

Protein Analysis with the Agilent 2100 Bioanalyzer System

An overview of the protein assay portfolio

Technical Overview



Introduction

The Agilent 2100 Bioanalyzer system is a microfluidic system for the electrophoresis-based analysis of biomolecules. It has developed into a key platform for routine QA/QC of protein samples by supporting three distinguished protein analysis kits:

- Protein 80 kit – for protein analysis in the low molecular weight range
- Protein 230 kit – for general protein analysis up to 230 kDa
- High Sensitivity Protein 250 kit – for picogram sensitivity and lowest level of impurity detection

Besides the ease-of-use of prevalidated reagents, this protein assay portfolio offers a number of advantages over techniques like slab-gel electrophoresis or standard CE-SDS methods:

- Wide molecular weight range, from 5 kDa to 250 kDa
- Broad protein loading capacity from pg/ μ L to μ g/ μ L



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- Protein detection with up to four orders of magnitude in linear dynamic range
- Minimal sample consumption
- Activated 2100 Security Pack enables 21 CRF Part 11 compliance

This Technical Overview compares the general performance features of the three protein analysis assays and provides guidance for selecting the assay best suited for the sample type at hand.

Experimental

Human myeloma IgG2 (Ab) was chosen as a model protein for this performance study. Samples of IgG2 (1 µg/µL) and myoglobin were obtained from Sigma (St. Louis, MO, USA). Protein 80 (P80), Protein 230 (P230) and High Sensitivity Protein 250 (HSP-250) kits were from Agilent Technologies (Waldbronn, Germany). All reduced and non-reduced IgG2 separations were performed on the Agilent 2100 Bioanalyzer system in combination with the respective protein assay. Unless otherwise mentioned, all chemicals were of analytical grade. Chips were prepared according to the respective kit protocol^{1,2,3}.

P80 and P230 assays

Four microliters of antibody sample were mixed with 2 µL of sample buffer (with or without DTT as reducing agent). The antibody solution and ladder were placed at 95°C for 5 min and further diluted with 84 µL of water. Six microliters were applied to the protein chip for analysis.

HSP-250 assay

First, ladder and antibody samples were covalently modified with a fluorescent dye as described in the High Sensitivity Protein 250 kit guide. Upon 1:200 dilution with distilled water, 4 µL of sample or ladder were mixed with 2 µL of sample buffer (with or without DTT as reducing agent). The solution was placed at 95 °C for 5 minutes before on-chip analysis.

Impurity level detection experiments were performed by spiking myoglobin to IgG2 samples followed by on-chip analysis under reducing conditions. Molecular weight resolution across the size range of the assays was studied with the respective protein sizing standards supplied with the kits. The Agilent 2100 Expert software (version B.02.07) was used for run control and data analysis.

Results and discussion

The three Bioanalyzer system protein assays differ in their specifications as well as their sample preparation workflows. The P80, P230 and HSP-250 assays cover protein size ranges from 5–80 kDa, 14–230 kDa and 10–250 kDa, respectively. The P80 and P230 assays employ on-chip staining where a fluorescent dye becomes associated with proteins in SDS micelles. This offers the advantage of direct and convenient sample preparation but results in background

fluorescence due to the omnipresent dye in the system. With the HSP-250 assay, in contrast, samples are first covalently labeled with dye before being diluted and loaded onto the chip for analysis. This direct labeling approach allows the HSP-250 assay to detect pg/µL levels of protein by completely eliminating background fluorescence.

We used the three protein assays to analyze identical sample sets. Figures 1 and 2 show reducing and non-reducing electrophoretic profiles of an IgG2 preparation.

