

TECHNICAL MANUAL

# Raji (HT-HiBiT) TCK Bioassay

Instructions for Use of Products  
JA1211 and JA1215

# Raji (HT-HiBiT) TCK Bioassay

All technical literature is available at: [www.promega.com/protocols/](http://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](mailto:techserv@promega.com)

1. Description .....	3
2. Product Components and Storage Conditions .....	10
3. Before You Begin .....	11
4. PBMC ADCC Assay Protocol .....	11
4.A. Preparing Assay Reagents and Samples .....	14
4.B. Plate Layout Design .....	15
4.C. Thawing Human PBMC, ADCC-Qualified Effector Cells .....	16
4.D. Thawing Raji (HT-HiBiT) Target Cells .....	16
4.E. Day 2: Preparing 3X Antibody Stock Solutions .....	17
4.F. Day 2: Preparing 3X Digitonin for Maximum Release Control .....	18
4.G. Day 2: Preparing Raji Cells (HT-HiBiT) .....	19
4.H. Day 2: Preparing Human PBMC, ADCC-Qualified Effector Cells .....	19
4.I. Day 2: Adding Target Cells, Effector Cells and Antibody .....	19
4.J. Day 2: Preparing and Adding Bio-Glo-NB™ TCK Reagent .....	20
4.K. Data Analysis .....	21
5. CD8+ T Cell TDCC Assay Protocol .....	22
5.A. Preparing Assay Reagents and Samples .....	24
5.B. Plate Layout Design .....	25
5.C. Preparing 3X Stock Solutions of Biologic Drugs .....	26
5.D. Preparing Raji Cells (HT-HiBiT) .....	27
5.E. Preparing Human CD8+ T Cells, TDCC Qualified Effector Cells (T Cells) .....	28
5.F. Adding Raji Cells (HT-HiBiT), Effector Cells and Biologic Drugs .....	28
5.G. Preparing and Adding Bio-Glo-NB™ TCK Reagent .....	29
5.H. Data Analysis .....	30



- 6. CAR-T Assay Protocol ..... 31**
  - 6.A. Preparing Assay Reagents ..... 33**
  - 6.B. Plate Layout Design..... 34**
  - 6.C. Preparing Raji Cells (HT-HiBIT) ..... 34**
  - 6.D. Preparing 2X Stock Solutions of CAR-T Cells..... 35**
  - 6.E. Adding Target Cells and CAR-T Cells to Assay Plates ..... 36**
  - 6.F. Preparing and Adding Bio-Glo-NB™ TCK Reagent ..... 36**
  - 6.G. Data Analysis ..... 37**
- 7. Troubleshooting ..... 38**
- 8. References ..... 40**
- 9. Appendix..... 40**
  - 9.A. Composition of Buffers and Solutions ..... 40**
  - 9.B. Related Products..... 41**

## 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 (HT-HiBiT) TCK Bioassays measure the death of target cells engineered to express a HiBiT fusion protein. HiBiT is an 11 amino acid peptide that binds with high affinity to LgBiT to form NanoBiT<sup>®</sup> 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 (Fz) 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 (HT-HiBiT) TCK Bioassay<sup>(a-d)</sup> (Cat.# JA1211, JA1215) is provided in a thaw-and-use format as cryopreserved cells that can be thawed, plated and used in an assay without the need for cell culture and propagation.

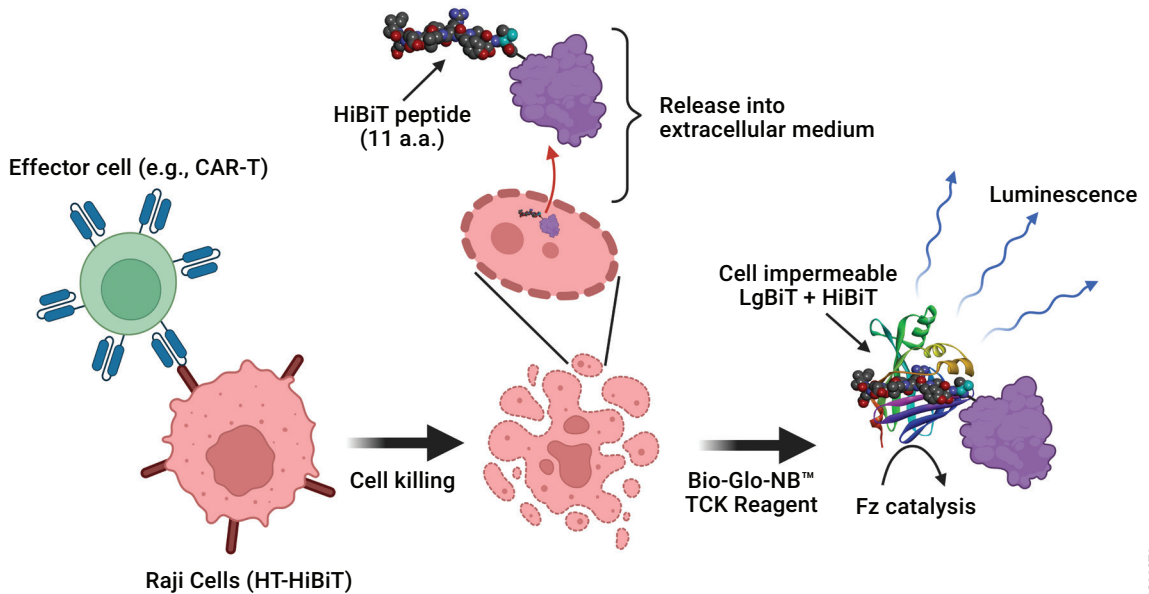
When Raji (HT-HiBiT) target cells are cocultured with appropriate effector cells (e.g., NK cells, or cytotoxic T cells), they can be used to specifically monitor the activity of biologics directed against receptors expressed on the Raji Cells (HT-HiBiT), such as CD19, CD20 or CD22. When Raji Cells (HT-HiBiT) are used in assays with biologics against TAAs they do not express, target cells are not lysed and no increase in luminescent signal is observed (Figure 2).

Raji Cells (HT-HiBiT) have 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 very sensitive, enabling the use of low numbers of Raji Cells (HT-HiBiT) (2,500–5,000 per well.) The cells have a low rate of spontaneous release of the HiBiT fusion protein, and the fusion protein is stable once released into the extracellular medium. Together, these features make the Raji Cells (HT-HiBiT) optimal targets for measuring the potency of biologic drugs and cell therapy products. For cell therapy, users can test a wide range of E:T (effector-to-target) ratios while conserving test material for experiments ranging from 4–72 hours (Figure 5).

