APPLICATION NOTE

Quantify low abundant mIL-17A homodimer in mouse serum and plasma.

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Abstract

Revvity offers a wide variety of AlphaLISA™ detection kits that can be used to quantify biomarkers in biochemical solutions, cell culture media, cell lysates, serum, plasma, and tissue homogenates [1]. AlphaLISA kits are homogenous (no wash) immunoassays that require as little as 5 µL of sample and enable rapid and accurate measurement of biomarkers. AlphaLISA mIL-17A homodimer detection kit is designed to measure mouse IL-17A homodimer in a wide variety of matrices including serum and plasma. This application note demonstrates the quantification of low levels of mIL-17A homodimer in mouse serum and plasma samples using AlphaLISA mIL-17A detection kit.

Introduction

Interleukin-17A (IL-17A) homodimer protein is produced mainly by T cells and has a molecular weight of ~30 kDa. It plays critical roles in lupus erythematosus (LE), rheumatoid arthritis (RA), multiple sclerosis (MS), and inflammatory skin diseases. IL-17A acts on fibroblasts and macrophages by binding to its receptors to promote proinflammatory mediators such as TNF-α, IL-1β, IL-6, and IL-8. IL-17A inhibitors and antibodies have been developed for possible treatment of autoimmune conditions such as RA, psoriasis, and inflammatory bowel disease (IBD). IL-17A is also a target for anti-inflammatory therapies to improve post-stroke recovery and reduce the formation of skin cancer. Monitoring IL-17A in biological samples is critical for the determination of disease status. In mice, IL-17A levels in circulating blood (serum or plasma) are reported to be <20 pg/mL [2-5] and the volumes of serum or plasma samples collected from a mouse are small (< 20 µL) in longitudinal studies.

For research purposes only. Not for use in diagnostic procedures.

Therefore, measuring low levels of mIL-17A in a small volume of serum or plasma samples is challenging because commercial ELISA assays require 100 µL sample/well. Mouse IL-17A AlphaLISA assays require only 5 µL sample/well with the sensitivity (lower detection limit or LDL) reported to be 3.5 pg/mL in AlphaLISA Immunoassay Buffer (IAB). Since the levels of mIL-17A are low (< 20 pg/mL) in mouse serum and plasma samples, it is not feasible to dilute samples with IAB to reduce the sample interferences and still detect mIL-17A in diluted serum and plasma. Testing undiluted mouse serum or plasma samples along with the standard curve prepared in biologically compatible matrices such as 100% fetal bovine serum (FBS) or analyte-depleted mouse plasma or serum may be feasible. Preparing the analyte-depleted serum or plasma as a diluent for immunoassays is difficult and expensive. Instead, biologically more relevant matrices such as FBS have been used routinely as a diluent. To detect < 20 pg/mL mIL-17A in serum and plasma samples, the sensitivity of the assay in 100% FBS or in analyte-depleted serum must be < 5 pg/mL. The sensitivity of mIL-17A AlphaLISA detection kit in serum and plasma compatible matrices such as 100% FBS or analyte-depleted mouse serum or plasma is not known. Here, we determined the sensitivities of mIL-17A detection kit in biologically compatible matrices (100% FBS and 30KDmP), which can be used to quantify low levels of mouse IL-17A in small amounts of non-diluted serum and plasma samples.

AlphaLISA technology assay principle

AlphaLISA™ technology allows for the detection and quantification of molecules of interest in buffer, cell culture media, serum, plasma, and other biological matrices in a homogeneous (no wash), highly sensitive, easy-to-use, and reproducible manner [1]. In an AlphaLISA assay, a biotinylated anti-analyte antibody binds to the streptavidin-coated Alpha Donor beads, while another anti-analyte antibody is conjugated to AlphaLISA Acceptor beads. In the presence of the analyte, the beads come into proximity. The excitation of the Donor beads at 680 nm converts ambient oxygen into singlet oxygen molecules, triggering a cascade of energy transfer within the Acceptor beads and subsequent light emission at 615 nm which can be detected on an Alpha-enabled plate reader, such as the EnVision™ Multilabel Plate Reader. The amount of light emission is proportional to the concentration of analyte in the test samples. Figure 1 illustrates the AlphaLISA mIL-17A kit in which the analyte of interest, mIL-17A, is recognized by two antibodies: a biotinylated anti-mIL-17A antibody and anti-mIL-17A antibody conjugated AlphaLISA Acceptor bead.

