Assessing Effects of Freeze-Thaw on Biotinylated Protein Molecules Using Gyrolab™

Xiaodong F. Liu[1], Roni Weaver[1], Xun Wang[1], Charles Hottenstein[2], Matthew Szapacs[2], Lee Abberly[2], William Lorelli[2], LingSing Chen[1]

[1] QPS, One Innovation Way, Suite 200, Newark, DE 19711 [2] GlaxoSmithKline plc., 709 Swedeland Rd, King of Prussia, PA 19406

QPS helps you navigate

INTRODUCTION

Recent advances in medicine reveal that the immune system plays a role in reduction of insulin producing cells and that Type 1 diabetes is an autoimmune disease. Thus, immunosupression may be a promising disease-modifying approach to correct insufficient production of insulin. Otelixizumab is a chimeric humanized monoclonal antibody which is an investigational immunomodulatory drug targeting CD3 and reducing T cell activation and cytokine release and is being developed for the treatment of Type 1 diabetes and other autoimmune diseases (Chatenoud, L & Bluestone, JA. Nat Rev Immunol. 7(8):622, 2007).

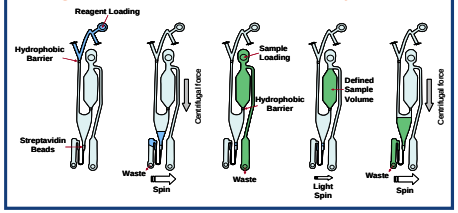
The technology used in this study, Gyrolab™, represents a recent breakthrough for large molecule bioanalysis to support biologic drug development. The advantages of this innovative platform include fully automated nanoscale immunoassay capability, better assay reproducibility and data quality, small reagent and sample volumes, no cross-talk and hook effect, and rapid assay development and validation as a result of reduced run time. Gyrolab has been increasingly used in pharmaceutical industry for immuno-bioanalysis. A fully validated Gyrolab assay for large molecule PK/TK bioanalysis has been reported recently (Liu, XF et. al. J Pharm Tox Method 65(3), 2012).

At present, the Gyrolab immunoassay system requires the capture reagent to be biotinylated as the solid phase comprises a streptavidin-coated-bead column. The performance of a Gyrolab assay relies on properties of the biotinylated molecule. Biotinylation adds biotin/spacer moiety onto the molecule and may affect the functional activity of the labeled molecule. In this study, we report that freeze-thaw of a biotinylated reagent and the type of biotin spacers affected the overall response and assay sensitivity. With proper treatment and careful selection of biotinylated reagents, variability in assay performance could be reduced. In addition, Gyrolab was proved to be a useful tool in evaluating properties of biotinylated molecules.

EXPERIMENTAL

Fig.1. A Fully Validated Gyrolab Workstation at QPS

Fig. 2. Functional Microstructures on a Gyrolab CD



The Gyrolab workstation (Fig. 1) used in this study, including the interface with Watson laboratory information management system (LIMS), was fully validated through the process of installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ) to meet the requirement for compliance with 21 CFR Part 11.

Gyrolab Bioaffy™ CD contains streptavidin-bead packed micro-structures. Reagents and samples were delivered separately to the microstructures through a volume-defined nanofluidic system (Fig 2). Otelixizumab was captured on the microstructure by a biotinylated monoclonal antibody and then detected by an Alexa-labeled monoclonal antibody (Fig. 3).

Fig. 3. Assay Design

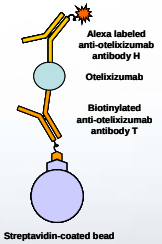


Fig. 4. Test Capture and Detection Reagents After Freeze-Thaw

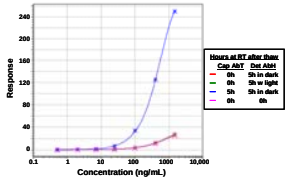


Fig. 5. Test Biotin-PEG4-Antibody T After Freeze-Thaw

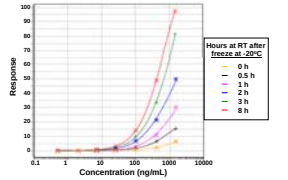


Fig. 6. Storage of Biotin-PEG4-Antibody T

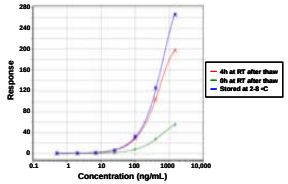
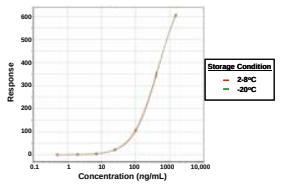


