

## Increased Throughput for Fragment Analysis on an ABI PRISM® 377 Automated Sequencer Using a Membrane Comb and STRand Software

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### ABSTRACT

*This manuscript outlines our protocol for using a freely downloadable fragment analysis software package (STRand) together with a 96+4 RapidLoad membrane comb to increase throughput of samples for fragment analysis on ABI sequencers without costly upgrades from the manufacturer. We outline how using these products allows one to score 90 lanes of sample per gel on an ABI PRISM® 377XL (64-lane sequencer), saving both time and money in the processing of samples. This protocol is a major modification to those suggested by the manufacturer. This protocol gives more consistent results that are easier to score than standard protocols, and it reduces reagent costs. Interest in fragment analysis (primarily microsatellites and AFLPs) is steadily increasing among both population ecologists and geneticists, and methods that simultaneously increase sample throughput while reducing costs associated with these analyses by over 50% per gel should prove useful to anyone using an ABI, MJ Base-station™, or LI-COR® automated sequencer for fragment analysis.*

### INTRODUCTION

Interest in microsatellites and AFLPs as genetic markers of choice for population and ecological genetics studies is steadily growing, and the advantages of fragment analysis on automated sequencers has increased demands on available systems concomitantly. Given increased demands on available sequencers, issues of optimization of multiplex reactions, sample throughput and cost have become the focus of many researchers when deciding which detection technique to use for scoring fragment polymorphisms. Upgrades of the ABI PRISM® 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA) can increase sample throughput to 96 lanes per gel but are expensive and often difficult to justify because of the substantial cost of matching peripheral equipment to the 96-well format. This article describes how we use STRand software (5) and RapidLoad membrane combs (4) from The Gel Company (San Francisco, CA, USA) to score up to 90 samples per gel using GeneScan® (Applied Biosystems) collection software on an ABI 377XL (64-lane) DNA sequencer. This method will work with either ABI 373 or 377 sequencers and provides a cheap alternative to increase the throughput of fragment analysis with only slight modification to existing protocols and without requiring any expensive equipment upgrade.

### MATERIALS AND METHODS

#### PCR and Multiplex Optimization

Using 5'-end labeled Phosphoramidite (6-FAM, HEX, and NED) custom primers from Applied Biosystems, dye-labeled amplification products are generated via PCR. PCR protocols were modified from the GeneScan Reference Guide provided by Applied Biosystems (1) to optimize amplification efficiency for marine crabs. ABI slab gel sequencers read four-colored dyes simultaneously, but one dye (ROX) is used for the internal size standard. Hence, three overlapping loci can be scored simultaneously in an individual via multiplex PCR amplification. Where loci with non-overlapping allelic ranges exist within the limitations of the size standard used (Applied Biosystems markets a variety of size standards), two (or more) loci can be labeled with the same dye to increase the number of loci scored in a single reaction with multiple primer pairs. Because the allelic range in the marine crabs *Petrolisthes cinctipes* and *Cancer magister* is unusually large (up to 190 bp), a maximum of seven loci can be simultaneously amplified in a single multiplex reaction depending on the degree of overlap among the allelic range of amplification products.

Although the primer front can be cut from the gel in STRand or GeneScan,



the presence of unincorporated primer often obscures small amplification products. This requires either the removal of unincorporated primer from the PCR products via a clean-up step or optimization of the PCR to minimize the amount of unincorporated primer remaining after amplification. We have chosen the latter strategy to eliminate the cost of PCR clean up and increase the speed of scoring samples following PCR. By sequentially halving the primer concentration until the amount of unincorporated primer is minimized, amplification products can often be improved, and the template-to-primer ratios of each locus can be adjusted as necessary to equalize the intensity of amplification among loci. Elimination of the majority of the primer front reduces interference with scoring small products (<100 bp) while saving money on dye-labeled primer use, ROX size standard, and PCR clean-up kits. This procedure also reduces the steps involved and reduces background, thereby making the resulting gel file easier to score.

