SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Cardiac Myofibril and cTnI Protein Preparation

Human Left ventricular transmural tissue samples were obtained from explanted end-stage failing hearts hearts with ISHD and IDCM during heart transplantation surgery as well as from unutilized healthy donor hearts (n = 10 per group), rapidly frozen and stored in liquid nitrogen. Ethics approval was provided by St. Vincent's Hospital (#H03/118), Sydney Australia and by The University of Sydney (#09-2009-12146). To preserve the endogenous phosphorylation status, frozen tissue samples each 0.1 g weight were used to purify cardiac myofibrils. Briefly, 0.1 g each of frozen LV heart tissue was collected in fresh relaxing buffer (containing 75 mM KCl, 2 mM MgCl2, 2 mM EGTA, 1 mM NaN3, 10 mM imidazole, pH 7.2, 4 mM Phosphocreatine, 1 mM ATP, 50 mM BDM, 1 mM DTT, 1 mM benzaamidine-HCI, 0.1 mM PMSF, 1 μg/ml leupeptine, 1 μg/ml pepstatin, and 1% Triton X-100) rapidly minced using a scissor and homogenized on ice for 10 s in the buffer containing 0.1 M EDTA plus the complete protease and phosphatase inhibitor cocktail tablets (Roche) using a Polytron homogenizer at 55% max speed. Resultant homogenates were centrifuged at 3,000 × g for 8 min at a temperature of 4 ºC and the supernatant fraction removed. The pellet was then suspended in a rigor buffer (containing 75 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 1 mM NaN_3, 10 mM imidazole, pH 7.2) with 1% Triton X-100. Transferred the solution into a Duall-glass homogenizer for homogenization and centrifuged two times at 3,000 × g for 8 min, followed by washing two times with the rigor buffer without Triton X-100. Following washes the pellet was suspended in a K-60 buffer (containing 60 mM KCl, 20 mM MOPS, and 2 mM $MgCl_2$) and centrifuged two

times at 2,000 × g for 8 min. Purified myofibril proteins were extracted in buffer (4 M urea, 0.3 M NaCI, 4% SDS, and 50 mM Tris-HCI pH 8.8) and centrifuged twice at 12, 000 × *g* for 15 min. The resultant soluble fraction was frozen at -80ºC until analysis.

Canine Model of HF and Reverse Remodeling after CRT

Left ventricle tissue (LV) was collected from adult mongrel dogs (n = 4 per group); groups included: normal (Control), dyssynchronous pacing-induced heart failure (HF_{dys}), or cardiac resynchronization therapy (CRT) as previously published.[1] All protocols were approved by the Animal Care and Use Committee of the Johns Hopkins Medical Institutions. Briefly, a left bundle-branch ablation (LBBB) by radiofrequency and subsequent right atrial pacing for 6 weeks at 200 bpm was used to induce HF_{dys}. LBBB was confirmed by intra-cardiac electrograms, with surface QRS widening from 50±7 to 104±7 ms ($P < 0.001$). For CRT, the HF_{dys} protocol was stopped after 3 weeks, at which time biventricular pacing was carried out 5 for 3 weeks at the same pacing rate of 200 bpm. At terminal study the hearts were extracted under cold cardioplegia, dissected into endocardial and mid/epicardial segments from the septum (i.e. LV and RV septum) and LV lateral wall, and frozen in liquid nitrogen. Tissue samples obtained from the upper third of the LV lateral wall were used in the present study.

Protein Quantification and Digestion and Peptide Extraction

Accurate determination of protein concentration was assessed by a bicinchoninic acid (BCA) assay kit form from Pierce (Rockford) according to the company's instruction. Extracted myofilament proteins were separated on 1D SDS-PAGE using 4-12% NuPAGE Bis-Tris gels (1mm, Invitrogen). Electrophoresis was performed according to manufacturer's protocols and individual gels were stained using the coomassie blue R-250**.** Protein bands relevant to cTnI

were excised from fresh gels and digested as described previously. Samples were monitored to ensure consistent and complete digestion after optimization of the method.

Briefly, protein bands were excised, cut into 1 mm^3 pieces, and washed 3 times with 50% acetonitrile/25 mM ammonium bicarbonate for 15 min with shaking. Gel pieces were incubated with 25 mM ammonium bicarbonate +10 mM dithiothreitol for 60 min at 55 ºC, washed with acetonitrile (ACN), then incubated with 25 mM ammonium bicarbonate + 55 mM iodoacetamide (freshly made) for 30 minutes in the dark. After these incubations, the gel pieces were washed with acetonitrile, dried, and rehydrated with 10 ng/μl trypsin (Promega, sequencing grade) in 25mM ammonium bicarbonate then placed on ice for 30 min. Excess trypsin was removed then 20 µL of 25 mM ammonium bicarbonate was added. Samples were digested at 37 ºC for 18 hr. The liquid was transferred to a clean tube. The peptides were extracted twice using 50% ACN+0.1 TFA% for 20 min at 25 ºC with a shaking, and combined two times of extraction with the liquid from the previous step. The final peptide mixture was performed off-line cleanup or desalting with a C18 reversed-phase chromatography in C18 ZipTips (Millipore) using 0.1% TFA. The peptides were then eluted three times with 10 μl of 70% ACN, 0.1% TFA. The combined solution was dried using a vacuum centrifuge SpeedVac (Thermo Electron) and frozen at -80 ºC until analysis.

Quantitative Comparison of Sample Processing.

Comparison between direct digestion with trypsin or first separated by SDS PAGE with cTnI band excised and undergo in-gel digestion was carried out in five replicates to ensure extent of phosphorylation was preserved during processing. Comparison of the phosphorylated and unphosphorylated peptides containing T142 was between a gel-based and direct homogenization-based protein digestion by MRM. In the gel-based method, 30 μg of purified myofilament

proteins from heart failure LV tissues was applied to 4-12% Bis-Tris NuPAGE gels as above, followed by Coomassie staining and tryptic in-gel digestion. Alternatively, whole proteins were extracted directly from the purified myofilament, and then 30 μg of the protein mixture was treated as above without the separation by NuPAGE gels. All peptide mixtures were desalted prior to analysis by MRM assays of peptides (RPTLR or $RP_{(p)}TLR$) and compared to the peptide NITEIADLTQK which thus normalizes to the total protein concentration.

MALDI-TOF MS and Orbitrap Mass Spectrometry

To ensure the correct band with cTnI was excised from 1 D gels, we performed protein identifications by peptide mass fingerprint (PMF) technique for all major 12 protein bands from a heart failure sample using a mass spectrometry 4800 MALDI-Tof/Tof mass spectrometry (ABI) MALDI-TOF/TOF for protein identification by peptide mass fingerprint (PMF) technique. MS spectra were acquired on the 4800 MALDI TOF/TOF Analyzer (ABI) using 1000 shots/spectra and a laser power between 4300–4700 units. PMF was conducted with the database search tool Mascot. Peptides were searched in against the SwissProt human database using the following criteria: fixed modification carbamidomethyl; variable modifications_oxidation (methionine); maximum missed cleavages_2; peptide tolerance_ ± 0.5Da; MS/MS tolerance_ ± 0.8 Da. The band 9 was successfully identified as cTnI about 70% sequence coverage with Mascot score 160 (***Supplemental*** Fig.17).

LTQ Orbitrap (Thermo Scientific) mass spectrometer was used for initial verification of the phosphorylated sites. For the LTQ Orbitrap, desalted peptides were reconstituted in 10 μl 0.1% v/v aqueous formic acid (FA). Each 5 μl sample was injected and analyzed on an Agilent 1200 nano-LC system (Agilent) connected to an LTQ-Orbitrap mass spectrometer equipped with a nanoelectrospray ion source (Thermo Scientific). Peptides were separated on a BioBasic (New

Objective, Woburn, MA) C18 RP-HPLC column (75 μm x 10 cm) using a linear gradient from 5% B to 65% B in 60 minutes at a flow rate of 300 nl/min, where mobile phase A was composed of 0.1% v/v aqueous FA and mobile phase B was 90% acetonitrile, 0.1 % FA in water. MS/MS data from the LTQ LC/MS/MS (Thermo Scientific) were analyzed using a Sorcerer 2™-SEQUEST®, with post-search analysis performed using Scaffold (Proteome Software). All raw data peak extraction was performed using Sorcerer 2™-SEQUEST® default settings. Data was searched against the Swissprot human database, using a full trypsin digestion, with the following criteria: fixed modification_carbamidomethyl; variable modifications_phosphorylation (Ser, Thr, and Tyr) and oxidation (Met); Peptide mass tolerance was set to either 100 ppm or 0.1 amu, fragment mass tolerance set to 1 amu, fragment mass type set to monoisotopic, maximum number of modifications set to 4 per peptide. All MS/MS spectra were manually examined using Scaffold (Proteome Software). The analyses for biological and technical replicates were conducted in triple.

Synthesized Internal Standards (SIS)

A series of SIS peptides were designed on detection of the appropriate peptide for the known and novel to be phosphorylated including mono or di-phosphorylated and chemically produced using solid-phase peptide synthesis (>95% pure) (New England Peptide; NEP). The corresponding non-phosphorylated sequences were isotopically labeled by incorporating $^{15}N/^{13}C$ in either Lys (K) or Arg (R) amino acid residue at the C-terminus (mass difference of 8 and 10 daltons, respectively for K or R). The phosphorylated peptides were unlabeled. The synthetic reference peptides were useful for confirming the identity of the target peptide through perfect co-elution and by exhibiting identical fragmentation patterns between the internal standards and endogenous analytes. Amino acid analysis was carried out both on labeled and unlabeled

peptides, and all were dried for storage and delivered to us by company. The peptide was suspended at 10 pmol/μl with 0.1% formic acid in HPLC water and aliquot each 10-50 μl for a storage at -80 ºC.

The optimized parameters included declustering potential (DP), collision energy (CE), and Collision Cell Exit Potential (CXP) for the synthetic peptides. Optimization was obtained by ramping the parameters DP (0-400 volts), CE (5-130 volts) and CXP (0-66 volts) from low to high with a step of 1 for all parameters and a fixed setting of 10 volts for entrance potential (EP) and are listed in table. A total 30 assays and 145 transitions were designed based on detection of the appropriate peptide and matching transition (Q1/Q3) for each mono or di-phosphorylated and the corresponding unphosphorylated sequence.

Standard curves

Using these optimized MRM parameters, we made calibration curves for each mono or di-phosphorylated and the corresponding non-phosphorylated tryptic peptide by nano-LC-MS/MS in triplicate. For each phosphorylation site, a series dilution of light/heavy peptides were made to produce 6-point calibration curves at 0.125, 0.25, 0.5, 1, 5, and 10 fmol/μl. All analytes were spiked in with or without a mix matrix containing 10 μg digest of the donor, ISHD, and IDCM LV at a six-point dilution ratio of different concentration 0.125, 0.25, 0.5, 1, 5, and 10 fmol/μl in triplicates. A mixture containing 10 heavy labeled peptides of cTnI at known concentrations (fixed at 1 fmol/μl) to mimic a digest matrix of human cTnI, then followed analysis by a 4000 QTRAP hybrid triple quadrupole/linear IT mass spectrometer (AB SCIEX) operating with Analyst 1.4.2 software, scheduled experiments in the optimized parameters and positive ion mode. Each standard curve for the phosphorylated peptides was then generated from

precise quantities of each of these internal standard peptides. The lower limit of detection and quantification (LLOD and LLOQ) was determined for each peptide.

Nano-LC/MS/MS Analysis

Digested protein samples were analyzed using a 4000 QTRAP hybrid triple quadrupole/linear IT mass spectrometer (AB SCIEX) operating with Analyst 1.4.2 software scheduled experiments in positive ion mode. The cleanup mixture of peptides from the in gel digestion of cTnI proteins were reconstituted with 20 μl water containing 0.1% Formic acid. Peptides were separated by an Eksigent Tempo nano-LC system (Eksigent Technology) onto a BioBasic C18 reverse-phase PicoFrit column (300 Å, 5 μm, 75 µm ×10 cm, 15 μm tip, New Objective). Peptides were eluted 36-min linear gradient from 5 to 40% B (mobile phase A: 2% v/v ACN containing 0.1% v/v formic acid; mobile phase B: 98% v/v ACN containing 0.1% v/v formic acid) at 500 nl/min flow rate. The column eluent solution was directed into the NanoSpray source spray head *via* coupling to a distal coated PicoTip fused silica spray tip (360 µm od, 75 μm id, 15 µm diameter emitter orifice; New Objective). Samples were analyzed using the following settings curtain gas (CUR):15; collision gas (CAD): high; ion spray voltage (IS): 2.5 kV; ion source gas1 (GS1): 25; ion source gas 2 (GS2): 0; resolution Q1 and Q3: unit; heater interface temperature: 150 ºC. Each sample was run in triplicates.

Data Processing and Statistical Analysis

Peak detection and quantification of peak area was determined with Multiquant software version 2.0 (AB SCIEX) and inspected manually to ensure correct peak identification and quantification. Measurements were performed in triplicate and then averaged to reduce technical variation. In cases where the peptide was detected, but not quantifiable (above LLOD but below

LLOQ), the quantity was tagged as LLOQ and was replaced with 100 as a lower cutoff point. If the peptide was not observed it was recorded as not detected (ND).The quantity of each peptide (in fmoles) was determined based on the corresponding standard curve using data points above the LLOQ. Values were then normalized against the total quantity of cTnI (phosphopeptide (fmol)/ cTnI (fmol)), and then the fold change between control and the heart failures groups was calculated. For human samples, data was analyzed using one-way analysis of variance (ANOVA) on ranks, followed by Dunnett's multiple comparisons post-hoc test. The canine samples were analyzed using a one-way ANOVA, followed by Bonferroni multiple comparisons post-hoc test. All calculations were done using SigmaPlot v11 (Systat), with a *P*-value less than 0.05 denoting significance.

Validation of MRM Quantification by Immunoblotting Analysis

Immunoblotting analysis was optimized for each antibody, by altering blocking conditions and concentrations of antibodies (primary and secondary). The phosphorylation status of cTnI was monitored by western blotting a specific antibody against phosphorylated PKA-sites anti-pS22 and/or 23 and Phospho-Tyrosine Mouse mAb (P-Tyr-100) (used at 1:1000) (Cell Signaling). For normalization, after visualizing the S22/23, membranes were striped and re-probed using a mouse antibody clone 8I-7 (used at 1:10000) from International Point of Care Inc, Toronto, Canada. Phosphorylated cTnI (S22/23) was calculated as phosphor signal/total protein signal in triple experiments.

Secondary antibodies were alkaline phosphatase conjugated donkey anti-rabbit and alkaline phosphatase conjugated goat anti-mouse, both from Jackson Immuno Research (1:10,000 dilution). Briefly, SDS-PAGE gels were incubated in transfer buffer (25 mM bis-Tris

and 1mmol/L EDTA, pH 7.2) for 10 min prior to being transferred to PVDF (Millipore, 45 μm) via the Mini-Transblot Cell (Bio-Rad) for 1h at 100V. Membranes were blocked with 5% v/v western blocking reagent (Roche) in TBST (20 mM Tris, 150 mM NaCl and 0.1% v/v Tween, pH 7.5) at 4°C for 1h. Blots were then incubated with primary antibody (1:5,000) for 1h at room temperature, washed for 30 min with TBST, incubated with secondary antibody for 1h and then washed for 1h with TBST (200 mM Tris, 1.5M NaCl, 0.1% v/v Tween-20, pH 7.5). Chemiluminescence was used in its linear range. Densitometrical analysis was done with a Progenesis image software (Nonlinear Dynamics), and the ratio of phosphor cTnI to total cTnI was calculated by Microsoft Excel software.

Limitations of the Study

The potential limitations of this study include the fact that human samples may have lost of phosphorylation (or have artificially-induced phosphorylation) during harvesting or while in -80^{o}C storage. However, there is no reason to suspect a priori differential changes in donor and heart failure samples and the MRM quantitative data shows that different amino acid residues either increase or decrease in failing and donor hearts. Thus, this is difficult to rationalize as a storage effect.

