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ADCC Reporter Bioassay:

A Novel, Bioluminescent Cell-Based Assay for Quantifying Fc Effector Function of Antibodies

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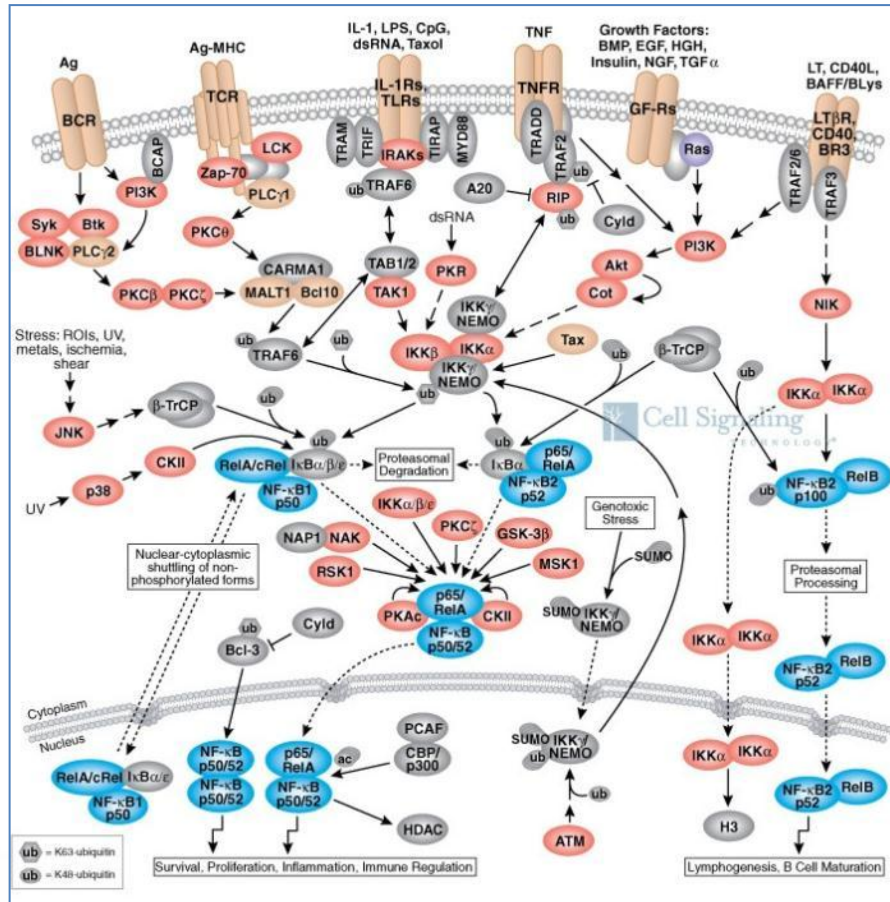
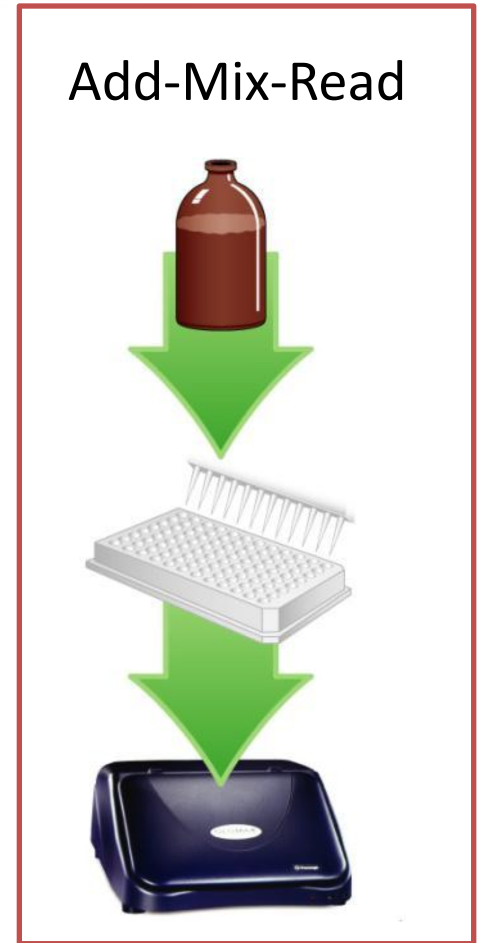
October 2012

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

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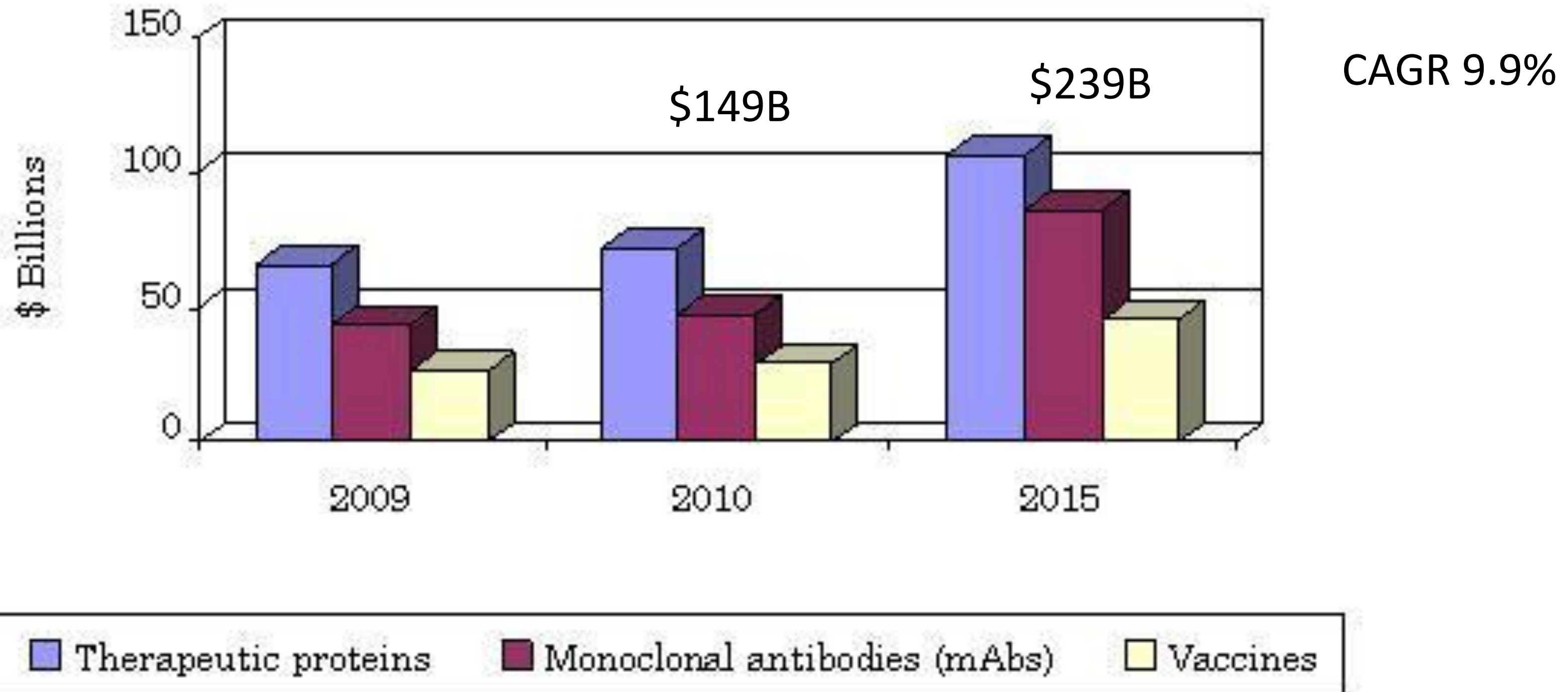

- Monoclonal antibodies (mAb) ~ 1/3rd of total biologics market
- mAb = $48 billion in 2010, expected $86 billion by 2015 (CAGR) of 12.4%

Source: BCC Research

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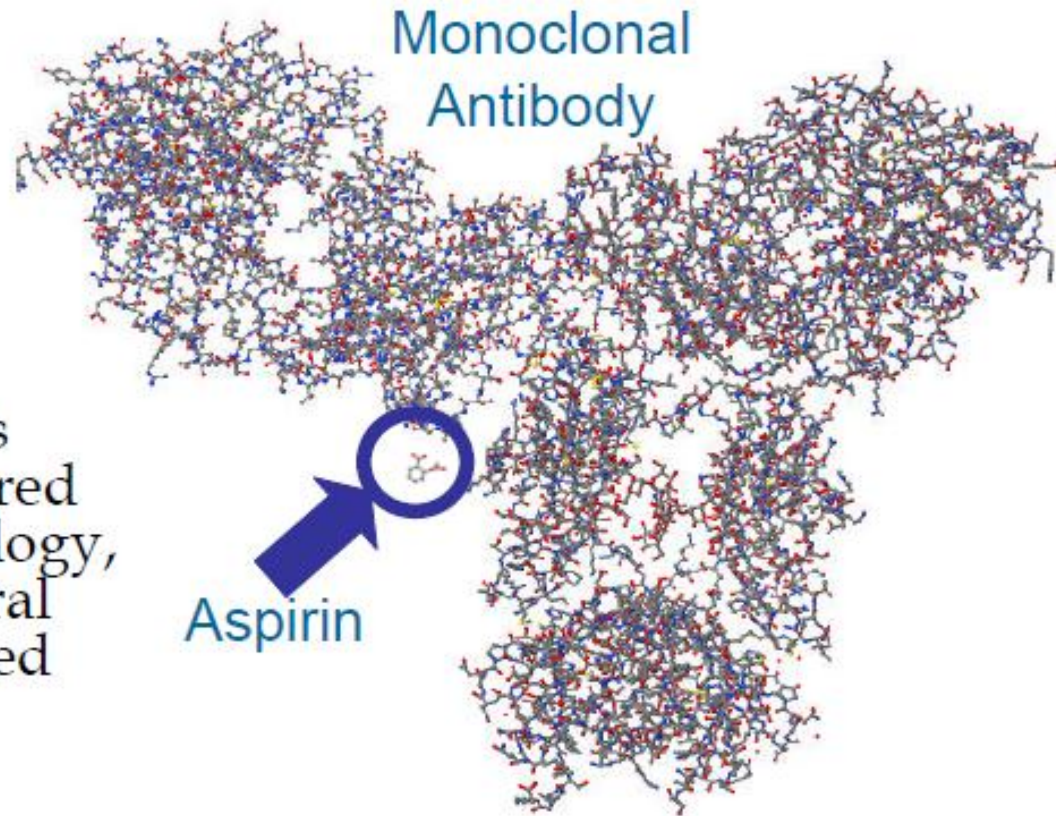
Biological Product – Monoclonal Antibody (mAb)

