Validation Workshop

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http://www.cstl.nist.gov/biotech/strbase/validation.htm

ISFG Pre-Conference Workshop
Buenos Aires, Argentina
September 15, 2009

Presentation Outline

• Introduction to Validation Terms and Principles
• Review of SWGDAM Revised Validation Guidelines
BREAK
• Detailed Example (presented by Dr. Peter Vallone, NIST)
  – Validation of NIST 26plex assay
• Suggestions for Documentation and Implementation
• Questions

NIST and NIJ Disclaimer

Funding: Interagency Agreement 2008-DN-R-121
between the National Institute of Justice and NIST
Office of Law Enforcement Standards

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best available for the purpose.

My Background

• PhD (Analytical Chemistry) from University of Virginia
  Research conducted at FBI Academy under Bruce
• NIST Postdoc – developed STRBase website
• GeneTrace Systems – private sector experience
  validating assays and developing new technologies
• NIST Human Identity Project Leader since 1999
• Invited guest to FBI’s Scientific Working Group on DNA
  Analysis Methods (SWGDAM) since 2000
• Member of SWGDAM Validation
  Subcommittee – resulting in Revised Validation Guidelines
• Served on WTC KADAP and helped evaluate and
  validate new miniSTR, mtDNA, and SNP assays

Current Areas of NIST Effort with Forensic DNA

• Standards
  – Standard Reference Materials
  – Standard Information Resources (STRBase website)
  – Interlaboratory Studies
• Technology
  – Research programs in SNPs, miniSTRs, Y-STRs, mtDNA, qPCR
  – Assay and software development
• Training Materials
  – Review articles and workshops on STRs, CE, validation
  – PowerPoint and pdf files available for download

http://www.cstl.nist.gov/biotech/strbase/training.htm
Stages of Technology for Forensic DNA Typing

- Idea
- Demonstration of feasibility
- Research and development
- Commercialization
- Validation by forensic labs
- Routine use by the community

TIME  MONEY

Decision to Switch/Upgrade to New Technology

- Hard to calculate
- COST to Change
- Improved Capabilities
- Validation time & effort
- Impact on legacy data

New multiplex STR kit
New detection technology
New DNA markers

Decisions about Changing Technologies

- Cost to change
- Comfort and experience levels
  - court approved methods must be used in forensic labs
- Capabilities…Enhancements
  - Are they really needed?
  - Will legacy data be impacted?

Where Is the Future Going for DNA Technology That Can Be Applied to Forensic DNA Typing?

Constant state of evolution (like computers)

- Higher levels of multiplexes
- More rapid DNA separations
- Better data analysis software
- New DNA Markers

Validating new technologies will always be important in progressive forensic DNA labs…

Importance of Validation

My Purpose in Teaching This Workshop

- I believe that many forensic laboratories, in an effort to be cautious, are taking too long to perform their validation studies and thereby delaying initiation of casework and contributing to backlogs in labs that are already overburdened

- Technology will continue to advance and thus validation of new methodologies will always be important in forensic DNA laboratories

There will always be something to “validate”…
Questions to Keep in Mind…

- Why is validation important?
- How does validation help with quality assurance within a laboratory?
- What are the general goals of analytical validation?
- How is method validation performed in other fields such as the pharmaceutical industry?
- How do accuracy, precision, sensitivity, stability, reproducibility, and robustness impact measurements?

Why is Method Validation Necessary?

- It is an important element of quality control.
- Validation helps provide assurance that a measurement will be reliable.
- In some fields, validation of methods is a regulatory requirement.
- …
- The validation of methods is **good science**.

Definition of Validation

- **Validation** is confirmation by examination and provision of objective evidence that the particular requirements for a specified intended use are fulfilled.
- **Method validation** is the process of establishing the performance characteristics and limitations of a method and the identification of the influences which may change these characteristics and to what extent. It is also the process of verifying that a method is fit for purpose, i.e., for use for solving a particular analytical problem.

More Validation Definitions

**ISO 17025**

5.4.5.1 Validation is the **confirmation by examination** and the provision of objective evidence that the particular requirements for a specific intended use are fulfilled

**DAB Quality Assurance Standards for Forensic DNA Testing Laboratories**

2 (ff) Validation is a process by which a procedure is evaluated to determine its efficacy and reliability for forensic casework analysis and includes:

To demonstrate that a method is suitable for its intended purpose…
Definitions


• Quality assurance (QA) – planned or systematic actions necessary to provide adequate confidence that a product or service will satisfy given requirements for quality

• Quality control (QC) – day-to-day operational techniques and activities used to fulfill requirements of quality

• Validation – the process of demonstrating that a laboratory procedure is robust, reliable, and reproducible in the hands of the personnel performing the test in that laboratory

Definitions

Robust method – successful results are obtained a high percentage of the time and few, if any, samples need to be repeated

Reliable method – the obtained results are accurate and correctly reflect the sample being tested

Reproducible method – the same or very similar results are obtained each time a sample is tested

General Levels of Validation

Developmental Validation – commonly performed by commercial manufacturer of a novel method or technology (more extensive than internal validation)

Internal Validation – performed by individual lab when new method is introduced

Performance Checks – can be performed with every run (set of samples)

The lifecycle of a method of analysis

Revised SWGDAM Validation Guidelines

(2004)


Validation Section of the DNA Advisory Board Standards


STANDARD 8.1 The laboratory shall use validated methods and procedures for forensic casework analyses (DNA analysis).

