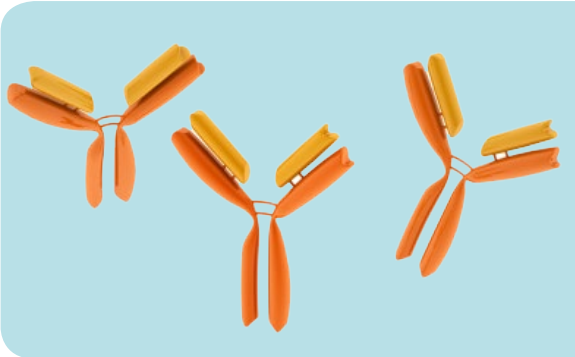
Molecular Devices



Homogeneous antibody binding assays with high content imaging

The development of homogeneous antibody binding assays has increased the efficiency for high throughput, high content analysis of cell and bead assays. These no wash, fluorometric microvolume assay technology (FMAT)-based assays allow rapid screening with fewer cells, since cell loss is reduced when multiple wash steps required by traditional assays are eliminated.

As part of the antibody discovery process during diagnostic, vaccine and therapeutic development, researchers screen hybridoma cells or colonies to select high expressing clones, measure ligand binding on cell surfaces or observe ligand or antibody internalization in cells. In addition to the benefits of traditional FMAT assays, homogeneous antibody binding assays performed on the ImageXpress® Micro Widefield High Content Screening System allow scientists to detect ligand binding on cell surfaces and multiplex different cells in the same plate well. The system also provides a pathway for experimental scale-up to 1536-well plates as only 2-3 µL of sample is required, and use lower secondary antibody concentrations while maintaining or even increasing existing detection limits.

Assay format

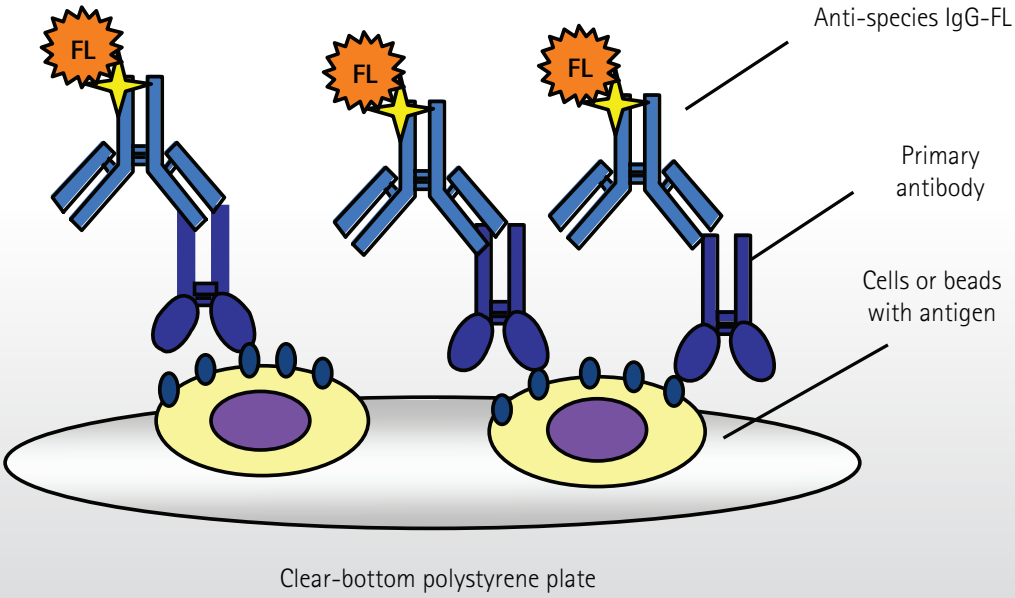
Antibodies are captured on a bead surface and then tagged with fluorescently-labeled secondary antibody. Fluorescent imaging is used to measure the amount of captured antibodies. A similar assay can be performed to measure ligand binding on cell surfaces, and for the internalization of a ligand or antibody.

