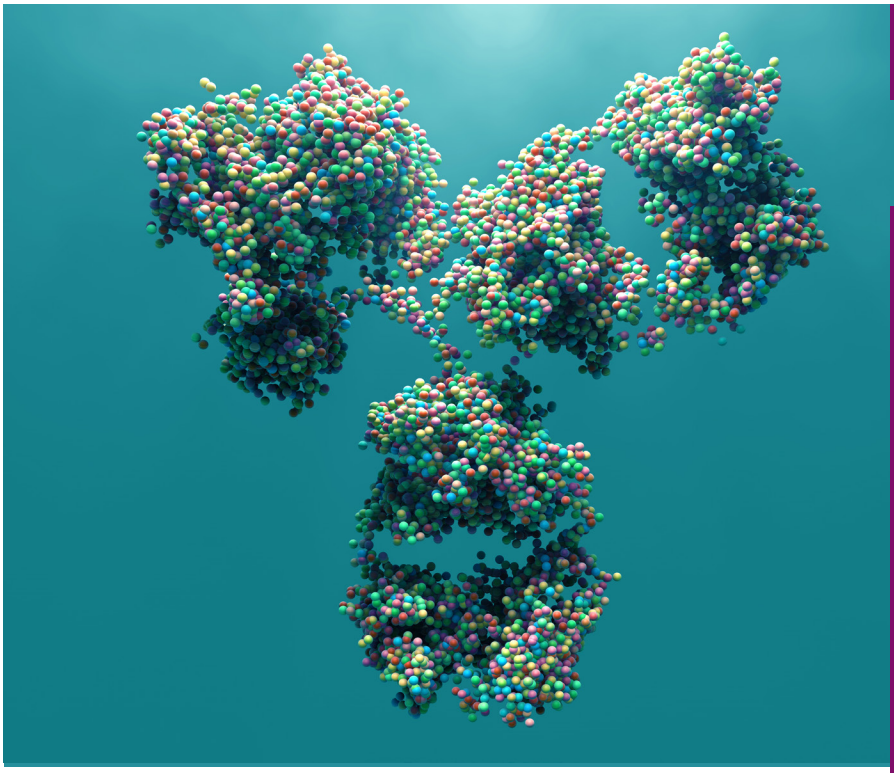

APPLICATION NOTE

Alpha Technology

Authors:

Laura Morra

René Moser

IBR Inc.,
Institute for Biopharmaceutical Research
Matzingen, Switzerland

Published in collaboration with IBR Inc.

Alpha Technology: A Fast and Sensitive Orthogonal Approach to Cell-based Potency Assays

Introduction

In biopharmaceutical development, potency estimation assumes a central role, being the most important parameter to confirm lot to lot consistency and equivalence. Reliable potency estimates are based on dilutional similarity (parallelism), which implies that the potency is dose-independent. The need for orthogonal systems to cell-based assays is made explicit in the EMA/FDA guidelines, which require cross-validation with independent but complementary approaches to prove and strengthen the reliability of results.

Bevacizumab (trade name Avastin®) is a recombinant humanized monoclonal IgG1 antibody that binds to Human Vascular Endothelial Growth Factor (VEGF), thereby inhibiting its biological function.[1] The mechanism of action of Bevacizumab has been elucidated using *in vitro* VEGF neutralization assays (antiproliferation assays) based on primary Human Umbilical Vein Endothelial Cells (HUVEC), which have been shown to give the most sensitive response.[2]

PerkinElmer®
For the Better

Alpha technology represents an ideal cell-free orthogonal system to determine Bevacizumab/$VEGF_{165}$ binding due to its high sensitivity, low reagent consumption, and ease of use. Taking advantage of the great versatility of Alpha technology in assay design, we developed an AlphaLISA assay that permits a fast and sensitive potency estimation of Bevacizumab. The assay was developed, qualified, and analyzed following the guidelines on the design and development, validation and analysis of biological assays described in the 2010 United States Pharmacopeial Convention (USP).[3-5] We demonstrate that the proximity-based and homogeneous AlphaLISA® assay represents an ideal orthogonal approach to cell-based potency assays.

