Everything You Need to Know about Validation

Validation Workshop

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ISFG Pre-Conference Workshop
Buenos Aires, Argentina
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Presentation Outline

- Introduction to Validation Terms and Principles
- Review of SWGDAM Revised Validation Guidelines

BREAK

- **Detailed Example** (presented by Dr. Peter Vallone, NIST)
  - Validation of NIST 26plex assay
- Suggestions for Documentation and Implementation
- Questions

NIST and NIJ Disclaimer

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

Points of view are mine and do not necessarily represent the official position or policies of the US Department of Justice or the National Institute of Standards and Technology.

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My Background

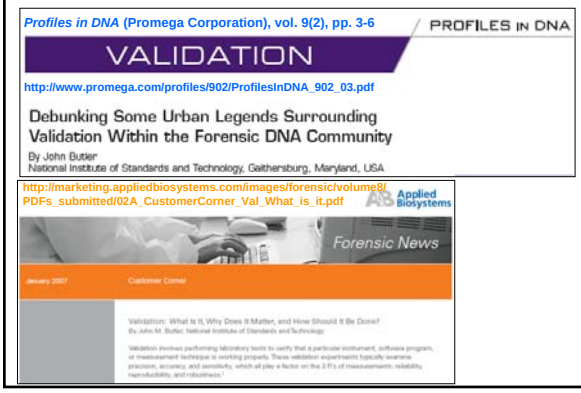
- PhD **(Analytical Chemistry)** from University of Virginia Research conducted at FBI Academy under Bruce McCord doing CE for STR typing (May 1993 - Aug 1995)
- NIST Postdoc – developed STRBase website
- GeneTrace Systems – private sector experience validating assays and developing new technologies
- NIST Human Identity Project Leader since 1999
- Invited guest to FBI's Scientific Working Group on DNA Analysis Methods (SWGDAM) since 2000
- **Member of SWGDAM Validation Subcommittee** – resulting in Revised Validation Guidelines
- Served on WTC KADAP and helped evaluate and validate new miniSTR, mtDNA, and SNP assays

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

Current Areas of NIST Effort with Forensic DNA

- **Standards** http://www.cstl.nist.gov/biotech/strbase/
  - Standard Reference Materials
  - Standard Information Resources (STRBase website)
  - Interlaboratory Studies
- **Technology**
  - Research programs in SNPs, miniSTRs, Y-STRs, mtDNA, qPCR
  - Assay and software development
- **Training Materials**
  - Review articles and workshops on STRs, CE, validation
  - PowerPoint and pdf files available for download

Some Articles I Have Written on Validation

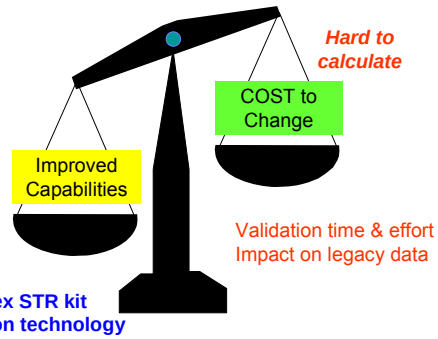


Stages of Technology for Forensic DNA Typing

- 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

My Purpose in Teaching This Workshop

- I believe that many forensic laboratories, in an effort to be cautious, are taking too long to perform their validation studies and thereby delaying initiation of casework and contributing to backlogs in labs that are already overburdened
- Technology will continue to advance and thus validation of new methodologies will always be important in forensic DNA laboratories

There will always be something to "validate"…

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

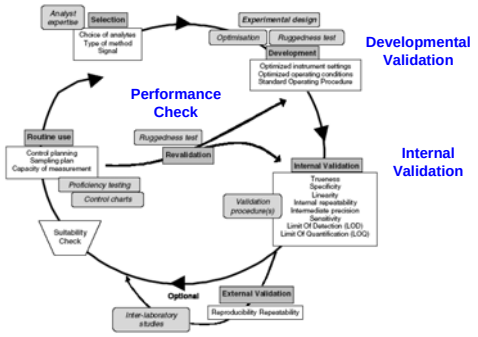
J.M. Butler (2005) *Forensic DNA Typing*, 2nd Edition, p. 391

- **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 July 2004 – Volume 6 – Number 3

Standards and Guidelines

Revised Validation Guidelines

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Scientific Working Group on DNA Analysis Methods (SWGDAM)

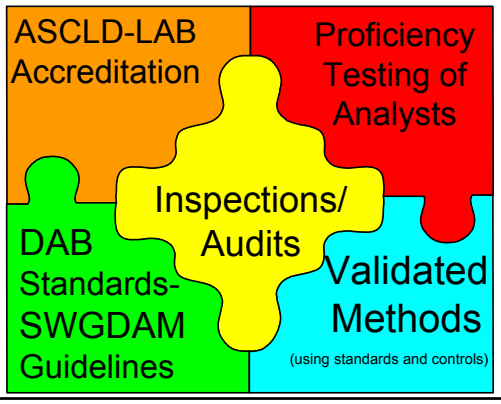
Introduction | Validation Considerations | Developmental Validation | Internal Validation | Material Modification | Performance Check | Definitions

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.

