Electronic Supplementary Material (ESI) for Analytical Methods.

Electronic Supplementary Information

Analysis of therapeutic monoclonal antibodies by imaged capillary isoelectric focusing (icIEF)

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Table S1 Details of 11 commercially available therapeutic mAbs

Generic Name	Brand Name	Subclass	Expiration Date
Adalimumab	Humira	IgG1	May 2018
Bevacizumab	Avastin	IgG1	Jul 2017
Ipilimumab	Yervoy	IgG1	Dec 2015
Omalizumab	Xolair	IgG1	Jul 2019
Pertuzumab	Perjeta	IgG1	Apr 2021
Tocilizumab	Actemra	IgG1	Aug 2019
Denosumab	Prolia	IgG2	Nov 2018
Panitumumab	Vectibix	IgG2	Oct 2015
Nivolumab	Opdivo	IgG4	Nov 2019
Pembrolizumab	Keytruda	IgG4	Feb 2022
Eculizumab	Soliris	IgG2/4	May 2018

Table S2 iCE3 system configuration

iCE Analyzer	
iCE Analyzer Model	iCE3
Lamp Type	Deuterium
Sample Transfer Time	70 sec
Autosampler	
Autosampler Model	720NV
Syringe Size	250 µL
Syringe Load Rate	5 µL/sec
Syringe Inject Rate	7 µL/min
Withdraw Rate	5 µL/sec
Load Duration	6 sec
Sample Injection Duration	70 and 90 sec
Transfer Time Delay	0 min
Rinse Rate	50 µL/sec
Tray Type	48 Vial Tray with 4 Rinse Vials
Needle Depth	48 mm

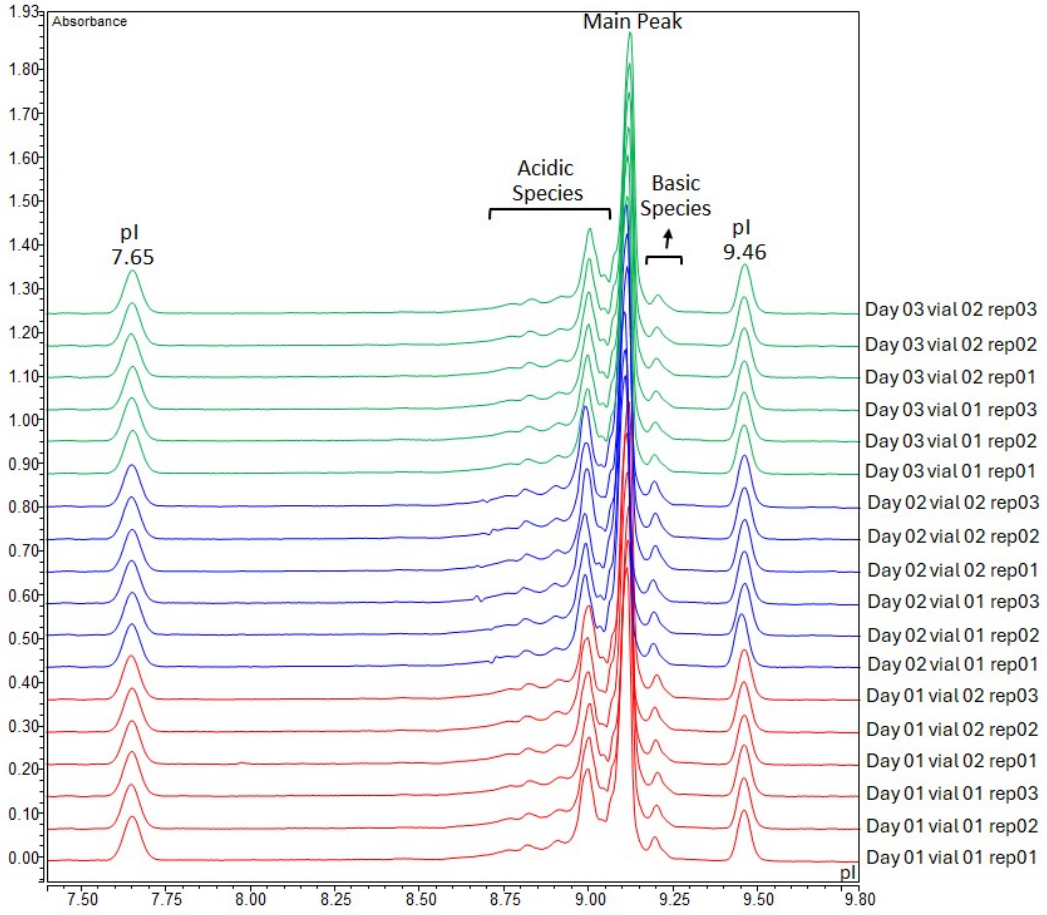

Fig. S1 Overlaid icIEF electropherograms of mAb-1 demonstrating reproducibility

(**Experimental conditions:** 5% Pharmalyte pH 3-10, mAb-1 concentration 0.4 mg/mL, focusing time: 8 min at 3 kV)

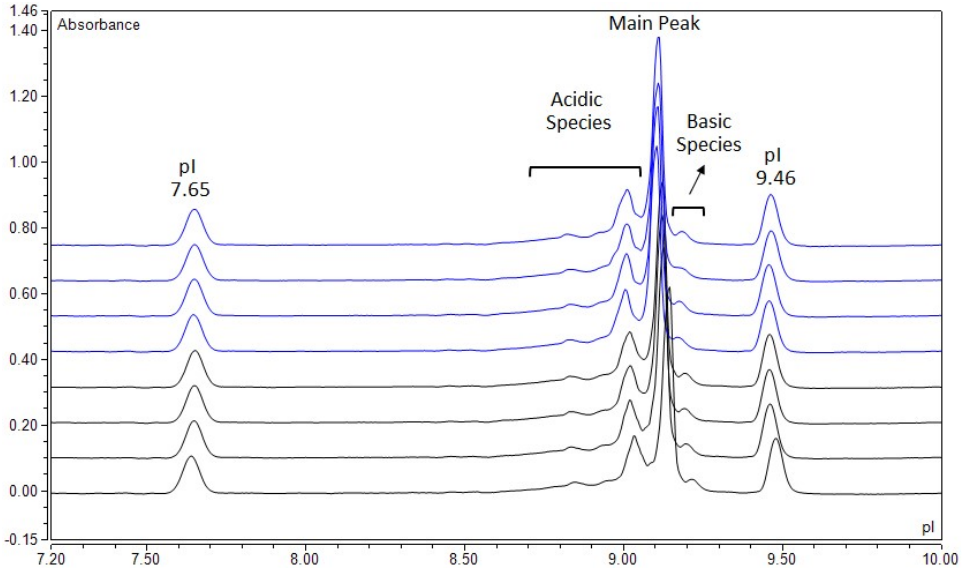

Fig. S2 Overlay of icIEF electropherograms of mAb-1 analyzed in replicates at two different time points: 0 hours (black traces) and 24 hours (blue traces).

