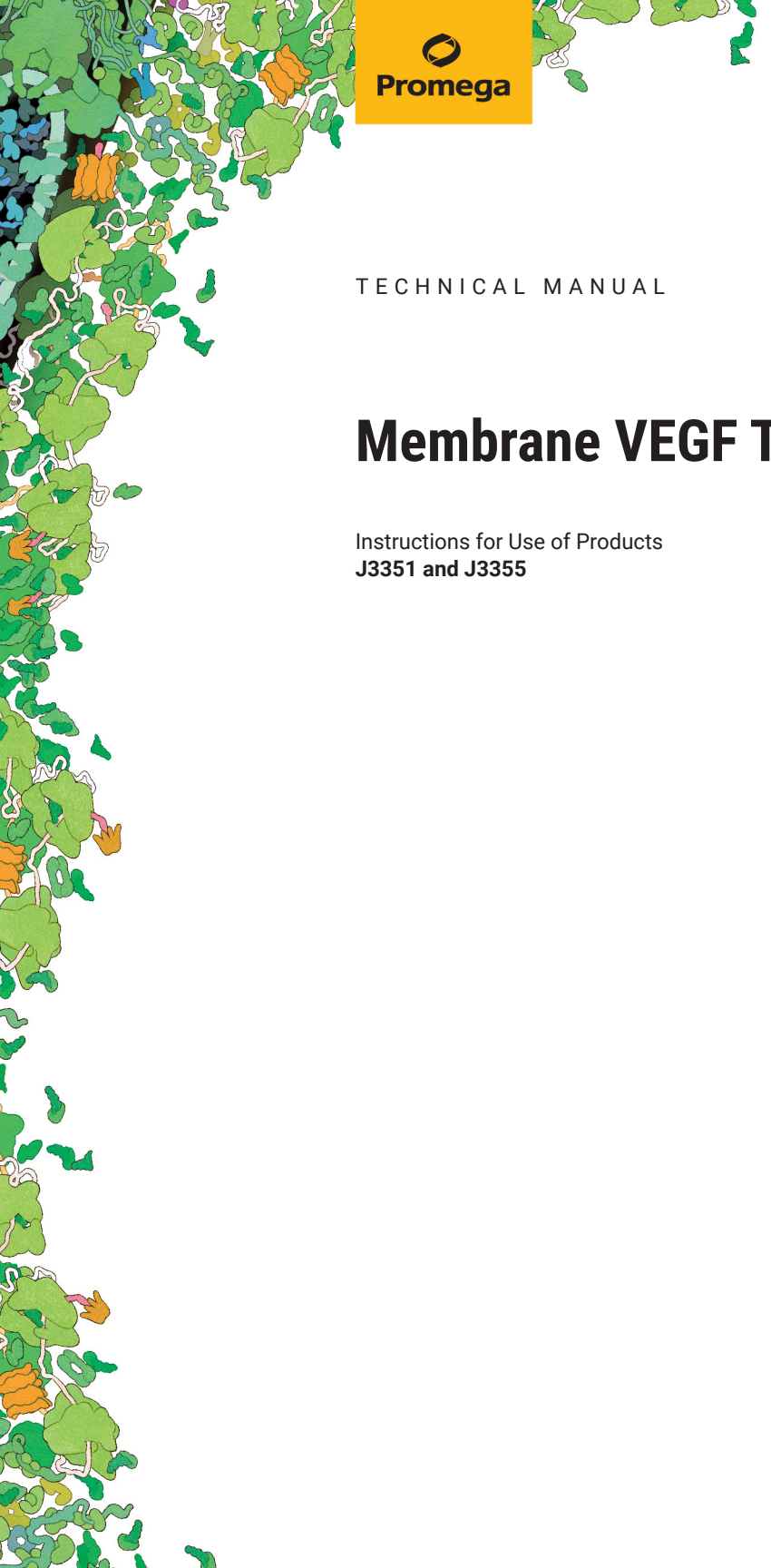

TECHNICAL MANUAL

Membrane VEGF Target Cells

Instructions for Use of Products
J3351 and J3355

Revised 1/25
TM678

Membrane VEGF Target Cells

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

1. Description

Vascular endothelial growth factor (VEGF) is an important signaling protein that is secreted from epithelial cells, tumor cells and macrophages. It has many functions, including stimulating angiogenesis, increasing vascular permeability, enhancing tumor invasion and survival and inhibiting antitumor response in regulatory T cells (Treg). There are several VEGF receptor subtypes: VEGFR1 (Flt-1), VEGFR2 (Flk-1/KDR) and VEGFR3. KDR mediates almost all known cellular responses to VEGF (1). VEGF occurs in four isoforms, including VEGF-121, VEGF-165, VEGF-189 and VEGF-206, of which VEGF-121 and VEGF-165 are diffusible forms. VEGF-165 is the predominant isoform in the body (2).

Expression of VEGF in tumors promotes angiogenesis and supports tumor growth, invasion and metastasis (2). VEGF overexpression can cause vascular disease in the retina of the eye as well as other parts of the body. Studies have shown that inhibiting VEGF signaling can effectively inhibit angiogenesis and treat various cancers and eye diseases (3,4). There are currently several therapeutics available that control these diseases by blocking VEGF signaling, such as the anti-VEGF antibodies bevacizumab and ranibizumab and the anti-KDR antibody ramucirumab (5).

Because some VEGF isoforms are membrane-bound (mVEGF), the Fc effector function of VEGF-targeted antibodies needs to be characterized, particularly its potential to induce antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). However, determining the ability of novel and biosimilar VEGF antibodies to induce cellular cytotoxicity is hampered by the lack of model cell lines naturally expressing mVEGF.

