Analyte Depletion of Serum for Quantitative AlphaLISA® Assays

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Introduction

The recently introduced AlphaLISA technology is an ELISA-like immuno-assay used for the detection of various analytes and biomarkers. Unlike ELISA, AlphaLISA assays are homogeneous, require no wash steps, offer a wide dynamic range without sample dilution, and are easy to miniaturize and automate, thus enabling efficient High Throughput Screening.

AlphaLISA technology allows the detection of molecules of interest in buffer, cell culture media, serum and plasma in a highly sensitive, quantitative, reproducible and user-friendly manner. As shown in ***Figure 1***, a biotinylated anti-analyte antibody binds to streptavidin-coated donor beads while another anti-analyte antibody is conjugated to AlphaLISA acceptor beads. The analyte is recognized by both antibodies, thus allowing the beads to come into close proximity. The excitation of the donor beads at 680 nm provokes the release of singlet oxygen molecules that trigger an energy transfer cascade in the acceptor beads. Consequently, a sharp peak of light emission is generated at 615 nm.

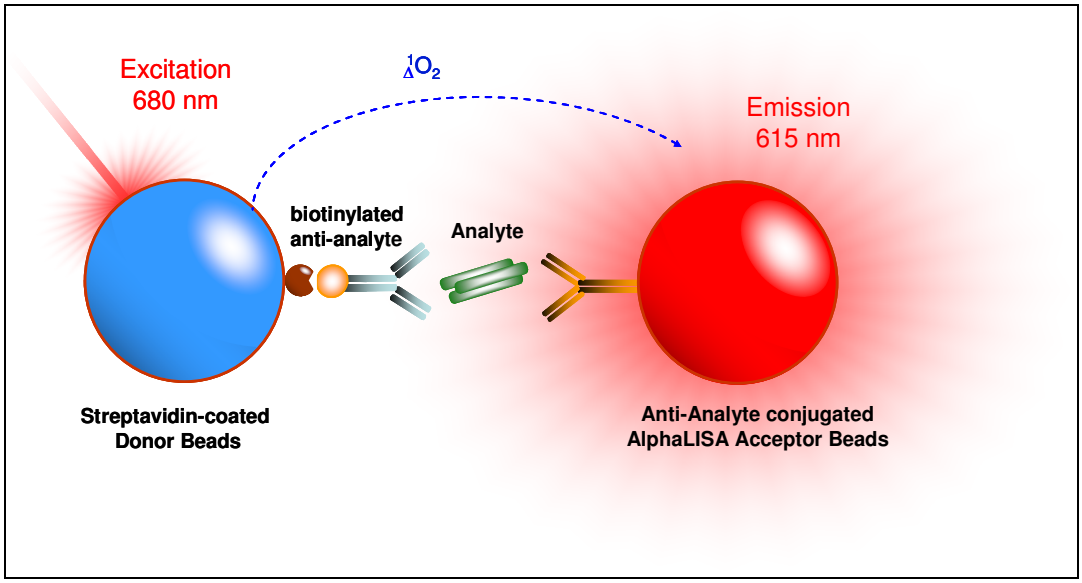

Figure 1: Principle of AlphaLISA technology

Quantitation of analyte in buffer and cell culture media samples can simply be performed by preparing a standard curve using the analyte diluted in analyte-free buffer or cell culture media and interpolating the unknown sample concentration from the standard curve. The same approach can be used for the detection of analyte in serum or plasma samples. However, as these biological fluids may naturally contain some level of the corresponding analyte, the latter needs to be removed from serum or plasma prior to performing an assay. This application note provides an

analyte depletion protocol that can be used to deplete most analytes from serum samples using biotinylated anti-analyte antibody and Streptavidin-Sepharose beads (SA-Sepharose). The protocol was tested with two analytes naturally occurring at different levels in serum, TNFα and VEGF. TNFα is found at approximately 1 – 15 pg/mL (1, 2) in normal human serum, whereas VEGF levels may range from 10 – 690 pg/mL (3, 4). Serum depletion was performed for both analytes and control samples were prepared (analyte spiked in depleted serum).

