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Technical tips for ELISA and multiplex immunoassay development

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Contents

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Assay development and optimization

Developing a high-performance sandwich ELISA or multiplex immunoassay can be complicated and high performance requires careful development and validation. To help, we've created this guide based on what we have learned during the development of over 500 matched antibody pairs, ELISA kits, and multiplex immunoassay kits.

Antibody pairs

- A high-performance antibody pair is essential for a high-performance immunoassay
- Use recombinant monoclonal-monoclonal pairs if reproducibility is critical
- Screen for pairs during antibody development – this allows you to screen more combinations for maximum performance
    - Use an immunogen and screening strategy designed for purpose; unfolded peptides don't make good ELISA antibodies
- Balance the cost of taking multiple pairs into full validation with the risk of late failure
    - When screening antibody pairs, use a full-length protein and a native biological sample and always screen with a different protein to the immunogen
    - Select a capture antibody that pairs with multiple detectors, and vice-versa, to ensure pair specificity
    - Screen for pairs using your buffers and assay format – small changes can impact pair performance
    - If developing a multiplex assay, expect a higher pair failure rate

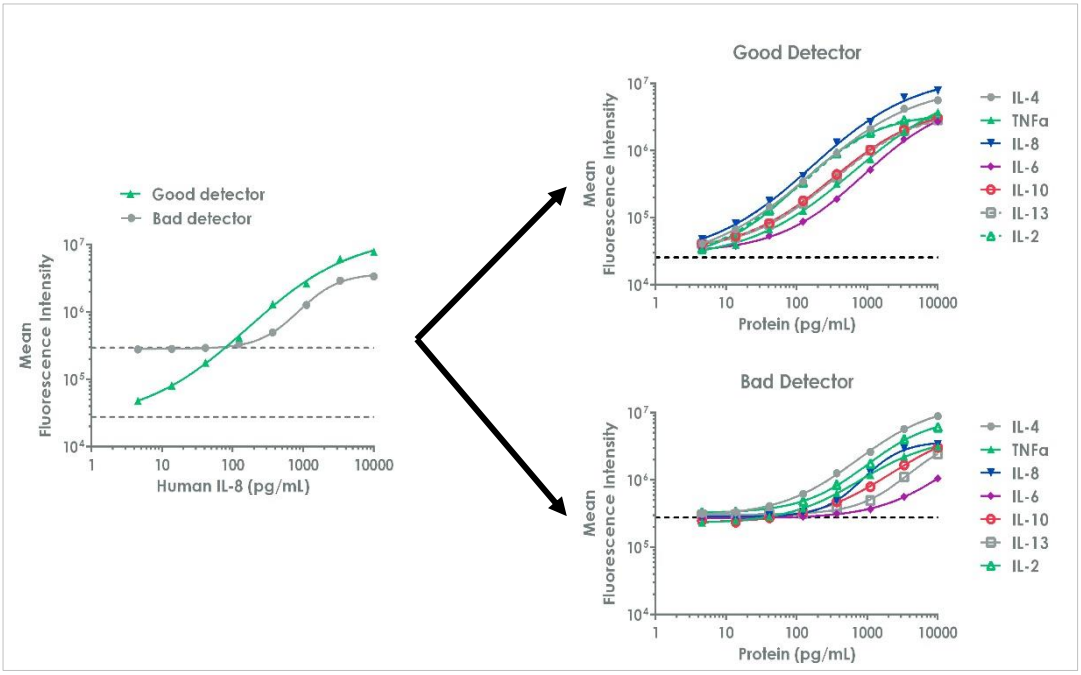


Figure 1. Good and bad antibodies can drastically affect data output. These results from a multiplex assay show non-specific binding of a good and bad detector. The bad detector negatively affected the assay by elevating the signal of all antibody pairs, rendering the 7-plex unusable.

Protein standards

- Immunoassays quantify proteins relative to the protein standard – the standard is critical
- Differences between standard and native proteins in buffer, complexing with proteins/peptides, folding, and post-translational modification can all affect reported results
- When selecting a standard, replicate the native protein and its environment as closely as possible; balance this against cost and long-term consistency
- To calibrate to an existing standard, perform a mass calibration against a benchmark protein by adjusting the amount of the standard to match signals
- Don't assume that two batches of a protein will perform the same – always recalibrate

Assay platform and format

- The assay platform contributes to assay sensitivity and dynamic range and determines sample volume and sample throughput
- Identify likely upper and lower limits of analyte concentration in your sample type
- Homogenous assay formats suffer from high dose hook and narrower dynamic ranges
    - This is caused by the analyte binding each antibody separately, blocking pair formation – avoid this with careful optimization

- If close to the limits of platform sensitivity, consider increasing incubation times as this may increase background signal and require optimization of wash steps
- When planning your standard curve, minimize the number of dilutions to reduce CV%s, particularly at the low end of the curve

