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Research paper

# Developmental validation of the ANDE© rapid DNA system with FlexPlex™ assay for arrestee and reference buccal swab processing and database searching

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ABSTRACT

A developmental validation was performed to demonstrate reliability, reproducibility and robustness of the ANDE System with the FlexPlex assay, including an integrated Expert System, across a number of laboratories and buccal sample variations. Previously, the related DNAscan™/ANDE 4C Rapid DNA System using the PowerPlex®16 assay and integrated Expert System Software received NDIS approval in March 2016. The enhanced ANDE instrument, referred to as ANDE 6C, and the accompanying 6-dye, 27-locus STR assay, referred to as FlexPlex, have been developed to be compatible with all widely used global loci, including the expanded set of the CODIS core 20 loci.

Six forensic and research laboratories participated in the FlexPlex Rapid DNA developmental validation experiments, testing a total of 2045 swabs, including those obtained from 1387 unique individuals. The goal of this extensive and comprehensive validation was to thoroughly evaluate and document the ANDE System and its internal Expert System to reliably genotype reference buccal swab samples in a manner compliant with the FBI's Quality Assurance Standards and the NDIS Operational Procedures.

The ANDE System, including automated Expert System analysis, generated reproducible and concordant results for buccal swabs when testing various instruments at different laboratories by a number of different operators. When testing a number of non-human DNAs, including oral bacteria, the ANDE System and FlexPlex assay demonstrated limited cross-reactivity. Potential PCR inhibitors were evaluated as part of the validation and no inhibition was detected. Reproducible and concordant profiles were generated from buccal swab samples collected with a limit of detection appropriate for buccal swab collections from arrestees. The precision and resolution of the System met industry standards for detection of microvariants and single base resolution.

The integrated Expert System appropriately demonstrated the ability to correctly pass or fail profiles for CODIS upload without human review. During this comprehensive developmental validation, the ANDE System successfully interpreted over 2000 samples tested with over 99.99% concordant alleles. The data package described herein led to the ANDE System with the FlexPlex assay receiving NDIS approval in June 2018.

## 1. Introduction

On August 18, 2017, the Rapid DNA Act (H.R. 510) became US Federal law [1], having been passed unanimously by both the US House and Senate. The legislation amends the DNA Identification Act of 1994 to "establish a system for integration of Rapid DNA instruments for use by law enforcement to reduce violent crime and reduce the current DNA analysis backlog" [2]. Specifically, the law permits criminal justice agencies to use Rapid DNA instruments approved by the Director of the Federal Bureau of Investigation. The practical impact is that Rapid DNA

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testing of arrestees will be possible in police booking stations in the more than 30 states. The FBI is preparing policies, IT infrastructure, and CODIS [3], to implement the new legislation. The impact of the new Law and Rapid DNA on public safety may be substantial by revolutionizing the speed and manner in which suspects are identified.

Two aspects of the Rapid DNA Act are of particular importance from technical, operational, and legal perspectives. First the Act requires that the generation and analysis must be fully-automated, without human intervention. Accordingly, a compliant system must have a fully-automated Expert System that functions without human data review. In light of the huge volume—potentially millions of arrestee samples that will be run at all hours of the day and night—and urgency to generate the results for searching (before the arrestee is released in non-charging states), the automated interpretation requirement is just as important as the automated processing requirement. Second, the Act tasks the FBI with the approval of Rapid DNA systems and the establishment of standards and procedures for their use outside of laboratories.

Taken together, these related issues provide a roadmap for the regulatory requirements for Rapid DNA processing of known reference samples in the police booking station. In 2016, the ANDE 4C System received the first and only National DNA Index System (NDIS) Approval for a Rapid DNA System [4]. The FBI concluded that "the system could confidently be used in an accredited laboratory for the rapid analysis of known exemplars and could be used by non-scientists in non-laboratory settings with appropriate protocols, quality assurance and quality control measures in place" [5]. As expected, and soon thereafter, the FBI expanded the CODIS core requirements from 13 to 20 loci. In order to receive NDIS Approval for the police booking station and be compliant with the Rapid DNA Act, three considerations are of paramount importance: 1) The Rapid DNA System must generate DNA profiles without human sample processing or data interpretation; 2) The system must interrogate the 20 STR loci of the Expanded CODIS core; and 3) The system must undergo a developmental validation in accordance with the FBI's Quality Assurance Standards and NDIS Operational Procedures [6–8]. The work reported herein describes the developmental validation studies that led to the approval of the ANDE 6C instrument, single-use consumable performing the FlexPlex assay, and automated Expert System—together comprising the ANDE Rapid DNA Identification System.

### 1.1. Overview of the ANDE rapid DNA identification system

The ANDE System [9] consists of three components (Fig. 1): the ANDE swab, the A–C hip (designed for buccal swab processing), and the ANDE instrument.

The A–C hip is a single use, disposable consumable which includes all reagents, materials and waste containment required to perform a fully-automated STR analysis. DNA purification reagents, FlexPlex STR PCR reagents, buffers, and separation polymer are all pre-loaded on the chip and have been optimized for the microfluidic environment to ensure consistent, balanced, and precise results. There is no direct contact between the instrument and the sample or the reagents; all liquids within the chip are driven by pneumatic pressure. This closed system design, coupled with swabs that lock and seal into the chips, minimizes the potential for cross-contamination.

The FlexPlex assay (Fig. 2) was developed to support compatibility with DNA databases around the world [9]. FlexPlex contains 23 autosomal STR loci (D1S1656, D2S1338, D2S441, D3S1358, D5S818, D6S1043, D7S820, D8S1179, D10S1248, D12S391, D13S317, D16S539, D18S51, D19S433, D21S11, D22S1045, FGA, CSF1PO, Penta E, TH01, vWA, TPOX, and SE33), three Y-chromosomal STR loci (DYS391, DYS570, and DYS576), and Amelogenin. FlexPlex generates data compatible with databases around the world, including the following:

- CODIS core 20 loci
- ENFSI/EDNAP Expanded European Standard set
- Australia's National Criminal Investigation DNA Database
- Canada's National DNA Data Bank
- China's National DNA Database
- Germany's DNA-Analyze-Datei
- New Zealand's National DNA profile databank
- United Kingdom's National DNA Database

The ANDE instrument is comprised of a set of subsystems, including a pneumatic subsystem for driving fluids throughout the chip, a thermal subsystem for performing multiplexed amplification, a high voltage subsystem for electrophoresis, a 6-color optical subsystem for exciting and detecting fluorescently labeled STR fragments during electrophoresis, and a ruggedization subsystem to allow transport and field forward operation without recalibration or optical realignment. A single board computer that is integrated with the instrument controls subsystems functions, performs data processing, interfaces with the user through an integrated VGA touch panel, and provides ethernet and USB connectivity. Note that the mechanical interface between the instrument and chips, pneumatic subsystem, thermal cycling subsystem, and high voltage subsystems are of identical design and construction to the 4-color ANDE System that previously received NDIS Approval.

