Application Notes

The AcroWell™ Plate: Low Fluorescence Background Using the DELFIA® System

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WHY FLUORESCENCE DETECTION?

One of the most powerful tools for receptor research and drug discovery is the use of ligand/receptor affinity screening of combinatorial libraries (Liu et, al., 1998). Early work involved the use of radioactive ligands to identify a binding event; however, there are numerous limitations involved in the use of radioactivity for High Throughput Screening (HTS). These limitations include but are not limited to health concerns for researchers, as well as problems with waste disposal resulting from the numerous samples processed. These factors have led to the development of highly sensitive non-radioactive alternatives to investigate ligand/receptor interactions. A sensitive and reliable labeling and detection system has been designed and patented by PerkinElmer Life Sciences (Turku, Finland). It uses lanthanide chelates that give an intense and long-lived fluorescence emission (millisecond range), making it possible to measure fluorescence after a delay time. This eliminates the background counts from short-lived fluorescent emissions from organic fluorophores that accompany the sample, because they will have decayed prior to detection. This time-delayed fluorescence, in combination with a large Stokes' shift (340 - 615 nm), effectively reduces background emissions to a level that allows measurement sensitivity to rival and possibly exceed that achieved using expensive and dangerous radioactive tags.

Numerous reports exist discussing the successful use of the lanthanide chelate labeling system to label antibodies for immunoassays as well as ligands for HTS receptor binding assays (Hemmilä and Webb, 1997; Appell et. al., 1998; Liu et. al., 1998; Inglese et. al., 1998). The DELFIA® system from PerkinElmer Life Sciences allows researchers to detect binding of a labeled ligand to a receptor after washing to remove unbound-labeled ligand. Cell-based assays are particularly well suited to the DELFIA system because of its broad detection range and high sensitivity (below 1.0 fmol europium). This degree of sensitivity can only be achieved when wash steps effectively remove unbound ligand and background is low. The most efficient way to remove binding and wash solutions, and minimize target loss, is through the use of a filter plate. Use of filter plates in fluorescent assay systems has, however, been limited because of high background fluorescence from the filter material.

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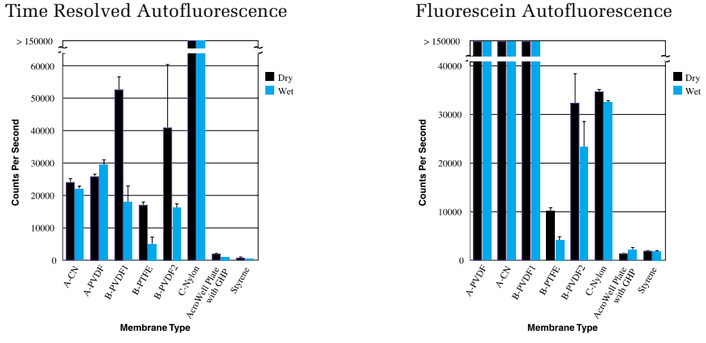
EVALUATION OF THE ACROWELL LOW-FLUORESCENCE 96-WELL FILTER PLATE

Whole cell, membrane fragment, or bead-based screening assays work best if the solutions can be washed by filtration rather than by the standard flushing of a plate washer. The need for a minimum population of cells as well as the requirement for the highest sensitivity does not lend itself well to further miniaturization or fluorophore proximity systems. Cell-based assays, particularly low-affinity assays, require high sensitivity and a 96-well filter plate that has a low fluorescence background.
AcroWell Filter Plate features:

- Low background fluorescence
- No crosstalk between wells
- No weeping of solution
- Robotic compatibility
- High chemical resistance
- Low non-specific binding

