

Assay of IgG Purity and Heterogeneity using High-Resolution Sodium Dodecyl Sulfate Capillary Gel Electrophoresis

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Overview

In this application note, we present the development of both a new polymer formulation and standardized methodology to assess the purity and heterogeneity of IgG and its isoforms. This methodology provides increased resolution of the IgG isoforms from typical developmental impurities like non-glycosylated heavy chain and low-molecular-weight impurities associated with the IgG light chain. The assay includes an artificially engineered IgG control with a fixed percentage of non-glycosylated heavy chain to provide assay suitability determination prior to the analysis of unknowns. All aspects of the methodology from preparing samples to automating data analysis will be discussed.

Introduction

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a commonly used analytical technique for the molecular weight estimation and purity assessment of proteins.¹ However, SDS-PAGE suffers from several limitations, including the use of neuro-toxic reagents, the manual labor-intensive methodology, and the lack of direct, accurate quantification. Alternatively, capillary gel electrophoresis (CGE) in the presence of sodium dodecyl sulfate (SDS) has been applied for automated quantitative protein analysis.²⁻⁸ Compared to SDS-PAGE, CGE offers automation, high-speed separation, enhanced resolution, and direct, on-line quantification using UV and fluorescence detection. In SDS-CGE, the SDS is bound to all the proteins at a constant weight ratio such that the SDS-protein complexes have approximately equivalent charge densities. The constant mass-to-charge property of the SDS-bound proteins allows separation according to differences in molecular weight. As the logarithm of the molecular mass of a protein is linear with its electrophoretic mobility, the molecular weight of a protein can be estimated from a series of well-chosen standards.

Replaceable gels, comprising linear polymers in solution, are commonly used as the separation matrix in SDS-CGE. The low viscosity of the linear polymer solutions allows the replacement of the sieving matrix after each analysis, improving the assay precision and robustness. Many different polymer matrices

have been applied to the separation of SDS-proteins in CGE. Demorest⁹ described the use of linear polyacrylamide polymer solutions for the separation of SDS proteins and DNA. Karger³ further described the use of a gel matrix of UV transparent polymers, such as Dextran and PEO. Since this time, several commercial products have been introduced using this approach.

We have developed a new, replaceable polymer matrix to improve the current resolving power of SDS-CGE and have developed this matrix into a commercial IgG purity/heterogeneity assay. The methodology involves heat denaturing a specified concentration of IgG (both reduced and nonreduced) in the presence of SDS. This application bulletin reviews the fundamental components of this assay development.

Materials and Methods

Instrumentation

All experiments were performed on the PA 800 Plus Pharmaceutical Analysis System (SCIEX). Bare, fused-silica capillaries of 50 μ m ID \times 20 cm to detection were used for the separation.

Reagents

SDS-gel buffer, SDS sample buffer, SDS protein size standards, 10 kD protein internal standard, and the IgG suitability standard were all manufactured at SCIEX (Fullerton, CA). Sigma (St. Louis, MO) was the source for 2-mercaptoethanol.

The SDS gel buffer creates a physical gel of an entangled polymer network for separation of the SDS-protein complexes. The gel buffer comprises a proprietary polymer buffer formulation (at pH 8.0) with 0.2% SDS.

The SDS sample buffer is used to prepare the SDS-protein complex for the IgG assay. The sample buffer is composed of 100 mM Tris-HCl, pH 9.0, with 1% SDS.

The acidic wash solution is a high-purity reagent comprised of 0.1 N HCl. The basic wash solution is a high-purity reagent composed of 0.1 N NaOH.

Preparation of SDS-Protein Complex

When preparing the SDS-protein complex, we diluted the protein sample solution (2–40 mg/mL concentration) with SDS sample buffer to give a final concentration of 1 mg/mL. The IgG-SDS complex was reduced by adding 5% neat 2-mercaptoethanol v/v, and then heating in a 70° C water bath for 10 minutes. For a non-reduced sample, the IgG-SDS complex was first alkylated with iodoacetamide by adding 5% of a 250 mM iodoacetamide v/v solution into the protein SDS buffer mixture and then heated in a 70° C water bath for 10 minutes.

Separation and Analysis

An optimized separation method and sequence were set up for batch analysis of 24 samples at a time. For each separation cycle, the capillary was first preconditioned with 0.1 N NaOH , 0.1 N HCl, deionized water, and SDS gel buffer. All gel buffers were degassed for 2 minutes under vacuum prior to use. Samples were electrokinetically introduced by applying voltage at -5 kV for 20 seconds. Electrophoresis was performed at constant voltage with applied field strength of (-) 497 volts/cm with a capillary thermostatted to 25° C using recirculating liquid coolant. The current generated was approximately 27 μ Amps. The system was programmed to automatically replenish all reagents through an increment of the buffer array after every eight cycles.

Results and Discussion

Resolving Power

To effectively resolve non-glycosylated from glycosylated IgG heavy chain, it was first necessary to formulate a gel of exceptionally high resolving capacity. A protein sizing ladder containing recombinant proteins of 10, 20, 35, 50, 100, 150, and 225 kDa was developed to assist both in the gel formulation phase to optimize the resolving capacity of the gel and to estimate the MW of unknowns. A recombinant 10 kD protein was used as an internal reference standard for mobility determination to accurately assign protein identification. Figure 1 illustrates a typical electropherogram of the analysis of the protein-sizing ladder using this method. The inset in Figure 1 illustrates a plot of the Log MW versus 1/Mobility and demonstrates good linearity of response over the standards sizing range. Figure 2 illustrates a different formulation of the protein sizing ladder in which we

changed the concentration of the individual components and further spiked the mixture with an 11 kDa protein. The baseline separation of the 10 and 11 kDa proteins highlights the good resolving power in this size region. Differences in detection response are due to the concentration of the individual proteins used in the two size standard formulations.