Membrane VEGF Target Cells[a,b] (Cat.# J3351, J3355) are genetically-engineered cells stably expressing a cleavage-resistant form of mVEGF that enforces its surface expression. mVEGF Target Cells are provided in thaw-and-use format, as cryopreserved cells that are ready to be thawed for use as target cells in assays that measure their effector functions in ADCC and CDC for anti-VEGF blockers. In addition, they can be used to measure antibody binding affinity to mVEGF.

The Membrane VEGF Target Cells, Propagation Model, are also available in cell propagation model (CPM) format (Cat.# J3342), which includes cryopreserved cells that can be thawed, propagated and banked for long-term use.

The mVEGF Target Cells express VEGF on the cell surface, as demonstrated by flow cytometry (Figure 1). The assay signal is specific to anti-VEGF antibodies in both ADCC and CDC assays. When used in the ADCC Reporter Bioassay (Cat.# G7010), luminescence increases following the addition of anti-VEGF antibodies but not following addition of anti-TNFα or anti-CD20 antibodies (Figure 2). In a CDC assay, mVEGF Target Cell death is detected following the addition of anti-VEGF antibodies but not following addition of anti-TNFα or anti-CD20 antibodies (Figure 3). The ADCC Reporter Bioassay using mVEGF Target Cells 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 4). The bioassay can be performed in a two-day timeframe, 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 5).

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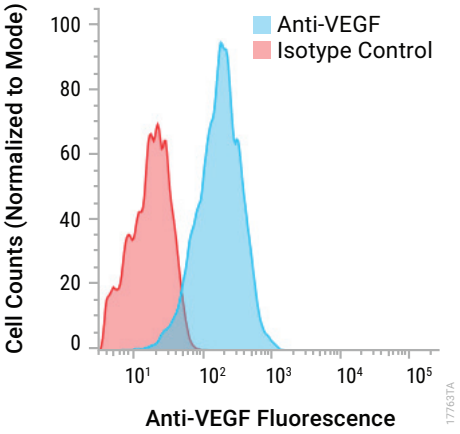

Figure 1. Surface expression of VEGF on mVEGF Target Cells. mVEGF Target Cells were labeled with isotype control or anti-VEGF (bevacizumab) followed by Alexa Fluor® 488-conjugated goat anti-human IgG (H+L). Cells were analyzed on a BD LSRFortessa™ X-20 flow cytometer. Data analysis was performed with FlowJo™ software.

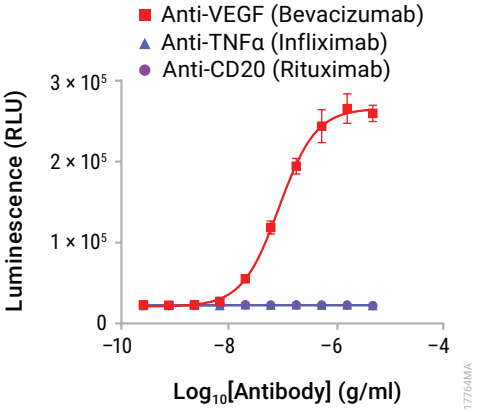

Figure 2. The ADCC Reporter Bioassay performed with mVEGF Target Cells reflects the mechanism of action (MOA) and shows specificity for antibodies designed to bind VEGF. ADCC Effector Cells were cocultured with mVEGF Target Cells in the presence of serial titrations of antibodies, as indicated. After a 6-hour induction, Bio-Glo™ 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)

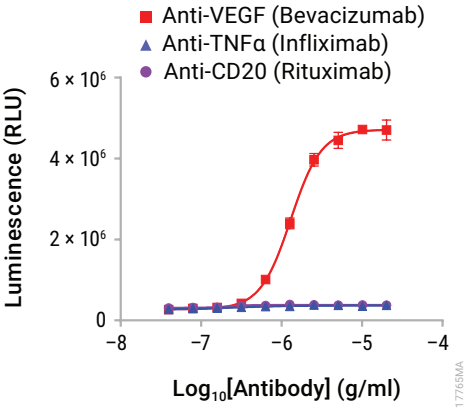

Figure 3. CDC assay with mVEGF Target Cells reflects the MOA and shows specificity for antibodies designed to bind VEGF. mVEGF Target Cells were incubated with 10% normal human serum complement in the presence of serial titrations of antibodies, as indicated. After a 6-hour incubation, CytoTox-Glo™ Reagent 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.

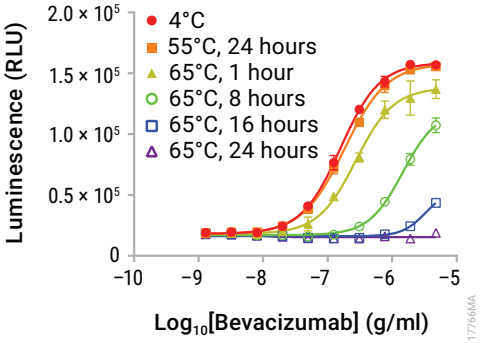

Figure 4. The ADCC Reporter Bioassay with mVEGF Target Cells is stability indicating. Samples of anti-VEGF (bevacizumab) were maintained at 4°C (control) or heat-treated at the indicated times and temperatures, then analyzed using the ADCC Reporter Bioassay with mVEGF Target Cells. Bio-Glo™ Reagent was 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. ADCC Reporter Bioassay using mVEGF Target Cells Shows Precision, Accuracy and Linearity.

Parameter	Results	
Accuracy	% Expected Relative Potency	% Recovery
	50	47.5
	70	71.3
	100	97.2
	140	148.1
	200	211.8
Repeatability (% CV)	100% (Reference)	2.1
Intermediate Precision (% CV)		5.0
Linearity (r^2)		0.9981
Linearity (y = mx + b)		y = 1.098x – 7.794

A 50–200% simulated potency series of bevacizumab was analyzed in triplicate in three independent experiments performed on three days by two analysts using mVEGF Target Cells in the ADCC Reporter Bioassay. Bio-Glo™ 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.

