MANUAL

Using the AlphaScreen™ TruHits™ kit

Catalog numbers: 6760627D, 6760627M

For Laboratory Use Only
Research Chemicals for Research Purposes Only

I. Before starting

Receiving the AlphaScreen™ TruHits™ kit

Upon receiving the AlphaScreen TruHits kit, ensure that the kit is on blue ice and that the ice packs are not completely melted. Verify that all components are present in the kit using the table on the next page. Store the kit at 4°C.

Provided Reagents and Materials

The following kit sizes are available:

- 1,000 assay points kit* (catalog number # 6760627D)
- 10,000 assay points kit* (catalog number # 6760627M)

* The number of assay points is based on an assay volume of 25 1L using the AlphaScreen TruHits beads at the recommended concentrations.

The reagents and materials provided in the AlphaScreen TruHits kits are listed in the table on the next page:

Kit components	6760627D (1,000 assay points)	6760627M (10,000 assay points)	Shelf-life and Storage Conditions
Biotinylated Acceptor beads stored in PBS, 0.05% Proclin- 300, pH 7.2	50 µL @ 2,5 mg/mL	0.5 mL @ 2,5 mg/mL	2 - 8°C until expiry date stated on the vial label. Do not freeze. Store protected from light
Streptavidin-Donor beads stored in PBS, 0.05% Proclin-300, pH 7.2	50 µL @ 5 mg/mL	0.5 mL @ 5 mg/mL	2 - 8°C until expiry date stated on the vial label. Do not freeze. Store protected from light

Important Note:

For maximum recovery of contents, briefly centrifuge the vials prior to removing the caps. Resuspend the beads by vortexing before use.

Additional Reagents and Materials

AlphaScreen TruHits assays can be performed in all commonly used physiological buffers, including buffers based on Tris, HEPES, or PBS. We recommend performing the AlphaScreen TruHits assays using the same buffer used for the high-throughput screening (HTS) assay under which the hits were generated. The following materials were used for buffers and reagents preparation during assay development. Equivalent sources can be substituted.

Item	Suggested source	Catalog #
PBS 1×	Invitrogen™ Corp.	14190078
Proclin™ -300	Sigma-Aldrich™ Co.	48126
D-biotin	Sigma-Aldrich™ Co.	B4501
Milli-Q® water		
OptiPlate™-384 (white opaque 384- well microplate)	Revvity®, Inc.	6007290
TopSeal-A Adhesive Sealing Film	Revvity®, Inc.	6005185
Single-channel Pipettors§		

§ For lower volume plate additions (2.5-10 µL), we recommend a pipettor precision ≤ 2%. For higher volume additions (25-1000 µL), we recommend a pipettor precision of ≤ 1%.

II. Introduction

"True hits" are compounds which act at the level of the therapeutic target subject to screening. These active molecules are usually enzyme inhibitors or receptor agonists or antagonists. "False positives" are compounds interfering with the HTS assay in ways that are unrelated to the screen target. When the false positive rate in an assay is too high, the quality of the results and the reliability of the whole screening process become questionable.

Characterizing each of the hits generated during an HTS campaign can be time-consuming and expensive. For this reason, it is essential to identify false positives as early as possible in the screening process. The AlphaScreen TruHits kit has been especially designed for this purpose and allows the early identification of false positives in a costeffective manner. In addition, the AlphaScreen TruHits kit allows for the determination of the root cause of compound interference. Using this kit will allow the AlphaScreen user to rapidly focus on true hits, and thus significantly increase the value of screening campaigns.

AlphaScreen™ TruHits™ and Compound Interference

The AlphaScreen TruHits kit contains Streptavidin Donor (SA-D) beads and biotinylated Acceptor (b-A) beads. Biotinylated Acceptor beads are coated with biotinylated bovine serum albumin (BSA). Biotin molecules conjugated on the BSA will bind tightly to the streptavidin on the Donor beads to form a bead complex generating an AlphaScreen signal upon irradiation at 680 nm (Fig. 1). The two beads interact together in the absence of any probe or bridging component.

False positives arise in all HTS campaigns, no matter the technological platform used. These compounds interfere with one of the assay components in a non-specific manner, or with assay signal generation and/or detection.

