

TECHNICAL MANUAL

# H929 (HiBiT) TCK Bioassay

Instructions for Use of Products  
JA1511 and JA1515

# H929 (HiBiT) TCK Bioassay

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## 1. Description

Tumor cell lysis induced 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). Chimeric antigen receptor T cells (CAR-T) 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.

The H929 (HiBiT) TCK Bioassay measures 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.

H929 (HiBiT) TCK Bioassay<sup>(a-d)</sup> (Cat. # JA1511, JA1515) 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.

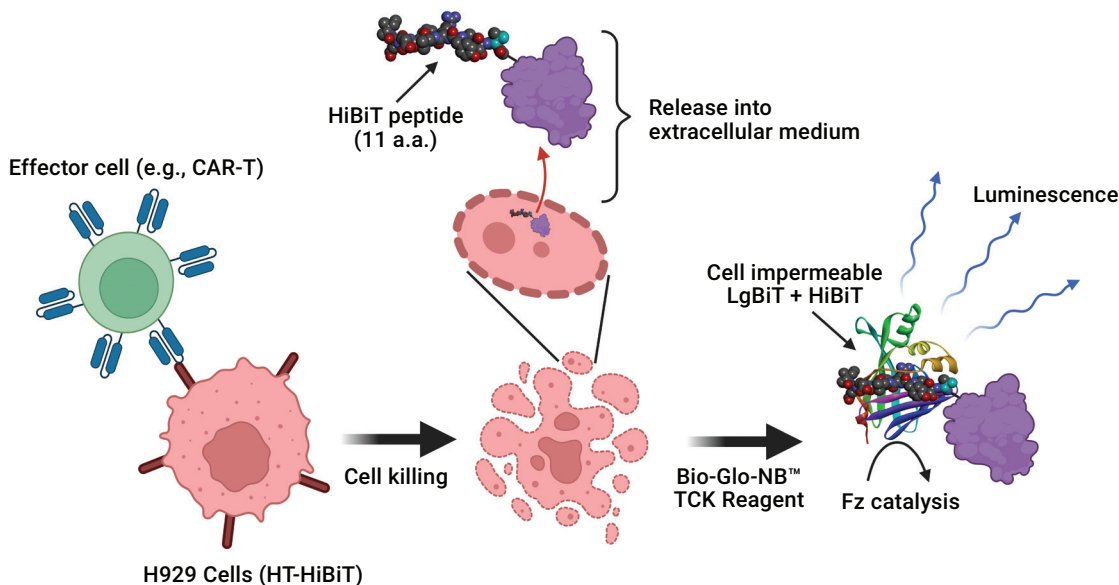
When H929 (HT-HiBiT) target cells are cocultured with appropriate effector cells (e.g., NK cells or cytotoxic T cells) they can be used to specifically monitor the activity of biologics directed against receptors expressed on the H929 Cells (HT-HiBiT), such as CD38 or BCMA. When H929 Cells (HT-HiBiT) are used in assays with biologics against TAAs they do not express, the target cells are not lysed and no increase in luminescent signal is observed (Figure 2).

H929 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 H929 Cells (HT-HiBiT) (2,500–5,000 cells 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. Together, these features make the H929 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) and timepoints ranging from 4–72 hours (Figure 5).

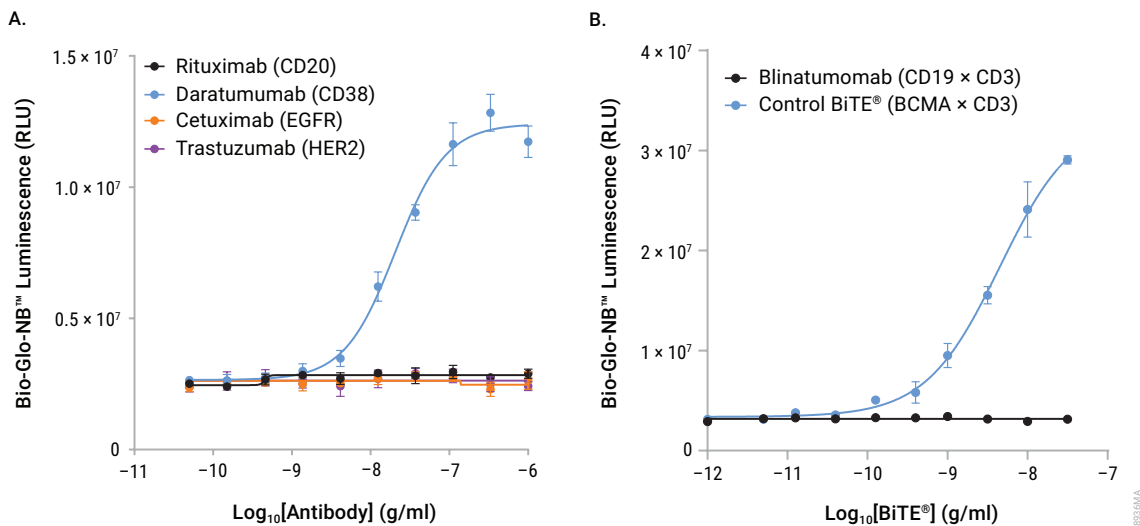
This technical manual provides sample protocols for using H929 Cells (HT-HiBiT) in an ADCC Bioassay with Human PBMC, ADCC-Qualified; a TDCC Bioassay with Human T Cell (CD8+), TDCC-Qualified; and a CAR-T Bioassay with user-defined CAR-T cells. Additional optimization may be necessary for use with your biologic product of interest.

