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Compound-Mediated Assay Interferences in Homogenous Proximity Assays

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Abstract

Homogeneous proximity assays are widely implemented in high-throughput screening (HTS) of small-molecule libraries for drug and probe discovery. Representative technologies include amplified luminescent proximity homogeneous assays (ALPHA, which is trademarked by PerkinElmer; also informally referred to as Alpha), Förster/fluorescence resonance energy transfer (FRET), time-resolved FRET (TR-FRET) and homogeneous time-resolved fluorescence (HTRF, which is trademarked by CisBio), bioluminescence resonance energy transfer (BRET), and scintillation proximity assays (SPA). While highly useful, these assay technologies are susceptible to a variety of technology-related compound-mediated interferences, most notably signal attenuation (e.g., through quenching, inner-filter effects, light scattering), signal emission (e.g., auto-fluorescence), and disruption of affinity capture components such as affinity tags and antibodies. These assays are also susceptible to more generalized compound-mediated interferences such as nonspecific reactivity and aggregation. This chapter describes (a) the basic principles of proximity assays, (b) common sources of compound-mediated assay interference in homogenous proximity assays, and (c) counter-screens and other strategies to classify compound-mediated assay interferences in homogenous proximity assays. This information should be useful for prioritizing bioactive compounds in homogenous proximity assays for drug and chemical probe discovery.

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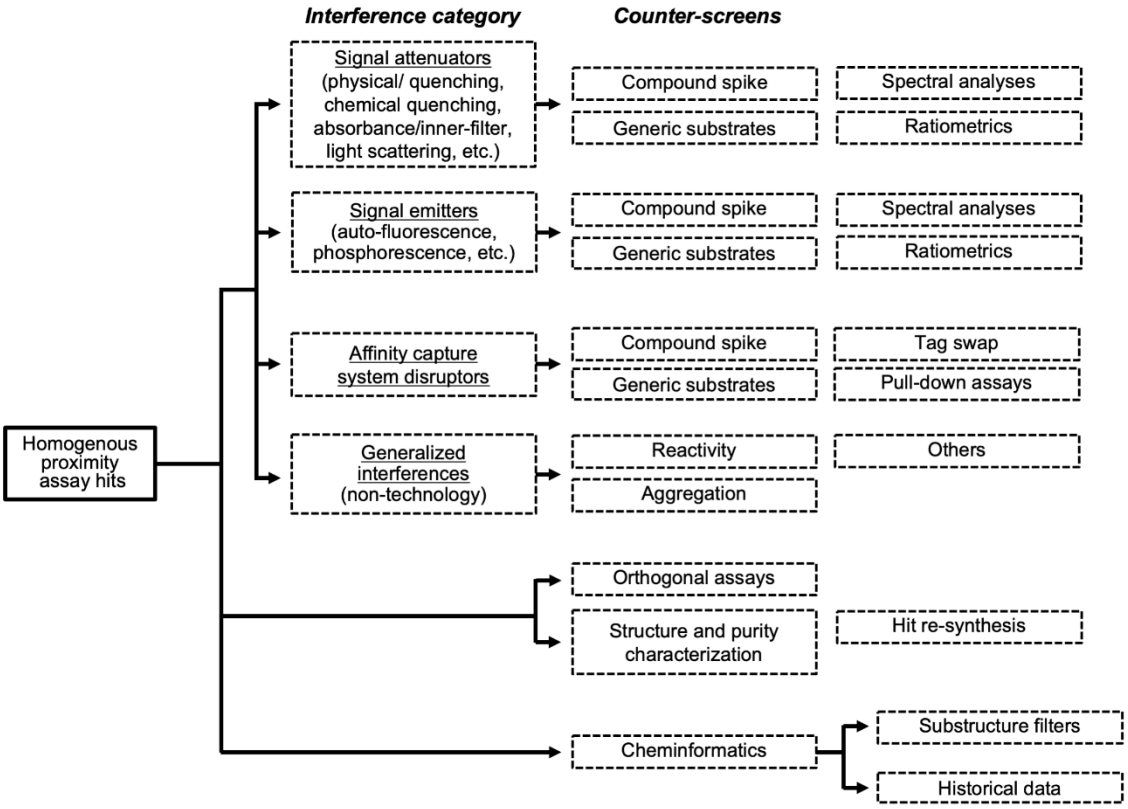
Flowchart



Abbreviations

ALPHA (Alpha)	amplified luminescent proximity homogeneous assay
BRET	bioluminescence resonance energy transfer
CAC	critical aggregation concentration
DELFIA	dissociation-enhanced lanthanide fluorescence immunoassay
ELISA	enzyme-linked immunosorbent assay
FRET	fluorescence/Förster resonance energy transfer
GSH	glutathione
GST	glutathione *S*-transferase
His	histidine
HTS	high-throughput screening
IC_{50}	half-maximal inhibitory value
SIR	structure-interference relationships
TR-FRET	time-resolved FRET

Introduction to Proximity Assays

Proximity assays are widely utilized for the discovery and optimization of small-molecule therapeutics and chemical probes (1). This broad range of assay technologies relies on close proximity between detection components for optimal signal production. These assays are highly amenable to HTS and quantitative HTS

(qHTS) because they do not require separation steps and many key reagents are available commercially and/or amenable to customization. Many proximity assays may be classified as homogenous, where all assay components are combined without any further separation steps ("mix and read"), or heterogeneous as with enzyme-linked immunosorbent assays (ELISA) or dissociation-enhanced lanthanide fluorescence immunoassays (DELFIA) that require wash steps to separate unbound components. Advantages of wash steps include higher signal-to-background that can be achieved and reduced spectroscopic interferences from test compounds due to the removal of these compounds prior to the signal acquisition. However, in general wash steps increase the time it takes to perform and automate an assay, increase waste generated, and increase imprecision.

The main advantage of homogenous proximity assays from an HTS perspective is that they do not require washing or separation steps. However, a key disadvantage of the mix-and-read approach is that potential interference compounds are not removed prior to signal acquisition. When not diagnosed, compounds that interfere with homogenous proximity assay readouts can waste scientific resources and even lead to flawed scientific conclusions (2,3).

In order to better understand the potential sources of compound-mediated interference, this section describes the general principles of several common homogenous proximity assays including amplified luminescent proximity homogeneous assays (ALPHA, Alpha) technology, fluorescence/Förster resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET), time-resolved FRET (TR-FRET), and scintillation proximity assay (SPA). Also discussed are common affinity capture systems such as affinity tags and antibody-analyte combinations that are often used in these proximity assays. Understanding the underlying chemical and physical principles of each technology, including commonly used affinity capture components, allows one to better predict the potential sources of compound-mediated interferences and to design effective counter-screens and orthogonal assays for de-risking such interferences.

Basic Principles of Homogenous Proximity Assays

Proximity assays are highly useful for measuring interactions between biomolecules, including protein-protein interactions, protein-nucleic acid interactions, post-translational modifications (e.g., protein phosphorylation), protein-ligand interactions, and specific analytes (e.g., cytokines, hormones). Such assays utilize two general components – a donor and an acceptor – to indicate proximity by a specific signal (Figure 1A). When the donor and acceptor components are sufficiently proximal, a signal from the donor is transmitted to the acceptor for emission of a signal indicative of proximity. Biomolecules amenable to study by homogenous proximity assays include macromolecules, such as proteins or nucleic acids, as well as smaller ligands. Target biomolecules are often coupled to affinity capture systems, such as antibodies or affinity tags, that can be introduced chemically or by recombinant engineering. Alternatively, target biomolecules could be amenable to radiolabeling for SPA. Measurements can be based on direct (e.g., a discreet protein-protein interaction) or indirect (e.g., displacement of a labelled ligand by a related ligand) interactions. These proximity assays can be developed for the detection of an increased signal (such as assays for activators or inhibitors of analyte modification) or a decreased signal (such as inhibitors of protein-protein interactions). Common types of homogenous proximity assays for HTS include: FRET, BRET, TR-FRET, Alpha, and SPA. Each technology has advantages and disadvantages (Table 1), which are described in more detail below.

FRET. Förster or fluorescence resonance energy transfer (FRET) is initiated by a donor fluorophore (also referred to as chromophore) that is excited to a higher electronic state by a lower-wavelength laser (approximately 350-550 nm). When there is significant overlap between the emission spectrum of the donor and the excitation spectrum of a proximal acceptor fluorophore (i.e., the initial excitation for the donor fluorophore does not excite the acceptor fluorophore), the acceptor can be excited by non-radiative, non-contact dipole–dipole coupling (Figure 1B). The dipole-dipole interaction gives a distance-dependence of $1/r^6$ where r = radius of the interaction, and this distance is approximately 1-10 nm. This excited acceptor fluorophore can then emit

longer-wavelength photons (approximately 500-650 nm) as it returns to its ground electronic state (4). For many biological assays, FRET donor and acceptor fluorophores are protein-based (e.g., CFP → YFP). There are a variety of donor and acceptor fluorophores amenable for FRET (Table 2).

TR-FRET. Time-resolved FRET (TR-FRET) combines the proximity features of the aforementioned FRET assays with time-resolved fluorometry (Figure 1B) (46,47). This variant of FRET assays utilizes donor fluorophores with longer emission times (1-2 ms), such as lanthanide ion complexes, to reduce potential interferences caused by the excitation energy. The optimal distances between donor and acceptor fluorophores are similar to FRET pairs (Tables 2 and 3); however, TR-FRET assays utilize a time-delay between fluorophore excitation and signal acquisition (approximately 50-150 µs in duration). This acquisition delay is sufficient to avoid interference by the usual short-lived fluorescence from test compounds, contaminants, matrix proteins, and aromatic biomolecules. After the delay, the fluorophore:background signal is typically enhanced relative to conventional FRET assays. Many TR-FRET reagents are commercially available (e.g., LANCE, Lanthanide chelate excite, and LANCE *Ultra* TR-FRET from PerkinElmer; HTRF, Homogeneous Time-Resolved Fluorescence from Cisbio). Advantages of TR-FRET are that it is not light sensitive, allows repeated measurements, and does not require radioactive isotopes like SPA.

BRET. A related assay technology is bioluminescence resonance energy transfer (BRET), which typically utilizes bioluminescent proteins and their substrates (e.g., luciferases and luciferin) to generate an initial excitation event. Analogous to FRET, a proximal acceptor fluorophore (e.g., YFP) can be excited through resonance energy transfer to produce the final emission readout (Figure 1C) (48,49). Bioluminescent proteins are typically introduced as fusion proteins. Common acceptor/donor pairs are RLuc8/GFP2 (BRET2) and NanoLuc and HaloTag (NanoBRET) (50-52). An advantage of BRET is that there is no need for bulk illumination of the sample with an excitation light source. This results in lower background signal through reduction of autofluorescent events, which can improve the sensitivity and ultimate performance of the assay.

ALPHA. AlphaScreen (amplified luminescent proximity homogeneous assay) is a proprietary proximity assay technology that utilizes singlet oxygen ($^1O_2$) transfer from an excited donor bead to an acceptor bead within a range of approximately 200 nm (Figure 1D) (53). Singlet oxygen is a short-lived, higher-energy state of molecular oxygen whereby the two normally unpaired HOMO electrons with the same spin state occupy the same degenerate π^* molecular orbital with opposite spin states. Donor and acceptor latex beads containing special chemical reagents are directed to biomolecules of interest through conjugation to capture reagents such as antibodies or capture tags. Phthalocyanine is a photosensitizer within the donor bead that excites ambient oxygen into a singlet state when irradiated by a high-wavelength laser (680 nm). This process produces singlet oxygen ($^1O_2$). Excitation of the standard donor beads generates about 60,000 oxygen singlets per second that can diffuse to a maximum distance of about 200 nm, due to the low microsecond half-life of singlet oxygen in solution and its diffusion rate (54). If the excited donor bead is in close enough proximity to the acceptor bead, a chemiluminescent signal is generated. The emission wavelength of this chemiluminescent signal depends on the composition of the acceptor bead.

Within AlphaScreen acceptor beads, thioxene forms an endoperoxide (ROOR) upon reaction with singlet oxygen. The endoperoxide which then decays and generates light that is transferred to anthracene and then to rubrene which results in chemiluminescence at a *lower* wavelength (520-620 nm; Figure 1D). Within AlphaLISA and AlphaPlex acceptor beads, anthracene and rubrene are replaced by either europium (λ_{Em} = 615 nm), terbium (λ_{Em} = 545 nm), or samarium (λ_{Em} = 645 nm) chelates that are directly excited upon fragmentation of the dioxetane intermediate formed by the reaction of thioxene with singlet oxygen (Table 4). This results in a more intense, narrower emission window optimized for biological matrices such as serum or plasma. In this chapter, discussion of AlphaScreen should be applicable to AlphaLISA and AlphaPlex. In addition to providing a large dynamic range and high sensitivity, Alpha technology has the advantage of being compatible with greater

distances than any other proximity approach, which enables larger molecular complexes to be studied. It also operates over a wider range of affinities of binding partners (K_D's or K_A's).

SPA. Scintillation proximity assay (SPA) is a solid-phase technique that links a scintillation matrix (beads or coated plates, FlashPlate) to a capture system (e.g., antibody, affinity tag) for measurement of a radioactive analyte by scintillation, a light-based readout (Figure 1E) (55,56). When a radiolabeled product (usually containing ^{3}H, ^{125}I, ^{33}P, or ^{14}C) is in close proximity to the scintillation matrix, the signal from radioactive decay of the radioisotope is amplified. In FlashPlate formats, radiolabeled analytes can be localized to the scintillation matrix by plate-bound capture reagents such as streptavidin or antibodies, or under certain circumstances nonspecific analyte absorption (57). The use of radiolabels enables a large dynamic range and increased sensitivity, and compared to more traditional radiolabeled filter-binding assays, SPA does not require a separation step. Important considerations for SPA include obtaining a radiolabeled reagent, obtaining institutional/regulatory approval for radioisotope work, specialized instrumentation (e.g., scintillation counters), disposal costs, and safety protocols.

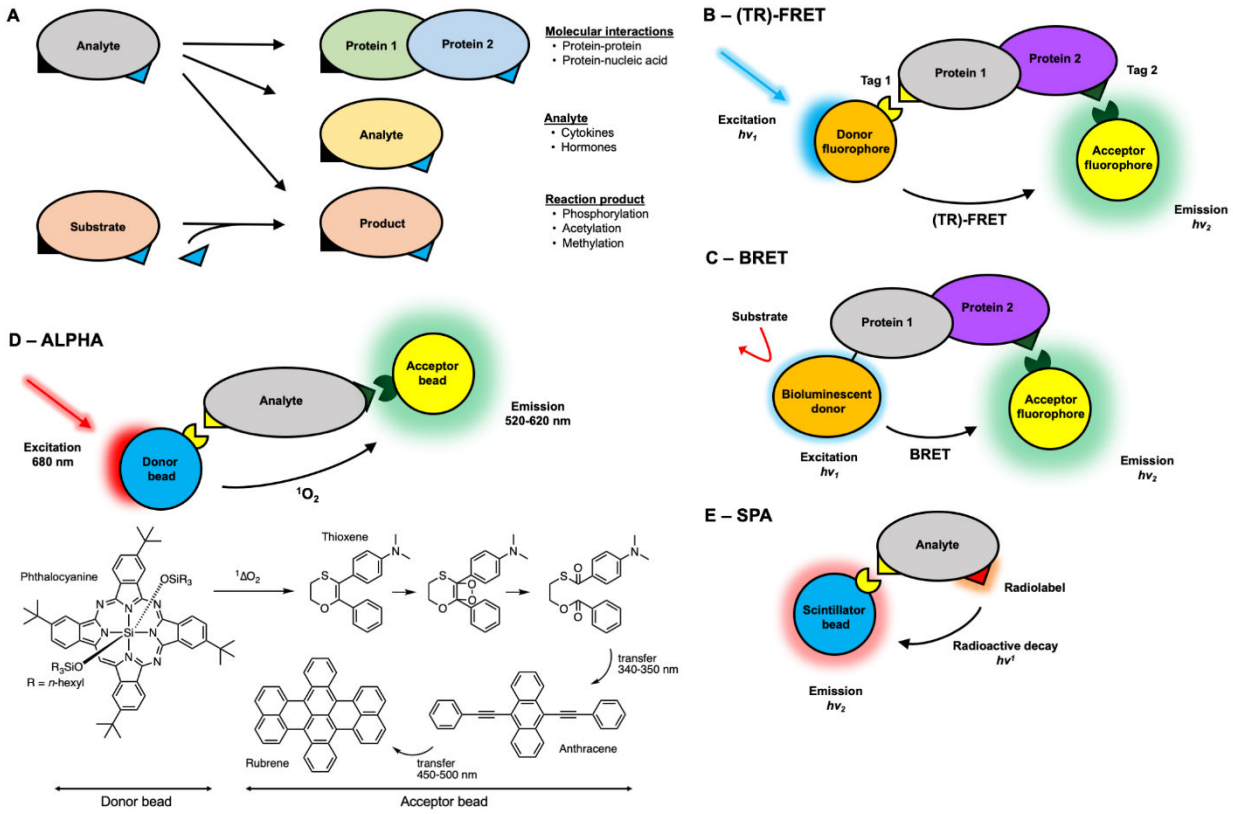


Figure 1 Schematics of common proximity assays. (A) The analyte promoting a signal from a proximity assay can be of many types, including a biomolecular interaction, a single molecule, or a reaction product, such as a protein post-translational modification. (B) FRET and TR-FRET. (C) BRET. (D) AlphaScreen, AlphaLISA, AlphaPlex. Additional details of the underlying Alpha chemistry are provided. (E) SPA. With proximity assays, proximal donor and acceptor components comprise a signal chain (*via* FRET/BRET, singlet oxygen transfer, or radioactive decay, respectively) for the assay readout. The donor and acceptor components can connect with analytes through affinity tags, antibodies, chemical modification, or fusion proteins, depending on assay requirements.

