Designing your own AlphaLISA assay: Selecting toolbox bead pairs to avoid potential bead-bead interactions.

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AlphaScreen™ and AlphaLISA™ are no-wash, bead-based assay technologies used to study biomolecular interactions in a microplate format. The acronym "Alpha" stands for Amplified Luminescent Proximity Homogeneous Assay. Binding of proteins or other binding partners captured on the beads leads to an energy transfer from one bead to the other, ultimately producing a luminescent signal.

Alpha assays require two bead types: Donor beads and Acceptor beads. Each bead type contains a different proprietary mixture of chemicals, which are key elements of the Alpha technology. Donor beads contain a photosensitizer, phthalocyanine, which converts ambient oxygen to an excited and reactive form of O_2, singlet oxygen, upon illumination at 680 nm. Within its four µs half-life, singlet oxygen can diffuse approximately 200 nm in solution. If an Acceptor bead is within that distance, energy is transferred from the singlet oxygen to thioxene derivatives within the Acceptor bead, resulting in light production at 520 - 620 nm (AlphaScreen) or at 615 nm (AlphaLISA). If the Donor bead is not in proximity of an Acceptor bead, the singlet oxygen falls to ground state and no signal is produced.

Revvity offers a variety of Alpha toolbox beads for designing assays in a fast and convenient way. The most commonly developed assays using Alpha toolbox beads are either indirect sandwich immunoassays, where toolbox reagents are used to capture antibodies that recognize an analyte of interest (Figure 1A), or protein-protein interaction assays where toolbox reagents and antibodies are used to capture tagged recombinant proteins (Figure 1B).

