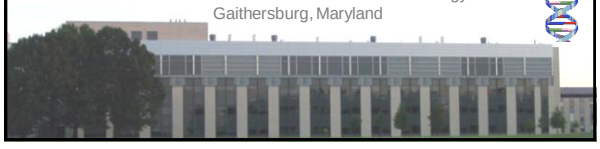
The Copenhagen Forensic Genetic Summer School
Advanced Topics in STR DNA Analysis
June 27-28, 2012

Overview of Validation

Mike Coble and Becky Hill

NIST Applied Genetics Group
National Institute of Standards and Technology
Gaithersburg, Maryland



Some Articles Written on Validation

Profiles in DNA (Promega Corporation), vol. 9(2), pp. 3-6

PROFILES IN DNA

VALIDATION

http://www.promega.com/profiles/902/ProfilesInDNA_902_03.pdf

Debunking Some Urban Legends Surrounding Validation Within the Forensic DNA Community

By John Butler
National Institute of Standards and Technology, Gaithersburg, Maryland, USA

http://marketing.appliedbiosystems.com/images/forensic/volume8/PDFs_submitted/02A_CustomerCorner_Val_What_is_it.pdf

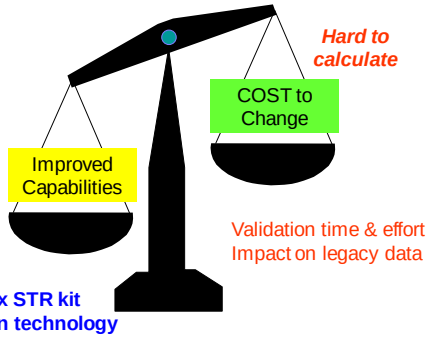
Applied Biosystems

Stages of Technology for Forensic DNA Typing

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

TIME MONEY

Decision to Switch/Upgrade to New Technology



New multiplex STR kit
New detection technology
New DNA markers

Decisions about Changing Technologies

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

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

Constant state of evolution (like computers)

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

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

Importance of Validation

Purpose of Validation

- 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"…

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)

Questions to Keep in Mind…

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

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

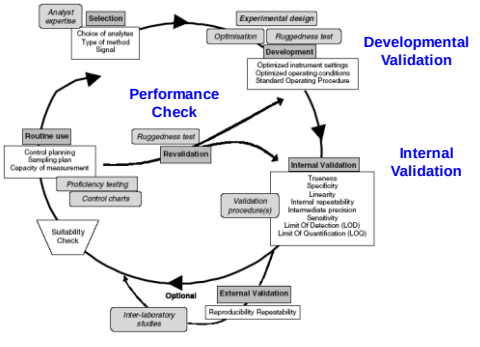
J.M. Butler (2005) *Forensic DNA Typing*, 2nd Edition, p. 391

- **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

General Levels of Validation

- **Developmental Validation** – commonly performed by commercial manufacturer of a novel method or technology (more extensive than internal validation)
- **Internal Validation** – performed by individual lab when new method is introduced
- **Performance Checks** – can be performed with every run (set of samples)

The lifecycle of a method of analysis



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

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

Revised SWGDAM Validation Guidelines

(July 2004)

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

FORENSIC SCIENCE COMMUNICATIONS

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.

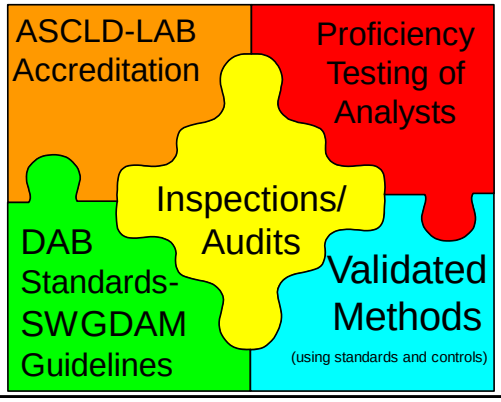
ENFSI Validation Guidelines (November 2010)

http://www.enfsi.eu/get_doc.php?uid=630

ENFSI

Recommended Minimum Criteria for the Validation of Various Aspects of the DNA Profiling Process			
DOCUMENT TYPE :	REF. CODE:	ISSUE NO:	ISSUE DATE:
POLICY	ENFSI DNA WORKING GROUP	001	November 2010

Ensuring Accurate Forensic DNA Results

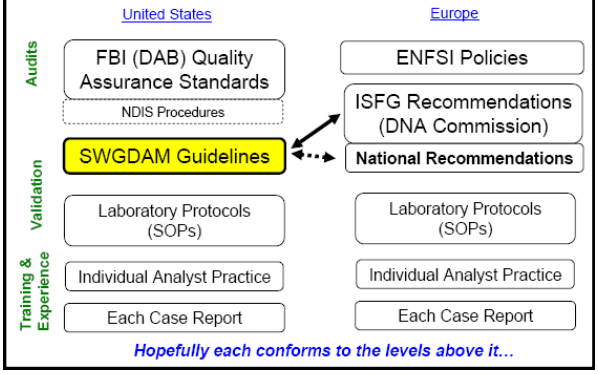


Checks and Controls on DNA Results

Community	FBI DNA Advisory Board's Quality Assurance Standards *(also interlaboratory studies)* ISO17025
Laboratory	ASCLD/LAB Accreditation and Audits
Analyst	Proficiency Tests & Continuing Education
Method/Instrument	**Validation of Performance** ***(along with traceable standard sample)***
Protocol	Standard Operating Procedure is followed
Data Sets	Allelic ladders, positive and negative amplification controls, and reagent blanks are used
Individual Sample	Internal size standard present in every sample
Interpretation of Result	Second review by qualified analyst/supervisor
Court Presentation of Evidence	Defense attorneys and experts with power of discovery requests

My perspective…

Hierarchy of Rules for Forensic DNA Labs



Hopefully each conforms to the levels above it…

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

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

The Community Benefits from Training

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

How do you validate a method?

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

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.

Urban Legends of Validation…

Butler, J.M. (2006) *Profiles in DNA* vol. 9(2), pp. 3-6

#1: HUNDREDS OR THOUSANDS OF SAMPLES ARE REQUIRED TO FULLY VALIDATE AN INSTRUMENT OR METHOD

#2: VALIDATION IS UNIFORMLY PERFORMED THROUGHOUT THE COMMUNITY

#3: EACH COMPONENT OF A DNA TEST OR PROCESS MUST BE VALIDATED SEPARATELY

#4: VALIDATION SHOULD SEEK TO UNDERSTAND EVERYTHING THAT COULD POTENTIALLY GO WRONG WITH AN INSTRUMENT OR TECHNIQUE

#5: LEARNING THE TECHNIQUE AND TRAINING OTHER ANALYSTS ARE PART OF VALIDATION

#6: VALIDATION IS BORING AND SHOULD BE PERFORMED BY SUMMER INTERNS SINCE IT IS BENEATH THE DIGNITY OF A QUALIFIED ANALYST

#7: DOCUMENTING VALIDATION IS DIFFICULT AND SHOULD BE EXTENSIVE

#8: ONCE A VALIDATION STUDY IS COMPLETED YOU NEVER HAVE TO REVISIT IT

Validation Philosophy

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?

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

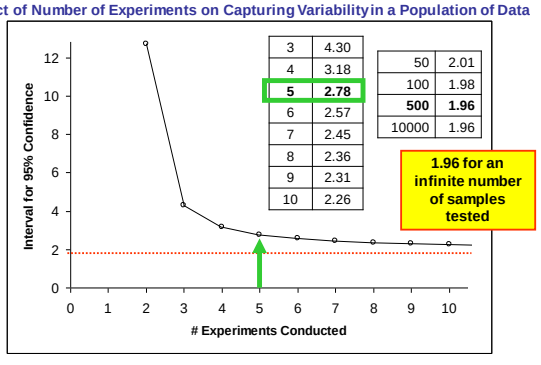
"Student" (real name: W. S. Gossett [1876-1937]) developed statistical methods to solve problems stemming from his employment in a brewery.

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



3	4.30
4	3.18
5	**2.78**
6	2.57
7	2.45
8	2.36
9	2.31
10	2.26

50	2.01
100	1.98
500	**1.96**
10000	1.96

1.96 for an infinite number of samples tested

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)

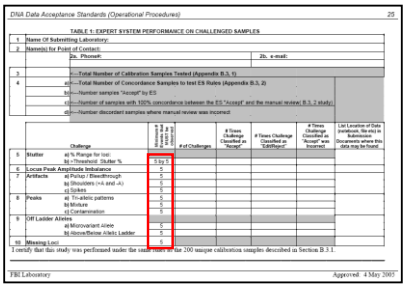

DNA Data Acceptance Standards (Operational Procedures) 25

TABLE 1: EXPERT SYSTEM PERFORMANCE ON CHALLENGED SAMPLES

	Challenge	Minimum # of Challenges to be Observed	# of Challenges	# Times Challenge Classified as "Accept"	# Times Challenge Classified as "Edit/Reject"	# Times Challenge Classified as "Accept" was Incorrect	List Location of Data Internal Validation Documents where this data may be found
5	Stutter	5					
6	Locus Peak Amplitude Imbalance	5					
7	Artifacts	5					
		5					
8	Peaks	5					
		5					
9	Off Ladder Alleles						
		5					
10	Missing Loci	5					

I certify that this study was performed under the same rules as the 100 sample calibration samples described in Section B.3.1.

FBI Laboratory Approved: 4 May 2005

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
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*

Most common allele

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

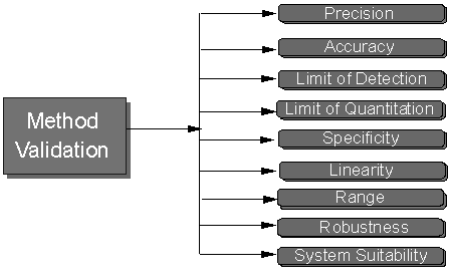
Validation in Other Fields
(Besides Forensic DNA Testing)

Pharmaceutical Industry and FDA Follows ICH Validation Documents

- ICH (**International Conference on Harmonization** of Technical Requirements for Registration of Pharmaceuticals for Human Use)
  - http://www.ich.org
  - **Q2A**: 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."

Useful Resources 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

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

DNA Advisory Board Quality Assurance Standards

Section 2. Definitions

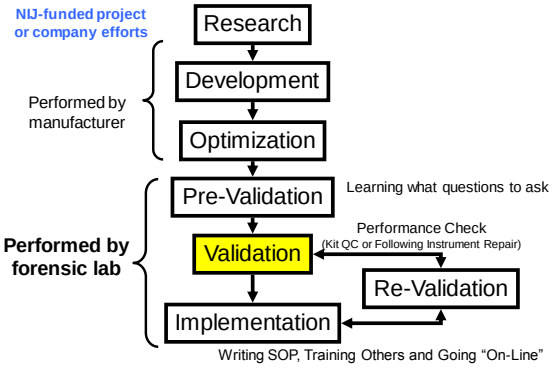
- (ff) Validation is a process by which a procedure is evaluated to determine its **efficacy and reliability** for forensic casework analysis *(DNA analysis)* and includes:

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

How an Assay Evolves



Overview of Developmental Validation Studies

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

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

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

PowerPlex Y Developmental Validation Experiments

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

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

Revised SWGDAM Validation Guidelines (July 2004)

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

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Scientific Working Group on DNA Analysis Methods (SWGDAM)

3. Internal Validation
…a total of **at least 50 samples**
(some studies may not be necessary…)

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).

