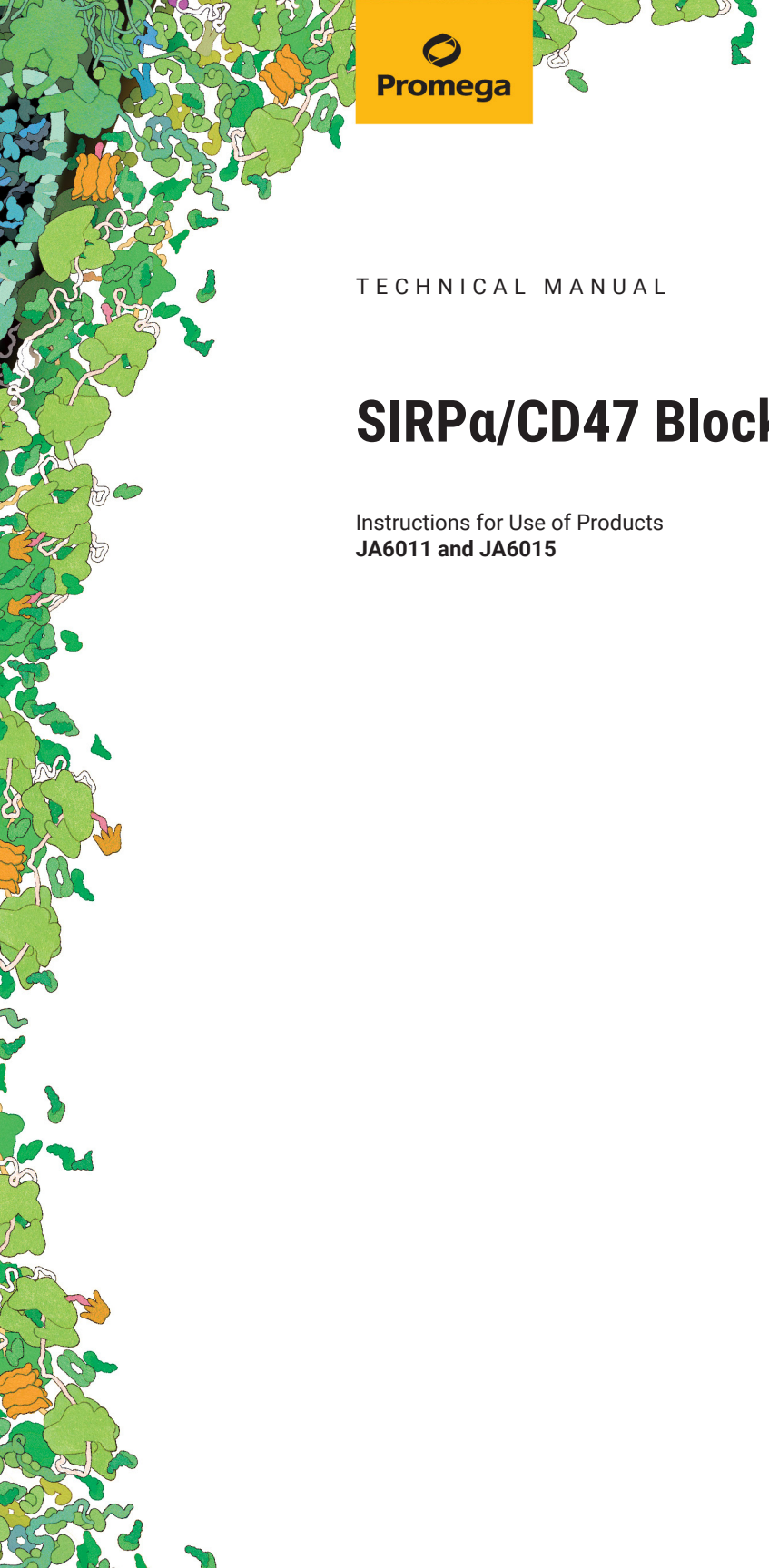

TECHNICAL MANUAL

SIRPα/CD47 Blockade Bioassay

Instructions for Use of Products
JA6011 and JA6015

Revised 9/24
TM699

Promega

SIRPα/CD47 Blockade Bioassay

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

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1. Description

Myeloid cells, including monocytes, macrophages and dendritic cells, play a central role in cancer immunosurveillance by eliminating tumor cells via phagocytosis and presenting tumor antigens to naïve T cells. These cell types express IgG receptors (FcγRs) that drive recognition and phagocytosis of antibody-opsonized cells. This process, termed antibody-dependent cellular phagocytosis (ADCP), is an important mechanism of action (MOA) for antibody-based immunotherapies. Therapeutic strategies designed to elicit or enhance ADCP have shown increased efficacy against a variety of human cancers (1–4).