### Gel Preparation

Long Ranger™ Singel packs (BMA, Rockland, ME, USA) are cast according to the manufacturer's protocol (3) using a casting comb (0.2 mm mylar no. 401913; Applied Biosystems). Once the gel is completely polymerized, the casting comb is removed and the well is washed with deionized (DI) water to remove any remaining bits of polymerized acrylamide. The well is visually inspected for a sharp, clean, and straight interface, the gel is held at a 45° angle, and a Kimwipe® is then used on one corner to wick all remaining DI water from the well (while avoiding contact with the gel surface). To ensure the well is completely dry before loading, the gel is left in a rack upside-down (with the well at the bottom) while preparing samples for loading.

Although unnecessary, we have found that pre-warming the equipment produces more consistent and easily scorable gels. We pre-warm the sequencer by using the gel and buffer from the previous run; the sequencer is simply restarted in PreRun mode during sample preparation to bring the system to temperature in advance of gel

loading. Once the sequencer is pre-warmed, the new gel comes to running temperature very quickly after being inserted into the machine, which precludes a lengthy pre-run.

### Sample Preparation

Dye-labeled amplification products are generated in advance as outlined above, and 1 µL of the PCR amplification is combined with 1.1 µL loading buffer, which consists of 0.16 µL GeneScan-500 ROX size standard (Applied Biosystems), 0.68 µL deionized formamide (Sigma, St. Louis, MO, USA), and 0.26 µL blue dextran/EDTA loading buffer (Applied Biosystems; supplied with GeneScan-500 ROX size standard). This loading mixture is greatly diluted relative to the recommendations of the Applied Biosystems protocol provided with the GeneScan-500 ROX size standard and is the lowest amount of size standard per lane we could load with reliable results. At this loading volume, the size standard is detected at 600–1500 relative fluorescence units (rfu). If higher signal strength is needed, then the volume of the ROX standard in the loading buffer can be easily increased. Some experimentation is required to minimize unincorporated primer in the reactions and to optimize the template-to-primer ratios for each locus to produce an amplification product detected at between 1000 and 4000 rfu (as described above). Once accomplished, this minimum loading volume (0.16 µL/lane) of the ROX size standard works very well and uses only about one-third the suggested amount of this expensive reagent per gel.

After the PCR and loading buffer are mixed, the PCR plate is sealed and the samples are denatured at 95°C for 3 min on a GeneAmp® 9700 thermal cycler (Applied Biosystems) before being transferred directly onto ice. Samples are kept on ice while transferring 0.9 µL of the denatured mixture from a 96-well PCR plate into the 96+4 well RapidLoad membrane comb dipping tray (The Gel Company). The company recommends loading only 0.4–0.7 µL sample onto each tooth of the comb, but the extra volume is sometimes desirable, and the volume of sample loaded can be easily controlled during dipping

(explained below). Despite the increased volume transferred to the dipping tray—among evaporation, denaturing, and pipettor error—very little of the denatured samples will be left in the PCR plate after transfer, and reloading is generally not possible in case of sample loss. If multiple loads are frequently necessary, then you may wish to increase your sample preparation volume.

### Gel Loading and Running

The denatured samples in the Rapid-Load tray are loaded onto the 96+4 membrane comb immediately before being loaded onto the gel. Samples are applied to the comb by dipping the comb into the numbered wells on the tray until the sample is absorbed. If the sample wicks up beyond three-quarters of the height of any tooth, then decrease either the time that you allow samples to wick into the comb (we simply withdraw the comb as soon as the samples cover approximately three-quarters of the tooth) or the amount of the denatured sample transferred to the dipping tray for future loads. In practice, three-quarters of the tooth on the 96+4 Rapid-Load membrane comb translates to approximately 0.7 µL sample and ensures no mixing of samples that is due to wicking between lanes.