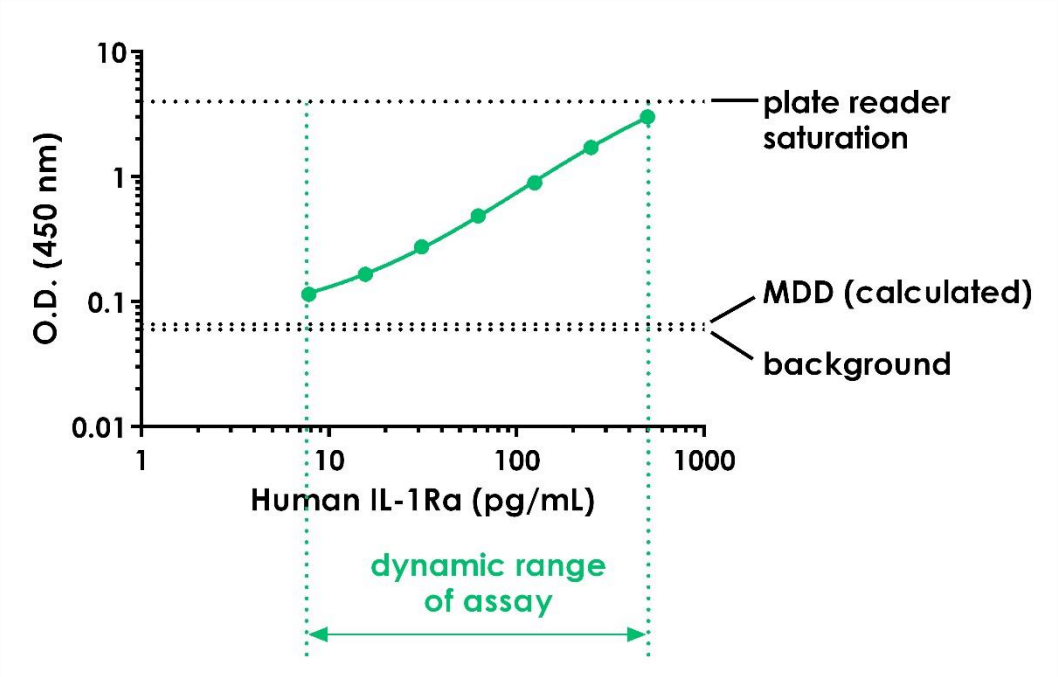


Figure 2. Understanding the dynamic range of your assay. MDD = minimum detectable dose.

Titrate antibodies

- Optimize your assay sensitivity and dynamic range by titrating capture and detector antibodies
- If your assay has a separate detection enzyme/dye, titrate this with the antibodies
- Titrate with high, medium, and low analyte concentrations to get a view across the whole dynamic range
- The signal-to-noise ratio should be at least 1:4 at the lowest analyte concentration
- To maximize sensitivity, minimize absolute background level by adjusting wash steps

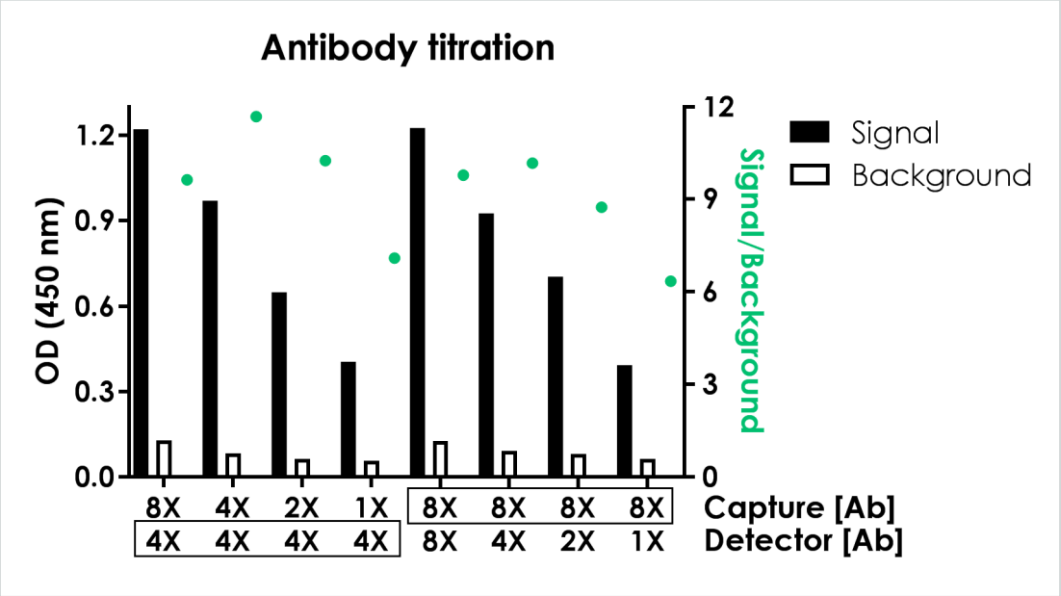


Figure 3. Using different concentrations of capture and detector antibodies allows you to optimize the signal-to-noise ratio.

