Validation Workshop

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NIST and NIJ Disclaimer

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I do not endorse Applied Biosystems' products although I do use them…

My Background

- PhD **(Analytical Chemistry)** from University of Virginia (Aug 1995)
- Research conducted at FBI Academy under Bruce McCord doing CE for STR typing
- NIST Postdoc – developed STRBase website
- GeneTrace Systems – private sector experience validating assays
- NIST Human Identity Project Leader since 1999
- Invited guest to SWGDAM since 2000
- **Member of SWGDAM Validation Subcommittee**
- Served on WTC KADAP and helped evaluate and validate new miniSTR, mtDNA, and SNP assays
- Author of *Forensic DNA Typing: Biology, Technology, and Genetics of STR Markers* (2nd Edition)
- Married with 6 children – I have "validated" that they are mine using STR typing…

Workshop Goal

To improve participants understanding of the value of validation and how to perform forensic DNA validation studies in a practical and efficient fashion

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"…

Presentation Outline

Theory
- Importance of Validation
- Validation Philosophy & Resources

BREAK
- History of Forensic Validation Guidelines
- **SWGDAM Revised Validation Guidelines**
- Summary of Literature & 2004 Validation Survey

BREAK

Practice
- Practical Examples
- Documentation

Validation Workshop (Aug 24-26, 2005 at NFSTC)

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

COURSE CONTENTS

Day #1
- Validation Overview (John)
- Introduction to DAB Standards (Robyn & John)
- Developmental Validation (John)

Day #2
- Inconsistency in Validation between Labs (John)
- Internal Validation (Robyn)
- Method Modifications and Performance Checks (Robyn)

Day #3
- Practical Exercises (Robyn)

Was filmed and is being made into a training DVD as part of the President's DNA Initiative…

Importance of Validation

Overview of the Theory Section

- 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?
- Define accuracy, precision, sensitivity, stability, reproducibility, and robustness as applied to general measurements

What is **Validation** and Why Should It Be Done?

- Part of overall quality assurance program in a laboratory
- **We want the correct answer when collecting data…**
  - We want **analytical measurements made in one location to be consistent with those made elsewhere** (without this guarantee there is no way that a national DNA database can be successful).
- If we fail to get a result from a sample, we want to have confidence that the sample contains no DNA rather than there might have been something wrong with the detection method…

Want no false negatives…

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**.

Roper, P., *et al.* (2001) *Applications of Reference Materials in Analytical Chemistry*. Royal Society of Chemistry, Cambridge, UK, pp. 107-108.

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.

EURACHEM Guide (1998) *The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics*; available at http://www.eurachem.ul.pt/guides/valid.pdf

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

J.M. Butler (2005) *Forensic DNA Typing*, 2nd Edition, p. 389, 391

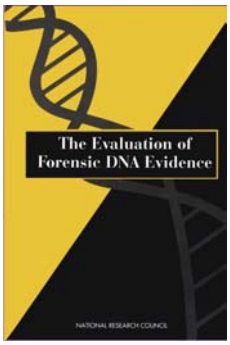
- **Quality assurance** (QA) – planned or systematic actions necessary to provide adequate confidence that a product or service will satisfy given requirements for quality
- **Quality control** (QC) – day-to-day operational techniques and activities used to fulfill requirements of quality
- **Validation** – 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

J.M. Butler (2005) *Forensic DNA Typing*, 2nd Edition, p. 391

- **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

NRC II Recommendation 3.1



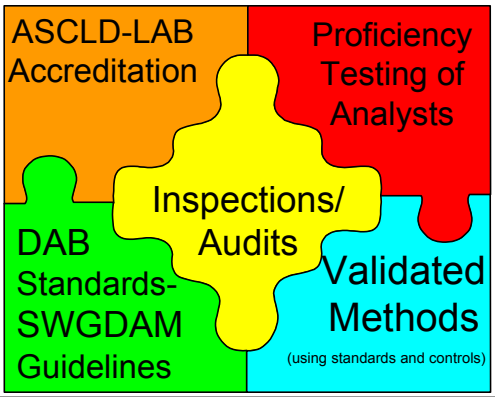
- Laboratories should adhere to high quality standards (such as those defined by TWGDAM and the DNA Advisory Board) and make every effort to be accredited for DNA work (by such organizations as ASCLD-LAB).

Some Desirable QC and QA Guidelines

Noted in NRC I pp. 104-105

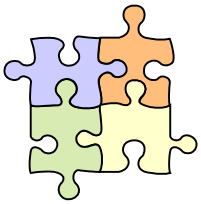
- Reagents and equipment are properly maintained and monitored.
- **Procedures used are generally accepted in the field and supported by published, reviewed data that were gathered and recorded in a scientific manner**.
- Appropriate controls are specified in procedures and are used.
- **New technical procedures are thoroughly tested to demonstrate their efficacy and reliability** for examining evidence material before being implemented in casework.

Ensuring Accurate Forensic DNA Results



Elements for Guaranteeing Quality Results in Forensic DNA Testing

- Accepted Standards and Guidelines for Operation
- Laboratory Accreditation
- Proficiency Testing of Analysts
- Standard Operating Procedures
- **Validated Methods**
- Calibrated Instrumentation
- Documented Results
- Laboratory Audits
- **Trustworthy Individuals**



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

L. Huber (2001) Validation of Analytical Methods: Review and Strategy. Supplied by www.labcompliance.com

Costs/Benefits of Validation and Quality Assurance

Costs

- Direct
  - Test materials
  - Standards
  - Quality assurance equipment
  - Analysis of QA/QC samples
  - Quality assurance official
  - Committee Work
  - Interlab Studies
  - Travel to meetings

Benefits

- More efficient outputs
- Fewer replicates for same reliability
- Fewer do-overs
- Greater confidence of:
  - Staff
  - Laboratory
  - Customers

Table 26.2 in J.K. Taylor (1987) *Quality Assurance of Chemical Measurements*. Lewis Publishers: Chelsea, MI.

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…

J.K. Taylor (1987) *Quality Assurance of Chemical Measurements*. Lewis Publishers: Chelsea, MI, p. 193

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.

Roper P *et al.* (2001) *Applications of Reference Materials in Analytical Chemistry*. Royal Society of Chemistry: Cambridge UK, p. 2

Community Needs 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

Pathway to Improved DNA Validation

- Collection of Current Philosophy on Validation
  - Community survey
  - Interviews
  - Literature summary
- Training **VALIDATION WORKSHOP**
  - Auditors must be consistent in treatment of labs
- Providing Tools to Enable Improved Validation
  - Sample set(s)
  - Workbook – provide specific examples
  - Standard report form – documentation standardization
- Collection of Validation Data from Labs
  - NIJ-funded labs to submit data to STRBase validation website

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

Roper, P., *et al.* (2001) *Applications of Reference Materials in Analytical Chemistry*. Royal Society of Chemistry, Cambridge, UK, pp. 108-109.

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.

Roper, P., *et al.* (2001) *Applications of Reference Materials in Analytical Chemistry*. Royal Society of Chemistry, Cambridge, UK, pp. 110-111.

Tools of Method Validation

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

Roper, P., *et al.* (2001) *Applications of Reference Materials in Analytical Chemistry*. Royal Society of Chemistry, Cambridge, UK, p. 110.

SWGDAM Revised Validation Guidelines

Section 1.1 Validation is the process by which the scientific community acquires the necessary information to

(a) Assess the ability of a procedure to obtain reliable results.

(b) Determine the conditions under which such results can be obtained.

(c) Define the limitations of the procedure.

The validation process identifies aspects of a procedure that are critical and must be carefully controlled and monitored.

Reliability, Reproducibility, Robustness, Range

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:

Manufacturer

  - (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.

Forensic Lab

Common Perceptions of Validation

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

"You cannot mimic casework because every case is different."

Effort

Lots of experiments are required

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

Time

Number of Samples Needed

Relationship between a sample and a population of data

How do you relate these two values?

Data collected in your lab as part of validation studies → All potential data that will be collected in the future in your lab

Student's *t*-Test associates a sample to a population

"Sample" of Typical Data

"Population" of All Data Obtained

Student's *t*-Tests

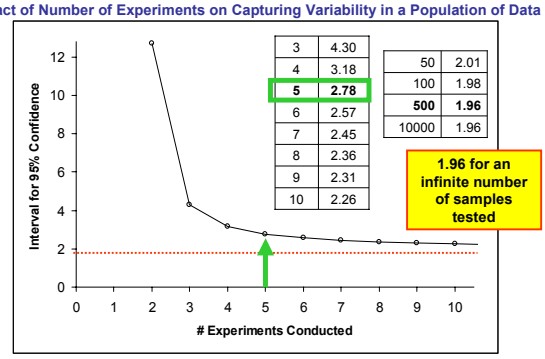
"Student" (real name: W. S. Gossett [1876-1937]) developed statistical methods to solve problems stemming from his employment in a brewery.

Student's *t*-test deals with the problems associated with inference based on "small" samples: the calculated mean (X_{avg}) and standard deviation (σ) 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

Student's t-Test Curve

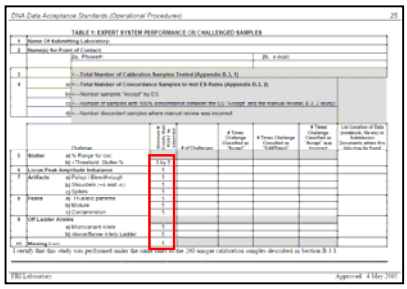
Impact of Number of Experiments on Capturing Variability in a Population of Data



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)



Allele Frequency Tables

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.

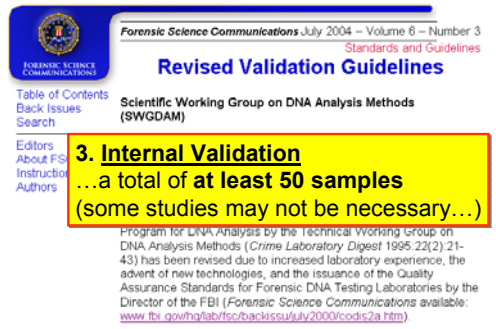
D3S1358	Allele	Caucasian (Butler *et al.* (2003) *JFS* 48(4):908-911) N= 302	Caucasian (Einum *et al.* (2004) *JFS* 49(6): 1381-1385) *N= 7,636*
	11	0.0017*	0.0009
	12	0.0017*	0.0007
	13	--	0.0031
Most common allele	14	0.1027	0.1240
	15	**0.2616**	**0.2690**
	15.2	--	--
	16	0.2533	0.2430
	17	0.2152	0.2000
	18	0.15232	0.1460
	19	0.01160	0.0125
	20	0.0017*	0.0001*

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

Revised SWGDAM Validation Guidelines
(July 2004)

http://www.fbi.gov/hq/lab/fsc/backissu/july2004/standards/2004_03_standards02.htm



The document provides validation guidelines and definitions approved by SWGDAM July 10, 2003.

General Steps for Internal Validation

- **Review literature and learn the technique**
- Obtain equipment/reagents, if necessary
- Determine necessary validation studies (there can be overlap and you only need to run a total of 50 samples)
- Collect/obtain samples, if necessary
- **Perform validation studies maintaining all documentation**
- Summarize the studies and submit for approval to Technical Leader
- Write-up the analytical procedure(s). Include quality assurance (controls, standards, critical reagents and equipment) and data interpretation, as applicable
- Determine required training and design training module(s)
- Design qualifying or competency test

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

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**

Steps Surrounding "Validation" in a Forensic Lab

Effort to Bring a Procedure "On-Line"

This is what takes the time…

- **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

Points for Consideration

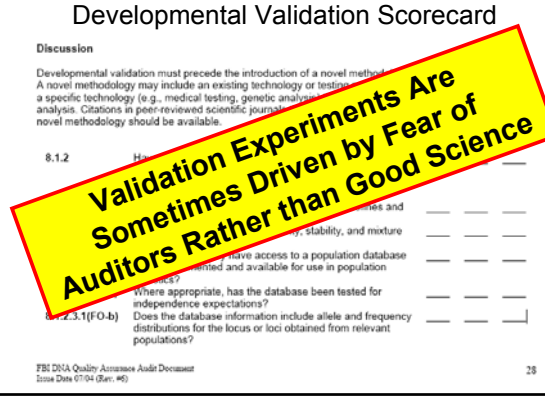
- Remove as many variables as possible in testing an aspect of a procedure
  - e.g., create bulk materials and then aliquot to multiple tubes rather than pipeting separate tubes individually during reproducibility studies
- Who can do (or should do) validation…
  - Outside contractor?
  - Summer intern
  - Trainee
  - Qualified DNA analyst

What are the goals of validation studies involving a new STR typing kit?

- Stutter product amounts
  Why?: aids in mixture interpretation guidelines (how often does laboratory call peaks below 15% of an adjacent allele?
- Precision studies
  Why?: aids in defining allele bin wind change the ±0.5 bp from th
- Sensitivity studies
  Why?: aid
- M of detecting the minor component
  hat new primer sets get the same results as original sets – potential of polymorphism causing allele dropout…
- eak height ratio studies
  Why?: aids in mixture interpretation guidelines (how often does your laboratory call peaks below a 60% heterozygote peak height ratio?)

Too often validation experiments are performed but observations are not considered for implementation purposes

FBI DNA Quality Assurance Audit Developmental Validation Scorecard



Discussion

Developmental validation must precede the introduction of a novel method
A novel methodology may include an existing technology or testing
a specific technology (e.g., medical testing, genetic analysis
analysis. Citations in peer-reviewed scientific journals
novel methodology should be available.

8.1.2 H

…nies and
…y, stability, and mixture
…have access to a population database
…ained and available for use in population
…ics?

8.1.2.3.1(FO-b) Where appropriate, has the database been tested for independence expectations?
Does the database information include allele and frequency distributions for the locus or loci obtained from relevant populations?

