Reference Standards for Bioassays

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The basics

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• If unknown & standard sufficiently similar, variations in the assay system affect them to the same extent

➢ Activity of unknown relative to reference standard remains constant
➢ “Relative potency”
Biological activity, potency

ICH Q6B – Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products

**Biological activity** - the specific ability or capacity of the product to achieve a defined biological effect

**Potency** – the measure of the biological activity using a suitably quantitative biological assay (also called potency assay or bioassay), based on the attribute of the product which is linked to the relevant biological properties
Potency data required

Throughout product development
• Formulation
• Stability
• Process optimization
• Specifications and release
• Post-licensing comparability
• .......... etc

Need reference standard from early stage product development
Reference standards

ICH Q6B section 2.2.1

For drug applications for new molecular entities, it is unlikely that an international or national standard will be available. At the time of submission, the manufacturer should have established an appropriately characterized in-house primary reference material, prepared from lot(s) representative of production and clinical materials.

In-house working reference material(s) used in the testing of production lots should be calibrated against this primary reference material.

Where an international or national standard is available and appropriate, reference materials should be calibrated against it.
While it is desirable to use the same reference material for both biological assays and physicochemical testing, in some cases, a separate reference material may be necessary. Also, distinct reference materials for product-related substances, product-related impurities and process-related impurities, may need to be established. When appropriate, a description of the manufacture and/or purification of reference materials should be included in the application. Documentation of the characterization, storage conditions and formulation supportive of reference material(s) stability should also be provided.
Reference standards

ICH Q6B  section 2.2.1 summarized

• in-house reference material, prepared from lot(s) representative of production and clinical materials
• calibrated against international or national standard if available and appropriate
• separate reference material may be necessary for biological and physicochemical assays
• documentation of manufacture, purification, characterization, storage, formulation, stability
General requirements of a standard

- Integrity  (of relevant properties, eg activity)
- Suitability
- Homogeneity (initial & continued)
- Stability
- Sufficient number of aliquots
- Continuity  (replacement)
Functional similarity

Functional similarity of the reference standard and sample in the bioassay is a fundamental condition for determination of a valid relative potency

- the preparations have an identical action in the assay
- the dose-response curves are identical except for a possible displacement along the concentration axis
Functional similarity

The preparations have an identical action in the assay. Functional similarity of the reference standard and sample is a fundamental condition for assay validity.

Example = linear log dose-response
Without functional similarity ... 

![Graph showing response vs log dose]

.... relative potency = ???
Relative potency can depend on bioassay system

😊 If standard and all batches of test material were identical, their relative responses would be identical in every system, but .......

😊 even small differences between preparations (drug or formulation) may result in differences in their behavior in the bioassay system

😊 relative potencies may differ between assay systems

😊 dose-response curves may no longer be similar
Assay systems may differ in sensitivity to various molecular features.

cell-based assay: signal = receptor phosphorylation
Assay systems may differ in sensitivity to various molecular features

- Cell-based assay: signal = receptor phosphorylation
  - Signal
  - Signal
  - Signal
  - No signal

- Biological
  - Signal
Assay systems may differ in sensitivity to various molecular features

- A change in the bioassay (deliberate or possibly not even realized, such as different batch of reagent) may make the assay sensitive to a difference between reference standards and/or unknowns that was not detected previously.

- Using a different bioassay system may reveal unsuspected differences between standard 1 and standard 2.

- However, using a different bioassay system may permit use of a standard which has proved unsuitable in the current bioassay system.
Unfortunate facts

😊 Different reference standards unlikely to be identical (in-house initial, in-house replacement, external initial, external replacement, ....)

😊 Reference standard unlikely to be identical to product sample

😊 Control samples unlikely to be identical to reference standard or product sample (batch, storage, ....)

😊 Even nominally identical assay systems may vary

⚠️ Suitability of reference standard must be established after any deliberate change & continuously monitored
Homogeneity of reference standard

- Homogeneity of bulk
- Precision of dispensing (if applicable)
- Identical containers, stoppers (at source & processing)
- Stability of material during dispensing & processing
- Stability of dispensing equipment (adsorption, etc)
- Homogeneity of processing (freeze-drier shelves, etc)
- Homogeneity of storage
In-house standard – beware homogeneity

A not necessarily = B not necessarily = C not necessarily = D
Stability of reference standards

How do you know your product isn’t changing if your reference standard could be changing?
Measures to maximize stability of WHO International Standards

May include, as appropriate:

• Freeze-drying - reduce water content (< 1%)
• Heat-sealed glass ampoules - prevent gas exchange, moisture entry, reaction with stopper
• Filling with inert gas - residual O2 < 45µmol/L
• Stored -20°C in the dark

Some of these measures may be appropriate for a particular in-house standard
Monitoring stability of reference standards for potency

• accelerated degradation - extrapolation from rates at high T to predict rate at lower T (short term data)

