

TECHNICAL MANUAL

Ramos (HiBiT) TCK Bioassay, Propagation Model

Instructions for Use of Products
M2472 and GA6070

Ramos (HiBiT) TCK Bioassay, Propagation Model

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1. Description	3
2. Product Components and Storage Conditions	10
3. Before You Begin	10
3.B. Ramos Cells (HT-HiBiT) Thawing and Initial Cell Culture	12
3.C. Ramos Cells (HT-HiBiT) Propagation and Maintenance	12
3.D. Ramos Cells (HT-HiBiT) Freezing and Banking	13
4. PBMC ADCC Assay Protocol	13
4.A. Preparing Assay Reagents and Samples	16
4.B. Plate Layout Design	17
4.C. Thawing and Plating Human PBMC, ADCC-Qualified Effector Cells	18
4.D. Preparing 3X Antibody Stock Solutions	18
4.E. Preparing 3X Digitonin for Maximum Release Control	18
4.F. Preparing Ramos Cells (HT-HiBiT)	19
4.G. Preparing Human PBMC, ADCC-Qualified Effector Cells	20
4.H. Adding Target Cells, Effector Cells and Antibody	20
4.I. Preparing and Adding Bio-Glo-NB™ TCK Reagent	20
4.J. 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 Ramos Cells (HT-HiBiT)	27
5.E. Preparing Human T Cells (CD8+), TDCC-Qualified Effector Cells	28
5.F. Adding Ramos Cells (HT-HiBiT), Effector Cells and Biologic Drugs	28
5.G. Preparing and Adding Bio-Glo-NB™ TCK Reagent	28
5.H. Data Analysis	30

6. CAR-T Assay Protocol	30
6.A. Preparing Assay Reagents.....	33
6.B. Plate Layout Design.....	34
6.C. Preparing Ramos 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.....	35
6.F. Preparing and Adding Bio-Glo-NB™ TCK Reagent	36
6.G. Data Analysis	37
7. Troubleshooting	38
8. References	40
9. Appendix.....	41
9.A. Composition of Buffers and Solutions	41
10. Summary of Changes	41

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.

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® luciferase (4). When dead or dying cells release the HiBiT fusion protein into the extracellular medium, it binds to the cell impermeable LgBiT, which is provided with the furimazine substrate in a nonlytic, homogeneous detection reagent (Figure 1). In coculture experiments, the luminescent signal will be proportional to target cell death alone, with no signal contribution arising from the death of effector cells.

Ramos (HiBiT) TCK Bioassay, Propagation Model^(a) (Cat.# M2472), is provided in a cell propagation model (CPM) format, which includes cryopreserved cells that can be thawed, propagated and banked for long-term use. A Ramos (HiBiT) Cell Bank (Cat.# GA6070) is also available.

When Ramos (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 TAA expressed on the Ramos Cells (HT-HiBiT), such as CD19, CD20 or CD22. When Ramos 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).

Ramos 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 Ramos 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 Ramos 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 instructions for culturing and banking Ramos Cells (HT-HiBiT), as well as sample protocols for using these cells in an ADCC Bioassay with Human PBMC, ADCC-Qualified; a TDCC Bioassay with Human T Cells (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)

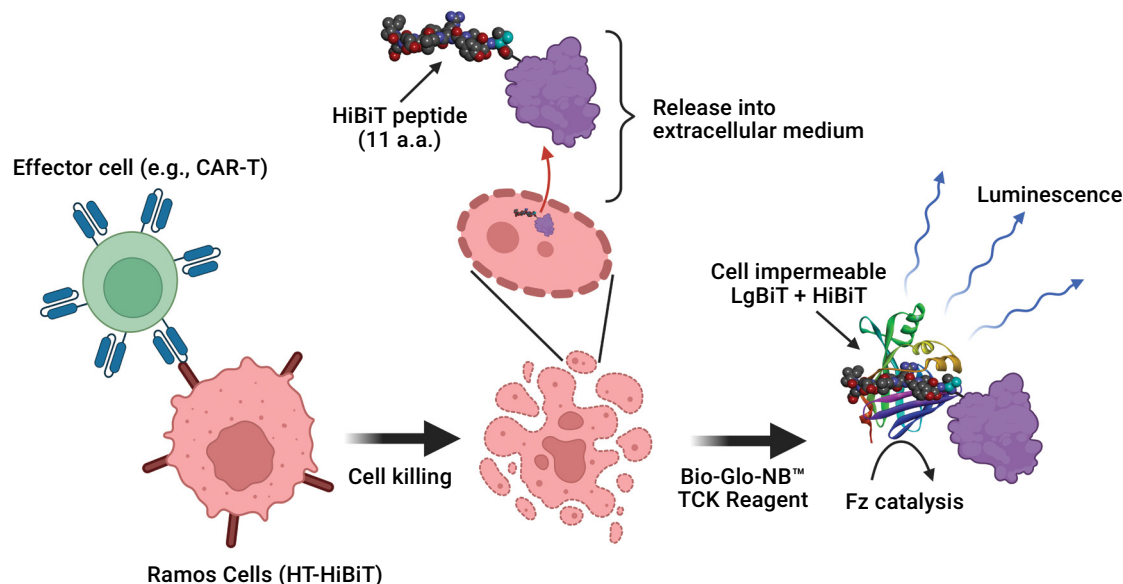


Figure 1. Representation of the Ramos (HiBiT) TCK Bioassay. Ramos Cells (HT-HiBiT) stably expressing a HiBiT fusion protein are cocultured with effector cells (CAR-T, T cells, NK cells, etc.). Effector cell-mediated killing of Ramos Cells (HT-HiBiT) leads to release of the HiBiT fusion protein into the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

