Development of a Bioluminescent Cell-based Bioassay to Measure Fc Effector Function in ADCC

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A new bioassay for Fc effector functionality in ADCC

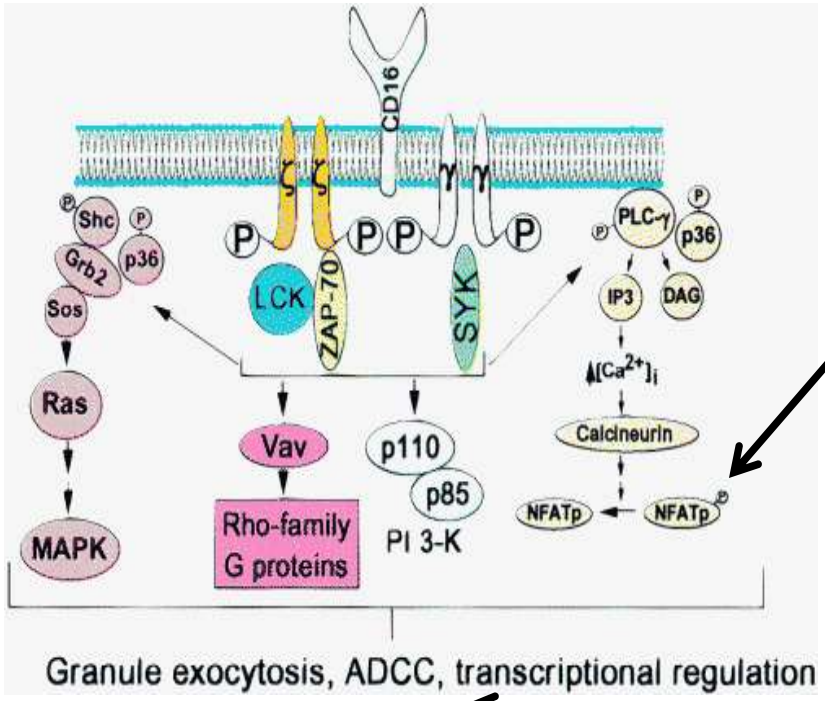
Highlights of a novel bioluminescent cell-based bioassay:

- A cell-based bioassay to quantify potency of Fc effector function in ADCC MOA pathway
- Based on target cell-bound antibody activation of FcγIIIa receptor signaling pathways in the effector cell
- Bioluminescent NFAT-RE-luciferase reporter bioassay readout is in engineered effector cells

- The bioassay is robust, specific, precise and accurate
- Suitable for stability studies, lot release and antibody characterization
- Effector cells are reagents -in frozen, thaw-and-use format

Signaling pathways activated in effector cells when FcγRIIIa is bound by target cell-bound antibody in ADCC

FcγRIII initiated signaling events



Leibson-PJ, *Immunity* 1997

New reporter gene bioassay uses an earlier step in the pathway

- To quantify biological activity of Abs in this MOA pathway
- Avoids preparation and high variability of primary effector cells
- Avoids high background and variability of spontaneous lysis

Traditional readout is cytotoxicity, the ultimate endpoint

Introduction to the reporter bioassay for Fc effector function in MOA pathway

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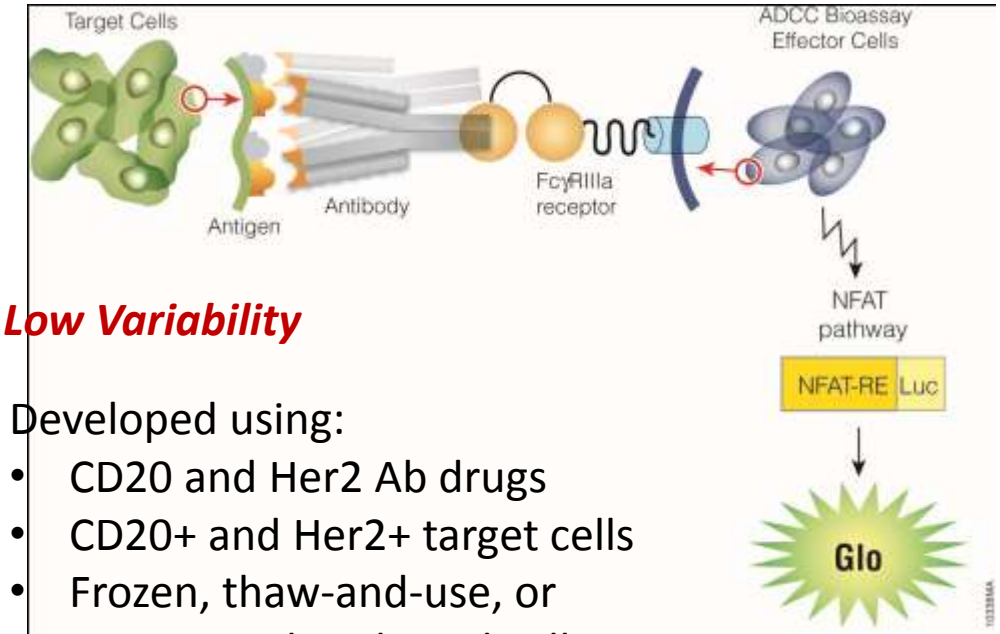
NFAT-RE luciferase bioassay



Low Variability

Developed 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

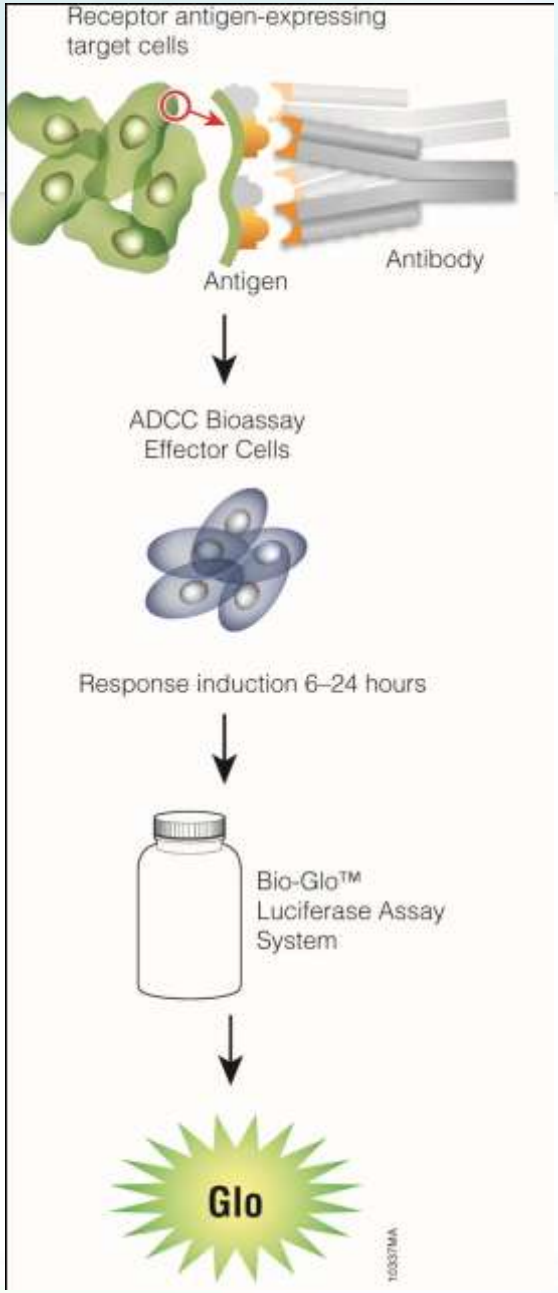
1. Effector cells are engineered to express FcγRIIIa (V158) and NFAT-RE-luc2 luciferase
2. 'Cells as reagents' (thaw-and-use)
3. Homogeneous assay format – simple 'add-mix-read' bioluminescent assay.
4. Optimized and robust assay reagents and protocol
5. Performance characteristics that meet needs of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

Protocol

1. Reference or test antibody is added to target cells.

2. Effector cells are added and response is induced in as little as 6 hours. Effector cells consist of a Jurkat cell line engineered to express FcγRIIIa (V158) receptor and NFAT-RE luc2 luciferase.

