TECHNICAL MANUAL

VEGF Bioassay

Instructions for Use of Products
GA2001 and GA2005

Revised 5/26
TM544

Promega

VEGF 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

1. **Description** 2
2. **Product Components and Storage Conditions** 9
3. **Before You Begin** 10
   - 3.A. Materials to Be Supplied by the User 10
4. **Assay Protocol** 11
   - 4.A. Preparing Bio-Glo™ Reagent, Assay Buffer, and Test and Reference Samples 13
   - 4.B. Plate Layout Design 14
   - 4.C. Preparing Serial Dilutions 15
   - 4.C. Preparing Serial Dilutions (continued) 16
   - 4.D. Preparing and Plating KDR/NFAT-RE HEK293 Cells 17
   - 4.E. VEGF/Inducer Stimulation Assay 17
   - 4.F. Antibody Blockade Assay 18
   - 4.G. Adding Bio-Glo™ Reagent 18
   - 4.H. Data Analysis 18
5. **Troubleshooting** 19
6. **References** 20
7. **Appendix** 20
   - 7.A. Representative Assay Results 20
   - 7.B. Assay Troubleshooting 23
   - 7.C. Composition of Buffers and Solutions 24
8. **Summary of Changes** 24

Promega

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 stimulation of angiogenesis, increase of vascular permeability, enhancement of tumor invasion and survival, and inhibition of antitumor response in Treg cells. There are several VEGF receptor subtypes—VEGFR1, VEGFR2 and VEGFR3. VEGFR2 (also known as 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).

All members of the VEGF family stimulate cellular responses by binding to receptors of the receptor tyrosine kinase family, namely VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR). When VEGF binds to KDR, the receptor dimerizes and becomes activated through transphosphorylation (2).

Expression of VEGF in tumor cells allows new blood vessels to form, which supports tumor growth, invasion and metastatic dissemination (2). Overexpression of VEGF can also cause vascular disease in the retina of the eye as well as other parts of the body. Studies have shown that inhibition of the VEGF signaling pathway can effectively inhibit angiogenesis and treat various cancers and eye diseases (3,4). There are currently several drugs available that can inhibit VEGF or KDR to control these diseases, such as the anti-VEGF drugs aflibercept, bevacizumab, ranibizumab and the anti-KDR drug ramucirumab (5).

The VEGF Bioassay is faster (completed in hours) and easier than other commonly used angiogenesis assays that use primary human umbilical vein endothelial cells (HUVEC), such as endothelial cell proliferation and differentiation assays. Also, the VEGF Bioassay does not use radioactive ^{3}H-thymidine often required in other assays. HUVEC assays, on the other hand, are time-consuming (completed in 4–5 days), challenging to run (due to limitations around cell senescence) and difficult to analyze (can be difficult to distinguish whether a decrease in cell number is due to cell death rather than decreased cell proliferation) (3,6).

The VEGF Bioassay[(a–c)] (Cat.# GA2001, GA2005) is a bioluminescent cell-based assay that measures VEGF stimulation and inhibition of KDR (VEGFR2) using the NFAT-RE as a readout. This assay overcomes many of the limitations of the current endothelial cell proliferation assays, and can be used for the discovery and development of novel biologic therapies aimed at either inducing or inhibiting the VEGF response. The KDR/NFAT-RE HEK293 Cells are provided in a thaw-and-use format as cryopreserved cells that can be thawed, plated and used in an assay without the need for cell propagation (also offered as a cell propagation model; CPM, Cat.# GA1082).

The KDR/NFAT-RE HEK293 Cells have been engineered to express the NFAT response element upstream of Luc2P as well as exogenous KDR. When VEGF binds to the KDR/NFAT-RE HEK293 Cells, the KDR transduces intracellular signals resulting in NFAT-RE-mediated luminescence (Figure 1). The bioluminescent signal is detected and quantified using Bio-Glo™ Luciferase Assay System (Cat.# G7940, G7941) and a standard luminometer, such as the GloMax® Discover System.

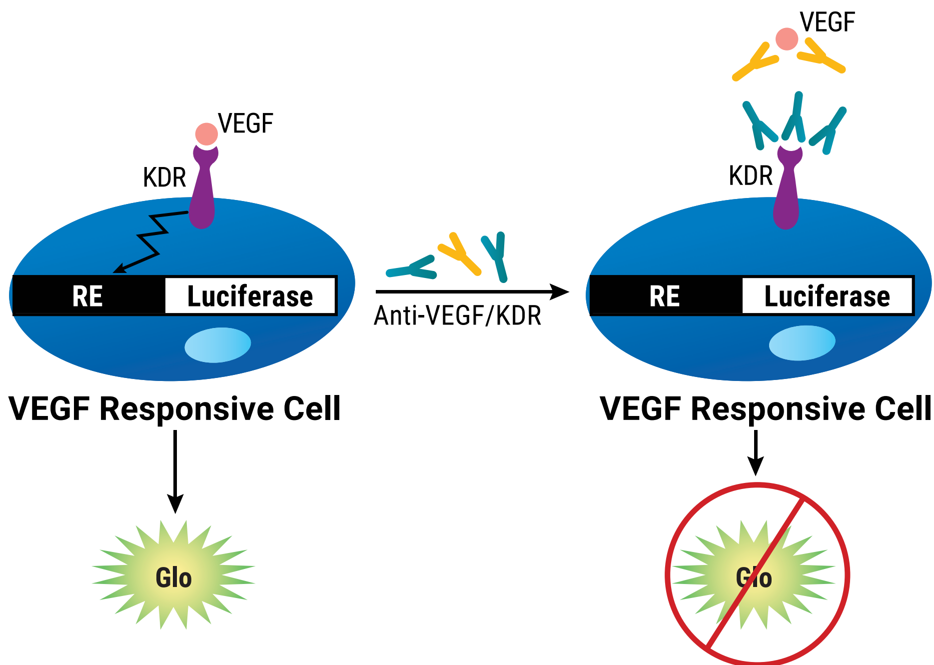

Figure 1. Representation of the VEGF Bioassay. The VEGF Bioassay consists of a genetically engineered cell line, KDR/NFAT-RE HEK293 Cells. When VEGF binds to KDR, receptor-mediated signaling induces luminescence that can be detected by adding Bio-Glo™ Reagent and quantitated with a luminometer. Inhibition of VEGF binding to KDR by either anti-VEGF or anti-KDR antibodies results in a decrease in luminescence.

