Host humoral immunological response plays an essential role in protection against pathogens. Identification of B-cell epitopes on antigens is required for accurate diagnosis and vaccine development. To map SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) spike linear epitopes, we developed a protocol of profiling sera from patients with COVID-19 (coronavirus disease 2019) via a peptide microarray designed according to spike protein. The protocol is also applicable for other antigens or sample types. This protocol is rapid, high throughput, and the cost is acceptable while it needs specialized microarray facilities.
Protocol
SARS-CoV-2 spike linear epitope scanning via a peptide microarray through sera profiling

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SUMMARY
Host humoral immunological response plays an essential role in protection against pathogens. Identification of B-cell epitopes on antigens is required for accurate diagnosis and vaccine development. To map SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) spike linear epitopes, we developed a protocol of profiling sera from patients with COVID-19 (coronavirus disease 2019) via a peptide microarray designed according to spike protein. The protocol is also applicable for other antigens or sample types. This protocol is rapid, high throughput, and the cost is acceptable while it needs specialized microarray facilities.

For complete details on the use and execution of this protocol, please refer to Li et al. (2020, 2021a, 2021b).

BEFORE YOU BEGIN
This protocol was used in recent publications (Li et al., 2020, 2021a, 2021b) in which we have profiled linear epitopes of SARS-CoV-2 Spike protein using the sera from COVID-19 patients.

Design and synthesize peptides

★ Timing: 1 day

To decode the linear epitopes for a given target protein, the protein is split into overlapped peptides to largely cover all potential linear epitopes.

1. Peptide library design
   a. Download the amino acid sequence of the target protein (the Spike protein of SARS-CoV-2) from Uniprot or other resources.
   b. Split the protein sequence into 12 aa peptides with 6 aa overlapped (a total of 211 peptides for the Spike protein).
   c. Add a Cysteine at the N terminus of each peptide.

△ CRITICAL: The Cysteine is necessary for conjugation to carrier protein through Sulfo-SMCC (sulfosuccinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate) reagent that is reactive to sulfhydryl group.
2. Peptide synthesis. The peptides of Spike were synthesized by GL Biochem (Shanghai) Ltd.

*Note:* The highly hydrophobic peptides may fail to be synthesized. For SARS-CoV-2 Spike, 10 out of 211 peptides failed to be synthesized.

**Positive sera sample preparation**

*© Timing: 1 h*

Epitope mapping usually requires the analysis of dozens to thousands of serum samples. Systematic error is inevitable for microarray technology, which partly results from variances among slides. To largely reduce the variances, one sample as a positive control should be detected alone with other samples for each slide and used for normalization among slides.

3. Randomly select 5–10 sera from pathogen infected patients. Specifically, we chose 50 SARS-CoV-2 infected patients who were confirmed by nucleic acid test.

4. Centrifuge at 12,000 × *g* for 20 min at 4°C.

5. Take out 10 μL supernatant of each serum and pool together. The pooled sample is then set as the positive control.

6. The positive control was then aliquoted to 10 μL per vial and stored at −80°C.

*Note:* 1) 50 μL positive control are good for around 200 slides, i.e., about 2,800 (200 × 14) sera samples. 2) Mixing of a set of samples instead of selecting only one as a positive control is to avoid bias.

**Microarray layout design and positive probe preparation**

*© Timing: 1 day*

Microarray layout design is to assign a position in an array for each probe and would be used to set printing parameters. The probes include not only the peptides of target antigen(s) but also some negative and positive controls which are used to justify the results of the microarray experiment.

7. Preparation of negative and positive control probes. Negative/blank controls: BSA (0.3 mg/mL), Printing buffer; Positive control: Anti-Human IgG (0.1 mg/mL), Anti-Human IgM (0.1 mg/mL), the antigen (Spike); Land marker (a probe with fluorescence used to indicate a given position in an array or subarray): Cy3 labeled Anti-Mouse antibodies (0.1 mg/mL), Cy5 labeled Anti-Mouse antibodies (0.1 mg/mL).

8. Design the layout of microarray.
   a. Design subarrays. A slide (75 mm × 25 mm) can contain a total of 14 (2 × 7) identical subarrays or blocks. About 200 peptides could fit in one of the subarrays.

