mirrorball®
application note

simultaneous determination of antibody binding, specificity and titer on the mirrorball fluorescence cytometer

introduction

The antibody discovery process relies on consecutive screens to test the same antibody sample for (1) binding affinity, (2) target specificity and (3) antibody titer. Conventionally such screens are done by ELISA or flow cytometry, but both techniques have technical limitations that restrict their utility in high-throughput screening environments:

- both assay formats require multiple wash steps, with significant hands-on time from the operator that restricts throughput
- ELISA assays generate only singleplex readouts, so each sample must be tested in separate experiments
- flow cytometry can generate multiplex assay readouts, but the sample throughput rate and plate capacity of most flow cytometers is incompatible with screening environments

Here we present a simple homogenous (no-wash) assay to screen antibody samples for binding to a target cell line, non-specific binding to a control cell line and antibody titer by binding to a sol-R™ bead. Such a setup allows clear distinction between affinity and titer, which is easily obscured in singleplex experiments. The assay was set up in 384-well plates, then read and analysed using the mirrorball fluorescence cytometer. Many of the screening components (cells and beads) can be prepared and stored in advance of the screen, to enable a rapid plate setup with validated and batch controlled reagents.

assay principle (Fig 1.):

A mixture of target and control cells, anti-IgG capture beads, detection antibody and antibody sample is added to the wells of an assay plate. The two cells lines are stained with different amounts of orange and red fluorescent dye; the anti-IgG capture beads have a brighter red fluorescence than either cell type. Cell or bead type classification is readily accomplished using a 2D scatter plot of orange versus red fluorescence intensity. The amount of binding to each cell type or bead is then determined using a single, green-fluorescent detection antibody directed against the antibody sample. This setup allows simultaneous determination of target-specific binding, non-specific binding, and antibody titer in a single assay.

materials

- sol-R5 streptavidin-coated beads (TTP Labtech, #4150-09041)
- goat anti-mouse capture antibody (biotinylated, F(ab')2 domain specific, JIR # 109-006-097); 1.4 mg/mL stocks
- A431 cells (brought into suspension by trypsinisation)
- Jurkat suspension cells
- Cell Tracker Orange CMTMR (Thermo Fisher Scientific #C2927)

key benefits

- 3-plex mixed cell and bead protocol for simultaneous determination of
  - specific binding (cell based)
  - nonspecific binding (cell based)
  - titer (bead based)
- no-wash assay protocol
- compatible with high density microplates
- automation friendly

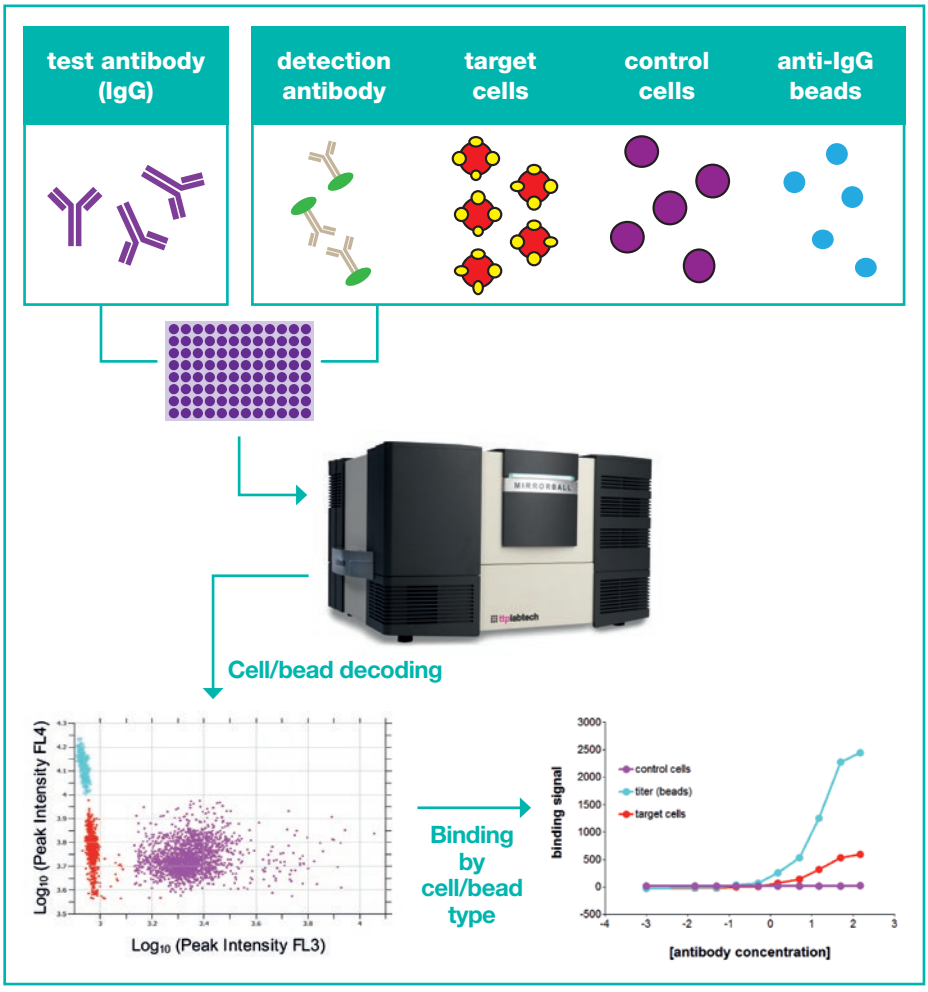

Fig 1. Assay Principle.

