Dialogue with NIST:
Standard Reference Materials for Molecular Diagnostics

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Presentation Outline

• NIST
  – location, role, organizational structure, funding

• Applied Genetics Group
  – members, expertise, equipment, funding
  – programs, projects, priorities

• Standard Reference Materials (SRMs)
  – Purpose, principles, production process, pricing
  – Differences from WHO standards (certification vs consensus)

• Clinical DNA SRMs
  – Pipeline, plans, and path forward
  – Partnerships: needs/priorities from the community
NIST Background
National Institute of Standards & Technology (NIST)

• Non-regulatory agency established in 1901 in the US Department of Commerce.

• Mission to promote US innovation and industrial competitiveness by advancing measurement science, standards & technology.

• NIST is at the top of the US standards pyramid for a wide variety of physical standards, test methods, and calibrations.
Functions and Activities include:

- Custody and dissemination of national standards
  - Calibrations, Certified Reference Materials, Reference Data, Reference Methods
- Determination of physical constants and properties of materials
- Comparison of US National standards with those of the world
- Solution of standards and measurement problems for industry and other agencies
Early Driver for U.S. Standards

1904
- Out-of-town fire companies arriving at a Baltimore fire cannot couple their hoses to the hydrants. 1526 buildings razed.

1905
- National Fire Protection Association adopted NBS-developed national hose coupling standard.
Since its inception NBS/NIST has focused its research and measurement services activities on contemporary societal needs.

1901

2010

Standards for train tracks, couplings, steel manufacturing ...

Standards for clinical analytes, medical imaging, cybersecurity ...

Cholesterol
NIST Today

Major Assets
- ~ 2,900 employees
- ~ 2600 associates and facilities users
- ~ 400 NIST staff on about 1,000 national and international standards committees
- 3 Nobel Prizes in past 15 years

Major Programs
- **NIST Laboratories**
- Baldridge National Quality Program
- Hollings Manufacturing Extension Partnership
- Technology Innovation Program

Joint NIST/University Institutes:
- JILA
- Joint Quantum Institute
- Institute for Bioscience & Biotechnology Research
- Hollings Marine Laboratory
Role of NIST

NIST is a non-regulatory federal agency within the Technology Administration of the U.S. Department of Commerce. NIST’s primary mission is to promote economic growth by working with industry to develop and apply technology, measurements, and standards.

NIST carries out its mission through a portfolio of four programs:

- **Measurement and Standards Program**
  Planned and conducted in cooperation with industry and focused on infrastructural technologies.

- **Baldridge National Quality Program**
  An outreach program recognizing organizational performance excellence.

- **Hollings Manufacturing Extension Partnership Program**
  A nationwide network of extension centers that provides hands-on technical assistance to smaller manufacturers.

- **Technology Innovation Program**
  Planned to provide cost-shared awards to industry, universities and consortia for research on potentially revolutionary technologies that address critical national and societal needs.
Traditionally focused research and measurement service activities on physical science and engineering disciplines

Bioscience and Health identified as a new area for significant emphasis for NIST labs
NIST Applied Genetic Group
Applied Genetics
Group Mission Statement

*Advancing technology and traceability* through quality genetic measurements to aid work in

- forensic DNA testing
- *clinical diagnostics*
- cell line authentication
- agricultural biotechnology
- DNA biometrics
APPLIED GENETICS Group

Major Programs Currently Underway

• Forensic DNA
  – New loci and assays (26plex)
  – STR kit testing
  – Ancestry SNP assays
  – Low-template DNA studies
  – Mixture interpretation
  – STR nomenclature
  – Variant allele cataloging and sequencing
  – Expert systems review
  – Training workshops to forensic DNA laboratories
  – Validation information and software tools
  – Textbook – 3rd ed. (2 vol.)

• Clinical Genetics
  – Huntington’s Disease SRM
  – CMV SRM
  – Exploring future needs

• Ag Biotech
  – “universal” GMO detection/quantitation (35S promoter)

• DNA Biometrics
  – Rapid PCR methods
  – Efforts to standardize testing of future portable DNA systems
  – Kinship analysis

• Cell Line Authentication
Group Expertise and Funding Sources

**Group Expertise**

- Reference Material Characterization
- Standard Information Resource Development
- Rapid Multiplex PCR Assay Construction
- Short Tandem Repeat (STR) Genotyping
- Single Nucleotide Polymorphism (SNP) Genotyping
- DNA Sequencing
- Training Materials and Workshops (validation info)

**Current Funding Sources**

- **National Institute of Justice** (Forensic DNA)
- **FBI Science & Technology Branch** (DNA Biometrics)
- **NIST SRM Program** (SRM development and production)
- **Base funding from Congress** (clinical DNA)
Applied Genetics Group

Instrumentation

• **ABI 3130xl** for Sanger sequencing, SNP analysis, and STR genotyping

• **ABI 7500, Qiagen Roto-Gene Q, Roche LightCycler**, for qPCR (DNA quantitation)

• **ABI 9700** and Veriti thermal cyclers for PCR

• **Fluidigm BioMark** for digital PCR (copy number determination)
NIST Digital PCR Instrument

Arrived Spring 2010
NIST Standard Reference Materials (SRMs)
SRM Information

• Purpose & use
• Decision to create & design of components
• Pricing: how determined?
• Characterization
  – Stability
  – Homogeneity
  – Value assignment
• Certified value: how determined?
• Difference in approach from WHO (NIBSC)
What are Standards and Metrology?

