Ella 48-Digoxigenin Cartridge Binding Assay Development

Introduction

Ella from ProteinSimple is a hands-free, low volume, microfluidic immunoassay platform. ProteinSimple now offers 48-Digoxigenin (48-Dig) cartridges that allow researchers to develop custom binding assays with their own reagents. With 48-Dig cartridges, up to 48 unique immunoassays can be run on each cartridge, with each reaction providing three technical replicates. Simple protein labeling reactions allow traditional plate-based assays to be quickly and easily transferred to the Simple Plex assay format. Simple Plex assays developed on Ella take advantage of an automated workflow, better data reproducibility, a 75-minute runtime, and use less sample and reagent than traditional plate-based assay formats.

Adalimumab is a biological drug used to treat rheumatoid arthritis, Crohn's disease and psoriasis. Adalimumab reduces inflammatory responses by inhibiting the binding of TNFα to its receptor[1]. It was the best-selling biopharmaceutical until its patent expired in 2016, reaching $16 billion in global sales annually. Several biosimilars are already on the market. Here, we develop an assay on the 48-Dig cartridge using adalimumab and its biosimilars (**Figure 1**). We show that the assay is fast and easy to develop, and the data generated agree with those that have been previously reported.

Materials

In addition to the Ella instrument, the materials used in this technical note are listed in **Table 1**.

REAGENT	VENDOR	PRODUCT NUMBER
48-Digoxigenin Cartridge (5-pack)	ProteinSimple	992-485
Ancillary Diluent Pack #1	ProteinSimple	992-522
Ancillary Diluent Pack #2	ProteinSimple	992-523
rhTNFα	R&D Systems	210-TA/CF
rhLIGHT/TNFSF14	R&D Systems	664-LI/CF
Lymphotoxin-α/TNFβ/TNFSF1	R&D Systems	211-TBB/CF
Fetal Bovine Serum - Premium	Atlanta Biologicals	S11195
Digoxigenin NHS-ester	Enzo Life Sciences	ENZ-45022
Goat anti-Human IgG Fc, Biotin Conjugate	ImmunoReagents	GtxHu-004-F2BIO
CHO-S media	External collaborator	N/A
Adalimumab (innovator)	External collaborator	N/A
Biosimilar 1	External collaborator	N/A
Biosimilar 2	External collaborator	N/A

TABLE 1. Reagents used in this technical note.

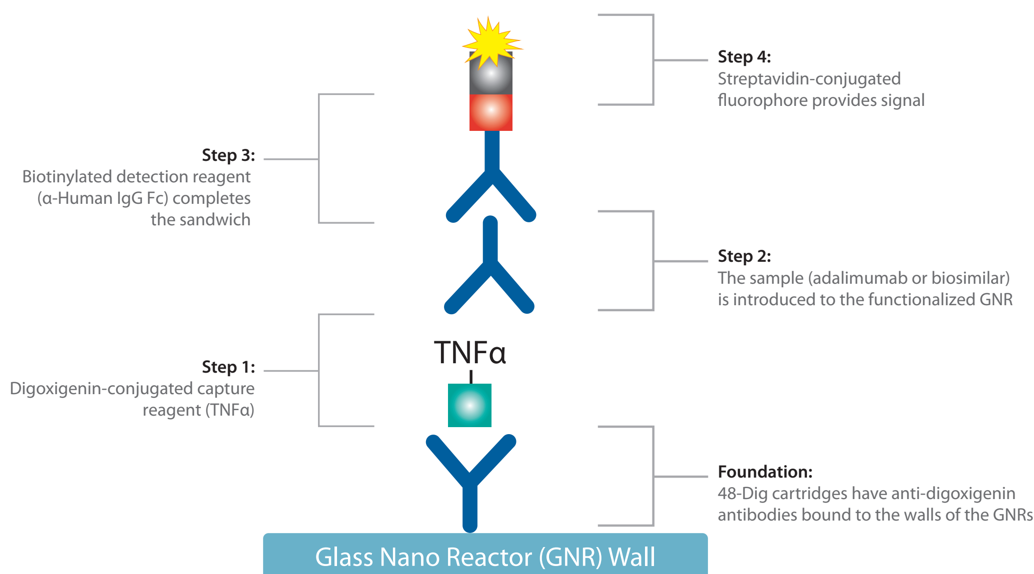


FIGURE 1. Ella performs immunoassays in four steps using the 48-Dig microfluidic Simple Plex cartridge.

