Microsatellite Analysis Manual

Model 4300 DNA Analyzer

LI-COR®
Biosciences

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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 (Hamilton Co., Part #84511) has syringe needles 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. 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.

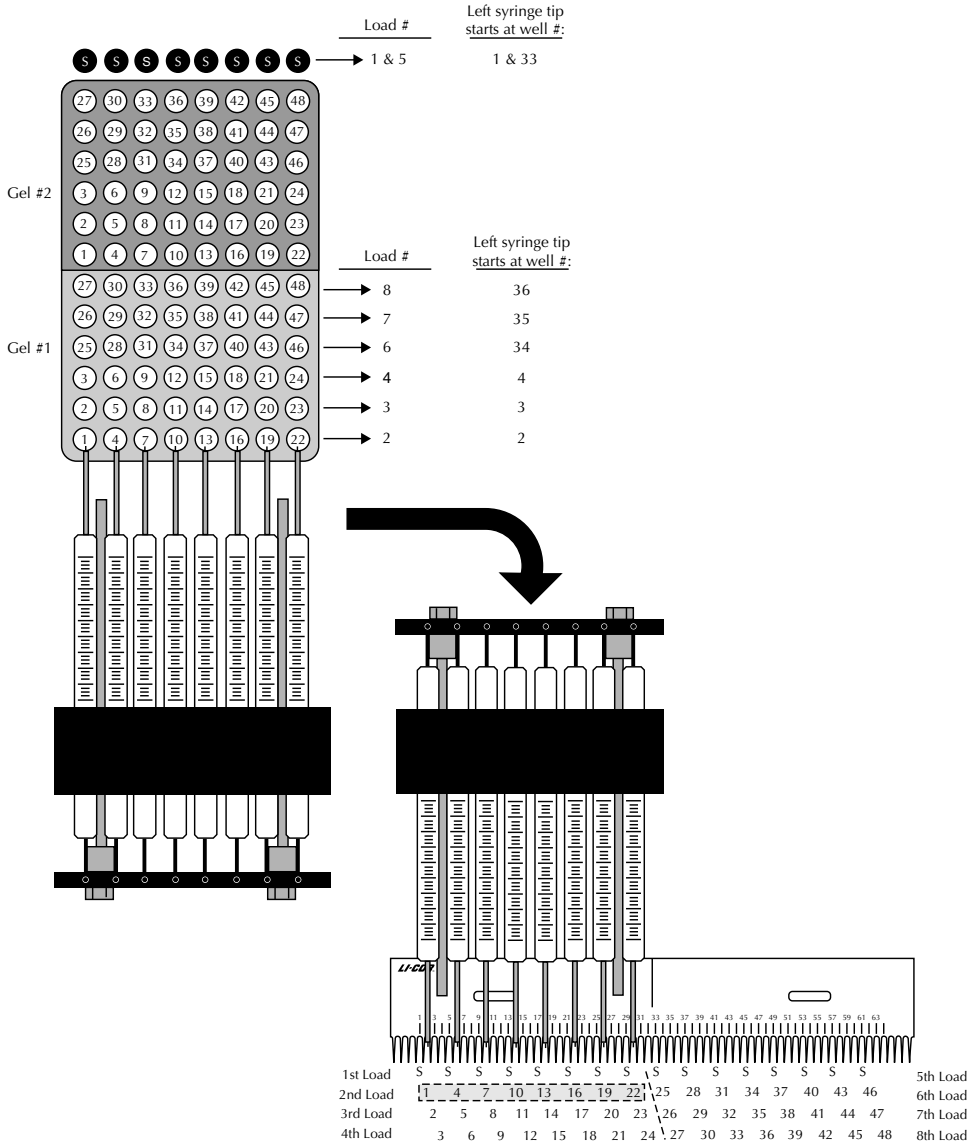


Figure 1-1. *Microplate showing 48 sample loading format. Size standards are loaded from a second plate or set of strip tubes.*

Loading 60 Samples on a 64-well Comb

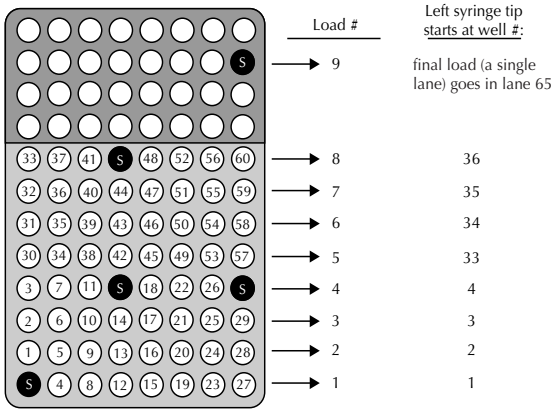


Figure 1-2. *Microplate showing 60 sample loading format.*

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, Liquinox, 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.