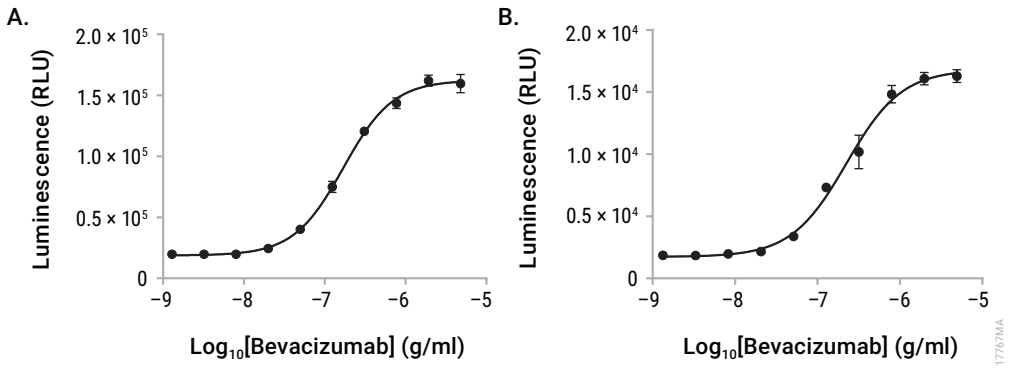

Figure 5. The ADCC Reporter Bioassay with mVEGF Target Cells is amenable to 384-well plate format. Panel A. The ADCC Reporter Bioassay with mVEGF Target Cells was performed in 96-well plates as described in this technical manual with a titration of anti-VEGF (bevacizumab). **Panel B.** The ADCC Reporter Bioassay with mVEGF Target Cells was performed in 384-well plates as briefly described here. mVEGF Target Cells were thawed and plated at 6×10^3/15µl/well 20 hours prior to the assay, in a 384-well white assay plate. On the day of the assay, 5µl of a threefold serial dilution of 5X concentrated bevacizumab was added to the wells, followed by the addition of 1.5×10^4/5µl/well of ADCC Effector Cells. After a 6-hour induction at 37°C, 5% CO_2, 25µl of Bio-Glo™ 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 17.5 and 22.6ng/ml for 96-well and 384-well formats, respectively, and the fold induction was 8.2 and 8.8 for 96-well and 384-well formats, respectively. Data were generated using thaw-and-use cells.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Membrane VEGF Target Cells	**1 each**	**J3351**

Not for Medical Diagnostic Use. **Includes:**

- 1 vial Membrane VEGF Target Cells (0.5ml)

PRODUCT	SIZE	CAT.#
Membrane VEGF Target Cells 5X	**1 each**	**J3355**

Not for Medical Diagnostic Use. **Includes:**

- 5 vials Membrane VEGF Target Cells (0.5ml)

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 procedure before beginning.

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

Note: mVEGF Target Cells are intended to be used with user-provided antibodies or other biologics designed to bind to VEGF. Data generated using bevacizumab (Avastin®) are shown in Section 8.A, Representative Assay Results.

To measure ADCC activity, mVEGF Target Cells can be used in conjunction with the thaw-and-use ADCC Reporter Bioassay (Cat.# G7010, G7018) or ADCC Reporter Bioassay, Propagation Model (Cat.# G7102), to detect ADCC function of anti-VEGF antibodies. To measure CDC activity, we recommend using CytoTox-Glo™ Cytotoxicity Assay (Cat.# G9290) with normal human serum complement.

mVEGF 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 recommended cell plating densities, induction time and assay buffer components described in Sections 4 and 5 were established using bevacizumab and ADCC Effector Cells (ADCC Reporter Bioassay) or in a CDC assay using the CytoTox-Glo™ Cytotoxicity Assay and complement-preserved human serum. You may need to adjust the parameters provided here and optimize assay conditions for your own assay readout and antibodies.

The ADCC Reporter Bioassay and the CDC assay with CytoTox-Glo™ Cytotoxicity Assay produce a bioluminescent signal and require a sensitive luminescence plate reader. Bioassay development and performance data included in this Technical Manual were generated using the GloMax® Discover System (see Section 8.B, Related Products). An integration time of 0.5 second/well was used for all readings. These bioassays are compatible with most other plate-reading luminometers, though relative luminescence unit (RLU) readings will vary with the sensitivity and settings of each instrument. If your luminometer or plate reader requires gain adjustment for luminescence, use the well with the highest antibody concentration.

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3. Before You Begin (continued)

Materials to Be Supplied by the User

Reagents

- user-defined anti-VEGF antibodies or other biologics samples (e.g., bevacizumab NDC 50242-060-01)
- Ham's F-12 Medium with L-glutamine (e.g., GIBCO® Cat.# 11765062)
- fetal bovine serum (FBS; e.g., GIBCO® Cat.# 35-015-CV or HyClone Cat.# SH30071.03)
- **optional:** ADCC Reporter Bioassay (Cat.# G7102, G7010, or G7018; for ADCC Bioassay)
- **optional:** Bio-Glo™ Luciferase Assay System (Cat.# G7940, G7941; for ADCC Bioassay)
- **optional:** Normal Human Serum Complement (Quidel, Cat.# A113; for CDC assay)
- **optional:** CytoTox-Glo™ Cytotoxicity Assay (Cat.# G9290; for CDC assay)

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.# 3570) for plating and reading luminescence
- sterile clear V-bottom 96-well plate with lid (e.g., Costar® Cat.# 3896) for preparing antibody dilutions
- sterile dilution reservoirs with lid (e.g., Dilux™ Cat.# D-1002) for higher volume 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., Costar®/Corning® 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, Cat.# GM3000, or equivalent)

4. Assay Protocol for ADCC Reporter Bioassay using mVEGF Target Cells

This assay protocol requires two engineered cell lines: ADCC Bioassay Effector Cells (Cat.# G7010, G7018) and mVEGF Target Cells (Cat.# J3351, J3355). ADCC Bioassay Effector Cells and Membrane VEGF Target Cells are also available in Propagation Model format (ADCC, Cat.# G7102; mVEGF, Cat.# J3342).