Ensuring Accurate Forensic DNA Results

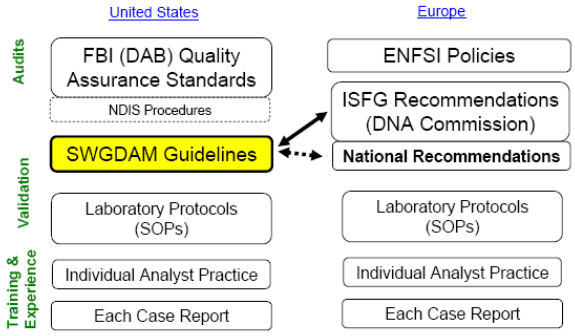


Checks and Controls on DNA Results

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

My Philosophy towards Validation

Ask first: Does the new method improve your capability?

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

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

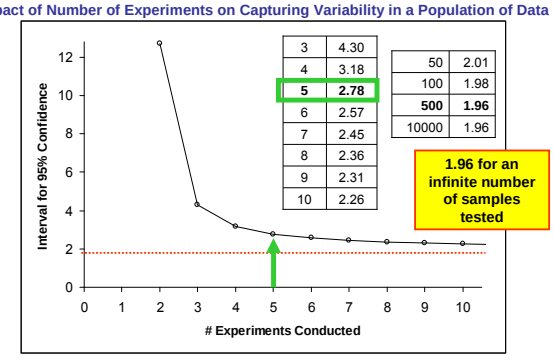
"Student" (real name: W. S. Gossett [1876-1937]) developed statistical methods to solve problems stemming from his employment in a brewery.

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

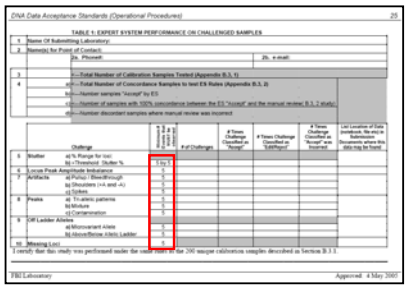
50	2.01
100	1.98
500	**1.96**
10000	1.96

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)



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

Minimum Allele Frequency = **5**/2N

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

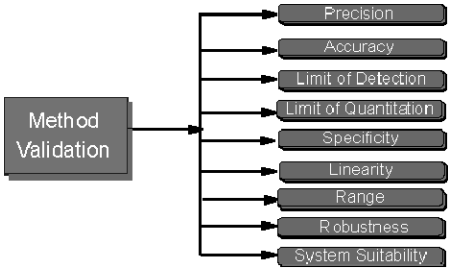
Validation in Other Fields
(Besides Forensic DNA Testing)

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

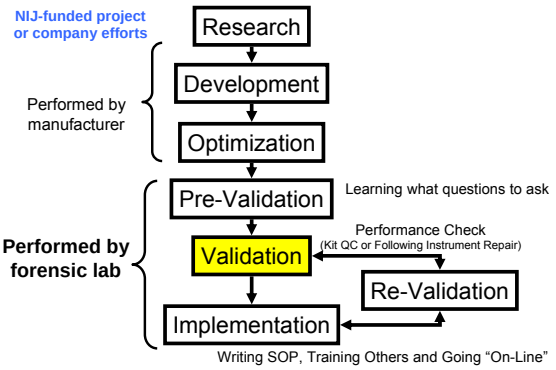
- (ff) Validation is a process by which a procedure is evaluated to determine its **efficacy and reliability** for forensic casework analysis *(DNA analysis)* and includes:

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

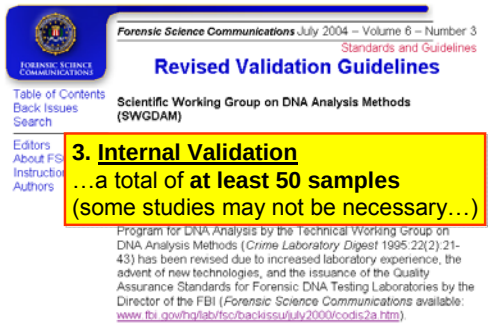
General Steps for Internal Validation

- **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



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

Overview of Internal Validation Studies

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

- 3.1 Known and nonprobative evidence samples
- 3.2 Reproducibility and precision
- 3.3 Match criteria
- 3.4 Sensitivity and stochastic studies
- 3.5 Mixture studies
- 3.6 Contamination
- 3.7 Qualifying test

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

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

Validation Workshop
26plex Internal Validation

Dr. Peter M. Vallone
US National Institute of Standards and Technology

23rd World Congress
ISFG Buenos Aires
September 15th, 2009

Outline

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

Aren't the Current STR Loci Good Enough?

