Validation Summary of Methods for Use of Identifiler With the ABI Prism 310 Genetic Analyzer

DEVELOPMENTAL VALIDATION

The following references describe the elements of developmental validation of the AmpFℓSTR Identifiler Amplification Kit with the ABI Prism 310 Genetic Analyzer. A copy of each is included in the applicable sections of this binder.

1) Components of the Identifiler User's Manual (excerpts)

   a. Section 1: Introduction
      i. Loci amplified by the kit
   b. Section 4: Experiments and Results
      i. Developmental Validation
      ii. Accuracy, Precision, and Reproducibility
      iii. Stutter
      iv. Characterization of Loci
      v. Species Specificity
      vi. Sensitivity
      vii. Stability
      viii. Mixture Studies
      ix. Population Data
      x. Mutation Rate
      xi. Probability of Identity
      xii. Probability of Paternity Exclusion
   c. Section 5: Genotyping
      i. Identifiler Allelic Ladder
      ii. Kazam (20% Filter)

2) Population Genetics

   a. Budowle B, Shea B, Niezgoda S, Chakraborty R. CODIS STR loci data from 41 sample populations. J Forensic Sci 2001:46;(3): 453-489
   b. Budowle B, Moretti TR, Baumstark AL, Defenbaugh DA, Keys KM. Population data on the thirteen CODIS core short tandem repeat loci in African Americans, U.S. Caucasians, Hispanics, Bahamians, Jamaicans, and Trinidadians. J Forensic Sci 1999;44(6): 1277-1286.
   c. Budowle B, Sprecher CJ. Concordance study on population database samples using the PowerPlex™ kit and AmpFℓSTR® COfiler™ kit. J Forensic Sci 2001;46(3): 637-641.

INTERNAL VALIDATION

3) For the instrument itself, sections 8.1.2.2, 8.1.3.1(a), 8.1.3.1(b), and 8.1.3.4 of the guidelines to complete internal validation requirements.

a. Known and Non-Probative Samples
    i. Characterized and compared known laboratory samples to results from the 3100.
    ii. Characterized and compared the results of non-probative casework samples, standard and questioned stains, from a variety of cases to previous determinations.
b. Reproducibility Studies
    i. Amplified and characterized known DNA (9947A) across a three day period.
    ii. Characterized reproducible artifacts over a 2 month period of time.
c. Precision Studies
    i. Determined precision of alleles from known DNA (9947A) across a three day period.
    ii. Determined precision of allelic ladder alleles in 7, 10, and 15 injection periods for both day and night conditions.
d. Match Criteria
e. Sensitivity and Stochastic Studies
    i. Characterized variation in sensitivity across multiple analysts.
    ii. Evaluated allele determination capability for different template amounts between two analysts and RFU thresholds of 50, 75, 100, and 150 each.
    iii. Observed pull-up in dye color.
f. Mixture Studies
    i. Calculated peak height ratios of laboratory known samples across all loci.
    ii. Determined required contribution of minor component to allow confident allele calls at 5 and 9 second injection times.
g. Carry-over Contamination Study
    i. Evaluated instrument for any cross contamination between samples within a run.
    ii. Characterized camera noise in blank samples occurring outside the size calling region.
h. Qualifying Test
    i. External proficiency test Distribution 2301, Lab #235 (Orchid)
    ii. NIST SRM 2391b
i. Material Modification Studies (injection time)
    i. Evaluated allele determination capability using different template amounts of DNA versus injection time used.

The validation studies referenced above have been reviewed and provide the necessary documentation required by the FBI Director's "Quality Assurance Standards for Forensic DNA Testing Laboratories" for a DNA typing method to be used in the forensic casework section of the Alabama Department of Forensic Sciences Birmingham DNA laboratory.

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8.1.3.1(a)

Has the procedure been tested using known and non-probative evidence samples?

