# Analysis of Potential Compound Interference of AlphaScreen™ Signal

## Introduction

In order to evaluate the effect of various compounds commonly used in assay development and high throughput screening, we analyzed their potential interference on AlphaScreen signal. A series of chemicals, such as color quenchers, antioxidants, detergents, salts, metal ions and others, were tested on the PT66 phosphotyrosine detection assay kit. This generic assay, used to measure tyrosine kinase activities, is based on the detection of a biotinylated phosphotyrosyl peptide by Streptavidin-Donor beads and Acceptor beads conjugated to PT66 anti-phosphotyrosine antibodies.

To discriminate between the effect of the compounds on AlphaScreen chemistry and that related to the binding of the peptide to PT66 antibodies, we conducted a control study in parallel using Streptavidin-Donor and biotin-BSA conjugated Acceptor beads. Since the data obtained using the two systems were similar, only those obtained with PT66 are reported here.

## Principles of AlphaScreen Technology

AlphaScreen is a bead based non-radioactive **A**mplified **L**uminescent **P**roximity **H**omogeneous **A**ssay. When a biological interaction brings the beads together, a cascade of chemical reactions acts to produce a greatly amplified signal. On laser excitation, a photosensitizer in the Donor bead converts ambient oxygen to a more excited singlet state. The singlet state oxygen molecules diffuse across to react with a thioxene derivative in the Acceptor bead generating chemiluminescence at 370 nm that further activates fluorophores contained in the same bead. The fluorophores subsequently emit light at 520-620 nm.

In the absence of a specific biological interaction, the singlet state oxygen molecules produced by the Donor bead go undetected without the close proximity of the Acceptor bead. As a result only a very low background signal is produced.

AlphaScreen provides a highly versatile, sensitive, time-resolved, homogeneous and miniaturizable means to efficiently perform assay development and HTS resulting in higher throughput at lower costs.

To maximize AlphaScreen signal detection, the AlphaQuest™-HTS and Fusion™α Microplate Analyzers were developed with the capability to measure assays in multi-well plates. These instruments use a highly efficient laser diode emitting at 680 nm, fiber optics and specially optimized photomultiplier tubes. Use of the Optiplate-384 NEW microplates is also recommended for best performance.

Fusion™α, AlphaQuest™-HTS microplate analyzers and Optiplate-384 NEW microplates are available from Packard BioScience company.

For further details on the AlphaScreen technology, refer to ASC-001: Principles of AlphaScreen.

## Materials and Methods

The AlphaScreen PT66 Assay Kit is composed of 10x control buffer, biotinylated phosphotyrosyl peptide (biotin-pY-peptide), Streptavidin-Donor and Acceptor beads conjugated to PT66 anti-phosphotyrosine antibodies.

The biotin-pY-peptide was used at 10 nM final concentration, and Donor and Acceptor beads were used at 20 mg/mL each. Compounds were tested at various concentrations within their working range. Ions were tested in 25 mM HEPES pH 7.4 without additives, while the other compounds were tested in HEPES buffer supplemented with 100 mM NaCl.

The protocol used was the following:

1. Mix 5 µL biotin-pY-peptide with 5 µL test compounds in a white opaque 384-well microplate.
2. Add 5 µL Acceptor beads along with 5 µL Donor beads; incubate at room temperature for 1 hour.
3. Detect AlphaScreen signal using either an AlphaQuest™-HTS or a Fusion™-α microplate analyzer.

Data were plotted as the relative AlphaScreen signal measured in the presence of a representative concentration of compound with respect to the positive control signal obtained in the absence of any compound. Non-specific binding (NS) is the signal measured in absence of biotin-pY-peptide.

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## Results

### Antioxidants

The AlphaScreen technology is based on the formation of excited singlet state oxygen molecules. Since antioxydants are well-characterized quenchers of reactive oxygen species (i.e. radicals) and could potentially interfere with AlphaScreen chemistry, the effect of different antioxidants on AlphaScreen signal was tested.

As shown in Figure 1, none of the antioxidants tested had a significant effect on signal at typical screening concentrations (1-10 µM).