Cleaning the Plates

Materials needed:

- 2% laboratory detergent solution made from a concentrate such as Micro (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 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 multiple 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). If you are using a squaretooth comb, 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 (other combs do not require back plate silane treatment). Allow the solution to dry before gel assembly.

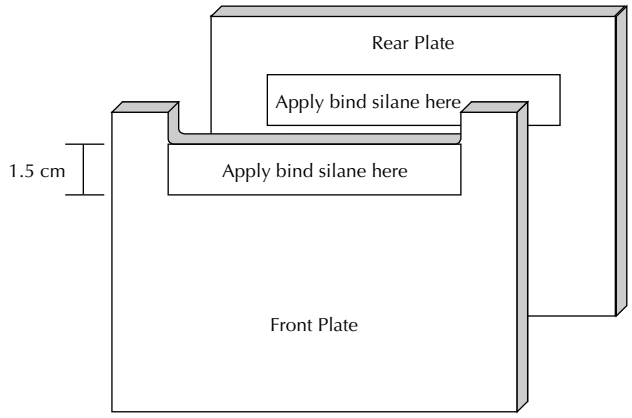


Figure 2-1. *Apply bind silane to the plates as shown. Note that only squaretooth combs require back plate silane treatment.*

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

Assembling the Gel Sandwich

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

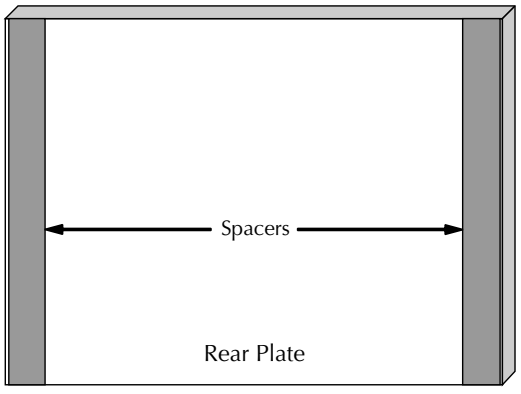
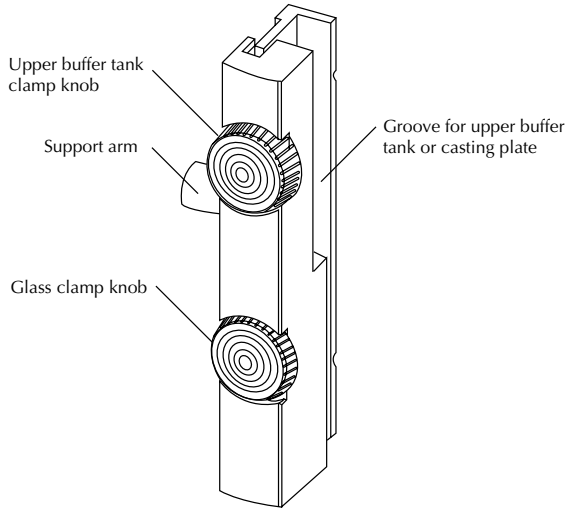
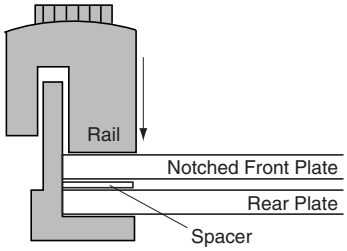
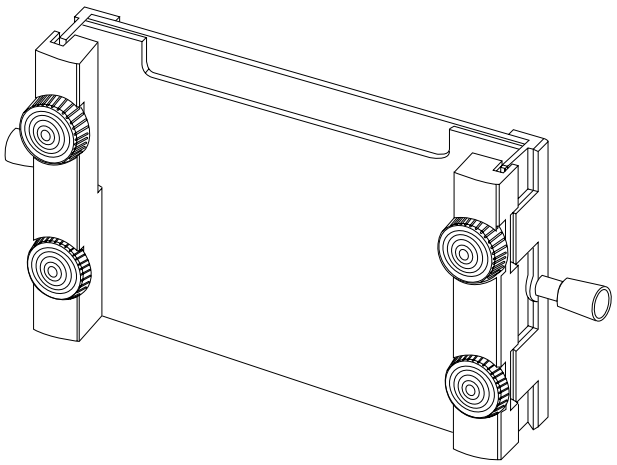