• parallel stability - comparison of different preparations of similar types of material stored under similar conditions

• real time monitoring - comparison of samples held at normal storage T with samples stored at ultra-low T (-150°C, -70°C)

• natural reference - comparison with eg. pooled samples from a population where the activity is defined
Nine years storage IS for IFN-α2b (95/566)

<table>
<thead>
<tr>
<th>Storage °C</th>
<th>-70</th>
<th>-150</th>
<th>FZB</th>
<th>4</th>
<th>20</th>
<th>37</th>
<th>45</th>
<th>56</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>antiviral</td>
<td>1.08</td>
<td>1.14</td>
<td>0.92</td>
<td>1.00</td>
<td>0.89</td>
<td>0.59</td>
<td>0.34</td>
<td>0.05</td>
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<tr>
<td>reporter</td>
<td>0.97</td>
<td>0.98</td>
<td>-</td>
<td>0.96</td>
<td>0.89</td>
<td>0.58</td>
<td>0.31</td>
<td>0.05</td>
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<tr>
<td>gene</td>
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<td></td>
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<td></td>
</tr>
</tbody>
</table>

Predicted yearly loss of activity at -20°C: 0.008%

Real time monitoring
4th IS FVIII vWF plasma

Data from A Hubbard, NIBSC

<table>
<thead>
<tr>
<th>Ampoule / Assay</th>
<th>Potency of -20°C ampoules as % -150°C ampoules</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.75 yrs</td>
</tr>
<tr>
<td>1</td>
<td>102</td>
</tr>
<tr>
<td>2</td>
<td>96</td>
</tr>
<tr>
<td>3</td>
<td>101</td>
</tr>
<tr>
<td>4</td>
<td>103</td>
</tr>
<tr>
<td>Mean (%CV)</td>
<td>100 (3.1)</td>
</tr>
</tbody>
</table>
## Comparison with normal plasma

2nd IS for FVIII/VWF plasma

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Potency U/ampoule vs 1st IS</th>
<th>Potency U/ampoule vs ‘N’</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVIII:C</td>
<td>0.61</td>
<td>0.59</td>
</tr>
<tr>
<td>FVIII:Ag</td>
<td>0.92</td>
<td>0.89</td>
</tr>
<tr>
<td>VWF:RCo</td>
<td>0.84</td>
<td>0.83</td>
</tr>
<tr>
<td>VWF:Ag</td>
<td>0.90</td>
<td>0.91</td>
</tr>
</tbody>
</table>

N : mean of fresh normal plasma in 25 labs (> 350 donors)

Comparison of vials with ampoules

Lyophilized albumin in ampoules, stoppered vials & screw-top vials tested for moisture & oxygen ingress under conditions of stress.

12 months, -70°C to + 56°C

- Ampoules – no change detected
- Vials – moisture ingress, possible oxygen ingress

Formulation of reference standards

- Standard may require formulation for stability, compatibility with assay, need for bulking agents if small quantity of API


  - Preservation of Factor VIII activity improved by stabiliser, best = glycine + Hepes. Plasma lyophilized alone, stability improved by further desiccation over phosphorus pentoxide

- Formulation of standard may differ from that of drug sample

- Formulation of drug product may be incompatible with bioassay

- External standard unlikely to be the same as in-house standard or sample

⚠ Any difference between reference & sample is a potential source of problems
Interference by standard excipient

• External standard, eg. WHO standard, may contain only small quantity (few microgram) of API together with bulking agent, protein carrier, stabilizers, etc.
• Excipient can interfere, particularly at the higher concentrations at the top end of dose-response curve

\[ \text{Response} \rightarrow \text{Log dose} \]

\textit{inhibition by excipient at high dose}
Interference by standard excipient

- In the assay, may be possible to
  - adjust assay medium to compensate
  - increase sensitivity of assay to API, reducing quantity of excipient introduced

- In preparation of standards,
  - potential formulations can be tested for interference in (currently) common assays
  - reduce volume of standard (same quantity of API, same concentration excipient, so higher API:excipient ratio, less excipient introduced into assay)
Harmonization

- Various sources of reference standards (primary & working) for biological activity: eg. WHO, USP, EP
- Use of a particular standard may be specified in regulatory requirements, eg pharmacopeial monograph
- Various standards may be different materials
- Different assays may be used to establish potency unitage
- May have more than one biological activity
- Particular impact on replacement of an existing standard – eg. possible impact on potency labelling of existing products

> Initiatives to promote harmonization
Harmonization of standardization of unfractionated heparin (UFH) - a case history

1st IS then 2nd IS (porcine mucosal) used to calibrate pharmacopeial reference standards – global harmonization

1973: 3rd IS (bovine lung) versus 2nd IS: USP method gave 7% lower activity than BP method

USP assigned value to next USP standard (& subsequent USP standards calibrated against predecessor) using USP method; WHO assigned value (& for 4th & 5th IS calibrated against previous IS) derived from all methods used in collaborative study

Disparity of USP unit & IU for heparin

2009: USP reference standard (Lot F) & 6th IS calibrated against 5th IS in same collaborative study.