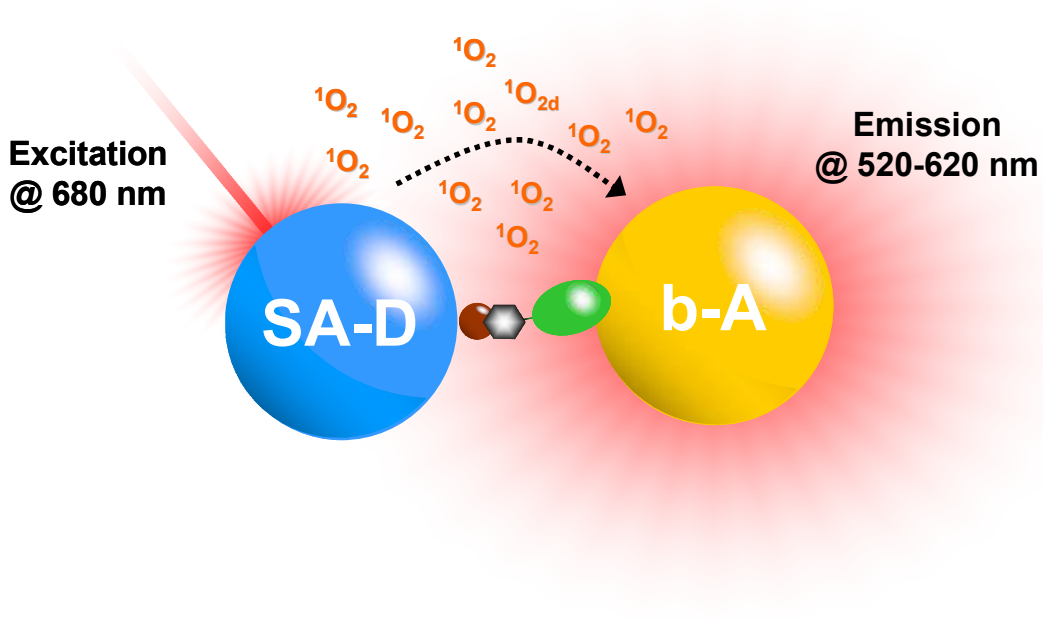


Figure 1. Schematic Illustration of the AlphaScreen TruHits Kit. The Streptavidin Donor (SA-D) bead and biotinylated Acceptor (b-A) beads form a complex that emits light between 520 and 620 nm upon laser irradiation at 680 nm.

Compounds which interfere with AlphaScreen assays belong to one of the following classes:

1) **Singlet Oxygen Quenchers.** These types of compounds actually bind to, or react with, singlet oxygen molecules generated by the irradiation of the donor bead at 680 nm, preventing singlet oxygen from reaching the acceptor bead and generating a signal. Examples of singlet oxygen quenchers include azide, ascorbate, and some transition metal ions such as Zn^{2+}, Cu^{2+}, Fe^{2+} and Fe^{3+}.

2) **Color Quenchers (also known as Inner Filters).** These colored molecules absorb light either at 680 nm (wavelength used for Donor bead excitation) or between 520-620 nm (range of emission of the Acceptor beads). These compounds are usually blue/green or blue, and are often easy to detect by simply examining the plates of compounds. Examples of dyes acting as color quenchers in AlphaScreen assays include malachite green and dextran blue. Color quenchers or inner filters are found in all HTS technologies that are based on light detection.

3) **Light Scatterers.** Light scattering is produced by insoluble compound aggregates. A significant proportion of compounds from HTS libraries falls out of solution to various extent when diluted in aqueous buffers. Compound aggregates diffract light at the excitation and/or emission wavelengths. Light scatterers interfere with all HTS technologies based on light detection.

4) **Biotin Mimetics.** AlphaScreen assays involve the binding of a biotinylated element, or probe, to the Streptavidin Donor beads. Some rare library compounds possess biotin-like binding properties, and will decrease the AlphaScreen signal by competing for this interaction. These compounds are either small molecules with biotin-like moieties, or short peptides. Biotin mimetic compounds will generate false positives with all technologies using the interaction of a biotinylated probe with streptavidin.

5) **Acceptor Bead Competitors.** Acceptor bead competitors interact directly with the binding partner conjugated to the Acceptor beads. These compounds prevent the capture of the assay probe by the Acceptor bead, resulting in signal decrease. This type of interference is assay dependent but occurs more commonly with the AlphaScreen Nickel Chelate kit. The positive control component available with each AlphaScreen kit is a valuable tool for the detection of false positives that are Acceptor bead competitors. These compounds will generate a signal drop when incubated with the kit components in the presence of the positive control probe. Positive control probes for the different AlphaScreen kits can be purchased from Revvity, Inc. The AlphaScreen Tru- Hits kit is not intended for the identification of Acceptor bead competitors.

The AlphaScreen TruHits kit will detect compounds that belong to the first four classes of interfering compounds (Fig. 2), and will help determine if active compounds in HTS assays are potential active hits or false positives. In TruHits assays, library compounds which interfere with the AlphaScreen signal are considered false positives while compounds which exhibit no effect on the signal are potential true hits.