1. Description (continued)



**Figure 1. Representation of the H929 (HiBiT) TCK Bioassay.** H929 Cells (HT-HiBiT) stably expressing an HT-HiBiT fusion protein are cocultured with effector cells (CAR-T, T cells, NK cells, etc.). Effector cell-mediated killing of H929 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. HT = HaloTag, the fusion partner for the HiBiT tag. (Created with BioRender.com.)

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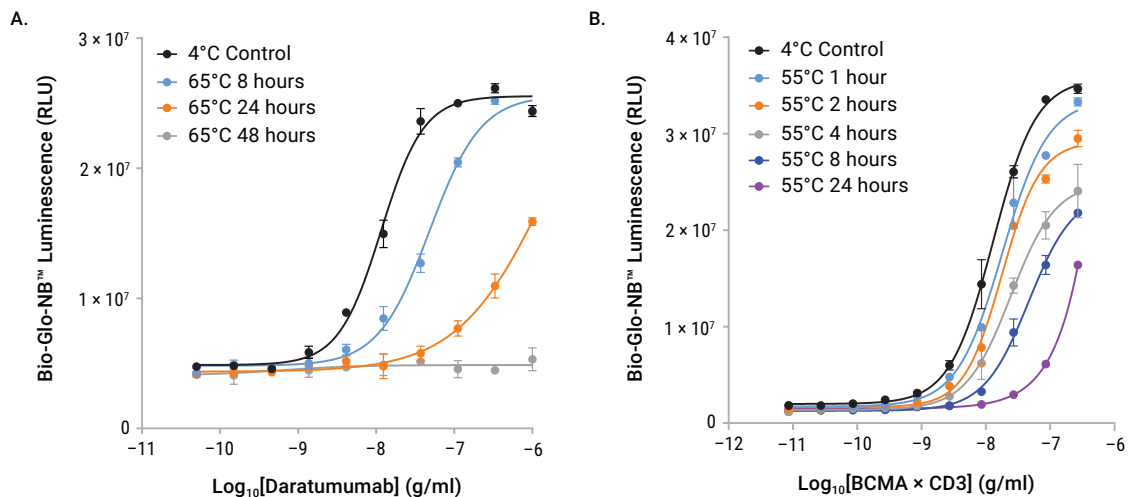


**Figure 2. HiBiT TCK Bioassays reflect the MOA of biologic drugs and demonstrate specificity for cell surface antigens. Panel A.** H929 Cells (HT-HiBiT) were incubated with Human PBMC, ADCC-qualified effector cells in the presence of serial titrations of antibodies, as indicated. After a 5-hour induction, Bio-Glo-NB™ TCK Reagent was added and luminescence quantified using the GloMax® Discover System (Cat.# GM3000). **Panel B.** H929 Cells (HT-HiBiT) were incubated with Human T Cells (CD8+) in the presence of bispecific T cell engagers (BiTE®). 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 tag.

