JOURNAL OF FORENSIC SCIENCES

J Forensic Sci, November 2009, Vol. 54, No. 6
doi: 10.1111/j.1556-4029.2009.01173.x
Available online at: interscience.wiley.com

TECHNICAL NOTE

Hiron Poon,[1] M.Sc.; Jim Elliott,[2] B.Sc.; Jeff Modler,[2] M.Sc.; and Chantal Frégeau,[3] Ph.D.

The Use of Hemastix® and the Subsequent Lack of DNA Recovery Using the Promega DNA IQ™ System

ABSTRACT: Following implementation of our automated process incorporating the Promega DNA IQ™ system as a DNA extraction method, a large number of blood-containing exhibits failed to produce DNA. These exhibits had been tested with the Hemastix® reagent strip, commonly used by police investigators and forensic laboratories as a screening test for blood. Some exhibits were even tainted green following transfer of the presumptive test reagents onto the samples. A series of experiments were carried out to examine the effect of the Hemastix® chemistries on the DNA IQ™ system. Our results indicate that one or more chemicals imbedded in the Hemastix® reagent strip severely reduce the ability to recover DNA from any suspected stain using the DNA IQ™ magnetic bead technology. The 3,3′,5,5′-tetramethylbenzidine (TMB) used as the reporting dye appears to interact with the magnetic beads to prevent DNA recovery. Hydrogen peroxide does not seem to be involved. The Hemastix® chemistries do not interfere in any way with DNA extraction performed using phenol-chloroform. The incompatibility of the Hemastix® chemistries on the DNA IQ™ system forced us to adopt an indirect approach using filter paper to carry out the presumptive test.

KEYWORDS: forensic science, Hemastix®, DNA recovery, DNA IQ™, presumptive test, magnetic beads, DNA extraction

Blood is probably the most commonly searched body fluid in forensic casework. The Takayama or Hemochromogen test (1) is used by some forensic laboratories as a confirmatory test for blood. A positive result is characterized by the formation of the characteristic feathery hemoglobin crystals in the presence of sodium hydroxide, pyridine, and reducing sugar. Unfortunately, this test requires the presence of relatively large quantities of blood which might not be readily available in forensic samples. Owing to their enhanced sensitivities, numerous catalytic tests have been developed and used to screen for the presence of blood. Among them, the luminol, leucomalachite green, phenolphthalein, and the Hemastix® tests have been commonly used (2,3). These tests are based on the catalytic action of hemoglobin on a peroxide substrate, causing it to react with a number of organic compounds to yield colored reaction products. However, any biological material that also possesses peroxidase activity, or any material that hydrolyzes the hydrogen peroxide in these chemical reactions, also reacts positively using the screening tests. Therefore, these tests are generally considered as presumptive tests for blood. The sensitivity of these presumptive tests varies considerably and is reported elsewhere (4). The Hemastix® Reagent strips, manufactured by Bayer HealthCare LLC, were developed to detect the presence of blood in urine (5). The test is based on the same general principle of detecting the peroxidase-like activity of hemoglobin using diisopropylbenzene dihydroperoxide as the substrate and 3,3′,5,5′-tetramethylbenzidine (TMB) as the reporting dye. Some police investigators and forensic laboratories, including those of the Royal Canadian Mounted Police (RCMP), are using the Hemastix® as the screening test for blood, owing to its ease of use, portability, and sensitivity.

With the introduction of DNA typing technologies, the effects of most of the commonly used presumptive tests on DNA analysis have been evaluated and found to have little or no detrimental effect on DNA typing (6–8). However, these published validation studies were primarily based on blood samples extracted using the phenol-chloroform procedure. In recent years, affinity-based DNA extraction procedures not requiring organic solvents are being adopted into the mainstream for automation and are becoming the method of choice for extracting DNA from forensic samples. The BioRobot EZ1 Genomic DNA Kit from Qiagen® (9–11) and the DNA IQ™ system from Promega (12–14) are the two most widely used affinity-based DNA extraction systems.

The DNA IQ™ system is based on the adsorption of DNA molecule to siliceous oxide-coated paramagnetic beads in the presence of chaotropic salts. The DNA-magnetic bead complex is then subsequently washed and the DNA eluted under low salt and high pH conditions. Although contaminants such as denim dyes and proteins will initially compete with DNA for the binding sites on the magnetic beads, these contaminants are removed during the washing steps, yielding in the final eluant purified DNA molecules suitable for subsequent polymerase chain reaction (PCR) analysis. Unlike the organic solvent-based phenol-chloroform procedure, which is more suitable for the recovery of large quantities of DNA, the DNA IQ™ system has a predefined DNA binding capacity and is more suitable for the recovery of a small quantity of DNA. The DNA IQ™ system requires the binding of DNA to the magnetic beads; chemicals and/or conditions that prevent either the binding of DNA or the elution of DNA from the beads will have a negative impact on the efficiency of DNA recovery using this procedure.

[1]Royal Canadian Mounted Police, National Forensic Services—Vancouver, Biology Services, 5201 Heather Street, Vancouver, BC, V5Z 3L7 Canada.

[2]Royal Canadian Mounted Police, National Forensic Services—Ottawa, Biology Services, 1200 Vanier Parkway, Ottawa, ON, K1G 3M8 Canada.

[3]Royal Canadian Mounted Police, National Services and Research, 1200 Vanier Parkway, Ottawa, ON, K1G 3M8 Canada.

Received 2 May 2008; and in revised form 20 Nov. 2008; accepted 6 Dec. 2008.

© 2009 American Academy of Forensic Sciences

The RCMP National Forensic Services implemented the Automated Protocols for processing biological samples for "Break and Enter" type cases in May 2004 and for forensic casework samples in the fall of 2005. The DNA IQ™ system is an integral part of the Automated Protocols (12,15,16). During the course of routine casework file reviews, the Hemastix® chemistry was suspected to have a significant effect on the ability to recover DNA from biological samples. The purpose of this communication is to report on findings regarding the incompatibility of the Hemastix® chemistries with the DNA IQ™ magnetic bead technology.

Materials and Methods

Samples

Blood, saliva, buccal swabs, scalp hair, urine, and clothing for the source of contact DNA used in these experiments were contributed by volunteers from the laboratory personnel (see Experimental Setup for details on sample types and sizes used).

Hemastix® Test

The Hemastix® reagent strips (Bayer HealthCare LLC) are used as a screening test for blood. In the Indirect testing procedure, a small piece of filter paper (Whatman) is folded into a point and the pointed end is gently rubbed against the questioned stain. The reagent pad of the Hemastix® is lightly moistened with de-ionized or reverse osmosis prepared (RO) water. The strip is lightly shaken to remove any excess water. Next, the test strip is lightly pressed on the transferred material on the filter paper. A positive test is indicated by a color change from yellowish orange to green or blue within 10 sec. In the Direct testing procedure, a moistened Hemastix® strip is used directly on the exhibit material. A positive test is also indicated by a color change from yellowish orange to green or blue within 10 sec.