FBI DNA Quality Assurance Audit Document
Issue Date 07/04 (Rev. #6)
28

Validation Experiments Are Sometimes Driven by Fear of Auditors Rather than Good Science

Organizations Involved in International Quality Assurance Issues

- International Standards Organization (ISO) **ISO 17025**
  - http://www.iso.ch
- AOAC International (Association of Official Analytical Chemists)
  - http://www.aoac.org
- Eurachem
  - http://www.eurachem.ul.pt
- VAM (Valid Analytical Measurement)
  - http://www.vam.org.uk
- CCQM (Comité Consultatif pour la Quantité de Matière; Consultative Committee for Amount of Substance – Metrology in Chemistry)
  - http://www.bipm.org/en/committees/cc/ccqm/
- CITAC (Co-operation on International Traceability in Analytical Chemistry)
  - http://www.citac.cc

Organizations Involved in International Quality Assurance Issues

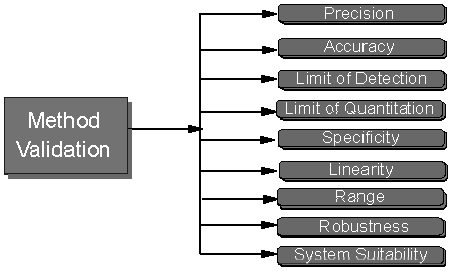
- ASTM International (American Society for Testing and Materials)
  - http://www.astm.org
- CLSI (Clinical and Laboratory Standards Institute)
  - http://www.clsi.org
- ANSI (American National Standards Institute)
  - http://www.ansi.org
- ILAC (International Laboratory Accreditation Cooperation)
  - http://www.ilac.org
- FDA (U.S. Food and Drug Administration)
  - http://www.fda.gov

ICH Validation Documents

- ICH (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use)
  - http://www.ich.org
  - **Q2A**: Text on Validation of Analytical Procedures (1994)
    - http://www.fda.gov/cder/guidance/ichq2a.pdf
  - **Q2B**: Validation of Analytical Procedures : Methodology (1996)
    - http://www.fda.gov/cder/guidance/1320fnl.pdf
- From Q2B:
  - "For the establishment of linearity, **a minimum of five concentrations is recommended**"
  - "Repeatability should be assessed using (1) **a minimum of 9 determinations covering the specified range for the procedure** (e.g., 3 concentrations/3 replicates each); or (2) a minimum of 6 determinations at 100 percent of the test concentration."

ICH Method Validation Parameters

http://www.waters.com/watersdivision/contentd.asp?watersit=JDRS-5LT6WZ



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."

Precision

- "The closeness of agreement between independent test results obtained under stipulated conditions."
- "Precision depends only on the distribution of random errors and does not relate to the true value or specified value. The measure of precision is usually expressed in terms of imprecision and computed as a standard deviation of the test results."
- "A measure for the reproducibility of measurements within a set, that is, of the scatter or dispersion of a set about its central value."

EURACHEM Guide (1998) *The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics*, p. 45; available at http://www.eurachem.ul.pt/guides/valid.pdf

Accuracy

- "The closeness of agreement between a test result and the accepted reference value."
- "Accuracy of a measuring instrument is the ability of a measuring instrument to give responses close to a true value."

EURACHEM Guide (1998) *The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics*, pp. 39, 41; available at http://www.eurachem.ul.pt/guides/valid.pdf

Sensitivity

- Limit of detection (**LOD**) – "the lowest content that can be measured with reasonable statistical certainty."
- Limit of quantitative measurement (**LOQ**) – "the lowest concentration of an analyte that can be determined with acceptable precision (repeatability) and accuracy under the stated conditions of the test."
- How low can you go?

EURACHEM Guide (1998) *The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics*, p. 43; available at http://www.eurachem.ul.pt/guides/valid.pdf

Threshold Settings for the ABI 310/3100

Detection Limit: 3x the standard deviation of the noise.
Estimated using **2x peak to peak noise**. (approximately 35 - 50 RFUs)

Limit of Quantitation: 10x the standard deviation of the noise
Estimated using **7x peak to peak noise** (150-200 RFUs)
Below this point estimates of peak area or height are unreliable.

Dynamic Range: The range of sample quantities that can be analyzed from the lowest to the highest **(linear range is also important)**

Stochastic Threshold: Level of quantifiable DNA below which peaks can show severe imbalance (peak height ratios below 60%) Approximately 150 -200 RFUs. Enhanced stutter also occurs at these signal levels.

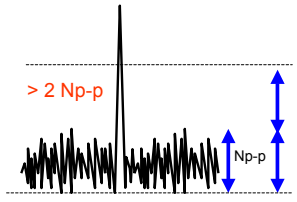
The Scientific Reasoning behind the Concept of an Analytical Threshold (limit of detection)

- This is fundamentally an issue of reliability
- **For a peak intensity three times the standard deviation of the noise there is a limited chance that such a signal is the result of a random fluctuation**
- This is because 99.7 percent of all noise signals fall below this value (from the definition of a Gaussian curve)
- **Below this point the very real possibility exists that what you think is a peak is simply a statistical fluctuation in the baseline noise**.

Limit of Detection (LOD)

- Typically 3 times the signal-to-noise (based on standard deviation of the noise) or 2x Np-p

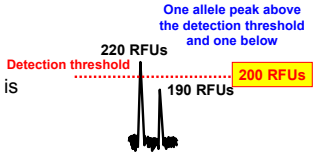
Is this peak real? *Yes, it is a peak but you cannot rely on it for concentration determinations as it is **not >10 S/N***



Types of Results at Low Signal Intensity
(Stochastic amplification potential)

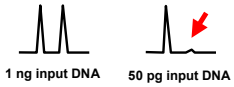
Straddle Data

- Only one allele in a pair is above the laboratory stochastic threshold



Allelic Drop-out

- one or more sets of alleles do not amplify



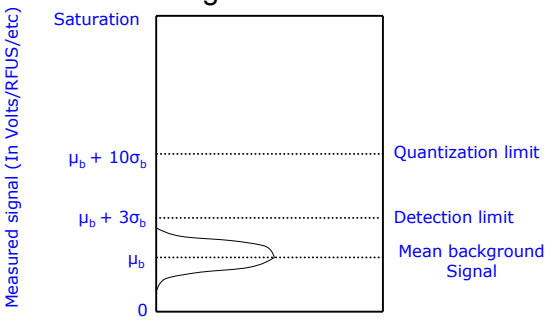
Forensic Bioinformatics – Defense Expert Attack on Detection Thresholds

Objective threshold determination

- The limit of detection is an extrapolated value.
- While easy to use, carte blanche thresholds make assumptions that may not be valid for a particular experiment/run.
- FBS study (currently unpublished)
  - Study characterizes noise signal in 42 runs taken from 7 cases analyzed by the FBI.
  - Each run contains a reagent blank, a positive control, and a negative control.
  - Output signal data was collected only from regions of the electropherogram free of analyte signal (positive control peaks, ROX peaks, +/-4 stutter) in all channels.
    - In-line reagent blanks/controls

Travis Doom, "Background Noise in STR Testing," Presentation at *The Science of DNA Profiling: A National Expert Forum*; Held at Wright State (Dayton, OH), August 12, 2005; available at http://www.bioforensics.com/conference05/Doom_BackgroundNoise.ppt

Signal Measure



Travis Doom, "Background Noise in STR Testing," Presentation at *The Science of DNA Profiling: A National Expert Forum*; Held at Wright State (Dayton, OH), August 12, 2005; available at http://www.bioforensics.com/conference05/Doom_BackgroundNoise.ppt

Study Results

Noise Characterization and Thresholds of Detection/Quantization (RFUs)

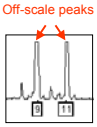
		μ	σ	μ + 3σ	μ + 10σ
Reagent Blank	**Run Type**				
	Maximum (Noisy)	15.4	6.65	35.4	81.9
	Average (n=43)	6.51	4.62	20.4	52.7
	Minimum	5.17	3.52	15.7	40.3
Negative Control	**Run Type**	μ	σ	μ + 3σ	μ + 10σ
	Maximum (Noisy)	16.3	24.5	89.9	262
	Average (n=43)	6.61	5.39	22.8	60.5
	Minimum	5.16	3.47	15.6	39.9
Positive Control	**Run Type**	μ	σ	μ + 3σ	μ + 10σ
	Maximum (Noisy)	15.4	6.00	33.4	75.4
	Average (n=43)	6.22	4.09	18.5	47.1
	Minimum	4.85	3.46	15.2	39.4

Travis Doom, "Background Noise in STR Testing," Presentation at *The Science of DNA Profiling: A National Expert Forum*; Held at Wright State (Dayton, OH), August 12, 2005; available at http://www.bioforensics.com/conference05/Doom_BackgroundNoise.ppt

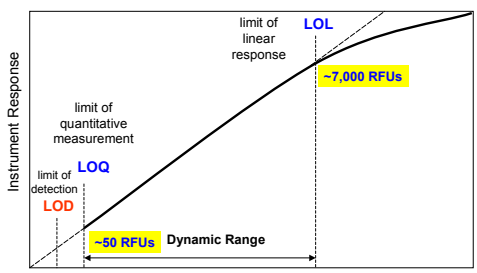
Limit of Linear Response (LOL)

- Point of saturation for an instrument detector so that higher amounts of analyte do not produce a linear response in signal
- In ABI 310 or ABI 3100 detectors, the CCD camera saturates leading to flat-topped peaks.

Off-scale peaks



Useful Range of an Analytical Method



LOD = 3x SD of blank
LOQ = 10x SD of blank

Adapted from Figure 1-7 in Skoog, D.A., *et al.* (1998) *Principles of Instrumental Analysis* (5th Edition). Thomson Learning, Inc.

Linearity and Range

- Linearity "defines the ability of the method to obtain test results proportional to the concentration of analyte."
- "The Linear Range is by inference the range of analyte concentrations over which the method gives test results proportional to the concentration of the analyte."
- Working range is a "set of values of measurands for which the error of a measuring instrument is intended to lie within specified limits."

EURACHEM Guide (1998) *The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics*, pp. 43, 46; available at http://www.eurachem.ul.pt/guides/valid.pdf

Specificity

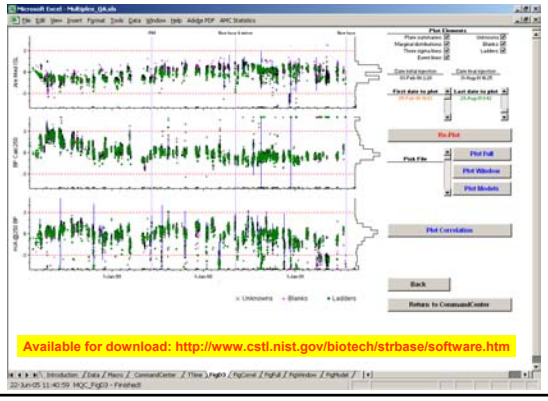
- "The ability of a method to measure only what it is intended to measure."
- "Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc."
- The primers in PCR amplification provide specificity in forensic DNA testing.

EURACHEM Guide (1998) *The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics*, p. 51; available at http://www.eurachem.ul.pt/guides/valid.pdf

Stability

- Will the method produce a result reliably over time?
- Control charts are an effective tool for monitoring stability and quality assurance over time
  - Dave Duewer at NIST has developed a software program called **Multiplex_QA** that permits a view of sensitivity and resolution of STR data in order to monitor instrument performance over time.
  - The program is available for download on the NIST STRBase website: http://www.cstl.nist.gov/biotech/strbase/software.htm

NIST Multiplex_QA Program for Monitoring Performance Over Time



Reproducibility

- "Precision under reproducibility conditions, i.e. conditions where test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment."
- Will you get the same result each time you test a sample?
- Different from **repeatability**, which is the "precision under repeatability conditions, i.e. conditions where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time."

EURACHEM Guide (1998) *The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics*, pp. 47-48; available at http://www.eurachem.ul.pt/guides/valid.pdf

Robustness (Ruggedness)

- "The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage."
- The method works routinely…
- You do not want the method to fail when you only have enough material for a single try.

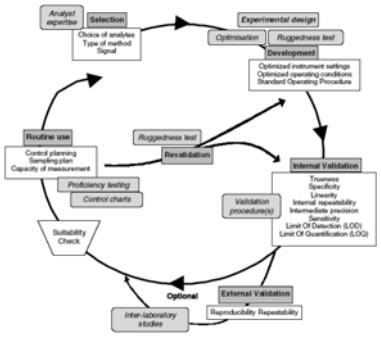
EURACHEM Guide (1998) *The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics*, p. 49; available at http://www.eurachem.ul.pt/guides/valid.pdf

System Suitability

- Fitness for purpose is the "degree to which data produced by a measurement process enables a user to make technically and administratively correct decisions for a stated purpose."

EURACHEM Guide (1998) *The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics*, p. 42; available at http://www.eurachem.ul.pt/guides/valid.pdf

The lifecycle of a method of analysis



Feinberg *et al.* (2004) *Anal. Bioanal. Chem.* 380: 502-514

How an Assay Evolves

NIJ-funded project or company efforts

Performed by manufacturer

- Research
- Development
- Optimization

Performed by forensic lab

- Pre-Validation — Learning what questions to ask
- Validation — Performance Check (Kit QC or Following Instrument Repair)
- Re-Validation
- Implementation — Writing SOP, Training Others and Going "On-Line"

Validation Resources

http://www.promega.com/profiles/403/ProfilesInDNA_403_14.pdf

CURRENT EVENTS

Requirements for Complete Validation of an STR Product

By Thomas J. Mozer, Ph.D.
Promega Corporation
tmozer@promega.com

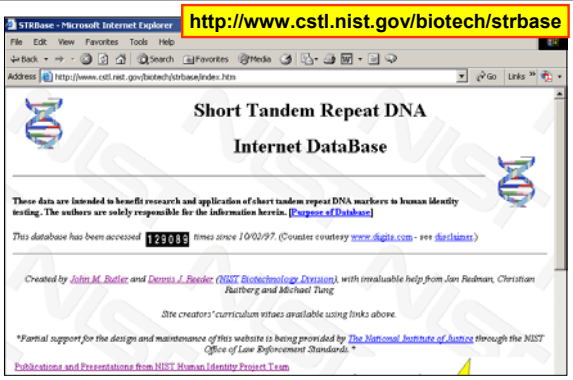
One of the inherent problems when developing a plan for product validation is that different stakeholders of the process have different views and agendas.

The subject of what constitutes proper validation of a DNA typing product has been a frequent point of discussion in the field of forensic science for well over a decade. The issue of validation has developed into a controversial problem for the field as a whole due to the varying opinions voiced by different members of the community. Occasionally, this controversy has spilled over into the courtroom as the judicial system wrestles with this issue (1,2). It is probable that these cases will eventually be resolved favorably and the results of DNA typing will be accepted in all courts. However, courtroom acceptance does not truly measure the success of a given validation procedure.

