer should be 18 MOhm-cm or greater.

Preparing KB^{Plus} Buffer

Gel and running buffer solutions are prepared from a standard 10x TBE buffer. For best results, KB^{Plus} buffer is recommended for use with KB^{Plus} gel matrix.

10x TBE Buffer:

1.	Pour about 600 ml of distilled water (18MΩ) into a 1 l beaker.
2.	Add about one-third of the contents of the KB^{Plus} 10X TBE pouch to the water in the 1 liter beaker.
3.	Stir the solution well using a stir bar and stir plate or a stir rod until all of the solids have gone into solution and the solution is clear.
4.	Repeat steps 2 and 3 until finished.
5.	Add enough water to bring the final volume to 1000 ml.

Store at room temperature. Note that some precipitation may occur during prolonged storage.

1X Running Buffer: (Use this buffer for electrophoresis.)

1.	Add 100 ml of 10X TBE prepared as above to 900 ml of distilled water (18MΩ).
2.	Mix well.

Preparing Ammonium Persulfate Solution

APS provides a source of free radicals needed for polymerization of the gel (Sambrook, 1989). A 10% APS solution is made by adding 0.1 g APS to 1.0 ml deionized water in a small test tube. **Prepare APS daily to ensure a fresh working solution.** "Stale" APS will adversely affect band quality and give bands a “fuzzier” appearance with less distinct edges.

Preparing Gel Solutions From Other Manufacturers

Deionized Water Requirements

The conductivity of the water used in the gel and buffer should be 18 MOhm-cm or greater.

Preparing Buffer

Gel and running buffer solutions are prepared from a standard 10x TBE buffer.

Gels generally contain 1.0x-1.2x TBE while the running buffer is 0.8x-1.0x TBE.

Prepare 10x TBE as follows:

1.	Add the following to a 1000 ml beaker: **Component** / **Amount** / **Molarity** Tris Base — 107.8 g — 0.89 M Boric Acid — 55.0 g — 0.89 M EDTA (disodium salt) — 7.4 g — 0.02 M Distilled water — 950 ml TOTAL VOLUME — 1000 ml
2.	Stir to dissolve. Bring to a final volume of 1000 ml.
3.	Check the freshly prepared 10x TBE to make sure that it has a pH of 8.3 at 25 °C.
4.	Store at room temperature. Note that some precipitation may occur during prolonged storage.

Preparing Ammonium Persulfate Solution

APS provides a source of free radicals needed for polymerization of the gel (Sambrook, 1989). A 10% APS solution is made by adding 0.1 g APS to 1.0 ml deionized water in a small test tube. **Prepare APS daily to ensure a fresh working solution.** "Stale" APS will adversely affect band quality and give bands a “fuzzier” appearance with less distinct edges.

Acrylamide

The tables below give examples for mixing gel solutions:

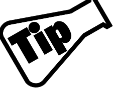
Long Ranger™ Gel Components

	41 cm		18 or 25 cm
	0.25 mm, 6%	0.20 mm, 5.5%	0.25 mm 6.5%
Urea (7M)	12.6 g	12.6 g	8.4 g
50% Long Ranger™ acrylamide	3.6ml	3.3 ml	2.6 ml
10X TBE buffer	3.0 ml	3.0 ml	2.0 ml
dd water	to 30.0 ml	to 30.0 ml	to 20 ml

Page Plus™ Gel Components

	41 cm		18 or 25 cm
	0.25 mm, 6%	0.20 mm, 5.5%	0.25 mm 6.5%
Urea (7M)	12.6 g	12.6 g	8.4 g
40% Page Plus™ acryla-mide	4.5 ml	4.125 ml	3.25 ml
10X TBE buffer	3.0 ml	3.0 ml	2.0 ml
dd water	to 30.0 ml	to 30.0 ml	to 20 ml

A number of other commercial acrylamides can be used to cast gels for the LI-COR system including RapidGel-XL (Affymetrix) and Sequagel (National Diagnostics).



To mix the gel solution in one beaker: Place a 100 ml beaker with a stir bar on a balance and tare the balance. Measure 12.6 grams of urea into the beaker, then add the acrylamide solution and 10x TBE buffer. Add dd water to target volume of 30 ml gel solution).

Mixing the Gel Solution

1.	Add 150 µl of 10% APS per 20 ml of gel solution and swirl gently.
2.	Just before pouring, add 15 µl TEMED. You have approximately 3-5 minutes to pour the gel before it polymerizes.

Pouring a 6.5% KB^{Plus} Gel

The following items are required to pour the gel:

- 20 ml of 6.5% KB^{Plus} Gel Matrix for Genotyping
- KB^{Plus} 1X TBE buffer (recommended for use with KB^{Plus} gel matrix)
- 150 µl of 10% Ammonium persulfate (APS)
- Comb
- 60 cc syringe with 14 gauge tip
- Pasteur pipette
- Assembled gel sandwich and casting stand (optional)
- 15 µl of TEMED

1.	Bring 20 ml of KB^{Plus} to room temperature (10-15 minutes) and prepare glass plates for gel injection while KB^{Plus} warms.
2.	Add 150 µl of 10% APS and 15 µl TEMED when ready to inject gel solution.
3.	Mix to homogenate and draw the gel solution into a 60 cc syringe with 14 gauge tip.

<table>
<tr><td>4.</td><td>For 25 cm gels, a notch on the back of each rail allows the apparatus to rest on the uppermost metal posts on the casting stand (Figure 2-6). This slight incline improves the flow of the gel between the plates.

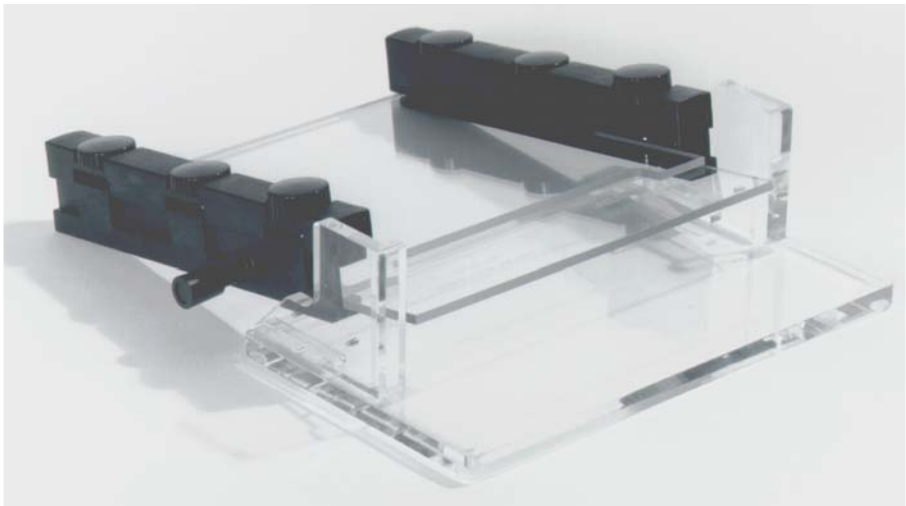


Figure 2-6. Rest the apparatus on the casting stand (25 cm plates).

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. Periodically 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.</td></tr>
<tr><td>5.</td><td>Remove any bubbles that form during gel pouring using a bubble hook (Figure 2-7).

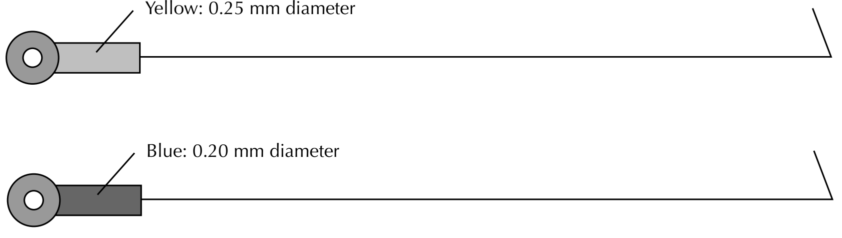
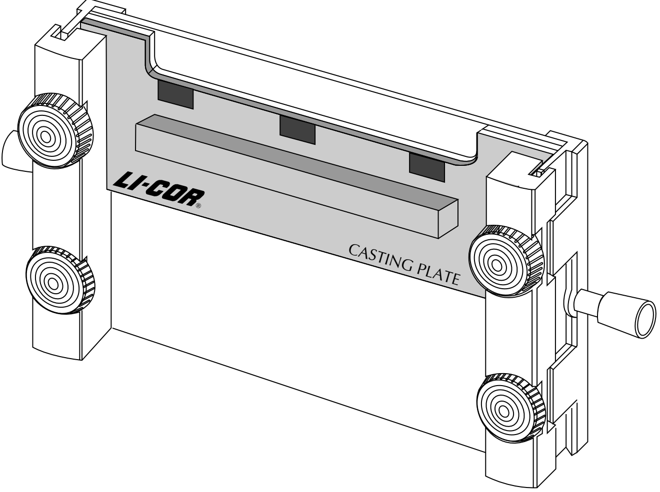


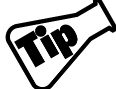
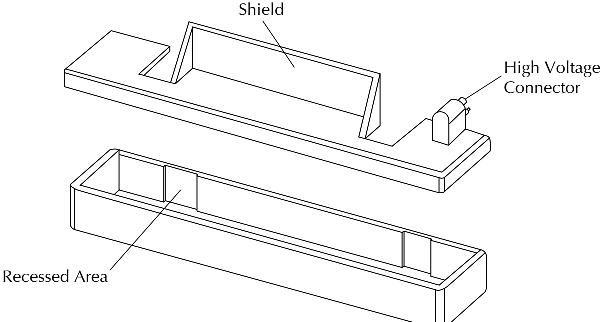
Figure 2-7. Bubble Hooks.</td></tr>
</table>

6.	Insert the comb. Figures 2-8 and 2-9 show how to insert the mylar sharkstooth and rectangular tooth combs after pouring the gel. 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 2-8**. 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 2-9**. 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.
7.	Place the casting plate into the grooved area in the rails normally occupied by the upper buffer tank. Tighten the two tank clamp knobs until finger tight. Alternatively, the upper buffer tank (with gasket) can be used in place of the casting plate. If you insert the upper buffer tank, be careful not to spill gel solution into the tank during gel pouring.  ***Figure 2-10.** Insert the casting plate and tighten the knobs.*

8.	Allow the gel to polymerize for at least 1 hour before use. Check the tightness of the clamp knobs after polymerization to make sure they are still tight.

