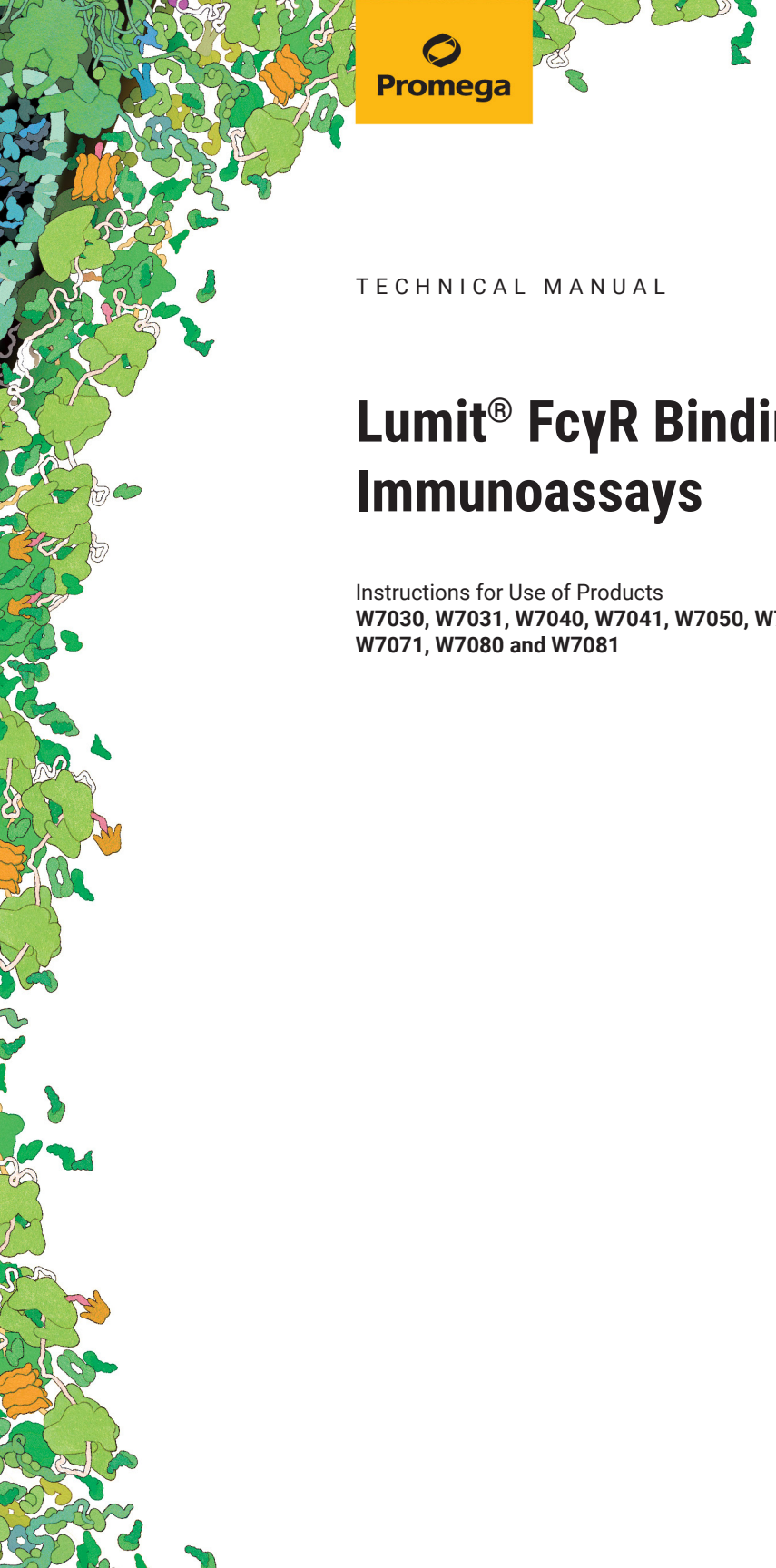

TECHNICAL MANUAL

Lumit® FcγR Binding Immunoassays

Instructions for Use of Products
W7030, W7031, W7040, W7041, W7050, W7051, W7060, W7061, W7070, W7071, W7080 and W7081

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TM750

Lumit® FcγR Binding Immunoassays

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

Fc gamma receptors (FcγRs) are a group of receptors that bind to the Fc region of immunoglobulin G (IgG) antibodies and are pivotal for mediating immune responses. There are many distinct FcγRs, including FcγRI, FcγRIIa, FcγRIIb, FcγRIIIa and their respective variants, each with specific expression patterns across immune cells such as macrophages, neutrophils and natural killer (NK) cells. The binding of IgG to these receptors can trigger a range of effector functions, including antibody-dependent cellular cytotoxicity (ADCC), phagocytosis and modulation of immune responses. In the context of antibody drug development, assessing the binding affinity for these FcγRs is crucial, as this interaction influences the therapeutic antibody's mechanism of action, safety profile and clinical efficacy. Evaluating the binding affinity to FcγRs provides a better understanding of the antibody's potential immune effector function, aiding in the optimization of therapeutic antibodies for desired clinical outcomes.

The Lumit® FcγR Binding Immunoassays are novel homogeneous (no-wash) competition assays, based on NanoBiT® technology (1), used to measure the interaction between human FcγRs and Fc proteins, including antibodies (Figure 1). NanoLuc® Binary Technology (NanoBiT) is a structural complementation reporter designed for biomolecular interaction studies. The NanoBiT® system consists of two subunits: the 18kDa Large BiT (LgBiT) and the 11 amino acid peptide Small BiT (SmBiT), which have been optimized for stability and minimal self-association.

