Homogeneous Binding Assay: Comparing Imaging Systems and Optimizing Acquisition Parameters for High-Throughput Screening

By Jayne Hesley, Ming Yu, and Paula Gedraitis from Molecular Devices, Inc., 1311 Orleans Drive, Sunnyvale, CA 94089

Abstract

Hybridoma cell lines are cultured for production of monoclonal antibodies for use in diagnostics, vaccine development or therapeutics. One well-accepted assay for screening the antibody-containing supernatant of hybridomas is to capture the antibody on the surface of beads (<10 um diameter microspheres). When tagged with a fluorescently labeled secondary antibody, the beads' fluorescence can be measured using an imager. A similar assay can be done to measure ligand-binding on cell surfaces.

Robust homogeneous assays are straight-forward to develop, compatible with automation and can be scaled-up to 1536-well plates since no removal of excess antibody or label is required. These studies show that the IsoCyte Scanning Cytometer achieves sensitive and reproducible detection of both cell and bead surface fluorescence while rejecting fluorescent background from the solution in the well. In addition, a plate of any well density can be scanned and analyzed on-the-fly in 5-10 minutes. Optimization of assay and acquisition conditions such as fluorophore wavelength and scan settings are presented as well as a comparison between the traditional ABI 8200 Cellular Detection System, IsoCyte Cytometer and ImageXpress® Ultra Confocal Imager.

Introduction

For decades, colorimetric and then fluorescent ELISA was used to screen for specific antibodies in solution. These labor-intensive assays required multiple reagent additions with thorough wash steps between additions. A homogeneous (no wash) assay, called Fluorometric Microvolume Assay Technology (FMAT), was developed that enabled antibody screening of cell supernatant by imaging of fluorescent particles. The antibody is captured on the surface of beads (<10 µm diameter microspheres) and then tagged with a fluorescently labeled secondary antibody. A similar assay can be designed to measure ligand-binding on cell surfaces. If the optical properties of the detection instrumentation are suitable, the background fluorescent signal is rejected by the system and only fluorescent objects are detected in the well images. The IsoCyte Scanning Cytometer is a bench top imager containing such optics. The system rapidly acquires whole-well images and analyzes them simultaneously. Although the capabilities of the ImageXpress Ultra Confocal Imaging System exceed what is necessary for this uncomplicated, low magnification application, its ability to focus in one discrete plane while rejecting out-of-focus background fluorescence make it completely suitable for this assay format.

Materials

- Goat anti-Mouse IgG Coated Polystyrene Particles (6-8 µm) – Spherotech P/N MPFc-60-5 (other species also available)
- Mouse IgG – Jackson ImmunoResearch P/N 015-000-003
- DyLight-649 conjugated Goat anti-Mouse IgG – Jackson ImmunoResearch P/N 115-495-071
- Cy5 AffiniPure Goat Anti-Mouse IgG – Jackson ImmunoResearch P/N 115-175-071
- DyLight-488 conjugated Goat anti-Mouse IgG – Jackson ImmunoResearch P/N 115-486-071
- Cells expressing target receptor
- Hybridoma supernatant containing target-specific human antibodies
- Cy5 AffiniPure Goat Anti-Human IgG – Jackson ImmunoResearch P/N 109-175-098
- Assay buffer - PBS with 5% FBS and 0.001M EDTA
- Black-walled polystyrene microplates – Corning P/N 3712 or Greiner P/N 781096 (384 well) and Greiner P/N 783092 (1536 well)

Methods

1. Add cells or antibody-coated beads to microplate (8,000 cells or 10,000 beads in 384-well plate, 4,000 beads in 1536-well plate)
2. Add analyte to microplate (hybridoma supernatant or mouse IgG) in various concentrations to determine detection range.
3. Incubate at room temperature for 1 hour or 4ºC overnight.
4. Add labeled secondary antibody (250-300 ng/mL of DyLight-649, DyLight-488, or Cy5 labeled goat anti-species antibody).
5. Incubate at room temperature 1 hour.
6. Image plate on 8200 Cellular Detection System, IsoCyte DL Cytometer or ImageXpress Ultra Confocal System.

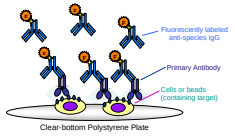
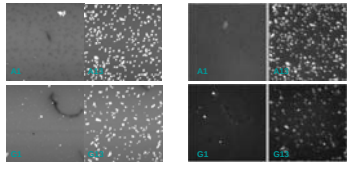


Figure 1. Schematic of homogeneous antibody binding assay.



8200 Cellular Detection System IsoCyte DL Cytometer

Figure 2. Images from a 384-well plate containing beads (top) and cells (bottom) detected with DyLight-649. Images from ABI 8200 System on left are pre-processed and IsoCyte Cytometer images on right are raw images. Since the IsoCyte Cytometer actually captures the entire well in one scan, this image is zoomed to compare at similar magnification.

Results

Several IsoCyte Cytometer acquisition and analysis parameters were examined to find the settings which gave the clearest image and best segregation of particles from background. For example, during acquisition the instrument can scan the same area multiple times and average the intensity of each pixel to improve image quality. This results in a file of the same size as one with non-averaged pixels but increases the time it takes to scan the plate. We looked for effect on precision (percent coefficient of variation of replicates) and sensitivity (lower detection limit) as well as overall curve shape of a dilution series. All concentrations were run in replicates of 4. The average %CV for Cy5 labeled beads scanned in 4 minutes on the IsoCyte Cytometer was 11% compared to 11% with the ABI 8200 Cellular Detection System. For DyLight 649 the average %CV was 11% using IsoCyte compared to 20% using the ABI 8200 Cellular Detection System. See Table 1 for a summary of these results.