(**Experimental conditions:** Same as Figure S1)

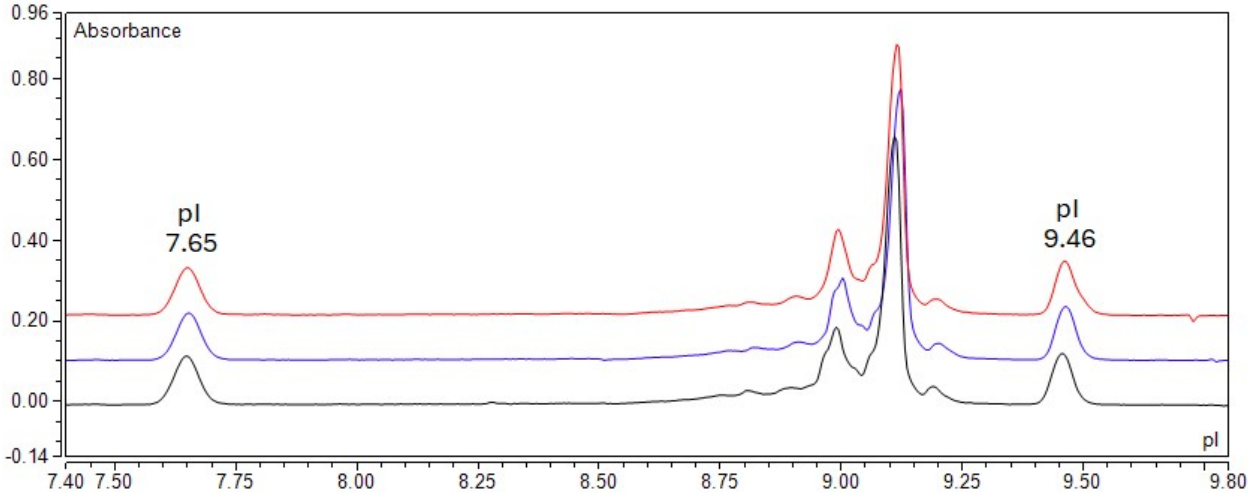

Fig. S3 Overlaid icIEF electropherograms of mAb-1 at three different concentrations of Pharmalyte pH 3-10: 4.75% (black), 5.0% (blue), and 5.25% (red)

(**Experimental conditions:** mAb-1 concentration, 0.4 mg/mL; focusing time: 8 min at 3.0 kV)

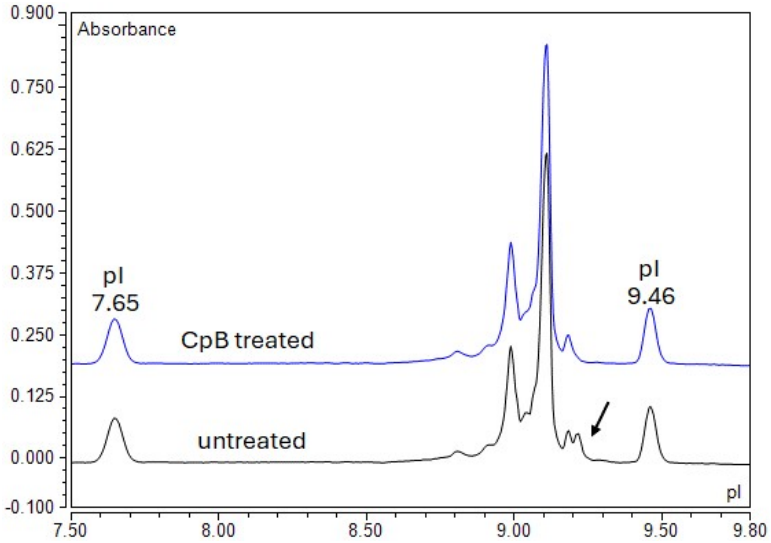

Fig. S4 icIEF electropherograms of an EU-Herceptin, untreated (black trace) and treated with carboxypeptidase B (blue trace)

Note: Carboxypeptidase (CpB) selectively cleaves Lys residues at the C-terminal end of proteins.

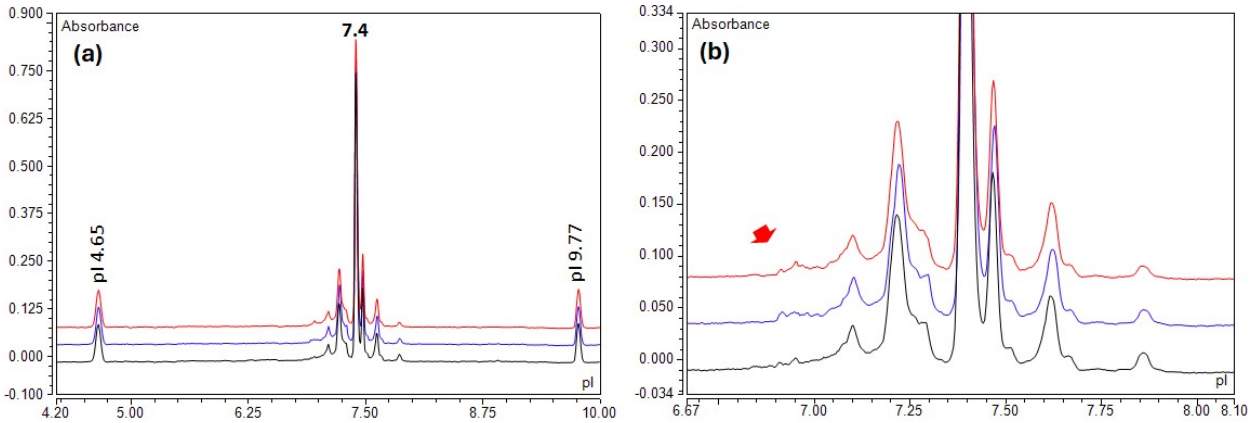

Fig. S5 Overlay of three replicate injections of pembrolizumab (Keytruda) icIEF electropherograms with focusing time of 6 min **(a)** Full view **(b)** zoomed-in view.

Note: The arrow indicates spikes, formed possibly due to protein precipitation.

(**Experimental conditions**: 0.35% methyl cellulose, 4% Pharmalyte pH 3-10, mAb concentration 0.6 mg/mL)

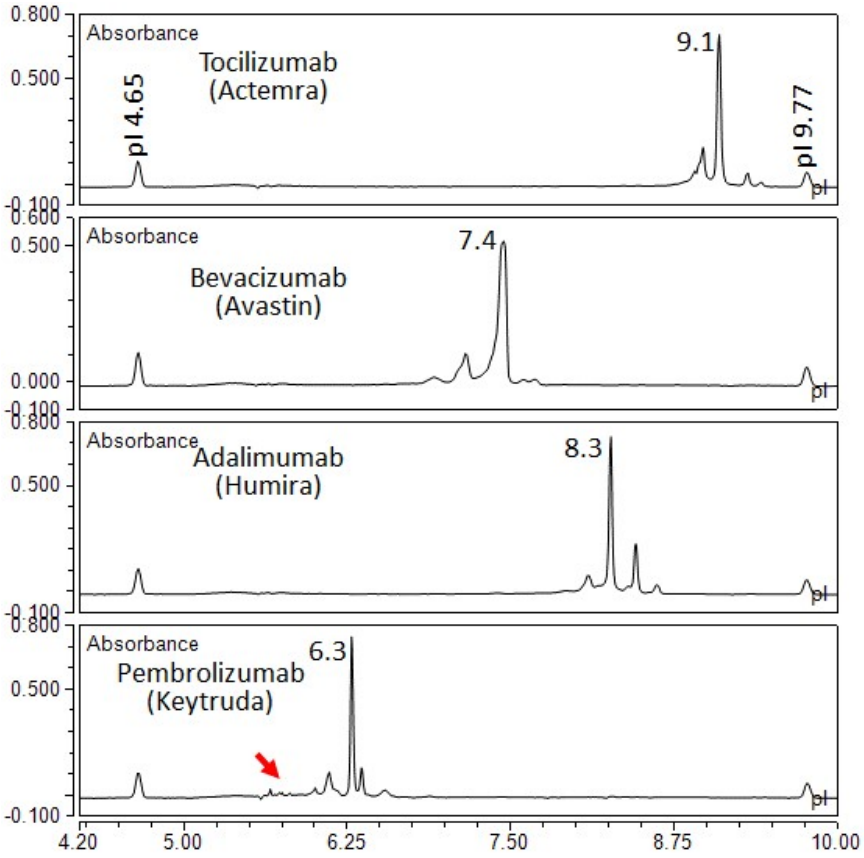

Fig. S6 icIEF electropherograms of four therapeutic mAbs

Note: The arrow indicates spikes, possibly due to protein precipitation.