1. Description (continued)

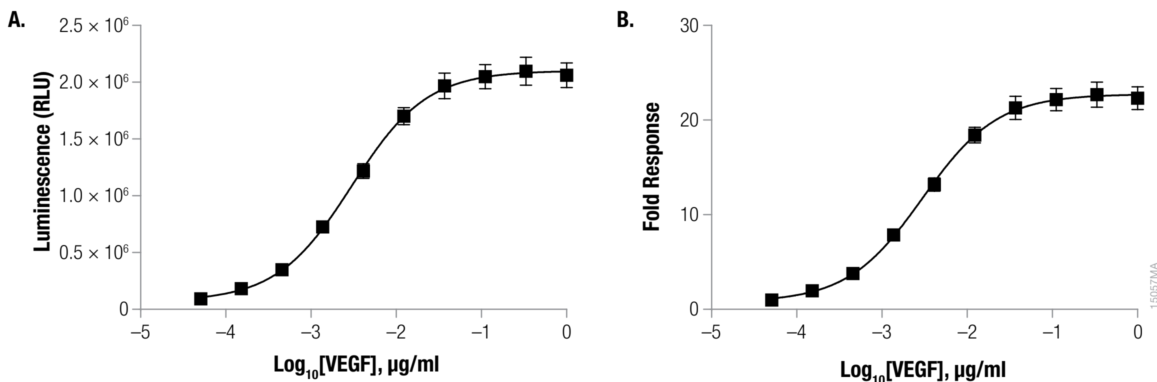

Figure 2. The VEGF Bioassay response to recombinant VEGF. KDR/NFAT-RE HEK293 Cells were incubated with serial dilutions of recombinant VEGF. After a 6-hour incubation, Bio-Glo™ Reagent was added and luminescence was quantified using the GloMax® Discover System. Data were fitted to a 4PL curve using GraphPad Prism® software. Data was generated using thaw-and-use cells.

An important application of the VEGF Bioassay is the discovery and development of biologics designed to bind to either VEGF or KDR, and therefore inhibit the VEGF signaling pathway. Figure 3, Panel A, shows the effect of several VEGF blockers. Bevacizumab is a recombinant, humanized monoclonal antibody that binds to VEGF-A, and has been approved for the treatment of various cancers. Ranibizumab is a Fab fragment derived from bevacizumab and has a higher affinity for VEGF-A compared to the parental antibody. Aflibercept is a novel decoy receptor which binds multiple isoforms of VEGF-A as well as some related VEGFR1 ligands (5). When incubated with KDR/NFAT-RE HEK293 Cells and an EC_{80} concentration of recombinant VEGF, these VEGF blockers show a dose-dependent decrease in luminescence. Importantly, adalimumab (an anti-TNFα monoclonal antibody) shows no response, demonstrating the specificity of the assay.

Figure 3, Panel B, shows the effect of several anti-KDR antibodies. Ramucirumab is a human monoclonal antibody directed against KDR, and works as a receptor antagonist to block the binding of VEGF. The anti-KDR Antibody (Creative Biolabs, Cat.# IMC-1C11) is a research-grade neutralizing antibody that binds to KDR, and blocks the binding of KDR to VEGF.

The bioassay is prequalified according to ICH guidelines and shows the precision, accuracy and linearity required for routine use in potency and stability studies (Table 1 and Figure 4). In addition, the bioassay workflow is simple and robust, and compatible with both 96-and 384-well plate formats used for antibody screening in early drug discovery (Figure 7). Finally, the bioassay can be used in the presence of up to 33.3% human serum (final concentration) with an excellent assay window (Figure 8), indicating potential for further development into a neutralizing antibody bioassay.

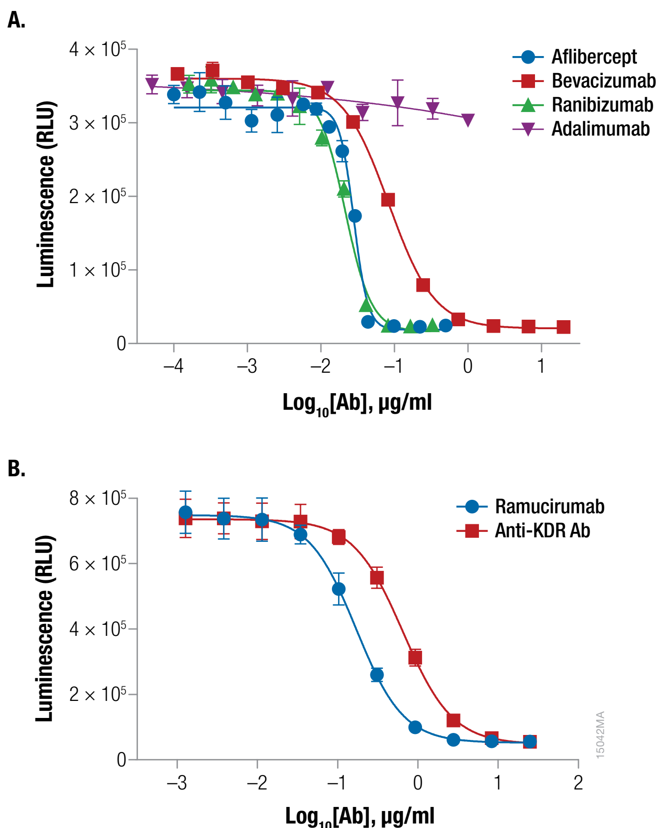

Figure 3. The VEGF Bioassay can be used to measure the activity of antibodies to either VEGF or KDR. KDR/NFAT-RE HEK293 Cells were incubated with serial dilutions of antibodies to either VEGF (**Panel A**) or KDR (**Panel B**), in the presence of an EC_{80} concentration of recombinant VEGF. After a 6-hour incubation, Bio-Glo™ Reagent was added and luminescence was quantified using the GloMax® Discover System. Data were fitted to a 4PL curve using GraphPad Prism® software. Data was generated using thaw-and-use cells. IC_{50} values were as follows: aflibercept 0.028µg/ml, bevacizumab 0.084µg/ml, ranibizumab 0.021µg/ml, ramucirumab 0.17µg/ml and anti-KDR Ab 0.65µg/ml.

Table 1. The VEGF Bioassay Shows Precision, Accuracy and Linearity.

Parameter	Results	
Accuracy	% Expected Relative Potency	% Recovery
	50	100.01
	75	96.67
	150	102.75
	200	107.25
Repeatability (% CV)	100% (Reference)	8.73
Intermediate Precision (% CV)		9.28
Linearity (r^2)		0.9984
Linearity (y = mx + b)		y = 1.1x − 7.792

A 50–200% theoretical potency series of bevacizumab (anti-VEGF antibody) was analyzed in triplicate in three independent experiments performed on three days by each of two analysts (for a total of six independent experiments). 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.

