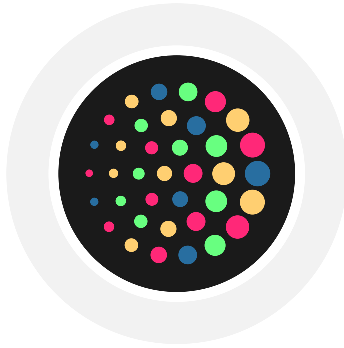

FluoroSpot Path:
SARS-CoV-2 (Spike+RBD) Human IgG

FSP-05R-RS1-1

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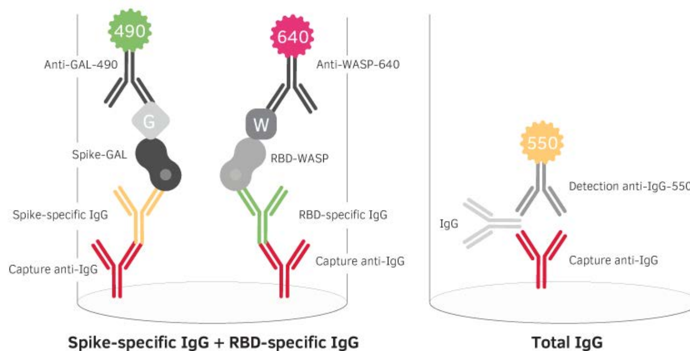
INTRODUCTION

Intended use

For enumeration of B cells secreting IgG antibodies specific for the SARS-CoV-2 Spike protein and Receptor Binding Domain (RBD). Enumeration of all cells secreting IgG (total IgG) can also be made.

Assay principle

Please read all instructions prior to starting the assay. Note that the reagents should not be prepared long in advance. It is recommended to consult the section Tips for a successful assay.



Spike-specific IgG + RBD-specific IgG **Total IgG**

Cells are added to a FluoroSpot plate pre-coated with anti-IgG monoclonal capture antibodies (mAbs). During incubation, IgG secreted by B cells will bind to the capture mAbs. After cell removal, Spike-specific IgG spots, RBD-specific IgG spots and total IgG spots can be detected. Spike-specific IgG spots are detected using Spike-GAL, i.e., recombinant Spike protein with peptide tag GAL*, followed by fluorophore-conjugated mAb anti-GAL-490. RBD-specific IgG spots are detected using RBD-WASP, i.e., recombinant RBD with peptide tag WASP**, followed by fluorophore-conjugated mAb anti-WASP-640. Total IgG spots are detected by addition of fluorophore-conjugated anti-IgG detection mAbs-550.

*Spike-GAL: Recombinant SARS-CoV-2 Spike protein prefusion ectodomain trimer based on uniprot P0DTC2 (aa 1-1208) with proline substitutions at residues 986 and 987, a GSAS substitution at the furin cleavage site (residues 682-685), a C-terminal T4 fibritin trimerization motif (Wrapp et al., Science, Feb 19, 2020), followed by GAL peptide tag used for detection (YPGQAPPGAYPGQAPPGA), and a C-tag (EPEA) used for purification.
**RBD-WASP: Recombinant SARS-CoV-2 Receptor Binding Domain (RBD) of the Spike protein, based on uniprot P0DTC2 (aa 319-541) with a C-terminal WASP peptide tag (PDYRPYDWASPDYRD).

CONTENTS AND STORAGE

Contents		Amount	Storage
For detection of Spike-specific IgG spots	Antigen: Spike-GAL, lyophilized	1 vial	-20 °C
	anti-GAL-490	60 µl	4-8 °C
For detection of RBD-specific IgG spots	Antigen: RBD-WASP, lyophilized	1 vial	-20 °C
	anti-WASP-640	60 µl	4-8 °C
For detection of total IgG spots	MT78/145-550	25 µl	4-8 °C
Polyclonal activators	R848, 1 mg/ml	100 µl	-20 °C
	Lyophilized recombinant human IL-2	1 µg	-20 °C
Buffer	Standard reconstitution buffer A5	2 x 1 ml	4-8 °C
Fluorescence enhancer	Ready-to-use Fluorescence enhancer-II	25 ml	4-8 °C
Pre-coated plate	Plate pre-coated with mAbs MT91/145	1 plate	Room temperature

Vials have been overfilled to ensure recovery of the specified amount.

