Total Laboratory Harmonization for Precision Laboratory Medicine

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Laboratory medicine: A hidden treasure in health care

<table>
<thead>
<tr>
<th>Percentage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>94%</td>
<td>objective data in medical records</td>
</tr>
<tr>
<td>60-70%</td>
<td>clinical decisions influenced</td>
</tr>
<tr>
<td>37%</td>
<td>of practice guidelines</td>
</tr>
<tr>
<td>23%</td>
<td>different disease areas &amp; growing number of companion diagnostics</td>
</tr>
</tbody>
</table>

Laboratory total testing procedure that enables harmonisation
When laboratory errors are happened?

- Preanalytical errors still account for nearly 60%–70% of all mistakes occurring in laboratory diagnostics, most of them attributable to mishandling during collection, handling and preparing the specimens for testing.

## Sample problems

<table>
<thead>
<tr>
<th>Type of sample error</th>
<th>Total (%)</th>
<th>Outpatients (%)</th>
<th>Inpatients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Routine</td>
</tr>
<tr>
<td>Hemolyzed</td>
<td>49.7</td>
<td>4.7</td>
<td>43.7</td>
</tr>
<tr>
<td>Clotted</td>
<td>9.1</td>
<td>1.8</td>
<td>53.1</td>
</tr>
<tr>
<td>Icteric/lipemic</td>
<td>2.0</td>
<td>4.0</td>
<td>96.0</td>
</tr>
<tr>
<td>Incorrect filling level</td>
<td>7.3</td>
<td>0</td>
<td>42.9</td>
</tr>
<tr>
<td>Incorrect</td>
<td>3.8</td>
<td>8.5</td>
<td>78.7</td>
</tr>
<tr>
<td>Inadequate quantity</td>
<td>24.3</td>
<td>14.8</td>
<td>84.3</td>
</tr>
<tr>
<td>Lost/not received</td>
<td>3.5</td>
<td>9.1</td>
<td>90.9</td>
</tr>
</tbody>
</table>

Key preanalytical steps identified by EFLM NS as the most critical and in need of immediate harmonization

Variation factors of laboratory data

1. Physiological variation (Inter-individual and intra-individual)
2. Specimen sampling, handling and storage
3. Patients’ conditions
4. Measurement procedure
1. Physiological variation

- Age and gender
- Life style
- Circadian rhythm
  - Serum iron is higher in the morning and lower in the evening. It is dramatically fluctuated.
  - Serum inorganic phosphorus is lower in the morning.
- Daily and seasonal variation, menstrual cycle
- Diet: blood glucose, triglyceride, insulin, etc.
- Exercise: skeletal muscle injury (CK), leukocytes
- Posture
  - High molecular weight molecules: standing > sitting > spine
Reference Interval of Alkaline Phosphatase (ALP)

By health check-up in Shizuoka prefecture

http://www.shizuoka.med.or.jp/documents/240208.pdf
Example of the Biological Variation (BV)

<table>
<thead>
<tr>
<th>Testing items</th>
<th>Within-subject BV</th>
<th>Between-subject BV</th>
<th>Reference change value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFP</td>
<td>12.2</td>
<td>45.6</td>
<td>0.27</td>
</tr>
<tr>
<td>CA19-9</td>
<td>16.0</td>
<td>130.5</td>
<td>0.12</td>
</tr>
<tr>
<td>CEA</td>
<td>12.7</td>
<td>55.6</td>
<td>0.23</td>
</tr>
<tr>
<td>CA15-3</td>
<td>6.1</td>
<td>62.9</td>
<td>0.10</td>
</tr>
<tr>
<td>CA125</td>
<td>24.7</td>
<td>54.6</td>
<td>0.45</td>
</tr>
<tr>
<td>ALP</td>
<td>6.45</td>
<td>26.1</td>
<td>0.25</td>
</tr>
<tr>
<td>TC</td>
<td>5.95</td>
<td>15.3</td>
<td>0.39</td>
</tr>
<tr>
<td>Na</td>
<td>0.6</td>
<td>0.7</td>
<td>0.86</td>
</tr>
</tbody>
</table>

https://www.westgard.com/biodatabase1.htm#11
Adequate Laboratory Conditions

- Patients’ conditions (including medication)
- Physiological variation and circadian rhythm
  - Medication
  - Diet, exercise, smoking, alcohol drinking
  - Circadian rhythm, seasonal variation
  - Age and gender
  - Posture

Individual variations can be diminished by adequate sampling conditions all times, and thus petit abnormalities can be detected earlier.
Recommendation: Precision laboratory medicine needs individual reference interval

- Not reference interval from groups, from the second examination
  - but needs harmonized results

- Use individual reference interval
  - it is useful for small reference change value (RCV)
Posture variation of laboratory testings