In the Lumit® FcγR Binding Immunoassays, a human IgG labeled with LgBiT (Tracer-LgBiT) is used as the tracer, while a human FcγR bound to SmBiT (FcγR-SmBiT) serves as the target (2). In the absence of an antibody analyte, Tracer-LgBiT binds to the FcγR-SmBiT target, resulting in maximum luminescent signal. When an antibody analyte is present, nonlabeled IgG competes with Tracer-LgBiT for binding to the FcγR target, leading to a concentration-dependent decrease in the luminescent signal. This decrease in luminescence provides a quantitative measure of the binding affinity between the Fc region of the antibody and the Fcγ receptor.

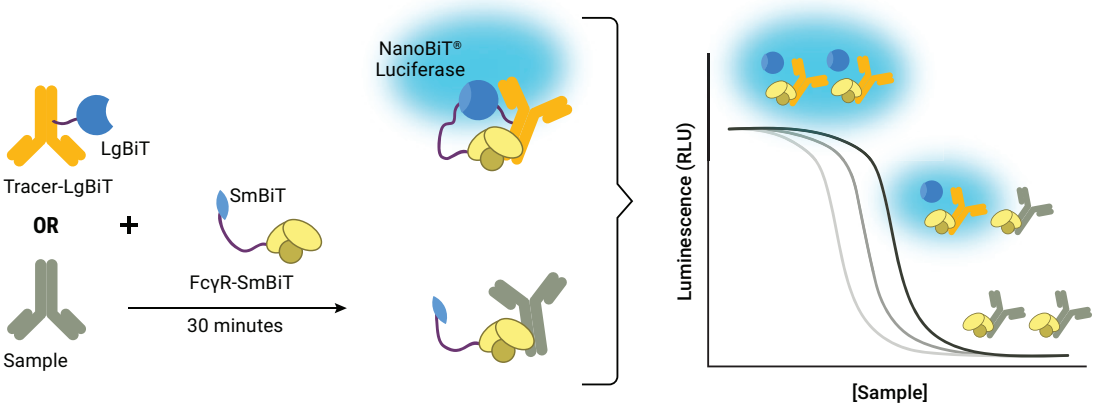

Figure 1. Schematic of a Lumit® FcγR Binding Immunoassay.

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2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Lumit® FcγRI Binding Immunoassay	**1 each**	**W7080**

Not for Medical Diagnostic Use. Each kit contains sufficient reagents for 100 assays using a 96-well plate. Includes:

- 25µl FcγRI-SmBiT
- 120µl FcγR Control Antibody
- 10µl Tracer-LgBiT
- 75µl Lumit® Detection Substrate A
- 25ml FcγR Assay Buffer

PRODUCT	SIZE	CAT.#
Lumit® FcγRI Binding Immunoassay 10X	**1 each**	**W7081**

Not for Medical Diagnostic Use. Each kit contains sufficient reagents for 1,000 assays using a 96-well plate. Includes:

- 10 × 25µl FcγRI-SmBiT
- 10 × 120µl FcγR Control Antibody
- 10 × 10µl Tracer-LgBiT
- 10 × 75µl Lumit® Detection Substrate A
- 10 × 25ml FcγR Assay Buffer

PRODUCT	SIZE	CAT.#
Lumit® FcγRIIa (H131) Binding Immunoassay	**1 each**	**W7070**

Not for Medical Diagnostic Use. Each kit contains sufficient reagents for 100 assays using a 96-well plate. Includes:

- 20µl FcγRIIa (H131)-SmBiT
- 120µl FcγR Control Antibody
- 60µl Tracer-LgBiT
- 75µl Lumit® Detection Substrate A
- 25ml FcγR Assay Buffer

PRODUCT	SIZE	CAT.#
Lumit® FcγRIIa (H131) Binding Immunoassay 10X	**1 each**	**W7071**

Not for Medical Diagnostic Use. Each kit contains sufficient reagents for 1,000 assays using a 96-well plate. Includes:

- 10 × 20µl FcγRIIa (H131)-SmBiT
- 10 × 120µl FcγR Control Antibody
- 10 × 60µl Tracer-LgBiT
- 10 × 75µl Lumit® Detection Substrate A
- 10 × 25ml FcγR Assay Buffer

PRODUCT	SIZE	CAT.#
Lumit® FcγRIIa (R131) Binding Immunoassay	**1 each**	**W7060**

Not for Medical Diagnostic Use. Each kit contains sufficient reagents for 100 assays using a 96-well plate. Includes:

- 20µl FcγRIIa (R131)-SmBiT
- 120µl FcγR Control Antibody
- 60µl Tracer-LgBiT
- 75µl Lumit® Detection Substrate A
- 25ml FcγR Assay Buffer

PRODUCT	SIZE	CAT.#
Lumit® FcγRIIa (R131) Binding Immunoassay 10X	**1 each**	**W7061**

Not for Medical Diagnostic Use. Each kit contains sufficient reagents for 1,000 assays using a 96-well plate. Includes:

- 10 × 20µl FcγRIIa (R131)-SmBiT
- 10 × 120µl FcγR Control Antibody
- 10 × 60µl Tracer-LgBiT
- 10 × 75µl Lumit® Detection Substrate A
- 10 × 25ml FcγR Assay Buffer