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DOCUMENT TYPE :	REF. CODE:	ISSUE NO:	ISSUE DATE:
POLICY	ENFSI DNA WORKING GROUP	001	November 2010

[1] When conducting an internal validation, the SWGDAM Revised Validation Guidelines recommend running a total of at least 50 samples—not 50 samples per experiment. (Debunking Some Urban Legends Surrounding Validation Within the Forensic DNA Community by John Butler National Institute of Standards and Technology, Gaithersburg, Maryland, USA – Promega, Profiles in DNA, September 2006)

ENFSI Validation Guidelines (November 2010)

Minimum parameters to be validated : New STR Kits…

- Repeatability: 5 replicates of the same sample.
- Reproducibility : 5 replicates of the same sample (as in the repeatability test) amplified at another time by another person(--> if manually processed).
- Sensitivity (limit of detection) : a series of 5 dilutions tested in three replicates.
- Mixture analysis (not necessary if only reference samples are processed with this kit) : a series of different laboratory defined mixture ratios should be tested in three replicates.
- Analysis of peak balance : check the peak balance of heterozygote alleles within a locus and of alleles between all loci. Acceptable peak balance ratios are > 60% for good quality samples.
- Check stutter ratios by calculating the ratio of the stutter peak height or area compared to the corresponding allele peak height or area. In general, stutter peaks have to be lower than the % of the allele peak height indicated by the manufacturer of the kit to be ignored as a biological artefact of the sample.
- Concordance study : a concordance study must be have been done using PCR products that have previously given full, balanced profiles.

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

Design of Experiments Conducted for Validation Studies

- Before performing a set of experiments for validation, ask yourself:
  - **What is the purpose of the study?**
  - **Do we already know the answer?**
  - **Can we write down how we know the answer?**
- Think before you blindly perform a study which may have no relevance (e.g., extensive precision studies)
- **Too often we do not differentiate learning, validation, and training**

Points for Consideration

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

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

Steps Surrounding "Validation" in a Forensic Lab

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

New NIST Software Tools

Developed by Dave Duewer (NIST)

From NIST STRBase Website:

http://www.cstl.nist.gov/biotech/strbase/tools/STR_MatchSamples.xls

STR_MatchSamples

- An Excel-based tool developed to aid comparison of STR genotypes from two or more data sets.

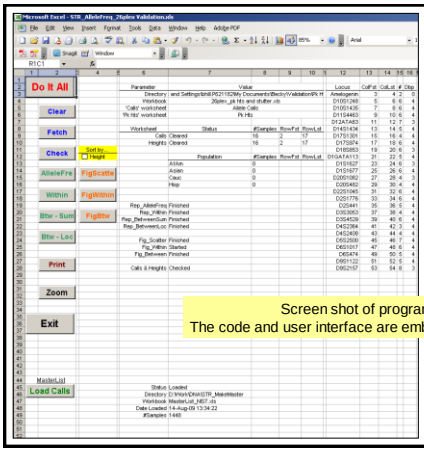
Tools under development (to aid validation studies)

- Peak height ratio
- Inter-locus balance
- Stutter percentages
- Allele frequency

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

Analysis Software

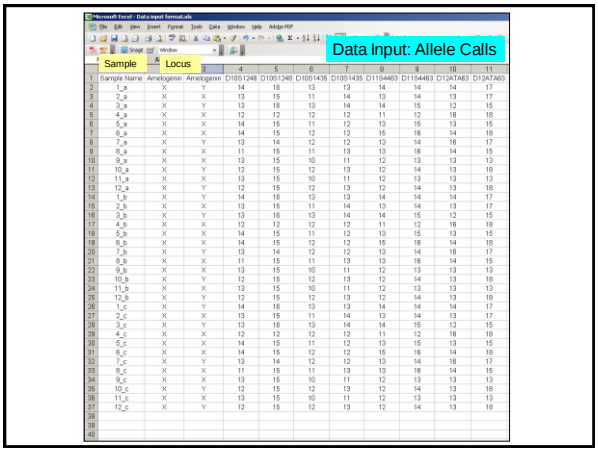
- Currently under development at NIST by Dr. David Duewer
- Performs calculations for
  - Allele frequencies
  - Intralocus signal balance (heterozygotes)
  - Interlocus signal balance ('multiplex balance')
  - Stutter
- Enables rapid analysis of internal validation data

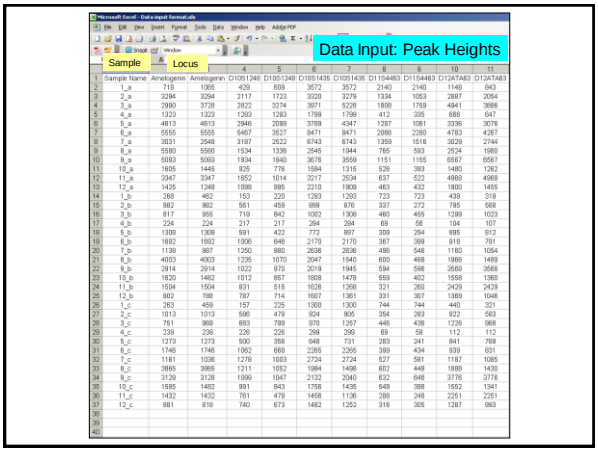


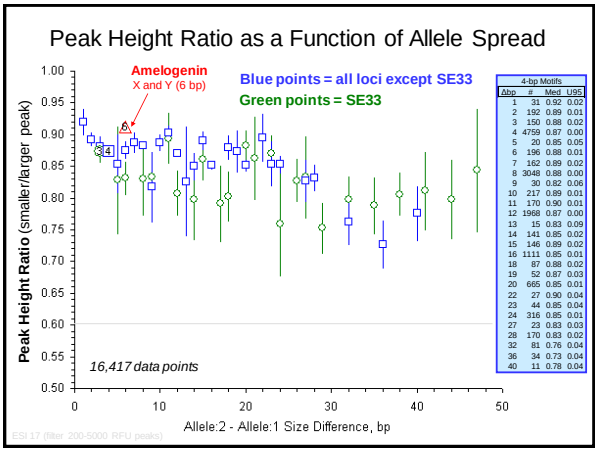
Screen shot of program
The code and user interface are embedded in excel

Program Data Input

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

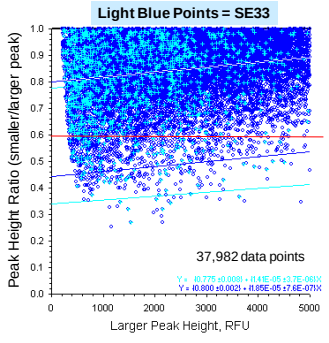

Data Input: Allele Calls
Sample
Locus


Data Input: Peak Heights
Sample
Locus


Peak Height Ratio as a Function of Allele Spread
Amelogenin
X and Y (6 bp)
Blue points = all loci except SE33
Green points = SE33
4-bp Motifs
Abp # Med U95
1 31 0.92 0.02
2 192 0.89 0.01
3 150 0.88 0.02
4 4759 0.87 0.00
5 20 0.85 0.05
6 196 0.88 0.01
7 162 0.89 0.02
8 3048 0.88 0.00
9 30 0.82 0.06
10 217 0.89 0.01
11 170 0.90 0.01
12 1968 0.87 0.00
13 15 0.83 0.09
14 141 0.85 0.02
15 146 0.89 0.02
16 1111 0.85 0.01
18 87 0.88 0.02
19 52 0.87 0.03
20 665 0.85 0.01
22 27 0.90 0.04
23 44 0.85 0.04
24 316 0.85 0.01
27 23 0.83 0.03
28 170 0.83 0.02
32 81 0.76 0.04
36 34 0.73 0.04
40 11 0.78 0.04
Peak Height Ratio (smaller/larger peak)
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
16,417 data points
0
10
20
30
40
50
Allele:2 - Allele:1 Size Difference, bp
CSI 17 (filter 200-5000 RFU peaks)

Peak Height Ratios with Full Data Set

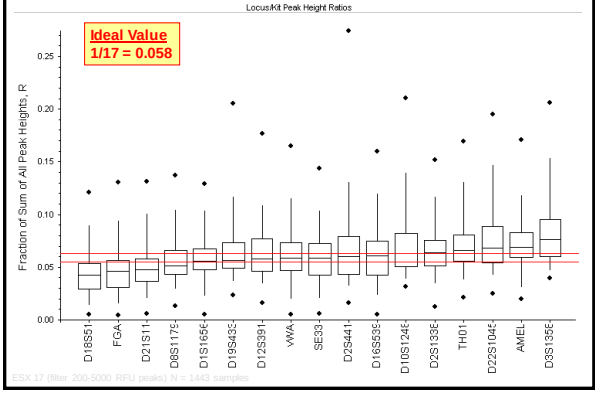
All Loci Included



SE33 Data (Average = 0.80)

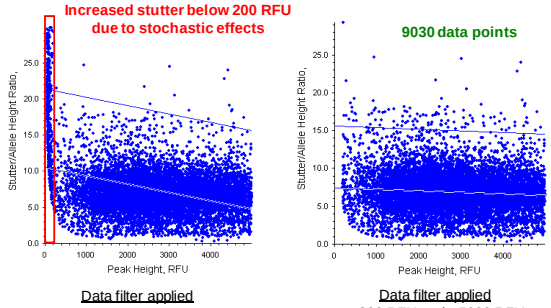
Locus	Δbp	#	Mean X	Mean s(X)
SE33	3	138	0.831	0.145
	5	12	0.795	0.146
	6	123	0.790	0.147
	8	13	0.775	0.144
	9	97	0.808	0.144
	11	26	0.855	0.144
	12	65	0.801	0.144
	14	28	0.784	0.145
	15	49	0.821	0.145
	17	33	0.755	0.145
	18	42	0.794	0.143
	20	59	0.859	0.143
	21	23	0.812	0.146
	23	54	0.846	0.145
	24	21	0.767	0.147
	26	67	0.796	0.146
	27	11	0.817	0.146
	29	68	0.735	0.147
	32	63	0.768	0.143
	35	63	0.776	0.146
	38	44	0.808	0.142
	41	41	0.787	0.146
	44	18	0.797	0.132
	47	11	0.798	0.146

PowerPlex® ESX 17 Inter-Locus Balance



PowerPlex® ESI 17 Stutter Ratios

by Peak Height across All Loci



Data filter applied
>50 RFU and <5000 RFU

Data filter applied
>200 RFU and <5000 RFU

Trinucleotide **D22S1045** Stutter Percentages

ESX 17

Allele	Size	Stutter #	Stutter Median
10	84.5	21	1.8
11	87.4	134	3.0
12	90.4	37	4.2
14	96.4	51	7.2
15	99.4	165	8.9
16	102.4	120	10.5
17	105.5	105	14.7
	Avg	633	7.2
	SD		4.6

633 data points

Avg + 3SD 21.0%

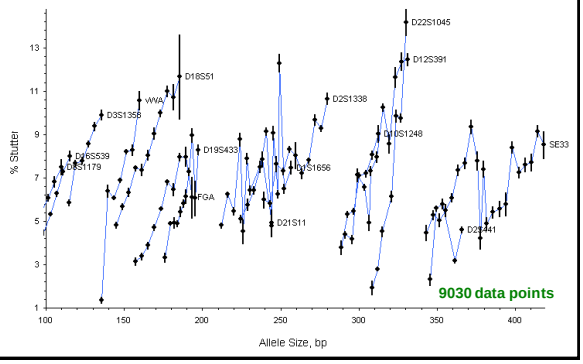
ESI 17

Allele	Size	Stutter #	Stutter Median
10	308.7	22	1.9
11	311.8	98	2.8
12	314.8	32	4.5
14	321.0	36	6.1
15	324.0	150	9.9
16	327.1	94	9.8
17	330.1	95	14.2
	Avg	527	7.0
	SD		4.4

