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# VALIDATION OF THE OHIO ATTORNEY GENERAL'S CENTER FOR THE FUTURE OF FORENSIC SCIENCE AUTOMATE EXPRESS™ ROBOT

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# VALIDATION OF THE OHIO ATTORNEY GENERAL'S CENTER FOR THE FUTURE OF FORENSIC SCIENCE AUTOMATE *EXPRESS*™ ROBOT

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## Abstract

The discovery of Deoxyribonucleic acid (DNA) extraction by Friedrich Miescher was pivotal for the scientific community as new research, technology, and methodology were developed as a result. As more information was discovered about DNA, these developments became more specialized within different fields. Alec Jeffreys and his colleagues transformed the field of Forensic Science by utilizing the Restriction Fragment Length Polymorphism (RFLP) method and the Southern blotting technique to develop discriminating profiles using an organic extraction. Solid-phase extractions widened the range of abilities for DNA extraction as well as provided a method for automation in forensic laboratories. One instrument, the AutoMate™ *Express* robot, performs solid-phase DNA extractions with greater efficiency than manual methods. This validation of the AutoMate™ *Express* instrument adheres to the Quality Assurance Standards (QAS) developed by the Federal Bureau of Investigation (FBI) to maintain accuracy of methodology as well as uphold accreditation standards. Results of the validation found that the AutoMate™ Express instrument is a reliable, efficient method that is well suited for daily laboratory use. Use of the AutoMate™ Express instrument would provide analysts with faster extraction of samples, as well as lower possibilities of contamination. Overall, the AutoMate™ Express instrument proves to be a practical option for use in conjunction with daily laboratory work.

**Introduction**

Extraction of Deoxyribonucleic Acid (DNA) from cells has been widely used in a variety of disciplines for many years. DNA extraction was first utilized for the purpose of determining the chemical components of cells[1]. This research was performed in 1869 by a Swiss physician named Friedrich Miescher, who was investigating proteins in leucocytes. Miescher observed a molecule in the leucocyte cells that differed in properties from the proteins he was studying. Specifically, the molecule would precipitate from solution in the presence of acid and would dissolve in alkaline solution. Using many wash steps and novel protocols, Miescher was able to isolate the molecule from the leucocyte cells. From his isolation, he discovered that the molecule was derived from the nucleus of cells, contained a high composition of phosphorus, and was slightly acidic. Miescher named this molecule "nuclein". Since his discovery, numerous scientists have experimented and studied Miescher's molecule to acquire the structure, function, and other important properties that the substance possessed. Nuclein was later changed to its proper name, Deoxyribonucleic acid (DNA), with Russian biochemist Phoebus Levene's discovery of the three main components of nucleotides that comprise the molecule: a ribose sugar, a phosphate group, and one of four nitrogenous bases[2]. Erwin Chargaff, Rosalind Franklin, James Watson, Francis Crick, and Maurice Wilkins are some of the notable scientists that then expanded on Levene's work to determine the complete double-helix structure of DNA. While Miescher was unable to fully identify the substance he was isolating from cells, his experiments were the first recorded examples of DNA extraction from its cellular components. The method that he had used provided the foundation for more complex extraction procedures, which have a wide variety of different purposes today. Modern-day isolation of DNA is used for

paternity testing, ancestry tracking, medical testing, genetic engineering, vaccines, hormone development using recombinant DNA, forensic testing, and many other applications[3].

While the use of DNA extraction has been around for many years, the application of the procedure for forensic science is relatively new. The first recorded use of DNA isolation with the intent of forensic field utilization was an experiment by a British geneticist named Alec Jeffreys in 1985. Jeffreys and his colleagues used the Restriction Fragment Length Polymorphism (RFLP) method and the Southern blotting technique to propose a more discriminating method for forensic testing of suspects[4]. Previously, biological profiles in crime laboratories were developed with different blood typing tests that identified blood group markers based on cellular antigens, such as ABO, ADA, AK, etc. Analysts were able to use these tests to eliminate suspects with some degree of discriminating power, but not enough to make a confident identification. The proposed technique that Jeffreys and his colleagues used in their experiment isolated large sections of DNA with motifs of repeating base pairs, known as Variable Number Tandem Repeats (VNTRs). The DNA was isolated using an organic extraction followed by a phenol-chloroform (PCl) cleanup step. VNTRs would then be excised from large segments of DNA in the sample using restriction enzymes and run on native gel electrophoresis to separate the fragments by size. The Southern blot method would then drive the DNA out of the gel into a membrane where $^{32}$P-labelled minisatellite probes were hybridized to the single-stranded DNA at complementary sequences. Bands were produced in the membrane, which would then be developed by an autoradiograph. These bands were found to be individualized to the DNA from a single contributor. From their analysis, Jeffreys and his colleagues deduced that DNA does not change over the course of an individual's lifetime, that degraded DNA will still produce results, and that all individuals will have different band sequences from one another. These results lead

to a higher power of discrimination; Jeffreys determined that using two separate probes in his procedure would decrease the chance association from 0.014 (blood testing) to $5x10^{-19}$ (RFLP/Blotting technique). The article further discusses how the developed autoradiographs could compare known and unknown samples. This was a great discovery for the forensic field because analysts could have greater confidence in making inclusions in their analyses among suspects. Lastly, the discovery of a differential extraction was a noteworthy accomplishment by Jeffreys and his colleagues in this article. In differential extractions, male sperm cells are separated from female vaginal epithelial cells to extract DNA from the sperm independently. Differential extractions are still frequently performed in laboratories today and are especially beneficially in specific cases such as sexual assault cases, which are generally mixtures of these two cell types. The discoveries made by Jeffreys and his colleagues transformed forensic testing of biological materials since many methodologies used today were developed directly from their findings.

The techniques used by Jeffreys and his colleagues were significant for the application of forensic testing, but still had many limitations. Autoradiographs, for instance, were only able to be developed from the method if high molecular weight DNA were used. Specifically, fragments of DNA analyzed needed to be greater than 20,000 base pairs in length. Also, the amount of DNA necessary for fingerprint production was between 50-500 ng. Many forensic samples do not contain this amount of DNA, so they would be unlikely to prove useful in a case. Mixture samples were also unable to be differentiated from this method, and profile development from single contributors took anywhere from two to four weeks. Overall, the method had a greater discrimination power than the testing used in crime laboratories previously, but it still was a lengthy and demanding procedure that required improvement for greater efficiency.

Since the implementation of DNA extraction in crime laboratories, many different methods have been developed to better suit forensic specialties. Organic extraction, used in Jeffreys experiment, is the oldest extraction method developed. Jeffreys also invented the differential extraction, a special kind of organic extraction that is used specifically for sperm cells. Organic extraction is highly susceptible to contamination, is time consuming, and can cause problems with downstream analysis if not properly performed. To increase efficiency of the organic extraction and potentially reduce limitations, the Chelex® extraction was developed synonymous with the invention of the Polymerase Chain Reaction (PCR) method[5]. The newer PCR process is able to develop profiles only using 0.25-2ng of DNA, in only 2-4 days or less, can be used to distinguish mixture profiles, and can tolerate up to 400 base pairs in the DNA. For these reasons, many laboratories have moved away from traditional RFLP and shifted to PCR-based methods. In Chelex® extraction, the use of styrene divinyl-benzene copolymer beads that contain two amphoteric iminodiacetate ion functional groups help to purify the extract through an ion exchange mechanism. This process is quick and has a higher efficiency than organic extraction but is not as efficient in removing inhibitors. Inhibitors cause issues in downstream DNA analysis like PCR amplification, so it is critical that inhibition is limited or reduced completely during an extraction method. Following Chelex® extraction utilization in laboratories, other PCR-based methods were later developed for specific, but not necessarily common, forensic samples. These include direct amplification, hair extraction, and bone/teeth extraction. Another more common method, solid-phase extraction, was also implemented into forensic laboratories for better efficiency and reduction of inhibition in analyses.

Solid-phase extraction, while similar to previous methods, is the only analysis that can be automated. The manual version of the solid-phase method uses beads---made of different

substances depending on the kit--- that bind to the DNA once it is released from its cellular container. The procedure continues with multiple wash steps to purify the DNA from proteins and other debris. DNA is then eluted from the beads and isolated from solution into a clean test tube for further analysis. Multiple kits have been developed with procedures that slightly vary from one another. These include the Promega DNA IQ™ System kit, Qiagen extraction kit, Applied Biosystems PrepFiler™ Forensic DNA Extraction Kit, as well as others. The procedure outlined in this validation uses the PrepFiler *Express*™ Forensic DNA Extraction Kit to prepare samples that will be run on the AutoMate™ *Express* instrument. The kit uses magnetic beads with an outer silica coating and follows the bind-wash-elute method of DNA extraction. Automation of methods has been desired in the forensic field because it would reduce time, analyst error, and contamination as well as increase efficiency in laboratories.

