A Practical Example of CLSI C60 in Action: Vitamins and Hormones

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Disclosures

- Thermo Fisher Scientific, Consulting

CLSI C60 “In Action” Presentation Notes

- Data shown was obtained BEFORE CLSI C60 draft guidelines were developed
- The presented data does not reflect draft guidelines in all cases, but will be used to frame discussions of draft CLSI C60 recommendations
- Draft CLSI C60 experimental design strategies are still under development and may not represent the final guidelines
- CLSI C60 Hierarchical Best Practice Recommendations will be highlighted, if available
CLSI C60 "In Action": Topics to be Covered

Stability
Blank Matrix, S/N, LLoQ
Precision, Accuracy
Linearity, Dilution
Carryover
Matrix Effects
Interferences
Quality Assurance

CLSI C60 "In Action": References

• Current Versions of Clinical and Laboratory Standards Institute (CLSI) Guidelines
• FDA Guidance for Industry: Bioanalytical Method Validation, May 2001
• European Union (EU) Directive 2002/647/EC: Analytical Methods and Interpretation of Results
• European Medicines Agency (EMA): Guideline on Bioanalytical Method Validation, Feb 2012
• Current CLIA Guidelines and CAP Checklist Items
• CLSI C60 Document Development Committee Consensus

CLSI C60 Recommendations: Stability Assessments

C60: Assess analyte stability in native matrix under appropriate storage conditions

Short-term stability at RT (Bench top)
Long-term stability at 2-8°C, -20°C or -70°C
Max # of Freeze/Thaw cycles, if applicable

FDA Experimental Design:
• General: Assess bias, imprecision - stored vs. fresh samples
  3 aliquots of 2 conc (Max 3x LLoQ and "near" ULoQ (EMA)),
• Short-term: RT for 4-24hrs
• Long-term: 3 storage durations
• Freeze/thaw: Thaw unassisted, assess at least 3 F/T cycles

**CLSI Guideline Under Dev: C55, Protocols for Establishment of Sample Stability in Clinical Chemistry and Toxicology; Est release June 2013**
C60: Assess analyte stability during all phases of the analytical measurement process

- Determine max bench-top processing time (ex: extracts at RT)
- Determine max storage duration of extracts/preparations in the autosampler (evaporation effects, analyte degradation)

C60: Assess stability of reagents (extraction reagents, mobile phases, IS, stock solutions)

- Monitor bias and imprecision of QC or other matrix approp test materials over duration of reagent storage

**CLSI EP25 – Evaluation of Stability of In Vitro Diagnostic Reagents**

- Monitor for extraction efficiency changes over time
- Monitor for degradation of IS signal, hydrogen-deuterium exchange

Determine max bench-top processing time (ex: extracts at RT)

Determine max storage duration of extracts/preparations in the autosampler (evaporation effects, analyte degradation)

CLSI C60 Recommendations: Stability Assessments

Stability of Extracts Stored in Autosampler

25-OH D3

Injected extracted cal, QC and native patient samples at T = 0

Extracts re-injected after 17hr and 24hr storage in autosampler (2-8°C)