8.1.1 Developmental validation that is conducted shall be appropriately documented.

8.1.2 Novel forensic DNA methodologies shall undergo developmental validation to ensure the accuracy, precision and reproducibility of the procedure. The developmental validation shall include the following:

8.1.2.1 Documentation exists and is available which defines and characterizes the locus.

8.1.2.2 Species specificity, sensitivity, stability and mixture studies are conducted.

8.1.3 Internal validation shall be performed and documented by the laboratory.

8.1.3.1 The procedure shall be tested using known and non-probative evidence samples (known samples only). The laboratory shall monitor and document the reproducibility and precision of the procedure using human DNA control(s).

8.1.3.2 The laboratory shall establish and document match criteria based on empirical data.

8.1.3.3 Before the introduction of a procedure into forensic casework (database sample analysis), the analyst or examination team shall successfully complete a qualifying test.

8.1.4 Where methods are not specified, the laboratory shall, wherever possible, select methods that have been published by reputable technical organizations or in relevant scientific texts or journals, or have been appropriately evaluated for a specific or unique application.

http://www.cstl.nist.gov/biotech/strbase/training.htm
Ensuring Accurate Forensic DNA Results

<table>
<thead>
<tr>
<th>ASCLD-LAB Accreditation</th>
<th>Proficiency Testing of Analysts</th>
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<tbody>
<tr>
<td>Inspections/Audits</td>
<td>Validated Methods</td>
</tr>
<tr>
<td>DAB Standards-SWGDAM Guidelines</td>
<td>(using standards and controls)</td>
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Checks and Controls on DNA Results

<table>
<thead>
<tr>
<th>Community</th>
<th>FBI DNA Advisory Board’s Quality Assurance Standards (also interlaboratory studies)</th>
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<tbody>
<tr>
<td>Laboratory</td>
<td>ASCLD/LAB Accreditation and Audits</td>
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<tr>
<td>Analyst</td>
<td>Proficiency Tests &amp; Continuing Education</td>
</tr>
<tr>
<td>Method/Instrument</td>
<td>Validation of Performance (along with traceable standard sample)</td>
</tr>
<tr>
<td>Protocol</td>
<td>Standard Operating Procedure is followed</td>
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<tr>
<td>Data Sets</td>
<td>Allelic ladders, positive and negative amplification controls, and reagent blanks are used</td>
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<tr>
<td>Individual Sample</td>
<td>Internal size standard present in every sample</td>
</tr>
<tr>
<td>Interpretation of Result</td>
<td>Second review by qualified analyst/supervisor</td>
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<tr>
<td>Court Presentation of Evidence</td>
<td>Defense attorneys and experts with power of discovery requests</td>
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My perspective...

Validation Philosophy

When is Validation Needed?

- Before introduction of a new method into routine use
- Whenever the conditions change for which a method has been validated, e.g., instrument with different characteristics
- Whenever the method is changed, and the change is outside the original scope of the method

Some Purposes of Validation

- To accept an individual sample as a member of a population under study
- To admit samples to the measurement process
- To minimize later questions on sample authenticity
- To provide an opportunity for resampling when needed

Sample validation should be based on objective criteria to eliminate subjective decisions...

The VAM Principles

VAM = Valid Analytical Measurement

1. Analytical measurements should be made to satisfy an agreed requirement.
2. Analytical measurements should be made using methods and equipment that have been tested to ensure they are fit for their purpose.
3. Staff making analytical measurements should be both qualified and competent to undertake the task.
4. There should be a regular and independent assessment of the technical performance of a laboratory.
5. Analytical measurements made in one location should be consistent with those made elsewhere.
6. Organizations making analytical measurements should have well defined quality control and quality assurance procedures.


The Community Benefits from Training

• To better understand what validation entails and how it should be performed (why a particular data set is sufficient)
• Many labs already treat DNA as a “black box” and therefore simply want a “recipe” to follow
• People are currently driven by fear of auditors and courts rather than scientific reasoning
• Many different opinions exist and complete consensus is probably impossible


How do you validate a method?

• Decide on analytical requirements
  – Sensitivity, resolution, precision, etc.
• Plan a suite of experiments
• Carry out experiments
• Use data to assess fitness for purpose
• Produce a statement of validation
  – Scope of the method

Assumptions When Performing Validation

• The equipment on which the work is being done is broadly suited to the application. It is clean, well-maintained and within calibration.
• The staff carrying out the validation are competent in the type of work involved.
• There are no unusual fluctuations in laboratory conditions and there is no work being carried out in the immediate vicinity that is likely to cause interferences.
• The samples being used in the validation study are known to be sufficiently stable.


