Report Title: Automated Processing of FTA Samples

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Abstract:
The goal of this project is to design a system to automate the processing of FTA cards in forensic DNA databanking applications. The system allows a user to load a stack of FTA EasiCollect cards in the system, and then get out a 96-well plate of punches ready for PCR. The prototype includes the following subsystems: card manipulation, well plate handling, sample locating, well plate static removal, card punching, fluid addition, and dust removal. The subsystems were integrated and the resulting prototype was tested for sample loss, cross contamination, and compatibility with downstream STR analysis.

The system was found to be effective in reducing the risk of sample loss from static effects. The final estimated error rate was less than 0.15%. Tests to evaluate cross-contamination indicate that a cleaning punch between samples reduces the cross contamination risk and that the current vacuum system needs some improvements to prevent general dust buildup. In a final test, the system was used to punch FTA cards containing buccal swabs. All the samples tested resulted in full STR profiles. The results of the prototype tests were then used to develop a conceptual design for a next-generation prototype.

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1 Executive Summary

FTA cards are used in forensics for the collection and storage of DNA containing samples such as buccal swabs. The benefits of using the FTA cards include long term stability of the DNA at room temperature and easy integration with the PCR workflow [1]. To perform an STR analysis on a FTA card sample, a portion of the card is first punched out. This punched out portion is then added to the PCR reagents and amplification occurs on-punch. There are no current solutions to automate the entire pre-PCR workflow. As the number of DNA samples being sent to forensic DNA databanking labs is increasing, there is a need for reliable automation. The objective of this project was to design an automated system for the processing of buccal swabs on FTA cards for STR analysis. The focus of the design was to 1) improve the overall throughput by automating all steps prior to PCR and 2) reduce the risk of losing a sample.

The methods for FTA card processing vary widely between different forensic labs. For this project the focus was on a workflow that 1) used the EasiCollect device to collect DNA samples onto FTA, 2) used a 1.2mm punch from the FTA card, and 3) performed on-punch PCR. The EasiCollect version of the FTA card was chosen because the card's frame makes it amenable to automation. The 1.2mm punch size was chosen because this is the smallest punch size typically used in forensic labs, and therefore is the most affected by static electricity build up in the well plates. Static electricity is a major cause of sample loss, as it can cause samples to "jump" out of a well or stick to the side of well where they may not be processed correctly.

For the FTA workflow described above, an automated prototype was designed and fabricated. The system allows a user to load in a stack of buccal swabs on FTA EasiCollect cards, and then get out a 96-well plate of punches ready for PCR. The prototype includes subsystems for card manipulation, well plate

handling, sample locating, well plate static removal, card punching, fluid addition, and dust removal.

Initially manual tests were performed to evaluate methods of static removal. It was found that pretreating a 96-well plate with an ionizing bar with an attached air knife allowed a greater percentage of 1.2mm punches to fall to the bottom of the wells. Based on the results of these tests, an initial anti-static treatment of the well plates was included in the automated device. Of the ~2600 punches performed on the system >99.85% of punches were observed to end up in the appropriate well. Cross contamination experiments suggest that cleaning punches can be used to eliminate the risk of carryover from one sample to the next. Additional experiments indicated that an improved dust removal system was required, to avoid cross-contamination from general dust build-up. In the final experiment, the system was used to punch FTA cards containing buccal swabs and all 11 cards yielded full STR profiles with no evidence of cross contamination.

1 Introduction

1.1 Background

FTA cards are used in forensics for the collection and storage of DNA containing samples such as buccal swabs. The benefits of using the FTA cards include long term stability of the DNA at room temperature, and easy integration with the PCR workflow [1]. To perform an STR analysis on a FTA card sample, a portion of the card must first be cut out. The cut out portion (also known as the punch) is treated to remove PCR inhibitors and then added to the PCR reagents.

The number of DNA samples being sent to forensic DNA databanking labs is increasing. Laws such as The DNA Fingerprint Act of 2005 calls for collecting a DNA sample from anyone arrested for a federal crime or detained and then determined to be illegally in the United States [2,3]. As the number of samples to be processed increases, so does the need for reliable automation. There are some devices on the market to automate the punching step. In this step, a 1.2- to 3mm diameter circle is cut out from the card. These "card-in and punch-out" devices result in a 96-well plate, each well containing a small paper punch, that must be transferred to a liquid handling robot for punch washing and PCR reagent addition. There are concerns over losing the paper punches in the punching device, in the liquid handling system, and while transferring the plates between instruments. One of the biggest causes of punch loss is the static that builds up in the 96-well plate. This can cause the punches to "jump" out of the well or even stick to the sides of the well, which can prevent them from being processed correctly downstream.

The objective of this project was to design an automated system for the processing of buccal swabs on FTA cards for STR analysis. The focus of the design was to 1) improve the overall throughput by automating all steps prior to PCR and 2) reduce the risk of losing a sample.

1.2 Approach

The method of FTA card processing varies widely between different forensic labs. The size of the punch varies from 1.2 to 6mm and the method of processing the cards also varies. For the smaller punches, the PCR is often performed directly on the punch, as recommended by the manufacturer. For larger punches, the labs extract the DNA off of the punch, purify it, and then do in-solution PCR.

One of the focuses of this project was to reduce the risk of lost punches. Currently, the smallest punch size (1.2mm) has the greatest risk and reported occurrence of lost or "jumping" punches. Therefore it was decided that the in-

house prototype would use the smaller sized 1.2mm punch. For this sized punch it is necessary to use the "on-punch" amplification method. For PCR amplification of a single STR, Ampdirect Plus® (Nacalai, #07199-20) was used in the PCR reagent mix. Ampdirect Plus allows on-punch PCR without any washing steps. For full STR analysis, the PowerPlex 18D kit (Promega, #DC1802) was used which also allows direct amplification without washing. By removing the washing steps, the fluid handling is simplified and the overall throughput is increased.

The type of FTA card used in the in-house prototype was the card contained in the EasiCollect device. The EasiCollect device includes an indicating FTA card in a plastic housing with an attached swab (for buccal cell collection). The plastic housing is designed to allow a more even transfer of cells to the FTA card and the card indicates where saliva has touched the card (see Figures 1). The stiff cardboard frame around the FTA card gives the card rigidity, minimizing bending, which is necessary for the card to be reproducibly handled by a robotic system.

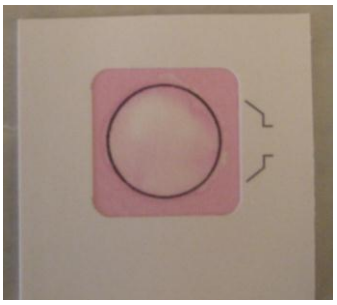
A  B 

Figure 1. A) EasiCollect device B) EasiCollect card with applied buccal swab. White area within printed circle indicates where saliva has been applied to card.

Discussions with our collaborators in forensics labs indicate that having punches "jump" out of their well, or not even go into the correct well to start with, is a common problem for the 1.2mm punches. As mentioned earlier, the most common cause is a build up of static charge (on the well plate and in the robotic device). Looking at the problem from a systems level, three elements were identified as being necessary to reduce the risk of lost punches. 1) Ensure the punch enters the well, 2) Ensure the punch falls to the bottom of the well, and 3) Ensure the punch remains at the bottom of the well.

Ensuring that the punch enters the well was addressed with careful design of both the puncher and the interface between the puncher and the well plate. The punch will have no other path but into the well plate. Ensuring the punch falls to the bottom of the well requires that static build-up on the well plate is removed prior to punching. To hold the punch in position at the bottom of the well, liquid was used. Reagents are dispensed into the well prior to punching.

The summary of the proposed workflow for the automated processing of FTA cards is summarized in Figure 2 below.

