M1072

Conjugated Antibody Characterization Is Critical for Reducing Variability in Immunogenicity and Biomarker Assays

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❶ Abstract

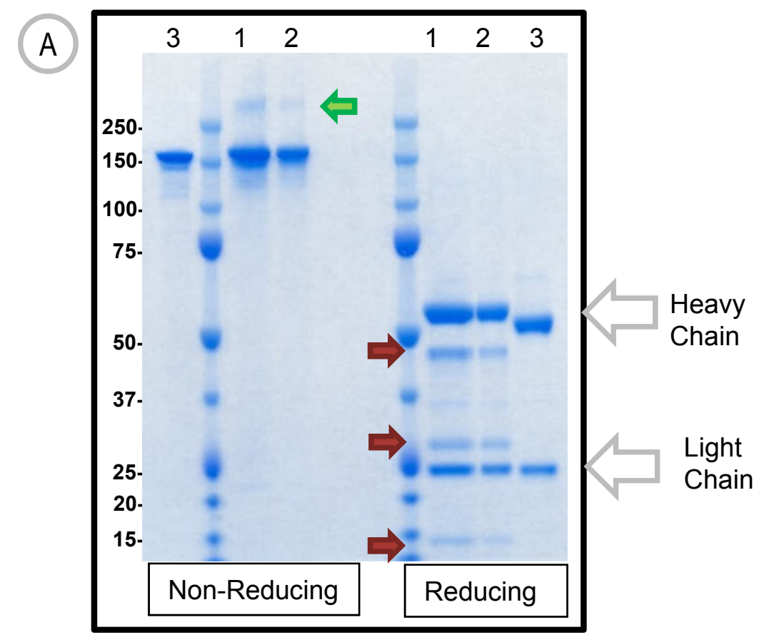
Purpose: To ensure lot-to-lot assay reproducibility and sustainability, all critical reagents must be characterized, conjugated, tested, and stored appropriately. Aggregation, contamination, or insufficient purity of conjugated antibodies can cause significant variability in assay performance.

Methods: Antibodies used in MESO SCALE DISCOVERY's MULTI-ARRAY® electrochemiluminescent assays were characterized using non-reducing and reducing SDS-PAGE, dynamic light scattering (DLS), and capillary isoelectric focusing (cIEF) to identify patterns that may indicate changes. The antibodies were then conjugated with biotin and MSD® SULFO-TAG, an electrochemiluminescent label, at multiple challenge ratios to optimize the immunoassay. Biotin incorporation was measured using a HABA assay; SULFO-TAG™ incorporation was measured using spectrophotometry. Purification was performed using various gel filtration columns to determine the best purification methods.

Results: Lot-to-lot variability in antibody aggregation (raw material) detected by SDS-PAGE and DLS can lead to non-specific background problems in immunoassays. Purification of conjugated antibodies through size exclusion chromatography eliminated background issues observed. In some cases, cIEF revealed significant differences between two lots from the same vendor, possibly attributable to changes in purification methods or a change in the polyclonal antibody. Standardization of the conjugation procedure proved to be a significant factor in assay reproducibility. In immunogenicity assays, unconjugated antibodies cause signal suppression. In one example, we found that incorporating 4 biotin or SULFO-TAG molecules reduced label-free antibodies to undetectable levels. Increasing biotin incorporation beyond 4–5 biotin molecules per antibody did not improve assay performance. Signals are proportional to the number of SULFO-TAG molecules incorporated; therefore, assays requiring the greatest sensitivity may have 10–15 SULFO-TAG molecules conjugated to an antibody. We developed optimized procedures for thorough removal of unincorporated biotin or SULFO-TAG after conjugation to antibodies or other proteins.

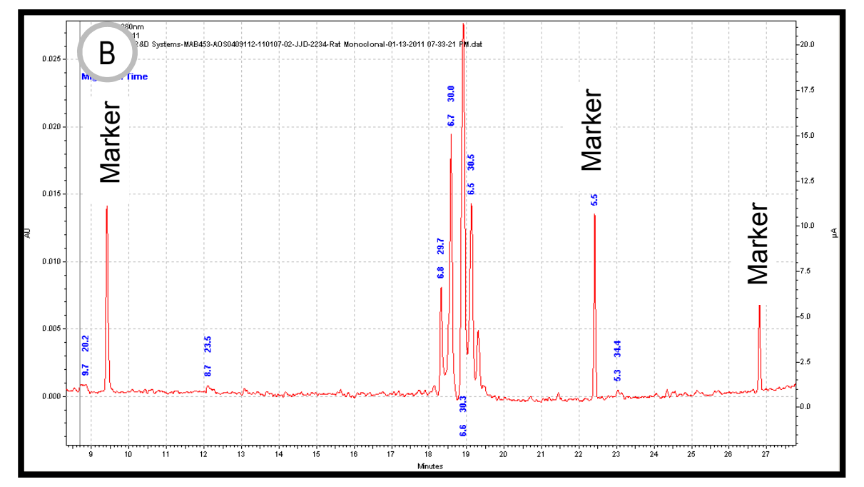
Conclusion: Variability in assay signals or background levels can come from antibody aggregation, from the presence of unconjugated antibody, or from free labeling compound in the conjugated antibody preparations. Thorough analytical characterization of raw material and the conjugated antibody is necessary to ensure lot-to-lot reproducibility of immunoassays.

