

Using Fluorescent Labels to Increase the Sensitivity of IgG Purity and Heterogeneity Assay on PA 800 plus

Marcia R Santos
SCIEX Separations, USA

When tasked with the assessment of critical quality attributes of protein biologic products, many analytical characterization approaches are currently available. Separation based techniques such as size exclusion chromatography, analytical ultracentrifugation, capillary isoelectric focusing, ion-exchange chromatography and Capillary electrophoresis sodium dodecyl sulfate (CE-SDS) are common.

CE-SDS has been successfully employed for the quantification of clipped products, host cell proteins, and aggregation products, glycosylation site occupancy analysis, monomer purity analysis, to name a few. Typically, CE-SDS is performed using UV absorbance detection at 214 or 220 nm.

However, the LOD of this assay in most commercial instruments is only 2.5 µg/mL and many impurities may fall below this range and impose a risk on product safety and efficacy. Therefore, there is an unmet need to improve the sensitivity of CE-SDS based assays.

This technical note demonstrates the advantages of CE-SDS-LIF (Laser Induced Fluorescence) using the NIST antibody reference material, labeled with two well-known amine reactive dyes; 3-2-(furoyl quinoline-2-carboxaldehyde (FQ) and 5-Carboxytetramethylrhodamine-succinimidyl ester (5-TAMRA.SE).

Workflow parameters pertaining to sample preparation and cleanup, ease of labeling, reagent toxicity, similarity with UV (separation profile) and sensitivity are discussed.



Key Advantages of Method

- Increased sensitivity for quantitation of species sub µg/mL
- 500 µg of sample is required
- Same Separation Gel used for UV detection of IgG Purity and Heterogeneity Assay
- Amine reactive fluorescent labels are used to increase sensitivity of CE-SDS based assays
- An increase in 3 orders of magnitude sensitivity is demonstrated (0.15 mg/ml of Intact antibody to 1000-fold dilution of spiked α-lactalbumin).

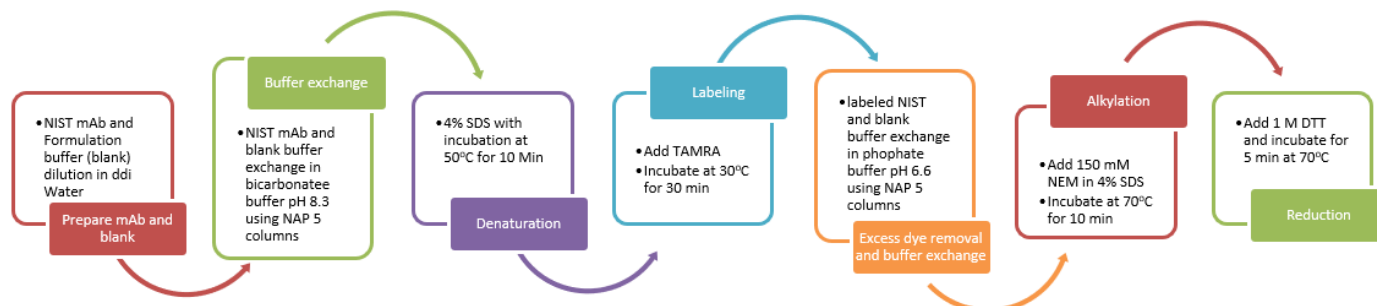


Figure 1. TAMRA Sample Preparation Scheme.

Methods

Sample Preparation: NIST mAb was purchased from NIST (Rockville, MD). 50 μ L aliquots were made and stored at -80 °C to avoid multiple freeze-thaw cycles.

Materials for TAMRA Labeling Protocol: Sodium bicarbonate, Sodium phosphate dibasic, Sodium Phosphate Monobasic, Sodium Dodecyl Sulfate, Dithiothreitol, Dimethyl Sulfoxide were purchased from Sigma Aldrich (St Louis, MO) and used without purification. Illustra NAP 5 columns from GE Healthcare (Boston, MA) were used to buffer exchange sample and to remove excess unbound dye. 5-TAMRA, SE (5-Carboxytetramethylrhodamine, Succinimidyl Ester), single isomer, was obtained from Life Technologies (Carlsbad, CA).

