Better Cell-Based Assays to Measure Co-Inhibitory and Co-Stimulatory Receptors in the Development of Therapeutic Antibodies

Richard Somberg, Ph.D. Strategic Collaborations Manager

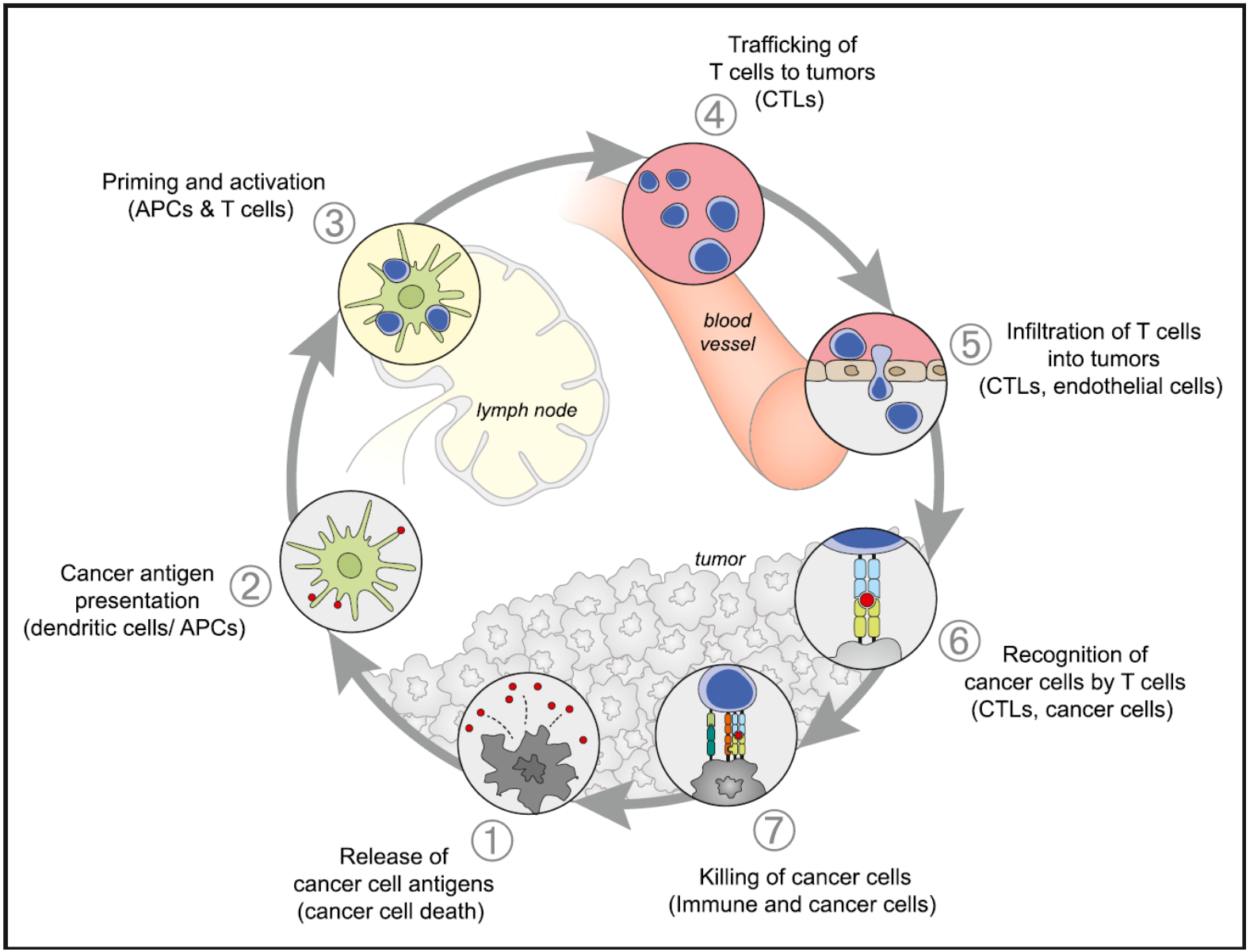
Outline

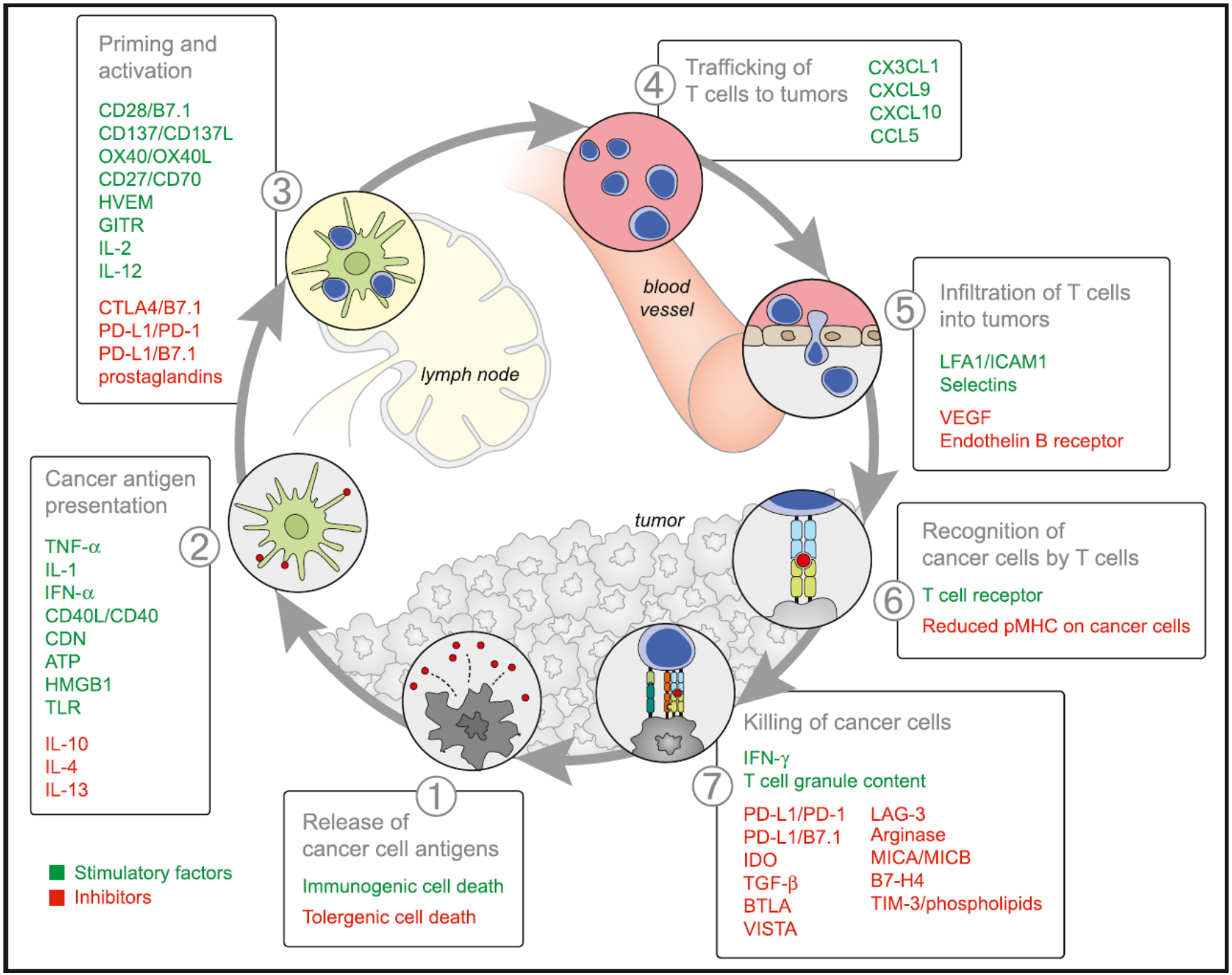
- Overview of Immunotherapy Targets
- General Development of Bioassays
- Co-inhibitory receptor bioassays: PD-1, CTLA-4, LAG3, Tim3
- Co-stimulatory receptor bioassays: GITR, CD40, OX40, 4-1BB
- T cell activation bioassays: CD3, CTLA-4 Fc
- Commercial formats - *Kits & Cell Propagation Model*

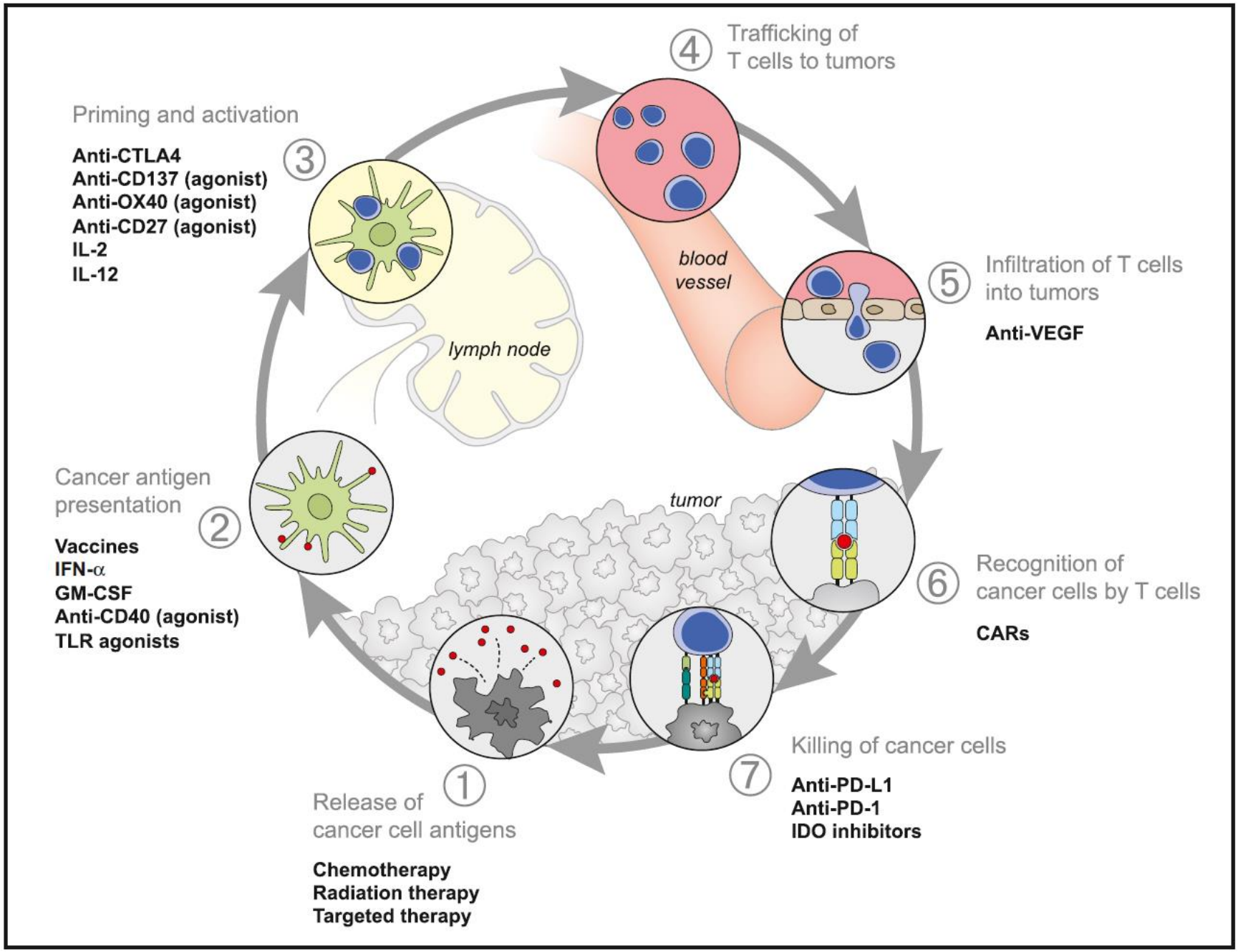
Oncology Meets Immunology: The Cancer Immunity Cycle

