A Simple, Sensitive System to Measure Blockade of Immune Checkpoint Targets with Promega Bioassays and GloMax® Discover

Promega Corporation

Materials Required

- CTLA-4 Blockade Bioassay (Cat.# JA3001 JA3005)
- GloMax® Discover System (Cat.# GM3000)
- White, 96-well assay plates (Corning Cat.# 3917)
- Control Ab, Anti-CTLA-4 (Cat.# J1020)

Caution: We recommend the use of gloves, lab coats and eye protection when working with these or any chemical reagents.

Protocols: *GloMax® Discover System Technical Manual* #TM397 available at: **www.promega.com/protocols/**

Immune checkpoint pathways are common targets for immunotherapy drug development. CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) is an immune inhibitory receptor and a homolog of the T-cell co-stimulatory receptor CD28. It is expressed on activated CD4+ and CD8+ T cells after TCR signaling. CTLA-4 binds CD80 and CD86, the ligands for CD28, but with greater avidity and affinity. CTLA-4 can efficiently attenuate T cell response by out-competing CD28 ligand binding and also via CTLA-4 mediated intracellular signaling.

The CTLA-4 Blockade Bioassay provies a non-radioactive, plate-based, homogeneous bioluminescent method for measuring the biological activity of CTLA-4 blocking antibodies during immunotherapy drug development. The assay consists of two cell lines—a CTLA-4 Effector Cell line and an antigen presenting cell line. CTLA-4 Effector cells stably express CTLA-4 receptor and a luciferase reporter that responds to TCR and CD28 activation. The artificial antigen presenting cells (aAPC/Raji) are Raji cells stably expressing a T cell activator protein, which activates the CTLA-4 Effector cells in an antigen-independent manner, and also expressing endogenous CD80 and CD86. Co-incubation of CTLA-4 Effector cells with aAPC/Raji cells blocks CD28-activated luciferase activity. Addition of an anti-CTLA-4 blocking antibody releases the inhibitory signal, restoring CD28-activated expression of luciferase activity.

The CTLA-4 Blockade Bioassay assay was developed and optimized using the GloMax® Discover System due to its luminescence sensitivity, dynamic range, cross-talk, and reproducibility. This Application Note describes a protocol for measuring CTLA-4 blockade response using the CTLA-4 Blockade Bioassay and the GloMax® Discover System. We also present results showing the sensitivity and reliability of the GloMax® Discover System compared to other plate readers.

CTLA-4 Blockade Bioassay Protocol

For detailed instructions and assay notes for various assay volumes and plate formats, see the *CTLA-4 Blockade Bioassay Technical Manual,* TM518.

1. Plate 25µl thaw-and-use CTLA-4 Effector cells into white, 96-well assay plates.
2. Serially dilute anti-CTLA-4 antibody and add 25µl to the assay wells already containing Effector Cells.
3. Add 25µl of thaw-and-use aAPC/Raji cells.
4. Incubate for 6 hours at 37°C, 5% CO_2.
5. Add 75µl of Bio-Glo™ Luciferase Assay Reagent and incubate for 10 minutes.
6. Measure luminescence on the GloMax® Discover using the Bio-Glo protocol.

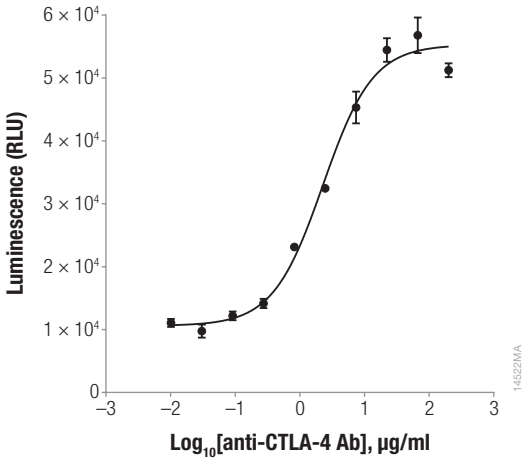


Figure 1. CTLA-4 Blockade Assay (6-hour) response to Ipilimumab, Anti-CTLA-4 Ab (Yervoy) using CTLA-4 Effector Cells and aAPC/Raji Cells. Luminescence was determined using GloMax Discover® System. Four-parameter logistic curve analysis was performed with GraphPad Prism® software. EC_{50} was 2.27µg/ml.