ttplabtech

- mouse anti-EGFR monoclonal antibody (Merck #GR01)
- DRAQ5 fluorescent dye (Biostatus #DR50200). 5 mM stocks
- goat anti-mouse polyclonal detection antibody (AF488-conjugated, Fc domain specific, JIR# 115-546-071); 1.5 mg/mL

method

1. coat 10^6 streptavidin-coated sol-R beads with 2 µg/mL of the biotinylated capture antibody according to the user information sheet supplied with the beads. Resuspend in 500 µL PBS/BSA.
2. harvest 10^6 Jurkat cells and stain with 5 µM Cell Tracker Orange CMTMR according to the manufacturer's instructions. Resuspend in 500 µL PBS/BSA.
3. harvest 10^6 A431 cells, spin down and then also re-suspended in 500 µL PBS/BSA.
4. prepare singleplex and multiplex detecting mixes according to the table below (sufficient for 1 x 384-well plate)

component	detection mixture (singleplex)	detection mixture (multiplex)
A431 cells	100 µL (one component)	100 µL
Stained Jurkat cells		100 µL
Coated sol-R beads		100 µL
DRAQ5	4 µL	4 µL
Detection Antibody	4.4 µL	4.4 µL
PBS/BSA	3.9 mL	3.7 mL

5. to each well of the assay plate add 10 µL of α EGFR mAb sample, then overlay with a further 10 µL of detecting mixture (singleplex or multiplex)

Each well will now contain: 500 cells or beads of each type, 2.5 µM DRAQ5, 800 ng/mL detection antibody in a total volume of 20 µL

6. cover the plate with a seal or lid and incubate for 2 hours at room temperature (no light)
7. read plate on the mirrorball fluorescence cytometer

results

cell type classification

2D scatter plots of orange (FL-3) versus red (FL-4) fluorescence peak intensity (Fig 2) were used to classify the cell and bead populations in the Cellista software. The two cell types (A431 and Jurkat) are both counterstained in red with DRAQ5, but only the Jurkat cells have the additional Cell Mask Orange CMTMR counterstain. This allows ready distinction of the two cell types based on their orange (FL-3) fluorescence intensity. The sol-R beads can be distinguished from either cell type based on their brighter red (FL-4) fluorescence intensity.

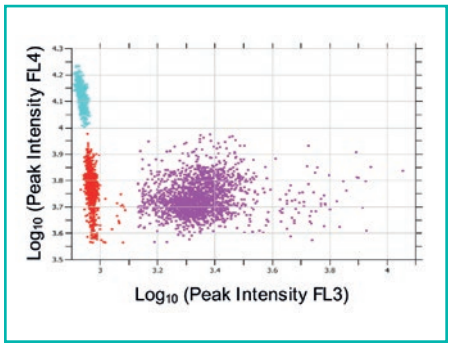

Fig 2. 2D scatter plot of orange (FL-3) versus red (FL-4) fluorescence peak intensity used for cell and bead classification. The purple population corresponds to the Jurkat cells, which have been counterstained in orange and red. The red population corresponds to the A431 cells, which have been counterstained in red only. The teal population corresponds to the sol-R5 beads, which have a brighter red fluorescence compared to either cell type.

binding analysis

Next, the amount of anti-EGFR antibody binding to each cell or bead type was determined by measuring the green fluorescence intensity [median (mean intensity FL-2)]. Fig 3. shows concentration-dependent binding of the anti-EGFR sample to the coated sol-R beads and the A431 cell line (expressing the EGF receptor). No non-specific binding was observed with the Jurkat control cell line, as expected.

summary

Here we have presented a simple binding assay that enables multiplexed antibody screening for affinity, specificity and titer in a single experiment. The homogenous (no-wash) assay format minimises the "hands-on" time for the operator and is compatible with simple liquid handling dispensers for setup. These features make this method ideally placed to accelerate the pace of antibody screening, whilst minimising the costs associated with setting of multiple washed assays.

about mirrorball

The mirrorball plate-based fluorescence cytometer uses TTP Labtech's laser scanning technology to provide HTS-friendly workflows that deliver gold standard data quality. The mirorball's proprietary background rejection optics enable the use of streamlined no-wash assay formats for multiplexed cell, and bead-based applications that provide process efficiencies over standard ELISA and flow cytometry.

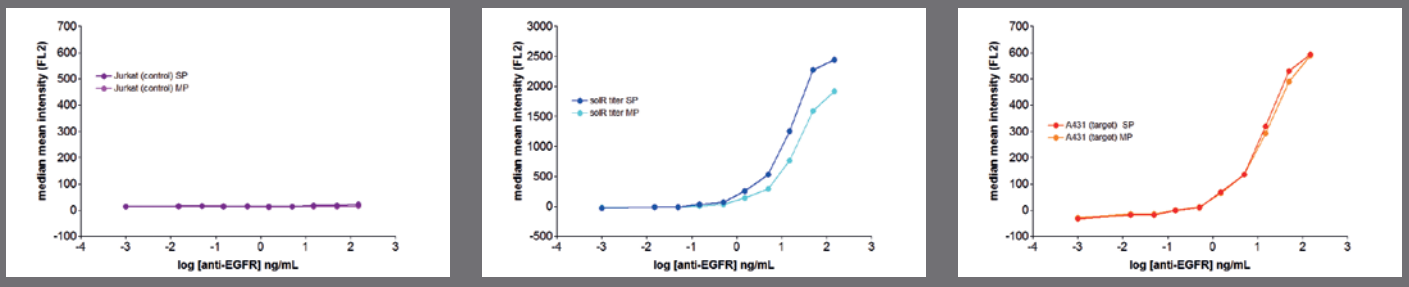

Fig 3. Binding comparison by cell or bead type and assay format. SP = singleplex; MP = multiplex

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