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Automated Processing of Forensic Casework Samples Using Robotic Workstations Equipped with Nondisposable Tips: Contamination Prevention

ABSTRACT: An automated process has been developed for the analysis of forensic casework samples using TECAN Genesis RSP 150/8 or Freedom EVO liquid handling workstations equipped exclusively with nondisposable tips. Robot tip cleaning routines have been incorporated strategically within the DNA extraction process as well as at the end of each session. Alternative options were examined for cleaning the tips and different strategies were employed to verify cross-contamination. A 2% sodium hypochlorite wash (1/5th dilution of the 10.8% commercial bleach stock) proved to be the best overall approach for preventing cross-contamination of samples processed using our automated protocol. The bleach wash steps do not adversely impact the short tandem repeat (STR) profiles developed from DNA extracted robotically and allow for major cost savings through the implementation of fixed tips. We have demonstrated that robotic workstations equipped with fixed pipette tips can be used with confidence with properly designed tip washing routines to process casework samples using an adapted magnetic bead extraction protocol.

KEYWORDS: forensic science, contamination, robotics, TECAN robots, casework, magnetic beads, DNA IQ™, fixed tips, D3S1358, HumvWA, HumFGA, D8S1179, D21S11, D18S51, D5S818, D13S317, D7S820

The potential benefits of automation for processing biological samples were first recognized in clinical settings (1–5). Currently, laboratories engaged in large-scale molecular diagnostics programs routinely purify nucleic acids from multiple sample types with varying levels of throughput (6–9). Large reference laboratories providing core chemical analyses or pathogen screening have greatly enhanced their performance by adopting an automated approach (10–18). In the forensic community, automation became the ultimate choice with the creation of large convicted offender DNA data banks in order to meet the highest standards of quality control and achieve enhanced efficiency with reduced operational cost (19–22). The operational success of forensic investigative DNA data banks led to the adoption of robotics for the processing of high volume casework samples (23–30). Some considerations for reducing operational cost when implementing a robotic process include the use of low-volume reactions to minimize reagent consumption and the use of nondisposable tips to minimize consumables. The latter is especially relevant for an automated process involving many pipetting or aspiration steps. The reduction in reagent volume is easily attainable with samples that are consistent in nature such as those collected using a standard collection kit with trained personnel and submitted to a forensic investigative DNA data bank. However, the use of nondisposable tips can be more challenging than the adoption of disposable tips. Typically, clinical as well as forensic investigators have been cautious about equipping their robotic workstation with nondisposable tips for processing biological samples due to potential cross-contamination issues (10,25–29,31–35) despite the fact that nondisposable tips offer a high degree of precision when aspirating or dispensing liquids due to their specific manufacturing tolerances and engineered standards (7,36–38). In addition, Teflon-coated stainless steel tips infrequently get plugged or damaged and therefore the tip replacement cost is minimal.

The National DNA Data Bank of Canada became operational in June 2000, and has since processed more than 120,000 biological samples (blood, buccal, or hair samples submitted on Whatman FTA® Sample Collection Cards [Fitzco Inc., Spring Park, MN; 21]; 120,000 punched disks processed for AmpF*l*STR® Profiler Plus™ PCR Amplification system and 120,000 punched disks processed for AmpF*l*STR® COfiler™ PCR Amplification system) using TECAN robotic workstations fitted with fixed low-volume Teflon-coated steel tips. In 7.5 years of operation, the short tandem repeat (STR) profiles produced using automation have indicated no sample carryover during the pre-PCR sample processing (washes of the FTA® disks) or during the post-PCR sample processing (preparation of the amplicons for their detection on the ABI Prism® 377 DNA Sequencer). The protocol developed to wash the fixed tips for the convicted offender sample process was simple yet highly efficient in preventing sample contamination (see Results section below). The pressing demand for processing high volume casework such as break and enter (B&E) cases catalyzed the development of an automated DNA extraction protocol to accommodate these types of samples on robotic workstations. The initial success experienced using nondisposable tips for convicted offender samples submitted to the national DNA data bank motivated our program to examine the possibility of using fixed tips for extracting DNA from challenging biological evidence left at the scene of the crime using a magnetic bead-based protocol.

This report presents an automated process that integrates DNA extraction, DNA quantification, DNA normalization, amplification

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setup, and post-PCR setup for the generation of STR profiles. Experiments carried out on the TECAN Genesis RSP 150/8 robotic workstations led to the development of an efficient nondisposable tip washing routine to prevent carryover during the automated sample processing of forensic material.

Materials and Methods

Sample Preparation

Although forensic laboratories process a wide variety of biological evidence, obtaining DNA from soil-contaminated exhibits likely represent the most challenging biological samples. The studies conducted in this paper focused primarily on this type of test samples both from a practical point of view and to subject our process to the most critical and aggressive evaluation.

Sterile cotton-tipped applicators (Puritan Medical Products Company LLC, Guilford, ME) were used to prepare samples with and without soil (water and soil mixture) tainted with human blood as follows: Approximately 3 mL of filtered autoclaved and deionized (FAD) water were added to 3 mL of soil designated V16 (from a Vancouver farmland site, packet #16) in a 15 mL Falcon tube and mixed vigorously before dipping the swabs into the mud. Swabs with soil were dried for 60 min before applying blood (various volumes from 40 μL down to 0.01 μL as indicated in the text). Blood was either collected fresh, or removed from the 4°C fridge and mixed on the Vari-Mix (Barnstead/Thermolyne, Dubuque, IA) for 15 min unless otherwise stated. For volumes of blood lower than 1 μL, blood was diluted with phosphate-buffered saline (PBS) and transferred to the swabs in either 1 or 10 μL aliquots. Swabs were set to dry at room temperature before being placed (swab tip only) into 2 mL Spin-eZe™ tubes (Fitzco Inc.) for immediate use, or stored at −20°C.

Blood (different volumes as stated in the text or tables) was also applied onto a variety of surfaces (glass, metal, wood) or substrates (blue denim, black denim). Blood samples applied onto Schleicher & Schuell (S&S) paper (20 or 30 μL per stain) or Whatman FTA® paper (50 μL per stain) were also processed. A Harris punch (Fitzco Inc.) equipped with a head equivalent in size to 2.5 mm was used for samples on S&S paper and 1.5 mm for samples on Whatman FTA® paper.

An important source of biological evidence often found at crime scenes originates from saliva (buccal cells) and shed epithelial cells which represent samples with disparate quantity and quality of material (may be degraded). To evaluate our extraction process, three common sources of such material, i.e., cigarette butts, chewing gums, and trace swabs from manipulated objects were tested. Cigarette butts were collected from known volunteer smokers. Filters were discarded and approximately 2 cm × 2 cm of the filter paper (cut into small pieces) from each cigarette butt was processed for DNA extraction. Chewing gums were collected from various individuals and stored at −20°C. An equivalent to 0.07–0.1 g of gum was used for DNA extraction. Trace swabs were prepared by wiping pop cans (soft drinks), drink bottles (essentially water bottles), telephone receivers, computer mouse, and chair arms belonging to different individuals.

Also relevant to forensic cases are mixtures of various human biological fluids such as blood, saliva, and semen. These fluids were collected from volunteers (n = 13 for blood [six females, seven males], n = 1 for saliva [female] and n = 2 for semen) and a series of mixtures were prepared in volume ratios ranging from 19:1 to 1:19. Some of the mixtures were prepared on S&S paper while others were applied to cotton swabs and black denim cuttings.

More challenging mixtures are those composed of animal blood and human blood. Such mixtures were prepared using various ratios of pig or horse blood and human blood with total blood volumes equivalent to 20, 10, and 1 μL. Animal blood originating from six pigs and six horses (4 mL each) were obtained from Maxxam Analytics Inc./Human Genetic Identification Division (Guelph, ON, Canada).

Vaginal swabs from a few female donors spiked with various size aliquots of 1/100th diluted semen (1–39 μL) or neat semen were used to challenge the robot tip washing routines. These samples normally yield very high amounts of DNA.

A series of blanks (low TE pH 7.5 or RCMP Lysis Buffer [LB] [10 mM Tris pH 8.0, 10 mM EDTA, 100 mM NaCl, 0.5% sarkosyl, 40 mM DTT] or FAD alone or either of these solutions applied on a blank swab) were always included during experimentations to evaluate the effectiveness of the various robot tip washing routines tested during the development of the automated extraction process.

Sample Lysis and Preparation for DNA Extraction

Samples assigned for "direct" DNA extraction were lysed overnight at 56°C in the presence of 250 μL up to 600 μL of LB supplemented with 0.5–1.5 mg/mL of proteinase K (Prot. K). Samples were pulse-centrifuged to bring down condensation, and the swabs were transferred to Spin-eZe™ baskets (Fitzco Inc.) using either sterile wooden sticks or sterile forceps. The tubes were centrifuged for 3 min at 13,200 rpm (16,300 × g) to pellet debris, and the baskets containing the swabs were discarded to waste. Lysates were either processed immediately, or stored frozen at −20°C.

Samples assigned for "differential" DNA extraction were lysed for 2 h at 37°C in the presence of 350 μL of LB without DTT supplemented with 0.2 mg/mL of Prot. K. Samples were pulse-centrifuged and the swabs were transferred to Spin-eZe™ baskets using either sterile wooden sticks or sterile forceps. The tubes were centrifuged for 10 min at 15,000 rpm (21,000 × g) to pellet sperm cells, and the baskets containing the swabs were discarded to waste. A volume equivalent to 315 μL of the 350 μL epithelial cell (EC) lysates was removed to an intermediate tube and replaced with 315 μL of fresh buffer (LB without DTT supplemented with 0.2 mg/mL Prot. K), vortexed and centrifuged for 10 min at 15,000 rpm before extraction.

Sample and Blank Sample Layouts for Contamination Checks

To assess cross-contamination of samples processed on our robotic workstations equipped with nondisposable Teflon-coated tips, the zebra-stripe format (alternating columns of samples containing an abundant source of DNA and reagent blank samples) and checkerboard format (alternating samples containing abundant DNA with reagent blanks in a checkerboard pattern across a 96 deep-well plate [DWP]) were used. Swabs with large aliquots of blood (20 or 40 μL) were purposely used in these experiments in order to challenge the robot tip washing routine and determine its effectiveness. Alternatively, sample batches were created with a variety of biological samples (blood swabs ± soil [different blood aliquots], bloodstains on black denim, cigarette butts, chewing gums, trace swabs) resembling a true casework sample batch and positioned in the plate as per the zebra-stripe or checkerboard format. In a few experiments, blanks were positioned in the center or the end of the sample batch as a block.

"Direct" DNA Extraction—Protocol 1 with Bead Percolations

Samples to be extracted using magnetic bead percolations (unpublished data) were loaded in tube strip racks on a TECAN

Genesis work surface (TECAN US, Research Triangle Park, NC). Lysates were transferred robotically (with two additional volumes of Promega Lysis Buffer [PLB] from the DNA IQ™ kit [Promega Corporation, Madison, WI]) to a 96-DWP (2 mL well capacity; polypropylene square well with conical bottom from VWR, Ville Mont Royal, QC, Canada) containing 12 or 16 μL of Promega DNA IQ™ resin robotically dispensed from eight vials without washing between dispenses. Binding of the DNA present in the lysate to the resin was achieved through bead percolations, i.e., half the liquid column (lysate + PLB) was aspirated and released with tracking back in the same well. A high speed liquid dispense was used to lift the beads up from the bottom of the well and allow them to slowly percolate down the liquid column in order to achieve maximum DNA binding to beads. Four rounds of bead percolations were deemed necessary to capture a maximum amount of DNA. The bead/DNA complexes were initially pelleted by quick centrifugation (ramping to 4000 rpm, [2254 *g*], 20 sec) followed by a magnetic bead attraction using a LifeSep™ 96F flat magnet (Dexter Magnetic Technologies, Chicago, IL). The large volume of liquid (e.g., 600 μL of lysate supplemented with 1200 μL of PLB) required an initial centrifugation step to ensure that all beads would be collected to the bottom of the wells in a timely fashion. For centrifugation, the 96-DWP was covered with a MicroAmp™ Clear Adhesive Film (Applied Biosystems, Foster City, CA). The liquid column was removed to the waste station using "liquid detection" and the bead/DNA complexes were washed consecutively once with PLB and twice with Promega Wash Buffer (PWB, containing 50% alcohol) by placing the plate on the magnet between wash steps to aspirate off the solution. The bead/DNA complexes were air-dried and DNA eluted in low TE buffer pH 9.0 twice at 65°C for a total of 16 min (8 min each time) with vortexing for 10 sec between elutions.

