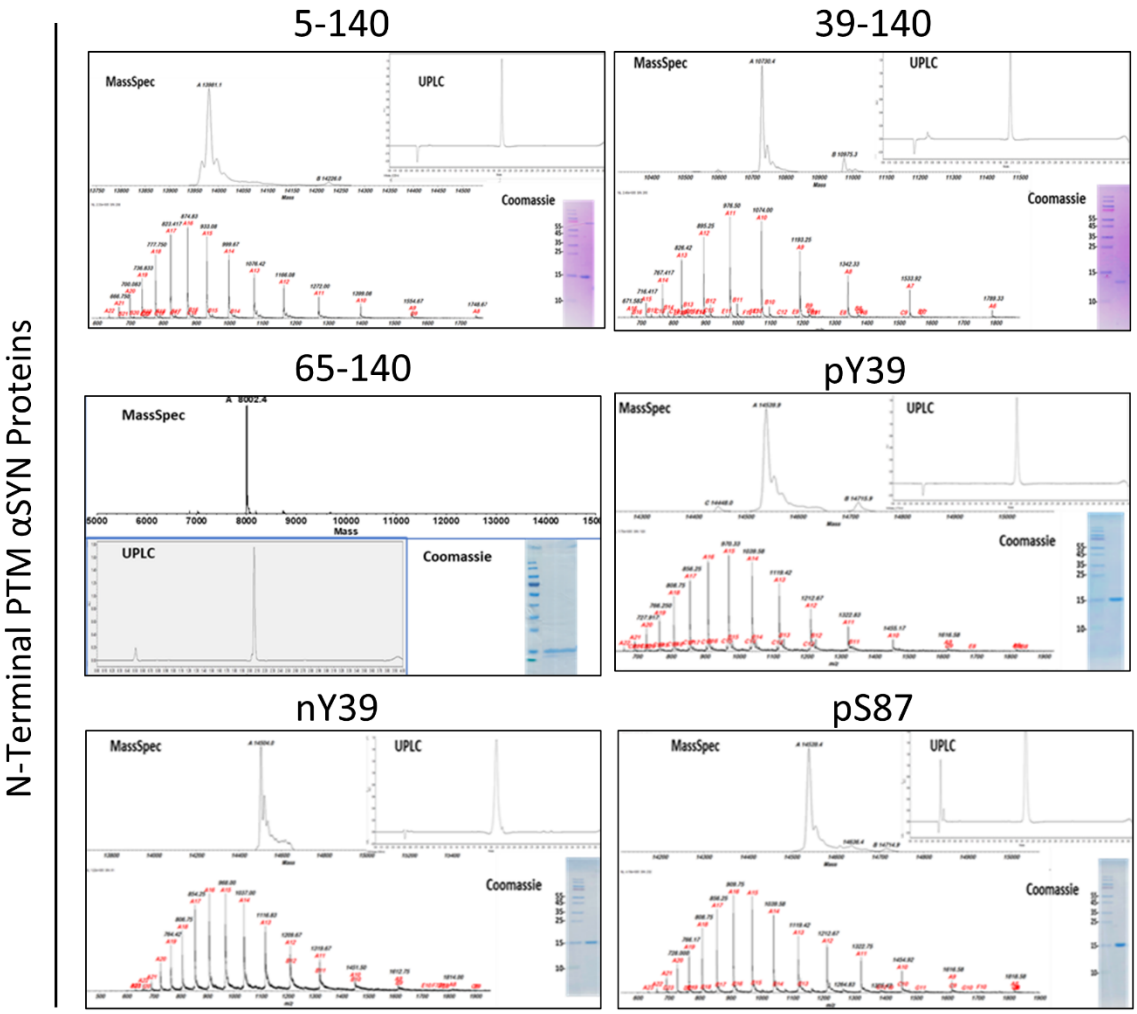
Supplementary Material

Comparative Analysis of Total Alpha-Synuclein (αSYN) Immunoassays Reveals That They Do Not Capture the Diversity of Modified αSYN Proteoforms

Evaluated parameter	Euroimmun	MSD	Biolegend
Well Format	96	96	96
Readout	Absorbance 450 nm	ECL	Luminescence
Dynamic Range pg/ml	5988-150	10'000-2,44	1500-6,1
Internal Calibrator controls	✓	can be purchased	✗
Easy to handle	✓	✓	✗
Assay running time	5 hrs over 1 day	4 hrs over 1 day	2 days
Final reaction stability	for 1 hr	✗	✗
Reading of partial plate	✓	✓	✗

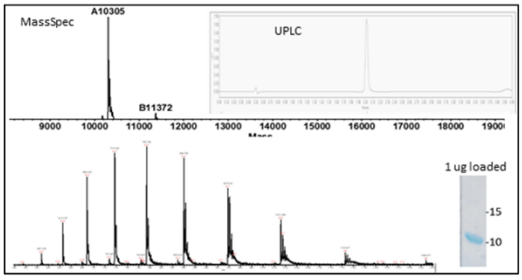
Supplementary Figure 1. Summary table of general technical and handling considerations of the analyzed total αSYN immunoassay kits.