Phagocytosis is regulated by a complex network of checkpoints that facilitate removal of aberrant or infected cells while preserving healthy tissues. CD47, a "marker-of-self" protein expressed on virtually all cell types, engages the myeloid-specific receptor signal regulatory protein alpha (SIRPα) to prevent phagocytosis of healthy cells (5). Despite its important physiological role, this SIRPα/CD47 checkpoint contributes to immune escape by tumor cells, many of which overexpress CD47 and thereby evade phagocytosis. Biologics that inhibit SIRPα/CD47 interaction enhance tumor cell phagocytosis in vitro and have shown promising therapeutic efficacy. Given the broad expression of CD47 in vivo, many biologics targeting the SIRPα/CD47 checkpoint are engineered to ablate or eliminate FcγR-binding (i.e., Fc-silent or Fc-null) to minimize on-target, off-tumor toxicities (6).

Current methods for assessing the activity of SIRPα/CD47 checkpoint inhibitors rely on primary monocyte-derived macrophages and direct measurement of phagocytosis. These assays are laborious and highly variable due to their reliance on donor cells, complex assay protocols and nonqualified assay reagents. As a result, these assays are difficult to establish in a quality-controlled setting.

The SIRPα/CD47 Blockade Bioassay[a–e] (Cat.# JA6011, JA6015) is a bioluminescent reporter cell-based assay that overcomes the limitations of existing assays. The bioassay is easy-to-use, quantitative and demonstrates the functional response of SIRPα/CD47 inhibitors. It can be used to measure the potency and stability of Fc-silent antibodies and other Fc-null biologics that block SIRPα/CD47 interaction. The assay consists of two genetically engineered cell lines:

- **SIRPα Effector Cells**: A monocytic cell line with a stably integrated NanoLuc® (NL) luciferase reporter driven by FcγR and SIRPα/CD47 pathway-dependent response elements.
- **CD47/FcγR-A CHO-K1 Target Cells**: CHO-K1 cells engineered to express human CD47 and an engineered cell surface protein designed to activate FcγR.

The SIRPα Effector Cells and CD47/FcγR-A CHO-K1 Target Cells are provided in 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 the two cell types are cocultured, SIRPα/CD47 interaction inhibits FcγR signaling and promoter-driven luminescence. Addition of Fc-silent anti-SIRPα or anti-CD47 antibody that blocks SIRPα/CD47 interaction releases the inhibitory signal and results in FcγR activation and promoter-driven luminescence (Figure 1). The bioluminescent signal is quantified using the Bio-Glo-NL™ Luciferase Assay System and a standard luminometer such as the GloMax® Discover System (Section 7.B, Related Products).

Note: The SIRPα/CD47 Blockade Bioassay, was designed specifically for Fc-silent or Fc-null SIRPα/CD47 checkpoint inhibitors. For Fc-functional inhibitors, we recommend using the SIRPα/CD47 Blockade Bioassay, Fc-Dependent (Cat.# JA4801, JA4805).

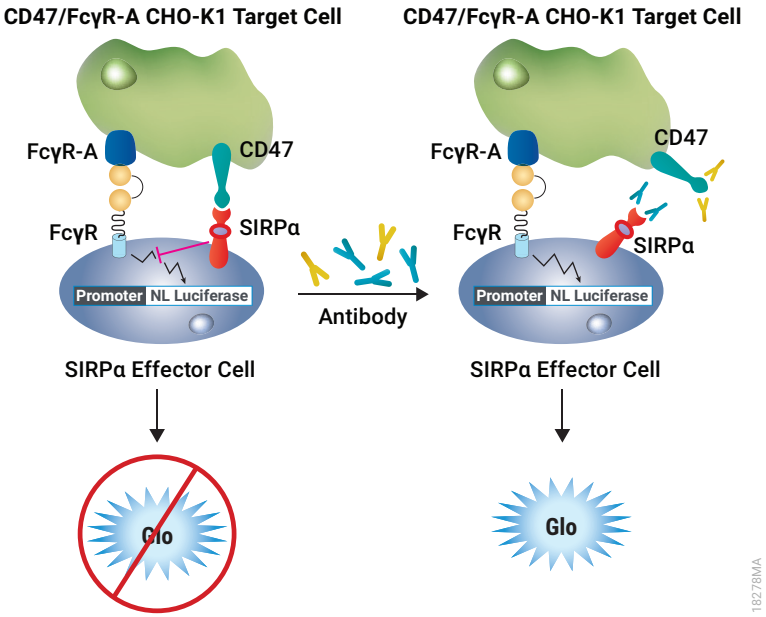

Figure 1. Representation of the SIRPα/CD47 Blockade Bioassay. The SIRPα/CD47 Blockade Bioassay consists of two cell lines, SIRPα Effector Cells and CD47/FcγR-A CHO-K1 Target Cells. When cocultured, the SIRPα/CD47 interaction inhibits FcγR-mediated luminescence. When the SIRPα/CD47 interaction is disrupted, FcγR activation triggers promoter-driven luminescence that can be detected and quantified using Bio-Glo-NL™ Reagent.

The SIRPα/CD47 Blockade Bioassay reflects the MOA of biologics designed to block the SIRPα/CD47 interaction. Specifically, FcγR-mediated luminescence is detected following anti-SIRPα blocking antibody addition, but not after anti-4-1BB, anti-ICOS or anti-PD-1 blocking antibody addition (Figure 2).