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 TBE buffer 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 TBE buffer 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.	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.)  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.
7.	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).
8.	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.
9.	Tighten the upper clamp knobs "finger tight". The electrophoresis apparatus is now fully assembled.
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 2-11**. 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 they are free of smears, dust, or spots that may interfere with detection.

Filling the Buffer Tanks

13.	For KB^{Plus} gels, use 1X TBE running buffer (see Gel Preparation). For other gel formulations use 10x TBE buffer stock solution to make 1000 ml of TBE running buffer. For example: To make 0.8x running buffer for 0.2mm gels, measure 80 ml of 10x TBE buffer into a one liter graduated cylinder and add dd water to 1000 ml. Seal the top of the cylinder with Parafilm and invert the cylinder several times (carefully!) to mix completely before filling the tanks.
14.	Fill the upper buffer tank to the Max Fill line. Do not fill past the Max Fill line. Pour the remainder of the buffer into the lower buffer tank. (Pour to the left of the left rail.) The level of buffer solution must be high enough to immerse the platinum electrodes (attached to the tank lids), so power can be applied to the gel.
15.	Fill a 20cc syringe with buffer from the upper tank, add a 22 gauge needle and flush the wells with buffer to remove crystallized urea and air bubbles. Be careful not to dislodge the teeth when flushing wells around the sharkstooth comb.
16.	Place the upper and lower buffer tank lids onto the tanks. Insert the power cable on the upper buffer tank and connect it to the high voltage connector on the instrument chassis, as shown in Figure 5-14. Make sure that both connectors are fully inserted.

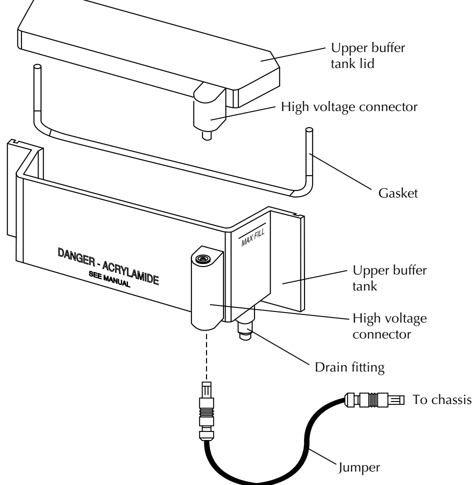
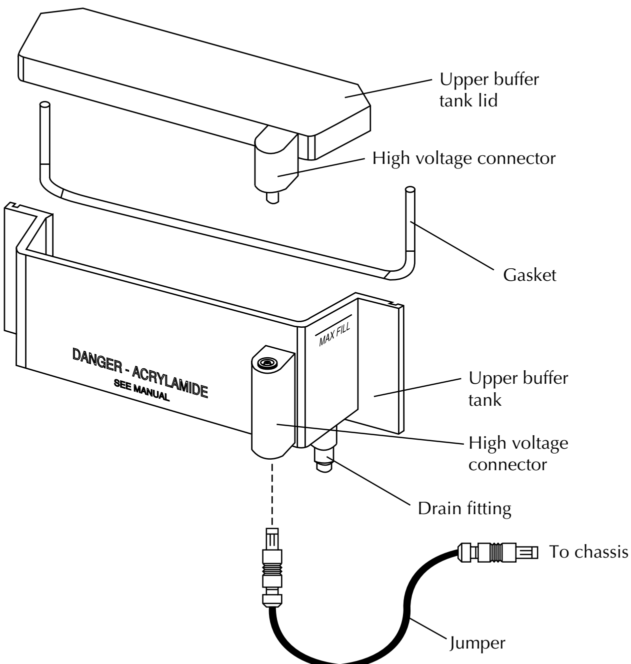


Figure 5-14. *Upper buffer tank connections.*

1.	Pour approximately 500 ml of KB^{Plus} 1X TBE running buffer (see Gel Preparation above) into both the upper and lower tanks. *Do not* fill past the Max Fill line on the upper tank. Pour the buffer to the left of the left rail in the lower tank. The level of buffer solution must be high enough to immerse the platinum electrodes (attached to the tank lids), so power can be applied to the gel.
2.	Flush the wells with a 20cc syringe to remove any urea or other particulate matter. Be careful not to dislodge the teeth when flushing wells around the sharkstooth comb.
3.	Place the upper and lower buffer tank lids onto the tanks. Insert the jumper on the upper buffer tank and connect it to the high voltage connector on the instrument chassis, as shown below. Make sure that both connectors are fully inserted.  ***Figure 2-11***. *Upper buffer tank connections.*

Starting Runs

Pre-electrophoresis and electrophoresis can be started using, Saga^{GT} Microsatellite Analysis Software, Saga Lite Electrophoresis Software, or through the browser interface of the DNA analyzer. Information on starting runs can be found in the following places:

- Saga^{GT}: Saga^{GT} Tutorial Manual and Saga^{GT} User Guide
- Saga Lite: Saga Lite help system
- Browser Interface: Model 4300 Operator's Manual.

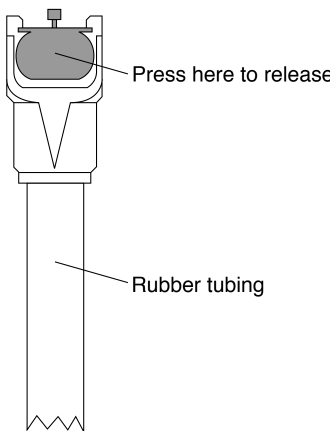
These manuals also discuss the procedure for reloading gels. Starting runs on reloaded gels is different because the pre-run is not necessary.

Sample Loading

Materials required: 20 cc syringe with 25 gauge tip.

1.	Denature samples at 94 °C for 3 minutes. After 3 minutes, immediately put the samples on ice and cover to reduce exposure to light.
2.	Open the instrument door and remove the upper buffer tank lid. Remove particulate matter by flushing the wells with running buffer using a 20 cc syringe.
3.	For a 0.25 mm gel, load with the 8-channel 0.2 mm Hamilton syringe or a pipette with flat 0.2 mm micropipette tips.
4.	Carefully position the tip(s) between the glass plates and slowly release the mixture into the wells. Load up to 1.0 µl per well depending on comb size and gel thickness. **Loading Tips:** • Do not load wells that are deformed or contain air bubbles that cannot be removed. • Do not overload the wells – samples could flow into adjoining wells. • Avoid injecting an air bubble into the well after the sample is loaded, as this can force the load into adjoining wells. • When loading rectangular tooth combs with a single pipette, flush four or five wells, load them, and repeat. If you wait too long after flushing the wells, urea will leach into the wells and make them even more transparent. • One of the accessories for the Model 4300 is a well visualization aid that can help you see rectangular wells. This aid has a mylar sheet that slides in behind the rear plate and can make the wells more visible. **Don't forget to remove the visualization aid before electrophoresis.** • One way to visualize rectangular wells is to look for the reflection of the wells in the upper surface of the notched front plate. Load by putting the tip in so it touches the back plate, then down slightly.
5.	After sample loading, replace the upper buffer tank lid (Figure 2-11), close the instrument door, and start the run using Saga^{GT} or the browser software.

Disassembly

1.	Remove the buffer tank lids.
2.	**For 18 cm gels**, remove the entire gel apparatus from the instrument and carefully dispose of the buffer solution.
3.	**For 25 cm or larger gels**, the upper buffer tank has a fitting for draining the buffer solution, while the apparatus is still secured to the instrument. A coupling attached to a length of gum rubber tubing is provided to facilitate draining the tank (Figure 2-12). *Make sure the tubing is placed in a proper receptacle, as buffer will begin to drain immediately upon connection of the coupling.* Insert the coupling until it snaps into place.  ***Figure 2-12**. Buffer tank drain fitting.* Depress the metal ring on the coupling and pull straight down to remove the drain hose when finished.

Cleanup

1.	After removing the upper and lower buffer tank lids and disconnecting the power cable, take the gel assembly off the sequencer and remove the upper buffer tank and rails.
2.	Remove the lower buffer tank and dispose of the buffer solution.
3.	Rinse the rails, spacers, and comb, and allow to air dry.
4.	Rinse and air dry the upper and lower buffer tanks and lids.
5.	Clean any spills on the heater plate, chassis, or front panel.

Cleaning the Plates

Materials needed:

- 2% laboratory detergent solution made from a concentrate such as Micro-90® (International Products Corp., Burlington NJ), or Liqui-Nox® (Alconox® Inc., New York, NY).
- Spray bottle capable of spraying a fine mist, containing 70% ethanol.

1.	A small black plastic wedge is included in the spare parts kit to aid in separating the gel plates. Insert the wedge between the plates to pry them apart for cleaning. **Caution:** Do not pry on the left and right sides of the front plate where the glass is narrow. *Never use metal tools to pry plates apart.* Lab spatulas, razor blades, or similar tools can chip plates.
2.	Dispose of the acrylamide in compliance with local regulations. Try to clean gels within 1-2 hours after the run is complete. If the gel has recently cooled to room temperature, acrylamide will adhere to paper towels, which can be used to lift the acrylamide off the plates. If gels have been left for more than 4 hours, use a razor blade to scrape the acrylamide from the plates.
3.	Pour a small amount (6 cm circle) of 2% detergent onto the side of the plate that will contact the gel.
4.	Work the solution into a lather with a nylon bristle brush and thoroughly scrub the entire plate. Remove any dried-on polyacrylamide with a razor blade, if necessary. Rinse well with water.

5.	Pour distilled water onto the plate. Work the water around the plate with a gloved hand and watch for bubble formation from detergent residue. Repeat until no bubbles form.
6.	Repeat steps 3-5 above with the second plate.
7.	Rinse the plate with deionized distilled water. Use a pump spray bottle to spray a fine mist of 70% ethanol/ddH_2O for a final rinse. Stand plates in a rack to air dry.

Section 3 PCR Protocols

dNTP Recommendation

We recommend using a dNTP mix containing 7-deaza-dGTP to minimize anomalies in migration that may cause difficulties in band sizing. Stock dNTP concentration is recommended to be 2 mM, whereas the concentration used in each reaction is 2 nmol for up to 6 loci per reaction.

PCR Optimization

As with any PCR, IRDye-labeled primer pairs may require optimization. Conditions employed in radioactive studies may need to be altered to be used for fluorescently-labeled primers.