Validation of STR Systems Reference Manual by Promega Corporation
http://www.promega.com/techserv/apps/hmnid/referenceinformation/powerplex/ValidationManual.pdf

Written from the perspective of only validating a STR kit…
(in this case PowerPlex 16)

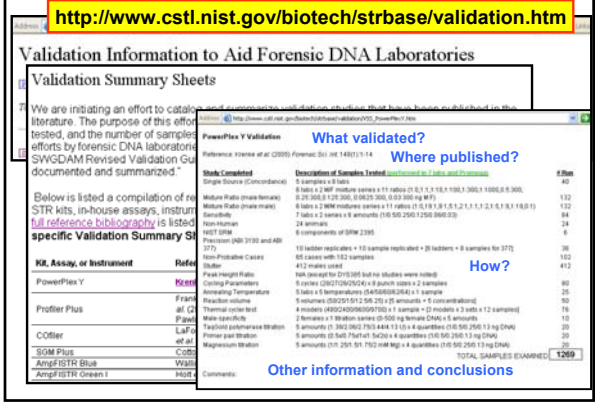
http://www.cstl.nist.gov/biotech/strbase



A Human Identity Testing Community Resource…

New Validation Homepage on STRBase

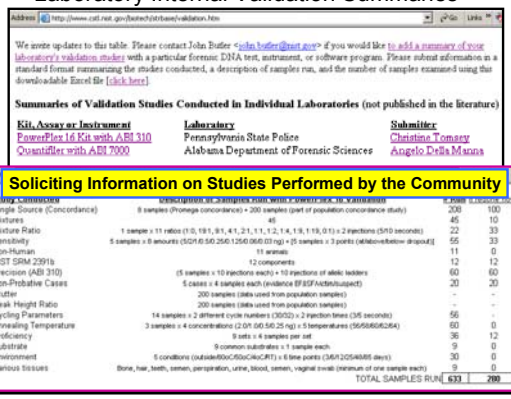
http://www.cstl.nist.gov/biotech/strbase/validation.htm



Validation Summary Sheet for PowerPlex Y

Study Completed (17 studies done)	Description of Samples Tested (performed in 7 labs and Promega)	# Run
Single Source (Concordance)	5 samples x 8 labs	40
Mixture Ratio (male:female)	6 labs x 2 M/F mixture series x 11 ratios (1:0,1:1,1:10,1:100,1:300,1:1000,0.5:300, 0.25:300,0.125:300, 0.0625:300, 0.03:300 ng M:F)	132
Mixture Ratio (male:male)	6 labs x 2 M/M mixtures series x 11 ratios (1:0, 19:1, 9:1, 5:1, 2:1, 1:1, 1:2, 1:5, 1:9, 1:19, 0:1)	132
Sensitivity	7 labs x 2 series x 6 amounts (1/0.5/0.25/0.125/0.06/0.03)	84
Non-Human	24 animals	24
NIST SRM	6 components of SRM 2395	6
Precision (ABI 3100 and ABI 377)	10 ladder replicates + 10 sample replicated + [8 ladders + 8 samples for 377]	36
Non-Probative Cases	65 cases with 102 samples	102
Stutter	412 males used	412
Peak Height Ratio	N/A (except for DYS385 but no studies were noted)	
Cycling Parameters	5 cycles (28/27/26/25/24) x 8 punch sizes x 2 samples	80
Annealing Temperature	5 labs x 5 temperatures (54/58/60/62/64) x 1 sample	25
Reaction volume	5 volumes (50/25/15/12.5/6.25) x [5 amounts + 5 concentrations]	50
Thermal cycler test	4 models (480/2400/9600/9700) x 1 sample + [3 models x 3 sets x 12 samples]	76
Male-specificity	2 females x 1 titration series (0-500 ng female DNA) x 5 amounts each	10
TaqGold polymerase titration	5 amounts (1.38/2.06/2.75/3.44/4.13 U) x 4 quantities (1/0.5/0.25/0.13 ng DNA)	20
Primer pair titration	5 amounts (0.5x/0.75x/1x/1.5x/2x) x 4 quantities (1/0.5/0.25/0.13 ng DNA)	20
Magnesium titration	5 amounts (1/1.25/1.5/1.75/2 mM Mg) x 4 quantities (1/0.5/0.25/0.13 ng DNA)	20
Krenke *et al.* (2005) *Forensic Sci. Int.* 148:1-14	TOTAL SAMPLES EXAMINED	1269

Laboratory Internal Validation Summaries



Resources to Aid Future Validation Studies

- STRBase Validation Website
  - **http://www.cstl.nist.gov/biotech/strbase/validation.htm**
  - Validation summary sheets
  - *Helpful information on aspects of validation studies*
- Multiplex_QA Program (Dave Duewer, NIST)
  - Software to monitor STR electropherogram performance (resolution, sensitivity) over time – **can aid performance checks**
  - Available for download: http://www.cstl.nist.gov/biotech/strbase/software.htm
- NIST Calibration Data Set (MIX05 data set is a prototype)
  - **We may construct a set of ~200 sample data files** that can be used to evaluate common STR typing "artifacts" such as stutter, non-template addition, spikes, peak imbalance, tri-allelic patterns, variant alleles, single base resolution

Useful Papers on Validation

- Taylor JK. (1981) Quality assurance of chemical measurements. *Analytical Chemistry* 53(14): 1588A-1596A.
- Taylor JK. (1983) Validation of analytical methods. *Analytical Chemistry* 55(6): 600A-608A.
- Green JM. (1996) A practical guide to analytical method validation. *Analytical Chemistry* 68: 305A-309A.
- EURACHEM Guide (1998) *The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics*; available at http://www.eurachem.ul.pt/guides/valid.pdf

Helpful Resource Books on Validation

- P. Roper, *et al.* (2001) *Applications of Reference Materials in Analytical Chemistry*. Royal Society of Chemistry, Cambridge, UK
- J.K. Taylor (1987) *Quality Assurance of Chemical Measurements*. Lewis Publishers: Chelsea, MI
- H. Gunzler, ed. (1996) *Accreditation and Quality Assurance in Analytical Chemistry*. Springer: New York
- J.K. Taylor (1990) *Statistical Techniques for Data Analysis*. Lewis Publishers: Chelsea, MI
- H.Y. Aboul-Enein, *et al.* (2001) *Quality and Reliability in Analytical Chemistry*. CRC Press: Washington, DC
- G.D. Christian (2004) *Analytical Chemistry* (6th Ed.). John Wiley & Sons, Inc.: Hoboken, NJ

Validation Workshop (Aug 24-26, 2005 at NFSTC)

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

COURSE CONTENTS

Day #1
- Validation Overview (John)
- Introduction to DAB Standards (Robyn & John)
- Developmental Validation (John)

Day #2
- Inconsistency in Validation between Labs (John)
- Internal Validation (Robyn)
- Method Modifications and Performance Checks (Robyn)

Day #3
- Practical Exercises (Robyn)

Was filmed and is being made into a training DVD as part of the President's DNA Initiative…

BREAK

History of Forensic DNA Validation Guidelines

Timeline Regarding Forensic Validation Information

- 1989 *Casto* case – concern over quality in forensic DNA cases
- 1989 TWGDAM – focus on RFLP
- 1991 TWGDAM – updated with PCR info
- 1995 TWGDAM – updated with more PCR info
- **1998/99 DNA Advisory Board Standards**
- 2004 Revised SWGDAM Validation Guidelines

Brief Historical Overview

Profiles in DNA (Sept 1999) 3(2): 10-11


CURRENT EVENTS

The Evolution of Quality Standards for Forensic DNA Analyses in the United States

By Special Agent Lawrence A. Presley, MS, MA
Federal Bureau of Investigation Laboratory, Washington, DC
lpresley@fbi.gov

Quality problems in late 1980s with DNA testing
TWGDAM established under FBI Lab sponsorship in 1988
NRC I (1992) and NRC II (1996) issued reports recommending formal QA programs
DNA Identification Act of 1994 lead to formation of DNA Advisory Board (DAB)
DAB Standards issued in Oct 1998 and Apr 1999
When DAB was dissolved in 2000, SWGDAM assumed leadership role

DNA Identification Act (1994)

Public Law 103-322

42 § 14131. Quality assurance and proficiency testing standards

(a) Publication of quality assurance and proficiency testing standards

(1) (A) Not later than 180 days after September 13, 1994, the Director of the Federal Bureau of Investigation shall appoint an advisory board on DNA quality assurance methods from among nominations proposed by the head of the National Academy of Sciences and professional societies of crime laboratory officials.

(B) The advisory board shall include as members scientists from State, local, and private forensic laboratories, molecular geneticists and population geneticists not affiliated with a forensic laboratory, and a representative from the National Institute of Standards and Technology.

(C) **The advisory board shall develop, and if appropriate, periodically revise, recommended standards for quality assurance**, including standards for testing the proficiency of forensic laboratories, and forensic analysts, in conducting analyses of DNA.

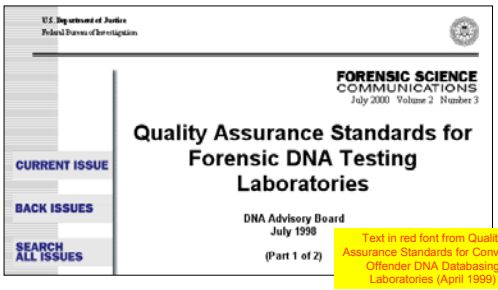
DNA Advisory Board (DAB)

DNA Advisory Board (DAB) Members

- **Joshua Lederberg** (Rockefeller University) – chair 1995-1998
- **Arthur Eisenberg** (University of North Texas Health Science Center) – chair 1998-2000
- **John Hicks** (Alabama Department of Forensic Sciences)
- **Shirley Abrahamson** (Wisconsin State Supreme Court)
- **Ranajit Chakraborty** (University of Texas Health Science Center)
- **Bruce Budowle** (FBI Laboratory)
- **Larry Presley** (FBI Laboratory)
- **Jack Ballantyne** (Suffolk County Crime Lab)
- **Jay Miller** (FBI Laboratory)
- **Dennis Reeder** (National Institute of Standards and Technology)
- **Margaret Kuo** (Orange County Sheriff's Office)
- **Bernard Devlin** (Carnegie Mellon University)
- **Marcia Eisenberg** (Laboratory Corporation of America)
- **Paul Ferrara** (Virginia Division of Forensic Science)
- **Terry Laber** (Minnesota State DNA Lab)
- Dwight Adams, Randall Murch, Barry Brown (FBI Laboratory)
- David Coffman (Florida Department of Law Enforcement)
- Fred Bieber (Harvard Medical School)
- Mary Gibbons (Oakland Police Department)
- Eric Juengst (Case Western Reserve University)
- Susan Narveson (Phoenix Police Department)
- Mohammad Tahir (Indianapolis-Marion County Crime Lab)
- Dawn Herkenham (FBI Laboratory)

Existed from 1995-2000

DAB Standards


U.S. Department of Justice
Federal Bureau of Investigation

FORENSIC SCIENCE COMMUNICATIONS
July 2000 Volume 2 Number 3

CURRENT ISSUE
BACK ISSUES
SEARCH ALL ISSUES

Quality Assurance Standards for Forensic DNA Testing Laboratories

DNA Advisory Board
July 1998

(Part 1 of 2)

Text in red font from Quality Assurance Standards for Convicted Offender DNA Databasing Laboratories (April 1999)

http://www.fbi.gov/hq/lab/fsc/backissu/july2000/codis2a.htm

http://www.fbi.gov/hq/lab/fsc/backissu/july2000/codis1a.htm

Outline of DAB Standards

Quality Assurance Standards (QAS)

1. SCOPE
2. DEFINITIONS
3. QUALITY ASSURANCE PROGRAM
4. ORGANIZATION AND MANAGEMENT
5. PERSONNEL
6. FACILITIES
7. EVIDENCE *(SAMPLE)* CONTROL
8. **VALIDATION**
9. ANALYTICAL PROCEDURES
10. EQUIPMENT CALIBRATION AND MAINTENANCE
11. REPORTS
12. REVIEW
13. PROFICIENCY TESTING
14. CORRECTIVE ACTION
15. AUDITS
16. SAFETY
17. SUBCONTRACTOR OF ANALYTICAL TESTING FOR WHICH VALIDATED PROCEDURES EXIST

Validation Section of the DNA Advisory Board Standards

issued October 1, 1998 and April 1999; published in *Forensic Sci. Comm.* July 2000

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

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.

8.1.2.3 Population distribution data are documented and available.

8.1.2.3.1 The population distribution data would include the allele and genotype distributions for the locus or loci obtained from relevant populations. Where appropriate, databases should be tested for independence expectations.

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.

8.1.3.4 Material modifications made to analytical procedures shall be documented and subject to validation testing.

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.

FORENSIC SCIENCE COMMUNICATIONS JULY 2000 VOLUME 2 NUMBER 3

Developmental Validation Overview

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.

8.1.2.3 Population distribution data are documented and available.

8.1.2.3.1 The population distribution data would include the allele and genotype distributions for the locus or loci obtained from relevant populations. Where appropriate, databases should be tested for independence expectations.

Internal Validation Overview

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.

8.1.3.4 **Material modifications** made to analytical procedures shall be documented and subject to validation testing.

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.

Material Modification

- Decrease in reaction volume from manufacturer's specifications
- Centricon tube membrane change
- Minimum peak threshold
- Injection times for genetic analyzers
- Increased amplification cycle numbers
- Others?

Performance Check

- Relocation of lab to a new facility
- Change of laser or other critical component on a genetic analyzer
- Software changes
  - Mac-based GS/GT to NT-based GS/GT
  - Mac-based collection software to NT or Windows-based collection software
- Additional instrumentation (i.e., 2nd 3130)

How would you evaluate each of these?

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

Other DAB Standards to Consider

9.1.1 The laboratory shall have an ***standard protocol*** for each analytical technique used.

9.1.2 The procedures shall include ***reagents, sample preparation, extraction, equipment and controls***, which are standard for DNA analysis and data interpretation.

9.2.3 The laboratory shall identify ***critical reagents*** (if any) and evaluate them prior to use in casework……

9.4 The laboratory shall monitor the analytical procedures using appropriate ***controls and standards***.

10.2 The laboratory shall identify ***critical equipment*** and shall have a documented program for calibration of instruments and equipment.

10.3 The laboratory shall have a ***documented program*** to ensure that instruments and equipment are properly maintained.