Materials and Methods

Reagents

Avastin® (Bevacizumab) antibody (Cat #10157814 CH 1712) and the negative control (NC) antibody MabThera® (Rituximabum or Rituximab) (Cat #10138638 CH 1111 1570) were purchased from Roche. AlphaLISA anti-VEGF Acceptor beads, streptavidin coated Donor beads (SA-Donor), biotinylated antibody anti-Human Vascular Endothelial Growth Factor (VEGF) and AlphaLISA immunoassay buffer were from PerkinElmer (Human Vascular Endothelial Growth Factor (VEGF) kit, Cat #AL201C/F). Recombinant Human $VEGF_{165}$ was purchased from PeproTech (Cat #100-20). Half-area 96-well white microplates were from PerkinElmer (Cat #6005560).

Data Analysis

Data were evaluated with Excel software equipped with the add-in XLFit for curve fitting Version 5.5.0.5. The data were fitted according to the four-parameter MMF equation (Model 606) based on the Levenberg–Marquardt algorithm.

AlphaLISA Assay

The Bevacizumab AlphaLISA assay was developed using a competitive assay configuration. The assay is dependent on bivalent binding of anti-VEGF antibodies to recombinant Human $VEGF_{165}$ molecules captured by biotinylated goat polyclonal anti-VEGF bound to streptavidin (SA) Donor beads and by mouse monoclonal anti-VEGF immobilized on the Acceptor beads. Laser excitation of the Donor beads provokes the release of singlet oxygen molecules that trigger a cascade of energy transfer in surrounding Acceptor beads, thus resulting in a time-delayed sharp peak of light emission at 615 nm. The presence of Bevacizumab prevents the recombinant Human $VEGF_{165}$ from bridging two anti-VEGF antibodies and, consequently, the two beads from coming into close proximity, resulting in a decrease in signal which is proportional to increasing Bevacizumab concentrations (Figure 1).

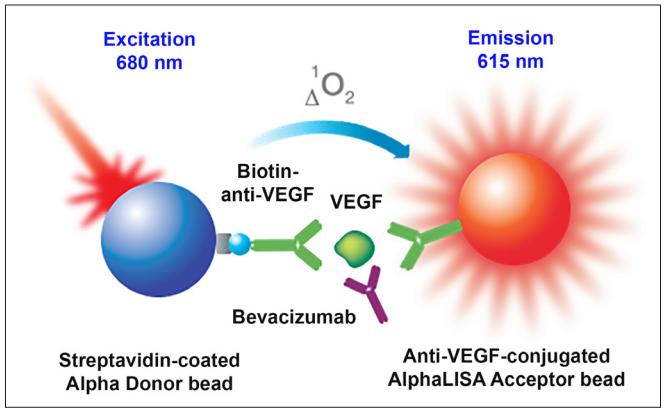

Figure 1. Competitive Bevacizumab AlphaLISA assay configuration.

Protocol

The initial assay protocol was developed following the manufacturer's recommendations for the Human Vascular Endothelial Growth Factor (VEGF) kit and the AlphaLISA Assay Development Guide.[6,7]

Since "order of addition" is a well-known optimization parameter in Alpha technology, we compared two sequences of reagent addition in this study:

A. Sequence "A", where Bevacizumab was pre-incubated for 0 and 30 minutes with VEGF and had more time to bind than the other two competing antibodies (Figure 2, Sequence A).

B. Sequence "B", where VEGF was added in the last step (Figure 2, Sequence B) and therefore all three competing antibodies had the same amount of time to bind (Figure 2, Sequence B).

The experiments to qualify the Bevacizumab AlphaLISA assay (accuracy, precision and linearity) were performed using reagent addition sequence "B" as follows:

20 µL of AlphaLISA anti-VEGF-Acceptor beads (10 µg/mL final) and biotinylated anti-VEGF antibody (1 nM final) were dispensed in a half-area 96-well white microplate, followed by 10 µL of Bevacizumab or NC sample dilutions (ranging from 100000.0 – 0.3 ng/mL final). Finally, 20 µL of SA-Donor beads diluted in assay buffer (40 µg/mL final) was added to each well. The plate was then centrifuged at 300 x *g* for 10 seconds, then incubated at room temperature (21 °C), in the dark, for 90 minutes (Figure 3). After this incubation, the plate was read using the EnVision® 2103 Multilabel Plate Reader to measure the AlphaLISA counts. Total measurement time was set to 200 ms and excitation time to 80 ms.