❷ Analytical Methods

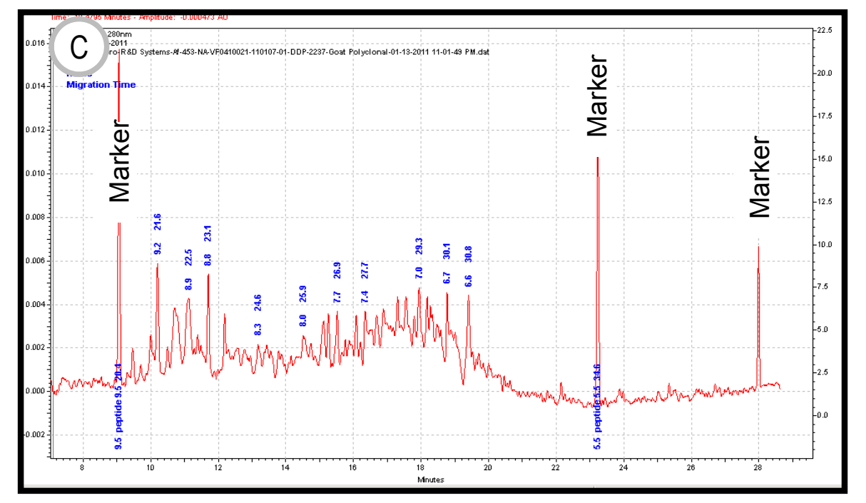


Reducing and non-reducing SDS-PAGE (Figure A) or Experion-automated electrophoresis is used to identify degradation of antibodies and impurities. In Figure A, two lots of an antibody (lanes 1 and 2) show degradation products in the reducing conditions (indicated by red arrows) that are not present in the non-reducing conditions. However the non-reducing conditions show higher molecular weight contaminants or aggregates (green arrow). Dynamic light scattering is another technique that can be used to identify aggregation. Lane 3 is the reference antibody.

Figures B and C below represent typical capillary isoelectric focusing graphs of a monoclonal and polyclonal antibody. These graphs are a "fingerprint" of the antibody based on its isoelectric point and can be used to identify changes to an antibody, including, but not limited to changes in purification, aggregation, degradation, and changes in the antibody source.



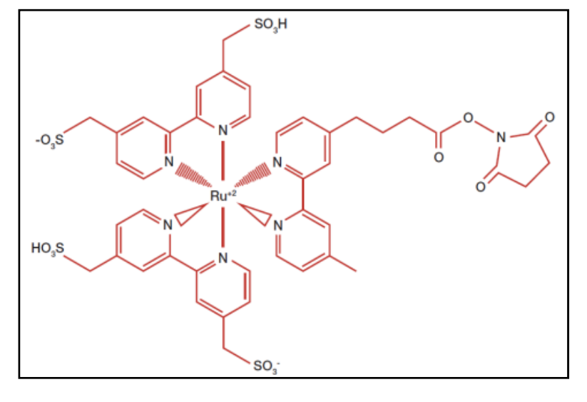
Typical Monoclonal CIEF Profile: Single to 3-4 well defined peaks



Typical Polyclonal CIEF Profile: Broad profile with multiple peaks.

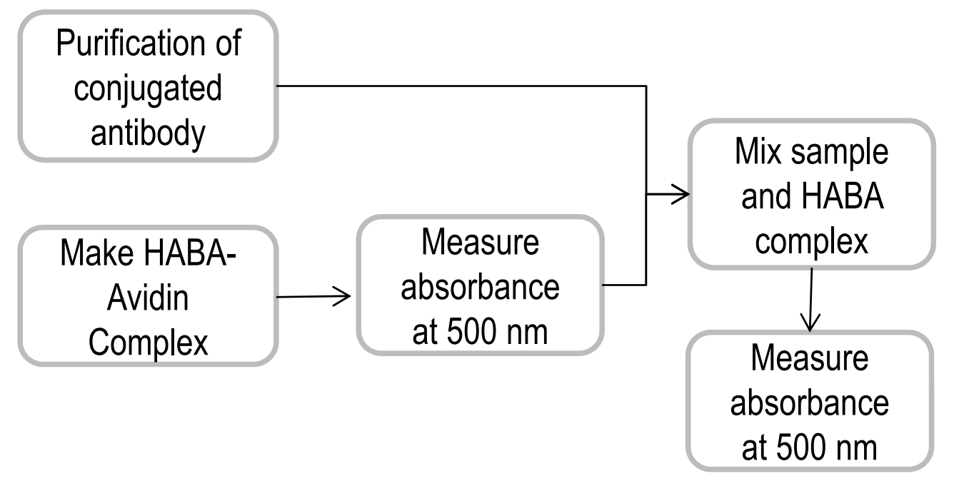
SULFO-TAG absorbance at 455nm can be used to determine the number of SULFO-TAG molecules conjugated to a protein.

Molar concentration of SULFO-TAG =
A_{455}/ (15400 $M^{-1}cm^{-1}$ X Path length)



SULFO-TAG NHS Ester

Biotin incorporation was measured using a kit from Thermo Scientific (catalog 28005). The kit measured the amount of biotin by displacement of 4'-hydroxyazobenzene-2-carboxylic acid (HABA) bound to an avidin complex. We found the results varied from operator to operator and day to day. Control samples and a rigorous SOP were required to reduce variability.



