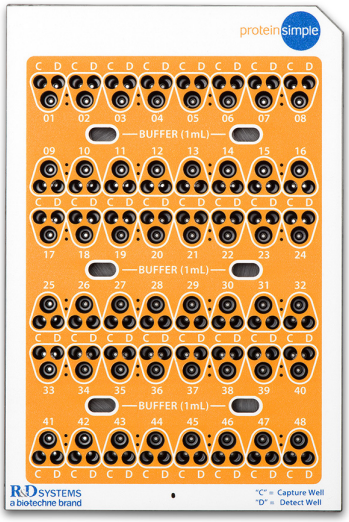
Open Access to Ella: Developing a Pharmacokinetic Assay Using the 48-Digoxigenin Cartridge



Introduction

A drug's pharmacokinetic (PK) profile provides researchers with a better understanding of how it may act in the body as a measure of the drug's absorption, distribution, metabolism and excretion. As a collection of measurements from various fluids such as serum, plasma, urine and/or saliva, PK studies are an indispensable part of the drug development process; they provide rationale and guidance for the advancement of a selected dosage through both preclinical and clinical evaluations. Today's drug development efforts are dominated by biotherapeutics. Monoclonal antibodies, biosimilars, antibody-drug conjugates and recombinant proteins, among others, are increasingly favored for their high target specificity and applicability to a number of therapeutic areas. But because their structure and manufacturing are remarkably complex and the product itself is human or humanized, safety and efficacy need to be thoroughly and continually assessed using the appropriate bioanalytical assays.

Highly sensitive and quantitative drug monitoring PK immunoassays are necessary for accurate modeling and analysis, and now you can develop them on Ella. Through a series of straightforward protein-labeling steps, Simple Plex immunoassays using the 48-Digoxigenin (48-Dig) cartridge can be tailored to fit your custom PK assay needs. The approach is not only specific and reliable but robust and efficient as well. In this technical note, we'll walk you through the simple proof-of-principle experiments that break down to just four steps required to get started.

How the Simple Plex 48-Dig Cartridge Works

Glass nano reactors (GNRs) within the 48-Dig cartridge are functionalized with an anti-digoxigenin antibody coating. This allows the GNRs to bind capture reagents labeled with a digoxigenin tag. The resulting tag–antibody interaction is of strong affinity, forming a stable and consistent target capture, which is, essentially, the foundation for the assay. Once the sample is introduced into 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, which is built into every cartridge and is the standard detection reagent for Simple Plex immunoassays (**Figure 1**).

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 up to 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 to the cartridge's wash buffer troughs. 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. However, ProteinSimple recommends screening a number of sample diluents when working with complex biological matrices.

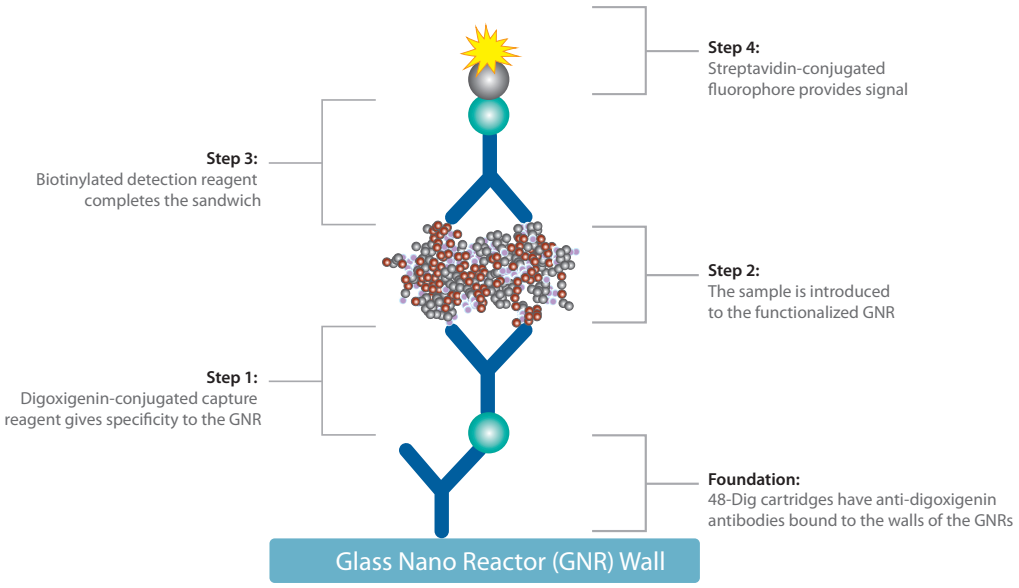


FIGURE 1. Ella performs immunoassays in four steps using the 48-Dig microfluidic Simple Plex cartridge.

