Technical Overview

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Protein Sizing with the Agilent ProteoAnalyzer System

Introduction

Characterization of proteins is crucial for understanding their expression and function. This characterization includes aspects such as purification level and identification of the protein of interest, especially in the field of biopharmaceuticals. As such, assessment of protein size is an important and routine aspect of many laboratories. Sizing of proteins has historically been assessed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to denature the samples, providing them with a consistent mass-to-charge ratio, and allowing for separation of the proteins based on their molecular weight. Today, capillary electrophoresis SDS (CE-SDS) instruments are available to make this process easier and faster.

The Agilent ProteoAnalyzer system is an automated, multi-capillary CE-SDS system that allows for analysis of up to 12 samples in parallel and the ability to program several consecutive runs. The system provides fast separation times, higher resolution, and increased sensitivity compared to traditional SDS-PAGE analysis[1,2]. This facilitates precise and accurate measurements for both sizing and purity of most proteins. Data from the ProteoAnalyzer is presented as both a digital gel and an electropherogram image of each sample. The Protein Broad Range P240 kit used with the system has a sizing range of 10 to 240 kDa (Lower marker (LM)-only method). An optional upper marker (UM) can be used with the kit to provide for better alignment from 10 to 200 kDa (LM & UM method)[1]. The system is compatible with the Agilent ProSize data analysis software, which automatically analyzes the data. Each peak in the sample is assigned a size based upon a ladder composed of known sizes that is run in parallel with the samples.

The analytical specifications of the ProteoAnalyzer system for sizing are based on model protein samples BSA, CAII, GREMLIN-1, and NIST mAb[1,3] (Table 1). However, it is well known that variations in the amino acid sequence, isoelectric point (pI), charge, hydrophobicity, and other protein characteristics can influence the SDS binding ratio to the protein, altering the mass-to-charge ratio and thus the apparent size of the protein during electrophoretic analysis. To further demonstrate the sizing capabilities of the ProteoAnalyzer, samples other than the model proteins used for establishing the kit specifications were analyzed and compared to traditional SDS-PAGE under reduced conditions. These include many commercially available proteins and enzymes. This technical overview highlights the sizing accuracy, precision, and reproducibility of the ProteoAnalyzer system to analyze a variety of protein samples.

Methods

Sample preparation

A variety of commercially available proteins were used in this study. Proteins used for accuracy and precision studies include: Albumin from chicken egg white (Sigma, p/n A7642), β-Galactosidase from *Escherichia coli* (Sigma, p/n G8511), β-Lactoglobulin from bovine milk (Sigma, p/n L3908), Lysozyme from chicken egg white (Sigma, p/n L4631), Myosin Heavy Chain from rabbit muscle (Sigma, p/n M7659), Phosphorylase b from rabbit muscle (Sigma, p/n P4649), and Trypsin inhibitor from *Glycine max* (soybean) (Sigma, p/n T9767). Enzymes include: Exonuclease I (*E. coli*) (ExoI) (NEB, p/n M0293), Optimase (Transgenomic, p/n 703010), T7 Endonuclease I (T7E1) (nzytech, p/n MB21201), Phire Hot Start II DNA Polymerase (Thermo Fisher Scientific, p/n F122), Taq DNA Polymerase (Thermo Fisher Scientific, p/n EP0402), GoTaq G2 DNA Polymerase (Promega, p/n M784A), FastDigest MssI (Thermo Fisher Scientific, p/n FD1344), Eco32I (Thermo Fisher Scientific, p/n FD303), and Fast Digest BsuRI (Thermo Fisher Scientific, p/n FD0154).

Lyophilized protein samples were reconstituted and diluted to 1 mg/ml in 1x PBS for analysis. Enzymes were used at the supplied concentration.

SDS-PAGE analysis

The samples were also analyzed with SDS-PAGE using precast gels (Bio-Rad, p/n 4569036) under reduced conditions. Each sample was diluted 3:1 with 4x Laemmli buffer (Bio-Rad, p/n 161-0747), with a final concentration of 50 mM DTT. The samples were heat denatured at 90 °C for five minutes, then 10 µL of each concentration was loaded onto the SDS-PAGE gels. 10 µL of Bio-Rad Precision Plus Protein Dual Color Standards (p/n 161-0374) was added to the wells flanking the sample lanes. Separation was conducted at 200 V for approximately 40 minutes. The gels were fixed (10% acetic acid, 40% ethanol, 50% water) for 15 minutes with rocking, then rinsed with water. The gels were stained overnight in Bio-Rad QC Colloidal Coomassie stain (p/n 1610803) and destained with de-ionized (DI) water for three hours. Analysis was performed using GelAnalyzer software[4] for sizing and quantification.

