TECHNICAL MANUAL

VEGF Bioassay, Propagation Model

Instructions for Use of Product
GA1082

Revised 5/26
TM545

Promega

VEGF Bioassay, Propagation Model

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 9
   3.A. Materials to Be Supplied by the User 10
   3.B. Preparing VEGF Bioassay cells 10
4. Assay Protocol 12
   4.A. Preparing and Plating KDR/NFAT-RE HEK293 Cells 14
   4.B. Preparing Bio-Glo™ Reagent, Assay Buffer, and Test and Reference Samples 15
   4.C. Plate Layout Design 16
   4.D. Preparing Serial Dilutions 17
   4.E. Preparing Cells for Assay 19
   4.F. VEGF/Inducer Stimulation Assay 19
   4.G. Antibody Blockade Assay 20
   4.H. Adding Bio-Glo™ Reagent 20
   4.I. Data Analysis 20
5. Troubleshooting 21
6. References 23
7. Appendix 23
   7.A. Representative Assay Results 23
   7.B. Assay Troubleshooting 26
   7.C. Composition of Buffers and Solutions 29
8. Summary of Changes 29

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, Propagation Model[a,b] (Cat.# GA1082) 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 Cell Propagation Model (CPM) format, which includes cryopreserved cells that can be thawed, propagated and banked for long-term use (also offered in a thaw-and-use format; Cat.# GA2001, GA2005).

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 .

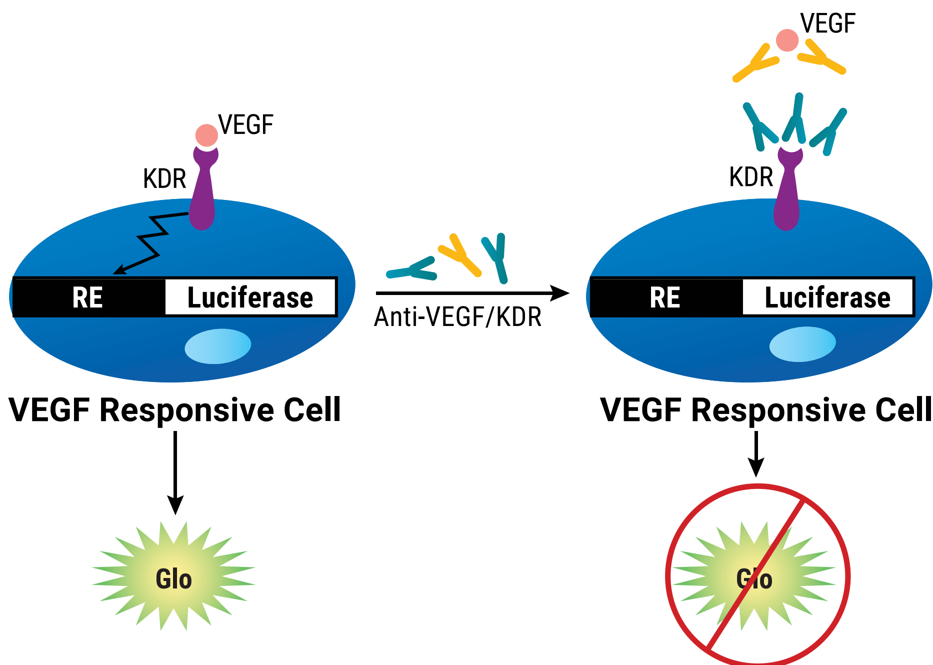

Figure 1. Representation of the VEGF Bioassay. The VEGF Bioassay consists of a genetically engineered cell line, KDR/NFAT-RE HEK293 Cells. When VEGF binds to KDR, receptor-mediated signaling induces luminescence that can be detected by adding Bio-Glo™ Reagent and quantitated with a luminometer. Inhibition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

1. Description (continued)

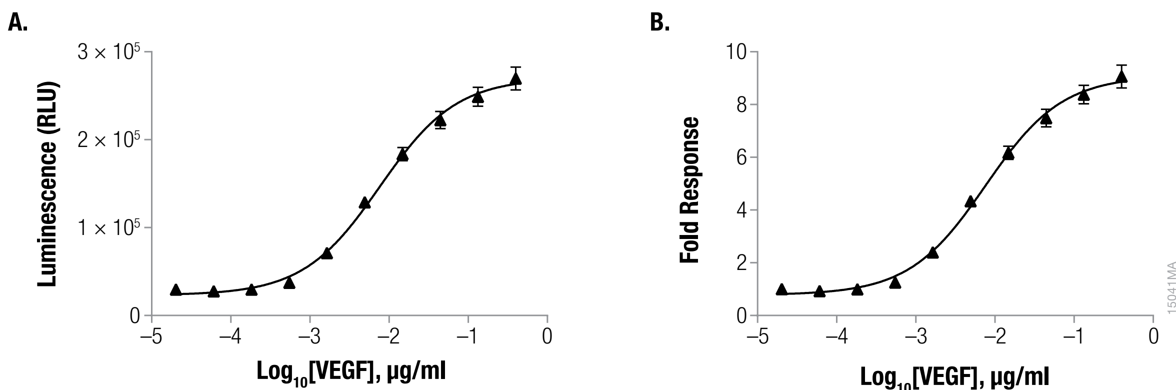

Figure 2. The VEGF Bioassay response to recombinant VEGF. KDR/NFAT-RE HEK293 Cells were grown as described in this protocol, and incubated with serial dilutions of recombinant VEGF. After a 6-hour incubation, Bio-Glo™ Reagent was added and luminescence was quantified using the GloMax® Discover System.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