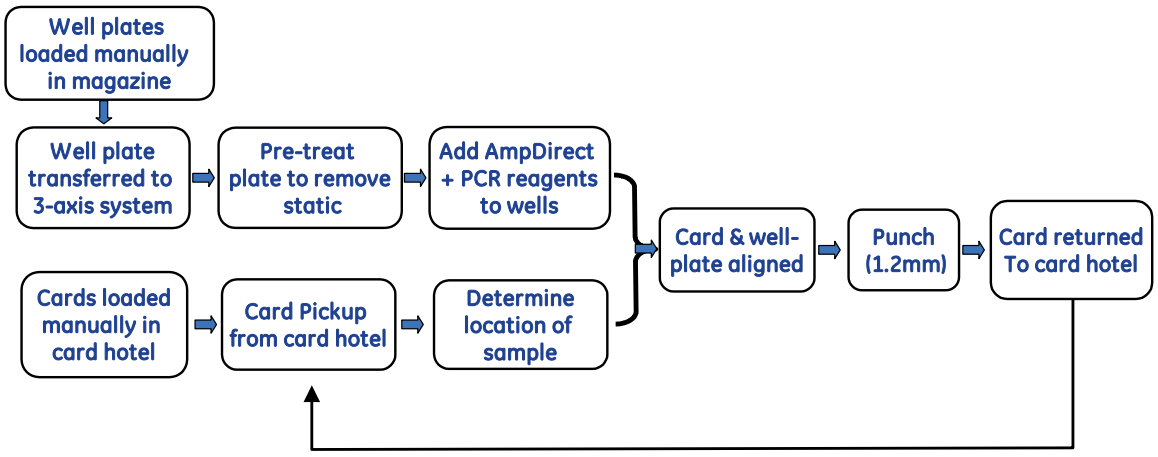


Figure 2. Proposed workflow for TRL4 system

2 System

2.1 Overall

The prototype system is shown in Figure 3 below. The system includes seven subsystems connected to a central control computer. The subsystems and their functions are:

1) Card handling subsystem: Picks up and manipulates the FTA cards.
2) Vision subsystem: Acquires an image of the FTA card for position calibration, to determine the location of the sample on the card, and to determine where punches have already been taken.
3) Well plate handling subsystem: Picks up, moves, and aligns 96-well plates.
4) Static removal subsystem: Pre-treats plate to remove static charge.
5) Liquid handling subsystem: Adds Ampdirect Plus and PCR reagents to the 96-well plate for STR analysis.
6) Punching subsystem: Punches a 1.2mm sample from the card and transfers it to the well of a 96-well plate.
7) Dust-removal subsystem: Removes dust from puncher and prevents buildup of dust.

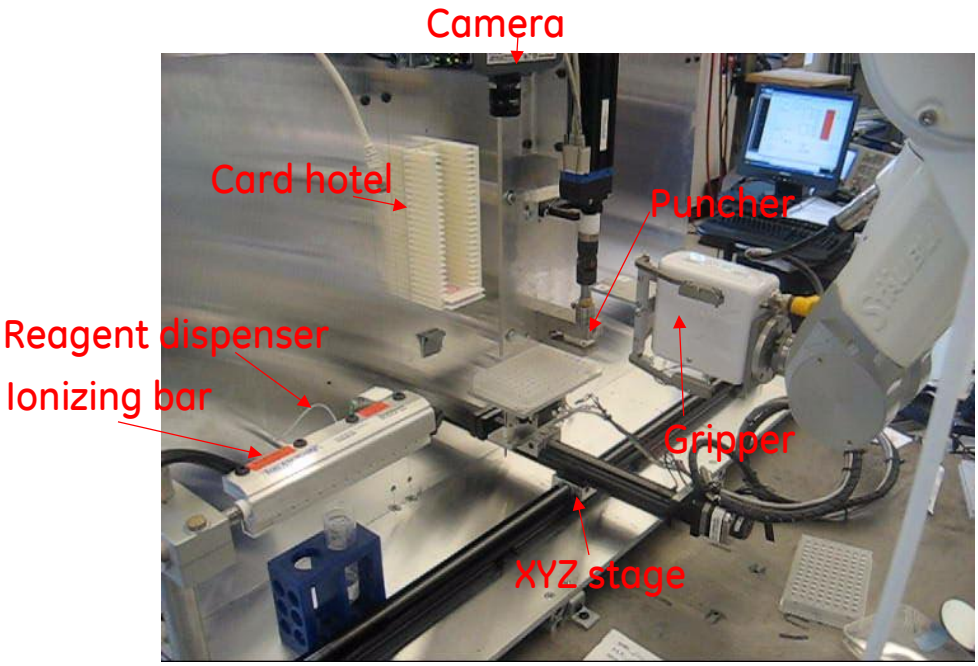


Figure 3. Close up of the automated system with subsystems labeled.

2.2 Subsystems

2.2.1 Card handling subsystem

The card handling subsystem removes the cards from the card hotel, puts them in position for imaging and punching, and then returns the card to the hotel. This prototype utilizes a 6-axis Staubli robot arm, a smart gripper (Applied Robotics Inc, SG2.1-75MM-10N), and a custom designed end-effectors for gripping cards as well as 96-well plates. The gripper has an on-board single axis controller for parallel gripping with the capability for standalone operation. In addition to controlling grip distance, speed, and velocity, the gripper can be programmed to do a variety of other tasks, such as detecting the presence of an object within its grasp and modulate the grip force if necessary. The different gripping tasks (e.g., open for card, grip card, open for well-plate, grip well-plate, etc.) are controlled using a simple custom parallel communication protocol over digital input lines. Feedback to the robot (e.g., information such as object found or motion complete) is indicated using individual digital output lines. It should be noted that the 6-axis Staubli arm and smart gripper are used for this proof-of-concept prototype because they allow significant flexibility in system design, workflow changes and for testing alternate card handling tools. In the conceptual design for the next generation prototype, simpler methods for card manipulation are recommended.

The customized end effectors for picking up cards and also well plates are shown in Figure 4. The card handling end effector consists of two rectangular plates with six threaded holes in each plate for mounting setscrews with sharp ends. These

setscrews act as 'teeth' to prevent the cards from slipping during card handling. For the card hotel, the design included support for the card on all 3 sides. Some of the cards were seen to have a slight curvature, which could interfere with the gripper picking up the card. The support grooves at the back of the hotel were found to help keep the cards straight. The slots are designed to be slightly larger than the card dimensions, as this was found to make manual loading of the hotel easier.

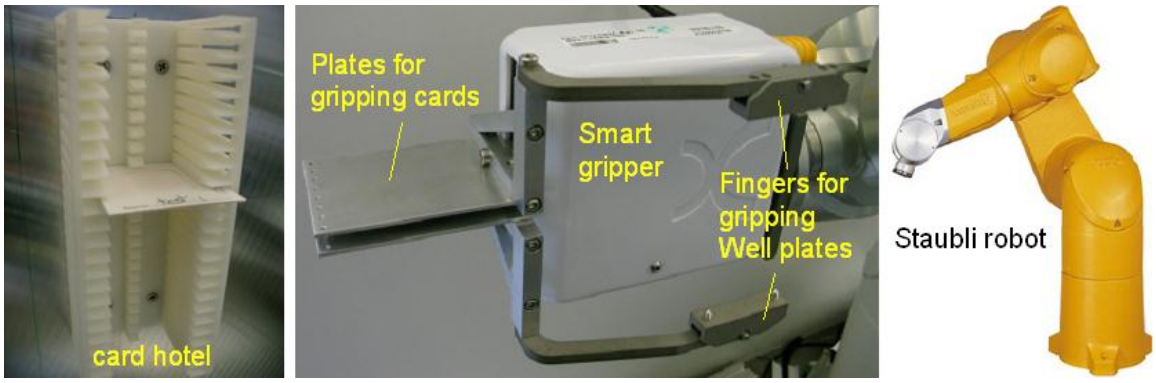


Figure 4. Components of card handling subsystem.

