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Design of Total Anti-Vegf Assay Provides Simple Data Interpretation of Anti-Vegf Therapeutic Study

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Purpose

Vascular Endothelial Growth Factor (VEGF) is an angiogenic factor associated with an array of diseases, including several cancers and retinopathy, making this protein both a valuable biomarker and therapeutic target. The development of a VEGF assay in the presence of an anti-VEGF therapeutic could be informative, indicating therapeutic efficacy by measuring "free" VEGF, or tracking the expression of VEGF throughout disease progression with "total" VEGF.

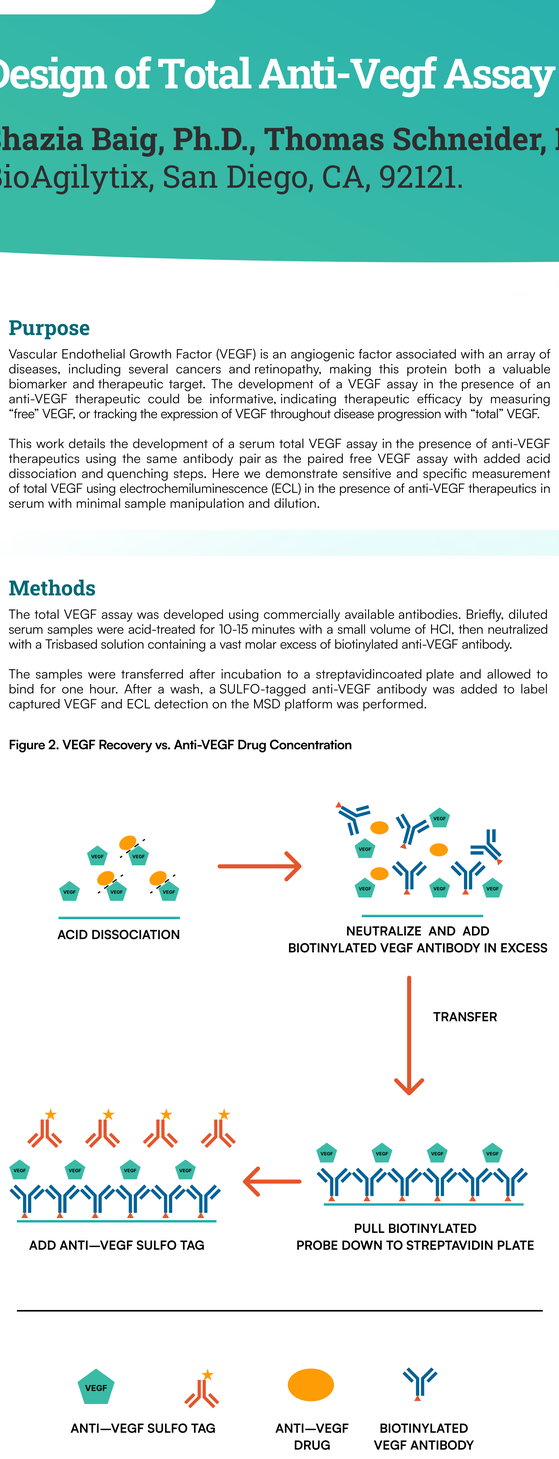
This work details the development of a serum total VEGF assay in the presence of anti-VEGF therapeutics using the same antibody pair as the paired free VEGF assay with added acid dissociation and quenching steps. Here we demonstrate sensitive and specific measurement of total VEGF using electrochemiluminescence (ECL) in the presence of anti-VEGF therapeutics in serum with minimal sample manipulation and dilution.

Methods

The total VEGF assay was developed using commercially available antibodies. Briefly, diluted serum samples were acid-treated for 10-15 minutes with a small volume of HCl, then neutralized with a Trisbased solution containing a vast molar excess of biotinylated anti-VEGF antibody.

The samples were transferred after incubation to a streptavidincoated plate and allowed to bind for one hour. After a wash, a SULFO-tagged anti-VEGF antibody was added to label captured VEGF and ECL detection on the MSD platform was performed.