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

9. ANALYTICAL PROCEDURES

STANDARD 9.1 The laboratory shall have and follow written analytical procedures approved by the laboratory management/technical manager.

9.1.1 The laboratory shall have a standard operating protocol for each analytical technique used.

9.1.2 The procedures shall include reagents, sample preparation, extraction, equipment, and controls, which are standard for DNA analysis and data interpretation.

9.1.3 The laboratory shall have a procedure for differential extraction of stains that potentially contain semen.

FORENSIC SCIENCE COMMUNICATIONS JULY 2000 VOLUME 2 NUMBER 3

Suitable Reagents

STANDARD 9.2 The laboratory shall use reagents that are suitable for the methods employed.

9.2.1 The laboratory shall have written procedures for documenting commercial supplies and for the formulation of reagents.

9.2.2 Reagents shall be labeled with the identity of the reagent, the date of preparation or expiration, and the identity of the individual preparing the reagent.

9.2.3 **The laboratory shall identify critical reagents** *(if any)* and evaluate them prior to use in casework. These critical reagents include but are not limited to: *(THIS LAST PORTION NOT IN CONVICTED OFFENDER DATABASING STANDARDS)*

(a) Restriction enzyme
(b) Commercial kits for performing genetic typing
(c) Agarose for analytical RFLP gels
(d) Membranes for Southern blotting
(e) K562 DNA or other human DNA controls
(f) Molecular weight markers used as RFLP sizing standards
(g) Primer sets
(h) Thermostable DNA polymerase

Human DNA Quantitation

STANDARD 9.3 The laboratory shall have and follow a procedure for **evaluating the quantity of the human DNA in the sample where possible**. *(NOT IN CONVICTED OFFENDER DATABASING STANDARDS)*

9.3.1 For casework RFLP samples, the presence of high molecular weight DNA should be determined.

Appropriate Controls and Standards

STANDARD 9.4 The laboratory shall monitor the analytical procedures using appropriate controls and standards.

9.4.1 The following controls shall be used in RFLP casework analysis:

9.4.1.1 Quantitation standards for estimating the amount of DNA recovered by extraction. *(When required by the analytical procedure, standards for estimating the amount of DNA recovered by extraction shall be used.)*

9.4.1.2 K562 as a human DNA control. (In monitoring sizing data, a statistical quality control method for K562 cell line shall be maintained.)

9.4.1.3 Molecular weight size markers to bracket known and evidence samples. *(Molecular weight size markers to bracket samples on an analytical gel. No more than five lanes shall exist between marker lanes.)*

9.4.1.4 *A* Procedure *shall be available* to monitor the completeness of restriction enzyme digestion. *(Interpretation of the autoradiluminigraph is the ultimate method of assessment but a test gel or other method may be used as necessary.)*

9.4.2 The following controls shall be used for PCR casework analysis *(database analysis)*:

9.4.2.1 **Quantitation standards**, which estimate the amount of human nuclear DNA recovered by extraction. *(When required by the analytical procedure, standards which estimate the amount of human nuclear DNA recovered by extraction shall be used.)*

9.4.2.2 **Positive and negative amplification controls**.

9.4.2.3 **Reagent blanks**. *(Contamination controls.)*
9.3.2.3.1 Samples extracted prior to the effective date of these standards without reagent blanks are acceptable as long as other samples analyzed in the batch do not demonstrate contamination.

9.4.2.4 **Allelic ladders and/or internal size makers** for variable number tandem repeat sequence PCR based systems.

Traceability to NIST Standard Reference Material

STANDARD 9.5 The laboratory shall check its DNA procedures annually or whenever substantial changes are made to the protocol(s) against an appropriate and available NIST standard reference material or standard traceable to a NIST standard.

ISO 17025 requires calibration to a national metrology laboratory

Written Guidelines for Data Interpretation

STANDARD 9.6 The laboratory shall have and follow written general guidelines for the interpretation of data.

9.6.1 The laboratory shall verify that **all control results are within established tolerance limits**.

9.6.2 Where appropriate, visual matches shall be supported by a numerical match criterion. *(NOT IN CONVICTED OFFENDER DATABASING STANDARDS)*

9.6.3 For a given population(s) and/or hypothesis of relatedness, the **statistical interpretation** shall be made following the recommendations 4.1, 4.2 or 4.3 as deemed applicable of the National Research Council report entitled "The Evaluation of Forensic DNA Evidence" (1996) and/or court directed method. These calculations shall be derived **from a documented population database appropriate for the calculation**. *(NOT IN CONVICTED OFFENDER DATABASING STANDARDS)*

10. EQUIPMENT CALIBRATION AND MAINTENANCE

STANDARD 10.1 The laboratory shall use equipment suitable for the methods employed.

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Instrument Calibration

STANDARD 10.2 The laboratory *(shall identify critical equipment and)* shall have a documented program for calibration of instruments and equipment.

10.2.1 Where available and appropriate, **standards traceable to national or international standards shall be used for the calibration**.

10.2.1.1 Where traceability to national standards of measurement is not applicable, the laboratory shall provide **satisfactory evidence of correlation of results**.

10.2.2 The frequency of the calibration shall be documented for each instrument requiring calibration. Such documentation shall be retained in accordance with applicable Federal or state law.

Instrument Maintenance

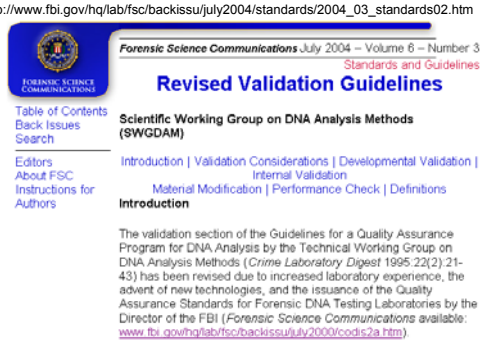
STANDARD 10.3 The laboratory shall have and follow a documented program to ensure that instruments and equipment are properly maintained.

10.3.1 New *(critical)* instruments and equipment, or *(critical)* instruments and equipment that have undergone repair or maintenance, shall be calibrated before being used in casework analysis.

10.3.2 **Written records or logs shall be maintained for maintenance service performed on instruments and equipment**. Such documentation shall be retained in accordance with applicable Federal or state law.

Revised SWGDAM Validation Guidelines
(July 2004)

http://www.fbi.gov/hq/lab/fsc/backissu/july2004/standards/2004_03_standards02.htm



The document provides validation guidelines and definitions approved by SWGDAM July 10, 2003.

Previous Guidelines Regarding Validation

Technical Working Group on DNA Analysis Methods (TWGDAM)

- TWGDAM (1995) – *Crime Lab Digest* 22(2):20-43
  - Budowle *et al.* "Guidelines for a quality assurance program for DNA analysis"
- TWGDAM (1991) – *Crime Lab Digest* 18(2):44-75
  - Kearney *et al.* "Guidelines for a quality assurance program for DNA analysis"
- TWGDAM (1989) – *Crime Lab Digest* 16(2):40-59
  - Kearney *et al.* "Guidelines for a quality assurance program for DNA restriction fragment length polymorphism analysis"

AABB standards (1989) – first standards adopted by an organization dealing with DNA testing impacting human identification; the standards are not intended to provide the details of a technique but rather to give an overview of general policies that when followed will help guarantee reliable results…

From more information on American Association of Blood Banks (AABB) – see http://www.aabb.org

Differences between 1991 and 1995 TWGDAM Guidelines

Crime Lab Digest 1991; 18(2):44-75
Crime Lab Digest 1995; 22(2):20-43

Validation

4.1.3	Expanded upon RFLP and added information on STRs
4.1.5.10	Added "where appropriate"
4.4.2.1	Added (b) "when a PCR product is characterized by direct sequencing…"

Equipment, Materials, and Facilities

5.3.2	Added "an extraction area for samples containing low DNA levels…"

Analytical Procedures

7.2.2	Changed "regular use" to "periodic use" and removed "cellular"
7.3	Added "where appropriate"
7.5.1.3	Removed "substrate" and "(e.g. unstained areas adjacent…)"
7.5.1.4	Deleted original 7.5.1.4 and moved 7.5.1.5 (1991) to 7.4.1.4 (1995)

Audits

10.1	Changed from "annually" to "at least once every 2 years"

At that time, it was not possible to quantify DNA down to the level where DNA could be amplified. Sections 4.4.2.1 and 5.3.2 were added to accommodate mtDNA sequencing needs.

Differences between 1991 and 1995 TWGDAM Guidelines

Crime Lab Digest 1991; 18(2):44-75
Crime Lab Digest 1995; 22(2):20-43

(1991) 4.1.3 Each locus to be used must go through the necessary validation.

(1995) 4.1.3 Once an RFLP procedure has been validated, **appropriate studies of limited scope** (*e.g.*, population studies, human DNA control value determination) must be available for each new locus used. A similar standard should be maintained when adding new loci to the different PCR-based techniques (*e.g.*, addition of short tandem (STR) locus to a validated STR procedure).

Comparison of DAB Standards and Previous Validation Guidelines

DNA Loci

TWGDAM 1989	TWGDAM 1991/1995	DAB (1998)	SWGDAM 2004
Inheritance	Inheritance (4.2.1)	Defined	Inheritance (2.1.1)
Gene mapping	Gene mapping (4.2.2)	Characterized	Mapping (2.1.2)
Polymorphism type	Polymorphism type (4.2.4)		Polymorphism type (2.1.4)
Probe available	Primers known (4.4.1.1)		Primer publication not required (2.10)
	Detection basis (4.2.3)		Detection basis (2.1.3)

PCR Considerations

TWGDAM 1989	TWGDAM 1991/1995	DAB (1998)	SWGDAM 2004
(Not discussed)	Minimum sample (4.1.5.10)	Sensitivity	Sensitivity studies (2.3)
	Primer sequence (4.4.1.1)		Primer publication not required (2.10)
	Contamination control (4.4.1.2)		
	PCR conditions (4.4.1.3)		PCR conditions (2.10.1)
	PCR cycle # (4.4.1.4)		
	Differential PCR (4.4.1.5)		Differential PCR (2.10.2)
	Positive & negative controls (4.4.2)		Positive & negative controls (2.10.4)
			Coamplification assessed (2.10.3)

Comparison of DAB Standards and Previous Validation Guidelines

Developmental Validation

TWGDAM 1989	TWGDAM 1991/1995	DAB (1998)	SWGDAM 2004
Standard specimens	Standard specimens	Standard specimens	
Different tissues	Different tissues		Sensitivity (2.3)
Consistency	Consistency		
Population studies	Population studies	Population studies	Population studies (2.7)
Reproducibility	Reproducibility	Reproducibility	Reproducibility (2.5)
Time/Temp	Environmental	Stability	Stability studies (2.4)
Degradation/Matrix	Degradation/Matrix		
Non-probative	Non-probative		Case-type samples (2.6)
Non-human	Non-human	Species specificity	Species specificity (2.2)
On-site (alpha/beta)	On-site (alpha/beta)		
	Mixed specimens	Mixture	Mixture studies (2.8)
		Accuracy	Precision & accuracy (2.9)
		Precision	
			PCR based procedures (2.10)

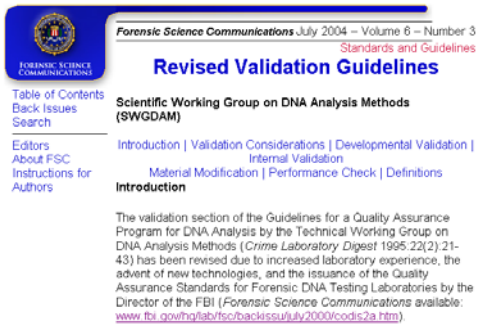
Comparison of DAB Standards and Previous Validation Guidelines

Internal Validation

TWGDAM 1989	TWGDAM 1991/1995	DAB (1998)	SWGDAM 2004
Known samples	Known samples	Known samples	Known & non-probative (3.1)
Proficiency tests	Proficiency tests		
Precision	Precision		
	Contamination control		
		Reproducibility	Reproducibility & precision (3.2)
		Non-probative	
		Match criteria	Match criteria (3.3)
			Sensitivity & stochastic effects (3.4)
			Mixture studies (3.5)
			Contamination (3.6)
			Qualifying test (3.7)

Revised SWGDAM Validation Guidelines
(July 2004)

http://www.fbi.gov/hq/lab/fsc/backissu/july2004/standards/2004_03_standards02.htm


Forensic Science Communications July 2004 – Volume 6 – Number 3
Standards and Guidelines

Revised Validation Guidelines

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Scientific Working Group on DNA Analysis Methods (SWGDAM)

Introduction | Validation Considerations | Developmental Validation | Internal Validation
Material Modification | Performance Check | Definitions

Introduction

The validation section of the Guidelines for a Quality Assurance Program for DNA Analysis by the Technical Working Group on DNA Analysis Methods (*Crime Laboratory Digest* 1995:22(2):21-43) has been revised due to increased laboratory experience, the advent of new technologies, and the issuance of the Quality Assurance Standards for Forensic DNA Testing Laboratories by the Director of the FBI (*Forensic Science Communications* available: www.fbi.gov/hq/lab/fsc/backissu/july2000/codis2a.htm).

The document provides validation guidelines and definitions approved by SWGDAM July 10, 2003.

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

SWGDAM Revised Validation Guidelines
http://www.fbi.gov/hq/lab/fsc/backissu/july2004/standards/2004_03_standards02.htm

Summary of 2004 Validation Survey

Organized by
John Butler and Margaret Kline (NIST),
Chris Tomsey (PASP)

Contacting the Community

- **Validation Standardization Questionnaire** handed out at NIJ DNA Grantees meeting (June 28-30, 2004)
- Emails sent to >200 scientists (July-Aug 2004)
  - Attendees from the NIJ DNA Grantees meeting
  - Participants in NIST interlaboratory studies
  - Contacts through STRBase website
- Responses from **52 scientists** were compiled
  - Covering 27 states + Puerto Rico, 4 companies, 2 outside US
- **Specific interviews were conducted** to gain perspectives from a small lab, a large lab, a private lab, and court testimony experience

Representative Labs Interviewed

- **Montgomery County Crime Lab** – small lab, 3 analysts, ~180 cases/year; using PP16 and ABI 310
- **Orchid Cellmark** – private contract lab, 40 analysts and technicians, ~5,000 cases/year; Profiler Plus/ COfiler and Identifiler with ABI 310 and ABI 3100; extensive court experience
- **AFDIL** – large federal lab, ~120 analysts/technicians, remains identification rather than strictly forensic cases, >1,000 cases/year (mtDNA & STRs); Profiler Plus/COfiler and PP16 with ABI 377 and ABI 3100

Information from interviews is included in the written report of this project…

Validation Standardization Questionnaire (conducted June-August 2004)

Review of Survey Questions

- What is validation?
- **How do you know when you are finished validating** a kit, instrument, software, or procedure?
- What steps are needed in internal validation and how many samples should be run at a minimum?
- **How many total samples do you think it takes to internally "validate" a new forensic kit?**
- How many different sets of samples are needed? Over what time period?
- Where do you look for guidance currently in terms of validation?
- **What are some kits, software, instruments that you are considering for validation in the next year?**
- How are validation, training, and proficiency testing related to one another?
- Do you think that the process of validation can be standardized?
- If a standard protocol or set of guidelines existed for validation, would you use it?
- If a standard set of samples existed for performing validation testing, would you use them?