2.2.2 Vision subsystem

The vision subsystem determines the position of the card within the card gripper and the positions of any prior punches on the card. For these tasks, a smart camera and real-time calibration method are being used. The camera used in the POC prototype is the National Instruments 1744 Smart Camera. This camera has a 1280 x 1024 CCD image sensor with integrated 533-MHz PowerPC processor in a package slightly larger than a pocket dictionary. The processing and analysis were implemented using LabVIEW®.

Figure 5 shows a schematic representation of the vision system architecture. The camera has two Ethernet ports, one connecting to a host computer where a graphical user interface displays real-time results, the other to the robot controller. A communication protocol implemented in LabVIEW is configured so that the camera can serve as a closed-loop feedback device to the robot controller. The camera is prompted via a TCP instruction when a sample is presented for inspection, triggering image capture. The image is then processed to recognize and locate standard position markers along with any prior punches to the sample. When complete, a message containing a positioning directive and list of available punching locations is sent back via TCP to the robot controller.

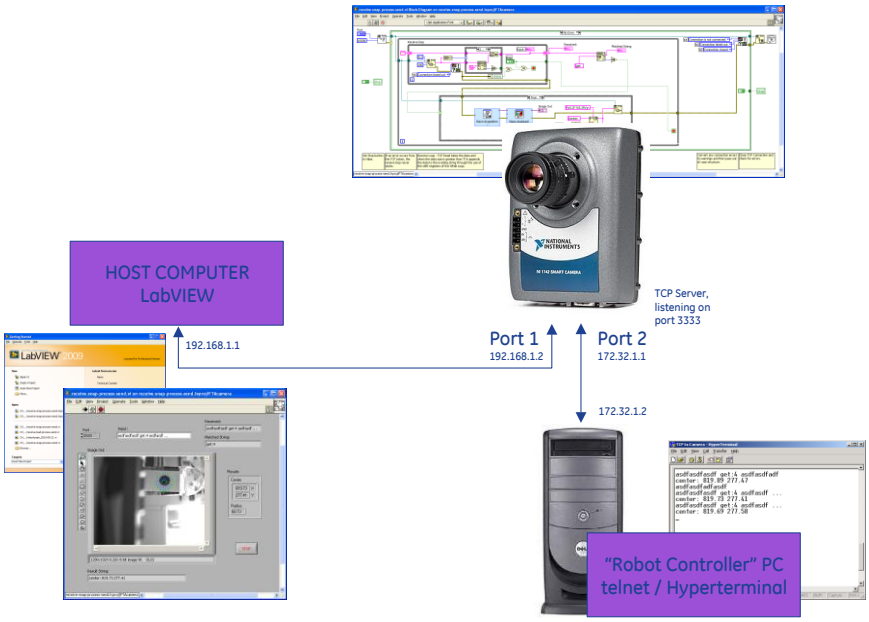


Figure 5. Vision system architecture.

The vision system is programmed to recognize markers on the robot gripper, which allows for automatic calibration to the punching system reference frame. This calibration is robust to any in-plane camera movement, though such movement is expected to be minimal due to rigidity of the overall structure. More importantly, the vision system can correct for variability of the card position within the gripper, which arises due to sizing the card hotel slots a bit larger than the card dimensions to permit ease of manual loading. Figure 6 shows a set of images representing different stages in a grid punching sequence. The top image is of a sample card taken immediately after punching 5 holes in a partial grid pattern. In the middle image, the same sample card is presented to the vision system for inspection. The card was deliberately re-oriented in the gripper to exaggerate the effects of position variability after retrieval from the card hotel. The vision system is asked to determine the card orientation and identify the set of pre-punched grid locations. By extension, a set of unused grid locations is also determined. In the bottom image, the robot and punching system have completed the grid using the positioning coordinates computed by the vision system. As a result of feedback from the vision system, the robot and punching system are successful in confining all punches within the circle that indicates where the swab contacts the sample.

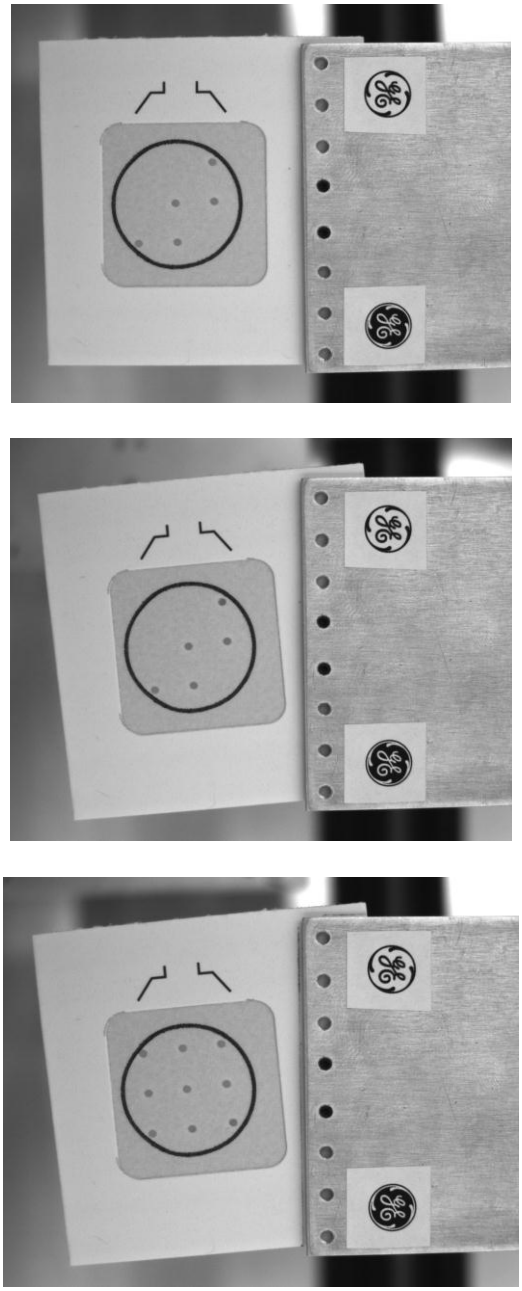


Figure 6. Grid punching experiment. The top image shows an EasiCollect card with 5 punches. The middle image shows the card after its position has been manually shifted. The bottom image shows that the system was able to detect the shift in position and complete punching the grid pattern.

2.2.3 Well plate handling subsystem

The task of the well plate handling subsystem is to move the well plate between the various process stations, i.e., fluid filling, punching, plate transfer and static control. It is a three degree-of-freedom (x, y, z) motion system, which consists of two linear slides and a custom designed slider crank mechanism. The motion along the x and y axes (horizontal, planar motion) is actuated by the linear slides (see Fig. 7). The x-axis slide has 30” travel while the y-axis side has 8” travel.

The x and y linear slides utilize Nema 17 motor and the slider crank is actuated by Nema 11 motor from *Lin Engineering*. The linear slides were ordered from *Velmex Inc* and available under the commercial name Xslide.

The well plate sits on the top plate, which can be moved vertically along the z-axis up to 20 mm by the slider crank mechanism (see Fig. 7). The slider crank system serves several important functions, one of which is related to punch control. Before punching, the well plate will be moved up until it is touching, or almost touching the bottom plane of the puncher. This ensures that the punch cannot fall into any well but the well directly below the puncher.