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

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)

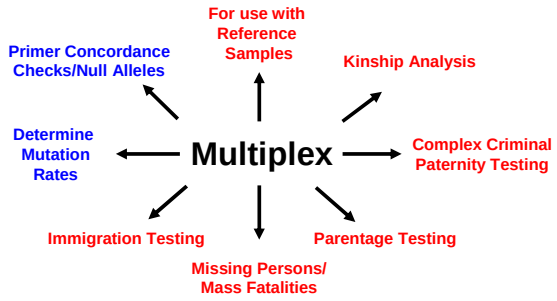
Utility of miniplexes
- Degraded DNA
- Low copy number analysis

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

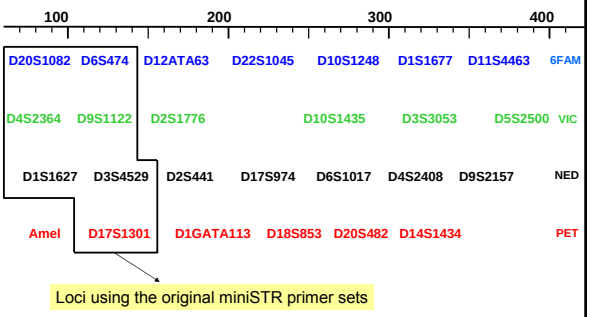
Why Build a Large Multiplex?



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



Loci using the original miniSTR primer sets

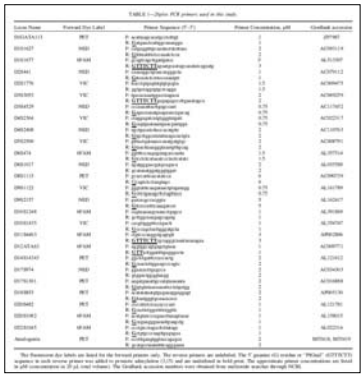
Developmental Validation

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

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)

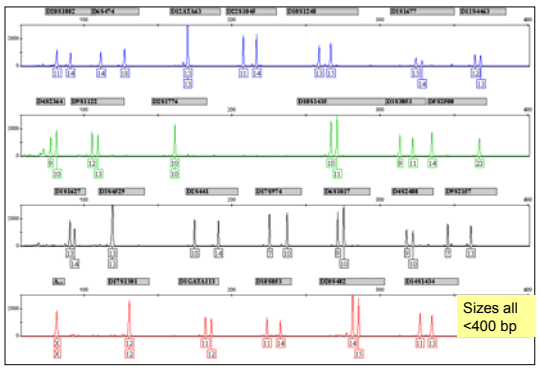
- 95˚C Hot Start for 11 min
- 30 cycles
  - 94˚C for 45 sec Denaturation
  - 59˚C for 2 min Annealing
  - 72˚C for 1 min Elongation
- 60˚C soak for 60 min
- 25˚C hold

~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)

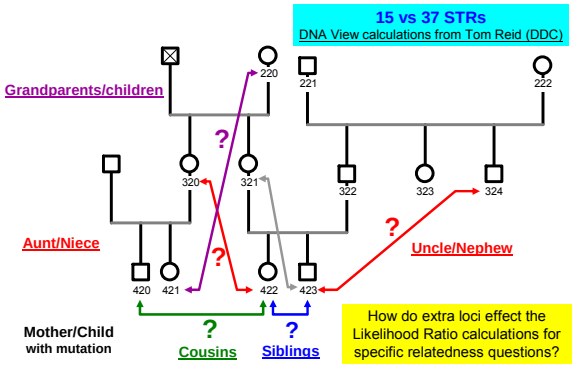
26plex Profile for 9947A



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…
Sib…		
Un…		
Cousins	0.45	**2.25**
Grandparents/ Grandchildren	0.53	**1.42**

For more on the uses of the 26plex see Poster P-022
"Uses of the NIST 26plex STR Assay for Human Identity Testing"
Peter M. Vallone, Carolyn R. Hill, Kristen E. Lewis, Toni M. Diegoli, Michael D. Coble, and John M. Butler

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

- 3.3 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)

Forensic Science Communications July 2004 – Volume 6 – Number 3

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

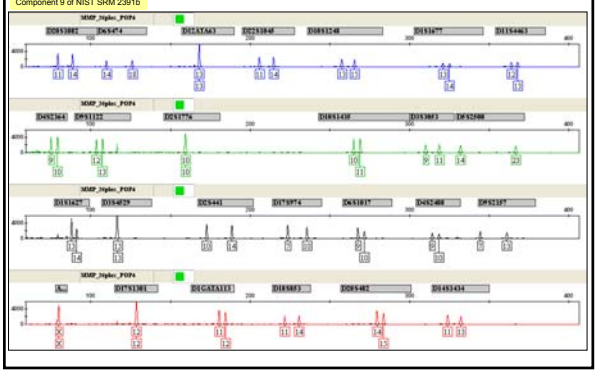
Concordance Study

NIST SRM 2391b

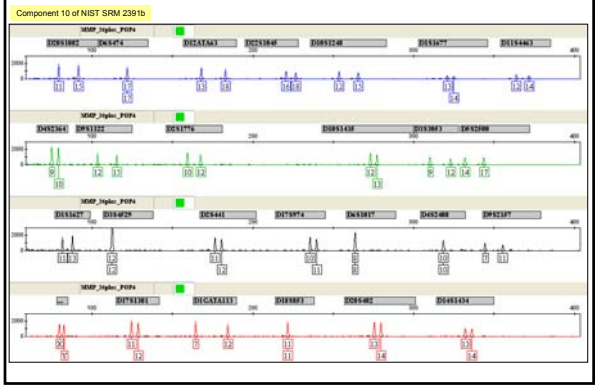
- 12 components in SRM 2391b
  - 9947A and 9948
- Material certified for the 25 STR loci
  - as of 2008
- 25 STRs X 12 samples = 300 genotypes
- 1 discordant allele call (drop out) 99.7% concordance