Automated Protocols

The Automated Protocols developed, validated and implemented for casework sample processing integrate a Laboratory Information Management System (JusticeTrax® LIMS-Plus® 3.52.20; JusticeTrax, Mesa, AZ) on all aspects of DNA analysis. The automated process includes DNA extraction, DNA quantification, sample normalization, PCR setup and amplification as well as analysis of the PCR products by capillary electrophoresis. The Tecan Freedom EVO and/or Genesis robotic workstations (Tecan US, Research Triangle Park, NC) were used to operate the liquid handling portions of the automated protocols. All samples were processed according to the procedures as described below.

DNA Extraction

Stains and swabs prepared from all body fluids were extracted using the Automated Direct Extraction Protocol (15,16). Samples were incubated overnight at 56°C in 350 μL of lysis buffer (LB; 10 mM Tris, pH 8.0, 10 mM EDTA, 100 mM NaCl, 0.5% sarkosyl, 40 mM DTT) with 1.5 mg/mL of Proteinase K (Prot. K). Lysates were supplemented with two volumes of Promega Lysis Buffer (PLB); these supplemented lysates are referred to as lysate liquid columns in the text. Twelve microliters of DNA IQ™ magnetic beads was used to capture the DNA from the lysate liquid column using a TE-Shake unit (TECAN US) for constant swirling of the beads. Following three separate washes of the beads complexed with DNA, the DNA was eluted in 60 μL of low Tris-EDTA (10 mM Tris pH 8.0, 0.1 mM EDTA) buffer at 65°C (2×8 min incubation).

DNA Quantification

DNA quantification was performed using the AB Quantifiler™ Human DNA Quantitation Assay (Applied Biosystems, Foster City, CA) on an ABI Prism™ 7000 Sequence Detection System. A 2.5 μL aliquot of extracted DNA or standard DNA was added to 10 μL of primer/probe solution and 12.5 μL of reaction mix (containing the Taq Gold polymerase). DNA from the cell line K562 (Promega Corporation, Madison, WI) was used as the quantification reference standard with data points between 32 and 0.0156 ng/μL. Amplification was carried out for 40 cycles using parameters as specified by the manufacturers.

PCR Amplification

DNA amplification was carried out using 1 ng of template DNA and the AmpF*l*STR® Profiler Plus® PCR Amplification kit (Applied Biosystems) in an MJ Research DNA Engine Peltier Thermal Cycler (PTC-200). Samples were amplified in a 96-well amplification plate with a final volume of 15 μL (6.0 μL of template DNA, 5.7 μL of AmpF*l*STR® PCR reaction mix, 3.0 μL of AmpF*l*STR® PCR Profiler Plus™ primer set, and 0.3 μL of AmpliTaq Gold® DNA Polymerase) following a hot start at 95°C/11 min, 28 cycles of denaturation at 94°C/60 sec, annealing at 59°C/90 sec, and extension at 72°C/90 sec, with a final non-template extension at 60°C/75 min.

Capillary Electrophoresis

Electrophoresis of the amplified DNA fragments was performed on an ABI Prism® 3100 Genetic Analyzer using the ABI Prism® 3100 Data Collection Software (version 2.0). Amplicons (0.25 μL), denatured in 20 μL of Hi-Di Formamide and 0.5 μL of Genescan-500 (ROX), were electrokinetically injected at 3 kV for 10 sec using POP-4 as the polymer. Sample file information generated by the 3100 Genetic Analyzer was analyzed using GeneMapper® *ID* Software (version 3.2).

Manual Processing of Samples

The procedure for the manual extraction of DNA using the phenol-chloroform method has been published elsewhere (6). The extracted DNA was quantified using the slot blot hybridization procedure and the alkaline phosphatase conjugated D17Z1 α-satellite probe (17). One and a half nanograms of DNA was amplified in tubes using the AmpF*l*STR® Profiler Plus® multiplex system in a final volume of 25 μL (10.0 μL of template DNA, 9.5 μL of AmpF*l*STR® PCR reaction mix, 5.0 μL of AmpF*l*STR® PCR Profiler Plus® primer set, and 0.5 μL of AmpliTaq Gold® DNA Polymerase) in an ABI GeneAmp® PCR System 9600 for 28 cycles (hot start at 95°C/11 min, denaturation at 94°C/60 sec, annealing at 59°C/90 sec, extension at 72°C/90 sec, final non-template extension at 60°C/45 min). The DNA fragments were separated in Tris-Borate buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) on a 0.2 mm thick, 36 cm 4% acrylamide vertical denaturing gel containing 6 M urea. Gel electrophoresis was performed on an ABI Prism® 377 DNA Sequencer for 2.5 h at 3000 V using the ABI Prism® 377 Data Collection Software (version 2.5.1). Analysis of the DNA profiles was performed using the Genescan® Analysis Software (version 3.1.2) and Genotyper® Software (version 2.5).

Experimental Setup

Several experiments were designed to investigate the adverse effects of Hemastix® on DNA recovery by the DNA IQ™ system. Other experiments specifically addressed the nature of the interference.

Experiment 1—Preliminary Experiments Using Hemastix®—Blood swabs (0.5, 1 and 20 μL) from one individual were prepared in duplicate and (i) placed in tubes in the presence of one or three Hemastix® strips before lysis; or (ii) tested with an Hemastix® strip using a direct contact method (usual method employed by the Evidence Recovery Unit [ERU] personnel of the RCMP) which transfers some of the Hemastix® chemistry onto the blood stain; or (iii) tested using an indirect method, i.e., pressing a filter paper lightly on the blood swab first to transfer some blood cells and then applying a moistened Hemastix® strip on the filter paper. Using the indirect method the original blood swab never comes in contact with the Hemastix® chemistry.

Two sets of duplicated blood swabs (1 and 20 μL) from the same individual were also prepared to investigate the relationship between the size and intensity of the green area noted on Hemastix®-tested swabs and the amount of DNA recovered. For one set, only the corner of the pre-wetted Hemastix® strip was pressed lightly on the edge of the suspected stain. For the other set, the entire area of the pre-wetted Hemastix® strip was pressed on the suspected stain.

