TECHNICAL NOTE

MultiMode Detection

Choosing the Right Measurement Conditions for Alpha Experiments

Introduction

Alpha (amplified luminescent proximity homogeneous assay) is a versatile, bead-based platform for efficient, highly adaptable assays to study a variety of targets – all in one well, with no wash steps. It is part of a complete assay platform solution that includes detection and automation instrumentation, and custom assay development; providing everything needed to ensure consistent, reproducible results across a wide variety of applications.

The first step is to find an Alpha assay optimized for your biological question. Assay kits for a large amount of cellular targets are already available from PerkinElmer, but if this should not be sufficient, it is possible to create a customized assay.

Beyond the optimization of the assay biology itself, an Alpha-capable plate reader is required. PerkinElmer microplate readers come with predefined protocols, but it is helpful to understand which parameters and settings are important.

- Reading times per plate are influenced by the protocol parameters, but so is sensitivity. How do they interact with each other?
- Are there differences in the optical setups of PerkinElmer microplate readers?
- Which plate is the best for an Alpha assay?
  - Does the plate colour really play a role?
  - And what is the advantage of PerkinElmer's AlphaPlate?

This technical note will provide answers to these questions.

Table 1. Current availability of the two different Alpha setups on PerkinElmer's Microplate Readers.

	Standard Alpha	HTS Alpha
EnVision®	+	+
EnSight®	-	+
VICTOR® Nivo™	+	-

PerkinElmer® For the Better

What is the Advantage of PerkinElmer's High-throughput Screening (HTS) Setup?

PerkinElmer microplate readers offer two options for Alpha measurements: Standard Alpha and HTS Alpha (see Table 1). The Standard Alpha technology is based on an epifluorescence system, known from standard microscopy. Other technologies follow the same principle, e.g. Fluorescence and Time-Resolved Fluorescence. The Standard Alpha Technology setup is shown in Figure 1 (a).

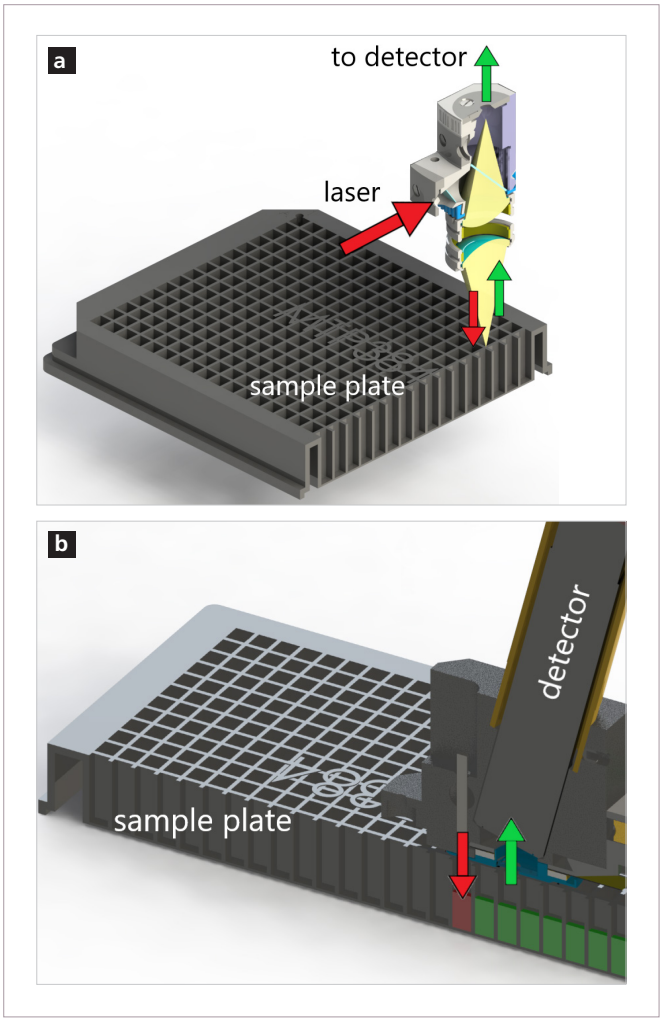

Figure 1. (a) Standard Alpha technology setup. The mirror module guides the laser excitation light (red arrow) to the sample and the emission light towards the detector (green arrow). (b) In the HTS Alpha setup the optical path of the laser excitation (red) is split from the detection path (green arrow) allowing extremely efficient detection of the emitted light .

