PCR Product Cleanup
Methods for Capillary
Electrophoresis

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Capillary electrophoresis technology has reduced electrophoresis time and eliminated slab gel casting and sample loading (1). However, analysis of PCR products by capillary electrophoresis can be compromised by the presence of contaminants such as salts, unincorporated dNTPs, primers, and primer dimers, causing variable electrokinetic injections and migration times. Another variation seen in capillary electrokinetic injection is bias toward injection of smaller fragments in the presence of higher salt concentrations. These factors significantly reduce the amount of PCR product injected, resulting in much lower signal strength, which is particularly problematic when initial signal strength is low.

A number of alternative post-PCR cleanup techniques have been developed. For the first time, we formally compare and contrast a variety of different cleanup methods routinely used before capillary electrophoresis. Conclusions are drawn as to the applicability of each method and in particular to single-cell multiplexing when PCR product is reduced.

Multiplex fluorescent PCR of Amelogenin (2) and short tandem repeats

DYS14 (3), D21S11 (4), D13S631 (5), D13S258 (5), D18S51 (6), D18S851 (<http://www.gdb.org/>), D13S317 (7), and D18S391 (8), was performed on extracted DNA from 96 venous blood samples collected with patient informed consent and relevant human research ethics committee approval.

DNA was extracted using QIAamp® DNA Blood Mini Kits (Qiagen, Valencia, CA, USA) as per the manufacturer's protocol. Multiplex reactions were carried out in 75 µL reaction volume to provide sufficient product to compare cleanup methods simultaneously. Each reaction contained 5 µL extracted DNA, forward and reverse primers, 1× PCR buffer (Applied Biosystems, Foster City, CA, USA), 1.5 mM MgCl₂ (Applied Biosystems), 1.25 mM each dNTP (Invitrogen, Melbourne, Australia), and 1.6 U AmpliTaq Gold® DNA polymerase (Applied Biosystems). PCR conditions were denaturation for 15 min at 94°C, followed by 39 cycles of denaturation for 30 s at 94°C, annealing for 45 s at 59°C, extension for 1 min at 72°C, and a final extension for 10 min at 72°C.

PCR product was processed using five various manual and automated methods. For dilution (9), dilute 2 µL PCR product with 8 µL autoclaved filtered water. For gel filtration, following the manufacturers' protocols, add the required amount of Sephadex® G-75 (Sigma, St. Louis, MO, USA) to each well of a MAGV N22 filter plate using the Multiscreen Column Loader (both

from Millipore, Bedford, MA, USA). Comparisons were also made with a Commercial Sephadex plate (Amersham Biosciences, Piscataway, NJ, USA), following the manufacturer's protocols. For magnetic separation, wash 15 µL magnetic beads (Magna-sil™ Paramagnetic Particles; Promega, Annandale, Australia) three times with 100 µL water. This is achieved following the manufacturer's protocols using the Promega PMP Concentrator and the CRS Robotics system (CRS Robotics, Burlington, ON, Canada) to attach the beads in place and the Hydra™ 96 (Robbins Scientific, Sunnyvale, CA, USA) to add and remove wash. Ammonium acetate/ethanol cleanup of 12 or 25 µL PCR product was performed using Amersham Biosciences protocols. Reagents required are 7.5 M ammonium acetate (Amersham Biosciences) and ethanol. For magnesium sulfate/ethanol cleanup, allow the PCR plate to come to room temperature and then add 75 µL 0.2 mM magnesium sulfate (BDH, Dorset, UK) in 70% ethanol to 12 or 25 µL PCR product. Vortex mix thoroughly and let stand for 15 min. Centrifuge for 15 min at 2318× g. Blot plate, and centrifuge upside down on paper towel 179× g for 1 min. Air-dry and then add 20 µL water to each well to resuspend DNA and vortex mix briefly.

In all cases, post-cleanup processing involved adding 2 µL cleaned-up product to 3 µL loading buffer (Amersham Biosciences). Samples were denatured and placed immediately on ice. Analy-

Table 1. Comparisons of Cleanup Methods

	Crude Product	Magnetic Separation	Dilution	Gel Filtration	CS	Magnesium Sulfate/Ethanol	Ammonium Acetate/Ethanol
Percentage of successful wells	58.0	63.9	84.0	92.4	97.9	100 (100)	93.4 (100)
Total rox size standard peak height	4499	3684	6687	15650	10513	10553 (11368)	14583 (13449)
Total peak height for all peaks (%)	100	100	77	364	51	27 (26)	221 (147)
Total target peak height (%)	100	335	240	829	390	290 (106)	695 (600)
Number of alleles per reaction (%)	100	150	172	208	188	181 (57)	162 (142)
Preferential injection of small rox fragment	2	6	28	16	107	167 (167)	98 (168)
Preferential injection of small peaks (%)	100	495	417	1003	1220	506 (441)	1664 (1500)
Background peak levels (%)	100	447	342	684	770	197 (129)	674 (154)
Primer removal (%)	100	428	412	1021	1257	429 (441)	1858 (1500)
Best methods are in bold. Values in brackets indicate 12 µL initial volume. Higher values indicate more efficient cleanup.							