Other Supplies Required for Assay Development

CONJUGATION MATERIALS

We've qualified all the materials you need to conjugate your capture and detect reagents. For a full list of materials and conjugation procedures, please refer to the 48-Digoxigenin Quick Start 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 MATERIALS

For developing PK assays using the 48-Dig Simple Plex Cartridge, ProteinSimple offers a selection of sample diluents recommended for matrix optimization (**Table 2**).

SAMPLE DILUENT	PART NUMBER
SD06	896096
SD10	896097
SD13	896098
SD19	896103
SD20	896104
SD42	896352
Ancillary Diluent Pack #1 (includes SD06, SD10, SD13)	992522
Ancillary Diluent Pack #2 (includes SD19, SD20, SD42)	992523
48-Digoxigenin Training Kit	992514

TABLE 2. Sample diluents compatible with various biological fluid sample types and a training kit are available from ProteinSimple. When considering a diluent for use in a PK assay, ProteinSimple recommends screening as broadly as possible. SD19, SD20 and SD42 have been successfully used in PK assays. However, SD06, SD10 and SD13 are also viable, and in the case of our experimentation here, SD06 performed the best. The Reagent Diluent included with the cartridge kits can also be used for some model systems but may not be optimal for eliminating matrix effects.

Materials & Methods Used in this Technical Note

Adalimumab is a TNF-blocking monoclonal antibody FDA-approved for the treatment of arthritis[1]. In this application note, we describe PK assay development for in-serum adalimumab using an anti-idiotype capture antibody and an anti-human detection antibody.

During sample and antibody preparation, all stocks were kept on ice, and working stocks were kept at room temperature.

CAPTURE ANTIBODIES

Recombinant monoclonal rabbit anti-adalimumab antibody (anti-idiotype; R&D Systems PN MAB9616) is referred to as Capture Antibody 1 herein. Mouse monoclonal anti-adalimumab antibody (anti-idiotype; R&D Systems, PN MAB9546) is referred to as Capture Antibody 2 herein. Both antibodies were labeled with Digoxigenin according to the protocol in the Quick Start Guide, and diluted in 1X Reagent Diluent, where 1X Reagent Diluent alone was used as a no-capture antibody control and dispensed as 25 µL per well. Optimal capture antibody concentrations were determined in a series of titration experiments using the innovator product, adalimumab, as the antigen, prepared in 100% matrix (or in 1X Reagent Diluent for no-matrix control) and dispensed as 50 µL per well. These experimental methods are described in STEP 1.

DETECTION ANTIBODY

Goat polyclonal IgG anti-human IgG (Fc)-biotin conjugate (ImmunoReagents, Inc., PN GtxHu-004-F2BIO) was diluted in 1X Reagent Diluent. Samples were added at 25 µL per well in a series of titration experiments to determine optimal concentration. These experimental methods are described in STEP 3.

MONOCLONAL ANTIBODIES

The innovator product, adalimumab, was provided by a pharmaceutical collaborator.

Anti-human IL-5 is provided as a part of the 48-Dig cartridge training kit listed in **Table 2**. This antibody is recommended for use as a negative control during PK assay development.

48-Dig PK Assay Development: Proof-of-Principle Experiments to Get Started

STEP 1. IDENTIFY THE OPTIMAL CAPTURE ANTIBODY AND CONCENTRATION

i. Start by diluting your antigen in 100% matrix (in this case human serum) at a concentration 20X more than the final assay concentration. Dilute your antigen/matrix sample 1:20 using the 1X Reagent Diluent provided to achieve 5% matrix, corresponding to final concentrations of 10 ng/mL, 1 ng/mL and 0 ng/mL of antigen (**Table 3**). Because the matrix may inhibit binding activity, create the same antigen dilution series using only the Reagent Diluent, leaving out the matrix. Add 50 µL of the final dilutions to the appropriate cartridge inlet.

ANTIGEN CONCENTRATION IN 100% MATRIX OR REAGENT DILUENT	FINAL ANTIGEN CONCENTRATION IN 5% MATRIX OR REAGENT DILUENT
200 ng/mL	10 ng/mL
20 ng/mL	1 ng/mL
0 ng/mL	0 ng/mL

TABLE 3. Dilution table listing starting and final concentrations of antigen for addition to the 48-Dig cartridge.

ii. Next, create a three-fold dilution series of each capture antibody to be tested using the 1X Reagent Diluent. Produce final concentrations of 10 µg/mL, 3.33 µg/mL, 1.11 µg/mL and 0 µg/mL. Add 25 µL to 50 µL of capture antibody per well.

iii. Dilute the detection antibody to 3.33 µg/mL in 1X Reagent Diluent. Add 25 µL to 50 µL of detection antibody per well.

iv. Finally, spin the 48-Dig cartridge via centrifuge for 15 seconds at 50 x g at room temperature. Add 1 mL of Wash Buffer per trough, insert the cartridge into the instrument and follow the setup instructions in the Runner software.