Used to help define specific examples …

How I felt after taking on this project…

Me

Literature, Validation Data, Survey Responses

Validation Standardization Questionnaire (conducted June-August 2004)

How do you know when you are finished with a validation study? (1)

- "When you have demonstrated that it works as expected over a range of samples that is representative of what is seen in casework"
- "When repeat performance gave the same result"
- "**When you pull the toothpick out and it is dry?...** Meet at least minimum expectations and DAB guidelines"
- "You are very comfortable that you know how it works and your documentation will convince a reviewer you have put the kit thru a rigorous review/test."

Validation Standardization Questionnaire (conducted June-August 2004)

How do you know when you are finished with a validation study? (2)

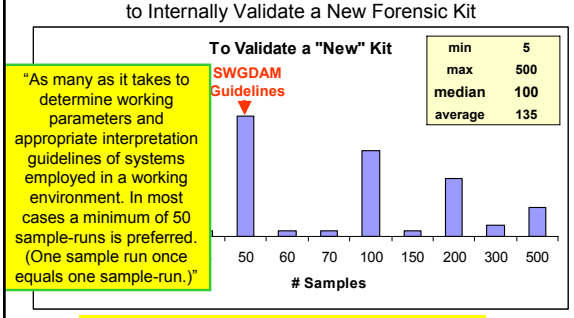
- "Once a reasonable body of data has been assembled and analyzed, quirks have been revealed, and the upper and lower limits of the system have been challenged using a range of samples that one could expect to encounter in the everyday operation of the system"
- "When you achieve accuracy and precision to the desired statistical level of certainty"
- "You can never know…but it is always nice to have more samples!"
- "Validation is never complete"

Validation Standardization Questionnaire (conducted June-August 2004)

Survey Summary for Recommended **Total Number of Samples**

to Internally Validate a New Forensic Kit

"As many as it takes to determine working parameters and appropriate interpretation guidelines of systems employed in a working environment. In most cases a minimum of 50 sample-runs is preferred. (One sample run once equals one sample-run.)"



Choices in survey were: **10, 50, 500, or other ____**

Validation Standardization Questionnaire (conducted June-August 2004)

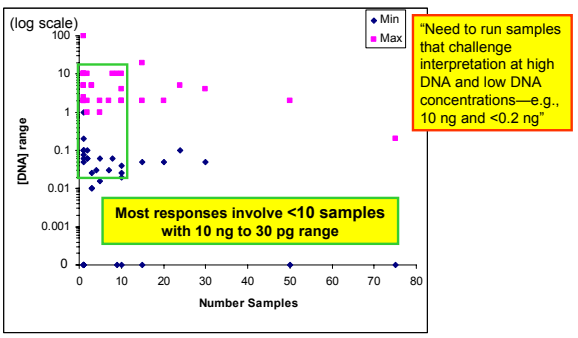
Survey Summary for Recommended **Precision Studies**

A few of the responses:

- "100 allelic ladder injections"
- "1 allelic ladder with 10 injections"
- "Depends upon the system being tested. For a databanking system, 50-100 runs of 50-100 specimens. Again, stats tell you when you've processed enough specimens to understand the system."
- "Minimum: Run one sample at least 8 times. Recommended: Run at least two samples plus allelic ladder at least 8 times." (24 sample-runs)

Validation Standardization Questionnaire (conducted June-August 2004)

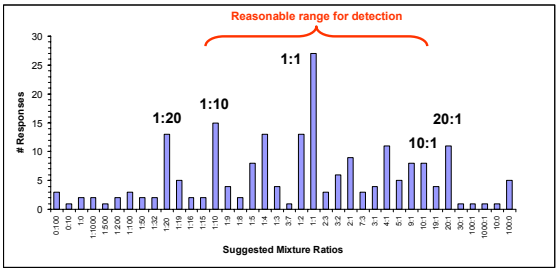
Survey Summary for Recommended **Sensitivity Studies**



"Need to run samples that challenge interpretation at high DNA and low DNA concentrations—e.g., 10 ng and <0.2 ng"

Validation Standardization Questionnaire (conducted June-August 2004)

Survey Summary for Recommended **Mixture Studies**



Some Recommended Numbers of Samples:
5 different 2-person mixtures
50 amplifications from at least 10 different mixtures
1 set of samples (ranging from 1:10 to 10:1)

Validation Standardization Questionnaire (conducted June-August 2004)

Survey Summary for Recommended **Non-Human Cases**

A few of the responses:

- "10-20 food animals, companion animals, local wildlife, ferrets"
- "I don't believe this is necessary in internal validation if external results are published. This would not be expected to vary in different analysts' hands."
- "I've trusted system manufacturers to handle this. Should I have?"
- "Minimum: Include information from developmental studies. If performing developmental studies, include at least bacterial and yeast/fungal example, plus mammalian and non-mammalian examples."

Validation Standardization Questionnaire (conducted June-August 2004)

Survey Summary for Recommended **Non-Probative Cases**

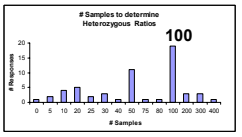
A few of the responses:

- Most responses were between 5-10 cases (range 3-25)
- "More important than the number of cases is the range of forensic samples that are typed during validation."
- "Complete cases are not required to test a system. Recommended: **Run at least 8 mock non-probative samples**. Note: Non-probative samples are not guaranteed to provide complete profiles. They are needed only to show that false results are not generated. Lack of results or incomplete results do not affect the validity of a validation."

Validation Standardization Questionnaire (conducted June-August 2004)

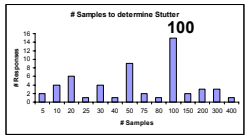
Survey Summary for Recommended **Numbers of Samples**

to Determine Heterozygote Peak Height Ratios and Stutter Values



min	0
max	400
median	50
average	85

Heterozygote Peak Height Ratios



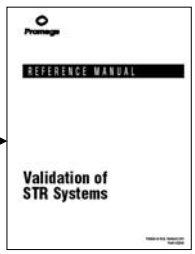
min	5
max	400
median	63
average	88

Stutter Values

Validation Standardization Questionnaire (conducted June-August 2004)

Where do you look for guidance currently in validation?

- SWGDAM
- DAB standards and ISO 17025
- Other scientists
- Literature publications
- Presentations at meetings
- Promega's validation guide
- FBI studies and publications
- NIST studies and publications
- Previous scientific training
- Common sense



Published in March 2001

Validation Standardization Questionnaire (conducted June-August 2004)

Can Validation be Standardized?

Statements from survey responders…

Over 86% (45/52) said yes

Those who responded "no" said

- "to some degree it can be, however, validation is specific to the platform, kits, …",
- "a start-up lab should do much more than an experienced lab…",
- "validation builds on previous work by lab or published data",
- "parts of it can be standardized; I don't think the non-probative cases could be", and
- "only in a general way, as with the SWGDAM guidelines. The uniqueness of each new procedure would make standardization difficult."

Our Conclusion…

to a certain extent it can…but everyone will always have a different comfort level…and **inflexible, absolute numbers for defined studies will not likely be widely accepted**

Validation Standardization Questionnaire (conducted June-August 2004)

If a Standard Protocol or Set of Guidelines Existed for Validation, Would You Use It?

90% (47/52) said yes

Some responses

- "No-I would reference them. I may not completely abide by them but I would certainly review them",
- "No-but it would be taken into consideration",
- "Yes-we would have to or there would be problems in court",
- "Yes-as long as they remain updated, relevant and feasible guidelines and do not become dogma",
- "Yes-if it would pass an audit for validation", and
- "Yes-unless they were far less stringent than current practice."

Validation Standardization Questionnaire (conducted June-August 2004)

If a Standard Set of Samples Existed for Performing Validation Testing, Would You Use Them?

90% (47/52) said yes

Some responses

- "Yes-would love to have something like that available; we are always eager to have benchmarks for assessment",
- "Yes-these types of samples would cut down on time for validation. It would be efficient if they were ready for the particular type of validation…",
- "Yes-as long as they are readily available at a reasonable price",
- "No-this approach is not recommended. It is most important that systems work with the materials available in individual laboratories. Laboratories should be allowed, even encouraged, to select their own preferred materials. Choices for such selection of standard materials for within laboratory analyses and cross-laboratory comparison already exist from a variety of government and commercial entities."

A Thoughtful Comment from One Interviewee

Before a set of validation experiments is performed…

- The question should be asked "Do we already know the answer to this question from the literature or a previous study performed in-house?"
- If the answer is "yes" and we document how we know this answer, **then there is no need to perform that set of validation experiments**.

A good example of this scenario is non-human DNA studies.

Validation Summary Sheet for PowerPlex Y

Study Completed (17 studies done)	Description of Samples Tested (performed in 7 labs and Promega)	# Run
Single Source (Concordance)	5 samples x 8 labs	40
Mixture Ratio (male:female)	6 labs x 2 M/F mixture series x 11 ratios (1:0,1:1,1:10,1:100,1:300,1:1000,0.5:300, 0.25:300,0.125:300, 0.0625:300, 0.03:300 ng M:F)	132
Mixture Ratio (male:male)	6 labs x 2 M/M mixtures series x 11 ratios (1:0, 19:1, 9:1, 5:1, 2:1, 1:1, 1:2, 1:5, 1:9, 1:19, 0:1)	132
Sensitivity	7 labs x 2 series x 6 amounts (1/0.5/0.25/0.125/0.06/0.03)	84
Non-Human	24 animals	24
NIST SRM	6 components of SRM 2395	6
Precision (ABI 3100 and ABI 377)	10 ladder replicates + 10 sample replicated + [8 ladders + 8 samples for 377]	36
Non-Probative Cases	65 cases with 102 samples	102
Stutter	412 males used	412
Peak Height Ratio	N/A (except for DYS385 but no studies were noted)	
Cycling Parameters	5 cycles (28/27/26/25/24) x 8 punch sizes x 2 samples	80
Annealing Temperature	5 labs x 5 temperatures (54/58/60/62/64) x 1 sample	25
Reaction volume	5 volumes (50/25/15/12.5/6.25) x [5 amounts + 5 concentrations]	50
Thermal cycler test	4 models (480/2400/9600/9700) x 1 sample + [3 models x 3 sets x 12 samples]	76
Male-specificity	2 females x 1 titration series (0-500 ng female DNA) x 5 amounts each	10
TaqGold polymerase titration	5 amounts (1.38/2.06/2.75/3.44/4.13 U) x 4 quantities (1/0.5/0.25/0.13 ng DNA)	20
Primer pair titration	5 amounts (0.5x/0.75x/1x/1.5x/2x) x 4 quantities (1/0.5/0.25/0.13 ng DNA)	20
Magnesium titration	5 amounts (1/1.25/1.5/1.75/2 mM Mg) x 4 quantities (1/0.5/0.25/0.13 ng DNA)	20
Krenke *et al.* (2005) *Forensic Sci. Int.* 148:1-14	TOTAL SAMPLES EXAMINED	1269

A Comparison to Y-PLEX 12 Validation

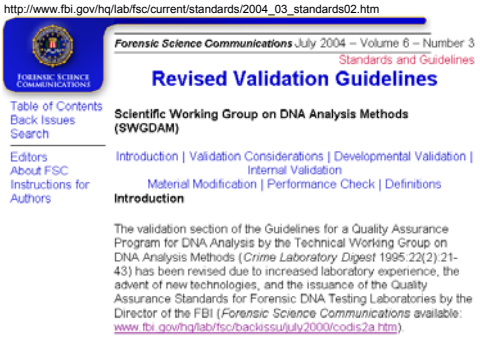
Shewale, J. G., Nasir, H., Schneida, E., Gross, A. M., Budowle, B., and Sinha, S. K. (2004) Y-chromosome STR system, Y-PLEX 12, for forensic casework: development and validation. *J Forensic Sci.* 49(6): 1278-1290.

Study Completed (26 experiments cited)	Description of Samples Tested	TOTAL
Single Source (Concordance)	[50 male + 30 female] mentioned in materials and methods; IPATIMUP,Humboldt shared samples	
Mixtures		
Mixture Ratio (male:female)	6 ratios (1:0/1:100/1:200/1:400/1:600/1:800) x 1 series (0.5 ng male with variable female DNA)	6
Mixture Ratio (male:male)	6 ratios (1:0/1:5/1:10/1:20/1:30/1:40) x 1 series (0.2 ng male-1 with increasing level of male-2)	6
Sensitivity	15 males x 5 amounts (0.05/0.1/0.2/0.5/1/2 ng)	75
Non-Human	9 mammals + 5 bacteria/virus	14
NIST SRM	6 components of SRM 2395	6
Precision (ABI 310, 377, 3100)	50 ladders (310) + 49 ladders (377) + 58 ladders (3100)	157
Non-Probative Cases	19 cases (comprising 45 samples by my calculations)	45
Stutter	34 males (part of another study?)	34
Peak Height Ratio	N/A	
Cycling Parameters	3 males x 4 cycles (28/30/32/34) x 1 amount (1 ng)	12
Annealing Temperature	1 sample x 5 temperatures (56/58/60/62/64) x 1 amount (1 ng)	5
Proficiency		
Substrate	*SEE Y-PLEX 6 and Y-PLEX 5 papers*	
Environment	*SEE Y-PLEX 6 and Y-PLEX 5 papers*	
Various tissues		
Reaction volume	3 volumes (12.5/25/50) x 4 males x 1 amount (1 ng)	12
Thermal cycler test	3 models (9600/9700/MJ PTC-200) x 1 sample	3
Male-specificity	46 unrelated female samples ranging up to 700 ng in amount	46
TaqGold polymerase titration	4 amounts (0.625/1.25/2.5/3.75 U) x 1 sample	4
Primer pair titration	3 amounts (0.25x/0.5x/1x) x 1 sample	3
Magnesium titration	at least 4 amounts (1.0/1.5/1.8/2.2 mM Mg) x 1 sample	4
	TOTAL SAMPLES EXAMINED	432

This Y-PLEX 12 developmental validation was performed in only one lab? (rather than 8) and had one-third the number of samples tested as the PowerPlex Y kit (432 vs. 1269). The study also shares two authors (Ann Marie Gross and Bruce Budowle) with the Krenke *et al.* (2005) PowerPlex Y study.