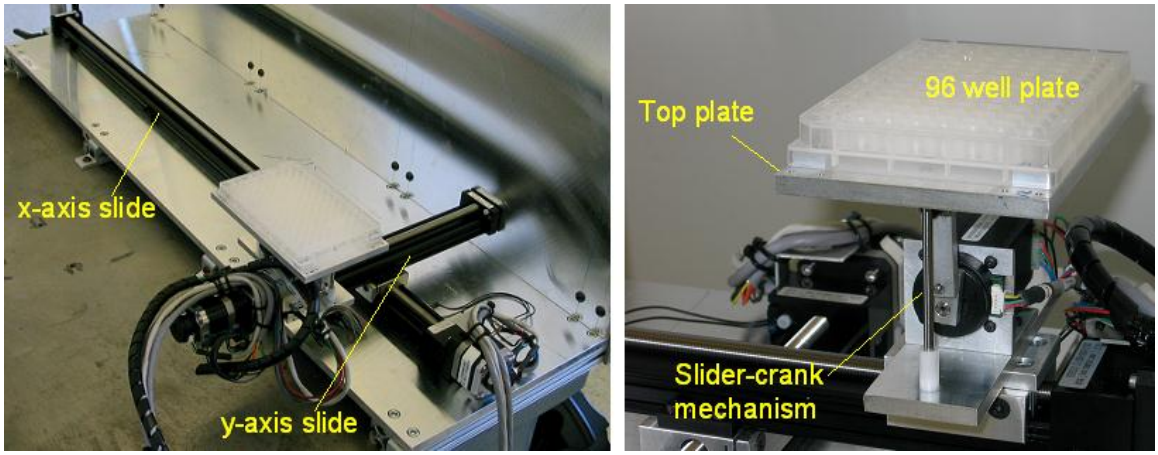


Figure 7. Well plate handling subsystem. The subsystem includes two linear slides (for x and y axis motion) and a custom designed z-axis actuator.

2.2.4 Static removal subsystem

The static removal subsystem is used to remove static charge from the 96-well plate prior to liquid addition and punching. This will help to ensure the punch is able to fall to the bottom of the well after punching. To achieve this, the subsystem must remove static both from the surface of the 96-well plate as well as from inside the wells themselves. For the proof-of-concept system being built here, an ionizing bar with an attached air knife was used. The ionizing bar (Exair Ion Air Knife, model 7106) produces both positive and negative ions and the size used here (6") covers the full length or width of the 96-well plate.

The Ion Air Knife has a row of 12 ionization nozzles spaced 0.5" apart. When powered on, the ionization nozzles generate localized high electrical field, which ionizes surrounding air. The device can operate in two modes: with or without forced airflow. Without forced airflow (ionizing bar mode), the generated ions diffuse from ionization source to surrounding area and recombine with oppositely charged particles. With forced airflow (ionizing bar + air knife mode), a continuous air sheet is created in front of the ionization nozzles from a pressurized air source. This air sheet is continuously ionized by the ion nozzles.

The generated ions are forced to move downward by the pressure source, creating an ion curtain. It was hypothesized that this forced ion motion (ionizing bar + air knife mode) would help move ions into the wells of the plate and therefore more effectively remove static from inside the wells.

2.2.5 Liquid handling subsystem

For liquid handling and dispensing a TECAN Cavro XLP 6000 syringe pump with a replaceable 2.5ml syringe and 4-port valve has been programmed for fluid dispensing. The pump has a 60 mm stroke distance and is driven by a stepper motor (6000 steps). With a 2.5ml syringe, it has a resolution of 0.4 ul. The pump is connected to a reservoir bottle and a dispensing tip by 0.5mm ID Teflon FEP tubing. The dead volume of the fluidic path, including the filling and dispensing tubing and the pump, will be controlled to be less than 100ul (so a full 2.5ml syringe will be able to fill a 96 well plate with 25ul in each well). This will be a single tip dispensing unit. For the final suggested design, a multi-tip dispensing unit would be suggested.

To evaluate different integration schemes, the Cavro XLP 6000 pump was tested for dispensing accuracy in three different mounting orientations, including upright, horizontal and inverted positions. The orientation of the pump in the system affects the tubing length (or dead volume) needed to connect the pump to the PCR reagent reservoir and the well plate. In this experiment, the syringe was first filled with water and the fluidic path was primed to remove air bubbles. Then twenty 25ul samples were taken under each pump orientation. The results indicate that while upright orientation produces slightly better accuracy than others, the dispensing accuracy is not very sensitive to pump orientation when the system is properly primed.

2.2.6 Punching subsystem

For the 1.2mm puncher, a tool and die based punch is used that also incorporates the ability to hold a card in place during the punching. The punching system is designed to reduce the possibility of punch loss. The cut disc is pushed through the entire length of the die by the punch head. The die hole is just slightly larger than the cut disc, thus the disc cannot get hung-up as it progresses through the die.

The actuator of the puncher is what provides the movement and force for punching. To allow us to test a range of acceleration speeds and forces, a linear motor (Copley Controls, Servotube STA1108) was chosen for the actuator. The control architecture is shown in Fig. 8. It uses the available fourth channel on the Arcus motion controller to generate the motion commands. Note that the other three channels are already used for controlling the three-axis well plate handling subsystem.

The motion command signals generated from the Arcus are in the standard pulse/direction form. This signal is converted by the Accelnet drive into an

appropriate trapezoidal position trajectory based on the programmed motion parameters such as high and low speeds and acceleration on the Arcus software. The Accelnet drive sends the motion signal to the servo tube actuator and receives the motion feedback signals via sin/cos encoder. Accelnet drive provides the closed-loop control via this feedback signal and also sends the motion signal to the Arcus for reference only. Fig 8 also shows the mockup of the system used for programming and testing the actuator before mounting on the back plane of the integrated device. The setup uses a mechanical home switch with a lever arm. During homing, the linear drive moves the thrust rod vertically until it comes into contact with the lever arm and activates the switch thereby, setting the reference position for subsequent motion.

