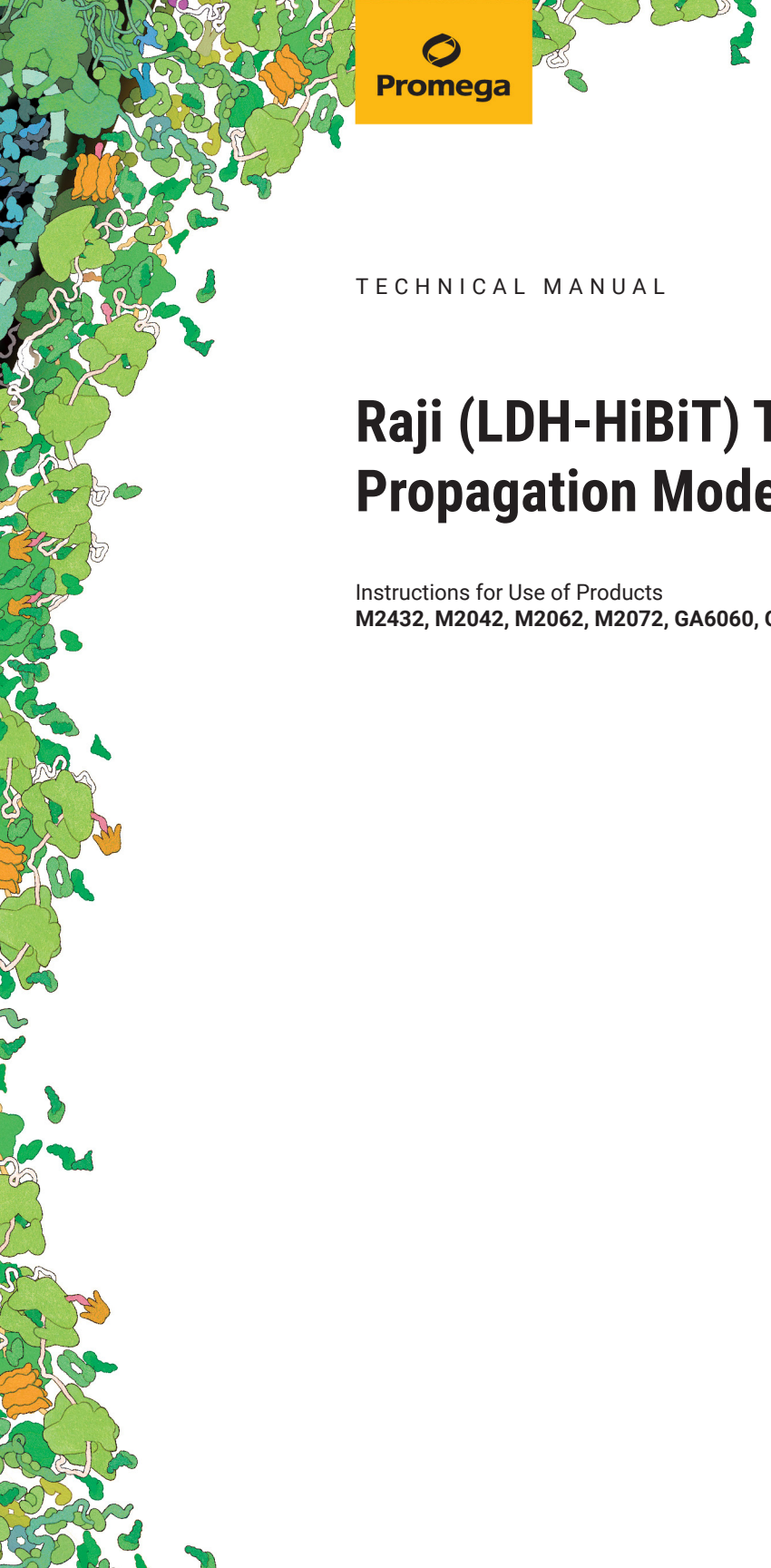

TECHNICAL MANUAL

Raji (LDH-HiBiT) TCK Bioassays, Propagation Model

Instructions for Use of Products
M2432, M2042, M2062, M2072, GA6060, GA6100, GA6120 and GA6130

Revised 5/25
TM737

Promega

Raji (LDH-HiBiT) TCK Bioassays, Propagation Model

All technical literature is available at: www.promega.com/protocols/
Visit the website to verify that you are using the most current version of this Technical Manual.
Email Promega Technical Services if you have questions on use of this system: techserv@promega.com

Promega

1. Description

Inducing tumor cell lysis by immune effector cells is a primary mechanism of action (MOA) for many immuno-oncology drugs. Monoclonal antibodies against tumor-associated antigens (TAA) can induce antibody-dependent cellular cytotoxicity (ADCC) by natural killer (NK) cells (1). Next-generation biologics, such as bispecific antibodies, can redirect T cells to lyse tumor cells (2). CAR-T cells and other cellular therapies are living drugs that directly lyse tumor cells as a component of their MOA (3). HiBiT Target Cell Killing (TCK) Bioassays provide a flexible, reproducible, label-free, luminescent system for measuring the potency of a variety of cellular and immuno-oncology drug products where target cell killing is a MOA.

Raji (LDH-HiBiT) TCK Bioassays, Propagation Model[a–e] measure the death of Raji cells engineered to express a fusion of HiBiT to the C terminus of the lactate dehydrogenase (LDHB) gene product (LDH-HiBiT). HiBiT is an 11 amino acid peptide that binds with high affinity to LgBiT to form NanoBiT® luciferase (4). When dead or dying cells release the HiBiT fusion protein into the extracellular medium, it binds to the cell-impermeable LgBiT, which is provided with the furimazine substrate in a nonlytic, homogeneous detection reagent (Figure 1). In coculture experiments, the luminescent signal will be proportional to target cell death alone, with no signal contribution arising from the death of effector cells.

Raji (LDH-HiBiT) TCK Bioassays, Propagation Model, are a suite of four individual bioassays comprised of the parental Raji line and three different KO variants (see Section 2). Each bioassay contains a clonal cell line in the cellular propagation model (CPM) and Cell Bank formats, which can be thawed, propagated and banked for long-term use. All cell lines express the LDH-HiBiT reporter, making them suitable for analysis using the HiBiT TCK technology.

The Raji (LDH-HiBiT) TCK Bioassay has been prequalified in a T cell-dependent cellular cytotoxicity (TDCC) bioassay following International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines and exhibit the precision, accuracy and linearity required for routine use in potency and stability studies (Table 1 and Figure 3). HiBiT TCK Bioassay workflows are simple, robust and compatible with both 96- and 384-well plate formats used for early biologic drug discovery and lot release settings (Figure 4).

Detection of released HiBiT fusion protein is sensitive, enabling the use of low numbers of HiBiT target cells (2,500–5,000 per well). The cells have a low rate of spontaneous HiBiT fusion protein release and the fusion protein is stable once released into the extracellular medium. These features enable use of the Raji (LDH-HiBiT) TCK Bioassays, Propagation Model, to measure the potency of biologic drugs and cell therapy products, as well as to monitor the specificity of drugs targeting CD19, CD20, or both receptors. For cell therapy, you can test a wide range of effector-to-target (E:T) ratios while conserving test material for experiments ranging from 4–72 hours (Figure 5).

When parental and knockout cells are cocultured with effector cells (e.g., NK cells, cytotoxic T cells, etc.) CD19-, CD20- or CD19/CD20-dependent killing can be monitored. For example, biologic drugs that target antigens expressed on the cell surface show dose-dependent signal increases (Figure 6). In contrast, when knockout cells are assayed with biologic drugs that target antigens no longer expressed on the cell surface, dose-dependent signal increases are absent (Figure 6).

This technical manual provides protocols for the suite of Raji (LDH-HiBiT) TCK Bioassays, Progagation Model, including sample protocols for antibody-dependent cellular cytotoxicity (ADCC) bioassays using Human PBMC, ADCC-Qualified cells (Section 4); TDCC bioassays using Human T Cell (CD8+), TDCC-Qualified cells (Section 5); and CAR-T bioassays with user-provided CAR-T cells (Section 6). Optimization may be necessary for use with your biologic product of interest.

1. Description (continued)

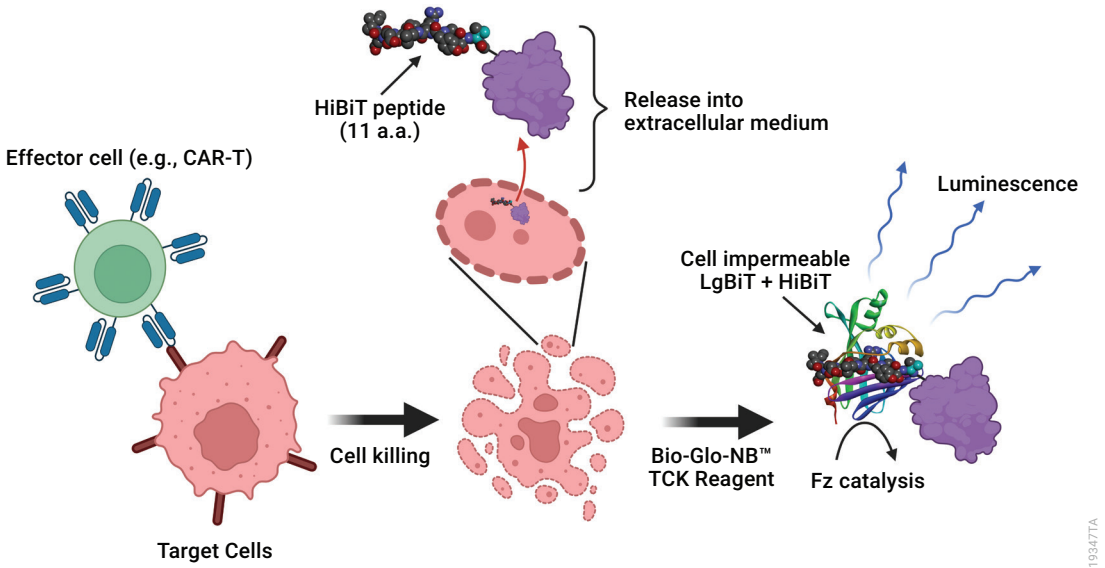

Figure 1. Representation of the HiBiT TCK Bioassay. Target cells stably expressing a HiBiT fusion protein are cocultured with effector cells (CAR-T, T cells, NK cells, etc.). Effector cell-mediated killing of target cells releases the HiBiT fusion protein into the extracellular medium. Cell-impermeable LgBiT and furimazine substrate (Fz) are added as components of the Bio-Glo-NB™ TCK Reagent. HiBiT complementation with LgBiT generates NanoBiT® luciferase, a bright, luminescent enzyme. (Created with BioRender.com.)

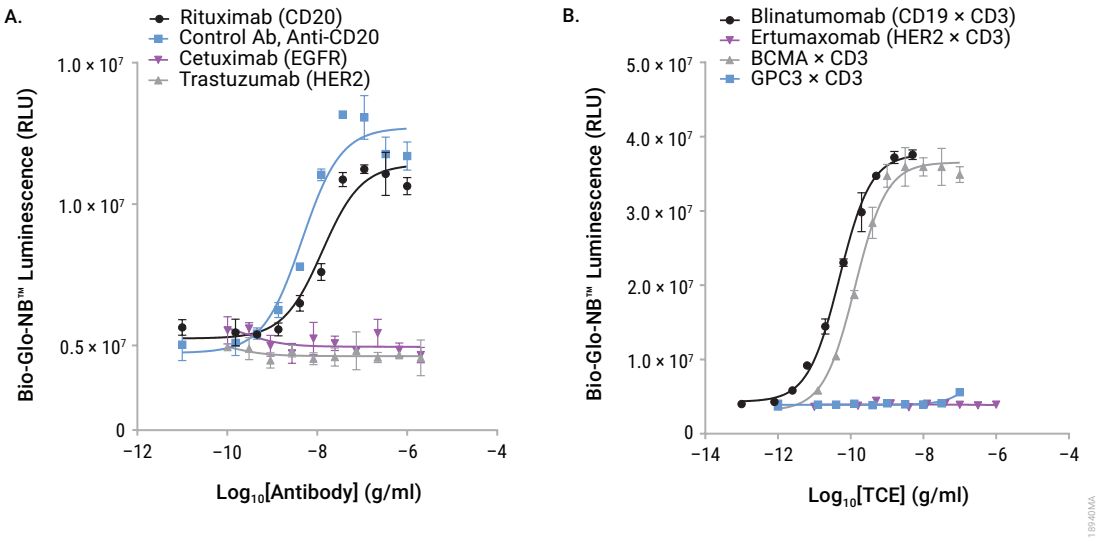

Figure 2. Raji (LDH-HiBiT) TCK Bioassays reflect the MOA of biologic drugs and demonstrates specificity for cell surface antigens. Panel A. Raji Cells (LDH-HiBiT) were incubated with Human PBMC, ADCC-Qualified effector cells in the presence of serial antibody titrations, as indicated. After a 5-hour induction, Bio-Glo-NB™ TCK Reagent was added and luminescence quantified using the GloMax® Discover System. Raji Cells (LDH-HiBiT) express CD19 and CD20, but they do not express EGFR or HER2 on the cell surface. **Panel B.** Raji Cells (LDH-HiBiT) were incubated with Human T Cells (CD8+) in the presence of serial T cell engager (TCE) titrations, as indicated. After a 24-hour induction, Bio-Glo-NB™ TCK Reagent was added and luminescence quantified using the GloMax® Discover System. Raji Cells (LDH-HiBiT) express CD19 and BCMA, but they do not express HER2 or GPC3 on the cell surface. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

1. Description (continued)

Table 1. Precision, Accuracy and Linearity of TDCC Bioassay with Raji Cells (LDH-HiBiT) as Targets.

