

Characterization of Biotinylated Antibodies and Other Proteins

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1 Abstract

PURPOSE

Biotin-conjugated antibodies and other proteins are critical reagents for bridging immunogenicity assays, pharmacokinetics and pharmacodynamics (PK/PD) assays, and other immunoassays that use avidin or streptavidin. When creating biotinylated proteins of consistent quality, composition, and performance, it is important to have reproducible methods for measuring biotin-protein ratios (B/P) and other characteristics of biotin-protein conjugates. The HABA (4'-hydroxazobenzene-2-carboxylic acid) assay is the most widely used method for measuring B/P, but it has insufficient sensitivity and reproducibility for use in quality control of biotinylated proteins. We looked for a method that could provide superior performance compared to the HABA assay.

METHODS

We compared HABA Biotin Quantitation Kit (Thermo Scientific) with FluoReporter® Biotin Quantitation Kit (Life Technologies) and Quant*Tag™ Biotin Kit (Vector Laboratories). We found that the methods differed in sensitivity, dynamic range, reproducibility, and ease of use. We adapted the Quant*Tag method to a 384-well format for increased throughput, sensitivity, and reproducibility. We also used capillary isoelectric focusing to characterize distribution of B/Ps and the presence of unconjugated protein in the final biotinylated product.

RESULTS

The modified Quant*Tag method was suitable for reliable small-scale (≥0.1 nmol/well in a 384-well plate) measurements of biotin-antibody conjugates with low B/P (<5), for comparing day-to-day variability in biotinylation processes, and for comparing lot-to-lot reproducibility of biotinylated products. We found that biotin incorporation can vary from 30% to 70% between different antibodies, and we present the distribution of B/Ps at different challenge ratios and for various antibody types. We also show that the presence of as little as 10% of unconjugated protein can be detected by capillary isoelectric focusing.

CONCLUSIONS

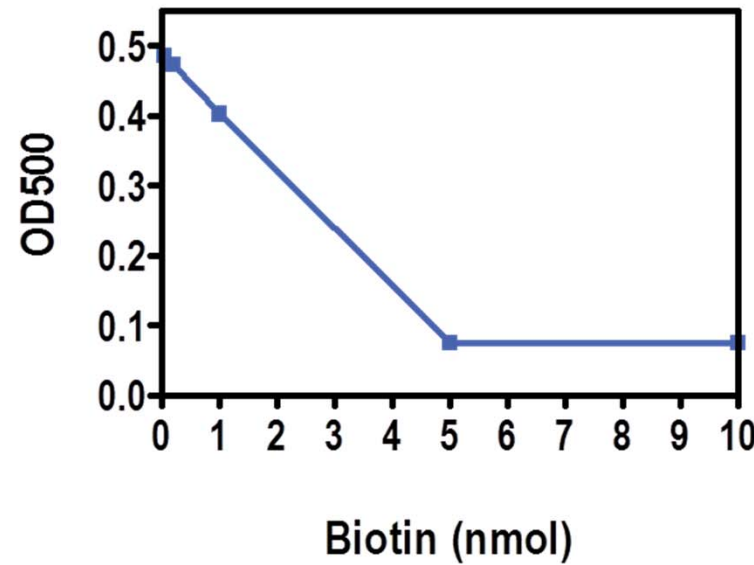
The methods for characterizing biotin-protein conjugates presented here have sufficient reproducibility and sensitivity for monitoring and optimizing biotinylation reactions and for quality control testing of the final biotinylated products.