Acceptability Criteria: ± 10% or ≤ 2 ng/mL

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Time 1</th>
<th>Time 2</th>
<th>Mean Response</th>
<th>%CV</th>
<th>Unit Dev.</th>
<th>% Diff.</th>
<th>% Diff.</th>
<th>% Diff.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>4.9</td>
<td>5.7</td>
<td>5.3</td>
<td>2.3</td>
<td>5.3</td>
<td>7.9</td>
<td>9.9</td>
<td>10.4</td>
</tr>
<tr>
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<td>5.5</td>
<td>5.3</td>
<td>2.3</td>
<td>5.3</td>
<td>7.9</td>
<td>9.9</td>
<td>10.4</td>
</tr>
<tr>
<td>Patient 3</td>
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<td>6.1</td>
<td>5.8</td>
<td>2.6</td>
<td>5.6</td>
<td>7.5</td>
<td>9.6</td>
<td>10.7</td>
</tr>
<tr>
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<td>5.7</td>
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<td>5.6</td>
<td>7.7</td>
<td>9.6</td>
<td>10.7</td>
</tr>
<tr>
<td>Patient 5</td>
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<td>6.2</td>
<td>5.9</td>
<td>2.3</td>
<td>5.8</td>
<td>7.3</td>
<td>9.0</td>
<td>10.2</td>
</tr>
<tr>
<td>Patient 6</td>
<td>5.0</td>
<td>6.2</td>
<td>5.9</td>
<td>2.3</td>
<td>5.8</td>
<td>7.4</td>
<td>9.3</td>
<td>10.6</td>
</tr>
<tr>
<td>Patient 7</td>
<td>5.5</td>
<td>6.3</td>
<td>5.9</td>
<td>2.2</td>
<td>5.9</td>
<td>7.3</td>
<td>9.1</td>
<td>10.3</td>
</tr>
<tr>
<td>Patient 8</td>
<td>5.0</td>
<td>6.3</td>
<td>5.9</td>
<td>2.2</td>
<td>5.9</td>
<td>7.3</td>
<td>9.1</td>
<td>10.3</td>
</tr>
<tr>
<td>Patient 9</td>
<td>5.5</td>
<td>6.4</td>
<td>6.0</td>
<td>2.1</td>
<td>6.0</td>
<td>7.2</td>
<td>9.0</td>
<td>10.2</td>
</tr>
<tr>
<td>Patient 10</td>
<td>5.0</td>
<td>6.4</td>
<td>6.1</td>
<td>2.0</td>
<td>6.1</td>
<td>6.9</td>
<td>8.9</td>
<td>10.0</td>
</tr>
<tr>
<td>Patient 11</td>
<td>5.5</td>
<td>6.5</td>
<td>6.1</td>
<td>2.0</td>
<td>6.2</td>
<td>7.0</td>
<td>9.0</td>
<td>10.1</td>
</tr>
<tr>
<td>Patient 12</td>
<td>5.0</td>
<td>6.5</td>
<td>6.1</td>
<td>2.0</td>
<td>6.1</td>
<td>7.0</td>
<td>9.0</td>
<td>10.1</td>
</tr>
</tbody>
</table>

CLSI C60 Recommendations: Validation of a Blank Matrix

C60: Validate a blank matrix for use in subseq validation procedures (Cal prep, LLoQ, AMR, recovery...ect)

- Ensure low bkg noise in solvent/extraction reagent
- Meas peak area of a double blank matrix (no analyte/no IS)
  - Can use BSA or stripped serum if no analyte-free native matrix

C60 Best Practice Acceptability Criteria:

- No peak OR
- Blank resp < 20% peak area of LLoQ resp and <5% of IS resp in 5-6 lots of blank material
CLSI C60 Recommendations: Signal-to-Noise (S/N)

CLSI EP17 LoD Limitations:
- LoD design is resource intense (40-60 reps/sample, 3-5 samples, 5 runs)
- LoD is not generally relevant for LC-MS/MS

C60: Calc S/N using blank matrix vs. matrix at LLoQ

C60 Best Practice Acceptability Criteria:
- S/N at LLoQ of 20:1 to ensure ruggedness

C60 Best Practice Acceptability Criteria:
- S/N at LLoQ of 16:1

S/N Verification at the LLoQ

Testosterone spiked into 2 lots of stripped serum
N=6 independent extracts, analyzed over 2 runs

<table>
<thead>
<tr>
<th>Target (ng/dL)</th>
<th>Mean (ng/dL)</th>
<th>SD (ng/dL)</th>
<th>CV (%)</th>
<th>Bias (%)</th>
<th>S/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>1.3</td>
<td>0.46</td>
<td>30.7</td>
<td>28.9</td>
<td>3.2</td>
</tr>
<tr>
<td>2.5</td>
<td>3.1</td>
<td>0.56</td>
<td>18.4</td>
<td>20.3</td>
<td>2.7</td>
</tr>
<tr>
<td>5.0</td>
<td>5.1</td>
<td>0.49</td>
<td>9.2</td>
<td>19.8</td>
<td>12.3</td>
</tr>
</tbody>
</table>