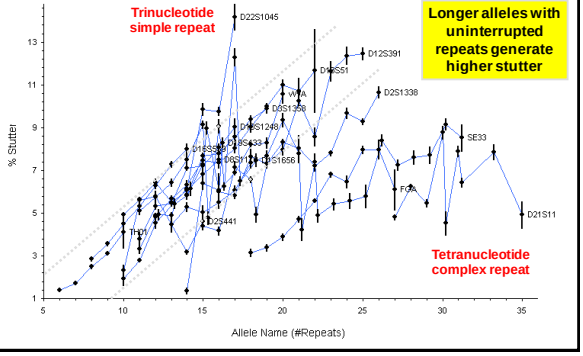
527 data points

Avg + 3SD 20.2%

PowerPlex® ESI 17 Stutter Ratios
by PCR Product Size across All Loci



PowerPlex® ESI 17 Stutter Ratios
by Repeat Number across All Loci



Finer Detail on the Stutter Calculations

D22S1045 Stutter

Allele	Size	Percentages (ESX 17) #	Stutter Median
10	84.5	21	1.8
11	87.4	134	3.0
12	90.4	37	4.2
14	96.4	51	7.2
15	99.4	165	8.9
16	102.4	120	10.5
17	105.5	105	14.7
	Avg	633	7.2
	SD		4.6

633 data points

Avg + 3SD
21.0%

D1S1656 Stutter

Allele	Size	Percentages (ESI 17) #	Stutter Median
11	224.6	39	5.1
12	228.9	75	5.8
13	232.9	55	6.4
14	237.0	92	7.5
15	241.0	92	9.1
15.3	244.2	29	4.8
16	245.2	90	9.1
16.3	248.3	47	6.2
17	249.2	31	12.3
17.3	252.4	53	6.5
18.3	256.5	27	7.5
	Avg	630	7.3
	SD		2.2

Avg + 3SD
13.9%

An Example Multiplex STR Assay Validation

- The NIST 26plex assay
- Uses of the assay
- Internal Validation
  - Experiments and Results

Work performed by Becky Hill and Pete Vallone (NIST)

More Loci are Useful
in Situations Involving Relatives

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

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

Additional loci were originally selected as candidates for miniSTR assays

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

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

Coble, M.D. and Butler, J.M. (2005) Characterization of new miniSTR loci to aid analysis of degraded DNA. *J. Forensic Sci.* 50: 43-53
Hill, C.R., Kline, M.C., Coble, M.D., Butler, J.M. (2008) Characterization of 26 miniSTR loci for improved analysis of degraded DNA samples. *J. Forensic Sci.* 53(1):73-80

NC Miniplexes

NC01	NC02	NC03	NC04
D10S1248	D1S1677	D3S3053	D1GATA113
D14S1434	D2S441	D6S474	D2S1776
D22S1045	D4S2364	D20S482	D4S2408

NC05	NC06	NC07	NC08
D1S1627	D3S4529	D9S1112	D17S1301
D8S1115	D9S2157	D12ATA63	D18S8534
D9S324	D10S1430	D14S1280	D20S1082

NC09	NC10
D10S2327	D3S3053
D11S4463	D6S474
D17S974	D20S482

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

26 New STR Loci for Human Identity Testing

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

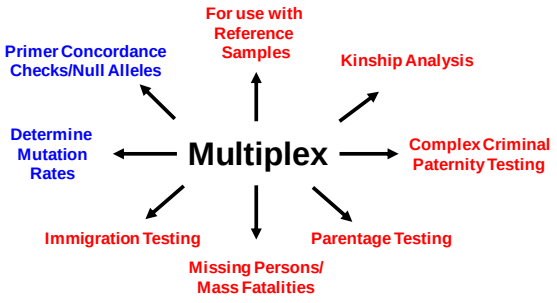
Utility of miniplexes
- Degraded DNA
- Low copy number analysis

EDNAP degraded DNA study shows value of miniSTR assay
Dixon *et al. Forensic Sci. Int.* 164: 33-44

Europe adopts new loci D10S1248, D14S1434 and D22S1045
Gill *et al. Forensic Sci. Int.* 2006;156:242-244
D2S441 replaces D14S1434
Gill *et al. Forensic Sci. Int.* 2006;163:155-157

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

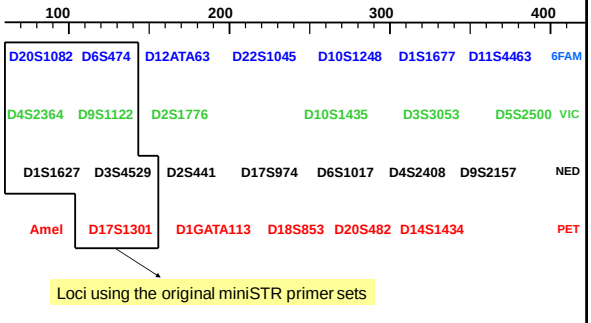
Why Build a Large Multiplex?



Reference Multiplex

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

26plex Schematic



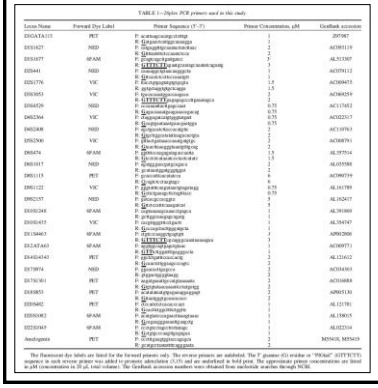
Developmental Validation

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

http://www.cstl.nist.gov/biotech/strbase/pub_pres/Promega2007_NewSTRloci.pdf

Hill, C.R., Butler, J.M., and Vallone, P.M. (2009) A new 26plex assay for use in human identity testing. *J. Forensic Sci. 54:* 1008-1015

PCR Primers and Concentrations



52 primers
26 dye labeled

6FAM (7)
VIC (6)
NED (7)
PET (6)
Purchased from (Applied Biosystems)

Primer concentrations specified in the paper

Range 0.75 to 6 μM

Hydrated in low salt 10 mM Tris-HCl 0.1 mM EDTA buffer (to suppress dye artifacts)

PCR Conditions

- Master Mix (final concentrations listed)
  - 2 mM $MgCl_2$
  - 1x PCR Buffer (supplied with Taq Gold)
  - 1 Unit TaqGold
  - ~0.2 μM Primer mix (varies by locus)
  - 250 mM dNTPs
  - 0.16 mg/mL BSA
- 20 μL reaction volume
  - target input DNA ~1 ng

PCR Conditions

	Stock	08-10-2009 26plex	Desired PCR conc	Volumes		# of Reactions
	conc.	Total volume of Reaction	20	to add		14
mM	25	Mg concentration (micromolar)	2	1.6	uL	22.4
uM	2	Primer concentration (micromolar)	0.2	2	uL	28
U/uL	5	units of Taq (units)	1	0.2	uL	2.8
mM	10	dNTP concentration (micromolar)	250	0.5	uL	7
x	10	PCR Buffer	1	2	uL	28
	3.2	BSA	0.16	1	uL	14
		Water to add		11.7	uL	163.8
		Master Mix volume		19		266
		Volume of added template (uL)	1			

add 19µL MM +
1µL sample = 20 µL rxn

Protocol template in excel

Thermal Cycling Conditions

Conditions for GeneAmp 9700 (9600 emulation mode)

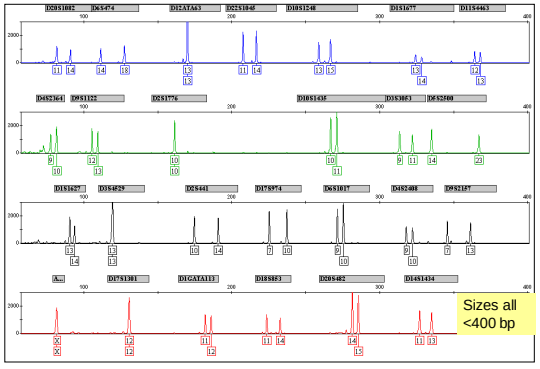
- 95˚C Hot Start for 11 min
- 30 cycles
  - 94˚C for 45 sec Denaturation
  - 59˚C for 2 min Annealing
  - 72˚C for 1 min Elongation
- 60˚C soak for 60 min
- 25˚C hold

~3.5 hours

CE Conditions

- Amplification products were diluted in Hi-Di formamide and GS500-LIZ internal size standard
- Analyzed on the 16-capillary ABI Prism 3130xl Genetic Analyzer
- Prior to electrophoresis, a 5-dye matrix was established under the "G5 filter" with the five dyes of 6FAM, VIC, NED, PET, and LIZ.
- POP-6 polymer was utilized for separations on a 36 cm array
- Samples were injected electrokinetically for 10 sec at 3 kV
- Fragments separated at 15 kV at a run temperature of 60ºC
- Data analyzed using GeneMapperID v3.2
- Bins and panels for the multiplex are available on STRBase
  (http://www.cstl.nist.gov/biotech/strbase/str26plex.htm#Bins-and-Panels)

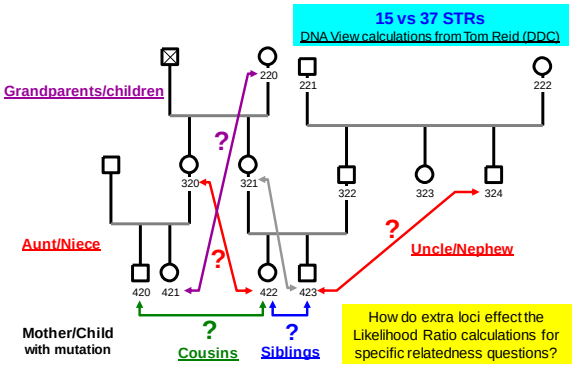
26plex Profile for 9947A



Example Use of the Assay

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

Extended Family Sample Testing



Comparison of Likelihood Ratios

Relationship Examined	**15 STRs** (Identifiler, ID15)	ID15 + 22 NC STRs = **37 loci** (A37)
Mother/Child* (*with single mutation)	0.214	**5,200,000** Extra loci help…
Siblings	477	**113,000** Extra loci help…
Uncle/Nephew	824	**247,000** Extra loci help…
Cousins	0.45	**2.25**
Grandparents/ Grandchildren	0.53	**1.42**

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

Use of the 26plex in Your Lab?

Perform an Internal Validation

- Review the literature on the 26plex assay
- Purchase primers
- TaqGold polymerase + buffers
- Prepare primer mix
  - Proper concentrations (follow paper)
  - Use a low salt tris buffer (dyes)
- Use the NIST SRM (9947A & 9948)

Revised Validation Guidelines
Scientific Working Group on DNA Analysis Methods (SWGDAM)

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

We are using these guidelines as a starting point for designing our internal validation experiments

These should be modified as appropriate for specific laboratory requirements

- 3.2 Reproducibility and precision: The laboratory must document the reproducibility and precision of the procedure using an appropriate control(s).
  - Examination of sizing precision on identical alleles
- 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.
  - Multiple injections and implementing sizing precision (bins and panels)

Forensic Science Communications July 2004 – Volume 6 – Number 3

Revised Validation Guidelines
Scientific Working Group on DNA Analysis Methods (SWGDAM)

- 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.
  - Sensitivity study
- 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., postcoital vaginal swabs).
  - Simple mixture study
- 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.
  - Negative controls
- 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.
  - Another analyst will run 12 samples (the NIST SRM)

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Experiments

71 amplification reactions
16 unique samples
8 injections on 3130

Mixture

	1	2	3	4	5	6	7	8	9	10	11	12
A	neg	neg	neg	neg	neg	neg	neg	SRM_08	neg		neg	SRM_08
B	1 ng	1 ng	1 ng	1 ng	1 ng	1 ng	SRM_01	SRM_09	Mix 0_1		SRM_01	SRM_09
C	0.5 ng	0.5 ng	0.5 ng	0.5 ng	0.5 ng	0.5 ng	SRM_02	SRM_10	Mix 1_9		SRM_02	SRM_10
D	0.25 ng	0.25 ng	0.25 ng	0.25 ng	0.25 ng	0.25 ng	SRM_03	SRM_11	Mix 1_3		SRM_03	SRM_11
E	0.125 ng	0.125 ng	0.125 ng	0.125 ng	0.125 ng	0.125 ng	SRM_04	SRM_12	Mix 1_1		SRM_04	SRM_12
F	0.060 ng	0.060 ng	0.060 ng	0.060 ng	0.060 ng	0.060 ng	SRM_05		Mix 3_1		SRM_05	
G							SRM_06		Mix 9_1		SRM_06	
H							SRM_07		Mix 1_0		SRM_07	