Tools of Method Validation

• Standard samples
  – positive controls
  – NIST SRMs
• Blanks
• Reference materials prepared in-house and spikes
• Existing samples
• Statistics
• Common sense

Urban Legends of Validation…


#1: HUNDREDS OR THOUSANDS OF SAMPLES ARE REQUIRED TO FULLY VALIDATE AN INSTRUMENT OR METHOD
#2: VALIDATION IS UNIFORMLY PERFORMED THROUGHOUT THE COMMUNITY
#3: EACH COMPONENT OF A DNA TEST OR PROCESS MUST BE VALIDATED SEPARATELY
#4: VALIDATION SHOULD SEEK TO UNDERSTAND EVERYTHING THAT COULD POTENTIALLY GO WRONG WITH AN INSTRUMENT OR TECHNIQUE
#5: LEARNING THE TECHNIQUE AND TRAINING OTHER ANALYSTS ARE PART OF VALIDATION
#6: VALIDATION IS BORING AND SHOULD BE PERFORMED BY SUMMER INTERNS SINCE IT IS BENEATH THE DIGNITY OF A QUALIFIED ANALYST
#7: DOCUMENTING VALIDATION IS DIFFICULT AND SHOULD BE EXTENSIVE
#8: ONCE A VALIDATION STUDY IS COMPLETED YOU NEVER HAVE TO REVISIT IT


http://www.cstl.nist.gov/biotech/strbase/training.htm
My Philosophy towards Validation

**Ask first**: Does the new method improve your capability?

- **Concordance** – are the same typing results obtained with the new technique as with an older one?
- **Constant Monitoring** – check multiple allelic ladders in a batch against one another to confirm precision and consistent lab temperature
- **Common Sense** – are replicate tests repeatable?

Common Perceptions of Validation

The goal is not to experience every possible scenario during validation...

- Lots of experiments are required
- “You cannot mimic casework because every case is different.”
- Many labs are examining far too many samples in validation and thus delaying application of casework and contributing to backlogs...
- Significant time is required to perform studies

Number of Samples Needed

**Relationship between a sample and a population of data**

Data collected in your lab as part of validation studies

- **“Sample” of Typical Data**

All potential data that will be collected in the future in your lab

- **“Population” of All Data Obtained**

Student’s t-Tests

“Student” (real name: W. S. Gosset [1876-1937]) developed statistical methods to solve problems stemming from his employment in a brewery.

Student’s t-test deals with the problems associated with inference based on “small” samples: the calculated mean ($\bar{X}$) and standard deviation ($\sigma$) may by chance deviate from the “real” mean and standard deviation (i.e., what you’d measure if you had many more data items: a “large” sample).

http://www.physics.csbsju.edu/stats/t-test.html

The Number “5” in Forensic Validation

**NDIS Appendix B Expert System Validation Requirements**

- At least 5 challenge events must be observed for each issue (e.g., pullup, shoulders, spikes, tri-allelic patterns, mixtures, contamination, variant alleles)

http://www.cstl.nist.gov/biotech/strbase/training.htm
Allele Frequency Tables

Ennom et al. (2006) JFS 49(6): 1381-1385

Allele frequencies denoted with an asterisk (*) are below the 5/2N minimum allele threshold recommended by the National Research Council report (NRCII) The Evaluation of Forensic DNA Evidence published in 1996.

Minimum Allele Frequency = 5/2N

Want to sample at least 5 chromosomes to provide a somewhat reliable estimate of an allele's frequency in a population.

Pharmaceutical Industry and FDA Follows ICH Validation Documents

ICH (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use)
- http://www.ich.org
- Q2A: Test on Validation of Analytical Procedures (1994)
- Q2B: Validation of Analytical Procedures: Methodology (1996)

From Q2B:
- “For the establishment of linearity, a minimum of five concentrations is recommended.”
- “Repeatability should be assessed using (1) a minimum of 6 determinations covering the specified range for the procedure (e.g., 3 concentrations/3 replicates each); or (2) a minimum of 8 determinations at 100 percent of the test concentration.”

ICH Method Validation Parameters

Method Validation provides an assurance of reliability during normal use, and is sometime referred to as “the process of providing documented evidence that the method does what it is intended to do.”

Useful Resources on Validation


See also STRBase Validation Section:
http://www.cstl.nist.gov/biotech/strbase/validation.htm

DNA Advisory Board Quality Assurance Standards

Section 2. Definitions
- (ff) Validation is a process by which a procedure is evaluated to determine its efficacy and reliability for forensic casework analysis (DNA analysis) and includes:
  - (1) Developmental validation is the acquisition of test data and determination of conditions and limitations of a new or novel DNA methodology for use on forensic samples;
  - (2) Internal validation is an accumulation of test data within the laboratory to demonstrate that established methods and procedures perform as expected in the laboratory.
Overview of Developmental Validation Studies

2. Developmental Validation: The developmental validation process may include the studies detailed below. Some studies may not be necessary for a particular method.

2.1 Characterization of genetic markers
2.2 Species specificity
2.3 Sensitivity studies
2.4 Stability studies
2.5 Reproducibility
2.6 Case-type samples
2.7 Population studies
2.8 Mixture studies
2.9 Precision and accuracy
2.10 PCR-based procedures

Overview of Internal Validation Studies

3. Internal Validation: The internal validation process should include the studies detailed below encompassing a total of at least 50 samples. Some studies may not be necessary due to the method itself.

3.1 Known and nonprobative evidence samples
3.2 Reproducibility and precision
3.3 Match criteria
3.4 Sensitivity and stochastic studies
3.5 Mixture studies
3.6 Contamination
3.7 Qualifying test
Design of Experiments Conducted for Validation Studies

- Before performing a set of experiments for validation, ask yourself:
  - What is the purpose of the study?
  - Do we already know the answer?
  - Can we write down how we know the answer?