The AutoMate™ *Express* instrument, developed by Applied Biosystems, extracts DNA very similarly to the manual solid-phase extraction. Samples are first subjected to lysis buffer, provided by the PrepFiler *Express*™ Forensic DNA Extraction Kit[6] containing a variety of different chemicals. These chemicals help to promote the rupture of the lipid membrane in cells, protein denaturation, and inhibit the binding of cofactors that promote nuclease activity. DNA is isolated from the cell packaging in solution. The instrument is then setup according to the user guide provided[7]. The protocol card, cartridge rack, and tip and tube rack are properly inserted into the instrument before samples are run. The cartridge rack contains three wash buffers and the magnetic beads that are used to bind, wash, and elute the DNA. The tip and tube racks provide sterile supplies for the robot to perform the procedure. Samples are then processed on the instrument and can be subjected to later quantification, amplification, and capillary electrophoresis to determine interpretable data for analysis.

The AutoMate *Express*™ instrument has been analyzed in numerous validations and research studies to determine whether laboratories should incorporate it in their daily operations. Jason Liu and colleagues, for instance, analyzed the efficiency and purpose of the AutoMate *Express*™ instrument by specifically examining its ability to remove a broad range of PCR inhibitors while maintaining a high DNA recovery rate[8]. Forensic evidence poses unique challenges to DNA extraction technologies because of the broad and sometimes unpredictable nature of evidence sample types. PCR inhibitors also naturally occur on both the biological samples and the substrate (the material in which the sample was deposited on). Therefore, extraction technology like the AutoMate *Express*™ must be effective in removing these inhibitors for further analysis and development of profiles. Results of the study show that the AutoMate *Express*™ provides reliable results at different DNA input amounts and is effective in maximizing the amount of DNA obtained from samples that contain both small and large quantities of biological material.

While studies conducted on instrumentation/methodology provide excellent background for laboratories to consider, they still must undergo a validation process before implementing it into daily practice. Any accredited crime laboratory in the United States must adhere to the Quality Assurance Standards (QAS) developed by the Federal Bureau of Investigation (FBI). Benefits of an accreditation approval for a laboratory is use of the Combined DNA Index System (CODIS) for performing forensic DNA testing, therefore many crime laboratories seek accreditation. Extraction procedures must adhere to the standards outlined in the QAS document for a laboratory to uphold their accreditation. Standard 8 in the document provides the parameters surrounding validation in the laboratory. It states that developmental validation should precede the implementation of any new methods used for forensic DNA analysis and

internal validations should be completed for any manual and robotic The Scientific Working Group on DNA Analysis Methods (SWGDAM) has also outlined more specific guidelines, that still adhere to the FBI-QAS, for validations of any DNA analysis methods in crime laboratories[10]. The intention of this document was not to create new standards for laboratories, but to encourage laboratories to review their current standards and update procedures as needed. Another SWGDAM document attempts to clarify and explain what specific studies and other parts of a validation are required that are not thoroughly explained by the FBI-QAS standards[11]. Internal validations, for instance, must have known and nonprobative evidence samples/mock evidence samples, sensitivity and stochastic studies, precision and accuracy evaluations, mixture studies, and contamination assessments. This validation performed these studies for the AutoMate *Express*™ instrument.

Previous validations of quantification, amplification, and capillary electrophoresis methodology and instrumentation have been performed at the Ohio Attorney General's Center for the Future of Forensic Science. These processes follow the extraction of DNA from samples to provide interpretable data to confirm the efficiency of the AutoMate *Express*™ as applicable to the FBI-QAS standards. The validation report for the Quantifiler® Trio DNA Quantification Kit and GlobalFiler® Amplification Kit determined the efficacy for quantifying and amplifying samples on their respective instruments: the QS5 Real-Time PCR and the MiniAmp™ Thermal Cycler[12]. The validation report for the 3500 Genetic Analyzer provided the efficacy of the instrument in separation of loci after amplification and to develop STR (Short Tandem Repeat) profiles for analysis[13]. The validation reports of these instruments/kits provide confidence in the data analysis for the extraction of samples in this experiment. The studies done in these validations illustrate that the instruments are efficient and reliable in the profile development

process, meaning that data analyzed in the validation of the AutoMate *Express*™ instrument is reliable as well.

**Materials**

Instrumentation

Previously validated instrumentation: the Applied Biosystems QS5 Real-Time PCR System (SN: 272522743), the Applied Biosystems MiniAmp™ Thermal Cycler (SN: 2280418045000), and the Applied Biosystems 3500 Genetic Analyzer (SN: 31189-060).

The Applied Biosystems AutoMate Express™ Instrument (Protocol Card version 1.1, SN: PFX 2102B1605) was validated in this procedure.

Kits

Previously validated kits: the Quantifiler® Trio DNA Quantification Kit, and the GlobalFiler® PCR Amplification Kit.

The PrepFiler™ Forensic DNA Extraction Kit (Manual), the PrepFiler *Express*™ Forensic DNA Extraction Kit (Robotic) were validated in this procedure. Additional Dithiothreitol (DTT) was prepared in the laboratory for use in conjunction with kits.

Analysis Software

Previously validated analysis software included: the HID Real-Time PCR Analysis Software v1.3, and the GeneMapper® *ID-X* Software v1.5/1.6.

Equipment

Equipment used during the procedure included: a vortex, centrifuge/microcentrifuge/plate centrifuge, thermomixer, 37°C oven/incubator, Invitrogen DynaMag-2 Magnetic Stand, tube racks, 96-well heat block, freezer/96-well snap cooling block, 3500 96-well plate base (black) and cover (white), 20 mL syringe, beakers, graduated cylinder, biohazard trash receptacle, lab

coats, gloves, face masks, autoclave, balance, sample storage boxes, pipette tips (10, 200, and 1000uL), micropipettes (10, 200, and 1000uL), 10% bleach, Kimwipes, aluminum foil, dolphin nosed tubes, spin baskets, sterile toothpicks/wooden applicator sticks, 1.5 mL microfuge tubes, MicroAmp® Optical 96 well plate, MicroAmp® Optical adhesive film, foil adhesive cover, 3500 Genetic analyzer 8 capillary array, 3500 reservoir septa, 96-well plate septa, and press/seal wrap.

**Methods**

Two sets of samples underwent manual or robotic extraction methods. The first set had 26 samples of various biological fluids deposited from many different contributors. Four reagent blanks were analyzed with the samples through the entire process. The Ohio Attorney General's Center for the Future of Forensic Science Forensic Biology & DNA Standard Operating Procedures (SOP) Manual[14], derived from Applied Biosystems' protocols, was followed for the "Manual PrepFiler™" extraction procedure. The second set of 29 samples contained nearly the same samples as the first set, except for the addition of a few additional samples. Four reagent blanks were also analyzed for this set of samples. These samples were prepared using the PrepFiler *Express*™ Forensic DNA Extraction Kit and run on the AutoMate *Express*™ instrument. The procedure performed followed the "AutoMate Express™ with PrepFiler *Express*™ Forensic DNA Extraction Kit" procedure outlined in the SOP Manual, based on the protocols developed by Applied Biosystems. Following extraction, samples were quantified on the QS5 Real-Time PCR System, amplified on the MiniAmp™ Thermal Cycler, and run on the 3500 Genetic Analyzer for capillary electrophoresis. These steps also followed the procedures outlined in the SOP Manual that were derived from Applied Biosystems protocols.

Between quantification and amplification steps, data was obtained to determine the amount of DNA in each sample using a standard curve with HID Real-Time PCR Analysis

Software. This data also determined the amount of each sample that was amplified. After capillary electrophoresis, STR electropherograms were developed and peaks were analyzed for potential artifacts or contamination. STR interpretation procedure was followed according to the SOP Manual, which closely adhered to the FBI-QAS guidelines interpreted by SWGDAM in the interpretation guidelines document[15]. PDFs of each sample electropherogram were printed and data was exported into an Excel spreadsheet. Data was further organized and analyzed using all samples from the validation, as well as samples used in other studies, to determine instrument efficiency and functionality in a forensic laboratory.

**Results**

Electropherograms were produced for the samples and controls using the GeneMapper® *ID-X* Software v1.5 directly after capillary electrophoresis (CE). For a single-source profile, two peaks at a single locus indicate heterozygous alleles for that locus and a single peak represents a homozygous allele for that locus. The boxes underneath each peak provide the allele call, size in base pairs, and the height in relative fluorescent units (RFUs). The base pair size for each peak is determined using an Internal Lane Standard (ILS). The ILS is a small fragment of manufactured DNA, mixed in wells with samples, with a known size in base pairs (bp) that is used as a standard for comparison of sample fragments of unknown sizes. The height is determined through the direct proportionality of fluorescence detection and amount of DNA. The greater amount of DNA fragments copied at a locus during PCR, the greater number of fluorescent tags that will be present during CE, corresponding to taller peaks. The allele calls are determined using an allelic ladder, which is an individual sample consisting of an artificial mixture of common alleles present in a population for STR markers. The software converts the size of a

peak to an allele repeat number by comparing to the allelic ladder. An example of a partial sample electropherogram is provided in **Figure 1**.

**Figure 1**. *Partial electropherogram for the Robot "10R" Sample.* Blue and green dye channels are shown for the sample as well as the loci at each channel. Boxes with a diagonal line through them represent an artifact peak edited and removed from the data.