The bioassay is prequalified following International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines and shows the precision, accuracy and linearity required for routine use in potency and stability studies (Table 1 and Figure 3). The bioassay can be performed in a two-day time frame, and the workflow is simple, robust and compatible with both 96- and 384-well plate formats used for antibody screening in early drug discovery (Figure 4).

In addition to the SIRPα/CD47 Blockade Bioassay, we offer Control Ab, Anti-SIRPα (Cat.# K1251), for use as a positive control for assay optimization and routine quality control.

1. Description (continued)

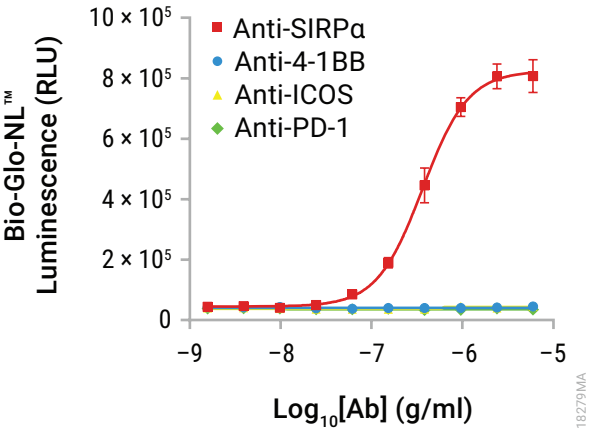

Figure 2. The SIRPα/CD47 Blockade Bioassay reflects the MOA and shows specificity for antibodies designed to block the SIRPα/CD47 interaction. SIRPα Effector Cells were incubated with CD47/FcγR-A CHO-K1 Target Cells in the presence of serial titrations of blocking antibodies as indicated. After a 4-hour induction, Bio-Glo-NL™ 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.

Table 1. SIRPα/CD47 Blockade Bioassay Shows Precision, Accuracy and Linearity.

Parameter	Results	
Accuracy	% Expected Relative Potency	% Recovery
	50	95.6
	70	93.9
	100	97.9
	130	95.8
	150	99.5
Repeatability (% CV)	100% (Reference)	5.0
Intermediate Precision (% CV)		4.7
Linearity (r^2)		0.998
Linearity (y = mx + b)		y = 1.006x – 3.570

A 50–150% theoretical potency series of Control Ab, Anti-SIRPα, was analyzed in triplicate in three independent experiments performed on three days by two analysts using the SIRPα/CD47 Blockade Bioassay. Bio-Glo-NL™ 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.

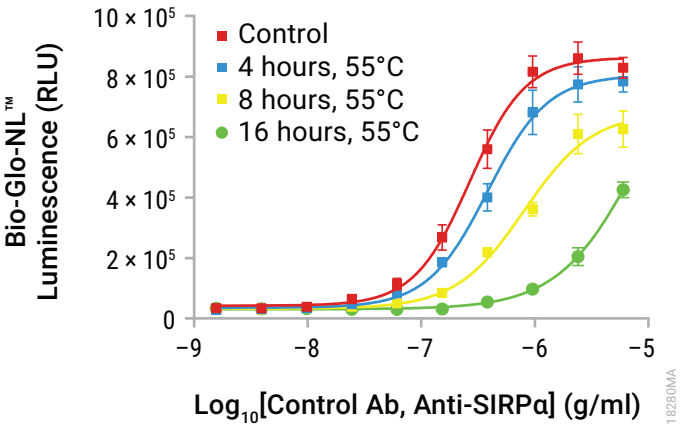

Figure 3. The SIRPα/CD47 Blockade Bioassay is stability-indicating. Samples of Control Ab, Anti- SIRPα, were maintained at 4°C (control) or heat-treated at the indicated times and temperatures, then analyzed using the SIRPα/CD47 Blockade Bioassay. Bio-Glo-NL™ Reagent was added and luminescence quantified 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)

