ADCC Reporter Bioassay:
A Novel, Bioluminescent Cell-Based Assay for Quantifying Fc Effector Function of Antibodies

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Outline

- Introduction to ADCC – *Problem with classic ADCC assays*
- Principle of the ADCC Reporter Bioassay
- “Cells as reagents” – *Frozen, thaw-and-use format*
- Performance – *Specific, Linear, Precise, Accurate, Reproducible, as well as Potency & Stability Indicating*
- Testing Ab variants – *Glycosylation & Fucosylation*
- Commercial formats - *Kits & Cell Propagation Model*
Complicated Biology — Simple Assay
Global Biologics Market & Forecast

- Monoclonal antibodies (mAb) ~ 1/3rd of total biologics market
- mAb = $48 billion in 2010, expected $86 billion by 2015 (CAGR) of 12.4%

Source: BCC Research
Biological Product – Monoclonal Antibody (mAb)

- 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
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 novel ADCC Reporter Bioassay fulfills each of these elements
Mechanism of Action (MOA) for mAb
Introduction to ADCC
What is ADCC?

**Antibody-dependent cell-mediated cytotoxicity (ADCC)** is the main MOA of antibodies through which virus-infected or other diseased cells are targeted for destruction by components of the cell-mediated immune system, such as NK cells.

Antibodies bind antigens on the surface of target cells → NK cell CD16 Fc receptors recognise cell-bound antibodies → Cross-linking of CD16 triggers degranulation into a lytic synapse → Tumour cells die by apoptosis

Classic ADCC Assays

Effector cells
- PBMCs (peripheral blood mononuclear cells)
- NK from PBMCs
- NK cell lines

Target cells
- Load with chromium-51 or Eu
- Monitor cell lysis (LDH, Calcein AM, GAPDH, CytoTox-Glo™)

CytoTox-Glo™ Cytotoxicity Assay
The Problem with Current ADCC assays
Case Study – ADCC Challenge

- A company acquired a late-stage mAb drug and needed to switch the manufacturing cell line and process to fit into their standard process.

- FDA requested that an assay examining the mAb mechanism of action (ADCC) be used to demonstrate similarity between process change.

- Classic ADCC assays had poor reproducibility, high variability, and were not suitable for use.

- The company developed a reporter assay with low variability, high reproducibility, and used it to successfully demonstrate similarity and make the manufacturing cell line change.

mAbs 4:3, 1-9; May 2012
Solution: A Better ADCC Bioassay
**Classic ADCC assay vs ADCC Reporter Bioassay**

**Classic ADCC assay**

- **Signal is from target cell**
  - High variability of assay - mainly due to primary NK cells
  - Spontaneous lysis of target & effector cells results in high background

**Reporter-based ADCC bioassay**

- **Signal is from effector cell**
  - Reduced variability by replacing NK cells with genetically engineered stable cell line
    - *FcγRIIIa (V158)*
    - *NFAT-RE luc2*
  - Improved bioassay performance with robust reagents and assay design
Principle of the ADCC Reporter Bioassay
**Scientific Basis of ADCC Reporter Bioassay**

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

ADCC Reporter Bioassay - Development

**Low Variability NFAT-RE luciferase bioassay**

1. Effector cells are engineered to express FcγRIIIa (V158) and NFAT-RE luc2 luciferase
2. ‘Cells as reagents’ (thaw-and-use)
3. Homogeneous assay format – simple ‘add-mix-read’ bioluminescent assay
4. Optimized and robust assay reagents and protocol
5. Performance characteristics that meet needs of stability testing, lot release and Ab characterization

Developed & tested using:
- CD20 and Her2 Ab drugs
- CD20+ and Her2+ target cells
- Frozen, thaw-and-use, or continuously cultured cells
- Extensive ‘alpha’ evaluations: - tested in multiple global biopharma & biotechs - tested in multiple systems
Bioassay Characteristics - ICH Guideline Q2 [R1]

Validation 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 150%

Relative potency

Repeatability

Linearity

Y = 1.026X - 5.126
R² = 0.995
Simple Protocol
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)
   - 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 – Initial Results

**Assay protocol:**

- CD20+ WIL2-S cells
  + Rituximab dilution series
  + Engineered Jurkat effectors
  ↓ Induction (22 hours)
  ↓ Quantification of luciferase activity

**Specifics**

- **E:T ratio = 6:1**
  (150k effector cells:25k WIL2-S target cells, per well)

**Bioluminescence (RLU)**

- EC$_{50}$ = $4.8 \times 10^{-9}$ g/ml
- FI = 30

**Log$_{10}$ [Rituximab], g/ml**

-11 -10 -9 -8 -7 -6

0 1$\times$10$^0$ 2$\times$10$^0$ 3$\times$10$^0$ 4$\times$10$^0$ 5$\times$10$^0$ 6$\times$10$^0$ 7$\times$10$^0$
Cell Selection and Frozen, Thaw-and-Use Format
Engineered Effector Cell Clone Selection