Parameter	Results	
Accuracy	% Expected Relative Potency	% Recovery
	50	102.9
	75	104.5
	100	104.8
	125	108.5
	150	99.7
Repeatability (% CV)	100% (Reference)	16
Intermediate Precision (% CV)		9.5
Linearity (r^2)		0.93
Linearity (y = mx + b)		y = 1.014x + 2.520

A 50–150% theoretical potency series of blinatumomab was analyzed in triplicate in three independent experiments performed on three days by two analysts using Raji Cells (LDH-HiBiT) and Human T Cells (CD8+), TDCC-Qualified effectors. Bio-Glo-NB™ TCK Reagent was added and luminescence quantified using the GloMax® Discover System. Data were analyzed and relative potencies calculated after parallelism determination using JMP® software. Data were generated using thaw-and-use cells.

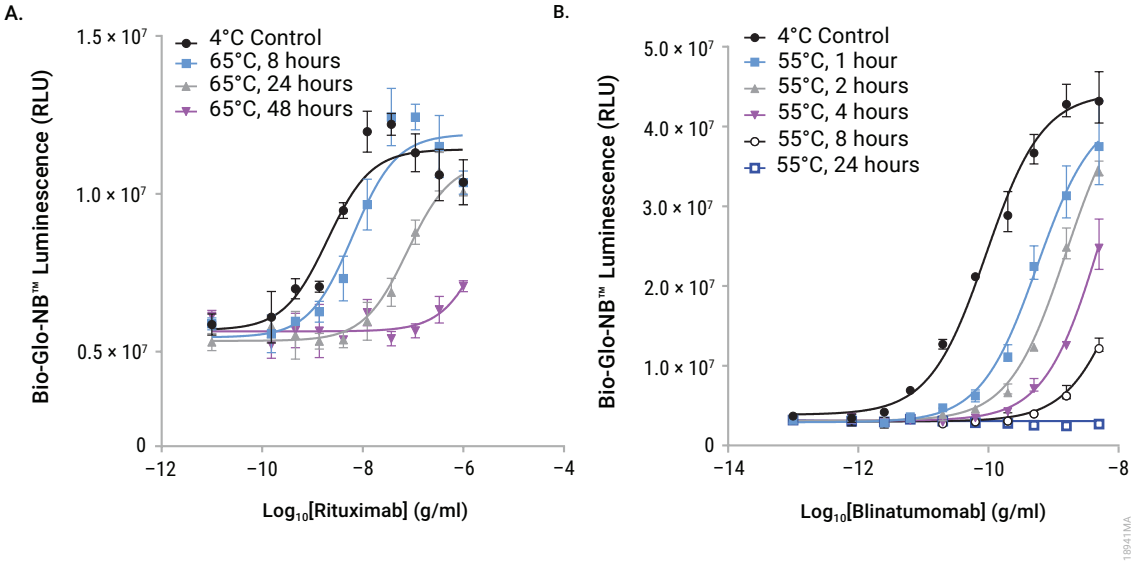

Figure 3. Raji (LDH-HiBiT) TCK Bioassays are stability-indicating. Panel A. Samples of rituximab were maintained at 4°C (control) or heat-treated at 65°C for the indicated times, then analyzed in an ADCC Bioassay with Raji Cells (LDH-HiBiT) and Human PBMC, ADCC-Qualified effector cells. **Panel B.** Samples of blinatumomab (CD19 × CD3) were maintained at 4°C (control) or heat-treated at 55°C for the indicated times, then analyzed in a TDCC Bioassay with Raji Cells (LDH-HiBiT) and Human T Cells (CD8+), TDCC-Qualified. For both panels, Bio-Glo-NB™ TCK Reagent was added and luminescence quantified using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

1. Description (continued)

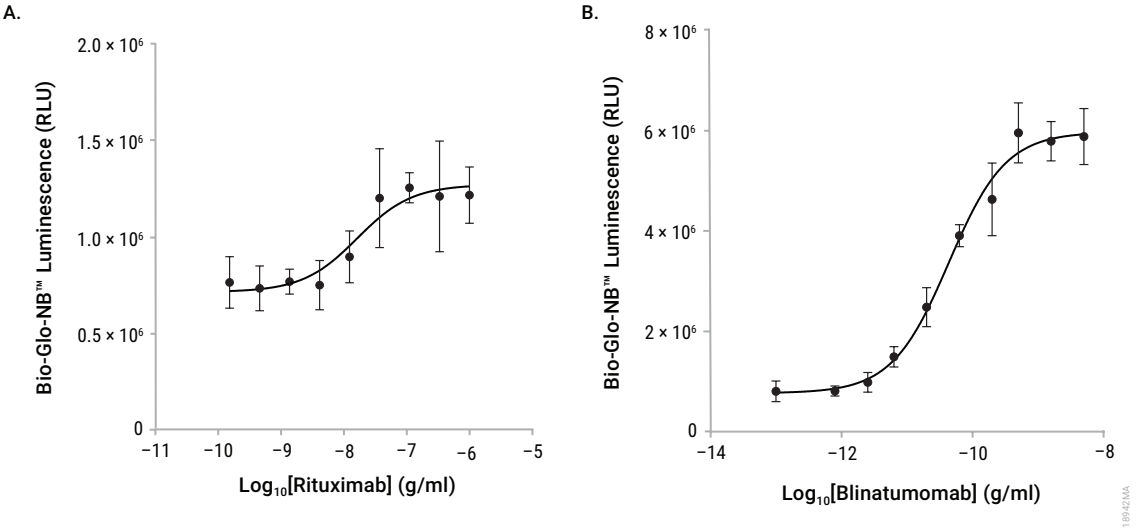

Figure 4. Raji (LDH-HiBiT) TCK Bioassays are amenable to 384-well plate format. Panel A. Raji Cells (LDH-HiBiT) were harvested after overnight culture and 1 × 10^3 cells/5µl/well plated in a 384-well round-bottom white assay plate (e.g., Corning® Cat.# 4512, low volume). Next, 5µl of 3X serially-diluted rituximab was added, then 2.5 × 10^4/5µl/well of Human PBMC, ADCC-Qualified effector cells from overnight culture were added. After a 5-hour incubation at 37°C, 5% CO_2, 5µl of 4X Bio-Glo-NB™ TCK Reagent was added to each well and luminescence quantified. **Panel B.** Raji Cells (LDH-HiBiT) were plated at 5 × 10^2 cells/5µl/well in a 384-well flat-bottom white assay plate (e.g., Corning® Cat.# 3570). Next, 5µl of 3X serially diluted blinatumomab was added, followed by 5 × 10^3/5µl/well of Human T Cells (CD8+), TDCC-Qualified. After a 24-hour incubation at 37°C, 5% CO_2, 15µl of Bio-Glo-NB™ TCK Reagent was added per well and luminescence quantified. Luminescence was measured using the GloMax® Discover System. Data were fitted to four-parameter logistic curves using GraphPad Prism® software. Data were generated using thaw-and-use cells.

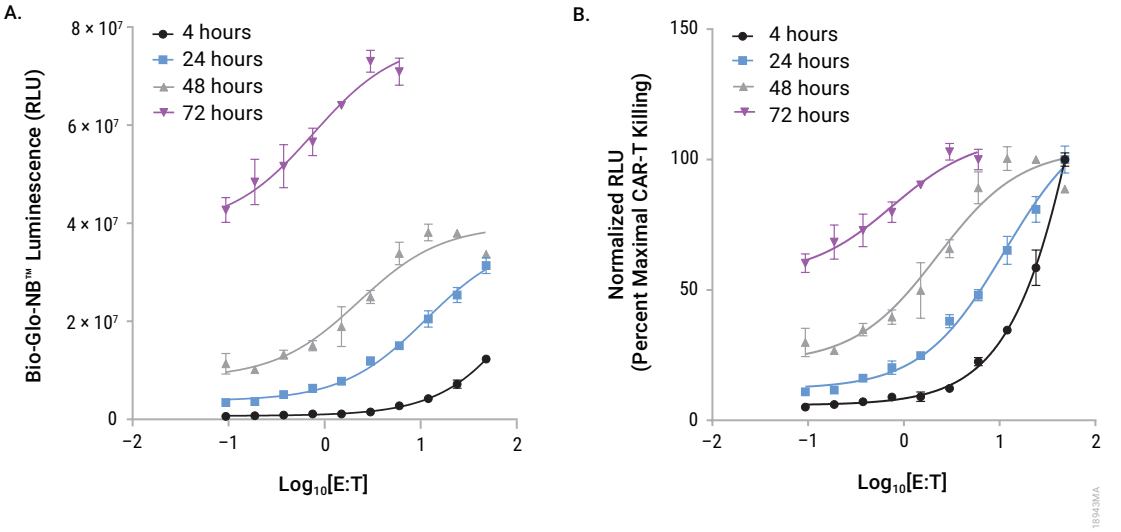

Figure 5. Raji (LDH-HiBiT) TCK Bioassays are amenable to use with CAR-T effector cells. Panel A. Raji Cells (LDH-HiBiT) were incubated with serially diluted human CD19 CAR-T cells at the indicated effector-to-target (E:T) ratios. After a 4-, 24-, 48- or 72-hour induction, Bio-Glo-NB™ TCK Reagent was added and luminescence quantified using the GloMax® Discover System. **Panel B.** The target cell killing data are represented as a percent of the maximum CAR-T-dependent lysis at each time point. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. Luminescence increased over time (**Panel A**) and EC_{50} values decreased (data not shown) with longer incubation periods (**Panel B**). Data were generated using thaw-and-use cells.

1. Description (continued)

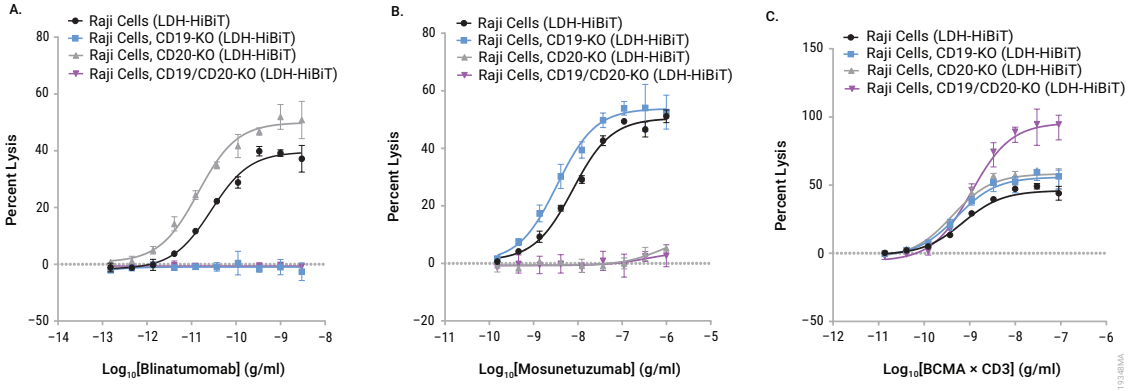

Figure 6. Raji (LDH-HiBiT) TCK Bioassays reflect biologic drug activity in wild-type and surface antigen knockout cells. Parental and knockout cells were incubated with Human T Cells (CD8+) in the presence of serial titrations of biologic drugs for 24 hours. **Panel A.** Raji cell lines expressing CD19 show a dose-dependent increase in percent specific lysis values for blinatumumab (CD19 × CD3), whereas CD19 KO cell lines do not. **Panel B.** Raji cell lines expressing CD20 show a dose-dependent increase in percent specific lysis values for mosunetuzumab (CD20 × CD3), whereas CD20 KO cell lines do not. **Panel C.** Raji cells express BCMA and show a dose-dependent increase in percent specific lysis values. Bio-Glo-NB™ TCK Reagent was added and luminescence measured using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Raji (LDH-HiBiT) TCK Bioassay, Propagation Model	**1 each**	**M2432**

Not for Medical Diagnostic Use. Includes:

- 2 vials Raji Cells (LDH-HiBiT) (CPM), 4 × 10^6 cells/ml (1.0ml per vial)

PRODUCT	SIZE	CAT.#
Raji (LDH-HiBiT) Cell Bank	**1 each**	**GA6060**

Not for Medical Diagnostic Use. Includes:

- 50 vials Raji Cells (LDH-HiBiT) (CPM), 4 × 10^6 cells/ml (1.0ml per vial)

PRODUCT	SIZE	CAT.#
Raji (LDH-HiBiT) CD19-KO TCK Bioassay, Propagation Model	**1 each**	**M2042**

Not for Medical Diagnostic Use. Includes:

