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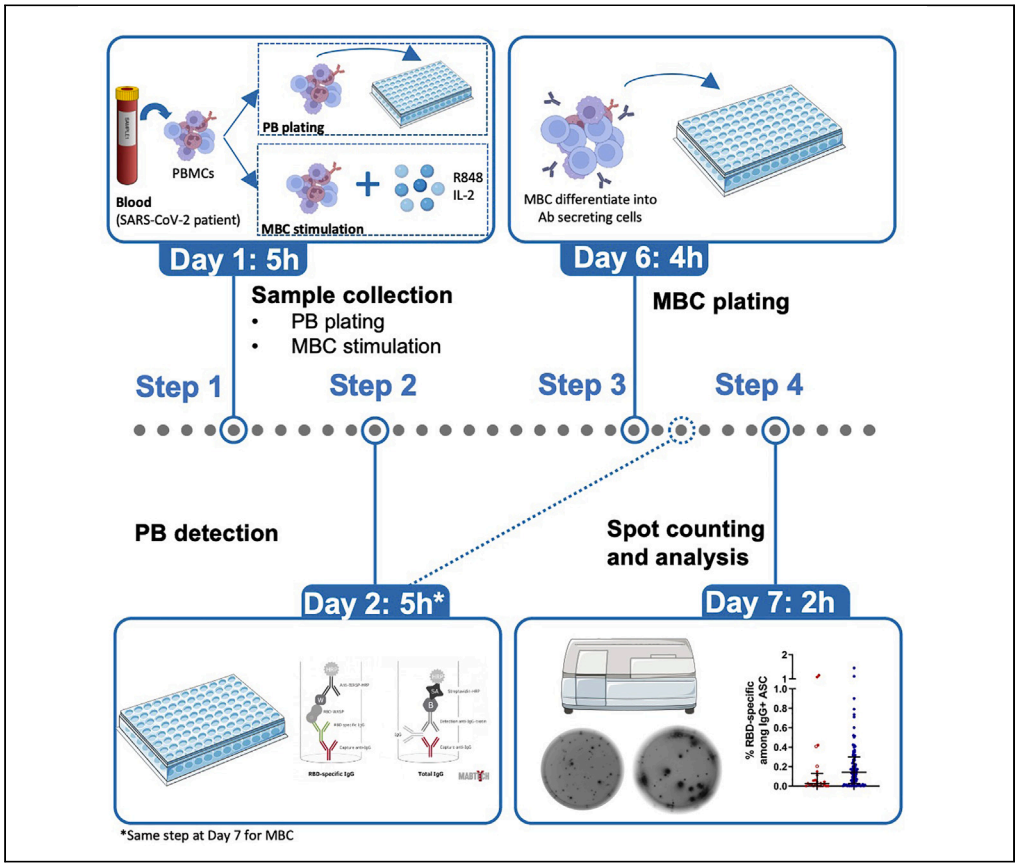
B-cell ELISpot assay to analyze human memory B cell and plasmablast responses specific to SARS-CoV-2 receptor-binding domain



B-cell ELISpot is an extremely sensitive assay based on the secretion of antibodies by B cells that requires the differentiation of B cells into antibody-secreting cells. Here, we describe the procedure to analyze both plasmablast (PB) and memory B cell (MBC) responses specific to SARS-CoV-2 receptor-binding domain (RBD) in the context of acute SARS-CoV-2 infection and vaccination. We detail steps for MBC stimulation, MBC and PB plating, detection, and counting of total IgG and RBD-specific spots.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Differentiation *in vitro* of memory B cells into antibody-secreting cells

Detection of plasmablasts and memory B cells specific to SARS-CoV-2 RBD

Monitoring of antigen-specific B cell responses following infection or immunization

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B-cell ELISpot assay to analyze human memory B cell and plasmablast responses specific to SARS-CoV-2 receptor-binding domain

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SUMMARY

B-cell ELISpot is an extremely sensitive assay based on the secretion of antibodies by B cells that requires the differentiation of B cells into antibody-secreting cells. Here, we describe the procedure to analyze both plasmablast (PB) and memory B cell (MBC) responses specific to SARS-CoV-2 receptor-binding domain (RBD) in the context of acute SARS-CoV-2 infection and vaccination. We detail steps for MBC stimulation, MBC and PB plating, detection, and counting of total IgG and RBD-specific spots.

For complete details on the use and execution of this protocol, please refer to Tay et al. (2022).[1]

BEFORE YOU BEGIN

Our protocol is based on optimization of original assays developed by others to detect IgG vaccine-induced B cell responses.[2] The protocol below describes the procedure using fresh PBMCs from acute SARS-CoV-2 samples. However, we have also used this protocol with frozen PBMCs from recovered patients and vaccinated volunteers. Depending on the user needs, plasmablast or memory B cell populations may also be analysed.