The procedure below illustrates the use of the mVEGF Target Cells in the ADCC Reporter Bioassay to test two anti-VEGF antibody samples against a reference sample in a single assay run, using the mVEGF Target Cells. Each test and reference antibody is run in triplicate, in a 10-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 5µg/ml as a starting concentration (1X) and a 2.5-fold serial dilution when testing bevacizumab.

4.A. Preparing ADCC Assay Reagents

1. **mVEGF Target Cell Plating Medium:** On the day before the assay, prepare an appropriate amount of Cell Plating Medium (90% Ham's F-12/10% FBS). Thaw the FBS overnight at 4°C or in a 37°C water bath on the day of use. Mix well and warm to 37°C before use. For reference, 30ml of Cell Plating Medium is typically sufficient for 120 wells in a 96-well assay format using the inner 60 wells.

2. **ADCC Assay Buffer:** On the day of the assay, prepare an appropriate amount of ADCC assay buffer (96% RPMI 1640/4% super low IgG FBS). Mix well and warm to 37°C before use. For reference, 30ml of ADCC assay buffer is typically sufficient for 120 wells in a 96-well assay format using the inner 60 wells.

   Note: The recommended ADCC assay buffer contains 4% super low IgG FBS. This concentration and type of FBS works well for the anti-VEGF antibody that we tested. If you experience assay performance issues when using this assay buffer, we recommend testing different serum concentrations and types, in the range of 0.5–10%.

3. **Bio-Glo™ Reagent:** For reference, 10ml of Bio-Glo™ Reagent is sufficient to assay 120 wells in a 96-well assay format. Thaw the Bio-Glo™ Luciferase Assay Buffer at 4°C overnight or in a room temperature water bath on the day of assay. Equilibrate the Bio-Glo™ Luciferase Assay Buffer to ambient temperature, protected from light. Transfer all of the Bio-Glo™ Luciferase Assay Buffer into the amber bottle containing the Bio-Glo™ Luciferase Assay Substrate and mix by inversion until the substrate is thoroughly dissolved. Equilibrate and store the reconstituted Bio-Glo™ Reagent at ambient temperature (22–25°C) protected from light before adding to assay plates.

   Note: The ADCC Reporter Bioassay is compatible only with the Bio-Glo™ Luciferase Assay System. **Do not** use the Bio-Glo-NL™ Luciferase Assay System with the ADCC Reporter Bioassay.

4. **Test and Reference Samples:** Using ADCC assay buffer as the diluent, prepare stock starting dilutions (dilu1, 3X final concentration) of two test antibodies (200µl each) and one reference antibody (400µl) in 96-well dilution plate. Store the plate containing antibody starting dilutions appropriately before making antibody serial dilutions.

   Note: If you are using bevacizumab (25mg/ml stock) as a reference antibody in your assay, prepare a 1mg/ml working stock of anti-VEGF antibody (bevacizumab), by adding 4µl of bevacizumab stock (25mg/ml) to 96µl of ADCC assay buffer. Prepare a 400µl starting dilution of 15µg/ml of bevacizumab (dilu1, 3X final concentration) by adding 6µl of bevacizumab working stock to 394µl of ADCC assay buffer.

4.A. Preparing ADCC Assay Reagents (continued)

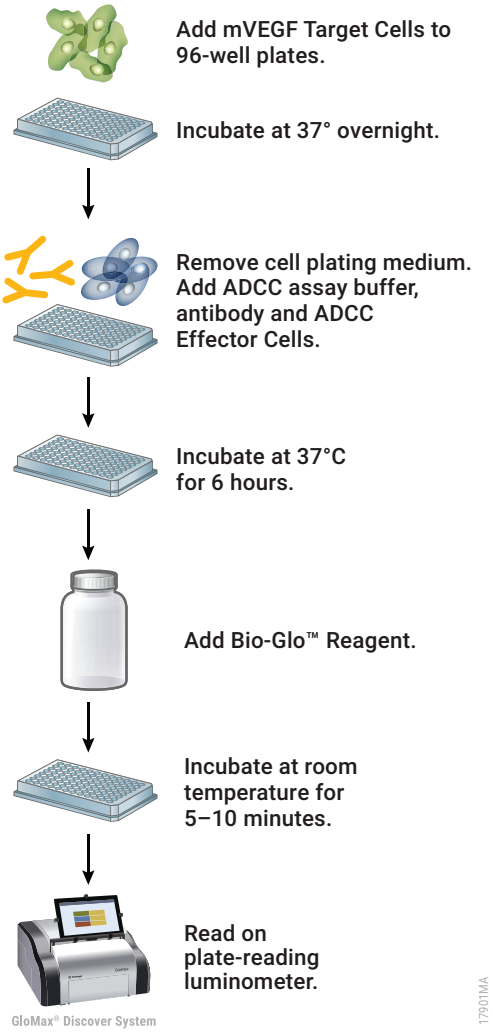

Figure 6. Schematic protocol for the ADCC Reporter Bioassay with mVEGF Target Cells.

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 antibody to generate two 10-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 7. Example plate layout showing non-clustered sample locations of test antibody and reference antibody dilution series and wells containing ADCC assay buffer alone (“B”).

4.C. Plating mVEGF Target Cells

Thaw-and-use mVEGF 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.

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

1. On the day before performing the assay, prepare mVEGF Target Cell Plating Medium (90% Ham’s F-12/10% FBS) as described in Section 4.A.
2. Transfer 14.5ml of cell plating medium to a 50ml conical tube.