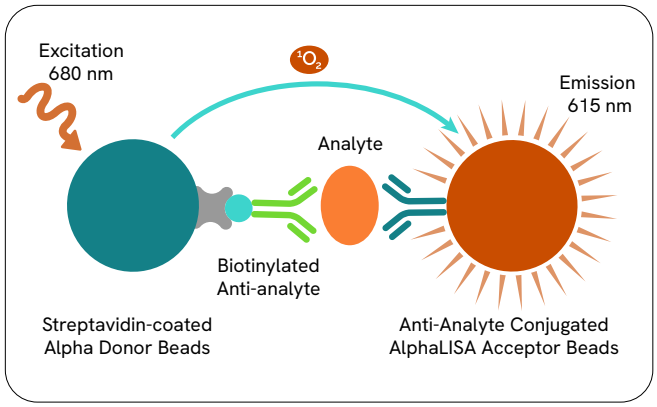


Figure 1. AlphaLISA mIL-17A assay principle.

Materials and methods

AlphaLISA mIL-17A detection kit (Revvity, AL540), FBS (FBS, Sigma, F2442), Charcoal Stripped FBS (csFBS, Gibco, A3382101), Heat-inactivated FBS (hiFBS, Gibco, A3840101), Dialyzed FBS (dzFBS, Gibco, A3382001), Ultra-Low IgG FBS (ul-IgG FBS, Gibco, A3382901), Ultrafiltered (30kDa) BALB/C Pooled Mouse Plasma (30KDmP, BioIVT, Custom no: MSE02PLNH-0106699), mIL-17A (R&D system, 421-mL-025/CF), 10 mL Balb/C serum (Innovative Research, IMJSBCSER), and 10 mL Balb/C plasma (Innovative Research, IMSBCPLAK2E) were purchased and stored at the recommended temperature until they were used for the assays. In addition to the mouse serum and plasma samples mentioned above, three mouse serum (S93-1, S93-2, and S94) and three mouse plasma (P1, P2, and P3) samples were also used in the experiments. OptiPlate-384, White Opaque 384-well Microplate (Revvity, 6007290), TopSeal™-A Plus Adhesive Sealing Film (Revvity, 6050185), and Revvity EnVision™ 2105 Multilabel Plate Reader were used to run the assays and collect the assay data.

Determining the sensitivity of AlphaLISA mIL-17A assays in 100% FBS

AlphaLISA mIL-17A assays were performed following the protocols provided in the kit technical data sheet (TDS) with modifications. In addition to preparing mIL-17A analyte standard curves (STD) in IAB, the analyte standard curves were also prepared in five different types of FBS such as FBS, csFBS, hiFBS, dzFBS, and ul-IgG FBS, and a 30KDmP to determine the sensitivities of mIL-17A assays. The assays were performed in 384-well Microplates with the total assay volume of 50 µL/well. The TDS recommended a 2-step assay protocol where 5 µL analyte standard dilutions or 5 µL test samples were dispensed into the plate and then 20 µL (2.5x)

biotinylated anti-mIL-17A antibody (bAb) and anti-mIL-17A Acceptor bead (Acc) mix was added. To reduce variability of triplicates, the test samples were spun 10 minutes at 1000 rpm and 5 µL supernatant (clear top portion) were dispensed into the assay plate. The assay plates were gently tapped, sealed with top-seal, and incubated for 60 min at 23°C. After incubation, 25 µL of freshly prepared (2x) streptavidin Donor beads (SA-DB) were added and incubated for an additional 30 min. In addition to running the 2-step protocol, the sensitivities in a 3-step protocol (5 µL STD dilutions or 5 µL test samples, 10 µL 5x Acc (30 min incubation), 10 µL 5x bAb (60 min incubation), and 25 µL 2x SA-DB (30 min incubation)) and a 2-step high sensitivity (2-step HS) protocol (5 µL STD dilutions or 5 µL test sample, 5 µL 10x bAb and Acc mix (60 min incubation), and 40 µL 1.25x SA-DB (30 min incubation)) were also determined to assess the suitability for detection of mIL-17A in serum or plasma samples. The final concentration of beads and antibodies were kept the same as in the kit manual. After incubation with SA-DB, the assay plates were read on a Revvity EnVision 2105 Multilabel Plate Reader equipped with an ALPHA option using the following settings: Total Measurement Time: 550 ms, Laser: 680 nm, Excitation Time: 180 ms, Mirror: 640 as (Barcode# 444), Emission Filter: Wavelength 570 nm, Bandwidth: 100 nm, Transmittance 75%, (Barcode# 244).