Institutional permissions

Use of human biological samples requires regulatory approval. Ensure that all ethical requirements are fulfilled. A minimum of 5 mL of whole blood collected in Vacutainer® CPT™ or 2×10^6 PBMCs is recommended for this procedure.

Acute SARS-CoV-2 infected samples need to be handled with proper PPE, please ensure compliance with all health and safety regulations.

Preparation of complete medium (R10)

Timing: 15 min

1. Supplement RPMI-1640 medium with 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin (10,000 units/mL of penicillin and 10,000 µg/mL streptomycin, Gibco 15140122).

Isolation of PBMCs from whole blood

Timing: 2–3 h

2. Centrifuge CPT™ tube containing whole blood at 1,800 × *g* for 20 min at 4°C without brake.

Alternatives: Dilute whole blood with PBS 1× (add 1 volume of PBS 1× for 1 volume of blood) and gently layer the diluted blood on Ficoll solution (volume of Ficoll equal to diluted blood). Centrifuge at 1,800 × *g* for 20 min at 4°C without brake.

3. Carefully collect the mononuclear cells and platelets layer above the polyester gel and transfer into a 50 mL conical tube.
4. Top up at 20 mL with Phosphate-buffered saline (PBS) 1× and centrifuge down at 500 × *g* for 5 min at 18°C–22°C.
5. Inverse the tube to remove the supernatant and resuspend the cells pellet in 6 mL of PBS 1×.
6. Count the cells using a hematocytometer.
7. Resuspend the cells at 2 × 10^6 cells/mL in R10 medium and keep at 37°C until use. 2.6 × 10^6 cells are required for the following procedure.

Optional: Freeze surplus PBMCs in human serum/10% DMSO.

Coating of ELISpot plate

Timing: 45 min (at least the day before)

8. Pre-wet the ELISpot plate by adding 50 µL of 35% ethanol solution in each well for 1 min.
9. Flick the plate to remove the ethanol and wash 4 times with 200 µL distilled water.
10. Prepare anti-human IgG purified antibody (mAb clone MT91/145, Mabtech) at 15 µg/mL and add 100 µL per well.
11. Wrap the plate in parafilm and keep it at 4°C 18–24 h.

⚠ CRITICAL: Coat only the number of wells required with the antibodies. Protect the rest of the wells with adhesive plastic film. Plates can be kept for maximum 1 week in the coating solution and are ready to use during this timeframe.

Alternatives: Use pre-coated plate (Pre-coated MSIP white plate (mAb clone MT91/145, Mabtech).

Blocking of ELISpot plate

Timing: 30 min (before starting plating)

12. Flick the plate to remove the coating solution.
13. Wash 4 times with 200 µL PBS 1×.
14. Add 200 µL complete medium (R10) in each well for minimum 30 min at 18°C–22°C.

Note: This step can be done just before starting the cells plating. The plating will take sufficient time to allow the minimum of 30 min incubation. Alternatively, the plate containing the blocking buffer can be prepared in advance and kept no more than 2 days at 4°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-human IgG mAbs MT91/145 purified (working concentration: 15 µg/mL)	Mabtech AB	Cat#3850-3-1000
anti-human IgG mAbs MT78/145 biotinylated (working concentration: 1 µg/mL)	Mabtech AB	Cat#3850-6-1000
Human anti-WASP mAb 36-5-10-ALP (working dilution: 1 in 275)	Mabtech AB	Cat#3745-9A-1000
Chemicals, peptides, and recombinant proteins		
RPMI 1640 media	Gibco	Cat#22400121
Fetal bovine serum (FBS)	R&D Systems	Cat#S12550H
Penicillin/Streptomycin	Gibco	Cat#15140122
Phosphate-buffered saline (PBS)	Gibco	Cat#10010023
Ethanol	Sigma-Aldrich	CAS 64-17-5
BCIP/NBT-plus substrate	Mabtech AB	Cat#3650-10
StimPack Memory B cells (R848+IL-2)	Mabtech AB	Cat#3660-1
SARS-CoV-2 RBD-WASP (working dilution: 1 in 25)	Mabtech AB	Cat# 3654-TP-10
Streptavidin-ALP (working dilution: 1 in 1000)	Mabtech AB	Cat#3310-10-1000
Rely+On™ Virkon™ Powder	Lanxess (VWR)	Cat#148-0201
Critical commercial assays		
ELISpot PVDF plates clear	Mabtech AB	Cat#3654-TP-10
BD Vacutainer® CPT™ Mononuclear Cell Preparation Tube - Sodium Citrate	BD Biosciences	Cat#362760
Deposited data		
Decreased memory B cell frequencies in COVID-19 delta variant vaccine breakthrough infection	EMBO Molecular Medicine	https://doi.org/10.15252/emmm.202115227

MATERIALS AND EQUIPMENT

RPMI complete medium (R10)

Reagent	Final concentration	Amount
RPMI (Gibco 22400121)	89%	445 mL
FBS (R&D Systems S12550H)	10%	50 mL
Penicillin/Streptomycin (Gibco 15140122)	1%	5 mL
Total	**N/A**	**500 mL**

The media can be stored up to 3 months at 2°C–8°C.