Purification of conjugated antibody

Make HABA-Avidin Complex

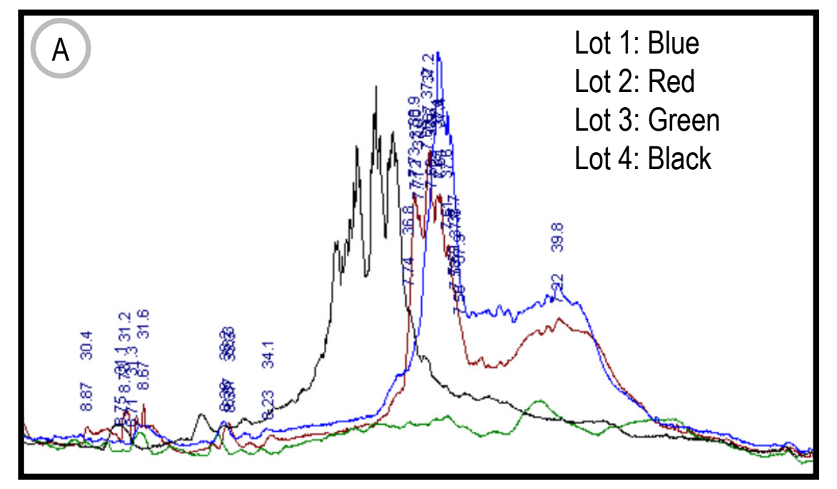
Measure absorbance at 500 nm

Mix sample and HABA complex

Measure absorbance at 500 nm

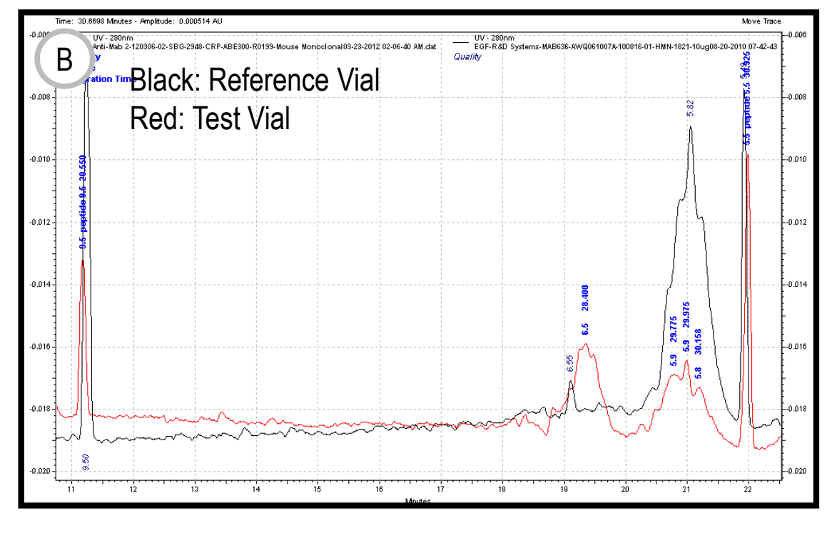
❸ Identifying Antibody Changes

We used cIEF to identify alterations to polyclonal antibodies that may indicate a change in functional performance. Figure A shows cIEF graphs from 4 different lots of supposedly the same polyclonal antibody. The performance of lots 1 and 2 was functionally similar. Lot 3 worked in western blot, but not in an MSD immunoassay. Lot 4 worked in the MSD assay, but gave different results from previous lots of antibody. Lots 1 and 2 were from early bleeds from one animal while lot 3 was the last bleed from the same animal. Lot 4 was from a different animal, but the vendor did not originally provide this information.



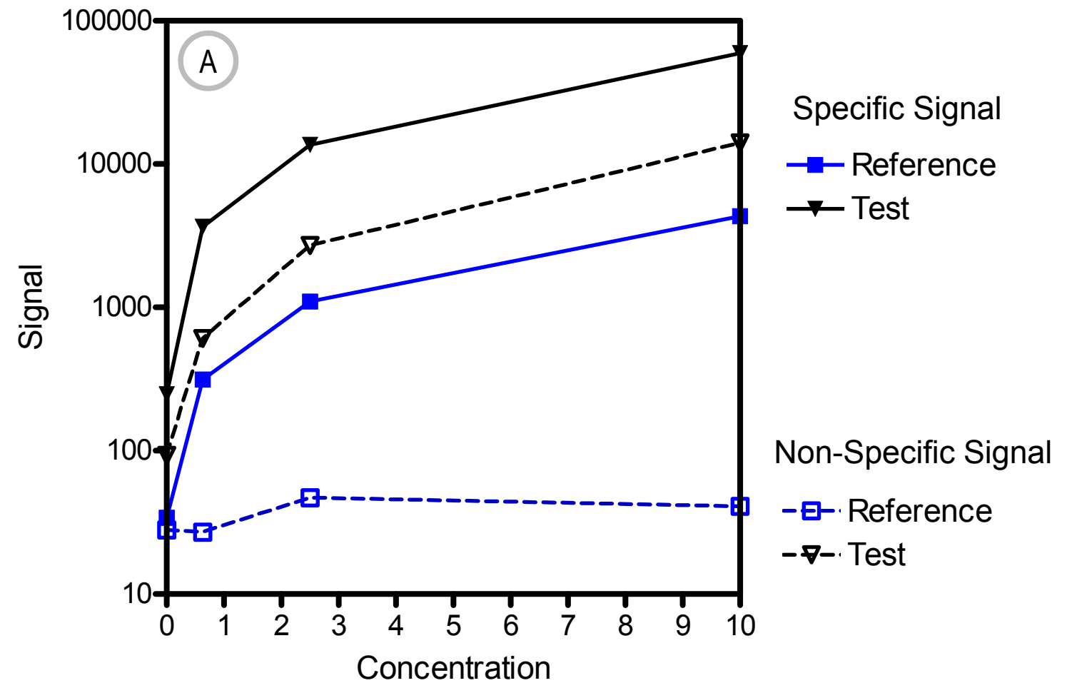
Working stocks of antibodies can degrade over time when stored improperly. DLS and cIEF can be used to detect aggregation, degradation, and basic or acidic shifts of monoclonal antibodies. In the example below, the functional performance of a working stock (test vial) of a monoclonal antibody degraded over time (performance compared to the reference antibody in the table below). The cIEF of the reference antibody (Figure B) has a single peak (black line), whereas a second peak is present in the cIEF of the test vial. This second peak is likely due to degraded or aggregated protein. The cIEF can be used to show qualitative changes in an antibody.