In homogeneous antibody binding assays (Figure 1), adherent cell, suspension cell or antibody-coated bead samples are added to 96-, 384- or 1536-well plates. Next, a primary antibody for the marker of interest is added, followed by a secondary antibody to detect any primary antibody binding to the cells or beads.

Benefits

- Increase efficiency and eliminate waste with no-wash assays
- Run assays with adherent cells, suspension cells or beads
- Use fluorescent labels of any wavelength
- Exquisite sensitivity for detecting low-abundance antigens

Figure 1. Homogeneous antibody binding assay



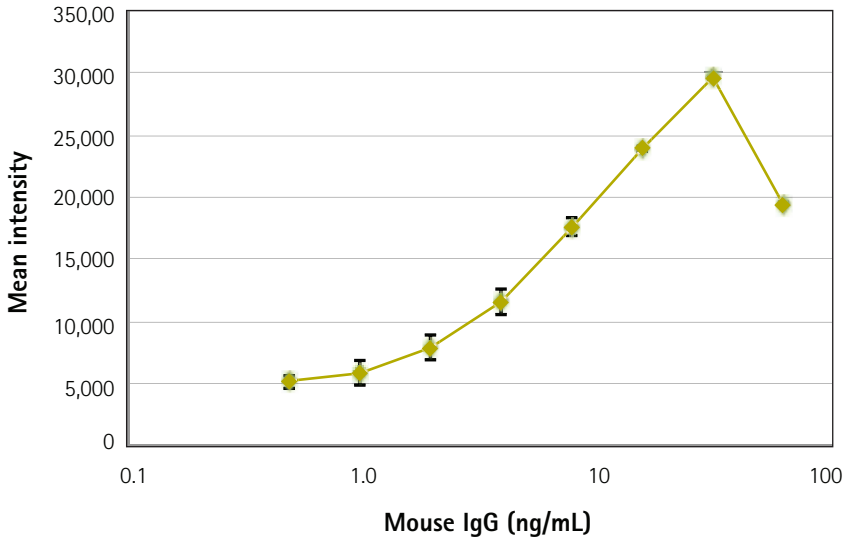
All assay reagents are added to the microplate well with no wash steps in between. Cells or antibody-coated beads that sit at the bottom surface of the plate well are identified and measured even in wells with high background fluorescence.