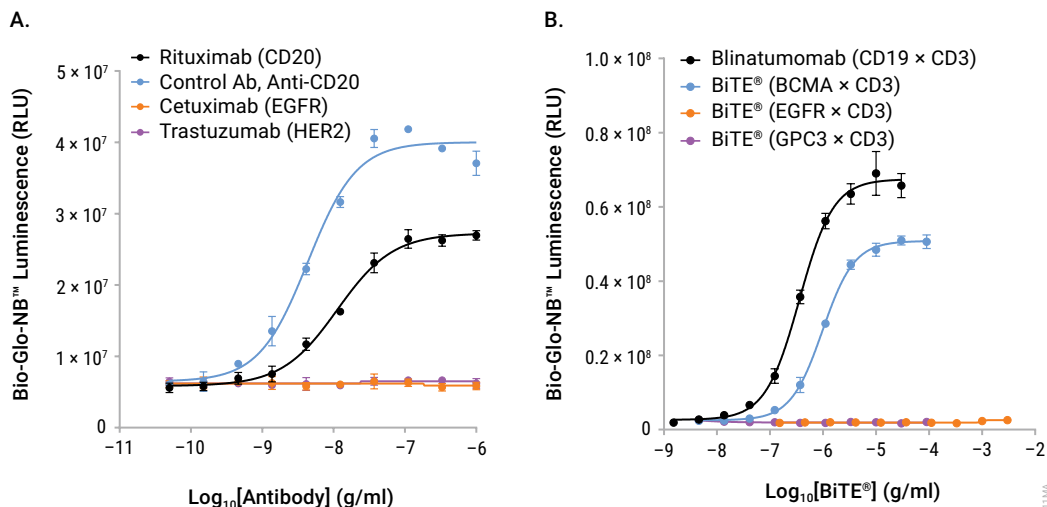


Figure 2. HiBiT TCK Bioassays reflect the MOA of biologic drugs and demonstrate specificity for cell surface antigens.

Panel A. Ramos Cells (HT-HiBiT) were incubated with Human PBMC, ADCC-qualified effector cells in the presence of serial titrations of antibodies, including Control Ab, Anti-CD20 (Cat.# GA1130), 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. Ramos 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 Ramos Cells (HT-HiBiT) as Targets.

Parameter	Results	
Accuracy	% Expected Relative Potency	% Recovery
	50	86.9
	75	99.8
	100	101.9
	125	100.7
	150	99.7
Repeatability (% CV)	100% (Reference)	8
Intermediate Precision (% CV)		10
Linearity (r^2)		0.94
Linearity ($y = mx + b$)		$y = 1.052x + 6.04$
A 50–150% theoretical potency series of blinatumomab was analyzed in triplicate in three independent experiments performed on three days by two analysts using Ramos 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® Discover System. Data were analyzed and relative potencies calculated after parallelism determination using JMP® software. Data were generated using thaw-and-use cells.		

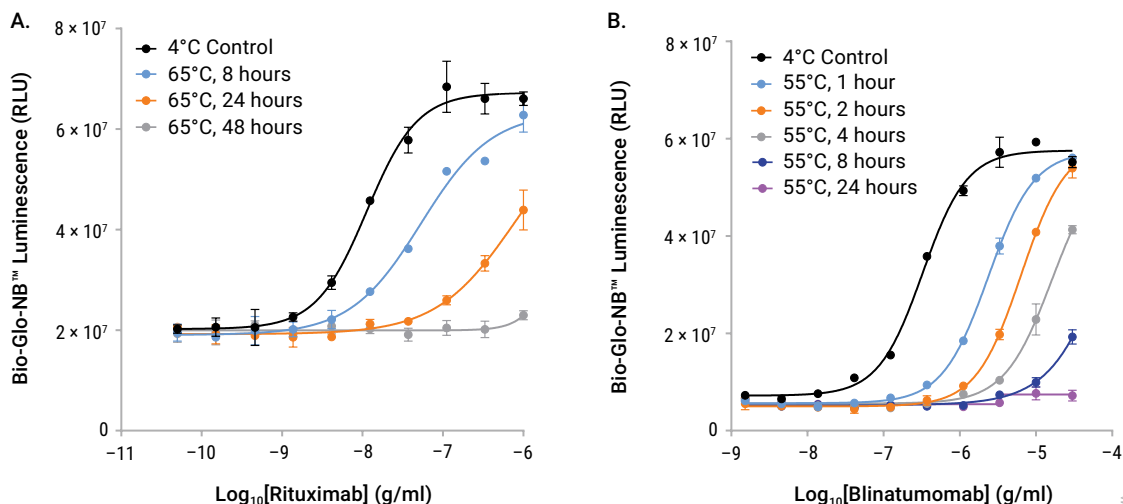


Figure 3. HiBiT TCK Bioassays are stability-indicating. Panel A. Samples of rituximab were maintained at 4°C (control) or heat-treated at 65°C for the indicated times, then analyzed in an ADCC Bioassay with Ramos Cells (HT-HiBiT) and Human PBMC, ADCC-Qualified effector cells. **Panel B.** Samples of blinatumomab were maintained at 4°C (control) or heat-treated at 55°C for the indicated times, then analyzed in a TDCC Bioassay with Ramos 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)

