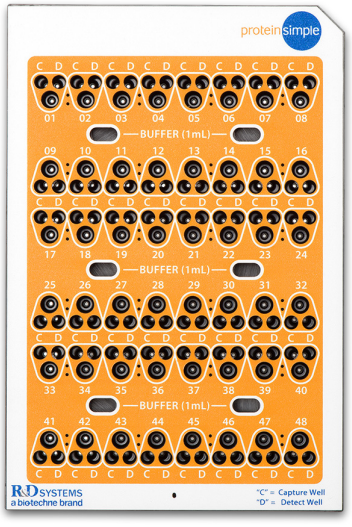
Open Access to Ella: Developing a Cytokine Immunoassay Using the 48-Digoxigenin Cartridge



Introduction

That's right. We're giving you open access to target detection on Ella. With the 48-Digoxigenin (48-Dig) Simple Plex cartridge, you'll have the freedom to choose your reagents, utilize the best immunoassay workflow out there, and detect the analytes most suitable for your research. All assay steps happen within a disposable Simple Plex cartridge similar in design to our standard format, but now your assay options are expanded beyond the preset menu. With straightforward protein labeling workflows, building your own sandwich assays on Ella is a breeze. And you'll get the same quality results! Other ELISA technologies have nothing on the reproducibility and detection sensitivity of Ella. So, whether you're a current Ella user or a newcomer looking for better immunoassay results, the 48-Dig cartridge is here to accommodate a wide range of target analytes. In this technical note, we'll show you how to get started with developing your own cytokine detection assay using the 48-Dig cartridge.

How the Simple Plex 48-Dig Cartridge Works

Glass nanoreactors (GNRs) within the 48-Dig cartridge are functionalized with an anti-digoxigenin antibody coating. This allows the GNRs to bind capture reagents that have been labeled with a digoxigenin tag prior to sample incubation. The resulting tag-antibody interaction is of strong affinity, forming a stable and consistent target capture—essentially, the foundation for the assay. Once the sample is introduced to the cartridge, the sandwich assay is completed with the addition of a biotinylated detection antibody. Signal recognition occurs by way of a streptavidin-conjugated fluorophore, the standard detection reagent for Simple Plex cartridge-based immunoassays (**Figure 1**).

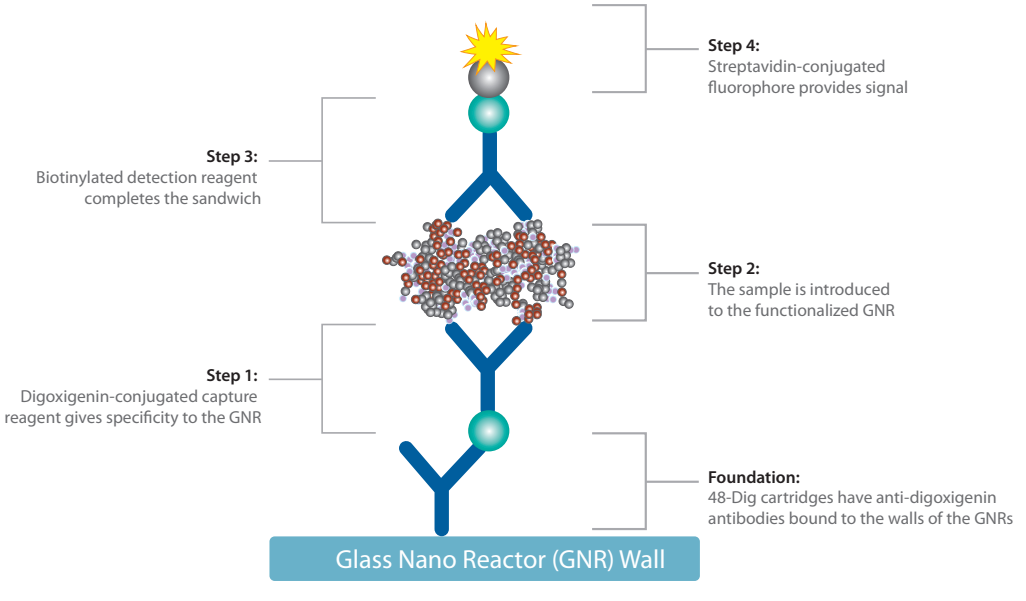


FIGURE 1. Ella performs immunoassays in four steps using the 48-Dig microfluidic Simple Plex cartridge.