35% ethanol

Reagent	Final concentration	Amount
Ethanol 100% (CAS 64-17-5)	35%	33.94 mL
Distilled water	65%	66.05 mL
Total	**N/A**	**100 mL**

The solution must be prepared **fresh** to pre-wet the plate. Dilution is calculated according to Gay Lussac's table.

⚠ CRITICAL: Ethanol is a flammable liquid and must be handled with care. It can cause serious eye damage/irritation. The 100% solution should be handled within a chemical cabinet to prepare the 35% solution.

STEP-BY-STEP METHOD DETAILS

Note: This protocol is designed to analyze both plasmablasts (PBs) and memory B cells (MBCs) in samples from acute SARS-CoV-2 infected patient. The principle is the same for PBs and MBCs in both plating and detection, but MBCs require an additional step of stimulation into antibody-secreting cells for their detection, whereas PBs can be plated directly in the ELISpot plate.

Step:	Plasmablasts	Memory B cells
Stimulation	–	Day 1
Plating	Day 1	Day 6
Detection	Day 2	Day 7

Total IgG-secreting B cells and Receptor Binding Domain (RBD)-specific IgG-secreting B cells are detected by B-cell ELISpot. Quantifying total IgG cells requires plating a small number of cells to avoid overlapping spots, whereas RBD-specific IgG cells, due to the rarity of the population, requires a higher number of cells to be plated to detect a sufficient number of spots. However, it is not recommended to plate more than 5×10^5 cells per well to avoid multilayer of cells which will affect the detection.

⚠ CRITICAL: Acute SARS-CoV-2 samples are infectious, and the plates need to be decontaminated before reading the spots on IRIS ELISpot reader (Mabtech). This protocol includes the decontamination steps by UV exposure and incubation with virucidal solution (Virkon). However, if the cells are from recovered patients or healthy volunteer (i.e.: vaccine monitoring) these steps are optional.

Plasmablast plating [Day 1]

Timing: 2 h

Plasmablasts constitutively secrete antibodies and must be analysed by B-cell ELISpot straight after the isolation of PBMCs from whole blood. The ELISpot plate needs to be ready to use before plating the cells. This includes the coating with anti-human-IgG and blocking with R10 medium as described earlier.

1. Take out PBMCs: aliquot 6×10^5 cells in an Eppendorf tube from the 2×10^6 cells/mL PBMCs suspension (**Tube A**) (Figure 1).
2. Dilute the cells for the "Total IgG" condition (Figure 1).
   a. Prepare a new tube with 656 µL of R10 (**Tube B**).
   b. Transfer 10 µL from Tube A into Tube B.
3. Plate cells into ELISpot plate.
   Each sample will be distributed into 4 wells: 2 concentrations of cells for each condition "Total IgG" and "RBD".
   a. Remove the blocking medium from the plate.
   b. Fill the wells with fresh R10 medium, the volume to add depends on the conditions (Figure 2).
   c. Add the cells:
      i. "RBD" (Tube A): 50 µL (1×10^5 cells) and 200 µL (4×10^5 cells).
      ii. "Total IgG" (Tube B): 50 µL (0.15×10^4 cells) and 100 µL (0.3×10^4 cells).

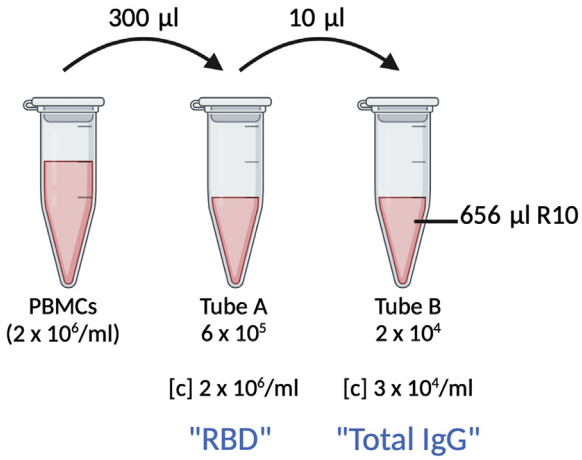


Figure 1. Schematic representation of PBMC aliquoting for plating of plasmablasts

⚠ CRITICAL: Do not let the membrane of the well dry during the procedure. During the plating, it is recommended to plate the highest concentration of the "RBD" condition (200 µL) first since there is no R10 added to keep the membrane wet for this condition. Alternatively, the different cell concentrations can be prepared in a separate 96-wells plate before transferring 200 µL using a multi-channel pipette.

iii. Wrap the plate in aluminum foil to limit evaporation and incubate at 37°C–5% CO_2 for 18–24 h.

