Evaluation of Parameters for Confident Phosphorylation Site Localization using an Orbitrap Fusion Tribrid Mass Spectrometer

Samantha Ferries[1,3], Simon Perkins[2], Philip J. Brownridge[1], Amy Campbell[1,3], Patrick A. Eyers[3], Andrew R. Jones[2], Claire E. Eyers[1,3].*

[1]Centre for Proteome Research, [3]Department of Biochemistry, Institute of Integrative Biology, University of Liverpool, Crown Street, Liverpool, L69 7ZB, United Kingdom.

[2] Department of Functional and Comparative Genomics, Institute of Integrative Biology, University of Liverpool, Crown Street, Liverpool, L69 7ZB, United Kingdom

Table of Contents

	HCD OT	HCD IT	EThcD OT	EThcD IT	HCD nl EThcD IT	HCD nl ETcaD IT	HCD OT nl EThcD IT	HCD OT nl ETcaD IT
MS1								
Orbitrap Resolution	60K	120K	60K	120K	120k	120k	60k	60k
RF Lens	60	60	60	60	60	60	60	60
Scan range (*m/z*)	350-2000	350-2000	350-2000	350-2000	350-2000	350-2000	350-2000	350-2000
AGC	2.00E+05	2.00E+05	2.00E+05	2.00E+05	2.00E+05	2.00E+05	2.00E+05	2.00E+05
Injection time	50 ms	50 ms	50 ms	50 ms	50 ms	50 ms	50 ms	50 ms
MIPS	Peptide	Peptide	Peptide	Peptide	Peptide	Peptide	Peptide	Peptide
Intensity	5.00E+04	5.00E+03	5.00E+04	5.00E+03	5.00E+03	5.00E+03	5.00E+04	5.00E+04
Charge states	2+ to 5+	2+ to 5+	2+ to 5+	2+ to 5+	2+ to 5+	2+ to 5+	2+ to 5+	2+ to 5+
Dynamic exclusion	60 s/ exclude isotopes	60 s/exclude isotopes	60 s/ exclude isotopes	60 s/exclude isotopes	60 s/exclude isotopes	60 s/exclude isotopes	60 s/exclude isotopes	60 s/exclude isotopes
Cycle time	3 s	3 s	3 s	3 s	3 s	3 s	3 s	3 s
MS2								
Isolation mode	Quadrupole	Quadrupole	Quadrupole	Quadrupole	Quadrupole	Quadrupole	Quadrupole	Quadrupole
Isolation window	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6
NCE	32	32	Cal. ETD reaction time, HCD 25%	Cal. ETD reaction time, HCD 25%	32	32	32	32
Detector	Orbitrap	Ion Trap	Orbitrap	Ion Trap	Ion Trap	Ion Trap	Orbitrap	Orbitrap
Resolution	30k	Rapid	30k	Rapid	Rapid	Rapid	30k	30k
First mass	110 *m/z*	110 *m/z*	110 *m/z*	110 *m/z*	110 *m/z*	110 *m/z*	110 *m/z*	110 *m/z*
Target value	5.00E+04	1.00E+04	5.00E+04	1.00E+04	1.00E+04	1.00E+04	5.00E+04	5.00E+04
Max. injection time	100 ms	35 ms	70 ms	50 ms	35 ms	35 ms	100 ms	100 ms
Data Type	Profile	Centroid	Profile	Centroid	Centroid	Centroid	Profile	Profile
Neutral Loss Trigger								
Targeted trigger (amu)					M=97.9763, M=80	M=97.9763, M=80	M=97.9763, M=80	M=97.9763, M=80
Mass tolerance					0.5 *m/z*	0.5 *m/z*	20 ppm	20 ppm
Isolation mode					Quadrupole	Quadrupole	Quadrupole	Quadrupole
Isolation width					1.6	1.6	1.6	1.6
Activation type					ETD cal. parameters	ETD cal. parameters	ETD cal. parameters	ETD cal. parameters
SA Collision energy					EThcD 25%	ETcaD 15%	EThcD 25%	ETcaD 15%
Detector					Ion Trap	Ion Trap	Ion Trap	Ion Trap
Scan rate					Rapid	Rapid	Rapid	Rapid
First mass					110 *m/z*	110 *m/z*	110 *m/z*	110 *m/z*
AGC					1.00E+04	1.00E+04	1.00E+04	1.00E+04
Max injection time					50 ms	50 ms	50 ms	50 ms

Table S1. Orbitrap Fusion Tribrid MS acquisition parameters for the eight methods assessed. Cal. ETD refers to the fact that the ETD reaction time was calibrated according to precursor ion charge state using angiotensin. AGC: automatic gain control; IT: Ion trap; MIPS: monoisotopic precursor selection; NCE: normalised collision energy; NL: Neutral loss; OT: Orbitrap; SA: Supplemental activation.

Search Engine		HCD OT	HCD IT	EThcD OT	EThcD IT	HCD OT nl EThcD	HCD OT nl ETcaD	HCD IT nl EThcD	HCD IT nl ETcaD
Andromeda (1% FDR)	**# PSM**[a]	705 ± 4	984 ±16	407 ± 18	515 ± 88	625 ± 194	650 ± 30	838 ± 37	745 ± 36
	# unique phosphopeptides	153	160	146	153	156	154	154	155
	# phosphosites	167	173	160	167	170	168	168	170
	# phosphosites correctly localised with PTM-score	153	155	155	159	152	154	147	150
	% phosphosites correctly localised with PTM-score	92%	90%	97%	95%	89%	92%	88%	88%
Andromeda (1% FDR, no score filter)	**# PSM**[a]	743 ± 25	987 ± 25	423 ± 15	540 ± 82	640 ± 210	668 ± 31	848 ± 45	744 ± 28
	# unique phosphopeptides	160	163	151	152	155	159	155	155
	# phosphosites	175	178	165	166	170	174	169	169
	# phosphosites correctly localised with PTM-score	160	154	158	156	149	159	151	152
	% phosphosites correctly localised with PTM-score	91%	87%	96%	94%	88%	91%	89%	90%

Table S2. Evaluation of Andromeda score cut-off using synthetic phosphopeptides. *For each of the eight Orbitrap Fusion MS acquisition methods (Table 1, Table S1) the number of peptide spectrum matches (PSMs) are presented (n = two technical replicates), together with the number of unique peptides (out of a total of 171) and phosphosites (of 185 total), as well as the number and percentage of correctly localized phosphosite using Andromeda with PTM-score (bottom) with either default settings, invoking a score cut off of 40 for modified peptides (top), or with this score filter removed (bottom).* [a]*Mean values are presented ± S.D.*

