Accelerating Antibody Drug Development
Using Novel ADCC Reporter Bioassays

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Research Manager
Outline

• Antibody drugs and mechanisms of action (MOA)

• Introduction to ADCC and ADCC Reporter Bioassays

• “Cells as reagents” – *thaw-and-use format*

• ADCC Reporter Bioassays, *V and F variants*

• Therapeutic Ab Testing - *using V and F variants*

• Bioassay using Glomax® Discover - *CFR 21 Part 11*
Biological Product – Monoclonal Antibody (mAb) Drug

- Biological products are generally produced using a living system or organism.

- Biological products may be manufactured through biotechnology, derived from natural sources, or produced synthetically.

Source: FDA webinar, Feb 15, 2012
Mechanism of Action (MOA) for mAb drugs

In vitro bioassays of MAbs during drug development need to be reflective of in vivo MOA.
Traditional ADCC bioassays using primary effector cells

**ADCC** (antibody-dependent cell-mediated cytotoxicity)

**Biology of ADCC**
- NK cells are the primary effector cell *in vivo*
- NK cells require target cell-bound Ab to mediate ADCC
- Granzyme and perforin secreted lead to target (tumor) cell lysis

**Bioassay of therapeutic Ab in ADCC MOA**
- Primary NK cells are used in an *in vitro* cell-based bioassay
- Blood donor effector cell source is highly variable and results in high assay variability
An Ideal Bioassay...

- Reflective of the mechanism of action (MOA) of the biological product
- Well controlled (precise, accurate, robust, reproducible)
- Stability-indicating
- Usable as a QC lot-release assay

Modified from Chana Fuchs (DMA/CDER)

In this webinar, we will demonstrate how the ADCC Reporter Bioassay fulfills these elements
ADCC Reporter Bioassay principle

Target-cell bound Ab binds to FcγRIIIa on effector cell – activating pathway

Luciferase reporter is readout of pathway activation state

New reporter gene bioassay measures a step earlier in the pathway

Traditional readout is cytotoxicity, the ultimate endpoint

Granule exocytosis, ADCC, transcriptional regulation

ADCC Reporter Bioassay – FcγRIIIa V and F variants

**FcγRIIIa**

<table>
<thead>
<tr>
<th>158F/V or F/F</th>
<th>158 V/V</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;85% population</td>
<td>~10-15% population</td>
</tr>
<tr>
<td>Less efficient Ab binding and ADCC</td>
<td>More efficient Ab binding and ADCC</td>
</tr>
</tbody>
</table>
**ADCC Reporter Bioassay – FcγRIIIa V and F variants**

- Surrogate assays that are reflective of *in vivo* MOA
- Precise, accurate, robust, reproducible, stability-indicating
- Suitable for biocomparability studies, QC stability studies etc. and other uses where the traditional assay falls short
- Complement primary cell-based ADCC bioassays in Ab drug development
ADCC Reporter Bioassay protocol

**Single day bioassay**

1. Incubate control, reference or test antibody with target cells.

2. Add engineered effector cells containing:
   - FcγRIIIa (V158) or (F158)
   - NFAT-RE luc2 luciferase

3. Incubate to allow for pathway activation (as short as 6 hours).

4. Add luciferase detection reagent and measure luminescence.
ADCC Reporter Bioassay development

1. Developing low variability ADCC MOA Reporter Bioassays
   - Thaw-and-Use cells
   - Critical parameters and DoE optimization
   - Robust reagents and assays
   - Simple, homogeneous

2. Bioassay performance testing
   - Accuracy
   - Precision
   - Specificity
   - Linearity and range
   - Robustness
   - Stability-indicating
   - Benchmarking
   - Bioassay of multiple Abs

Assay qualification
Cells as critical reagents in thaw-and-use format
Cells as critical reagents

Traditional use of fresh cells from cell culture: slow and variable

- Thaw for culture
- Cell culture
- Cell count, harvest, prepare for assay
- Read plates in e.g. GloMax Discover

Time: 1-2 weeks

Thaw-and-use cells: fast, simple, and improved reproducibility

- Thaw-and-Use
- Resuspend in assay buffer, plate for assay
- Read plates in e.g. GloMax Discover

Time: < 24hr
No cell culture required

1. **Human cell lines (Jurkat, WIL2-S, Raji, U937, HEK293)**
   - Developed for immediate thaw-and-use in bioassay
   - Designed for good recovery and robust response upon thawing