Optimize buffers and assay specificity

- Assay diluent optimization should be carried out prior to beginning your assay
- Diluent elements to vary include bovine serum albumin (BSA), detergents, PBS/Tris buffer, and protease inhibitors
- For multiplex assays, check for interference between antibody pairs
    - Using a combinatorial system with small pools of detector antibodies is the most efficient way to screen large panels

Determine the optimal sample dilution

- Run a series of test samples using the diluent used for the standard curve to determine the best sample dilution
- The optimal dilution balances diluting out factors causing the matrix effect and changing the effective assay dynamic range
- Large sample dilutions can increase CV% due to pipetting error
- For multiplex assays, ensure that the dynamic range, sample dilutions and analyte concentrations align so all analytes can be quantified in the same well

Evaluate assay specificity with crude biological samples

- Use spike-recovery or linearity-of-dilution experiments
- Spike-recovery experiments measure the concentration of protein spiked into various biological samples
    - Ideally, after subtracting the native protein concentration, the remaining quantity should equal the input
- Linearity-of-dilution experiments measure protein in biological samples across multiple dilution points
    - Calculated levels, adjusted for dilution, should vary by less than +/- 20%

- Both report on performance in the biological sample matrix (biological sample + sample diluent), containing factors that disrupt the assay, ie cause the matrix effect

The matrix effect

- Caused by antibodies, proteins, small molecules, etc, and particularly common in plasma samples
- Use custom blocking buffers and assay diluents to mimic the biological sample and prevent interference
    - Create a mimic by adding isolated constituents of the sample to a buffer, eg your base culture media or, for serum or plasma, IgGs, albumin, and other high-expression serum or plasma proteins
    - Check how well your mimic replicates your sample with linearity of dilution tests
    - Using a mimic is better for long-term assay reproducibility as you can use purified standardized constituents
    - An alternative serum or plasma diluent would contain animal serum or plasma from a divergent species as the main constituent
        - Be aware that high variability exists for animal serum and plasma batches
- False positive interference affects assay performance and occurs as a result of
    - Heterophilic antibodies (polyspecific, multiple binding specificities arise naturally)
    - To correct for heterophilic blockers, try commercially available heterophilic blockers based on immunoglobulin mixes to block all of the interfering antibodies before early optimization or establishing the dynamic range
    - Autoimmune-like interfering factors such as rheumatoid factor and human anti-mouse antibody (HAMA)
        - To correct for HAMA, add mouse Ig as a blocker