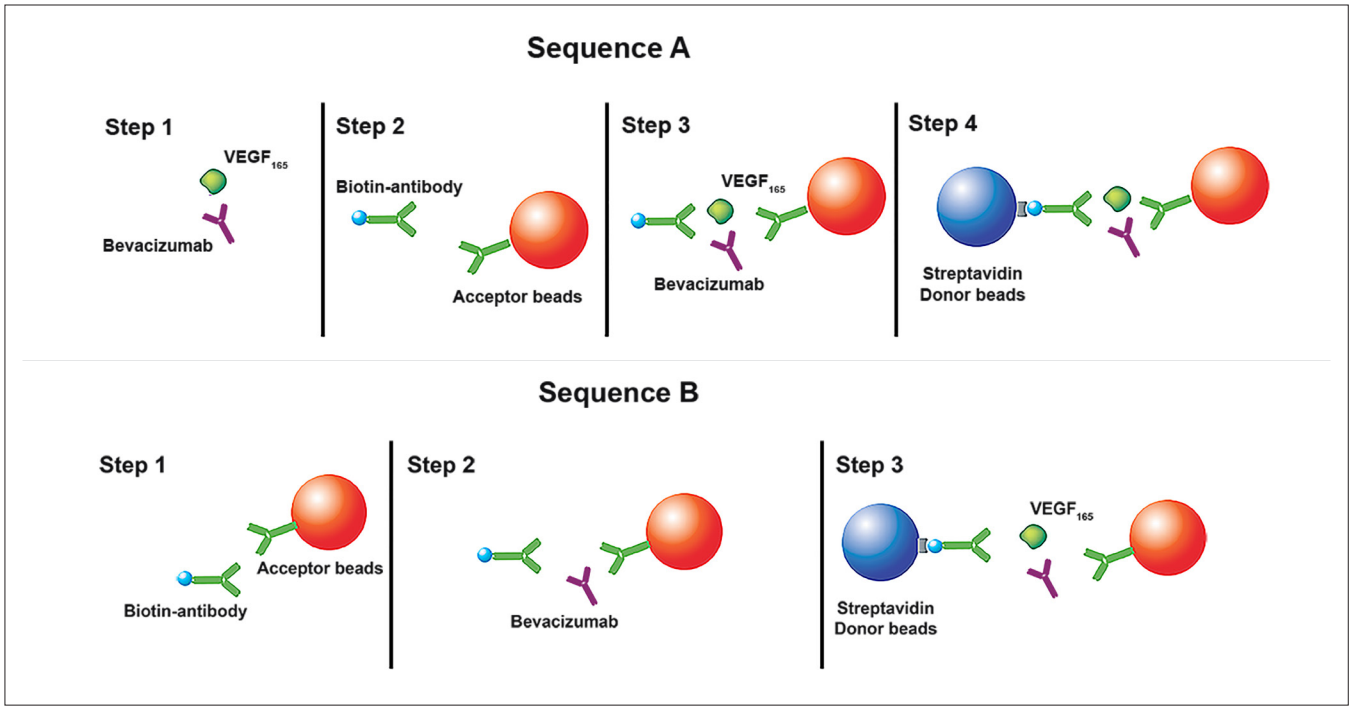

Figure 2. Sequences of reagent addition.

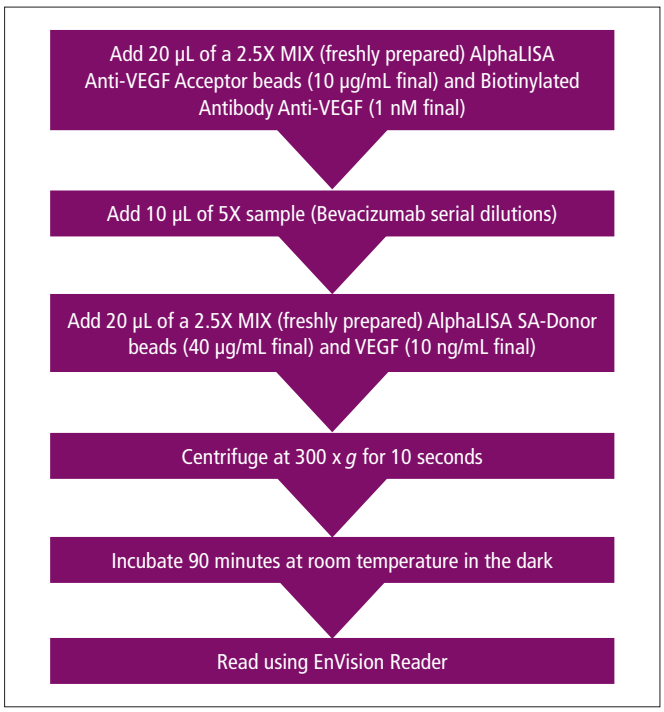

Figure 3. AlphaLISA assay protocol.

