Sample dilution

A bead-based flow cytometry method for determining the sample dilution factor for a bead-based SARS-CoV-2 ELISA

Keywords
Dynabeads, ELISA, SARS-CoV-2, spike trimer, IgG, IgM, Attune NxT Flow Cytometer

- A simple, 35-minute bead-based flow cytometry screening protocol was developed to determine appropriate sample dilution factors for an accurate quantitative ELISA to detect SARS-CoV-2 antibodies.
- A simple, 45-minute bead-based ELISA protocol was developed to quantify SARS-CoV-2 antibodies isolated from human serum or plasma.

Introduction

Detection of SARS-CoV-2 antibodies in serum or plasma can confirm that an individual has been exposed to the virus and generated an immune response to it. The immune response to SARS-CoV-2 varies significantly among individuals. The concentration of antibodies in serum and plasma varies substantially from person to person, which can potentially lead to inaccurate test results. An enzyme-linked immunosorbent assay (ELISA) can be used to identify a specific immunoglobulin (Ig), quantify the immune response, and help stratify serum and plasma samples collected from exposed individuals for downstream research. However, a key limitation of ELISA-based methods is that they have narrow linear dynamic ranges imposed by the optical density readout method.

In previous work, SARS-CoV-2 antibodies were spiked into samples that tested negative for SARS-CoV-2 at concentrations of 2,000 pg/mL or 4,000 pg/mL. These concentrations were within the linear detection range of the Invitrogen™ Dynabeads™ SARS-CoV-2 Spike Ig Total ELISA Kit (Figure 1). Other samples were spiked with antibodies at a concentration of 8,000 pg/mL, which was outside the linear dynamic range of the assay. Total Ig concentrations at the edge or within the range of the standard curve were accurately measured using the Thermo Scientific™ Varioskan™ LUX multimode microplate reader and Thermo Scientific™ SkanIt™ Software for Microplate Readers (ELISA). Ig recoveries that fell outside the linear range had to be calculated manually based on the slope of the standard curve, which generated inaccurate results (Figure 2). The data indicated it was necessary to extensively test samples with unknown antibody concentrations prior to ELISA analysis to establish appropriate dilution factors. This is a time-consuming and costly process, so a rapid test for determining appropriate dilution factors would be beneficial.

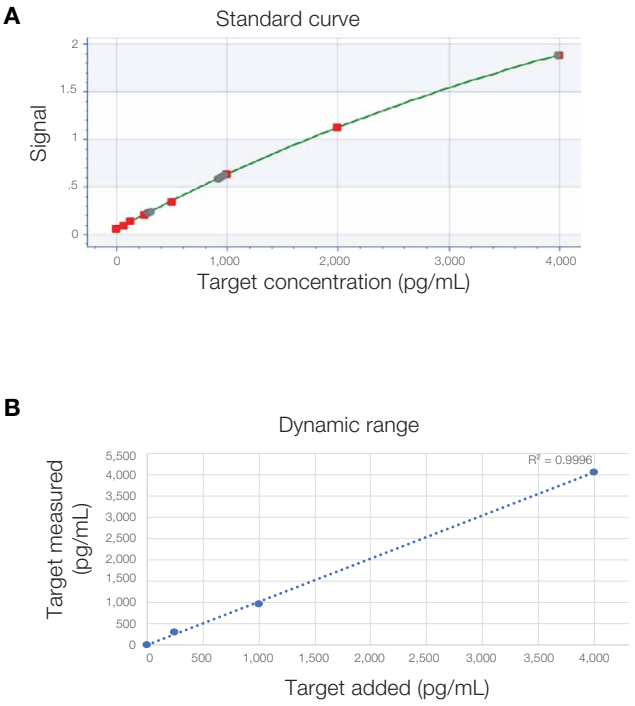

Figure 1. Standard curve and dynamic range for total Ig in serum. **(A)** The standard curve was analyzed using 4PL regression. Analysis was performed using the Varioskan LUX multimode microplate reader and SkanIt Software. The standard curve had a good model curve fit with an $R^2 > 0.999$. **(B)** Good correlation between the amount of target added and the amount of target measured was observed within the dynamic range of the assay.