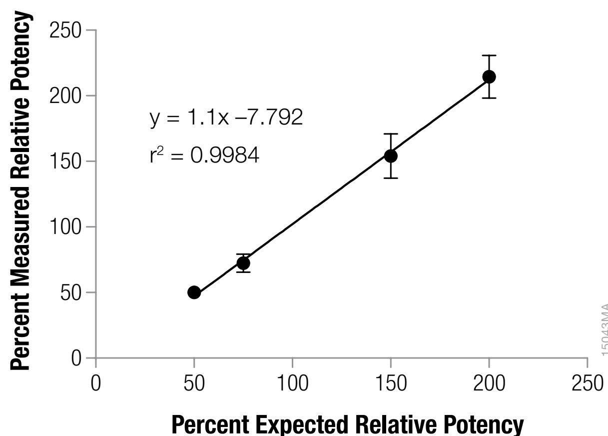

Figure 4. The VEGF Bioassay shows precision, accuracy and linearity. A 50–200% theoretical potency series of bevacizumab (anti-VEGF antibody) was analyzed in triplicate in three independent experiments performed on three days by each of two analysts using the VEGF Bioassay (for a total of six independent experiments). Linearity and r^2 values were determined using GraphPad Prism® software. Data were generated using thaw-and-use cells.

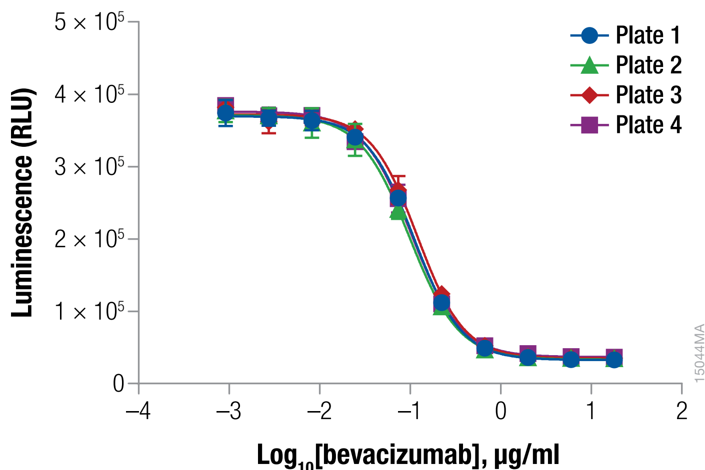

Figure 5. The VEGF Bioassay demonstrates repeatability. Four separate serial dilution series of bevacizumab (anti-VEGF antibody) were analyzed on four individual assay plates using the VEGF Bioassay. Bio-Glo™ Reagent was added and luminescence was quantified using the GloMax® Discover System. Data were fitted to a 4PL curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

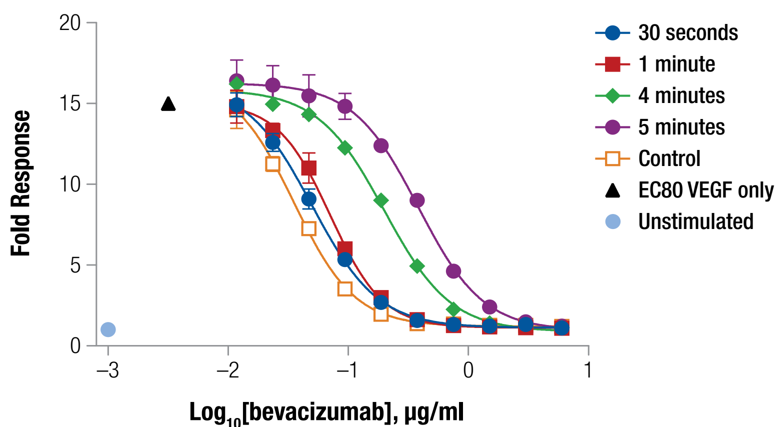

Figure 6. The VEGF Bioassay indicates stability. Samples of bevacizumab were maintained at 4°C (control) or heat-treated (80°C) for increasing lengths of time (30 seconds–5 minutes), then analyzed using the VEGF Bioassay. Bio-Glo™ Reagent was added and luminescence was quantified using the GloMax® Discover System. Data were fitted to a 4PL curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

Promega

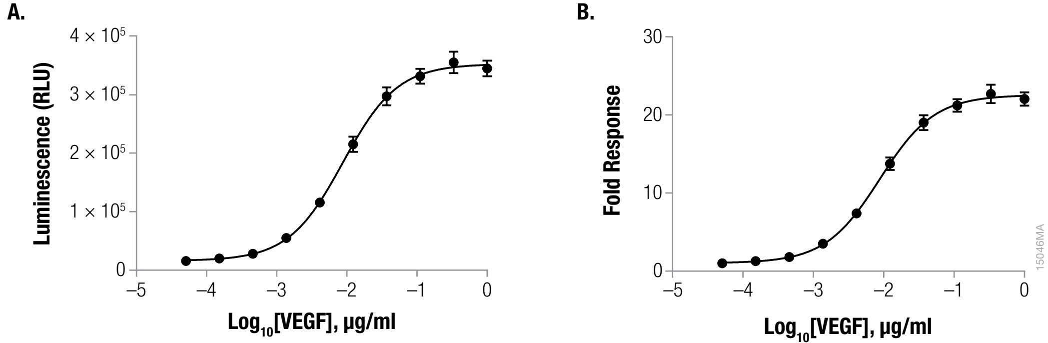

Figure 7. The assay is amenable to 384-well plate format. The VEGF Bioassay was tested in a 384-well format. KDR/NFAT-RE HEK293 Cells were plated at 15,000 cells/well in 10µl volume. A threefold serial dilution of recombinant VEGF was added at 10µl per well, and 10µl of media was added to bring the volume up to 30µl/well. After 6 hours of stimulation with VEGF, 30µl of Bio-Glo™ Reagent was added and luminescence was quantified using the GloMax® Discover System. Data were fitted to a 4PL curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