Known samples:

Ten known liquid blood laboratory samples (Angelo, Carolynn, Dot, Ken, Larry, Lauren, Lisa, Melissa, Rickey, and Stacy) were amplified in duplicate at 0.5ng and 0.25ng each, for a total of 40 separate amplifications. Allele determinations generated following separation on the 310 (75 RFU threshold) were compared to allele calls generated from Identifiler data on the 3100. All allele calls were identical between the two instruments. An allele table is included for both the 310 generated data, and the 3100 generated data. Genotyped data displaying allele calls are also included for the 310 instrument.

Non-probative evidence samples:

Nine cases, for a total of 35 non-probative evidence samples, were amplified and characterized using the Identifiler kit and User's Guide procedures, and compared to original case conclusions. Case samples consisted of: blood; bloodstains; saliva; sperm and cell fractions of vaginal, rectal, and genital swabbing, as well as sheets, panties and dried secretions; and fingernail scrapings. Analysis was conducted using a 100 RFU threshold. The peak height of all alleles was above the 100 RFU threshold set, therefore all allele calls would also be reproduced using a 75 RFU threshold.

All case results using Identifiler materials and methods matched original case conclusions for the information available. Allele tables and genotyped data with allele calls are included for each case.

8.1.3.1(b)

Has the *reproducibility* and precision of the procedure been monitored and documented using human DNA control(s)?

Experiment: Reproducibility using human DNA controls (9947A)

Twenty samples of 1ng human DNA control 9947A were amplified separately with Identifiler. All twenty samples were run on three separate days (6/10/03, 6/11/03, 6/12/03). Samples were analyzed at a 100 RFU threshold.

Results:

All allele calls were identical across 20 separate amplifications, and three days of separation runs (for a total of 60 comparisons). Day three, sample 16, exhibited an off-ladder allele at D19 and was subsequently re-injected and analyzed producing the correct allele calls. The off ladder peak may be a result of poor separation in the capillary due to changing room parameters (temperature control). Re-injection and analysis of the sample supports this conclusion. A table of allele calls and genotyped data for each day are included.

The peak height of all alleles was above the 100 RFU threshold set, therefore all allele calls would also be reproduced using a 75 RFU threshold.

Reproducible artifacts:

Reproducible artifacts have been identified and documented using Identifiler material and methods. Genotyper data with base pair size labels are included for runs conducted over a three month period (April – June).

8.1.3.1(b)

Has the reproducibility and *precision* of the procedure been monitored and documented using human DNA control(s)?

Experiment: Precision using human DNA controls (9947A)

Twenty 1ng samples of human DNA control 9947A were amplified separately with Identifiler kit components and methods. Identical 1ng 9947A samples were loaded onto the capillary on each of three separate runs spanning three days (6/10/03, 6/11/03, 6/12/03). Each of the twenty 1ng samples was re-injected throughout the three runs and the size determinations were conducted with a 100 RFU threshold.

Results:

Size determinations for all alleles (60 sample observations over three separate days) did not exceed a standard deviation of 0.35 base. 20 of 26 alleles were less than 0.25 base. The largest fragments observed the greatest standard deviation among the loci. CSF1PO generated a 0.28 and 0.31 base standard deviation; D2S1338 generated a 0.34 and 0.31 base standard deviation; D18S51 generated a 0.23 and 0.35 base standard deviation; and FGA generated a 0.20 and 0.20 base standard deviation (respectively for peak one and peak two of each locus). Base pair labeled tables for days 1-3 are included.

The peak height of all alleles was above the 100 RFU threshold set, therefore all allele calls would also be reproduced using a 75 RFU threshold.

Experiment: Precision using Identifiler allelic ladder

Seven, ten, and fifteen injections of Identifiler allelic ladder were separated over a day and night period each. Size determinations were conducted on all alleles of the ladder for all separations, and a standard deviation in base pair sizing was calculated for each allele of the ladder for all loci and each separate set of injections. A final day and night total precision was calculated for the total 30 injections of the ladder using the mean base pair size for each allele of each ladder injection over 15 injections of day + night.

