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Detection of bovine inflammatory cytokines IL-1β, IL-6, and TNF-α with a multiplex electrochemiluminescent assay platform

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ABSTRACT

Commercially available bovine-specific assays are limited in number, and multiplex assays for this species are rare. Our objective was to develop a multiplex assay for the bovine inflammatory cytokines IL-1β, IL-6, and TNF-α using the Meso Scale Discovery U-PLEX platform. "Do-It-Yourself" ELISA kits that contained polyclonal antibodies, both unlabeled and biotinylated, and the specific recombinant bovine cytokine standard, were purchased for each of these three cytokines. The biotinylated antibodies were coupled to linkers that bind to specific locations within each well of the U-PLEX plate. Unique linkers were used for each of the cytokines. The unlabeled antibodies were conjugated with electrochemiluminescent labels to serve as detection antibodies. Each cytokine assay was optimized individually prior to performing an optimization on the multiplex assay containing reagents for all three cytokines. To calculate cytokine concentrations, standard curves were developed using the recombinant cytokines and were run concurrently on each plate. Standard curves for IL-1β and TNF-α were run at concentrations ranging from 0 to 50,000 pg/mL, and for IL-6 from 0 to 10,000 pg/mL. The average lowest level of detection concentration measured by the standard curves were 5.3 pg/mL, 0.92 pg/mL, and 22.34 pg/mL for IL-1β, IL-6, and TNF-α respectively, as determined by data from seven plates containing bovine plasma samples from a combination of healthy and diseased cattle. The U-PLEX platform was a viable means to develop custom analyte- and species-specific multiplex assays using privately developed or purchased sets of commercially available reagents.

1. Introduction

Multiplex immunoassays are an efficient alternative to running individual assays for each metabolite of interest. Less sample can be utilized which is important for rare or difficult to obtain samples, much less time is spent on setting up plates and dilutions, and variation between different assays can be reduced by running them concurrently rather than over different plates and/or days (Dudal et al., 2014; Lea et al., 2011; Leng et al., 2008). Unfortunately, there are few multiplex bovine cytokine assays commercially available "off the shelf". These include the Milliplex® Bovine Cytokine/Chemokine 15-plex Panel 1 which includes IFNγ, IL-1α, IL-1β, IL-4, IL-6, IL-10, IL-17A, MIP-1α, IL-36RA, IP-10, MCP-1, MIP-1β, TNF-α, and VEGF-A (https://www.emdmillipore.com); the Abcam Bovine Cytokine Antibody Array which includes IFNα, IFNγ, IL-13, IL-1α, IL-1F5, IL-21, IP-10, MIG, MIP-1β, and TNF-α (https://Abcam.com); and the RayBiotech Quantibody® Bovine Cytokine Array 1 which includes ANGPT1, CCL5, CD40 L, DCN, IFNβ1, IL-10, IL-17A, IL-18, IL-1-β, and LIF (https://www.raybiotech.com/). This "one-size--fits-all" selection of metabolite assays may result in researchers paying for more information than they require or may not include the specific metabolite assays needed.

Platforms currently available for multiplexing include bead-based

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systems - also known as multiplexed bead immunoassays (MBIA) or bead-based multiplexed assays (BBMA) - where antibodies against specific analytes are bound to fluorescent beads and the samples are analyzed by a dual-laser, flow-based reader (Burgos-Ramos et al., 2012), glass slide arrays where the antibodies are bound to glass slides which are analyzed on specialized plate scanners, and the Meso Scale Discovery (MSD) platform where antibodies are bound within wells of a 96-well plate that includes electrodes that when stimulated in the specialized plate reader, results in electrochemiluminescent (ECL) emission. These different systems all have pros and cons as reviewed by Dudal et al. (2014) and Tighe et al. (2015), however one of the greatest considerations and costs is generally the "reader" required for each platform.

As with the ELISA, the gold standard of protein assay systems (Burgos-Ramos et al., 2012; Dernfalk et al., 2007; Dudal et al., 2014; Leng et al., 2008), all the multiplex systems available require the use of antibodies specific to the proteins being measured. Great progress in developing immune reagents such as antibodies for the study of veterinary immunology has been made through the efforts of individual research laboratories and groups such as the US Veterinary Immune Reagent Network (https://www.umass.edu/vetimm/) and The Immunological Toolbox maintained in the UK by The Pirbright Institute (https://www.immunologicaltoolbox.co.uk/). Researchers test lab-developed antibodies from domestic animals and in cooperation with companies such as Kingfisher Biotech (St. Paul, MN), these reagents are made commercially available to other research laboratories. Also available are "Do-It-Yourself" ELISA kits containing the antibody pairs required to perform a sandwich ELISA and a recombinant protein to serve as the standard/calibrator. Utilizing these kits eliminates the time and cost of identifying antibody pairs that bind to different epitopes thus allowing one to serve as the binding antibody and one as the reporter antibody.