Materials and Methods

Materials

Table 1: Reagents and consumables

Product	Supplier	Cat. #
Human serum	Cambrex Corporation	14-402E
Biotinylated Antibody Anti-TNFα	PerkinElmer, Inc.	From kit # AL208
Biotinylated Antibody Anti-VEGF	PerkinElmer, Inc.	From kit # AL201
AlphaLISA human TNFα (0.1 µg), lyophilized	PerkinElmer, Inc.	# AL208S
AlphaLISA human VEGF (0.3 µg), lyophilized	PerkinElmer, Inc.	# AL201S
Streptavidin Sepharose beads	GE Healthcare, Inc.	17-5113-01
Phosphate-Buffered Saline (PBS) pH 7.4 (1X)	Invitrogen Corporation	10010-023
Charcoal depleted human serum	Innovative Research, Inc.	IPLA-SER
AlphaLISA Immunoassay Buffer (10X)	PerkinElmer, Inc.	AL000C
AlphaLISA TNFα Kit	PerkinElmer, Inc.	AL208C
AlphaLISA VEGF Kit	PerkinElmer, Inc.	AL201C
AlphaLISA Insulin Kit	PerkinElmer, Inc.	AL204C
AlphaLISA EPO Kit	PerkinElmer, Inc.	AL206C
OptiPlate™-384, white	PerkinElmer, Inc.	6007290
EnVision® HTS Reader, model 2101	PerkinElmer, Inc.	N/A

Methods

A. Calculations

1. Determine the natural level of the analyte in serum (e.g. literature).
2. Calculate the amount of analyte to be removed from the required volume of serum (plasma):

$$n_{analyte} = \frac{c_{analyte} \times V_{serum}}{M.W._{analyte}}$$

with:

$n_{analyte}$ = amount analyte [pmol]
$c_{analyte}$ = natural level of analyte in serum [pg/mL]
V_{serum} = volume of serum to be depleted [mL]
$M.W._{analyte}$ = molecular weight of analyte [pg/pmol]

3. Calculate the volume of biotinylated anti-analyte antibody (bio-Ab) stock needed to deplete the analyte from the serum using an ***antibody : analyte*** ratio (R_{AA}) of ≥ 20.
(Note: Depending on the antibody affinity, a higher ratio (e.g. 50 – 100) is suggested).

$$V_{bio-Ab} = \frac{n_{bio-Ab}}{c_{bio-Ab}} = \frac{n_{analyte} \times R_{AA}}{c_{bio-Ab}}$$

with:

V_{bio-Ab} = volume of bio-Ab stock [µL]
n_{bio-Ab} = amount of bio-Ab [pmol]
$n_{analyte}$ = amount of analyte [pmol] (see step 2)
R_{AA} = bio-Ab to analyte ratio, ≥ 20
c_{bio-Ab} = concentration of bio-Ab stock [pmol/µL]

4. Calculate the volume of Streptavidin Sepharose (SA-Sepharose) slurry needed for the depletion based on a ***sepharose binding sites : antibody*** ratio of ≥ 20:

* Assumption 1: 1 mL sedimented SA-Sepharose corresponds to 300 nmol biotin-binding capacity; 1 mL slurry corresponds to 0.3 mL sedimented SA-Sepharose or 90 nmol (or 90,000 pmol/mL) binding capacity.
* Assumption 2: 10 biotin molecules per anti-analyte antibody (if no other information available).

$$V_{slurry} = \frac{R_{SA} \times n_{bio-Ab} \times B}{Cap_{slurry}}$$

with:

R_{SA} = ratio of sepharose binding sites (pmol) to bio-Ab (pmol)
n_{bio-Ab} = amount of bio-Ab [pmol]
B = number of biotin molecules per bio-Ab
Cap_{slurry} = binding capacity of Sepharose beads [pmol/mL]