Figure 2. VEGF Recovery vs. Anti-VEGF Drug Concentration



Results

Assay Range

Calibration Range:

- 0-4600 pg/mL
- LLOQ: 1.12 pg/mL
- ULOQ: 4600 pg/mL
- MRD: 2X

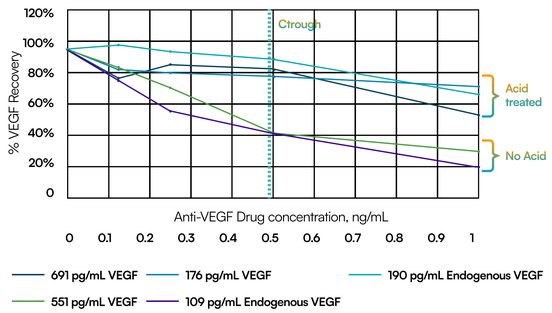
Table 1. VEGF Recovery vs. Anti-VEGF Drug Concentration

STD	Conc (pg/mL)	RLU Mean	RLU CV%	% Recovery	Calc Conc Mean	Calc Conc CV %
S001	4600	115646	1.1%	100.2	4610	1.2%
S002	1150	30280	3.2%	99.0	1139	3.2%
S003	288	7702	1.8%	101.0	290	1.8%
S004	71.9	1928	1.8%	100.6	72.3	1.9%
S005	18.0	531	0.9%	100.1	18.0	1.1%
S006	4.49	188	3.8%	94.3	4.24	6.9%
S007	1.12	119	0.0%	122.2	1.37	0.0%
S008	0	86	0.8%	N/A	N/A	N/A

Drug Tolerance

Drug tolerance of the assay was tested by spiking serum samples with an anti-VEGF therapeutic and observing VEGF recovery with and without acid treatment. The samples without acid were "treated" and "neutralized" with additional sample diluent for comparison. Results show higher tolerance for the drug in serum in acid treated samples. Both endogenous VEGF and spiked recombinant VEGF serum samples displayed the same behavior for drug tolerance, shown in Figure 2.

Figure 2. VEGF Recovery vs. Anti-VEGF Drug Concentration



Total Clinical Sample Testing

Figure 3 shows the total VEGF results in all clinical samples tested. The acid-treated samples ranged in concentration from ~100 pg/mL to >10,000 pg/mL VEGF, and the diluent treated samples ranged from ~ 6 to 300 pg/mL. An unpaired t-test shows a significant difference between the two groups with P value <0.0001. This demonstrates acid treatment can significantly increase the amount of measurable VEGF.

Figure 2. VEGF Recovery vs. Anti-VEGF Drug Concentration

VEGF Sequestration

Clinical serum samples were tested with acid treatment (total VEGF) and control diluent treatment (free VEGF) to assess VEGF sequestration in the serum. Table 2 shows free and total VEGF with fold change difference. Five samples showed virtually no difference between the two treatments, while the other samples ranged from 2 to 326-fold difference in VEGF concentration.

The degree of sequestration could be due to: circulating drug, disease stage, concurrent therapy, etc.

Table 2. VEGF Sequestration in Clinical Serum Samples, pg/mL

Sample	Sample	Acid Treated	Fold Change
Sample 1	27.0	8808	326
Sample 2	66.3	11668	176
Sample 3	116.1	1270	10.9
Sample 4	216.4	247	1.1
Sample 5	5.8	390	66.8
Sample 6	6.0	467	77.7
Sample 7	42.7	220.6	5.2
Sample 8	97.7	227.2	2.3
Sample 9	116.6	180.1	1.5
Sample 10	34.9	6948	199
Sample 11	37.4	8300	222
Sample 12	32.7	5552	170
Sample 13	167.0	8296	49.7
Sample 14	166.8	1214	7.3
Sample 15	100.1	9883	99
Sample 16	54.1	13378	247
Sample 17	266.4	307	1.2
Sample 18	122.6	3043	24.8
Sample 19	295.1	319	1.1
Sample 20	92.9	15519	167

