Qualitative immunoassay for the detection of anti-SARS-COV-2 spike antibody in human milk samples

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Protocol
Qualitative immunoassay for the detection of anti-SARS-COV-2 spike antibody in human milk samples

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SUMMARY
Antibodies in milk obtained from those previously SARS-CoV-2-infected or vaccinated against COVID-19 may provide passive immunity to the breastfed infant. Few assays have been established to measure antibodies in human milk, despite the public health importance of this topic. In the present protocol, we describe an optimized indirect ELISA assay aimed to measure SARS-CoV-2-reactive antibodies in human milk, which can be used as a rapid screen on undiluted samples or to designate samples as relatively low, moderate, or high titer.

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

BEFORE YOU BEGIN
Human milk samples used for this assay were collected from fully consented individuals under a protocol approved by the Institutional Review Board (IRB) at Mount Sinai Hospital (IRB 19-01243). Use of this protocol may require specific institutional approval.

1. Produce or purchase SARS-CoV-2 Spike antigen. We recommend recombinant production following the published protocol (Stadlbauer et al., 2020); however, any commercially-available SARS-CoV-2 Spike antigen (including S1, Receptor Binding Domain (RBD)) can be used, as long as positive cutoffs are established using negative controls. Even other SARS-CoV-2 immunogens such as Nucleocapsid can be used.
2. Collect human milk at least 7 days after symptom onset or positive COVID-19 diagnostic test, or 14 days after the first dose of a COVID-19 vaccine (note these are minimal intervals based on Pace et al., 2021).

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-human IgA-HRP (Ω1.6000) | EMD Millipore | Cat#401135 |
| Anti-human IgG-HRP (Ω1.2000) | Southern Biotech | Cat#2040-05 |
| Anti-human SC-HRP (Ω1.2000) | Nordic MuBio | Cat#GAHu/SC/PO |
| Anti-human IgM-HRP (Ω1.2000) | Southern Biotech | Cat#2020-05 |
| Biological samples  |        |            |
| Human milk samples  | Study participants | N/A |

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### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE CHARACTERISTIC | SOURCE | IDENTIFIER |
|-----------------------------------|--------|------------|
| Chemicals, peptides, and recombinant proteins | | |
| Recombinant SARS-CoV-2 Spike protein produced in house (full-length trimer, S1, or Receptor Binding Domain (RBD)) | N/A | N/A |
| **Other** | | |
| Clear, polystyrene, flat-bottom half-area plates | Corning | Cat#3690 |
| Goat serum | MP Biomedicals | Cat#191356 |
| Milk powder | Fisher Scientific | Cat#A614-1000 |
| Goat serum | MP Biomedicals | Cat#191356 |
| Bovine Serum Albumin | Rockland | Cat#BSA-30-0050 |
| Flat bottom half-area 96-well polypropylene plates | Costar | Cat#3879 |
| 1-Step Ultra TMB substrate | Thermo Fisher Scientific | Cat#34028 |
| 1N HCl | Fisher Scientific | Cat#SA48-4 |
| Plate sealers | Thermo Fisher Scientific | Cat#3501 |
| 50 mL conical tubes | Thermo Fisher Scientific | Cat#339653 |
| Tween-20 | Gibco | Cat#10010-023 |
| Reservoirs | Costar | Cat#4870 |
| Pipettes – Eppendorf Research Plus (P2.5, P P20, P100, P200, P1000) | Eppendorf | Various |
| Electronic multichannel pipettes – Sartorius Picus NxT (P300, P1200) | Sartorius | Various |
| Pipet tips – Eppendorf epT.I.P.S, Sartorius Safety Space filter tips –20°C or –80°C Freezer | Eppendorf, Sartorius | Various |
| Centrifuge | Thermo Fisher Scientific | Sorvall Legend XFR |
| Plate washer | Tecan | HydroSpeed |
| Plate reader | BioTek | Powerwave HT |
| **Software and algorithms** | | |
| Plate reader software | BioTek | GenS 3.08 |
| Spreadsheet software | Microsoft Office | Excel |
| Data analysis software | GraphPad | Prism 9.2.0 |

