Introduction

The High-Throughput Screening (HTS) approach to drug discovery has gained widespread popularity over the last 15 years. Because of the need to process thousands of assays per day, HTS relied upon multiple-well microplates and robotic processing technologies. Pressure for increased throughput and reduction in cost led to the adoption of high-density, lower-volume microplates as well as fast, homogeneous, miniaturizable screening assays.

BMG LABTECH’s PHERAstar® FS combines rapid plate reading necessary for HTS with enhanced performance and sensitivity needed to read small liquid volumes. The performance and features of the PHERAstar FS are presented in this application note with commercially available HTS kits such as Far Red PolarScreen™, AlphaScreen®, and HTRF®.

Far Red PolarScreen™ Kinase Assay

In fluorescence polarization mode, the Far Red PolarScreen™ assay (Life Technologies) for tyrosine kinase (Csk) was used in low volume 384-well plates (from Corning) with a final assay volume of 20 μL according to the kit protocol. The Far Red fluorophore was excited with 200 flashes at 610 nm and emission was detected in both polarization planes simultaneously at 670 nm.

AlphaScreen® cAMP competition assay

The cAMP assay kit (PerkinElmer) was performed in AlphaScreen® mode in accordance with the kit protocol in white 384-well small volume (SV) plates (Greiner Bio-One) with a final assay volume of 25 μL. The AlphaScreen® beads were excited with 400 flashes at 680 nm and emission was detected simultaneously in the range of 520 nm to 620 nm. To avoid evaporation, all plates were sealed with transparent microplate sealers during incubation or storage and the sealer was not removed during reading of the microplate.

HTRF® TNFα immunoassay

The time-resolved fluorescence mode was explored using the HTRF® reader control kit (Cisbio) that is designed for the calibration of HTRF® compatible readers. Results were obtained in 384-well small volume plates (Greiner) according to the kit protocol. The incubation took place overnight at room temperature and the final assay volumes were 20 μL. The kit is based on a Tumor Necrosis Factor alpha [TNFα] assay and may also be used for reader validations. On the flash lamp-based PHERAstar FS, the assays were run with 200 or 400 flashes per well. The FRET donor europium cryptate was excited at 337 nm and emission was simultaneously read at 620 nm and 665 nm.

Results & Discussion

Far Red PolarScreen™ Kinase Assay

The Far Red PolarScreen™ assays employ a proprietary Far Red fluorophore in homogeneous fluorescence polarization assays. Protein kinases [PKs] are a diverse group of enzymes involved in many areas of cell signalling. These include cell growth and proliferation and neural functions. The keen interest in PKs arises from their role in regulating biological mechanisms. Through phosphorylation, PKs participate in many cellular signal transduction processes. Research focused on kinase activity could ultimately identify targets that can be used to develop new pharmaceutical agents to treat many of these diseases.

In a Far Red kinase assay, kinase, substrate, and ATP are allowed to react in the presence of library compounds. After the reaction is complete, antibody and Far Red labelled tracer are added. The antibody can associate with either the labelled tracer [resulting in a high FP value] or the kinase-produced phosphorylated substrate [resulting in a lower FP value]. The amount of antibody that binds to the tracer is inversely related to the amount of phosphorylated product present, and in this manner, kinase activity can be detected and measured by a decrease in FP value. Thus, library compounds that inhibit the reaction are identified as wells that have a high polarization value.

To demonstrate the use of the PHERAstar FS with Invitrogen’s Far Red PolarScreen™ kinase assay, a tyrosine kinase (Csk) titration curve (n = 3) was performed, in the concentration range of 0.5 pg/mL to 2 μg/mL Csk, as shown in Fig. 1.
**AlphaScreen® cAMP competition assay**

Cyclic AMP (cAMP) is one of the most important intracellular mediators. Detection of cAMP with AlphaScreen® is based on the competition between cAMP produced by cells and a biotinylated cAMP probe that is sandwiched by streptavidin-donor and anti-cAMP antibody conjugated acceptor beads. A decrease in signal is observed with an increase in intracellular cAMP produced. In the absence of intracellular cAMP, a maximum signal is detected. To demonstrate the functionality of the AlphaScreen® assays and the performance of the PHERAstar FS, a titration curve [n = 3] with cAMP was performed (Fig. 2).

Final assay volume was 25 μL per well in a 384-well SV plate and after 12 hours incubation at room temperature the plate was read on PHERAstar FS using an integration time of 0.3 seconds per well. The concentration of cAMP was in the range of 1 μM to 10 pM and the cAMP titration curve reveals a high S/B = 82 value. MARS evaluation software, including a 4-parameter-fit function, was used for curve fit and IC50 determination. The calculated IC50 = 24 nM value complies with the literature. These experimental evaluations show that the PHERAstar FS can produce high quality data for the AlphaScreen® assay technology.

**HTRF® TNFα immunoassay**

Cisbio’s HTRF® assays employ fluorescent Eu³⁺ cryptates (donor) and XL665 (acceptor) in homogeneous time-resolved FRET-based assays. Tumor Necrosis Factor alpha (TNFα), a 17 kDa cytokine, is an important mediator secreted by activated macrophages and monocytes with a large spectrum of antiviral immunoregulation, metabolic and inflammatory properties. The HTRF® TNFα assay is a single step double-site immunometric assay involving two MABs conjugated either with europium cryptate or to XL665. The HTRF® TNFα assay principle is shown in Fig. 3.

Under its final configuration, free TNFα from calibrators or samples is sandwiched by mouse MAb IPM2-Eu cryptate (IPM2-K) and mouse MAb IPM3-XL665 (IPM3-XL665) conjugates. The FRET signal generated by the simultaneous binding of the two conjugates is proportional to the amount of TNFα present in the sample. Both 665 nm and 620 nm signals were measured simultaneously on the PHERAstar. Delta F is a value calculated from the 665 nm / 620 nm ratios which enables the data to be normalized with respect to between-assay variations. In addition, delta F is reader independent and can be used for indicating and comparing the quality of a reader. The PHERAstar FS results in an excellent delta F value (> 1100 % High calibrator).

**Conclusion**

The discovery of new leads through HTS is based on the ability to precisely measure biomolecular interactions and find successful detection strategies that are compatible with miniaturized HTS. The PHERAstar FS multimode reader proved to have a wide range of possible applications for HTS needs.