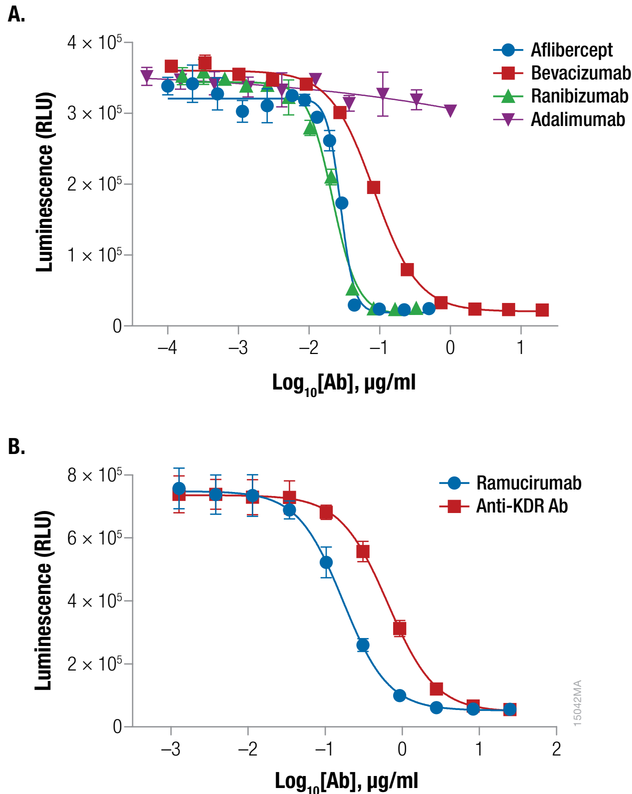

Figure 3. The VEGF Bioassay can be used to measure the activity of antibodies to either VEGF or KDR. KDR/NFAT-RE HEK293 Cells were incubated with serial dilutions of antibodies to either VEGF (**Panel A**) or KDR (**Panel B**), in the presence of an EC_{80} concentration of recombinant VEGF. After a 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.

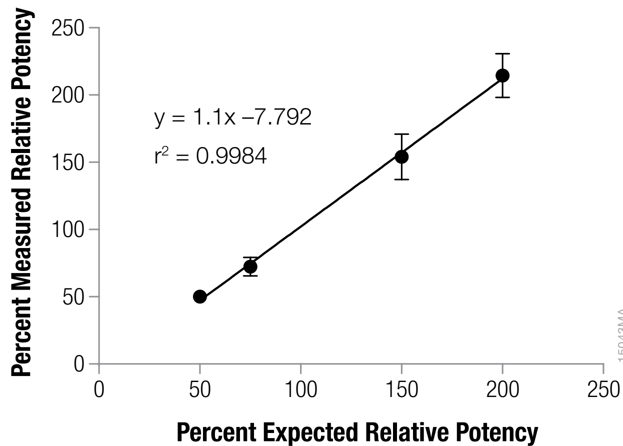

Figure 4. The VEGF Bioassay shows precision, accuracy and linearity. A 50–200% theoretical potency series of bevacizumab (anti-VEGF antibody) was analyzed in triplicate in three independent experiments performed on three days by each of two analysts using the VEGF Bioassay (for a total of six independent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

Promega

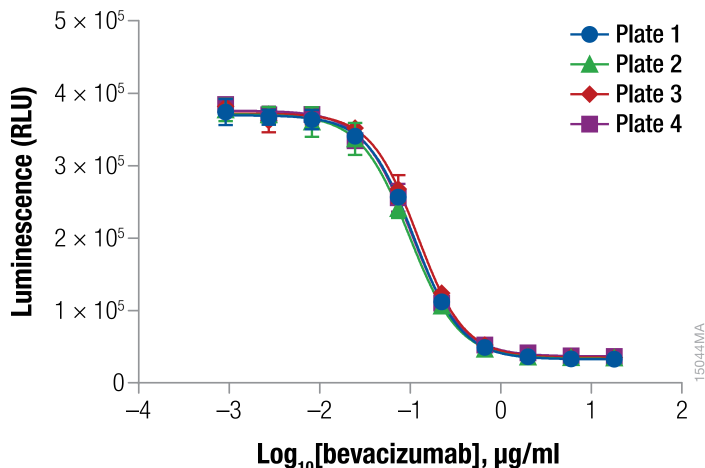

Figure 5. The VEGF Bioassay demonstrates repeatability. Four separate serial dilution series of bevacizumab (anti-VEGF antibody) were analyzed on four individual assay plates using the VEGF Bioassay. Bio-Glo™ Reagent was added and luminescence was quantified using the GloMax® Discover System. Data were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

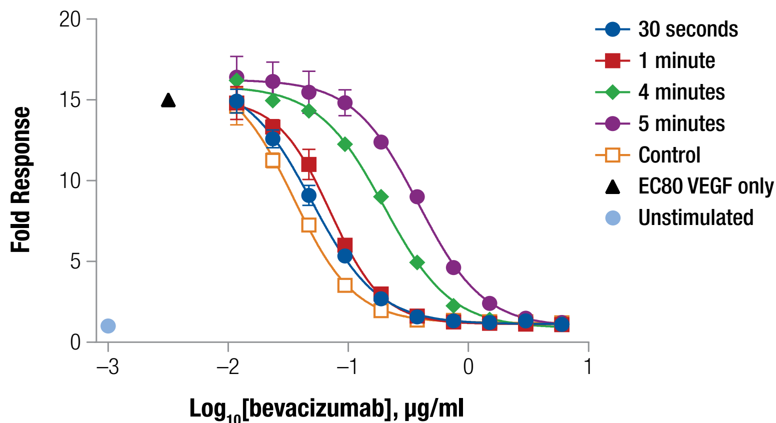

Figure 6. The VEGF Bioassay indicates stability. Samples of bevacizumab were maintained at 4°C (control) or heat-treated (80°C) for increasing lengths of time (30 seconds to 5 minutes), then analyzed using the VEGF Bioassay. Bio-Glo™ Reagent was added and luminescence was quantified using the GloMax®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