B. Preparation of anti-analyte sepharose beads

1. Gently resuspend the SA-Sepharose beads to generate homogeneous slurry by inverting the bottle several times (do not vortex).
2. Use a pipette to remove the required volume of slurry (see step A.4) and transfer to an appropriate container / tube.
(Note: Consult step 4 of this section to determine the appropriate tube size.)
3. Sediment the matrix at 1000 × *g* (Beckman GS-6R G-3.8 rotor, 2000 rpm, or equivalent) for 5 minutes. Quickly decant the supernatant to prevent disturbance of the beads.
4. Add 5 – 10 mL PBS for each mL of slurry used in step 2 and gently invert the tube several times until all beads are completely resuspended (do not vortex).
5. Sediment the matrix at 1000 × *g* for 5 minutes. Quickly decant the supernatant to prevent disturbance of the beads.
6. Repeat steps 4 to 5, twice.
7. Gently resuspend the sedimented SA-Sepharose beads with an equal volume of PBS (do not vortex).
(Note: Each mL of slurry used in step 2 results in approximately 0.3 mL sedimented beads.)
8. Mix the 50 % SA-Sepharose slurry with the required volume of biotinylated anti-analyte antibody (refer to step A.3).
9. Incubate for 2 hours at 4 °C with gentle agitation (e.g. rocking platform).
10. Sediment the beads at 1000 × *g* for 5 minutes. Quickly decant the supernatant to prevent disturbance of the beads or use a pipette if the tube walls are covered with a significant amount of beads.
11. Add 5 – 10 mL PBS for each mL of slurry used in step 2 and gently invert the tube several times until all beads are completely resuspended (do not vortex).

12. Sediment the beads at 1000 × *g* for 5 minutes. Quickly decant the supernatant to prevent disturbance of the beads.
13. Repeat steps 11 and 12 once.

C. Analyte depletion from serum

1. Add the required volume of serum (or plasma) and gently invert the tube several times until all beads are completely resuspended (do not vortex).
2. Incubate overnight at 4 °C with gentle agitation (e.g. rocking platform).
3. Sediment the beads by centrifugation at maximum speed (e.g. Beckman GS-6R G-3.8 rotor at 3000 rpm; an Eppendorf centrifuge at 12,000 – 13,000 rpm is recommended for small volumes) for 10 minutes.
4. Recover as much sample as possible using a pipette while avoiding disturbance of the sedimented beads and transfer into a new tube(s).
5. Sediment remaining beads by repeating step 3.
6. Recover as much sample as possible using a pipette while avoiding aspiration of any beads and transfer into a new tube(s).
7. Sediment remaining beads in the new tubes by repeating step 3.
8. Collect the depleted serum and store in aliquots at -20 °C.

D. AlphaLISA assay

Refer to the corresponding AlphaLISA Technical Data Sheet for detailed product and assay information (references 7 and 8). **Volumes of all reagents must be adjusted depending on the number of samples to be tested.**

1. Resuspend lyophilized analyte from the AlphaLISA kit in 100 µL depleted serum. Wait 15 minutes for complete dissolution before using the stock solution.
2. Dilute the analyte stock solution (prepared in step 1) 10-fold in depleted serum for the first standard curve point (TNFα: 100 ng/mL, VEGF: 300 ng/mL).
3. Prepare 11 additional standard curve points by performing serial dilutions of each standard dilution in depleted serum (two dilutions per log, i.e. 3X or 3.33X dilutions) (down to 0.3 pg/mL for TNFα and 1 pg/mL for VEGF) as shown in ***Table 2***.

