A Novel MOA-based Bioluminescent Bioassay for Quantitative Measurement of anti-VEGF Antibody Potency and Stability

Jennifer Wilkinson, Matt Robers, Frank Fan and Mei Cong

Promega Corporation, 2800 Woods Hollow Rd, Madison, WI 53711

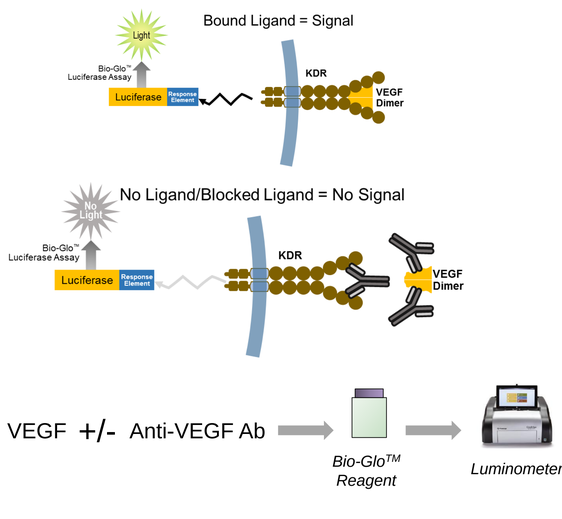
1. Introduction

Vascular endothelial growth factor (VEGF) is an important signaling molecule that stimulates angiogenesis, increases vascular permeability, and enhances tumor invasion and survival. Overexpression of VEGF can cause vascular disease in many parts of the body. Cancers that express VEGF are able to grow and metastasize, since angiogenesis is fundamental to tumor growth, invasion and metastatic dissemination. Therefore, inhibition of the VEGF signaling pathway has proven to be effective treating various cancers and eye diseases, and is currently an attractive target for cancer therapeutics and treatment of age-related macular degeneration.

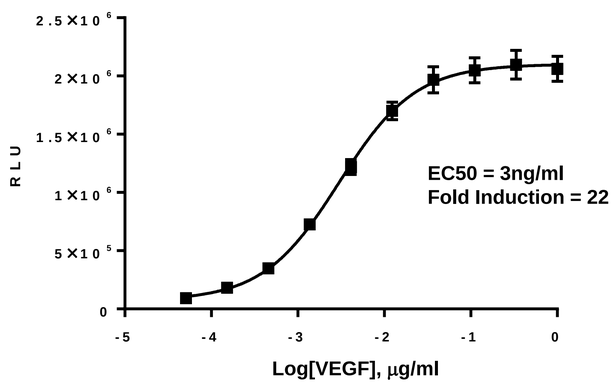
Currently, the commonly used angiogenesis assays are endothelial cell proliferation and differentiation assays, which use primary human umbilical vein endothelial cells (HUVEC). These assays are time consuming (4-5 days), technically challenging (due to limitations around cell senescence), and can be difficult to analyze (it can be difficult to determine if a decrease in cell number is due to cell death rather than a decrease in cell proliferation). Also, many of these assays still use radioactive ^{3}H-thymidine.

We have developed a bioluminescent reporter-based bioassay that measures VEGF stimulation/inhibition of KDR (VEGFR2). 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 bioassay utilizes cryopreserved thaw-and use cells, eliminating the need for cell culture. This assay format offer the benefits of convenience, reproducibility, and transferability. The assay cab be performed in less than 7 hours, which is a significant improvement over a 4-5 day traditional cell proliferation assay. Finally, using the anti-VEGF antibody drug bevacizumab, we demonstrate that the assay is suitable for potency and stability studies per ICH guidelines.