Materials for FQ Labeling Protocol: Sodium Phosphate monobasic, Sodium Phosphate dibasic, Dithiothreitol (DTT), N-Ethylmaleimide (NEM), sodium dodecyl sulfate (SDS), were purchased from Sigma Aldrich (St Louis, MO) and used without purification. Illustra NAP 5 from GE Healthcare - PN 17085301 (Boston, MA). ATTO-TAG™ FQ Amine-Derivatization Kit from Life Technologies (Carlsbad, CA) was the dye reagent kit. The 20 mM FQ dye stock solution was prepared in DMSO. This solution was further diluted in ddi water to a final concentration of 2.5 mM and was used as the FQ dye working solution. The nucleophile reagent used was potassium cyanide at a concentration of 30 mM.

Instrument and Software: The capillary electrophoresis instrument used was a PA 800 plus equipped with LIF detection with solid state laser with excitation wavelength at 488 nm and the emission filters were 560 nm for TAMRA (SCIEX P/N 149068) and 600 nm/80 nm band pass (Edmund Optics P/N 65736). EZCartridge was used for the separation of NIST antibody in both assays (Figure 3, SCIEX P/N A55625). Separations were performed at 500 V/cm and injection was electrokinetic at 20s/-5kV. The separation gel used was from the IgG Heterogeneity and Purity Assay (SCIEX P/N A10663). Data



Figure 3. The EZ-CE Cartridge. EZCartridge was used for the separation of NIST antibody in both assays.

acquisition and analysis was performed using 32Karat software V10.2.

Principles of the Labeling Reaction

Both FQ¹ and TAMRA² are amine-reactive fluorescent tags. The conjugation of these fluorescent tags with the antibody molecule occurs under either the near neutral pH of a phosphate buffer for FQ or at high pH of bicarbonate buffer for TAMRA.

Both labeling schemes (Figure 1 and 2) require buffer exchange of the antibody sample especially if the formulation buffer is comprised of components containing amino groups (i.e. Tris, histidine), as these are prone to labeling as well.

The reaction with FQ requires a strong nucleophile reagent - KCN - which due to the high toxicity, requires extra care to keep lab personnel safe. The FQ labeling reaction does not have an end point, thus quenching is required and can be easily performed by adding 1% SDS solution to the reaction mix. No further purification step is needed afterwards.

Meanwhile, TAMRA dye is a highly fluorescent molecule when both unbound and bound, for this reason it is necessary to treat the labeled sample with an efficient sample cleanup process which is accomplished by using NAP 5 columns.

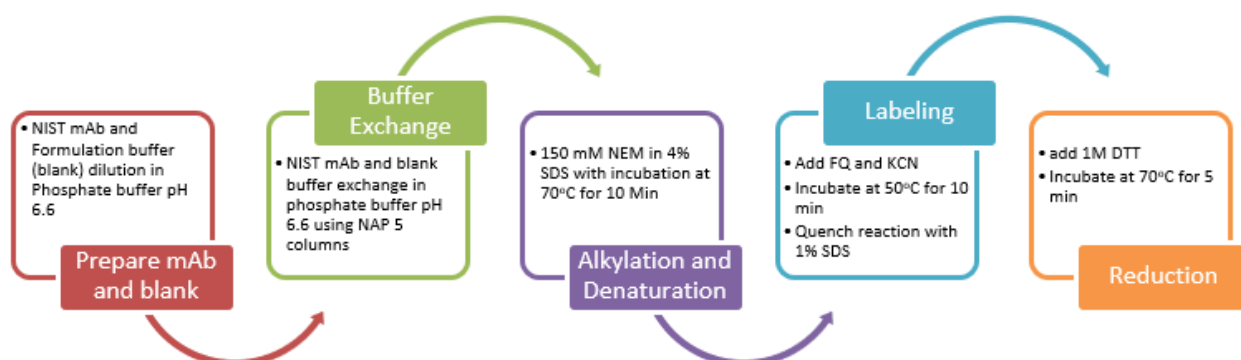


Figure 2. FQ Sample Preparation Scheme.

Either by quenching the reaction or by buffer exchange, sample clean-up to remove free dye is not 100% efficient. Therefore, it is extremely important to always treat the antibody's formulation buffer as blank and submit it through the same dilution and labeling process as the antibody to avoid taking the free dye peaks into account during data analysis of the antibody peaks.

Sensitivity of the Assay

To investigate the sensitivity of the assay, α -lactalbumin was chosen because it is small, and migrates as a sharp peak well before the light chain (LC), making it also a good candidate as a mobility marker replacing 10 kDa used in absorbance-based assays. Figure 4 shows α -lactalbumin spiked into the NIST sample labeled with FQ at 10, 100 and 1000-fold less concentrated relative to the antibody (0.15 mg/mL). The S/N for α -lactalbumin at 1000-fold dilution was 35.9 well above limit of quantitation, demonstrating that a combination of a sensitive assay and a considerably lower noise at the baseline level results in lower limits of detection and quantitation.