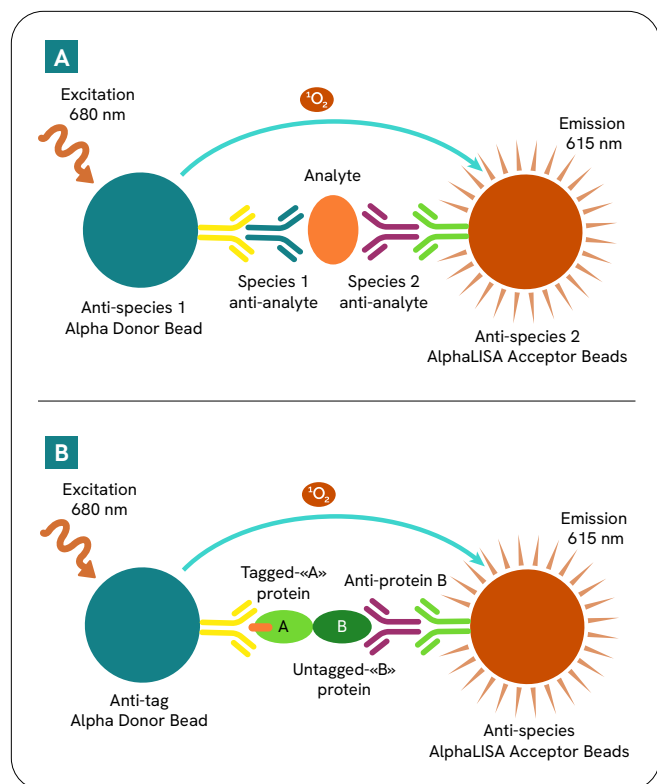For research purposes only. Not for use in diagnostic procedures.
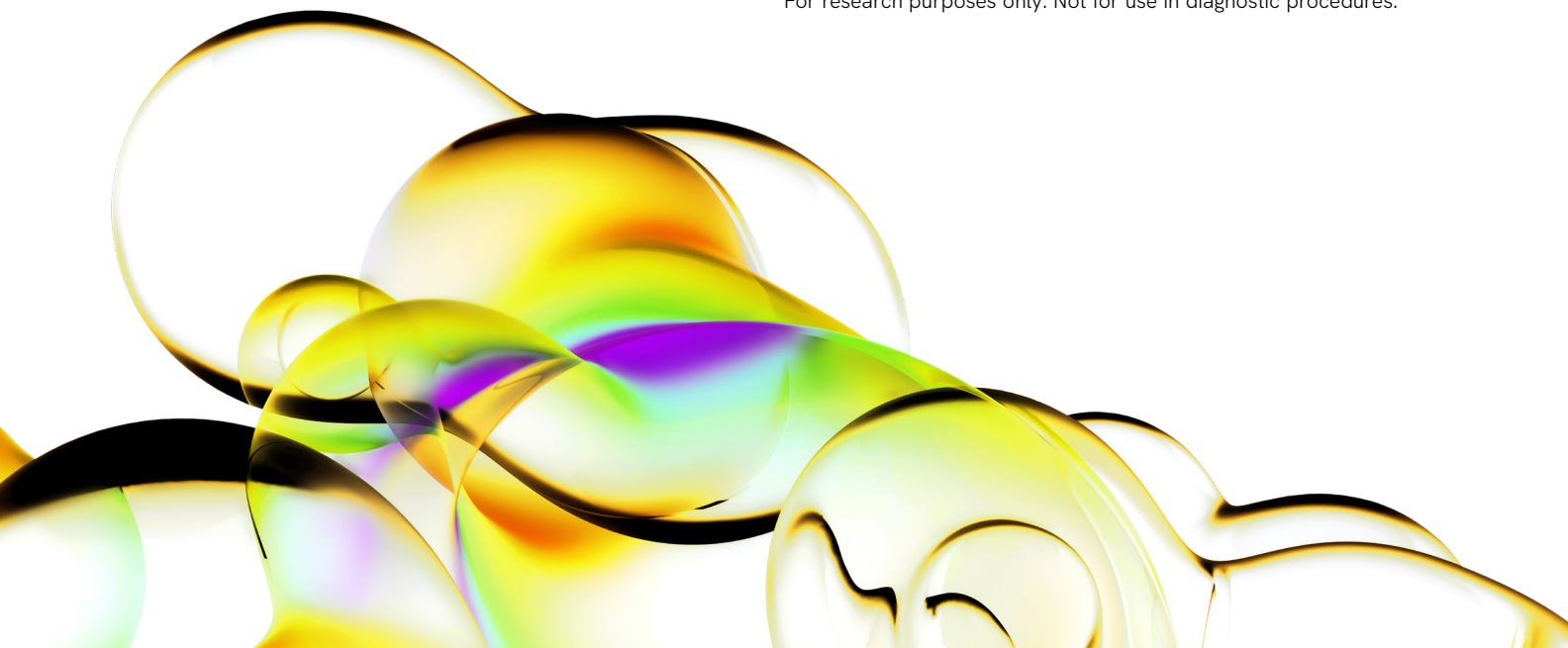
Figure 1. **Common Assay Setups using alpha toolbox beads.** In an indirect immunoassay (1A), primary antibodies of two different species are used to capture an analyte of interest and anti-species Alpha beads are used to capture the primary antibodies. When using Alpha to detect protein-protein interactions, the proteins of interest can be used either tagged or untagged. In the case shown in 1B, protein "A" is tagged and captured by anti-tag Alpha Donor beads, while protein "B" is captured by a primary anti-protein B antibody which is then captured by an anti-species AlphaLISA Acceptor bead. When protein "A" and "B" interact, the Donor and Acceptor beads are brought in close proximity. In both cases shown, it is important to know the species of the antibody conjugated to the Alpha Acceptor beads to be sure there is no cross-reactivity with the antibody found on the Alpha donor beads.

Regardless of assay format, toolbox bead selection is an important aspect of designing an AlphaLISA™ assay. It can impact assay performance, and occasionally, can mean the difference between a successful assay and an unworkable assay. For example, in some cases, a particular pairing of donor and acceptor beads can lead to a false positive signal when the antibodies or proteins coated on the two beads have affinity for one another. The purpose of this technical note is to give recommendations on different bead pairings by showing the various host species of the antibodies on each toolbox bead and to show data generated on each bead pair in the absence of analyte and other assay components.