SUPPLEMENTAL TABLES

Supplemental Table 1. Characteristics of samples in human heart failure

Code #	Type	Gender	Age	LVEF (%)	NYHA
1	ISHD	M	31	23	III
2	ISHD	M	45	-	IV
3	ISHD	M	46	25	III
4	ISHD	M	47	20	III/IV
5	ISHD	M	50	-	-
6	ISHD	M	52	30-35	III/IV
7	ISHD	M	54	-	-
8	ISHD	M	54	35	III/IV
9	ISHD	F	43	35	IIIB
10	ISHD	F	49	35	III
11	IDCM	M	43	-	III
12	IDCM	M	46	15-20	III
13	IDCM	M	56	15	IV
14	IDCM	M	57	10	III
15	IDCM	M	58	20	III/IV
16	IDCM	M	60	15	IV
17	IDCM	F	23	15	III
18	IDCM	F	31	20	III
19	IDCM	F	53	20	III
20	IDCM	F	54	22	III
21	Donor	M	19	-	-
22	Donor	M	23	-	-

23	Donor	M	23	-	-
24	Donor	M	26	-	-
25	Donor	M	39	-	-
26	Donor	M	44	-	-
27	Donor	M	52	-	-
28	Donor	F	27	-	-
29	Donor	F	41	-	-
30	Donor	F	45	-	-

The average age of the ISHD, IDCM, and donor group is 47, 48, and 34, respectively. M: male; F: female; LVEF: left ventricular ejection fraction, normal range 55-79%; NYHA: New York Heart Association; A functional classification of cardiac failure according to severity of disease and the need for therapeutic intervention. I Asymptomatic heart disease; II Comfortable at rest; symptomatic with normal activity; III Comfortable at rest; symptomatic with < normal activity; IV Symptomatic at rest—Criteria Committee, NYHA, Inc: Diseases of Heart & Blood Vessels, 6th ed, Little Brown, Boston 1964.

Supplemental Table 2. List of all peptides and transitions used for MRM assays to monitor the known phosphorylation of cTnI site

Residue	Peptide	Q1[1]	Q3[2]	RT[3]	ID	CE[4]
S22	$_{(p)}$SSNYR	353.9^{+2}	175.3^{+1}	22.0	y1	32.2
		353.9^{+2}	304.9^{+2}	22.0	y5-98	13.6
		353.9^{+2}	338.4^{+1}	22.0	y2	22.7
		353.9^{+2}	452.4^{+1}	22.0	y3	22.1
		353.9^{+2}	539.5^{+1}	22.0	y4	22.2
S23	S$_{(p)}$SNYR	353.6^{+2}	338.4^{+1}	22.0	y2	22.6
		353.6^{+2}	452.5^{+1}	22.0	y3	22.0
		353.6^{+2}	521.5^{+1}	22.0	y4-98	23.0
		353.6^{+2}	549.5^{+1}	22.0	y4-80	21.5
		353.6^{+2}	619.5^{+1}	22.0	y4	23.0
S22/23	$_{(p)}$S$_{(p)}$SNYR	394.0^{+2}	296.1^{+2}	22.0	y5-2×98	18.9
		394.0^{+2}	338.3^{+1}	22.0	y2	27.2
		394.0^{+2}	345.0^{+2}	22.0	y5-98	14.0
		394.0^{+2}	452.3^{+1}	22.0	y3	26.0
		394.0^{+2}	521.8^{+1}	22.0	y4-98	27.4
S22/23	SSNYR	313.2^{+2}	338.4^{+1}	22.0	y2	21.6
		313.2^{+2}	452.5^{+1}	22.0	y3	17.0
		313.2^{+2}	539.5^{+1}	22.0	y4	18.4
S22/23	SSNYR*	318.2^{+2}	185.1^{+1}	22.0	y1	28.1
		318.2^{+2}	175.1^{+1}	22.0	b2	16.2
		318.2^{+2}	348.4^{+1}	22.0	y2	21.6
		318.2^{+2}	462.5^{+1}	22.0	y3	17.0
		318.2^{+2}	549.5^{+1}	22.0	y4	18.4
S22/23	RRSSNYR	469.8^{+2}	338.2^{+1}	22.0	y2	28.5
		469.8^{+2}	539.2^{+1}	22.0	y4	28.5
		469.8^{+2}	269.6^{+1}	22.0	y4	28.5
S22	RR$_{(p)}$SSNYR	509.8^{+2}	338.2^{+1}	22.0	y2	30.5
		509.8^{+2}	619.2^{+1}	22.0	y4	30.5
		509.8^{+2}	310.1^{+1}	22.0	y4	30.5
S41	I$_{(p)}$SASR	306.9^{+2}	258.4^{+2}	22.0	y5-98	15.5
		306.9^{+2}	262.3^{+1}	22.0	y2	25.7
		306.9^{+2}	333.4^{+1}	22.0	y3	19.9
		306.9^{+2}	402.4^{+1}	22.0	y4-98	21.8
		306.9^{+2}	500.5^{+1}	22.0	y4	16.4
S43	ISA$_{(p)}$SR	307.0^{+2}	333.4^{+1}	22.0	y3-80	19.2
		307.0^{+2}	342.2^{+1}	22.0	y2	27.7
		307.0^{+2}	402.3^{+1}	22.0	y4-98	20.3
		307.0^{+2}	420.3^{+1}	22.0	y4-80	25.1
		307.0^{+2}	500.4^{+1}	22.0	y4	14.8

S41/43	$I_{(p)}SA_{(p)}SR$	347.5^{+2}	298.3^{+1}	22.0	y3-98-17	25.0
		347.5^{+2}	384.7^{+1}	22.0	y4-2×98	24.8
		347.5^{+2}	412.4^{+1}	22.0	y3	21.4
		347.5^{+2}	482.5^{+1}	22.0	y4-98	20.0
		347.5^{+2}	580.2^{+1}	22.0	y4	16.1
S41/43	ISASR	267.0^{+2}	262.2^{+1}	22.0	y2	12.0
		267.0^{+2}	333.6^{+1}	22.0	y3	17.0
		267.0^{+2}	420.4^{+1}	22.0	y4	17.0
S41/43	ISASR*	272.0^{+2}	185.3^{+1}	22.0	y1	27.1
		272.0^{+2}	272.2^{+1}	22.0	y2	12.0
		272.0^{+2}	254.2^{+1}	22.0	y2-18	26.0
		272.0^{+2}	343.6^{+1}	22.0	y3	17.0
		272.0^{+2}	430.4^{+1}	22.0	y4	17.0
S41/43	SKISASRK	438.2^{+2}	381.3^{+1}	22.0	y4	25.0
		438.2^{+2}	225.7^{+1}	22.0	y5	25.0
		438.2^{+2}	563.4^{+1}	22.0	y6	25.0
S41/43	ISASRKLQLK	572.9^{+2}	515.3^{+1}	22.0	b5	25.0
		572.9^{+2}	643.4^{+1}	22.0	b6	25.0
		572.9^{+2}	872.6^{+1}	22.0	y7	25.0
S41	$SKI_{(p)}SASRK$	478.2^{+2}	461.3^{+1}	22.0	y4	25.0
		478.2^{+2}	265.7^{+1}	22.0	y5	25.0
		478.2^{+2}	643.4^{+1}	22.0	y6	25.0
S41	$I_{(p)}SASRKLQLK$	612.9^{+2}	595.3^{+1}	22.0	b5	25.0
		612.9^{+2}	723.4^{+1}	22.0	b6	25.0
		612.9^{+2}	872.6^{+1}	22.0	y7	25.0
S76	$AL_{(p)}STR$	313.9^{+2}	276.3^{+1}	22.0	y2	27.3
		313.9^{+2}	345.3^{+1}	22.0	y3-98	22.1
		313.9^{+2}	443.2^{+1}	22.0	y3	15.5
		313.9^{+2}	458.5^{+1}	22.0	y4-98	21.1
		313.9^{+2}	556.5^{+1}	22.0	y4	16.6
T77	$ALS_{(p)}TR$	313.9^{+2}	256.4^{+1}	22.0	y2-98	16.3
		313.9^{+2}	345.4^{+1}	22.0	y3-98	19.2
		313.9^{+2}	443.4^{+1}	22.0	y3	14.6
		313.9^{+2}	458.5^{+1}	22.0	y4-98	21.6
		313.9^{+2}	556.6^{+1}	22.0	y4	15.8
S76/T77	$AL_{(p)}S_{(p)}TR$	354.6^{+2}	256.7^{+1}	22.0	y2-98	18.4
		354.6^{+2}	327.5^{+1}	22.0	y3-2×98	24.1
		354.6^{+2}	425.4^{+1}	22.0	y3-98	18.3
		354.6^{+2}	523.4^{+1}	22.0	y3	14.9
		354.6^{+2}	538.7^{+1}	22.0	y4	18.9
S76/T77	ALSTR	274.2^{+2}	276.2^{+1}	22.0	y2	17.7
		274.2^{+2}	363.2^{+1}	22.0	y3	16.1
		274.2^{+2}	476.3^{+1}	22.0	y4	17.5
S76/T77	ALSTR*	278.9^{+2}	286.3^{+1}	22.0	y2	17.7
		278.9^{+2}	373.3^{+1}	22.0	y3	16.1

		278.9^{+2}	486.5^{+1}	22.0	y4	17.5
T142	$P_{(p)}$TLR	283.7^{+2}	279.1^{+1}	22.0	b2	19.2
		283.7^{+2}	392.2^{+1}	22.0	b3	19.2
		283.7^{+2}	469.3^{+1}	22.0	y3	19.2
T142	PTLR	243.7^{+2}	199.1^{+1}	22.0	b2	17.2
		243.7^{+2}	312.2^{+1}	22.0	b3	17.2
		243.7^{+2}	389.3^{+1}	22.0	y3	17.2
T142	$RP_{(p)}$TLR	362.2^{+2}	288.4^{+1}	22.0	y2	22.9
		362.2^{+2}	313.3^{+2}	22.0	y5-98	21.2
		362.2^{+2}	370.3^{+1}	22.0	y3-98	22.1
		362.2^{+2}	468.5^{+1}	22.0	y3	26.2
		362.2^{+2}	565.8^{+1}	22.0	y4	22.8
T142	RPTLR	322.2^{+2}	288.2^{+1}	22.0	y2	29.3
		322.2^{+2}	389.4^{+1}	22.0	y3	28.7
		322.2^{+2}	486.6^{+1}	22.0	y4	24.0
T142	RPTLR*	327.2^{+2}	185.2^{+1}	22.0	y1	29.3
		327.2^{+2}	298.2^{+1}	22.0	y2	29.3
		327.2^{+2}	327.2^{+2}	22.0	y5	12.9
		327.2^{+2}	399.4^{+1}	22.0	y3	28.7
		327.2^{+2}	496.6^{+1}	22.0	y4	24.0
S149	$I_{(p)}$SADAMMQALLGAR	764.4^{+2}	1061.6^{+1}	22.0	y10	43.2
		764.4^{+2}	1176.6^{+1}	22.0	y11	43.2
		764.4^{+2}	1247.6^{+1}	22.0	y12	43.2
		764.4^{+2}	1414.7^{+1}	22.0	y13	43.2
S149	ISADAMMQALLGAR	724.4^{+2}	1061.6^{+1}	22.0	y10	41.2
		724.4^{+2}	1176.6^{+1}	22.0	y11	41.2
		724.4^{+2}	1247.6^{+1}	22.0	y12	41.2
		724.4^{+2}	1334.7^{+1}	22.0	y13	41.2
S165	$AKE_{(p)}$SLDLR	506.8^{+2}	288.4^{+1}	22.0	y2	23.2
		506.8^{+2}	458.2^{+2}	22.0	y8-98	25.9
		506.8^{+2}	507.2^{+2}	22.0	y8	14.7
		506.8^{+2}	585.6^{+1}	22.0	y5-98	33.0
		506.8^{+2}	715.8^{+1}	22.0	y6-98	31.0
S165	AKESLDLR	466.7^{+2}	288.2^{+1}	22.0	y2	32.2
		466.7^{+2}	603.8^{+1}	22.0	y5	31.0
		466.7^{+2}	732.9^{+1}	22.0	y6	24.9
S165	AKESLDLR*	471.7^{+2}	298.5^{+1}	22.0	y2	32.3
		471.7^{+2}	436.3^{+2}	22.0	y7	26.4
		471.7^{+2}	526.6^{+1}	22.0	y4	25.1
		471.7^{+2}	613.8^{+1}	22.0	y5	26.7
		471.7^{+2}	742.9^{+1}	22.0	y6	24.9
Total	NITEIADLTQK*	627.4^{+2}	612.9^{+1}	22.0	y5	38.3
		627.4^{+2}	683.8^{+1}	22.0	y6	28.1
		627.4^{+2}	797.0^{+1}	22.0	y7	30.0
		627.4^{+2}	926.0^{+1}	22.0	y8	29.1

		627.4^{+2}	1027.1^{+1}	22.0	y9	28.0

Note: For MRM transition, characteristic parameters were used in assay including: 1. Q1, parental ion and its charge is in superscript; 2. Q3, fragment ion and it charge in superscript; 3. Retention time (RT), unit in msec; 4. Collision energy (CE). Each transition was optimized, as described in ***Supplemental*** Methods. Star (*) indicates the isotopically labeled by incorporating $^{15}N/^{13}C$ in either Lys (K) or Arg (R) amino acid residue at the C-terminus, in mass difference of 8 and 10 daltons respectively for K or R. Total means unmodified tryptic peptide used to quantify the total amount of cTnI present in samples.

Supplemental Table 3. List of all peptides and transitions used for MRM assays to monitor the novel phosphorylation sites

Residue	Peptide	Q1[1]	Q3[2]	RT[3]	ID	CE[4]
S4	Ac-ADG$_{(p)}$SSDAAR	486.7^{+2}	430.3^{+2}	22.0	y8	17.0
		486.7^{+2}	437.8^{+2}	22.0	y9-98	17.0
		486.7^{+2}	686.5^{+1}	22.0	y6	18.0
		486.7^{+2}	645.6^{+1}	22.0	y7-98	21.0
		486.7^{+2}	743.6^{+1}	22.0	y7	16.0
S5	Ac-ADGS$_{(p)}$SDAAR	486.7^{+2}	317.5^{+1}	22.0	y3	36.1
		486.7^{+2}	599.5^{+1}	22.0	y5	17.7
		486.7^{+2}	686.8^{+1}	22.0	y6	17.5
		486.7^{+2}	743.7^{+1}	22.0	y7	20.0
		486.7^{+2}	430.4^{+2}	22.0	y8	18.7
S4S5	Ac-ADGS$_{(p)}$S$_{(p)}$DAAR	526.7^{+2}	363.4^{+2}	22.0	y7-98	30.3
		526.7^{+2}	412.2^{+2}	22.0	y7	26.5
		526.7^{+2}	421.2^{+2}	22.0	y8-98	24.5
		526.7^{+2}	627.9^{+1}	22.0	y7-2×98	31.8
		526.7^{+2}	570.6^{+1}	22.0	y6-98	29.9
S4S5	Ac-ADGSSDAAR	446.7^{+2}	606.6^{+1}	22.0	y6	21.3
		446.7^{+2}	663.7^{+1}	22.0	y7	18.9
		446.7^{+2}	778.8^{+1}	22.0	y8	18.3
S4S5	Ac-ADGSSDAAR*	451.7^{+2}	442.8^{+1}	22.0	y4	13.9
		451.7^{+2}	529.6^{+1}	22.0	y5	18.7
		451.7^{+2}	616.6^{+1}	22.0	y6	21.3
		451.7^{+2}	673.7^{+1}	22.0	y7	18.9
		451.7^{+2}	788.8^{+1}	22.0	y8	18.3
T50	$_{(p)}$TLLLQIAK	490.6^{+2}	441.8^{+2}	22.0	y8-98	17.6
		490.6^{+2}	459.6^{+1}	22.0	y4	26.2
		490.6^{+2}	572.7^{+1}	22.0	y5	26.0
		490.6^{+2}	685.9^{+1}	22.0	y6	23.9
		490.6^{+2}	799.0^{+1}	22.0	y7	23.8
T50	TLLLQIAK	450.2^{+2}	572.7^{+1}	22.0	y5	22.6
		450.2^{+2}	685.9^{+1}	22.0	y6	21.3
		450.2^{+2}	799.0^{+1}	22.0	y7	22.4
T50	TLLLQIAK*	454.2^{+2}	339.5^{+1}	22.0	y3	30.0
		454.2^{+2}	467.6^{+1}	22.0	y4	26.0
		454.2^{+2}	580.7^{+1}	22.0	y5	22.6
		454.2^{+2}	693.8^{+1}	22.0	y6	21.3
		454.2^{+2}	807.0^{+1}	22.0	y7	22.4
T180	ED$_{(p)}$TEK	351.6^{+2}	293.6^{+2}	22.0	y5-98	17.2
		351.6^{+2}	227.4^{+2}	22.0	y3	24.8
		351.6^{+2}	284.6^{+2}	22.0	y4	19.2
		351.6^{+2}	359.6^{+1}	22.0	y3-98	21.9
		351.6^{+2}	474.5^{+1}	22.0	y4-98	23.6