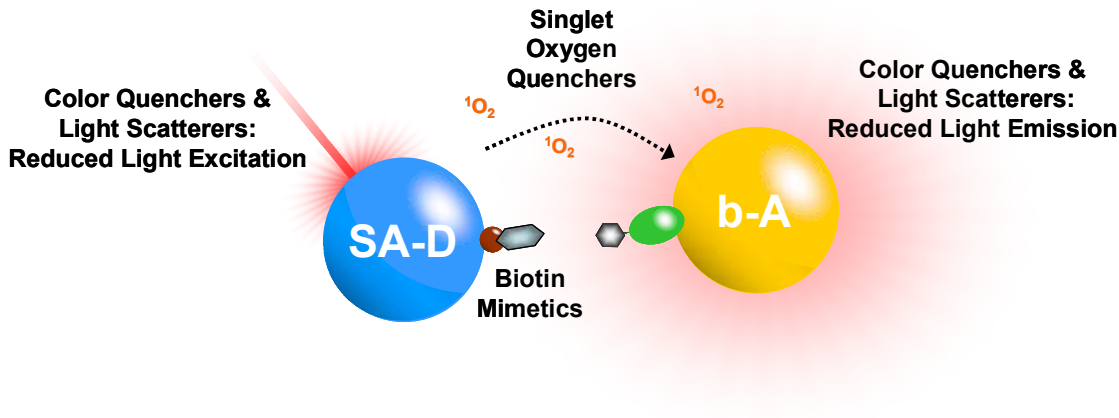


Figure 2. Classes of Compound Detected with the AlphaScreen TruHits Kit. Compounds which interfere with the AlphaScreen TruHits signal can be singlet oxygen quenchers, color quenchers, light scatterers, or biotin mimetics.

Strategies for Assessing Compound Interference

Two TruHits assay configurations can be used for the identification of false positives. The first configuration will determine if the compounds are singlet oxygen quenchers, color quenchers or light scatterers. The second configuration will identify biotin mimetics.

- **Testing for Singlet Oxygen Quenching / Color Quenching / Light Scattering.** In this assay configuration, SA-D and b-A beads are pre-incubated together prior to the addition of hit compounds to the assay wells. A drop in signal is an indication that the compound being tested is either a singlet oxygen quencher, a color quencher, or a light scatterer. Biotin mimetics cannot be identified under this assay configuration. The interaction between biotin and streptavidin is so strong that once it is formed during pre-incubation, it cannot be disrupted, even by biotin itself.

- **Testing for Biotin Mimetics.** Compounds that did not alter the TruHits signal using the first assay configuration could be biotin mimetics. The TruHits kit allows for the specific identification of biotin mimetics using a procedure where compounds are pre-incubated with the SA-D beads, prior to adding the b-A beads. Biotin mimetic compounds will bind to the streptavidin on Donor beads and generate a signal loss. Note that biotin mimetics will not interfere with the AlphaScreen cAMP assay (catalog number # 6760625). The biotinylated cAMP probe in the AlphaScreen cAMP assay is preincubated with the SA-D beads prior to bead addition to cells stimulated by the compounds.

If a compound identified as a hit in an AlphaScreen primary screen does not reduce the TruHits signal in one of the two assay configurations, it can be considered to be a potential true hit.

III. Screening for singlet oxygen quenchers, color quenchers and light scatterers

The final concentration of compounds in the TruHits assay should be the same as the final concentration used in the primary screen that lead to the identification of the hit compounds (usually 10 µm or below). It is recommended to vortex and then quick spin the AlphaScreen TruHits beads prior to pipetting from the stock suspensions. The assay is performed in a volume of 25 µL in white 384-well OptiPlates (Revvity® catalog number # 6007290).

Reagent preparation

- Prepare 1× assay buffer
- Prepare 5× intermediate dilutions of hit compounds in assay buffer (1× being the final concentration used in the original screen)
- Prepare a TruHits bead premix as follows*:

Reagent	Volume	[Premix]	[Final]
1× assay buffer	1.99 mL	-	-
SA-D beads	5 µL	12.5 µg/mL	10 µg/mL
b-A beads	5 µL	6.25 µg/mL	5 µg/mL

- Incubate premix for 30 min at room temperature.

** This volume of premix is sufficient for ~ 90 assay points. Adjust volumes according to the number of compounds to be tested. Prepare the bead premix fresh.*

Reagent Addition

- Add to the wells of a 384-well microplate:
  - 20 µL of bead premix
  - 5 µL of the 5× compound solution or buffer (as a control)
- Cover the plate with a TopSeal-A sealing film
- Incubate for 10 min at room temperature
- Read plate using an AlphaScreen multiplate reader

Interpreting the Data

In this assay configuration, the Donor and Acceptor beads are premixed prior to compound addition. Any signal decrease will result from either color quenching, light scattering or singlet oxygen quenching. A signal lower than the average of control wells by more than two standard deviations indicates an interfering compound. An example of interference by singlet oxygen quenching and color quenching is presented in Figure 3. As explained earlier, biotin mimetics cannot be detected using this assay configuration due to the strong association between biotin and streptavidin established during pre-incubation.