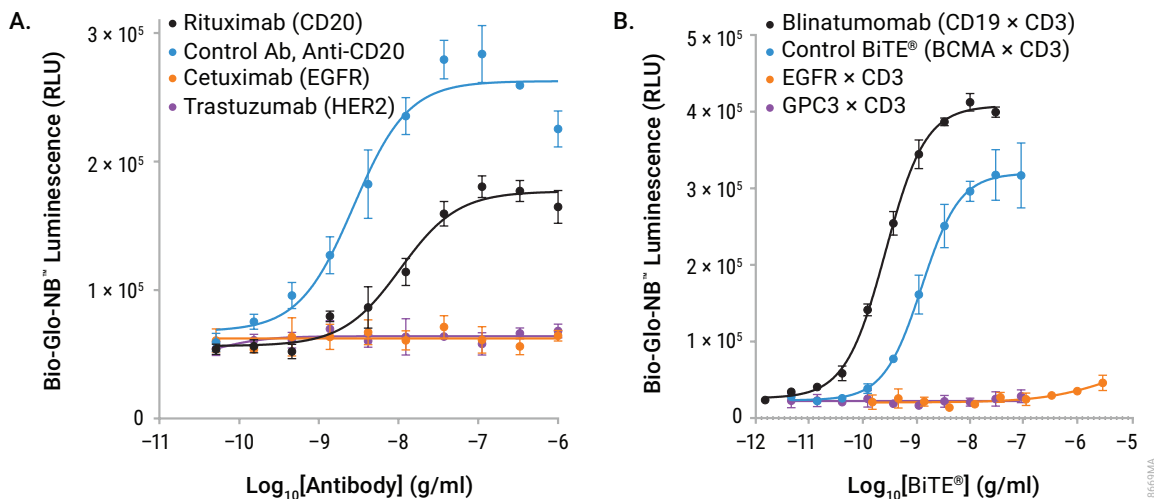
This technical manual provides instructions for the Raji (HT-HiBiT) TCK Bioassay, as well as sample protocols for use of the bioassay with an ADCC Bioassay with Human PBMC, ADCC-qualified; a TDCC Bioassay with Human T Cell (CD8+), TDCC-qualified; and a CAR-T Bioassay with user-defined CAR-T cells. Additional optimization may be necessary for use with your biologic product of interest.

1. Description (continued)



**Figure 1. Representation of the Raji (HT-HiBiT) TCK Bioassay.** Raji Cells (HT-HiBiT) stably expressing a HiBiT fusion protein are cocultured with effector cells (CAR-T, T cells, NK cells, etc.). Effector cell-mediated killing of Raji Cells (HT-HiBiT) leads to release of 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.)

186687A

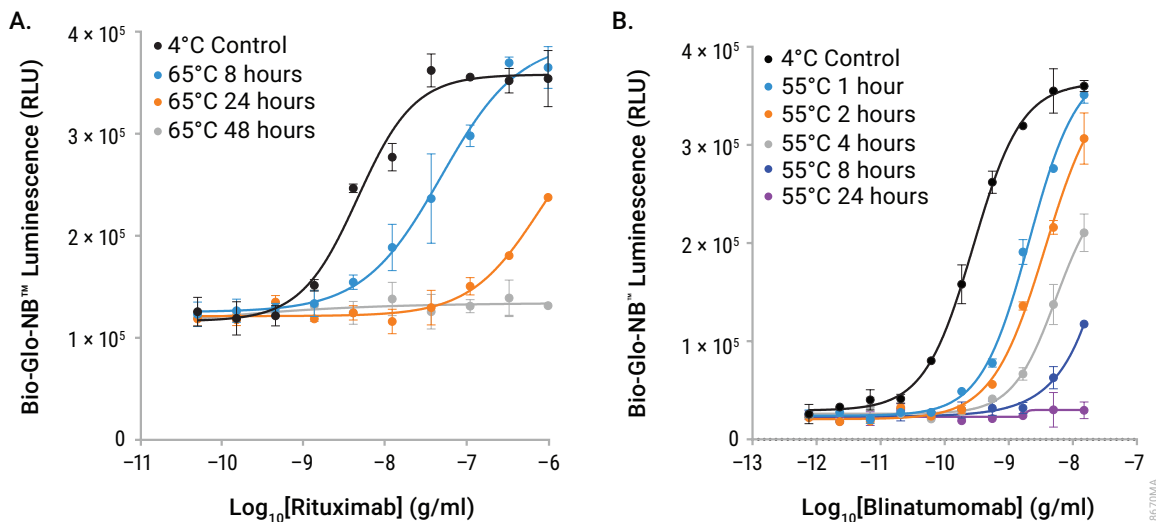


**Figure 2. Raji (HT-HiBiT) TCK Bioassays reflect the MOA of biologic drugs and demonstrate specificity for cell surface antigens. Panel A.** Raji Cells (HT-HiBiT) were incubated with Human PBMC, ADCC-qualified effector cells in the presence of serial titrations of antibodies as indicated. After a 5-hour induction, Bio-Glo-NB™ TCK Luciferase Reagent was added and luminescence quantified using the GloMax® Discover System. **Panel B.** Raji Cells (HT-HiBiT) were incubated with Human T Cells (CD8+) in the presence of serial titrations of biologic drugs as indicated. After a 24-hour induction, 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. HT = HaloTag, the fusion partner for the HiBiT epitope.

**1. Description (continued)**

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

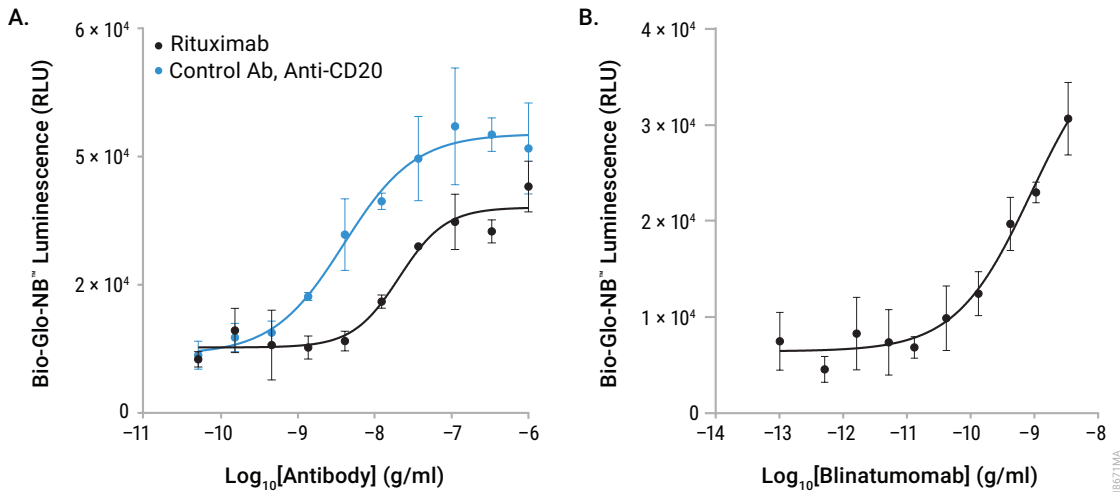
Parameter	Results	
Accuracy	% Expected Relative Potency	% Recovery
	50	102.1
	75	96.3
	100	99.7
	125	95.9
	150	103.1
Repeatability (% CV)	100% (Reference)	
Intermediate Precision (% CV)	13	
Linearity (r <sup>2</sup> )	0.91	
Linearity (y = mx + b)	y = 1.02x – 2.4	
<p>A 50–150% theoretical potency series of blinatumomab was analyzed in triplicate in three independent experiments performed on three days by two analysts using Raji Cells (HT-HiBiT) targets 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.</p>		