Without the 96-lane upgrade on the ABI 377XL, the path of the laser is not wide enough to take advantage of the entire 100-lane capacity of the Rapid-Load 96+4 membrane comb. The laser detection zone reads only the lanes numbered 4–93 on the RapidLoad dipping tray. However, using STRand, it is possible to take advantage of the full read path (maximum of 90 lanes) of the laser on the ABI 377XL sequencer. The drawback of increasing the number of lanes scored per gel is a reduced number of channels per sample (7). However, in comparisons of 128 multiplex reactions run with the method outlined here to those same samples run using a traditional 64-lane GeneScan protocol (1), we found not a single difference in allele sizes called for a given sample. Unfortunately, the 96+4 RapidLoad combs do not fit the entire width of the gel as do the standard shark's-tooth combs; this is problematic because 90 lanes are the absolute maximum width of the data-gath-

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ering region, and any slight variation in centering the comb in the read region will cause either the first or last lane to be missed on the gel file. Therefore, it is useful to mark the comb position on the plate to ensure the same position in every run and to run positive controls in lanes 1 and 90 of each gel so that a slight shift in the comb position does not result in lost data. With the two positive controls, 88 lanes of data can be collected reliably on each gel.

Although it does not appear to be necessary, we leave the RapidLoad dipping tray on ice throughout sample transfer and comb loading because in our experience it increases band resolution and uniformity slightly. Once the samples are transferred into the dipping tray, the gel from the previous run (used to pre-warm the sequencer, as described above) is removed and the new gel is loaded onto the sequencer. If any additional liquid has appeared in the well while the plate is inverted, then the gel is again tilted at a 45° angle and the liquid is removed as previously described. After confirming a flat baseline with a plate check, the front heating plate is attached and buffer is added to the correct level in the bottom buffer tray only. The upper buffer chamber is attached but left empty. The gel run (module GS 36D-1200, 36-cm WTR XL Scan, 2.5-h collection time) is started using a “dummy” sample sheet (we use a 64 sample sheet each time, but the sample sheet information and lane number collected for the gel are irrelevant) and then immediately paused (a No EP Current Error will pause the machine automatically if you fail to do it manually).

A pipettor is used to add 400–500 µL of 5% Ficoll® loading solution (diluted with PCR-quality water from The Gel Company’s 20% Ficoll loading solution) to completely fill the well to the point where the solution just begins to bead between the plates. However, it is particularly important to avoid getting any gel loading solution on the back plate while filling the well. Any liquid on the back plate will cause the comb to stick and make loading it awkward, greatly increasing the likelihood of damaging the comb teeth during insertion. Bubbles in the loading solution should be removed with a gel-loading tip or micro-syringe. Once the well is

filled with loading solution, we try to minimize the amount of time taken to apply the samples to the comb, insert the comb into the well, and start the electrophoresis run (it should not take more than a minute or two).

After samples have been applied to the membrane comb, it is ready for immediate loading into the loading solution-filled well. This is most easily accomplished by holding the comb at a 45° angle to the back plate and allowing the teeth to first penetrate the bead of loading solution left between the plates (as described above). As the teeth penetrate the bead and then come into contact with the back plate, the angle of the comb can be increased toward 90° to allow the teeth to easily slide down between the plates until they rest just above or on the gel surface. Over-insertion of the comb can damage the gel matrix and lead to irregular spacing between lanes or “bent” lanes that are much more difficult to score. Although the dilute loading solution can have extra blue dextran dye added to increase the resolution of the gel interface, if the gel was poured using the casting comb recommended above (Applied Biosystems), then the teeth of the membrane comb will just barely reach the gel surface when the upper edge is flush with the top of the back plate. After only a few loads to familiarize ourselves with the technique, we found that this method of loading becomes much faster and easier than traditional methods of loading a shark’s-tooth comb using a multichannel syringe.