- 2 vials Raji Cells, CD19-KO (LDH-HiBiT) (CPM), 4 × 10^6 cells/ml (1.0ml per vial)

PRODUCT	SIZE	CAT.#
Raji (LDH-HiBiT) CD19-KO Cell Bank	**1 each**	**GA6100**

Not for Medical Diagnostic Use. Includes:

- 50 vials Raji Cells, CD19-KO (LDH-HiBiT) (CPM), 4 × 10^6 cells/ml (1.0ml per vial)

PRODUCT	SIZE	CAT.#
Raji (LDH-HiBiT) CD20-KO TCK Bioassay, Propagation Model	**1 each**	**M2062**

Not for Medical Diagnostic Use. Includes:

- 2 vials Raji Cells, CD20-KO (LDH-HiBiT) (CPM), 4 × 10^6 cells/ml (1.0ml per vial)

PRODUCT	SIZE	CAT.#
Raji (LDH-HiBiT) CD20-KO Cell Bank	**1 each**	**GA6120**

Not for Medical Diagnostic Use. Includes:

- 50 vials Raji Cells, CD20-KO (LDH-HiBiT) (CPM), 4 × 10^6 cells/ml (1.0ml per vial)

PRODUCT	SIZE	CAT.#
Raji (LDH-HiBiT) CD19/20-KO TCK Bioassay, Propagation Model	**1 each**	**M2072**

Not for Medical Diagnostic Use. Includes:

- 2 vials Raji Cells, CD19/20-KO (LDH-HiBiT) (CPM), 4 × 10^6 cells/ml (1.0ml per vial)

PRODUCT	SIZE	CAT.#
Raji (LDH-HiBiT) CD19/20-KO Cell Bank	**1 each**	**GA6130**

Not for Medical Diagnostic Use. Includes:

- 50 vials Raji Cells, CD19/20-KO (LDH-HiBiT) (CPM), 4 × 10^6 cells/ml (1.0ml per vial)

Note: Thaw and propagate one vial to create frozen cell banks before use in an assay. The remaining vial(s) should be reserved for future use.

Storage Conditions: Upon arrival, immediately transfer the cell vials to below –140°C (freezer or liquid nitrogen vapor phase) for long-term storage. **Do not** store cell vials submerged in liquid nitrogen. Do not store cell vials at –80°C because this will decrease cell viability and cell performance.

3. Before You Begin

Please read through the entire protocol to become familiar with the components and the assay procedures before beginning.

In this technical manual, we provide example protocols for using the Raji (LDH-HiBiT) TCK Bioassay, Propagation Model, in three different HiBiT TCK Bioassays: PBMC ADCC, TDCC and CAR-T. Please refer to the sections specified for your assay of interest: Section 3, Preparing Raji Cells (LDH-HiBiT); Section 4, PBMC ADCC Assay; Section 5, CD8+ T Cell TDCC Assay; Section 6, CAR-T Killing Assay. Recommendations for including knockout cell line controls are noted at the end of each section. For other applications, use these protocols as a guide and optimize the assay for your specific application.

Note the catalog number, lot number and dispensed lot number from the cell vial box label. This information can be used to download specific documents from **www.promega.com**, such as the Certificate of Analysis.

Note: HiBiT TCK Bioassays use the Bio-Glo-NB™ TCK Luciferase Assay System (Cat.# JB1001, JB1002, JB1003) for detection. **Do not** use the Bio-Glo-NL™ Luciferase Assay System (Cat.# J3081, J3082, J3083) or the Bio-Glo™ Luciferase Assay System (Cat.# G7940, G7941).

Raji (LDH-HiBiT) TCK Bioassays, Propagation Model, are intended for use with effector cells capable of binding to and killing target cells alone or in combination with an appropriate biologic drug. Human PBMC, ADCC-Qualified, or Human T-Cell (CD8+), TDCC-Qualified, are available separately for use in ADCC and TDCC assays, respectively. For ADCC assays, Control Ab, Anti-CD20 (Cat.# GA1130) is available separately.

Cell thawing, propagation and banking should be performed exactly as described in Section 3. Cell seeding and propagation densities have been optimized to ensure stable cell growth, which is reflected in a steady cell doubling rate, to achieve optimal, consistent performance. An accurate, reliable and reproducible cell counting method is required for routine cell culturing and optimal bioassay performance.

Raji (LDH-HiBiT) TCK Bioassays, Propagation Model, produce a bioluminescent signal and require a sensitive luminescence plate reader. Bioassay development and performance data included in this technical manual were generated using the GloMax® Discover System (see Section 9.C, Related Products). An integration time of 0.5 second/well was used for all readings.

Raji (LDH-HiBiT) TCK Bioassays, Propagation Model, are compatible with most other plate-reading luminometers, though relative light unit (RLU) readings will vary with the sensitivity and settings of each instrument. If using a reader with adjustable gain, we recommend a high-gain setting. The use of different instruments and gain adjustment will affect the magnitude of the raw data but should not affect the measured relative potency of test samples.

Materials to Be Supplied By the User
(Composition of Buffers and Solutions is provided in Section 9.A.)

Reagents

- RPMI 1640 medium with L-glutamine and HEPES (e.g., Corning® Cat.# 10-041-CV or GIBCO® Cat.# 22400105)
- fetal bovine serum (FBS; e.g., VWR Cat.# 89510-194 or GIBCO® Cat.# 16000044)
- Trypan blue solution (e.g., Sigma Cat.# T8154)
- DMSO (e.g., Sigma Cat.# D2650)

Supplies and Equipment

- pipettes
- sterile 15ml and 50ml conical tubes
- T75 cell culture flasks (e.g., Corning® Cat.# 430641U)
- 37°C, 5% CO_2 incubator
- 37°C water bath

3.A. Raji Cells (LDH-HiBiT) Thawing and Initial Cell Culture

The following protocol steps refer to thawing, propagation and freezing of Raji Cells (LDH-HiBiT), the parental cell line associated with the Raji (LDH-HiBiT) TCK Bioassay, Propagation Model. The same procedures should be followed when culturing CD19-, CD20- and CD19-/CD20- cells.

Follow institutional guidelines for handling, including use of personal protective equipment (PPE) and waste disposal for biohazardous material.

1. Prepare 20ml of cell growth medium by adding 2ml of FBS to 18ml of RPMI 1640 medium prewarmed to 37°C. This cell growth medium will be used for culturing the cells immediately after thawing.
2. Transfer 9ml of prewarmed cell growth medium to a 15ml conical tube.
3. Remove one vial of Raji Cells (LDH-HiBiT) from storage at –140°C and thaw in a 37°C water bath with gentle agitation (do not invert cell vial) until just thawed (typically 2–3 minutes).
4. Transfer all of the cells (approximately 1ml) to the 15ml conical tube containing 9ml of prewarmed cell growth medium.
5. Centrifuge at 150 × *g* for 10 minutes.
6. Carefully aspirate the medium and resuspend the cell pellet in 10ml of prewarmed cell growth medium. The initial cell density will be approximately 4×10^5 viable cells/ml.
7. Transfer the cell suspension to a T75 cell culture flask and place the flask horizontally in a humidified 37°C, 5% CO_2 incubator.
8. Incubate for 24–48 hours.

3.A. Raji Cells (LDH-HiBiT) Thawing and Initial Cell Culture (continued)

9. Passage the cells by adding fresh growth medium. For the initial passage, seed cells at a density no less than 3×10^5 viable cells/ml.

   Note: If needed, centrifuge at 150 × *g* for 10 minutes and resuspend in fresh growth medium to concentrate the cells to 3×10^5 viable cells/ml.

10. Incubate for approximately 48 hours before passaging the cells according to the schedule outlined in Section 3.B.

3.B. Raji Cells (LDH-HiBiT) Propagation and Maintenance

For cell propagation and maintenance, use the growth medium and monitor cell viability and doubling rate during propagation. The cell growth rate will stabilize by 7–10 days after thawing, at which time cell viability is typically >90%, and the average cell doubling rate is 24 hours. Passage number should be recorded for each passage. In our experience, cells maintain their functionality for at least 25 passages or 60 cell doublings, if passaging is performed on a Monday–Wednesday–Friday schedule.

1. On the day of cell passage, measure cell viability and density by Trypan blue staining.
2. Seed the cells at a density of $2–3 \times 10^5$ cells/ml if passaging every two days (e.g., Monday–Wednesday or Wednesday–Friday) or 2×10^5 cells/ml if passaging every three days (e.g., Friday–Monday). Always maintain the flasks in a horizontal position in the incubator. Do not allow the cells to grow to a density greater than 1.5×10^6 cells/ml. Duplicate flasks can be passaged at higher or lower cell density to ensure optimal cell density at harvest.
3. Maintain the cell culture by adding fresh cell growth medium to the cell suspension in the original flask or by transferring the cells to a new flask while maintaining a consistent ratio of culture volume to flask surface area (e.g., 25ml volume per T75 flask or 50ml volume per T150 flask).
4. Place the flasks horizontally in a humidified, 37°C, 5% CO_2 incubator.

3.C. Raji Cells (LDH-HiBiT) Freezing and Banking

1. On the day of cell freezing, prepare fresh cell freezing medium (Section 9.A) and keep on ice.
2. Gently mix the cells with a pipette to create a homogeneous cell suspension.
3. Remove a sample for cell counting by Trypan blue staining. Calculate the volume of cell freezing medium needed based on desired cell freezing densities of $2 \times 10^6–2 \times 10^7$ cells/ml.
4. Transfer the cell suspension to 50ml sterile conical tubes or larger sized centrifuge tubes, and centrifuge at 150 × *g*, 4°C for 10–15 minutes.
5. Gently aspirate the medium, taking care not to disturb the cell pellet.
6. Carefully resuspend the cell pellet in ice-cold cell freezing medium to a final cell density of $2 \times 10^6–2 \times 10^7$ cells/ml. Combine the cell suspensions into a single tube and dispense into cryovials.
7. Freeze the cells using a controlled-rate freezer (preferred), or a Mr. Frosty® or a Styrofoam® rack in a –80°C freezer overnight. Transfer the vials to –140°C or below for long-term storage.

4. PBMC ADCC Assay Protocol

This procedure illustrates the use of Raji Cells (LDH-HiBiT), the parental cell line associated with the Raji (LDH-HiBiT) TCK Bioassay, Propagation Model, to test two antibody samples against a reference sample in a single PBMC ADCC assay. Each test and reference antibody is run in triplicate, in an eight-point dilution series, in two 96-well assay plates using the inner 60 wells. Recommendations for including knockout cell line controls are at the end of this section. Other experimental and plate layouts are possible but may require further optimization.

Note: When preparing test and reference antibodies, choose an appropriate starting concentration and dilution scheme to achieve a complete dose-response curve with proper upper and lower asymptotes and sufficient points on the slope. For reference, we use 1µg/ml as a starting concentration (1X) and threefold serial dilutions when testing Control Ab, Anti-CD20 (Cat.# GA1130).

Maximum release (MR) and spontaneous release (SR) controls should be included in your experiment. The MR control uses digitonin to permeabilize target cells, providing the maximal luminescent signal. The SR control is a measure of background cell death in presence of effector cells but in the absence of a monoclonal antibody.

Materials to Be Supplied By the User

(Composition of Buffers and Solutions is provided in Section 9.A.)

Reagents

- Human PBMC, ADCC-Qualified (e.g., **www.promega.com/products/reporter-bioassays/primary-cell-bioassays/pbmc-adcc-bioassay/**; please enquire)
- antibodies or other biologics capable of inducing ADCC
- RPMI 1640 medium with L-glutamine and HEPES (e.g., Corning® Cat.# 10-041-CV or GIBCO® Cat.# 22400105)
- heat-inactivated (HI) fetal bovine serum (FBS) (e.g., GIBCO® Cat.# 16140)
- recombinant human IL-2 (e.g., Sigma Cat.# I2644)
- β-mercaptoethanol (e.g., GIBCO® Cat.# 21985)
- sodium pyruvate (e.g., GIBCO® Cat.# 11360)
- digitonin (e.g., Cat.# G9441)
- Bio-Glo-NB™ TCK Luciferase Assay System (Cat.# JB1001, JB1002, JB1003)
- Trypan blue solution (e.g., Sigma Cat.# T8154)
- **optional:** Control Ab, Anti-CD20 (Cat.# GA1130)

4. PBMC ADCC Assay Protocol (continued)

Supplies and Equipment

- solid-white, U-bottom 96-well assay plates (e.g., Corning® Cat.# 3355) or 384-well assay plates (e.g., Corning® Cat.# 4512, 4513) for ADCC assay applications
- sterile clear V-bottom 96-well plate with lid (e.g., Costar® Cat.# 3896) for preparing antibody dilutions
- pipettes (single-channel and 12-channel; for best results use both manual and electronic pipettes as needed)
- sterile 15ml and 50ml conical tubes
- sterile reagent reservoirs (e.g., Corning®/Costar® Cat.# 4870)
- T75 cell culture flasks (e.g., Corning® Cat.# 430641U)
- 37°C, 5% CO_2 incubator
- 37°C water bath
- sensitive plate reader with glow luminescence measuring capability or luminometer (e.g., GloMax® Discover System or equivalent)

Note: Corning® Cat.# 3355 plates are supplied without a lid and are marked as nonsterile. Use a lid from another Corning® plate type (e.g., Cat.# 3917). Although marked as nonsterile, we had no issues with sterility when using these plates while developing this product.