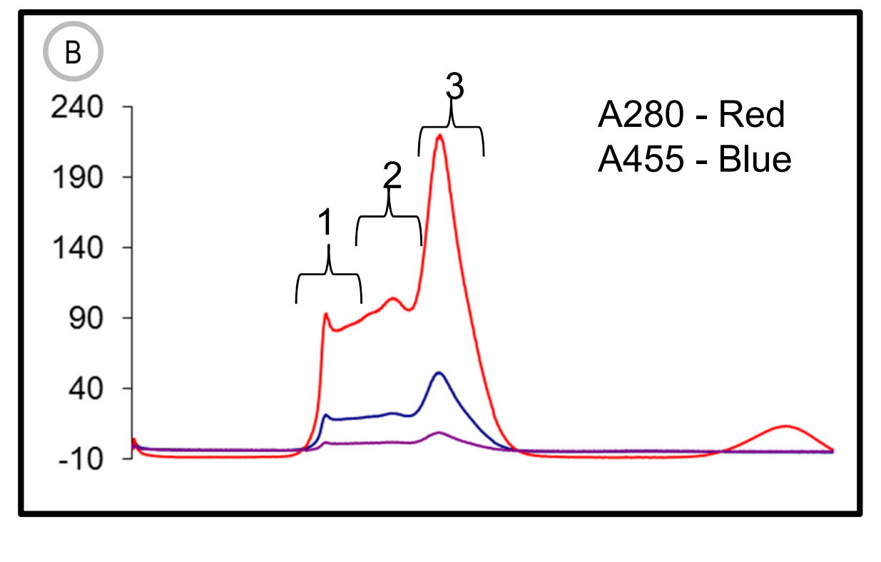
	Reference Vial		Test Vial		
Calibrator	Average Signal	Intra-Plate %CV	Average Signal	Intra-Plate %CV	Test Lot/ Ref. Lot
Standard 1	637302	4%	367794	5%	58%
Standard 2	139771	2%	63854	1%	46%
Standard 3	35082	5%	9824	0%	28%
Standard 4	5009	5%	1939	4%	39%
Standard 5	1128	8%	479	11%	43%
Standard 6	392	4%	202	8%	52%
Standard 7	187	%	134	19%	72%
Blank	106	9.3%	112	11%	106%

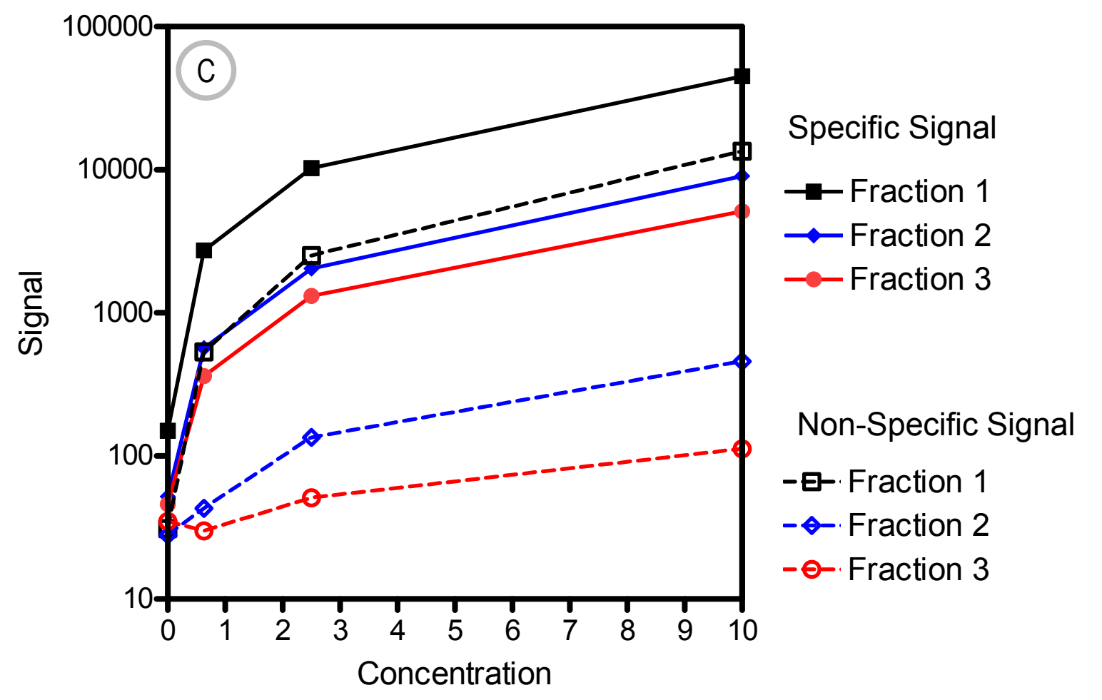


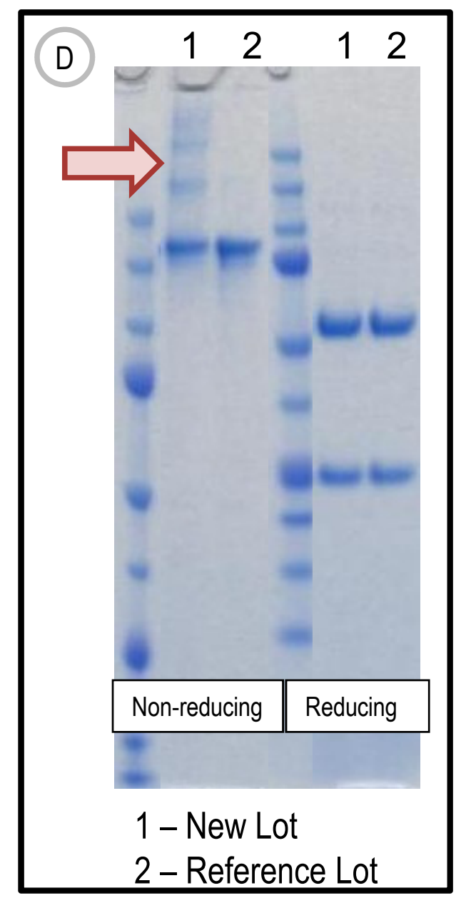
❹ Aggregated Detection Antibody Can Cause Higher Background

The example below shows that elevated backgrounds can be caused by large aggregates of detection antibody. A new lot of conjugated antibody was identified as having 2–3 times higher specific (solid lines) signals and 100 times higher non-specific signals (dashed lines) when compared to the reference antibody lot (Figure A). The non-specific signals (dashed lines) were generated from the detection antibody with a non-specific capture antibody in the same MULTI-SPOT® well as the specific capture antibody. The SDS-PAGE gel (Figure D) shows aggregated proteins (red arrow) in the raw material for the new lot. Size exclusion chromatography (SEC) of the conjugated material (Figure B) shows 3 peaks with a large amount of oligomers (peak #1), probable dimers (peak #2) and the monomer (peak #1).Three fractions of the antibody were collected from SEC and run in the functional assay. Fraction 1 (largest oligomers) generated non-specific signals that were greater than the specific signals from the other fractions. The monomeric fraction (#3) was within 20% of the signals generated from the reference lot (Figure C).