Experiment 2—Determining Whether or Not the Reagents Imbedded in the Hemastix® Strip Bind or Damage DNA—To initiate our investigation on the mechanism of interference of Hemastix®, an experiment was conducted to determine whether or not the reagents on the Hemastix® strip covalently bind or damage DNA. To this end, 2, 4, and 6 Hemastix® strips were immersed with mild agitation in 200 μL filtered, autoclaved, and deionized (FAD) water for 20 sec. The strips were then placed into Spin-eZe baskets (Fitzco Inc., Spring Park, MN) and spun for 2.5 min at $21.000 \times g$. Visual inspection of the resulting Hemastix® solutions indicated an increase in yellow color intensity with a green tinge noted in some of the solutions. The solutions were intensely green by the time the solutions were used for experimentation (45 min after preparation). A 2 μL aliquot of each Hemastix® solution was added to 0.5 μL of purified K562 DNA (5 ng/μL diluted stock; original stock from Promega Corporation), in triplicate, in quantification plates, and incubated at room temperature for 4 min before the addition of the quantification reagents (see DNA Quantification above).

Another set of prepared samples was further treated by passing through Microcon-100 size-exclusion columns (Millipore, Billerica, MA). For these samples, the 2.5 μL aliquot comprised of K562 DNA + Hemastix® solution was added to 397.5 μL FAD and filtered according to the manufacturer's instructions. The recovered colorless material was adjusted to a final volume of 2.5 μL and quantified as detailed above.

Experiment 3—Activated Versus Non-activated Hemastix®—To assess if the adverse effect noted on DNA recovery originated strictly from activated reagents, i.e., when exposing the *Hemastix®* strip to blood-positive samples or could also be observed when exposing the strip to blood-negative samples, i.e., as a result of transferring non-activated reagents onto the samples, the following experiment was carried out. DNA previously extracted from buccal swabs from one female volunteer was applied to sterile cotton swabs, in triplicate, using 10, 50, 100, and 200 ng of DNA. These samples were air-dried overnight prior to testing under the following conditions: (i) no treatment (control); (ii) direct contact with non-activated Hemastix®; and (iii), direct contact with activated Hemastix®. For condition (ii), a Hemastix® strip was pre-wetted with 10 μL of FAD water and then pressed directly onto the DNA swabs for 10 sec. No color reaction was observed from the Hemastix® strips. For condition (iii) a pre-wetted Hemastix® strip was activated by pressing for 2 sec against a moistened piece of filter paper, which had previously been pressed firmly for 5 sec against a control blood stain. The activated strip (now of a green color) was then pressed firmly against the DNA swabs for 10 sec to transfer the activated chemistry.

In addition, "contact DNA" swabs prepared by swabbing used pop cans and drinking bottles of two volunteers ($N = 2$ from donor #1 and $N = 6$ from donor #2) were also tested under the same conditions. No color change was observed on the Hemastix® strips that were applied directly on the contact swabs. All of the prepared swabs were processed according to the validated Automated Protocols.

Experiment 4—Effect of "Compromised" DNA IQ™ Magnetic Beads on Subsequent DNA Extraction—The next set of experiments was designed to see if the interfering components from the Hemastix® strips could be easily washed off from the magnetic beads after their interaction with them. Blood swabs (20 μL) were prepared in duplicate, air-dried overnight, and treated with Hemastix® pressing lightly (1 sec) or firmly (5 sec) against the blood spot. The treated swabs were further air-dried for 2 h before lysis overnight. These swabs were then used in an experiment that explores the feasibility of re-using "compromised" DNA IQ™ magnetic beads (recovered at different stages during an initial extraction of Hemastix®-tested blood swabs) to extract DNA from lysates prepared from blood swabs not exposed to Hemastix®. The points at which the magnetic beads were recovered were: (i) after DNA binding and lysate liquid column removal; (ii) after the first wash step with PLB; and (iii) after the last wash step with Promega Wash Buffer. The "recycled" or potentially compromised magnetic beads were then used to extract a new series of 20 μL control blood swabs.

Experiment 5—Re-extraction of "Compromised" Lysate Liquid Columns Using Fresh Aliquots of DNA IQ™ Magnetic Beads—The following experiments were conducted to determine if the interfering components from the Hemastix® strips could eventually be titrated out from the lysate liquid columns (lysates + two volumes of PLB). Selected sets of frozen lysate liquid columns stored at −20°C following a first DNA extraction of Hemastix®-tested blood swabs on the robotic workstation were thawed at room temperature and re-extracted robotically using a fresh aliquot (12 μL) of DNA IQ™ magnetic beads. Lysate liquid columns were kept after each session on the robot and extracted up to 15 times using new aliquots of beads every time.

Experiment 6—Determination of the Causative Agent(s) Responsible for Preventing DNA Recovery from Hemastix®-Treated Samples—This experiment attempted to determine the causative agent or agents responsible for the prevention of DNA recovery from Hemastix®-treated samples. The major reagents comprised in the Hemastix® strip, i.e., hydrogen peroxide and TMB, were purchased as separate chemicals (hydrogen peroxide:

30% w/w, Sigma-Aldrich, St. Louis, MO; and TMBZ: Sigma-Aldrich or 3,3′,5,5′- tetramethylbenzidine, dihydrochloride, dihydrate i.e., TMBZ HCl: Sigma-Aldrich) and tested on 20 μL blood swabs using different concentrations (0.5, 1.0, 2.0, 3.0, and 4.0% for TMB and 0.5, 1.0, 2.0, 4.0, and 6.8% for hydrogen peroxide) and various combinations (i.e., used separately or in combination) for their ability to prevent DNA recovery during the Automated DNA Extraction Protocol using DNA IQ™ magnetic beads. TMB was first prepared in heated (65°C) methanol and 10 μL volume applied on each blood swab. No color change was noted on the swabs before lysis. In the second set of experiments conducted, TMBZ HCl was prepared in FAD water and 5–40 μL volumes applied on each blood swab. No color change was noted on the swabs before lysis. Hydrogen peroxide (30% stock) was diluted in FAD water and 10 μL volume applied on each blood swab. Bubbling action was observed locally on each swab. For combinations tested, a dark color developed over the blood spot where chemicals were applied. For the last two sets of experiments, TMB was prepared in 50% DMSO and 10–40 μL volumes applied on each blood swab. No color change was noted on the swabs before lysis. Hydrogen peroxide (30% stock) was diluted in PBS and 10 μL volume applied on each blood swab. Bubbling action was observed. For combinations tested, a dark color developed over the blood spot where chemicals were applied.