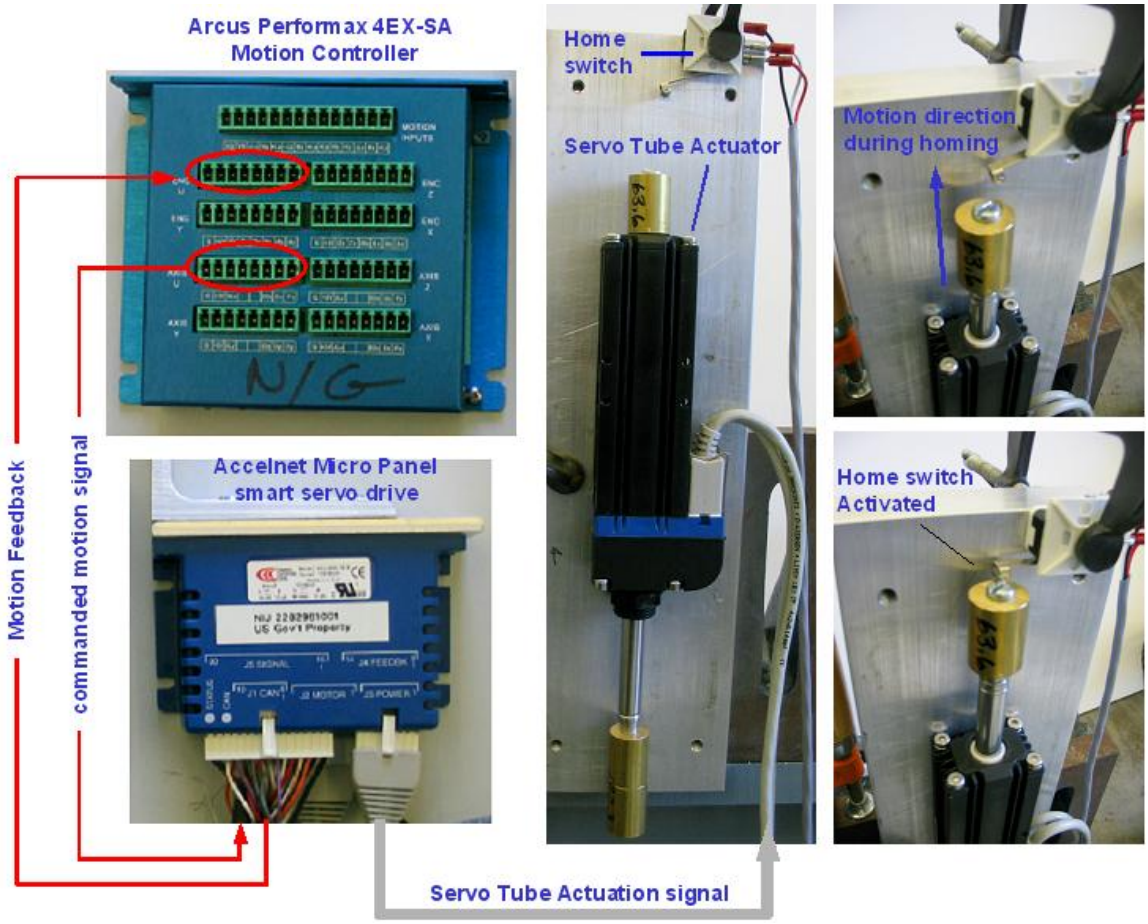
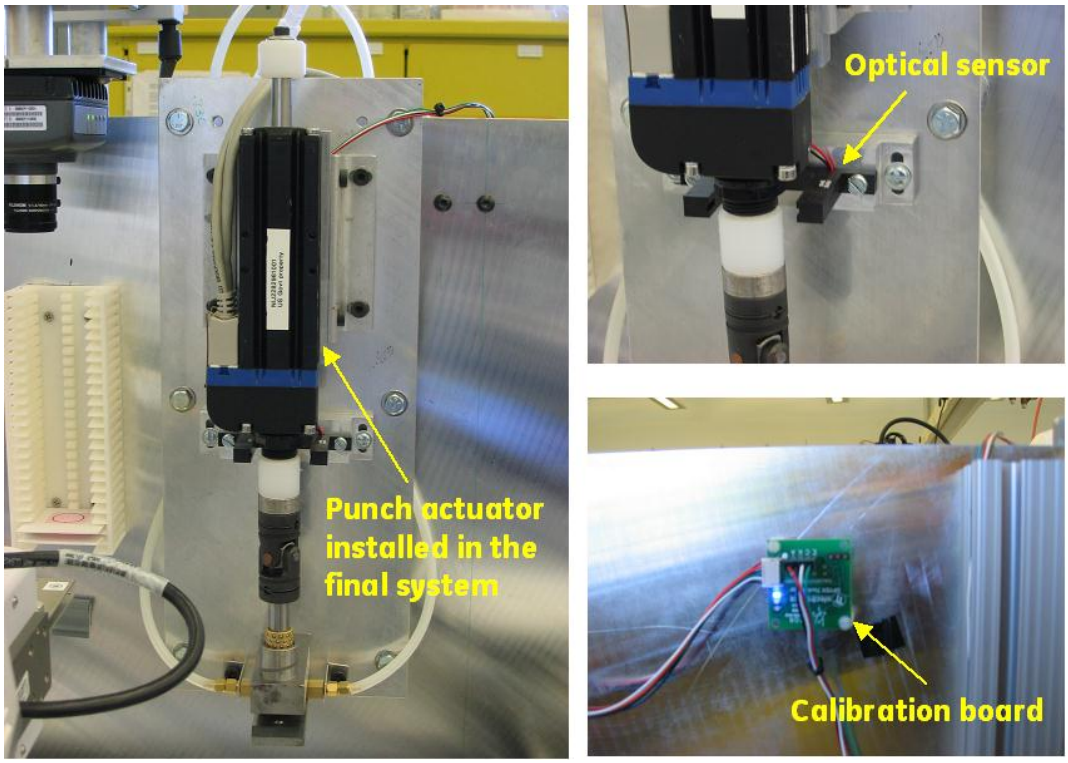


Figure 8. Control architecture for actuator.

The mechanical home switch comes into physical contact with the punch and is therefore, prone to damage. Therefore, in the final implementation, the mechanical home switch was replaced by an optical sensor (part # OPB819Z) from OPTEK Technology. The optical sensor comes with an auto-calibrating board (part# OCB350L062Z). The final configuration including the optical sensor and calibration board is shown in Fig. 9.



Figures 9. Sensing and calibration for actuator as installed on prototype.

2.2.7 Dust removal subsystem

Dust build-up is one of the leading causes of cross contamination. In addition to reducing dust formation via tighter tolerances on the puncher, our design also includes a subsystem to remove the dust as it is formed. The subsystem used here removes the dust using airflow across bottom of puncher. The intake vents are located at the rear of the puncher and the airflow is driven by light vacuum.

2.3 System Integration

To integrate the subsystems together, an aluminum base and back plane were constructed. As seen in Figure 2 the well plate handling system is attached to the base, while the card hotel, fluid handling subsystem, punching subsystem, and vision subsystem are attached to the back plane. A solid back plane was used to minimize vibrations, which could reduce the quality of both the imaging and punching steps. The ionizing bar is attached to base, but in such a way that the height of the ionizing bar above the 96-well plates can be modified for later optimization.

3 Methods

3.1 Static Removal Tests

A series of bench top experiments were performed to test the static removal system and optimize its method of operation. For these tests, static is induced on the 96-well plate by rubbing the plate with a cloth. The static charge on the upper surface of the plate can then be measured with a static meter (Electro-Tech Systems, Inc, model 212). The static charge inside the wells, however, cannot be accurately measured due to the small line-of-sight sidewall area. These experiments were performed before the ionizing bar was integrated into the automated system, so all steps were performed manually.

3.1.1 Experiment – Determine effect of ionizing bar and air knife on percentage of punches falling to bottom of well

The well plate is given a surface static charge of 10 to 20 kV/inch and then passed under the ionizing bar for a total exposure time of ~15 seconds. The conditions tested include: ionizing bar and air knife off (control); ionizing bar on, but air knife off; and both ionizing bar and air knife on. An untreated card (Whatman Protein Saver 903 card) was then manually punched using a 1.2mm Harris punch. The punch was released into the well, with the end of the punch either above or below the upper lip of the well by ~2-3mm. A total of 20 punches were performed for each condition, each punch being ejected into a separate well. The number of punches falling to the bottom of the well was recorded.

3.1.2 Experiment – Determine effect of ionizing bar distance and air knife pressure

The well plate is given a surface static charge of 10 to 20 kV/in^2 and then passed under the ionizing bar for a total exposure time of ~15 seconds. The distance between the ionizing bar and the bottom of the well plate was varied between 2 and 9 in. The air pressure used in the air knife is varied between 1 and 5 psi. An untreated card (Whatman Protein Saver 903 card) was then manually punched using a 1.2mm Harris punch. The punch was released into the well with the end of the punch 2-3mm above the lip of the well. A total of 10 punches were performed for each condition, each punch being ejected into a separate well. The number of punches falling to the bottom of the well was recorded.

3.2 Buccal swab sample collection

Buccal swabs were anonymously collected from volunteers. They were collected using the swabbing method/time recommended by the EasiCollect manufacturer. The swab is wetted by running it along the gumline and under the tongue. The swab is then rubbed for ~15 seconds on the inside of each cheek. The swab is pushed into the contact position (where it touches FTA card) for ~10 seconds. The FTA cards are dried and then stored in a desiccator until used.