Table 1 lists all of the toolbox beads that Revvity offers highlighting the host species and subclass of each antibody conjugated to each bead. Knowing the host species of the antibodies on the beads can provide insight into designing a good assay to avoid situations where the antibody bound donor and acceptor beads cross-react in the absence of the target of interest. For example, since the anti-HA acceptor beads (AL170) have a rabbit monoclonal antibody, we see high signal with the anti-rabbit IgG donor beads (AS105) and the protein A donor beads (AS102), since protein A is known to have a strong affinity for rabbit antibodies. An illustration of this cross-reactivity is shown in Figure 2. Also of note, the anti-rat beads not only show cross-reactivity with those beads containing rat antibodies, but also show some cross-reactivity with beads containing mouse antibodies.

Table 1. Available Toolbox Beads with Species and Clonality of Bead-Conjugated Antibody.

Bead coating	Alpha donor beads	AlphaLISA acceptor beads	Monoclonal/ polyclonal	Host	Subclass
Protein A	AS102	AL101	N/A	N/A	N/A
Protein A		6760137 (AlphaScreen)	N/A	N/A	N/A
Protein G		AL102	N/A	N/A	N/A
Anti-Human IgG		AL103	Polyclonal	Mouse	(Fab')2 fragment
Anti-Rabbit IgG	AS105	AL104	Polyclonal	Goat	(Fab')2 fragment
Anti-Mouse IgG	AS104	AL105	Polyclonal	Goat	(Fab')2 fragment
Anti-Mouse IgG		AL164	Polyclonal	Goat	nd
Anti-Rat IgG	AS110	AL106	Polyclonal	Goat	(Fab')2 fragment

Designing your own AlphaLISA assay: Selecting toolbox bead pairs to avoid potential bead-bead Interactions.

| Bead coating	Alpha donor beads	AlphaLISA acceptor beads	Monoclonal/ polyclonal	Host	Subclass
Anti-Goat IgG		AL107	Polyclonal	Rabbit	(Fab')2 fragment
Ni Chelate	AS101	AL108	N/A	N/A	N/A
Glutathione	6765300	AL109	N/A	N/A	N/A
Anti-GST		AL110	Polyclonal	Goat	nd
Anti-cMyc		AL111	Monoclonal	Mouse	IgG1k
Anti-FLAG	AS103	AL112	Monoclonal	Mouse	IgG1
Anti-DIG	AS108	Polyclonal	Sheep	Fab fragments	
Anti-DIG		AL113	Monoclonal	Mouse	IgG1k
Streptavidin	6760002	AL125	N/A	N/A	N/A
Protein L		AL126	N/A	N/A	N/A
Anti-FITC		AL127	Monoclonal	Mouse	IgG2a
Anti-His		AL128	Monoclonal	Mouse	IgG1
Anti-V5		AL129	Monoclonal	Mouse	IgG2a
Anti-Mouse IgM		AL130	Monoclonal	Rat	IgG2a,k
Anti-Chicken IgY		AL131	Polyclonal	Rabbit	nd
Anti-Sheep IgG		AL132	Polyclonal	Rabbit	nd
Anti-GFP	AS112	AL133	Monoclonal	Mouse	IgG2a
Anti-Maltose Binding Protein (MBP)		AL134	Monoclonal	Rat	IgG2a
Strep-Tactin™	AS106	AL136	N/A	N/A	N/A
Anti-DNP	AS111	AL173	Polyclonal	Goat	nd
Anti-HRP	AS109	AL171	Polyclonal	Goat	nd
Anti-Protein C		AL172	Monoclonal	Mouse	IgG1,k
Anti-HA		AL170	Monoclonal	Rabbit	nd
Anti-Human IgG1		AL141	Monoclonal	Rat	IgG2a
Anti-Human IgG1		AL153 (isotyping)	Polyclonal	Goat	nd
Anti-Human IgG2		AL154 (isotyping)	Monoclonal	Mouse	IgG1
Anti-Human IgG4		AL142	Monoclonal	Mouse	IgG1
Anti-Human IgG4		AL156 (isotyping)	Monoclonal	Mouse	IgG1
Anti-Mouse IgE		AL161 (isotyping)	Polyclonal	Goat	nd
Anti-Mouse IgG1		AL157 (isotyping)	Monoclonal	Rat	IgG1,k
Anti-Mouse IgG2a		AL158 (isotyping)	Polyclonal	Goat	nd
Anti-Mouse IgG2b		AL159 (isotyping)	Monoclonal	Rat	IgG2a,k
Anti-Mouse IgG3		AL160 (isotyping)	Polyclonal	Rabbit	nd
Anti-Mouse IgM		AL162 (isotyping)	Polyclonal	Goat	nd
Lens Culinaris Agglutinin (LCA)		AL140	N/A	N/A	N/A
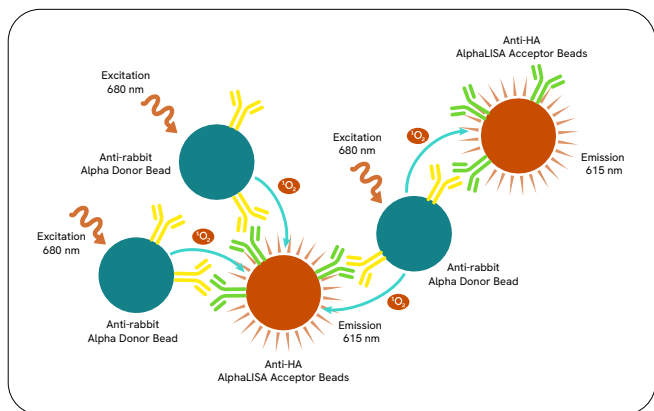
Figure 2. **Cross-reactivity of Antibodies leads to bridging of donor and acceptor beads.** The antibody conjugated to anti-HA AlphaLISA Acceptor beads is a rabbit monoclonal. Therefore, when paired with anti-rabbit donor beads, the donor and acceptor beads can bind in the absences of any analyte causing a false positive Alpha signal. In order to avoid this effect, it is important to check the host species of all of the antibodies and toolbox reagents being engaged in an Alpha assay by using the information found in Table 1.