Methods

STEP 1: CAPTURE PROTEIN OPTIMIZATION

The 48-Dig cartridge is functionalized with anti-digoxigenin antibodies that provide the foundation for building a custom binding assay. First, we identify the optimal concentration of the capture protein, a digoxigenin labeled TNFα molecule (Dig-TNFα) and the minimal required dilution (MRD) of media. The plate layout for this step is shown in **Figure 2**.

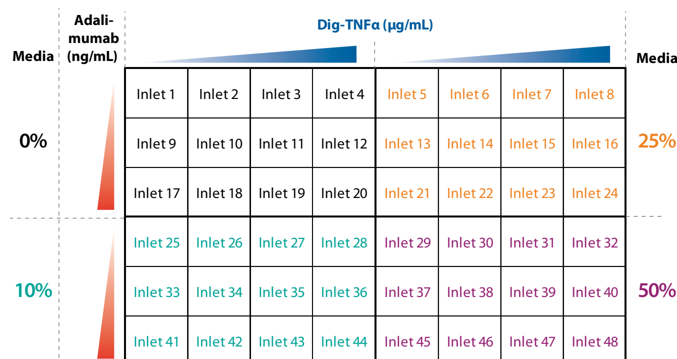


FIGURE 2. Plate layout for capture protein optimization. This plate was also loaded with 3.5 µg/mL biotinylated anti-human IgG Fc detection antibody. Two plates were assayed in parallel, one with serum-free media and the other supplemented with 10% FBS.

STEP 2: SAMPLE DILUENT EVALUATION

With the optimal capture protein concentration and MRD from Step 1, we next evaluate different sample diluents. The plate layout for this step is shown in **Figure 3**.

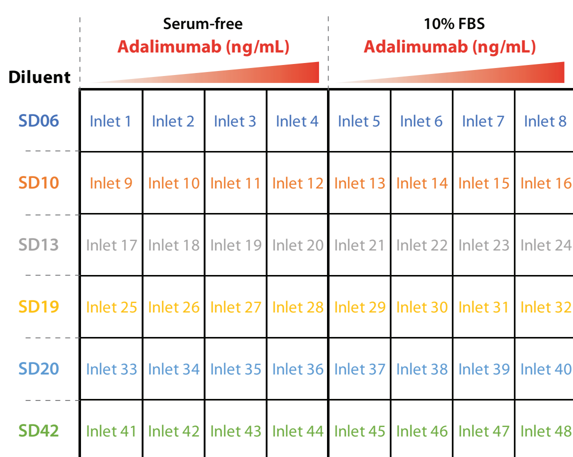


FIGURE 3. Plate layout for sample diluent evaluation. The sample was diluted in 50% CHO-S serum-free media (left) and with 10% FBS (right). This plate contained 3.5 µg/mL of biotinylated anti-human IgG Fc detection antibody.

STEP 3: DETECTION ANTIBODY OPTIMIZATION

Last, we identify the optimal concentration of detection antibody (biotinylated anti-human IgG Fc) with and without media. The plate layout for this step is shown in **Figure 4**.

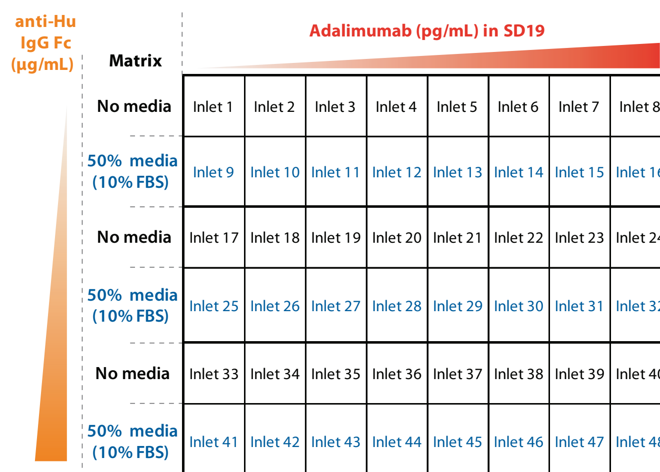


FIGURE 4. Plate layout to optimize detection antibody.

