Collaborative Study to Evaluate the Proposed 1st WHO International Standard for Epstein-Barr Virus (EBV) for Nucleic Acid Amplification (NAT)-Based Assays

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Rationale

- Viral load measurements (using NAT) are important in the prevention, diagnosis and monitoring of EBV infections, particularly in the immunocompromised.

- Range of commercial and laboratory-developed methods/controls leads to variability in viral load measurements between laboratories. Difficult to compare clinical practice and standardise patient management.

- Need for higher order reference to calibrate EBV secondary references and standardise assays.

- Proposal discussed at SoGAT Clinical Diagnostics meeting, June 2008:
  - Whole virus (to standardise entire assay – including extraction)
  - EBV B95-8 (candidate strain) – prototype laboratory strain, published sequence
  - Formulate in universal buffer for further dilution in sample matrix appropriate to assay
  - Concentration of ~1x10^7 copies/mL (IU when established)
  - Evaluate alongside Namalwa and Raji cell preparations
Preparation of bulk and production summary

- Candidate virus grown in B95-8 cells incubated with PMA (Jung-Chung Lin. Meth Mol Med 24; Antiviral methods and protocols), virus harvested from clarified culture medium by ultracentrifugation
- Concentration of virus stock evaluated at NIBSC using commercial assays and in UK clinical laboratories
- Bulk formulated in 10mM Tris.HCl buffer (pH7.4), 0.5% HSA, 0.1% Trehalose

<table>
<thead>
<tr>
<th>NIBSC code</th>
<th>09/260</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product name</td>
<td>Epstein-Barr virus</td>
</tr>
</tbody>
</table>
| Dates of processing | Filling; 25 January 2010  
Lyophilisation; 25-29 January 2010  
Sealing; 29 January 2010 |
| Presentation | Freeze-dried preparation in 5 mL screw-cap glass vial |
| Mean fill weight (CV) | 0.9997 g (0.25%) |
| Mean residual moisture, n=11 (CV) | 0.7 % Karl Fischer, 0.48 % NIR (9.5%) |
| Mean oxygen content, n=12 (CV) | 0.18 % (26.6%) |
| No. of vials available to WHO | 5677 |
Stability of EBV 09/260 at 4 and 8 months (accelerated degradation)

No evidence for degradation at temperatures up to +45°C for 8 months

Mean concentration (log_{10} copies/mL)

Temperature (°C)

-70, -20, +4, +20, +37, +45

4 months
8 months
Collaborative study

- 28 participants from 16 countries, selected for experience in EBV NAT and geographic distribution; include IVD manufacturers, clinical, reference, and research laboratories
- 4 study samples
  - Sample 1; EBV B95-8 whole virus, freeze dried preparation
  - Sample 2; EBV B95-8 whole virus, frozen liquid bulk
  - Sample 3; Namalwa cells, frozen liquid preparation
  - Sample 4; Raji cells, frozen liquid preparation
- Study protocol
  - Quantitative assays: min 2 dilutions / sample, 4 separate occasions, report copies/mL
  - Qualitative assays: min of 5 dilutions around the assay end-point for each sample, 4 separate occasions, report results as +/-
  - Dilutions prepared in matrix appropriate to assay, e.g. plasma, whole blood, etc
Participant methods and statistical analysis

- 38 datasets; 36 quantitative assays, 2 qualitative assays
  - Dilutions mainly in plasma or whole blood
  - Range of automated and manual extraction
  - All real-time PCR assays; commercial (n=21), laboratory-developed (n=17)

- Statistical analysis:
  - Quantitative assays: determined log_{10} copies/mL for each assay run, then mean log_{10} copies/mL for each assay.
  - Qualitative assays: determined no. +ve/ no. tested at each dilution, then single end-point ‘NAT-detectable units/mL’
  - Intra-assay variability expressed as SD and %GCV of individual mean estimates
  - Overall mean estimate determined across all datasets (inter-assay variability expressed as SD and %GCV of overall mean)
  - Potency relative to candidate = difference in estimated log_{10} units/mL + candidate assigned value
## Inter- and intra-laboratory variation

### Inter-laboratory:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Assay</th>
<th>No. data sets</th>
<th>Mean, log₁₀ (range)</th>
<th>SD</th>
<th>%GCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>qualitative</td>
<td>2</td>
<td>6.04 (5.49-6.59)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>quantitative</td>
<td>36</td>
<td>6.71 (5.03-7.56)</td>
<td>0.58</td>
<td>277</td>
</tr>
<tr>
<td>S2</td>
<td>qualitative</td>
<td>2</td>
<td>5.97 (5.41-6.20)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>quantitative</td>
<td>36</td>
<td>6.75 (5.02-7.54)</td>
<td>0.57</td>
<td>270</td>
</tr>
<tr>
<td>S3</td>
<td>qualitative</td>
<td>2</td>
<td>4.64 (3.83-5.46)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>quantitative</td>
<td>36</td>
<td>5.92 (4.41-6.82)</td>
<td>0.60</td>
<td>294</td>
</tr>
<tr>
<td>S4</td>
<td>qualitative</td>
<td>2</td>
<td>6.20 (5.96-6.45)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>quantitative</td>
<td>36</td>
<td>7.45 (5.96-8.56)</td>
<td>0.63</td>
<td>327</td>
</tr>
</tbody>
</table>