ProteoAnalyzer analysis

Samples were analyzed under reduced conditions with the Agilent ProteoAnalyzer system using the Agilent Protein Broad Range P240 kit (p/n 5191-6640) with the LM only method and the LM & UM method.

Table 1. Kit specifications of the Agilent Protein Broad Range P240 kit used with the Agilent ProteoAnalyzer system.

Analytical Specifications		**ProteoAnalyzer Protein Broad Range P240 kit**
Sizing Range	LM only LM and UM	10 to 240 kDa 10 to 200 kDa
Typical Sizing Accuracy (% Sizing Error)	LM only LM and UM	< 15% for BSA, CAII (using reduced conditions) < 10% for BSA, CAII (using reduced conditions)
Typical Resolution		< 10% molecular weight resolution between 15 to 150 kDa (based on ladder) R ≥ 1 NIST mAb NGHC/HC (using reduced conditions)
Sizing Precision	LM only	< 8% CV for BSA, CAII, GREMLIN-1, and NIST mAb (using reduced conditions) < 10% CV for intact NIST mAb (using non-reduced conditions)
	LM and UM	< 5% CV for BSA, CAII, GREMLIN-1 and NIST mAb (using reduced conditions)
Quantitative Range		2 ng/µL to 2,000 ng/µL for BSA in PBS
Sensitivity (Signal/Noise > 3)		1 ng/µl for BSA, CAII in PBS
Quantification Reproducibility		<15 %CV for 20 – 2,000 ng/µL BSA <25 %CV for 2 – 20 ng/µL BSA
Physical Specifications		
Total Run Time		30 minutes
Samples Per Run		11 samples + ladder in well 12
Sample Volume Required		1 µL
Kit Stability		Minimum 4 months

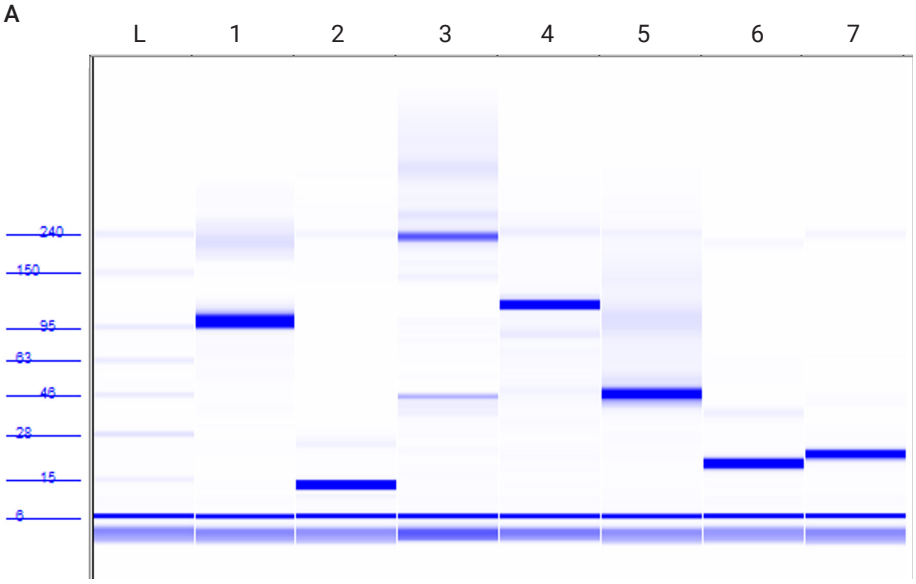
LM = Lower Marker, UM = Upper Marker

Results and discussion

Accuracy

Several commercially available proteins of known molecular weight were analyzed on the ProteoAnalyzer to examine the ability of the system to accurately and reliably size samples other than the model proteins on which the kit specifications were based. The expected sizes of the samples tested range from 14 to 220 kDa to provide examples across the sizing range of the Protein Broad Range P240 kit. Samples were assessed with both the LM & UM method and the LM-only method on the ProteoAnalyzer. The peak observed with myosin, which has an expected size of 220 kDa, overlaps with the UM, so was analyzed with the LM-only method. The reported size of each sample was compared to both the expected molecular weight and the size determined by SDS-PAGE for comparison. The gel images from each system are shown in Figure 1.

