Scope and Limitations of Bioassays
Workshop Session Two in Grand Ballroom Dvorak
Session Co-Chairs: Thomas Schreitmüller, F. Hoffmann-La Roche Ltd. and Lance Smallshaw, UCB Pharma SA

13:40 – 14:05

EU Expectations with Potency Assays
Margarida Menezes Ferreira, INFARMED – National Authority of Medicines and Health Products, Lisbon, Portugal

- What is so great about bioassays?
- What product attribute(s) do bioassay results confirm?
- What is the perspective concerning receptor/target binding assays versus bioassays?
- What is the perspective concerning results from surrogate product attributes versus bioassay results?
- Under what circumstances a bioassay may be omitted during development?
- Under what circumstances a bioassay may be omitted for a commercial product?

Margarida Menezes Ferreira
Senior Assessor / INFARMED, National Authority for Medicines and Health Products – PORTUGAL
PT expert at BWP/CHMP – EMA
CAT member - EMA
(margarida.menezes@infarmed.pt)
Figure 1: Selected assays of 30 evaluated areas most urgently requiring new or improved testing methods

Bioassays to assess potency for release of drugs
Aggregation
Better stability assays
Biotech drug comparability (for in-house manufacturing changes, as well as biosimilars)
Glycosylation

2013
42.4%
40.7%
39.8%
39.8%
37.3%

2012
Bioassays to assess potency for release of drugs
Aggregation
Better stability assays
Biotech drug comparability (for in-house manufacturing changes, as well as biosimilars)
Glycosylation

28.1%
31.6%
31.4%
32.7%
• **Biological Activity**
  - describes the specific ability or capacity of a product to achieve a defined biological effect

• **Potency** (expressed in units) is 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.
  - [Quantity (expressed in mass) is a physicochemical measure of (protein) content.]

• Mimicking biological activity in the clinical situation is not always necessary.

• Correlation between clinical response and biological activity should be established in pharmacodynamic or clinical studies.
• biological assay with appropriate precision and accuracy serves as a complement to physicochemical analysis, e.g., as a surrogate assay for higher order structure

    = suitable approach

to confirm that change in specific higher order structure has not occurred

• Where physicochemical or biological assays are not considered adequate to confirm that the higher order structure is maintained, it might be appropriate to conduct a nonclinical or clinical study.
Microheterogeneity in recombinant proteins

- Point mutations
- charge
- Oxidation
- Glycosylation

- Impact on tertiary / quaternary structure?
- How to address?

BIOASSAYS ADDRESS INTERACTIONS OF HIGH ORDER STRUCTURES
ICH Q6B – Product related substances / impurities in biotech proteins

a) **Truncated forms**: Hydrolytic enzymes or chemicals may catalyse the cleavage of peptide bonds.

may be detected by HPLC or SDS-PAGE. Peptide mapping may be useful, depending on the property of the variant.

b) **Deamidated, isomerised, mismatched S-S linked, oxidised or altered conjugated forms** (e.g., glycosylation, phosphorylation)

may be detected and characterised by chromatographic, electrophoretic and/or other relevant analytical methods (e.g., HPLC, capillary electrophoresis, mass spectroscopy, circular dichroism).

c) **Aggregates**: dimers and higher multiples of the desired product.

generally resolved and quantitated by appropriate analytical procedures (e.g., size exclusion chromatography, capillary electrophoresis).
# Physico-chemical analytical program for a MAb