Sensitivity

2 samples
5 dilutions
triplicate

Concordance
NIST SRM 2391b
12 components

Injected 3 times for Precision

Qualifying run

SRM run by
different analyst

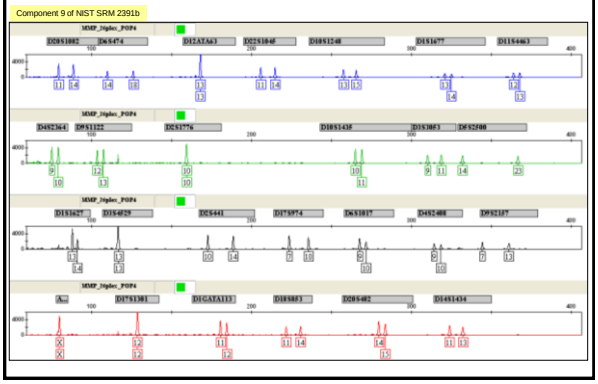
Detection threshold 50 RFUs

Concordance Study

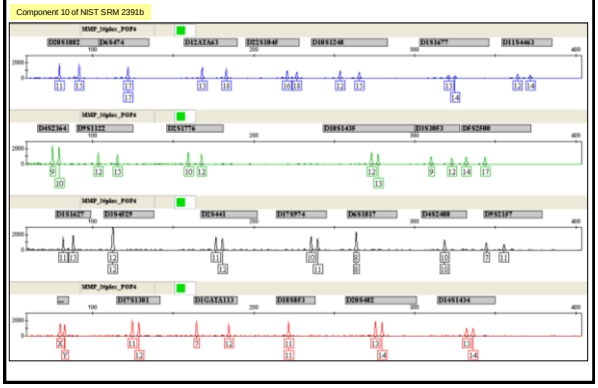
NIST SRM 2391b

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

9947A



9948



SRM 2391b

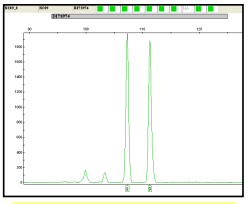
Sample Name	Amelogenin	D10S1248	D10S1435	D11S4463	D12ATA63	D14S1434	D17S1301	D17S974	D18S853	D1GATA113	D1S1627	D1S1677	D20S1082
Component 1	X,Y	14,16	13,13	14,14	14,17	13,14	11,11	9,11	11,14	11,11	10,14	12,13	11,15
Component 2	X,X	13,15	11,14	13,14	13,17	11,13	11,12	9,10	11,11	12,13	13,14	14,16	14,15
Component 3	X,Y	13,16	13,14	14,15	12,15	14,15	11,12	9,9	11,11	11,11	13,14	14,17	11,11
Component 4	X,X	12,12	12,12	11,12	16,18	10,11	12,13	7,9	11,13	13,13	11,12	14,15	14,15
Component 5	X,X	14,15	11,12	13,15	13,15	13,14	11,11	11,12	10,15	11,12	14,15	13,14	11,14
Component 6	X,X	14,15	12,12	15,16	14,18	13,14	11,11	9,9	11,14	11,12	11,13	13,14	11,15
Component 7	X,Y	13,14	12,12	13,14	16,17	10,14	11,12	11,11	14,14	10,12	11,14	12,13	14,15
Component 8	X,X	11,15	11,13	13,16	14,15	13,13	12,12	8,8	12,13	10,12	13,14	14,16	11,15
Component 9	X,X	13,15	10,11	12,13	13,13	11,13	12,12	7,10	11,14	11,12	13,14	13,14	11,14
Component 10	X,Y	12,15	12,13	12,14	13,18	13,14	11,12	10,11	11,11	7,12	11,13	13,14	11,15
Component 11	X,X	13,15	10,11	12,13	13,13	11,13	12,12	7,10	11,14	11,12	13,14	13,14	11,14
Component 12	X,Y	12,15	12,13	12,14	13,18	13,14	11,12	10,11	11,11	7,12	11,13	13,14	11,15

Sample Name	D20S482	D22S1045	D2S1776	D2S441	D3S3053	D3S4529	D4S2364	D4S2408	D5S2500	D6S1017	D6S474	D9S1122	D9S2157
Component 1	14,14	14,15	11,12	11,14	9,12	14,15	9,9	10,10	17,18	10,10	15,17	11,12	8,13
Component 3	15,15	15,16	8,10	10,14	9,11	14,16	9,10	8,9	17,18	10,12	14,15	12,12	11,13
Component 4	14,15	17,18	11,12	12,14	11,11	15,16	9,9	9,10	17,18	7,10	14,16	12,12	11,11
Component 5	14,15	11,14	12,13	11,14	11,11	13,15	9,10	10,11	14,15	8,9	15,18	11,13	7,14
Component 6	14,14	11,15	11,12	10,11	9,9	15,17	8,9	9,9	14,18	10,10	14,17	11,12	11,13
Component 7	14,14	11,15	11,12	11,14	11,11	14,16	9,9	8,11	14,20	7,12	15,17	11,12	12,15
Component 8	15,16	16,17	11,12	11,11.3	9,9	14,14	9,9	11,11	14,18	10,12	17,17	13,13	11,11
Component 9	14,15	11,14	10,10	10,14	9,11	13,13	9,10	9,10	14,23	9,10	14,18	12,13	7,13
Component 10	13,14	16,18	10,12	11,12	9,12	12,12	9,10	10,10	14,17	8,8	17,17	12,15	7,11
Component 11	14,15	11,14	10,10	10,14	9,11	13,13	9,10	9,10	14,23	9,10	14,18	12,13	7,13
Component 12	13,14	16,18	10,12	11,12	9,12	12,12	9,10	10,10	14,17	8,8	17,17	12,15	7,11

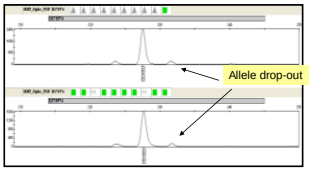
Concordance check

One Discordant Sample

Genomic component 8
8,9 at D17S974
As certified in the NIST SRM 2391b
Amplified with miniSTR primers



Genomic component 8
8,8 at D17S974
Amplified with 26plex primers

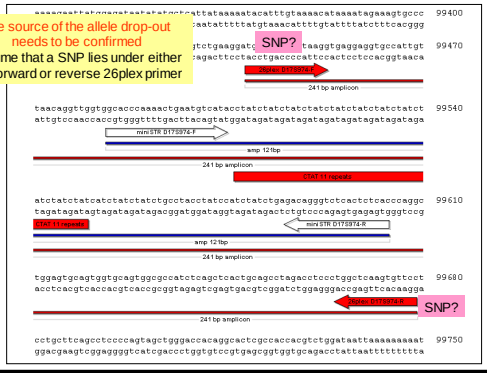


Run in duplicate

Alleles confirmed by sequencing

Map of D17S974

The source of the allele drop-out needs to be confirmed
Assume that a SNP lies under either the forward or reverse 26plex primer



Genotypes for some common samples

	Standard DNA Template Genotypes				SRM 2391b Components							
Locus	9947A	9948	ABI 007	K562	Genomic 1	Genomic 2	Genomic 3	Genomic 4	Genomic 5	Genomic 6	Genomic 7	Genomic 8
D1GATA113	11,12	7,12	12,12	11,12	11,11	12,13	11,11	13,13	11,12	11,12	10,12	10,12
D1S1627	13,14	11,13	11,14	10,14	10,14	13,14	13,14	11,12	14,15	11,13	11,14	13,14
D1S1677 (NC02)	13,14	13,14	13,13	13,14	12,13	14,16	14,17	14,15	13,14	13,14	12,13	14,16
D2S441 (NC02)	10,14	11,12	14,15	10,14	11,14	11,14	10,14	12,14	11,14	10,11	11,14	11,11.3
D2S1776	10,10	10,12	8,10	11,11	11,12	11,11	8,10	11,12	12,13	11,12	11,12	11,12
D3S3053	9,11	9,12	9,9	12,12	9,12	10,11	9,11	11,11	11,11	9,9	11,11	9,9
D3S4529	13,13	12,12	13,13	14,14	14,15	13,16	14,16	15,16	13,15	15,17	14,16	14,14
D4S2364 (NC02)	9,10	9,10	9,10	9,9	9,9	9,10	9,10	9,9	9,10	8,9	9,9	9,9
D4S2408	9,10	10,10	10,11	10,11	10,10	9,9	8,9	9,10	10,11	9,9	8,11	11,11
D5S2500	14,23	14,17	17,18	14,14	17,18	17,24	17,18	17,18	14,15	14,18	14,20	14,18
D6S474	14,18	17,17	14,14	15,18	15,17	14,17	14,15	14,16	15,18	14,17	15,17	17,17
D6S1017	9,10	8,8	10,10	8,11	10,10	10,12	10,12	7,10	8,9	10,10	7,12	10,12
D8S1115	9,18	15,17	15,17	16,16	16,16	16,16	16,17	9,17	9,15	9,16	9,18	15,16
D9S1122	12,13	12,15	12,12	10,14,15	11,12	12,13	12,12	12,12	11,13	11,12	11,12	13,13
D9S2157	7,13	7,11	13,13	13,13	8,13	9,11	11,13	11,11	7,14	11,13	12,15	11,11
D10S1248 (NC01)	13,15	12,15	12,15	12,12	14,16	13,15	13,16	12,12	14,15	14,15	13,14	11,15
D10S1435	10,11	12,13	11,13	10,12	13,13	11,14	13,14	12,12	11,12	12,12	12,12	11,13
D11S4463	12,13	12,14	14,14	13,14	14,14	13,14	14,15	11,12	13,15	15,16	13,14	13,16
D12ATA63	13,13	13,16	13,17	17,17	14,17	13,17	12,15	16,18	13,15	14,18	16,17	14,15
D14S1434 (NC01)	11,13	13,14	11,14	10,10	13,14	11,13	14,15	10,11	13,14	13,14	10,14	13,13
D17S974	7,10	10,11	9,10	8,8	9,11	9,10	9,9	7,9	11,12	9,9	11,11	8,9*
D17S1301	12,12	11,12	12,13	11,12	11,11	11,12	11,12	12,13	11,11	11,11	11,12	12,12
D18S853	11,14	11,11	11,11	12,15	11,14	11,11	11,11	11,13	10,15	11,14	14,14	12,13
D20S482	14,15	13,14	14,15	15,15	14,14	14,16	15,15	14,15	14,15	14,14	14,14	15,16
D20S1082	11,14	11,15	12,14	11,11	11,15	14,15	11,11	14,15	11,14	11,15	14,15	11,15
D22S1045 (NC01)	11,14	16,18	11,16	16,16	14,15	11,16	15,16	17,18	11,14	11,15	11,15	16,17

*allele drops out with 26plex

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

Previous Concordance Study

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

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

Sensitivity Study

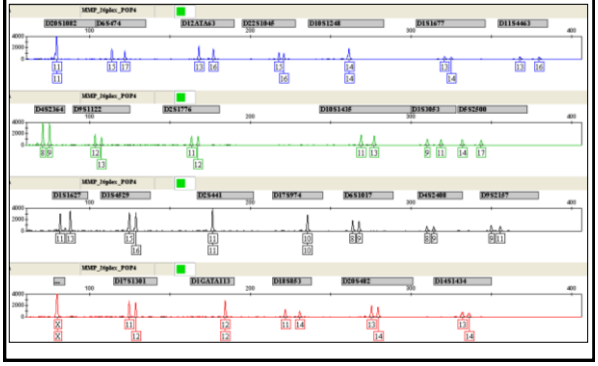
- Run 2 unique samples in triplicate
  1. 1 ng
  2. 0.5 ng
  3. 0.25 ng
  4. 0.125 ng
  5. 0.060 ng
- Sample concentration determined with Quantifiler prior to sensitivity study