PRODUCT	SIZE	CAT.#
Lumit® FcγRIIIa (V158) Binding Immunoassay	**1 each**	**W7050**

Not for Medical Diagnostic Use. Each kit contains sufficient reagents for 100 assays using a 96-well plate. Includes:

- 20µl FcγRIIIa (V158)-SmBiT
- 120µl FcγR Control Antibody
- 60µl Tracer-LgBiT
- 75µl Lumit® Detection Substrate A
- 25ml FcγR Assay Buffer

PRODUCT	SIZE	CAT.#
Lumit® FcγRIIIa (V158) Binding Immunoassay 10X	**1 each**	**W7051**

Not for Medical Diagnostic Use. Each kit contains sufficient reagents for 1,000 assays using a 96-well plate. Includes:

- 10 × 20µl FcγRIIIa (V158)-SmBiT
- 10 × 120µl FcγR Control Antibody
- 10 × 60µl Tracer-LgBiT
- 10 × 75µl Lumit® Detection Substrate A
- 10 × 25ml FcγR Assay Buffer

2 . Product Components and Storage Conditions (continued)

PRODUCT	SIZE	CAT.#
Lumit® FcγRIIIa (F158) Binding Immunoassay	**1 each**	**W7040**

Not for Medical Diagnostic Use. Each kit contains sufficient reagents for 100 assays using a 96-well plate. Includes:

- 30µl FcγRIIIa (F158)-SmBiT
- 120µl FcγR Control Antibody
- 120µl Tracer-LgBiT
- 75µl Lumit® Detection Substrate A
- 25ml FcγR Assay Buffer

PRODUCT	SIZE	CAT.#
Lumit® FcγRIIIa (F158) Binding Immunoassay 10X	**1 each**	**W7041**

Not for Medical Diagnostic Use. Each kit contains sufficient reagents for 1,000 assays using a 96-well plate. Includes:

- 10 × 30µl FcγRIIIa (F158)-SmBiT
- 10 × 120µl FcγR Control Antibody
- 10 × 120µl Tracer-LgBiT
- 10 × 75µl Lumit® Detection Substrate A
- 10 × 25ml FcγR Assay Buffer

PRODUCT	SIZE	CAT.#
Lumit® FcγRIIb Binding Immunoassay	**1 each**	**W7030**

Not for Medical Diagnostic Use. Each kit contains sufficient reagents for 100 assays using a 96-well plate. Includes:

- 40µl FcγRIIb-SmBiT
- 120µl FcγR Control Antibody
- 60µl Tracer-LgBiT
- 75µl Lumit® Detection Substrate A
- 25ml FcγR Assay Buffer

PRODUCT	SIZE	CAT.#
Lumit® FcγRIIb Binding Immunoassay 10X	**1 each**	**W7031**

Not for Medical Diagnostic Use. Each kit contains sufficient reagents for 1,000 assays using a 96-well plate. Includes:

- 10 × 40µl FcγRIIb-SmBiT
- 10 × 120µl FcγR Control Antibody
- 10 × 60µl Tracer-LgBiT
- 10 × 75µl Lumit® Detection Substrate A
- 10 × 25ml FcγR Assay Buffer

Storage Conditions: Store kits at –30°C to –10°C. Tracer-LgBiT and FcγR-SmBiT components do not freeze at –30°C to –10°C. Before using a kit, thaw reagents and centrifuge tubes briefly to collect the reagents at the tube bottom.

3. Before You Begin

This technical manual contains protocols for six related Lumit® FcγR Binding Immunoassays. Please identify the relevant section for your assay and refer to that protocol. Before conducting experiments, please read through the entire assay protocol to become familiar with the components and the assay procedure.

The Lumit® FcγR Binding Immunoassays are intended for use with user-provided antibodies or Fc fusion proteins. A polyclonal antibody is provided in the kit to use as a positive control. If a true standard is required, the NISTmAb reference material is available from the National Institute of Standards and Technology (NIST Cat.# RM8671). Affinity between Human IgG and FcγR can be relatively weak. To get a full sigmoidal dose-response curve, you may need to adjust the dilution series suggested in Tables 1 and 2.

The Lumit® FcγR Binding Immunoassays produce a bioluminescent signal and require a sensitive luminescence plate reader for signal detection. Performance data included in this technical manual were generated using the GloMax® Discover System (Section 13, Related Products). An integration time of 0.5 seconds/well was used for all readings. The assay is compatible with most other plate-reading luminometers; however, relative luminescence unit (RLU) readings may vary due to the sensitivity and settings of each instrument. The use of different instruments should not affect the measured relative potency of test samples.

Each kit has sufficient reagents to run a full 96-well plate assay. However, the assay can be miniaturized to a 384-well plate, while maintaining the same ratio of reagents.

3.A. Materials to Be Supplied By the User

- white multiwell assay plates, polypropylene or nonbinding (e.g., Corning® Cat.# 3605)
- multichannel pipette or automated pipetting station
- luminometer capable of reading multiwell plates (e.g., GloMax® Discover System, Cat.# GM3000)
- reagent reservoirs (e.g., Thermo Fisher Scientific Cat.# 8093-11)
- 15ml conical tubes
- plate shaker (e.g., IKA MTS 2/4 digital microtiter shaker, ID.# 0003208001)
- plate sealer or plate lid (e.g., Microtiter® Plate Sealer, Thermo Fisher Scientific Cat.# 3501)
- **optional:** NISTmAb reference material (NIST Cat.# RM8671)

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3.B. Preparing the FcγR Control Antibody

A twelve-point dilution series is recommended for each plate. Use Table 1 or Table 2 as a guide to prepare Control Antibody dilutions. There will be enough antibody for two replicates per dilution.