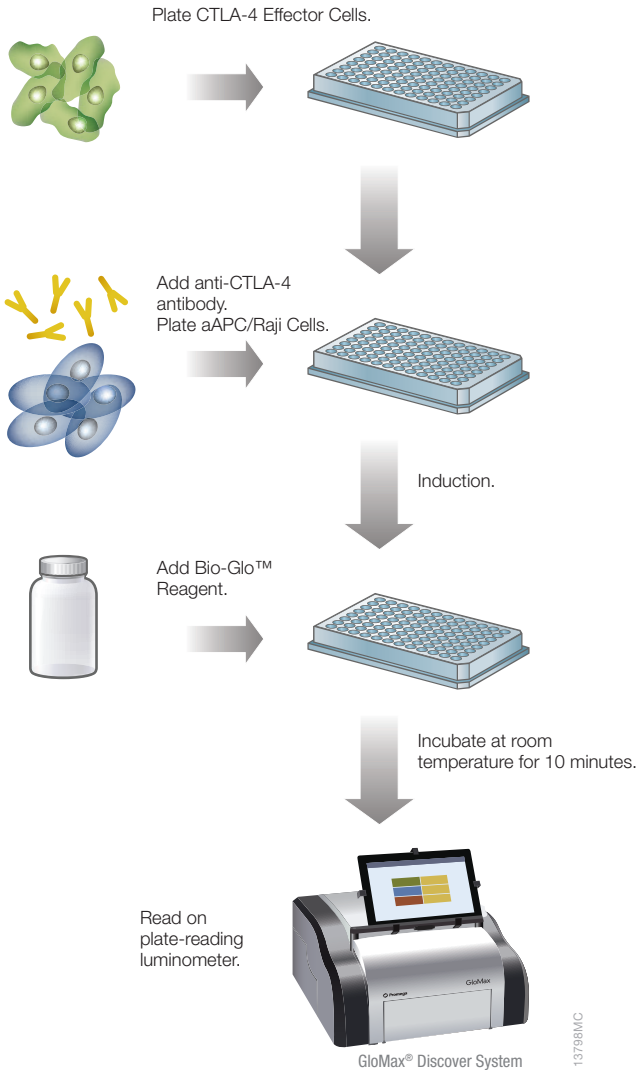


Figure 2. Schematic workflow for the CTLA-4 Blockade Bioassay.

Plate Reader Comparison

Senstivity: Assay performance can be greatly affected by the plate reader used. We tested assay sensitivity using firefly luciferase and the Bio-Glo™ Luciferase Assay System, and compared performance of the GloMax® Discover and GloMax® Navigator instruments with that of several other plate readers.

Serial dilutions of QuantiLum® Recombinant Luciferase (Cat.# E1701) in 1X Passive Lysis Buffer (Cat.# E1941) with 1mg/ml BSA (Cat.# W3841) were used to assess instrument sensitivity (Figure 3). For each instrument, 100µl of each dilution was added to a 96-well white opaque plate in triplicate. All test plates were prepared at the same time using the same reagents and then frozen. For each instrument, three plates were thawed and assayed using the Bio-Glo™ Luciferase Assay System. Test plates and Bio-Glo™ Luciferase Assay Reagent were brought to room temperature and 100µl of Bio-Glo™ Luciferase Assay Reagent added to each well. After brief mixing, the plates were incubated inside the instrument for 10 minutes, and then luminescence was measured (Figure 3).

The limit of detection (LOD) was determined for each instrument based on a luciferase concentration of 4.1×10^{-18} moles of luciferase, which was within the linear range of the detection limit for all instruments. The following industry-recognized formula was applied to determine the instrument LOD:

$$\text{LOD} = \frac{[4.18\text{E-}18]}{\text{Mean RLU at 4.1E-18} - \text{Mean RLU at blank}} \times (3 * \text{StDev of blank})$$

Potency Profiles: Potency curves for the CTLA-4 Blockade Bioassay were also compared to assess the ability of each plate reader to generate quality potency profiles. The CTLA-4 Blockade Bioassay was prepared in 96-well plate format according to the protocol described above and measured on GloMax® Discover, BMG CLARIostar® and BMG PHERAstar® plate readers. In addition, the CTLA-4 Blockade Bioassay was prepared in 384-well plate format using 1/5th the volume per well described above and measured on GloMax® Discover, Tecan Infinite® M1000 and Tecan Spark® 20. The same 96-well or 384-well plate was read on all instruments to eliminate any plate-to-plate variability. Since the GloMax® Discover automatically provides optimal gain, no adjustment was needed. For the BMG and Tecan instruments, multiple gain settings were tested to determine the optimal setting. To determine the variation due to the time it takes to measure the assay plate on each plate reader, the GloMax® Discover was used for the first and the last measurement in the 96-well plate assay. Response curves for each instrument are shown in Figure 4.