Shipping

Shipped at ambient temperature. On arrival, store components as specified above.
Fluorophore conjugates and Fluorescence enhancer contain 0.002% Kathon CG.

Expiry date

The expiry date indicates how long unopened products are recommended for use when stored according to instructions.

ASSAY PROTOCOL

Cells

In vivo activated B cells (e.g., during acute infection) may be analyzed directly in the FluoroSpot plate without prior stimulation.

Memory B cells may require pre-stimulation *in vitro* with a mixture of R848 (1 µg/ml) and IL-2* (10 ng/ml) in tubes for 3-4 days to secrete detectable amount of antibody. After pre-stimulation, wash the cells extensively to remove secreted antibodies in the supernatant.

* Reconstitute IL-2 with 1 ml PBS to obtain 1 µg/ml. Leave for 15 min and then vortex. Use directly or store in aliquots at -20°C.

A. Plate preparation (sterile conditions)

1. Remove the plate from the sealed package and wash the plate three times with sterile PBS (200 µl/well).

2. Block/condition the plate by adding cell incubation medium containing 10% fetal calf serum (200 µl/well). Incubate for at least 30 minutes at room temperature.

B. Cell incubation (sterile conditions)

1. Remove the medium and add the cell suspension. Spike-specific IgG and RBD-specific IgG are set up and analyzed in the same well. Samples for total IgG should be set up in separate wells. For detection of Spike-specific IgG spots and RBD-specific IgG spots add 100,000-500,000 cells/well and for total IgG spots 25,000-50,000 cells/well. Set up samples in triplicates or duplicates.

2. Incubate the cells in the plate at 37 °C in a humidified incubator with 5% CO_2 for 18-24 hours. Do not move the plate during incubation and avoid evaporation, for example by wrapping the plate in aluminum foil.

C. Detection

1. Remove the cells by emptying the plate and then wash the plate five times with PBS (200 µl/well).

2. **Spike-specific IgG spots and RBD-specific IgG spots:** Prepare Spike-GAL and RBD-WASP by adding 500 µl standard reconstitution buffer to the lyophilized Spike-GAL and 500 µl standard reconstitution buffer to the lyophilized RBD-WASP. Allow it to dissolve for 5 minutes and mix thoroughly. Dilute the Spike-GAL and RBD-WASP solutions 1:25 in the same tube, e.g. by adding 500 µl Spike-GAL and 500 µl RBD-WASP to 11.5 ml PBS containing 0.1% BSA (PBS-0.1% BSA). Add the dilution to the plate, 100 µl/well. **Total IgG spots:** Add 100 µl/well of PBS-0.1% BSA.
   Incubate for 2 hours at room temperature.

3. Wash the plate five times with PBS (200 µl/well).

4. **Spike-specific IgG spotsand RBD-specific IgG spots:** Dilute the anti-GAL-490 (1:200) and anti-WASP-640 (1:200) in the same tube in PBS-0.1% BSA and add 100 µl/well.
   Total IgG spots: Dilute MT78/145-550 (1:500) in PBS-0.1% BSA and add 100 µl/well.
   Incubate for 1 hour at room temperature.

5. Wash the plate five times with PBS (200 µl/well).

6. Empty the plate, add Fluorescence enhancer (50 µl/well), and leave for 5-15 minutes at room temperature.

7. Empty by flicking the plate to remove the Fluorescence enhancer. Do not wash. It is important that the plate is not tapped against paper towels since dust particles may interfere with the assay.

8. Remove the underdrain (the soft plastic drain under the plate), for example using pliers. Dry the plate protected from light. The plate should be completely dry before analysis. Store plate in the dark at room temperature.