Experiment 7—Effect of Hemastix® on Various Sample Types and Other Chemically Treated Samples Using DNA IQ™ or Phenol-Chloroform for DNA Extraction—We were interested in determining if the adverse effect seen with blood-containing samples would also be noted for other sample types and if the interference in DNA recovery would be observed with other chemicals commonly used in the laboratory. Blood stains (white cotton) and blood swabs (2 and 20 μL), saliva stains (white cotton) and saliva swabs (20 μL), scalp hairs and samples for contact DNA (sweat band from baseball cap from one volunteer) were prepared in duplicate and dried overnight. The samples were treated with (i) no chemicals (control samples); or (ii) Hemastix® (direct contact) in combination with other screening and confirmatory tests that are routinely used by ERU personnel (see Table 1 for details). Hemastix® (Bayer HealthCare LLC) strip is used as a screening test for blood. Hemochromogen (20% dextrose, 2% NaOH, 20% pyridine) is used as a confirmatory test for blood. Azostix® (Bayer Corporation, Elkhart, IN) is used as a screening test for urine. Fast Blue (0.1% w/v Fast Black salt, 1.2% w/v anhydrous sodium acetate, 1% v/v glacial acetic acid, 0.08% w/v sodium α-naphthylphosphate) is used as a screening test for semen. When possible, each sample was divided in half, with each portion processed either manually using the phenol-chloroform protocol or robotically using the validated Automated Protocols.

TABLE 1—*Chemical tests used in the preparation of biological samples for Experiment #7.*

Sample Type	Chemical Test
Blood stain or swab	Hemastix® (direct contact)
	Hemochromogen (5 μL)
	Hemastix® (direct contact) + Hemochromogen (5 μL)
	Azostix® (direct contact)
Blood swab + urine	Azostix® (direct contact)
Saliva stain or swab	Hemastix® (direct contact)
	Fast Blue (5 μL)
Scalp hairs (shaft or root)	Hemastix® (direct contact)
Possible biological material (e.g., sweat band from baseball cap)	Hemastix® (direct contact)

Results and Discussion

Preliminary Experiments Using Hemastix®

Results shown in Table 2 indicate that the Hemastix® chemistry has an adverse impact on DNA recovery using the DNA IQ™ system. All samples that came in direct contact with the Hemastix® chemistry showed either no DNA or significantly reduced DNA yields based on the Quantifiler real-time PCR assay, whereas all control swabs generated expected yields. In addition, the internal positive control (IPC) co-amplified with each sample during quantification showed no sign of depression in any of the samples tested (data not shown). This suggests that failure to provide a quantification result is likely due to an absence of DNA rather than PCR inhibition.

The relationship between the size and intensity of the green area noted on tested swabs and the amount of DNA recovered was also examined using another set of blood swabs. The amount of DNA recovered decreased proportionally to the size and intensity of the tested area (data not shown). Swabs that were barely touched by the Hemastix® strip yielded similarly reduced DNA yields as observed for swabs with a larger and darker green spot. For 1 μL blood swabs, DNA yields ranged from 4.7–8.8 ng for swabs showing a 1 mm^2 light green spot to 0.04–0.16 ng for those with a 5 mm^2 dark green tested area. For 20 μL blood swabs, DNA yields ranged from 2.5–3.8 ng for swabs showing a 2 mm^2 light green spot to 1.0–4.0 ng for those with a 5 mm^2 dark green area.

Determining Whether or Not the Reagents Imbedded in the Hemastix® Strip Bind or Damage DNA

We postulated that the major reduction in DNA recovery noted for Hemastix®-tested samples could be a result of (i) Hemastix® chemicals interacting with the DNA preventing it from binding to the magnetic beads, (ii) Hemastix® chemicals modifying the structure of the DNA preventing it from binding to the magnetic beads, or (iii) Hemastix® chemicals acting as a competitor for the binding sites on the magnetic beads. Results shown in Table 3 indicate that the chemicals present on the Hemastix® strip are not covalently attached to the DNA because a simple filtration step, such as the use of Microcon-100 filter units, proved sufficient to allow full recovery of the DNA. The chemicals present on the Hemastix® strip interfering with the DNA binding process are small enough to pass through the Microcon-100 filter units. In addition, the DNA is

TABLE 2—*Interference from Hemastix® with DNA recovery using the Promega DNA IQ™ system.*

Blood on Swab (μL)	Treatment	Average DNA Yield (ng) $N = 2$* or $N = 4 \pm$ SD
0.5	None	3.8
1		10.0
20		180.0 ± 20.0
0.5	1 or 3 Hemastix® strip(s)	0.04 ± 0.09
20		0.09 ± 0.05
1	Direct contact with Hemastix® strip	0.00
20		0.10
1	Indirect test/filter paper	6.1
20		210.0

*No standard deviation is given for $N = 2$.

TABLE 3—*Effect of Hemastix® chemistry on purified DNA.*

Purified K562 DNA (μL)	Number of Hemastix® Strips Used to Prepare Solution	Average DNA Yield (ng ± SD) $N = 3$	Average DNA Yield After Using Microcon-100 Filter Units (ng ± SD) $N = 3$
0.5	2 strips	38.0 ± 3.6	50.7 ± 2.1
	4 strips	0.0 ± 0.0	48.7 ± 1.2
	6 strips	0.0 ± 0.0	49.0 ± 8.5
	None	61.7 ± 7.6	ND

ND, not done.

not considered structurally damaged as the DNA can be amplified using the Quantifiler assay. However, the IPC included in the real-time PCR quantification assay for all samples not filtered through Microcon-100 filter units was depressed, indicating that the colored solution prevented efficient amplification of the IPC. In previously quantified samples, the IPC never showed any sign of inhibition. The amount of Hemastix® chemistry in the current experiment likely exceeded that normally found on Hemastix®-tested samples, thus the IPC inhibition. The fact that DNA can be recovered despite the excess of chemistry reinforces the notion that the DNA is not covalently associated with any of the reagents present on the Hemastix® strip and is of a degree of quality good enough to be amplified. It is therefore likely that the Hemastix® chemicals act as a competitor for the binding sites on the magnetic beads during DNA extraction using DNA IQ™.

Activated versus Non-activated Hemastix®

We then wanted to determine if the Hemastix® chemicals needed to be activated in order to exert their negative effect on DNA recovery or if the presence of the non-activated reagents would produce a similar adverse effect. All swabs prepared and treated with activated or non-activated reagent strips produced no DNA, indicating an interference effect on DNA recovery regardless of the status of the strip (data not shown). Similar results were also noted for trace swabs (no DNA versus 4.4–12.9 ng for control trace swabs). These observations suggest that any suspected stain (blood-positive or blood-negative) tested by a moistened Hemastix® strip will show a significant reduction in DNA yield even though no green color was produced on the exhibit material.

All control swabs provided DNA which represented 1/3 to 1/4 of input DNA. The yields were as follows: for the 10 ng DNA swab, 3.4 ± 0.1 ng; for the 50 ng DNA swab, 20.7 ± 5.0 ng; for the 100 ng DNA swab, 33.3 ± 4.5 ng; and for the 200 ng DNA swab, 50.7 ± 5.5 ng. This is consistent with observations made by Promega Corporation (Alan Tereba, personal communication) which indicated that purified DNA is not efficiently captured on magnetic beads and efficient DNA recovery on DNA IQ™ beads likely requires the presence of some protein.