2 Methods for Biotinylation Measurement

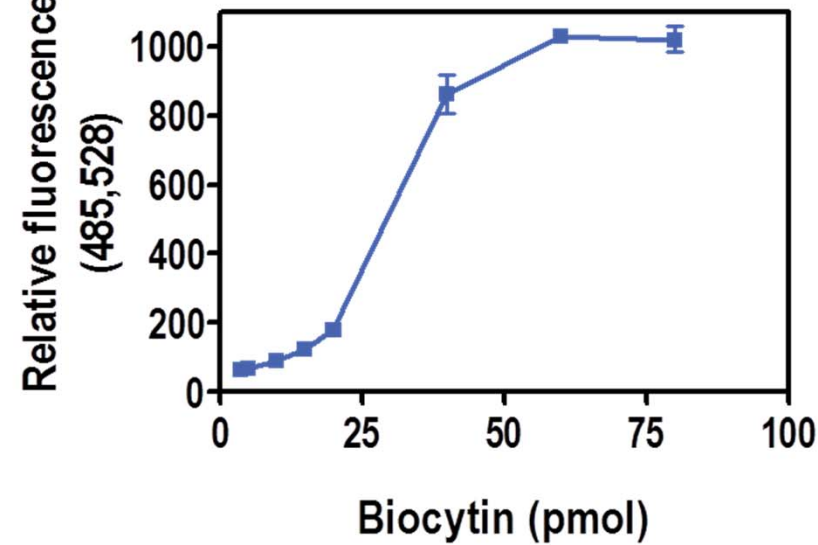
Several approaches to measurement of biotin content are available. One simple and popular method is based on HABA. HABA binds to avidin to produce a colored complex that absorbs at 500 nm. Due to its higher affinity, HABA displaces HABA, which leads to a decrease in absorbance. Another group of methods is based on a change in fluorescence in fluorescently tagged avidin upon biotin binding. This is achieved by displacement of a fluorescence quencher (e.g. HABA). The third approach is based on a specific chemical modification of free or bound biotin resulting in a colored product that can be quantified using a spectrophotometer.

Kits representing the different approaches to biotin measurement were purchased and compared. Specifically, HABA Biotin quantification kit (Panel A) and FluoReporter Biotin Quantification Kit (Panel B) were compared to Quant*Tag Biotin Quantification Kit (Panel C) using vendor recommended protocols. The FluoReporter kit uses biocytin as a standard and the other two kits use biotin. The difference between biocytin and biotin is discussed in Section 4. In our testing, the Quant*Tag Biotin Kit has a favorable dynamic range (0.1 to 5 nmol/well) compared to the HABA assay and the FluoReporter assay. Note that in the plots corresponding to the HABA and Quant*Tag assays, the error bars are smaller than the markers.

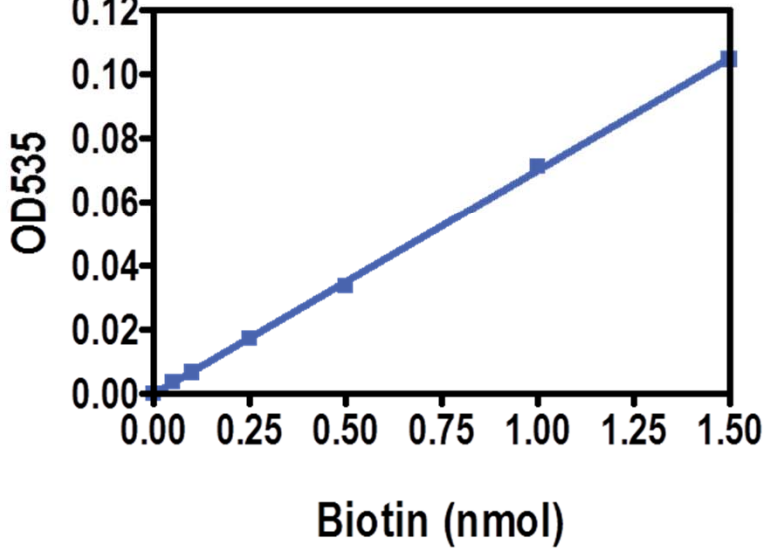
A HABA Biotin Quantitation Kit (Thermo Scientific)



B FluoReporter Biotin Quantitation Kit (LifeTech)



C Quant*Tag Biotin Quantitation Kit (Vector Laboratories)