IgG Suitability Standard

Our criteria for the development of the IgG purity/ heterogeneity assay include both resolution specifications for reduced and non-reduced IgG and quantitation specifications for detecting impurities. To ensure good control in both the manufacture and implementation of this assay, we have developed an IgG suitability standard that will test both the resolution

and quantitation requirements. As the percent of non-glycosylated heavy chain is an important assessment parameter, yet is variable in quantity and difficult to resolve, it became a natural component to focus upon in the creation of a suitability standard. The suitability standard has been designed to contain a controlled quantity of the non-glycosylated heavy chain (9.5% of total heavy chain), which allows us to test both the resolving capacity of the gel and to ensure the quantitation variability is within an acceptable range before beginning the assay of unknowns. Figure 3 illustrates the analysis of the IgG suitability standard in both the reduced and non-reduced forms. Under reducing conditions, a very good separation between light and heavy chain is obtained, while the glycosylated heavy chain is baseline resolved from non-glycosylated (NG) heavy chain.

Assay Precision

Table 1 summarizes the results of six consecutive analyses of the reduced IgG standard. The relative standard deviation (% RSD) of both the light chain and heavy chain mobility was < 1%, while the quantitative determination of the % NG heavy chain was also < 1%. Assay precision was also evaluated with the non-reduced IgG (Table 2). All the impurities, such as light chain (L), heavy chain (H), heavyheavy chain (HH), and 2 heavy 1 light chain (HHL), are well resolved from the intact antibody. The nonglycosylated heavy chain was also baseline separated from the IgG monomer (Figure 3). From six replicate analyses, the RSD value of IgG mobility is < 1% (Table 2). The % RSD of the quantity determination of the minor impurities is < 3%.

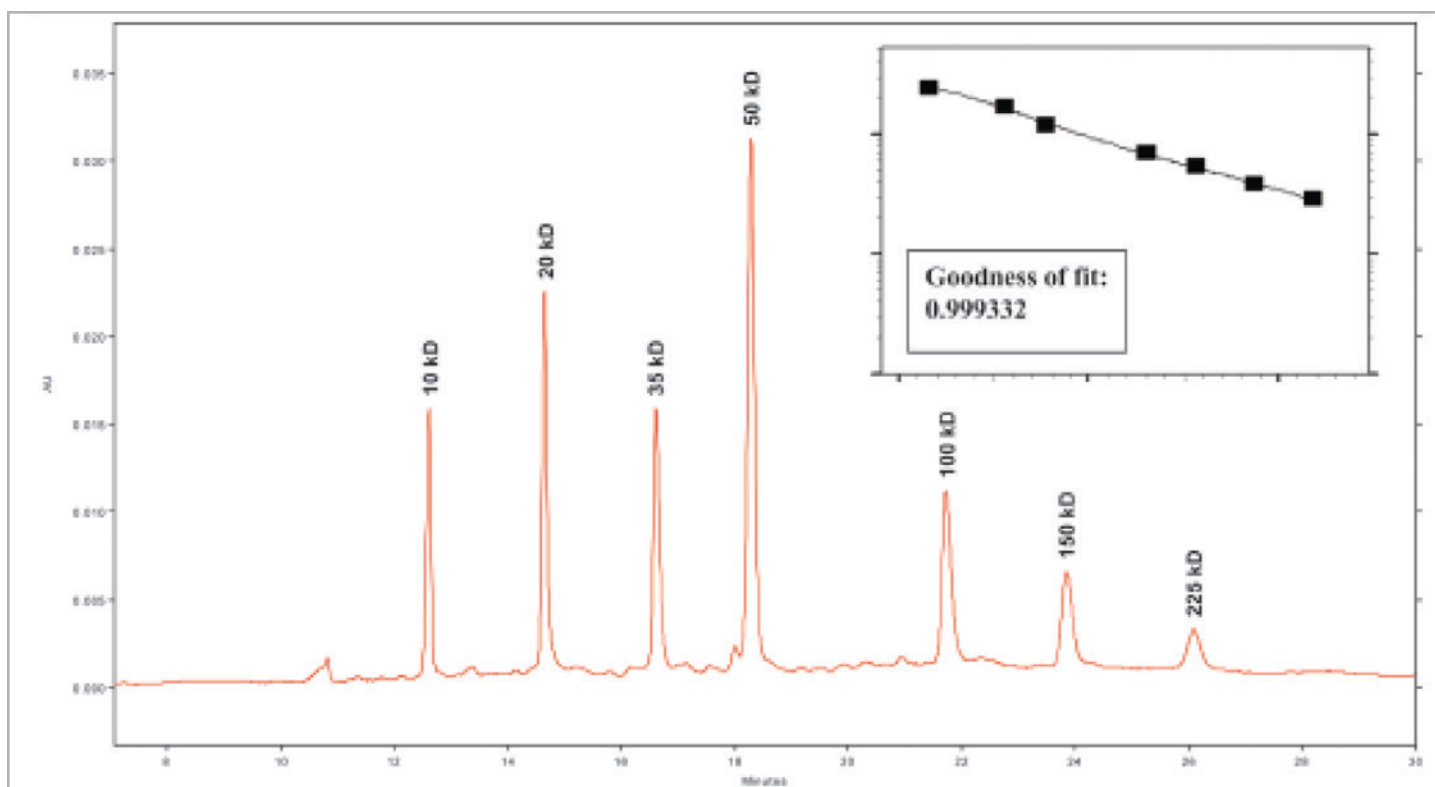


Figure 1. Resolution of the SDS-Gel MW standards. The inset plot illustrates the good linearity of the sizing standards plotted as Log MW versus 1/Mobility.