<table>
<thead>
<tr>
<th>Primary Structure</th>
<th>Peptide mapping</th>
<th>Characterisation, Stability</th>
<th>peptide sequence, and identity</th>
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<tbody>
<tr>
<td>Amino acid analysis</td>
<td>Characterisation</td>
<td>amino acid composition</td>
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<tr>
<td>N-terminal sequencing</td>
<td>Characterisation</td>
<td>N-terminal identity</td>
<td></td>
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<tr>
<td>C-terminal sequencing</td>
<td>Characterisation</td>
<td>C-terminal identity</td>
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<tr>
<td>Disulfide bonds</td>
<td>Characterisation</td>
<td>disulfide bond location</td>
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<td>Free thiol analysis</td>
<td>Characterisation</td>
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<tr>
<th>Higher Order Structure</th>
<th>FTIR</th>
<th>Characterisation</th>
<th>secondary structure</th>
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<tr>
<td>CD</td>
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<td>DSC</td>
<td>Characterisation</td>
<td>thermal transition temperatures</td>
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<table>
<thead>
<tr>
<th>Mass and Weight</th>
<th>Intact mass by ES-MS</th>
<th>Characterisation, Stability</th>
<th>molecular weight for intact IgG by mass spectrometry</th>
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<tr>
<td>SEC MALS</td>
<td>Characterisation</td>
<td>size distribution and molecular weight</td>
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<tr>
<td>SE-AUC</td>
<td>Characterisation</td>
<td>molecular weight by sedimentation equilibrium</td>
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<tr>
<td>SV-AUC</td>
<td>Characterisation</td>
<td>sedimentation coefficient</td>
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<table>
<thead>
<tr>
<th>Purity</th>
<th>SE-HPLC</th>
<th>Release, Stability</th>
<th>Monomeric purity and aggregate content</th>
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<tbody>
<tr>
<td>CE-SDS</td>
<td>Release, Stability</td>
<td>electrophoretic mobility and purity under non-reducing and reducing conditions</td>
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</tr>
<tr>
<td>SDS-PAGE</td>
<td>Characterisation</td>
<td>electrophoretic mobility and purity under non-reducing and reducing conditions</td>
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</table>

<table>
<thead>
<tr>
<th>Charge Variants</th>
<th>IEF</th>
<th>Release, Stability</th>
<th>isoelectric point</th>
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<tr>
<td>IE-HPLC</td>
<td>Release, Stability</td>
<td>charge variant distribution</td>
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<table>
<thead>
<tr>
<th>Glycosylation</th>
<th>Monosaccharide analysis</th>
<th>Characterisation</th>
<th>neutral and amino sugar composition</th>
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<tbody>
<tr>
<td>Sialic acid analysis</td>
<td>Characterisation</td>
<td>sialic acid content</td>
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<tr>
<td>N-linked glycan analysis</td>
<td>Characterisation</td>
<td>oligosaccharide structure and distribution</td>
<td></td>
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<tr>
<td>Oligosaccharide profiling</td>
<td>Characterisation, Stability</td>
<td>glycosylation pattern (G0, G1, G2)</td>
<td></td>
</tr>
</tbody>
</table>
bioassays

• Receptor/antigen binding assays = molecular recognition no function associated

• *In vitro* assays - requires susceptible cells = specific response require involvement of receptors/target surface molecules:
  - stimulation of cell proliferation,
  - inhibition of cell proliferation,
  - induction of cytotoxicity/apoptosis,
  - induction of differentiation,
  - induction of antiviral or antimicrobial activity, and
  - up-regulation of expression of intracellular, secreted and surface membrane proteins

• *In vivo* assays = out of fashion - 3R’s

In accordance with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes and Directive 2010/63/EU on protection of animals used for scientific purposes, the 3R principles (replacement, reduction and refinement) should be applied to production and control testing of pharmaceuticals.
### TABLE

<table>
<thead>
<tr>
<th>Type of analytical procedure characteristics</th>
<th>IDENTIFICATION</th>
<th>TESTING FOR IMPURITIES</th>
<th>ASSAY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accuracy</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Precision</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Repeatability</td>
<td>-</td>
<td>+ (1)</td>
<td>+ (1)</td>
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<tr>
<td>Interm. Precision</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Specificity (2)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Detection Limit</td>
<td>-</td>
<td>- (3)</td>
<td>+</td>
</tr>
<tr>
<td>Quantitation Limit</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Linearity</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Range</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Cell based assay - validation strategy – parallel assay

- **Specificity**
  - Compare to diluent with no product
  - Stress / denatured product
  - use unrelated proteins

- **Linearity and Range**
  - Verify linear response across the dose range used

- **Precision** – several plates same day/ different technicians different days

- **Accuracy** - corrected specific activity should be within variability of standard
  - Samples of different strengths (eg 50%, 100%, 150%).