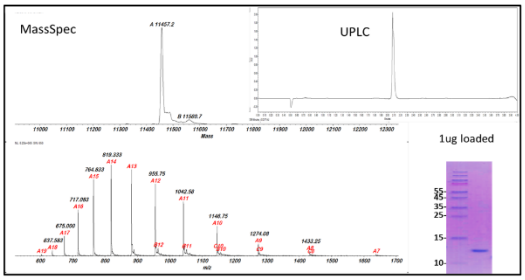
N-Terminal PTM Human αSYN Proteins	
Protein	**Molecular Weight (Da)**
αSYN 5-140	13967.524
αSYN 39-140	10599.583
αSYN 64-140	8103.7896
αSYN pY39	14540.094
αSYN nY39	14505.000
αSYN pS87	14540.094

C-Terminal PTM αSYN Proteins

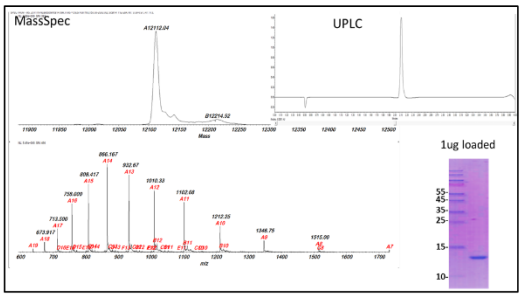
1-103



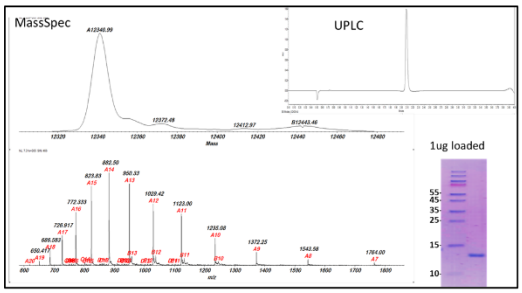
1-114



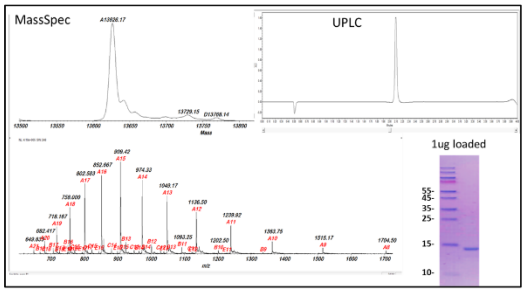
1-120



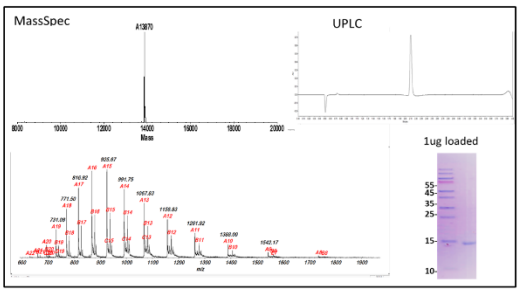