Effect of "Compromised" DNA IQ™ Magnetic Beads on Subsequent DNA Extraction

Table 4 indicates that the beads collected at different stages during the initial extraction of Hemastix®-tested blood swabs remained compromised throughout the initial extraction process and their capacity to bind DNA in subsequent extractions of non-tested samples was greatly compromised. The chemistry compromising the beads was not washed off during the bead washing steps carried out following the usual DNA capture in the extraction protocol. Control blood swabs and blood swabs tested with Hemastix® (light and firm touch) extracted using fresh DNA IQ™ beads generated the expected DNA yields that are inversely related to the extent of direct contact to the Hemastix® strips.

TABLE 4—*Capacity of "compromised" magnetic beads to recover DNA with automated DNA IQ™.*

Beads Used in Initial DNA Extraction or in Subsequent Extraction of Non-tested 20 μL Blood Swabs	Hemastix® Test Carried Out for Initial DNA Extraction	Average DNA Yield (ng) $N = 2$
Fresh beads used for the initial DNA IQ™ DNA extraction of Hemastix®-tested swabs	None	175.0
	Direct contact Light press	33.5
	Direct contact Firm press	0.54
Compromised beads collected after DNA binding and lysate liquid column removal of the initial DNA IQ™ DNA extraction of Hemastix®-tested swabs	Direct contact Light press	8.4
	Direct contact Firm press	0.21
Compromised beads collected after the first wash step of the initial DNA IQ™ DNA extraction of Hemastix®-tested swabs	Direct contact Light press	1.7
	Direct contact Firm press	0.95
Compromised beads collected after the last wash step of the initial DNA IQ™ DNA extraction of Hemastix®-tested swabs	Direct contact Light press	13.8
	Direct contact Firm press	0.77

Re-extraction of "Compromised" Lysate Liquid Columns from Hemastix®-Tested Samples Using Fresh Aliquots of Magnetic Beads

The experiments performed to determine if the Hemastix® chemicals binding to the magnetic beads could be titrated out from the lysate liquid columns revealed that it is indeed the case. For 1 μL blood control swabs, most DNA was recovered in the first three extractions performed (94%); but for Hemastix®-tested swabs (Direct contact), only 43% of DNA was detected in extraction numbers 1 to 3 (Table 5). In fact, most of the DNA was detected in extraction number 3 and beyond (data not shown). Interestingly, close to 22 ng of DNA was extracted in both cases which suggests that the DNA remains available in the lysates but is simply not capable of efficiently binding to the beads due to the presence of the Hemastix® chemistry. In the case of 20 μL blood swabs tested with Hemastix® (Direct contact), the DNA was recovered in extractions #1 to #15 compared to #1 to #8 for control swabs. Only 46% of DNA was detected in extraction numbers 1 to 3 for the Hemastix®-tested swabs while 85% of DNA was detected in the control swabs. In both 1 and 20 μL test groups, swabs that were tested using the indirect method exhibited results indistinguishable from those of the corresponding control groups. This observation reaffirms our hypothesis that the reduction in DNA recovery from

TABLE 5—*Re-extractions of "compromised" lysate liquid columns with fresh aliquots of DNA IQ™ beads.*

Sample		Hemastix® Test	Total DNA Yield (ng)	Extraction Number Where ≥0.2 ng DNA Was Detected	% of DNA Recovered from Extraction Number†	
					1	1 to 3
Blood swab 1 µL		None	21.7	1 to 5	46	94
		Indirect test/filter paper	16.3	1 to 3	37	98
	Direct contact	Different sizes and intensities of tested area	20.7 ± 8.9 (n = 6)	1 to 8	12	43
		Untested portion	5.5	1 to 2	95	100
		+ Hemastix® strip	ND*	>15	0	0
Blood swab 20 µL		None	483.6	1 to 8	37	85
		Indirect test/filter paper	488.0	1 to 10	43	91
	Direct contact	Different sizes and intensities of tested area	257.6 ± 101.6 (n = 6)	1 to 15	1	46
		Untested portion	245.4	1 to 5	56	96
		+ Hemastix® strip	ND*	>15	0	9

*ND = not determined as the Hemastix® reagents still interfering with DNA binding.
†% of DNA recovered is calculated by dividing the amount of DNA recovered from the indicated number of extractions by the total DNA yield.

Hemastix®-treated samples is caused by Hemastix® chemicals interacting directly with the magnetic beads. The interfering action of the Hemastix® chemicals is so potent that samples taken adjacent to the tested areas also demonstrated a moderate DNA yield reduction (Table 5, 1 and 20 µL untested portion). Presumably the chemicals in the tested area diffused to the untested region, thus exhibiting the interference.

The number of extractions required to recover the total amount of DNA present in the lysate liquid column appears to be dependent on the amount of Hemastix® chemistry transferred on the swab. Indeed, the remaining lysates from blood swabs lysed in the presence of a Hemastix® strip have been extracted up to 20 times without any recovery of DNA.

This set of results suggests that the compromising component of the Hemastix® strip binds to the beads and gets gradually depleted from the lysate liquid column to finally allow binding of the DNA to fresh aliquots of beads. DNA is not lost but rather competes for sites, albeit inefficiently on the beads at every extraction. DNA is