Daniel S. Chen and Ira Mellman, *Immunity* **39**(1), 25 July 2013, Pages 1–10

Promega

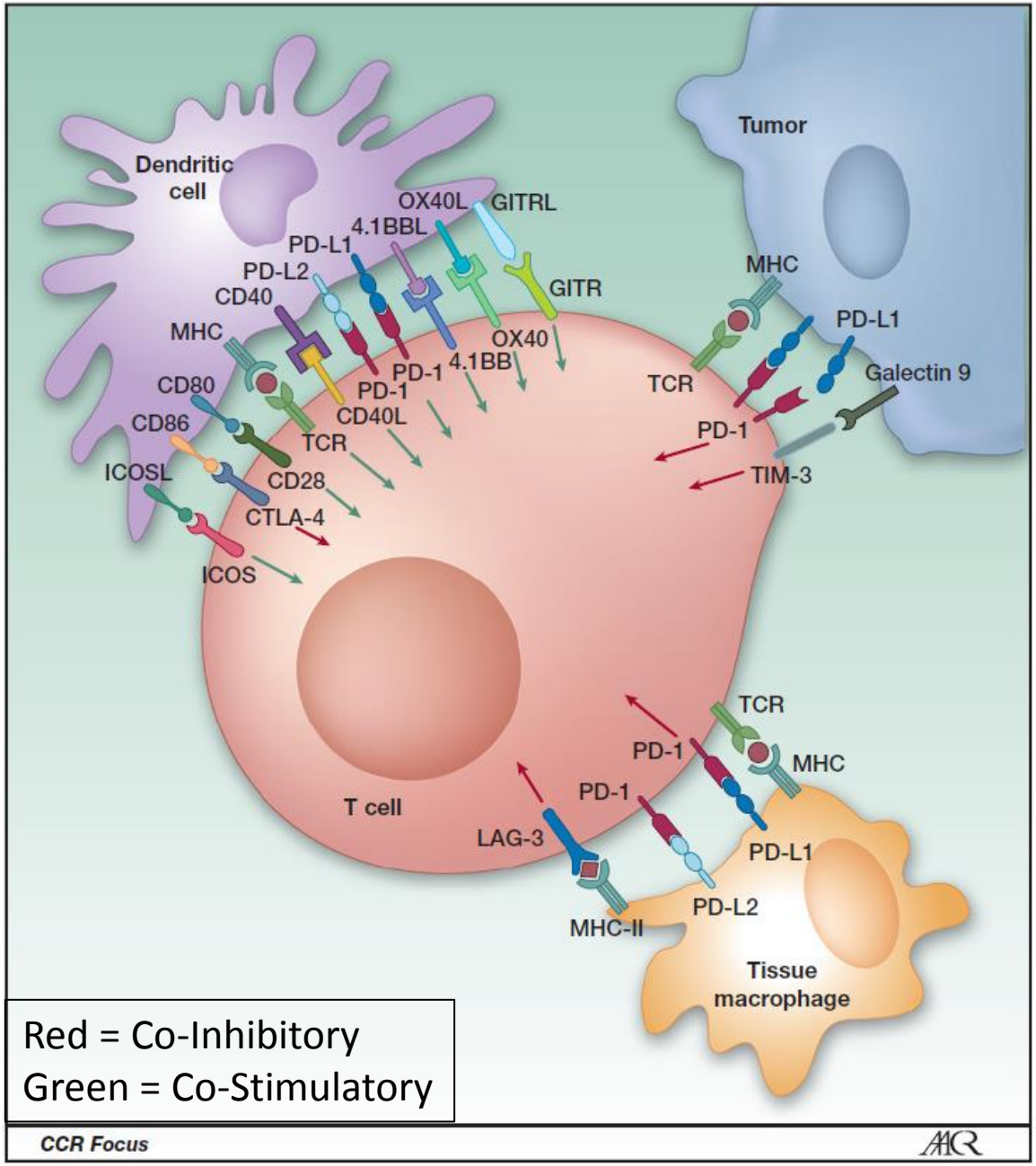



Priming and activation
CD28/B7.1
CD137/CD137L
OX40/OX40L
CD27/CD70
HVEM
GITR
IL-2
IL-12
CTLA4/B7.1
PD-L1/PD-1
PD-L1/B7.1
prostaglandins
3
4
Trafficking of T cells to tumors
CX3CL1
CXCL9
CXCL10
CCL5
blood vessel
lymph node
5
Infiltration of T cells into tumors
LFA1/ICAM1
Selectins
VEGF
Endothelin B receptor
Cancer antigen presentation
TNF-α
IL-1
IFN-α
CD40L/CD40
CDN
ATP
HMGB1
TLR
IL-10
IL-4
IL-13
2
tumor
6
Recognition of cancer cells by T cells
T cell receptor
Reduced pMHC on cancer cells
1
Release of cancer cell antigens
Immunogenic cell death
Tolergenic cell death
7
Killing of cancer cells
IFN-γ
T cell granule content
PD-L1/PD-1
PD-L1/B7.1
IDO
TGF-β
BTLA
VISTA
LAG-3
Arginase
MICA/MICB
B7-H4
TIM-3/phospholipids
Stimulatory factors
Inhibitors


4 Trafficking of T cells to tumors
Priming and activation
Anti-CTLA4
Anti-CD137 (agonist)
Anti-OX40 (agonist)
Anti-CD27 (agonist)
IL-2
IL-12
3
blood vessel
5 Infiltration of T cells into tumors
Anti-VEGF
lymph node
Cancer antigen presentation
2
Vaccines
IFN-α
GM-CSF
Anti-CD40 (agonist)
TLR agonists
tumor
6 Recognition of cancer cells by T cells
CARs
7 Killing of cancer cells
Anti-PD-L1
Anti-PD-1
IDO inhibitors
1 Release of cancer cell antigens
Chemotherapy
Radiation therapy
Targeted therapy

"Immune checkpoint blockade has established a new standard for the treatment of cancer with prospects for clinical benefit durability in patients with melanoma. Our improved understanding of the mechanisms involved with immune regulation now provides a strong foundation for the development of combinatorial approaches of immune therapeutic, small molecule targeted, and antiangiogenic strategies to improve patient outcomes."

CTLA-4 and PD-1/PD-L1 Blockade: New Immunotherapeutic Modalities with Durable Clinical Benefit in Melanoma Patients. Patrick A. Ott, F. Stephen Hodi, and Caroline Robert. Clin Cancer Res; 19(19); 5300–9 (October 1, 2013).



Revenue Projections for Immune checkpoint drugs…

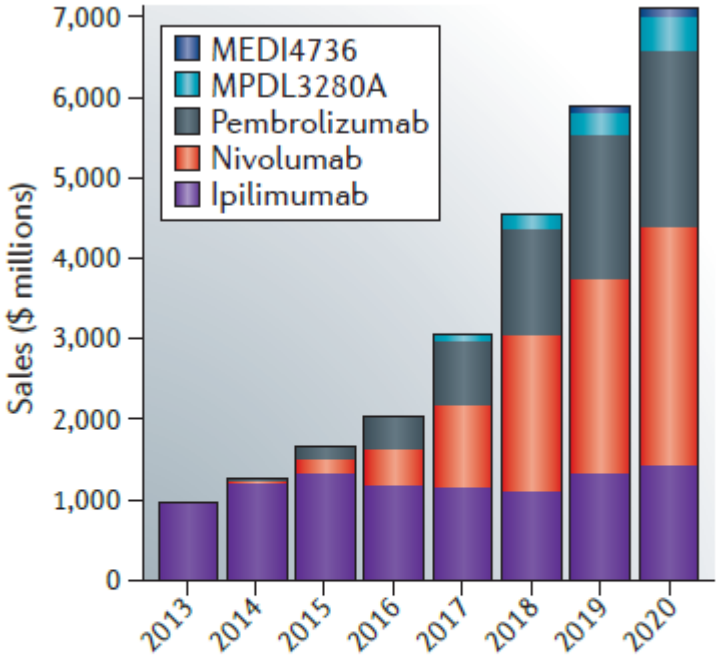


Figure 1 | **Major-market sales of select immune checkpoint inhibitors (estimated).** Sales forecast in the seven major markets (United States, France, Germany, Italy, Spain, United Kingdom and Japan) from 2013 to 2020. Sales shown in US$ million.

Projections correspond to 33% annual growth

Many companies are exploring combinatorial approaches

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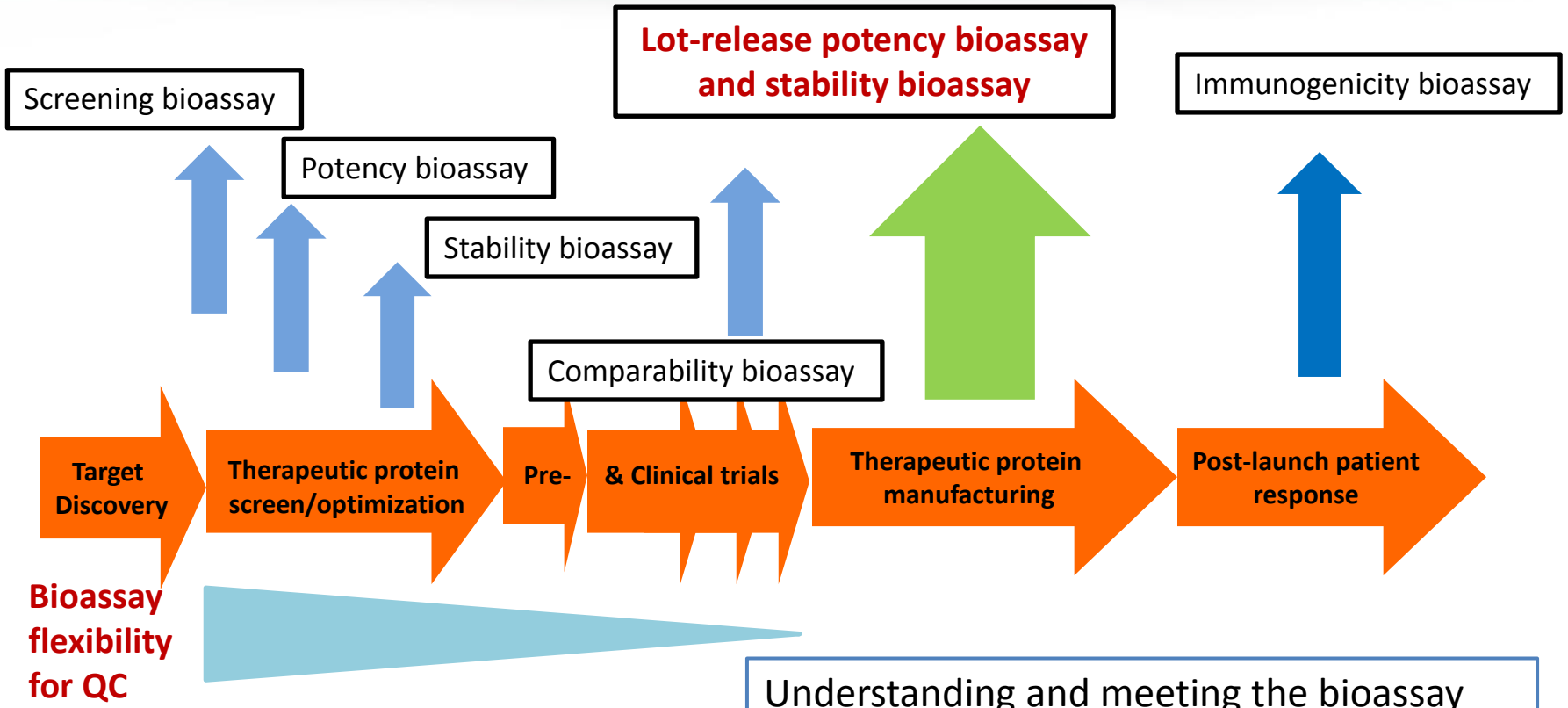
Rachel M. Webster

Bioassay Development

Bioassays in Antibody Drug Development

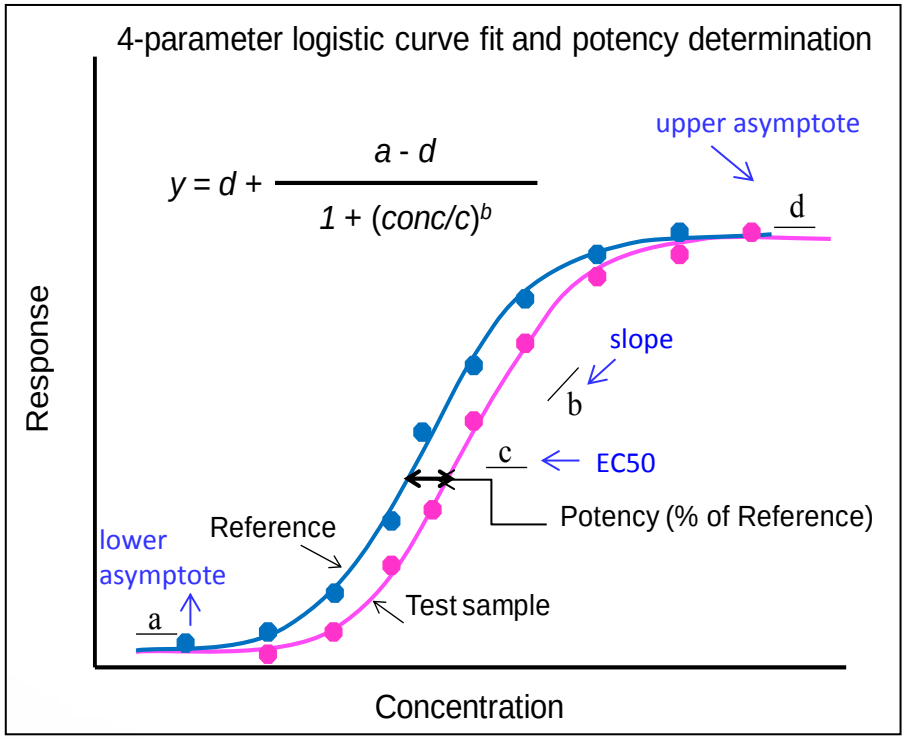


Understanding and meeting the bioassay needs of different areas during biologic drug development and manufacture

- Less variability
- Higher reproducibility

Lot Release Bioassays Determine Relative Potency

Relative Potency: the potency activity of unknown biologic ***relative*** to a reference standard



Relative potency can only be determined when:

- The upper and lower asymptotes as well as the slopes of the curves are not significantly different. Hence the curves are parallel
- Only the EC_{50}s differ
- Relative potency calculation:

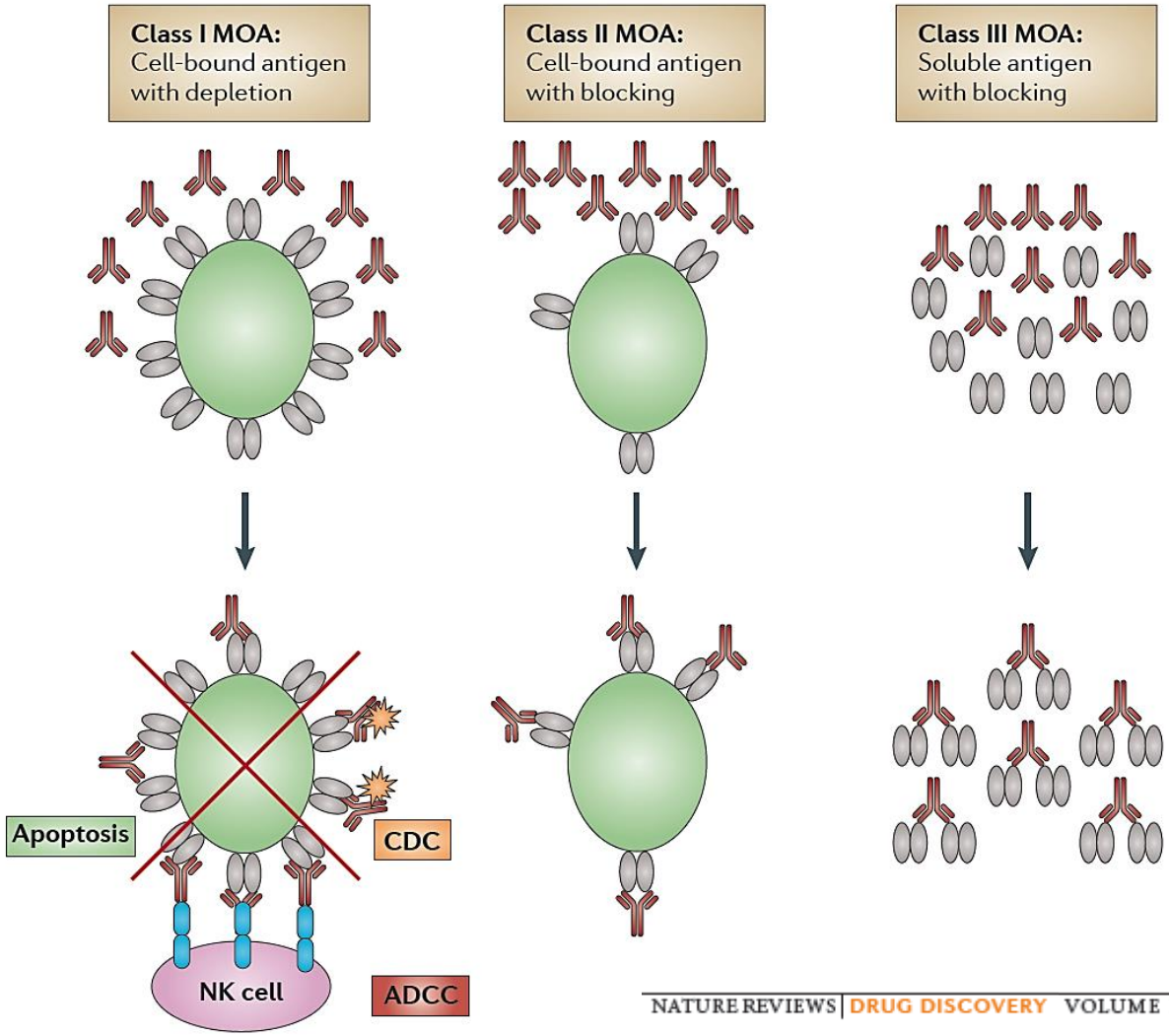
$$\frac{EC_{50}\ Reference}{EC_{50}\ Test\ sample}$$