3. Luciferase detection reagent is added and luminescence is measured immediately.



Cells as reagents in bioassays

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 (overgrown, etc.)
   - Indefinite storage
   - Identical cells in bioassay, day-to-day

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

FcγRIIIa antibody blocking of ADCC reporter bioassay response demonstrates FcγRIIIa expression

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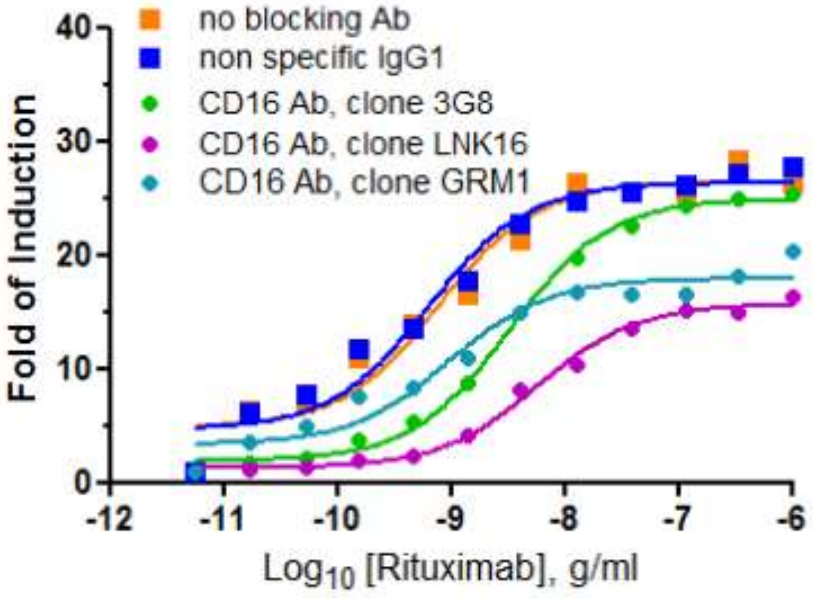
Effector cell line is a Jurkat cell line engineered to express FcγRIIIa (V158) receptor and NFAT-RE luc2 luciferase



	EC50
no blocking Ab	7.612e-010
non specific IgG1	6.189e-010
CD16 Ab, clone 3G8	3.164e-009
CD16 Ab, clone LNK16	5.563e-009
CD16 Ab, clone GRM1	9.218e-010

Assay used WIL2-S target cells

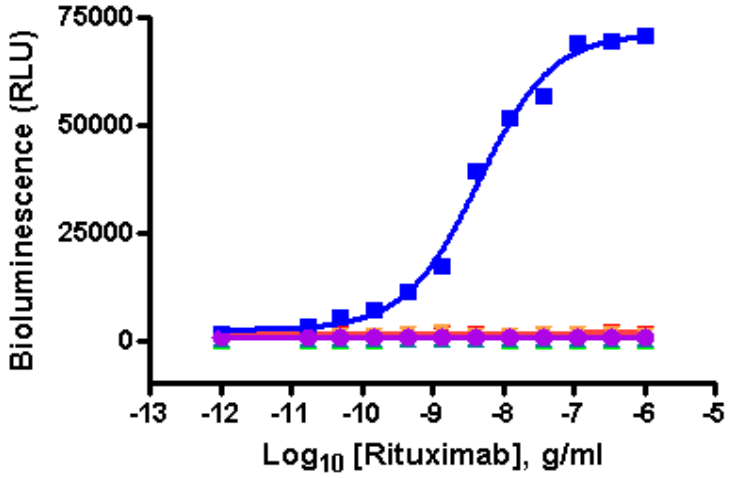
ADCC Reporter Bioassay demonstrates appropriate specificity

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



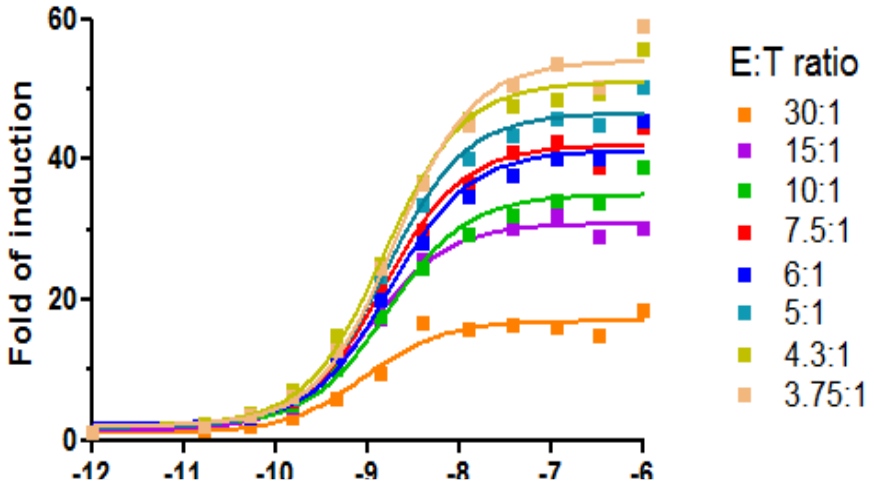
Assay signal is dependent on:

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

Bioassay development: Optimizing Effector:Target (E:T) ratio

An example: Evaluation of different E:T ratios for assay optimization using frozen, thaw-and-use engineered Jurkat Effector cells (FcγRIIIA (V158) / NFAT-RE-luc2) with fresh-from-culture target cells (WIL2-S, CD20+).