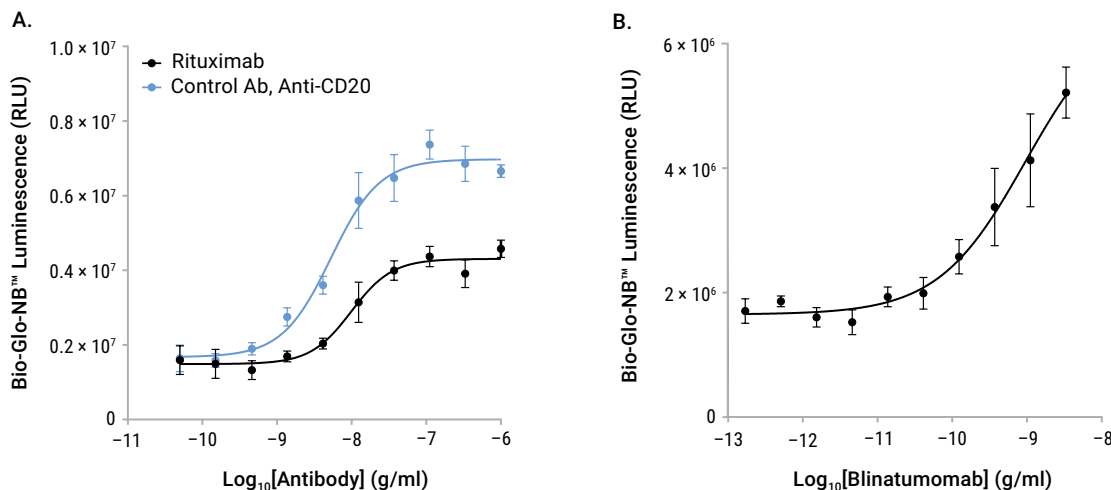


Figure 4. HiBiT TCK Bioassays are amenable to 384-well plate format. Panel A. Ramos Cells (HT-HiBiT) were harvested after overnight culture and 1×10^3 cells/5 μ l/well plated in a 384-well round-bottom white assay plate (e.g., Corning® Cat.# 4512, low volume). Next, 5 μ l of serially-diluted 3X Control Ab, Anti-CD20 (Cat.# GA1130), or anti-CD20 antibody rituximab, was added, then 2.5×10^4 /5 μ l/well of Human PBMC, ADCC-Qualified effector cells from overnight culture were added. After a 5-hour incubation at 37°C, 5% CO₂, 5 μ l of 4X Bio-Glo-NB™ TCK Reagent was added per well and luminescence quantified. **Panel B.** Ramos Cells (HT-HiBiT) were plated at 5×10^2 cells/5 μ l/well in a 384-well flat-bottom white assay plate (e.g., Corning® Cat.# 3570). Next, 5 μ l of 3X serially-diluted blinatumomab was added, followed by 5×10^3 /5 μ l/well of Human T Cells (CD8+), TDCC-Qualified. After a 24-hour incubation at 37°C, 5% CO₂, 15 μ 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.

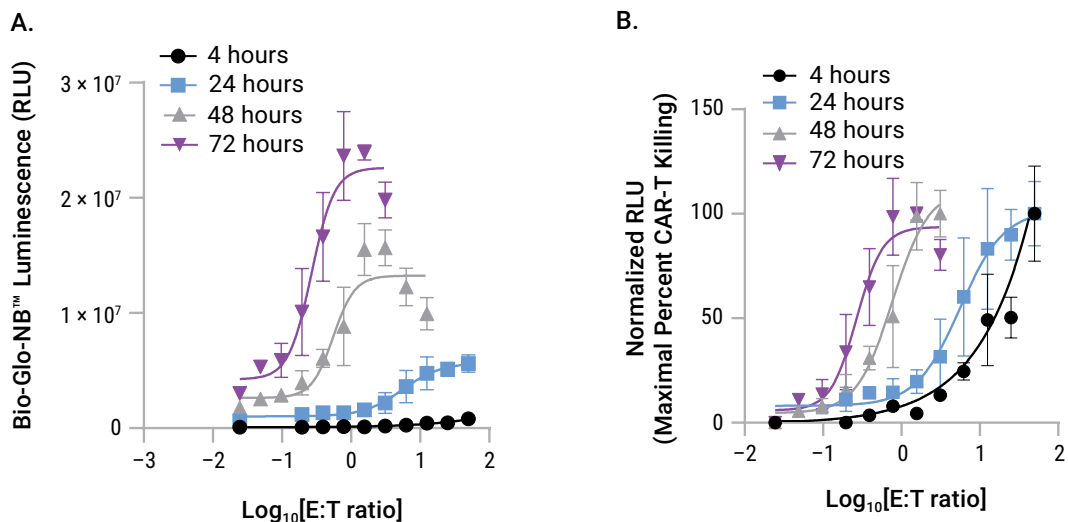


Figure 5. Ramos Cells (HT-HiBiT) are amenable to use in HiBiT TCK Bioassays using CAR-T effector cells. Panel A.