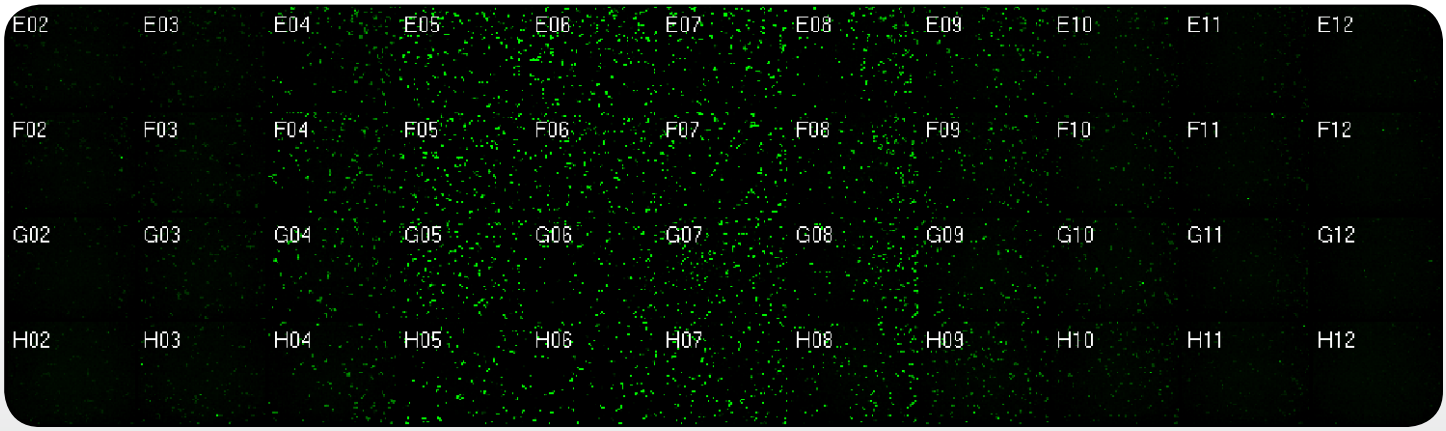
Linearity and detection limits

In this first experiment, primary antibody was titrated at various concentrations and then bound to 7 µm beads. Analyte was then added, followed by a secondary antibody, and the beads were imaged with the ImageXpress Micro System. With traditional FMAT assays, detection is limited to the red (Cy5) channel. For homogeneous assays run on the ImageXpress Micro System, any secondary antibody tag can be used since multiple wavelengths can be detected. For this titration experiment, the secondary antibody was labeled with DyLight 488.

Image analyses using the Count Nuclei Module in MetaXpress® High Content Image Acquisition and Analysis Software determined a lower limit of detection (LLD) of 0.5 ng/mL (30 pg/well), and a linear response of approximately 1–31 ng/mL. The curve of this primary antibody titration experiment displays the well-known prozone or 'hook effect' associated