- **Robustness** - Calculated specific activity not affected (within intermediate precision)
  - Vary conditions: Cell seeding density; reporter incubation time; Incubation temperature; Cell passage number; cell confluence
Some examples
Well characterised MP
no biological characterisation / release

Highly controlled and experienced manufacturing process
High physico-chemical consistency in quality control
Expressed in IU or units for historical reasons

**INSULIN ASPART**

Insulinum aspartum

H·Gly·Ile·Val·Glu·Gln·Cys·Cys·Thr·Ser·Ile·Cys·Ser·Leu·Tyr·Gin·Leu·Glu·Asn·Tyr·Cys·Asn·OH

H·Phe·Val·Asn·Gln·His·Leu·Cys·Gly·Ser·His·Leu·Val·Glu·Ala·Leu·Tyr·Leu·Val·Cys·Gly·Ser·His·Leu

**SOMATROPIN FOR INJECTION**

Somatropinum injectabile

**Eur Phar Assay – HPLC**

Content: 90.0 per cent to 104.0 per cent of insulin aspart C 256H381N65O79S6 plus A21Asp insulin aspart, B3Asp insulin aspart, B3isoAsp insulin aspart and B28isoAsp insulin aspart (dried substance).

0.0350 mg of insulin aspart is equivalent to 1 unit

**Eur Phar Assay – SEC**

**Bioassay for process validation** – (growth promotion test = in vivo pharmacology)

1 mg of anhydrous somatropin (C\(_{990}\)H\(_{1528}\)N\(_{262}\)O\(_{300}\)S\(_{7}\)) is equivalent to 3.0 IU of biological activity.
Interferons – multiple targets

surrogate bioassay addressing common anti viral effect

General Eur Phar. method for interferons = Reduction of cytopathic viral effect on susceptible human cell line

- Encephalomyocarditis virus (EMCV) with the A549 epithelial lung carcinoma cell line,

- Semliki Forest virus or Sindbis virus with human fibroblasts

- Vesicular Stomatitis Virus with human diploid fibroblasts or Madin-Darby bovine kidney cell line.

Need high sensitivity and parallel response compared to International Standard

Not relevant for IFN beta-2a – Multiple Sclerosis!

But potency measured by antiviral activity - *vitro* cytopathic effect bioassay using lung carcinoma cells (A549) and Encephalomyocarditis virus (EMCV).

-Receptor binding assay
cAMP – surrogate marker for Thyrogen bioassay

Thyrogen dosed on mass units not bio units
Evolution of Bioassay for G-CSF
- increase simplicity / increase sensitivity

1. Cell proliferation assay using murine cell line NFS-60 and 3H-TdR
2. Cell proliferation assay using murine 32D clone3 grown in G-CSF + Alamar Blue readout
3. Rapid assay based on reporter gene expression using 32D clone transfected with huG-CSFR and reporter gene + luciferase

Filgrastim and the reference drug Neupogen were compared for ability to interact with the G-CSF receptor in vitro NFS-60 cell assay. (EPAR)
Erythropoietins – glycoprotein – differences in glycosylation not apparent with in vivo normocythaemic mouse assay

rhEPO Eur Phar: specific bioactivity should be at least 100,000 IU/mg rhEPO. The estimated potency should not be less than 80% and not more than 125% of the stated potency (119,000 IU/mg).