Table 1. Control Antibody Dilutions for FcγRI, FcγRIIa (H131), FcγRIIa (R131), FcγRIIIa (V158) and FcγRIIIa (F158) Binding Immunoassays.

Tube/Well #	FcγR Assay Buffer (µl)	Volume and Tube # of Control Antibody (µl)	Dilution Series Concentration (µg/ml)	In-Well Concentration (µg/ml)
1	60	40 of FcγR Control Antibody	4,000	1,000
2	75	25 of #1	1,000	250
3	75	25 of #2	250	62.5
4	75	25 of #3	62.5	15.6
5	75	25 of #4	15.6	3.9
6	75	25 of #5	3.9	1.0
7	75	25 of #6	1.0	0.25
8	75	25 of #7	0.24	0.06
9	75	25 of #8	0.061	0.015
10	75	25 of #9	0.015	0.004
11	75	25 of #10	0.004	0.001
12	75	0 of #11	0	0

Table 2. Preparing Control Antibody Dilutions for FcγRIIb Binding Immunoassay.

Tube/Well#	FcγR Assay Buffer (µl)	Volume and Tube # of Control Antibody (µl)	Dilution Series Concentration (µg/ml)	In-Well Concentration (µg/ml)
1	0	100 of FcγR Control Antibody	10,000	2,500
2	75	25 of #1	2,500	625
3	75	25 of #2	625	156.3
4	75	25 of #3	156.3	39.1
5	75	25 of #4	39.1	9.8
6	75	25 of #5	9.8	2.4
7	75	25 of #6	2.4	0.61
8	75	25 of #7	0.61	0.15
9	75	25 of #8	0.15	0.038
10	75	25 of #9	0.038	0.0095
11	75	25 of #10	0.0095	0.0024
12	75	0 of #11	0	0

3.C. Protocol Schematic

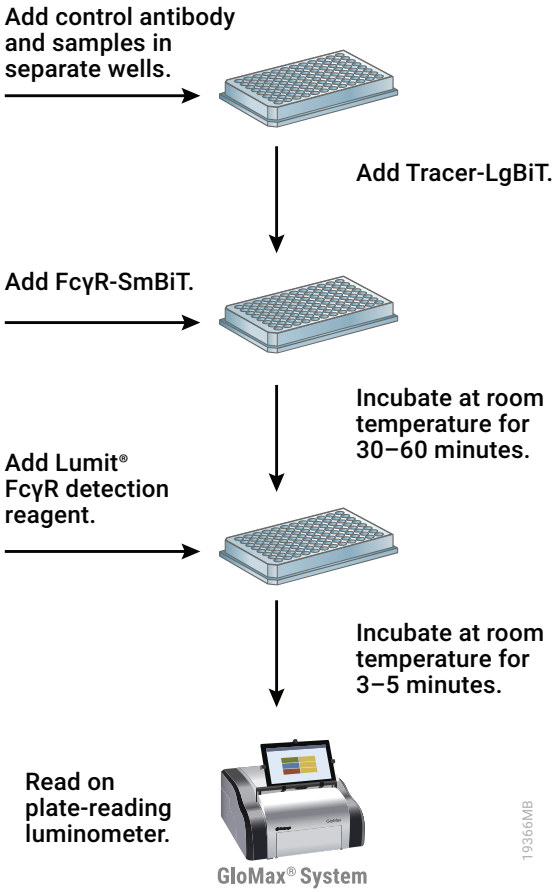

Figure 2. Lumit® FcγR Binding Immunoassay workflow

4. Lumit® FcγRI Binding Immunoassay

4.A. Preparing Control

Prepare a control antibody dilution series according to Table 1 in Section 3.B.

4.B. Preparing Samples

When preparing samples, choose an appropriate starting concentration and dilution scheme to achieve a full dose-response curve with the proper upper and lower asymptotes and sufficient points on the slope. For reference, we find that a sample range of 0–1.0mg/ml with a serial fourfold dilution in FcγR Assay Buffer achieves a full dose-response curve as a twelve-point series. Concentration ranges and dilution schemes may need to be optimized for your samples.

4.C. Preparing Reagents

Preparing Tracer-LgBiT

1. Add 3ml of FcγR Assay Buffer to a conical tube. Add 2.3µl of Tracer-LgBiT.
2. Mix by inverting the tube.
3. Transfer to reagent reservoir.

Preparing FcγRI-SmBiT

4. Add 6ml of FcγR Assay Buffer to a conical tube. Add 18µl of FcγRI-SmBiT.
5. Mix by inverting the tube.
6. Transfer to reagent reservoir.

4.D. Protocol

1. Add 25µl of FcγR Control Antibody or sample to wells of a white 96-well plate.
2. Add 25µl of Tracer-LgBiT solution to each well.
3. Add 50µl of FcγRI-SmBiT solution to each well.
4. Cover plate with a lid or plate seal and mix on a plate shaker (300–400rpm) for 30–60 minutes at room temperature.
5. Add 60µl of Lumit® Detection Substrate A to 3ml of FcγR Assay Buffer in a conical tube to generate Lumit® FcγR detection reagent.
6. Mix by inverting the tube. Transfer to reagent reservoir.
7. Add 25µl of Lumit® FcγR detection reagent to each well.
8. Incubate the plate at room temperature for 3–5 minutes.
9. Read the plate on a luminometer.