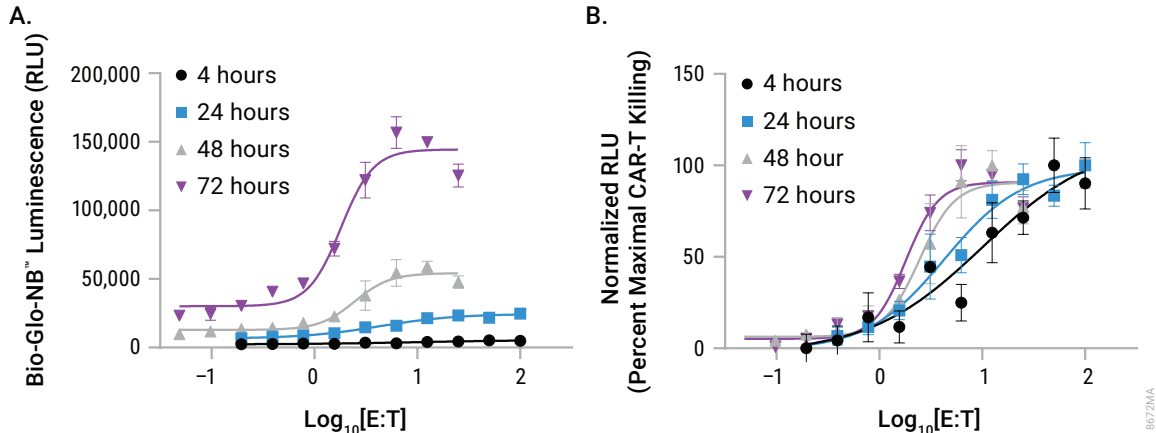
**Figure 3. Raji (HT-HiBiT) TCK Bioassays are stability-indicating. Panel A.** Samples of rituximab were maintained at 4°C (control) or heat-treated at 65°C for the indicated times, then analyzed in an ADCC Bioassay with Raji Cells (HT-HiBiT) and Human PBMC, ADCC-qualified effector cells. **Panel B.** Samples of blinatumomab 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 (HT-HiBiT) and Human T Cells (CD8+), TDCC-Qualified. For both **Panel A** and **Panel B**, 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 (HT-HiBiT) TCK Bioassays are amenable to 384-well plate format. Panel A.** Raji Cells (HT-HiBiT) were harvested after overnight culture and  $1 \times 10^3$  cells/5 $\mu$ l/well plated in a 384-well round-bottom white assay plate (e.g., Corning® Cat. # 4512, low volume). Next, 5 $\mu$ l of 3X serially-diluted Control Antibody, Anti-CD20 (Cat. # GA1130) was added, then  $2.5 \times 10^4$ /5 $\mu$ l/well of Human PBMC, ADCC-Qualified effector cells from overnight culture were added. After a 5-hour incubation at 37°C, 5% CO<sub>2</sub>, 5 $\mu$ l of 4X Bio-Glo-NB™ TCK Reagent was added to each well and luminescence quantified. **Panel B.** Raji Cells (HT-HiBiT) were plated at  $5 \times 10^2$  cells/5 $\mu$ l/well in a 384-well flat-bottom white assay plate (e.g., Corning® Cat. # 3570). Next, 5 $\mu$ l of 3X serially diluted blinatumomab was added, followed by  $5 \times 10^3$ /5 $\mu$ l/well of Human T Cells (CD8+), TDCC-Qualified. After a 24-hour incubation at 37°C, 5% CO<sub>2</sub>, 15 $\mu$ l of Bio-Glo-NB™ TCK Reagent was added per well and luminescence quantified 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 (HT-HiBiT) TCK Bioassays are amenable for use with CAR-T effector cells.** **Panel A.** Raji Cells (HT-HiBiT) were incubated with serially diluted human CD19 CAR-T cells at the indicated E:T (effector-to-target) 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<sub>50</sub> values (data not shown) decreased with longer incubation periods (**Panel B**). Data were generated using thaw-and-use cells.



## 2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
<b>Raji (HT-HiBiT) TCK Bioassay</b>	<b>1 each</b>	<b>JA1211</b>

Not for Medical Diagnostic Use. Each kit contains sufficient reagents for 120 assays using the inner 60 wells of two 96-well plates. Includes:

- 1 vial Raji (HT-HiBiT) Cells (0.5ml per vial)
- 3 × 36ml RPMI 1640 Medium
- 2 × 4ml Fetal Bovine Serum
- 1 vial Bio-Glo- NB<sup>®</sup> TCK Luciferase Assay Substrate
- 1 vial Bio-Glo- NB<sup>®</sup> TCK LgBiT Protein
- 10ml Bio-Glo- NB<sup>®</sup> TCK Luciferase Assay Buffer

PRODUCT	SIZE	CAT.#
<b>Raji (HT-HiBiT) TCK Bioassay, 5X</b>	<b>1 each</b>	<b>JA1215</b>

Not for Medical Diagnostic Use. Each kit contains sufficient reagents for 600 assays using the inner 60 wells of ten 96-well plates. Includes:

- 5 vials Raji (HT-HiBiT) Cells (0.5ml per vial)
- 15 × 36ml RPMI 1640 Medium
- 10 × 4ml Fetal Bovine Serum
- 5 vials Bio-Glo-NB<sup>®</sup> TCK Luciferase Assay Substrate
- 5 vials Bio-Glo-NB<sup>®</sup> TCK LgBiT Protein
- 5 × 10ml Bio-Glo-NB<sup>®</sup> TCK Luciferase Assay Buffer


**Storage Conditions:** Upon arrival, immediately transfer the cell vials to below  $-140^{\circ}\text{C}$  (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  $-80^{\circ}\text{C}$  because this will decrease cell viability and cell performance. Store Bio-Glo-NB<sup>®</sup> TCK Luciferase Assay Substrate, Bio-Glo-NB<sup>®</sup> TCK LgBiT Protein, Bio-Glo-NB<sup>®</sup> TCK Luciferase Assay Buffer and Fetal Bovine Serum (FBS) at  $-30^{\circ}\text{C}$  to  $-10^{\circ}\text{C}$ . Avoid multiple freeze-thaw cycles of the serum. The Bio-Glo-NB<sup>®</sup> TCK Luciferase Assay Substrate and Bio-Glo-NB<sup>®</sup>-TCK LgBiT Protein remain as liquids and do not freeze. Store RPMI 1640 Medium at  $+2^{\circ}\text{C}$  to  $+10^{\circ}\text{C}$ , protected from fluorescent light.

### 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 Raji Cells (HT-HiBiT) 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, Before You Begin (preparing materials and reagents); Section 4, PBMC ADCC Assay; Section 5, CD8+ T Cell TDCC Assay; Section 6, CAR-T Killing assay. For other applications, use these protocols as a guide and optimize the assay for your specific application.

Remove the product label from the box containing vials of cells or note the catalog number, lot number and dispensed lot number from the label. This information can be used to download specific documents from [www.promega.com](http://www.promega.com), such as the Certificate of Analysis.

 **Note:** The Raji (HT-HiBiT) TCK Bioassay uses 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 Cells (HT-HiBiT) 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.

Raji Cells (HT-HiBiT) are provided in frozen, thaw-and-use format and are ready to be used without any additional cell culture or propagation. When thawed and diluted as instructed, the cells will be at the appropriate concentration for the assay. The cells are sensitive, and care should be taken to follow cell thawing and plating procedures as described. Do not overmix or overwarm the cell reagents.

