mirrorball®
application note

multiplexed fluorescence ELISA assays on mirrorball®

introduction

Enzyme-linked immunosorbant assays (ELISA) can be used in multiple stages of the drug discovery process to help identify and measure the cellular responses to therapeutic or toxic molecules. The most common ELISA format is the sandwich assay. Briefly, a capture antibody is immobilised onto a solid support surface, the analyte to be quantified (e.g. cytokine, peptide, protein) binds to the capture antibody and can then be detected by a biotinylated secondary antibody. Incubation with streptavidin-conjugated HRP (horse radish peroxidise), followed by a colorimetric substrate facilitates assay quantitation.

Although ELISAs are very sensitive, they are time-consuming, require multiple separate wash steps and incubation steps and are not amenable to multiplexing.

In this application note, we demonstrate the transfer of standard commercially available colorimetric IL-8 and IL-6 ELISA kits onto a no-wash fluorescence based ELISA on the mirrorball. The resulting ELISA format offers significant process efficiency improvements through use of a single incubation step, without the requirement for wash steps, and is amenable to multiplexing.

materials and methods

bead coating

IL-8 capture beads were prepared as follows: $4x10^6$ carboxy-coated, 8.5 µm diameter, sol-R2 beads (TTP Labtech, Carboxy sol-R2) were washed three times by centrifugation at 13,000 rpm with 500 µL 50 mM MES pH 5.2 (Sigma, M8250). Final wash was removed and beads were resuspended in 500 µL 50 mM MES pH 5.2. The bead surface was activated for 15 minutes at room temperature in the dark by incubation with 50 µL of 20 mg/mL EDAC (N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride) (Sigma, E1769). Mouse anti-human IL-8 capture antibody (R&D Systems, DY208) was added to give 4 µg/mL working concentration. Beads were incubated on tube rotator in the dark for 1 hour to allow covalent coupling to occur. Beads were washed three times by centrifugation at 13,000 rpm with 500 µL PBS, 0.05% Tween 20, 0.05% BSA. After final wash, the beads were resuspended in 500 µL PBS, 0.05% Tween 20, 0.05% BSA, 0.05% sodium azide. Final bead density was determined by haemocytometer. Beads were stored at 4°C in the dark until required. Identical procedure was used to prepare IL-6 capture beads except sol-R4 beads (TTP Labtech, Carboxy sol-R4) were used and coated with mouse anti-human IL-6 capture antibody (R&D Systems, DY206).

fluorescence ELISA process – simpler than ELISA

colorimetric ELISA

Immobilise capture Ab **(OVERNIGHT)**
↓
Wash 3x
↓
Block buffer for **(1 HOUR)**
↓
Wash 3x
↓
Test sample **(2 HOURS)**
↓
Wash 3x
↓
Biotinylated 2° Detection Ab **(2 HOURS)**
↓
Wash 3x
↓
Streptavidin-HRP **(20 MIN)**
↓
Wash 3x
↓
TMB substrate **(20 MIN)**
↓
2N H_2SO_4 stop solution and read Absorbance

no-wash ELISA on mirrorball

Immobilise capture Ab onto sol-R beads **(1.25 HOURS)**
↓
Wash 3x
↓
Add 10 µL test sample to assay well
↓
Add 10 µL containing:
Capture -Ab-coated beads (one or more bead types), biotinylated 2° Detection Ab and streptavidin-AF488
↓
Incubate **(4 HOURS)**
↓
Scan on mirrorball

Fig 1. No-wash mirrorball ELISA is a much simpler workflow relative to standard colorimetric ELISA and is flexible enough to enable multiplex assay format.

key benefits

TTP Labtech's mirrorball facilitates transfer of ELISA to no-wash fluorescent ELISA assay format resulting in throughput and automation advantages:

- single incubation step and no washes required
- smaller sample volume required due to assay miniaturisation
- multiplexing capability gives more information per sample
- overall process efficiency improvement freeing up valuable time and sample for other tests

cytokine preparation

Halving dilution series of IL-8 and IL-6 (R&D Systems, DY208 and DY206 respectively) in PBS, 1% BSA were prepared.

colorimetric ELISA

Colorimetric IL-8 and IL-6 assays (R&D systems, DY208 and DY206 respectively) were carried out according to manufacturer instructions.

no-wash mirrorball IL-8 ELISA

Prepared a bead mixture in PBS, 1% BSA containing 50,000 beads/mL of anti-IL-8-coated beads, 40 ng/mL biotinylated anti-IL-8 detection antibody (R&D Systems, DY208) and 100 ng/mL Alexa Fluor® 488-conjugated streptavidin [(AF488) JIR cat# 016-540-084]. 10 µL of IL-8 standard and bead mixture was added to the 384-well assay plate (Corning, 3712). The plate was incubated at room temperature in the dark for 4 hours and scanned on mirrorball (Fig 2).

ttplabtech

no-wash mirrorball IL-6 ELISA

Prepared a bead mixture in PBS,1%BSA containing 50,000 beads/mL of anti-IL-6-coated beads, 100 ng/mL biotinylated anti-IL-6 detection antibody (R&D Systems, DY206) and 200 ng/mL AF488 (JIR cat# 016-540-084). 10 µL of IL-6 standard and bead mixture was added to the 384-well assay plate (Corning, 3712). The plate was incubated at room temperature in the dark for 4 hours and scanned on mirrorball (Fig 2).