2. **Thaw-and-Use format**
   - Cell propagation conditions & defined freezing protocol control assay performance for a consistent bioassay response
   - No pre-culturing prior to assay means less variability introduced
   - Same cells in bioassay, day to day

3. **Minimizes pre-assay planning, time & labor**
   - Ample cell banks provide long-term supply

*means no cell culture required*
Biological performance equivalent to fresh cells

**Fresh cells from continuous culture**

- EC₅₀ = 3.5 ng/ml
- FL = 34-fold

**Frozen, thaw-and-use cells**

- EC₅₀ = 2.9 ng/ml
- FL = 41-fold

**Assay specifics:**
- E:T ratio = 6:1; fresh WIL2-S cells as target cells; Bio-Glo™ reagent;
- 20 hr induction for fresh V variant effector cell assay;
- 6 hr induction for thaw-and-use effector cell assay.
Cells as reagents (Thaw-and-use): Complete QC tests

- STR analysis – cell ID profile (human)
- CO1 analysis (cytochrome oxidase) – test for presence of species (human and other potential contaminants)
- Cell doubling time under propagation conditions
- Mycoplasma (Hoechst)
- Mycoplasma (Direct culture)
- Sterility
- Cell density
- Cell viability after thaw
- Fill volume
- ADCC Reporter Bioassay (EC50 and fold induction)
V variant ADCC Reporter Bioassay
Identification of critical assay parameters for bioassay optimization

Induction hours

E: T ratio with Effector cell number constant

Other parameters tested:
✓ Assay buffer: serum concentration, use of low IgG serum
✓ Cell numbers per well
✓ Pre-plating and incubation time: target cell plating, antibody/target cells incubation
✓ Assay plates: White flat, V- or U-bottom plates
Bioassay development using DoE

**Design of Experiments**
- Allow understanding of the interactions between critical assay factors
- Minimizes amount of work needed to develop robust assays

**Variables:**
- Induction time
- Target/Ab pre-incubation
- Effector cell number
- Target cell number

**Outputs & Results:**
Good response (fold induction) = 19-27
Good (low) L-term* values = 0.1-0.2

* L-term is a measure of assay precision around the EC$_{50}$ determination (log width of the 95% confidence interval around logEC$_{50}$)
Bioassay qualification and performance testing

- **Determination of parallelism** and measurement of **Potency** relative to the reference antibody
- **Linearity & Range:** demonstration across the desired range of potencies
- **Accuracy** of observed versus expected potencies across the desired working range of potencies
- **Precision:** intra-assay precision (repeatability) and intermediate (inter-assay) precision
- **Specificity** to show response is dependent on specific antibody and not other components
- **Stability-indicating** to show the bioassay is capable of detecting loss of structural integrity of an antibody
- **Robustness** to demonstrate that the assay is not affected by small changes in protocol (e.g., induction time)

ICH Guideline Q2 [R1]
Demonstration of measurement of relative potency and repeatability

**Design:**
- Two analysts
- Three days
- Four plates per day
- 100% vs 50%
- 100% vs 75%
- 100% vs 125%
- 100% vs 150%

**Measure relative potency and parallelism**

<table>
<thead>
<tr>
<th>Antibody Sample</th>
<th>100%-1</th>
<th>100%-2</th>
<th>100%-3</th>
<th>100%-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50</td>
<td>4.230e-008</td>
<td>4.693e-008</td>
<td>4.323e-008</td>
<td>4.245e-008</td>
</tr>
<tr>
<td>relative potency</td>
<td>102%</td>
<td>92%</td>
<td>100% control</td>
<td>102%</td>
</tr>
</tbody>
</table>

Assay with thaw-and-use effector and WIL2-S target cells

*Parallelism and relative potency determined with JMP Software*
**Precision, accuracy, linearity and range of ADCC Reporter Bioassay**