T180	EDTEK	310.9^{+2}	276.4^{+1}	22.0	y2	16.9
		310.9^{+2}	377.4^{+1}	22.0	y3	18.7
		310.9^{+2}	500.5^{+1}	22.0	y4	19.9
T180	EDTEK*	314.9^{+2}	284.4^{+1}	22.0	y2	16.9
		314.9^{+2}	385.4^{+1}	22.0	y3	18.7
		314.9^{+2}	500.5^{+1}	22.0	y4	19.9
S198	$NIDAL_{(p)}SGMEGR$	622.8^{+2}	414.6^{+1}	22.0	y7	27.8
		622.8^{+2}	549.7^{+1}	22.0	y5	25.9
		622.8^{+2}	716.8^{+1}	22.0	y6	33.0
		622.8^{+2}	918.8^{+1}	22.0	y9-98	12.1
		622.8^{+2}	1016.9^{+1}	22.0	y9	34.8
S198	NIDALSGMEGR	582.8^{+2}	636.8^{+1}	22.0	y6	27.8
		582.8^{+2}	820.8^{+1}	22.0	y8	34.8
		582.8^{+2}	936.9^{+1}	22.0	y9	26.1
S198	$NIDAL_{(p)}SGMEGR$*	627.8^{+2}	726.8^{+1}	22.0	y6	28.4
		627.8^{+2}	559.6^{+1}	22.0	y5	27.8
		627.8^{+2}	839.7^{+1}	22.0	y7	35.3
		627.8^{+2}	928.8^{+1}	22.0	y9-98	12.5
		627.8^{+2}	1026.9^{+1}	22.0	y9	26.2
Total	NITEIADLTQK*	627.4^{+2}	612.9^{+1}	22.0	y5	38.3
		627.4^{+2}	683.8^{+1}	22.0	y6	28.1
		627.4^{+2}	797.0^{+1}	22.0	y7	30.0
		627.4^{+2}	926.0^{+1}	22.0	y8	29.1
		627.4^{+2}	1027.1^{+1}	22.0	y9	28.0

Note: For MRM transition, these characteristic parameters were used in assay including: 1. Q1, parental ion and it charge in superscript; 2. Q3, fragment ion and it charge in superscript; 3. Retention time (RT), unit in msec; 4. Collision energy (CE). Each transition was optimized, as described in ***Supplemental*** Methods. Star (*) indicates the isotopically labeled by incorporating $^{15}N/^{13}C$ in either Lys (K) or Arg (R) amino acid residue at the C-terminus, in mass difference of 8 and 10 daltons respectively for K or R. Total means unmodified tryptic peptide used to quantify the total amount of cTnI present in samples.

Supplemental Table 4. Identification results of major LV myofibril proteins by MALDI-TOF/TOF mass spectrometry analysis

Band #	Protein name	Peptide Sequence	Observed m/z	Calculated m/z	Error ppm	Score	Expect	Queries /matched
1	Titin	LIPGQEYIPR	1234.70	1234.67	28.5	37	6.6e-03	2/10
		EILGYWVEYR	1326.70	1326.66	34.5	36	8.3e-03	6/10
		WEQFYVMPLPR	1464.76	1464.76	28.5	51	2.0e-04	8/10
		VTGLVEGLEYQFR	1509.82	1509.78	26	37	6.0e-03	10/10
		VENLTEGAIYYFR	1573.82	1573.78	31.7	56	7.2e-05	14/10
		IPGPPETLQIFDVSR	1667.91	1667.88	18.2	31	1.8e-02	16/10
		IDQLQEGCSYYFR	1677.80	1677.74	33.0	51	2.1e-04	18/10
		TLEATISGLTAGEEYVFR	1956.01	1956.01	17.5	55	7.1e-05	21/10
		LPYTTPGPPSTPWVTNVTR	2083.11	2083.07	18	57	2.0e-03	27/10
		NLTEGEEYTFQVMAVNSAGR	2215.06	2215.02	21.7	36	5.2e-03	29/10
2	Myosin	ILYGDFR	882.46	882.45	3.0	46	8.6e-04	1/9
		MFNWMVTR	1083.50	1083.49	1.0	44	1.5e-03	3/9
		DSLLVIQWNIR	1355.75	1355.75	2.4	57	7.0e-05	5/9
		NNLLQAELEELR	1440.76	1440.75	4.6	106	8.6e-10	7/9
		IEELEEELEAER	1487.71	1487.69	10.8	94	1.2e-08	9/9
		VIQYFAVIAAIGDR	1534.85	1534.85	3.5	55	8.5e-05	11/9
		ILNPAAIPEGQFIDSR	1739.93	1739.92	5.8	63	1.5e-05	20/9
		DLEEATLQHEATAAALR	1837.93	1837.93	10.3	130	2.8e-12	22/9
		SEAPPHIFSISDNAYQYMLTDR	2554.21	2554.18	14.0	106	3.9e-10	29/9
3	α-Actinin	TIPWLENR	1027.57	1027.54	29.6	61	3.0e-05	2/10
		EGLLLWCQR	1173.62	1173.59	26.2	60	4.4e-05	4/10
		LASELLEWIR	1228.71	1228.68	27.2	63	1.9e-05	6/10
		GITQEQMNEFR	1351.65	1351.61	26.5	70	3.8e-06	9/10
		VGWELLLTTIAR	1370.82	1370.79	25.9	78	3.5e-07	11/10
		GYEEWLLNEIR	1420.73	1420.69	26.4	71	2.4e-06	15/10
		ILASDKPYILAEELR	1730.01	1729.96	30.5	96	4.7e-09	20/10

		ETADTDTAEQVIASFR	1752.86	1752.81	28.9	118	4.2e-11	22/10
		ISSSNPYSTVTMDELR	1798.89	1798.84	29.7	95	7.4e-09	24/10
		ACLISMGYDLGEAEFAR	1901.92	1901.86	30.7	91	1.9e-08	28/10
4	Desmin	VELQELNDR	1114.53	1114.56	25.2	31	3.3e-02	8/8
		VAELYEEELR	1249.61	1249.61	5.4	71	3.2e-06	10/8
		FASEASGYQDNIAR	1527.68	1527.69	9.1	94	9.8e-09	16/8
		RIESLNEEIAFLK	1560.82	1560.85	14.5	30	2.7e-02	18/8
		EINLPIQTYSALNFR	1648.89	1648.89	0.7	92	1.5e-08	21/8
		FLEQQNAALAAEVNR	1672.85	1672.85	0.2	132	1.9e-12	23/8
		TFGGAPGFPLGSPLSSPVFPR	2087.09	2087.08	3.9	96	5.1e-09	27/8
		LQEEIQLKEEAENNLAAFR	2245.15	2244.13	2.2	142	1.2e-13	29/8
5	Actin	GYSFVTTAER	1129.50	1129.54	30.3	69	4.6e-06	4/7
		AVFPSIVGRPR	1197.66	1197.69	23.7	49	5.0e-04	6/7
		QEYDEAGPSIVHR	1499.65	1499.70	29.4	99	3.2e-09	10/7
		SYELPDGQVITIGNER	1789.85	1789.88	14.3	97	5.1e-09	13/7
		VAPEEHPTLLTEAPLNPK	1954.99	1955.03	20.5	94	8.3e-09	17/7
		DLYANNVLSGGTTMYPGIADR	2227.02	2227.05	14.4	122	1.3e-11	22/7
		TTGIVLDSGDGVTHNVPIYEGY ALPHAIMR	3195.57	3195.60	7.3	98	1.8e-09	29/7
6	Troponin T	YEINVLR	905.49	905.49	4.2	47	9.0e-04	1/3
		VLAIDHLNEDQLR	1534.79	1534.81	7.3	90	3.3e-08	12/3
		DLNELQALIEAHFENR	1910.94	1910.94	4.1	107	5.1e-10	25/3
7	Tropomyosin	LVIIESDLER	1185.64	1185.66	15.1	78	7.1e-07	3/5
		IQLVEEELDR	1242.63	1242.64	7.1	85	1.3e-07	5/5
		SIDDLEDELYAQK	1537.67	1537.71	23.8	94	9.2e-09	16/5
		IQLVEEELDRAQER	1726.86	1726.88	12.6	58	4.7e-05	19/5
		AISEELDHALNDMTSI	1757.78	1757.81	18.6	62	1.4e-05	21/5
8	Translocase	GAWSNVLR	901.46	901.47	13.6	45	2.1e-03	3/3
		EQGFLSFWR	1168.55	1168.56	7.7	65	1.2e-05	16/3
		YFPTQALNFAFK	1445.70	1445.73	17.3	53	1.0e-04	23/3
9	Troponin I	AYATEPHAK	986.46	986.48	20.2	50	3.0e-04	1/7
		AKESLDLR	930.59	930.56	32.2	57	1.3e-05	2/7

		TLLLQIAK	898.71	898.78	70.7	43	5.3e-03	3/7
		NITEIADLTQK	1244.69	1244.66	40.1	64	3.0e-05	3/7
		NIDALSGMEGR	1161.43	1161.44	8.6	57	1.1e-06	4/7
		ISADAMMQALLGAR	1446.78	1446.73	29.5	77	1.6e-06	10/7
		CQPLELAGLGFAELQDLCR	2189.05	2189.06	4.5	118	6.6e-11	25/7
10	MLC-2	EAFMLFDR	1027.45	1027.47	22.2	58	4.1e-05	1/7
		ITYGQCGDVLR	1280.65	1280.61	26.5	76	7.6e-07	7/7
		ALGQNPTQAEVLR	1395.80	1395.74	39.3	96	6.4e-09	10/7
		DTGTYEDFVEGLR	1500.72	1500.67	35.4	74	1.3e-06	14/7
		IEFTPEQIEEFK	1508.77	1508.73	22.1	42	2.0e-03	16/7
		AAPAPAPPPEPERPK	1523.87	1523.80	40.1	52	1.0e-04	18/7
		NKDTGTYEDFVEGLR	1742.85	1742.81	27.1	79	3.3e-07	23/7
11	MLC-1	DTFAALGR	849.36	849.43	85.3	76	7.3e-02	2/4
		DGFIDKNDLR	1191.60	1191.58	15.0	50	3.0e-03	13/4
		EAFTIMDQNR	1223.58	1223.56	18.4	38	4.7e-03	15/4
		GADPEETILNAFK	1403.70	1403.69	10.7	65	9.8e-06	19/4
12	Troponin C	SEEELSDLFR	1223.57	1223.56	5.7	84	1.1e-07	17/1

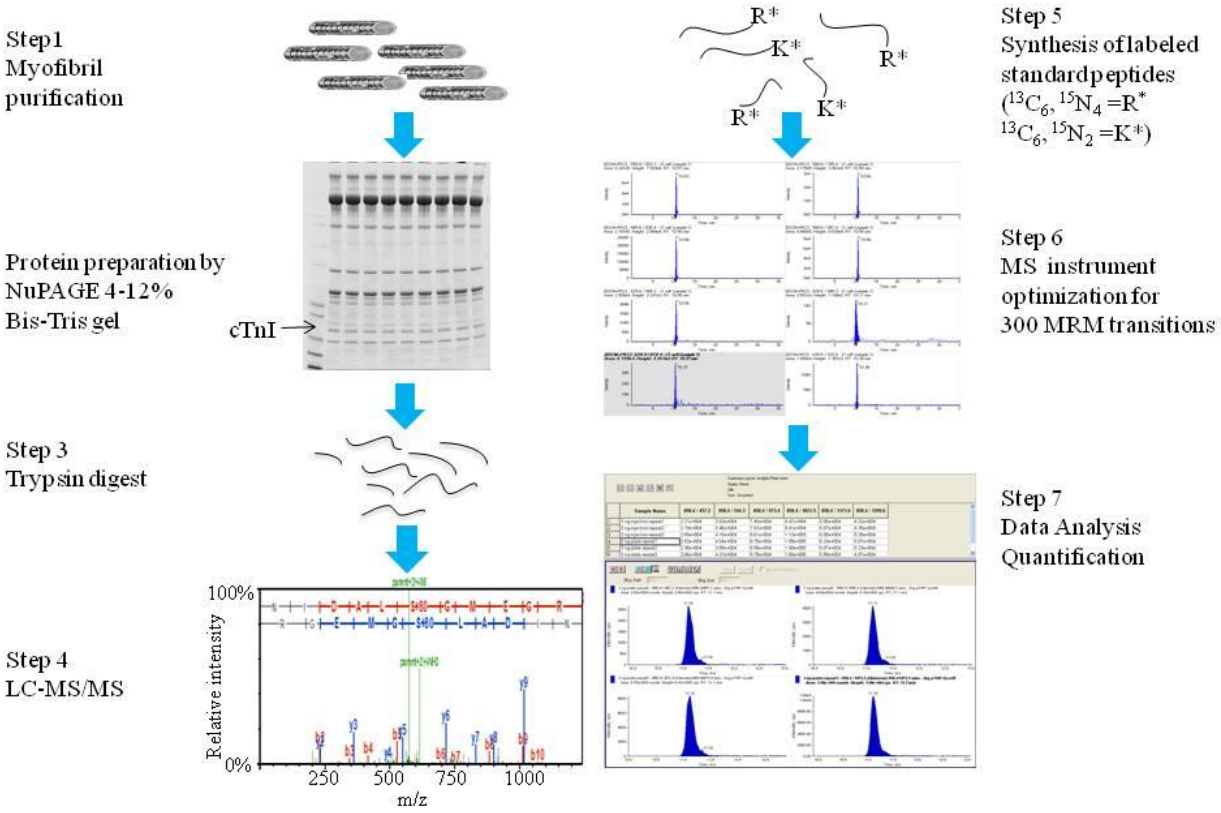
Note: Human left ventricular proteins of heart failure (30µg/lane) were resolved by NuPAGE 4-12% Bis-Tris gel and stained with Coomassie R-250. The protein identification was based on Mascot search results by peptide mass fingerprint (PMF) technique. MS spectra were acquired on the 4800 MALDI TOF/TOF Analyzer (ABI) using 1000 shots/spectra and a laser power between 4300–4700 units. MLC: Myosin light chain

Supplemental Table 5. Summary for the quantitation of site-specific cTnI phosphorylation by MRM assay in ISHD and IDCM failing and normal human heart (*n* = 10 for each group)

