Bioassays Reflecting Fc Effector Functions – Selecting the Most Relevant Assay Type Considering the Molecules Mode of Action and the Assays Performance Characteristics

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The importance of functional and target-binding assays

**Functional assays** serve multiple purposes (...)*:

- Confirm integrity of the higher order structures
- Should reflect clinically relevant MoA(s)
- Indicator of manufacturing process consistency, product purity, potency and stability
- If a reference product exhibits multiple functional activities, sponsors should perform a set of appropriate assays designed to evaluate the range of relevant activities for that product.

**Target binding**:

- When binding is part of the activity (...) analytical tests should be performed to characterize the (...) specific binding properties

*FDA Draft Guidance, Development of Therapeutic Protein Biosimilars: Comparative Analytical Assessment and Other Quality-Related Considerations, May 2019
### Complexity according to MoA

**IgG:**

<table>
<thead>
<tr>
<th>MoA</th>
<th>Binding to soluble antigen</th>
<th>Cell-bound antigen with blocking</th>
<th>Cell-bound antigen with depletion</th>
<th>Cell-bound antigen with blocking and depletion; cell and/or tissue dependent MULTIPLE MoA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fab domain</strong></td>
<td>Target binding</td>
<td>Target binding</td>
<td>Target binding</td>
<td>Target binding Multiple Fab cell-based assay(s)</td>
</tr>
<tr>
<td></td>
<td>Fab cell-based assay</td>
<td>Fab cell-based assay(s)</td>
<td>Fab cell-based assay(s)</td>
<td>Fab cell-based assay(s)</td>
</tr>
<tr>
<td><strong>Fc domain</strong></td>
<td>FcγRs, FcRn C1q</td>
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</tr>
</tbody>
</table>

*Figures show different binding and interaction scenarios for IgG antibodies.*

*[Image of IgG molecule with different binding modes]*
IgG1 and IgG3 are strong inducers of Fc-mediated effector functions

Functional assay
- CDC

Binding by SPR, BLI
- C1q

FcγRI, IIa (H/R131), IIb, IIIa (F/V158), IIIb

ADCC is mediated by NK cells, monocytes or macrophages via FcγRIIIa

ADCP can be mediated by monocytes, macrophages, neutrophils and dendritic cells primarily via FcγRIIa but also via FcγRI and FcγRIIIa.

Functional & surrogate assay
- ADCP
- ADCC

Binding by SPR, BLI
- FcRn

TRIM21 triggers polyubiquitinylation of the opsonized particles (e.g., virus, intracellular bacteria) and proteasomal degradation. TRIM21 mediates antibody-dependent intracellular neutralization (ADIN).

Both receptors are found on B cells suggesting a role in regulation of humoral immune responses. The exact mechanism and significance for human immune responses has not been elucidated.
Intermediate precision of bioassays is typically between 2 and 20%
Antibody Dependent Cellular Cytotoxicity is an important mode of action for IgG1/3 molecules and needs special attention due to the high sensitivity towards changes in glycosylation.
Available ADCC assay formats

<table>
<thead>
<tr>
<th>Assay Type</th>
<th>Primary Donor</th>
<th>Physiological Relevance</th>
<th>Intermediate Precision (SD)*</th>
<th>Sensitivity to Glycan Change</th>
<th>Robustness / Effort</th>
<th>Suitability</th>
</tr>
</thead>
<tbody>
<tr>
<td>primary donor peripheral blood mononuclear cells (PBMCs)</td>
<td>Peripheral Blood</td>
<td>high</td>
<td>13 – 36%</td>
<td>high</td>
<td>low / high</td>
<td>similarity (+)</td>
</tr>
<tr>
<td>immortalized natural killer (NK) cells</td>
<td>NK Cells</td>
<td>high</td>
<td>5 – 25%</td>
<td>high</td>
<td>high / medium</td>
<td>development (++), similarity (++), release (+)</td>
</tr>
<tr>
<td>reporter gene assay (RGA)</td>
<td></td>
<td>medium</td>
<td>7 – 30%</td>
<td>high (F variant), low (V variant)</td>
<td>high / low</td>
<td>development (++), similarity (++/+), release (++)</td>
</tr>
</tbody>
</table>

