Analysis of Cas9 by capillary electrophoresis sodium dodecyl sulfate (CE-SDS) and imaged capillary isoelectric focusing (icIEF) using Maurice

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Introduction

CRISPR/Cas9 has become a revolutionary tool for precise genome editing in a wide variety of prokaryotes and eukaryotes, and multiple Cas9 variants have been engineered to further broaden its functionality. Purity, heterogeneity and stability are critical properties in the biophysical characterization, chemical modification and structural investigation of Cas9 and its variants. At the forefront of techniques to monitor these properties are capillary electrophoresis sodium dodecyl sulfate (CE-SDS) and capillary isoelectric focusing (cIEF). Maurice™ from ProteinSimple enables both CE-SDS and imaged cIEF (icIEF) in a single unit, with several key advantages over other CE-SDS and cIEF systems, such as ease-of-use, high data quality, and speed. In addition, the highly sensitive native fluorescence whole column detector affords analysis of low concentration samples.

In this study, Cas9 samples were obtained from three vendors and analyzed by CE-SDS and icIEF on Maurice. For all three samples, purity and charge heterogeneity of the Cas9 samples were evaluated. The impact of stress on sample stability was also evaluated. These results demonstrate that Maurice is a powerful tool for monitoring purity, heterogeneity and stability of Cas9.

Materials and Methods

Instruments: Maurice CE instrument, icIEF cartridge and CE-SDS cartridge.

Maurice icIEF has two whole-column detectors: 1) UV absorption at 280 nm and 2) fluorescence excited at 280 nm, that detects native fluorescence emission of proteins.

icIEF cartridge

CE-SDS cartridge

Samples and Reagents:

1. Cas9 proteins were purchased from three vendors, labeled as Vendor A, Vendor B, and Vendor C.
2. Reagents used for Maurice icIEF analysis: Maurice cIEF Method Development Kit (ProteinSimple), 1% methyl cellulose, (ProteinSimple). pI marker 7.05 and pI marker 10.17 (ProteinSimple), Urea (Electrophoresis grade), Pharmalytes 3-10 (GE Healthcare), and Arginine (500 mM solution prepared in deionized water).
3. Reagents used for Maurice CE-SDS analysis: Maurice CE-SDS Application Kit (ProteinSimple), SDS Sample Buffer (ProteinSimple), Molecular weight standard ladder (ProteinSimple), Molecular weight internal standard (IS, ProteinSimple), Iodoacetamide (IAM), 250 mM solution prepared in deionized water, and β-mercaptoethanol (β–ME).

Methods

icIEF Method: Cas9 proteins (~0.02 mg/mL) were diluted in 4% Pharmalytes (3-10), 8M Urea with pI markers 7.05 and 10.17.

Samples were focused at 1.5 kV for 1 min, followed by 3 kV for 8 min.

CE-SDS Method: Cas9 proteins, both reduced (R) and non-reduced (NR), were run at 0.4 mg/mL for Vendors A and B, 0.08 mg/mL for Vendor C (due to its low sample concentration), using EK sample injection (20 seconds at 4600 V) followed by separation at 5750 V for 40 minutes.

Results and Discussion

icIEF: Fluorescence improves Cas9 Detection

The Cas9 samples obtained for the study came at relatively low concentrations, ranging from 0.2–2 mg/mL. The samples were further diluted by 10–20X into the final sample solution in order to control the final salt concentration. At these final Cas9 concentrations, the sensitivity of the UV absorption detector was insufficient to accurately distinguish Cas9 from the baseline noise (**Figure 1A**). Concentrating the sample can be a challenge, due to the relatively low stability of the protein (as shown later).

In addition to absorbance, Maurice icIEF is equipped with a UV fluorescence whole-column detector that can detect the native fluorescence emission of proteins when excited at 280 nm, with 3-5X higher sensitivity compared to absorbance. Using fluorescence detection, the Cas9 samples could be analyzed (**Figure 1B**).

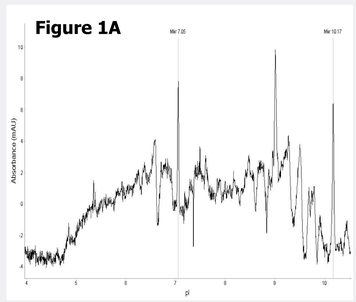
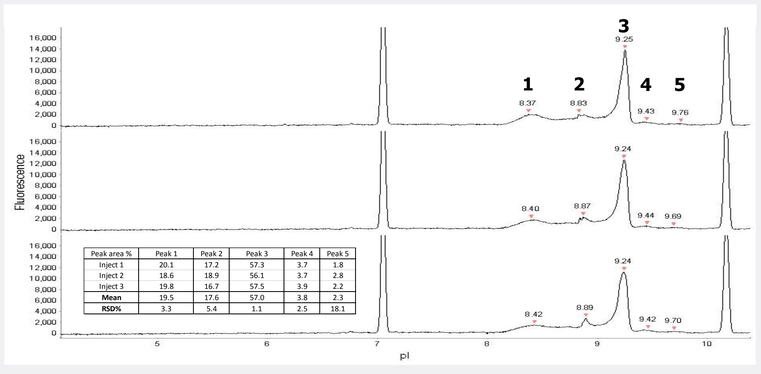