- 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

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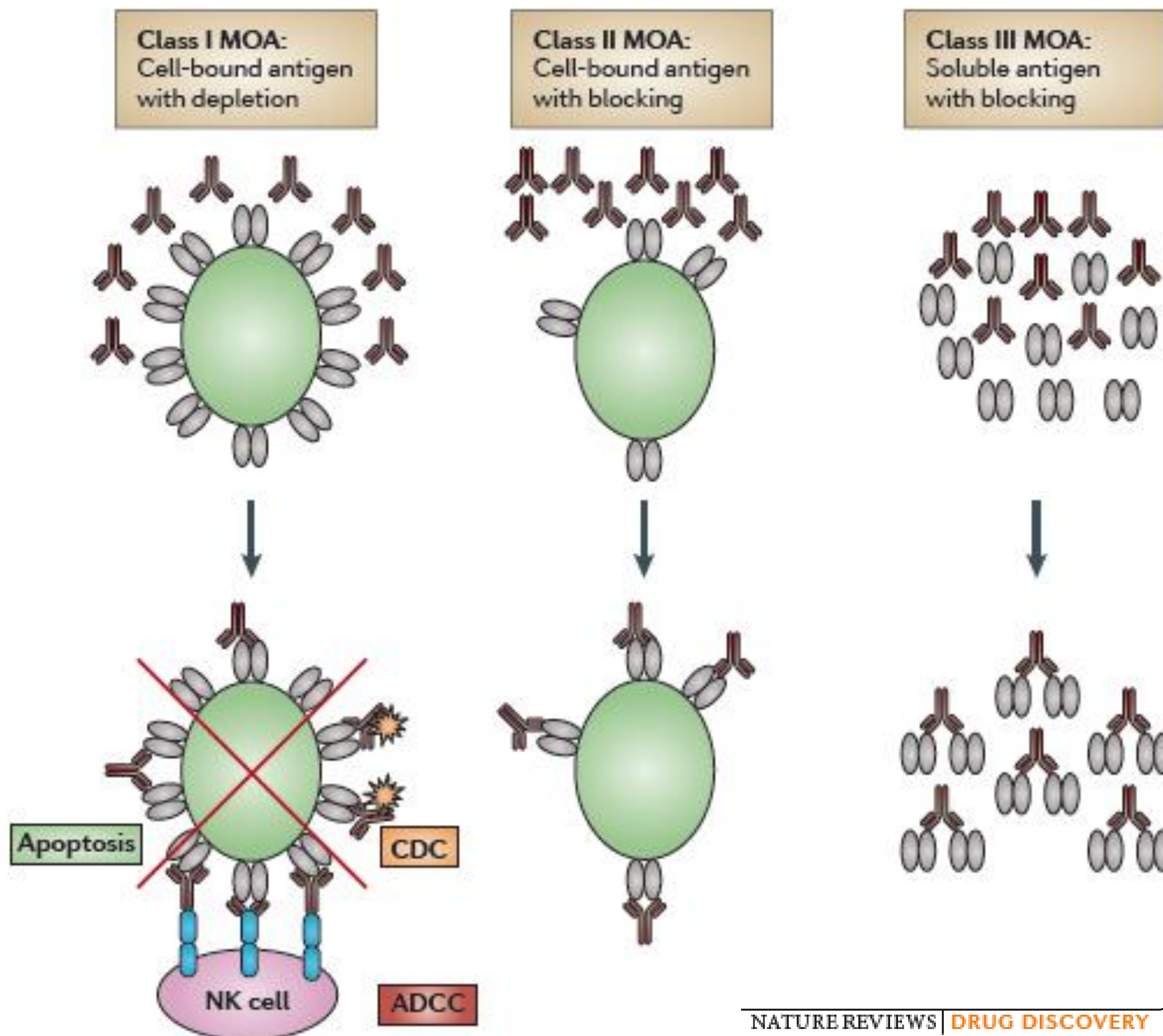
Mechanism of Action (MOA) for mAb



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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

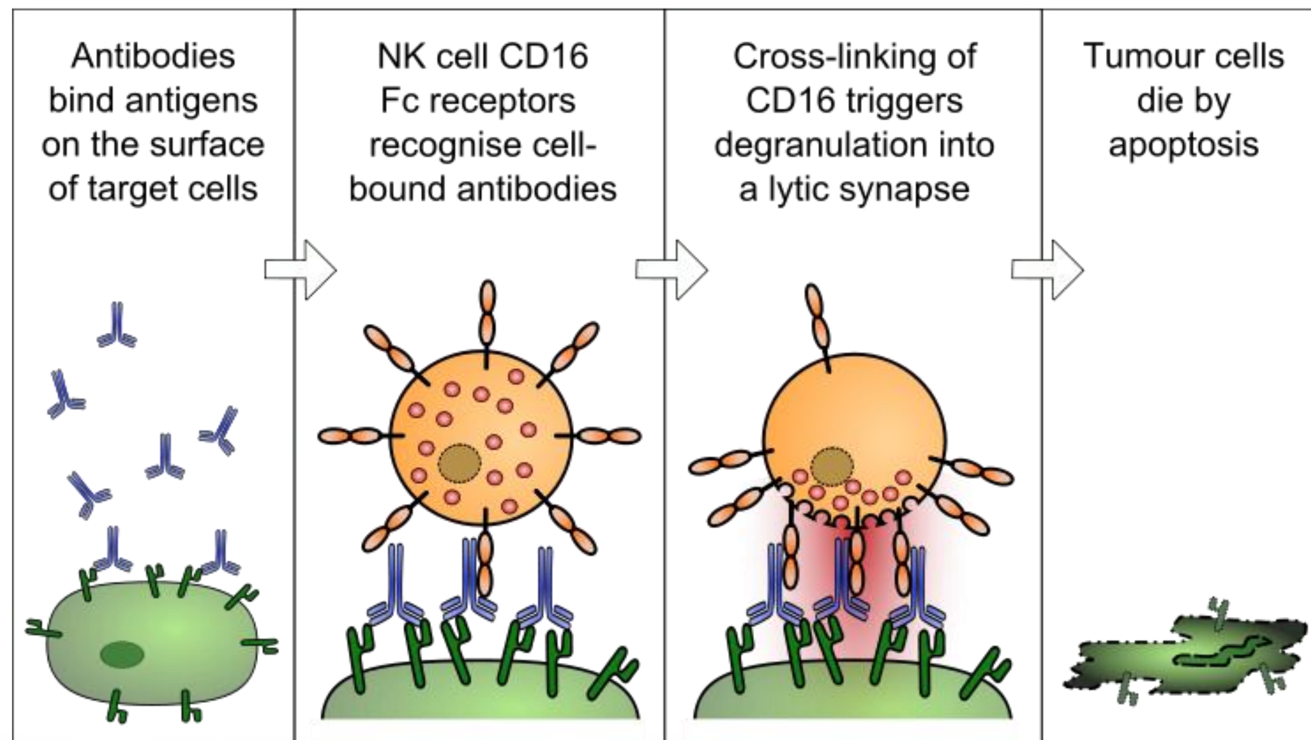


Image source: Wikipedia

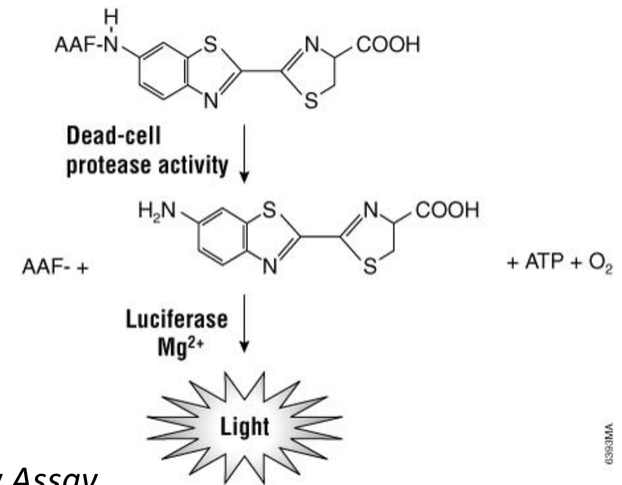
Classic ADCC Assays

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

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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

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Solution: A Better ADCC Bioassay

Classic ADCC assay vs ADCC Reporter Bioassay

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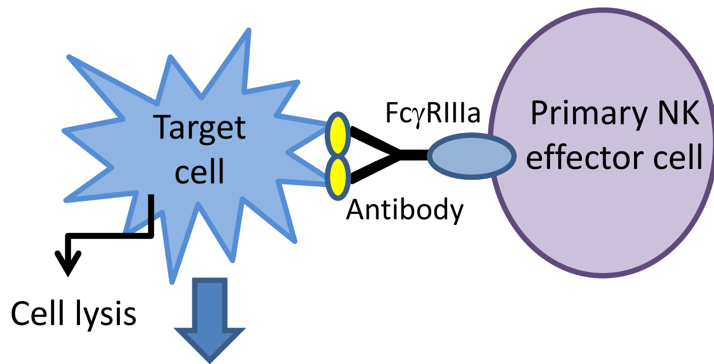
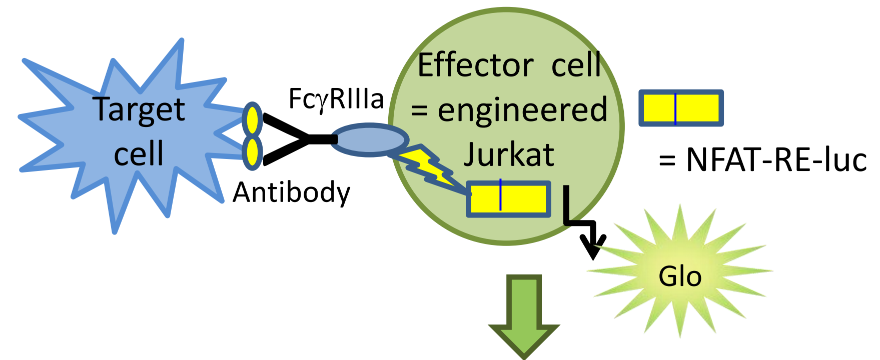
Classic ADCC assay



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

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Principle of the ADCC Reporter Bioassay