Christopher-Henning et al., state "…it is useful to have multiplexed cytokine assays that can objectively assess the biological immune responses to pathogens, vaccines, and therapeutics or other interventions for controlling animal diseases." (2012) By adapting "Do-It-Yourself" ELISA kits, we developed a multiplex assay for the bovine inflammatory cytokines IL-1β, IL-6, and TNF-α using the MSD U-PLEX platform which can concurrently perform up to ten separate assays in a single well. The MSD U-PLEX platform utilizes a unique linker technology to capture biotinylated reagents at specific locations within the wells of microtiter plates, and users can develop customized multiplex assays at the bench. Herein we summarize our process and the efficacy of this multiplex assay.

2. Methods

2.1. Samples

Two types of samples were used as unknowns to develop the multiplex assay. The first samples were tissue culture supernatants from bovine monocytes that were isolated from three feedlot animals and either treated with LPS (N = 3) to stimulate cytokine production or were untreated (N = 3). Monocytes were isolated as previously described (Chitko-McKown et al., 2004) and were cultured in RPMI 1640 (Gibco, Thermo Fisher Scientific, Grand Island, NY; 22,400,105) containing 5% FBS (HyClone Laboratories, Logan, UT; 16777−530) and antibiotic/antimycotic (Thermo Fisher Scientific; 15,240,062). LPS (055:B5, Sigma-Aldrich, St. Louis, MO; L6529) was added to the media at a concentration of 1 μg/mL, and the cells cultured for 24H at 37℃ with 5% CO_2. The second set were plasma samples obtained from cattle enrolled on a longitudinal study of bovine respiratory disease complex (BRDC; DeDonder et al., 2015). These samples were from both healthy animals (N = 28) and those suffering from respiratory disease (N = 28). Plasma was collected from blood obtained from four time points from the healthy animals (D0, D1, D9, and D28), and six time points (D0, D1, D 9, date of diagnosis and treatment for respiratory disease, 5 days after diagnosis of respiratory disease, and D28). Isolation of bovine monocytes for stimulation with LPS was approved by the USMARC Institutional Animal Care and Use Committee (#2.1), and blood collection from the BRDC study cattle was approved by the KSU Institutional Animal Care and Use Committee (#3338).

2.2. Multiplex U-PLEX assay development

2.2.1. Reagent preparation

The U-Plex Development Pack (Catalog # K15228N-2) was purchased from MSD (Rockville, MD) and contained 3-Assay SECTOR Plates (96-well format), a set of U-PLEX linkers (for well locations 1, 3, and 10), U-PLEX Stop Solution, and 4X Read Buffer T. MSD SULFO-TAG Conjugation Pack (#R31AA-1) which utilizes NHS-Ester coupling chemistry, was also used to conjugate detection antibodies with SULFO-TAG, following manufacturer's instructions. "Do-It-Yourself ELISA" kits for Bovine TNF-α, IL-6, and IL-1β were purchased from Kingfisher Biotech, Inc., (Saint Paul, MN, USA; DIY0675B-003, DIY0670B-003, DIY1111B-003). Additional MSD Diluents used in the assays were purchased separately and included Diluent 2 (#R51BB-3), Diluent 7 (#R54BB-3) which were tested as diluents for the standard curves and the samples, and Diluent 8 (#R54BA-3) used to dilute the detection antibody mix.

The Biotinylated polyclonal antibodies from the "Do-It-Yourself ELISA" kits which would normally be used as reporter antibodies in a traditional ELISA were instead used as capture antibodies in the U-PLEX assay. In order to specifically bind to one of the 10 binding locations within each U-PLEX plate well, each antibody was diluted in 1% BSA/PBS-T to a concentration of 10ug/mL and coupled with a unique MSD Linker as per the manufacturer's instructions to result in three coating solutions with final antibody concentrations of 1μg/mL. For singleplex coating solutions, linked antibodies were diluted with MSD Stop Solution 10-fold to 1μg/mL final concentration; for multiplex coating solution, the three linked antibodies were combined and diluted with MSD Stop Solution so that the final concentration of each antibody was 1μg/mL. Linker 1 was coupled with biotinylated anti-bovine TNF-α polyclonal antibody, Linker 3 was coupled with biotinylated anti-bovine IL-1β polyclonal antibody, and Linker 10 was coupled with biotinylated anti-bovine IL-6 polyclonal antibody.

Recombinant protein standards/calibrators for all three cytokines were reconstituted at 5μg/mL in 1% BSA/PBS-T. In addition to preparing individual calibrators, a blended calibrator was created by combining the three recombinant proteins in a sub-stock at a concentration of 50,000 pg/mL each. The highest calibrator point (STD1) was prepared at a concentration of 10,000 pg/mL and 4-fold serial dilutions were carried out to a concentration of 2.44 pg/mL (STD7). The lowest calibrator point (STD8) was 1% BSA/PBS-T buffer only.