- Standards and Metrology are the things needed to supply good data that can be used to confidently support decision making.
  - in a good world, good decisions are informed with good data which are the results of excellent measurements!

- Metrology and standards comprise the ‘formal’ system that tells us how well we trust those data

- Establishing confidence in data
Examples of Standards

1. **Documentary Standard**: Guideline documentation that reflects agreements in practices by governmental bodies or nationally or internationally recognized industrial, professional, trade associations.

2. **Certified or Standard Reference Material**: A physical entity to serve as a reference in measuring quantities or qualities, establishing practices or procedures, or evaluating results.
Importance of Reference Materials

Dr. Karen Mann, President of the Association of Molecular Pathology, in her testimony before Congress (Feb 24, 2010):

“Reference materials are important to ensure the necessary sensitivity, specificity and level of reproducibility of intra- and inter-laboratory test results. The best approach to achieve consistent and comparable quantitative data amongst laboratories is by the use of internationally established reference reagents.”

Source: http://www.amp.org
Comparing Results

- Results are only useful when compared
  - to other results
    - e.g., to observe a trend
  - to limits
    - e.g., a threshold for action
  - different results in different places or measured at different times…
    - “comparability over space-and-time”
Comparability of results

• Results linked to a common reference can be compared

• Scope of reference defines scope of comparability
  – global network
  • SI
Measurement Uncertainty

• Are these results the same?

• How well do you know the result?
  – essential part of being able to compare!

• Are these results good enough?
  – fit-for-purpose
“Quality” In Measurements

Method Validation

am I measuring what I set out to measure?

Uncertainty

how well do I know the result of what I’ve measured?

Traceability of Result
(Reference Materials)

can I compare this result with other results?
Applied Genetics

Standard Reference Materials (SRMs)

http://www.nist.gov/srm

Traceable standards to ensure accurate and comparable measurements between laboratories

SRM 2391b – autosomal STRs
SRM 2392 &-I – mtDNA sequencing
SRM 2395 – Y-STRs
SRM 2372 – DNA quantitation
SRM 2394 – mtDNA heteroplasmy
SRM 2399 – Fragile X

Calibration with SRMs enables confidence in comparisons of results between laboratories

Helps meet ISO 17025 needs for traceability to a national metrology institute
How Do We Know an SRM is Needed?

• May be mandated or recommended by a scientific society or even Congress
  – SRMs are useful when there is a need for between laboratory comparability of measurements; especially if the labs use different measure equipment and/or methods

• Other agencies may come to NIST requesting an SRM
  – it is valuable to document who is requesting the reference material and why
  – The National Institute of Justice (NIJ) requested we develop SRMs for the Forensic DNA Testing Community when DNA technology started entering the courtroom
  – For NIST in general other agencies include: CDC, EPA, NIH…
  – The FDA when inspecting looks at the calibration of critical instruments and may stipulate the use of a higher order standard when available
NIST and other National Metrology Institutes (NMIs) worldwide:
- provide and maintain primary standards
- provide linkages for traceability to the international system of measurement
- disseminate these realizations in a manner and of a quality that is consistent with the needs of the measurement community

The typical role of an NMI is to establish and maintain:

Scientifically-Sound, Metrologically-Based Competencies and Measurement Capabilities that are Internationally Vetted and Recognized

To provide calibration and measurement services disseminated to Customers via mechanisms such as:

- Validated Reference Methods
- Certified Reference Materials
- Reference Data
- Value-assignment of customer-provided samples or materials
- Value-assignment of Proficiency Testing samples
- Measurement Services for other Government Agencies
- Etc.

Although physical quantities such as length, mass, temperature, time, etc. are the first to come to mind when one thinks about metrology and measurement standards, chemical measurement research and standards have been a major activity at NIST since its inception in 1901.
Metre Convention
1875

General Conference on Weights and Measures
(CGPM)
meets every four years and consists of delegates from Member States

International Committee for Weights and Measures
(CIPM)
consists of eighteen individuals elected by the CGPM
It is charged with supervision of the BIPM and affairs of the Metre Convention
The CIPM meets annually at the BIPM

Consultative Committees (CCs)
Ten CCs each chaired by a member of CIPM; to advise the CIPM; act on technical matters and take important role in CIPM MRA; comprise representatives of NMIs and other experts.

Bureau International des Poids et Mesures
(BIPM)
International centre for metrology
Laboratories and offices at Sèvres with an international staff of about seventy

Associate States and Economies
of the CGPM

Governments of Member States

International organizations

Diplomatic Treaty

National metrology institutes (NMIs)
CIPM Mutual Recognition Arrangement

**Objectives:**

- Establish the degree of equivalence of national measurement standards maintained by NMIs
- Provide for the mutual recognition of both calibration and CRM certificates issued by NMIs
- Provide a secure technical foundation for wider agreements related to international trade, commerce and regulatory affairs

The CIPM Mutual Recognition Arrangement (MRA) was signed in October, 1999 by the directors of the NMIs of 47 member states of the Metre Convention, and representatives of two international organizations.
CIPM Mutual Recognition Arrangement

**National Metrology Laboratories Must:**

- Declare measurement capabilities that underpin services delivered to customers
- Participate in relevant International Key comparisons to validate claims
- Provide evidence of competence and **Quality systems** that underpin delivery of measurement services

**Outcome:**

- Statements of the measurement capabilities of each NMI in a database publicly available on the Web
Requirements for a NIST SRM

Material must be fit for purpose:

- **Homogeneity**
  - All tubes are the same
  - Test random samples

- **Stability**
  - Will withstand shipping and normal storage and is periodically tested over the life time of the SRM

- **Recoverability**
  - What went in the tubes comes out
  - Appropriate storage containers

- **Traceability**
  - Values assigned are traceable to the designated certification method

- **Commutability**
  - Is the SRM what the intended user needs?
  - Inter-laboratory study
Steps Involved in SRM Production

Receive input on priorities for projects and potential SRMs

Research potential properties and samples to be characterized and measurement method to be used

Obtain candidate components/make measurements

Decide on number of SRM units to produce (impacts price/unit), sample packaging, concentration, etc.