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)

Mechanism of Action (MOA) for mAb



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Bioassay Qualification – ICH guideline Q2 [R1]

- **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 the presence of target cells and FcγRIIa on effector cells, 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)

Bioassay Development Using DOE

DOE = Design of Experiments

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

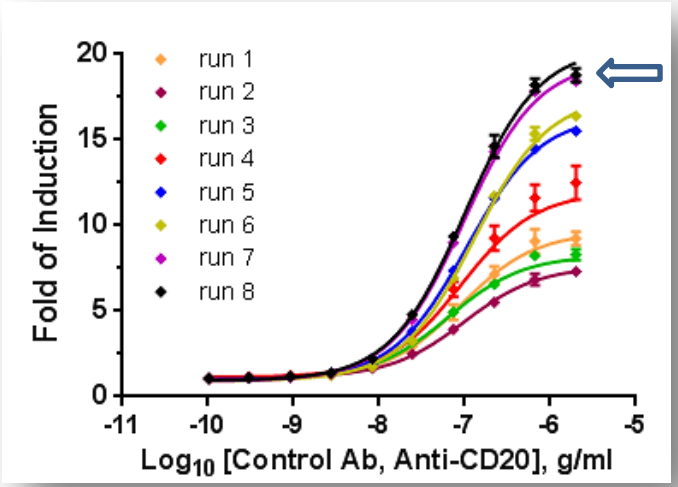
Run/plate	JMP#	cell density (K)	incubation time @37°C	Cell density (k)	induction time hr
1	−−−−	7.5	0	75	5
2	+−+−	12.5	0	60	5
3	−−++	7.5	0	60	6
4	+−−+	12.5	0	75	6
5	−++−	7.5	30	60	5
6	++−−	12.5	30	75	5
7	−+−+	7.5	30	75	6
8	++++	12.5	30	60	6

Factors:

- Target cell density
- Target cell/antibody incubation time
- Effector cell density
- Induction time

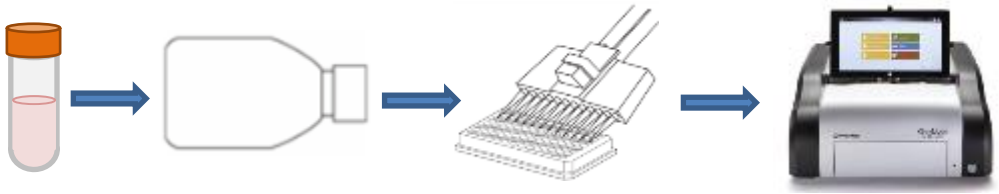
Outputs:

- Fold of induction
- EC_{50}
- EC_{50} difference between F and V variant Jurkat effector cells



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

Time: 1-2 weeks

Frozen, thaw-and-use cells: fast, simple, and improved reproducibility

Thaw-and-Use → Resuspend in assay buffer, plate for assay → Read plates

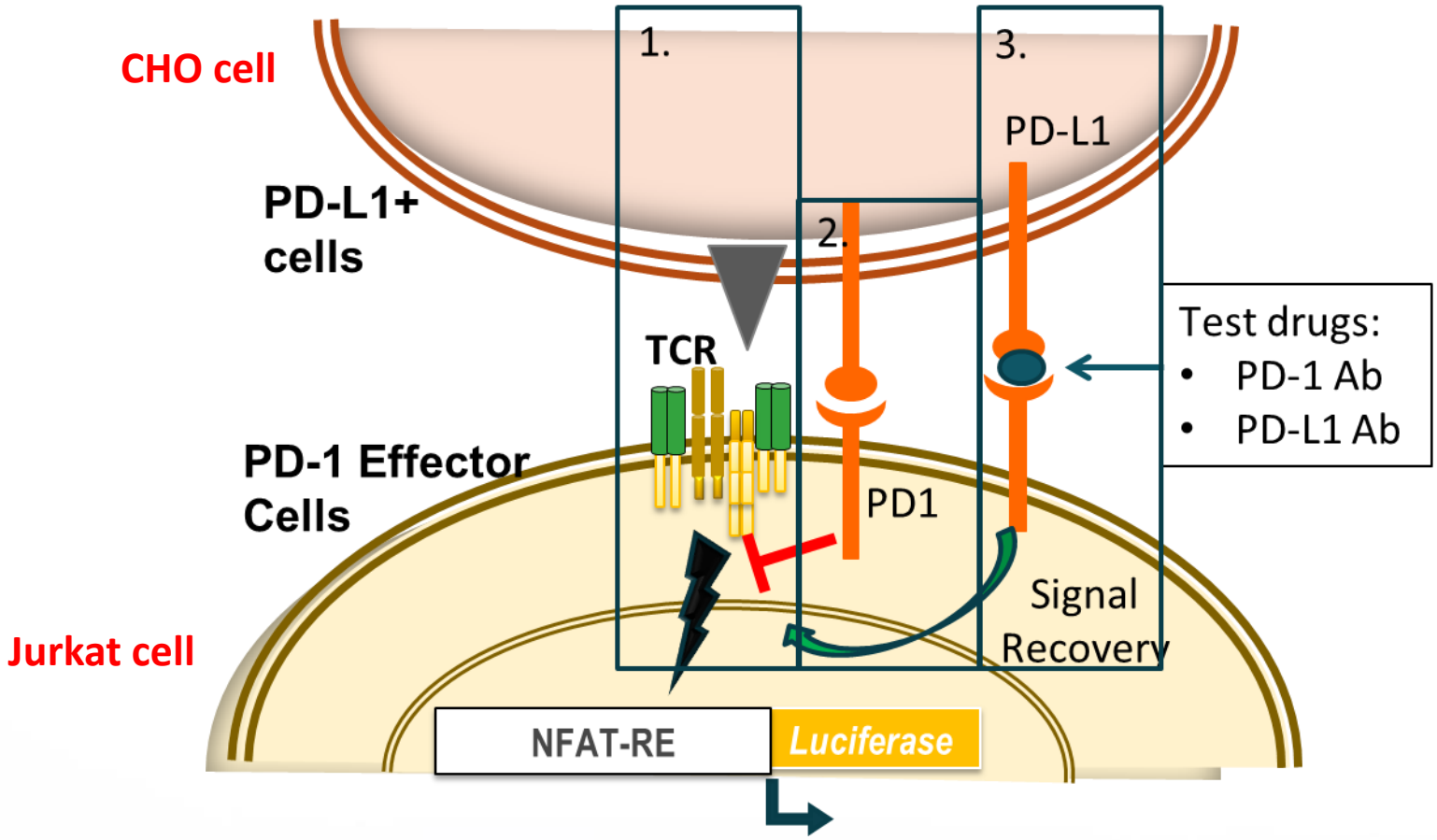
Time: < 24hr

Co-Inhibitory Receptor Bioassays

Customer Pain Points

- A functional cell-based assay is a key requirement in any drug discovery program. Building one to monitor an immune response is extremely challenging.
- Possible readouts include T-cell proliferation, cytokine production, FACS analysis, and target cell death.
- All are highly relevant, yet problematic due to multiple cell types being present in the assay, long assay times, and variability with sources of primary cells.
- Promega bioassays are designed to reproduce the MOA in a much less variable format, thereby allowing these cell-based assays to be used in QC lot release potency testing.

PD-1 / PD-L1 Bioassay Principle



PD-1/PD-L1 Blockade Bioassay Protocol

Bioassay starts with an overnight incubation of PD-L1+ CHO cells, then proceeds like a traditional reporter bioassay.

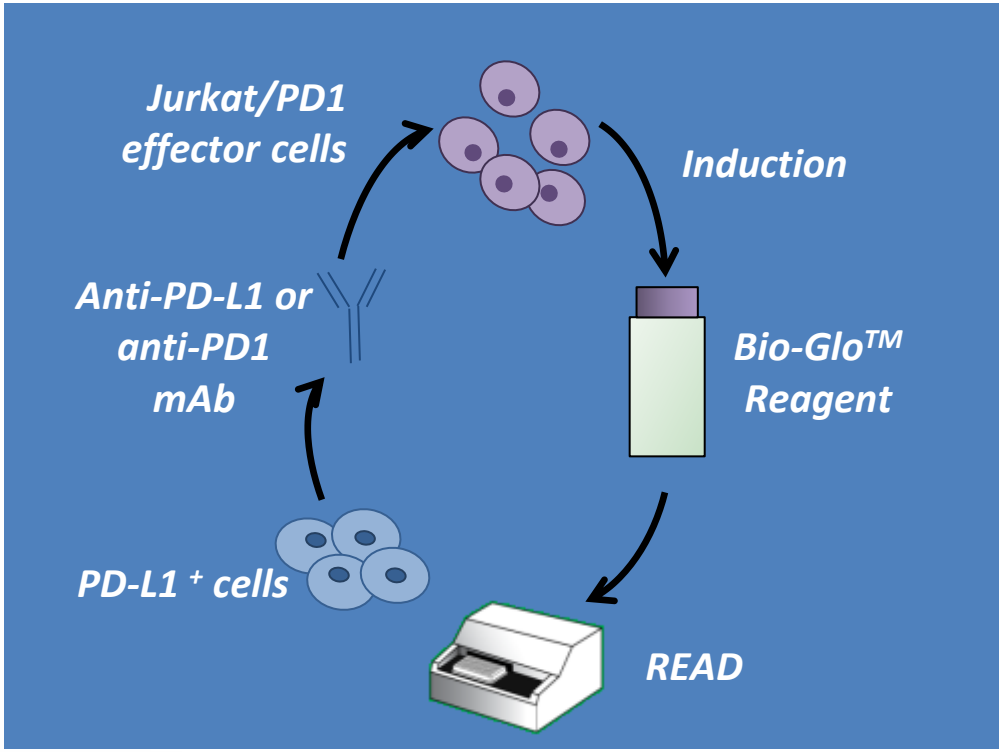


Plate PD-L1+ cells overnight

↓

Add anti-PD-1 or anti-PD-L1 mAb at serial dilution

↓

Add PD-1 effector cells

↓

6hr induction

↓

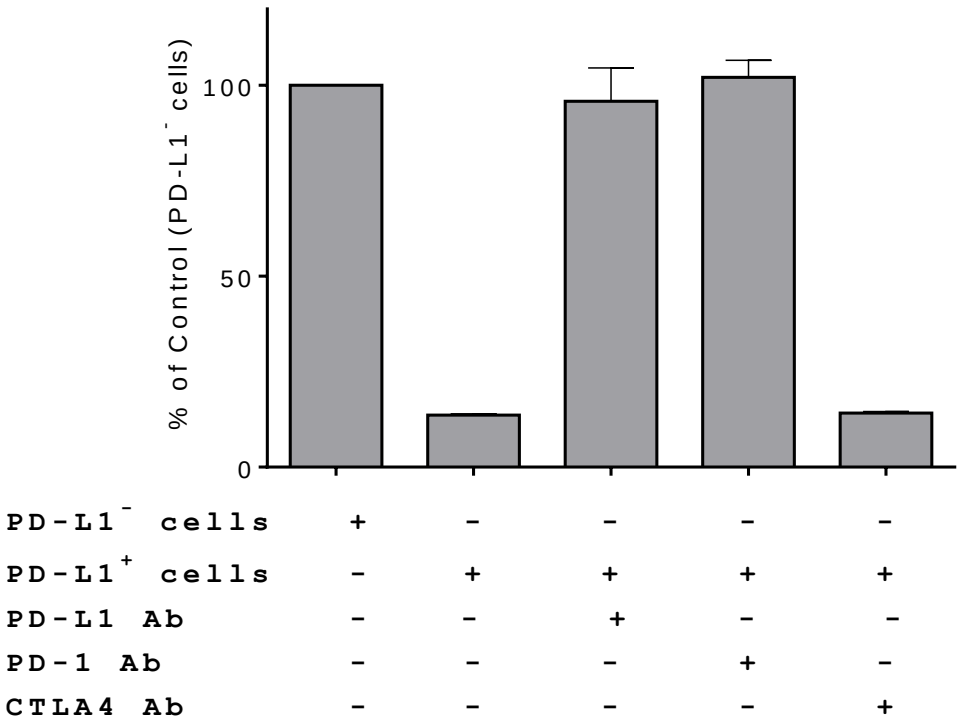
Add Bio-Glo™ detection reagent, measure luciferase activity

PD-1/PD-L1 Blockade Bioassay Results

1. Cross linking of TCR on NFAT Jurkat cells by the “TCR activator” on the CHO cell results in expression of luciferase.

2. The amount of luciferase expression is reduced when PD-1 and PD-L1 are present.

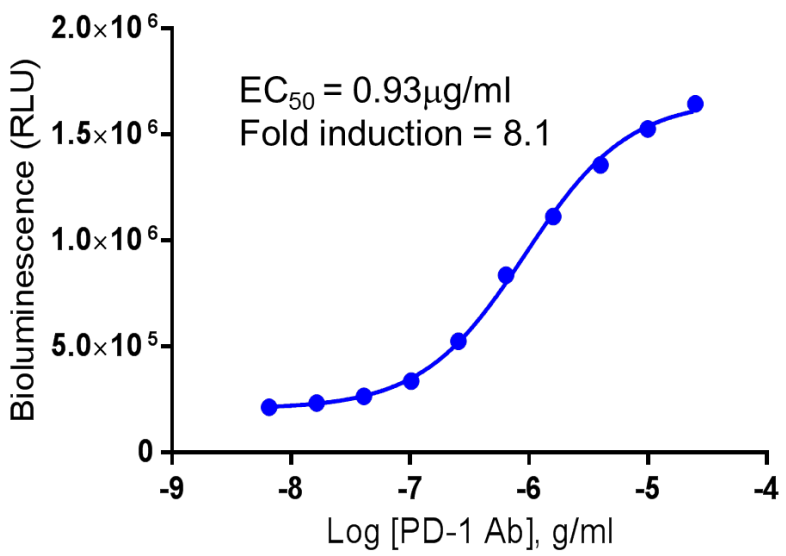
3. Agents that block the interaction of PD-1 and PD-L1 are able to recover the levels of luciferase expression.