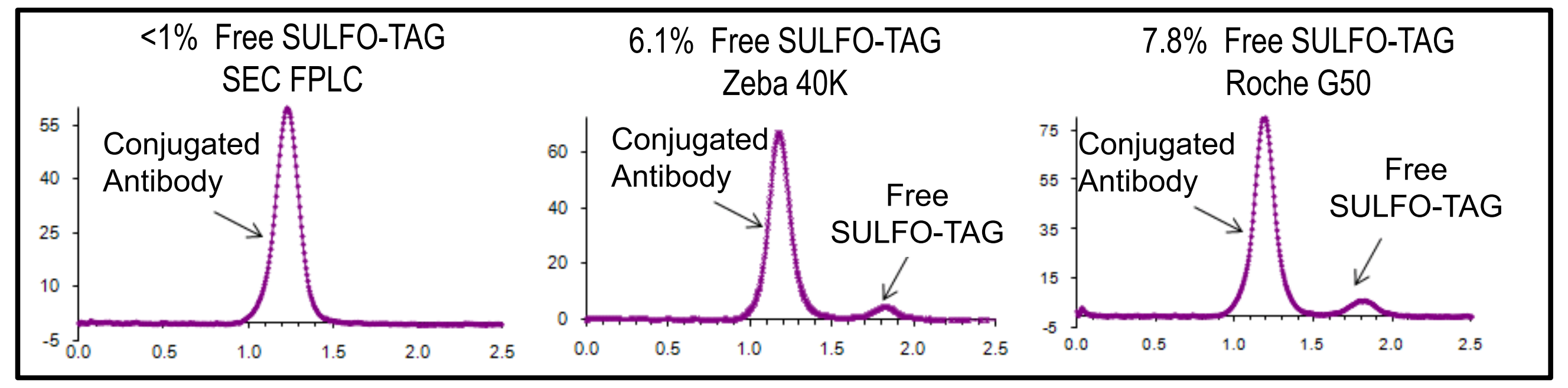




❺ Purification of Free Biotin and SULFO-TAG Labels

Removal of unincorporated labels (NHS-biotin or NHS-SULFO-TAG) after the conjugation step may be critical for good assay reproducibility. SULFO-TAG and biotin are both less than 1.5 kDa and should be easily purified by many different columns. We found that not all purification columns and conditions were able to completely remove unconjugated labels. We measured the residual SULFO-TAG (through analytical SEC) after purification through different chromatographic media. All columns were loaded with 2 mg/ml of conjugated antibody. We observed that the Thermo Scientific columns (TS) outperformed the Pierce, Sigma, and Roche columns. The best results were obtained when no more than one-quarter of the column size (volume) was loaded. Passing the conjugated material through two columns further reduced the amount of unincorporated label.

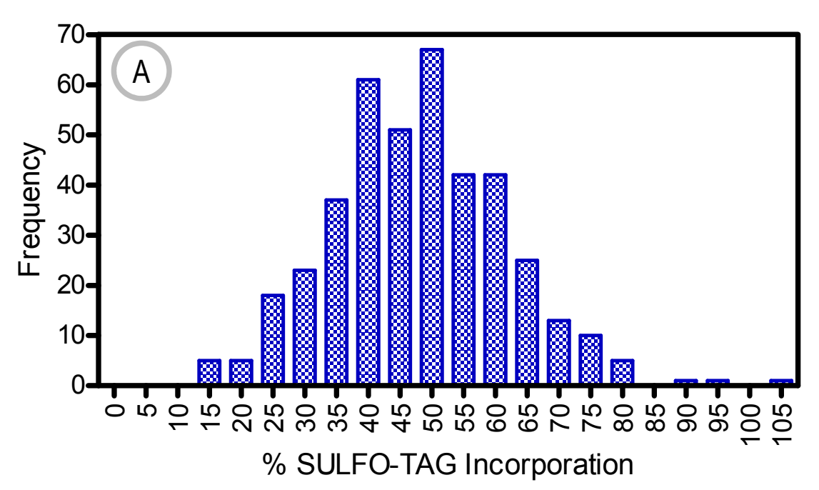
							OD455	
	Source	Description	Cat #	Column Size	Recommended Sample vol.	STAG loaded (ml)	Mean	SD
1	Thermo Sci	Zeba 40K	87768	2ml	200-900ul	0.45	0.00	0.00
2	Thermo Sci	Zeba 40K	87768	2ml	200-900ul	0.9	0.16	0.00
3	PIERCE	Zeba 7K	89892	5ml	300-2000ul	1	0.29	0.01
4	PIERCE	Zeba 7K	89892	5ml	300-2000ul	2	0.99	0.03
5	Thermo Sci	Zeba 40K	87772	10ml	1000-4000ul	2	0.00	0.00
6	Thermo Sci	Zeba 40K	87772	10ml	1000-4000ul	4	0.15	0.01
7	Sigma	Self packed	G5080	10ml	1000-4000ul	2	0.21	0.01
8	Sigma	Self packed	G5080	10ml	1000-4000ul	4	0.50	0.00
9	Roche	G-50 Sephadex	3117928001	3ml	100-500ul	0.25	0.15	N/A
10	Roche	G-50 Sephadex	3117928001	3ml	100-500ul	0.5	0.35	0.01
11	Unprocessed	N/A	N/A	N/A	N/A	N/A	2.51	0.01