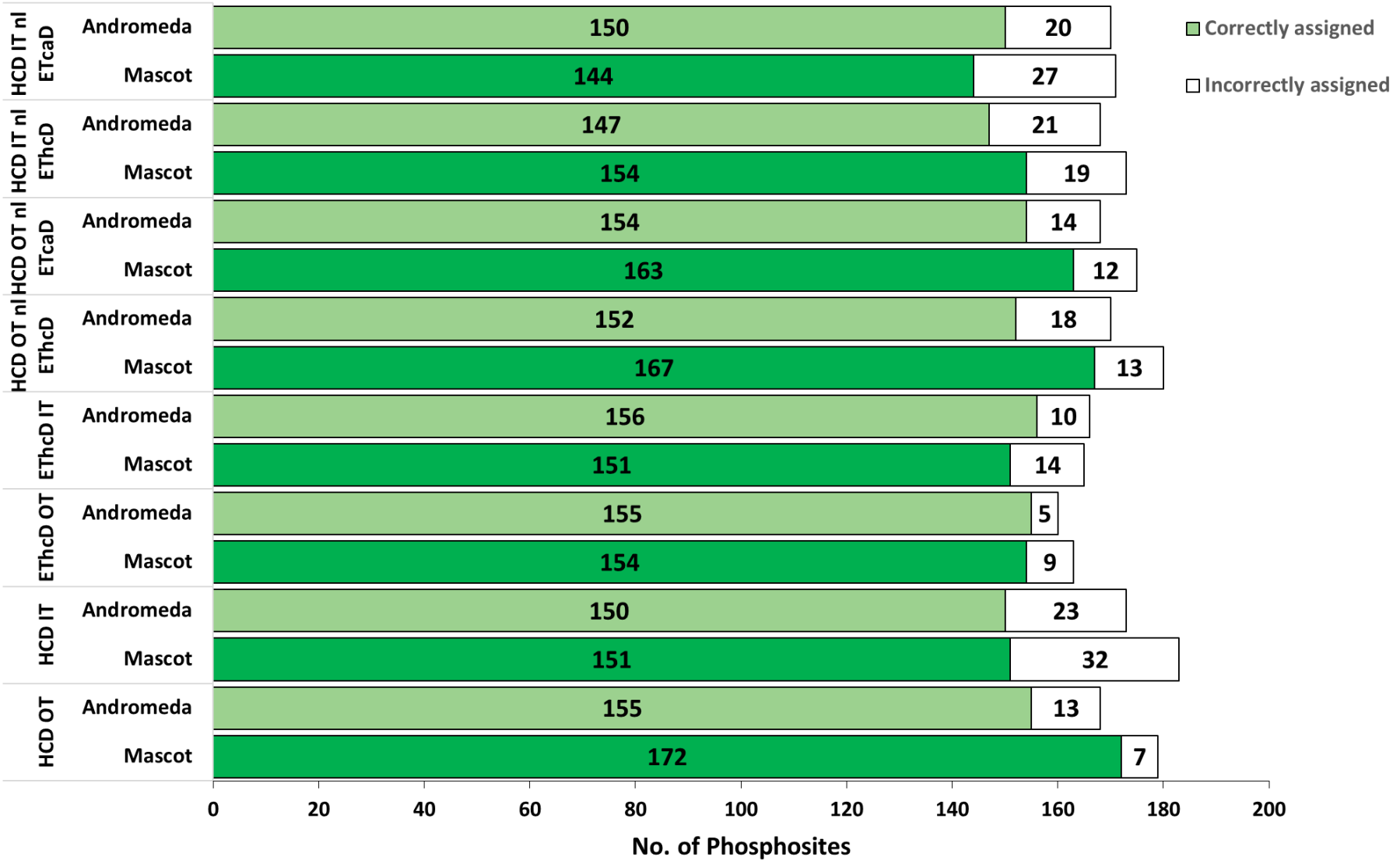

Figure S1. Acquisition method-specific phosphosite localization. Number of correctly assigned (green) and incorrectly assigned (white) phosphosites from the synthetic phosphopeptide library for each of the eight MS acquisition methods using either Andromeda or MASCOT (Table 1; Table S1).

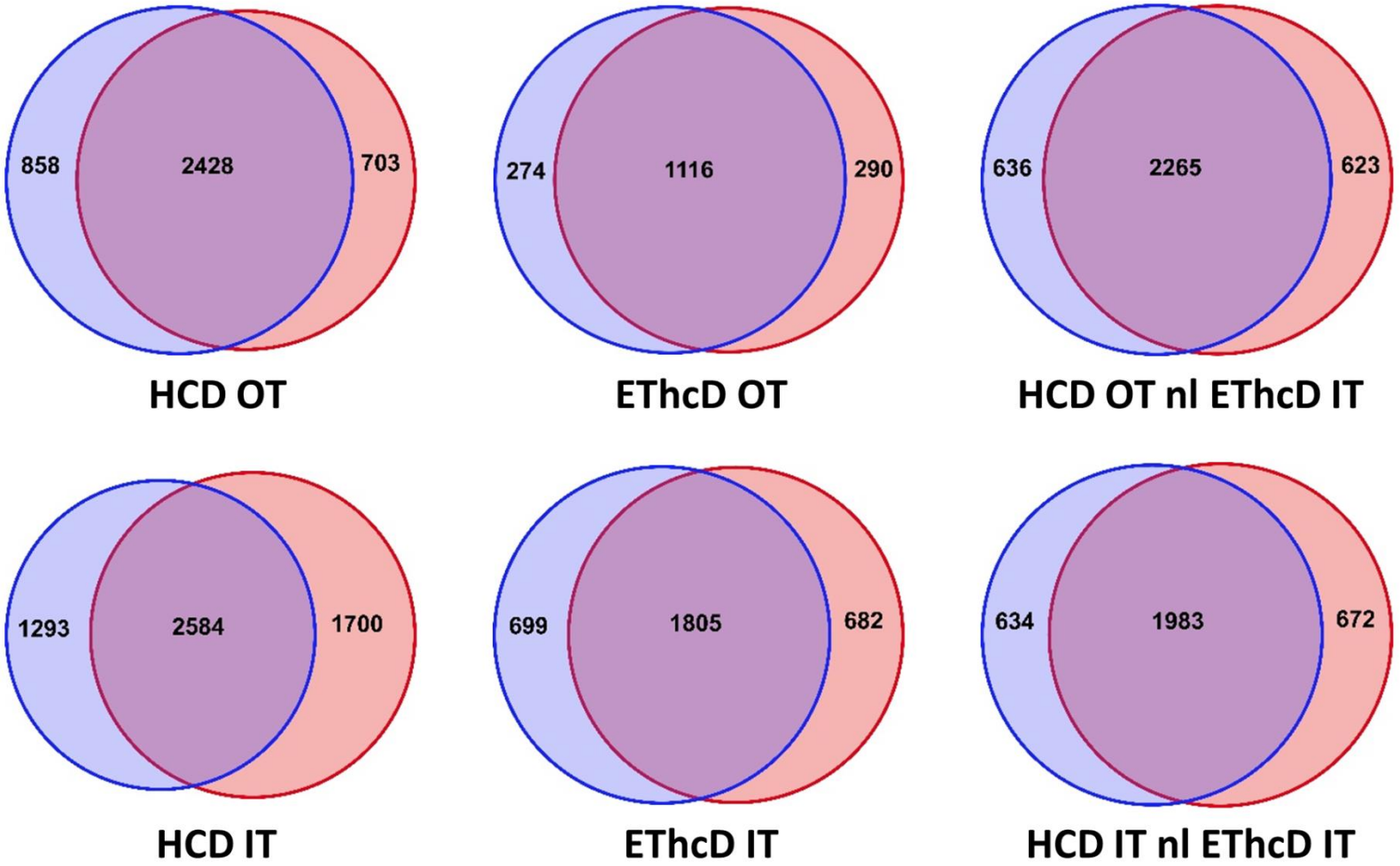

Figure S2. Overlap between technical replicates processed using Andromeda. U2OS phosphopeptide enriched cell lysate was analysed in duplicate using each of six Orbitrap Fusion MS acquisition methods as indicated. Venn diagrams present the overlap in the number of identified phosphopeptides between replicate analyses for each of the methods. See Table S1 for full details of MS methods.