To demonstrate this set of optimization steps, **Figure 2A** presents an example cartridge setup in table format for capture antibody optimization. Two capture antibodies can be tested at four concentrations against three concentrations of antigen and in two matrix conditions, all while keeping the detection antibody concentration constant: 2 x 4 x 3 x 2 = 48 inlets total. **Figure 2B** shows an example dataset from capture antibody titration experiments as outlined above. In this example, our two capture antibodies tested—rabbit and mouse anti-adalimumab antibodies (anti-idiotype)—are diluted in 1X Reagent Diluent to final concentrations of 3 µg/mL, 1 µg/mL and 0.33 µg/mL against 1ng/mL of the innovator product—adalimumab—as antigen, also diluted in 1X Reagent Diluent (**Figure 2B**). Our results indicate that 3 µg/mL of Capture Antibody 2 is optimal for our assay, as it produced a slightly higher signal-to-NSB ratio than Capture Antibody 1 at the same concentration (**Figure 2B**). For ease of assay preparation, a 1:200 dilution of Capture Antibody 2 (~3.5 µg/mL) was used moving forward.

A

		CAPTURE ANTIBODY 1				CAPTURE ANTIBODY 2			
	ANTIGEN	10 µg/mL	3.33 µg/mL	1.11 µg/mL	0 µg/mL	10 µg/mL	3.33 µg/mL	1.11 µg/mL	0 µg/mL
Matrix 1	10 ng/mL	01	02	03	04	05	06	07	08
	1 ng/mL	09	10	11	12	13	14	15	16
	0 ng/mL	17	18	19	20	21	22	23	24
Matrix 2	10 ng/mL	25	26	27	28	29	30	31	32
	1 ng/mL	33	34	35	36	37	38	39	40
	0 ng/mL	41	42	43	44	45	46	47	48

Note: Numbers in the table refer to inlet numbers on the cartridge.

B

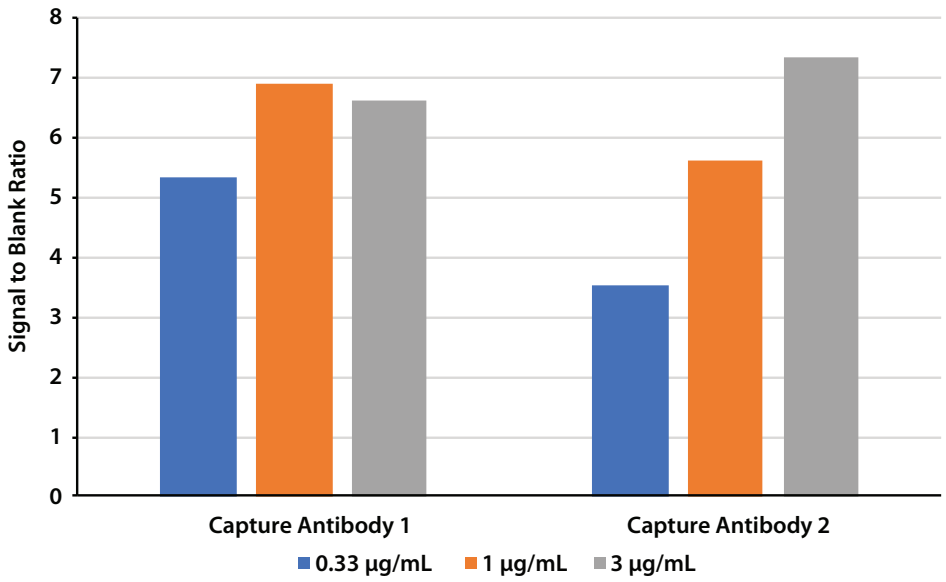


FIGURE 2. Capture antibody identification and concentration optimization. In this setup, four concentrations of two capture antibodies can be tested against three concentrations of any antigen in two different matrices (A). Titration experiment using three concentrations of anti-adalimumab Capture Antibody 1 or Capture Antibody 2 (anti-idiotype) with 1 ng/mL adalimumab and 3.33 µg/mL detection antibody diluted in 1X Reagent Diluent (B).

STEP 2. IDENTIFY THE TWO BEST-PERFORMING SAMPLE DILUENTS

i. Start by diluting your antigen in 100% matrix at a concentration 20X more than the final assay concentration. This time, dilute your antigen/matrix sample 1:20 using each Sample Diluent (SD) from the Ancillary Diluent Packs to achieve 5% matrix, corresponding to concentrations of 10 ng/mL, 1 ng/mL, 0.1 ng/mL and 0 ng/mL of antigen. Refer back to the methodology outlined in STEP 1 and **Table 3** for more detail. Add 50 µL of the final dilutions to the appropriate sample inlet.

ii. Based on your results from STEP 1, dilute the selected capture antibody in 1X Reagent Diluent to the determined optimal concentration. Add 25 µL to 50 µL per well.

iii. Next, dilute the detection antibody in 1X Reagent Diluent at 0.1 µg/mL and 10 µg/mL. Add 25 µL to 50 µL of detection antibody per well.

iv. Spin the 48-Dig cartridge via centrifuge for 15 seconds at 50 x g at room temperature. Add 1 mL of Wash Buffer per trough, insert the cartridge into the instrument and follow the setup instructions in the Runner software.