To complement the instrument and A–C hip, the ANDE System includes integrated software packages for instrument control, data collection, and Expert System interpretation of STR profiles. The instrument integrated touchscreen features a graphical user interface with workflow driven instructions. After the A–C hip containing swab samples has been inserted into the instrument and the door closed, sample processing starts automatically. Following electrophoretic separation and laser-based detection of amplified STR fragments, the Expert System software automatically analyzes and interprets the data and provides rapid feedback on the usability of the STR profiles for database enrollment and searching. A visual indicator in the form of a green checkmark, yellow checkmark, or red "X" visual display indicates if the result of each lane is successful. In all cases, the output files, including an electropherogram (Fig. 3), are available for review by a qualified DNA analyst. All output files are encrypted and can be exported and decrypted by FAIRS™ (ANDE Corporation), a multi-function, multi-tiered user privilege software package. FAIRS integrates database generation and management, search and match, and kinship determination functionalities.

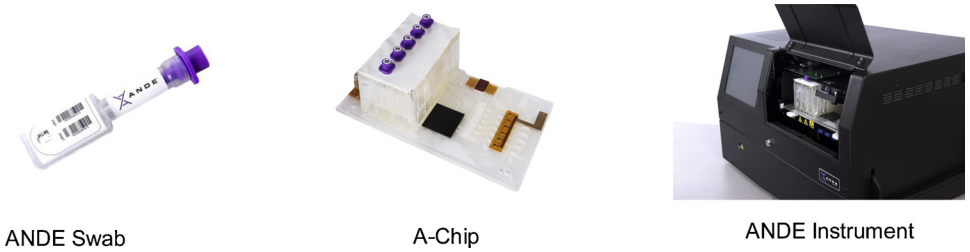


**Fig. 1.** Three components of the ANDE Rapid DNA Identification System.

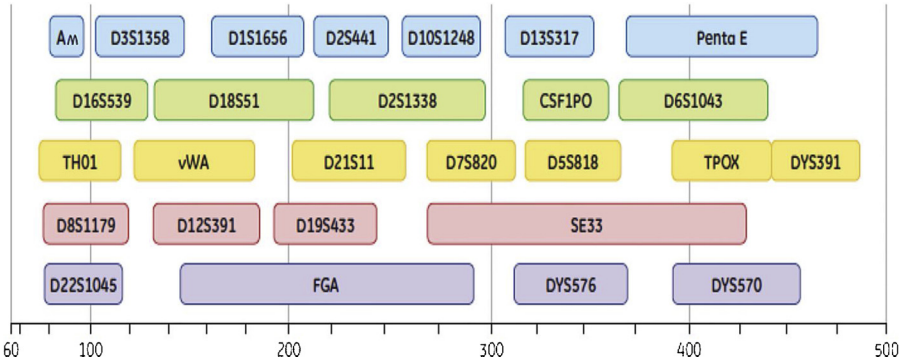


**Fig. 2.** Configuration of the FlexPlex STR assay.

## 2. Materials and methods

Five independent forensic laboratories (four of which are accredited) plus ANDE, the developer of the ANDE System, tested swabs as part of the developmental validation experiments and all data was compiled and analyzed. A total of 13 separate ANDE instruments were used in the study, run at different laboratories, by a number of individual users. All ANDE Systems represented the same product number operating identical software versions.

Buccal samples were collected on ANDE swabs, the swabs were placed into the A–C hip, and the chip was then placed into the ANDE instrument for fully-automated sample processing, allele calling, and data interpretation with the integrated ANDE Expert System.

The study was approved by an Institutional Review Board; all buccal swab donors reviewed a Research Subject Information Sheet and provided informed consent.

### 2.1. Characterization of genetic markers and population studies

The loci found in the FlexPlex assay include the expanded CODIS core 20 loci (CSF1PO, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, FGA, TH01, TPOX, vWA, D1S1656,