Raji (HT-HiBiT) TCK Bioassays 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 (HT-HiBiT) TCK Bioassays 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

### 4. PBMC ADCC Assay Protocol

This procedure illustrates the use of the Raji (HT-HiBiT) TCK Bioassay 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. Other experimental and plate layouts are possible but may require further optimization.

#### 4. PBMC ADCC Assay Protocol (continued)

**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 1 µg/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 target 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.)

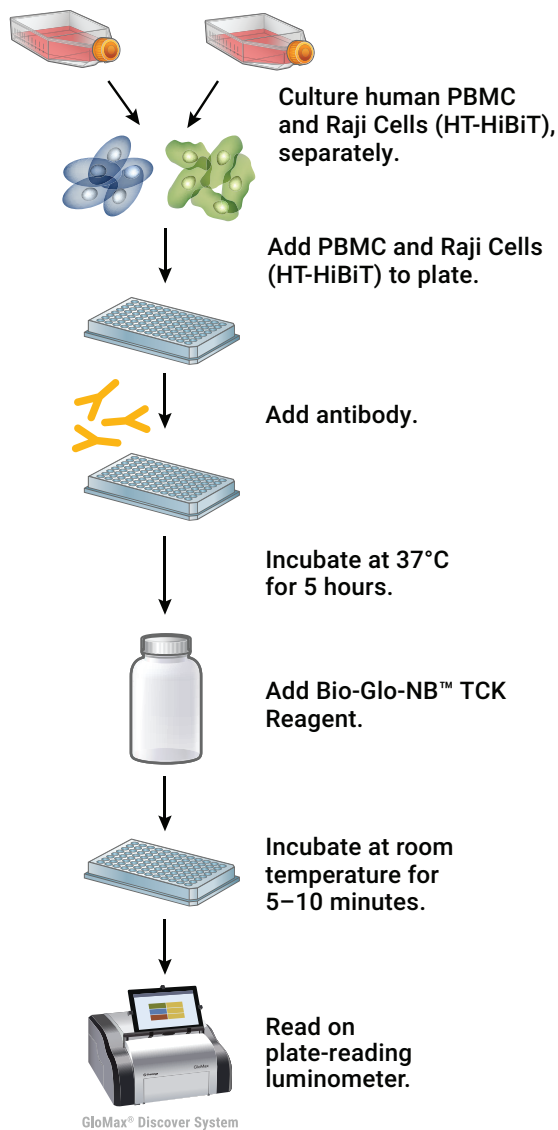
#### Reagents

- Human PBMC, ADCC-Qualified (e.g., [www.promega.com/products/reporter-bioassays/primary-cell-bioassays/pbmc-adcc-bioassay](http://www.promega.com/products/reporter-bioassays/primary-cell-bioassays/pbmc-adcc-bioassay); please enquire)
- antibodies or other biologics samples capable of inducing ADCC
- 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)
- Trypan blue solution (e.g., Sigma Cat.# T8154)
- **optional:** Control Ab, Anti-CD20 (Cat.# GA1130)

#### 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 flask (e.g., Corning® Cat.# 430641U)
- 37°C, 5% CO<sub>2</sub> incubator
- 37°C water bath
- sensitive plate reader with glow luminescence measuring capability or luminometer (e.g., GloMax® Discover System [Cat.# GM3000] or equivalent)

**Note:** Corning® Cat.# 3355 plates are supplied 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 6. Schematic protocol for the Raji (HT-HiBiT) TCK Bioassay in PBMC ADCC assay application.**

#### 4.A. Preparing Assay Reagents and Samples

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

**Heat-Inactivated (HI) FBS:** Thaw the provided FBS overnight at +2°C to +10°C or in a 37°C water bath on the day of use. Heat inactivate the FBS for 30 minutes at 56°C. Mix well and adjust to 37°C before use.


**Raji Cells (HT-HiBiT) Recovery Medium:** On the day before the assay, prepare 15ml of Raji Cells (HT-HiBiT) recovery medium (see Section 9.A). Thaw both vials of FBS overnight at +2°C to +10°C or in a 37°C water bath on the day of use. Heat inactivate the FBS for 30 minutes at 56°C. Mix well and adjust to 37°C before use. To prepare Raji Cell (HiBiT) recovery medium, add 1.5ml of FBS to 13.5ml of 37°C RPMI 1640 Medium. For reference, 15ml of Raji Cell (HiBiT) recovery medium is sufficient to thaw and plate one vial of Raji HiBiT Target Cells. If multiple vials will be thawed, then scale the amount of Raji Cell (HiBiT) cell recovery medium appropriately. Store the remaining FBS at +2°C to +10°C for use in preparing the assay buffer on the day of the assay.

**PBMC Culture Medium:** On the day before the assay, prepare an appropriate amount of PBMC culture medium: 90% RPMI 1640 with 10% FBS with IL-2,  $\beta$ -mercaptoethanol and sodium pyruvate.

**PBMC ADCC Assay Buffer:** On the day of the assay, prepare an appropriate amount of assay buffer: 95% RPMI 1640 medium with 5% HI-FBS with IL-2 and  $\beta$ -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. Store the Bio-Glo-NB™ TCK Luciferase Assay Substrate and 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) during the 5-hour assay induction period. We recommend preparing the reconstituted Bio-Glo-NB™ TCK Reagent immediately before use.

 **Note:** The Raji (HT-HiBiT) TCK Bioassay is compatible only with Bio-Glo-NB™ TCK Reagent. **Do not** use Bio-Glo™ Reagent or Bio-Glo-NL™ Reagent with the Raji (HT-HiBiT) TCK Bioassay.

**Test and Reference Samples:** Using assay buffer as the diluent, prepare stock starting dilutions (dilu1, 3X final concentration) of two test antibodies (300 $\mu$ l each) and one reference antibody (600 $\mu$ l) 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 300 $\mu$ l of a starting dilution at 3 $\mu$ g/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 7 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 7. Example plate layout showing nonclustered sample locations of test antibody and reference antibody dilution series.** Maximum Release (MR) control wells contain Raji Cells (HT-HiBiT), PBMC effector cells and digitonin. Spontaneous Release (SR) control wells contain Raji Cells (HT-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

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

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

1. Remove one vial ( $\sim 2 \times 10^7$  cells) of PBMC from storage at  $-140^\circ\text{C}$  and thaw in a  $37^\circ\text{C}$  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 cell culture flask and place the flask horizontally in a humidified  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  incubator.
5. Incubate for approximately 16–24 hours before assay setup.

#### 4.D. Thawing Raji (HT-HiBiT) Target Cells

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

Thaw-and-use Raji Cells (HT-HiBiT) are in a sensitive state immediately post-thaw and care should be taken to follow the thawing and plating procedures exactly as described. Handle the cells gently and do not overmix the cells or overwarm the reagents. Do not vortex the cells or centrifuge at speeds  $>150 \times g$ . It is important to keep pipette tips away from tube surfaces during delivery or mixing steps to minimize mechanical stress on the cells. We recommend that you thaw and dilute a maximum of two vials of thaw-and-use cells at any one time.