In the past, filter-bottom plates had a number of shortcomings that prevented them from being useful for fluorescent HTS applications. The primary problem was the high fluorescence background of the membrane. Currently-available filter-bottom plates contain membranes such as hydrophilic PVDF (polyvinylidene fluoride) or nylon that give an unacceptably high fluorescent background. A low-fluorescence hydrophilic PTFE (polytetraflouroethylene) containing plate has been available but of limited use because its large pore size (5 µm) allows cells or membrane fragments to pass through the membrane. Low affinity binding is impossible to detect if the plate has high background fluorescence.
Problems with currently-available filter plates include:
1. Robotic handling incompatibility or actual jamming in the detector (two piece-part plates are particularly problematic).
2. Loss of detection solution due to weeping during incubation and detection (leakage prevents the implementation of full automation).
3. Loss of flow during filtration due to either low bubble point (membrane allows air to pass) or inconsistent seals. This requires either the filling of all wells or the addition of plugs to the well that empties early and relieves the vacuum.
4. Incorporation of materials with limited chemical resistance, high background fluorescence, or which promote non specific binding of labeled compounds (such as styrene or glass-impregnated polypropylene).
The AcroWell™ filter plate with GHP membrane avoids the common filter plate problems described above. In this paper we will describe the primary attributes of the AcroWell plate and report its utility in receptor/ligand binding assays.

Figure 1



All 96 wells of the AcroWell filter plate and competitor plates (A, B or C) were read on a Wallac VICTOR™ multilabel counter using wavelength settings optimized for either Fluorescein (excitation @485 nm, emission @535 nm) or Time Resolved Fluorescence (excitation @340 nm, emission @615 nm). Dry plates were read directly, while wet plates were read after vacuum (10 in. Hg) filtration of 200 µL distilled sterile water. Autofluorescence values for the AcroWell plate with GHP membrane were not significantly higher then values for the plain styrene plates (no filtration membrane).

***A-CN** = 0.45 µm Cellulose Nitrate, **A-PVDF** = 0.45 µm PVDF, **B-PVDF1** = 0.22 µm PVDF with an opaque plate, **B-PTFE** = 5.0 µm hydrophilic PTFE, **B-PVDF2** = 0.22 µm PVDF, **C-Nylon** = 1.2 µm Nylon, **AcroWell Plate with GHP** = AcroWell plate with 0.45 µm GHP membrane, **Styrene** = standard microtiter plate without a filtration membrane.*

LOW FLUORESCENCE BACKGROUND MEANS GREATER SENSITIVITY

The AcroWell filter plate consists of two parts, a clear polystyrene lid and a chemically resistant/biologically inert polypropylene filter plate assembly. The plate assembly's rigid single-piece construction meets the design recommendations of the Society of Biomolecular Screening (Danbury, CT). The plates are stackable with or without the lid in place, and fit most standard vacuum manifolds. Two membrane layers are sealed to the bottom of each well using a patented process that minimizes well-to-well crosstalk and weeping. The upstream GHP membrane has a nominal pore size of 0.45 µm. The downstream membrane layer is hydrophobic Emflon® (PTFE) membrane, which protects the GHP membrane and acts as a barrier to flow, allowing longer incubations while the wells are filled with solution.

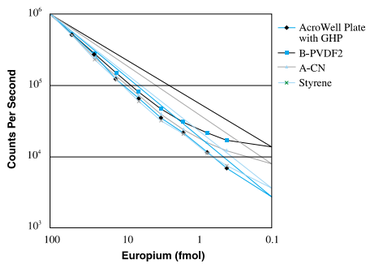
Background Fluorescence: The background fluorescence of the AcroWell™ and competitor plates was determined. Measurements were made before (dry) and after (wet) filtration of water to determine if any extractables were present that either masked or caused autofluorescence. Some of the competitor PVDF plates did have fluorescent extractables; but even after rinsing, the background was still extremely high with some background emissions consistently above 150,000 counts per second.

Our data show conclusively that the GHP membrane-containing AcroWell plate has a background fluorescence that is many times lower than the nearest competitor (Figure 1). Its fluorescent background emissions are similar to that of a plain styrene 96-well plate without a filter membrane. The extremely low fluorescence background for the AcroWell plate was seen for both time-resolved (left graph, excitation Q340 nm, emission Q615 nm) and fluorescein (right graph, excitation Q485 nm, emission 8535 nm) wavelengths.

Europium Detection: The benefit of extremely low background fluorescence is that significantly lower concentrations of fluorescent label can be detected. We verified this by comparing the ability of the AcroWell plate and competitor plates to detect serial dilutions of Wallac's Europium Standard Solution. The data show that at europium levels above 100 fmol/well the competitor and AcroWell plates perform equivalently. However, at levels below 100 fmol/well, the linearity of the measurements, and thus the ability to accurately detect trace europium, is impaired by the high background emissions of competitor plates (Figure 2).