### MATERIALS AND EQUIPMENT

**Wash Buffer: 1× PBS/0.1% Tween-20**

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| 1× PBS pH 7.4 | 99.9% | 3,996 mL |
| Tween-20 | 0.1% | 4 mL |
| **Total** | 100% | 4,000 mL |

Storage: 4°C up to one week

**Block Buffer: 0.1% PBS-T/3% goat serum/0.5% milk powder**

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| 1× PBS pH 7.4 | 96.4% | 96.4 mL |
| Tween-20 | 0.1% | 0.1 mL |
| Goat serum | 3% | 3 mL |
| Milk powder | 0.5% | 0.5 g |
| **Total** | 100% | 100 mL |

Storage: 4°C up to one day
CRITICAL: Hydrochloric acid is toxic if inhaled and should be handled under a fume hood. Handler should wear appropriate PPE to cover skin and eyes.

STEP-BY-STEP METHOD DETAILS

Day 1: prepare experimental plates

⊙ Timing: 30 min preparation, 12 h incubation

The experimental plates can be stored at −80°C for up to a week.

1. Coat plates with 50 μL of SARS-CoV-2 Spike protein at 0.5 ug/mL diluted in 1× PBS (pH 7.4).
   a. Cover plates using plate sealers.

Day 2: ELISA experiment

⊙ Timing: 6 h

For this step, an indirect ELISA is performed - the proteins reactive to SARS-CoV-2 Spike in human milk would bind to SARS-CoV-2 Spike coating, then the HRP-conjugated secondary antibody (Anti-IgG/IgA/IgM/SC) is added to target the human proteins, TMB and HCl (1N) is added for a colorimetric reaction that can be read on the plate reader.

2. Retrieve and thaw human milk samples from freezer.
   a. Fresh human milk samples can also be used in this protocol and all samples would be processed the same prior to storage as described in step 2 below.
   b. When choosing negative control samples, we recommend including at least 10 prepandemic (pre-Dec 2019) milk samples to determine cutoff values. If these samples are not available, samples obtained after Dec 2019 from participants with no history of confirmed or suspected infection or known exposure to close contacts, though in this case more control samples (20–30) may be needed in order to remove significant outliers from the control dataset as these may be due to unknown previous SARS-CoV-2 infection.
      i. Pre-pandemic control samples may be pooled to be used as a negative control in all experiments.
      ii. Similarly, positive samples can be pooled as a positive control to be used in each assay.
      iii. Screened controls should be included on every plate of each subsequent experiment to reduce plate-to-plate variation.
   3. Centrifuge thawed samples in 50 mL conical tubes at 805×g for 10 min at 20°C–25°C.
   4. Remove any remaining fat from the top of each sample.
      a. Remove fat by scooping with a sterile spatula or by pouring off fat layer.
      b. Samples can be frozen or used immediately.
   5. Prepare 1× PBS/0.1% Tween-20 wash buffer.
      a. We recommend preparing at least 4 liters wash buffer in a bottle that can be connected to the Tecan HydroSpeed plate washer, or similar to uniformly wash all wells of each plate.

| Reagent          | Final concentration | Amount  |
|------------------|---------------------|---------|
| BSA 30%          | 1%                  | 3.3 mL  |
| 1× PBS pH 7.4 99%| 99%                 | 96.7 mL |
| Total            | 100%                | 100 mL  |