The "Do-It-Yourself ELISA" kit polyclonal antibodies that would traditionally have been used as capture antibodies in a sandwich ELISA were used as detection antibodies in the U-PLEX assay. These antibodies were buffer exchanged to remove sodium azide using Zeba™ Spin Desalting Columns (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturer's Buffer Exchange protocol. Recovered antibodies were then SULFO-TAG conjugated following MSD GOLD SULFO-TAG NHS-Ester Conjugation Pack 1 protocol. SULFO-TAG detection antibodies were diluted individually and in a multiplex format, all at a final concentration of 1μg/mL in 1% BSA/PBS-T.

2.2.2. Experiment one

Our first experiment was to test the sensitivity of each cytokine assay in singleplex on U-PLEX plates along with comparing the assays in multiplex using a simple diluent. Additionally, we sought to test the detection antibody cross-reactivity by testing each detection antibody individually and with the multiplex assay components. The assay was performed following a generic protocol provided in the MSD U-PLEX Development pack. All assays described in the following experiments

were performed in 8 rows by 12 columns, 96-well U-PLEX plates. Standards were run on every plate and all samples and standards were run in duplicate. Briefly, MSD 3-Assay 10-Spot SECTOR Plate and reagents were equilibrated to RT for 30 min prior to beginning the assay. The plate was blocked by adding 150μL 3% BSA prepared in phosphate buffered saline with 0.05 % Tween 20 (PBS-T) per well, sealed with adhesive plate sealing film and incubated with shaking at 700 RPM at RT for 1H on a benchtop microplate shaker. Alternately, plates were blocked overnight by adding 150μg/mL 3% BSA/PBS-T per well, sealed, and refrigerated overnight at 4 °C, with no agitation.

Blocking solution was removed and wells were washed three times with 150μl PBS-T. Fifty μl of coating solution containing capture antibodies coupled to a unique linker for TNF-α was added to columns 1 & 2 of the plate. Columns 3 & 4 were used for IL-6, and columns 5 & 6 for IL-1β. Columns 7–12 were coated with 50 μl containing all three capture antibodies. The plate was sealed and incubated for 1H at RT with shaking as described above. Plates were then washed three times with 150 μl wash solution.

Calibrators were prepared in 1% BSA/PBS-T and 4-fold serial dilutions were prepared with a highest calibrator concentration of 10,000 pg/mL for each recombinant protein. Seven serial dilutions of standard were plated in duplicate started with the highest calibrator (STD1) in row A, and the lowest concentration in row G (STD7). Row H contained 1% BSA/PBS-T only. The individual cytokine standards (50 μl/well) were plated as described above for the capture antibodies. The combined three standards were plated in descending concentrations in columns 7–11, and TNF-α STD 3 was plated in column 12 rows A–B, IL-6 STD 3 in rows C–D, IL-1β STD 3 in rows E–F, and 1% BSA/PBS-T was added to rows G–H. The plate was sealed and incubated at RT with shaking for 1H, followed by washing 3 times as described above.

Detection antibodies were added at 1 μg/mL in 25 μl using the following format: TNF-α, IL-6, and IL-1β were individually added to columns 1–2, 3–4, and 5–6, respectively. The three detection antibodies were combined and added to columns 7−8. The individual detection antibodies were also added to columns 9–11 similar to columns 1–6, and the combined antibodies were added to column 12. The plate was sealed and incubated for 1H at RT with shaking, followed by washing as described above. 150μl of 2X Read Buffer T was added to each well of the plate which were immediately analyzed on the SQ120 QuickPlex reader (MSD).

2.3. Experiment two

A second experiment was designed to compare the performance of each assay in different diluents and a titration of IL-6 detection antibody was performed. Capture antibodies were prepared and added to the plate as described for Experiment One followed by incubation for 1H at RT with shaking and washing as previously described. Calibrators were prepared as described however IL-6 STD 1 for this experiment was 10,000 pg/mL, and IL-1β and TNF-α STD 1 were increased to 50,000 pg/mL. Four-fold serial dilutions of the calibrators were performed in 1% BSA/PBS-T and were added to columns 1–6, and calibrators serially diluted with Diluent 7 were added to columns 7−12. Plates were sealed, incubated with shaking at RT for 2H and were washed as previously described.