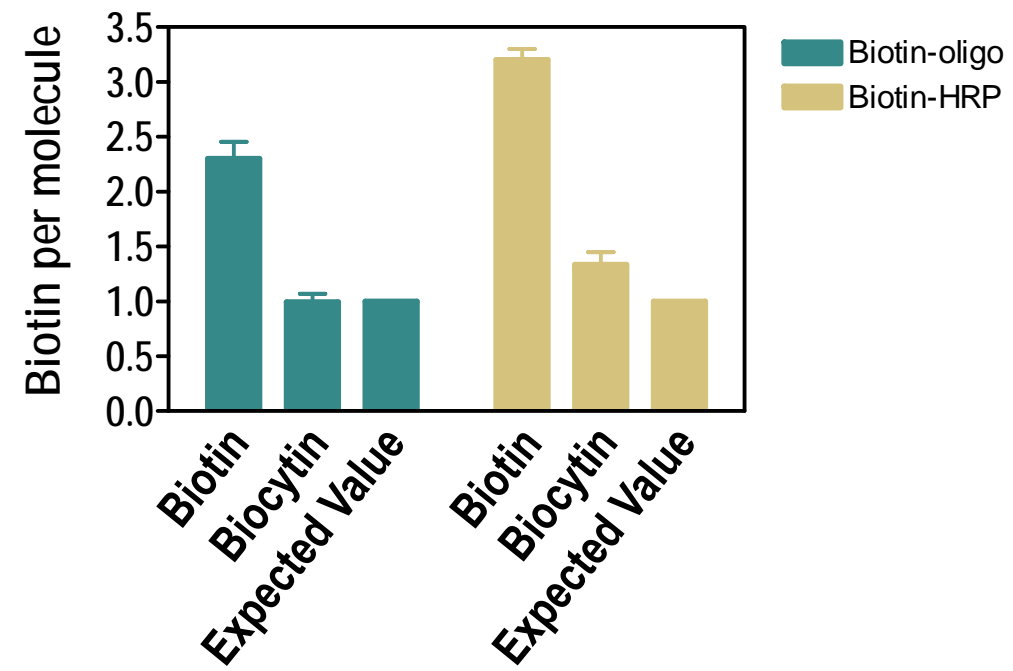
3 Reproducibility of Biotinylation Measurement

For all three assays, reproducibility was calculated based on independent measurements (n) of the biotinylated proteins that are used as controls. %CV for the Quant*Tag assay performed with modifications described in this poster is reported. We introduced a specific mouse monoclonal antibody biotinylated at the challenge ratio of 10 [expected biotin/protein ratio (B/P) between 3 and 7] as the control for the assay.

	Biotinylated Protein	Expected B/P	Average B/P	n	%CV
HABA	HRP	1	0.74	28	29.1
FluoReporter	GAM	4.7-4.8	4.8	17	39.9
QuantTag	mAb	3-7	5.2	12	3.4

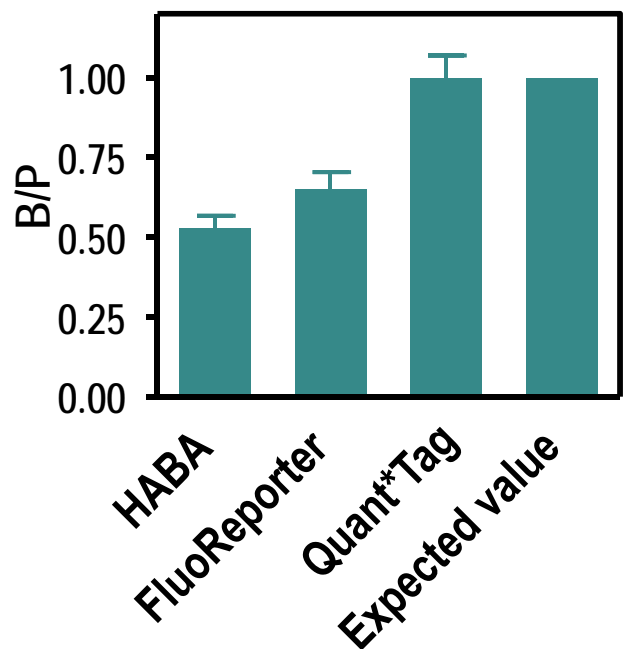
4 Selection of Standard for the Quant*Tag Assay: Biotin vs Biocytin

Biocytin is an amide formed from biotin and the amino acid L-lysine. We investigated whether biocytin is a better standard than biotin for measurement of biotin bound to macromolecules. Model DNA and protein reagents containing 1 biotin per molecule were used as controls. They included 3' biotinylated 12 mer oligonucleotide (IDT DNA) with biotinylation approaching 100% as confirmed by ESI mass spec and >95% purity by electrophoresis, and ImmunoPure® Biotinylated Horseradish Peroxidase (Thermo). We show that when biocytin is used as a standard, the amount of incorporated biotin is closer to the expected value for both protein and DNA.