Scientific Basis of ADCC Reporter Bioassay

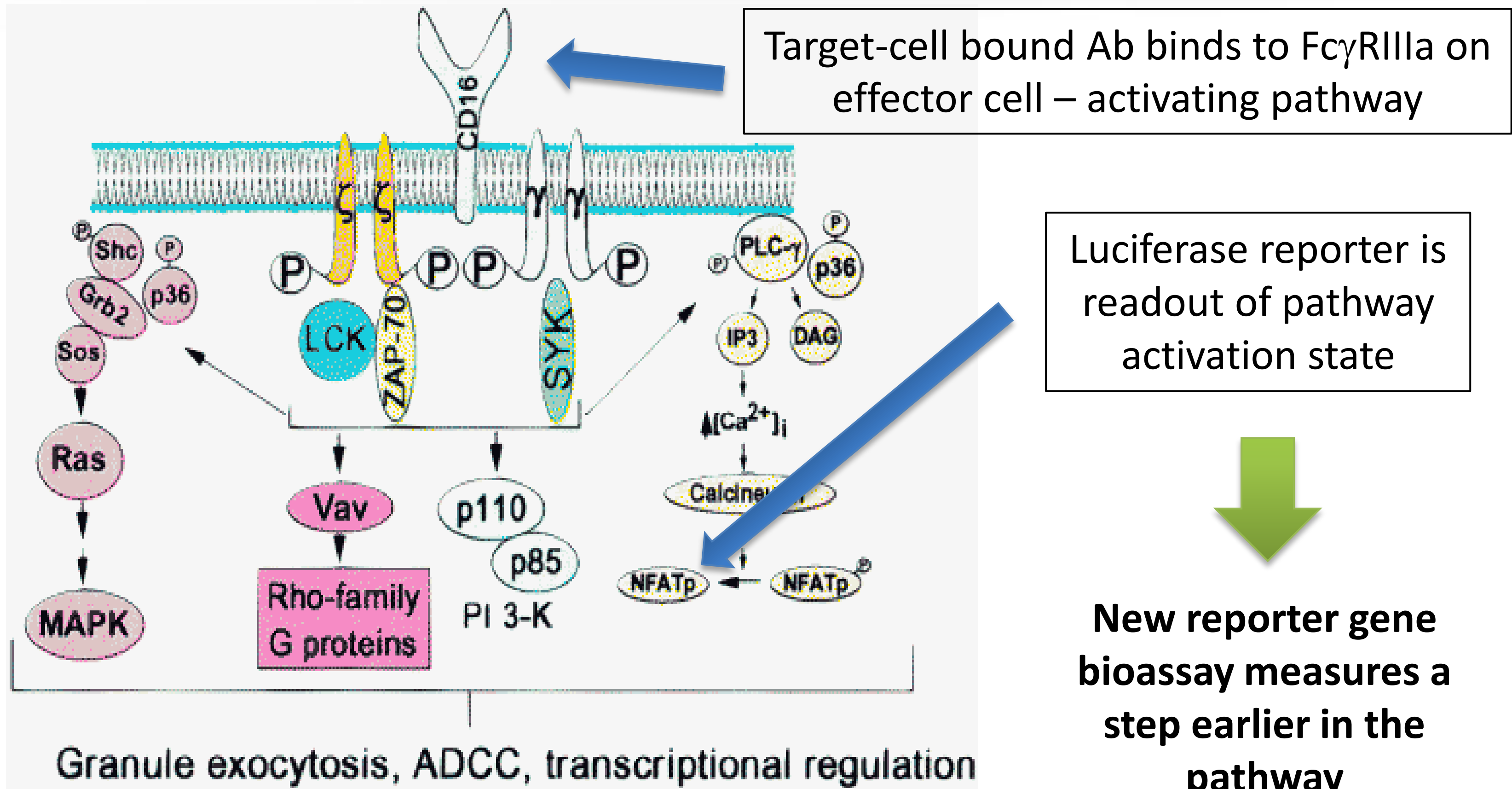
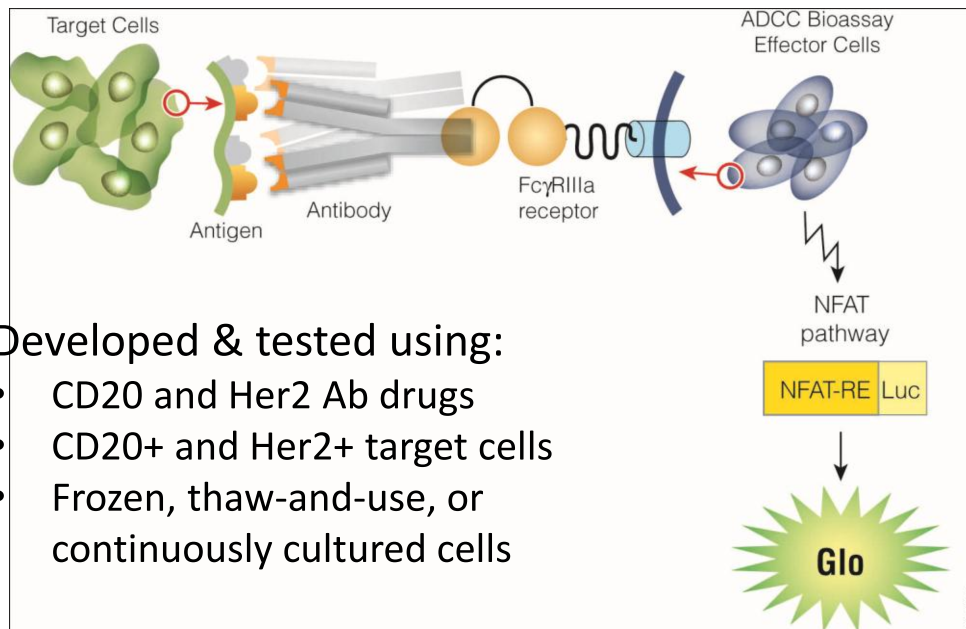


Image source: Leibson-PJ, *Immunity* 1997

ADCC Reporter Bioassay - Development

Low Variability NFAT-RE luciferase bioassay



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

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

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Bioassay Characteristics - ICH Guideline Q2 [R1]

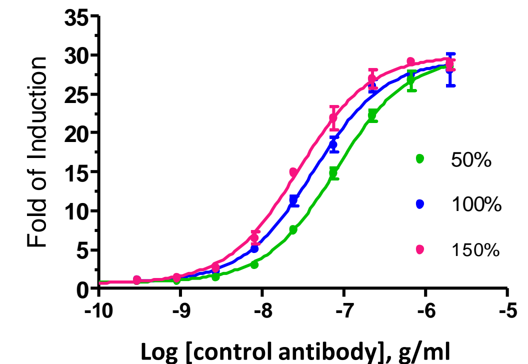
Validation of Analytical Procedures

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

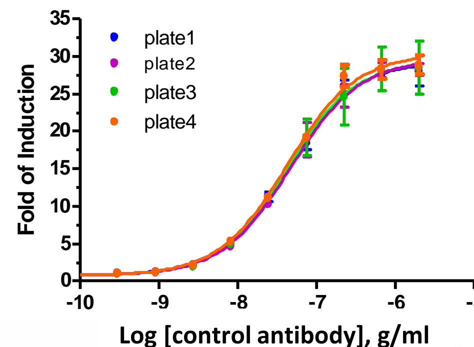
Design:
- Two analysts
- Three days
- Four plates per day

✓100% vs 50%
✓100% vs 75%
✓100% vs 125%
✓100% vs 150%

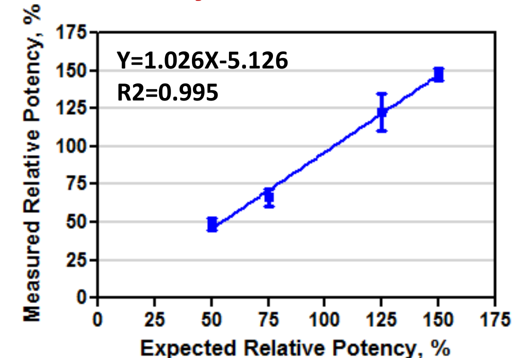
Relative potency



Repeatability



Linearity



Simple Protocol

ADCC Reporter Bioassay Protocol

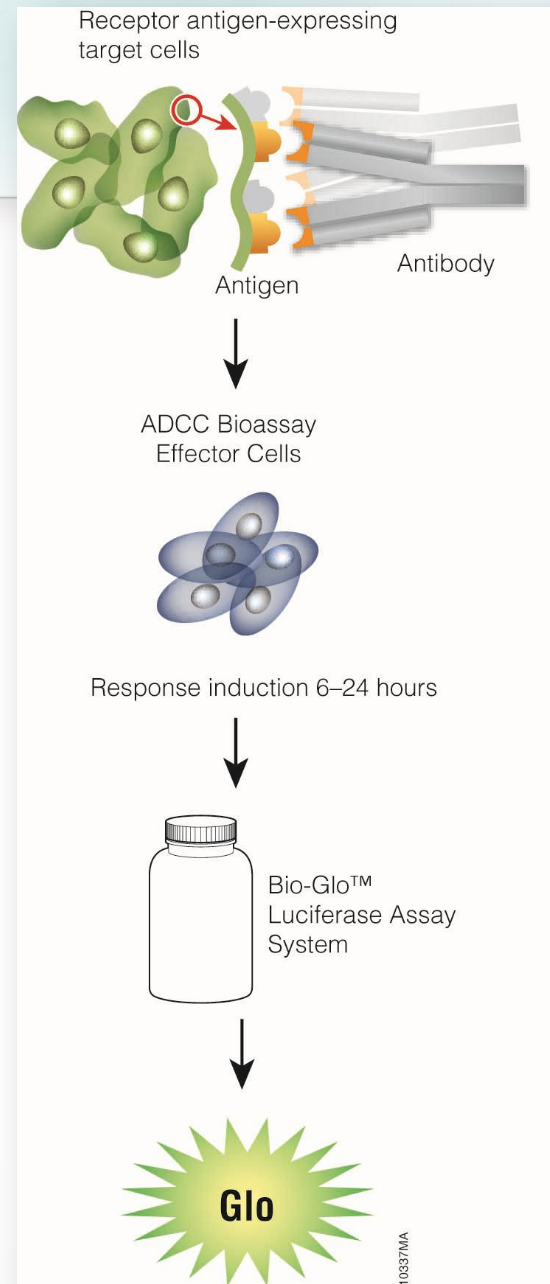
Single day bioassay

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.



