Development and Qualification of a Sensitive and High Throughput Cell-Based Neutralizing Antibody Assay for Bevacizumab

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Introduction

Bevacizumab (Avastin®) is a humanized monoclonal antibody that blocks angiogenesis by inhibiting VEGF-A. Protein-based therapeutics like Bevacizumab have the potential to induce immune responses in the body. One adverse immune response is the generation of neutralizing antibodies (NAbs) that can bind to the drug and reduce efficacy and potentially safety of the therapeutic. Cell-based assays have emerged as the industry standard in detection of NAbs because they mimic the method by which these antibodies exert their effect in a living biological system. The US FDA and the European Medicines Agency (EMA) have recognized the importance of these cell-based NAb assays and have recommended them whenever possible. We have successfully qualified a cell-based Bevacizumab NAb detection assay based on Promega's KDR-NFAT assay technology. The assay is fast, high-throughput, sensitive, and tolerates high levels of human serum for neutralizing antibody detection in human serum samples.

Methods

The reporter gene assay is based on a HEK293 cell line stably expressing vascular endothelial growth factor receptor 2 (VEGFR-2 or KDR) and the luciferase reporter gene (Luciferase) under regulation of the nuclear factor of activated T cells (NFAT) response element (RE).

Figure 1. KDR-NFAT Assay Principle (Promega Corp.)

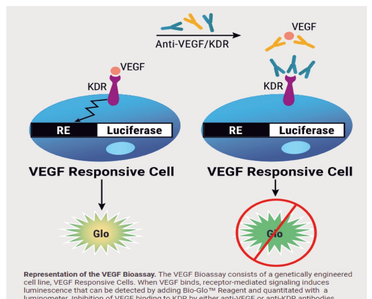


Figure 2. Neutralizing Antibody Assay Workflow

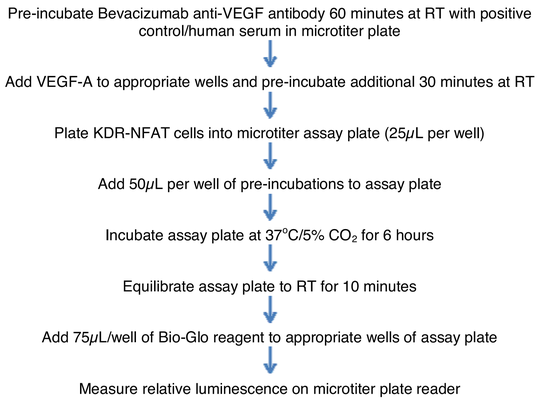
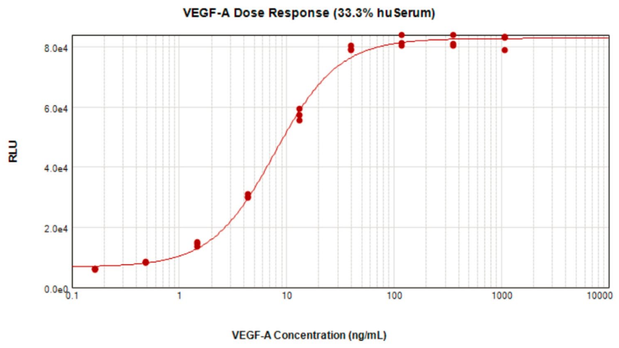
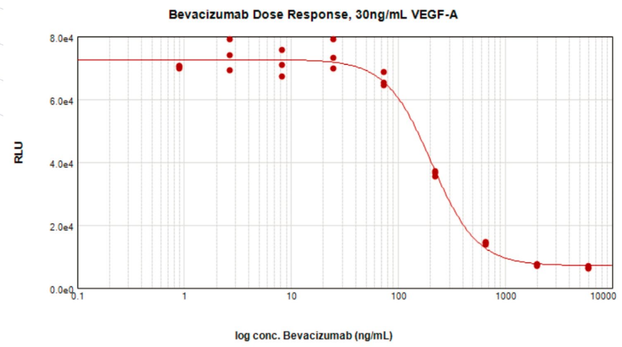