Standing > Sitting > Spine posture

Ichihara and Kohguchi (edited): Laboratory Diagnosis Manual. 2011
Dietary interference on HbA1c

%  

6.5 6.0 5.5 5.0 4.5 4.0 3.5

6.1 5.2

9 h 10 h 11 h 3 h 6 h

Hours after dinner  Breakfast  Hours after breakfast

女①

男①

男②

男③

男④

Breakfast
Dietary interference on blood glucose

![Graph showing blood glucose levels over time](image-url)
Preanalytical variables in miRNA analysis

Associations of miRNAs with age, BMI and sex

Association q-values of miRNAs in two-step regression models with adjustment for technical and biological parameters. The -log10(q) values of the linear regression analysis of miRNA levels and phenotypes age (blue rectangle), BMI (green triangle) and sex (red circle) are depicted. Q-values were obtained via Benjamini-Hochberg (BH) multiple testing correction of raw p-values. The dotted line marks the significance threshold of q = 0.05. Plasma miRNAs are lexicographically arranged on the x-axis (though not labelled individually).

Ameling et al. Associations of circulating plasma microRNAs with age, body mass index and sex in a population-based study. BMC Medical Genomics (2015) 8:61
miRNAs up and down regulated by exercise training in different tissues and in the circulation

*Other conditions included spinal cord lesion and brain phenotypes.

MicroRNAs as Important Regulators of Exercise Adaptation. Prog Cardiovascular Diseases 2017; 60: 130-151.
2. Specimen sampling, handling and storage

- **Specimen**
  - Whole blood, plasma, serum
  - RBC (hemolysis), WBC, Plt, anti-coagulant
  - Other biological fluid

- **Stability**

- **Infusion, transfusion, medication**

- **Incorporation of tissue fluid**

- **Patient identification**

- **Transportation (temperature, time, vibration)**

- **Centrifugation (temperature, speed and gravity, time)**

- **Deposit and storage (temperature, time and period, tube, condition, freeze and thaw counts, etc.)**
# Anti-coagulants and their purpose

<table>
<thead>
<tr>
<th>Anti-coagulant and others</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>heparin</td>
<td>Blood gas</td>
</tr>
<tr>
<td>Sodium fluoride</td>
<td>Blood glucose</td>
</tr>
<tr>
<td>EDTA–2K</td>
<td>Blood counts</td>
</tr>
<tr>
<td>EDTA–2Na</td>
<td>Renin, ACTH</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>Coagulation, ESR</td>
</tr>
</tbody>
</table>
Effect of NaF on blood glucose

![Graph showing the effect of NaF on blood glucose levels over time. The graph has two lines, one for NaF (+) and one for NaF (-), showing a decrease in blood glucose levels as time progresses from 0 to 6 hours.]
Hemolysis

- Molecules rich in red blood cells strongly affect laboratory data.

<table>
<thead>
<tr>
<th>Test</th>
<th>RBC/plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>LD</td>
<td>160</td>
</tr>
<tr>
<td>K</td>
<td>23</td>
</tr>
<tr>
<td>AST</td>
<td>20</td>
</tr>
</tbody>
</table>

Degree of hemolysis
Contamination of infusion fluid containing glucose might lead to misdiagnosis and unnecessary therapy.
Effect of time from blood sampling to centrifugation at 4°C and room temperature

AST

Just after AST (U/L)
Effect of time from centrifugation to analysis at 4°C and room temperature.

Graph showing the effect of time from centrifugation to analysis at 4°C and room temperature on ALT levels:
- **4°C**
- **Room Temperature (RT)**

- **Just after**
- **24hrs**
- **48hrs**
- **72hrs**

(U/L)
Storage stability on different temperatures

ALT

LD

PSA

Insulin

Ikeda K: Specimen stability. Guideline of clinical laboratory testings. 2015
Sample matrix effect on circulating miRNAs determination