Results:

Size determinations for all alleles of the ladder analyzed for day *or* night as a group, for 7, 10, or 15 injections did not exceed 0.12 base. The largest deviations were again observed in the larger fragment alleles of CSF1PO, D2S1338, D18S51, and FGA. Standard deviation of greater than 0.50 base were observed in the combination of day *and* night ladder separations at locus D18S51 (alleles 21, 22, 23, and 24 exhibited STDs of 0.52, 0.54, 0.55, and 0.53 respectively) and FGA (alleles 42.2, 43.2, 44.2, 45.2, 46.2, and 47.2 exhibited STDs of 0.64, 0.63, 0.64, 0.67, 0.64, and 0.59 respectively). These are some of the largest fragments in the multiplex. Precision tables for all loci and injection groups are included. Genotyped data with allele and base pair size labels are also included.

Remarks:

Precision data presented by Applied Biosystems, represented in the User's Manual on pages 4-7 through 4-17, is based on 7 injections of ladder (reference 8.1.2 of binder). The instrument is spec'd for precision of less than 0.5 base pair when analyzed with a ladder spaced every 10 injections. Internal experimental results indicate that precision on the Alabama Department of Forensic Sciences Birmingham Laboratory DNA ABI Prism 310 CE instrument exceed specifications as exhibited by a standard deviation of less than 0.13 base for groups of 15 injections day or night. However, results indicate a noticeable increase of standard deviation as a whole across some alleles from 10 to 15 injections in both the day and night groups. Although this increase is much less than the specified precision of the instrument, it is recommended that ladders be spaced every 10 samples for optimum typing results. When separation crosses the day/night threshold (~5pm/6pm) it is recommended to space ladders every 7 injections.

8.1.3.1(b)

*Reference Standard 8.1.2.2

Have species' specificity, *sensitivity*, stability and mixture studies been conducted?

Experiment: Sensitivity using human DNA controls (9947A)

Nine samples of human control DNA 9947A were amplified in duplicate by two separate analysts at varying concentrations ranging from 0.0125ng to 1ng. Following separation samples were analyzed at 150, 100, 75, and 50 RFU thresholds for each analyst's group.

Results:

As the amount of input DNA decreases, signal decreases. As threshold parameters are lowered both true allele peaks and artifacts or baseline can become evident. Full or partial profiles were also observed with all concentrations amplified, although as the concentration fell below 0.1ng many fewer loci gave complete results. Pull-up was characterized at the 1ng concentration in the D3 locus for analyst 1 (SO). Reproducible artifacts referenced in section 8.1.3.1(b) of this binder, and by Applied Biosystems, were observed in the interpretable region of 75 RFU analyses to a larger degree.

Analyst 1 (SO) observed sensitivity to 0.2ng with full profile results when analyzed with a 150 RFU threshold. A partial profile was observed at 0.1ng with allele 17 of vWA falling below threshold.

Analyst 2 (DKD) observed sensitivity to 0.2ng with full profile results when analyzed with a 150 RFU threshold. However, sample 0.2-1 experienced low signal due to an apparent amplification error and is not considered for the final results. A partial profile was observed with 0.1-1 sample (0.1ng) with dropout observed at allele 19 of the D2 locus and allele 23 of the FGA locus. Sample 0.1-2 had additional drop-out, but allele peaks were visually present.

Analyst 1 (SO) observed sensitivity to 0.1ng with full profile results when analyzed with a 100 RFU threshold. No appreciable increase in artifacts or baseline was noted decreasing threshold from 150 RFUs to 100 RFUs.

Analyst 2 (DKD) observed sensitivity to 0.1ng on sample 0.1-2 with full profile results when analyzed with a 100 RFU threshold. Sample 0.1-1 (0.1ng) exhibited drop-out at allele 19 of the D2 locus. This locus shows a high degree of imbalance in peak heights for this sample.