Serial Dilution

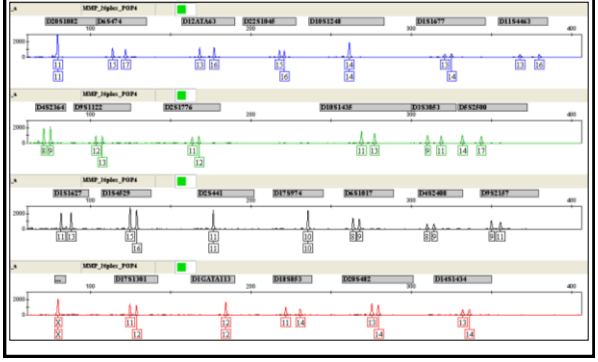
- Prepare serial dilution to use 2 μL volume per PCR reaction
- Prepare 20 μL of each concentration point (enough volume to run triplicate experiments)
- Example for stock sample 4.5 ng/μL

ng in 2 uL	ng/uL	Stock conc	Vol to add (uL)	Water	Total Volume
1	0.5	4.5 ng/uL	2.2	17.8	20
500	0.25	0.5 ng/uL	10	10	20
250	0.125	0.25 ng/uL	10	10	20
125	0.0625	0.125 ng/uL	10	10	20
60	0.03	0.0625 ng/uL	9.6	10.4	20

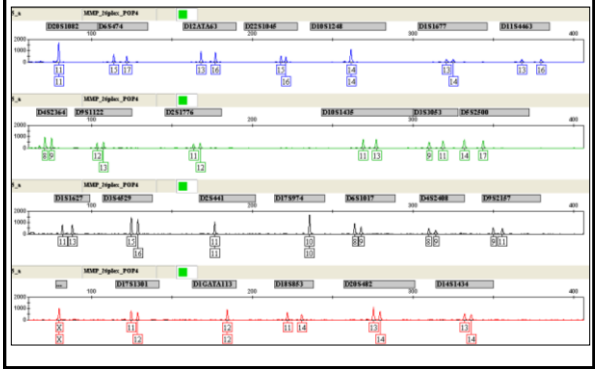
Sensitivity (Sample 1) 1 ng



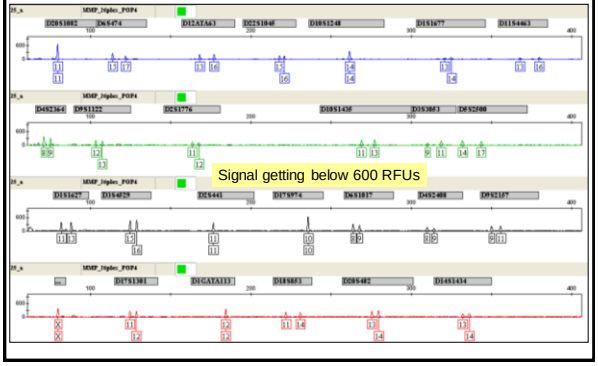
Sensitivity (Sample 1) 0.5 ng



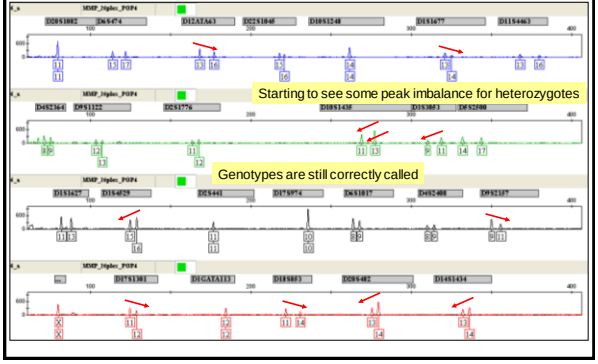
Sensitivity (Sample 1) 0.25 ng



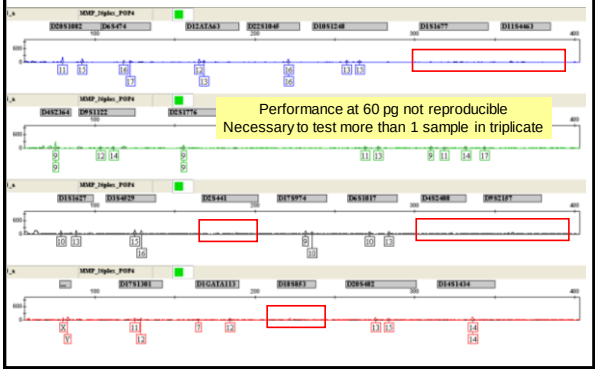
Sensitivity (Sample 1) 0.125 ng



Sensitivity (Sample 1) 0.06 ng



Sensitivity (Sample 2) 0.06 ng



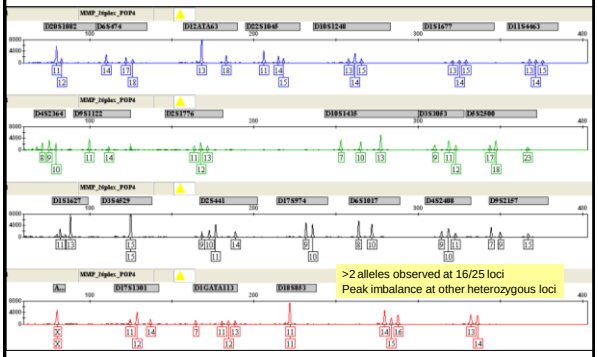
Sensitivity Study Conclusions

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

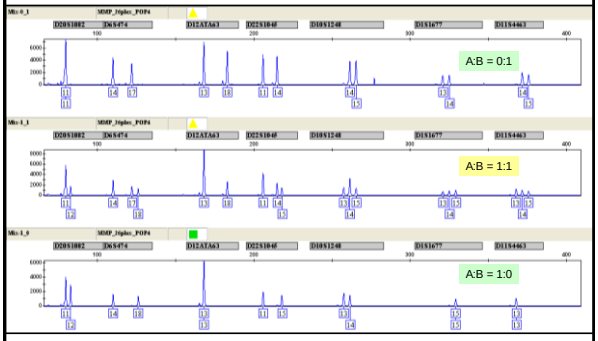
Mixture Study

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

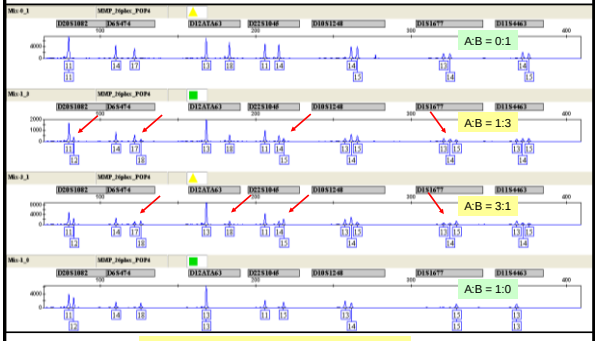
1:1 Mixture



Comparing Controls to 1:1 mixture

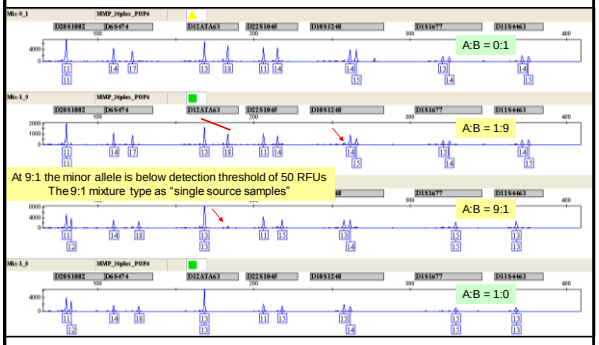


Comparing Controls to 3:1 mixtures



The minor allele is detectable in 3:1 mixtures

Comparing Controls to 9:1 mixtures

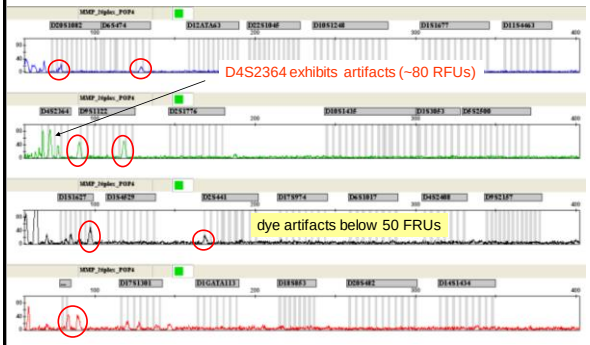


Mixture Study Conclusions

- The 26plex is capable of detecting a mixture ratio of 1:1 and 3:1
- At 9:1 the minor alleles are not called (detection threshold 50 RFUs)
- The assay is fit for our purposes - running single source reference samples (but we should be able to detect a significant mixture)

Negative Controls

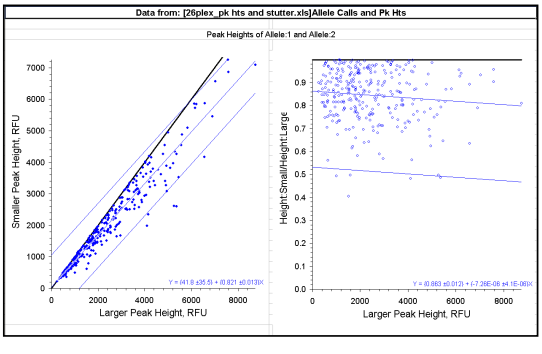
Negative Control



Qualifying Run

- Someone else (qualified person!) in the lab should run the assay on the same samples used in the validation experiments
  - Provided analyst with 26plex primer mix and assay protocol
- 12 components of the NIST SRM 2391b
- 100% concordance was observed with previously called genotypes

Peak Height Ratios



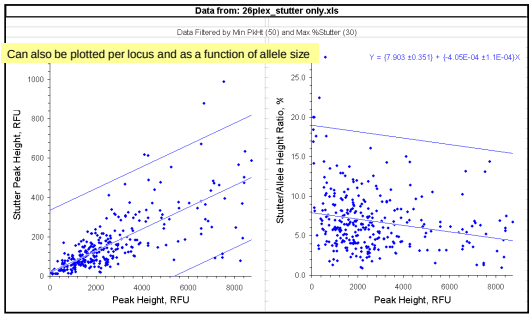
Peak Height Ratios

- An example data table

Locus	Δbp	#	Mean X	Mean s(X)
D10S1248	4	11	0.82	0.10
	8	8	0.83	0.01
	12	5	0.89	0.06
	16	1	0.87	na

Locus	Δbp	#	Mean X	Mean s(X)
D11S4463	4	8	0.88	0.08
	8	4	0.85	0.08
	12	2	0.82	0.07

Stutter



Stutter

- An example data table

D10S1248

Sample	Allele	Size S	Size P	Height S	Height P	S/P Ratio
12_a	15	261.52	265.51	103	995	10.352
10_a	15	261.58	265.50	77	776	9.923
8_a	15	261.40	265.43	127	1336	9.506
3_a	16	265.42	269.39	307	3274	9.377
11_a	15	261.43	265.46	90	1014	8.876
9_a	15	261.44	265.40	161	1840	8.750
2_a	13	253.49	257.49	184	2117	8.692
2_a	15	261.43	265.44	143	1723	8.299
3_a	13	253.55	257.50	229	2822	8.115
9_a	13	253.46	257.48	156	1934	8.066
11_a	13	253.46	257.47	107	1652	6.477
12_a	12	249.60	253.57	65	1099	5.914
10_a	12	249.61	253.63	53	925	5.730
4_a	12	249.53	253.47	73	1283	5.690
8_a	11	245.54	249.47	79	1534	5.150
6_a	17	269.83	272.94	12	916	1.310
					avg	7.514
					std	2.331919