Table 2: Standard curve dilutions for TNFα and VEGF

TNFα Standard Curve				VEGF Standard Curve			
Tube	**Volume of TNFα**	**Volume of diluent**	**[TNFα] (g/mL)¥**	**Tube**	**Volume of VEGF**	**Volume of diluent**	**[VEGF] (g/mL)¥**
A	10 µL of TNFα stock (1)	90 µL	1E-7	A	10 µL of VEGF stock (1)	90 µL	3E-7
B	60 µL of A	140 µL	3E-8	B	60 µL of A	120 µL	1E-7
C	60 µL of B	120 µL	1E-8	C	60 µL of B	140 µL	3E-8
D	60 µL of C	140 µL	3E-9	D	60 µL of C	120 µL	1E-8
E	60 µL of D	120 µL	1E-9	E	60 µL of D	140 µL	3E-9
F	60 µL of E	140 µL	3E-10	F	60 µL of E	120 µL	1E-9
G	60 µL of F	120 µL	1E-10	G	60 µL of F	140 µL	3E-10
H	60 µL of G	140 µL	3E-11	H	60 µL of G	120 µL	1E-10
I	60 µL of H	120 µL	1E-11	I	60 µL of H	140 µL	3E-11
J	60 µL of I	140 µL	3E-12	J	60 µL of I	120 µL	1E-11
K	60 µL of J	120 µL	1E-12	K	60 µL of J	140 µL	3E-12
L	60 µL of K	140 µL	3E-13	L	60 µL of K	120 µL	1E-12
M – P*	0 µL	100 µL	0	M – P*	0 µL	100 µL	0

¥ this concentration refers to the concentration of the analyte in the analyte dilutions (i.e. in 5 µL), and not in the final volume of the assay (i.e. 50 µL).
* background (signal of diluent without analyte)

4. In a white Optiplate-384, add 5 µL of each standard dilution (in triplicate) (rows 1 – 12). Add 5 µL of 1X AlphaLISA Immunoassay Buffer to the last four rows (in triplicate) to determine the background level and LDL value.
5. Prepare (5X = 50 µg/mL) AlphaLISA Anti-Analyte Acceptor beads by diluting them 100-fold in 1X AlphaLISA Immunoassay Buffer and add 10 µL to each well (10 µg/mL final assay concentration).
6. Incubate 30 minutes at 23 °C.
7. Prepare (5X = 5 nM) Biotinylated Anti-Analyte Antibody by diluting 100-fold in 1X AlphaLISA Immunoassay Buffer and add 10 µL to each well (1 nM final assay concentration).
8. Incubate 60 minutes at 23 °C.
9. Under subdued (100 lux or less) or green lighting, prepare Streptavidin Donor beads by diluting 62.5-fold in 1X AlphaLISA Immunoassay Buffer and add 25 µL to each well (40 µg/mL final assay concentration).
10. Incubate 30 minutes at 23 °C in the dark.
11. Read using EnVision instrument.
    a. Example for instrument settings: Total Measurement Time: 550 ms, Excitation Time: 180 ms, Mirror: D640as, Emission Filter: M570w, Center Wavelength 570 nm, Bandwidth 100 nm, Transmittance 75 %
12. Analyze the data as follows:
    a. Calculate the average count values for background wells (from M to P), standards (from A to L) and samples.
    b. Generate a standard curve by plotting the averaged counts as a function of the amount of standard.
    c. Transform the X-axis to log10 scale.
    d. Analyze the data using non-linear regression fit with a $1/Y^2$ data weighting. The data points at maximal concentrations of analyte after the hook point should be removed for correct analysis (keep the highest one for the regression).
    e. The lower detection limit (LDL) is calculated by interpolating the average background (from the 12 wells without analyte) plus 3 times the standard deviation value from the standard curve [LDL = Avge.Backgr + (3 × SD)].