Ramos Cells (HT-HiBiT) were incubated with serially-diluted CD19 human 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 EC₅₀ values decreased with longer incubation periods (**Panel B**) due to cumulative target cell killing over time. Data were generated using thaw-and-use cells.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT. #
Ramos (HiBiT) TCK Bioassay, Propagation Model	1 each	M2472

Not for Medical Diagnostic Use. Includes:

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

PRODUCT	SIZE	CAT. #
Ramos (HiBiT) Cell Bank	1 each	GA6070

Not for Medical Diagnostic Use. Includes:

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

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

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

3. Before You Begin

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

In this technical manual, we provide example protocols for using Ramos 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, Preparing Ramos Cells (HT-HiBiT); 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.

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



Note: Ramos (HiBiT) TCK Bioassay, Propagation Model, 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).

Ramos 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.

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

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. An integration time of 0.5 second/well was used for all readings.

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.

3.A. Materials to Be Supplied By the User

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

Reagents

- RPMI 1640 medium with L-glutamine and HEPES (e.g., Corning® Cat.# 10-041-CV or GIBCO® Cat.# 22400105)
- fetal bovine serum (FBS; e.g., VWR Cat.# 89510-194 or GIBCO® Cat.# 16000044)
- Trypan blue solution (e.g., Sigma Cat.# T8154)
- Blasticidin (e.g., GIBCO® Cat.# A11139)
- DMSO (e.g., Sigma Cat.# D2650)

Supplies and Equipment

- T75 cell culture flask (e.g., Corning® Cat.# 430639)
- pipettes (single-channel; for best results use manual and electronic pipettes as needed)
- 15ml and 50ml conical tubes
- 37°C, 5% CO₂ incubator
- 37°C water bath

3.B. Ramos Cells (HT-HiBiT) Thawing and Initial Cell Culture

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

1. Prepare 20ml of initial cell culture medium by adding 2ml of FBS to 18ml of RPMI 1640 medium prewarmed to 37°C. This initial cell culture medium will be used for culturing the cells immediately after thawing.
 2. Transfer 9ml of prewarmed initial cell culture medium to a 15ml conical tube.
 3. Remove one vial of Ramos Cells (HT-HiBiT) from storage at -140°C and thaw in a 37°C water bath with gentle agitation (do not invert cell vial) until just thawed (typically 2–3 minutes).
 4. Transfer all of the cells (approximately 1ml) to the 15ml conical tube containing 9ml of prewarmed initial cell culture medium.
 5. Centrifuge at $150 \times g$ for 10 minutes.
 6. Carefully aspirate the medium and resuspend the cell pellet in 10ml of prewarmed initial cell culture medium. Initial cell density will be 4×10^5 viable cells/ml.
 7. Transfer the cell suspension to a T75 cell culture flask and place the flask horizontally in a humidified 37°C, 5% CO₂ incubator.
 8. Incubate for approximately 24 hours before passaging the cells.
 9. Passage the cells at a seeding density of $1-3 \times 10^5$ viable cells/ml using cell growth medium containing selection antibiotics (see Section 9.A).
- Note:** When passaging cells for the first time after thawing, it is critical to use a minimum seeding density of 3×10^5 viable cells/ml. Lower densities may reduce cell viability and growth.

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

3.C. Ramos Cells (HT-HiBiT) Propagation and Maintenance

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

1. On the day of cell passage, measure cell viability and density by Trypan blue staining.
2. Seed the cells at a density of $2-3 \times 10^5$ cells/ml if passaging every 2 days (e.g., Monday–Wednesday or Wednesday–Friday) or $1-2 \times 10^5$ cells/ml if passaging every 3 days (e.g., Friday–Monday). Always maintain the flasks in a horizontal position in the incubator. Do not allow the cells to grow to a density greater than 1.5×10^6 cells/ml. Duplicate flasks can be passaged at higher or lower cell density to ensure optimal cell density at harvest.
3. Maintain the cell culture by adding fresh cell growth medium to the cell suspension in the original flask or by transferring the cells to a new flask while maintaining a consistent ratio of culture volume to flask surface area (e.g., 25ml volume per T75 flask or 50ml volume per T150 flask).
4. Place the flasks horizontally in a humidified, 37°C, 5% CO₂ incubator.

3.D. Ramos Cells (HT-HiBiT) Freezing and Banking

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

4. PBMC ADCC Assay Protocol

This procedure illustrates the use of Ramos Cells (HT-HiBiT) 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 $1\mu\text{g/ml}$ 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 the presence of effector cells but in the absence of a monoclonal antibody.