Table 1 Summary of common homogeneous proximity assays. Listed are general advantages, disadvantages, and potential technology-related sources of compound-mediated interference.

Assay type	Advantages	Disadvantages	Potential technology-related compound-mediated interferences
FRET	Commercial reagents available Compatible with intracellular readouts Relatively inexpensive Compatible with standard instrumentation	Lower dynamic range compared to Alpha or SPA Very short distance resulting in limited use	Excitation attenuation (quenching, inner-filter) FRET quenching Emission attenuation (quenching, inner-filter) Compound emission (auto-fluorescence) Affinity capture system disruption
TR-FRET	Commercial reagents available Generally larger dynamic range compared to FRET Reduced interference from excitation (due to Ratiometric readout reduces artifacts	Cost of reagents	Excitation attenuation (quenching, inner-filter) FRET quenching Emission attenuation (quenching, inner-filter); less likely given larger Stokes shift Compound emission (auto-fluorescence); less likely given time delay Affinity capture system disruption

Table 1 continued from previous page.

Assay type	Advantages	Disadvantages	Potential technology-related compound-mediated interferences
BRET	Commercial reagents available Compatible with intracellular readouts Donor is protein-encoded No issues with photobleaching Background fluorescence does not affect donor Spectral separation between donor and acceptor not needed	BRET signal can be weaker that FRET Donor is protein-encoded Expression levels of donor must be matched to acceptor.	Bioluminescence attenuation (quenching, inner-filter, luciferase inhibition) Luciferase stabilizers and inhibitors BRET quenching Emission attenuation (quenching, inner-filter) Compound emission (auto-fluorescence)
ALPHA (AlphaScreen, AlphaLISA, AlphaPlex)	Large dynamic range Commercial reagents available Highly amenable to miniaturization (1536-well plates) Compatible with distances up to 200 nm Works with wide range of affinities, even weaker ones	Cost of reagents Specialized plate reader required	Excitation attenuation (light quenching, light scattering, inner-filter) Singlet oxygen quenching (physical, chemical) Emission attenuation (light quenching, light scattering, inner-filter) Compound emission (auto-fluorescence) Affinity capture system disruption
SPA	Large dynamic range Commercial reagents available	Radioactive (additional safety, waste disposal) Specialized instrumentation required (e.g., CCD or microplate scintillation counter) Requires available radiolabeled reagent	Scintillation quenching Affinity capture system disruption

Table 2 Properties of select FRET pairs. Several donor and acceptor fluorophores are listed for each FRET pair with corresponding excitation and emission wavelengths and the R0 distance for FRET.

Donor fluorophore	λ_{Ex} (nm)	λ_{Em} (nm)	Acceptor fluorophore	λ_{Ex} (nm)	λ_{Em} (nm)	R_0 (nm)	Ref
EDANS	335	493	Dabcyl	453		3.3	(5)
IAEDANS	337	490	Fluorescein	490	525	4.6	(5)
Sirius	355	424	mseCFP	434	474	3.7	(6)
mTagBFP	402	457	sfGFP	485	510	4.6	(7-10)
mAmetrine	406	526	tdTomato	554	581	6.6	(7,11-14)
ECFP	433	475	EYFP	513	527	4.9	(13,15-17)
ECFP	433	475	mVenus	515	528	5.0	(13,16-20)
ECFP	439	476	YPet	517	530	N/A	(13,16, 21)
ECFP	433	475	mCherry	587	610	3.5	(13,16,22-24)
mTurquoise2	434	474	mVenus	515	528	5.8	(17,19,20,22,25,26)
mTurquoise2	434	474	sEYFP	515	528	5.9	(26-28)
CyPet	435	477	YPet	517	530	N/A	(13,29,30)
LSSmOrange	437	572	mKate2	588	633	7.0	(7,12,31)
mTFP1	462	492	mCitrine	516	529	5.7	(11,32,33)
EGFP	488	507	ShadowG	486	510	4.7	(13,34)

Table 2 continued from previous page.

Donor fluorophore	λ_{Ex} (nm)	λ_{Em} (nm)	Acceptor fluorophore	λ_{Ex} (nm)	λ_{Em} (nm)	R_0 (nm)	Ref
EGFP	488	507	PA-GFP	504	517	4.4	(7,13,35)
EGFP	488	507	Phanta	506	516	5.8	(7,13,36)
EGFP	488	507	sREACh	517	531	5.8	(13,34)
EGFP	488	507	mCherry	587	610	5.4	(13,24)
Fluorescein	490	525	Tetramethylrhodamine	557	576	5.5	(5)
Fluorescein	490	525	QSY 7 & QSY 9	560		6.1	(5)
Alexa Fluor 488	490	525	Alexa Fluor 555	555	580	7.0	(37)
CyOFP1	497	589	mCardinal	604	659	6.9	(7,31,38)
BODIPY FL	503	512	BODIPY FL	503	512	5.7	(5)
Clover	505	515	mRuby2	559	600	6.3	(13,27)
mClover3	506	518	mRuby3	558	592	6.5	(39)
mNeonGreen	506	517	mRuby3	558	592	6.5	(10)
EYFP	513	527	mCherry	587	610	5.7	(13,17,22,24)
mVenus	515	528	mKOκ	551	563	6.3	(7,17,19,20,40,41)
mOrange	548	562	mCherry	587	610	6.3	(21,42,43)
Alexa Fluor 594	590	617	Alexa Fluor 647	650	665	8.5	(37)
eqFP650	592	650	iRFP	690	713	5.8	(7,44,45)

Table 3 Properties of select TR-FRET reagents. Donors containing europium or terbium cryptates are paired with different acceptors for detection at 665 nm or 520 nm. Data adapted from www.cisbio.com.

Donor	λ_{Ex} (nm)	λ_{Em} (nm)	Acceptor	λ_{Ex} (nm)	λ_{Em} (nm)	R_0 (nm)
Europium (III) cryptate	320 - 340	615	XL665		665	9
Europium (III) cryptate	320 - 340	615	Allophycocyanine		665	9
Europium (III) cryptate	320 - 340		d2	620	665	9
Lumi4-Tb	320 - 340	490, 545, 620	d2	620	665	5.8
Lumi4-Tb	320 - 340	490, 545, 620	Fluorescein	490	520	4.6
Terbium (III) cryptate	320 - 340	490	GFP	488	520	7

Table 4 Properties of select Alpha reagents. Alpha proximity assays all utilize the same donor beads that are excited with a 680 nm laser. Signal emission depends upon the acceptor bead type and requires proximity within approximately 200 nm.

Donor	λ_{Ex} (nm)	Acceptor	λ_{Em} (nm)	Distance (nm)
Donor bead	680	AlphaScreen	520 - 620	200
Donor bead	680	AlphaPlex (Terbium)	545	200
Donor bead	680	AlphaLISA (Europium)	615	200
Donor bead	680	AlphaLISA (Samarium)	645	200

Affinity Capture Systems and Chemical Modifications

Proximity assays can utilize several different capture approaches to couple the donor and acceptor assay components to the analyte. Popular examples include biotin/streptavidin, glutathione (GSH)/glutathione-*S*-transferase (GST), nickel-NTA/polyhistidine (Ni-NTA/polyHis), FLAG/anti-FLAG, HA/anti-HA, Myc/anti-Myc, and antibodies/analytes. Each capture system has distinct advantages, disadvantages, and other nuances that must be considered (Table 5) (58). Many of these systems utilize tags that can be genetically engineered into recombinant proteins (e.g., 6xHis, GST, FLAG, Myc, or biotinylation in *Escherichia coli*), eliminating the need for any post-translational chemical modifications (59). Most commercial proximity assay technologies incorporate reagents specifically developed for common capture systems, such as donor and/or acceptor beads conjugated to streptavidin, nickel chelate, or antibodies to FLAG, GST, HA, or species-specific secondary immunoglobulins (e.g., anti-mouse IgG, anti-rabbit IgG). Post-translational modifications (e.g., histone methylations, phosphorylated proteins) or other analytes can also be targeted in proximity assays using custom antibodies; however, such antibodies must be carefully validated for specificity (60,61). For example, polyclonal or monoclonal antibodies can be conjugated to donor or acceptor beads with conventional techniques such as reductive amination. Proteins or peptides can also be directly conjugated to donor or acceptor beads for certain applications. The optimum affinity capture system and parameters for a particular proximity assay depends on multiple factors including the molecular target(s) and is beyond the scope of this chapter.

While highly useful, affinity capture components introduce several potential sources for compound-mediated assay interference, notably compounds that disrupt tag-capture system interactions. However, the variety of affinity capture systems can be exploited to develop certain interference counter-screens, discussed in subsequent sections.

Table 5 Summary of common affinity capture systems for homogenous proximity assays. Advantages, disadvantages, and miscellaneous comments pertinent to homogenous proximity assays utilizing test compounds are listed.

Capture reagent	Advantages	Disadvantages	Other comments
Biotin-strepavidin	Streptavidin or biotinylated tags can be genetically encoded Biotin amenable to chemical conjugation to various biomolecules Many commercial kits available Strong affinities (K_d ~ 10^{-15} M)	Steric bulk streptavidin tags Biotin-streptavidin interaction susceptible to biotin and biotin mimetics Biotin-streptavidin interaction can be sensitive to protein denaturants	Certain tissues contain biotin and avidins Certain bacteria produce avidins Streptavidin has increased specificity, lower nonspecific binding than avidin
GSH-GST	GST tag genetically encoded Tagged proteins very easy to purify Many commercial kits available	Steric bulk GST tags GSH/GST interaction susceptible to GSH mimetics GSH/GST interaction can be sensitive to protein denaturants GST can dimerize Potential for increased protein aggregation	Potentially redox sensitive
Anti-GST-GST	GST tag genetically encoded Tagged proteins very easy to purify Many commercial kits available	Steric bulk GST tags Anti-GST/GST interaction susceptible to mimetics Sensitive to protein denaturants GST can dimerize Potential for increased protein aggregation	

Table 5 continued from previous page.

Capture reagent	Advantages	Disadvantages	Other comments
Antibodies-analyte	Quality antibodies can possess high specify and sensitivity Many antibodies commercially available	Polyclonal and monoclonal antibodies can show lot-dependent variations Antibody-analyte interactions susceptible to analyte mimetics Sensitive to protein denaturants	Need to verify specificity of antibody
Polyhistidine-tag	His tag genetically encoded Many commercial kits available Tagged proteins are very easy to purify	Metal ion/His tag interactions are susceptible to chelators	Zinc and cobalt can be used in place of nickel ions
FLAG tag	FLAG tag genetically encoded Many commercial kits available	FLAG-Anti-FLAG interactions susceptible to mimetics	Needs other tags for protein purification
Myc tag	Myc tag genetically encoded	Myc-Anti-Myc interactions susceptible to mimetics	

Section Summary

Homogenous proximity assays are widely used in HTS and follow-up assays for testing hits and compounds with bioactivity. Common technologies include Alpha, FRET, BRET, TR-FRET, and SPA, each of which exploits the proximity of a donor and acceptor source for optimal signal production. However, each technology, including commonly employed affinity capture systems, is susceptible to a variety of compound-mediated assay interferences that are discussed in detail in the subsequent section.

Chemical Mechanisms of Assay Interference in Homogenous Proximity Assays

Homogenous proximity assay technologies are susceptible to a range of compound-mediated assay interferences. This section describes the chemical mechanisms that can alter proximity assay readouts. Technology-related interferences can be attributed to different mechanisms related to the properties of compounds in solution and their impact on the assay technology. Such interferences can ultimately manifest as reductions of the assay signal due to mechanisms such as absorbance, quenching, light scattering, reporter enzyme modulation, and disruption of affinity capture components. For example, compounds that interfere with excitation of the donor are encountered in FRET- and Alpha-based proximity assays, as compared to SPA and BRET where initial excitation is caused by radioactive decay of a radioisotope or luciferase activity, respectively. Alternatively, depending on the assay technology employed, assay signals can be increased by interference mechanisms including auto-fluorescence, phosphorescence, and singlet oxygen generation. Sources of non-technology-related interferences that can constitute poorly tractable sources of bioactivity, such as nonspecific compound reactivity and aggregation, are also discussed. Each mechanism is discussed below, as understanding fundamental chemical mechanisms of assay interference allows the design of more effective counter-screens and the subsequent identification of likely interference compounds from homogenous proximity assays utilized in HTS and follow-up campaigns (62).

Mechanisms of Interference

Fluorescence quenching. Quenching is a phenomenon by which fluorescence is decreased in intensity due to another chemical species. *Dynamic quenching* can occur through (1) FRET, whereby energy is transferred from excited donor to a proximal quencher with significant spectral overlap through nonradiative dipole–dipole interactions; (2) Dexter electron transfer, a short-range process dependent on molecular orbital overlap between the excited donor and a proximal quencher, and (3) exciplex formation. *Static* or *contact quenching* is a process

where the ground state donor comes into physical contact with the quencher to form a complex with altered spectral properties. Each of these quenching processes can induce a quencher to an excited state. As the excited quencher returns to the ground state, this can result in light emission through emissive decay, albeit at lowered intensities relative to quencher-free conditions. Other quenchers act through *dark quenching*, whereby the energy released from the excited to ground state transition is dissipated through molecular vibrations (heat). The end result can be a perturbation in the assay signal independent of the desired bioactivity. Signal attenuators can interfere with several stages in most proximity assays: (1) donor signal excitation/transmission; (2) donor to acceptor signal transmission; and (3) acceptor emission (Figure 2A).

The efficiency of such quenchers is directly related to their extinction coefficient (ε), a factor that represents the capacity of a compound to absorb light at a specific wavelength under a specific set of experimental conditions. The higher ε is, the more efficient the compound at absorbing light. This absorption capacity is also directly related to compound concentration as expressed by the classic Beers-Lambert law (Equation 1):

$$\text{Absorbance} = \text{Concentration} \times \text{Extinction coefficient } (\varepsilon) \times \text{path length} \qquad \text{Equation 1}$$

Chemical quenching. For Alpha-based proximity assays, compounds can interfere with the readout by quenching singlet oxygen, which defined broadly can include (1) energy and charge transfer mechanisms between singlet oxygen and the compound quencher that returns singlet oxygen to the ground state without oxygen consumption (physical quenching); and (2) direct chemical reaction of singlet oxygen with the quenching compound to consume oxygen (chemical quenching) (Figure 2B) (63,64). In organic synthesis, singlet oxygen classically reacts with cunsaturated chemotypes such as alkenes (ene reactions) and also certain heteroatoms to form various oxidation products such as peroxides (65). Singlet oxygen is also capable of oxidizing various phenols, sulfides, and amines. Some examples of singlet oxygen quenchers include carotenes, tocopherols, and tertiary amines like 1,4-diazabicyclo[2.2.2]octane (DABCO; Figure 2B) (66-68). There are also lingering questions about the quenching potential of certain chemotypes prevalent in screening collections including unsaturated carbonyls, and thiones (69-72). As much of the singlet oxygen chemistry has been performed in the context of organic synthesis under non-assay-like conditions (i.e., organic solvents, non-physiological temperatures), the chemical mechanisms, prevalence, and overall significance of singlet oxygen quenching by test compounds in Alpha-based assays are relatively uncharacterized and should be confirmed in conditions mimicking the parent assay.