Promega

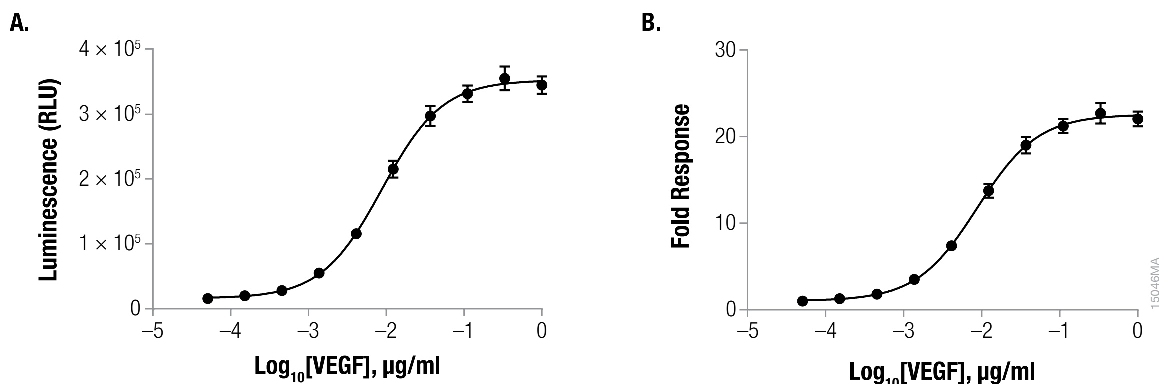

Figure 7. The assay is amenable to 384-well plate format. The VEGF Bioassay was tested in a 384-well format. KDR/NFAT-RE HEK293 Cells were plated at 15,000 cells/well in 10µl volume. A threefold serial dilution of recombinant VEGF was added at 10µl per well, and 10µl of media was added to bring the volume 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.

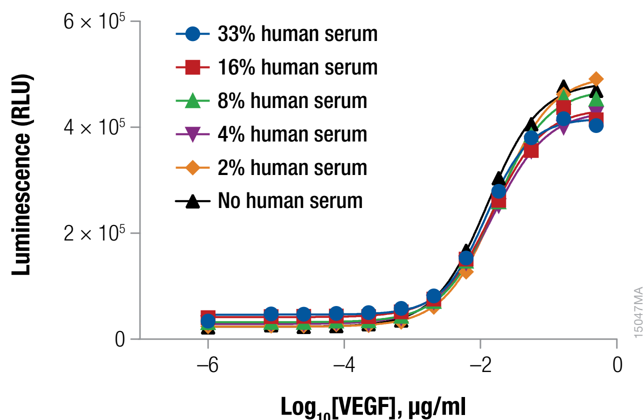

Figure 8. The VEGF Bioassay tolerates human serum. KDR/NFAT-RE HEK293 Cells were tested with a dose-response of recombinant VEGF in the absence or presence of increasing concentrations of pooled normal human serum, resulting in final assay concentrations of human serum (0–33%). Bio-Glo™ Reagent was added 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.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
VEGF Bioassay, Propagation Model	**1 each**	**GA1082**

Not for Medical Diagnostic Use. Includes:

- 2 vials KDR/NFAT-RE HEK293 Cells, 1×10^7 cells/ml (1ml per vial)

Note: Thaw and propagate one vial to create frozen cell banks before use in an assay. Reserve the second vial for future use.

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

3. Before You Begin

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

Cell thawing, propagation and banking should be performed exactly as described in Section 3.B. Cell seeding and propagation densities have been optimized to ensure stable cell growth, which is reflected in a steady cell doubling rate, to achieve optimal, consistent performance.

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.

Promega

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 (121aa or 165aa isoform)
- user-defined anti-VEGF or KDR antibodies or other biologics samples
- DMEM medium with 4.5g/L glucose, L-glutamine and sodium pyruvate (e.g., Corning® Cat.# 10-013-CV or GIBCO® Cat.# 11995)
- fetal bovine serum (e.g., HyClone Cat.# SH30070.02 or Corning® Cat.# 35-015-CV)
- hygromycin B (e.g., GIBCO® Cat.# 10687-010)
- G418 sulfate solution (e.g., GIBCO® Cat.# 10131035)
- DPBS Ca^{++}, Mg^{++} free (e.g., GIBCO® Cat.# 14190)
- Accutase® solution (e.g., Sigma Cat.# A6964)
- DMSO (e.g., Sigma Cat.# D2650)
- Trypan blue solution (e.g., Sigma Cat.# T8154)
- Bio-Glo™ Luciferase Assay System (Cat.# G7940 or G7941)

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)

3.B. Preparing VEGF Bioassay cells

Cell Thawing and Initial Cell Culture

1. Prepare 40ml of initial cell culture medium by adding 4ml of FBS to 36ml of DMEM prewarmed to 37°C. This initial cell culture medium will be used for culturing the cells immediately after thawing.
2. Transfer 9ml of prewarmed initial cell culture medium to a 15ml conical tube.
3. Remove one vial of KDR/NFAT-RE HEK293 Cells from storage at –140°C and thaw in a 37°C water bath with gentle agitation (no inversion) until just thawed (typically 2–3 minutes).
4. Spray vial with 70% ethanol and transfer to cell culture hood.
5. Transfer all of the cells (approximately 1ml) to the 15ml conical tube containing 9ml of prewarmed initial cell culture medium.

6. Centrifuge at 200 × *g* for 5 minutes.
7. Carefully aspirate the medium, and resuspend the cell pellet in 30ml of prewarmed initial cell culture medium.
8. Count cells with trypan blue and determine cell number and viability.
9. Transfer the cell suspension to a T150 tissue culture flask, and place the flask horizontally in a humidified 37°C, 5% CO_2 incubator.

   Note: We do not recommend seeding cells below 1.5×10^4 cells/cm^2. If there are fewer viable cells than necessary for a T150 flask (~2.25×10^6 cells), seeding in a T75 flask is a better option.
10. Incubate for 1–3 days, until cells are approximately 80–90% confluent before passaging the cells. Check flasks daily to ensure cells do not get overly confluent.

Cell Maintenance and Propagation

Note: For cell maintenance and propagation starting from the second cell passage, use growth medium containing selection antibiotics (see Section 7.C), and monitor cell viability and doubling rate during propagation. The cell growth rate will stabilize by approximately 1 week post-thaw. At this time, the cell viability is typically >95% and the average cell doubling rate is 22–25 hours. Passage number should be recorded for each passage. Cells are expected to retain their functionality for greater than 35 passages.