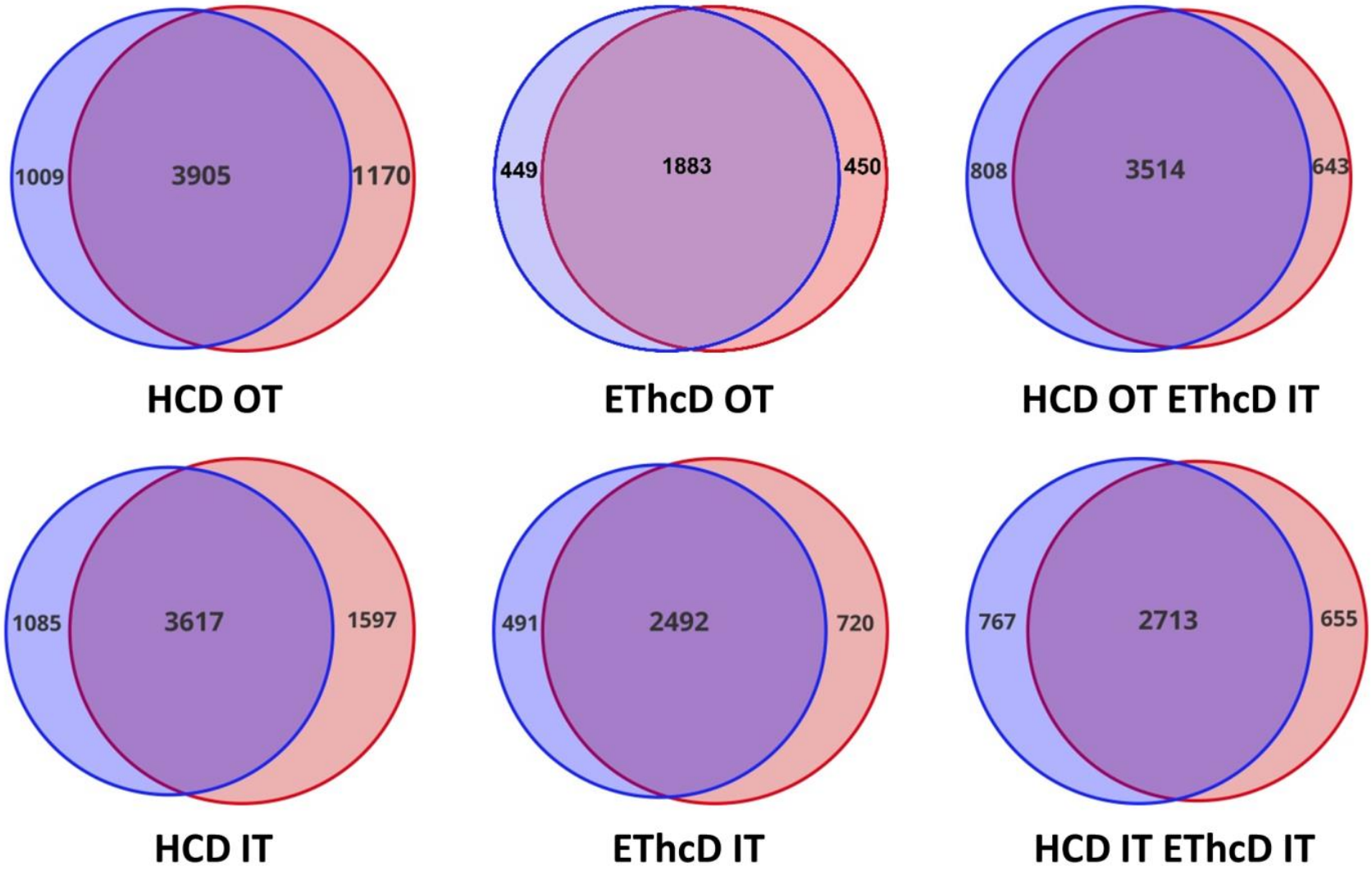

Figure S3. Overlap between technical replicates processed using Mascot. U2OS phosphopeptide enriched cell lysate was analysed in duplicate using each of six Orbitrap Fusion MS acquisition methods as indicated in duplicate. Venn diagrams present the overlap in the number of identified phosphopeptides between replicate analyses for each of the methods. See Table S1 for full details of MS methods.

