Application Forum

Therapeutic protein analysis with the Agilent 2100 Bioanalyzer

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Introduction

The Agilent 2100 Bioanalyzer is a microfluidic system for the electrophoresis-based analysis of biomolecules. In recent years it has developed into a key platform for routine QA/QC of protein samples by supporting three distinguished protein analysis kits (1):

- 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 kit portfolio offers a number of advantages over techniques like slab-gel electrophoresis or standard CESDS methods:

- Wide molecular weight range, from 5 kDa to 250 kDa
- Broad protein loading capacity from pg/ μ L to μ g/ μ L
- Protein detection with up to four orders of magnitude in linear dynamic range
- Minimal sample consumption
- Electronic data
- CFR 21 Part 11 compliance

This Technical Note compares the general performance features of the three protein analysis kits and provides guidance for selecting the kit 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 μ g/ μ L) and myoglobin were obtained from Sigma-Aldrich (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 nonreduced IgG2 separations were performed on the Agilent 2100 Bioanalyzer 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 (2–4).

P80 and P230 assays. Four microliters of antibody sample were mixed with 2 μ L 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 μ L 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 μ L sample or ladder were mixed with 2 μ L sample buffer (with or without DTT as reducing agent). The solution was heated to 95°C for 5 minutes before on-chip analysis.

Figure 1. Analysis of an IgG2 preparation under reducing conditions. One representative electropherogram per assay is shown as generated with the Agilent 2100 Bioanalyzer. (A) Analysis with the Protein 80 assay. (B) Analysis with the Protein 230 assay. (C) Analysis with the High Sensitivity Protein 250 assay. LC, light chain; HC, heavy chain.

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. (A) Analysis with the Protein 230 assay. (B) Analysis with the High Sensitivity Protein 250 assay. LC, light chain; HC, heavy chain; ngAb, nonglycosylated antibody.

Table 1. Assay precision for protein purity determination.

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

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 results (CV < 6%).

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 protein kits differ in their specifications as well as their sample preparation workflows. The P80, P230, and HSP-250 kits cover protein size ranges of 5–80 kDa, 14–230 kDa, and 10–250 kDa, respectively.

The P80 and P230 kits employ on-chip staining in which a fluorescent dye becomes associated with proteins in SDS micelles (5). 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 novel HSP-250 kit, 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 kits to analyze identical sample sets. Figures 1 and 2 show reducing and nonreducing electrophoretic profiles of an IgG2 preparation.

Under nonreducing 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 ~150 kDa. The P230 and HSP-250 kits clearly resolve light chain (LC), heavy chain (HC) and a mixture of LC and HC peaks including the nonglycosylated form of IgG2 (P230, 144.5-kDa peak; 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 (i.e., 250 kDa). High molecular weight aggregates or impurities are thus analyzed for size and quantity as well.

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 be 2.3% and 6.0%, respectively (Table 1).

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 μg/μL and analyzed with the three protein assays for the

Figure 3. Impurity detection with the Bioanalyzer Protein Kit 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 shown an expanded view on the myoglobin peaks detected and list the corresponding spike level. (A) Analysis with the Protein 80 assay. (B) Analysis with the Protein 230 assay. (C) Analysis with the High Sensitivity Protein 250 assay. LC, light chain; HC, heavy chain.

Agilent 2100 Bioanalyzer (Figure 3). The limit of detection for the myoglobin spike with a signal-to-noise ratio > 3 was determined to be 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) (6,7). 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 kit specifications with CV values of $\sim 1.5\%$ (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 kit. Molecular weight resolution (R_s 0.8) was calculated as a percentage for each pair of protein ladder peaks. Generally, a molecular weight resolution of below 10% is achieved except for the lowest molecular weight region from 10 to 30 kDa.

Highest protein resolving power was observed with the P80 kit, which is specifically designed for the analysis of low molecular weight proteins up to 80 kDa. The P230 and HSP-250 kits, in contrast, sacrifice some resolving power in the low molecular weight range for a larger size range up to 230 kDa and beyond.

Conclusion

The combination of the Agilent 2100 Bioanalyzer and Agilent protein kits forms a versatile tool for the validation of protein preparations. For rapid, routine assessment of impurities at higher levels, the P80 kit is an excellent choice for the low-molecular-weight range, while the P230 kit is better for analyzing larger proteins. If more sensitivity is needed for impurity detection, the HSP-250 kit is the best choice.

References

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