(**Experimental conditions**: 0.35% methyl cellulose, 2% Pharmalyte pH 3-10, 2% Pharmalyte pH 8-10.5, mAb concentration 0.6 mg/mL, focusing time: 10 min at 3 kV)

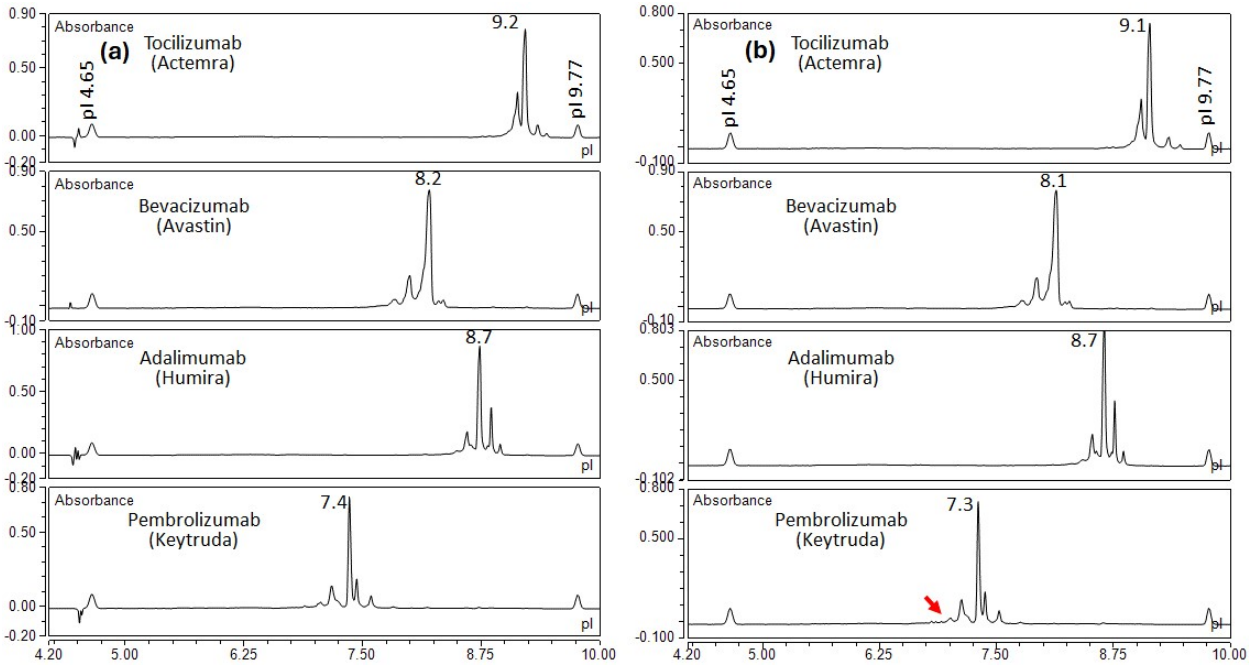

Fig. S7 icIEF electropherograms of four therapeutic mAbs **(a)** focusing time 8 min at 3 kV, **(b)** focusing time 10 min at 3 kV

Note: The arrow indicates spikes, possibly due to protein precipitation.

(**Experimental conditions**: 0.35% methyl cellulose, 4% Pharmalyte pH 3-10, 4 mM arginine, mAb concentration 0.6 mg/mL)

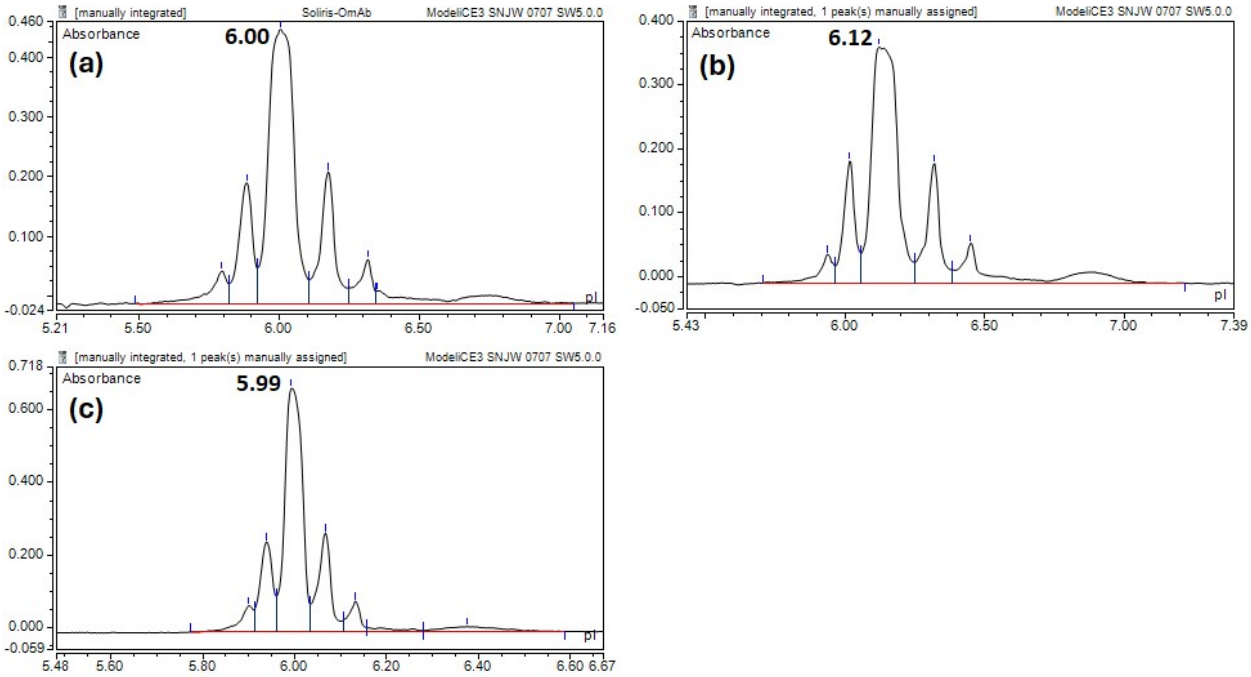

Fig. S8 Zoomed-in view of icIEF electropherograms of eculizumab (Soliris) under **(a)** native condition with 4% Pharmalyte pH 3-10, 4 mM arginine, **(b)** denaturing condition with 3.2 M urea, 4% Pharmalyte pH 3-10, and **(c)** denaturing condition with 3.2 M urea, 2% Pharmalyte pH 3-10 and 2% Pharmalyte pH 8-10.5 mixture.

(mAb concentration: 0.6 mg/mL and focusing time: 8 min for native and 10 min for denaturing conditions)

Note: The broad peak in the basic region could be contributed by aggregates because size exclusion chromatography analysis showed 9.2% of high molecular weight species. No low molecular weight species were detected.