<table>
<thead>
<tr>
<th>Antibody Sample</th>
<th>Measured Potency (%)</th>
<th>Mean Potency (%)</th>
<th>SD %</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>day 1</td>
<td>48.5</td>
<td>48.9</td>
<td>3.9</td>
<td>97.7</td>
<td>7.9</td>
</tr>
<tr>
<td>day 2</td>
<td>50%</td>
<td>45.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 3</td>
<td>52.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 1</td>
<td>63.1</td>
<td>66.4</td>
<td>5.9</td>
<td>88.5</td>
<td>8.9</td>
</tr>
<tr>
<td>day 2</td>
<td>75%</td>
<td>62.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 3</td>
<td>73.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 2</td>
<td>125%</td>
<td>136.3</td>
<td>12.3</td>
<td>98.4</td>
<td>10.0</td>
</tr>
<tr>
<td>day 3</td>
<td>120.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 1</td>
<td>148.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 2</td>
<td>150%</td>
<td>150.4</td>
<td>3.6</td>
<td>98.4</td>
<td>2.4</td>
</tr>
<tr>
<td>day 3</td>
<td>143.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Intermediate Precision**
(Av of RSD(%))
7.3%

**Accuracy**
(Av of % Recovery)
95.8%

**Linearity**
- $y = 1.016x - 0.052$
- $R^2 = 0.995$
- in the range of 50-150% relative potency

Assay with thaw-and-use effector and WIL2-S target cells and anti-CD20 B1 antibody

![Graph showing observed potency vs. expected potency with the equation $y = 1.0161x - 0.0516$ and $R^2 = 0.9949$.]
Two independent assay characterizations were performed using ADCC reporter bioassay effector cells and:

- CD20+ WIL2-S frozen, thaw-and-use target cells and anti-CD20 B1 antibody
- CD20+ Raji frozen, thaw-and-use target cells and anti-CD20 B1 antibody

Performance characteristics support suitability of ADCC Reporter Bioassay for validation
ADCC Reporter Bioassay is specific

Target cells, effector cells and specific antibody

No Target cells

No Effector cells or no FcγRIIIa

No antibody or non-specific antibody

Assay signal is dependent on:

- Presence of Target cells
- Presence of FcγRIIIa receptor
- Appropriate specific antibody

Graph showing bioluminescence (RLU) vs. log₁₀ [Rituximab], g/ml.
ADCC Reporter Bioassay is stability-indicating

Activity of heat-stressed Rituximab

<table>
<thead>
<tr>
<th>Temperature</th>
<th>EC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C</td>
<td>2.133e-009</td>
</tr>
<tr>
<td>65°C, 1 day</td>
<td>2.148e-008</td>
</tr>
<tr>
<td>65°C, 3 days</td>
<td>1.411e-007</td>
</tr>
<tr>
<td>65°C, 5 days</td>
<td>9.528e-007</td>
</tr>
</tbody>
</table>

Activity of heat-stressed Trastuzumab

<table>
<thead>
<tr>
<th>Temperature</th>
<th>EC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C</td>
<td>1.284e-008</td>
</tr>
<tr>
<td>65°C, 1 day</td>
<td>1.902e-008</td>
</tr>
<tr>
<td>65°C, 3 days</td>
<td>2.031e-008</td>
</tr>
<tr>
<td>65°C, 5 days</td>
<td>3.139e-008</td>
</tr>
</tbody>
</table>

Bioluminescence (RLU) vs. Log$_{10}$ [Rituximab], g/ml

Bioluminescence (KLU) vs. Log$_{10}$ [Trastuzumab], g/ml
ADCC Reporter Bioassay is robust

### Time of induction

<table>
<thead>
<tr>
<th>Run</th>
<th>Induction time</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.0hr</td>
<td>3.15x10&lt;sup&gt;-8&lt;/sup&gt; g/ml</td>
</tr>
<tr>
<td>2</td>
<td>5.5hr</td>
<td>3.83x10&lt;sup&gt;-8&lt;/sup&gt; g/ml</td>
</tr>
</tbody>
</table>

### E:T ratio and cell # per well

<table>
<thead>
<tr>
<th>Run</th>
<th>E:T ratio</th>
<th>E cell #</th>
<th>T cell #</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.5:1</td>
<td>75k</td>
<td>10k</td>
<td>3.09x10&lt;sup&gt;-8&lt;/sup&gt; g/ml</td>
</tr>
<tr>
<td>2</td>
<td>6:1</td>
<td>90k</td>
<td>15k</td>
<td>3.83x10&lt;sup&gt;-8&lt;/sup&gt; g/ml</td>
</tr>
</tbody>
</table>
A series of trastuzumab glycosylation blend mixtures were prepared by blending PNGase F – treated (deglycosylated) and untreated trastuzumab (N-glycosylated).