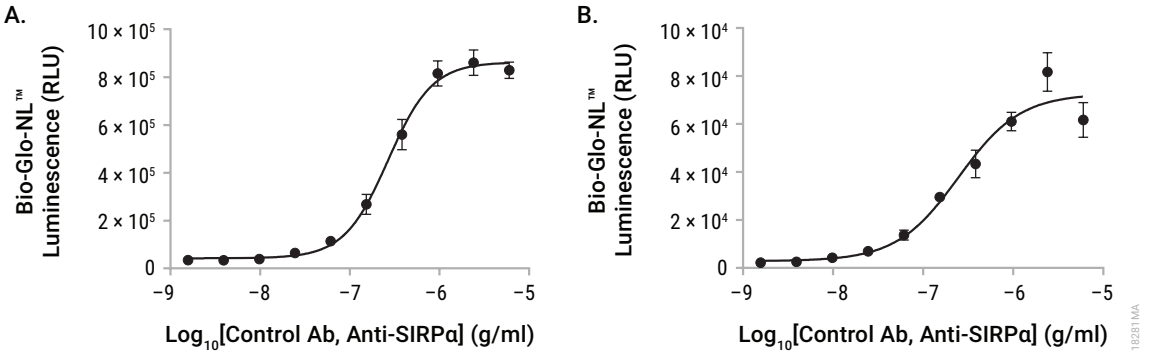

Figure 4. The SIRPα/CD47 Blockade Bioassay is amenable to 384-well plate format. Panel A. The SIRPα/CD47 Blockade Bioassay was performed in 96-well plates as described in this technical manual with a titration of Control Ab, Anti- SIRPα. **Panel B.** The SIRPα/CD47 Blockade Bioassay was performed in 384-well format as briefly described here. CD47/FcγR-A CHO-K1 Target Cells were harvested and 2.5 × 10^3 cells/15µl/well plated 16–24 hours prior to assay, in a 384-well white assay plate (e.g., Corning® Cat.# 3570). On the day of the assay, 5µl of 5X serially diluted Control Ab, Anti- SIRPα was added, followed by the addition of 2 × 10^4/5µl/well of SIRPα Effector Cells. After a 4-hour incubation at 37°C, 5% CO_2, 25µl of Bio-Glo-NL™ Reagent was added per well and luminescence quantified using the GloMax® Discover System. Data were fitted to four-parameter logistic curves using GraphPad Prism® software. The EC_{50} values were 0.27 and 0.25µg/ml for the 96- and 384-well formats, respectively, and the fold induction was 25 and 33 for 96- and 384-well formats, respectively. Data were generated using thaw-and-use cells.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
SIRPα/CD47 Blockade Bioassay	**1 each**	**JA6011**

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 SIRPα Effector Cells (0.5ml per vial)
- 1 vial CD47/FcγR-A CHO-K1 Target Cells (0.5ml per vial)
- 25ml Ham's F12 Medium
- 36ml RPMI 1640 Medium
- 4ml Fetal Bovine Serum
- 1 vial Bio-Glo-NL™ Luciferase Assay Substrate
- 10ml Bio-Glo-NL™ Luciferase Assay Buffer

PRODUCT	SIZE	CAT.#
SIRPα/CD47 Blockade Bioassay, 5X	**1 each**	**JA6015**

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 SIRPα Effector Cells (0.5ml per vial)
- 5 vials CD47/FcγR-A CHO-K1 Target Cells (0.5ml per vial)
- 5 × 25ml Ham's F12 Medium
- 5 × 36ml RPMI 1640 Medium
- 5 × 4ml Fetal Bovine Serum
- 5 vials Bio-Glo-NL™ Luciferase Assay Substrate
- 5 × 10ml Bio-Glo-NL™ Luciferase Assay Buffer

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.

Store Bio-Glo-NL™ Luciferase Assay Substrate, Bio-Glo-NL™ Luciferase Assay Buffer and Fetal Bovine Serum (FBS) at –30°C to –10°C. Avoid multiple freeze-thaw cycles of the serum. The Bio-Glo-NL™ Luciferase Assay Substrate remains liquid and does not freeze.

Store RPMI 1640 Medium and Ham's F12 Medium at +2°C to +10°C, protected from fluorescent light.

3. Before You Begin

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

Remove the product label from the box containing vials with cells or note the catalog number, lot number and dispensed lot number from the label. This information can be used to download documents for the specified product from the web site, such as the Certificate of Analysis.

Note: The SIRPα/CD47 Blockade Bioassay, uses the Bio-Glo-NL™ Luciferase Assay System (Cat.# J3081, J3082, J3083) for detection. **Do not** use the Bio-Glo™ Luciferase Assay System (Cat.# G7940, G7941).

The SIRPα/CD47 Blockade Bioassay, is intended for use with user-provided, Fc-silent antibodies or other Fc-null biologics designed to inhibit SIRPα/CD47 interaction. Control Ab, Anti-SIRPα (Cat.# K1251) is available separately for assay optimization and routine quality control. We strongly recommend including Control Ab, Anti- SIRPα, along with the required CD47/FcγR-A CHO-K1 Target Cells, as a positive control in the first few assays to gain familiarity with the assay. Data generated using Control Ab, Anti- SIRPα are shown in Section 7.A, Representative Assay Results.

The SIRPα Effector Cells and CD47/FcγR-A CHO-K1 Target Cells are provided in frozen, thaw-and-use format and are ready to be used without any additional cell culture or propagation. When thawed and diluted as instructed, the cells will be at the appropriate concentration for the assay. The cells are sensitive, and care should be taken to follow cell thawing and plating procedures as described. Do not overmix or overwarm the cell reagents.

The SIRPα/CD47 Blockade Bioassay produces a bioluminescent signal and requires a sensitive luminescence plate reader. Bioassay development and performance data included in this Technical Manual were generated using the GloMax® Discover System (see Section 7.B, Related Products). An integration time of 0.5 second/well was used for all readings.

The bioassay is compatible with most other plate-reading luminometers, though relative luminescence 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.B.)

