RePLiCal: A QconCAT protein for retention time standardisation in proteomics studies – Supporting Information

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Supplementary experimental

RP-nano uHPLC-MS^E experiment to identify candidate peptides and check performance of SPOT synthesised peptides

RP-nuHPLC-MS^E was performed using a nanoACQUITY ultra-performance liquid chromatograph (Waters Ltd., Elstree, UK) and coupled to a Synapt HDMS quadrupole-time-of-flight mass spectrometer (Waters Ltd., Elstree UK). Both instruments were operated under the control of MassLynx version 4.1. Initially, the samples were loaded onto a Symmetry C18 trapping column (5 µm packing material, 180 µm x 20 mm) (Waters Ltd., Elstree UK). Trapping and desalting was performed for 3 minutes at a flow rate of 5 µL min^{-1}. The solvent conditions were 99.9 % A:0.1% B (A = 0.1 % formic acid, B = 0.1 % formic acid in MeCN). Post-trapping, the peptides were eluted onto a HSS T3 nanoACQUITY C18 analytical column (1.7 µm packing material, 75 µm x 150 mm) (Waters Ltd., Elstree, UK) and separated using gradient elution. The gradient starting conditions were 97 % A: 3% B. The column was developed to 40 % B over 90 min, then to 85 % B over 3 min, held at 85 % B for 5 min, reset to starting conditions over 1 min and then re-equilibrated over 21 min. The flow rate was constant at 300 nL min^{-1}. The column oven temperature was 35 ^{o}C and the autosampler temperature was 4 ^{o}C. A lock mass solution of 500 fmol µL^{-1} of glu-fibrinopeptide B (Sigma-Aldrich Ltd., Poole, UK) in 0.1 % formic acid in H_2O:MeCN [50:50, v/v] was infused into the nano-electrospray ionisation (nESI) source from an auxiliary pump at a flow rate of 300 nL min^{-1}. All solvents were LC-MS grade (Sigma-Aldrich Ltd., Poole, UK). The column effluent was introduced into a nano-ESI source fitted with a PicoTip emitter (New Objective, Woburn, MA, USA). The polarity of the ionisation source set to positive. The ionisation source was operated under the following conditions; capillary voltage, 3.0 kV; cone voltage, 25 V; extraction cone, 4 V; source temperature, 70 ^{o}C; trap gas 1.5 mL min^{-1}; detector 2000 V. The instrument was calibrated using the product ion

spectrum of glu-fibrinopeptide B (500 fmol μL^{-1} in 0.1 % formic acid in H_2O:MeCN [50:50, v/v]), which was infused directly into the nano-ESI source at a constant flow rate of 500 nL min^{-1}. Data acquisitions were performed using a MS^E experiment.[1] A survey scan was performed over the *m*/*z* range 50-2000 with a scan time of 1 sec and a trap cell collision energy of 6 eV. Product ion spectra were then acquired over the *m*/*z* range 50-2000 with a scan time of 1 sec. The trap cell collision energy was ramped from 15 to 40 eV over the course of the scan. The transfer cell collision was maintained at 4 eV for both the survey and product ion scans. The lock mass was sampled every 30 sec using a trap cell collision energy of 6 eV and a cone voltage that facilitated a detector response of between 100-200 counts-per-second.

Data processing was performed using ProteinLynx Global Server version 2.4 (Waters, Elstree, UK).[2, 3] The data preparation stage used a low energy threshold of 250 counts, an elevated energy threshold of 100 counts and an intensity threshold of 1500 counts. The lock mass was defined as glu-fibrinopeptide B ($[M + 2H]^+$, *m*/*z* 785.8426). The processed spectra were searched against the UniProt reference complete proteome set for either *E. coli* or *S. cerevisiae* (for the identification of candidate peptides) or an in-house generated database of the sequences of the SPOT peptides. The search used the default settings for precursor and product ion mass tolerance, and the thresholds for a match were as follows; minimum fragment ion matches per peptide, 3; minimum fragment ion matches per protein, 7; minimum peptide matches per protein, 1. For the searches against the *E. coli* or *S. cerevisiae* databases, fixed carbamidomethyl modification for cysteine and variable modifications for methionine oxidation and asparagine and glutamine deamidation were specified. For the search against the SPOT peptides database, variable modification of asparagine and glutamine deamidation was specified. For all searches, two trypsin missed cleavages were

allowed. The threshold score/expectation value for the acceptance of individual spectra was the default value for the program, such that the false positive rate was 4 %.

RePLiCal expression and purification

A gene encoding for RePLiCal was synthesised and cloned into the expression vector pET21a and provided as 3 μg of lyophilised powder (PolyQuant GmbH, Bad Abbach, Germany). The powder was solubilised in 30 μL of 10 mM Tris-HCl, 1 mM EDTA pH 8.0. One microlitre of the solution was taken and the RePLiCal gene was transformed into *E. coli* BL21 (λDE3) competent cells. A 25 μL aliquot of the *E. coli* cells was streaked onto a Luria agar (LA) with 50 μg mL^{-1} ampicillin selection plate and incubated at 37 ^{o}C overnight as previously described.[4] A single colony was picked and inoculated into 5 mL of Luria broth (LB) with 50 μg mL^{-1} ampicillin and incubated at 37 ^{o}C overnight with shaking at 220 rpm. This culture was transferred to 500 mL of fresh LB medium and incubated at 37 ^{o}C with shaking at 220 rpm. One millilitre samples were removed at hourly intervals and the absorbance at 600 nm measured using visible light spectrophotometry. Once an OD_{600nm} of between 0.6-0.8 had been reached, expression of RePLiCal was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Five hours post-induction, cells were harvested by centrifugation at 2,000 x g and the cell pellets stored at -20 ^{o}C prior to purification. Harvested cells were lysed in 2 mL of BugBuster Protein Extraction Reagent (Novagen, Nottingham, UK) containing 600 units of benzonase nuclease (Millipore, Watford, UK), Complete EDTA-free protease inhibitor cocktail (Roche, Burgess Hill, UK) and 50 μg lysozyme (Sigma-Aldrich, Poole, UK). The sample was centrifuged at 15,000 x g to pellet the lysed sample. The supernatant fraction was removed and stored on ice. The cell pellet was resolubilised in 2.5 mL of lysis buffer with 50 μg lysozyme and incubated for 5 min at room temperature. A further 15 mL of diluted (1:10) lysis

buffer:water was added and the sample vortexed for 1 min. The sample was centrifuged at 15,000 x g to pellet the inclusion bodies. A 10 µL portion of the supernatant fraction and approximately 1 mg of the pellet fraction were mixed with 2 x sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (60 mM Tris-HCl, pH 6.8, 0.2 % [w/v] SDS, 1 % [v/v] glycerol, 0.1 % [w/v] bromophenol blue, 100 mM dithiothreitol), heated at 95 ^{o}C for 5 min and loaded onto a 12 % gel and separated at a constant voltage of 200 V (Figure S1). From the gel, it was determined that RePLiCal was present in the pellet fraction (contained in inclusion bodies). Further purification was by virtue of the hexa-histidine tag using a nickel affinity column according to the manufacturer's protocol (G.E. Healthcare, Amersham, UK) in the presence of 6M guanidinium chloride. (Figure S2). The inclusion body pellet was resuspended in 10 mL of bind buffer (500 mM NaCl, 50 mM sodium phosphate, 10 mM imidazole pH 8.0 and 6 M guanidinium chloride) and filtered using a 1.2 µm filter. The sample was applied to a 1 mL HisTrap^{TM} HP column and the unbound sample was collected. Ten microlitres of the starting material and unbound fraction were retained for SDS-PAGE analysis. Ten millilitres of bind buffer was applied to the column, followed by 6 mL of wash buffer (500 mM NaCl, 50 mM sodium phosphate, 20 mM imidazole pH8.0 and 6 M guanidinium hydrochloride). RePLiCal was eluted using 6 mL of elution buffer (500 mM NaCl, 50m M sodium phosphate, 500 mM imidazole pH8.0 and 6M guanidinium hydrochloride). One millilitre fractions were collected and 10 microlitres of each were analysed by SDS-PAGE to identify the fractions containing RePLiCal. To remove the guanidinium hydrochloride before gel electrophoresis, the protein from each aliquot was bound to 10 µL of StrataClean Resin (Agilent Technologies, UK) and centrifuged at 500 x g. The supernatant fraction was discarded and the resin washed before adding 2 x SDS-PAGE sample buffer. The resin and sample buffer was loaded directly onto a 12% gel (Figure S2). RePLiCal predominantly eluted in elution fraction 1 (E1), which was dialysed against