"Direct" DNA Extraction—Protocol 2 Using a TE-Shake™ Unit

Samples to be extracted were loaded in tube strip racks on a TECAN Genesis work surface configured with a TE-Shake™ unit (TECAN US) (unpublished data). Resin from the Promega DNA IQ™ kit was manually deposited in the last column of a DWP (A12-H12; 144 or 192 μL per well) and further distributed to each column of the plate (12 or 16 μL per well) by the robot while shaking at 1200 rpm on a TE-Shake™ unit. With the TE-Shake™ unit still activated, lysates were transferred robotically (with two additional volumes of PLB) to the DWP. Following a 15-min-shaking period, the resin/DNA complex was pelleted, the liquid column removed to waste, and the resin/DNA complexes washed as described in protocol 1. The resin/DNA complex was air-dried and eluted as per protocol 1.

"Differential" DNA Extraction—Protocol Using a TE-Shake™ Unit

EC lysates (10% of the original 350 μL lysates used only) to be extracted using the adapted DNA IQ™ protocol utilizing a TE-Shake™ unit (described above) were placed in the appropriate robot strip racks and brought to the TECAN Genesis RSP 150/8 robot deck. The magnetic beads and lysates were transferred robotically as described above. While the DNA from the EC lysates was being captured by the beads during a 10-min-shaking period, 50 μL of LB (see "Sample Lysis and Preparation for DNA Extraction," no DTT with 0.2 mg/mL Prot. K) was added to each of the original tubes on the robot containing the sperm pellets. Samples were vortexed 5–8 sec then centrifuged for 5 min at 21,000 × *g* before being returned to the robot deck. The wash supernatants were discarded to waste, then 350 μL of heated (65°C) PLB with DTT was added to the sperm cell pellets by the robot and the samples were left on the robot deck to lyse at room temperature for 5 min. The sperm cell lysates were then transferred to the DWP alongside the EC lysate/PLB liquid columns and extraction continued according to the original "direct" protocol. The capture of the sperm cell DNA was carried out for 5 min only. The bead/DNA complexes were pelleted and washed as described in the previous section. DNA elution was carried out as per the original "direct" protocol.

DNA Quantification

All extracted DNA samples and blank samples were quantified by real-time PCR (Q-PCR) with the AB Quantifiler™ Human DNA Quantitation assay (Applied Biosystems) on an ABI Prism® 7000 Sequence Detection System (7000 SDS software v1.0 for real-time data collection and analysis) as outlined in the AB draft protocol. One exception was the use of the K562 cell line DNA (200 ng/μL stock solution, range of DNA standards used was 8–0.0078 ng/μL) as the quantification standard instead of the DNA Quantification Standard provided by the manufacturers. It was found at the time of experimentation that the K562 cell line was providing more accurate values than the AB Quantification Standard provided in the manufacturer's prototype kit (data not shown). All PCR reactions were set up robotically. Amplification was carried out for 40 cycles.

Amplification Conditions for DNA Extracted from Biological Samples

A Quattro-Pro® spreadsheet template was written in-house to evaluate the quantification output file from the real-time PCR assay and translate the values directly into a robot worklist for the preparation of appropriate dilutions from the concentrated DNA eluates (DNA normalization). DNA amplification using the AmpF*l*STR® Profiler Plus™ PCR Amplification kit (Applied Biosystems) was carried out in a final PCR volume of 25 μL using 1 ng of DNA as per the RCMP Biology Operations standard procedures for manual processing or in a final PCR volume of 15 μL using 0.5 ng of DNA or less as indicated in the text.

When the sample volume needed for amplification exceeded 6 μL (for the 15 μL PCR reaction) or 10 μL (for the 25 μL PCR reaction), Microcon-100 size-exclusion columns (Amicon Inc., Beverly, MA) or Montage PCR Filter Units (Millipore, Bedford, MA) were used to reduce the volume according to the manufacturer's protocols. Amplifications were set up manually (for 25 μL reactions) in 0.2 mL tubes and carried out in a GeneAmp PCR System 9600 thermal cycler (Perkin Elmer Cetus; Applied Biosystems) or were set up by the robot (for 15 μL reactions) in 96-well plates in a DNA Engine PTC-200 Peltier thermal cycler (MJ Research Inc., Waltham, MA). Only the 15 μL PCR reactions were topped off with 5 μL of oil before being subjected to the following cycling parameters: 95°C, 11 min followed with 28 cycles of denaturation for 60 sec at 94°C, annealing of primers for 90 sec at 59°C and extension for 90 sec at 72°C. A final extension at 60°C for 45 min followed by an overnight incubation at room temperature was also included.

Amplification Conditions for Blank Samples

For the purpose of our validation, regardless of the presence or absence of a Q-PCR signal in the blanks, the eluates from the vast

majority of reagent blank samples were further processed to determine if carryover had taken place during extraction. Initially, blank eluates (25 or 35 μL of low TE pH 7.5) were concentrated to 10 μL using vacuum centrifugation (Eppendorf Vacufuge™; Brinkmann Instruments Inc., Westbury, NY) and amplified in a final PCR volume of 25 μL using 28 cycles of amplification as stated in the previous paragraph.

This approach was discontinued as it was discovered that DNA eluates (derived from a DNA IQ™-based extraction) containing known amounts of DNA failed to produce an STR profile following their concentration using vacuum centrifugation (data not shown). The use of vacuum centrifugation was potentially concentrating an inhibitor of the PCR. It was suspected that EDTA or guanidium isothiocyanate (GTC) or a combination of both reagents may have been responsible for the failure to amplify DNA. Experiments carried out with increased amounts of GTC in the DNA eluates completely inhibited amplification (data not shown). GTC is present in the PLB but also in small concentration in the PWB to maintain DNA binding with the resin throughout the extraction process. Blank eluates tested initially failed to produce STR allele peaks on two different detection platforms (ABI Prism® 377 DNA Sequencer and ABI Prism® 3100 Genetic Analyzer) which may have been the result of PCR inhibition as opposed to absence of carryover DNA molecules. These initial results were not included in this report.

The next approach adopted was to use the maximum volume of the blank eluate (35 or 40 μL) that could be accommodated in the amplification reaction, i.e., 6 μL in a final PCR volume of 15 or 10 μL eluate in a 25 μL PCR volume. This approach was only used for experiments pertaining to wash routine #5 (referred to in Tables 1 and 2) and B&E sample batches processed by the B&E DNA Processing Unit (see Table 2). Alternatively, the blanks processed (60 μL eluate in low TE pH 9.0) were subjected to filtration through Microcon-100 size-exclusion columns (Amicon Inc.) or Montage PCR Filter Units (Millipore) according to the manufacturer's protocols. This approach was adopted for all experiments carried out with wash routines #6 and above (referred to in Tables 1 and 2) including experiments performed using the automated differential protocol (see Table 2). For Microcon-100 filtered eluates, DNA was recovered in a final 8.5 or 12.5 μL of low TE pH 7.5. For Montage filtered eluates, DNA was recovered in 20 μL of low TE buffer pH 7.5. This 20 μL volume was further reduced to 8.5 or 12.5 μL by vacuum centrifugation. In all instances, an aliquot of 2.5 μL was taken for a second real-time quantitative PCR assay and the remaining 6 or 10 μL was added to 9 or 15 μL of cocktail mix for STR amplification in a final PCR volume of 15 or 25 μL.

Analysis of Fluorescently Labeled Amplification Products

Amplicons from the 15 and 25 μL PCR reactions were analyzed either by gel electrophoresis on the ABI Prism® 377 DNA Sequencer or by capillary electrophoresis on the ABI Prism® 3100 Genetic Analyzer.

For gel electrophoresis, aliquots of 2–2.5 μL of amplified products from the 15 μL PCR reaction were extracted in butanol to eliminate oil from the sample and robot tips, mixed with blue dextran/salt before ethanol precipitation and resuspended in 4.2 μL of a cocktail containing formamide, FAD, and GeneScan 500 (in the final proportions of 4:1:1 or 66.6%:16.7%:16.7%) in a 96-well plate. Samples were denatured for 2 min at 90°C, snap-cooled at 4°C for 3 min using the robot cool block, and then aliquots of 0.9 μL were spotted by the robot on 96 tab-membrane combs prior to insertion into 5% Long Ranger acrylamide/6 M urea gels heated to 51°C. Electrophoresis was conducted for 2.5 h at constant voltage (3000 V) in TTE buffer (2X TTE in the upper reservoir and 1X TTE in the lower reservoir) with the laser power set at 40 mW.

For the 25 μL reaction, aliquots of 1–1.5 μL of amplified products were mixed with 4 μL denaturing loading buffer (20 mg/mL blue dextran, 7.3 M urea, 2X TBE, 20 mM EDTA) and 0.5 μL GeneScan 500 in individual tubes. Samples were heated for 2 min at 95°C, snap-cooled at 4°C for 2 min, and a 1.5 μL aliquot was loaded on a 4% (19:1) polyacrylamide gel containing 6 M urea prerun at constant voltage (1000 V) for 30 min and equilibrated to 51°C. Electrophoresis was conducted for 2.5 h at constant voltage (3000 V) in TBE buffer with the laser power set at 40 mW.

A highly discriminating and sensitive test employed to assess carryover in the blanks used a "boosting" strategy (39). The test is essentially the RCMP Biology standard operating procedure for enhancing the fluorescence peak heights of samples. However, for the blanks, the entire extract was used for amplification instead of a small aliquot. The strategy uses an equivalent of 6 μL of the 15 μL amplified extracts or 10 μL of the 25 μL PCR reactions which are vacuum-centrifuged and reconstituted in 4 μL of gel loading buffer. From these sample preparations, a 1.5 μL aliquot (maximum volume capacity of the wells) is loaded on gels and run on an ABI Prism® 377 DNA Sequencer. This volume of amplified extracts analyzed (i.e., 15% of the PCR reaction) is equivalent to the 10X "boosted" aliquot processed occasionally by operational units in the RCMP Biology program.

The lack of cross-contamination was ascertained from an absence of peaks in the 10X "boosted" samples, i.e., when the entire eluate from blank swabs or reagent blanks was filtered through Microcon-100 or Montage units, amplified and 15% of the amplified material loaded on gels.

For analysis using capillary electrophoresis, aliquots equivalent to 0.5 or 1 μL of the amplified material (from the 15 or 25 μL PCR reactions) were mixed by the robot with 0.5 μL GeneScan 500 and 20 μL of HiDi formamide (Applied Biosystems), heated for 2 min at 90°C, and snap-cooled at 4°C for 3 min using the robot cool block. Samples were electrokinetically injected in 10 sec at 3 kV and electrophoresis was carried out at 15 kV and 60°C.