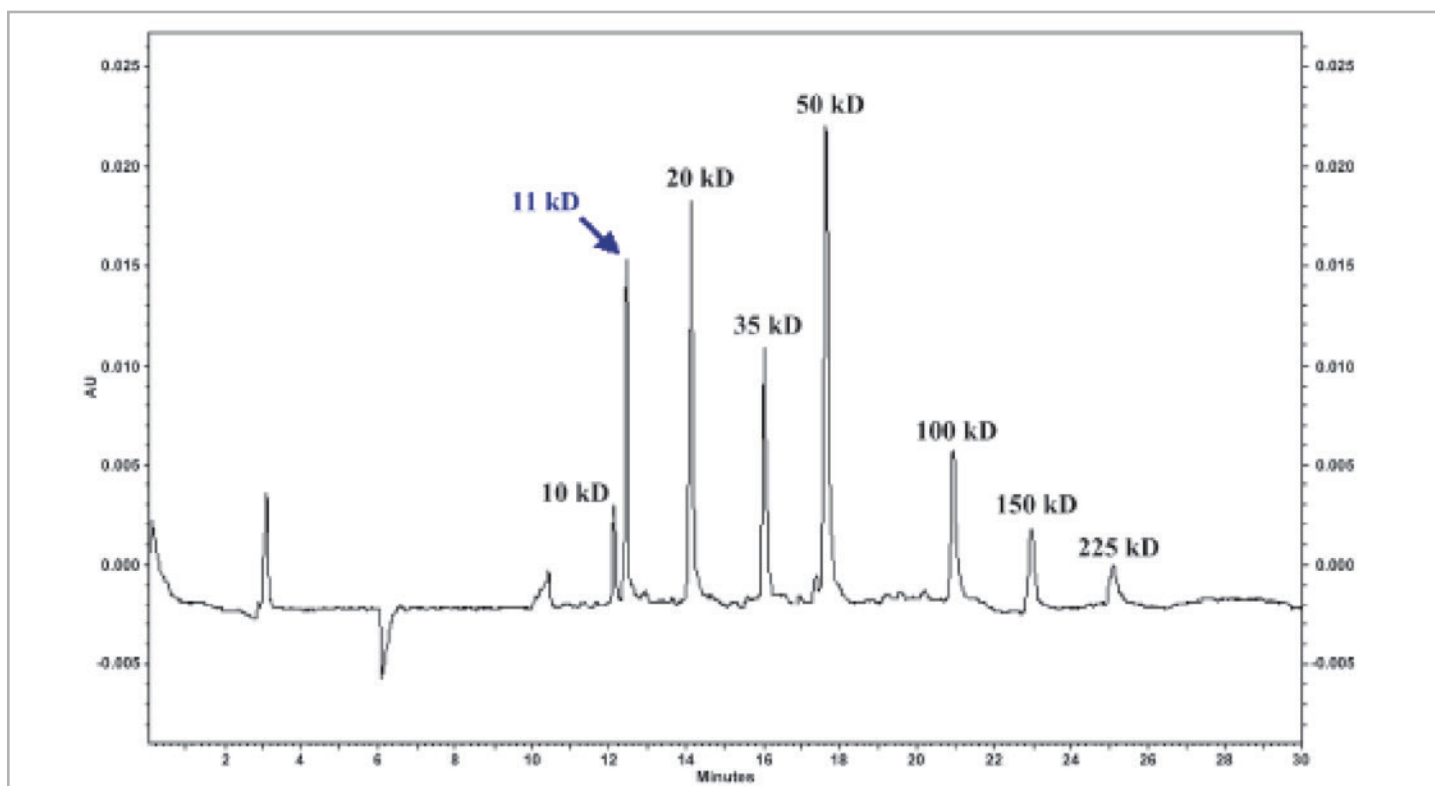


Figure 2. A different formulation of the protein size standards spiked with an 11 kDa protein.

Detection Linearity

The linearity of assay detection was also investigated. Figure 4 illustrates the linearity of the detector response using the corrected peak area versus protein concentration obtained from introducing increasing concentrations of the recombinant protein mobility marker (0.02 to 2 mg/mL) into the assay. Good linearity with a coefficient of determination (r^2) of 0.9994 was achieved over this concentration range.

Low-Level Impurity Detection

To demonstrate the low-level detection of impurities, we spiked the 10 kDa mobility marker with lysozyme at both 1% and 0.1% of the total loaded protein. Figure 5A illustrates the resolution and detection of this added protein at both concentration levels. The signal-to-noise ratio of 0.1% lysozyme is approximately 6:1. We subsequently introduced a second protein, the 11 kDa recombinant protein, at 0.1% into the IgG control standard. By doubling the injection time to 40 seconds, we could improve the signal-to-noise ratio to as high as 10:1 at the 0.1% level of contamination. This effect is illustrated in Figure 5B. Both examples used reducing conditions.

Impact of Sample Preparation on IgG Fragmentation Artifacts

A 1% SDS sample buffer solution was used to prepare the SDS-protein complexes for CE-SDS separation. During sample preparation, heating the sample solution at high temperature is required to accelerate SDS-binding. As shown in Figure 6A, no IgG signal was observed when non-reduced IgG was prepared at room temperature for 1 hour. Figure 6B shows a separation profile of non-reduced mouse IgG treated at 60° C for 5 minutes. The whole antibody was well separated from all the impurities such as the light chain, heavy chain, heavy-heavy chain, and 2-heavy-1-light chain. However, heating a non-reduced IgG sample at high temperature may introduce fragmentation and aggregation, and thus alter the accuracy of the sample analysis. A significant increase in IgG fragment peaks was observed when the IgG sample was heated at 95° C, indicating broken disulfide bonds between both the light and the heavy chains (Figure 6C). This temperature-associated fragmentation may be significantly reduced through sample alkylation procedures.