Results

Following the protocol described above, we first optimized the conditions for the capture protein. To do so, we tested binding of adalimumab in a 10X dilution series against a 3X dilution series of the capture protein. The signal to nonspecific background (NSB) was calculated from the no-adalimumab control at each capture protein concentration. Two plates were run in parallel containing serum-free media and media supplemented with 10% FBS. From this analysis, the capture protein performed best across all conditions when diluted to a concentration of 10 µg/mL (**Figure 5**). Also, the MRD was determined to be 50% media with or without FBS supplement (**Figure 5**).

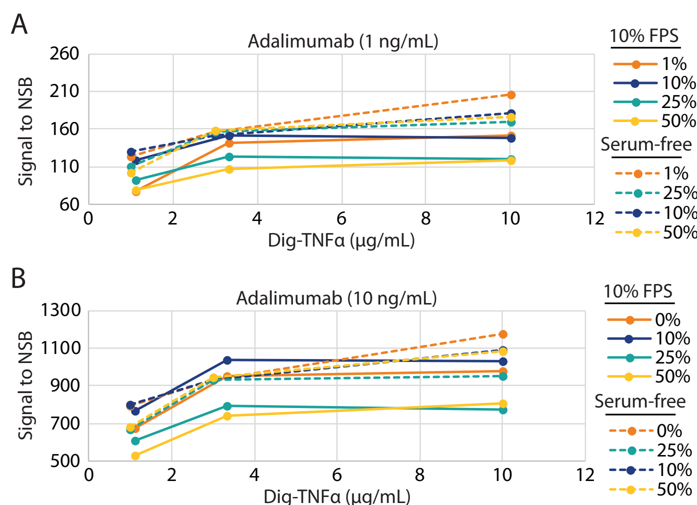


FIGURE 5. Identification of the optimal capture protein concentration and MRD at (**A**) 1 ng/mL adalimumab and (**B**) 10 ng/mL adalimumab.

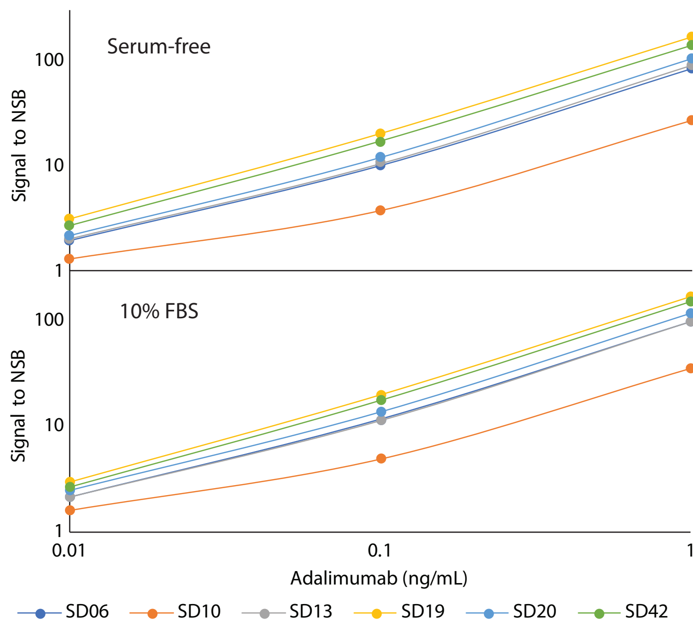


FIGURE 6. Binding activity of adalimumab diluted in different sample diluents available from ProteinSimple. Three dilutions, 0.01, 0.1 and 1 pg/mL of adalimumab were tested in each sample diluent.

Next, we evaluated which sample diluent performed best in our assay. The sample was tested in a 10X dilution series (1, 0.1, 0.01, and 0 ng/mL) of 50% CHO-S media with and without 10% FBS supplement. The NSB was calculated using 0 pg/mL of adalimumab as the background control. From this analysis, SD19 performed the best in both serum-free media and media supplemented with 10% FBS (**Figure 6**).

Finally, we optimized the detection antibody (biotinylated anti-human IgG Fc) concentration. Our findings show that the 1 µg/mL of detection antibody was optimal regardless of the presence or absence of 50% media (**Figure 7**). Concentrations greater than 1 µg/mL negatively impact the assay sensitivity.