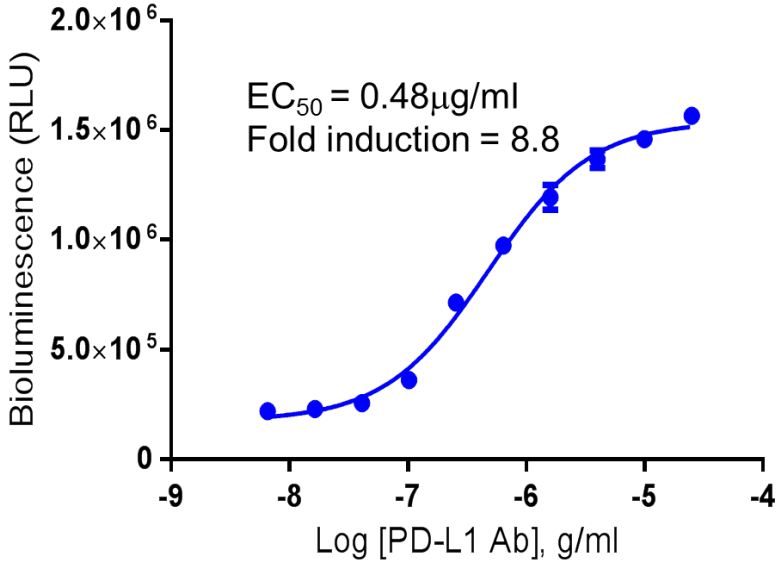
<u>Goal</u>: find blocker Abs that result in the highest levels of recovery with the least amount of drug

Dose Response with PD-1 and PD-L1 Ab

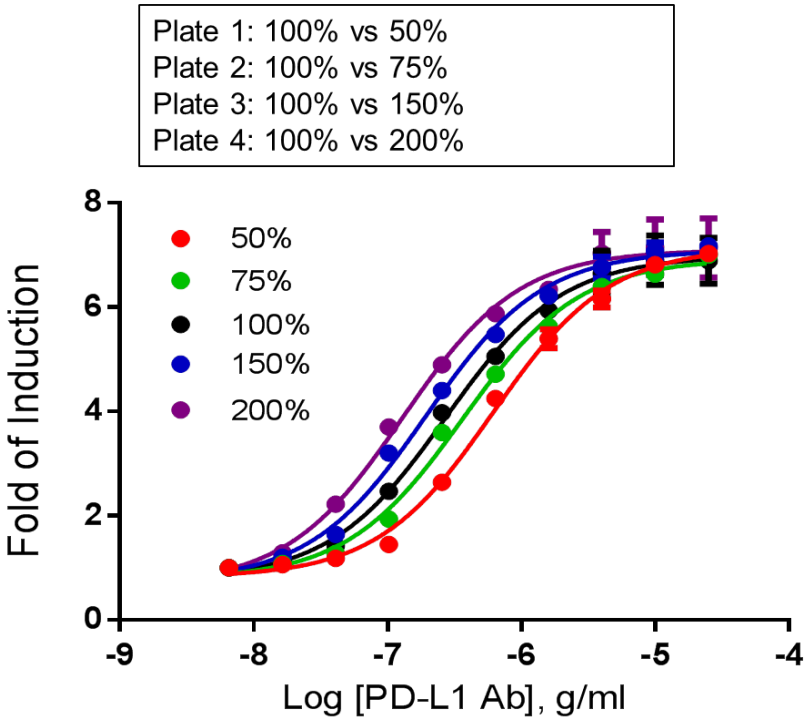
Anti-PD-1 antibody



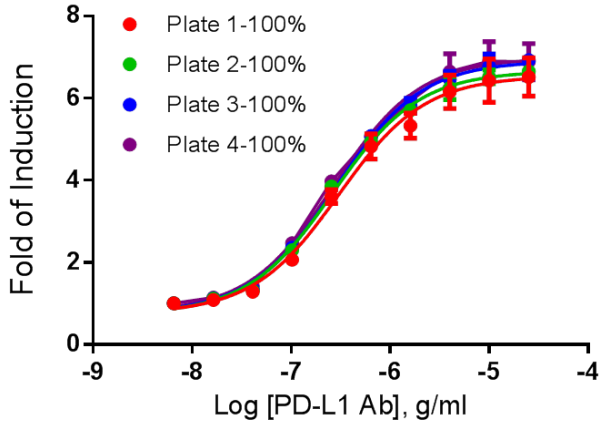
Anti-PD-L1 antibody



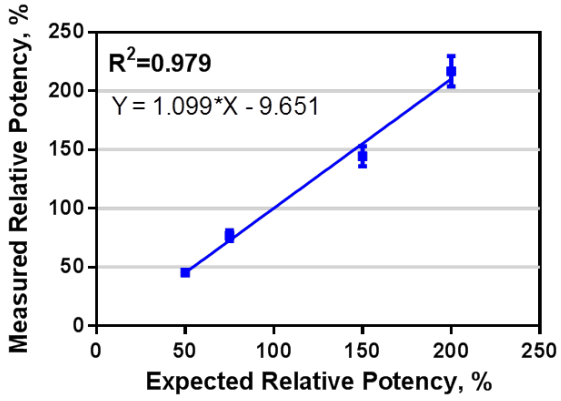
PD-1 / PD-L1 Qualification Results



Repeatability



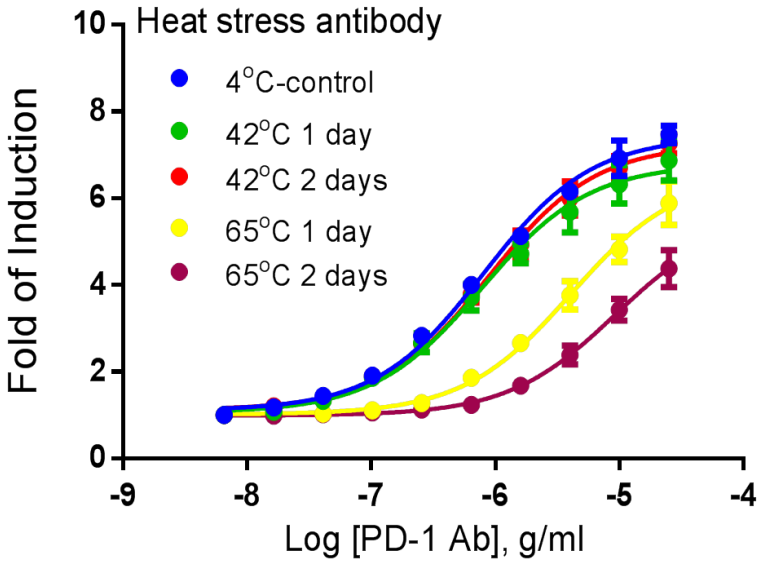
Linearity



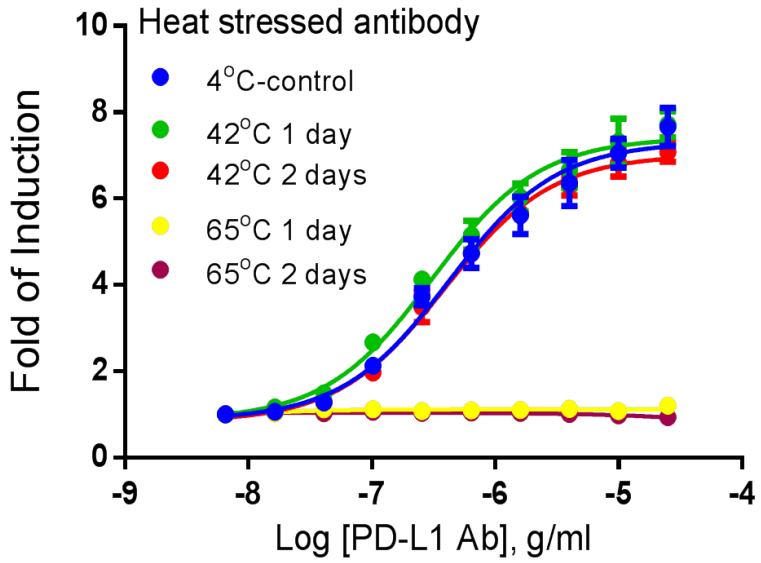
Measuring relative potency for PD-L1 Ab showing assay repeatability and linearity in the range of 50% - 200%.

PD-1 / PD-L1 Bioassay is Stability Indicating

Anti-PD-1 antibody



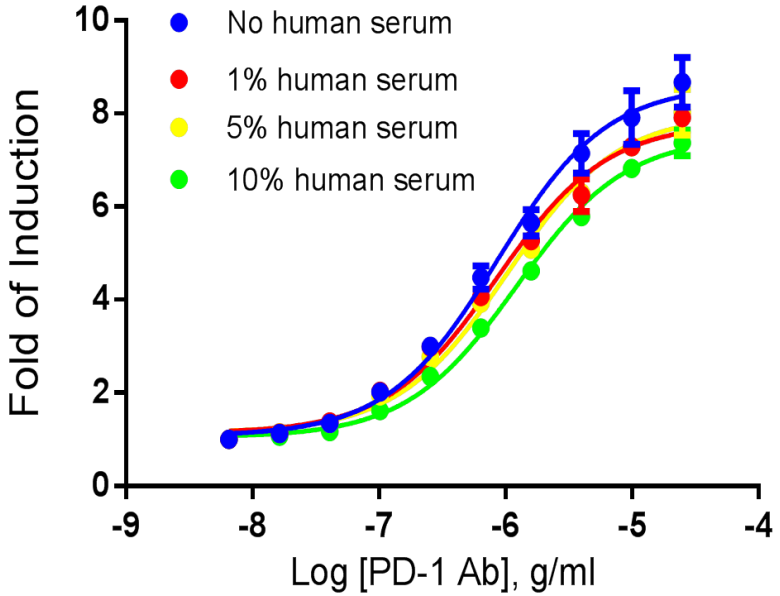
Anti-PD-L1 antibody



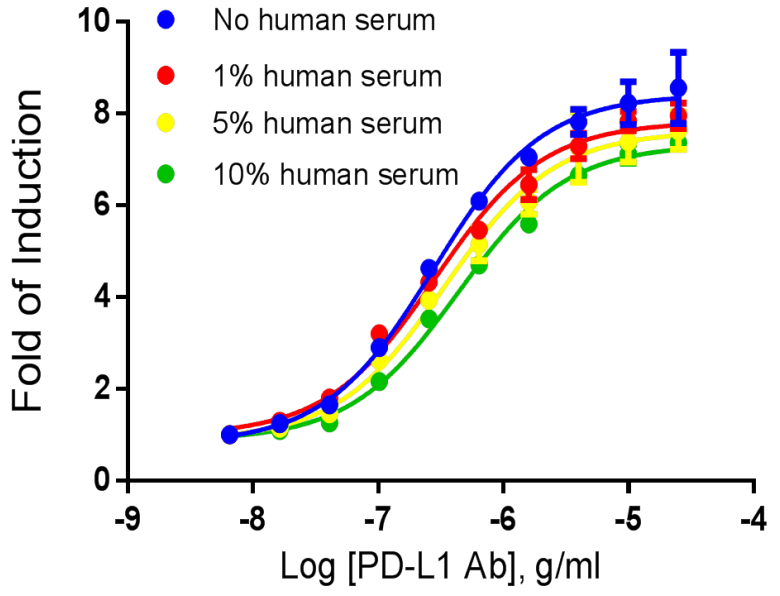
Detecting the loss of reporter response to PD-1 or PD-L1 blocking antibody after heat-treatment in PD-1 Effector cells.

PD-1 / PD-L1 Bioassay – test with Human Serum

Anti-PD-1 antibody

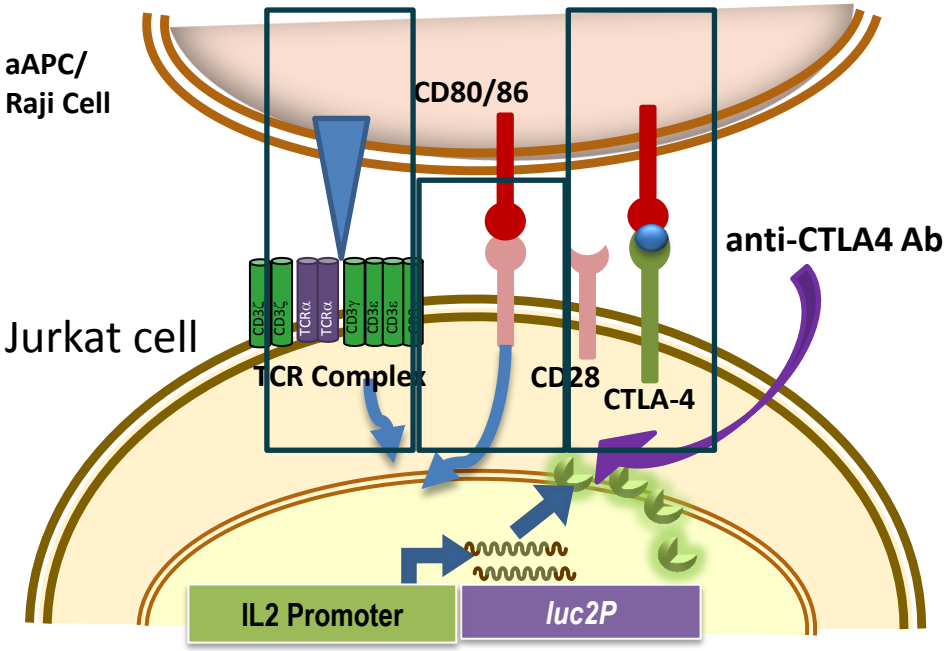


Anti-PD-L1 antibody



The assay is minimally impacted by human serum up to 10% and is suitable for further development to a neutralizing antibody assay.