100 mM ammonium bicarbonate. To quantify RePLiCal in the purified preparation, SDS-PAGE was performed as described above using a bovine serum albumin (BSA) standard curve as a reference (Figure S3). Using the 1 in 4 dilution of purified RePLiCal, it was estimated that the final concentration was ~2 μg μL^{-1}, which is equivalent to ~50 pmol μL^{-1}.

RP-nUHPLC-SRM-MS analysis of RePLiCal

All experiments involving RePLiCal were performed as detailed below unless otherwise stated. The transitions determined during the initial characterisation of RePLiCal were used to monitor the peptides in scheduled SRM assays. The SRM assays used, along with those used for the iRT-Kit, Peptide Retention Time Calibration Mixture and MS RT Calibration Mix peptides, can be found in the supporting information spreadsheet. In addition, the transitions selected from SRMAtlas[5] for the 100 yeast peptides in the RT prediction experiment are detailed in the supporting information spreadsheet. Samples were diluted to the required concentration in 0.1% TFA in H_2O:MeCN [97:3, v/v] and analysed by RP-nUHPLC-nESI-SRM-MS/MS on a nanoACQUITY LC instrument coupled to a Xevo TQMS tandem quadrupole mass spectrometer (Waters Ltd., Elstree, UK). The samples were loaded onto a Symmetry C18 trapping column (5 μm packing material, 180 μm x 20 mm) (Waters Ltd., Elstree UK). Trapping and desalting was performed for 3 min at a flow rate of 5 μL min^{-1}. The solvent conditions were 99.9 % A: 0.1% B (A = 0.1 % FA [v/v], B = 0.1 % FA in MeCN [v/v], except for the ion-pairing agent comparison experiment where acetic acid was used at 0.5 % [v/v]). Post-trapping and desalting, the peptides were eluted onto a HSS T3 nanoACQUITY C18 analytical column (1.8 μm packing material, 75 μm x 150 mm) (Waters Ltd., Elstree, UK) and separated using gradient elution. The gradient starting conditions were 97 % A: 3% B. The column was developed to 40 % B over either 10, 30, 60 or 90 min, then to 95 % B over 2 min, held at 95 % B for 2 min, reset to starting conditions over 1 min and

re-equilibrated over 15 min. For the direct injection experiments, the peptides were loaded onto the analytical column in 99.9 % A: 0.1 % B at 400 nL min^{-1} for 15 min. At 15.01 min, the mobile phase composition was changed to 97 % A: 3% B and the gradient started as described above. In all experiments, the flow rate for the separation was constant at 300 nL min^{-1} and the column oven temperature was 35 ^{o}C. The column effluent was introduced into a nESI source fitted with a PicoTip emitter (New Objective, Woburn, MA, USA). The polarity of the ionisation source was set to positive. The ionisation source was operated under the following conditions; capillary voltage, 3.0 kV; cone voltage, 30 V; source temperature, 70 ^{o}C, nanoflow gas, 0.1 bar; cone gas, 50 L hr^{-1}. SRM analysis was performed in scheduled mode with time windows of 1, 2, 3 and 4 min for the 10, 30, 60 and 90 min gradients respectively. Collision-induced dissociation (CID) was performed using argon as the collision gas. The collision energy to dissociate each peptide was calculated using a linear regression optimised for the tandem quadrupole instrument used.[6]

Transferability of RePLiCal across LC instrumentation

Fifty femtomoles of RePLiCal was analysed by RP-nUHPLC-nESI-MS/MS on a Ultimate 3000 RSLC LC instrument coupled to a QExactive quadrupole-Orbitrap mass spectrometer (ThermoFisher Scientific, Hemel Hempstead, UK). The sample was loaded onto a PepMap100 C18 µ-Precolumn (5 µm packing material, 300 µm x 5 mm) (ThermoFisher Scientific, Hemel Hempstead, UK). Trapping and desalting was performed for 5 min at a flow rate of 5 µL min^{-1} using 0.1 % TFA in H_2O:MeCN [98:2, v/v] as the solvent. Post-trapping and desalting, the peptides were eluted onto an Easy-Spray C18 analytical column (2 µm packing material, 75 µm x 500 mm) (ThermoFisher Scientific, Hemel Hempstead, UK) and separated using gradient elution. The gradient starting conditions were 96.2 % A: 3.8 % B (A = 0.1 % FA [v/v], B = 0.1 % FA in 80:20 MeCN:H_2O [v/v]). The column was

developed to 50 % B over either 30 or 90 min, then to 99 % B over 2 min, held at 99 % B for 5 min, reset to starting conditions over 0.1 min and then re-equilibrated for 15 min. The flow rate was constant at 300 nL min^{-1} and the column oven temperature was 35 ^{o}C. The column effluent was directed through the integrated nESI emitter with the ionisation source polarity set to positive. The ionisation source was operated under the following conditions; spray voltage, 2.0 kV; S-lens RF level, 50 %; capillary temperature, 250 ^{o}C; sheath gas, 0 arbitrary units; auxiliary gas, 0 arbitrary units; sweep gas, 0 arbitrary units. Data-dependent acquisition (DDA) was performed consisting of the acquisition of a full scan mass spectrum between *m*/*z* 300-2000 at a mass resolution of 70,000 FWHM at *m*/*z* 200. The automatic gain control was set to $1e^6$ ions with a maximum fill time of 250 ms. The top 10 most abundant peaks were selected for MS/MS using higher-energy collisional dissociation (HCD) with nitrogen as the collision gas.[7] Product ion data was acquired at a mass resolution of 35,000 FWHM at *m*/*z* 200. The automatic gain control was set to $1e^5$ ions with a maximum fill time of 100 ms. The ion selection window was 2 *m*/*z* units and a normalised collision energy of 30 % was used. Dynamic exclusion was applied with a 20 s window. The data was converted to .mgf format using ProteoWizard and searched using Mascot.[8] The data was searched with a precursor ion mass tolerance of 10 ppm and a product ion mass tolerance of 10 mmu. No fixed or variable modifications were selected, and no missed cleavages were allowed. The data was searched against an in-house generated database containing sequences of several QconCATs, including RePLiCal. The peptide ion scores from the three replicates acquired using the 30 min gradient are shown in Table S5.