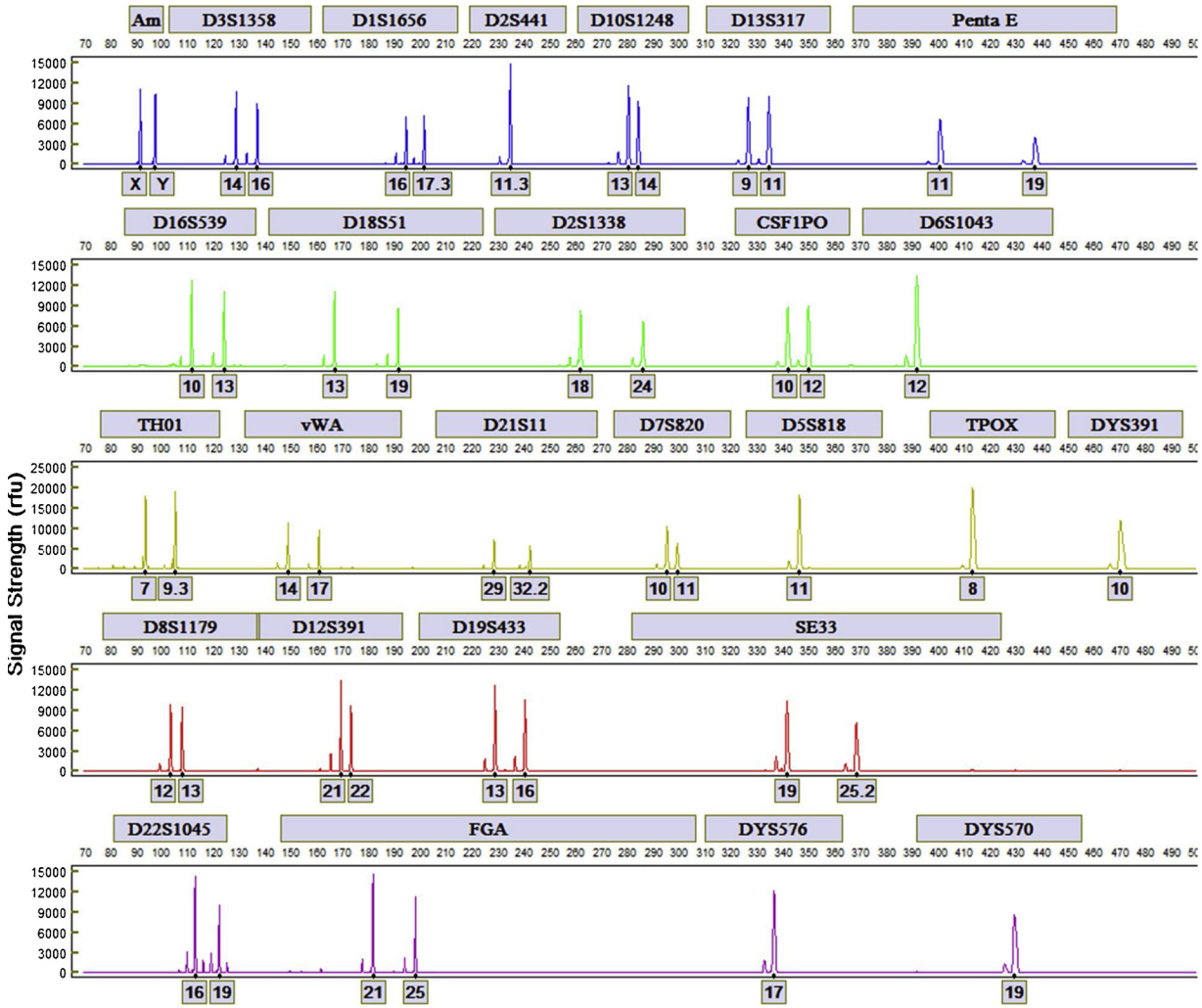


**Fig. 3.** Representative electropherogram developed by the ANDE System.

D2S441, D2S1338, D10S1248, D12S391, D19S433, and D22S1045), plus Amelogenin, Penta E, D6S1043, SE33, DYS391, DYS570, and DYS576. The characterization of these genetic markers is well understood [10–28]. The National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 2391c has certified types for all markers [29,30], and population studies evaluating allele frequencies have been conducted and published [17,18,28,31–38].

Updated information detailing variants and observed tri-allelic patterns can be found on the NIST STRBase website [39]. Numerous regional population studies have also been published and the references compiled and listed on the NIST STRBase website [40].

### 2.2. Species specificity

Testing of a total of ten different species, including animals and bacteria, was performed in duplicate. For each of the following, chimpanzee, gorilla, orangutan, dog, mouse, horse, and ferret, 500 ng of genomic DNA in 50 µL of $TE^{-4}$ were deposited on individual ANDE swabs. Genomic DNA from *Streptococcus pneumoniae* ($4.89 \times 10^7$ genome equivalents), *Staphylococcus aureus* ($3.49 \times 10^7$ genome equivalents), and *Lactobacillus plantarum* ($2.92 \times 10^7$ genome equivalents), in 50 µL of $TE^{-4}$ were placed on individual ANDE swabs.

### 2.3. Limit of detection

Buccal swabs were collected from 5 unique donors using 1 swipe, 3 swipes, and 6 swipes collection where a swipe is defined as a single up-and-down motion. Each donor was collected by inserting the cotton tip of the ANDE swab into the mouth and pressing against the inside of the cheek to cause the cheek to protrude. The swab was then swiped along the cheek the defined number of times for the limit of detection study.

Swabs spiked with purified DNA at 2.0 µg, 1.0 µg, 0.5 µg, 0.25 µg and 0.1 µg quantities were tested in duplicate. Purified DNA samples were created by processing the entire tip of one swab using a guanadinium-based extraction [41] which is essentially a tube-based version of the ANDE System protocol and quantified using a Nanodrop™ 2000C Spectrophotometer (Thermo Scientific). Purified DNA from the swabs was pooled to produce a stock DNA solution. The stock DNA solution was diluted in $TE^{-4}$, pH 8 buffer to prepare 500 µL solutions of 40, 20, 10, 5 and 2 ng/µL. Swab samples were then spiked by pipetting 50 µL of diluted DNA solution onto the swab head.

### 2.4. Stability

Buccal swabs were collected from two unique donors on ANDE swabs and stored in their protective plastic cover for 0 (tested immediately), 1, 2, or 7 days in environmental chambers at both 22°C (room temperature) and 4°C (refrigerated). Immediately after collection, swabs were placed in the protective cover without being dried.

Sixteen chips were stored in environmental chambers at 22°C. Eight chips were run with buccal swabs after three months of storage and the remaining eight chips were run following six months of storage.

### 2.5. Inhibitors

Ten potential PCR-inhibitory substances likely to be found in the oral cavity were tested in duplicate (mint, beer, bloody swab, cigarette, coffee, gum, mouthwash, tea, tobacco dip, and toothpaste). Each substance was consumed by the donor in a manner similar to reasonable use prior to buccal swab collection, except the “bloody swab” which was created by depositing 10 µL from a finger stick onto a freshly collected buccal swab.

### 2.6. Reproducibility and standard reference material

Ten donors donated buccal samples which were tested in duplicate by all six laboratories. Three laboratories also tested swabs spiked with 2 µg of purified DNA from 3 unique donors. Purified DNA samples were created by processing the entire tip of one swab using a guanadinium-based extraction [41] which is essentially a tube-based version of the ANDE System protocol and quantified using a Nanodrop™ 2000C Spectrophotometer (Thermo Scientific). Purified DNA from the swabs was pooled to produce a stock DNA solution. Swab samples were then spiked by pipetting 50 µL containing 2 µg of diluted DNA solution onto the swab head.

A NIST-traceable swab was created and run on the ANDE System [42]. The commercially available NIST SRM 2391c (PCR-Based DNA Profiling Standard) commonly used for validation of forensic DNA applications contains four Components (A–D) of genomic DNA in $TE^{-4}$ buffer and two paper matrices, Components E and F [29]. Since the ANDE System was optimized for buccal swabs and not purified DNA, the Components within NIST SRM 2391c are not suitable for testing on the ANDE System due to their insufficient quantity of DNA template.

### 2.7. Mixtures

Swabs containing mixed DNA were created by collecting buccal swabs from 2 donors and each was extracted by processing the entire tip of one swab using a guanadinium-based extraction [41] and quantified using a Nanodrop 2000C Spectrophotometer (Thermo Scientific). Once quantified, the purified DNA was mixed in the ratios of 19:1, 5:1, 1:1, 1:5, and 1:19 yielding a total of two micrograms (2 µg) of DNA in 50 µL of $TE^{-4}$.