1. Add 9.5ml of Raji Cells (HT-HiBiT) recovery medium to a 15ml conical tube and warm to  $37^\circ\text{C}$ .
2. Remove one cryovial of Raji Cells (HT-HiBiT) from storage at  $-140^\circ\text{C}$  and thaw in a  $37^\circ\text{C}$  water bath with gentle agitation (do not invert) until just thawed (typically 2–3 minutes).
3. Using a 2ml serological pipette or a 1ml wide-bore pipette tip, transfer 1ml of prewarmed Raji Cells (HT-HiBiT) recovery medium, one drop at a time, to the cryovial. Using the same pipette, gently mix the contents of the cryovial by slowly pipeting approximately 1ml, once. Gently transfer the contents of the cryovial to the 15ml conical tube containing prewarmed Raji Cells (HT-HiBiT) recovery medium.
4. Pellet the cells at  $150 \times g$  for 5 minutes.
5. Aspirate the supernatant, leaving 100–300 $\mu\text{l}$  of Raji Cells (HT-HiBiT) recovery medium above the cell pellet.
6. Resuspend the cell pellet by gently flicking the tube bottom 2–3 times. Add 5ml of prewarmed Raji Cells (HT-HiBiT) recovery medium to the side of the tube. Do not pipet Raji Cells (HT-HiBiT) recovery medium directly onto the cell pellet. Pipet once to mix.
7. Transfer the cell suspension to a T25 cell culture flask and place the flask horizontally in a humidified  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  incubator.
8. Incubate for approximately 16–24 hours before assay setup.

#### 4.E. Day 2: Preparing 3X Antibody Stock Solutions

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 150 $\mu$ l stock of each dilution provides sufficient volume for analysis in triplicate. To prepare threefold serial dilutions, you will need 300 $\mu$ l of reference and test 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 300 $\mu$ l of reference antibody starting dilution (dilu1, 3X final concentration) to wells A10 and B10 (Figure 8).
3. Add 300 $\mu$ l of test antibodies 1 and 2 starting dilution (dilu1, 3X final concentration) to wells E10 and G10, respectively (Figure 8).
4. Add 200 $\mu$ l of PBMC ADCC assay buffer to the other wells in these four rows, from column 9 to column 2.
5. Transfer 100 $\mu$ l 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 200 $\mu$ l of PBMC ADCC assay buffer without antibody as a negative control for delivery to SR control wells..
7. Proceed immediately to Section 4.F.

**4.F. Day 2: Preparing 3X Digitonin for Maximum Release Control**

1. Dilute digitonin stock in PBMC ADCC assay buffer to 300µg/ml (3X). Prepare 100µl for each antibody to be tested.
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 in a humidified, 37°C 5% CO<sub>2</sub> incubator until Section 4.I.

Recommended Plate Layout for Antibody Dilutions Prepared from a Single 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 8. 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.G. Day 2: Preparing Raji Cells (HT-HiBiT)

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

1. Gently mix and harvest the Raji Cells (HT-HiBiT) that were plated overnight.
2. Transfer into a 15ml centrifuge tube.
3. Centrifuge cells at  $150 \times g$  for 5 minutes.
4. Wash cells by removing supernatant, and gently resuspending in 10ml of prewarmed assay buffer.
5. Centrifuge cells at  $150 \times g$  for 5 minutes, then remove and discard supernatant.
6. Gently resuspend the cell pellet in 10ml of prewarmed assay buffer.
7. Incubate the Raji Cells (HT-HiBiT) at ambient temperature until Section 4.I.

#### 4.H. Day 2: 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  $300 \times g$  for 5 minutes at ambient temperature.
4. Gently but thoroughly resuspend the pellet in PBMC ADCC assay buffer to cell density of  $5 \times 10^6$  cells/ml.
5. Incubate the PBMC at ambient temperature until Section 4.I.

#### 4.I. Day 2: 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 (HT-HiBiT) suspension to a sterile reagent reservoir.
2. Using a multichannel pipette, immediately dispense 25 $\mu$ l 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  $5 \times 10^3$  cells/well.
3. Using a multichannel pipette, 25 $\mu$ l of the appropriate antibody dilution to wells B3–G10 of the assay plate according to Figure 7. Add 25 $\mu$ l of PBMC ADCC assay buffer to SR control wells (B2–G2). Add 25 $\mu$ l of PBMC ADCC assay buffer plus 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 25 $\mu$ l of the cell suspension to each of wells B2–G11 of the assay plates. The final cell number in each well should be  $1.25 \times 10^5$  cells/well.
6. Add 75 $\mu$ l of PBMC ADCC assay buffer to each of the outside wells of the assay plates.

#### 4.I. Day 2: Adding Target Cells, Effector Cells and Antibody (continued)


7. Cover the assay plates with lids and incubate in a 37°C, 5% CO<sub>2</sub> 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.J. Day 2: 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:** The Raji (HT-HiBiT) TCK Bioassay is compatible only with Bio-Glo-NB™ TCK Reagent. **Do not** use Bio-Glo™ Reagent or Bio-Glo-NL™ Reagent with the Raji (HT-HiBiT) TCK Bioassay.

1. Remove the Bio-Glo-NB™ TCK Luciferase Assay Substrate from 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 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. 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 the Bio-Glo-NB™ TCK Reagent to all 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 incubation time will affect the raw relative light unit (RLU) values but should not significantly change the EC<sub>50</sub> value and maximum fold induction.

#### 4.K. Data Analysis

The Raji (HT-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:

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

2. Calculate percent specific lysis:

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

MR (Maximum Release) control wells contain Raji Cells (HT-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 (HT-HiBiT) plus PBMC effector cells (no test or reference antibody added). These wells measure background target cell death in the absence of a test or reference antibody.

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

## 5. CD8+ T Cell TDCC Assay Protocol

This procedure illustrates the use of Raji Cells (HT-HiBiT) Bioassay 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.

**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

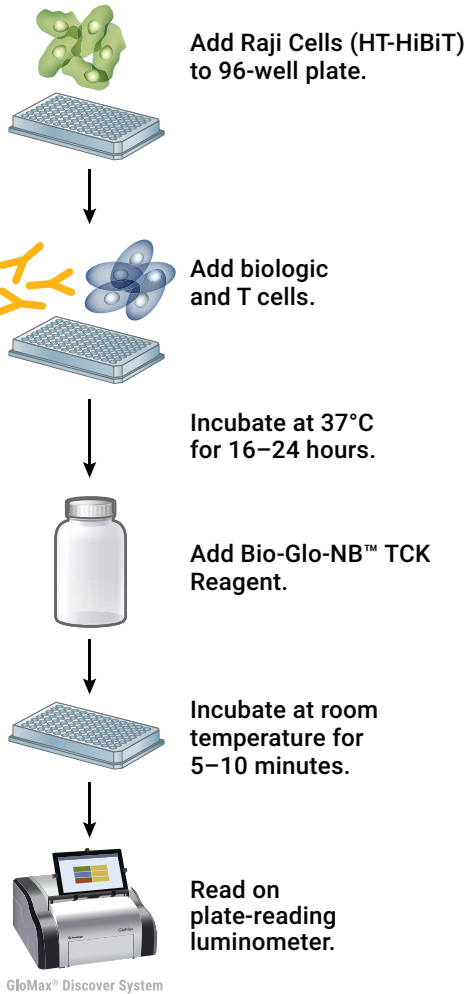
(Composition of Buffers and Solutions is provided in Section 9.A.)