Optimisation of LC gradients to maximise peptide and protein identification rates

Tryptic digests of RePLiCal (100 fmol) were analysed by RP-nUHPLC-nESI-MS/MS on a nanoACQUITY LC instrument (Waters Ltd., Elstree, UK) coupled to an Orbitrap Velos

linear ion trap-Orbitrap mass spectrometer (ThermoFisher Scientific, Hemel Hempstead, UK). Trapping and separation of peptides was performed as described for the RP-nUHPLC-nESI-SRM-MS analyses using nanoACQUITY instrumentation above using gradient lengths of 30, 60 and 90 min. The column effluent was introduced into a nESI source fitted with a PicoTip emitter (New Objective, Woburn, MA, USA). The polarity of the ionisation source was set to positive. The ionisation source was operated under the following conditions; spray voltage, 3.0 kV; S-Lens RF level, 69 %; capillary temperature, 200 ^{o}C; sheath gas, 0 arbitrary units; auxiliary gas, 0 arbitrary units; sweep gas, 0 arbitrary units. Data-dependent acquisition (DDA) was performed by acquiring a full scan mass spectrum between *m*/*z* 300-2000 at a mass resolution of 30,000 FWHM at *m*/*z* 400. The automatic gain control was set to 1e^{6} ions with a maximum fill time of 500 ms. The top 20 most abundant peaks were selected for MS/MS in the linear ion trap using CID with helium as the collision gas. The automatic gain control was set to 1e^{4} ions with a maximum fill time of 100 ms. The ion selection window was 1.2 *m*/*z* units, the activation Q was 0.250 and the activation time was 10 ms. A normalised collision energy of 35 % was used and WideBand activation was switched on. Dynamic exclusion was applied with a 20 s window. The retention times of the RePLiCal peptides were used to generate "*in silico*-optimized" and "Custom distribution" non-linear gradients of 30, 60 and 90 minutes (3-40 % B) using the GradientOptimizer program.[9, 10] One microgram of a whole cell yeast lysate tryptic digest was separated using linear and both non-linear gradients in triplicate using the same MS conditions as described above. The acquired data was processed using MaxQuant v.1.5.1.0[11] and searched against the reference proteome *S. cerevisiae* strain S288C protein database downloaded from UniProt (accessed 04 February 2015) using Andromeda 1.4.0.0.[12] The data was searched with a precursor ion mass tolerance of 40 ppm for the first search and 10 ppm for the main search, and a product ion mass tolerance of 0.6 Da. Carbamidomethylation of Cys was selected as a

fixed modification and *N*-terminal acetylation and Met oxidation were chosen as variable modifications. Two missed cleavages were allowed. Matching between runs was activated with a matching time window of 0.7 min and an alignment window of 20 min. The false discovery rate (FDR) was set to 1 % at both the peptide- and protein-level. Peptides and proteins not identified in all three replicate injections were discarded from the data set.

Recalibration of m/z scale using RePLiCal

RePLiCal was spiked into a tryptic digest of a whole cell yeast lysate at ratio of 25 fmol:250 ng per µL. Four microlitres of sample was analysed by RP-nUHPLC-nESI-MS/MS on a nanoACQUITY LC instrument (Waters Ltd., Elstree, UK) coupled to an Orbitrap XL linear ion trap-Orbitrap mass spectrometer (ThermoFisher Scientific, Hemel Hempstead, UK). Trapping and separation of the peptides was performed as described for the RP-nUHPLC-nESI-SRM-MS analyses using nanoACQUITY instrumentation above except that the gradient length was either 90 min or 270 min. The column effluent was introduced into a nESI source fitted with a PicoTip emitter (New Objective, Woburn, MA, USA). The polarity of the ionisation source was set to positive. The ionisation source was operated under the following conditions; spray voltage, 2.8 kV; capillary voltage, 45 V; capillary temperature, 200 ^{o}C; sheath gas, 0 arbitrary units; auxiliary gas, 0 arbitrary units; sweep gas, 0 arbitrary units; tube lens, 140 V. Data-dependent acquisition (DDA) was performed with a full scan mass spectrum acquisition between *m*/*z* 300-1700 at a mass resolution of 60,000 FWHM at *m*/*z* 400. The automatic gain control was set to $2e^5$ ions with a maximum fill time of 500 ms. The top 5 most abundant peaks were selected for MS/MS in the linear ion trap using CID with helium as the collision gas. The automatic gain control was set to $1e^5$ ions with a maximum fill time of 25 ms. The ion selection window was 3 *m*/*z* units, the activation Q was 0.250 and the activation time was 30 ms. A normalised collision energy of 35 % was used

and WideBand activation was switched on. Dynamic exclusion was applied with a 60 s window. The data was miscalibrated by 0.125 *m*/*z* units and sliced into 27 sections, each containing a RePLiCal peptide, using the RecalOffline functionality in Xcalibur (ThermoFisher Scientific, Hemel Hempstead, UK). The data was recalibrated using the exact *m*/*z* value of the RePLiCal peptide and converted to .mgf format using ProteoWizard.[8] The .mgf files were combined, and with the .mgf file from the original data, searched against the reference proteome *S. cerevisiae* strain S288C protein database downloaded from UniProt (accessed 04 February 2015) using Mascot v2.5.1. The data was searched with a precursor ion mass tolerance of 10 ppm and a product ion mass tolerance of 0.6 Da. Carbamidomethylation of Cys was selected as a fixed modification and *N*-terminal acetylation and Met oxidation were chosen as variable modifications. Two missed cleavages were allowed. The FDR was set to 1 %.