1-122



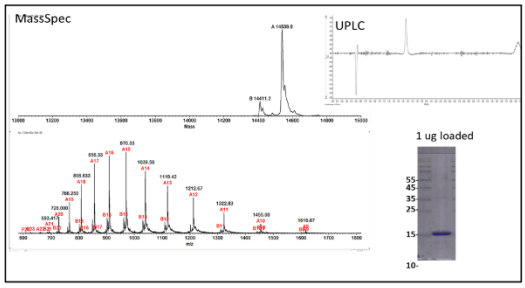
1-133



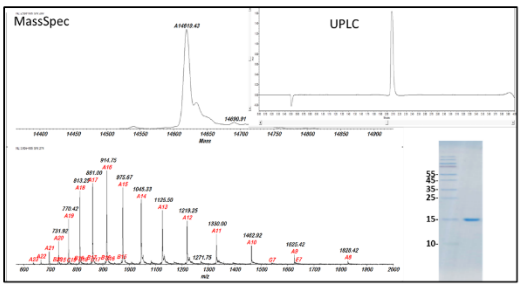
1-135



pY125

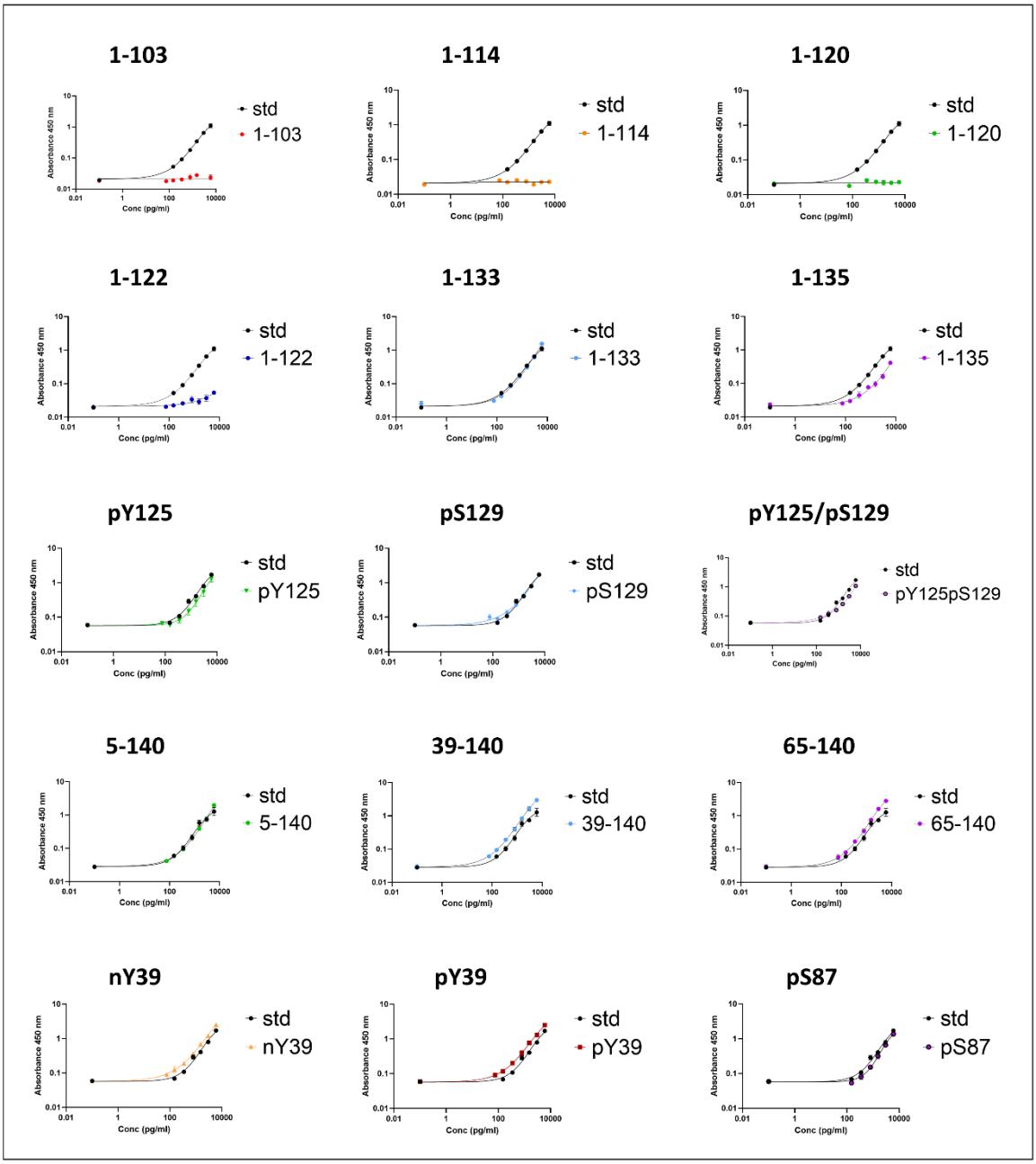


pS129/pY125

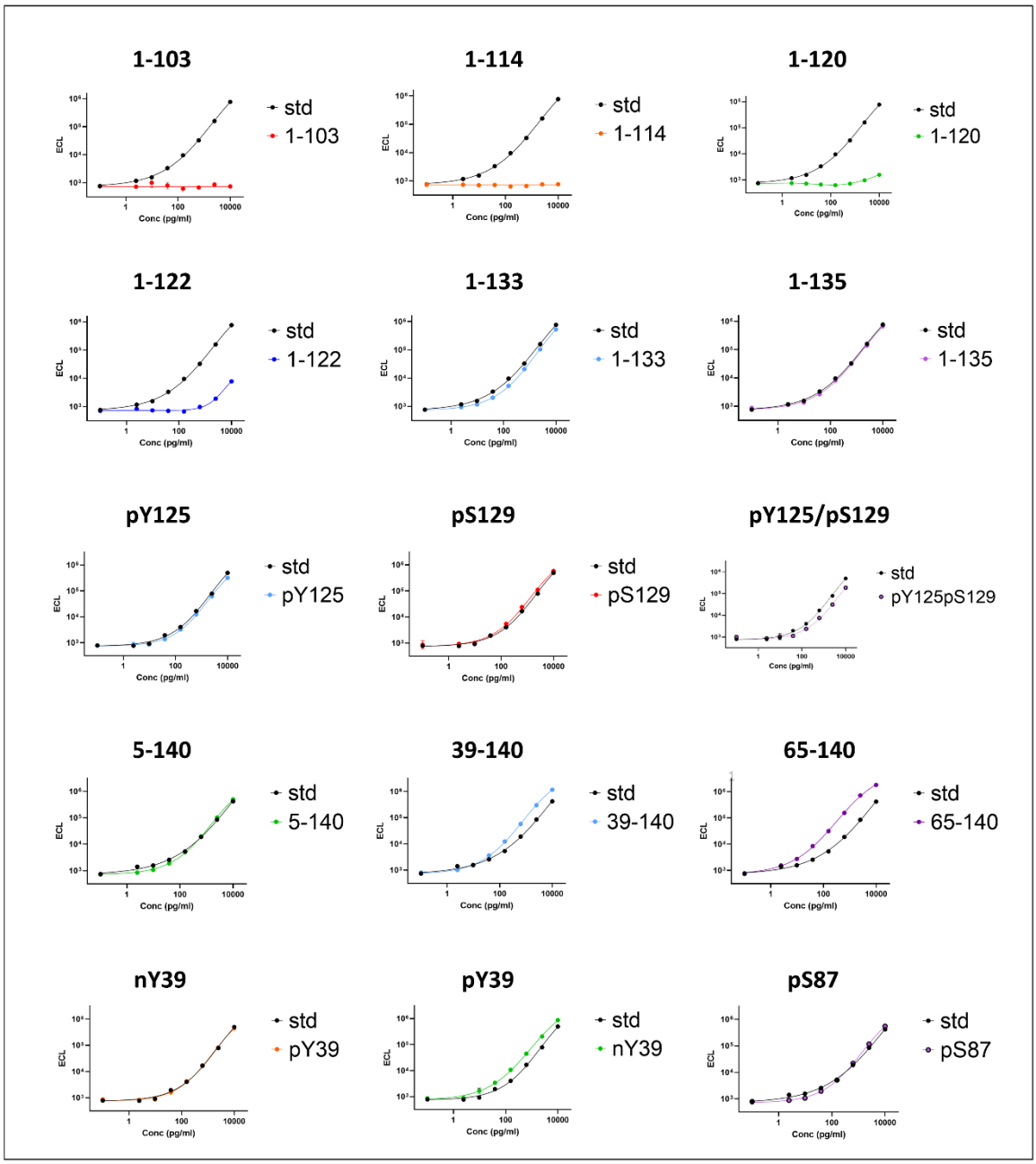


C-Terminal PTM Human αSYN Proteins	
Protein	**Molecular Weight (Da)**
αSYN 1-103	10303.833
αSYN 1-114	11457.040
αSYN 1-120	12111.774
αSYN 1-122	12340.966
αSYN 1-133	13627.294
αSYN 1-135	13870.513
αSYN pY125	14540.000
αSYN pY125 pS129	14620.074