Excitation light from a 680 nm diode laser is guided via an optical fibre to the Alpha mirror module. A dedicated dichroic mirror reflects the laser light to the sample, and a lens focuses it into the well. If donor and acceptor Alpha beads are in close proximity to each other, emission light is created with a wavelength in the green / red range. This light is transmitted by the mirror module, passes an emission filter and is finally guided onto the detector. This setup is available on the EnVision and VICTOR Nivo multimode plate readers as an optional technology.

The HTS Alpha setup makes use of the several seconds long emission time of the excited alpha sample and establishes an extremely sensitive light detection mode. In this setup, the excitation and emission beam paths are separated from each other. As can be seen in Figure 1(b), the laser fibre (in red) is placed next to the detector unit. When a measurement is started, the sample in a well is first excited by the laser, with the laser fibre a short distance directly above the well. After this excitation, the plate is moved and the next well is excited. With the same movement, the previously excited well is placed under the detector and is measured right after the excitation of the second well. As the entrance aperture of the detector is almost in contact to the sample carrier surface, all light emerging from the sample can be collected by the detector system, while scattered light from neighboring wells is blocked.

An advantage which is discussed in more detail elsewhere is the capability for Standard Alpha to run MultiPlex Assays.[1] The emission light can be split into two different wavelength channels, so that two different types of acceptor bead types can be distinguished in a single well simultaneously. This requires some space for light-guiding and filtering elements and is therefore not available on the HTS option.

Compared to Standard Alpha, the delay time between excitation and emission is longer in the HTS setup. Alpha signal decay occurs on a predictable time scale, making such measurements possible. The advantage of the HTS setup is the absence of long optical pathways and optical elements inside such a light path. A greater amount of light can be collected at the input aperture and less light is lost, for example on lens surfaces or at apertures on the way to the detector. As a result, the amount of light required to achieve excellent sensitivity can be collected in much shorter time, thus reducing the time per plate. Alternatively, sensitivity will improve significantly when the measurement time per well is not reduced. The HTS technology setup is unique in PerkinElmer instruments.

The difference in performance can be seen in Figure 2 which shows the sensitivities of Omnibeads (PerkinElmer, #760626D) for Standard Alpha vs. HTS Alpha. In this example, 25 µl of 20 µg/ml Omnibead solution was pipetted into white 384-well AlphaPlates, with 18 high samples and 100 background samples in total. The total measurement time (excitation time + emission time) and emission times were set to identical values of 550 ms / 180 ms, respectively in a first experiment (shown in blue) and 200 ms / 100 ms shown in red for a second experiment. Due to the more sensitive optical setup, the sensitivity can be reduced from 200 pg/well to 22 pg/well for the shorter measurement times per well (red bars), and from 44 to 10 pg/well for the longer measurement times (blue bars) when switching from Standard to the HTS Alpha technology. The graph in Figure 2 also enables comparison of the effect of different measurement times per well for the same technology: a longer measurement time per well improves the sensitivity. This effect is much larger for Standard Alpha than for HTS.

In many cases the sensitivity is limited by the assay itself, so the instrument's intrinsic sensitivity is not fully utilized. In those cases it is possible to reduce the measurement time per well to increase the measurement speed – this approach is especially useful when measuring many plates. In the example shown in Figure 2, the measurement time for a full 384-well plate with an assured sensitivity < 50 pg/well was two minutes for HTS Alpha and four minutes for Standard Alpha – the throughput is doubled with the Alpha HTS setup. Another benefit of a higher measurement speed is an increased temperature tolerance. The temperature in the measurement chamber is controlled to be at ambient and on the EnVision with Standard Alpha. The dedicated temperature control module may be used for more specific temperature control. Signal levels of Alpha Assays are to great extent affected by temperature changes, and if a plate was not properly equilibrated before the measurement, this could lead to false readings. Increasing measurement speed allows the plate to finish before a possible temperature difference between sample and instrument chamber becomes detectable, making later mathematical correction unnecessary.