Example raw data from a single experiment performed to determine which bead pairs might be able to interact on their own, leading to a false-positive signal, is demonstrated in Table 2. The heat map shows the raw signals ranging from low (green) to moderate (yellow) to high (red). In some cases, even though the raw signal is yellow for a particular bead pair, if the signal in the absence of donor is also yellow, the signal to background may still be low. Most of the cross-reactivity can be rationalized by looking back at the host species table (see Table 1). In addition, donor and acceptor bead pairing experiments were performed twice and read on both a standard 2103 EnVision™ and an HTS EnVision, with one hour incubation and an overnight incubation (data not shown). Some bead pairings were tested with multiple lots. The bead-bead interactions were assessed by the counts measured and the signal to background determined from the acceptor bead/donor bead pair and acceptor beads alone.

Table 2. **Bead pairings test.** Example raw data for one lot of each bead, read on a 2103 standard EnVision after one hour. Each data point is the average of three replicates. The heat map shows the signal which ranges from low (in green) to moderate (yellow) to high (red). experiments were performed with 20 µg/mL of donor bead and 20 µg/mL of acceptor bead in 1X universal buffer (Revvity catalog number AL001). Counts may vary in other buffers and at varied bead concentrations.

Acceptor beads	Donor beads													
	No donor beads	Streptavidin-6760002	Anti-FLAG (AS103)	Anti-Mouse (AS104)	Anti-Rabbit (AS105)	Streptactin (AS106)	Ni chelate (AS101)	Glutathione (6765300)	Protein A (AS102)	Anti-DNP (AS111)	Anti-HRP (AS109)	Anti-Rat (AS110)	Anti-DIG (AS108)	Anti-GFP (AS112)
No acceptor beads	8	8	344	19	6	30	52	147	6	20	128	22	56	10
Streptavidin (AL125)	433	1201	1030	795	509	438	645	1179	655	1876	1223	1597	2320	2082
Protein L (AL126)	1375	1628	2441	1446	1480	1099	36168	2473	1398	1715	2151	4426	3498	7031
Anti-FITC (AL127)	297	900	1033	81510	499	349	812	1793	85703	2029	1347	11043	1772	2359
Anti-His (AL128)	134	562	901	105869	366	333	461	621	501	1326	1227	15871	1734	1619
Anti-V5 (AL129)	192	798	1015	32581	470	330	694	997	42438	1051	1378	1161	2209	2378
Anti-Mouse IgM (AL130)	1037	1520	1606	2264	1238	941	1113	1516	1369	1629	1962	107763	2966	2378