4. PBMC ADCC Assay Protocol (continued)

Materials to Be Supplied By the User

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

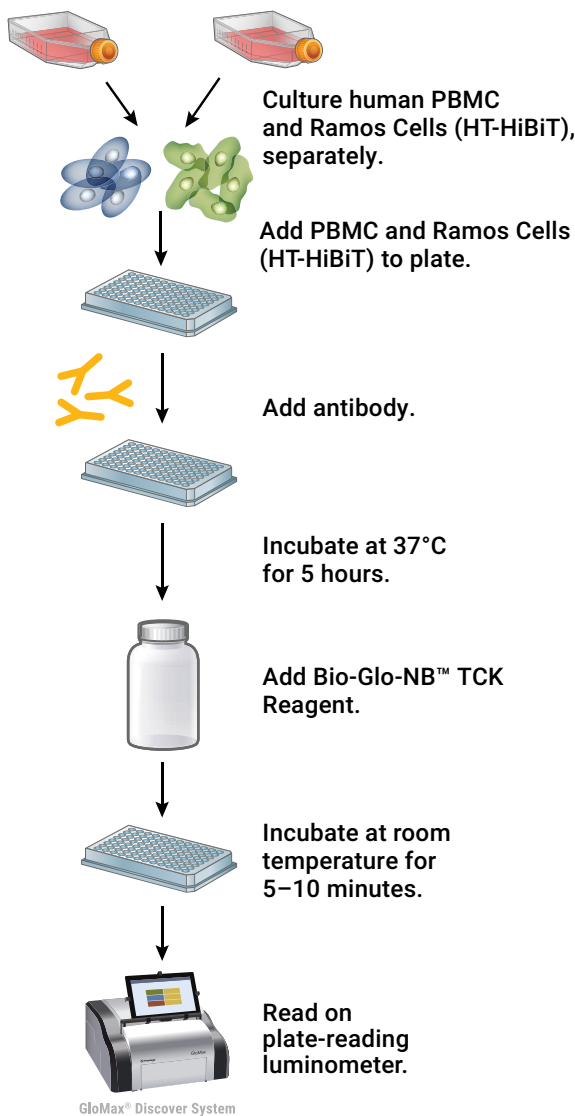
Reagents

- Human PBMC, ADCC-Qualified (e.g., www.promega.com/products/reporter-bioassays/primary-cell-bioassays/pbmc-adcc-bioassay/; please enquire)
- antibodies or other biologics samples capable of inducing ADCC
- RPMI 1640 medium with L-glutamine and HEPES (e.g., Corning® Cat.# 10-041-CV or GIBCO® Cat.# 22400105)
- heat-inactivated fetal bovine serum (HI-FBS; e.g., GIBCO® Cat.# 16140)
- recombinant human IL-2 (e.g., Sigma Cat.# I2644)
- β-mercaptoethanol (e.g., GIBCO® Cat.# 21985)
- sodium pyruvate (e.g., GIBCO® Cat.# 11360)
- digitonin (e.g., Cat.# G9441)
- 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)
- 37°C, 5% CO₂ incubator
- 37°C water bath
- sensitive plate reader with glow luminescence measuring capability or luminometer (e.g., GloMax® Discover System (Cat.# GM3000 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.



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Figure 6. Schematic protocol for the Ramos (HiBiT) TCK Bioassay, Propagation Model in PBMC ADCC assay application.

4.A. Preparing Assay Reagents and Samples

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

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

PBMC Culture Medium: On the day before the assay, prepare an appropriate amount of PBMC culture medium: 90% RPMI 1640 with 10% FBS and IL-2, β -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, β -mercaptoethanol. Mix well and warm to 37°C before use.

Note: The recommended assay buffer contains 5% HI-FBS. This concentration of FBS works well for the Control Ab, Anti-CD20 (Cat.# GA1130), that we tested. If you experience assay performance issues when using this assay buffer, we recommend testing 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: Ramos Cells (HT-HiBiT) are compatible only with Bio-Glo-NB™ TCK Reagent. **Do not** use Bio-Glo™ Reagent or Bio-Glo-NL™ Reagent with Ramos Cells (HT-HiBiT).

Test and Reference Samples: Using assay buffer as the diluent, prepare stock starting dilutions (dilu1, 3X final concentration) of two test antibodies (300 μ l each) and one reference antibody (600 μ 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 μ l of a starting dilution at 3 μ 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 Ramos Cells (HT-HiBiT), PBMC effector cells and digitonin. Spontaneous release (SR) control wells contain Ramos 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 and Plating Human PBMC, ADCC-Qualified Effector Cells



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°C and thaw in a 37°C water bath with gentle agitation (do not invert cell vial) until just thawed (typically 2–3 minutes).
2. Transfer all of the cells (approximately 1ml) to a 50ml conical tube containing 24ml of prewarmed PBMC ADCC culture medium.
3. Gently resuspend the PBMC using a 25ml pipette.
4. Transfer the cell suspension to a T75 cell culture flask and place the flask horizontally in a humidified 37°C , 5% CO_2 incubator.
5. Incubate for approximately 16–24 hours before assay setup.

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

7. Proceed immediately to Section 4.E.

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

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

Recommended Plate Layout for Antibody Dilutions Prepared from a Single 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.F. Preparing Ramos Cells (HT-HiBiT)

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



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

1. Gently mix and count the Ramos Cells (HT-HiBiT) by Trypan blue staining.
2. Harvest sufficient cells for assay into 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 PBMC ADCC assay buffer.
5. Centrifuge cells at $150 \times g$ for 5 minutes, then remove and discard supernatant.
6. Gently resuspend the cell pellet in PBMC ADCC assay buffer to achieve a concentration of 2×10^5 viable cells/ml.
7. Keep the Ramos Cells (HT-HiBiT) at ambient temperature until Section 4.H.