*Note:* The number of format of subarrays are largely dependent on the multiple-well chamber which is used to create isolated wells for multiple sera detection on a single slide.

   b. Design the array layout by including all the peptide as well as control probes using Command Centre™ for Marathon v2.2, the software of the Super Marathon microarray printer. Triplicates for each probe in a subarray are recommended.

   c. According to the instruction of the software Command Centre™ for Marathon v2.2, transform the layout of the array to the layout for 384-well plate.
KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Cy3-Goat Anti-Human IgG | Jackson ImmunoResearch | Cat# 109-165-008 |
| Alexa Fluor 647 Donkey Anti-Human IgM | Jackson ImmunoResearch | Cat# 709-605-073 |
| Anti-Human IgG       | Sigma  | Cat# I2136 |
| Anti-Human IgM       | Sigma  | Cat# I2386 |
| Anti-BSA antibody    | Sangon Biotech | Cat# D220272-0025 |
| Cy3-Goat Anti-Mouse IgG | Jackson ImmunoResearch | Cat# 115-165-071 |
| Cy5-Goat Anti-Mouse IgM | Jackson ImmunoResearch | Cat# 115-175-075 |
| Chemicals, peptides, and recombinant proteins |        |            |
| SARS-CoV-2 Spike protein | Our lab (Li et al., 2021a) | N/A |
| BSA                  | Yeasen Biotech | Cat# B27371 |
| Sulfo-SMCC           | Thermo Fisher | Cat# 22322 |
| SARS-CoV-2 Spike S1  | Hangzhou Bioeast Biotech Co., Ltd. | N/A |
| QuickBlock blocking buffer | Beyotime | Cat# P0220 |
| Software and algorithms |        |            |
| ImmunomeBrowser      | IEDB   | http://tools.iedb.org/immunomebrowser/ |
| Other                |        |            |
| Microarrayer         | Arrayjet | Super Marathon |
| LuxScan scanner      | CapitalBio | 10K-A |
| GenePix scanner      | Molecular Devices | 4200A |
| Centrifuge for slides | CapitalBio | SlideWasher™ 8 |
| 16-Well chamber (with gasket) | RayBiotech | AA-FRAME-G16 |
| Slide container      | Our lab | This paper |
| PATH substrate slide | Grace Bio-Labs | 805025 |
| 384-Well plates      | Arrayjet | AJC010 |

MATERIALS AND EQUIPMENT

PBS buffer (10×)

Combine buffer ingredients (1,370 mM NaCl, 27 mM KCl, 81 mM Na₂HPO₄, and 18 mM KH₂PO₄) in dH₂O and adjust the pH to 7.4. This solution can be stored at 4°C for at least 6 months.

Blocking buffer

Dissolve 3 g BSA into 100 mL 1 × PBS buffer to a final concentration of 3% (wt/vol). This solution should be freshly prepared before use.

Wash buffer

Mix 0.5 mL Tween-20 into 500 mL 1 × PBS to a final concentration of 0.1% (vol/vol). This solution can be stored at 4°C for at least 6 months.

Incubation buffer

Dissolve 0.1 g BSA into 10 mL Wash buffer or Mix 1 mL 10% (wt/vol) BSA (stored at −20°C) into 9 mL Wash buffer. This solution should be freshly prepared before use.

Printing buffer
Mix 2.5 mL glycerol and 1 mL 10× PBS buffer into 6.5 mL dH₂O to a final concentration of 25% (vol/vol) glycerol. This solution can be stored at 4°C for at least 6 months.

**STEP-BY-STEP METHOD DETAILS**

**Conjugation of peptides with BSA**

© Timing: 2 days

To efficiently immobilize the peptide on the substrate slide, the peptides are conjugated to a large carrier protein. Here we used BSA as the carrier. Other protein can be used. However, the protein may bring non-specific binding so as to generate high background or false positive signals. One strategy to lower this effect is that the carrier protein is added into the Blocking buffer and serum Incubation buffer.

1. Reconstitute the lyophilized peptides with double distilled water (ddH₂O) to 4 mg/mL. Notably, for hydrophobic peptides which is difficult to be dissolved with water, DMSO (20 µL) could be added to 1 mg peptide followed by adding 230 µL ddH₂O. However, a few peptides that still cannot be completely dissolved would have a compromised concentration.

2. BSA activation by Sulfo-SMCC.
   a. Dissolve 10 mg BSA with 2.5 mL 1× PBS (phosphate buffer saline).
   b. Dissolve 2 mg Sulfo-SMCC with 100 µL ddH₂O and pipette up and down to mix to generate homogenous solution.
   c. Add 97.7 µL dissolved Sulfo-SMCC into the BSA solution, gently shake and incubate for 30 min at room temperature (22°C–27°C).

   **Note:** The molar ratio of BSA to Sulfo-SMCC is 1:30.

   d. Dialyze in 1× PBS at 4°C to remove the excess Sulfo-SMCC. The dialysis buffer (1× PBS) should be changed at least twice after 3–4 h of dialysis each.