- Fixed Effector cell number
- Varied Target cell number and E:T ratios
- 6hr induction
- Bio-Glo™ Assay Reagent

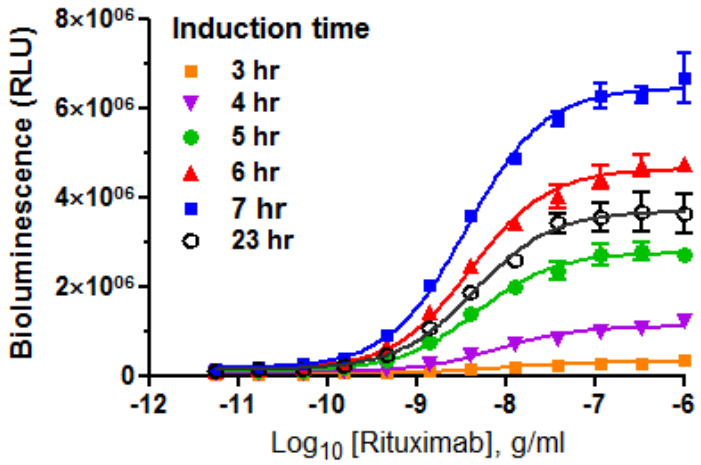


Higher target cell numbers generate greater fold induction

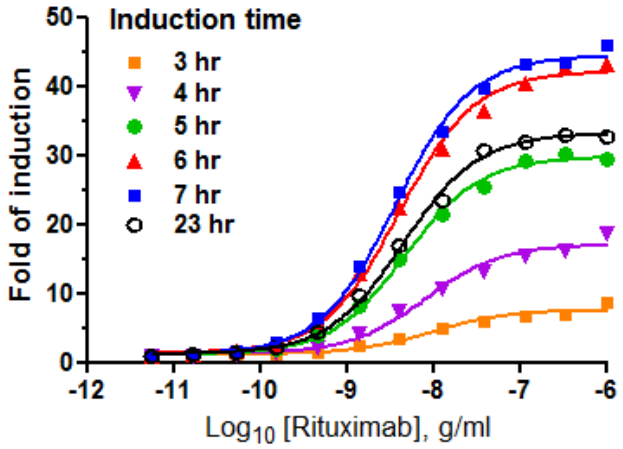
Bioassay development: Evaluating bioassay duration for response (fold induction)

Evaluation of different induction hours for assay optimization using frozen, thaw-and-use Jurkat Effector cells with fresh-from-culture target cells (WIL2-S, CD20+)

Bioluminescence



Fold Induction



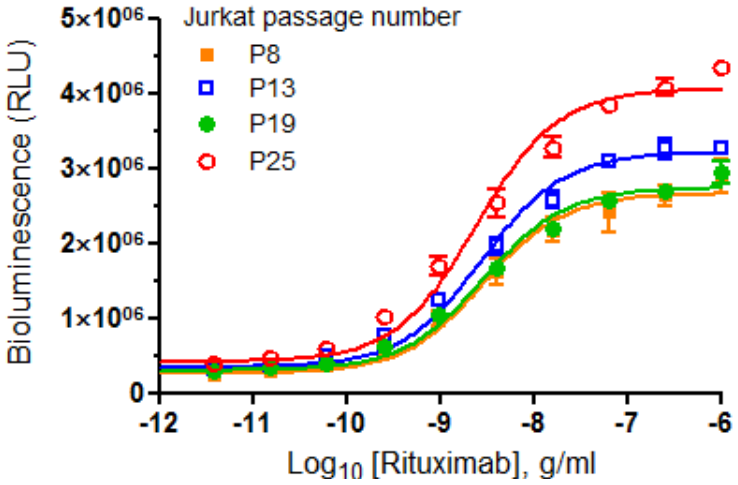
	3 hr	4 hr	5 hr	6 hr	7 hr	23 hr
EC50	8.978e-009	7.755e-009	4.605e-009	3.968e-009	3.577e-009	4.352e-009

Bioassay can be conducted with as few as 5-6hr induction using frozen, thaw-and-use Effector cells.

Bioassay development: Evaluating clone stability for engineered Jurkat effector cells

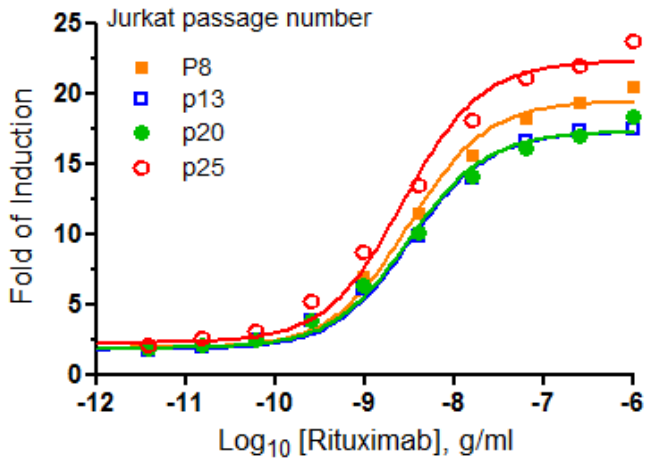
Evaluation of stable effector cell line clone stability for assay optimization using frozen, thaw-and-use effector cells with fresh-from-culture WIL2-S target cells.

Bioluminescence



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

Fold Induction



Frozen, thaw-and-use effector cell responsiveness was stable up to passage 25.
(Current stability > passage 50)

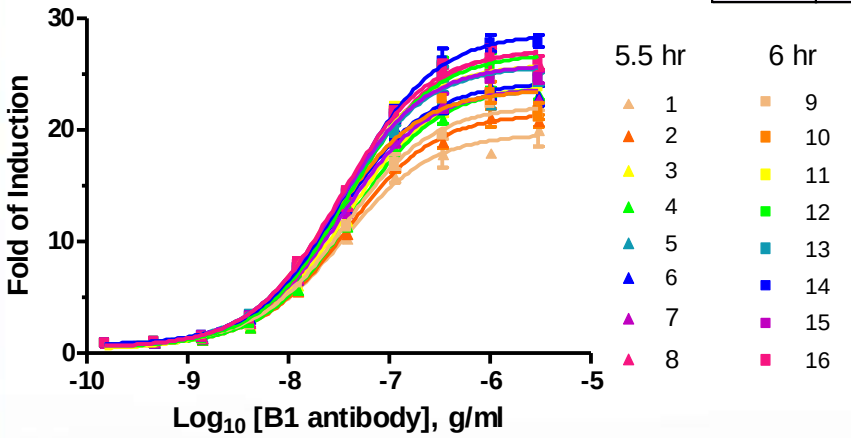
- E:T ratio = 7.5:1
- 6hr induction
- Bio-Glo™ Assay Reagent

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 and Results:

Good response (fold induction) = 19-27

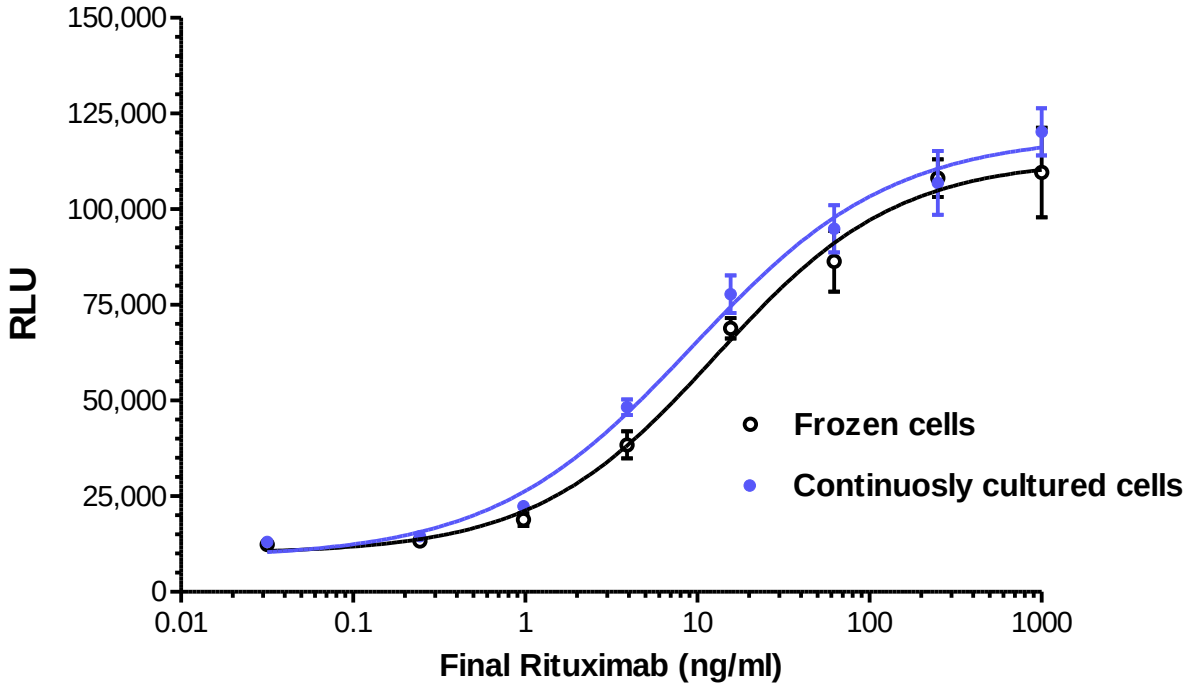
Good (low) L-term values = 0.1-0.2*

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

Target cells can also be frozen, thaw-and-use format

WIL2-S target cells can be used in frozen, thaw-and-use format

Direct comparison of target cell format: continuously-cultured vs. frozen, thaw-and-use cells

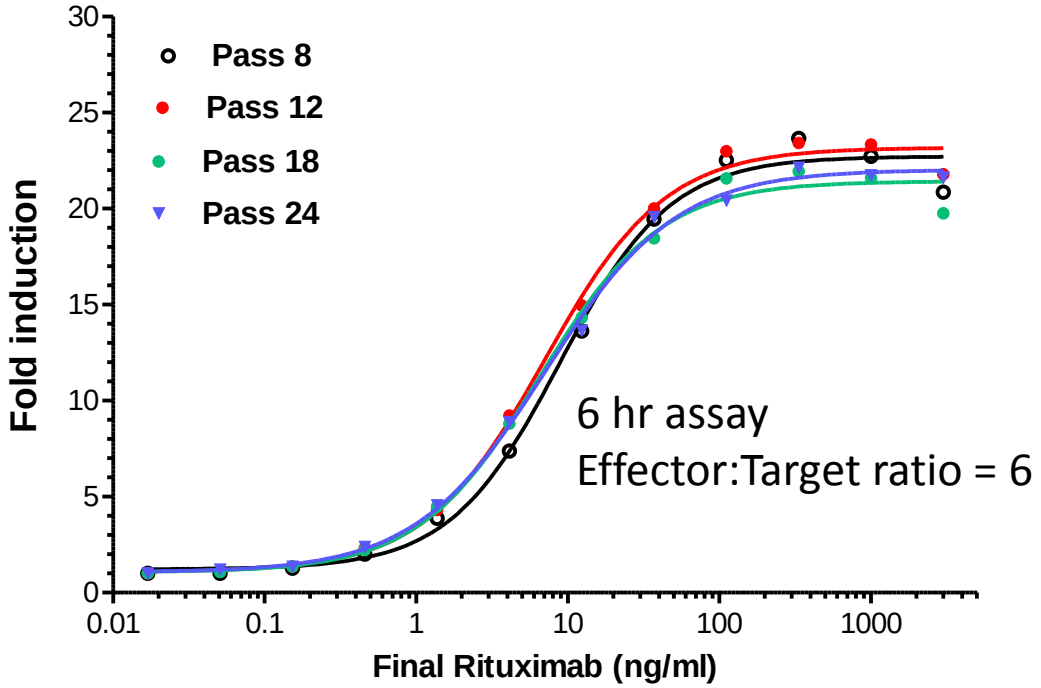


Effector cells: FcγRIIIa/NFAT-luc Jurkat (frozen, thaw-and use); Ab: Rituximab

Evaluating passage and cell density at freezing for WIL2-S (CD20+) Target cells

Establishment of optimized frozen, thaw-and-use Target cells includes establishment of acceptable passage range and cell growth conditions prior to freezing.

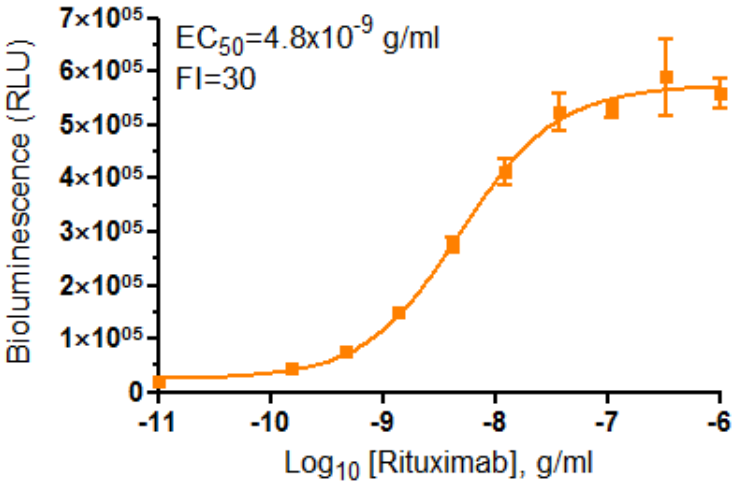
Important for establishing cell banks and manufacturing capabilities



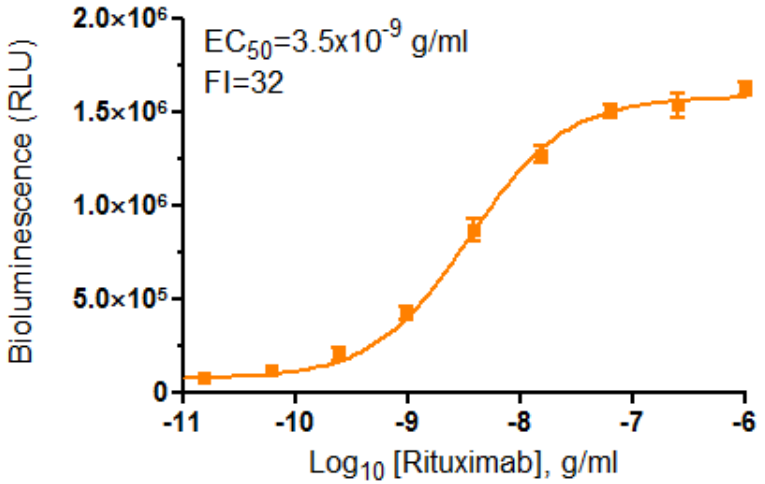
	Pass 8	Pass 12	Pass 18	Pass 24
EC50	8.851	7.057	6.520	7.122