As an example, **Figure 3A** shows a cartridge setup in table format for the assessment of SD performance. Top performing SDs can be identified by testing six diluents against four concentrations of antigen and two concentrations of detection antibody while keeping the amount of capture antibody constant at its previously optimized concentration: 6 x 4 x 2 = 48 inlets total. **Figure 3B** demonstrates the results of this setup using a spiked sample of adalimumab (1 ng/mL or 10 ng/mL) into 5% human serum, diluted using the various SDs. We tested samples diluted in Reagent Diluent, SD06, SD10, SD13, SD20 and SD42 with two concentrations of detection antibody (0.1 µg/mL and 10 µg/mL) to measure the signal-to-non-specific binding (NSB) ratio (y-axis). The optimal concentration of capture antibody, determined in the previous step, was fixed at 3.5 µg/mL. Based on this evaluation, SD06 and SD20 were chosen as they exhibited the greatest sensitivity under these conditions.

STEP 3. IDENTIFY THE OPTIMAL CONCENTRATION OF DETECTION ANTIBODY AND GENERATE ANTIGEN-BASED STANDARD CURVES

i. Create a standard curve based on a five-fold dilution series of the antigen in 100% matrix. Refer to the methodology outlined in STEP 1 and **Table 3** for more detail. Using the two best-performing SDs identified in STEP 2, dilute toward a final matrix concentration of 5%, with final antigen concentrations corresponding to 100,000 pg/mL, 20,000 pg/mL, 4,000 pg/mL, 800 pg/mL, 160 pg/mL, 32 pg/mL, 6.4 pg/mL and 0 pg/mL. Add 50 µL per well.

ii. Dilute the capture antibody in 1X Reagent Diluent to the optimal concentration as identified in STEP 1. Add 25 µL to 50 µL per well.

iii. Create a five-fold dilution series of the detection antibody prepared using 1X Reagent Dilution to final concentrations of 0.5 µg/mL, 0.1 µg/mL and 0.02 µg/mL. (If STEP 2 results in higher signal-to-NSB at the 10 µg/mL detection antibody, then optimize at final concentrations of 10 µg/mL, 2 µg/mL and 0.4 µg/mL.) Add 25 µL to 50 µL of detection antibody per well.

iv. Spin the 48-Dig cartridge via centrifuge for 15 seconds at 50 x g at room temperature. Add 1 mL of Wash Buffer per trough, insert the cartridge in the instrument and follow the setup instructions in the Runner software.

In **Figure 4A**, we've demonstrated an example cartridge setup for detection antibody optimization using a table format. The optimal detection antibody concentration can be identified by testing three concentrations of detection antibody against eight concentrations of antigen in the two top-performing SDs selected and the optimal capture antibody concentration: 3 x 8 x 2 = 48 inlets total. The results from STEP 2 showed higher signal-to-NSB with the 0.1 µg/mL detection antibody than with the 10 µg/mL antibody. Based on these results, we chose to test three concentrations of anti-human IgG Fc biotin conjugate detection antibody: 0.02 µg/mL, 0.1 µg/mL and 0.5 µg/mL. For the standard curve, adalimumab was titrated in human serum and then diluted to a final concentration of 5% using SD06 (left) and SD20 (right) as shown in **Figure 4B**. The capture antibody was introduced at 3.5 µg/mL as determined in STEP 1. Looking at the standard curves generated in **Figure 4** and assessing the raw RFU values obtained, we identified 0.25 µg/mL as the optimal concentration of detection antibody for our assay. When analyzing the signal-to-NSB ratio (**Figure 5**), the three concentrations of detection antibody we tested performed similarly when comparing SD06 and SD20. Therefore, we chose the 0.25 µg/mL concentration, because it produced the highest signal with a relatively low NSB (<50 RFU) in both SD06 and SD20.

A

	DETECTION ANTIBODY (10 µg/mL)				DETECTION ANTIBODY (0.1 µg/mL)			
ANTIGEN	10 ng/mL	1 ng/mL	0.1 ng/mL	0 ng/mL	10 ng/mL	1 ng/mL	0.1 ng/mL	0 ng/mL
Reagent Diluent	01	02	03	04	05	06	07	08
SD06	09	10	11	12	13	14	15	16
SD10	17	18	19	20	21	22	23	24
SD13	25	26	27	28	29	30	31	32
SD20	33	34	35	36	37	38	39	40
SD42	41	42	43	44	45	46	47	48

Note: Numbers in the table refer to inlet numbers on the cartridge.