Testing mouse serum and plasma samples using the standard curves prepared in 100% FBS

After determining the sensitivities of AlphaLISA mIL-17A kit in different types of 100% FBS as diluent for analyte standard curves, the mIL-17A concentrations were measured in undiluted mouse serum and plasma samples using the standard curves prepared in different types of 100% FBS. To measure IL-17A, 5 µL undiluted mouse serum and plasma samples (n=3 to 6) were assayed along with the standard curves (5 µL/dilution, n=3) prepared in different types of 100% FBS and IAB. The AlphaLISA signals from serum and plasma samples were then interpolated to the standard curves to obtain the mIL-17A concentrations in the samples. The addition of antibodies, SA-DB, incubation time and temperature, and plate reading were identical to those described in the previous section. To confirm the compatibility of 100% FBS as diluent for mIL-17A standard curve preparation, undiluted mouse serum and plasma samples were spiked with known amounts of mIL-17A (20, 10, and 5 pg/mL). The mIL-17A analyte spiked samples were assayed along the standard curves prepared in 100% ul-IgG FBS. AlphaLISA signals from the mIL-17A spiked serum and plasma samples were then interpolated to the standard curves to obtain mIL-17A concentration in the samples. The percent (%) spike recoveries were calculated after the endogenous concentration was subtracted.

Quantification of mIL-17A levels in mouse serum and plasma samples using mIL-17A standard curve prepared in 30KDmP

The 30KDmP is a mouse plasma solution that does not contain molecules > 30kDa (e.g., IgGs, albumin, globulin) including mIL-17A. In the assays, three serum and three plasma samples were tested along with the standard prepared in 30KDmP. The assay was performed using the 2-step HS protocol. After dispensing 5 µL of standard curve dilutions or 5 µL of test samples to a 384-well assay plate, 5 µL of the 10x biotinylated anti-mIL-17A antibody and anti-mIL-17A Acceptor bead mix were added, the plates were spun for 10 to 15 seconds at 1000 rpm, and then sealed with top seal and incubated for 60 min at 23°C. Then, 40 µL 1.25x SA-DB was added and incubated for 30 minutes at 23°C. The plates were read as described in the previous sections. A mouse IL-17A spike recovery experiment was also performed to find out the recovery when the standard was prepared in 30KDmP with the 2-step HS protocol. Three known amounts of mIL-17A were spiked into normal mouse serum (n=3) and plasma (n=3) samples (30, 10, and 3 pg/mL mIL-17A in spiked samples). The spiked and undiluted samples were assayed along with the standard prepared in 30KDmP. The concentrations of mIL-17A in spiked and undiluted samples were obtained by interpolating the Alpha signals to the standard curve and the spike recoveries (%) were calculated as described above.

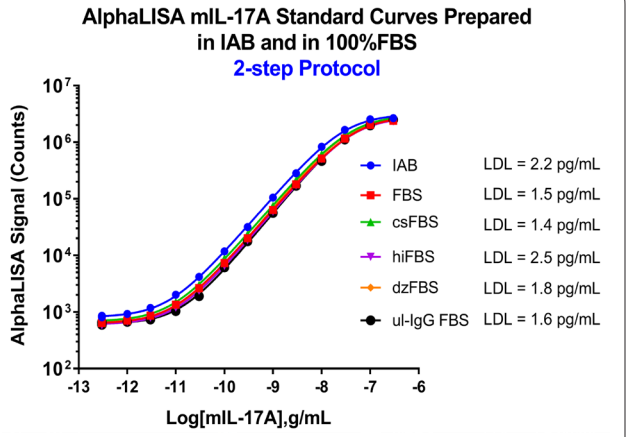
Data analysis

Data analysis was performed using Microsoft Excel and GraphPad Prism. AlphaLISA mIL-17A standard curve fitting (AlphaLISA signals vs. mIL-17A concentrations) was completed using a 4-parameter logistic equation (sigmoidal dose-response curve with variable slope) with a 1/Y2 data weighting. The lower limit of detection (LDL) was calculated by interpolating the average background signals (12 wells without analyte) + 3x standard deviation value (average background counts + (3xSD)) on the standard curve.