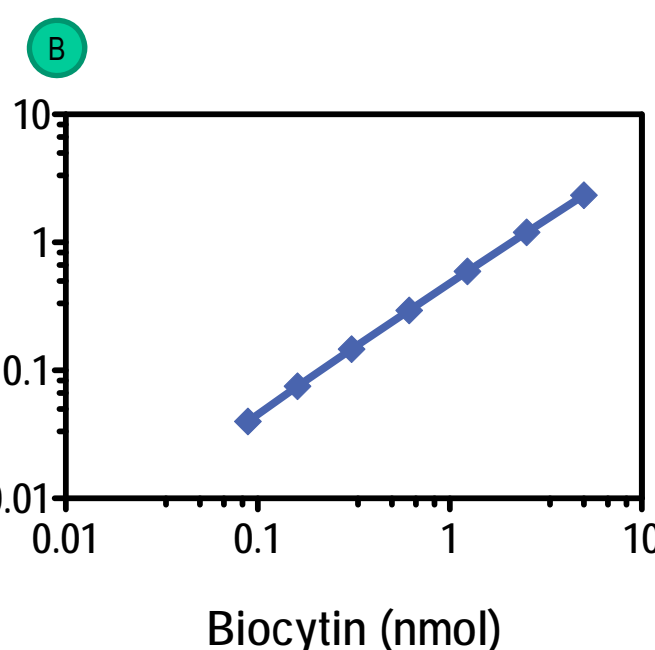
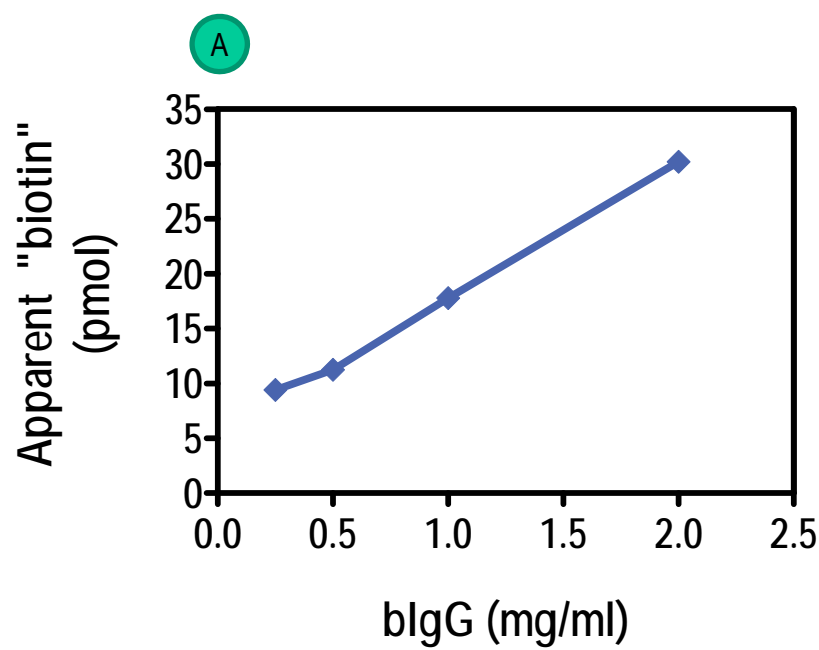
5 Accuracy of Biotinylation Ratio Determination

A 12-mer 3' biotinylated oligo produced by IDT DNA was used as a model system for comparison of the accuracy of of biotin measurements. Oligo biotinylation approaching 100% was confirmed by ESI mass spec. Based on capillary electrophoresis measurements, the oligonucleotide was >95% pure. Biotin levels were measured at concentrations in the linear ranges of the corresponding assays, using vendor recommended protocols. Results obtained by Quant*Tag assay were closest to the expected biotin to oligonucleotide ratio of 1.



