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# Development of Method for Detection of Anti-Drug Antibodies to Bevacizumab in the Presence of Soluble VEGF

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## Abstract

**PURPOSE:**
Bevacizumab (Avastin®) is a recombinant humanized monoclonal antibody that inhibits angiogenesis by binding to the vascular endothelial growth factor A (VEGF-A). Bevacizumab is currently licensed to treat various cancers, including colorectal, lung, breast, glioblastoma, kidney and ovarian. Because serum levels of bevacizumab may be as high as 50 µg/mL, it is important that immunogenicity assays for this compound have excellent drug tolerance. In addition, bridging assays for such antibodies may be prone to positive interference caused by soluble target.

**METHODS:**
The ADA method is a solution phase bridging assay with acid dissociation on an electrochemilumenesent (ECL) platform. Acid treated samples are neutralized in a reaction containing biotinylated and ruthenlyated bevacizumab, and specific additives to reduce soluble VEGF interference. After incubation, bridging complexes of labeled drug and ADA are captured on a streptavidin plate and visualized on the MesoScale Discovery (MSD) instrument.

**RESULTS:**
The assay method was qualified for use in human serum. Method demonstrates suitable precision, sensitivity, drug tolerance, and robustness. Performance characteristics are as are shown in Table 1.

**CONCLUSION:**
A sensitive method to detect anti-drug antibodies was qualified to support the development of biosimilar bevacizumab. The bridging ECL format with acid dissociation demonstrates up to100 µg/mL of bevacizumab tolerance. Specific additive overcomes soluble VEGF-A interference up to 100 ng/mL.

## Purpose:

Bevacizumab (Avastin®) is a recombinant humanized monoclonal antibody that inhibits angiogenesis by binding to the vascular endothelial growth factor A (VEGF-A). Bevacizumab contains human IgG1 framework regions (93%) and the complementarity-determining regions (CDR) of a murine antibody (7%) that binds to VEGF. Bevacizumab binds to VEGF extracellularly to prevent interaction with VEGF receptors (VEGFR) on the surface of endothelial cells, thereby inhibiting stimulation of new blood vessel formation. Bevacizumab is currently licensed to treat various cancers, including colorectal, lung, breast (outside the USA), glioblastoma (USA only), kidney and ovarian.

Evaluation of the comparability of bevacizumab biosimilars to the innovator drug should follow the guidelines laid out by the FDA and EMA. The analysis should be multifactorial, taking into account both the physicochemical characteristics and clinical performance of the biosimilar compared to the innovator. Eurofins Bioanalytical Services offers a range of off-the-shelf bevacizumab assays for comparability testing within biosimilar programs including Pharmacokinetic (PK) and Anti-Drug Antibody (ADA) Immunogenicity testing.

The purpose of this study was to develop a sensitive and precise assay for the detection of antibodies to bevacizumab in human serum to support clinical studies. As VEGF-A is a soluble homodimer it has the potential to interfere in standard bridging assays by causing false positives. This novel solution phase bridging assay is designed to prevent interference from VEGF-A, while maintaining a high level of drug tolerance.

## Materials & Methods:

The analytical method is a solution phase bridging assay with acid dissociation on an MesoScale Discovery electrochemiluminescent (ECL) platform where the anti-drug antibodies (ADA) in human sera are dissociated from any bevacizumab by acid treatment. The acid treated samples are then neutralized in a reaction containing biotinylated and ruthenlyated bevacizumab, and with specific additives to reduce soluble VEGF interference. After incubation any bridging complexes of labeled drug and ADA are captured on a streptavidin plate and visualized on the ECL instrument.

Thirty (30) samples from normal human donors were assayed in four separate runs by two analysts. This data was used to establish a preliminary floating cut point factor using statistical methods as recommended in Shankar, et al.

An anti- idiotypic monoclonal antibody to bevacizumab was used as a positive control antibody. Low, Mid and High positive controls were prepared by spiking the anti-bevacizumab into pooled normal human serum.

### Assay Sensitivity

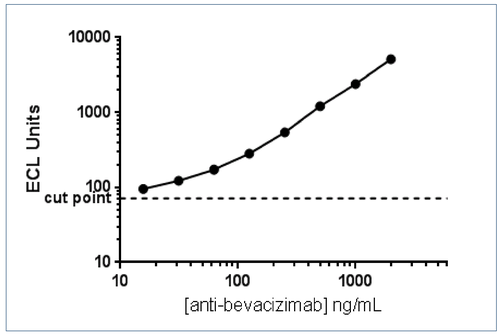


**Figure 1:** Assay sensitivity was determined by diluting anti-Bevacizumab Antibody in negative human serum . The estimated cut point falls below 15.6 ng/mL.