Benchmarks

Table 2. Evaluation of Cleanup Methods

Method	Time (min)	Automation Potential	Cost/Plate (\$US)	Analysis Quality	Suitability for Reduced Product
Crude Product	(0) ***	***	(0) ***	*	*
Dilution	(5) ***	***	(0) ***	**	**
Magnetic Separation	(100) *	***	(10) **	*	*
Ammonium Acetate/Ethanol	(45) **	**	(0.10) ***	***	***
Magnesium Sulfate/Ethanol	(45) **	**	(0.10) ***	**	*
Gel Filtration	(180) *	*	(60) *	***	***

***, highly preferable; **, average; *, not preferable

sis was completed using the Megabace™ 1000 capillary electrophoresis system with Genetic Profiler™ Version 1.5 software (Amersham Biosciences). Injection parameters were -3 kV for 45 s, and run parameters were -10 kV for 75 min at 44°C.

A wide range of allele base pair sizes, different peak heights, various fluorophores, and peak base size differences were seen within each single multiplex reaction. Cleanup methods were evaluated by comparing different colors, base pair sized peak heights, and total product availability. Three plates were simultaneously cleaned up using dilution, gel filtration, magnetic separation, and ammonium acetate/ethanol. A further three plates were cleaned up using ammonium acetate/ethanol and magnesium sulfate/ethanol, the relative values for these cleanups is extrapolated from known ammonium acetate/ethanol data.

Table 1 shows the mean values across all three plates for each cleanup method. The numbers of failed wells for each method was compared to determine the level of salts present after cleanup. The total height of all rox peaks injected from each plate was compared to determine injection efficiency. The amount of rox (size standard) contained within the injection mixture should be consistent between all plates and cleanup methods, since rox is contained within the loading

buffer. The sum of the total and target peak heights injected, calculated as a percentage of the sum of the crude product injection peak height, from each cleanup method can be compared to determine injection efficiency.

Allele dropout levels due to injection efficiency were determined by comparing of the total number of alleles analyzed, shown as a percentage of the total number of alleles analyzed when injecting crude product, for each cleanup method. To determine preferential injection of smaller peaks, rox peak heights between 100 and 150 bp were compared to those greater than 150. For simplicity, the value of total peak heights less than 150 bp divided by total peak heights greater than 150 has been assigned as 100 when no preferential injection has occurred. Correspondingly, preferential injection of peaks less than 150 would result in values less than 100, and preferential injection of peaks greater than 150 would result in values greater than 100. To further explore preferential injection, the sum of all peak heights from fragments with base pair size of 100–150 bp was divided by the sum of all the peak heights from fragments with a base pair size greater than 150 bp. This value was then calculated as a percentage of the value obtained when injecting crude product.

To determine the levels of background and artifact peaks present after

cleanup, the sum of the total peak heights was compared to the sum of the peak heights of the target short tandem repeats. This value was then calculated as a percentage of the value obtained when injecting crude product. PCR cleanup methods were compared to determine removal of very small peaks (primer dimer peaks). Peak heights less than 100 bp (primer front) were compared to the peak heights greater than 100 bp. This value was then calculated as a percentage of the value obtained when injecting crude product.

Each of the different cleanup methods was analyzed for failed size standard injection, size standard peak height, total and target peak heights, primer removal, preferential injection of small fragment, and the number of short tandem repeat alleles analyzed (Table 1). Table 2 contains a summary of the cleanup methods comparing relative time, automation, cost, amount of product available for analysis, and suitability for reduced levels of product.

Dilution of the PCR product directly reduces the concentrations of contaminants and also significantly reduces the concentration of target DNA. Therefore, when product is low, analysis is likely to be significantly compromised.

Gel filtration columns can be purchased pre-hydrated or can be dispensed and hydrated manually and show comparable results (Table 1). Gel filtration successfully reduced the amount of contaminants; however, a small amount of preferential injection of smaller peaks was exhibited, and primer removal was not as successful when compared to ammonium acetate/ethanol.

Magnetic separation has the advantage of full automation but only slightly reduced the difficulties observed with crude product injection.

Ammonium acetate/ethanol cleanup successfully reduced interference from contaminants. This method can be applied to low yield reactions, can be partially automated, and is low cost.

Magnesium sulfate/ethanol cleanup also successfully reduced the concentration of contaminants except primer dimer. However, overall product available for analysis was also reduced, making it unlikely that this cleanup could be applied to low-yield samples.

Comparisons between all five clean-up methods conclude that only ammonium acetate/ethanol is clearly superior in reducing PCR contaminants without compromising product analysis. Ammonium acetate/ethanol cleanup is low in cost, can be partially automated with no detectable reduction in quality of the analysis, and is therefore the method of choice for fragment analysis in multiplex PCR, particularly when product yield may be low.

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