11. On the day of cell passage, visualize cells under microscope and estimate confluency.
12. To harvest cells for passaging, aspirate medium and gently rinse cell monolayer with DPBS, being careful not to disrupt the cell monolayer. Carefully aspirate DPBS and add prewarmed Accutase® to flask.

    Note: We recommend using 3ml for a T75 flask, 6ml for a T150 flask, or 9ml for a T225 flask. Scale according to surface area of flask.
13. Incubate with Accutase® for 2–5 minutes at 37°C, inspecting flask periodically to see if cells have started to detach. Gently pat the sides of the flask to detach cells.
14. Once cells detach, add an equal amount of growth medium to the Accutase® and pipet to gently break up cell clumps. Count cells with trypan blue and seed cells in a new flask according to the steps outlined below.
15. For daily cell maintenance, adjust cell seeding density based on the length of cell culture (2 days, 3 days or 4 days). Maintain cells between 5%–95% confluency. Do not allow cells to become 100% confluent prior to passaging as this can affect performance in subsequent passages.
16. Recommended seeding density for passaging cells is as follows:
    a. For 2-day culture: ~50,000–60,000 cells/cm^2 of flask (i.e., 4.5 million cells in a T75 flask)
    b. For 3-day culture: ~20,000–30,000 cells/cm^2 of flask (i.e., 2.25 million cells in a T75 flask)
    c. For 4-day culture: ~10,000–15,000 cells/cm^2 of flask (i.e., 1 million cells in a T75 flask)

    Note: We recommend using the following media volumes for routine cell propagation: 15ml for a T75, 30ml for a T150 and 45ml for a T225. Scale according to surface area of flask.
17. Place the flasks horizontally in a humidified 37°C, 5% CO_2 incubator.

Note: It is important to maintain a consistent passaging regimen to obtain consistent assay results.

Cell Freezing and Banking

Note: We recommend making a cell bank at the earliest possible passage.

18. On the day of cell freezing, make new cell freezing medium and keep on ice.
19. Grow cells as indicated in protocol above. When cells are approximately 50–80% confluent, harvest cells as described above.
20. Remove a sample for cell counting by trypan blue staining. Calculate the volume of freezing medium needed based on desired cell freezing density. We recommend a range of 2×10^6–2×10^7 cells/ml.
21. Transfer cells to 50ml sterile conical tubes or larger sized centrifuge tubes, and centrifuge cells at 200 × *g* for 5–15 minutes.
22. Gently aspirate the supernatant, being careful not to disturb the cell pellet.
23. Carefully resuspend cell pellet in ice cold Freezing Medium to desired final cell density. Combine the cell suspensions into a single tube and dispense 1ml into cryovials.
24. Freeze using a controlled-rate freezer (or use an insulated Mr. Frosty® or a Styrofoam® type of cell freezing container at –80°C overnight)
25. Transfer to –140°C or below for long-term storage.

4. Assay Protocol

The VEGF Bioassay can be used in two different formats: VEGF Stimulation (described in Section 4.F) or Antibody Blockade (described in Section 4.G). 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.

KDR/NFAT-RE HEK293 Cells must be cultured in a very specific way for several days prior to the day of the assay (see Section 4.A). If this preculture protocol is not followed, the assay will be altered dramatically.

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. While comparisons across experiments are made, it is important to compare similarly performed experiments with respect to preculture protocols followed.

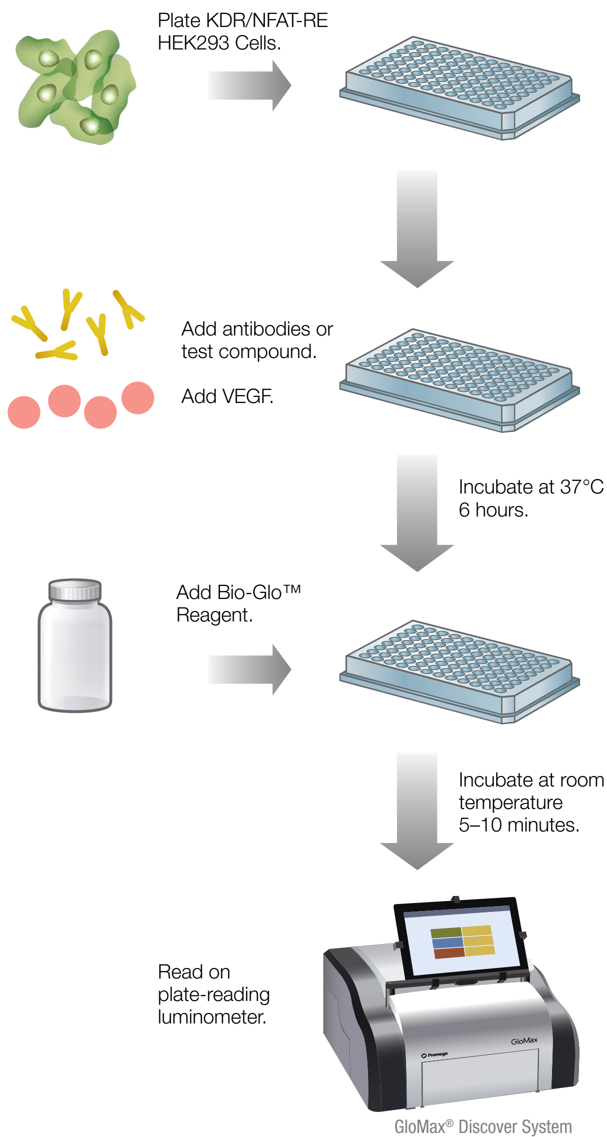

Figure 9. VEGF Bioassay schematic protocol.

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

While maintaining the KDR/NFAT-RE HEK293 Cells in culture, follow the recommended cell seeding density during routine propagation, because changes in cell culture volume or seeding density could affect the cell growth rate and assay performance. Only use cells in this assay after the doubling rate has stabilized during propagation.

Preparing cells for Assay

These cells need to be precultured in a precise way prior to using in an assay. If this exact preculture protocol is not followed, the assay results will be dramatically altered, and possibly the assay could fail (see Figure 17). This step is absolutely critical for a successful assay.