- Think before you blindly perform a study which may have no relevance (e.g., extensive precision studies)

- Too often we do not differentiate learning, validation, and training

Points for Consideration

- Remove as many variables as possible in testing an aspect of a procedure
  - e.g., create bulk materials and then aliquot to multiple tubes rather than pipetting separate tubes individually during reproducibility studies

- Who can do (or should do) validation...
  - Outside contractor?
  - Summer intern?
  - Trainee?
  - Qualified DNA analyst

From a validation standpoint, having an outside group perform the validation studies on your instruments is legitimate, but valuable experience and knowledge are lost...

Steps Surrounding “Validation” in a Forensic Lab

- Installation – purchase of equipment, ordering supplies, setting up in lab
- Learning – efforts made to understand technique and gain experience troubleshooting; can take place through direct experience in the lab or vicariously through the literature or hearing talks at meetings
- Validation of Analytical Procedure – tests conducted in one’s lab to verify range of reliability and reproducibility for procedure
- SOP Development – creating interpretation guidelines based on lab experience
- QC of Materials – performance check of newly received reagents
- Training – passing information on to others in the lab
- Qualifying Test – demonstrating knowledge of procedure enabling start of casework
- Proficiency Testing – verifying that trained analysts are performing procedure properly over time

Outline

- The NIST 26plex assay
- Uses of the assay
- Internal Validation

Validation Workshop

26plex Internal Validation

Dr. Peter M. Vallone
US National Institute of Standards and Technology
23rd World Congress
ISFG Buenos Aires
September 15th, 2009

Aren’t the Current STR Loci Good Enough?

- For general forensic matching of evidence to suspect, core STR loci are usually sufficient
  - e.g. the 13 CODIS U.S. core loci
- For other human identity/relationship testing questions, more autosomal loci can be beneficial or even necessary

http://www.cstl.nist.gov/biotech/strbase/training.htm
More Loci are Useful
in Situations Involving Relatives

- Missing Persons and Disaster Victim Identification (kinship analysis)
- Immigration Testing (often limited references)
  - Recommendations for 25 STR loci
- Deficient Parentage Testing
  - often needed if only one parent and child are tested

Relationship testing labs are being pushed to answer more difficult genetic questions

Additional loci were originally selected as candidates for miniSTR assays

- Certain CODIS and existing kit loci are not amenable to miniSTR assay design
  - Large allele range (FGA)
  - STR flanking region sequence that results in larger amplicons (D7S820 and D21S11)
- In 2004 - 2005 Dr. Mike Coble performed a survey of autosomal STRs to find candidate loci
  - Heterozygosity > 0.7
  - Moderate allele range (= low mutation rates)
  - Tri & Tetra nucleotide repeat motifs
  - Not linked to CODIS/kit loci

26 candidates were selected and termed NC for non-CODIS/Core loci

NC Miniplexes

<table>
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<tr>
<th>NC01</th>
<th>NC02</th>
<th>NC03</th>
<th>NC04</th>
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<tr>
<td>D10S1248</td>
<td>D1S1677</td>
<td>D3S3053</td>
<td>D1GATA113</td>
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<tr>
<td>D14S1434</td>
<td>D2S441</td>
<td>D6S474</td>
<td>D2S1776</td>
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<td>D4S2364</td>
<td>D20S482</td>
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<td><strong>NC07</strong></td>
<td><strong>NC08</strong></td>
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<td>D1S1627</td>
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<td><strong>D14S1280</strong></td>
<td><strong>D20S1082</strong></td>
</tr>
</tbody>
</table>

4 Loci removed because they were problematic: 30 – 4 = 26!!

26 New STR Loci for Human Identity Testing

Initial miniSTR work
- Small multiplex assays developed (10 miniplexes)
- Intended for use on degraded samples
- Sensitivity down to 100 pg (with 30 cycles)

Utility of miniplexes
- Degraded DNA
- Low copy number analysis

US NIST Standard Reference Materials
- The 26 loci are certified for NIST SRM 2391b

Why Build a Large Multiplex?

- Primer Concordance Checks/Null Alleles
- Determine Mutation Rates
- Immigration Testing
- Missing Persons/ Mass Fatalities
- For use with Reference Samples
- Complex Criminal Paternity Testing
- Kinship Analysis

Reference Multiplex

- Goal: to type all 26 loci in a single reaction
- 65 to 400 base pair amplicons
- Majority of PCR primers redesigned
  - no longer miniSTRs
- D8S1115 was omitted from the final reference multiplex
- 26plex = 25 STRs + Amelogenin

http://www.cstl.nist.gov/biotech/strbase/training.htm
26plex Schematic

Loci using the original miniSTR primer sets

Developmental Validation

- Vary number of cycles during amplification
- Optimize annealing temperature
- Vary post PCR soak time (adenylation)
- Optimize primer pair concentration
- Perform sensitivity study
- Determine mutation rates
- Determine genotype concordance with data obtained from miniplex primer sets