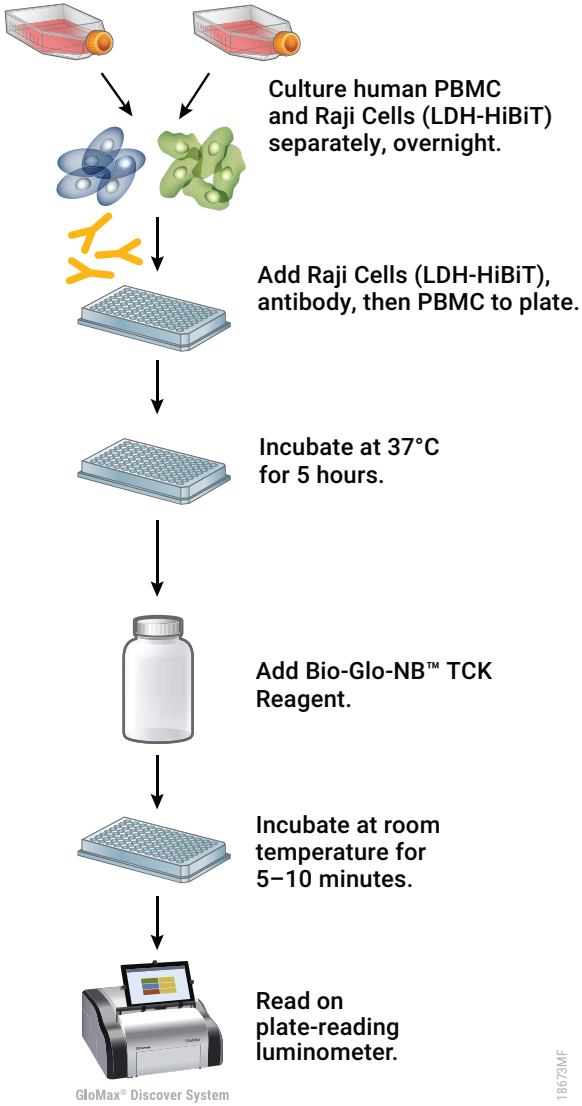

Figure 7. Schematic protocol for the Raji (LDH-HiBiT) TCK Bioassay, Propagation Model in a PBMC ADCC assay application.

4.A. Preparing Assay Reagents and Samples

Complete formulas for the following reagents are provided in Section 9.A.

PBMC Culture Medium: On the day before the assay, prepare an appropriate amount of PBMC culture medium: 90% RPMI 1640 with 10% HI-FBS and IL-2, β-ME, Na+ pyruvate.

PBMC ADCC Assay Buffer: On the day of the assay, prepare an appropriate amount of assay buffer: 95% RPMI 1640 with 5% HI-FBS and IL-2, β-mercaptoethanol. Mix well and warm to 37°C before use.

Note: The recommended assay buffer contains 5% HI-FBS. This concentration of FBS works well for the Control Ab, Anti-CD20 (Cat.# GA1130) that we tested. If you experience assay performance issues when using this assay buffer, we recommend testing different serum concentrations in the range of 0.5–10%.

Bio-Glo-NB™ TCK Reagent: For reference, 10ml of Bio-Glo-NB™ TCK Reagent is sufficient to assay 120 wells in a 96-well assay format. The Bio-Glo-NB™ TCK Luciferase Assay Substrate and LgBiT Protein should always be stored at –30°C to –10°C. Thaw the Bio-Glo-NB™ TCK Luciferase Assay Buffer at room temperature (do not exceed 25°C) during the 5-hour assay induction period. We recommend preparing the reconstituted Bio-Glo-NB™ TCK Reagent immediately before use.

Note: HiBiT TCK Bioassays are compatible only with Bio-Glo-NB™ TCK Reagent. **Do not** use Bio-Glo™ Reagent or Bio-Glo-NL™ Reagent.

Test and Reference Samples: Using assay buffer as the diluent, prepare stock starting dilutions (dilu1, 3X final concentration) of two test antibodies (300µl each) and one reference antibody (600µl) in tubes. Store the tubes containing antibody starting dilutions appropriately before making antibody serial dilutions.

Note: If you are using Control Ab, Anti-CD20 (Cat.# GA1130), as a reference antibody in your assay, prepare 300µl of a starting dilution at 3µg/ml (dilu1, 3X final concentration) in PBMC ADCC assay buffer.

4.B. Plate Layout Design

For the protocol described here, use the plate layout illustrated in Figure 8 as a guide. The protocol describes serial replicate dilutions (n = 3) of test and reference antibodies to generate two eight-point dose-response curves for each plate.

Recommended Plate Layout Design													
	1	2	3	4	5	6	7	8	9	10	11	12	
A	B	B	B	B	B	B	B	B	B	B	B	B	Assay Buffer (B)
B	B	SR	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	MR	B	Reference Ab
C	B	SR	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	MR	B	Test Ab
D	B	SR	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	MR	B	Reference Ab
E	B	SR	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	MR	B	Test Ab
F	B	SR	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	MR	B	Reference Ab
G	B	SR	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	MR	B	Test Ab
H	B	B	B	B	B	B	B	B	B	B	B	B	Assay Buffer (B)

Figure 8. Example plate layout showing nonclustered sample locations of test antibody and reference antibody dilution series. Maximum release (MR) control wells contain Raji Cells (LDH-HiBiT), PBMC effector cells and digitonin. Spontaneous release (SR) control wells contain Raji Cells (LDH-HiBiT) and PBMC effector cells. Test or reference antibodies are not added to MR and SR control wells. Wells containing assay buffer alone are denoted by "B".

4.C. Thawing Human PBMC, ADCC-Qualified Effector Cells

Note: Perform the following steps in a sterile cell culture hood.

1. Remove one vial (~2 × 10^7 cells) of PBMC from storage at –140°C and thaw in a 37°C water bath with gentle agitation (do not invert cell vial) until just thawed (typically 2–3 minutes).
2. Transfer all of the cells (approximately 1ml) to a 50ml conical tube containing 24ml of prewarmed PBMC ADCC culture medium.
3. Gently resuspend the PBMC using a 25ml pipette.

4. Transfer the cell suspension to a T75 tissue culture flask and place the flask horizontally in a humidified 37°C, 5% CO_2 incubator.
5. Incubate for approximately 16–24 hours before assay setup.

4.D. Preparing 3X Stock Solutions of Antibody

The instructions described here are for the preparation of 3X stocks of test and reference antibody. A threefold dilution series is made for each antibody, where a single 150µl stock of each dilution provides sufficient volume for analysis in triplicate. To prepare threefold serial dilutions, you will need 300µl of each test antibody and 600µl of reference antibody at 3X the highest antibody concentration in your dose-response curve. For other dilution schemes, adjust the volumes accordingly. For instance, prepare three independent stocks of each dilution for analysis in triplicate.

1. On the day of the assay, prepare an appropriate amount of PBMC ADCC assay buffer as described in Section 9.A.
2. To a sterile clear V-bottom 96-well plate, add 300µl of reference antibody starting dilution (dilu1, 3X final concentration) to wells A10 and B10 (Figure 9).
3. Add 300µl of test antibodies 1 and 2 starting dilution (dilu1, 3X final concentration) to wells E10 and G10, respectively (Figure 9).
4. Add 200µl of PBMC ADCC assay buffer to the other wells in these four rows, from column 9 to column 2.
5. Transfer 100µl of the antibody starting dilutions from column 10 into column 9. Mix well by pipetting. Avoid creating bubbles.
6. Repeat equivalent threefold serial dilutions across the columns from right to left through column 3. Do not dilute into column 2.

   Note: Wells A2, B2, E2 and G2 contain 200µl of PBMC ADCC assay buffer without antibody as a negative control.
7. Proceed immediately to Section 4.E.

4.E. Preparing 3X Digitonin for Maximum Release Control

1. Dilute digitonin stock in PBMC ADCC assay buffer to 300µg/ml (3X). Prepare 100µl for each test antibody and 200µl for the reference antibody.
2. Transfer 100µl of the digitonin solution to wells A11, B11, E11 and G11 of the antibody dilution plate.
3. Cover the antibody dilution plate with a lid and incubate at 37°C in a humidified 5% CO_2 incubator until Section 4.H.

Recommended Plate Layout for Antibody Dilutions Prepared from a Single Antibody Stock.													
	1	2	3	4	5	6	7	8	9	10	11	12	
A		no Ab	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	digitonin		Reference Ab
B		no Ab	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	digitonin		Reference Ab
C													
D													
E		no Ab	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	digitonin		Test Ab 1
F													
G		no Ab	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	digitonin		Test Ab 2
H													

Figure 9. Antibody dilution plate. Test and reference antibodies are serially diluted prior to addition to assay plates. Digitonin-containing samples in column 11 are added to MR control wells. No antibody samples in column 2 are added to SR control wells.

4.F. Preparing Raji Cells (LDH-HiBiT)

While maintaining the Raji Cells (LDH-HiBiT), follow the recommended cell seeding density (see Section 3). Changes in cell culture volume or seeding density will affect cell growth rate and assay performance. Do not allow the cells to overgrow. Only use the cells in the assay after the cell doubling rate has stabilized during propagation.

Note: Perform the following steps in a sterile cell culture hood.

1. Gently mix and count the Raji Cells (LDH-HiBiT) by Trypan blue staining.
2. Harvest sufficient cells for assay into a 15ml or 50ml centrifuge tube.
3. Centrifuge cells at 150 × *g* for 5 minutes.
4. Wash cells by removing supernatant, and gently resuspending in 10ml of PBMC ADCC assay buffer.
5. Centrifuge cells at 150 × *g* for 5 minutes, then remove and discard supernatant.
6. Gently resuspend the cell pellet in PBMC ADCC assay buffer to achieve a concentration of 2×10^5 viable cells/ml.
7. Keep the Raji Cells (LDH-HiBiT) at ambient temperature until Section 4.H.

4.G. Preparing Human PBMC, ADCC-Qualified Effector Cells

1. Gently resuspend and harvest PBMC using a 25ml pipette.
2. Transfer all of the PBMC to a 50ml conical centrifuge tube.
3. Pellet the cells at 300 × *g* for 5 minutes at ambient temperature.
4. Gently but thoroughly resuspend the pellet in 4ml of PBMC ADCC assay buffer to a cell density of 5×10^6 cells/ml.
5. Keep the PBMC at ambient temperature until Section 4.H.

4.H. Adding Target Cells, Effector Cells and Antibody

Note: Use only white assay plates with U-shaped well bottoms.

1. Gently resuspend and transfer the Raji Cells (LDH-HiBiT) suspension to a sterile reagent reservoir.
2. Using a multichannel pipette, immediately dispense 25µl of the cell suspension to wells B2–G11 of a 96-well white U-bottom assay plate. The final cell number in each well should be 5×10^3 cells/well.
3. Using a multichannel pipette, 25µl of the appropriate antibody dilution to wells B3–G10 of the assay plate according to Figure 7. Add 25µl of PBMC ADCC assay buffer to SR control wells (B2–G2). Add 25µl of PBMC ADCC assay buffer plus 300µg/ml digitonin to MR control wells (B11–G11).
4. Gently resuspend and transfer the PBMC effector cell suspension to a sterile reagent reservoir.
5. Using a multichannel pipette, immediately dispense 25µl of the cell suspension to each of wells B2–G11 of the assay plates. The final cell number in each well should be 1.25×10^5 cells/well.
6. Add 75µl of PBMC ADCC assay buffer to each of the outside wells of the assay plates.
7. Cover the assay plates with lids and incubate in a 37°C, 5% CO_2 incubator for 5 hours.

   Note: The 5-hour incubation was optimized using Control Ab, Anti-CD20. We recommend optimizing assay time (3–24 hours) with your antibody or other biologic samples.
8. During assay incubation, warm Bio-Glo-NB™ TCK Luciferase Assay Buffer to ambient temperature.

4.I. Preparing and Adding Bio-Glo-NB™ TCK Reagent

We recommend preparing the Bio-Glo-NB™ TCK Reagent immediately before use. Ensure that Bio-Glo-NB™ TCK Luciferase Assay Buffer is equilibrated to room temperature (do not exceed 25°C) before reconstituting the reagent. Do not store the reconstituted reagent. Once reconstituted, the reagent will lose about 15% activity over 8 hours and about 60% activity over 24 hours at room temperature.

Note: HiBiT TCK Bioassays are compatible only with Bio-Glo-NB™ TCK Reagent. **Do not** use Bio-Glo™ Reagent or Bio-Glo-NL™ Reagent.

1. Remove the Bio-Glo-NB™ TCK Luciferase Assay Substrate from –30°C to –10°C storage and mix by pipetting. Briefly centrifuge the tubes if the substrate has collected in the cap or on the sides of the tubes.
2. Remove the Bio-Glo-NB™ TCK LgBiT Protein from –30°C to –10°C storage and mix by pipetting. Briefly centrifuge the tubes if the solution has collected in the cap or on the sides of the tubes.