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What Comes with the Simple Plex 48-Dig Cartridge Kit?

The 48-Dig Cartridge Kit (PN 952927) contains several reagents that support the operation of 48 immunoassays. The first reagent is the Wash Buffer (PN 896055), a standard requirement for all Simple Plex cartridge formats. The Wash Buffer is supplied as a working concentration and is added directly to the wash buffer troughs on the cartridge. The other buffer included is the Reagent Diluent (PN 895182), recommended for introducing the capture and detection reagents to the cartridge at their working concentrations. It can also be used as a sample diluent (SD), however, ProteinSimple recommends screening several SDs when working with complex biological matrices.

Other Supplies Required for Assay Development

CONJUGATION MATERIALS

We've qualified all the materials you need to conjugate your capture and detection reagents. For a full list, please refer to the 48-Digoxigenin Method Development Guide. The materials used in this technical note are listed in **Table 1**.

REAGENT	VENDOR	PRODUCT NUMBER
Digoxigenin-NHS ester	Sigma-Aldrich	55865
Biotin-XX, SE	Sigma-Aldrich	B3295
N,N-Dimethylformamide (DMF)	Sigma-Aldrich	270547
Dimethyl Sulfoxide (DMSO), ≥ 99% Pure	Sigma-Aldrich	D2650
Sodium Bicarbonate	Sigma-Aldrich	S8875
ELISA Plate-Coating Buffer – PBS	R&D Systems	DY006
Hinge-Cap Polypropylene Vials (1.7 mL)	United Laboratory Plastics	UP 2061
Disposable Glass Tubes with Plain end (12x75 mm)	Fisher Scientific	14-961-26
UV-Star® Transparent Microplates (96-well, flat-bottom)	Greiner Bio-One	655801
Zeba™ Spin Desalting Columns (40K MWCO, 0.5 mL)	Thermo Fisher	87766

TABLE 1. Reagents used in this technical note.

ANCILLARY DILUENTS

For developing cytokine and other biomarker assays using the 48-Dig Simple Plex Cartridge, ProteinSimple offers a selection of SDs recommended for use with a variety of biological sample types (**Table 2**).

SAMPLE DILUENT	VENDOR	PART NUMBER
SD06	ProteinSimple	896096
SD10	ProteinSimple	896097
SD13	ProteinSimple	896098
Ancillary Diluent Pack #1 (includes all of the above)	ProteinSimple	992522

TABLE 2. Commercially available SDs compatible with a variety of sample types. Abbreviation: SD, sample diluent.

48-Dig Assay Development

ANTIBODY PAIR EVALUATION

To increase the likelihood of a successful assay, as many antibody pairs as possible should be taken through the following screening procedure. When evaluating an antibody pair against a chosen recombinant antigen control at or near the total antigen binding capacity of the cartridge, you should, in general, look for an emission signal greater than 300 relative fluorescence units (RFU). In **Figure 2**, we've screened three antibodies for all nine possible sandwiches, which were prepared using the Reagent Diluent included with the 48-Dig Cartridge Kit. This diluent is suitable for preparing the recombinant antigen standard.

To evaluate the general antigen sensitivity of the antibody pairs chosen, you should perform a titration experiment. For example, in **Figure 3**, we've serially diluted the recombinant form of our analyte ranging from 100,000 pg/mL down to 6.4 pg/mL using Simple Plex Sample Diluent SD13 (PN 896098). This allows for the full characterization of the assay range. From our titration data, the upper and lower LOQ values of the assay could be derived. The acceptance criteria used were a recovery of 80% to 120% based on backfit with calibrator replicate intra-assay CVs <20%.