There are two possible preculture protocols for this assay: a 2-day incubation period and a 3-day incubation period. Each protocol will give similar results when compared to similar protocols followed, and these two options will allow more convenience for assays to be performed on desired days. See Figure 16 for example data generated using the different preculture protocols. Please note the cell seeding density for each incubation period, as they are different, and cannot be interchanged.

- For the 2-day incubation: cells can be seeded on Monday for assay on Wednesday, seeded Tuesday for assay on Thursday, or seeded Wednesday for assay on Friday.
- For the 3-day incubation: cells can be seeded on Monday for assay on Thursday, seeded Tuesday for assay on Friday, or seeded Friday for assay on Monday.

Day 1: Cell Seeding Day

1. Remove medium from cells, wash cells gently with DPBS (Ca^{++}, Mg^{++} free), harvest cells with Accutase®. Resuspend cells in 10ml growth medium.
2. Count cells by Trypan blue staining.
3. Plate cells in growth medium without selection antibiotics at the density indicated in the table below, for the desired incubation period. Cells must be seeded in this density range. If cells are seeded too sparsely, the assay window will suffer dramatically. It is preferable to have a slightly higher density as opposed to a lower density at this step (see Figure 17).
4. Incubate the cells for either 2 or 3 days (depending on seeding density) in a humidified 37°C, 5% CO_2 incubator. Do not change the media during this time. To ensure consistent results, cells must be incubated for the indicated number of days prior to assay. Cell seeding densities or incubation periods outside the indicated range will result in inconsistent results and possibly a failed assay.

Number of days to incubate:	Cell density to seed: (can be scaled according to flask size)	Expected cell density at harvest
2 days	250,000 cells/cm^2 (i.e., 19 million cells in a T75 flask)	500,000 to 600,000 cells/cm^2
3 days	66,000 cells/cm^2 (i.e., 5 million cells in a T75 flask)	200,000 to 350,000 cells/cm^2

Day 3 or 4: Assay day: Preparing reagents for assay

We recommend preparing all needed assay reagents prior to harvesting the cells. After reagents are prepared, continue on to Section 4.E.

4.B. 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 –20°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.C. 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.

4.D. 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:

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.

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 all wells have 100µl volume. Do not dilute into column 2.
6. Cover the plate with a lid and set aside.

4.D. 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.

Promega

4.E. Preparing Cells for Assay

1. Prepare assay medium. Store at 37°C until use.
2. Harvest cells for assay. Remove medium from cells, wash cells gently with DPBS (Ca^{++}, Mg^{++} free), and harvest cells with prewarmed Accutase®. Do not use trypsin to dissociate cells at this step. We recommend using 3ml for a T75 flask or 6ml for a T150 flask. Incubate cells with Accutase® at 37°C for approximately 2–3 minutes. Shorter exposure to Accutase® will lead to better assay results. Once cells begin detaching from the flask, firmly tap the flask to dislodge cells, add twice the volume of assay medium, and gently pipet several times to break up clumps (i.e., if 3ml of Accutase® was used to harvest cells, add 6ml assay medium). Count cells by Trypan blue staining. The optimal cell viability should be above 85%, and the cell density upon harvest should be within a range of approximately 200,000 to 350,000 cells/cm^2 (for 3 days preculture) or 500,000 to 600,000 cells/cm^2 (for 2 days preculture) to ensure optimal cell performance in the assay.
3. Centrifuge cells at 200 × *g* for 5 minutes. Carefully aspirate supernatant and resuspend cell pellet with assay medium to a density of 1.6×10^6 cells/ml. Dispense 25µl cells to each of the inner 60 wells of a white-bottom, 96-well assay plate, resulting in 4×10^4 cells per well.

   Note: Overly harsh centrifugation could adversely affect the cells at this step.
4. Add 75µl of prewarmed assay buffer into the outermost wells, labeled "B" in Figure 10.
5. Cover the plates with lids and keep at ambient temperature (22–25°C). Continue on to either Section 4.F for VEGF/Inducer Stimulation Assay or Section 4.G for Antibody Blockade Assay.

4.F. VEGF/Inducer Stimulation Assay

1. Prepare 3X inducer (VEGF) serial dilution in assay buffer as described in Section 4.D. 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.H.

4.G. Antibody Blockade Assay

1. Prepare 3X antibody serial dilution in assay buffer as described in Section 4.D. 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.H.

4.H. 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.I. Data Analysis

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

   Note: When calculating fold induction, if the no-antibody control sample RLUs 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 cell viability can lead to low luminescence readout and variability in assay performance.
	Low activity of Bio-Glo™ Reagent leads to low RLU. Store and handle the Bio-Glo™ Reagent according to the instructions.
	Ensure that cells are not incubated with Accutase® for more than 2–3 minutes when harvesting on the day of assay. Longer Accutase® incubation times will result in decreased RLUs and fold responses. If cells are not sufficiently detached after a 2–3 minute incubation period, firmly tap the flask to dislodge cells.
Assay performance is variable	Ensure that incubation times are consistent between assays.
	Ensure that the preculture protocol is strictly followed for either 2-day or 3-day incubation period. These two are not interchangeable and data generated from the 2-day protocol will be different from the data generated from the 3-day protocol.
	Cells must be treated the same way prior to assay for each assay. Variability in cell growth rates and preculture plating densities will result in variable assay results.
	Ensure that Accutase® incubation times are consistent when harvesting cells on the day of assay. Variability in Accutase® incubation times will result in variable assay results.

Promega

5. Troubleshooting (continued)

Symptoms	Causes and Comments
Weak assay response (low fold induction)	Optimize the concentration range of your test sample(s) to achieve a full dose response with complete upper and lower asymptotes. The EC_{50} value obtained 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 18).
	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 19).
	Ensure that the preculture protocol is followed exactly, and that cells are doubling approximately every 24 hours prior to starting the pre-culture period. Cells that are growing too slowly or plated too sparsely will not reach the appropriate cell densities during the pre-culture period (see Figure 17).
	Ensure that the media is not changed during the preculture period.
	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.
	Ensure that cells are not incubated with Accutase® for more than 2–3 minutes when harvesting on the day of assay. Longer Accutase® incubation times will result in decreased RLUs and fold responses. If cells are not sufficiently detached after a 2–3 minute incubation period, firmly tap the flask to dislodge cells.