Profile determination was performed using GeneScan Analysis 3.1 and Genotyper 2.5 for samples run on gels. GeneScan Analysis 3.7 and Genotyper 3.7 programs were used for samples run on capillaries. The peak detection threshold used during profile analysis for both the ABI Prism® 377 DNA Sequencer and ABI Prism® 3100 Genetic Analyzer was set at 20 relative fluorescence units (RFUs) to maximize the detection of peaks in all samples including blanks. The peak detection threshold adopted for the interpretation of STR profiles generated during development and validation of the robot tip cleaning routine was set at 20 RFUs. Profiles generated by the B&E DNA Processing Unit were interpreted using a peak detection threshold of 40 RFUs (referred to in Table 2 for wash routine #18).

Results

Tip Cleaning Routines Evaluated Using the Automated DNA Extraction Process with Bead Percolations

A simple wash routine with the robot's system liquid (reverse osmosis [RO] water) has proven sufficient to clean tubing and fixed tips to prevent contamination of convicted offender samples submitted to the National DNA Data Bank (NDDB) of Canada. In this process, only one wash station is used and tips are washed with 2 mL RO water in the waste reservoir of the station and 2 mL RO

TABLE 1—*Wash routines evaluated on the TECAN Genesis RSP series during the development of the automated DNA extraction and automated DNA quantification (Q-PCR) process.*

	"Clean" Wash Station						"Dirty" Wash Station						
Wash Routine Number	Waste Reservoir (mL)	Shallow Reservoir (mL)	Deep Reservoir	MPO* Setting (%)	Flow Rate via FWP (μL/sec)	Waste Reservoir	Shallow Reservoir (mL)	Deep Reservoir (mL)	Flow Rate via Diluters (μL/sec)	MPO* Setting (%)	Flow Rate via FWP (μL/sec)	Bleach in Trough	Comment
1	2	2		15	357								NDDB wash routine
2	4	2		15	357								
3	4	4		15	357								
4	4	4		50	ND								
5	4	4		15	357		5	5	600	OFF			
6	4	4		15	357		5	5	600	OFF		0.2% After extraction and Q-PCR setup	Percolation used in process
7	4	4		15	357		5	5	600	OFF		0.6% After extraction and Q-PCR setup	Septa membrane used in process
8	4	4		15	357		5	5	600	OFF		0.6% After extraction and Q-PCR setup + within extraction	Septa membrane used in process
9	4	4		15	357		5	5	600	OFF		0.6% After extraction and Q-PCR setup + within extraction	Percolation used in process
10	10	10		100	ND		10			100	ND	0.6% After extraction and Q-PCR setup No bleach within process	4 mL delivered in each reservoir
11	5	5		50	ND		5			50	ND	0.6% After extraction and Q-PCR setup No bleach within process	2 mL delivered in each reservoir
12	5	5		50	ND		5			50	ND	0.6% After extraction and Q-PCR setup No bleach within process	Resin manually pipetted, centrifugation steps
13	4	10		15	357		10			15	357	6% After extraction and Q-PCR setup + within extraction	Resin manually pipetted, centrifugation steps
14	4	10		15	357		10			15	357	2% After extraction and Q-PCR setup + within extraction	Resin pipetted by robot, centrifugation steps
15	4	10		15	357		10			15	357	DNA Away™† After extraction and Q-PCR setup + within extraction	Resin pipetted by robot, centrifugation steps
16	4	10		15	357		10			15	357	2% After extraction and Q-PCR setup + within extraction	Resin manually pipetted, centrifugation steps
17	4	10		15	357		10			15	357	2% After extraction and Q-PCR setup + within extraction	TE-shake unit Resin manually pipetted
18	4	10		15	357		10			15	357	2% After extraction and Q-PCR setup + within extraction	TE-shake unit Resin distributed by robot

*MPO = monitored pump option.
†DNA Away™ = commercially available chemical solution tested as a replacement for bleach.

TABLE 2—*Cross-contamination checks performed as the automated process evolved.*

Wash Routine Number	Volume of Blood Used and Blank Sample Layout in Plate	Number of Blanks Showing Real-time PCR Values	Real-time-PCR Values Obtained (ng/μL)	Theoretical Amount of DNA and Final Volume of DNA Extract	Number of Blanks Showing a Ct Value <36	Instrument Used for Profile Detection	STR Results[§]
2	10, 20 or 40 μL Zebra-stripe	59 out of 60	Lowest = 0.010 Highest = 0.130	0.23 ng (25 μL) 2.9 ng (25 μL)	All		Not processed
3	10, 20 or 40 μL Zebra-stripe	13 out of 24	Lowest = 0.010 Highest = 0.020	0.23 ng (25 μL) 0.45 ng (25 μL)	9		Not processed
4	10, 20 or 40 μL Zebra-stripe	17 out of 48	Lowest = 0.002 Highest = 0.020	0.045 ng (25 μL) 0.45 ng (25 μL)	9		Not processed
5	10, 20 or 40 μL Zebra-stripe	5 out of 24	Lowest = 0.002 Highest = 0.005	0.045 ng (25 μL) 0.11 ng (25 μL)	0		Not processed
5 used with "liquid tracking" approach for extraction	10, 20 or 40 μL Zebra-stripe or Middle or end of sample block	9 out of 59	Lowest = 0.001 Highest = 0.020	0.035 ng (35 μL) 0.7 ng (35 μL)	2 33.18 32.37	3100	1 with no profile 1 with partial profile (7 loci, matches sample previously aspirated)
6	10, 20 or 40 μL Zebra-stripe or 0.01 μL to 20 μL or Middle or end of sample block	9 out of 137	Lowest = 0.001 Highest = 0.017	0.035 ng (35 μL) 0.60 ng (35 μL)	3 34.58 34.37 32.66	377 3100	2 with no profile 1 with partial profile (7 loci, matches one of the analysts working on the robot)
7	10, 20 or 40 μL Checkerboard Use of septa during extraction	32 out of 56	Lowest = <0.001 Highest = 0.007	<0.060 ng (60 μL) 0.42 ng (60 μL)	26 16 from one exp. 33.16–35.94 10 from the other 33.56–35.80	3100 377	16 with full or partial profiles (match samples from within batch) 10 with no profile
8	10, 20 or 40 μL Checkerboard Use of septa during extraction	9 out of 24	Lowest = 0.001 Highest = 0.004	0.060 ng (60 μL) 0.24 ng (60 μL)	9 33.60–35.62	3100	9 with full or partial profiles (match samples from within batch)
9	10, 20 or 40 μL Checkerboard or 0.1, 1 or 5 μL Checkerboard or Middle or end of sample block	9 out of 89	Lowest = <0.001 Highest = 0.002	<0.060 ng (60 μL) 0.12 ng (60 μL)	3 35.49 35.16 34.57	3100	2 with no profile 1 with partial profile (matches one of the analysts handling the blanks post-robotically)
10	20 or 40 μL Checkerboard	7 out of 38	Lowest = 0.001 Highest = 0.035	0.060 ng (60 μL) 2.1 ng (60 μL)	4 31.57–35.14	377	4 with full or partial profiles (match samples from within batch)

TABLE 2—*Continued*

Wash Routine Number	Volume of Blood Used and Blank Sample Layout in Plate	Number of Blanks Showing Real-time PCR Values	Real-time-PCR Values Obtained (ng/μL)	Theoretical Amount of DNA and Final Volume of DNA Extract	Number of Blanks Showing a Ct Value <36	Instrument Used for Profile Detection	STR Results[§]
11	10, 20 or 40 μL Zebra-stripe	29 out of 96	Lowest = <0.001 Highest = 0.004	<0.06 ng (60 μL) 0.24 ng (60 μL)	13 33.95–35.94	377	5 with no profile 8 with full or partial profiles (match samples from within batch)
12	10, 20 or 40 μL Zebra-stripe or Middle or end of sample block	22 out of 64	Lowest = 0.001 Highest = 0.011	0.06 ng (60 μL) 0.66 ng (60 μL)	17 33.00–35.91		17 with full or partial profiles (match samples from within batch)
13	20 or 40 μL Zebra-stripe	1 out of 56	<0.001	<0.060 ng (60 μL)	0	377	No profile at 1X or 10X
14	20 μL Zebra-stripe	1 out of 38	0.001	0.060 ng (60 μL)	0	377	No profile at 1X or 10X
15	40 μL Zebra-stripe	22 out of 112	Lowest = <0.001 Highest = 0.004	<0.060 ng (60 μL) 0.24 ng (60 μL)	2 34.30, 35.54	377	Partial profiles at 10X
16	20, 10, 5, 1 μL Zebra-stripe or Casework-like samples* or Animal:human mixture[†] or Human:human mixture[†]	4 out of 91	Lowest = <0.001 Highest = 0.001	<0.060 ng (60 μL) 0.060 ng (60 μL)	0	377	No profile at 1X or 10X
17	10, 20 μL Zebra-stripe or 10–0.01 μL + casework or Animal:human mixture or Human:human mixture	7 out of 120	Lowest = <0.001 Highest = 0.002	<0.060 ng (60 μL) 0.12 ng (60 μL)	0	377	No profile at 1X or 10X
18	20 or 40 μL Zebra-stripe or 10–0.01 μL + casework or Human:human mixture	38 out of 514	Lowest = <0.001 Highest = 0.025	<0.060 ng (60 μL) 1.5 ng (60 μL)	4 32.60–35.48 then none upon re-quantification	377	No profile at 1X or 10X

TABLE 2—*Continued*

Wash Routine Number	Volume of Blood Used and Blank Sample Layout in Plate	Number of Blanks Showing Real-time PCR Values	Real-time-PCR Values Obtained (ng/μL)	Theoretical Amount of DNA and Final Volume of DNA Extract	Number of Blanks Showing a Ct Value <36	Instrument Used for Profile Detection	STR Results[§]
18 B&E batches	Variety of swabs Mainly with blood 46 batches	0 out of 46	0	0	0	377 membrane comb	6 μL maximum volume for PCR; No profile
18 Samples extracted using differential protocol[‡]	Saliva:semen mixtures or Blood:semen or Semen alone or Vaginal:semen or Buccal:semen	46 out of 423	30X at <0.001 10X at 0.001 6X at 0.002	<0.060 ng (60 μL) 0.12 ng (60 μL)	2 35.59 35.95	3100	No profile

*Casework-like samples refer to cigarette butts, chewing gums, blood stains on black denim, blood swabs and trace swabs processed during the development of the automated process (see Materials and Methods).

†For animal: human mixtures and human: human mixtures refer to Materials and Methods.

‡For details on the samples subjected to the automated differential DNA extraction, see Materials and Methods.

§The peak detection threshold used for interpreting the STR results was 20 RFUs for all wash routines with the exception of the B&E batches (wash routine #18) for which a peak detection threshold of 40 RFUs was used. These sample batches represent actual casework batches and a more conservative peak detection threshold was adopted.