The detection antibody mix was prepared with three different concentrations of IL-6 (1.0, 0.5, or 0.25 μg/mL) in two different buffers, either 1%BSA/PBS-T or Diluent 8 from MSD. Concentrations of the IL-1β and TNF-α antibodies remained at 1μg/mL. The detection antibody mix diluted in 1% BSA/PBS was added to columns 1–6, and the mix diluted in Diluent 8 was added to columns 7−12. IL-6 detection antibody was 1μg/mL for the mixes used in columns 1–2, and 7–8, 0.5μg/mL for the mixes used in columns 3–4, and 9–10, and 0.25μg/mL for the mixes used in columns 5–6, and 11−12. The detection antibody mix was added to each well at 25μL/well, the plate was sealed and incubated with shaking for 1H at RT and was washed as described above followed by the addition of 150μL of 2X Read Buffer T to each well of the plate with immediate analysis on the SQ120 QuickPlex reader.

2.4. Experiment three

We designed this experiment to test the performance of the multiplex when using 1% BSA/PBS-T as the diluent as compared to RPMI + 5% FBS. Samples tested in this experiment included supernatants from bovine monocytes cultured in RPMI 1640 with 5% FBS which were either untreated (N = 3) or treated with 1μg/mL LPS (N = 3) to stimulate inflammatory cytokine production. As described for Experiments One and Two, the capture antibodies were prepared at 10μg/mL in 1% BSA/PBS-T, the calibrators were prepared with STD 1 at 50,000 pg/mL for IL-1β and TNF-α and 10,000 pg/mL for IL-6. These were diluted in either 1% BSA/PBS-T or RMPI + 5%FBS. Supernatant samples were run undiluted. Detection antibodies were used at 1μg/mL for IL-1β and TNF-α, and at 0.25μg/mL for IL-6. The assay was run as described for Experiments One and Two.

2.5. Experiment four

This experiment was designed to test if plasma samples obtained from cattle either healthy or suffering from BRDC could be better assayed by diluting samples and calibrators with Diluent 2 or Diluent 7. The capture antibody mix was prepared with 1% BSA/PBS-T, and the detection antibody mix was prepared with Diluent 8. The plasma samples were assayed neat and at 2-fold, 4-fold, 8-fold, and 16-fold dilutions. The assay was run with all other conditions as described for Experiment Three.

2.6. Experiment five

Two hundred fifty unique bovine plasma samples were run undiluted using optimized conditions determined by the prior four experiments to determine the percent intra- and inter-assay variation.

2.7. Statistics

The data for experiments one through four were analyzed using MSD Workbench 4.0 software. The software calculates the working range of the assays by determining the Lower Level of Detection (LLOD) and the Upper Level of Detection (ULOD). Concentrations within these levels fall within the working range.

For experiment five, variability was assessed by estimating the Coefficient of Variation CV;(Reed et al., 2002) Intra-assay CV were calculated from 36 available animal, day, and plate combinations with duplicate assays on the same plate and having values from 2 to 100, 000 pg/mL. Five IL-1β combinations were removed for values greater than 100,000 pg/mL, one TNF-α sample did not produce a duplicate value, and three IL-6 values were less than 2 pg/mL. Assay CV were calculated from the SD of the two duplicates divided by the mean of the duplicates. The CVs for an assay were squared, summed, and then divided by number of samples. The square root of this value was an estimate of the intra-assay CV. Inter-assay CV was calculated from 16 available animal and day combinations that had duplicate assays on different plates. Three IL-1 β duplicates were removed for values greater than 100,000 pg/mL, and two IL-6 duplicates had less than 2 pg/mL. Duplicates within a plate were averaged and SD of these averages across plates were calculated for each combination. The SD was divided by the average to estimate a CV for the 16 inter-plate assays. These CV were squared, summed, and divided by the number of combinations. The square root of this value was an estimate of the inter-assay CV.

3. Results

Experiment One: The dynamic range and sensitivity for both IL-1β

and TNF-α were better in singleplex than multiplex format (Table 1). The signal to background ratio (S:B; Relative Light Units - RLU) were 30 and 70 RLU for IL-1β and TNF-α in singleplex, respectively, which decreased to 14 and 20 RLU respectively, for the multiplex. Background increased from 290 to 590 RLU from singleplex to multiplex for IL-1β and from 250 to 650 RLU for TNF-α. However, the sensitivities of the singleplex and multiplex formats were similar for both cytokines. The dynamic range and sensitivity of the bovine IL-6 assays were comparable in both formats, with the highest S:B of 30 RLU for singleplex and 14 RLU for multiplex. The background signals were similar in both formats for this cytokine, as were the sensitivities. The sensitivities for all assays were in the pg/mL range, and the standard curves each had an r^2 of greater than 0.99. All detection antibodies had a low non-specific binding to the IL-6 capture antibody; however, this was within a manageable level.

Experiment Two: Decreasing the concentration of IL-6 detection antibody increased the S:B for both the IL-1β and TNF-α antibodies in all diluents tested, and 0.25μg/mL was determined to be the optimal concentration for the IL-6 detection antibody. However, compared to 1% BSA/PBS-T, using Diluent 7 or 8 resulted in higher background signals and lower sensitivity for TNF-α but lower background signals for IL-1β (Fig. 1).