Selecting the antibody pairs appropriate for further testing will depend on the nature of the analyte(s) being investigated. For low abundance analytes with

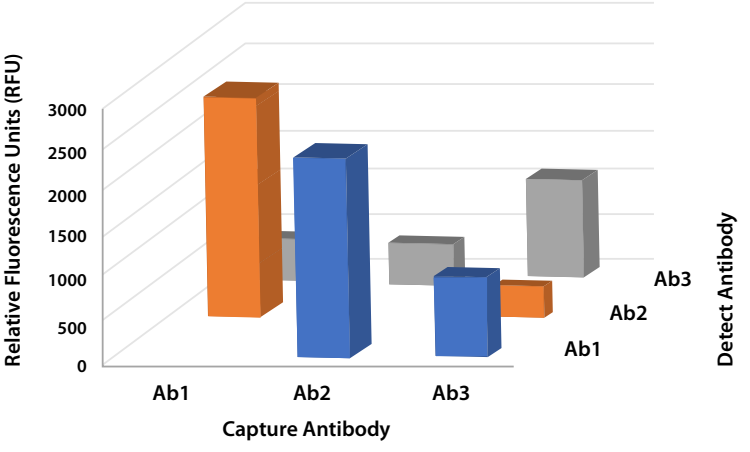
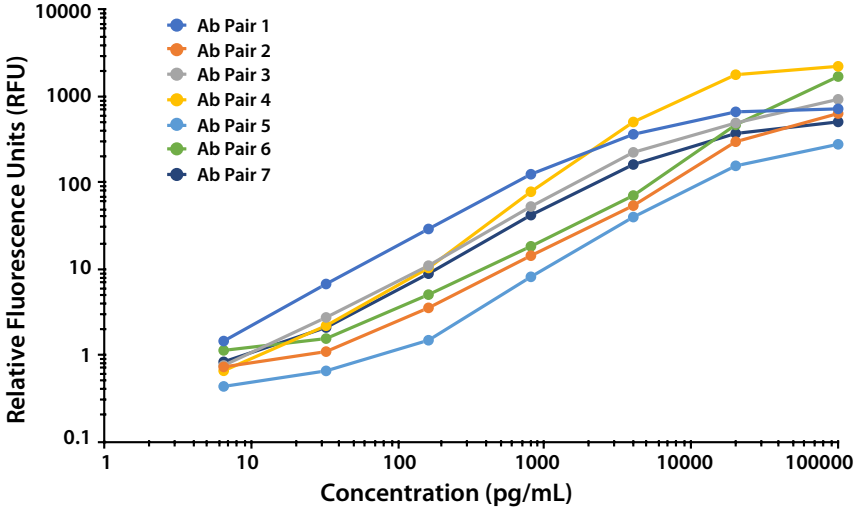


FIGURE 2. Capture and detection antibody pair screening against a recombinant standard, as a measure of RFU (y-axis). Abbreviation: Ab, antibody.



Ab PAIR	CAPTURE	DETECT
1	Ab 1	Ab 2
2	Ab 1	Ab 3
3	Ab 2	Ab 1
4	Ab 2	Ab 3
5	Ab 3	Ab 1
6	Ab 3	Ab 2
7	Ab 3	Ab 3

FIGURE 3. Capture and detection antibody pair screening against a recombinant standard, as a measure of RFU (y-axis). Abbreviation: Ab, antibody.

challenging endogenous detection, the lower limit of quantitation (LLOQ) should be a major consideration. For higher abundance analytes or analytes with highly variable expression levels, a broad assay range may take precedence over low-end sensitivity. In our screen, antibody pairs 1 and 2 were chosen based on observed LLOQ values (**Figure 3**). Once pairs have been selected, it can be useful to screen those pairs in multiple candidate diluents to confirm their performance, as the sample diluent can impact the range and sensitivity of the antibody pair.