Figure 3. VEGF-A Dose Response activity of KDR-NFAT Cells



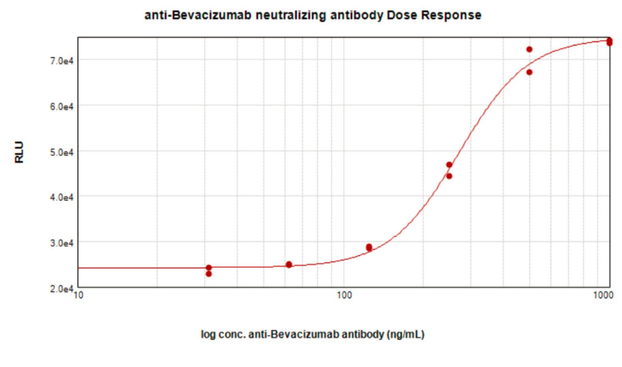
The VEGF-A (R&D Systems) was serially diluted across the range of expected activity in the presence of 33.3% pooled human serum (30 Sera) in order to determine the EC_{50} and range of the response in KDR-NFAT cells. The EC_{50} was calculated to be 7.8 ng/mL (n=3) and the EC_{90} of 30 ng/mL was determined to be optimal for Bevacizumab dose response analysis (see fig. 4).

Figure 4. Bevacizumab Dose Response with 30 ng/mL VEGF-A



Bevacizumab (Somru) was serially diluted and tested in the presence of 30 ng/mL VEGF-A and 33.3% pooled human serum (30 Sera) to determine the EC_{50} and range of the response in KDR-NFAT cells. The EC_{50} was calculated to be 200.4 ng/mL (n=3) and a concentration of 300 ng/mL was determined to be optimal for testing of the anti-Bevacizumab antibody (Bio-Rad), (see fig. 5).

Figure 5. Bevacizumab Neutralizing Antibody Dose Response



A monoclonal anti-Bevacizumab neutralizing antibody (Bio-Rad) was serially diluted in pooled human serum (30 Sera) and tested in the presence of 30 ng/mL VEGF-A, 300 ng/mL Bevacizumab in order to determine the EC_{50} of the response and calculate the assay sensitivity at the cut point. The EC_{50} of the response was determined to be 272 ng/mL (n=3) and an assay sensitivity was calculated to be 409 ng/mL at the assay cut point (see table 2).

Table 1. Assay Cut Point Calculation

	Batch 1 (RLU)	Batch 2 (RLU)	Mean (RLU, n=30 Sera, 2 Batches)
Avg. (n=15)	19127	17288	18208
STDDev (n=15 Sera)	518	359	439
%CV (n=15 Sera)	2.7	2	2.35
Median (n=15)	20438	17687	19063
MAD (n=15)	1934	2638	2286
MADn (n=15)	2867	3911	3389
Batch Cut Point	25154	24120	24637
NegC (no VEGF-A)	20207	16593	18400
Blank (30ng/mL VEGF -A)	66182	57183	61683
Signal : Background	3.3	3.4	3.35

Individual human serums (n=30) were tested in replicate in two separate batches in order to determine a mean assay cut point. Data was normally distributed by Shapiro-Wilkes analysis. Cut point was determined for each batch based on the normalized median average deviation of the individual serums (MAD x 1.486) added to each batch Median. The data is shown is Table 1.

Table 2. Assay Sensitivity at Calculated Cut Point

	anti-Bevacizumab antibody Concentration						
Batch	4500 ng/mL	2250 ng/mL	1125 ng/mL	562.5 ng/mL	281.3 ng/mL	140.6 ng/mL	Batch Cut Pt.
1 (RLU)	73807	69668	45564	28598	24784	23489	25154
2 (RLU)	65388	59738	35825	24957	20486	18989	24120

Shaded cells are RLU higher than the batch cut point
Mean Sensitivity at the batch cut point = 409 ng/mL

The anti-Bevacizumab antibody was serially diluted in pooled human serum (30 Sera) across the range of expected assay sensitivity and tested in duplicate in batches 1 and 2. The antibody concentration at the assay cut point is calculated and the mean is shown in Table 2.

