Nanoparticle Immunogenicity Assay Development Guide

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This guide outlines steps to develop a highly sensitive assay that can detect all potential antigen (Ag)-specific positive samples, i.e., minimize false negatives, even at the cost of detecting false positives. This general guide covers the main principles of immunogenicity testing for various types of antigens. When assessing a nanomaterial's immunogenicity, the specific assay design and controls should be determined on a case-by-case basis depending on the goal of the assay, e.g., screening for an antibody response against an immunogenic-targeting moiety of a nanoparticle, a biotechnology drug formulated on the nanoparticle, a structural component (e.g., coating polymer), or the entire nanoparticle construct if made of a potentially immunogenic material. Therefore, the experimental details will vary in each study. This guide is intended to help with understanding the general principles and identifying the most critical steps and common resources.

Screening Assay

A. Preparatory Step*

Using an ELISA plate and at least 6 animal or 10 human individual sera (or plasma—the same matrix you will screen for antibodies) from untreated animals or healthy donors, test them at different dilutions to determine a dilution at which you stop seeing a serum background effect.

This is one example of how to do this:

1. Pre-wet the plate† using coating buffer‡ (300 µL/well)
2. Add blocking buffer‡ (300 µL/well) and incubate the plate at room temperature for 1 hr
3. Wash the plate with wash buffer‡ (300 µL/well)
4. Add sample diluent to all wells, 240 µL/well in Row A and 125 µL/well in Rows B–H
5. Add sera (10 µL/well) from each animal/donor to designated wells. For example:
    a. Row A, wells 1 and 2: assay diluent‡
    b. Row A, wells 3 and 4: sera 1
    c. Row A, wells 5 and 6: sera 2
    d. Row A, wells 7 and 8: sera 3
    e. Row A, wells 9 and 10: sera 4
    f. Row A, wells 11 and 12: sera 5 (*Prepare additional plates for screening additional sera.*)
6. Pipette up and down 3 times and transfer 125 µL to Row B
7. Repeat step 6 down to Row H and discard 125 µL after mixing so the final volume in all wells is 125 µL
8. Incubate the plate at room temperature for 1 hr
9. Wash the plate using wash buffer (300 µL/well)

10. Add conjugate (125 µL/well) and incubate the plate at room temperature for 1 hr
11. Add substrate (125 µL/well) and incubate the plate at room temperature for 10–30 min
12. Add stop solution (30 µL/well)
13. Read the plate at 450 nm (assuming you use HRP)
14. Analysis
    a. Compare the OD in wells 1 and 2 across all rows. These wells did not receive serum, so they will show the general background.
    b. Compare the OD in wells of individual sera. As you move from row A downward, the signal will decrease and begin to fluctuate as it approaches the background levels observed in wells 1 and 2. The goal is to find a minimal dilution at which the ODs do not decrease with all subsequent dilutions (in this example, Row A is dil25, Row B is dil50, Row C is dil100, etc.); this dilution will be used in the Screening Step described below. If you see no titration effect of the background, the tested dilutions are too high; in this case, start row A with a lower dilution or undiluted serum.
    c. Using the OD from the minimum dilution selected in step b, , calculate the mean value from all animals/donors and add two standard deviations. This will result in an assay cut-off one can use to evaluate test sera in the Screening Step.

B. Screening Step*

1. Coat test plate with antigen in coating buffer (125 µL/well) overnight at 4°C (1 hr at room temperature or 37°C can suffice for some antigens). Be sure to include 2–4 uncoated wells as a negative control and 2–4 wells coated with mouse IgG or IgM (or IgG+IgM, depending on what you are screening for). The IgG/IgM-coated wells will serve as a temporary positive control, which will help ensure the assay detects antibody if it is present, i.e., a control for your conjugate and substrate.
2. Coat a second, control plate with just coating buffer (125 µL/well). This is the negative control plate.
3. Add blocking buffer (300 µL/well) to both plates and incubate the plate at room temperature for 1 hr
4. Wash both plates with wash buffer (300 µL/well)
5. To all wells—except the 2–4 uncoated wells and 2–4 wells coated with IgG/IgM—add sera (100 µL/well) from your test animals/donors at the dilution determined from the Preparatory Step above and incubate 1 hr at room temperature
6. Wash both plates using wash buffer (300 µL/well)
7. Add conjugate (125 µL/well) to both plates and incubate at room temperature for 1 hr
8. Add substrate (125 µL/well) to both plates and incubate at room temperature for 10–30 min
9. Add stop solution (30 µL/well) to both plates
10. Read the plates at 450 nm (assuming you use HRP)
11. Analysis

a. The signal in wells coated with IgG/IgM should be high; this is the positive control for the presence of antibodies.

b. The signal in the uncoated wells should be low; this is the background.

c. Compare the OD from the coated, test and uncoated, control plates; select wells from the test plate that have a signal greater than that for the same serum on the control plate. Generally, when OD values on the coated plate are above those on the uncoated plate, they are also above the assay cut-off calculated in step 14c from the Preparatory Step above. Selecting sera with OD above both the uncoated plate and assay cut-off will reduce false-positive results; however, it may also screen out samples with a weak positive signal. Therefore, the decision whether to compare the signal on the coated plate to that on the uncoated plate or to both the uncoated plate and the assay cut-off should be made on a case-by-case basis depending on the study design, the type of the antigen, and the overall goal of the study.