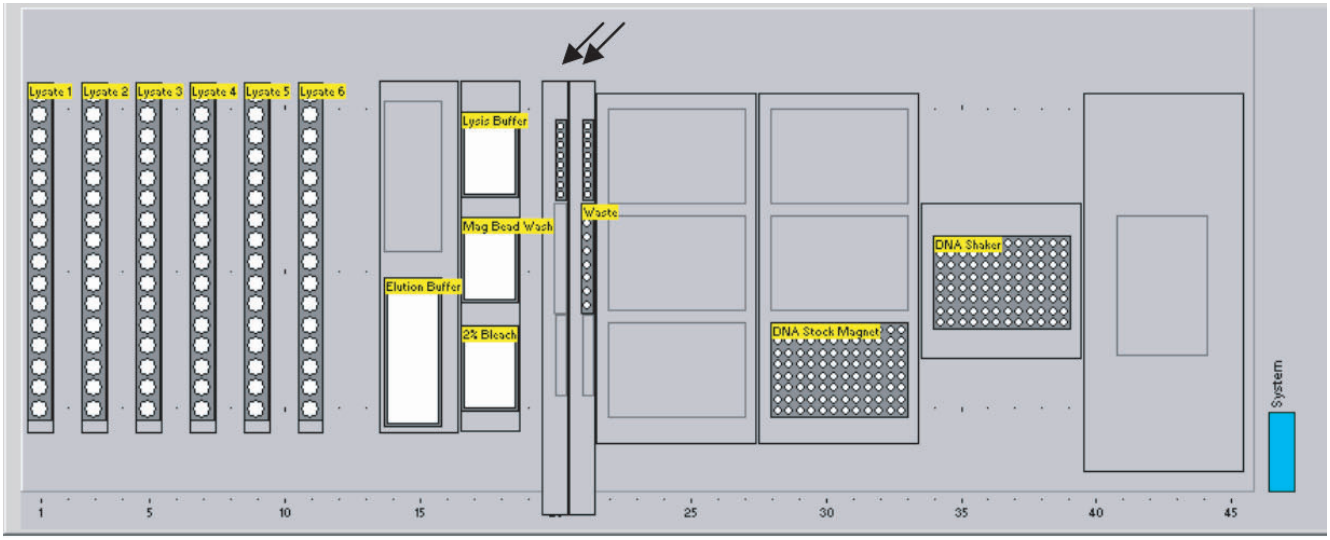
water in the shallow reservoir invoking the Fast-Wash Pump (FWP) with the flow rate set at 15% (actual flow rate calculated at 357 μL/sec [10 mL RO water going through robot tip in 28 sec]). The largest volumes ever pipetted by the robot during the pre-PCR processing steps, i.e., cleaning of the FTA® disks are 100 and 200 μL for the FTA® wash and FAD washes, respectively. The use of a total of 4 mL of RO water dilutes the aspirated liquids by a factor greater than 40 and 20, respectively. This proved to be an effective wash routine as none of the samples processed to date (over 240,000 FTA® punched disks [120,000 for AmpF*l*STR® Profiler Plus™ and 120,000 for AmpF*l*STR® COfiler™]) have shown mixed profiles that could have been attributed to biological material remaining inside the robot tip or tubing that could have been carried over to other samples in the 96-well plate. In addition, the blank FTA® disks included in each sample batch were amplified and always failed to produce an STR result.

For high volume casework samples such as nonsuspect break and enter (B&E) specimens, the wash routine was designed to be more stringent because the biological samples and their substrates are greatly diverse and not submitted on any controlled substrates such as Whatman FTA® Sample Collection paper. The automated DNA extraction, automated DNA quantification, and automated PCR setup incorporate steps where DNA is released into solution and, therefore, travels through the robot tubing and fixed tips. The various wash routines evaluated during the development of the automated process for high volume casework are presented in Table 1. Table 2 summarizes the DNA quantification results for blanks (blank swab plus buffer; see "Sample Preparation" section under Materials and Methods) extracted using the automated process as it evolved and combined with the various wash routines tested. Blank samples with real-time PCR signals were amplified using the AmpF*l*STR® Profiler Plus™ system and the outcome of these amplifications is also indicated in Table 2.

Our initial intent was to adapt the simple wash routine implemented for the NDDB convicted offender samples (processed on standard FTA® Sample Collection Cards) for general casework samples. This was carried out first by increasing the volume of RO water flowing through the tubing and tips to a total of 6 or 8 mL in contrast to the usual 4 mL. As indicated in Table 2 (compare wash routines #2 and #3), these initial trials were unsuccessful at preventing carryover. Many blanks tested showed Q-PCR values (72 out of 84) and the number of blanks with a Ct <36 was still significant (68 out of 72). Based on our internal validation performed using the AB Quantifiler™ Assay on the ABI Prism® 7000 Sequence Detection System, blank samples with a Ct <36 usually showed either complete or partial STR profiles (unpublished data), consequently, significant emphasis was placed on blank samples as an indication of contamination. No further improvement was noted in the cleaning of the tips using a higher FWP monitored pump option (MPO) setting (see wash routine #4).

Experiments performed to determine if the cross-contamination was originating from the DNA extraction process and/or the DNA quantification reaction setup (both carried out on the robotic workstation) indicated that the source of the contamination was the extraction process (data not shown). Consequently, the need for a second wash station was identified. This additional station was labeled the "clean" wash station while the original wash station was used as the "dirty" station (see Fig. 1). A new wash routine was tested (see wash routine #5) which significantly improved the cleaning of the robot line and tips. Only five out of the 24 blanks examined had very low real-time PCR values with corresponding Ct >36 (see Table 2). The incorporation of the "liquid detection"

A. B&E DNA extraction robotic deck layout (two standard wash stations)



B. Wash routine for fixed tips/B&E pre-PCR process

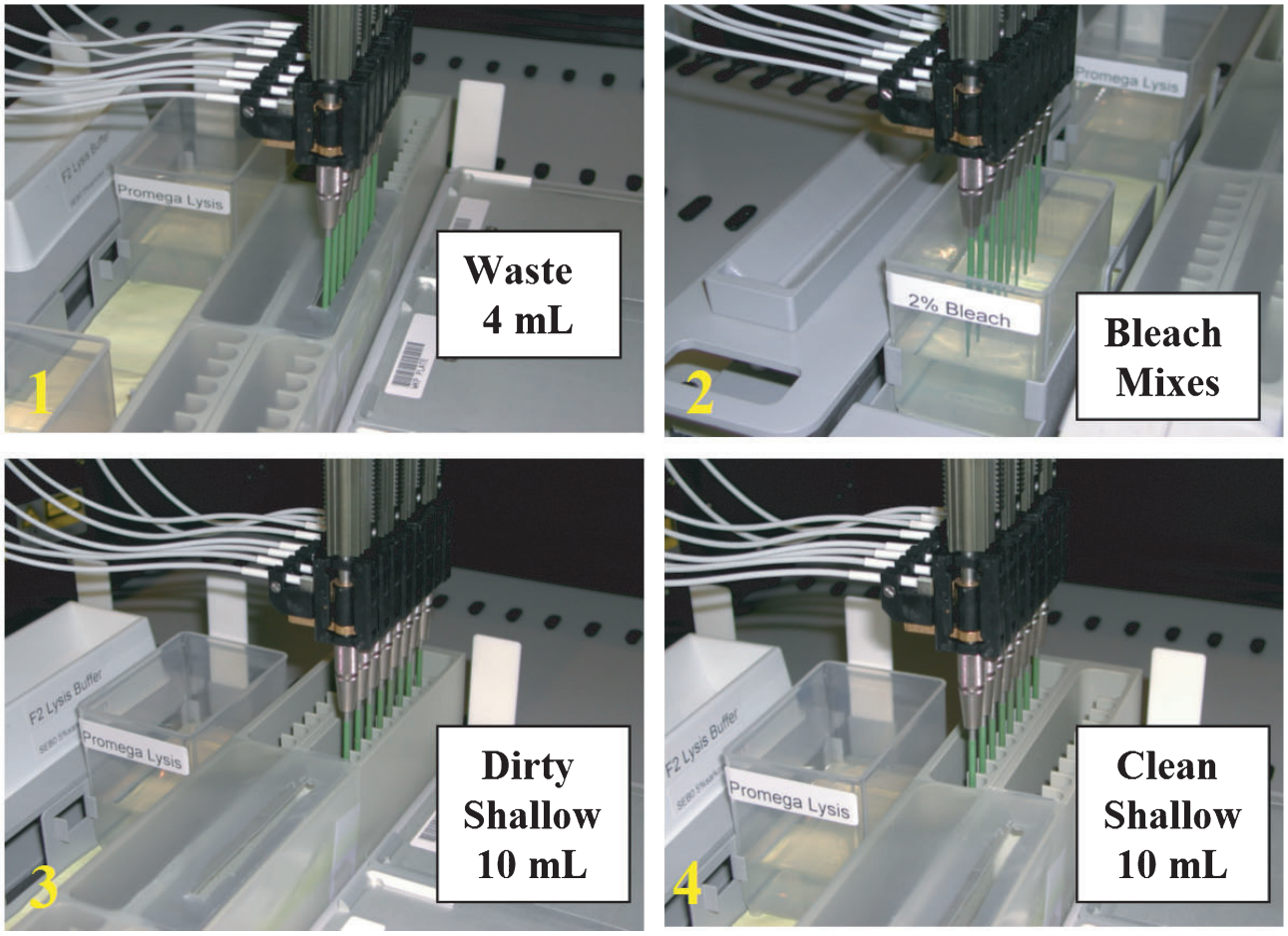


FIG. 1—*Wash routine for the B&E pre-PCR process utilizing Teflon-coated stainless steel fixed tips developed for TECAN Genesis RSP 150/8 robotic workstations. Panel A: Robotic deck layout showing the location of the two wash stations used to wash tips for the automated process developed for nonsuspect B&E cases. Panel B: Current wash routine developed for the B&E automated process (see text for details).*

option in all liquid classes invoked for the aspiration of solutions prevented dipping the robot fixed tips into the lysate column and the wash solution containing DNA reduced the level of cross-contamination even further. Q-PCR signals were noted in blanks 15% of the time (nine out of 59 blank samples processed). This wash routine (#5) was eventually replaced as washing the robot line

and tips without invoking the FWP increased washing time significantly. It could also induce excessive wear and tear over time on the diluter motors and syringes which are expensive to replace. Furthermore, this washing routine when used in combination with major modifications introduced in the automated DNA extraction process to enhance DNA yields and reduce processing time led to elevated carryover. Q-PCR signals were detected in 44 out of 89 (50%) blanks processed in these experiments (data not shown).

Other wash routines were evaluated using either 0.2% or 0.6% sodium hypochlorite (stock from the manufacturer at 10.8%) within the extraction process, i.e., during lysate transfer (from sample tube to DWP) and at lysate column removal (from DWP to waste reservoir) and/or after extraction and Q-PCR setup (see wash routines #6–#9). The new wash regimens were evaluated in combination with the use of a 96-well plate septa membrane (Innovative Microplate, Chicopee, MA) to reduce the extraction process by permitting the simultaneous mixing of all samples in a batch during the DNA binding step onto the magnetic beads. Although the use of the septa reduced the extraction process by at least 50 min, the ability to seal the plates properly was questionable based on the increased number of contaminated blanks noted in the experiments carried out. As shown in Table 2, marginal Q-PCR values were still noted in many of the blanks processed and a large number of blanks ($n = 41$) showed Ct <36 using these washing conditions. Many of these blanks produced complete or partial STR profiles (matching either samples from within a batch or the analyst mending the robotic workstation) when processed on the ABI Prism® 377 DNA Sequencer or ABI Prism® 3100 Genetic Analyzer.

Further modifications were made to the washing conditions (see Table 1, routines #10 and up) including the following: (i) the FWP program was altered to run at 100% or 50% capacity instead of the usual 15%, (ii) the shallow wash stations were exclusively used throughout the process and the deep reservoir condemned from use, and (iii) the liquid classes and volumes were modified to eliminate foaming during lysate transfer, lysate removal, and waste dispenses. In these experiments, the 0.6% bleach step within the extraction process was not included but the bleach steps normally set after the extraction session and after the Q-PCR setup were retained. As noted in Table 2, several blanks gave positive signals for the real-time quantification assay with 17 blank samples showing a Ct <36 with many of these producing complete or partial profiles.