3.3 Creation and Evaluation of Model Human DNA Samples

In a buccal swab sample, there is some variation in the amount of DNA collected from different volunteers. For some of the system evaluation experiments, a consistent amount of DNA is desired. For these cases, model samples were fabricated where human genomic DNA (dissolved in TE Buffer, pH=8) was directly applied to the cards. 100uL of 5 ng/uL human genomic DNA was applied to each card, resulting in approximately 1.2ng of DNA being present in a 1.2mm punch. The cards were allowed to dry for 3 hours and then stored in a dessicator until used.

The distribution of model DNA across the spotted area was evaluated. Nine punches (1.2mm diameter) were taken from the card from different locations (Figure xxx).

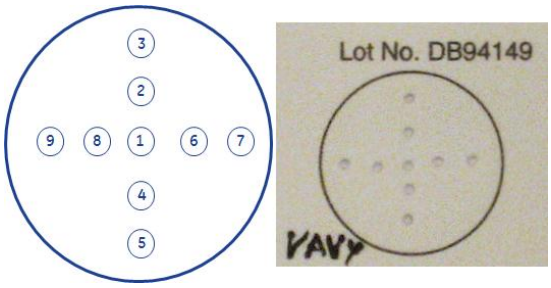


Figure XXX: Image showing where punches were taken from a model Human DNA sample.

A bulk (14X) PCR mix was prepared that contained:

Component	1X	X14
Water	7.5	105
THO1 or F13B Oligos	2.5	35
2X Ampdirect Plus (Nacalai USA, Inc.)	12.5	175
ROX Dye (3 pmol/µl)	1	14
1:100 SYBR Green I (Invitrogen)	0.25	3.5
NovaTaq (Novagen, 5 U/µl)	0.25	3.5
Total Volume	24 µl	336

24 µl of the bulk PCR mix was aliquoted into separate 0.2 ml reaction tubes. Each reaction tube had the following components added:

- 1 µl of water (No Template Control), or
- 1 µl containing either 1 ng, 10 ng or ~90 ng of human genomic DNA, or
- 1 µl of water and one 1.2 mm punch containing human genomic DNA

The mixture was thermal cycled using the following conditions in a qPCR machine (Applied Biosystems 7500):

1. 95°C for 10 minutes
2. 94°C for 30 seconds

3. 60°C for 30 seconds
4. 70°C for 1 minute
5. Go to Step #2 39 times
6. Hold at +4°C

When the thermal cycling reactions were complete, 2.5 µl of each reaction was mixed with 2.5 µl of Gel Loading Buffer II (Invitrogen), heated at 95°C for two minutes and quenched on ice. The entire sample was then loaded into a well of a 6% TBE-Urea acrylamide gel (Invitrogen) and electrophoresed at 160 volts (constant voltage) at between 60°C to 63°C until the XCFF dye was approximately 2.5 cm from the bottom. The gel was stained for 15 minutes with a 1:200 dilution of SYBR Gold (Invitrogen) in TE Buffer and visualized using a Typhoon 9410 Variable Mode Imager (Amersham Biosciences).

3.4 Creation of Other Model DNA Samples

During the final evaluation tests, it became necessary to develop a non-human model DNA sample. Although initial tests did not have any contamination issues, over the course of the project contamination with human DNA became a persistent problem. Even with the experimental protocol only analyzing a single STR region, the data demonstrated that the contamination was not due to the DNA being punched on the cards. Over the course of this project, the room where the Staubli robotic arm was located had a larger number of people working in it, and the ventilation system was not able to control the amount of general dust in the air. As the Staubli system could not be moved, the project moved to an alternative DNA for remaining system testing. For these cases, model samples were fabricated where *Bacillus Subtilis* DNA (Ehrenberg) Cohn genomic DNA 168 from ATCC (#23857D-5) (dissolved in TE Buffer, pH=8) was directly applied to the cards. 100uL of 5 ng/uL genomic DNA was applied to each card, resulting in approximately 1ng of DNA being present in a 1.2mm punch.

3.5 Automated System Evaluation – Sample Loss

The ionization bar with air knife is used to remove static from the 96-well plate, which should then reduce the risk of sample loss. The plate was first passed under the ionizing bar (with air knife turned on) and then 25uL of water was dispensed into each well. The well plate was then moved to the punching subsystem. After each set of punches, the plates were examined to confirm that there was only 1 punch in each well, and record any errors (e.g. no punches in a well).

3.6 Automated System Evaluation - Cross contamination

One of the largest potential sources of cross-contamination is any dust that is produced during the card handling and punching. Three potential sources of dust and contamination were identified. 1) Contamination from dust falling during punching. 2) Dust remaining on the Punch/Die and traveling from one punch to

the next. 3) General dust buildup leading to random cross-contamination. Conversations with scientists working in forensics labs indicated that the 3rd risk was currently the most problematic.

3.6.1 Dust falling during punching

Slow motion videos taken of the puncher show that there is some dust falling along with the punch. There was a concern that this dust could get into the surrounding wells of the wellplate. The system is designed to minimize this risk, by moving the plate in the z-axis until it is touching the underside of the punch. To determine if dust was still getting into the surrounding wells, a punching pattern was set up so that every other well had a punch placed into it. The pattern was staggered so that each empty well was surrounded by 4 wells with punches (above, below, to the left, and to the right). These experiments were initially performed with model human DNA samples and then model *B. Subtilis* samples.

The wells that did not contain a punch were amplified with PCR. The PCR mixture added to these wells contained the following components:

Component	1X
Water	13.7
Primers (5pmol/uL)	1
2X Ampdirect Plus (Nacalai USA, Inc.)	15
NovaTaq (Novagen, 5 U/µl)	0.3
Total Volume	30 µl

The PCR conditions were as follows:

1. 95°C for 10 minutes
2. 95°C for 30 seconds
3. 60°C for 30 seconds
4. 72°C for 1.5 minute
5. Go to Step #2 49 times
6. Hold at +4°C

26uL of the post-PCR reagents were added to 250uL of a Quant-iT™ PicoGreen dsDNA reagent (Invitrogen #P7581) diluted 1:200 in TE Buffer, pH 7.4. This mixture was added to a Costar Assay Plate (flat bottom, white polystyrene, #3912), mixed and incubated for 20 minutes at RT in the dark. The plate was then read on a Tecan SNiPer plate reader from Amersham Pharmacia Biotech. The remaining 4 µl of each PCR was saved, mixed with 1 µl 100 mM EDTA and stored at –20°C for subsequent gel analysis (15% acrylamide TBE-Urea, Invitrogen). The SNiPer analysis was performed with an excitation setting of 485nm, an emission setting of 535nm, an automated optimization of the gain setting, 10 flashes with no lag time, and a 40µs integration time.

3.6.2 Dust transferred by the puncher

It is common practice in labs to take 1-2 punches from a "clean" area of a card after each sample punch. The thinking is that this will remove any dust built up on the punch or die. To determine if any dust or DNA is transferred by the puncher itself, the instrument was set up to take a punch from a card containing *B. Subtilis* and then three punches from cards with no DNA. The punches from the blank card are then put through the analysis described in Section 3.6.1, with 32 cycles of PCR are performed. These experiments were performed with model *B. Subtilis* samples

3.6.3 Contamination from general dust buildup

As mentioned previously, discussions with scientists in forensics lab indicate that the greatest source of cross contamination is general dust buildup in the system. The system here is designed to reduce dust buildup, by using a vacuum source at the rear of the puncher. Several different types of human DNA were used, with each one having a different THO1 STR profile. First 10x96 punches were taken from cards containing DNA #1 (VAVY). The assumption is that if there was dust-buildup, this would provide enough homogenous DNA to be seen in a later punch. Then 50 punches were taken from cards containing DNA #2 (EHM). Some wells were also kept empty (no punches added). 15 of the empty wells and 5 of the wells containing punches were then analyzed using the method described in 3.3 (for the THO1 STR region).