Revised SWGDAM Validation Guidelines (July 2004)

http://www.fbi.gov/hq/lab/fsc/current/standards/2004_03_standards02.htm



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The document provides validation guidelines and definitions approved by SWGDAM July 10, 2003.

Revised Validation Guidelines Additions

3.0 The internal validation process includes the studies detailed below *(following slides)* encompassing a total of at least **50** samples. Some studies may not be necessary due to the method itself.

Can the same samples be used to cover different studies in the same validation? What about other validations?

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.1 Known and non-probative 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.*

- Known samples
- Authentic case samples *or*
- Simulated case samples
- Use previous data

Why do we do this? *To show that the technique works in our hands*

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.1 Known and non-probative evidence samples:

- **Profiler Plus validation (JFS 2001) :** Analyzed nineteen non-probative cases that included blood standards for comparison to semen stains or bloodstains. Nine of these were previously analyzed in PM and D1280.
- **PowerPlex 2.1 validation (JFS 2002):** Analyzed eleven proficiency tests as well as thirty samples for which previous PowerPlex 1.1 data was available as well as thirty-two cases for which previous RFLP, CTT or PowerPlex 1.1 data was available.
- **Identifiler Validation (Internal 2004):** Analyzed ten known samples of lab employees on 310 and 3100 genetic analyzers and compared results. Also analyzed nine cases and compared to the original case conclusions.

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.1 Known and non-probative evidence samples:

- **DNA extraction with DNA IQ (Internal 2003):** Twenty-four sets of body fluids (blood, semen, saliva, and vaginal fluid) as well as hair (n=12) from known individuals were extracted. All gave the expected results following DNA analysis demonstrating that the technique worked on the commonly seen samples in DNA. Mixed samples (post-coital) as well as samples applied to a variety of substrates were also extracted and demonstrated the expected results following DNA analysis.
- **3100 Validation (Internal 2003):** Thirty-four known samples were analyzed and compared to the previous platform.

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.1 Known and non-probative evidence samples:

- **Quantifiler Validation (Internal 2004):** Eleven samples were quantitated and compared with previous QF results. Also participated in the NIST Quantitation study (8 additional samples). All samples were amplified with Identifiler and analyzed on a 310.
- **Quantifiler Validation (Internal 2004):** Fifty two samples quantitated in Quantifiler, Quantiblot and AluQuant, amplified in PP/CF and analyzed on a 310 or 3100.

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.1 Known and non-probative evidence samples:

- **GMID Validation (Internal 2005):** One thousand twenty-six samples were analyzed and compared to GS/GT results.

Why such a large number when only 50 required?

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

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

What are these?

Reproducibility is being able to obtain the same results under the same conditions

- the IPC in QF or the allelic ladder used in STR analysis

Precision is the "tightness" or closeness of the results

- the range of the CT for the IPC of the base pair size of the alleles in the allelic ladder

You need a method that will give you the same result consistently with the same level of "tightness"

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.2 ***Reproducibility*** and precision:

- **Profiler Plus validation (JFS 2001) :** Interlaboratory reproducibility was assessed by analyzing fifty samples at two different sites; compared ten samples separated by gel electrophoresis versus capillary electrophoresis; evaluated results from twenty samples extracted organically and non-organically.
- **PowerPlex 2.1 validation (JFS 2002):** Concordance studies with 100 convicted offender samples and analyzed at four different sites *(one site only analyzed 25 samples)* . Also compared results of 25 of the samples with results obtained with Profiler Plus and Cofiler at a fifth site.
- **Identifiler Validation (Internal 2004):** Twenty samples of control 9974A were separately amplified at 1 ng target DNA and analyzed on 3 separate days.

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.2 ***Reproducibility*** and precision:

- **DNA extraction with DNA IQ (Internal 2003):** Same sample set as the known samples. Also, neat blood samples extracted under the same parameters yielded equivalent quantitation results.
- **3100 Validation (Internal 2003):** Same single source samples utilized for ***3.1 Known and non-probative evidence samples.*** Each of thirty-four samples was injected independently on each of the 16 capillaries.

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.2 ***Reproducibility*** and precision:

- **Quantifiler Validation (Internal 2004):** A sample of K562 was diluted from 2 ng/ul to 0.06 ng/ul and quantitated in replicates of 4 (or more) by two separate analysts on two separate days for at least 3 runs. Select samples from the reproducibility study were amplified and the average peak heights determined.
- **Quantifiler Validation (Internal 2004):** Twenty single source samples were quantified on three different days. Each of the twenty samples was also quantified in triplicate on a single run. Male: female mixtures were also prepared and quantitated in triplicate *(one time in duplicate)* over several days. (Same samples as precision samples)

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.2 ***Reproducibility*** and precision:

- **GMID Validation (Internal 2005):** Positive control samples from Profiler Plus and CoFiler demonstrated the expected results over numerous runs on numerous days from several different capillary electrophoresis platforms from 6 different labs.

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.2 Reproducibility and ***Precision***:

- **Profiler Plus validation (JFS 2001) :**
  - **Precision of allele determination:** Five known samples were injected twenty times and the base pair size and genotype data collected for one allele at each locus. Sizing data was also collected for the first allele of the allelic ladder for D3, amelogenin and D5 from 100 allelic ladder runs.
  - **Precision of relative peak height:** Used samples from reproducibility, stutter and above precision studies were used to determine the average heterozygote peak height ratio.

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.2 Reproducibility and ***Precision***:

- **PowerPlex 2.1 validation (JFS 2002):** Not discussed
- **Identifiler Validation (Internal 2004):** Twenty samples of control 9974A were separately amplified at 1 ng target DNA and analyzed on 3 separate days. Each of the samples was re-injected throughout the three runs and base pair size determinations conducted.

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.2 Reproducibility and ***Precision***:

- **DNA extraction with DNA IQ (JFS 2004):** Same as reproducibility samples
- **3100 Validation (Internal 2003):** Profiler Plus and Cofiler ladders were injected numerous times (Profiler Plus 944 injections and Cofiler 1600 injections) and the average base pair size for each allele determined and from that the mean for each locus as well as standard deviation determined. ***Note: The average base pair size from the previous samples utilized in the reproducibility study may also have been used.***

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.2 Reproducibility and ***Precision***:

- **Quantifiler Validation (Internal 2004):** A set of 8 standard dilutions of Quantifiler human DNA standards was made ranging in concentrations of 50 ng to 0.023 ng. These were run in 3 separate plates on 2 separate days. The CT values were complied, averages and SD determined. Also, the CT values for 330 IPCs were complied, averaged, and the SD determined.
- **Quantifiler Validation (Internal 2004):** Twenty single source samples were quantified on three different days. Each of the twenty samples was also quantified in triplicate on a single run. Male: female mixtures were also prepared and quantitated in triplicate *(one time in duplicate)* over several days. (Same samples as reproducibility samples)

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.2 Reproducibility and ***Precision***:

- **GMID Validation (Internal 2005):** Positive control samples from Profiler Plus and CoFiler demonstrated the expected results over numerous runs on numerous days from several different capillary electrophoresis platforms from 6 different labs. Also, the one thousand plus samples yielded concordant allelic calls when compared to results obtained with the previous analysis software. These samples were also run on numerous days from several different capillary electrophoresis platforms from 6 different labs.

What does this tell us relative to algorithms used to define a peak? About stutter filters? Allelic bins?

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.3 Match criteria: ***For procedures that entail separation of DNA molecules based on size, precision of sizing must be determined by repetitive analyses of appropriate samples to establish criteria for matching or allele designation.***

What does that mean?????

Concerns procedures that involve DNA separation
- need to determine the precision of that separation
- the reliability of the separation

Why?????

- so that the criteria used for matching alleles (to the allelic ladder) or determining an allelic designation are sound.

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.3 Match criteria:

- **Profiler Plus validation (JFS 2001) :** Data is addressed in the precision study
- **PowerPlex 2.1 validation (JFS 2002):** Not addressed
- **Identifiler Validation (Internal 2004):**Data is addressed in the precision study
- **DNA extraction with DNA IQ (Internal 2003):** Not addressed
- **3100 Validation (Internal 2003):**Data is addressed in the precision study
- **Quantifiler Validation (Internal 2004):** Not applicable
- **Quantifiler Validation (Internal 2004):** Not applicable
- **GMID Validation (Internal 2005):** Same 1000+ samples utilized.

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.4 Sensitivity and stochastic studies: ***The laboratory must conduct studies that ensure the reliability and integrity of results. For PCR-based assays, studies must address stochastic effects and sensitivity levels.***

- Must determine the sensitivity of the method being validated to ensure reliability and integrity of the results -
- If the method is a PCR-based assay, you must determine how (if) stochastic effects and sensitivity levels have an affect on your data.

Why?????

so that you know the limits of the method being validated

Only related to low level samples? What happens in STR amplification if a sample is seriously overloaded? Does this correlate to RT PCR? What about extraction methods like magnetic bead technology?

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.4 Sensitivity and stochastic studies:

- **Profiler Plus validation (JFS 2001) :** Prepared dilutions from 10 ng to 36 pg, amplified the samples and ran on 3 separate 310s. Also examined injection times ranging from five to twenty seconds on samples containing 0.6 ng to 36 pg of input DNA.
- **PowerPlex 2.1 validation (JFS 2002):** Prepared dilutions ranging from 25 ng down to 0.03125 ng, amplified samples and analyzed using gel electrophoresis.
- **Identifiler Validation (Internal 2004):** Nine samples of 9947A were amplified in duplicate by 2 separate analysts in concentrations ranging from 0.0125 to 1 ng and analyzed at 50 to 150 rfus.

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.4 Sensitivity and stochastic studies:

- **DNA extraction with DNA IQ (Internal 2003):** Extracted blood dilutions from neat to $1x10^{-4}$ in triplicate to determine the sensitivity of the extraction method. Also varied the elution volume. Also extracted timed mock sexual kits to determine the limits of detecting sperm in a mixed sample.
- **3100 Validation (Internal 2003):** Samples from known sources (volunteers or positive controls) were quantitated and amplified in PP and/or CF targeting 0.06 to 2 ng of input DNA.

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.4 Sensitivity and stochastic studies:

- **Quantifiler Validation (Internal 2004):** Not addressed
- **Quantifiler Validation (Internal 2004):** Profiler Plus positive control was diluted from neat to 1:200. Also quantitated dilutions of DNA extracted from saliva, bloodstains and semen with various extraction methods. Also tested approximately 85 reagent blanks from previous training and proficiency tests as well as low level and high level samples and inhibited samples
- **GMID Validation (Internal 2005):** Not addressed

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.5 Mixture studies: ***When appropriate, forensic casework laboratories must define and mimic the range of detectable mixture ratios, including detection of major and minor components. Studies should be conducted using samples that mimic those typically encountered in casework (e.g., post-coital vaginal swabs).***

Labs need to look at how mixtures affect results and need to design mixture interpretation guidelines based on these studies. These guidelines need to be utilized in casework.

What would be some good samples to use to help define your mixture guidelines?

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.5 Mixture studies:

- **Profiler Plus validation (JFS 2001) :** Two samples were mixed together at known proportions (1:200, 1:100, 1:20, 1:10, 1:2, and 1:1) to determine the ratio at which the major and minor components of a mixture could be resolved. Amplified 2 ng of target DNA
- **PowerPlex 2.1 validation (JFS 2002):** Preparations of a series of DNA:DNA ratios from already quantified samples were utilized as well as mixtures of body fluids in known volumes prior to DNA extraction and quantification. Amplified 1 ng of target DNA.

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.5 Mixture studies:

- **Identifiler Validation (Internal 2004):**
  - Peak Height ratio study: Ten single source samples were amplified in duplicate and analyzed
  - Five second injection study: Two known DNA samples (male and female) were mixed in a variety of ratios and injected for 5 seconds
  - Nine second injection study: same as above
- **DNA extraction with DNA IQ (Internal 2003):** Extracted 4 timed mock sexual assault kits to determine when the male component of the mixture could no longer be determined.
- **3100 Validation (Internal 2003):** Prepared 2 sets of mixtures from 1:1 to 1:16 with male and female major components.

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.5 Mixture studies:

- **Quantifiler Validation (Internal 2004):** Not performed
- **Quantifiler Validation (Internal 2004):** Female to male mixtures were made utilizing various body fluids and quantitated in both total human and total Y to determine the lowest amount of male DNA that could still be amplified and detected in the presence of female DNA (total DNA)
- **GMID Validation (Internal 2005):** Looked at numerous mixtures and compared results to those obtained in previous analysis with GenoTyper.

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.6 Contamination: *The laboratory must demonstrate that its procedures minimize contamination that would compromise the integrity of the results. A laboratory should employ appropriate controls and implement quality practices to assess contamination and demonstrate that its procedure minimizes contamination.*

Demonstrate that procedures minimize this -

HOW?????

Use of accepted controls and established procedures.

The accepted controls must consistently yield the expected results.

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.6 Contamination:

- **Profiler Plus validation (JFS 2001) :** Not discussed
- **PowerPlex 2.1 validation (JFS 2002):** Not discussed
- **Identifiler Validation (Internal 2003):** Although more instrument related that kit related, the lab put 9 sets of sample tubes in the sample tray for the 310 in a set pattern with some containing excessive size standard and injected in a specific order.
- **Automated extraction with DNA IQ (JFS 2004):** Use of appropriate controls (blanks) through out the validation study demonstrated no instances of contamination.