ADCC Reporter Bioassay using frozen, thaw-and-use Effector and Target cells

A. Fresh cells from continuous culture



B. Frozen, thaw-and-use cells



Target cells are CD20+ WIL2-S

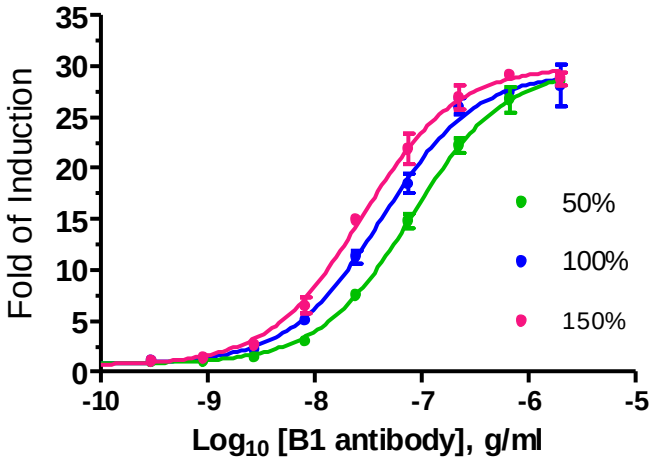
EC50s and fold induction of responses are equivalent

Performance characteristics of ADCC Reporter Bioassay

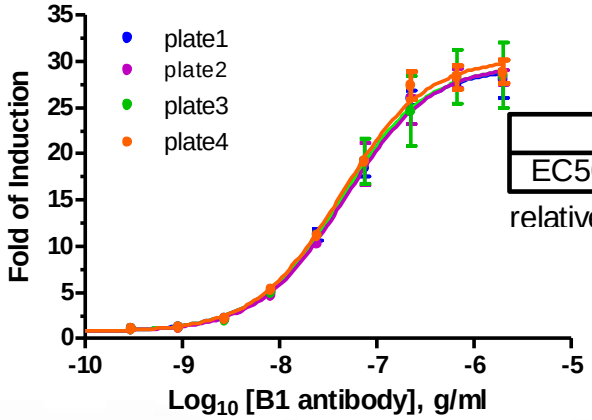
Design:
- Two analysts
- Three days
- Four plates per day

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

Measure relative potency and parallelism



Measure repeatability



	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%

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

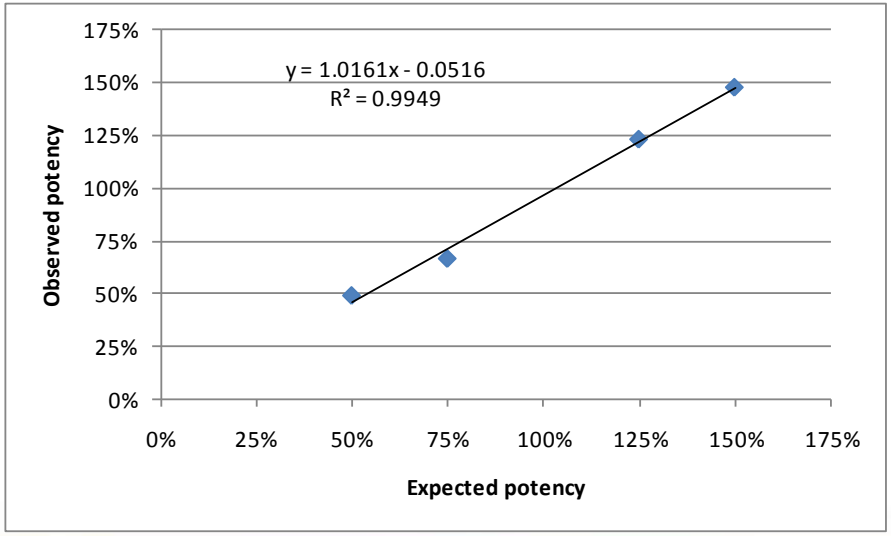
Precision, accuracy and linearity of ADCC Reporter Bioassay

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

Precision
(Av of RSD(%))
7.3%

Accuracy
(Av of % Recovery)
95.8%

Linearity
y= 1.016x – 0.052
R^2 = 0.995



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

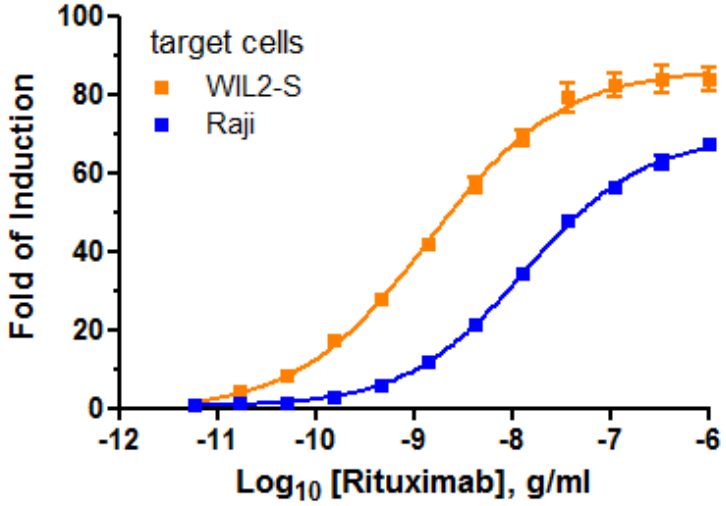
ADCC Reporter Bioassay can measure potency of antibody drugs in different systems

Suspension or adherent target cells can be used

Rituximab (anti-CD20)

CD20⁺ B cell lines (suspension) as target cells (plated directly from suspension)

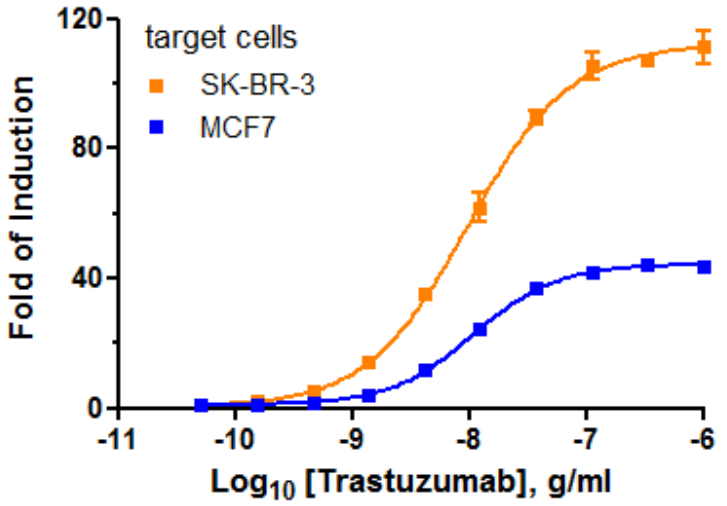
	EC50
Wil2S	1.395e-009
Raji	1.307e-008



Trastuzumab (anti-Her-2)

Her2⁺ breast cancer cell lines (adherent) as target cells (plated 16-18hr prior)