Analyst 1 (SO) observed sensitivity to 0.05ng on sample 0.05-2 with full profile results when analyzed with a 75 RFU threshold.

Analyst 2 (DKD) observed sensitivity to 0.1ng with full profile results when analyzed with a 75 RFU threshold. 0.05ng concentrations gave a more complete partial profile than when analyzed at a 100 RFU threshold.

Analyst 1 (SO) observed sensitivity to 0.05ng on sample 0.05-2 with full profile results when analyzed using a 50 RFU threshold. An appreciable increase of "OL Allele?," including artifacts and baseline noise was observed with the 50 RFU analysis parameter.

Analyst 2 (DKD) observed sensitivity to 0.1ng with full profile results when analyzed with a 50 RFU threshold. 0.05ng samples gave almost a complete profile at 50 RFUs. An appreciable increase of "OL Allele?," including artifacts and baseline noise was observed with the 50 RFU analysis parameter.

Allele tables for each level of analysis, for each analyst, at all concentrations are included. Allele call and peak height data are included in the tables. Peak height graphs for the largest loci are included for each analyst's results, as well as GeneScan panel printouts and raw data electropherograms.

Remarks:

The number of artifacts or baseline noise does not increase significantly on the Alabama Department of Forensic Sciences Birmingham DNA ABI 310 CE instrument when lowering the RFU threshold from 150 to 100, or 100 to 75 RFUs. Confident allele calls can be made from 75 RFUs on this instrument, in consideration of reproducible artifacts previously mentioned in section 8.1.3.1(b), to a sensitivity of 0.1ng of DNA. This threshold is also supported by the manufacturer suggestion of three times the noise of the instrument (noise = ~25 RFUs). Significant "noise" is encountered in the interpretable region when analyzing from a 50 RFU threshold. A threshold of 150 RFUs is recommended for standard reference samples, and 75 RFUs for forensic samples to produce confident and reproducible allele calls. A flexible system of threshold settings based on the circumstances at hand and the analyst's experience is supported by this experiment for confident and reproducible allele calls.

8.1.3.1(b)

Have mixture studies been conducted?

Experiment: Peak Height Ratio Study
Ten (10) single source laboratory known samples were collected and amplified in duplicate at ~0.5ng using the Identifiler Amplification Kit. Combined with the positive control DNA 9947A – a total of 21 separate amplifications were compared for peak height analysis. All samples were genotyped and labeled with allele and peak height designations. Peak height ratios were calculated for all heterozygous loci, for each sample, and averaged for each locus.

Results:
Peak height ratios for each locus, with the exception of vWA, were above 0.80 with most falling in the mid-to-high range of 85% balance between the two alleles. This was in concordance with previous Applied Biosystems experiments as shown in the included table. A 0.79 ratio was observed for the vWA locus, which is within the range of experimental results determined by Applied Biosystems. Pages 4-38 through 4-41 from the Identifiler User's Manual, peak height ratio tables, peak height ratio graph, and Genotyper printouts are included.

Experiment: Mixture Study (5 Second Injection)
Two known samples, one male (A) and one female (B), were mixed in the following A:B ratios: 1:0, 19:1, 9:1, 4:1, 3:2, 1:1, 2:3, 1:4, 1:9, 1:19, 0:1, labeled M1 through M11 respectively, and injected for 5 seconds. Each sample was subsequently amplified with Identifiler, and interpreted with a 75 RFU threshold.

Results:
Detection of the minor component for samples M2 – M10 was successful when analyzed with a 75 RFU threshold. Minimal allelic dropout was noted. Individual results for these samples, as well as a description of the 9 loci analyzed where A and B do not share alleles, is as follows:

M2 minor component (19:1) produced a complete profile at 5 of 9 possible loci where no alleles are shared, and a partial profile at 3 of 9 possible loci. Only D18 for M2 revealed no minor component. All un-shared alleles were shown by the minor component at the applicable loci for the remaining mixture samples.