Storage: 4°C up to one week
6. Prepare blocking buffer using 0.1% PBS-T/3% goat serum/0.5% milk powder.
7. Wash plates using the Tecan HydroSpeed plate washer using prepared wash buffer.
   a. Set up a program with 3 rounds of washing/aspiration filling to max capacity of your plate.
   b. Note, plate washing can be performed manually while ensuring proper aspiration of all liquid
      before moving forward.
8. Add 50 µL blocking buffer to each well of your washed experimental plates.
9. Incubate experimental plates for 1 h at room temperature.
10. During the one hour incubation, set up 4-fold titrations in separate round-bottom polypro-
    pylene dilution plates with samples and any relevant controls (Figure 1) at set concentrations
    in 1% BSA diluted in 1 x PBS, pH 7.4.
    a. Start titrations with undiluted processed milk to maximize sensitivity.
    b. Include pre-pandemic/naïve and positive controls in the assay.
    c. We recommend leaving a few wells without any samples and a few wells without any second-
        ary antibody to ensure reagents are performing as expected.
11. After incubating for 1 h in blocking buffer, wash plates as described in step 6.
12. Transfer 50 µL of samples prepared in dilution plates to washed experimental plates.
13. Incubate covered experimental plates containing relevant samples for 2 h at room temperature.
14. Prepare goat anti-human secondary antibodies in 1 x PBS, pH 7.4
    a. We recommend using the following ratios of antibody to 1 x PBS if using the ones specified
       above:
       i. Goat anti-human IgA 1:6000
       ii. Goat anti-human IgG 1:2000
       iii. Goat anti-human IgM 1:2000
       iv. Goat anti-human secretory component (SC) 1:2000
15. After the experimental plates complete the 2 h incubation with samples, wash as described in
    step 6 using the Tecan HydroSpeed plate washer.
16. Add 50 µL prepared secondary antibodies to relevant experimental plates.
    a. Note, one secondary is tested per set of samples. To test multiple secondary antibodies, set
       up relevant additional experimental plates.
17. Incubate covered experimental plates for 1 h at room temperature.
    a. During this incubation, it is recommended to remove the TMB Substrate from 4°C to accli-
       mate to room temperature.
18. After incubation with secondary antibodies, wash as described in step 6 using wash buffer.
19. Add 50 µL TMB Substrate to each well and allow plates to develop for 5–10 min, maintaining
    timing consistency with any future experiments.

![Sample plate layout](image-url)
a. If successful, you should see relevant saturation based on the dilutions set up to help determine the best amount of time needed to develop under your specific conditions for future experiments.

20. After the TMB Substrate has developed, add 25 μL 1N HCl to stop the reaction.

21. Promptly read endpoint data from experimental plates at an absorbance of 450 nm on BioTek Powerwave HT plate reader using BioTek Gen5 3.08 software, or similar.

   a. Plate readers with the same specifications could be used at user’s discretion.

22. Export data as an Excel file for further analysis.

**EXPECTED OUTCOMES**

Figure 2 illustrates an example of expected ELISA data using the described protocol. (A) shows the full titration against Spike; (B) gives an example of endpoint dilution titers.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Data analysis**

© Timing: 1 h

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**Figure 2. Sample of expected results for an anti-IgA Spike ELISA assay**

(A) Full titration against Spike. NEG (i.e., negative)/segmented lines: pre-pandemic controls. COV/solid lines: milk from COVID-19-recovered donors.

(B) Endpoint dilution titers. Experiments were performed in duplicate and repeated twice. Mean with SEM is shown. Dotted lines indicate positive cutoff value (mean OD or endpoint titer of negative control milk samples + 2*SD).
In this step, the data is analyzed using GraphPad Prism, and the calculation of endpoint titer is determined.

1. Copy plate reader data into a GraphPad Prism ‘XY table/graph’.
   a. Be sure to properly include replicate values in the XY table.
2. Label each sample data column used in the experiment.
3. Include the appropriate X values as increasing titer (=1/milk dilution).
4. Examine the XY graph produced by the software to ensure curves appear as expected and OD values decrease with increasing titer.
5. Determine the positive cutoff value.
   a. Using COVID-naïve control milk samples, ODs at the lowest titer tested (we recommend undiluted milk for this calculation, determine the mean and standard deviation values (can be done easily in excel).
      i. Positive cutoff should be set as two standard deviations above this mean, based on our published work (Fox et al., 2020).
6. In the XY graph, double-click the Y axis, and insert a dotted line at the positive cutoff value.
7. If determination of endpoint titer is desired:
   a. On the XY table in the first empty row after the final OD value, add your endpoint OD value.
      We recommend using an OD of 1.0 based on our published work (Fox et al., 2020).
      i. This represents the dilution of the highest analyte that provides a reading above the cutoff chosen at an absorbance best fit for these data.
   b. Using the ‘Analysis’ button, transform the XY table data as X=LogX.
   c. Using the ‘Analysis’ button on this transformed XY table, select non-linear regression and then 4-parameter (sigmoidal; X is log(concentration).
      i. Select ‘interpolate unknowns from standard curve’ at bottom of options window.
   d. In the nonlinear fit results sheet, select the ‘Interpolated X mean values’ tab.
   e. Using the ‘Analysis’ button, transform data as X=10^x to obtain final endpoint titer values.

