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A Novel Derivatization-Based CGE Assay for Quantifying Free Proteins in Conjugate Vaccines

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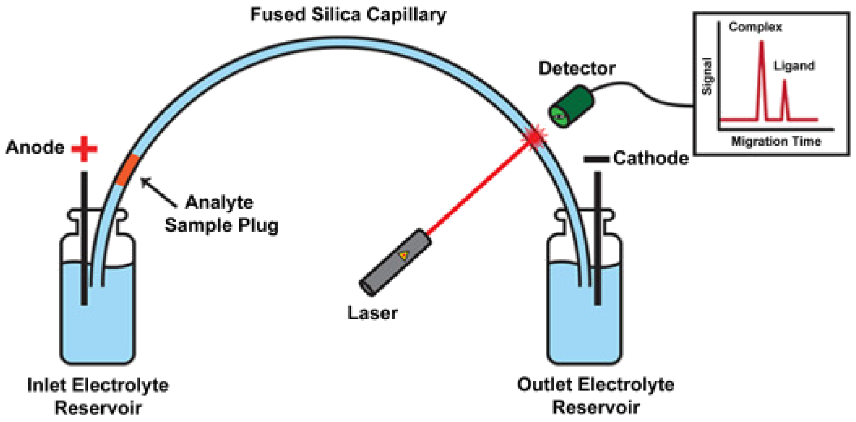
Outline

- ❑ General Introduction of CE
- ❑ History of Conjugated Vaccine
- ❑ Limitation of the historical method
- ❑ Development of the derivatization-based CGE method
- ❑ Assay qualification
- ❑ Complex sample matrix screening
- ❑ Summary

General mechanism of capillary electrophoresis (CE)



- Bare fused silica capillary (or with other coatings)
- Internal diameter: 20 to 200 µm
- Length usually 25-50 cm
- Normal polarity means
  - Left: Anode (positive)
  - Right: Cathode (negative)

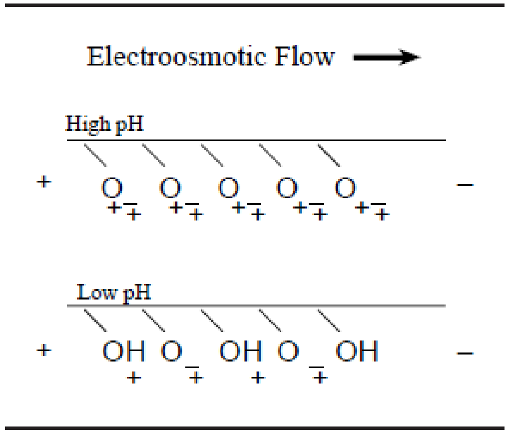
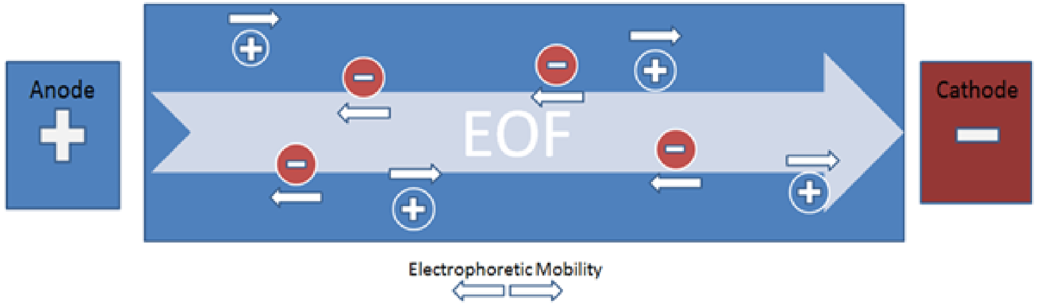
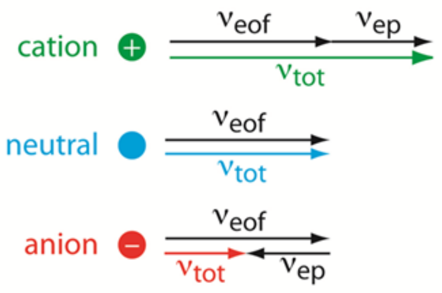


Figure 2. Effect of pH on the Electroosmotic Flow

- Electrical double layer
- Liquid gets dragged as cation moves towards cathode
- Robust, fast electroosmotic flow highly dependent on pH of BGE





$$v_{tot} = v_{eof} + v_{ep}$$

$$\mu_{i,app} = \frac{V_i}{E} = \frac{L_d / t_m}{V / L_t}$$

Length of the capillary to the detector (m)

Migration time (s)

Total length of the capillary (m)

Voltage (V)