Results

TNFα and VEGF serum depletion

The SA-Sepharose depleted serum protocol described in the Method section was used to deplete TNFα or VEGF from serum. The SA-Sepharose depleted serum was then tested in an AlphaLISA assay using the corresponding kit. To validate the SA-Sepharose serum depletion protocol, AlphaLISA assays were performed in parallel in AlphaLISA Immunoassay Buffer from the AlphaLISA kit and in commercially available charcoal depleted serum (charcoal stripping reduces the level of e.g. hormones, thus minimizing lot to lot serum variability). In addition to the generation of standard curves prepared in the 3 diluents, two types of controls were included in the experiments. All three diluents were spiked with known amounts of analyte and assayed as positive control. These samples were expected to result in 100 % analyte recovery when interpolated from the standard curve prepared in the same diluent. Moreover, human serum was spiked with known

amounts of analyte followed by SA-Sepharose depletion. The purpose of this experiment was to verify the efficiency of the depletion protocol provided with this application note as the AlphaLISA signal was expected to be comparable to the background signal (signal of diluent without analyte) of the depleted serum standard curve.

To ensure complete analyte removal from the serum, the following ratios were used (based on 15 pg/mL TNFα and 690 pg/mL VEGF in human serum; see also ***Table 7***):

Table 3: Serum depletion parameters for the standard curve preparations

Analyte	**R_{AA}** (see step A.3)	**R_{SA}** (see step A.4)
TNFα	18	26,000
VEGF	249	991

R_{AA} = bio-Ab to analyte ratio
R_{SA} = ratio of sepharose binding sites (pmol) to bio-Ab (pmol)

As VEGF is found at significantly higher levels in human serum (see ref. 3, 4) compared to TNFα, it would render the process too costly to deplete at a higher R_{SA} than indicated in Table 3. Thus, this parameter was adjusted to approximately 1000, which was expected to be efficient for VEGF depletion from human serum.

The TNFα and VEGF standard curves prepared in both, SA-Sepharose depleted and charcoal depleted sera, resulted in the lowest maximum counts as shown in ***Figure 2*** and ***Table 4*** (approximately 2-fold lower than buffer). Compared to AlphaLISA Immunoassay Buffer, the LDL (Lower Detection Limit) was found to be approximately 10-fold higher and the S/B ratio 10-fold lower for the SA-Sepharose depleted TNFα standard curve. The background (signal in the presence of diluent only) however was significantly higher than in charcoal depleted serum as well as buffer. For VEGF, the LDL was less than 2-fold higher in SA-Sepharose depleted serum and the S/B ratio comparable to AlphaLISA Immunoassay Buffer. Background was lower with SA-Sepharose depleted serum than with the Immunoassay buffer (Fig.2D). This observation is most likely due to mild quenching from serum components that affects the AlphaLISA detection per se.