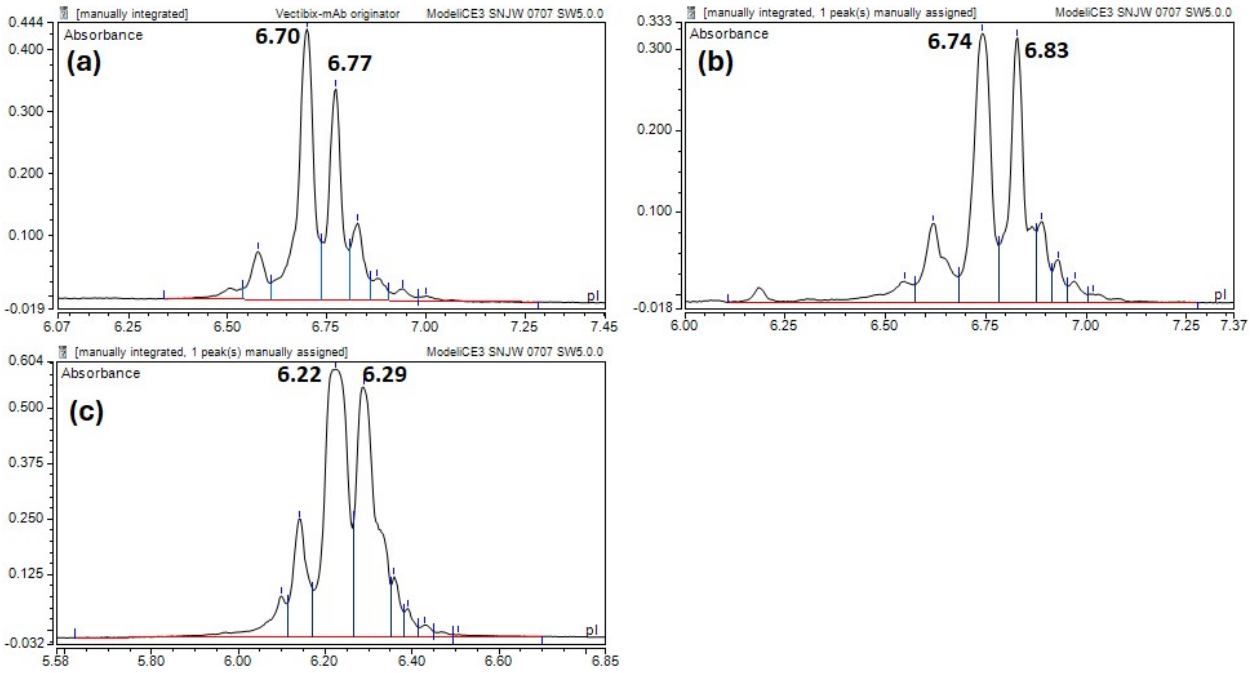

Fig. S9 Zoomed-in view of icIEF electropherograms of panitumumab (Vectibix) under **(a)** native condition with 4% Pharmalyte pH 3-10, **(b)** denaturing condition with 3.2 M urea, 4% Pharmalyte pH 3-10, and **(c)** denaturing condition with 3.2 M urea, 2% Pharmalyte pH 3-10 and 2% Pharmalyte pH 8-10.5 mixture.

(mAb concentration: 0.6 mg/mL and focusing time: 10 min for native and denaturing conditions)

Note: The two main peaks are possibly due to formation of disulfide isoforms (**Reference:** A. Resemann, L. Liu-Shin, G. Tremintin, A. Malhotra, A. Fung, F. Wang, G. Ratnaswamy and D. Suckau, *MAbs*, 2018, **10**, 1200–1213)

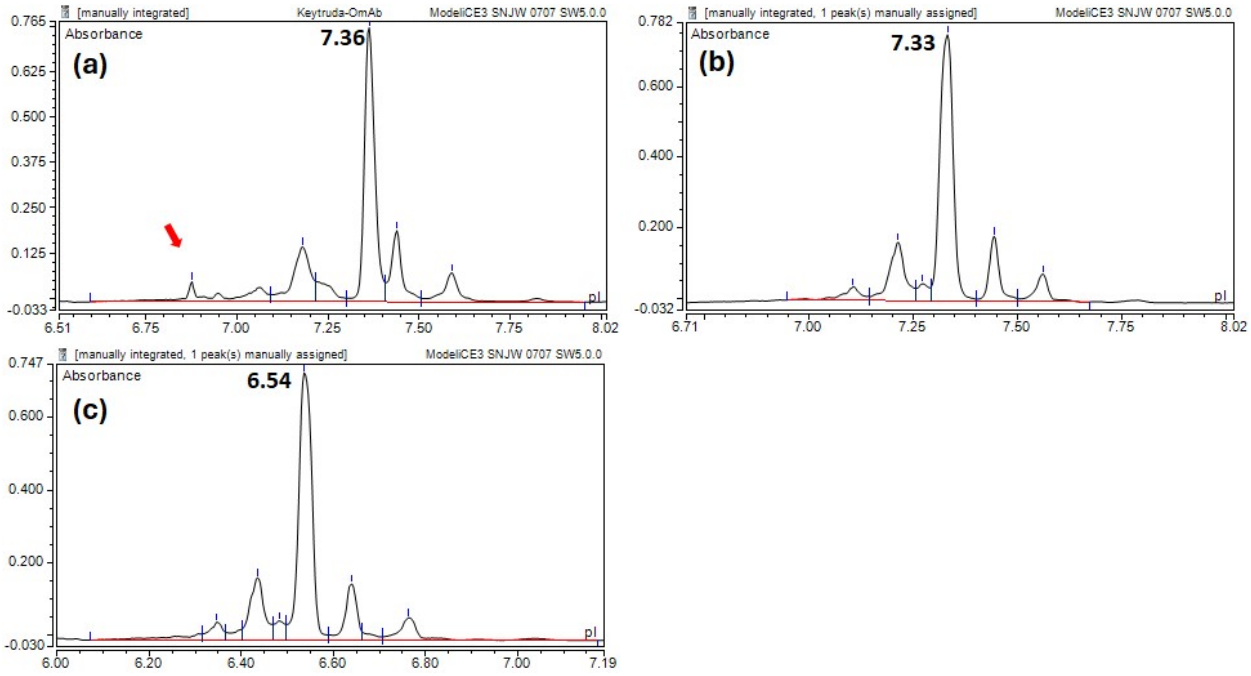

Fig. S10 Zoomed-in view of icIEF electropherograms of pembrolizumab (Keytruda) under **(a)** native condition with 4% Pharmalyte pH 3-10, 4 mM arginine, **(b)** denaturing condition with 3.2 M urea, 4% Pharmalyte pH 3-10, and **(c)** denaturing condition with 3.2 M urea, 2% Pharmalyte pH 3-10 and 2% Pharmalyte pH 8-10.5 mixture.

(mAb concentration: 0.6 mg/mL and focusing time: 8 min for native and 10 min for denaturing conditions)

Note: The arrow indicates spikes, possibly due to protein precipitation.

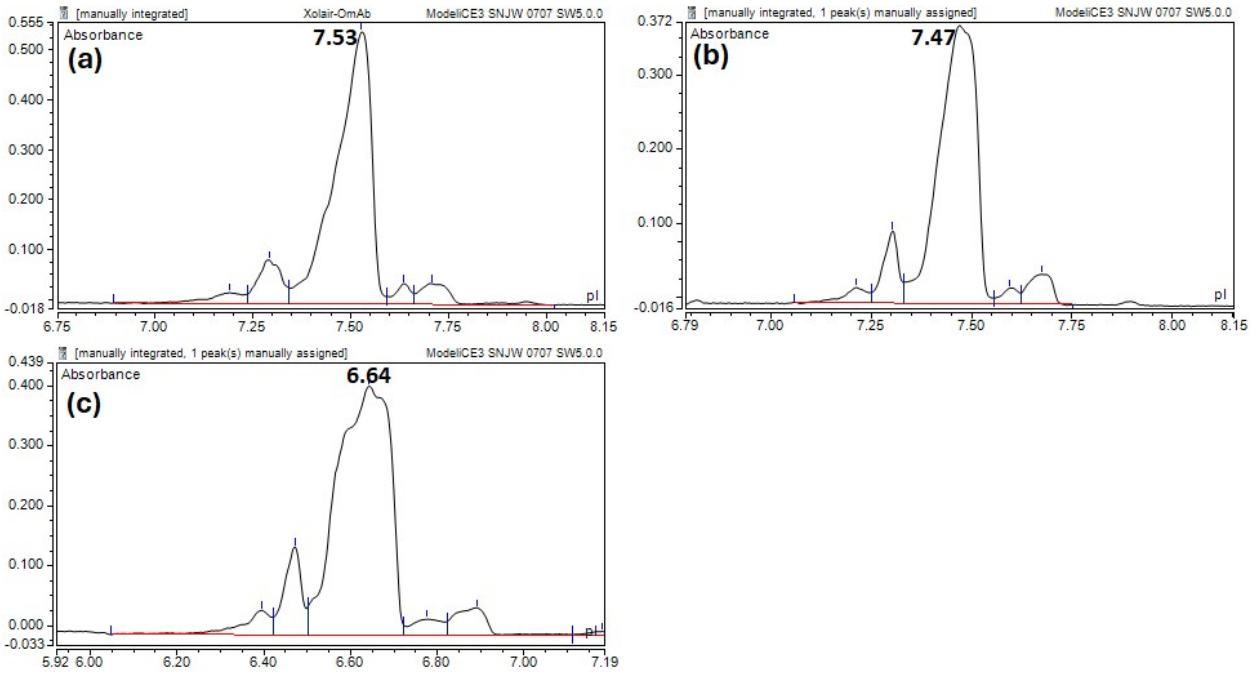

Fig. S11 Zoomed-in view of icIEF electropherograms of omalizumab (Xolair) under **(a)** native condition with 4% Pharmalyte pH 3-10, 4 mM arginine, **(b)** denaturing condition with 3.2 M urea, 4% Pharmalyte pH 3-10, and **(c)** denaturing condition with 3.2 M urea, 2% Pharmalyte pH 3-10 and 2% Pharmalyte pH 8-10.5 mixture.