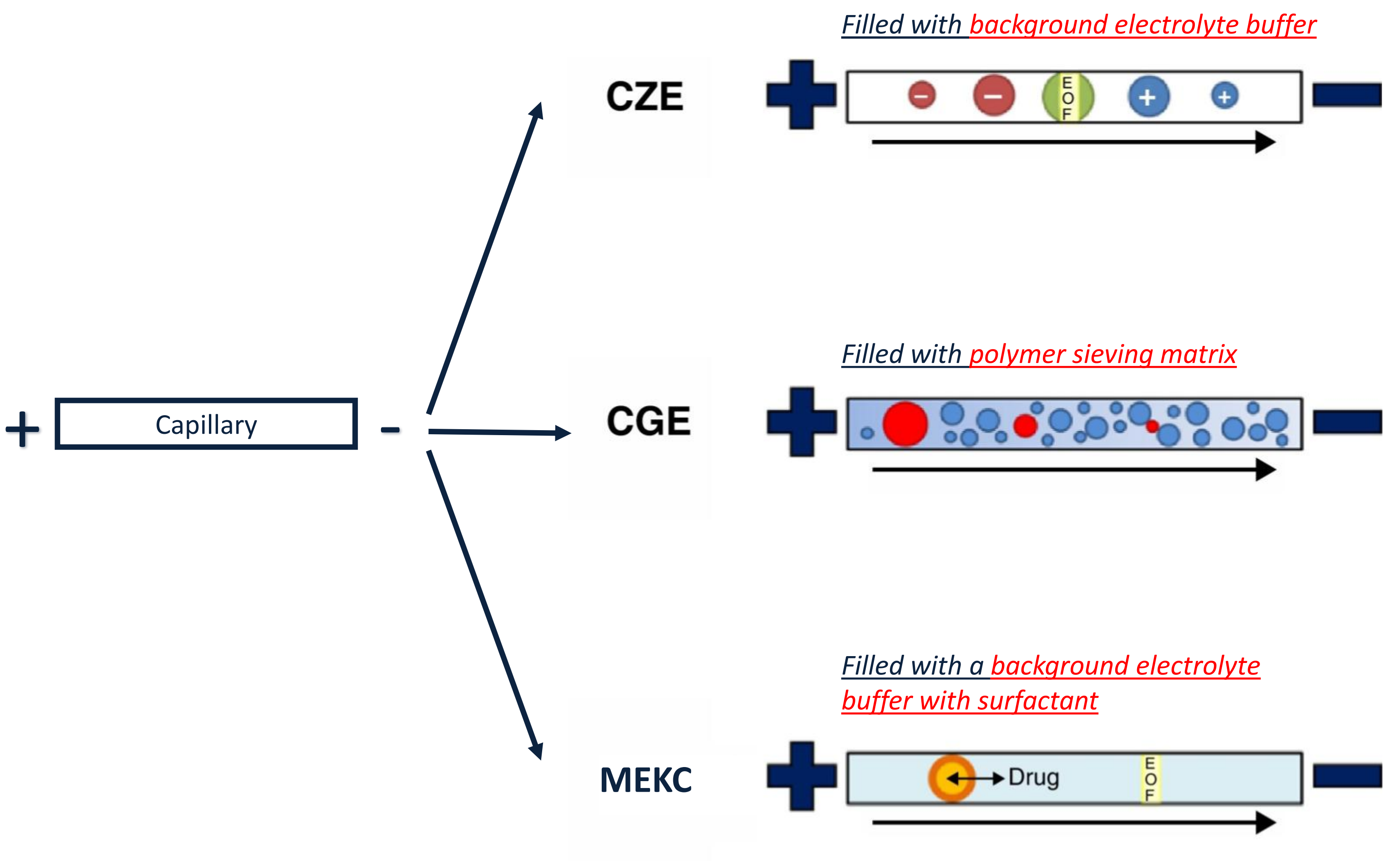
$$v_{eo} = \frac{\epsilon \zeta}{4\pi\eta} E$$

Dielectric constant

Zeta potential

Buffer viscosity

Main types of Electrophoresis



- Separate based on charge to size (friction) ratio

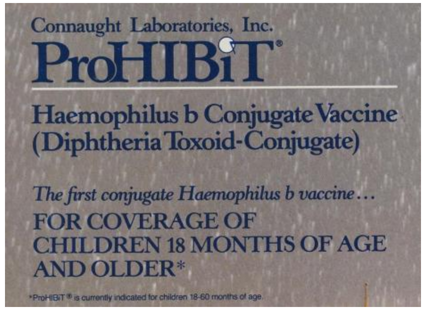
$$\mu \approx \frac{Charge}{Friction}$$

- Small ion quantification – K^+, Na^+, Mg^{2+}
- Large particle (VLP) separations

- Separate solely based on size
- Protein, DNA, RNA separation

- Separate based on the charge to size (friction) ratio and the partition between the analyte and the surfactant micelle
- Neutral species, chiral, protein separation

History of Conjugate Vaccine(s)…



1987

1989

PedvaxHIB®
[Haemophilus b Conjugate Vaccine (Meningococcal Protein Conjugate)]

2000

2005

Menactra®
Meningococcal (Groups A,C,Y and W-135) Polysaccharide Diphtheria Toxoid Conjugate Vaccine

2015
MenAfriVac

2024

CAPVAXIVE™
Pneumococcal 21-valent Conjugate Vaccine

- **Weak antigens (polysaccharides)** often fail to trigger a sufficient immune response, but conjugation to a highly immunogenic **protein carrier** enhances their effectiveness.
- This enables **T–B cell cooperation**, leading to **class switching, high-affinity antibodies, and memory B cell formation**.
- As a result, **infants and young children**—whose immune systems respond poorly to polysaccharides—can develop robust and lasting immunity.
- Detection of trace amounts of unconjugated protein remains challenging in complex multivalent vaccine products.

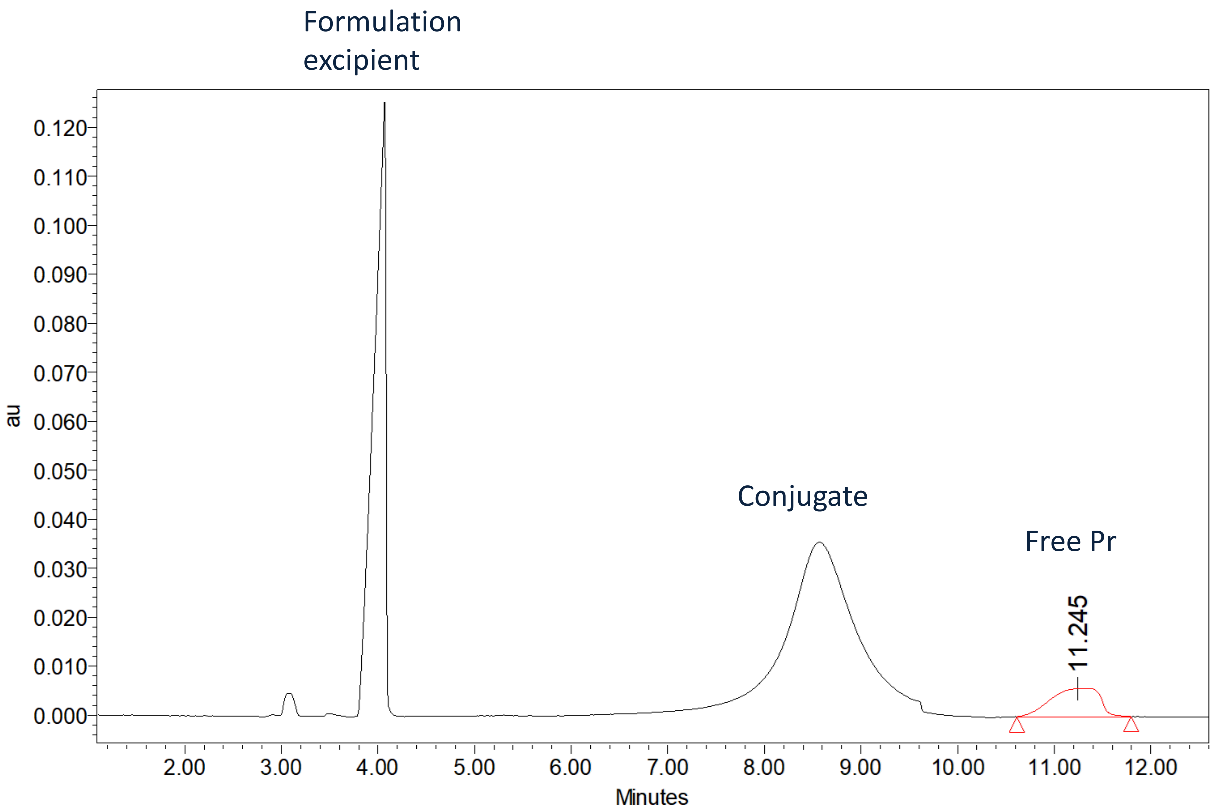
Limitations of the historical method…

MEKC assay for free Pr detection

- LOQ: 5ug/mL
- Only for mono-valent sample

New method requirement:

- LOQ at 0.1ug/mL
- Able to evaluate multi-valent sample

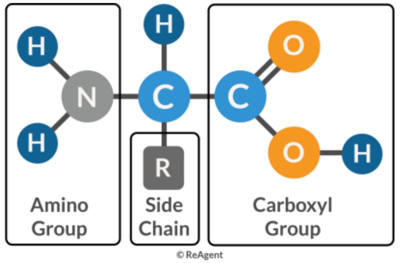


MEKC free Pr assay we have for DS

Fluoroprobe choice and Pr labeling strategy

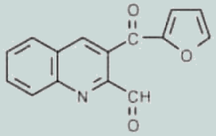
Pr derivatization strategy

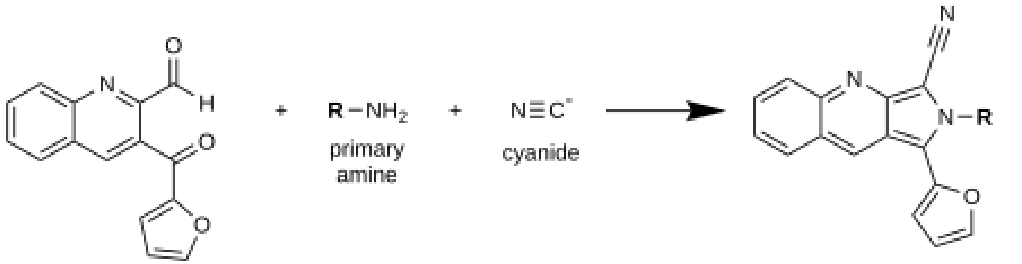
- Unreacted amino groups from **amino acids**



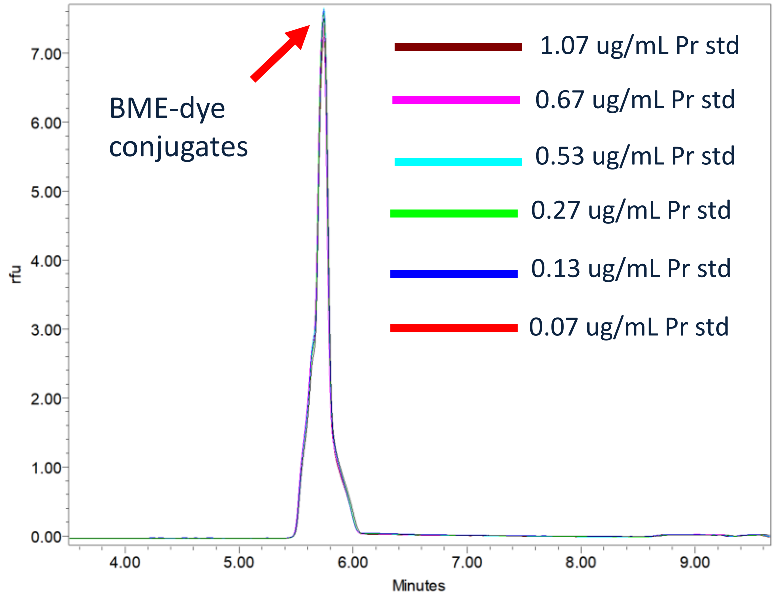
PA800 Laser Induced Fluorescence (LIF) detector

- 488nm laser
- 600nm ± 60nm bandpass filter manually installed

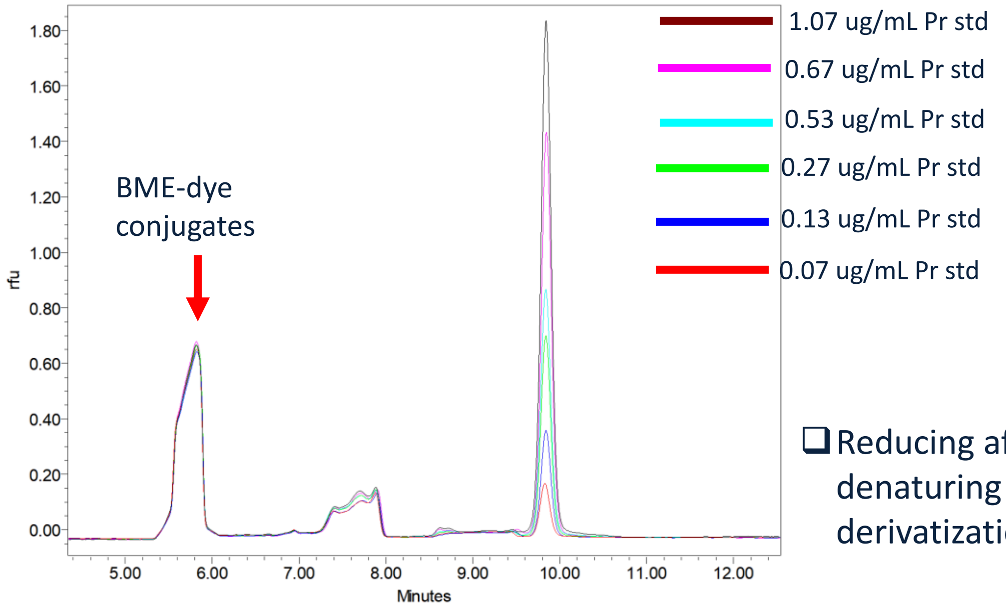
Dye	Structure	Excitation/Emission	Mw
ATTO-TAG dye		480/600	305.3



Where we started…



- Reducing after denaturing, ***before*** derivatization



- Reducing after denaturing and ***after*** derivatization

Initial conditions tested for pure Pr (PA800):