Certain assay components or contaminants may also interfere with Alpha-based technologies. For example, azide (N_3^-), a common antimicrobial additive in biological reagents, may interfere with singlet oxygen transmission at sufficiently high concentrations. Certain metals (Al^{3+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, Ni^{2+}, Zn^{2+}) may also quench singlet oxygen, albeit at low millimolar concentrations (73). Notably, metals can be present as contaminates within test compound solutions (74).

Scintillation can be susceptible to chemical quenching. Compound disruption of the scintillation process causes non-radiative dissipation of energy manifesting as an apparent reduction of the energy of the decay event. This translates to a decrease in the number of photons produced (75). Some liquid scintillation counters have built-in functions for quenchers, often prepared by obtaining standard curves of known quenchers (75-78). However, it should not be assumed that such strategies completely correct for scintillation interference.

Absorbance. Test compounds can interfere with proximity assay readouts by absorbing light. Compounds that interfere with this assay step by inner-filter effects will absorb light in the excitation wavelength. For Alpha-based assays, these are usually blue-colored compounds that strongly absorb in the near-infrared region (Table 6 and Figure 3A). By contrast, the yellow and orange compounds more prevalent in screening libraries do not usually absorb in the light regions relevant to Alpha technology. For FRET-based assays, these types of interfering compounds usually absorb at shorter wavelengths corresponding to overlap with the FRET donor fluorophore. With SPA, some compounds, usually colored, can interfere with scintillation (also referred to as

'color quenching') (79). As with quencher, the ability of compounds to interfere with light can be expressed by the Beers-Lambert law (Equation 1), the consequence being that absorbance is concentration-dependent and dependent on the extinction coefficient for a particular compound at a given wavelength and under defined experimental conditions. For additional details on compound interference *via* light absorbance, refer to Interference with Fluorescence and Absorbance.

Light scattering. Another related mechanism of signal quenching is light scattering, whereby insoluble materials such as screening compounds effectively diffract light (Figure 2C) (80,81). Many of these compounds have high cLog*P* values and other poor physicochemical properties related to solubility in assay conditions. A characteristic of light-scattering compounds is a relatively continuous absorption spectrum (i.e., no apparent λ_{max}). Light scattering is most efficient at shorter wavelengths (decreases by factor of n^4), and scattered light is relatively polarized (81). This phenomenon can interfere with the transmission of the initial excitation light source (e.g., laser) and the final light excitation. Such compounds can occasionally be identified by simple visual inspection for cloudy suspensions or by centrifuging a sample and looking for gross precipitate. More quantitatively, this can be accomplished by nephelometry, turbidimetry, or dynamic light scattering. In certain cases, their apparent activity can be attenuated by the inclusion of detergents such as 0.1% Tween-20 (v/v) (Figure 3B; see also subsequent section Strategies to Mitigate Interference by Light-based Interferences).

Case example: Interpreting light-based interferences can be complex. For example, Figure 4 shows test compound activities in a TR-FRET assay for bromodomain inhibitors where binding of a His-tagged bromodomain to a biotinylated peptide is coupled to a Eu-labeled anti-His antibody (donor) and APC-labeled streptavidin (acceptor) for detection. Examining the acceptor and donor signals separately shows tight distributions for the active (AC) and neutral (NC) controls, whereas many test compounds interfere with the fluorescence signal (Figure 4A) and some would be classified as hits due to a reduction in the TR-FRET normalized ratio (Figure 4B). For this example, taking compounds that show a signal reduction greater than three standard deviations from the donor channel suggests that 30% of the putative hits are likely fluorescent quenchers. However, understanding the many interference mechanisms involved is complicated and building evidence for genuine biological activities of individual test compounds would benefit from orthogonal assay technologies and interference counter-screens.

Luciferase modulators. Certain proximity assays use luminescence reporters such as either BRET (Renilla luciferase and GFP) or NanoBRET (Nanoluciferase and Halotag enzymes) or split reporter enzymes as employed in NanoBiT technology that uses split forms of Nanoluciferase. Compounds that attenuate the luciferases signal either through inhibition of the enzyme or through light absorbance will interfere with these assay formats. For a review of luciferase inhibitors refer to Interferences with Luciferase Reporter Enzymes. For ratiometric assays such as BRET (or TR-FRET as mentioned above), it is important to examine both donor and acceptor channels in addition to the ratio data which is discussed in more detail in this chapter (see subsequent section Ratiometric Data Analysis).

Affinity capture system disruption. The next major category of compound-mediated proximity assay interference is disruptors of affinity capture components. These compounds interfere with affinity capture systems, leading to a disruption of the assay signal. Depending on the assay configuration, such interference could be misinterpreted as bioactivity. The chemical mechanisms of affinity capture interference depend on the specific donor and acceptor components.

One of the more popular affinity capture systems for proximity assays is the His-Ni^{2+} system. The popularity of this tag is given by its dual use for protein purification and affinity capture for proximity assays. While highly useful, several chemotypes have been shown to interfere with the His-Ni^{2+} interaction, including in AlphaScreen assays (Figure 5A and Table 4) (82). The most common motif in one explicit analysis of His-Ni^{2+} disruptors was the NCCN chemotype (Table 7). It is hypothesized that many of these chemotypes form bifurcated hydrogen bonds with NH-donating histidine side chain residues (present in His-tag) and/or chelate metal ions (present on

beads) (Figure 5B) (83-85). If bound to either the histidine tag side chains or nickel, proper tag function could be disrupted and the assay signal could be attenuated. A second prevalent category of His-Ni^{2+} frequent fitters are chelating agents. Examples for these chemotypes are picolylamines or pyridines (Table 4). Certain hydrazones may also interfere by a chelation mechanism (68).

The streptavidin-biotin interaction is another popular choice for coupling an analyte to a detection technology. Compounds with significant affinities (including fast kinetics) for avidins such as biotin analogs or isosteres may in principle compete with biotin to disrupt the intended streptavidin-biotin interaction (Figure 5C) (87). Biotin-avidin interactions are well characterized, and while biotin analogs have been described, the likelihood of such compounds actually interfering through this mechanism is probably low due to the remarkably strong affinity of the streptavidin-biotin interaction ($K_d \sim 10^{-15}$ M) (88-90). In one report, 2 of 24,779 (0.008%) compounds interfered by this mechanism in a TR-FRET HTS versus ROCK-II inhibitors (87). As another example, several compounds were found to interfere with the streptavidin-biotin interaction among 1,199 hits evaluated from a primary screen with an AlphaLISA assay (Figure 6) (91). In proximity assays utilizing streptavidin-biotin, the buffer should be otherwise free of biotin (as a contaminant, as an intended ingredient in the media such as RPMI, or as an endogenous component of biological samples such as serum). This biotin will compete with the affinity capture system and disrupt the intended streptavidin-biotin interaction. Furthermore, test compounds with structural similarity to biotin including the imidazoline-2-one motif can disrupt the affinity capture system (Figure 6) (92,93).

The GSH-GST interaction has also been successfully utilized to couple an analyte to detection reagents. There are several potential chemical mechanisms for compound-mediated interference of GSH-GST interactions: (a) binding of a compound with similar molecular features as GSH to the GSH-binding site on GST, (b) covalent modification of GST or GSH by electrophilic compounds, and (c) reversible binding to non-active sites on GST (Figure 4D) (94). Note that most reagents utilizing GSH will immobilize GSH at its *S*-position, so compound reactivity with GSH would presumably occur at its terminal *N*- or *C*- positions.

Several chemotypes have been shown to interfere with the GSH-GST interaction in the context of AlphaScreen technology (Table 8) (94). Note a similar affinity capture system utilizes an anti-GST antibody and a complimentary GST tag. In theory, compounds that bind GST or the anti-GST antibody could disrupt the intended antibody (anti-GST)-analyte (GST) interaction.

A subset of affinity capture systems susceptible to compound-mediated interference is antibody-analyte systems. Compounds with chemotypes similar to the antibody antigen may compete with the desired antigen, effectively preventing the antibody-antigen interaction. A notable example of this interference are acetamide-containing compounds that were enriched in an AlphaScreen HTS which utilized an acetylated lysine/anti-acetylated lysine antibody system (Figure 5E) (86). In another example, following a screen of ~350,000 with an AlphaLISA primary assay, 1,199 hits were further evaluated by a battery of counter-screens (95). By comparing results from TruHit counter-screen configurations with a counter-screen utilizing the primary assay and purified analyte, it was possible to rule out any interferences by antibody-mediated capture of the analyte (N. P. Coussens, unpublished data).

Auto-fluorescence. Auto-fluorescence can be induced by: (1) absorption of the donor excitation light, (2) absorption of the energy transferred from the donor to acceptor, and (3) compounds that absorb the signal from the acceptor (Figure 7A). Such compounds can interfere with the final assay readout by producing measurable signal even in the absence of the intended analyte.

Compared to other interferences, most compounds that emit light in the assay readout can be rather easily identified by signal intensities that are outside the range of the controls. This is especially apparent in assays where desired bioactivity produces an attenuated signal. With such an assay, a compound that emits light, but also has genuine bioactivity might not be identified and would thus be a false negative. By contrast, in assays

where desired bioactivity produces an augmented signal, emitters have the potential to function as false positives. Finally, it should be noted that compounds with genuine on-target activity but that also fluoresce can display a range of patterns, depending on the assay conditions and the compound properties (Figure 7B).

Singlet oxygen generators. In Alpha-based assays, aside from fluorescent compounds, certain compounds may produce singlet oxygen *in situ* (Figure 7C) (96,97). These photosensitizing agents characteristically possess high absorption coefficients in the excitation light spectral region, contain a triplet state allowing for efficient energy transformation to ground state oxygen, have high quantum yields for the triplet state, and have high photostabilities (64). Prototypic agents include organic dyes such as rose bengal, eosin derivatives, and methylene blue, as well as porphyrin derivatives (Figure 7C). These compounds are relatively uncommon in screening collections as they are non-lead-like by conventional metrics. Naphthalenes, anthracenes, quinones, and anthraquinones are aromatic hydrocarbons chemotypes that may be screening collections, and notably these and related derivatives have been shown to produce singlet oxygen in certain aqueous conditions (98,99). The significance of photosensitizing compounds in the context of biological screening conditions and Alpha assay interference is relatively unexplored, however. Additionally, note that some compounds that produce singlet oxygen *in situ* have been shown to damage SPA reagents (100).

Phosphorescence. During an evaluation of hit compounds, Erythrosin B was identified as an outlier by the EPIgeneous HTRF Methyltransferase assay (Cisbio), with a signal 10-fold beyond the 100% inhibition control (N. P. Coussens, unpublished observation). The assay measured SAH, which competitively displaces d2-labeled SAH that is pre-bound to anti-SAH labeled with Lumi4-Tb, resulting in a loss of FRET signal. In the absence of SAH (100% inhibition control), excitation of the terbium donor results in a long emission at 620 nm capable of exciting the d2 acceptor which emits at 665 nm. In the case of Erythrosin B, the donor emission (normalization control) was consistent with the other controls; however, the signal at 665 nm was 10-fold higher. It has been reported that excitation of Erythrosin B at 490-560 nm results in an emission at 665 nm that can persist for ~700 μs (101). The major emission peak for terbium is centered at 540 nm, which is in the excitation range of Erythrosin B.

Other mechanisms of assay interference in proximity assays. Recently it was reported that salicylic acids can interfere with TR-FRET assays by causing a large signal enhancement attributed to the interaction between the salicylate and the cryptand-ligated europium (102). In SPA, compounds that emit radiation may interfere via signal emission, though such compounds are not routine components of screening libraries and would be extremely rare. However, such compounds could occur as contaminants in screening centers making use of radioisotope-based assays and reagents.

Proximity assays are susceptible to a variety of generalized compound-mediated assay interferences, including nonspecific reactivity and aggregation (103). Reactive test compounds, including redox-active compounds that produce H_2O_2 *in situ*, can interfere with biological assays by reacting nonspecifically with assay reagent(s), substrate(s), target(s), and/or unrelated target(s) (104,105). Aggregating test compounds can interfere with biological assays by forming compound aggregates that nonspecifically interact with assay proteins, leading to altered protein structure and dynamics (106-109).

A common pitfall is to assume bioactivity confirmed by an orthogonal assay represents tractable bioactivity and excludes the possibility of false positives. In many cases, apparent bioactivity from aggregating or nonspecific reactive compounds is *highly reproducible* and *can be confirmed by orthogonal assays*. This is because reactive or aggregating compounds disrupt protein structure and dynamics nonspecifically in susceptible systems. However, unlike more desirable mechanisms of bioactivity, this reactivity and general protein perturbation is generally poorly tractable and difficult to optimize for specificity. Depending on the biological system and assay, aggregating compounds can display bell-shaped curves and some compounds can fail to reach maximal (100%) inhibition regardless of compound concentration (J. Walsh, unpublished observations) (110,111). Therefore, it is

crucial to perform appropriate orthogonal assays *and* counter-screens to rule-out generalized compound-mediated assay interference (Table 9).

For additional details on assay interference by aggregators in HTS, refer to Assay Interference by Aggregation. For additional details on assay interference by reactivity in HTS, refer to Assay Interference by Chemical Reactivity.

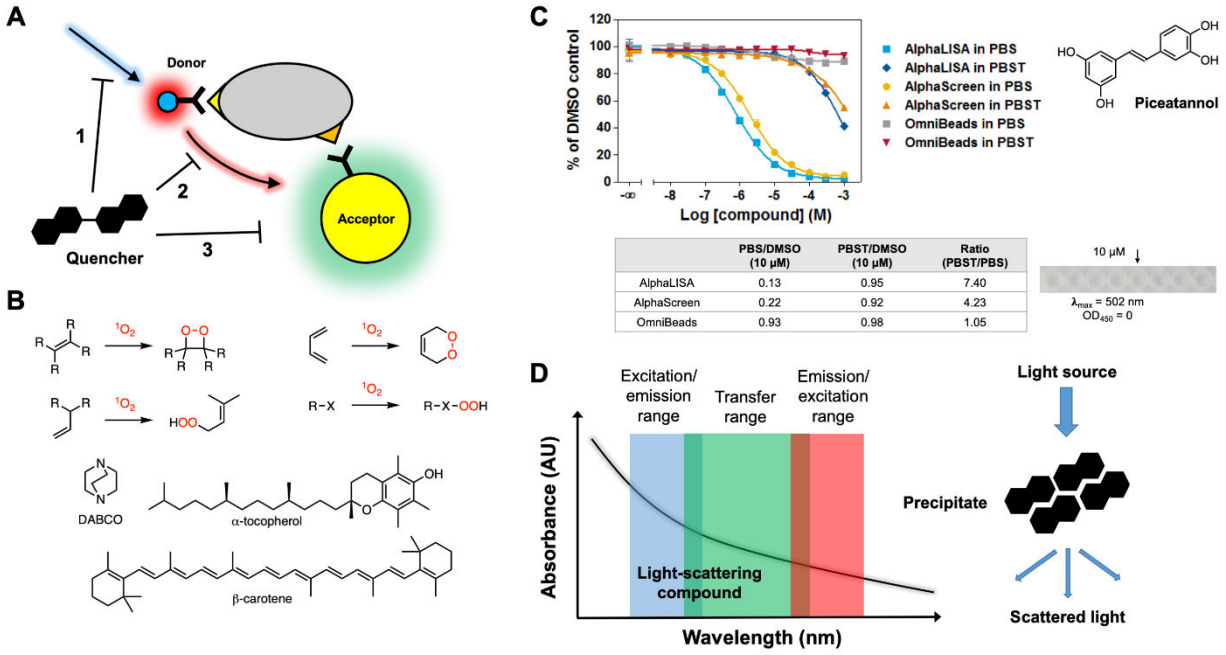


Figure 2 General schematic of compound-mediated interference in homogenous proximity assays by signal-attenuating compounds. (A) Compounds can attenuate the signal of proximity assay readouts at several points: (1) excitation of donor (Note: for BRET this could be a luciferase inhibitor), (2) signal transmission from the donor to the acceptor, and (3) final emission event. Note: for SPA, interference can be observed at points (2) and (3). (B) Singlet oxygen quenching by test compounds. (left) Prototypical reactions of singlet oxygen. (right) Prototypical singlet oxygen quenchers. (C) Example of likely singlet oxygen quenching in Alpha technology by piceatannol. Compounds were tested in concentration-response format using AlphaScreen, AlphaLISA, and Omnibead formats in the presence and absence of Tween-20 detergent. The compound does not interfere significantly in the Omnibead format, as the singlet oxygen transmission occurs within the beads. Again note the interference presumably by light scattering is attenuated by the addition of 0.1% Tween-20 (v/v). Data courtesy P. Roby (PerkinElmer). (D) Light-scattering compounds can also attenuate assay light transmission through diffraction. Such compounds are often insoluble in assay conditions and have absorbance spectra with characteristically continuous light absorption.