6 Modified Quant*Tag Assay

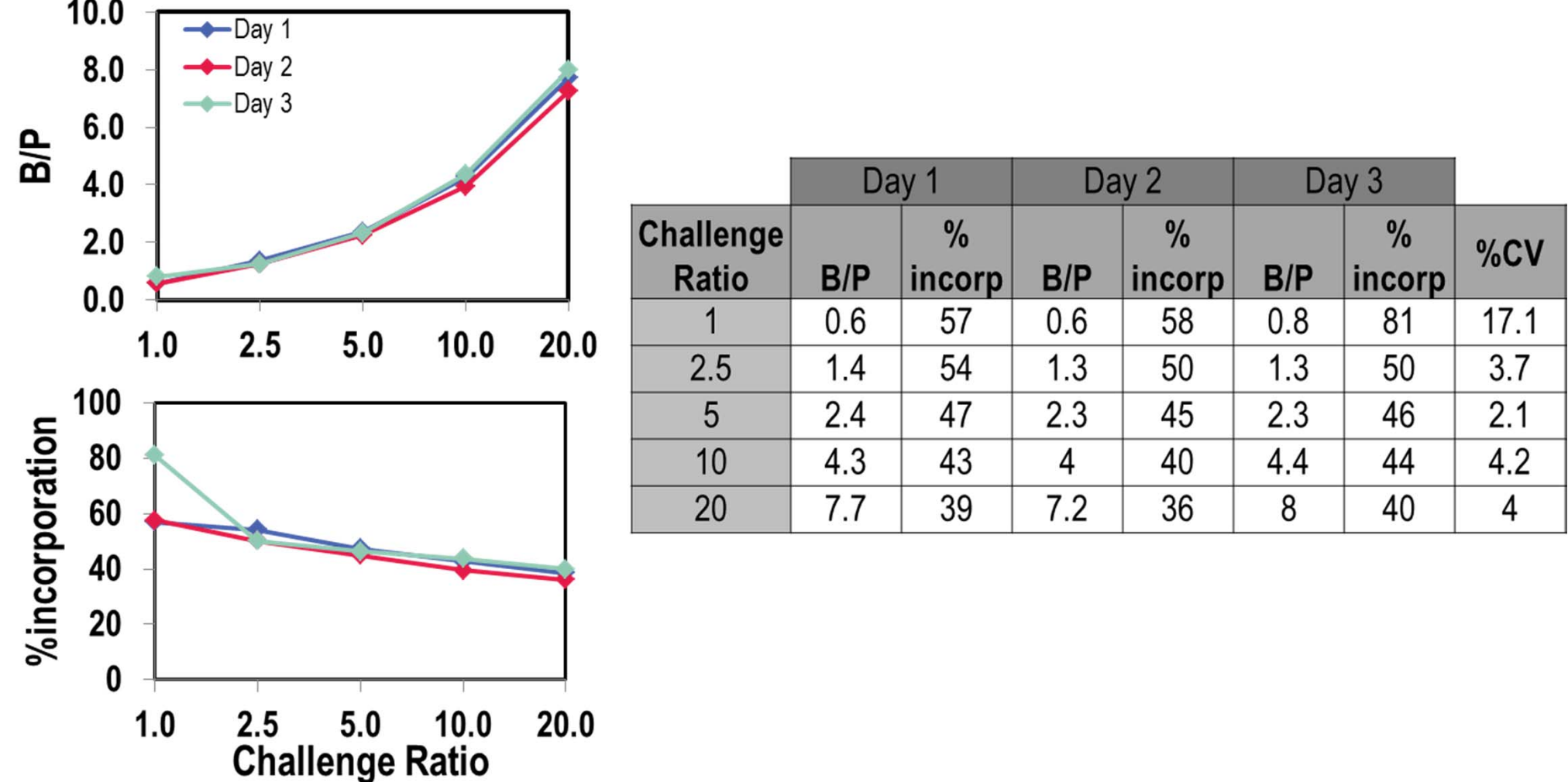
The chemical reagent used in the Quant*Tag kit that reacts with free or bound biotin creating a color change also creates a background signal when incubated with unbiotinylated antibodies (Panel A). This could be due to non-specific binding to proteins or, alternatively, due to low level of intrinsic biotinylation of native antibodies. This effect is independent on the clonality or host of the antibodies. To compensate for this non-specific effect, the standard curve is built in the presence of 0.5 mg/ml bovine IgG (Panel B). Antibody solutions are diluted to 0.5 mg/ml for biotin measurements. The modified Quant*Tag assay can read ≥ 0.1 nmol/well of biotin (0.3 mg/mL of biotinylated antibody at B/P = 1) and has superior sensitivity to the HABA assay in our testing.