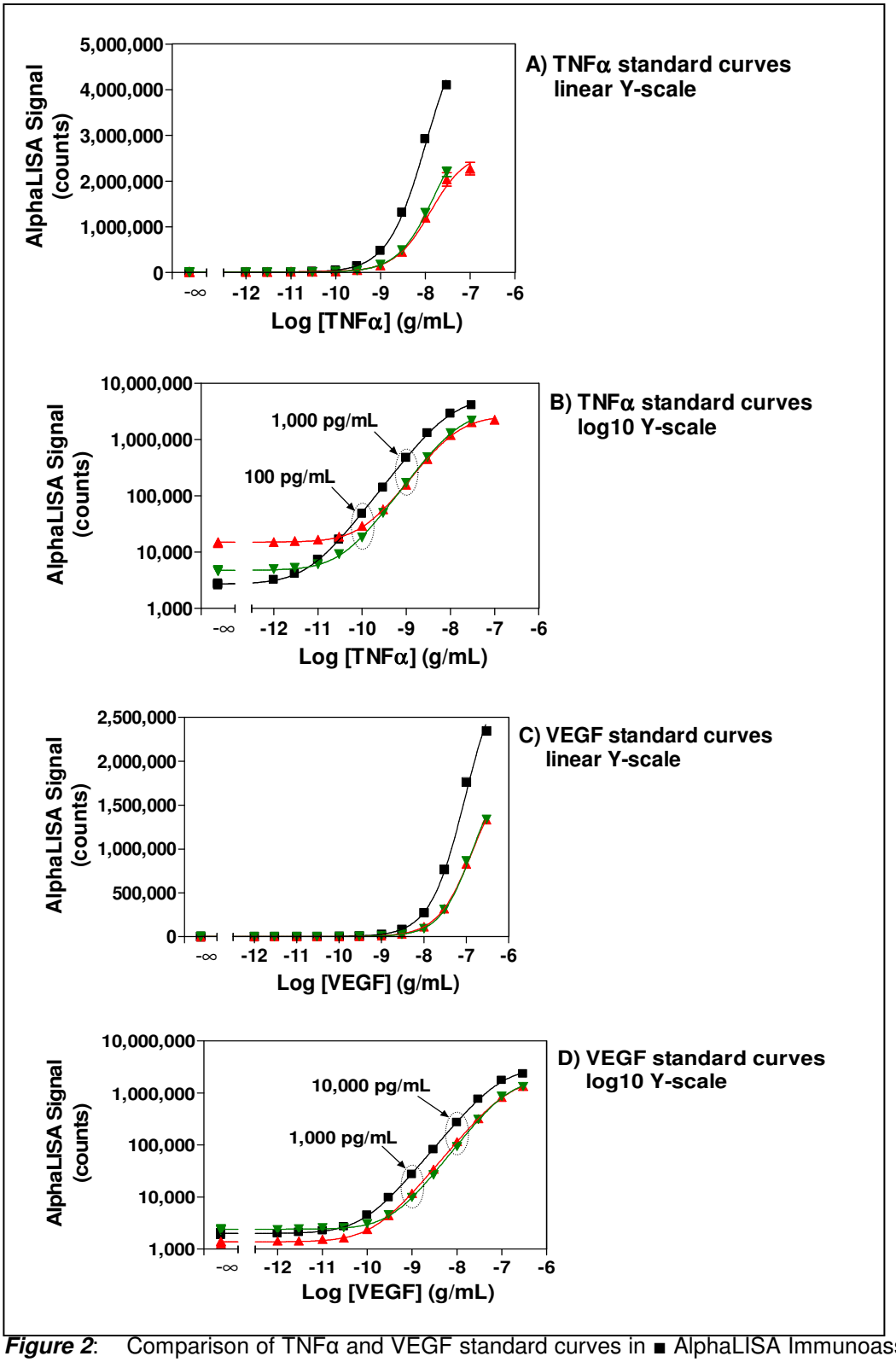

Figure 2: Comparison of TNFα and VEGF standard curves in ■ AlphaLISA Immunoassay Buffer, ▲ SA-Sepharose and ▼ charcoal depleted serum

Table 4: TNFα and VEGF standard curve parameters in different diluents

Diluent	Standard curve parameters TNFα				
	Min counts	Max counts	S/B**	EC_{50}	LDL***
Buffer	2,693	4.10×10^6	1,521	10 nM	1.0 pg/mL
Serum (SA-Sepharose*)	14,782	2.27×10^6	154	13 nM	10.3 pg/mL
Serum (Charcoal*)	4,686	2.20×10^6	469	15 nM	3.7 pg/mL
Diluent	**Standard curve parameters VEGF**				
	Min counts	Max counts	S/B**	EC_{50}	LDL***
Buffer	1,983	2.34×10e6	1,181	93 nM	15.0 pg/mL
Serum (SA-Sepharose*)	1,361	1.33×10e6	978	142 nM	25.2 pg/mL
Serum (Charcoal*)	2,384	1.34×10e6	561	139 nM	47.8 pg/mL

* depletion method
** Signal-to-Baseline ratio
*** Lower Detection Limit (average background + 3 × SD)

Analyte recovery in samples spiked with known amounts of TNFα or VEGF was close to 100 % (85 – 106 %) when interpolating the controls with the standard curve prepared with the same diluent as shown in ***Table 5*** (blue boxes). Samples analyzed on the standard curves prepared in a different diluent (non-shaded boxes) showed aberrant recovery values, as expected. Analyte recovery was also acceptable when using different sera for control and standard curve preparation (light green boxes, 70 – 115 %) within the overlapping range of the standard curves (≥ 300 pg/mL) (refer to ***Figure 2***).