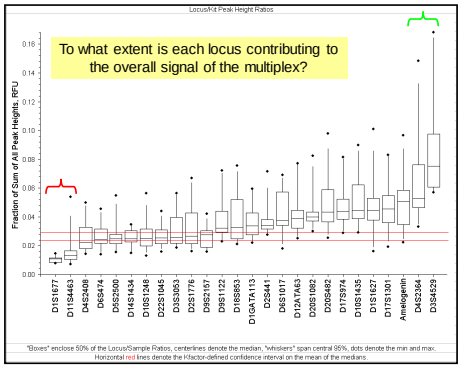
D11S4463

Sample	Allele	Size S	Size P	Height S	Height P	S/P Ratio
8_a	16	375.44	379.43	60	593	10.118
5_a	15	371.52	375.43	84	1061	7.917
1_a	14	367.61	371.48	169	2140	7.897
10_a	14	367.85	371.66	28	393	7.125
8_a	13	363.85	367.75	54	765	7.059
12_a	12	359.85	363.71	32	463	6.911
5_a	13	363.73	367.57	87	1297	6.708
12_a	14	367.67	371.60	28	432	6.481
10_a	12	359.83	363.76	30	526	5.703
					avg	7.324
					std	1.249151

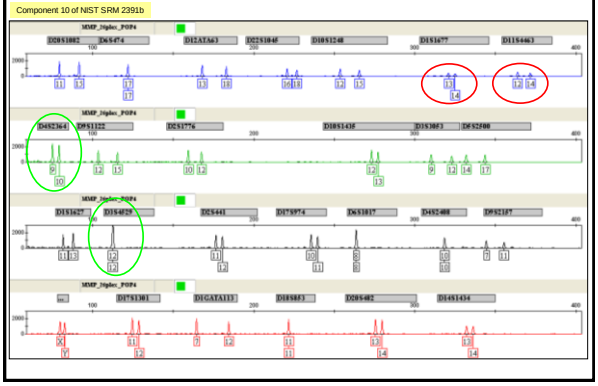
Interlocus Balance

- Signal intensity between loci
- Qualitatively described as 'balance' of the multiplex
- The cumulative signal is normalized to 1 and the fractional contribution of each locus is calculated

Interlocus Balance

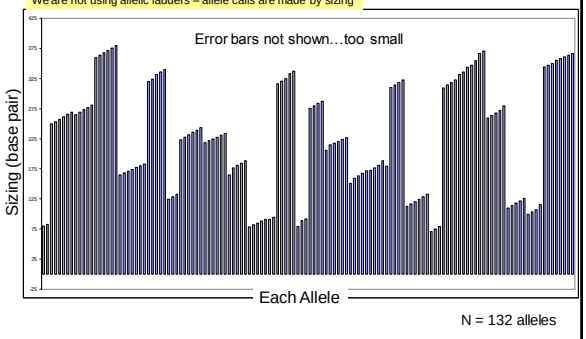


9948



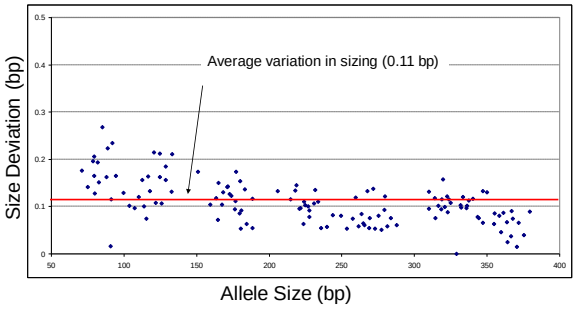
Sizing Precision

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



N = 132 alleles

Sizing Precision

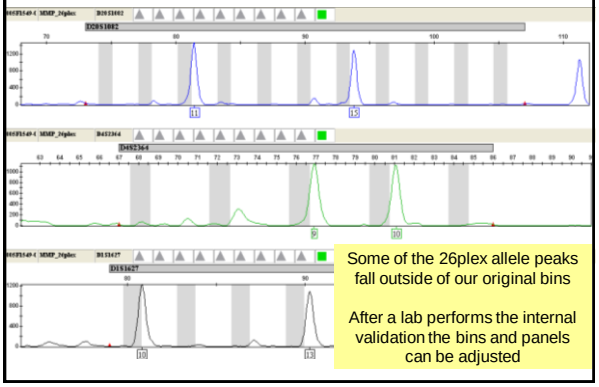


26plex Bins and Panels

- For Genemapper IDv3.2
- Written for POP4 and POP6
- We can provide the bins and panels on STRBase, but you must check them…
  - Use 9947A & 9948

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

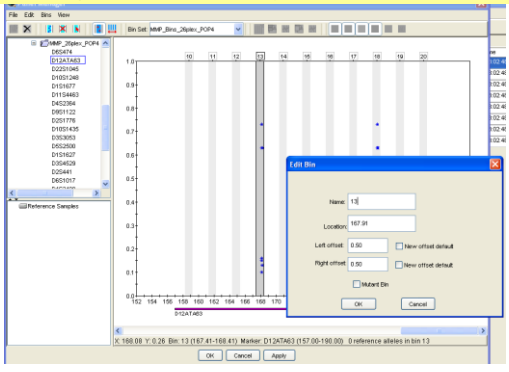
26plex Data from Collaborator (POP6)



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

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

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



Experiments Day 1

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

Experiments Day 2

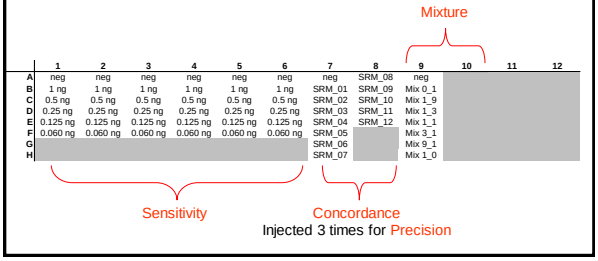
- 2 samples are amplified in triplicate for sensitivity study
- The mixture study samples are amplified

Experiments Day 3

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

Alternative Approach…

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



Data Analysis

The programs for data analysis are still under development, but the following information can be tabulated

- Stutter for each locus (and allele size)
- Heterozygote balance at each locus
- Interlocus balance (multiplex balance)
- Precision (sizing reproducibility)
- Concordance (allele drop out?)
- Sensitivity (down to 125 ng)
- Mixture (a 3:1 mixture can be detected)
- Qualifying run (concordance)

Conclusions

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

71 amplification reactions
16 unique samples
8 injections on 3130

Some Other Examples

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)**

Instrument/Software Upgrades or Modifications

- What should be done to "validate" new upgrade?
  - ABI 7000 to ABI 7500
  - ABI 3100 to ABI 3130xl
  - GeneScan/Genotyper to GeneMapper*ID*
- Try to understand what is different with the new instrument or software program compared to the one you are currently using (e.g., ask other labs who may have made the switch)
- If possible, try to retain your current configuration for comparison purposes for the validation period

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

ABI 3130xl vs ABI 3100

What NIST did to "validate" a 3130xl upgrade

- Ran plates of samples on both instruments with same injection and separation parameters and compared results
  - Data Collection version 1.0.1 (3100) vs 3.0 (3130xl)
  - POP-6 (3100) vs POP-7 (3130xl)
  - 36 cm array (3100) vs 50 or 80 cm array (3130xl)
- Ran several plates of 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 – is the new instrument "fit for purpose"?

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

Suggestions for an Internal Validation of an STR Kit

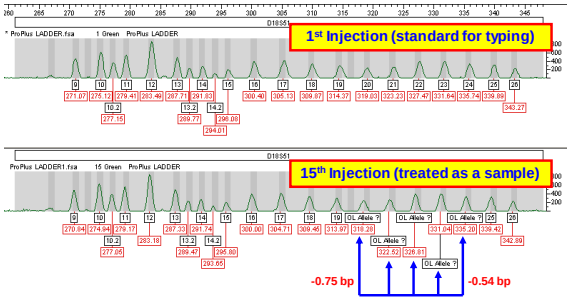
- Standard samples (3.1) **Between 1 and ~20 samples**
  - Verify correct type with positive control or NIST SRM samples
  - Concordance study with 5-10 (non-probative casework) samples previously typed with other kit(s)
- Precision samples (3.2) **5-10 samples**
  - Run at least 5-10 samples (allelic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

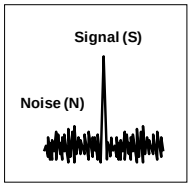


These alleles have drifted outside of their genotyping bins due to temperature shifting over the course of the sample batch

Setting Thresholds

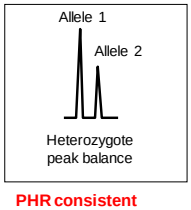
What is a true peak (allele)?

Peak detection threshold



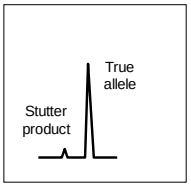
Signal > 3x sd of noise

Peak height ratio (PHR)



PHR consistent with single source Typically above 60%

Stutter percentage



Stutter location below 15%

Setting Thresholds

- **Detection (analytical) threshold**
  - Dependent on instrument sensitivity ~50 RFU
  - Impacted by instrument baseline noise

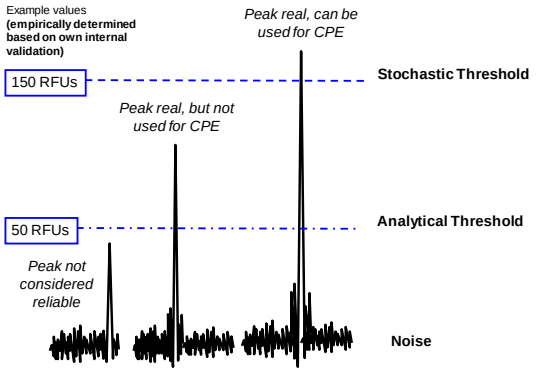
what is a peak?

- **Dropout (stochastic) threshold**
  - Dependent on biological sensitivity ~150-200 RFU
  - Impacted by assay and injection parameters

what is reliable PCR data?

Validation studies should be performed in each laboratory

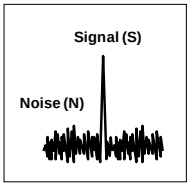
Different Thresholds



Analytical threshold

- The Laboratory should establish an analytical threshold based on signal-to-noise analyses of internally derived empirical data.

Peak detection threshold



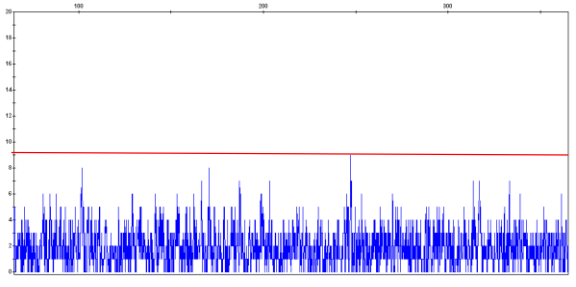
Signal > 3x sd of noise

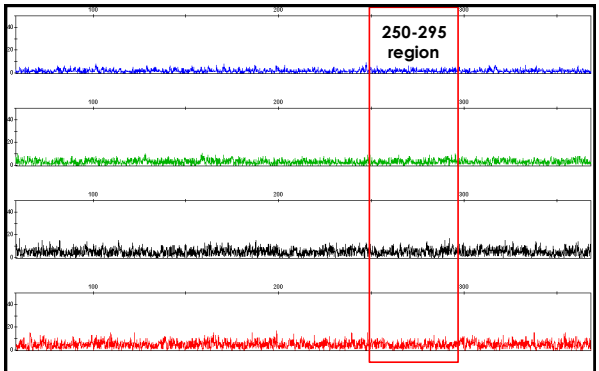
1. Preliminary Evaluation of Data

- An analytical threshold defines the minimum height requirement at and above which detected peaks can be reliably distinguished from background noise. Because the analytical threshold is based upon a distribution of noise values, it is expected that occasional, non-reproducible noise peaks may be detected above the analytical threshold.

1. Preliminary Evaluation of Data

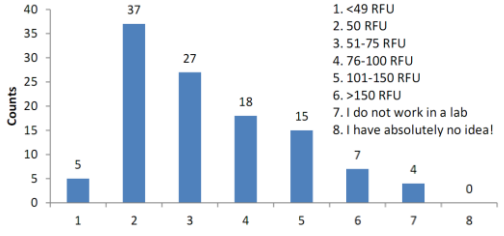
- An analytical threshold should be sufficiently high to filter out noise peaks. Usage of an exceedingly high analytical threshold increases the risk of allelic data loss which is of potential exclusionary value.