**1. Description (continued)**

**Table 1. Precision, Accuracy and Linearity of TDCC Bioassay with H929 Cells (HT-HiBiT) as Targets.**

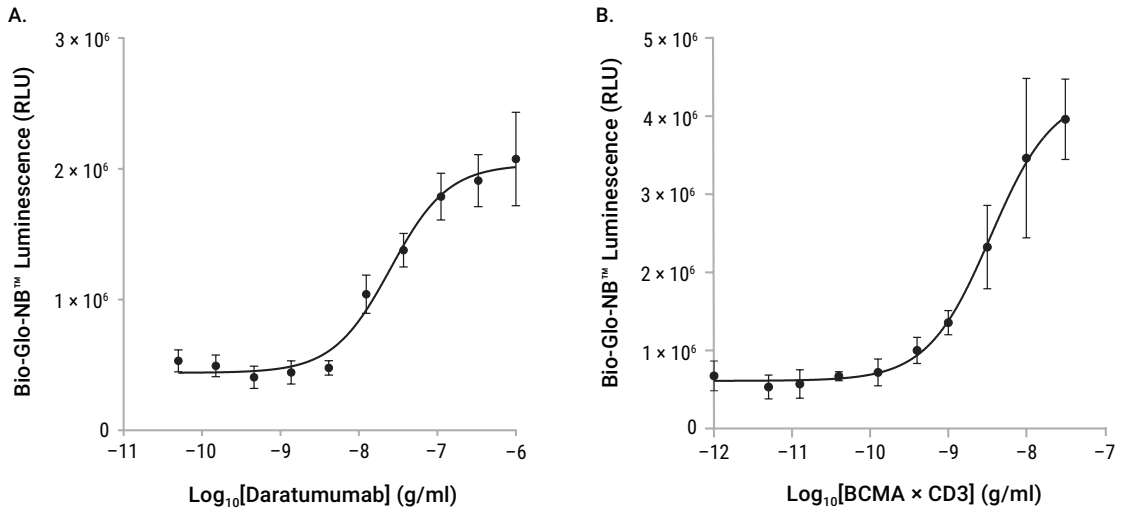
<b>Parameter</b>	<b>Results</b>	
Accuracy	% Expected Relative Potency	% Recovery
	50	109.2
	75	111.0
	100	93.1
	125	105.1
	150	102.7
Repeatability (% CV)	100% (Reference)	6
Intermediate Precision (% CV)		8
Linearity (r <sup>2</sup> )		0.93
Linearity (y = mx + b)		y = 0.988x + 4.48
<p>A 50–150% theoretical potency series of BCMA × CD3 BiTE<sup>®</sup> was analyzed in triplicate in three independent experiments performed on three days by two analysts using H929 Cell (HT-HiBiT) targets and Human T Cells (CD8+), TDCC-Qualified effectors. Bio-Glo-NB™ TCK Reagent was added, and luminescence quantified using the GloMax<sup>®</sup> Discover System. Data were analyzed and relative potencies calculated after parallelism determination using JMP<sup>®</sup> software. Data were generated using thaw-and-use cells.</p>		



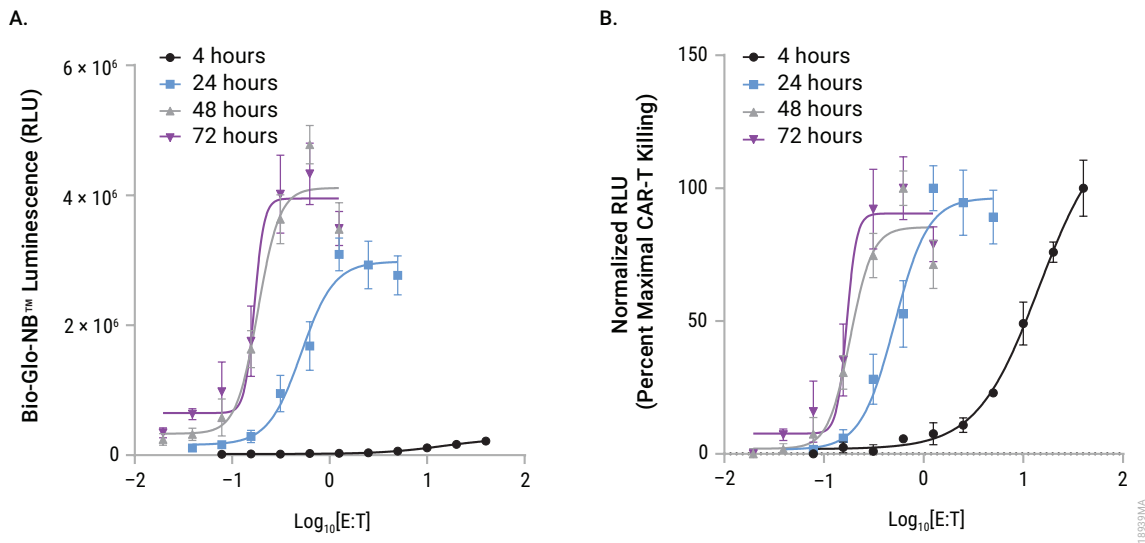
**Figure 3. HiBiT TCK Bioassays are stability-indicating. Panel A.** Samples of daratumumab were maintained at 4°C (control) or heat-treated at 65°C for the indicated times, then analyzed in an ADCC Bioassay with H929 Cells (HT-HiBiT) and Human PBMC, ADCC-qualified effector cells. **Panel B.** Samples of BCMA x CD3 BiTE® were maintained at 4°C (control) or heat-treated at 55°C for the indicated times, then analyzed in a TDCC Bioassay with H929 Cells (HT-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. HiBiT TCK Bioassays are amenable to 384-well plate format. Panel A.** H929 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 antibody daratumumab 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.** H929 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 BCMA x CD3 BiTE® 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. 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. H929 Cells (HT-HiBiT) are amenable to use in HiBiT TCK Bioassays using CAR-T effector cells.** **Panel A.** H929 Cells (HT-HiBiT) were incubated with serially-diluted human BCMA CAR-T cells at the indicated 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<sub>50</sub> values 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>H929 (HiBiT) TCK Bioassay</b>	<b>1 each</b>	<b>JA1511</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 H929 Cells (HT-HiBiT) (0.5ml per vial)
- 3 × 36ml RPMI 1640 Medium
- 2 × 4ml Fetal Bovine Serum
- 1 vial Bio-Glo-NB™ TCK Luciferase Assay Substrate
- 1 vial Bio-Glo-NB™ TCK LgBiT Protein
- 10ml Bio-Glo-NB™ TCK Luciferase Assay Buffer