7 Reproducibility of Antibody Biotinylation Measurement

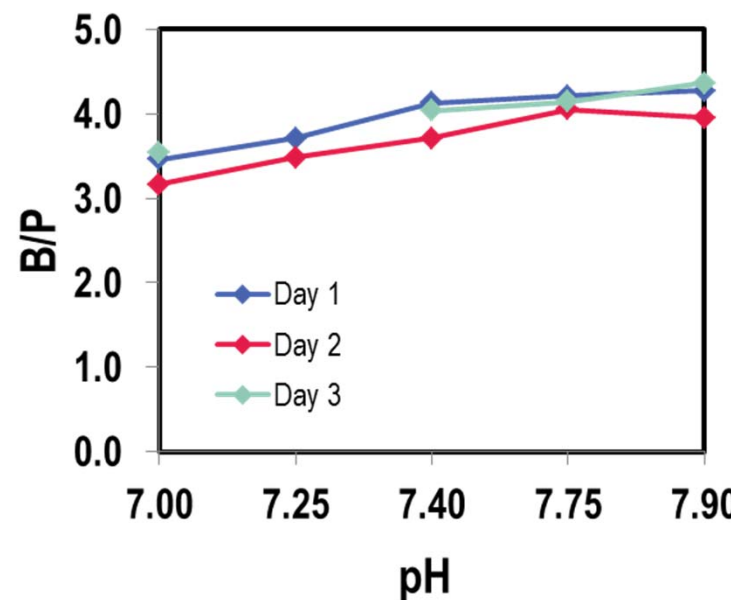
An antibody was conjugated to biotin using Sulfo-NHS-LC-Biotin (Thermo) at 1 mg/ml and various challenge ratios (CR) of biotin to protein. Unincorporated biotin derivatives were removed by buffer exchange using Zeba 40K columns (Thermo). Concentration of bound biotin was measured by the modified Quant*Tag method, and protein concentration was measured by absorbance at UV280. Based on these measurements, % incorporation and average amount of biotin residues per antibody (B/P) were calculated.

Biotinylation measurements were carried out on 3 different days and yielded similar results. At each challenge ratio, %CV did not exceed 4.2%, except for CR 1 (Table).



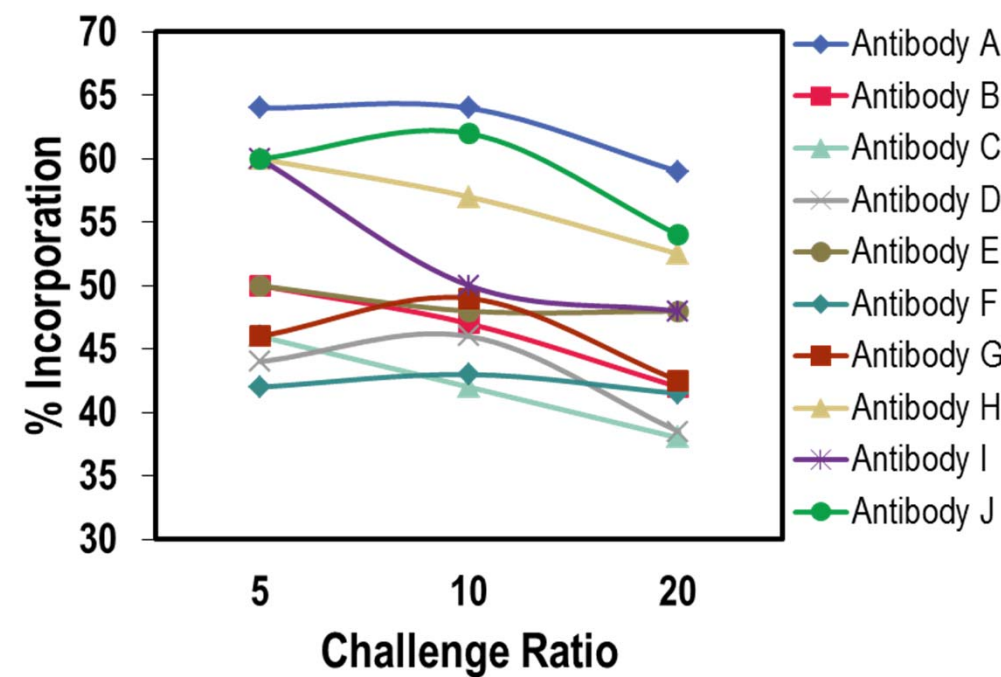
8 Antibody Biotinylation pH Dependence

An antibody was conjugated to biotin using Sulfo-NHS-LC-Biotin at 1 mg/ml, challenge ratio 10 in PBS with pH ranging from 7 to 7.9. Unincorporated biotin derivatives were removed by buffer exchange using Zeba 40K columns. Concentration of bound biotin was measured by the modified Quant*Tag method, and protein concentration was calculated. Biotinylation measurements were carried out on 3 different days and yielded similar results. At each pH point, %CV did not exceed 4.7%. As expected, % incorporation increases as the pH becomes increasingly basic.