- Non-reduced;
- Reduction after denaturing and derivatization to avoid BME interference with dye;
- Conditions tested:

Denature
- **Heat**
- **SDS**

→

Reduction
- **BME**
- **Heat**

→

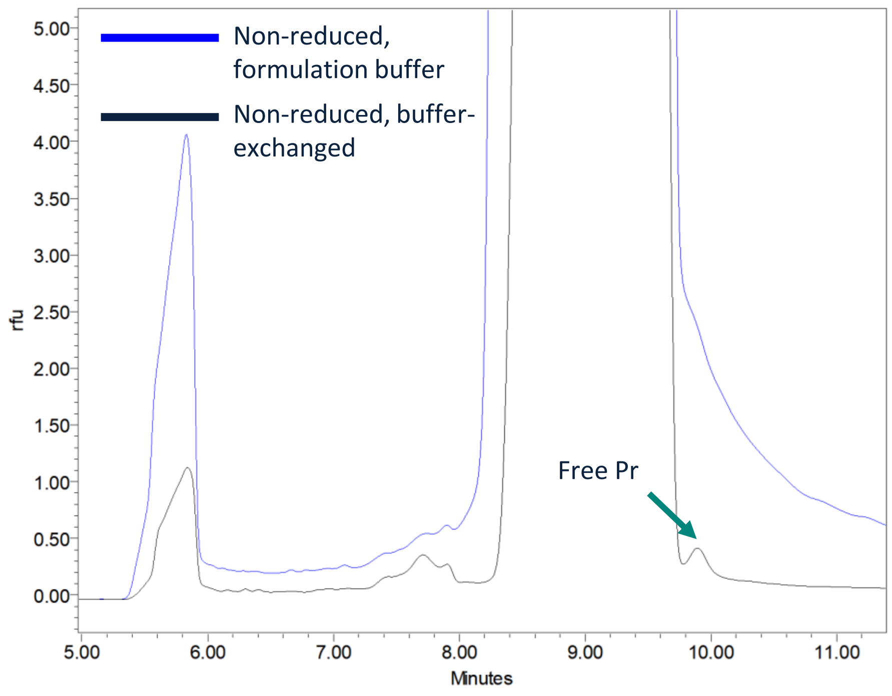
Derivatization
- **Heat**

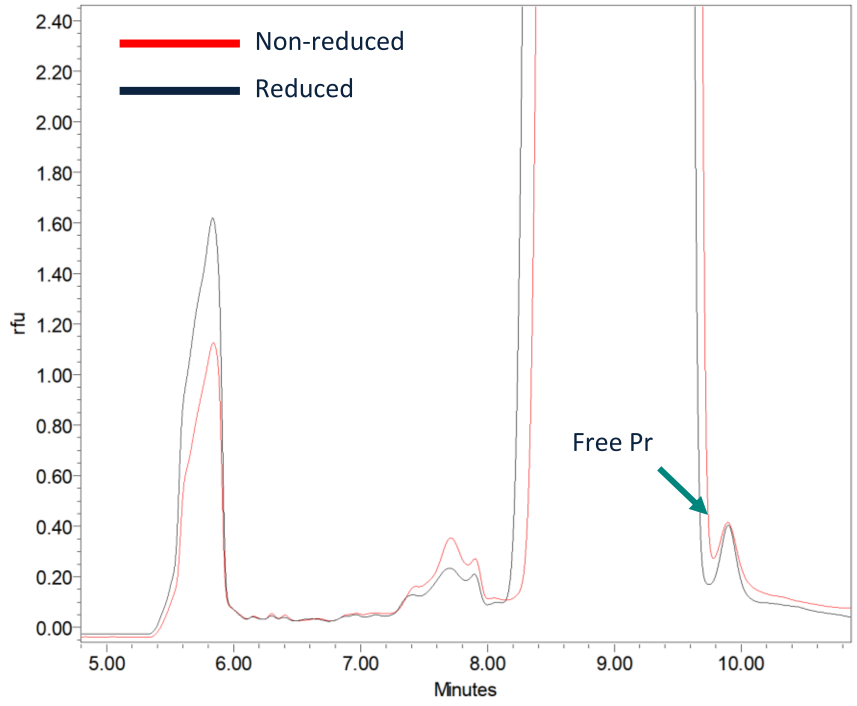
→

Separation
- **EK injection**
- **15 kV**

MERCK

Conditions screened…

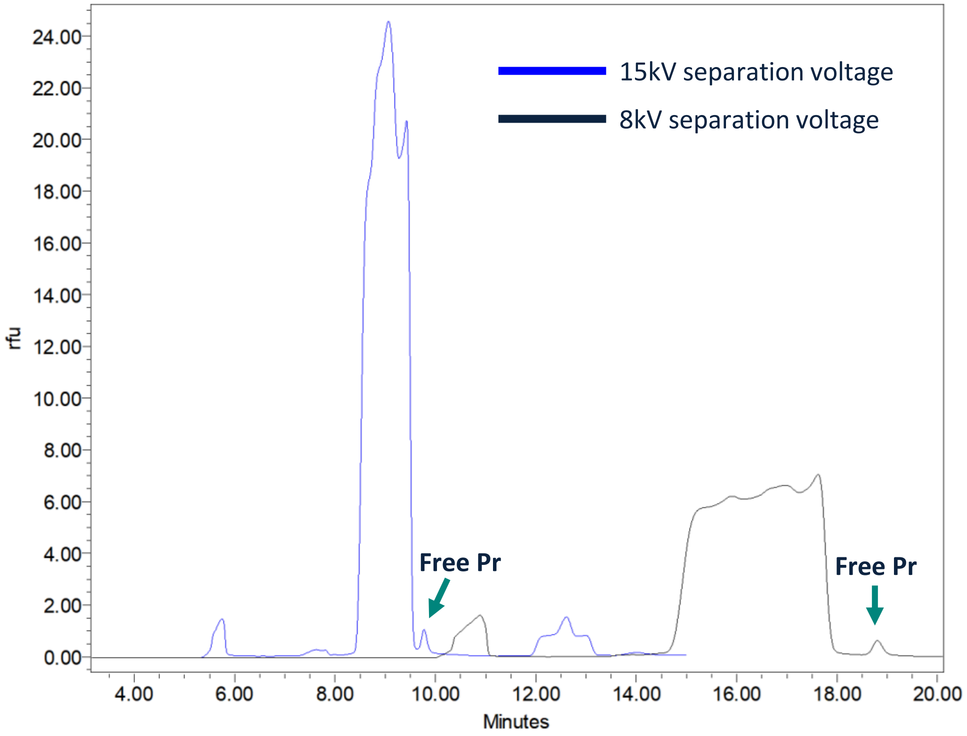


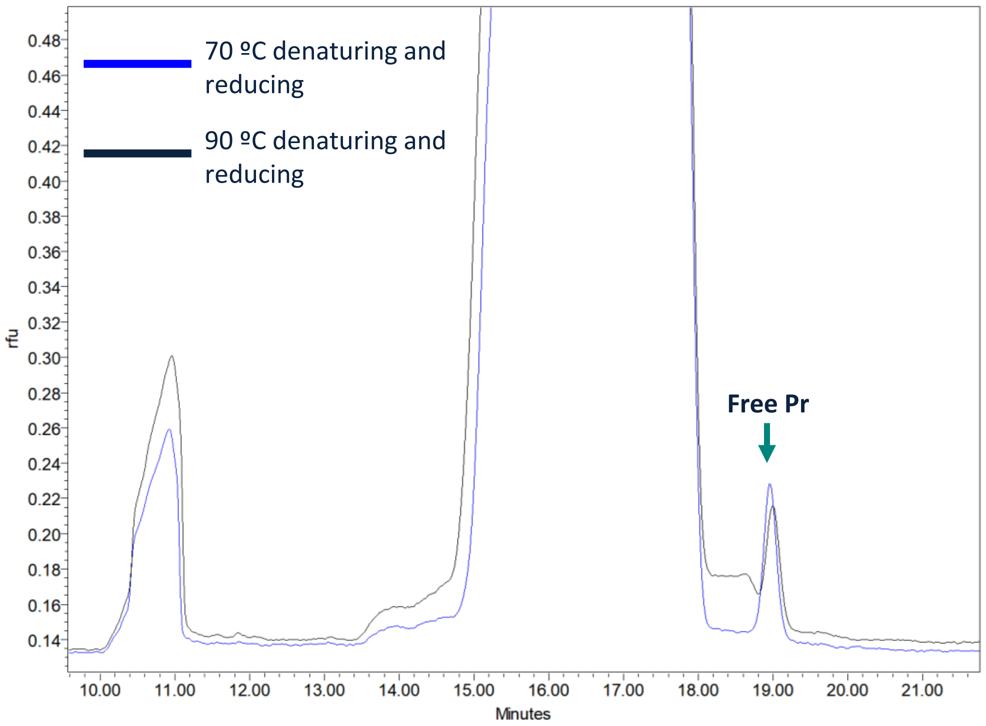


Conditions tested:

- Buffer-exchanged vs non-buffer-exchanged (Formulation buffer)