PRODUCT	SIZE	CAT.#
<b>H929 (HiBiT) TCK Bioassay, 5X</b>	<b>1 each</b>	<b>JA1515</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 H929 Cells (HT-HiBiT) (0.5ml per vial)
- 15 × 36ml RPMI 1640 Medium
- 10 × 4ml Fetal Bovine Serum
- 5 vials Bio-Glo-NB™ TCK Luciferase Assay Substrate
- 5 vials Bio-Glo-NB™ TCK LgBiT Protein
- 5 × 10ml Bio-Glo-NB™ 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™ TCK Luciferase Assay Substrate, Bio-Glo-NB™ TCK LgBiT Protein, Bio-Glo-NB™ 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™ TCK Luciferase Assay Substrate and Bio-Glo-NB™ 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. Store the Bio-Glo-NB™ TCK Luciferase Assay Substrate protected from 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 H929 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 H929 (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).

H929 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.

H929 Cells (HT-HiBiT) are provided in frozen, thaw-and-use format and are ready to be used without 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.

H929 (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.B, Related Products). An integration time of 0.5 second/well was used for all readings.

H929 (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 H929 (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.

**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 daratumumab.

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

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.)

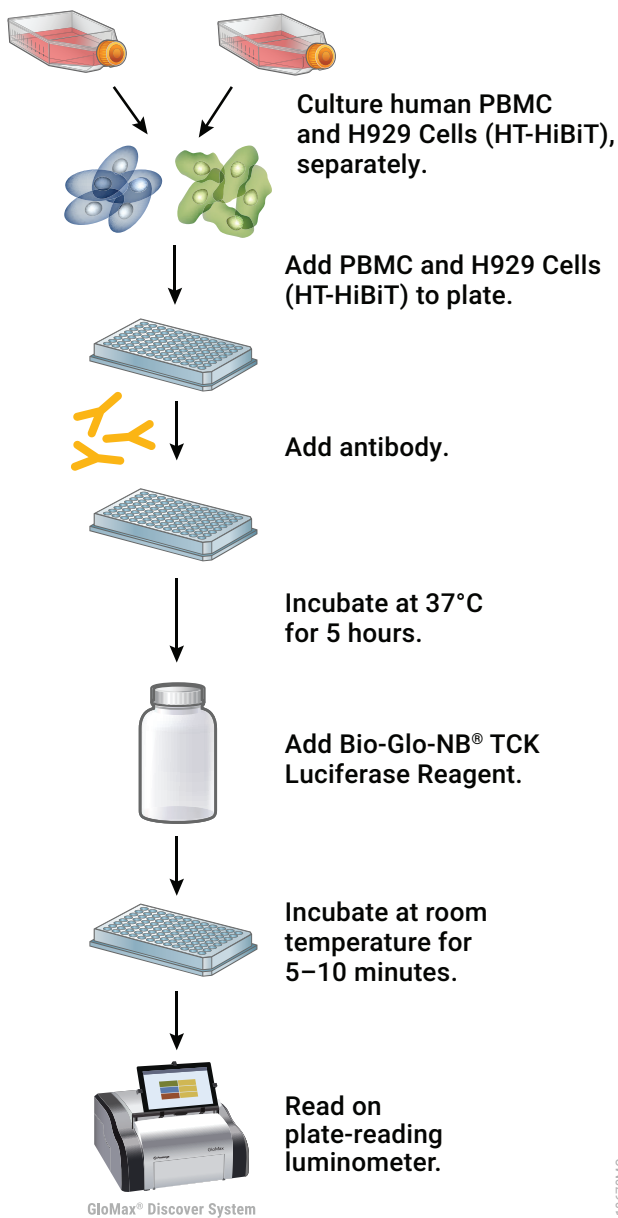
#### Reagents

- 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)
- $\beta$ -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:** anti-CD38 antibody daratumumab or other appropriate control antibody