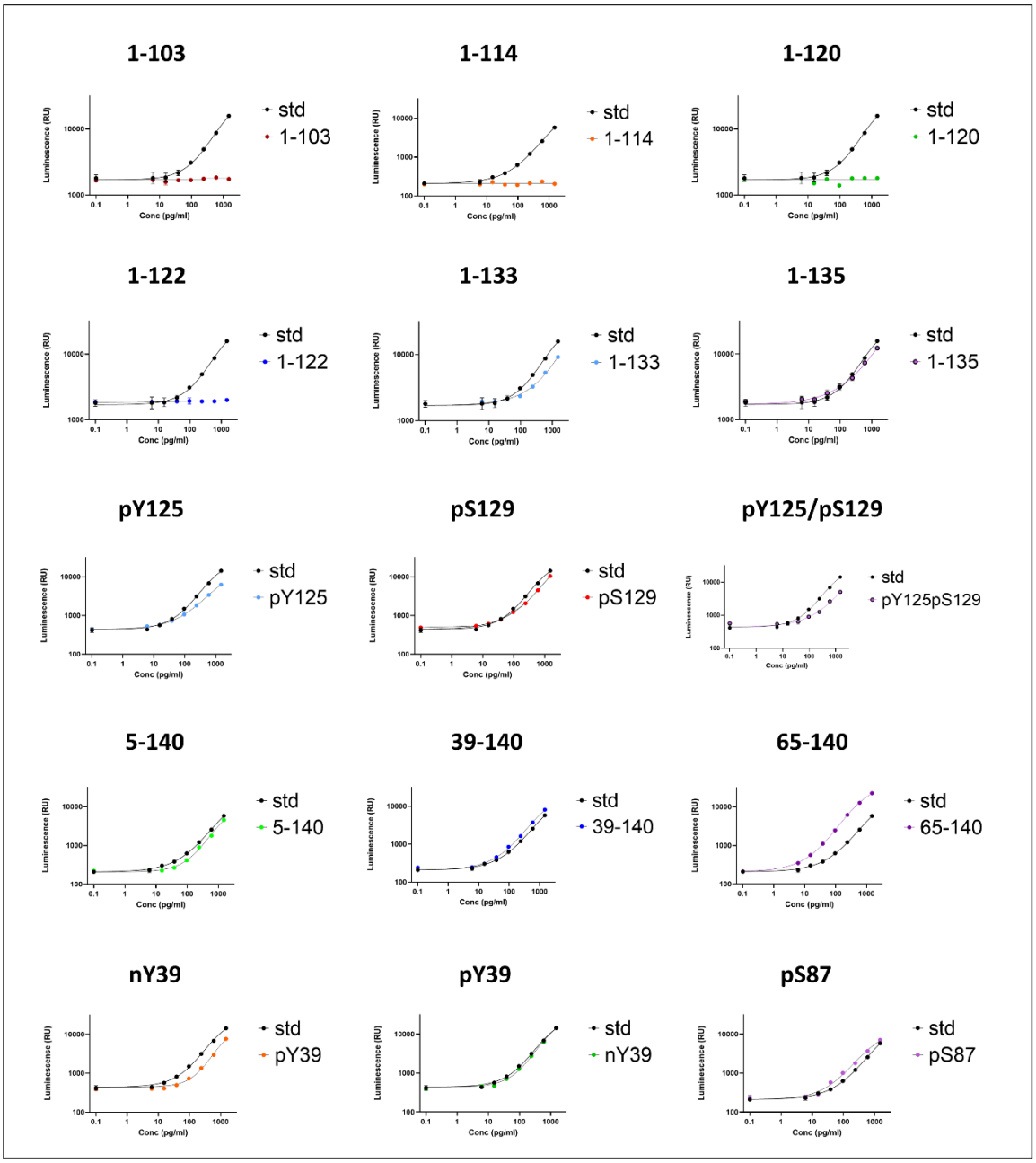
Supplementary Figure 2. Characterization of the generated library of αSYN proteins bearing the most commonly occurring N-terminal and C-terminal PTMs. Mass spectrometry, ultra-performance liquid chromatography (UPLC) and Coomassie staining were performed to establish the purity and integrity of all generated proteins.