High variability – requires adequate statistical approach

Practice Research & Innovation
34 • Volume 15 • 2009/2 [www.ejhp.eu](http://www.ejhp.eu)
Carsten Brockmeyer, PhD; Andreas Seidl, PhD
Sandoz Biopharmaceutical Development

Brink et al. Pharm Res. 2011 February; 28(2): 386–393
IgG – complex structure multiple interactions

(Micro) heterogeneity / variants due to:
glycoforms,
charge,
cysteine-related,
oxidation,
size
Aa point mutation variants

Possible impact on :
Target antigen recognition
Antigen binding affinity,
ADCC – affinity to Fc receptor(s)
ADC – complement binding,
Immunogenicity related to aggregates
pharmacokinetics
Mabs – Cancer indication

target growth factor receptors / tumor cell marker

**Fab function:**
*Target Receptor XGFR - soluble / cell bound binding affinity -Surface Plasmon Resonance*
*Susceptible cell proliferation inhibition assay*

**Fc function:**
*Cell based ADCC assay* - Proliferation inhibition assay human PBMC’s (peripheral blood mononuclear cells)
*binding affinity -Surface Plasmon Resonance to:*
*FcγRIA*
*FcγRIIA or FcγRIIB*
*FcγRIIIA*
*FcRn*
*Cell based CDC assay*
*ELISA-based complement-binding assay - C1q*
*Apoptosis assay*
# Mabs – anti TNFα

target TNFα / rheumatoid arthritis / psoriasis /

## Table 1: Currently licenced anti-TNF biologic drugs.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Infliximab</th>
<th>Adalimumab</th>
<th>Golimumab</th>
<th>Etanercept</th>
<th>Certolizumab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fully human Ligand</td>
<td>TNF</td>
<td>TNF</td>
<td>TNF</td>
<td>TNF &amp; LTα3</td>
<td>TNF</td>
</tr>
<tr>
<td>Molecular weight (kDa)</td>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
<td>95</td>
</tr>
<tr>
<td>Half-life (days)</td>
<td>8–10</td>
<td>10–14</td>
<td>12 ± 3</td>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td>Dosing route and frequency</td>
<td>I.v every 8 weeks following loading at weeks 0,2 and 6</td>
<td>Sub-cut every 2 weeks</td>
<td>Sub-cut monthly</td>
<td>Sub-cut weekly</td>
<td>Sub-cut every 2 weeks</td>
</tr>
</tbody>
</table>

| Most immunogenic | ADCC       | ADCC       | ADCC       | least immunogenic |
| Stable TNF-Mab   | TNF        | TNF        | TNF        | NO ADCC         |
| TNFm              | TNFm       | TNFm       | TNFm       | NO CDC          |
| Reverse signal   | Reverse signal | Reverse signal | Reverse signal | NO CDC          |

- **ADCC:** Activated phagocytes kill target cells
- **CDC:** Complement dependent cytotoxicity
- **TNFs (3:1):** Three TNF molecules per Mab
- **No Crohn’s indication:**
Oligosaccharide structures in Mab's

<table>
<thead>
<tr>
<th>Structure</th>
<th>Terminal Man</th>
<th>Reduce FcRIIIa binding</th>
<th>Increase CDC activity</th>
<th>No interference on ADCC nor antigen recognition</th>
<th>Decrease binding of Mab to C1q and reduced CDC activity</th>
<th>Serum half-life</th>
</tr>
</thead>
<tbody>
<tr>
<td>NANA-Gal-GlcNAc-Man</td>
<td>G2S2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reduce FcRIIIa binding affinity</td>
</tr>
<tr>
<td>NANA-Gal-GlcNAc-Man</td>
<td>G2S1 (α,6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;5% increase Mab serum half-life but Reduce FcRIIIa binding</td>
</tr>
<tr>
<td>Gal-GlcNAc-Man</td>
<td>G2S1 (α,3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Gal-GlcNAc-Man</td>
<td>G1 (α,6)</td>
<td></td>
<td></td>
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<tr>
<td>Gal-GlcNAc-Man</td>
<td>G1 (α,3)</td>
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<tr>
<td>GlcNAc-Man</td>
<td>G0</td>
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</table>