During our studies, a crucial observation was made that had a tremendous impact on the design of the automated DNA extraction process. After replacing a plugged tip, magnetic beads were observed at the junction where the robot tip and the plastic tubing connect (a bead-like suspension that was attracted to a magnet). This material (magnetic beads/DNA complexes) was identified as a possible source of contamination in some blanks. As shown in Fig. 2, the current fixed tip design with a narrower upper portion and a wider lower portion of the tip head makes it easier to connect the plastic pipette tubing to a tip upon replacement. However, the small space at the junction of the robot tubing and tip is a potential source of significant contamination using our current extraction process. A potential option to eliminate carryover was to avoid having magnetic beads in the lines at any time during the extraction process. This implied having to either manually pipet the magnetic resin into the DWP at the beginning of the process or having the resin pipetted by the robot using single aspirations with a maximum aspiration volume of no more than 35 µL (the maximum a fixed tip can hold for the specific fixed tips that are used in our process) in order to avoid the junction. As additional precautions, a centrifugation step was incorporated before initiating a new bead percolation and before collecting the lysate column and the bead wash solution in order to pellet any fine magnetic material. It was noted during our investigations that the paramagnetic beads included in the Promega DNA IQ™ kit may not be uniform in size (C.J. Frégeau and C.M. Lett, personal observations). It is not known how this shape or size variation could affect the final yield of DNA recovered or how it could present challenges with accumulation at key tubing/tip interfaces.

The experiments carried out under new parameters (resin manually pipetted to plate and centrifugation steps included) and wash routine #12 revealed that 22 out of 64 blanks tested showed positive Q-PCR values with 17 showing Ct <36 (Table 2). All 17 blanks produced complete or partial STR profiles. This approach, despite all precautions taken, was unsuccessful at preventing contamination. It became apparent that a more stringent wash routine was required in order to eliminate cross-contamination. The 0.2% or 0.6% bleach routines as detailed in previous experiments appeared effective in reducing carryover when used within the process but could not completely eliminate the contamination.

As a means to increase the stringency of the wash routine, a new 6% sodium hypochlorite wash (1.8-fold dilution of the 10.8% bleach stock) was strategically incorporated within the extraction process for every aspiration from the plate where DNA could be present (i.e., at lysate transfer, after each percolation, at lysate column removal, at lysis wash solution removal). The bleach wash steps incorporated after the extraction session and after Q-PCR setup were also carried out using a 6% bleach solution (wash routine #13). The optimized bleach process directed the robot tips to the waste reservoir first after the transfer of the lysate from the sample tube to the DWP to get rid of the remaining lysate in the tips, then to the bleach trough for a few mixes (5×400 µL) followed by the "dirty shallow" reservoir to remove any residual bleach solution and the "clean shallow" reservoir for optimal cleanup (Fig. 1). This approach reduced the chance of an exothermic reaction creating foam at the surface of the trough containing the 6% bleach solution and prevented rapid neutralization of the bleach. Liquid detection and tracking were used for every aspiration in the washing process and reduced exposure of the tips in the bleach solution by minimizing exterior surface contact to only a few millimeters. The interior walls of the tips were washed with a volume of bleach solution equivalent to the volume of specific solution aspirated in the previous step in the process. For example, if 400 µL lysate was transferred to the DWP at "lysate transfer," tips were washed using 400 µL of bleach incorporating mixing. After each percolation, tips were washed using 600 µL of bleach incorporating mixing as half the lysate/PLB liquid column was aspirated. At "lysate column removal," tips were washed using 1000 µL of bleach incorporating mixing as the entire liquid column was aspirated. At "lysis wash removal," tips were washed using 250 µL of bleach incorporating mixing. The bleach wash step was not incorporated at "wash solution removal" since the large majority of DNA was bound to the beads and not free in the wash solution. The design of an efficient automation process must take into account the length of time required to perform the task and the desired goal. The incorporation of a bleach wash at every step in the process would lengthen the process with no additional enhancement of quality. However, as an additional precaution, parafilm was used to cover the wash stations to prevent aerosol contamination of samples that were placed in proximity of the wash carriers on the robotic worktable.

Collectively, these modifications involving the washing conditions (routine #13) were implemented and were found to be very effective in preventing cross-contamination. Only one blank sample out of the 56 tested gave a marginal signal (<0.001 ng/µL) with a

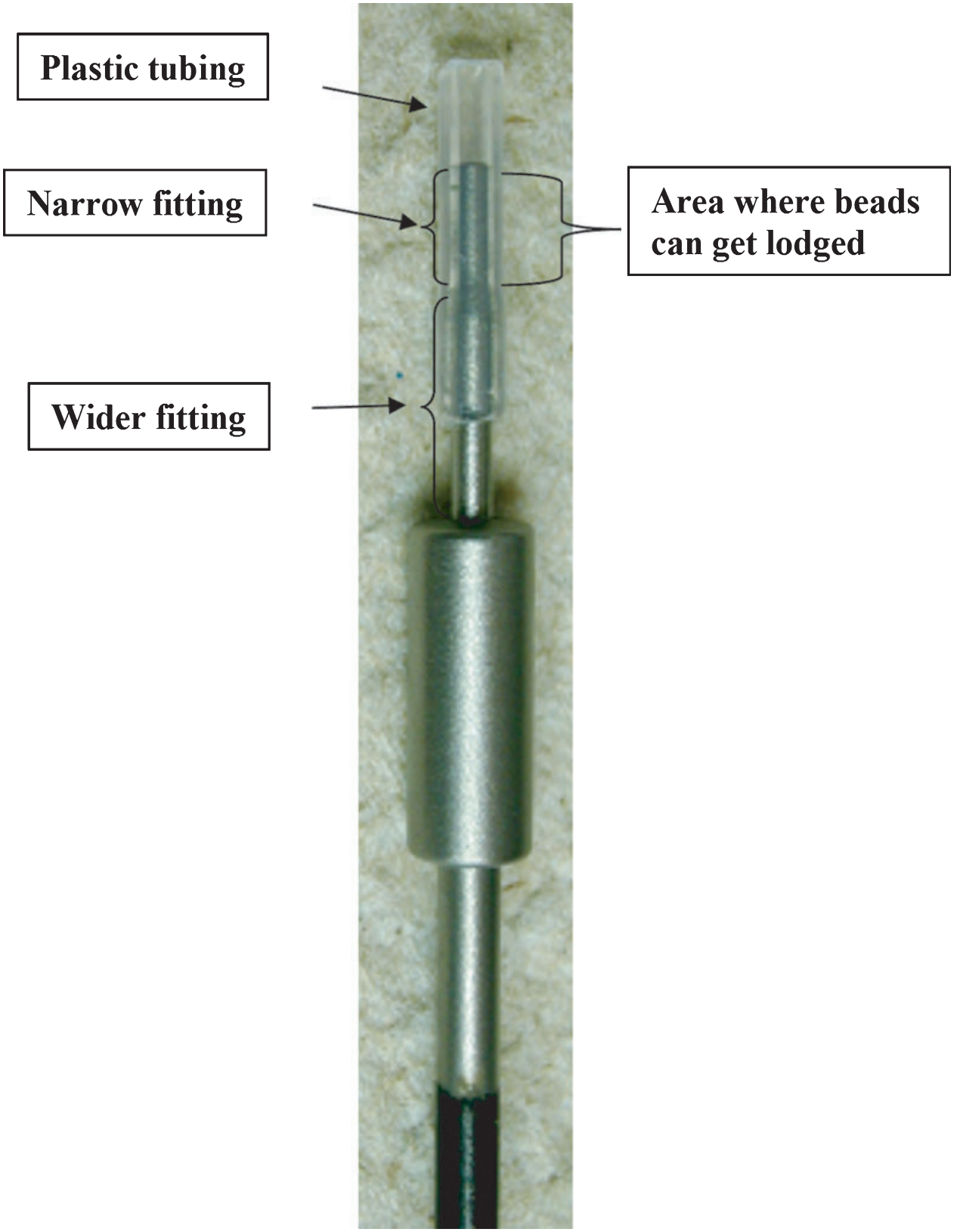


FIG. 2—*Tapered end of the low-volume Teflon-coated stainless steel tips used in the automated DNA extraction. Magnetic beads were noted at the junction of tubing and tip.*

Ct >36 when subjected to Q-PCR. No STR profile was derived from this sample using a 1X or 10X boosted aliquot of the amplified reaction on the ABI Prism® 377 DNA Sequencer.

Automation development should also consider sustainability of process by reviewing maintenance issues and long-term adverse problems introduced by repetitive action or exposure to reagents.

The corrosive 6% sodium hypochlorite solution could potentially damage the Teflon-coated steel tips over time. Consequently, the effectiveness of a 2% bleach solution to eliminate carryover was also evaluated. To enhance the automation process, robotic pipetting of bead slurry was evaluated. The magnetic beads were dispensed from tubes to the DWP by the robot ensuring that the bead volume aspirated was below the tubing/tip interface to prevent contamination. As shown in Table 2 for wash routine #14, only one blank sample out of the 38 tested gave a marginal signal (0.001 ng/μL) with a Ct >36 when subjected to Q-PCR. No STR profile was derived from this sample using a 1X or 10X boosted aliquot of the amplified reaction on the ABI Prism® 377 DNA Sequencer. The 2% bleach solution appeared to be as effective as the 6% bleach at preventing carryover.

It was noted that a systemic repetition of bead aspiration could potentially introduce contamination concerns due to bead shape and lack of uniformity. Over time, i.e., after five subsequent extraction sessions, some beads were detected in the bleach trough likely due to the initial mixes of the beads (8 × 150 μL) carried out by the robot before aspirating single bead aliquots for the DWP. The paramagnetic beads from Promega Corporation settle down very quickly at the bottom of tubes and it was crucial to incorporate these initial mixes to ensure bead homogeneity before their distribution by the robot to each well of the plate. These observations and the analysis of the blank samples tested during these experiments demonstrated that the creation of a magnetic bead suspension that could be confidently pipetted by the robot without contamination would require extensive trial and error development. This step could be performed in a timely manner with complete reliability by manual hand pipetting once in the first stage of the DNA extraction process. An important consideration that should be noted with all automation development for a DNA process is that not all steps need to be performed by the robot. The risk of achieving the desired end result must be balanced with the time and effort required to achieve success and the overall gain in efficiency for the system.

One other commercially available nucleic acid neutralizing chemical, i.e., DNA Away™ (Molecular BioProducts Inc., San Diego, CA), was also evaluated as a replacement for the bleach solution. As shown in Table 2 for wash routine #15, many blanks turned out positive for Q-PCR and showed partial profiles on the ABI Prism® 377 DNA Sequencer using the "boosting" approach. Preliminary results using DNA Away™ suggested that this chemical may not be as stringent as bleach in eliminating DNA based on our robotic configuration and automated process. One explanation for this weak performance is the fact that DNA Away™ may require several minutes to react with the DNA and, when used on the robot, it sat in the lines very briefly.