Figure 2

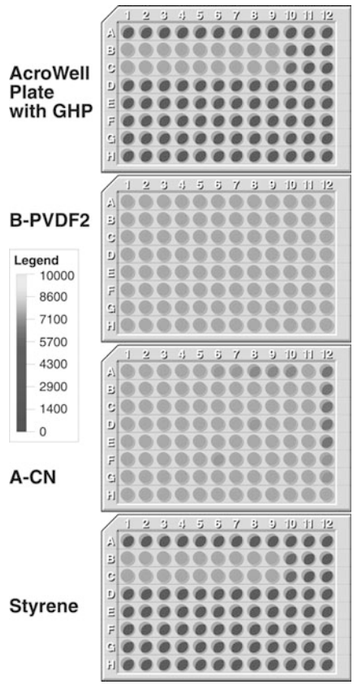
AcroWell Filter Plate Dilution Series



Serial dilutions of Wallac's 1.0 nmol/L Europium Standard Solution was read in AcroWell and competitor plates using a VICTOR™ multilabel counter. A master mix containing a stepwise dilution series beginning at 200 fmol/well and ending at 0.4 fmol/well was placed in duplicate in rows B and C of each plate. The graphic image on the right was taken directly from the VICTOR™ software window showing the false color image over a linear range of 0 to 10,000 CPS. The sequence of captured images is AcroWell Plate with GHP, B-PVDF2, A-CN, and Styrene from top to bottom respectively.

The graph shows the normalized data for the respective plates. The AcroWell plates and plain styrene plates can reliably detect to 1.5 fmol of europium standard while the competitor plates can only distinguish europium concentrations above 12 fmol because of high background fluorescence.

AcroWell Plate with GHP *= AcroWell plate with 0.45 µm GHP membrane,* ***B-PVDF2*** *= 0.22 µm PVDF,* ***A-CN*** *= 0.45 µm Cellulose Nitrate,* ***Styrene*** *= standard microtiter plate without a filtration membrane.*



However, even at levels below 10 fmol, the AcroWell plate is capable of quantitatively detecting the europium fluorescence emissions. This data is consistent with the background fluorescence data (Figure 1); the AcroWell plate has the same background and sensitivity characteristics as the plain styrene plate. The AcroWell plate and plain styrene plates allow reliable detection of as little as 1.5 fmol of europium standard while the competitor plates can only detect europium concentrations greater than 12 fmol because of their high background fluorescence.

An equally important factor in low level fluorescence detection is the signal-to-noise ratio. At signal-to-noise ratios below 5:1 it is difficult to be sure that the measurement is a real signal above background. The AcroWell plate and styrene plate do not have a signal-to-noise ratio below 5:1 until europium levels are below 1 fmol/well (Table 1). Competitor filter plate signal-to-noise ratios drop below 5:1 at europium levels below 13 fmol/well.

Table 1

Signal-to-Noise Ratios of Dilute Europium Samples[1]

Europium (fmol/well)[2]	Plate Type: AcroWell Plate	B-PVDF	A-CN	Styrene
13	53:1	8:1	6:1	60:1
6	27:1	4:1	3:1	30:1
3	14:1	3:1	2:1	16:1
2	8:1	2:1	1:1	10:1
1	5:1	1:1	1:1	5:1

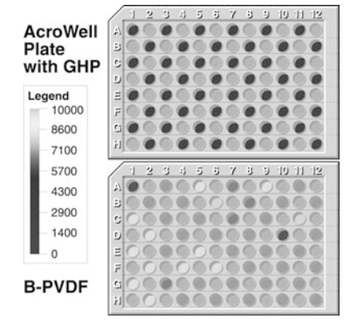
1. Signal-to-noise ratios were calculated from the measurements illustrated in Figure 2 by dividing the total activity at each Europium concentration by the fluorescence background activity of the respective plates.

2. Based on the assumption that when using the DELFIA system, each labeled antibody has an average of 10 europium conjugates bound, detection of 1.0 fmol of europium should be equivalent to the detection of 0.1 fmol of labeled antibody.