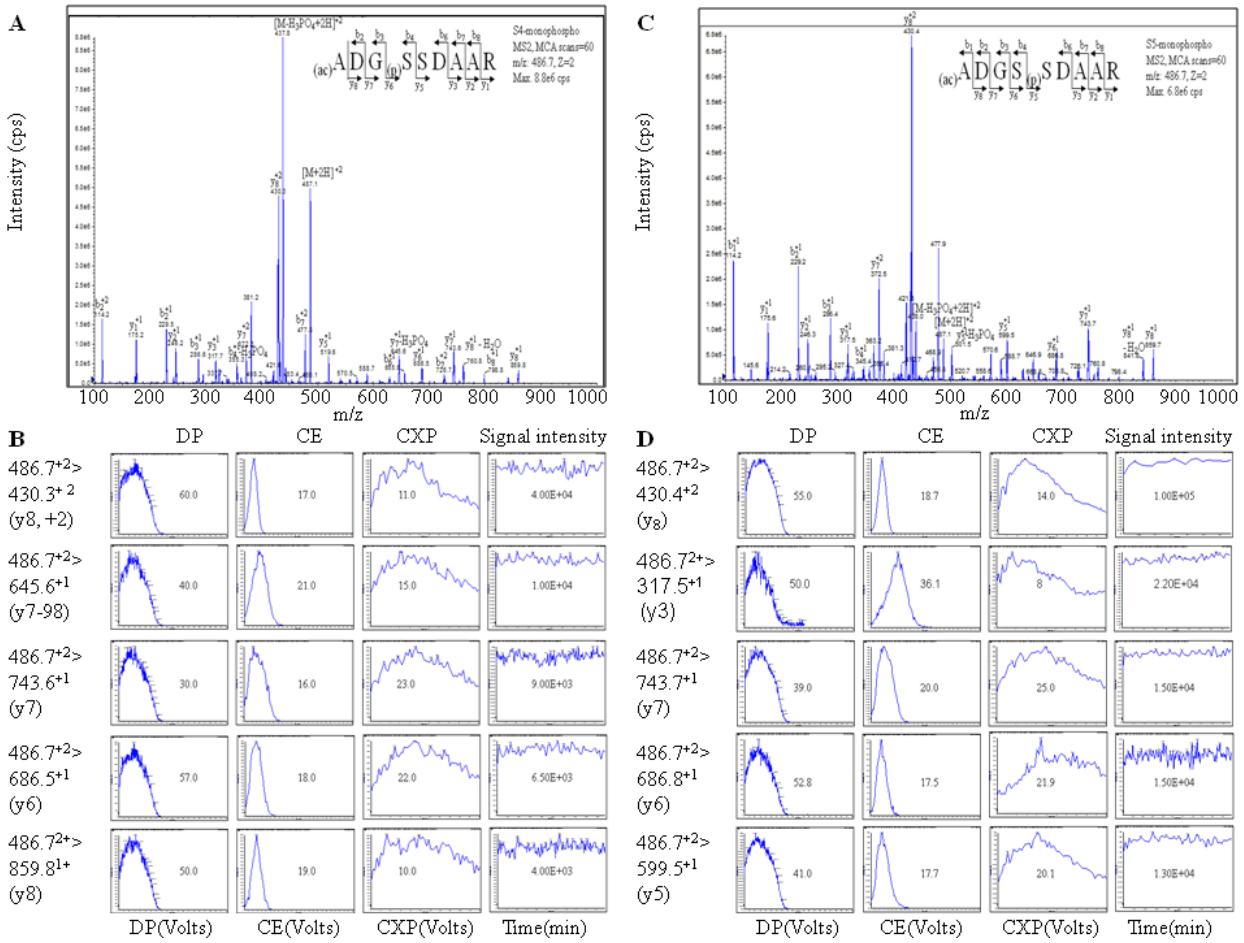
	Donor		ISHD		IDCM	
Site	Median	Q_1, Q_3	Median	Q_1, Q_3	Median	Q_1, Q_3
S4	0.0052	0.0049, 0.0058	0.0039 *	0.0036, 0.0045	0.0045	0.0043, 0.0049
S5	0.0046	0.0045, 0.0049	0.0036 *	0.0035, 0.0037	0.0041 *	0.0038, 0.0044
S4 & S5	0.0078	0.0075, 0.0082	N.D.		N.D.	
S22	0.0022	0.0021, 0.0024	0.0015 *	0.0014, 0.0016	0.0017	0.0016, 0.0019
S23	0.0212	0.0208, 0.0222	0.0083 *	0.0081, 0.0092	0.0051 *	0.0045, 0.0053
S22 & S23	0.0070	0.0066, 0.0074	LLOQ		LLOQ	
Y25	0.0770	0.0714, 0.0830	0.0444 *	0.0425, 0.0468	0.0436 *	0.0307, 0.0458
T30	N.D.		N.D.		N.D.	
S41	0.0094	0.0090, 0.0096	0.0115 *	0.0111, 0.0128	0.0126 *	0.0108, 0.0141
S43	0.0039	0.0026, 0.0050	0.0040	0.0037, 0.0043	0.0060 *	0.0056, 0.067
S41 & S43	0.0027	0.0026, 0.0029	0.0027	0.0026, 0.0028	0.0028	0.0026, 0.0032
T50	0.0223	0.0214, 0.0231	0.0230	0.0204, 0.0241	0.0244	0.0204, 0.0281
S76	0.0774	0.0763, 0.0789	0.1115 *	0.1069, 0.1210	0.1070 *	0.1051, 0.1080
T77	0.0301	0.0300, 0.0309	0.0360 *	0.0355, 0.0368	0.0389 *	0.0362, 0.0456
S76 & T77	0.0034	0.0031, 0.0036	0.0040 *	0.0037, 0.0041	0.0045 *	0.0040, 0.0055
T142	0.0974	0.0756, 0.1188	0.1534 *	0.1329, 0.1651	0.1776 *	0.1589, 0.2036
S149	N.D.		N.D.		N.D.	
S165	0.0023	0.0021, 0.0027	0.0032	0.0022, 0.0038	0.0064 *	0.0051, 0.0098
T180	0.0337	0.0330, 0.0362	0.0487 *	0.0465, 0.0490	0.0460 *	0.0447, 0.0498
S198	0.0017	0.0017, 0.0019	0.0040 *	0.0034, 0.0048	0.0039 *	0.0036, 0.0041

The ratio of each phosphorylated residue of cTnI (fmol phosphorylation/fmol total protein) determined by MRM assay for Donor, ischemic heart failure (ISHD) and dilated cardiomyopathy (IDCM) (*n* = 10 per group) for all of the sites. Phospho-peptides observed, but below lower limited of quantification (LLOQ); Phospho-peptides that were not detected by MS are denoted as not detected (N.D.). Q_1, first quartile; Q_3, third quartile; *, $P < 0.05$ ISHD or IDCM versus Donor by one-way ANOVA on ranks followed by a Dunnett's multiple comparisons post-hoc test.

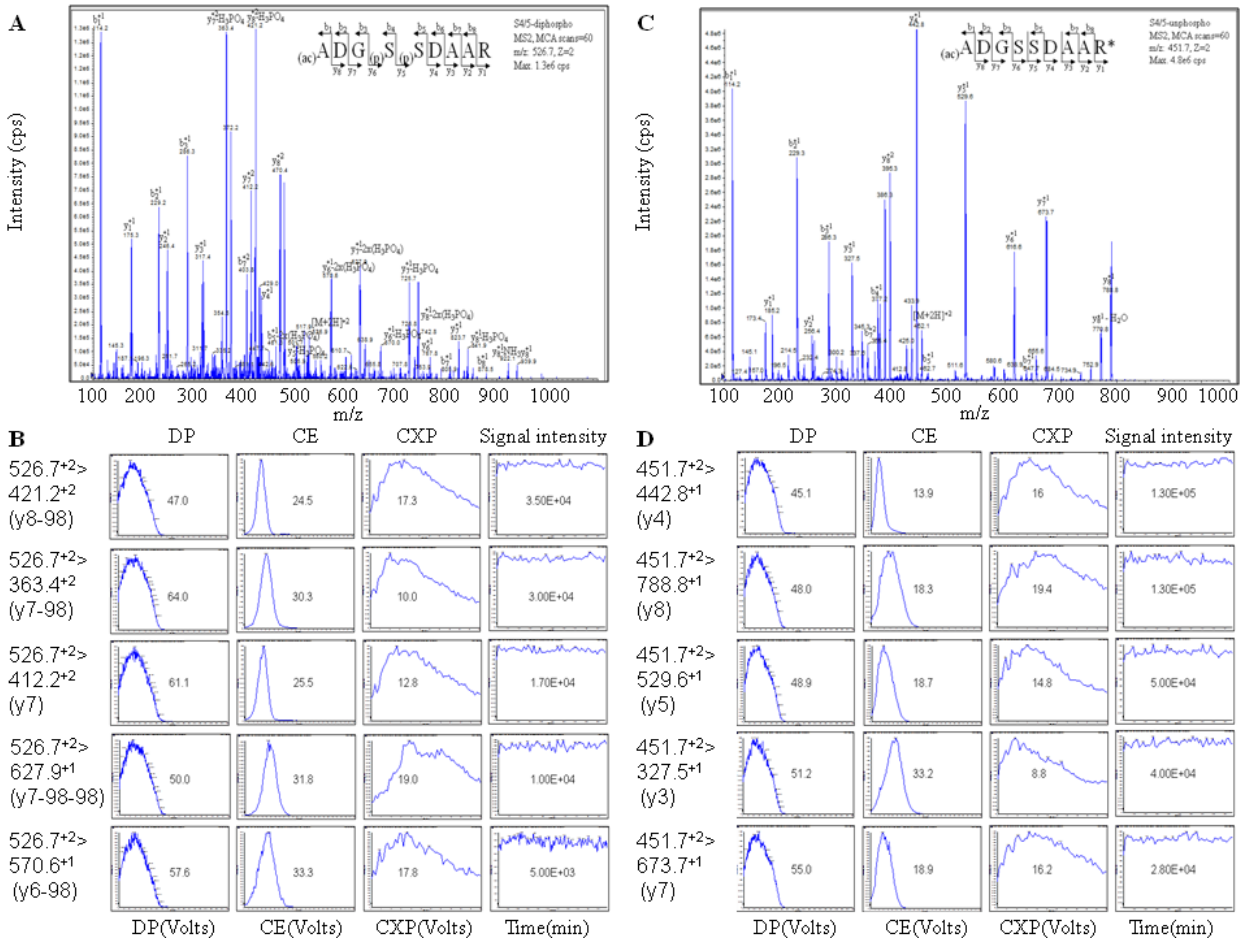
SUPPLEMENTAL FIGURES



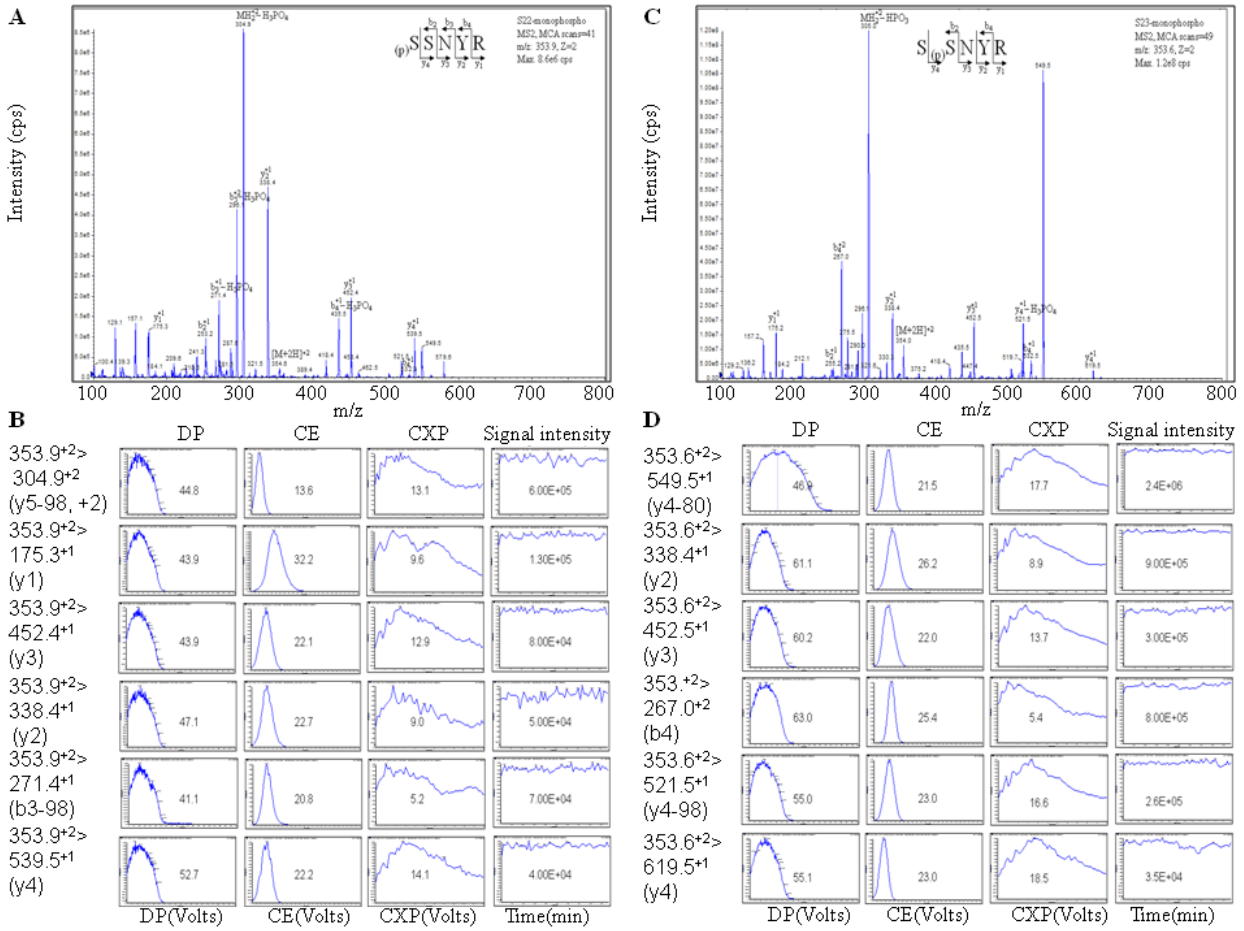
Supplemental Figure 1. Schematic process in developing multiple reaction monitoring (MRM) assays for cardiac troponin I phosphorylation.



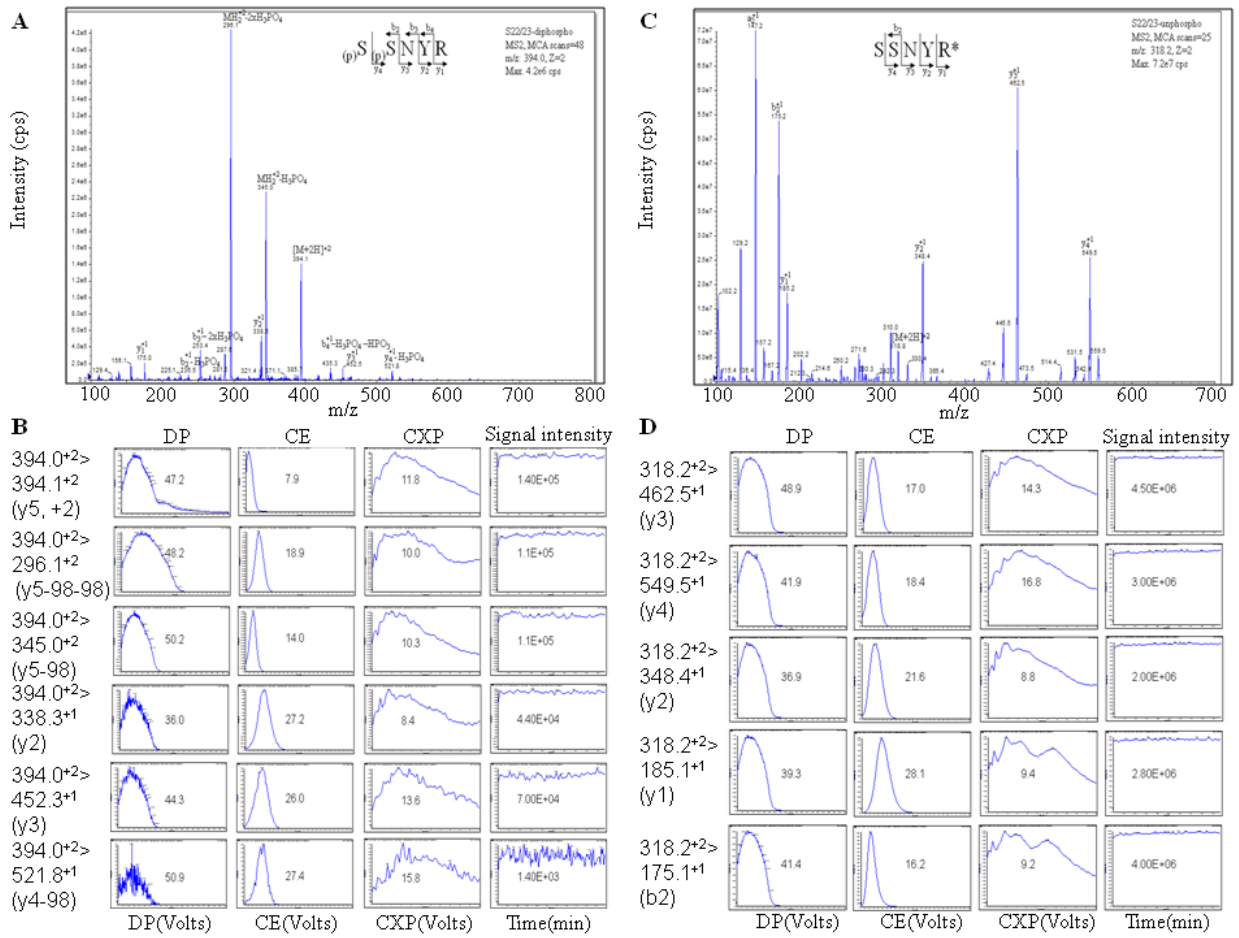
Supplemental Figure 2. Optimization of the MRM assay for phosphorylated peptides containing S4 or S5. Panel A: MS/MS spectrum of the novel monophosphorylated site S4 in vitro. MS/MS of the doubly charged 486.7 ion allows for selection of product ions for monitoring the native (light) S4 monophosphorylated peptide ($_{(ac)}$ADG$_{(p)}$SSDAAR). Panel B: Optimization of MRM on the S4 monophosphorylated peptide ($_{(ac)}$ADG$_{(p)}$SSDAAR). Panel C: MS/MS spectrum of the novel monophosphorylated site S5 in vitro. MS/MS of the doubly charged 486.7 ion allows for selection of product ions for monitoring the native (light) S6 monophosphorylated peptide ($_{(ac)}$ADGS$_{(p)}$SDAAR). Panel D: Optimization of MRM on the S5 monophosphorylated peptide ($_{(ac)}$ADGS$_{(p)}$SDAAR). Optimization was obtained by ramping the parameters declustering potential (DP)(0-400 volts), collision energy (CE)(5-130 volts), and Collision Cell Exit Potential (CXP)(0-66 volts) from low to high with a step of 1 for all parameters and a fixed setting of 10 volts for entrance potential (EP).