B

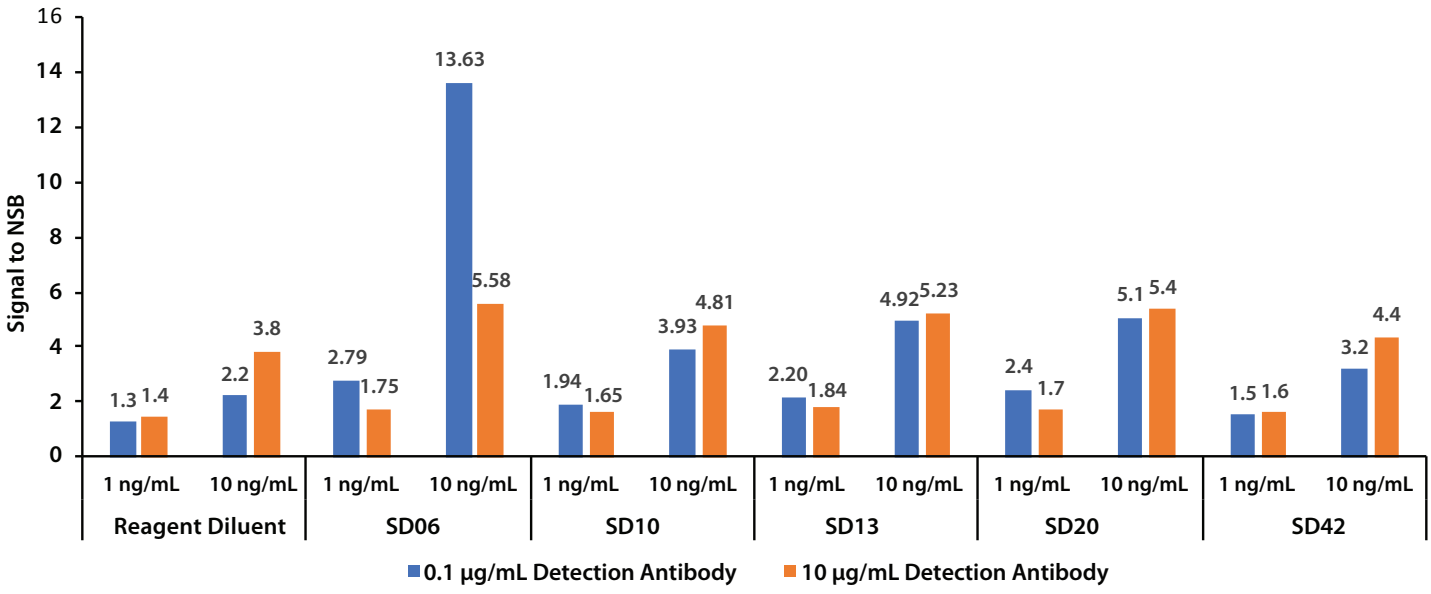


FIGURE 3. SD performance assessment. In this setup, six diluents can be tested against four concentrations of antigen and two concentrations of detection antibody (A). 1 ng/mL or 10 ng/mL of adalimumab antigen spiked in 5% human serum in combination with either 0.1 µg/mL or 10 µg/mL of detection antibody at a constant concentration of capture antibody was used to test the various SDs (B). Using SD06, the 0.1 µg/mL detection antibody had a lower background than the 10 µg/mL, which led to a better signal-to-NSB and the most favorable assay condition tested.

A

		ANTIGEN CONCENTRATION							
	DETECTION ANTIBODY	100,000 pg/mL	20,000 pg/mL	4,000 pg/mL	800 pg/mL	160 pg/mL	32 pg/mL	6.4 pg/mL	0 pg/mL
SD06	0.5 µg/mL	01	02	03	04	05	06	07	08
	0.1 µg/mL	09	10	11	12	13	14	15	16
	0.02 µg/mL	17	18	19	20	21	22	23	24
SD20	0.5 µg/mL	25	26	27	28	29	30	31	32
	0.1 µg/mL	33	34	35	36	37	38	39	40
	0.02 µg/mL	41	42	43	44	45	46	47	48

Note: Numbers in the table refer to inlet numbers on the cartridge.