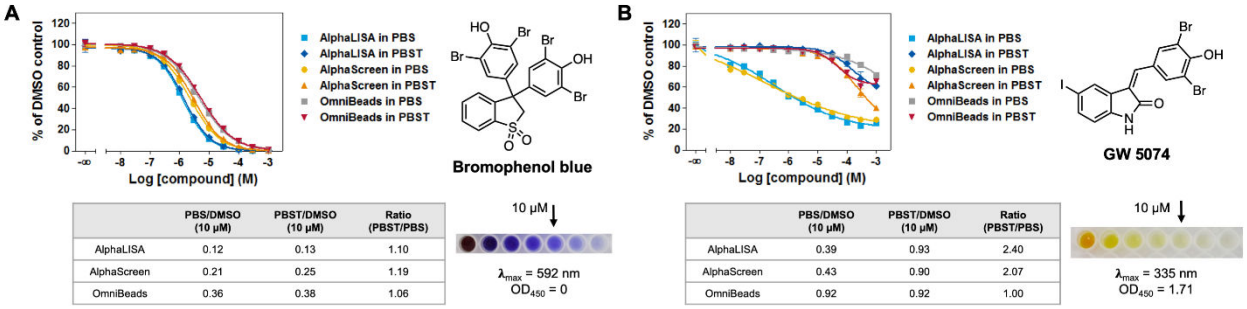


Figure 3 Examples of colored and scattering interferences in AlphaScreen. Compounds were tested in concentration-response format using AlphaScreen, AlphaLISA, and Omnibead (beads containing all necessary Alpha chemistry reagents) formats in the presence and absence of Tween-20 detergent. (A) Example of a colored compound, bromophenol blue, interfering with Alpha technology. The compound interferes similarly across all assay formats, as each format requires light transmission that overlaps the compound absorption. (B) Example of a poorly soluble compound, GW 5074, interfering with Alpha technology readouts. Note the interference presumably by light scattering can be attenuated by the addition of 0.1% Tween-20 (v/v). The compound does not interfere significantly in the Omnibead format, as the required light transmission occurs within the beads. Data courtesy P. Roby (PerkinElmer).

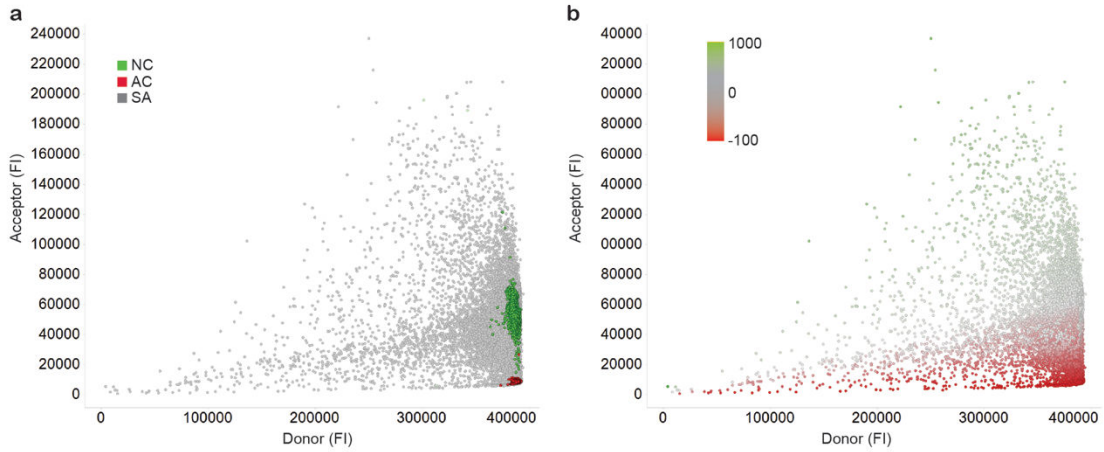


Figure 4 Example compound interference in a TR-FRET assay. (A) Neutral controls (DMSO treated, NC; green) and active controls (unlabeled peptide; red) show a tight distribution in both donor and acceptor fluorescent signals, while compound samples (grey data) significantly skew these signals with both quenching effects on the donor as well as increases in the acceptor signal, likely due to light scattering effects. (B) The same data colored by the normalized ratio (A/D) percent activity (-100% = 100% inhibition, red). Many of the quenchers are scored as hits. Data courtesy D. Auld (Novartis Institutes of Biomedical Research).

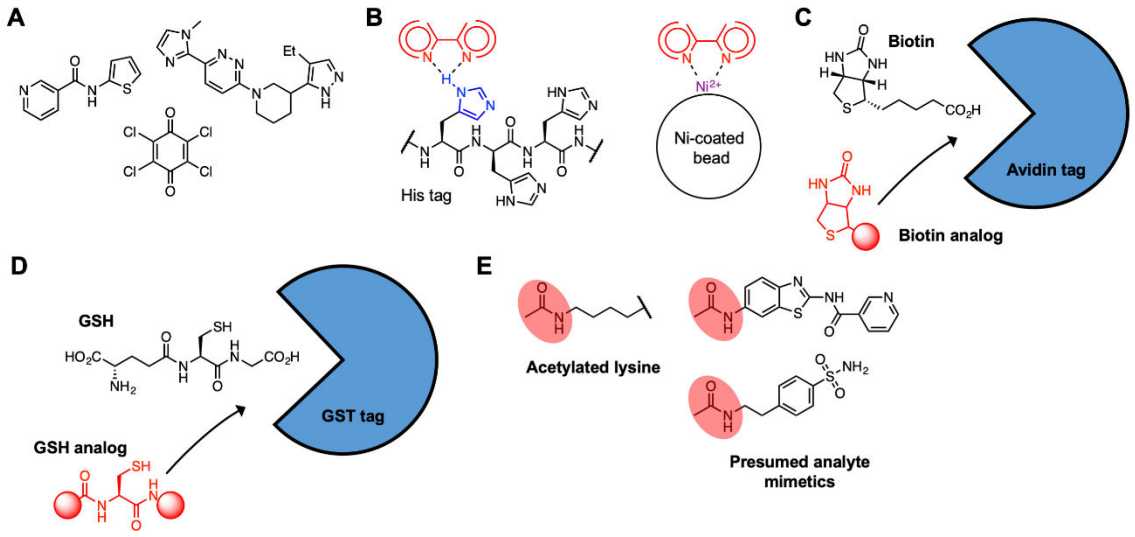


Figure 5 Compound-mediated interference of affinity capture systems in proximity assays. (A) Examples of reported His- Ni^{2+} interaction disrupters (82). (B) Potential chemical mechanisms of His-Ni^{2+} interference include interfering compounds (red) that are proposed to form hydrogen bonds with NH-donating histidine side chains (blue, left schematic) or coordinate with nickel ions (purple, right schematic). (C) Potential chemical mechanisms of biotin-streptavidin capture interference include compounds such as biotin analogs (red) that compete with biotin for binding. (D) Potential chemical mechanisms of interference of GSH-GST-mediated capture interference include compounds such as GSH analogs (red) that compete with GSH for the GSH-binding site on GST. (E) Potential chemical mechanisms of interference of antibody-analyte capture interference include analyte mimicry. In this example from an AlphaScreen for inhibitors of the MOZ histone acetyltransferase, the assay quantified the amount of acetylated lysine residues using an anti-acetyl lysine antibody conjugated to an acceptor bead (86). Compounds with an acetamide motif were enriched among the HTS primary actives and were triaged using a combination of counter-screens (product-spiking, antibody swap).

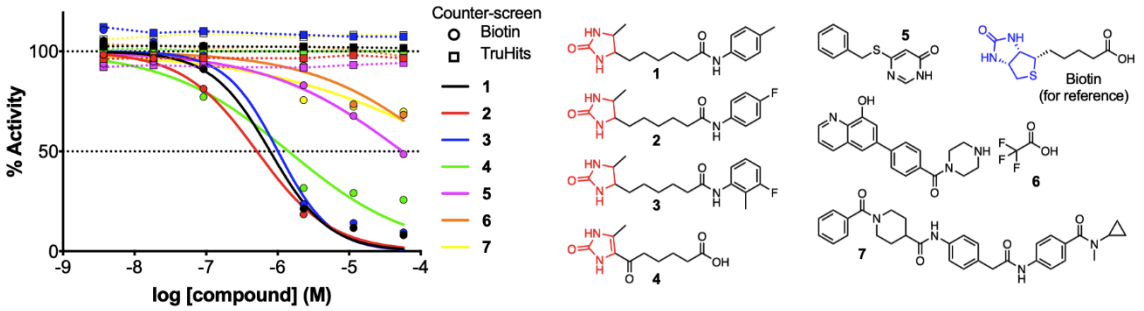


Figure 6 TruHit counter-screen flags compounds that disrupt the streptavidin-biotin interaction. Compounds **1**-**7** disrupt the streptavidin-biotin interaction were identified after performing both configurations of the TruHit counter-screen. Compounds **1**-**7** only showed activity when pre-incubated with streptavidin donor beads prior to the addition of biotinylated acceptor beads (circles), whereas no activity was observed when the beads were premixed before compound addition (squares). Note **1**-**4** contain the imidazoline-2-one motif (red) common to biotin (blue).

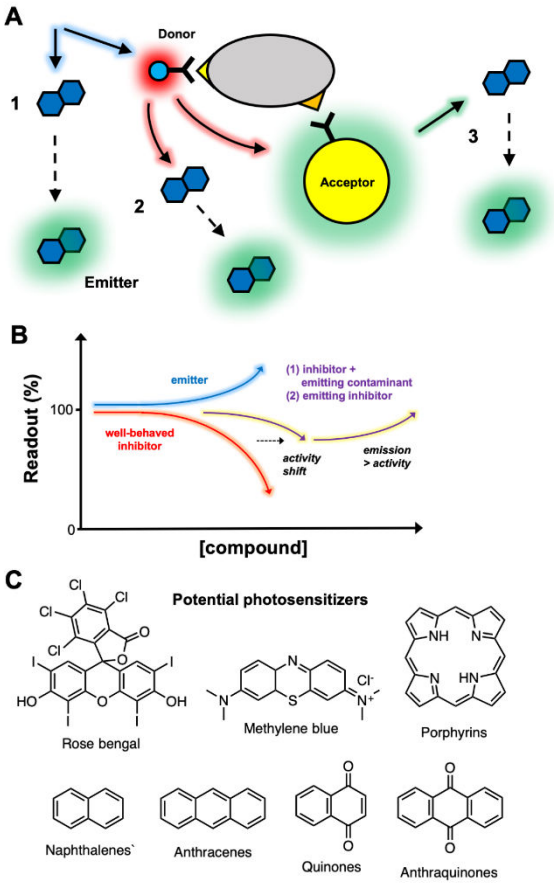


Figure 7 Compound-mediated interference in proximity assays by compound emission. (A) Compounds (blue hexagons) can interfere with proximity assay readouts through compound emission at several points: (1) compound excitation by initial excitation event, (2) compound excitation by signal transmission from donor to acceptor, and (3) compound excitation by final emission event. Interference can result when the test compound emits signal (usually light) in the same wavelength as the final assay readout. (B) Compounds that emit light can be easier to identify from assays with negative readouts (compare red and blue lines); however, emission from a compound with on-target activity could display a variety of patterns and result in a false-negative result (orange lines). (C) With Alpha-based assays, compound-mediated emission could result from photosensitizing agents that produce singlet oxygen under aqueous conditions causing emission from the acceptor bead. Structures of representative photosensitizing agents are shown.

Table 6 Examples of reported AlphaScreen quenchers via light absorbance. Colored compounds have the potential to interfere with AlphaScreen and FRET readouts due to their light absorbance properties.

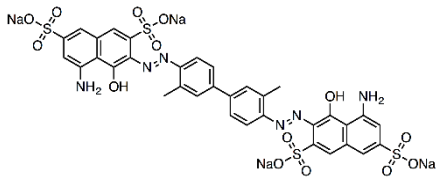
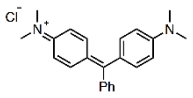
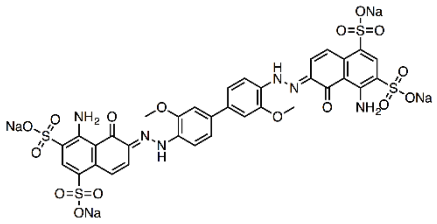
Compound name	Chemical structure	Concentration	% interference	λ_{max} (nm)	Reference
Trypan blue		1 μM	~90	607	(73)
Malachite green		1 μM	~20	615	(73)
Chicago sky blue		1 μM	~40	618	(73)
Sodium azide		0.01% (w/v)	~60		(73)

Table 7 Reported AlphaScreen His-Ni2+ interaction disrupting chemotypes. Chemotypes were derived from an AlphaScreen-based counter-screen (82). Many of the His-tag disruptors are thought to interfere by a chelation mechanism. See also https://ochem.eu (AlphaScreen-HIS-FHs) for SMARTS strings and online substructure screening tool.

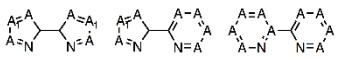
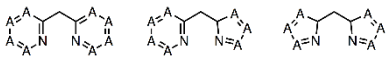
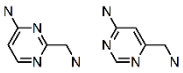
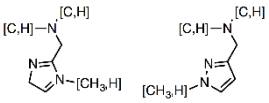
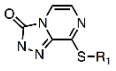
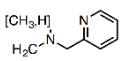
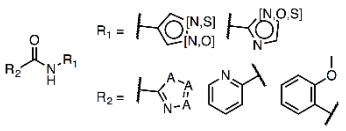
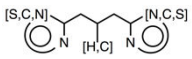
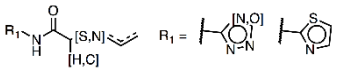
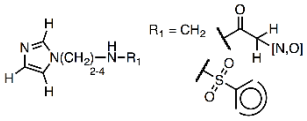
Name	Chemotype	Name	Chemotype
NCCN	 A = aromatic C or N; A1 = aromatic C, N or S	Diarylmethanes	 A = aromatic C or N
Picolylamines_A		Aminals	 Dashed line = any type of covalent bond
Picolylamines_B		Heterylsulfides	 R_1 = any atom, group
Picolylamines_C		Heterylamides_A	 A = any aromatic atom
Bis-picolylamines		Heterylamides_B	
Alkylimidazoles_A		Pyrimidinetriones	

Table 7 continued from previous page.

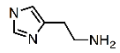
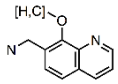
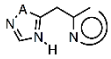
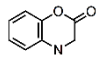
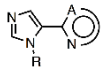
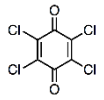
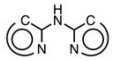
Name	Chemotype	Name	Chemotype
Alkylimidazoles_B		8-Quinolinols	
Alkylimidazole_C	 A = any aromatic atom	Benzoxozinones	
Heterylimidazoles	 R = any atom, group; A = any aromatic atom	Perchloroquinone	
Diarylamines			

Table 8 Reported AlphaScreen GST-GSH interaction disrupting chemotypes (94). Chemotypes were derived from an AlphaScreen-based counter-screen. See also https://ochem.eu (AlphaScreen-GST-FHs) for SMARTS strings and online substructure screening tool.

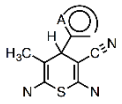
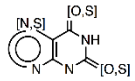
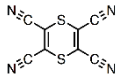
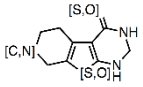
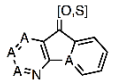
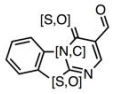
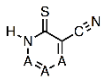
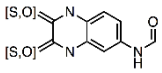
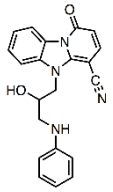
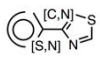
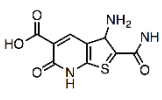
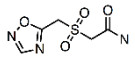
Name	Chemotype	Name	Chemotype
Cyanothiopyrans	 A = any aromatic atom	Pyrimidinodiones	
Cyanodithiine	 Singleton	Thienopyrimidinone_A	
Azafluorenones	 A = aromatic C or N	Thienopyrimidinone_B	
Cyanothioazinones	 A = aromatic C or N	Piperazinediones	
Cyanopyridinones		4-Heteryl-thiazoles	
Thienopyridinones		Oxadiazoles	

Table 8 continued from previous page.