### 2.8. Accuracy and concordance

Buccal swabs from 1387 unique donors, collected following the recommended collection protocol of 6 swipes of the cheek, were tested for accuracy and concordance during the validation. The swabs were stored at room temperature immediately after collection and then at approximately 4 °C before being shipped to the laboratories for testing. Accuracy samples were used to calculate and evaluate concordance, signal strength, peak height ratio, first pass success rate, and resolution.

To evaluate concordance, conventional laboratory STR testing was completed at an accredited, forensic laboratory (The Bode Technology Group, Inc.) and allele calls were compared to the results of the ANDE System. The conventional laboratory completed analysis by clipping approximately ¼ of the swab, purifying the DNA, and using approximately 0.5–4 ng of DNA for amplification with both the PowerPlex® Fusion 6C System (Promega Corporation) and the PowerPlex® 21 System (Promega Corporation; utilized to provide data on the D6S1043 locus). Separation and detection was conducted with an Applied Biosystems® 3130xl Genetic Analyzer (Thermo Fisher Scientific) following lab validated or manufacturer’s protocols.

Additionally, to resolve discrepancies between ANDE System profiles and comparator profiles, the VeriFiler Express (ThermoFisher Scientific) and PowerPlex ESX 17 (Promega Corporation) assays were used on five samples.

The conventional data was analyzed using Genemapper® ID, version 3.2.1 (ThermoFisher Scientific) and interpreted by two qualified analysts and reviewed by a qualified technical reviewer. Additional techniques such as re-injection, re-extraction, quantification, and re-amplification were employed for conventionally-processed samples that did not initially pass technical specifications for reporting.

### 2.9. Contamination assessment

The potential for cross-contamination between sample lanes within a chip was examined by performing analysis with two patterns of alternating buccal swabs and blank swabs. Buccal swabs were collected using the standard 6 swipe collection protocol. Blank swabs were new ANDE swabs that were removed from the packaging, labeled as blanks,

and subjected to the same handling as the buccal swab samples.

All 6 laboratories tested contamination assessment and a total of 84 blank swabs were typed across both patterns.

## 3. Results and discussion

### 3.1. Species specificity

The species specificity of STR typing systems is essential to ensure that interpretation of STR profiles derived from human subjects is not complicated by other species' cross-reactivity with the assay under study. This study is of particular importance when testing buccal swabs because quantification of human DNA is not required for reference samples [7]. The ANDE System is intended for human single source buccal samples making microorganisms prevalent in the oral cavity those most relevant for evaluation of cross-reactivity. Additional macroorganisms, including primates and domestic animals, were also evaluated.

The ANDE Expert System did not generate passing STR profiles for any of the ten species tested. No amplification peaks were called for dog, mouse, horse, ferret, *Streptococcus pneumonia*, *Lactobacillus plantarum*, and *Staphylococcus aureas*. Six low-level peaks were observed but not labeled for mouse. Due to the genetic similarities between humans and other primates, it is not surprising that peaks were detected for the primate samples and, as expected, all detected peaks were failed by the Expert System.

### 3.2. Limit of detection

Reference and arrestee buccal swabs contain inconsistent amounts of DNA and are subject to additional variability when collected in the field, such as booking stations. The limit of detection of the ANDE System was evaluated to determine robustness of the system when used on field-collected buccal swabs.

All 30 swabs, collected with 1, 3, or 6 swipes, generated full profiles containing all CODIS core 20 loci. The STR profiles were examined for the presence of expected alleles, signal strength, and peak height ratio. The peak height ratio for each locus was determined for each set of samples that were collected with 6 swipes, 3 swipes, and 1 swipe (Fig. 4). The average peak height ratio across all autosomal loci was 82%, 81%, and 78% for 6 swipes, 3 swipes, and 1 swipe respectively, demonstrating excellent intra-locus balance for all heterozygous loci across all dye channels.

The ANDE System was not optimized for processing purified DNA but is capable of processing purified DNA samples nonetheless. When processed in the ANDE System, purified DNA is subjected to a redundant purification in the chip and, in the absence of other cellular materials normally found in a buccal swab sample, the process is quite inefficient. Nevertheless, it is valuable to characterize the quantity of purified DNA required to generate a profile in the ANDE System and the quantities of 2.0 µg, 1.0 µg, 0.5 µg, 0.25 µg and 0.1 µg were evaluated. These quantities were selected to bracket the typical amount of DNA expected recovered on a buccal swab sample [43].

Replicates containing 2.0 µg of DNA generated full profiles. Partial profiles containing less than the CODIS core 20 loci were generated for the remaining quantities and were properly flagged by the Expert System for analyst review.

The ANDE System was designed to yield full CODIS profiles with arrestee and reference buccal swabs. As expected, the results of this testing confirm that the system is less efficient in processing purified DNA (which is subjected to an inefficient redundant purification in the chip). A chip for processing forensic samples with lower amounts of input DNA, the ANDE I-Chip, has been designed and is compatible with the ANDE System [44].

### 3.3. Stability

The ANDE System is designed to accelerate and simplify STR profile generation, allowing samples to be collected and analyzed immediately with a DNA test result obtained in 94 min. This is in contrast to conventional forensic laboratory STR testing where a reference buccal sample may be stored for a lengthy amount of time prior to analysis. Nonetheless, stability testing was performed to examine the possible effects of short-term storage of ANDE swab samples on the ANDE System performance.

Full CODIS core 20 loci profiles were generated for all samples included in the stability experiment and no degradation was observed under all storage conditions. This indicates freshly collected moist swabs can be reliably stored at 4°C or room temperature for at least 7 days in the ANDE swab plastic protective cover and produce successful typing results. For longer storage at room temperature, a protective swab cover containing desiccant should be used.

We have previously demonstrated room temperature stability of chips with PowerPlex16 chemistry over at least six months of room

