Application Protocol: Klickmer® for Detection of SARS-CoV-2 Spike-specific B cells

Intended use	Preparation of Spike-Klickmer® by attaching Spike protein to Klickmer®-PE or Klickmer®-APC to detect and analyze SARS-CoV-2 Spike-specific B cells by flow cytometry.
Materials Provided	Klickmer® PE, DX01K PE (160 nM) Klickmer® APC, DX01K APC (160 nM)
Materials Required (not provided)	Mono-biotinylated recombinant SARS-CoV-2 Spike protein (ACRO Biosystems, cat. no. SPN-C82E9) in aqueous buffer (pH 7.0-7.5) with a biotinylation level > 75% and no excess of free biotin Human Fc block reagent, BD Biosciences D-biotin solution 100 µM, Avidity, BIO200 Fixable Viability Stain 575V, BD Biosciences Antibodies for staining of other surface markers, BV421 mouse anti-human CD19, V500 mouse anti-human CD3, PerCP-Cy5.5 mouse anti-human CD14, BV786 mouse anti-human CD27, FITC mouse anti-human IgD, BD Biosciences PBB buffer: PBS, 1% BSA, pH 7.0 Wash buffer: PBS, 5% FCS, pH 7.4 Brilliant blue stain buffer, BD Biosciences

Procedure

1. **Preparation of Spike-Klickmer® Solutions**

   The following protocol describes the preparation of 20 µL Spike-Klickmer®-PE and Spike-Klickmer®-APC solutions in a ratio of 7 Spike molecules per Klickmer® molecule. The concentration of recombinant Spike protein in this example is 2000 nM.
   Adapt the protocol to your experiment by inserting the concentration of your recombinant Spike protein, Klickmer® stock volume, and no. of ligands in the below formula. See examples in Table 1.

   1. Calculate the volumes of Klickmer® and recombinant Spike protein using the equation:

$$\text{Recombinant Spike protein volume (µL)} = \frac{\text{(Stock Klickmer® volume (µL) * Stock Klickmer® concentration (nM) * Number of ligands per Klickmer®)}}{\text{Recombinant protein concentration (nM)}}$$

$$\text{Recombinant protein volume } (\mu L) = ((4\ \mu L) * (160\ nM) * (7)) / (2000\ nM)$$

   2. Add the calculated volume of Spike protein into two light protected reaction tubes, labelled PE-Klickmer®-Spike and APC-Klickmer®-Spike, respectively.

3. Add Klickmer®-PE or Klickmer®-APC to the tubes to a final concentration of the Klickmer® at 32 nM, corresponding to 1/5 of the total volume of the desired Spike-Klickmer® solution. Pipette mix gently 5x.
4. Add the calculated volume of PBB buffer as shown below and pipette mix gently 5x.

PBB Buffer Volume (µL) =

(Desired Spike-Klickmer® volume (µL) – Recombinant Spike-Protein Volume (µL) – Stock Klickmer® Volume (µL))

5. Incubate for 30 min in the dark at room temperature.
6. Store the Spike-Klickmer® solutions in the dark at 2-8°C until use. The reagent can be stored for up to 1 week at 2–8 °C, protected from light.

2. Preparation of PBMCs for Staining of Spike-specific B cells

1. Thaw PBMCs (up to 1-3 x 10^6) and resuspend in 10 mL wash buffer.
2. Centrifuge at 300 x g for 10 minutes and remove supernatant. Repeat washing for a total of 2 washes.
3. Resuspend cell pellet in 1 mL wash buffer and incubate with 2 µL of BD Fixable Viability Stain 575V stock solution for 15 min at room temperature.
4. Add 10 mL wash buffer and centrifuge at 300 x g for 10 min. Remove supernatant.
5. Resuspend cell pellet in a suitable volume of BD brilliant blue stain buffer.