PCR Primers and Concentrations

- 52 primers
- 26 dye labeled
- 6FAM (7)
- VIC (6)
- NED (7)
- PET (6)
- Purchased from Applied Biosystems
- Primer concentrations specified in the paper
- Range 0.75 to 6 μM
- Hydrated in low salt 10 mM Tris-HCl 0.1 mM EDTA buffer (to suppress dye artifacts)

PCR Conditions

- Master Mix (final concentrations listed)
  - 2 mM MgCl₂
  - 1x PCR Buffer (supplied with Taq Gold)
  - 1 Unit TaqGold
  - ~0.2 μM Primer mix (varies by locus)
  - 250 mM dNTPs
  - 0.16 mg/mL BSA
  - 20 μL reaction volume
  - target input DNA ~1 ng

Thermal Cycling Conditions

Conditions for GeneAmp 9700 (9600 emulation mode)

- 95°C Hot Start for 11 min
- 30 cycles
  - 94°C for 45 sec Denaturation
  - 59°C for 2 min Annealing
  - 72°C for 1 min Elongation ~3.5 hours
- 60°C soak for 60 min
- 25°C hold
CE Conditions

- Amplification products were diluted in Hi-Di formamide and GS500-LIZ internal size standard.
- Analyzed on the 16-capillary ABI Prism 3130xl Genetic Analyzer.
- Prior to electrophoresis, a 5-dye matrix was established under the “G5 filter” with the five dyes of 6FAM, VIC, NED, PET, and LIZ.
- POP-6 polymer was utilized for separations on a 36 cm array.
- Samples were injected electrophoretically for 10 sec at 3 kV.
- Fragments separated at 15 kV at a run temperature of 60°C.
- Data analyzed using GeneMapperID v3.2.
- Bins and panels for the multiplex are available on STRBase.

Example Use of the Assay

- Kinship Testing.
- Samples were typed with Identifiler and the NIST assay.
- Note: at the time of this analysis the assay was only a 23plex (22 STRs + Amelogenin).

Comparison of Likelihood Ratios

<table>
<thead>
<tr>
<th>Relationship Examined</th>
<th>15 STRs (Identifiler, ID15)</th>
<th>ID15 + 22 NC STRs = 37 loci (A37)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mother/Child* (with single mutation)</td>
<td>0.214</td>
<td>5,200,000 Extra loci help...</td>
</tr>
<tr>
<td>Sister</td>
<td></td>
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<tr>
<td>Uncle/Niece</td>
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<tr>
<td>Cousin</td>
<td>0.45</td>
<td>2.25</td>
</tr>
<tr>
<td>Grandparents/Grandchildren</td>
<td>0.53</td>
<td>1.42</td>
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</table>

Conclusions: Longer distance multi-generational questions cannot usually be solved with additional autosomal STRs...

Extended Family Sample Testing

For more on the uses of the 26plex see Poster P-022

"Uses of the NIST 26plex STR Assay for Human Identity Testing" Peter M. Vallone, Carolyn R. Hill, Kristen E. Lewis, Toni M. Diegoli, Michael D. Coble, and John M. Butler

Use of the 26plex in Your Lab?

Perform an Internal Validation

- Review the literature on the 26plex assay
- Purchase primers
- TaqGold polymerase + buffers
- Prepare primer mix
  - Proper concentrations (follow paper)
  - Use a low salt tris buffer (dyes)
- Use the NIST SRM (9947A & 9948)
Revised Validation Guidelines
Scientific Working Group on DNA Analysis Methods (SWGDAM)

3. Internal Validation: The internal validation process should include the studies detailed below encompassing a total of at least 50 samples. Some studies may not be necessary due to the method itself.

3.1 Known and nonprobative evidence samples: The method must be evaluated and tested using known samples and, when possible, authentic case samples; otherwise, simulated case samples should be used. DNA profiles obtained from questioned items should be compared to those from reference samples. When previous typing results are available, consistency as to the inclusion or exclusion of suspects or victims within the limits of the respective assays should be assessed. – 12 component NIST SRM + 2 sensitivity study + 2 mixture = 16 samples

3.2 Reproducibility and precision: The laboratory must document the reproducibility and precision of the procedure using an appropriate control(s).

Experiments

71 amplification reactions
16 unique samples
8 injections on 3130

<table>
<thead>
<tr>
<th>Sample</th>
<th>Component 1</th>
<th>Component 2</th>
<th>Component 3</th>
<th>Component 4</th>
<th>Component 5</th>
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<td>SRM_07</td>
<td>Mix 1_0</td>
</tr>
</tbody>
</table>

Mixture

Sensitivity

2 samples
5 dilutions
triplicate

Concordance Study

NIST SRM 2391b

- 12 components in SRM 2391b
  - 9947A and 9948
- Material certified for the 25 STR loci
  - as of 2008
- 25 STRs X 12 samples = 300 genotypes
- 1 discordant allele call (drop out) 99.7% concordance

9947A

http://www.cstl.nist.gov/biotech/strbase/training.htm
Component 1 of NIST SRM 2391b

Sample Name

Component 1

<table>
<thead>
<tr>
<th>X,Y</th>
<th>14,14</th>
<th>13,13</th>
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Sample Name

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<th>12,12</th>
<th>9,10</th>
<th>10,10</th>
<th>14,17</th>
<th>8,8</th>
</tr>
</thead>
</table>