Figure 2-2. *Place a spacer on the long edges of the plate.*

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.
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.

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.	Empty the contents of the KB^{Plus} 10X TBE pouch into a 1 liter beaker.
2.	Add enough distilled water (18MΩ) to bring volume to about 800 ml.
3.	Stir the solution well until all of the solids have gone into solution and the solution is clear.
4.	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™ acrylamide	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 (USB) 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.	Mix well at room temperature and filter the solution using a membrane filtration system (syringe top or bottle top from any supplier).
2.	Add 150 µl of 10% APS per 20 ml of gel solution and swirl gently.
3.	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.

4. For 25 cm gels, 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.

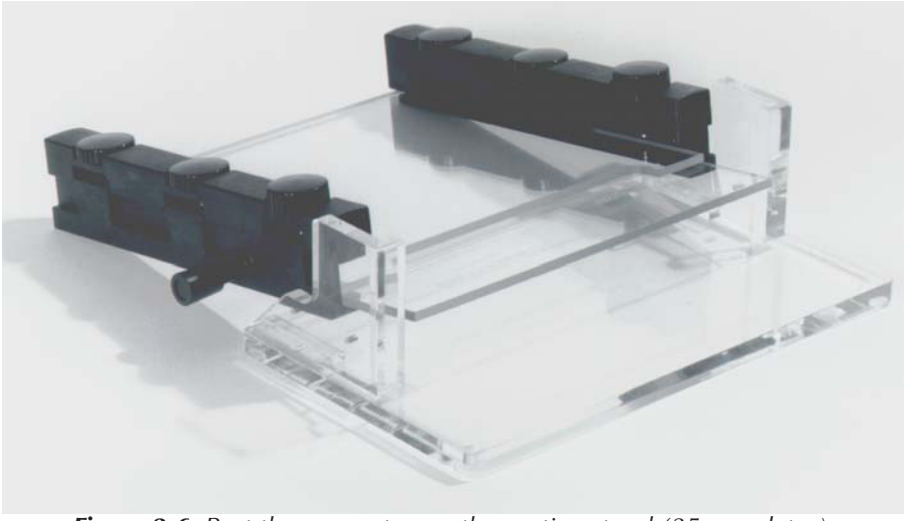


Figure 2-6. *Rest the apparatus on the casting stand (25 cm plates).*

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.

5. Remove any bubbles that form during gel pouring using a bubble hook (Figure 2-7).

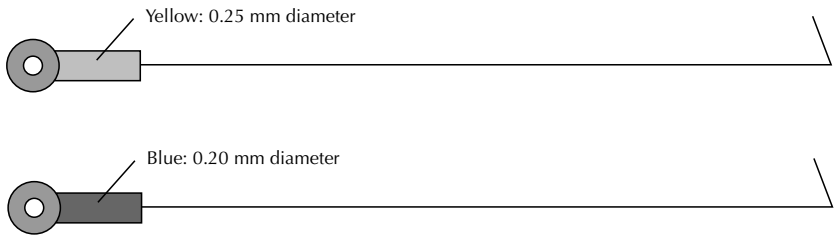
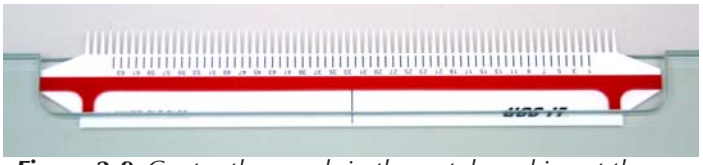
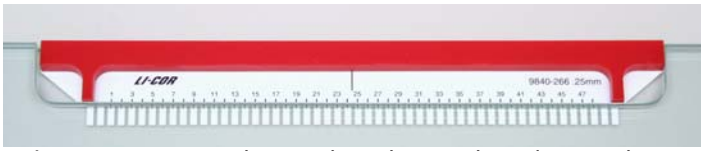


Figure 2-7*. Bubble Hooks.*

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. Instructions for inserting paper combs are given in the Appendices (Section 5). 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.