⚠ CRITICAL: Do not move the plate during the incubation to avoid shifting of the cells in the well.

Memory B cells stimulation [Day 1]

Timing: 2 h

Unlike plasmablasts, memory B cells require *in vitro* stimulation to differentiate into antibody-secreting cells. The same suspension of PBMCs is used as a starting point (at a concentration of 2 × 10^6 cells/mL).

4. Take out PBMCs: aliquot 2 × 10^6 cells in an Eppendorf tube from the 2 × 10^6 cells/mL PBMCs suspension.
5. Prepare 1 mL of stimulation medium for each sample. Add R848 at a final concentration of 1 µg/mL. Add IL-2 at a final concentration of 10 ng/mL.

Note: R848 (TLR7 /8 agonist) directly stimulates memory B cells that constitutively express TLR7 and myeloid cells expressing TLR7 that will bring additional support to B-cell proliferation and differentiation.[3] IL-2 is a growth factor for various immune cells, including activated B cells.[4]

6. Resuspend PBMCs in the stimulation medium.
   a. Centrifuge down the PBMCs at 500 × *g* for 5 min at 18°C–22°C.
   b. Discard the supernatant and add 1 mL of stimulation medium.
   c. Transfer into one well of a 48-wells plate and incubate at for 5 days.

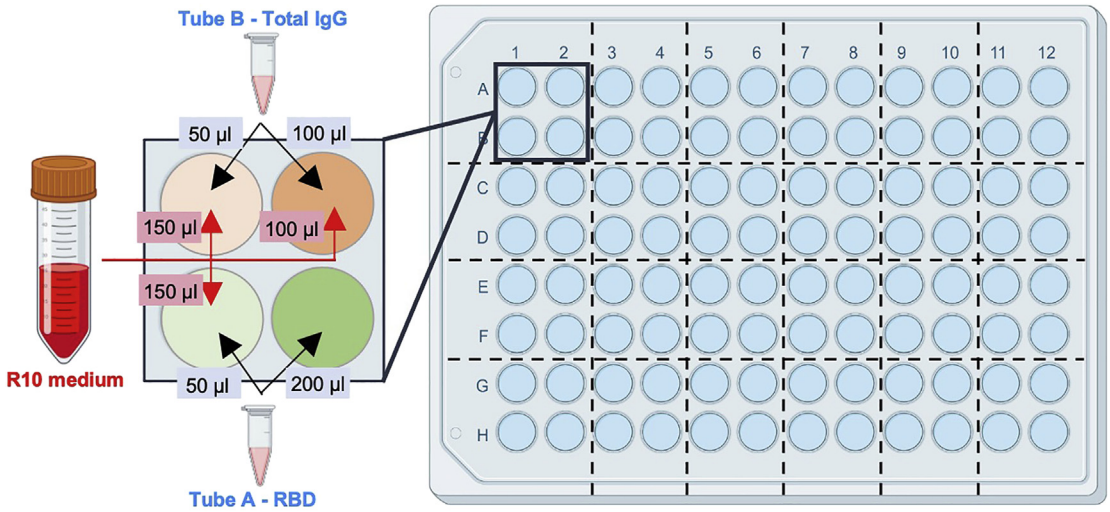


Figure 2. Schematic representation of cell plating
Each sample is distributed into four wells. The final volume of each well is 200 µL.

Note: Do not use edge wells on the plate. Instead, fill the empty wells around your wells containing the cells with sterile water to limit sample well evaporation.

Note: Check the culture after 2–3 days. If some of the wells turned yellow, it is recommended to add fresh medium.

Memory B cells plating [Day 6]

Timing: 3 h

After collecting the cells at day 6, the plating of memory B cells follows the same principle as plasmablasts as described in Figure 2.