Experiment Three: The analytes could be detected at below 50 pg/mL for all assays and excellent reproducibility was observed with both RPMI + 5% FBS and 1% BSA/PBS-T with % CVs of 11.1 and 7.8, respectively. (Tables 2 and 3).

Experiment Four: Diluent 2 performed better than Diluent 7 when analyzing plasma samples. The differences noted between the two diluents were observational. However, because it was determined that by diluting the plasma samples, we would no longer be able to measure IL-6 in many samples due to the lower concentrations of IL-6 as compared to IL-1β and TNF-α, all plasma samples were analyzed undiluted (Table 4).

Experiment five: Analysis of 280 plasma samples each run in duplicate, half from healthy cattle and half from cattle presenting with BRDC, resulted in intra-assay CVs of 5.6 %, 3.6 %, and 4.2 %, and inter-assay CVs of 32.9 %, 28.0 %, and 18.0 % for IL-1β, IL-6, and TNF-α, respectively. The CV became greater at the extreme ends of the standard curves. Only 37 out of 280 unique bovine serum samples assayed had IL-1β or TNF-α concentrations above the ULOD of these standard curves thus requiring the samples to be diluted, and only three samples had IL-6 concentrations below the LLOD (Fig. 2).

4. Discussion

There are examples in the literature of researchers using the MSD platform to study bovine analytes. Mayers and Sawyer (2012) used this system to detect antibodies against four viral bovine respiratory disease pathogens. Jones et al. (2010) used an MSD multiplex to measure seven analytes important in *Mycobacterium bovis* infection in cattle. Coad et al. (2010) developed a bovine seven-plex to study the effect of repeated tuberculin skin testing on naturally infected tuberculous cattle. However, in these studies, the researchers relied on the company to custom prepare the assay plates. Tang et al. (2019) describe using the U-PLEX system to develop a multiplexed drug level assay thus showing the utility of this system for preparing custom assays within a research laboratory as compared to in MSD's manufacturing facilities.

Christopher-Henninge et al. (2013) list the numerous assay optimization steps that should be considered when developing immunoassays. These include identification of specific antibody pairs (either monoclonal or polyclonal), the identification of optimal buffering conditions, incubation times, optimal range of standards/calibrators, and comparisons made between singleplex and multiplex formats. The steps followed in the development of the multiplex assay described in this study cover all these considerations. We obtained Do-It-Yourself ELISA kits that used the same polyclonal antibody for both the capture and the detection steps, thus eliminating the concern that two monoclonal antibodies against the same analyte may bind to the same epitope (Christopher-Hennings et al., 2013). Using the U-PLEX chemistry and platform, the roles of these antibodies were reversed from the standard sandwich ELISA format. Various dilution buffers were tested for all stages of our U-PLEX assay, incubation times were validated, the range of our standards/calibrators were adjusted, and we compared the sensitivity of our assays in both singleplex and multiplex formats.

Samples used to develop and validate this assay included cell culture supernatants from bovine monocytes that were either un-treated, or treated with LPS to stimulate inflammatory cytokine production, and plasma samples obtained from a group of feedlot cattle under study for BRDC. We chose this cohort of cattle because values for inflammatory cytokines should be low in healthy cattle and elevated in sick cattle. This provided us with a broad range of concentrations to measure in the assay and would indicate if our "optimal" concentrations selected for the standard curve were sufficient and would give us an indication if the dynamic range of the multiplex was sufficient to suit most samples.