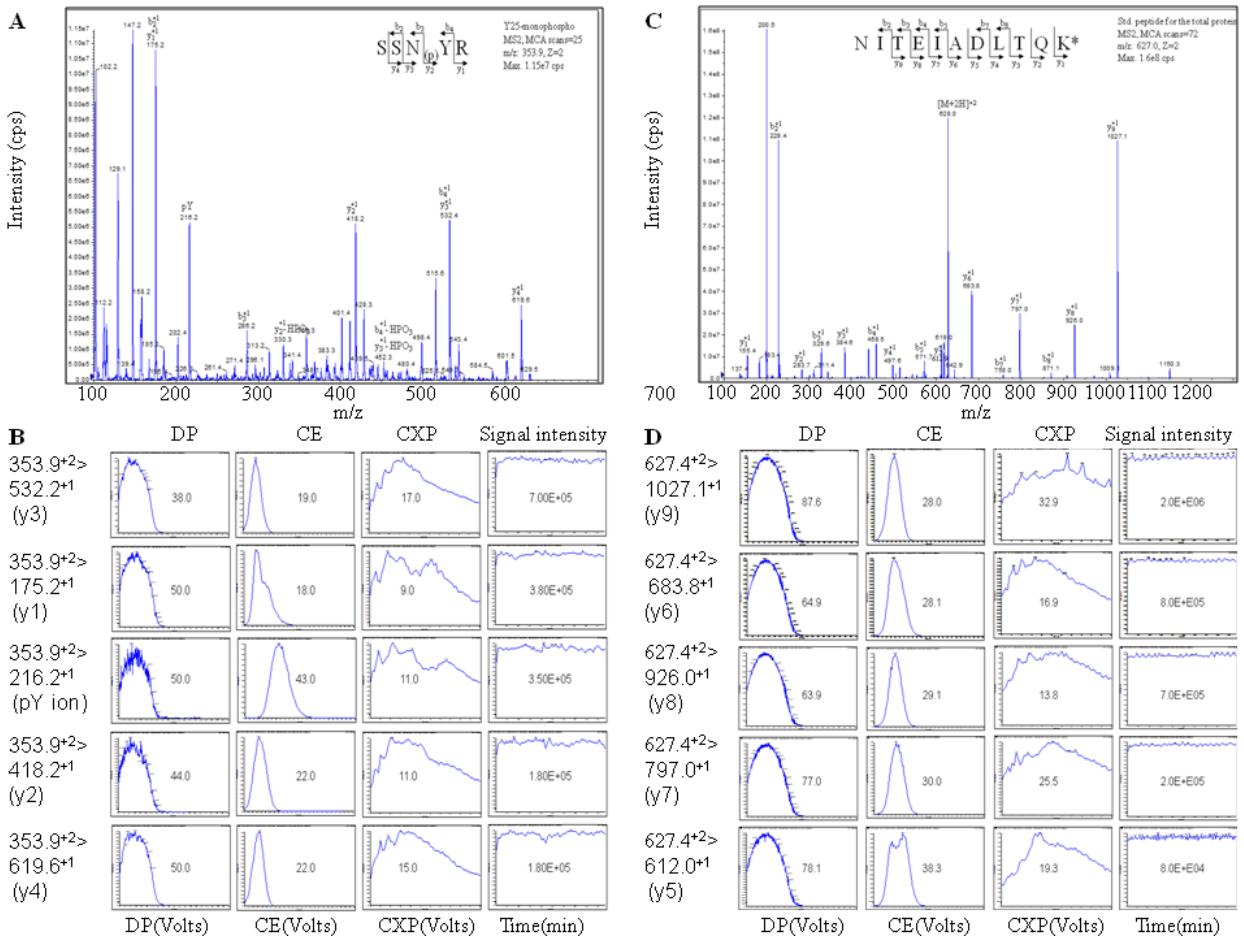
Supplemental Figure 3. Optimization of the MRM assay for phosphorylated and unphosphorylated peptides containing S4/5. Panel A: MS/MS spectrum of the novel diphosphorylated sites S4/5 in vitro. MS/MS of the doubly charged 526.7 ion allows for selection of product ions for monitoring the native (light) S4/5 diphosphorylated peptide ($_{(ac)}$ADG$_{(p)}$S$_{(p)}$SDAAR). Panel B: Optimization of MRM on the S4/5 diphosphorylated peptide ($_{(ac)}$ADG$_{(p)}$S$_{(p)}$SDAAR). Panel C: MS/MS of the doubly charged 415.7 ion allows for selection of product ions for monitoring the labeled (heavy) S4/5 unphosphorylated peptide ($_{(ac)}$ADGSSDAAR). Panel D: Optimization of MRM on the S4/5 unphosphorylated peptide ($_{(ac)}$ADGSSDAAR). Optimization was obtained by ramping the parameters declustering potential (DP)(0-400 volts), collision energy (CE)(5-130 volts), and Collision Cell Exit Potential (CXP)(0-66 volts) from low to high with a step of 1 for all parameters and a fixed setting of 10 volts for entrance potential (EP).



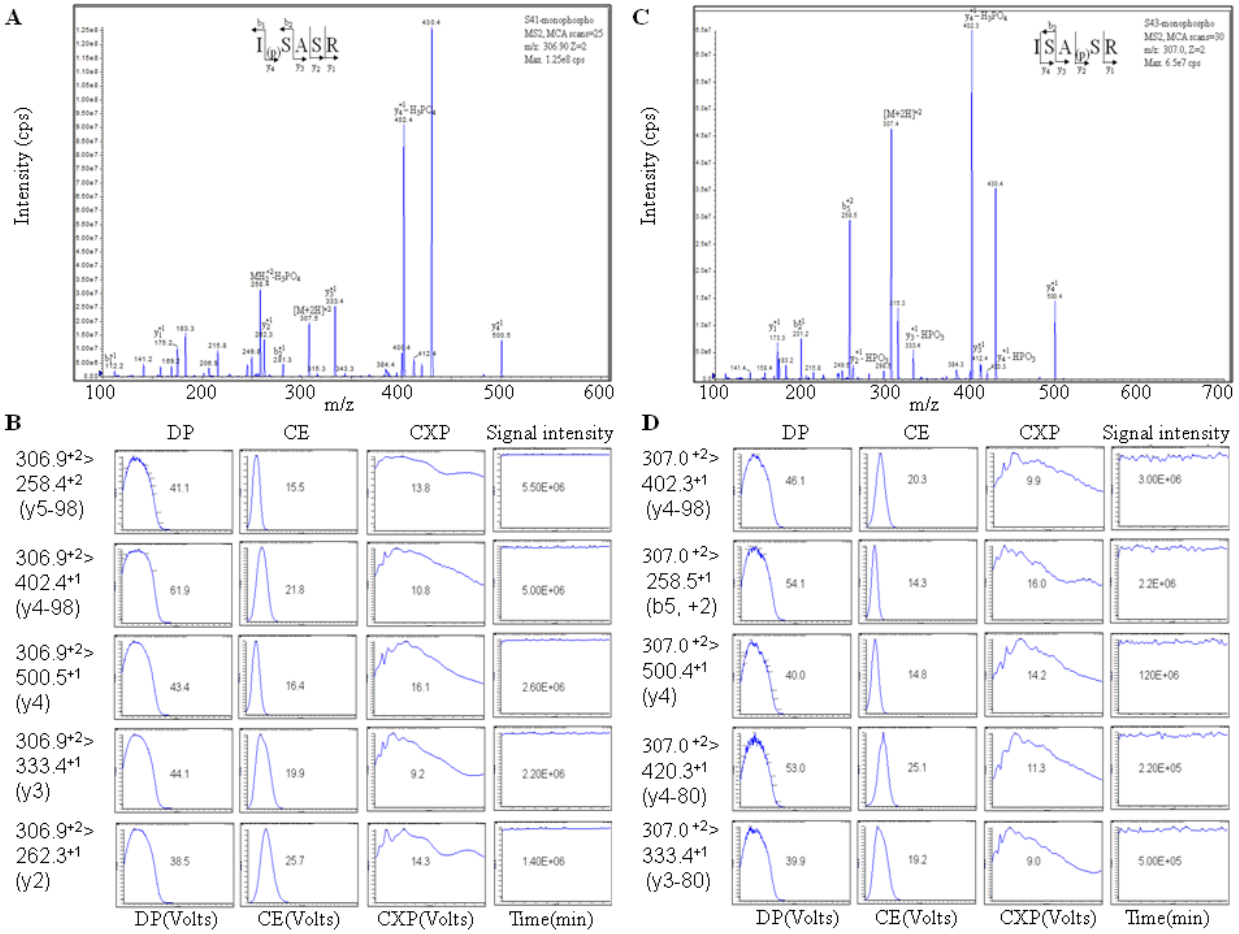
Supplemental Figure 4. Optimization of the MRM assay for phosphorylated peptides containing S22 or S23. Panel A: MS/MS spectrum of the monophosphoylated site S22 in vitro. MS/MS of the doubly charged 353.9 ion allows for selection of product ions for monitoring the native (light) S22 monophosphorylated peptide ($_{(p)}$SSNYR). Panel B: Optimization of MRM on S22 monophosphorylated peptide ($_{(p)}$SSNYR). Panel C: MS/MS spectrum of the monophosphoylated site S23 in vitro. MS/MS spectrum of the doubly charged 353.6 ion allows for selection of product ions for monitoring the native (light) S23 monophosphorylated peptide (S$_{(p)}$SNYR). Panel D: Optimization of MRM on S23 monophosphorylated peptide (S$_{(p)}$SNYR). Optimization was obtained by ramping the parameters declustering potential (DP)(0-400 volts), collision energy (CE)(5-130 volts), and Collision Cell Exit Potential (CXP)(0-66 volts) from low to high with a step of 1 for all parameters and a fixed setting of 10 volts for entrance potential (EP).



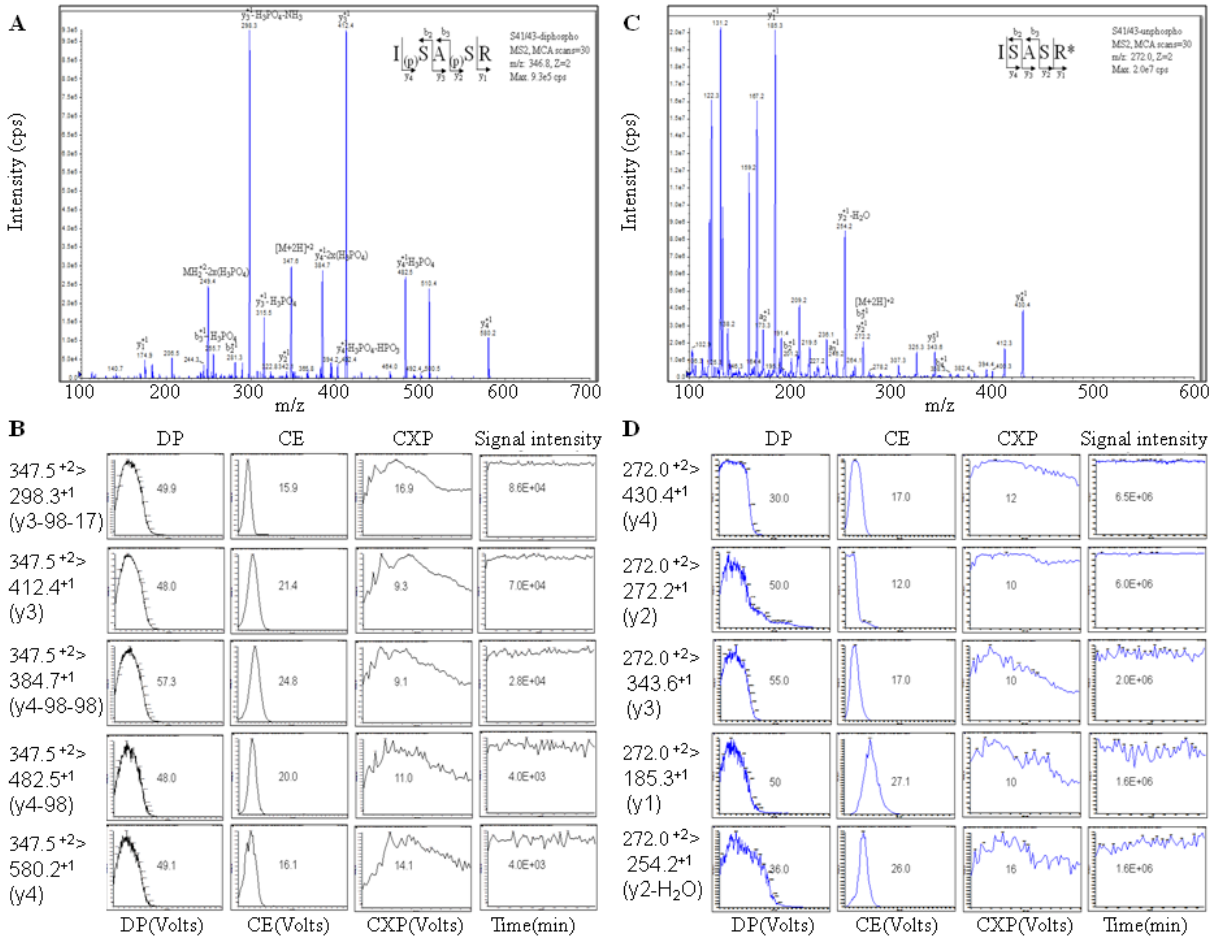
Supplemental Figure 5. Optimization of the MRM assay for phosphorylated and unphosphorylated peptides containing S22/23. Panel A: MS/MS spectrum of the diphosphorylated sites S22/23 *in vitro*. MS/MS spectrum of the doubly charged 394.0 ion allows for selection of product ions for monitoring the native (light) S22/23 diphosphorylated peptide ($_{(p)}$S$_{(p)}$SNYR). Panel B: Optimization of MRM on the signature S22/23 diphosphorylated peptide ($_{(p)}$S$_{(p)}$SNYR). Panel C: MS/MS spectrum of the doubly charged 318.2 ion allows for selection of product ions for monitoring the labeled (heavy) S22/23 unphosphorylated peptide (SSNYR*). Panel D: Optimization of MRM on S22/23 unphosphorylated peptide (SSNYR*). Optimization was obtained by ramping the parameters declustering potential (DP)(0-400 volts), collision energy (CE) (5-130 volts), and Collision Cell Exit Potential (CXP)(0-66 volts) from low to high with a step of 1 for all parameters and a fixed setting of 10 volts for entrance potential (EP).