Reagents

- user-defined, Fc-silent anti-SIRPα or anti-CD47 blocking antibodies or other Fc-null biologics samples
- solid-white, flat-bottom 96-well assay plates (e.g., Corning® Cat.# 3917) or 384-well assay plates (e.g., Corning® Cat.# 3570) for plating and reading luminescence
- sterile clear V-bottom 96-well plate with lid (e.g., Costar® Cat.# 3896) for preparing antibody dilutions
- pipettes (single-channel and 12-channel; for best results use both manual and electronic pipettes as needed)
- sterile 15ml and 50ml conical tubes
- sterile reagent reservoirs (e.g., Corning®/Costar® Cat.# 4870)
- 37°C, 5% CO_2 incubator
- 37°C water bath
- sensitive plate reader with glow luminescence measuring capability or luminometer (e.g., GloMax® Discover System or equivalent system)
- **optional:** Control Ab, Anti- SIRPα (Cat.# K1251)

4. Assay Protocol

This procedure illustrates the use of the SIRPα/CD47 Blockade Bioassay to test two antibody samples against a reference sample in a single assay run (Figure 5). Each test and reference antibody is run in triplicate, in a ten-point 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 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 6µg/ml as a starting concentration (1X) and 2.5-fold serial dilution when testing Control Ab, Anti-SIRPα (Cat.# K1251).

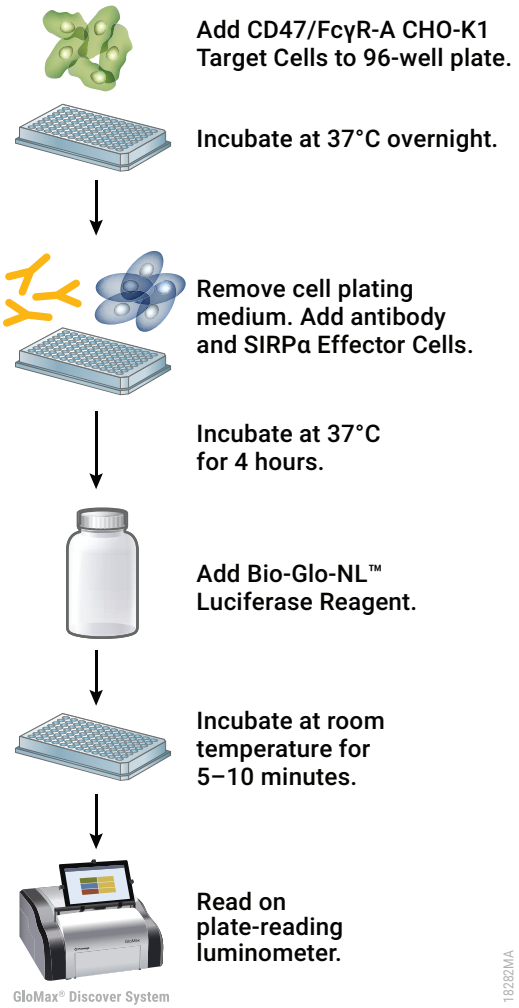

Figure 5. Schematic protocol for the SIRPα/CD47 Blockade Bioassay.

4.A. Preparing Cell Recovery Medium, Assay Buffer, Bio-Glo-NL™ Reagent and Antibody Samples

CD47/FcγR-A CHO-K1 Cell Recovery Medium: On the day before the assay, prepare 14.5ml of cell recovery medium (90% Ham's F-12/10% FBS). Thaw the FBS overnight at +2°C to +10°C or in a 37°C water bath on the day of use. Mix well and warm to 37°C before use. To prepare cell recovery medium, add 1.5ml of FBS to 13ml of Ham's F-12 Medium. For reference, 14.5ml of cell recovery medium is sufficient to thaw and plate 1 vial of CD47/FcγR-A CHO-K1 Target Cells. If multiple vials will be thawed, then scale the amount of cell recovery medium appropriately. Warm the remaining Ham's F-12 Medium to 37°C. Store the remaining FBS at +2°C to +10°C for use in preparing the assay buffer on the day of the assay.

Assay Buffer: On the day of the assay, prepare 20ml of assay buffer (98% RPMI 1640/2% FBS). Add 0.4ml of FBS to 19.6ml RPMI 1640 Medium. Mix well and warm to 37°C before use. Warm the remaining RPMI 1640 Medium to 37°C.

Note: The recommended assay buffer contains 2% FBS. This concentration of FBS works well for the Control Ab, Anti-SIRPα, that we tested. If you experience assay performance issues when using this assay buffer, we recommend testing different serum concentrations in the range of 0.5–10%.

Bio-Glo-NL™ Luciferase Reagent: For reference, 10ml of Bio-Glo-NL™ Reagent is sufficient to assay 120 wells in a 96-well assay format. The Bio-Glo-NL™ Luciferase Assay Substrate should always be stored at –30°C to –10°C. Thaw the Bio-Glo-NL™ Luciferase Assay Buffer at room temperature (do not exceed 25°C) during the 4-hour assay induction period. We recommend preparing the reconstituted Bio-Glo-NL™ Luciferase Assay Reagent immediately before use. For instructions on use of the Bio-Glo-NL™ Luciferase Assay System, please refer to the *Bio-Glo-NL™ Luciferase Assay System Quick Protocol*, #FB227.