3. Transfer the desired amount of ambient temperature Bio-Glo-NB™ TCK Luciferase Assay Buffer to a 15ml or 50ml centrifuge tube.
4. Add Bio-Glo-NB™ TCK LgBiT Protein (1:100 dilution) and Bio-Glo-NB™ TCK Luciferase Assay Substrate (1:50 dilution) to the Bio-Glo-NB™ TCK Luciferase Assay Buffer. For example, if the experiment requires 10ml of reagent, add 100µl of Bio-Glo-NB™ TCK LgBiT Protein and 200µl of Bio-Glo-NB™ TCK Luciferase Assay Substrate to 10ml of buffer. Ten milliliters (10ml) of Bio-Glo-NB™ TCK Reagent is sufficient for 120 wells (two assay plates, using the inner 60 wells of each plate).
5. Remove assay plates from the incubator after the incubation period and equilibrate to room temperature for 10–15 minutes.
6. Using a multichannel pipette, add 75µl of Bio-Glo-NB™ TCK Reagent to all assay wells, taking care to not create bubbles. This includes MR and SR control wells.
7. Wait 10 minutes, then measure the luminescence in a GloMax® Discover System or a plate reader with glow-type luminescence reading capabilities. The luminescence intensity will decay gradually, with a signal half-life of 1–2 hours at room temperature.

   Note: Varying the Bio-Glo-NB™ TCK Reagent incubation time will affect the raw relative light unit (RLU) values but should not significantly change the EC_{50} value and maximum fold induction.

4.J. Data Analysis

The HiBiT TCK Bioassay is a nonlytic bioassay that measures HiBiT released from dead or dying target cells using Bio-Glo-NB™ TCK Reagent. Luminescence signal is expressed as RLU.

1. Calculate fold induction:

$$\text{Fold Induction} = \frac{RLU_{induced}}{RLU_{SR\ control}}$$

2. Calculate percent specific lysis:

$$\text{Percent Specific Lysis} = \frac{RLU_{induced} - RLU_{SR\ control}}{RLU_{MR\ control} - RLU_{SR\ control}} \times 100$$

   MR (maximum release) control wells contain Raji Cells (LDH-HiBiT) plus PBMC effector cells (no test or reference antibody added). These wells are treated with digitonin to measure the total amount of HiBiT protein in each well.

   SR (spontaneous release) control wells contain Raji Cells (LDH-HiBiT) plus PBMC effector cells (no test or reference antibody added). These wells measure background killing in the absence of a test or reference antibody.

3. Graph data as RLU versus Log_{10}[antibody], fold induction versus Log_{10}[antibody] and percent specific lysis versus Log_{10} [antibody]. Fit curves and determine the EC_{50} value of antibody response using appropriate curve fitting software (such as GraphPad Prism® software).

4.K. Using Knockout Controls

Knockout cells should be plated at the same density as the parental cell line and handled identically during bioassay setup. You should continue to include test and reference antibodies on the same plate, using separate plates for parental and knockout cell lines. An antibody that targets an antigen remaining on the surface of knockout cells (e.g., CD22 or CD38) can be included as a positive control for both parental and knockout cell lines. Always calculate percent specific lysis when comparing between parental and knockout controls, as clones may vary in raw RLU.

5. CD8+ T Cell TDCC Assay Protocol

This procedure illustrates the use of Raji Cells (LDH-HiBiT), the parental cell line associated with the Raji (LDH-HiBiT) TCK Bioassay, Propagation Model, to test two samples against a reference sample in a single TDCC assay. Each test and reference sample is run in triplicate, in an eight-point dilution series, in a single 96-well assay plate using the inner 60 wells (Figure 10). Other experimental and plate layouts are possible but may require further optimization. See Section 5.I for recommendations on how to include knockout cell line controls.

Note: When preparing test and reference samples, choose an appropriate starting concentration and dilution scheme to achieve a complete dose-response curve with proper upper and lower asymptotes and sufficient points on the slope. For reference, we use 15ng/ml as a starting concentration (1X) and threefold serial dilutions when testing blinatumomab.

Maximum release (MR) and spontaneous release (SR) controls should be included in your experiment. The MR control uses digitonin to permeabilize target cells, providing the maximal luminescent signal. The SR control is a measure of background cell death in the absence of a biologic drug.

Materials to Be Supplied By the User

(Composition of Buffers and Solutions is provided in Section 9.A.)

Reagents

- Human T Cells (CD8+), TDCC-Qualified (please enquire: **EarlyAccess@promega.com**)
- bispecific antibodies or related biologic drugs capable of inducing TDCC
- RPMI 1640 medium with L-glutamine and HEPES (e.g., Corning® Cat.# 10-041-CV or GIBCO® Cat.# 22400105)
- heat-inactivated fetal bovine serum (e.g., GIBCO® Cat.# 16140)
- digitonin (e.g., Cat.# G9441)
- Bio-Glo-NB™ TCK Luciferase Assay System (Cat.# JB1001, JB1002, JB1003)
- Trypan blue solution (e.g., Sigma Cat.# T8154)

Supplies and Equipment

- solid-white, flat-bottom 96-well assay plates (e.g., Corning® Cat.# 3917) or 384-well assay plates (e.g., Corning® Cat.# 4512, 4513)
- sterile clear V-bottom 96-well plate with lid (e.g., Costar® Cat.# 3896) for preparing antibody dilutions
- pipettes (single-channel and 12-channel; for best results use both manual and electronic pipettes as needed)
- sterile 15ml and 50ml conical tubes
- sterile reagent reservoirs (e.g., Corning®/Costar® Cat.# 4870)
- 37°C, 5% CO_2 incubator
- 37°C water bath
- sensitive plate reader with glow luminescence measuring capability or luminometer (e.g., GloMax® Discover System or equivalent)

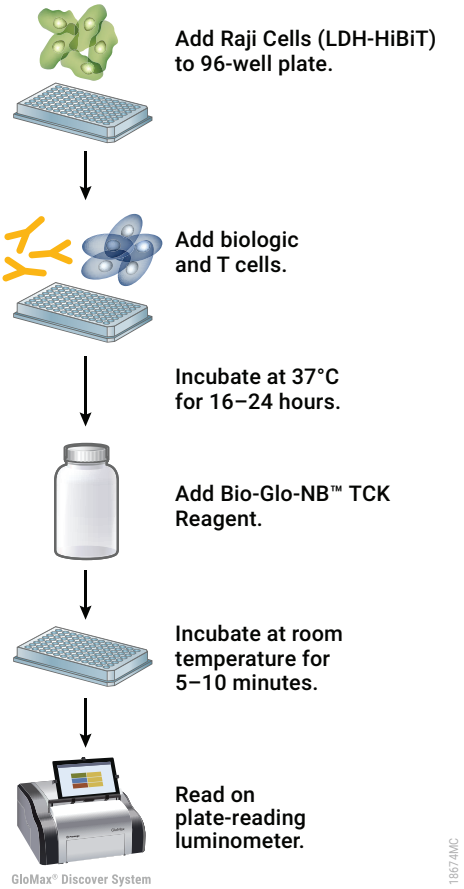

Figure 10. Schematic protocol for the Raji (LDH-HiBiT) TCK Bioassay, Propagation Model, in a TDCC bioassay application.

5.A. Preparing Assay Reagents and Samples

CD8+ T Cell TDCC Assay Buffer: On the day of the assay, prepare an appropriate amount of assay buffer (90% RPMI 1640, 10% HI-FBS). Mix well and warm to 37°C before use.

Note: The recommended assay buffer contains 10% HI-FBS. This concentration of FBS works well for use with blinatumomab. If you experience assay performance issues when using this assay buffer, we recommend testing serum concentrations in the range of 0.5–10%.

Bio-Glo-NB™ TCK Reagent: For reference, 10ml of Bio-Glo-NB™ TCK Reagent is sufficient to assay 120 wells in a 96-well assay format. The Bio-Glo-NB™ TCK Luciferase Assay Substrate and the Bio-Glo-NB™ TCK LgBiT Protein should always be stored at –30°C to –10°C. Thaw the Bio-Glo-NB™ TCK Luciferase Assay Buffer at room temperature (do not exceed 25°C) during the overnight assay induction period. We recommend preparing the reconstituted Bio-Glo-NB™ TCK Reagent immediately before use.

Note: HIBiT TCK Bioassays are compatible only with Bio-Glo-NB™ TCK Reagent. **Do not** use Bio-Glo™ Reagent or Bio-Glo-NL™ Reagent.

Test and Reference Samples: Using assay buffer as the diluent, prepare stock starting dilutions (dilu1, 3X final concentration) of two test biologic drugs (300µl each) and one reference biologic (600µl) in tubes. Store the tubes containing antibody starting dilutions appropriately before making antibody serial dilutions.

5.B. Plate Layout Design

For the protocol described here, use the plate layout illustrated in Figure 11 as a guide. The protocol describes serial replicate dilutions (n = 3) of test and reference antibodies to generate two eight-point dose-response curves for each plate.

Recommended Plate Layout Design													
	1	2	3	4	5	6	7	8	9	10	11	12	
A	B	B	B	B	B	B	B	B	B	B	B	B	Assay Buffer (B)
B	B	SR	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	MR	B	Reference Biologic
C	B	SR	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	MR	B	Test Biologic
D	B	SR	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	MR	B	Reference Biologic
E	B	SR	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	MR	B	Test Biologic
F	B	SR	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	MR	B	Reference Biologic
G	B	SR	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	MR	B	Test Biologic
H	B	B	B	B	B	B	B	B	B	B	B	B	Assay Buffer (B)

Figure 11. Example plate layout showing nonclustered sample locations of test and reference dilution series of biologic drugs. Maximum release (MR) and spontaneous release (SR) control wells contain Raji Cells (LDH-HiBiT) and CD8+ T cells but lack test or reference biologic drugs. MR control wells receive Bio-Glo NB™ TCK Reagent containing 200µg/ml digitonin. Wells containing assay buffer alone are denoted by "B".

5.C. Preparing 3X Stock Solutions of Biologic Drugs

The instructions described here are for preparation of 3X stocks of test and reference biologic drugs. A threefold dilution series is made for each biologic drug, where a single 150µl stock of each dilution provides sufficient volume for analysis in triplicate. To prepare threefold serial dilutions, you will need 300µl of test and 600µl of reference biologic drugs at 3X the highest concentration used in your dose-response curve. For other dilution schemes, adjust the volumes accordingly. For instance, prepare three independent stocks of each dilution for analysis in triplicate.

Note: Perform the following steps in a sterile cell culture hood.

1. On the day of the assay, prepare an appropriate amount of TDCC assay buffer as described in Section 9.A.
2. To a sterile clear V-bottom 96-well plate, add 300µl of reference biologic starting dilution (dilu1, 3X final concentration) to wells A10 and B10 (Figure 12).
3. Add 300µl of test biologics 1 and 2 starting dilution (dilu1, 3X final concentration) to wells E10 and G10, respectively (Figure 12).
4. Add 200µl of TDCC assay buffer to dilu2 through dilu8 in rows A, B, E and G, from column 9 to column 3. Add 200µl of TDCC assay buffer per well to no biologic drug controls wells (columns 2 and 11).
5. Transfer 100µl of the biologic drug starting dilutions from column 10 into column 9. Mix well by pipetting. Avoid creating bubbles.
6. Repeat equivalent threefold serial dilutions across the columns from right to left through column 3. Do not dilute into column 2.

   Note: Wells A2, B2, E2 and G2 contain 200µl of TDCC assay buffer without biologic drug for delivery to SR control wells. Wells A11, B11, E11 and G11 contain 200µl of TDCC assay buffer without biologic drug or digitonin for delivery to MR control wells. Digitonin is added to MR control wells at the time of luminescence measurement (Section 5.G).
7. Cover the biologic dilution plate with a lid and incubate at 37°C in a humidified 5% CO_2 incubator until Section 5.F while preparing the Raji Cells (LDH-HiBiT) and TDCC Effector cells.

Recommended Plate Layout for Biologic Dilutions Prepared from a Single Antibody Stock.													
	1	2	3	4	5	6	7	8	9	10	11	12	
A		No Biologic	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	No Biologic		Reference Biologic
B		No Biologic	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	No Biologic		Reference Biologic
C													
D													
E		No Biologic	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	No Biologic		Test Biologic 1
F													
G		No Biologic	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	No Biologic		Test Biologic 2
H													

Figure 12. Biologic drug dilution plate. Test and reference biologic drugs are serially diluted prior to addition to assay plates. No biologic drug samples in columns 2 and 11 are added to SR and MR control wells, respectively. Do not add digitonin to the wells in column 11. Digitonin is added to MR control wells at the time of luminescence measurement (Section 5.G).

5.D. Preparing Raji Cells (LDH-HiBiT)

While maintaining the Raji Cells (LDH-HiBiT), follow the recommended cell seeding density (see Section 3). Changes in cell culture volume or seeding density will affect cell growth rate and assay performance. Do not allow the cells to overgrow. Only use the cells in the assay after the cell doubling rate has stabilized during propagation.