Comparison of LIF based Assay to the Traditional UV based Assay

In Figure 5, the top, middle and bottom panels show the typical profile of a non-reduced NIST antibody unlabeled, labeled with TAMRA and FQ respectively, In Figure 6, the top, middle and bottom panels show the typical profile of a reduced NIST antibody unlabeled, labeled with TAMRA and FQ respectively, To the right of each figure shows the zoomed view of the low abundant peaks.

The data under reduced conditions reveals that both labeling schemes and unlabeled antibody show a resolution between non-glycosylated Heavy Chain (ng-HC) and Heavy Chain (HC) is 1.4 or better and the ratio HC:LC is 2.1, consistent to what has been reported for NIST mAb using absorbance detection^{4,5}.

The data under non-reducing conditions also shows a lot of similarities between UV and LIF based detections, however it is clear that with LIF detection the clipped products and LC, HC, HL, HH chain dimer and HHL chain products^{4,5} is clearly more evident thus allowing for improved integration and quantitation.

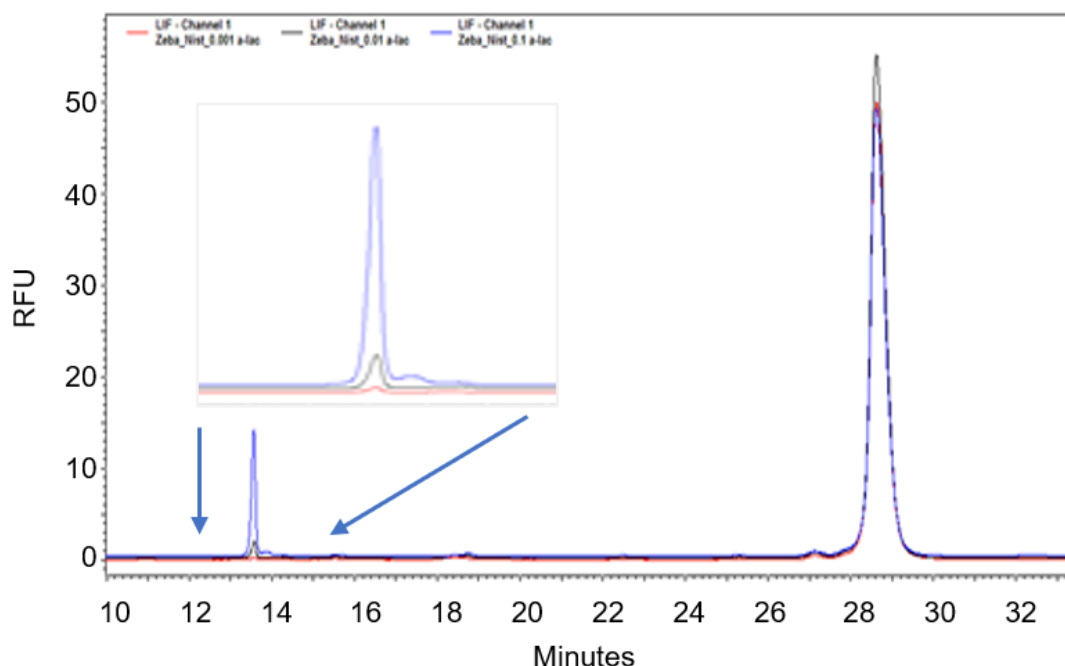


Figure 4. Sensitivity of Assay. Overlay of e-grams of NIST antibody labeled with FQ under non-reducing conditions spiked with α -lactalbumin at 10, 100- and 1000-fold dilution relative to NIST.