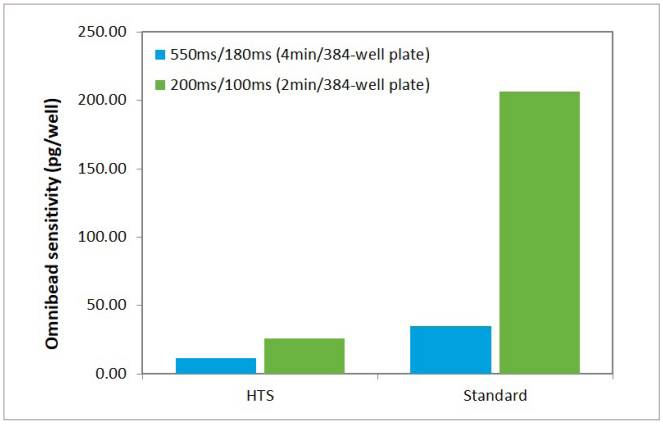

Figure 2. Omnibead sensitivity for HTS Alpha and Standard Alpha and its dependence on the measurement time per well (exc 550 ms/ em180 ms and exc 200 ms/ em 100 ms) and hence time per plate. Sensitivities of < 50 pg/well can be reached after two min per full 384-well plate using HTS Alpha and four min per full 384-well plate using Standard Alpha.

If measurement times per plate are kept comparable, the HTS setup offers increased sensitivity compared to the Standard setup. Alternatively, it can be used to speed up the measurement process and increase the plate throughput of the instrument while maintaining a comparable level of sensitivity to the Standard Alpha setup. For a summary, please see Table 2.

Table 2. Summary of advantages (+), disadvantages (-) and balanced (o) properties of the different Alpha setups.

	Standard Alpha	HTS Alpha
Sensitivity	o	+
Speed	o	+
Multiplexing	+	-

What is the Advantage of PerkinElmer's AlphaPlate?

Microplates have important influence on assay quality in general. For example, Fluorescence-based assays benefit from the suppression of background light from black plates. On the other hand, assays working with a generally lower emission light intensity and with a technology where the excitation light does not contribute to the background noise work best in white plates. The white well surface has higher reflectance, increasing the signal emerging from the well. This is particularly the case for Luminescence applications, where no excitation is required. These considerations generally hold true for Alpha Assays as well, since the excitation light is switched off at the time of detection. Therefore, white plates are usually favoured for Alpha assays.

An important parameter affecting the quality of Alpha assays is the well-to-well crosstalk. It is firstly caused by wells which were previously excited by the laser and still emit a small amount of signal. Secondly, wells partially excited by scattered laser light contribute to the crosstalk signal. Such an unwanted excitation causes a bleaching of the Alpha samples before the actual measurement, in turn causing a decreased signal. Therefore, unwanted excitation of neighboring wells containing Alpha samples can result in signal inhomogeneity over the plate. Such an effect is typically more pronounced in wells of the outer rows or columns. With the typical wavelengths of around 600 nm, emission light of Alpha samples can, to a small extent, penetrate through the material of white plates, which are optimized for luminescence applications. The emitted light from bright wells can either directly or indirectly excited, spill into neighbor wells and may lead to signal overestimation of darker wells. Competitive binding assays are especially affected by this.

Avoiding this requires significant effort to arrange the plate maps such that samples expected to show high counts are not located next to samples expected to be low, or to include blank wells in between. Such an arrangement is not possible in all cases and reduces the amount of samples per plate, thereby reducing throughput.

Crosstalk can be automatically corrected by applying certain algorithms at the end of a measurement. Nevertheless, it is far more preferable to avoid it in the first place.

A solution to circumvent these crosstalk issues is to use plates that are designed to keep light from passing through the plate material, like PerkinElmer's AlphaPlates. In Figure 3 and Figure 4, the Signal/Background (S/B) and Crosstalk are compared between white OptiPlates (PerkinElmer, #6007290) and AlphaPlates (PerkinElmer #6005350). Measurements with both setups were taken as above with 25 µl of 20 µg/ml Omnibead solution per well with 18 high samples and 100 background samples in total.