### Drug Tolerance to Bevacizumab

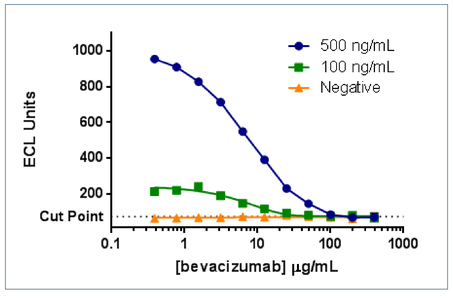


**Figure 2:** Drug Tolerance was assessed by adding increasing amounts of bevacizumab to samples containing no antibody, 100 ng/mL and 500 ng/mL anti-bevacizumab. For the 500 ng/mL anti-bevacizumab sample, the addition of 100 µg/mL bevacizumab did not drop the signal below the cut point.

### Selectivity of Normal Human Serum

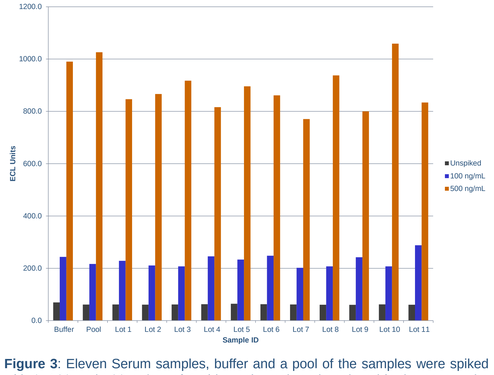


**Figure 3**: Eleven Serum samples, buffer and a pool of the samples were spiked with 0, 100 and 500 ng/mL of Anti-bevacizumab and analyzed in the assay. The resulting signals from the serum samples were compared to the signal in buffer. 10 of 11 samples were within 30% of the buffer response at all levels.

### Assay Precision

| Sample | Run # | Result 1 | Result 2 | Result 3 | Result 4 | Result 5 | Result 6 | n | Mean | Std Dev | %CV |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Negative | Run 1 | 61 | 65 | 58 | 60 | 63 | 70 | 6 | 63 | 4.0 | 6.3 |
| | Run 2 | 78 | 79 | 82 | 95 | 78 | 73 | 6 | 81 | 8.0 | 9.9 |
| | Run 3 | 68 | 69 | 69 | 67 | 65 | 68 | 6 | 68 | 2.0 | 2.9 |
| | Run 4 | 77 | 54 | 68 | 80 | 62 | 73 | 6 | 69 | 10.0 | 14.5 |
| | **Intra-Assay (within-run) Statistics (Pooled):** | | | | | | | **6.00** | **70** | **6.8** | **9.7** |
| | **Inter-Assay (between-run) Statistics (ANOVA):** | | | | | | | **24** | **70** | **9.8** | **14.0** |
| Sample | Run # | Result 1 | Result 2 | Result 3 | Result 4 | Result 5 | Result 6 | n | Mean | Std Dev | %CV |
| Low | Run 1 | 251 | 253 | 330 | 257 | | | 4 | 273 | 38.0 | 13.9 |
| | Run 2 | 292 | 276 | 341 | 308 | | | 4 | 304 | 28.0 | 9.2 |
| | Run 3 | 369 | 236 | 272 | 251 | | | 4 | 282 | 60.0 | 21.3 |
| | Run 4 | 316 | [a]220 | 276 | 264 | | | 3 | 285 | 27.0 | 9.5 |
| | **Intra-Assay (within-run) Statistics (Pooled):** | | | | | | | **4** | **286** | **38.8** | **13.5** |
| | **Inter-Assay (between-run) Statistics (ANOVA):** | | | | | | | **15** | **286** | **38.8** | **13.5** |
| Sample | Run # | Result 1 | Result 2 | Result 3 | Result 4 | Result 5 | Result 6 | n | Mean | Std Dev | %CV |
| Mid | Run 1 | 824 | 933 | 909 | 905 | | | 4 | 893 | 47.0 | 5.3 |
| | Run 2 | 1016 | 939 | 1138 | 982 | | | 4 | 1019 | 86.0 | 8.4 |
| | Run 3 | 1075 | 1021 | 917 | 844 | | | 4 | 964 | 104.0 | 10.8 |
| | Run 4 | 1230 | [a]754 | 1189 | 899 | | | 3 | 1106 | 180.0 | 16.3 |
| | **Intra-Assay (within-run) Statistics (Pooled):** | | | | | | | **4** | **988** | **107.1** | **10.8** |
| | **Inter-Assay (between-run) Statistics (ANOVA):** | | | | | | | **15** | **992** | **126.1** | **12.7** |
| Sample | Run # | Result 1 | Result 2 | Result 3 | Result 4 | Result 5 | Result 6 | n | Mean | Std Dev | %CV |
| High | Run 1 | 3190 | 3196 | 3859 | 3583 | | | 4 | 3457 | 325.0 | 9.4 |
| | Run 2 | 3734 | 3886 | 4204 | 3454 | | | 4 | 3820 | 313.0 | 8.2 |
| | Run 3 | 4086 | 3406 | 5180 | 3792 | | | 4 | 4116 | 762.0 | 18.5 |
| | Run 4 | 4740 | 3110 | 4537 | 3658 | | | 4 | 4011 | 763.0 | 19.0 |
| | **Intra-Assay (within-run) Statistics (Pooled):** | | | | | | | **4** | **3851** | **583.4** | **15.1** |
| | **Inter-Assay (between-run) Statistics (ANOVA):** | | | | | | | **16** | **3851** | **583.4** | **15.1** |

