STAR Protocols

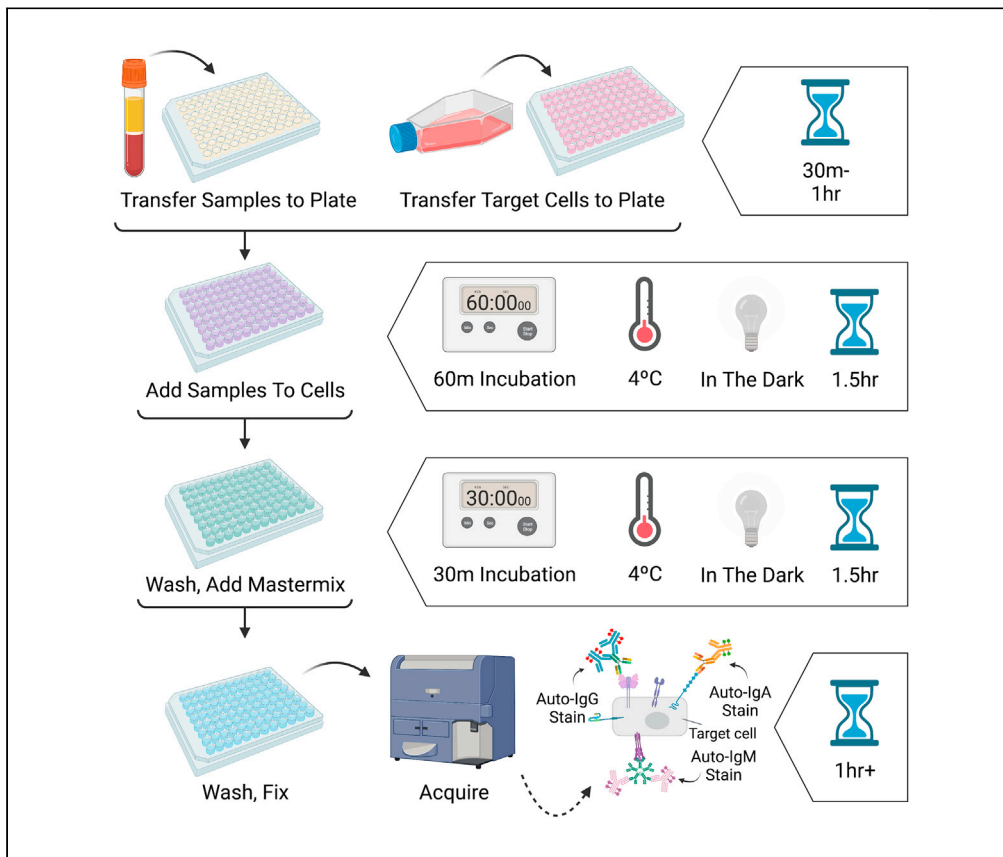**Protocol**

A flow-cytometry-based protocol using diverse cell types for detecting autoantibodies from human plasma and serum samples

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Highlights

A flow-cytometry-based protocol to detect the presence of autoantibodies

Uses input cells to screen plasma or serum samples and identify hits

Hits are samples reactive with cellular surface antigens, and may be studied further

Here, we describe a protocol for cell-based detection of autoantibodies from human plasma and serum samples using a standard flow cytometer. The protocol allows detection of autoantibodies against a wide array of extracellular antigens. Antigen coverage is limited to the cell types tested, and researchers will need to further determine if autoantibody-positive samples correlate with cytotoxic or clinical outcomes. This protocol is less expensive and faster to perform when compared to protein microarrays and requires no prior knowledge of potential targets.

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Protocol

A flow-cytometry-based protocol using diverse cell types for detecting autoantibodies from human plasma and serum samples

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SUMMARY

Here, we describe a protocol for cell-based detection of autoantibodies from human plasma and serum samples using a standard flow cytometer. The protocol allows detection of autoantibodies against a wide array of extracellular antigens. Antigen coverage is limited to the cell types tested, and researchers will need to further determine if autoantibody-positive samples correlate with cytotoxic or clinical outcomes. This protocol is less expensive and faster to perform when compared to protein microarrays and requires no prior knowledge of potential targets.