9947A

Component 9 of NIST SRM 2391b



9948



SRM 2391b

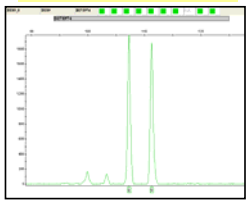
Sample Name	Amelogenin	D10S1248	D10S1435	D11S4463	D12ATA63	D14S1434	D17S1301	D17S974	D18S853	D1GATA113	D1S1627	D1S1677	D20S1082
Component 1	X,Y	14,16	13,13	14,14	14,17	13,14	11,11	9,11	11,14	11,11	10,14	12,13	11,15
Component 2	X,Y	13,15	11,14	13,14	13,17	11,13	11,12	9,10	11,11	12,13	13,14	14,16	14,15
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,Y	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	D2OS482	D22S1045	D2S1776	D2S441	D3S3053	D3S4529	D4S2364	D4S2408	D5S2500	D6S1017	D6S474	D8S1122	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

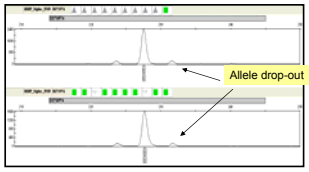
One Discordant Sample

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



Alleles confirmed by sequencing

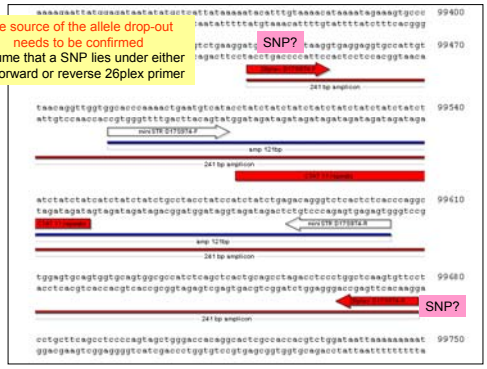
Genomic component 8
8,8 at D17S974
Amplified with 26plex primers



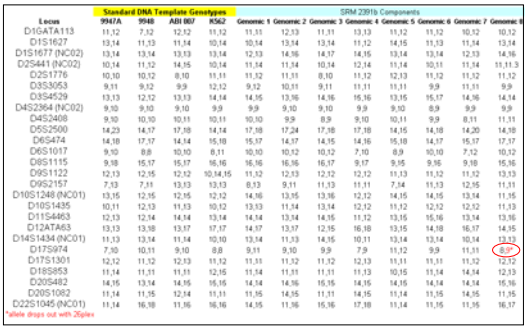
Run in duplicate

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



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

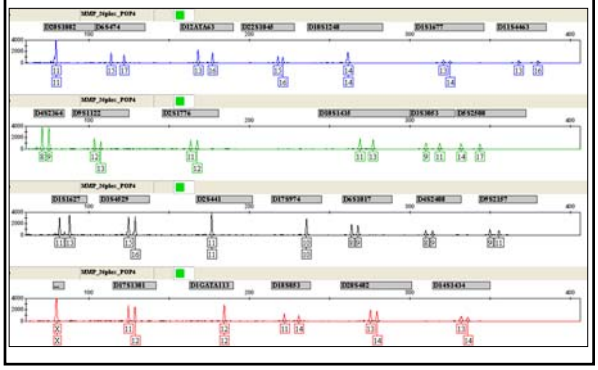
- Run 2 unique samples in triplicate
  1. 1 ng
  2. 0.5 ng
  3. 0.25 ng
  4. 0.125 ng
  5. 0.060 ng
- Sample concentration determined with Quantifiler prior to sensitivity study

Serial Dilution

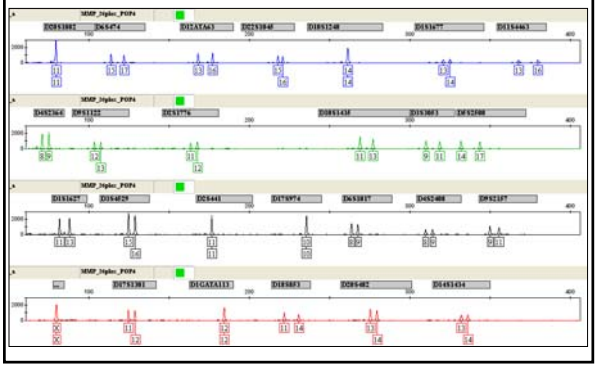
- Prepare serial dilution to use 2 μL volume per PCR reaction
- Prepare 20 μL of each concentration point (enough volume to run triplicate experiments)
- Example for stock sample 4.5 ng/μL