  - Buffer exchange required for removing the interference from the sample matrix
- Reduced vs non-reduced
  - Reducing condition using BME increases the resolution between the free Pr and the sample matrix peak

Conditions screened – cont'd…

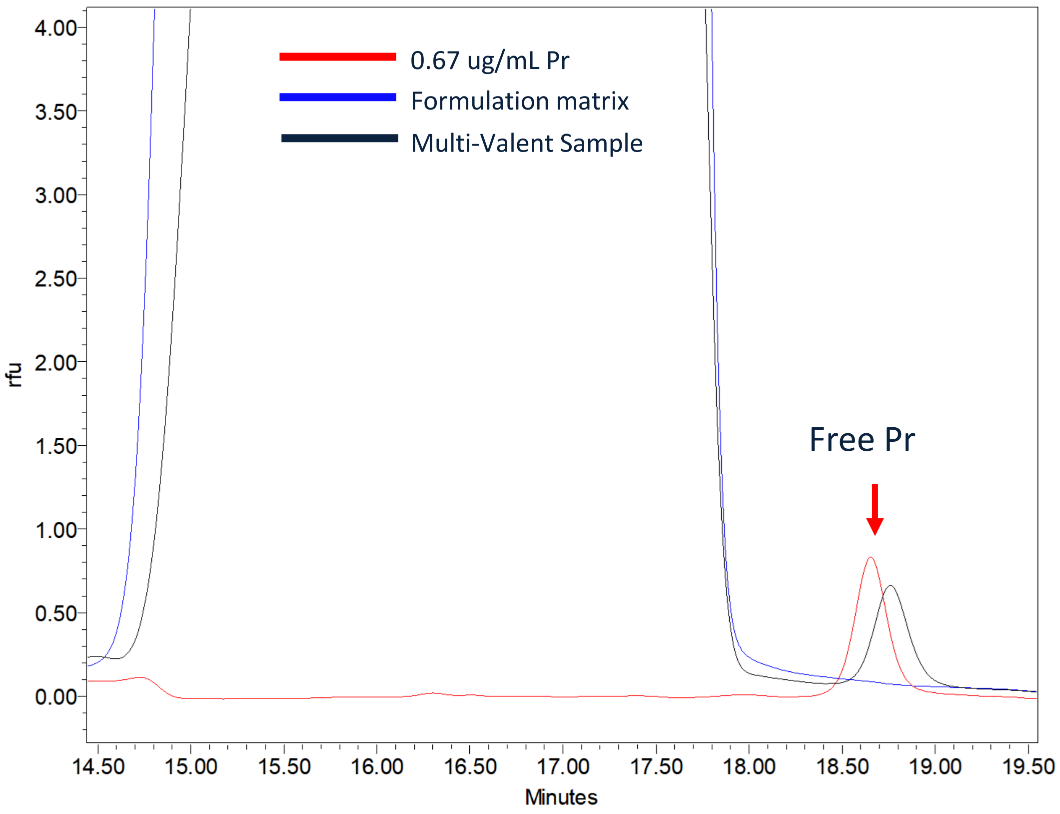




<u>Conditions tested:</u>

- 8kV vs 15kV separation voltage
  - Increased resolution at 8kV with limited increase in separation time
- Reducing and denaturing temperatures
  - Lower resolution in 90 ºC compared to 70 ºC

Final condition confirmed on CE-SDS



Final condition: (PA800)

