A serological assay is simple to assemble and qualify, given quality reagents, proper planning, and expectations. To assist labs that are in search of a rapid multiplex solution for detecting antibodies against SARS-CoV-2 in serum, this brief addendum to the xMAP® Cookbook can help address many of the questions that can arise when developing a serological assay.

1. Which antigen(s) do I want to use as the capture molecule(s)?

Some anecdotal feedback from developers indicates that using more than just one antigen in serological testing may be critical to achieving good sensitivity. These antigens (spike, nucleocapsid, membrane, envelope) are listed in order of most-to-least desirable as the capture molecule in the assay.

a. Spike proteins – monomer 140kD

Spike proteins are the most obvious antigen for antibody capture, as they are the predominant external protein of the virus. Further, spike proteins are one of the more variable proteins across the coronaviruses, improving the opportunity for assay specificity. There are several options for representative spike proteins to use in bead coupling:

i. Whole homo-trimer
   1. Likely to maintain correct conformation
   2. Full spectrum of available epitopes

ii. S1
   1. Full subunit elicited neutralizing antibodies in MERS
   2. N-Terminal Domain (NTD)
      a. Can elicit neutralizing antibodies
      b. Candidate for specific capture due to less conservation across CoVs
   3. C-Terminal Domain (CTD)/Receptor Binding Domain (RBD)
      a. Multiple neutralizing epitopes in SARS/MERS
      b. Is a primary target of subunit vaccines
      c. Thus will be relevant to many vaccine trials
      d. More conserved than S1

iii. S2/Fusion Protein (FP)
   1. Fusion protein with RBD elicits high Ab titer
b. Nucleocapsid – 50kD

As the most abundant protein in the virus, the nucleocapsid is a strong candidate as a capture antigen on the beads.

i. Most abundant overall
ii. Highly antigenic in SARS patients
iii. Highly conserved = risk for cross-reactivity
c. Membrane – 25kD

Less research is available using membrane protein in serological assays and as a vaccine candidate. Ensure you acquire high-quality, biologically relevant antigens from a reputable source.

i. Most abundant on SARS surface

ii. Immunization of SARS M protein elicited efficient neutralizing antibodies

iii. Highly conserved across species = risk for cross-reactivity

d. Envelope – 10kD

The envelope protein is quite small, and this can cause issues both for the immune response and for use in assay development. Special adaptations to the coupling procedure are recommended for a protein this size, and those may skew any relative detection aspect of a multiplex serological assay.

i. Too small to have significant immunogenicity

ii. Virulence factor that plays into cytokine storm

iii. Likely to add little value to assay

2. Where should I buy my antigens?

a. What is the assay purpose? Check any end-user restrictions or licensing requirements from the supplier.

b. How long will the assay be needed? Will the antigen supply remain available and of consistent quality from lot to lot?

c. Is the supplier reputable? Have I worked with them before or do I have peers who can speak to their quality?

d. Is the antigen biologically relevant? Is it the most representative version of the virus the body sees during infection (e.g., folded/glycosylated/know to be immunogenic)?

e. How is the antigen purified? Some tags, such as Fc tags, may cause non-specific binding with serum samples.

f. Is the antigen in an easy-to-work-with format and buffer system?

g. Are there any other labs who have used commercially available antigens and can share their experience?

3. How do I couple the antigens to the beads?

a. Check the molecular weight.

i. Greater than 15kD should be ok with traditional carbodiimide coupling, see xMAP Cookbook, Edition 4, chapter 4.2.1 for information.

ii. Smaller could require a modified coupling chemistry/linker, see xMAP Cookbook, Edition 4, chapter 4.4 for options and applications.

b. Check the sequence and 3D structure of the protein.

i. Reactive groups

   1. Most chemistries make use of reactive groups in the protein, attaching these at random to the bead surface.

   2. This can happen regardless of whether a reactive group is present within the epitope.

   3. If so, there can be reduced reactivity because some epitopes may be positioned toward the bead surface, making them unavailable for antibodies.

ii. Protein folding can also lead to reduced availability of reactive groups for coupling, reducing reactivity.

iii. Issues could be resolved with spacer or alternative chemistry, see xMAP Cookbook, Edition 4, page 45 for decision tree and below for general chemistry guidelines.

c. Choose your chemistry – see xMAP Cookbook, Edition 4, chapter 4 for protein conjugation chemistry.

i. Carbodiimide (covalent with MagPlex® beads) for larger proteins with limited conflicts in sequence/structure.

ii. Biotin-avidin (with MagPlex®-Avidin beads) for proteins of all sizes with biotin tags.