The antibody samples were tested with SK-BR-3 target cells, using untreated antibody as the 100% activity reference.
ADCC Reporter Bioassay can measure potency of different antibody drugs

Suspension or adherent target cells can be used. Optimize E:T ratio and dose-range for different Ab and target cells

**Rituximab**

- **CD20**⁺ WIL2-S B cell line (suspension) as target

**Trastuzumab**

- Her2⁺ SK-BR-3 breast cancer cell line (adherent) as target
The ADCC Reporter Bioassay can be used in 96-well and 384-well plate format

A. WIL2-S target cells

B. Raji target cells

<table>
<thead>
<tr>
<th>Assay volume per well</th>
<th>Target cells</th>
<th>Antibody</th>
<th>Effector cells</th>
<th>Bio-Glo</th>
</tr>
</thead>
<tbody>
<tr>
<td>96-well plate</td>
<td>25 µl</td>
<td>25 µl</td>
<td>25 µl</td>
<td>75 µl</td>
</tr>
<tr>
<td>384-well plate</td>
<td>5 µl</td>
<td>5 µl</td>
<td>5 µl</td>
<td>15 µl</td>
</tr>
</tbody>
</table>
F variant ADCC Reporter Bioassay
Why is an F Variant assay needed?

- The human FcγRIIIa receptor has a polymorphism at amino acid 158 resulting in either an F or V amino acid
- In the general population, 85% are F/V and F/F, while only 15% are V/V
- The V variant has a higher affinity for antibody Fc regions than the F variant, resulting in better ADCC activity
- In clinical trials with antibodies with ADCC activity, better responsiveness by V variant patients has been observed
- Developing Ab that are more effective at mediating ADCC through the F variant receptor should help patients that lack the V variant
- An F variant ADCC Reporter Bioassay can be used to evaluated engineered Abs for improvements in F variant ADCC activity
**Bioluminescent ADCC Reporter Bioassay, F variant**

**Protocol**

1. Incubate control, reference or test antibody with target cells.

2. Add engineered effector cells expressing:
   - FcγRIIIa(F158)
   - NFAT-RE luc2 luciferase upon ADCC pathway activation

3. Induce response in as little as 6 hours.

4. Add luciferase detection reagent and measure luminescence immediately in e.g. a Glomax® Discover.
Assay development critical parameter: Effector to Target Ratio

**Suspension target cells**

- E : T ratio
  - 30 : 1
  - 15 : 1
  - 10 : 1
  - 6 : 1
  - 4 : 1
  - 2 : 1

**Adherent target cells**

- E : T ratio
  - 50 : 1
  - 30 : 1
  - 15 : 1
  - 10 : 1
  - 6 : 1
  - 4 : 1

Fold of induction vs. Log$_{10}$ [rituximab], g/ml

Fold of induction vs. Log$_{10}$ [trastuzumab], g/ml

Promega Corporation
Assay development critical parameter: Induction Time

Fresh-from-culture effector cells

Thaw-and-use effector cells
Bioassay Characteristics - ICH Guideline Q2 [R1]

Qualification of Analytical Procedures

- Accuracy
- Precision:
  - Repeatability (intra-assay precision)
  - Intermediate precision (day to day, analyst-to-analyst)
  - Reproducibility (lab to lab)
- Specificity
- Linearity
- Range
- Robustness

Design:
- Two analysts
- Three days
- Four plates per day
  - 100% vs 50%
  - 100% vs 75%
  - 100% vs 125%
  - 100% vs 200%

Glomax® Discover provides technical elements for laboratories to comply with 21CFR part 11