9. Spot analysis is performed with an automated FluoroSpot reader equipped with filters for the fluorophores used. Filters should have high specificity to avoid bleed-through artifacts.

Analysis of	Required filter
Spike-specific IgG spots	Excitation 490 nm/emission 510 nm (FITC)
RBD-specific IgG spots	Excitation 640 nm/emission 660 nm (Cy5)
Total IgG spots	Excitation 550 nm/emission 570 nm (Cy3)

TIPS FOR A SUCCESSFUL ASSAY

Buffers
PBS for washing and dilution should be filtered (0.2 µm) to remove any particles. We do not recommend the inclusion of Tween or other detergents in the washing and incubation buffers.

Washing
Plates can be washed using a multi-channel micropipette. A regular plate washer can be used in washing steps when sterile conditions are not required, provided that the washing head is adapted to ELISpot/FluoroSpot plates.

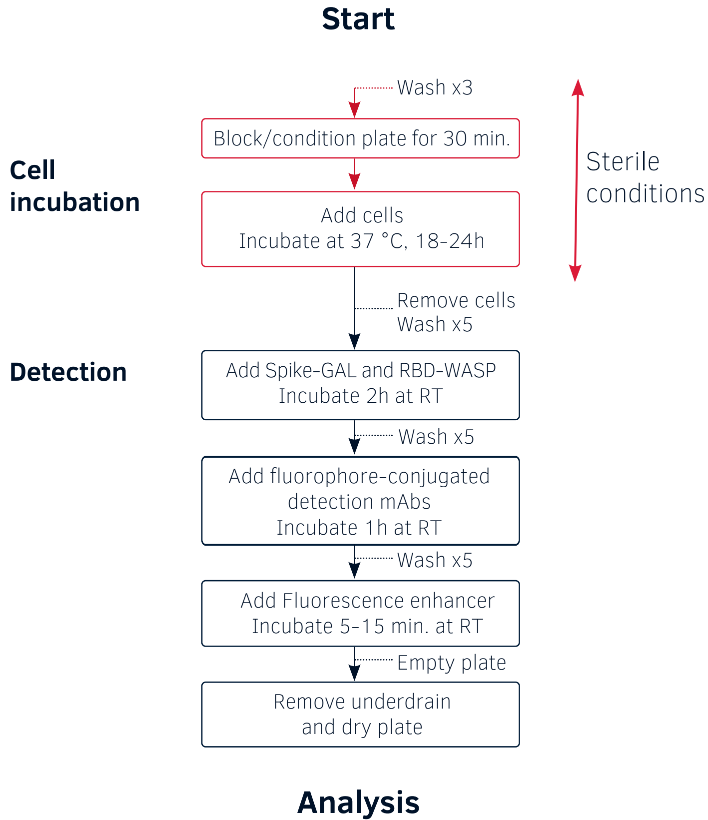
Cells
Freshly prepared and cryopreserved cells may be used. Let the latter rest for at least one hour at 37 °C to allow removal of cell debris.

Assay controls
Preferably include sample collected pre-SARS-CoV-2 infection. Enumeration of all cells secreting IgG (total IgG) can be included as a control of assay functionality.

Serum
The serum should be selected to support cell culture and give low background staining. We recommend the use of fetal calf serum. Alternatively serum-free medium evaluated for cell culture can be used. Human serum should not be used as it contains intrinsic IgG which will interfere with the assay.

Analysis
An automated FluoroSpot reader with separate filters is required for analysis. The reader should be equipped with filters for the fluorophores used. Filters should be selective for the specific wavelengths to avoid bleed-through artifacts. Spots from cells secreting multiple analytes are identified by coordinates, i.e. based on spot position. In order to obtain accurate measurements of cells secreting multiple analytes, the automated reader must be able to create an overlay analysis from the filters. To avoid fading, the plates should be kept dark and should preferably be analyzed within 1 week of development.

PROTOCOL SUMMARY



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