Genotypes for some common samples

http://www.cstl.nist.gov/biotech/strbase/miniSTR/miniSTR_NC_loci_types.htm

• Performed during developmental validation (~2007)
• 639 samples compared
• 14,058 total types (639 x 22 STR loci)
• 28 types discordant (0.20%)
• 99.80% concordance
• Discordance has not yet been confirmed by sequencing

http://www.cstl.nist.gov/biotech/strbase/NullAlleles.htm
Sensitivity Study

- Run 2 unique samples in triplicate
  1. 1 ng
  2. 0.5 ng
  3. 0.25 ng
  4. 0.125 ng
  5. 0.060 ng
- Sample concentration determined with Quantifiler prior to sensitivity study

Serial Dilution

- Prepare serial dilution to use 2 μL volume per PCR reaction
- Prepare 20 μL of each concentration point (enough volume to run triplicate experiments)
- Example for stock sample 4.5 ng/μL

<table>
<thead>
<tr>
<th>ng in 2 μL</th>
<th>ng/μL</th>
<th>Stock conc</th>
<th>Vol to add (μL)</th>
<th>Water</th>
<th>Total Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>4.5 ng/μL</td>
<td>2.2</td>
<td>17.8</td>
<td>20</td>
</tr>
<tr>
<td>500</td>
<td>0.25</td>
<td>0.5 ng/μL</td>
<td>10</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>250</td>
<td>0.125</td>
<td>0.25 ng/μL</td>
<td>10</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>125</td>
<td>0.0625</td>
<td>0.125 ng/μL</td>
<td>10</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>60</td>
<td>0.03</td>
<td>0.0625 ng/μL</td>
<td>9.6</td>
<td>10.4</td>
<td>20</td>
</tr>
</tbody>
</table>

http://www.cstl.nist.gov/biotech/strbase/training.htm
Sensitivity Study Conclusions

- The 26plex assay provides full profiles down to 125 pg of pristine DNA template
- Partial profiles with > 20 loci are obtained down to 60 pg
- Remember: quality of sample will effect assay performance

Mixture Study

- We are primarily using the 26plex for databasing single source samples
- Performing a minimal mixture study with 2 unique samples
- Mixture ratios: $0:1$, $1:9$, $1:3$, $1:1$, $3:1$, $9:1$, $1:0$

1:1 Mixture

Comparing Controls to 1:1 mixture

>2 alleles observed at 16/25 loci
Peak imbalance at other heterozygous loci

http://www.cstl.nist.gov/biotech/strbase/training.htm
Mixture Study Conclusions

- The 26plex is capable of detecting a mixture ratio of 1:1 and 3:1

- At 9:1 the minor alleles are not called (detection threshold 50 RFUs)

- The assay is fit for our purposes - running single source reference samples (but we should be able to detect a significant mixture)

Negative Controls

- D4S2364 exhibits artifacts (~80 RFUs)

- Dye artifacts below 50 FRUs

Qualifying Run

- Someone else (qualified person!) in the lab should run the assay on the same samples used in the validation experiments
  - Provided analyst with 26plex primer mix and assay protocol

- 12 components of the NIST SRM 2391b

- 100% concordance was observed with previously called genotypes

http://www.cstl.nist.gov/biotech/strbase/training.htm
Analysis Software

- Currently under development at NIST by Dr. David Duewer
- Performs calculations for
  - Allele frequencies
  - Intralocus signal balance (heterozygotes)
  - Interlocus signal balance ('multiplex balance')
  - Stutter
- Enables rapid analysis of internal validation data

Program Data Input

- Tables are exported from Genemapper Format:
  - Allele calls
  - Peak heights
- Data formatted in Excel
- Data is read by the program

Peak Height Ratios
Peak Height Ratios

• An example data table

<table>
<thead>
<tr>
<th>Locus</th>
<th>Δbp</th>
<th>#</th>
<th>X</th>
<th>s(X)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D10S1248</td>
<td>4</td>
<td>11</td>
<td>0.82</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td></td>
<td>0.83</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>5</td>
<td>0.89</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>1</td>
<td>0.87</td>
<td>na</td>
</tr>
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<table>
<thead>
<tr>
<th>Locus</th>
<th>Δbp</th>
<th>#</th>
<th>X</th>
<th>s(X)</th>
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Stutter

• An example data table

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<th>S Height</th>
<th>P Height</th>
<th>S/P Ratio</th>
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<td>261.52</td>
<td>265.51</td>
<td>103</td>
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<tr>
<td>5_a</td>
<td>15</td>
<td>371.52</td>
<td>375.43</td>
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<tr>
<td>3_a</td>
<td>16</td>
<td>265.42</td>
<td>269.39</td>
<td>127</td>
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<td>11_a</td>
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<td>265.46</td>
<td>90</td>
</tr>
<tr>
<td>9_a</td>
<td>15</td>
<td>261.44</td>
<td>265.40</td>
<td>161</td>
</tr>
<tr>
<td>2_a</td>
<td>15</td>
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<td>265.44</td>
<td>143</td>
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<tr>
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<td>257.50</td>
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<td>253.57</td>
<td>257.50</td>
<td>184</td>
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Avg                  | 7.514    |
Std                  | 2.331919 |

Interlocus Balance

• Signal intensity between loci

• Qualitatively described as ‘balance’ of the multiplex

• The cumulative signal is normalized to 1 and the fractional contribution of each locus is calculated

Interlocus Balance

To what extent is each locus contributing to the overall signal of the multiplex?
Sizing Precision

We are not using allelic ladders – allele calls are made by sizing.