#### 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)
- T25 cell culture flasks (e.g., Corning® Cat.# 430639)
- T75 cell culture flasks (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 or equivalent system)

**Note:** Corning® Cat.# 3355 plates come 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 6. Schematic protocol for the H929 (HiBiT) TCK Bioassay in a PBMC ADCC assay application.**

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

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

**Heat-Inactivated FBS (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. Store any unused HI-FBS at +2°C to +10°C for use in preparing assay buffer on the assay day.


**H929 Cells (HT-HiBiT) Recovery Medium:** On the day before the assay, prepare 15ml of H929 Cells (HT-HiBiT) recovery medium (see Section 9.A). Add 1.5ml of HI-FBS to 13.5ml of 37°C RPMI 1640 Medium. For reference, 15ml of H929 Cell (HT-HiBiT) recovery medium is sufficient to thaw and plate one vial of H929 (HT-HiBiT) target cells. If multiple vials will be thawed, then scale the amount of H929 Cells (HT-HiBiT) recovery medium appropriately.

**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,  $\beta$ -mercaptoethanol, sodium 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,  $\beta$ -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 daratumumab that we tested. 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. 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) during the 5-hour assay induction period. We recommend preparing the reconstituted Bio-Glo-NB™ TCK Reagent immediately before use.

 **Note:** The H929 (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 H929 (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 daratumumab 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 H929 Cells (HT-HiBiT), PBMC effector cells and digitonin. Spontaneous release (SR) control wells contain H929 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 H929 (HT-HiBiT) Target Cells

Thaw-and-use H929 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.

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

1. Add 9.5ml of H929 Cells (HT-HiBiT) recovery medium to a 15ml conical tube and warm to  $37^\circ\text{C}$ .
2. Remove one cryovial of H929 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, drop-by-drop, 1ml of prewarmed H929 Cells (HT-HiBiT) recovery medium 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 H929 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 H929 Cells (HT-HiBiT) recovery medium above the cell pellet.
6. Resuspend the cell pellet by gently flicking the tube bottom 2–3 times, then add 5ml of prewarmed H929 Cells (HT-HiBiT) recovery medium to the side of the tube. Do not pipet H929 (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

These instructions 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 300 $\mu$ g/ml (3X). Prepare 100 $\mu$ l for each antibody to be tested.
2. Transfer 100 $\mu$ l 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<sub>2</sub> incubator until Section 4.I.

#### 4.F. Day 2: Preparing 3X Digitonin for Maximum Release Control (continued)

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 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 H929 Cells (HT-HiBiT)



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

1. Gently mix and harvest the H929 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 H929 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. Keep 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 H929 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, add 25 $\mu$ l of the appropriate antibody dilution to wells B3–G10 of the assay plate according to Figure 8. 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.
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 daratumumab. 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 H929 (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 H929 (HiBiT) TCK Bioassay.

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 5-hour incubation (Section 4.H, Step 7) and equilibrate to room temperature for 10–15 minutes.
6. Using a multichannel pipette, add 75µl of 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 H929 (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 H929 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 H929 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 H929 (HiBIT) TCK 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 BCMA × CD3 BiTE®.

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 a biologic drug.

### Materials to Be Supplied By the User

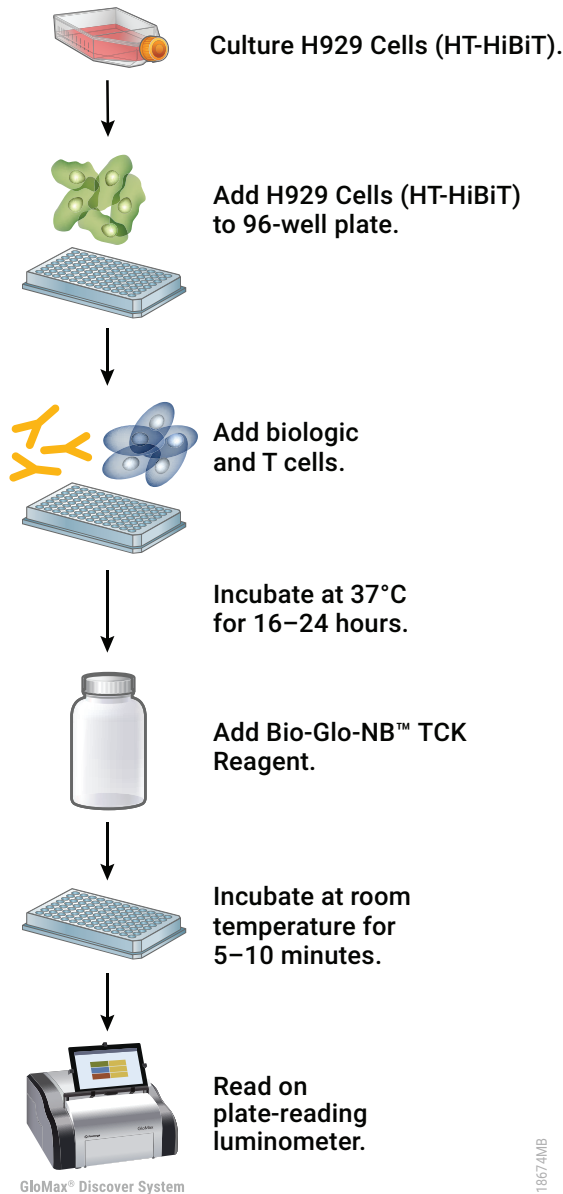
(Composition of Buffers and Solutions is provided in Section 9.A.)

