



## Optimization of Perkin Elmer's AlphaScreen® cAMP Assay on the Synergy™ H4 Hybrid Multi-Mode Microplate Reader

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*PerkinElmer's AlphaScreen® product line represents an important and widely used assay technology for the study of biomolecules, including kinases, GPCRs, biomarkers, etc. The technology uses streptavidin-coated donor beads and acceptor beads typically with an antibody coating specific to the analyte of interest or an affinity tag for generic assays. Assays can be developed using biotinylated antibodies or biotinylated analyte. Diode lasers operating at 680 nm are a common excitation source, but here we demonstrate that the Tungsten lamp component of Synergy™ readers provides satisfactory AlphaScreen performance, as demonstrated using the AlphaScreen cAMP kit.*

### Introduction

AlphaScreen® is a bead-based assay technology used to study biomolecular interactions in a microplate format. The acronym "Alpha" stands for amplified luminescent proximity homogeneous assay. This platform provides a non-radioactive, homogeneous assay which has low background and high signal:background ratios. The assay can be easily automated and miniaturized to a variety of well formats. The assay incorporates two sets of beads, donor and acceptor beads, to generate a signal. The donor bead is illuminated at 680 nm. If the acceptor bead is in close proximity to the donor bead, energy is transferred to the acceptor bead, and light is produced in the 520-620 nm range. If an acceptor bead is not in close proximity to the donor bead, no energy is transferred, and the donor bead falls back to its ground state. The energy necessary to excite the donor bead is commonly provided by a laser which is incorporated into the microplate reader. While this technology works well for AlphaScreen® measurements, it is costly, and cannot always be used with other assay technologies. The Tungsten Halogen lamp used in the Synergy™ H4 Hybrid Multi-Mode Microplate Reader, by comparison, represents a less expensive solution to read AlphaScreen® assays, while still delivering the same high quality data. In addition, the Tungsten lamp is a broad band source useful for a wide wavelength range of excitation and can also be used with other detection modes including fluorescence intensity and fluorescence polarization assays.

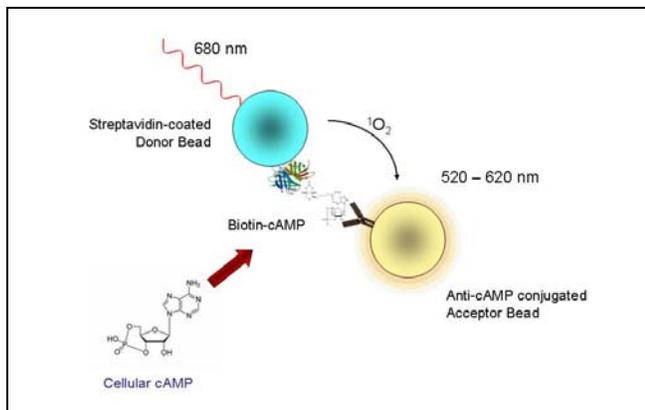
### Synergy™ H4 Hybrid Multi-Mode Reader

The BioTek Synergy™ H4 Hybrid Multi-Mode Microplate Reader was used to excite and measure the signal from the AlphaScreen® cAMP assay. The filter-based system, which incorporates deep blocking filters and a dichroic mirror, along with the Tungsten Halogen lamp, was used with the assay.



**Figure 1.** Synergy™ H4 Hybrid Multi-Mode Microplate Reader. The instrument was used to quantify the signal from all assay plates.

## AlphaScreen® cAMP Assay Chemistry



**Figure 2.** AlphaScreen® cAMP Assay Diagram

Streptavidin-coated donor beads contain a photosensitizer, phthalocyanine, which produces singlet oxygen, an excited and reactive form of  $O_2$ , upon illumination at 680 nm. This form of oxygen has a limited lifetime prior to falling back to ground state. Within its 4  $\mu\text{sec}$  half-life, singlet oxygen can diffuse approximately 200 nm in solution. In the absence of cellular cAMP, the biotinylated-cAMP will bind to the anti-cAMP conjugated acceptor bead, bringing them in close proximity to one another. Energy is transferred from the singlet oxygen to thioxene derivatives within the acceptor bead, subsequently culminating in light production at 520-620 nm. As cellular cAMP is produced, the anti-cAMP antibody will preferentially bind to this form of cAMP, displacing the donor bead/biotin cAMP complex. In the absence of an acceptor bead, singlet oxygen falls to ground state and no signal is produced.