Reproducibility: Read-to-read reproducibility was also tested to determine instrument reliability. Because GloMax ® instruments exhibited the best limit of detection, GloMax® Discover was selected to evaluate read-to-read reproducibility. The CTLA-4 Blockade Bioassay was performed in a single 96-well assay plate and luminescence determined. Seven measurements were collected over the course of 30 minutes. EC_{50} was determined for each plate read (Figure 5).

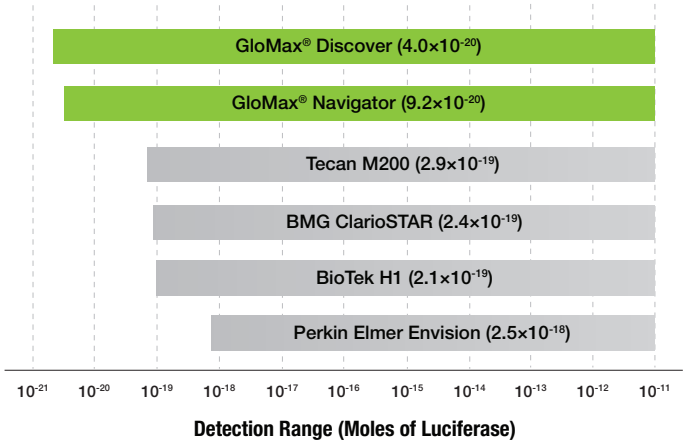
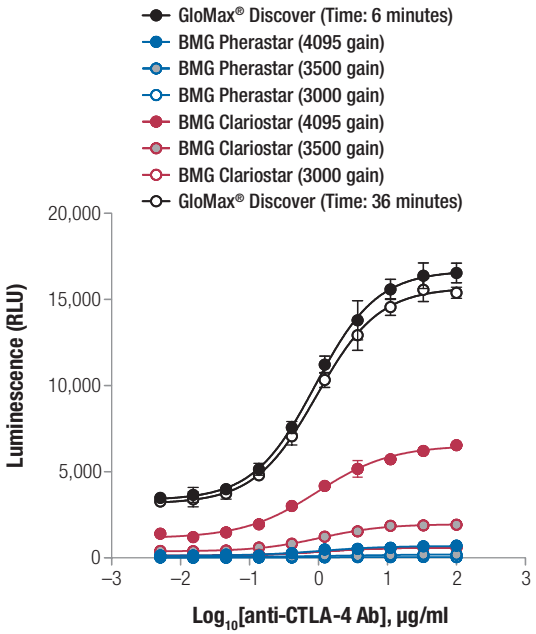


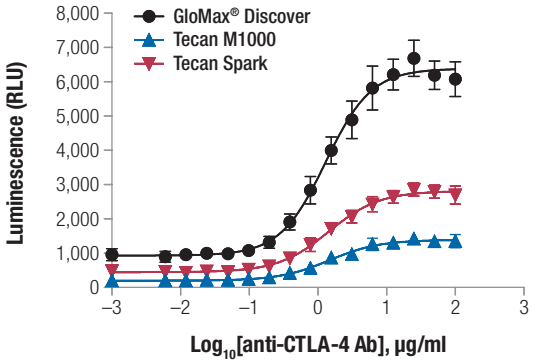
Figure 3. Sensitivity of Bio-Glo™ detection on GloMax® Systems and other commercially available plate readers.

A.



Instrument	EC_{50}
GloMax® Discover (6 minutes)	0.8996
BMG Pherastar (4095 gain)	1.122
BMG Pherastar (3500 gain)	2.085
BMG Pherastar (3000 gain)	0.7653
BMG Clariostar (4095 gain)	0.9672
BMG Clariostar (3500 gain)	1.079
BMG Clariostar (3000 gain)	1.083
GloMax® Discover (36 minutes)	0.9402

B.