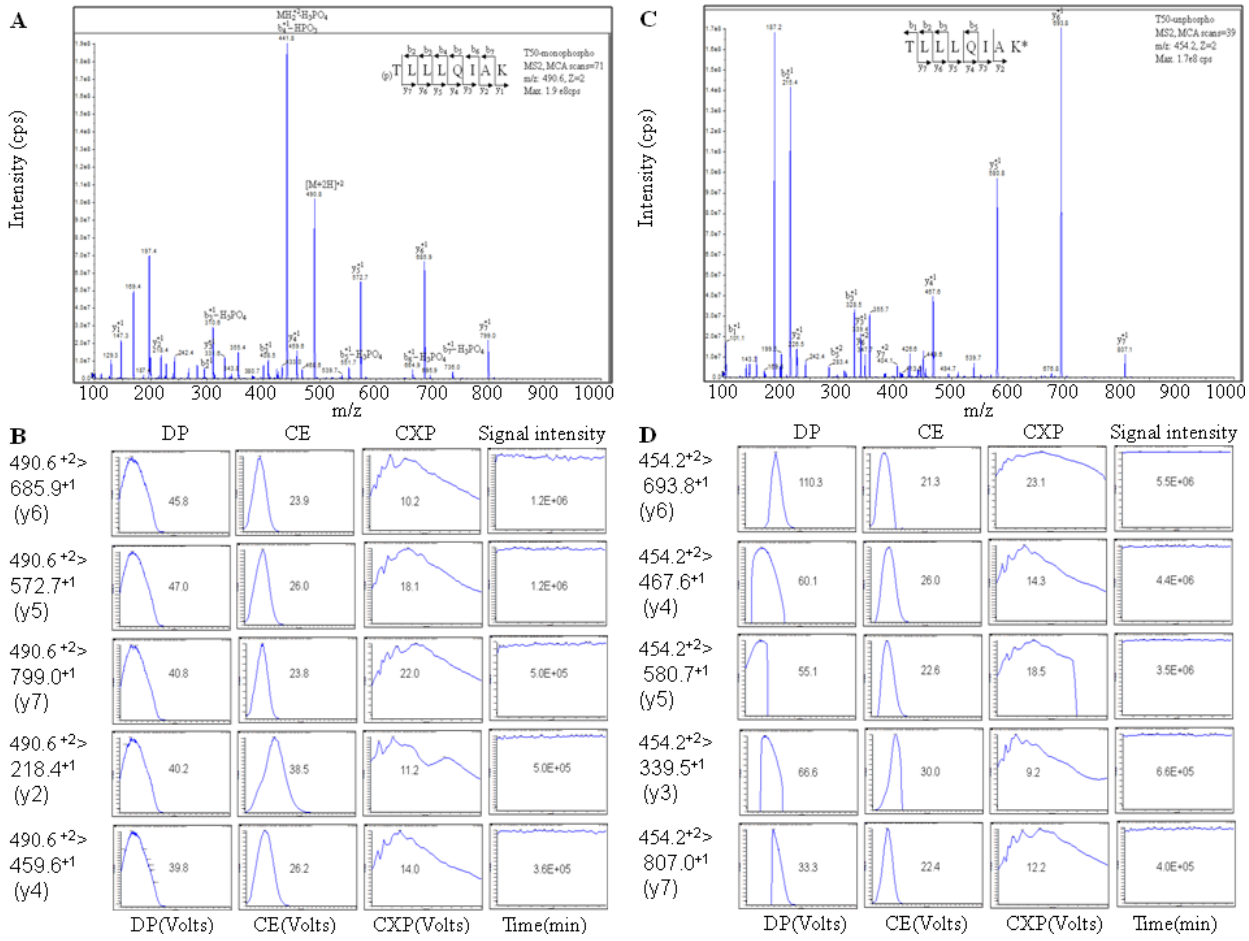
Supplemental Figure 6. Optimization of the MRM assay for phosphorylated peptide containing Y25 and an unmodified peptide for quantification of the total protein. Panel A: MS/MS spectrum of the novel monophosphorylated site Y25 *in vitro*. MS/MS of the doubly charged 353.9 ion allows for selection of product ions for monitoring the native (light) Y25 monophosphorylated peptide ($SSN_{(p)}YR$). Panel B: Optimization of MRM on the Y25 monophosphorylated peptide ($SSN_{(p)}YR$). Panel C: MS/MS spectrum of the doubly charged 627.4 ion allows for selection of product ions for monitoring the labeled (heavy) peptide (NITEIADLTQK*) for quantification of the total protein. Panel D: Optimization of MRM on the peptide (NITEIADLTQK*) for the total protein. Optimization was obtained by ramping the parameters declustering potential (DP)(0-400 volts), collision energy (CE)(5-130 volts), and Collision Cell Exit Potential (CXP)(0-66 volts) from low to high with a step of 1 for all parameters and a fixed setting of 10 volts for entrance potential (EP).



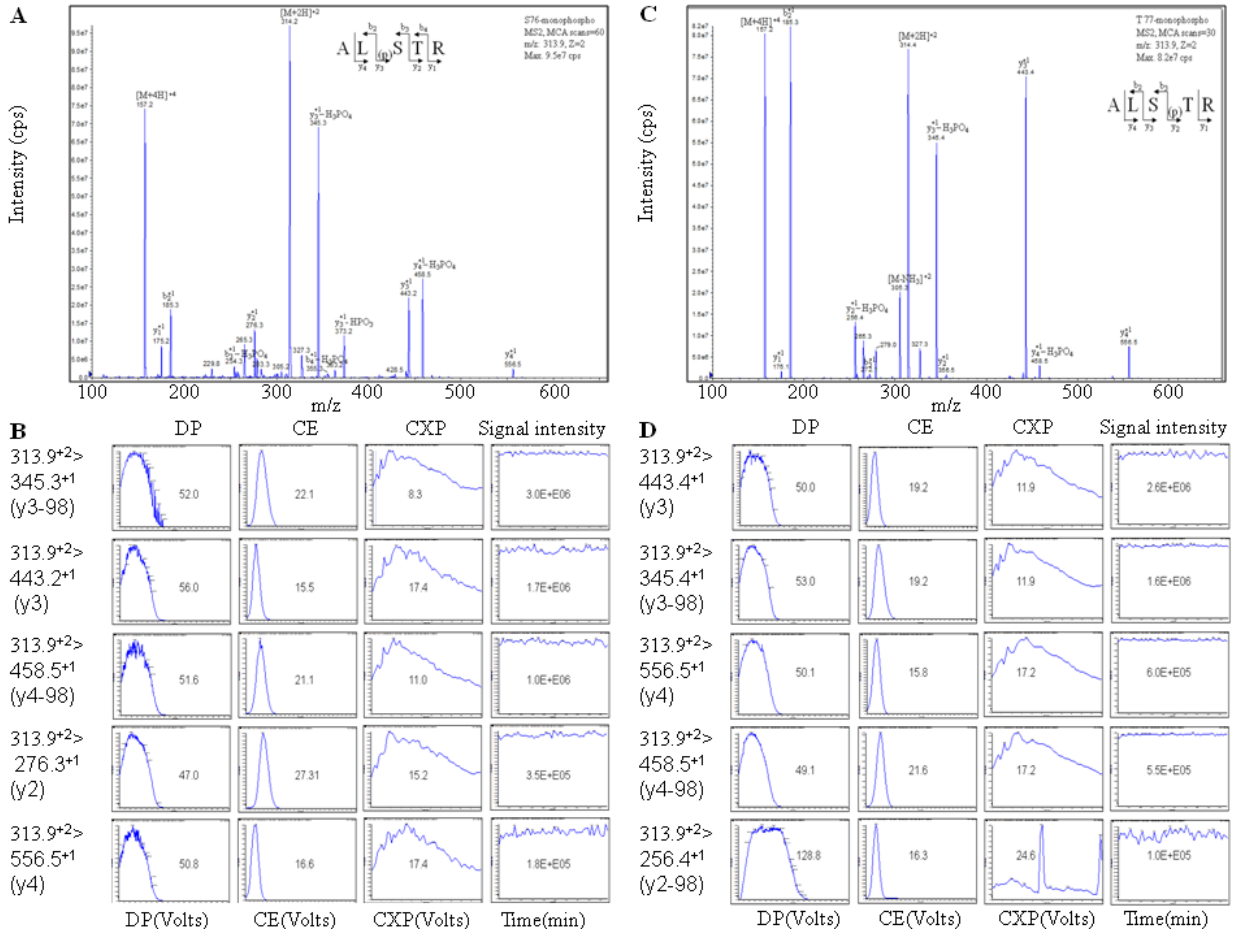
Supplemental Figure 7. Optimization of the MRM assay for phosphorylated peptides containing S41 or S43. Panel A: MS/MS spectrum of the monophosphoylated site S41 *in vitro*. MS/MS spectrum of the doubly charged 306.9 ion allows for selection of product ions for monitoring the native (light) S41 monophosphorylated peptide ($I_{(p)}$SASR). Panel B: Optimization of MRM on S41 monophosphorylated peptide ($I_{(p)}$SASR). Panel C: MS/MS spectrum of the monophosphoylated site S43 *in vitro*. MS/MS spectrum of the doubly charged 307.0 ion allows for selection of product ions for monitoring the native (light) S43 monophosphorylated peptide ($ISA_{(p)}SR$). Panel D: Optimization of MRM on the S43 monophosphorylated peptide ($ISA_{(p)}SR$). Optimization was obtained by ramping the parameters declustering potential (DP)(0-400 volts), collision energy (CE)(5-130 volts), and Collision Cell Exit Potential (CXP)(0-66 volts) from low to high with a step of 1 for all parameters and a fixed setting of 10 volts for entrance potential (EP).



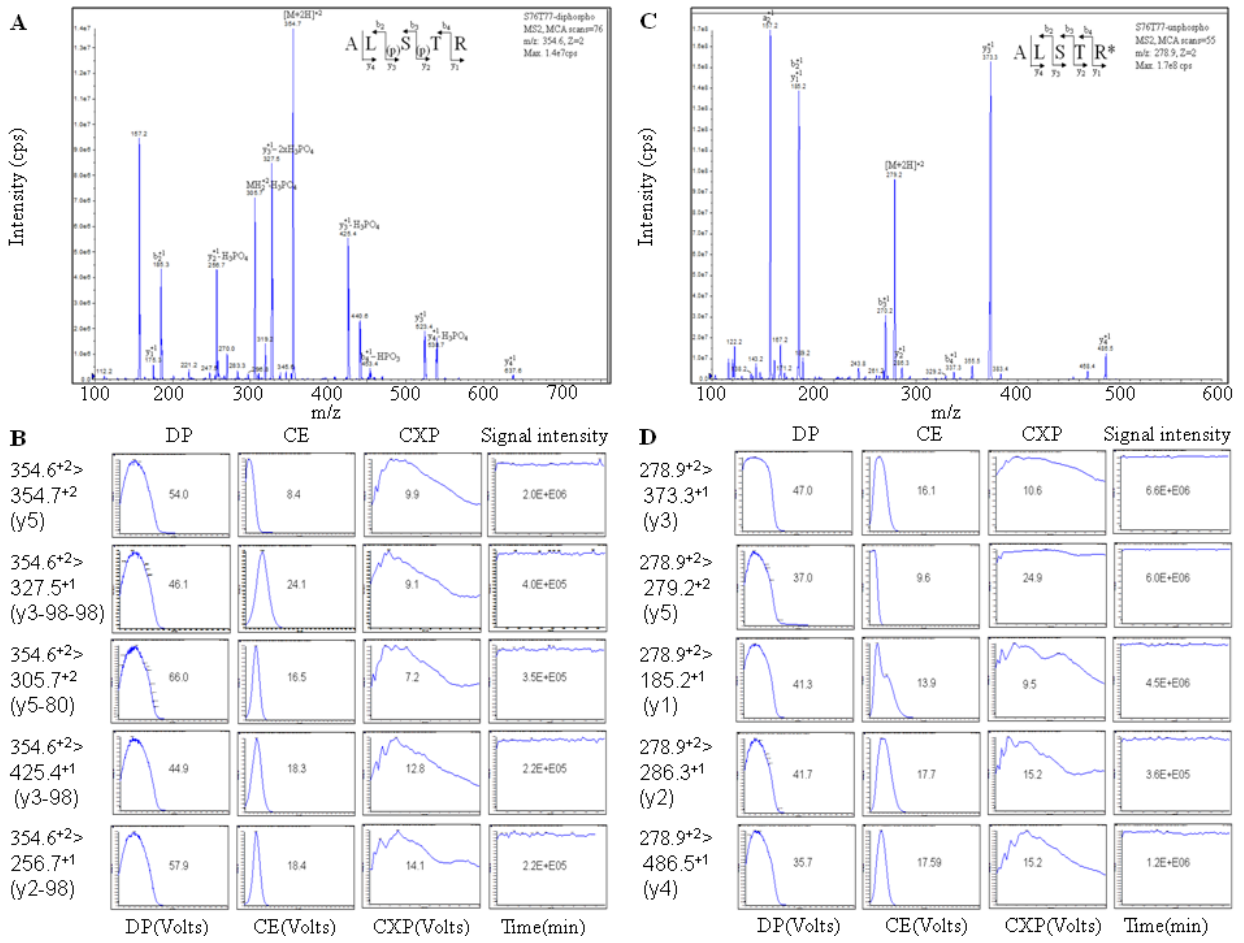
Supplemental Figure 8. Optimization of the MRM assay for phosphorylated and unphosphorylated peptides containing S41/43. Panel A: MS/MS spectrum of diphosphorylated sites S41/43 *in vitro*. MS/MS spectrum of the doubly charged 346.8 ion allows for selection of product ions for monitoring the native (light) S41/43 diphosphorylated peptide ($I_{(p)}SA_{(p)}SR$). Panel B: Optimization of MRM on the S41/43 diphosphorylated peptide ($I_{(p)}SA_{(p)}SR$). Panel C: MS/MS spectrum of the doubly charged 272.0 ion allows for selection of product ions for monitoring the labeled (heavy) S41/43 unphosphorylated peptide (ISASR*). Panel D: Optimization of MRM on the S41/43 unphosphorylated peptide (ISASR*). Optimization was obtained by ramping the parameters declustering potential (DP)(0-400 volts), collision energy (CE)(5-130 volts), and Collision Cell Exit Potential (CXP)(0-66 volts) from low to high with a step of 1 for all parameters and a fixed setting of 10 volts for entrance potential (EP).



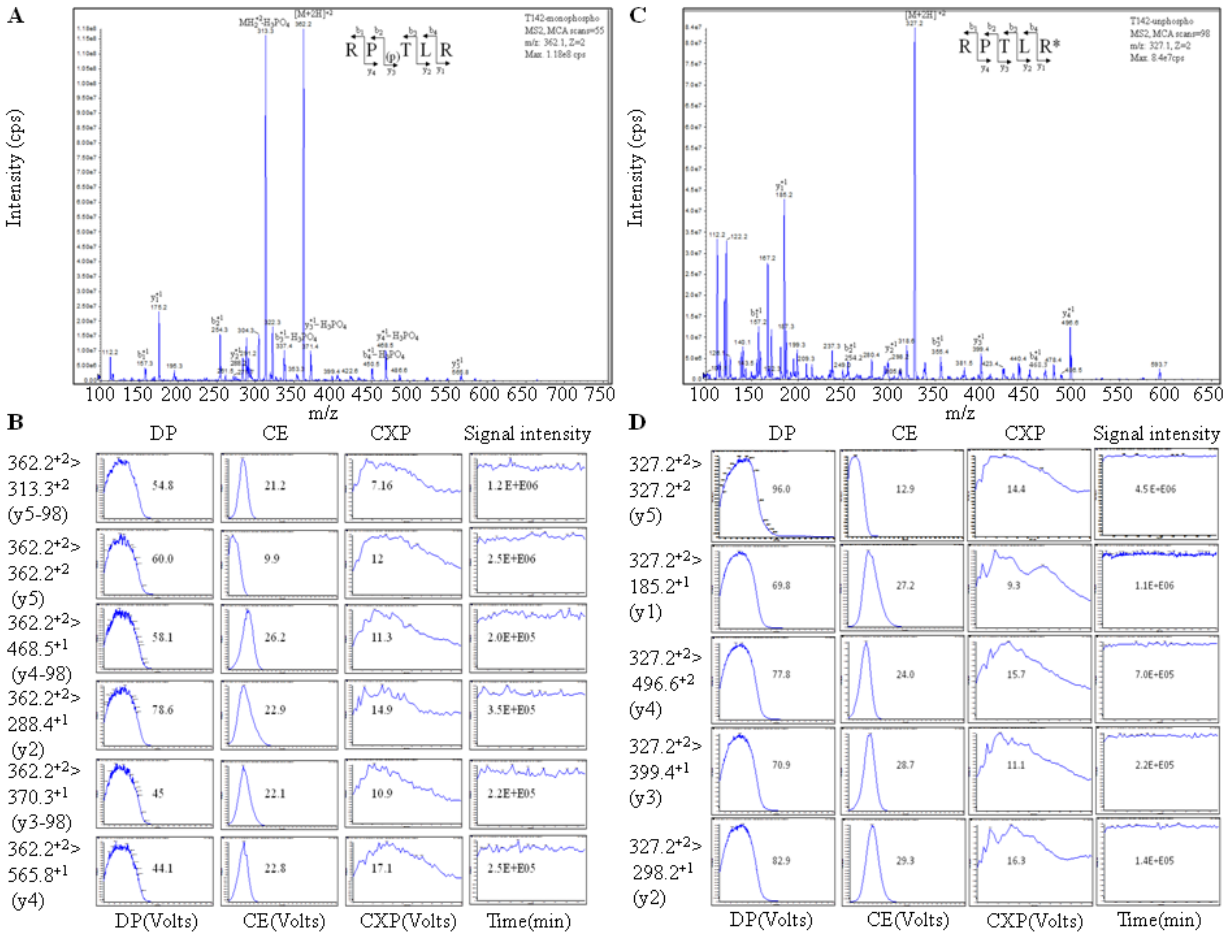
Supplemental Figure 9. Optimization of the MRM assay for phosphorylated and unphosphorylated peptides containing T50. Panel A: MS/MS spectrum of the novel monophosphorylated site T50 *in vitro*. MS/MS of the doubly charged 490.6 ion allows for selection of product ions for monitoring the native (light) T50 monophosphorylated peptide ($_{(p)}$TLLLQIAK). Panel B: Optimization of MRM on the T50 monophosphorylated peptide ($_{(p)}$TLLLQIAK). Panel C: MS/MS of the doubly charged 454.2 ion allows for selection of product ions for monitoring the labeled (heavy) T50 unphosphorylated peptide (TLLLQIAK*). Panel D: Optimization of MRM on the T50 unphosphorylated peptide (TLLLQIAK*). Optimization was obtained by ramping the parameters declustering potential (DP)(0-400 volts), collision energy (CE)(5-130 volts), and Collision Cell Exit Potential (CXP)(0-66 volts) from low to high with a step of 1 for all parameters and a fixed setting of 10 volts for entrance potential (EP).