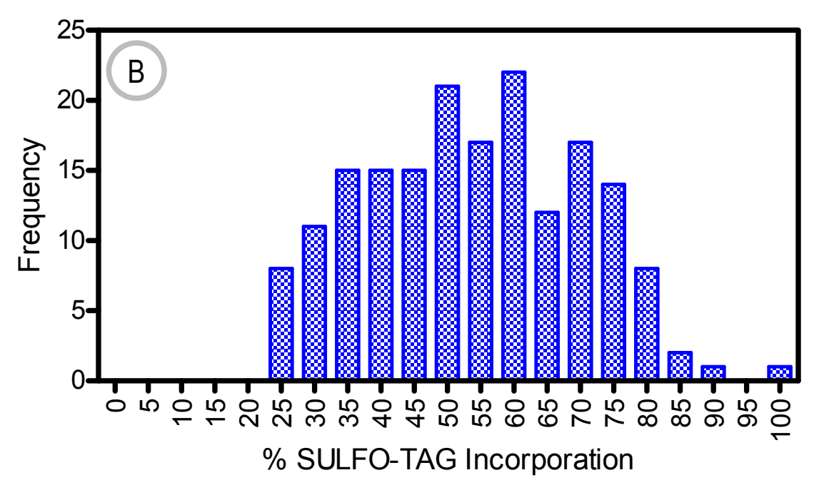
❻ Incorporation of SULFO-TAG and Conjugation Consistency

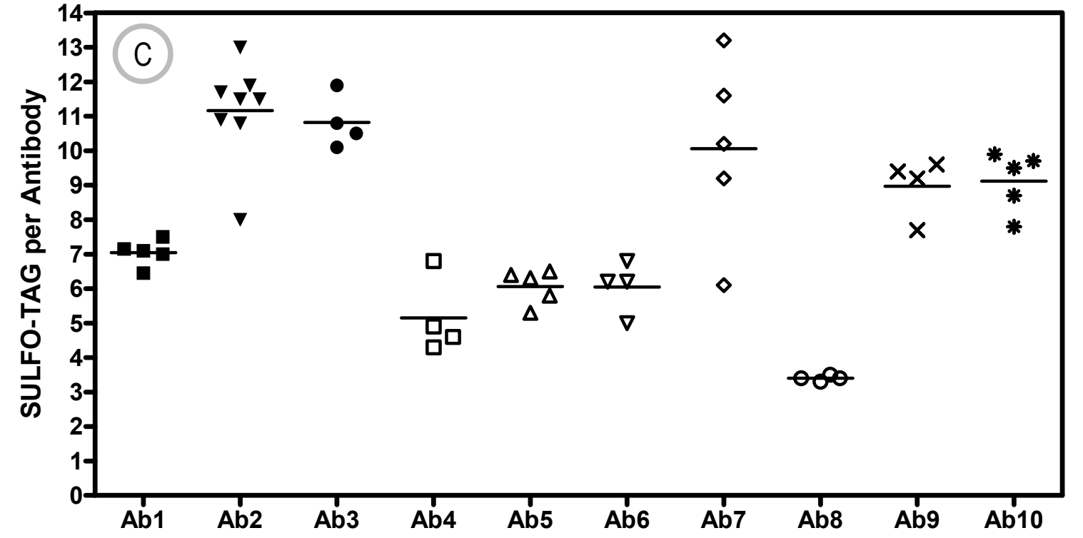
The % incorporation of SULFO-TAG and consistency of conjugation is dependent on the specific antibody and the challenge ratio (CR). Figure A shows a histogram of the % incorporation for 400 different antibodies conjugated at a challenge ratio of 20. The median % incorporation (amount of label incorporated) is 48% and the distribution looks Gaussian. We conjugated over 150 different antibodies at challenge ratios of less than 20. The distribution of the % incorporation was much wider. Figure C shows 10 different antibodies conjugated multiple times (at the same challenge ratio) during a 2.5 year period. Conjugation of most antibodies was very reproducible, however some antibodies exhibited greater variability in the incorporation of SULFO-TAG.