(mAb concentration: 0.6 mg/mL and focusing time: 8 min for native and 10 min for denaturing conditions)

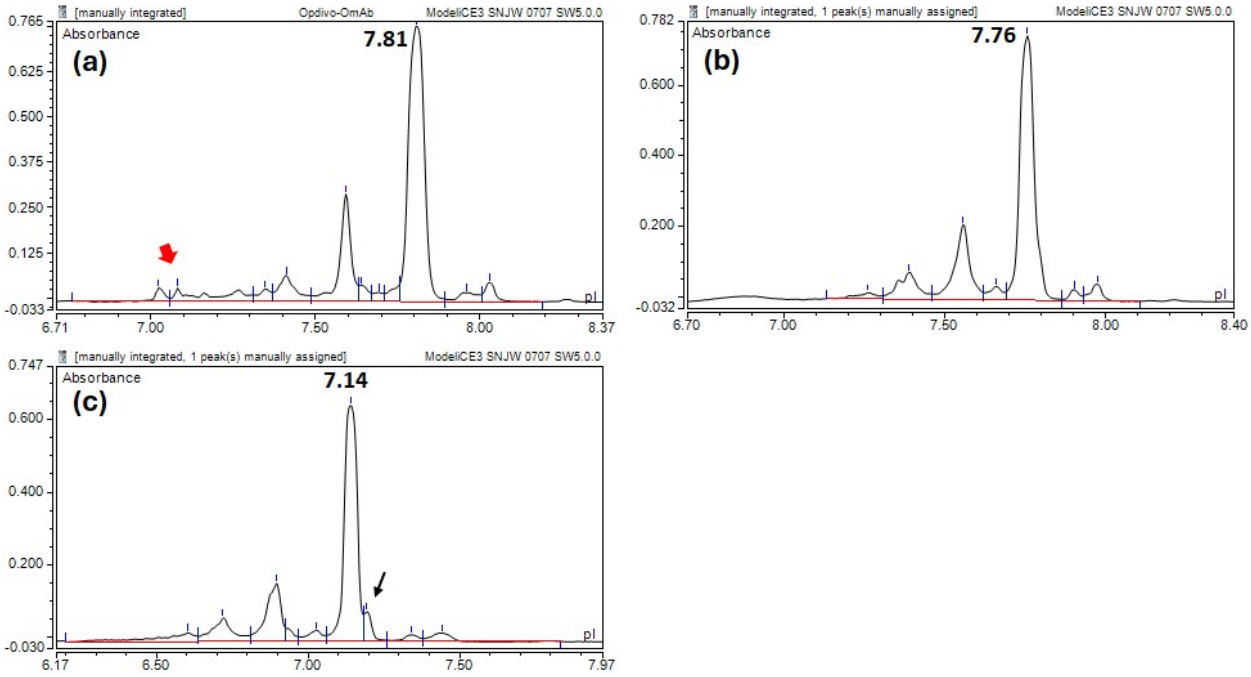

Fig. S12 Zoomed-in view of icIEF electropherograms of nivolumab (Opdivo) under **(a)** native condition with 4% Pharmalyte pH 3-10, 4 mM arginine, **(b)** denaturing condition with 3.2 M urea, 4% Pharmalyte pH 3-10, and **(c)** denaturing condition with 3.2 M urea, 2% Pharmalyte pH 3-10 and 2% Pharmalyte pH 8-10.5 mixture.

(mAb concentration: 0.6 mg/mL and focusing time: 8 min for native and 10 min for denaturing conditions)

Note: The red arrow in panel (a) shows spikes, possibly due to protein precipitation and the black arrow in panel (c) shows clearly visible shoulder basic peak.

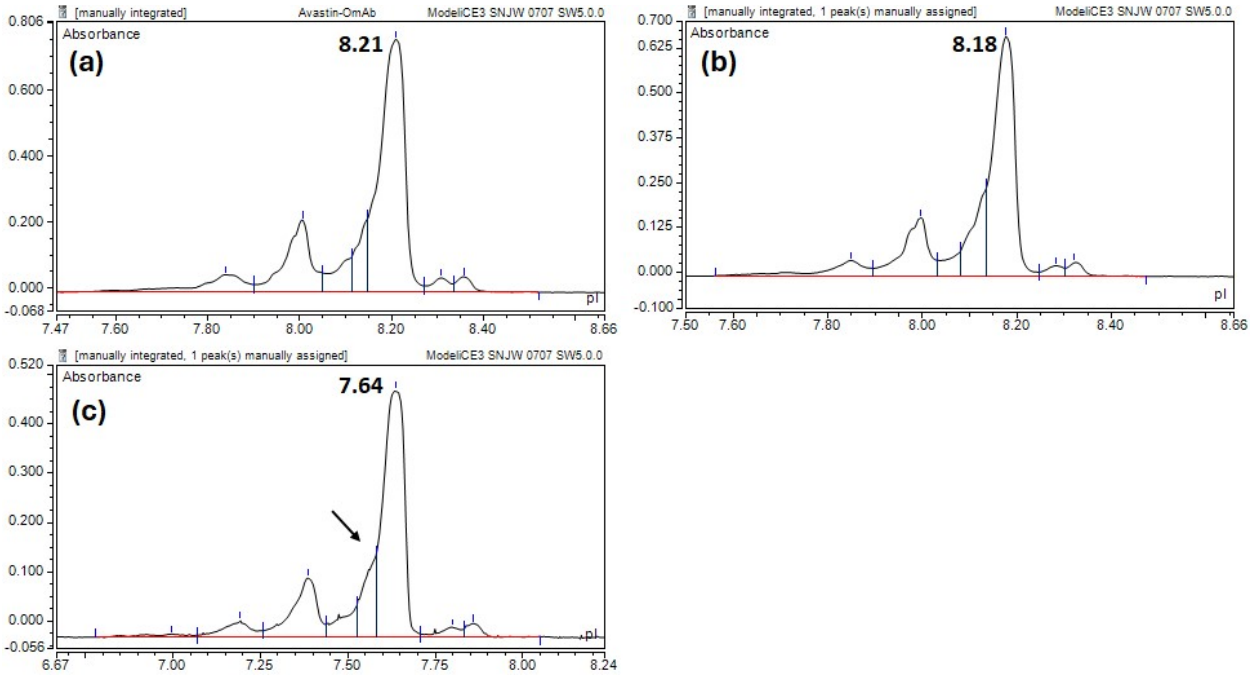

Fig. S13 Zoomed-in view of icIEF electropherograms of bevacizumab (Avastin) under **(a)** native condition with 4% Pharmalyte pH 3-10, 4 mM arginine, **(b)** denaturing condition with 3.2 M urea, 4% Pharmalyte pH 3-10, and **(c)** denaturing condition with 3.2 M urea, 2% Pharmalyte pH 3-10 and 2% Pharmalyte pH 8-10.5 mixture.