7. Collect and count the stimulated cells.
   a. Collect the cells from the 48-wells plate and transfer into Eppendorf tubes.
   b. Centrifuge down the cells at 500 × *g* for 5 min at 18°C–22°C.
   c. Discard supernatant (Optional: collect the supernatant in new tubes to analyze antibodies secreted by stimulated B cells).
   d. Resuspend the cells in 200 µL of R10 medium and count nucleated cells: take out 10 µL of cells to mix with 40 µL of methylene blue (dilution 1:5).
8. Resuspend the cells at 2 × 10^6 cells/mL in R10 medium. 5.5 × 10^5 cells are needed to fill all the conditions (troubleshooting 1). This suspension corresponds to Tube A ("RBD" condition) as described in Figure 1.
9. Dilute the cells for the "Total IgG" condition.
   a. Prepare a new tube with 656 µL of R10 (Tube B).
   b. Transfer 10 µL from Tube A into Tube B.
10. Plate the cells as described in step 3 and Figure 2.

ELISpot plate detection [PB: Day 2 / MBC: Day 7]

Timing: 4–5 h

The detection steps are the same for plasmablasts and memory B cells. The plate for plasmablasts is detected on day 2 while the plate for memory B cells is detected on day 7.

11. Prepare the detection reagents:
    a. Anti-IgG-biotin (detection step A): prepare 100 µL /well in PBS 1× at a final concentration of 1 µg/mL.
    b. RBD-WASP (detection step A): prepare 100 µL /well in PBS 1× at a 1/25 dilution.
    c. Streptavidin-ALP (detection step B): prepare 100 µL /well in PBS 1× at 1/1000 dilution.
    d. Anti-WASP-ALP (detection step B): prepare 100 µL /well in PBS 1× at 1/275 dilution.

    Note: Keep the diluted reagent on ice until use.

12. Flick the plate upside down over a waste container. Be careful to avoid splashing of bleach or other liquid in the plate (troubleshooting 2).
13. Wash the plate with 200 µL of PBS 1× per well for a total of 4 times. Use a pipette to flush up and down in each well for the first wash (to detach all the cells from the wells). Flick the plate upside down for the following washes.

    Optional: Place the plate (containing PBS 1×) under UV for 5 min to decontaminate the wells (only in the case of acute samples). Protect the unused wells from UV with aluminum foil. Remove the PBS before adding the detection reagents.

14. Add 100 µL of detection reagents (detection step A, **see detail in** Figure 3) in each well and incubate 2 h at 18°C–22°C.
15. Wash the plate 4 times with 200 µL of PBS 1×.
16. Add 100 µL of detection reagents (detection step B, **see detail in** Figure 3) in each well and incubate 1 h at 18°C–22°C.
17. Wash the plate 4 times with 200 µL of PBS 1×.
18. Filter the BCIP/NBT-plus substrate before usage and add 100 µL in each well.

    ⚠ CRITICAL: protect from light since BCIP/NBT-plus substrate is sensitive to light which may affect the spots development.

19. Incubate 4–5 min and stop the reaction by adding 250 µL of distilled water (troubleshooting 3).
20. Wash the plate 4 times with 200 µL of distilled water.

    Optional: Add 200 µL of Virkon-S (21.41% Potassium peroxymonosulfate, 1.5% sodium chloride) disinfectant[5] to decontaminate the wells (only in case of acute samples). Incubate 5 min and wash 4 times with 200 µL of distilled water.

21. Remove the plastic rubber bottom and let the plate dry at 18°C–22°C before reading.

    ⚠ CRITICAL: Shake the plate upside down on a tissue between each washing step to remove as much liquid as possible (troubleshooting 3). Removal of the water at the last step will allow the plate to dry faster.

Reading of plate

Timing: 1–2 h

The IRIS ELISpot reader was used to count the spots. More details can be found on Mabtech website (https://www.mabtech.com/iris). Other readers can be used but parameters will have to be set up for adequate spot detection.