Primer Design Considerations:

1. Forward and Reverse primers should not be complimentary, especially at the 3' ends. The last six bases on the 3' end should be approximately 50% G+C, with a G or C at the end if possible (no T's).
2. Primers should not be less than 17 bases long.
3. Primers should have a T_m greater than 60 °C. A general approximation for T_m is T_m (°C) = 2(#A + #T) + 4(#G + #C)
4. Primers should not have more than 4 consecutive bases of any one base.
5. No palindrome at the 3' end.
6. No stable hairpin loops at the 3' end.

Multiplexing Loci

If you need to design a screening set for an application, all loci should be separated into groups according to allele size ranges, annealing temperature, $MgCl_2$ concentration requirements, and possible primer/dimer combinations. Markers that can be clearly separated on gels are placed in groups of three to six loci. The remaining loci can be run in groups of two or individually if needed. Generally three markers can be multiplexed conveniently by either combining

PCR reactions prior to loading the gel (pooling) or by including compatible primer pairs in the same PCR reaction (single tube).

When selecting compatible primer pairs, the size range of the PCR products (smallest and largest alleles) must be such that loci are adequately spaced without overlap. Choose each locus range generously and allow 10-12 bp between adjacent locus ranges.

Pooling

When reactions are pooled, each primer pair can be run under optimum PCR conditions without making compromises in PCR that would decrease yields. Separate PCR reactions produce fewer nonspecific amplification products in comparison with multiplexing in single tube reactions. Also, with separate reactions, the reaction volumes can be adjusted to avoid band saturation for strong reactions, or to increase signal intensity for weak reactions. Saturated bands decreases accuracy when bands are sized. The disadvantages of pooling separate reactions are the additional reagent costs, labor and pipetting time.

Single Tube Reactions

Multiplexing will require some optimization, and the final conditions may not be optimal for all primer pairs. For example, the Marshfield Clinic web site (http://www.marshfieldclinic.org/research/genetics/) provides information on multiplexing primer pairs for various screening sets. Multiplexed reactions reduce pipetting steps and save on reagent costs, but may require more primer to yield the same band intensity. Since the amount of primer provided is usually more than adequate to complete even a large study, these advantages may justify the effort required to define conditions for multiple primer sets in single tube reactions.

Microsatellite Optimization

There are four considerations when optimizing thermocycling and reaction conditions for amplifying microsatellites: 1) primer amount; 2) $MgCl_2$ concentration; 3) T_m; and 4) DNA concentration. All four considerations should be simultaneously addressed for optimum results.

Primer Amount

Microsatellite primers have an inherent "quality" due to their base composition, primer length (ideal length is 20-24 bases), GC clamp (the last 6 bases should be 50% GC*),* salt requirements, T_m, primer-to-primer interactions in monoplex and multiplex (multiple loci amplified in the same tube) reactions, primer-dimers, etc. This "quality" results in the need to use varying amounts of primer to amplify a particular locus and obtain an acceptable signal intensity. In multiplex reactions, one primer set may need only 55 fmol of each primer, whereas

another may need 1.1 pmol to achieve the same band intensity. Even in monoplex reactions, a few primers may need 2-10 pmol of primer to achieve optimal results. These primers have relatively poor quality and should be redesigned if possible.

To begin optimization, start the initial amplification with 0.1 to 0.5 pmol of primer for either monoplex or multiplex conditions. If you want to multiplex a group of primers, the initial optimization should be performed as a multiplex reaction – the conditions used in a monoplex reaction may not be maintained in a multiplex reaction due to primer-primer interactions, primer-template interactions, competition for dNTP's, M13 primers, etc.

If M13 primers are used for the "M13 tailed primer protocol", start with 0.3 pmol of primer (work up to 1.0 pmol). See *Tailed Primers* protocol on page 3-9 in this section.

If bands on the electrophoresis image look fuzzy or smeared, too much primer has been used. Dilute the reaction with stop buffer before loading or load a smaller amount. If this does help, decrease the primer concentration by 50% and reamplify. Primer concentration should also be reduced when excess non-specific products are present, along with allele bands that are intense, but fuzzy or smeared.

If there is no amplification, or very weak signal, check for a strong band at the primer front, which indicates non-incorporated primer. In this case, make sure that <u>all</u> reagents are added to the reaction. If this is true, make sure the primer sequence is correct. Also check the T_m and make sure the cycling program is in the range of the T_m (a good starting point is 3 degrees below the theoretical T_m (or T_a)). If there are weak bands and weak or no primer front, double the primer concentration and continue until the signal is seen. Increase the concentration up to 10 pmol if needed.

$MgCl_2$ Concentration (ranges from 1.0 to 4.0 mM)

Most loci can be amplified using 1.5 to 2.0 mM of $MgCl_2$. If you have poor results after running different primer concentrations with 1.5 to 2.0 mM of $MgCl_2$, set up reactions using 1.0 mM, 1.5 mM, 2.0 mM, 2.5 mM, 3.0 mM, 3.5 mM and 4.0 mM. Using $MgCl_2$ tolerant polymerases, such as Tfl, may reduce the need to optimize $MgCl_2$.

T_m (ranges from 48 °C to 60 °C)

Monoplex Reactions: Most loci can be amplified using standard thermocycling parameters with the annealing temperature set in the range T_m to T_m - 3 °C (i.e., the annealing temperature (T_a)). For some primers, it may be necessary to go down to T_m - 5 °C, though the result may increase the amount of non-specific amplification products.

Multiplex Reactions: For multiplexing, the "touchdown" protocol (54 °C High Temperature Enhanced) allows you to mix primer sets that vary widely in T_m. A "touchdown" protocol starts with a series of decreasing annealing temperatures for several cycles (usually decreasing1-2 °C per cycle for 5-10 cycles). These initial cycles allow primers with higher annealing temperatures to start amplifying before primers with lower melting temperatures. The final cycles (25-35) are performed near the lowest T_m. With this protocol, higher T_m primers are given an early start, thereby producing a series of highly specific products. These products "overwhelm" any non-specific products that may be produced at the lower annealing temperatures. The primers in the multiplex with lower annealing temperatures amplify as they would in the standard thermocycling protocol.

When the results are not satisfactory under standard conditions, test each primer separately in reactions varying the following conditions:

1. [Mg^{+2}]: Test 1.5 mM, 2 mM, 2.5 mM, 3.0 mM, 3.5 mM and 4.0 mM.
2. Annealing temperature (determined from T_m of both primers). Refer to the Appendices for calculation of T_m.
3. Standard PCR methods vs "touchdown" methods.

Template DNA Amount

20-50 ng of DNA is recommended for genotyping, although as little as 5 ng of DNA can be used with primers of average to high quality. For primers of normal to lower quality, up to 50 ng of DNA may be necessary. For multiplexing, it is normally not necessary to use more than 50 ng of DNA total in the same reaction. As with any amplification of genomic DNA, purity should be 1.7 to 2.0 (spectrophotometrically determined 260nm/280nm ratios).

Lab Organization

Any lab performing routine PCR reactions should consider the potential for contamination. Laboratories performing PCR on a regular basis generally establish three separate stations: template isolation, a pre-PCR area and a post-PCR area. We strongly recommend this system. It is preferable that the stations be in separate labs, though this may not always be possible due to space limitations. If stations are in the same lab, separate the pre-PCR and post-PCR areas as much as possible. In the protocol below, the PCR reaction is (if possible) prepared in an isolated pre-PCR hood with UV lighting. The use of UV lighting will eliminate any cross contamination from previous samples. If possible, the thermocycler(s) and the DNA analyzer should be in a second location to reduce the chance of post-PCR contamination in subsequent samples.

Preparing Genomic DNA

DNA Quality

Most DNA preps will work, but as with all PCR, the DNA should have a 260/280 ratio of 1.7 or greater. If the DNA was isolated from blood, the heme group can inhibit the DNA polymerase if not removed.

DNA and Primer Amounts

The amplification reactions can be approached in two ways, depending on the availability of DNA and the laboratory budget for primers. If primer cost conservation is critical, reactions can be run with higher amounts of DNA (20-50 ng) and lower primer amounts (>0.5 pmole). The protocols in this manual were developed on this basis. Alternatively, if DNA is limiting, the PCR can be run with significantly less DNA (2-5 ng) at the expense of higher primer amounts (0.5 - 4.0 pmoles), depending on the quality of the primer reactions. In either case, the best data will require optimization for each new primer pair. Note that the quantity of DNA must be uniform for proper optimization.

Size Standards

Size standards are composed of IRDye-labeled DNA fragments with equal banding intensities in 90% formamide solution with bromophenol blue. The fragment sizes were selected to allow rapid identification of bands and accurate fragment sizing over the entire range. The fragments cover the size ranges of 50 to 350 bp and 50 to 700.

Description	# Lanes	Dye	Cat. Number
50-1500 bp DNA Sizing Standard 700	100-125	IRDye 700	829-05350
50-1500 bp DNA Sizing Standard 800	100-125	IRDye 800	829-05351
50-1500 bp Two-Color DNA Sizing Standard	100-125	IRDye 700 & 800	829-05352
700 bp Size Standards	125	IRDye 700	4200-60
700 bp Size Standards	125	IRDye 800	4000-45
700 bp Size Standards	2500	IRDye 700	829-05345
700 bp Size Standards	2500	IRDye 800	829-05346
350 bp Size Standards	125	IRDye 700	4200-44
350 bp Size Standards	125	IRDye 800	4000-44B
350 bp Size Standards	2500	IRDye 700	829-05343
350 bp Size Standards	2500	IRDye 800	829-05344

The following concentrated size standards are also available.

Description	Volume	Dye	Cat. Number
350 bp Size Standards	500 µl	IRDye 700	829-06157
350 bp Size Standards	500 µl	IRDye 800	829-06158

The fragment sizes are listed below:

Figure 3-1. *Fragment sizes for 50-350 and 50-700 size standard sets.*

Figure 3-2. *Fragment sizes for the concentrated 50-350 size standard set.*

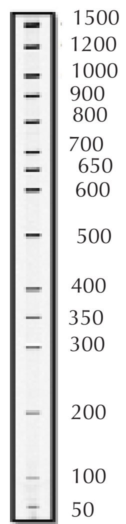


Figure 3-3. *Fragment sizes for 50-1500 size standard sets.*

Precautions

The IRDye-labeled DNA should be stored in the dark at -20 °C. For best performance, exposure to light should be minimized.