4.E. Representative Data

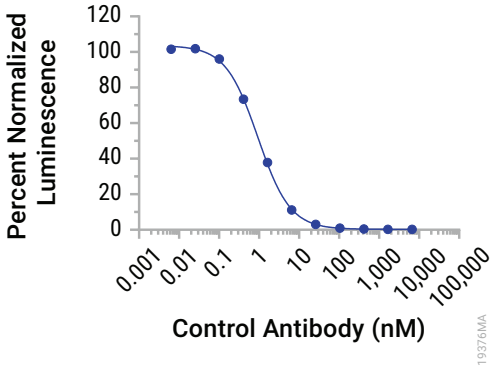

Figure 3. Lumit® FcγRI Binding Immunoassay. A representative standard curve of FcγR Control Antibody is plotted as normalized data. Normalized luminescence is calculated by setting the maximum bioluminescent signal (observed in the absence of an analyte) as 100%, and expressing the signal in the presence of an analyte as a percentage of this maximum. Data were fitted to a four-parameter logistic regression equation with $1/y^2$ weighting to calculate IC_{50}.

5. Lumit® FcγRIIa (H131) Binding Immunoassay

5.A. Preparing Control

Prepare a control antibody dilution series according to Table 1 in Section 3.B.

5.B. Preparing Samples

When preparing samples, choose an appropriate starting concentration and dilution scheme to achieve a full dose-response curve with the proper upper and lower asymptotes and sufficient points on the slope. For reference, we find that a sample range of 0–1.0mg/ml with a serial fourfold dilution in FcγR Assay Buffer achieves a full dose-response curve as a twelve-point series. Concentration ranges and dilution schemes may need to be optimized for your samples.

5.C. Preparing Reagents

Preparing Tracer-LgBiT

1. Add 3ml of FcγR Assay Buffer to a conical tube. Add 45µl of Tracer-LgBiT.
2. Mix by inverting the tube.
3. Transfer to reagent reservoir.

Preparing FcγRIIa (H131)-SmBiT

4. Add 6ml of FcγR Assay Buffer to a conical tube. Add 11µl of FcγRIIa (H131)-SmBiT.
5. Mix by inverting the tube.
6. Transfer to reagent reservoir.

5.D. Protocol

1. Add 25µl of FcγR Control Antibody or sample to wells of a white 96-well plate.
2. Add 25µl of Tracer-LgBiT solution to each well.
3. Add 50µl of FcγRIIa (H131)-SmBiT solution to each well.
4. Cover plate with a lid or plate seal and mix on a plate shaker (300–400rpm) for 30–60 minutes at room temperature.
5. Add 60µl of Lumit® Detection Substrate A to 3ml of FcγR Assay Buffer in a conical tube to generate Lumit® FcγR detection reagent.
6. Mix by inverting the tube. Transfer to a reagent reservoir.
7. Add 25µl of Lumit® FcγR detection reagent to each well.
8. Incubate the plate at room temperature for 3–5 minutes.
9. Read on a luminescence plate reader.

5.E. Representative Data

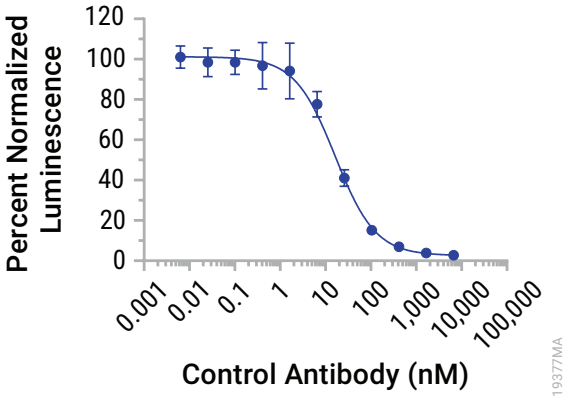

Figure 4. Lumit® FcγRIIa (H131) Binding Immunoassay. A representative standard curve of FcγR Control Antibody is plotted as normalized data. Normalized luminescence is calculated by setting the maximum bioluminescent signal (observed in the absence of an analyte) as 100%, and expressing the signal in the presence of an analyte as a percentage of this maximum. Data were fitted to a four-parameter logistic regression equation with $1/y^2$ weighting to calculate IC_{50}.

6. Lumit® FcγRIIa (R131) Binding Immunoassay

6.A. Preparing Control

Prepare a control antibody dilution series according to Table 1 in Section 3.B.

6.B. Preparing Samples

When preparing samples, choose an appropriate starting concentration and dilution scheme to achieve a full dose-response curve with the proper upper and lower asymptotes and sufficient points on the slope. For reference, we find that a sample range of 0–1.0mg/ml with a serial fourfold dilution in FcγR Assay Buffer achieves a full dose-response curve as a twelve-point series. Concentration ranges and dilution schemes may need to be optimized for your samples.

6.C. Preparing Reagents

Preparing Tracer-LgBiT

1. Add 3ml of FcγR Assay Buffer to a conical tube. Add 45µl of Tracer-LgBiT.
2. Mix by inverting the tube.
3. Transfer to reagent reservoir.