Supplementary Results

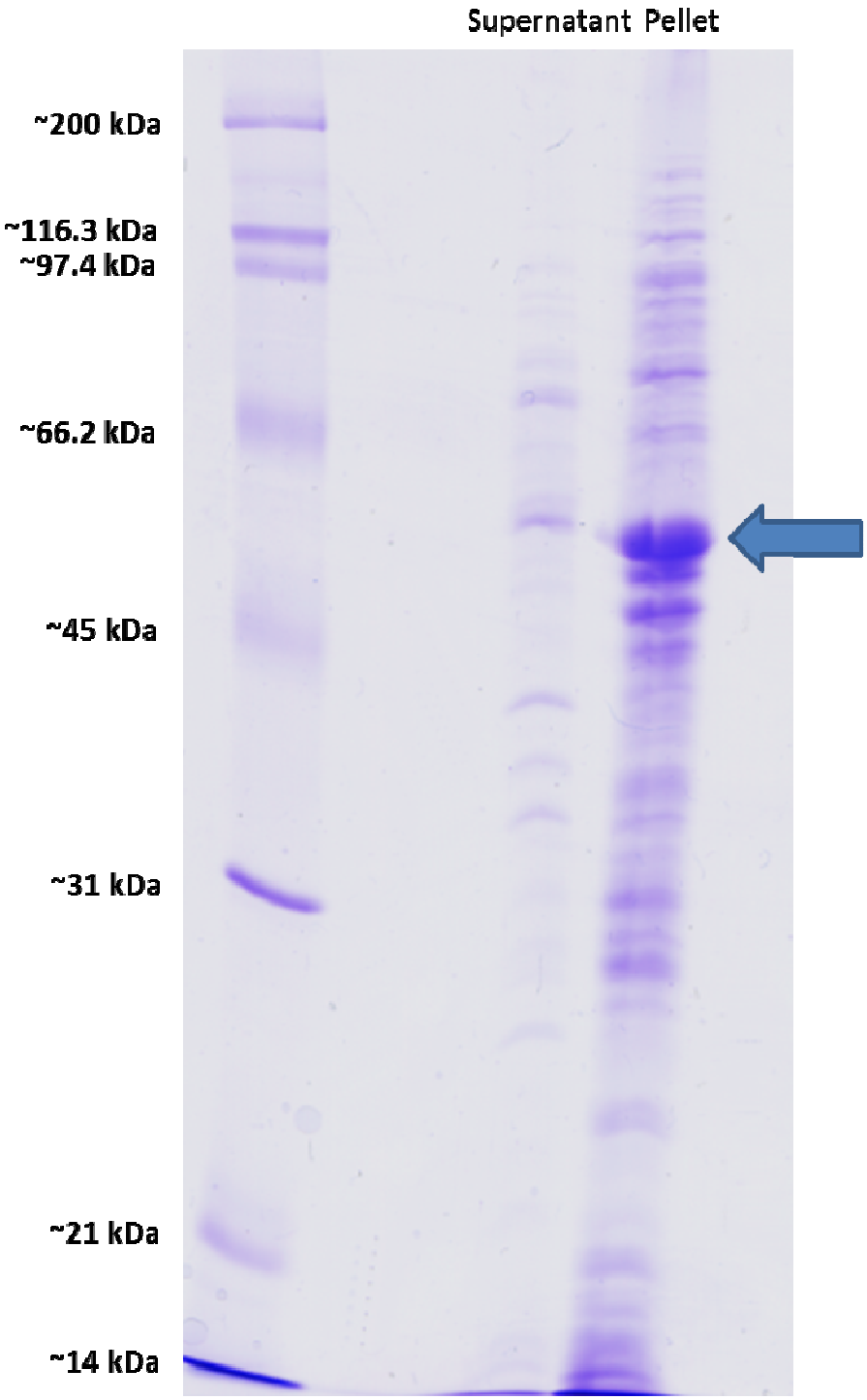

Figure S1 SDS-PAGE gel of the soluble fraction and cell pellet from the lysed *E. coli* cells post-expression of RePLiCal. The arrow indicates the band associated with RePLiCal

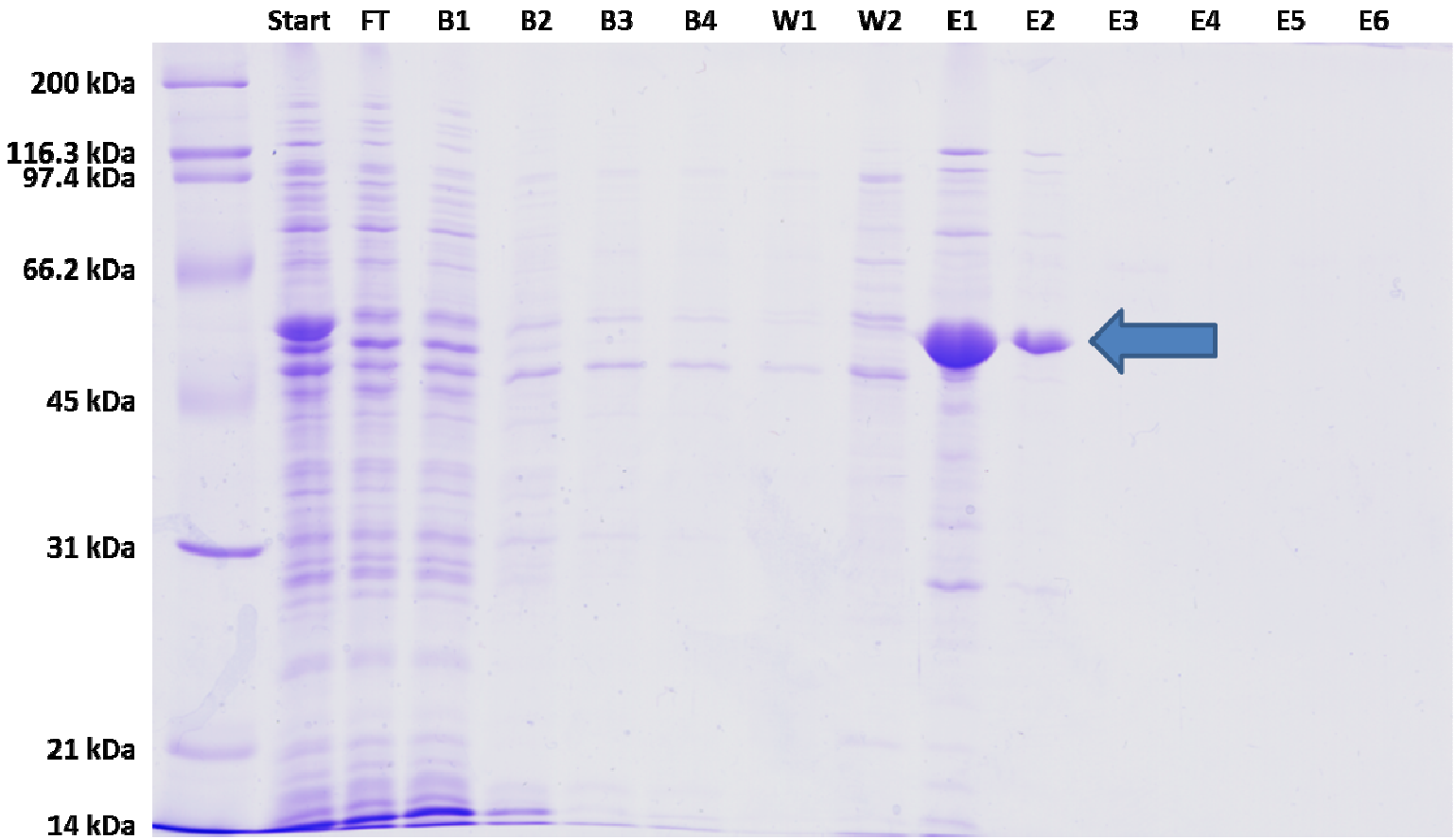

Figure S2 SDS-PAGE gel of collected fractions from the purification of RePLiCal using a 1 mL HisTrap™ HP column. Aliquots bound to StrataClean resin form the starting material (Start), starting material flow-through (FT), bind buffer (B1-B4), wash buffer (W1-W2) and elution buffer (E1-E6). The arrow indicates the band associated with RePLiCal