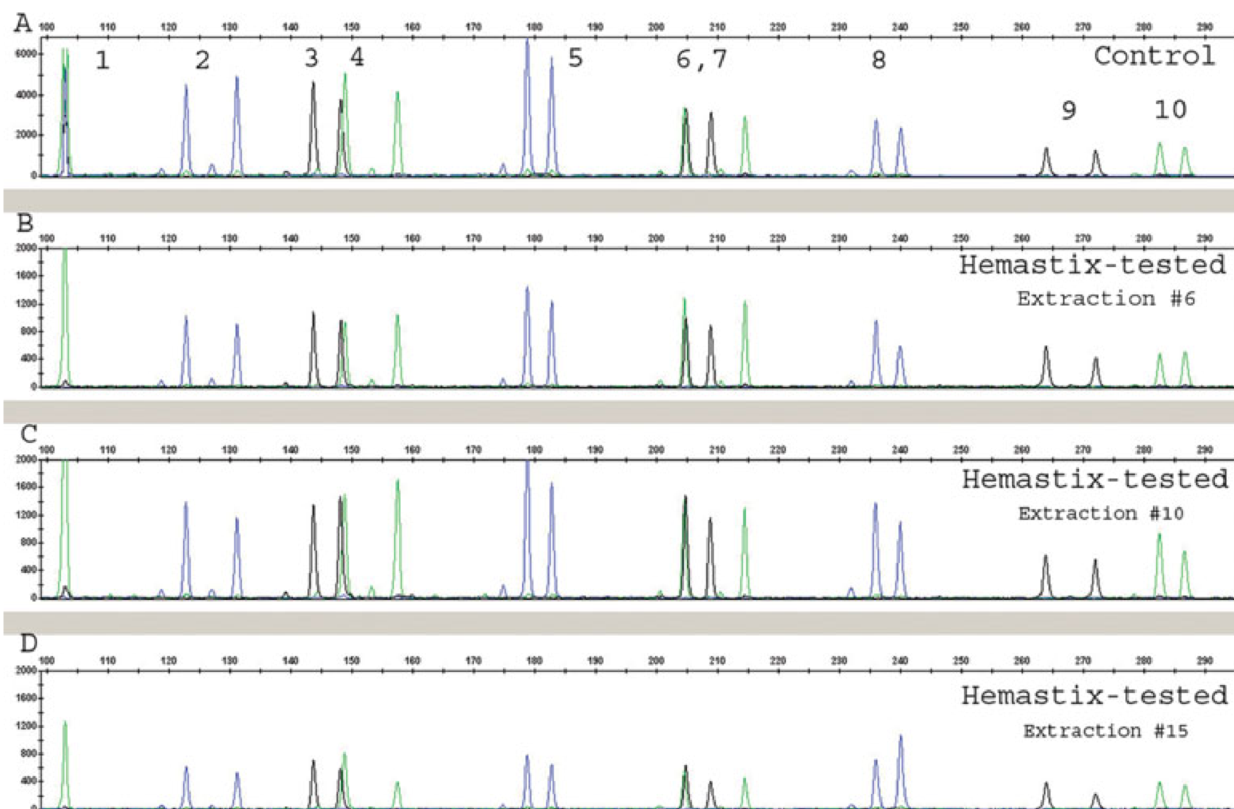

FIG. 1 —*AmpFl STR® Profiler Plus® profiles generated from 20 µL blood swabs tested with Hemastix® using the direct contact approach and extracted multiple times using DNA IQ™. Panel A refers to the control blood swabs not tested with Hemastix®; DNA originated from extraction session #2, 1 ng target DNA amplified. Panel B presents profiles of Hemastix®-treated blood swabs from DNA obtained in extraction session #6, 0.44 ng target DNA amplified. Panel C presents profiles of Hemastix®-treated blood swabs from DNA obtained in extraction session #10, 0.78 ng target DNA amplified. Panel D presents profiles of Hemastix®-treated blood swabs from DNA obtained in extraction session #15, 0.78 ng target DNA amplified. Each panel depicts the relative fluorescence intensity (Y-axis) and the size estimate in bases (X-axis). The AmpFl STR® Profiler Plus® genetic markers, as observed from left to right, in order of size, are: Amelogenin (1), D3S1358 (2), D5S818 (3), D8S1179 (4), HumvWA (5), D13S317 (6), D21S11 (7), HumFGA (8), D7S820 (9), and D18S51 (10).*

still captured on the beads without replenishing the lysate with any solution, even after 15 consecutive extractions (see Table 5). The quality of the DNA is such that very good Profiler Plus profiles can be obtained following testing with Hemastix® and multiple rounds of DNA extraction using robotics (Fig. 1). However, it is noted that the signal intensities from the profiles obtained from multiple extractions are reduced. This reduction in signal intensity observed can be attributed to (i) lower quantities of input DNA used (1.0 ng for panel A versus 0.44, 0.78, and 0.78 ng for panels B, C, and D, respectively), and/or (ii) potential degradation of the input DNA after multiple rounds of freezing and thawing of the lysate liquid column between re-extraction sessions.

Determination of the Causative Agent(s) Responsible for Preventing DNA Recovery from Hemastix®-Treated Samples

Results from Experiments 1 to 5 indicate that one or more chemicals from the Hemastix® strips are responsible for the non-recovery of DNA from DNA IQ™ magnetic beads. Diisopropylbenzene dihydroperoxide and TMB are the prime suspect chemicals for this interference since they are the main reactive components of the strip. However, results from Table 6 suggest that the interference is likely due to the chemical interactions between multiple components including the TMB.

Kiruthiga *et al.* (18) in studying the formation and decay of the N,N,N′,N′-form of TMB (N-TMB) by using various oxidants, including hydrogen peroxide, reported that the peroxide-driven oxidation of N-TMB resulted in the formation of a cation radical (N-TMB$^{\bullet+}$). This intermediary can undergo further oxidation to attain the more stable di-cation quinoidal form (N-TMB^{++}) or undergo further reactions with other compounds including isocyanates (19). Thus, it was initially believed that the intermediates of TMB reaction were preferentially bound to the DNA IQ™ magnetic beads, preventing DNA from binding to the beads during the DNA isolation procedure. However, as illustrated in Table 6, neither TMB (when prepared in water; experiment #2), hydrogen peroxide, nor a combination of the two chemicals in different proportions exhibited any significant interference on DNA recovery when using the DNA IQ™ system.

It is worth noting that the methanoic preparation of TMB exhibited a moderate interference (up to 20%) of DNA recovery (experiment #1). This interference is probably caused by the solvent (methanol) rather than the native TMB as the water soluble form of TMB does not have any apparent adverse effect on DNA recovery.

According to the Hemastix® patent application filed by Miles Laboratories Inc. (20), the reagent strip contains many other chemicals whose functions are to stabilize the reactive ingredients as well as to enhance the color development of the oxidized tetramethylbenzidine. In fact, the product monogram (5) indicates that the buffer (48.0% w/w) and other non-reactive (41.2% w/w) components constitute the majority of the ingredients of the Hemastix® strip. Since one of the diluents used is dimethyl sulfoxide (DMSO), part of this experiment also investigated whether DMSO, singularly or in conjunction with TMB and hydrogen peroxide, had any adverse effect on DNA recovery by DNA IQ™. The results of this experiment were somewhat surprising.

Little or no interference was detected when blood swabs that were treated with TMB and DMSO, with or without additional reaction with hydrogen peroxide, were subjected to DNA extraction using the DNA IQ™ system (experiment #3). However, a considerable amount of DNA was "lost" (average 42.6%) when an identical set of swabs was treated with freshly prepared TMB in DMSO. A similar degree of interference was also observed regardless of the presence of hydrogen peroxide (36.9%). Moreover, little or no interference was detected when the blood swabs were treated with DMSO and/or hydrogen peroxide (experiment #4). The 8.7% reduction observed in the hydrogen peroxide PBS preparation is likely due to statistical variation between two populations of samples with different means. Therefore, the observed interference in DNA recovery by DNA IQ™ system on samples directly exposed to Hemastix® chemicals likely involves the chemical interactions between TMB and the magnetic beads (resin) in the presence of one or more chemical compounds imbedded in the chemical strip.