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

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

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



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

1. Gently resuspend and transfer the Ramos Cells (HT-HiBiT) suspension to a sterile reagent reservoir.
2. Using a multichannel pipette, immediately dispense 25 μ 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 5×10^3 cells/well.
3. Using a multichannel pipette, 25 μ l of the appropriate antibody dilution to wells B3–G10 of the assay plate according to Figure 7. Add 25 μ l of PBMC ADCC assay buffer to SR control wells (B2–G2). Add 25 μ 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 μ l of the cell suspension to each of wells B2–G11 of the assay plates. The final cell number in each well should be 1.25×10^5 cells/well.
6. Add 75 μ 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% CO₂ incubator for 5 hours.

Note: The 5-hour incubation was optimized using Control Ab, Anti-CD20 (Cat.# GA1130). We recommend optimizing assay time (3–24 hours) with your antibody or other biologic samples.

8. During assay incubation, warm Bio-Glo-NB™ TCK Luciferase Assay Buffer to ambient temperature.

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

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



Note: Ramos Cells (HT-HiBiT) are compatible only with Bio-Glo-NB™ TCK Reagent. **Do not** use Bio-Glo™ Reagent or Bio-Glo-NL™ Reagent with Ramos Cells (HT-HiBiT).

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 EC₅₀ value and maximum fold induction.

4.J. Data Analysis

The Ramos (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 Ramos 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 Ramos 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 Log₁₀[antibody], fold induction versus Log₁₀[antibody] and percent specific lysis versus Log₁₀ [antibody]. Fit curves and determine the EC₅₀ 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 Ramos Cells (HT-HiBiT) to test two samples against a reference sample in a single TDCC assay (Figure 9). 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: TRSupport@Promega.com)
- bispecific antibodies or related biologic drugs capable of inducing TDCC
- RPMI 1640 medium with L-glutamine and HEPES (e.g., Corning® Cat.# 10-041-CV or GIBCO® Cat.# 22400105)
- heat-inactivated fetal bovine serum (HI-FBS; e.g., GIBCO® Cat.# 16140)
- digitonin (e.g., Cat.# G9441)
- Bio-Glo-NB™ TCK Luciferase Assay System (Cat.# JB1001, JB1002, JB1003)
- Trypan blue solution (e.g., Sigma Cat.# T8154)

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% CO₂ incubator
- 37°C water bath
- sensitive plate reader with glow luminescence measuring capability or luminometer (e.g., GloMax® Discover System or equivalent system)

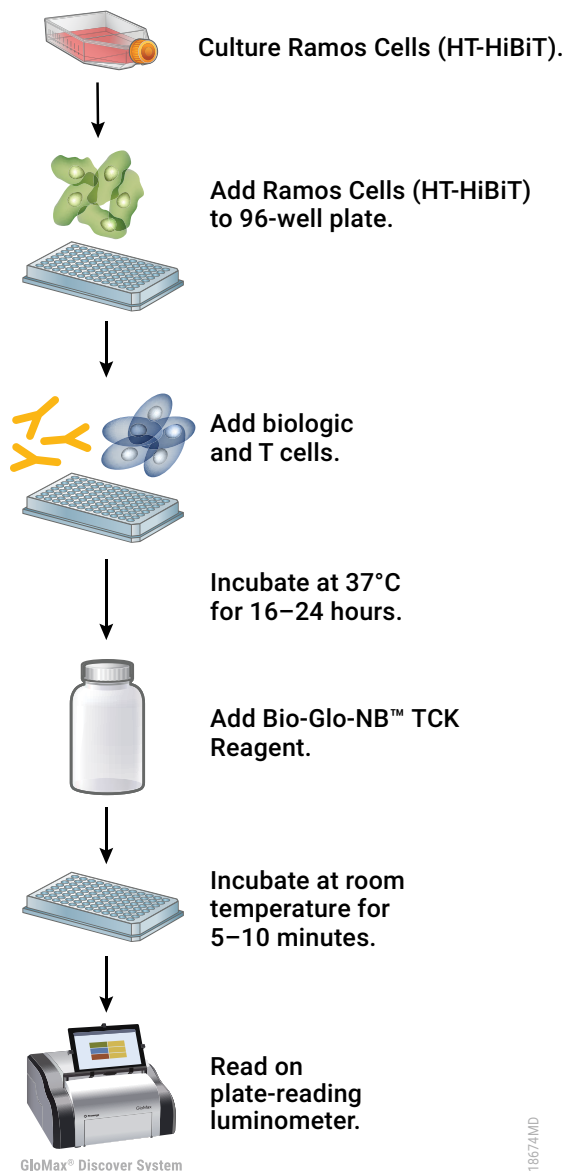


Figure 9. Schematic protocol for the TDCC Bioassay with Ramos Cells (HT-HiBiT).

5.A. Preparing Assay Reagents and Samples

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

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

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

 **Note:** Ramos Cells (HT-HiBiT) are compatible only with Bio-Glo-NB™ TCK Reagent. **Do not** use Bio-Glo™ Reagent or Bio-Glo-NL™ Reagent with Ramos Cells (HT-HiBiT).

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 Ramos 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 150µl stock of each dilution provides sufficient volume for analysis in triplicate. To prepare threefold serial dilutions, you will need 300µl 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 300µl of reference biologic starting dilution (dilu1, 3X final concentration) to wells A10 and B10 (Figure 11).
3. Add 300µl 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.