Alkylation of the non-reduced IgG sample serves to stabilize the disulfide bond. Iodoacetamide (IAM) at 12.5 mM was added into

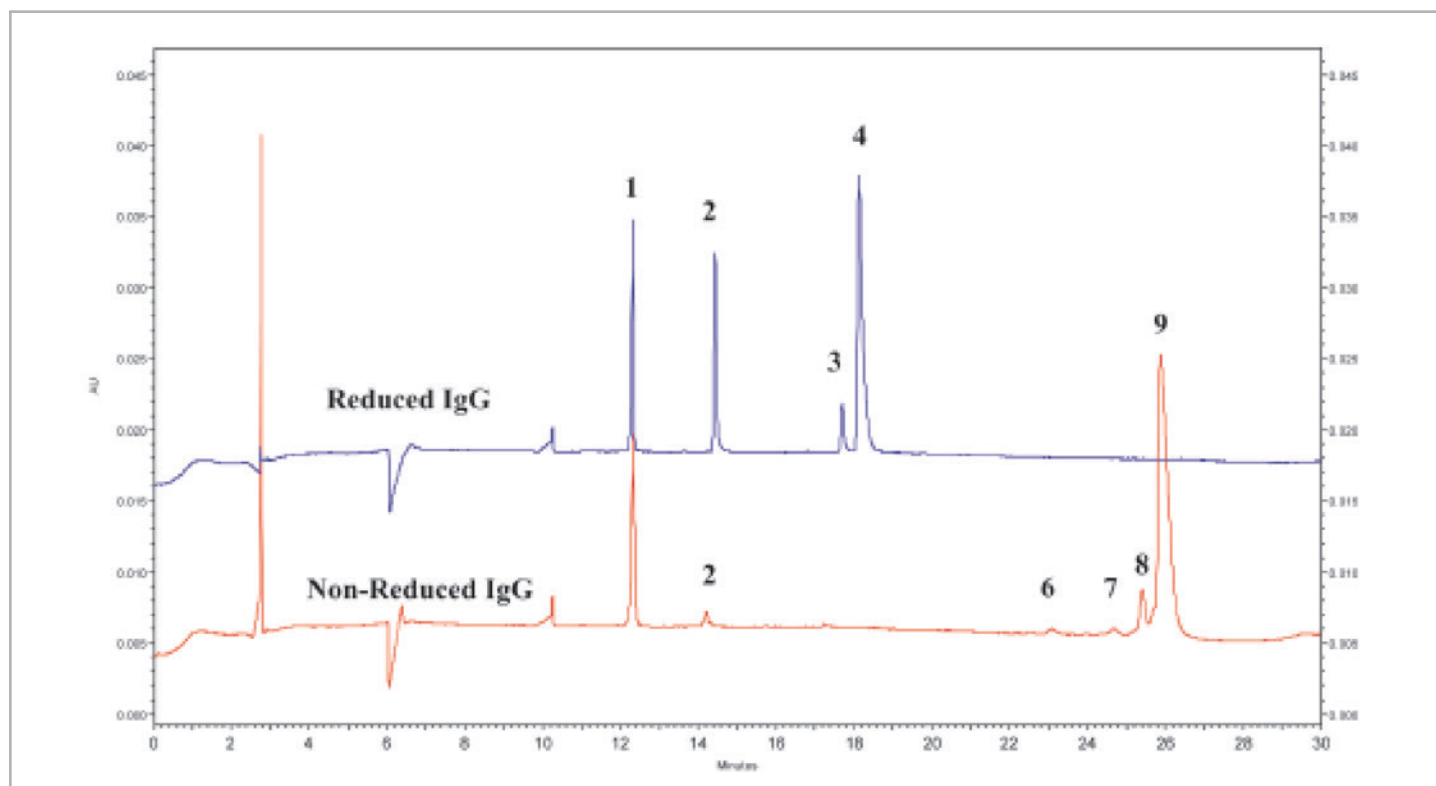


Figure 3. Analysis of both the reduced and non-reduced IgG suitability standard using the IgG purity method. Peak 1: Internal standard (10 kDa); 2: Light Chain (L); 3: NG heavy chain; 4: Heavy chain (H); 6: Heavy-heavy chain (2H); 7: 2 heavy 1 light chain (2H1L); 8: NG HC; 9: IgG monomer.

the protein sample buffer prior to heating. The sample was then treated at 70° C and 95° C for 10 minutes. Figure 7 compares the separation profile of the IgG control standard with and without alkylation. Better resolution was observed with the alkylated IgG sample simply because more efficient SDS binding was achieved at the higher temperature treatment. Furthermore, the disulfide bond of the IgG molecule was stabilized after alkylation, so no fragmentation was observed, even when the sample was treated at 95° C for 10 minutes.

Effect of High Salts

Since the assay uses electrokinetic sample introduction, the signal intensity and resolution of the analytes may be affected if the salt concentration in the sample becomes too high. Figure 8 shows the effect of a mouse IgG1K sample that contains 150 mM NaCl in the final sample preparation. Without desalting, using the standard sample introduction conditions, very low signal was obtained, and nonglycosylated heavy chain remained undetectable. However, after desalting the sample, the signal intensity was significantly improved, and the nonglycosylated

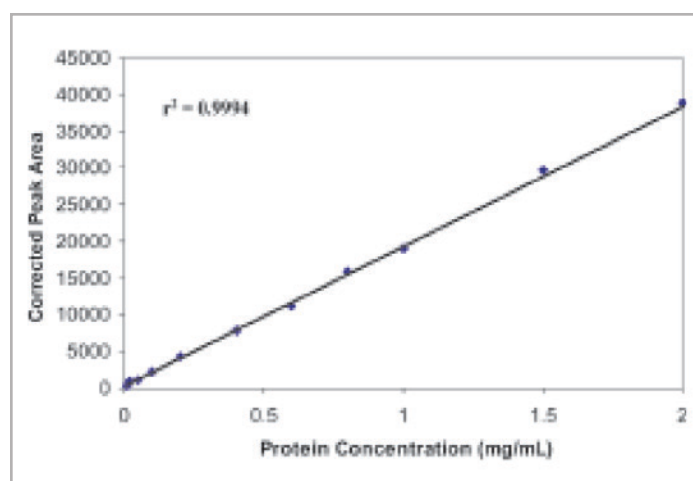


Figure 4. Linearity of detection response, plotting velocity-corrected peak area versus protein concentration over the range of 0.02 to 2 mg/mL of the 10 kDa standard.

heavy chain was well detected and separated from the glycosylated heavy chain. Generally, if the final salt concentration is below 50 mM, no desalting steps are required.