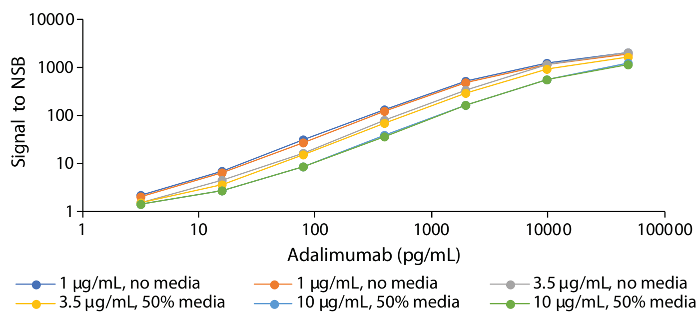
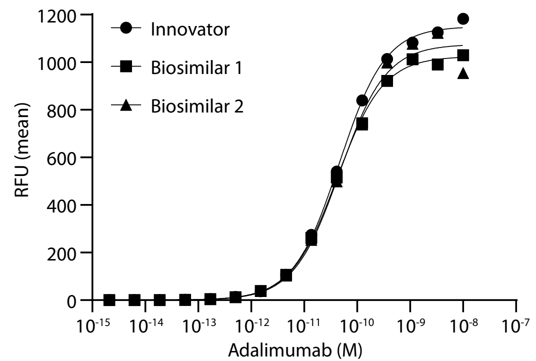


FIGURE 7. Binding activity of adalimumab with different detection antibody conditions. A titration of adalimumab was created, and each dilution series was tested with 1, 3.5, and 10 µg/mL detection antibody, with and without matrix.

Below is a summary of the optimized conditions for the TNFα binding assay:

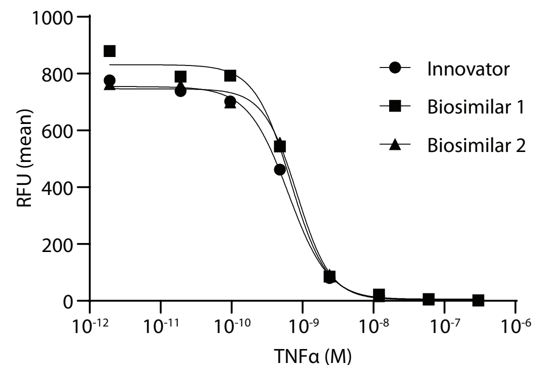
- Capture protein: 10 µg/mL Dig-TNFα
- MRD: 50% CHO-S media (+/- 10% FBS)
- Sample diluent: SD19 (ProteinSimple, PN 896-103)
- Detection antibody: 1 µg/mL biotinylated anti-human IgG Fc

With these conditions optimized, we performed saturation binding analysis (**Figure 8**) and competitive binding analysis (**Figure 9**) with adalimumab and biosimilars to TNFα. In addition, to demonstrate specificity to TNFα and not related proteins, we performed saturation binding (**Figure 10**) and competitive binding assays (**Figure 11**) with TNFα-related proteins, LIGHT/TNFSF14 and TNFβ.



	INNOVATOR	BIOSIMILAR 1	BIOSIMILAR 2
Kd (M)	4.615×10^{-11}	4.178×10^{-11}	4.698×10^{-11}

FIGURE 8. Saturation binding assay of adalimumab innovator and biosimilars to TNFα.



	INNOVATOR	BIOSIMILAR 1	BIOSIMILAR 2
IC50 (M)	6.308×10^{-10}	6.826×10^{-10}	8.410×10^{-10}

FIGURE 9. Competitive binding assay of adalimumab innovator and biosimilars to TNFα.