#### 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)
- T25 cell culture flasks (e.g., Corning® Cat.# 430639)
- 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 or equivalent system)



**Figure 9. Schematic protocol using the H929 (HiBiT) TCK Bioassay with T cells.**



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

**Heat-Inactivated FBS (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. Store any unused HI-FBS at +2°C to +10°C for use in preparing assay buffer on the assay day.

**H929 Cells (HT-HiBiT) Recovery Medium:** On the day before the assay, prepare 15ml of H929 Cells (HT-HiBiT) recovery medium (see Section 9.A). Add 1.5ml of HI-FBS to 13.5ml of 37°C RPMI 1640 Medium. For reference, 15ml of H929 Cell (HT-HiBiT) recovery medium is sufficient to thaw and plate one vial of H929 (HT-HiBiT) target cells. If multiple vials will be thawed, then scale the amount of H929 Cells (HT-HiBiT) recovery medium appropriately.

**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 the BCMA × CD3 BiTE<sup>®</sup> that we tested. 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 H929 (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 H929 (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 H929 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. Thawing H929 (HT-HiBiT) Target Cells

Thaw-and-use H929 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.

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

1. Add 9.5ml of H929 Cells (HT-HiBiT) recovery medium to a 15ml conical tube and warm to 37°C.
2. Remove one cryovial of H929 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).
3. Using a 2ml serological pipette or a 1ml wide-bore pipette tip, transfer 1ml of prewarmed H929 Cells (HT-HiBiT) recovery medium, drop-by-drop, 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 H929 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 H929 Cells (HT-HiBiT) recovery medium above the cell pellet.
6. Resuspend the cell pellet by gently flicking the tube bottom 2–3 times, then add 5ml of prewarmed H929 Cells (HT-HiBiT) recovery medium to the side of the tube. Do not pipet H929 (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°C, 5%  $\text{CO}_2$  incubator.
8. Incubate for approximately 16–24 hours before assay setup.

### 5.D. Day 2: 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 $\mu\text{l}$  stock of each dilution provides sufficient volume for analysis in triplicate. To prepare threefold serial dilutions, you will need 300 $\mu\text{l}$  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 $\mu\text{l}$  of reference biologic starting dilution (dilu1, 3X final concentration) to wells A10 and B10 (Figure 11).
3. Add 300 $\mu\text{l}$  of test biologics 1 and 2 starting dilution (dilu1, 3X final concentration) to wells E10 and G10, respectively (Figure 11).

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<sub>2</sub> incubator until Section 5.G while preparing the H929 Cells (HT-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 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.E. Preparing H929 Cells (HT-HiBiT)

 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. Gently mix and harvest the H929 Cells (HT-HiBiT) that were plated overnight.
2. Transfer cells to a 15ml or 50ml 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 H929 Cells (HT-HiBiT) at ambient temperature until Section 5.G.

### 5.F. Preparing Human CD8+ T Cells, TDCC Qualified Effector Cells

1. 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).
2. Transfer all of the cells into 3.5ml of prepared TDCC assay buffer for a concentration of  $1 \times 10^6$  cells/ml.
3. Incubate the T cells at ambient temperature until Section 5.G.

### 5.G. Adding H929 Cells (HT-HiBiT), Effector Cells and Biologic Drugs

1. Gently resuspend and transfer the H929 Cells (HT-HiBiT) suspension to a sterile reagent reservoir.
2. Using a multichannel pipette, immediately dispense  $25\mu\text{l}$  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.
3. Using a multichannel pipette, add  $25\mu\text{l}$  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  $25\mu\text{l}$  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.H).