with homogenous antibody binding assays. This signal fall-off at the top of the curve is caused by excess unbound primary antibody in the media binding secondary antibody, making it unavailable to bind to cells or beads (Figure 2).

Figure 2. Primary antibody titration curve





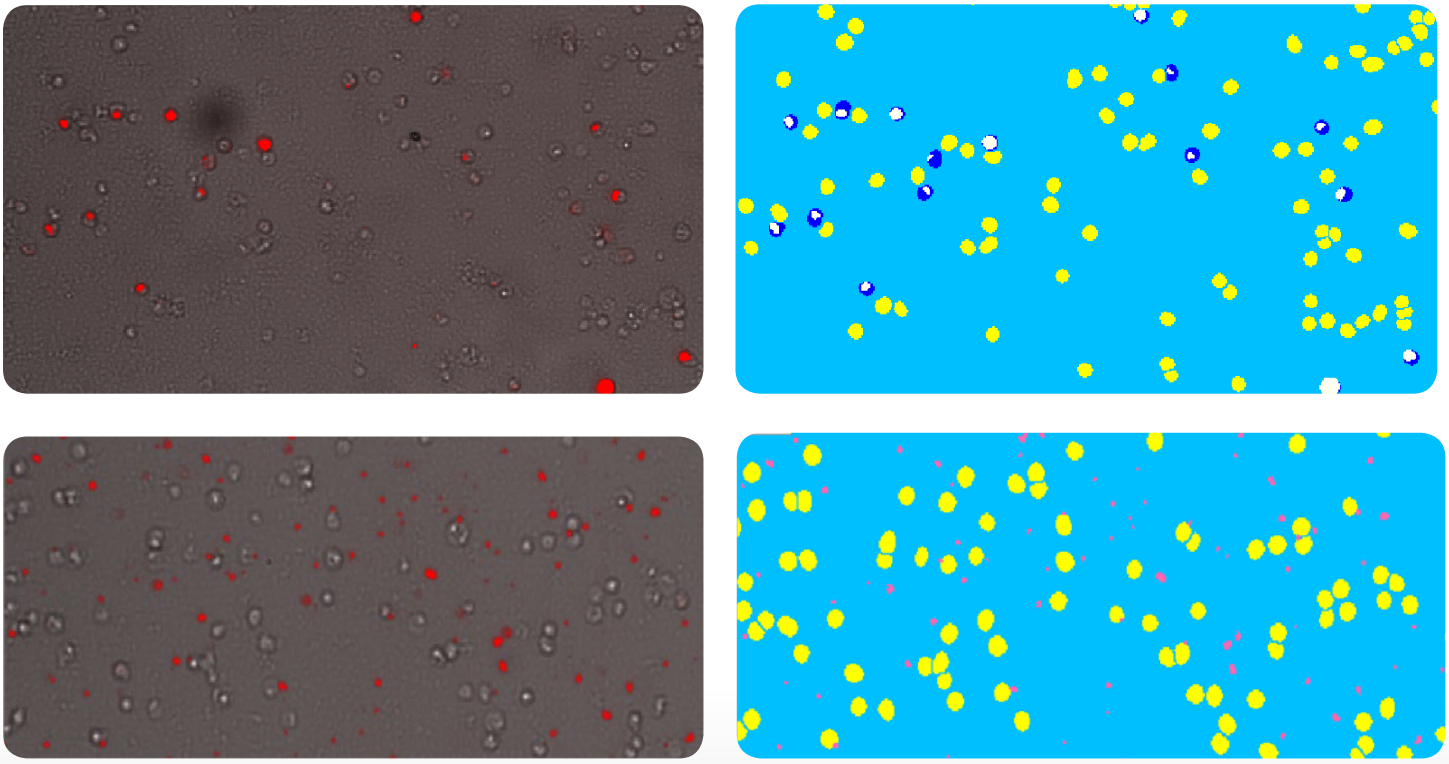
Primary antibody binding curve for bead homogeneous assay (top). Wells were run in quadruplicate, with an isotype antibody control in column 12. 7 µm beads coated with goat anti-mouse antibody were combined with varying concentrations of mouse IgG analyte and added to the wells of a microplate followed by AlexaFluor488-labeled secondary antibody and incubated at room temperature for one to two hours. Plates were imaged with a 10X PlanFluor objective. Thumbnail images of the wells (bottom) clearly shows dose-response of binding. Analysis resulted in a 0.5 ng/mL (30 pg/well) LLD and a linear response between 1 and 31 ng/mL.