Note: The SIRPα/CD47 Blockade Bioassay is compatible only with Bio-Glo-NL™ Luciferase Assay Reagent. **Do not** use Bio-Glo™ Luciferase Assay Reagent with the SIRPα/CD47 Blockade Bioassay.

Test and Reference Samples: Using assay buffer as the diluent, prepare stock starting dilutions (dilu1, 1.5X final concentration) of two test antibodies (300µl each) and one reference antibody (600µl) in 1.5ml tubes. Store the tubes containing antibody starting dilutions appropriately before making antibody serial dilutions.

Note: If you are using Control Ab, Anti-SIRPα (Cat.# K1251) as a reference antibody in your assay, prepare a 600µl starting dilution of 9µg/ml Control Ab, Anti-SIRPα (dilu1, 1.5X final concentration) by adding 10.8µl of Control Ab, Anti-SIRPα working stock to 589.2µl of assay buffer.

4.B. Plate Layout Design

For the protocol described here, use the plate layout illustrated in Figure 6 as a guide. The protocol describes serial replicate dilutions (n = 3) of test and reference antibody to generate two ten-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	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference Ab
C	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test Ab
D	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference Ab
E	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test Ab
F	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference Ab
G	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test Ab
H	B	B	B	B	B	B	B	B	B	B	B	B	Assay Buffer (B)

Figure 6. Example plate layout showing nonclustered sample locations of test antibody and reference antibody dilution series and wells containing assay buffer only (denoted by "B").

4.C. Plating CD47/FcγR-A CHO-K1 Target Cells

Thaw-and-use CD47/FcγR-A CHO-K1 Target Cells are sensitive and care should be taken to follow the cell thawing and plating procedures **exactly** as described. Do not overmix or overwarm the cell reagents. No additional cell culture or manipulation is required or recommended. We recommend that you thaw and dilute a maximum of two vials of thaw-and-use cells at any one time.

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

Perform the following steps in a sterile cell culture hood.

1. On the day before performing the assay, prepare 14.5ml of cell recovery medium (90% Ham's F-12/10% FBS) in a sterile 50ml conical tube, as described in Section 4.A.

2. Remove one vial of CD47/FcγR-A CHO-K1 Target Cells from storage at –140°C and transfer to the bench on dry ice. Thaw the cells in a 37°C water bath until just thawed (about 2 minutes). While thawing, gently agitate and visually inspect the vial. Do not invert.
3. Gently mix the cell suspension by pipetting, then transfer 0.5ml cells to the tube containing 14.5ml of cell recovery medium. Mix well by gently inverting the tube 1–2 times.
4. Transfer the suspension to a sterile reagent reservoir. Using a multichannel pipette, immediately dispense 100µl of the cell suspension to each of the inner 60 wells of two 96-well white flat-bottom assay plates.
5. Add 100µl of prewarmed (37°C) Ham's F12 Medium to each of the outside wells of the assay plates.
6. Place lids on the assay plates and incubate in a 37°C, 5% CO_2 incubator overnight (18–22 hours).

4.D. Preparing Antibody Serial Dilutions

The instructions described here are for preparation of a single stock of 2.5-fold serial dilutions of a single antibody for analysis in triplicate (180µl of each dilution provides a sufficient volume for analysis in triplicate). Alternatively, you can prepare three independent stocks of serial dilutions to generate triplicate samples. To prepare 2.5-fold serial dilutions, you will need 600µl of reference antibody at 1.5X the highest antibody concentration in your dose-response curve. You will need 300µl of each test antibody at 1.5X the highest antibody concentration in each of the test antibody dose-response curves. For other dilution schemes, adjust the volumes accordingly.

Note: If you are using Control Ab, Anti- SIRPα (Cat.# K1251) as a control in the assay, follow the instructions below to prepare 2.5-fold serial dilutions. A 2.5-fold serial dilution for test antibodies is listed as an example below as well.

1. On the day of the assay, prepare an appropriate amount of assay buffer as described in Section 4.A.
2. To a sterile clear V-bottom 96-well plate, add 300µl of reference antibody starting dilution (dilu1, 1.5X final concentration) to wells A11 and B11 (Figure 7).
3. Add 300µl of test antibodies 1 and 2 starting dilution (dilu1, 1.5X final concentration) to wells E11 and G11, respectively (Figure 7).
4. Add 180µl of assay buffer to other wells in these four rows, from column 10 to column 2.
5. Transfer 120µl of the antibody starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
6. Repeat equivalent 2.5-fold 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 180µl of assay buffer without antibody as a negative control.
7. Cover the antibody dilution plate with a lid and keep at ambient temperature (22–25°C) while preparing the SIRPα Effector Cells.