A



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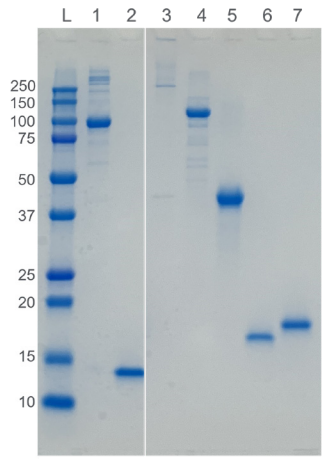

Sample ID	Sample Name
L	Ladder
1	Phosphorylase b from rabbit muscle
2	Lysozyme from chicken egg white
3	Myosin heavy chain from rabbit muscle
4	β-Galactosidase from E. coli
5	Albumin from chicken egg white
6	β-Lactoglobulin
7	Trypsin inhibitor from *Glycine max*

Figure 1. A) Digital gel results of random proteins assessed on the Agilent ProteoAnalyzer system under reduced conditions with the LM-only method. B) SDS-PAGE results are shown for comparison.

Representative electropherogram images of some of the samples are shown in Figure 2 as examples of the data reported by the ProteoAnalyzer.

The sizing analysis of these proteins is summarized in Table 2. The average size and percent error compared to the expected molecular weight, as well as the percent difference compared to the SDS-PAGE results are shown for each sample. Both the SDS-PAGE and the ProteoAnalyzer results demonstrated high levels of accuracy, with less than 15% error for each sample. However, SDS-PAGE consistently sized most of the proteins smaller than the ProteoAnalyzer, resulting in a larger percent difference between the two systems.