<table>
<thead>
<tr>
<th>Sample matrix</th>
<th>miRNA behavior</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma-EDTA</td>
<td>Best anticoagulant for PCR-based (miRNA) profiling</td>
<td>[84]</td>
</tr>
<tr>
<td></td>
<td>Higher levels of miR-223 compared to the other matrices</td>
<td>[85]</td>
</tr>
<tr>
<td>Plasma-heparin</td>
<td>Interferes with enzyme activity in PCR-based assays</td>
<td>[14]</td>
</tr>
<tr>
<td></td>
<td>Lowest levels of both miR-16 and miR-223</td>
<td>[85]</td>
</tr>
<tr>
<td>Plasma NaF/KOx</td>
<td>Suitable alternative to EDTA, but it can determine increased miRNA detection</td>
<td>[85]</td>
</tr>
<tr>
<td></td>
<td>compared to the other matrices</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Higher levels of miR-223 compared to the other matrices</td>
<td>[85]</td>
</tr>
<tr>
<td></td>
<td>When NaF/KOx are added to frozen samples, levels of miR-16 doubled in EDTA-plasma, and tripled in serum</td>
<td>[85]</td>
</tr>
<tr>
<td>Plasma-citrate Serum</td>
<td>Interferes with enzyme activity in PCR-based assays</td>
<td>[14]</td>
</tr>
<tr>
<td></td>
<td>Stable and reproducible</td>
<td>[17]</td>
</tr>
<tr>
<td></td>
<td>Higher variability of miR-16 and miR-223 compared to the other matrices</td>
<td>[85]</td>
</tr>
</tbody>
</table>
Relationship between free haemoglobin (Hb) and miRNA content of plasma samples

A dilution series of lysed RBCs in plasma (top) was prepared and Hb content measured by absorbance at 414 nm [33]. RNA was isolated from the samples indicated by the box and levels of miR-16 and miR-451 were quantified using a standard curve. While a change in plasma colour is only clearly visible from a RBC concentration of 0.125% (v/v) the amount of free Hb as well as this of both miR-451 and miR-16 already substantially increased at a RBC concentration of 0.031% (v/v).

Paired plasma (left 3 columns) and serum (right 3 columns) samples were obtained from 10 healthy individuals. We spiked 400 L of the uncentrifuged (Unspun) and 2 supernatant aliquots with C. elegans cel-miR-54, extracted the RNA, and analyzed the samples for miR-15b (A), miR-16 (B), miR-24 (C), and cel-miR-54 (D). The resulting miRNA concentrations are reported as raw Cq values. The boxes represent the 25th and 75th percentiles; the horizontal line in each box represents the mean; the error bars indicate the range. Significant differences (P< 0.05, Wilcoxon signed rank test) from the unspun control (*) and significant differences between the 15 000g and 355 000g centrifugation steps (**) are indicated.

# MicroRNA Expression in Stepwise Processed Plasma Samples Using qRT-PCR Profiling

Plasma Processing Conditions Substantially Influence Circulating microRNA Biomarker Levels. 
Stability study of circulating miRNAs in plasma at different storage time and temperature conditions. A and B: cel-miR-39. C and D: hsa-miR-21. E and F: hsa-miR-16. The results are presented as differences in raw Cq values from the 0-hour control (0h) (positive differences represent decreases and negative differences represent increases in miRNA concentrations) and as percentage recoveries from the 0h for each miRNA. The data represent the means SD of three independent experiments. Statistical evaluation was based on the U-test. *P<0.05.

Quantification of Circulating miRNAs in Plasma Effect of Preanalytical and Analytical Parameters on Their Isolation and Stability. J Mol Diag 15(6), 2013
3. Patients’ conditions

- Pathological states
- Dehydration and dilution
  - Malnutrition is often associated with dehydration.
  - Large molecules might be concentrated.
- Anabolism and catabolism, input and output
  - Serum albumin concentration decreases not only by synthesis reduction due to hepatic dysfunction, but also excretion elevation from kidney, digestive tract and skin.
Possible mechanism of elevation of tumor marker levels in sera

Healthy

Cancer
Inflammation
Hepatic disorders
Medication

Chronic Kidney Dis.
Dialysis

Tumor marker level

Not 0

Normal

Synthesis elevation

Reduction of catabolism or excretion
## Cut-off values of tumor marker in dialysis patients

<table>
<thead>
<tr>
<th>Test</th>
<th>MW</th>
<th>Correction of cut-off value</th>
<th>Related cancers</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEA</td>
<td>180,000</td>
<td>twice</td>
<td>colorectal, gastric, lung, breast</td>
</tr>
<tr>
<td>CA19-9</td>
<td>&gt; 3,000,000 (cancer)</td>
<td>twice</td>
<td>pancreas, colorectal</td>
</tr>
<tr>
<td>ProGRP</td>
<td>10,000</td>
<td>2 - 3 fold</td>
<td>lung small cell</td>
</tr>
<tr>
<td>SCC</td>
<td>46,000</td>
<td>2.5 – 3 fold</td>
<td>uterine cervix, lung</td>
</tr>
<tr>
<td>PSA</td>
<td>34,000</td>
<td>-</td>
<td>prostate</td>
</tr>
<tr>
<td>AFP</td>
<td>65,000</td>
<td>-</td>
<td>HCC, germ cell</td>
</tr>
<tr>
<td>CA15-3</td>
<td>90,000</td>
<td>-</td>
<td>breast</td>
</tr>
</tbody>
</table>
Total and some miRNAs are decreased in CKD