99.4	100.0	97.4	3.1	93.6	104.3	104.5	103.6	106.7	104.9	101.9	**51.0**
2.4	99.0	95.6	97.4	96.4	97.6	97.1	98.8	97.8	100.7	102.9	101.0
99.0	98.5	**44.0**	95.2	95.6	96.3	94.7	98.1	1.6	96.6	96.6	100.7
97.9	**39.9**	101.9	95.5	96.2	93.9	93.6	93.0	95.6	88.7	2.6	91.7
96.8	110.7	96.1	96.2	108.2	96.4	96.0	94.3	95.5	115.9	95.2	98.6
99.5	96.5	97.2	95.6	96.7	**40.5**	2.2	**41.4**	94.6	94.4	93.5	100.6
98.5	97.0	96.4	96.8	96.1	97.1	95.6	104.4	114.1	2.7	98.5	100.5
97.4	96.9	98.4	98.1	100.0	98.4	101.2	99.4	102.7	99.7	98.2	**49.9**

Figure 3. Detection of AlphaScreen Signal Interference with Premixed TruHits Beads. Premixed beads from the TruHits kit, prepared as described in Section III, were dispensed in 96 wells of a white 384-well OptiPlate. A total of 12 wells were spiked randomly with either a singlet oxygen quencher (2 mM sodium azide; in bold) or a color quencher (2 µM malachite green; shaded). The volume of the remaining 84 wells was completed with 5 µL of assay buffer. Signal was detected using an EnVision™ multiplate reader. Results are expressed as percentage of signal measured from the average of control wells.

Spectrophotometric Analysis of Interfering Compounds

Compounds which decrease the AlphaScreen signal when added to premixed TruHits beads can be further analyzed in order to determine the root cause of signal interference. An absorption spectrum of the compounds in solution will allow for the determination of the type of interference involved, and will differentiate light interfering compounds from singlet oxygen quenchers. Samples for spectrophotometric analysis are prepared as follows:

- Dilute compounds to the concentration used in the original screening assay
- Transfer to a spectrophotometer cuvette
- Perform a spectrophotometric scan between 350 and 800 nm

The absorption spectrum of hit compounds can be of three types:

1) with a defined peak of absorption which overlaps with the excitation (680 nm) or emission (520-620 nm) wavelengths of the AlphaScreen assays. This type of spectrum is typical of compounds that are color quenchers. Figure 4 shows the absorption spectra of malachite green, a blue compound that quenches a large fraction of the AlphaScreen red light emission.

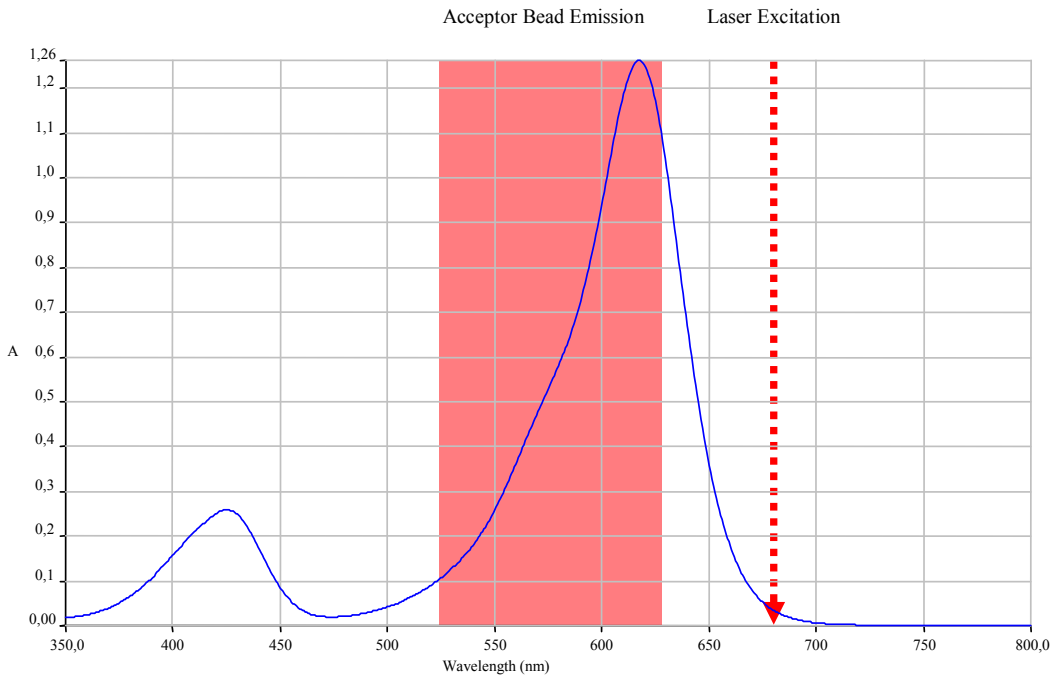


Figure 4. Absorption Spectra of Malachite Green. The dye was dissolved in PBS at a concentration of 10 µM. The shaded area between 520 and 620 nm represents the emission wavelengths of AlphaScreen Acceptor beads while the dotted arrow at 680 nm indicates the wavelength of excitation of Donor beads. Note the significant overlap of the absorption peak of malachite green with the emission of the Acceptor beads.