3.7 Automated System Evaluation – STR analysis

The system was used to punch 11 EasiCollect cards containing buccal swabs from volunteers. Initially, 25μL aliquots of the reaction mixture (15uL water, 5uL 5x Master mix, and 5uL Primer Pair) from the PowerPlex 18D kit were added to each well that was going to receive a punch. The amplification mixture was also added to a neighboring well, which was not going to receive a punch (referred to as blanks). Then, the system was used to take one 1.2mm diameter punch from each card and transfer it to the well plate. After the punching was complete, the plate was covered with an adhesive seal and transferred to a thermocycler. The amplification conditions, shown below, were those recommended by the kit manufacturer:

Thermal Cycling Conditions

1. 96°C for 2 minutes
2. 94°C for 10 seconds
3. 60°C for 1 minute
4. Go to Step #2 26 times (27 total cycles)
5. 60°C for 20 minutes
6. Hold at +4°C

The samples were then kept frozen at -20ºC before being shipped overnight on dry ice to an external lab (Strand Analytical Laboratories) for STR analysis on an ABI 3130xl Genetic Analyzer.

4 Results

4.1 Evaluation of Static Removal

As described in Section 3.1, a series of experiments were done near the beginning of the project to determine if the combination of ionizing bar and air knife would reduce the static charge in the wells of a 96-well plate. These experiments were performed on the bench with manual punching. The results, shown in Table 2, suggest that the most static is removed when the ionizing bar is used along with the air knife.

Table 2. Effect of ionizing bar and air knife on well plate static removal (manual punching)

	% of 1.2mm punches dropping to bottom of well	
	Released above well	Released in well
Ionizing bar off	10%	30%
Ionizing bar on – air knife off	65%	90%
Ionizing bar on – air knife on	80%	100%

An additional set of preliminary experiments helped to determine the offset distance and air pressure needed to reduce static in the wells. These experiments, summarized in Table 3, suggest that a distance of about 2-5" between the plate and ionizing bar and a minimum pressure of ~3 psi for the air knife will remove the most static from the well plate, allowing the most punches to fall to the bottom of the wells. These values were fed into the design of the automated system prototype.

Table 3. Percent of punches falling to bottom of well as a function of air pressure and distance (manual punching).

		Distance between ionizing bar and bottom of well plate		
		2"	5"	9"
Air pressure used with air knife (psi)	1	100%	100%	90%
	2	90%	100%	80%
	3	100%	100%	100%
	4	100%	100%	100%
	5	100%	100%	100%

Control (no ionizing bar or air knife used)	20%

4.2 Evaluation of Model DNA System

Nine punches were taken from a card containing the model DNA sample, as described in Section 3.2. The gel image of the amplified product, along with corresponding Ct values, are show in Figure xxx below. The Ct values for the 9 punches indicate a consistent concentration across the card.

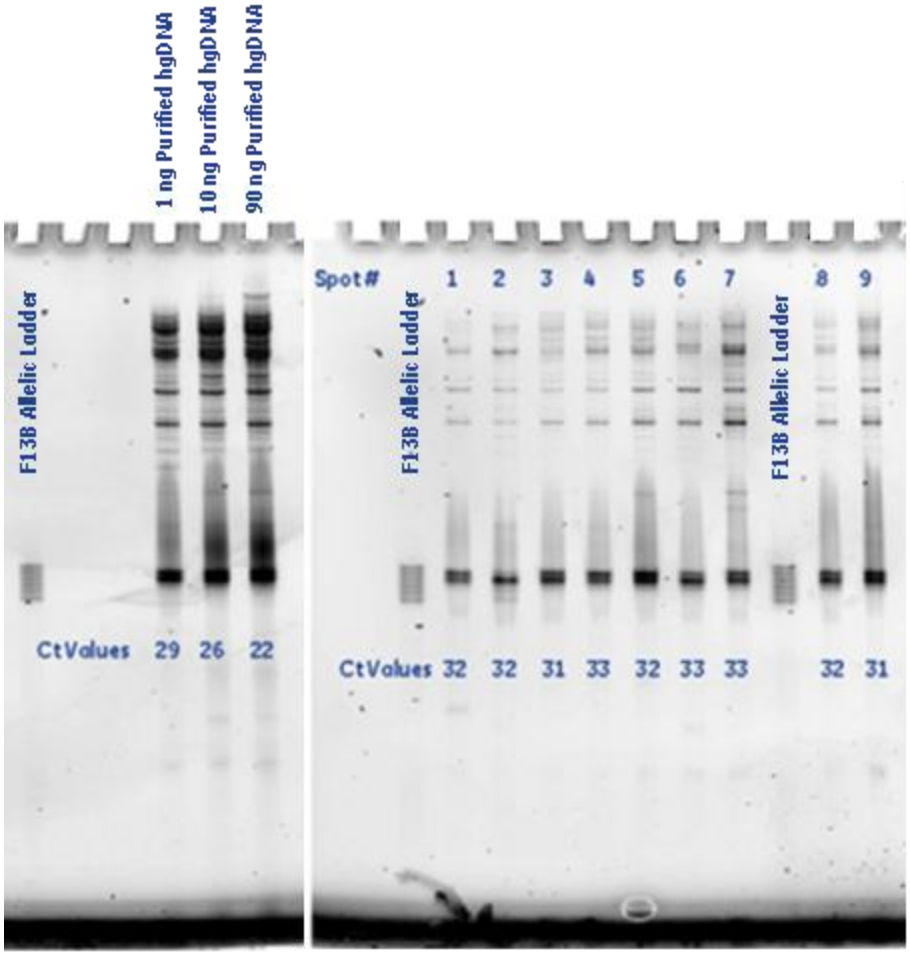


Figure xxx: Gel image of amplified DNA taken from different areas of a model DNA sample, along with the Ct value for the amplification.

4.3 Evaluation of Punching and Sample Loss

The puncher was tested and shown to successfully punch. A grid pattern of up to 96 punches could be punched out of a single EasiCollect card, as shown below in Figure 10. One of the lessons learned during the initial punch set-up and testing was the importance of the vision system. If a new punch partially overlapped with a previous punch, the tool part of the punch would be deflected enough that an obvious “hitting” sound could be heard and damage could be

seen on the punch head. Our vision system is able to look at a card and determine which positions on the punching grid do NOT overlap with previous punches.

Figure 10. EasiCollect card that has been punched 96 times using the automated system. The punches have a diameter of 1.2mm.

Initially there were some problems with punches not freely falling from the puncher. It was hypothesized that this was caused by static in the punched bit of card. The system was modified to try and minimize this issue. The modifications include:

- Using a tool/die set with a tighter fit. This should result in a cleaner cut and therefore less fibers hanging off the edge of the punched card to stick to surfaces.
- Addition of fluid to the wells prior to punching. This was found to help keep punches in the wells, even when the well plate was being lifted out of the system for inspection.
- Modifying the trajectory of the punch. A number of profile trajectory modifications were tried to remedy the static issue. The original punch trajectory profile included a tap stroke after the punch stroke. After passing the punching tool all the way through the die, it is partially retracted and then passed all the way through the die again. If any card punches are inside or outside the die, this will “tap” them off. The punch profile was modified to include two taps after the main punch stroke.

These modifications did work as evident by a number of successful runs. In a total of ~2600 punches, there were only 4 instances when a punch was not in the appropriate well at the end of the run. For three of these, the position of the punch indicates that it may have jumped out when the plate was removed from the system for inspection. This gives an estimated error rate of between 0.04% and 0.15%.

Some magnified images of the card (post-punching) are shown in Figure 11. They show an overall clean punch with a reproducible size and only an occasional hanging fiber.