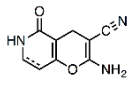
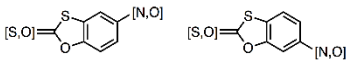
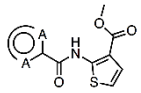
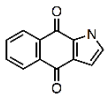
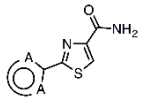
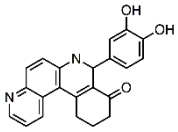
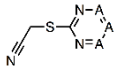
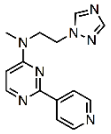
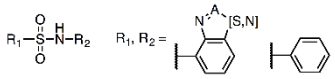
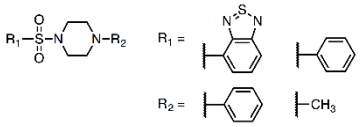
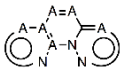
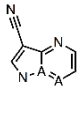
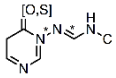
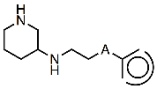
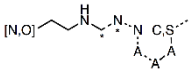
Name	Chemotype	Name	Chemotype
Pyranopyridinones	 Dashed line = double or aromatic bond	Cycloheptenones	
Benzoxathiolones		Thiophenes	 A = any aromatic atom
Indoloquinones		Thiazoles	
Catechols		Cyanothioethers	 A = aromatic C or N
Pyrazolones		Aminopyrimidines_A	
Sulfonylamide_A	 A = any atom	Aminopyrimidines_B	
Sulfonylamide_B		Fused azacycles_A	 A = aromatic C or N
Thiopyrylium		Fused azacycles_B	 A = aromatic C or N
Pyrimidinones_A	 * = bond also can be aromatic	Ethylenediamines	 A = any aliphatic atom
Pyrimidinones_B		Arylethanolamines	 Dashed line = aromatic bond; * = atoms that must belong to 6-membered ring
Pyrimidinones_C		Azaspironones	

Table 9 Interpretation of technology-independent compound-mediated assay interference in proximity assays. Well-behaved active compounds will show activity in both the proximity assay and the orthogonal assay. Technology-related artifacts will typically be inactive in truly orthogonal assays. Note compounds active through nonspecific mechanisms of action such as reactivity and aggregation of target assay components may show activity in both the proximity and orthogonal assays. Active compounds should be assessed for common sources for nonspecific bioactivity by additional counter-screens.

Mode of action	Proximity assay	Orthogonal assay	Interpretation
Well-behaved active compound	Active	Active	Rule-out nonspecific activity with aggregation, reactivity counter-screens
Quencher, capture system disruptor	Active	Inactive	Proximity assay-specific artifact
Nonspecific reactivity	Active	Active	Rule-out nonspecific activity with aggregation, reactivity counter-screens
Aggregation	Active	Active	Rule-out nonspecific activity with aggregation, reactivity counter-screens

Prevalence of Interferences in Homogenous Proximity Assays

There are multiple factors that can influence the prevalence of interferences in a given homogenous proximity assay, including:

- Compound library composition and concentration of test compounds (112)
- Assay technology
- Biological target (e.g., is it intrinsically sensitive to thiol-reactive compounds or aggregators?)
- Buffer/media components (detergent, scavenging reagents, chelating agents, salts, metal ions)
- Enzyme, substrate, and co-factor concentrations
- Reagent concentrations
- Spectrophotometric settings (wavelengths, filter widths, acquisition delay)
- Temperature, pH, oxygen saturation, ambient room fluorescent lights (for certain fluorophores and microplates)
- Assay procedure (incubation times, order of compound and reagent addition)

Section Summary

Based on the assay technology, proximity assays are susceptible to several sources of compound-mediated assay interferences. The main categories of technology-related compound-mediated assay interference include compounds that (1) attenuate signal transmission, (2) disrupt capture systems, (3) emit signal. Proximity assays are also susceptible to non-technology-related sources of assay interference, notably aggregation and nonspecific reactivity including thiol reactivity and redox activity. A sound knowledge of these chemical mechanisms enables the design of useful counter-screens and orthogonal assays for assessing compound-mediated assay interference, as discussed in the next section.

Strategies to Identify Compound-Mediated Interference in Homogenous Proximity Assays

This section describes a number of counter-screens that can be devised for the identification of compound-mediated interference mechanisms associated with homogenous proximity assays. Example counter-screen strategies include target-independent configurations, bypassing a reaction or process to only evaluate analyte detection, switching affinity capture components, and profiling studies, such as absorbance and emission tests. Data from these counter-screens can help to link technology-related interference mechanisms to test compounds; however, it is important to keep in mind that such compounds might also have on-target activity,

which should be evaluated by an orthogonal assay. The implementation of counter-screens will help to triage assay-specific artifacts, confirm truly bioactive compounds, and identify potentially useful bioactive technology-related interference compounds. Suggestions are given on the workflow design for both technology-related and generalized counter-screens, as well as orthogonal assays. Data analysis strategies are also discussed, including cheminformatics analyses to flag compounds associated with promiscuity and/or assay interference.

Employment of Counter-Screens in Workflows

An important question is, "How much effort should be devoted to investigating compound-mediated interferences?" We recommend performing at least one orthogonal assay to confirm bioactivity. In addition, we also recommend performing specific counter-screens for the primary biological assay interferences. The rationale for performing interference counter-screens even if the intended bioactivity is confirmed by orthogonal assays is to determine how much of the readout is due to true bioactivity versus interference. Note that certain counter-screens such as product spiking can efficiently identify a variety of interference mechanisms. More detailed counter-screens for specific interference mechanisms (e.g., pull-downs, emission spectral analyses) may be warranted under certain circumstances including further de-risking prior to more involved studies or compound progression.

Post-HTS screening triage should include counter-screens for technology-related assay interference and also orthogonal assays (62). Counter-screens and orthogonal assays can either be performed serially or in parallel. The exact order usually depends on the number of hits marked for follow-up, the throughput of each follow-up assay, the number of replicates and doses to be tested, and the pre-test probability of each interference type (the latter can be estimated by examining the structure of actives, and the behavior of the primary assay in response to known interference compounds). In practice, with sufficient planning, including pre-HTS optimization, technology-related counter-screens can be performed in a matter of weeks.

Compounds with confirmed bioactivity in orthogonal assay(s) and minimal interference in assay-related counter-screens should be considered for progression in a project. Compounds without confirmed bioactivity in orthogonal assay(s) that demonstrate interference in assay-specific counter-screens should generally be triaged. Compounds with confirmed bioactivity in orthogonal assay(s) and confirmed interference in counter-screens can be progressed, with the caveat that subsequent experiments should continuously monitor assay interference in addition to bioactivity. For target-based assays, biophysical measurements such as surface-plasma resonance should be performed to confirm compound binding and stoichiometry to target molecule and target-engagement in cell-based assay can be confirmed using techniques such as cellular thermal shift assays (CETSA). Additional details on CETSA can be found at the related Assay Guidance Manual chapter Screening for Target Engagement using the Cellular Thermal Shift Assay.

Target-Independent Configurations as Counter-Screens

Counter-screens can be performed to determine whether test compounds modulate the signal independent of the target. One approach is to utilize linker substrates to induce proximity between the donor and acceptor components. For example, a linker substrate might be a biotinylated peptide containing a polyhistidine tag (Figure 8A). Ideally the linker incorporates the same affinity capture components that are utilized in the primary assay. This enables the identification of compounds that disrupt the interactions between the affinity capture components in addition to other technology-related interference mechanisms. If strong-affinity capture components (e.g., streptavidin-biotin) are involved, two counter-screens can be performed to specifically identify disruptors of this interaction. For the first counter-screen, the test compounds should be added *before* the complementary capture component is added (see sample protocol below). For the second counter-screen, compounds are added after the capture components have bound the analyte. Greater activity in the first counter-screen would indicate interference with the affinity capture components, whereas similar activities in both counter-screens would be indicative of another technology-related interference mechanism. The necessary

reagents for target-independent counter-screen can sometimes be prepared in-house through standard recombinant and protein chemistry techniques; however, in many cases they are commercially-available or can be produced by vendors.

For Alpha-based assays, there are several commercially-available reagents that can be used to identify and distinguish technology-related interference compounds. The TruHit reagent includes streptavidin donor beads and biotinylated acceptor beads (Figure 8B). The TruHit beads can be used to develop counter-screen to identify different types of interference compounds, including biotin mimetics, light scattering compounds, singlet oxygen quenchers, light quenchers, and emitters. Omnibeads contain everything required to generate an AlphaScreen signal within each bead, and thus are not susceptible to interference by affinity capture disruption or singlet oxygen quenching (Figure 8B). Therefore, also including a counter-screen with Omnibeads allows one to distinguish light-based interference mechanisms (light absorbance, light scattering, quenchers, and emitters) from others that might be identified with the TruHit counter-screens.

Sample steps are provided below to illustrate how these counter-screens are performed and some points to consider. While counter-screens take time to develop and optimize, target-independent counter-screens can be applied to triage hits from many different screens performed with the same primary assay technology. Therefore, this strategy is likely to be well worth the initial investment.

Sample steps | Alpha Counter-Screens.

Using TruHit streptavidin-donor and biotin-acceptor beads

Configuration 1: Screening for singlet oxygen quenchers, color quenchers and, light scatters

Incubate donor (streptavidin) and acceptor (biotin) beads in assay buffer for 30 min.

Add test compounds, control compound (Malachite green), and vehicle control to corresponding reaction vessels from Step 1 using acoustic dispenser (e.g., Echo) or pintool transfer. Alternatively, transfer solutions from Step 1 to reaction vessels containing pre-dispensed compounds and vehicle controls.

Incubate beads and compounds from Step 2 for approximately 30 min.

Record assay readout (λ_{Ex} 680 nm/ λ_{Em} 615 nm).

Compare signals from test compounds to those of the controls.

Configuration 2: Screening for biotin mimetics (*Note that biotin mimetics are only identified after comparing the results of Configuration 2 against those of Configuration 1, because both configurations are susceptible to interference by singlet oxygen quenchers, color quenchers and light scatters*)

Incubate donor beads with test compounds, control compound (biotin), and vehicle control in assay buffer for 30 min.

Add acceptor (biotin) beads to corresponding reaction vessels from Step 1.

Incubate beads and compounds from Step 2 for 30 min.

Record assay readout (λ_{Ex} 680 nm/ λ_{Em} 615 nm).

Compare signals from test compounds to those of the controls.

Researchers should consider the following technical points:

continues on next page...

continued from previous page.

Beads can usually be prepared as working solutions in 1X assay buffer.

Titrate the beads to match the TruHit signal with the primary Alpha assay.

Incubation times should be altered to approximate the conditions of the parent assay

The concentration of test compounds should be the same as in the primary assay.

Ensure compound and bead mixtures are equilibrated.

Consider testing compounds in multiple concentrations.

In Configuration 1, the pre-formed biotin-streptavidin complex is difficult to disrupt. To investigate capture component interferences, use Configuration 2.

Compounds that decrease the assay readout in Configuration 2, but not in Configuration 1, are likely biotin mimetics.

Using Omnibeads

Incubate Omnibeads in assay buffer for 15 min.

Add test compounds, control compound (Malachite green), and vehicle control to corresponding reaction vessels from Step 1 using acoustic dispenser (e.g., Echo) or pintool transfer. Alternatively, transfer solutions from Step 1 to reaction vessels containing pre-dispensed compounds and vehicle controls.

Incubate beads and compounds from Step 2 for approx. 30 min.

Record assay readout (λ_{Ex} 680 nm/ λ_{Em} 615 nm).

Compare the signals from test compounds to those of the controls. Significant changes in signal likely result from light-based interference mechanisms.

Researchers should consider the following technical points:

Include positive and negative controls for relevant interferences (Table 10).

Beads can usually be prepared as working solutions in 1X assay buffer.

Incubation times should be altered to approximate the testing conditions of the parent assay.

Ensure compound and bead mixtures are equilibrated.

Titrate the Omnibeads to match the signal with the primary Alpha assay.

Consider testing compounds in multiple concentrations.

Compounds that decrease the assay readout are likely signal quenchers, while compounds that increase the assay readout are likely emitters.

A comparison between the results of the TruHit and the Omnibead counter-screens would distinguish compounds that interfere by quenching singlet oxygen.

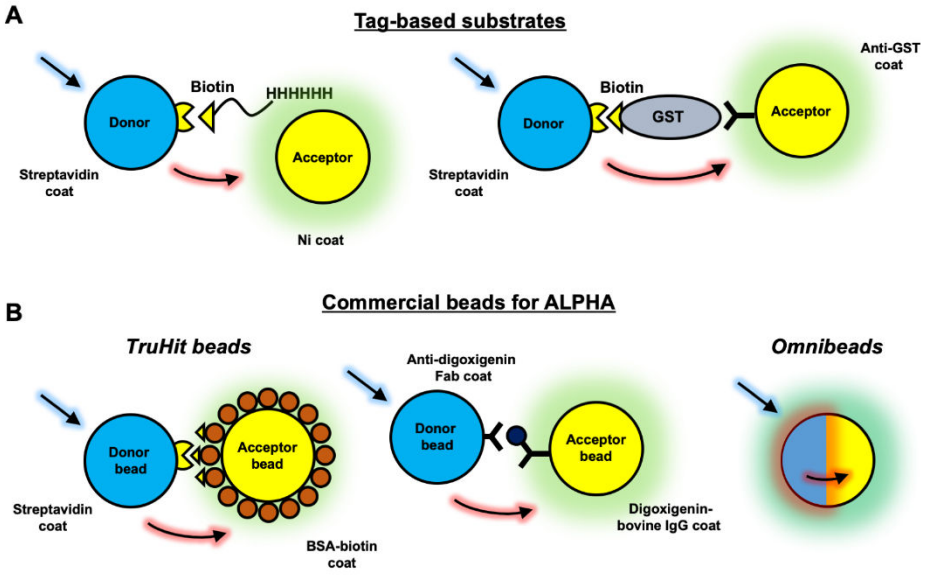


Figure 8 Schematic of reporter-based and tag substrate counter-screens for compound-mediated interference in proximity assays. (A) Reagents containing capture components such as a biotinylated peptide with a His tag (left) or biotinylated GST (right) can be constructed with standard recombinant and protein chemistry technologies or obtained commercially. This approach is applicable to most proximity assays. As with the TruHit beads, the addition of these substrates to complementary capture components will generate a stable assay signal in the absence of interfering compounds (see also **Figure 9**). To identify interference with strong affinity interactions, such as the biotin-streptavidin interaction, compounds can be added before or after reporter/analyte addition and equilibration (with strong-affinity tag interactions, compounds should be added *before* the complementary capture component is added). (B) For Alpha-based assays, TruHit and Omnibeads are commercially-available control beads to assess for target-independent changes in assay readout. TruHit beads contain streptavidin donor and biotinylated acceptor beads that generate a stable assay signal in the absence of interfering compounds. Omnibeads contain all the necessary reagents for the Alpha signal and thus can identify compounds that reduce the excitation light or emission signal.

Table 10 Suggested interference controls for homogenous proximity assays.

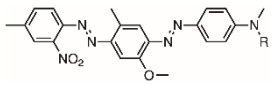
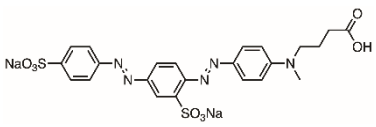
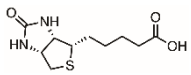
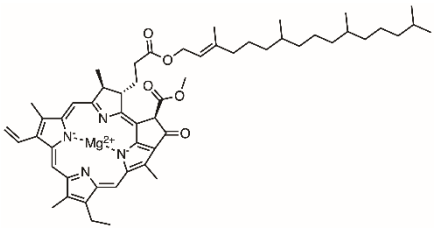
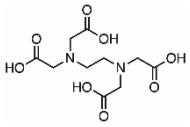
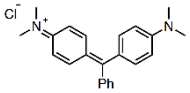
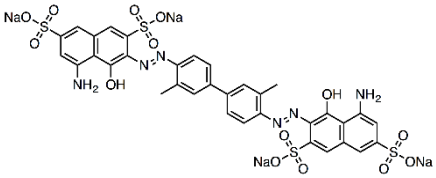
Compound name	Chemical structure	Mechanism	Notes
BHQ-1		Light absorbance	Absorbs light between 480-580 nm; amine/ carboxylic acid versions (at R position) commercially available Contains azo moiety – may be susceptible to singlet oxygen interference
BHQ-10		Light absorbance	Better water solubility than BHQ-1 (due to carboxylic acid) Contains azo moiety – may be susceptible to singlet oxygen interference
Biotin		Affinity reagent disruptor	Use for biotin-avidin systems
Chlorophyll A		Light absorbance	Absorbs at Alpha emission wavelength

Table 10 continued from previous page.