In summary, the final automated extraction protocol incorporated a manual dispense of the beads into the DWP, centrifugation steps (ramp to 1500 rpm [317 *g*], 10 sec) before each percolation and (ramp to 4000 rpm [2254 *g*], 20 sec) after the final percolation and the use of the 2% sodium hypochlorite within the process as well as after the extraction session. The bleach step following Q-PCR setup was also retained as an adequate precaution for contamination prevention. This protocol was tested on 11 separate experiments involving a variety of samples (animal:human mixtures, human: human mixtures, casework-like samples [cigarette butts, chewing gums, swabs from pop cans, blood swabs ± soil, blood stains on denim]). As shown in Table 2 for wash routine #16, four blank samples out of 91 gave marginal signals (<0.001 or 0.001 ng/μL) with none showing a Ct <36. No STR profiles were derived from these samples using a 1X or 10X boosted aliquot of the amplified reaction on the ABI Prism® 377 DNA Sequencer and a peak detection threshold of 20 RFUs. STR profiles generated from any of the biological samples tested were consistent with expected results (see examples in the section below that presents the quality of the STR profiles generated following the use of the 2% bleach tip cleaning routine).

Evaluating the 2% Bleach Tip Cleaning Routine Using the Automated DNA Extraction Process Incorporating a TE-Shake™ Unit

The final automated DNA extraction process as detailed previously was carried out in 3.5 h for a full sample batch (88 samples) with more than 45 min of bead percolation conducted in four separate steps. To reduce percolation time, an orbital mixer from TECAN, i.e., the TE-Shake™ was evaluated. Constant shaking created a uniform suspension of beads in all wells of the plate which enhanced DNA binding and eliminated time-consuming multiple percolation steps and associated tip flushing and cleanup. A single centrifugation step following the 15 min DNA binding step was all that was necessary to effectively pellet the beads and fine particles thereby preventing potential subsequent plugging at tubing/tip interfaces.

To verify that the established robot tip cleaning routine (wash routine #17) was effective with the modified extraction protocol using the TE-Shake™ unit, a series of experiments were set up using the zebra layout for swabs with large volumes of blood (10, 20 μL). In addition, casework-like samples (cigarette butts, chewing gums, swabs from pop cans, blood stains on black denim, blood swabs ± soil) and animal:human or human:human blood mixtures were used to determine the robustness of the bleach routine. In these experiments, blank samples were either positioned in the plate according to the zebra-stripe or checkerboard format or at the center or the end of the sample batch. As shown in Table 2 for wash routine #17, seven out of 120 blanks processed in 19 separate experiments showed marginal Q-PCR values following quantification and no blanks had a Ct <36. No STR profile was derived from any blank samples even when using a 10X boosted aliquot of the amplified reaction. The 2% bleach routine developed appeared robust. It was noted that many blanks that had a marginal Q-PCR value (i.e., 0.0003 ng/μL) failed to yield a positive result with subsequent retesting.

Additional modifications were incorporated in the TE-Shake™-based extraction protocol to further reduce processing time as well as limit human intervention to promote quality assurance. The bead dispense was modified to involve the robot as well as the TE-Shake™ unit for an efficient suspension of the beads before their distribution to the wells. The appropriate volume of beads for an entire row was manually pipetted in each well of the last column (wells A12-H12) of a DWP at the beginning of the process, the plate was then put on the TE-Shake™ unit and while the beads were uniformly mixing on the shaker, the robot (eight tips working simultaneously) aspirated the appropriate volume of resin from the last column of the plate and dispensed it into each well of the plate. The small volume of resin pipetted at any one time by the robot tips did not cross the tubing/tip junction where the beads could get lodged and create problems. These washes were then performed using a multidispense option to speed up buffer distribution to the wells. Blank samples were included in all experiments carried out using this modified protocol. Blanks were positioned in the sample plate according to the zebra-stripe format or in the center of the batch. As shown in Table 2 for routine #18, 38 blanks out of 514 blanks extracted in 18 separate experiments showed marginal Q-PCR values following quantification. On subsequent

quantifications, four blanks out of the original 38 turned out positive again with marginal values using either straight aliquots of the eluates or the concentrated eluates (data not shown). None had a Ct <36. Interestingly, one blank that was shown to be negative when subjected to two subsequent quantifications, gave a marginal value in the third assay and turned out negative when the concentrated eluate was subjected to Q-PCR (data not shown). Noteworthy, the amplification plots for blanks with marginal Q-PCR values were not indicative of the presence of DNA and rather revealed unexpected patterns (data not shown). One possible explanation for the marginal and spurious Q-PCR results would be the instability of the fluorochrome marker and subsequent release from the probe following high levels of amplification (more than 36 cycles and up to 40 cycles). As complementary evidence of such possibility, no STR alleles were detected for any of the blanks tested using a peak detection threshold of 20 RFUs even after 40% of the amplification reaction (i.e., 6 μL of the 15 μL volume containing the entire concentrated blank eluate) was vacuum-centrifuged, reconstituted in 4 μL of gel-loading buffer from which a 1.5 μL aliquot (15% of the original PCR reaction) was run on the ABI Prism® 377 DNA Sequencer. This data indicated that blanks were not contaminated with DNA and suggested that marginal Q-PCR values with a Ct very close to 36 should be interpreted with caution. These results confirmed the effectiveness of the robot fixed tip cleaning routine using 2% bleach.

Evaluating the Effectiveness of the 2% Bleach Tip Cleaning Routine for the Automated Processing of B&E Samples and Sexual Assault Samples Submitted for Automated Differential DNA Extraction

The effectiveness of the optimized robot fixed tip cleaning routine was further established when the results of 46 batches of samples processed by the B&E DNA Processing Unit were examined. The automated protocol implemented for B&E sample batches does not incorporate the use of a TECAN Vacuum Separation Module (Te-VacS) to concentrate DNA eluates before PCR setup. Therefore, the maximum volume of the 40 μL blank eluate that can be accommodated in the 15 μL amplification reaction is 6 μL. As shown in Table 2 for the B&E sample batches, none of the blanks processed showed Q-PCR values. No STR products were detected on the ABI Prism® 377 DNA Sequencer using a peak detection threshold of 40 RFUs (more conservative threshold used in actual sample batches) when the maximum eluate volume of 6 μL was used for amplification.

The optimized robot fixed tip cleaning routine was also incorporated into our automated differential DNA extraction protocol with great success. As shown in Table 2 for differential extracted sample batches, 46 blanks out of 423 blanks extracted in 33 separate experiments carried out during the development and validation of the protocol showed marginal Q-PCR values following quantification. Two of these blanks had Ct values that were very close to 36 cycles (i.e., 35.59 and 35.95). None of the blanks processed manually produced STR products when using the entire eluate (i.e., 60 μL eluates filtered through Montage PCR Filter Units as detailed in Materials and Methods) for amplification and a peak detection threshold of 20 RFUs.

Evaluating the Effect of Using a 2% Bleach Tip Cleaning Routine on DNA Yields and Quality of STR Profiles

Although the cross-contamination issues were addressed using the robot tip cleaning routine with 2% bleach within the process, it was important to establish that the use of such a solution would not have any deleterious effect on DNA yields detected by the Q-PCR assay. This was particularly true for samples with low amounts of DNA. Eight series of 24 biological samples were prepared as stated in Materials and Methods and the use of 2% bleach at lysate transfer, lysate column removal, and lysis wash solution removal was omitted for four of the series. Table 3 revealed that the use of 2% bleach within the extraction process did not have any adverse effect on the amount of DNA recoverable from large (10 μL) or small (1, 0.1, 0.05, 0.01 μL) blood aliquots (swabs or stains), cigarette butts, chewing gums, and trace swabs. DNA yields calculated for all samples extracted in the presence of bleach were very similar to those obtained from the same samples extracted without bleach. This was especially true for samples prepared in the laboratory such as the blood swabs and blood stains. The large standard deviations noted for the 0.01 μL blood swabs ± soil could be attributed to the variations in the aliquots taken from the blood dilutions prepared for this experiment (10 day-old blood stored in the fridge). More variation was observed between the +/− bleach series for cigarette butts, chewing gums, and trace swabs which undoubtedly would present different amounts of genetic material just by their nature.

These results indicated that the use of bleach was not accompanied by a loss in DNA yield. Equally as important was our finding that equivalent quality of results was noted between DNA extracted with the bleach wash steps as compared to without bleach. STR profiles were of high quality and had balanced alleles across all loci (see Fig. 3).

Comparison Between Manual and Automated Processing of Samples Using Fixed Tips or Disposable Tips

As shown in Table 4, manually processing a full batch of samples (i.e., 88 samples) through DNA extraction (magnetic bead-based protocol), DNA quantification setup, and PCR setup required approximately 11 h (manual process B, see table legend for details). Processing the same number of samples using the manual process A, i.e., extracting using phenol/chloroform, setting up samples for slot blot quantification, then setting up for PCR reactions, took 12 h (see table legend for details). Processing 88 samples using the automated process adapted for the TECAN Genesis RSP 150 workstation equipped with fixed tips (DNA extraction with the TE-Shake™ unit, DNA quantification setup, and PCR setup; automated process B) took approximately 4.25 h including sample tracking and incorporating the optimized tip washing routine detailed in this report. Processing 88 samples using the automated process (DNA extraction with bead percolations, DNA quantification setup, and PCR setup; automated process A) required approximately 5.25 h including sample tracking and incorporating the optimized tip washing routine. The use of a TECAN Genesis RSP 150 workstation equipped with disposable tips to process 88 samples through DNA extraction with bead percolations and DNA quantification setup was estimated at 4 h excluding the automated PCR setup (not performed robotically; E. Alimkulov, RCMP FLS-Vancouver, personal communication). This estimated time includes some time spent emptying the disposable tips in the waste reservoir of the wash station after an aspiration before their ejection into the biohazardous waste container. Taking into account the DNA extraction and quantification steps only, the estimated difference in processing time between fixed tips and disposable tips is not significant (about 30 min) but the processing cost using disposable tips is significantly greater than that of fixed tips. An additional 51–55 US$ per sample batch (88 samples) would be needed just to cover

TABLE 3—*Effect of 2% bleach on DNA yields.*

Sample Type	Number of Samples Used for Each Condition Tested (*n*)	Average Total Amount of DNA (ng ± SD)			
		250 µL Lysate		350 µL Lysate	
		− Bleach	+ Bleach	− Bleach	+ Bleach
Blood swab 10 µL	4	165.0 ± 37.0	232.5 ± 132.2	170.0 ± 39.2	160.0 ± 24.5
Blood swab 1 µL	4	14.8 ± 1.5	24.5 ± 5.3	8.4 ± 1.5	18.0 ± 2.9
Blood swab 0.1 µL	4	0.91 ± 0.41	0.80 ± 0.53	0.69 ± 0.27	0.57 ± 0.15
Blood swab 0.05 µL	8	0.59 ± 0.24	0.35 ± 0.27	0.17 ± 0.11	0.35 ± 0.20
Blood swab 0.01 µL	12	0.15 ± 0.12	0.08 ± 0.09	0.04 ± 0.05	0.13 ± 0.12
Blood swab 10 µL + soil	4	119.3 ± 42.1	113.3 ± 46.1	105.5 ± 20.0	94.0 ± 19.6
Blood swab 1 µL + soil	4	4.8 ± 2.0	7.9 ± 2.4	10.1 ± 4.9	9.9 ± 5.5
Blood swab 0.1 µL + soil	4	0.49 ± 0.34	0.24 ± 0.21	0.36 ± 0.19	0.41 ± 0.09
Blood swab 0.01 µL + soil	4	0.06 ± 0.05	0.08 ± 0.02	0.05 ± 0.04	0.26 ± 0.22
Blood stain on black denim 10 µL	4	78.5 ± 18.4	134.5 ± 48.2	138.0 ± 56.6	155.5 ± 63.4
Cigarette butts	4	49.5 ± 32.1	42.8 ± 19.4	25.2 ± 12.3	116.0 ± 153.6
Chewing gums	4	156.8 ± 59.3	326.8 ± 138.8	322.5 ± 78.5	222.5 ± 28.7
Trace swabs (pop cans, water bottles)	4	18.0 ± 8.4	61.8 ± 102.9	7.9 ± 4.9	27.0 ± 8.0

the cost of disposable tips required during the automated DNA extraction alone (456 tip changes × 0.112 US$ to 0.122 US$ per filtered tip [1000 or 200 µL]). Laboratories equipped with robotic workstations capable of re-using disposable tips during specific steps of the process have the potential to reduce the cost of plastic consumables.