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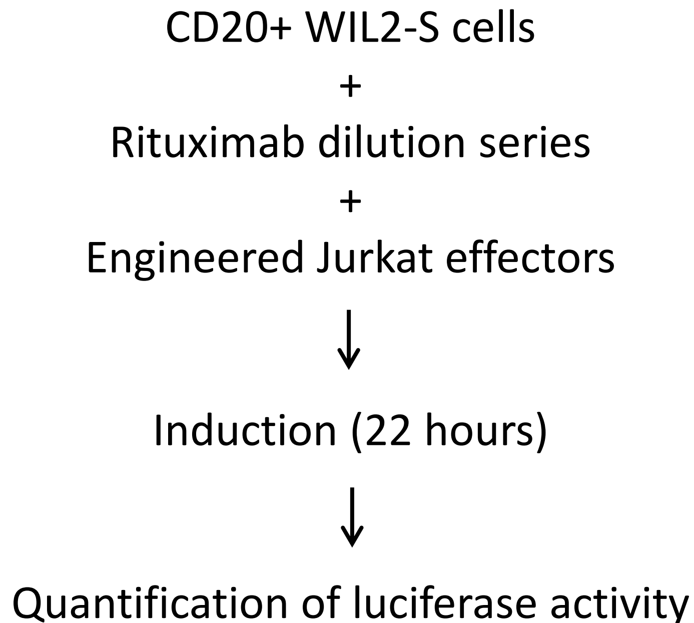
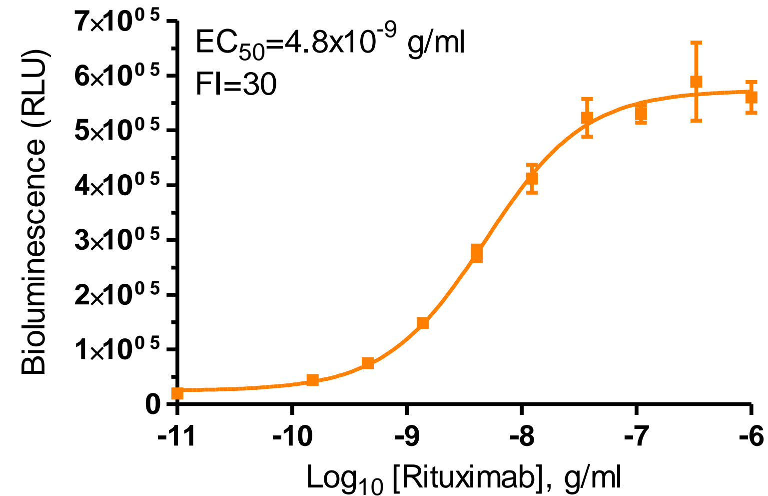
ADCC Reporter Bioassay – Initial Results

Assay protocol:





Specifics

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

Cell Selection and Frozen, Thaw-and-Use Format

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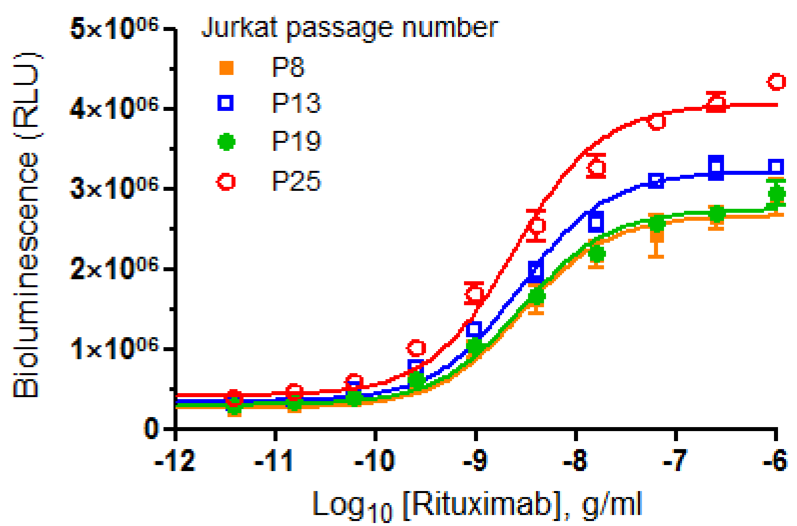
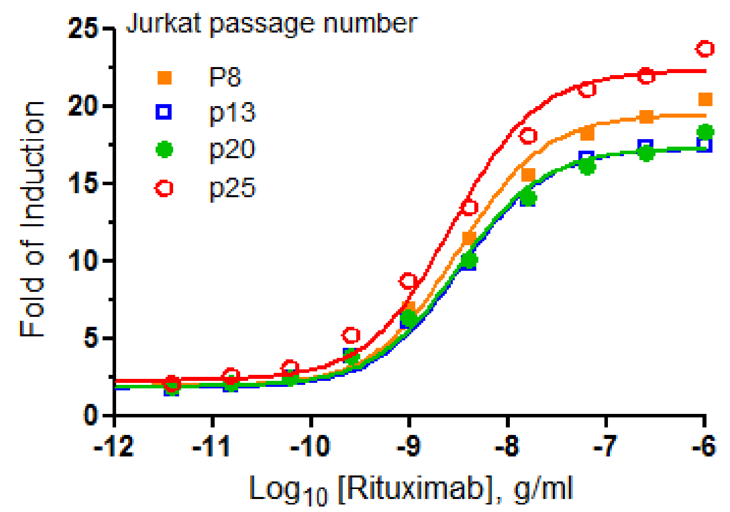
Engineered Effector Cell Clone Selection

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

Bioluminescence



Fold Induction



	P8	p13	p20	p25
EC50	3.094e-009	3.211e-009	3.077e-009	2.665e-009

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

Cells as Reagents

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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)

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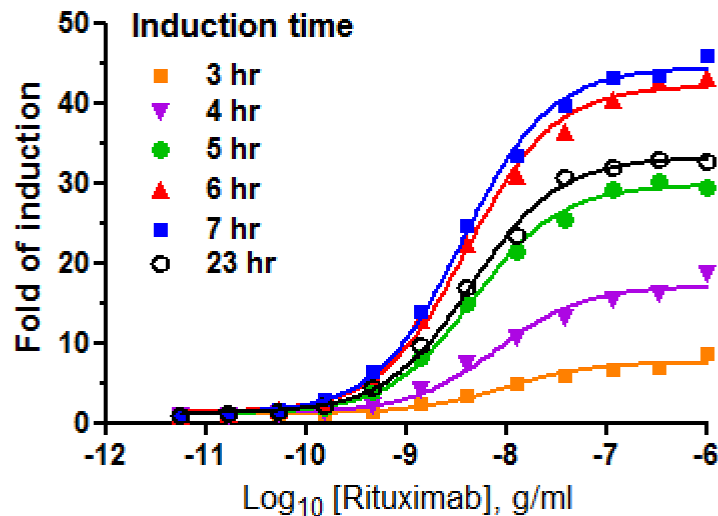
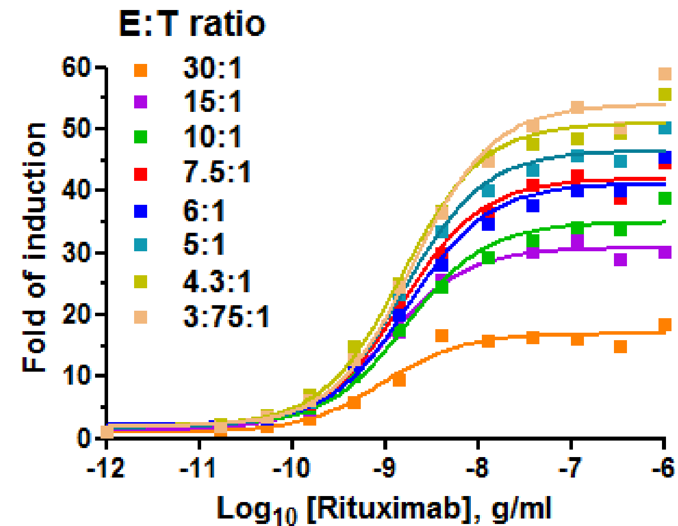
Optimization Studies

Critical Assay Parameters

Induction time



E:T ratio with constant Effector cell number



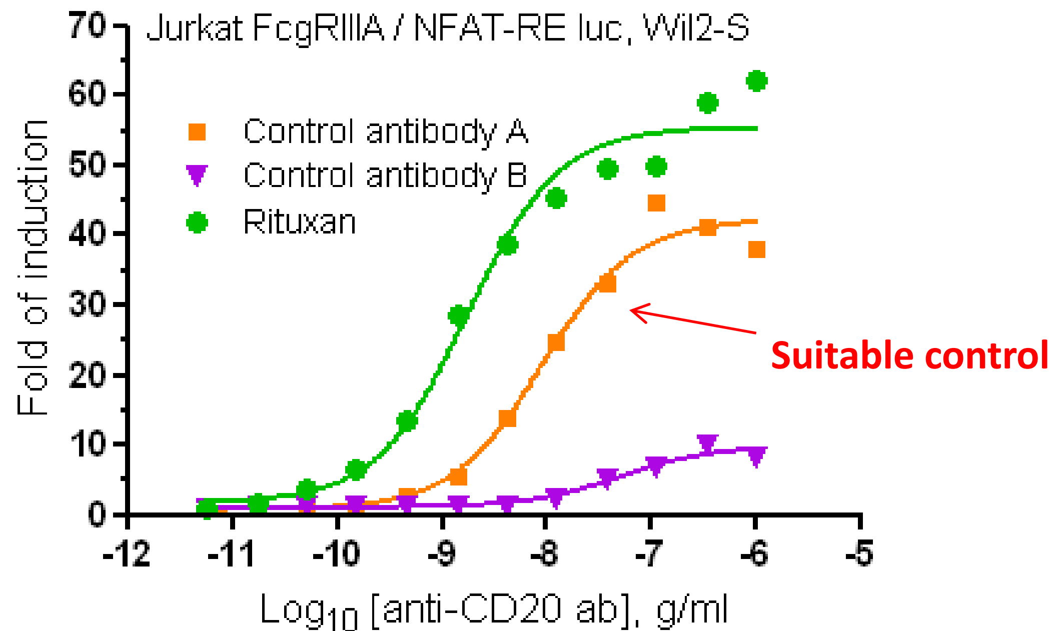
Other parameters tested:

- 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



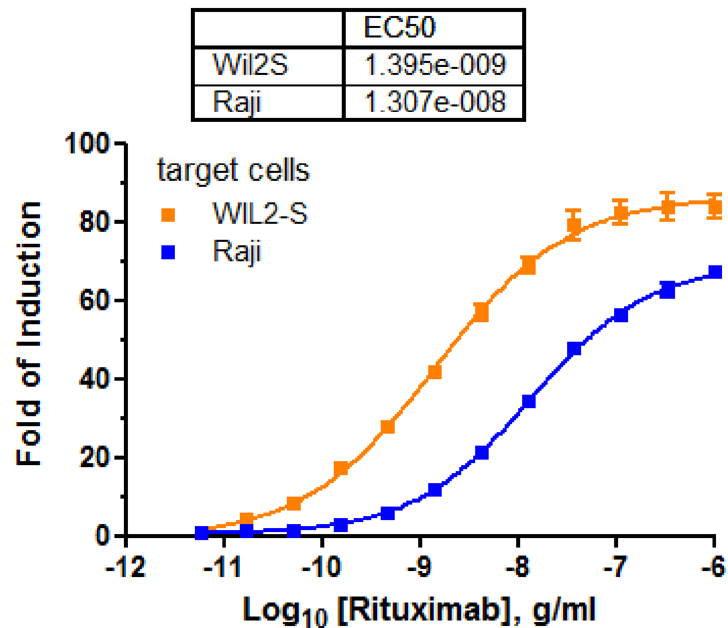
Anti-CD20	Rituximab	Control Ab (A)	Control Ab (B)
EC_{50} (g/ml)	$1.7x10^{-9}$	$8.9x10^{-9}$	$47.3x10^{-9}$
Fold Induction	62	38	8

Use of Different Target Cells

Suspension or adherent target cells can be used

Rituximab (anti-CD20)

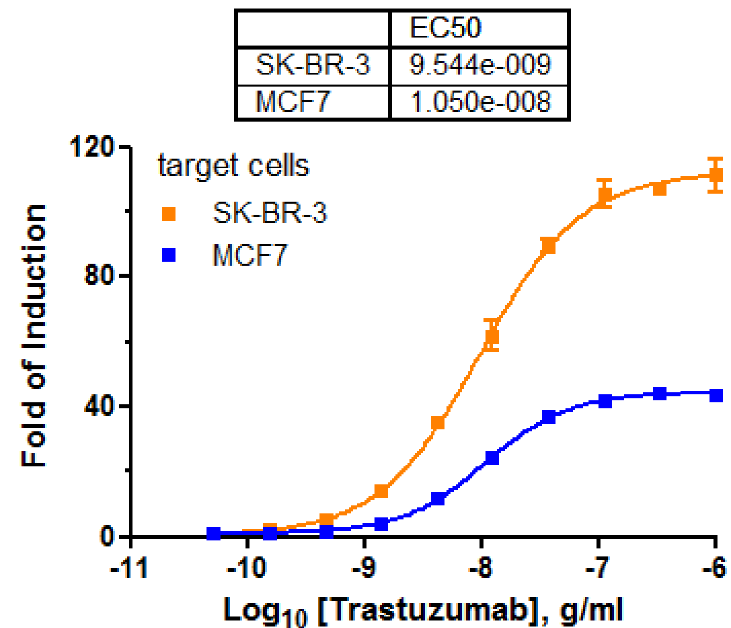
CD20+ B cell lines (suspension) as target cells



	EC50
Wil2S	1.395e-009
Raji	1.307e-008

Trastuzumab (anti-Her-2)

Her2+ breast cancer cell lines (adherent) as target cells

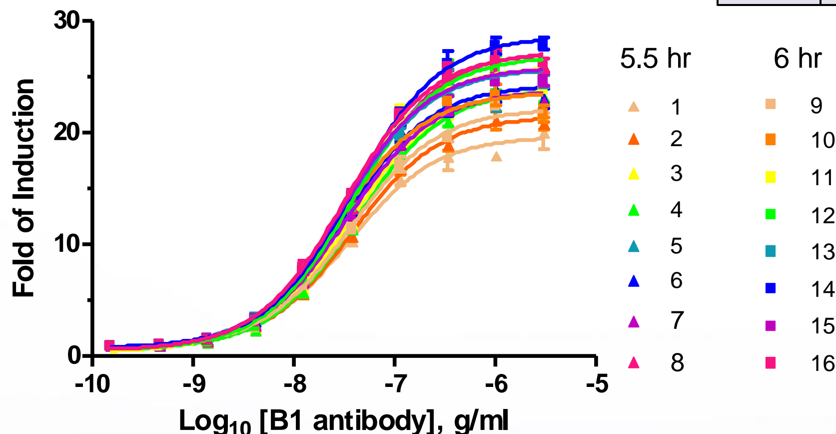


	EC50
SK-BR-3	9.544e-009
MCF7	1.050e-008

Bioassay Development: Optimization Using DOE

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

run	induction time hr	Target cell / Ab incubation time(mins)	Jurkat cell plating number (K)	Target cell plating number (K)
1	5.5	30	75	10
2	5.5	30	75	12.5
3	5.5	30	90	12
4	5.5	30	90	15
5	5.5	45	75	10
6	5.5	45	75	12.5
7	5.5	45	90	12
8	5.5	45	90	15
9	6	30	75	10
10	6	30	75	12.5
11	6	30	90	12
12	6	30	90	15
13	6	45	75	10
14	6	45	75	12.5
15	6	45	90	12
16	6	45	90	15



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

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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+FcγRIIIa, Rituximab

No Target cells — NO Wil2-S, Jurkat/NFAT-luc+FcγRIIIa, Rituximab

No Effector cells or no FcγRIIIa:
- Wil2-S, Jurkat/NFAT-luc (NO FcγRIIIa), Rituximab
- Wil2-S, NO Jurkat/NFAT-luc+FcγRIIIa, Rituximab

No antibody or non-specific antibody:
- Wil2-S, Jurkat/NFAT-luc+FcγRIIIa, NO Rituximab
- Wil2-S, Jurkat/NFAT-luc+FcγRIIIa, Trastuzumab

Assay signal is dependent on:

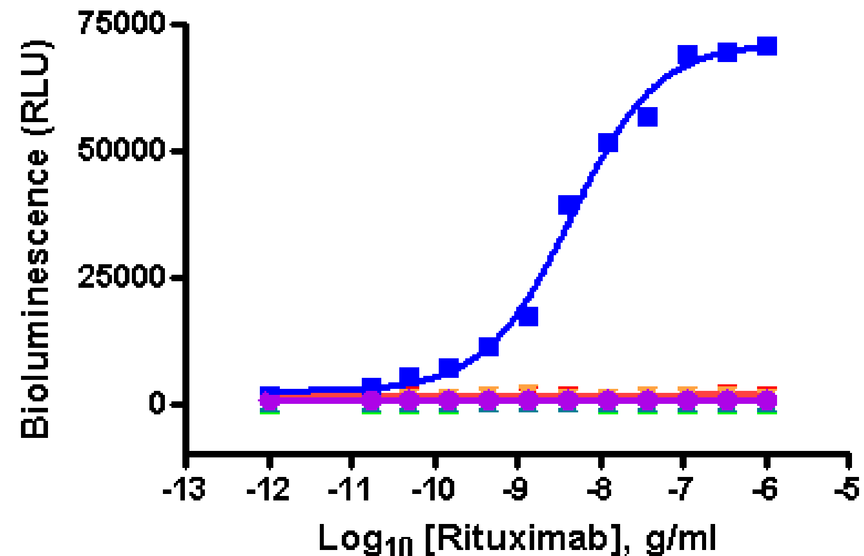
Presence of Target cells

+

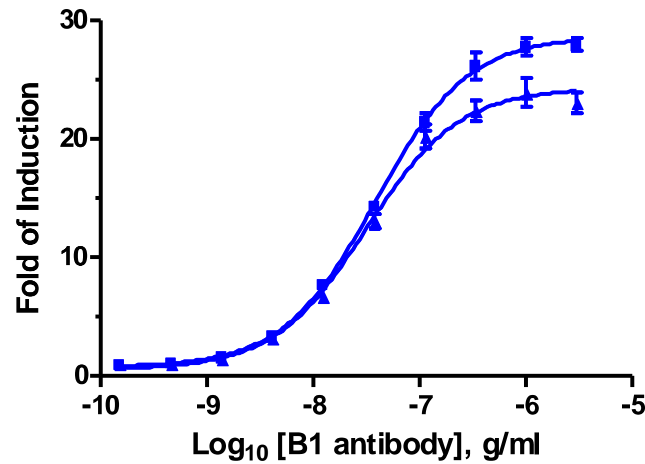
Presence of FcγRIIIa receptor

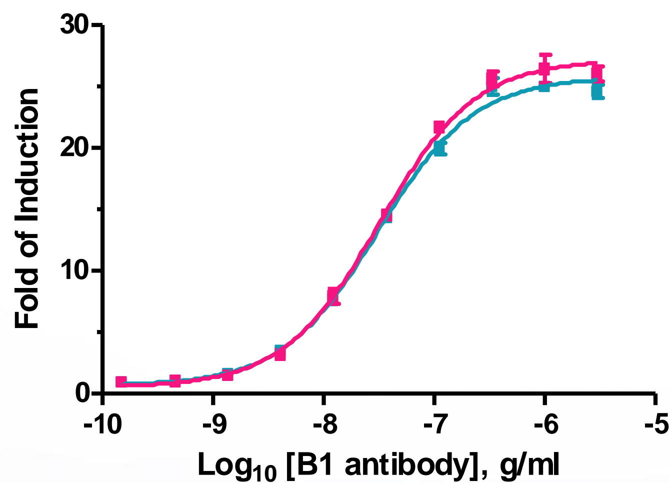
+

Appropriate specific antibody



ADCC Reporter Bioassay is Robust





Time of induction

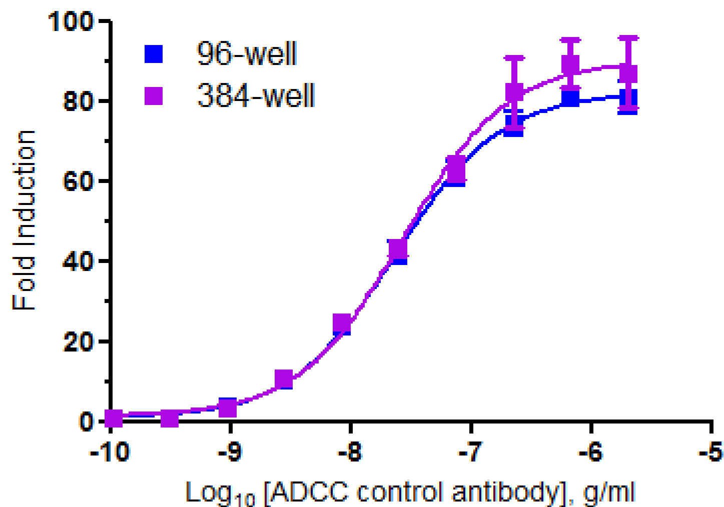
Run	Induction time	EC_{50}
1	6.0 hr	3.15×10^{-8} g/ml
2	5.5 hr	3.83×10^{-8} g/ml