Table 3. Assay Interference by Matrix Components

	Unspiked			LQC 220 ng/mL			HQC 330 ng/mL		
Batches	Lot#	Mean RLU	> CP	Lot#	Mean RLU	> CP	Lot#	Mean RLU	> CP
3,4	ME Blank 1	19004	Neg/No	ME LQC 1	41181	Pos/Yes	ME HQC 1	60339	Pos/Yes
	ME Blank 2	24268	Neg/No	ME LQC 2	52014	Pos/Yes	ME HQC 2	78989	Pos/Yes
	ME Blank 3	17557	Neg/No	ME LQC 3	35548	Pos/Yes	ME HQC 3	54108	Pos/Yes
	ME Blank 4	18016	Neg/No	ME LQC 4	38322	Pos/Yes	ME HQC 4	65059	Pos/Yes
	ME Blank 5	20434	Neg/No	ME LQC 5	44282	Pos/Yes	ME HQC 5	65280	Pos/Yes
	ME Blank 6	19346	Neg/No	ME LQC 6	40920	Pos/Yes	ME HQC 6	65855	Pos/Yes
	ME Blank 7	20806	Neg/No	ME LQC 7	43710	Pos/Yes	ME HQC 7	71822	Pos/Yes
	ME Blank 8	27239	Neg/No	ME LQC 8	52807	Pos/Yes	ME HQC 8	84718	Pos/Yes
	ME Blank 9	24841	Neg/No	ME LQC 9	54578	Pos/Yes	ME HQC 9	84885	Pos/Yes
	ME Blank 10	23495	Neg/No	ME LQC 10	56005	Pos/Yes	ME HQC 10	61159	Pos/Yes
	Mean	21501	Neg/No	Mean	45937	Pos/Yes	Mean	69221	Pos/Yes
	Cut Pt. for batch 4		28779.4	Cut Pt. for batch 3		32485.2	Cut Pt. for batch 3		32485.2

Individual human serums were tested (10 Sera) either spiked or unspiked with 220 ng/mL (LQC) and 330 ng/ml (HQC) of anti-Bevacizumab antibody in order to determine the serum matrix effect on the assay positive controls. Data show that all spiked individual serums tested positive (> CP) for the anti-Bevacizumab antibody as expected.

Table 4. Assay Drug Tolerance (Bevacizumab)

Bevacizumab Drug Concentration		781ng/mL		391 ng/mL	
anti-Bevacizumab Antibody		0 ng/mL	220ng/mL	0 ng/mL	220ng/mL
Mean (n=3)		29816	31116	29816	38063
% CV		7.8	2.4	7.8	2.5
Batch Cut Pt.	36272				
Acceptance Criteria					
% CV ≤ 20			Pass		Pass
mean response > CP			Fail		Pass

Positive control anti-Bevacizumab antibody in pooled serum (30 Sera) was tested across a range of excess Bevacizumab concentrations in order to determine the effect of excess drug on the assay sensitivity. The assay positive control tolerates up to 391 ng/mL excess Bevacizumab in the human serum background.

Results

The KDR-NFAT assay is robust with a dynamic range for $VEGF_{165}$ response of 1 – 100 ng/mL in the presence of 33.3% pooled human serum (30 serum lots) and an EC_{50} of the response of 7.8 ng/mL (n=3). At an optimal VEGF-A ($VEGF_{165}$, R&D Systems) concentration of 30 ng/mL (EC_{90}), Bevacizumab (Somru) dose response assays yield an IC_{50} of 200 ng/mL (n=3) with a dynamic range of 30 ng/mL – 2 ug/mL when tested in the presence of 33.3% pooled human serum. At an optimal Bevacizumab concentration of 300ng/mL (EC_{70} EC_{80}) and at 30 ng/mL VEGF-A, an anti-Bevacizumab neutralizing antibody (Bio-Rad) was tested in a human serum background of 33.3% and demonstrated an EC_{50} of 276 ng/mL (n=3). At optimized assay concentrations for VEGF-A and Bevacizumab, 30 individual lots of human serum were tested in order to determine a specific cut point and sensitivity for the detection of anti Bevacizumab neutralizing antibodies in the KDR-NFAT reporter assay. The results indicate an assay specific cut point yielding overall sensitivity of 409 ng/mL for anti-Bevacizumab antibodies. Drug tolerance assays were also performed in the optimized assay by testing positive controls of anti- Bevacizumab antibody dosed with increasing amounts of Bevacizumab in pooled human serum. The assay was tolerant of up to 391 ng/mL Bevacizumab without deleterious effect on the positive control QCs.

Conclusion

We have successfully developed a cell-based neutralizing antibody assay for the detection of anti-bevacizumab antibodies that is fast, high-throughput, and sensitive. The assay is highly reproducible and suitable for the comparative assessment of neutralizing antibody (NAb) detection to support biosimilar studies.

Future work

The qualification of this assay relied on a limited data set and we intend to further optimize this assay as part of a full validation plan to improve drug tolerance and reassess the assay cut point and sensitivity.

References

Development of a robust reporter-based assay for the bioactivity determination of anti-VEGF therapeutic antibodies. Lan Wang, Gang-lingXu, Kai Gao, Jennifer Wilkinson et al. Journal of Pharmaceutical and Biomedical Analysis 125 (2016) 212-218.
Recommendations for the Development and Validation of Neutralizing Antibody Assays in Support of Biosimilar Assessment. D. Gouty, C.C. Cai, X.Y. Cai et al. AAPS Journal (December 28, 2017).

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