Results and discussion

Sensitivities of mIL-17A standard curves prepared in different types of 100% FBS

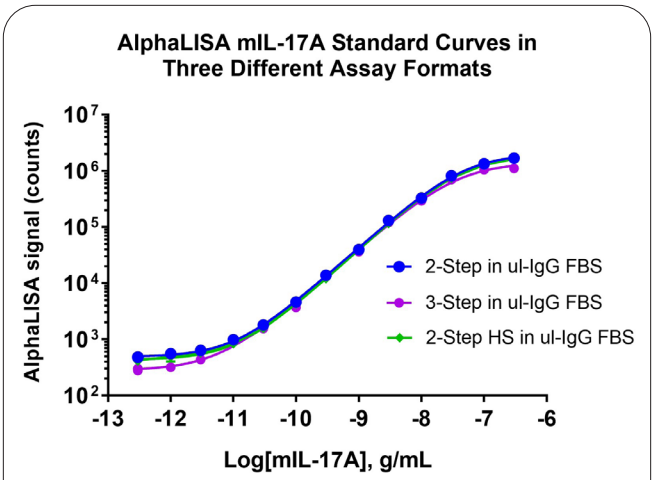
The results showed that AlphaLISA mIL-17A detection kit had excellent sensitivity (≤ 2.5 pg/mL) not only in IAB, but also in different types of 100% FBS (Figure 2). The standard curve parameters in the table below Figure 2 show that the AlphaLISA mIL-17A detection kit had a dynamic range of 3 to 4 logs, good signal to background ratio (S/B > 3000) with an EC50 of 30 to 50 ng/mL. There were noticeable differences in AlphaLISA signal among the standard curves prepared in IAB and in different types of 100% FBS. Overall, these results suggest that different types of 100% FBS can be used as a diluent to detect low levels of mIL-17A (as low as 3 pg/mL) in mouse serum and plasma samples.



mIL-17A	IAB	IAB	csFBS	hiFBS	dzFBS	ul-IgG FBS
LDL (pg/mL)	2.2	1.5	1.4	2.5	1.8	1.6
EC_{50} (ng/mL)	30	47	40	44	38	43
Max Signal	2657158	2392141	2530057	2323521	2447899	2487268
Min Signal	835	640	691	612	654	601
S/B Ratio	3184	3740	3662	3798	3742	4136

Figure 2. AlphaLISA mIL-17A standard curves in IAB and five different types of100% FBS showing the sensitivity and assay parameters.

The sensitivity of AlphaLISA mIL-17A kit using the 2-step, 3-step, and 2-step HS protocols is presented in Figure 3. The results showed that there was no difference in sensitivity (~2 pg/mL), EC50 (~40 ng/mL), and S/B ratio (~3400) among the different assay formats: 2-step (recommended in TDS) vs. 3-step vs. 2-step HS protocols. These results indicate that all three protocols can be used to measure low levels of mIL-17A in mouse serum and plasma samples by using the standards prepared in 100% FBS.



mIL-17A	2-Step	3-Step	2-Step HS
LDL (pg/mL)	1.9	2.0	1.9
EC50 (ng/mL)	47	38	45
Max Signal	1692875	1107285	1624380
Min Signal	487	291	430
S/B Ratio	3478	3804	3778

Figure 3. Sensitivity of AlphaLISA mIL-17A detection kit in three different assay formats (2-step, 3-step, and 2-step HS protocols). The analyte standard curve prepared in 100% ul-IgG FBS is shown as an example.

Detection of mIL-17A in mouse serum and plasma samples

Multiple experiments were conducted to measure the levels of mIL-17A in undiluted mouse serum and plasma samples and to determine the recovery of mIL-17A spiked into mouse serum and plasma samples. The first set of assays were performed to measure mIL-17A in serum and plasma samples along with the analyte standard curves prepared in IAB and different types of 100% FBS. The concentration of mIL-17A measured in serum and plasma samples is summarized in Figure 4. As expected, the levels of mIL-17A detected in samples varied when mIL-17A was quantified using the analyte standard curves prepared in different matrices (Figure 4). Approximately 4 to 5 pg/mL mIL-17A was detected in serum and plasma samples when STD prepared in IAB was used. In contrast, 7 to 15 pg/mL mIL-17A was detected when STD prepared in 100% FBS was used to quantify mIL-17A in serum and plasma samples. Overall, the STD curves prepared in 100% FBS gave 2- to 3-fold higher mIL-17A concentrations than the IAB (Figure 4). These results showed that higher levels of mIL-17A in mouse serum and plasma can be detected when the standard curve prepared in 100% FBS was used.