E:T ratio and cell # per well

Run	E:T ratio	E cell #	T cell #	EC_{50}
1	7.5:1	75k	10k	3.09×10^{-8} g/ml
2	6:1	90k	15k	3.83×10^{-8} g/ml

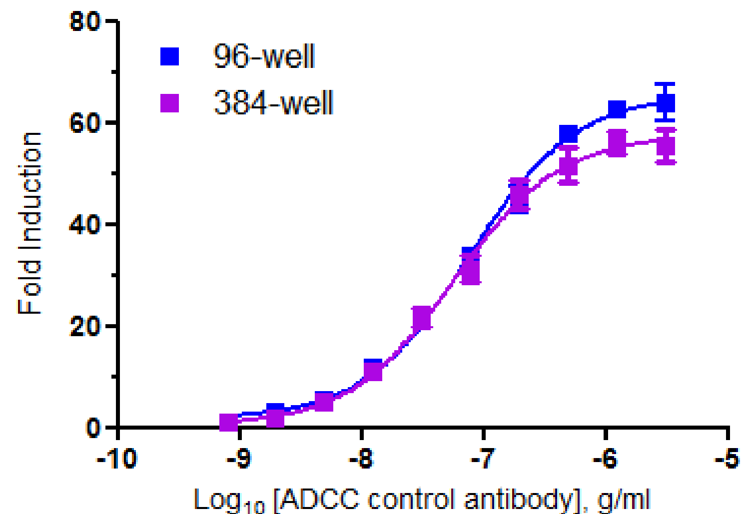
Miniaturization to 384-well Plates

WIL2-S target cells



	96-well	384-well
EC50	2.286e-008	2.651e-008

Raji target cells



	96-well	384-well
EC50	7.434e-008	5.583e-008

Assay volume per well	Target cells	Antibody	Effector cells	Bio-Glo™
96-well plate	25µl	25µl	25µl	75µl
384-well plate	5µl	5µl	5µl	15µl

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Assay Qualification Results

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

Design:

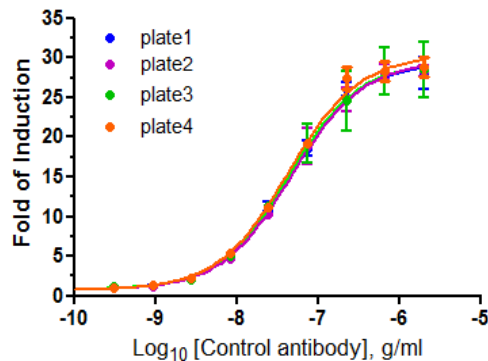
- Three days
- Four plates per day

✓100% vs 50%
✓100% vs 75%
✓100% vs 125%
✓100% vs 150%

Representative plate layout

	1	2	3	4	5	6	7	8	9	10	11	12	Plate1
A													
B		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		100%
C		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		50%
D		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		100%
E		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		50%
F		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		100%
G		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		50%
H													

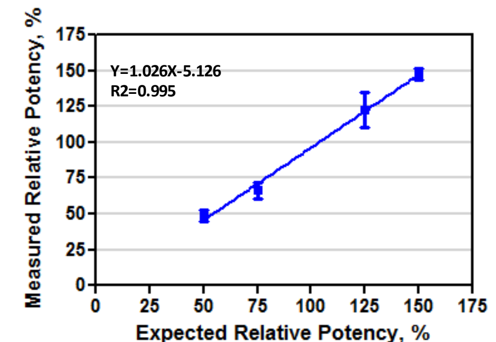
Repeatability



Precision = average of RSD (%) = 7.3%
Accuracy = average of Recovery (%) = 95.8%

	Antibody Sample	Measured Potency (%)	Mean Potency (%)	SD %	Recovery (%)	RSD (%)
day 1	50%	48.5	48.9	3.9	97.7	7.9
day 2		45.2				
day 3		52.9				
day 1	75%	63.1	66.4	5.9	88.5	8.9
day 2		62.9				
day 3		73.2				
day 1	125%	112.1	123.0	12.3	98.4	10.0
day 2		136.3				
day 3		120.5				
day 1	150%	148.8	147.6	3.6	98.4	2.4
day 2		150.4				
day 3		143.6				

Linearity



Good repeatability, accuracy, precision and linearity were obtained

Assay Qualification Results with Raji Target Cells

Analyst 1

Day	Antibody Sample	Measured Potency (%)	Mean Potency (%)	SD (%)	Recovery (%)	CV (%)
1	50%	49.9	51	0.7	102	1.4
2		51.3				
3		50.5				
1	75%	78.9	76	5.11	101	6.7
2		78.8				
3		70				
1	125%	118.6	117	1.19	94	1
2		116.9				
3		116.3				
1	150%	143.2	145	3.91	97	2.7
2		142.5				
3		149.6				

Precision: 2.95%

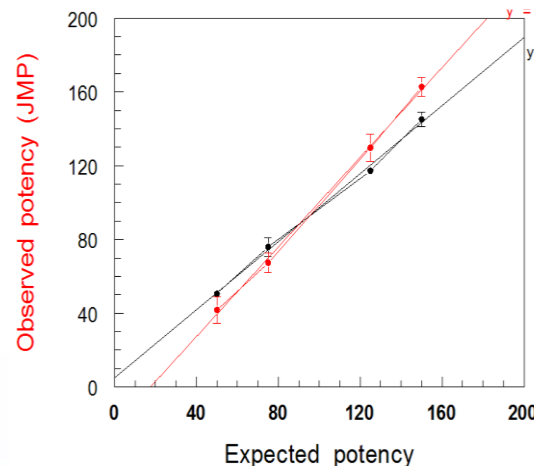
Accuracy: (recovery average): 98.5%

Linearity: Y=0.922x+5.0

Analyst 2

Day	Antibody Sample	Measured Potency (%)	Mean Potency (%)	SD (%)	Recovery (%)	CV (%)
1	50%	38.4	41.8	7.2	83.5	17.2
2		47.2				
3		33.2				
4		48.2				
1	75%	59.6	67.4	5.2	89.9	7.7
2		70.2				
3		69.3				
4		70.5				
1	125%	120	129.7	7.5	103.7	5.8
2		132.3				
3		137.8				
4		128.6				
1	150%	160.2	162.8	5.2	108.5	3.2
2		158.2				
3		162.7				
4		170				

Linearity



Precision: 8.47%

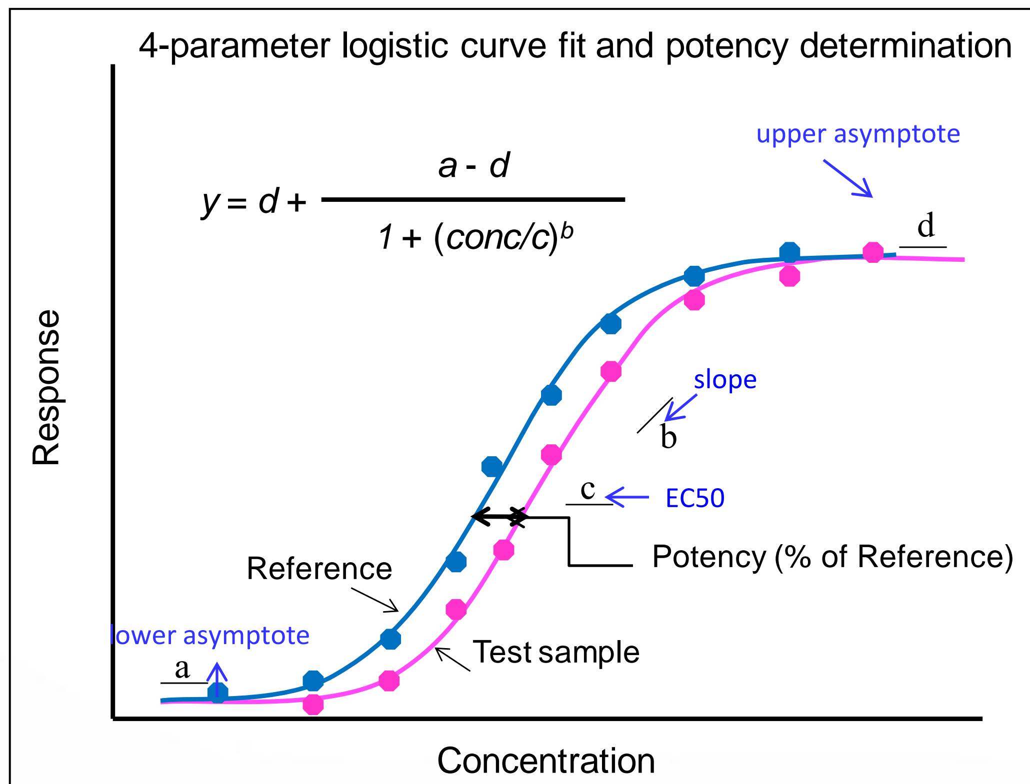
Accuracy (recovery average): 96.4%

Linearity: Y=1.22x-21.3

Potency Determinations Using Quantitative Bioassays

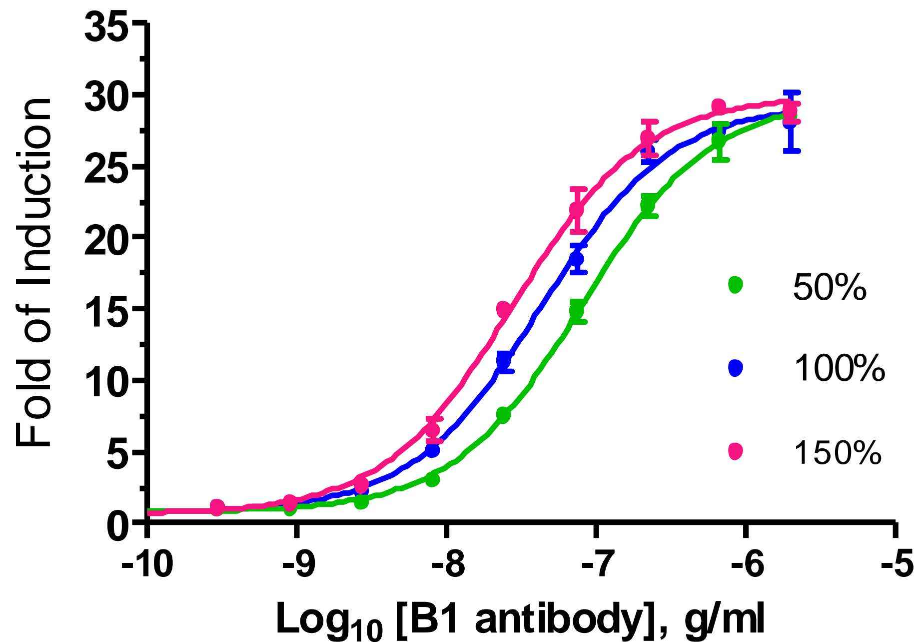
A test sample of unknown biological activity is compared with a reference sample of established biological activity in a dose-response study in the test system.