Bottle components and conduct homogeneity and stability studies; finalize uncertainty

Write Report of Analysis and Certificate of Analysis

Certificate Reviewed and Approved by NIST Measurement Services Division

SRM Made Available for Purchase
http://www.nist.gov/srm

Attend conferences, read the literature, talk to potential customers

Sequence & Copy Number

TAGATAGATAGATA

100

110
Pipeline for SRM Production

1. Request for SRM and internal decision to go forward
2. Understanding needs and scope of SRM (research)
3. SRM design
   what formats and amounts are best?
4. Gather materials for SRM components
5. Characterization of components
   Measure amounts with uncertainties, homogeneity, stability
6. Complete paperwork
   Report of Analysis (ROA) and Certificate of Analysis
7. Paperwork reviewed and SRM approved
8. SRM released (http://www.nist.gov/srm)
9. Customers use SRM and provide feedback

SD (standards development)  WCF (working capital funds)  SRM office
How is NIST SRM Cost Determined?

- SRM price is based on cost recovery of working capital funds requested in order to produce the SRM components
## Estimated Cost of an SRM

### $50K - 500 units produced

<table>
<thead>
<tr>
<th>Calculate SRM Unit Price</th>
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<tbody>
<tr>
<td><strong>Laboratory</strong></td>
<td><strong>CSTL</strong></td>
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<td><strong>SRM Category</strong></td>
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<td><strong>Production Costs</strong></td>
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<tr>
<td><strong>Units</strong></td>
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<td><strong>NIST Unit Production Cost</strong></td>
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<tr>
<td><strong>MSD SRM Operating Cost (Surcharge)</strong></td>
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<tr>
<td><strong>Cold Storage Fee</strong></td>
<td><strong>AD VALOREM Surcharge</strong></td>
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<tr>
<td>49% Service Development:</td>
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<tr>
<td>1% Obsolescence:</td>
<td>$1.00</td>
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<tr>
<td><strong>Dept. of Commerce surcharge</strong></td>
<td>$4.32</td>
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<td><strong>Lab Operations, if applicable</strong></td>
<td>$48.00</td>
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</table>

**Total Price** | **$389.32** | **Total Price** | **$989.32** |

**Rounded to Nearest Dollar** | **$389.00** | **Rounded to Nearest Dollar** | **$989.00**

### $50K - 100 units produced

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**Total Price** | **$989.32** | **Total Price** | **$989.00** |

**Rounded to Nearest Dollar** | **$989.00** | **Rounded to Nearest Dollar** | **$989.00**
How does NIST Determine the Number of Units to Produce?

We (the staff producing the SRM) are responsible for determining the appropriate number of units to produce considering items such as:

- the perceived demand for each SRM based on historical sales rates of the SRM or of similar SRMs,
- the technical difficulty of future renewals,
- the marginal cost of producing additional units,
- the cost of storage space,
- material stability

The sales price is set to collect all production costs (no profit) and repay the WCF in no more than five years.
If we build it, will they come?

Will clinical DNA labs or commercial calibrant suppliers consider the cost of the NIST material too expensive or not important to their work?
Lessons Learned from Forensic DNA Experience

• Mandate use of SRMs
  – This mandates NIST participation & SRM production

• Consistent, significant funding
  – Enables research as well as SRM production
  – National Institute of Justice partnership

• Experienced staff and appropriate equipment

• Standard techniques and targets
  – Short tandem repeat (STR) typing and core loci

• Information sharing
  – Creation of STRBase website to help community

• Inter-laboratory studies
  – Lessons learned can help with SRM needs and design
STANDARD 9.5

The laboratory shall check its DNA procedures annually or whenever substantial changes are made to the protocol(s) against an appropriate and available NIST standard reference material or standard traceable to a NIST standard.
1995: SRM 2391 PCR-based DNA Profiling Standard

- Size & D1S80 Allelic Ladders
- Amplified D1S80 products
- Genomic DNA extracts
- Cell lines on 903 paper
2003: NIST SRM 2391b

Driven primarily by commercial kit loci...

22 autosomal STRs characterized across 12 DNA samples
NIST Standard Reference Material (SRM) for Forensic DNA Testing

**SRM 2391b (2003-2011)**
- 48 autosomal STR loci with certified values
- 10 liquid genomic DNA components + 2 punches (cells on 903 paper)
- All single source samples
- 4 males + 6 females
- 9947A & 9948 included

**SRM 2391c (2011-future)**
- 23 autosomal STR loci and 17 Y-STRs certified
- 4 liquid genomic DNA components + 2 punches (cells on FTA & 903 paper)
- 5 single source + 1 mixture
- 3 males + 2 females (unique)
- All new samples
  - no 9947A or 9948
How to use an SRM...

• The SRM material has been assigned a value through vigorous analysis methods.
• The appropriate SRM is used to value assign/validate materials and/or methods or calibrate.
• Specific SRM usage instructions are included in the Certificate of Analysis supplied with each SRM material.
• The format of the SRM determines the analysis step for its use. (carried through the complete analysis, or only at a later stage of analysis)
How to Create NIST Traceable Materials?