LIMITATIONS
ELISA can be subject to changes in OD values due to temperature fluctuations, and it is best to perform this assay in a temperature-controlled environment. Positive and negative pooled control samples previously-aliquoted and stored as well as uncoated plate wells can serve as tools to ensure assay-to-assay consistency. It is important to develop all plates at the same temperature and to be stopped after the same amount of time each experiment for accurate comparison. This ELISA cannot determine if antibodies are neutralizing or capable of additional Fc-mediated function.

TROUBLESHOOTING
Problem 1
Control samples do not yield consistent OD values (ELISA Experiment step 7 Data Analysis).

Potential solution
Be sure to aliquot small volumes of control samples. Human milk samples should be stored at –20°C, or 4°C for less than 5 days. Ensure that all reagents are stored according to manufacturer recommendations. Temperature can affect OD readings and the lab should be kept at a consistent temperature.

Problem 2
Plates do not change color or are very light when TMB is added (ELISA Experiment step 22).

Potential solution
Typically this is due to accidentally omission of a step in the protocol, such as improper plate coating, failure to add secondary antibody, or incorrect calculation of the appropriate amount of stock antibody. This issue can also occur if TMB does not acclimate to room temperature before use.
Problem 3
Plate well color does not titrate and wells are similarly colored (ELISA Experiment step 22).

Potential solution
Typically this is due to use of excess secondary antibody or accidental coating of excessive Spike protein. Improper washing can also lead to this issue, or omission of the blocking step. Be sure to stop the TMB reaction using 1N HCl in a timely manner to avoid over-developing plates.

Problem 4
Plate well color and OD values exhibit high background (ELISA Experiment step 1/Data Analysis).

Potential solution
This can be resolved by ensuring proper washing procedure to remove any residual buffer remaining in wells by forcefully tapping to absorb. Store experiment plates in dark areas away from light exposure during incubations and limit light exposure throughout. Adhere to recommended incubation times to prevent high background.

Problem 5
Control samples do not yield consistent ODs over time (ELISA Experiment step 1/Data Analysis).

Potential solution
After long-term storage (>6 months) at –20°C, we have found that particularly the IgG in milk is not as reactive compared to when the milk was initially stored, thus yielding lower OD values. One solution is to keep milk at –80°C. Another solution is to replace controls every 3 months with a fresh pool of samples.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Rebecca Powell (Rebecca.Powell@mssm.edu).

Materials availability
No novel materials were generated for this study.

Data and code availability
No code was generated for this study. Sample data from this protocol can be shared upon email request.

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AUTHOR CONTRIBUTIONS

A.F. performed all the experiments described in the paper, assisted with data analysis, and drafted this manuscript. X.Y. assisted with drafting this manuscript and creating figures. R.P. conceived of the protocol and its optimization, oversaw data collection, performed data analysis and interpretation, and revised this manuscript.

DECLARATION OF INTERESTS

Mount Sinai has filed for patent protection for this assay.
REFERENCES

Fox, A., Marino, J., Amanat, F., Krammer, F., Hahn-Holbrook, J., Zolla-Pazner, S., and Powell, R.L. (2020). Robust and specific secretory IgA against SARS-CoV-2 detected in human milk. iScience, 101735. https://doi.org/10.1016/j.isci.2020.101735.

Pace, R.M., Williams, J.E., Jarvinen, K.M., Belfort, M.B., Pace, C.D.W., Lackey, K.A., Gogel, A.C., Nguyen-Contant, P., Kanagaiah, P., Fitzgerald, T., et al. (2021). Characterization of SARS-CoV-2 RNA, antibodies, and neutralizing capacity in milk produced by women with COVID-19. mBio 12. https://doi.org/10.1128/mBio.03192-20.

Stadlbauer, D., Amanat, F., Chromikova, V., Jiang, K., Strohmeier, S., Arunkumar, G.A., Tan, J., Bhavsar, D., Capuano, C., Kirkpatrick, E., et al. (2020). SARS-CoV-2 seroconversion in humans: a detailed protocol for a serological assay, antigen production, and test setup. Curr. Protoc. Microbiol. 57, e100. https://doi.org/10.1002/cpmc.100.