4.C. Plating mVEGF Target Cells (continued)

3. Remove one vial of mVEGF 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.
4. Gently mix the cell suspension by pipetting, then transfer 0.5ml cells to the tube containing 14.5ml of cell plating medium. Mix well by gently inverting the tube 1–2 times. 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 a 96-well white flat-bottom assay plate.
5. Add 100µl of mVEGF Target Cell plating 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 (100µl 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 400µl of reference antibody at 3X the highest antibody concentration in your dose-response curve. You will need 200µl of each test antibody at 3X the highest antibody concentration in each of the test antibody dose-response curves. For other dilution schemes, adjust the volumes accordingly.

Note: The instructions below use bevacizumab; 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.

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

1. On the day of the assay, prepare an appropriate amount of ADCC assay buffer as described in Section 4.A.
2. To a sterile clear V-bottom 96-well plate, add 200µl of reference antibody starting dilution (dilu1, 3X final concentration) to wells A11 and B11 (Figure 8).
3. Add 200µl of test antibodies 1 and 2 starting dilution (dilu1, 3X final concentration) to wells E11 and G11, respectively (Figure 8).
4. Add 120µl of ADCC assay buffer to other wells in these four rows, from column 10 to column 2.
5. Transfer 80µl 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 120µl of ADCC 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).

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 8. Example plate layout showing antibody serial dilutions.

4.E. Adding Antibody to mVEGF Target cells in Assay Plates

1. Take the 96-well assay plate containing mVEGF Target Cells out of the incubator. Invert the assay plate 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 25µl of ADCC assay buffer to the inner 60 wells of both 96-well assay plates.
3. Using a multichannel pipette, add 25µl of the appropriate antibody dilution (Figure 8) to the assay plates according to the plate layout in Figure 7.
4. Add 75µl of ADCC assay buffer to each of the outside wells of the assay plate.
5. Cover the assay plates with lids and keep the plates on the bench before adding ADCC Bioassay Effector cells at the next step.

4.F. Plating ADCC Bioassay Effector Cells

The thaw-and-use ADCC Effector Cells in the ADCC Reporter Bioassay (Cat.# G7010) 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 3.6ml of prewarmed (37°C) ADCC assay buffer to the 15ml conical tube.

2. Remove one vial of ADCC 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. Do not invert the vial.
3. Gently mix the cell suspension by pipetting, then transfer 630µl of cells to the 15ml conical tube containing 3.6ml of ADCC assay buffer. Mix well by gently inverting the tube.
4. 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.
5. Cover the assay plates with lids and incubate in a 37°C, 5% CO_2 incubator for 6 hours.

   Note: The 6-hour assay time was optimized using bevacizumab. We recommend optimizing the assay time (5–24 hours) with your antibody or other biologic samples.

4.G. Adding Bio-Glo™ Reagent

The ADCC Reporter Bioassay is compatible only with the Bio-Glo™ Luciferase Assay System. **Do not** use Bio-Glo-NL™ Luciferase Assay System with the ADCC Reporter Bioassay.

Bio-Glo™ Reagent should be at ambient temperature (22–25°C) when added to plates.

1. Remove assay plates from the 37°C incubator and equilibrate to ambient temperature (22–25°C) for 15 minutes.
2. Using a manual multichannel pipette, add 75µl of Bio-Glo™ Reagent to the inner 60 wells of the assay plates; avoid creating any bubbles.
3. Add 75µl of Bio-Glo™ Reagent to wells B1, D1 and F1 in each assay plate to determine plate background.
4. Incubate at ambient temperature for 5–20 minutes.
5. Measure luminescence using a plate reader with glow-type luminescence reading capabilities.

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. Assay Protocol for CDC Assay using mVEGF Target Cells

The procedure below illustrates the use of the mVEGF Target Cells in a CDC assay (using CytoTox-Glo™ Cytotoxicity Assay, Cat.# G9290) to test two anti-VEGF antibody samples against a reference sample in a single assay run using the Membrane VEGF Target Cells. Each test and reference antibody is run in triplicate, in a 10-point dilution series, in a single 96-well assay plate. Other experimental and plate layouts are possible but may require further optimization.

Notes: 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 20µg/ml as a starting concentration (1X) and twofold serial dilutions when testing bevacizumab.

5.A. Preparing CDC Assay Reagents

1. **mVEGF Target Cell Plating Medium/CDC Assay Buffer:** On the day before the assay, prepare an appropriate amount of cell plating medium/CDC assay buffer (90% Ham's F-12/10% FBS). Thaw the FBS overnight at 4°C or in a 37°C water bath on the day of use. Mix well and warm to 37°C before use. For reference, 50ml of cell plating medium/CDC assay buffer is typically sufficient for 132 wells in a 96-well assay format (Figure 10). After plating mVEGF Target Cells, remaining assay buffer can be stored at 4°C overnight for use on the day of the assay.

2. **CytoTox-Glo™ Reagent:** Prepare an appropriate amount of CytoTox-Glo™ Reagent on the day of the assay. Thaw the CytoTox-Glo™ Assay Buffer in a room temperature water bath, and equlibrate to ambient temperature, protected from light. Thirty minutes prior to the end of the assay, transfer 5ml of buffer into one amber bottle containing AAF-Glo™ Substrate and mix by inversion, until the substrate is thoroughly dissolved. For reference, 10ml of CytoTox-Glo™ Reagent is enough for 132 assay wells in a 96-well assay format.

   For optimal results, use freshly prepared CytoTox-Glo™ Reagent. Use within 12 hours if stored at room temperature. The CytoTox-Glo™ Reagent can be stored at 4°C for up to 7 days with no appreciable loss of performance. The CytoTox-Glo™ Reagent can be stored in single-use aliquots for up to 4 months at –70°C. Freezing and thawing will damage the reagent and should be avoided.

3. **Normal Human Serum Complement:** Follow the manufacturer's instructions for storage, preparation and handling. The recommended assay conditions include 10% normal human serum complement. This concentration and type of complement works well for the anti-VEGF antibodies we tested. If you experience assay performance issues when using normal human serum complement, we recommend testing different concentrations in the range of 5–20%.