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Acceptor beads	Donor beads													
	No donor beads	Streptavidin-6760002	Anti-FLAG (AS103)	Anti-Mouse (AS104)	Anti-Rabbit (AS105)	Streptactin (AS106)	Ni chelate (AS101)	Glutathione (6765300)	Protein A (AS102)	Anti-DNP (AS111)	Anti-HRP (AS109)	Anti-Rat (AS110)	Anti-DIG (AS108)	Anti-GFP (AS112)
Anti-Chicken IgY (AL131)	1743	1728	1857	1659	376086	1372	1468	2444	89594	3661	2457	2196	4700	1879
Anti-Sheep IgG (AL132)	244	562	916	732	311710	428	565	947	95141	5219	2365	894	6834	1021
Anti-GFP (AL133)	451	1225	1080	62761	570	497	669	1451	1051	1118	915	8475	2007	1206
Anti-MBP (AL134)	1478	1651	1942	3622	1291	1347	1465	2214	1438	1946	1732	208494	3847	3022
Streptactin (AI136)	158	627	890	412	372	422	439	921	417	1021	1202	671	2215	1458
Protein A (AL101)	56	515	650	406	336	378	428	673	330	700	1177	787	2348	1041
Protein G (AL102)	767	828	1171	42236	49475	830	1099	954	936	21633	3367	39018	55976	1063
Anti-Human IgG (AL103)	64	431	715	86236	326	369	475	917	1675	1100	936	10288	3036	1456
Anti-Rabbit IgG (AL104)	161	457	762	392	396	453	383	681	381	1019	774	534	2270	1008
Anti-Mouse IgG (AL105)	57	529	36433	366	238	269	324	542	245	630	616	661	713	9735
Anti-Rat IgG (AL106)	257	734	17879	527	390	447	575	836	519	1244	1031	722	1828	3745
Anti-Goat IgG (AL107)	76	756	761	350	3313	261	442	513	270	21991	5136	508	2623	1610
Ni Chelate (AL108)	1136	915	1229	811	825	861	883	1049	927	943	1071	912	967	733
Glutathione (AL109)	142	909	995	487	451	304	674	839	490	2082	1267	1220	3024	2094
Anti-GST (AL110)	293	688	858	559	406	399	535	591	518	720	890	770	1322	923
Anti-cMyc (AL111)	169	773	1015	52098	333	301	539	925	650	599	1055	7907	1416	1156

Acceptor beads	Donor beads													
	No donor beads	Streptavidin-6760002	Anti-FLAG (AS103)	Anti-Mouse (AS104)	Anti-Rabbit (AS105)	Streptactin (AS106)	Ni chelate (AS101)	Glutathione (6765300)	Protein A (AS102)	Anti-DNP (AS111)	Anti-HRP (AS109)	Anti-Rat (AS110)	Anti-DIG (AS108)	Anti-GFP (AS112)
Anti-FLAG (AL112)	173	498	1119	81778	319	294	389	927	349	689	675	13471	1242	852
Anti-Digoxigenin (AL113)	100	341	659	66938	207	211	270	566	267	364	666	8676	837	625
Anti-DNP (AL173)	68	461	729	321	90	280	547	769	294	752	683	767	1100	1054
Anti-HRP (AL171)	3278	2992	2655	2615	2435	2331	2629	2741	2584	2679	2632	2602	3578	2506
Anti-Protein C tag (AL172)	685	901	1114	144525	1002	595	954	1316	9520	1001	1180	19739	1461	1495
Anti-HA (AL170)	127	550	874	464	245489	277	881	1019	65992	1001	1106	819	1301	1485
Anti-Mouse IgG (AL164)	521	840	21280	679	603	582	801	1022	702	1254	1246	1018	1585	5549
Anti-Human 1gG1 (AL153)	118	808	2294	524	416	247	517	751	481	1075	953	954	1285	799
Anti-Human 19G2 (AL154)	211	959	907	116110	484	427	513	800	473	1124	832	14989	1242	880
Anti-Human 1gG4 (AL156)	79	851	784	81501	335	322	380	753	410	1574	916	11068	1095	897
Anti-Mouse IgE (AL161)	129	575	1148	469	445	258	557	893	582	1236	1624	602	1216	1689
Anti-Mouse IgG1 (AL157)	310	706	395337	2510	511	381	664	1010	528	707	861	24817	1761	86675
Anti-Mouse IgG2a (AL158)	268	637	1127	586	519	382	607	934	600	1458	875	1708	1611	949
Anti-Mouse IgG2b (AL159)	208	417	1013	2490	553	257	410	591	493	807	982	168933	996	688
Anti-Mouse 19G3 (AL160)	397	765	1144	772	366267	475	717	837	125200	1937	1005	1064	1317	1402
[Anti-Mouse IgM (AL162)	223	579	1193	570	448	283	497	694	565	1841	1565	1183	1656	913
LCA (AL140)	301	488	963	417	383	309	531	678	456	625	875	766	1008	894