Crosstalk Minimization: A 96-well filterbottom plate also needs to be able to filter and incubate solutions while preventing cross contamination. The AcroWell plate uses a patented sealing technology that eliminates both well-to-well crosstalk (a result of lateral flow of solution between wells) and weeping of solution through the membrane due to capillary action or gravity. A hydrophobic Emflon® membrane is welded together with the GHP membrane to the bottom of each well. The hydrophobic Emflon membrane layer contains small holes in the center of each well which allow the fluid to pass only when vacuum is applied. These holes are small enough to prevent solution loss during incubation and detection. At incubations up to 24 hours (Figure 3), no solution was lost.

Absence of crosstalk was confirmed by loading alternating wells with either Enhancement Solution alone or Enhancement Solution containing approximately 10 fmol europium. The plates were then incubated for 24 hours at room temperature. Average counts for AcroWell plates (n=4) for wells containing europium were 95,000 ± 16,000 CPS (counts per second); adjacent wells without added europium had 2400 ± 880 CPS. These data indicate that no lateral flow (crosstalk) of europium occurred (Figure 3) because the background fluorescence of the Enhancement Solution is typically 2000 to 3000 CPS when detected in either plain styrene or the AcroWell plate.

Figure 3
AcroWell Plates Minimize Crosstalk



The Acrowell and a competitor PVDF plate were read using Wallac VICTOR™ multilabel counter after the addition of Europium Standard Solution (10 fmol per well) to every other well creating a checkerboard pattern. Blank wells contain only Enhancement Solution. The graphic image above was taken directly from the VICTOR software window showing the false color image over a linear range of 0 to 10.000 CPS.

Average counts for the competitor plate (n=2 plates) for wells containing europium were 67,000 + 8,400 CPS; adjacent wells without added europium had 27,000 ± 5100 CPS. Signal-to-noise ratios at 10 fmol europium were 40:1 for the AcroWell™ plate and 2.5:1 for the competitor plate. This poor signal-to-noise ratio prevents us from conclusively determining whether crosstalk occurred in the competitor plate. During incubation, it was also observed that a number of wells in competitor plates leaked (weeped) solution out of the wells, potentially skewing the data. The image taken from the program for the AcroWell and competitor PVDF plates illustrates the problem in discriminating signal from noise when the background is high (Figure 3).

DELFIA® ASSAYS USING THE ACROWELL FILTER PLATE

The utility of a low fluorescence filter plate is best demonstrated by measuring a receptor/ ligand interaction and verifying that the same sensitivity is seen with the AcroWell plate as with the plain styrene plate. When filtration is not used, as with the plain styrene plate, receptor/ligand binding occurs in the wells and unbound label is removed by washing with a plate washer. Plate washing only works effectively if the cells, beads, or membranes are tightly bound to the plate being washed. Even if the target is not washed away by the plate washer, inconsistent removal of unbound label can give false positive results. Using the AcroWell plate, targets are retained on the 0.45 µm filtration membrane and easily washed. A low fluorescence filter plate allows immediate detection within the plate, eliminating the requirement to transfer the samples to a plain styrene plate.

A high-throughput researcher performed a binding assay using a previously characterized receptor/ligand binding system that was developed in the absence of filtration. (The identity of the receptor/ligand system has been withheld at the request of the researcher.) During this research, several key questions needed to be addressed to determine the utility of the AcroWell plate for receptor/ligand binding assays:

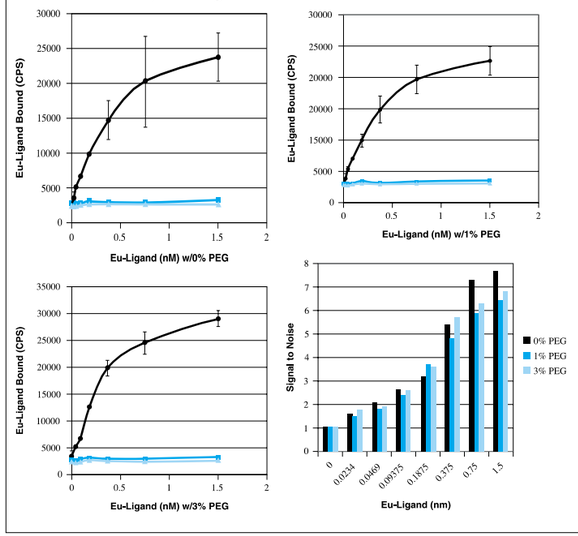
1. Does the AcroWell plate have a uniform low fluorescence background in all 96 wells under binding assay conditions?