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



ADCC Reporter Bioassay is stability-indicating for Fc effector function

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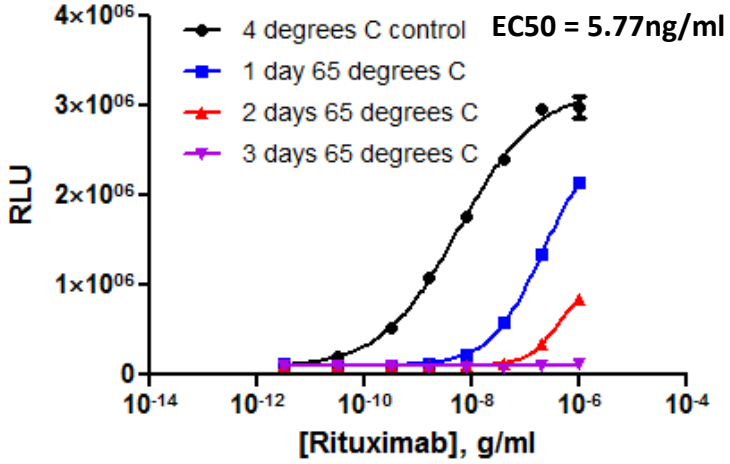
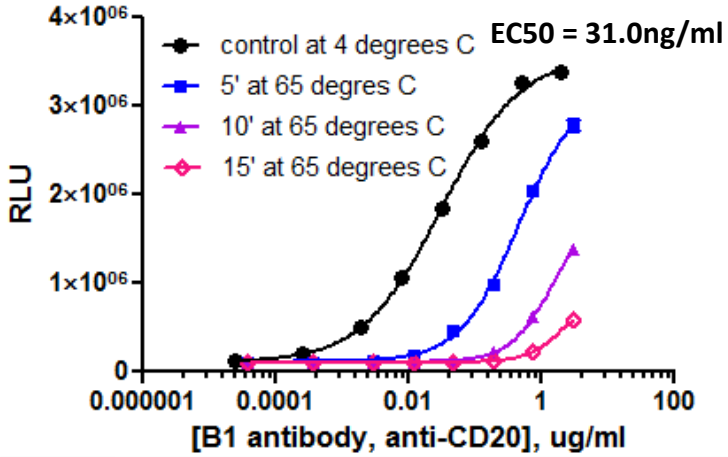
Rituximab



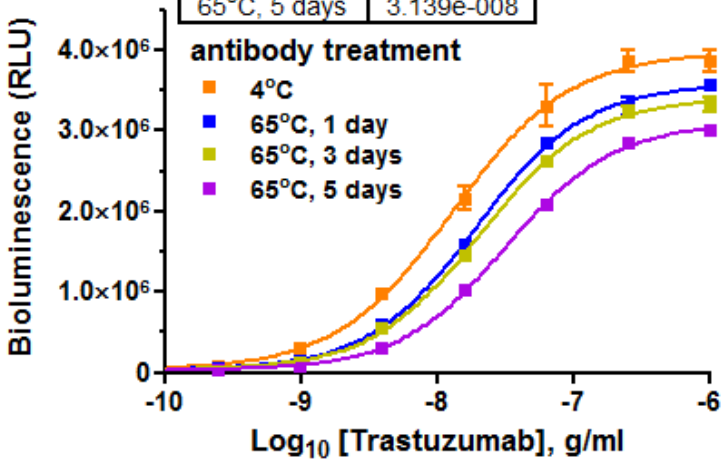
Tositumomab



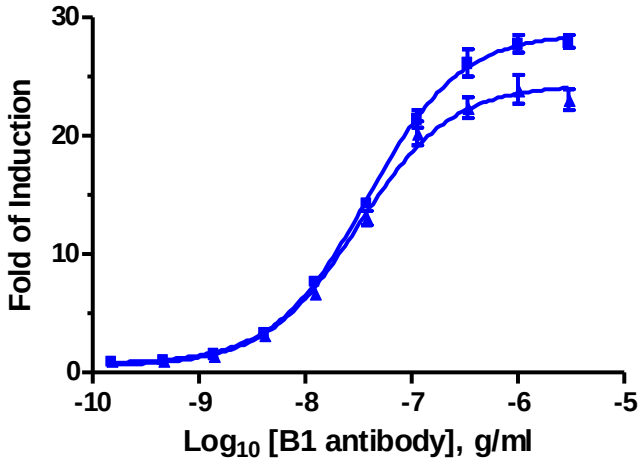
Activity of heat-treated antibody drugs

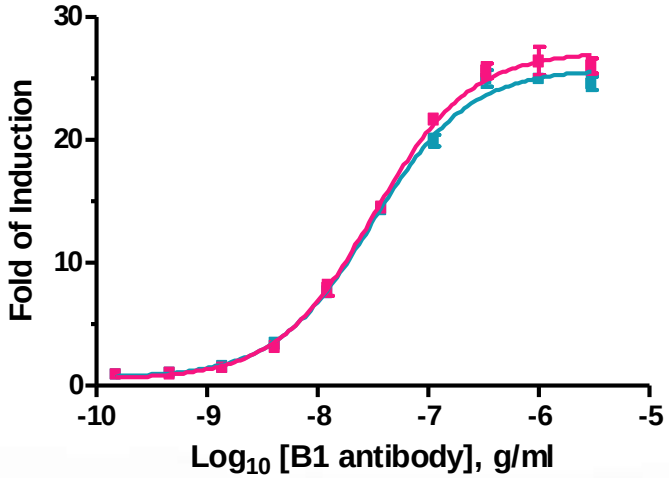
Trastuzumab

	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



ADCC Reporter Bioassay is robust





Time of induction

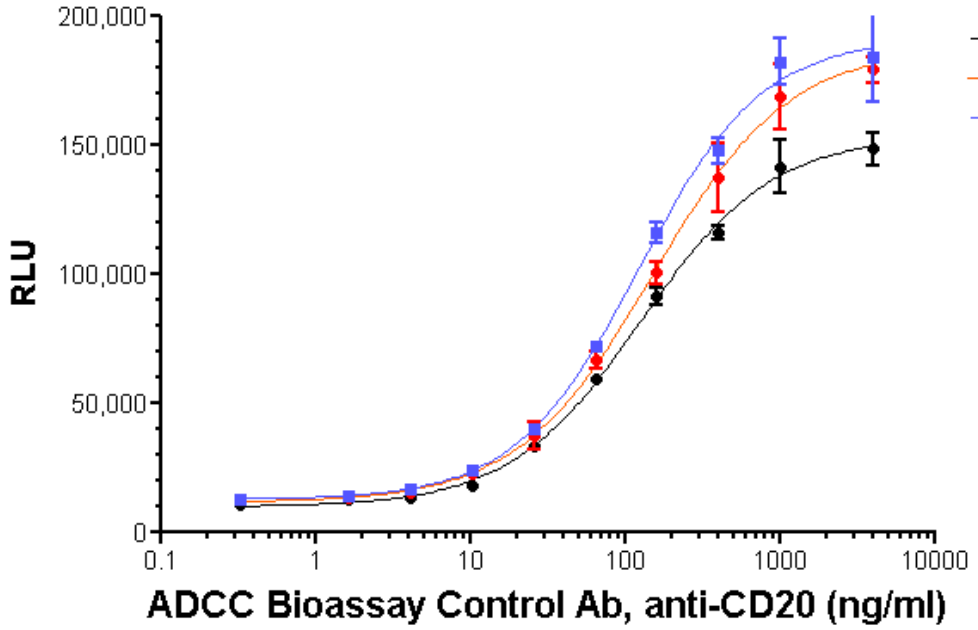
Run	Hr induction	EC50
1	6 hr	3.15 x 10e-8 g/ml
2	5.5 hr	3.83 x 10e-8 g/ml