Injection	LV Mobility	HC Mobility	LC%	HC%	NG%	HC/LC
1	-0.0000448	-0.0000347	31.36	62.10	9.53	1.98
2	-0.00004482	-0.00003477	31.42	61.96	9.66	1.97
3	-0.00004478	-0.00003469	31.31	62.06	9.65	1.98
4	-0.0000448	-0.00003471	31.35	62.06	9.60	1.98
5	-0.00004489	-0.00003488	32.34	61.07	9.74	1.89
6	-0.00004488	-0.00003487	31.37	62.06	9.58	1.98
Mean:	-0.00004487	-0.00003488	31.52	61.88	9.63	1.96
Std Dev:	0.00000008	0.00000017	0.4007	0.4007	0.0729	0.0369
%RSD	0.17	0.49	1.27	0.68	0.757	1.46

Table 1. Assay precision for the reduced IgG suitability standard. Percent quantity is determined from the velocity corrected peak area of the analyte/total velocity-corrected peak area of all IgG analytes (excludes 10 kDa mobility marker).

Injection	Mobility of IgG	LMW%	Main%	NG%
1	-0.2585	6.36	85.11	9.11
2	-0.2601	6.67	84.68	9.27
3	-0.2602	6.79	84.49	9.36
4	-0.2616	6.75	84.50	9.38
5	-0.2615	6.63	84.54	9.46
6	-0.262	6.79	84.75	9.08
Mean:	-0.2607	6.66	84.68	9.28
Std Dev:	0.00130958	0.16	0.24	0.15
%RSD	0.66	2.4	0.28	1.62

Table 2. Assay precision of the non-reduced IgG suitability standard.

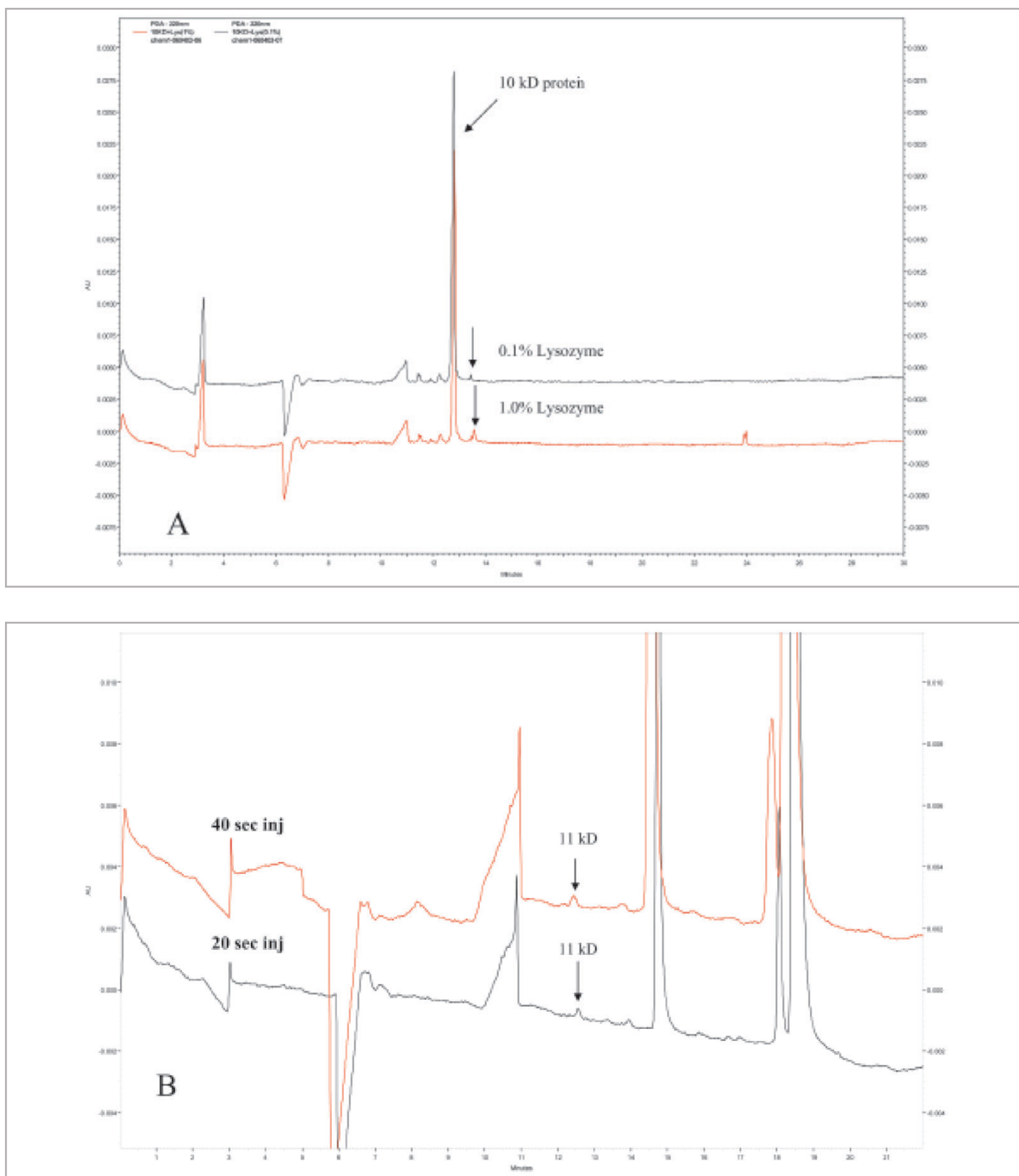


Figure 5. Panel A illustrates the detection of lysozyme spiked into the mobility marker at 1% and 0.1% levels. Panel B illustrates an 11 kDa protein spiked into our IgG suitability standard at 0.1%. The impact of changing the injection time from 20 to 40 seconds is indicated.