CTLA-4 Bioassay Principle



1. By co-cultivating the two cell lines, Jurkat IL2 pathway is activated via antibody crosslinking of TCR activator/TCR complex.

2. CTLA-4 binds to CD80/86 with greater affinity than CD28, resulting in blocking T-cell activation.

3. Blockade of CTLA-4:CD80/86 interaction with anti-CTLA-4 mAb can reactivate IL2 pathway in a dose-dependent manner.

CTLA-4 Bioassay Protocol

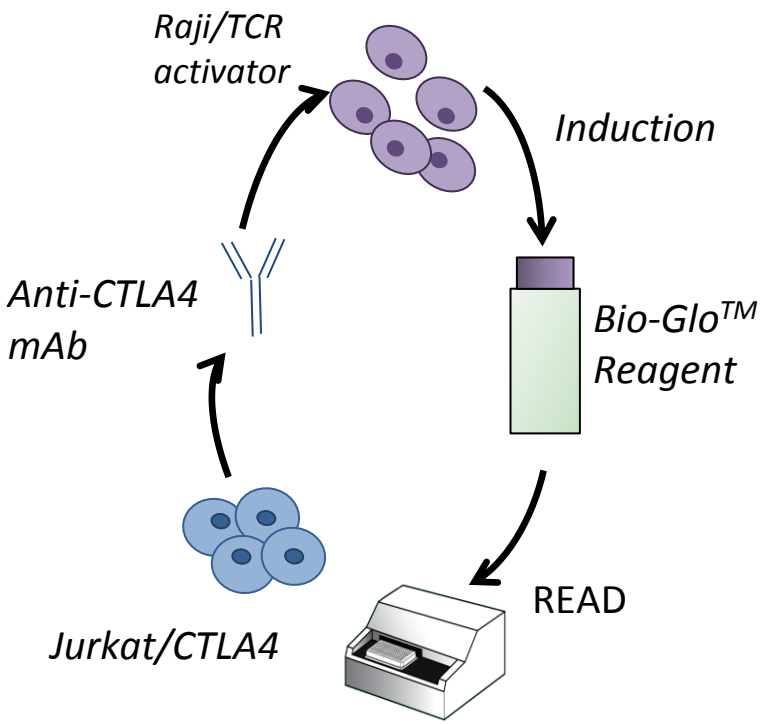


Plate Jurkat/CTLA4 cells

↓

Add anti-CTLA4 antibody at serial dilution

↓

Add Raji/ TCR activator cells

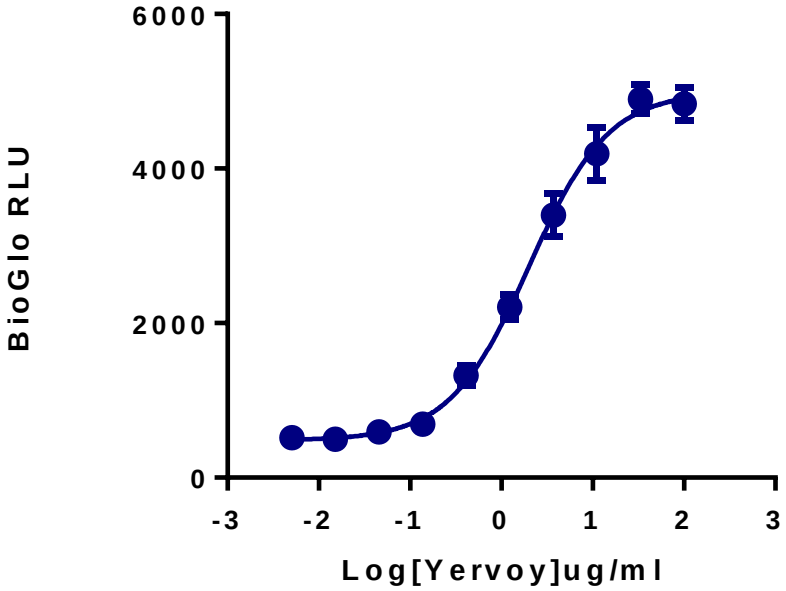
↓

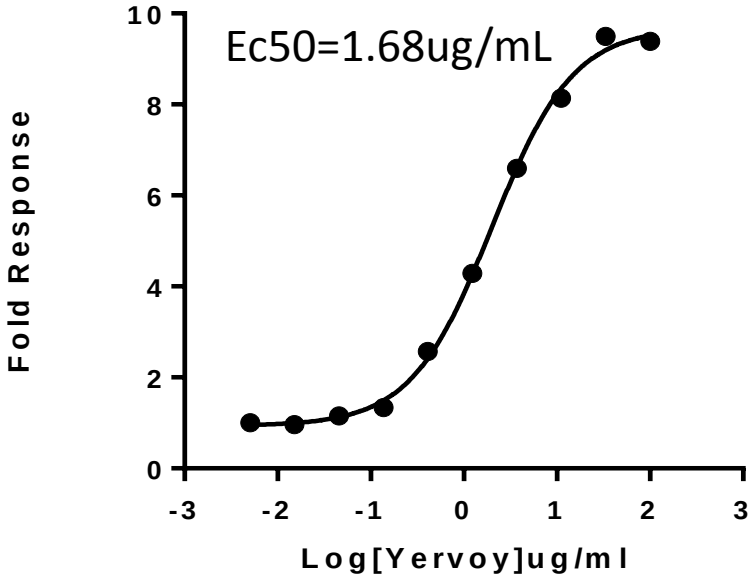
16 hr Induction

↓

Add Bio-Glo™ detection reagent, measure luciferase activity

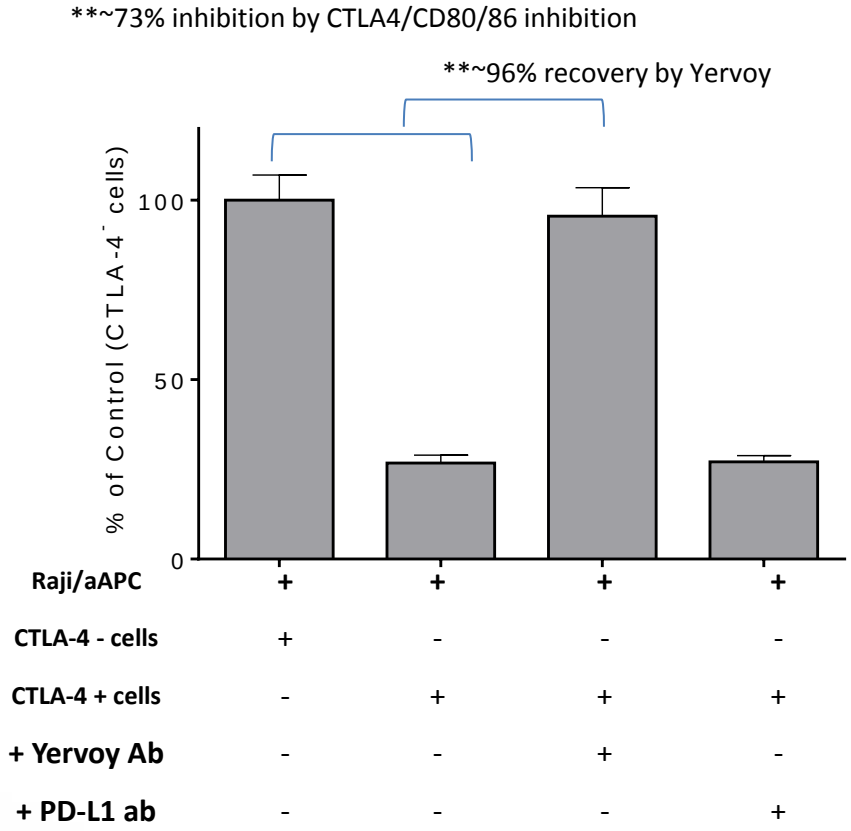
Measure the Potency of Ipilimumab (Yervoy)

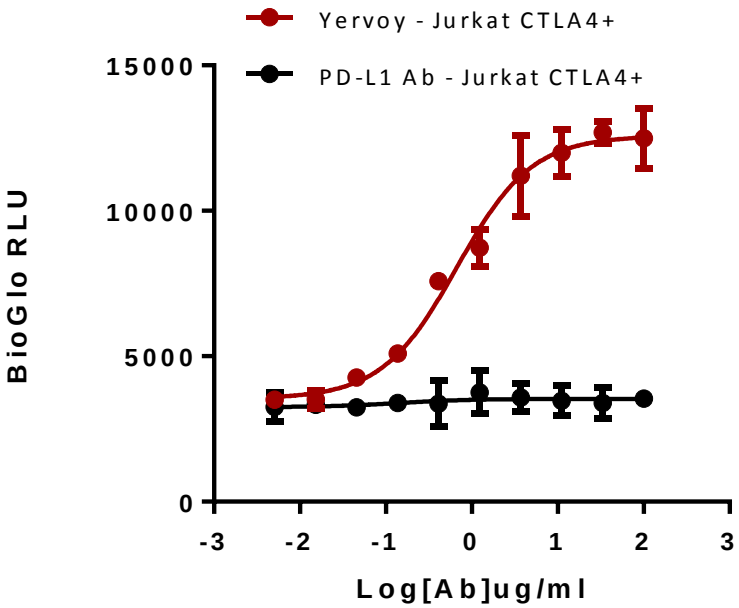




- Plate Jurkat/Il2-Luc2P/CTLA-4, 100K per well in 96 well plate
- Add serial dilution of Ipilimumab
- Add Raji/TCR activator cells, 5K per well
- Induction for 16hrs
- Assay using Bio-Glo™ Luciferase Assay System