E:T ratio and cells/well

Run	E:T ratio	T cells	E cells	EC50
1	7.5:1	10K	75K	3.09 x 10e-8 g/ml
2	6:1	15K	90K	3.83 x 10e-8 g/ml

Additional target cell option: Raji cells

Frozen, thaw-and-use FcγRIIIa/NFAT-luc2 Effector Cells (75K per well)



Raji cells/well

	10K	12.5K	15K
EC50	125.5	150.3	122.3

Accuracy, linearity & precision using frozen, thaw-and-use Raji cells and CD20 Ab

Analyst 1

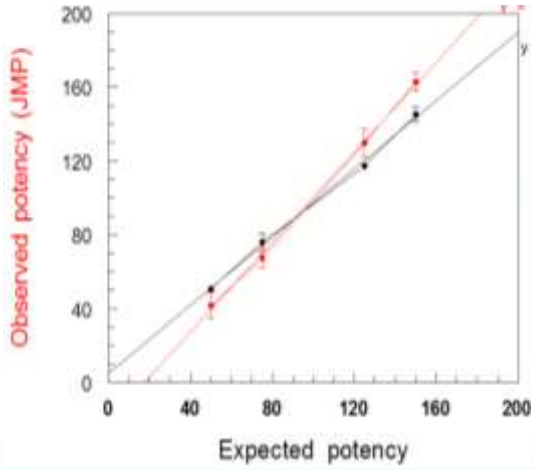
Day	Antibody Sample	Measured Potency (%)	Mean Potency (%)	SD (%)	Recovery (%)	CV (%)
1	50%	49.9	51	0.7	102	1.4
2		51.3				
3		50.5				
1	75%	78.9	76	5.11	101	6.7
2		78.8				
3		70				
1	125%	118.6	117	1.19	94	1
2		116.9				
3		116.3				
1	150%	143.2	145	3.91	97	2.7
2		142.5				
3		149.6				

Precision: 2.95%

Accuracy: (recovery average): 98.5%

Linearity: Y=0.922x+5.0

Linearity



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				

Precision: 8.47%

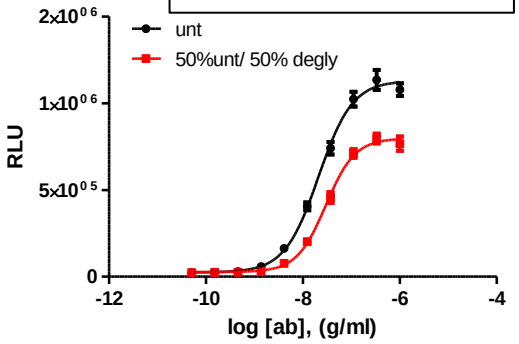
Accuracy (recovery average): 96.4%

Linearity: Y=1.22x-21.3

Detection of small differences in Fc effector biological activity of therapeutic antibody preps

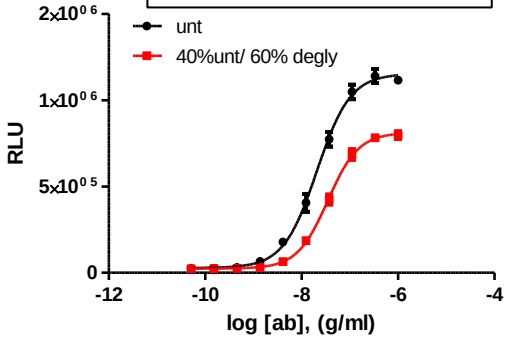
Differentiation of blended % mixes of fully N-glycosylated & fully deglycosylated Trastuzumab

50% deglycosylated



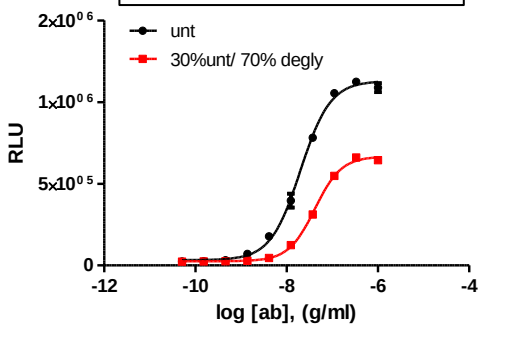
	unt	50%unt/ 50% degly
EC50	2.110e-008	2.990e-008

60% deglycosylated



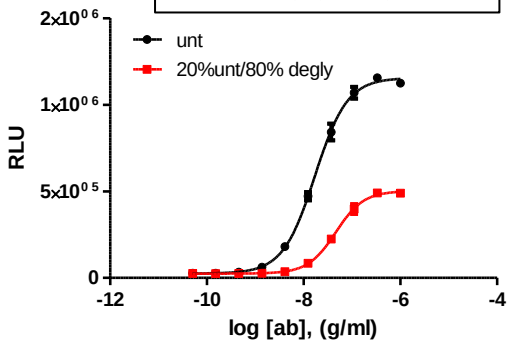
	unt	40%unt/ 60% degly
EC50	2.082e-008	3.486e-008

70% deglycosylated



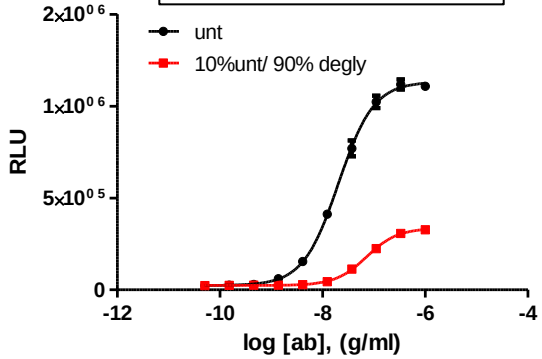
	unt	30%unt/ 70% degly
EC50	2.002e-008	4.153e-008

80% deglycosylated



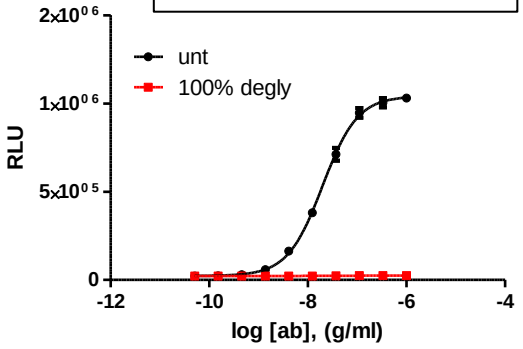
	unt	20%unt/80% degly
EC50	1.720e-008	4.626e-008