(mAb concentration: 0.6 mg/mL and focusing time: 8 min for native and 10 min for denaturing conditions)

Note: arrow indicates shoulder acidic peak is clearly visible

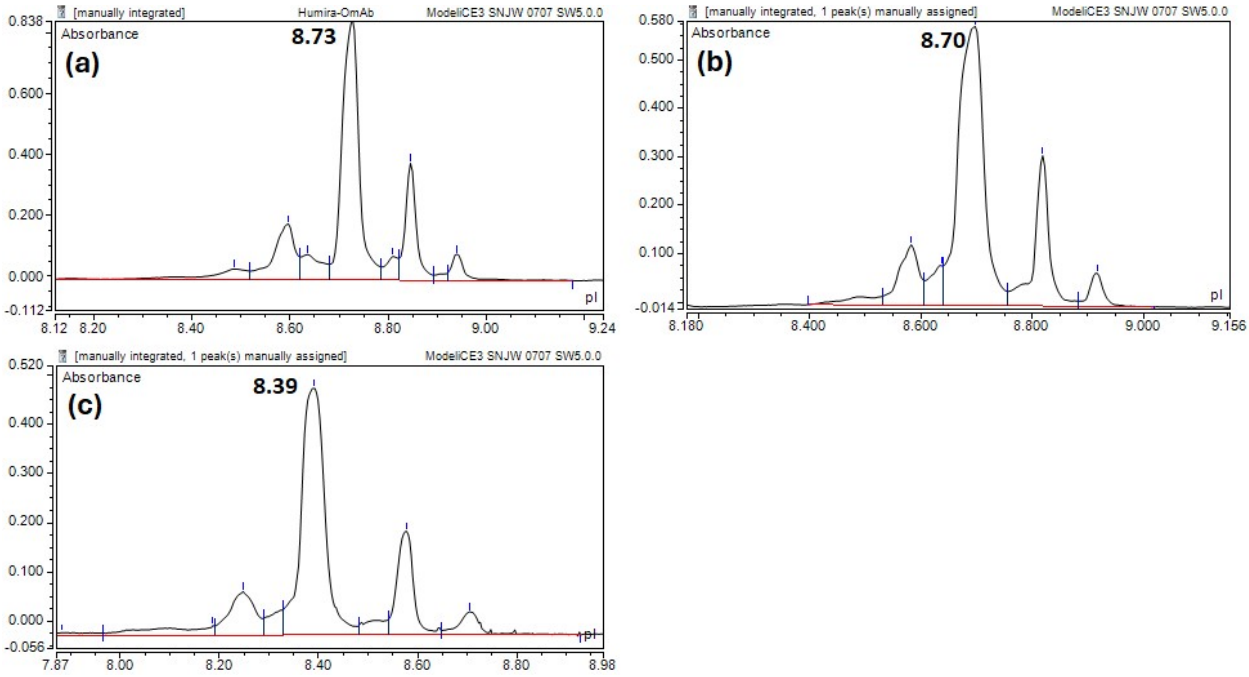

Fig. S14 Zoomed-in view of icIEF electropherograms of adalimumab (Humira) under **(a)** native condition with 4% Pharmalyte pH 3-10, 4 mM arginine, **(b)** denaturing condition with 3.2 M urea, 4% Pharmalyte pH 3-10, and **(c)** denaturing condition with 3.2 M urea, 2% Pharmalyte pH 3-10 and 2% Pharmalyte pH 8-10.5 mixture.

(mAb concentration: 0.6 mg/mL and focusing time: 8 min for native and 10 min for denaturing conditions)

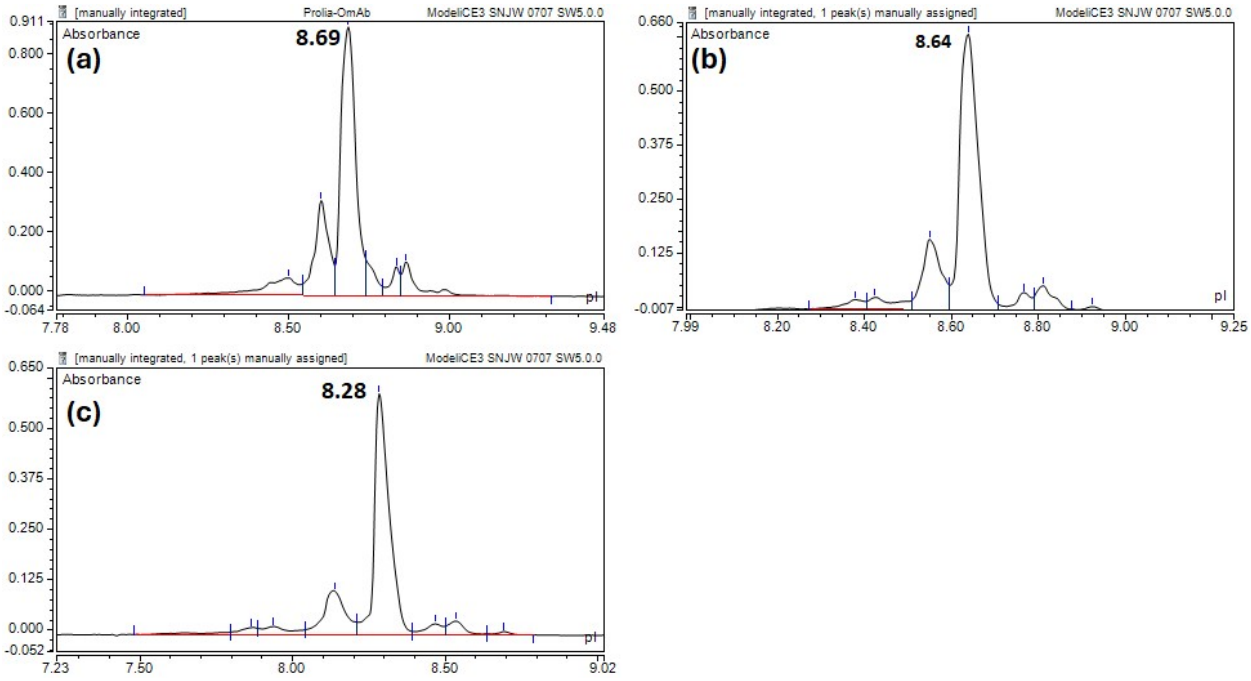

Fig. S15 Zoomed-in view of icIEF electropherograms of denosumab (Prolia) under **(a)** native condition with 4% Pharmalyte pH 3-10, 4 mM arginine, **(b)** denaturing condition with 3.2 M urea, 4% Pharmalyte pH 3-10, and **(c)** denaturing condition with 3.2 M urea, 2% Pharmalyte pH 3-10 and 2% Pharmalyte pH 8-10.5 mixture.

(mAb concentration: 0.6 mg/mL and focusing time: 8 min for native and 10 min for denaturing conditions)