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

BEFORE YOU BEGIN

◉ Timing: 1 h

Studies relying on resources from human patients or volunteers must be performed with institutional permission and oversight. This study was approved by our Institutional Review Board.

Working quickly whilst ensuring cells and reagents are cold will minimize adhesion of cells to plasticware, minimize non-specific binding of autoantibodies to cells, and uphold high cell viability, which also ensures lower non-specific binding. It is therefore advised that reagents, commonly stored at ambient temperature, be adequately cooled, and the time spent handling samples at ambient temperature be kept to a minimum.

1. Pre-chill PBS to 4°C, and if taken out to the bench, keep small aliquots on ice.
2. Retrieve biological samples, typically plasma or serum, and cool to 4°C if fresh, or thaw on ice or at 4°C if previously frozen.
3. Transfer up to 55 μL of cooled plasma or serum into a 96-well U-bottom plate; 50 μL will be eventually transferred onto cells, and the 5 μL overage provides greater assurance of method uniformity. This is the sample plate, and after it has been prepared, the plate should be kept cold, either by placement on ice, or movement to a refrigerator. Make detailed notes of sample positions; these samples will be moved onto cells plated out in corresponding wells of a separate plate.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Human anti-CD98 (20 μg/mL for 5×10^4 cells)	Absolute Antibody	Ab00361–10.0
Goat anti-Human IgG DyLight 650 (6.25 μg/mL for 5×10^4 cells)	ThermoFisher	SA5-10137; RRID:AB_2556717
Goat anti-Human IgA FITC (1:1000 dilution for 5×10^4 cells)	ThermoFisher	A18788; RRID:AB_2535565
Mouse anti-Human IgM BV650 (1:40 dilution for 5×10^4 cells)	BioLegend	314526; RRID:AB_2563836
Critical commercial assays		
Live/Dead Aqua	ThermoFisher	L34957
Anti-murine IgG Kappa Beads for compensation	BD Biosciences	552843
ArC Amine-reactive beads for compensation	ThermoFisher	A10346
Biological samples		
Human plasma and serum samples (age range 24–92 yrs; 52% male)	Wong et al. (2021)	https://doi.org/10.1016/j.xcrm.2021.100321
Experimental models: Cell lines		
HULEC-5a	ATCC	CRL-3244; RRID:CVCL_0A11
Chemicals, peptides, and recombinant proteins		
TrypLE Express	ThermoFisher	12604021
PBS 1×	Lonza	7–516F
MCDB 131 medium	ThermoFisher	10372019
Epidermal Growth Factor	Sigma Aldrich	E5036-500UG
Hydrocortisone	StemCell Technologies	07925
L-glutamine	Sigma Aldrich	G7513-100ML
Fetal Calf Serum	ThermoFisher	10099
Paraformaldehyde (PFA)	Electron Microscopy Sciences	15710
Deposited data		
Source data	Wong et al. (2021)	https://data.mendeley.com/datasets/k33vwbdfsn/3
Other		
96-well U bottom plate	Corning	3799
T175 Flask	Corning	431306
50 mL Tube	Corning	352070
70 μm Cell Strainer	Corning	352350
Flow Cytometer	BD Biosciences	LSRFortessa

MATERIALS AND EQUIPMENT

Cell culture medium for HULEC-5a cells		
Reagent	Final concentration	Amount
MCDB 131 medium	1×	419.8 mL
Epidermal Growth Factor	10 ng/mL	5208 μL
Hydrocortisone	1 μg/mL	27.6 μL
L-glutamine	10 mM	25 mL
Fetal Calf Serum	10% (v/v)	50 mL
Store at 4°C for up to a month.		

STEP-BY-STEP METHOD DETAILS

This protocol refers specifically to analyses utilizing an adherent cell line of pulmonary origin. However, it is recommended to incorporate multiple cell types into analyses as this will broaden the scope of cell-surface markers that can be candidate targets for autoantibodies. If cells are purchased

from a commercial supplier, always refer to suppliers' recommendations for optimal culturing practices, and ensure cells are cultured at sub-confluent densities. It may also be prudent to determine if the cell lines have Fc receptors. Cells lacking Fc receptors would be more reflective of the potential targets of circulating autoantibodies and thus are representative of targets which are vulnerable to the cytotoxic effects of autoantibodies binding, such as complement-dependent cytotoxicity.