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



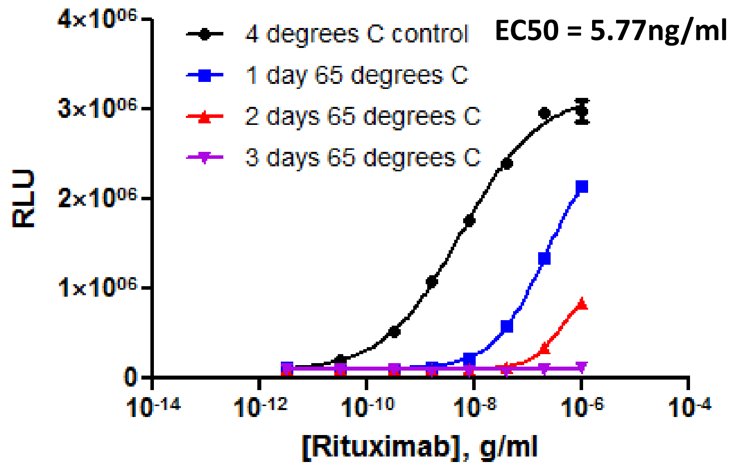
	100%-1	100%-2	100%-3	100%-4
EC50	4.230e-008	4.693e-008	4.323e-008	4.245e-008
relative potency	102%	92%	100% control	102%

Parallelism and relative potency determined with JMP Software

Stability Indicating

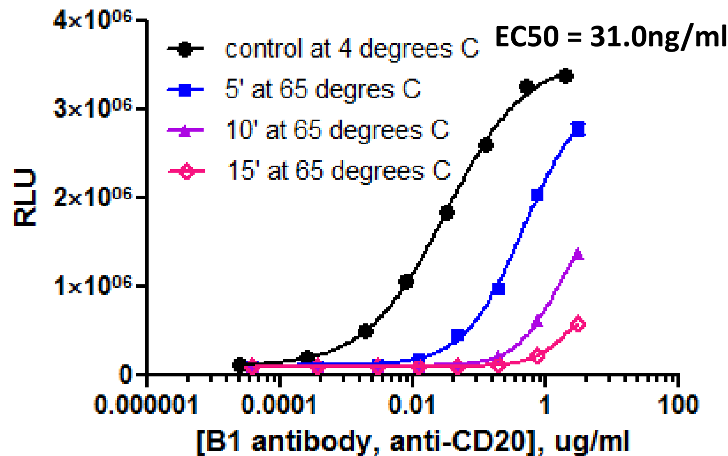
Stability Indicating for Fc Effector Function

Rituximab



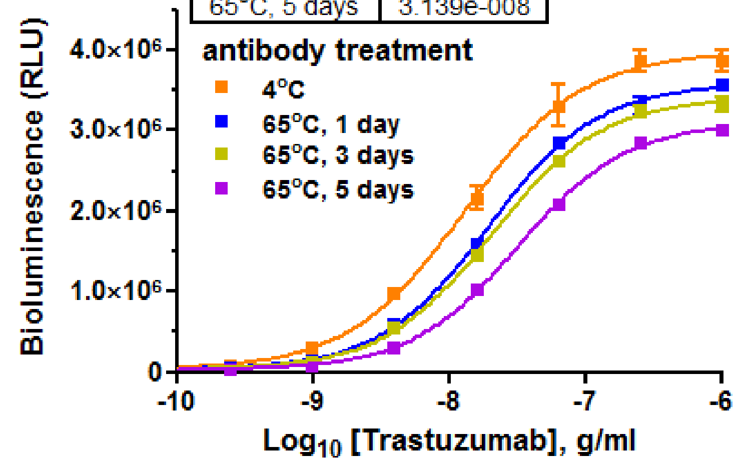
Activity of heat-treated antibody drugs

Tositumomab



Trastuzumab

	EC50
4°C	1.284e-008
65°C, 1 day	1.902e-008
65°C, 3 days	2.031e-008
65°C, 5 days	3.139e-008



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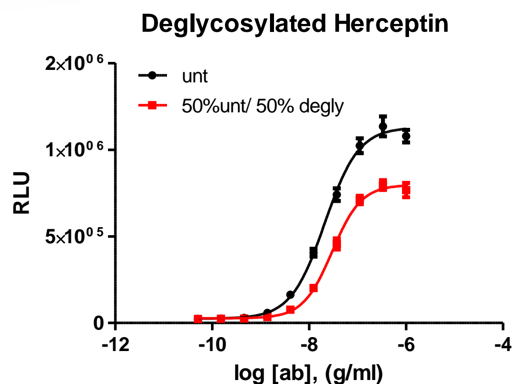
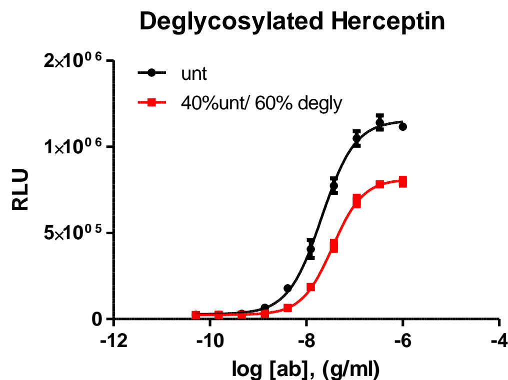
Antibody Variants

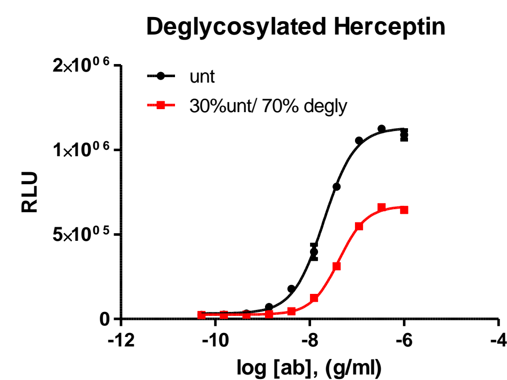
Analysis of Mixed Glycosylation mAbs



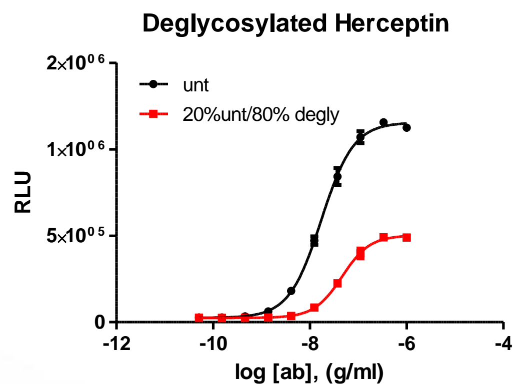
	unt	50%unt/ 50% degly
EC50	2.110e-008	2.990e-008



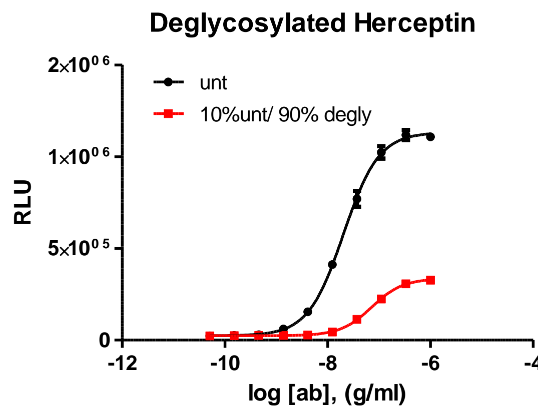
	unt	40%unt/ 60% degly
EC50	2.082e-008	3.486e-008



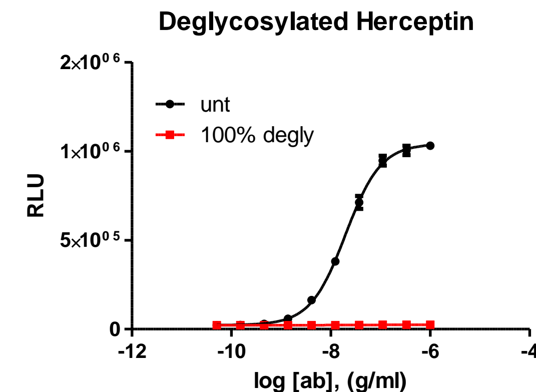
	unt	30%unt/ 70% degly
EC50	2.002e-008	4.153e-008



	unt	20%unt/80% degly
EC50	1.720e-008	4.626e-008



	unt	10%unt/ 90% degly
EC50	2.037e-008	7.174e-008



	unt	100% degly
EC50	1.988e-008	3.202e-008

Target cells: SKBR3; Unt = 100% glycosylated

ADCC Reporter Bioassay Activity Correlates with Amount of Antibody N-glycosylation

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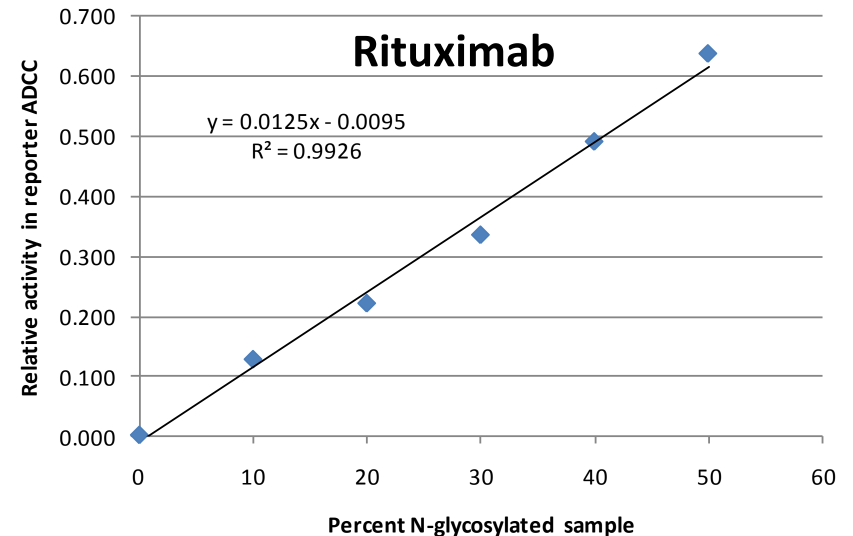
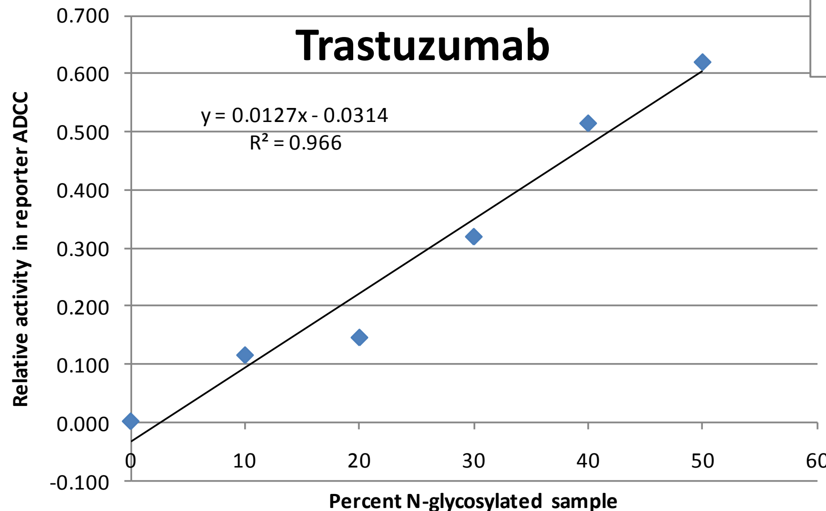
Rituximab and Trastuzumab:
Linear correlation obtained between percentage of N-glycosylated antibody in blended antibody samples and relative luciferase reporter activity in ADCC reporter bioassay