The sample electropherograms were edited to remove any artifacts that misrepresented actual DNA in the sample. Data interpretation of artifacts was determined using the SOP manual[14]. Some of the artifacts removed included stutter, pull-up, and split/shoulder peaks caused from non-template strand adenine addition by the Taq polymerase (-A). Editing was performed for all samples, both manual and robotic, and the resulting single-source electropherograms were saved as PDF files for reference.

After electropherogram editing, the data provided by the software was exported into an Excel spreadsheet. Data from both manual and robotic procedures were organized into tables by sample and peak heights for alleles at each of the 24 STR loci analyzed during CE. These tables provide a general overview of data collection as well as provide a broad demonstration of peak heights for the entire method. Same type descriptions are also provided for more in-depth

analysis. **Table 1** provides an example for the robotic samples data organization. A similar table was also constructed for the manual procedure samples.

**Table 1.**
*Robot Extraction Sample Data*. Example of data organization in Excel spreadsheet. Robot sample descriptions and corresponding names are located on the left of the table. Average peak heights for either heterozygous or homozygous alleles at each locus are reported horizontally for each sample. The peak heights correspond to both sample and STR locus.

| Description | Sample | D3S1358 | vWA | D16S539 | CSF1PO | TPOX | Yindel | AMEL | D8S1179 | D21S11 | D18S51 | DYS391 | D2S441 | D19S433 | TH01 | FGA | D22S1045 | D5S818 | D13S317 | D7S820 | SE33 | D10S1248 | D1S1656 | D12S391 | D2S1338 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CO Hair | 1R | 2798 | 2634.5 | 2121.5 | 2715 | 2323.5 | | 5557.5 | 6023 | 3969 | 3326 | | 4105.5 | 2807 | 3276.5 | 2307 | 4942.5 | 3877.5 | 4448.5 | 4870 | 3643.5 | 4179.5 | 5170.5 | 4387.5 | 3867.5 |
| JRC Buccal | 2R | 7149 | 5794 | 4151 | 4454 | 3844.5 | 9158 | 10510 | 10204 | 6072 | 5871.5 | 6533 | 5877 | 5798.5 | 5736 | 4016.5 | 7546 | 6302 | 7919.5 | 5335 | 6950.5 | 9365 | 9274.5 | 6373 | 6891.5 |
| CO Buccal | 3R | 5686.5 | 3839 | 3889 | 4277.5 | 3360.5 | | 8873.5 | 7996.5 | 5006.5 | 4500 | | 5584.5 | 5323.5 | 4804 | 4285 | 7091 | 5063.5 | 5407 | 4438 | 5357 | 7696 | 7005.5 | 5047.5 | 7376 |
| Twin A Buccal on Flocked Swab | 4R | 11537.5 | 10196 | 10580.5 | 10615.5 | 8112 | | 14530 | 20601 | 16869.5 | 13552.5 | | 12326.5 | 12048.5 | 12771 | 10732 | 15499 | 15867 | 19107 | 15329.5 | 12765 | 17877.5 | 20692 | 14648.5 | 19271.5 |
| Twin B Buccal on Flocked Swab | 5R | 4297.5 | 4144 | 3984 | 3545.5 | 2821 | | 10107 | 8356.5 | 6875 | 5116.5 | | 3321.5 | 2560 | 3317 | 2530 | 7385 | 6757 | 6929 | 5343.5 | 4650.5 | 9609.5 | 8716.5 | 7032.5 | 6071 |
| Neat Male Saliva (Lot # M5632 LS6620273) | 6R | 2928.5 | 1969 | 1399 | 1104.5 | 1079 | 7482 | 5676.5 | 3813 | 2021.5 | 2166 | 1414 | 2914 | 3180 | 2270 | 1993.5 | 5381 | 3506.5 | 3061.5 | 2024.5 | 1442 | 5607.5 | 3563 | 1800.5 | 2074 |
| Neat Male Saliva (Lot # M5632 LS6620273) with 0.1 g/mL Humic Acid | 7R | 4454 | 2837 | 2358 | 2201 | 1399.5 | 8191 | 8309 | 4672 | 3202.5 | 4264.5 | 1888 | 4246 | 4479.5 | 2890 | 3483.5 | 6688 | 4316 | 4443 | 4252.5 | 2835.5 | 7525.5 | 4537 | 2718 | 3626 |
| Neat Male Blood HMN461141 | 8R | 5130 | 4563 | 5239.5 | 6421 | 5532.5 | 8224 | 10599.5 | 6655.5 | 5598 | 7918 | 5288 | 3309.5 | 3445.5 | 6388 | 4665 | 10713 | 9095 | 11055 | 5529.5 | 6868.5 | 9238.5 | 10531 | 4376.5 | 13884.5 |
| Male Blood HMN481614 (CM33) – 25 uL @ 110,000 cells | 9R | 2749 | 2806 | 3212 | 3210.5 | 2843.5 | 3991 | 6295 | 4691.5 | 5646 | 4093.5 | 3438 | 3313 | 2896 | 3453 | 2895 | 4527.5 | 5983 | 5674 | 4233 | 4511.5 | 4499.5 | 5504 | 3520 | 5691.5 |
| Male Blood HMN481614 (CM33) – 50 uL @ 220,000 cells | 10R | 3106 | 2623.5 | 3082.5 | 3815 | 3480.5 | 4715 | 6191.5 | 5648.5 | 4159 | 3891 | 4307 | 3842 | 3250.5 | 5797 | 4043 | 5418.5 | 4536 | 6500 | 3769.5 | 3457 | 3764 | 4751 | 2482 | 5507 |
| Male Blood HMN481614 (CM33) – 75 uL @ 330,000 cells | 11R | 3784.5 | 3899.5 | 3344.5 | 5373 | 3460.5 | 7091 | 9127 | 5788 | 5520 | 5804.5 | 5685 | 2539 | 2780 | 5961 | 3938 | 7546.5 | 7099.5 | 7837 | 3645 | 4842.5 | 6573 | 7530 | 3687 | 8448.5 |
| Male Blood HMN481614 (CM33) – 100 uL @ 440,000 cells | 12R | 2979.5 | 4476 | 3072.5 | 4736 | 2803.5 | 9638 | 9964 | 4903.5 | 5683 | 3941 | 3754 | 2063 | 2055 | 6783.5 | 4999.5 | 10802 | 8713.5 | 10306.5 | 2596 | 3341 | 6917 | 7078 | 2499.5 | 7249 |
| Male Blood HMN509395 (BM72) @ 328,000 cells on cotton | 13R | 2071.5 | 2454.5 | 2238.5 | 3778 | 2369.5 | 3188 | 4965 | 2334 | 2098.5 | 2497 | 2238 | 1478.5 | 1028 | 3031.5 | 2130.5 | 4532.5 | 3944.5 | 4154.5 | 1192 | 1583 | 4964 | 3452.5 | 1197 | 4037.5 |
| Male Blood HMN509395 (BM72) @ 328,000 cells on flocked | 14R | 5404 | 6026.5 | 6752 | 9216 | 4788.5 | 9540 | 16214.5 | 11535 | 6126.5 | 8578 | 7063 | 4892.5 | 4846.5 | 10865 | 10848 | 10807 | 15392.5 | 13700 | 7224 | 6567 | 10539 | 9224.5 | 5917 | 11545 |
| Male Blood HMN509395 (BM72) @ 328,000 cells on dissolvable | 15R | 1514.5 | 11051 | 3132 | 10361 | 4421.5 | 8440 | 21193 | 3547.5 | 4446.5 | 3269.5 | 6637 | 1504 | 995 | 6814 | 17306 | 14818 | 14651.5 | 14872 | 2620.5 | 1118 | 15859 | 13555.5 | 2299 | 7586 |
| NIST-E (2391d) on FTA paper | 16R | 6804.5 | 5884 | 6000 | 5321.5 | 3649.5 | | 13097.5 | 7449.5 | 6749 | 6408 | | 4448.5 | 3684 | 4181.5 | 4060.5 | 8168 | 8431.5 | 9217.5 | 7092 | 6103.5 | 10004 | 11025 | 6558.5 | 8804 |
| NIST Traceable Male Blood (LS2411372) | 17R | 3125.5 | 6353 | 5150 | 7106.5 | 4338 | 9357 | 16074.5 | 5760.5 | 6005.5 | 4722.5 | 7986 | 3430.5 | 2205.5 | 6408 | 7025 | 11525 | 12159.5 | 12406.5 | 2969.5 | 4113.5 | 11193.5 | 10801 | 4228 | 8940 |
| NIST Traceable Female Blood (LS2411373) | 18R | 14139.5 | 16009 | 19136.5 | 12286 | 13049.5 | | 13239 | 26450.5 | 24777.5 | 11853 | | 24814 | 21873.5 | 14431.5 | 30089.5 | 14787.5 | 24958.5 | 25707.5 | 14005.5 | 12892 | 7338.5 | 22388.5 | 10147.5 | 27522 |
| NIST Traceable Male Blood (LS2411372) with 0.1 g/mL Humic Acid | 19R | 22965.5 | 20873.5 | 18375 | 13013 | 13914.5 | 28993 | 29477.5 | 28433 | 29841.5 | 24095 | 21560 | 15260.5 | 12007.5 | 13352.5 | 14279.5 | 31509.5 | 29560.5 | 28785 | 23439 | 16046.5 | 30843.5 | 31999.5 | 29831 | 25826.5 |
| NIST Traceable Female Blood (LS2411373) with 0.1 g/mL Humic Acid | 20R | 17562.5 | 16070.5 | 21681 | 11760 | 14269.5 | | 14112.5 | 27991.5 | 28969 | 12012.5 | | 30521.5 | 28920.5 | 11099 | 30308 | 15233.5 | 20402.5 | 21254 | 16050.5 | 11209.5 | 13814 | 20404 | 11545 | 27587 |
| Neat Semen (Lot # 2771-01 IRHUSMS1mL) | 21R | 3508.5 | 2914 | 2259 | 3915 | 1343 | 4188 | 3855 | 3156 | 4299.5 | 2835.5 | 3531 | 2052.5 | 1159 | 2062 | 2590.5 | 3737.5 | 4383 | 3513.5 | 3770.5 | 3319.5 | 5184 | 5342.5 | 2666 | 4720 |
| 1:10 Semen (Lot # 20-01-595 T6464) | 22R | 5177.5 | 4343 | 3998 | 4610.5 | 4357 | 6675 | 8360.5 | 6632 | 5508.5 | 6678.5 | 6262 | 3941 | 3540.5 | 5200 | 3861 | 5807 | 6184 | 8292 | 6885 | 5951.5 | 8403.5 | 8358.5 | 5938.5 | 9810 |
| 1:100 Semen (Lot # 2771-01 IRHUSMS1mL) | 23R | 3172.5 | 3251.5 | 2469 | 2141 | 2335.5 | 5665 | 4879 | 4216 | 4172.5 | 3787.5 | 3467 | 2887.5 | 2389 | 2663 | 2405.5 | 4222 | 4427 | 4553 | 4010 | 3428.5 | 5962 | 4593.5 | 3606.5 | 5861.5 |
| 1:1000 Semen (Lot # 2771-01 IRHUSMS1mL) | 24R | 4211.5 | 4027.5 | 3784.5 | 3568.5 | 3440 | 7500 | 7056.5 | 5905 | 4669.5 | 4936.5 | 5934 | 4442.5 | 3951.5 | 4420 | 4355 | 4735.5 | 4803.5 | 5920 | 5176 | 3757 | 6169 | 6496 | 4097.5 | 7937.5 |
| 1:10,000 Semen (Lot # 2771-01 IRHUSMS1mL) | 25R | 872 | 891.5 | 756.5 | 1152.5 | 777 | 1420 | 1325 | 1475 | 1219.5 | 1334.5 | 1803 | 1525.5 | 1639.5 | 1621 | 1313.5 | 1331 | 1244 | 1009.5 | 1020 | 1068.5 | 702 | 1183.5 | 822 | 1304.5 |