CLSI C60 Recommendations: Assessment of LLoQ & Precision

C60: Use CLSI EP17 to validate LLoQ
- 40 reps/sample, 3-5 samples/run over 5 runs

C60 Best Practice Acceptability Criteria:
- CV < 20% at LLoQ, < 15% bias if using a CRM

C60: Use CLSI EP5 to validate Precision
- 2 conc, 2 runs/d, in dupilc for 20d
- Calc Within-run ("Repeatability") and Between-run ("Within-Laboratory") CVs using ANOVA

C60 Best Practice Acceptability Criteria:
- CV ≤ 15% except at LLoQ where < 20% is acceptable
Precision Assessment (CLSI EP5-A2)

<table>
<thead>
<tr>
<th>25-OH D3</th>
<th>% CVwr</th>
<th>% CVbr</th>
</tr>
</thead>
<tbody>
<tr>
<td>(P% BSA)</td>
<td>1.0</td>
<td>7.1</td>
</tr>
<tr>
<td>(P)</td>
<td>18.8</td>
<td>3.2</td>
</tr>
<tr>
<td>(P%)</td>
<td>38.9</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Imprecision Profile

Imprecision profile generated to determine %CV across AMR
25-OHD3 spiked into various matrices
Analyzed in quadruplicate/run for 2 runs

CLSI C60 Recommendations: Accuracy

C60: Assess accuracy using multiple approaches

C60 Best Practice (Exper described in CLSI EP15, EP9):
Validate "true"ness using a "reference of higher order" (CLSI X5, ISO 17511) listed by JCTLM (approved RMPs, ref labs and RMs)

Described in CLSI EP15
- Method Comp vs. a JCTLM-approved RMP
- Matrix-approp CRMs (pref commutable)
  (limited # of samples, RMs not necessarily commutable)
- Spike and Recovery – if RMP or RMs are unavail

C60 Alternative Approaches:
- Accuracy-Based PT Materials (limited # of samples)
- Method Comp vs. Previous Method (Bias vs. prev method only, not trueness)

> Use native patient samples whenever possible
> Analyze a min of 40 patient samples in duplicate over 5d (CLSI EP9-A2)
Accuracy: Method Comp with RMP - VDSP

- CDC/CDC VDSP
  (www.cdc.gov/labstandards/fsc.html)
  (www.cdc.gov/Research/VitaminD.aspx#vdsp)

**Suggested TEs based on published biological variation data**

Accuracy: Certified Reference Material (CRM)

<table>
<thead>
<tr>
<th>Single Run Analyzed</th>
<th>NIST SRM 1951</th>
<th>NIST SRM 1952</th>
<th>NIST SRM 1953</th>
<th>NIST SRM 1954</th>
</tr>
</thead>
<tbody>
<tr>
<td>9 replicates of NIST SRMs</td>
<td>(1.1) 6.2</td>
<td>(1.1) 6.2</td>
<td>(1.1) 6.2</td>
<td>(1.1) 6.2</td>
</tr>
<tr>
<td>CLSI EP15-A2: 2 reps/run, 3-5 runs</td>
<td>(1.1) 6.2</td>
<td>(1.1) 6.2</td>
<td>(1.1) 6.2</td>
<td>(1.1) 6.2</td>
</tr>
</tbody>
</table>

Acceptability Criteria: ±10% or 2 ng/mL bias vs. NIST

Accuracy: Spike and Recovery

- 25-OH D3 Spiked into 8% BSA (Low Conc)
- 25-OH D3 Spiked into Stripped Serum with back-calculation