### Reagents

- Human T Cells (CD8+), TDCC-Qualified (please enquire: [EarlyAccess@promega.com](mailto:EarlyAccess@promega.com))
- bispecific antibodies or related biologic drugs capable of inducing TDCC
- digitonin (e.g., Cat.# G9441)

### 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<sub>2</sub> incubator
- 37°C water bath
- sensitive plate reader with glow luminescence measuring capability or luminometer (e.g., GloMax® Discover System [Cat.# GM3000] or equivalent)



**Figure 9. Schematic protocol for the Raji (HT-HiBiT) TCK Bioassay in a TDCC bioassay application.**




### 5.A. Preparing Assay Reagents and Samples

**Heat-Inactivated (HI) FBS:** Thaw the provided FBS overnight at +2°C to +10°C or in a 37°C water bath on the day of use. Heat inactivate the FBS for 30 minutes at 56°C. Mix well and adjust to 37°C before use.

**CD8+ T Cell TDC 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:** The Raji (HT-HiBiT) TCK Bioassay is compatible only with Bio-Glo-NB™ TCK Reagent. **Do not** use Bio-Glo™ Reagent or Bio-Glo-NL™ Reagent with the Raji (HT-HiBiT) TCK Bioassay.

**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 10 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 10. 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 (HT-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 150 $\mu$ l stock of each dilution provides sufficient volume for analysis in triplicate. To prepare threefold serial dilutions, you will need 300 $\mu$ l of test and 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 300 $\mu$ l of reference biologic starting dilution (dilu1, 3X final concentration) to wells A10 and B10 (Figure 11).
3. Add 300 $\mu$ l of test biologics 1 and 2 starting dilution (dilu1, 3X final concentration) to wells E10 and G10, respectively (Figure 11).
4. Add 200 $\mu$ l of TDCC assay buffer to dilu2 through dilu8 in rows A, B, E and G, from column 9 to column 3. Add 200 $\mu$ l of TDCC assay buffer per well to no biologic drug controls wells (columns 2 and 11)
5. Transfer 100 $\mu$ l 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 200 $\mu$ l of TDCC assay buffer without biologic drug for delivery to SR control wells. Wells A11, B11, E11 and G11 contain 200 $\mu$ l 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 in a humidified 37°C, 5% CO<sub>2</sub> incubator while preparing the Raji Cells (HT-HiBiT) and TDCC Effector cells (Section 5.F).

Recommended Plate Layout for Biologic Dilutions Prepared from a Single 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 11. 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 (HT-HiBiT)

Thaw-and-use Raji Cells (HT-HiBiT) are in a sensitive state immediately post-thaw and care should be taken to follow the thawing and plating procedures exactly as described. Handle the cells gently and do not overmix the cells or overwarm the reagents. Do not vortex the cells or centrifuge at speeds  $> 150 \times g$ . It is important to keep pipette tips away from tube surfaces during delivery or mixing steps to minimize mechanical stress on the cells. We recommend that you thaw and dilute a maximum of two vials of thaw-and-use cells at a time.

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

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

1. Add 9.5ml of Raji Cells (HT-HiBiT) recovery medium to a 15ml conical tube and warm to 37°C.
2. Remove one vial of Raji Cells (HT-HiBiT) from storage at  $-140^{\circ}\text{C}$  and thaw in a 37°C water bath with gentle agitation (do not invert) until just thawed (typically 2–3 minutes).

#### 5.D. Preparing Raji Cells (HT-HiBiT) (continued)

- Using a 2ml serological pipette or a 1ml wide-bore pipette tip, transfer 1ml of prewarmed Raji Cells (HT-HiBiT) recovery medium, one drop at a time, to the cryovial. Using the same pipette, gently mix the contents of the cryovial by slowly pipeting approximately 1ml, once. Gently transfer the contents of the cryovial to the 15ml conical tube containing prewarmed Raji Cells (HT-HiBiT) recovery medium.
- Pellet the cells at  $150 \times g$  for 5 minutes.
- Aspirate the supernatant, leaving 100–300 $\mu$ l of Raji Cells (HT-HiBiT) recovery medium above the cell pellet.
- Resuspend the cell pellet by gently flicking the tube bottom 2–3 times. Add 5ml of prewarmed Raji Cells (HT-HiBiT) recovery medium to the side of the tube. Do not pipet Raji Cells (HT-HiBiT) recovery medium directly onto the cell pellet. Pipet once to mix.
- Pellet the cells at  $150 \times g$  for 5 minutes.
- Aspirate the supernatant, leaving 100–300 $\mu$ l of Raji Cells (HT-HiBiT) recovery medium above the cell pellet.
- Resuspend the cell pellet by gently flicking the bottom of the tube 2–3 times, then add 20ml of prewarmed Raji Cells (HT-HiBiT) recovery medium to the side of the tube for a concentration of approximately  $1 \times 10^5$  cells/ml. Do not pipet Raji Cells (HT-HiBiT) recovery medium directly onto the cell pellet. Pipet once to mix.
- Keep the Raji Cells (HT-HiBiT) at ambient temperature until Section 5.F.

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

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

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

- Gently resuspend and transfer the Raji Cells (HT-HiBiT) suspension to a sterile reagent reservoir.
- Using a multichannel pipette, immediately dispense 25 $\mu$ l 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  $2.5 \times 10^3$  cells/well.
- Using a multichannel pipette, add 25 $\mu$ l of the appropriate biologic drug dilution (Figure 11) to wells B3–G10 of the assay plate according to the plate layout in Figure 10. Add 25 $\mu$ l 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).

- Gently resuspend and transfer the T cell suspension to a sterile reagent reservoir.
- Using a multichannel pipette, immediately dispense 25 $\mu$ l of the T cell suspension to each of wells B2–G11 of the assay plates. The final cell number in each well should be  $2.5 \times 10^4$  cells/well.
- Add 75 $\mu$ l of TDCC assay buffer to each of the outside wells of the assay plates.

- Cover the assay plates with lids and incubate in a 37°C, 5% CO<sub>2</sub> 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.
- 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:** The Raji (HT-HiBiT) TCK Bioassay is compatible only with Bio-Glo-NB™ TCK Reagent. **Do not** use Bio-Glo™ Reagent or Bio-Glo-NL™ Reagent with the Raji (HT-HiBiT) TCK Bioassay.

- Remove the Bio-Glo-NB™ TCK Luciferase Assay Substrate from storage and mix by pipetting. Briefly centrifuge the tubes if the substrate has collected in the cap or on the sides of the tubes.
- Remove the Bio-Glo-NB™ TCK LgBiT Protein from storage and mix by pipetting. Briefly centrifuge the tubes if the solution has collected in the cap or on the sides of the tubes.
- Transfer the desired amount of ambient temperature Bio-Glo-NB™ TCK Luciferase Assay Buffer to a 15ml or 50ml centrifuge tube.
- 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).
- Prepare the Bio-Glo-NB™ TCK Reagent with 200µg/ml digitonin for detection of MR controls as follows:
  - Calculate the volume of Bio-Glo-NB™ TCK Reagent required for MR control wells.
  - Transfer the calculated volume of fully reconstituted Bio-Glo-NB™ TCK Reagent to a separate tube.
  - Add digitonin to a final concentration of 200µg/ml.
- Remove assay plates from the incubator after the incubation period and equilibrate to room temperature for 10–15 minutes.
- Using a manual multichannel pipette, add 75µl the Bio-Glo-NB™ TCK Reagent to wells treated with test or reference biologic drugs and SR controls, taking care not to create bubbles.
- Add 75µl of the Bio-Glo-NB™ TCK Reagent with 200µg/ml digitonin (from Step 5) to MR control wells, taking care not to create bubbles.