Assay validation by the customer
Linearity and range with CD20 Ab

WIL2S

Expected Relative Potency, %

Measured Relative Potency, %

R square 0.9965

$Y = 1.071X - 12.04$

Raji

Expected Relative Potency, %

Measured Relative Potency, %

R square 0.9925

$Y = 1.017X + 0.5956$
## Accuracy, precision, linearity and range

<table>
<thead>
<tr>
<th>Accuracy</th>
<th>% Expected Relative Potency</th>
<th>WIL2-S Target Cells</th>
<th>Raji Target Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Recovery</td>
<td>% Recovery</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>87.7</td>
<td>101.3</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>90.4</td>
<td>99.2</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>95.1</td>
<td>107.8</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>103.1</td>
<td>99.3</td>
</tr>
<tr>
<td>Repeatability (%CV)</td>
<td>100% (Reference)</td>
<td>3.4</td>
<td>5.7</td>
</tr>
<tr>
<td>Intermediate Precision (%CV)</td>
<td></td>
<td>6.2</td>
<td>7.7</td>
</tr>
<tr>
<td>Linearity ($r^2$)</td>
<td></td>
<td>0.997</td>
<td>0.993</td>
</tr>
<tr>
<td>Linearity ($y=mx+b$)</td>
<td></td>
<td>$y=1.071x - 12.04$</td>
<td>$y = 1.017x + 0.596$</td>
</tr>
</tbody>
</table>
Specificity

Assay signal is dependent on:
- Target cells expressing relevant antigen
- Specific Ab
- Effector cells expressing FcγRIIIa receptor and NFAT-RE/luc2

No target cells

No Ab or wrong Ab

No FcγRIIIa
Specificity – Antibody isotype

Order of response: hu IgG1, hu IgG3, mouse IgG2a >> hu IgG2, hu IgG4, mouse IgG1
Stability-indicating – heat stressed samples

Potency and Efficacy

EC$_{50}$ increases (right shift)

Fold induction decreases
Measure potencies of different mAb biologic drugs

Suspension or adherent target cells can be used.
Optimize E:T ratio and dose-range for different Abs and target cells

Three best-selling mAb biologic drugs that possess ADCC as a main MOA

**Rituximab**, anti-CD20, chimeric IgG1.
**Trastuzumab**, anti-HER2, humanized IgG1.
**Cetuximab**, anti-EGFR, chimeric IgG1.
V and F variant ADCC Reporter Bioassays for better characterization of Ab during drug development

Results align with expected differences in biological activity via V and F receptors

### Trastuzumab

**HER2+ SKBR3 target cells**

<table>
<thead>
<tr>
<th>Effector cell</th>
<th>EC50 (g/ml)</th>
<th>Fold Induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>V</td>
<td>4.1e-008</td>
<td>107.4</td>
</tr>
<tr>
<td>F</td>
<td>1.3e-007</td>
<td>58.1</td>
</tr>
</tbody>
</table>

### Cetuximab

**EGFR+ A431 target cells**

<table>
<thead>
<tr>
<th>Effector cell</th>
<th>EC50 (g/ml)</th>
<th>Fold Induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>V</td>
<td>1.2e-008</td>
<td>24.4</td>
</tr>
<tr>
<td>F</td>
<td>2.7e-008</td>
<td>10.1</td>
</tr>
</tbody>
</table>
Suitable instrumentation for ADCC Reporter Bioassays across Ab drug development workflow

GloMax® Discover

Superior Performance
• Broader dynamic range to measure extreme ranges
• Better sensitivity for low-level samples
• Lower well-to-well cross talk for confidence

Flexible
• Modular design so you choose the capability you need
• Automation-friendly to support your workflow
• 6 to 384-well plate formats

Integrated with Promega Assays
• Optimized and Pre-loaded Promega Assays
• Spend less time optimizing your experiment

21CFR part 11
• GloMax® Discover provides required technical elements of a part 11 compliant system to be used with the appropriate laboratory workflow
Summary of ADCC Reporter Bioassays

**ADCC Reporter Bioassays**

- Alternative cell-based bioassays to quantify potency of antibodies reflective of ADCC mechanism of action
- Based on target cell-bound antibody activation of FcγIIIa receptor signaling pathways in the effector cell
- Bioluminescent NFAT-RE-luciferase reporter bioassay with readout from engineered effector cells

- The bioassays are simple, robust, specific, precise and accurate
- Suitable for stability studies, use in biocomparability assays, antibody engineering to improve efficacy, reflective of *in vivo* MOA
- Effector cells are well-controlled reagents in thaw-and-use format
References:


   i. Measuring the ADCC Reporter Bioassay Complete Kit (WIL2-S) Signal on the GloMax Discover System
   ii. Validation and Comparability Studies with ADCC Reporter Bioassays
      Ulrike Herbrand, Sabrina Schöbel, Sascha Kramm, Simone Scotti, Charles River Biopharmaceutical Services GmbH, Erkrath, Germany
THANK YOU!

Questions?