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, Cell Propagation Model using VEGF to stimulate (Figure 12); or bevacizumab as an antibody blockade (Figure 15). The 3-day preculture incubation protocol was used.

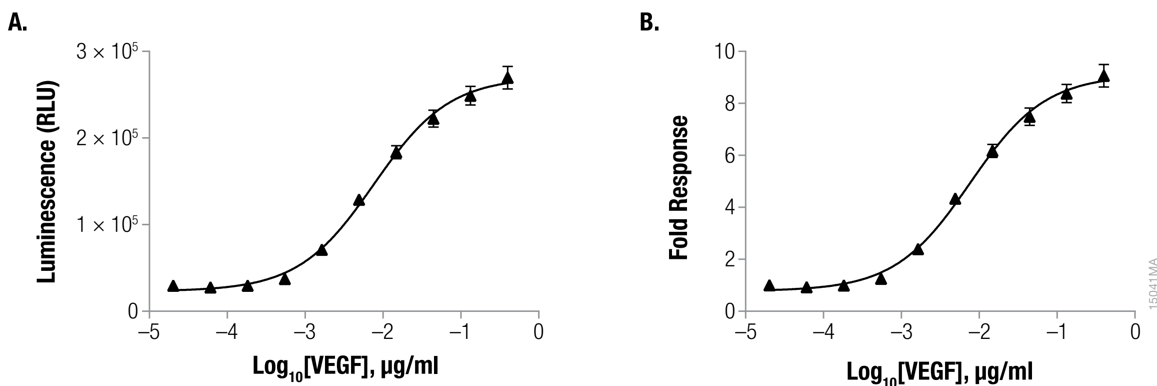

Figure 12. The VEGF Bioassay measures the potency of recombinant VEGF. KDR/NFAT-RE HEK293 Cells were plated and data was generated as indicated in the protocol. Cells were incubated with increasing concentrations of recombinant VEGF for 6 hours before luminescence measurement. Bio-Glo™ Reagent was added and luminescence was quantified using the GloMax® Discover System. Data were analyzed using GraphPad Prism® software. Data were plotted as RLU (Relative Light Units; **Panel A**) or fold response over untreated (**Panel B**). The EC_{50} of VEGF was approximately 7ng/ml.

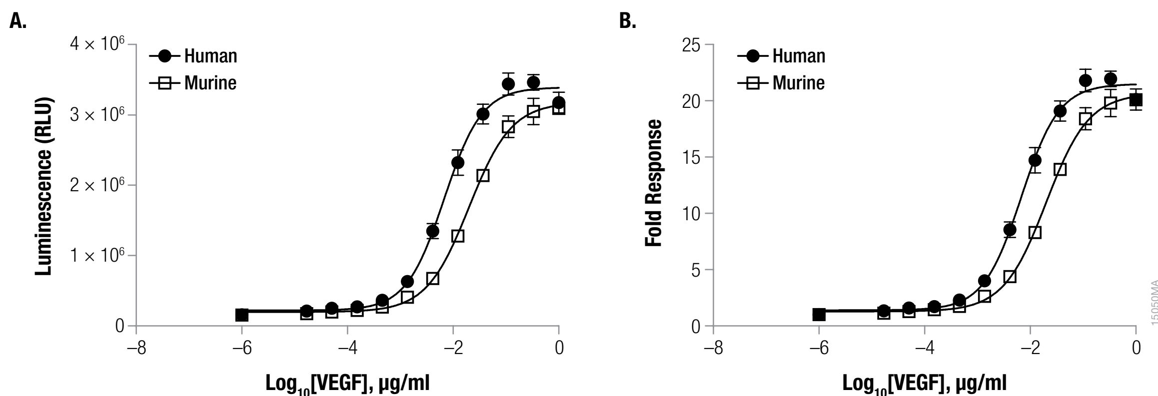

Figure 13. The VEGF Bioassay measures the potency of murine VEGF. KDR/NFAT-RE HEK293 Cells were plated and data was generated as indicated in the protocol. Cells were incubated with increasing concentrations of either recombinant human VEGF or recombinant murine VEGF for 6 hours before luminescence measurement. 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 (Relative Light Units; **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 were generated using thaw-and-use cells.

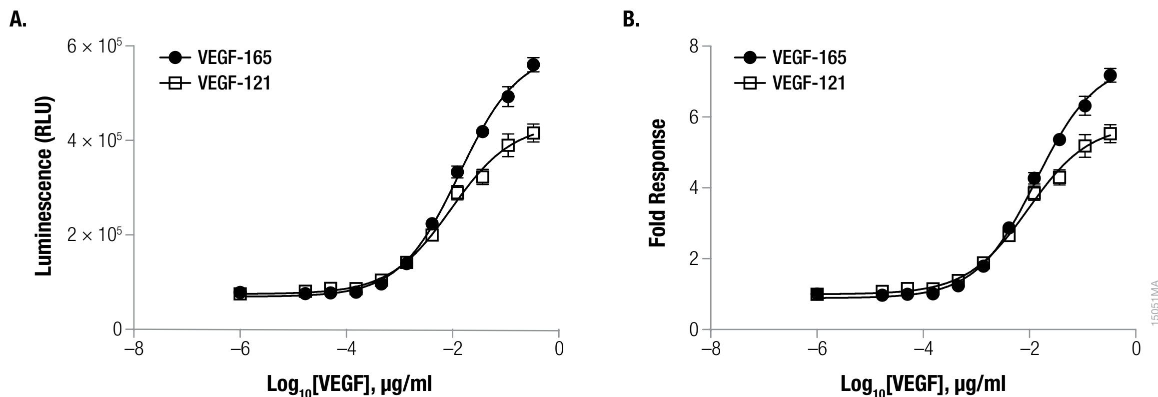

Figure 14. The VEGF Bioassay measures the potency of various VEGF isoforms. KDR/NFAT-RE HEK293 Cells were plated and data was generated as indicated in the protocol. Cells were incubated with increasing concentrations of either recombinant human VEGF-165 or recombinant human VEGF-121 for 6 hours before 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 (Relative Light Units; **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.)