4. **Test and Reference Samples:** Using CDC assay buffer as the diluent, prepare stock starting dilutions (dilu1, 1.5X final concentration) of two test antibodies (400µl each) and one reference antibody (800µl) in 1.5ml tubes. Store the tubes containing antibody starting dilutions appropriately before making antibody serial dilutions.

   Note: If you are using bevacizumab (25mg/ml stock) as a reference antibody, prepare a 1mg/ml working stock of anti-VEGF antibody (bevacizumab) by adding 4µl of bevacizumab stock (25mg/ml) to 96µl of CDC assay buffer. Prepare 800µl starting dilution of 30µg/ml of bevacizumab (dilu1, 1.5X final concentration) by adding 24µl of bevacizumab working stock to 776µl of CDC assay buffer.

5. **Digitonin:** Prior to completion of the assay, prepare 1mg/ml digitonin in CytoTox-Glo™ Assay Buffer from 20mg/ml stock provided in the CytoTox-Glo™ Cytotoxicity Assay (Cat.# G9290) by adding 5µl of digitonin stock to 95µl of CytoTox-Glo™ Assay Buffer.

5.A. Preparing CDC Assay Reagents (continued)

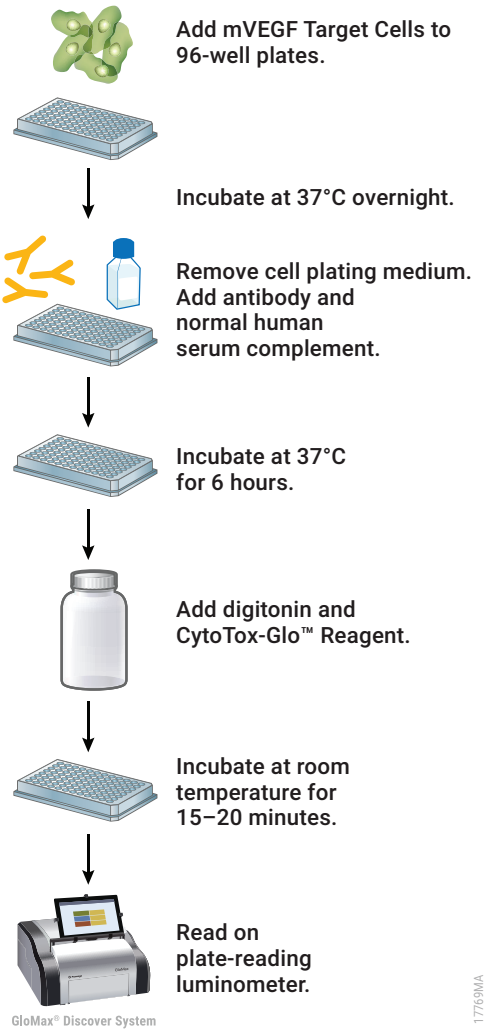

Figure 9. Schematic protocol for the CDC assay with mVEGF Target Cells.

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 antibody to generate two 10-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	D	Reference Ab
C	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	D	Test Ab
D	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	D	Reference Ab
E	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	D	Test Ab
F	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	D	Reference Ab
G	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	D	Test Ab
H	B	B	B	B	B	B	B	B	B	B	B	B	Assay Buffer (B)

Figure 10. Example plate layout showing non-clustered sample locations of test antibody and reference antibody dilution series and wells containing digitonin ("D") or CDC assay buffer ("B").

5.C. Plating mVEGF Target Cells

Thaw-and-use mVEGF 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.

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

1. On the day prior to performing the assay, prepare an appropriate amount of mVEGF Target Cell plating medium by combining 45ml of Ham's F-12 with 5ml of FBS to yield 90% Ham's F-12/10% FBS. Mix well and warm to 37°C prior to use.
2. Transfer 14.5ml of mVEGF Target Cell plating medium to a 50ml conical tube.

5.C. Plating mVEGF Target Cells (continued)

3. Remove one vial of mVEGF 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.
4. Gently mix the cell suspension by pipetting, then transfer 0.5ml cells to the tube containing 14.5ml of cell plating medium. Mix well by gently inverting the tube 1–2 times. Transfer the suspension to a sterile reagent reservoir.
5. Using a multichannel pipette, immediately dispense 100µl of the cell suspension B2 through G12 of a 96-well white flat-bottom assay plate (Figure 10).
6. Add 100µl of mVEGF Target Cell plating medium to each of the empty wells of the assay plates (Figure 10).
7. Place lids on the assay plates and incubate in a 37°C, 5% CO_2 incubator overnight (18–22 hours).

5.D. Preparing Antibody Serial Dilutions

The instructions described here are for preparation of single stocks of twofold serial dilutions of a single antibody for analysis in triplicate (200µl 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 twofold serial dilutions, you will need 800µl of reference antibody at 1.5X the highest antibody concentration in your dose-response curve. You will need 400µl 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: The instructions below use bevacizumab follow the instructions below to prepare twofold serial dilutions. A twofold serial dilution for test antibodies is also listed as an example below.

1. On the day of the assay, warm CDC assay buffer prepared the day before to 37°C. Otherwise, prepare an appropriate amount of CDC assay buffer as prepared in Section 5.A.
2. To a sterile 12-well reservoir labeled “reference”, add 800µl of an appropriate reference antibody starting dilution (dilu1, 1.5X final concentration) to well 11 (Figure 11).
3. To two additional sterile 12-well reservoirs labeled “test 1” and “test 2”, add 400µl of test 1 and 2 antibodies starting dilutions (dilu1, 1.5X final concentration) to well 11 (Figure 11).
4. For reference antibody, add 400µl of CDC assay buffer to wells 2 through 10 and 12 (well 1 is empty).
5. For test antibodies 1 and 2, add 200µl of CDC assay buffer to wells 2 through 10 and 12 (well 1 is empty).
6. Transfer 400µl of the reference antibody starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
7. Repeat equivalent reference antibody twofold serial dilutions across the columns from right to left through column 3. Do not dilute into column 2.