(a) Sample removed due to High %CV between replicates

**Figure 4:** Negative Human serum was spiked with Low, Mid, and High level of Anti-bevacizumab and analyzed multiple times in multiple assays. The Inter-Assay Precision was 14.0, 13.5, 12.7 and 15.1 %CV for the Negative, Low, Mid, and High Samples, respectively.

### VEGF Tolerance due to Assay Additive

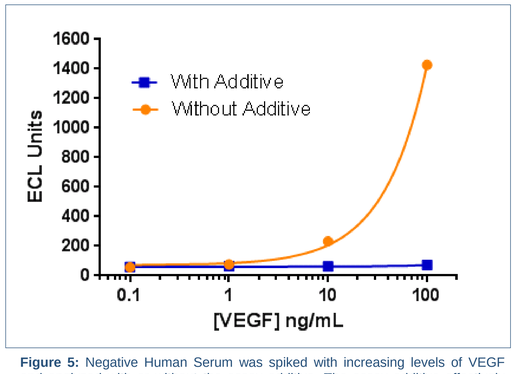


**Figure 5:** Negative Human Serum was spiked with increasing levels of VEGF and analyzed with or without the assay additive. The assay additive effectively removed false positive signal from up to 100 ng/mL VEGF in negative samples.

### Assay Cut Point

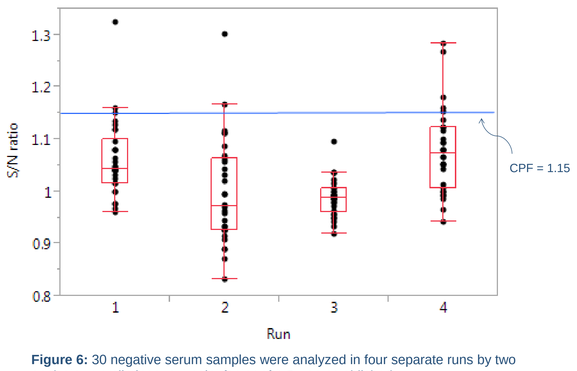


**Figure 6:** 30 negative serum samples were analyzed in four separate runs by two analysts. A preliminary cut point factor of 1.5 was established.

### Table 1: Summary of Performance Characteristics

| Performance characteristic | Results |
|---|---|
| Sensitivity | <15.6 ng/mL |
| Cut Point Assessment | Screening:Cut point Factor 1.15<br>Confirmatory Cut point:25% inhibition |
| Selectivity (Matrix recovery) | 10 /11 normal human serum samples spiked with 500 ng/mL anti-Avastin report within ±30% of reference |
| Precision<br>Intra-assay<br>Inter-assay | Range: 9.7 to 15.1 % CV<br>Range: 12.7 to 15.1 % CV |
| Drug Tolerance | 500 ng/mL ADA is detectable in the presence of 100 µg/mL of bevacizumab |
| Target Tolerance | Up to 100 ng/mL of VEGF-A does not interfere in the assay |

## Summary

- **A bridging assay for detection of antibodies to bevacizumab was developed and qualified on the MSD electrochemiluminescence (ECL) platform**
- **Improved drug tolerance was achieved through the application of an acid dissociation step. Positive control anti-bevacizumab antibodies prepared at 500 ng/mL, the required sensitivity limit for ADA methods, could be detected in the presence of up to 100 µg/mL of bevacizumab.**
- **In order to overcome the challenge of soluble VEGF in human serum, a specific additive was used to block interference from soluble VEGF. This effectively manages the risk of soluble VEGF interference, which could otherwise cause a false-positive response. Using this approach, VEGF levels of at least 100 ng/mL did not interfere in the assay.**
- **The assay exhibits excellent sensitivity, with capacity to detect anti-bevacizumab antibodies at 15.6 ng/mL or less.**

## Conclusion

**A sensitive, robust, selective and precise method was qualified to support the development of biosimilar bevacizumab. The bridging ECL format with acid dissociation demonstrates up to100 µg/mL of bevacizumab tolerance. Specific additive overcomes soluble VEGF-A interference up to 100 ng/mL**

*1. Shankar G et al. 2008. Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products. Journal of Pharmaceutical and Biomedical Analysis 48, 1267-1281.*

*2. Mire-Sluis AR et al. 2004. Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology. products. J Immunol Methods 289, 1-16*

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