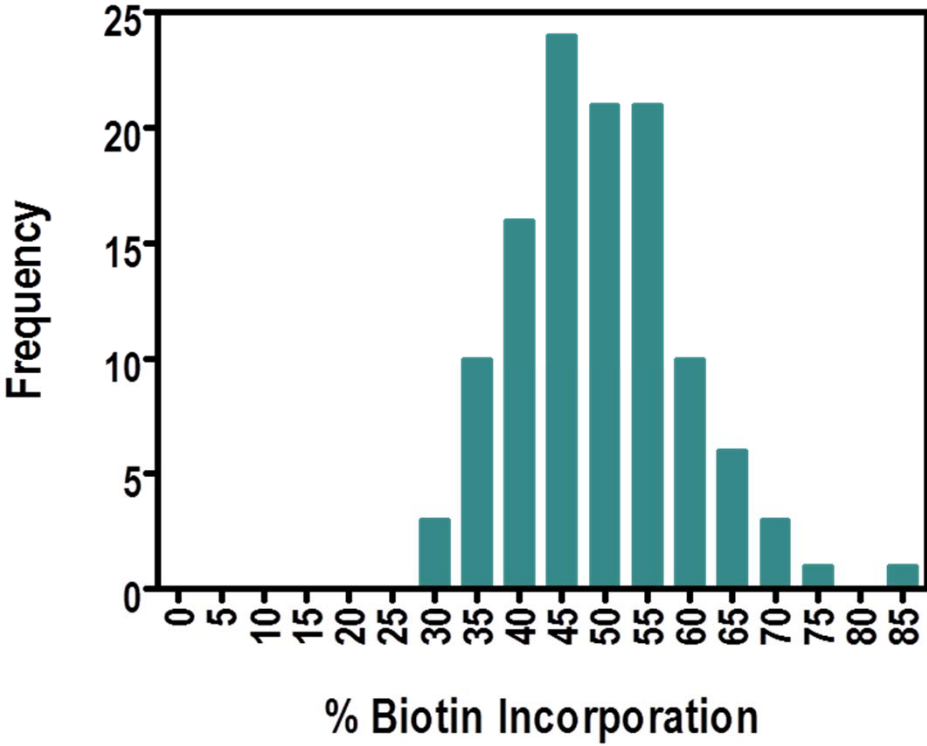
9 Antibody Biotinylation Challenge Ratio Optimization

Challenge ratio optimization is an important step in development of an immunoassay reagent. Antibodies were conjugated to biotin using Sulfo-NHS-LC-Biotin at 2 mg/ml, pH 7.9, and 3 challenge ratios (CR) of biotin to protein: 5, 10, 20. Unincorporated biotin derivatives were removed by buffer exchange using Zeba 40K columns. Concentration of bound biotin was measured by the modified Quant*Tag method, and protein concentration was measured by absorbance at UV280. Based on these measurements, % incorporation and average amount of biotin residues per antibody were calculated.



10 Incorporation of Biotin and Conjugation Consistency for Antibodies

The % incorporation of biotin and consistency of conjugation is dependent on the specific antibody and the challenge ratio (CR). Antibodies were conjugated to biotin using Sulfo-NHS-LC-Biotin at 2 mg/ml and CR 10. The figure shows a histogram of the % incorporation for 140 different antibodies. The median % incorporation (amount of biotin incorporated) is 46%.