Additional Cleaning Routines to Prevent Contamination During Automated Processing

The 2% bleach washing step performed after a DNA extraction session and after DNA quantification setup was implemented to eliminate any trace of DNA before moving to the next step in the protocol (see Fig. 4). This bleach routine takes approximately 15 min to execute and is easily incorporated in the normal workflow. The routine is executed while DNA is eluted off the magnetic beads for 16 min at 65°C or while aliquots of the DNA eluates are placed in the ABI Prism® 7000 SDS unit for real-time quantification. This inter-process bleach routine may be considered superfluous for some clinical diagnostic applications but was considered important to carry out for forensic DNA processing in order to justify the final results when processing limited evidence from criminal investigations.

The robotic workstations are also cleaned thoroughly every week with a mild detergent (RoboScrub; TECAN, Raleigh, NC). This cleaning routine includes a 20 min soak period for carboys, tubing, tips, and wash stations. It takes approximately 40 min to run and is normally executed early in the week or late in the week.

A robot tip check routine is run before the first session on any robot. It verifies that the tips are not plugged and flushes the system to remove any air bubbles that could be lodged in the system's tubing. To minimize bubble formation, the 20 L carboy filled with RO water that feeds the robotic workstation has been placed about 3 feet off the bench counter on a custom-built shelf. This robot tip check routine also checks for 2 and 20 µL volumes dispense (visual check by the analyst). A separate precision check routine is also carried out daily before any Q-PCR setup session on the robot. If the expected level of precision was not achieved, the necessary adjustments are made (e.g., replacement of valves or adjusting syringes) before proceeding with the pipetting of the 2.5 µL volume for real-time PCR quantification.

A summary of the various tip checks and wash routines employed during the automated pre-PCR and post-PCR processes for B&E samples is presented in Fig. 5.

Discussion

The use of robotics in clinical or forensic laboratories offers key advantages regarding sample throughput, quality assurance, and safety considerations. When adopting robotics with fixed tips, the design of thorough cross-contamination tests for a specific application and appropriate tip washing routines should promote the reliability and accountability of the results generated using the automated approach.

The data presented in this report indicate that bleach is required to prevent carryover when processing biological samples on a liquid handling robotic system equipped with nondisposable tips. Wash routines utilizing forceful flushing of the tubing and tips using only the system's liquid (RO water) were not sufficient and signs of contamination were noted in the STR profiles derived from the DNA samples processed robotically. This was true despite the full optimization of the process including the magnetic bead dispense. The 2% sodium hypochlorite solution effectively destroys any remaining DNA in the robot line and tips and RO water used in high volumes dilutes the traces of bleach remaining in the tubing/tips. The 2% bleach routine has been used on a few of our robotic workstations for the past 5 years and, to date, no sign of corrosion has been noted on or inside the Teflon-coated steel tips. Fixed tips typically are replaced only when deemed necessary i.e.,

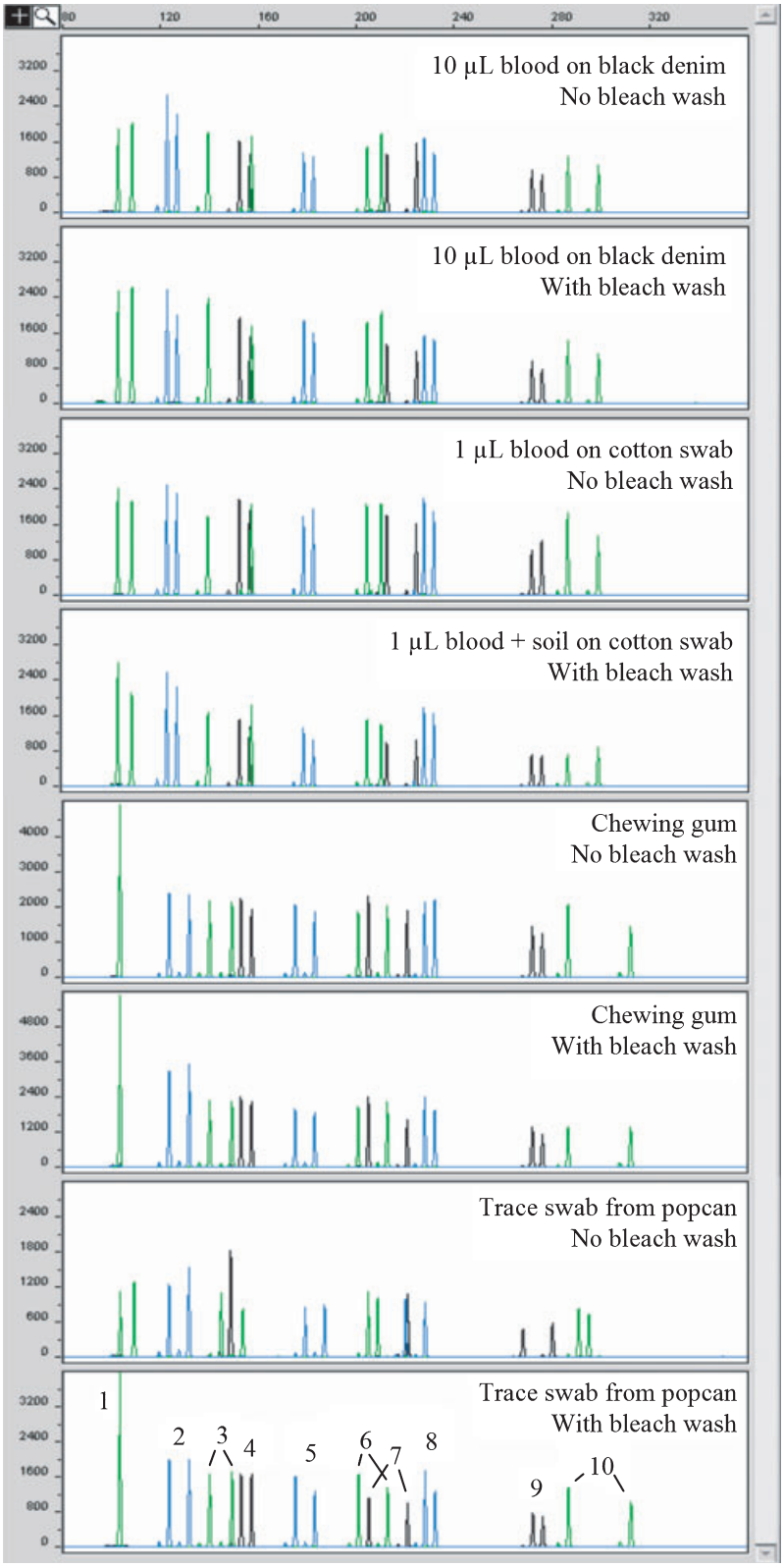


FIG. 3—*Electropherograms showing the AmpFlSTR Profiler Plus™ profiles derived from DNA extracted from various casework-like samples using the automated process incorporating or not the 2% bleach steps within the DNA extraction. PCR amplifications were performed using 0.5 ng of template DNA in a 15 µL PCR reaction volume as detailed in the Materials and Methods section. Amplified products were analyzed on an ABI Prism® 3100 Genetic Analyzer as detailed in the Materials and Methods section. Each panel depicts the relative fluorescence intensity (RFU,* Y*-axis) and the size estimate in bases (*X*-axis) derived from the internal lane standard GeneScan-500 [ROX] using the ABI GeneScan Analysis version 3.7 software. The genetic markers observed from left to right, labeled 1–10, are: Amelogenin, D3S1358, D8S1179, D5S818, HumvWA, D21S11, D13S317, HumFGA, D7S820, and D18S51.*

TABLE 4—*Estimated processing time using the manual versus automated protocol (disposable or fixed tips).*

Process	Processing Time for a Full Batch (n = 88 Samples)		
	Extraction* (h)	Quantification Setup (h)	PCR Setup (h)
Manual			
Process A	5	4	3
1) Phenol-chloroform DNA extraction[†]			
2) Slot blot DNA quantification setup[‡]			
3) Preparation of DNA dilutions or filtration of samples and PCR setup			
Process B	7	1	3
1) Magnetic bead-based DNA extraction[§]			
2) AB Quantifiler™ real-time DNA quantification setup[¶]			
3) Preparation of DNA dilutions or filtration of samples and PCR setup			
Automated			
Process A	4	0.5	0.75
1) DNA extraction (bead percolations) using fixed tips plus bleach within extraction including sample tracking			
2) AB Quantifiler™ real-time DNA quantification setup[¶]			
3) Preparation of DNA dilutions and PCR setup			
Process B	3	0.5	0.75
1) DNA extraction (TE-Shake™ unit) using fixed tips plus bleach within extraction including sample tracking			
2) AB Quantifiler™ real-time DNA quantification setup[¶]			
3) Preparation of DNA dilutions and PCR setup			
Process C	3.25	0.70	ND
1) DNA extraction (bead percolations) using disposable tips including sample tracking			
2) AB Quantifiler™ real-time DNA quantification setup[¶]			

*The extraction excludes sample lysis (overnight at 56°C).

[†]The manual organic extraction was carried out on a batch of mock-questioned samples destined for ''direct'' extraction, no tracking carried out (RCMP Laboratory Information Management System and RCMP ''PCR forms'' not used) and the analyst had access to at least two or three microcentrifuges.

[‡]The 1 h exposure time is not included.

[§]The manual DNA IQ™ extraction was carried out on a batch of mock-questioned samples destined for ''direct'' extraction, no tracking carried out and the analyst was only able to process 12 samples simultaneously due to a limited access to magnetic stands.

[¶]The 1 h 46 min running time is not included.

when bent or plugged. In our situation, a few tips had to be replaced during the development of the automated DNA extraction process due to the presence of soil trapped inside. The convicted offender set of tips used to process FTA® Sample Collection Cards have been used in continual operation for 7 years with no loss of quality.

Bleach (6–10% sodium hypochlorite commercial stock) has been recognized as one of the best chemical disinfectancts available. It effectively destroys nearly all disease-producing micro-organisms (40–42) and causes oxidative damage to nucleic acids (43–47). As such, bleach has often been used to eliminate PCR carryover, i.e., inadvertent transfer of amplified DNA into a DNA sample waiting to be amplified (46,47). Bleach-treated DNA molecules are not proper targets for the Taq DNA polymerase in subsequent PCR reactions. Sample to sample carryover is a primary concern in forensics. The consequence of an inadvertent transfer of biological fluid or DNA from one sample to another while being processed on a robotic workstation would have major repercussions. It is therefore important to design efficient routines that can be incorporated easily in the automated process to ensure the integrity of results.