Data comparison among samples in this validation, as well as two additional experiments, were conducted in parallel with the internal validation studies recommended by the SWGDAM guideline document[11]. Known profiles of the samples analyzed in this validation were previously obtained and used to determine concordance for the robotic and manual methods. Two tables were created to demonstrate the comparison among both methods using peak heights and peak height ratios (PHRs). **Table 2** provides comparison of peak height averages among different loci, dye channels, and overall averages between both manual and robotic samples. For loci with heterozygous peaks, the average was taken among the two peak heights. For the homozygous peak, however, the single height value was divided by two. Evaluation of peak height values

among the two methods can give information regarding the efficiency of the AutoMate *Express*™ instrument.

**Table 2.**
*Comparison of Average Peak Heights for Manual and Robotic Methods. Note.* Peak height averages were determined at each locus among all samples within each method. Total averages were also calculated for each dye channel and the overall spectrum for robotic and manual methods.

| | Robot | | Manual | |
|---|---|---|---|---|
| By Loucs | Avg. Hetero. Peak Height | Std. Dev. | Avg. Hetero. Peak Height | Std. Dev. |
| D3S1358 | 5885.18 | 5273.46 | 6119.95 | 2727.33 |
| vWA | 5997.24 | 5019.11 | 5334.64 | 2678.00 |
| D16S539 | 5686.64 | 5666.29 | 5137.07 | 2813.60 |
| CSF1PO | 5627.92 | 3544.79 | 5448.81 | 2868.30 |
| TPOX | 4564.54 | 3788.99 | 4181.48 | 2505.09 |
| Yindel | 7969.78 | 5768.10 | 11173.94 | 5682.42 |
| AMEL | 10383.60 | 6062.67 | 11703.52 | 4968.07 |
| D8S1179 | 8969.54 | 7956.92 | 8995.64 | 4474.10 |
| D21S11 | 7980.22 | 8040.40 | 7252.98 | 3845.95 |
| D18S51 | 6298.12 | 4843.56 | 7491.05 | 3740.77 |
| DYS391 | 5488.22 | 4475.66 | 3527.21 | 2061.62 |
| D2S441 | 6185.62 | 7226.07 | 5613.81 | 2890.06 |
| D19S433 | 5554.56 | 6658.55 | 4969.02 | 2473.03 |
| TH01 | 5983.84 | 3705.61 | 5433.38 | 2555.20 |
| FGA | 7218.42 | 7972.24 | 5400.74 | 3074.78 |
| D22S1045 | 8990.16 | 6149.25 | 8459.43 | 3725.58 |
| D5S818 | 9266.36 | 7153.95 | 9090.88 | 4529.45 |
| D13S317 | 9842.92 | 7188.70 | 9454.00 | 5128.79 |
| D7S820 | 6272.82 | 5342.07 | 6619.10 | 3136.55 |
| SE33 | 5512.90 | 3889.72 | 6350.02 | 3602.96 |
| D10S1248 | 8953.14 | 5956.27 | 10135.31 | 3990.55 |
| D1S1656 | 9727.10 | 7153.13 | 10394.50 | 5023.02 |
| D12S391 | 5897.04 | 5950.45 | 6278.36 | 2870.54 |
| D2S1338 | 9657.58 | 7521.95 | 9907.07 | 4768.86 |
| | | | | |
| Blue | **5552.30** | **4684.75** | **5244.39** | **2741.59** |
| Green | **7963.46** | **6539.76** | **8425.65** | **4953.04** |
| Yellow | **6235.61** | **6520.80** | **5354.24** | **2719.28** |
| Red | **7977.03** | **6223.50** | **7994.69** | **4207.85** |
| Purple | **8558.72** | **6766.29** | **9178.81** | **4500.06** |
| Overall | **7258.76** | **6244.27** | **7268.36** | **4274.87** |

PHR represents the balance between alleles that provide analysts with information for the consideration of the alleles as "true" DNA. A PHR balance greater than 70% (50-60%) among two peaks can conclude that they are true DNA alleles[14]. The peak height ratios were determined

for loci with heterozygous alleles using **Equation 1**. Peak height ratio data for manual and robotic samples was organized and compared in **Table 3.**

$$\text{PHR} = \frac{RFUs\ of\ shorter\ allele/peak}{RFUs\ of\ taller\ allele/peak} * 100\% \qquad \textbf{(Equation 1)}$$

**Table 3.**
*Comparison of Average Peak Height Ratios (PHRs) for Manual and Robotic Methods. Note.* Peak height ratios were determined at each locus among all samples within each method. Total averages were also calculated for each dye channel and the overall spectrum for robotic and manual methods.

| | Robot | | Manual | |
|---|---|---|---|---|
| By Loucs | PHR | Std. Dev. | PHR | Std. Dev. |
| D3S1358 | 0.86 | 0.11 | 0.88 | 0.06 |
| vWA | 0.91 | 0.08 | 0.89 | 0.10 |
| D16S539 | 0.89 | 0.09 | 0.91 | 0.07 |
| CSF1PO | 0.88 | 0.08 | 0.88 | 0.10 |
| TPOX | 0.81 | 0.11 | 0.86 | 0.08 |
| AMEL | 0.76 | 0.14 | 0.84 | 0.12 |
| D8S1179 | 0.86 | 0.11 | 0.87 | 0.08 |
| D21S11 | 0.85 | 0.11 | 0.88 | 0.09 |
| D18S51 | 0.86 | 0.09 | 0.89 | 0.08 |
| D2S441 | 0.87 | 0.11 | 0.84 | 0.21 |
| D19S433 | 0.87 | 0.09 | 0.88 | 0.08 |
| TH01 | 0.86 | 0.05 | 0.82 | 0.04 |
| FGA | 0.93 | 0.07 | 0.86 | 0.11 |
| D22S1045 | 0.83 | 0.23 | 0.87 | 0.09 |
| D5S818 | 0.90 | 0.06 | 0.86 | 0.14 |
| D13S317 | 0.91 | 0.07 | 0.89 | 0.09 |
| D7S820 | 0.85 | 0.10 | 0.87 | 0.09 |
| SE33 | 0.80 | 0.16 | 0.86 | 0.10 |
| D10S1248 | 0.86 | 0.12 | 0.90 | 0.07 |
| D1S1656 | 0.83 | 0.15 | 0.84 | 0.14 |
| D12S391 | 0.85 | 0.11 | 0.89 | 0.10 |
| D2S1338 | 0.84 | 0.12 | 0.88 | 0.10 |
| | | | | |
| Blue | **0.87** | **0.10** | **0.89** | **0.09** |
| Green | **0.83** | **0.12** | **0.87** | **0.09** |
| Yellow | **0.89** | **0.09** | **0.86** | **0.13** |
| Red | **0.85** | **0.14** | **0.87** | **0.10** |
| Purple | **0.84** | **0.13** | **0.88** | **0.11** |
| Overall | **0.86** | **0.12** | **0.87** | **0.10** |

Data was compared among individual samples in the same way as the previous examples. Comparing peak height and PHR averages among samples for both robotic and manual methods offers additional information for the evaluation of instrument efficiency. **Table 4** evaluates peak

height averages for each sample analyzed by both manual and robotic methods. Some additional samples were analyzed with the robotic technique, so no comparison was completed. These additional robotic samples were two twin buccal samples on flocked swabs (4R and 5R) and a NIST-E sample on FTA paper (16R); all other samples were duplicates. Data was determined by averaging the peak heights for the entire sample, including every locus.