Preparing FcγRIIa (R131)-SmBiT

4. Add 6ml of FcγR Assay Buffer to a conical tube. Add 11µl of FcγRIIa (R131)-SmBiT.
5. Mix by inverting the tube.
6. Transfer to reagent reservoir.

6.D. Protocol

1. Add 25µl of FcγR Control Antibody or sample to wells of a white 96-well plate.
2. Add 25µl of Tracer-LgBiT solution to each well.
3. Add 50µl of FcγRIIa (R131)-SmBiT solution to each well.
4. Cover plate with a lid or plate seal and mix on a plate shaker (300–400rpm) for 30–60 minutes at room temperature.
5. Add 60µl of Lumit® Detection Substrate A to 3ml of FcγR Assay Buffer in a conical tube to generate Lumit® FcγR detection reagent.
6. Mix by inverting the tube. Transfer to a reagent reservoir.
7. Add 25µl of Lumit® FcγR detection reagent to each well.
8. Incubate the plate at room temperature for 3–5 minutes.
9. Read on a luminescence plate reader.

6.E. Representative Data

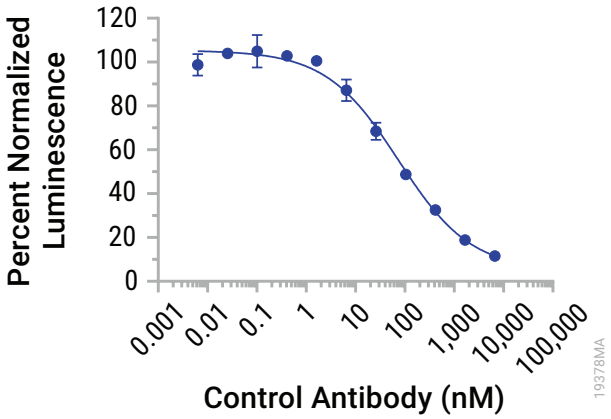

Figure 5. Lumit® FcγRIIa (R131) Binding Immunoassay. A representative standard curve of FcγR Control Antibody is plotted as normalized data. Normalized luminescence is calculated by setting the maximum bioluminescent signal (observed in the absence of an analyte) as 100%, and expressing the signal in the presence of an analyte as a percentage of this maximum. Data were fitted to a four-parameter logistic regression equation with $1/y^2$ weighting to calculate IC_{50}.

7. Lumit® FcγRIIIa (V158) Binding Immunoassay

7.A. Preparing Control

Prepare a control antibody dilution series according to Table 1 in Section 3.B.

7.B. Preparing Samples

When preparing samples, choose an appropriate starting concentration and dilution scheme to achieve a full dose-response curve with the proper upper and lower asymptotes and sufficient points on the slope. For reference, we find that a sample range of 0–1.0mg/ml with a serial fourfold dilution in FcγR Assay Buffer achieves a full dose-response curve as a twelve-point series. Concentration ranges and dilution schemes may need to be optimized for your samples.

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7.C. Preparing Reagents

Preparing Tracer-LgBiT

1. Add 3ml of FcγR Assay Buffer to a conical tube. Add 45µl of Tracer-LgBiT.
2. Mix by inverting the tube.
3. Transfer to a reagent reservoir.

Preparing FcγRIIIa (V158)-SmBiT

4. Add 6ml of FcγR Assay Buffer to a conical tube. Add 11µl of FcγRIIIa (V158)-SmBiT.
5. Mix by inverting the tube.
6. Transfer to a reagent reservoir.

7.D. Protocol

1. Add 25µl of FcγR Control Antibody or sample to wells of a white 96-well plate.
2. Add 25µl of Tracer-LgBiT solution to each well.
3. Add 50µl of FcγRIIIa (V158)-SmBiT solution to each well.
4. Cover plate with a lid or plate seal and mix on a plate shaker (300–400rpm) for 30–60 minutes at room temperature.
5. Add 60µl of Lumit® Detection Substrate A to 3 ml of FcγR Assay Buffer in a conical tube to generate Lumit® FcγR detection reagent.
6. Mix by inverting the tube. Transfer to reagent reservoir.
7. Add 25µl of Lumit® FcγR detection reagent to each well.
8. Incubate the plate at room temperature for 3–5 minutes.
9. Read on a luminescence plate reader.

7.E. Representative Data

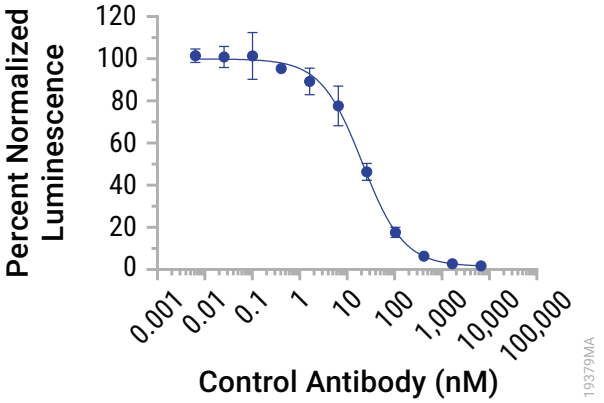

Figure 6. Lumit® FcγRIIIa (V158) Binding Immunoassay. A representative standard curve of FcγR Control Antibody is plotted as normalized data. Normalized luminescence is calculated by setting the maximum bioluminescent signal (observed in the absence of an analyte) as 100%, and expressing the signal in the presence of an analyte as a percentage of this maximum. Data were fitted to a four-parameter logistic regression equation with $1/y^2$ weighting to calculate IC_{50}.