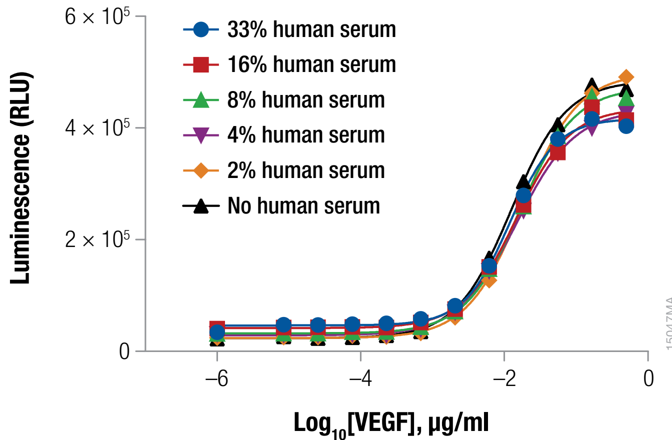

Figure 8. The VEGF Bioassay tolerates human serum. KDR/NFAT-RE HEK293 Cells were tested with a dose-response of recombinant VEGF in the absence or presence of increasing concentrations of pooled normal human serum, resulting in final assay concentrations of human serum (0–33%). Bio-Glo™ Reagent was added and luminescence was quantified using the GloMax® Discover System. Data were fitted to a 4PL curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

Promega

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
VEGF Bioassay	**1 each**	**GA2001**

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 KDR/NFAT-RE HEK293 Cells, 2×10^7 cells/ml (0.5ml per vial)
- 60ml DMEM Medium
- 4ml Fetal Bovine Serum
- 1 vial Bio-Glo™ Luciferase Assay Substrate (lyophilized)
- 10ml Bio-Glo™ Luciferase Assay Buffer

PRODUCT	SIZE	CAT.#
VEGF Bioassay 5X	**5 each**	**GA2005**

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 KDR/NFAT-RE HEK293 Cells, 2×10^7 cells/ml (0.5ml per vial)
- 5 × 60ml DMEM Medium
- 5 × 4ml Fetal Bovine Serum
- 5 vials Bio-Glo™ Luciferase Assay Substrate (lyophilized)
- 5 × 10ml Bio-Glo™ Luciferase Assay Buffer

Note: VEGF Bioassay components are shipped separately because of differing temperature requirements. The KDR/ NFAT-RE HEK293 Cells are shipped on dry ice. The Bio-Glo™ Luciferase Assay System and Fetal Bovine Serum are shipped on dry ice, separately from the cells. The DMEM Medium is shipped at ambient temperature.

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™ Luciferase Assay Substrate, Bio-Glo™ Luciferase Assay Buffer and Fetal Bovine Serum at –30°C to –10°C. Avoid multiple freeze-thaw cycles of the serum.
- For optimal performance, use reconstituted Bio-Glo™ Reagent on the day of preparation. Once reconstituted, Bio-Glo™ Reagent can be stored at –30°C to –10°C for up to 6 weeks.
- Store DMEM Medium at +2°C to +10°C protected from light. Minor variations in the color of the DMEM Medium may be observed. The color change will not impact assay performance.

3. Before You Begin

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

Note the catalog number and lot number from the cell vial box label. This information can be used to download documents for the specified product from the website, such as Certificate of Analysis.

The VEGF Bioassay is intended to be used with user-provided antibodies or other biologics designed to activate or inhibit the VEGF/KDR signaling pathway. The recommended cell plating density, induction time and assay buffer components described in Section 4 were established using research-grade recombinant human VEGF and bevacizumab (anti-VEGF monoclonal antibody). You may need to adjust the parameters provided here and optimize assay conditions for your own antibodies or other biologic samples. Data generated using these reagents is shown above and below in Section 7.A, Representative Assay Results.

The VEGF Bioassay produces a bioluminescent signal and requires a sensitive luminometer or luminescence plate reader for the detection of luminescence. Bioassay development and performance data included in this Technical Manual were generated using the GloMax® Discover System and GloMax® Discover System. An integration time of 0.5 second/well was used for all readings. The bioassay is compatible with most other plate-reading luminometers; however, relative luminescence unit 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.

3.A. Materials to Be Supplied by the User

Composition of buffers and solutions is provided in Section 7.C.

Reagents

- Recombinant VEGF (Cat.# J2371) or equivalent recombinant human VEGF-165 or 121aa.
- user-defined anti-VEGF or KDR antibodies or other biologics samples

Supplies and Equipment

- white, flat-bottom 96-well assay plates (e.g., Corning® Cat.# 3917)
- sterile clear 96-well plate with lid (e.g., Corning® Cat.# 3896 or Linbro Cat.# 76-223-05) for preparing sample dilutions
- pipettes (single-channel and 12-channel)
- sterile 15ml and 50ml conical tubes
- sterile reagent reservoirs (e.g., Corning® Cat.# 4870)
- 37°C, 5% CO_2 humidified incubator
- 37°C water bath
- plate reader that measures glow luminescence or luminometer (e.g., GloMax® Discover System)

4. Assay Protocol

The VEGF Bioassay can be used in two different formats: VEGF Stimulation (described in Section 4.E) or Antibody Blockade (described in Section 4.F). This protocol illustrates the use of the VEGF Bioassay to test two test samples against a reference sample in a single assay run. Each test and reference sample 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.

Notes:

a. When preparing test and reference samples, choose an appropriate starting concentration and dilution scheme to achieve a full 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 threefold serial dilutions when testing bevacizumab to achieve full dose-response curves. Antibody dilution schemes may need to be optimized for your antibodies/samples.

b. To minimize assay setup time, prepare antibody serial dilutions prior to thawing and plating cells.

c. When diluted as directed, each kit containing medium, serum, and 1 vial of KDR/NFAT-RE HEK293 Cells is sufficient for 120 wells (two 96-well plates using inner 60-well format). The thaw-and-use cells are for single use only and cannot be cultured or refrozen for second time use. Please plan your experiments accordingly to optimize the use of the thaw-and-use cells.