   Note: Well 2 contains 400µl of CDC assay buffer without antibody, as a negative control. Well 12 contains 400µl of CDC assay buffer without antibody and will be used for digitonin addition at end of the assay.
8. Transfer 200µl of the test 1 and 2 antibody starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.

9. Repeat equivalent test 1 and 2 antibody twofold serial dilutions across the columns from right to left through column 3. Do not dilute into column 2.

   Note: Well 2 contains 200µl of CDC assay buffer without antibody, as a negative control. Well 12 contains 200µl of CDC assay buffer without antibody and will be used for digitonin addition at end of the assay.

10. Cover the antibody dilution reservoirs with their lids and keep at ambient temperature (22–25°C) while preparing the normal human serum complement.

Recommended 12-well Reservoir Layouts for Antibody Dilutions Prepared from a Single Antibody Stock.												
1	2	3	4	5	6	7	8	9	10	11	12	
	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	no Ab	Reference Ab

1	2	3	4	5	6	7	8	9	10	11	12	
	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	no Ab	Test Ab 1

1	2	3	4	5	6	7	8	9	10	11	12	
	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	no Ab	Test Ab 2

Figure 11. Example 12-well reservoir layouts showing antibody serial dilutions.

5.E. Preparing Normal Human Serum Complement

Note: Normal human serum complement is heat labile. Take care to ensure it is fully intact when used in the assay. Follow the manufacturer's instructions for storage and handling.

1. Dilute the normal human serum complement with CDC assay buffer, to achieve a 30% solution. Once diluted in the assay plate, this gives a 10% final concentration. You will need at least 4ml of 30% complement to fill 120 assay wells, or 60 wells of two assay plates.

5.F. Adding Antibody and Complement to Assay Plates

1. Remove the 96-well assay plates containing mVEGF Target Cells from the incubator. Invert to remove 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 appropriate antibody dilution to the assay plates according to the plate layout in Figure 10.
3. Using a multichannel pipette, add 25µl of the 30% normal human serum complement to each of the inner 60 wells of the assay plates (Figure 10).
4. Using a multichannel pipette, add 25µl of CDC assay buffer to wells B12–G12 (digitonin wells).

5. Add 75µl of CDC assay buffer to each of the empty outside wells of the assay plates.
6. Cover the assay plates with lids and incubate in a 37°C, 5% CO_2 incubator for 6 hours.

   Note: The 6-hour assay time was optimized using bevacizumab. We recommend optimizing assay time (3–24 hours) with your own antibody or other biologic samples.

5.G. Adding Digitonin and CytoTox-Glo™ Reagent

1. During the 6-hour incubation time, reconstitute the CytoTox-Glo™ Reagent according to the instructions in Section 5.A.
2. At the end of the 6-hour incubation, remove the assay plates from the incubator and immediately add 8µl of 1mg/ml digitonin (prepared in Section 5.A) to wells B12–G12, per plate layout in Figure 10.
3. Gently shake plate briefly to mix the digitonin in the well.
4. Allow plate to equilibrate to ambient temperature (15–20 minutes).
5. Add 40µl of CytoTox-Glo™ Reagent (at ambient temperature) to all wells containing mVEGF Target Cells (wells B2–G12).
6. Add 40µl of CytoTox-Glo™ Reagent to wells B1, D1 and F1 to determine plate background.
7. Incubate at ambient temperature for 15 minutes.
8. Measure luminescence using a plate reader.

5.H. Data Analysis

1. Determine the plate background by calculating the average RLU from wells B1, D1 and F1.
2. Determine the maximum killing by calculating the average RLU from wells B12–G12.
3. Calculate fold induction:

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

4. Calculate percent specific lysis:

$$\text{Percent Specific Lysis} = \frac{\text{RLU (antibody} - \text{background)}}{\text{RLU (digitonin} - \text{background)}} \times 100$$

5. Graph data as RLU versus Log_{10}[antibody], fold induction versus Log_{10}[antibody] and percent specific lysis 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.

6. 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 a sensitive instrument designed for luminescence detection. Instruments designed primarily for fluorescence detection are not recommended. Luminometers measure and report luminescence as relative values, and actual 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™ Reagent or CytoTox-Glo™ Reagent can lead to low RLU. Store and handle the reagents according to the instructions.
Weak assay response (low fold induction)	Optimize the concentration range of your test sample(s) to achieve a complete dose response with complete upper and lower asymptotes. The EC_{50} values obtained in the ADCC Reporter Bioassay or CDC assay with mVEGF Target Cells may vary from the values obtained using other methods.
	Optimize the ADCC Reporter Bioassay incubation time within a range of 5–24 hours or the CDC assay within a range of 3–24 hours, and choose the incubation time that gives optimal response.
	Optimize the super low IgG FBS concentration from 0.5–10% in ADCC assay buffer if ADCC Reporter Bioassay performance is not ideal.
Weak CDC assay response (low percent lysis)	Optimize the normal human serum complement from 5–20% if CDC assay performance is not ideal.
Variability in assay performance	Ensure that incubation times are consistent between assays.
	Ensure that the Preparing and Plating protocols (Section 5) are strictly followed.