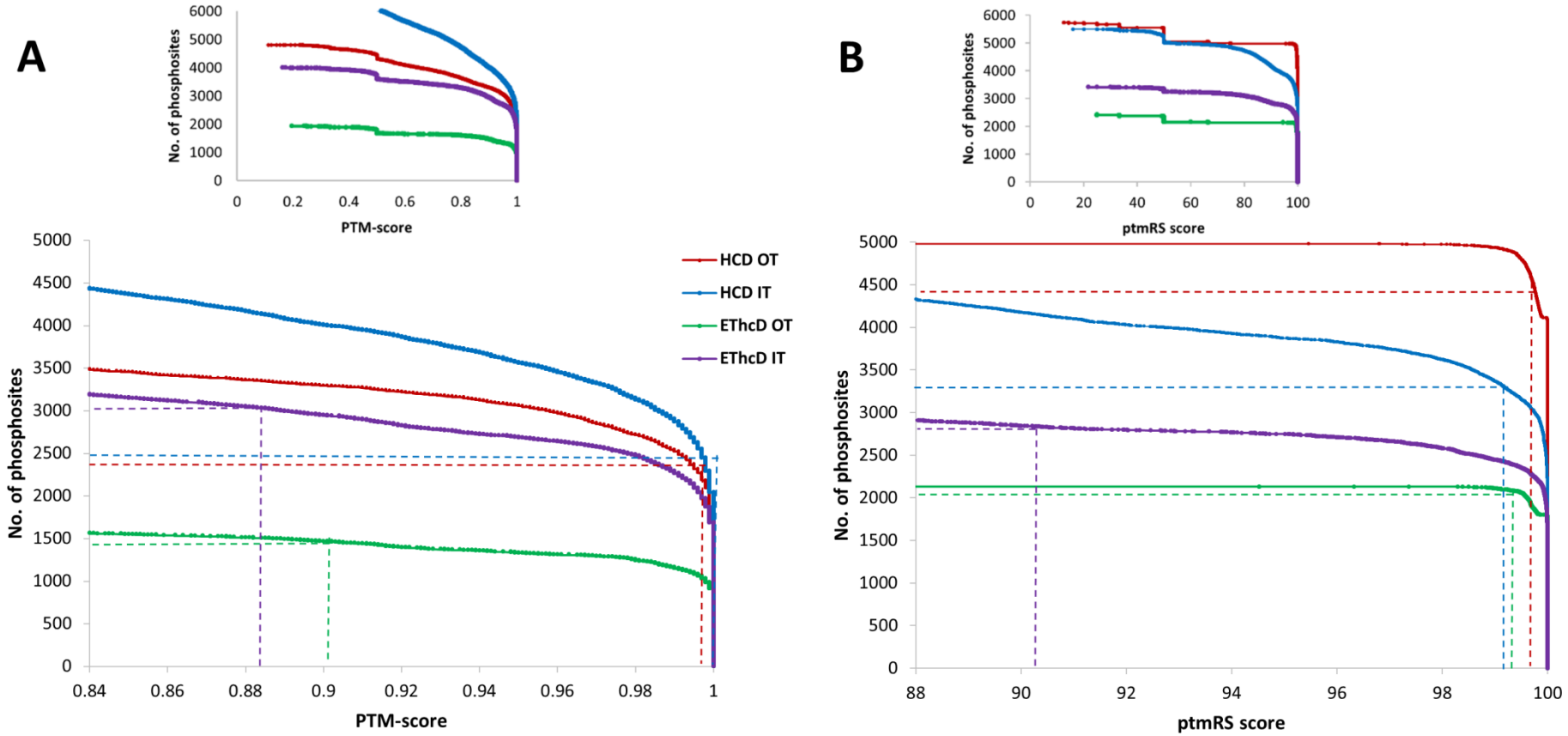

Figure S4. Distribution of phosphosite localisation scores for either PTM-score (A) or ptmRS (B) from cell lysate-derived phosphopeptides analysed using either HCD OT (red), HCD IT (blue), EThcD OT (green) or EThcD IT (purple). Dotted lines represent the value equivalent to 1% FLR (0.7% FLR for EThcD OT). Insets depict the complete score distribution for each site localisation algorithm.

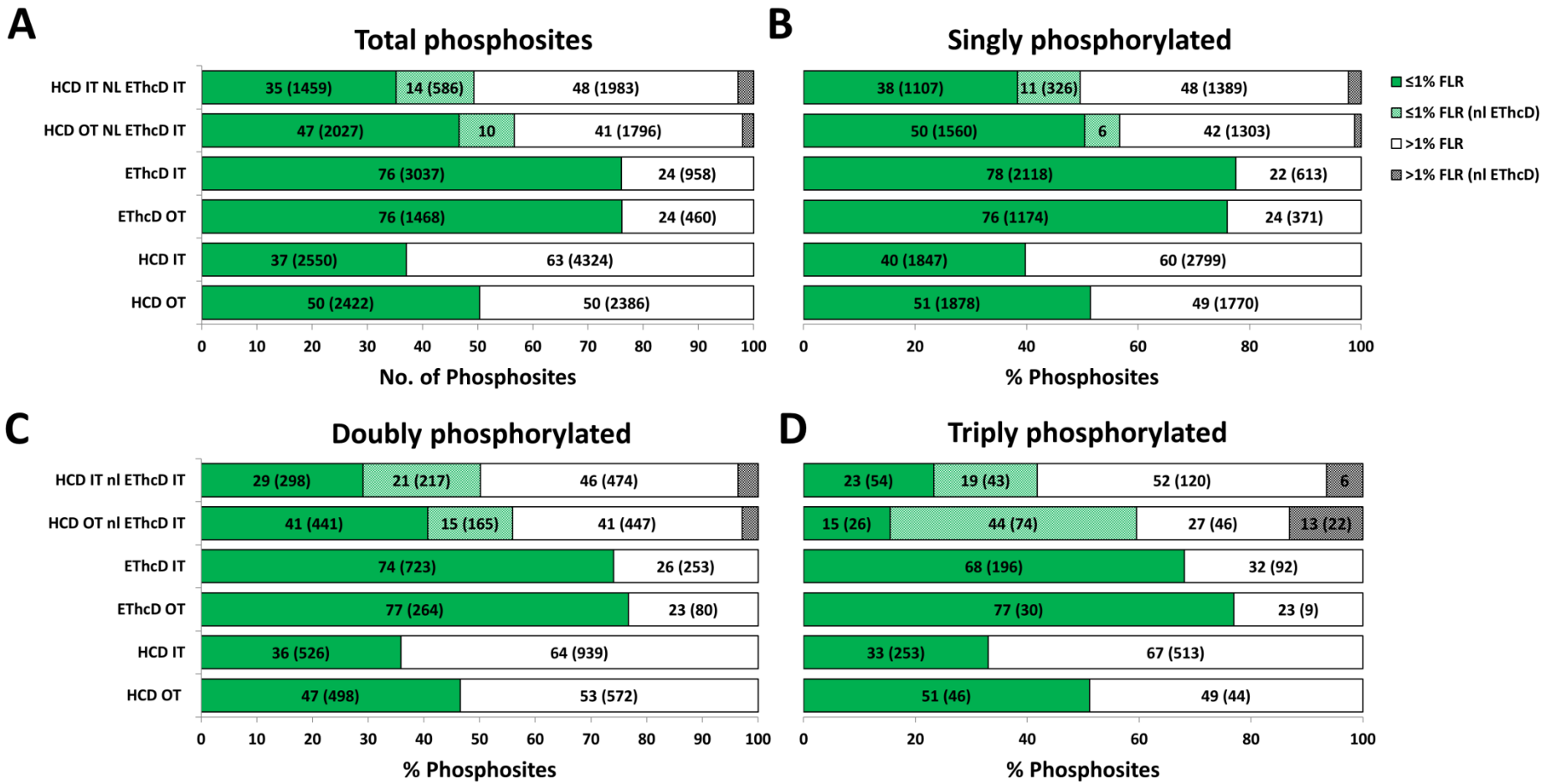

Figure S5. Phosphosite localisation confidence with Andromeda/PTM-score. Percent correctly site localised phosphopeptides (FLR ≤1%, green) or site ambiguous (FLR >1%, white/grey) phosphopeptides is presented for (A) all identified phosphorylation sites; (B) singly phosphorylated peptides; (C) doubly phosphorylated peptides; (D) triply phosphorylated peptides.