3. Staining of Spike-specific B cells in PBMC sample with Spike-Klickmer®

1. Spin the Spike-Klickmer® solutions from Step 1 at 10,000 x g for 1 min.
2. Add 0.4 µL 100 µM d-Biotin to an empty flow tube, add 10 µL Spike-Klickmer®-PE and 10 µL Spike-Klickmer®-APC and vortex briefly.
3. Add 50 µL cell suspension to the mixture and vortex briefly.
4. Incubate for 20 min in the dark at room temperature.
5. Add Fc block reagent (0.5 mg/mL) to cells to a final amount of 2.5 µg according to manufacturer's instructions and incubate for 10 min at room temperature.
6. Add 5 µL of each fluorochrome-conjugated antibody. Pipette mix 5x.
7. Incubate for 20 min. in the dark at room temperature.
8. Wash cells by adding 2 mL wash buffer. Centrifuge at 300 x g for 5 min. and remove the supernatant. Repeat washing for a total of 2 washes.
9. Resuspend cell pellet in 100 µL of wash buffer
10. Proceed to analyze the samples on a flow cytometer and make sure to acquire a minimum of 50,000 to 100,000 B cells to detect a sufficient number of Spike-specific B cells.

4. Data Analysis

1. Gate on the lymphocyte population in the forward scatter (FSC)/side scatter (SSC) plot.
2. Gate on singlets in the FSC-A/FSC-H and SSC-A/SSC-H plots.
3. Gate on viable $CD19^+$ cells in the CD19/viability stain plot to identify living B cells.
4. Exclude $CD3^+$ and $CD14^+$ cells by gating on $CD3^-$ and $CD14^-$ cells in the CD3/CD14 plot.
5. To exclude false positive cells, use double discrimination with a two-parameter plot with Spike-Klickmer®, PE and Spike-Klickmer®, APC to gate on Spike-specific B cells on the diagonal of the dot plot.

Optionally, to investigate the memory status of the Spike-specific B cells use a CD27/IgD plot

Technical Support

For Tips & Tricks, FAQs and protocols, please visit https://www.immudex.com/resources/ or contact our support team at customer@immudex.com
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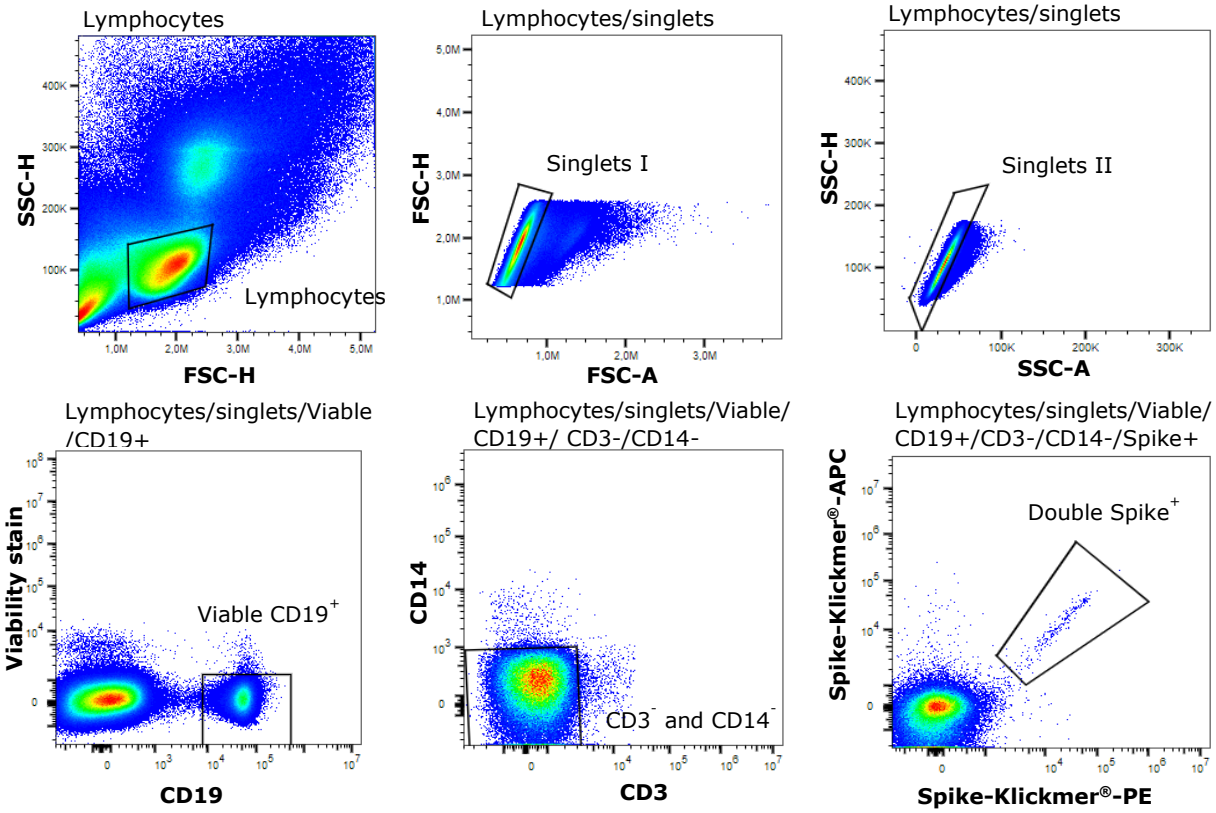