1. Gently mix and count the Raji Cells (LDH-HiBiT) by Trypan blue staining.
2. Harvest sufficient cells for assay into a 15ml or 50ml centrifuge tube.
3. Centrifuge cells at 150 × *g* for 5 minutes.
4. Wash cells by removing supernatant and gently resuspending in 10ml of TDCC assay buffer.
5. Centrifuge cells at 150 × *g* for 5 minutes.
6. Gently resuspend the cell pellet in TDCC assay buffer to a concentration of 1×10^5 viable cells/ml.
7. Incubate the Raji Cells (LDH-HiBiT) at ambient temperature until Section 5.F.

5.E. Preparing Human CD8+ T Cells, TDCC-Qualified Effector Cells (T Cells)

1. Remove one vial of T cells from storage at –140°C and thaw in a 37°C water bath with gentle agitation (do not invert cell vial) until just thawed (typically 2–3 minutes).
2. Transfer all of the cells into 3.5ml of prewarmed TDCC assay buffer for a concentration of 1×10^6 cells/ml.
3. Incubate the T cells at ambient temperature until Section 5.F.

5.F. Adding Raji Cells (LDH-HiBiT), Effector Cells and Biologic Drugs

1. Gently resuspend and transfer the Raji Cells (LDH-HiBiT) suspension to a sterile reagent reservoir.
2. Using a multichannel pipette, immediately dispense 25µl of the cell suspension to each of wells B2–G11 of a 96-well, white, flat-bottom assay plate. The final cell number in each well should be 2.5×10^3 cells/well.
3. Using a multichannel pipette, add 25µl of the appropriate biologic drug dilution (Figure 12) to wells B3–G10 of the assay plate according to the plate layout in Figure 11. Add 25µl of TDCC assay buffer to MR and SR control wells (B2–G2 and B11–G11, respectively).

   Note: Digitonin will be added to MR control wells just prior to measuring luminescence (Section 5.G).
4. Gently resuspend and transfer the T cell suspension to a sterile reagent reservoir.
5. Using a multichannel pipette, immediately dispense 25µl of the T cell suspension to each of wells B2–G11 of the assay plates. The final cell number in each well should be 2.5×10^4 cells/well.
6. Add 75µl of TDCC assay buffer to each of the outside wells of the assay plates.
7. Cover the assay plates with lids and incubate in a 37°C, 5% CO_2 incubator for 16–24 hours.

   Note: The incubation period was optimized using blinatumomab. We recommend optimizing assay time (3–24 hours) with your biologic drug of interest.
8. Near the end of the assay incubation, equilibrate the Bio-Glo-NB™ TCK Luciferase Assay Buffer to ambient temperature.

5.G. Preparing and Adding Bio-Glo-NB™ TCK Reagent

We recommend preparing the Bio-Glo-NB™ TCK Reagent immediately before use. Ensure that the Bio-Glo-NB™ TCK Luciferase Assay Buffer is equilibrated to room temperature (do not exceed 25°C) before reconstituting the reagent. Do not store the reconstituted reagent. Once reconstituted, the reagent will lose approximately 15% activity over 8 hours and approximately 60% activity over 24 hours at room temperature.

Note: HiBiT TCK Bioassays are compatible only with Bio-Glo-NB™ TCK Reagent. **Do not** use Bio-Glo™ Reagent or Bio-Glo-NL™ Reagent.

1. Remove the Bio-Glo-NB™ TCK Luciferase Assay Substrate from –30°C to –10°C storage and mix by pipetting. Briefly centrifuge the tubes if the substrate has collected in the cap or on the sides of the tubes.
2. Remove the Bio-Glo-NB™ TCK LgBiT Protein from –30°C to –10°C storage and mix by pipetting. Briefly centrifuge the tubes if the solution has collected in the cap or on the sides of the tubes.
3. Transfer the desired amount of ambient temperature Bio-Glo-NB™ TCK Luciferase Assay Buffer to a 15ml or 50ml centrifuge tube.
4. Add Bio-Glo-NB™ TCK LgBiT Protein (1:100 dilution) and Bio-Glo-NB™ TCK Luciferase Assay Substrate (1:50 dilution) to the Bio-Glo-NB™ TCK Luciferase Assay Buffer. For example, if the experiment requires 10ml of reagent, add 100µl of Bio-Glo-NB™ TCK LgBiT Protein and 200µl of Bio-Glo-NB™ TCK Luciferase Assay Substrate to 10ml of Bio-Glo-NB™ TCK Luciferase Assay Buffer. Ten milliliters (10ml) of Bio-Glo-NB™ TCK Reagent is sufficient for 120 wells (two assay plates, using the inner 60 wells of each plate).
5. Prepare the Bio-Glo-NB™ TCK Reagent with 200µg/ml digitonin for detection of MR controls as follows:
   a. Calculate the volume of Bio-Glo-NB™ TCK Reagent required for MR control wells.
   b. Transfer the calculated volume of fully reconstituted Bio-Glo-NB™ TCK Reagent to a separate tube.
   c. Add digitonin to a final concentration of 200µg/ml.
6. Remove assay plates from the incubator after the incubation period and equilibrate to room temperature for 10–15 minutes.
7. Using a manual multichannel pipette, add 75µl of Bio-Glo-NB™ TCK Reagent to wells treated with test or reference biologic drugs and SR controls, taking care to not create bubbles.
8. Add 75µl of Bio-Glo-NB™ TCK Reagent with 200µg/ml digitonin (from Step 5) to MR control wells, taking care to not create bubbles.
9. Wait 10 minutes, then measure the luminescence in a GloMax® Discover System or a plate reader with glow-type luminescence reading capabilities. The luminescence intensity will decay gradually, with a signal half-life of approximately 120 minutes at room temperature.

   Note: Varying the Bio-Glo-NB™ TCK Reagent incubation time will affect the raw RLU values but should not significantly change the EC_{50} value and maximum fold induction.

5.H. Data Analysis

The HiBiT TCK Bioassay is a nonlytic bioassay that measures HiBiT released from dead or dying cells using Bio-Glo-NB™ TCK Reagent. The luminescent signal is expressed as RLU.

1. Calculate fold induction:

$$\text{Fold Induction} = \frac{RLU_{Induced}}{RLU_{SR\ control}}$$

2. Calculate percent specific lysis:

$$\text{Percent Specific Lysis} = \frac{RLU_{Induced} - RLU_{SR\ control}}{RLU_{MR\ control} - RLU_{SR\ control}} \times 100$$

   Maximum release (MR) control wells contain Raji Cells (LDH-HiBiT) plus CD8+ T cells (no test or reference biologic drug added). These wells are treated with Bio-Glo-NB™ TCK Reagent supplemented with 200µg/ml digitonin to measure the total amount of HiBiT protein in each well.

   Spontaneous release (SR) control wells contain Raji Cells (LDH-HiBiT) plus CD8+ T cells (no test or reference biologic drug added). These wells measure background killing in the absence of a test or reference biologic drug.

3. Graph data as RLU versus Log_{10} [biologic drug], fold induction versus Log_{10} [biologic drug] and percent specific lysis versus Log_{10}[biologic drug]. Fit curves and determine the EC_{50} value of the biologic drug response using appropriate curve fitting software (such as GraphPad Prism® software).

5.I. Using Knockout Controls

Knockout cells should be plated at the same density as the parental cell line and handled identically during bioassay setup. Users should continue to include test and reference antibodies on the same plate, using separate plates for parental and knockout cell lines. A biologic drug that targets an antigen remaining on the surface of knockout cells (e.g., BCMA) can be included as a positive control for both parental and knockout cell lines (Figure 6). Always calculate percent specific lysis when comparing between parental and knockout controls, as clones may vary in raw RLU.

6. CAR-T Assay Protocol

This procedure illustrates the use of Raji Cells (LDH-HiBiT), the parental cell line associated with the Raji (LDH-HiBiT) TCK Bioassay, Propagation Model, to test CAR-T effector cells against a reference sample in a single cytotoxicity assay (Figure 13). Each test and reference CAR-T is assayed in triplicate, in an eight-point effector-to-target (E:T) ratio dilution series, in a single 96-well assay plate using the inner 60 wells. Other experimental and plate layouts are possible but may require further optimization. See Section 6.H for recommendations on including knockout cell line controls.

Note: When preparing test and reference CAR-T dilutions, choose an appropriate starting concentration and dilution scheme to achieve a complete dose-response curve with proper upper and lower asymptotes and sufficient points on the slope. For reference, we use a 100:1 E:T ratio as a starting concentration (1X) and twofold serial dilutions when testing CAR-T cells. Appropriate dilution schemes may vary significantly depending on the potency of your CAR-T cells.

Maximum release (MR) and spontaneous release (SR) controls should be included in your experiment. The MR control utilizes digitonin to permeabilize target cells, providing the maximal luminescent signal. The SR control is a measure of background cell death in the absence of CAR-T cells.

Materials to Be Supplied By the User

(Composition of Buffers and Solutions is provided in Section 9.A.)

Reagents

- user-provided test and reference CAR-T effector cells recognizing antigen expressed on Raji Cells (LDH-HiBiT) (CD19, CD20, CD22, etc.)
- RPMI 1640 medium with L-glutamine and HEPES (e.g., Corning® Cat.# 10-041-CV or GIBCO® Cat.# 22400105)
- heat-inactivated fetal bovine serum (HI-FBS; e.g., GIBCO® Cat.# 16140)
- digitonin (e.g., Cat.# G9441)
- Bio-Glo-NB™ TCK Luciferase Assay System (Cat.# JB1001, JB1002, JB1003)
- Trypan blue solution (e.g., Sigma Cat.# T8154)

6. CAR-T Assay Protocol (continued)

Supplies and Equipment

- solid-white, U-bottom 96-well assay plates (e.g., Corning® Cat.# 3355)
- sterile clear V-bottom 96-well plate with lid (e.g., Costar® Cat.# 3896) for preparing CAR-T cell dilutions
- pipettes (single-channel and 12-channel; for best results use both manual and electronic pipettes as needed)
- sterile 15ml and 50ml conical tubes
- sterile reagent reservoirs (e.g., Corning®/Costar® Cat.# 4870)
- 37°C, 5% CO_2 incubator
- 37°C water bath
- sensitive plate reader with glow luminescence measuring capability or luminometer (e.g., GloMax® Discover System or equivalent system)

Notes:

a. Solid-white flat-bottom assay plates can also be used for CAR-T assays. However, with flat-bottom plates we generally observe a right-shifted dose-response curve compared to U-bottom assay plates. The optimal plate type will vary depending on the properties of the CAR-T cells used in your experiment.

b. Corning® Cat.# 3355 plates are provided without lids and are marked as nonsterile. Use a lid from another Corning® plate type (e.g., Cat.# 3917). Although marked as nonsterile, we had no issues with sterility when using these plates while developing this product.

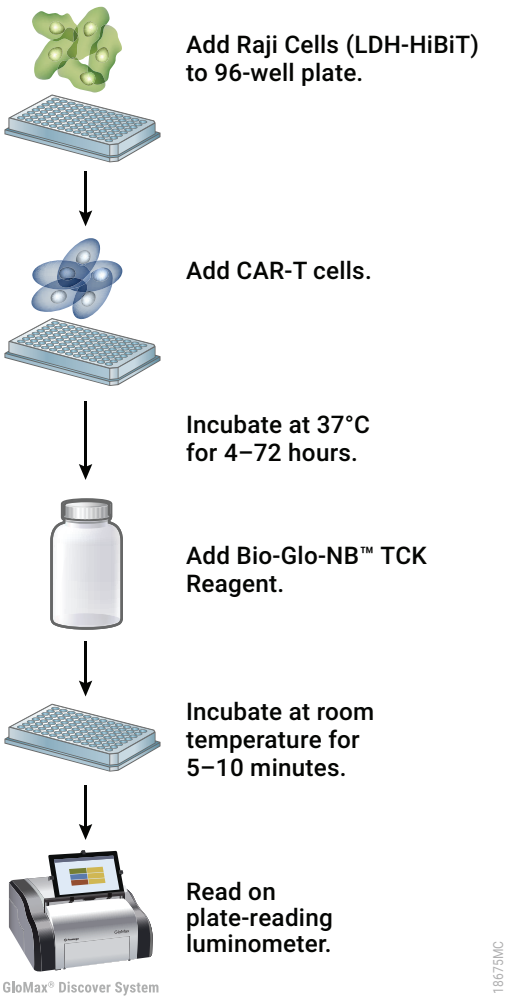

Figure 13. Schematic protocol for the Raji (LDH-HiBiT) TCK Bioassay, Propagation Model, in a CAR-T assay application.

6.A. Preparing Assay Reagents

CAR-T Assay Buffer: On the day of the assay, prepare an appropriate amount of CAR-T assay buffer (90% RPMI 1640 with 10% HI-FBS). Mix well and warm to 37°C before use.

Note: The recommended assay buffer contains 10% HI-FBS. This concentration of FBS works well for the CAR-T that we have tested. If you experience assay performance issues when using this assay buffer, we recommend testing different serum concentrations in the range of 0.5–10%.