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

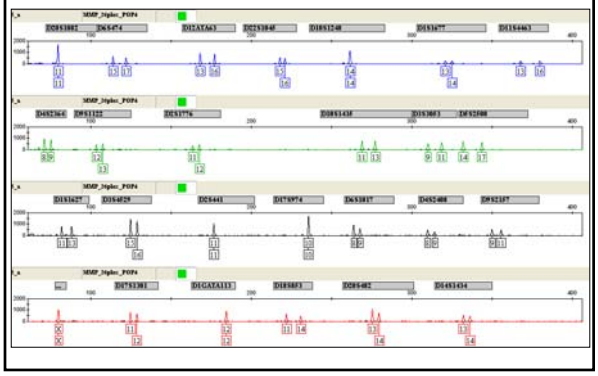
Sensitivity (Sample 1) 1 ng



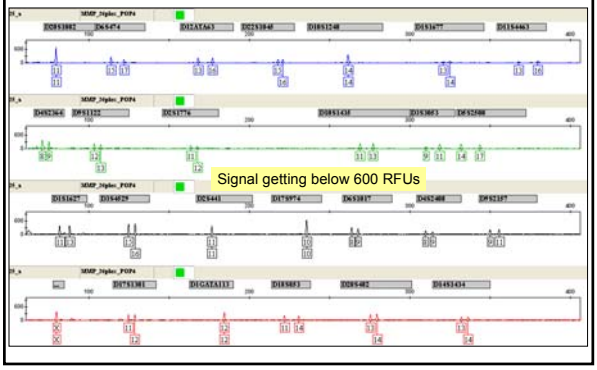
Sensitivity (Sample 1) 0.5 ng



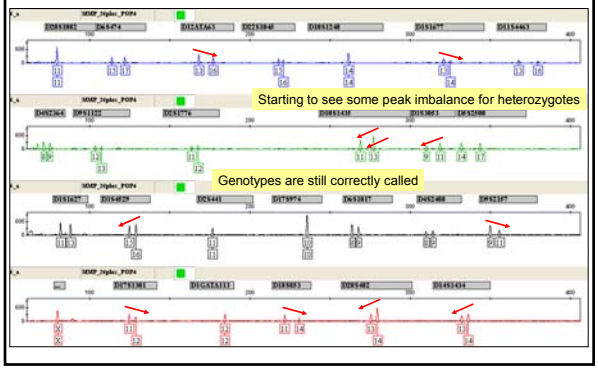
Sensitivity (Sample 1) 0.25 ng



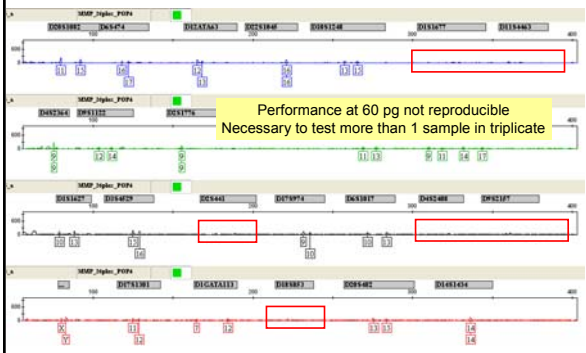
Sensitivity (Sample 1) 0.125 ng



Sensitivity (Sample 1) 0.06 ng



Sensitivity (Sample 2) 0.06 ng



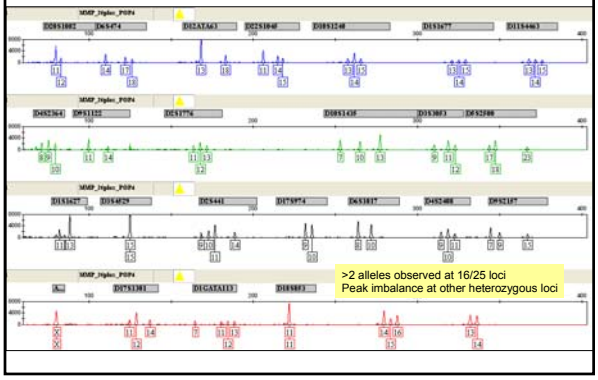
Sensitivity Study Conclusions

- 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

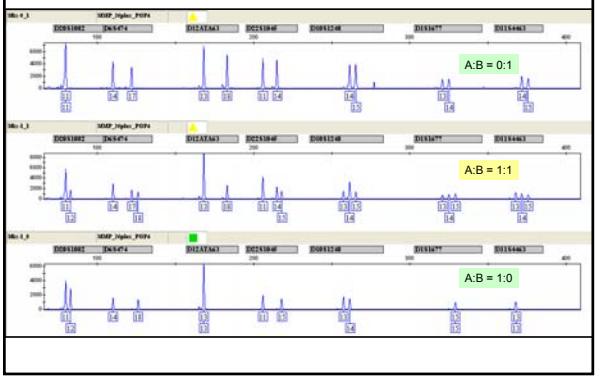
Mixture Study

- 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

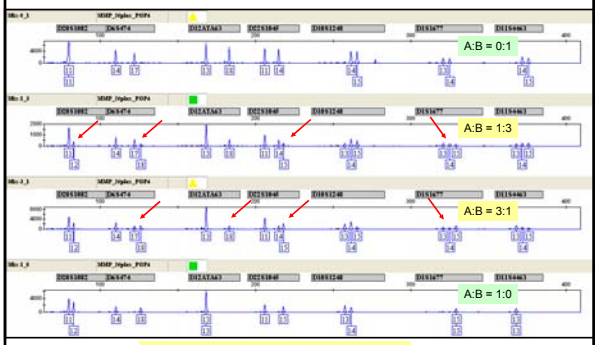
1:1 Mixture



Comparing Controls to 1:1 mixture