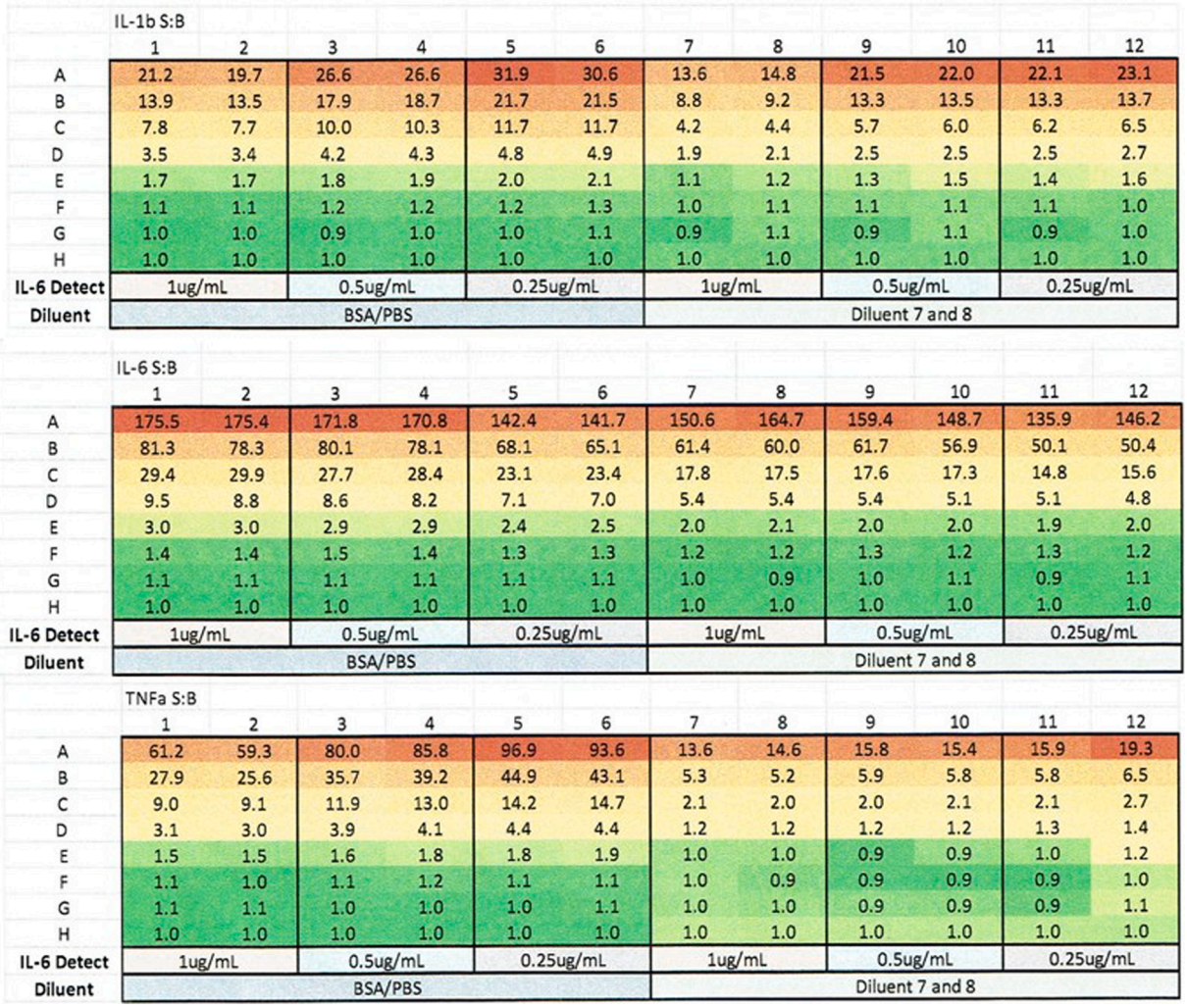
The MSD Workbench 4.0 software default calculates the ULOD as the highest standard concentration unless there is an obvious plateau in the top of the curve in which case it will calculate 2.5 Std deviations below the top standard. The LLOD is calculated as 2.5 Std deviations above the background signal, so this will change for each run based on the precision of those background signals. In our experiments the ULOD of the standards/calibrators were consistently listed as the highest concentration included in the standard curves. Several plasma samples fell below the LLOD of the IL-6 assay however, no sample were below LLOD for IL-1β and TNF-α. In contrast, several samples were above the ULOD for IL-1β and TNF-α. Diluting serum samples can result in a reduction of matrix background (Juncker et al., 2014; Leng et al., 2008), but due to the differences in plasma concentrations between IL-6 and the other two cytokines, dilution of our samples at even 1:2 would have resulted in many samples being below the LLOD for IL-6. The differences in ranges of analyte concentrations should be considered when developing multiplex assays for this reason (Dudal et al., 2014; Leng et al., 2008). It appears that STD 1 for IL-1β and TNF-α may require a higher concentration than 50,000 pg/mL when analyzing samples from animals suffering from inflammation, however, we did not attempt it in these experiments. For studies where concentrations are hypothesized to be higher than in normal samples, this increase should be considered. We used four-fold serial dilutions to prepare our standard curves, and it may be possible to start with a higher maximum concentration and use a larger dilution to increase the range of the standards for IL-1β and TNF-α while still using seven calibrator dilutions and one with buffer only.

Table 1
Summary of the limits of detection (LLOD, ULOD) for each assay format and shows the dynamic range and reproducibility of the standards for each assay.