Promega

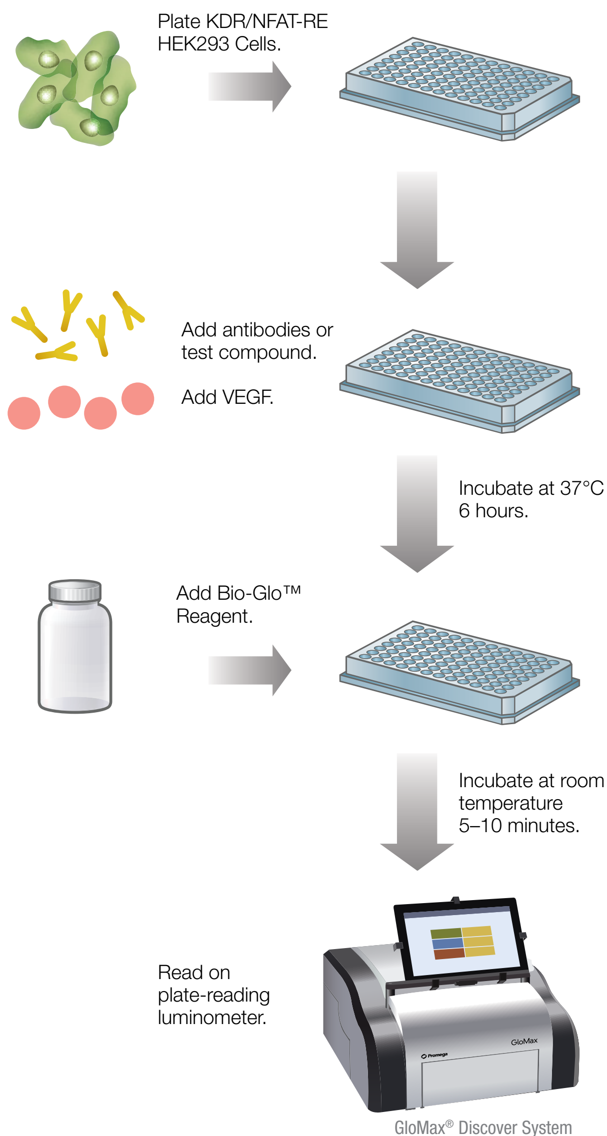

Figure 9. VEGF Bioassay schematic protocol.

Promega

4.A. Preparing Bio-Glo™ Reagent, Assay Buffer, and Test and Reference Samples

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 in a refrigerator 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.

If you are using a large (100ml) size of Bio-Glo™ Luciferase Assay System, dispense the reconstituted Bio-Glo™ Reagent into 10ml aliquots and store at –30°C to –10°C for up to 6 weeks. Avoid repeated freeze-thaw cycles. On the day of the assay, thaw the appropriate amount of reconstituted Bio-Glo™ Reagent in a room temperature water bath for at least 1–2 hours before use. Approximate stability of Bio-Glo™ Reagent after reconstitution is 18% loss of luminescence after 24 hours at ambient temperature and 12% loss of luminescence after 5 days at 4°C.

Assay Buffer: Ensure that an appropriate amount of assay buffer is prepared for the assay. Thaw the fetal bovine serum (FBS) overnight at 4°C, or in a 37°C water bath taking care not to overheat it. To make 40ml of assay buffer, add 4ml of FBS to 36ml DMEM medium to yield 90% DMEM/10% FBS. Mix well and warm to 37°C prior to use. For reference, 40ml of assay buffer is typically sufficient for 120 wells in a 96-well assay format using the inner 60 wells.

Note: The recommended assay buffer contains 10% FBS. This concentration of FBS works well for all of the anti-VEGF and anti-KDR antibodies we have tested. If you experience assay performance issues when using this assay buffer, we recommend testing serum concentrations in the range of 0.5–10%.

Test and Reference Samples: Prepare starting dilutions (dilu1, 3X final concentration) of test and reference samples (see Figures 10 and 11). Using assay buffer as the diluent, prepare 300µl of reference sample starting dilution and 150µl of each test sample starting dilution in 1.5ml tubes. Store the tubes containing starting dilutions appropriately before making serial dilutions.

4.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 samples 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/drug
C	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test Ab/drug
D	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference Ab/drug
E	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test Ab/drug
F	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference Ab/drug
G	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test Ab/drug
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 and reference antibody/drug dilution series and wells containing assay buffer (denoted by "B") alone.

On the morning of the VEGF Bioassay, prepare an appropriate amount of assay buffer as described in Section 4.A.

1. Dispense 75µl of prewarmed assay buffer into the outermost wells, labeled "B" in Figure 10, of both assay plates.
2. Cover the plates with lids and proceed to Section 4.C.

4.C. Preparing Serial Dilutions

Note: Serial dilutions should be prepared on the day of assay.

The instructions described here are for preparation of a single stock of threefold serial dilutions of a single sample 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 threefold serial dilutions, you will need 300µl of a reference sample at 3X the highest concentration in your dose-response curve. You will need 150µl of each test sample at 3X the highest concentration in each of the test sample dose-response curves. For other dilution schemes, adjust the volumes accordingly.

Notes on recommended starting concentrations of reference samples:

a. For VEGF Stimulation using Promega Recombinant VEGF (Cat.# J2371) as your reference sample, we recommend a 3X starting concentration of 3µg/ml and performing threefold serial dilutions.

b. For Antibody Blockade using bevacizumab as your reference sample, we recommend a 3X starting concentration of 18µg/ml and performing threefold serial dilutions.

1. To a sterile clear 96-well plate, add 150µl of reference sample starting dilution (dilu1, 3X final concentration) to wells A11 and B11 (see Figure 11).
2. Add 150µl of test samples 1 and 2 starting dilution (dilu1, 3X final concentration) to wells E11 and G11, respectively.
3. Add 100µl of assay buffer to other wells in these four rows, from column 10 to column 2.
4. Transfer 50µl of the sample starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
5. Repeat equivalent threefold serial dilutions across the columns from right to left until you reach column 3. Remove 50µl from column 3 so that all wells contain 100µl. Do not dilute into column 2.
6. Cover the plate with a lid and set aside.

4.C. Preparing Serial Dilutions (continued)

Recommended Plate Layout for Sample Dilutions Prepared from a Single Sample 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 sample
B		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Reference sample
C													
D													
E		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Test sample 1
F													
G		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Test sample 2
H													

Figure 11. Example plate layout showing reference and test sample serial dilutions.

Note: Wells A2, B2, E2 and G2 contain 100µl of assay buffer without sample as a negative control.

4.D. Preparing and Plating KDR/NFAT-RE HEK293 Cells

The thaw-and-use KDR/NFAT-RE HEK293 Cells included in this kit are sensitive; carefully 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. We recommend that you thaw and dilute a maximum of two vials of thaw-and-use cells at a time.

Follow the steps below using aseptic technique in a sterile cell culture hood.

1. Remove one vial of KDR/NFAT-RE HEK293 Cells from storage at –140°C and transfer to the bench on dry ice.
2. Add 4.6ml of prewarmed (37°C) assay buffer to a 15ml conical tube.
3. Warm the cells in a 37°C water bath until just thawed (about 2 minutes). While thawing, gently agitate and visually inspect. Try not to submerge the vial completely. Do not invert.
4. Gently mix the cell suspension by pipetting, then transfer 0.4ml of the cells to the 15ml conical tube containing 4.6ml of assay buffer. Mix well by gently pipetting or inverting 1–2 times.
5. Transfer the cell suspension to a sterile reagent reservoir.
6. Using a multichannel pipette, immediately dispense 25µl of the cell suspension to each of the inner 60 wells of two 96-well assay plates. Optimal results depend on gently keeping the cells evenly resuspended during the plating process.
7. Cover each assay plate with a lid. Continue on to either Section 4.E for VEGF/Inducer Stimulation Assay or Section 4.F for Antibody Blockade Assay.