2) Undefined “absorption spectrum” from insoluble compounds. Although they do not actually absorb light, insoluble compounds show a typical absorption spectrum similar to the one presented in Figure 5. This peculiar spectrum shows optical density (OD) values above 0.05 for all wavelengths between 350 and 800 nm. This type of spectrum is not truly produced by absorption, but arises from light scattering caused by compound crystals or micelles floating in solution. This interference can be avoided in some cases by adding trace amounts of detergent, such as Tween-20, to the AlphaScreen detection buffer. Increased solubilization of compounds by detergent should reduce or eliminate the signal interference observed in the TruHits and other AlphaScreen assays.

3) Spectrum showing no light absorption at the wavelengths of excitation (680 nm) or emission (520-620 nm) of AlphaScreen assays. Compounds of this type are most likely singlet oxygen quenchers.

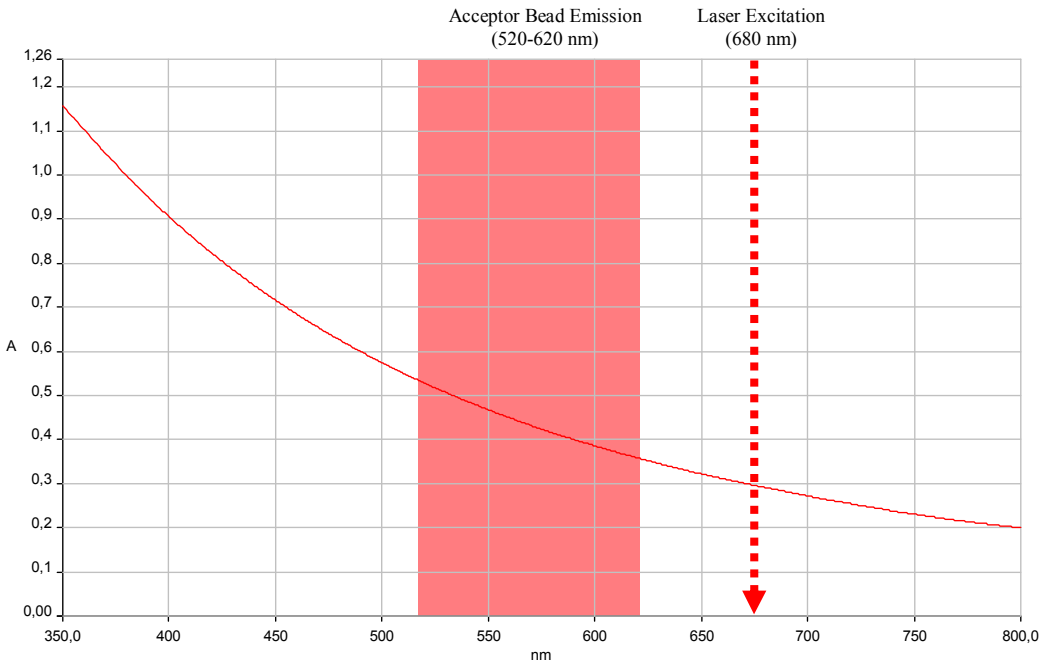


Figure 5. Absorption Spectra of an Insoluble Compound. The insoluble compound was mixed in PBS at a concentration of 50 µM. The shaded area between 520 and 620 nm represents the emission wavelengths of AlphaScreen Acceptor beads while the dotted arrow at 680 nm indicates the wavelength of excitation of Donor beads. Note the continuous diffraction of the insoluble compound at all wavelengths of the spectra.

IV. Screening for biotin mimetics

The final concentration of compounds tested in the TruHits assay should be the same as the concentration used in primary screen that lead to the identification of the hit compounds (usually 10 µm or below). It is recommended to vortex and then quick spin the AlphaScreen TruHits beads prior to pipetting from the stock suspensions. This assay is performed in a volume of 25 µL in white 384-well OptiPlates (Revvity® catalog number # 6007290).

Reagent preparation

- Prepare 1× assay buffer
- Prepare 5× intermediate dilutions of hit compounds in assay buffer (1× being the final concentration used in the original screen)
- Prepare a 5× biotin control solution as follows:
  - Dissolve 24.4 mg of D-biotin in 10 mL of DMSO (10 mM stock solution).
  - Further dilute to 50 µM by adding 5 µL of the stock solution to 995 µL of assay buffer
- Prepare dilutions of TruHits bead stock suspensions as follows:
  - SA-D beads*: Dilute to 25 µg/mL by adding 5 µL of the bead stock solution to 995 µL of assay buffer
  - b-A beads*: Dilute to 12.5 µg/mL by adding 5 µL of the bead stock solution to 995 µL of assay buffer.