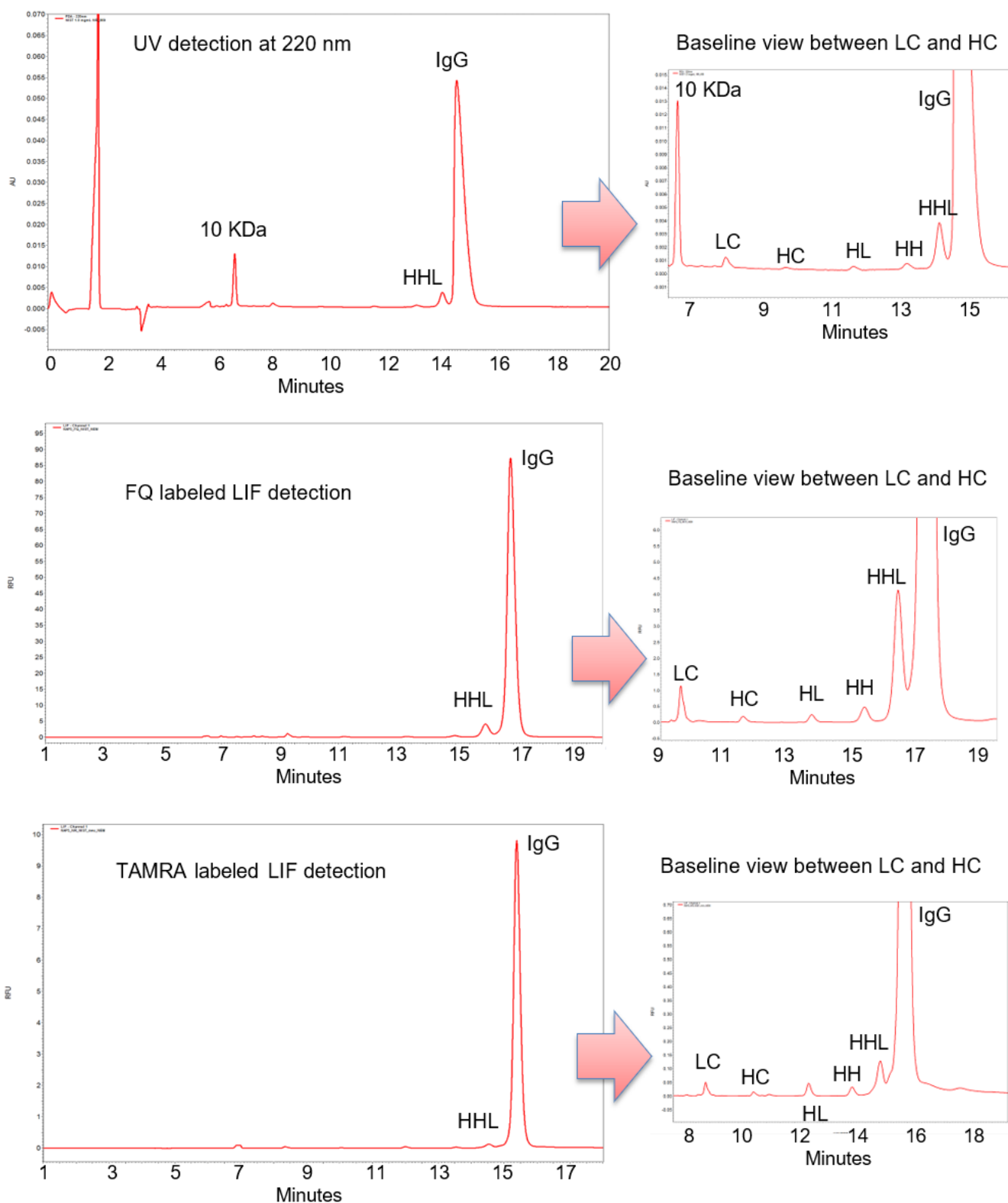


Figure 5. Comparison of LIF Based Assays to UV Assays for the Non-Reduced NIST mAb. The separation of an unlabeled NIST antibody using UV detection (top) was compared to the separation of the same antibody labeled with FQ (middle) and TAMRA (bottom). The right figure for each represents the zoomed view of the y-axis to highlight separation of the low abundant species.