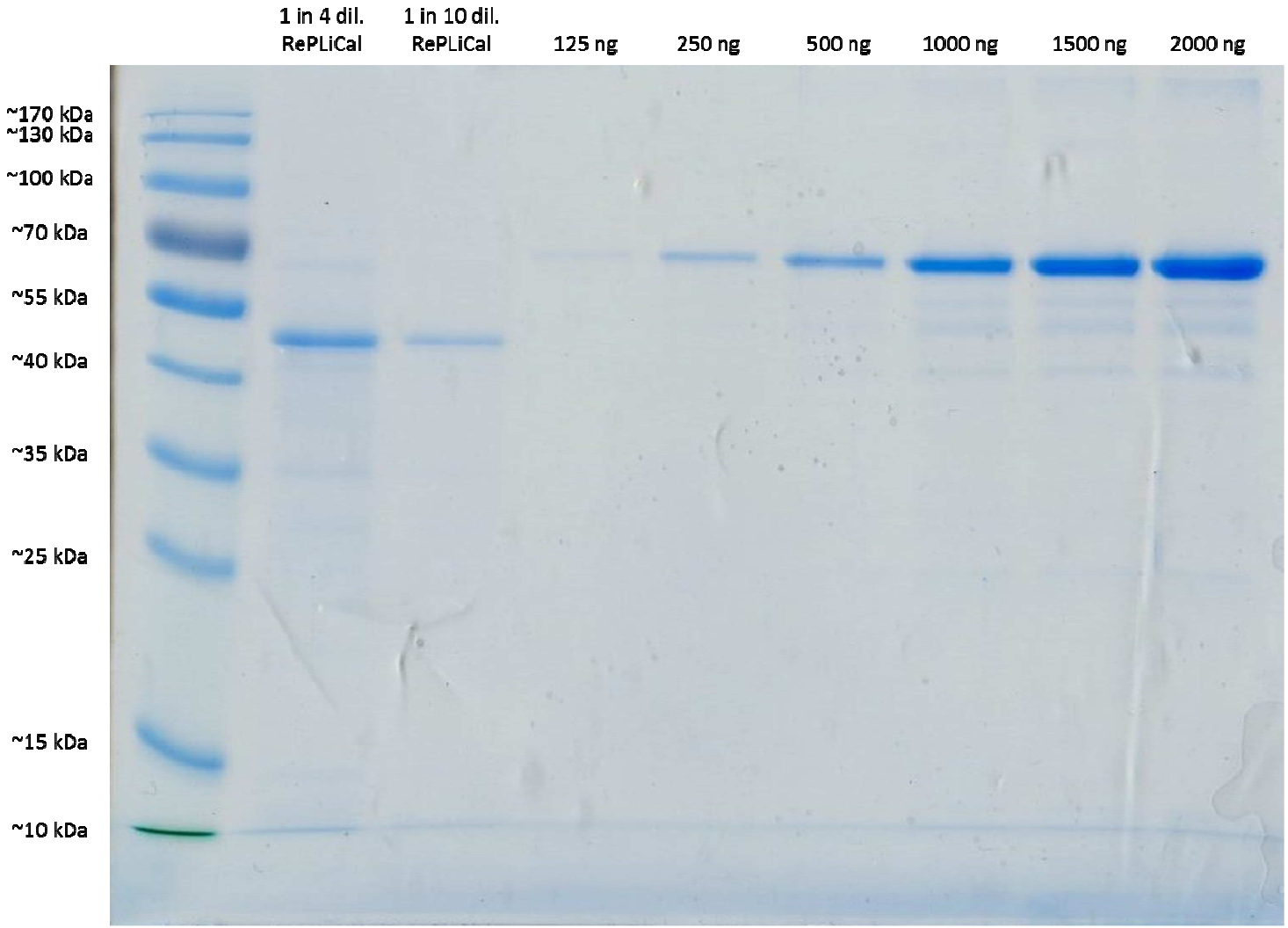

Figure S3 SDS-PAGE gel of purified RePLiCal (1 in 4 and 1 in 10 dilutions of purified material) alongside a BSA standard curve

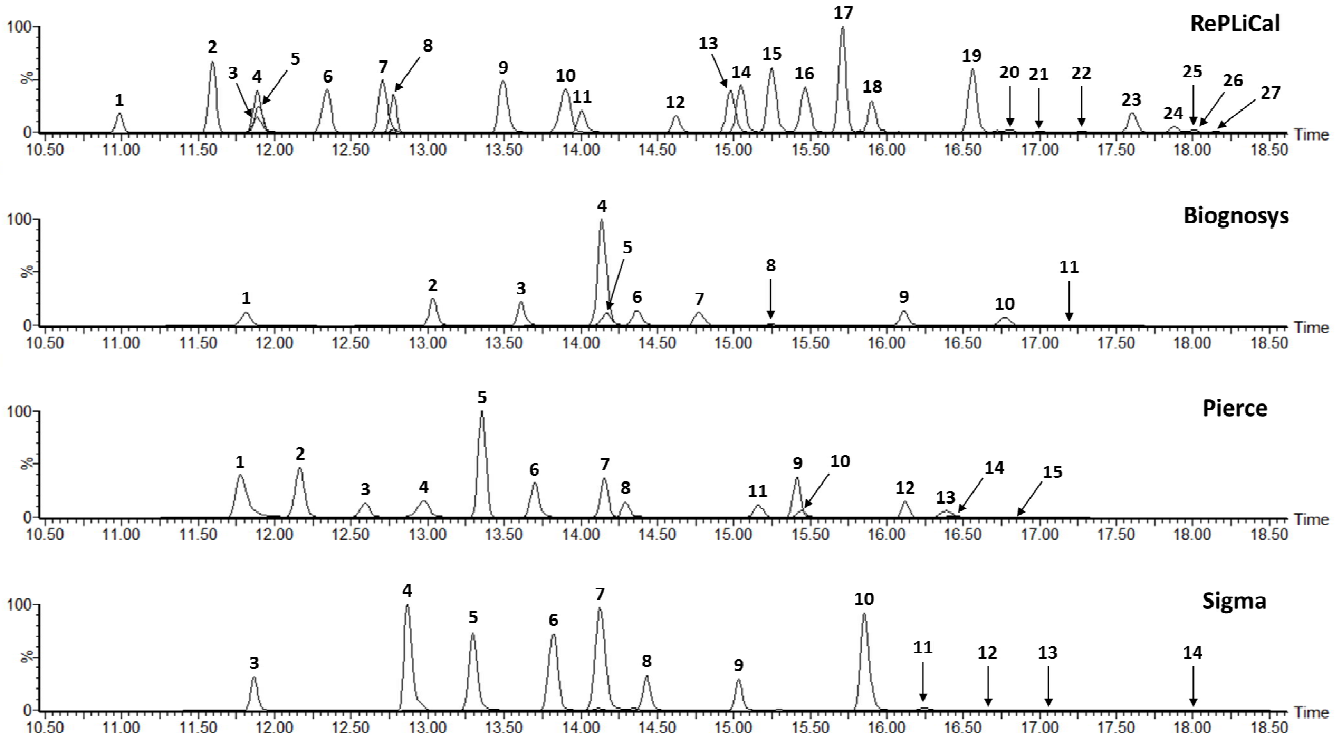

Figure S4 Comparison of chromatograms on a 10 min LC gradient (3-40 % 0.1 % formic acid in MeCN) with nESI-SRM-MS data acquisition for RePLiCal and three commercially available retention time standards. The numerical annotations represent the elution order as provided by the manufacturer. *N.B.* Peptides 1 and 2 from the MS RT Calibration Mix were not observed. These peptides, RGDSPASSPK and GLVK, are very hydrophilic and are not trapped efficiently, having been designed to test LC configurations without a trapping column