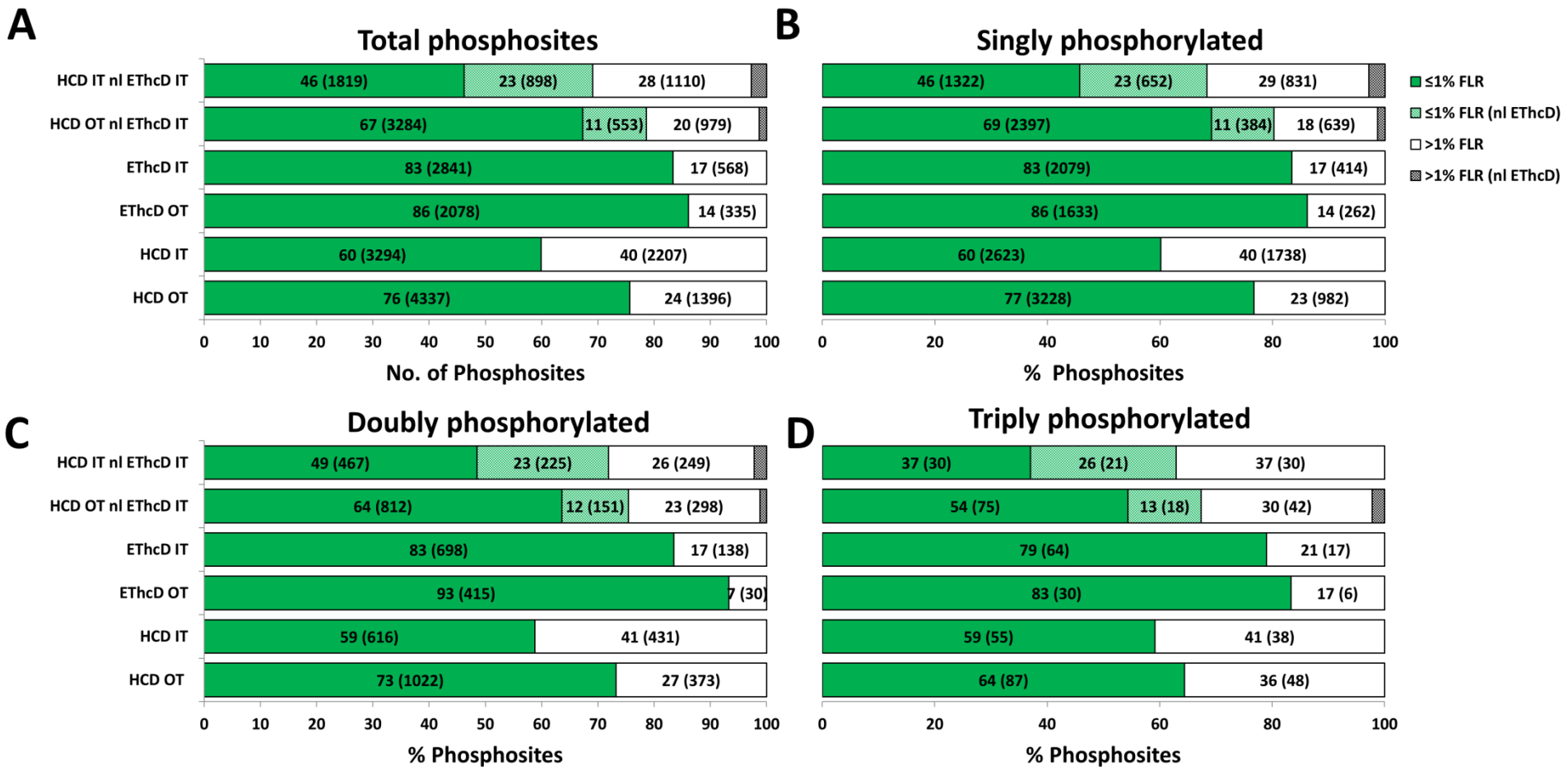

Figure S6. Phosphosite localisation confidence with MASCOT/ptmRS. Percent correctly site localised phosphopeptides (FLR ≤1%, green) or site ambiguous (FLR >1%, white/grey) phosphopeptides is presented for (A) all identified phosphorylation sites; (B) singly phosphorylated peptides; (C) doubly phosphorylated peptides; (D) triply phosphorylated peptides

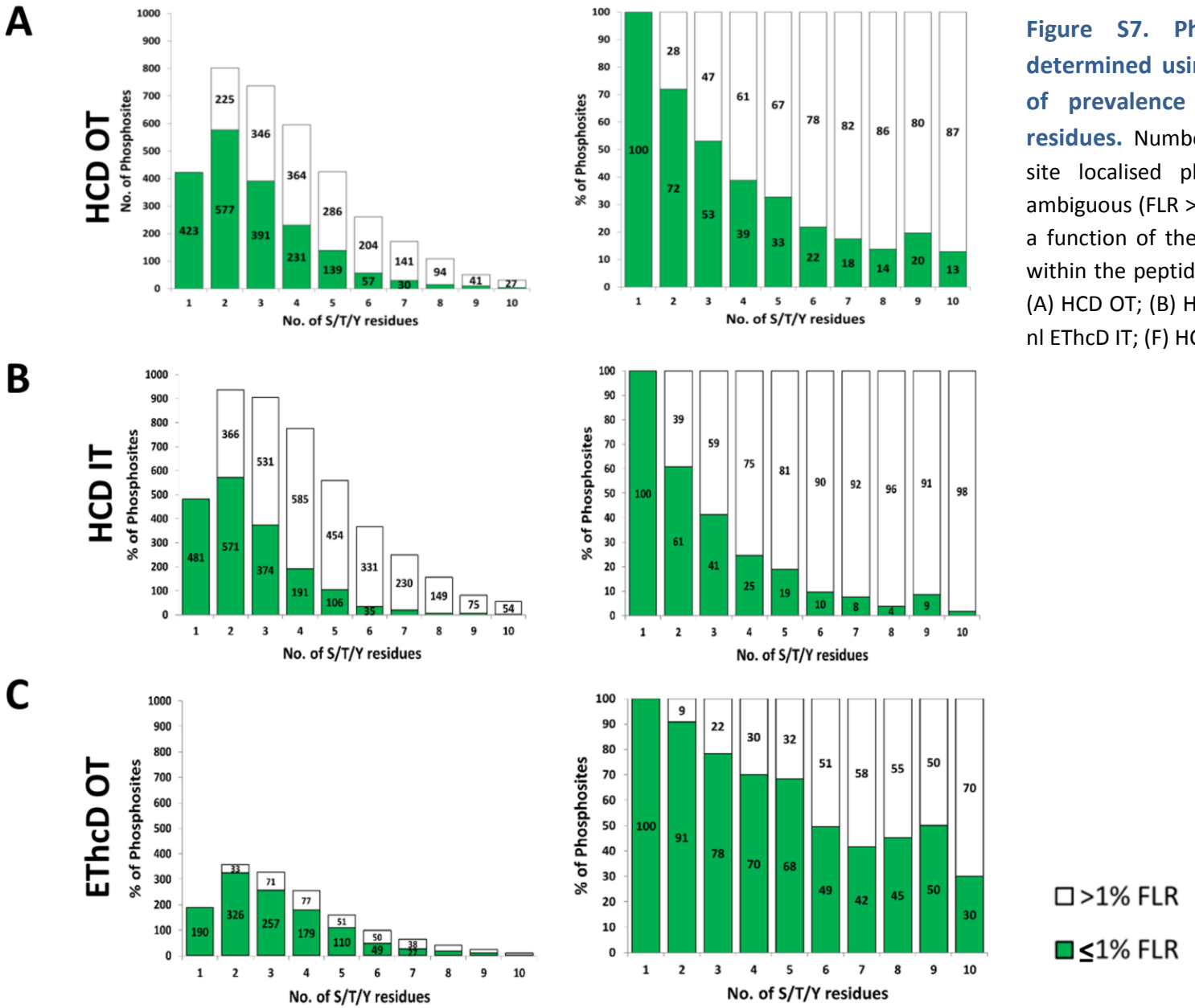

Figure S7. Phosphosite localisation confidence as determined using Andromeda/PTM-score, as a function of prevalence of common putative phosphorylated residues. Numbers (left) and percentage (right) of correctly site localised phosphopeptides (FLR ≤1%, green) or site ambiguous (FLR >1%, white) phosphopeptides are presented as a function of the number of Ser (S), Thr (T) or Tyr (residues) within the peptide for each of the six MS acquisition methods: (A) HCD OT; (B) HCD IT; (C) EThcD OT; (D) EThcD IT; (E) HCD OT nl EThcD IT; (F) HCD IT nl EThCD IT.