**Table 4.**
*Comparison of Average Peak Heights for Manual and Robotic Samples. Note.* Average peak heights were determined for each sample. White cells indicate that a sample was not analyzed for a particular method.

| Robot | | | Manual | | |
|---|---|---|---|---|---|
| Sample | Avg. Hetero. Peak Height | Std. Dev. | Sample | Avg. Hetero. Peak Height | Std. Dev. |
| 1R | 3788.68 | 1105.69 | 1M | 9167.27 | 2522.71 |
| 2R | 6713.58 | 1893.24 | 2M | 3084.38 | 1125.86 |
| 3R | 5541.25 | 1495.56 | 3M | 5962.95 | 2449.58 |
| 4R | 14342.25 | 3585.66 | | | |
| 5R | 5612.27 | 2314.86 | | | |
| 6R | 2911.29 | 1656.52 | 4M | 4654.15 | 2298.81 |
| 7R | 4159.04 | 1872.32 | 5M | 3093.88 | 1424.08 |
| 8R | 7094.52 | 2764.36 | 6M | 8722.98 | 3269.29 |
| 9R | 4153.21 | 1140.77 | 7M | 7657.92 | 2606.52 |
| 10R | 4255.71 | 1110.11 | 8M | 8125.79 | 2926.67 |
| 11R | 5471.02 | 1892.27 | 9M | 11514.79 | 4222.55 |
| 12R | 5473.10 | 2803.91 | 10M | 7981.88 | 3141.17 |
| 13R | 2789.94 | 1195.46 | 11M | 7747.19 | 3057.98 |
| 14R | 8900.50 | 3297.14 | 12M | 5407.35 | 2283.22 |
| 15R | 8000.50 | 6054.87 | 13M | 7863.43 | 8529.92 |
| 16R | 6961.00 | 2449.45 | | | |
| 17R | 7224.35 | 3622.35 | 14M | 13201.98 | 5273.69 |
| 18R | 18268.02 | 6616.39 | 15M | 11151.05 | 2336.38 |
| 19R | 23095.10 | 6946.80 | 16M | 14613.52 | 3937.16 |
| 20R | 19217.18 | 6951.52 | 17M | 3307.00 | 1109.01 |
| 21R | 3346.04 | 1098.74 | 18M | 5756.13 | 1876.89 |
| 22R | 6032.29 | 1703.02 | 19M | 3177.83 | 895.31 |
| 23R | 3773.56 | 1130.30 | 20M | 9177.88 | 2813.81 |
| 24R | 5053.94 | 1276.60 | 21M | 4790.42 | 2183.70 |
| 25R | 1200.46 | 302.08 | 22M | 4382.10 | 1574.44 |

The PHRs were also compared for individual samples among methods. This is demonstrated in **Table 5**.

**Table 5.**
*Comparison of Average Peak Height Ratios (PHRs) for Manual and Robotic Samples*. *Note.* Peak height ratios were determined for each sample. White cells indicate that a sample was not analyzed for a particular method.

| Robot | | | Manual | | |
|---|---|---|---|---|---|
| Sample | PHR | Std. Dev. | Sample | PHR | Std. Dev. |
| 1R | 0.84 | 0.10 | 1M | 0.90 | 0.07 |
| 2R | 0.89 | 0.08 | 2M | 0.91 | 0.09 |
| 3R | 0.87 | 0.12 | 3M | 0.88 | 0.09 |
| 4R | 0.93 | 0.06 | | | |
| 5R | 0.88 | 0.06 | | | |
| 6R | 0.81 | 0.16 | 4M | 0.88 | 0.08 |
| 7R | 0.83 | 0.14 | 5M | 0.86 | 0.11 |
| 8R | 0.85 | 0.18 | 6M | 0.85 | 0.16 |
| 9R | 0.83 | 0.11 | 7M | 0.85 | 0.08 |
| 10R | 0.84 | 0.08 | 8M | 0.87 | 0.08 |
| 11R | 0.83 | 0.10 | 9M | 0.89 | 0.08 |
| 12R | 0.87 | 0.11 | 10M | 0.88 | 0.07 |
| 13R | 0.79 | 0.14 | 11M | 0.87 | 0.08 |
| 14R | 0.87 | 0.09 | 12M | 0.83 | 0.14 |
| 15R | 0.86 | 0.13 | 13M | 0.51 | 0.44 |
| 16R | 0.88 | 0.08 | | | |
| 17R | 0.85 | 0.10 | 14M | 0.91 | 0.06 |
| 18R | 0.92 | 0.08 | 15M | 0.92 | 0.06 |
| 19R | 0.94 | 0.04 | 16M | 0.88 | 0.22 |
| 20R | 0.85 | 0.23 | 17M | 0.86 | 0.12 |
| 21R | 0.78 | 0.13 | 18M | 0.88 | 0.08 |
| 22R | 0.89 | 0.09 | 19M | 0.81 | 0.10 |
| 23R | 0.85 | 0.11 | 20M | 0.87 | 0.10 |
| 24R | 0.87 | 0.08 | 21M | 0.87 | 0.10 |
| 25R | 0.85 | 0.08 | 22M | 0.87 | 0.08 |

Nonprobative/mock evidence samples were provided by an experiment previously conducted and used for an evaluation of comparable results compared to known data by the instrument. This data was also used as part of the precision study. The samples collected were individual buccal swabs collected from eighteen students analyzed using the same manual procedure as the samples in this validation. **Table 6** depicts the data compared for the robot and manual samples (same as before) with the “unknown” buccal swabs.

**Table 6.**
*Comparison of Average Peak Heights for Knowns and Unknowns. Note.* Peak heights were determined at each locus for all known and unknown samples. Unknowns (buccal samples) were provided from an external experiment. Total averages were also calculated for each dye channel and the overall spectra.

| | Robot | | Manual | | Buccal Samples | |
|---|---|---|---|---|---|---|
| By Loucs | Avg. Hetero. Peak Height | Std. Dev. | Avg. Hetero. Peak Height | Std. Dev. | Avg. Hetero. Peak Height | Std. Dev. |
| D3S1358 | 5885.18 | 5273.46 | 6119.95 | 2727.33 | 9727.06 | 6733.92 |
| vWA | 5997.24 | 5019.11 | 5334.64 | 2678.00 | 7336.75 | 5305.34 |
| D16S539 | 5686.64 | 5666.29 | 5137.07 | 2813.60 | 7799.94 | 5536.92 |
| CSF1PO | 5627.92 | 3544.79 | 5448.81 | 2868.30 | 7511.97 | 5210.12 |
| TPOX | 4564.54 | 3788.99 | 4181.48 | 2505.09 | 6928.72 | 4836.27 |
| Yindel | 7969.78 | 5768.10 | 11173.94 | 5682.42 | 8725.75 | 5184.20 |
| AMEL | 10383.60 | 6062.67 | 11703.52 | 4968.07 | 10819.11 | 4545.83 |
| D8S1179 | 8969.54 | 7956.92 | 8995.64 | 4474.10 | 11839.75 | 7025.37 |
| D21S11 | 7980.22 | 8040.40 | 7252.98 | 3845.95 | 9690.64 | 6803.75 |
| D18S51 | 6298.12 | 4843.56 | 7491.05 | 3740.77 | 9389.97 | 7106.54 |
| DYS391 | 5488.22 | 4475.66 | 3527.21 | 2061.62 | 7523.00 | 4943.40 |
| D2S441 | 6185.62 | 7226.07 | 5613.81 | 2890.06 | 8844.36 | 4786.87 |
| D19S433 | 5554.56 | 6658.55 | 4969.02 | 2473.03 | 8538.39 | 4803.97 |
| TH01 | 5983.84 | 3705.61 | 5433.38 | 2555.20 | 9120.22 | 4569.56 |
| FGA | 7218.42 | 7972.24 | 5400.74 | 3074.78 | 8196.78 | 3892.35 |
| D22S1045 | 8990.16 | 6149.25 | 8459.43 | 3725.58 | 10682.31 | 7282.74 |
| D5S818 | 9266.36 | 7153.95 | 9090.88 | 4529.45 | 9746.67 | 6468.80 |
| D13S317 | 9842.92 | 7188.70 | 9454.00 | 5128.79 | 12019.86 | 8543.39 |
| D7S820 | 6272.82 | 5342.07 | 6619.10 | 3136.55 | 9661.58 | 6508.59 |
| SE33 | 5512.90 | 3889.72 | 6350.02 | 3602.96 | 9279.81 | 6233.16 |
| D10S1248 | 8953.14 | 5956.27 | 10135.31 | 3990.55 | 13113.89 | 9742.86 |
| D1S1656 | 9727.10 | 7153.13 | 10394.50 | 5023.02 | 13442.81 | 9739.09 |
| D12S391 | 5897.04 | 5950.45 | 6278.36 | 2870.54 | 10450.75 | 8711.19 |
| D2S1338 | 9657.58 | 7521.95 | 9907.07 | 4768.86 | 13950.97 | 8959.49 |
| | | | | | | |
| Blue | **5552.30** | **4684.75** | **5244.39** | **2741.59** | **7860.89** | **5523.36** |
| Green | **7963.46** | **6539.76** | **8425.65** | **4953.04** | **10203.82** | **6263.14** |
| Yellow | **6235.61** | **6520.80** | **5354.24** | **2719.28** | **8674.94** | **4445.19** |
| Red | **7977.03** | **6223.50** | **7994.69** | **4207.85** | **10278.04** | **6968.55** |
| Purple | **8558.72** | **6766.29** | **9178.81** | **4500.06** | **12739.60** | **9202.69** |
| Overall | **7258.76** | **6244.27** | **7268.36** | **4274.87** | **9877.86** | **6800.01** |