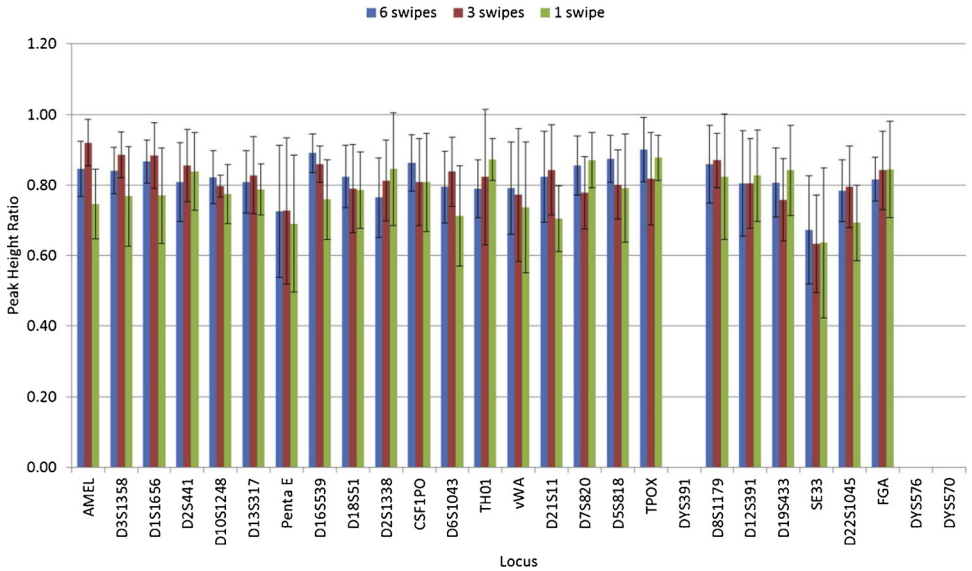


**Fig. 4.** Peak height ratio for all autosomal loci with standard deviation. Limit of detection buccal samples yielding a full profile for the CODIS core loci were included in the calculation.

temperature storage [4]. To extend this result to chips with FlexPlex chemistry, following three and six months of room temperature storage, chips were evaluated by running with buccal swabs. At three months, the first-pass success was 92.5% and at six months was 90%, both essentially the same as for chips immediately following manufacture.

### 3.4. Inhibitors

Potentially PCR-inhibitory substances are often found in the mouths of arrestees and other reference sample donors when collected in the field. Most buccal swab collection protocols recommend abstaining for food and beverage consumption prior to collection for DNA testing, but adherence to that strict requirement is not always possible. Therefore, it is important to evaluate the most common substances that may be present in oral cavities.

Ten potential PCR inhibitors (mint, beer, bloody swab, cigarette, coffee, gum, mouthwash, tea, tobacco dip, and toothpaste) were tested to determine their potential impact on the ability to develop an STR profile using the ANDE System. All samples yielded passing profiles and demonstrated no negative impact on the amplification reactions, demonstrating the ANDE System is robust and reliable in the presence of tested potential oral inhibitors.

### 3.5. Reproducibility

Reproducibility of ten single source reference samples (tested in duplicate) on the ANDE System with integrated Expert System was evaluated within and between all 6 participating laboratories. The reproducibility of STR profiles generated from the same single source reference samples ensures the inter-operability of nationwide DNA databases and instills confidence in the downstream searching capability of DNA profiles generated from individual reference samples.

Full CODIS profiles with the expected genotypes were obtained for all replicates (n = 20) of all reproducibility buccal swabs (Fig. 5) as well as the replicates of swabs spiked with purified DNA (n = 9).

A NIST-traceable swab was created and run in parallel with the NIST SRM 2391c Components A–C and the results were concordant with the certified values recorded on the Certificate of Analysis (03 April 2015) provided by NIST.

### 3.6. Mixtures

It is important for the Rapid DNA Identification of reference and arrestee buccal swabs that any potential mixed DNA samples are appropriately detected and failed by the Expert System. Although mixtures are not commonly encountered in the testing of reference samples, it is important to assess whether the ANDE System with its integrated Expert System will effectively detect mixed STR profiles in the event a true mixed DNA sample is collected, or contamination occurs.

Mixed profiles are failed by the Expert System when one or more of the following conditions are met:

- Two or more heterozygous loci with 3 alleles
- One or more heterozygous loci with 4 alleles
- One or more hemizygous loci with 2 alleles

Mixed samples at five ratios from 19:1 to 1:19 were analyzed using the ANDE System and all detected mixed profiles were reliably detected and appropriately failed by the Expert System.

### 3.7. Precision and accuracy

#### 3.7.1. Precision

Allelic ladders from 402 runs on 13 ANDE instruments were used to calculate inter-run precision. The standard deviation in base pairs was calculated for each allele in the allelic ladder (Fig. 6) and ranged from 0.011 at D7S820 11 to 0.063 at Penta E 25. The variation at three standard deviations range from 0.033 bases to 0.188 bases and are well below the acceptable target value of 0.5 bases.

#### 3.7.2. Concordance

Concordance at the CODIS core 20 loci was evaluated for a total of 1338 unique donors. Concordance was evaluated by comparing each allele call generated by the ANDE System with the corresponding allele call generated by conventional laboratory testing. The accuracy allele calling rate was 99.998% for the CODIS core 20 loci with allelic drop-out observed at a single CODIS core locus in one sample. No instances of allelic drop-in were observed. Five instances of primer binding site mutations (single base substitutions), two in vWA and three in D6S1043, were observed and confirmed by DNA sequencing.

#### 3.7.3. Signal strength

Signal strength for homozygous and heterozygous loci for 1338 Accuracy buccal swabs was determined by summing the signal strengths of all called peaks within the locus and dividing by two. The signal strength of hemizygous loci was determined by the signal strength of the single peak.

The average peak height ranged from 5376 rfu at D6S1043 to 17,928 rfu at TH01. This data demonstrates the ANDE System is capable of generating CODIS suitable profiles while dealing with wide fluctuations of DNA input and resulting signal strengths. These fluctuations have previously been characterized using the guanidine method on the ANDE swabs in tube-based experiments. Buccal swabs were found to contain approximately 1266.8 ng DNA (713.7 standard deviation, n = 90), with an approximately 15-fold range (304.8–4455.3 ng/swab) [43].

#### 3.7.4. Peak height ratio

The peak height ratio for each heterozygous locus was determined for each of the 1338 donor samples that yielded results at all 20 CODIS core loci. The peak height ratio was calculated as the signal strength of the weaker peak divided by the signal strength of the stronger peak (Fig. 7). The results range from 0.721 at D22S1045 to 0.894 at Amelogenin. The average peak height ratio across all loci was 81%. Heterozygous peak height ratios in this range are more than adequate for the ANDE System which is designed for use with single source samples.

#### 3.7.5. Stutter

The percentage of stutter at each locus was determined for 1338 Accuracy samples by calculating the ratio of the peak height of the minor peak over the peak height of the called allele. The minor peak was identified as the peak 3 bases smaller than the called trinucleotide loci, 4 bases smaller than the called allele for tetranucleotide loci, or 5 bases smaller than the called alleles for pentanucleotide loci.

The minor peaks were included if they had a peak height of 250 rfu or greater at Penta E or a peak height 300 rfu or greater at the remainder of the loci. The average stutter varied from 0.030 at TPOX to 0.166 at D21S391 (Fig. 8). These results confirm the stutter settings for the Expert System are appropriate for use with FlexPlex on the ANDE System.