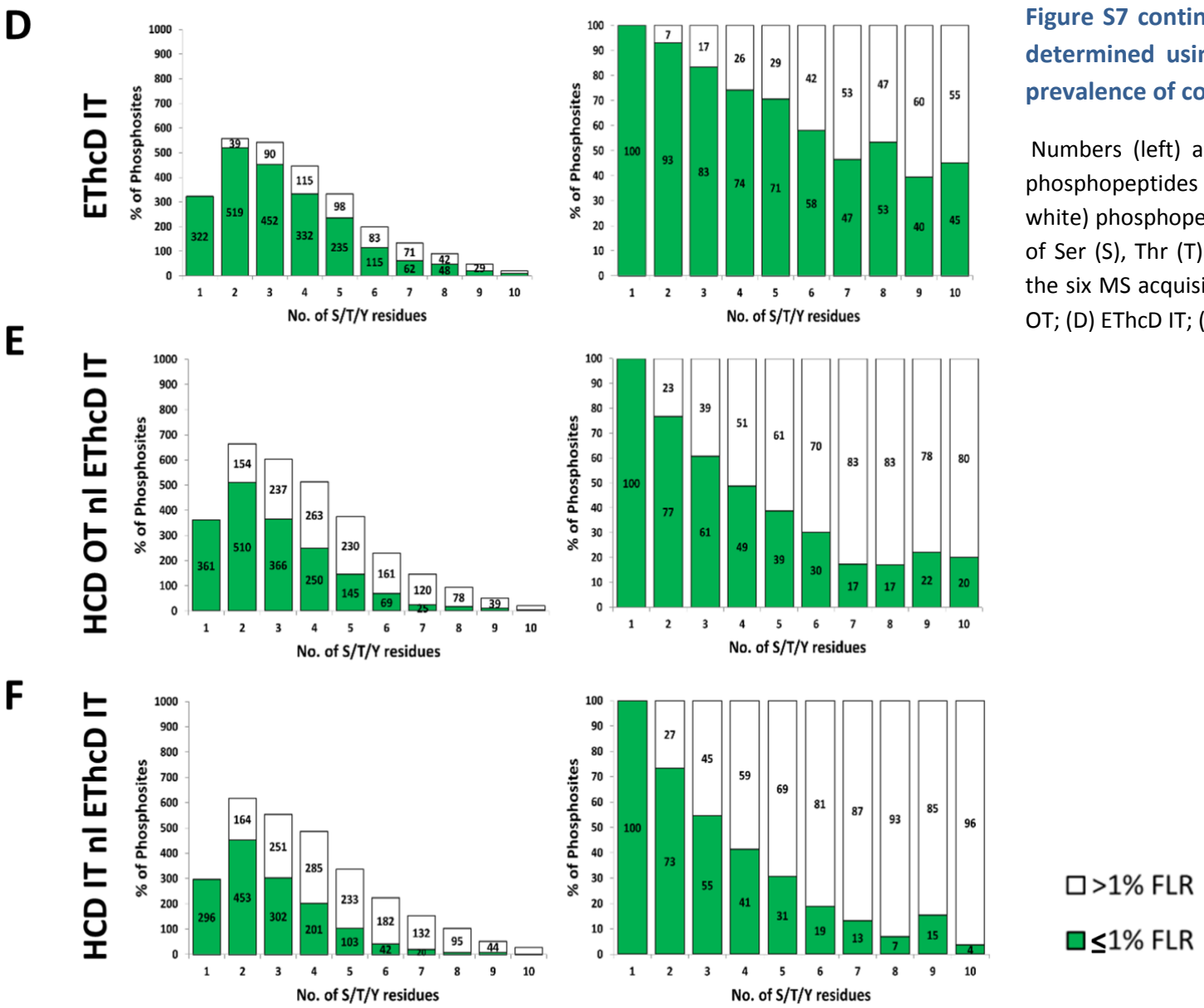

Figure S7 continued. Phosphosite localisation confidence as determined using Andromeda/PTM-score, as a function of prevalence of common putative phosphorylated residues.

Numbers (left) and percentage (right) of correctly site localised phosphopeptides (FLR ≤1%, green) or site ambiguous (FLR >1%, white) phosphopeptides are presented as a function of the number of Ser (S), Thr (T) or Tyr (residues) within the peptide for each of the six MS acquisition methods: (A) HCD OT; (B) HCD IT; (C) EThcD OT; (D) EThcD IT; (E) HCD OT nl EThcD IT; (F) HCD IT nl EThCD IT.