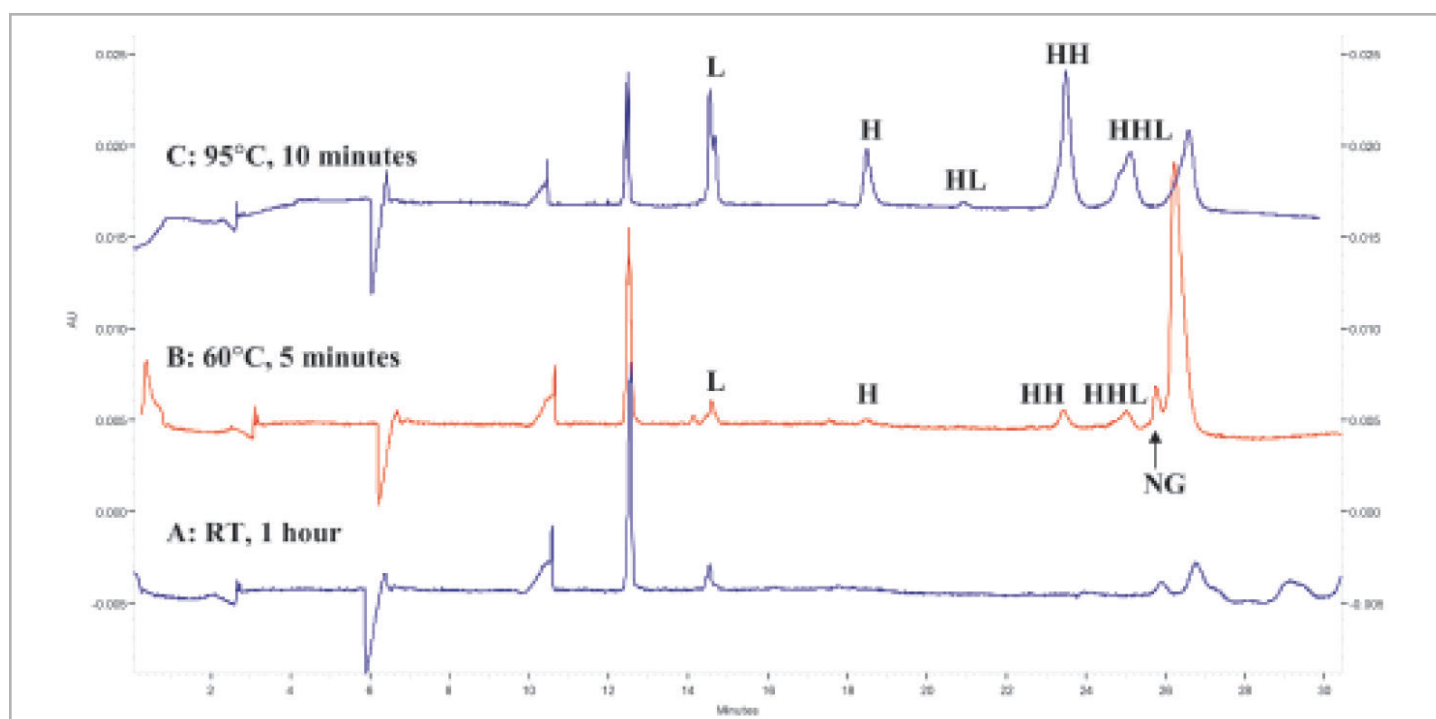


Figure 6. Separation of non-reduced IgG standard prepared at different temperatures. A: Sample incubated at room temperature for 1 hour; B: Sample heated at 60° C for 5 minutes; C: Sample heated at 95° C for 10 minutes.

Analysis of Unknowns

Using the methodology described in this Application Bulletin, we have screened several different mouse and human IgG samples. The percent of non-glycosylated heavy chain varied between the different IgG samples, ranging from 0 to 2% of the total heavy chain. Figure 9 illustrates an example of the analysis of a representative recombinant human IgG preparation taken from a bulk production at 40 mg/mL. Even though the salt present in the bulk sample was high, the dilution generated during sample preparation removed the need for sample desalting. Light chain, low-molecular-weight impurities, heavy chain, and non-glycosylated heavy chain were all automatically detected and identified by the software based on the known mobilities of these species. In this example, the non-glycosylated heavy chain represented about 1.6% of the total heavy chain, while the low-molecular-weight impurities accounted for about 0.3% of the total protein load (excluding mobility marker). The inset in Figure 9 highlights a zoomed in portion of the electropherogram, providing an indication of the stability of the baseline and the resolution of the low-level impurities.

Summary

We have developed a new, replaceable polymer matrix to improve the current resolving power of SDS-CGE, and have developed this matrix into a commercial IgG Purity/Heterogeneity assay. In doing this, we are providing a quality-controlled assay to the biotechnology industry, with specifications developed to assess the purity and heterogeneity of IgG in both a reduced and non-reduced state. This assay will detect impurities as low as 0.1% and includes an IgG control with a designated quantity of non-glycosylated heavy chain. The IgG control is used to test the suitability of the assay prior to running unknowns. With non-reduced IgG, the assay will resolve the intact IgG from: 1) IgG dimer, 2) 2 heavy chains and 1 light chain, 3) 2 heavy chains, 4) 1 light chain, 1 heavy chain, 5) 1 heavy chain, 6) light chains. With reduced IgG, the assay will resolve the IgG heavy chain, light chain, non-glycosylated heavy chain, and the lower-molecular-weight impurities that fall between the heavy and light chains, as well as higher-molecular-weight impurities that are larger than the intact heavy chain.