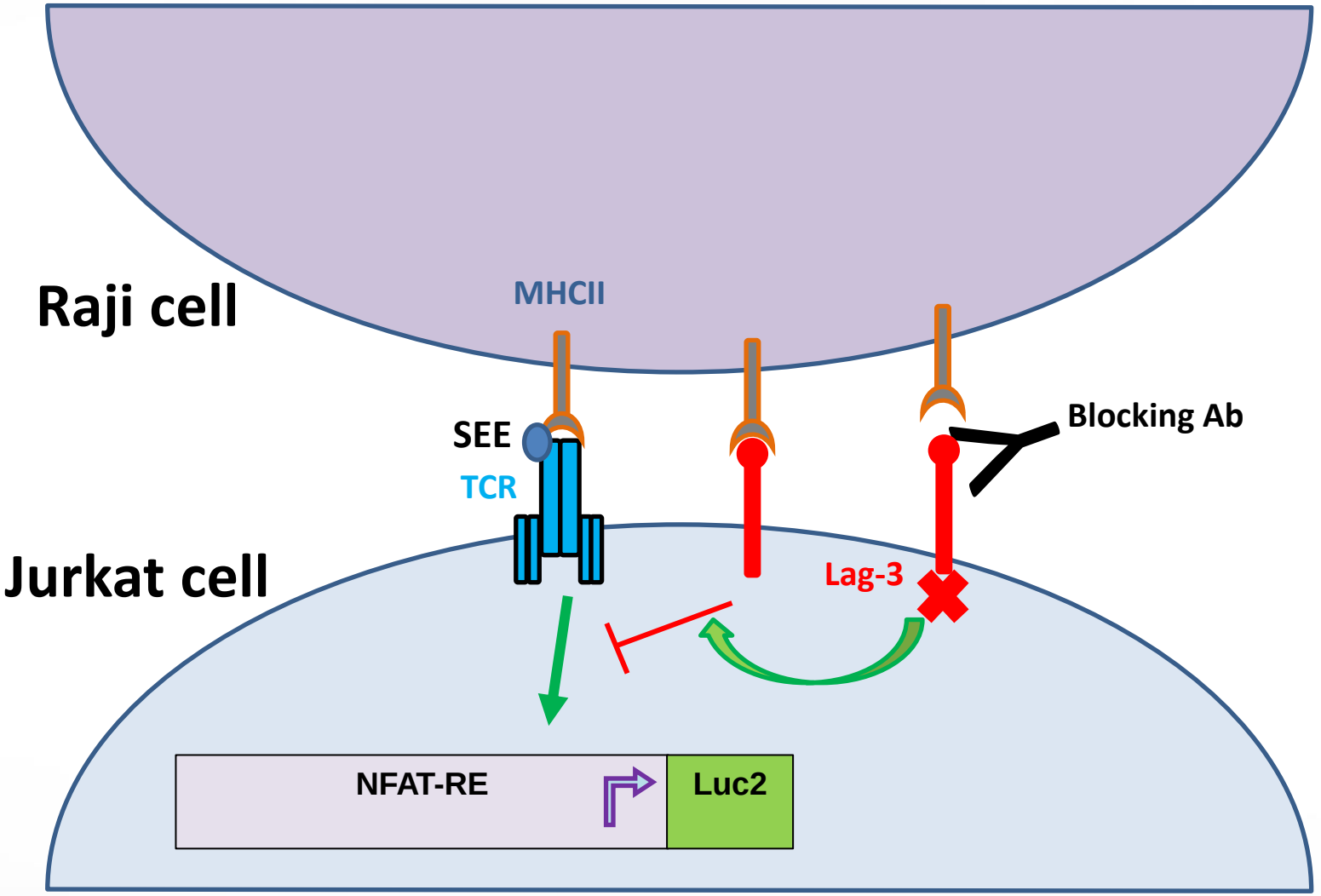
CTLA-4 Bioassay is Specific



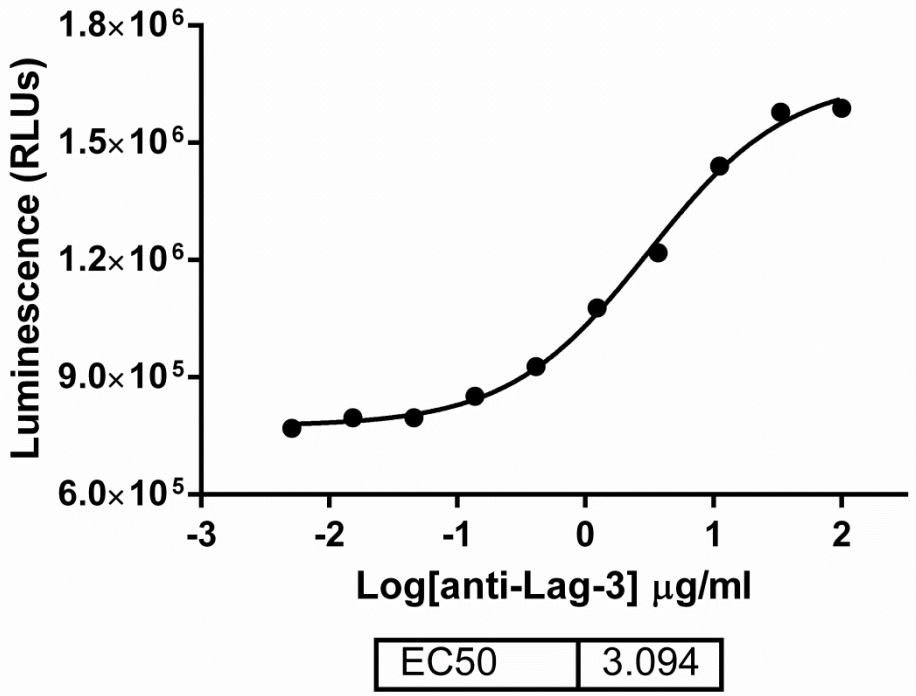


* Assay was done with 6hr Yervoy incubation

Lag-3 / MHC II Bioassay Principle



Lag-3 / MHCII Bioassay Results



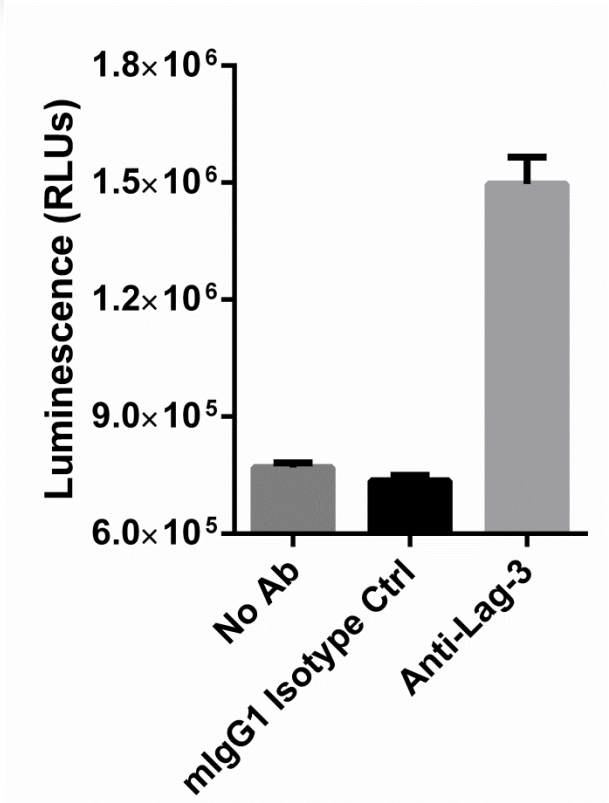
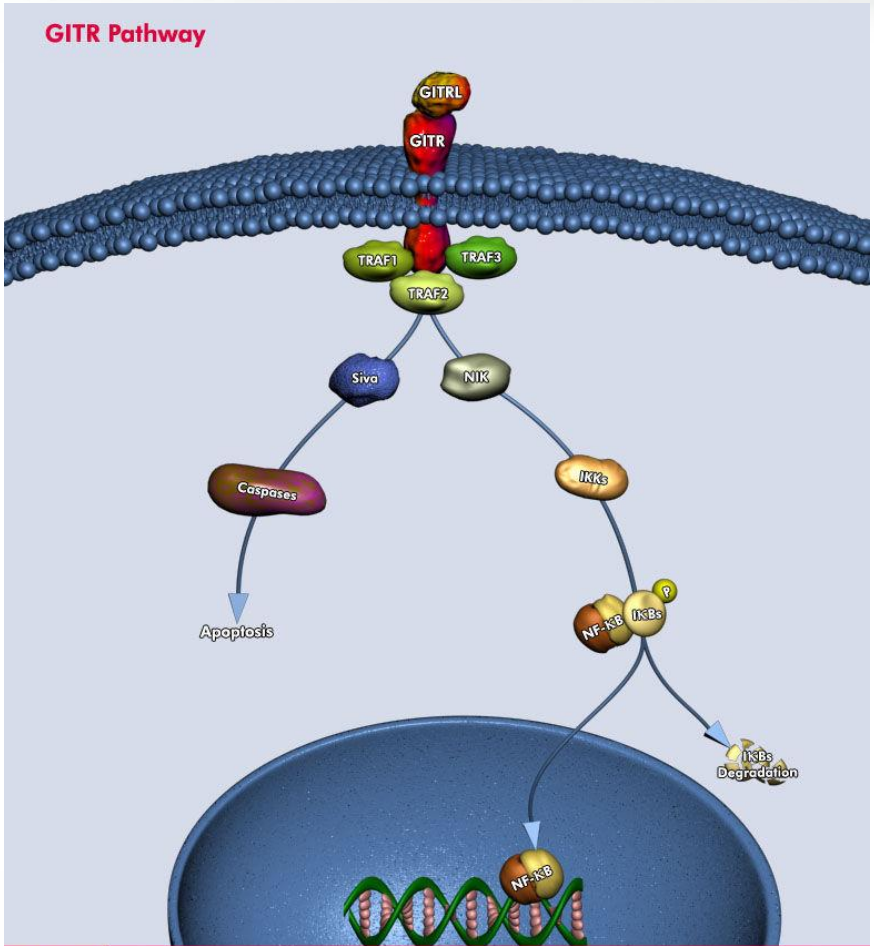


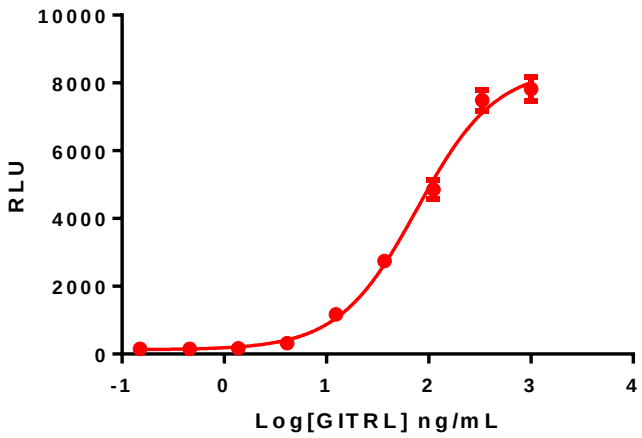
Plate Raji cells, add SEE, add blocking Ab (clone 17B4), add Lag-3+ NFAT Jurkat cells, incubate for 6 hours and then add Bio-Glo™ Reagent.

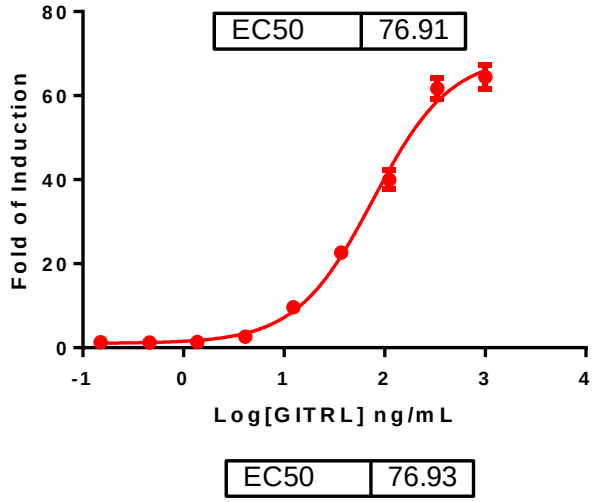
Co-Stimulatory Receptor Bioassays

GITR Bioassay – Principle and Results



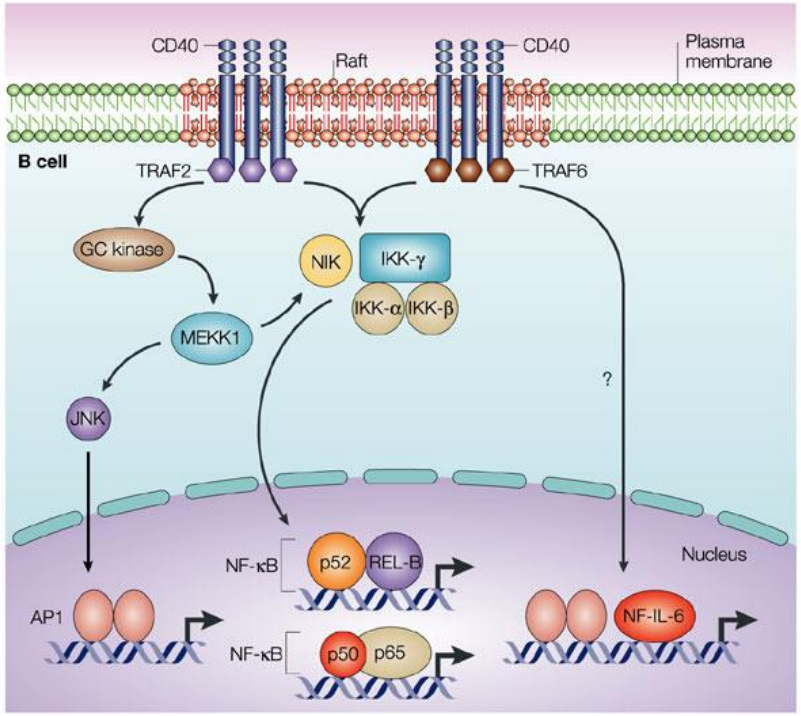
NFkB pathway activation by GITRL / GITR





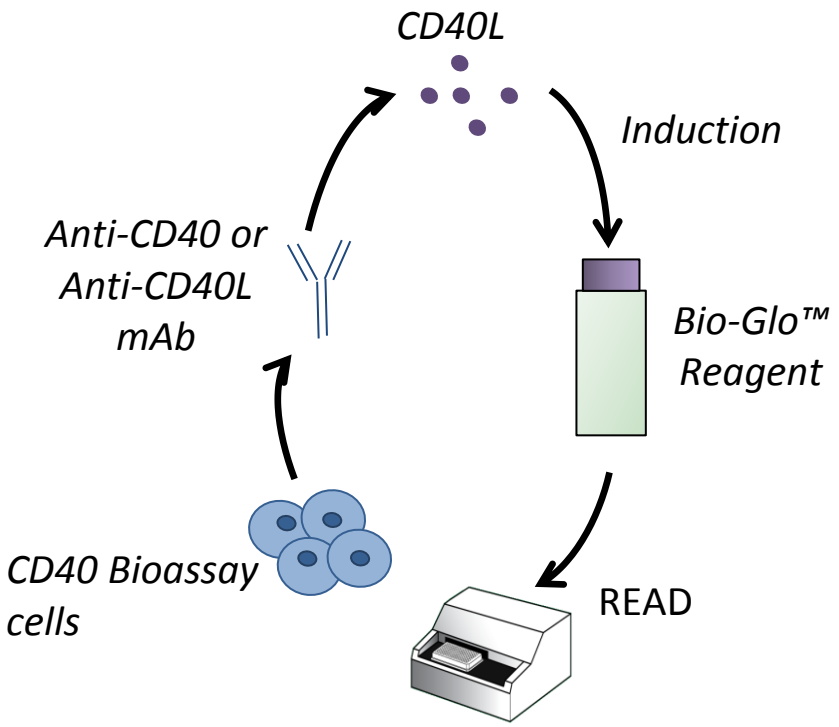
CD40 Bioassay Principle and Protocol

CD40/CD40L Mechanism of Action



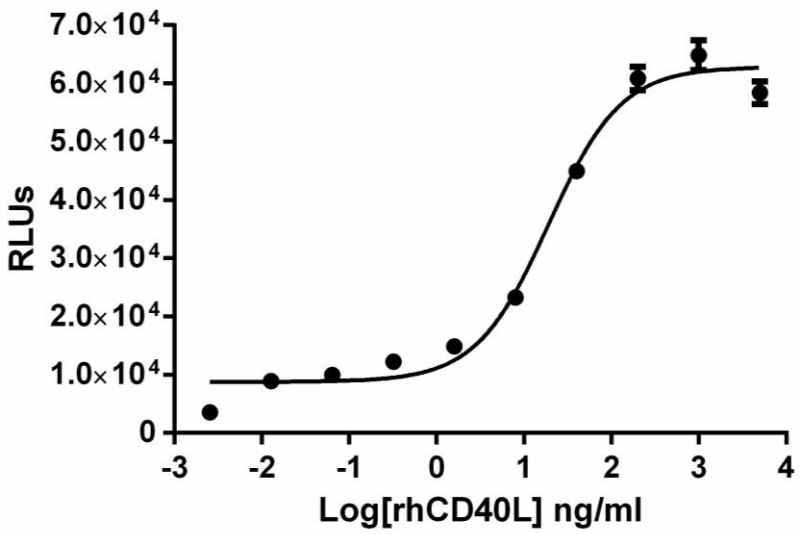
AP1/NFκB pathway activation by CD40 stimulation