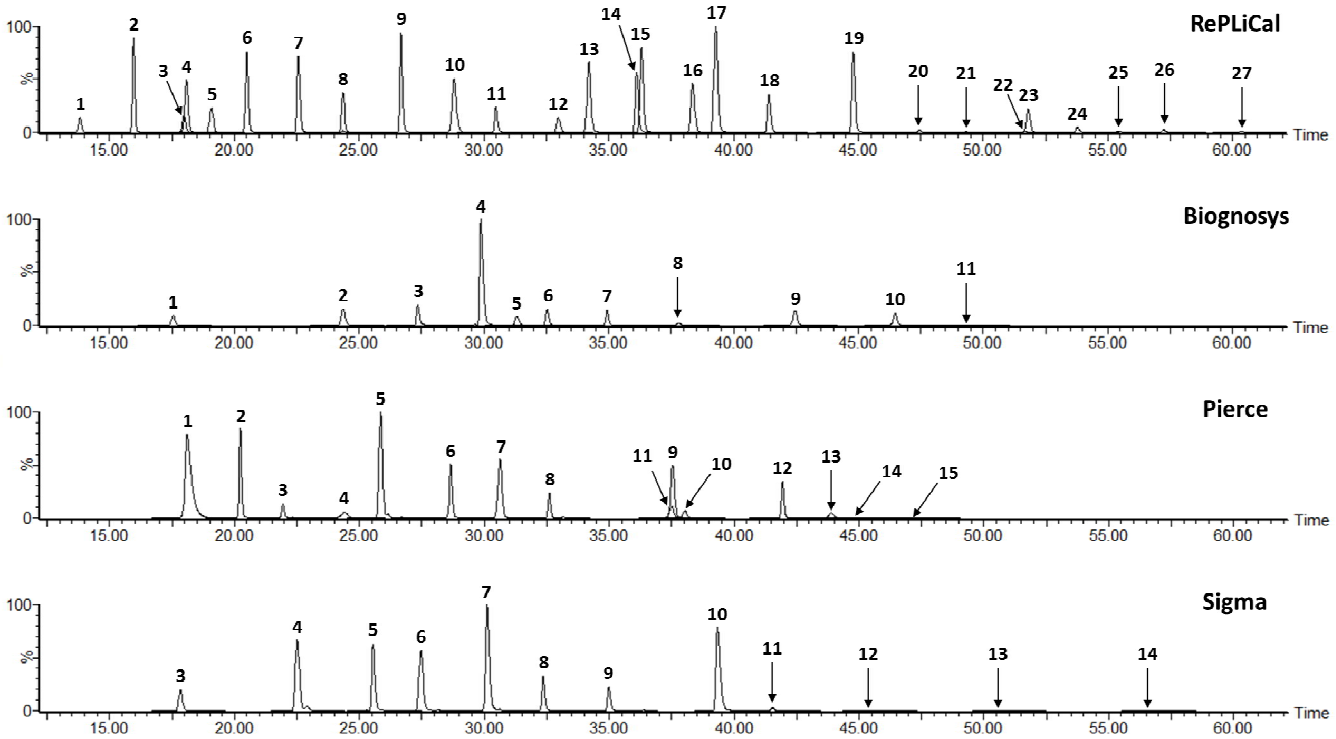

Figure S5 Comparison of chromatograms on a 60 min LC gradient (3-40 % 0.1 % formic acid in MeCN) with nESI-SRM-MS data acquisition for RePLiCal and three commercially available retention time standards. The numerical annotations represent the elution order as provided by the manufacturer. *N.B.* Peptides 1 and 2 from the MS RT Calibration Mix were not observed. These peptides, RGDSPASSPK and GLVK, are very hydrophilic and are not trapped efficiently, having been designed to test LC configurations without a trapping column

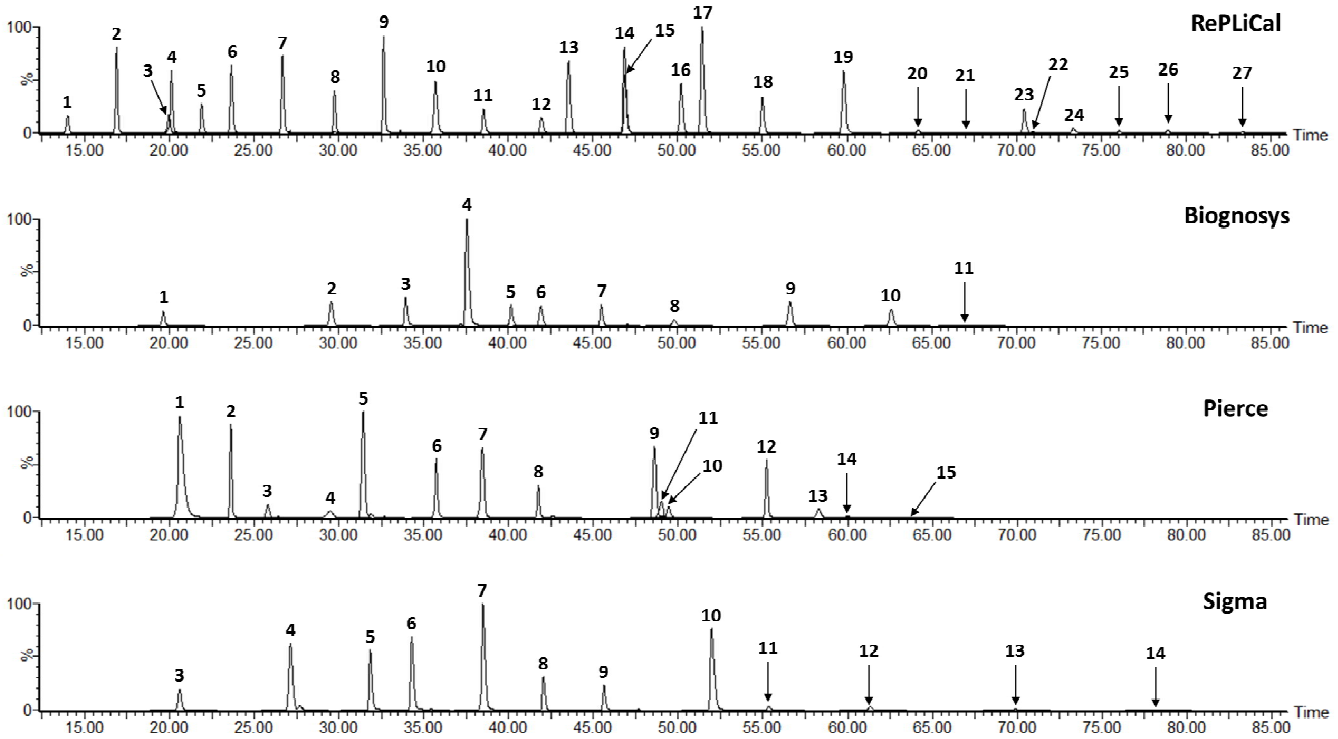

Figure S6 Comparison of chromatograms on a 90 min LC gradient (3-40 % 0.1 % formic acid in MeCN) with nESI-SRM-MS data acquisition for RePLiCal and three commercially available retention time standards. The numerical annotations represent the elution order as provided by the manufacturer. *N.B.* Peptides 1 and 2 from the MS RT Calibration Mix were not observed. These peptides, RGDSPASSPK and GLVK, are very hydrophilic and are not trapped efficiently, having been designed to test LC configurations without a trapping column