Under non-reducing conditions (Figure 2) the intact antibody (Ab) is detected at 156.6 kDa with the HSP-250 assay, which is in close agreement with its theoretical molecular mass of about 150 kDa. The P230 and HSP-250 assays clearly resolve light chain (LC), heavy chain (HC) and a mixture of LC and HC peaks including the non-glycosylated form of IgG2 (P230: 144.5 kDa peak and HSP-250: 143.3 kDa peak). A unique feature of the HSP-250 assay is the size and concentration measurement beyond the size range of the ladder, that is, 250 kDa. High molecular weight aggregates or impurities are thus analyzed for size and quantity as well.

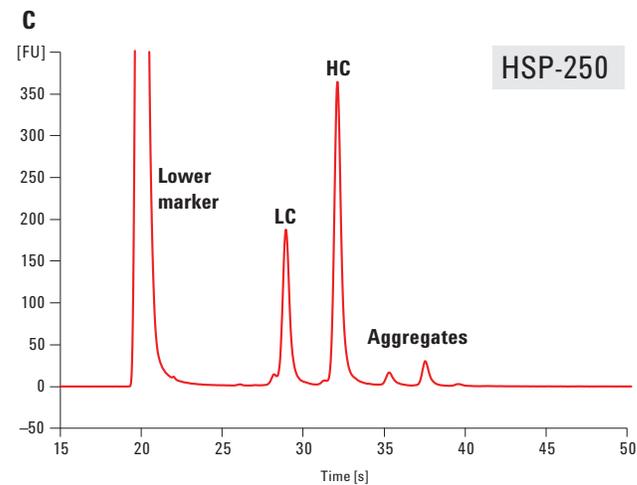
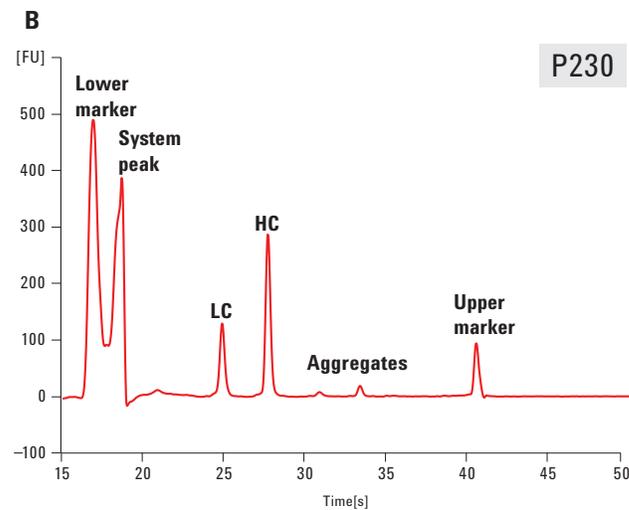
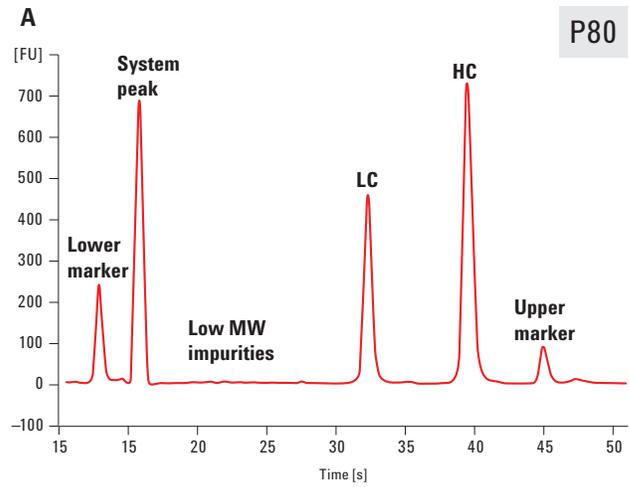


Figure 1
Analysis of an IgG2 preparation under reducing conditions. One representative electropherogram per assay is shown as generated with the Agilent 2100 Bioanalyzer system.
Abbreviations: LC: Light Chain; HC: Heavy Chain.