<table>
<thead>
<tr>
<th>N = 10 reps/sample over 2 runs</th>
</tr>
</thead>
<tbody>
<tr>
<td>8% BSA</td>
</tr>
<tr>
<td>Expected (ng/mL)</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>1.5</td>
</tr>
<tr>
<td>2.5</td>
</tr>
<tr>
<td>5.0</td>
</tr>
<tr>
<td>8.0</td>
</tr>
<tr>
<td>10.0</td>
</tr>
<tr>
<td>15.0</td>
</tr>
<tr>
<td>20.0</td>
</tr>
<tr>
<td>25.0</td>
</tr>
<tr>
<td>30.0</td>
</tr>
<tr>
<td>40.0</td>
</tr>
<tr>
<td>50.0</td>
</tr>
<tr>
<td>60.0</td>
</tr>
<tr>
<td>80.0</td>
</tr>
</tbody>
</table>

C60 Best Practice:
Spike into native matrix, 3 conc (L,M,H)/5 reps (FDA)

Acceptability Criteria: ±10% or 2 ng/mL bias vs. NIST
Accuracy: Accuracy-Based PT Programs

<table>
<thead>
<tr>
<th></th>
<th>Immunoassay (ng/mL)</th>
<th>LC-MS/MS (ng/mL)</th>
<th>% Diff LC-MS/MS vs. IA</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB-D-00</td>
<td>32.4</td>
<td>29.1</td>
<td>13.0</td>
</tr>
<tr>
<td>AB-D-07</td>
<td>32.2</td>
<td>31.9</td>
<td>4.9</td>
</tr>
<tr>
<td>AB-D-08</td>
<td>34.7</td>
<td>35.7</td>
<td>2.9</td>
</tr>
<tr>
<td>AB-D-10</td>
<td>34.0</td>
<td>36.4</td>
<td>6.4</td>
</tr>
<tr>
<td>AB-D-12</td>
<td>18.6</td>
<td>14.4</td>
<td>13.3</td>
</tr>
<tr>
<td>AB-D-03</td>
<td>11.0</td>
<td>5.6</td>
<td></td>
</tr>
</tbody>
</table>

Note that 3-epi D3 is NOT included in the Target Value

- CAP Accuracy Based Vitamin D Survey
- CAP Testosterone and Estradiol Accuracy Survey
CLSI C60 Recommendations: Linearity

**C60 Linearity: Assess Linearity Using CLSI EP6**

- 9-11 (min 5) points with 2-4 reps each
- Evaluate linearity using the Polynomial Regression Method (EP6-A)

**C60 Best Practice:**
Use matrix-appropriate material
Serial dilutions should be avoided (prop of pipetting errors)

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**Linearity (CLSI EP6-A) – Polynomial Regression Approach**

25-OH D3 analyzed used matrix-appropriate commercial linearity materials

Nonlinearity should be assessed for clinical significance

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**Linearity (CLSI EP6-A) – Polynomial Regression Approach**

High Testosterone sample diluted with stripped serum

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CLSI C60 Recommendations: Validation of Dilution Protocol

C60: Assess Dilutions Within and Outside of AMR

- Assess dilution effects using Recovery Experiments
  CLSI EP15-A2: 2-3 reps/sample, over 3-5 runs
- Use analyte free matrix as the dilution buffer
- Sample extracts should be diluted to avoid ME when diluting unextracted serum

Validation of Dilution Protocol

According to SOP 25-OH D3 > ULoQ (150 ng/mL), extracts are diluted 1:2

Conc tested w/in and outside of AMR
Used Recovery Experiment, 2 reps each over a single run

Acceptability Criteria: Recovery ±10% or 2 ng/mL

C60 Best Practice Acceptability Criteria:
  ➢ Recovery ±115%, CV ≤ 15%

CLSI C60 Recommendations: Validation of Carryover

C60: Assess carryover using CLSI EP10

- 3 conc (L, M, H), 10 reps/run, 1 run/vid for 5d
  (**or 20 d for manuf)
- Seq: Mid, High, Low, Mid, Low, Low, High, High, Mid

C60: Also assess carryover for a high conc sample followed by blank (FDA, EMA)