Preparation of samples

⏱ Timing: 1.5 h

1. Prepare cells for staining.
   a. Aspirate expended media from culture vessels.
   b. Wash cells once with ambient temperature PBS. Swirl flask carefully whilst ensuring the cellular monolayer has made thorough contact with the PBS. Aspirate the PBS.
   c. Dissociate subconfluent (<85%) target cells from T-175 culture flasks by adding 5 mL undiluted TrypLE Express reagent (Thermo Fisher Scientific, Catalog number 12604021) or an alternate dissociating reagent (like trypsin) following the manufacturer's instructions. Transfer flasks to 37°C incubator. Check flasks periodically to ensure cells have been dissociated. For a majority of cells this should occur well within five minutes; however, it is possible to incubate cells with TrypLE Express reagent for up to 20 min if they are refractory to detachment. However, prolonged exposure to TrypLE Express reagent may result in loss of certain surface antigens.
   d. Collect cells with 40 mL cold PBS and run through a 70 µm cell strainer into a new sterile 50 mL tube. Fill tube containing cells with cold PBS. Spin cells down at 500 × g for 3 min.

   △ CRITICAL: Human plasma that contacts cell culture media may clot, and this will prevent recovery of the sample for analysis. It is therefore important to wash cells with ample PBS.

   e. Resuspend cells at a concentration of 5.0×10^5 cells/mL in cold PBS and dispense 100 µL into a well of a U-bottom 96 well plate (5.0×10^4 cells/well). Dispense as many wells as there are biological samples, and an additional 9 wells for in-experimental controls. As a guide, refer to Figure 1 for an example of a recommended plate format. Work with plates on a bed of ice to prevent cellular adhesion and upkeep of high cell viability.

   △ CRITICAL: Ascertain cell viability; this can be made through automated counting methods or manually through trypan blue exclusion. For these experiments to be informative, cell viabilities must be greater than 90%. Dying cells are more likely to nonspecifically bind antibodies, including secondary antibodies, leading to false positive results. If cell viabilities are lower than 90%, return cells to culture following distributors' guidelines, and reattempt experiments once cells have recovered.

2. Transfer up to 50 µL of biological sample, undiluted, from the previously prepared sample plate to corresponding wells containing the target cells.
3. To one of the control wells (i.e., the IgG positive control), add 48 µL PBS and 2 µL anti-CD98 antibody (20 µg/mL final concentration. Human anti-CD98 IgG; Absolute Antibody Clone CP1-5, Catalog number: Ab00361–10.0). CD98, an amino acid transport protein, is widely expressed on the surfaces of many mammalian cell types.

   Note: Ensure that the final volume per well is 150 µl. Alternate reagents may be used as the positive control in lieu of the human anti-CD98 antibody; however, these alternative reagents will need to be tested for binding to target cell types, and for their subsequent reactivity with the anti-human IgG secondary antibody.
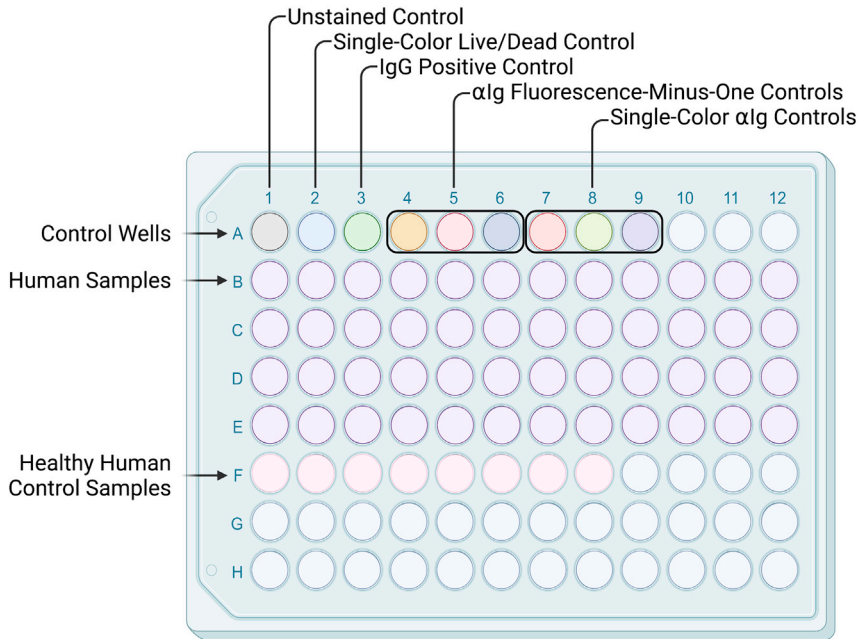
Figure 1. Sample plating layout
To aid in the differentiation of control reactions from sample reactions, designate a row within a plate to controls (top row), followed by samples. Cells within the control wells will not be used to screen biological samples. The IgG Positive control will receive both the anti-CD98 antibody, as well as the staining cocktail. Single color controls, in concert with the unstained control will provide preliminary guidance as to where to draw positive-event gates. The placements of these gates are later refined by the boundaries set by the fluorescence-minus-one controls. We separated healthy human control samples from biological human samples of interest. These healthy human controls can further refine the lower limits of positive-event gates, as it is hypothesized that healthy human controls lack or have minimal circulating autoantibodies.