### Intra-laboratory:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>SD</td>
<td>%GCV</td>
<td>SD</td>
<td>%GCV</td>
</tr>
<tr>
<td></td>
<td>0.14</td>
<td>38</td>
<td>0.15</td>
<td>42</td>
</tr>
</tbody>
</table>

Inter-laboratory variability significantly higher than intra-laboratory variability – highlights the urgent need for standardisation of EBV NAT assays
Sample 1 – B95-8 virus, freeze-dried preparation (09/260)

2.5 $\log_{10}$ range in viral load measurements compares with other studies
Sample 2 – B95-8 virus, frozen liquid preparation

Sample 2

Sample 2 – Relative to Sample 1
Sample 3 – Namalwa cells, frozen liquid preparation

Sample 3

Sample 3 – Relative to Sample 1
Sample 4 – Raji cells, frozen liquid preparation

Sample 4

Sample 4 – Relative to Sample 1
<table>
<thead>
<tr>
<th>Sample</th>
<th>Assay</th>
<th>No. data sets</th>
<th>Mean (range)</th>
<th>SD</th>
<th>%GCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>S2</td>
<td>All labs</td>
<td>36</td>
<td>6.74 (6.56-6.91)</td>
<td>0.09</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Excluding 12 and 17</td>
<td>34</td>
<td>6.74 (6.56-6.91)</td>
<td>0.09</td>
<td>23</td>
</tr>
<tr>
<td>S3</td>
<td>All labs</td>
<td>36</td>
<td>5.90 (4.45-7.05)</td>
<td>0.46</td>
<td>187</td>
</tr>
<tr>
<td></td>
<td>Excluding 12 and 17</td>
<td>34</td>
<td>5.92 (5.13-6.74)</td>
<td>0.30</td>
<td>99</td>
</tr>
<tr>
<td>S4</td>
<td>All labs</td>
<td>36</td>
<td>7.43 (6.03-8.48)</td>
<td>0.46</td>
<td>187</td>
</tr>
<tr>
<td></td>
<td>Excluding 12 and 17</td>
<td>34</td>
<td>7.45 (6.68-8.12)</td>
<td>0.36</td>
<td>128</td>
</tr>
</tbody>
</table>

SD without standardisation

- S2: 0.57
- S3: 0.60
- S4: 0.63
Virus vs. cell reference to standardise NAT assays for cell-based samples

Sample 4 - Relative to Sample 3

<table>
<thead>
<tr>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 4</td>
<td>7.45</td>
</tr>
<tr>
<td>Sample 4, relative to 1</td>
<td>7.43</td>
</tr>
<tr>
<td>Sample 4, relative to 3</td>
<td>7.53</td>
</tr>
</tbody>
</table>
Neither the quantification of EBV in study samples 1-4 nor the relative potencies of samples 2-4 relative to 1 (candidate) were affected by the type of matrix used as a diluent in the assay.
Summary and proposal

- Overall mean estimate for sample 1 (candidate) was $5 \times 10^6$ (6.7 log$_{10}$) 'copies/mL'.
- The overall range in laboratory mean estimates for all study samples was 2.5 log$_{10}$.
- Agreement between laboratories for virus sample 2 was markedly improved when the potencies was expressed relative to the candidate standard (sample 1).
- The agreement between laboratories for cell samples 3 and 4 was also improved when the potencies were expressed relative to the candidate standard (sample 1), however, the improvement was less marked than for sample 2. This may be due to greater variability in extraction efficiencies for cellular samples.
- Results from accelerated thermal degradation studies up to 8 months indicate that the candidate is stable and suitable for long-term use.
- Proposed that the candidate standard (NIBSC code 09/260) is established as the 1st WHO International Standard for EBV with an assigned potency of $5 \times 10^6$ International Units when reconstituted in 1 mL of nuclease-free water.
Comparison of assay performance for laboratories participating in both HCMV and EBV studies

- 10 laboratories participated in both HCMV and EBV collaborative studies
- Laboratory means for EBV sample 1 plotted against those for HCMV sample 1
- Correlation coefficient; $R=0.393$, $R^2=0.154$. Significance of testing $R=0$; $p=0.057$