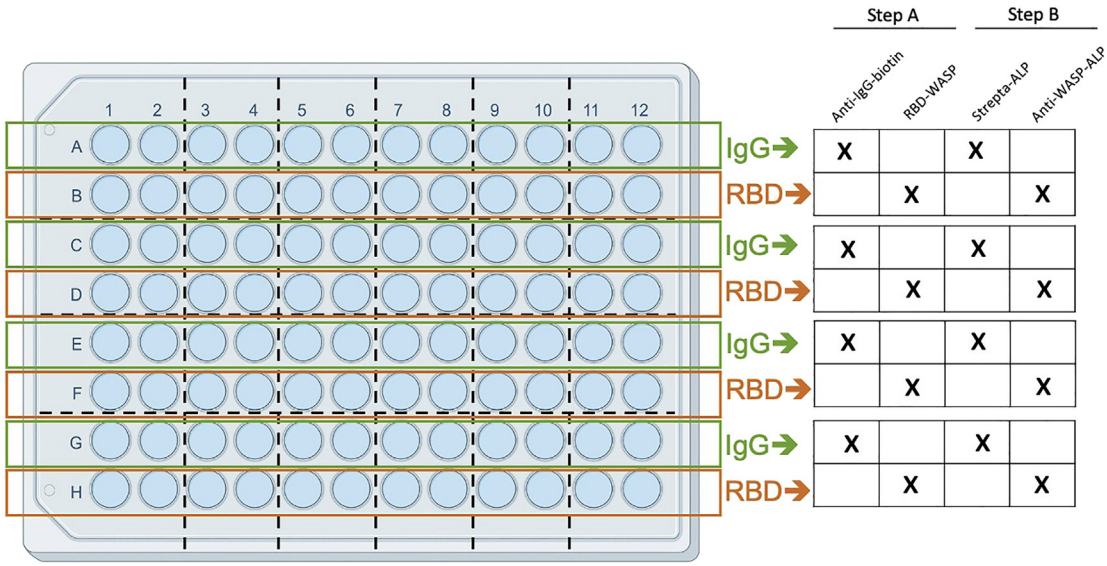


Figure 3. Template for adding of detection reagents

The protocol below provides the parameter used to count RBD and IgG spots using the MabTech reader.

22. Select all the wells to read and start the reading in the IRIS software.
23. Select all wells from the "Total IgG" condition: choose "tiny" emphasis and change the intensity to 80-90 (depend on the development of the spots) in the parameters (troubleshooting 4, troubleshooting 5).
24. Select all wells from the "RBD" condition: choose "big" emphasis and change the intensity to 10-20 (depend on the development of the spots) in the parameters (troubleshooting 4, troubleshooting 5).

EXPECTED OUTCOMES

The procedures described here allow to quantify the RBD-specific plasmablasts and memory B cells following natural infection or vaccination. Plasmablasts are expected to be detected for few days to 2 weeks after immune activation. Memory B cells are detected from 2-3 weeks after immune activation and can last for several months to years in certain context. The response is usually presented in Spot Forming Unit (SFU) per million of cells or as a frequency of RBD-specific B cells among total-IgG secreting cells (Figure 4).

QUANTIFICATION AND STATISTICAL ANALYSIS

The data can be exported exported as an image of the plate (Figure 5) and a Microsoft Excel table (Figure 6). The counted spots can be easily handled to calculate the SFU per million of cells or the frequency among total IgG.

LIMITATIONS

The sensitivity of the B-cell Elispot assay is high and allow to detect an extremely low frequency of antigen-specific memory B cells. However, the area of the wells limits the cell loading to 5×10^5 per well (higher cells numbers may result to multiple layer of cells and affect the detection). Thus, a frequency of antigen-specific MBCs <0.0002% among total cells will be undetectable.

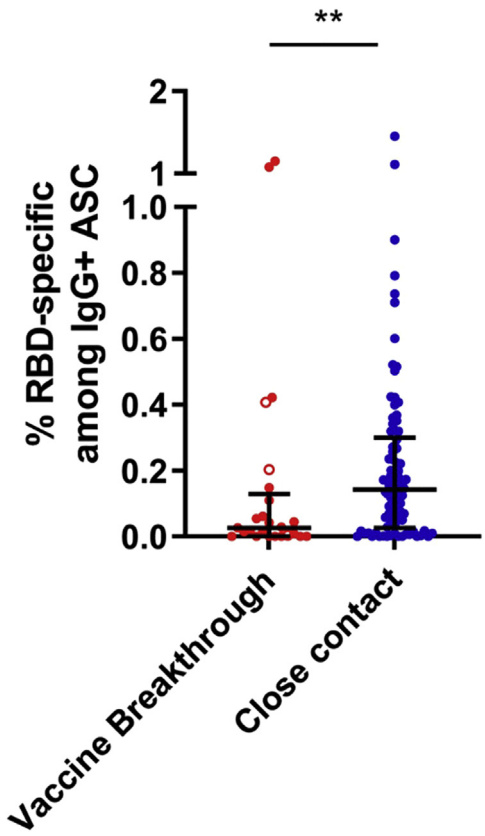


Figure 4. Figure issued from Tay M., Rouers A. et al. (https://doi.org/10.15252/emmm.202115227) representing the memory B cells specific to RBD in vaccine breakthrough COVID-19 patients compared to a group of close contact

p value was determined by two-tailed Mann–Whitney U-test, **p < 0.01. Error bars indicate median and interquartile range.

B-cell ELISpot does not detect the frequency of the antigen-specific MBCs *ex vivo* (as Flow cytometry assays allow) but after stimulation. The data obtained reflect a relative frequency of antigen-specific MBCs. Others have reported a correlation between MBC detected by flow cytometry and B-cell ELISpot[6] Nonetheless, B-cell ELISpot has the advantage to reflect the ability of MBCs to secrete antibodies on top of detecting their frequency.