> *Note:* At least six samples from healthy individuals should be included to serve as healthy controls, however the utilization of as many samples as possible is recommended.

4. Mix samples by gentle pipetting, then transfer plates to 4°C for one hour.

Staining cells

⏱ Timing: 1.5 h

5. Whilst plates are incubating at 4°C, prepare a cell staining mastermix using PBS as a diluent; this is to increase the signal to noise of the viability stain. It is advised to prepare a 10% overage to account for pipetting errors.

Mastermix for counterstaining cells (volumes per reaction)		
Reagent	Final concentration	Amount
Goat anti-Human IgG DyLight 650	6.25 μg/mL for 5×10^4 cells	2 μL
Goat anti-Human IgA FITC	1:1000 dilution for 5×10^4 cells	0.1 μL
Mouse anti-Human IgM BV650	1:40 dilution for 5×10^4 cells	2.5 μL
Live/Dead Aqua	1:400 (reconstitute reagent initially with 12.5 μL DMSO)	0.25 μL
PBS	1×	95.15 μL

Note: We used most stains at amounts that were below the manufacturer's per-reaction recommendation (generally 0.5× the recommended volume) given that we were staining 5×10^4 cells.

Note: Where applicable, include stains for other cellular markers as a means of providing assurances of cellular phenotype and/or cell health following culture. As a starting point, the minimal panel includes a viability dye, a stain for IgG, a stain for IgM, and a stain for IgA.

⚠ CRITICAL: As controls, ensure the inclusion of single-color stains and fluorescence-minus-one reactions. These control stains are performed in the absence of patient samples, and are used to inform where gates should be placed for positively-stained cellular populations during analyses. Single-color stain controls should also be prepared to aid in the placement of gates during data analysis and to control for non-specific binding of anti-Ig secondary antibodies to target cells. To this end, add only secondary anti-Ig antibody stains to cells (e.g., to create the single-color stain control for add only anti-IgG to cells that have not been exposed to patient samples). For fluorescence-minus-one control stains, add two anti-Ig antibodies, but omit the third (e.g., to create the anti-IgM fluorescence-minus-one reaction, omit the secondary anti-IgM antibody, but include the secondary anti-IgG and anti-IgA antibodies). Furthermore, these stains will provide assurances that cells tested lack Fc receptor expression.