Fig. 7. Test Biotin-LC-Antibody T With or Without Freeze-Thaw



The capture antibody (AbT)(GSK) was biotinylated using EZ-Link® NHS-PEO Solid Phase Biotinyl-ation kit (Thermo Scientific) following manufacturer's instructions. The antibody was first buffer-exchanged with phosphate-buffered saline (PBS) on a Nanosep® 30K OMEGA™ Centrifugal Device (Pall Life Sciences) before biotinylation. For the biotin spacer comparison, the AbT was also biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific) at a challenge molar ratio of 12:1 (biotin:protein). The biotin/protein ratio of the produced conjugate was determined by the 4'-hydroxyazo-benzene-2-carboxylic acid (HABA) assay using EZ™ Biotin Quantification Kit (Thermo Scientific). The biotin/protein ratios of Biotin-PEG4-AbT and Biotin-LC-AbT were 4.4 and 5.4, respectively. Protein concentration was determined by absorbance at 280 nm using 1.35 as the molar extinction coefficient (ε) in cm-1(mg/mL)-1 of a typical IgG (Harlow, E & Lane, D. Antibodies, pp658, 1988). The detection antibody (AbH)(GSK) was labeled with Alexa Fluor®, 100 µg antibody per batch, using Alexa Fluor 647 Labeling Kit (Life Tech) following manufacturer's instructions. The reagent was buffer-exchanged with 0.1 M bicarbonate solution prior to labeling. The Alexa-labeled AbH with an Alexa/protein ratio of 7.7 was used for the study.

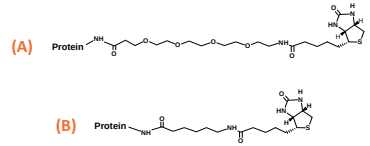
The assay conditions were evaluated and optimized on a Bioaffy 1000 CD, which provided 2-4 fold more sensitivity than a Bioaffy 200 CD for the assay. The combination of 150 µg/mL of the capture and 25 nM of the detection reagents provided the highest signal-to-background ratio. The capture reagent was diluted in PBST. The detection reagent was centrifuged at 16000xg for 2 min and diluted in Rexxip F™ buffer (Gyros). Otelixizumab (12 mg/mL, GSK) was aliquoted and stored at –70°C. Calibration standards were prepared at the concentrations of 2.5, 10, 35, 125, 500, 2000, 7500 ng/mL by spiking otelixizumab in pooled human serum (Bioreclamation) with at least 95% matrix in the final volume and were stored at -70°C. All samples in neat human sera were diluted 5 fold in Rexxip H™ buffer (Gyros) before loaded on CD. Thus, the on-CD concentrations for the standards were 0.5, 2, 7, 25, 100, 400, 1500 ng/mL, respectively (Fig. 4-7). All frozen standards (stored at -70°C) and reagents (stored at -20°C) were thawed unassisted at room temperature. Data acquisition at 1% photomultiplier-tube (PMT) level was found appropriate for the assay conditions described above. Regression was performed by Gyrolab Evaluator (v 3.1.5.137, Gyros AB, Sweden) with 5-parameter logistic fit without including the blank. Weighting was applied for response (1/Y).

RESULTS

A Gyrolab assay for otelixizumab in human serum was developed with a quantification range of 2.5 – 2000 ng/mL with MRD of 5. During the method development, significant variability in total response and sensitivity was observed. We noticed that the variability was related to the reagents' time at room temperature or reagents' time under light. Frozen standards, capture and detection reagents were all tested for room temperature stability. The detection reagent was tested for light sensitivity as well. The results indicate that standards and the detection reagent were all stable at room temperature under light but the activity of biotin-PEG4-AbT capture reagent increased significantly (Fig. 4). The increase of the activity was time-dependent (Fig. 5). The overall response increased 10 fold after 8-hour incubation and the signal-to-background increased 8 fold. The thawed capture reagent was compared to the capture reagent that had never been frozen. The result showed that the activity of the thawed reagent improved greatly after 4 hour incubation but was still lower than the activity of the reagent stored at 2-8°C (Fig. 6) suggesting that the biotin-PEG4-AbT reagent lost activity rather than gained additional activity upon freeze-thaw and the lost activity could be recovered gradually at room temperature. When the same antibody was biotinylated with hydrocarbon spacers, the conjugate biotin-LC-AbT was not affected by freeze-thaw (Fig. 7) indicating that although hydrophilic polyethylene glycol (PEG) spacer enhances water solubility of the biotinylated reagent its activity was more likely to be affected by freeze-thaw compared to antibodies labeled with reagents having only hydrocarbon spacers (for spacer structures, see Fig. 8).

Since plate-based assays require prolonged and stepwise incubations before the endpoint can be reached, time-critical assay parameters can not be easily evaluated in these assays. In contrast, Gyrolab assays can be particularly useful for assessing the time-dependent properties of biotinylated reagent because data acquisition can occur minutes after reagents and samples are loaded onto the machine.

Fig. 8. Biotin-PEG4-AbT (A) and Biotin-LC-AbT (B)



CONCLUSIONS

- The biotin spacer could affect performance of an immunoassay and can be quickly evaluated by a Gyrolab assay.
- PEG4 spacers were more likely to be affected by freeze-thaw than hydrocarbon spacers.
- Extended incubation periods might be necessary for biotinylated reagents after freeze-thaw to avoid potential variability in the assay.
- For short-term storage of PEG4-reagents, 2-8°C is preferable.
- Since the Gyrolab assay does not require incubation, it provides an effective tool for assessing critical reagents in assay development and optimization, especially for evaluating time-dependent parameters.

Correspondence: frank.liu@qps.com Tel: +1 302 455 9945