* Volumes of bead dilutions are sufficient for ~ 90 assay points. Adjust volumes according to the number of compounds to be tested. Prepare bead dilutions fresh.

Reagent Addition

- Add to the wells of a 384-well OptiPlate:
  - 10 µL of SA-D beads
  - 5 µL of compounds to test

Note: Include a few wells with buffer only as well as some wells spiked with the 50 µM D-biotin control solution

- Cover plate with another opaque microplate
- Incubate for 30 min at room temperature
- Add 10 µL of b-A beads
- Cover plate with a TopSeal-A sealing film
- Incubate for 30 min at room temperature
- Read plate on an AlphaScreen reader

Interpreting the Data

In the TruHits assay, preincubating the SA-D beads with potential biotin mimetics will allow these compounds to saturate streptavidin binding sites and thus compete for the interaction with the Acceptor bead. A signal lower than the average of control wells by more than two standard deviations indicates a biotin-mimetic compound. An inhibition curve of the TruHits signal by D-biotin is presented in Figure 8.

In HTS campaigns performed with regular AlphaScreen kits, biotin mimetics prevent biotinylated assay probes from being captured by the SA-D beads. With most AlphaScreen assays, it is possible to eliminate the interference by biotin mimetics by pre-incubating the SA-D beads with the biotinylated assay probe for 30 min prior to compound addition. This simple adjustment to the protocol requires an extra incubation step, but completely eliminates the interference by biotin mimetics.

V. Characterizing interfering compounds

False positives interfere with the AlphaScreen signal at the compound concentration used in the original HTS assay. It might be possible to rescue some of the false positives by using them at a concentration that will not interfere with the AlphaScreen signal. Dose-response curves using the TruHits kit will indicate the concentration range at which a false positive interferes non-specifically with the assay, and will allow the user to determine at which concentration the compound could be used in HTS. The following protocols describe the set-up of inhibition curves using the two assay configurations described earlier for the various classes of interfering compounds.

1) Inhibition Curves with Singlet Oxygen Quenchers and Color Quenchers

Reagent Preparation

It is recommended to vortex and then quick spin the AlphaScreen TruHits beads prior to pipetting from the stock suspensions. The assay is performed in triplicate in a volume of 25 µL in white 384-well OptiPlates (Revvity® catalog number # 6007290).

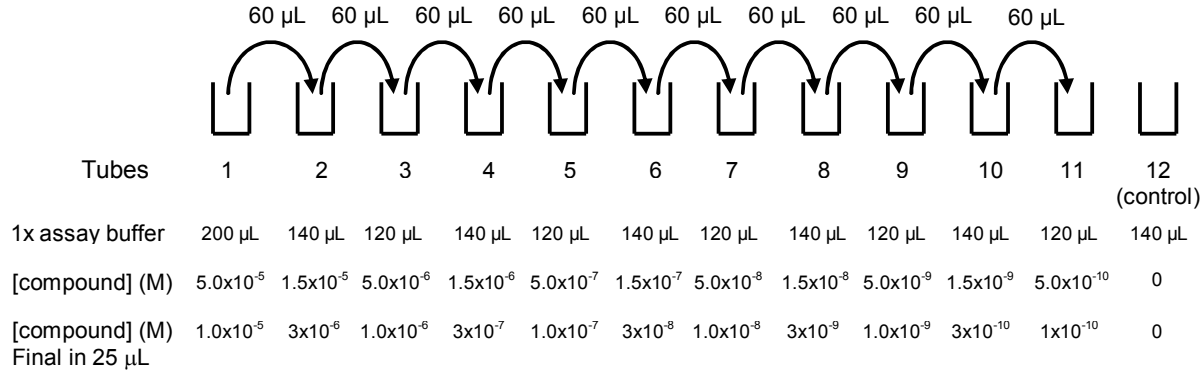
- Prepare 2 mL of assay buffer for each compound to be evaluated
- Prepare a TruHits bead premix as follows*:

Reagent	Volume	[Premix]	[Final]
1× assay buffer	1.99 mL	-	-
SA-D beads	5 µL	12.5 µg/mL	10 µg/mL
b-A beads	5 µL	6.25 µg/mL	5 µg/mL

- Incubate the premix for 30 min at room temperature

* This volume of premix is sufficient for ~ 90 wells. Adjust volumes according to the number of compounds to be tested. Prepare the bead premix fresh.

- Prepare initial 5× compound dilutions in assay buffer (1× being the final concentration used in the original screen)
- Serially dilute compounds to yield concentrations ranging from 5 × 10^{-5} M (50 µM) to 5 × 10^{-10} M (500 pM) using halflog dilutions, as illustrated in Figure 6. Include a buffer control (buffer only; identified as tube #12).