6. Retrieve plates containing cells from 4°C. Top each well with 100 μL of cold PBS and spin down at 500 × *g* for 3 min.
7. Aspirate supernatants. Wash cells once with 200 μL of cold PBS and repeat spin.
8. Aspirate supernatants and add mastermixes to all wells except for wells designated for no-stain, single-color and fluorescence-minus-one reactions. Mastermix and other well volumes should be maintained to 100 μL. Gently mix by pipetting. Return plates to 4°C, in the dark, for 30 min.
9. Whilst cells are undergoing staining, prepare a 1% (v/v) PFA solution by diluting PFA from stock, commonly available in 16% or 32% (v/v) stocks, in PBS, and store on ice.
10. Retrieve cells from 4°C and top up with 100 μL of cold PBS. Spin plates at 500 × *g* for 3 min.
11. Wash cells two more times in cold PBS, centrifuging cells at 500 × *g* for 3 min between each wash.
12. Aspirate PBS and fix cells with 100 μL 1% PFA. Resuspend cells gently by pipetting. Plates should be immediately analyzed on a flow cytometer, however we have not observed decreases in fluorescence from plate stored for up to three hours at 4°C prior to acquisition. Record as many events as possible. Ensure height and if present, width parameters on side and forward scatter are recorded, as this would enable identification and exclusion of doublet events during analysis.

⚠ CRITICAL: Polychromatic flow cytometry often requires the creation of a compensation matrix to correct spectral overflow from partially overlapping fluorophores. Selecting fluorophores that minimally fluoresce at wavelengths of light as others within the same antibody panel will aid in limiting the effects of spectral overlap and improve resolution. Refer to the manufacturer of the flow cytometer to determine the correct steps in performing compensation. To perform compensation, we utilized non-fluorescent beads (BD Comp-Beads Anti-Mouse Ig, BD Biosciences, catalog number 552843 for antibody reagents; and ArC Amine reactive compensation beads, Thermo Fisher Scientific, catalog number A10346 for viability dye reagents). As some of the antibody reagents used in this panel were not of murine origin, they were incompatible with the bead set. Thus, these reagents were substituted with murine isotype control antibodies (from the same vendor) conjugated to the same fluorophore.

13. For acquisitions, ensure a main cellular population (identified in Forward Vs. Side scatter) can be determined. Draw Forward scatter thresholds to eliminate the recording of background 'noise'
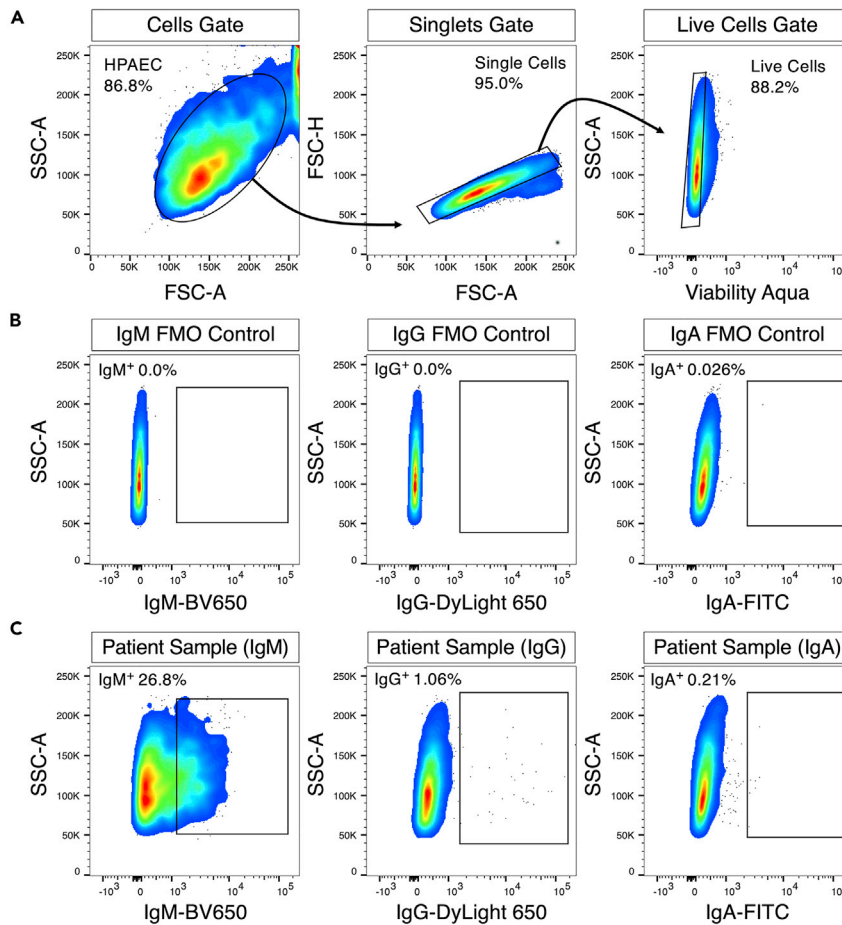
Figure 2. Example staining of human primary airway cells
In (A), the gating strategy allows for inclusion of single viable cells. Fluorescence-minus-one controls are shown in (B). Cells positive for IgM, IgG or IgA antibodies are demonstrated in (C).