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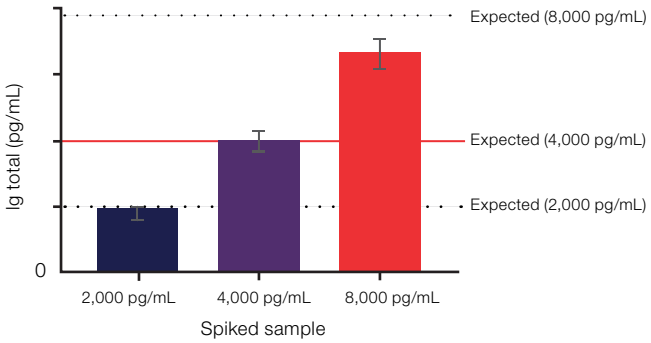


Figure 2. Detection of anti–SARS-CoV-2 antibodies in plasma using the Dynabeads SARS-CoV-2 Spike Ig Total ELISA Kit. The samples had to be diluted to bring the Ig concentrations within the linear range of the standard curve to obtain reliable quantitative results. SARS-CoV-2 antibodies were spiked into the samples at concentrations within and outside the linear range of the standard curve. The upper limit of the curve was 4,000 pg/mL (red line). Antibody concentrations above 4,000 pg/mL had to be calculated manually using the slope of the curve.

Flow cytometry signals at known SARS-CoV-2 antibody concentrations in plasma

We developed a fast flow cytometry method for determining appropriate dilution factors to bring SARS-CoV-2 antibody concentrations in plasma closer to the linear dynamic range of our ELISA (Figure 3). SARS-CoV-2 antibodies were spiked into plasma at concentrations of 2,000 pg/mL, 4,000 pg/mL, or 8,000 pg/mL. Invitrogen™ Dynabeads™ SARS-CoV-2 Spike beads (12 mg) conjugated to SARS-CoV-2 trimeric spike protein were incubated with the spiked samples in assay buffer in a 96-well plate for 15 minutes at room temperature with mixing. The Invitrogen™ HulaMixer™ Sample Mixer can be used for mixing if samples are incubated in tubes. Incubating samples for 10 minutes resulted in slightly lower fluorescence intensity (data not shown).

After the Dynabeads beads were coated with SARS-CoV-2 antibodies, the plate was placed on the Invitrogen™ DynaMag™-96 Side Skirted Magnet. The beads were washed three times with the wash buffer for the Dynabeads SARS-CoV-2 Spike Ig Total ELISA Kit. The beads were then incubated with goat anti–human IgG conjugated to Invitrogen™ Alexa Fluor™ 488 dye for 15 minutes. The unbound antibody conjugates were washed away, and the bead–antibody complexes were analyzed directly on an Invitrogen™ Attune™ NxT Flow Cytometer. The geometric means of the fluorescence signals are shown in Figure 4. In the absence of SARS-CoV-2 antibodies, the geometric mean was 190. Spiking in 2,000 pg/mL of SARS-CoV-2 antibodies increased the geometric mean to 490. The upper limit of the linear detection range of the Dynabeads SARS-CoV-2 Spike Ig Total ELISA was reached after spiking in 4,000 pg/mL of SARS-CoV-2 antibodies, which resulted in a geometric mean of 704. Spiking in 8,000 pg/mL of SARS-CoV-2 antibodies yielded a geometric mean of 1,287, which was outside the linear detection range of the assay.