B

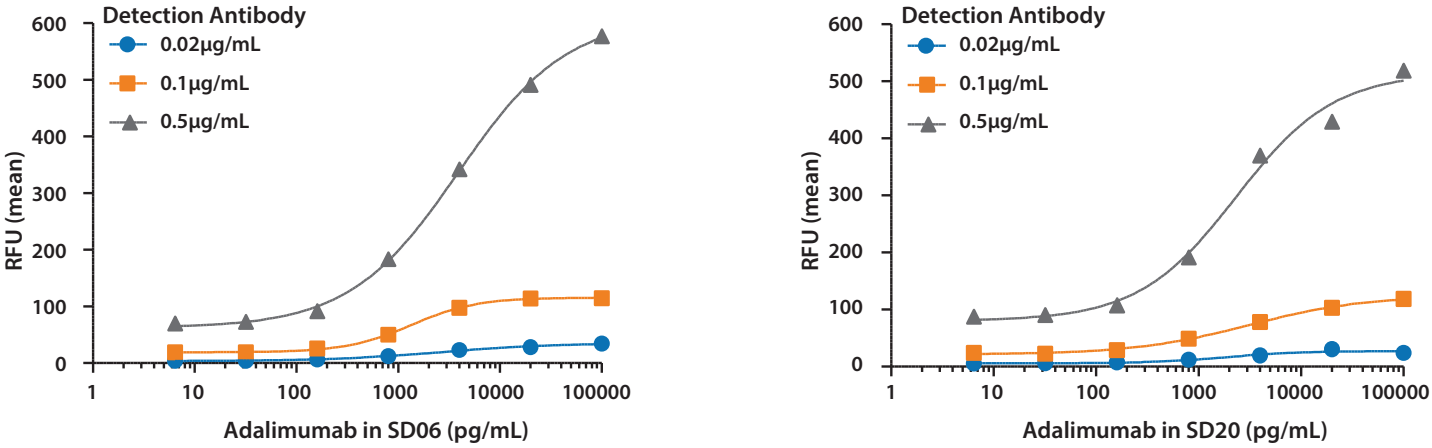


FIGURE 4. Detection antibody optimization. Three concentrations of detection antibody can be tested against eight concentrations of antigen in the two top-performing SDs (A). Titration of adalimumab (x-axis) with 5% human serum in either SD06 (left) or SD20 (right) as a measure of RFU (y-axis) (B). Three concentrations (0.02 µg/mL, 0.1 µg/mL and 0.5 µg/mL) of anti-human IgG Fc biotin conjugate detection antibody were tested and standard curves generated. Capture antibody concentration used was 3.5 µg/mL.

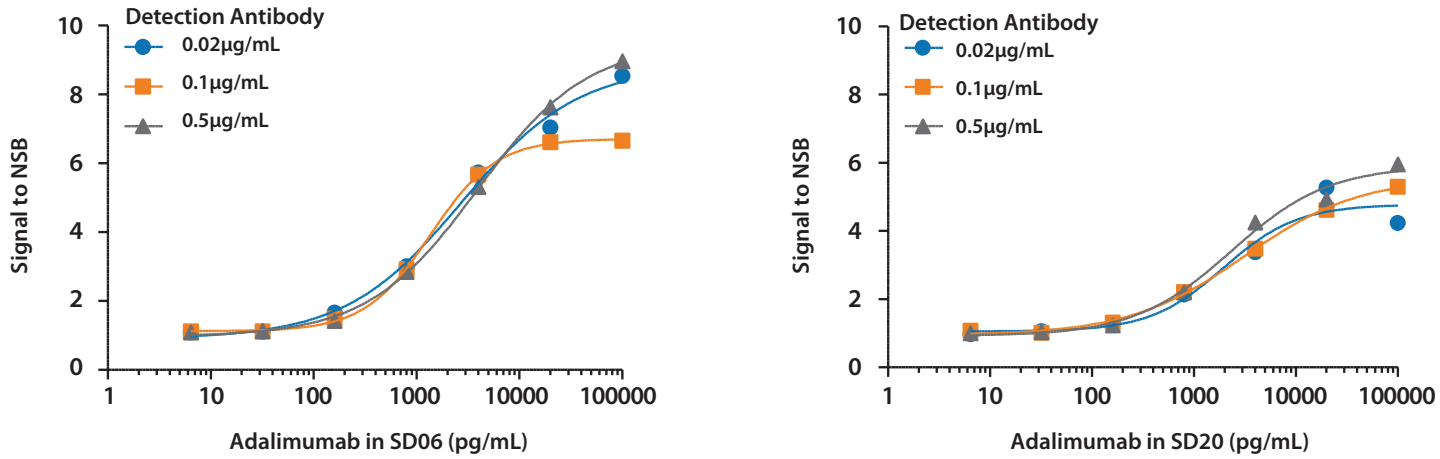


FIGURE 5. Detection antibody concentration optimization. Titration of adalimumab (x-axis) with 5% human serum in either SD06 (left) or SD20 (right) as a measure of signal-to-NSB (y-axis). Three concentrations (0.02 µg/mL, 0.1 µg/mL and 0.5 µg/mL) of anti-human IgG Fc biotin conjugate detection antibody were tested and standard curves generated. Capture antibody concentration used was 3.5 µg/mL (B).