#### 3.7.6. Non-template addition

The percentage of incomplete Non-Template Addition (iNTA) at each locus was determined for 1338 Accuracy samples. Non-template addition was calculated as the ratio of the peak heights of the minor peak over the peak height of the called allele. The minor peak was identified as 1 base smaller than the called allele. The minor peaks were required to have a peak height of 250 rfu or greater to be included in the calculation. No iNTA was observed on D10S1248, D13S317, Penta E, CFS1PO, D6S1043, D7S820, D5S818, TPOX, DYS391, SE33, DYS576, and DYS570. For loci where iNTA was observed, the iNTA varied from 0.037 at FGA to 0.141 at D2S1338 (Fig. 9). These results confirm the

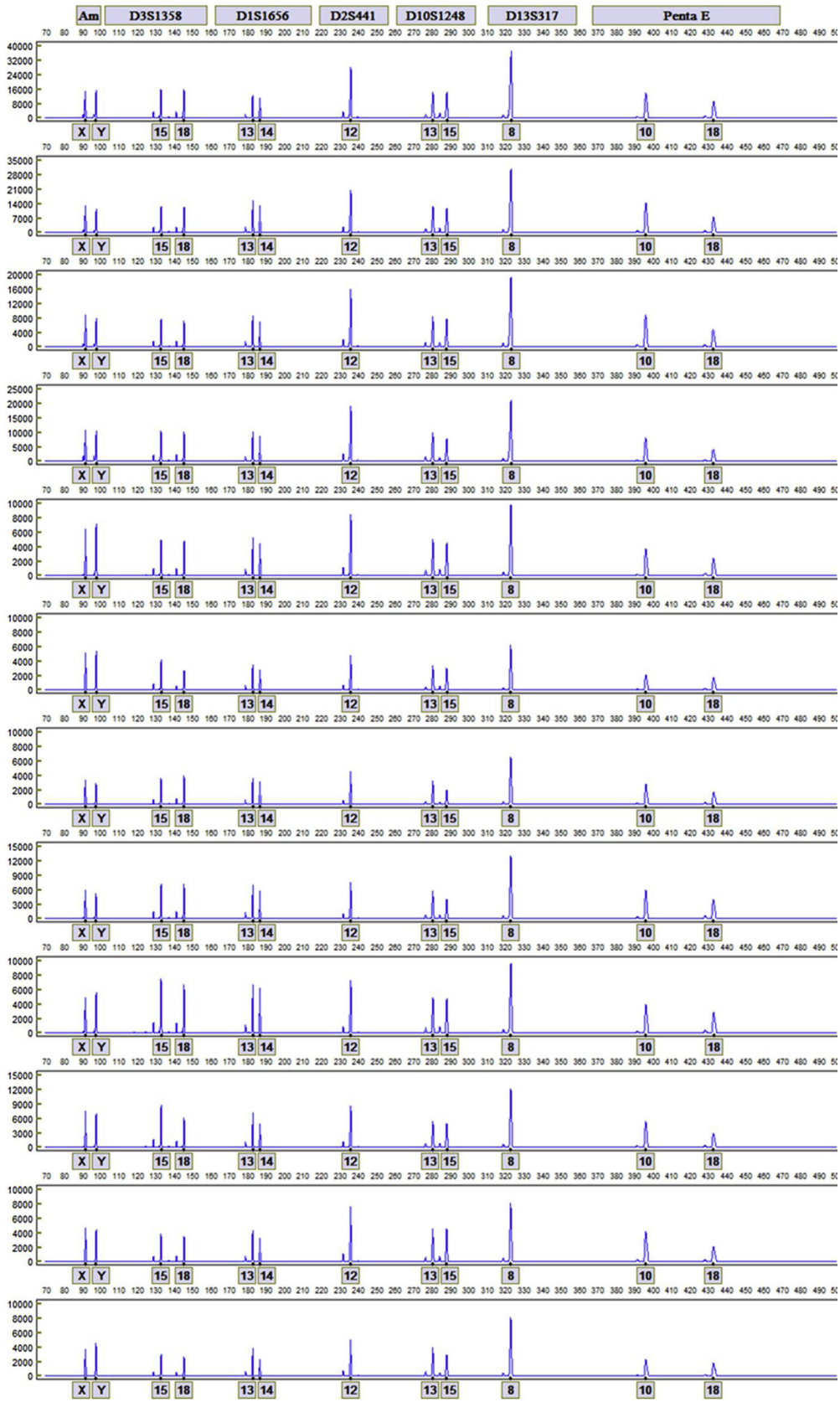


**Fig. 5.** Profiles from twelve reproducibility buccal samples of NB1968 from six laboratories. Data for the Fluorescein (FAM) channel is shown.