Table 3. IgG-Bound VEGF Samples

Sample	IgG Bound VEGF (pg/mL)
Sample 1	42.9
Sample 2	31.4
Sample 3	<LLOQ
Sample 4	35.4
Sample 5	17.3
Sample 6	10.7
Sample 7	5.6
Sample 8	2767
Sample 9	3044
Sample 10	1768
Sample 11	39.2
Sample 12	82.6
Sample 13	468
Sample 14	438
Sample 15	97.6
Sample 16	75.2
Sample 17	48.6
Sample 18	349
Sample 19	897
Sample 20	<LLOQ
Sample 21	7.14
Sample 22	<LLOQ
Sample 23	6.09
Sample 24	693
Sample 25	44.94
Sample 26	<LLOQ
Sample 27	8.61
Sample 28	8.21
Sample 29	5.90
Sample 30	<LLOQ
Sample 31	369

IgG-Bound VEGF

Patient serum samples were processed through Protein G spin columns to evaluate ADA originally, but the IgGbound eluate was analyzed with the total VEGF assay to assess any IgG-VEGF interactions in patient samples.

Concentrations in Table 3 are reported as found in the eluted volume—they were not adjusted for dilution in the final volume of buffer.

The results indicate there was VEGF bound to IgG, indicating it could be complexed to endogenous IgG or an IgGbased anti-VEGF therapeutic.

VEGF Scavenger Treatment

Serum samples were treated with an excess (7.5 µg/mL) VEGF scavenger, a different anti-VEGF antibody, prior to acid treatment and compared to initial acid dissociated measurements to confirm specificity to VEGF.

Table 4 shows many samples treated with the excess competing antibody were below the detection limit of the assay, while other samples showed >1000-fold difference in VEGF levels, indicating a specific response to VEGF in serum.

Table 4. Total and Inhibited VEGF in Clinical Samples

Sample	Total VEGF	Ab-Treated
Sample 1	8808	9.03
Sample 2	11668	11.2
Sample 3	1270	< LLOQ
Sample 4	247	< LLOQ
Sample 5	390	< LLOQ
Sample 6	467	< LLOQ
Sample 7	220.6	< LLOQ
Sample 8	227.2	< LLOQ
Sample 9	180.1	< LLOQ
Sample 10	6948	8.49
Sample 11	8300	< LLOQ
Sample 12	5552	< LLOQ
Sample 13	8296	11.5
Sample 14	1214	NaN
Sample 15	9883	9.39
Sample 16	13378	14.3
Sample 17	307	< LLOQ
Sample 18	3043	< LLOQ
Sample 19	319	< LLOQ
Sample 20	15519	13.9

Conclusions

The total VEGF assay revealed interfering substances not attributable to the drug of interest, making the Free/Total paired assays less indicative of therapeutic efficacy. The high concentration of total VEGF in some clinical samples adds another dimension to the data analysis of the clinical samples for using VEGF as a biomarker for therapeutic efficacy and disease progression.

The detection of VEGF in IgG bound fractions suggests the possibility of higher order molecular immune complexes present in serum, potentially confounding VEGF measurements or tracking with an immune response. This approach allowed for the same critical reagents in the total and free assays with only a few additional steps to dissociate and re-bind the target for detection, a strategy that can be employed with other Free/Total paired assays.

Although this approach has its limitations in drug tolerance, it minimizes the impact of sample processing and dilution on the assay bias and sensitivity, allows for detection and quantitation of samples through disease progression and treatment, and provides more direct data analysis of clinical samples.

References

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2. Takahashi H, Nomura Y, Nishida J, Fujino Y, Yanagi Y, Kawashima H. Vascular endothelial growth factor (VEGF) concentration is underestimated by enzyme-linked immunosorbent assay in the presence of anti-VEGF drugs. Invest Ophthalmol Vis Sci. 2016; 57: 462—466.

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