events whilst leaving the main cellular population untouched. Draw a gate capturing the main cellular population and utilize the newly drawn gate as a stopping gate. Record at least 5000 events, or until IgM, IgA and/or IgG signals have stabilized, whichever is higher.

14. For analysis, first identify the cellular population in the Forward Vs. Side scatter area plot (Figure 2A; left panel).

15. Next, draw a gate in the Forward scatter (Area) Vs. Forward scatter (Height) plot and draw a secondary gate around single events by identifying those with proportional Area and Height signals (Figure 2A; middle panel).

16. Following the previous step, select live cells based on the viability dye staining (Figure 2B, right panel).

17. Lastly, determine limits for events positive for Ig staining by first using single-color controls to define negative populations (Figure 2B), and refine these gates based upon the fluorescence-minus-one controls. Next, apply these gating strategies to samples (Figure 2C).

EXPECTED OUTCOMES

It should not be anticipated that a sample results in 100% of cells being stained, as there is a limited amount of autoantibodies in each sample. In contrast, cells stained with the anti-CD98 control antibody can be gated so that close to 100% of events are deemed positive. Figure 2 depicts a stain of human primary airway cells. The gating strategy is shown in a. Fluorescence-minus-one controls are

shown in **b**. In **c**, examples of patient plasma screened on cells are shown, alongside gate placements that were informed by single-color negative, fluorescence-minus-one controls and gating outside healthy human controls; these gates were retroactively applied to **b**.

In this example, the percentage of cells positively stained for IgM can be considered to be high (26.8%), and thus the sample was indeed of clinical interest. It should be expected that samples that contain autoantibodies stain will variably target cells. In challenging cell types, especially those with low copy numbers of autoantibody targets on the cell surface, it may be preferred to analyze data through changes in median fluorescence intensity rather than pursue gating analyses. Conversely, a researcher may find that a small subset of samples obtained from patients do highly react with the tested cell types. Ultimately this assay functions as a screen, and highly reactive samples simply indicate that those samples should be considered for further characterizations, such as a protein target microarray, or functional assays such as a complement fixation assay.

LIMITATIONS

This assay determines the presence of auto-reactive antibodies that can target cell surface markers. Furthermore, in the absence of formally determining the specific cell surface marker population of target cells, this assay is semi-quantitative. Samples with high reactivities with target cells could indicate high autoantibody titer, and/or diverse autoantibody species. An alternative explanation is that these samples led to false positive results. Whilst a key strength of this assay is its enablement of rapid screens for potential autoantibody reactivity with cells that are of strong interest to the researcher, orthogonal assays are highly recommended to confirm the presence of autoantibodies.

On the other hand, it is also possible to discover false negative results, for which we have hypothesized two main reasons. Firstly, markers that are low or not expressed on the cell type of choice may not be sufficiently stained and thus lead to false negative results. Secondly, limited autoantibodies numbers in small sample volume sizes may again insufficiently stain cells and also lead to false negative results. To counter these limitations, include as many relevant and complementary cell types as possible, and consider increasing the input sample volume, where possible. A third potential reason is that the dissociation reagent, TrypLE Express, is digesting potential ligands. To address this latter limitation, consider the utility of cells grown in suspension, and/or an orthogonal cell disassociation protocol (though, non-enzymatic disassociation reagents may lower cell viability).

Additionally, the detection of autoantibodies may not necessarily indicate pathology. This will need to be determined in secondary assays to examine whether autoantibody binding has effects such as complement fixation or inhibition of key cell surface receptors and subsequent disruption of cell signaling pathways.

TROUBLESHOOTING
Problem 1
Coagulated cells-sample mixtures (steps 2–6).

Potential solution
Coagulation may occur if cell media remains with cell samples and was insufficiently washed out with PBS. Perform a second cell washing step using PBS.