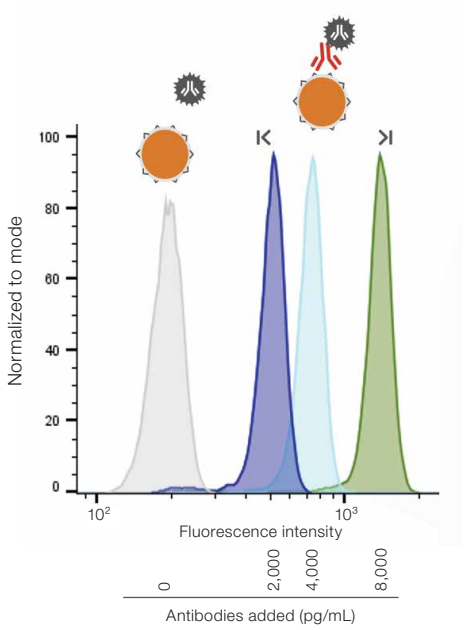


Figure 4. Detection of SARS-CoV-2 antibodies in plasma after incubation with Dynabeads magnetic beads and goat anti–human IgG conjugated to Alexa Fluor 488 dye. SARS-CoV-2 antibodies were spiked into samples at concentrations within the linear range: 2,000 pg/mL (purple peak) or 4,000 pg/mL (light blue peak). Samples were also spiked with 8,000 pg/mL of SARS-CoV-2 antibodies, which was outside the linear range of the ELISA standard curve (green peak). The spiked samples were then incubated with Dynabeads magnetic beads coated with SARS-CoV-2 spike trimer. After the beads were coated with SARS-CoV-2 antibodies and incubated with secondary antibodies conjugated to Alexa Fluor 488 dye, they were analyzed by flow cytometry. For a negative control, Dynabeads SARS-CoV-2 Spike beads were incubated with plasma that did not contain SARS-CoV-2 antibodies prior to addition of the secondary antibody (gray peak).

Figure 3. Flow cytometry workflow.

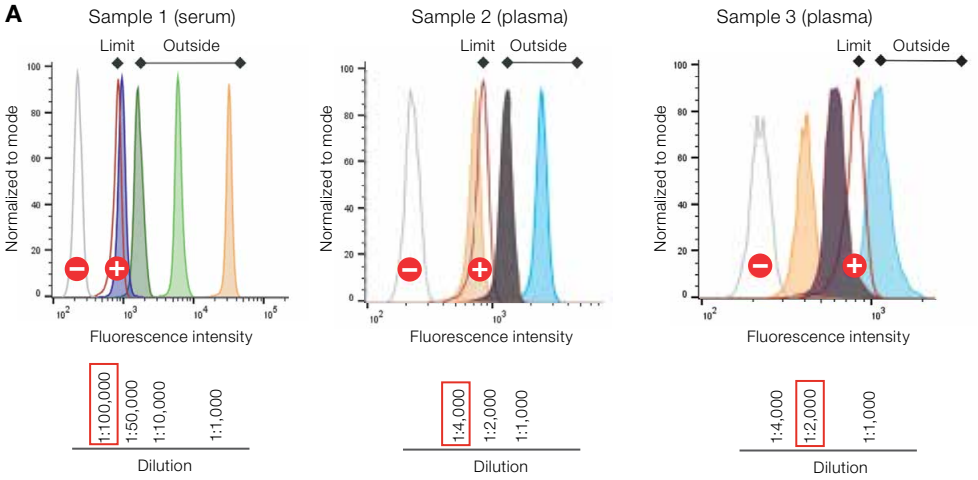
Flow cytometry signals for plasma and serum samples with unknown SARS-CoV-2 antibody concentrations

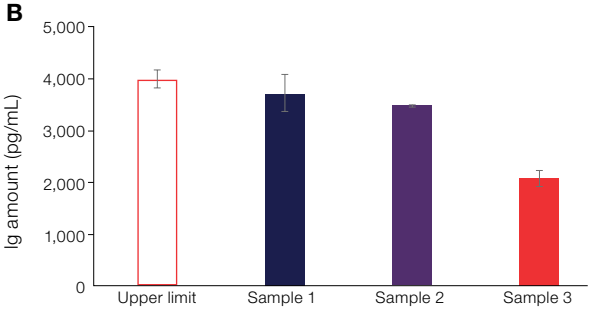
The SARS-CoV-2 total antibody concentrations in one serum sample and two plasma EDTA samples from three individuals suspected of being exposed to SARS-CoV-2 were analyzed using the new flow cytometry method. The serum sample was diluted 1:1,000, 1:10,000, 1:50,000, and 1:100,000 in assay buffer. The plasma EDTA samples were diluted 1:1,000, 1:2,000, and 1:4,000 in assay buffer. Dynabeads SARS-CoV-2 Spike beads were incubated with 100 µL of each diluted sample, washed three times with wash buffer, then incubated with goat anti–human IgG conjugated to Alexa Fluor 488 dye for 15 minutes prior to flow cytometry analysis. Positive controls were prepared by adding SARS-CoV-2 spike antibody to negative controls at 1 µg/mL, which corresponded to the upper limit of the ELISA standard curve for total Ig. Dynabeads SARS-CoV-2 Spike beads were also incubated with plasma from non-infected individuals to prepare negative controls.