Clone selection based on maximizing RLU, fold induction, and passage stability

**Bioluminescence**

*Jurkat passage number*

- P8
- P13
- P19
- P25

**Fold Induction**

*Jurkat passage number*

- P8
- p13
- p20
- p25

- E:T ratio = 7.5:1
- 6hr induction
- Bio-Glo™ Luciferase Assay System
Cells as Reagents

Frozen, Thaw-and-Use Cells

1. Human cell lines
   - Developed as Thaw-and-Use for immediate use in bioassay
   - Designed to give 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
   - Indefinite storage
   - Identical cells in bioassay, day-to-day

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

→ No cell culture required with cells in frozen, thaw-and-use format
Complete QC on Cells

Production cell batches are rigorously tested:

- 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 and direct culture)
- Sterility
- Cell density
- Cell viability after thaw
- Fill volume
- ADCC Reporter Bioassay ($EC_{50}$ and fold induction)
Optimization Studies
Critical Assay Parameters

Induction time

E:T ratio with constant Effector cell number

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
Selection of Control Antibody

Requirements:

- $EC_{50}$ close to the range of biologic Ab drugs
- Good fold induction
- Good stability

<table>
<thead>
<tr>
<th>Anti-CD20</th>
<th>Rituximab</th>
<th>Control Ab (A)</th>
<th>Control Ab (B)</th>
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<tr>
<td>$EC_{50}$ (g/ml)</td>
<td>$1.7 \times 10^{-9}$</td>
<td>$8.9 \times 10^{-9}$</td>
<td>$47.3 \times 10^{-9}$</td>
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<tr>
<td>Fold Induction</td>
<td>62</td>
<td>38</td>
<td>8</td>
</tr>
</tbody>
</table>

Suitable control
Use of Different Target Cells

Suspension or adherent target cells can be used

Rituximab (anti-CD20)
CD20⁺ B cell lines (suspension) as target cells

Trastuzumab (anti-Her-2)
Her2⁺ breast cancer cell lines (adherent) as target cells
Bioassay Development: Optimization Using DOE

Variables:
1. Induction time
2. Target/Ab pre-incubation
3. Effector cell number
4. Target cell number

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

* a measure of assay precision around the EC50 determination (log width of the 95% confidence interval around logEC50)
Performance
Qualification Studies

- **Parallelism** and measurement of *potency* relative to the reference antibody
- **Linearity & accuracy** of observed versus expected potencies across the desired working range of potencies
- **Precision**
  - intra-assay
  - intermediate (inter-assay) precision
- **Specificity** to show response is dependent on specific antibody and the presence of target cells and FcγRIIIa on effector cells, and not other components
- **Stability-indicating** to show the bioassay is capable of detecting loss of structural integrity of an antibody

*These qualification studies are critical to demonstrate a useful and effective ADCC bioassay*
ADCC Reporter Bioassay is Specific

Target cells, effector cells and specific antibody

- **Wil2-S, Jurkat/NFAT-luc+FcyRIIIa, Rituximab**
- **NO Wil2-S, Jurkat/NFAT-luc+FcyRIIIa, Rituximab**
- **Wil2-S, Jurkat/NFAT-luc (NO FcyRIIIa), Rituximab**
- **Wil2-S, NO Jurkat/NFAT-luc+FcyRIIIa, Rituximab**
- **Wil2-S, Jurkat/NFAT-luc+FcyRIIIa, NO Rituximab**
- **Wil2-S, Jurkat/NFAT-luc+FcyRIIIa, Trastuzumab**

**Assay signal is dependent on:**

- Presence of Target cells +
- Presence of FcyRIIIa receptor +
- Appropriate specific antibody
ADCC Reporter Bioassay is Robust

**Time of induction**

<table>
<thead>
<tr>
<th>Run</th>
<th>Induction time</th>
<th>EC$_{50}$</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>6.0 hr</td>
<td>3.15x10$^{-8}$ g/ml</td>
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<tr>
<td>2</td>
<td>5.5 hr</td>
<td>3.83x10$^{-8}$ g/ml</td>
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**E:T ratio and cell # per well**

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<th>Run</th>
<th>E:T ratio</th>
<th>E cell #</th>
<th>T cell #</th>
<th>EC$_{50}$</th>
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<tr>
<td>1</td>
<td>7.5:1</td>
<td>75k</td>
<td>10k</td>
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<td>2</td>
<td>6:1</td>
<td>90k</td>
<td>15k</td>
<td>3.83x10$^{-8}$ g/ml</td>
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Miniaturization to 384-well Plates

WIL2-S target cells

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>
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<tbody>
<tr>
<td>96-well plate</td>
<td>25μl</td>
<td>25μl</td>
<td>25μl</td>
<td>75μl</td>
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<tr>
<td>384-well plate</td>
<td>5μl</td>
<td>5μl</td>
<td>5μl</td>
<td>15μl</td>
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Assay Qualification Results

Bioassay uses frozen-thaw-and-use cells for both effector cells and WIL2-S target cells

