AlphaLISA™ in Biomarker Detection for Drug Discovery

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Enzyme-linked immunosorbent assays (ELISAs) have historically been among the most effective and widely adopted assays for use in detection and quantification of low analyte concentrations. The technology is selective, sensitive and versatile. Its usefulness, however, has been limited by low throughput due to wash steps, a generally narrow dynamic range and the inability to use low-affinity antibodies.

Because of these limitations, biomarker detection in drug discovery has necessarily employed more time- and resource-intensive processes. In general, a homogeneous (no wash) technology that could detect a wide range of biological analytes would be of considerable value to drug discovery programme.

Comparing E- and Alpha-LISA™

To utilise the advantages of ELISA assays, including their inherent simplicity and economy, yet reduce the disadvantages, PerkinElmer, Inc. (PKI) developed AlphaLISA™. The technology does not require wash steps and is easy to miniaturise and automate, enabling an efficient high-throughput screening set-up. AlphaLISA can be set up as either sandwich or competition immunoassays and be used to detect analytes without removal of biological matrices, such as serum, plasma or cell lysates.

Conventional ELISAs readily detect analytes in the low picomolar (pM) range, but have limitations, such as multiple washing steps to remove non-specifically absorbed reactants. These wash steps restrict ELISA use in high-throughput protocols because they are time-consuming and require additional reagent and reagent preparation as well as complex instruments and programming. ELISAs handle relatively low sample volumes adequately, but because of their limited sensitivity, larger volumes are usually needed. This can be restrictive in cases where the target analyte is present only in low concentrations. Furthermore, because ELISAs often exhibit approximately two orders of magnitude in dynamic range, the sample needs to be diluted several times to bring analyte levels within the range of detection (see Table 1).

Table 1: Comparison of AlphaLISA with ELISA

<table>
<thead>
<tr>
<th></th>
<th>Conventional ELISA</th>
<th>AlphaLISA</th>
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<tbody>
<tr>
<td>Selectivity</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>Sensitive (pM range)</td>
<td>Highly sensitive (sub-pM)</td>
</tr>
<tr>
<td>Assay nature</td>
<td>Heterogeneous: multiple washings</td>
<td>Homogenous: no washing</td>
</tr>
<tr>
<td>Labours</td>
<td>Very labour-intensive</td>
<td>Limited labour requirement</td>
</tr>
<tr>
<td>HTS format</td>
<td>Difficult to employ for HTS</td>
<td>Easy to miniaturise for HTS</td>
</tr>
<tr>
<td>Antibody req.</td>
<td>Requires high affinity antibodies</td>
<td>Can be used with high and low affinity antibodies</td>
</tr>
<tr>
<td>Assay volume</td>
<td>Large, 25–50 l</td>
<td>&gt; 5 l</td>
</tr>
<tr>
<td>Analytical range</td>
<td>Limited (2 orders of mag.)</td>
<td>Large (4 orders of mag.)</td>
</tr>
<tr>
<td>Instrumentation</td>
<td>Standard microplate reader</td>
<td>More restricted</td>
</tr>
</tbody>
</table>

AlphaLISA for Biomarker Analysis

AlphaLISA is specifically designed to meet requirements for high-throughput assays for detecting analytes of various sizes – from a small molecule such as oestrogen, to protein–protein complexes, to full-size phages. AlphaLISA is ideal for complex samples such as serum and plasma.

The AlphaLISA is a bead-based homogenous proximity assay. Two beads, the donor (blue bead) and the acceptor (red bead), are coated with analyte-specific antibodies. The beads are brought into proximity through binding to the analyte. When excited by laser (680nm), the donor bead releases singlet oxygen. This reaction is highly amplified with 60,000 singlet oxygen molecules produced. The singlet oxygen can travel 200nm in solution before decay so if the donor and acceptor beads are within that distance, energy transfer occurs. The singlet oxygen molecules react with chemicals in the acceptor beads to produce a luminescent response. A highly intense luminescence is given off at a wavelength of 615nm.

The technology is based on PKI’s Amplified Luminescent Proximity Homogenous Assay (AlphaScreen®) and employs oxygen channelling chemistry developed initially as a diagnostic detection assay platform under the name of LOC®. AlphaLISA exploits the short diffusion distance of singlet oxygen to initiate a chemiluminescent reaction near the site where it was formed. The technology comprises two discrete polystyrene beads, designated as ‘donor’ and ‘acceptor’. Each donor/acceptor pair can be separated by as much as 200nm and retain efficient energy transfer. This relatively large distance allows greater flexibility in the choice of analyte that can be studied, and thus accommodates assays for larger molecules such as full-length proteins, immunocomplexes and others (see Figure 1). Because the lifetime of the singlet oxygen reactive species in water is very short (approximately four microseconds), the donor and acceptor beads need to be bound to one another to generate a signal. Beads that do not bind exhibit a very low singlet oxygen concentration that contributes minimally to the background signal. AlphaLISA emission is intense and better defined spectrally (615nm) than traditional ELISA technology and is

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less prone to matrix interferences from compounds such as haemoglobin or transferring. AlphaLISA assays require only small sample volumes (5μl), yet their analytical range is more than 100-fold greater than ELISA. Because AlphaLISA is highly amplified and can employ the same antibody pairs, assays using this approach are as selective as conventional ELISAs, but more sensitive.