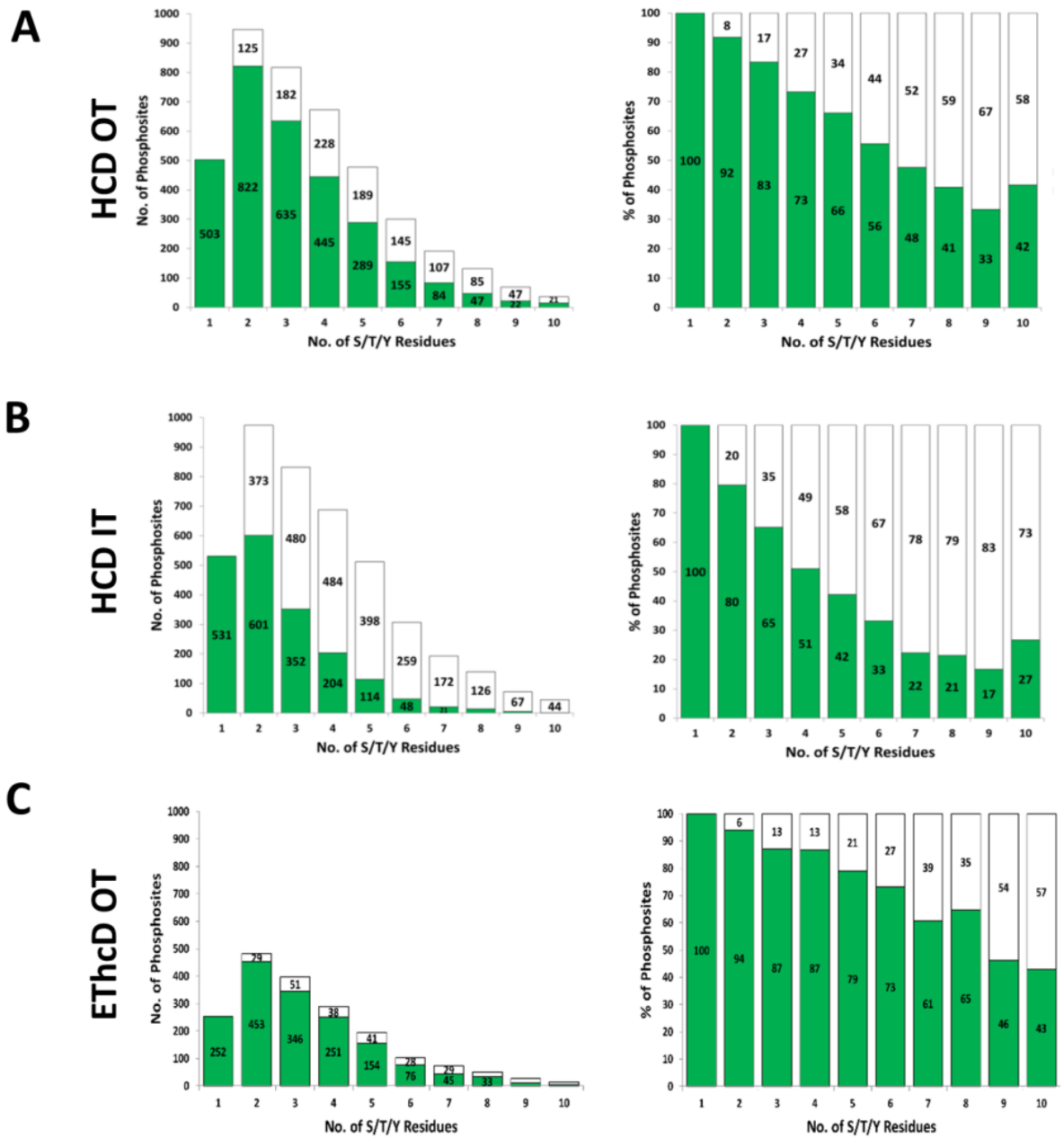

Figure S8. Phosphosite localisation confidence as determined using MASCOT/ ptmRS, as a function of prevalence of common putative phosphorylated residues.

Numbers (left) and percentage (right) of correctly site localised phosphopeptides (FLR ≤1%, green) or site ambiguous (FLR >1%, white) phosphopeptides are presented as a function of the number of Ser (S), Thr (T) or Tyr (residues) within the peptide for each of the six MS acquisition methods: (A) HCD OT; (B) HCD IT; (C) EThcD OT; (D) EThcD IT; (E) HCD OT nl EThcD IT; (F) HCD IT nl EThCD IT.

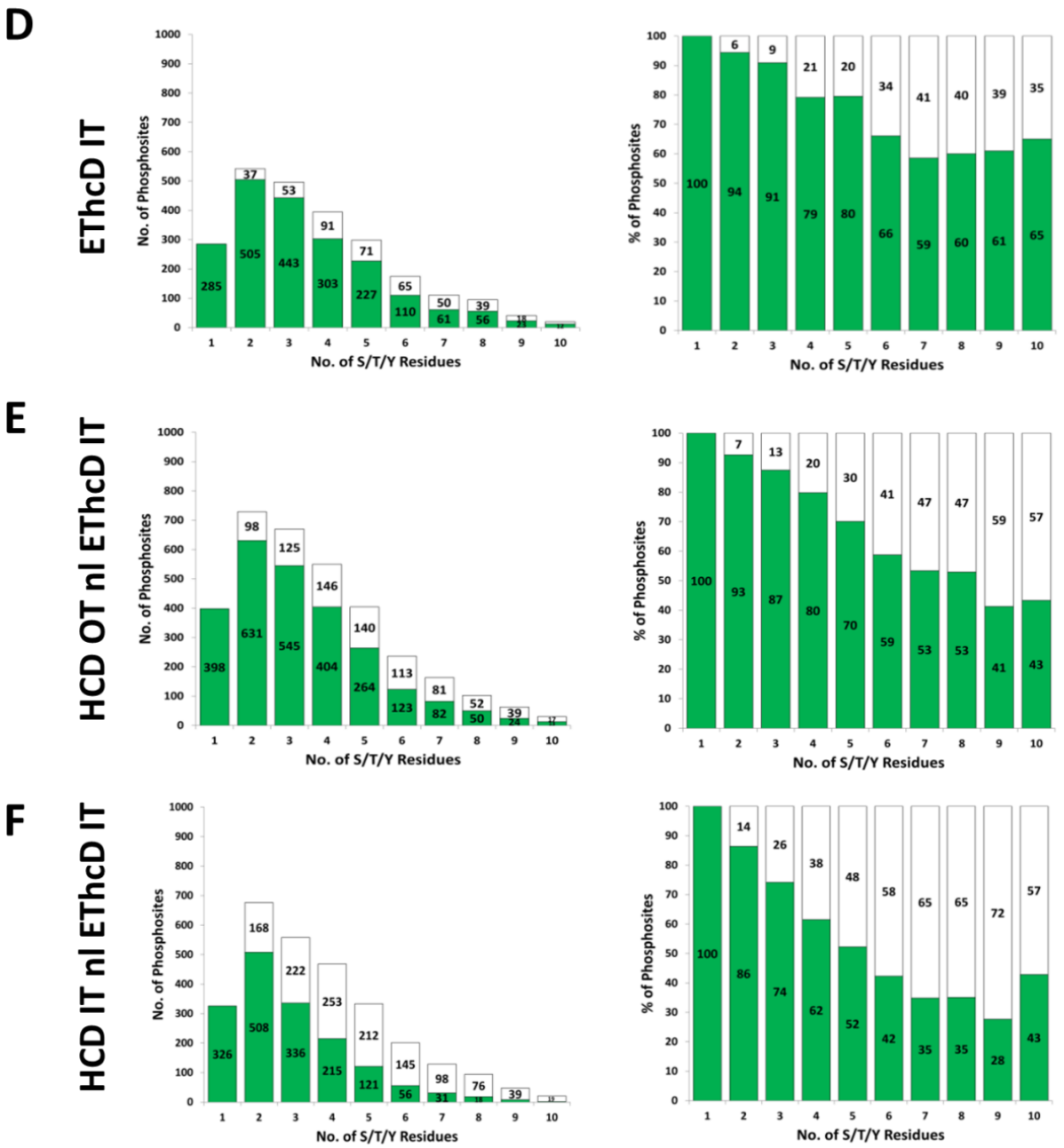

Figure S8 continued. Phosphosite localisation confidence as determined using MASCOT/ ptmRS, as a function of prevalence of common putative phosphorylated residues.

Numbers (left) and percentage (right) of correctly site localised phosphopeptides (FLR ≤1%, green) or site ambiguous (FLR >1%, white) phosphopeptides are presented as a function of the number of Ser (S), Thr (T) or Tyr (residues) within the peptide for each of the six MS acquisition methods: (A) HCD OT; (B) HCD IT; (C) EThcD OT; (D) EThcD IT; (E) HCD OT nl EThcD IT; (F) HCD IT nl EThCD IT.