Antibodies Conjugated at CR 20



Antibodies Conjugated at CR < 20

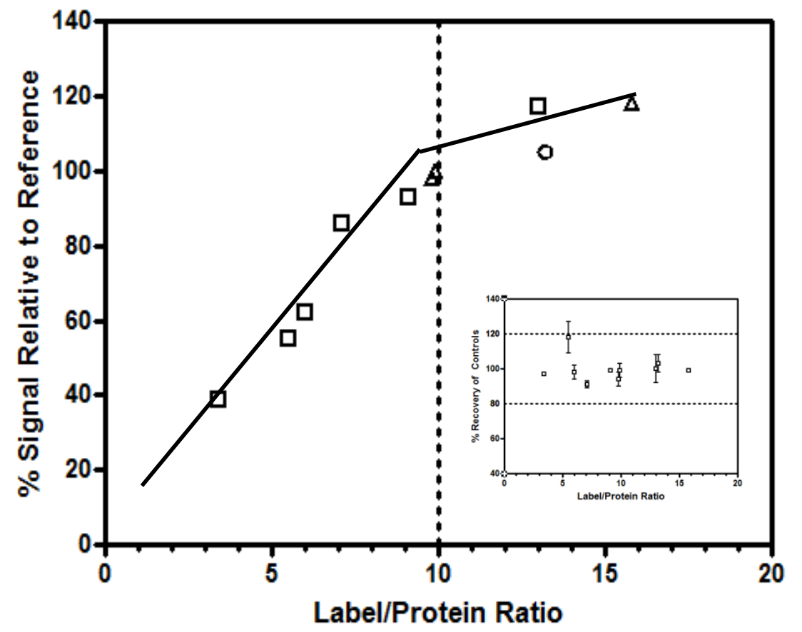




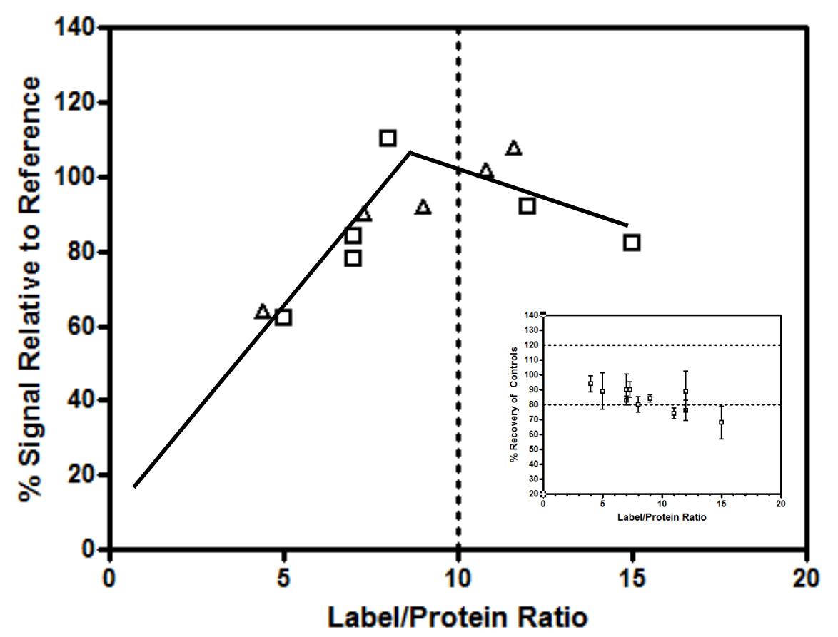
❼ Over Conjugation of Antibodies

Increasing the number of SULFO-TAG labels increases the signals and improves sensitivity for most antibodies. Antibody A, a typical antibody, shows increasing signal with increasing labels per protein (LP). The % recovery of controls is good throughout the range of labels incorporated (inset). For a small number of antibodies like Antibody B, the signals decrease as the label per protein increases beyond a certain point. For this group of antibodies, the recovery of controls worsens with increasing incorporation because the label may have been incorporated into the variable region of the antibody. The dashed lines represent the initial targeted label per protein for both antibodies. We reduced the label per protein target for Antibody B to 8.

Detection Antibody A



Detection Antibody B



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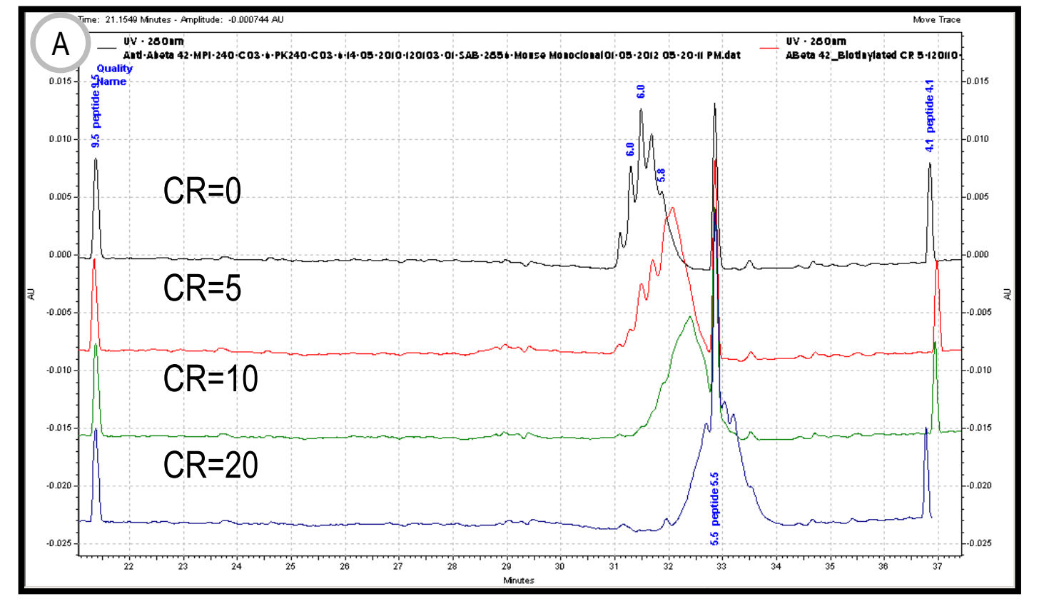
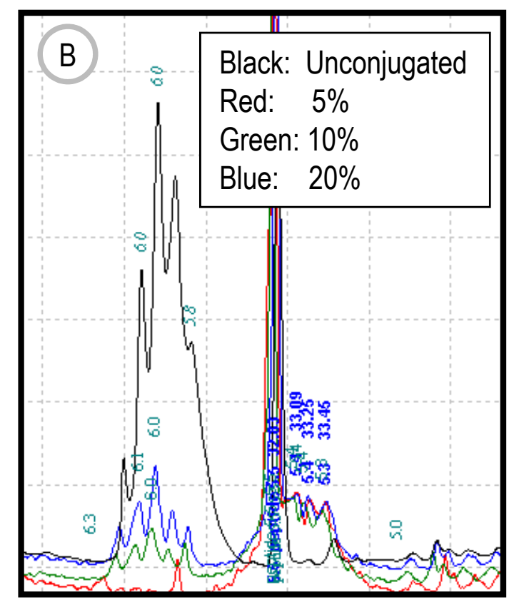
❽ Residual Unconjugated Antibody (Drug)

Residual unconjugated drug in a bridging immunogenicity assay acts as an interference that suppresses assay signals. We conjugated a monoclonal antibody with biotin at challenge ratios (CR) of 5, 10, and 20. The number of biotin molecules incorporated into the antibody measured by HABA is shown in the table below. The cIEF graphs of the conjugated antibodies (Figure A) shows an acidic shift in the conjugated antibodies. The cIEF of the CR20 material is well separated from the unconjugated material. The cIEF of the CR5 antibody shows two peaks that overlap with the unconjugated antibody, suggesting that a significant amount of antibody is unconjugated. Our data suggests that antibodies conjugated at a ratio of 5 or less would have unconjugated antibodies in the post conjugation solution. We spiked 5%, 10%, and 20% unconjugated material back into the CR20 material and found that the cIEF was sensitive to about 10% unconjugated material (Figure B). From this we estimated that the CR5 material had 10-20% unconjugated antibody.