11 Protein Biotinylation Measurements

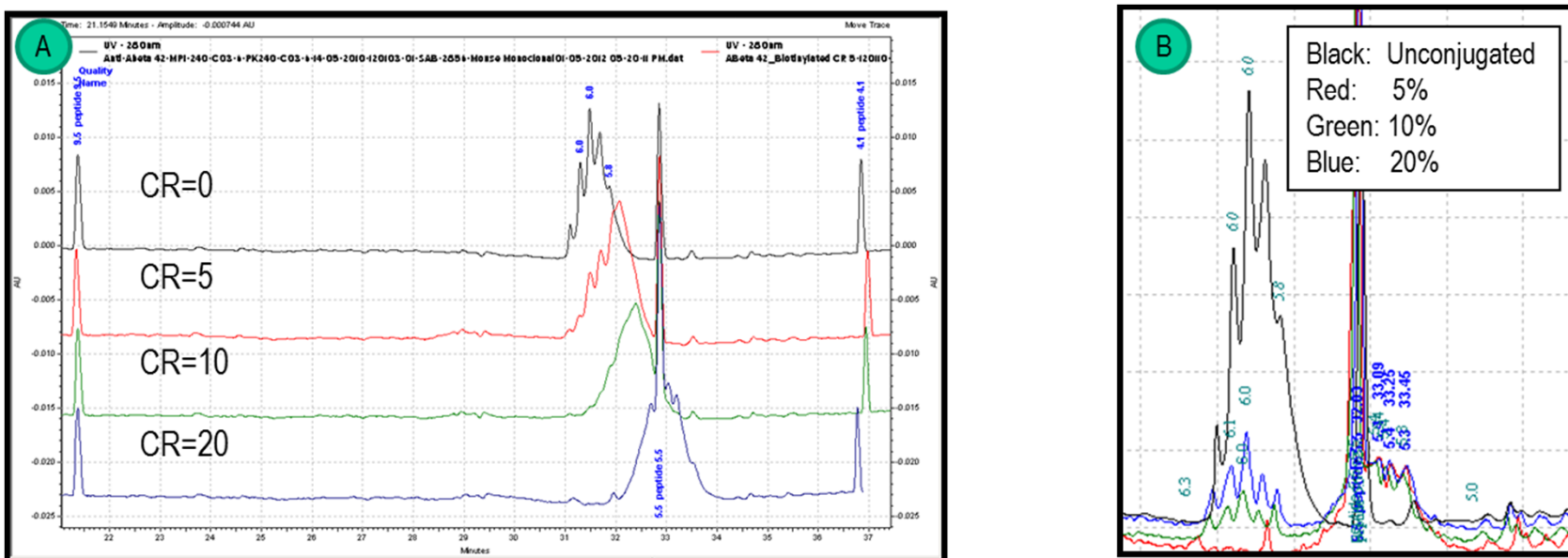
We used our modified Quant*Tag assay to measure the biotinylation ratio in a variety of proteins. We tested whether the proteins interact with the Quant*Tag reagent to produce a color change in the absence of biotinylation (Table A). We demonstrate that this effect depends on the nature of the protein. Our recommendation is to use the unbiotinylated material for background subtraction in the Quant*Tag assay. The proteins were conjugated to biotin using Sulfo-NHS-LC-Biotin at various challenge ratios (CR) of biotin to protein (Table B). Unincorporated biotin derivatives were removed by buffer exchange or dialysis. Concentration of bound biotin was measured by the modified Quant*Tag method, and protein concentration was measured by absorbance at UV280 or BCA. Based on these measurements, average amount of biotin residues per protein (B/P) was calculated. The incorporation of biotin is protein-dependent, which may be due to the amount of surface accessible Lys residues.

Protein	Concentration, mg/ml	Apparent B/P	%CV
mL1b	0.5	0.1	6.3
Streptavidin	0.5	0.7	1.5
Bovine IgG	0.5	0.5	13.7
BSA	0.5	0.1	35.6
Lysozyme	0.5	0.1	3.2

	MW	CR	# Lysines	B/P	%CV
hSTAT5A	80.0	15	42	4.0	4.5
mKc	7.0	6	7	3.0	8.4
mTNFα	17.4	15	9	3.6	5.5
mIFNγ	15.6	15	10	1.2	3.3
mL1β	17.5	15	15	3.0	0.9

12 Detection of Residual Unconjugated Antibody

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. cIEF graphs of the conjugated antibodies (Panel A) show 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 (Panel B). From this we estimated that the CR5 material had 10-20% unconjugated antibody.



13 Conclusion

Free biotin or unconjugated antibodies can create lot-to-lot variability in bioanalytical assays (e.g. assays for immunogenicity or pharmacokinetics). We have found that typical methods for measuring biotin incorporation are inaccurate and have low precision and limited dynamic range. In this work, we describe a highly reproducible method for measuring biotin incorporation and several other related techniques. This set of analytical methods can significantly improve the characterization of biotin-protein conjugates. The reproducibility and sensitivity of these methods are sufficient to monitor and optimize biotinylation reactions in-process and for quality control testing of final biotinylated products. More broadly, these methods provide a route to better bioanalytical assays by improving the quality and reproducibility of the underlying reagents.

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