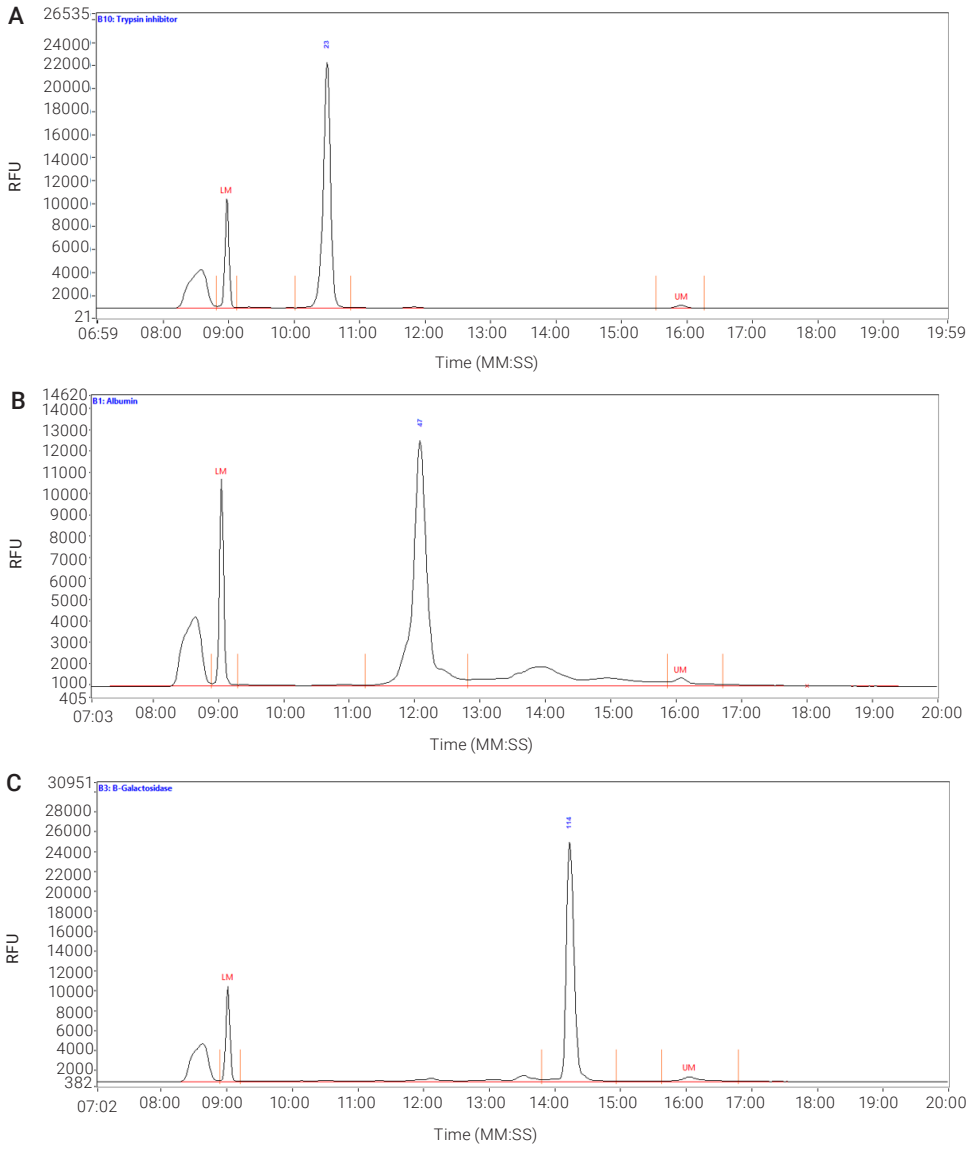

Figure 2. Electropherogram images of representative proteins assessed on the Agilent ProteoAnalyzer system under reduced conditions with the LM & UM method. A) Trypsin inhibitor. B) Albumin. C) β-Galactosidase.

Table 2. Protein sizing accuracy. The average size of commercially available proteins reported by the Agilent ProteoAnalyzer system compared to SDS-PAGE and known molecular weight. N = 7-10 replicates per sample.

Protein	Expected Size (kDa)	SDS-PAGE Size (kDa)	SDS-PAGE %error	ProteoAnalyzer Size (with LM & UM) (kDa)	LM UM %error	% Difference to SDS	ProteoAnalyzer Size (with LM only) (kDa)	LM only %error	% Difference to SDS
Lysozyme	14.3	13	9.09	14.14	1.10	8.42	14.00	2.10	7.41
β-Lactoglobulin	18.4	16	13.04	20.33	10.51	23.85	20.00	8.70	22.22
Trypsin inhibitor	20.1	18	10.45	23.00	14.43	24.39	23.00	14.43	24.39
Albumin	45	45	0.00	47.89	6.42	6.22	45.29	0.63	0.63
Phosphorylase b	97	91	6.19	107.57	10.90	16.69	100.00	3.09	9.42
β-Galactosidase	116	106	8.62	115.70	0.26	8.75	113.71	1.97	7.02
Myosin	220	248	12.73	NA	NA	NA	240.17	9.17	3.21

Reproducibility

The methods for the ProteoAnalyzer are preprogrammed to automatically clean and rejuvenate the capillaries in between separations, providing reliable data from run to run. To demonstrate this, several proteins were analyzed across subsequent runs. β-Lactoglobulin, albumin, β-Galactosidase, and myosin were analyzed three times in a row, and the sizes reported by the ProteoAnalyzer compared. For example, the reported size of albumin was identical at 48 kDa in each of the three runs, and β-Galactosidase demonstrated very close sizing across the three analyses, at 116, 115, and 116 kDa, respectively. As shown in Figure 3, the size of each sample remained very consistent across multiple separations, demonstrating the robust nature of the system.

Additionally, the system provides higher throughput than other CE-SDS systems, with the ability to analyze up to 12 samples in parallel. To demonstrate the reproducibility that can be achieved between capillaries in the same run, multiple proteins samples were analyzed in three individual capillaries simultaneously. The average size, standard deviation, and percent CV of each protein is shown in Table 3. The %CV values were less than 2.8 for each protein, with many of them being 0, far below the specifications of the kit using the model proteins. Together, this data shows the excellent sizing precision achieved with the ProteoAnalyzer and highlights the reproducibility of the system.