ESRD: end-stage renal disease

4. Measurement procedure

Not standardized
Not harmonized

Each immunoassay reagent is different from each other (antibody, labeling, buffer, time, calibrator, standard material).
Harmonization and standardization

• Harmonization
  – Equivalent results among different measurement procedures for the same laboratory test

• Standardization
  – Equivalent results are achieved by metrological traceability to a fit-for-purpose higher order reference system

• Equivalent
  – Equivalent does not mean identical
  – Equivalent means within a total allowable error consistent with an acceptable risk of harm from decisions based on a lab test result

Greg Miller, IFCC-GC in 2018
### Traceability class by ISO 17511

<table>
<thead>
<tr>
<th>Class</th>
<th>RMP</th>
<th>Primary RM (pure substance)</th>
<th>Secondary RM (valued substance)</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Electrolyte, Glu, cortisol</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>Enzyme</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Coagulation factor</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Protein, TM, HIV</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Protein, EBV, VZV</td>
</tr>
</tbody>
</table>
Metrological traceability: an unbroken chain of calibrations from a clinical sample result to a higher order reference system component (ISO 17511)

Greg Miller, IFCC-GC in 2018
Harmonization needs EQA feedback to the IVD industry

- We need a mechanism for EQA providers to cooperate to:
  1. Cover measurands on an annual or biennial cycle
  2. Prepare aggregated data summaries among schemes
EQA result of TSH (middle conc.)

2018 EQA program of Japan Medical Association
TSH belongs to class 4 of ISO 17511

- Value assignment
- Commutability

Secondary Reference Material (matrix)

Mfr Working Calibrator
Mfr Product Calibrator

Mfr Selected Procedure
Mfr Standing Procedure
Routine Procedure

Patient sample results are traceable to a reference material
Harmonization of Serum Thyroid-Stimulating Hormone Measurements Paves the Way for the Adoption of a More Uniform Reference Interval

Linda M. Thienpont,1,2* Katleen Van Uytfanghe,3 Linde A.C. De Grande,1 Dries Reynders,4 Barnali Das,5 James D. Faix,6 Finlay MacKenzie,7 Brigitte Decallonne,8 Akira Hishinuma,9 Bruno Lapauw,10 Paul Taelman,11 Paul Van Crombrugge,12 Annick Van den Bruel,13 Brigitte Velkeniers,14 and Paul Williams15 on behalf of the IFCC Committee for Standardization of Thyroid Function Tests (C-STFT)
Reference measurement system for TSH

• TSH analysis is “mixture” analysis
  – Serum TSH – intact, total, with glycosylation pattern encountered in specified diagnostic applications
  – Results in mIU/L defined by WHO IRP 80/558 & 81/565

• “The” problem
  – WHO IRP’s not commutable with TSH assays
  – Reference measurement procedure technically not to expect in the short- to midterm
Figure 1. Combined difference (%) plots to the APTM-4 before (A) and after recalibration (B).

- For each assay and sample, the difference of the mean from duplicate measurements is plotted
- Filled and colored circles: differences of the assays that were most discrepant before recalibration
- Open black circles: all other assays
- Red broken lines: 7.8% bias limits
- Blue broken lines: 15th and 85th centiles of the differences

Recalibration eliminates the calibration differences between the assays

APTM: all procedure trimmed mean

Detection of miRNAs in **plasma** by RT-qPCR, using different RNA extraction methods and fixed RNA volumes

(a) RNA was extracted from 200 μL of plasma (n = 6). Exosomes were isolated from 2 x 2 mL of plasma (n = 3), then subjected to RNA isolation. The results represent mean Cq values ± SD, using 2.5 μL of RNA/RT reaction. Expression levels of plasma mir-106a, mir-222 and mir-223 were normalized to mir-16 levels and expressed as fold change relative to miRNeasy® condition under the histograms.  (b) RNA was isolated from 200 μL of plasma (n = 3) containing 25 fmol of cel-mir-39-3p as a spike-in control directly added into the lysis solution before mixing it with the plasma sample. The results represent mean Cq values ± SD, using 2.5 μL of RNA/RT reaction. **P < 0.01 ***P < 0.001. 

Quality Assurance

- Examination Request
- Preanalytical Process
- Analytical Process
- Postanalytical Process

Validation

Internal Quality Control (IQC)

External Quality Assessment (EQA)

ISO 15189
Lab. director, Quality manager
Document (SOP, Worksheet, etc.)
Learning, Education
Equipment, Reagent, other tools
Laboratory **Total** Testing Procedure that enables **HARMONIZATION**

Anytime, anywhere, equivalent result, same interpretation (quality assured)

Thank you for your kind attention

Adapted from Plebani M. AACB conference 2013