Although the company protocol (4) states that samples will not diffuse into the loading solution, we try to start the run as quickly as possible after inserting the comb. That is why we start and then pause the electrophoresis run before filling the well and loading the samples onto the comb. Once the comb is inserted, the upper buffer chamber can be quickly (but gently) filled. As soon as the door is closed, the run can be resumed, thereby minimizing the time that samples sit in the comb. After restarting the run, we allow 2 min electrophoresis (at 200 W) to transfer the samples into the gel. The run is then paused, and the comb is removed and discarded. We have heard of some people reusing the membrane combs after

soaking in DI water to remove any residual sample, but we have not tried this technique. The 5% Ficoll loading solution is then gently rinsed from the well with 1× TBE from the upper buffer chamber using a 200-µL pipettor. It is essential that the loading solution be completely rinsed from the well, but it is also important that the rinsing be relatively gentle to avoid irregularly spaced or wavy gels. The standard method of vigorous rinsing with a 60-cc syringe invariably produced unscorable gels. The 5% dilution is much more easily dislodged than the original 20% Ficoll solution, so gentle rinsing several times across the width of the well is all that is needed to completely displace the loading solution. Once the well is thoroughly rinsed, the lid to the upper buffer chamber is put into place and the run is resumed.

## RESULTS

After the gel run is finished, the gel file is transferred to a PC running Microsoft® Windows 98 SE®, on which STRand (5) has been installed. The STRand software package is freely downloadable from the *BioTechniques* Software Library ([www.BioTechniques.com](http://www.BioTechniques.com)) or from the University of California, Davis Veterinary Genetics Laboratory Web page (<http://www.vgl.ucdavis.edu/STRand>); note that the server is case sensitive) and was installed and configured following the instruction manual (6). The unanalyzed gel file produced by the GeneScan collection software is copied from a Macintosh®-format zip disk onto a PC using MacDrive 2000 for Windows™ (Mediafour, West Des Moines, IA, USA), and the raw gel file is imported into STRand directly. Users can predefine the number of lanes to be found or let the software determine the number of visible lanes on the gel. The tracked gel is then analyzed using a set of user-defined rules for each locus included in the multiplex reaction. These locus rules are fully customizable for specific applications. The program calls both the size standards and the allele/band sizes for each lane of data and allows the user to examine and alter any of the peak or size designations. The program displays a



list of peak heights and alleles called for each locus. A separate window that provides user alerts (for problems such as having more than two alleles called for a locus) is also included along with the electropherogram for each sample in a single window, eliminating the need to switch between windows or programs to complete the analysis. Individual dye colors can be hidden as necessary, and both the x- and y-axes of the electropherogram are easily adjustable with sliders for cases when zooming in on the electropherogram can help with scoring.

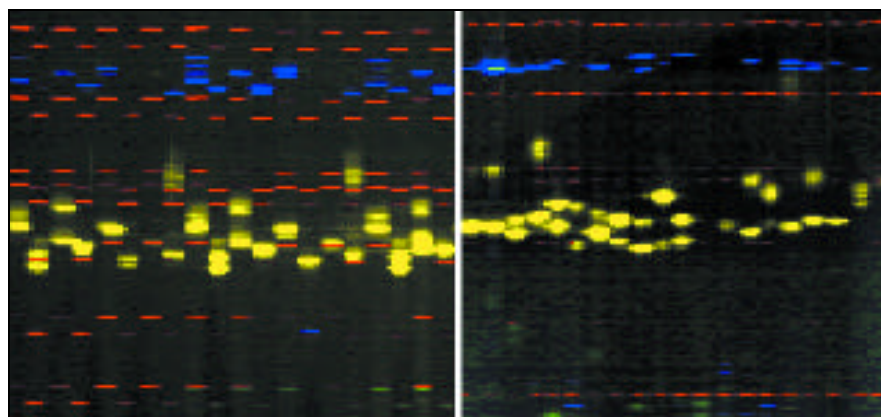
## DISCUSSION

In our experience, results from gels that are run using the protocol we outline here are generally indistinguishable from those using a traditional shark's-tooth comb (Figure 1). We believe that the RapidLoad comb offers three primary advantages over the shark's-tooth comb that are unrelated to data quality. First, all 90 lanes are loaded simultaneously with the RapidLoad comb, which not only decreases load times but also eliminates the need for stagger-loading typically required with multichannel syringes used with a shark's-tooth comb. Second, there is no possibility of samples leaking between wells with a RapidLoad comb. Finally, less product is required for running with the RapidLoad combs, which proves to be a significant cost savings over standard pro-

ocols using a shark's-tooth comb.