3. Peptide conjugation.
   a. Add 250 µL activated BSA to 1 mg reconstituted peptides (1:1 in w/w).
   b. Incubate at room temperature (22°C–27°C) for 4 h on a rotator.

   **Optional:** Conjugation efficiency can be estimated by electrophoresis separation (Figure 1A). It is highly recommended particularly for those peptides which do not dissolve completely.

**Peptide microarray fabrication**

© Timing: 2 days

High quality microarray is essential for high quality data acquisition. This part provides detailed procedures as well as considerations to fabricate peptide microarray. For the Spike peptides, we created an array of 32 × 20 features with triplicates for each probe.

4. Load the dissolved peptides and controls to a 384-well plate
   a. Dilute the BSA-peptide conjugates with Printing buffer (1× PBS with 25% glycerol) to 0.3 mg/mL.

   **Note:** The concentration refers to the peptides. The optimal concentration of peptides for serum detection is 0.1–1 mg/mL according to our experience. However, the optimal concentration that largely depends on the sample types or antibody concentrations, if required, it needs to be determined in a case-by-case manner.
b. According to the designed layout of the microarray, add the probes into the designated wells of the 384-well plate. For the Spike peptides, we created an array of 32 x 20 features with triplicates for each probe (Figure 1B). The minimal volume is 10 μL per well, while the maximal volume is 50 μL. The minimal and maximal volume would be different for alternative types of 384-well plates.

**Note:** the probes should be handled on ice.

c. Centrifuge the plate at 800 × g for 1 min at 4°C.

**Pause point:** the prepared plate could be stored at −80°C for long term (at least one year).

5. Microarray printing
a. Use the printer (Super Marathon) to print the probes on PATH substrate slides according to the manufacture’s instruction (https://www.arrayjet.co.uk/instruments/printers-in-detail).
b. Incubate the microarrays at 4°C with humidity less than 50% overnight (over 12 h).
c. Put the slides into the dedicated slide box (5 or 1 slide(s) per box), seal the box with Parafilm and store the box at −80°C.

Pause point: the microarrays can be stored at −80°C for several years.

6. GAL (GenePix Array List) file generation
   a. Create an Excel file and list the position of the probes on 384-well plate (Table 1).
   b. Create GAL file using the printer software by combining of this file with the printing program.

Note: The GAL file is for extracting the data from the microarray (step 17).

Optional: Microarray quality evaluation. The steps for this experiment are similar with the following part of Serum Profiling (step 9–17) except that 1) the Blocking buffer were replaced by a commercial blocking buffer (QuickBlock blocking buffer) and 2) the microarray was incubated with an Anti-BSA antibody (1:1000) followed by incubation with a Cy3 labeled Anti-Mouse secondary antibody (1:1000).

Serum profiling

© Timing: 1 day

7. Blocking.
   a. Take out the slides from −80°C and place them at 4°C for 30 min followed by room temperature for 15 min to enable temperature equilibrium. The slide box should be well sealed during this process.

△ CRITICAL: for PATH substrates, condensate water would cause merging of neighboring dots without the equilibrium step.
   b. Prepare the Blocking buffer (1 × PBS with 3% BSA).
   c. Add 30 mL Blocking buffer in the Slide Container at room temperature (22°C–27°C) (materials and equipment).
   d. Keep the face of the slide down. Immerse the barcode side into the buffer while keep the other side out, then keep barcode side and slowly lay down the other side until the whole side is immersed into buffer. Turn over the slide and leave the slide immersed in the blocking buffer.

△ CRITICAL: It is better to take 1.5 minutes or longer to finish this process to avoid trailing effect (a phenomenon that a big tail attached to the otherwise circular feature).
   e. Keep the slides at room temperature (22°C–27°C) and shake at 20–30 rpm (revolutions per minute) on a shaker for 3 h.

8. Wash and dry the slide.
   a. Discard the Blocking buffer, add 20–30 mL 1 × PBS and keep the slides shaken at 20–30 rpm for 5 min at room temperature (22°C–27°C).

| Plate | Column | Row | Name |
|-------|--------|-----|------|
| 1     | 1      | 1   | P1   |
| 1     | 1      | 2   | P2   |
| 1     | 1      | 3   | BSA  |
|       |        |     | ...  |
b. Discard the buffer, add 20–30 mL 0.2 × PBS and keep the slides shaken at 20–30 rpm for 5 min at room temperature (22°C–27°C).

c. Discard the buffer, add 20–30 mL ddH₂O and keep the slides shaken at 20–30 rpm for 5 min at room temperature (22°C–27°C).

d. Dry the slides by centrifugation. As list in materials and equipment table, we used a specialized slide centrifuge SlideWasher™ 8 with default parameters. Alternatively, a normal staining rack with a compatible centrifuge can be used (500 × g for 1–2 min).