- Inject one or more blank samples immediately after high sample to validate a known limit (ex: highest physiol conc)
- Inject a blank sample after increasingly high conc samples to determine the carryover limit
Carryover Assessment – CLSI EP10

CLSI EP10 Protocol

<table>
<thead>
<tr>
<th>Run</th>
<th>% Carryover</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.6</td>
</tr>
<tr>
<td>2</td>
<td>0.9</td>
</tr>
<tr>
<td>3</td>
<td>0.4</td>
</tr>
<tr>
<td>4</td>
<td>1.3</td>
</tr>
<tr>
<td>Mean</td>
<td>0.83</td>
</tr>
</tbody>
</table>

C60: Best Practice Acceptability Criteria

- Carryover data from EP10 should be eval in context of TEa

CLSI C60 Recommendations: Carryover (High Sample vs. Blank)

<table>
<thead>
<tr>
<th>Analysis Sequence</th>
<th>Sample Name</th>
<th>Sample Peak Area (pg)</th>
<th>Analyte Peak Area (pg)</th>
<th>Area Ratio</th>
<th>IS Peak Area (pg)</th>
<th>Conc (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Patient 1</td>
<td>15,000</td>
<td>1,000</td>
<td>15.0</td>
<td>10,000</td>
<td>10.0</td>
</tr>
<tr>
<td>2</td>
<td>Patient 2</td>
<td>15,000</td>
<td>1,000</td>
<td>15.0</td>
<td>10,000</td>
<td>10.0</td>
</tr>
<tr>
<td>3</td>
<td>Blank</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

C60 Best Practice Acceptability Criteria:

- No peak in blank or < 20% of LLoQ

CLSI C60 Recommendations: Evaluation of Matrix Effects

Common causes of Ion Suppression/Matrix Effects in LC-MS/MS:

- Salts, phospholipids, protein content
- Effect of binding proteins, pH, or ionic strength on extr efficiency
- Free fatty acids, metabolites, other organic molecules

- Matrix effects are highly method-dependent (esp depend on sample prep)
1) Pre vs. Post Extraction Spike and Recovery (Matuszewski/CLSI-C50)

\[
\text{A} \quad \text{Neat Standard in Solvent} \\
\text{B} \quad \text{Samples Spiked POST-Extraction} \\
\text{C} \quad \text{Samples Spiked PRE-Extraction}
\]

\[
\text{% Matrix Effect} = \frac{\text{B}}{\text{A}} \times 100 \\
\text{% Extraction Efficiency} = \frac{\text{C}}{\text{B}} \times 100 \\
\text{% Process Efficiency} = \frac{\text{C}}{\text{A}} \times 100
\]

N=6 indep matrices recommended (EMA)

2) Matrix Mixing (CLSI EP7-A2) - Admixtures of patient sample to characterize ME

3) T – column infusion experiment

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**C60 Best Practice Hierarchical Experimental Design Strategies:**

**Pre vs. Post-Extraction Spike and Recovery (CLSI-C50)**

10 ng/mL D3 spiked into Low Patient Pool (~1 ng/mL)

<table>
<thead>
<tr>
<th>Spiked Pre-Extraction</th>
<th>Spiked Post-Extraction</th>
<th>Dil in Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>EU spiked into Serum (pmol)</td>
<td>Endogenous D3 in Serum (pmol)</td>
<td>DIFF (pmol)</td>
</tr>
<tr>
<td>Rep 1</td>
<td>2050</td>
<td>2650</td>
</tr>
<tr>
<td>Rep 2</td>
<td>10050</td>
<td>2650</td>
</tr>
<tr>
<td>Rep 3</td>
<td>11500</td>
<td>2650</td>
</tr>
<tr>
<td>Rep 4</td>
<td>10050</td>
<td>2650</td>
</tr>
<tr>
<td>Rep 5</td>
<td>11500</td>
<td>2650</td>
</tr>
<tr>
<td>Mean</td>
<td>10450</td>
<td>2700</td>
</tr>
<tr>
<td>SD</td>
<td>292</td>
<td>179</td>
</tr>
</tbody>
</table>