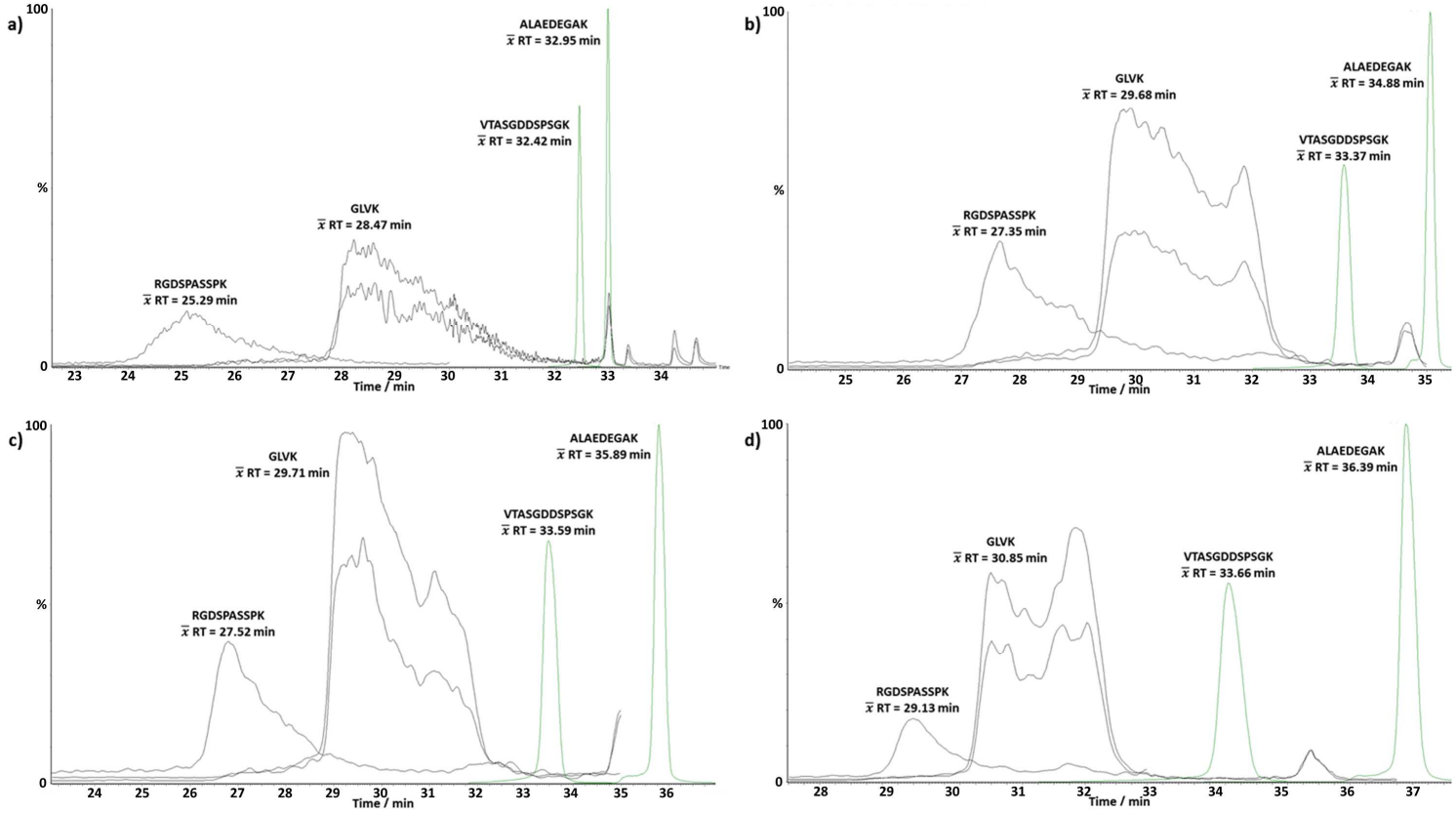

Figure S7 Separation following direct injection of early eluting peptides from Sigma (black traces) and RePLiCal (green traces) peptides on a nanoACQUITY LC instrument using a) 10 min, b) 30 min, c) 60 min and d) 90 min LC gradients (3-40 % 0.1 % formic acid in MeCN). The traces for the Sigma peptide have been magnified by x280 for the 10, 30 and 60 min gradients, and x124 for the 90 min gradient

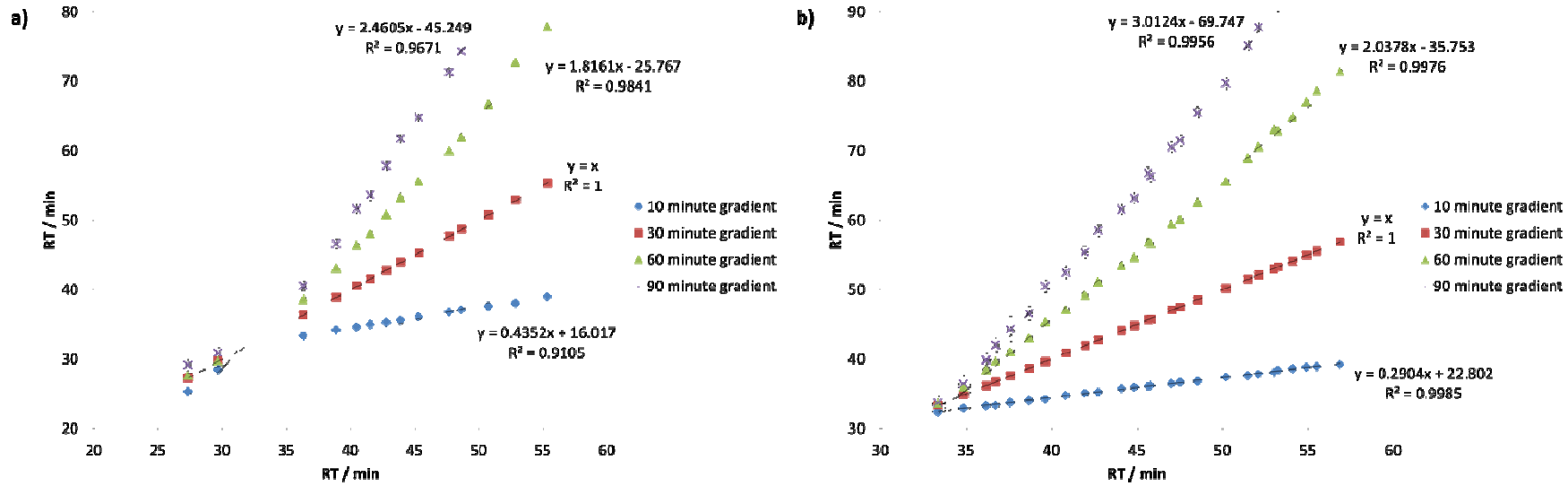

Figure S8 Comparison of retention times of a) Sigma and b) RePLiCal peptides on different LC gradients (3-40 % 0.1 % FA in MeCN) following direct injection using the 30 min gradient as a reference (n = 3). Error bars represented ± 2 standard deviations