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.6 Contamination:

- **3100 Validation (Internal 2003):**
  - Mechanical carryover (carryover from one injection to the next): wells of positive controls were injected followed immediately by injection of blanks
  - Optical carryover (signal from one capillary being detected and associated with the adjacent capillary by the detection cell):wells of positive control injected adjacent to wells of blanks
- **Quantifiler Validation (Internal 2004):** Not discussed
- **Quantifiler Validation (Internal 2004):** Not discussed
- **GMID Validation (Internal 2005):** Not discussed

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.7 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 that the laboratory routinely analyzes. This qualifying test may be administered internally, externally, or collaboratively.*

Test method in a hands on format -

like an old proficiency test

Written format? Laboratory format?

The audit document states that this can be either.

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.7 Qualifying test:

- **Profiler Plus validation (JFS 2001) :** Not discussed
- **PowerPlex 2.1 validation (JFS 2002):** Not discussed
- **Identifiler Validation (Internal 2004):** Analyzed a previously characterized external DNA proficiency test as well as NIST SRM 2391b.
- **DNA extraction with DNA IQ (Internal Validation 2003):** not discussed
- **3100 Validation (Internal 2003):** Analysts were required to run a set of previously characterized samples. Written examination also required.

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

3.7 Qualifying test:

- **Quantifiler Validation (Internal 2004):** Not discussed
- **Quantifiler Validation (Internal 2004):** Previously characterized samples were re-run and analyzed. Written test also required.
- **GMID Validation (Internal 2005):** Previously collected data was provided for analysis.

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

General Steps for Internal Validation

- Review literature and learn the technique
- Obtain equipment/reagents, if necessary
- Determine necessary validation studies (there can be overlap and you only need to run a total of 50 samples)
- Collect/obtain samples, if necessary
- Perform validation studies maintaining all documentation
- Summarize the studies and submit for approval to Technical Leader
- Write-up the analytical procedure(s). Include quality assurance (controls, standards, critical reagents and equipment) and data interpretation, as applicable
- Determine required training and design training module(s)
- Design qualifying or competency test

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

BREAK

My Philosophy towards Validation

Ask first: Does the new method improve your capability?

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

Practical Examples

Practical Examples

- Profiler Plus/COfiler kit switch to Identifiler
- ABI 3100 upgrade to ABI 3130xl
- GeneScan/Genotyper to GeneMapperID
- New allelic ladder provided by company
- Bringing Quantifiler "on-line" (from Quantiblot)
- DNA IQ
- Corbett robot
- FSS-i3 expert system software
- Reduced volume reactions

Suggestions for an Internal Validation of an STR Kit

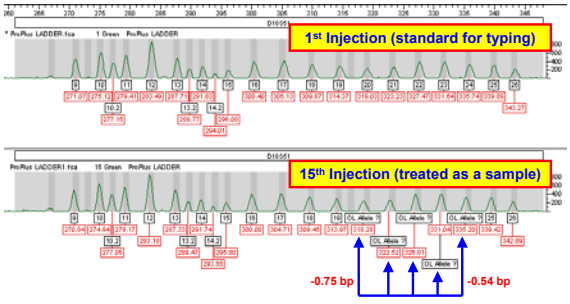
- Standard samples (3.1) **Between 1 and ~20 samples**
  - Verify correct type with positive control or NIST SRM samples
  - Concordance study with 5-10 (non-probative casework) samples previously typed with other kit(s)
- Precision samples (3.2) **5-10 samples**
  - Run at least 5-10 samples (allelic 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

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

Example with Identifiler STR Kit

- **Your lab is currently running ProfilerPlus/COfiler and wants to switch to Identifiler**. What is needed for your internal validation?
- **What is different between Identifiler and ProfilerPlus/COfiler**?
  - **Two new STR loci: D19S433 and D2S1338**
  - **Different fluorescent dyes**
  - **Additional fluorescent dye (5-dye vs 4-dye)**
  - **Different dye on internal size standard**
  - **More loci being amplified in the multiplex**
  - **Mobility modifiers to move allele sizes**

Different
Loci (2 extra STRs)
Dyes
Mobility Modifiers
Software (5-dye)

- PCR primer sequences are the same so potential allele discordance due to primer binding site mutations should not be an issue
- **What has been reported in terms of developmental validation for Identifiler?**

ABI Kit Validation Papers

J. Forensic Sci. 2002; 47(1): 66-96

Cydne L. Holt,[1] Ph.D.; Martin Buoncristiani,[2] M.P.H.; Jeanette M. Wallin,[1] M.P.H.; Theresa Nguyen,[1] B.S.; Katherine D. Lazaruk,[1] Ph.D.; and P. S. Walsh,[1] M.P.H.

TWGDAM Validation of AmpFℓSTR™ PCR Amplification Kits for Forensic DNA Casework

J. Forensic Sci. 2004; 49(6): 1265-1277

Patrick J. Collins,[1] B.A.; Lori K. Hennessy,[1] Ph.D.; Craig S. Leibelt,[1] A.B.; Rhonda K. Roby,[1] M.P.H.; Dennis J. Reeder,[1] Ph.D.; and Paul A. Foxall,[1] Ph.D.

Developmental Validation of a Single-Tube Amplification of the 13 CODIS STR Loci, D2S1338, D19S433, and Amelogenin: The AmpFℓSTR® Identifiler® PCR Amplification Kit

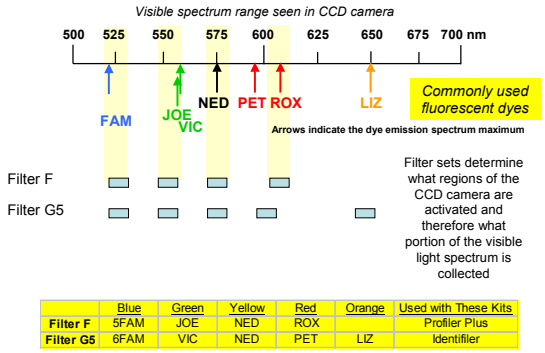
AmpFℓSTR® Identifiler™
PCR Amplification Kit
User's Manual

Population Studies with D2S1338 and D19S433

- These STR loci are part of the widely used SGM Plus kit
- Included in profile frequency calculator using 24 European populations and 5,700 individuals: http://www.str-base.org/calc.php

- Budowle, B. (2001) Genotype profiles for five population groups at the short tandem repeat loci D2S1338 and D19S433. *Forensic Sci. Comm.* 3(3); available at http://www.fbi.gov/hq/lab/fsc/backissu/july2001/budowle1.htm
- Budowle, B., *et al.* (2001) Population data on the STR loci D2S1338 and D19S433. *Forensic Sci. Comm.* 3(3); available at http://www.fbi.gov/hq/lab/fsc/backissu/july2001/budowle2.htm
- Butler, J.M., *et al.* (2003) Allele frequencies for 15 autosomal STR loci on U.S. Caucasian, African American, and Hispanic populations. *J. Forensic Sci.* 48(4):908-911; genotypes available at http://www.cstl.nist.gov/biotech/strbase/NISTpop.htm

Different Fluorescent Dyes



	Blue	Green	Yellow	Red	Orange	Used with These Kits
Filter F	5FAM	JOE	NED	ROX		Profiler Plus
Filter G5	6FAM	VIC	NED	PET	LIZ	Identifiler

Mobility Shift with Non-Nucleotide Linker "Mobility Modifiers"

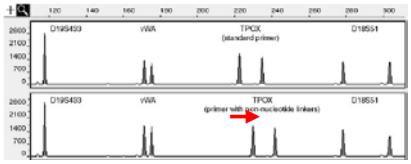


FIG. 1—*NED dye labeled loci from two amplifications of a single sample using TPOX primers both with and without non-nucleotide linkers. The X-axis indicates base pair size and the Y-axes RFU. The top panel depicts the amplification without non-nucleotide linkers. Sizes for the TPOX alleles for this panel were 222.93 and 234.81 bp. Sizes for the TPOX alleles in the amplification using the modified primer, depicted in the bottom panel, were 229.85 and 241.71 bp, indicating an average shift of 6.91 bp. Peaks heights, intralocus balance, and intracolor balance were similar in both amplifications.*

Collins PJ, Hennessy LK, Leibelt CS, Roby RK, Reeder DJ, Foxall PA. Developmental validation of a single-tube amplification of the 13 CODIS STR loci, D2S1338, D19S433, and amelogenin: the AmpFISTR Identifiler PCR amplification kit. *J. Forensic Sci.* 2004; 49(6): 1265-1277.

Mobility Modifiers

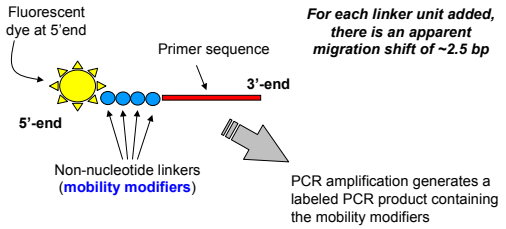


Figure 5.7, J.M. Butler (2005) *Forensic DNA Typing*, 2nd Edition © 2005 Elsevier Academic Press

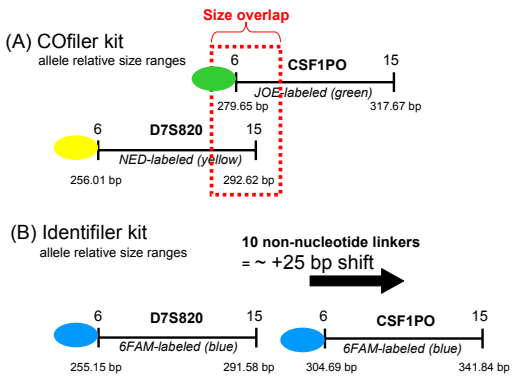


Figure 5.8, J.M. Butler (2005) *Forensic DNA Typing*, 2nd Edition © 2005 Elsevier Academic Press

Sizing Precision with Non-Nucleotide Linkers

TABLE 1—*Sizing shift and sizing precision data for loci incorporating non-nucleotide linkers on the ABI PRISM 310 Genetic Analyzer.*

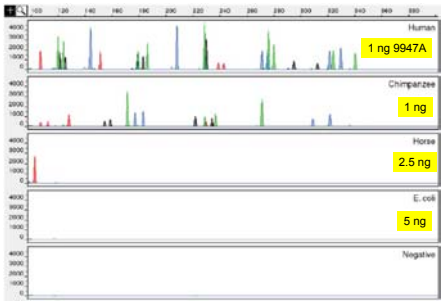
Locus	Increase in Detected Size (bp)	Range of Standard Deviation of Alleles for Identifiler Kit (bp)	Range of Standard Deviation of Alleles Previous Kit (bp)
CSF1PO	26	0.08–0.13*	0.03–0.10†
D2S1338	16	0.05–0.12*	0.02–0.15‡
D13S317	12	0.05–0.09*	0.02–0.09§
D16S539	23	0.06–0.09*	0.01–0.08‡
TPOX	7	0.03–0.08*	0.02–0.07†

No apparent significant decrease in precision with mobility modifiers…

Collins PJ, Hennessy LK, Leibelt CS, Roby RK, Reeder DJ, Foxall PA. Developmental validation of a single-tube amplification of the 13 CODIS STR loci, D2S1338, D19S433, and amelogenin: the AmpFISTR Identifiler PCR amplification kit. *J. Forensic Sci.* 2004; 49(6): 1265-1277.

Non-Human Studies (Species Specificity)

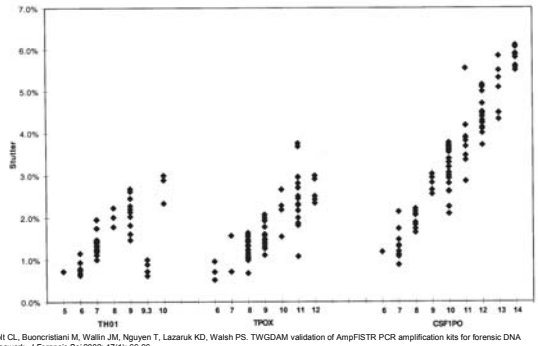
Identifiler STR Kit Developmental Validation



Collins PJ, Hennessy LK, Leibelt CS, Roby RK, Reeder DJ, Foxall PA. Developmental validation of a single-tube amplification of the 13 CODIS STR loci, D2S1338, D19S433, and amelogenin: the AmpFISTR Identifiler PCR amplification kit. *J. Forensic Sci.* 2004; 49(6): 1265-1277.

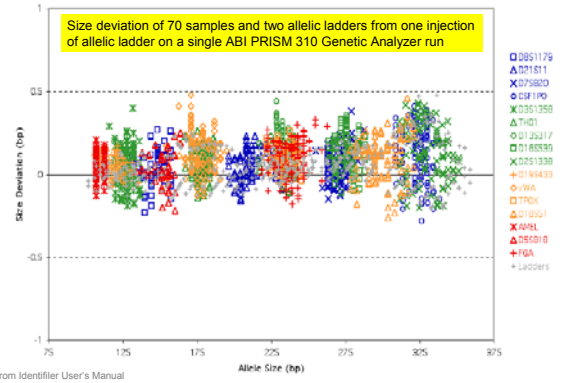
Measured Stutter Percentages

Variable by Allele Length and Composition



Holt CL, Buoncristiani M, Wallin JM, Nguyen T, Lazaruk KD, Walsh PS. TWGDAM validation of AmpFISTR PCR amplification kits for forensic DNA casework. *J Forensic Sci* 2002; 47(1): 66-96.

Precision from Run-to-Run on ABI 310



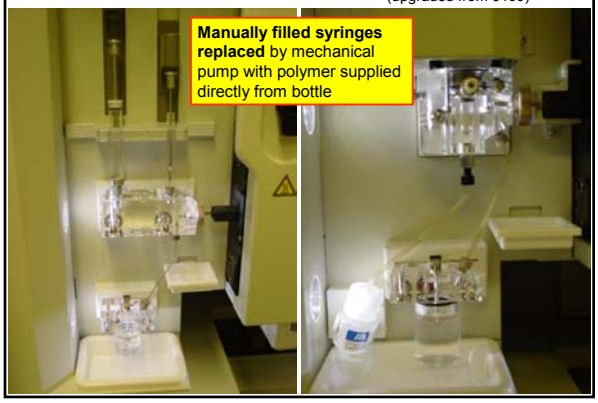
From Identifiler User's Manual

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 3100 | ABI 3130xl (upgraded from 3100)



ABI 3130xl vs ABI 3100

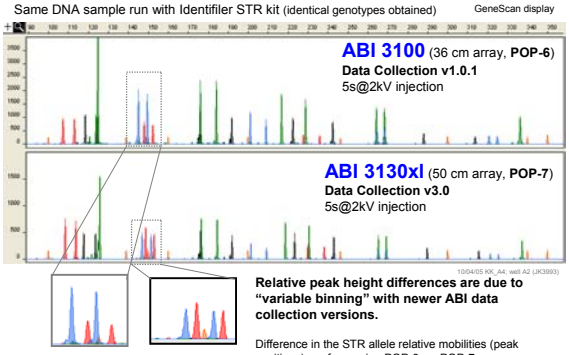
What NIST did to "validate" a 3130xl upgrade

- 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 Identifiler 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

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

Comparison of ABI 3100 Data Collection Versions

Same DNA sample run with Identifiler STR kit (identical genotypes obtained) GeneScan display



Relative peak height differences are due to "variable binning" with newer ABI data collection versions.