Bio-Glo-NB™ TCK Reagent: For reference, 10ml of Bio-Glo-NB™ TCK Reagent is sufficient to assay 120 wells in a 96-well assay format. Store the Bio-Glo-NB™ TCK Luciferase Assay Substrate and Bio-Glo-NB™ TCK LgBiT Protein at –30°C to –10°C. Thaw the Bio-Glo-NB™ TCK Luciferase Assay Buffer at room temperature (do not exceed 25°C) near the end of the assay induction period. We recommend preparing the reconstituted Bio-Glo-NB™ TCK Reagent immediately before use.

Note: HiBiT TCK Bioassays are compatible only with Bio-Glo-NB™ TCK Reagent. **Do not** use Bio-Glo™ Reagent or Bio-Glo-NL™ Reagent.

Test and Reference Samples: Using assay buffer as the diluent, prepare stock starting dilutions (dilu1, 2X final concentration of CAR-T cells for the highest E:T ratio) of each CAR-T before making serial dilutions.

6.B. Plate Layout Design

For the protocol described here, use the plate layout illustrated in Figure 14 as a guide. The protocol describes serial replicate dilutions (n = 3) of test and reference CAR-T cell preparations to generate two eight-point dose-response curves for each plate.

Recommended Plate Layout Design													
	1	2	3	4	5	6	7	8	9	10	11	12	
A	B	B	B	B	B	B	B	B	B	B	B	B	Assay Buffer (B)
B	B	SR	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	MR	B	Reference CAR-T
C	B	SR	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	MR	B	Test CAR-T
D	B	SR	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	MR	B	Reference CAR-T
E	B	SR	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	MR	B	Test CAR-T
F	B	SR	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	MR	B	Reference CAR-T
G	B	SR	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	MR	B	Test CAR-T
H	B	B	B	B	B	B	B	B	B	B	B	B	Assay Buffer (B)

Figure 14. Example plate layout showing nonclustered sample locations of test and reference CAR-T dilution series. Maximum release (MR) and spontaneous release (SR) control wells contain Raji Cells (LDH-HiBiT) alone (no effector cells). MR control wells receive Bio-Glo-NB™ TCK Reagent containing 200µg/ml digitonin. Wells containing assay buffer alone are denoted by "B".

6.C. Preparing Raji Cells (LDH-HiBiT)

While maintaining the Raji Cells (LDH-HiBiT), follow the recommended cell seeding density (see Section 3). Changes in cell culture volume or seeding density will affect cell growth rate and assay performance. Do not allow the cells to overgrow. Only use the cells in the assay after the cell doubling rate has stabilized during propagation.

Note: Perform the following steps in a sterile cell culture hood.

1. Gently mix and count the Raji Cells (LDH-HiBiT) by Trypan blue staining.
2. Harvest sufficient cells for assay into a 15ml or 50ml centrifuge tube.
3. Centrifuge cells at 150 × *g* for 5 minutes.
4. Wash cells by removing supernatant and gently resuspending in 10ml of CAR-T assay buffer.
5. Centrifuge cells at 150 × *g* for 5 minutes.
6. Gently resuspend the cell pellet in CAR-T assay buffer (Section 9.A) to achieve a concentration of 1.25×10^5 viable cells/ml. This results in a final concentration of 5×10^3 Raji Cells (LDH-HiBiT) per well.
7. Incubate the Raji Cells (LDH-HiBiT) at ambient temperature until Section 6.E.

6.D. Preparing 2X Stock Solutions of CAR-T Cells

1. Thaw or harvest CAR-T cells according to the protocol established in your lab.
2. Pellet cells according to your protocol and resuspend in CAR-T assay buffer at a concentration of 6.25×10^6 viable cells/ml, a 2X stock solution for a maximum E:T ratio of 50:1.
3. In a clear V-bottom 96 well plate or 12-well dilution reservoir, perform seven twofold serial dilutions of CAR-T cells using CAR-T assay buffer as diluent. Prepare enough of each cell suspension for 40µl per well of each cell density tested in triplicate.
4. Proceed immediately to Section 6.E.

6.E. Adding Target Cells and CAR-T Cells to Assay Plates

1. Gently resuspend and transfer the Raji Cells (LDH-HiBiT) suspension to a sterile reagent reservoir.
2. Using a multichannel pipette, immediately dispense 40µl of the cell suspension to each of wells B2–G11 of a 96-well white U-bottom assay plate. The final cell concentration should be 5×10^3 cells/well.
3. Using a multichannel pipette, add 40µl of the appropriate CAR-T dilution to wells B3–G10 of the assay plates according to the plate layout in Figure 14. Add 40µl of CAR-T assay buffer to MR and SR control wells (B2–G2 and B11–G11, respectively).

   Note: Digitonin will be added at the time of luminescence measurement (Section 6.F).
4. Add 80µl of CAR-T assay buffer to each of the outside wells of the assay plates.
5. Cover the assay plates with lids and incubate in a 37°C, 5% CO_2 incubator for 4–72 hours. We recommend testing several assay times as part of assay optimization for the CAR-T of interest.
6. Near the end of the assay incubation, warm Bio-Glo-NB™ TCK Luciferase Assay Buffer to ambient temperature.

6.F. Preparing and Adding Bio-Glo-NB™ TCK Reagent

We recommend preparing the Bio-Glo-NB™ TCK Luciferase Reagent immediately before use. Ensure that the Bio-Glo-NB™ TCK Luciferase Assay Buffer is equilibrated to room temperature (do not exceed 25°C) before reconstituting the reagent. Do not store the reconstituted reagent. Once reconstituted, the reagent will lose approximately 15% activity over 8 hours and approximately 60% activity over 24 hours at room temperature.

Note: HiBiT TCK Bioassays are compatible only with Bio-Glo-NB™ TCK Reagent. **Do not** use Bio-Glo™ Reagent or Bio-Glo-NL™ Reagent.

1. Remove the Bio-Glo-NB™ TCK Luciferase Assay Substrate from –30°C to 10°C storage and mix by pipetting. Briefly centrifuge the tubes if the substrate has collected in the cap or on the sides of the tubes.
2. Remove the Bio-Glo-NB™ TCK LgBiT Protein from –30°C to –10°C storage and mix by pipetting. Briefly centrifuge the tubes if the solution has collected in the cap or on the sides of the tubes.
3. Transfer the desired amount of ambient temperature Bio-Glo-NB™ TCK Luciferase Assay Buffer to a 15ml or 50ml centrifuge tube.
4. Add Bio-Glo-NB™ TCK LgBiT Protein (1:100 dilution) and Bio-Glo-NB™ TCK Luciferase Assay Substrate (1:50 dilution) to the Bio-Glo-NB™ TCK Luciferase Assay Buffer. For example, if the experiment requires 10ml of reagent, add 100µl of Bio-Glo-NB™ TCK LgBiT Protein and 200µl of Bio-Glo-NB™ TCK Luciferase Assay Substrate to 10ml of Bio-Glo-NB™ TCK Luciferase Assay Buffer. Ten milliliters (10ml) of the Bio-Glo-NB™ TCK Reagent is sufficient for 120 wells (two assay plates, using the inner 60 wells of each plate).
5. Prepare Bio-Glo-NB™ TCK Reagent with 200µg/ml digitonin as a detection reagent for MR controls:
   a. Calculate the volume of Bio-Glo-NB™ TCK Reagent required for the MR control wells.
   b. Transfer the calculated volume of Bio-Glo-NB™ TCK Reagent to a separate tube.
   c. Add digitonin to a final concentration of 200µg/ml.
6. Remove assay plates from the incubator after the incubation period and equilibrate to room temperature for 10–15 minutes.
7. Using a manual multichannel pipette, add 80µl of Bio-Glo-NB™ TCK Reagent to the wells treated with test or reference CAR-T cells and SR controls, taking care not to create bubbles.
8. Add 80µl of Bio-Glo-NB™ TCK Reagent plus 200µg/ml of digitonin (from Step 5) to MR control wells.
9. Wait 10 minutes, then measure the luminescence in a GloMax® Discover System or a plate reader with glow-type luminescence reading capabilities. The luminescence intensity will decay gradually, with a signal half-life of approximately 1–2 hours at room temperature.

   Note: Varying the Bio-Glo-NB™ TCK Reagent ™ incubation time will affect the raw relative light unit (RLU) values but should not significantly change the EC_{50} value and maximum fold induction.

6.G. Data Analysis

The HiBiT TCK Bioassay is a nonlytic bioassay that measures HiBiT released from dead or dying cells using Bio-Glo-NB™ TCK Reagent. Luminescence signal is expressed as RLU.

1. Calculate fold induction:

$$\text{Fold Induction} = \frac{RLU_{Induced}}{RLU_{SR\ control}}$$

2. Calculate percent specific lysis:

$$\text{Percent Specific Lysis} = \frac{RLU_{Induced} - RLU_{SR\ control}}{RLU_{MR\ control} - RLU_{SR\ control}} \times 100$$

   Maximum release (MR) control wells contain Raji Cells (LDH-HiBiT) alone (no CAR-T cells added). These wells are treated with Bio-Glo-NB™ TCK Reagent supplemented with 200µg/ml digitonin to measure the total amount of HiBiT protein in each well.

   Spontaneous release (SR) control wells contain Raji Cells (LDH-HiBiT) alone (no CAR-T cells added). These wells measure background target cell death in the absence of CAR-T cells.

3. Graph data as RLU versus Log_{10}(E:T ratio), fold induction versus Log_{10}(E:T ratio) and percent specific lysis versus Log_{10} (E:T ratio). Fit curves and determine the EC_{50} value of the CAR-T response using appropriate curve fitting software (such as GraphPad Prism® software).

6.H. Using Knockout Controls

Knockout cells should be plated at the same density as the parental cell line and handled identically during bioassay setup. Users should continue to include test and reference preparations of CAR-T cells on the same plate, using separate plates for parental and knockout cell lines. A CAR-T preparation that targets an antigen remaining on the surface of knockout cells (e.g., CD22 or BCMA) can be included as a positive control for both parental and knockout cell lines. Always calculate percent specific lysis when comparing between parental and knockout controls, as clones may vary in raw RLU values.

7. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. Email: **techserv@promega.com**

Symptoms	Causes and Comments
Low luminescence measurements (RLU readout)	Ensure that you are using Bio-Glo-NB™ TCK Reagent. Raji (LDH-HiBiT) TCK Bioassays, Propagation Model, are not compatible with Bio-Glo™ or Bio-Glo-NL™ Reagents.
	Choose an instrument designed for plate-reading luminescence detection. Instruments designed primarily for fluorescence detection are not recommended. Luminometers measure and report luminescence as relative values, and actual RLU numbers will vary between instruments.
	Some models of luminometers with low sensitivity should be avoided. If using a reader with an adjustable gain, we recommend a high-gain setting.
	Insufficient cells per well can lead to low RLU. Handle and plate cells according to the instructions to ensure a sufficient number of viable cells per well.
	Low activity of Bio-Glo-NB™ TCK Reagent leads to low RLU. Store and handle Bio-Glo-NB™ Reagent according to the instructions. For best results, prepare immediately before use.
Weak assay response (low fold induction)	Ensure that you are using Bio-Glo-NB™ TCK Reagent. Raji (LDH-HiBiT) TCK Bioassays, Propagation Model, are not compatible with Bio-Glo™ or Bio-Glo-NL™ Reagents.
	Optimize the concentration range of your test sample(s) to achieve a full dose response with complete upper and lower asymptotes. The EC_{50} value obtained with the HiBiT TCK Bioassay may vary from the EC_{50} value obtained using other methods.
	The assay is sensitive to the concentration of FBS in assay buffer. Optimize the FBS concentration from 0.5–10% in assay buffer if assay performance is not ideal. Always use heat-inactivated FBS.
	Optimize the assay incubation time within a range of 3–24 hours (or longer) for the CAR-T assay.
	Performance of PBMC ADCC assays is highly donor-dependent. Raji Cells (LDH-HiBiT) have been optimized for use with Promega Human PBMC ADCC-Qualified.

7. Troubleshooting (continued)

Symptoms	Causes and Comments
Weak assay response (low fold induction, continued)	Always use white, round-bottom plates for PBMC ADCC assays. In most cases, TDCC and CAR-T assays can be performed in round- or flat-bottom plates, though the performance characteristics of your biologic drug or engineered effector cells may vary with plate type.
	If spontaneous release RLU is less than 100-fold above plate reader background RLU, subtract plate background RLU from all samples before calculating fold induction.
High spontaneous release	Insufficient washing of target cells prior to assay setup leads to high spontaneous release values due to carryover of free HiBiT from culture. Always wash target cells thoroughly, according to the protocols listed in this technical manual.
	Low cell viability causes high spontaneous release values because dead and dying cells release HiBiT into the medium. Target cells should be >95% viable at the time of assay setup. Handle cells carefully and according to the instructions in this protocol. Centrifuge at low speeds and resuspend cell pellets gently.
Specific lysis greater than 100%	In assays longer than 6 hours, Raji Cells (LDH-HiBiT) may divide before being lysed by effector cells, leading to an increase in the total HiBiT present in the well. In short (same-day) assays, add digitonin to maximum release wells at the time of assay setup. For overnight assays (or longer) add digitonin when adding detection reagent.
	Digitonin at concentrations greater than 200µg/ml and alternative detergents can interfere with NanoBiT® complementation and reduce luminescence in maximum release wells. Always use digitonin at the recommended final concentration of 100µg/ml.