- (A) Analysis with the Protein 80 assay**
- (B) Analysis with the Protein 230 assay**
- (C) Analysis with the High Sensitivity Protein 250 assay**

Under reducing conditions (Figure 1), LC and HC are well resolved with all three assays. Aggregates of higher molecular weight are observed with the P230 and HSP-250 assays whereas the P80 assay resolves low molecular weight impurities associated with the IgG2 sample.

We assessed reproducibility of the results on sizing, purity and relative concentration, by analyzing samples repeatedly over multiple chips (n = 5). All three assays returned precise sizing and purity information with the highest coefficients of variation (CVs) determined to 2.3% and 6.0%, respectively (Table 1). Reproducibility of the relative protein concentration was within the specified limit of 20% CV (data not shown). The relative protein concentration is determined by comparing protein peak areas to assay standards; the upper marker in the P80 and P230 assays and the ladder in the HSP-250 assay. Quantitation reproducibility is therefore affected by pipetting and mixing inconsistencies.

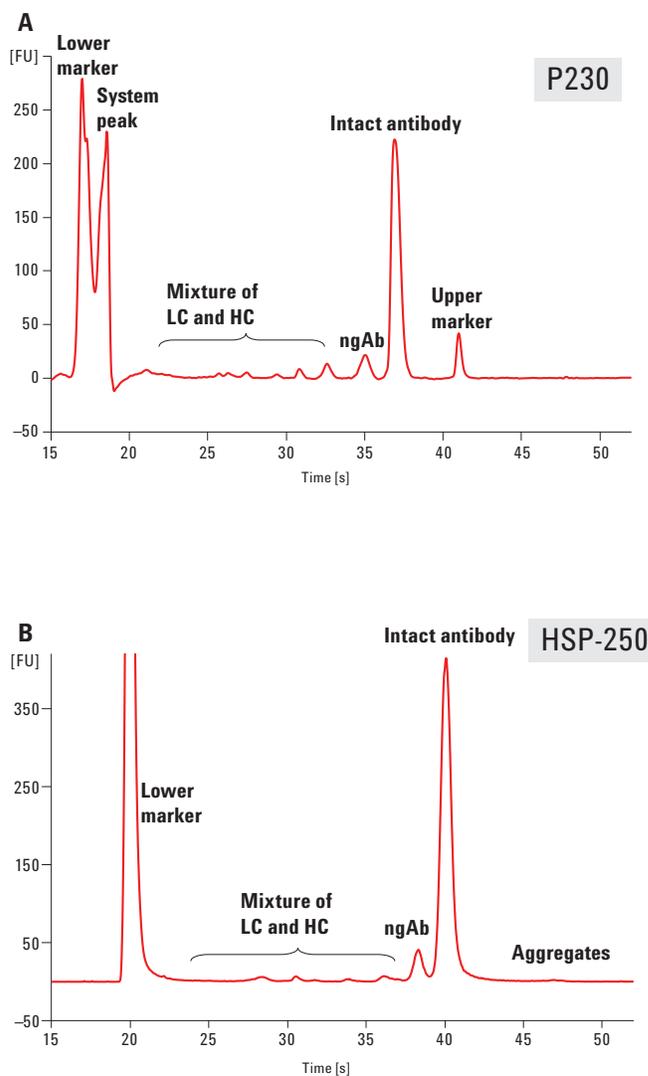


Figure 2
Analysis of an IgG2 preparation under non-reducing conditions. One representative electropherogram per assay is shown as generated with the Agilent 2100 Bioanalyzer system. Abbreviations: LC: Light Chain; HC: Heavy Chain; ngAb: non-glycosylated antibody.