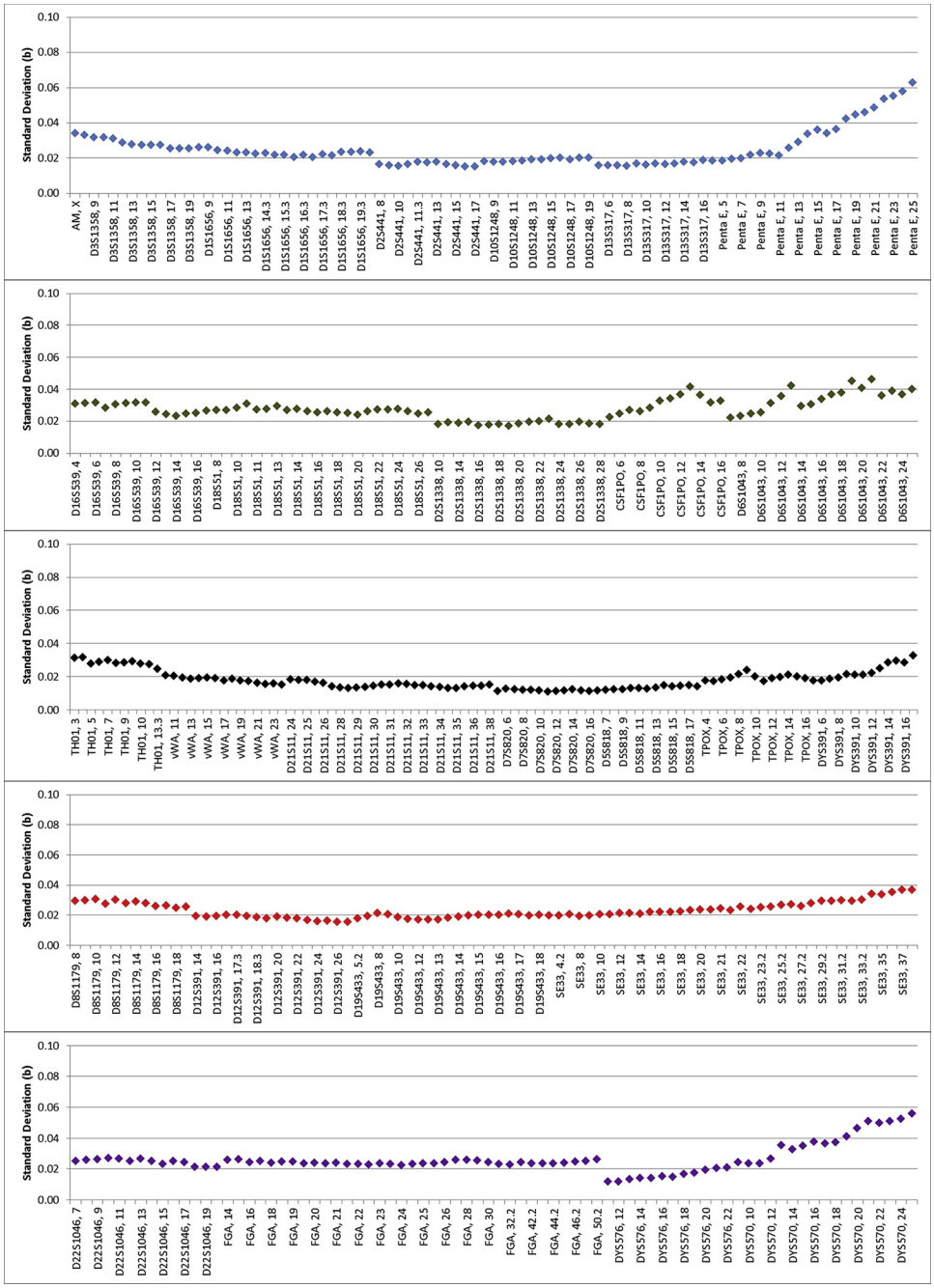


**Fig. 6.** Sizing variation at a single standard deviation for each allele in the allelic ladder calculated for 402 runs on 13 ANDE instruments.

Expert System settings used to identify iNTA are appropriate for use with FlexPlex on the ANDE System.

### 3.7.7. Success rate

The first pass success rate was calculated from buccal swab samples from donors in the Accuracy study. Samples were classified as a pass for first pass success rate if they generated a full CODIS core 20 on the first attempt. The first pass success rate for the ANDE System was 92%. The first pass success rate for the corresponding sample testing at the conventional laboratory was 77.2%.

### 3.7.8. Resolution

The separation and detection of STR fragments on the ANDE System consists of filling the separation channels with sieving polymer, filling the anode and cathode chambers with electrophoresis buffer, completing an electrokinetic injection, and conducting separation across a short, plastic separation channel. Data evaluation and calculations were completed to determine the resolution of this microfluidic separation and detection system. The evaluation of resolution evaluates the system's ability to resolve to a single base pair difference, such as the 9.3 and 10 alleles at TH01, as well as properly detect and label microvariants present within a locus.

Effective resolution was calculated based on the set of 1338 samples used for accuracy calculations (Fig. 10). The equation for effective resolution was adapted by setting R* = 0.2 as the measure of single base resolution [45].

$$R_{eff} = 0.2\frac{\Delta b(w1 + w2)}{2(t2 - t1)}$$

In Gaussian distribution:

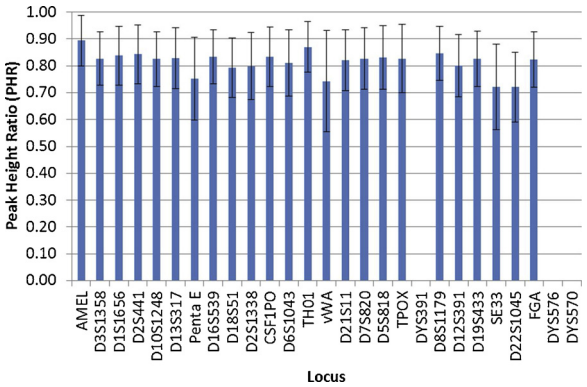


**Fig. 7.** Average peak height ratio by locus, for all autosomal loci, with standard deviation accuracy buccal swab samples. Samples with results at a minimum of the 20 CODIS core loci were included in the calculation.

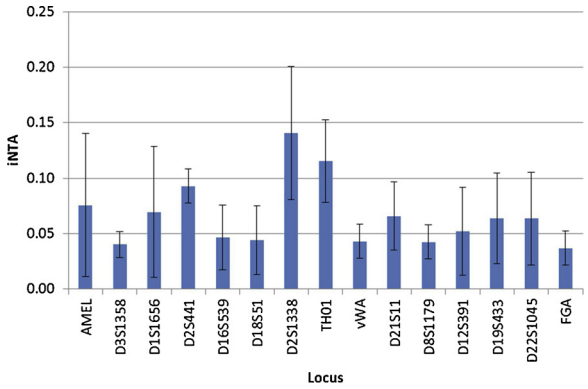


**Fig. 9.** iNTA ratio by locus with standard deviation for 1338 accuracy buccal swab samples. Samples with results at a minimum of the 20 CODIS core loci were included in the calculation. Loci with no iNTA are omitted from this figure.

$w = 4\sigma$, $FWHM = 2(\ln 2)^{0.5} = 2.355\sigma$

$\sigma = Area/[(2\pi)^{0.5} \times Height]$

The results demonstrate the system has sufficient precision and resolution across all loci for effectively sizing microvariants present within all loci.

### 3.8. Contamination assessment

A total of 84 blank swabs were typed across both patterns. The blank swabs generated background noise with no labeled alleles.

### 3.9. PCR-based studies

The ANDE System is a closed system wherein the laboratory performing testing is unable to alter any of the thermal cycling parameters including cycle number, and anneal, extend, and denature times and temperatures. Similarly, the reaction mix cannot be adjusted by the laboratory. Reaction conditions for the ANDE System with FlexPlex were optimized by ANDE during development and no further studies were performed in association with the Developmental Validation.