2. Can saturation binding be measured in a standard binding assay performed at low signal strengths?

3. Is the signal-to-noise ratio at low signal strengths adequate to identify a positive "hit"?

4. Is it possible to measure competitive inhibition of ligand binding without interference from high background fluorescence?

Figure 4
DELFIA® Assays Using the AcroWell Plate



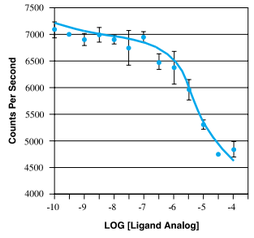
Europium-labeled ligand was bound to a target receptor found in lysed cellular membrane fragments (specific receptor and ligand identities are currently confidential). Three independent readings for each data point were made on AcroWell plates. Increasing concentrations of labeled ligand were added to determine signal to noise at saturation in three solutions, each containing the indicated concentration of polyethylene glycol (PEG). The total binding activity (blue) approached saturation at 1.5 nM Eu-ligand while the non-specific binding (red) and background plate fluorescence (yellow) remained extremely low. Signal-to-noise ratios were calculated as total binding/ nonspecific binding and plotted for all three PEG concentrations in the lower right corner.

Receptor/Ligand Binding Assays: A binding assay was set up using targets embedded in cell membrane fragments. Three concentrations of polyethylene glycol (PEG) were added to the assay buffer and, following the binding of europium labeled ligand, the samples were washed thoroughly. Increasing concentrations of labeled ligand were added from 0 to 24 nM. Saturation was achieved at 1.5 nM and the data at higher concentrations are not shown (Figure 4). Filtration was performed at 1000 x g using a low-speed centrifuge with a rotor designed for 96-well plates. Subsequent ligand binding assays have been performed using vacuum (10 in. Hg) with similar results (data not shown).

Signal uniformity was confirmed by adding a constant quantity of target and ligand across the plate during binding assays. Under these conditions, the AcroWell plate exhibited a well-to-well variation of less than 10%. Overall, the data demonstrate tight standard errors and saturation binding kinetics curves similar to those seen previously using a plain styrene plate for detection (data not shown).

Using AcroWell plates, the signal-to-noise ratio was 7:1 at 1.5 nM labeled ligand. This signal-to-noise ratio was achieved at a fluorescent count of around 18,000 CPS using a VICTOR™ multilabel counter. It is important to note that following filtration washes and addition of Enhancement Solution, competitor plates produce background fluorescence counts near 15,000 CPS while the AcroWell plate background fluorescence remains below 2500 CPS. Therefore, under these typical assay conditions a competitor plate would only give a 1:1 signal-to-noise ratio, making detection of positive hits unlikely. In contrast, the use of the AcroWell plate allows researchers to easily detect new drug candidates.

Figure 5
Binding Inhibition Using Ligand Binding Agonists



Europium-labeled ligand was bound to the same target receptors as in Figure 4 at a ligand concentration of 0.2 nM Eu-ligand. Increasing concentrations of a ligand analog were added to separate wells and each data point was assayed as before in triplicate directly in the AcroWell™ plate.

Competitive Inhibition: When a new drug lead is detected it is always important to verify that the positive hit is actually binding to the target and not to some other receptor or non-specific binding activity. Because the AcroWell plate is made of inert polypropylene it is unlikely that the plate itself will bind labeled ligands. To eliminate the contribution of other non-specific interactions, a second assay must be performed. The most effective way to verify target-binding specificity is to set up an assay series at ligand concentrations well below saturation and add increasing concentrations of a second molecule that is known to compete for binding to the specific target receptor. In order to accurately measure competitor inhibition, it is critical that filter plate background fluorescence does not mask the Europium signal as signal strength declines.
A low affinity binding assay was set up using labeled ligand concentration of 0.2 nM and 3% PEG, increasing concentrations of inhibitor (ligand analog) were added to the assay mixture and allowed to interact. Following washes and the addition of Enhancement Solution, the assays were counted directly in the AcroWell plate. The resulting data indi-

cate that the analog specifically competes with the labeled ligand binding and the binding constant was calculated at 1.27 x 10^{-6} nM inhibitor (Figure 5). Note that the detection of europium labeled ligand ranged from 7300 to 5000 CPS. Competitor plates would not allow detection of binding and inhibition at this level due to their high background fluorescence.

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