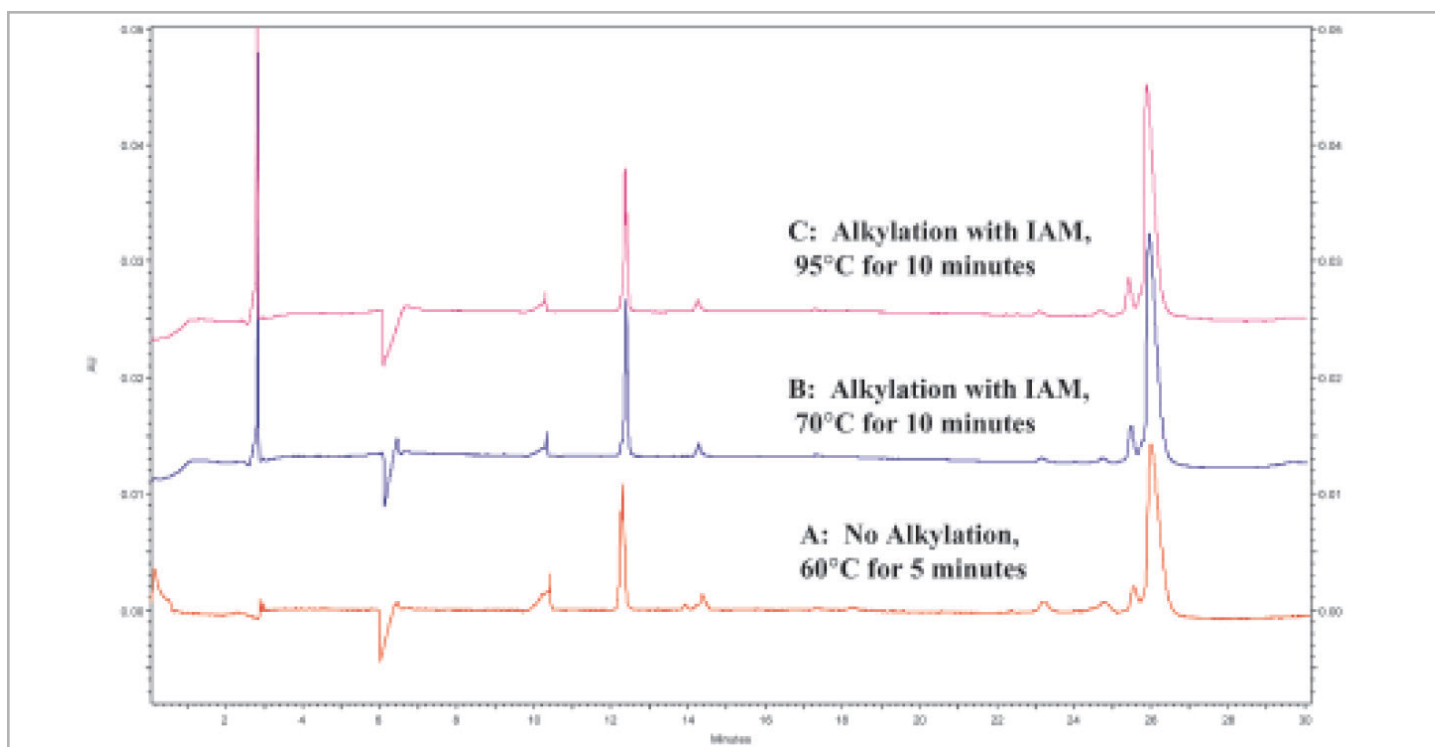


Figure 7. Separation of non-reduced IgG standard alkylated with iodoacetamide (IAM). A: Sample treated at 60° C for 5 minutes without alkylation; B: Sample alkylated with IAM and then heated at 70° C for 10 minutes; C: Sample alkylated with IAM and then heated at 95° C for 10 minutes.