Table 5: TNFα and VEGF recovery in control samples

Control sample prepared in:	TNFα: Recovery controls – Analyte standard curve prepared in:					
	Buffer		Serum (SA-Seph.*)		Serum (Charc.*)	
	Control A	Control B	Control A	Control B	Control A	Control B
Buffer	94 %	91 %	294 %	225 %	275 %	269 %
Serum (SA-Sepharose*)	29 %	55 %	85 %	106 %	85 %	162 %
Serum (Charcoal*)	32 %	33 %	93 %	27 %	92 %	94 %
Control sample prepared in:	**VEGF: Recovery controls – Analyte standard curve prepared in:**					
	Buffer		**Serum (SA-Seph.*)**		**Serum (Charc.*)**	
	Control A	Control B	Control A	Control B	Control A	Control B
Buffer	92 %	97 %	234 %	238 %	256 %	297 %
Serum (SA-Sepharose*)	35 %	36 %	86 %	93 %	101 %	115 %
Serum (Charcoal*)	28 %	30 %	70 %	77 %	83 %	94 %

TNFα controls: Control A = 1,000 pg/mL; Control B = 100 pg/mL
VEGF controls: Control A = 10,000 pg/mL; Control B = 1,000 pg/mL
* depletion method (SA-Sepharose or Charcoal)

To validate the depletion protocol, samples from other TNFα depletion batches (using different serum lots) carried out with antibody-to-analyte ratios (R_{AA}) of 6 – 38 and SA-Sepharose-to-antibody ratios (R_{SA}) of 82,000 – 580,000 (see ***Table 6,*** # 1 – 4) were included in the AlphaLISA assay and interpolated from the serum (SA-Sepahrose) standard curve shown in ***Figure 2***. No TNFα could be detected in these samples.

In addition, human serum was spiked with TNFα (1,000 pg/mL; see ***Table 6***, # 5 – 7) and VEGF (10,000 pg/mL, 1,000 pg/mL; see ***Table 6***, # 9 – 11), respectively, depleted using SA-Sepharose and finally interpolated using the corresponding serum standard curve. In both cases, a R_{AA} value of approximately 10 (see ***Table 6***, # 7) was not enough to completely remove the analyte from the serum sample, even though in the case of TNFα, previous depletion experiments with a R_{AA} of 6 had been successful. This result indicates that it is important to choose a sufficiently high R_{AA} value to successfully deplete analyte from different serum batches containing possibly varying amounts of analyte.

A non-depleted human serum sample resulted in the detection of 11.6 pg/mL TNFα (# 8) and 196 pg/mL VEGF (# 12) using the corresponding AlphaLISA kit, results that are in the range found in literature, demonstrating the ability of the AlphaLISA technology to provide physiologically meaningful results.

Table 6: TNFα and VEGF detection in depleted human serum samples (SA-Sepharose depletion)

#	Sample (TNFα)	Depletion parameters R_{AA}	Depletion parameters R_{SA}	TNFα detection (pg/mL)
1	Depleted h serum*	38	115,000	< LDL***
2	Depleted h serum*	38	82,000	< LDL***
3	Depleted h serum*	6	580,000	< LDL***
4	Depleted h serum*	6	117,000	< LDL***
5	h serum spiked (1,000 pg/mL), depleted**	100	1,050	< LDL***
6	h serum spiked (1,000 pg/mL), depleted**	100	984	< LDL***
7	h serum spiked (1,000 pg/mL), depleted**	10	9,847	83
8	h serum	N/A	N/A	11.6
#	**Sample (VEGF)**	**Depletion parameters R_{AA}**	**Depletion parameters R_{SA}**	**VEGF detection (pg/mL)**
9	h serum spiked (10,000 pg/mL), depleted**	89	900	<LDL****
10	h serum spiked (10,000 pg/mL), depleted**	9	2,250	34.5
11	h serum spiked (1,000 pg/mL), depleted**	57	2,250	<LDL****
12	h serum	N/A	N/A	196