4.E. VEGF/Inducer Stimulation Assay

1. Prepare 3X inducer (VEGF) serial dilution in assay buffer as described in Section 4.C. For Recombinant VEGF (Cat. # J2371), we recommend a 3X starting concentration of 3µg/ml.
2. Using a multichannel pipette, dispense 25µl of the sample/inducer dilutions to the 25µl of preplated cells according to the plate layout in Figure 10.
3. Dispense an additional 25µl of assay buffer to each well to bring the volume in the assay well up to 75µl.
4. Cover each assay plate with a lid and incubate in a humidified 37°C, 5% CO_2 incubator for 6 hours.
5. After the 6-hour incubation is over, proceed to Section 4.G.

Promega

4.F. Antibody Blockade Assay

1. Prepare 3X antibody serial dilution in assay buffer as described in Section 4.C. If you are using bevacizumab as a reference sample, we recommend a 3X starting concentration of 18µg/ml, although this may need to be optimized for your particular reference sample.
2. Prepare 3X VEGF at EC_{80}–EC_{90} concentration in assay buffer. This concentration should be determined using the VEGF Stimulation Assay protocol above, as each batch of VEGF may have slightly different potency. The expected EC_{80} of VEGF is approximately 20ng/ml (3X = 60ng/ml), and the expected EC_{90} is approximately 50ng/ml (3X = 150ng/ml).
3. Using a multichannel pipette, dispense 25µl of the 3X antibody serial dilutions prepared in Step 1 to the 25µl of preplated cells according to the plate layout in Figure 10.
4. Using a multichannel pipette, dispense 25µl of the 3X EC_{80} VEGF mixture prepared in Step 2 to the inner 60 assay wells. The final assay volume is 75µl.
5. Cover each assay plate with a lid and incubate in a humidified 37°C, 5% CO_2 incubator for 6 hours.
6. After the 6-hour incubation is over, proceed to Section 4.G.

4.G. Adding Bio-Glo™ Reagent

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

1. Remove the assay plates from the incubator, remove the plate lid, and equilibrate to ambient temperature for 10–15 minutes.
2. Using a multichannel pipette, add 75µl of Bio-Glo™ Reagent to the inner 60 wells of the assay plates, taking care not to create bubbles.
3. Add 75µl of Bio-Glo™ Reagent to wells B1, C1 and D1 of each assay plate to measure the background signal if desired.
4. Incubate at ambient temperature for 5–10 minutes.

   Note: Varying the incubation time will affect the raw RLU values but should not significantly change the EC_{50} value and fold induction.
5. Measure luminescence using a luminometer or luminescence plate reader.

4.H. Data Analysis

1. Measure plate background by calculating the average relative light units (RLU) from wells B1, C1 and D1.
2. Calculate fold induction = $\frac{\text{RLU (induced–background)}}{\text{RLU (no antibody control–background)}}$

   Note: When calculating fold induction, if the no-antibody control sample RLU are at least 100X the plate background RLUs, there is no need to subtract plate background from sample RLU.
3. Graph data as RLU versus Log_{10} [sample] and fold induction versus Log_{10} [sample]. 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-reading luminescence detection. Instruments designed primarily for fluorescence detection are not recommended. Luminometers measure and report luminescence as relative values, and actual RLU numbers will vary between instruments.
	Insufficient cells per well can lead to low RLU. Handle and plate the cells according to the instructions to ensure a sufficient number of viable cells per well.
	Low activity of Bio-Glo™ Reagent leads to low RLU. Store and handle the Bio-Glo™ Reagent according to the instructions.
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 in the VEGF Bioassay may vary from the EC_{50} obtained using other methods such as primary HUVEC-based assays.
	Ensure the assay incubation period is 6 hours and not overnight. Overnight assay incubation does not work for this assay (see Figure 16).
	Ensure that cells are plated in the assay plate immediately prior to performing the assay. Cells cannot be plated in the assay plate overnight prior to assaying (see Figure 17).
	If untreated control RLU is less than 100X above plate reader background RLU, subtract plate reader background RLU from all samples prior to calculating fold induction.
Assay performance is variable	Ensure that incubation times are consistent between assays.

6. 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 antiangiogenic tumor therapy. *Biol. Pharm. Bull.* **34**, 1785–88.
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**, 212–18.
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.
6. Staton, C.A. *et al.* (2009) A critical analysis of current in vitro and in vivo angiogenesis assays. *Int. J. Exp. Path.* **90**, 195–221.

7. Appendix

7.A. Representative Assay Results

The following data were generated using the VEGF Bioassay, thaw-and-use cells using VEGF to stimulate (Figure 12); or bevacizumab as an antibody blockade (Figure 15).

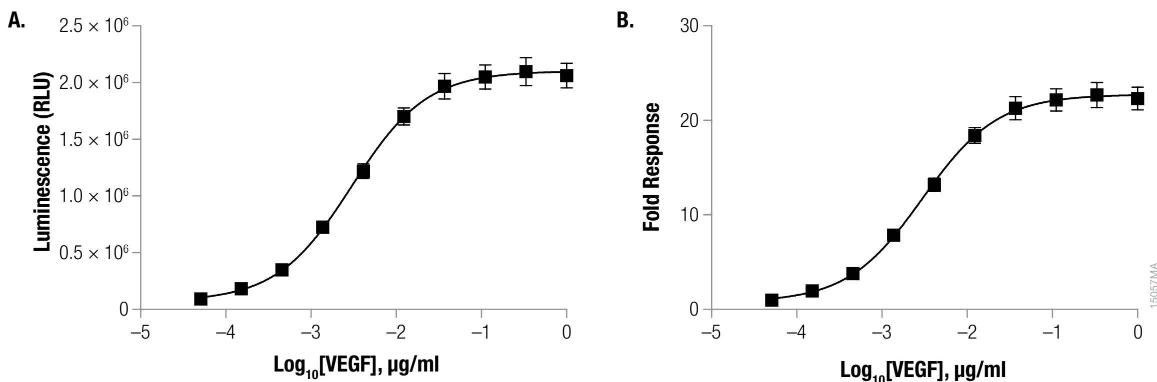

Figure 12. The VEGF Bioassay measures the potency of recombinant VEGF. KDR/NFAT-RE HEK293 Cells were plated and data was generated as indicated in the protocol. Cells were incubated with increasing concentrations of recombinant VEGF for 6 hours before luminescence measurement. Bio-Glo™ Reagent was added and luminescence was quantified using the GloMax® Discover System. Data were analyzed using GraphPad Prism® software. Data are plotted as RLU (**Panel A)** or fold response over untreated (**Panel B**). The EC_{50} of VEGF was approximately 3ng/ml.