Analytical Thresholds can be determined for each dye channel

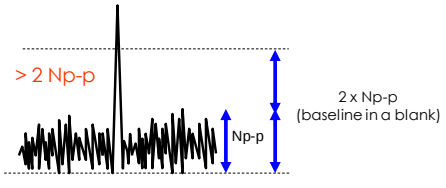
What is your AT? Question at the Promega meeting 10/2010 (n=113)



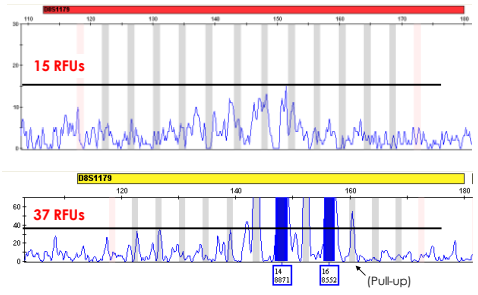
Slide Courtesy of Robin Cotton

1.1. Analytical threshold

- As an example, an analytical threshold may be based on two times the intensity difference between the highest peak and lowest trough within the instrumental noise data. *Other scientific methods may be used.*



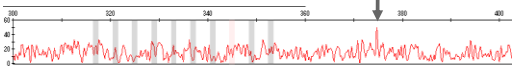
Sample Source – Negatives? Positives?



New Instruments, New Thresholds…

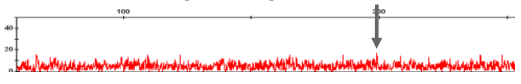
ABI 3500
1.2 kV, 15 sec (Default)

50 RFUs



ABI 3130
3.0 kV, 10 sec (Default)

16 RFUs



Calculations Using Negative Controls

Identifiler								
	Average RFU	Stdev	Min RFU	Max RFU	Method 1	Method 2	Method 3	Method 4
Blue	9	3.3	2	22	19	19	44	42
Green	13	3.6	5	27	24	23	54	49
Yellow	20	4.9	8	31	35	34	62	69
Red	27	7.1	10	50	49	48	100	99

Identifiler Plus								
	Average RFU	Stdev	Min RFU	Max RFU	Method 1	Method 2	Method 3	Method 4
Blue	9	3.1	3	20	18	18	40	39
Green	13	3.4	4	26	23	23	52	47
Yellow	20	5.1	7	37	36	35	74	72
Red	28	7.2	11	54	49	48	108	99

If calculating analytical threshold using negative controls:
Identifiler: 100 RFU

If calculating analytical threshold using negative controls:
Identifiler Plus: 100 RFU

Calculations Using DNA Dilution Series

Identifiler								
	Average RFU	Stdev	Min RFU	Max RFU	Method 1	Method 2	Method 3	Method 4
Blue	9	8.4	1	66	34	33	132	93
Green	13	11.5	3	84	48	47	168	128
Yellow	22	11.6	4	88	57	56	176	138
Red	28	8.8	10	80	54	53	160	116

Identifiler Plus								
	Average RFU	Stdev	Min RFU	Max RFU	Method 1	Method 2	Method 3	Method 4
Blue	10	4.6	3	68	23	23	136	55
Green	16	5.6	3	78	33	32	156	72
Yellow	24	7.9	7	63	48	47	126	103
Red	31	8.9	7	81	57	56	162	120

If calculating analytical threshold using a DNA dilution series
Identifiler: 140 RFU

If calculating analytical threshold using a DNA dilution series
Identifiler Plus: 120 RFU

Single Analytical Threshold Summary

	Negative Controls	Positive Controls
Identifier	100 RFU	140 RFU
Identifiler Plus	100 RFU	120 RFU

How to set an analytical threshold (AT)? Some Examples…

SWGDAM: Two times the intensity difference between the highest peak and lowest trough (as an example).

"The Ballpark": Three times the highest peak.

Gilder et al. (2007): Determined LOD by examining Pos, Neg, RB from 150 cases.

$$LOD = \mu_b + 3\sigma_b$$

J Forensic Sci, January 2007, Vol. 52, No. 1
doi:10.1111/j.1556-4029.2006.00318.x
Available online at: www.blackwell-synergy.com

TECHNICAL NOTE

Jason R. Gilder,[1] M.S.; Travis E. Doom,[2] Ph.D.; Keith Inman,[3] M. Crim.; and Dan E. Krane,[4] Ph.D.

Run-Specific Limits of Detection and Quantitation for STR-based DNA Testing

Gilder et al. (2007)

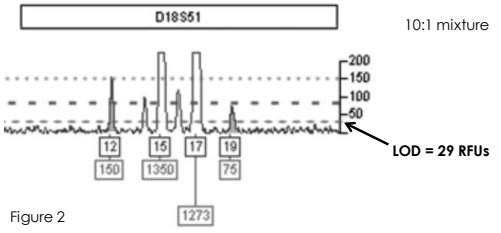


Figure 2

Gilder et al. (2007)

TABLE 1—*Maximum, minimum, and average baseline levels observed in the set of reagent blanks, negative controls, and positive controls (determined from controls in 50 different runs).*

	μ_b	σ_b	$\mu_b+3\sigma_b$	$\mu_b+10\sigma_b$
Positive Control				
Maximum	6.7	6.9	27.4	75.7
Average	5.0	3.7	16.1	42.0
Minimum	3.7	2.4	10.9	27.7
Negative Control				
Maximum	13.4	13.2	53.0	145.4
Average	5.4	3.9	17.1	44.4
Minimum	4.0	2.6	11.8	30.0
Reagent Blank				
Maximum	6.5	11.0	39.5	116.5
Average	5.3	4.0	17.3	45.3
Minimum	4.0	2.6	11.8	30.0
All three controls averaged				
Maximum	7.1	7.3	29.0	80.1
Average	5.2	3.9	16.9	44.2
Minimum	3.9	2.5	11.4	28.9

All values are in RFUs.

How to set an analytical threshold (AT)? Some Examples…

- SWGDAM: Two times the intensity difference between the highest peak and lowest trough (as an example).
- "The Ballpark": Three times the highest peak.
- Gilder et al. (2007): Determined LOD by examining Pos, Neg, RB from 150 cases.
- Catherine Grgicak (Boston U.) presentation at the 2010 ISHI (Promega) mixture workshop. (http://www.cstl.nist.gov/biotech/strbase/mixture.htm)

Multiple methods for determining AT

- Method 1.
  - Kaiser (IUPAC 1976)
    - Winefordner 1983 and Krane 2007
- Method 2.
  - Currie (IUPAC 1995)
    - Winefordner 1983
- Method 3.
  - Example in SWGDAM Guidelines

Negative Controls (at least 20)

- Method 4.
  - Miller & Miller. *Statistics for Analytical Chemistry (Ellis Horwood & Prentice Hall)*
    - IUPAC 1997 ElectroAnalytical Committee

DNA Dilution Series

- Method 5.
  - 1997 IUPAC ElectroAnalytical Committee Recommendations

Courtesy of Catherine Grgicak

Multiple methods for determining AT

$$AT_{M1} = \bar{Y}_{bl} + ks_{bl}$$

$$AT_{M3} = 2(Y_{\max} - Y_{\min})$$

$$AT_{M2} = \bar{Y}_{bl} + t_{1-\alpha,v}\frac{s_{bl}}{\sqrt{n}}$$

Negative Controls (at least 20)

$$AT_{M4} = b + 3S_y$$

$$AT_{M5} = b + t_{n-1,\alpha}S_y$$

(http://www.cstl.nist.gov/biotech/strbase/mixture.htm)

Courtesy of Catherine Grgicak

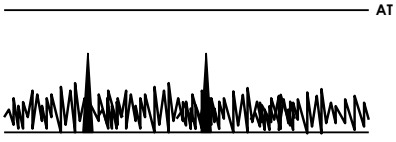
Multiple methods for determining AT

Method	Origin	Analytical Threshold for green 5s injection example
1	Negatives	7
2	Negatives	4
3	Negatives	20
4	DNA Series	31
5	DNA Series	39

Courtesy of Catherine Grgicak

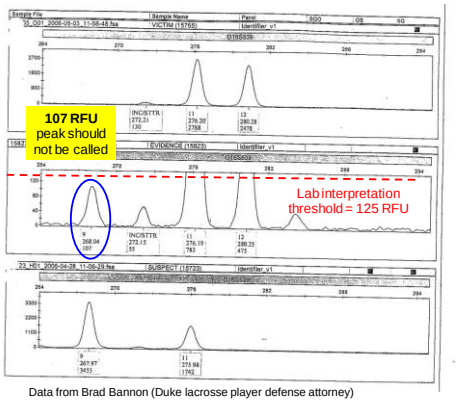
What about peaks below AT?

- The Analytical Threshold is the "floor" of the EPG. Peaks below the AT are not to be trusted!



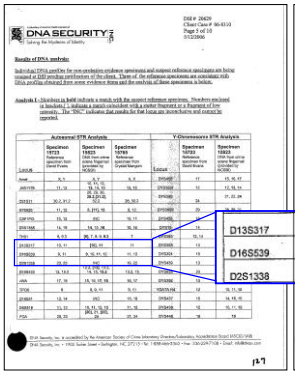
"The Sixth Peak"

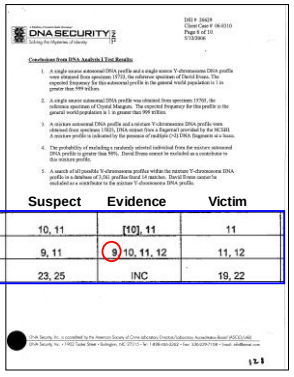
Don't Call Peaks Below Your Validated Threshold!



Data from Brad Bannon (Duke lacrosse player defense attorney)

May 12, 2006: DNA Security Report





	Suspect	Evidence	Victim
D13S317	10, 11	[10], 11	11
D16S539	9, 11	9, 10, 11, 12	11, 12
D2S1338	23, 25	INC	19, 22

Data from Brad Bannon (Duke lacrosse player defense attorney)

Setting Thresholds

- **Detection (analytical) threshold**
  - Dependent on instrument sensitivity ~50 RFU
  - Impacted by instrument baseline noise

what is a peak?

- **Dropout (stochastic) threshold**
  - Dependent on biological sensitivity ~150-200 RFU
  - Impacted by assay and injection parameters

what is reliable PCR data?

Validation studies should be performed in each laboratory

Determining the Dropout (Stochastic) Threshold

Gill *et al.* (2008) *FSI Genetics* 2(1): 76–82

- The dropout threshold can be determined experimentally for a given analytical technique from a series of pre-PCR dilutions of extracts of known genotype technique (it will probably vary between analytical methods). These samples can be used to determine the point where allelic dropout of a heterozygote is observed relative to the size of the survivor companion allele. The threshold is the maximum size of the companion allele observed. This is also the point where Pr(D) approaches zero…

Dropout threshold will change depending on instrument and assay conditions (e.g., longer CE injection will raise dropout threshold)

Modeling of Stochastic Thresholds

Forensic Science International: Genetics 3 (2009) 104–111

Contents lists available at ScienceDirect

Forensic Science International: Genetics

journal homepage: www.elsevier.com/locate/fsig

The *low-template-DNA* (stochastic) threshold—Its determination relative to risk analysis for national DNA databases

Peter Gill [a,b,*], Roberto Puch-Solis [c], James Curran [d]

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[b] Institute of Forensic Medicine, University of Oslo, 0027 Oslo, Norway
[c] Forensic Science Service, Trident Court, Solihull B37 7YN, UK
[d] Department of Statistics, University of Auckland, Private Bag 92019, New Zealand

Gill, P. et al. (2009) The low-template-DNA (stochastic) threshold—Its determination relative to risk analysis for national DNA databases. *Forensic Science International: Genetics* 3: 104–111.