Cycling Programs

1. Standard

Step	Temperature (°C)	Time
1.	95	5 minutes
2.	95	20 seconds
3.	55*	20 seconds
4.	72	30 seconds
5.	72	3 minutes
6.	4	hold
7.	end	

* Note: Annealing temperatures will alter depending on the T_m of the primers used.

2. 54 °C "Touchdown" PCR

Step	Temperature (°C)	Time	
1.	95	5 minutes	
2.	95	45 seconds	5 cycles total
3.	68 °C minus 2°C/cycle*	5 minutes	
4.	72	1 minute	
5.	95	45 seconds	
6.	58	2 minutes	
7.	72	1 minute	
8.	95	45 seconds	
9.	56	2 minutes	
10.	72	45 seconds	
11.	95	45 seconds	10 cycles total
12.	54	2 minutes	
13.	72	1 minute	
14.	95	45 seconds	23 cycles total
15.	54 **	2 minutes	
16.	72	1 minute	
17.	72	10 minutes	
18.	4	hold	
19.	end		

* Annealing temperatures are 68, 66, 64, 62 and 60, respectively, for the five cycles.
** Or, lowest annealing temperature of primers used.

3. 47 °C "Touchdown" PCR

Step	Temperature (°C)	Time	
1.	95	5 minutes	
2.	95	45 seconds	5 cycles total
3.	68 °C minus 2°C/cycle*	5 minutes	
4.	72	1 minute	
5.	95	45 seconds	11 cycles total
6.	54	2 minutes	
7.	72	1 minute	
8.	95	45 seconds	24 cycles total
9.	47	2 minutes	
10.	72	1 minute	
11.	72	10 minutes	
12.	4	hold	
13.	end		

* Annealing temperatures are 68, 66, 64, 62 and 60, respectively, for the five cycles.

Tailed Primers

In this approach, one of the unlabeled STR primers is synthesized with an M13 forward or reverse primer sequence on the 5'-end. An IRDye-labeled M13 Primer is included in the PCR reaction. The M13 primer is added to the PCR product during the first few cycles of amplification. The labeled M13 primer is incorporated in subsequent cycles, thus labeling the PCR product. This method requires no purification prior to gel analysis.

Important: When using tailed primers to amplify a locus that will be analyzed with Saga^{GT}, the modified minimum and maximum locus boundaries in Saga's Locus Manager should be increased by the length of the primer incorporated in the PCR product.

The M13 primer sequence can be used on both 800 and 700 channels. LI-COR M13 forward and reverse primer sequences are as follows:

4000-20B Forward (-29)/IRDye 800-labeled Primer, 19-mer: 5' - CACGACGTTGTAAAACGAC - 3'

4000-21B Reverse/IRDye 800-labeled Primer, 20-mer: 5' - GGATAACAATTTCACACAGG - 3'

Protocol

1.	Enter the *Standard* program on the thermocycler (given earlier in this Section).
2.	Add 20-50 ng of genomic DNA to a microcentrifuge tube or a microplate well.
3.	Determine the quantity of each component required using the following table, based on the total number of reactions. Substitute water for the other primers if only a single locus is being run. If $MgCl_2$ is not premixed in the PCR buffer, account for $MgCl_2$ as necessary.

Component	Stock Concentration	Qty Per Reaction
10X Buffer		1.0
dH_2O		3.3
dNTPs	2.0 mM	1.0
Primer 1fwd	1.0 pmol/µl	0.5
Primer 1rev	1.0 pmol/µl	0.5
Primer 2fwd	1.0 pmol/µl	0.5
Primer 2rev	1.0 pmol/µl	0.5
Primer 3fwd	1.0 pmol/µl	0.5
Primer 3rev	1.0 pmol/µl	0.5
M13f-29 primer	1.0 pmol/µl	1.0
Taq	5.0 unit	0.2
Total Volume		**9.0 µl**

4.	Pipette gently to mix.

5.	Add 9 µl of the STR mixture from above to each tube or well and pipette gently to mix.
6.	If the thermocycler does not have a heated lid, add one drop of mineral oil to each well.
7.	Place the tubes or plate in the thermocycler. Start the cycling program.
8.	After completion of the program, add 2 µl of stop buffer to each tube or well and mix gently.
9.	Heat samples at 95°C for 3 minutes and snap cool on ice before loading.
10.	Load gel (volume depends on comb and gel thickness.

Labeled Primers

One of the STR primer pairs can be synthesized with a 5'-IRDye label. In this manner one strand of the resulting PCR product is labeled during amplification. The method is very robust and no purification is required prior to gel analysis. Custom primers are available from LI-COR.

Protocol

<table>
<tr><td>1.</td><td>Enter the Standard program, or a custom program on the thermocycler (given earlier in this Section).</td></tr>
<tr><td>2.</td><td>Add 20-50 ng of genomic DNA to a microcentrifuge tube or a microplate well.</td></tr>
<tr><td>3.</td><td>Determine the quantity of each component required using the following table, based on the total number of reactions. Substitute water for the other primers if only a single locus is being run. If $MgCl_2$ is not premixed in the PCR buffer, account for $MgCl_2$ as necessary.

<table>
<tr><th>Component</th><th>Stock Concentration</th><th>Qty Per Reaction</th></tr>
<tr><td>10X Buffer</td><td></td><td>1.0</td></tr>
<tr><td>dH_2O</td><td></td><td>3.8</td></tr>
<tr><td>dNTPs</td><td>2.0 mM</td><td>1.0</td></tr>
<tr><td>Primer 1 labeled</td><td>1.0 pmol/µl</td><td>0.5</td></tr>
<tr><td>Primer 1unlabeled</td><td>1.0 pmol/µl</td><td>0.5</td></tr>
<tr><td>Primer 2labeled</td><td>1.0 pmol/µl</td><td>0.5</td></tr>
<tr><td>Primer 2unlabeled</td><td>1.0 pmol/µl</td><td>0.5</td></tr>
<tr><td>Primer 3labeled</td><td>1.0 pmol/µl</td><td>0.5</td></tr>
<tr><td>Primer 3unlabeled</td><td>1.0 pmol/µl</td><td>0.5</td></tr>
<tr><td>Taq</td><td>5.0 unit</td><td>0.2</td></tr>
<tr><td>Total Volume</td><td></td><td>9.0 µl</td></tr>
</table>
</td></tr>
<tr><td>4.</td><td>Pipette gently to mix.</td></tr>
<tr><td>5.</td><td>Add 9 µl of the STR mixture from above to each tube or well and pipette gently to mix.</td></tr>
<tr><td>6.</td><td>If the thermocycler does not have a heated lid, add one drop of mineral oil to each well.</td></tr>
<tr><td>7.</td><td>Place the tubes or plate in the thermocycler. Start the cycling program.</td></tr>
<tr><td>8.</td><td>After completion of the program, add 2 µl of stop buffer to each tube or well and mix gently.</td></tr>
<tr><td>9.</td><td>Heat the samples at 95°C for 3 minutes and snap cool on ice before loading.</td></tr>
<tr><td>10.</td><td>Load gel (volume depends on comb and gel thickness.</td></tr>
</table>

Troubleshooting

Problem	Cause	Solution
Multiplexing		
Not all loci amplify.	Unamplified locus has lower quality than others.	Choose different locus. Increase primer concentration.
When using M13 tailed primers, no loci amplify or a weak signal is seen.	M13 labeled primer has been exposed to light.	Replace with fresh primer.
Reactions		
Some tubes are empty after cycling without oil.	Caps are not sealing tightly.	Seal caps only once. Change tube suppliers to match with cap supplier. Switch to the CycleSeal Film for sealing.
One locus in a multiplex reaction is very weak.	Lower quality primer, subop-timal annealing temperature.	Needs higher concentration of primer. Need to alter annealing tem-perature or change to an enhanced protocol.
A "smeared" band is seen where the loci should be visible.	High primer concentration.	Use primer with lower con-centration.
	High concentration of DNA.	Reduce DNA concentration and/or spectrophotometri-cally analyze DNA to keep concentration between 20-50 ng/reaction (no need to increase concentration in a multiplex reaction).
A particular locus appears "wavy" across the gel.	Possible overload.	Reduce loading volume and/or dilute samples.
	Dinucleotide repeat of lower MW that is not fully resolved.	Reload using 25-33 cm gel plates.
No bands appear, consistently.	Degenerated primers.	Try new primers.
	dNTP's are bad.	Try dNTP's with 7-Deaza.

Problem	Cause	Solution
Bands in the 800 channel are consistently weaker than the 700 channel.	IRDye 700 is more sensitive than IRDye 800.	With the 800 image displayed, select **Alter Intensity** from the **View** menu in e-Seq and adjust the image until the bands are easier to see.
Bands are weak.	Primer concentration too low.	Increase primer concentra-tion.
	Mg concentration too low.	Increase Mg concentration.
	DNA concentration too low.	Increase DNA concentration.

Section 4 Appendices

T_m Calculation

There are two different formulas presented below that can estimate the melting temperature (T_m) of a primer. The first is a very simple method (AT + GC)T_m that will give a rough estimate. This estimate is based on the following:

4 °C for every G or C and 2 °C for every A or T.

Example:

5'-TAC CTG GTT GAT CCT GCC AGT AG-3'

This primer is a 3-mer containing 12 G and C's, 11 A and T's. Therefore,

$(12 \times 4) + (11 \times 2) = 48 + 22 = 70$ °C.

The second method (based on thermodynamic T_m) results in a closer prediction to the actual melting temperature and is the preferred formula. This method is as follows:

69.3 °C + 0.41x - 650/Primer length,

where x is the GC percentage.

Example:

Using the same primer shown above, the GC percentage is 52.2% and primer length is 23 bases, so

69.3 °C + 0.41(52.2) - 650/23 = 69.3 + 21.4 -28.26 = 62.4 °C.

Calculation of Oligonucleotide Concentration (nmol) Given Optical Density (O.D.)

Some DNA synthesis laboratories will include the O.D. and possibly the molecular weight (g/mol) with the primer that was synthesized. Others will calculate the concentration and report it in nmol. To calculate DNA concentration in nmol, both the O.D. and the molecular weight must be known. If the molecular weight was not reported, it can be estimated by the following:

Each dATP = 313.21 g/mol
Each dCTP = 289.19 g/mol
Each dGTP = 329.21 g/mol
Each dTTP = 304.20 g/mol

Added to the calculation is 1 molecule of water (18.02 g/mol).