Buffer Exchange → Denature
- Heat
- SDS

→ Derivatization
- Heat

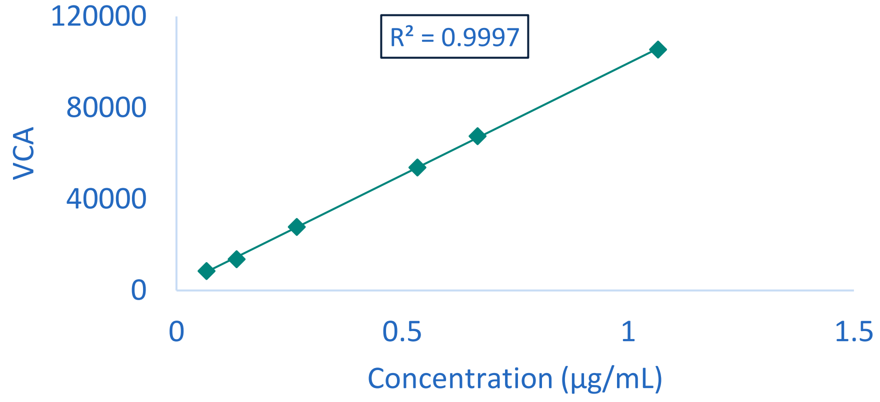
→ Reduction
- BME
- Heat

→ Separation
- EK injection
- 8 kV

Assay Qualification

Pure Pr linearity and sample dilutional linearity

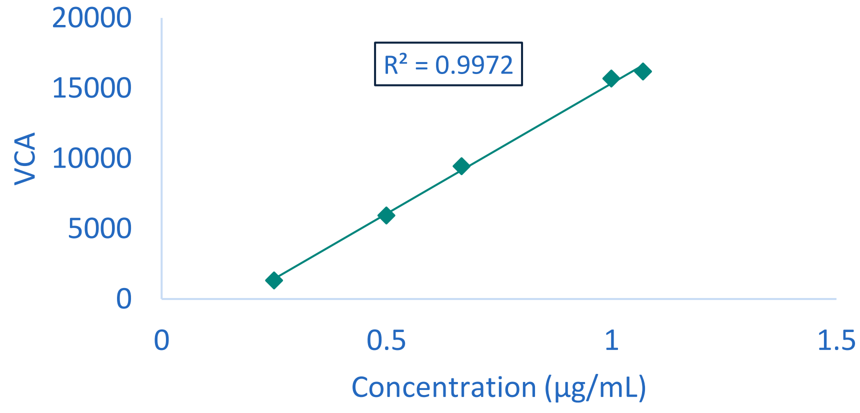
CALIBRATION CURVE



Pure Pr standard linearity

- Pure Pr diluted at 6 levels
- Range: 0.07 - 1.07 ug/mL
- R^2 = 1.000

DILUTIONAL LINEARITY



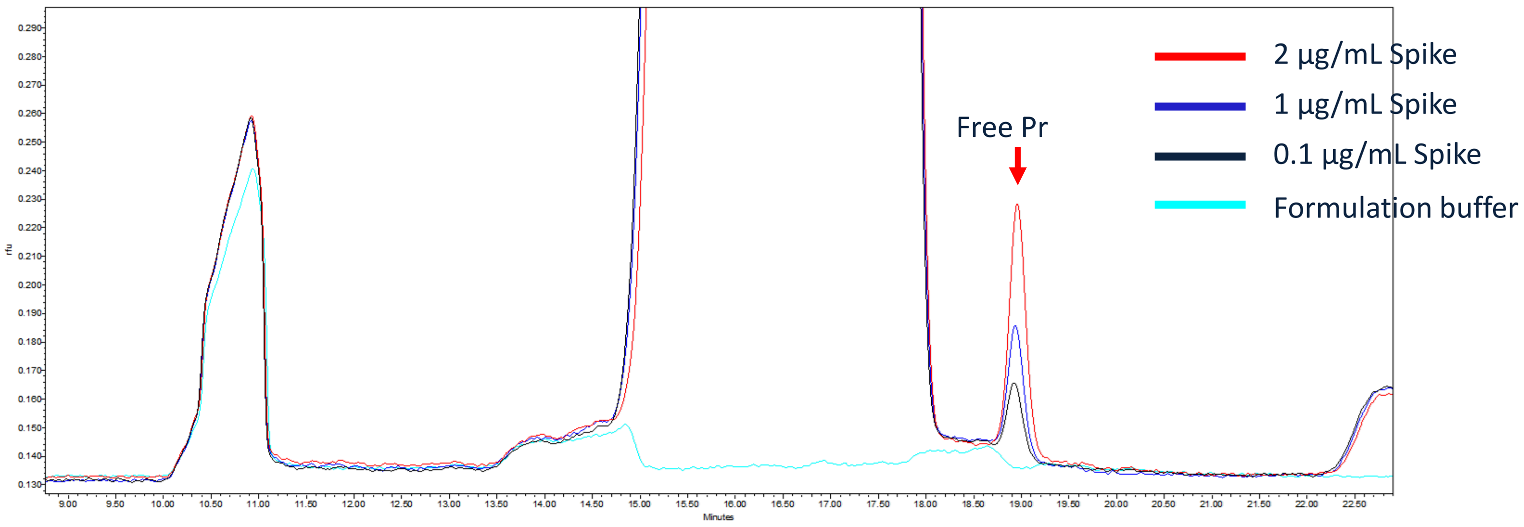
Multi-valent sample dilutional linearity

- Multi-Valent sample diluted at 5 levels
- Range: 25% formulated concentration (4x dilution) – 107% formulated concentration
- R^2 = 0.997

Accuracy

	Target (Theoretical) Spike Concentration (µg/mL)	Measured Free Pr concentration (µg/mL)	%recovery
0.1 µg/mL Spike	0.10	0.09	90%
1 µg/mL Spike	1.00	0.92	92%
2 µg/mL Spike	2.00	2.03	102%