## Materials and Methods

384-well assay plates were purchased from Corning Life Sciences. AlphaScreen cAMP assay kits were purchased from PerkinElmer. Complete plate and kit descriptions are listed in Table 1.

| Component  | Vendor                | Part Number |
|--|-----------------------|-------------|
| 384-Well White, Flat Bottom, Non-Treated Assay Plate | Corning Life Sciences | 3572        |
| AlphaScreen® cAMP Assay Kit                          | PerkinElmer           | 6760625D    |

**Table 1.** Project component list

The Synergy™ H4 was set up using the components and settings listed in Table 2. For optimum assay performance, it is essential that the 680/30 nm filter and filter wheel plug are placed next to each other on the excitation wheel (Ex. 680/30 nm filter in position 1, and filter wheel plug in position 2). The same should be done for the 570/100 nm filter and filter plug on the emission wheel (Ex. 680/30 nm filter in position 1, and filter wheel plug in position 2). The purpose for the dual read, and incorporation of the filter wheel plugs, is to allow the proper delay time between excitation of the donor beads and capture of the signal from the acceptor beads.

Filter switching per well will move the excitation and emission filters, and filter wheel plugs into the correct position for each read.

| Instrument                         |                                       |                  |
|------------------------------------|---------------------------------------|------------------|
| Detection Component                |                                       | BioTek Catalog # |
| Excitation Filter 1                | 680/30 nm                             | 7082229          |
| Excitation Filter 2                | Plug                                  | 708673           |
| Emission Filter 1                  | Plug                                  | 708673           |
| Emission Filter 2                  | 570/100 nm                            | 7082264          |
| Dichroic                           | 635 nm Cutoff                         | 7139635          |
| Optimized Instrument Settings      |                                       |                  |
| Light Source: Tungsten             | Delay after Plate Movement : 250 msec |                  |
| Measurements per Data Point: 15    | Dynamic Range: Extended               |                  |
| Filter Switching per Well: Enabled |                                       |                  |

**Table 2.** Synergy™ H4 AlphaScreen® Assay Instrument Settings

Here we show the utility of the Synergy™ H4 to read the cAMP assay under normal assay conditions. cAMP standard curves were used to compare the signal:background and  $IC_{50}$  value attained using these settings to those listed in the assay manual.

## Experimental Design

- Donor beads and biotinylated cAMP were combined in 1X control buffer to final concentrations of 1 Unit/15  $\mu\text{L}$ .
- Anti-cAMP acceptor beads were diluted in 1X control buffer to a final concentration of 1 Unit/5  $\mu\text{L}$ .
- A 12-point standard curve was setup by serially diluting cAMP in  $\frac{1}{2}$  log increments. The cAMP concentrations of the curve ranged from  $5.0 \times 10^{-6}$  M to  $5 \times 10^{-11}$  M, including a 0 M cAMP positive control.
  - The standard curve was used to generate an  $IC_{50}$  value for cAMP.
  - The  $5.0 \times 10^{-6}$  M and 0 M points on the curve were used to determine the signal:background ratio and  $Z'$ -Factor value for the assay.
- 5  $\mu\text{L}$  replicates of anti-cAMP acceptor beads were added to the Corning 384-well assay plate.
- Fifteen 5  $\mu\text{L}$  replicates of each standard curve point were then added to the wells already containing acceptor beads.
- The plate was mixed for 30 seconds on an orbital shaker, covered, and incubated in the dark for 30 minutes at RT.
- 15  $\mu\text{L}$  replicates of the biotinylated cAMP/donor bead mix were then added to each well.
- The plate was mixed for 30 seconds on an orbital shaker, covered, and incubated in the dark for 1 hour at RT, and then read on the Synergy™ H4.
- Following the initial read, the plate was recovered, incubated for an additional hour in the dark, and then read again on the Synergy™ H4.

## Results and Discussion

The intent of this application note is to demonstrate the capability of the Synergy™ H4 to excite and read the signal from AlphaScreen® assay chemistries with acceptable assay performance using the incubation times prescribed in the assay manual.

### One Hour Incubation Data

The assay plate was read at one hour following the addition of the biotinylated cAMP/donor bead mix to the assay plate, which is the prescribed incubation time described in the assay manual. An inhibition curve and IC<sub>50</sub> value was generated using the replicate data from each point on the standard curve. The signal:background ratio was determined by dividing the average signal of the 0 M cAMP by the average signal of the 1.0x10<sup>-6</sup> M cAMP (final concentration) points on the curve. Finally, a Z'-Factor value was also determined using the average of the two points just described. The data for the one hour incubation time point is shown in Figure 3.