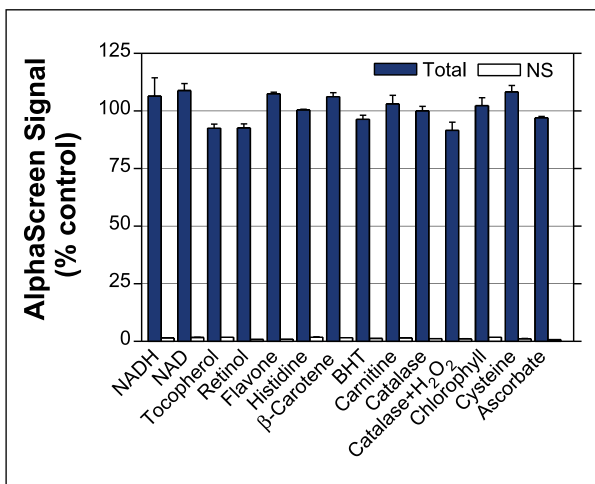


***Figure 1.***
Effect of Antioxydants. All compounds at 10 µM except chlorophyll and β-carotene at 1 µM (not soluble at higher concentrations)

### Color Quenchers

A variety of colored compounds were tested as potential quenchers of AlphaScreen signal. As expected, green and blue dyes that absorb light in the AlphaScreen signal emission range (520-620 nm) led to a decrease in AlphaScreen signal that varied with the molar extinction coefficient of the dyes (Figure 2).

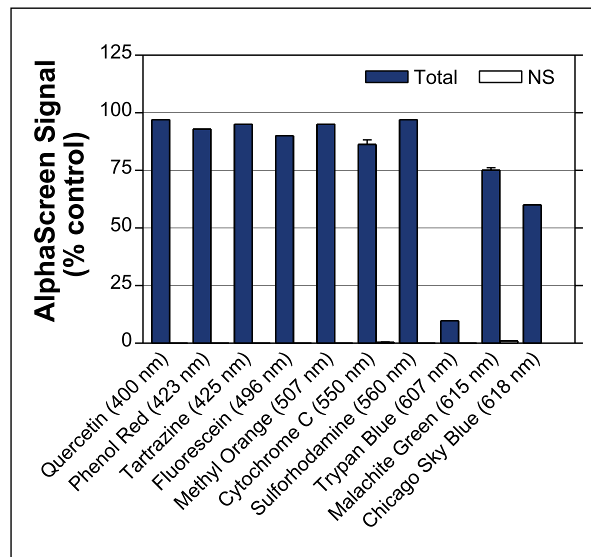


***Figure 2.***
Effect of Color Quenching by Chemicals. All compounds at 1 µM, listed according to their $\lambda_{max}$ of absorption.

### Other Compounds and Chemicals

We tested a set of compounds commonly used in biological assays with potential interference properties (Figure 3).

Most compounds had no significant effect on AlphaScreen signal. However, the preservative sodium azide ($NaN_3$), a well-known singlet oxygen quencher, was shown to strongly interfere with the AlphaScreen signal when used at its recommended concentration (0.01 %) and therefore should not be included in AlphaScreen assays. Proclin-300 is recommended as an alternative preservative. EDTA, staurosporine (generic tyrosine kinase inhibitor), as well as glycerol and glucose, did not significantly affect total signal at concentrations reported.

The culture medium RPMI 1640 did not interfere with the system when used at 1%. However, the presence of 10% culture medium led to a signal reduction of about 30% (data not shown), presumably due to the presence of excess biotin and iron in this medium. MEM, which lacks these components, does not affect AlphaScreen assays.