For example, the sequence 5'-GTA CTG ATT TAA TTC ACA TTT CCC-3' contains 6 A's, 10 T's, 6 C's, and 2 G's. Therefore,

A's	6 × 313.21 = 1,879.26 g/mol
T's	10 × 304.20 = 3,042.00 g/mol
C's	6 × 289.19 = 1,735.14 g/mol
G's	2 × 329.21 = 658.42 g/mol

Total molecular weight = 7,314.82 g/mol, plus 18.02 (one molecule of water) = 7332.84 g/mol.

Since 1.0 O.D. is equal to approximately 33.0 µg, then if 1 O.D. of the aforementioned oligo was synthesized, the calculation would be as follows:

$$\frac{33\times10^{-6}g}{1OD}\times\frac{1mol}{7333g}\times\frac{10^{9}nmol}{1mol}=\frac{3.3\times10^{4}nmol}{7333\times1OD}=\frac{4.5nmol}{1OD}$$

Therefore, the synthetic oligo yield is 4.5 nmol, or 4,500 pmol. If 1.0 ml of TE (10 mM Tris, 1 mM EDTA, pH 8.0) was added, the concentration would be 4.5 pmol/µl.

Protocol for 96-well Paper Combs

96 well paper sharkstooth combs are used for microsatellite, AFLP®, and other genotyping applications. These combs are capable of being inserted, loaded, and subsequently reloaded several times without removal.

Protocol

1.	After pouring the gel, invert the 0.25 mm casting comb to create a trough that will form the bottom of each well.
2.	After gel polymerization, remove the casting comb from the gel and clean the single trough that was created. Use a small amount of water and a razor blade to clean urea and gel debris from the trough area. Remove the water in the trough by absorbing with a Kim Wipe®.
3.	Mark the bottom of the trough with two dots from a Sharpie®. This will help to identify the bottom when inserting the comb.
4.	Add 1-2 ml of 1X TBE to the trough.
5.	Insert the comb into the trough until the teeth just touch the gel. Use the two dots from step 3 as a guide (Since the comb will swell as it absorbs the buffer, placement of the comb should be completed in less than 10 seconds).
6.	Set up your gel for pre-electrophoresis, adding the 1X TBE buffer to the upper and lower buffer tanks.
7.	Pre-run your gel for the desired amount of time.
8.	Using an 8-channel Hamilton syringe (optional), load samples. The Hamilton syringe loads every 6th well as described in Section 1.
9.	To re-load gels, stop electrophoresis, flush the wells (without removing the comb), and load again. **Note:** Gel may not be reloaded after 6 hours of its initial insertion.

AFLP® Manual

Model 4300
DNA Analyzer

LI-COR®

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Section 3. Appendix

IRDye® Fluorescent AFLP Protocol for Large Plant Genome Analysis
AFLP Expression Analysis Protocol

Section 1 AFLP® Analysis Notes

Introduction

The majority of the instructions required for AFLP® analysis can be found in the *IRDye® Fluorescent AFLP® Protocol For Large Plant Genome Analysis* and the *AFLP® Expression Analysis Protocol*. Copies of both documents are included at the end of this AFLP Manual. The most recent editions are also available at http://biosupport.licor.com.

Instructions for gel assembly, gel pouring, sample loading, or cleanup are given in Section 2 of this manual.

Hamilton 8-Channel Syringe

The Hamilton 8-channel 0.2 mm syringe (LI-COR, Part #870-05848) has syringe needles that are spaced 9 mm apart. Since the wells formed by LI-COR® 64-well combs are on 2.25 mm centers, the 8-channel syringe can be used to load every fourth well of 0.25 mm gels. For 48-well combs, every third lane can be loaded. LI-COR's ClickIR™ Assembly for the Hamilton 8-channel syringe assures consistent volume delivery through tactile feedback. The ClickIR assembly attaches to the 8-channel syringe and allows the user to feel clicks that correspond to 0.3 µl, 0.4 µl, or 0.5 µl (selectable) as sample is dispensed.

Microplate Configurations

The arrangement of samples and standards in microplates should be predefined in order to achieve the desired order on the gel. Figure 1-1 shows a microplate configuration designed for the Hamilton 8-channel syringe that loads standards in the two outside lanes, with 64 sample lanes in between the standards. The numbers in the microplate wells indicates the lane number that will be loaded.

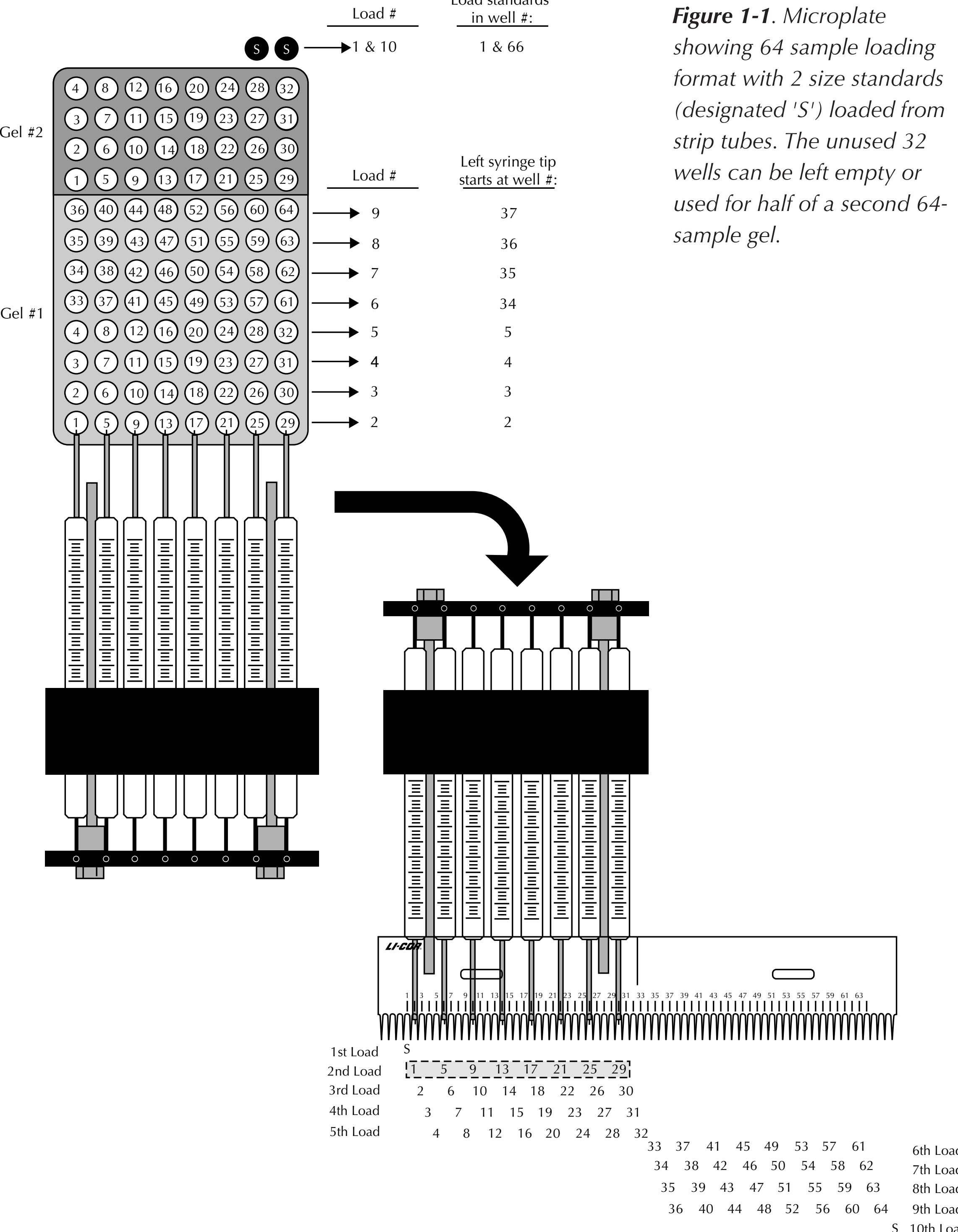


***Figure 1-1**. Microplate showing 64 sample loading format with 2 size standards (designated 'S') loaded from strip tubes. The unused 32 wells can be left empty or used for half of a second 64-sample gel.*

Size Standards

Size standards are composed of DNA fragments labeled with IRDye® infrared dye with equal banding intensities in 90% formamide solution with bromophenol blue. The fragment sizes were selected to allow rapid identification of bands and accurate fragment sizing over the entire range. The fragments cover the size ranges of 50 to 350 bp and 50 to 700.

Description	# Lanes	Dye	Part Number
50-1500 bp DNA Sizing Standard 700	100-125	IRDye 700	829-05350
50-1500 bp DNA Sizing Standard 800	100-125	IRDye 800	829-05351
50-1500 bp Two-Color DNA Sizing Standard	100-125	IRDye 700 & 800	829-05352
700 bp Size Standards	125	IRDye 700	4200-60
700 bp Size Standards	125	IRDye 800	4000-45
700 bp Size Standards	2500	IRDye 700	829-05345
700 bp Size Standards	2500	IRDye 800	829-05346
350 bp Size Standards	125	IRDye 700	4200-44
350 bp Size Standards	125	IRDye 800	4000-44B
350 bp Size Standards	2500	IRDye 700	829-05343
350 bp Size Standards	2500	IRDye 800	829-05344

The following concentrated size standards are also available.