Results

Sequence of Reagent Addition

To determine the most suitable sequence of reagent addition for the assay protocol, two different sequences were compared (Figure 2). The goal was to have as little impact of order of reagent addition on IC_{50} as possible. In Sequence "A", Bevacizumab was pre-incubated for 0 and for 30 minutes with VEGF, while in Sequence "B", VEGF was added in the last step without pre-incubation with Bevacizumab. Therefore, in Sequence "B" Bevacizumab had no temporal advantage over the other anti-VEGF antibodies for VEGF binding and the IC_{50} could not be influenced by the duration of pre-incubation time. Conversely, in sequence "A", the IC_{50} was influenced by the pre-incubation time Bevacizumab/VEGF. The IC_{50} was on average (three experiments) 11-fold lower after 30 minutes pre-incubation (data not shown). The qualification of Bevacizumab AlphaLISA (accuracy, precision and linearity) was therefore performed using sequence "B" of reagent addition.

Qualification of Bevacizumab AlphaLISA Assay

Accuracy, precision, and linearity of the Bevacizumab AlphaLISA assay were assessed by comparing the response of the Reference Item Bevacizumab at 100% potency (applied doses: 100000.0, 25000.0, 6250.0, 1000.0, 370.4, 137.2, 50.8, 18.8, 4.7, 1.2, 0.3 ng/mL) to five nominal potency levels of Bevacizumab: 50%, 75%, 100%, 125% and 150%. The five potency levels were simulated by applying 50%, 75%, 100%, 125% and 150% of Bevacizumab concentration at 100% potency (Reference Standard, Figure 4).