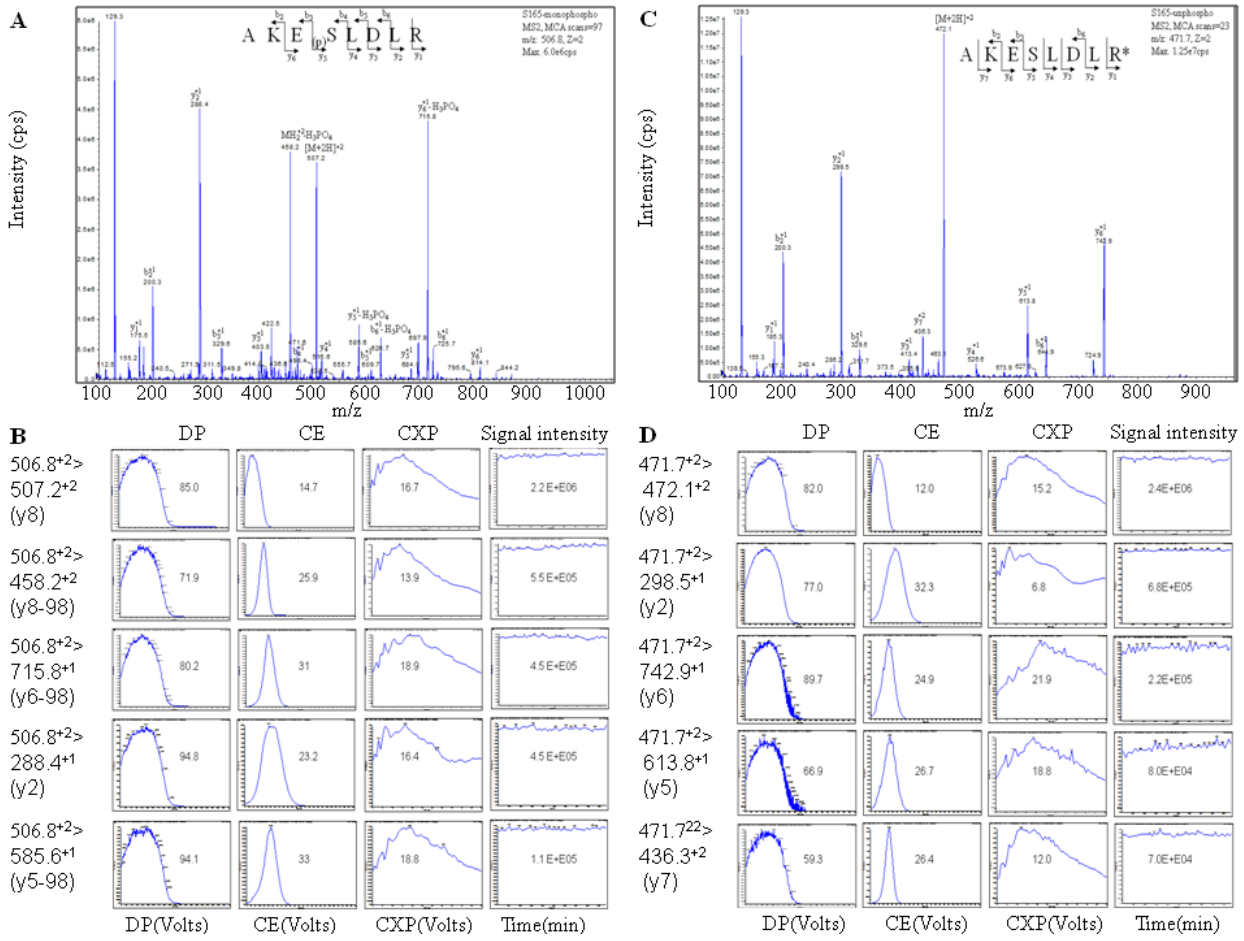
Supplemental Figure 10. Optimization of the MRM assay for phosphorylated peptides containing S76 or T77. Panel A: MS/MS spectrum of the novel monophosphorylated site S76 *in vitro*. MS/MS of the doubly charged 313.9 ion allows for selection of product ions for monitoring the native (light) S76 monophosphorylated peptide ($AL_{(p)}STR$). Panel B: Optimization of MRM on the S76 monophosphorylated peptide ($AL_{(p)}STR$). Panel C: MS/MS spectrum of the novel monophosphorylated site T77 *in vitro*. MS/MS of the doubly charged 313.9 ion allows for selection of product ions for monitoring the native (light) T77 monophosphorylated peptide ($ALS_{(p)}TR$). Panel D: Optimization of MRM on the T77 monophosphorylated peptide ($ALS_{(p)}TR$). Optimization was obtained by ramping the parameters declustering potential (DP)(0-400 volts), collision energy (CE)(5-130 volts), and Collision Cell Exit Potential (CXP)(0-66 volts) from low to high with a step of 1 for all parameters and a fixed setting of 10 volts for entrance potential (EP).



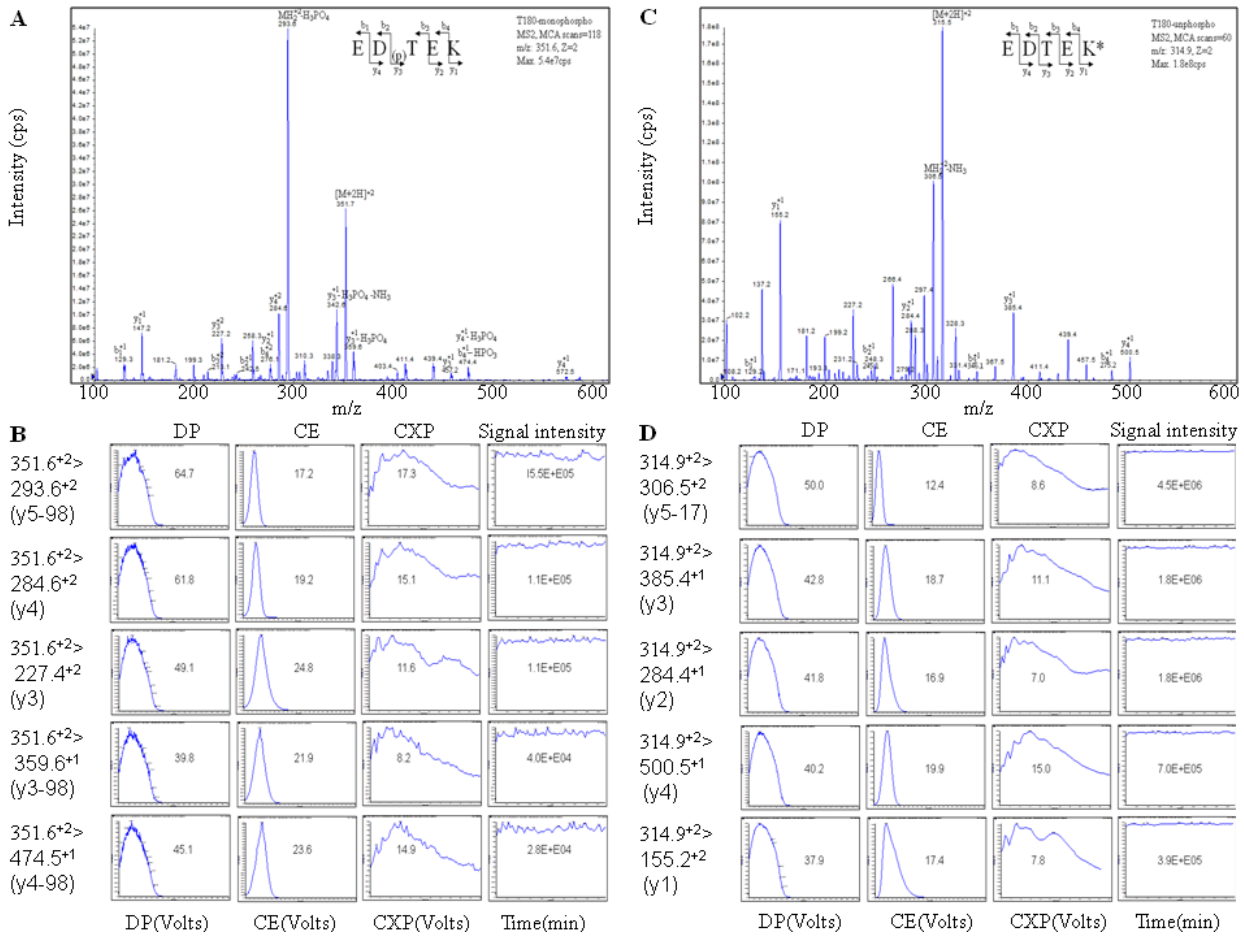
Supplemental Figure 11. Optimization of the MRM assay for phosphorylated and unphosphorylated peptides containing S76/T77. Panel A: MS/MS spectrum of the novel diphosphorylated sites S76/T77 *in vitro*. MS/MS of the doubly charged 354.6 ion allows for selection of product ions for monitoring the native (light) S76/T77 diphosphorylated peptide ($AL_{(p)}S_{(p)}TR$). Panel B: Optimization of MRM on the S76/T77 diphosphorylated peptide ($AL_{(p)}S_{(p)}TR$). Panel C: MS/MS of the doubly charged 278.9 ion allows for selection of product ions for monitoring the labeled (heavy) S76/T77 unphosphorylated peptide (ALSTR*). Panel D: Optimization of MRM on the S76/T77 unphosphorylated peptide (ALSTR*). Optimization was obtained by ramping the parameters declustering potential (DP)(0-400 volts), collision energy (CE)(5-130 volts), and Collision Cell Exit Potential (CXP)(0-66 volts) from low to high with a step of 1 for all parameters and a fixed setting of 10 volts for entrance potential (EP).



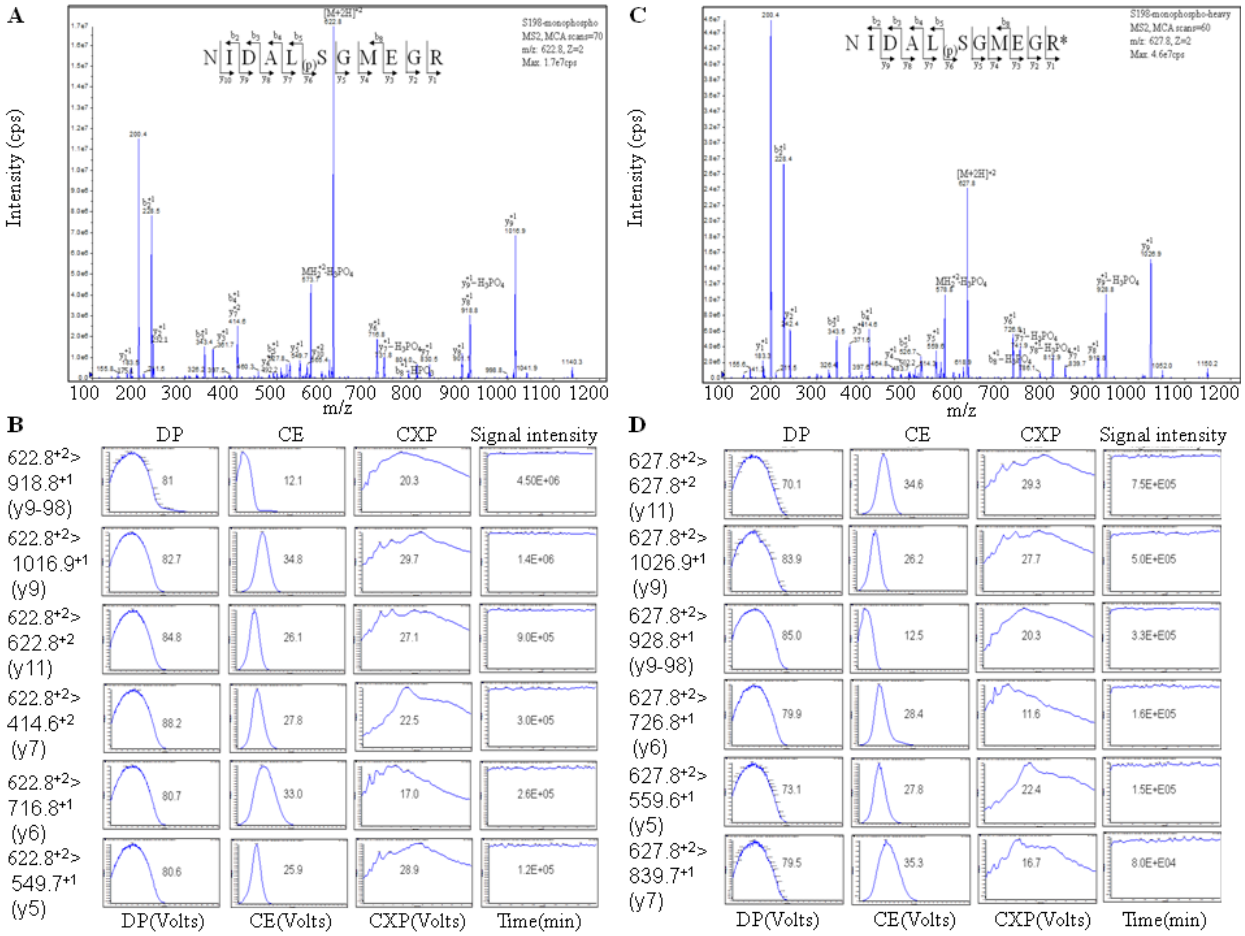
Supplemental Figure 12. Optimization of the MRM assay for phosphorylated and unphosphorylated peptides containing T142. Panel A: MS/MS spectrum of the monophosphoylated site T142 *in vitro*. MS/MS spectrum of the doubly charged 362.2 ion allows for selection of product ions for monitoring the native (light) T142 monophosphorylated peptide ($RP_{(p)}TLR$). Panel B: Optimization of MRM on T142 monophosphorylated peptide ($RP_{(p)}TLR$); Panel C: MS/MS spectrum of the doubly charged 327.2 ion allows for selection of product ions for monitoring the labeled (heavy) T142 unphosphorylated peptide (RPTLR*). Panel D: Optimization of MRM on T142 unphosphorylated peptide (RPTLR*). Optimization was obtained by ramping the parameters declustering potential (DP)(0-400 volts), collision energy (CE)(5-130 volts), and Collision Cell Exit Potential (CXP)(0-66 volts) from low to high with a step of 1 for all parameters and a fixed setting of 10 volts for entrance potential (EP).