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	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Reference Ab
B		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Reference Ab
C													
D													
E		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Test Ab 1
F													
G		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Test Ab 2
H													

Figure 7. Example plate layout showing antibody serial dilutions.

4.E. Preparing SIRPα Effector Cells

Thaw-and-use SIRPα Effector Cells are sensitive and care should be taken to follow the cell thawing and plating procedures exactly as described. Do not overmix or overwarm the cell reagents. No additional cell culture or manipulation is required or recommended. We recommend that you thaw and dilute a maximum of two vials of thaw-and-use cells at any one time.

1. Label a sterile 15ml conical tube "Effector Cells". Add 4.5ml of prewarmed (37°C) assay buffer to the 15ml conical tube.
2. Remove one vial of SIRPα Effector Cells from storage at –140°C and transfer to the bench on dry ice. Thaw the cells in a 37°C water bath until just thawed (about 2 minutes). While thawing, gently agitate and visually inspect the vial.
3. Gently mix the cell suspension by pipetting, then transfer 0.5ml cells to the 15ml conical tube containing 4.5ml of assay buffer. Mix well by gently inverting the tube.

4.F. Adding SIRPα Effector Cells and Antibody to Assay Plates

1. Take the 96-well assay plates containing CD47/FcγR-A CHO-K1 Target Cells out of the incubator. Invert each assay plate above a sink to remove the medium. Then, place the inverted plate on paper towels for 5–10 seconds to drain any remaining medium. Alternatively, remove 95µl of medium from each of the wells using a manual multichannel pipette.

2. Using a multichannel pipette, add 50µl of the appropriate antibody dilution (Figure 7) to the assay plates according to the plate layout in Figure 6.
3. Mix the SIRPα Effector Cells by inverting the tube, then transfer the suspension to a sterile reagent reservoir. Using a multichannel pipette, immediately dispense 25µl of the cell suspension to each of the inner 60 wells of the assay plates. Gently swirl the assay plates to ensure mixing of the Effector Cells and antibody.
4. Add 75µl of assay buffer to each of the outside wells of the assay plates.
5. Cover the assay plates with lids and incubate in a 37°C, 5% CO_2 incubator for 4 hours.

   Note: The 4-hour incubation was optimized using the Control Ab, Anti-SIRPα. We recommend optimizing assay time (3–24 hours) with your antibody or other biologic samples.

4.G. Preparing and Adding Bio-Glo-NL™ Reagent

We recommend preparing the Bio-Glo-NL™ Luciferase Assay Reagent immediately before use. Ensure that the Bio-Glo-NL™ 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 10% activity in approximately 8 hours at room temperature.

Note: The SIRPα/CD47 Blockade Bioassay is compatible only with Bio-Glo-NL™ Luciferase Assay Reagent. **Do not** use Bio-Glo™ Luciferase Assay Reagent with the SIRPα/CD47 Blockade Bioassay.

1. Remove the Bio-Glo-NL™ 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. Prepare the desired amount of reconstituted Bio-Glo-NL™ Luciferase Assay Reagent by combining one volume of substrate with 50 volumes of buffer. For example, if the experiment requires 10ml of reagent, add 200µl of substrate to 10ml of buffer. Ten milliliters (10ml) of Bio-Glo-NL™ Reagent is sufficient for 120 wells (two assay plates, using the inner 60 wells of each plate).
3. Remove assay plates from the incubator after the incubation period and equilibrate to room temperature for 10–15 minutes.
4. Using a manual multichannel pipette, add 75µl of Bio-Glo-NL™ Reagent to the inner 60 wells of the assay plates, taking care not to create bubbles.
5. Add 75µl of Bio-Glo-NL™ Luciferase Assay Reagent to wells B1, D1, and F1 of each assay plate to measure background signal.
6. Incubate at room temperature for 5–10 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-NL™ incubation time will affect the raw relative light unit (RLU) values but should not significantly change the EC_{50} value and maximum fold induction.

4.H. Data Analysis

1. Determine the plate background by calculating the average RLU from wells B1, D1 and F1.
2. Calculate fold induction:

$$\text{Fold Induction} = \frac{\text{RLU (induced – background)}}{\text{RLU (no antibody control – background)}}$$

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

5. 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)	Choose an instrument designed for plate-based 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 low-sensitivity luminometer models 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-NL™ Reagent leads to low RLU. Store and handle Bio-Glo-NL™ Reagent according to the instructions. For best results, prepare immediately before use.

Symptoms	Causes and Comments
Weak assay response (low fold induction)	Optimize the concentration range of your test sample(s) to achieve a full dose response with complete upper and lower asymptotes. The EC_{50} value obtained with the SIRPα/CD47 Blockade Bioassay may vary from the EC_{50} value obtained using other methods such as primary macrophage-based assays.
	The SIRPα/CD47 Blockade Bioassay was designed specifically for Fc-silent or Fc-null inhibitors. Inhibitors containing a functional Fc domain may interfere with assay performance. For Fc-functional inhibitors, we recommend using the SIRPα/CD47 Blockade Bioassay, Fc-Dependent (Cat.# JA4801).
	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.
	Optimize the assay incubation time within a range of 3–24 hours.
	If untreated control RLU is less than 100-fold above plate reader background RLU, subtract plate background RLU from all samples before calculating fold induction.
Variability in assay performance	Ensure that you are using Bio-Glo-NL™ Reagent, which is designed for NanoLuc® Luciferase reporter bioassays. The SIRPα/CD47 Blockade Bioassay is not compatible with Bio-Glo™ Reagent, which is designed for firefly luciferase reporter bioassays.