- (A) Analysis with the Protein 230 assay**
- (B) Analysis with the High Sensitivity Protein 250 assay**

In a second study, we assessed assay performance with regard to detection of low level impurities: myoglobin (17 kDa) was spiked at various levels into the IgG2 preparation of 1 $\mu\text{g}/\mu\text{L}$ and analyzed with the three protein assays for the Agilent 2100 Bioanalyzer system (Figure 3). The limit of detection for the myoglobin spike with a $S/N > 3$ was determined to 0.03% for the HSP-250 assay. Therefore, the HSP-250 assay meets the impurity detection limit of 0.05% imposed by the Food and Drug Administration (FDA)⁴. As expected, detection limits were significantly higher with the standard protein assays P80 and P230 and were determined to 0.8% and 1.6%, respectively. Reproducibility of the data was well within the boundaries described in assay specifications with %CV values around 1.5% (Figure 3).

Finally, the three protein assays were compared on their protein resolution capacity across their size range using the corresponding protein ladders of each assay. Molecular weight resolution (R_s 0.8) was calculated as a percentage for each pair of protein ladder peaks (Figure 4). Generally, a molecular weight resolution of below 10% is achieved except for the lowest molecular weight region from 10 to 30 kDa. Highest protein resolution was observed with the P80 assay which is specifically designed for the analysis of low molecular weight proteins up to 80 kDa. The P230 and HSP-250 assays, in contrast, sacrifice some resolving power in the low molecular weight range for a larger size range up to 230 kDa and beyond.

Peak	Light Chain			Heavy Chain		
Assay	Protein 80	Protein 230	HSP 250	Protein 80	Protein 230	HSP 250
Average %Total	28.9	27.1	31.5	60.3	63.3	59.1
%CV	3.8	5.9	4.4	1.8	1.6	1.5

Table 1

Assay precision for protein purity determination: Repetitive analysis of an IgG2 preparation with the three Bioanalyzer protein assays as shown in Figure 1. Listed are average and relative standard deviation (%CV) for the contribution (% total) of light chain and heavy chain to all proteins detected with each assay. The three assays return similar and highly reproducible (%CV < 6%) results.