Sensitivity studies were performed using the data from samples ran in this validation as well as data from samples in a separate external experiment previously conducted. The samples 9R-12R and 7M-10M (refer to **Table 1** and **Table 4**) were ran as a sensitivity study within the analysis. The "R" refers to robot analysis and the "M" refers to manual analysis, but the same samples were used for both methods. These samples contained swabs with the same male blood deposit, varying in concentration. Concentration was measured in both $\mu$L and cell count, ranging from 25 $\mu$L at 110,000 cells to 100 $\mu$L at 440,000 cells. Another set of samples, 23R-

25R and 20M-22M contain the same semen sample set up as a dilution series among the swabs. The dilutions used range from 1:10 to 1:10,000, decreasing in semen concentration from sample to sample. Interpretable data can be found in **Table 4** and **Table 5** corresponding to each sample. A second sensitivity study analyzed for comparison was based on samples provided from a different external experiment using the AutoMate *Express*™ instrument. Samples contained varying concentrations of both carrier and 007 (standard) DNA. **Figure 2** provides comparison among differing starting DNA input amounts for PCR and total DNA recovered after complete analysis.

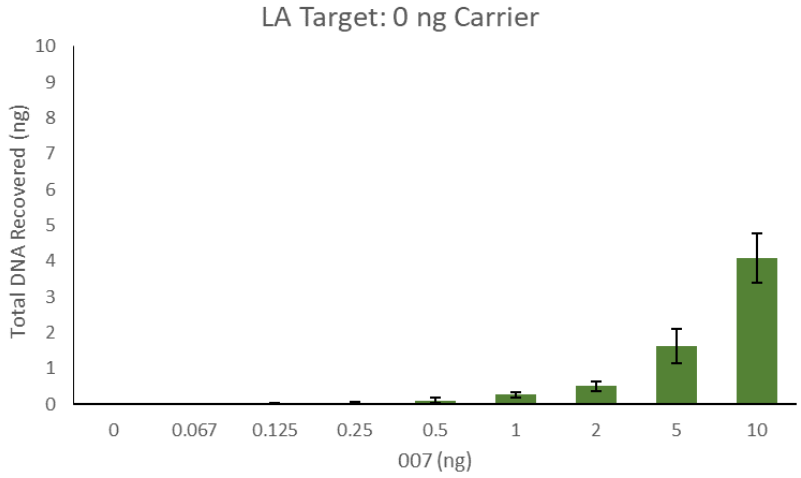


**Figure 2**. *Total DNA Recovered (ng) Compared to Input Concentration of 007 DNA*. Data collected was based on samples containing only the 007 DNA and no carrier DNA. Data was provided from an additional external experiment.

Additional data provided from this second external study included the average percent recovery of DNA from varying concentrations of starting DNA input. This data helps provide information for consistency of methods used, as the percent recovery should not be affected by varying concentrations. **Figure 3** analyzes this phenomenon.

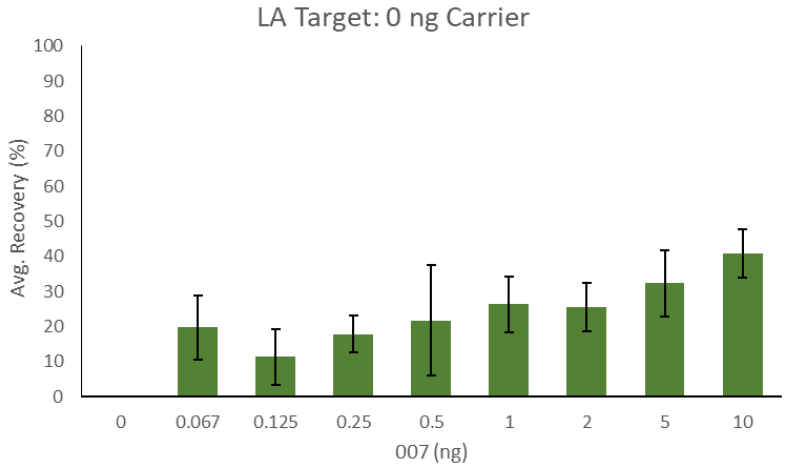


**Figure 3**. *Average Percent Recovery of DNA Compared to Input Concentration of 007 DNA.* Data collected was based on samples containing only the 007 DNA and no carrier DNA. Data was provided from an additional external experiment.

Sample peaks were compared to negative and positive control data for the contamination and reliability/accuracy studies. Contamination can be analyzed using negative/reagent blank data and comparing to sample data. If any peaks are observed in the negative controls, it could be an indication for possible contamination of samples. **Figure 4** displays an example of a negative control profile at two dye channels run in conjunction with the samples during CE.

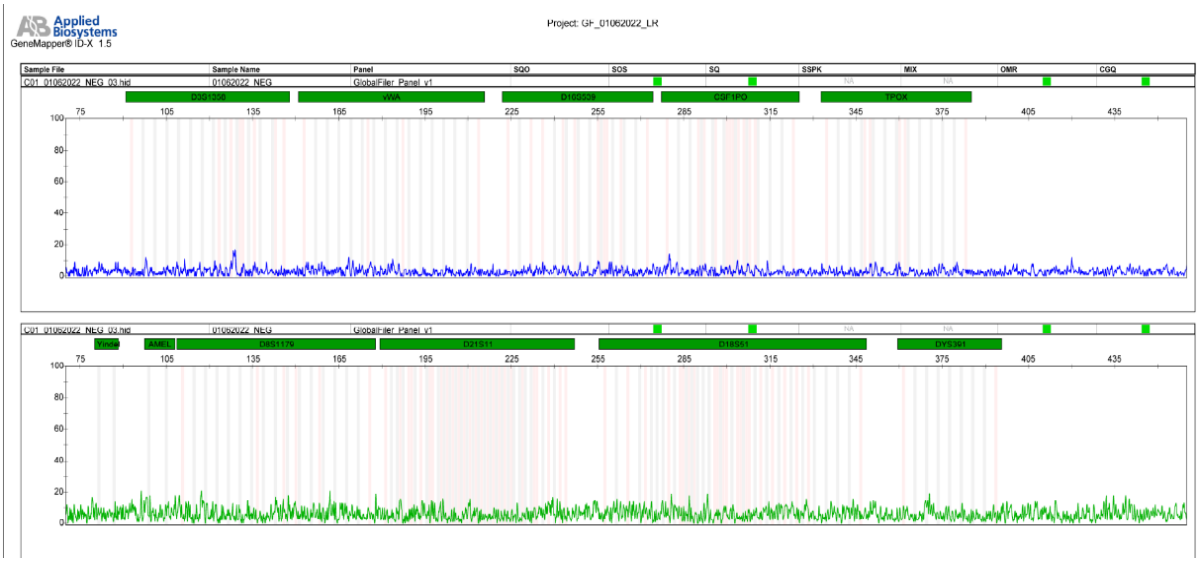


**Figure 4**. *Partial Negative Control Profile.* The blue and green dye channels are shown. Small peaks are indicative of instrument noise, large peaks are indicative of contamination.

Positive control data can aid in the evaluation of CE method efficiency; sample data reliability is based on the efficiency of the CE. The positive control is a DNA sample with a known profile that is used to determine if the instrument is working properly. The resulting profile should look similar to an electropherogram that would be generated for a sample. Positive controls, like negative controls, are run with samples during CE. A partial positive control profile is represented in **Figure 5**. The same negative and positive control were used for both manual and robotic samples.