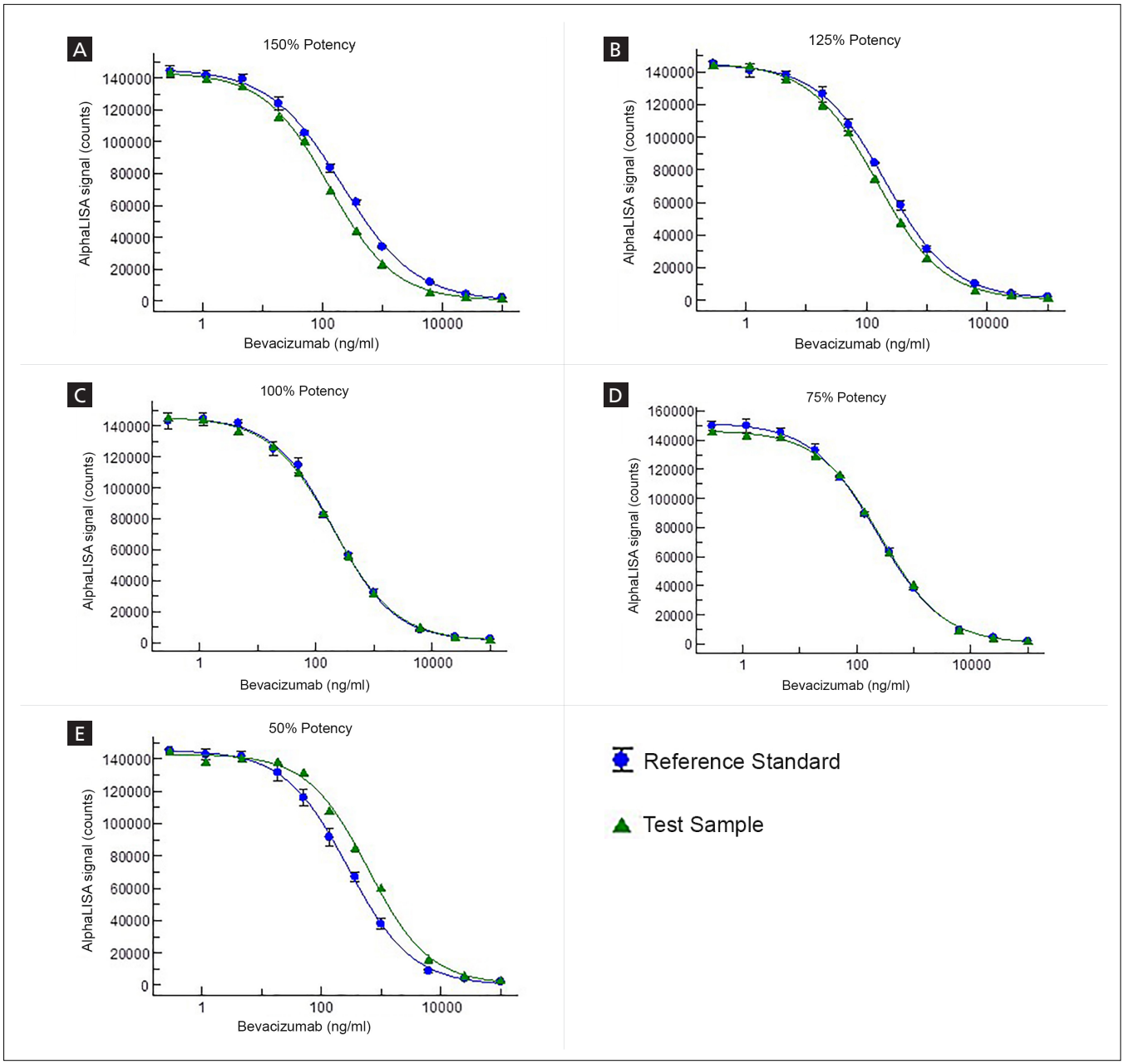

Figure 4. Dose-response curves (one of three experiments). Inhibitory response of the competitive Bevacizumab AlphaLISA to increasing Bevacizumab concentrations. Reference Standard (blue) and the corresponding test sample (150%, 125%, 100%, 75%, 50% potency levels of Bevacizumab; green). Graphics originate from the Excel software add-in XLFit using the MMF equation derived from the Levenberg-Marquardt algorithm. X-axis: concentration in ng/mL, Y-axis: response values (AlphaLISA signal in counts). A) Bevacizumab Reference Standard and 150% potency level; B) Bevacizumab Reference Standard and 125% potency level; C) Bevacizumab Reference Standard and 100% potency level; D) Bevacizumab Reference Standard and 75% potency level; E) Bevacizumab Reference Standard and 50% potency level.

The system suitability of the AlphaLISA assay to measure binding of Bevacizumab to VEGF was confirmed by the decreasing AlphaLISA signal in response to increasing Bevacizumab concentrations.

The inhibitory Bevacizumab response in the competitive AlphaLISA showed minimum effect at the lowest potency level (50%) and maximum effect at the highest potency level (150%, Figure 4) to increasing Bevacizumab concentrations.

Relative Potency and Overall Inter-Assay Accuracy

To calculate the half-maximal inhibitory concentration (IC_{50}) and estimate potency, data were fitted according to the MMF four-parameter equation based on the Levenberg–Marquardt algorithm. The MMF equation of the S-shaped response curve is based on a four-parameter equation characterized by the upper and lower asymptotes, the slope factor, and the inflection point ($logIC_{50}$ or IC_{50} factor). The Relative Potency comparing the Reference Standard (Reference Item at 100% potency) to the Test Samples (50-150% potency levels of Bevacizumab) represents the shift in the slope factor ($logIC_{50}$).

The MMF equation is represented in XLFit as follows:

$$y = (A*B+C*x^D)/(B+x^D)$$

Where

A = Maximum Y value

B = IC_{50} factor

C = Minimum Y value

D = Slope factor

The IC_{50} value is calculated as follows:

$$IC_{50}=B^1/D$$

The Relative Potency was calculated using the formula described below, based on the IC_{50} of Reference Standard and Test Sample:

Relative Potency (%) = (IC_{50} Reference Standard x 100) / (IC_{50} Test Sample)

The Intra-Assay Accuracy (Recovery) was calculated based on the ratio Relative Potency / Nominal Potency in one independent assay. In the example below, the calculation of potency level 50%:

Intra-Assay Accuracy $_{(50\%)}$ = Relative Potency $_{(50\%)}$ x100 / Nominal Potency $_{(50\%)}$

Acceptance criterion for the Intra-Assay Accuracy was 100 ± 20%.

The Relative Potencies of Bevacizumab (estimated for five potency levels ranging from 50 to 150%) were recovered with an accuracy to the Nominal Potencies within the acceptance criterion of 100±20% for each potency level in each independent assay.