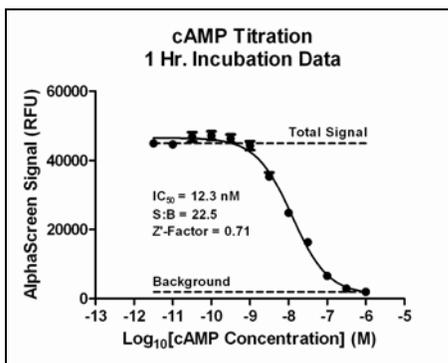


Figure 3. AlphaScreen® cAMP Assay 1 Hour Incubation Data

The results show that the data generated using the Synergy™ H4 compare positively to that listed in the assay manual.

| Validation Component   | Synergy™ H4 Generated Value | PerkinElmer Value |
|------------------------|-----------------------------|-------------------|
| IC <sub>50</sub> Value | 12.3 nM                     | ~7.5–10 nM        |
| Signal:Background      | 22.5                        | ~12.5             |
| Z'-Factor Value        | 0.71                        | NA                |

Table 3. Synergy™ H4/PerkinElmer Validation Data Comparison

Table 3 shows that the determined IC<sub>50</sub> value of 12.3 nM, is well within acceptable experimental variation of the listed range of ~7.5 – 10.0 nM. The signal:background value also exceeds that listed by the kit manual. Finally, the Z'-Factor value of 0.71 is indicative of an excellent assay using the settings previously described.

### Two Hour Incubation Data

The assay plate was also read at two hours post biotinylated cAMP/donor bead mix addition. This was done in order to determine whether a longer incubation time could substantially improve assay results. Figure 4 shows the data from the two hour incubation time point.

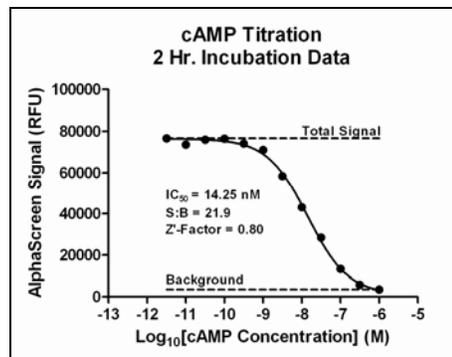


Figure 4. AlphaScreen® cAMP Assay 2 Hour Incubation Data

The results show that while the total signal from the 0 M cAMP point does increase, the signal from the 1.0x10<sup>-6</sup> M cAMP point also increases. Therefore, the signal:background ratio is not affected by the change in signal. What is also evident is that the IC<sub>50</sub> value does not substantially shift with the increased incubation time. If the data from the one and two hour incubation time points is normalized, the inhibition curves show the same curve shape, and the IC<sub>50</sub> values are equivalent.

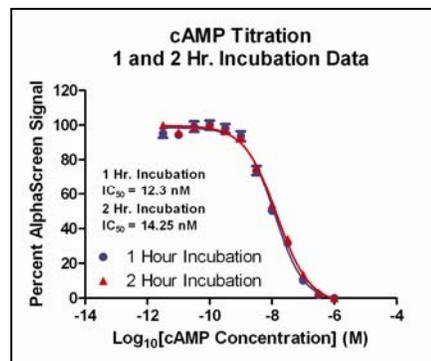


Figure 5. Normalization of AlphaScreen® cAMP Assay 1 and 2 Hour Incubation Data

What is apparent is that the variability among the replicates at each point used for the Z'-Factor determination decreases with the longer incubation time. Because of this, the Z'-Factor value for the two hour incubation time point increases from 0.71 to 0.80. Therefore a longer incubation time may yield higher quality data.

## Conclusions

1. The Synergy™ H4 is able to generate the same data quality as that seen by other AlphaScreen® capable readers.
2. The use of the Tungsten Halogen lamp on the Synergy™ H4 provides a lower-cost, and more flexible solution to read AlphaScreen® assays.
3. The data seen using the recommended assay procedure meets or exceeds that listed in the assay manual.
4. The incorporation of a two hour incubation following the addition of the biotinylated cAMP/donor bead mix can help to improve data quality without sacrificing assay performance.

## References

PerkinElmer AlphaScreen® cAMP Functional Assay  
Technical Manual.

Rev. 3/18/10, AN031810\_08