From all the data gathered, we put together a bead pairing recommendation table (Table 3). The green check marks show recommended pairings that gave low signal under all conditions tested. The yellow exclamation marks show pairs with higher background due to moderate interactions between the conjugated proteins/antibodies on the beads. In these cases, users should exercise caution as the signal seen may be usable on a particular instrument with a short incubation time, but may provide too high of a background signal under other conditions, and most importantly, could vary based on lot of antibody (and Alpha bead). Pairings indicated in red gave particularly high signals under all conditions tested and should be avoided.

Table 3. **Bead Pairing Recommendations.** The green check marks show recommended pairings that gave low signal under all conditions tested. The yellow exclamation marks show pairs with higher background due to moderate interactions between the conjugated proteins/antibodies on the beads. In cases where a yellow exclamation mark is shown, it is important to note that the level of interaction could potentially vary as the lots of assay components change. This could mean while low background alpha signal is observed initially, if different lots of antibody (and toolbox reagents) are used over time, the background Alpha signal could increase or decrease significantly. Pairings indicated in red gave particularly high signals under all conditions tested and should be avoided.

Acceptor Beads	Donor beads												
	Streptavidin-6760002	Anti-FLAG (AS103)	Anti-Mouse (AS104)	Anti-Rabbit (AS105)	Streptactin (AS106)	Ni chelate (AS101)	Glutathione (6765300)	Protein A (AS102)	Anti-DNP (AS111)	Anti-HRP (AS109)	Anti-Rat (AS110)	Anti-DIG (AS108)	Anti-GFP (AS112)
Streptavidin (AL125)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Protein L (AL126)	✓	✓	✓	✓	✓	✗	✓	✓	✓	✓	!	✓	✗
Anti-FITC (AL127)	✓	✓	✗	✓	✓	✓	✓	✗	✓	✓	✗	!	✓
Anti-His (AL128)	✓	✓	✗	✓	✓	✓	✓	✓	✓	✓	✗	!	✓
Anti-V5 (AL129)	✓	✓	✗	✓	✓	✓	✓	✗	✓	✓	✓	!	!
Anti-Mouse IgM (AL130)	✓	✓	!	✓	✓	✓	✓	✓	✓	✓	✗	✓	✓
Anti-Chicken IgY (AL131)	✓	✓	✓	✗	✓	✓	✓	✗	✓	✓	✓	✓	✓
Anti-Sheep IgG (AL132)	✓	✓	✓	✗	✓	✓	✓	✗	✗	!	✓	✗	✓
Anti-GFP (AL133)	✓	✓	✗	✓	✓	✓	✓	✓	✓	✓	✗	✓	✓
Anti-MBP (AL134)	✓	✓	!	✓	✓	✓	✓	✓	✓	✓	✗	✓	✓
Streptactin (AI136)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	!	✓
Protein A (AL101)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	!	✓
Protein G (AL102)	✓	✓	✗	✗	✓	✓	✓	✓	✗	✓	✗	✗	✓
Anti-Human IgG (AL103)	✓	✓	✗	✓	✓	✓	✓	!	✓	✓	✗	!	✓
Anti-Rabbit IgG (AL104)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	!	✓
Anti-Mouse IgG (AL105)	✓	✗	✓	✓	✓	✓	✓	✓	✓	✓	!	✓	✗
Anti-Rat IgG (AL106)	✓	✗	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	!
Anti-Goat IgG (AL107)	✓	✓	✓	✗	✓	✓	✓	✓	✗	✗	✓	!	!
Ni Chelate (AL108)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Glutathione (AL109)	✓	✓	✓	✓	✓	✓	✓	✓	!	✓	✓	!	!
Anti-GST (AL110)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Anti-cMyc (AL111)	✓	✓	✗	✓	✓	✓	✓	✓	✓	✓	✗	✓	✓
Anti-FLAG (AL112)	✓	✓	✗	✓	✓	✓	✓	✓	✓	✓	✗	✓	✓