4. Gently resuspend and transfer the T cell suspension to a sterile reagent reservoir.
5. Using a multichannel pipette, immediately dispense  $25\mu\text{l}$  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.
6. Add  $75\mu\text{l}$  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  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$  incubator for 16–24 hours.

**Note:** The incubation period was optimized using a BCMA  $\times$  CD3 BiTE<sup>®</sup>. 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.H. 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 H929 (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 H929 (HiBiT) TCK Bioassay.

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 16–24 hour incubation period and equilibrate to room temperature for 10–15 minutes.
7. 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.
8. 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.
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<sub>50</sub> value and maximum fold induction.

## 5.1. 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:

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

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 H929 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 H929 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.

3. Graph data as RLU versus  $\text{Log}_{10}$ [biologic drug], fold induction versus  $\text{Log}_{10}$ [biologic drug] and percent specific lysis versus  $\text{Log}_{10}$ [biologic drug]. Fit curves and determine the  $\text{EC}_{50}$  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 H929 Cells (HT-HiBiT) 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 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 50:1 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 H929 Cells (HT-HiBiT) (CD38, BCMA, etc.)
- digitonin (e.g., Cat.# G9441)

### Supplies and Equipment

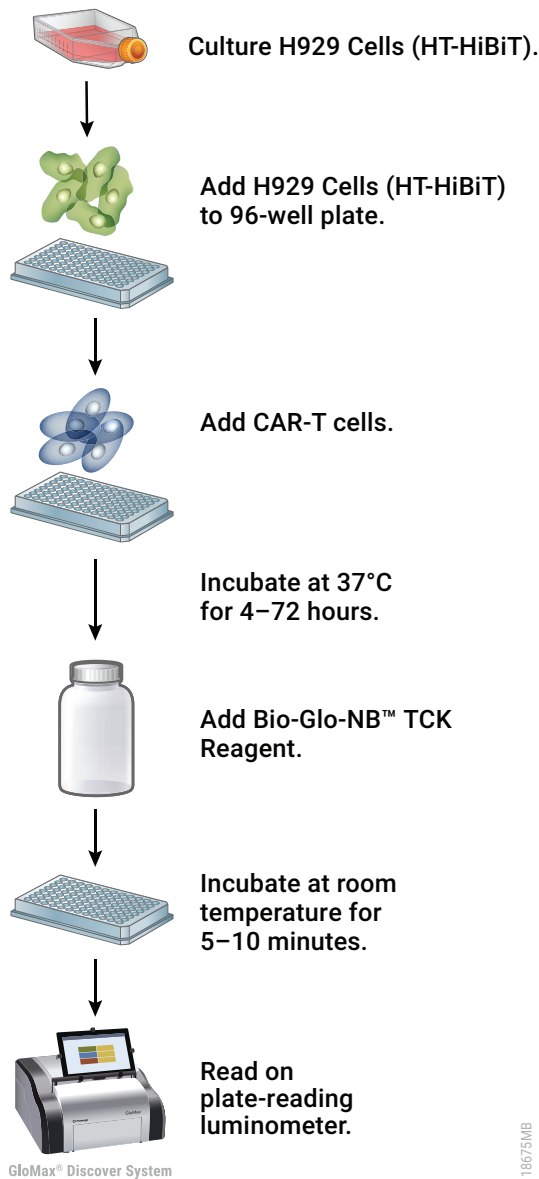
- solid-white, U-bottom 96-well assay plates (e.g., Corning® Cat.# 3355)
- sterile clear V-bottom 96-well plate with lid (e.g., Costar® Cat.# 3896) for preparing CAR-T cell dilutions
- pipettes (single-channel and 12-channel; for best results use both manual and electronic pipettes as needed)
- sterile 15ml and 50ml conical tubes
- sterile reagent reservoirs (e.g., Corning®/Costar® Cat.# 4870)
- T25 cell culture flasks (e.g., Corning® Cat.# 430639)
- 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 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. 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 using the H929 (HiBiT) TCK Bioassay with CAR-T cells.**

## 6.A. Preparing Assay Reagents and Samples


**Heat-Inactivated FBS (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. Store any unused HI-FBS at +2°C to +10°C for use in preparing assay buffer on the assay day.

**H929 Cells (HT-HiBiT) Recovery Medium:** On the day before the assay, prepare 15ml of H929 Cells (HT-HiBiT) recovery medium (see Section 9.A). Add 1.5ml of HI-FBS to 13.5ml of 37°C RPMI 1640 Medium. For reference, 15ml of H929 Cell (HT-HiBiT) recovery medium is sufficient to thaw and plate one vial of H929 (HT-HiBiT) target cells. If multiple vials will be thawed, then scale the amount of H929 Cells (HT-HiBiT) recovery medium appropriately.