Multi-wavelength analysis for more accurate determination of cell response

In addition to fluorescent images, the ImageXpress Micro System can also capture transmitted light (TL) images to identify cells more precisely. In this cell-based assay example, both a TL image of cells and a fluorescent image were acquired, and cells were identified using a custom module in MetaXpress Software.

A fluorescent image of the same well identify where antibody was bound to cell surface, but overlaying the fluorescent and transmitted light images shows that not every cell contained bound antibody (Figure 3, top left). Subsequent image analysis properly identifies cells that were positive for antibody binding (Figure 3, top right). The Cy5 image captured for a different well displays high fluorescent signal, indicating positive antibody binding. However, the transmitted light and fluorescent image overlay indicates the fluorescence in this well is not associated with the cells (Figure 3, bottom left). Full image analysis confirms the detected fluorescent signal was caused by artifacts not associated with cells, therefore these cells were scored as negative (Figure 3, bottom right).

Figure 3. More accurate image analysis using transmitted light



Top: Overlay of Cy5 (red) and transmitted light (translucent) images shows presence of cells that are both positive and negative for antibody binding (left). Analysis segmentation shows cells that were negative for antibody binding in yellow, and cells that were positive for antibody binding (in the Cy5 channel) in blue, with areas of overlap in white (right).

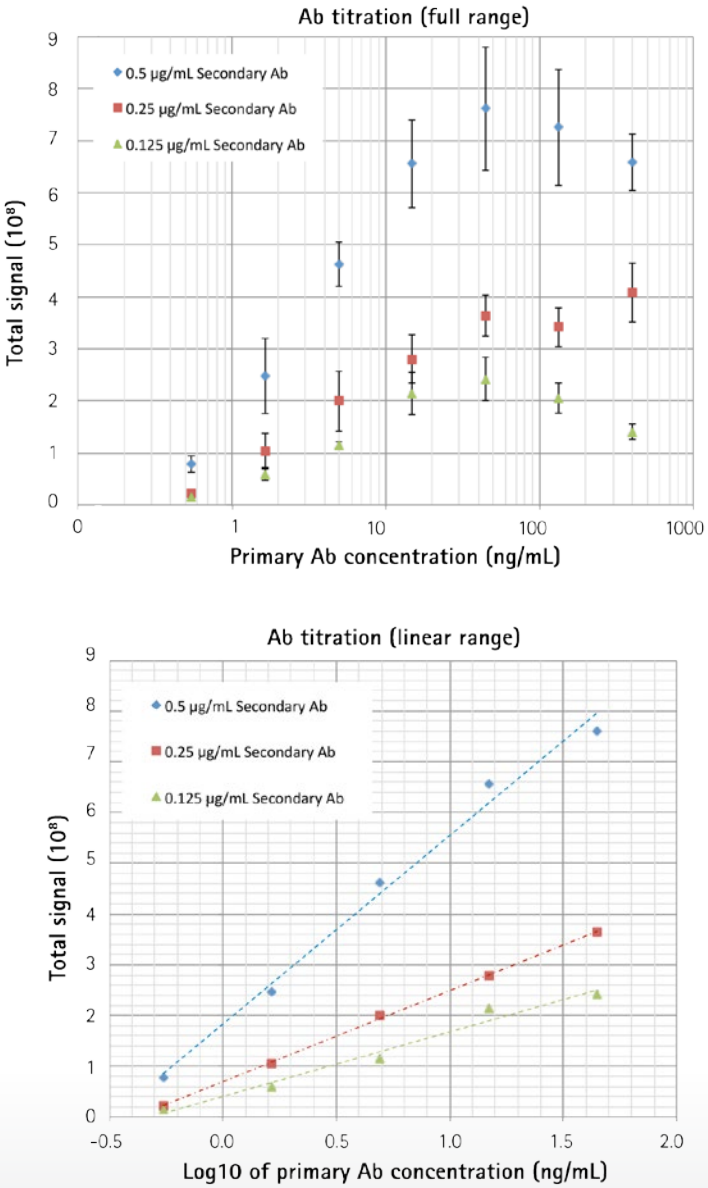
Bottom: Overlay of Cy5 (red) and transmitted light (translucent) images shows presence of fluorescent artifacts (left) which are not associated with cells. Analysis shows cells that were negative for antibody binding in yellow, and objects positive for Cy5 with no associated cell in pink (right), to be artifacts.

Assay development experiments can easily be performed for optimization of secondary antibody concentrations. In this example, three different concentrations of secondary antibody were run over a large range of primary antibody concentrations. The plotted results also display the same 'hook effect' as the previous bead assay (Figure 4, top). The lower limit of detection at the 0.25 µg/mL secondary antibody concentration was 0.5 ng/mL, the same as a standard FMAT read. All three concentrations of secondary antibody were found to be linear over the titrated range (Figure 4, bottom).

Imaging homogeneous assays enables more flexibility

High content imaging of homogeneous antibody binding assays using the ImageXpress Micro System and MetaXpress Software provides additional information than traditional FMAT assays. The ability to quickly and more definitively identify cellular response combined with the elimination of common assay artifacts produces cleaner, more accurate results. In addition, scientists are able to maintain the ability to detect ligand binding on cell surfaces while gaining flexibility to detect secondary labels at any wavelength, multiplex different cells in the same plate well, and scale up to 1536-well plate experiments without compromising assay sensitivity.

Figure 4. Optimizing detection reagents for best sensitivity



Top: Primary antibody titration (shown on x-axis) using three different secondary antibody concentrations.

Bottom: Results indicate a linear response over five dilutions of analyte (primary antibody), which yielded an LLD of 0.5 ng/mL.

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