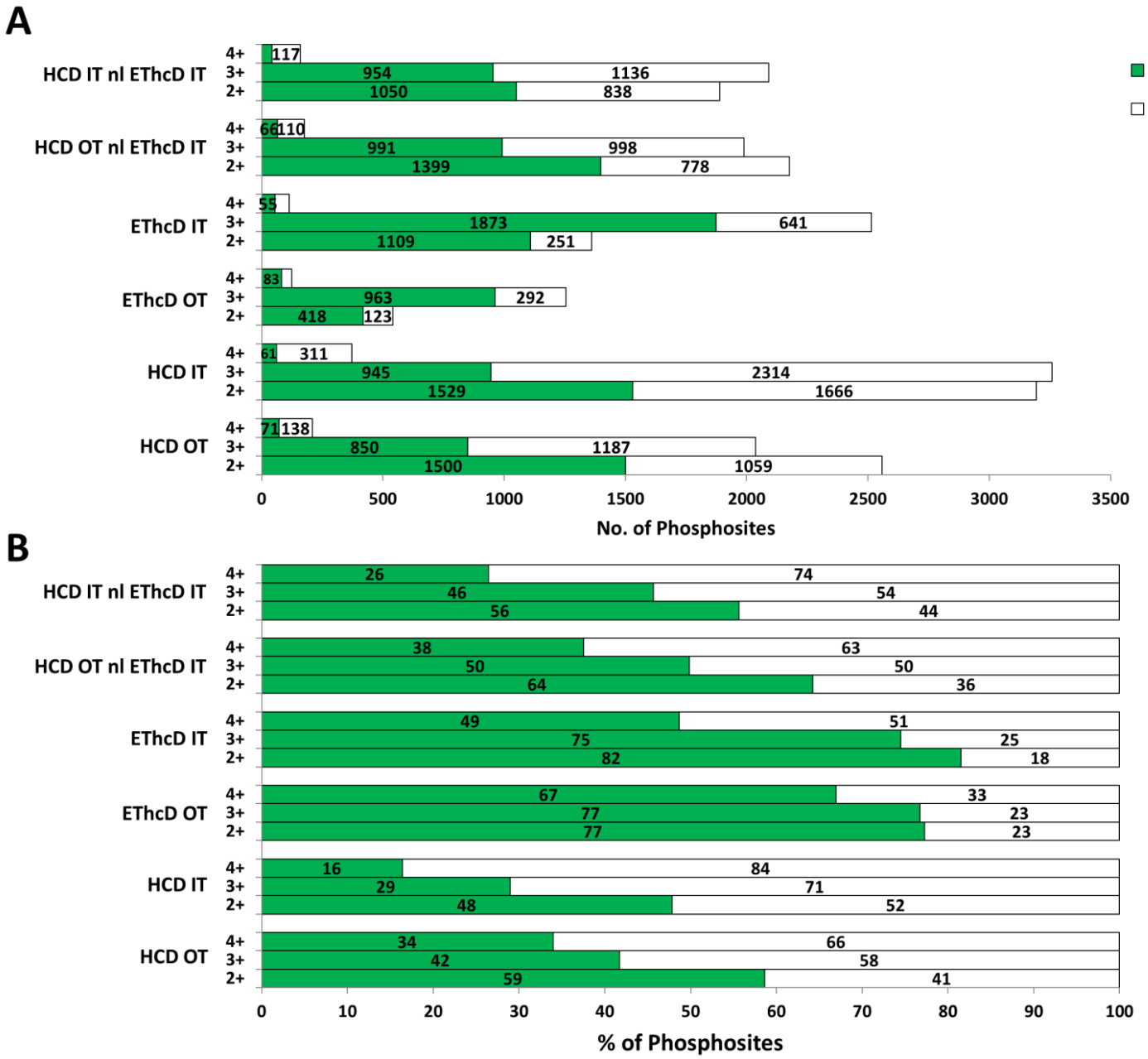

Figure S9. Phosphosite localisation determined using Andromeda/PTM-score, as a function of peptide ion charge state. Numbers (A) and percentage (B) of correctly site localised phospho-peptides (FLR ≤1%, green) or site ambiguous (>1%, white) phosphopeptides are presented as a function precursor ion charge state for each of the six MS acquisition methods.

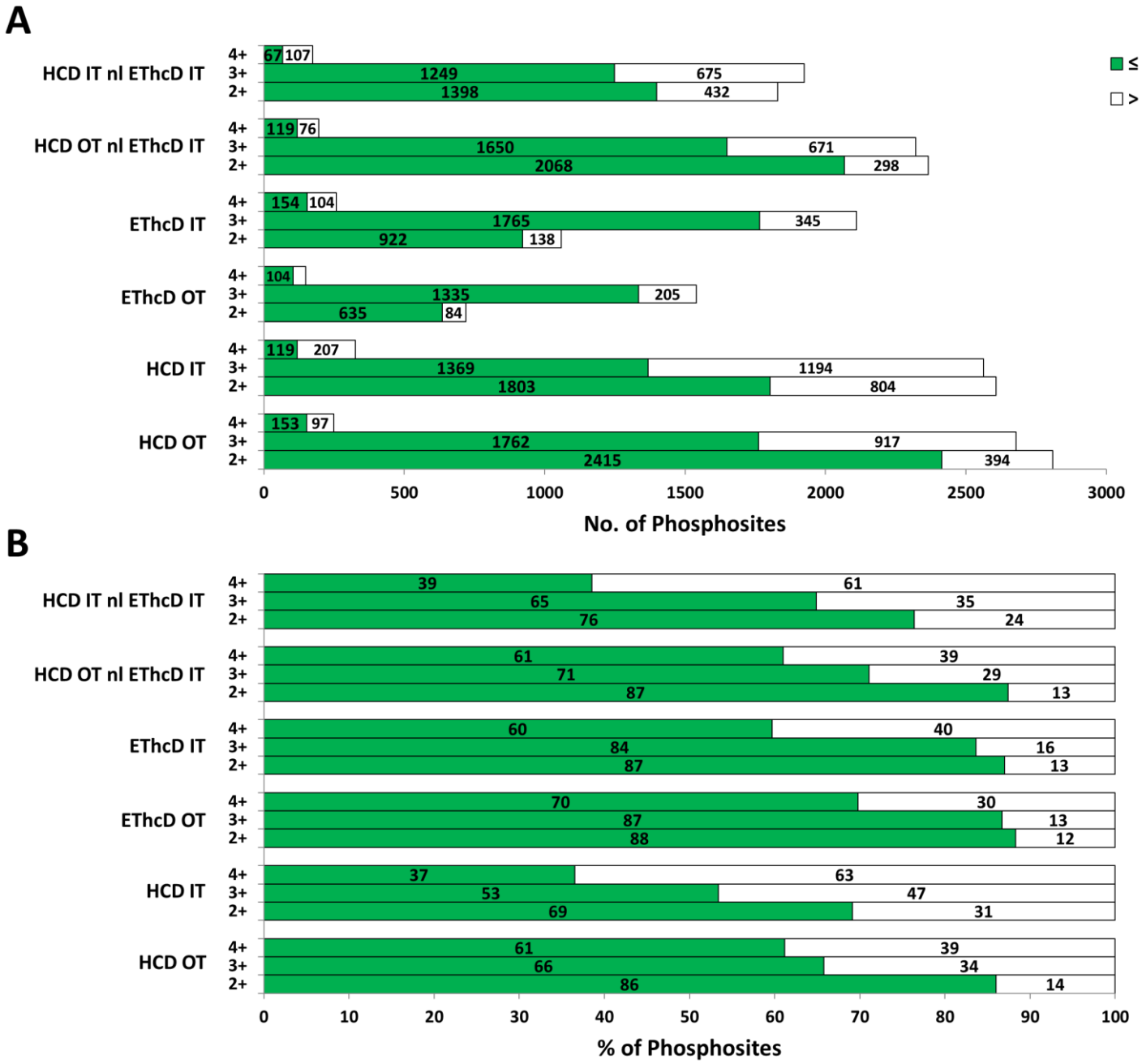

Figure S10. Phosphosite localisation determined using MASCOT/ptmRS, as a function of peptide ion charge state. Numbers (A) and percentage (B) of correctly site localised phosphopeptides (FLR ≤1%, green) or site ambiguous (>1%, white) phospho-peptides are presented as a function precursor ion charge state for each of the six MS acquisition methods.