Figure 1. Example of Cas9 absorption (A) and fluorescence (B) icIEF e-grams from Vendor A.

icIEF: Cas9 Method gives Reproducible Results



Peak area %	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
Inject 1	20.1	17.2	57.3	3.7	1.8
Inject 2	18.6	18.9	56.1	3.7	2.8
Inject 3	19.8	16.7	57.5	3.9	2.2
Mean	19.5	17.6	57.0	3.8	2.3
RSD%	3.3	5.4	1.1	2.5	18.1

Figure 2. Reproducibility of fluorescence detection of Cas9. As an example, three injections of the Cas9 obtained from Vendor B are shown to demonstrate the reproducibility of the method. Inset: Relative % peak areas, mean and %RSD for each Cas9 peak.

icIEF: Comparison of Charge Heterogeneity Profiles of Cas9 from 3 Vendors

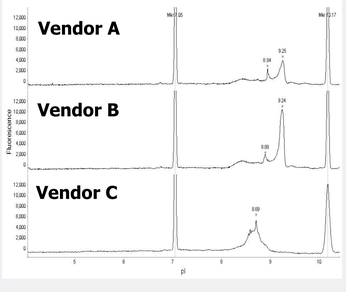


Figure 3. Comparison of Cas9 obtained from three commercial sources.

Different charge heterogeneity profiles were obtained by Maurice icIEF for the various commercially available Cas9 proteins.

icIEF: Analysis of Cas9 Protein Stability

The icIEF method can be used as a protein stability indicator. The Cas9 proteins were heat stressed by incubation at room temperature for 3 hours before analysis. For all three Cas9 proteins, significant changes in the peak profiles were observed.

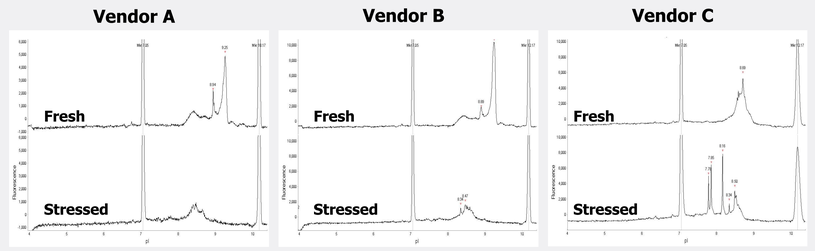


Figure 4. Comparison of Cas9 from three vendors, before and after stressing Cas9 proteins for 3hrs at room temperature.

CE-SDS: Cas9 Purity and Apparent Molecular Weight Analysis

CE-SDS is used to detect protein purity and size heterogeneity. The Cas9 proteins were run under non-reducing (NR) and reducing (R) conditions, and analyzed on Maurice by CE-SDS alongside the Molecular weight ladder (L), shown in **Figure 5**. The purity and apparent molecular weight of Cas9 obtained from the three vendors are shown in **Table 1**. The apparent molecular weight determined by the CE-SDS is consistent with the reported values (~160 kDa).

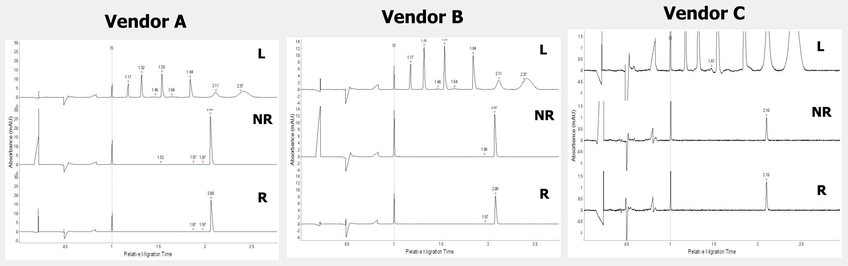


Figure 5. Non-reduced and reduced CE-SDS analysis of the Cas9 from the three vendors.

	Apparent MW (kDa)		Purity (%)	
	Non-reduced	Reduced	Non-reduced	Reduced
Vendor A	162	164	98.50	98.60
Vendor B	165	167	99.30	99.50
Vendor C	176	174	*	*

Table 1. Measurements of apparent molecular weight and purity for Cas9 proteins.
*indicates concentration too low for purity analysis.

Conclusions

- The icIEF and CE-SDS methods for Cas9 analysis were optimized on Maurice.
- For analysis of Cas9 by icIEF, the enhanced sensitivity of the native fluorescence detection overcame challenges in detecting protein in low-concentration samples and eliminated the need to concentrate the samples.
- The icIEF method was reproducible, and could be used as a stability indicator for Cas9.
- Differences in the charge profile between Cas9 from the three vendors were observed.
- Maurice CE-SDS determined apparent molecular weight values of Cas9 that were consistent with reported values. Purity of Cas9 from different vendors was determined quantitatively.