Notes:

- a. 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.
- !**
- b. 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% CO₂ incubator until Section 5.F while preparing the Ramos Cells (HT-HiBiT) and TDCC Effector cells.

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 Ramos Cells (HT-HiBiT)

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

1. Gently mix and count the Ramos Cells (HT-HiBiT) by Trypan blue staining.
2. Harvest sufficient cells for assay into 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 TDCC assay buffer.
5. Centrifuge cells at $150 \times g$ for 5 minutes.
6. Gently resuspend the cell pellet in TDCC assay buffer to a concentration of 1×10^5 viable cells/ml.
7. Incubate the Ramos Cells (HT-HiBiT) at ambient temperature until Section 5.F.

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

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

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

1. Gently resuspend and transfer the Ramos Cells (HT-HiBiT) suspension to a sterile reagent reservoir.
2. Using a multichannel pipette, immediately dispense 25 μl of the cell suspension to each of wells B2–G11 of a 96-well, white, flat-bottom assay plate. The final cell number in each well should be 2.5×10^3 cells/well.
3. Using a multichannel pipette, add 25 μl 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 μl of TDCC assay buffer to MR and SR control wells (B2–G2 and B11–G11, respectively).

Note: Digitonin will be added to MR control wells just prior to measuring luminescence (Section 5.G).

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

Note: The incubation period was optimized using blinatumomab. We recommend optimizing assay time (3–24 hours) with your biologic drug of interest.

8. Near the end of the assay incubation, equilibrate the Bio-Glo-NB™ TCK Luciferase Assay Buffer to ambient temperature.

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

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



Note: Ramos Cells (HT-HiBiT) are compatible only with Bio-Glo-NB™ TCK Reagent. **Do not** use Bio-Glo™ Reagent or Bio-Glo-NL™ Reagent with Ramos Cells (HT-HiBiT).

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 of 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. The final digitonin concentration will be 100µg/ml.
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 EC₅₀ value and maximum fold induction.

5.H. Data Analysis

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

1. Calculate fold induction:

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

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 Ramos 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 Ramos 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 Log₁₀[biologic drug], fold induction versus Log₁₀[biologic drug] and percent specific lysis versus Log₁₀[biologic drug]. Fit curves and determine the EC₅₀ 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 Ramos 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 utilizes digitonin to permeabilize target cells, providing the maximal luminescent signal. The SR control is a measure of background cell death in the absence of CAR-T cells.

Materials to Be Supplied By the User

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

Reagents

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

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% CO₂ incubator
- 37°C water bath
- sensitive plate reader with glow luminescence measuring capability or luminometer (e.g., GloMax® Discover System or equivalent system)

Note: 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.

6. CAR-T Assay Protocol (continued)

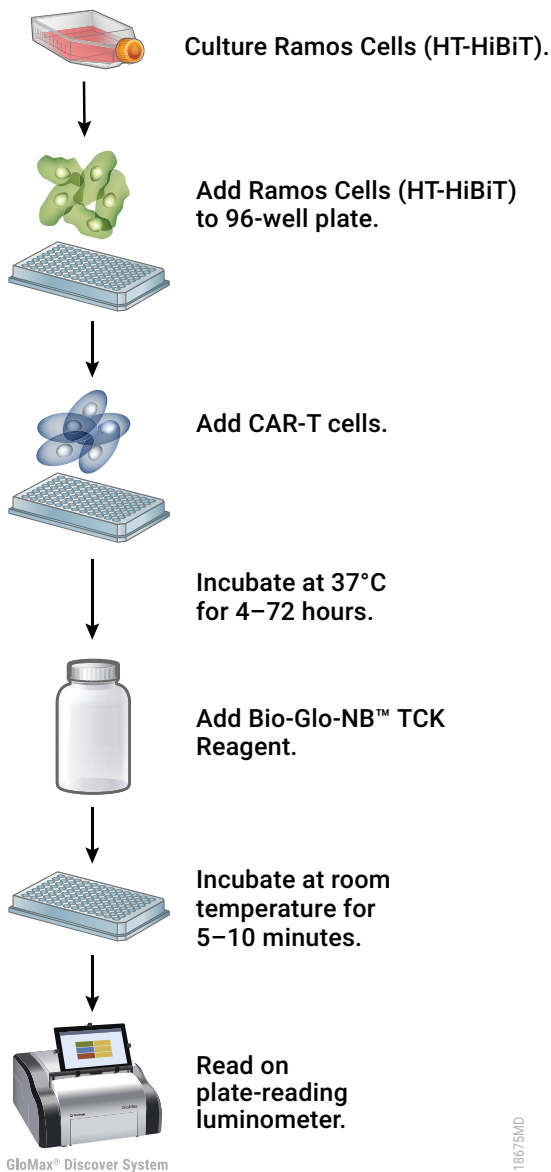


Figure 12. Schematic protocol for CAR-T assay using Ramos Cells (HT-HiBiT).