8. Lumit® FcγRIIIa (F158) Binding Immunoassay

8.A. Preparing Control

Prepare a control antibody dilution series according to Table 1 in Section 3.B.

8.B. Preparing Samples

When preparing samples, choose an appropriate starting concentration and dilution scheme to achieve a full dose-response curve with the proper upper and lower asymptotes and sufficient points on the slope. For reference, we find that a sample range of 0–1.0mg/ml with a serial fourfold dilution in FcγR Assay Buffer achieves a full dose-response curve as a twelve-point series. Concentration ranges and dilution schemes may need to be optimized for your samples.

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8.C. Preparing Reagents

Preparing Tracer-LgBiT

1. Add 3ml of FcγR Assay Buffer to a conical tube. Add 90µl of Tracer-LgBiT.
2. Mix by inverting the tube.
3. Transfer to reagent reservoir.

Preparing FcγRIIIa (F158)-SmBiT

4. Add 6ml of FcγR Assay Buffer to a conical tube. Add 21.7µl of FcγRIIIa (F158)-SmBiT.
5. Mix by inverting the tube.
6. Transfer to reagent reservoir.

8.D. Protocol

1. Add 25µl of FcγR Control Antibody or sample to wells of a white 96-well plate.
2. Add 25µl of Tracer-LgBiT solution to each well.
3. Add 50µl of FcγRIIIa (F158)-SmBiT solution to each well.
4. Cover plate with a lid or plate seal and mix on a plate shaker (300–400rpm) for 30–60 minutes at room temperature.
5. Add 60µl of Lumit® Detection Substrate A to 3ml of FcγR Assay Buffer in a conical tube to generate Lumit® FcγR detection reagent.
6. Mix by inverting the tube. Transfer to reagent reservoir.
7. Add 25µl of Lumit® FcγR detection reagent to each well.
8. Incubate the plate at room temperature for 3–5 minutes.
9. Read on a luminescence plate reader.

8.E. Representative Data

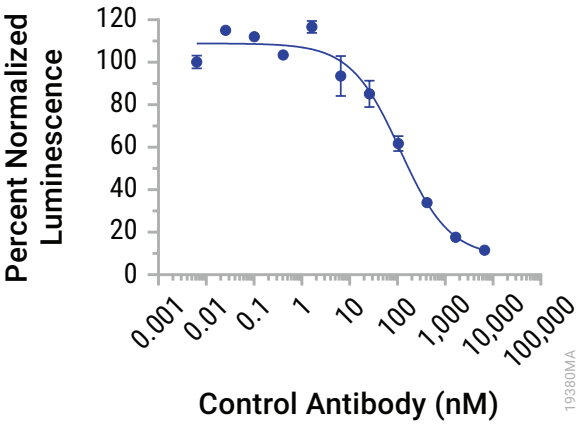

Figure 7. Lumit® FcγRIIIa (F158) Binding Immunoassay. A representative standard curve of FcγR Control Antibody is plotted as normalized data. Normalized luminescence is calculated by setting the maximum bioluminescent signal (observed in the absence of an analyte) as 100%, and expressing the signal in the presence of an analyte as a percentage of this maximum. Data were fitted to a four-parameter logistic regression equation with $1/y^2$ weighting to calculate IC_{50}.

9. Lumit® FcγRIIb Binding Immunoassay

9.A. Preparing Control

Prepare a control antibody dilution series according to Table 2 in Section 3.B.

9.B. Preparing Samples

When preparing samples, choose an appropriate starting concentration and dilution scheme to achieve a full dose-response curve. Due to the weak binding of FcγRIIb with IgG it is difficult to achieve lower asymptotes. Therefore, it is important to have sufficient points on the slope to compare relative IC_{50} values. For reference, we find that a sample range of 0–2.5mg/ml in the well with a serial twofold dilution in FcγR Assay Buffer can provide satisfactory response. Concentration ranges and dilution schemes may need to be optimized for your samples.

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9.C. Preparing Reagents

Preparing Tracer-LgBiT

1. Add 3ml of FcγR Assay Buffer to a conical tube. Add 45µl of Tracer-LgBiT.
2. Mix by inverting the tube.
3. Transfer to reagent reservoir.

Preparing FcγRIIb-SmBiT

4. Add 6ml of FcγR Assay Buffer to a conical tube. Add 29µl of FcγRIIb-SmBiT.
5. Mix by inverting the tube.
6. Transfer to a reagent reservoir.

9.D. Protocol

1. Add 25µl of FcγR Control Antibody or sample to wells of a white 96-well plate.
2. Add 25µl of Tracer-LgBiT solution to each well.
3. Add 50µl of FcγRIIb-SmBiT solution to each well.
4. Cover plate with a lid or plate seal and mix on a plate shaker (300–400rpm) for 30–60 minutes at room temperature.
5. Add 60µl of Lumit® Detection Substrate A to 3ml of FcγR Assay Buffer in a conical tube to generate Lumit® FcγR detection reagent.
6. Mix by inverting the tube. Transfer to reagent reservoir.
7. Add 25µl of Lumit® FcγR detection reagent to each well.
8. Incubate the plate at room temperature for 3–5 minutes.
9. Read on a luminescence plate reader.