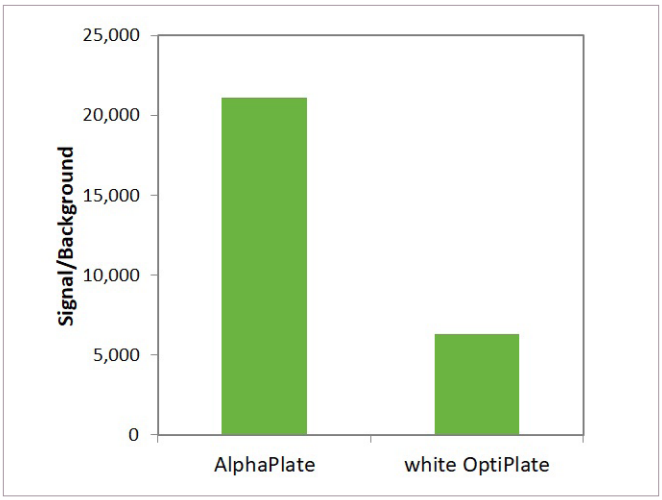

Figure 3. Comparison of Signal to Background of AlphaPlates vs. white OptiPlates.

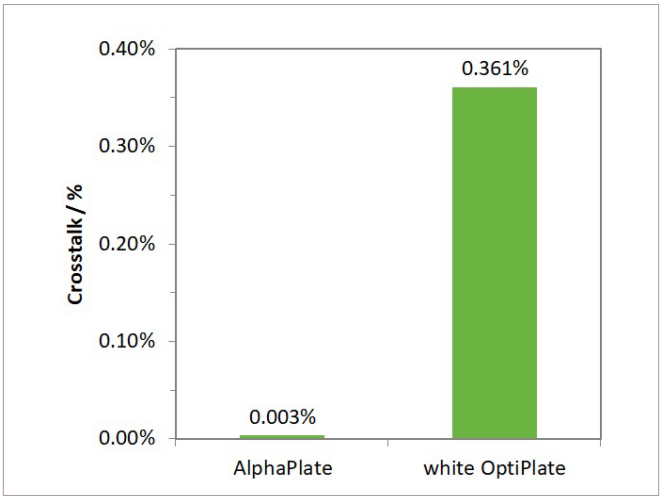

Figure 4. Comparison of Crosstalk for AlphaPlates vs. white OptiPlates.

The grey walls of the AlphaPlate significantly reduce background light, but also show a moderate decrease of the absolute signal intensity. However, in combination this leads to a signal/background increase in this example here by more than a factor of 3 (Figure 3).

At the same time, crosstalk effects were greatly reduced by a factor of more than 100 (Figure 4). Both effects lead to a better assay quality: in this example the dynamic range of the measurement is be limited to ~1:280 for the white plate (assuming relevant dim well close to a bright one) while for the AlphaPlate the dynamic range is 1:33000 (Table 3).

Table 3. Summary of advantages (+), disadvantages (-) and balanced (o) effects of the different plates used for Alpha measurements.

	White OptiPlate	AlphaPlate
Sensitivity	o	+
Crosstalk	o	+
S/B	o	+
z'	+	+

Conclusion

While the Standard setup provides excellent results for Alpha assays and allows Alpha Multiplex applications, the HTS module improves the sensitivity while simultaneously providing a much faster read time per plate. This is especially important for HTS applications, but it can also reduce the time the instrument is occupied in busy labs.

Choosing the right plate is an important aspect when planning Alpha assays. White plates give a very good performance and are often already available in the lab. However, if crosstalk must be reduced and the dynamic range increased to enhance assay quality, PerkinElmer's AlphaPlates are recommended. They combine the two advantages of white, signal-enhancing plate material and crosstalk-supressing properties usually found with black plates.

In conclusion, Alpha HTS technology on either the EnVision (optional module) or the EnSight microplate readers and the use of AlphaPlates are shown to achieve the best speed, sensitivity and crosstalk suppression.

Reference

1. AlphaPlex for EnVision, PerkinElmer Literature no. 011882_01 (2015)

Authors

Alexander Ehlers

Norbert Garbow

PerkinElmer, Inc.
Hamburg, Germany

PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

PerkinElmer

75958 PKI