- Make a “lot” of material that you want to use as your control material.
- Test your control material for homogeneity and stability
- Analyze your control material in parallel with the SRM material, using the SRM material to assign the value to the control material.
- This “lot” of control material is now traceable to the NIST SRM.
- When this “lot” of control material is consumed or yields a different result it can no longer be used
- New “lots” of control materials must be analyzed in parallel with the SRM; not to one another
What is Documented When Creating a NIST SRM?
What is Documented When Creating a NIST SRM?

• Homogeneity, Stability, Fit for Purpose, Purity

• Well characterized material
  – 3 levels of certification: certified, reference, or informational values

• Well documented:
  • An established paper trail that would allow a future NIST scientist to reproduce the material if needed
  – ROA: Report of Analysis
    • An internal NIST document that is very detailed
  – COA: Certificate of Analysis
    • What is released with the reference material that describes the contents of the SRM along with the certified values
SRM 2395 Documentation

Store all data in a central, secure location
SRM 2395 file drawer contents
Paper trail leads to a Certificate

This certificate will be provided with the reference material and describes what is certified.
REPORT OF ANALYSIS

25-June-2007

Submitted to: Laurie E. Locascio, Chief
Biochemical Science Division

Authors: Margaret C. Kline
Amy E. Decker
David L. Dueser
Peter M. Vallone

Title: Preparation and Homogeneity Testing of Materials for SRM
2372 Human DNA Quantitation Standard

Constituents: Human Genomic DNA

This ROA is 54 pages

The ROA is kept on file at the SRM Office (and the Division) and describes in great detail experimental data supporting the specified certified values.
Some of the details of SRM 2372

Material Qualification

Preliminary absorption spectra of the bulk materials over the wavelength range 220 nm to 345 nm were obtained using the BioCary 100 spectrophotometer located in 227/B261.

The molecular mass of the DNA in the bulk materials was assessed with a FlashGel (Cambrex Bio Science Rockland, Inc., Rockland, ME). The gel was loaded with 1 µL of each component and the proprietary FlashGel DNA Marker. The gel was electrophoresed for 7 minute at 275 Volts. Results of the analysis were imaged with the system’s proprietary gel-stain and recorded as a digital photograph on the FlashGel Dock with viewing light.

Selection of Vials for Analysis

Vials of each component were pulled for four different sets of analysis: UV/vis certification measurements, UV/vis homogeneity assessment, qPCR homogeneity assessment, and interlaboratory validation. The certification measurements required the total volume of 51 vials; three vials were taken from three of the corner cells (coded red in the diagram to the right) of each of the 17 boxes. The UV/vis homogeneity assessment required one vial from each box; this was taken from the “near center” cells (coded teal green in the diagram). The qPCR homogeneity assessment also required one vial from each box; this was taken from one of the edge cells (along one edge between the red cells). The interlaboratory validation study consumed 34 vials of the A, B, and C materials; these were selected from the “near center” and the remaining corner.

UV/vis Verification and Homogeneity

Absorbance homogeneity for all components was assessed using the BioCary 100 spectrophotometer located in 227/B261. Seventeen vials, one from each of the storage boxes, were assayed for each component using 80 µL cuvettes. The temperature of the cuvettes was 22 °C ± 1 °C, the same as used in the HAS II certification measurements.

The detailed UV/Vis homogeneity protocol is given in Appendix B.
qPCR Commutability Studies.

While the interlaboratory study supplied some information about systematic differences in response to the A, B, and C materials among the available qPCR methods, studies were conducted at NIST to provide greater detail for specific assays [6,7,8] and to evaluate Component B. Four commercially available DNA quantitation standards were used for these commutability studies. These studies are documented in MCK Notebook QPCR 05 pages 38 – 43, SRM2372\2372Certification\qPCR\qPCR_Usage.

One 96-well plate was used for each of the three qPCR methods evaluated. The design of the studies is shown schematically in Figure 4.

![Figure 4. qPCR Commutability Design](image)

Note book details

The SRM must be useful to the community. 2372 was checked against the most frequently used qPCR methods for the forensic Human Id community. Additional commercial stds were also tested.

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Note: “X” represents the component A, B or C is the 1:10 diluted material, X is a 1:5 dilution of X, X is a 1:2 dilution of X, X is a 1:2 dilution of X, X is a 1:2 dilution of X. “SP” represents one of the four commercially available DNA calibration materials. “SP” denotes a buffer-only negative control. SP Applied Biosystems (Foster City, CA).

Quantifier Human DNA Standard Part # 4343895 Lot #0412010, SP Applied Biosystems
Quantifier Human DNA Standard Part # 4343895 Lot #0602018, SP Applied Biosystems
Quantifier Human DNA Standard Part # 4343895 Lot #0604020, SP Applied Biosystems
Quantifier Human DNA Standard Part # 4343895 Lot #13636102. Applied Biosystems Quantifier Human DNA Standards reported [DNA] 200 ng/μL Promega Human Genomic DNA Male reported [DNA] 262 ng/μL.
Pipettes are Checked

When equipment is used for CRITICAL Measurements
The calibration of the equipment must be checked!

In this case with qPCR the pipettes used to aliquot the samples were verified to be functioning properly.