9.E. Representative Data

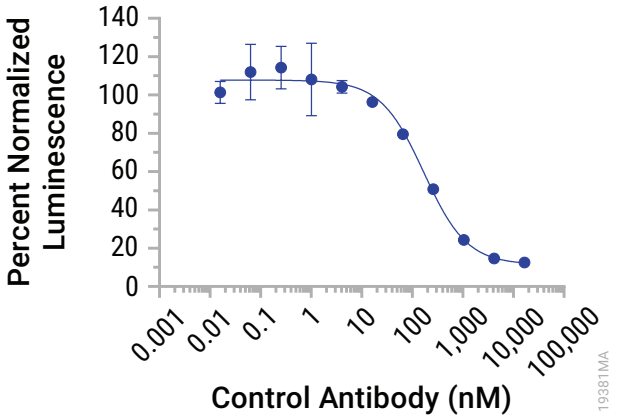

Figure 8. Lumit® FcγRIIb Binding Immunoassay. A representative standard curve of FcγR Control Antibody is plotted as normalized data. Normalized luminescence is calculated by setting the maximum bioluminescent signal (observed in the absence of an analyte) as 100%, and expressing the signal in the presence of an analyte as a percentage of this maximum. Data were fitted to a four-parameter logistic regression equation with $1/y^2$ weighting. Because of the difficulty of achieving a lower asymptote due to weak IgG binding, accurate determination of IC_{50} values may be challenging. However, relative binding affinities can still be determined.

10. Reagent Preparation Quick Reference

Table 3. Amount of Tracer-LgBiT and FcγR-SmBiT Reagents Added to Respective Lumit® FcγR Binding Immunoassays.

Receptor	Volume of Tracer-LgBiT added to 3ml of FcγR Assay Buffer	Volume of FcγR-SmBiT added to 6ml of FcγR Assay Buffer
FcγRI	2.3µl	18µl
FcγRIIa (H131)	45µl	11µl
FcγRIIa (R131)	45µl	11µl
FcγRIIIa (V158)	45µl	11µl
FcγRIIIa (F158)	90µl	21.7µl
FcγRIIb	45µl	29µl

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11. Troubleshooting

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

Symptoms	Causes and Comments
High RLU for entire plate	The Lumit® FcγR Binding Immunoassays are competition assays. The order of reagent addition is very important. Follow the protocol for reagent addition order: sample, Tracer-LgBiT, then FcγR-SmBiT.
Steep drop in signal with high asymptote, indicative of nonspecific binding.	Some FcγR Binding Immnoassays are sensitive to aggregates present in antibody samples. To minimize effects of nonspecific binding due to antibody aggregation, centrifuge antibody samples prior to using in the assay.
Low RLU or compressed assay window.	Check assay plates and ensure use of nonbinding surface or polypropylene plates.
Incomplete competition or lack of lower asymptote.	Check order of addition and ensure FcγR-SmBiT is added last.
	Increase concentration of competitor if binding affinity may be weak.
Inconsistent data points	Pipetting errors.

12. References

1. Dixon, A.S. *et al.* (2016) NanoLuc complementation reporter optimized for accurate measurement of protein interactions in cells. *ACS Chem. Biol.* **11(2)**, 400–8.
2. Nath, N. *et al.* (2022) A homogeneous bioluminescent immunoassay for parallel characterization of binding between a panel of antibodies and a family of Fcγ receptors. *Sci. Rep.* **12**, 12185.

13. Related Products

Lumit® Immunoassays

Product	Size	Cat.#
Lumit® FcRn Binding Immunoassay*	100 assays	W1151
Lumit® Immunoassay Labeling Kit	1 each	VB2500
Lumit® Immunoassay Cellular System – Starter Kit	200 assays	W1220
Lumit® Immunoassay Cellular System – Set 1*	100 assays	W1201
Lumit® Immunoassay Cellular System – Set 2*	100 assays	W1331
Lumit® Immunoassay Lysis and Detection Kit*	100 assays	W1231

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

Lumit® Immunoassay Detection Reagents

Product	Size	Cat.#
Lumit® Immunoassay Detection Reagent A	500 assays	VB2010
Lumit® Immunoassay Detection Reagent B	100 assays	VB4050

Not for Medical Diagnostic Use.
Additional sizes are available.

Lumit® Immunoassay Reagents

Product	Size	Cat.#
Lumit® Anti-Mouse Ab-LgBiT	30µl	W1021
Lumit® Anti-Rabbit Ab-SmBiT	30µl	W1031
Lumit® Anti-Rabbit Ab-LgBiT	30µl	W1041
Lumit® Anti-Mouse Ab-SmBiT	30µl	W1051
Lumit® Anti-Goat Ab-LgBiT	30µl	W1061
Lumit® Anti-Goat Ab-SmBiT	30µl	W1071

13. Related Products (continued)

Fc Effector Bioassays

Product	Size	Cat.#
ADCC Reporter Bioassay, Complete Kit (Raji)*	1 each	G7015
ADCC Reporter Bioassay, Target Kit (Raji)*	1 each	G7016
ADCC Reporter Bioassay, Core Kit*	1 each	G7010
ADCC Reporter Bioassay, F Variant, Core Kit**	1 each	G9790
FcγRIIa-H ADCP Reporter Bioassay, Complete Kit**	1 each	G9901
FcγRIIa-H ADCP Reporter Bioassay, Core Kit**	1 each	G9991

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

Luminometers

Product	Size	Cat.#
GloMax® Discover System	1 each	GM3000

For Research Use Only. Not For Use in Diagnostic Procedures.