The Overall Inter-Assay Accuracy (Recovery) was calculated based on the ratio Relative Potency / Nominal Potency estimated for five potency levels ranging from 50 to 150% in three independent assays. The example below shows the calculation of Overall Inter-Assay Accuracy at potency level 50%:

Overall Inter-Assay Accuracy $_{(50\%)}$ = Mean Relative Potency $_{(50\%)}$ (3 assays) x 100 / Nominal Potency $_{(50\%)}$

Acceptance criterion for the Overall Inter-Assay Accuracy was 100 ± 20%.

The Relative Potencies of Bevacizumab are recovered with a Mean Accuracy to the Nominal Potencies that ranged from 94.7 to 110.8% in three independent experiments (Table 1), within the acceptance criterion of 100 ± 20%.

Table 1. Overall Accuracy and Inter-Assay Precision (three experiments). The Relative Potencies of Bevacizumab are recovered with an accuracy of 94.7 - 110.8% to the Nominal Potencies in three independent experiments. The Inter-Assay Precision (CV) for the estimation of the Relative Potencies is within a range of 0.9 - 11.7%.

Nominal Potency	Relative Potency			Mean Relative Potency	SD	CV	Mean Recovery
	Replicate						
	1	2	3				
	%						
150	162.8	159.7	148.5	157.0	7.5	4.8	104.7
125	139.8	137.3	138.4	138.5	1.3	0.9	110.8
100	102.5	91.0	103.4	99.0	6.9	7.0	99.0
75	85.8	69.6	86.0	80.5	9.4	11.7	107.3
50	46.0	46.4	49.7	47.4	2.0	4.3	94.7

Intra- and Inter-Assay Precision

The Intra-Assay Precision was calculated based on the Coefficient of Variation (CV) of the quadruplicate response measurements at each concentration of the dose-response curve. The CV was calculated as the ratio of the standard deviation to the mean response:

$$CV\ (\%)_{(concentration\ x)} = SD_{(concentration\ x)} \times 100 / Mean\ Response\ Value_{(concentration\ x)}$$

Acceptance criterion for the Intra-Assay Precision was CV ≤ 20%. The AlphaLISA signal was measured in quadruplicate and at least three of four replicates were used for the data analysis. The Intra-Assay Precision was within the acceptance criterion of CV ≤ 20% for all concentration points in the three independent assays.

The Overall Intermediate Precision (Inter-Assay Precision) was calculated as the ratio of the Relative Potency standard deviation to the mean Relative Potency in three independent assays:

$$CV\ (\%) = SD_{(Relative\ Potency\ 3\ assays)} \times 100 / Mean_{(Relative\ Potency\ 3\ assays)}$$

Acceptance criterion for the Inter-Assay Precision was CV ≤ 20%. The Inter-Assay Precision for the estimation of the Relative Potencies was within a range of 0.9 – 11.7% (Table 1), within the acceptance criterion of CV ≤ 20%.

Inter-Assay Precision of IC_{50}

The inter-assay variation of the effective half-maximal inhibitory concentration (IC_{50}) of Bevacizumab was estimated by the mean IC_{50} of the Reference Standard (Bevacizumab at 100% potency level) in all three independent assays. Acceptance criterion for the inter-assay variation of the IC_{50} was CV ≤ 20%. The inter-assay variation of the IC_{50} in all three independent assays was within the acceptance criterion of CV ≤ 20% (Table 2).

Table 2. Inter-assay variation of IC_{50}. The inter-assay variation for the estimation of Bevacizumab effective half-maximal inhibitory concentration (IC_{50}) at 100% potency is < 20% (CV=19.22%; n=15).

Mean Bevacizumab IC_{50} effective	SD	CV
ng/ml		%
285.19	54.80	19.22

Overall Linearity

Linearity was assessed for each experiment based on the ratio of the Nominal Potency to the Relative Potency at two potency levels. The Linearity was calculated using the formula below:

$$Linearity: (Nominal\ Potency_{(75\%)} / Nominal\ Potency_{(50\%)}) / (Relative\ Potency_{(75\%)} / Relative\ Potency_{(50\%)}) \times 100$$

Acceptance criterion for the Linearity was 100 ± 20%.

The Overall Linearity was calculated based on the ratio of the Nominal Potency to the Mean Relative Potency at two potency levels. The Overall Linearity was calculated using the formula below:

$$Overall\ Linearity: (Nominal\ Potency_{(75\%)} / Nominal\ Potency_{(50\%)}) / (Mean\ Relative\ Potency_{(75\%)}\ (3\ assays) / Mean\ Relative\ Potency_{(50\%)}\ (3\ assays)) \times 100$$

Acceptance criterion for the Overall Linearity was 100±20%.