6.A. Preparing Assay Reagents

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

Note: The recommended assay buffer contains 10% HI-FBS. This concentration of FBS works well for the CAR-T 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:** Ramos Cells (HT-HiBiT) are compatible only with Bio-Glo-NB™ TCK Reagent. **Do not** use Bio-Glo™ Reagent or Bio-Glo-NL™ Reagent with the 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 Ramos 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 Ramos Cells (HT-HiBiT)

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



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

1. Gently mix and count the Ramos Cells (HT-HiBiT) by Trypan blue staining.
2. Harvest sufficient cells for assay into 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 CAR-T assay buffer.
5. Centrifuge cells at $150 \times g$ for 5 minutes.
6. Gently resuspend the cell pellet in CAR-T assay buffer (Section 9.A) to achieve a concentration of 1.25×10^5 viable cells/ml. This results in a final concentration of 5,000 Ramos Cells (HT-HiBiT) per well.
7. Incubate the Ramos Cells (HT-HiBiT) at ambient temperature until Section 6.E.

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

1. Thaw or harvest CAR-T cells according to the protocol established in your lab.
2. Pellet cells according to your protocol and resuspend in CAR-T assay buffer at a concentration of 6.25×10^6 viable cells/ml, a 2X stock solution for a maximum E:T ratio of 50:1.
3. In a clear V-bottom 96-well plate or 12-well dilution reservoir, perform seven twofold serial dilutions of CAR-T cells using CAR-T assay buffer as diluent. Prepare enough of each cell suspension for 40 μ l 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 Ramos Cells (HT-HiBiT) suspension to a sterile reagent reservoir.
2. Using a multichannel pipette, immediately dispense 40 μ 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 5×10^3 cells/well.
3. Using a multichannel pipette, add 40 μ 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 μ 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.F).

4. Add 75 μ 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% CO₂ 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:** Ramos Cells (HT-HiBiT) are compatible only with Bio-Glo-NB™ TCK Reagent. **Do not** use Bio-Glo™ Reagent or Bio-Glo-NL™ Reagent with the 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 EC₅₀ value and maximum fold induction.

6.G. Data Analysis

The Ramos (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 Ramos 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 Ramos 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 Log₁₀ (E:T ratio), fold induction versus Log₁₀ (E:T ratio) and percent specific lysis versus Log₁₀ (E:T ratio). Fit curves and determine the EC₅₀ 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. Email: techserv@promega.com

Symptoms	Causes and Comments
Low luminescence measurements (RLU readout)	Ensure that you are using Bio-Glo-NB™ TCK Reagent. Ramos Cells (HT-HiBiT) are not compatible with Bio-Glo™ or Bio-Glo-NL™ Reagents. Choose an instrument designed for plate-reading luminescence detection. Instruments designed primarily for fluorescence detection are not recommended. Luminometers measure and report luminescence as relative values, and actual RLU numbers will vary between instruments. Some models of luminometers with low sensitivity should be avoided. If using a reader with an adjustable gain, we recommend a high-gain setting. Insufficient cells per well can lead to low RLU. Handle and plate cells according to the instructions to ensure a sufficient number of viable cells per well. Low activity of Bio-Glo-NB™ TCK Reagent leads to low RLU. Store and handle Bio-Glo-NB™ TCK 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 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 EC₅₀ value obtained with the HiBiT TCK Bioassay may vary from the EC₅₀ value obtained using other methods. The assay is sensitive to the concentration of FBS in assay buffer. Optimize the FBS concentration from 0.5–10% in assay buffer if assay performance is not ideal. Always use heat-inactivated FBS. Optimize the assay incubation time within a range of 3–24 hours (or longer for the CAR-T assay). Performance of PBMC ADCC assays is highly donor-dependent. Ramos Cells (HT-HiBiT) have been optimized for use with Promega Human PBMC, ADCC-Qualified.

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.

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 Ramos 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 medium. Ramos 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 gently resuspend cell pellets.

Specific lysis greater than 100%

In assays longer than 6 hours, Ramos 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/well.

Variability in assay performance

Variations in cell growth conditions including cell plating, harvest density, cell viability and cell doubling time can cause low assay performance and high assay variation. Avoid one-day cell passages whenever possible. Use high-quality cell culture reagents (especially serum) and plasticware for maintaining cells in culture. Ensure consistent cell growth by handling the cells exactly according to the instructions.

Inappropriate cell handling during cell harvest, including long centrifuge times and high centrifuge speeds, can cause low assay performance and high assay variation. Centrifuge the cells exactly according to the instructions.

7. Troubleshooting (continued)

Symptoms	Causes and Comments
Variability in assay performance (continued)	Inappropriate cell freezing/DMSO exposure can cause low assay performance and high assay variation. Freeze the cells exactly according to the instructions.
	Inappropriate cell counting methods can lead to variation in cell numbers in culture and assays and cause high assay variation.
	Ensure consistent and accurate cell counting methods.

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

Ramos Cells (HT-HiBiT) initial cell culture medium

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

Ramos Cells (HT-HiBiT) cell growth medium

90% RPMI 1640 with L-glutamine and HEPES
10% FBS
3µg/ml blasticidin

Ramos Cells (HT-HiBiT) cell freezing medium

85% RPMI 1640 with L-glutamine and HEPES
10% FBS
5% DMSO

CAR-T assay buffer

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

PBMC ADCC assay buffer

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

PBMC culture medium

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

TDCC assay buffer

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

10. Summary of Changes

The following changes were made to the 7/26 revision of this document:

1. Removed Section 9.B, Related Products.
2. Made minor text edits.



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