iii. ADH (modified chemistry) with MagPlex beads for smaller proteins, flipping chemistry to putting amine on beads and attaching to COOH groups on protein.

iv. MPBH (modified chemistry) with MagPlex beads for smaller proteins, using a maleimide group on beads and attaching to cysteine sulphydryls on protein.

d. Make sure antigen storage buffer is coupling-friendly.

i. See xxMAP Cookbook, Edition 4, Appendix C for buffer notes and buffer exchange options.
4. What other supplies will I need?
   a. General
      i. Select a different bead ID for each antigen you plan to use/screen.
      ii. Select a biotinylated or PE-labeled detection antibody that is against the species of serum you will be testing.
      iii. Consider isotypes and select an appropriate detection antibody.
         1. IgG will ultimately be the most abundant Ig in the sample and could interfere with detection of other isotypes.
         2. IgM will be detectable earlier than IgG, but early studies indicate that this is within a more narrow window than seen in similar infections.
         3. IgA relevance is still in debate.
         4. Total antigen-specific Ig can be evaluated by mixing detection antibodies against specific isotypes together into one detection cocktail.
      iv. Consider coupling chemistry - use of biotin coupling precludes use of SAPE for detection, thus detection antibodies must have direct phycoerythrin label.
      v. Qualified serum
         1. Tested via another serological method to confirm
         2. Paired with an RT-PCR positive swab test
         3. Multiple time points
         4. Specificity panels of sera with similar pathogens (other coronaviruses, other respiratory diseases)
      vi. Quantitative assays – “calibrator” material is difficult to come by or have confidence in, currently.
      vii. SAFETY – need an inactivation method for pre-treating samples. Generally, 1 hr at 56°C accepted.
   b. Coupling
      i. See Chapter 4.2.1 of the xMAP Cookbook for carbodiimide protein coupling and supplies needed.
      ii. See Chapter 4.4.1 of the xMAP Cookbook for biotin-based coupling and supplies needed.
      iii. See Chapter 4.4.2-3 of the xMAP Cookbook for ADH bead modification and coupling and supplies needed.
      iv. See Chapter 4.4.4-5 of the xMAP Cookbook for MPBH bead modification and coupling and supplies needed.
   c. Assays
      i. See Chapter 4.2.2 of the xMAP Cookbook for approximate supply list for antigen coupling confirmation.
         1. To perform coupling confirmation on antigen coupling, you must have an antibody specific for the antigen (i.e., if spike protein is coupled to the bead, you need an anti-spike antibody that is conjugated to biotin or PE).
         2. This antibody replaces the “PE or biotin-labeled anti-species detection antibody” from the xMAP Cookbook.
      ii. See Chapter 4.3.3 of the xMAP Cookbook for indirect immunoassay supplies needed.

5. How do I get started?
   a. Couple your antigens.
      i. Do a small-scale coupling of each antigen onto 1x10^6 beads.
         1. 5 µg/1x10^6 beads is our generic starting point.
         2. If using a molar coupling approach, use starting point of 100 pmol/1x10^6 beads.
      ii. Consider coupling a few concentrations of antigen in parallel to check if performance is enhanced by a certain protein amount per 1x10^6 beads.
         1. Titrate 2–4 points above and below the starting concentrations cited.
            a. For example: 0.2, 0.5, 1.5, 5, 15 µg/1x10^6 beads.
      iii. An Excel-based procedure guide and calculator is available from your Field Applications Scientist.
b. Confirm the coupling was successful.
   i. Be certain to include a blank in your plate setup to check for any reagent cross-reactivity to the beads.
   ii. This QC testing may not be feasible due to limited availability of antibodies against the SARS-CoV-2 antigens.
      1. The assay requires an antibody specific to the antigen coupled to the beads AND
      2. That antibody must be conjugated with biotin or PE
      OR
      3. You must also use a biotin or PE-conjugated secondary antibody that is specific to the species of the antibody
         in which #1 above is raised, in a second step of the confirmation assay.
   iii. If reagents are available, perform a coupling confirmation assay. See the protocol in the xMAP Cookbook
        Chapter 4.2.2 for reference.
        1. Use the antigen-specific antibody and dilute it as described for the “phycoerythrin-labeled anti-species IgG
           detection antibody” in the xMAP Cookbook.
        2. If the antibody has been conjugated with PE, follow the xMAP Cookbook procedure.
        3. If the antibody has been conjugated with biotin:
           a. After the washing step, add 100 µL of SAPE to each well and shake at 600-800 rpm for 30 minutes.
           b. Perform another wash, and then resuspend in 100 µL and read.
        4. If the antibody is unconjugated:
           a. After the washing step, add 100 µL of the biotin or PE-labeled secondary antibody at 6 µg/mL and
              shake at 600-800 rpm for 30 minutes.
           b. Proceed through the remainder of the assay following either the PE-conjugated or biotin-conjugated
              workflows from above steps 2 or 3.
   iv. If the blank MFIs are low (under 100) and there is a titration curve showing a dose response to dilution of the antibody
       (decreasing MFI proportional to the antibody concentration), then proceed to testing and optimization.