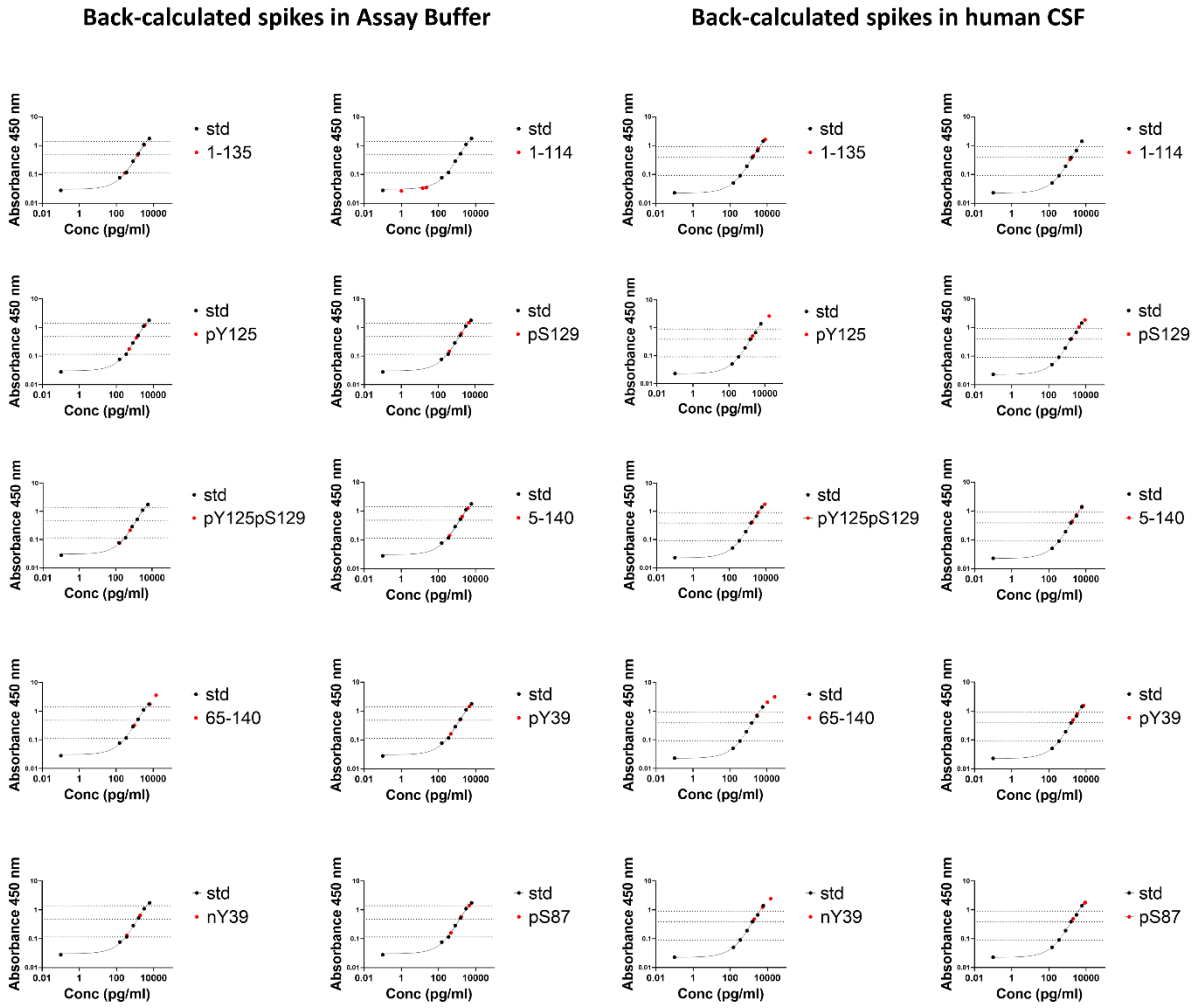
Supplementary Figure 3. Single graphs separately showing the detection of PTM αSYN proteins using the Euroimmun immunoassay. Full dilution curves of the proteins with the same dynamic range as the standard curve fitted to a 4-parameter sigmoid curve and plotted against the kit standard curve. C-terminal truncations ranging from 103 to 122 were not recognized.