	Scan time	4 min.	8 min.	16 min.	32 min.	8 min.	16 min.
	um Resolution/Pixel Averaging	5x5, 1 avg	5x5, 2 avg	5x5, 4 avg	5x5, 8avg	10x10, 4 avg	20x5, 16 avg
Cy5	LDL (ng/mL)	0.5	0.5	0.5	0.5	**1.0**	0.5
	EC50 (ng/mL)	5.8	6.4	5.5	6.8	6.3	6.0
DyLight649	LDL (ng/mL)	0.5	0.5	0.5	0.5	0.5	0.5
	EC50 (ng/mL)	4.8	4.9	5.0	5.1	6.6	**8.7**

Table 1. The LDL (lower detection limit) and curve shape (partly defined by the EC50 value) in this bead-based assay was generally unaffected by pixel averaging. The same plate run in the ABI8200 System yielded LDLs of 0.5 ng/mL for both fluorophores and equivalent curve shapes with EC50 of 9.2 and 6.5 ng/mL for Cy5 and DyLight 649 respectively.

Results

The IsoCyte Cytometer images the entire well so identifies all objects in the well. The ImageXpress Ultra Confocal Microscope with a 10X objective identifies up to 2000 objects in the field of view (See Figure 3). The same plate read on the ABI 8200 instrument contains up to 350 objects in the field of view. The output that is plotted for the assay is total intensity (i.e. the sum of intensities of all objects found) so the results were normalized when comparing between different instruments with varying fields of view or different fluorophore labels with differing intensities. The same 384-well microplate was read and analyzed on all three instrument platforms to examine sensitivity and dynamic range. Figure 4 shows the results of a primary antibody titration captured with beads and detected with Cy5 labeling.

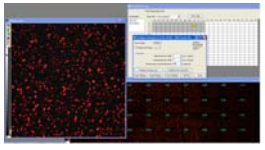


Figure 3. ImageXpress Ultra Confocal Microscope image of a well within the MetaXpress® Acquisition and Analysis Software.

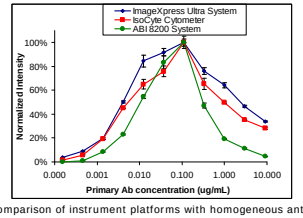


Figure 4. Comparison of instrument platforms with homogeneous antibody assay in 384-well microplates using Cy5 labeled secondary antibody. Lower Detection Limit was between 0.5-1 ng/mL. Results were normalized to the highest intensities seen with each instrument for ease of plotting on the same graph.

Although the ABI 8200 System operates with just one 635 nm laser for measuring red-shifted emission, both the IsoCyte and ImageXpress Ultra Systems are capable of multiple wavelength measurements. To illustrate flexibility and possible multi-plexing capabilities within a well, we tested two 635 nm and one 488 nm excitable fluorophores in a 384-well plate.

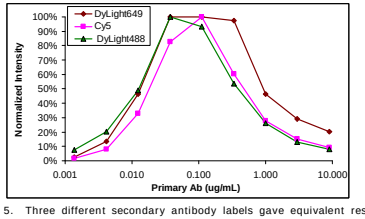


Figure 5. Three different secondary antibody labels gave equivalent results in a bead-based assay on both the IsoCyte Cytometer and ImageXpress Ultra System (data not shown). The lower detection limits were 1.0 ng/mL analyte in this experiment regardless of fluorophore. Cy5 and DyLight649 labeled beads were detected as low as 1.0 ng/mL in the ABI 8200 System as well.

Results

Homogeneous assays are generally easy to scale up. The hurdles of liquid handling can be overcome with modern equipment and centrifuging to eliminate any bubbles separating the reagents. The bead-based assay was run with a DyLight 649 labeled secondary antibody in a 1536-well plate and imaged on the IsoCyte Cytometer. Centrifuging tended to cause many beads to migrate to the well edges so a larger number of beads were used than might be expected in a well of this size and the region of interest was set up to omit the well edges. This allowed only the well-dispersed beads throughout the rest of the well to be analyzed. Figure 6 shows acceptable repeatability of two 1536-well plates. In Plate 1, the beads were allowed to settle for less than one hour by gravity and Plate 2 was centrifuged at 1000 RPM for a minute.

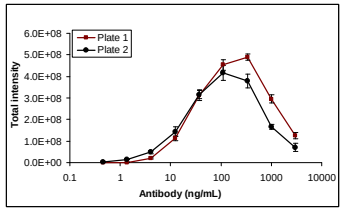


Figure 6. Two 1536-well microplates of primary antibody dilutions detected on beads with DyLight 649 gave similar results whether allowed to settle by gravity (Plate 1) or hastened with centrifugation (Plate 2). Plates were read and analyzed with the IsoCyte Cytometer. The lower detection limits were 4 ng/mL in Plate 1 and 0.5 ng/mL in Plate 2.

Summary

- Homogeneous antibody assay sensitivity, dilution series curve shapes, and EC50s obtained on the IsoCyte Cytometer and ImageXpress Ultra Confocal System were equivalent to results from the ABI 8200 Cellular Detection System.
- Sensitivities in the picomolar range are achievable with the homogeneous bead or cell-based imaging assay.
- Typical 384-well scan time is 15 minutes on ABI 8200 while IsoCyte with 5x5 µm resolution and 2 averaging requires <8 minutes for acquisition and image processing.
- Secondary antibody labels DyLight 488 and DyLight 649 can be used in place of Cy5 to give equal lower detection limits.
- The homogeneous assays can be quickly scaled up to a 1536-well format to achieve similar results as seen in a 384-well format.

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