Effect of Hemastix® on Various Sample Types and Other Chemically Treated Samples Using DNA IQ™ or Phenol/Chloroform for DNA Extraction

Also of interest was the determination if other chemicals used in the laboratory could produce a decrease in DNA yield when used in conjunction with DNA IQ™ and if other sample types other

TABLE 6—*Concentrations and combinations of chemicals evaluated to determine the causative agent(s) responsible for preventing DNA recovery using the DNA IQ™ beads.*

Experiment Number	Concentrations and Combinations of Chemicals Tested*	Average DNA Yield for 20 μL Blood Swabs (ng ± SD (*N*))	% Reduction†
# 1	None	163.3 ± 15.3 (3)	NA‡
	0.5/1.0/2.0/3.0/4.0% TMB Methanol	130.7 ± 23.4 (15)	20.0
# 2	None	250.0 ± 55.7 (3)	NA
	0.5/1.0/2.0/4.0/6.8% Hydrogen Peroxide Water	249.3 ± 32.8 (15)	0.3
	0.5/1.0/2.0/3.0/4.0% TMB HCl Water	286.7 ± 75.8 (15)	0.0
	0.5/4.0% TMB HCl Water + 0.5/6.8% Hydrogen Peroxide Water	260.8 ± 43.4 (12)	0.0
# 3	None	124.0 ± 36.7 (3)	NA
	0.5/1.0/2.0/3.0/4.0% TMB DMSO	145.0 ± 37.7 (15)	0.0
	0.5/1.0/2.0/3.0/4.0% fresh TMB DMSO	71.1 ± 26.1 (15)	42.6
	0.5/4.0% TMB DMSO + 0.5/6.8% Hydrogen Peroxide PBS	136.6 ± 21.6 (15)	0.0
	0.5/4.0% fresh TMB DMSO + 0.5/6.8% Hydrogen Peroxide PBS	78.3 ± 27.3 (15)	36.9
# 4	None	145.0 ± 7.1 (3)	NA
	2.0/4.0/6.8% Hydrogen Peroxide PBS	132.4 ± 8.0 (9)	8.7
	5.0/20.0/40.0 μL DMSO	156.8 ± 31.6 (9)	0.0
	5.0/20.0/40.0 μL DMSO + 6.8% Hydrogen Peroxide PBS	185.6 ± 43.5 (9)	0.0

*Except for the controls (no chemical tested), results of all concentrations from a single method were combined to generate the average DNA yields.

†% Reduction is the percent difference between the tested and control groups. No difference is reported if DNA yield from the tested group is higher than that from the control group.

‡Not applicable for the control group.

TABLE 7—*Effect of Hemastix® and other chemicals on DNA yields from different sample types using manual organic or automated DNA IQ™.*

			Average DNA Yield (ng) N = 2 for Each Value	
			Automated DNA IQ™ Extract	Manual Organic Extract
Sample Type	Chemical Test	Portion Submitted to Extraction	Q-PCR	Slot Blot
Blood swab 2 μL and 20 μL	None	Whole	20.8, 93.8*	36.5, 200.0*
	Hemastix®	Whole	0.04, 1.7	36.5, 250.0
		Untested	10.3, 86.8	21.9, 74.0
		Tested	0.0, 1.4	13.4, 124.0
	Hemochromogen	Whole	10.0, 151.0	20.0, 455.0
	Hemastix® + Hemo.	Whole	0.03, 0.17	18.8, 310.0
	Azostix®	Whole	16.0, 146.5	18.0, 655.0
		Untested	14.3, 73.3	14.0, 415.0
		Tested	9.2, 146.8	11.8, 240.0
Blood stain on white cotton 2 μL and 20 μL	None	Whole	17.5, 192.8*	37.0, 565.0*
	Hemastix®	Whole	0.0, 0.06	49.0, 740.0
		Untested	0.0, 111.0	19.5, 120.0
		Tested	0.0, 0.11	25.0, 245.0
	Hemochromogen	Whole	7.0, 201.8	9.5, 500.0
	Hemastix® + Hemo.	Whole	0.03, 0.30	12.5, 950.0
	Azostix®	Whole	20.2, 202.5	24.0, 925.0
		Untested	10.1, 143.5	6.8, 305.0
		Tested	10.8, 147.5	8.8, 275.0
Blood swab 20 μL + urine	Azostix®	Whole	215.0	150.0
Saliva swab or stain on white cotton 20 μL	None	Whole	48.9, 59.3†	86.0, 81.0†
	Hemastix®	Whole	0.06, 0.41	149.0, 74.0
		Untested	12.6, 7.9	22.0, 27.0
		Tested	0.66, 0.06	88.0, 102.0
	FastBlue	Whole	19.8, 67.0	15.0, 39.0
Scalp hairs shaft or root	None	Whole	174.3	189.0
	Hemastix®	Whole	86.3, 91.8‡	295.0, 325.0‡
Possible biological material§	None	Whole	0.73	23.2
	Hemastix®	Whole	0.0	22.0
		Untested	0.21	9.7

*For these sections, the first number refers to the DNA yield for the 2 μL blood volume and the second number refers to the DNA yield for the 20 μL blood volume.
†For this section, the first number refers to the DNA yield for the saliva swab and the second number refers to the DNA yield for the saliva stain.
‡The first number refers to the DNA yield for the hair shafts tested and the second number refers to the DNA yield for the hair roots.
§Possible biological material refers to contact DNA collected from the sweat band of a baseball cap.

than blood could be affected. None of the chemicals used by the ERU personnel other than Hemastix® interfered with DNA recovery using DNA IQ™ (Table 7). None of the chemicals used including Hemastix® resulted in reduced or no DNA yield using the manual organic extraction method. Except for one sample, all control samples as well as untreated areas of the test samples yielded expected quantities of DNA. Untested portions of blood and saliva stains prepared on white cotton cloth and extracted with DNA IQ™ showed reduced DNA yield. It is likely that the Hemastix® chemistry interfered with DNA recovery in these untested halves of samples as a result of the diffusion of the chemicals through the substrate.

A subset of organically extracted DNA was subjected to real-time PCR quantification while a subset of DNA IQ™-extracted DNA was subjected to slot blot quantification. All organically extracted DNA provided results using real-time PCR or slot blot quantification. For DNA IQ™-extracted DNA, samples with no DNA result based on real-time PCR quantification also showed no DNA based on slot blot quantification (data not shown). These results confirm that DNA recovery is compromised rather than the amplification or the probe hybridization.