STEP 4. IDENTIFY WHICH OF THE TWO SAMPLE DILUENTS PERFORMS BEST IN THE ASSAY BY SPIKE AND RECOVERY

i. Create a standard curve based on a five-fold dilution series of antigen in 100% matrix. Refer to the methodology outlined in STEP 1 and **Table 3** for more detail. Using the two previously identified best-performing SDs, dilute to final concentrations of 5% and 1% matrix, with final antigen concentrations corresponding to 100,000 pg/mL, 20,000 pg/mL, 4,000 pg/mL, 800 pg/mL, 160 pg/mL, 32 pg/mL, 6.4 pg/mL and 0 pg/mL. Add 50 µL per well.

ii. From the standard curves generated in STEP 3, choose three antigen concentrations that fall within the low, middle and high linear range of detection for use in a spike and recovery experiment. Again, create these samples by first diluting antigen in 100% matrix, and then diluting further using SD to final concentrations of 5% and 1% matrix. For the fourth spike sample control, pair the high-concentration antigen sample without a capture antibody or with an unrelated capture antibody. Add 50 µL per well.

iii. Dilute the capture and detection antibodies to their optimal concentrations as identified in STEP 1 and STEP 3, respectively, using 1X Reagent Diluent. Add 25 µL to 50 µL of capture and detection antibodies per well.

iv. Spin the 48-Dig cartridge via centrifuge for 15 seconds at 50 x g at room temperature. Add 1 mL of Wash Buffer per trough, insert the cartridge into the instrument and follow the setup instructions in the Runner software.

In **Figure 6A**, we've demonstrated an example cartridge setup in a table format for delineating which of the two SDs will perform better in your assay via a spike and recovery experiment. The setup can be evaluated by testing four spiked sample controls in two matrix conditions using the two SDs against eight concentrations of antigen in two matrix conditions using the two SDs: (4 x 2 x 2) + (8 x 2 x 2) = 48 inlets total. From the SD06 and SD20 standard curves in **Figure 6B** (left and right, respectively), we can conclude that SD06 (left) would perform better than SD20 in our assay based on smooth spike and recovery curves, and that sensitivity was similar in either matrix condition. However, the 5% serum saw a more truncated assay range with SD20.

Finally, both SD06 and SD20 were evaluated for their ability to properly recover a spike of adalimumab using 1% serum and the predetermined concentrations of capture and detection antibody. Low, medium and high concentrations of spiked antigen were generated as 1,000 pg/mL, 3,000 pg/mL and 9,000 pg/mL, respectively. In addition, a negative control was prepared using the anti-human IL-5 capture and detect antibodies available in the 48-Digoxigenin Training Kit. This unrelated antibody pair provides a negative control in the presence of a high concentration of adalimumab in the spike sample (**Figure 7**). These were then recovered against standard curves in the respective SD. All three sample controls in SD06 maintained a recovery between 80% to 120%, whereas the samples in SD20 were found to under- and over-recover.

A

		ANTIGEN CONCENTRATION							
		100,000 pg/mL	20,000 pg/mL	4,000 pg/mL	800 pg/mL	160 pg/mL	32 pg/mL	6.4 pg/mL	0 pg/mL
SD06	Matrix 1	01	02	03	04	05	06	07	08
	Matrix 2	09	10	11	12	13	14	15	16
	Spiked	17	18	19	20	21	22	23	24
SD20	Matrix 1	25	26	27	28	29	30	31	32
	Matrix 2	33	34	35	36	37	38	39	40
	Spiked	41	42	43	44	45	46	47	48
		SPIKE 1	SPIKE 2	SPIKE 3	SPIKE 4	SPIKE 1	SPIKE 2	SPIKE 3	SPIKE 4
		Matrix 1				Matrix 2			

Note: Numbers in the table refer to inlet numbers on the cartridge.