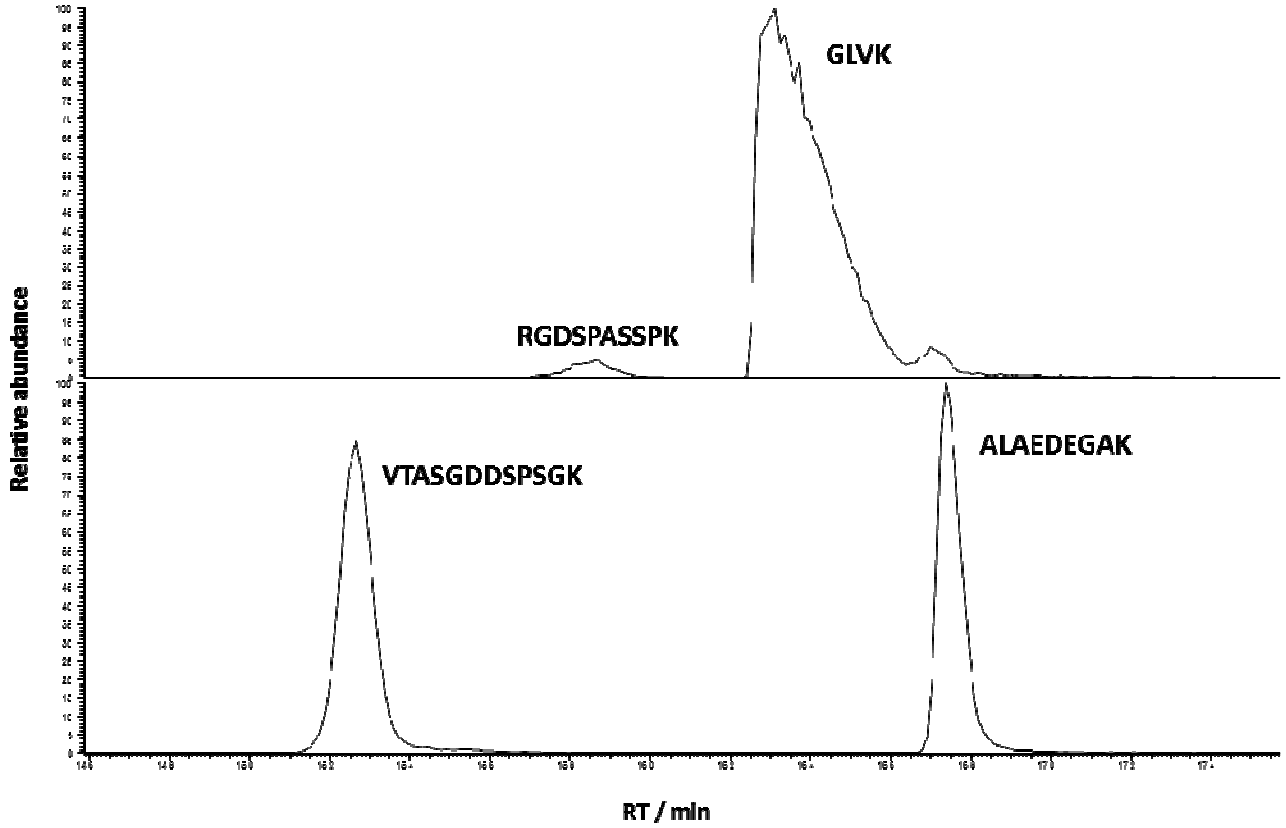

Figure S9 Separation following direct injection of early eluting peptides from Sigma (upper trace) and RePLiCal (lower trace) peptides on a RSLC LC instrument using 30 min LC gradient (3.8-50 % 0.1 % FA in MeCN:H_2O [80:20, v/v)

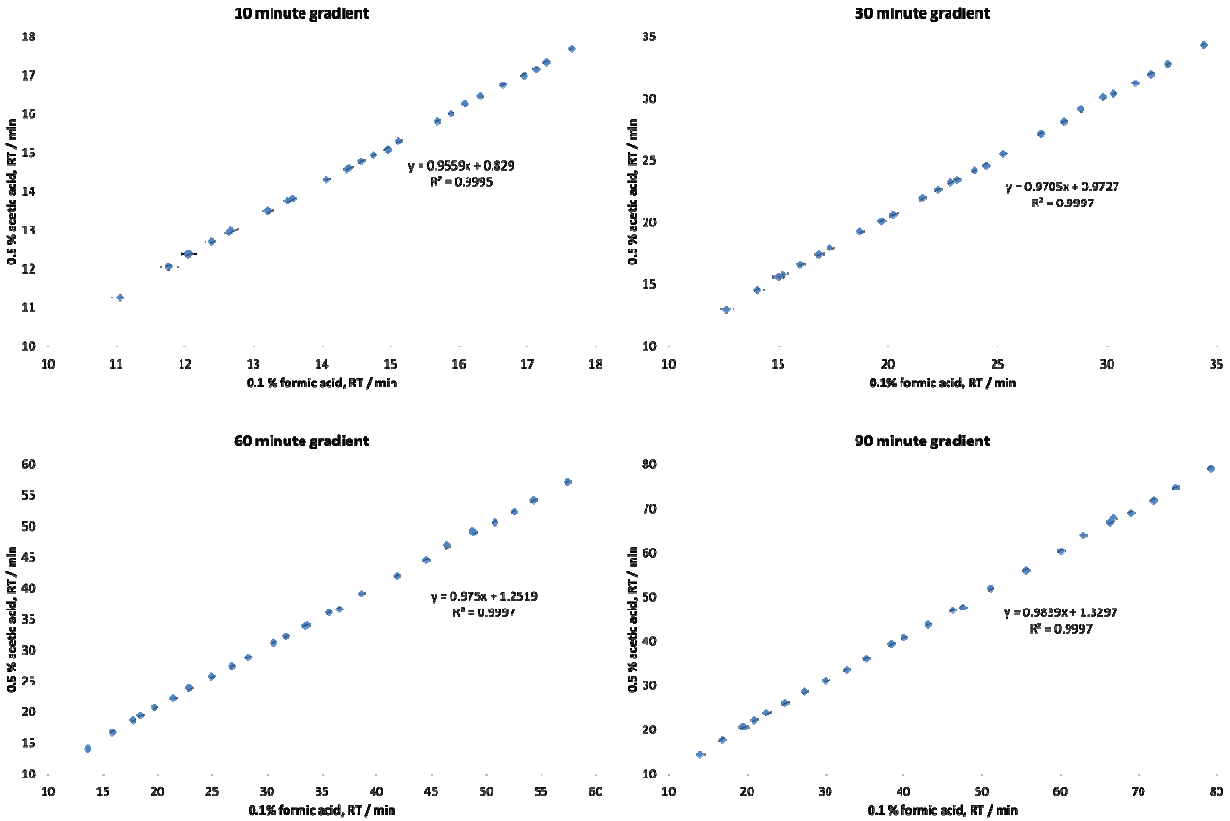

Figure S10 Comparison of the retention times of RePLiCal peptides using 0.1 % FA and 0.5 % acetic acid as the ion-pairing agent for four different LC gradient lengths (3-40 % 0.1 % FA or 0.5 % acetic acid in MeCN) (n = 3). Error bars represent ± 2 standard deviations

Table S1 Numbers of identified yeast peptides eluting pre-first and post-last standard peptides for four RT standards separated on a 90 min LC gradient (3-40 % 0.1 % FA) and percentages relative to the total number of identified peptides

Standard	# of yeast peptides eluting pre-first RT standard peptide	# of yeast peptides eluting post-last RT standard peptide	# of yeast peptides eluting outside RT range covered by RT standard	% of yeast peptides eluting pre-first RT standard peptide	% of yeast peptides eluting post-last RT standard peptide	% of yeast peptides eluting outside RT range covered by RT standard
RePLiCal	1	63	64	0.04	2.34	2.38
Biognosys	77	532	609	2.85	19.73	22.58
Pierce	125	182	307	4.64	6.75	11.39
Sigma	127	647	774	4.74	24.00	28.71

Table S2 Average peak areas and peak area RSDs from sixty injections of 50 fmol of RePLiCal in 1 µg of yeast tryptic peptides

Peptide #	Peptide sequence	Average peak area	RSD peak area
1	VTASGDDSPSGK	268,536	6.53
2	ALAEDEGAK	552,949	6.82
3	ASADLQPDSQK	132,621	8.16
4	SSYVGDEASSK	327,130	6.72
5	AAAPEPETETETSSK	214,102	10.58
6	IVPEPQPK	439,887	5.45
7	GAIETEPAVK	425,376	6.71
8	FHPGTDEGDYQVK (doubly protonated)	47,720	12.64
8	FHPGTDEGDYQVK (triply protonated)	189,429	6.80
9	VGYDLPGK	465,624	5.84
10	SAGGAFGPELSK	380,699	7.11
11	TASEFESAIDAQK	139,535	10.13
12	GVNDNEEGFFSAK	91,996	9.27
13	VGLFAGAGVGK	490,728	5.45
14	TQLIDVEIAK	326,305	7.81
15	LTVLESLSK	489,212	7.45
16	LAPDLIVVAQTGGK	402,835	9.19
17	LTIAPALLK	779,136	6.47
18	ILTDIVGPEAPLVK	232,130	10.66
19	LTIEEFLK	481,986	8.02
20	TSAESILTTGPVVPVIVVK	45,598	19.49
21	ISSIDLSVLDSPLIPSATTGTSK	18,538	29.63
22	AGLEFGTTPEQPEETPLDDLAETDFQTFSGK	11,655	37.10
23	VVSLPDFFTFSK	273,887	12.55
24	AVTTLAEAVVAATLGPK	78,396	15.12
25	IAFFESSFLSYLK	25,427	28.64
26	SSIPVFGVDALPEALALVK	40,144	17.86
27	FLSSPFAVAEVFTGIVGK	14,172	21.51

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