STANDARD CURVE GENERATION

Once the antibody pairs have been successfully narrowed based on the obtained antibody pair RFU values and analyte titration data, the detection range of the assay for that particular recombinant protein should be fully characterized. Simple Plex immunoassays routinely achieve sensitivity ranging from 3 to 4 logs. Therefore, ProteinSimple recommends that an extensive dilution series of the analyte be tested for standard curve generation. The goal is to make sure the upper and lower asymptotes of the standard curve are fully defined. The upper and lower LOQs are defined by the highest and lowest curve points to achieve backfit recovery between 80% to 120% and intracurve CVs less than 20%.

In **Figure 4**, we illustrate broadly generated standard curves for both antibody pairs, beginning with an analyte concentration of 50,000 pg/mL and further diluted five-fold to generate an eight-point curve of recombinant protein calibration concentrations. Antibody Pair 1 and Antibody Pair 2 were both evaluated with the same set of calibrators.

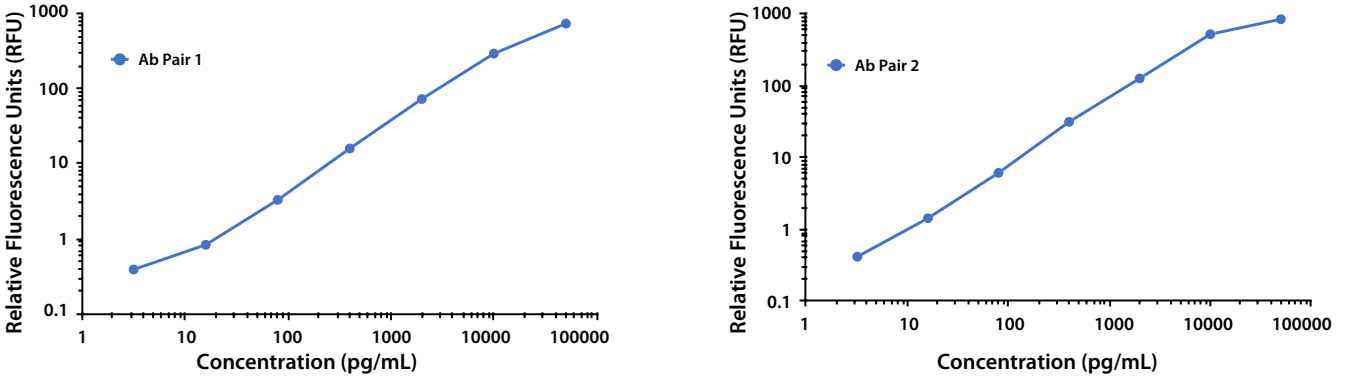


FIGURE 4. Standard curve for Antibody Pair 1 (left) and Antibody Pair 2 (right). Abbreviation: Ab, antibody.

SAMPLE PERFORMANCE OPTIMIZATION

Finally, once the standard curves are developed and the quantitative range of the assay is reasonably well defined, sample performance optimization can be achieved by evaluating SDs in different matrices according to two criteria: 1) spike recovery and 2) dilutional linearity. This is an important step in Simple Plex assay development, as selecting the correct diluent is critical for optimal sample performance, and there are several ancillary diluents that can be used to develop assays in conjunction with the 48-Dig cartridge. In this section, we outline one of several such screening approaches for choosing the proper diluent before proceeding with your assay.