M3 minor component (9:1) produced a complete profile at 7 of 9 possible loci where no alleles are shared, and a partial profile at 2 of 9 possible loci.

M4 minor component (4:1) produced a complete profile at 9 of 9 possible loci where no alleles are shared.

M5 minor component (3:2) produced a complete profile at 9 of 9 possible loci where no alleles are shared.

M7 minor component (2:3) produced a complete profile at 9 of 9 possible loci where no alleles are shared.

M8 minor component (1:4) produced a complete profile at 9 of 9 possible loci where no alleles are shared.

M9 minor component (1:9) produced a complete profile at 8 of 9 possible loci where no alleles are shared, and a partial profile at 1 of 9 possible loci.

M10 minor component (1:19) produced a complete profile at 7 of 9 possible loci where no alleles are shared, and a partial at 2 of 9 possible loci.

Experiment: Mixture Study (9 Second Injection)

Amplification product from the two known samples, one male (A) and one female (B), listed above were injected for 9 seconds on a separate run. Each sample was subsequently interpreted with a 75 RFU threshold to evaluate mixture interpretation results versus injection time, as compared to the study above.

Results:

Detection of the minor component for samples M2 – M10 was successful when analyzed with a 75 RFU threshold. Concordant allele results were obtained as compared to the 5 second injection mixture study above, with improvement noted at locus D18 (9:1 '14' allele called) and TPOX (1:19 '10' allele called) giving M3 an improved complete profile at 8 of 9 possible loci where no alleles are shared. TPOX has a shared allele, '8', in the mixture but the unshared minor component allele, 10, for that sample called with a peak height of 85 RFUs.

8.1.3.4

Have material modifications to analytical procedures been documented and subjected to validation testing?

Experiment: Variations of injection time with known human control DNA (9947A)

Human control DNA 9947A was amplified in two concentrations, 1 and 0.5 nanograms, with Identifiler materials and methods. The injection time for each concentration was varied from 1 to 9 seconds in increments of 1 second for 3 injections at each increment, for each concentration. Allele calls, peak height, and peak height increases for each allele were noted for the time frames of 1-5 seconds, 5-9 seconds, and 1-9 seconds.

Results:

All injection times from 1 to 9 seconds gave correct and reproducible allele calls. A missed injection occurred at one injection of the 1ng 1 second separation (raw data included). Decreasing the injection time from 5 seconds to 1 second decreased the peak height, on average (over 26 alleles), by 4.86 times its original height with a 0.16 standard deviation at the 1ng concentration. Increasing the injection time from 5 seconds to 9 seconds increased the peak height 1.44 times its original peak height with a 0.15 standard deviation at 1ng concentration. The total increase was 1 second injection time to 9 seconds was seen to be a 7.1 fold with a 0.87 standard deviation at 1ng of human control DNA concentration. This data is represented as a summary table that is included, as well as in a detailed allele and peak height table for each concentration and injection. Pull-up was observed at the D3 locus from 6 to 9 seconds, D19 at one injection at 9 seconds, and vWA from 8 to 9 seconds in the 1ng sample. Increase in pull-up can be 2-3 fold from 8 to 9 seconds at these loci for 1ng samples. At 0.5ng human control DNA concentration a 4.73 fold increase and 0.20 standard deviation was observed from 1-5 seconds; 1.35 fold and 0.08 standard deviation from 5-9 seconds; and a total increase of 6.37 fold and a standard deviation of 0.60. Raw data and Genotyper data electropherograms are included for all injections.