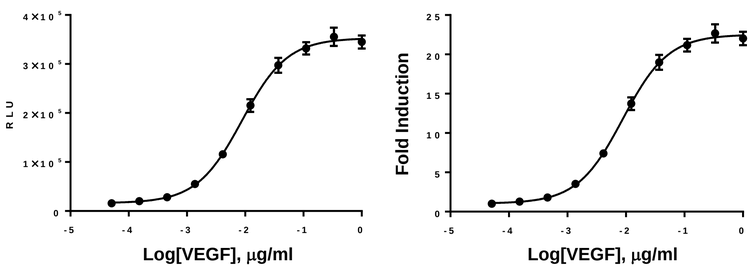


2. VEGF Induces Luciferase Activity in a Dose-dependent Manner



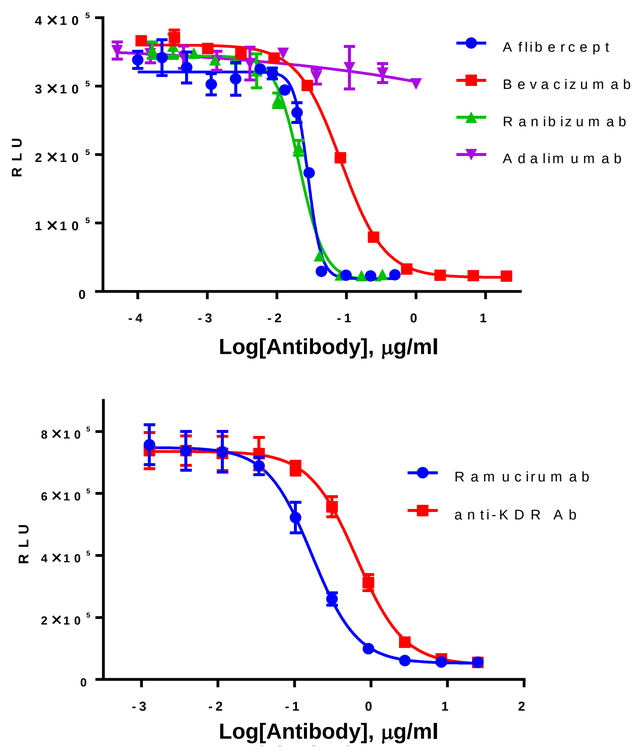
VEGF Responsive Cells were thawed, diluted and plated into a solid white 96-well plate. Serial dilutions of recombinant human VEGF-165 (Biolegend #583706) were prepared in assay buffer and added to cells. After a 6 hour incubation, Bio-Glo™ Reagent was added and luminescence was quantified using the GloMax® Discover Detection System.

3. The VEGF Bioassay is Compatible with 384-well Plate Format



VEGF Responsive Cells were plated at 15,000 cells/well in a 10 µl volume. A three-fold serial dilution of VEGF was added at 10 µl per well, and 10 µl of media was added to bring the total volume to 30 µl per well. After a 6 hour incubation, 30 µl of Bio-Glo™ Reagent was added and luminescence was quantified using the GloMax® Discover System.

4. The VEGF Bioassay is Specific

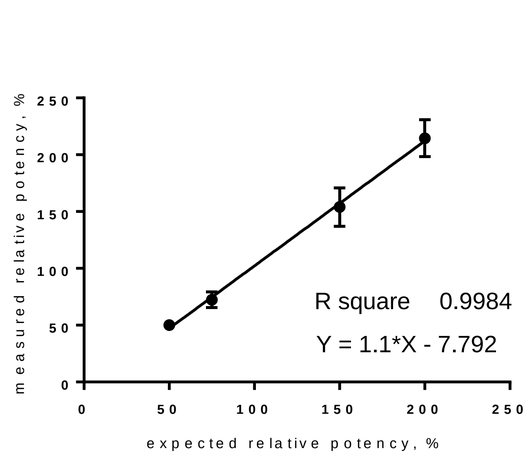


The VEGF Bioassay was used to measure the activity of anti-VEGF (top panel) or anti-KDR (VEGFR2) (bottom panel) blocking antibodies. VEGF Responsive Cells were incubated with increasing concentrations of the anti-VEGF blocking antibodies Aflibercept (trade name Eylea), Bevacizumab (trade name Avastin), Ranibizumab (trade name Lucentis) or the anti-KDR blocking antibody Ramucirumab (trade name Cyramza) or a research-grade anti-KDR antibody in the presence of an EC80 concentration of recombinant human VEGF-165. After a 6 hour incubation, Bio-Glo™ Reagent was added and luminescence was quantified using the GloMax® Discover Detection System. All three anti-VEGF antibodies and the two anti-KDR antibodies, but not the anti-TNFα blocking antibody Adalimumab, inhibited VEGF-induced luciferase activity in a dose-dependent manner.

IC50 values were as follows: Aflibercept 0.03 µg/ml, Bevacizumab 0.08 µg/ml, Ranibizumab 0.02 µg/ml, Ramucirumab 0.17 µg/ml, and anti-KDR Ab 0.65 µg/ml.