6. References

1. Overdijk, M.B. *et al.* (2015) Antibody-mediated phagocytosis contributes to the anti-tumor activity of the therapeutic antibody daratumumab in lymphoma and multiple myeloma. *MAbs* **7**, 311–21.
2. Shi, Y. *et al.* (2015) Trastuzumab triggers phagocytic killing of high HER2 cancer cells in vitro and in vivo by interaction with Fcγ receptors on macrophages. *J. Immunol.* **194**, 4379–86.
3. VanDerMeid, K.R. *et. al.* (2018) Cellular cytotoxicity of next-generation CD20 monoclonal antibodies. *Cancer Immunol. Res.* **6,** 1150–60.
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5. Oldenborg, P.A. *et. al.* (2000) Role of CD47 as a marker of self on red blood cells. *Science* **288**, 2051–4.
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7. Appendix

7.A. Representative Assay Results

The following data were generated using the SIRPα/CD47 Blockade Bioassay, with Control Ab, Anti-SIRPα (Figure 8).

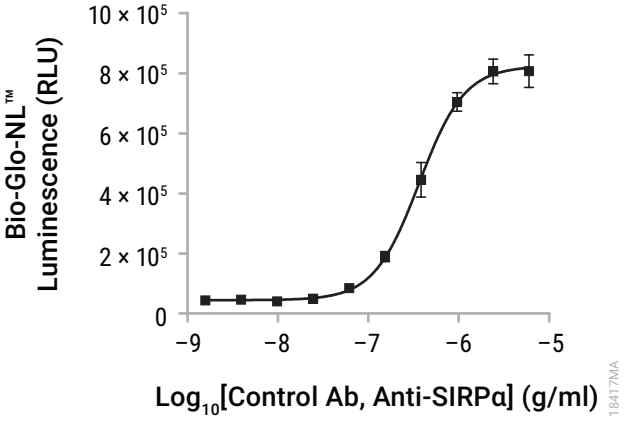

Figure 8. The SIRPα/CD47 Blockade Bioassay measures the activity of Control Ab, Anti-SIRPα. CD47/FcγR-A CHO-K1 Target Cells were added to a 96-well assay plate 20 hours prior to the assay. On the day of assay, SIRPα Effector Cells and a titration of Control Ab, Anti-SIRPα (Cat.# K1251), were added. After a 4-hour induction at 37°C, Bio-Glo-NL™ Reagent was added and luminescence measured using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. The EC_{50} was 0.37µg/ml and the fold induction was 19.

7.B. Related Products

Fc Effector Bioassays

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

*For Research Use Only. Not for use in diagnostic procedures.
**Not for Medical Diagnostic Use.
Additional kit formats are available.

Fc Effector Immunoassay

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

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

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7.B. Related Products (continued)

Immune Checkpoint Bioassays

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

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

T Cell Activation Bioassays

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

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

Cytokine and Growth Factor Bioassays

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

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

Macrophage-Directed Bioassays

Product	Size	Cat.#
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 and sizes 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

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7.B. Related Products (continued)

Detection Reagents

Product	Size	Cat.#
Bio-Glo™ Luciferase Assay System	10ml	G7941
Bio-Glo-NL™ Luciferase Assay System	10ml	J308

Not for Medical Diagnostic Use. Additional sizes are available.

Detection Instruments

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

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

Note: Additional Fc Effector, Immune Checkpoint, T Cell Activation, Cytokine, Macrophage, Primary Cell and Target Cell Killing Bioassays are available. To view and order Promega Bioassay products visit: **www.promega.com/products/reporter-bioassays/** or email: **EarlyAccess@promega.com**.

For information on custom bioassay development and services visit the Promega Tailored R&D Solutions website: **www.promega.com/custom-solutions/tailored-solutions/**

8. Summary of Changes

The following changes were made to the 9/24 revision of this document:

1. Text defining ICH was corrected in the Description.
2. Miscellaneous text edits were made.
3. Related Products, Section 7.B, was updated.
4. One patent statement and third party trademarks were updated.

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

[c]U.S. Pat. Nos. 8,557,970, 8,669,103, 9,777,311, 9,840,730, 9,951,373, 10,233,485, 10,633,690, 10,774,364, 10,844,422, 11,365,436, 11,661,623, 11,667,950; European Pat. Nos. 2456864, 2635595, 2990478, 3181687, 3409764; Japanese Pat. Nos. 6038649, 6155424, 6227615, 6374420, 6539689; and other patents and patents pending.

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