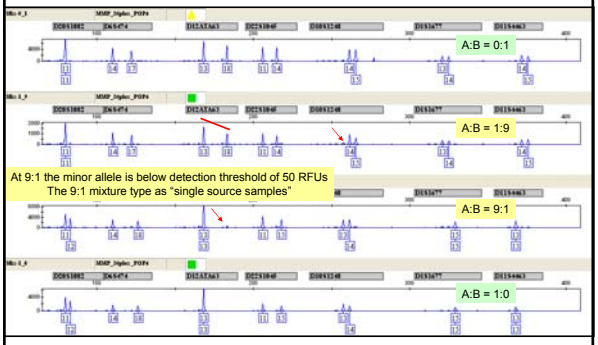


Comparing Controls to 3:1 mixtures



The minor allele is detectable in 3:1 mixtures

Comparing Controls to 9:1 mixtures

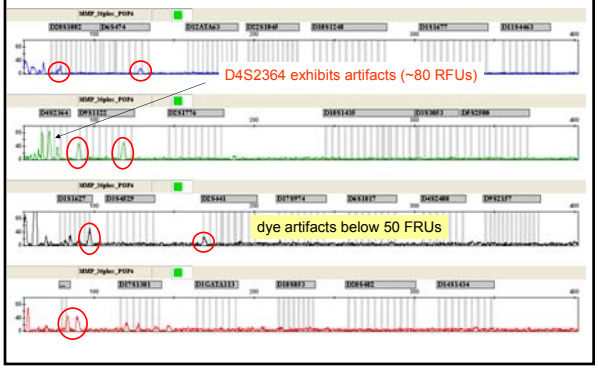


Mixture Study Conclusions

- 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

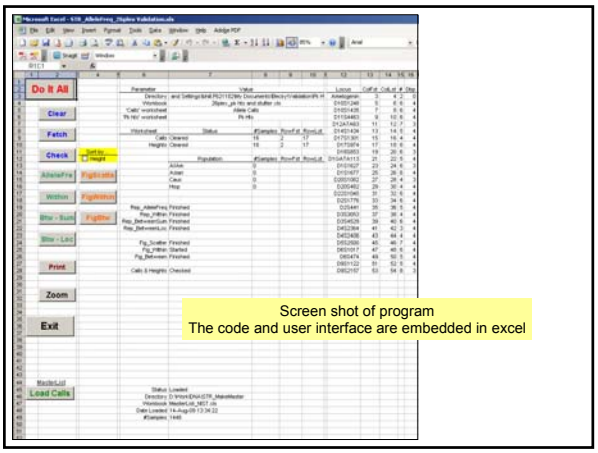


Qualifying Run

- 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

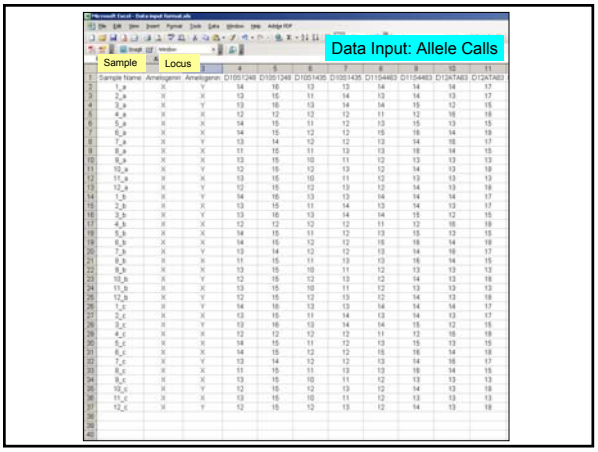
Analysis Software

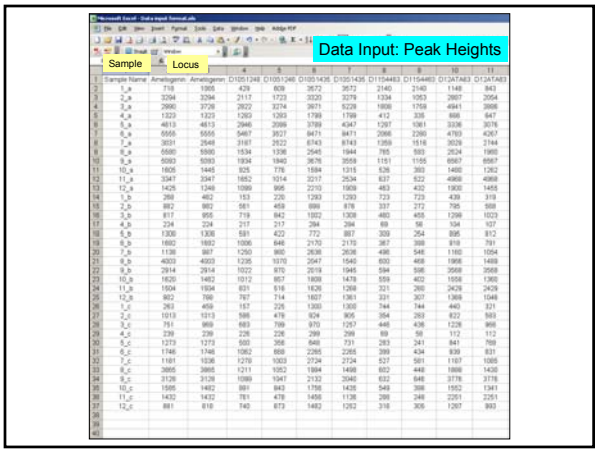
- Currently under development at NIST by Dr. David Duewer
- Performs calculations for
  - Allele frequencies
  - Intralocus signal balance (heterozygotes)
  - Interlocus signal balance ('multiplex balance')
  - Stutter
- Enables rapid analysis of internal validation data