The 2 µL is within -1.8 % of the setpoint

The 10 µL is within -0.25 % of the setpoint
Definition of RM vs CRM (SRM at NIST)

**reference material** RM
material, sufficiently homogeneous and stable with reference to specified properties, which has been established to be fit for its intended use in **measurement** or in examination of nominal properties (JCGM 200: 2008)

**certified reference material** CRM
reference material, accompanied by documentation issued by an **authoritative body** and providing one or more specified property values with associated uncertainties and traceabilities, using valid procedures. (JCGM 200: 2008)

**Standard Reference Material® (SRM®)**: A CRM issued by NIST that also meets additional NIST certification criteria. (NIST SP 260-136: 2000)
Definition of SRM Materials

Certified Reference Materials from NIST that are:

• Well-characterized
• Using state-of-the-art measurement methods and/or technologies
• For the determination of chemical composition and/or physical properties
Three Possible Data Quality Descriptors

• **NIST Certified Value**
  – Highest confidence in its accuracy
  – All known sources of bias have been investigated

• **NIST Reference Value**
  – Best estimate of the true value
  – All known sources of bias have not been fully investigated

• **NIST Information Value**
  – Value will be of interest and use
  – Insufficient information is available to assess uncertainty

*NIST Special Publication 260-136 “Definitions of Terms and Modes Used at NIST for Value-Assignment of Reference Materials for Chemical Measurements*
Modes Used at NIST for Value-Assignment of Reference Materials for Chemical Measurements to Obtain a NIST Certified Value

- Certification at NIST Using a Single Primary Method with Confirmation by Other Method(s)
- Certification at NIST Using Two Independent Critically-Evaluated Methods
- Certification/Value-Assignment Using One Method at NIST and Different Methods by Outside Collaborating Laboratories
Steps Involved in Production

- Tubes need to be purchased
- Boxes need to be put together
- Labels need to be ordered from the SRM Office for each component and the box (6 wk lead time)
- Samples need to be dispensed into separate tubes as uniformly as possible to maintain homogeneity
- Homogeneity studies need to be performed
- Data collected will be evaluated by a statistician from the NIST Statistical Engineering Division
Bottling SRM 2372 Materials

Teflon container holding $\approx 250 \text{ mL}$ of **Candidate** SRM 2372.

*It’s not an SRM until it passes all testing.*

With a multi-channel pipettor 8 tubes can be filled at a time. That’s $\approx 214$ reps to fill 1700 tubes per component.
The assembly line closing the recently filled tubes

Protecting the SRM Product from the Staff: Lab Coats, Masks and Hair nets or full face shields Personal Protective Equipment (PPE) or Product Protective Equipment.
Safety Considerations: The Blister Brigade

Closing the 1,700 component A tubes (SRM 2372) caused some blisters even while wearing gloves. **Safety resolution:** Band-aids applied prior to closing SRM component tubes the next session helped reduce the number of blisters formed!
World Health Organization (WHO)

National Institute for Biological Standards and Control (NIBSC)

http://www.nibsc.ac.uk/products/biological_reference_materials.aspx

- Endocrinological
- Bacteriological
- Virological
- Cytokine, Growth Factor,
- Allergens, Sera,
- Miscellaneous
- Blood Products,
- Haemostasis, Thrombosis,
- Transfusion Science,
- Immunohaematology
- Retroviral
The World Health Organization established a program in biological reference materials with the intended use for vaccine production, immunological and biological assays. **NIBSC provides the International Standards (IS)**

An important motivation for this work was the safety of the blood supply.

They were initially designed to be qualitative standards.

The first WHO International Standard, for HCV, was released in 1997.

IS include: HCV, HBV DNA, HIV1 RNA, HAV, HPV type 16 and 18, Parvovirus B19, CMV.
NIST and NIBSC have different models for reference materials production

<table>
<thead>
<tr>
<th>NIBSC</th>
<th>NIST</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Clinical material or whole cultured virus</td>
<td>• Pure viral DNA</td>
</tr>
<tr>
<td>• Consensus evaluation, assay dependent value</td>
<td>• Independent RM</td>
</tr>
<tr>
<td>• Quantity expressed as International Units unique to that material</td>
<td>• Units are genome copy number per volume, traceable to the International system of units or SI</td>
</tr>
<tr>
<td>• No uncertainty</td>
<td>• Reported uncertainty</td>
</tr>
</tbody>
</table>
WHO International Standards - IU/mL

• **An International Standard** is a collection of ampoules containing as far as is possible the same amount of the analyte in as stable a form as possible (e.g. lyophilised, low moisture, low oxygen, stored at -20°C)

• The material has a stable quantifiable biological activity. It is similar to the real life analyte.

• Stability studies are carried out before it is established and real time stability is monitored

• The lifetime is unspecified and depends on data or need. Replacement where necessary is an issue.

• The unit is arbitrarily defined as a fraction of the contents of an ampoule; when the collection of ampoules is exhausted the replacement is chosen to be as close to the old one as possible but there is uncertainty and strictly speaking the unit changes.

• *Information on this and the next few slides provided by Phil Minor, Head of Virology, NIBSC*
Process for establishing WHO International Standards

- Identify need for standard, including requirements for source material and formulation (in consultation with experts within the field)

- Proposal for development of standard presented to WHO ECBS for adoption into standardization program

- Source and evaluate candidate materials, process development (trial fills)

- Prepare candidate standard (definitive fills)

- Launch worldwide collaborative study to evaluate candidate standard, samples are sent to a number of high quality laboratories for their analysis

- Data return and statistical analysis

- Prepare report for approval by study participants

- Present report to WHO ECBS for establishment of standard (candidate standard assigned concentration in International Units (IU), usually based on the mean titer determined in the collaborative study)

- NIBSC acts as the custodian and worldwide distributor of established WHO International Standards
Two standards developing organizations are working on primary reference materials for molecular diagnostics for infectious diseases with a current focus on viral infections of immune-compromised patients.