The ANDE instrument contains a custom thermal cycler to perform fast cycling by allowing the PCR reaction solution temperatures to be heated and cooled rapidly, controllably, and reproducibly [46]. The ANDE thermal cycler consists of a high output thermoelectric cooler/heater mounted to a high efficiency heat sink. During the development of the FlexPlex chemistry, the optimal cycle number and anneal, extend, and denature temperatures and times were determined

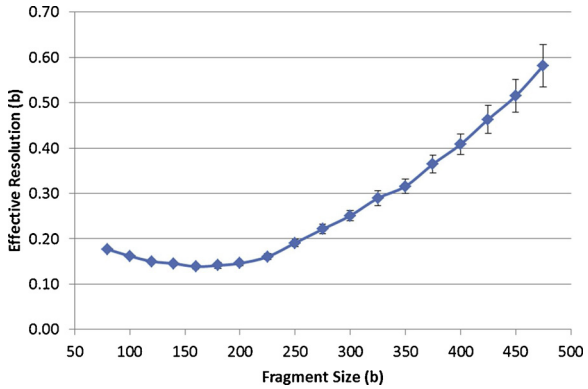


**Fig. 10.** Effective Resolution by fragment size in base pairs with standard deviation.

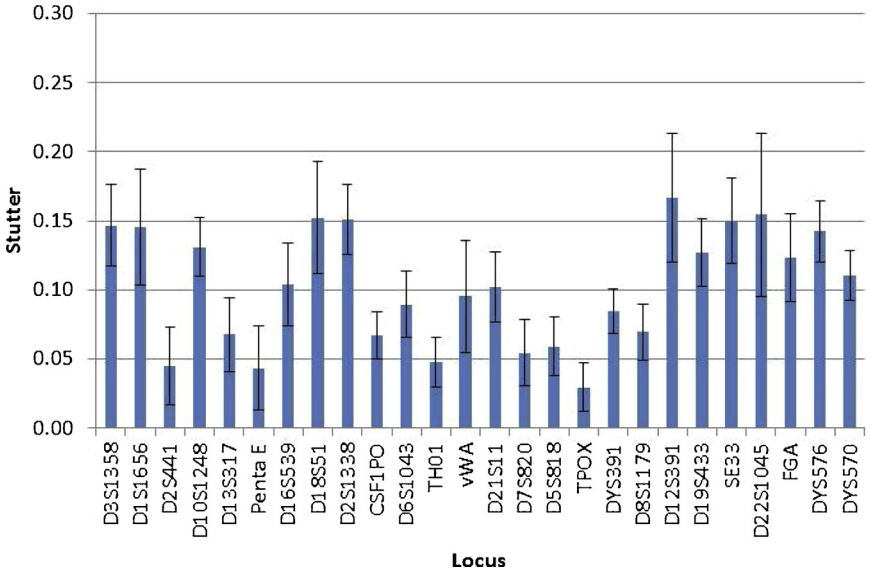


**Fig. 8.** Stutter ratio by locus with standard deviation for accuracy buccal swab samples. Samples with results at a minimum of the 20 CODIS core loci were included in the calculation.

empirically. For example, anneal time was varied in one second increments from 2 to 30 s, and anneal temperatures varied from 55 to 65°C in 0.5°C increments. Initial experiments were typically performed with 1 ng of substrate DNA, and, with final conditions established, the range of DNA tested was from 6 pg to 4 µg.

The creation of a robust and reliable multiplex STR amplification reaction requires careful primer design and extensive testing of reagents and thermal cycling parameters. The overall results of this developmental validation demonstrate FlexPlex is a well-balanced multiplex suitable for use in human identification.

## 4. The ANDE expert system

The ANDE Expert System Software processes the raw data, assigns allele designations, and employs rules to interpret the DNA profiles without human intervention. The ANDE System also automatically generates a run success output that provides a real-time summary of each sample in the form of green or yellow checkmarks and red X.

Following the completion of a run, the ANDE System automatically performs Expert System analysis on the raw data to generate an STR profile.

The Expert System interprets the profiles by applying a series of rules. These rules include Peak Evaluation, ILS Evaluation, Allelic Ladder Evaluation, Allele Assignment, Locus Evaluation, and Sample Evaluation. Peaks, alleles, and loci that pass each rule are processed further by subsequent rules while those that fail are not processed any further. Failed peaks, alleles, and loci will either be unlabeled or be labeled in red warning boxes. After all rules are applied, the sample is either classified as a pass, visible with a green checkmark, requiring further review (yellow checkmark), or failed (red X). Both. fsa and. png files are generated for all profiles. No. xml files are generated for failed profiles or profiles requiring review.

The following outputs are generated:

- Allele table listing allele calls for all passing samples
- .png file (electropherogram) for rapid output visualization
- .xml file for upload to CODIS (only for passing samples)
- .fsa file to permit review with conventional software packages

## 5. Conclusion

A developmental validation of the ANDE System was performed on over 2000 total swabs, including 1387 unique individuals, and the studies were based on the FBI's Quality Assurance Standards and SWGDAM recommendations. The ANDE System has been shown to provide high quality, concordant results for reference buccal swabs, including automated data analysis with an integrated Expert System. Five external laboratories participated in the validation testing along with ANDE, showing the reproducibility and reliability of the system and its successful use in different settings with numerous operators. The ANDE System shows limited cross-reactivity with other species, is stable, is resilient in the presence of numerous potential PCR inhibitors, and produced reproducible results for both buccal and purified DNA samples with limit of detection at a level appropriate for buccal swabs.

The ANDE integrated Expert System was examined as part of the developmental validation and demonstrated a concordance rate over 99.99% and a first pass success rate for the CODIS core 20 loci of 92%. The ANDE System identified and failed both mixed samples and samples with insufficient genetic information. The precision and resolution of the system is sufficient for detection of micro-variants and is capable of displaying single base resolution. PCR-based studies provide confidence that the System is robust and that the amplification reaction has been optimized to provide interpretable results for the Expert System. The ANDE System utilizes the FlexPlex assay, based on the Promega Fusion 6C System, and the results of this developmental validation are consistent with previous validations [47].

The Rapid DNA Act of 2017 has the potential to usher in the most significant change to forensic DNA processing since the introduction of CODIS in 1998. Over the past two decades, NDIS-participating laboratories laid the foundation for Rapid DNA Identification in the booking station by developing robust technologies, quality systems, and policies to allow reproducible generation, interpretation, and utilization of STR profiles. As a result, the CODIS DNA databases are highly reliable. By adopting the approaches, requirements, and regulations established by these laboratories, a clear roadmap for Rapid DNA has been developed. Rapid DNA Identification applications in military, immigration, homeland security, disaster victim identification will advance in parallel. Taken together, Rapid DNA has the potential to dramatically reduce recidivism and crime rates and broadly improve societal safety. NDIS Approval of the ANDE System and FlexPlex assay represents an important step towards realizing this potential.

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