TROUBLESHOOTING

Problem 1

Cell count is lower than 5.5 × 10^5 cells. It might be due to low concentration of cells stimulated (below a concentration of 1 × 10^6/mL) resulting in insufficient cells interaction and death or substandard quality of cells (related to step 8).

Potential solution

- The concentration of cells for the 5-day stimulation needs to be ideally between 1–2 × 10^6/mL. 1 mL can be plated in 48-well plates. If the cell number is too low and required to resuspend in a lower volume, you can use smaller wells (200 µL/well in 96-wells plate flat bottom).
- We used R848 and IL-2 in this procedure. Other stimulation cocktails containing alternative stimulants (i.e.: CpG) can be explored.[2]
- If there are insufficient cells to plate after the 5-day stimulation: reduce the number of cells to plate per condition but we recommend keeping a minimum of 5 × 10^4 for "RBD" condition. Consider plating all cells in one well for "RBD" instead of the two wells at different concentrations.

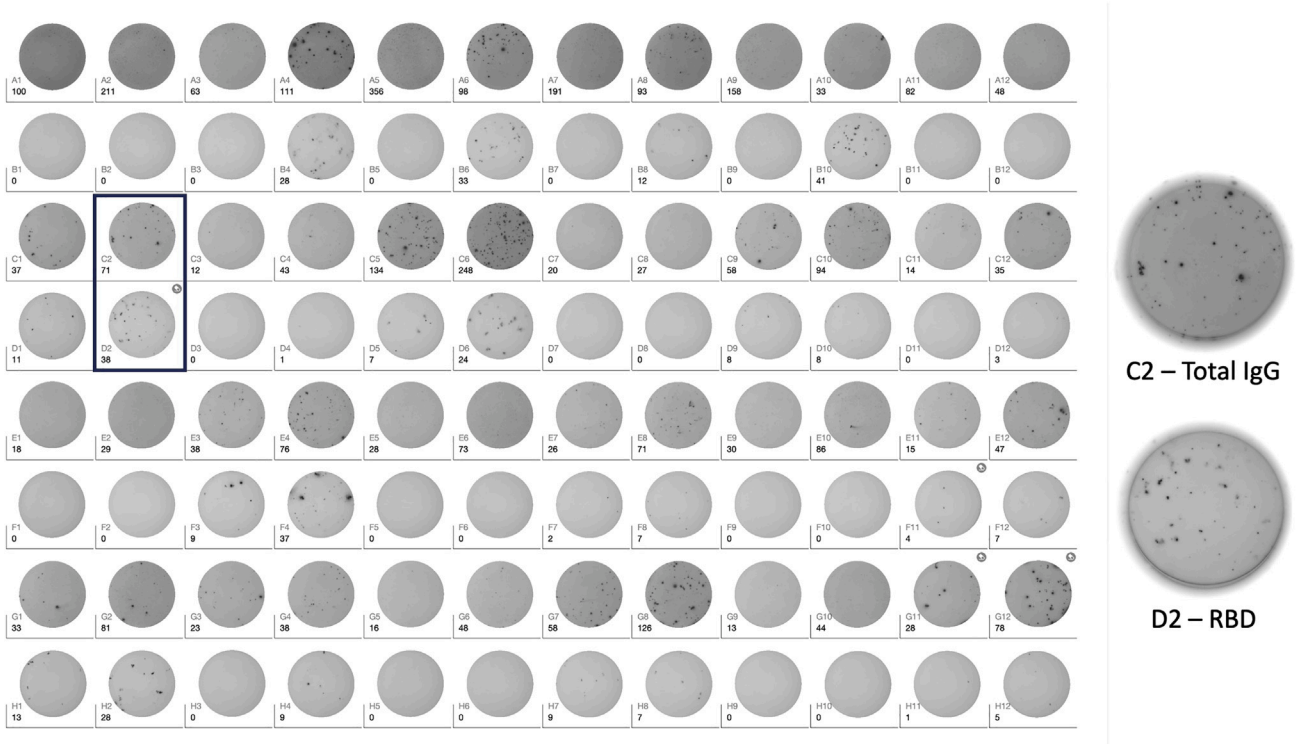


Figure 5. Image exported from the IRIS software (Mabtech) after reading of the plate

Number of spots counted is indicated at the bottom left corner of each well. Eraser icon on top right of certain wells indicate the manual removing of fiber or artifact.

Problem 2

Splashing of detergent in the plate during washing (related to step 12).

Potential solution

Place tissue at the surface of the liquid in the waste container when you flick the plate upside down. Other option is to remove the content from the plate by using a multichannel pipette (but it may increase the likelihood of damaging the plate membrane with the tips (see troubleshooting 4).