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

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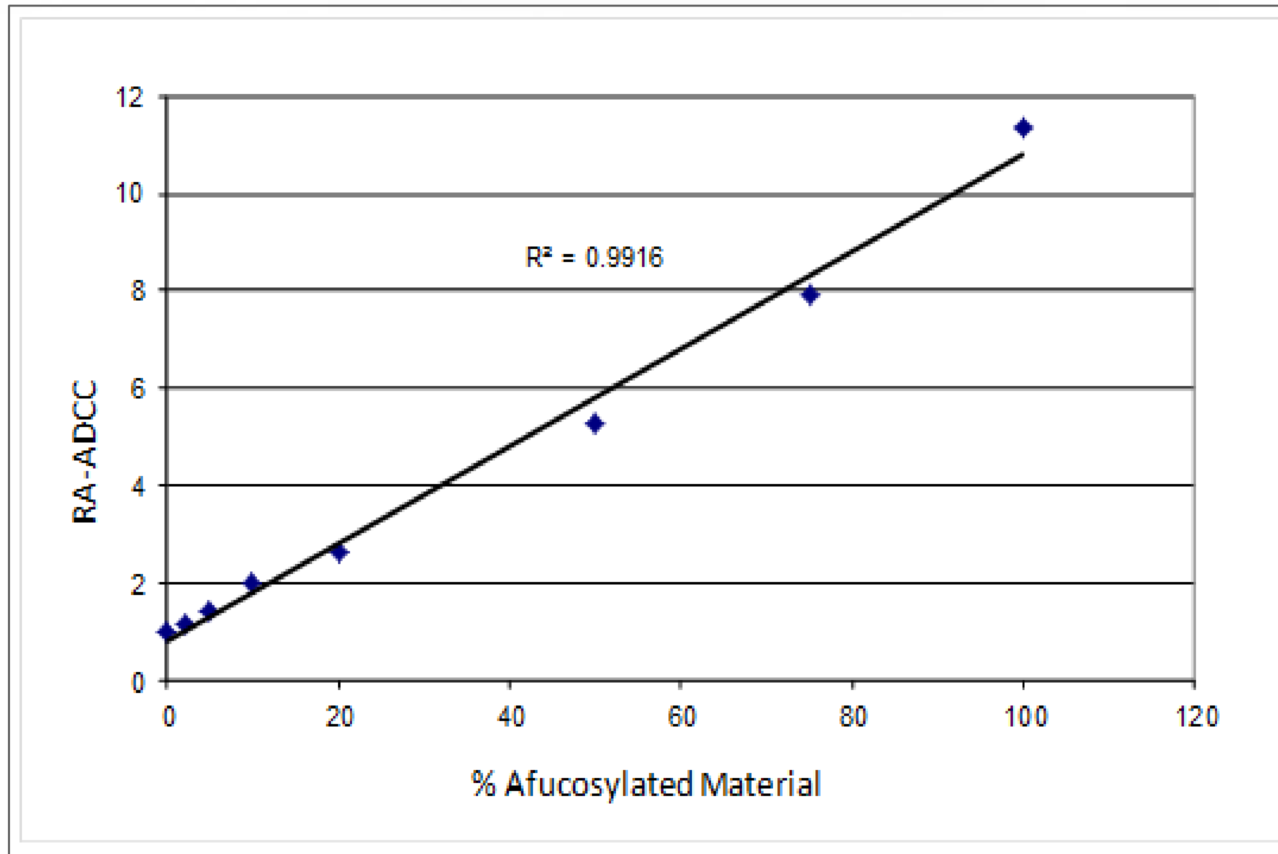

Linear correlation shown between percentage of afucosylated antibody in blended antibody samples and relative luciferase reporter activity in ADCC reporter assay

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Bioassay Characteristics - ICH Guideline Q2 [R1]

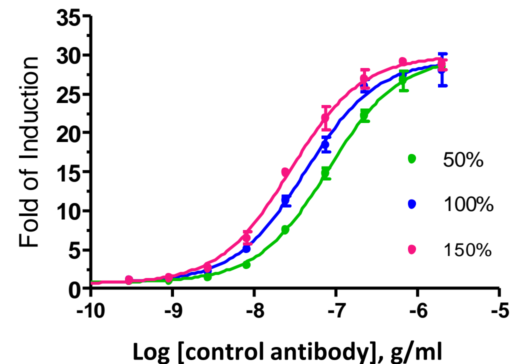
Validation of Analytical Procedures:

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

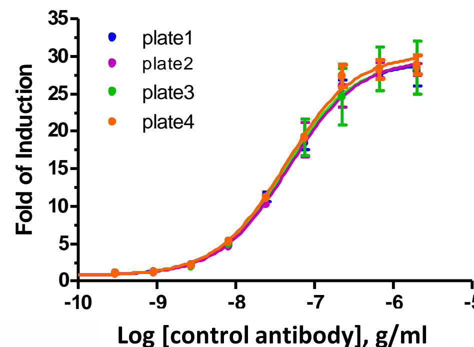
Design:
- Two analysts
- Three days
- Four plates per day

✓100% vs 50%
✓100% vs 75%
✓100% vs 125%
✓100% vs 150%

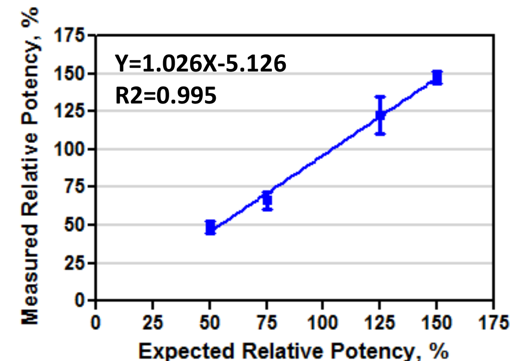
Relative potency



Repeatability



Linearity



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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

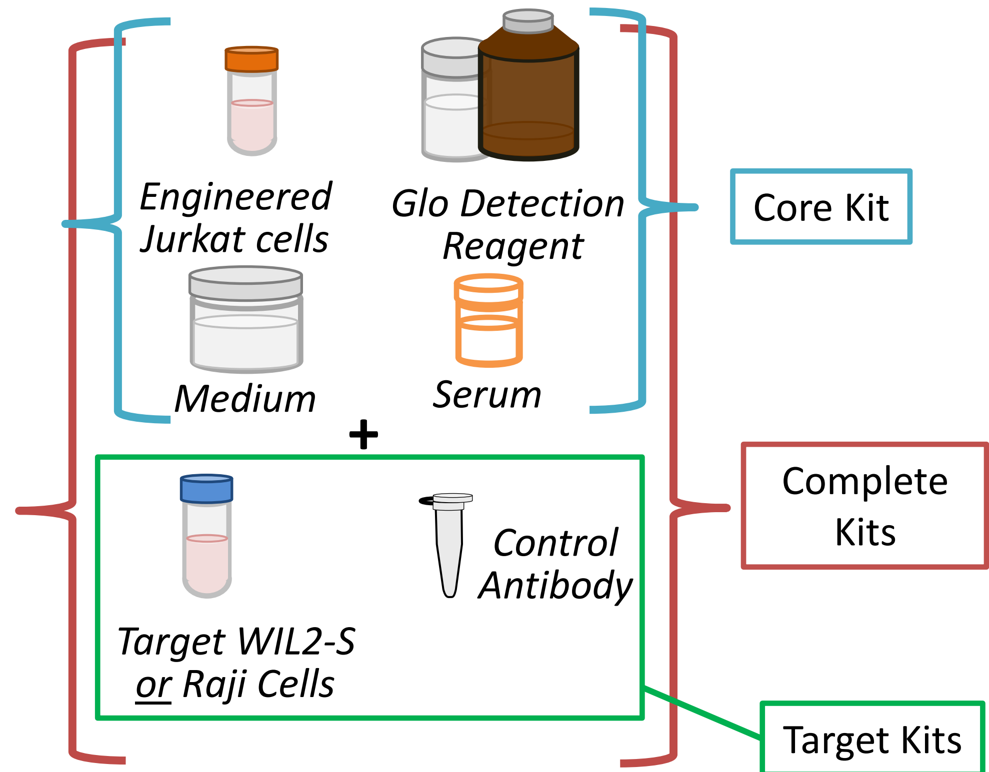
ADCC Reporter Bioassay Kit Configurations

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*

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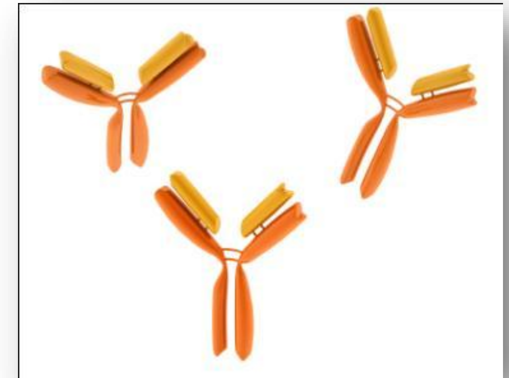
Summary of the ADCC Reporter Bioassay



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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Acknowledgements:
Frank Fan, Terry Surowy, Jey Cheng, Rich Moravec, Denise Garvin, Aileen Paguio, Pete Stecha