c. Perform the assay with diluted “normal” serum.
   i. Multiplex the beads for this assay.
   ii. Follow xMAP Cookbook Chapter 4.3.3 for general starting-point recommendations for serological assays.
   iii. Try serum diluted 1:100, 1:250, 1:500, and 1:1000 for first assay, as well as just assay buffer as control wells.
   iv. Be especially diligent about thorough washing to help drive down background signal.
      1. Use an automated washer if available. Protocols for some washers are available from your
         Field Applications Scientist.
      2. Otherwise, you can use the inversion method rather than manually pipet to remove liquid from the wells.
      3. For improved washing, shake the plate at 600–800 rpm for 2 minutes with wash buffer before recapturing
         the beads on the magnetic separator and decanting the wash buffer.
   v. If you have multiple options for detection antibodies, you can test these in parallel starting at this point, using them
      against the same bead mix and same samples to provide head-to-head data about performance.
   vi. If your detection antibody is biotinylated, follow the xMAP Cookbook procedure and use SAPE. If your antibody is
       PE-conjugated, you can skip this step and proceed directly to washing and resuspending after the detection
       antibody incubation.
   vii. Normal sera should not react with any of your antigen-coated beads. You will likely see some non-specific MFI
        on some or all of your beads, which should decrease as the dilution increases. This can be addressed
        in assay optimization.
6. What are my next steps?

a. Optimize the assay – more information for all can be found in xMAP Cookbook Chapter 4.5.
   i. Coupling – feel free to incorporate several bead sets of a given antigen coupled at different concentrations, as long as they are coupled to different bead IDs. This gives performance data in the most uniform way possible.
   ii. Detection antibody – test multiple antibody options for each isotype of interest. Test these antibodies both mixed together in a multiplex and in parallel in singleplex to check for cross-reactivity. Titrate the concentration of the antibody(ies) up and down to find the concentration for your desired performance.
   iii. Cross-reactivity – check that reagents behave well together and nothing is binding nonspecifically. This is a smaller task for a serological assay format.
   iv. Incubation times, temperatures, and shaking speeds – adjust all three up to increase sensitivity and signal, but also at the risk of increasing background and non-specific binding. Do not shake higher than 1000 rpm to avoid splashing in general, and ensure you follow your specific equipment’s performance recommendations.
   v. Minimum Required Dilution (MRD) – titrate the serum dilution factor up and down to determine the minimum dilution of serum needed to reduce background and maintain desired sensitivity.
   vi. Positive sera – positive sera for both SARS-CoV-2 antibodies (or, at a minimum, those from patients with a positive RT-PCR result) are critical for assay performance evaluation and should be incorporated as early in the development as possible to identify any assay performance requiring optimization. Sera for related infections are highly recommended for interrogating specificity of the assay.

b. Scale up production – coupling scale-up isn't linear.
   i. Make larger bead coupling batches with approximately the same starting ratio of protein to beads, but expect to titrate protein concentration up and down to find the best match to the smaller scale coupling.
   ii. If possible, do this on a different bead ID so you can mix it with your “gold standard” smaller scale bead for head-to-head comparison.
   iii. See the xMAP Cookbook page 21 for scale-up suggestions at a variety of bead inputs.

c. Verify/Confirm/Validate the assay.
   i. Once assay is working, interrogate performance characteristics with a fit-for-purpose approach:
      1. Sensitivity
      2. Specificity
      3. Precision
      4. Linearity
      5. Parallelism (if assay has a quantitative component)
   ii. Use the data from verification and from positive sera to establish an analysis algorithm.
      1. Confirmed sera are critical for accuracy here.
      2. Many labs set a threshold MFI above which samples are considered to have antibodies present.
      3. Other labs choose a signal-to-noise ratio in place of this MFI threshold.
      4. Other options include multi-variate algorithms incorporating results of several beads into the final result.

To learn more, please visit: luminexcorp.com/covid19-testing-solutions/#serology

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