* additional depletion batches
** h serum spiked with TNFα or VEGF and depleted thereafter
*** LDL = 10 pg/mL
**** LDL = 25 pg/mL

Other depletion experiments

Additional SA-Sepharose depletion experiments were carried out with TNFα, VEGF, EPO and Insulin. In contrast to TNFα and EPO, VEGF and Insulin occur at relatively high natural levels in human serum (see ***Table 7***), requiring significant quantities of SA-Sepharose matrix for the analyte depletion. To optimize the depletion protocol for these analytes, R_{SA} of 500, 200, 50 and 20 were tested for Insulin. Overlapping standard curves (graphs not shown here) and similar LDL values indicated efficient analyte depletion in all cases. Consequently, the R_{SA} was reduced to 20 – 30 for

Insulin and VEGF in the final protocol. All control samples (set of 3 for each experiment as indicated in ***Table 7***) resulted in recoveries close to 100 % confirming successful analyte depletion.

Compared to the results for TNFα and VEGF shown previously (see paragraph **TNFα and VEGF serum depletion**), higher S/B ratios and lower LDL values were obtained due to the higher R_{AA} employed. In addition, the VEGF depletion data presented below demonstrate that the R_{SA} can be significantly reduced without impacting the depletion success.

Table 7: AlphaLISA assay performance for different analytes (SA-Sepharose depleted serum)

Analyte	**Level in normal human serum***	**Depletion parameters**		**AlphaLISA assay parameters**		
		R_{AA}	R_{SA}	S/B	LDL	Recovery**
TNFα	1 – **15** pg/mL (ref. 1, 2)	320	1727	599	3.6 pg/mL	89 – 97 %
VEGF	10 – **690** pg/mL (ref. 3, 4)	166	30	1323	8.7 pg/mL	101 – 106 %
EPO	5 – **25** mU/mL (25 – 125 pg/mL) (ref. 5)	320	720	259	5.8 mIU/mL	98 – 107 %
Insulin	0 – **70** µU/mL (0 – 2414 pg/mL) (ref. 6)	200	21	906	1.3 µIU/mL	94 – 112 %

* maximum level (bold) used to calculate depletion parameters
** controls samples: TNFα, VEGF: 3,000, 300, 30 pg/mL; EPO: 3,000, 300, 30 mIU/mL; Insulin: 300, 30, 3 µIU/mL

Conclusions

This application note provides a protocol for an efficient depletion of analytes from sera using SA-Sepharose beads. Complete analyte removal, as well as analyte recoveries close to 100 % in depleted serum samples spiked with known concentrations, was demonstrated for TNFα and VEGF. To ensure precise quantitation of an analyte of interest, the corresponding standard curve should be prepared in the same diluent as the sample to be tested. As shown for TNFα and VEGF, SA-Sepharose- and charcoal-depleted sera are possibly interchangeable if the mid to high range of the standard curve is used for quantification. However, this assumption has to be confirmed and the limits precisely defined for each analyte.

Based on the data obtained, the following recommendations are made:

- Base calculations on maximum analyte concentration found in literature.
- Depletion conditions suggested: R_{AA} = 20 – 100
  R_{SA} = ≥ 20
- Include control samples (depleted serum spiked with a known concentration of analyte)

The protocol may have to be adapted to the requirements of the AlphaLISA assay (background, sensitivity), thus we suggest to increase R_{AA} if lower background and better sensitivity (LDL) are needed (e.g. R_{AA} ≥ 200) or when the antibody affinity is low.

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