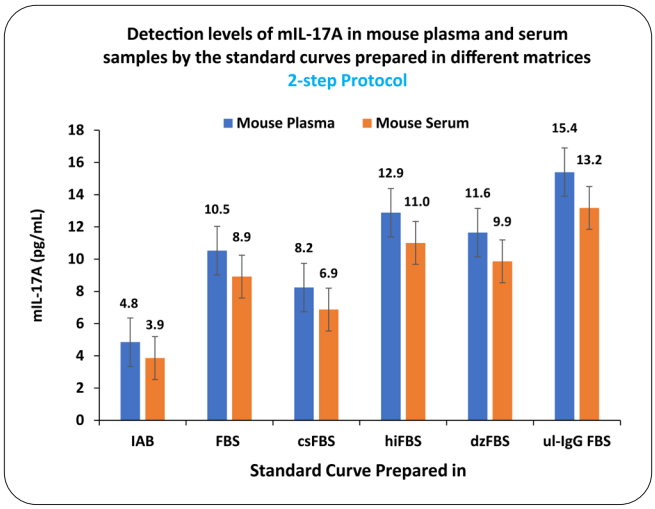


Figure 4. Mouse IL-17A measured in mouse serum and plasma samples using mIL-17A AlphaLISA detection kit. The levels were quantified by mIL-17A analyte standards prepared in IAB and various types of 100% FBS. The assays were performed using the regular 2-step protocol.

The results of the spike recovery experiment showed 76% recovery in mouse plasma and 71% in mouse serum (Table 1) after deducting the endogenous levels (15.4 pg/mL in plasma and 11.4 pg/mL in serum). Overall recoveries were acceptable (>70%). However, it indicated that standard curve prepared in 100% FBS (ul-IgG FBS) was not 100% compatible with the mouse serum or plasma samples as roughly 25 to 30% of spiked analyte was not recovered. It also implied that the levels of mIL-17A detected in mouse serum and plasma samples using 100% FBS (ul-IgG FBS) as a STD diluent were ~25 to 30% lower than the actual mIL-17A concentrations in mouse serum and plasma samples.

Table 1. Spike recovery of mIL-17A in mouse serum and plasma samples using ul-IgG FBS as STD diluent (2-step regular protocol)

Spiked samples	Plasma	Serum	Plasma	Serum
Spiked (pg/mL)	Recovered (pg/mL)		Recovery (%)	
20	15.1	13.9	76	70
10	7.4	6.4	74	64
5	3.9	4.0	77	81
Average	N/A	N/A	76	71

Quantification of mIL-17A in Mouse Serum and Plasma Samples Using the Standard Curve Prepared in 30KDmP

As indicated in the previous sections, the standard prepared in 100% FBS can be used to measure low levels of mouse IL-17A in undiluted mouse serum and plasma samples. However, the recovery of spiked mIL-17A (76% and 71%) in plasma and serum suggested that 100% FBS was not 100% compatible.

Additional experiments were performed to quantify the levels of mIL-17A in mouse serum and plasma samples using the standard curve prepared in 30KDmP (see materials section). The AlphaLISA mIL-17A assays with the 2-step HS protocol were performed. Figure 5 showed the sensitivity (<2 pg/mL) of the standards prepared in 30KDmP, IAB, and three different types of FBS. The results suggest that 30KDmP can be used as a diluent for the detection of low concentrations of mIL-17A (as low as 3 pg/mL) in serum and plasma samples.

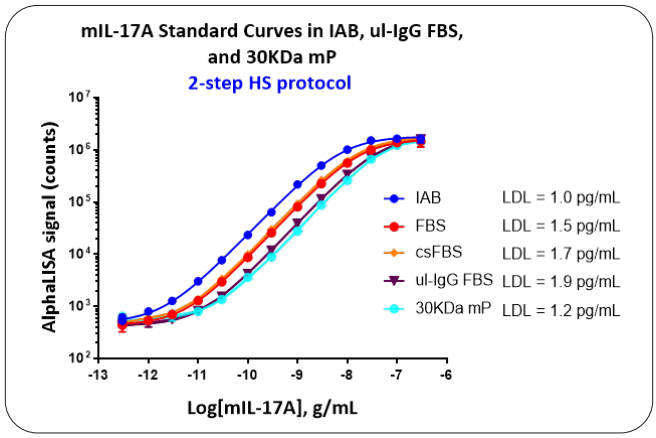


Figure 5. Sensitivity of the mIL17A AlphaLISA kit in IAB, 100% FBS, and 30KDmP in the 2-step HS protocol.