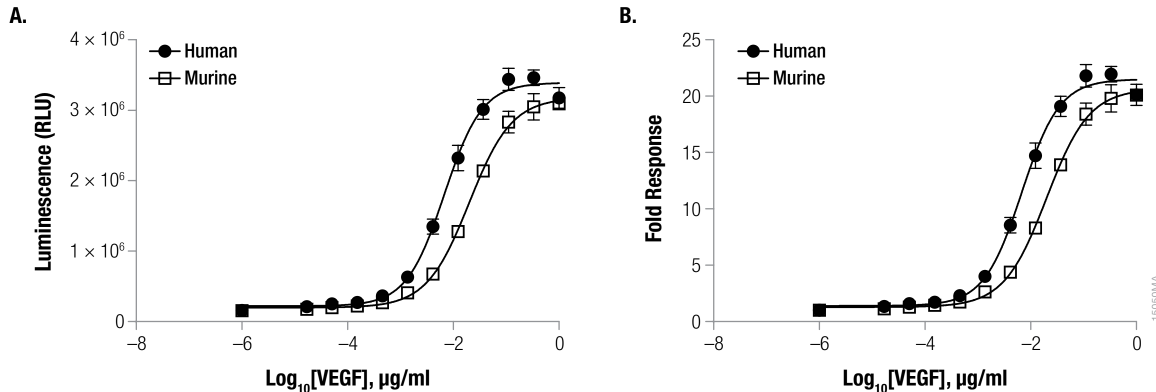

Figure 13. The VEGF Bioassay measures the potency of murine VEGF. KDR/NFAT-RE HEK293 Cells were plated and data was generated as indicated in the protocol. Cells were incubated with increasing concentrations of either recombinant human VEGF or recombinant murine VEGF for 6 hours before luminescence measurement. Bio-Glo™ Reagent was added and luminescence was quantified using the GloMax® Discover System. Data were analyzed using GraphPad Prism® software. Data are plotted as RLU (**Panel A**) or fold response over untreated (**Panel B**). The EC_{50} of human VEGF was approximately 6.8ng/ml, and the EC_{50} of murine VEGF was approximately 20ng/ml. Data was generated using thaw-and-use cells.

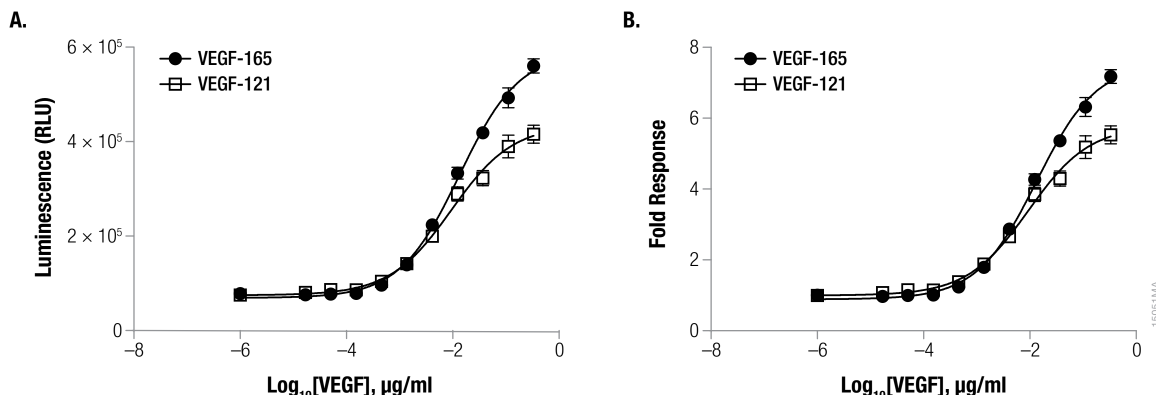

Figure 14. The VEGF Bioassay measures the potency of various VEGF isoforms. KDR/NFAT-RE HEK293 Cells were plated and data was generated as indicated in the protocol. Cells were incubated with increasing concentrations of either recombinant human VEGF-165 or recombinant human VEGF-121 for 6 hours before luminescence measurement. Bio-Glo™ Reagent was added and luminescence was quantified using the GloMax® Discover System. Data were analyzed using GraphPad Prism® software. Data are plotted as RLU (**Panel A**) or fold response over untreated (**Panel B**). VEGF-165 resulted in a fold response of 7 and an EC_{50} of approximately 13ng/ml. VEGF-121 resulted in a fold response of 5 and an EC_{50} of approximately 9ng/ml. Data were generated using cell propagation model cells.

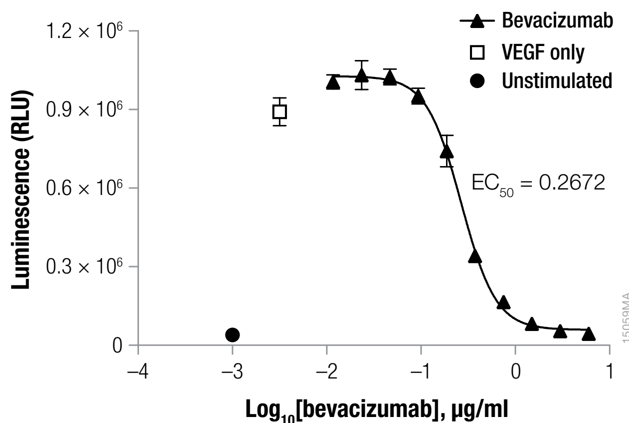

Figure 15. The VEGF Bioassay measures the potency of bevacizumab antibody. KDR/NFAT-RE HEK293 thaw-and-use cells were thawed and data was generated as indicated in the protocol. Cells were incubated with increasing concentrations of bevacizumab in the presence of an EC_{90} concentration of recombinant VEGF for 6 hours before luminescence measurement. Bio-Glo™ Reagent was added and luminescence measured using the GloMax® Discover System. Data were fitted to a 4PL curve using GraphPad Prism® software. The IC_{50} of bevacizumab was approximately 0.26µg/ml.

7.B. Assay Troubleshooting

The following data were generated using the VEGF Bioassay, demonstrating optimal assay incubation time (Figure 16) and the requirement for plating cells on the day of the assay (Figure 17).