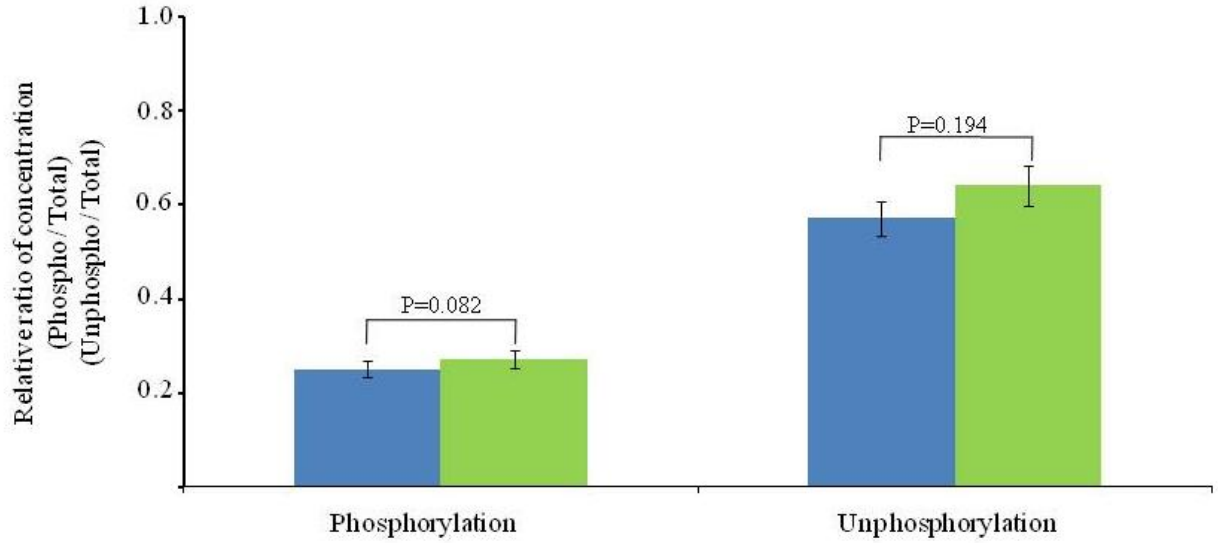
Supplemental Figure 13. Optimization of the MRM assay for phosphorylated and unphosphorylated peptides containing S165. Panel A: MS/MS spectrum of the novel monophosphorylated site S165 *in vitro*. MS/MS of the doubly charged 506.8 ion allows for selection of product ions for monitoring the native (light) S165 monophosphorylated peptide ($AKE_{(p)}SLDLR$). Panel B: Optimization of MRM on the S165 monophosphorylated peptide ($AKE_{(p)}SLDLR$). Panel C: MS/MS of the doubly charged 471.7 ion allows for selection of product ions for monitoring the labeled (heavy) S165 unphosphorylated peptide ($AKE_{(p)}SLDLR^*$). Panel D: Optimization of MRM on the S165 unphosphorylated peptide ($AKE_{(p)}SLDLR^*$). Optimization was obtained by ramping the parameters declustering potential (DP)(0-400 volts), collision energy (CE) (5-130 volts), and Collision Cell Exit Potential (CXP)(0-66 volts) from low to high with a step of 1 for all parameters and a fixed setting of 10 volts for entrance potential (EP).



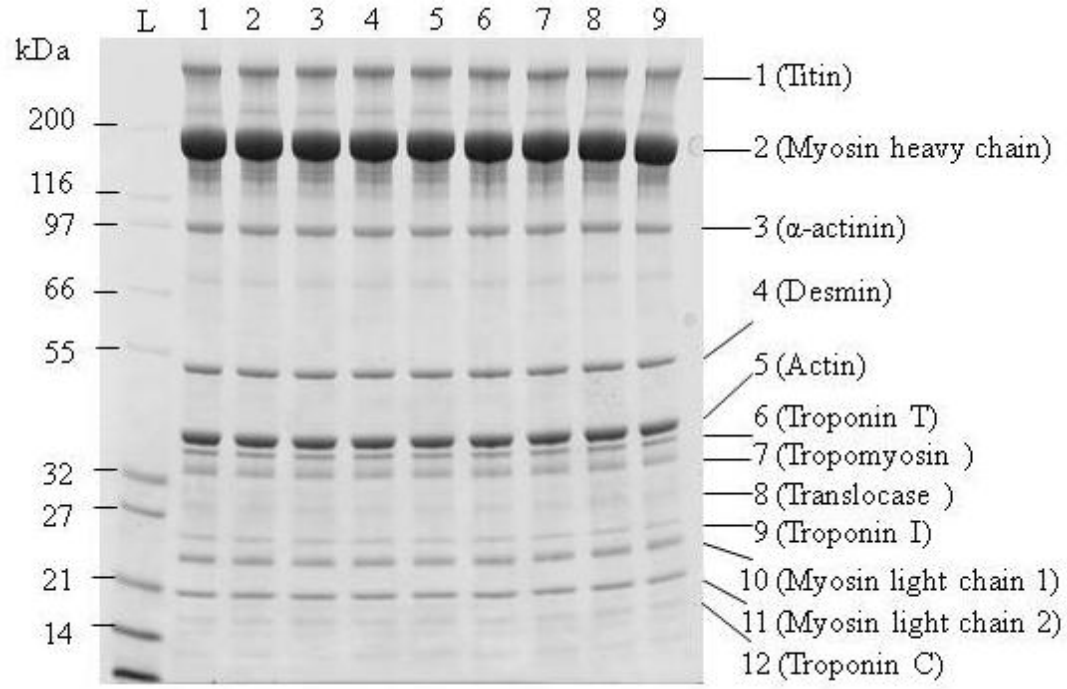
Supplemental Figure 14. Optimization of the MRM assay for phosphorylated and unphosphorylated peptides containing T180. Panel A: MS/MS spectrum of the novel monophosphorylated site T180 *in vitro*. MS/MS of the doubly charged 351.6 ion allows for selection of product ions for monitoring the native (light) T180 monophosphorylated peptide ($ED_{(p)}TEK$). Panel B: Optimization of MRM on the T180 monophosphorylated peptide ($ED_{(p)}TEK$). Panel C: MS/MS of the doubly charged 314.9 ion allows for selection of product ions for monitoring the labeled (heavy) T180 unphosphorylated peptide (EDTEK*). Panel A: Optimization of MRM on the T180 unphosphorylated peptide (EDTEK*). Optimization was obtained by ramping the parameters declustering potential (DP)(0-400 volts), collision energy (CE)(5-130 volts), and Collision Cell Exit Potential (CXP)(0-66 volts) from low to high with a step of 1 for all parameters and a fixed setting of 10 volts for entrance potential (EP).



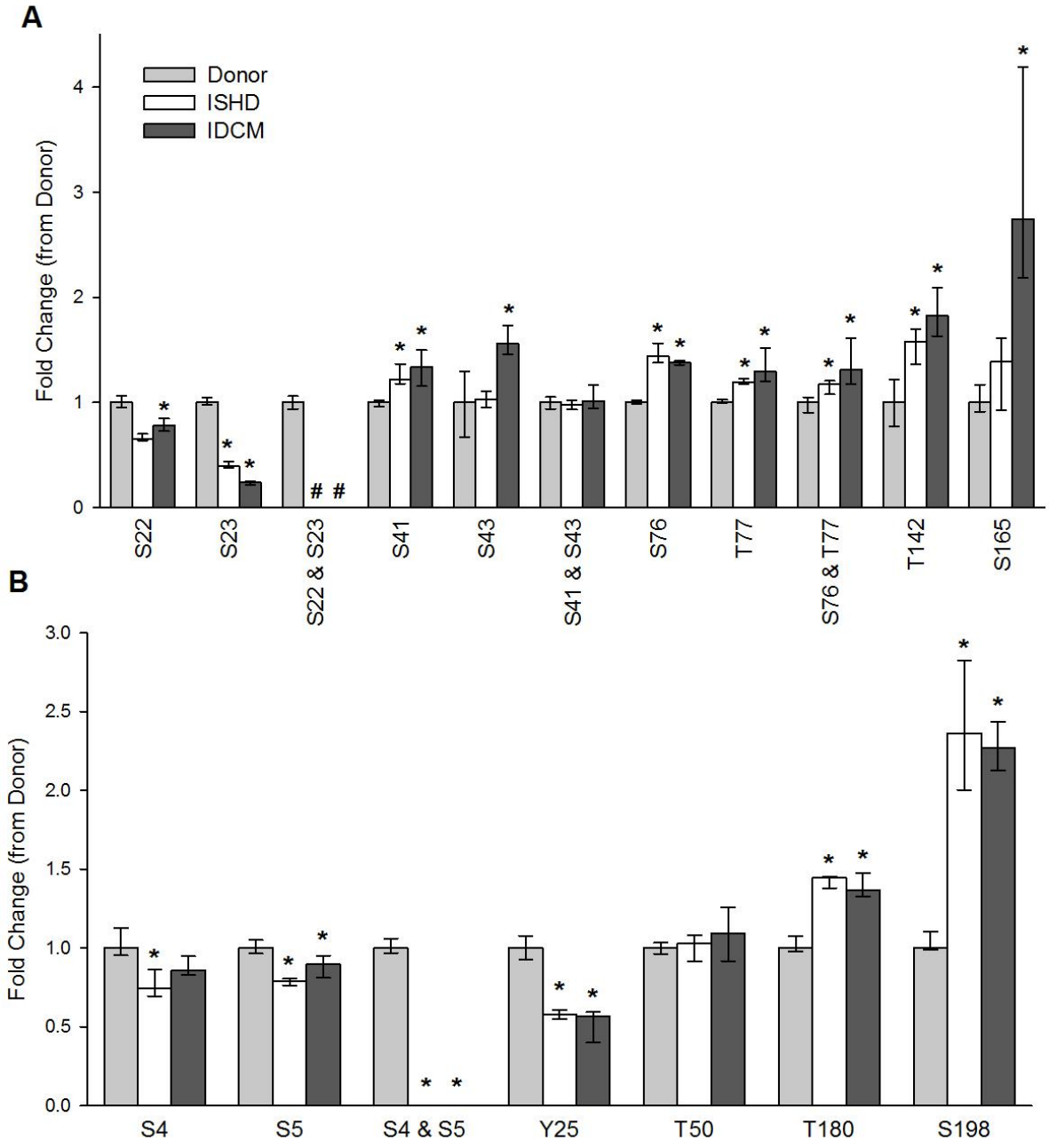
Supplemental Figure 15. Optimization of the MRM assay for phosphorylated peptides containing S198. Panel A: MS/MS spectrum of the monophosphoylated site S198 *in vitro*. MS/MS spectrum of the doubly charged 622.8 ion allows for selection of product ions for monitoring the native (light) S198 monophosphorylated peptide (NIDAL$_{(p)}$SGMEGR). Panel B: Optimization of MRM on S199 monophosphorylated peptide (NIDAL$_{(p)}$SGMEGR). Panel C: MS/MS spectrum of the doubly charged 627.8 ion allows for selection of product ions for monitoring the labeled (heavy) S198 monophosphorylated peptide (NIDAL$_{(p)}$SGMEGR*). Panel D: Optimization of MRM on the heavy labeled S198 monophosphorylated peptide (NIDAL$_{(p)}$SGMEGR*). Optimization was obtained by ramping the parameters declustering potential (DP)(0-400 volts), collision energy (CE)(5-130 volts), and Collision Cell Exit Potential (CXP)(0-66 volts) from low to high with a step of 1 for all parameters and a fixed setting of 10 volts for entrance potential (EP).



Supplemental Figure 16. Quantitative comparison of sample processing. Comparison of the phosphorylated and unphosphorylated peptides containing T142 was between a gel-based and direct homogenization-based protein digestion by MRM. In the gel-based method (green), 30 μg of purified myofilament proteins from heart failure LV tissues was applied to 4-12% Bis-Tris NuPAGE gels (Invitrogen), followed by Coomassie staining and tryptic in-gel digestion. Alternatively, whole proteins were extracted directly from the purified myofilament, and then 30 μg of the protein mixture was treated as above without the separation by NuPAGE gels (blue). All peptide mixtures were desalted prior to analysis by MRM assays of peptides (RPTLR or $RP_{(p)}TLR$) to the peptide NITEIADLTQK for the total protein. All the data shown was an average value of five replicates with standard errors. The result of MRM assays in five replicates showed no significant difference between two sample preparation methods. As well, the gel-based method demonstrated greater intensity of signals and reduced percent coefficient of variation (CV% = 5-6%) compared to the direct protein digestion.

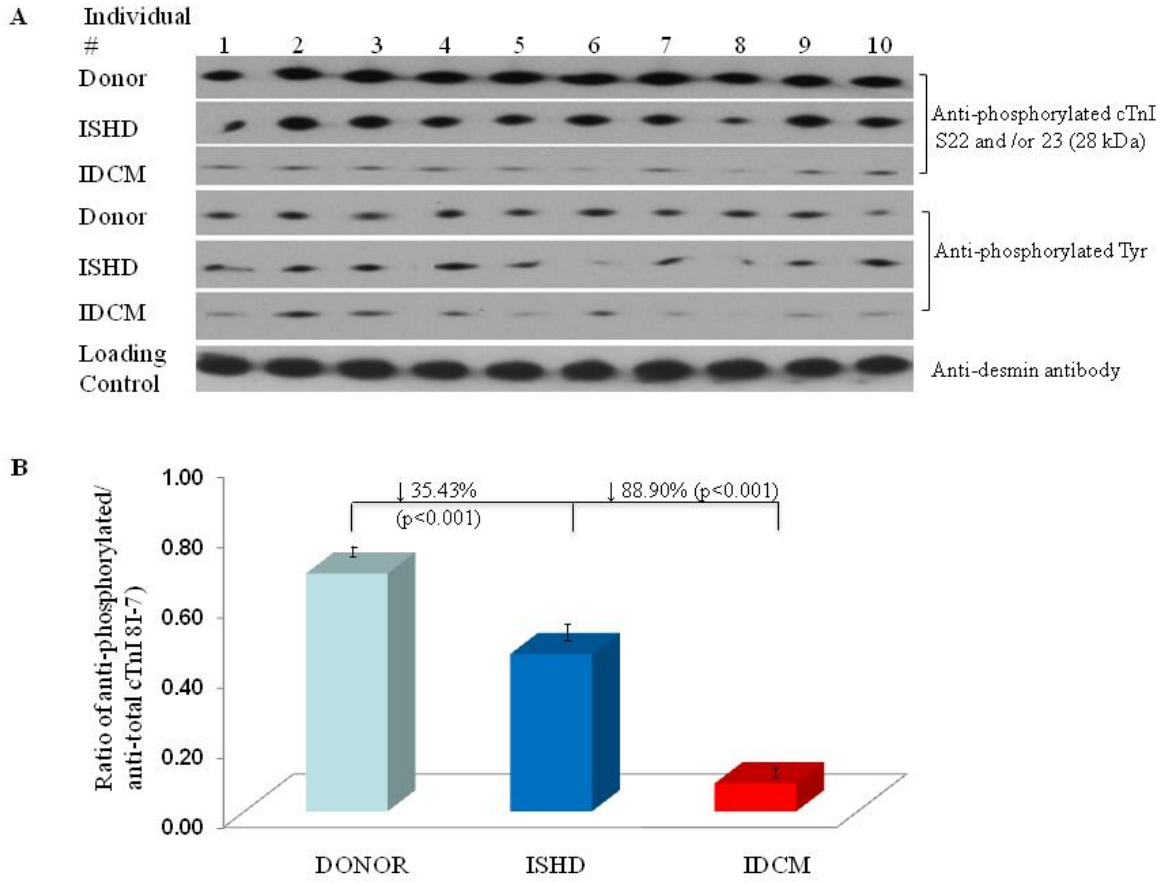


Supplemental Figure 17. Preparation and identification of cTnI. Lane L is the protein marker of molecular size; lanes 1-9 are proteins of human left ventricular tissues with heart failure. Proteins of 30 μg were loaded for each lane and resolved by NuPAGE 4-12% Bis-Tris gel and stained by Coomassie R-250. Protein identification was completed by peptide mass fingerprint (PMF) technique on MALDI-TOF/TOF instrument (see ***Supplemental*** Table 4).



Supplemental Figure 18. Quantitation of the fold change of phosphorylation sites in myocardium obtained from donor and failing hearts. The fold change of the phosphorylation sites of cTnI by MRM assay in Donor (light gray), ISHD (white) and IDCM (dark gray) heart

(Panel A); The fold change of the novel phosphorylation sites of cTnI (*n* = 10 per group) (Panel B). Values are median, 1st quartile and 3rd quartile. The quantity of each peptide was determined in the linear range of standard curve and then the fold change was calculated between the various groups. S149 phosphorylation was not detected**.** Note: S22 & S23 diphosphorylated peptide was detected but below LLOQ in the failing heart (indicated with a #).



Supplemental Figure 19. Quantitatively determine phosphorylation of cTnI at S22/23 by immunoblot. Panel A: Quantitation of the phosphorylation was verified by immunoblot for PKA-sites of S22 and/or 23 and the potential Y25. Image of immunoblot result of extracts from human left ventricular (LV) tissues using anti-phospho-troponin I (Cardiac) (Ser22 and/or 23) antibody and Phospho-Tyrosine Mouse mAb (P-Tyr-100) (Cell signaling). The loading control is monoclonal anti-desmin antibody (Sigma). Panel B: Average ratio of phosphorylated PKA-sites of S22 and/or 23 decreased in ISHD and IDCM. Experiment results were acquired in triplicate on 1 μg each of proteins from LV myofibrils isolated from the end-stage failing hearts of ISHD, IDCM, and non-failing donor hearts (n = 10). Densitometrical analysis was done with a Progenesis image software (Nonlinear Dynamics). Phosphorylated cTnI (S22/23) was calculated as phospho signal/total protein signal in triple experiments.

SUPPLEMENTAL REFERENCES

1. Spragg DD, Leclercq C, Loghmani M, Faris OP, Tunin RS, DiSilvestre D, McVeigh ER, Tomaselli GF, Kass DA. Regional alterations in protein expression in the dyssynchronous failing heart. *Circulation*. 2003; 108: 929–932.