The results of mIL-17A quantifications in mouse serum and plasma samples using STD prepared in 30KDmP and the 2-step HS protocol are shown in Figure 6. The levels of mIL-17A measured in serum and plasma samples were 13 to 25 pg/mL using the STD prepared in 30KDmP for quantifications, which shows that mIL-17A standard curve prepared in 30KDmP can also be utilized to measure low levels of mIL-17A in mouse serum and plasma samples.

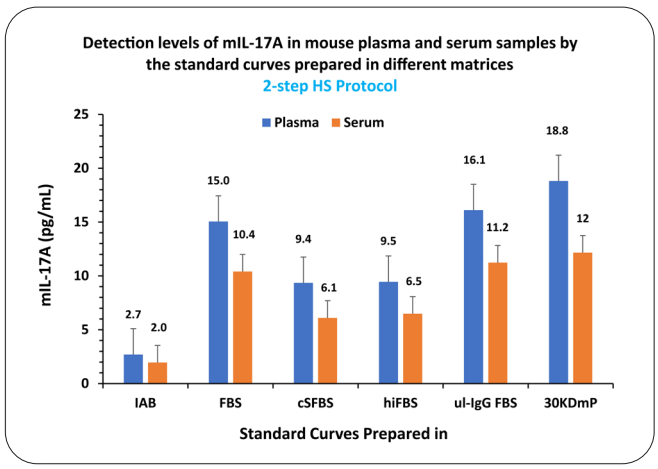


Figure 6. Mouse IL-17A measured in mouse serum and plasma samples using mIL-17A AlphaLISA detection kit. The levels were quantified by mIL-17A analyte standards prepared in IAB, four different types of 100% FBS, and 30KDmP. The assays were performed using the 2-step HS protocol.

The results of the spike recovery assay showed that the average recoveries were 80% and 75% in plasma and serum samples, respectively (Table 2). These results are nearly identical to the recoveries obtained using a standard curve prepared in 100% ul-IgG FBS in the regular 2-step protocol (Table 2). The recovered levels shown in Table 2 are the levels after subtracting the endogenous levels of 18.2 pg/mL in plasma and 14.7 pg/mL in serum.

Table 2. Spike recoveries of mIL-17A in mouse serum and plasma samples using 30KDmP as STD diluent (2-step HS protocol)

Spiked samples	Plasma (n=3)	Serum (n=3)	Plasma (n=3)	Serum (n=3)
Spiked (pg/mL)	Recovered (pg/mL)		Recovery (%)	
30	22.0 ± 3.9	22.9 ± 1.0	73 ± 7.4	76 ± 3.4
10	8.4 ± 1.2	7.1 ± 1.7	84 ± 12.3	71 ± 2.5
3	2.4 ± 1.2	2.3 ± 1.1	81 ± 2.9	77 ± 13.5
Average	N/A	N/A	79 ± 7.6	75 ± 7.6

Summary and Conclusion

Measuring low levels of biomarkers in complex biological samples such as serum and plasma is always challenging. This is further complicated by low sample volumes collected from mice which are widely used in the early stages of drug discovery and development. Here we report the sensitivity (LDL) of AlphaLISA mIL-17A detection kit in biologically compatible matrices such as FBS and 30KDmP that can be used to quantify mIL-17A in undiluted mouse serum and plasma samples. The results showed that AlphaLISA mIL-17A detection kit has excellent sensitivity (~ 2 pg/mL) not only in IAB, but more importantly in different types of 100% FBS and 30KDmP regardless of the assay format (2-step regular, 3-step regular, and 2-step HS). The low levels of IL-17A in undiluted mouse serum or plasma samples can be measured effectively using the standard curves for interpolation made in 100% FBS or in 30KDmP. The concentrations of mIL-17A detected in serum and plasma samples ranged from 6 to 25 pg/mL when the STD prepared in 100% FBS and 30KDmP were used for quantification. In conclusion, AlphaLISA mIL-17A detection kit can detect low levels of mIL-17A in 5 µL undiluted mouse serum or plasma samples when standard curves prepared in 100% FBS are used for quantification.

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