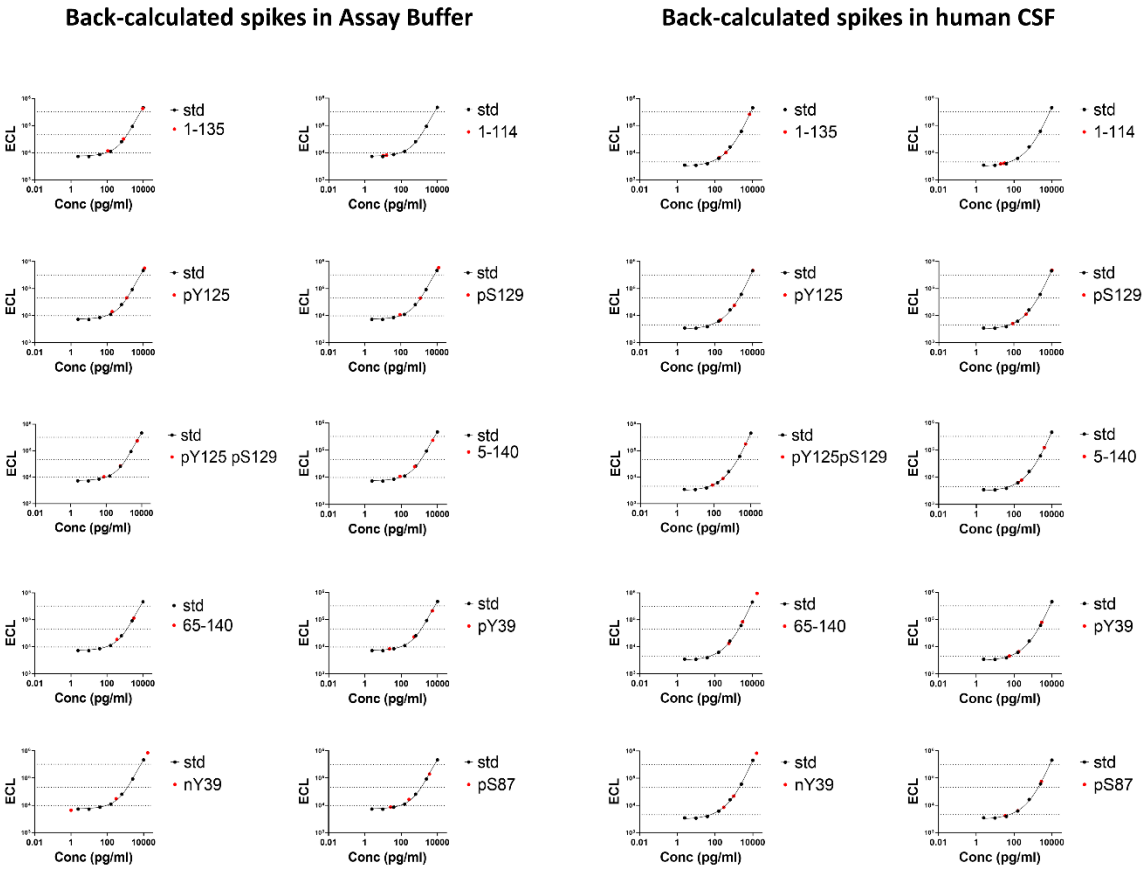
Supplementary Figure 4. Single graphs separately showing the detection of PTM αSYN proteins using the MSD immunoassay. Full dilution curves of the proteins with the same dynamic range as the standard curve fitted to a 4-parameter sigmoid curve and plotted against the kit standard curve. C-terminal truncations ranging from 103 to 122 were not recognized.