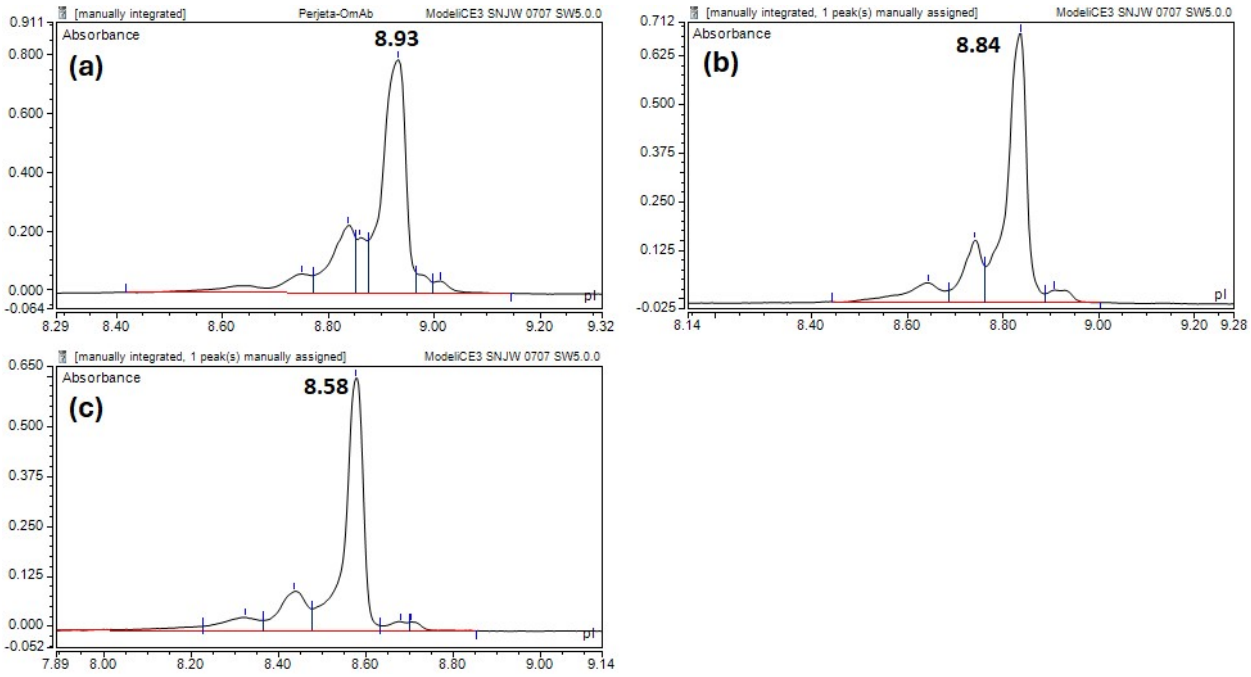

Fig. S16 Zoomed-in view of icIEF electropherograms of pertuzumab (Perjeta) under **(a)** native condition with 4% Pharmalyte pH 3-10, 4 mM arginine, **(b)** denaturing condition with 3.2 M urea, 4% Pharmalyte pH 3-10, and **(c)** denaturing condition with 3.2 M urea, 2% Pharmalyte pH 3-10 and 2% Pharmalyte pH 8-10.5 mixture.

(mAb concentration: 0.6 mg/mL and focusing time: 8 min for native and 10 min for denaturing conditions)

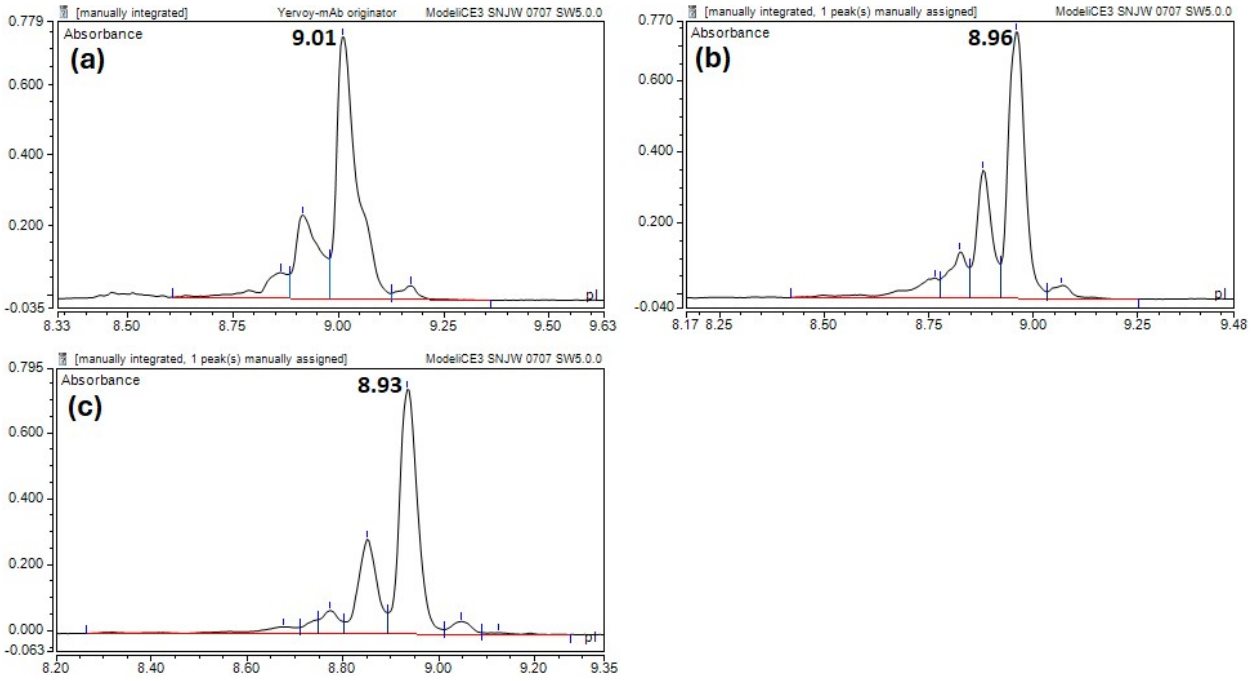

Fig. S17 Zoomed-in view of icIEF electropherograms of ipilimumab (Yervoy) under **(a)** native condition with 4% Pharmalyte pH 3-10, **(b)** denaturing condition with 3.2 M urea, 4% Pharmalyte pH 3-10, and **(c)** denaturing condition with 3.2 M urea, 2% Pharmalyte pH 3-10 and 2% Pharmalyte pH 8-10.5 mixture.

(mAb concentration: 0.6 mg/mL and focusing time: 10 min for native and denaturing conditions)

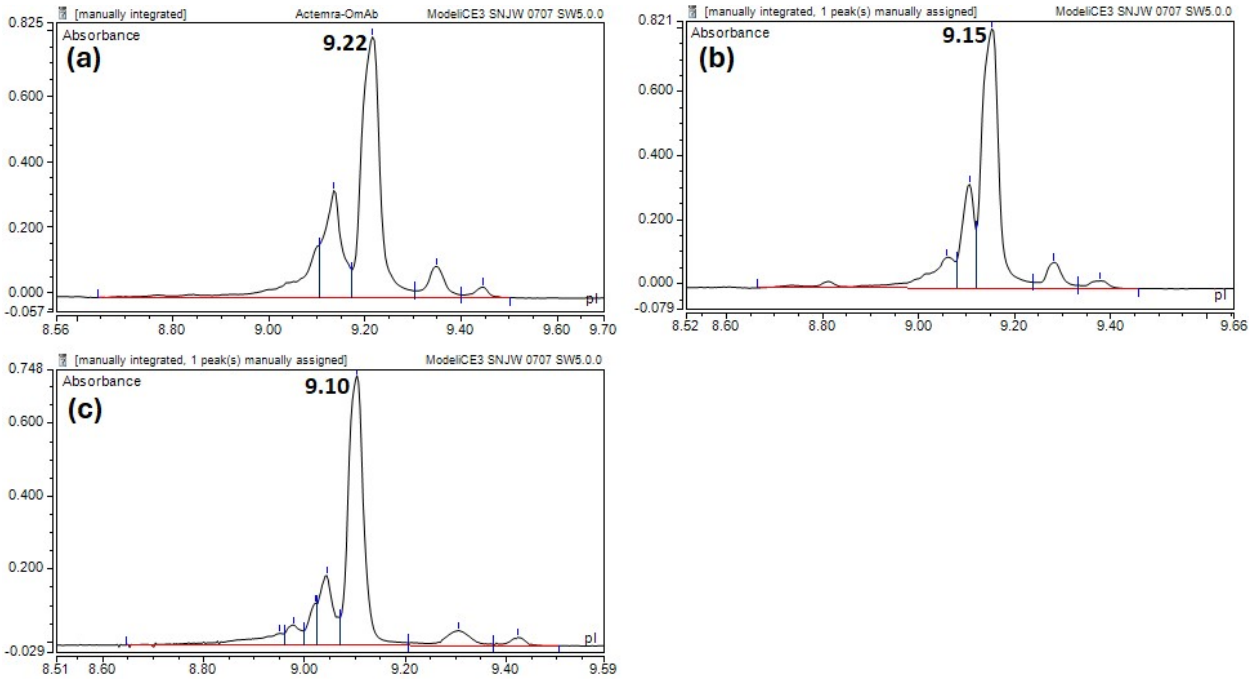

Figure S18 Zoomed-in view of icIEF electropherograms of tocilizumab (Actemra) under **(a)** native condition with 4% Pharmalyte pH 3-10, 4 mM arginine, **(b)** denaturing condition with 3.2 M urea, 4% Pharmalyte pH 3-10, and **(c)** denaturing condition with 3.2 M urea, 2% Pharmalyte pH 3-10 and 2% Pharmalyte pH 8-10.5 mixture.