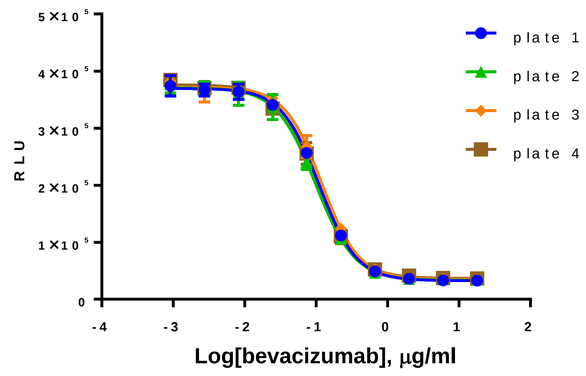
5. Qualification Study: Accuracy, Precision, and Linearity

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 two analysts. 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.		



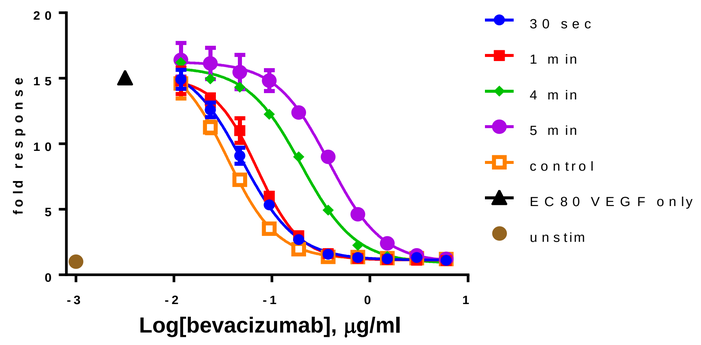
The VEGF Bioassay shows accuracy, precision, and linearity.
A 50–200% theoretical potency series of bevacizumab (anti-VEGF antibody) was analyzed in triplicate in three independent experiments performed on three days by two analysts using the VEGF Bioassay. Linearity and r^2 values were determined using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

6. Qualification Study: 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 Detection System.

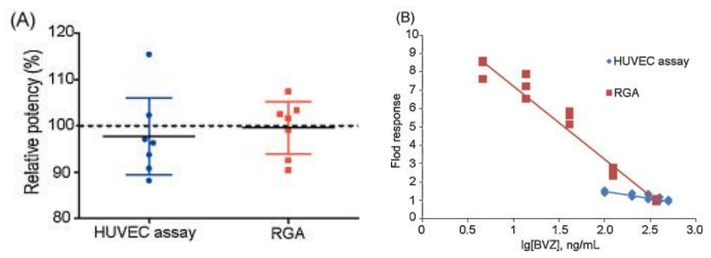
7. The VEGF Bioassay is Stability-indicating



	control	30 sec	1 min	4 min	5 min
EC50	0.0351	0.0498	0.0706	0.1991	0.3815

Samples of bevacizumab were maintained at 4°C (control) or heat-treated (80°C) for increasing amounts of time, as indicated. Samples were then analyzed using the VEGF Bioassay. Bio-Glo™ Reagent was added and luminescence was quantified using the GloMax® Discover Detection System.

8. Comparison to Primary HUVEC Assay



Parameter	VEGF Bioassay	HUVEC Assay
Experiment period	1 day	4 days
Regression line (R^2)	>0.99	>0.95
Linear Range	4.6-370 ng/ml	100-500 ng/ml
Fold Induction	>8	~1.5
Precision (intra- and inter-)	<10%	>15%

Relative biological activities of 7 batches of bevacizumab were measured using the VEGF Bioassay (denoted “RGA”) and a primary HUVEC assay. Each point represents the mean of 3 replicates. The VEGF Bioassay is superior in precision, sensitivity, and assay simplicity compared to the HUVEC assay.
(data published in Wang, et al. *Journal of Pharmaceutical and Biomedical Analysis.* 2016)

9. Conclusions

The Promega VEGF Bioassay:

- Is pre-qualified according to ICH guidelines for accuracy, precision, linearity, and reproducibility
- Can be used for anti-VEGF antibody potency and stability studies
- Demonstrates larger fold induction than a traditional HUVEC proliferation assay, can be used over a range of antibody concentrations, and can be completed within 1 day
- Can be performed in 96- and 384-well plates

Corresponding author: Jennifer.Wilkinson@promega.com