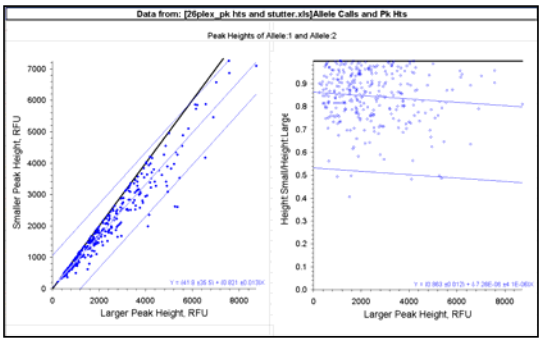
Program Data Input

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





Peak Height Ratios



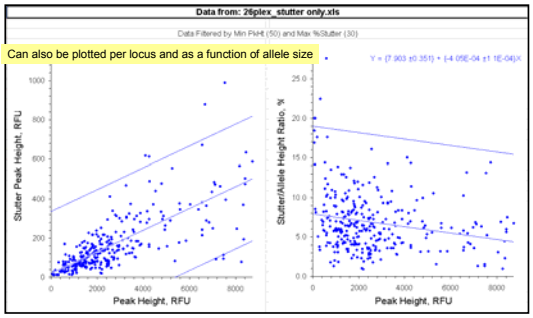
Peak Height Ratios

- An example data table

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

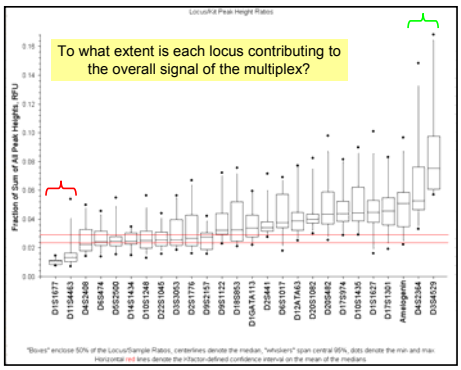
D11S4463

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

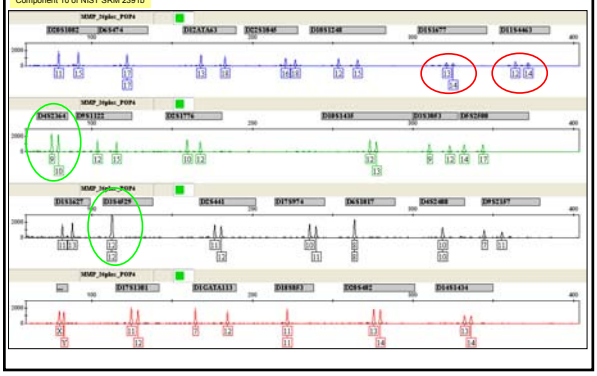
Interlocus Balance

- 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

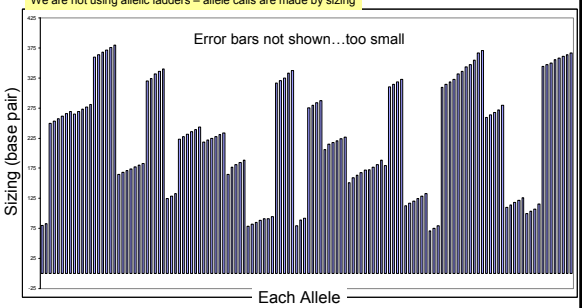


9948



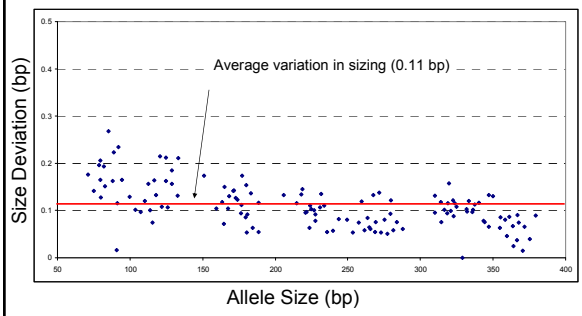
Sizing Precision

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



N = 132 alleles

Sizing Precision

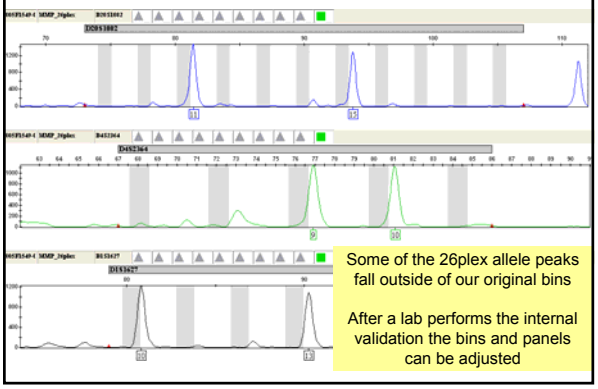


26plex Bins and Panels

- 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

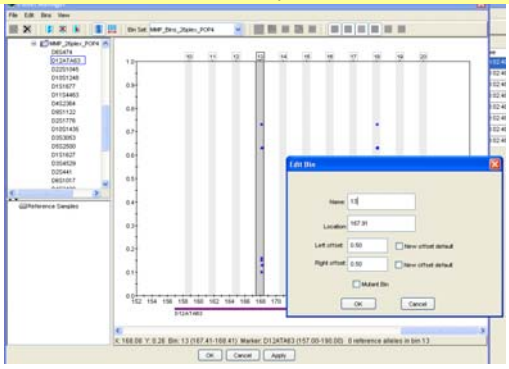
26plex Data from Collaborator (POP6)