Protocol

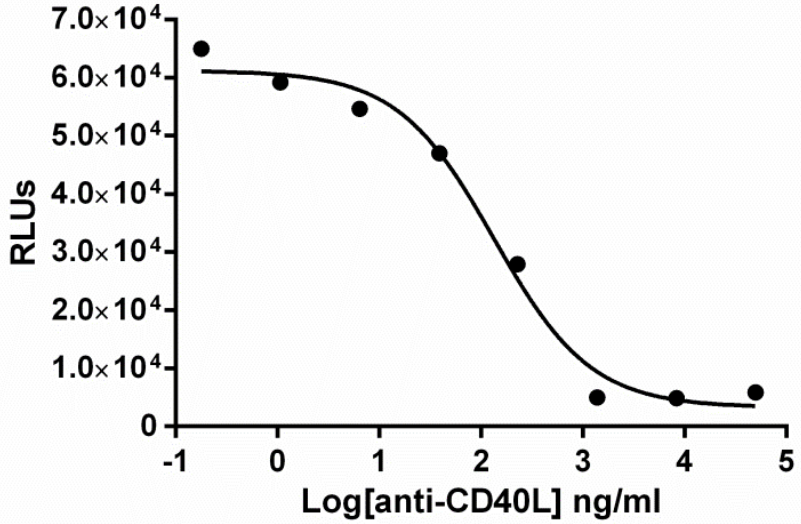


CD40 Bioassay Performance

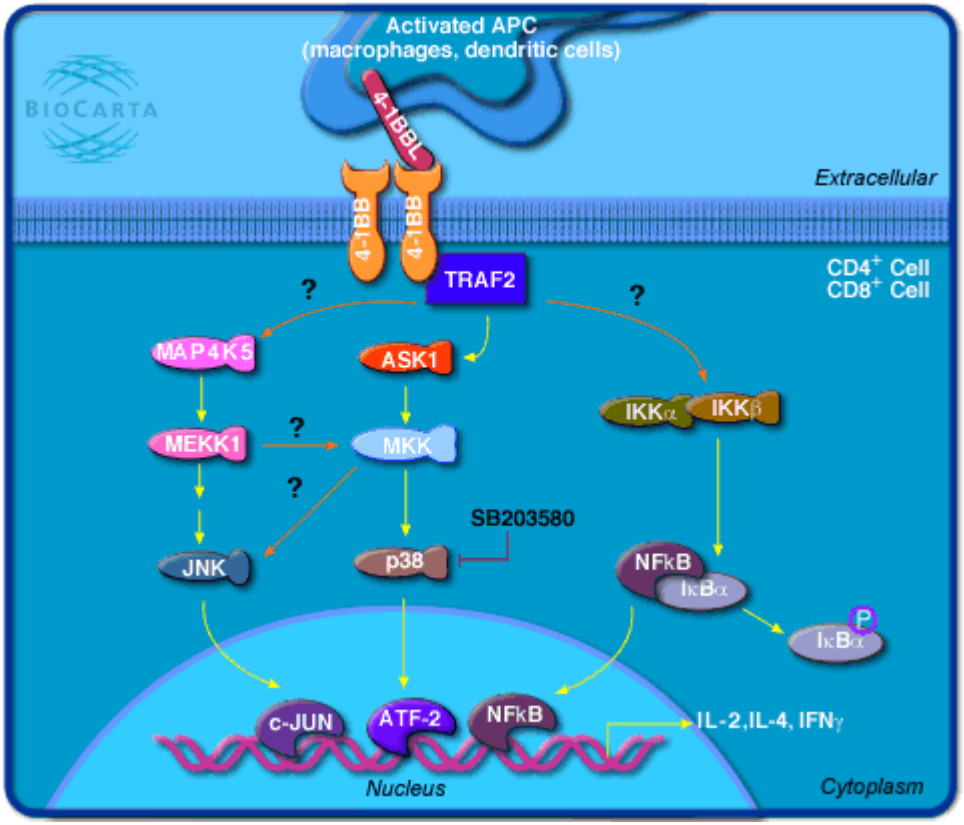
Activation by CD40L

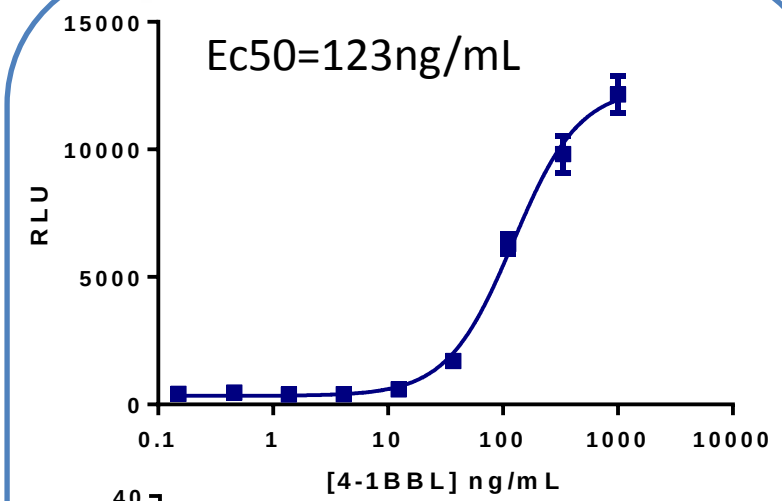


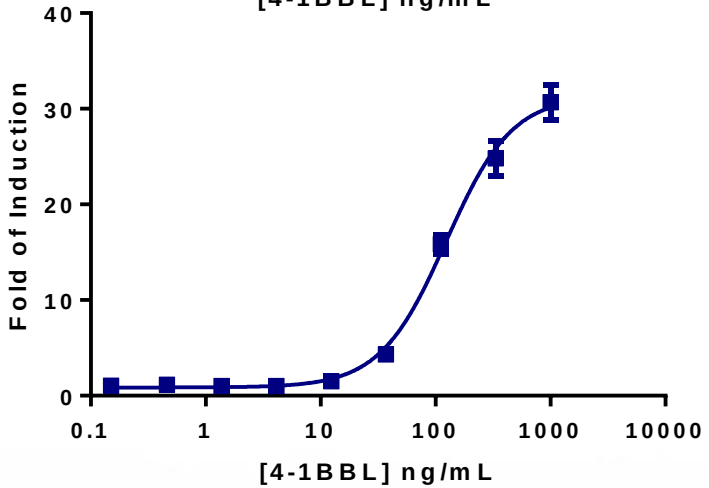
Inhibition by Anti-CD40L Ab



4-1BB Bioassay - NFkB-Luc2P Jurkat cells (pool)







OX-40 Bioassay - NFkB-Luc2P Jurkat cells (pool)

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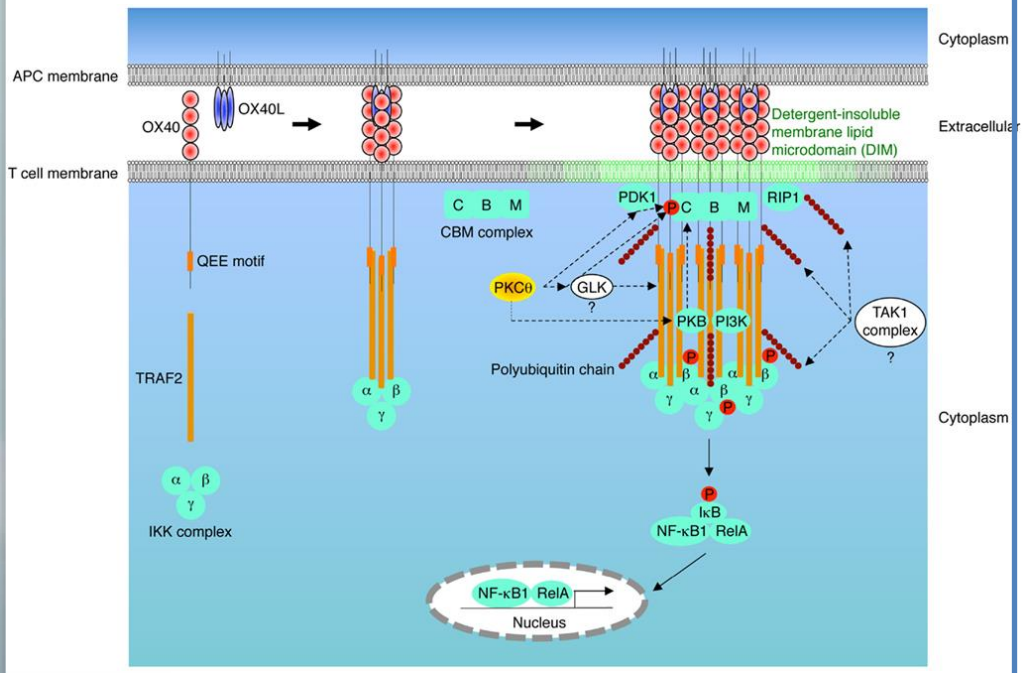
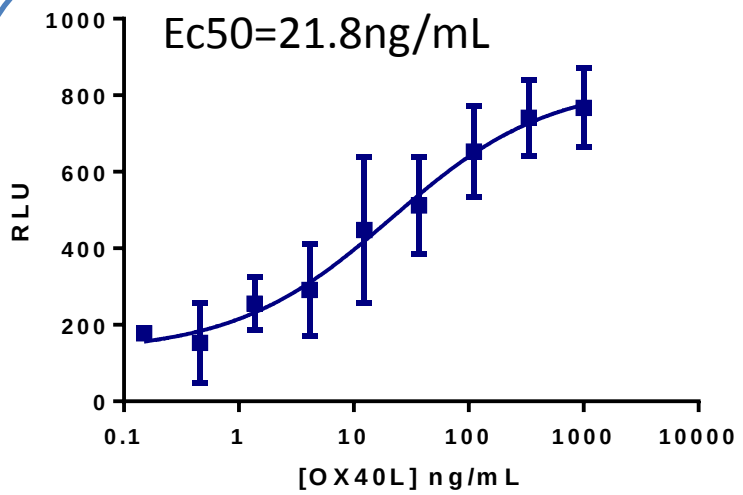
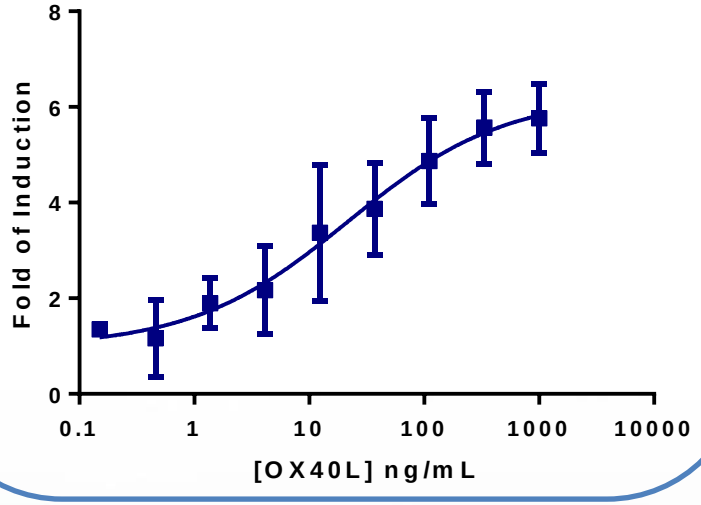
NF-κB1 Signaling Through OX40 is Essential for Activated/Effector T Cell Responses



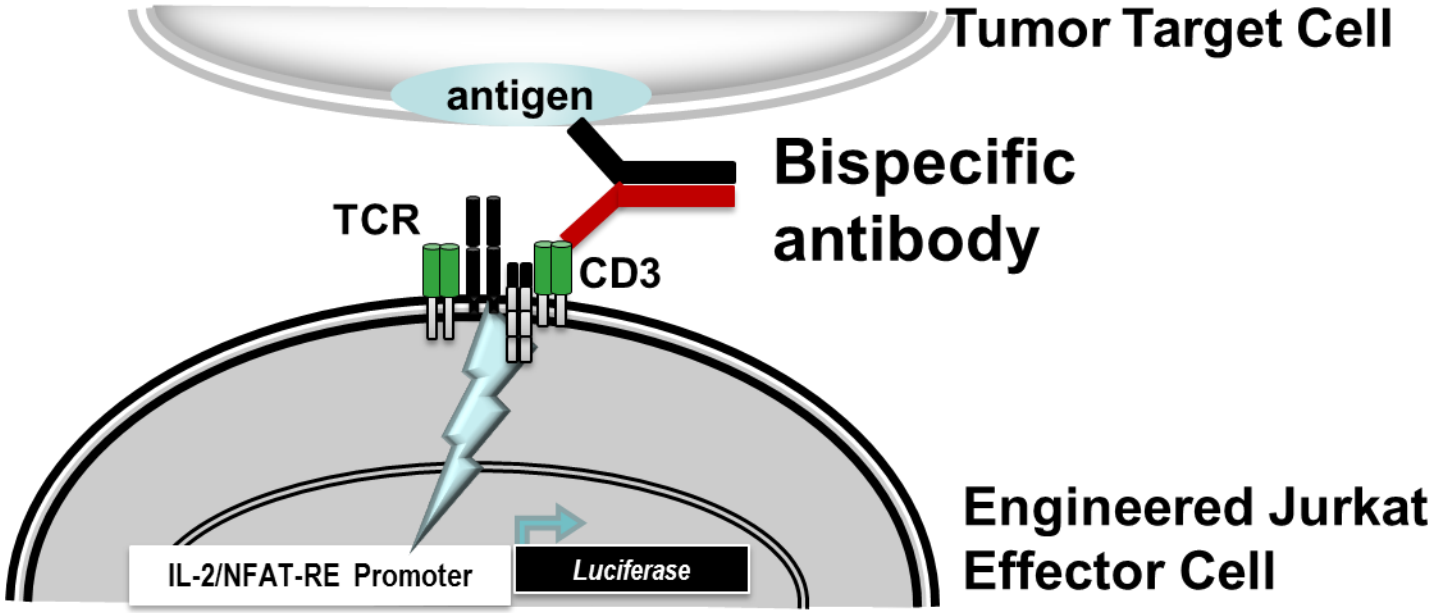




Takanori So et al, Front. Immunol., 28 May 2012

T cell Activation Bioassays

Anti-CD3 Bispecific Bioassay Principle



Responsive to CD3 cross-linking

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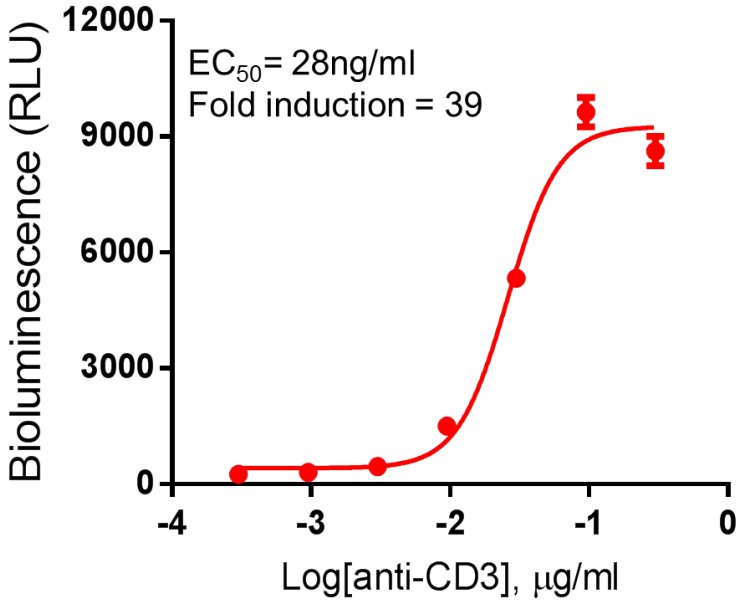
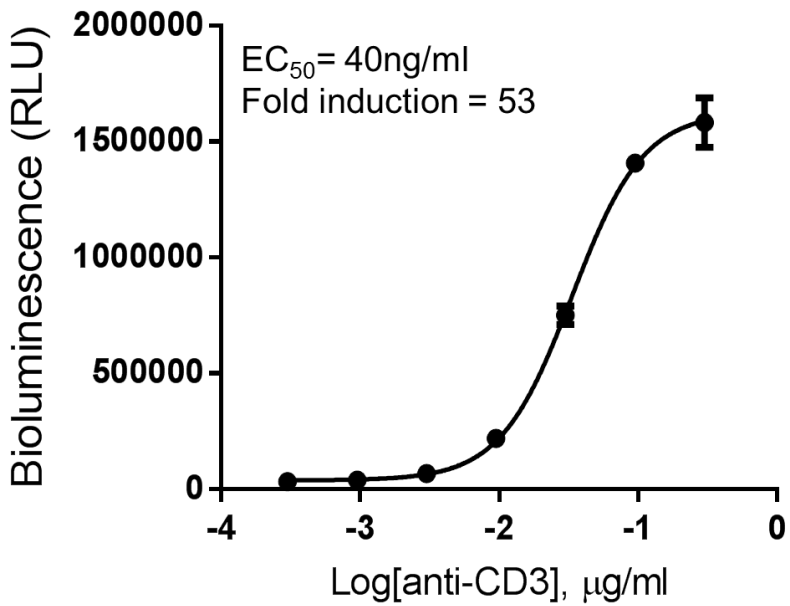
Jurkat / IL2 reporter cells