Description	Volume	Dye	Part Number
350 bp Size Standards	500 µl	IRDye 700	829-06157
350 bp Size Standards	500 µl	IRDye 800	829-06158

The fragment sizes are listed below:

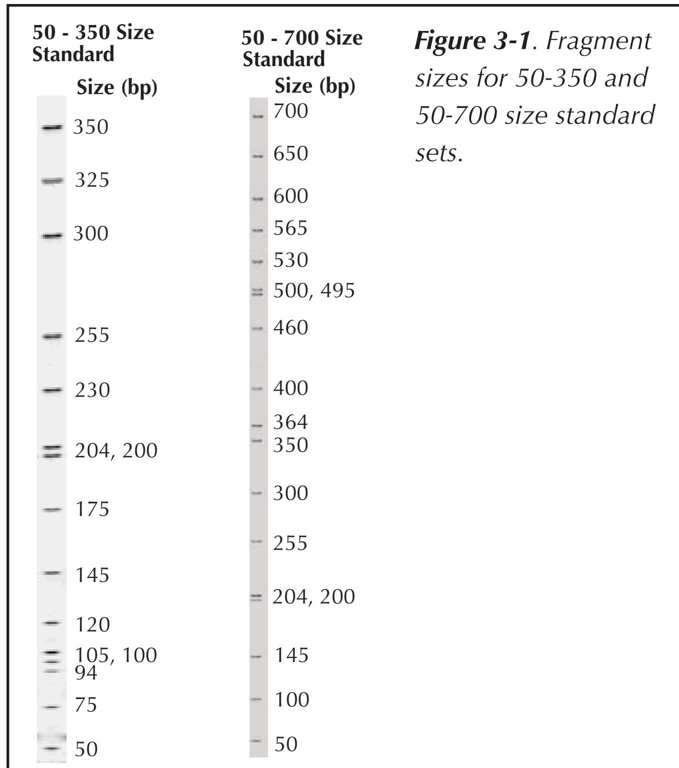


Figure 3-1. *Fragment sizes for 50-350 and 50-700 size standard sets.*

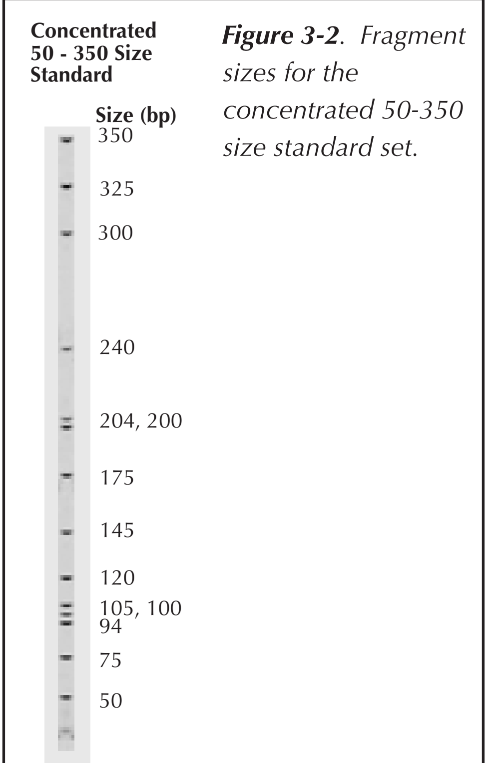


Figure 3-2. *Fragment sizes for the concentrated 50-350 size standard set.*

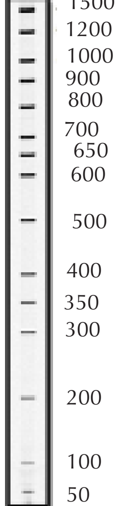


Figure 3-3. *Fragment sizes for 50-1500 size standard sets.*

Precautions

The DNA labeled with IRDye infrared dye should be stored in the dark at -20 °C. For best performance, exposure to light should be minimized. The dye will remain stable for 6 months under these conditions.

Section 2 Gel Preparation and Electrophoresis

Choosing a Comb

The 48-well rectangular tooth comb is recommended for AFLP® expression analysis because it gives added clarity between samples. 48-well combs can be loaded with a Hamilton 8-channel syringe (every third well) or a standard syringe. Notes on other combs are given below.

The 64-well square or sharkstooth comb works also well for many applications. For speed and convenience, a Hamilton 8-channel syringe can be used for gel loading. Section 1 discusses sample arrangement in 96-well plates to achieve the desired lane order when using an 8-channel syringe.

Gel Apparatus Assembly

Table 2-1. Gel Specifications.

	AFLP	AFLP Expression
Plate Length	25 cm	25 cm
Spacer Thickness	0.25 mm	0.25 mm
Gel Composition	6.5% LI-COR® KB^{Plus}	6.5 or 8%

The following items are required to assemble the electrophoresis apparatus:

- Gloves (non-powdered)
- Safety glasses
- Non-abrasive tissues (Kaydry and Kimwipes)
- 25 cm front plate (notched)
- 25 cm back plate (rectangular)
- 1 set of 0.25 mm spacers
- Comb
- 1 set of 25 cm rail assemblies
- Casting plate
- Casting stand (optional)
- Concentrated laboratory detergent solution (Micro-90®, Liqui-Nox®, etc. detergent and tap water)

- Deionized water (≈18.0 mOhm)
- 3-(trimethoxysilyl)propyl methacrylate 98% (Bind silane)
- 10% acetic acid
- Test tube or centrifuge tube (must hold 170 µl of liquid)
- Isopropanol (70 - 100%)

Caution: Always follow proper laboratory safety procedures. Always wear gloves and safety glasses when working with chemicals.

Caution: Newer Borofloat plates purchased from LI-COR are etched to show an ID number that reflects the level of fluorescence. Any two plates with the same ID number or whose numbers do not differ by more than a value of one may be used together as part of a set. Mismatched plates with ID numbers differing by more than one may result in focus errors during the focusing routine that the 4300 follows prior to every run.

Borofloat plate etched as Grade 5.

Cleaning the Plates

Materials needed:

- 2% laboratory detergent solution made from a concentrate such as Micro-90® (International Products Corp., Burlington NJ), or Liqui-Nox® (Alconox® Inc., New York, NY).
- Spray bottle capable of spraying a fine mist, containing 70% ethanol.

1.	Pour a small amount (6 cm circle) of 2% detergent onto the side of the plate that will contact the gel.
2.	Work the solution into a lather with the bristle brush included, and thoroughly scrub the entire plate. Remove any dried-on poly-acrylamide with a razor blade, if necessary. Rinse well with water. Buffer solution that has dried onto the plates can be removed with 0.1N NaOH.
3.	Pour distilled water onto the plate. Work the water around the plate with a gloved hand and watch for bubble formation from detergent residue. Repeat until no bubbles form.

4.	Repeat steps 1-3 above with the second plate.
5.	Rinse the plates with deionized distilled water. Use a pump spray bottle to spray a fine mist of 70% ethanol/ddH_2O for a final rinse. Stand plates in a rack to air dry.

Preparing Stock Bind Silane

1.	Add 50 µl of bind silane to 10 ml of 100% ethanol.
2.	Mix well and store at 4 °C in an amber colored bottle, or wrap the bottle in aluminum foil.

Silane treatments are used in autoradiography to facilitate removal of the gel from the plates for exposure to film after electrophoresis. Infrared fluorescence detection does not require repelling silane treatments because DNA bands are detected in the gel, in real time, during electrophoresis.

3-(trimethoxysilyl)propyl methacrylate (98%), however, is a binding silane used to covalently bind the gel to the glass in the area where the comb is inserted. This treatment helps maintain good well morphology when loading gels multiple times.

Applying the Bind Silane Solution

1.	Mix 25 µl of stock bind silane solution and 25 µl of 10% acetic acid in a 1.5 ml microcentrifuge tube. Mix thoroughly (pipette or vortex).

2.	Use a cotton swab to apply the solution on the inside of the short plate over the area below the edge of the notch where the wells will form (Figure 2-1). Apply solution to the rear plate using the front plate as a guide to determine where to place the bind silane onto the rear plate. Allow the solution to dry before gel assembly.

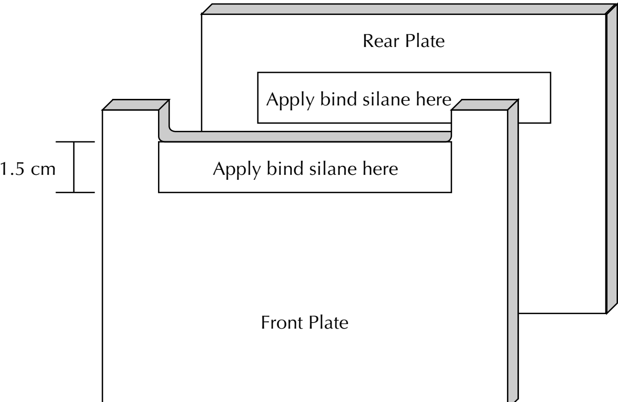
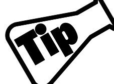


Figure 2-1. *Apply bind silane to the plates as shown.*



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.

Assembling the Gel Sandwich

1.	Lay the rear plate down (gel side up) and place the spacers along the edges, as shown in Figure 2-2.

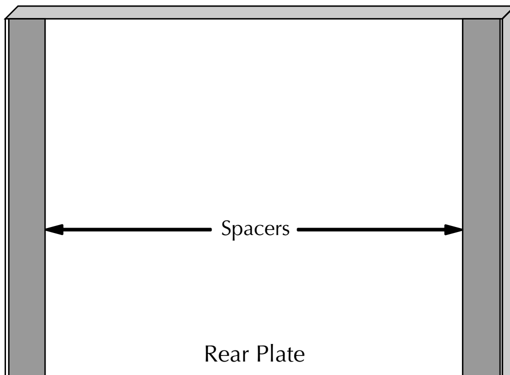
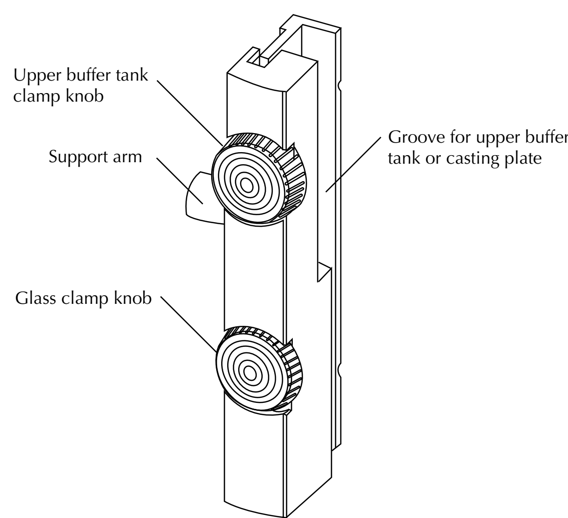
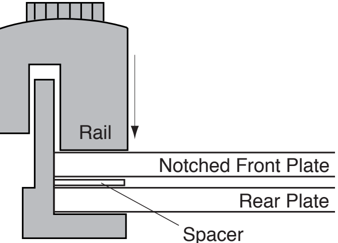
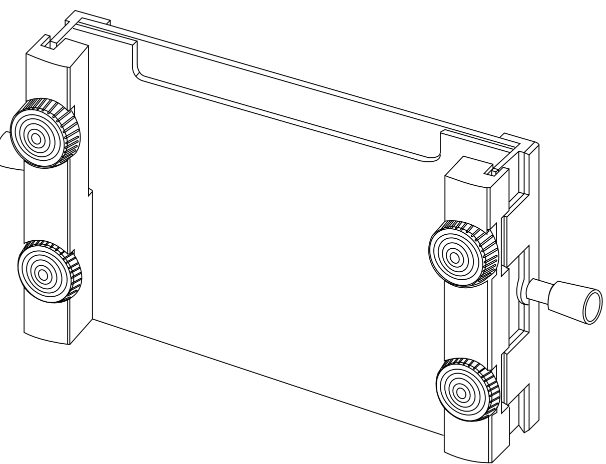
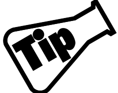


Figure 2-2. *Place a spacer along each edge of the plate.*

2.	Place the front plate on top of the rear plate (gel side down) and align the spacers with the outside edges of the plates. Make sure that the plates are aligned evenly at the bottom.