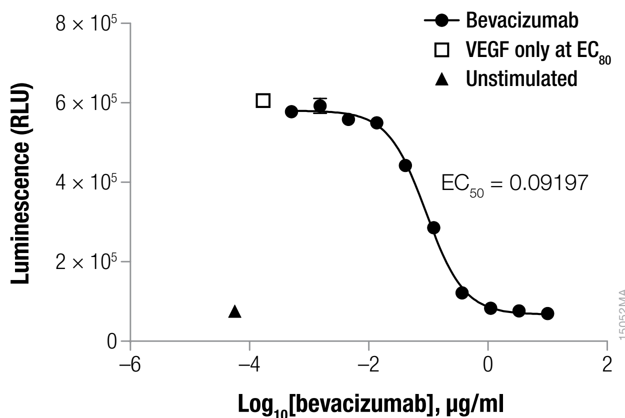

Figure 15. The VEGF Bioassay measures the potency of bevacizumab antibody. KDR/NFAT-RE HEK293 Cells were plated and data was generated as indicated in the protocol. Cells were incubated with increasing concentrations of bevacizumab in the presence of an EC_{80} concentration of recombinant VEGF (14ng/ml) 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 92ng/ml.

7.B. Assay Troubleshooting

The following data were generated using the VEGF Bioassay, Propagation Model. Figure 16 shows representative results from the 2-day and 3-day pre-culture protocol. Figure 17 demonstrates the requirement for precise cell density during the preculture period, and how the assay will fail if cells are not plated at high enough densities on day 1. Figure 18 demonstrates the optimal assay incubation time and Figure 19 shows the requirement for plating cells on the day of the assay.

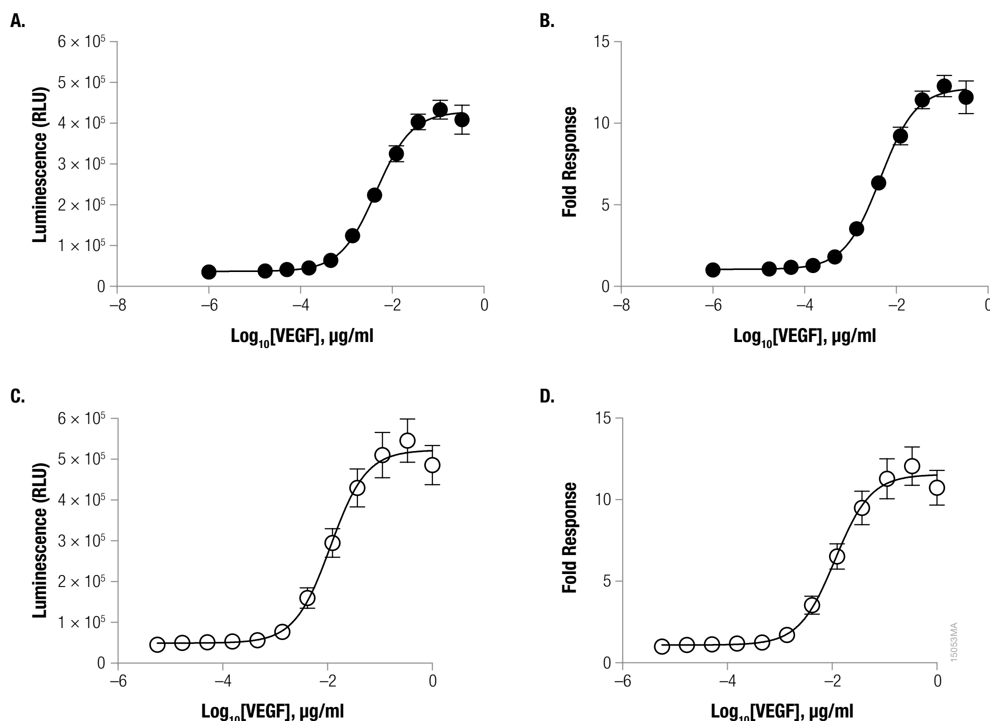

Figure 16. Assay results are similar using either the 2-day and 3-day pre-culture protocol. KDR/NFAT-RE HEK293 Cells were used (Cell Propagation Model) and data were generated as indicated in the protocol. Cells were precultured at the appropriate density for either 2 or 3 days, respectively. Cells were then harvested and plated in assay medium, and 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 (Relative Light Units; **Panels A** and **C**) or fold response over untreated (**Panels B** and **D**). Two-day preculture protocol results are shown in **Panels A** and **B**, and 3-day preculture protocol results are shown in **Panels C** and **D**. The EC_{50} of VEGF was between 4–10ng/ml.