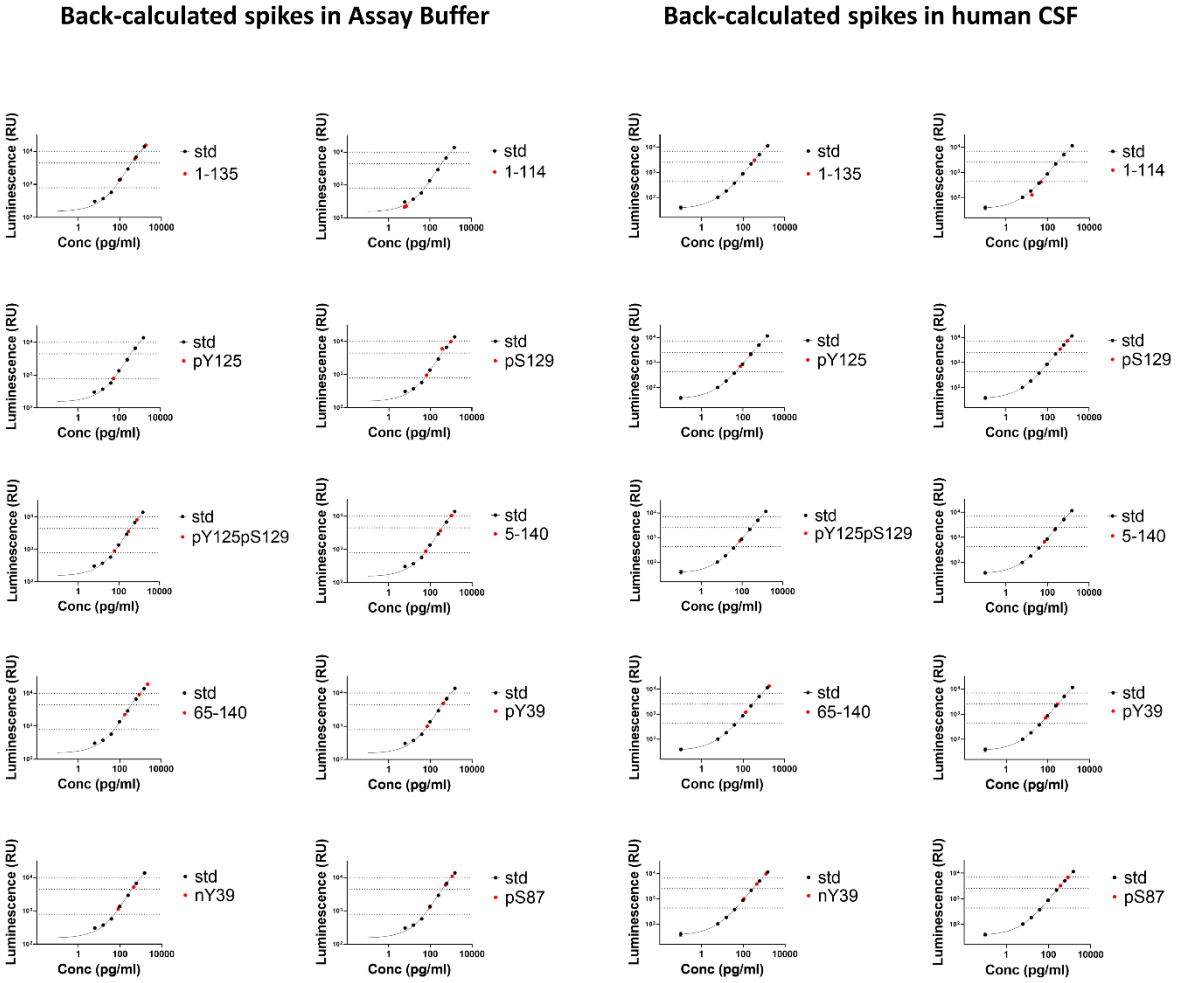
Supplementary Figure 5. Single graphs separately showing the detection of PTM αSYN proteins using the Biolegend immunoassay. Full dilution curves of the proteins with the same dynamic range as the standard curve fitted to a 4-parameter sigmoid curve and plotted against the kit standard curve. C-terminal truncations ranging from 103 to 122 were not recognized.