Although the use of the RapidLoad comb simplifies loading and reduces the cost of reagents per lane on the gel, it is the analysis software, STRand, that allows us to use the full width of the laser path on the gel and get 88–90 lanes of scorable data from the 64-lane ABI 377XL. STRand was originally designed to address the needs of the University of California, Davis Veterinary Genetics Laboratory (VGL) while developing its equine DNA test using 14 STR loci multiplexed into a single lane. The software available at the time was inefficient for that level of high-throughput genotyping and had a tedious user interface without any relational database integration. The goal for STRand was to maximize accuracy while minimizing the analysis time. Flexibility was also important; STRand is ready for new technologies through the easy addition of any gel image format, any number of lanes, any number of dyes, etc. Currently, ABI 373, 377, 310, 3100, and 3700, MJ BaseStation™ (MJ Research, Waltham, MA, USA), and LI-COR IR<sup>2</sup>® (LI-COR, Lincoln, NE, USA) formats are all supported.

STRand performs the full analysis from the original collection file to genotypes as opposed to using two programs such as GeneScan and Genotyper (Applied Biosystems). VGL found that STRand could cut analysis time by a factor of 4–6 because lane tracking is done automatically, sample sheets are pulled from a database, and



**Figure 1. Magnified sections of a typical 64-lane shark's-tooth comb (left) and 64+4 RapidLoad membrane comb (right).** Each lane is a four-locus multiplex reaction amplified from individual anomuran crabs, *Petrolisthes cinctipes*, and uses the GeneScan-500 ROX size standard from Applied Biosystems. We have zoomed in on the 200–450 bp portion of the center of each gel (lanes 22–42) for comparison.

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the analysis window shows the electropherogram along with the other data with no need to switch between windows or programs. This year VGL expects to complete 140 000 DNA types using 13 markers each for a total of almost 1.7 million genotypes, all analyzed using STRand. A 96-lane gel with 13 STR loci per lane takes a skilled VGL analyst about 45 min to complete. Over the years, STRand may have lost a little of its lead as Applied Biosystems product literature claims 60 min to analyze 96 lanes of 14 markers for GeneScan/Genotyper and 10 min using their new GeneMapper™ software (2). New features on the horizon for STRand should continue to keep it competitive in terms of performance. More attractive to individual research laboratories is the fact that STRand is licensed under the GNU Public License ([www.fsf.org](http://www.fsf.org)) from The Regents of the University of California, which means that it is freely available. The source code is also available so that you can modify and improve the program as

needed. The STRand homepage is located at <http://www.vgl.ucdavis.edu/STRand> (server is case sensitive), from which it is possible to download the software and a manual as well as to subscribe to the users e-mail list.

To manage all of the data generated, STRand stores all results in a relational database. The database can be either a Microsoft Access® database file or Microsoft SQL Server (other database systems may also work but have not been tested). Using a relational database server allows STRand users to reliably share the same database across a network so that all researchers have access to the same samples. Keeping the complete data set in a single location also provides easy access for data checking and for collecting statistics such as allele frequencies. STRand exports results in a text file format similar to that used by Genotyper, or results can be accessed directly from the database.

Although the protocol detailed here is specific to the ABI PRISM 377 automated sequencer, it should be readily adaptable to any automated sequencer that STRand supports. In summary, the method outlined here allows users to easily increase STR sample throughput on an automated sequencer without expensive equipment upgrades and, more importantly, without sacrificing data quality. The additional cost of the RapidLoad membrane combs over a reusable mylar shark's-tooth comb is easily offset by the savings on the GeneScan-500 ROX size standard consumed with each gel. Aside from gaining 24 additional lanes of data per gel, this protocol also reduces our cost; overall, our cost per gel is reduced by over 50% with this protocol relative to the standard 64-lane GeneScan protocol we followed originally (1). Together with the freely downloadable STRand fragment analysis software, the added benefits of the ease of sample loading, and the increased sample throughput of the RapidLoad membrane combs, this protocol should prove useful to anyone using an automated sequencer for fragment analysis.

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