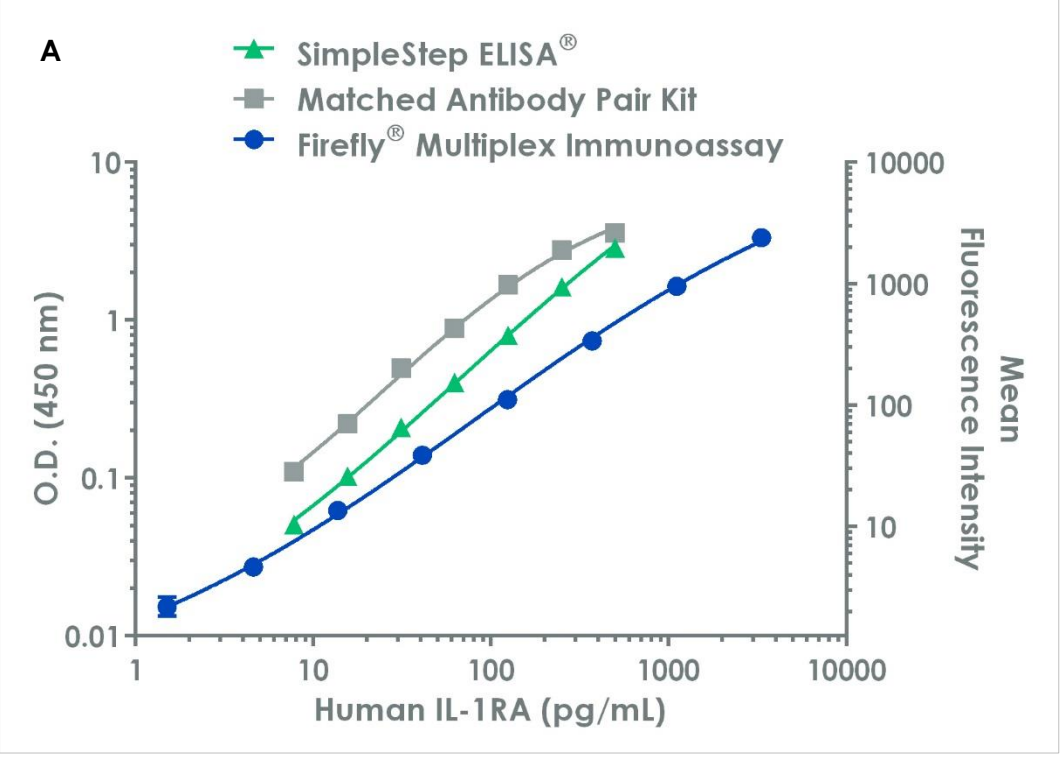
Plan for batch-to-batch variability

- A change in antibody or conjugate batches can dramatically affect assay performance
- Calibrate capture and detector antibody and protein standard batches to ensure consistent results over time and ensure that you calibrate across the full dynamic range of the assay
    - To ensure effective calibration, reserve a small quantity of the first batch to use for future calibration
    - Also, reserve a set of control biological samples with low, medium, and high expression of your chosen analytes
- Consider running control samples in each experiment to control for inter-assay variation

- You may need to titrate relative antibody amounts if performance changes – utilizing the first batch will enable easier and more accurate calibration

Plan carefully when switching assay platforms

- Run a comparison assay on your intended different platform before switching
- Use the same antibody pairs and protein standards across multiple platforms whenever possible
- Ensure correlation or regression coefficients are suitably high to demonstrate agreement of results between platforms
- Always expect correlation, not direct equivalence, in results between different assays, even on the same platform
    - Use benchmarks to compensate for this: for example, use reserved lots of biological samples or external reference standards, eg NIBSC, WHO, and ARUP international standards



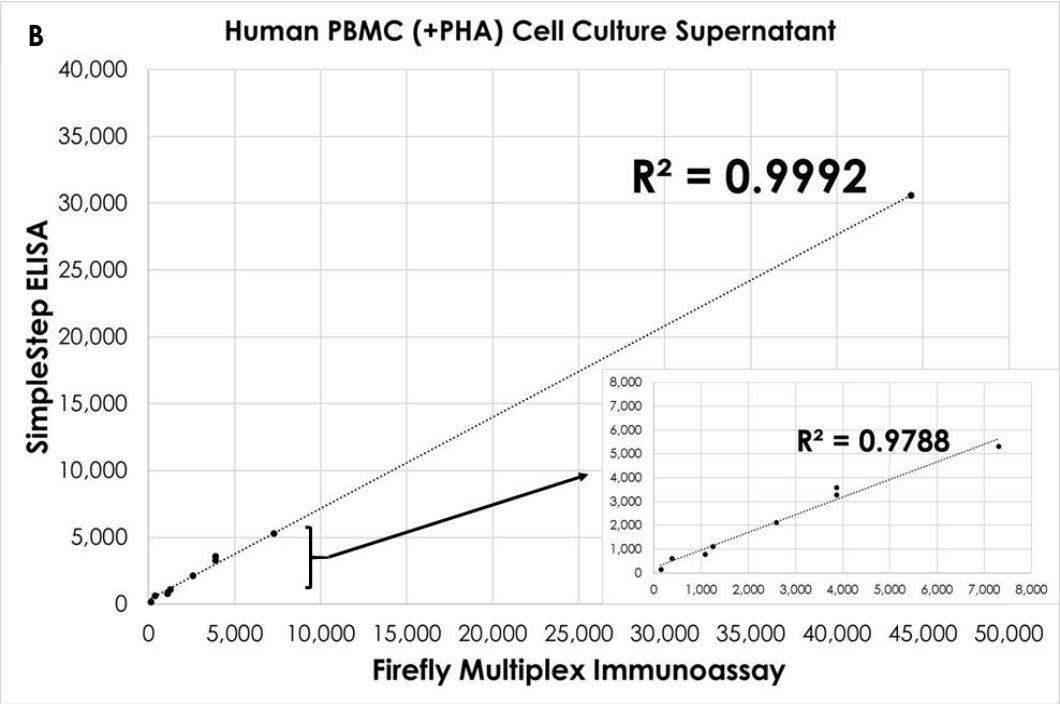


Figure 4. Comparing assay performance across three different platforms. **A** is a comparison of OD results using human IL-1RA matched antibody pairs in a SimpleStep ELISA® and a Firefly® multiplex immunoassay. **B** shows the high correlation between the two platforms.

Benefit from our expertise

We incorporate these tips into the development of our ELISA, matched antibody pairs, and multiplex immunoassay kits to ensure you achieve the most accurate, sensitive, and consistent results.

Browse our kits and resources here