3.	Make sure the rails are completely dry from prior runs before assembly. Place the left and right rail assemblies over the plate edges. Note that the top portion of each rail is notched for insertion of the upper buffer tank or casting plate (Figure 2-3). The uppermost clamp knob on each rail is larger than the other one, as well.  ***Figure 2-3**. Note orientation of rail assemblies (left rail shown).*
4.	Check to make sure the rails fit tightly against the edges of both glass plates (Figure 2-4). The spacer must also be tight against the rail. A leak will occur if there is a gap between the rail and either plate, or between the rail and the spacer.  ***Figure 2-4.** Bottom view of assembled gel apparatus showing the proper fit of the plates and spacer in the rail.*

5.	Tighten the glass clamp knob 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. Assemble the apparatus as shown in Figure 2-5.  ***Figure 2-5***. *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.
6.	Select a comb (sharkstooth or rectangular tooth comb) with thickness that matches the spacers. Clean plastic combs with water and/or ethanol if necessary. Make sure that the comb fits between the two plates at the top of the gel, behind the notch in the front plate. If it doesn't fit or is very loose, try another comb.

Mixing 6.5% KB^{Plus} Gel Matrix

The following items are required to mix the KB^{Plus} gel matrix:

- 20 ml LI-COR® 6.5% KB^{Plus} Gel Matrix for Genotyping
- LI-COR KB^{Plus} 1X TBE buffer (recommended for use with KB^{Plus} Gel Matrix)
- 150 µl of 10% Ammonium persulfate (APS)
- 15 µl TEMED

Deionized Water Requirements

The conductivity of the water used in the gel and buffer should be 18 MOhm-cm or greater.

Preparing KB^{Plus} Buffer for 6.5% KB^{Plus} Gel Matrix

Gel and running buffer solutions are prepared from a standard 10x TBE buffer. For best results, KB^{Plus} buffer is recommended for use with KB^{Plus} gel matrix.

KB^{Plus} 10x TBE Buffer:

1.	Pour about 600 ml of distilled water (18MΩ) into a 1 l beaker.
2.	Add about one-third of the contents of the KB^{Plus} 10X TBE pouch to the water in the 1 liter beaker.
3.	Stir the solution well using a stir bar and stir plate or a stir rod until all of the solids have gone into solution and the solution is clear.
4.	Repeat steps 2 and 3 until finished.
5.	Add enough water to bring the final volume to 1000 ml.

Store at room temperature. Note that some precipitation may occur during prolonged storage.

KB^{Plus} 1X Running Buffer: (Use this buffer for electrophoresis.)

1.	Into a one liter graduated cylinder, add 100 ml of KB^{Plus} 10X TBE (prepared as above) to 900 ml of distilled water (18MΩ).
2.	Seal the top of the cylinder with Parafilm and invert the cylinder several times (carefully!) to mix completely.

Preparing Ammonium Persulfate Solution (APS)

APS provides a source of free radicals needed for polymerization of the gel (Sambrook, 1989). A 10% APS solution is made by adding 0.1 g APS to 1.0 ml deionized water in a small test tube. **Prepare APS daily to ensure a fresh working solution.** "Stale" APS will adversely affect band quality and give bands a “fuzzier” appearance with less distinct edges.

Mixing 6.5% KB^{Plus} Gel Matrix

1.	Bring 20 ml KB^{Plus} gel matrix to room temperature (10-15 minutes). Glass plates can be prepared for gel injection as describe above while KB^{Plus} warms.
2.	Add 150 µl of 10% APS and 15 µl TEMED when ready to inject gel solution and proceed immediately to gel pouring steps below.

Mixing 8% Acrylamide Gel Matrix

Preparing Standard Buffer for Use With 8% Gels

For 8% gels used in AFLP expression protocols, the buffer solution in the gel is a standard 10X TBE buffer. The running buffer is a freshly prepared 1X solution from the 10X TBE stock solution. Prepare the standard 10X TBE as follows:

1.	Add the following to a 1000 ml beaker: **Component** / **10X Molarity** / **1X Molarity** Tris 107.8 g / 0.89 M / 89 mM Boric Acid 55.0 g / 0.89 M / 89 mM EDTA 7.4 g / 0.02 M / 2 mM ddH$_2$O / 950 ml
2.	Stir to dissolve. Bring to a final volume of 1000 ml.
3.	Store at room temperature. Note that some precipitation may occur during prolonged storage.

Component	10X Molarity	1X Molarity
Tris 107.8 g	0.89 M	89 mM
Boric Acid 55.0 g	0.89 M	89 mM
EDTA 7.4 g	0.02 M	2 mM
ddH$_2$O	950 ml	

Alternatively, premixed packages of reagents are available from a number of suppliers.

1X Running Buffer: (Use this buffer for electrophoresis.)

1.	Into a one liter graduated cylinder, add 100 ml of standard 10X TBE (prepared as above) to 900 ml of distilled water (18MΩ).
2.	Seal the top of the cylinder with Parafilm and invert the cylinder several times (carefully!) to mix completely.
3.	Check the freshly prepared 1X TBE to make sure that it has a pH of 8.3-8.7 at 50 °C.

Preparing Ammonium Persulfate Solution (APS)

APS provides a source of free radicals needed for polymerization of the gel (Sambrook, 1989). A 10% APS solution is made by adding 0.1 g APS to 1.0 ml deionized water in a small test tube. **Prepare APS daily to ensure a fresh working solution.** "Stale" APS will adversely affect band quality and give bands a “fuzzier” appearance with less distinct edges.

Mixing 8% Acrylamide Gel Matrix

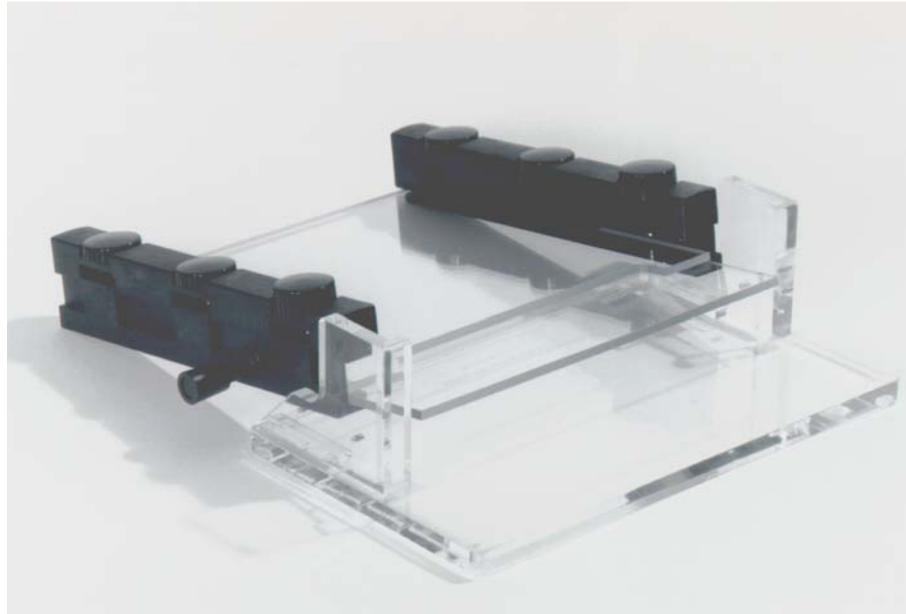
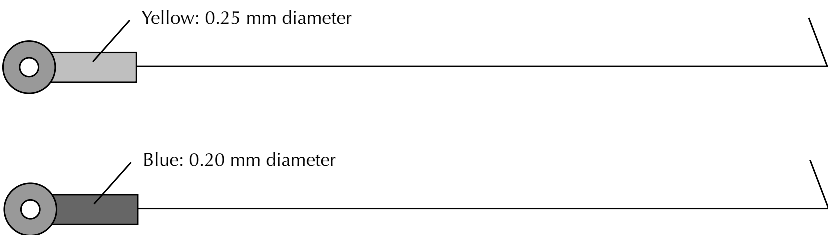
<table>
<tr><td>1.</td><td>Add the following components to a 100 ml beaker (20 ml of gel solution):
<table>
<tr><th>Component</th><th>8% Polyacrylamide</th></tr>
<tr><td>Urea</td><td>8.4 g</td></tr>
<tr><td>10X TBE buffer</td><td>2.0 ml</td></tr>
<tr><td>40% Acrylamide, or
50% Acrylamide</td><td>4.0 ml, or
3.2 ml</td></tr>
<tr><td>dH_2O</td><td>As needed to bring to 20 ml</td></tr>
</table>
</td></tr>
<tr><td>2.</td><td>Mix well and filter the solution (optional).</td></tr>
<tr><td>3.</td><td>Add 15 µl TEMED and 150 µl of 10% APS when ready to inject gel solution and proceed immediately to gel pouring steps below.</td></tr>
</table>

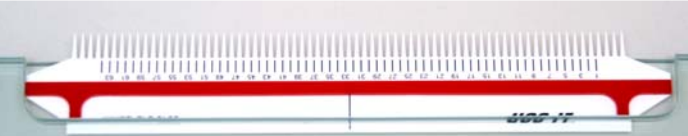
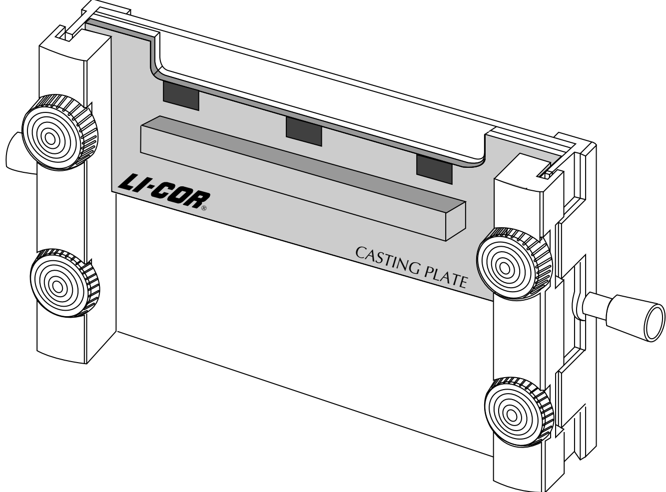
Pouring the Gel

The following items are required to inject the gel solution:

- Comb
- Assembled gel sandwich and casting stand
- 60 cc syringe with 14 gauge tip
- 20 cc syringe
- Pasteur pipette

1.	Mix gel solution to homogenate and draw into a 60 cc syringe with 14 gauge tip.

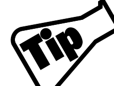
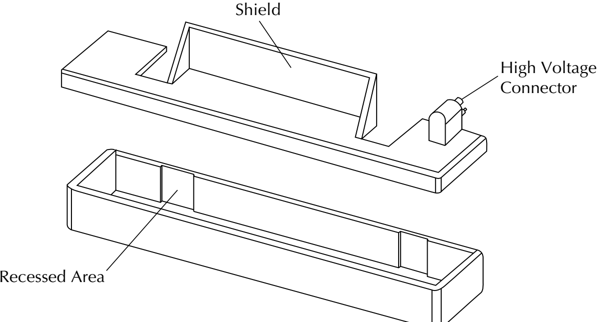
2.	There is a notch on the back of each rail that allows the apparatus to rest on the uppermost metal posts on the casting stand (Figure 2-6). This slight incline improves the flow of the gel between the plates.  ***Figure 2-6.*** *Rest the apparatus on the casting stand (25 cm or larger plates).* Start 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. Periodically 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.
3.	Remove any bubbles that form during gel pouring using a bubble hook (Figure 2-7).  ***Figure 2-7***. *Bubble Hooks.*

4.	Insert the comb. Figures 2-8 and 2-9 show how to insert the mylar sharkstooth and rectangular tooth combs after pouring the gel. 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 2-8***. *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 2-9***. *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.
5.	Place the casting plate into the grooved area in the rails normally occupied by the upper buffer tank. Tighten the two tank clamp knobs until finger tight. Alternatively, the upper buffer tank (with gasket) can be used in place of the casting plate. If you insert the upper buffer tank, be careful not to spill gel solution into the tank during gel pouring.  ***Figure 2-10.*** *Insert the casting plate and tighten the knobs.*

6.	Allow the gel to polymerize for at least 1 hour before use. Check the tightness of the clamp knobs after polymerization to make sure they are still tight.

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 TBE buffer 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 TBE buffer 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.	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.)  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.
7.	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).
8.	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.
9.	Tighten the upper clamp knobs "finger tight". The electrophoresis apparatus is now fully assembled.
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 2-11**. 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 they are free of smears, dust, or spots that may interfere with detection.

Filling the Buffer Tanks

<table>
<tr><td>13.</td><td>For KB^{Plus} gels, fill the upper buffer tank to the Max Fill line with KB^{Plus} 1X TBE running buffer (see *Mixing 6.5% KB^{Plus} Gel Matrix* above). Do not fill past the Max Fill line. Pour the remainder of the buffer into the lower buffer tank. (Pour to the left of the left rail.) The level of buffer solution must be high enough to immerse the platinum electrodes (attached to the tank lids), so power can be applied to the gel. **Note:** for 8% acrylamide gel matrix, substitute standard 1X TBE buffer prepared as described in *Mixing 8% Acrylamide Gel Matrix* above.</td></tr>
<tr><td>14.</td><td>Fill a 20cc syringe with buffer from the upper tank, add a 22 gauge needle and flush the wells with buffer to remove crystallized urea and air bubbles. Be careful not to dislodge the teeth when flushing wells around the sharkstooth comb.</td></tr>
<tr><td>15.</td><td>Place the upper and lower buffer tank lids onto the tanks. Insert the power cable on the upper buffer tank and connect it to the high voltage connector on the instrument chassis, as shown in Figure 2-12. Make sure that both connectors are fully inserted.

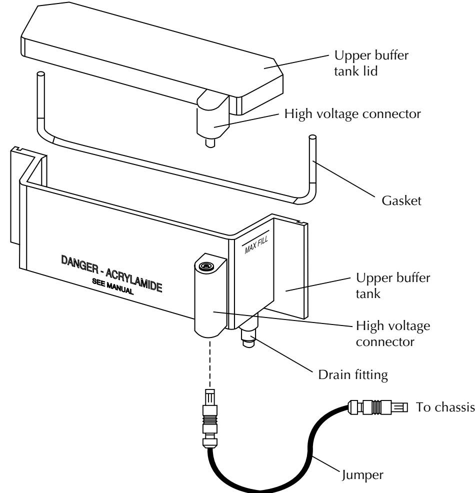


Figure 2-12. *Upper buffer tank connections.*</td></tr>
</table>

Starting Runs

Pre-electrophoresis and electrophoresis can be started using, Saga^{MX} AFLP® Analysis Software, Saga Lite Electrophoresis Software, or through the browser interface of the DNA analyzer. Information on starting runs can be found in the following places:

- Saga^{MX}: Saga^{MX} Tutorial Manual and Saga^{MX} User Guide
- Saga Lite: Saga Lite help system
- Browser Interface: Model 4300 Operator's Manual.

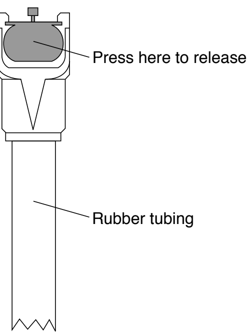
These manuals also discuss the procedure for reloading gels. Starting runs on reloaded gels is different because the pre-run is not necessary.

Sample Loading

Materials required: 20 cc syringe with 25 gauge tip.

1.	Denature at 94 °C for 3 minutes. After 3 minutes, immediately put the samples on ice and cover to reduce exposure to light.
2.	Open the door on the instrument and remove the upper buffer tank lid. Remove particulate matter by flushing the wells with running buffer using a 20 cc syringe.
3.	For a 0.25 mm gel, load with the 8-channel 0.2 mm Hamilton syringe or pipette with flat 0.2 mm micropipette tips.
4.	Carefully position the tip(s) between the glass plates and slowly release the mixture into the wells. Load up to 1.0 µl per well depending on comb size and gel thickness. **Loading Tips:** • Do not load wells that are deformed or contain air bubbles that cannot be removed. • Do not overload the wells – samples could flow into adjoining wells. • Avoid injecting an air bubble into the well after the sample is loaded, as this can force the load into adjoining wells. • When loading rectangular tooth combs with a single pipette, flush four or five wells, load them, and repeat. If you wait too long after flushing the wells, urea will leach into the wells and make them even more transparent. • One of the accessories for the Model 4300 is a well visualization aid that can help you see rectangular wells. This aid has a mylar sheet that slides in behind the rear plate and can make the wells more visible. **Don't forget to remove the visualization aid before electrophoresis.** • One way to visualize rectangular wells is to look for the reflection of the wells in the upper surface of the notched front plate. Load by putting the tip in so it touches the back plate, then down slightly
5.	After sample loading, replace the upper buffer tank lid (Figure 2-12), close the instrument door, and start the run.

Disassembly

1.	Remove the buffer tank lids.
2.	Disconnect the power cable.
3.	***For 18 cm gels***, remove the entire gel apparatus from the sequencer and carefully dispose of the buffer solution.
4.	***For 25 cm gels***, the upper buffer tank has a fitting for draining the buffer solution, while the apparatus is still secured to the sequencer. A coupling attached to a length of rubber tubing is provided to drain the tank (Figure 2-13) Make sure the tubing is placed in a proper receptacle, as buffer will begin to drain immediately upon connection of the coupling. Insert the coupling until it snaps into place.  ***Figure 2-13.*** *Buffer tank drain fitting.* Depress the metal ring on the coupling and pull straight down to remove the drain hose when finished.

Cleanup

1.	After removing the upper and lower buffer tank lids and disconnecting the power cable, take the gel assembly off the sequencer and remove the upper buffer tank and rails.
2.	Remove the lower buffer tank and dispose of the buffer solution.
3.	Rinse the rails, spacers, and comb, and allow to air dry.
4.	Rinse and air dry the upper and lower buffer tanks and lids.
5.	Clean any spills on the heater plate, chassis, or front panel.

Cleaning the Plates

Materials needed:

- 2% laboratory detergent solution made from a concentrate such as Micro-90® (International Products Corp., Burlington NJ), or Liqui-Nox® (Alconox® Inc., New York, NY).
- Spray bottle capable of spraying a fine mist, containing 70% ethanol.

1.	A small black plastic wedge is included in the spare parts kit to aid in separating the gel plates. Insert the wedge between the plates to pry them apart for cleaning. **Caution:** Do not pry on the left and right sides of the front plate where the glass is narrow. *Never use metal tools to pry plates apart.* Lab spatulas, razor blades, or similar tools can chip plates.
2.	Dispose of the acrylamide in compliance with local regulations. Try to clean gels within 1-2 hours after the run is complete. If the gel has recently cooled to room temperature, acrylamide will adhere to paper towels, which can be used to lift the acrylamide off the plates. If gels have been left for more than 4 hours, use a razor blade to scrape the acrylamide from the plates.
3.	Pour a small amount (6 cm circle) of 2% detergent onto the side of the plate that will contact the gel.
4.	Work the solution into a lather with a nylon bristle brush and thoroughly scrub the entire plate. Remove any dried-on polyacrylamide with a razor blade, if necessary. Rinse well with water.
5.	Pour distilled water onto the plate. Work the water around the plate with a gloved hand and watch for bubble formation from detergent residue. Repeat until no bubbles form.

6.	Repeat steps 3-5 above with the second plate.
7.	Rinse the plate with deionized distilled water. Use a pump spray bottle to spray a fine mist of 70% ethanol/ddH_2O for a final rinse. Stand plates in a rack to air dry.

Section 3

Appendix