AlphaLISA has been tested for detection and quantification of analytes from cell culture supernatants, cell lysates and serum/plasma samples from small molecules up to large complexes, immunoglobulin Gs (IgGs) to full-size phage particles. Excellent performance was demonstrated with dynamic ranges up to 4.5 log of analyte concentration in the sample, and sensitivities below 1pg/ml with high accuracy and precision. Among the specific assays that have been developed using AlphaLISA for these purposes are quantification of human insulin in serum, quantification of amyloid Beta 1-40 peptide (A-40) in cell culture supernatants, measurement of contaminant CHO host cell proteins (CHOP) in cell lysates and detection of bulky protein chondroitin sulphate proteoglycan.3

Poulsen and Jensen reported direct comparisons of AlphaLISA and ELISA assays to measure human insulin in plasma samples.4 The samples were simultaneously analysed using the AlphaLISA and a conventional ELISA method employing the same antibody pairs. Assay sensitivity was in the sub-picomolar range with 4 logs of dynamic range. The assay also had excellent intra- and inter-assay precision and was unaffected by plasma and serum matrices. Furthermore, AlphaLISA detected 15-fold lower levels of analyte than conventional ELISA, while using one-fifth of the sample volume. It also had more than 100-fold greater assay range with similar precision levels. The authors thus concluded that the AlphaLISA platform provided a viable alternative to ELISA and, being homogenous, was more amenable to automated fluid dispensing systems.

AlphaLISA and Cellular Kinase Assays

Protein kinases are a large family of enzymes that catalyse the phosphorylation of the target substrate by the addition of a phosphate group from adenosine triphosphate (ATP), converting ATP to adenosine diphosphate (ADP) in the process. Kinases comprise two major families – those that catalyse phosphorylation of tyrosine residues and those that phosphorylate serine or threonine residues.

Although cell signaling events in complex samples such as phosphorylation – as well as proteolysis, ubiquitination, sumoylation, and glycosylation – remain difficult to measure, AlphaLISA has shown excellent results and thus opens pathway mapping to new levels of simplicity, economy and precision.

Several technologies besides AlphaLISA have been developed as ELISA alternatives. AlphaScreen bead-based assays (PKI) were developed to detect the function of both kinase families.5 6 7 8. AlphaScreen can be separated by as much as 200nm and retain efficient energy transfer. Most are only usable in a cell-free context involving highly purified kinases or kinase domains. However, modified AlphaScreen assays have been developed to measure kinase phosphorylation of endogenous substrates, including other kinases in the signalling pathway. TGR Biosciences recently developed a technology, SureFire™, that utilises Alphascreen technology to measure endogenous protein phosphorylation in cell lysates, without washing or blotting. SureFire is thus an alternative to Western blotting and a wide range of assays are now available under an agreement with PKI. As a result this is one of the few technologies capable of studying kinase function in cellular context, particularly using protocols adaptable to high-throughput screening (HTS) instrumentation.

Two other technologies of note are the homogenous fluorometric microvolume assay technology (FMAT) and electrochemiluminescence (ECL).3 9 10 FMAT can be formatted such that biotinylated antigen interacts with streptavidin-coated beads. An AlexaFluor-647-tagged antibody binds to the antigen and a luminescent signal is emitted from the aggregated beads. The specific signal is then discerned from background using laser-scanning microscopy. In an ECL assay, biotinylated antigen is bound to streptavidin-coated electroplates, and binding of ruthenium-labelled anti-mouse antibodies in close proximity to an electroplate results in a chemiluminescent signal.

Like AlphaLISA, FMAT and ECL technologies are highly sensitive, do not require extensive washing and can be formatted for HTS. Beasley et al. have very recent preliminary data that compared FMAT, ECL and AlphaLISA methods to detect common biomarkers.11 Here, AlphaLISA exhibited higher sensitivity and required lower sample volumes than the other assays. They also indicated that ECL and FMAT techniques involved the use of specialised detection instrumentation (laser scanning microscopy for FMAT) and equipment (electrochemical plates for ECL) that limited their flexibility yet increased assay cost. ECL was also limited by the high costs of the consumable electrochemical plates required.

Summary

As with any technology there are advantages and limitations to the AlphaLISA platform. The main concern of AlphaLISA is that it is sensitive to intense light or long exposure to ambient light, a problem that is easily overcome by simple assay adjustments. Singlet oxygen can be sequestered by compounds in screening libraries that can scavenge radical oxygen. Donor bead photo bleaching can be a limitation as the system is effectively limited to a single read. Nonetheless, AlphaLISA has greater flexibility than technologies such as ECL and FMAT, all three of which require a high-energy laser excitation source.

The greatest advantage of AlphaLISA is that it is applicable to a very broad range of analytes. The assays are homogeneous (no wash), rapid, highly robust and more sensitive than previously reported immunossay methods. They are economic from both a reagent use and assay time perspective and are ideal for HTS applications. Furthermore, AlphaLISA assays do not require insertion of large fluorescent epitope tags that can sterically hinder the molecular interactions. AlphaLISA can be employed in crude biological fluids such as cell lysates, serum and plasma to measure enzyme activity and cellular/body fluid matrices that do not easily affect the assay read-out.

Conclusion

AlphaLISA can be used to measure a diverse range of molecular interactions of interest across drug discovery. The homogenous nature of the technique allows it to be an important tool in high-throughput screening of new small molecules and most recently of novel protein therapeutics. For example, it has been used for hybridoma-screening for thousands of clones that express antibodies for therapeutic development. Such screening presently involves use of conventional ELISAs, which, as noted above, are less adaptable for high-capacity screening and potentially more costly.
Cell signalling events in complex samples such as phosphorylation, proteolysis, ubiquitination, sumoylation and glycosylation remain difficult to measure. However, other AlphaScreen and SureFire technologies have now been used to successfully screen against these processes. Consequently, novel small molecules or biotherapeutics may be identified to treat disorders such as Alzheimer’s, Parkinson’s and Huntington’s disease, as well as oncology and various immunological disorders.