Difference in the STR allele relative mobilities (peak positions) are from using POP-6 vs. POP-7.

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 the kit used)**

Setting thresholds for the ABI 310/3100

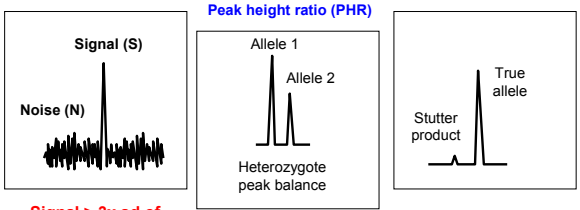
- Where do current ideas on instrument thresholds for the ABI 310/3100 come from?
- How do I set these values in my laboratory?
- Why might they vary from one instrument to the next?
- How do these thresholds affect data interpretation?

Future defense attacks will likely focus on detection thresholds – can you defend your current threshold (e.g., 50 RFU or 150 RFU)?

What is a true peak (allele)?

GeneScan function *Genotyper function*

Peak detection threshold **Peak height ratio (PHR)** **Stutter percentage**



Signal > 3x sd of noise

PHR consistent with single source Typically above 60%

Stutter location above 15%

TWGDAM validation of AmpFlSTR Blue

Wallin *et al.* (1998) *J. Forensic Sci.* 43(4): 854-870

- Minimum cycle # (27-30 cycles examined)
- Amplification adjusted to 28 cycles so that quantities of DNA below 35pg gave very low peaks or no peaks (below the analytical threshold!)
- 35 pg is approx 5 cells
- (but is 35pg the analytical threshold?) Determining this value might be a useful goal of a validation study

TWGDAM validation of AmpFISTR Blue

Wallin *et al.* (1998) *J. Forensic Sci.* 43(4): 854-870

Determination of Minimum Sample

- Goal: avoid situations where peak imbalance results in only one detectable allele from a heterozygous pair.
- Perform serial dilution (1ng- 8pg) of 2 control samples which were heterozygous at all 3 loci
  - **Samples above 125pg had peak height RFUs above 150**
  - **Below 125pg peak heights were not significantly above background**
  - **At 31 pg peaks were very low or undetectable**
- **"Peaks below 150 RFU should be interpreted with caution"** **Why? Noise and stochastic fluctuation!**

Sensitivity of Detection

Moretti et al, JFS, 2001, 46(3), 661-676

- Different 310 instruments have different sensitivities; determination of stochastic threshold should be performed following in-house studies
  - Variations in quantitation systems
  - Variations in amplification systems
  - Variations in instrument sensitivity
- Peaks with heights below the threshold should be interpreted with caution
  - Caution should be used before modification of
    - Amplification cycles
    - Electrophoretic conditions

Sensitivity Study

(Debbie Hobson-FBI)

- 25 Individuals
  - 63 pg to 1 ng amplifications with Profiler Plus and Cofiler
  - amplicon run on five 310s
  - GeneScan Analysis threshold sufficient to capture all data
  - GenoTyper: category and peak height
- Import data into Excel
  - peak height ratios determined for heterozygous data at each locus

Documentation

Documentation of Internal Validation Studies

What is the best way to do this? Standardized format?

Who needs to review?

Who needs to approve?

Should it be presented or published?

From Robyn Ragsdale (FDLE), Validation Workshop (Aug 24-26, 2005 at NFSTC)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

Appropriate Documentation…

- Publications in the Peer-Reviewed Literature
  - See provided reference list
  - http://www.cstl.nist.gov/biotech/strbase/validation.htm
- 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?

ABI 7500 Quantifiler Validation Documentation

http://www.appliedbiosystems.com

Applied Biosystems
an Applera Corporation business

Experimental data supports that the 7500 system with v1.2.3 software provides consistent performance when compared to the ABI PRISM® 7000 Sequence Detection System previously validated for forensic applications. Therefore, the 7500 system can be sold to Human Identification customers at this time. Further guidance for specific operating conditions will follow.

Validation of the Applied Biosystems 7500 Real-Time PCR System with v1.2.3 Software

Applied Biosystems scientists have conducted experiments following the guidance provided by the DAB/SWGDAM to validate the Applied Biosystems 7500 Real-Time PCR System with v1.2.3 software ("7500 System") for use in forensic applications using the Quantifiler Human and Quantifiler Y kits. We are pleased to let you know that the Applied Biosystems 7500 Real-Time PCR System equipped with v1.2.3 software is now validated for use in forensic sample testing pursuant to these guidelines using the Quantifiler kits. We conducted experiments, reviewed data, and determined that the 7500 Real-Time PCR System provides results that are robust, reliable, reproducible and provide accurate results when used in conjunction with the Quantifiler™ Human and Quantifiler™ Y kits for the analysis of genomic DNA samples.

Promega Material Modification Reported for PP16 Primer Mix Storage

http://www.promega.com/applications/hmnid/11072-AN-GI-final.pdf

promega
Application Notes

Amplifications Using the PowerPlex® 16 System and a 10X Primer Pair Mix Stored in TE^{-4} Buffer or in Water Yield Comparable Results

Abstract

Promega is changing the solution used to prepare the 10X Primer Pair Mix in the PowerPlex® 16 System from water to TE^{-4} buffer. We show that the amplification results with the 10X Primer Pair Mix prepared in TE^{-4} buffer are comparable to those with the 10X Primer Pair Mix prepared in water, even when we varied reaction parameters (the amount of DNA template, volume of TE^{-4} buffer, the thermal cycler used, amount of AmpliTaq Gold® DNA polymerase used and primer concentrations). Because the performance with both primer pair formulations is comparable, users of the PowerPlex® 16 System should see no adverse effects on amplification results.

Introduction

DNA is traditionally stored in water or a buffered solution, such as TE^{-4} (10mM Tris-HCl, 0.1mM EDTA [pH 8.0]). DNA stored in TE^{-4} buffer is more stable due to the buffering capacity of Tris and the presence of EDTA (1). For this reason, the 10X Primer Pair Mix for the

DNA polymerase and 1.5mM $MgCl_2$, unless otherwise indicated. DNA templates were diluted in 1× 2µl of TE^{-4} buffer, unless otherwise indicated. Amplifications were carried out using the Perkin-Elmer GeneAmp® PCR System 9600, unless otherwise indicated, for 32 cycles (10/32 cycling). Amplification products were detected using the ABI PRISM® 310 or 3100 Genetic Analyzer and the GeneScan® analysis software. Sample files were imported into the Genotyper® program and analyzed using the PowerTyper™ 16 Macro.

Variations in Amplification Reaction Conditions

Primers stored in TE^{-4} buffer are more stable than primers stored in water (data not shown), but we wanted to be sure that the use of TE^{-4} buffer to resuspend the primers did not affect amplification results. We varied the amplification conditions and compared the results obtained with the two primer pair formulations to determine if there were any effects due to the new formulation.

Validation Section of the DNA Advisory Board Standards

issued July 1998 (and April 1999); published in *Forensic Sci. Comm.* July 2000

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

8.1.1 Developmental validation that is conducted **shall be appropriately documented**.

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

FORENSIC SCIENCE COMMUNICATIONS JULY 2000 VOLUME 2 NUMBER 3

Why is Documentation of Validation Important?

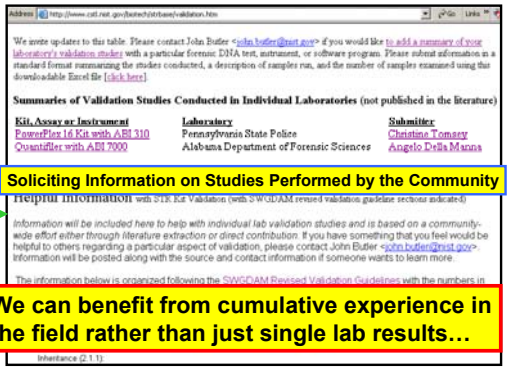
9. Documentation of Validated Methods

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.**

EURACHEM Guide (1998) *The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics*, p. 37; available at http://www.eurachem.ul.pt/guides/valid.pdf

Laboratory Internal Validation Summaries



We invite updates to this table. Please contact John Butler <john.butler@nist.gov> if you would like to add a summary of your laboratory's validation studies with a particular forensic DNA test, instrument, or software program. Please submit information in a standard format summarizing the studies conducted, a description of samples run, and the number of samples examined using this downloadable Excel file [click here].

Summaries of Validation Studies Conducted in Individual Laboratories (not published in the literature)

Kit, Assay or Instrument	Laboratory	Submitter
PowerPlex 16 Kit with ABI 310	Pennsylvania State Police	Christine Tomsey
Quantifiler with ABI 7000	Alabama Department of Forensic Sciences	Angelo Della Manna

Soliciting Information on Studies Performed by the Community

Helpful Information with STR Kit Validation (with SWGDAM revised validation guideline sections indicated)

Information will be included here to help with individual lab validation studies and is based on a community-wide effort either through literature extraction or direct contribution. If you have something that you feel would be helpful to others regarding a particular aspect of validation, please contact John Butler <john.butler@nist.gov>. Information will be posted along with the source and contact information if someone wants to learn more.

The information below is organized following the SWGDAM Revised Validation Guidelines with the numbers in

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

Inheritance (2.1.1)

Example of Validation Documentation

Alabama Department of Forensic Sciences
Birmingham DNA

ABI Prism® 7000 Validation

TABLE OF CONTENTS

TAB	TITLE
8.1.1	Developmental Validation
8.1.3.1(a)	Known and Non-Probative Samples
8.1.3.1(b)	Reproducibility • Quantitation • Peak Height Experiment – ABI 310
8.1.3.1(b)	Precision

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 quantitation method to be used in the forensic casework section of the Alabama Department of Forensic Sciences Birmingham DNA laboratory.

Angelo Della Manna, MSFS, D-ABC
Forensic Biology Discipline Chief
Statewide Technical Leader

Date

Available on STRBase Validation Website:
http://www.cstl.nist.gov/biotech/strbase/validation/ADFS-BH_7000val.pdf

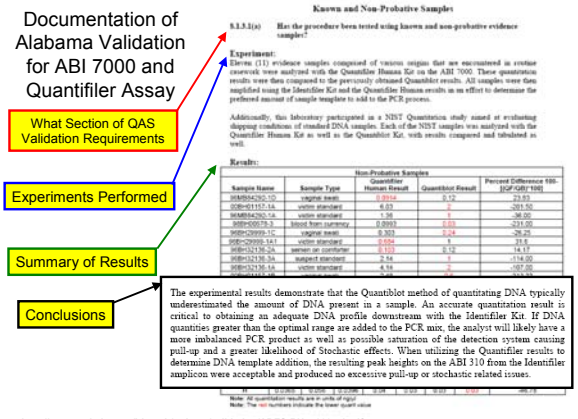
Documentation of Alabama Validation for ABI 7000 and Quantifiler Assay

What Section of QAS Validation Requirements

Experiments Performed

Summary of Results

Conclusions



Known and Non-Probative Samples

9.1.3.1(a) Has the procedure been tested using known and non-probative evidence samples?

Experiment:
Eleven (11) evidence samples composed of various origins that are encountered in routine casework were analyzed with the Quantifiler Human Kit on the ABI 7000. These quantitation results were then compared to the previously obtained Quantiblot results. All samples were then amplified using the Identifiler Kit and the Quantifiler Human results in an effort to determine the preferred amount of sample template to add to the PCR process.

Additionally, this laboratory participated in a NIST Quantitation study aimed at evaluating shipping conditions of standard DNA samples. Each of the NIST samples was analyzed with the Quantifiler Human Kit as well as the Quantiblot Kit, with results compared and tabulated as well.

Results:

Non-Probative Samples

Sample Name	Sample Type	Quantifiler Human Result	Quantiblot Result	Percent Difference 100-[(QF/QB)*100]
06MB04292-1D	vaginal swab	0.0914	0.12	23.83
06B=01157-1A	victim standard	6.83	?	-281.50
06MB04292-1A	victim standard	1.56	?	-36.00
06B=00078-3	blood from currency	0.0093	0.03	-231.00
06B=29999-1C	vaginal swab	0.303	0.24	-26.25
06B=29999-1A1	victim standard	0.654	?	35.6
06B=32136-2A	semen on comforter	0.103	0.12	14.17
06B=32136-3A	suspect standard	2.14	?	-114.00
06B=32136-1A	victim standard	4.14	?	-167.00

The experimental results demonstrate that the Quantiblot method of quantitating DNA typically underestimated the amount of DNA present in a sample. An accurate quantitation result is critical to obtaining an adequate DNA profile downstream with the Identifiler Kit. If DNA quantities greater than the optimal range are added to the PCR mix, the analyst will likely have a more imbalanced PCR product as well as possible saturation of the detection system causing pull-up and a greater likelihood of Stochastic effects. When utilizing the Quantifiler results to determine DNA template addition, the resulting peak heights on the ABI 310 from the Identifiler amplicon were acceptable and produced no excessive pull-up or stochastic related issues.

Note: All quantitation results are in units of ng/µl
Note: The red numbers indicate the lower quant value

http://www.cstl.nist.gov/biotech/strbase/validation/ADFS-BH_7000val.pdf

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.

So, 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)
http://www.cstl.nist.gov/biotech/strbase/validation/validationworkshop.htm

Acknowledgments

National Institute of Justice
The Research, Development, and Evaluation Agency of the U.S. Department of Justice

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- Dave Duewer (NIST)
- Kari Tontarski (Montgomery County Crime Lab)
- Robin Cotton (Orchid Cellmark)
- Tim McMahon (AFDIL)

- **Many members of forensic DNA typing community for their input on our 2004 validation questionnaire**