Error bars not shown...too small

Each Allele

N = 132 alleles

Sizing Precision

Average variation in sizing (0.11 bp)

Size Deviation (bp)

Allele Size (bp)

26plex Bins and Panels

• For Genemapper IDv3.2

• Written for POP4 and POP6

• We can provide the bins and panels on STRBase, but you must check them...
  – Use 9947A & 9948

26plex Data from Collaborator (POP6)

Some of the 26plex allele peaks fall outside of our original bins

After a lab performs the internal validation the bins and panels can be adjusted

Adjust bins for different separation polymers, instrument performance, laboratory environment, etc.

Experiments Day 1

• 12 SRM components for Concordance
  – Samples set up in 8-strip tubes
  – After confirming that data is on scale and that the assay is successfully performing the concordance samples can be injected 2 more times (overnight) for Precision (allele sizing)

http://www.cstl.nist.gov/biotech/strbase/str26plex.htm#Bins-and-Panels
Experiments Day 2

- 2 samples are amplified in triplicate for sensitivity study
- The mixture study samples are amplified

Experiments Day 3

- A qualified analyst amplified the NIST SRM 2391b (12 components)

Alternative Approach...

- Set up all the experiments on one 96-well sample plate (except the qualifying run)

Data Analysis

The programs for data analysis are still under development, but the following information can be tabulated:
- Stutter for each locus (and allele size)
- Heterozygote balance at each locus
- Interlocus balance (multiplex balance)
- Precision (sizing reproducibility)
- Concordance (allele drop out?)
- Sensitivity (down to 125 ng)
- Mixture (a 3:1 mixture can be detected)
- Qualifying run (concordance)

Conclusions

- The performance for this lot of 26plex primer mix has been characterized
- The same internal validation will be performed when a new lot of primer mix is prepared
  - Compared to previous lot performance
- The validation took about 3 days
  - The software tools greatly speed up the data analysis process

Some Other Examples

http://www.cstl.nist.gov/biotech/strbase/training.htm
Example: PowerPlex 16

- Switch from ProfilerPlus/COFiler kits to PowerPlex 16
- Retaining same instrument platform of ABI 310

Recommendations:
- Concordance study (somewhat, but better to review literature to see impact across a larger number of samples and which loci would be expected to exhibit allele dropout—e.g., D5S818)
- Stutter quantities, heterozygote peak height ratio
- Some sensitivity studies and mixture ratios
- Do not need precision studies to evaluate instrument reproducibility

Example: ABI 3130

- Evaluation of a new ABI 3130 when a laboratory already has experience with ABI 310
- STR kits used in lab will remain the same

Recommendations:
- Precision studies to evaluate instrument reproducibility
- Sensitivity studies
- Do not need new stutter, mixture ratio, peak height ratio, etc. (these relate to dynamics of the kit used)

Instrument/Software Upgrades or Modifications

- What should be done to “validate” new upgrade?
  - ABI 7000 to ABI 7500
  - ABI 3100 to ABI 3130xl
  - GeneScan/Genotyper to GeneMapper ID

- Try to understand what is different with the new instrument or software program compared to the one you are currently using (e.g., ask other labs who may have made the switch)

- If possible, try to retain your current configuration for comparison purposes for the validation period

run the same plate of samples on the original instrument/software and the new one

ABI 3130xl vs ABI 3100

What NIST did to “validate” a 3130xl upgrade

- Ran plates of samples on both instruments with same injection and separation parameters and compared results
  - Data Collection version 1.0.1 (3100) vs 3.0 (3130xl)
  - POP-6 (3100) vs POP-7 (3130xl)
  - 36 cm array (3100) vs 50 or 80 cm array (3130xl)

- Ran several plates of Identifier samples and compared allele calls (noticed a sensitivity difference with equal injections and relative peak height differences between dye colors) — all obtained allele calls were concordant

- Ran a plate of Profiler Plus samples and compared sizing precision — precision was not significantly different

- Also examined SNaPshot products and mtDNA sequencing data — is the new instrument “fit for purpose”?

Environmental conditions may change over time so original validation is no longer valid...

Suggestions for a Internal Validation of an STR Kit

- Standard samples (3.1) Between 1 and ~20 samples
  - Verify correct type with positive control or NIST SRM samples
  - Concordance study with 5-10 (non-probative casework) samples previously typed with other kit(s)

- Precision samples (3.2) 5-10 samples
  - Run at least 5-10 samples (allelic ladder or positive control)

- Sensitivity samples (3.4) 14 samples
  - Run at least 2 sets of samples covering the dynamic range
  - 5 ng down to 50 pg—e.g., 5, 2, 1, 0.5, 0.2, 0.1, 0.05 ng

- Mixture samples (3.5) 10 samples
  - Run at least 2 sets of samples
  - Examine 5 different ratios—e.g., 10:1, 3:1, 1:1, 1:3, 1:10

>50 samples

Additional Suggestions for Meeting the SWGDAM Revised Validation Guidelines

- Match Criteria (3.3)
  - As part of running a batch of samples (e.g., 10 or 96), run one allelic ladder at the beginning and one at the end
  - If all alleles are typed correctly in the second allelic ladder, then the match criteria (i.e., precision window of +/-0.5 bp) has likely been met across the entire size range and duration of the run

- Contamination Check (3.6)
  - Run negative controls (samples containing water instead of DNA) with each batch of PCR products

- Qualifying Test (3.7)
  - Run proficiency test samples

http://www.cstl.nist.gov/biotech/strbase/training.htm
Use of Second Allelic Ladder to Monitor Potential Match Criteria Problems

These alleles have drifted outside of their genotyping bins due to temperature shifting over the course of the sample batch.