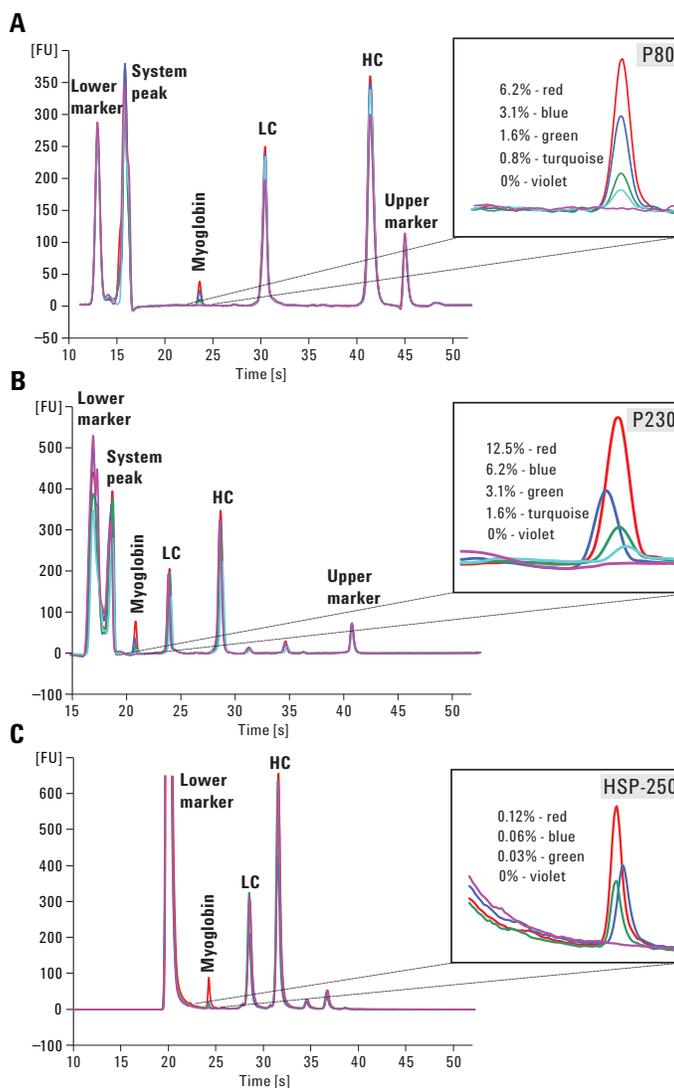


Figure 3

Impurity detection with the Bioanalyzer protein assay portfolio: Myoglobin (17 kDa) was spiked at the indicated percentages into an IgG2 preparation and analyzed under reducing conditions with the three Bioanalyzer protein assays. Inserts show an expanded view on the myoglobin peaks detected and list the corresponding spike levels.

(A) Analysis with the Protein 80 assay.

(B) Analysis with the Protein 230 assay.

(C) Analysis with the High Sensitivity Protein 250 assay.

Conclusion

The Agilent 2100 Bioanalyzer system in conjunction with its protein assay portfolio is a versatile tool for the validation of protein preparations. The results of the presented set of experiments are summarized in Table 2 to help with assay selection.

References

1. Agilent Protein 80 Kit Guide, Agilent reference number G2938-90062
2. Agilent Protein 230 Kit Guide, Agilent reference number G2938-90054
3. Agilent High Sensitivity Protein 250 Kit Guide, Agilent reference number G2938-90310.
4. Reviewer Guidance, Validation of Chromatographic Methods, Center for Drug Evaluation and Research, Food and Drug Administration, 1994.

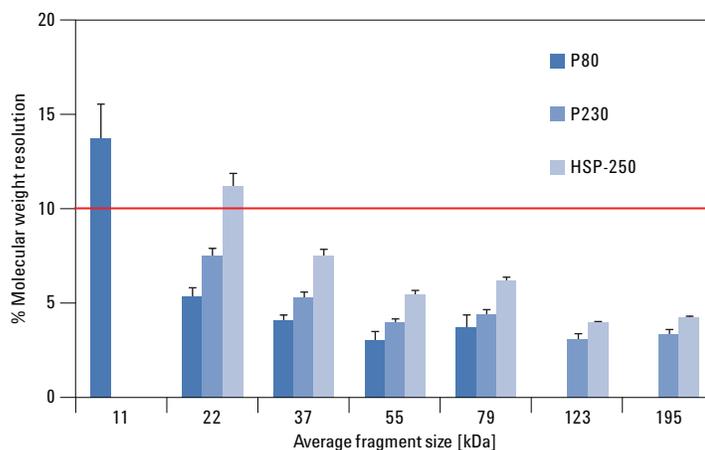


Figure 4
Molecular weight resolution across the size range of the three protein assays: The protein size standards of the corresponding assays were analyzed and the % molecular weight resolution was calculated for pairs of adjacent ladder peaks.

	P80 Assay	P230 Assay	HSP-250 Assay
Molecular weight range	5-80 kDa	14-230 kDa	10-250 kDa
Application area	Routine assessment of low MW impurities	Routine assessment of protein impurity and size in different molecular weight range	Highly sensitive detection of impurities
Precision⁺ for: Sizing	< 3%		< 3%
Relative concentration % Purity	< 20% < 6%		< 20% < 5%
Impurity detection*	≤ 1.5%		0.03%
Protein staining/labeling	On-chip		Off-chip
Sample preparation time	~ 10 min/10 samples		~ 45 min/10 samples
Run time	~ 30 min		
MW resolution (Rs = 0.8)	< 10% for higher MW proteins		

⁺ %CV (n = 5 chips)

* with IgG2

Table 2
Summary of the present study: Listed are the key characteristics of the three 2100 Bioanalyzer protein assays.

www.agilent.com/genomics/bioanalyzer

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© Agilent Technologies, Inc., 2010-2019
February 1, 2019
Publication Number 5990-5283EN



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