B

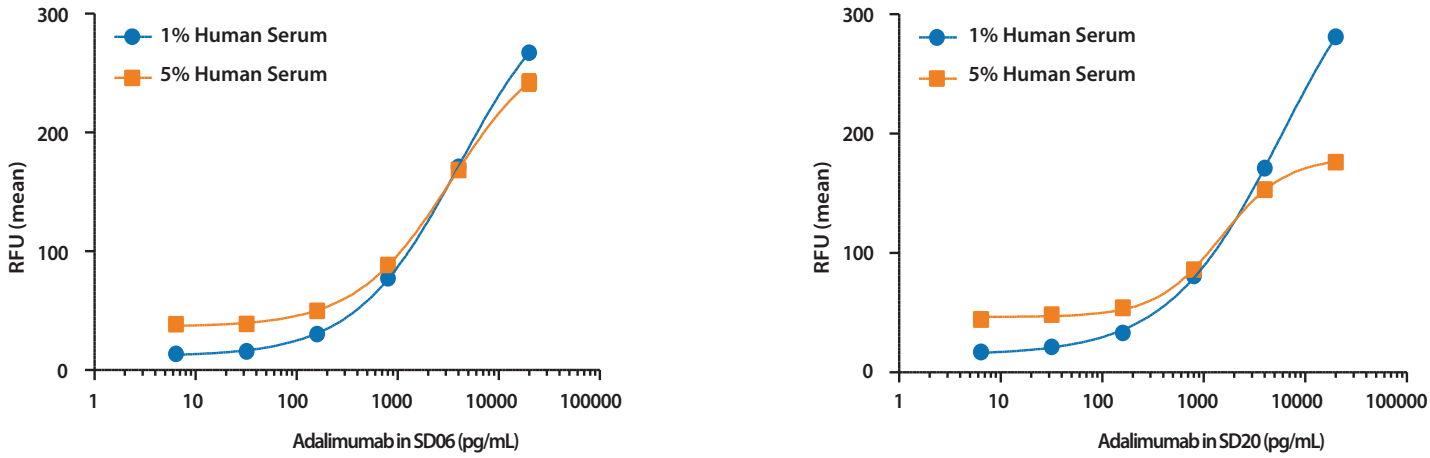


FIGURE 6. Choosing between the top two SDs. The top-performing SD can be identified by testing each with four spiked sample controls in two matrix conditions using eight concentrations of antigen (A). Standard curves using titrated adalimumab antigen and human serum diluted to 1% and 5% with either SD06 (left) or SD20 (right) (B). Standard curves in B were generated by using 3.5 µg/mL capture antibody and 0.25 µg/mL of detection antibody. Note that the RFU value obtained at 100,000 pg/mL was excluded from the graphs due to an observed Hook effect and interference with quantitative analysis.

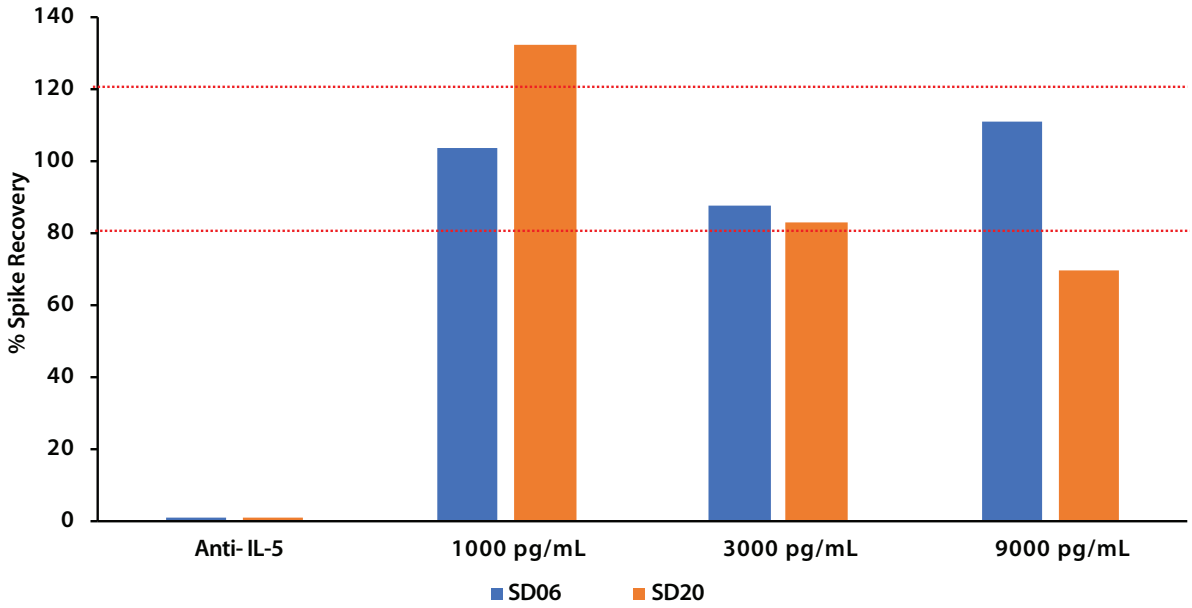


FIGURE 7. Adalimumab spike recovery using the top-two SDs. Spike and recovery experiments using titrated adalimumab antigen and human serum diluted to 1% with either SD06 (orange) or SD20 (blue). Capture antibody concentration used was 3.5 µg/mL, and the detection antibody was 0.25 µg/mL.

Conclusion

In this technical note, we've outlined how you can get started quickly with four preliminary assay optimization steps that will let you establish proof-of-principle for your PK assay including optimizing reagent concentrations, finding the best diluent and characterizing your assay's performance in a matrix. With an assay run time of just 80 minutes, Ella gets you the answers quick, so you can keep pushing your research forward. Get to a better PK assay faster with the 48-Digoxigenin cartridge on Ella.

References

1. Humira (adalimumab) label, Food and Drug Association, Available online at: https://www.accessdata.fda.gov/drugsatfda_docs/label/2011/125057s0276lbl.pdf. Accessed February 5, 2019. Updated March 2011.

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