- 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<sub>50</sub> value and maximum fold induction.

## 5.H. Data Analysis

The Raji (HT-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.

- Calculate fold induction:

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

- Calculate percent specific lysis:

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

Maximum release (MR) control wells contain Raji Cells (HT-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 (HT-HiBiT) plus CD8+ T cells (no test or reference biologic drug added). These wells measure background target cell death in the absence of a test or reference biologic drug.

- Graph data as RLU versus Log<sub>10</sub> [biologic drug], fold induction versus Log<sub>10</sub> [biologic drug] and percent specific lysis versus Log<sub>10</sub>[biologic drug]. Fit curves and determine the EC<sub>50</sub> value of the biologic drug response using appropriate curve fitting software (such as GraphPad Prism® software).

## 6. CAR-T Assay Protocol

This procedure illustrates the use of Raji (HT-HiBiT) TCK Bioassay to test CAR-T effector cells against a reference sample in a single cytotoxicity assay (Figure 12). 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 designs and plate layouts are possible but may require further optimization.

**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 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 uses digitonin to permeabilize target cells, providing the maximal luminescent signal. The SR control is a measure of background target 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 (HT-HiBiT) (CD19, CD20, CD22, etc.)
- digitonin (e.g., Cat.# G9441)

#### Supplies and Equipment

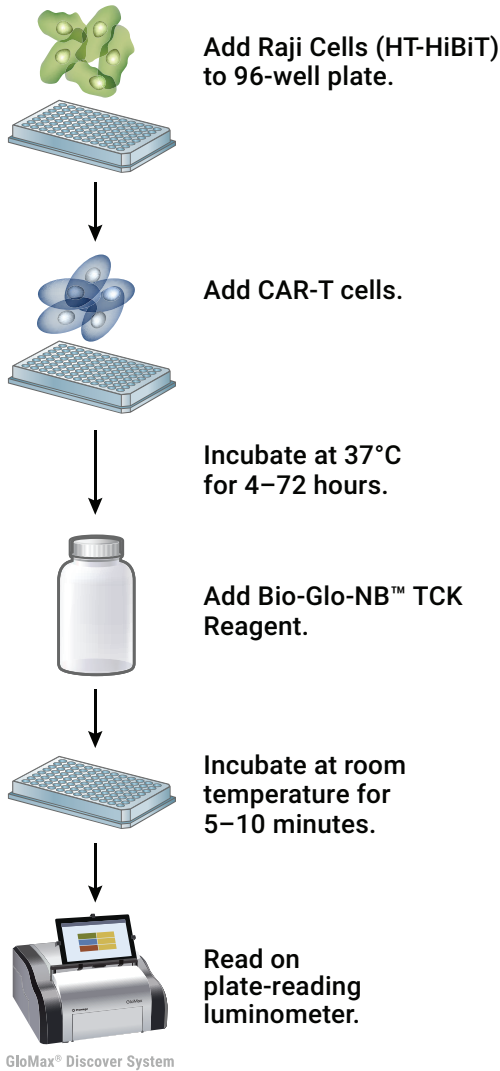
- solid-white, U-bottom 96-well assay plates (e.g., Corning® Cat.# 3355)
- sterile clear V-bottom 96-well plate with lid (e.g., Costar® Cat.# 3896) for preparing CAR-T cell dilutions
- pipettes (single-channel and 12-channel; for best results use both manual and electronic pipettes as needed)
- sterile 15ml and 50ml conical tubes
- sterile reagent reservoirs (e.g., Corning®/Costar® Cat.# 4870)
- 37°C, 5% CO<sub>2</sub> incubator
- 37°C water bath
- sensitive plate reader with glow luminescence measuring capability or luminometer (e.g., GloMax® Discover System [Cat.# GM3000] or equivalent)

#### 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. Optimal plate type used will vary depending on the properties of the CAR-T cells used.
- 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.



**6. CAR-T Assay Protocol (continued)**



**Figure 12. Schematic protocol for the Raji (HT-HiBiT) TCK Bioassay in a CAR-T assay application.**


## 6.A. Preparing Assay Reagents

**Heat-Inactivated (HI) FBS:** Thaw the provided FBS overnight at +2°C to +10°C or in a 37°C water bath on the day of use. Heat inactivate the FBS for 30 minutes at 56°C. Mix well and adjust to 37°C before use.

**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:** The Raji (HT-HiBiT) TCK Bioassay is compatible only with Bio-Glo-NB™ TCK Reagent. **Do not** use Bio-Glo™ Reagent or Bio-Glo-NL™ Reagent with the Raji (HT-HiBiT) TCK Bioassay.

**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 13 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 13. 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 (HT-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 (HT-HiBiT)

Thaw-and-use Raji Cells (HT-HiBiT) are in a sensitive state immediately post-thaw and care should be taken to follow the thawing and plating procedures exactly as described. Handle the cells gently and do not overmix the cells or overwarm the reagents. Do not vortex the cells or centrifuge at speeds > 150 × g. It is important to keep pipette tips away from tube surfaces during delivery or mixing steps to minimize mechanical stress on the cells. We recommend that you thaw and dilute a maximum of two vials of thaw-and-use cells at a time.

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

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

1. Add 9.5ml of Raji Cells (HT-HiBiT) recovery medium to a 15ml conical tube and warm to 37°C.
2. Remove one vial of Raji Cells (HT-HiBiT) from storage at -140°C and thaw in a 37°C water bath with gentle agitation (do not invert) until just thawed (typically 2–3 minutes).
3. Using a 2ml serological pipette or a 1ml wide-bore pipette tip, transfer 1ml of prewarmed Raji Cells (HT-HiBiT) recovery medium, one drop at a time, to the cryovial. Using the same pipette, gently mix the contents of the cryovial by slowly pipeting approximately 1ml, once. Gently transfer the contents of the cryovial to the 15ml conical tube containing prewarmed Raji Cells (HT-HiBiT) recovery medium.
4. Pellet the cells at 150 × g for 5 minutes.
5. Aspirate the supernatant, leaving 100–300µl of Raji Cells (HT-HiBiT) recovery medium above the cell pellet.
6. Resuspend the cell pellet by gently flicking the tube bottom 2–3 times. Add 5ml of prewarmed Raji Cells (HT-HiBiT) recovery medium to the side of the tube. Do not pipet Raji Cells (HT-HiBiT) recovery medium directly onto the cell pellet. Pipet once to mix.
7. Pellet the cells at 150 × g for 5 minutes.
8. Aspirate the supernatant, leaving 100–300µl of Raji Cells (HT-HiBiT) recovery medium above the cell pellet.
9. Resuspend the cell pellet by gently flicking the bottom of the tube 2–3 times, then add 20ml of prewarmed Raji Cells (HT-HiBiT) recovery medium to the side of the tube for a concentration of approximately 1 × 10<sup>5</sup> cells/ml. Do not pipet Raji Cells (HT-HiBiT) recovery medium directly onto the cell pellet. Pipet once to mix.
10. Transfer the cell suspension to a T25 cell culture flask and place the flask horizontally in a humidified 37°C, 5% CO<sub>2</sub> incubator.
11. Incubate the Raji Cells (HT-HiBiT) at 37°C 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<sup>6</sup> 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 (HT-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  $5 \times 10^3$  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 12. 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 75µl 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<sub>2</sub> 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:** The Raji (HT-HiBiT) TCK Bioassay is compatible only with Bio-Glo-NB™ TCK Reagent. **Do not** use Bio-Glo™ Reagent or Bio-Glo-NL™ Reagent with the Raji (HT-HiBiT) TCK Bioassay.