Instrument	EC_{50}
GloMax® Discover	1.3
Tecan M1000	1.471
Tecan Spark	1.442

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Figure 4. Potency curves for the CTLA-4 Bio-Glo™ Assay generated using various plate readers.

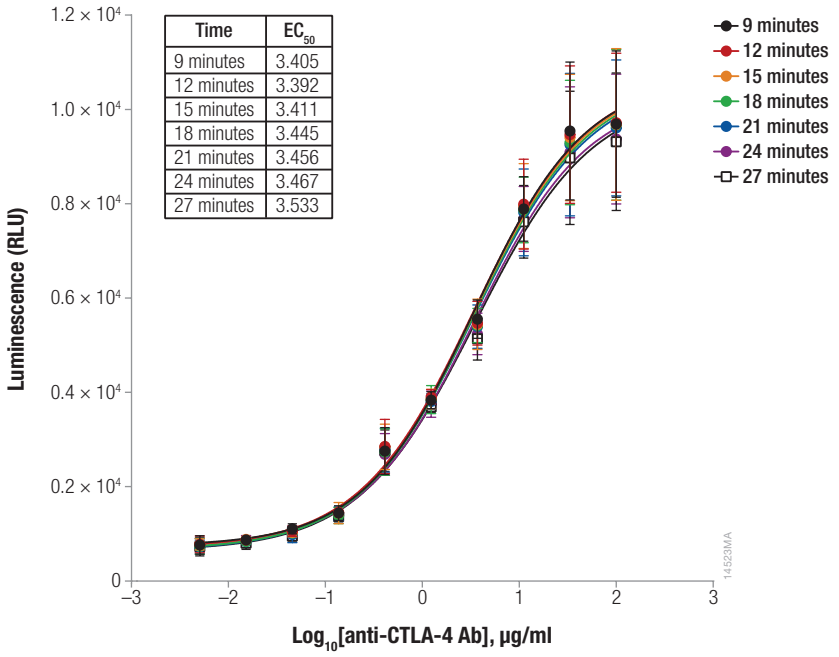

Figure 5. Sequential reads of CTLA-4 Blockade Bioassay using Bio-Glo™ Reagent measured by the Glomax® Discover System.

Conclusion

Here, we demonstrate use of the CTLA-4 Blockade Bioassay to and the GloMax® Discover System to detect and measure the cellular response to anti-CTLA-4 antibody (Figure 1). A sixfold signal-to-background induction was observed in a 6-hour assay using the GloMax® Discover System. Alternatively, the assay may be read after 16–17 hours, resulting in a higher assay window but lower luminescence readings (data not shown). Data was generated using Thaw-and-use cells, which are frozen cells that are plated right after thawing, eliminating the need to culture cells. This results in a convenient assay work-flow with high reproducibility and low variability.

GloMax® instruments were the most sensitive plate readers tested, exhibiting 10 to 100-fold greater sensitivity (Figure 3). Of all plate readers tested, GloMax® Discover provided the best potency curve for the assay (Figure 4), producing higher signals at the lowest antibody concentrations. The ability to measure the assay at these low antibody concentration levels increases the overall sensitivity of the assay. Plate readers with lower signals at these concentrations were less sensitive, limiting the utility of the assay. When measuring the assay at 6 minutes and 36 minutes on the GloMax® Discover, very little assay variation was observed.

GloMax® Discover also gave excellent reproducibility, with EC_{50} values remaining consistent after seven measurements collected over a 30-minute period (Figure 5).

In addition to these performance advantages, GloMax® provides ease-of-use, pre-loaded Promega Bioassay protocols, Instrument and Operational Qualification services (IQ and OQ), the technical elements to comply with part 11 regulations (user authentication and authorization, data integrity and protection, electronic signatures and audit trails), and multiple data export formats for use in both research and manufacturing environments.

The CTLA-4 Blockade Bioassay assay was developed and optimized using the GloMax® Discover System due to the superior performance for assay sensitivity, dynamic range, cross-talk, ease-of-use, and reproducibility. This integrated bioassay provides confidence that even low level cellular responses can be measured successfully. The CTLA-4 Blockade Bioassay and the GloMax® Discover System provide users a simple, sensitive, and reliable system for immune checkpoint pathway assessment.

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