(mAb concentration: 0.6 mg/mL and focusing time: 8 min for native and 10 min for denaturing conditions)

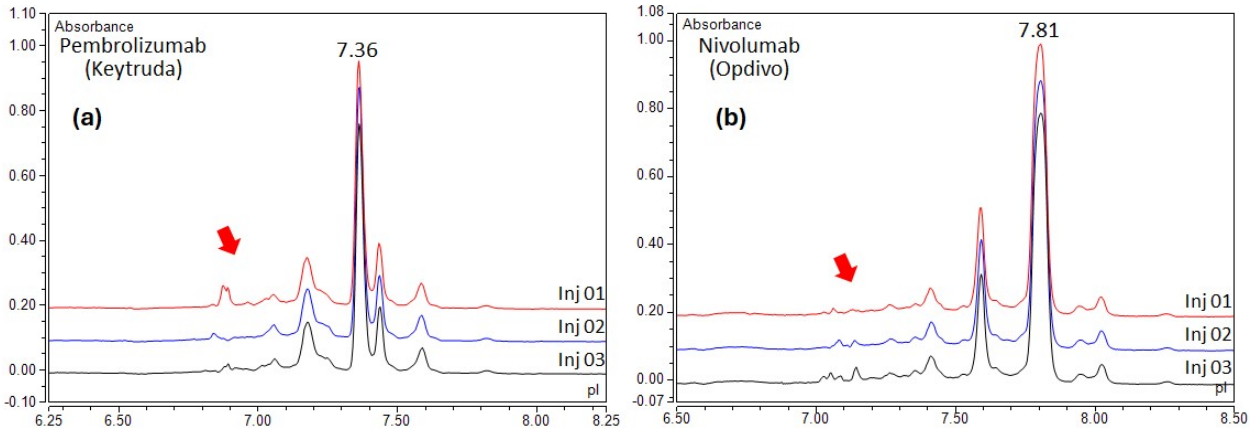

Fig. S19 icIEF electropherograms of therapeutics mAbs **(a)** pembrolizumab (Keytruda) and **(b)** nivolumab (Opdivo)

Note: The arrow indicates non-reproducible spikes in the acidic region of the electropherograms, possibly due to protein precipitation.

(**Experimental conditions**: 0.35% methyl cellulose, 4% Pharmalyte pH 3-10, 4 mM arginine, mAb concentration 0.6 mg/mL, focusing time 8 min at 3kV)

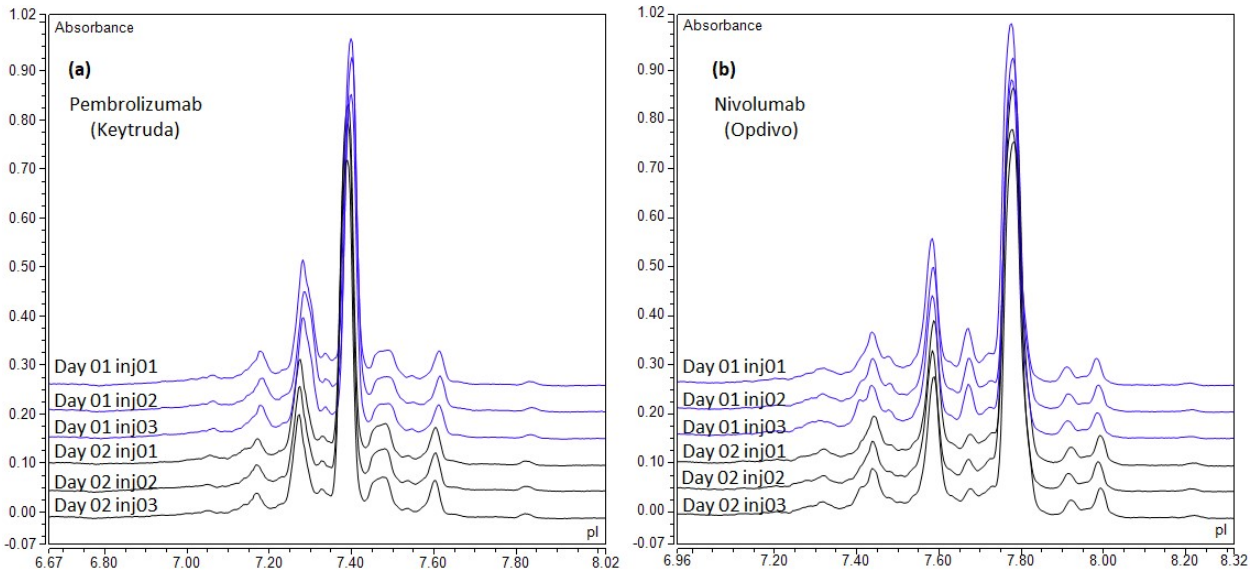

Fig. S20 icIEF electropherograms of therapeutics mAbs **(a)** pembrolizumab (Keytruda) and **(b)** nivolumab (Opdivo)

(**Experimental conditions**: 0.35% methyl cellulose, 4% Pharmalyte pH 3-10, mAb concentration 0.6 mg/mL, focusing time 10 min at 3kV)

Table S3 Main peak pIs of the 11 therapeutic mAbs

mAbs	pI Values (References: 5,19,20)	Observed Main Peak pI		
		Native icIEF	Denaturing icIEF Pharm. 3-10	Denaturing icIEF Pharmalyte 3-10 and 8-10.5
Eculizumab (Soliris)	6.1	6.00	6.12	5.99
Panitumumab (Vectibix)	6.8	6.70/6.77*	6.74/6.83*	6.22/6.29*
Pembrolizumab (Keytruda)	7.6	7.36	7.33	6.54
Omalizumab (Xolair)	7.3	7.53	7.47	6.64
Nivolumab (Opdivo)	8.0	7.81	7.76	7.14
Bevacizumab (Avastin)	8.3	8.21	8.18	7.64
Adalimumab (Humira)	8.9	8.73	8.70	8.39
Denosumab (Prolia)	8.9	8.69	8.64	8.28
Pertuzumab (Perjeta)	9.0	8.93	8.84	8.58
Ipilimumab (Yervoy)	9.2	9.01	8.96	8.93
Tocilizumab (Actemra)	9.2	9.22	9.15	9.10

*Panitumumab can show two main peaks due to formation of disulfide isoforms (**Reference:** A. Resemann, L. Liu-Shin, G. Tremintin, A. Malhotra, A. Fung, F. Wang, G. Ratnaswamy and D. Suckau, *MAbs*, 2018, **10**, 1200–1213)

Table S4 Percentages of acidic, main, and basic species of the 11 therapeutic mAbs

mAbs	Native icIEF			Denaturing icIEF Pharm. 3-10			Denaturing icIEF Pharmalyte 3-10 and 8-10.5		
	% Acidic	% Main	% Basic	% Acidic	% Main	% Basic	% Acidic	% Main	% Basic
Eculizumab (Soliris)	18.30	55.53	26.17	16.85	56.19	26.96	19.37	54.61	26.02
Panitumumab (Vectibix)	9.79	74.74*	15.47	19.20	67.81*	12.99	17.39	76.09*	6.52
Pembrolizumab (Keytruda)	31.55	47.01	21.46	23.14	59.34	17.52	25.72	54.94	19.33
Omalizumab (Xolair)	11.95	79.67	8.37	11.47	80.78	7.74	13.55	77.55	8.90
Nivolumab (Opdivo)	37.11	57.61	5.26	32.89	62.19	4.93	35.59	55.48	8.91
Bevacizumab (Avastin)	37.97	57.38	4.65	41.03	54.53	4.45	38.93	56.66	4.44
Adalimumab (Humira)	25.82	50.96	23.23	17.09	59.81	23.11	20.56	54.79	24.65
Denosumab (Prolia)	28.27	56.36	15.38	23.70	66.04	10.26	25.79	65.45	8.74
Pertuzumab (Perjeta)	34.02	61.01	4.97	25.01	71.10	3.89	26.03	69.52	4.45
Ipilimumab (Yervoy)	32.59	64.24	3.17	42.47	54.12	3.41	36.43	59.40	4.17
Tocilizumab (Actemra)	35.83	55.81	8.37	31.58	60.03	8.39	30.66	61.05	8.29

*sum of two main peaks for panitumumab.