Compound name	Chemical structure	Mechanism	Notes
EDTA		Chelator	Water-soluble (salt poorly soluble in DMSO)
Malachite green		Light absorbance	Absorbs at Alpha emission wavelength
Trypan blue		Light absorbance	Absorbs at Alpha emission wavelength Contains azo moiety – may be susceptible to singlet oxygen interference
Creamer	-	Light scattering	Contains hydrogenated vegetable oils and sodium casein Prepare as 10-20X stock solutions in water Suggested starting concentration: 1 mg/mL

Counter-Screens to Bypass a Reaction or Process

Another strategy for counter-screens involves the addition of test compounds to a solution of pre-formed or purified analyte (Figure 9). This approach should not be taken if the analyte of interest is an equilibrium species, such as a protein-protein interaction, which could instead be countered by a target-independent assay (see above). However, this strategy is well-suited to assays that detect a product of some process, such as an enzymatic reaction or a cellular response. This type of counter-screen can be carried out in two ways: (1) the test compound is added after the reaction or process is completed and the detection reagents have equilibrated, or (2) the test compound is added after a purified version of the analyte has equilibrated with detection reagents (without the other components involved in the process, such as an enzyme or cells). Either variation can be useful for identifying multiple types of compound-mediated assay interferences, including light absorbance, quenching, light emitters, and capture system disruption.

Compared to the first variation utilizing products formed *in situ*, assays using a purified analyte might have better reproducibility due to less confounding factors, such as incomplete reactions or the presence of cells. For instance, an AlphaLISA primary assay was performed to measure IL-1β secreted from stimulated cells (95). A counter-screen was subsequently conducted with the same primary assay and purified IL-1β in the absence of cells to identify technology related interferences. Depending on the assay, a similar approach could be taken with purified cytokines, hormones, or even peptides containing relevant post-translational modifications.

There are some limitations and important considerations for counter-screens designed to bypass a reaction or process. When conducting the first variation that follows a completed process, it is important to ensure that the assay readout is stable prior to spiking in test compounds. Changing readouts are typically due to incomplete/non-quenched reactions (for enzymatic systems), non-equilibrated binding (protein-protein, analyte-reagent complexes), and/or analyte instability. For the second variation using a purified analyte, one should note that certain analytes might be difficult to purify in sufficient quantities and/or have stability limitations.

Sample steps | Counter-Screens to Bypass a Reaction or Process.

In situ format

Perform the complete proximity assay using standard procedures, but omit vehicle control, and test compounds. Include negative control to determine background (e.g., no-enzyme control for enzymatic reaction).

Add test compounds and vehicle controls to microplate wells containing reactions from Step 1.

Allow system to equilibrate.

Obtain counter-screen readout.

Compare the signals from samples treated with test compounds with those of the controls.

Purified analyte format

Perform proximity assay using a modified procedure: mix purified reaction product (analyte) and donor/acceptor reagents, omit vehicle controls and test compounds. Include negative control to determine background (e.g., no-analyte control).

Add test compounds and vehicle controls to microplate wells containing reactions from Step 1.

Allow system to equilibrate.

Obtain counter-screen readout.

Compare the signals from samples treated with test compounds with those of the controls.

Researchers should consider the following technical points:

Include positive and negative controls for relevant interferences (Table 10).

While the sample steps are suggestive of an enzymatic assay, the same approach can be applied to cell-based assays, where the proximity assay detects an analyte produced by the cell following stimulation.

Ensure that the counter-screen signal is comparable to that of the primary assay. Note that the absence of vehicle control (e.g., DMSO) may alter the initial readout before compound/control spiking.

Verify readout stability by obtaining serial readouts (typically 30 to 60 min) prior to performing this counter-screen.

When spiking in a product, the concentration of the purified product should be comparable to what it would be under primary assay conditions.

Depending on the kinetics, this counter-screen format may not be appropriate for certain protein-protein interaction studies. True actives may disrupt the interaction of interest after spiking detection reagents.

Counter-Screens with Different Affinity Capture Components

Developing a counter-screen that incorporates orthogonal affinity capture components can help to identify test compounds that interfere with the coupling of the analyte to the detection system. Antibodies represent a notable subset of capture components in proximity assays. Antibodies are often used to target specific analytes including post-translational modifications (e.g., phosphorylation, acetylation, methylation) and can be monoclonal or polyclonal. An ideal antibody is highly sensitive (i.e., strong affinity) and most importantly specific for its target epitope. However, certain test compounds may interfere with the antibody-analyte interaction by mimicking the analyte pharmacophore. Depending on the similarities of biomolecular

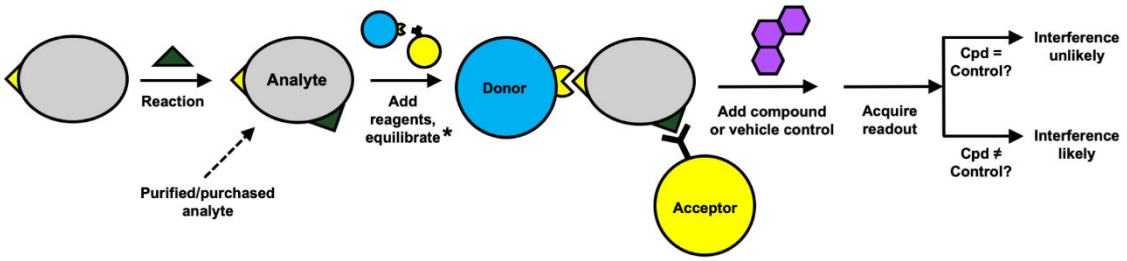


Figure 9 Schematic of product-spiking counter-screens for compound-mediated interference in homogenous proximity assays. Compounds can be added to a proximity assay system before affinity capture of the analyte. Readouts can be compared to vehicle controls, with significant changes in readout likely attributable to compound-mediated assay interference. Note: with strong-affinity capture components such as streptavidin-biotin, compounds can be added *before* streptavidin reagents are added (*).

recognition, these analyte analogs can prevent the intended antibody-analyte interaction. For proximity assays utilizing an antibody for a highly specific analyte, a potential counter-screen is to substitute the antibody used in the primary assay with an independently-derived antibody (Figure 10). Depending on the assay technology, the suitability of an antibody for a proximity assay might vary. For example, FRET pairs are much more sensitive to the distance and orientation of the donor and acceptor, than Alpha pairs. In principle, if two antibodies are suitable for an assay, then the observed activities for a given test compound should be independent of the antibody choice. However, differences in activities might be attributable to antibody-specific compound interference due to different target epitopes (and thus different affinities to the test compounds).

Similarly, substitution capture components are available for some tags. For example, Ni^{2+}-NTA beads or anti-6xHis-antibody beads are commercially available to capture 6x-His-tags for Alpha-based assays. Likewise, GSH beads or anti-GST-antibody beads will bind GST tags. Compounds that only inhibit reactions using Ni^{2+}-NTA beads, but not anti-6xHis-antibody beads are likely to be chelators and not genuine hits. In general, a genuine hit should affect the signal output independent of the capture component (i.e. for example Ni^{2+}-NTA vs. anti-6xHis-antibody) used.

Absorbance Counter-Screens

Counter-screens can be performed to measure the absorbance spectrum of test compounds in assay-like conditions. Compounds with the potential to interfere by light absorbance will absorb at the excitation and/or emission wavelengths (Figure 11). Such compounds might lead to false-negative interpretations, false-positive interpretations, or indeterminate interpretations. For example, upon evaluating active compounds identified from an AlphaScreen proximity assay, absorbance at 680 nm (excitation) and/or 520-620 nm (emission) would indicate the potential for interference via light absorbance. A similar counter-screen for (TR)-FRET would focus on the excitation and emission wavelengths of the donor and acceptor fluorophores, respectively. Recall that the degree of interference is related to the concentration of compound and its extinction coefficient (Beers Law). Whenever possible, measure compound absorption in assay-like conditions. Light properties such as fluorescence can depend on experimental conditions such as ionic strength, temperature, buffer composition, and buffer pH.

Emission Counter-Screens

Counter-screens can also be conducted to measure the emission properties of test compounds in assay-like conditions. Compounds that emit light at assay-relevant wavelengths might cause false-negative interpretations, false-positive interpretations, or indeterminate interpretations. *A caveat is that such compounds are rare in practice and specific counter-screens for emitters may not be the highest yield unless such a mechanism is suspected or must be de-risked.*

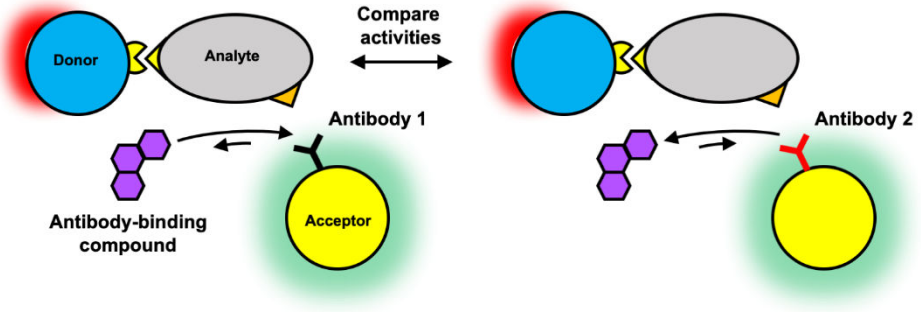


Figure 10 Schematic of an antibody-based counter-screen for compound-mediated interference in proximity assays. Certain test compounds may show antibody-dependent activity, which can be evaluated by comparing the activity of a suspected compound in otherwise identical assays constructed with independent antibodies to the target analyte.

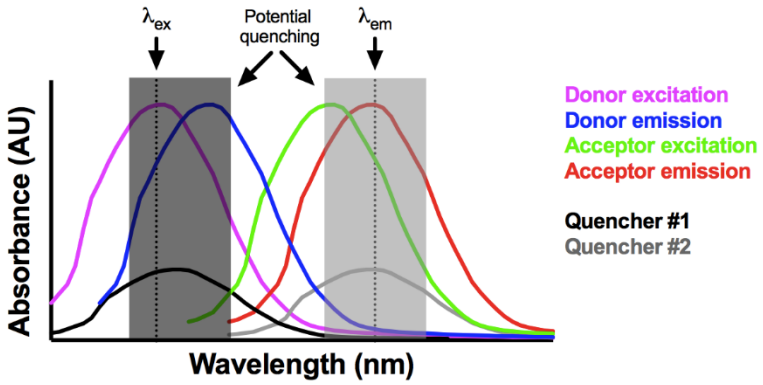


Figure 11 Schematic of absorbance counter-screen for compound-mediated interference in proximity assays. Shown in a hypothetical FRET assay with a donor fluorophore (purple, excitation spectra; blue, emission spectra) and acceptor fluorophore (green, excitation spectra; red, emission spectra). Compounds that absorb near the excitation (Quencher #1, dark grey) or emission wavelengths (Quencher #2, light grey) have the potential to quench the assay readout through inner-filter effects by effectively reducing the intensity of excitation and emission light, respectively.

A straightforward approach for flagging compound emission in homogenous proximity assays is to measure the emission of a test compound under assay-like conditions using a fixed excitation wavelength. The most relevant, high-yield experiment would be to excite a compound at the donor excitation wavelength and then monitor for light emission in the acceptor emission channel. The magnitude of light emission can be expressed relative to a positive and negative controls for the assay readout and/or a pure fluorescent standard (mean fluorescent concentration (112)). Note that other combinations of excitation and emissions can be tested (e.g., excite at the donor emission wavelength, then monitor emission in acceptor emission channel).

Other pragmatic counter-assays capable of identifying emitting compounds include compound spiking, the use of target-independent linker substrates, and bead-based reagents for Alpha assays (i.e., Omnibeads, TruHit beads). As above, the magnitude of light emission can be quantified relative to positive and negative assay controls. For fluorescent compounds, absorbance spectra (see above) can be correlated with emission (i.e., compounds absorb light at assay-relevant wavelengths).

In cellular fluorescence-based assays where the desirable activity is associated with an increased fluorescence signal (e.g., enhanced promotor activity), hits should be tested on parental cells without the incorporated reporter. If the parental cells also show increased fluorescence upon compound treatment, the hits are likely to be flagged as fluorescence emitters.

There are several considerations when performing emission counter-screens:

- Emission spectra can be normalized to a known quantity of standard (positive control) to control for fluctuations in instrumentation and/or reporter systems (112).
- Whenever possible, obtain emission spectra in assay-like conditions. Light properties such as fluorescence can vary depending on experimental conditions such as ionic strength, temperature, buffer composition, and buffer pH.
- Obtain emission at several compound concentrations, including above and below the activity concentration. For a compound with an AC_{50} of 10 μM, this might include taking emission spectra at 1, 10, and 100 μM compound concentrations.

Ratiometric Data Analysis

A high-yield experimental design for certain proximity assays is to measure the readout (emission) in the donor fluorophore *and* the acceptor fluorophore channels. Readouts can then be analyzed in the donor fluorophore emission channel, the acceptor fluorophore emission channel, and as a ratio of these donor:acceptor channels (Equation 2) (113). This type of analysis is applicable to (TR)-FRET, BRET, and Alpha.

$$\text{Ratio} = \frac{\text{Acceptor}}{\text{Donor}} \qquad \text{Equation 2}$$

Analyzing activity in terms of each channel separately, the ratio can correct for some well-to-well effects and also identify certain light-interference compounds (Figure 12). Plotting the control well data for both donor and acceptor channels to determine the variation without compound addition can be used to judge if decreases/increases in the ratio data are due to either genuine complex disruption/formation or aberrant signal modulation of the donor or acceptor channels.

Well-behaved (i.e., non-fluorescent, non-quenching) bioactive compounds should perturb the readout in the acceptor channel and not the donor channel. Compounds that quench light in the donor and acceptor channels show decreases in signal intensities in both channels. Typically, this ratio is similar to the unmodulated (vehicle control) ratio as it affects both the donor and acceptor channels equally. Compounds that fluoresce in both channels will show increased intensities in both the donor and acceptor channels, and the donor:acceptor ratios are again unchanged from vehicle controls. Compounds that selectively attenuate readout in either the donor or acceptor channels may appear as bioactive compounds depending on the assay setup. Similarly, compounds that selectively fluoresce in either the donor or acceptor channels may also appear as bioactive compounds depending on the assay setup.

When analyzed as concentration response curves, light-interfering compounds may show variable patterns that will depend on the assay setup (up- or down-readout), fluorescence behavior, quenching behavior, and on-target bioactivity. Therefore, it is highly recommended to analyze the single channel and ratiometric readouts. Such a protocol can be implemented as a counter-screen or as a built-in data acquisition step in the routine performance of a given assay. In cases where there may be appreciable compound light interference, orthogonal assays and counter-screens should be performed to further examine technology-based interferences and on-target bioactivity.