7. References

1. Goel, H.L. and Mercurio, A.M. (2013) VEGF targets the tumour cell. *Nat. Rev. Cancer* **13**, 871–82.
2. Takahashi, S. (2011) Vascular endothelial growth factor (VEGF), VEGF receptors and their inhibitors for anti-angiogenic tumor therapy. *Biol. Pharm. Bull.* **34**, 1785–8.
3. Wang, L. *et al.* (2016) Development of a robust reporter-based assay for the bioactivity determination of anti-VEGF therapeutic antibodies. *J. Pharm. Biomed. Anal.* **125**, 212–8.
4. MacDonald, D.A. *et al.* (2016) Aflibercept exhibits VEGF binding stoichiometry distinct from bevacizumab and does not support formation of immune-like complexes. *Angiogenesis* **19**, 389–406.
5. Papadopoulos, N. *et al.* (2012) Binding and neutralization of vascular endothelial growth factor (VEGF) and related ligands by VEGF Trap, ranibizumab and bevacizumab. *Angiogenesis* **15**, 171–85.

8. Appendix

8.A. Representative Assay Results

The following data were generated using mVEGF Target Cells.

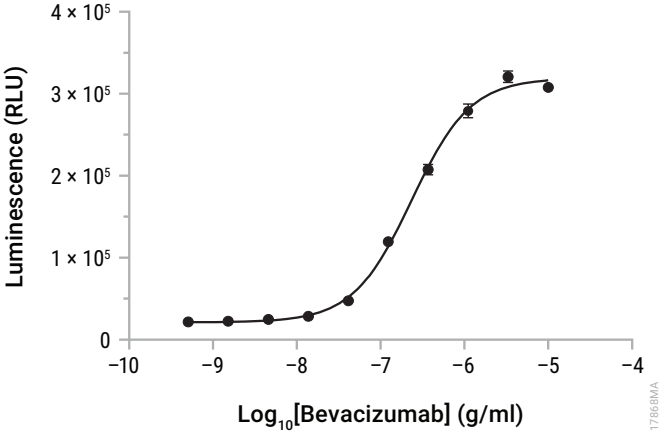

Figure 12. The ADCC Bioassay Effector Cells with mVEGF Target Cells measures the activity of the anti-VEGF antibody bevacizumab. mVEGF Target Cells were added to a 96-well assay plate 18 hours prior to the assay. On the day of the assay, ADCC Effector Cells and a titration of bevacizumab were added. After a 6-hour induction at 37°C, Bio-Glo™ 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 0.23µg/ml and fold induction was 14.9.

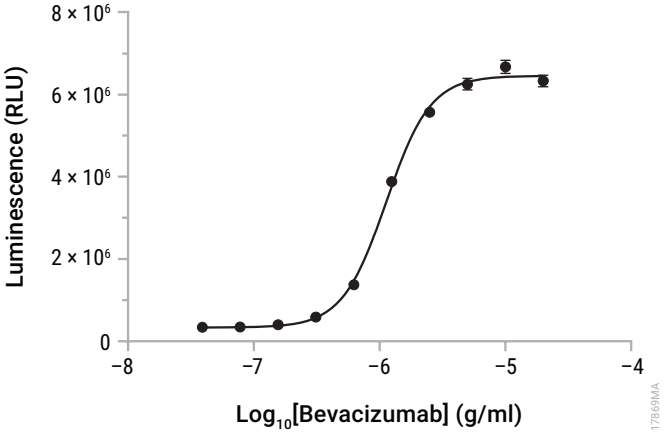

Figure 13. A CDC assay measures the activity of the anti-VEGF antibody bevacizumab. mVEGF Target Cells were added to a 96-well assay plate 18 hours prior to the assay. On the day of assay, 10% normal human serum complement and a titration of bevacizumab in CDC assay buffer were added. After a 6-hour induction at 37°C, CytoTox-Glo™ 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} for bevacizumab was 1.13µg/ml, the fold induction was 18.8 and percent specific lysis was 47.7%.

8.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

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**Not for Medical Diagnostic Use.

Additional kit formats are available.

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8.B. Related Products (continued)

Fc Effector Immunoassay

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

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

Immune Checkpoint Bioassays

Product	Size	Cat.#
4-1BB Bioassay	1 each	JA2351
CD28 Bioassay	1 each	JA6701
CD28 Blockade Bioassay	1 each	JA6101
CD40 Bioassay	1 each	JA2151
CTLA-4 Blockade Bioassay	1 each	JA3001
GITR Bioassay	1 each	JA2291
ICOS Bioassay	1 each	JA6801
ICOS Blockade Bioassay	1 each	JA6001
LAG-3/MHCII Blockade Bioassay	1 each	JA1111
OX40 Bioassay	1 each	JA2191
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 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 and sizes 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

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Macrophage-Directed Bioassays

Product	Size	Cat.#
SIRPα/CD47 Blockade Bioassay	1 each	JA6011
SIRPα/CD47 Blockade Bioassay, Fc-dependent	1 each	JA4801
TLR Bioassay	1 each	JA9011
ADCP Reporter Bioassay (THP-1)	1 each	JA9411

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Control Antibodies and Proteins

Product	Size	Cat.#
Control Ab, Anti-4-1BB	50µg	K1161
Control Ab, Anti-CD20	5µg	GA1130
Control Ab, Anti-OX40	50µg	K1191
Control Ab, Anti-CD40	50µg	K1181
Control Ab, Anti-CTLA-4	100µg	JA1020
Control Ab, Anti-LAG-3	100µg	K1150
Control Ab, Anti-PD-1	100µg	J1201
Control Ab, Anti-TIGIT	100µg	J2051
Control Ab, Anti-TIM-3	100µg	K1210
Recombinant VEGF ligand	10µg	J2371

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8.B. Related Products (continued)

Detection Reagents

Product	Size	Cat.#
Bio-Glo™ Luciferase Assay System	10ml	G7941
	100ml	G7940
Bio-Glo-NL™ Luciferase Assay System	10ml	J3081
	100ml	J3082
	1,000ml	J3083

Not for Medical Diagnostic Use.

Luminometers

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

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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/**

9. Summary of Changes

The following changes were made to the 1/25 revision of this document:

1. Corrected text defining ICH in the Description, Section 1.
2. Updated Related Products, Section 8.B.
3. Updated fonts and trademarks.

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