90% deglycosylated



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

100% deglycosylated

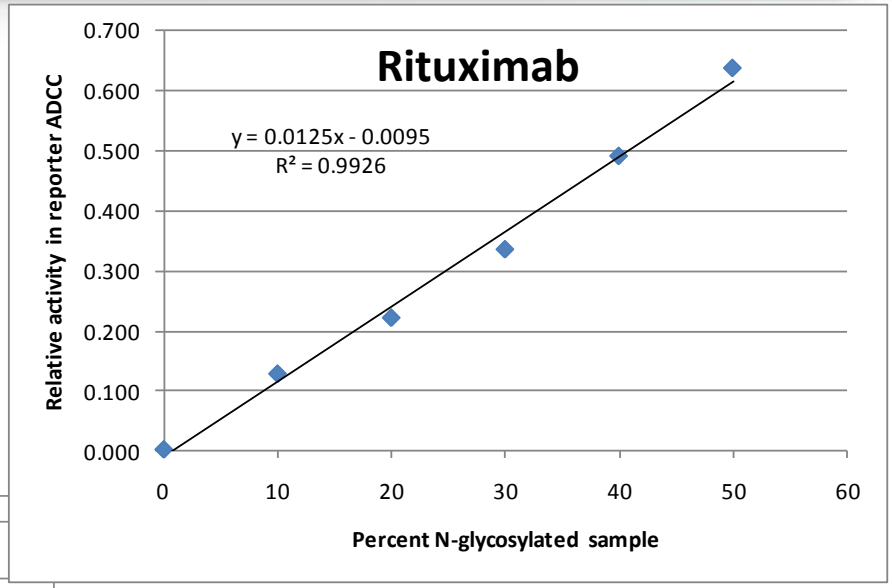


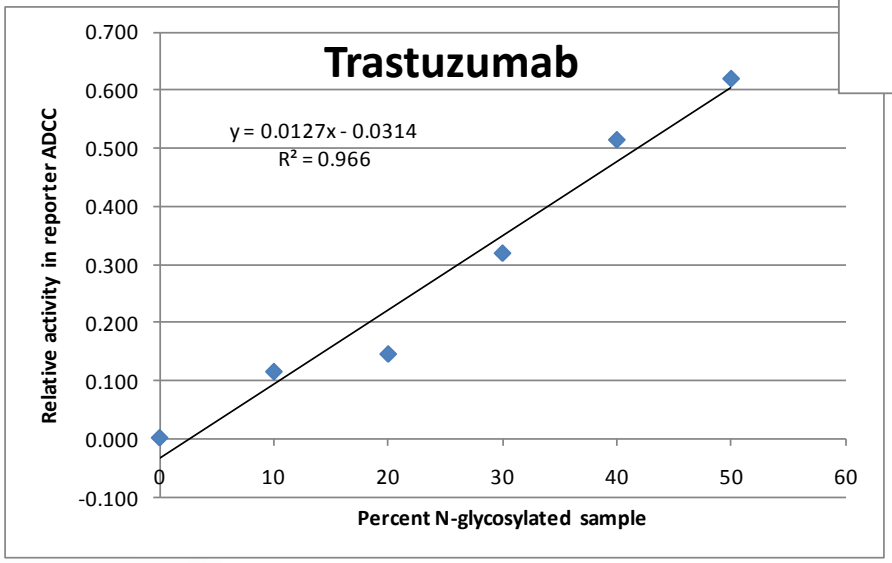
	unt	100% degly
EC50	1.988e-008	3.202e-008

Target cells: SKBR3; Unt = 100% glycosylated

Fc effector biological activity correlates well with antibody N-glycosylation

Rituximab and Trastuzumab:
Good correlation between blended % mixes of fully N-glycosylated and deglycosylated preparations and their relative biological activity in ADCC Reporter Bioassay





Small differences in Fc effector activity in ADCC MOA pathway are easily distinguished in the ADCC reporter bioassay

Planned ADCC Reporter Bioassay kit configurations

Core Kit

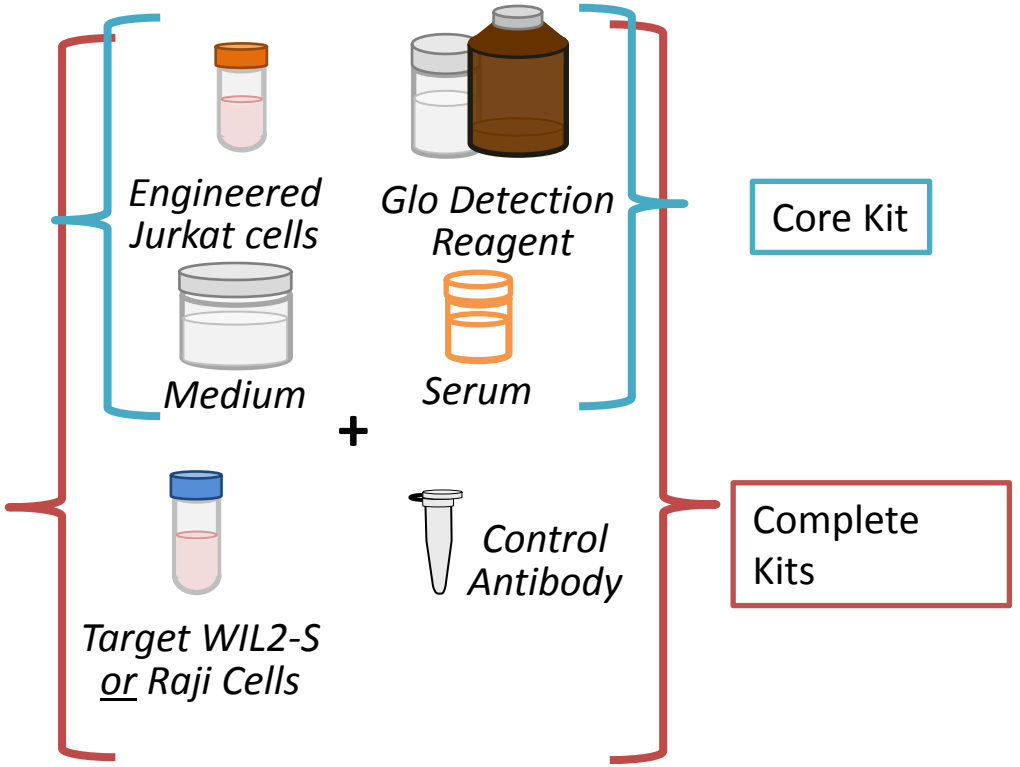
- Engineered Jurkat Effector cells (NFAT-RE-luc2 / FcγRIIIa); *frozen, thaw-and-use*
- Bio-Glo™ Luciferase Assay System
- RPMI Medium
- Low IgG Serum

Use with different Abs and target cells

Complete Kits (2 types)

- *Above PLUS:*
- Target WIL2-S cells; *frozen, thaw-and-use* or Target Raji cells; *frozen thaw-and-use*
- Control Ab (CD20)

Use as assay control, or when WIL2-S or Raji target cells are suitable or target is CD20



Summary of the ADCC Reporter Bioassay

Promega

Design Features

1. Low variability
2. Engineered effector cells to replace primary NK cells (Jurkat FcγRIIIa/NFAT-RE-luc2)
3. "Cells as reagents", frozen, thaw-and-use format – consistency & convenience
4. Simplicity and robustness of assay protocol and reagents
5. Broad applicability to 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 differences in relative biological activity of Fc effector function in ADCC MOA that result from small changes in Ab glycosylation

For more information

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