Single vs Multiple	IL1-β Single	IL1-β Multi	IL-6 Single	IL-6 Multi	TNF-α Single	TNF-α Multi
LLOD (pg/mL)	20.1	23.2	4.2	1.8	29.0	43.4
ULOD (pg/mL)	10,000	10,000	10,000	10,000	10,000	10,000
Dynamic Range (log)	2.7	2.6	3.4	3.7	2.5	2.4
%CV Signal	2.3	2.0	4.0	1.8	3.5	2.6

5. Conclusions

We show that commercially available "Do-It-Yourself" ELISA kits can be adapted to the Meso Scale Discovery U-PLEX platform to develop custom multiplex assays. Our U-PLEX assay for the bovine cytokines IL-1β, IL-6, and TNF-α produced consistent, high quality data with intra- and inter-assay variation within acceptable ranges. Although there are



IL-1b S:B

	1	2	3	4	5	6	7	8	9	10	11	12
A	21.2	19.7	26.6	26.6	31.9	30.6	13.6	14.8	21.5	22.0	22.1	23.1
B	13.9	13.5	17.9	18.7	21.7	21.5	8.8	9.2	13.3	13.5	13.3	13.7
C	7.8	7.7	10.0	10.3	11.7	11.7	4.2	4.4	5.7	6.0	6.2	6.5
D	3.5	3.4	4.2	4.3	4.8	4.9	1.9	2.1	2.5	2.5	2.5	2.7
E	1.7	1.7	1.8	1.9	2.0	2.1	1.1	1.2	1.3	1.5	1.4	1.6
F	1.1	1.1	1.2	1.2	1.2	1.3	1.0	1.1	1.1	1.1	1.1	1.0
G	1.0	1.0	0.9	1.0	1.0	1.1	0.9	1.1	0.9	1.1	0.9	1.0
H	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
IL-6 Detect	1ug/mL		0.5ug/mL		0.25ug/mL		1ug/mL		0.5ug/mL		0.25ug/mL	
Diluent	BSA/PBS						Diluent 7 and 8					

IL-6 S:B

	1	2	3	4	5	6	7	8	9	10	11	12
A	175.5	175.4	171.8	170.8	142.4	141.7	150.6	164.7	159.4	148.7	135.9	146.2
B	81.3	78.3	80.1	78.1	68.1	65.1	61.4	60.0	61.7	56.9	50.1	50.4
C	29.4	29.9	27.7	28.4	23.1	23.4	17.8	17.5	17.6	17.3	14.8	15.6
D	9.5	8.8	8.6	8.2	7.1	7.0	5.4	5.4	5.4	5.1	5.1	4.8
E	3.0	3.0	2.9	2.9	2.4	2.5	2.0	2.1	2.0	2.0	1.9	2.0
F	1.4	1.4	1.5	1.4	1.3	1.3	1.2	1.2	1.3	1.2	1.3	1.2
G	1.1	1.1	1.1	1.1	1.1	1.1	1.0	0.9	1.0	1.1	0.9	1.1
H	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
IL-6 Detect	1ug/mL		0.5ug/mL		0.25ug/mL		1ug/mL		0.5ug/mL		0.25ug/mL	
Diluent	BSA/PBS						Diluent 7 and 8					

TNFa S:B

	1	2	3	4	5	6	7	8	9	10	11	12
A	61.2	59.3	80.0	85.8	96.9	93.6	13.6	14.6	15.8	15.4	15.9	19.3
B	27.9	25.6	35.7	39.2	44.9	43.1	5.3	5.2	5.9	5.8	5.8	6.5
C	9.0	9.1	11.9	13.0	14.2	14.7	2.1	2.0	2.0	2.1	2.1	2.7
D	3.1	3.0	3.9	4.1	4.4	4.4	1.2	1.2	1.2	1.2	1.3	1.4
E	1.5	1.5	1.6	1.8	1.8	1.9	1.0	1.0	0.9	0.9	1.0	1.2
F	1.1	1.0	1.1	1.2	1.1	1.1	1.0	0.9	0.9	0.9	0.9	1.0
G	1.1	1.1	1.0	1.0	1.0	1.1	1.0	1.0	0.9	0.9	0.9	1.1
H	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
IL-6 Detect	1ug/mL		0.5ug/mL		0.25ug/mL		1ug/mL		0.5ug/mL		0.25ug/mL	
Diluent	BSA/PBS						Diluent 7 and 8					

Fig. 1. Signal to Background (S:B) showing the highest S:B in red and the lowest S:B in green. Maximum S:B for all cytokines was obtained when the IL-6 detection antibody was used at 0.25μg/mL.

Table 2
Summary of the lower, and upper limits of detection (LLOD, ULOD) for each assay format and shows the dynamic range and reproducibility of the standards for each assay when different sample diluents (1%BSA/PBS-T vs RPMI + 5% FBS).

U-PLEX 3-Assay	1%BSA/PBS-T			RPMI + 5%FBS		
	IL-1β	IL-6	TNF-α	IL-1β	IL-6	TNF-α
LLOD (pg/mL)	17.0	1.5	38.3	5.2	1.3	14.5
ULOD (pg/mL)	50,000	10,000	50,000	50,000	10,000	50,000
Dynamic Range (log)	3.5	3.8	3.1	4.0	3.9	3.5
Calc Conc. %CV	13.3	2.7	7.2	22.8	2.5	8.2

Table 3
Calculated concentration means and calculated concentration %CV for bovine monocyte tissue culture supernatant sample either unstimulated or stimulated with LPS.