The slope was calculated as steepness of the line obtained by plotting the Nominal Potency against the Relative Potency (linear regression line) using the regression line formula in Microsoft® Excel.

Acceptance criterion for the slope was: 0.80 ≤ slope ≤ 1.20.

The Correlation (r^2) was calculated using the correlation formula in Microsoft® Excel (Table 3).

Table 3. Overall Linearity, correlation and slope of the Relative Potencies (three experiments). The Mean Linearity of Bevacizumab Relative Potencies is recovered within a range of 89.0 - 108.2% (Mean Linearity) in three independent experiments. The Bevacizumab Relative Potencies are recovered with a squared correlation coefficient (r^2) of linear regression within the acceptance criterion of ≥0.90 and with a slope that ranges from 1.00 to 1.18 (within the acceptance criterion of 0.80 ≥ x ≥ 1.20).

Nominal Potency	Linearity			Mean Linearity	Correlation (r^2)			Slope		
	Replicate				Replicate			Replicate		
	1	2	3		1	2	3	1	2	3
	%									
150	n/a	n/a	n/a	n/a	0.99	0.98	0.97	1.15	1.18	1.00
125	103.1	103.2	111.8	105.9						
100	91.7	82.8	93.4	89.3						
75	111.6	102.1	110.9	108.4						
50	80.3	100.0	86.6	88.2						

Acceptance criterion for the Correlation was $r^2 \geq 0.90$.

The calculation of the Linearity for each potency level, correlation, and slope of the linear regression line enabled assessing the range of linearity, in which the assay gives a linear response in relation to different potency levels (Figure 5).

Specificity

The system specificity was assessed by comparing the response of the Bevacizumab AlphaLISA assay to increasing concentrations of the anti-VEGF antibody Bevacizumab and the anti-CD20 antibody Rituximab. The AlphaLISA signal was measured in triplicate and at least two of three replicates were used for data analysis. In contrast to Bevacizumab, the control antibody Rituximab did not inhibit the AlphaLISA signal in response to increasing antibody concentrations, confirming the specificity of the Bevacizumab AlphaLISA assay for the anti-VEGF antibody Bevacizumab (Figure 6). At the highest Rituximab concentration, the response value (AlphaLISA signal in counts) was 13.5% lower when compared to the mean response of all other Rituximab concentration points (25000.0-0.3 ng/mL). However, this slightly lower response at this concentration point (100000.0 ng/mL), was 57.5-fold higher than the response of Bevacizumab at the same concentration (Figure 6). Therefore, a specific effect of Rituximab at this concentration point can be excluded and the slightly lower response might be rather due to a steric hindrance effect.

Conclusions

The Bevacizumab AlphaLISA assay developed in this study is an *in vitro* method for the determination of Bevacizumab binding to VEGF. The adopted competitive assay configuration prevents hook effects, which are observed when employing a non-competitive setting (data not shown).

The settings have been optimized to achieve robustness by adopting an assay protocol that prevents influences on the IC_{50} due to discrepancies in the incubation times between the anti-VEGF antibodies and VEGF. When Bevacizumab is given temporal advantage over the other anti-VEGF antibodies for the binding to VEGF with a pre-incubation step, the IC_{50} variation might be of acceptable amplitude (below 20%) provided the incubation times are constant (data not shown). Keeping the incubation times constant (e.g. 30 ± 2 minutes) might be challenging due to operating times, when performing experiments with several plates. Therefore, a protocol that does not imply Bevacizumab / VEGF pre-incubation times is of advantage, since variations in pre-incubation times may cause fluctuations of the IC_{50}.