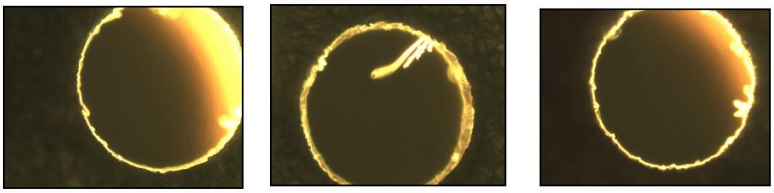


Figure 11. Several 4x magnification of 1.2mm punched areas on an EasiCollect card.

4.4 Evaluation of Cross-Contamination

4.4.1 Cross contamination during punching

These experiments were initially performed with model human DNA samples and, after contamination from the lab became an issue, they were performed with model *B. Subtilis* DNA samples. Out of 116 blank wells (each surrounded by at least 4 wells with DNA containing punches) only 1 well showed potential DNA contamination. This one instance was from the human DNA samples and with only one STR being analyzed, it's not certain if this potential contamination was due to the general lab issue being experienced or from the particular card being punched. There were no instances of contamination with the *B. Subtilis* DNA samples. Based on this, it is concluded that cross-contamination during punching is not a significant issue for this system.

4.4.2 Cross contamination from punch/die

Table 4 below shows the results of these experiments. As discussed in Section 3, one punch was taken from an FTA card with DNA applied, and then 3 punches were taken from FTA cards with no DNA. As shown in Table 4, the first "clean" punch taken after the DNA punch often shows DNA contamination. It is therefore recommended that at least one cleaning punch be taken.

Table 4. Summary of cross-contamination experiments focusing on punch to punch contamination

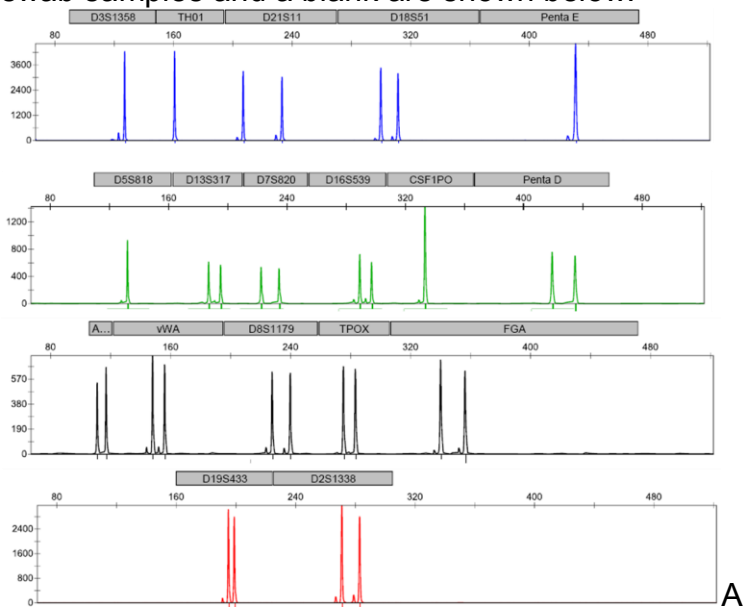
	1st Clean punch	2nd Clean punch	3rd Clean punch
# showing DNA contamination (out of 24 total)	10	2	0

4.4.3 Cross contamination from general dust buildup

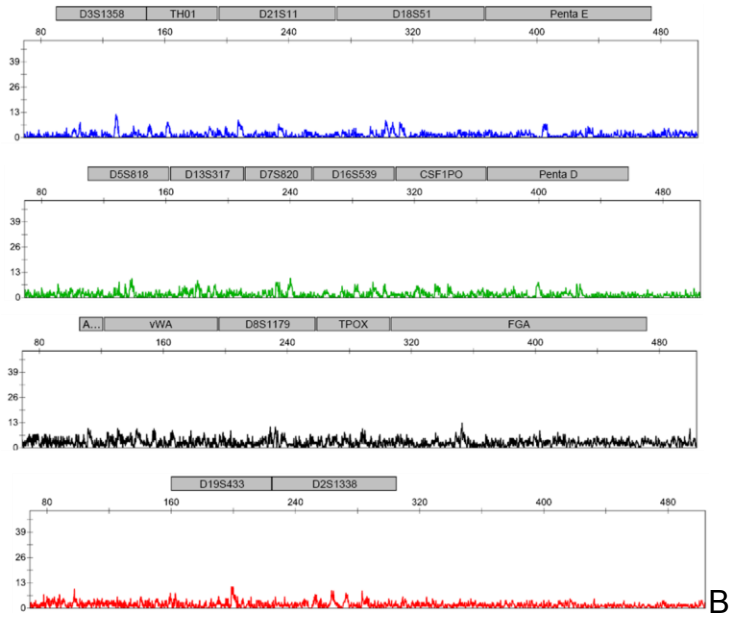
In this experiment 960 punches were taken from FTA cards with DNA #1, and then some punches were taken from FTA cards containing model DNA #2.The punches and blank wells from this second set of punches were then analyzed to see if there was any carryover of DNA#1. Of the fifteen empty wells tested, four were found to contain DNA#1. Of the five punches tested, two were found to contain both DNA #2 and DNA#1. This high number of wells/punches contaminated with DNA#1 indicate that the vacuum system was not effective enough in preventing dust buildup while the DNA #1 cards were being punched. This is addressed in the conceptual design for the next generation system.

4.5 Full STR analysis

All 11 buccal swab samples gave full STR profiles (all 18 STR regions detected) and in each sample only 1 person's STR profile was detected (no cross-contamination from punch to punch). All 11 of the nearby wells (where amplification buffer was added, but no punch was added) showed no detection of STR regions. This confirms that cross contamination from dust released during punching is not an issue in this system. Examples of the spectra from bucall swab samples and a blank are shown below.



A



B

Figure 12. A) Example STR profiles for buccal swab samples. B) Example STR profiles from nearby blank

5 Discussion and Conclusions

A system has been built which can automate the entire FTA card processing workflow. The seven subsystems have been fabricated and integrated to allow for a card-in and DNA-ready-for-PCR-out workflow. The design of the system was focused on reducing the risk of sample loss.

The initial experiments on reducing sample loss focused on evaluating the ability of an ionizing bar and air knife to remove static from the wells of a 96-well plate and allow a punch to fall to the bottom of the well. These experiments, shown in Tables 2 and 3, indicated that the device did remove static and would increase the probability of a punch falling to the bottom of a well plate.

The later experiments evaluating sample loss looked at how the automated system as a whole (including the static removal subsystem) enabled punches to fall to the bottom of the correct well and be held there by the previously dispensed fluid. Once characteristics such as punch profile were optimized, the punch loss was found to be less than 0.15%.

A series of experiments were also performed to evaluate the risk of cross-contamination in this system. The risk from contamination of dust created falling during punching was not found to be significant. The risk of contamination from punch to punch (due to carryover on the punch/die) was found to be significant, but could be overcome by performing a cleaning punch between samples. The risk of contamination from general dust build-up was also found to be significant and an improved dust removal system was designed to address this.

Based on what was learned from working with and testing the TRL4 automated prototype system, a conceptual design for a next generation prototype was developed. As a TRL6 design this prototype would take advantage of commercially available OEM parts, but would fit on a bench and could be tested in a real lab environment.

6 Implications for Policy and Practice

Having a system that enables automation of FTA card handling could improve the throughput of sample processing of DNA databanking forensic labs. By having the automated system work reliably with the smallest size punch (1.2mm) labs would also be able to minimize their reagent costs. This cost savings would come both from using smaller volumes of the PCR reagents and from having fewer “lost” punches (and thus fewer sample re-runs).

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