Structure of major N-glycans found in human IgG. The majority of glycans found in the Fc of human IgG are shown. These glycans are complex biotinyl structures with heterogeneity because of terminal galactose and/or sialic acid residues (NANA, N-acetylneuraminic acid; Gal, galactose; GlcNAc, N-acetylgalcosamine; Man, mannose; Fuc, fucose). About 10% of these glycans may not contain core Fuc residue (not shown) [G0: no Gal; G1: one Gal on either α,1,6-branch or α,1,3-branch; G2: two Gal; G2S1: two Gal and one NANA on either α,1,6-branch or α,1,3-branch; G2S2: two Gal and two NANA].
ADCC based on PBMC from donated blood – HIGH VARIABILITY

**ADCC mediated by FcIIla transfected NK-92 cells - TARGET Wil-2 CD20+ labeled**

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

**Development of a quantitative, cell-line based assay to measure ADCC activity mediated by therapeutic antibodies**

Schnueriger et al. Molecular Immunology 2011, 48: 1512–1517

**ADCC- NFAT reporter gene assay.**

Wil-2, a CD-20+ human B lymphoblastoma cell line, was seeded in a 96 well opaque tissue culture plate. Anti-CD20 antibody (IgGv) was serially diluted and incubated with the Wil-2 cells 1 h at 37°C, 5% CO2. Following incubation, Jurkat NFAT luciferase reporter cells, were added to the Wil-2/antibody mixture at 150,000 cells per well. The mixture was incubated 4 h at 37°C, 5% CO2, and measured for luciferase production using a luminescent substrate.

*Development and validation of an antibody-dependent cell-mediated cytotoxicity-reporter gene assay*

Parek et al. mAbs 4:3, 310-318; 2012

**ALTERNATIVES NO PBMC**
complement-mediated cytotoxicity CDC - classical methods

- $^{51}$Chromium
- Live cell / cell lysis: Alamar blue – fluorescence
- MTT
- Trypan blue

LABORIOUS – REQUIRE COMPLEMENT (HUMAN OR RABBIT)

Alternative:
ELISA-based complement-binding assay - C1q SPR?

Comparability equivalence testing

Tolerance interval
95/99 tolerance interval - range defined such that 99% of the reference batches lie within that range with 95% probability.

Comparability band
To confirm comparability the true difference or ratio of means including the 90% confidence interval must be within the comparability band – arbitrary number

Number of samples adequate?
Normal distribution applicable?
Adequate statistic approach? Justification required
Mab potency – inhibition of cancer cell line proliferation
Validation protocol

- parallel line – specificity / robustness – all valid
- Stress samples no effect - ?

### Analysis of Stressed Samples

<table>
<thead>
<tr>
<th>Samples and Conditions</th>
<th>Potency ($\times 10^4$ Units/mg)</th>
<th>RSD (%)</th>
<th>95% Lower Confidence Limit</th>
<th>95% Upper Confidence Limit</th>
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<tbody>
<tr>
<td>Control</td>
<td>$\leq -60^\circ C$</td>
<td>0.96</td>
<td>3</td>
<td>0.90</td>
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<tr>
<td>Thermal</td>
<td>3 weeks, 40$^\circ$ C</td>
<td>0.93</td>
<td>7</td>
<td>0.76</td>
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<tr>
<td></td>
<td>6 weeks, 40$^\circ$ C</td>
<td>0.94</td>
<td>9</td>
<td>0.72</td>
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<tr>
<td>Light Exposure</td>
<td>Foiled Control</td>
<td>0.93</td>
<td>14</td>
<td>0.62</td>
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<tr>
<td></td>
<td>Exposed to light (1.2 $\times 10^8$ lux-hours)</td>
<td>0.91</td>
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<td>0.86</td>
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<tr>
<td>pH Variation</td>
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<tr>
<td></td>
<td>pH 8.5</td>
<td>0.83</td>
<td>7</td>
<td>0.69</td>
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<tr>
<td>Oxidation</td>
<td>Oxidation Control</td>
<td>0.96</td>
<td>7</td>
<td>0.80</td>
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<tr>
<td></td>
<td>0.5% tBHP$^b$</td>
<td>1.09</td>
<td>20</td>
<td>0.56</td>
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</table>