1. Remove the Bio-Glo-NB™ TCK Luciferase Assay Substrate from 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 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<sub>50</sub> value and maximum fold induction.

## 6.G. Data Analysis

The Raji (HT-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:

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

2. Calculate percent specific lysis:

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

Maximum release (MR) control wells contain Raji Cells (HT-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 (HT-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<sub>10</sub> (E:T ratio), fold induction versus Log<sub>10</sub> (E:T ratio) and percent specific lysis versus Log<sub>10</sub> (E:T ratio). Fit curves and determine the EC<sub>50</sub> value of the CAR-T response using appropriate curve fitting software (such as GraphPad Prism® software).

## 7. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: [www.promega.com](http://www.promega.com). Email: [techserv@promega.com](mailto:techserv@promega.com)

### Symptoms

### Causes and Comments

Low luminescence measurements (RLU readout)

Ensure that you are using Bio-Glo-NB™ TCK Reagent. The Raji (HT-HiBiT) TCK Bioassay is 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, which is designed for nonlytic NanoBIT® luciferase reporter bioassays. The Raji (HT-HiBiT) TCK Bioassay is not compatible with Bio-Glo-NL™ Reagent, which is designed for use with lytic bioassays.

Optimize the concentration range of your test sample(s) to achieve a full dose response with complete upper and lower asymptotes. The EC<sub>50</sub> value obtained with the Raji (HT-HiBiT) TCK Bioassay may vary from the EC<sub>50</sub> 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 CART assay.

Performance of PBMC ADCC assays is highly donor-dependent. Raji Cells (HT-HiBiT) have been optimized for use with Promega Human PBMC ADCC-Qualified.

**Symptoms**

Weak assay response  
(low fold induction, continued)

**Causes and Comments**

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 test antibodies or engineered effector cells may vary.

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.

For TDCC and CAR-T assays, fold induction can be further enhanced by incubating the TCK cells for 16–24 hours followed by a thorough wash prior to assay setup (see Section 4.D).

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 Raji Cells (HT-HiBiT) thoroughly (twice, as recommended) before assay setup.

Low cell viability causes high spontaneous release values because dead and dying cells release HiBiT into the media. Raji Cells (HT-HiBiT) 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 (HT-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.

Variability in assay performance

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 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.



## 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.

## 9. Appendix

### 9.A. Composition of Buffers and Solutions

#### recovery medium

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

#### 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
FcγRIIIa-H ADCP Reporter Bioassay, Complete Kit**	1 each	G9901
FcγRIIIa-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 <sup>®</sup> FcRn Binding Immunoassay	100 assays	W1151

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

## 9.B. Related Products (continued)

### 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 (TCR $\alpha\beta$ -KO, CD4+)	1 each	GA1172
T Cell Activation Bioassay (TCR $\alpha\beta$ -KO, CD8+)	1 each	GA1162
T Cell Activation Bioassay (TCR $\alpha\beta$ -KO, CD4+, CD8+)	1 each	GA1182

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

### Cytokine and Growth Factor Bioassays

<b>Product</b>	<b>Size</b>	<b>Cat.#</b>
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

<b>Product</b>	<b>Size</b>	<b>Cat.#</b>
SIRP $\alpha$ /CD47 Blockade Bioassay	1 each	JA6011
SIRP $\alpha$ /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 (TCK) Bioassays

<b>Product</b>	<b>Size</b>	<b>Cat.#</b>
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/](http://www.promega.com/products/reporter-bioassays/) or email: [EarlyAccess@promega.com](mailto: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/](http://www.promega.com/custom-solutions/tailored-solutions/)

Ⓔ NOT FOR MEDICAL DIAGNOSTIC USE. FOR IN VITRO USE ONLY. BY USE OF THIS PRODUCT, RECIPIENT AGREES TO BE BOUND BY THE TERMS OF THIS LIMITED USE STATEMENT. If the recipient is not willing to accept the conditions of this limited use statement, and the product is unused, Promega will accept return of the unused product and provide the recipient with a full refund.

This product may not be further sold or transferred by the recipient and may be used only by the recipient, and then only for (1) research use; (2) discovery, development and monitoring of biologic drugs and vaccines; (3) quality assurance testing of biologic drugs and vaccines; and (4) product release assays for biologic drugs and vaccines. No other commercial use is allowed. "Commercial use" means any and all uses of this product by recipient for monetary or other consideration, including providing a service, information or data to unaffiliated third parties, and resale of this product for any use. Recipient has no right to propagate, modify, derivatize, genetically engineer or otherwise create variations of the cells or genes stably transfected within the cells. In addition, recipient must use Bio-Glo-NB™ TCK Luciferase Assay System purchased from Promega Corporation for all luminescence assays using this product or contact Promega to obtain a license for use of this product with reagents other than Promega's. PROMEGA MAKES NO REPRESENTATIONS OR WARRANTIES OF ANY KIND, EITHER EXPRESSED OR IMPLIED, INCLUDING AS TO MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE WITH REGARDS TO THIS PRODUCT. The terms of this agreement shall be governed under the laws of the State of Wisconsin, USA.

Ⓕ U.S. Pat. No. 8,809,529, European Pat. No. 2635582, Japanese Pat. No. 5889910 and other patents and patents pending.

Ⓖ U.S. Pat. Nos. 9,797,889, 9,797,890 and 10,648,971; European Pat. Nos. 2970412 and 3783011; Japanese Pat. Nos. 6654557 and 7280842; and other patents and patents pending.

Ⓖ Product cannot be used for proficiency testing.

© 2024 Promega Corporation. All Rights Reserved.

GloMax, Lumit, NanoBIT and NanoLuc are registered trademarks of Promega Corporation. Bio-Glo, Bio-Glo-NB and Bio-Glo-NL are trademarks of Promega Corporation.

Blinicyto is a registered trademark of Amgen, Inc. Corning and Costar are registered trademarks of Corning, Inc. GIBCO is a registered trademark of Life Technologies Corp. GraphPad Prism is a registered trademark of GraphPad Software, Inc. JMP is a registered trademark of SAS Institute, Inc.

Products may be covered by pending or issued patents or may have certain limitations. Please visit our website for more information.

All prices and specifications are subject to change without prior notice.

Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.