**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 cells 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 H929 (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 H929 (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 H929 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. Thawing H929 Cells (HT-HiBiT)


Thaw-and-use H929 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 H929 Cells (HT-HiBiT) recovery medium to a 15ml conical tube and warm to 37°C.
2. Remove one cryovial of H929 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).
3. Using a 2ml serological pipette or a 1ml wide-bore pipette tip, transfer 1ml of prewarmed H929 Cells (HT-HiBiT) recovery medium, drop-by-drop, 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 H929 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 H929 Cells (HT-HiBiT) recovery medium above the cell pellet.
6. Resuspend the cell pellet by gently flicking the tube bottom 2–3 times, then add 5ml of prewarmed H929 Cells (HT-HiBiT) recovery medium to the side of the tube. Do not pipet H929 (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°C, 5%  $\text{CO}_2$  incubator.
8. Incubate for approximately 16–24 hours before assay setup.

### 6.D. Day 2: Preparing H929 Cells (HT-HiBiT)

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

1. Gently mix and harvest the H929 Cells (HT-HiBiT) that were plated overnight.
2. Transfer to 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 H929 Cells (HT-HiBiT) at ambient temperature until Section 6.F.

### 6.E. 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  $6.25 \times 10^6$  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 40 $\mu$ l per well of each cell density tested in triplicate.
4. Proceed immediately to Section 6.F.

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

1. Gently resuspend and transfer the H929 Cells (HT-HiBiT) suspension to a sterile reagent reservoir.
2. Using a multichannel pipette, immediately dispense 40 $\mu$ l 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 40 $\mu$ l of the appropriate CAR-T dilution to wells B3–G10 of the assay plates according to the plate layout in Figure 12. Add 40 $\mu$ l 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.G).

4. Add 75 $\mu$ l 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.G. 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 H929 (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 H929 (HiBiT) TCK Bioassay.

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 4–72 hour 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.H. Data Analysis

The H929 (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 H929 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 H929 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  $\text{Log}_{10}(\text{E:T ratio})$ , fold induction versus  $\text{Log}_{10}(\text{E:T ratio})$  and percent specific lysis versus  $\text{Log}_{10}(\text{E:T ratio})$ . Fit curves and determine the  $\text{EC}_{50}$  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)

<b>Symptoms</b>	<b>Causes and Comments</b>
Low luminescence measurements (RLU readout)	<p>Ensure that you are using Bio-Glo-NB™ TCK Reagent. The H929 (HiBiT) TCK Bioassay is not compatible with Bio-Glo™ or Bio-Glo-NL™ Reagents.</p> <p>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.</p> <p>Some models of luminometers with low sensitivity should be avoided. If using a reader with an adjustable gain, we recommend a high-gain setting.</p> <p>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.</p> <p>Low activity of Bio-Glo-NB™ TCK Reagent leads to low RLU. Store and handle Bio-Glo-NB™ TCK Reagent according to the instructions. For best results, prepare immediately before use.</p>
Weak assay response (low fold induction)	<p>Ensure that you are using Bio-Glo-NB™ TCK Reagent, which is designed for nonlytic NanoBIT® luciferase reporter bioassays. The H929 (HiBiT) TCK Bioassay is not compatible with Bio-Glo-NL™ Reagent, which is designed for use with lytic bioassays.</p> <p>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 HiBiT TCK Bioassay may vary from the EC<sub>50</sub> value obtained using other methods.</p> <p>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.</p> <p>Optimize the assay incubation time within a range of 3–24 hours (or longer) for the CART assay.</p> <p>Performance of PBMC ADCC assays is highly donor-dependent. H929 Cells (HT-HiBiT) have been optimized for use with Promega Human PBMC, ADCC-Qualified.</p>



## 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 test antibodies or engineered effector cells may vary.
High spontaneous release	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 H929 Cells (HT-HiBiT) thoroughly (twice, as recommended) before assay setup.
High spontaneous release	Low cell viability causes high spontaneous release values because dead and dying cells release HiBiT into the medium. H929 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, H929 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.
Specific lysis greater than 100%	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.
Variability in assay performance	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

#### H929 Cells (HT-HiBiT) 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® 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

**Immune Checkpoint Bioassays (continued)**

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

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

## 9.B. Related Products (continued)

### 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 (TCK) 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.

### 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

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

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



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<sup>(b)</sup>U.S. Pat. Nos. 9,797,890 and 10,648,971; Japanese Pat. Nos. 6654557 and 7280842; and other patents and patents pending.

<sup>(c)</sup>Product cannot be used for proficiency testing.

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