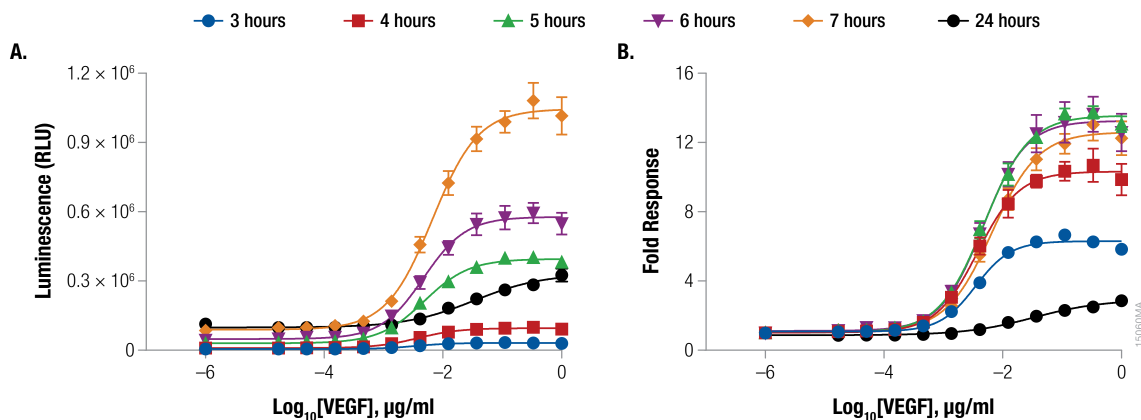

Figure 16. The VEGF Bioassay requires a 6 hour assay incubation. KDR/NFAT-RE HEK293 Cells were used (Cell Propagation Model) and data was generated as indicated in the protocol. Cells were incubated with increasing concentrations of recombinant VEGF for 3, 4, 5, 6, 7 or 24 hours before luminescence measurement. Bio-Glo™ Reagent was added and luminescence was quantified using the GloMax® Discover System. Data were analyzed using GraphPad Prism® software. Data are plotted as RLU (**Panel A**) or fold response over untreated (**Panel B**).

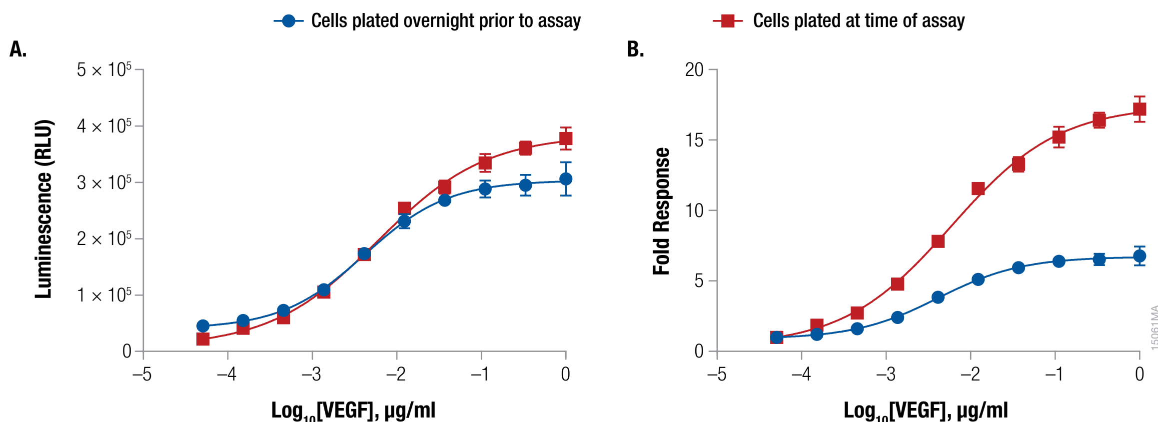

Figure 17. The VEGF Bioassay requires cells to be plated at time of assay. KDR/NFAT-RE HEK293 thaw-and-use cells were thawed according to protocol and plated in a 96-well assay plate either: 1) the day before the assay, followed by an overnight incubation at 37°C, 5% CO_2; or 2) immediately prior to assay. Data was generated as indicated in the protocol. Cells were incubated with increasing concentrations of recombinant VEGF for 6 hours before luminescence measurement. Bio-Glo™ Reagent was added and luminescence was quantified using the GloMax® Discover System. Data were analyzed using GraphPad Prism® software. Data are plotted as RLU (**Panel A**) or fold response over untreated (**Panel B**). The EC_{50} of VEGF was approximately 5ng/ml.

7.C. Composition of Buffers and Solutions

Assay Buffer

90% DMEM
10% FBS

8. Summary of Changes

The following changes were made to the 5/26 revision of this document:

1. Removed Section 7.D, Related Products.
2. Made minor text and formatting edits.

Promega

[a]NOT FOR MEDICAL DIAGNOSTIC USE. FOR IN VITRO USE ONLY. BY USE OF THIS PRODUCT, RECIPIENT AGREES TO BE BOUND BY THE TERMS OF THIS LIMITED USE STATEMENT. If the recipient is not willing to accept the conditions of this limited use statement, and the product is unused, Promega will accept return of the unused product and provide the recipient with a full refund.

This product may not be further sold or transferred by the recipient and may be used only by the recipient, and then only for (1) research use, (2) discovery, development and monitoring of biologic drugs and vaccines, (3) quality assurance testing of biologic drugs and vaccines, and (4) product release assays for biologic drugs and vaccines. No other commercial use is allowed. “Commercial use” means any and all uses of this product by recipient for monetary or other consideration, including providing a service, information or data to unaffiliated third parties, and resale of this product for any use. Recipient has no right to propagate, modify, derivatize, genetically engineer or otherwise create variations of the cells or genes stably transfected within the cells. In addition, recipient must use Bio-Glo™ Luciferase Assay System purchased from Promega Corporation for all luminescence assays using this product or contact Promega to obtain a license for use of this product with reagents other than Promega’s. PROMEGA MAKES NO REPRESENTATIONS OR WARRANTIES OF ANY KIND, EITHER EXPRESSED OR IMPLIED, INCLUDING AS TO MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE WITH REGARDS TO THIS PRODUCT. The terms of this agreement shall be governed under the laws of the State of Wisconsin, USA.

[b]U.S. Pat. No. 10,077,244 and other patents.

[c]HEK293 cells were obtained under license from AdVec Inc.

© 2018–2026 Promega Corporation. All Rights Reserved.

GloMax and Lumit are registered trademarks of Promega Corporation. Bio-Glo and Bio-Glo-NL are trademarks of Promega Corporation.

Accutase is a registered trademark of Innovative Cell Technologies, Inc. Corning is a registered trademark of Corning, Inc. GraphPad Prism is a registered trademark of GraphPad Software, Inc. JMP is a registered trademark of SAS Institute, Inc.

Products may be covered by pending or issued patents or may have certain limitations. Please visit our website for more information.

All prices and specifications are subject to change without prior notice.

Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.