Sample ID	Calculated Concentration Means			Calculated Concentration %CV		
	IL-1β	IL-6	TNF-α	IL-1β	IL-6	TNF-α
386 Control	30.1	20.5	60.4	21.1	2.8	18.9
386 LPS	137.5	63.5	2,244.9	5.6	0.7	2.0
455 Control	13.2	31.3	123.3	5.4	0.3	3.8
455 LPS	403.2	94.6	4,812.9	2.4	0.5	5.0
602 Control	81.2	87.1	316.9	0.4	0.1	1.9
602 LPS	1,932.0	214.5	7,935.2	1.6	1.0	0.1

few commercially available multiplex assays for species others than rodents and humans, the availability of monoclonal and polyclonal antibodies and recombinant proteins specific for other species are becoming more abundant. By using these reagents within the U-PLEX system, researchers can more easily develop multiplex assays for their specific needs.

Table 4
Calculated intra- and inter-assay %CV for 36 and 16 bovine plasma samples respectively, run over at least 2 plates.

U-PLEX 3-Assay	Calculated Concentration %CV		
	IL-1β	IL-6	TNF-α
Intra-assay %CV	5.6	3.6	4.2
Inter-assay %CV	32.9	28.0	18.0

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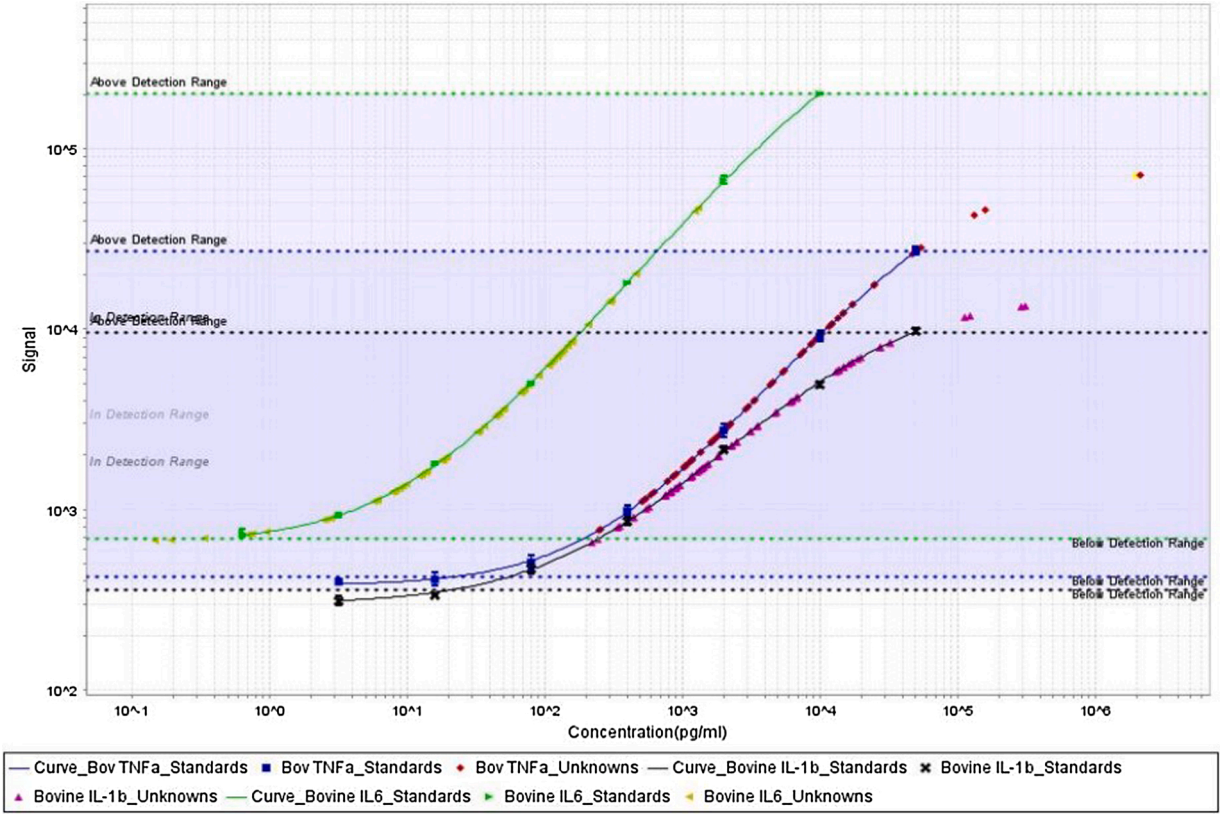

Fig. 2. Representative standard curves for the multiplex assay for bovine IL-1β, IL-6, and TNF-α with bovine plasma samples from healthy cattle, or animals presenting with bovine respiratory disease serving as unknowns.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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