1 USP unit = 1IU of UFH
WHO International Standards & Reference Reagents

- **International Standards (IS):** assigned potency in international units (IU)
- **Reference Reagents:** assigned potency in units, intended as interim for speedy establishment - more limited characterization
- **Other historical categories,** eg. International Reference Preparation

Used to calibrate local standards – NOT as working standards. Supplies limited, and replacement of IS risks causing discontinuity

catalogs:

- [www.nibsc.ac.uk](http://www.nibsc.ac.uk) ➔ products ➔ biological reference materials
WHO biological reference standards

Principles:

• assigned potency in arbitrary units

• unit directly traceable to a reference preparation with a physical existence

• unit unrelated to a specific method of determination
WHO biological reference standards – particular cases

• 1 standard : 2 activities

  eg. 2nd IS for LMW heparin, code 01/608
      1097 IU anti-Xa activity / ampoule
      326 IU anti-IIa activity / ampoule

• Separate standards for different activities

  eg. 2nd IS for FSH (rec) for bioassay, code 08/282
       126 IU / ampoule
  1st IS for FSH (rec) for immunoassay, code 92/510
       60 IU / ampoule
Pleiotropic molecules

Many biologicals have multiple biological activities

Scenario:

• Reference standard 1 assigned unitage
• Replacement standard: not identical preparation. Different ratio of activities A & B
• Assigned unitage to provide continuity for activity A ..... discontinuity for activity B
• And currently licensed products?
Replacing an international standard – a difficult case study

- Interferon alfa: multiple subtypes with biological activities
- Assay systems
- 1st IS: mixture of subtypes from human leukocytes
- Candidate replacement standards = individual recombinant species


“lack of assay independence and relative activity equivalence
........... individual, homologous standards, each with a separate unitage, were required for biological standardisation and potency determinations of individual IFN-alpha subtypes”
Replacement of in-house standard

- need reference standard at early stage product development
- better continuity of data if same standard used throughout

⚠️ but early batches of product may differ from later batches
  - fewer problems the more similar reference standard is to test samples
  - reference material should be prepared from lot(s) representative of production & clinical materials
  ..... so may need to replace initial in-house reference standard with different material

⚠️ or stocks of standard may simply be running out

➢ Direct comparison of both (all) in-house reference standards & any external reference standard using panel of suitable test samples & controls, in all bioassay systems to be used
“Standard creep” – sequential calibrations

⚠ Beware of sequential calibrations of successive standards
Characterization of in-house standard

• characterization of performance in bioassay system(s), including comparison with any external standards
• stability data from potency measurements
• physicochemical data, even if a separate standard is used for the biological activity
• physicochemical stability data can support potency data for stability characterization
• need to demonstrate similarity to production & clinical materials & assess any differences identified
More than one bioassay required

Most products will require at least two, preferably orthogonal, bioassays at various stages, even if only one is used routinely for release.

Products with multiple biological activities:

“When changes are made to a product with multiple biological activities, manufacturers should consider performing a set of relevant functional assays designed to evaluate the range of activities.” (ICH Q5E)

⚠️ Need to determine whether the reference standard is suitable for each assay system
Changing the bioassay

Problem: in-house standard was functionally same as external standard in original bioassay, but not in new bioassay

⚠️ Check IHS and product functionally same in new bioassay

• Test excipient

➢ Assign unitage to IHS using old bioassay (as done previously). Use this unitage to assign potency to product using new bioassay
In the future: SI-calibrated WHO International Standards …… ?

- Discussion on changing calibration of some proteins (insulin, rhGH) to SI units (mg)
- More readily applicable to bacterial polysaccharides used in vaccines. EP monographs and WHO Guidelines define potency in terms of mass of saccharide. There is no bioassay
- First example of SI-calibrated standard: Haemophilus influenzae b PRP,  [www.nibsc.ac.uk/documents/ifu/02-208.pdf](http://www.nibsc.ac.uk/documents/ifu/02-208.pdf)
- Different process for value assignment: WHO Guidelines - multiple methods yielding a consensus value; SI: single reference method
- Particular need to address Uncertainty of Measurement

How would SI or other form of mass unitage be applied to bioassay standards, especially for heterogeneous protein mixtures?
In conclusion

• Reference standards are essential for bioassays

• The reference standard must be shown to be suitable for the product and the bioassay for which it is being used in order to obtain valid potency measurements
... and I would like to thank colleagues from around the world for the interesting discussions on standardization issues we have had over many years, and particularly Elaine Gray and Chris Jones at NIBSC for their advice on this presentation
Reference standard catalogs

www.who.int/biologicals
www.nibsc.ac.uk
www.usp.org/referenceStandards/