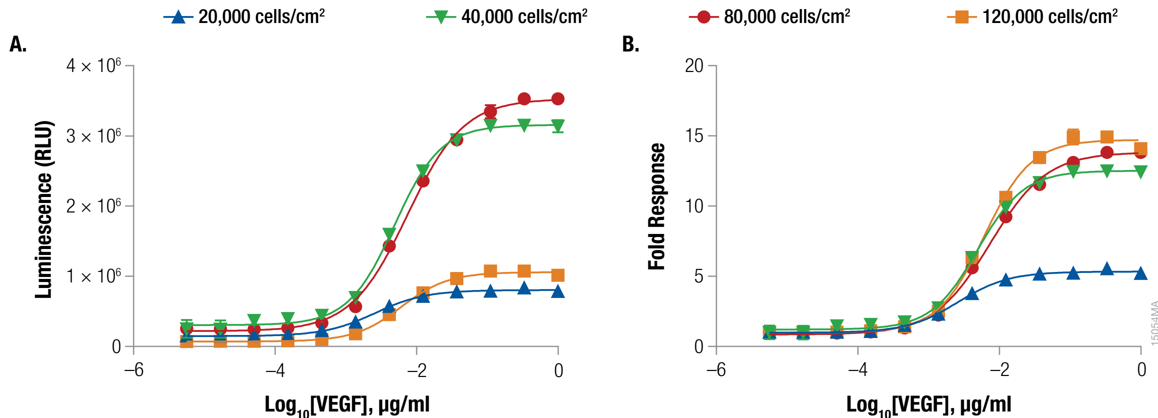

Figure 17. VEGF Bioassay requires precise cell densities during pre-culture period. KDR/NFAT-RE HEK293 Cells were used (Cell Propagation Model) and data was generated as indicated in the protocol. On day 1, KDR/NFAT-RE HEK293 Cells were plated in four flasks at various densities (expressed as cells/cm^2 of surface area) and grown without media change for 3 days. Cells were harvested on day 4 according to the assay protocol, and plated at 40,000 cells/well in assay medium in 96-well format. Cells were then 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 were plotted as RLU (Relative Light Units; **Panel A**) or fold response over untreated (**Panel B**). Cells plated at a density below 40,000 cells/cm^2 on day 1 result in a significantly reduced assay window.

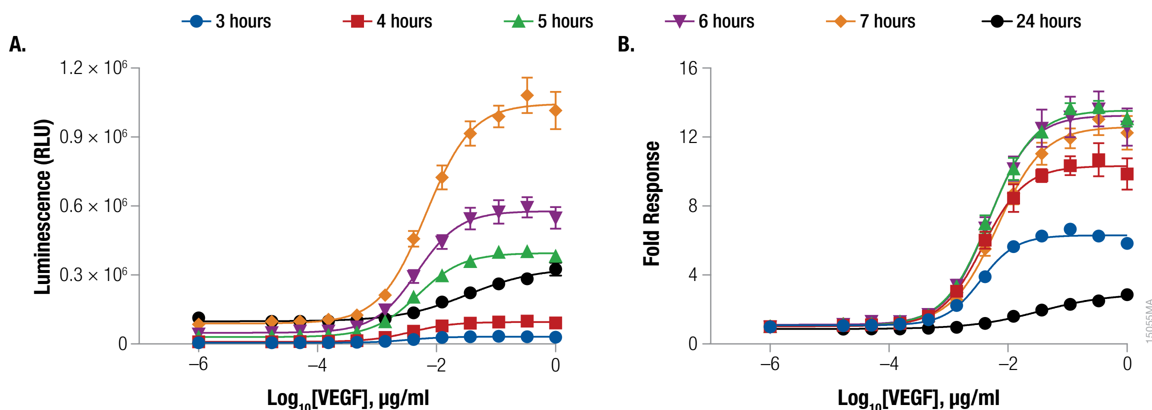

Figure 18. The VEGF Bioassay requires a 6-hour assay incubation. KDR/NFAT-RE HEK293 Cells were used (Cell Propagation Model) and data was generated as indicated in the protocol. Cells were incubated with increasing concentrations of recombinant VEGF for 3, 4, 5, 6, 7 or 24 hours before luminescence measurement. Bio-Glo™ Reagent was added and luminescence was quantified using the GloMax® Discover System. Data were analyzed using GraphPad Prism® software. Data were plotted as RLU (Relative Light Units; **Panel A**) or fold response over untreated (**Panel B**).

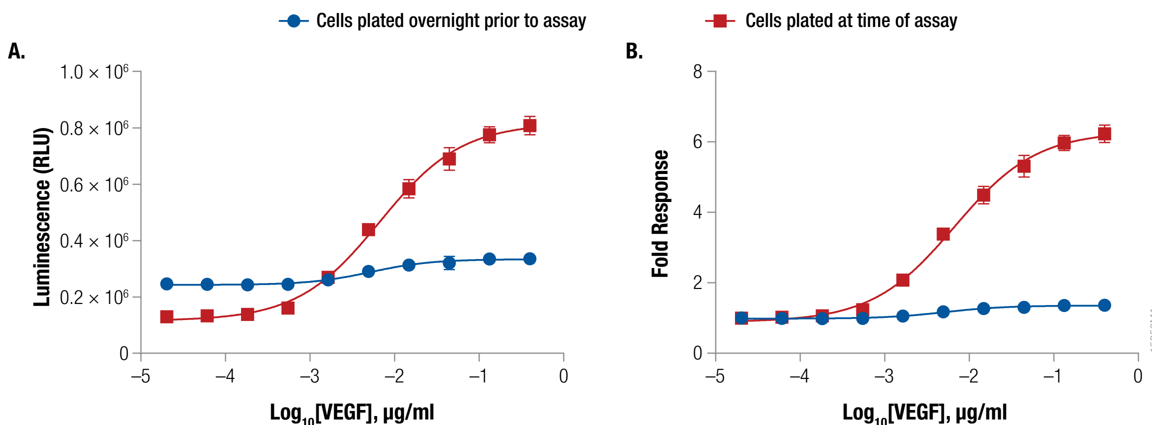

Figure 19. The VEGF Bioassay requires cells to be plated at time of assay. KDR/NFAT-RE HEK293 Cells were precultured according to protocol and plated in a 96-well assay plate either: 1) the day before the assay, followed by an overnight incubation at 37°C, 5% CO_2; or 2) immediately prior to assay. Data 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 were plotted as RLU (Relative Light Units; **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/initial cell culture medium

90%	DMEM
10%	FBS

complete cell culture medium

90%	DMEM
10%	FBS
100µg/ml	Hygromycin B (selection antibiotic)
250µg/ml	G-418 Sulfate Solution (selection antibiotic)

freezing medium

80%	DMEM
10%	FBS
10%	DMSO

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.

[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 modify, derivatize, genetically engineer or otherwise create variations of the cells or genes stably transfected within the cells except that recipient may propagate and store the cells for long-term use. 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]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. GIBCO is a registered trademark of Life Technologies Corporation. GraphPad Prism is a registered trademark of GraphPad Software, Inc. JMP is a registered trademark of SAS Institute, Inc. Mr. Frosty is a registered trademark of Nalge Nunc International Corporation. Styrofoam is a registered trademark of Dow Chemical Company.

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.