Fig. 1 Gating strategy identifying Spike-specific B cells by double-discrimination using Spike-Klickmer® reagents.

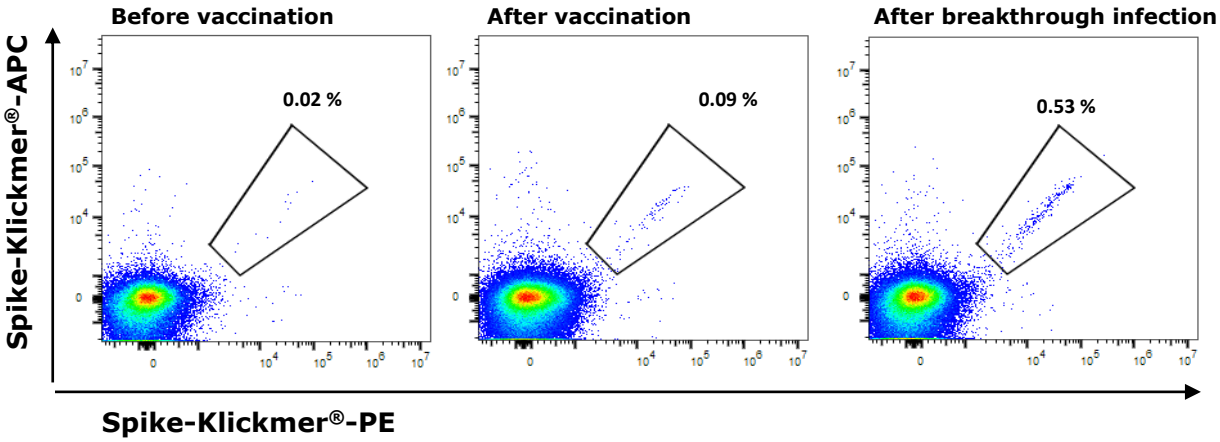

Fig. 2 Detection of Spike-specific B cells in one sample at baseline, after vaccination and breakthrough infection, using the Spike-Klickmer® reagents by flow cytometry.

Table 1. Volumes of Spike-Klickmer® reagents

No. of tests	***Recombinant Spike protein*** *(2000 nM)*	***Klickmer® stock solution*** *(PE or APC) (160 nM)*	***PBB buffer***
1	1.1 µL	2 µL	6.9 µL
5	5.6 µL	10 µL	34.4 µL
10	11.2 µL	20 µL	68.8 µL
20	22.4 µL	40 µL	137.6 µL

Each test in Table 1 corresponds to staining 1-3 x 10^6 PBMCs.