Assay Validation:
How a CFU Replacement Assay is Validated for Cord Blood, Mobilized Peripheral Blood and Bone Marrow
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1. What is Assay Validation?
Assay validation is defined as establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its predetermined specifications and quality attributes. Validation involves documenting, through the use of specific laboratory investigations, that the performance characteristics of the method are suitable for and reliable for the intended analytical applications.

The goal of assay validation is to confirm the operating characteristics of the procedure for its predetermined specifications and quality attributes. Assay validation is defined as establishing documented evidence which provides a high level of confidence that the method will provide accurate and reliable results.

Cross-Validation is a comparison of validation parameters when two or more methods are used to generate data within the same study or across different studies. Cross-validation would be a situation where an original validated method serves as a reference and the revised method is compared. The comparisons should be done in both ways.

An assay is validated when it demonstrates:
- Accuracy: the proportion of correct outcomes.
- Sensitivity: the proportion of correctly identified positive samples.
- Selectivity: the proportion of correctly identified negative samples.
- Precision (Reliability & Reproducibility): intra- and inter-laboratory variability.
- Robustness: the ability of the assay to withstand changes and transferability.

References:

2. The Principle of Bioluminomics™ Assays for Cellular Therapy
Chemical Energy can be used as a Biochemical Marker for Multiple Readouts

<table>
<thead>
<tr>
<th>Phenomenon</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferation</td>
<td>Increase in cell number</td>
</tr>
<tr>
<td>Cytotoxicity</td>
<td>Decrease in cell number</td>
</tr>
<tr>
<td>Cell Number</td>
<td>Consistent cell number</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>Increased cell death</td>
</tr>
</tbody>
</table>

Released IATP + Luciferin + Luciferase = Bioluminescence

Calibrated, Standardized & Validated Assays for Specific Cell Therapy Applications

3. The ATP Standard Curve and Controls

- ATP Standard Curve:
  - The ATP standard curve allows Relative Luminescence Units (RLU) to be converted to standardized ATP concentrations (μM).
  - The controls calibrate the instrument.
  - ATP correlation coefficient (r²) = 0.997.
  - ATP Standard Curve Slope = 0.937 (range 0.8-1.1).
- Assay ATP sensitivity: ≤0.01 μM.
- Low ATP Control range: 0.041 - 0.051 μM.
- High ATP Control range: 0.595 - 0.605 μM.
- Extra High ATP Control range: 1.408 - 1.501 μM.
- Lowest ATP value indicating unsustainable cell proliferation: ≤0.04 μM.
- ATP value below which cells are not metabolically viable: ≤0.01 μM.

4. ATP Bioluminomics™ CFU Replacement Assays that have been Validated

The colony-forming unit (CFU) assay (manual or image analysis colony counting) cannot be validated or standardized due to the lack of standards and controls and can therefore not be validated. In contrast, ATP Bioluminomics™ assays for hematopoietic stem and progenitor cells, which have been developed from the CFU assay, are not only fully validated, but some have FDA Master File status and can be referenced in a BLA or IND. ATP assays for hematopoietic stem and/or progenitor cells that have been validated include:

- HALO™ 96 CFU-C®:
  - A progenitor cell equivalent assay to CFU-GM.
- STEMPredict™:
  - A day 3 stem cell assay for cord blood and mobilized peripheral blood to predict stem cell functionality and viability.
- HALO™ 96 SPC-QC:
  - An assay to ensure stem cell quality during processing and cryopreservation. (Assay submitted for FDA Master File status).
- HALO™ 96 PQR:
  - A reference standard-based potency assay to predict engraftment potential of cord blood, mobilized peripheral blood and bone marrow prior to transplantation. (Assay has FDA Master File status).
- HALO™ 96 PMT:
  - An assay to predict time to engraftment and detect lympho-hematopoietic reconstitution.
- LUMENESC™ 96 QC:
  - Similar to HALO™ 96 SPC-QC, but for mesenchymal stem cells.
- LUMENESC™ 96 PQR:
  - Similar to HALO™ 96 PQR, but for mesenchymal stem cells.

5. Cross-Validation and Accuracy

ATP Assays Correlate Directly with CFU, Demonstrating Cross-Validation

ATP Standard Curve Concentrations

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>Mean (µM)</th>
<th>St. Dev. (µM)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0052</td>
<td>0.0052</td>
<td>0.00057</td>
<td>76</td>
</tr>
<tr>
<td>0.0201</td>
<td>0.0201</td>
<td>0.00343</td>
<td>76</td>
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<tr>
<td>0.0402</td>
<td>0.0402</td>
<td>0.00434</td>
<td>76</td>
</tr>
<tr>
<td>0.0804</td>
<td>0.0804</td>
<td>0.02318</td>
<td>76</td>
</tr>
<tr>
<td>0.1608</td>
<td>0.1608</td>
<td>0.04955</td>
<td>76</td>
</tr>
</tbody>
</table>

ATP correlation coefficient (R²) = 0.997.

Mean ATP Concentration (μM) = 0.057 ± 0.003 μM

N must be =<15% with a Lower Level of Quantitation (LLOQ) =<20%

6. Sensitivity and Selectivity

Recovery Operating Characteristic (ROC) curve fittings used to measure sensitivity and selectivity.

A total of 216 background values were compared with 214 hematopoietic CFU-GEMM stem cell and 207 lympho-hematopoietic CFU-SP primitive stem cell controls. A false positive is defined as a test result that is not true positive. The Area Under the Curve (AUC) is calculated and must be between 0.5 and 1 and will be to the right of the diagonal. The AUC value of 0.997 is the curve set to the right of the diagonal. This means that the assay cannot distinguish either false positives or false negatives. In other words, the assay would be useless. The AUC value for both curves is =0.75, indicating that the assay exhibits both sensitivity and selectivity for different stem cell populations.

7. Precision (Reliability & Reproducibility) and Robustness

Precision is defined as the closeness of the individual measures of an analyte or cell sample when the procedure is applied repeatedly to multiple aliquots. Precision is usually subdivided into:

- Within-run, intra-batch precision, which measures the precision during a single run, and
- Between-run, inter-batch precision, which measures the precision with time and could also involve several users performing the same test, equipment, reagents and laboratories.

**Within-run, intra-batch precision**

<table>
<thead>
<tr>
<th>Sample</th>
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<th>Mean (μM)</th>
<th>St. Dev. (μM)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>0.05</td>
<td>0.05</td>
<td>0.002</td>
<td>76</td>
</tr>
<tr>
<td>Sample 2</td>
<td>0.06</td>
<td>0.06</td>
<td>0.005</td>
<td>76</td>
</tr>
<tr>
<td>Sample 3</td>
<td>0.07</td>
<td>0.07</td>
<td>0.003</td>
<td>76</td>
</tr>
</tbody>
</table>

**Between-run, inter-batch precision**

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Correlation of HALO™96 SPC-C with HALO™96 PQR

- **Correlation of HALO™ 96 SPC-C with HALO™ 96 PQR**
  - The assay can be easily transferred from one laboratory to another and is therefore robust.
  - ATP concentrations less than 0.04 μM do not usually support sustained proliferation (see Panel 3). Therefore, this ATP value corresponds to the acceptance/rejection cutoff. The potency ratio of the reference standard is always 1. A cumulative potency ratio less than 1 and/or an ATP value below the cutoff will indicate that the cells have low or no engraftment potential and should not be released for use. Note that engraftment potential and therefore potency are not the same as time to engraftment and do not correlate with each other.