The organizations use two different models in the development of these reference materials.

NIST provides SRMs and NIBSC/WHO provide International Standards.

In the case of CMV, the NIST material is pure viral DNA (Towne strain) in buffer and the NIBSC material will be lyophilized intact virus (Merlin strain).

The SRM will be certified for sequence and quantity traceable to the SI and with calculated uncertainties.

The IS will have an assigned unitage that is derived from a collaborative study consensus value.

The proposed standards are to be used as primary standards to calibrate secondary standards that will be subsequently used as calibrants.
Clinical DNA SRMs: Current & Future
## Search Results

### November 12, 2010

**SRM/RM Number:** [Enter SRM/RM Number]

**Keywords:** clinical DNA

- **D** - Detail
- **T** - Table
- **C** - Certificate

### Archived Certificates

**Full Certificate Archive >>**

<table>
<thead>
<tr>
<th>SRM</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>C SRM 918</td>
<td>Potassium Chloride (Clinical Standard), January 22, 1971</td>
</tr>
<tr>
<td>C SRM 918a</td>
<td>Potassium Chloride (Clinical Standard), April 17, 1995</td>
</tr>
<tr>
<td>C SRM 919</td>
<td>Sodium Chloride (Clinical Standard), August 6, 1973</td>
</tr>
<tr>
<td>C SRM 919a</td>
<td>Sodium Chloride (Clinical Standard), April 23, 2004</td>
</tr>
<tr>
<td>C SRM 928</td>
<td>Lead Nitrate (Clinical Standard), Preprint, November 1, 1975</td>
</tr>
<tr>
<td>C SRM 933</td>
<td>Clinical Laboratory Thermometer</td>
</tr>
<tr>
<td>C SRM 934</td>
<td>Clinical Laboratory Thermometer, June 18, 1990</td>
</tr>
<tr>
<td>C SRM 937</td>
<td>Iron Metal (Clinical Standard), June 9, 1978</td>
</tr>
</tbody>
</table>
Clearly we need to improve our SRM offerings AND search engine!
Current NIST DNA Reference Materials

Forensic Applications

- **STR PCR DNA Profiling (SRM 2391b)** – 1995, r2008
- **Human Y-Chromosome DNA Profiling (SRM 2395)** – 2003, r2008
- **RFLP DNA Profiling (SRM 2390)** – 1992, r2001, *now obsolete*

Clinical Applications

- **Fragile X Human DNA Triplet Repeat (SRM 2399)** – 2004, r 2007
- **Huntington's Disease CAG Repeats (SRM 2393)**
- **Cytomegalovirus (CMV) Copy Number & Sequence (SRM 2366)**

Platform Testing

- **Human DNA Quantitation (SRM 2372)** - 2007
- **Heteroplasmic mtDNA Mutation Detection (SRM 2394)** - 2004
- **DNA Sequence Library for External RNA Controls (SRM 2374)**

*Several in development…*
SRM 2374 – DNA Sequence Library for External RNA Controls

• NIST SRM to contain 96 unique control sequences inserted in common plasmid DNA
  – engineered to be readily *in vitro* transcribed to make RNA controls
  – RNA controls intended to mimic mammalian mRNA
• Developed *sequence library* from submission by ERCC members, as well as synthesis
  – evaluated performance of RNA controls on variety of platforms
  – selected 96 well-performing sequences in collaborative study
    • from library of 176
• Array manufacturers modified products to include SRM sequences
• Prepared 400 units of SRM
  • 96 tubes in each

External RNA Control Consortium (ERCC)

Contact: Marc Salit to learn more
Alleles included in the SRM are:
15, 17, 29, 35, 36, 39, 40, 45, 50, & 75
NIST SRM projects for molecular diagnostics of infectious disease

Cytomegalovirus (CMV) – under development

Epstein-Barr virus and BK virus – in the planning stages
Who are our customers for infectious disease standards?

• Reagent and calibrant manufacturers
• Clinical laboratories that prepare their own calibrants
• NIST standards should be primary standards traceable to the SI
• NIST standards should be used to establish traceability for secondary standards/calibrants
• A central reference point to link the standards in use in clinical laboratories

Improved measurements through better calibration should lead to reduced variability among clinical labs
CMV Standard Reference Material

Type of material - pure viral DNA in buffer

- CMV DNA, Towne strain, cloned into a bacterial artificial chromosome (Towne Δ147)
  - Developed by Dr. Hua Zhu, New Jersey Medical School

- The BAC/CMV DNA is essentially a very large plasmid 240,000 bp, which is propagated and purified by a process that is similar to plasmids

- Advantages - Consistent genome size and ease of propagation
147 Towne BAC

- BAC DNA is considered stable and can accommodate up to 300,000 base pairs.

- DNA has been removed (20,000 base pairs) from the CMV viral genome to accommodate the BAC related DNA.

- DNA that has been removed is not in the regions used for amplification.
Properties Being Certified

- **DNA sequence** of regions of the CMV genome that are used as targets of PCR amplification
  - The following regions have been Sanger sequenced – UL34, UL54, UL55-56, UL80, UL83, UL97, UL122-126, UL132, & US17
  - Sequence to date matches GenBank Towne strain sequence

- **Copy number by direct measurement** using digital PCR
CMV DNA Sequence Characterization

qPCR assays for digital PCR measurement

Regions sequenced

Schematic map of the CMV genome. The CMV genome is organized as two regions of unique sequences, unique long (UL) and unique short (US), flanked by two sets of inverted repeats (light shaded boxes). Kotenko et al. (2000) PNAS 97(4): 1695-1700

Sequence alignments from available GenBank CMV strains being compared against published PCR primers and probes – this information is being included on the NIST Clinical DNA Information Resource website.

Sanger sequence result obtained at NIST from UL54 (section shown is the reverse primer region for the CP1 assay used in digital PCR and matches the Towne strain and published primer)
Certification of the CMV DNA - genome copy counting

Digital PCR – Quantify the amount of DNA (copies/volume) by counting amplification from single molecules

- Nano scale reactions (6 nL)
- DNA concentration where some reaction chambers are negative
- Based on Poisson statistics, number of copies is determined
- Thousands of replications/assay repeated with multiple assays targeting regions on the CMV genome
- Traceable to the SI via the Mole
- New tool, with active research at other National Metrology Institutes to validate this approach
NIST Digital PCR Instrument

Arrived Spring 2010
Digital PCR

Pressurized valve

Samples: 1-12
Water: H

HX 1 2 3 4 5 6 7 8 9 10 11 12 X H

765 individual chambers / panel
12 panels/chip
Preparation of the SRM

- Three levels of DNA concentration in buffer
- Packaging in Teflon tubes
- Certification for copy number/volume will be done on each of the concentrations
- Monitoring for homogeneity and stability for the life of the SRM (5 years from time of issue)
  - We currently have a stock that we have been monitoring for 18 months at three temperatures
Variability in Digital PCR Measurements Across Different CMV Targets (all three candidate components for SRM 2366)
Variability in Digital PCR Measurements Across Different CMV Targets
(single candidate component of SRM 2366)

ANOVA of the data showed no significant differences between groups

<table>
<thead>
<tr>
<th>TaqMan Assay</th>
<th>Average across all groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average: 2118 copies/μL</td>
</tr>
<tr>
<td></td>
<td>SD: 150 copies/μL</td>
</tr>
<tr>
<td></td>
<td>Uk=2: 300 copies/μL</td>
</tr>
</tbody>
</table>
QCMD CMV EQA 2010 Inter-laboratory Study

- NIST collaborated with Quality Control for Molecular Diagnostics (QCMD) for an inter-laboratory study conducted as part of the QCMD CMV 2010 EQA program

- NIST provided aliquots of CMV DNA, component B of the candidate CMV SRM, to QCMD

- A vial of DNA was sent out to each participating laboratory along with the 10 QCMD samples (lyophilized virus in plasma or VTM)

- While the QCMD samples required extraction, the NIST DNA was to be added directly to the assay. Participants were asked to run the assay in triplicate and report results in copies / mL.

- 181 data sets were submitted
# QCMD Data Analyzed by Assay Type

<table>
<thead>
<tr>
<th>QCMD CMV EQA - Participants and assays</th>
<th># Data sets</th>
<th>Median</th>
<th>MADe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Datasets</td>
<td>181</td>
<td>5.900</td>
<td>0.486</td>
</tr>
<tr>
<td>Conventional Commercial</td>
<td>5</td>
<td>5.854</td>
<td>0.872</td>
</tr>
<tr>
<td>Real-Time Laboratory developed - Total</td>
<td>78</td>
<td>6.002</td>
<td>0.650</td>
</tr>
<tr>
<td>Real-Time Commercial - Total</td>
<td>96</td>
<td>5.826</td>
<td>0.451</td>
</tr>
<tr>
<td>Argene CMV HHV6,7,8 R-gene</td>
<td>6</td>
<td>5.864</td>
<td>0.150</td>
</tr>
<tr>
<td>Argene CMV R-gene</td>
<td>15</td>
<td>6.205</td>
<td>0.332</td>
</tr>
<tr>
<td>Nanogen Q-CMV Real time Complete Kit</td>
<td>21</td>
<td>5.733</td>
<td>0.794</td>
</tr>
<tr>
<td>QIAGEN artus CMV PCR Kit (RG, LC, TM)</td>
<td>28</td>
<td>5.821</td>
<td>0.326</td>
</tr>
<tr>
<td>Roche LightCycler CMV Quant Kit</td>
<td>12</td>
<td>5.776</td>
<td>0.298</td>
</tr>
</tbody>
</table>
Information Resources for the Clinical Genetics/Healthcare Community

http://www.nist.gov/mml/biochemical/genetics/clinical_dna.cfm

Clinical DNA Informational Resource (CDIR)
http://www.nist.gov/mml/biochemical/genetics/clinical_dna.cfm

• In 2009, we started an internet based information resource – the goal is to provide information on DNA sequence alignments and primer and probes for clinical assays as a start

• We plan to load this resource with accessible and useful information for the clinical diagnostics community

• We welcome input from you on additions and changes to this information resource
Clinical DNA Informational Resource Website

http://www.nist.gov/mml/biochemical/genetics/clinical_dna.cfm
Sequence Alignments for CMV Strains

 Applied Genetics

Sequence Alignments for CMV Strains

Applied Genetics

Sequence Alignments for CMV Strains

Applied Genetics
Sequences from 14 CMV Strains

Sequence alignments

The qPCR assay below (Tanaka 2000 Journal of Medical Virology 60:455–462) has a large number of mismatches that cause a false negative rate of 24%. A little more time in the library, probably would have lead this researcher to redesign the primer and probe binding sites.

<table>
<thead>
<tr>
<th>Ref</th>
<th>Isolate</th>
<th>Forward primer</th>
<th>qPCR probe</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3]</td>
<td>AD169</td>
<td>gactagtgtgtgctggcccaag</td>
<td>agcctgaggttatcaagtatgaagcgcc</td>
<td>cagatgaggaagaggctattgtgac</td>
</tr>
<tr>
<td>[5]</td>
<td>Toledo</td>
<td>gactagtgtgtgctggcccaag</td>
<td>agcctgaggttatcaagtatgaagcgcc</td>
<td>cagatgaggaagaggctattgtgac</td>
</tr>
<tr>
<td></td>
<td>FIX</td>
<td>gactagtgtgtgctggcccaag</td>
<td>agcctgaggttatcaagtatgaagcgcc</td>
<td>cagatgaggaagaggctattgtgac</td>
</tr>
<tr>
<td>[7]</td>
<td>TB40/E Clin iso 1</td>
<td>gactagtgtgtgctggcccaag</td>
<td>agcctgaggttatcaagtatgaagcgcc</td>
<td>cagatgaggaagaggctattgtgac</td>
</tr>
<tr>
<td>[8]</td>
<td>Clin iso 2</td>
<td>gactagtgtgtgctggcccaag</td>
<td>agcctgaggttatcaagtatgaagcgcc</td>
<td>cagatgaggaagaggctattgtgac</td>
</tr>
<tr>
<td></td>
<td>Clin iso 3</td>
<td>gactagtgtgtgctggcccaag</td>
<td>agcctgaggttatcaagtatgaagcgcc</td>
<td>cagatgaggaagaggctattgtgac</td>
</tr>
<tr>
<td></td>
<td>Clin iso 4</td>
<td>gactagtgtgtgctggcccaag</td>
<td>agcctgaggttatcaagtatgaagcgcc</td>
<td>cagatgaggaagaggctattgtgac</td>
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<tr>
<td></td>
<td>Clin iso 5</td>
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<td>Clin iso 6</td>
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<td>agcctgaggttatcaagtatgaagcgcc</td>
<td>cagatgaggaagaggctattgtgac</td>
</tr>
</tbody>
</table>
Summary

• We are asking for your input on what we are currently doing and the relevance to the clinical community

• This is the first time that NIST has worked on standards for clinical diagnostics of infectious disease – we want to get it right

• Please come and talk to us during the meeting: we have three posters G09, ID19, and OTH05.
NIST Presentations at AMP 2010

- G09 (poster): Characterizing the Electrophoretic Mobility of Huntington Disease Alleles 13-77: Are the Deviations from the Theoretical Values Intrinsic or Internal Sizing Standard Artifacts?

- ID19 (poster): NIST Candidate Standard Reference Material (SRM): Cytomegalovirus DNA

- OTH05 (poster): Cautionary Considerations when Exploring Cell Lines as Potential Reference Materials
Contact Information

Group Members Working on Clinical DNA Projects

• Marcia Holden (x4162)
• Margaret Kline (x3134)
• Ross Haynes (x4469)
• John Butler (x4049) – Group Leader

Email: first name . last name @ nist.gov
Phone: 301-975-x

http://www.nist.gov/mml/biochemical/genetics/clinical_dna.cfm
Pipeline for SRM Production

1. Request for SRM and internal decision to go forward
2. Understanding needs and scope of SRM (research)
3. SRM design
   what formats and amounts are best?
4. Gather materials for SRM components
5. Characterization of components
   Measure amounts with uncertainties, homogeneity, stability
6. Complete paperwork
   Report of Analysis (ROA) and Certificate of Analysis
7. Paperwork reviewed and SRM approved
8. SRM released (http://www.nist.gov/srm)
9. Customers use SRM and provide feedback

SD (standards development)  WCF (working capital funds)  SRM office
Reference Materials Requested in Karen Mann’s Congressional Testimony (Feb 24, 2010)

- **Immediate**
  - CMV
  - BCR/ABL
  - KRAS
  - EGFR

- **Short term**
  - BK virus
  - Epstein Barr virus

- **Medium term**
  - Certified Gene Sequence Databases
  - Infectious agents: adenovirus, enterovirus, Hepatitis B virus, Herpes simplex, JC virus, …

Source: http://www.amp.org
CMV

- Materials being characterized for sequence and amount
  - Material: Towne strain
  - Components: 3 vials of liquid DNA containing the same sequence but different concentrations
BCR/ABL

• **Specific Needs:**
  – What is the measurement issue?

• **SRM Design:**
  – What format (e.g., genomic DNA, amount)?
  – How many variants (e.g., different types)?
  – How many components desired?

• **Source of Materials:**
  – Who can supply appropriate starting material of sufficient quantity for SRM production?
KRAS

• Specific Needs:
  – What is the measurement issue?

• SRM Design:
  – What format (e.g., genomic DNA, amount)?
  – How many variants (e.g., different types)?
  – How many components desired?

• Source of Materials:
  – Who can supply appropriate starting material of sufficient quantity for SRM production?
EGFR

• **Specific Needs:**
  – What is the measurement issue?

• **SRM Design:**
  – What format (e.g., genomic DNA, amount)?
  – How many variants (e.g., different types)?
  – How many components desired?

• **Source of Materials:**
  – Who can supply appropriate starting material of sufficient quantity for SRM production?