Pause point: The dried slides can be stored at 4°C in a dry environment for at least one month without compromised signal intensity for serum profiling.

9. Sera sample preparation. Centrifuge the tube at 12,000 × g at 4°C for 20 min.

10. Sample incubation
   a. Install a 16-well chamber with gasket on the slide.
   b. Dilute the sera sample as well as the positive control (1: 200) with Incubation buffer (1 × PBS with 0.1% tween-20 and 1% BSA).
   c. Add 200 μL diluted sample into one well that contains one subarray. For each slide, the last two blocks or wells are added with Incubation buffer and positive sera sample as blank control and positive control, respectively, i.e., block # 13 for blank control, and block #14 for positive control. A blank control is used to monitor unexpected contaminations.

   Note: 1) The samples can be added with a multichannel pipette. 2) Immediately record the sera number, the block position and the slide number.
   d. Incubate the slide at room temperature (22°C–27°C) for 2 h. It is recommended to keep them in a box to prevent evaporation.

11. Pre-wash and wash.
   a. Remove the solution from each well with a multichannel pipette, add 200 μL Wash buffer (1 × PBS with 0.1% tween-20) to each well, and pipette up and down for 15–20 times.
   b. Repeat the above step twice.
   c. Uninstall the 16-well chamber.

   CRITICAL: Do not leave the surface of the slide dry.
   d. Place the slide in a container with ~10 mL Wash buffer. Keep the container at room temperature (22°C–27°C) and shake for 90–100 rpm on a shaker for 10 min.
   e. Repeat the above step twice.

12. Secondary antibody incubation
   a. Dilute the secondary antibody (1: 1000) with Incubation buffer.
   b. Remove the Wash buffer from the container and add 15 mL diluted secondary antibodies. Depending on the isotypes of antibody of interest, multiple isotypes could be detected simultaneously, only if the isotype specific fluorescence labeled secondary antibodies are available and compatible with the microarray scanner. Here, we detected the IgG and IgM isotypes using Cy3 (532 nm excitation) and Alexa Fluor 647 (635 nm excitation) labeled isotype specific antibody, respectively (Figure 1C).

   Note: if only one slide is used, a smaller container would be suggested with a smaller incubation volume.
   c. Keep the container at room temperature (22°C–27°C) and shake for 20–30 rpm on a shaker for 1 h. Keep the slides shielded from light.

13. Wash and dry
a. Remove the solution from the container and add 30 mL Wash buffer. Keep the container at room temperature (22°C–27°C) and shake for 90–100 rpm on a shaker for 10 min.
b. Repeat the above step twice.
c. Wash twice with ddH₂O.
d. Dry the slide by centrifugation.

Note: From step 15 on, the slides should be kept under dark.

14. Microarray scanning (link to troubleshooting, problem 2–4)
   a. Open the Scanner GenePix 4200A and warm up for 15 min.
   b. Scan the slide with 532 nm (IgG) and 635 nm (IgM) laser, successively. Recommended scanning parameters: 95% power, PMT 200–300. However, the optimal scanning intensity should be adjusted according to the overall signal intensity.
   c. After scanning is completed, save the two images as one single TIF file.

△ CRITICAL: 1) All sides which belongs to the same experiment should apply the same parameters. 2) Avoid scraping or touching the surface of the microarrays in any way during the whole process.

Microarray data processing

⊙ Timing: 1 day

Quantitative signal intensity (indicating the antibody response) to each peptide for one sample could be acquired by transforming the color intensity of the microarray image to digital signals, which is microarray data extraction. Then, normalization is performed to enable comparisons of the signal intensities among the samples.

15. Extract microarray data
   a. Use the software GenePix 6.0 to align the features. The software enables automatic alignment of the GAL file (step 8) to the features of the microarray image, however, it is necessary to double check all aligned features and adjust the incorrectly aligned features. It is a time-consuming process.
   b. Extract the data of all features to generate a GPR file (raw data) for each slide.

16. Calculate signal intensity
   a. Calculate signal intensity for each feature: subtract Foreground Median (F median) with Background Median (B median). IgG (532 nm channel) and IgM (635 nm channel) are calculated separately.
   b. Calculate the average of the triplicates of each probe in the same subarray.