When detecting targets in serum or plasma samples, ProteinSimple recommends using the Ancillary Diluent Pack #1. This pack contains diluents SD06, SD10 and SD13 which are often appropriate for developing assays using serum, plasma and cell culture supernatant samples. Also note that when working with any matrix on the 48-Dig cartridge, the minimum required dilution is 1:2 and further dilution may be required to minimize matrix effects. To select an appropriate SD, in **Figure 5** we demonstrate a spike recovery experiment using the recombinant protein analyte and several human blood derivative sample types as a means for selecting the optimally performing SD. The chosen spike concentrations will vary depending on the range of your standard curves, but you should aim to cover a broad range while also avoiding the curve's limits. Based on our results in **Figure 5**, Sample Diluent SD10 was chosen because the spike recovery could be maintained between 80% to 120% across low, medium and high

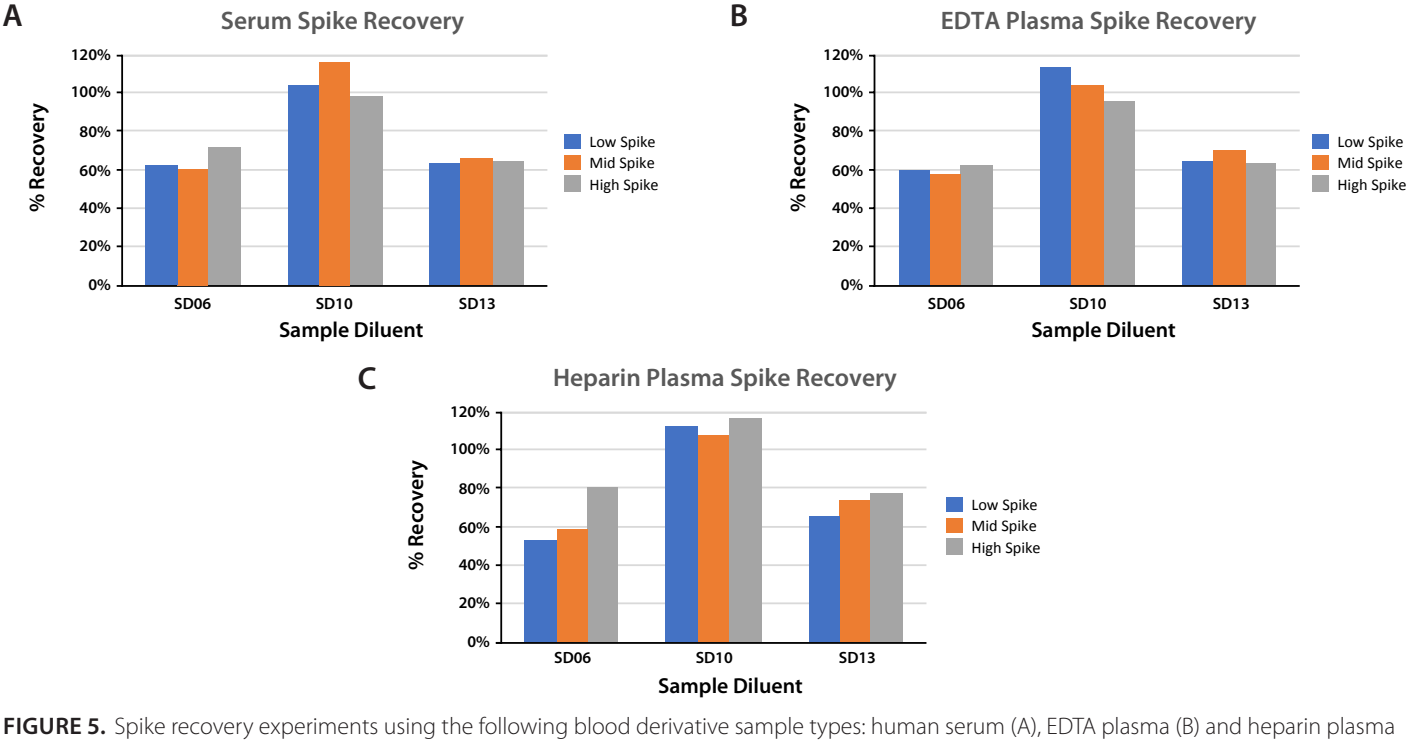


FIGURE 5. Spike recovery experiments using the following blood derivative sample types: human serum (A), EDTA plasma (B) and heparin plasma (C) sample types.

concentrations of spiked material for all human blood derivative samples analyzed.