no-wash mirrorball multiplexed IL-6, IL-8 and non-specific ELISA

Prepared a bead mixture in PBS, 1%BSA containing 50,000 beads/mL of each of anti-IL-6-coated beads, anti-IL-8-coated beads and non-coated red bead (TTP Labtech, sol-R3), 40 ng/mL biotinylated anti-IL-8 detection antibody (R&D Systems, DY208), 100 ng/mL biotinylated anti-IL-6 detection antibody (R&D Systems, DY206) and 250 ng/mL AF488 (JIR cat# 016-540-084). 10 µL of either IL-6 or IL-8 standard and 10 µL bead mixture was added to the 384-well assay plate (Corning, 3712). The plate was incubated at room temperature in the dark for 4 hours and scanned on mirrorball (Fig 2).

results

The multistep colorimetric IL-6 and IL-8 ELISAs were successfully transformed into a single 4 hour no-wash ELISA format on mirrorball, offering significant process efficiencies (Fig 1). Additionally, these assays were carried out in a multiplex format, offering even more information and process efficiency savings, with no significant reduction in assay performance over the singleplex format (Fig 2).

conclusions

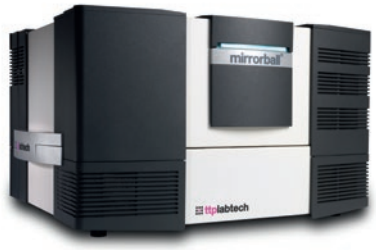
These data demonstrate the use of reagents from commercially available multistep ELISAs in a no-wash ELISA on the mirrorball in both singleplex and multiplex format. The mirrorball assay format offers significant advantages over ELISA:

- no wash steps required
- shorter overall assay time
- multiplexed assay capability
- significant overall process efficiency improvement

about mirrorball

The mirrorball fluorescence cytometer is applicable to many stages within biologics discovery from screening to identify hits to binding characterisation and monitoring phenotypic responses. Streamlined no-wash cell or bead-based assays provide process efficiencies over standard flow cytometry or ELISA formats, enabling you to do more with precious samples and time. Key assay capabilities are:

- biologics screening – hybridoma, phage display, antibody fragments, peptides, scFv, Fab
- quantification of soluble proteins – cytokines, hormones
- binding characterisation – Bmax and Kd determination
- phenotypic assays – cell health, cell cycle, proliferation, signalling, apoptosis



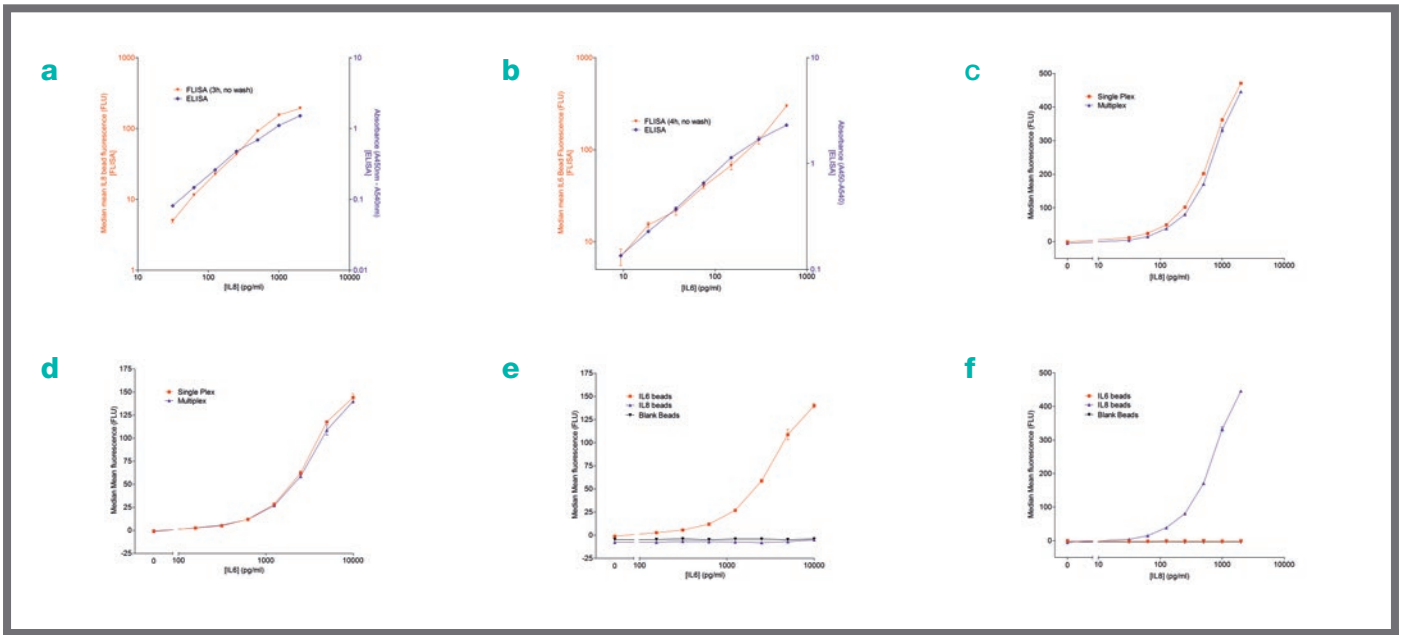


Fig 2. No-wash mirrorball ELISA gives comparable results to ELISA and produces similar results in singleplex and multiplex assay format. No-wash mirroball ELISA gives comparable results to ELISA for both IL-8 (a) and IL-6 (b). Very similar results are obtained in singleplex or multiplex assay format on mirrorball for IL-8 (c) or IL-6 (d) measurement. Multiplex assays clearly show concentration-dependent binding response to only IL-6 (e) or IL-8 (f) beads in the presence of the single cytokine, with no binding to the incorrect cytokine bead or blank bead.

designed for discovery

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