- % Matrix Effect (B/A*100) 85.7
- % Extr Eff (C/B*100) 76.4
- % Process Eff (C/A*100) 65.5
- % Matrix Bias -14.3
- % Matrix Bias Corr for IS -2.3

**C60 Best Practice Acceptability Criteria:**

- Eval based on TEa requirements (CLSI EP7 – partition TE into bias, imprecision and interference components)

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**Eval of Matrix Effects: Matrix Mixing (CLSI EP7)**

Experiment for purpose of validating surrogate matrices

But, this approach can also be used to validate ME in patient samples

Native Patient Serum mixed in proportion with Stripped Serum or 8% BSA

N=4 reps over a single run

**Native Patient Serum + Stripped Serum:**

- contribution from endogenous D3 in stripped serum removed

**Native Patient Serum + 8% BSA:**

- contribution from endogenous D3 in stripped serum removed
Evaluation of Matrix Effects: T - Infusion

Protein Precipitation

D3 in MeOH infused + injection of extracted Blk Serum

Exogenous and Other Endogenous Interferences

- Reagents and Disposables
- Collection Tube Additives
- Hemolysis, Lipemia, Icterus
- Drugs/Metabolites
- Physiological or Disease-Associated Interferences
  - Isobaric – Interferences resulting in shared product ions w/test analytes
  - Isotopomeric – Interferences that share a same precursor and product ion
  - Adducts – chemical modifications resulting in a shared precursor and product ion

CLSI C60 Recommendations: Interference Testing

C60: Check for interferences from reagents & disposables
Testo and IS in MeOH was extracted using sialized tubes

Interference obs in IS transition

C60: Check for potential endogenous interferences
†† Bile Salts in PBC patient

T2/T1 = -38% vs. Cal Curve
Interference from Collection Tubes

- Interfer is obs in 289/97 and 289/109
- Interfer is method/sample prep dependent

Interference from Preanalytical Phase: CLSI EP7

Spike and Recovery for Hemolysis
Using hemoglobin-equiv hemolysate

Patient Admixtures for Lipid Interference Testing

Note: Intralipid may contaminate the source

Dr Russell Grant and Brian Rappold
LabCorp inc.
[Slides 40 & 41]
**Interference Assessment: Ion Ratios and Isobaric Compounds**

- Aldosterone (361.2) 1.0
- Cortisol (363.2) 2.0
- Cortisone (361.2) 0.5
- Androstenedione (287.2) 0.7
- Testosterone (289.2) 0.8
- 17-OHProgesterone (331.2) 0.9
- Progesterone (315.2) 0.8
- Dihydrotesterone (291.2) 0.8
- 21-Desoxycortisol (347.2) 0.8
- Corticosterone (347.2) 0.4
- 11-Deoxycortisol (347.2) 0.8
- Deoxycorticosterone (331.2) 1

**Chromatographic Resolution is Required**

See Abstract: Steroid Interference Determination Versus Clinically Relevant Steroids – ASMS 2010 MP13 311*

**QA Monitoring – Run Acceptability and Sample Acceptability**

- Slope & Intercept PASS Range
- % Calibrator Recovery (ex: ±10%)
- $r^2$ or SE of Calibrator Curve
- Run-to-run IS Peak Area Recovery Criteria
- IS Recovery Criteria for each sample
- RT, Peak Resolution
- Presence of Interfering Peaks or Absent Peaks
- Peak Shape/Peak Symmetry
- T2/T1 Ratio Criteria (CLSI-C50)
Reagent Lot Change – Patient Comps and Linearity (CAP)

Plus chromatography acceptability criteria
Plus QC acceptability criteria (native pt based)

Periodic Accuracy Monitoring – Use of CRM

Using Patient Pools traceable to NIST SRM 972 (25-OHD)

1) Create Patient Pools near NIST SRM values
2) Calc Method Bias vs. NIST SRM
3) Apply a Correction Factor to Patient Pools to trace their values to NIST-assigned values

Q&A!