Conclusion

Experiments described in this paper demonstrate that one or more chemicals imbedded in the Hemastix® reagent strip severely impair the ability to recover DNA when used in conjunction with the Promega DNA IQ™ system. Although the exact mechanism of such an interference is not known, it is suspected that the reporting dye, TMB, plays a significant role in preventing DNA from binding to the magnetic beads. Moreover, our experiments also demonstrate that the interactions of the chemical components to the magnetic beads are irreversible as the chemicals cannot be washed off from the compromised magnetic beads. Unlike the interaction between denim dyes and the magnetic beads, the chemicals from the Hemastix® reagent strip are extremely effective in binding to the magnetic beads. The nature of this interaction, however, remains unknown. Furthermore, the chemicals exerted no detectable damage on the DNA molecule. The DNA remains in solution (lysate), which can be extracted subsequently with fresh aliquots of magnetic beads or with organic solvents. Once the DNA is extracted, full and good quality DNA typing profiles can be developed using the AmpF*l*STR® Profiler Plus® PCR Amplification kit.

Despite our findings that the Hemastix® chemistry severely reduces the efficacy of DNA recovery when using the DNA IQ™ system, Hemastix® strips can still be used as a presumptive test to screen for the presence of blood provided that the chemicals are not transferred onto the questioned stain itself. This can be achieved by rubbing a piece of dry filter paper, folded into a cone, on the unknown stain. The filter paper is then tested with a pre-wetted Hemastix® strip. Our experience in the RCMP forensic laboratories has shown that this indirect method is an effective way of screening suspected bloodstains. For example, diluted bloodstains (1/1000) on swabs, shoes, and clothing were successfully and

repeatedly detected using this indirect method during our training exercises (data not shown).

Unlike the organic procedure which partitions DNA into the aqueous phase during the extraction step, the Promega DNA IQ™ system relies on the successful binding and the subsequent elution of the DNA from the sample. Chemicals that interfere with the initial DNA binding step will have a profound effect on the efficacy of DNA recovery. Currently, further tests are being conducted to determine if other reagents and identification chemicals commonly used by Canadian law enforcement agencies have any negative impact on DNA recovery using the DNA IQ™ magnetic bead technology.

Acknowledgments

The authors would like to thank members of the Evidence Recovery Unit and Biology Operations Section of the RCMP for their involvement in the preparation or processing of some samples used in the experiments mentioned herein.

References

1. Takayama M. A method for identifying blood by hemochromogen crystallization. Kokka Igakkai Zasshi 1912;306:15–33.
2. Ponce AC, Pascual FAV. Critical revision of presumptive tests for bloodstains. Forensic Sci Commun 1999;1:1–5. http://www.fbi.gov/hq/lab/fsc/backissu/july1999/ponce.htm. Accessed August 26, 2009.
3. Gaensslen RE. Blood Identification—catalytic tests. In: Source book in forensic serology, immunology, and biochemistry. Washington, DC: National Institute of Justice, 1983;101–45.
4. Tobe SS, Watson N, Daeid NN. Evaluation of six presumptive tests for blood, their specificity, sensitivity, and effect on high molecular-weight DNA. J Forensic Sci 2007;52:102–9.
5. Hemastix® Reagent Strips. Bayer HealthCare LLC. 2004, Rev. 12/04.
6. Frégeau CJ, Germain O, Fourney RM. Fingerprint enhancement revisited and the effects of blood enhancement chemicals on subsequent Profiler Plus® fluorescent short tandem repeat DNA analysis of fresh and aged bloody fingerprints. J Forensic Sci 2000;45:354–80.
7. Grubwieser P, Thaler A, Kochl S, Teissl R, Rabl W, Parson W. Systematic study on STR profiling on blood and saliva traces after visualization of fingerprint marks. J Forensic Sci 2003;48:733–41.
8. Hochmeister MN, Budowle B, Baechtel FS. Effects of presumptive test reagents on the ability to obtain restriction fragment length polymorphism (RFLP) patterns from human and semen stains. J Forensic Sci 1991;36:656–61.
9. Kishore R, Hardy WR, Anderson VJ, Sanchez NA, Buoncristiani MR. Optimization of DNA extraction from low-yield and degraded samples using the BioRobot® EZ1 and BioRobot® M48. J Forensic Sci 2006;51:1055–61.
10. Montpetit SA, Fitch IT, O'Donnell PT. A simple automated instrument for DNA extraction in forensic casework. J Forensic Sci 2005;50:555–63.
11. Anslinger K, Bayer B, Rolf B, Keil W, Eisenmenger W. Application of the BioRobot EZ1 in a forensic laboratory. Leg Med 2005;7:164–8.
12. Frégeau CJ, Lett ML, Elliott J, Bowen KL, White T, Fourney RM. Adoption of automated DNA processing for high volume DNA casework: a combined approach using magnetic beads and real-time PCR. Int Congr Ser, 2006;1288:688–90.
13. Crouse C, Yeung S, Greenspoon S, McGuckian A, Sikorsky J, Ban J, Mathies R. Improving efficiency of a small forensic laboratory: validation of robotic assays and evaluation of microcapillary array device. Croat Med J 2005;46:563–77.
14. Greenspoon SA, Ban JD, Sykes K, Ballard EJ, Edler SS, Baisden M, Covington BL. Application of the BioMek® 2000 laboratory automation workstation and the DNA IQ™ system to the extraction of forensic casework samples. J Forensic Sci 2004;49:29–39.
15. Frégeau CJ, Lett ML, Elliott J, Yensen C, Fourney RM. Automated processing of forensic casework samples using robotic workstations equipped with non-disposable tips: contamination prevention. J Forensic Sci 2008;53:632–51.
16. Frégeau CJ, Yensen C, Elliott J, Fourney RM. Optimized configuration of fixed-tip robotic liquid handling stations for the elimination of biological sample cross-contamination. J Assoc Lab Automat 2007;12:339–54.
17. Waye JS, Presley LA, Budowle B, Shutler GG, Fourney RM. A simple and sensitive method for quantifying human genomic DNA in forensic specimen extracts. BioTechniques 1989;7:852–5.
18. Kiruthiga K, Aravindan P, Anandan S, Maruthamuthu P. Investigation on the formation and decay of the N,N,N′,N′-tetramethylbenzidine and radical cation using various oxidants and reductants by stopped-flow spectrophotometry. Res Chem Intermediates 2006;32:115–35.
19. Chemical Data Sheet—3,3′,5,5′-Tetramethylbenzidine, http://cameochemicals.noaa.gov/chemical/21089.
20. Lam CTW, inventor, Miles Laboratories Inc., assignee. Test composition and device for determining peroxidatively active substances and process for preparing same. US Patent Application 777,002. 1977 Mar 14.

Additional information and reprint requests:
Hiron Poon, M.Sc.
Royal Canadian Mounted Police
National Forensic Services—Vancouver
5201 Heather Street
Vancouver, BC
V5Z 3L7 Canada
E-mail: hiron.poon@rcmp-grc.gc.ca