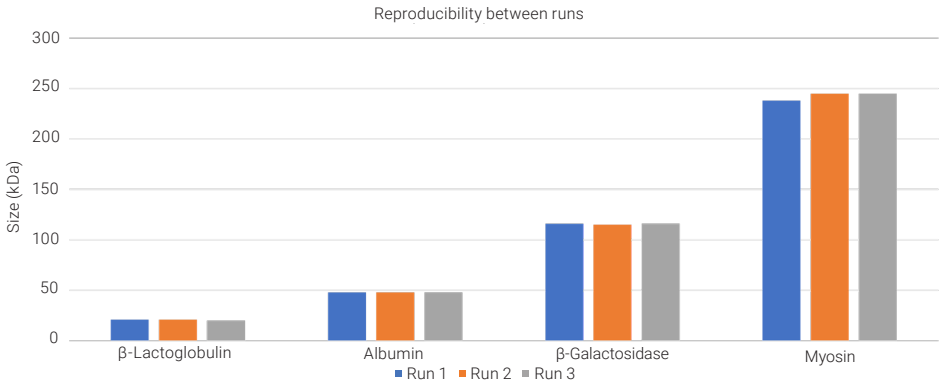

Figure 3. Run-to-run reproducibility. All proteins were analyzed with the Agilent ProteoAnalyzer system under reduced conditions with the LM & UM method, except for myosin, which used the LM-only method.

Table 3. Well-to-well reproducibility. N = 3. All proteins were analyzed with the Agilent ProteoAnalyzer system under reduced conditions with the LM & UM method, except for myosin, which used the LM-only method.

Protein	Average size (kDa)	Standard Deviation	%CV
Lysozyme	14.00	0.00	0.00
β-Lactoglobulin	20.33	0.57	2.84
Trypsin inhibitor	23.00	0.00	0.00
Albumin	48.00	1.00	2.08
Phosphorylase b	108.00	0.00	0.00
β-Galactosidase	115.67	0.57	0.50
Myosin	237.75	1.73	0.73

Enzyme analysis

Several commercially available enzymes were also assessed on the ProteoAnalyzer to demonstrate the capabilities of the system to size other sample types. Due to the proprietary nature of the enzymes, the exact size is unknown. The apparent size was estimated using SDS-PAGE under reduced conditions for comparison to the size reported by the ProteoAnalyzer (Table 4). Several of the enzymes displayed multiple bands on the SDS-PAGE gel and the ProteoAnalyzer digital gel images. The average size of the prominent bands from the ProteoAnalyzer was compared to the size of the corresponding band on the SDS-PAGE gel. The difference in sizing for each fragment was less than 15% between the two systems. In many cases, the ProteoAnalyzer was also able to identify the presence of other impurity peaks not detected on the SDS-PAGE gels, as shown on the representative electropherogram images of the samples, demonstrating the sensitivity and resolution of the system (Figure 4).

Table 4. Sizing of commercially available enzymes was compared between SDS-PAGE and the Agilent ProteoAnalyzer system.

Enzyme	Band	SDS-PAGE (kDa)	ProteoAnalyzer (kDa)	StDev	%CV	% Difference
ExoI	1	51	52.5	0.46	0.9%	3%
	2	66	68.7	0.49	0.7%	4%
	3	n.d.	148.1	0.81	0.5%	n/a
Optimase	1	57	52.5	0.17	0.3%	8%
	2	92	87.9	0.50	0.6%	5%
T7E1	1	66	71.1	0.76	1.1%	7%
	2	n.d.	147.3	0.60	0.4%	n/a
Phire II	1	8	9.2	0.10	1.1%	14%
	2	67	69.2	0.75	1.1%	3%
	3	99	94.1	0.75	0.8%	5%
	4	n.d.	147.8	0.32	0.2%	n/a
Taq	1	90	83.6	0.53	0.6%	7%
GoTaq	1	90	83.2	0.21	0.3%	8%
MssI	1	25	22.9	0.10	0.4%	9%
	2	27	30.1	0.17	0.6%	11%
	3	64	68.9	0.26	0.4%	7%
	4	n.d.	148.2	0.40	0.3%	n/a
Eco32I	1	27	27.2	0.12	0.4%	1%
	2	64	70.1	0.59	0.8%	9%
	3	n.d.	148.9	1.03	0.7%	n/a
BsuRI	1	65	68.2	0.21	0.3%	5%
	2	n.d.	148.9	0.35	0.2%	n/a