Supplementary Figure 6. Single graphs separately showing backcalculated spike values using the Euroimmun immunoassay. Single graphs separately showing the back-calculated values of the high-, medium- and low-PTM αSYN spikes in assay buffer or CSF interpolated to the assay standard curve (red dots). Nominal expected high, medium, and low spikes interpolated to the standard curve are shown by the dotted lines.



Supplementary Figure 7. Single graphs separately showing back-calculated spike values using the MSD immunoassay. Single graphs separately showing the back-calculated values of the high-, medium-, and low-PTM αSYN spikes in assay buffer or CSF interpolated to the assay standard curve (red dots). Nominal expected high, medium, and low spikes interpolated to the standard curve are shown by the dotted lines.



Supplementary Figure 8. Single graphs separately showing back-calculated spike values using the Biolegend immunoassay. Single graphs separately showing the back-calculated values of the high-, medium-, and low-PTM αSYN spikes in assay buffer or CSF interpolated to the assay standard curve (red dots). Nominal expected high, medium, and low spikes interpolated to the standard curve are shown by the dotted lines.

Spike Recovery %							
Sample	Spike	Euroimmun Assay		MSD Assay		Biolegend Assay	
		AB	hCSF	AB	hCSF	AB	hCSF
1-135	High	77	153	134	97	93	166
	Medium	92	104	80	121	73	104
	Low	85	50	104	133	111	112
1-114	High	ND	ND	ND	ND	ND	ND
	Medium	ND	ND	ND	ND	ND	ND
	Low	ND	ND	ND	ND	ND	ND
pY125	High	88	396	171	147	65	59
	Medium	81	290	121	104	81	64
	Low	75	131	185	156	104	68
pS129	High	113	185	177	44	107	94
	Medium	113	177	119	32	123	120
	Low	116	157	84	33	131	102
pY125pS129	High	44	178	80	73	84	60
	Medium	39	136	62	27	99	67
	Low	46	107	69	49	121	74
5-140	High	97	107	79	57	116	65
	Medium	126	89	55	21	101	61
	Low	116	48	81	127	122	59
65-140	High	360	627	396	254	248	196
	Medium	435	602	308	299	290	183
	Low	272	377	348	525	353	184
pY39	High	114	139	74	61	69	64
	Medium	94	106	50	75	140	82
	Low	131	99	17	59	139	72
nY39	High	112	333	131	175	163	133
	Medium	121	248	111	167	151	135
	Low	103	84	121	104	162	135
pS87	High	113	171	52	38	123	88
	Medium	107	132	27	61	178	111
	Low	133	99	21	95	185	103

Supplementary Figure 9. Summary table of single percentage recoveries obtained at high, medium, and low spikes of the analyzed total αSYN immunoassays in assay buffer and commercial human CSF. The high, medium, and low recoveries are assessed as the percentage of the back-calculated values of the spiked samples subtracted from the unspiked sample with respect to the nominal spiked protein amount. ND, not determinable.