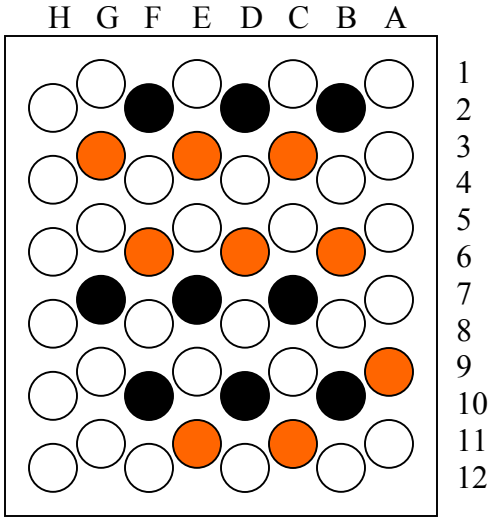
Remarks:

These results are consistent with notations in the Journal of Forensic Sciences' Technical Note that is included (i.e., little variation in Resolution Length (RSL) is seen up to a 9 second injection time, and only about a seven fold increase in RFU is seen when the results of the 1-second injection are compared with that obtained from a 9 second injection). Although pull-up is evident at 7-9 seconds in the 1ng sample, it is not a complicating factor in interpretation of the data. Injection times ranging from 1 to 9 seconds produce confident and reproducible allele calls while allowing the analyst control over signal intensity to aid in the analysis of a sample.

Carry-Over Contamination

The laboratory must demonstrate that its procedures minimize contamination that would compromise the integrity of the results.

Experiment: Carry-over Contamination Study
Nine (9) sets of sample tubes were placed in the sample tray for the ABI Prism 310 instrument in the following arrangement:



Where = Excessive Size Standard (4x the normal amount), and

= Blank (Formamide only)

The injection order for the applicable samples were: A9, B2, B6, B10, C3, C7, C11, D2, D6, D10, E3, E7, E11, F2, F6, F10, G3, G7.

The above tray arrangement was utilized to encompass the variety of sample location possibilities, and in an order of excessive size standard and blank to show no cross-contamination, or carryover, from one sample tube to the next in the run process.

Results:

Screen captures of the raw data results, as presented following TAB #28, show no carryover contamination from the excessive addition of LIZ internal lane standard followed by the subsequent injection of a sample tube with no size standard, only formamide (blank). Injection A represents the size standard injection, followed by injection B – the blank, in the following documents.

Camera noise is present outside the size calling region (~2000-3200 data points) for each injection, and is reproducible across all injections. Artifacts were observed in the injection of sample tube location B2 and could be dust, lint, or crystals (across all four

dyes, as these peaks do not fit necessary manufacturer parameters to be labeled as a true allele peak).

Qualifying Test

The method must be tested using a qualifying test. This may be accomplished through the use of proficiency test samples or types of samples that mimic those which the laboratory routinely analyzes. This qualifying test may be administered internally, externally, or collaboratively.

Experiment: External Proficiency Test Distribution 2301, Lab #235
Proficiency test distribution 2301, lab #235, was used to test the method. Crime sample "2311" (item 1A), and standards "2313", "2314", and "2315" (items 1C, 1D, and 1E respectively) were amplified using the Identifiler kit, and run on the ABI prism 310 Capillary Electrophoresis Instrument. The results were compared to the published results of the external proficiency provider Orchid Diagnostics.

Results

The DNA profiles obtained using the Identifiler kit on the ABI Prism 310 Genetic Analyzer for external proficiency test distribution 2301, lab #235, matched those results obtained from the previously run PowerPlex 1.1 FMBIO generated genetic profile. In addition, the Identifiler results are concordant with results seen in distribution 2301 as published by Orchid Diagnostics.

The allele table and published results are included in this section, and Genotyper printouts can be found following TAB #29.

Experiment: NIST SRM 2391b
Standard Reference Material 2391b was used to test the method and system under validation. Components 1-10 consisted of Genomic DNA at the concentration of ~1ng/uL. Components 11 and 12 were extracted using the organic method of extraction (1/2 of the paper disc was utilized for components 11 and 12). All 12 components were amplified with the Identifiler kit, and run on the 310 Genetic Analyzer.

Results

The DNA profiles obtained from components 1-12 on the 310 Genetic Analyzer match those provided by the National Institute of Standards and Technology. A copy of the NIST Certificate of Analysis is included in this section, and Genotyper printouts are included following TAB #29.