Confirmatory Assay

Samples that were identified as positive in the Screening Assay are re-analyzed in the Confirmatory Assay. The same format as above can be followed, but the serum should be screened side-by-side with unspiked and spiked with excess antigen. A significant reduction in the signal of the spiked sample confirms the presence of Ag-specific antibodies. Confirmed positive sera can then be used as a more specific positive control.

Titration Assay

Samples that are confirmed to be positive in the Confirmatory Assay are then subjected to the Titration Assay. This assay helps to better understand the relative concentration of the antibody in the sample. One can use NCL ITA-36 as an example of a Titration Assay and optimize it to adjust to the specifics of the antigen of interest: https://dctd.cancer.gov/drug-discovery-development/assays/nano/ncl-methods-ita36.pdf.

Curve-based Assay

The Curve-based Assay serves the same purpose as the Titration Assay but reports the results in µg or ng/mL instead of titer. Initially, purified total IgG or IgM are used to build the curve; this is less ideal because it relies on total immunoglobulins not specific to the antigen. Later, when positive sera are identified, they can be used to build this curve. Multiple sera can be mixed to increase the volume of the positive serum. It can also be used for affinity purification of the antigen-specific antibody and other characterization.

Neutralization Assay

The Neutralization Assay is used to determine the functional consequence of the antibodies detected in the above four assays. Usually, this is a cell-based assay in which the antigen is either cytotoxic, needed to maintain cell viability, induces proliferation, or induces a secreted biomarker in cells, etc. These cells are tested with and without the antigen-specific positive serum/sera identified above to see if the expected effect of the antigen on the cells (e.g., cytotoxicity, viability, proliferation, induction of a secreted biomarker) is neutralized in the presence of the positive serum.

Resources

***Incubation time, temperature, and volumes**

- The times, temperatures, and volumes mentioned throughout this guide are those most commonly used. However, depending on the antigen, buffer, detection reagent, and plate, these parameters can be optimized to better suit the given assay.

†Plates for ELISA (with surface modifications for better immobilization of the antigen)

- Maxisorp (ideal for proteins and peptides): https://www.biolegend.com/en-us/products/nunc-maxisorp-elisa-plates-uncoated-9606
- CovaLink (ideal for antigens with specific terminal groups): https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-assays-analysis/elisa/elisa-microplates-plasticware/covalent-crosslinking-immunoassay-plates.html
- Click-chemistry plates, i.e., plates coated with DBCO (cat # 006 90 651): https://www.poly-an.de/functionalized-microplates/functionalized-96-well-microplates/click-chemistry-microplates
- Hydrophobic, lipid-binding plates (ideal for lipids): https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-assays-analysis/elisa/elisa-microplates-plasticware/adsorption-immunoassay-plates/hydrophobic-lipid-binding-plates.html

‡Coating buffer, blocking buffer, wash buffer, and assay diluent

- Coating buffer, blocking buffer, wash buffer, and assay diluent are selected empirically for each antigen and antigen-antibody. It is advised to use the most commonly used buffers or buffers used for a similar antigen, then optimize as needed for your use.

Assays for anti-PEG antibodies

- Chen, B. M., Su, Y. C., Chang, C. J., Burnouf, P. A., Chuang, K. H., Chen, C. H., Cheng, T. L., Chen, Y. T., Wu, J. Y., & Roffler, S. R. Measurement of Pre-Existing IgG and IgM Antibodies against Polyethylene Glycol in Healthy Individuals. *Analytical Chemistry*. 2016:88(21);10661–10666. https://doi.org/10.1021/acs.analchem.6b03109 https://pubmed.ncbi.nlm.nih.gov/27726379/

Reagents for anti-PEG antibody ELISA

- https://www.ibms.sinica.edu.tw/steve-roffler/
- https://www.ibms.sinica.edu.tw/~sroff/anti-PEG/index.html

Reagents for ELISA (conjugates)

- For general search, https://www.jacksonimmuno.com/
- For anti-mouse IgG+IgM conjugate, https://www.jacksonimmuno.com/catalog/products/115-035-044

Immunoglobulin-free BSA for blocking buffers and diluents to avoid high background

- https://www.jacksonimmuno.com/catalog/products/001-000-161

Background Literature

- FDA Guidance for Industry: Immunogenicity Testing of Therapeutic Protein Products — Developing and Validating Assays for Anti-Drug Antibody Detection. January 2019. https://www.fda.gov/media/119788/download.
- NorthEast Biolab, A Complete Guide On Anti-Drug Antibody (ADA) Assay Development, Immunogenicity Assessment, and FDA Guidance. https://www.nebiolab.com/anti-drug-antibody-assay-immunogenicity-assessment-services-fda-guidance/
- Chen, B. M., Su, Y. C., Chang, C. J., Burnouf, P. A., Chuang, K. H., Chen, C. H., Cheng, T. L., Chen, Y. T., Wu, J. Y., & Roffler, S. R. Measurement of Pre-Existing IgG and IgM Antibodies against Polyethylene Glycol in Healthy Individuals. *Analytical Chemistry*. 2016:88(21);10661–10666. https://doi.org/10.1021/acs.analchem.6b03109 https://pubmed.ncbi.nlm.nih.gov/27726379/

Contact

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Please cite this as: Dobrovolskaia MA, NCL Immunogenicity Assay Development Guide, September 2026, version 1. https://dctd.cancer.gov/drug-discovery-development/assays/nano/protocols-and-capabilities