Tubes	1	2	3	4	5	6	7	8	9	10	11	12 (control)
1x assay buffer	200 µL	140 µL	120 µL	140 µL	120 µL	140 µL	120 µL	140 µL	120 µL	140 µL	120 µL	140 µL
[compound] (M)	$5.0x10^{-5}$	$1.5x10^{-5}$	$5.0x10^{-6}$	$1.5x10^{-6}$	$5.0x10^{-7}$	$1.5x10^{-7}$	$5.0x10^{-8}$	$1.5x10^{-8}$	$5.0x10^{-9}$	$1.5x10^{-9}$	$5.0x10^{-10}$	0
[compound] (M) Final in 25 µL	$1.0x10^{-5}$	$3x10^{-6}$	$1.0x10^{-6}$	$3x10^{-7}$	$1.0x10^{-7}$	$3x10^{-8}$	$1.0x10^{-8}$	$3x10^{-9}$	$1.0x10^{-9}$	$3x10^{-10}$	$1x10^{-10}$	0

Figure 6. Serial Compound Dilutions in Assay Buffer.

Reagent Addition

- To triplicate wells of a 384-well Optiplate, add the following:
  - 20 µL of bead premix
  - 5µL of compound dilution
- Cover with a TopSeal-A sealing film
- Incubate 10 min at room temperature
- Read on an AlphaScreen reader

Interpreting the Data

Compound inhibition curves are graphed as scatter plots, with compound concentration on the X-axis and AlphaScreen signal on the Y-axis. If there is a concentration-dependent inhibition of signal, the data will yield a sigmoidal curve showing a signal decrease in the presence of increasing concentrations of the interfering compound. The IC_{50} of the curve represents the concentration of compound leading to a 50% decrease in signal (Fig. 7).

Inhibition curves allow the user to determine if a false positive could be rescued and retested in the primary HTS assay at a lower concentration. This would be the case, for example, if the percentage of signal inhibition observed in the primary HTS assay was significantly higher than the percentage of inhibition observed with the TruHits assay at the same concentration. The compound could then be a true hit, interfering only partially with the AlphaScreen signal detection.

If the percentage of signal inhibition observed in the primary screen is similar to the percentage of signal inhibition observed in the TruHits assay at the same screening concentration, the possibility of rescuing a compound would then depend on its IC_{50} in the TruHits assay. If the IC_{50} is in the micromolar (µM) range or above, diluting the compound three to ten-fold should solve most of the interference issue. However, if the IC_{50} for the inhibition is in the nanomolar (nM) range, it is unlikely that diluting the compound will allow rescue for the primary HTS screen. Figure 7 shows an inhibition curve obtained with the singlet oxygen quencher sodium azide (NaN3). The IC_{50} of sodium azide for the TruHits assay is 0.84 mM (~ 0.005%). This very clearly illustrates why buffers containing sodium azide should not be used when working with AlphaScreen.

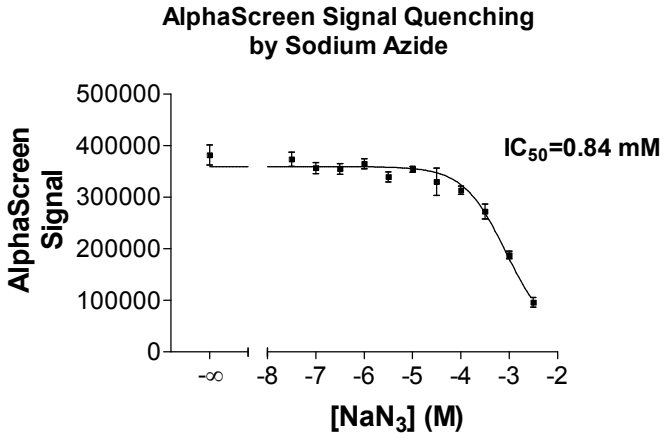


Figure 7. TruHits Signal Inhibition by the Singlet Oxygen Quencher Sodium Azide. Sodium azide (NaN3) was incubated at concentrations ranging from 3×10^{-2} M to 3×10^{-7} M in the presence of TruHits premixed beads prepared as recommended in text. Signal was detected using an EnVision™ multiplate reader.

2) Inhibition curves with biotin mimetics

Reagent preparation

It is recommended to vortex and then quick spin the AlphaScreen TruHits beads prior to pipetting from the stock suspensions. The assay is performed in triplicate in a volume of 25 µL in white 384-well OptiPlates (Revvity® catalog number # 6007290).

- Prepare 2 mL of assay buffer for each compound to be evaluated
- Prepare a 5× biotin control solution as follow:
  - Dissolve 24.4 mg of D-biotin in 10 mL of DMSO (10 mM stock solution).
  - Further dilute to 50 µM by adding 5 µL of the stock solution to 995 µL of assay buffer
- Prepare dilutions of TruHits bead stock suspensions as follows:
  - SA-D beads*: Dilute to 25 µg/mL by adding 5 µL of the bead stock solution to 995 µL of assay buffer
  - b-A beads*: Dilute to 12.5 µg/mL by adding 5 µL of the bead stock solution to 995 µL of assay buffer.