Appropriate Documentation...

- Publications in the Peer-Reviewed Literature
  - See provided reference list
- In terms of documentation, is the community doing too much? Too little?
  - Benefit of STRBase Validation website
- Should we be requesting more information from the manufacturers of commercial kits in terms of developmental validation studies?

Why is Documentation of Validation Important?

9. Documentation of Validated Methods

9.1 Once the validation process is complete it is important to document the procedures so that the method can be clearly and unambiguously implemented. There are a number of reasons for this. The various assessments of the method made during the validation process assume that, in use, the method will be used in the same way each time. If it is not, then the actual performance of the method will not correspond to the performance predicted by the validation data. Thus the documentation must limit the scope for introducing accidental variation to the method. In addition, proper documentation is necessary for auditing and evaluation purposes and may also be required for contractual or regulatory purposes.

9.2 Appropriate documentation of the method will help to ensure that application of the method from one occasion to the next is consistent.

Validation Summary Sheet for PowerPlex Y

<table>
<thead>
<tr>
<th>Study</th>
<th>Validation Type</th>
<th>Description of Samples Tested</th>
<th>Run(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single Source (Concentration)</td>
<td>5 samples at 5 data points</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>Mutant Ratio (male/female)</td>
<td>6 tubes at 2.5 x 10e6 copies x 110 ratios (10, 20, 50, 100, 250, 500, 1000, 2500, 5000, 10000)</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td>Mutant Ratio (male/male)</td>
<td>6 tubes at 2.5 x 10e6 copies x 110 ratios (10, 20, 50, 100, 250, 500, 1000, 2500, 5000, 10000)</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td>Sensitivity</td>
<td>7 tubes at 2 x 10e6 copies</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>Non-Human</td>
<td>20 human samples</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>NIST SRM</td>
<td>4 compounds of SRM 2260</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Precision (ABI 3700 and ABI 377)</td>
<td>105 samples replicated + 8 samples for ABI 377</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Non-Probative Cases</td>
<td>60 cases with 123 samples</td>
<td>103</td>
<td></td>
</tr>
<tr>
<td>Promoter</td>
<td>412 males tested</td>
<td>412</td>
<td></td>
</tr>
<tr>
<td>Peak Height Ratio</td>
<td>N/A (except for DYS385 but no studies were noted)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycling Parameters</td>
<td>5 cycles (30/30/18/18/18) &amp; 9 annealing/9 extension sizes x 2 samples</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>Annealing Temperature</td>
<td>5 cycles (30/30/18/18/18) &amp; 9 annealing/9 extension sizes x 1 sample</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Reaction volume</td>
<td>5 volumes (50/25/15/12.5/6.25) &amp; 5 amounts + 5 concentrations</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Thermal cycling test</td>
<td>5 replicates + 3 sets of 6 samples</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>Mike specificity</td>
<td>2 tubes at x 1 dilution range (250 ng/male DNA) &amp; 5 amounts each</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>TaqGold polymerase titration</td>
<td>6 amounts (0.05/0.1/0.2/0.5/1.0/10 ng) &amp; 5 concentrations</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Primer pair titration</td>
<td>6 amounts (0.05/0.1/0.2/0.5/1.0/10 ng) &amp; 5 concentrations</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Allele dropout</td>
<td>6 amounts (0.05/0.1/0.2/0.5/1.0/10 ng)</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Allele drop-in</td>
<td>6 amounts (0.05/0.1/0.2/0.5/1.0/10 ng) &amp; 5 concentrations</td>
<td>26</td>
<td></td>
</tr>
</tbody>
</table>


Example of Validation Documentation


Laboratory Internal Validation Summaries

We can benefit from cumulative experience in the field rather than just single lab results...

Soliciting Information on Studies Performed by the Community

We can benefit from cumulative experience in the field rather than just single lab results...

**Example of Validation Documentation**

Alabama Department of Forensic Sciences

Birmingham DNA

**TABLE OF CONTENTS**

1. Introduction and Objectives
2. Materials and Methods
3. Results
4. Conclusion

Implementation of the Newly Validated Procedure

Ok, the validation studies are complete and approved, the procedure is written and approved and the lab is ready to implement the new procedure into casework. What about training? Who needs to be trained and what is the extent of the training? How is the training documented? What constitutes completion of training? Per individual or per lab?

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)


**Acknowledgments**

- **NIJ Funding** through the NIST Office of Law Enforcement Standards
- **Robyn Ragsdale (FDLE)**

This presentation will be available at: [http://www.cstl.nist.gov/biotech/strbase/training.htm](http://www.cstl.nist.gov/biotech/strbase/training.htm)