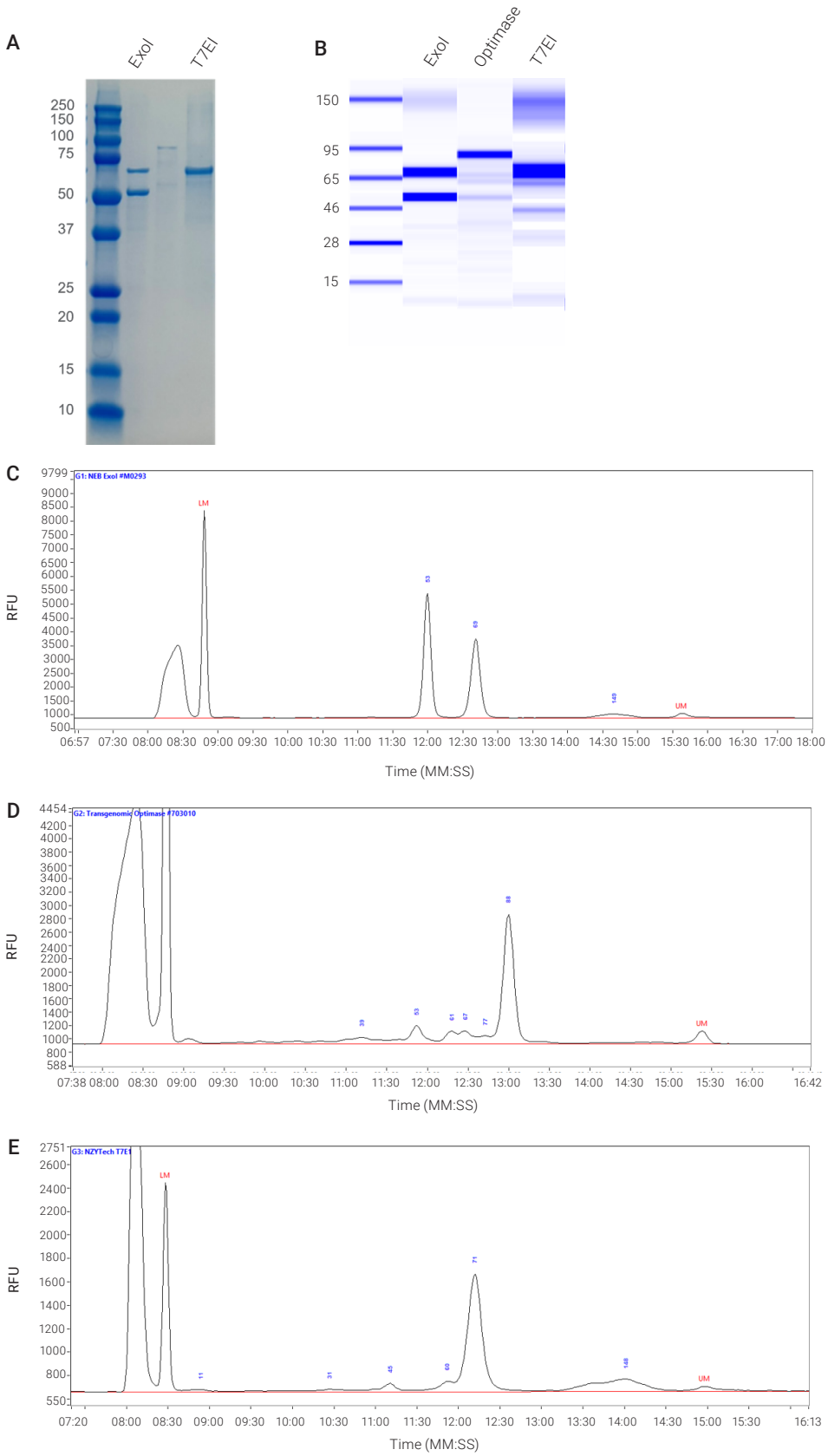

Figure 4. Representative examples of commercially available enzymes analyzed on A) SDS-PAGE and on B) the Agilent ProteoAnalyzer system for comparison. The ProteoAnalyzer data is displayed as B) a digital gel image of all samples, or individual electropherograms of C) ExoI, D) Optimase and E) T7EI. N = 3.

Conclusion

This technical overview described the sizing analysis of several commercially available proteins and enzymes on the Agilent ProteoAnalyzer system, an automated CE-SDS platform. While characteristics such as the amino acid sequence, charge, and hydrophobicity can impact sizing analysis, this overview provided examples of accurate and reliable analysis of many different proteins. The ProteoAnalyzer and SDS-PAGE both provided sizes close to the expected molecular weight of the protein. In some cases, the ProteoAnalyzer also revealed extra impurity peaks that were not visible on the SDS-PAGE gels, providing customers with valuable purity information for their samples. Further, the data presented here demonstrates that the system is highly robust, providing reliable precise data across each capillary as well as between runs, giving users confidence to use the ProteoAnalyzer system in higher-throughput protein analysis workflows.

References

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