** Volumes of bead dilutions are sufficient for ~ 90 wells. Adjust volumes according to the number of compounds to be tested. Prepare bead dilutions fresh.*

- Prepare initial 5× compound dilutions in assay buffer (1× being the final concentration used in the original screen)
- Serially dilute compounds to yield concentrations ranging from 5×10^{-5} M (50 µM) to 5×10^{-10} M (500 pM) using halflog dilutions, as illustrated in Figure 6. Include a buffer control (buffer only; identified as tube #12). If desired, a control curve can also be generated using D-biotin (Fig. 8).

Reagent Addition

- Add to triplicate wells of a 384-well OptiPlate:
  - 10 µL of SA-D beads
  - 5 µL of compound dilutions

Note: Include as controls three wells with buffer only as well as three wells with the 50 µM D-biotin solution if a curve with D-biotin is not performed in parallel.

- Cover plate with another opaque microplate
- Incubate for 30 min at room temperature
- Add 10 µL of b-A beads
- Cover plate with a TopSeal-A sealing film
- Incubate for 30 min at room temperature
- Read plate on an AlphaScreen reader

Interpreting the Data

Compound inhibition curves are graphed as scatter plots, with compound concentration on the X-axis and AlphaScreen signal on the Y-axis. If there is a concentration-dependent inhibition of signal, the data will yield a sigmoidal curve showing a signal decrease in the presence of increasing concentrations of the interfering compound. The IC_{50} of the curve represents the concentration of compound leading to a 50% decrease in signal.

As mentioned in Section IV, it is possible to eliminate the interference by biotin mimetics in most AlphaScreen assays by preincubating the SA-D beads with the biotinylated assay probe prior to compound addition. Biotin mimetics can also be rescued in a way similar to the rescue of color or singlet oxygen quenchers. Inhibition curves generated by biotin mimetics will help determine if a false positive can be rescued and retested in the primary HTS assay at a lower concentration. This would be the case, for example, if the percentage of signal inhibition observed in the primary HTS assay was significantly higher than the percentage of inhibition observed with the TruHits assay at the same compound concentration. The compound could then be a true hit, interfering only partially with the AlphaScreen signal detection.

If the percentage of inhibition is similar in the primary screen and in the TruHits assay at the same screening concentration, the possibility of rescuing a compound would then depend on its IC_{50} in the TruHits assay. If the IC_{50} is in the micromolar (µM) range or above, diluting the compound three to ten-fold should solve most of the interference issue. However, if the IC_{50} for the inhibition is in the nanomolar (nM) range, it is unlikely that diluting the compound will allow rescue for the primary HTS screen. Preincubation of the assay probe with the SA-D bead would then be indicated. Figure 8 shows an inhibition curve obtained with D-biotin. The IC50 for D-biotin is 17 nM, which represents approximately 50% of the biotin-binding capacity of streptavidin molecules conjugated to the Donor beads.

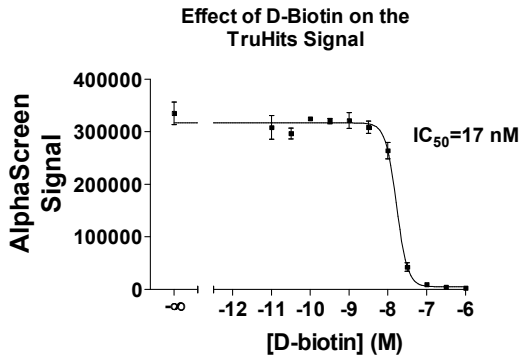


Figure 8. TruHits Signal Inhibition by Increasing Concentration of D-biotin. D-biotin final concentrations ranging from 1×10^{-6} M to 1×10^{-11} M were used. D-biotin was preincubated for 30 min with the SA-D beads as described in text. Acceptor beads were then added and the AlphaScreen signal was monitored 30 min later using an EnVision™.

VI. Troubleshooting guide

This section describes possible problems that could be encountered with the TruHits kit and proposes simple solutions. If more information is required, please consult the Revvity® technical support division (see last page for customer support information).

Low Counts

- Vortex and then quick spin the tubes of AlphaScreen TruHits beads using a micro-centrifuge in "Pulse" mode to ensure that all of the beads are recovered from the cap and walls of the tube.
- AlphaScreen beads are light sensitive. Pipet in a subdued light environment (≤100 lux) or use green filters on light sources.
- Ensure that the correct amount of Acceptor and Donor beads were used.
- Incubation temperature should be maintained at 21°C or above.

Day-to-day Variation

- Check room temperature variations. Variations in temperature will cause variations in signal.
- Work in subdued light environment (≤100 lux). Exposure of Donor beads to bright light will reduce signal.

www.revvity.com

Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA
www.revvity.com

For a complete listing of our global offices, visit www.revvity.com