17. Perform normalization among slides. Use the data of the positive sera sample (the data of block #14 for each slide) to calculate the normalization factor (Factor N) for each slide. IgG (532 nm channel) and IgM (635 nm channel) are calculated separately.
   a. With the data of block #14 of all slides, calculate the overall average signal intensity for each peptide (not for the control probes) to create a reference data set.
   b. Exclude the peptides with low signal intensity (< 300).
   c. Calculate the Pearson correlation coefficient between the reference data set and the data of block #14 for each slide. If the coefficient is less than 0.98, exclude some spots far away from the diagonal line.
   d. Perform linear fitting and calculate the Factor N for each slide using the following function in Excel software: LINEST (Y, Xi, FALSE), where the Y is the reference data set, the Xi is the data of block #14 from the slide i, and the FLASE means to make the constant term equal to 0.
   e. For each slide, all the signal intensities are normalized by multiplying the corresponding Factor N.
Linear epitope landscape
Due to potential non-specific binding, sera from healthy people were set as negative controls to define the basal signal intensity for each peptide, so response frequency could be specifically depicted for each peptide in the patient group. For the map, higher frequency value implies widespread epitopes of the target antigen. The peptides with higher response frequency imply epitopes that elicit stronger antibody responses.

18. Set threshold for each peptide
   a. Use serum samples from healthy people to define the baseline and calculate the threshold value as Mean value + 2 × SD (standard deviation).
   b. Calculate positive rate or frequency response for each peptide for the patient group.

19. Generate linear epitope map. For each peptide, provide the total patient sera number and positive number as well as the peptide sequence to the webpage-based tool ImmunomeBrowser (Dhanda et al., 2018). An epitope map would be generated (Figure 1E). For the map, higher frequency value implies widespread epitopes of the target antigen. For SARS-CoV-2 Spike, several hot areas in rich of linear epitopes were indicated in the map.

EXPECTED OUTCOMES
The BSA-peptide conjugation generates an obvious shift in gel electrophoresis when compared with BSA and Sulfo-SMCC activated BSA (Figure 1A). The quality of the peptide microarray can be evaluated by Anti-BSA antibody (Figure 1B). A qualified peptide microarray exhibits bright, uniform, organized and regular features. For serum derived from infected patients, the microarray image is expected to exhibit high differences among the peptides and normally most of the peptides are negative. The background is low and uniform (Figure 1C). High correlations could be achieved between the repeats of the positive sera samples analyzed on different slides. The normalization factor (Factor N) could be easily calculated by linear fitting (Figure 1D). An example (SARS-CoV-2 Spike) of linear epitope map generated by ImmunomeBrowser is shown (Figure 1E).

LIMITATIONS
Theoretically, this protocol cannot profile conformational epitopes of the target antigens. It may also fail to detect some linear epitopes that were not covered by set of 12-aa peptides on the microarray. Feature alignment (step 17) could dramatically affect the signals of the features, so apply of the same standard for all features during this process is important to achieve comparable and highly reliable quantitative data.

TROUBLESHOOTING
Problem 1
The peptides with highly hydrophobic properties might fail to be synthesized (step 2 in “before you begin” section).

Potential solution
One possible solution is that extend or shorten the peptides to reduce the number of hydrophobic amino acids. However, we did not try in our experiment.

Problem 2
False positive signals of the peptides were observed for the bank control sample (# 13 block) on some slide(s) (step 16).

Potential solution
The False positive on the control slide may be caused by one of the following reasons: 1) Contaminations from other serum samples are brought when loading serum samples; 2) Contaminations from other serum samples are brought when performing the pre-wash step. 3) The Blocking buffer,
Incubation buffer or Wash buffer is contaminated. Discard the current buffer and prepare new buffer, then re-analyze the samples. Carefully operate during the incubation and wash steps.

**Problem 3**
Very high background of the microarray (step 16).

**Potential solution**
Increase the blocking time or concentration of the BSA in Blocking buffer (step 9); Decrease the concentration of the serum samples (step 12); decrease the power of the scanner (step 16); Change the substrate slides.

**Problem 4**
Many merged features in the microarray (step 16).

**Potential solution**
Use a bigger spot-spot distance when printing (step 7); lower the concentration of the printed samples (step 12); extend the equilibrium time (step 9).

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sheng-ce Tao (taosc@sjtu.edu.cn).

**Materials availability**
This study did not generate new unique reagents.

**Data and code availability**
This study did not generate/analyze datasets/code.

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**AUTHOR CONTRIBUTIONS**
S.-c.T. supervised the project. Y.L. and D.-y.L. conducted the experiments and data analysis. Y.L. and S.-c.T. wrote the manuscript.

**DECLARATION OF INTERESTS**
The authors declare no competing interests.

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