**Figure 5**. *Partial Positive Control Profile.* The blue and green dye channels are shown. Peaks are represented the same way as the electropherogram in **Figure 1**.

The mixture studies were evaluated using four mixture samples analyzed with both manual and robotic methods. Different sample types were used to evaluate manual and robotic method competency analyzing varying sample types, compare the data between the methods, and determine which types (if any) are able to produce better results. The types of samples used were male and female blood, semen, and male saliva. After development of electropherograms for each of the mixtures, allele calls were recorded in **Figure 6** corresponding to individual loci. A

major and minor profile was determined as best as possible using the electropherograms and STR interpretation section of the SOP manual.

| Description | Extraction | D3S1358 | vWA | D16S539 | CSF1PO | TPOX | Yindel | AMEL | D8S1179 | D21S11 | D18S51 | DYS391 | D2S441 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Neat Semen (2771-01) & Male Saliva (M5632) Mix | Manual | 14 **16** | **16 17** | **10 12** | **10** 12 | **8** 9 **11** | **2** | **X Y** | **12** 15 **16** | **29** **30.2** | **12** 15 **17** | **11** | **11** **14** |
| | Robot | 14 **16** | **16 17** 18 | **10 12** | **10** 11 **12** | **8** **11** | **2** | **X Y** | **12** 15 **16** | **29** 30 **30.2** | **12** 15 **17** | **11** | **11** 13 **14** |
| Neat Semen (2771-01) & Female Blood (LS2411373) Mix | Manual | 14 16 18 | 16 17 | 9 10 11 12 | 10 11 12 | 8 11 12 | 2 | X Y | 12 13 16 | 29 30 30.2 31.2 | 12 14 17 18 | 11 | 10 11 14 |
| | Robot | 14 16 18 | 16 17 | 9 10 11 12 | 10 11 12 | 8 11 12 | 2 | X Y | 12 13 16 | 29 30 30.2 31.2 | 12 14 17 18 | 11 | 10 11 14 |
| Neat Male (LS2411372) Blood & Female Blood (LS2411373) Mix | Manual | **14** 17 **18** | **17** 19 | **9 11** | **11 12** | 8 11 **12** | 2 | **X** Y | 13 15 16 | 28 29 **30** **31.2** | 12 13 **14 18** | 11 | **10 11** |
| | Robot | **14** 17 **18** | **17** 19 | **9 11** | **11 12** | 8 11 **12** | 2 | **X** Y | **13** 15 **16** | 28 29 **30** **31.2** | 12 13 **14 18** | 11 | **10 11** |
| Male Saliva (M5632) & Female Blood (LS2411373) Mix | Manual | **14 18** | **17** | **9 11** | **11 12** | **12** | 2 | **X** | **13** 15 **16** | 28 **30** **31.2** | **14 18** | | **10 11** 13 14 |
| | Robot | **14 18** | **17** | **9 11** | **11 12** | **12** | | **X** | **13** 15 **16** | **30** **31.2** | **14 18** | | **10 11** |

Allele present in robot, not manual — Allele present in manual, not robot — **Major** — could not determine major

| D19S433 | TH01 | FGA | D22S1045 | D5S818 | D13S317 | D7S820 | SE33 | D10S1248 | D1S1656 | D12S391 | D2S1338 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **13 14.2** | **9 9.3** | 24 **25 26** | **15** | **11** 12 | **11** | **8** 11 **12** | **27.2 31.2** | **13** | **12** **17** | **18 24** | **19 23** |
| **13 14.2** 16 | **9 9.3** | 24 **25 26** | **15** | **11** 12 | 8 **11** | **8** 11 **12** | **27.2 31.2** 32.2 | **13** 16 | **12** 14 **17** | **18 24** | **19 23** |
| 13 14 14.2 15 | 9 9.3 | 20 21 25 26 | 15 | 9 11 12 | 11 12 | 8 11 12 | 16 27.2 30.2 31.2 | 13 15 17 | 12 14 17 | 17 18 20 24 | 18 19 23 25 |
| 13 14 14.2 15 | 9 9.3 | 20 21 25 26 | 15 | 9 11 12 | 11 12 | 8 11 12 | 16 27.2 30.2 31.2 | 13 15 17 | 12 14 17 | 17 18 20 24 | 18 19 23 25 |
| **14** 14.2 **15** | 9 9.3 | 20 21 | 11 **15** 16 | **9** 11 **12** | 9 **11 12** 13 | 9 **11 12** | **16** 17 **30.2** | 13 14 **15 17** | **14** 16 **17** 17.3 | 17 19 20 | **18** 20 23 **25** |
| **14** 14.2 **15** | **9** 9.3 | 20 21 | 11 **15** 16 | **9** 11 **12** | 9 **11 12** 13 | 9 **11 12** | **16** 17 **30.2** | 13 14 **15 17** | **14** 16 **17** 17.3 | **17** 19 **20** | **18** 20 23 **25** |
| **14 15** | **9** 9.3 | **20 21** | **15** | **9 12** | **11 12** | **11 12** | **16 30.2** | **15** 16 **17** | **14 17** | **17 20** | **18 25** |
| **14 15** | **9** 9.3 | **20 21** | **15** | **9 12** | **11 12** | **11 12** | **16 30.2** | **15** **17** | **14 17** | **17 20** | **18 25** |

**Figure 6**. *Allele Call Evaluation for Mixture Profiles.* Both manual and robotic methods were used to determine the major profiles from the mixture samples. The major allele(s) were determined and bolded; if no major could be determined, then the allele calls remained normal font. Alleles present in the robot and not the manual data were highlighted, and alleles present in the manual data exclusively were made red font. The figure is split in half for formatting convenience, and the bottom half corresponds horizontally with the top half (read left to right).

The total number of alleles for each sample, per corresponding method, were counted and recorded in **Figure 7**. The possible non-overlapping alleles were determined through analysis of the known electropherograms for each sample mixture. Allele counts less than the number of possible non-overlapping alleles indicate "dropout" within the analysis. Dropout is the phenomenon that occurs with low-level samples, instrument and stochastic errors, and other possible issues that arise with analysis. This causes the presence of a single peak at a locus when there should be two peaks.

| Description | Sample | Extraction | Allele Count | Possible (Non-Overlapping) Alleles |
|---|---|---|---|---|
| Neat Semen (2771-01) & Male Saliva (M5632) Mix | 23M | Manual | 46 | 66 |
| | 26R | Robot | 54 | |
| Neat Semen (2771-01) & Female Blood (LS2411373) Mix | 24M | Manual | 68 | 70 |
| | 27R | Robot | 68 | |
| Neat Male (LS2411372) Blood & Female Blood (LS2411373) Mix | 25M | Manual | 65 | 67 |
| | 28R | Robot | 65 | |
| Male Saliva (M5632) & Female Blood (LS2411373) Mix | 26M | Manual | 45 | 68 |
| | 29R | Robot | 40 | |

**Figure 7**. *Dropout Evaluation for Mixture Profiles.* Total alleles and possible non-overlapping alleles were determined and compared among samples/methods.

The analysis of internal validation studies provides the basis for thorough evaluation of the AutoMate *Express*™ instrument. Using the information from these studies, the efficiency of the instrument can be determined by analyzing results for precision, accuracy, repeatability, reproducibility, and mixture profile differentiation. The parameters surrounding the reliability of the instrument can also be evaluated based on contamination and sensitivity results. Furthermore, an overall conclusion can be made regarding whether the instrument is proficient and beneficial enough to be considered for daily laboratory use.

## Discussion

Validations for new methodology and/or instrumentation help determine the efficiency and necessity for use in the laboratory. A method or instrument must be as good (preferably better) than the method/instrument that precedes it. In this validation of the AutoMate *Express*™, the instrument's efficiency was evaluated to determine whether the solid-phase extraction method was at least as efficient as the manual solid-phase extraction method. Using the results from the studies performed in this validation as well as the two external experiments, efficiency and laboratory suitability of the AutoMate *Express*™ instrument can be identified along with limitations of the instrument.

Comparison of average peak heights/PHRs and standard deviations among both robotic and manual samples afforded an indication of concordance among methods in the known sample study. **Tables 2-5** compare both methods through analysis at each locus, dye channel, sample, and total profile. Peak heights were fairly comparable among both manual and robotic in **Table 2**, with some variation at a few loci. The dye channel averages were also very similar. The total peak height average for the full robot profile was 7,258.76 RFUs. The overall peak height average for the manual samples was 7,268.36 RFUs. This indicates that the methods produced very similar results and that there was no large discrepancy between proficiency of data among methods. In other words, the manual and robotic methods maintained high degree of concordance of results. The results of PHRs comparisons in **Table 3** provide similar information as that of **Table 2**. The average ratios were very similar among loci, dye channels, and total averages for entire sample profiles. The overall average PHRs was 0.86 (86%) for robotic samples and 0.87 (87%) for the manual samples. While PHRs help determine "true" DNA at a particular locus, they can also reveal consistency of balance among peaks. Profiles with high consistency among peak balance (number closer to 1.00) indicate greater quality of analysis. The results of **Table 3** offer a high concordance among PHRs for both manual and robotic methods. They also indicate decent quality among both methods, since 0.86 and 0.87 are fairly close to 1.00. **Table 4** provides the same information as **Table 2**, except that individual samples are evaluated rather than loci and dye channels. The case is the same for **Table 5** and **Table 3**. Results were very similar as before; average peak heights and PHRs were comparable among samples from both the robotic and manual methods overall. The results of this study advocate for the efficiency of the AutoMate *Express*™ instrument, as it is able to produce similar results to the manual method ran with the same samples.