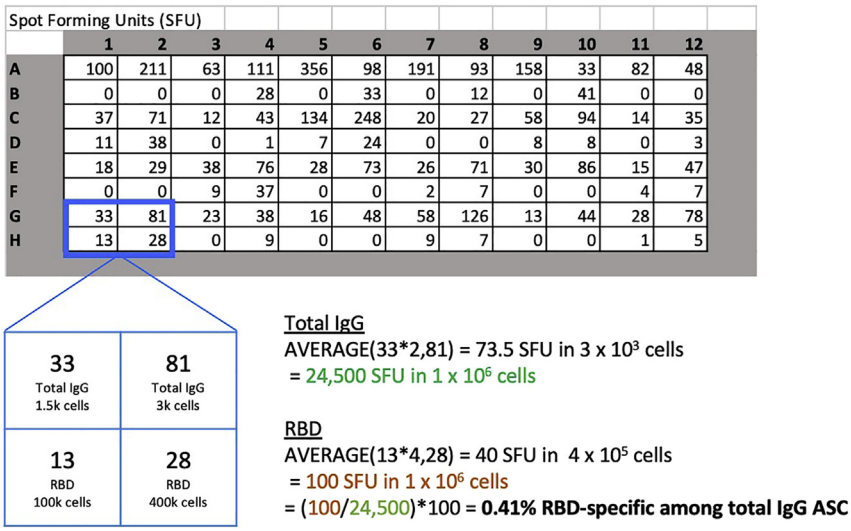
Problem 3

High background in spot signal (related to steps 19 and 21).

Potential solution

This problem could be caused by:

- Insufficient washing: wash at least 4 times the plate between each step. Increasing the number of rounds of washing may reduce the background if any. It is recommended to use PBS 1× with MSIP ELISpot plates.
- Plates were incubated too long with BCIP/NBT-plus substrate. Depending on your experimental setup, adjust the time of incubation. Note that final spot size after drying often appears larger than immediately after substrate incubation.
- Pre-wetting of the plate before the procedure is a critical step. High background could be due to missing this step or incubation for too long with ethanol (recommended for 1 min).
- The membrane is not dry: remove the rubber at the end of the procedure to dry the plate. A wet membrane will remain dark and difficult to read.



Spot Forming Units (SFU)

	1	2	3	4	5	6	7	8	9	10	11	12
A	100	211	63	111	356	98	191	93	158	33	82	48
B	0	0	0	28	0	33	0	12	0	41	0	0
C	37	71	12	43	134	248	20	27	58	94	14	35
D	11	38	0	1	7	24	0	0	8	8	0	3
E	18	29	38	76	28	73	26	71	30	86	15	47
F	0	0	9	37	0	0	2	7	0	0	4	7
G	33	81	23	38	16	48	58	126	13	44	28	78
H	13	28	0	9	0	0	9	7	0	0	1	5

Figure 6. Example of a table exported after reading and the method of calculation used to determine the frequency of RBD-specific MBCs

- High background could also be due to overloading of the wells (in that case the well will appear very dark): consider reducing the number of cells to plate.

Problem 4

Artifact and/or damage detected in the membrane (related to steps 23 and 24).

Potential solution

- Debris or fibers from damaged membranes are the most common artifact in ELISpot, work in sterile condition to limit this problem.
- The membrane can be damaged by scratching with the tips during pipetting. Be careful when pipetting in the wells, do not touch the bottom and always remain on the edge of the wells.
- The rubbers at the bottom of the plate need to be kept until the end of the procedure to avoid any accidental impact of the membrane from behind. Afterwards, they must be removed for proper drying.

Problem 5

No spot detected (related to steps 23 and 24).

Potential solution

- Context 1: No spot in both "Total IgG" and "RBD"

This is due to insufficient number of cells plated/unsuccessful stimulation. Data are not useable. Refer to troubleshooting 1.

- Context 2: There are spots in "Total IgG" but not in "RBD"

Total IgG is a control to ensure that the cells have been stimulated and differentiate into antibody-secreting cells. If there is no RBD spots but spots are present in Total IgG you can conclude that there is no RBD-specific response detectable.

- Context 3: There are spots in "RBD" but not in "Total IgG"

There may be a mistake with experimental procedure, cells may not have been plated in the "Total IgG" well. Alternatively, it may be that there are too many cells in "Total IgG" resulting in no detectable spots and overall darker aspect (See troubleshooting 3).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Prof Laurent Renia lcsrenia@ntu.edu.sg.

Materials availability

This study did not generate new unique reagents.

Data and code availability

The published article[1] includes all data presented in this protocol.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

A.R. wrote the original draft. M.Z.T, L.F.P.N., and L.R. edited and completed the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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