Jurkat / NFAT-RE reporter cells



Thaw-and-Use Jurkat reporter cells were incubated with cross-linked anti-CD3 antibody for 5 hours. Luciferase activities were assayed using Bio-Glo™ reagent.

T cell Activation Bioassay Protocol

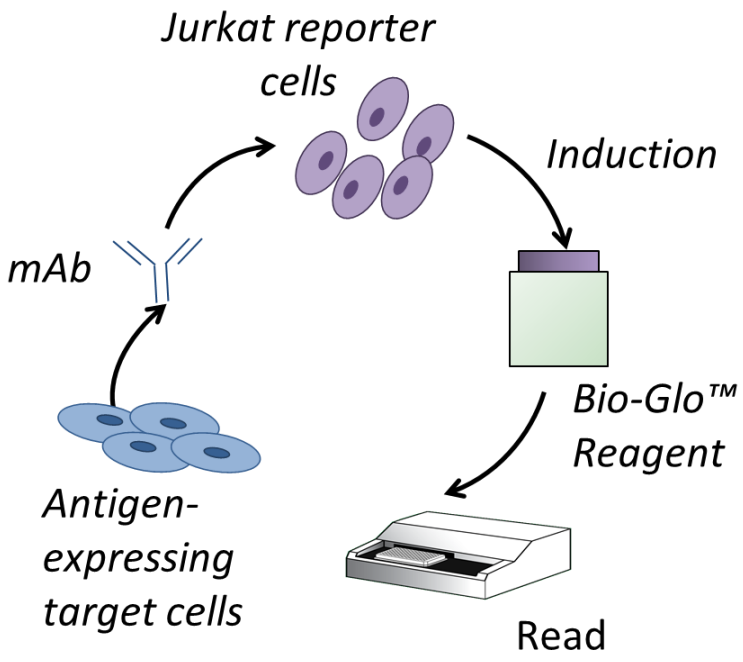


Plate antigen-expressing target cells

⇩

Add test antibody

⇩

Add Jurkat reporter cells

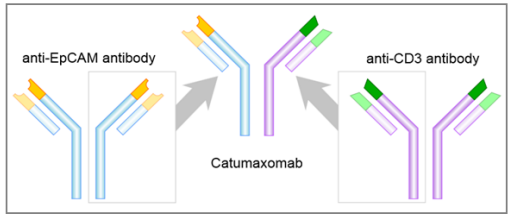
⇩

Induction for 5 hours

⇩

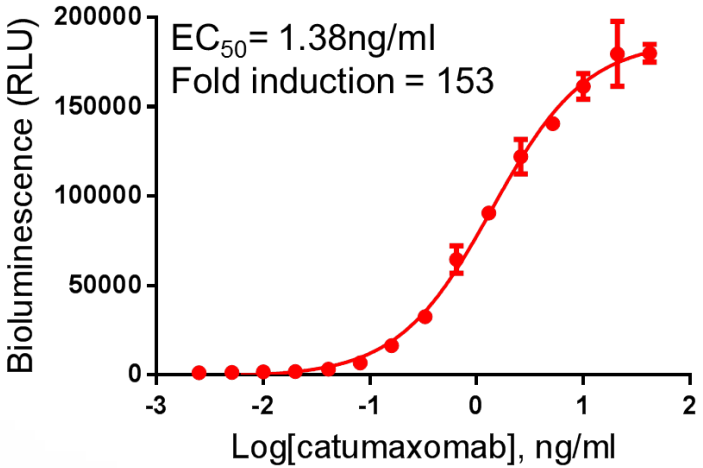
Add Bio-Glo™ detection reagent, read plates

Dose Response with Removab

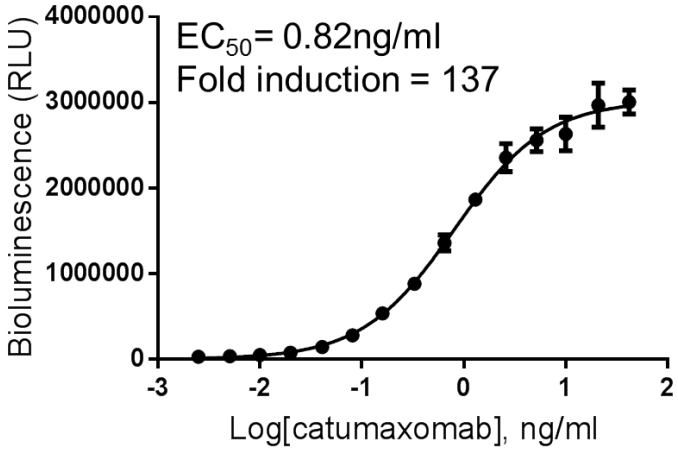


Catumaxomab (trade name: Removab) is a rat-mouse hybrid monoclonal antibody which is used to treat malignant ascites, a condition occurring in patients with metastasizing cancer. It binds to CD3 and EpCAM.

Jurkat / IL2 reporter cells

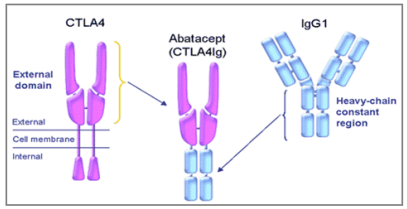


Jurkat / NFAT-RE reporter cells



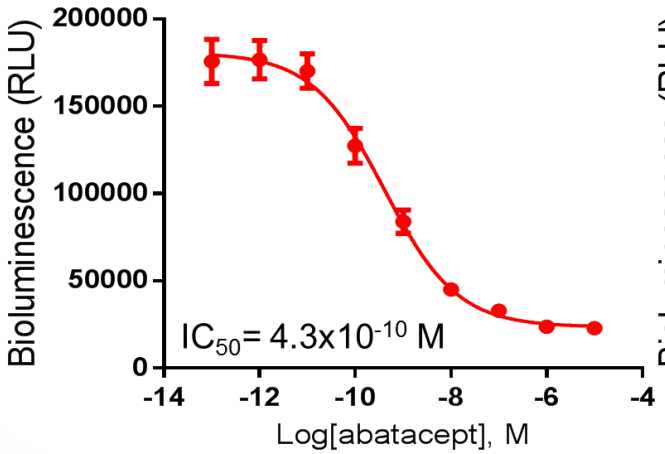
Dose-dependent reporter response to catumaxomab in Thaw-and-Use Jurkat reporter cells using EpCAM+ MDA-MB-231.

Dose Response with Orencia

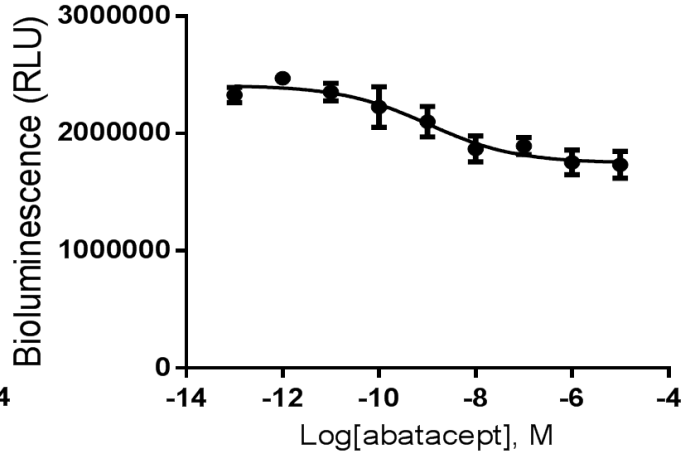


Abatacept (trade name: Orencia) is a fusion protein composed of the Fc region of the immunoglobulin IgG1 fused to the extracellular domain of CTLA-4. It is used for the treatment of rheumatoid arthritis in the case of inadequate response to anti-TNFα therapy.

Jurkat / IL2 reporter cells



Jurkat / NFAT-RE reporter cells



Dose-dependent inhibition of reporter signal by abatacept in Thaw-and-Use Jurkat/ IL-2 reporter cells stimulated with CD3 Ab and Raji B cells which express CD28/ CTLA4 ligand, B7.

T cell Activation Bioassay Qualification

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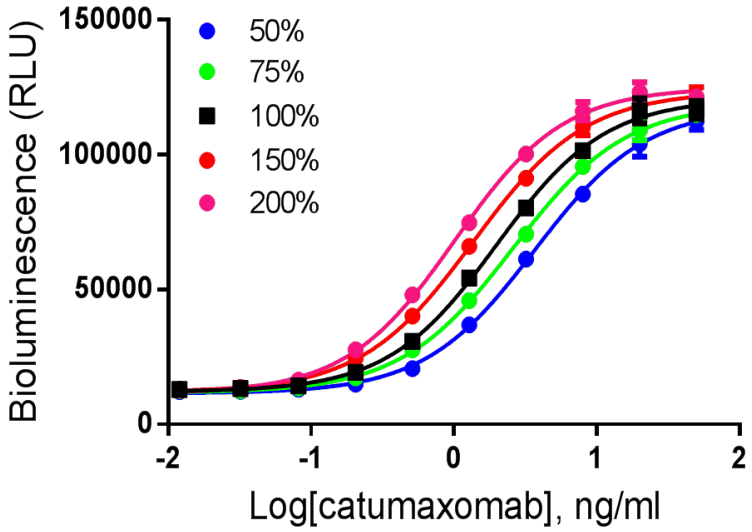
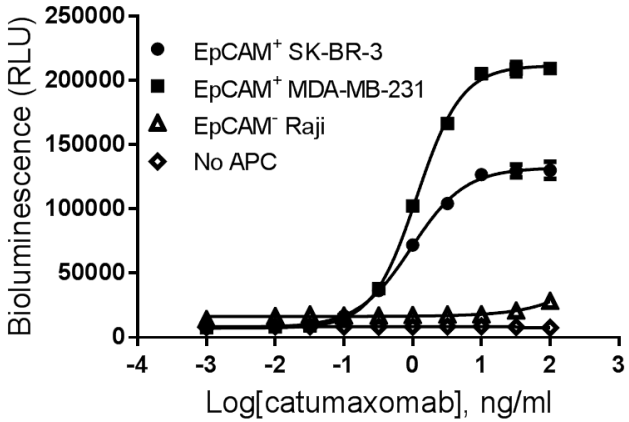
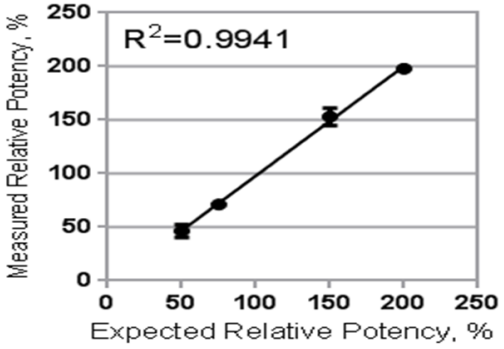
Measure relative potency



Assay specificity



Linearity



Summary - Cancer Immunotherapy Bioassays

Co-inhibitory receptors

- **PD-1 / PD-L1 bioassay**
- **CTLA-4 bioassay**
- LAG3/MHC II - *in dev*
- TIM-3 - *in dev*

Co-stimulatory receptors

- **GITR bioassay**
- **CD40 bioassay**
- OX40 - *in dev*
- 4-1BB – *in dev*

General T-Cell Activation

- **NFAT Jurkat bioassay**
- **IL-2 Jurkat bioassay**

Angiogenesis

- **VEGF bioassay**

Reporter Bioassay Formats Available

Kits

- Thaw-and-use cells (single use)
- Medium and serum
- Bio-Glo™ detection reagent
- No banking or propagation of cells

Cell propagation model (CPM)

- 2 vials of cells
- Banking and propagation of cells
- Required to use Bio-Glo™ detection reagent

Custom Assay Services

www.promega.com/cas

- Promega builds custom bioassays
- Projects are milestone based and payments are made based on completion of each milestone

Feasibility study

- Transient transfection
- Proof of concept
- Assay reproducibility

Stable cell line generation

- Stable pool generation
- Single clone selection

Assay optimization

- Cell number/well
- Agonist concentration
- Luciferase induction time
- Antibody concentration

Assay qualification

- Accuracy
- Precision
- Specificity
- Linearity

For more information…

Richard Somberg, PhD
Strategic Collaborations Manager
Richard.somberg@promega.com

Vanessa Ott, PhD
Senior Global Product Manager
Vanessa.ott@promega.com

Custom Order Department
COD@promega.com

Custom Assay Services
CAS@promega.com

Acknowledgements:

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