Symptoms	Causes and Comments
Variability in assay performance	Variations in cell growth conditions including cell plating, harvest density, cell viability and cell doubling time can cause low assay performance and high assay variation. Avoid one-day cell passages whenever possible. Use high-quality cell culture reagents (especially serum) and plasticware for maintaining cells in culture. Ensure consistent cell growth by handling the cells exactly according to the instructions.
	Inappropriate cell handling during cell harvest, including long centrifuge times and high centrifuge speeds, can cause low assay performance and high assay variation. Centrifuge the cells exactly according to the instructions.
	Inappropriate cell freezing/DMSO exposure can cause low assay performance and high assay variation. Freeze the cells exactly according to the instructions.
	Inappropriate cell counting methods can lead to variation in cell numbers in culture and assays and cause high assay variation. Ensure consistent and accurate cell counting methods.
RLU differ between parental and knockout cell lines	Use percent specific lysis to compare the functional response between the parental clone and one or more knockout clones if the small difference in light output the different cell lines is a concern.

8. References

1. Barnhart, B.C. and Quigley, M. (2017) Role of Fc-FcγR interactions in the antitumor activity of therapeutic antibodies. *Immunol. Cell Biol.* **95**, 340–6.
2. van de Donk, N.W.C.J. and Zweegman, S. (2023) T-cell-engaging bispecific antibodies in cancer. *Lancet* **402**, 142–58.
3. Haslauer, T. *et. al.* (2021) CAR T-cell therapy in hematological malignancies. *Int. J. Mol. Sci.* **22**, 8996.
4. Schwinn, M.K. *et. al.* (2018) CRISPR-mediated tagging of endogenous proteins with a luminescent peptide. *ACS Chem. Biol.* **13**, 467–74.

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9. Appendix

9.A. Composition of Buffers and Solutions

Raji Cells (LDH-HiBiT) growth medium

90%	RPMI 1640 with L-glutamine and HEPES
10%	FBS

Raji Cells (LDH-HiBiT) freezing medium

79%	RPMI 1640 with L-glutamine and HEPES
14%	FBS
7%	DMSO

CAR-T assay buffer

90%	RPMI 1640 with L-glutamine and HEPES
10%	HI-FBS

PBMC ADCC assay buffer

95%	RPMI 1640 with L-glutamine and HEPES
5%	HI-FBS
55µM	β-mercaptoethanol
5ng/ml	IL-2

PBMC culture medium

90%	RPMI 1640 with L-glutamine and HEPES
10%	HI-FBS
1X	sodium pyruvate
55µM	β-mercaptoethanol
5ng/ml	IL-2

TDCC assay buffer

90%	RPMI 1640 with L-glutamine and HEPES
10%	HI-FBS

9.B. Related Products

Fc Effector Bioassays

Product	Size	Cat.#
ADCC Reporter Bioassay, Complete Kit (Raji)*	1 each	G7015
ADCC Reporter Bioassay, Core Kit*	1 each	G7010
ADCC Reporter Bioassay, F Variant, Core Kit**	1 each	G9790
ADCC Reporter Bioassay, Target Kit (Raji)*	1 each	G7016
FcγRIIa-H ADCP Reporter Bioassay, Complete Kit**	1 each	G9901
FcγRIIa-H ADCP Reporter Bioassay, Core Kit**	1 each	G9991
Mouse FcγRIV ADCC Bioassay, Complete Kit**	1 each	M1201
Mouse FcγRIV ADCC Bioassay, Core Kit**	1 each	M1211
Membrane TNFα Target Cells**	1 each	J3331
Membrane RANKL Target Cells**	1 each	J3381

*For Research Use Only. Not for use in diagnostic procedures.

**Not for Medical Diagnostic Use.

Additional kit formats are available.

Fc Effector Immunoassay

Product	Size	Cat.#
Lumit® FcRn Binding Immunoassay	100 assays	W1151

Not for Medical Diagnostic Use. Additional kit formats and sizes are available.

Immune Checkpoint Bioassays

Product	Size	Cat.#
4-1BB Bioassay	1 each	JA2351
CD28 Bioassay	1 each	JA6701
CD28 Blockade Bioassay	1 each	JA6101
CD40 Bioassay	1 each	JA2151
CTLA-4 Blockade Bioassay	1 each	JA3001
GITR Bioassay	1 each	JA2291
ICOS Bioassay	1 each	JA6801
ICOS Blockade Bioassay	1 each	JA6001
LAG-3/MHCII Blockade Bioassay	1 each	JA1111
OX40 Bioassay	1 each	JA2191
PD-1/PD-L1 Blockade Bioassay	1 each	J1250
PD-1+TIGIT Combination Bioassay	1 each	J2211
PD-L1 Negative Cells	1 each	J1191
TIGIT/CD155 Blockade Bioassay	1 each	J2201

Not for Medical Diagnostic Use. Additional kit formats and sizes are available.

T Cell Activation Bioassays

Product	Size	Cat.#
T Cell Activation Bioassay (IL-2)	1 each	J1651
T Cell Activation Bioassay (NFAT)	1 each	J1621
T Cell Activation Bioassay (TCRαβ-KO, CD4+)	1 each	GA1172
T Cell Activation Bioassay (TCRαβ-KO, CD8+)	1 each	GA1162
T Cell Activation Bioassay (TCRαβ-KO, CD4+, CD8+)	1 each	GA1182

Not for Medical Diagnostic Use. Additional kit formats and sizes are available.

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9.B. Related Products (continued)

Cytokine and Growth Factor Bioassays

Product	Size	Cat.#
IL-2 Bioassay	1 each	JA2201
IL-6 Bioassay	1 each	JA2501
IL-12 Bioassay	1 each	JA2601
IL-15 Bioassay	1 each	JA2011
IL-23 Bioassay	1 each	JA2511
RANKL Bioassay	1 each	JA2701
VEGF Bioassay	1 each	GA2001

Not for Medical Diagnostic Use. Additional kit formats and sizes are available.

Macrophage-Directed Bioassays

Product	Size	Cat.#
SIRPα/CD47 Blockade Bioassay	1 each	JA6011
SIRPα/CD47 Blockade Bioassay, Fc-Dependent	1 each	JA4801
TLR Bioassay	1 each	JA9011
ADCP Reporter Bioassay (THP-1)	1 each	JA9411

Not for Medical Diagnostic Use. Additional kit formats are available.

HiBiT Target Cell Killing Bioassays

Product	Size	Cat.#
Raji (HT-HiBiT) TCK Bioassay	1 each	JA1211
Raji (LDH-HiBiT) TCK Bioassay	1 each	JA1311
Ramos (HiBiT) TCK Bioassay	1 each	JA1411
H929 (HiBiT) TCK Bioassay	1 each	JA1511

Not for Medical Diagnostic Use. Additional kit formats are available.

9.B. Related Products (continued)

Control Antibodies and Proteins

Product	Size	Cat.#
Control Ab, Anti-4-1BB	50µg	K1161
Control Ab, Anti-CD20	5µg	GA1130
Control Ab, Anti-CD40	50µg	K1181
Control Ab, Anti-CTLA-4	100µg	JA1020
Control Ab, Anti-LAG-3	100µg	K1150
Control Ab, Anti-OX40	50µg	K1191
Control Ab, Anti-PD-1	100µg	J1201
Control Ab, Anti-SIRPα	50µg	K1251
Control Ab, Anti-TIGIT	100µg	J2051
Control Ab, Anti-TIM-3	100µg	K1210
Recombinant VEGF ligand	10µg	J2371

Detection Reagents

Product	Size	Cat.#
Bio-Glo™ Luciferase Assay System	10ml	G7941
Bio-Glo-NL™ Luciferase Assay System	10ml	J3081
Bio-Glo-NB™ TCK Luciferase Assay System	10ml	JB1001

Not for Medical Diagnostic Use. Additional sizes are available.

Detection Instruments

Product	Size	Cat.#
GloMax® Navigator System	1 each	GM2000
GloMax® Discover System	1 each	GM3000
GloMax® Explorer System	1 each	GM3500

For Research Use Only. Not for use in diagnostic procedures.

Note: Additional Fc Effector, Immune Checkpoint, T Cell Activation, Cytokine, Macrophage, Primary Cell and Target Cell Killing Bioassays are available. To view and order Promega Bioassay products visit: **www.promega.com/products/reporter-bioassays/** or email: **EarlyAccess@promega.com**. For information on custom bioassay development and services visit the Promega Tailored R&D Solutions website: **www.promega.com/custom-solutions/tailored-solutions/**

10. Summary of Changes

The following changes were made to the 5/25 revision of this document:

1. Updated the Lumit trademark.
2. Revised text about the label in Section 3.

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Appendix A, Broad IP List

BROAD TECHID	APPLICANTS	EXEMPLARY FAMILY SERIAL NO
BI-2011/008	Broad, MIT, Harvard and Rockefeller	PCT/US2013/074611; WO2014093595
BI-2011/008B	Broad, MIT and Harvard	14/259,420
BI-2011/008A	Broad and MIT	PCT/US2013/074743; WO2014093661
BI-2011/008C	Broad, MIT, and Harvard	PCT/US2013/074790; WO2014093694
BI-2011/020	Broad, MIT and Harvard	PCT/US2013/051418; WO2014018423
BI-2012/084A	Broad and MIT	PCT/US2013/074825; WO2014093718
BI-2012/084B	Broad, MIT and Harvard	PCT/US2013/074812; WO2014093709
BI-2013/003	Broad, MIT and Harvard	PCT/US2013/074667; WO2014093622
BI-2013/003D	Broad, MIT and Harvard	PCT/US2014/041803; WO2014201425
BI-2013/004E	Broad, MIT and Harvard	PCT/US2013/074691; WO2014093635
BI-2013/004F	Broad, MIT and Harvard	PCT/US2013/074736; WO2014093655
BI-2013/004G	Broad, MIT and Harvard	PCT/US2013/074819; WO2014093712
BI-2013/007	Broad, MIT and Harvard	14/855,046; US20160068822
BI-2013/066	Broad, MIT and Harvard	PCT/US2014/041800; WO2014204724
BI-2013/073	Broad and MIT	PCT/US2014/041806; WO2014204727
BI-2013/085	Broad, MIT and Whitehead	15/141,348; US20160251648
BI-2013/087J	Broad, Editas*, Iowa and MIT	PCT/US2014/064663; WO2015070083
BI-2013/087M	Broad, Iowa and MIT	PCT/US2014/069902; WO2015089354
BI-2013/087V	Broad, Iowa and MIT	PCT/US2014/069897; WO2015089351
BI-2013/093	Broad, MIT and Tokyo	15/171,141; US20160340660
BI-2013/094	Broad, MIT and Rockefeller	PCT/US2014/070135; WO2015089465
BI-2013/098	Broad and MIT	PCT/US2014/070068; WO2015089427

BI-2013/099	Broad, MIT and Harvard	PCT/US2014/041804; WO2014204726
BI-2013/101	Broad and MIT	PCT/US2014/070127; WO2015089462
BI-2013/103	Broad and MIT	PCT/US2014/041808; WO201404728
BI-2013/105	Broad, MIT and Harvard	PCT/US2014/041809; WO2014204729
BI-2013/107	Broad and MIT	PCT/US2014/070057; WO2015089419
BI-2013/112	Broad and MIT	PCT/US2013/074800; WO2014093701
BI-2013/113	Broad, MIT and Harvard	PCT/US2014/070152; WO2015089473
BI-2014/005	Broad, MIT, Harvard and Tokyo	PCT/US2014/070175; WO2015089486
BI-2014/061	Broad, MIT and Harvard	PCT/US2015/045504; WO2016028682
BI-2014/069	Broad and MIT	15/467,888; US20180010134
BI-2014/071	Broad and MIT	15/349,603; US20170107536
BI-2014/072	Broad and MIT	15/467,949; US20180044662
BI-2014/084	Broad, MIT and Harvard	15/469,081; US20180057810
BI-2014/097	Broad, MIT and Harvard	PCT/US2015/067177; WO2016106244
BI-2014/100	Broad, MIT and Harvard	PCT/US2015/065385; WO2016094867
BI-2014/101	Broad, MIT and Harvard	15/632,067; US20170306335
BI-2014/103	Broad and MIT	PCT/US2015/067138; WO2016100974
BI-2014/106	Broad and MIT	PCT/US2015/065393; WO2016094872
BI-2014/107	Broad and MIT	15/619,735; US20170349894
BI-2014/108	Broad and MIT	15/619,737; US20170349914
BI-2014/113	Broad and MIT	15/640,103; US20180112255
BI-2015/002	Broad, MIT, Harvard and Tokyo	PCT/US2016/038252; WO2016205759
BI-2015/052	Broad and MIT	PCT/US2016/038034; WO2016205613
BI-2015/053	MIT	PCT/US2016/038205; WO2016205728
10086	Broad and MIT	PCT/US2017/047458; WO2018035387
10114	Broad and MIT	PCT/US2017/053795; WO2018064208
10125	Broad and MIT	62/502,064 62/564,102
10209	Broad, Harvard, MIT, New York University and NY Genome Center	62/529,573

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