Next, you'll need to test the performance of your antibody pairs in your sample matrix. The goal is to validate their ability to detect both the endogenous and recombinant forms of analyte in matrix. You should also note whether the analytes display parallelism.

In **Figure 6**, samples containing endogenous levels of the target analyte and samples spiked with the recombinant form were tested for parallel linear dilution. All samples were diluted two-fold in Sample Diluent SD10, after which a further two-fold dilution series was generated for a total of five dilution points. Each data point was back calculated by the dilution factor and compared with the first dilution point in the series. The linearity and recovery experiments performed led us to conclude that Antibody Pair 1 provided superior performance with both the endogenous and recombinant form of the analyte. When evaluating Antibody Pair 2, the natural analyte displayed a linear dilution whereas the recombinant spiked protein did not dilute linearly. This implies that for Antibody Pair 2,

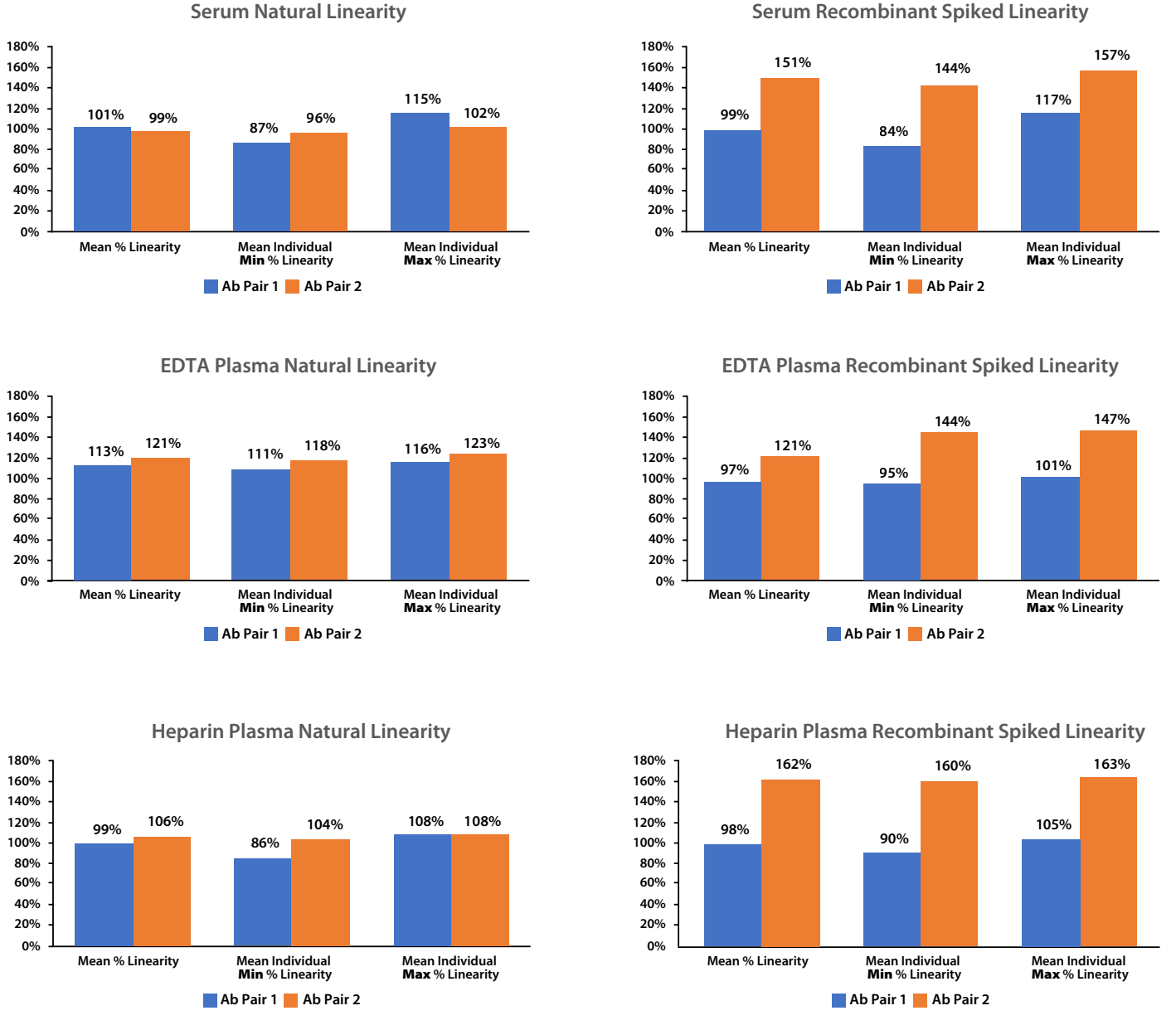


FIGURE 6. Spiked linearity (right column graphs) and natural linearity (left column graphs) experiments assessing the performance of the chosen antibody pairs in matrix using SD10. Abbreviation: Ab, antibody.

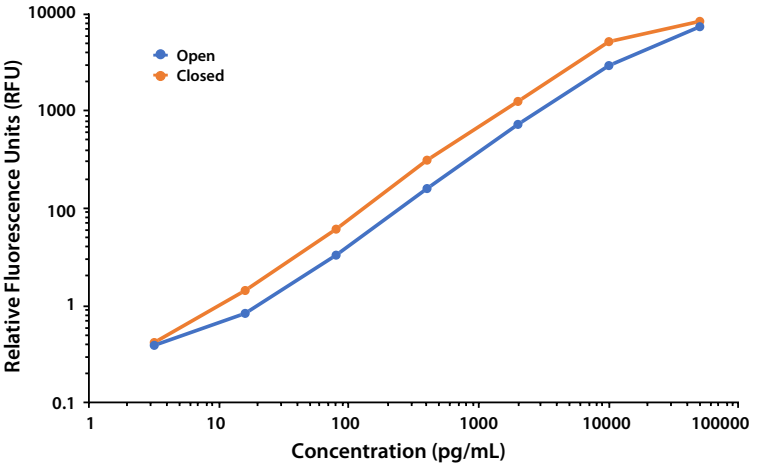


FIGURE 7. 48-Dig cartridge (Open, blue line) versus the closed format cartridge (Closed, orange line) standard curve comparison.

the recombinant protein could not be used for calibration against the natural molecule because they dilute differently in matrix. Antibody Pair 1 showed linear dilution for both the natural analyte and the recombinant spikes.

Comparison to the Closed Format

To validate our assay, using the same analyte, we compared the standard curve we generated with the 48-Dig cartridge to that of a factory calibrated one, provided with Simple Plex preset assay types, available in 72x1, 16x4 and 32x4 cartridge formats. Our results indicate a comparable performance with less than a two-and-a-half-fold difference in empirical LLOQ values between cartridge formats (**Figure 7**). For the same analyte, the closed format produced an LLOQ of 1.85 pg/mL, whereas it was 4.62 pg/mL for the open format.

Conclusion

The 48-Dig Simple Plex cartridge lets you take Ella's performance and workflow to a new level. With open access to targets that best suit your research, the possibilities are endless! Of course, as with any immunoassay, antibody pair screening is an essential preliminary step for success. Once you've identified the optimal pair for your assay, be sure to also screen for the best performing SD, as results may vary depending on your sample matrix. However, in the end, you'll enjoy results comparable to our preset closed cartridges, all with increased flexibility to accommodate your research needs. In this technical note, we've provided you with some guiding steps and example data to help you get started.

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