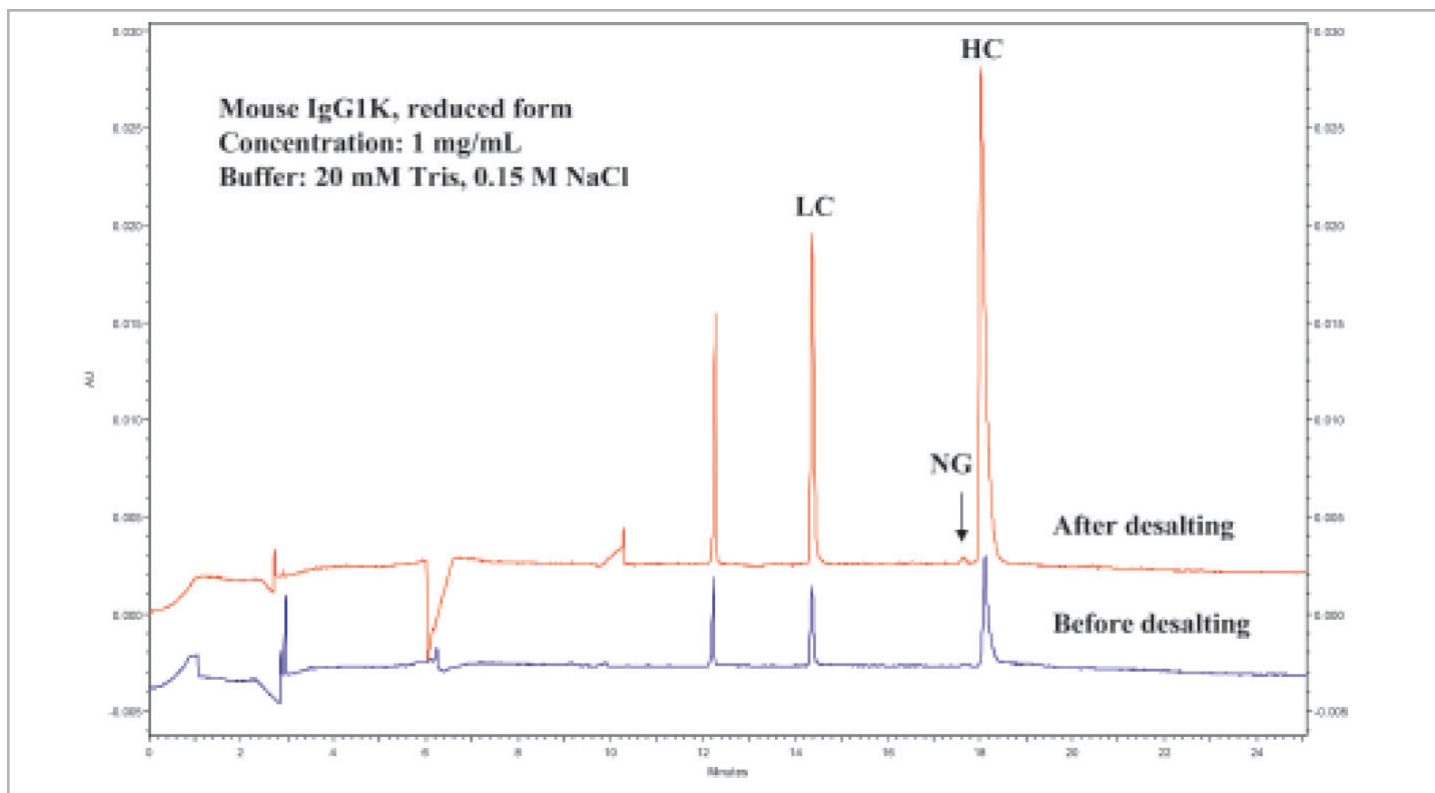


Figure 8. Effect of salt concentration on assay performance.

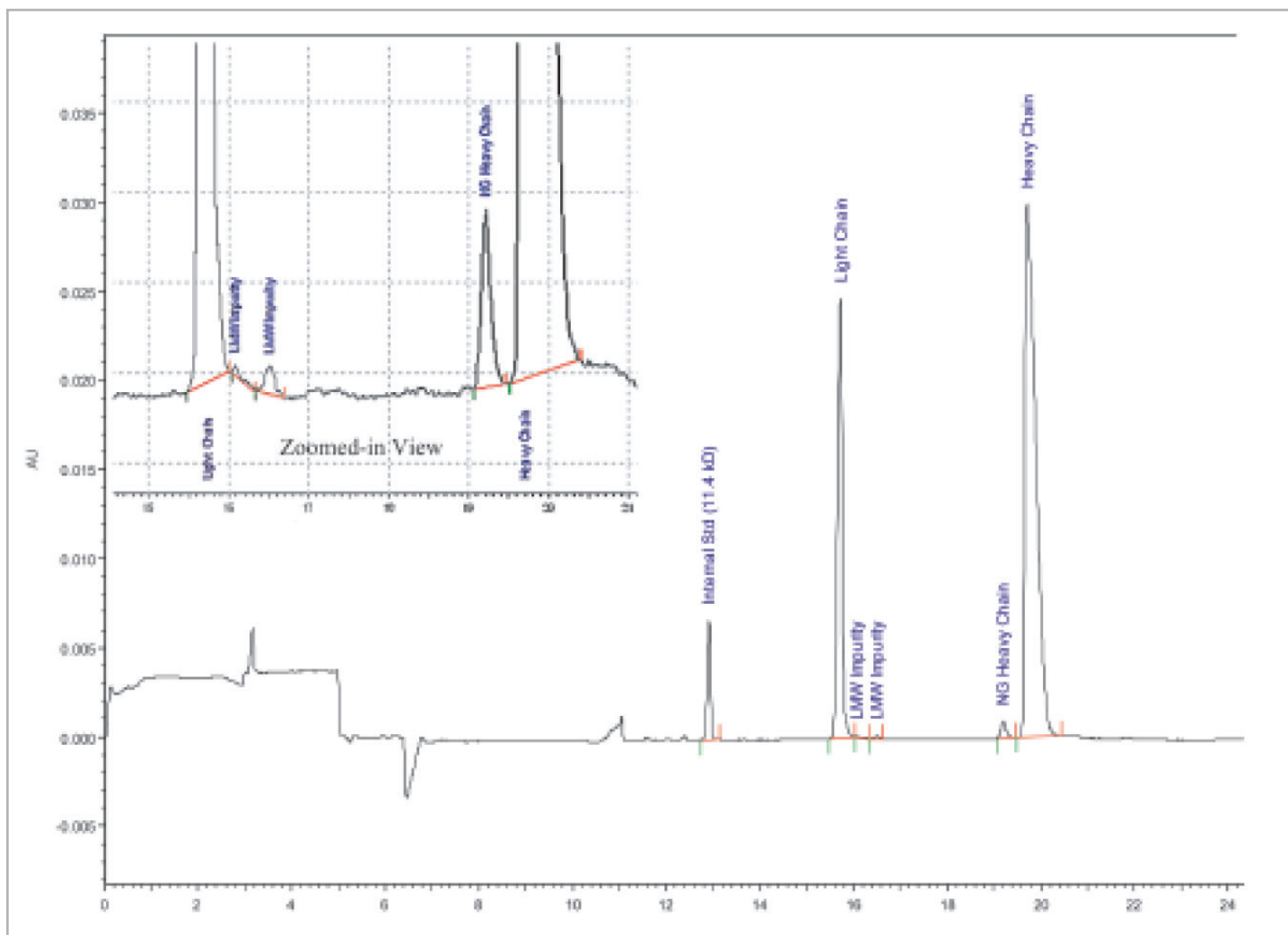


Figure 9. An electropherogram of the analysis of a typical reduced recombinant human IgG. The nonglycosylated heavy chain (NG) is well resolved from the heavy chain, while low-molecular-weight impurities are clearly discernable from the light chain. The inset simply highlights a zoomed-in region of the electropherogram.

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