Can it be stability indicating?
Potency Mab for Cancer indication: target cell binding (FACS) versus functional assay (CDC)

Comparison of FACS and CDC Potency Data after Storage at 40°C

<table>
<thead>
<tr>
<th>Batch</th>
<th>Test</th>
<th>Storage Time (Months)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>FACS</td>
<td>FACS</td>
<td>102</td>
</tr>
<tr>
<td>CDC Potency</td>
<td>FACS</td>
<td>107</td>
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<tr>
<td>FACS</td>
<td>CDC Potency</td>
<td>102</td>
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<tr>
<td>CDC Potency</td>
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<td>103</td>
</tr>
<tr>
<td>FACS</td>
<td>FACS</td>
<td>116</td>
</tr>
<tr>
<td>CDC Potency</td>
<td>FACS</td>
<td>102</td>
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<tr>
<td>FACS</td>
<td>CDC Potency</td>
<td>106</td>
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<td>FACS</td>
<td>FACS</td>
<td>104</td>
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<tr>
<td>CDC Potency</td>
<td>91</td>
<td>60</td>
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Comparison of FACS and CDC Potency Data after Storage under ICH Light Conditions

<table>
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<th>Batch</th>
<th>Test</th>
<th>Storage Time (Months)</th>
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<td></td>
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<td>CDC Potency</td>
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<td>86</td>
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<td>CDC Potency</td>
<td>97</td>
</tr>
<tr>
<td>CDC Potency</td>
<td>94</td>
<td>79</td>
</tr>
</tbody>
</table>

Functionality possibly more sensitive - stability indicating
- Potency has to be quantitative and related to relevant biological properties = drawn from development (and preclinical studies).
- Potency assay required for consistency, comparability, stability.
- Potency may evolve during development – define reference preparations as early as possible.
- Potency at release – requires validation for MA (+ transfer to EU).

Bioassays *in vitro* preferred to *in vivo* – 3R’ + validation + sensitivity.
- Functional *in vitro* assay more meaningful as it integrates specific receptor interaction with measurable response – *in vitro* cell based preferred.
- Receptor binding assays have less variability but may not reflect the cascade of events resulting in the biological effect.
- Kinetic studies to validate relevant biological activity(ies) – for comparability.
- Unrelated products / low potency fractions expected required for validation.
- Stressed samples to confirm stability indicating / comparability.
<table>
<thead>
<tr>
<th>QUESTION</th>
<th>ANSWER</th>
</tr>
</thead>
<tbody>
<tr>
<td>What is so great about bioassays?</td>
<td>Address intended biological interaction / may enable immunogenicity studies of neutralising antibodies</td>
</tr>
<tr>
<td>What product attribute(s) do bioassay results confirm?</td>
<td>Adequate conformation for the biologic interaction and response</td>
</tr>
<tr>
<td>What is the perspective concerning receptor/target binding assays versus bioassays?</td>
<td>More sensitive but dissociated from the biological response</td>
</tr>
<tr>
<td>What is the perspective concerning results from surrogate product attributes versus bioassay results?</td>
<td>Quantitative results on surrogate attributes suitable for release if validated to correlate to functional biological assay or no other choice …</td>
</tr>
<tr>
<td>Under what circumstances a bioassay may be omitted during development?</td>
<td>(Initial scientific rational already address biological effect. Bioassays evolve from there.) Biological characterisation is essential for comparability – mandatory to ensure use of evolving clinical data</td>
</tr>
<tr>
<td>Under what circumstances a bioassay may be omitted for a commercial product?</td>
<td>If well characterised / well known. Needed for comparability and stability. May be omitted at release if replaced by validated surrogate (above)</td>
</tr>
</tbody>
</table>
THANK YOU!