Validations allow for the analysis of a new instrument or method in the laboratory to determine how efficient and accurate it will be for casework and unknown testing. It is important, therefore, to provide a study of unknown/mock case samples to evaluate this information. Data from the buccal samples provided in the first external study was compared to the robotic and manual methods in **Table 6**. The average peak heights were found to be generally higher among loci for the unknown samples than the other methods. Possible reasons for this could be that buccal samples generally contribute more DNA than other samples. The robotic and manual samples in this validation analyzed many different types of samples, including blood, semen, and saliva. These sample types tend to contribute less DNA for analysis, which is reflected in lower peak height averages. Regardless, the data among the manual/robotic methods and the unknown data was relatively comparable, with no major outliers or issues. The manual and robotic methods, therefore, are able to provide relatively accurate results for casework as well as known profiles according to this study.

Sensitivity, precision and accuracy, and contamination studies allocate information regarding parameters and optimization of the instruments and/or methods in a validation. Sensitivity data for the robotic and manual methods was determined using different samples with varying concentrations during analysis, as well as data from the second external study. Peak height averages among samples 9R-12R and 7M-10M were compared using data in **Table 4** and **Table 5**. The concentration of blood increased with the increasing sample number (i.e. sample 9R had a lower concentration than 10R), which should produce higher average peak heights for each increasing sample number. The robot method, as seen in **Table 4**, was able to replicate this theory better than the manual method. Peak height averages increased from sample 9R to sample 12R, but sample 9M for the manual method produced a higher peak height average than sample

10M. While the other manual samples followed the trend, this sensitivity study showed that the robot had better accuracy of analyzation. The peak height ratios, however, were found to be better for the manual method as seen in **Table 5**. In general, the robot and manual data proved comparable as there were few discrepancies among values. For samples 23R-25R and 20M-22M, the opposite result occurred. The manual method was able to follow the trend for the dilution series better than the robotic method, which is shown in **Table 4**. With increasing sample numbers (i.e. 23R to 24R), the average peak heights should decrease because it is proportional to the smaller concentration of DNA found in sample. This was true for the manual samples, but the robot sample 24R increased in peak height from 23R, which does not follow the predicted trend. The PHRs in **Table 5** were greater for these samples analyzed with the robotic method rather than the manual method, indicated by values closer to 1.00. Again, the overall concordance among methods was consistently high for both peak height averages and PHRs. The second external study also provided a separate sensitivity study for analysis of the AutoMate *Express*™ instrument. While the intent of this study was not to determine the efficiency of the instrument, the data collected was used to compare to the data collected by the instrument in this validation to provide conclusions about the efficiency. **Figure 2** provides that the data for the total DNA recovery through analysis increases with greater starting PCR input amounts, which correctly corresponds to the trend of sensitivity. The instrument, therefore, is also capable of differentiating sample concentrations and also has the ability to generate CE detection as low as 0.125ng of DNA according to this study. The results of low input data such as 0.125ng developed poor results of recovery so, if possible, the starting extraction amount should be greater for better downstream analysis and results for the instrument. **Figure 3** compares the varying concentrations of input DNA with the frequency, on average, that the DNA is recovered.

The results of this figure indicate that the average percent recovery is about the same for all samples tested, no matter what the starting input amount was. This shows that the PrepFiler *Express*™ Forensic DNA Extraction Kit recovers DNA relatively well over many analyses. From the sensitivity studies conducted and corresponding data, the AutoMate *Express*™ instrument proves efficient in the comparability to the manual method, the ability to differentiate concentrations of DNA in samples, detect at low levels, and provide a proficient amount of DNA for downstream analysis.

The precision and accuracy of the AutoMate *Express*™ instrument was determined through the analysis of repeatability and reproducibility studies as well as standard deviations of results. The first external study contributed to the reproducibility of the manual method as the method used for the buccal swabs was the same manual procedure as the one performed for the samples in this validation. The comparable results among the two provide that the method is reliable and accurate. Since the manual method was found to be accurate through reproducibility of results, it can therefore be an accurate measure against the results provided by the robotic method. Both the manual and robotic samples produced comparable results, indicating that the AutoMate *Express*™ instrument is also reliable and accurate. In addition to sensitivity information, the second external study provided a repeatability study as well. The samples used to collect data were also run on the AutoMate *Express*™ instrument. The collection of reliable data by this study indicates that the instrument can be utilized by different analysts with different samples and still produce reliable and accurate results. Standard deviations for **Tables 2-6** were taken for all data collected. This data can help determine the precision of methods evaluated; lower standard deviation values indicate smaller spread of the data, which is proportional to a greater precision among the data. Generally, the standard deviations were low in comparison to

the averages in the tables, indicating a low degree of imprecision. The AutoMate *Express*™ instrument is therefore able to generate precise data according to this study.

Evaluation of an instrument/method potential contamination is examined in validation studies to determine if any issue occurred during analysis and if data is reliable. If contamination occurred during any point of the analysis, results could be incorrect and skew the conclusions made previously. The contamination study for this validation included utilizing reagent blanks as well as negative and positive controls. Reagent blanks are run with samples through extraction, quantification, amplification, and CE as a negative control contamination index. Negative and positive controls are just run with the CE method, to determine any instrument noise/stochastic effects. The partial negative control profile in **Figure 4** showed no signs of contamination, as no large or unusual peaks were observed. This result was consistent among the entire profile. The partial positive control profile in **Figure 5** provided that the instrument was running correctly and efficiently, as peaks were observed at every locus consistent with known results. There were some stochastic effects observed (indicated by boxes with diagonal lines through them), such as pull-up and stutter, indicative of PCR chemistry and CE instrument effects. These are unavoidable and random effects that give an indication for observation in sample profiles as well. Overall, the controls found the analysis to be reliable with little concern for contamination. Sample data and evaluation can therefore be considered reliable as well.

Mixture profiles can be very difficult to analyze for forensic samples. It is imperative, therefore, to have methodology/instrumentation efficient enough to provide differentiable data for these samples. Data in **Figure 6** demonstrated that the robotic method was able to detect more alleles for the first mixture sample than the manual method. For the other three mixture samples, the manual and robotic were able to detect nearly the same alleles. This study suggests

that for certain mixtures, the robotic method may be able to differentiate profiles better than the manual method. Data in **Figure 7** contributes to the analysis of dropout in method detection. For the first sample, the robotic method has less dropout than the manual, indicated by an allele count value closer to the possible non-overlapping alleles value. The second and third samples had the same analysis for both methods and the fourth had the opposite effect. Overall, both methods are nearly as efficient in distinguishing major and minor profiles from mixtures as well as detecting all possible alleles. The AutoMate *Express*™ instrument, according to this study, is able to provide analysis for mixture samples in the forensic laboratory comparable to the manual method.

The AutoMate *Express*™ instrument, as demonstrated by the results of this validation, is as efficient and in some cases better than the manual solid-phase extraction method. Previous studies have also confirmed this statement through research[8]. The instrument has a short operation time, low potential for contamination, lower detection limits for some samples, and the ability to produce accurate/reliable data for both known and unknown samples. It does, however, have a few limitations. The instrument is expensive to purchase, which could be an issue for some laboratories. The approximate cost of the instrument is $48,950, not including the cost of the PrepFiler *Express*™ Forensic DNA Extraction Kit required to perform extractions[16]. The instrument is also unable to perform certain types of extractions, such as differential extractions, and may not be able to provide as accurate of results for certain samples. It would be prudent, therefore, for laboratories to maintain the manual extraction method in the case of poor detection by the AutoMate *Express*™ instrument for those samples, and the organic extraction method for differentials. Even so, the instrument proves to be a practical option for use in conjunction with daily forensic laboratory. It has the ability to promote efficiency in the laboratory by cutting

down time for analysis, which in turn could reduce overall cost, and increase reliability of analysis as well due to low contamination probability.

**Conclusion**

New DNA technology and methods have been more prominent as more discoveries and advancements are being made. It is important, therefore, to standardize and evaluate these methods as they are being utilized to draw conclusions impactful to individual lives. This is especially true in the forensic science field. The data produced by instrumentation and subsequent evaluations based on the methodology by analysts can indirectly impact the decision of an individual's freedom. This validation of the AutoMate *Express*™ instrument provides an example of the extent of evaluation recommended by the FBI that should be performed for new methodology/instrumentation. By providing studies and validation analysis, further forensic reports can be conducted ensuring high certainty of results with instrumentation/methodology used. Overall, while validations provide information on the efficiency of a method, they more importantly contribute to the greater cause of maintaining high ethical standards in the forensic science field.

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