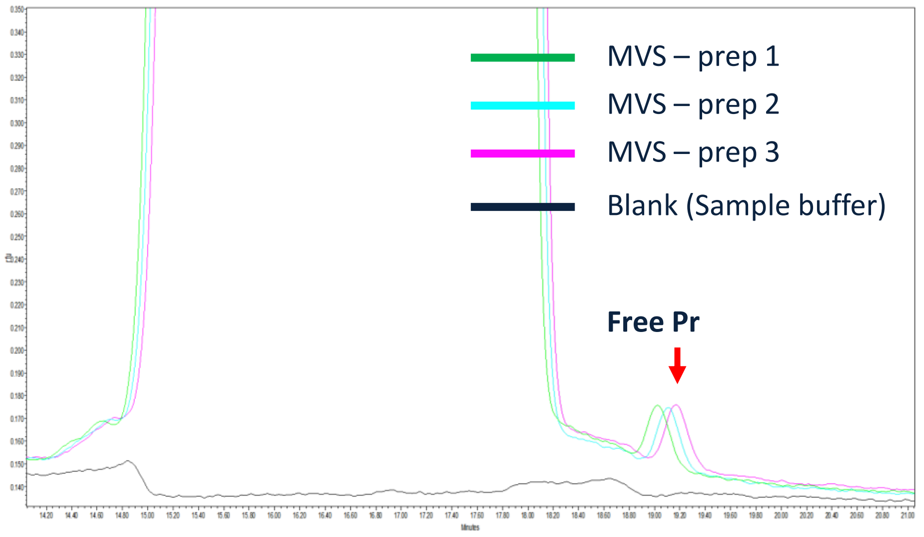
- Pure Pr was spiked into a control sample with an endogenous level of free Pr.
- Low/Med/High spike points were evaluated (0.1µg/mL/ 1µg/mL/ 2µg/mL)
- $\%Recovery = \frac{Spiked\ Sample - Control\ Sample}{Theoretical\ Free\ \mathrm{Pr}\ Spiked\ concentration} \times 100$
- 90-102% spiking recovery at the specific region with no interference from the formulation buffer, indicating good specificity.



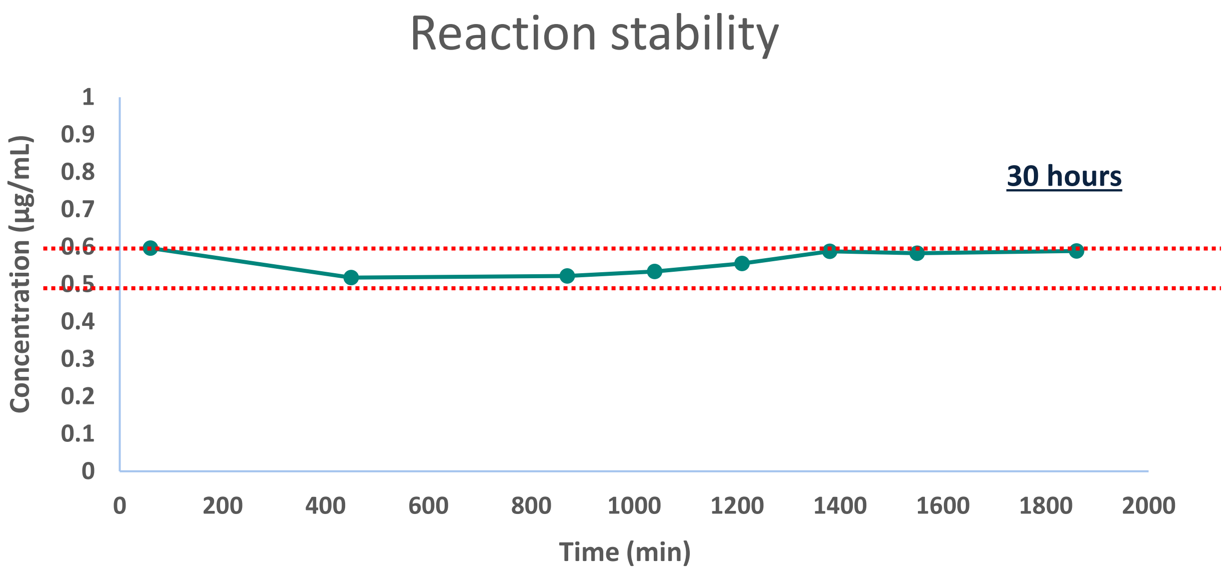
Repeatability and analyst variability

	Sample Name	Measured Free Pr concentration (µg/mL)	Average concentration (µg/mL)		Repeatability %RSD	Intermediate %RSD
Analyst 1	MVS – prep 1	0.52	0.54	0.57	2.6%	6.0%
	MVS – prep 2	0.53				
	MVS – prep 3	0.56				
Analyst 2	MVS – prep 1	0.61	0.60		1.5%	
	MVS – prep 2	0.60				
	MVS – prep 3	0.59				

- Multi-Valent Sample (MVS) was prepared three times and injected separately
- Both repeatability %RSD and analyst variability %RSD were evaluated and are both <10%
- Sample buffer matrix was derivatized and injected, no signal was observed at the position of interest



Derivatized sample stability over time



- Multi-Valent Sample (MVS) was injected one hour after the derivatization
- Same MVS was then repeatedly injected until 30 hours
- The average amount of free Pr is 0.56 ± 0.03 within 30 hours
- Derivatization is stable for at least 30 hours with a 5.4% concentration fluctuation

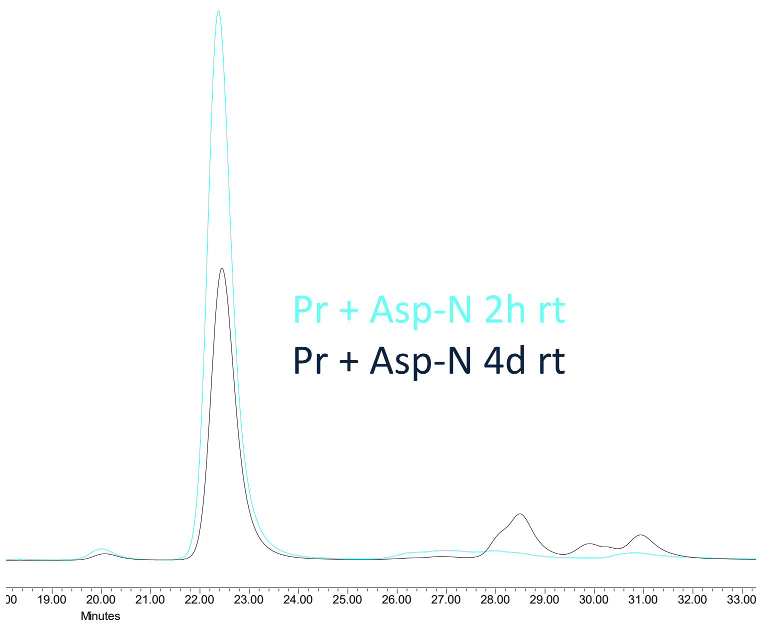
Assay qualification result

	Target Method Performance	Results
Linearity	The reference standard curve, $R^2 \geq 0.980$	$R^2 = 1.000$
Dilutional Linearity	The plot of peak area (of diluted samples) vs 1/dilution factor, $R^2 \geq 0.970$	$R^2 = 0.997$
Accuracy	50% - 200% at each level	90 – 102%
Precision	Intra-run precision, ≤ 30% RSD	2.6% RSD
Intermediate Precision	Intermediate precision must be ≤ 40% RSD.	Intermediate precision is RSD <10%.
Specificity	No peaks present in the placebo sample electropherograms that are greater than or equal to the lowest calibration standard of 0.10 µg/mL	Specificity is confirmed based on the absence of peaks in the placebo sample electropherogram, and also through the 90%-102% spiking recovery.
Range	The range of the assay will be determined from precision, accuracy and linearity results.	0.10 µg/mL – 1.07 µg/mL
Limit of Quantitation (LOQ)	The LOQ must meet the acceptance criteria for linearity, accuracy and precision.	0.10 µg/mL
Derivatization Stability	N/A	Stable for at least 30 hours