Based on the data presented in this report, a concentration of 2% sodium hypochlorite appears to be sufficient to prevent contamination when properly incorporated into the robot worklist with appropriate volumes to cover the contaminated inner and outer portions of the fixed tips. It was shown to be effective with samples of various DNA concentrations but also with samples that represent the highest DNA concentrations likely to be submitted for DNA profiling. Lower sodium hypochlorite concentrations, i.e., 0.2% and 0.6%, appeared insufficient at preventing sample carryover. As no corrosion has been noted in 5 years of use of the 2% sodium hypochlorite solution, it is preferable to have higher concentrations when processing forensic samples of unknown origin and concentration. Various concentrations of bleach have been used previously for eradicating disease transmission (6% sodium hypochlorite, soak; 41), for eliminating PCR carryover (2–10% bleach, overnight soak; 46), and for decontaminating the surface of ancient bones to get rid of exogenous nucleic acids (6% sodium hypochlorite, 15 min; 47). Laboratories engaged in automation that utilize robotic workstations equipped with nondisposable tips have adopted different approaches to prevent carryover. Researchers have used an excess volume of water to flush out potential contaminants (7,19,36,48–52) or have used a diluted version of RoboScrub (TECAN product) for cleaning the tips (53). Some researchers have also incorporated a 0.5% or 1% sodium hypochlorite tip wash step at the start of each day as a preventive measure to potential carry-over (7,36). These tip washing routines proved to be sufficient to prevent contamination for high throughput DNA databasing (19), high throughput plasmid DNA isolation for sequencing (52), robotic chromatography (50), biopharmaceutical applications (53), or clinical diagnostic applications (36,48,51). The effectiveness of a wash routine is dependent on the sensitivity of the test for which it is applied. For casework samples, an excess volume of water to flush out potential contaminants was shown to be insufficient at preventing contamination. Forty percent of the amplification reaction containing the entire bead eluate (filtered through a Montage membrane) was purposely used in the preparation of samples for gel analysis and an aliquot (representing 15% of the PCR reaction) was run on gels to ensure that no DNA remained in the robot tubings or tips.

In studies published on automation in the forensic field, investigators have used disposable tips to reduce concerns for potential

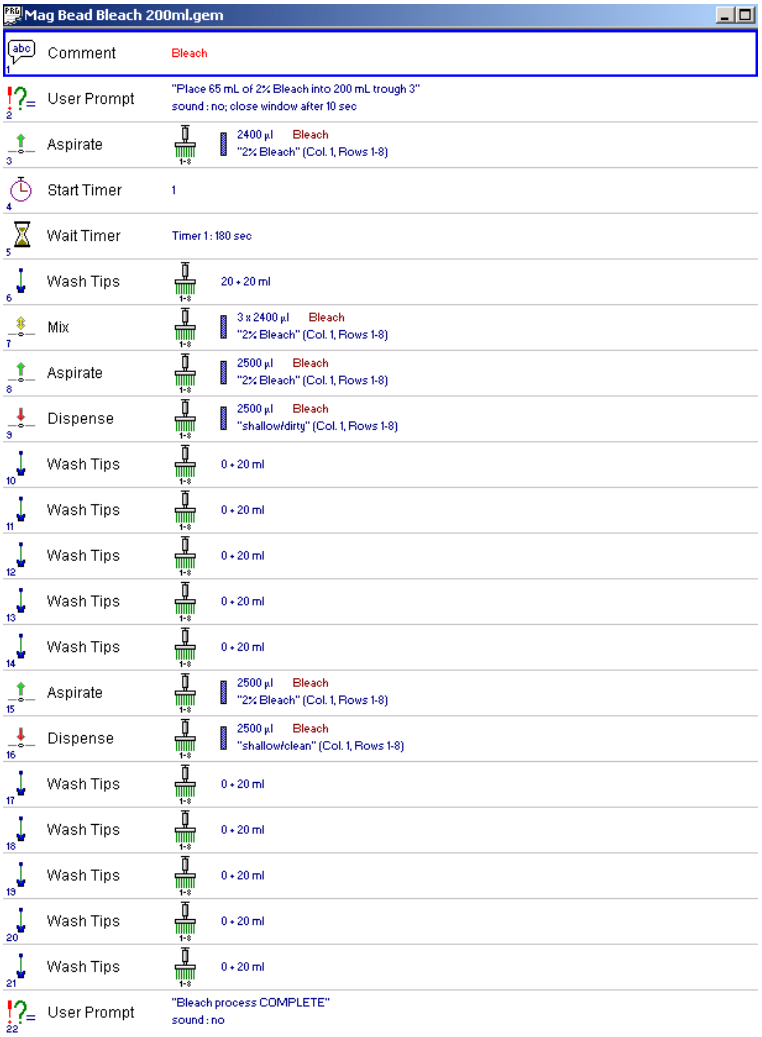


FIG. 4—*Script for the 2% bleach routine carried out between each processing step in the overall automation process. This routine takes approximately 15 min to run.*

carryover contamination on the robotic platform. These research groups have evaluated contamination by using the maximum volume of bead eluate that could be easily accommodated in the PCR reaction as a representative aliquot of the entire eluate and used the maximum volume allowable on the gel system for analysis (25,29). Other groups treated blanks as regular samples and the same volume as that used for regular samples was subjected to amplification (27). These approaches were adopted based on the genotyping systems, amplification conditions, and sensitivity level of the detection instruments used for DNA analysis and validated in these respective laboratories. In the clinical and diagnostic field, users of automation assess potential sample carryover by treating the negative samples as regular samples in their respective assays (33,35,36,38,48,51).

We found that the incorporation of a bleach wash within the DNA extraction process was more efficient than running a bleach wash at the end of an extraction session before starting the next process, i.e., DNA quantification. Although some robotic applications appeared to produce reliable results by incorporating a bleach wash at the start of each day (7,36), this regimen certainly was not optimal for forensic type samples extracted using a magnetic bead-based protocol with our robotic configuration. The additional bleach routine that we incorporated between processes may be superfluous; however, since no control can be placed on the nature of the casework samples submitted for automated extraction, it acts as a safety cushion.

The incorporation of the bleach washing steps within the automated DNA extraction (involving the TE-Shake™ unit) adds close to one hour to the overall process using 88 samples. We are currently modifying the washing conditions to reduce the amount of RO water required to carry out efficient washes and prevent carryover. Recently, TECAN has designed new wash carriers with shallower and narrower wells to enhance the efficiency of the washes to reduce the overall processing time. These carriers are equipped with a bottom release valve to evacuate contaminants from the tip wash stations as they are washed away from tips and reservoirs. Newly designed tips without the undesirable tubing/tip junction have also been evaluated. Preliminary work suggests that a simple modification to the FWP setting speeds up the wash process by 36 min. Additional changes in the wash volumes (rendered more effective because of the new wash station design) improved that performance even more. Time spent washing tips was minimized significantly and processing samples using fixed tips turned out to be almost as fast as an equivalent process that uses disposable tips (54).

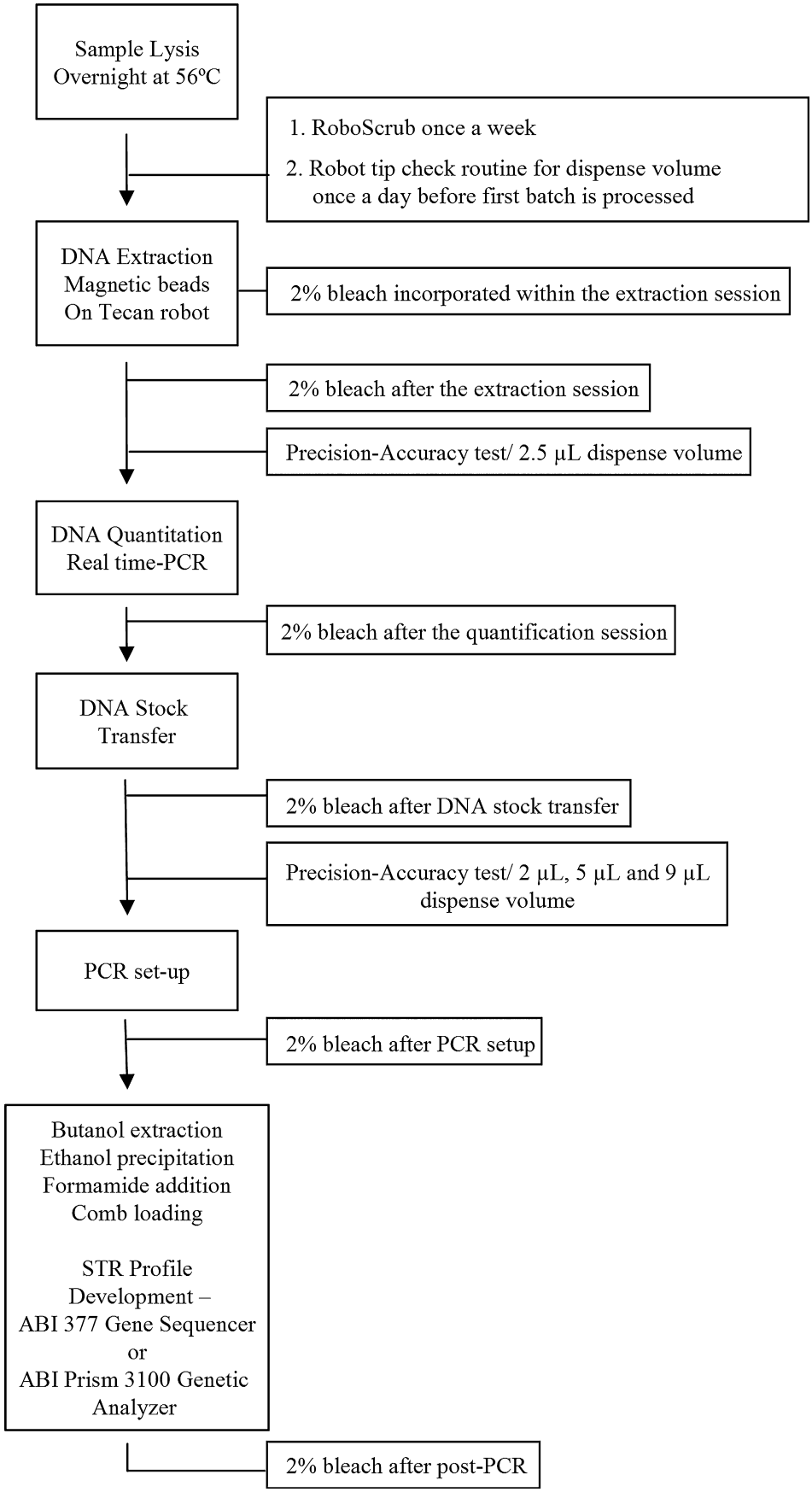


FIG. 5—*Cross-contamination prevention regimen for TECAN robotic workstations equipped with fixed tips and used during the automated process of B&E cases. This diagram summarizes the tip washing steps and robot tip checks carried out to prevent sample to sample carryover during the automated process of nonsuspect B&E cases.*

Conclusion

The robot fixed tip cleaning routine using 2% sodium hypochlorite incorporated within the DNA extraction and at the end of each of the automated processes, i.e., DNA extraction, DNA quantification setup, DNA stock transfer, PCR setup, and post-PCR process (for sample destined for capillary electrophoresis) between subsequent batches of samples was shown to be very effective in preventing sample to sample carryover. Blank control samples did not show any signs of DNA contamination despite high concentrated DNA samples being processed on the same plate. The implementation of the 2% sodium hypochlorite routine did not have any deleterious effect on DNA yields for samples with low as well as high amounts of DNA and the quality of the STR profiles produced was not compromised.

The use of low-volume fixed tips reduces the operational cost significantly. The increase in processing time resulting from the incorporation of the tip washing steps is anticipated to be much less significant when reviewing the washing routine in greater detail. Data presented in this report indicate that fixed tips combined with robust washing routines can be used in confidence for processing casework samples and is a viable and an effective alternative to disposable tips. Although our automated process and the development of a contamination verification and prevention protocol was primarily designed for forensic applications, the rigorous quality assurance and high standards for acceptance should be of interest to clinical diagnostic laboratories as well.

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