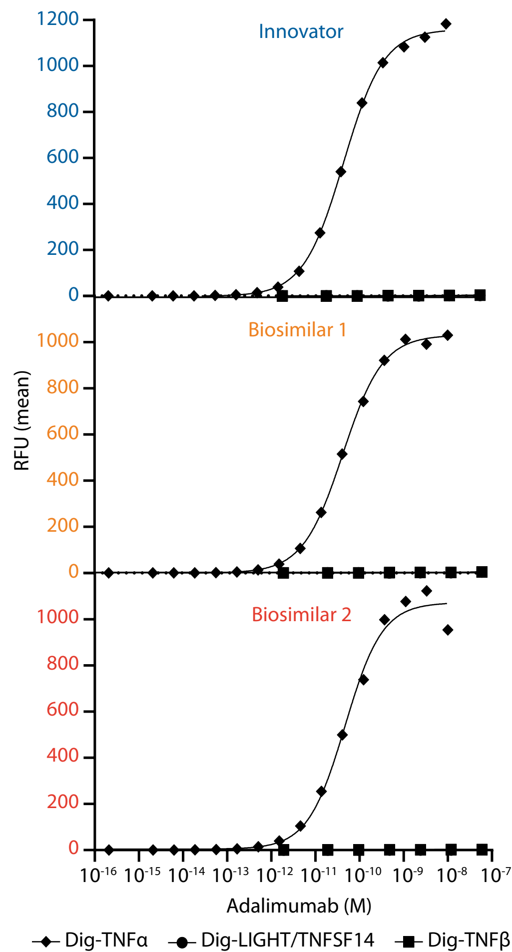


FIGURE 10. Saturation binding assay showing the specificity of adalimumab innovator and biosimilars to TNFα. Each plate contained 10 µg/mL capture protein and 1 µg/mL detection antibody.

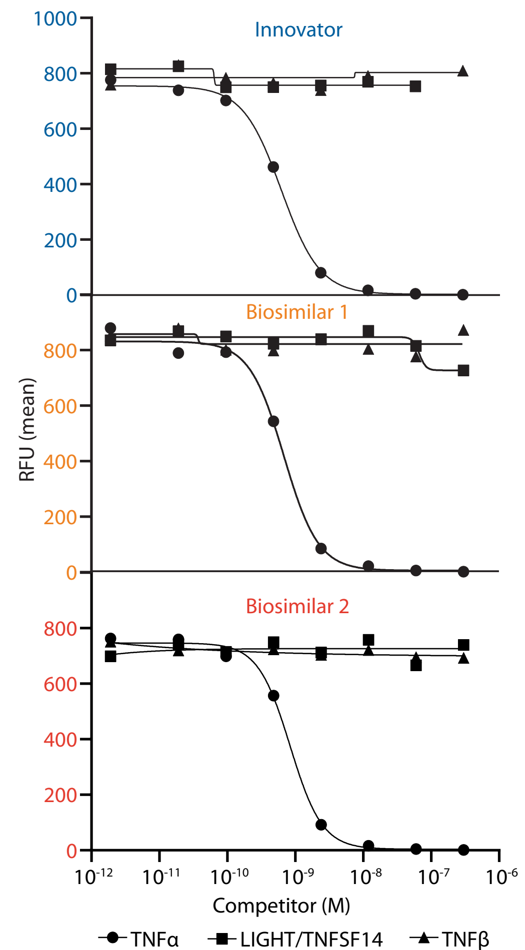


FIGURE 11. Competitive binding assay showing the specificity of adalimumab innovator and biosimilars to TNFα. Each plate contained 10 µg/mL capture protein and 1 µg/mL detection antibody.

Conclusion

With just four cartridges over two days, it was possible to develop a custom binding assay for adalimumab and its biosimilars to TNFα using the 48-Dig cartridge and Ella. With this platform-based approach, the specificity of adalimumab for rhTNFα was demonstrated by binding to human TNFα, but not human LIGHT/TNFSF14 or TNFβ. Furthermore, the Kd values obtained here were close to those previously published, which were between 5.8×10^{-11} M and 9.7×10^{-11} M[2]. Similarly, the IC50 values were close to those described previously, 10×10^{-10} to 10×10^{-11} M[2]. With the 48-Dig cartridge and Ella you can easily develop a high-quality binding assay on a reproducible and automated platform.

References

1. Adalimumab in the treatment of immune-mediated diseases, G Lapadula, A Marchesoni, A Armuzzi, C Blandizzi, R Caporali, S Chimenti, R Cimaz, L Cimino, P Gionchetti, G Girolomoni, P Lionetti, A Marcellusi, FS Mennini and C Salvarani, ***International Journal of Immunopathology and Pharmacology,*** 2014; 27:33–48.

2. Scientific discussion for the approval of Humira, European Medicines Agency; Available from: https://www.ema.europa.eu/en/documents/scientific-discussion/humira-epar-scientific-discussion_en.pdf

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