Alternatively, specific cell types may cause plasma to clot (Tormoen et al., 2012). It would be advised to either dilute the biological sample 1:2 or 1:3 in PBS, and reattempt stain.

Problem 2
Low number of recorded events (steps 11–13).

Potential solution

Some sample loss is to be expected. However, if too few events are recorded, this can lower confidence that autoantibody-bound cells are present. If sample wells are depleted following acquisition. increase the number of input cells by 50%, and further to 100% if low sample collection numbers persist. However, researchers should scale the amount of input biological sample accordingly. The secondary stains do not need adjustment; used at the concentrations listed, the stains would still be supplied in excess. Researchers may opt for the use of v-bottom plates to assist with retention of cells withing plates; however, if working with v-bottom plates, ensure thorough resuspension of cell pellets in buffers or stain mixtures after centrifugation. If no cells are observed during acquisitions, increase centrifugation times to 5 min, and forgo the use of vacuum-assisted aspirators; sample loss is likely due to suboptimal cell pelleting and/or aspiration. Double check that stopping gates have been drawn across dominant cell populations.

Problem 3

Low anti-CD98 signal in positive IgG control (step 17).

Potential solution

Increase anti-CD98 antibody to 5 μL, and/or ensure secondary anti-IgG antibody has not quenched fluorescence.

Problem 4

Observed populations or plots are shown occupying a 'negative' signal (step 16 and 17).

Potential solution

Double check reagents used to generate compensation matrices. If the antibody used in staining is not applied towards determining the compensation parameters (e.g., the antibody is incompatible with the antibody capture beads), replace the substitute fluorescent antibody with another from a different manufacturing lot or is against another target.

Problem 5

No observation of populations positively counterstained for IgA, IgG or IgM (step 17).

Potential solution

First ensure a signal for IgG (through the anti-CD98-treated positive control) is observed; the absence of any signal within this population should indicate an omission of fluorescent secondary antibodies in the staining mastermix. Not all patients will generate autoantibody responses. Test samples across additional cell types (especially from different tissues) to determine if non-reactivity is simply due to lack of binding partners. Revisit cell dissociation step. Whilst TrypLE Express is a mild dissociation reagent, prolonged exposure may lead to digestion of antibody targets. Consider decreasing TrypLE Express contact time, and alternative enzymatic (e.g., Accutase) and non-enzymatic dissociation reagents (EDTA).

Problem 6

Stain is present in healthy human samples, or all cell samples show high levels of staining (step 17).

Potential solution

Autoantibodies may be present in samples obtained from humans who are healthy or otherwise. The healthy human control samples are used predominately to set gates, but if high anti-Ig staining is still observed but at fluorescence intensities relatively close to the negative control (<10-fold brighter), it may be better to set gates more conservatively to capture the brightest events within test samples.

If all cell samples are positively stained, the cells may be expressing Fc receptors that are non-specifically binding the fluorescent antibodies. Use a Fc blocking reagent (or equivalent). If there is high

IgA reactivity that closely matches that of IgG or IgM, the secondary polyclonal antibody may be directly binding to IgG/IgM. In this scenario, consider requesting a separate reagent lot number from the manufacturer. Lastly, high levels of staining occurring from healthy samples may simply indicate either antibody cross-reactivity (stemming from prior exposure to a disease or as an effect of vaccination) or that those circulating autoantibodies are non-pathological. In such scenarios, the cell selection would be deemed unfit-for-purpose, and an alternative cell type/line should be sourced.

RESOURCE AVAILABILITY

Lead contact
Further information and requests should be directed to the lead contact, Cheryl Maier, at cheryl.maier@emory.edu.

Materials availability
Materials used in this study are commercially available.

Data and code availability
Source data in the paper is available in Wong et al. (2021) and at https://data.mendeley.com/datasets/k33vwbdfsn/3.

AUTHOR CONTRIBUTIONS

C.L.M. and G.S. conceived the study. A.K.H.W. designed the experiments and performed experiments. A.K.H.W. and C.L.M. wrote the original manuscript. D.A.K. provided technical guidance. All authors participated in the interpretation of study results and in the reporting of the data.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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