Enzymatic Degradation w/ Asp-N

Enzymatic conditions Asp-N (~ 24.5 kDa)

- Enzymatic digestion (Asp-N) is another option as observed degradant peaks w/ SEC
- Slow degradation at rt using recommended enzyme conc and Asp-N:Pr ratios (0.04 ug/uL, ~1:200)

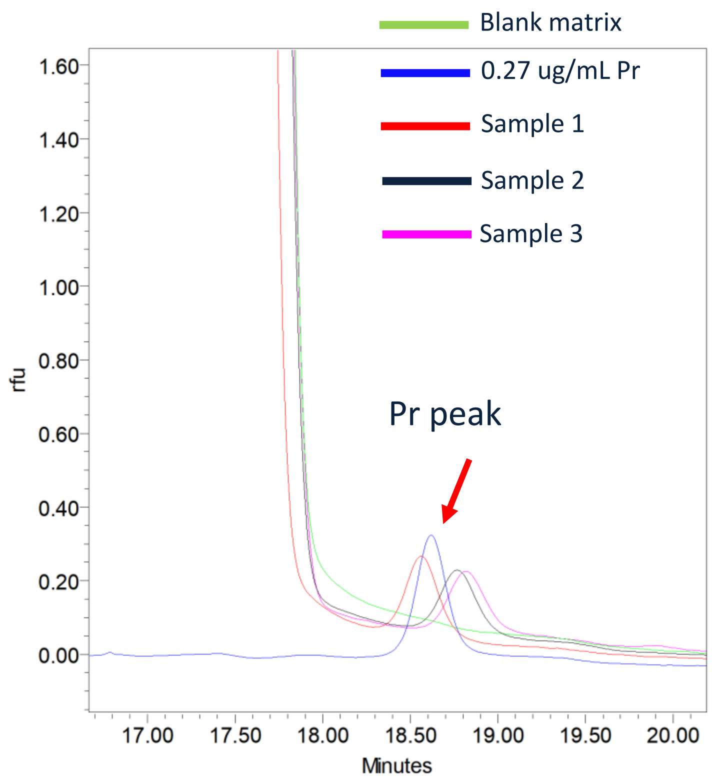


Sample	Theoretical conc (mg/mL)	SEC-FLD		CE-SDS	
		Exp conc (mg/mL)	% control	Exp conc (mg/mL)	% control
Pure Pr	0.89	0.90	N/A	0.89	N/A
Pure Pr + Asp-N T=2.5d, rt	N/A	0.79	89%	0.77	86%

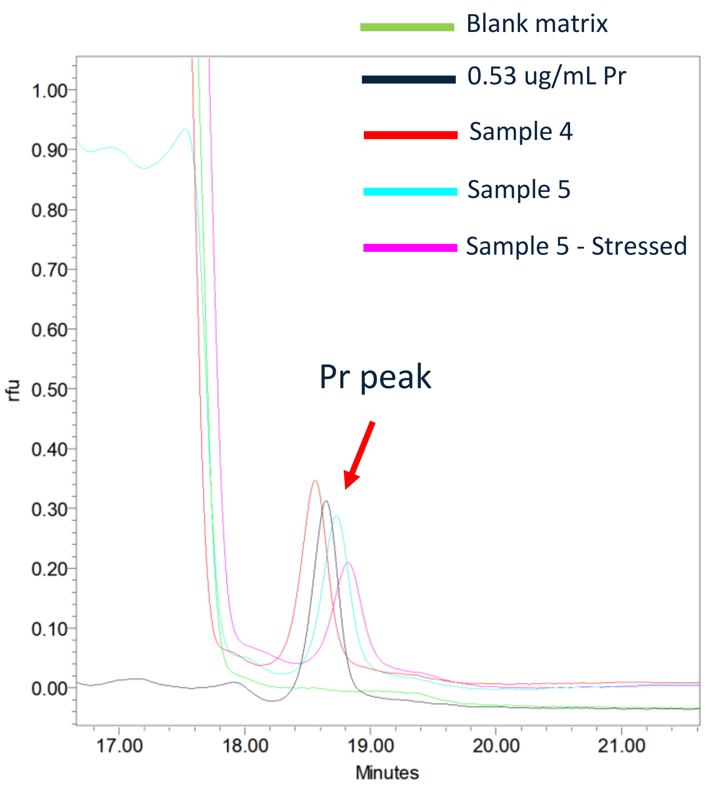
Trending:
- SEC-FLD and CE-SDS methods reveal similar % loss of Pr in degradation sample relative to control

Absolute values (accuracy):
- SEC-FLD agrees with CE-SDS on the free Pr degradation degree
- rCE-SDS is able to detect degradation of free Pr (Only if the decreasing amount is higher than the intermediate %RSD)

Complex multi-valent samples screening…



Multi-valent samples 1/2/3



Multi-valent samples (4 and 5)

	Free Pr conc (ug/mL)
Sample 1	0.57
Sample 2	0.45
Sample 3	0.42
Sample 4	1.75
Sample 5	1.70
Sample 5 – Stressed	1.15

- Successfully quantify the amount of free Pr at ng/mL level in various multi-valent samples.
- Assay is able to detect the stability change upon stressed condition.

Summary

•Key Features:

- Derivatization with ATTO-TAG FQ dye for enhanced sensitivity (LOQ 0.10 µg/mL)
- Capillary Gel Electrophoresis (CGE) separation optimized for complex samples
- Suitable for multi-valent drug formulations

•Performance Highlights:

- Excellent linearity ($R^2 > 0.999$) and accuracy (90–102% recovery)
- High precision (%RSD <10%) and specificity (no placebo interference)
- Stability of derivatized samples confirmed for 30+ hours
- Detects protein degradation

•Impact:
Enables characterization of the free Pr in the conjugate vaccines with improved sensitivity and applicability over previous assays.