	conc (mg/ml)	MW	biotin/ protein	% Incorporation
Challenge Ratio 5 (CR5)	1.77	150000	1.6	32
Challenge Ratio 10 (CR10)	1.79	150000	2.5	25
Challenge Ratio 20 (CR20)	1.73	150000	4.0	20

We simulated residual unconjugated drug in an immunogenicity assay. The biotin and SULFO-TAG conjugated drug was tested with 0, 10%, 25%, 50% and 80% unconjugated drug. At 25% unconjugated material, there is % inhibition of the immunogenicity assay at 500 ng/ml ADA. From this we would recommend less than 10% unconjugated materials for immunogenicity assays.

	% Unconjugated Biotin Drug										% Unconjugated SULFO-TAG Drug									
	0%		10%		25%		50%		80%		0%		10%		25%		50%		80%	
[ADA] ng/mL	Signal	CV	Signal	CV	Signal	CV	Signal	CV	Signal	CV	Signal	CV	Signal	CV	Signal	CV	Signal	CV	Signal	CV
500	114521	2%	98271	3%	78726	1%	47275	2%	11574	2%	115563	0%	89529	9%	76975	1%	45921	0%	13364	2%
125	26714	2%	22726	2%	18298	0%	11084	0%	2778	1%	25489	6%	22583	3%	18169	2%	10279	8%	3058	4%
31	6776	1%	5740	1%	4605	1%	2779	0%	736	0%	6590	1%	5634	2%	4441	1%	2598	2%	812	4%
8	1770	1%	1463	3%	1222	0%	768	2%	246	1%	1751	0%	1459	3%	1182	0%	729	0%	256	1%
2	493	3%	438	2%	367	0%	249	2%	116	1%	506	3%	433	3%	358	2%	239	4%	102	6%
0	70	5%	75	10%	76	3%	73	9%	70	4%	76	7%	72	3%	68	8%	66	1%	53	3%
% Inhibition	**0%**		**15%**		**31%**		**58%**		**89%**		**0%**		**16%**		**32%**		**60%**		**87%**	

Using the same reagents, we simulated residual unconjugated drug in step-wise PK assays with a wash after the capture antibody incubation. This format of assay is less sensitive to unbiotinylated drug because it is washed away before the sample is added. However we would expect more of an effect for lower avidity antibodies because less antibody is used to capture the sample.

	% Unconjugated Biotin Drug										% Unconjugated SULFO-TAG Drug									
	0%		10%		25%		50%		80%		0%		10%		25%		50%		80%	
[ADA] ng/mL	Signal	CV	Signal	CV	Signal	CV	Signal	CV	Signal	CV	Signal	CV	Signal	CV	Signal	CV	Signal	CV	Signal	CV
500	98655	4%	108783	0%	108901	2%	102860	1%	64877	5%	102138	2%	88387	2%	70852	4%	43109	1%	13362	1%
125	25418	1%	28344	1%	28728	0%	26264	1%	17448	5%	24512	5%	22467	0%	18262	3%	11150	5%	3450	4%
31	6540	3%	7146	3%	7464	1%	6731	3%	4782	5%	6524	1%	5975	3%	4883	6%	2808	0%	930	5%
8	1770	0%	1879	1%	1929	1%	1811	2%	1255	0%	1737	3%	1570	1%	1268	1%	793	3%	274	2%
2	491	3%	529	2%	537	1%	511	2%	360	4%	488	1%	455	0%	365	1%	248	4%	110	4%
0	73	6%	75	6%	71	1%	69	4%	70	1%	78	2%	77	4%	75	3%	68	5%	58	9%
% Inhibition	**0%**		**-9%**		**-12%**		**-3%**		**30%**		**0%**		**10%**		**27%**		**56%**		**86%**	

❾ Conclusion

We have demonstrated that variability in raw materials and conjugation can lead to assay variability. Characterization of all critical reagents is important for sustaining the assay over multiple lots of reagents. For assays developed at MSD, all antibodies are purified and characterized to ensure consistent quality of the raw materials used in our validated assays. Antibody degradation caused by instability, changes in purification techniques, or contamination can result in a loss of signal and sensitivity. Aggregated antibodies can lead to higher variability and increased non-specific backgrounds. For immunogenicity assays, anti-drug antibodies (ADA) may not recognize aggregated drug because of masked epitopes.

The conjugation step is a critical if an assay is to perform well. Improper removal of free biotin could cause blocking of the plates and free SULFO-TAG could cause improper assignment of labels per protein. For optimal removal of free label, only one-quarter of the total column volume should be used in purification columns. To get the most complete purification, we use size exclusion chromatography to purify out aggregates, degraded proteins, and unconjugated label.

Immunogenicity assays require low incorporation levels to reduce epitope masking. We recommend a biotin challenge ratio of 8–10 and incorporation of at least 3 biotin molecules per protein. SUFLO-TAG conjugation should be performed at a challenge ratio of 8–12 incorporating more than 4 SULFO-TAG molecules per antibody. As little as 10–20% unconjugated material can cause 15–30% suppression in assay performance.

Antibodies used in **pharmacokinetic (PK) and biomarker assays** should be conjugated at a higher challenge ratio. Biotin conjugation should be performed at a 10–15 challenge ratio incorporating more than 5 labels per protein. To get the most sensitivity, detection antibodies should be conjugated at a challenge ratio of 20 with a target label per protein between 8 and 15. Unconjugated capture antibody is less problematic when used in washed protocols. Unconjugated detection antibody results in a proportional reduction in signal and sensitivity. Achieving consistent conjugation is critical for sustaining precision and accuracy an assay's lifetime.

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