Acceptor Beads	Donor beads												
	Streptavidin-6760002	Anti-FLAG (AS103)	Anti-Mouse (AS104)	Anti-Rabbit (AS105)	Streptactin (AS106)	Ni chelate (AS101)	Glutathione (6765300)	Protein A (AS102)	Anti-DNP (AS111)	Anti-HRP (AS109)	Anti-Rat (AS110)	Anti-DIG (AS108)	Anti-GFP (AS112)
Anti-Digoxigenin (AL113)	✓	✓	✗	✓	✓	✓	✓	✓	✓	✓	✗	✓	✓
Anti-DNP (AL173)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Anti-HRP (AL171)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Anti-Protein C tag (AL172)	✓	✓	✗	✓	✓	✓	✓	!	✓	✓	✗	✓	✓
Anti-HA (AL170)	✓	✓	✓	✗	✓	✓	✓	✗	✓	✓	✓	✓	✓
Anti-Mouse IgG (AL164)	✓	✗	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✗
Anti-Human 1gG1 (AL153)	✓	!	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Anti-Human 19G2 (AL154)	✓	✓	✗	✗	✓	✓	✓	✓	✓	✓	✗	✓	✓
Anti-Human 1gG4 (AL156)	✓	!	✗	✓	✓	✓	✓	✓	!	!	✗	✓	✓
Anti-Mouse IgE (AL161)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Anti-Mouse IgG1 (AL157)	✓	✗	!	✓	✓	✓	✓	✓	✓	✓	✗	✓	✗
Anti-Mouse IgG2a (AL158)	✓	!	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Anti-Mouse IgG2b (AL159)	✓	✓	!	✓	✓	✓	✓	✓	✓	✓	✗	✓	✓
Anti-Mouse 19G3 (AL160)	✓	✓	✓	✗	✓	✓	✓	✗	✓	✓	✓	✓	✓
[Anti-Mouse IgM (AL162)	✓	!	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
LCA (AL140)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

In conclusion, the bead pair recommendation table shows pairings that should be avoided and those that may have moderate affinity for one another. It should be noted, however, that there are many factors that can influence the background signal observed by alpha bead pair cross-reactivity. The recommendations made in Table 3 take into account things such as incubation time (low background signal even after an overnight incubation) and type of instrument (low background on both HTS and Standard Envision 2103 models), but does not represent every assay condition possible. For example, the experiments in Figure 2 were carried out in Universal assay buffer. Any difference in assay buffer conditions could impact the relative assay background signal. Therefore, it is important that a "no analyte" control be performed using the precise assay conditions of interest (incubation time, temperate, assay buffer, etc.) to verify the level of bead cross-reactivity that may be occurring between a particular Alpha bead pairing.

The AlphaLISA Immunoassay conversion quick-start guide and the protein: Protein interaction assay development guide are more in-depth resources that provide further guidance on assay design using alpha toolbox reagents. Troubleshooting tips and other important assay advice can also be found by visiting www.revvity.com.

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