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

Representative plate layout

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<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tr>
<td>B</td>
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<td>dilu8</td>
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<td>dilu5</td>
<td>dilu4</td>
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<td>dilu2</td>
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<td>dilu8</td>
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</table>

Linearity

Y = 1.026X - 5.126
R² = 0.995

Repeatability

<table>
<thead>
<tr>
<th>Antibody Sample</th>
<th>Measured Potency (%)</th>
<th>Mean Potency (%)</th>
<th>SD %</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
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<tr>
<td>day 1</td>
<td>50%</td>
<td>48.5</td>
<td>4.9</td>
<td>97.7</td>
<td>7.9</td>
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<td>day 2</td>
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<td>45.2</td>
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<tr>
<td>day 3</td>
<td>150%</td>
<td>143.6</td>
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</table>

Accuracy = average of Recovery (%) = 95.8%

Precision = average of RSD (%) = 7.3%

Precision = average of RSD (%) = 7.3%

Accuracy = average of Recovery (%) = 95.8%

Good repeatability, accuracy, precision and linearity were obtained
## Assay Qualification Results with Raji Target Cells

### Analyst 1

<table>
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<tr>
<th>Day</th>
<th>Antibody Sample</th>
<th>Measured Potency (%)</th>
<th>Mean Potency (%)</th>
<th>SD (%)</th>
<th>Recovery (%)</th>
<th>CV (%)</th>
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### Analyst 2

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</table>

### Linearity

- **Precision:** 2.95%
- **Accuracy:** (recovery average): 98.5%
- **Linearity:** 
  \[ Y = 0.922x + 5.0 \]

- **Precision:** 8.47%
- **Accuracy (recovery average):** 96.4%
- **Linearity:** 
  \[ Y = 1.22x - 21.3 \]
Potency Determinations Using Quantitative Bioassays

A test sample of unknown biological activity is compared with a reference sample of established biological activity in a dose-response study in the test system. The bioassay establishes potency relative to a reference standard.

- Curve fitting and statistical methods determine parallelism.
- Parallel curves signify equivalent means of effecting biological activity.
- Relative potency is quantified through shift of response along the x-axis.
- Slope difference suggests non-equivalent means of effecting response if it falls outside of acceptance criteria; a manufactured lot would fail if this were so.
Measurement of Relative Potency & Parallelism

Parallelism and relative potency determined with JMP Software
Stability Indicating
Stability Indicating for Fc Effector Function

Rituximab

EC50 = 5.77ng/ml

Activity of heat-treated antibody drugs

Tositumomab

Trastuzumab

EC50

<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>
Antibody Variants
Analysis of Mixed Glycosylation mAbs

Target cells: SKBR3; Unt = 100% glycosylated
**ADCC Reporter Bioassay Activity Correlates with Amount of Antibody N-glycosylation**

**Rituximab and Trastuzumab:**
Linear correlation obtained between percentage of N-glycosylated antibody in blended antibody samples and relative luciferase reporter activity in ADCC reporter bioassay.

- **Rituximab**
  \[ y = 0.0127x - 0.0314 \]
  \[ R^2 = 0.966 \]

- **Trastuzumab**
  \[ y = 0.0125x - 0.0095 \]
  \[ R^2 = 0.9926 \]

*Small differences in Fc effector activity in ADCC pathway activation are easily distinguished in the ADCC reporter bioassay.*
ADCC Reporter Activity Correlates with Amount of Antibody Afucosylation

Linear correlation shown between percentage of afucosylated antibody in blended antibody samples and relative luciferase reporter activity in ADCC reporter assay
Bioassay Characteristics - ICH Guideline Q2 [R1]

Validation 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 150%

Relative potency

Repeatability

Linearity

Log \[\text{[control antibody]}, \text{g/ml}\]

Fold of Induction

Y=1.026X-5.126
R²=0.995
External Evaluations
Updates from Clients...

- Approved manufacturing cell line switch by a pharmaceutical company
- Submitted in an IND filing
- Being developed for lot-release testing
- Charles River Laboratories and Catalent are providing ADCC Reporter Bioassay services
- Adopted by major pharmaceutical companies
Kit Formats
To be more flexible to research needs, we offer multiple kit formats:

1. **Core Kits:**
   - 1X kit – Cat.# G7017
   - 5X kit – Cat.# G7018

2. **Complete Kits:**
   Available as Custom material

3. **Target Kits:**
   To be available later in year

**Note:** the ADCC Bioassay Effector Cells are available for propagation and banking under a unique purchase agreement
Summary of the ADCC Reporter Bioassay

**Features**
- Low variability
- Engineered effector cells to replace primary NK cells (Jurkat FcγRIIIa/NFAT-RE luc2)
- “Cells as reagents”, frozen, thaw-and-use format – consistency & convenience
- Simple & robust protocol & reagents
- Broad applicability in use with multiple target cells – suspension or adherent

**Benefits**
- Demonstrates precision, accuracy, linearity, robustness
- Can quantify potency and stability of therapeutic Ab drugs
- Can differentiate biological activity of Fc effector function in ADCC MOA resulting from small changes in Ab glycosylation
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