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

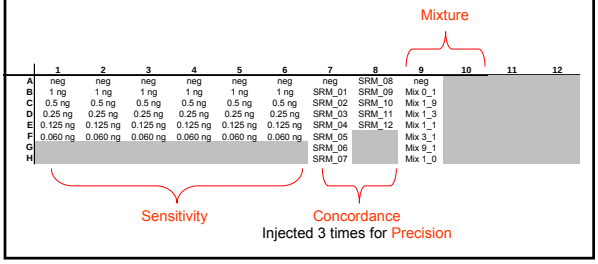
- 2 samples are amplified in triplicate for sensitivity study
- The mixture study samples are amplified

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

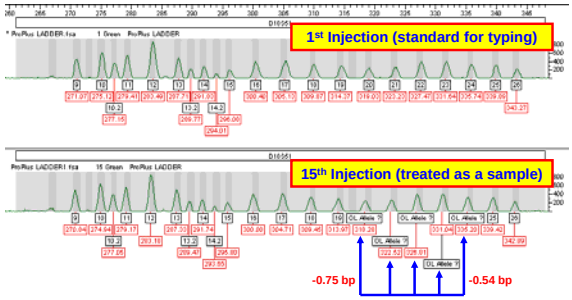
- Standard samples (3.1) **Between 1 and ~20 samples**
  - Verify correct type with positive control or NIST SRM samples
  - Concordance study with 5-10 (non-probative casework) samples previously typed with other kit(s)
- Precision samples (3.2) **5-10 samples**
  - Run at least 5-10 samples (allelic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

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

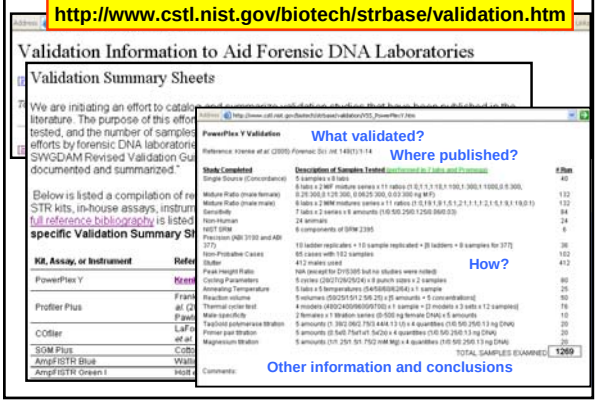
9.1 Once the validation process is complete it is important to document the procedures so that the method can be clearly and unambiguously implemented. There are a number of reasons for this. **The various assessments of the method made during the validation process assume that,in use, the method will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

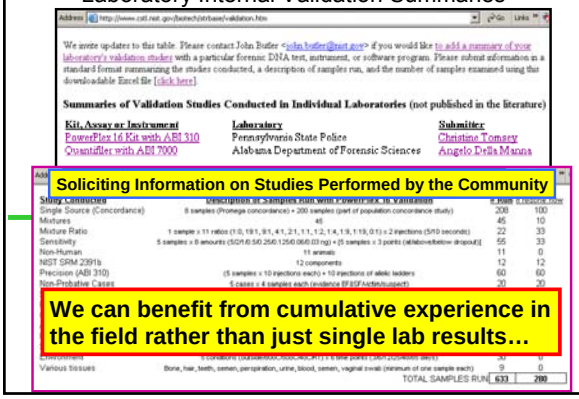
http://www.cstl.nist.gov/biotech/strbase/validation.htm



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

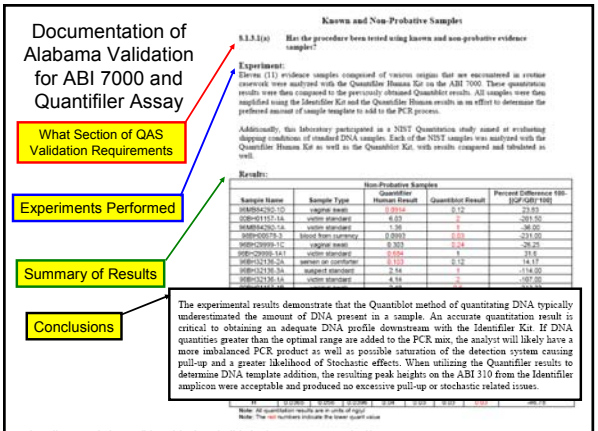


Example of Validation Documentation

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



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?

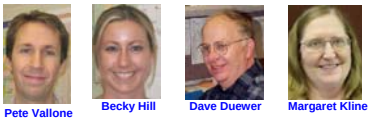
Who needs to be trained and what is the extent of the training? How is the training documented? What constitutes completion of training? Per individual or per lab?

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
...working with industry to foster innovation, trade, security and jobs
NIST

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



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