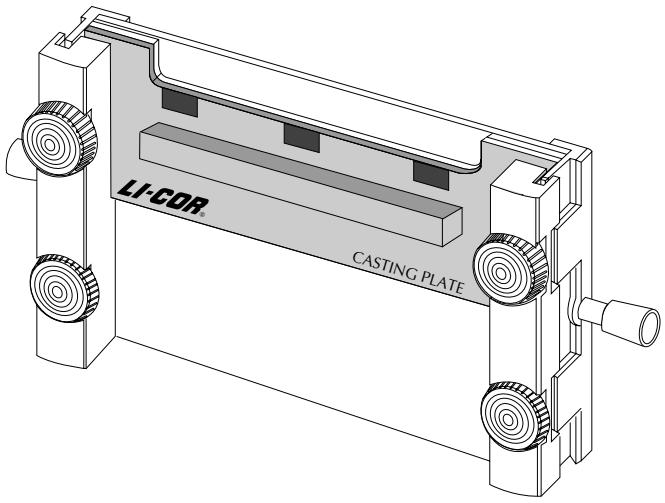


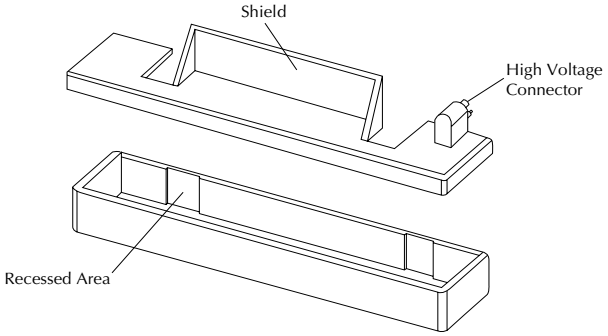
Figure 2-10. *Insert the casting plate and tighten the knobs.*

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.

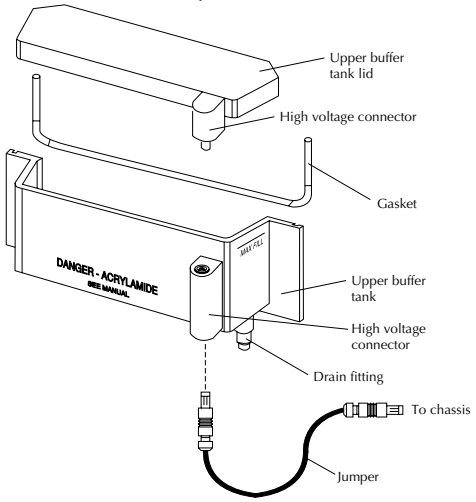


Figure 5-14*. Upper buffer tank connections.*

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.

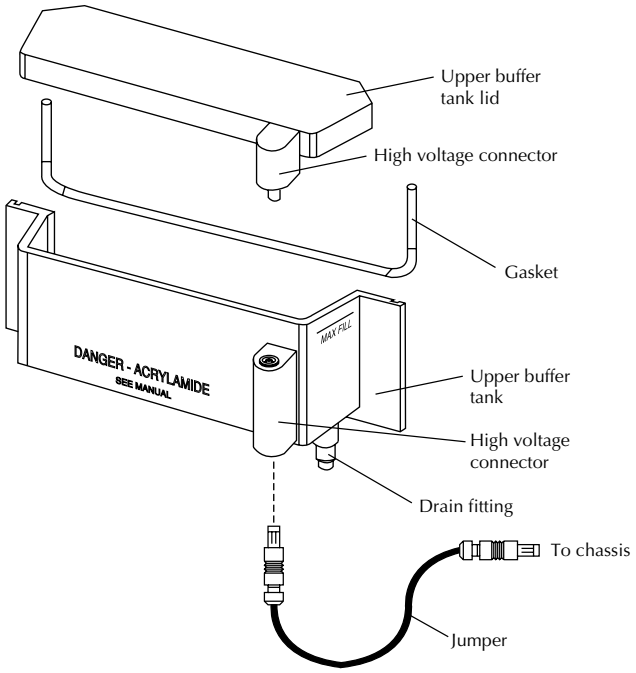


***Figure 2-11**. Upper buffer tank connections.*

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.

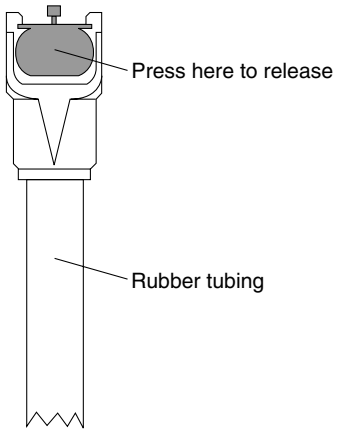
These manuals also discuss the procedure for reloading gels. Starting runs on reloaded gels is different because the pre-run is not necessary.

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 waiting too long after flushing the wells, urea will leach into the wells and makes 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 (International Products Corp., Burlington NJ), or Liquinox (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 your 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 all 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
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:

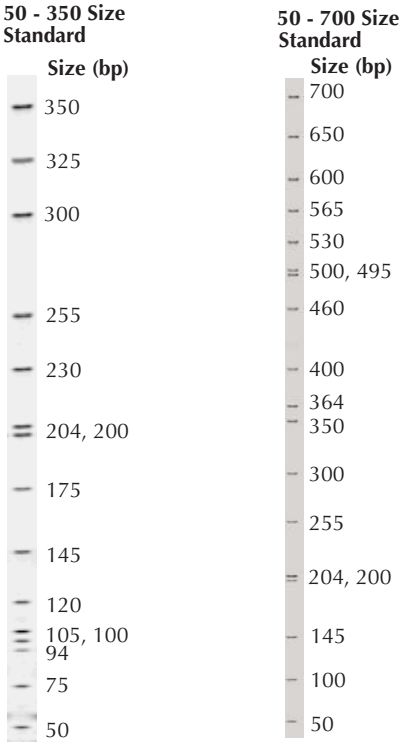


Figure 3-1. *Fragment sizes for 50-350 and 50-700 size standard sets.*

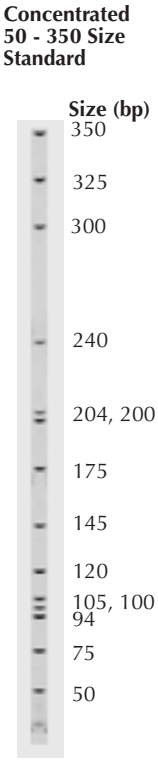


Figure 3-2. *Fragment sizes for the concentrated 50-350 size standard set.*

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

Cycle

Cycle

Cycle

* 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

1.	Enter the *Standard* program, or a custom 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		3.8
dNTPs	2.0 mM	1.0
Primer 1 labeled	1.0 pmol/µl	0.5
Primer 1unlabeled	1.0 pmol/µl	0.5
Primer 2labeled	1.0 pmol/µl	0.5
Primer 2unlabeled	1.0 pmol/µl	0.5
Primer 3labeled	1.0 pmol/µl	0.5
Primer 3unlabeled	1.0 pmol/µl	0.5
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 the samples at 95°C for 3 minutes and snap cool on ice before loading.
10.	Load gel (volume depends on comb and gel thickness.

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, suboptimal annealing temperature.	Needs higher concentration of primer. Need to alter annealing temperature or change to an enhanced protocol.
A "smeared" band is seen where the loci should be visible.	High primer concentration.	Use primer with lower concentration.
	High concentration of DNA.	Reduce DNA concentration and/or spectrophotometrically 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 concentration.
	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 × 4) + (11 × 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 + 0.41*x* - 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:

$$\frac{33\times10^{-6}\,g}{1\,O.D.}\times\frac{1\,\mu mol}{\sim 7333\,g}\times\frac{10^{3}\,nmol}{1\,\mu mol}=\frac{3.3\times10^{4}\,nmol}{7333\times1\,O.D.}=\frac{4.5\,nmol}{1\,O.D.}$$

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.

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