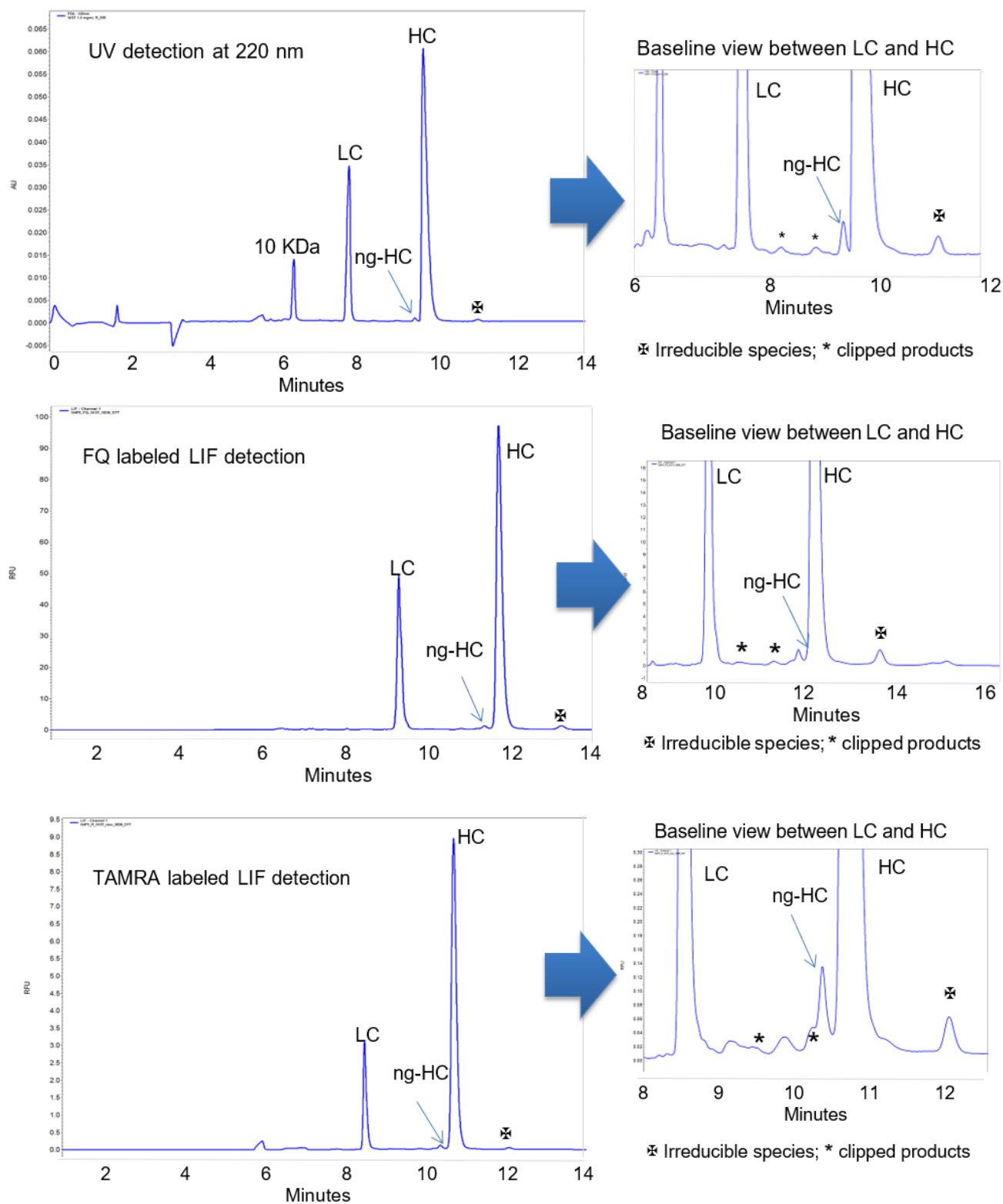


Figure 6. Comparison of LIF Based Assays to UV Assays for the Reduced NIST mAb. The separation of an unlabeled NIST antibody using UV detection (top) was compared to the separation of the same antibody labeled with FQ (middle) and TAMRA (bottom). The right figure for each represents the zoomed view of the y-axis to highlight separation of the low abundant species.

Conclusions:

Benefits of these labelling technique for enhancing the sensitivity of IgG Purity and Heterogeneity Assay:

- **Same IgG separation gel:** no changes to the separation gel currently used for PDA based separations.
- **A little goes a long way:** Typical concentration to work with is around 0.15 mg/mL which is below LOD of a typical UV based assay.
- **Assay offers exquisite sensitivity:** 3 orders of magnitude detection range (0.15 mg/ml of Intact antibody to 1000-fold dilution of spiked α -lactalbumin).
- **Stable baseline:** Because the mode of detection is fluorescence, the baseline is well behaved allowing for an improved and reproducible integration.
- **System peaks are no longer visible:** Because system peaks are not labeled.
- **Stability of Antibody-dye conjugate.** Both dyes provide a relatively stable conjugate. TAMRA seems to be more stable than FQ after storage of a few days at -20 °C (data not shown), both reaction, dyes and conjugation product need to be kept away from light.
- **Similarity with UV:** This work demonstrated good similarity between the two labeling strategies and UV (Figure 3) but better sensitivity than UV.

References

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Document number: RUO-MKT-02-8609-A