The flow cytometry signals for the serum sample indicated it contained a very high concentration of SARS-CoV-2 antibodies. Dilution to 1:100,000 was necessary to obtain a signal at the upper limit of the standard curve (Figure 5A). One of the plasma samples had to be diluted 1:4,000 to stay within the standard curve. The other plasma sample only required dilution to 1:2,000, which suggested it contained a lower concentration of total SARS-CoV-2 antibodies.

Rapid and quantitative Dynabeads SARS-CoV-2 Spike Ig Total ELISA

To estimate the total SARS-CoV-2 antibody concentrations in the plasma or serum samples, we used a quantitative Dynabeads beads–based ELISA method. The overall time required to perform the assay was just 45 minutes due to fast binding kinetics on the beads [1]. The rapid bead-based ELISA and the new flow cytometry method were used to quantify the total SARS-CoV-2 antibody concentrations in diluted plasma and serum from three individuals. We obtained accurate results that were within the linear range of the ELISA standard curve (Figure 5B). The dilution factors used for the calculations are shown in Figure 5C. The quantitative bead-based ELISA protocol is described in more detail in a different application note [2].





C

Sample	ELISA concentration (pg/mL)	ELISA dilution factor	Ig in sample (µg/mL)
1	3,688	100,000	368.8
2	3,497	4,000	13.99
3	2,054	2,000	4.1

Figure 5. Rapid flow cytometry–based method for determining appropriate dilution factors for a quantitative SARS-CoV-2 total Ig ELISA with Dynabeads SARS-CoV-2 Spike beads. (A) The gray peaks are from negative controls. The red peaks are from measurements at the upper limit of the ELISA standard curve for total Ig. The other peaks correspond to samples at indicated dilutions. **(B)** Concentrations of total Ig in the same three serum and plasma samples in panel A. The dilution factors outlined in red in panel A resulted in signals within the linear range of the ELISA standard curve for total Ig of sample 3. Absorbance was measured at 450 nm, and SkanIt software was used to calculate the amount of Ig (pg/mL) in the samples. **(C)** The dilution factors used to calculate the concentrations of total Ig (µg/mL) in the samples.

Summary

We developed a simple, rapid, and reliable flow cytometry method to quickly estimate SARS-CoV-2 antibody concentrations in plasma or serum to determine appropriate dilution factors for an accurate and quantitative SARS-CoV-2 ELISA. Dynabeads SARS-CoV-2 Spike beads capture anti–spike immunoglobulins in serum and plasma, and they are included in all qualitative and quantitative ELISA-based Invitrogen™ Dynabeads™ SARS-CoV-2 antibody detection kits. The quantitative ELISA can be performed manually in just 45 minutes or automated using the Thermo Scientific™ KingFisher™ Duo Prime Purification System [2].

Ordering information

Product	Cat. No.
Flow cytometry	
Dynabeads SARS-CoV-2 Spike	18100D
HulaMixer Sample Mixer	15920D
DynaMag-96 Side Skirted Magnet	12027
Attune NxT Flow Cytometer, blue	A24864
ELISA	
Dynabeads SARS-CoV-2 Spike Ig Total ELISA Kit	18020DFIVE
Dynabeads SARS-CoV-2 Spike IgM ELISA Kit	18010D
Dynabeads SARS-CoV-2 Spike IgG ELISA Kit	18000D
Clear Flat-Bottom Immuno Nonsterile 96-Well Plates	439454
Varioskan LUX Multimode Microplate Reader	VLBLATD2

References

1. https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0025847_Dynabeads_SARS-CoV-2_ELISA_Ig_Total_UG.pdf.
2. A rapid, fully automatable bead-based SARS-CoV-2 ELISA in only 45 minutes (COL35044). Thermo Fisher Scientific, Waltham, MA.

Learn more at **thermofisher.com/dynabeadselisa**