Peak Height Dependence on Allele Dropout

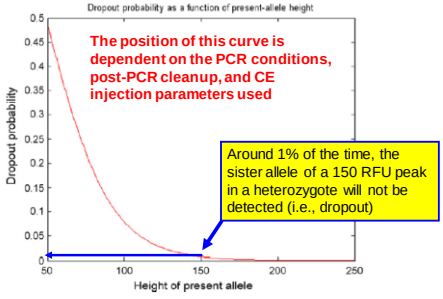
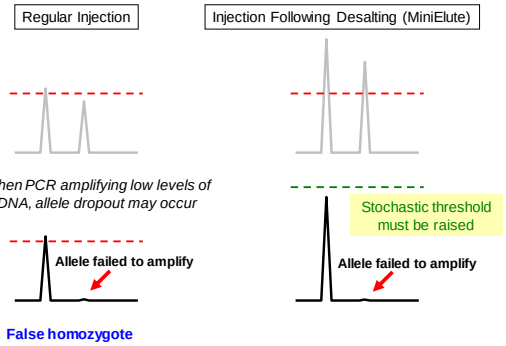


Fig. A.2. Probability drop-out as a function of present-allele height $Pr(D|h)$.

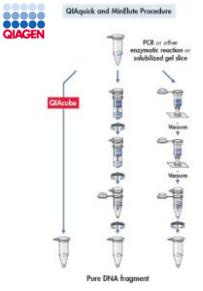
Gill, P. et al. (2009) The low-template-DNA (stochastic) threshold—Its determination relative to risk analysis for national DNA databases. *Forensic Science International: Genetics* 3: 104–111.

Stochastic Effects and Thresholds



Why MiniElute increases peak heights

- QIAGEN MiniElute **reduces salt levels in samples causing more DNA to be injected**
- **Requires setting a higher stochastic threshold** to account for the increased sensitivity



Smith, P.J. and Ballantyne, J. (2007) Simplified low-copy-number DNA analysis by post-PCR purification. *J. Forensic Sci.* 52: 820-829

Sample Conductivity Impacts Amount Injected

$$[DNA_{inj}] = \frac{Et(\pi r^2)(\mu_{ep} + \mu_{eof})[DNA_{sample}](\lambda_{buffer})}{\lambda_{sample}}$$

$[DNA_{inj}]$ is the amount of sample injected

E is the electric field applied

t is the injection time

r is the radius of the capillary

μ_{ep} is the mobility of the sample molecules

μ_{eof} is the electroosmotic mobility

$[DNA_{sample}]$ is the concentration of DNA in the sample

λ_{buffer} is the buffer conductivity

λ_{sample} is the sample conductivity

Cl- ions and other buffer ions present in PCR reaction contribute to the sample conductivity and thus will compete with DNA for injection onto the capillary

Butler *et al.* (2004) Electrophoresis 25: 1397-1412

Forensic Science International: Genetics 3 (2009) 222–226

Contents lists available at ScienceDirect

Forensic Science International: Genetics

journal homepage: www.elsevier.com/locate/fsig

Estimating the probability of allelic drop-out of STR alleles in forensic genetics

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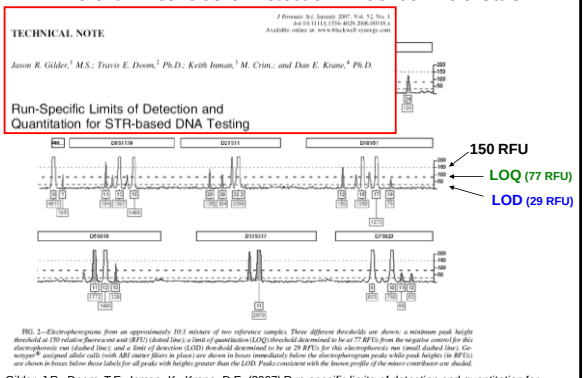
[a] Department of Mathematical Sciences, Aalborg University, Fredrik Bajers Vej 7G, DK-9220 Aalborg East, Denmark
[b] Section of Forensic Genetics, Department of Forensic Medicine, Faculty of Health Sciences, University of Copenhagen, Fredrik V's Vej 11, DK-2100 Copenhagen East, Denmark

Table 3
Mean peak heights (rfu) for various drop-out probabilities for 10 STR loci.

$P(\widehat{D}\widehat{H})$	D3	vWA	D16	D2	D8	D21	D18	D19	TH0	FGA	Overall
0.0001	556	577	622	562	558	461	531	722	723	692	648
0.0005	384	399	430	388	385	318	367	499	499	478	439
0.0010	327	340	366	331	328	271	313	425	426	407	371
0.0050	226	235	253	228	226	187	216	293	294	281	251
0.0100	192	200	215	194	193	159	184	250	250	239	212
0.0500	132	137	147	133	132	109	126	171	171	164	142
0.1000	111	115	124	112	111	92	106	144	144	138	119
0.2000	92	95	103	93	92	76	88	119	120	114	98
0.3000	81	84	91	82	81	67	78	105	106	101	86
0.4000	73	76	82	74	74	61	70	95	95	91	77
0.5000	67	69	75	68	67	55	64	87	87	83	70
0.6000	61	63	68	62	61	50	58	79	79	76	63
0.7000	55	57	62	56	55	46	53	71	71	68	57
0.8000	49	50	54	49	49	40	46	63	63	60	50
0.9000	40	42	45	41	40	33	39	52	52	50	41
0.9500	34	35	38	34	34	28	32	44	44	42	34
0.9900	23	24	26	23	23	19	22	30	30	29	23

Different Thresholds of Detection Influence Allele Calls



Gilder, J.R., Doom, T.E., Inman, K., Krane, D.E. (2007) Run-specific limits of detection and quantitation for STR-based DNA testing. *J. Forensic Sci*. 52(1): 97-101.

How to Determine the Stochastic Threshold

- Examine intensity and peak height ratio of 5 samples at three different low concentrations (e.g., 60, 75, and 125 pg)
- Observe variation in peak height ratio and peak intensity
- The stochastic threshold is the point at which this variation begins a rapid increase (change in slope of line relating std dev vs concentration)
- This can also be defined as the concentration at which a set percentage of peak height ratio values fall below 60%

Alternative Procedure (Mass State Police)

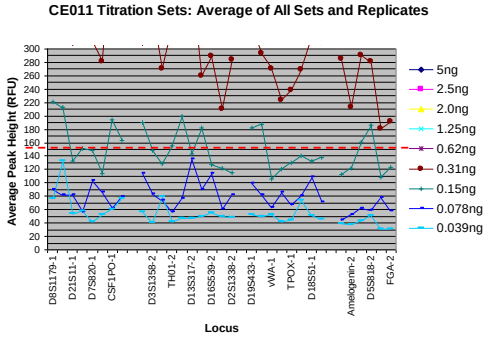
1. Since most estimates for LCN show up from 100-250 pg DNA, select a low level sample - say 150 pg as your stochastic limit.

2. Amplify 2 or more samples at a range of concentrations (1.0-0.005) ng multiple times and score the intensity

3. The stochastic limit is the intensity (RFUs) at which half the alleles have intensity above this value and half are below

4. In this way you define straddle data as at the point 50% of your alleles will be above this mark

Reporting Threshold set at **red line** for partial profile @ 0.15ng & 3X noise (baseline + background)



Slide from Joanne Sgueglia (Mass State Police) – AAFS 2008 Mixture Workshop
http://www.cstl.nist.gov/biotech/strbase/training/AAFS2008_MixtureWorkshop.htm

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?

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

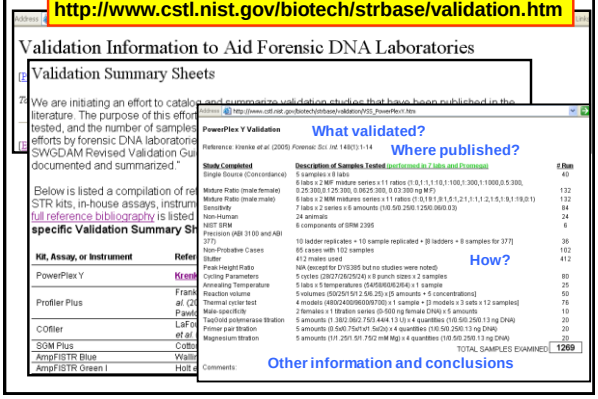
9.1 Once the validation process is complete it is important to document the procedures so that the method can be clearly and unambiguously implemented. There are a number of reasons for this. **The various assessments of the method made during the validation process assume that,in use, the method will 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

Validation Homepage on STRBase

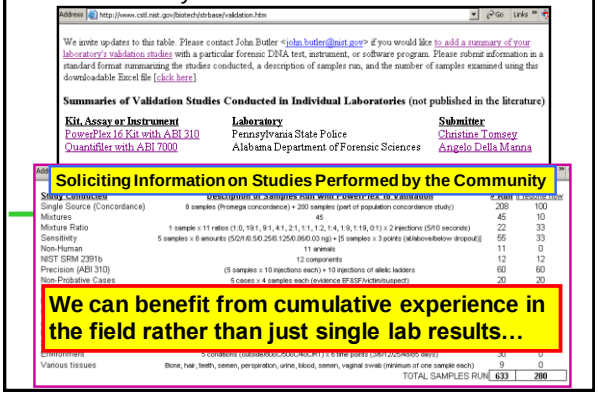
http://www.cstl.nist.gov/biotech/strbase/validation.htm



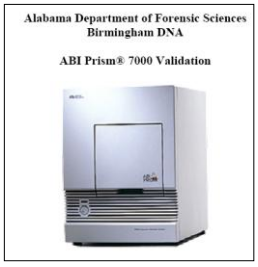
Validation Summary Sheet for PowerPlex Y

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



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

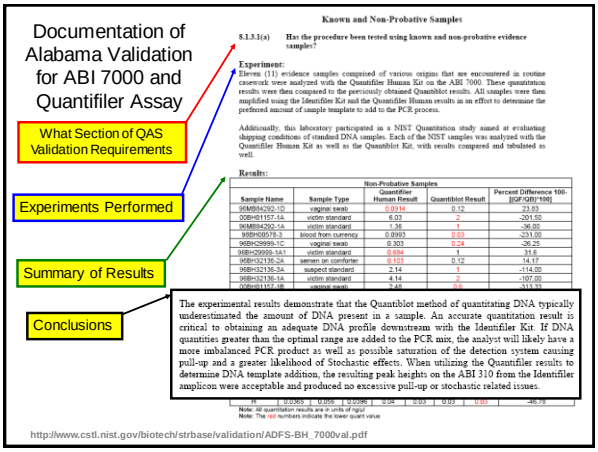
Documentation of Alabama Validation for ABI 7000 and Quantifiler Assay

What Section of QAS Validation Requirements

Experiments Performed

Summary of Results

Conclusions



Known and Non-Probative Samples

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

Experiment:

Eleven (11) evidence samples comprised 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]
06M894292-1D	vaginal swab	0.0914	0.12	23.83
00BH01157-1A	victim standard	6.03	2	-201.50
96M894292-1A	victim standard	1.36	1	-36.00
96BH00578-3	blood from currency	0.0993	0.03	-231.00
96BH29999-1C	vaginal swab	0.303	0.24	-26.25
96BH29999-1A1	victim standard	0.684	1	31.6
96BH32136-2A	semen on comforter	0.103	0.12	14.17
96BH32136-3A	suspect standard	2.14	1	-114.00
96BH32136-1A	victim standard	4.14	2	-107.00
00BH01157-1B	vaginal swab	2.48	0.6	-313.33
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
IN	0.0365	0.056	0.03395	0.04	0.05	0.03	0.03	-46.76

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

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.

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

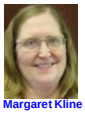
Acknowledgments

National Institute of Standards and Technology NIST
...working with industry to foster innovation, trade, security and jobs

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


Pete Vallone


Dave Duewer


Margaret Kline

Thank you for your attention

Acknowledgments: NIJ & FBI Funding

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Our team publications and presentations are available at:
http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm