Developing Protein Reference Materials and Reference Measurement Procedures

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NIST ... is an agency of the US Department of Commerce.

NIST Assets Include:

- 3,000 employees
- 1,600 guest researchers
- $971 million annual budget in FY16
- NIST Laboratories ($690 million budget in FY16)
- Standard reference material (SRM) program → > 1500 certified reference materials (CRM)
NIST Standards for Chemical Measurements

Chemical standards constitute over 2/3 of ~1,500 NIST SRM types

- High Purity Neat Chemicals
- Organic Solution Standards
- Inorganic Solution Standards
- Gas Mixture Standards

Complex Matrix Standards
- Advanced Materials
- Biological Fluids/Tissues
- Foods/Botanicals
- Geologicals
- Metals and Metal Alloys
- Petroleum/Fossil Fuels
- Sediments/Soils/Particulates
- Cements

- Molecular Spectrometry Standards
- Electrolytic Conductivity Standards
- pH / Ion Activity Standards
What is Measurement Standardization/Harmonization?

- Standardization improves the accuracy of measurements.
- Standardization and harmonization improve measurement equivalence over time and space.
- Implementation of a reference measurement system.
Accuracy and Equivalence in measurement is achieved through traceability.

“traceability is the property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty”

--JCGM200:2012 International vocabulary of metrology (VIM)

The reference can be an international unit (mole, kilogram, etc.), an international reference material, or an in-house reference material.
Measurement Traceability:

- **SI Unit (mole)**
  - **purity assessment**
  - **reference measurement procedure**
  - **value transfer procedure**
  - **routine measurement procedure**
  - **Measurement Result**

- **metrology institutes**
  - **primary certified reference materials**
  - **secondary certified reference materials**

- **assay manufacturers**
  - **calibrators**

- **laboratory**
  - **routine sample**

*Adapted from ISO 17511*
Elements of a Reference Measurement System:

- Definition of the Measurand
- Certified Reference Materials
- Reference Measurement Procedures
- Reference/Calibration Laboratory Networks
Definition of the Measurand

What are you measuring?

- For small molecules, defining the measurand is often straightforward.

- For large and chemically complex molecules such as proteins, defining the measurand remains a substantial challenge (confounded by the use of affinity reagents in routine measurement procedure).
Elements of a Reference Measurement System:

- Definition of the Measurand
- Certified Reference Materials
- Reference Measurement Procedures
- Reference/Calibration Laboratory Networks
Type of Reference Materials

Certified Reference Materials (CRMs):  

- Analyte concentration measured using one or several reference measurement procedure (ensure accuracy and precision) which provide traceability to an SI unit (e.g., mole, kg, etc)
- Heterogeneity and stability thoroughly characterized
- Certificate of Analysis provides information on certified analyte concentration and its measurement uncertainty
- Produced in large batches to produce a 5+ year supply to support continuity of use
- Provided by national metrology institutes (NIST, IRMM, LGC, NMIA, NMIJ, CENAM, etc) and some commercial suppliers (Fluka, Supelco, RTC, Cerilliant, etc.)
Type of Reference Materials

Reference Materials (RMs) :

- Analyte concentration measured using one or several measurement procedure but without traceability to an SI unit.
- Heterogeneity and stability thoroughly characterized
- Provided with reference concentration values and incomplete statement of measurement uncertainty
- Produced in large batches to produce a 5+ year supply to support continuity of use
- Provided by national metrology institutes (NIST, IRMM, LGC, NMIA, NMIJ, CENAM, etc.) and international organizations (USP, IAEA, etc.)
Type of Reference Materials

Reference Preparations/International Standards:

- Analyte concentration in arbitrary units without statements of uncertainty
- Stability and homogeneity may or may not be characterized
- Produced in large batches to produce a 5+ year supply to support continuity of use
- Produced by national metrology institutes (NIBSC) and international organizations (WHO)
Uses of Reference Materials

- Preparation of calibrators for reference measurement procedures (from pure substance CRMs)
- Value-assign calibrator concentration for routine measurement procedures
- QC materials for evaluating within-laboratory performance
- QC materials for evaluating between-laboratory performance
- QC materials for evaluating new measurement procedures
Development of Clinical Certified Reference Materials

Step 1: *Identify need through appropriate clinical partner*

- NIST works with professional societies (IFCC, AACC, CAP) and other government agencies (NIH, CDC) to assess the clinical need for standardization and define goals
Step 2: **Define the measurand**

- Highly heterogeneous analytes (i.e., proteins) may have forms that possess higher clinical relevance than other forms of the same analyte.

- Highly heterogeneous analytes may be reduced into simpler measurands by identifying a constant region (i.e., cardiac troponin I) or a single post-translational modification (i.e., hemoglobin A1c).
Development of Clinical Certified Reference Materials

Step 3: Development Reference Measurement Procedure

- Isotope dilution (ID) LC-MS/MS is the most commonly used method to measure well-defined small clinical molecules and some proteins.

- ID LC-MS/MS requires the availability of an isotopically-labeled internal standard for the analyte being measured.

- Well-characterized immunoassays are being explored as an alternative method for certification when ID LC-MS/MS cannot easily be used.
Step 4: Produce Certified Reference Material

- Primary (i.e., pure substance) certified reference materials need to be available or produced with purity assessment.
- For simple, well-characterized analytes (i.e., creatinine), matrix-based reference materials can be produced by spiking a primary CRM into the appropriate matrix.
- For most cases, certified reference materials can only be produced from pooled patient samples with endogenous levels of the analyte.
Development of Clinical Certified Reference Materials

Step 5: Evaluate Homogeneity and Stability of CRM

- For homogeneity assessment, the reference measurement procedure is used
- For stability assessment, a method is used that can highlight instability

Step 6: Perform Commutability Study of CRM

- Clinical partners are invaluable in providing access to the patient samples and routine assays needed to for a commutability study
CERTIFIED REFERENCE MATERIAL:  
**HUMAN CARDIAC TROPONIN**

- Human cardiac troponin complex has 3 subunits:
  - cardiac troponin T (cTnT)
  - cardiac troponin C (cTnC)
  - cardiac troponin I (cTnI)
- Troponin complex regulates the contraction of heart muscle
- Troponin complex released into the bloodstream upon injury to heart muscle leading to cardiomyocyte necrosis
- Measurement of cTnT and cTnI in blood is used to diagnose heart muscle damage and as a prognostic indicator
Certified Reference Material Development: 
*cardiac troponin I*

in heart muscle tissue

in the bloodstream
Troponin I
Troponin C
Sites of proteolytic degradation
Heparin
Phosphorylation

Other chemical modifications – phosphorylation, oxidation, reduction, N-acetylation

Figure kindly supplied by Dr Alexey Katrukha (Hytest)
Modified forms of cTnI

**Cardiac tissue**

- **kDa 30**
- **kDa 20**
- **kDa 14.4**

![Cardiac tissue gel image]

- cTnI
- Peptide 1
- Peptide 2
- Peptide 3
- Peptide 4
- Peptide 5
- Peptide 6
- Peptide 7

**Blood**

- **kDa 37**
- **kDa 25**
- **kDa 15**

![Blood gel image]

**Katrukha et al.**

**Labugger et al.**
Circulation 2000;102:1221-6
cTnI, acetylated, phosphorylated, w/o C-terminal PheGluSer (calc $M_r = 23,635$)
cTnI, acetylated, di-phosphorylated, w/o C-terminal PheGluSer (calc $M_r = 23,715$)
cTnI, acetylated, phosphorylated, w/o C-terminal GluSer (calc $M_r = 23,782$)
cTnI, acetylated, di-phosphorylated, w/o C-terminal GluSer (calc $M_r = 23,862$)
cTnI, acetylated, phosphorylated (calc $M_r = 23,997$)
cTnI, acetylated, di-phosphorylated (calc $M_r = 24,078$)
First Round Robin Study on Troponin I Standardization
(in collaboration with the AACC Subcommittee on Troponin I Standardization)

Starting with 10 Candidate Reference Materials...
  • extracted and recombinant Troponin CIT complexes
  • recombinant Troponin CI complex
  • lyophilized extracted/recombinant Troponin CIT complex
  • lyophilized recombinant Troponin CI complex

...sent to the manufacturers of 13 cTnI measurement assays
  (9 domestic, 4 international)

Results:
Statistical ranking (using the deviation from expected results) was used to choose 4 Candidate Reference Materials for further evaluation...
Second Round Robin Study on Troponin I Standardization

4 Candidate Reference Materials (cRM):
- extracted Troponin CIT complex
- recombinant Troponin CI complex
- recombinant Troponin CIT complex
- lyophilized extracted Troponin CIT complex
- lyophilized recombinant Troponin CI complex
- lyophilized recombinant Troponin CIT complex

9 pooled Patient Samples:
- target values of 0.4, 1.5, and 15 µg/L; AMI ≤ 24 hours
- target values of 0.4, 1.5, and 15 µg/L; AMI > 48 hours
- degraded recombinant Troponin CIT complex in serum; target values of 0.3, 1.0, and 12 µg/L

Manufacturers calibrated their assays using each of the 4 cRMs and then measured the 9 patient samples; commutability was assessed.
NIST SRM 2921: Human Cardiac Troponin Complex

- Human cardiac troponin complex, extracted from human hearts, prepared by HyTest Ltd. (Turku, Finland)
- Supplied as a dilute solution (31 mg/L) in 150 mM sodium chloride, 5 mM calcium chloride, and 20 mM tris buffer, pH 7.5
- Concentration of TnI determined through a combination of reversed-phase LC and amino acid analysis
- Relative purity and the evaluation of the structures of the troponin subunits, including post translational modifications made by:
  - LC/MS
  - MALDI-MS
  - Peptide mapping (with trypsin and a combination of endo Glu-C/Lys-C) followed by MALDI-MS and capillary LC/MS
- Commutability study performed in cooperation with the American Association for Clinical Chemistry and clinical cTnI assay manufacturers
Elements of a Reference Measurement System:

- Definition of the Measurand
- Certified Reference Materials
- Reference Measurement Procedures
- Reference/Calibration Laboratory Networks
International vocabulary of metrology (VIM) Definition of a Reference Measurement Procedure (RMP):

“Measurement procedure accepted as providing measurement results fit for their intended use in assessing measurement trueness of measured quantity values obtained from other measurement procedures for quantities of the same kind, in calibration, or in characterizing reference materials”

--JCGM200:2012 International vocabulary of metrology (VIM)

In simpler terms, a RMP is a measurement method(s) which:
• has been validated to measure what it is intended to measure
• provides measurements which have been thoroughly assessed for bias
Uses of Reference Measurement Procedures:

• value-assignment of certified reference materials and higher-order calibration solutions

• Assessing the performance characteristics of routine assay systems (instrumentation and reagents)

• Comparison of routine assays

• Detection of analytical influence quantities in routine samples
Reference Measurement Procedure:  
**ID-LC-MS/MS Quantification of Human Serum Albumin in Urine**

• A healthy kidney filters blood, retaining blood proteins and filtering small molecule waste into the urine

• With kidney disease, the kidney cannot filter properly and serum proteins enter the urine

• The clinical measurement of human serum albumin in urine is used to diagnose and monitor kidney disease
ID-LC-MS/MS Quantification of Human Serum Albumin in Urine

Quantification of Urinary Albumin by Using Protein Cleavage and LC-MS/MS
Jesse C. Seegmiller, David R. Barnidge, Bradley E. Burns, Timothy S. Larson, John C. Lieske, and Rajiv Kumar; Mayo Clinic, Rochester, MN, USA
*Clinical Chemistry June 2009 vol. 55 no. 6 1100-1107*
Urine Albumin peptides with demonstrated MRM transitions by LC-MS/MS

Underlined sequences are peptides whose masses have been observed by LC-MS/MS analysis.

DAHKSEVAHRFK\textbf{DLGEENFK}ALVLIAFAQYLQQCPFEDHVKL\textbf{LVNEVTETFAK}
TCVADESAENCDSKLHTLF6GKCTVATLRETYGEMADCCAKQEPEERNE
CFLQHKDDNPNLPLRVRPEVDVMCTAFHDNEETFLK\textbf{KYLEYIAR}RHPFYF
APELLFFAKRY\underline{KAFAFECQAAADKAACL}LPKLDLRLDEGKASSAKQRLKC
ASLQKFGERFAKAWAVA\underline{LSQ}RFPK\underline{AEFAEV}SLV\underline{DLT}KVHTECCHGD
LCEC\underline{ADDRA}DLAKY\underline{IC}ENQ\underline{DSI}SSKLKECCEKPLEKSHCIAEVENDEMPA
DLPSLAADFVESKD\underline{VC}KN\underline{AY}AE\underline{AK}DVFLGMFLYEYARR\underline{HPDYSVVL}LLR\underline{LA}
KTYETTLEK\underline{CC}AA\underline{AD}PHECYAKVFDEFKPLVEEPQNLIKQNC\underline{ELFEQLGE}
YK\underline{FQNALL}VR\underline{YRTK}KVPQVSTP\underline{LT}L\underline{VE}SRNLGKVGSKCC\underline{H}PEAKRMPCAE
DYLS\underline{VLNLQ}L\underline{CVLHEK}\underline{TP}V\underline{SDR}VT\underline{KCT}ESL\underline{VN}R\underline{RPCFSALEVD}ETYVPK
EF\underline{NAET}T\underline{TFHADICTL}E\underline{SEKERQ}IKK\underline{QTAL}VEL\underline{VK}HKPKAT\underline{KEQLKAVM}DD
\underline{FAAFVEK}CCK\underline{ADD}D\underline{KETCF}AE\underline{EGK}KLVA\underline{ASQALGL}
Albumin in Urine Reference Measurement Procedure

- Multiplexed LC-MS/MS quantitative and qualitative measurement of 8 tryptic peptides from human albumin
- Two MRM transitions per peptide are measured
- CV ≈ 1% for technical replicates

![Graph]

\[ y = 0.9116x + 0.2636 \]
\[ R^2 = 0.9979 \]

<table>
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<th>Sample</th>
<th>Avg. Area Ratio</th>
<th>Stdev</th>
<th>%CV</th>
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*n=3 (Technical Replicates)
RESOURCES

JCTLM Database

The Joint Committee for Traceability in Laboratory Medicine (JCTLM) maintains a database of vetted certified reference materials and reference measurement procedures for clinical measurand that have been submitted by national metrology institutes:

http://www.bipm.org/jctlm/

Papers and Guidelines

THANK YOU