Accuracy, precision, and linearity of the Bevacizumab AlphaLISA assay were within the acceptance criteria which were set based on the suggestions of EMA/FDA guidelines and the 2010 United States Pharmacopeial Convention regarding acceptance criteria

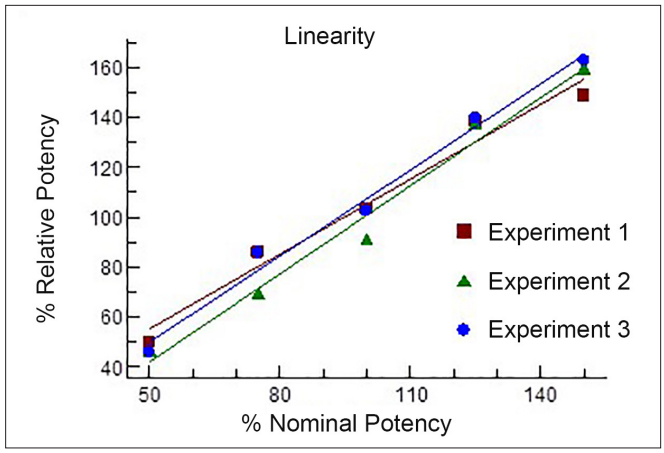

Figure 5. Linear regression line (three experiments). Linear regression line of Relative versus Nominal Potencies for the evaluation of the range of linearity for Bevacizumab potencies in three independent experiments: experiment 1 (red squares), experiment 2 (green triangles) and experiment 3 (blue circles). Graphics originate from the Excel software add-in XLFit. X-axis: Nominal Potency (%), Y-axis: Relative Potency (%).

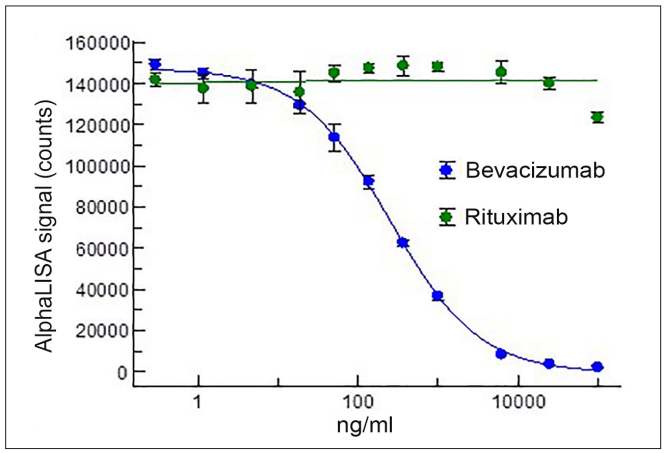

Figure 6. Specificity of the Bevacizumab AlphaLISA assay. In contrast to Bevacizumab (blue), the control antibody Rituximab (green) does not inhibit the AlphaLISA signal in response to increasing antibody concentrations. X-axis: concentration in ng/mL, Y-axis: response values (AlphaLISA signal in counts).

for validation of assays to characterize biologics and biosimilars. The AlphaLISA assay delivers a linear, accurate and precise response to increasing Bevacizumab concentrations within the explored range of potency (50%-150%). It is therfore suitable to assess lot-to-lot consistency and equivalence of Bevacizumab and biosimilars. The IC_{50} showed variations below the limit of 20% commonly accepted for biologics and biosimilars.

The specificity of the assay was confirmed by comparing the response to increasing concentrations of Bevacizumab, versus Rituximab (anti-CD20 mAb), which does not bind to VEGF.

Taken together these data qualify the competitive Bevacizumab AlphaLISA assay as a suitable cell-free method for potency estimation, orthogonal and complementary to cell-based assays.

Acknowledgements

We thank Dr. Michael Lässle and Dr. Dusan Zencak from PerkinElmer for excellent scientific support and providing AlphaLISA reagents and Dr. Michael Lässle for critical reading of the manuscript.

References

1. Presta, L. G., H. Chen, et al. (1997). Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. Cancer Res 57(20): 4593-9.
2. Yang, J., et al. Potent anti-angiogenesis and anti-tumor activity of a novel human anti-VEGF antibody, MIL60. Cell Mol Immunol, 2014. 11(3): p. 285-93.
3. USP (2010), 1032> Design and Development of Biological Assays. The United States Pharmacopeial Convention: 1032.
4. USP (2010), 1033> Biological Assay Validation. The United States Pharmacopeial Convention: 1033.
5. USP (2010), 1034> Analysis of Biological Assays. The United States Pharmacopeial Convention: 1034.
6. AlphaLISA Research Reagents Technical Data Sheet (TDS). Human Vascular Endothelial Growth Factor (VEGF) kit PerkinElmer Product No.: AL201 C/F.
7. AlphaLISA Assay Development Guide, PerkinElmer.

IBR Inc., a PerkinElmer Collaborator, is a Swiss GxP-compliant contract research organization offering bioanalytical services. www.ibr-inc.com

PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

PerkinElmer®

014315_01 PKI