* based on the NIBSC study on Rituximab standard and Novartis experience
NK ADCC assay shows good variability, high sensitivity to glycan changes and high physiological relevance

Assay precision

Assay sensitivity to glycans

Data from Prior et al., 2018 NIBSC study on Rituximab standard
recent Novartis projects
NK ADCC assay variability is stable long term

- RSD of a single determination is 7%
- $\Delta$ measured vs calculated ADCC gives a good estimate about the long-term assay stability (8 years)

### Intermediate precision from method validation

<table>
<thead>
<tr>
<th># of repeats</th>
<th>relative lower 95% CL</th>
<th>relative upper 95% CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>90</td>
<td>112</td>
</tr>
<tr>
<td>4</td>
<td>92</td>
<td>108</td>
</tr>
</tbody>
</table>

$\Delta$ measured / calculated ADCC $n=2$
Measuring binding property is a good surrogate for functional assays

Good correlation between FcγRIIIa binding by BLI and ADCC by NK assay

ADCC – BLI correlation Mab1

\[ R^2 = 0.83 \]

BLI RSD ≈ 8 %

ADCC – BLI correlation Mab2

\[ R^2 = 0.93 \]

BLI RSD ≈ 4 %
What can be done to increase assay precision?

1. Adherence to principles of good laboratory practice
   - Appropriate training of operators
   - Proper calibration/control of equipment, reagents and materials
   - Proper handling of reference materials
   - Adherence to standard operating procedures
   - Good documentation practice

2. Good assay design/setup (eg optimization of dilution steps)

3. Good study design (what is the purpose of the test and what precision do I need?)

4. Determination of method capability (eg number of replicates needed to achieve a defined precision, linearity, sensitivity to glycan changes etc)

5. Monitoring of assay characteristics (SST's eg % lysis) to check validity of the result

6. Implementation of a quality control sample (as relative potency is determined such a sample won’t pick up every assay issue)

7. Definition of max allowable difference between a technical replicate

8. Trending of assay characteristics and results to identify abnormal assay behaviour as soon as possible

9. Trending of assay results along with calculated relative results from glycan data to identify abnormal assay behaviour
The established structure-function relationship between glycans and ADCC can be useful for monitoring of the assay variability.

- High impact:
  - afucosylated glycans
- Medium impact:
  - high mannose glycans
- Low impact:
  - terminal galactosylated glycans
- Very low impact:
  - sialylated glycans

consistent impact across molecules
Calculated ADCC can be used to monitor the long term variability of the assay.

*calculated ADCC based on relative glycan level x impact/weight factor
Glycan structures taken into account: afucosylated, high mannose and galactosylated glycans
**Assay precision and sensitivity needs to be appropriate to detect potentially relevant changes in ADCC**


Kim S, et al. mAbs 2017; 9(4)


*N Post hoc analysis suggests that the downward drift in Herceptin was a contributing factor to lower event-free survival; Pivot X, et al. Eur J Cancer. 2019 Oct;120: 1-9*
Relative changes in ADCC can be estimated using a general structure-function formula. Calculated relative ADCC gives a good estimate about assay sensitivity to glycan changes.

\[ \text{Calc}_\text{rel}_{-}\text{ADCC} = 78\% \times \sum \text{afucosylated} + 19\% \times \sum \text{mannosylated} + 3\% \times \sum \text{galactosylated} \]

*average cal_rel_ADCC using the general structure-function formula
Summary

- Bioassays and cell binding assays show a wide range of variability between 2 and 35% depending on the exact method/setup/experience.
- High precision can be achieved when considering multiple factors of assay development/experimental conduction/control.
- Specific to ADCC, the NK based assay shows good precision, physiological relevance and long term stability. RGA assays can be valuable alternative if sensitivity to glycan changes can be demonstrated.
- Structure-function relationship between glycans and ADCC can be helpful to monitor assay stability.
- For routine release purpose, glycan analysis or binding assays may be a robust alternative for a cell-based ADCC assay.
- Sensitive and precise bioassays reflecting the molecules mode of action together with relevant phys-chem data allows a full characterization of IgG’s.
- Appropriately developed and conducted bioassays are more sensitive than clinical studies in picking up differences between products/processes and may replace clinical efficacy studies within the development of biosimilar products.
Thank you