Pull-Down Assays

Compounds that interfere with proximity assay capture components can also be flagged by conventional pull-down assays (94). In this counter-screen, test compounds are incubated with a target-independent substrate containing one of the capture components (e.g., GST-conjugated protein) in assay-like conditions, followed by the addition of the complementary solid-phase capture component (e.g., GSH-coated beads). Beads are washed to remove unbound compound and proteins, followed by protein elution from the washed beads, and the formerly bound proteins are quantified after electrophoresis separation by staining (e.g., Coomassie) or

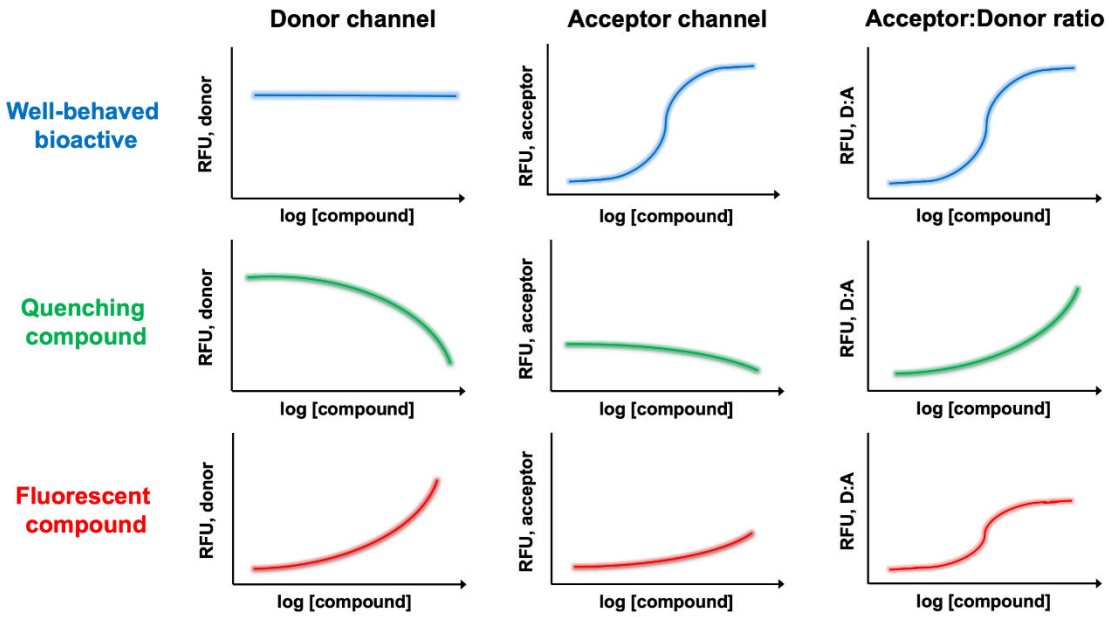


Figure 12 Ratiometric analysis of proximity assays to identify compound-mediated interferences. In this example, on-target bioactivity results in an increase in TR-FRET signal. Since light-based interferences by compounds are concentration-dependent, such compounds may be flagged by plotting the ratio of the acceptor:donor fluorescence as a function of compound concentration (see right-most panels). Depending on the magnitude of compound quenching and fluorescence in each fluorophore channel, quenching compounds (green) and fluorescent compounds (red) may show concentration-dependent changes in the acceptor:donor ratio. In such cases, orthogonal assays and counter-screens should be performed to further examine technology-based interferences and on-target bioactivity. Adapted from Imbert *et al* (113).

immunostaining (e.g., western blot). Compounds that disrupt the binding of the complementary binding reagents show decreased amounts of pulled-down product compared to vehicle controls and well-behaved compounds. Incubation of the test compound with capture component including stoichiometry and buffer conditions should attempt to mimic the parent assay. Unlike other counter-screens discussed in this chapter, these assays can be lower-throughput, semi-quantitative, and may not necessarily replicate assay-like conditions. Therefore, we recommend reserving for cases where previous counter-screens are inconclusive or other higher-throughput counter-screens cannot be performed for technical reasons.

Sample steps | Pull-Down Counter-Screen.

Prepare 100X compound stock solutions, (e.g., 1 mM in DMSO to test at 10 µM compound concentrations).

Prepare serial dilutions in stock solution solvent (e.g., DMSO).

Dilute non-target protein stock containing capture component acceptor (e.g., GST, His tag, antibody epitope) with 1X assay buffer.

Add 3 µL of 100X compound solution to reaction vessel such as standard Eppendorf tube with pipette.

Add 297 µL solution from Step 3 to reaction vessel from Step 4. Mix gently with inversion or brief vortex.

Incubate solution from Step 5 for 60 min at same temperature as parent assay.

Add beads containing immobilized complementary capture component to solution from Step 6 (e.g., agarose-GSH, agarose- Ni^{2+}, agarose-antibody).

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Incubate solution from Step 7 for additional 1-2 h at room temperature.

Centrifuge solutions from Step 8 for 5 min at 1000 *g* at room temperature. Remove supernatant. Replace supernatant with fresh 1X assay buffer.

Repeat Step 9 for 5 cycles.

Elute bound proteins with SDS-containing buffer (alternatively, low pH or high salt buffers).

Analyze eluates by standard SDS-PAGE followed by protein staining or Western blot.

Compare quantity of eluate proteins by treatment with test compounds versus vehicle control as well as relevant positive controls. Eluates with decreased amounts of protein are presumed capture tag disruptors.

Researchers should consider the following technical points:

Include positive controls that disrupt the interaction of interest (e.g., purified GSH, GST, anti-His, chelators).

Utilize assay buffer from proximity assay of interest, as compound behavior can depend on experimental conditions such as ionic strength, temperature, buffer composition, and buffer pH.

Keep in mind that the amount of protein used in pull down experiments will be much higher than in micro-well proximity assays.

For each system, experimental parameters such as the number of washes, wash buffer composition, and incubation should be optimized.

Consider testing compounds at multiple concentrations.

Include positive and negative controls. For example, nickel-chelating agents such as EDTA could be used as positive controls for Ni^{2+}-His pull-downs.

Orthogonal Assays

The purpose of performing orthogonal assays is to rule out technology-dependent activities that are usually hallmarks of compound-mediated assay interference, as useful bioactivity should ideally be independent of assay technology (Table 6). A typical high-throughput approach is to employ an independent proximity assay technology as the orthogonal assay (e.g., if Alpha was used for a primary screen, use SPA or TR-FRET as the orthogonal screen, as well as a different set of tags if feasible). However, efforts should also be placed in identifying non-homogenous proximity secondary assays to avoid the possibility of technology-related interference, such as light-based interference or tag disruption. Non-proximity formats could include mass spectrometry assays or biophysical target engagement assays such as surface plasmon resonance (note: while showing direct binding, such assays do not necessarily indicate functional readouts such as enzymatic modulation or tractable/optimizable bioactivity). Otherwise, orthogonal proximity-based assays could include separation steps at the expense of throughput (e.g., filter-binding, ELISA).

Generalized Interference Counter-Screens

Counter-screens for non-technology-related interferences such as nonspecific reactivity and aggregation should also be performed. For brevity, extensive details on counter-screens for these interferences can be found at the related Assay Guidance Manual chapters on Assay Interference by Aggregation and Assay Interference by Chemical Reactivity.

Time-Course Measurements

Another strategy to identify light-based interferences is to obtain multiple readout measurements over a range of compound concentrations to obtain a set of reaction rates (Figure 13) (113,114). Light-based interferences are relatively static and are generally not time-dependent (e.g., unless compound instability is an issue). Therefore, in an unquenched reaction (e.g., enzymatic assay), compounds that perturb the readout at the same rate, independent of compound concentration, are potential interference compounds. Bioactive compounds should induce concentration-dependent changes in reaction rates. Bioactive compounds that also interfere with the readout may show a mixed pattern at a particular time point, but when readouts are plotted as reaction rates, show more well-behaved bioactivity. This strategy is most appropriate for assays that exhibit readout stability, activity not disrupted by detection reagents, and show steady-state-like, time-dependent activity.

Note this assay format is usually most relevant to certain enzymatic proximity assays used to detect a product (e.g., a kinase assay with AlphaScreen to measure a phosphopeptide). Most binding assays occur too rapidly to measure kinetically. Additionally, technical factors such as instrumentation may prevent rapid measurements, or system instability may hinder delayed measurements.

Cheminformatics

Cheminformatics can be useful tools for flagging potential compound interferences from proximity assay data sets. One common cheminformatics approach for identifying interference compounds involves substructure filters derived from larger data sets or historical records of distinct compounds. The former enables flagging compounds that may not have been tested previously, but share a chemotype associated with a particular assay misbehavior (e.g., Tables 6-8), but this strategy runs the risk of overgeneralizing. The latter approach can be especially useful if the same compounds are routinely assayed in multiple unrelated proximity assays or if the primary hit list is very large and a chemoinformatics filtering helps to reduce the number of compounds to be tested in counter- as well as orthogonal assays. An alternative cheminformatics approach is to calculate frequent hitting behavior for each compound in a screening collection (many of which may owe their bioassay promiscuity to various interference mechanisms) using their historical performance, without explicitly considering compound structure (115).

There are several substructure filter sets for AlphaScreen-based interferences, including general interference (Table 11) as well as interferences with His-Ni^{2+} and GSH-GST interactions (Tables 7 and 8) (94,116). These filters may be highly useful to flag actives for either triage or additional counter-screening.

There are several considerations for the use of these chemoinformatics filters, however: (1) substructure filters have the potential to be specific for a given assay type or capture technology depending on the training set, whereas generalized filters (e.g., REOS, Lilly) may flag more generally promiscuous compounds; (2) computational methods provide a hypothesis about the promiscuity of compounds and thus need confirmation through appropriate assays; (3) for many specific interferences or assay technologies, these filters do not exist and the generation of these filters needs a large dataset of several independent targets employing the same detection and capture technology.

Cheminformatics has distinct advantages and disadvantages. Most obvious is the speed of these approaches and the reduction in required experiments. However, compound interference can be highly assay-dependent. For example, the observed spectroscopic properties of compounds can be dependent on experimental parameters such as ionic strength, pH, temperature, and other factors such as compound solubility. As with experimental approaches, cheminformatics approaches are also susceptible to potential false positives and false negatives. An overly strict method may triage potentially useful non-interfering compounds. By comparison, reliance on a less strict method may provide a false sense of confidence in filtered compounds. Therefore, we recommend using

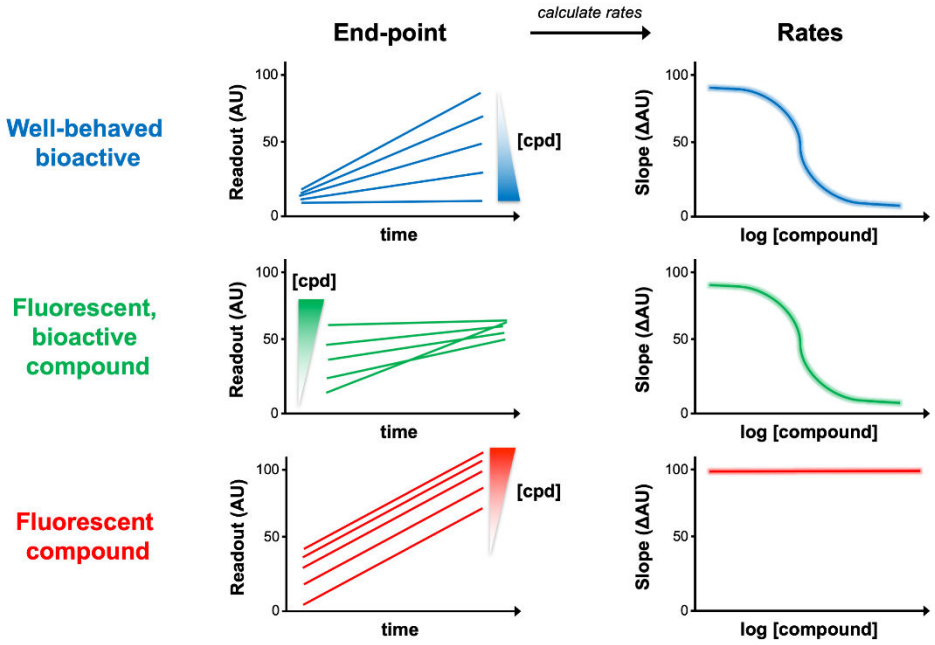
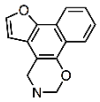
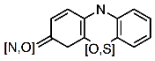
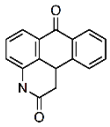
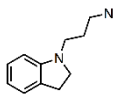


Figure 13 Time-course analysis of proximity assays to identify compound-mediated interferences. For certain proximity assays (usually enzymatic), readouts can be obtained at multiple time points to derive rates. Because light-based interferences are time-independent and concentration-dependent, some light-based interference compounds (red, bottom panel) can be identified by reaction rates independent of compound concentration. By contrast, well-behaved, bioactive compounds (blue, top panel) would show concentration-dependent changes in reaction rates. Certain bioactive light-based interference compounds (green) may also show concentration-dependent changes in reaction rates. Adapted from Imbert *et al* (113).

cheminformatics as a flagging tool as opposed to a triage tool to help prioritize compounds for interference counter-screens and orthogonal assays.

Table 11 Reported AlphaScreen frequent hitters. Chemotypes were derived from an AlphaScreen-based counter-screen (82). See also https://ochem.eu (AlphaScreen-FHs) for SMARTS strings and online substructure screening tool.

Name	Chemotype	Name	Chemotype
Naphthafurans		Imidazotriazoles	
Phenoxazines		Indenones	
Anthrones		Indolines	

Section Summary

Compound-mediated interferences in proximity assays can be identified through well-designed counter-screens. Users should also perform at least one orthogonal assay to confirm desired bioactivity, and counter-screens to identify compounds that interfere with capture components (affinity tag systems, antibody-analyte interactions)

and signal transmission (quenchers, emitters). Compounds should be triaged, progressed, or flagged for additional studies based on the combination of orthogonal and assay-specific interference counter-screens.

Strategies to Mitigate Compound-Mediated Interferences in Proximity Assays

This section describes several approaches to reduce the incidence of compound-mediated assay interference in proximity assays. Strategies to mitigate interference by light-based interferences such as the use of red-shifted fluorophores and ratiometric data analyses are highlighted. Steps to mitigate interference by aggregators and nonspecific reactive compounds are briefly discussed. The use of known interference compounds to guide the optimization of assay experimental conditions and to characterize the effects of various interference modalities is also discussed.

Strategies to Mitigate Interference by Light-Based Interferences

Many screening compound collections are enriched in sp^2-hybdridized atoms (117), and consequently absorb light in the UV light range. Such compounds can interfere with proximity assay readouts by auto-fluorescence and/or quenching. However, the use of red-shifted fluorophores can significantly reduce the burden of compound-mediated light interferences (81,103,112,118,119). This is because in general, the ability of a compound to absorb or be excited by a higher wavelength (lower energy) photon requires more extensive electronic delocalization. Such extensively conjugated compounds are usually blue by visible inspection and are relatively rare in conventional HTS collections.

Another strategy is to acquire multiple channel (donor, acceptor) signals to analyze individually and as ratios (113). Depending on factors such as the assay stability, available instrumentation, and overall throughput of data acquisition, generating data for multiple channels may be relatively straightforward (see above section, Ratiometric Data Analysis).

Finally, including detergents in assay buffer may attenuate the effects of light-scattering compounds. Detergents can effectively increase the solubility of certain compounds, attenuating the amount of suspended light-scattering particles. Care should be taken to remain below the critical micelle concentration (CMC) for the given detergent, however.

Special cases: TR-FRET time delay. One approach to reduce compound interference in TR-FRET assays is to measure apparent bioactivity as a function of the time delay of the TR-FRET measurement (113). Relatively short time delays (e.g., 25 µs) may still detect significant fluorescence from short-lived compound fluorescence. Increasing the time delay (e.g., 50 or 100 µs) typically reduces this background fluorescence (Figure 14). Compounds that show significant reductions in apparent bioactivity with increasing time delays are suspicious for fluorescence interference. While useful, certain long-lived fluorescent compounds may continue to show apparent bioactivity even with long time delays. However, as noted in the previous sections, TR-FRET will still be susceptible to compounds which absorb the donor fluorescence as well as light scattering effects.

Special cases: fluorescent lifetime (FLT) detection. Another method to mitigate against fluorescent compound interference is to perform rapid kinetic measurement of the fluorescence over time to characterize the fluorescent lifetime of a probe fluorophore (Figure 15A). Microtiter-plate readers are available which can perform these measurements in micro-well densities as high as 1536-well plates (TTP Lab Tech Ameon reader). Fluorescent probes such as FLEX17 (Figure 15B, 9-aminoacridine, from Almac; PT14, PT22, from GE Healthcare) with long lifetimes (τ = 14 to 22 ns) have been developed which show a change in fluorescent lifetime when the proximal environment changes due to events that occur in a variety of biochemical assays (Figure 15B). The long lifetime is rather easily distinguished from typical fluorophores and fluorescent compounds because such compounds show much faster fluorescent decay rates (τ = 1 to 5 ns). Therefore, the use

of longer-decaying fluorophores and/or analysis of fluorescent decay curves can be used to flag and even mitigate the burden of fluorescent compounds (120). Assays resulting in either longer or shorter (quenched) lifetimes can be configured for a variety of enzyme assays and have been developed for both serine/threonine and tyrosine kinases (Figure 15C), HATs, HMTs, HDACs, DUBs, and arginine-modifying enzymes.

Strategies to Mitigate Interference by Reactive Compounds

A popular and straightforward strategy to mitigate nonspecific compound reactivity in biochemical assays is to include scavenging reagents such as DTT (121). Often these assays use low millimolar concentrations (usually 0.5 to 1 mM). The principle of this strategy is that excess scavenging reagents can react with nonspecific reactive compounds, leaving the biomolecule(s) of interest unperturbed (104,122). Other scavenging agents can include β-mercaptoethanol (BME), L-cysteine, *tris*(2-carboxyethyl)phosphine (TCEP), and decoy proteins containing free cysteines such as BSA. However, addition of reducing agents can promote unwanted activity by redox cycling compounds (123). Therefore, compatibility of scavenging reagents with a given proximity assay and optimal concentrations should be verified experimentally. Ideally, the susceptibility of the protein target and assay system should be profiled against the suite of scavenging agent options in the presence of suitable control compounds for these mechanisms.

For additional details on mitigating interference by reactive compounds, refer to Assay Interference by Chemical Reactivity.

Strategies to Mitigate Interference by Aggregators

Several relatively facile strategies exist for mitigating the incidence of aggregators in biochemical assays, including proximity assays. Conveniently, utilizing detergents in assay buffer can reduce light-scattering compounds and aggregate formation. The inclusion of nonionic detergents such as Triton X-100 and decoy proteins such as BSA can usually increase the critical aggregation concentration (CAC) and saturate compound aggregates, respectively (106,107). As an example, Triton X-100 or Tween-20 is often used at 0.01% (v/v) concentrations in assay buffers to mitigate aggregation. As with scavenging reagents, the compatibility of detergent and/or decoy proteins with the assay technology and biological system should be verified, along with the optimal concentration. More specifically, detergents should usually be used below their corresponding critical micelle concentrations (CMCs), as detergent micelles themselves can perturb the assay system. Product literature indicates Alpha technology can typically tolerate the following detergents (73):

- Tween-20: up to 1% (v/v)
- Triton X-100: up to 1% (v/v)
- CHAPS: less than 0.1% (v/v)
- SDS: avoid (interference at 0.05% v/v)

Based on the stoichiometric model of enzyme inhibition for aggregators, increasing the concentration of enzymes may also mitigate the effects of aggregation (110,124). Finally, decreasing the concentration of test compounds such that more compound concentrations are below the CAC under testing conditions may also lower the incidence of aggregation. As an alternative to decreasing single-point screening concentrations, compounds can be tested in dose response by qHTS to sample testing concentrations below CACs (125). The final concentrations of enzymes (or other biomolecules of interest) and test compounds usually balance multiple competing factors including assay timing, readout intensity, and reagent costs.

For additional details on mitigating interference by aggregators, refer to Assay Interference by Aggregation.

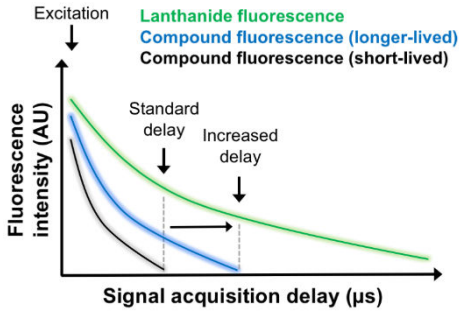


Figure 14 Effect of signal acquisition time delay on TR-FRET bioactivity. Many screening compounds can exhibit short-lived fluorescence (black). Increasing the signal acquisition delay can reduce interference from longer-lived fluorescent compounds (blue) provided the dynamic range is sufficient.

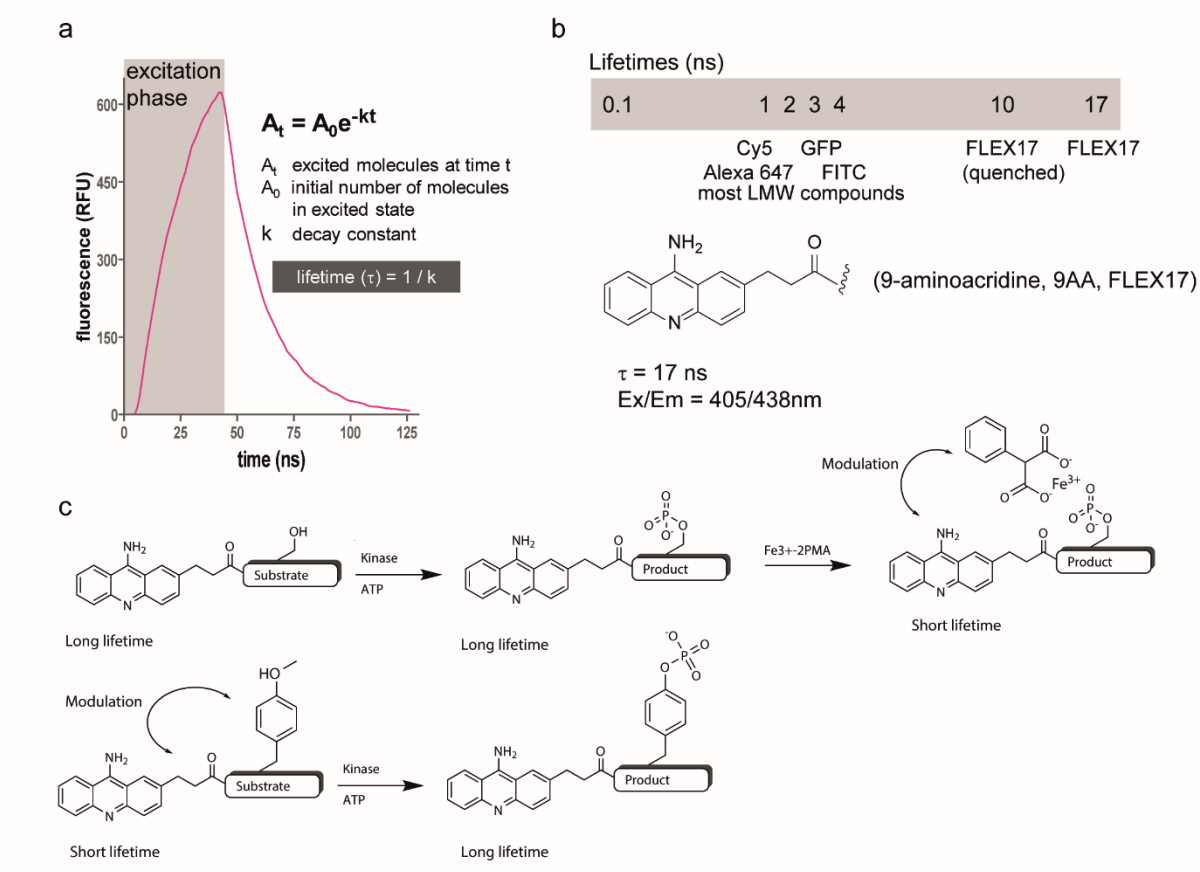


Figure 15 Principle of FLT and example assays. (A) Rapid detection of the fluorescent signal after excitation affords determination of the fluorescent lifetime through examining the slope of the fluorescent decay curve (fitting to the first-order equation to the data). (B) Standard fluorophores and typical fluorescent compounds show much shorter fluorescent lifetimes compared to FLT probes such as 9AA. (C) Assays for protein kinases can be developed where the phosphorylation event is monitored through the lifetime probe. Product formation can be followed when the by either a decrease or increase in fluorescent lifetime. Adapted from TTP Lab Tech material.

Interference Control Challenge to Mitigate Compound-Mediated Interference

During the assay design and validation phases, it is recommended to determine the effect(s) of known interference compounds on the assay readout (122,126). During the assay optimization process, experimental conditions can potentially be modified to reduce the effects of certain sources of compound-mediated assay interference (e.g., increasing detergent concentration in an assay prone to aggregator interference; increasing signal acquisition delay in a TR-FRET assay prone to auto-fluorescence compound interference). Challenging proximity assays with known interference compounds and key reagents permits the characterization of specific interference mechanisms on the assay readout (Table 10). For example, Ni^{2+}-His tag assays can be challenged with chelating agents (e.g., EDTA) while biotin-streptavidin assays can be challenged with biotin to gauge the interference phenotype and magnitude by capture component disruptors. Light-based interferences can be assessed using various colored, auto-fluorescent, and insoluble compounds. Several suggested electrophiles and aggregators can be obtained commercially (121,122,127). This information can facilitate compound triage, especially in complex assay systems, where the effects of interference compounds may not be immediately intuitive. Finally, the relative sensitivity of a given proximity assay to known interference compounds combined with preliminary estimates of specific interference incidences can help guide the design of post-HTS workflows (such as the selection and order of counter and orthogonal assays).

Section Summary

When combined with preliminary interference characterization on the assay readout, carefully designing certain assay features such as reagent selection, reagent concentration, and instrumentation settings can potentially reduce the burden of compound-mediated interference in proximity assays due to light-based interference, aggregation, and nonspecific reactivity. Some high-yield recommendations include (1) the use of red-shifted fluorophores, (2) the addition of detergents, decoy proteins, electrophilic scavenging reagents, and (3) the thorough characterization of known interference compounds on proximity assay readouts to inform on the appropriate counter-screens and orthogonal assays that will be required to characterize compound activity.

Conclusions

Homogenous proximity assays – including Alpha, FRET, BRET, TR-FRET, FLT, and SPA – are powerful tools for lead and chemical probe discovery and development. These technologies obviate the need for analyte separation steps and are amenable to customization with off-the-shelf commercial reagents. While highly useful, these assays are susceptible to compound-mediated interferences. Sources of technology-related compound-mediated interferences include signal quenchers, signal emitters, and capture system disruptors. Additionally, homogenous proximity assays are also susceptible to generalized (non-technology-related) compound-mediated interferences, including nonspecific reactivity and aggregation. Compound-mediated interferences in homogenous proximity assays are best approached by a combination of orthogonal assays to confirm activities and counter-screens to rule-out or distinguish any technology-related interferences. The establishment of a primary assay along with orthogonal and counter-screens should be the task of every initial assay development phase. Each counter-screen strategy has advantages and disadvantages (Table 12). A combination of orthogonal assays and counter-screens helps to (1) triage assay artifacts, (2) to identify desired bioactivity among active compounds, and (3) to characterize potentially tractable compounds with interference properties.

Table 12 Summary of counter-screens for compound-mediated assay interference in proximity assays. Certain counter-screens are appropriate for specific proximity assay technologies. For each counter-screen, susceptible interferences, and notable advantages and disadvantages are listed. Mechanisms of interference should be confirmed or ruled-out with multiple counter-screens.

Counter-screen	Homogenous proximity assay	Light / Fluorescence absorption	Quench	Singlet oxygen	Tag disruption[a]	Light/ Fluorescence emission	General[b]	Advantages, disadvantages
		Key interferences flagged						
TruHit beads	Alpha	+	+	+	± (usually Strep/ biotin)	+	±	Commercially available Straightforward protocol Expensive Limited tags available
Omni-beads	Alpha	+	+	?	-	+	-	Commercially available Straightforward protocol Expensive
Tag substrates	Alpha (TR)-FRET BRET SPA	+	+	+ (only Alpha)	+	+	±	Commercially available Synthesizable Straightforward protocol Can be used to identify specific tag interferences
Product spiking (*in situ*)	Alpha (TR)-FRET BRET, FLT SPA	+	+	+	+	+	±	Straightforward protocol May flag unstable and slow acting covalent compounds in addition to readout artifacts Requires stable product Excess product may place assay out of linear range Ensure system stability, equilibration

Table 12 continued from previous page.

Counter-screen	Homogenous proximity assay	Light / Fluorescence absorption	Quench	Singlet oxygen	Tag disruption[a]	Light/ Fluorescence emission	General[b]	Advantages, disadvantages
		Key interferences flagged						
Product spiking (purified product)	Alpha (TR)-FRET BRET, FLT SPA	+	+	+	+	+	±	Straightforward protocol May flag unstable and slow acting covalent compounds in addition to readout artifacts Requires stable product Excess product may place assay out of linear range Ensure system stability, equilibration
Antibody swap	Alpha (TR)-FRET SPA				+			Depends on antibody
Absorbance spectra	Alpha (TR)-FRET BRET, FLT SPA	+	+					Straightforward protocol
Emission spectra	Alpha (TR)-FRET BRET, FLT SPA					+		Straightforward protocol
Cheminformatics	Alpha (TR)-FRET BRET, FLT SPA	±	±	±	±	±	±	Inexpensive Rapid False positives False negatives Needs to be confirmed with experiments
Ratiometrics	Alpha (TR)-FRET BRET SPA	+	+			+		
Kinetics	Alpha (TR)-FRET BRET, FLT SPA	+	+			+		Useful for non-quenched/ continuous assays
Pull-down	Alpha (TR)-FRET BRET SPA				+			Low throughput High concentration of protein needed

[a] Depends on tags used in beads
[b] Generalized compound-mediated interference including nonspecific reactivity, aggregation, chelation

Suggested Readings (alphabetical order)

Baell J.B., Holloway G.A. J Med Chem. 2010;53:2719. PubMed PMID: 20131845.

Describes several hundred chemical substructures exhibiting enriched bioactivity across six independent AlphaScreen HTS assays. See Supplementary Information for in-depth discussions of likely AlphaScreen and generalized interference chemotypes.

Brenke J.K., Salmina E.S., Ringelstetter L., Dornauer S., Kuzikov M., Rothenaigner I., Schorpp K., Giehler F., Gopalakrishnan J., Kieser A., Gul S., Tetko I.V., Hadian K. J Biomol Screen. 2016;21:596. PubMed PMID: 27044684.

An excellent analysis of compound-mediated GSH-GST tag interference including several counter-screens.

Falk H., Connor T., Yang H., Loft K. J., Alcindor J. L., Nikolakopoulos G., Surjadi R. N., Bentley J. D., Hattarki M. K., Dolezal O., Murphy J. M., Monahan B. J., Peat T. S., Thomas T., Baell J. B., Parisot J. P., Street I.F. J Biomol Screen. 2011;16:1196. PubMed PMID: 22086725.

Describes AlphaScreen post-HTS triage, including the use of TruHit beads and orthogonal assays. Also reports several compounds that appear to disrupt an antibody-analyte interaction.

Imbert P.E., Unterreiner V., Siebert D., Gubler H., Parker C., Gabriel D. Assay Drug Dev Technol. 2007;5:363. PubMed PMID: 17638536.

Details multiple counter-screens and data analysis strategies to mitigate and identify compound-mediated assay interference in TR-FRET assays. Many of the strategies are applicable to other proximity assay technologies.

Schorpp K., Rothenaigner I., Salmina E., Reinshagen J., Low T., Brenke J.K., Gopalakrishnan J., Tetko I.V., Gul S., Hadian K. J Biomol Screen. 2014;19:715. PubMed PMID: 24371213.

An analysis of compound-mediated assay interference encountered during a series of AlphaScreen HTS campaigns including several counter-screens.

Watson V.G., Drake K.M., Peng Y., Napper A.D. Assay Drug Dev Technol. 2013;11:253. PubMed PMID: 23679849.

Illustrative example of an AlphaScreen HTS assay development and interference triage. Describes an original counter-screen for AlphaScreen artifacts using a donor-acceptor peptide. Also reports several examples of presumed AlphaScreen artifacts.

Suggested Web Resources

Online Chemical Modeling Environment (OCHEM). (Available at: http://ochem.eu/alerts).

An open-access, user-friendly cheminformatics resource for identifying potential proximity assay artifacts, based on Suggested Readings #2 and #5. Test compounds can be provided as lists (SMILES), files (SDF, MOL2), or drawn. Submitted compounds can be filtered according to one of three relevant filters: AlphaScreen-HIS-FHs (His-tag disruptors), AlphaScreen-GST-FHs (GST-tag disruptors), and AlphaScreen-FHs (generalized AlphaScreen™ artifacts).

Glossary

Artifact – A compound whose apparent bioactivity is due to compound interference with a particular assay technology

Counter-screen – An assay that is designed to flag compounds for specific technology or general interference

False-positive – Compounds, including artifacts, whose bioactivity cannot be reconfirmed in subsequent testing; not to be confused with poorly tractable compounds whose bioactivity is reproducible but *via* undesirable mechanisms

Generalized interference – Modulation of an assay readout by compound-target engagement via poorly tractable or undesirable mechanisms; usually independent of assay technology

Homogenous proximity assay – An assay that produces a detectable readout specifically when donor and acceptor components are in proximity, all without separation or filtration steps

Interference – Assay readout modulation by a compound due to mechanisms not related to the desired biological activity; due to technology and/or generalized interferences

Orthogonal assay – An assay that is designed to confirm compound bioactivity of interest compounds using an independent assay technology relative to the primary assay

Quenching, light – Attenuation of *fluorescence intensity* by a test compound; can be due to FRET, excitplex, or static (contact) mechanisms; distinct from light-based readout attenuation by absorption or light scattering

Quenching, singlet oxygen – Attenuation of singlet oxygen by a test compound; can be due to physical or chemical mechanisms

Technology-related interference – Modulation of an assay readout by compound that interferes with a specific assay technology

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Conflict of Interest Statement

The authors declare no conflicts of interest.

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