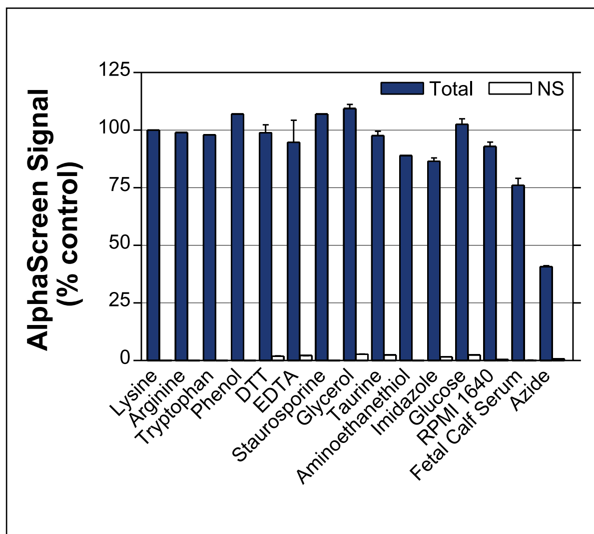


***Figure 3.***
Effect of Other Compounds and Chemicals

Similarly, fetal calf serum was shown to reduce total signal by about 25% when used at 1%. We therefore recommend rinsing cells grown in serum in an appropriate buffer such as PBS before they are used in AlphaScreen assays.

### DMSO

DMSO was tested at concentrations between 0.05 and 10%. As seen in Figure 4, it was found to be well tolerated and is suitable for compound addition in HTS. Interestingly, we noted an increase in counts at the higher DMSO concentrations, while S/B ratios remained constant.

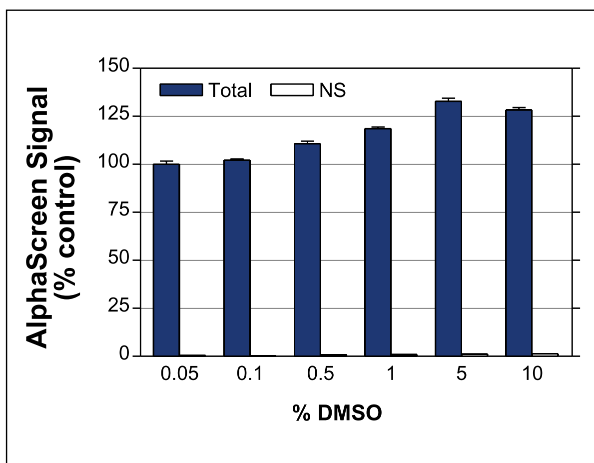


***Figure 4.***
Effect of DMSO

### Detergents

Tween-20 and Triton-X100 used at concentrations up to 1% did not affect AlphaScreen signal.
CHAPS could be used at concentrations of 0.1% or less.
SDS should be avoided in AlphaScreen assays in general, as it did have a significant effect on total signal even at low concentrations (0.05%).
To reduce non-specific binding between AlphaScreen beads, we recommend adding 0.1% BSA in the assay buffer. Tween-20 at concentrations up to 1% can also be used in most assays.

### Salts

We recommend performing AlphaScreen assays using physiological salt concentrations. Cations such as $Na^+$, $K^+$, $Mg^{++}$, $Mn^{++}$ and $Ca^{++}$, used at their respective physiological concentrations, were shown to have no effect on AlphaScreen signal generation.

Monovalent anions such as $Cl^-$, $I^-$, and $F^-$ were shown to be safe when used at concentrations as high as 100 µM.

Divalent anions however, such as $CO_3^{2-}$ and $SO_4^{2-}$, should be used at 1 mM or less in the PT66 assay. The sensitivity of AlphaScreen to divalent anions is attributed to their effect on anti-phosphotyrosine antibody affinity for phosphotyrosine (Ruff-Jamison, S. *et al.* JBC **266**(10):6607-6613).

Orthovanadate, when used as a phosphatase inhibitor at 1 mM, had no effect.

### Metal Ions

We strongly recommend avoiding the use of the following metal ions: $Fe^{2+}$, $Fe^{3+}$, $Cu^{2+}$, $Zn^{2+}$ and $Ni^{2+}$. These metals have been shown to be potent singlet oxygen quenchers in the mM and sub-mM ranges (100 µM for $Fe^{2+}$).

## Conclusion

The PT66 phosphotyrosine assay kit was used as a benchmark to assess potential compound interference on AlphaScreen signal generation. Among all the compounds tested, sodium azide, divalent ions and blue colored compounds were shown to be deleterious to AlphaScreen signal when used at high concentrations. All other chemicals tested, which are commonly used as biological assay components, were shown to be innocuous at typical working concentrations.

### Related Publications

- Application note ASC-001: Principles of AlphaScreen

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