Supplementary

Protein extracted from Pfu cell paste extract:
The total soluble protein extracted per 100 g of Pfu cell paste was 7g. By combining the extracts from multiple 100g batches a single large batch was generated and then divided into a large number of identical 500 mg vials. This large scale production was done to ensure a consistent supply of the Agilent Complex Proteomics Standard (CPS) for many years.

Reconstitution of lyophilized Pfu-based Complex Proteomics Standard:
Vials of the CPS were shipped to a distant location where they were reconstituted and the protein concentration was determined. The lyophilized material reconstituted completely in 50 ul of water resulting in a clear solution. The measured concentration was 10.2 (+/- 0.2) mg/ml demonstrating that essentially all of the protein lyophilized from the original 10 mg/ml extract was recovered in soluble form.

The stability of samples stored under conditions commonly encountered by 'working stocks' were assessed. CPS vials were reconstituted and stored for two weeks at 4C followed by ten additional weeks at -80C during which they were thawed and refrozen twice. There was no discernable change in the protein profile as assessed by SDS-PAGE (Figure S1), HPLC, or MS profiling (data not shown).

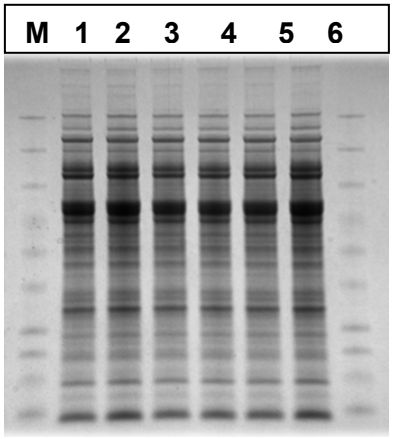

Figure S1: Production Lot stability after reconstitution and freeze-thaw cycles.

Proteins from organisms that naturally grow at extremely elevated temperatures, such as Pfu, are noted to be more resistant to degradation at lower temperatures than proteins from non-thermophiles. It is therefore not surprising, though advantageous, that Pfu extracts are stable when stored lyophilized or in solution at 4C.

Table S1. Numbers of identified *Pyrococcus furiosus* proteins, peptides, and spectra in five protein fractionation experiments using a QTOF mass spectrometer. False discovery rates are estimated to be below 0.5%.

	Number of identified proteins	**Number of identified peptides**	**Number of identified spectra**
25pfu-pepOGE	866	6980	28397
Long-new-pfu-oge	993	13377	42988
New65-pfu-oge	890	10415	51394
XL-new-pfu-oge	962	12188	51895
New-pfu-oge	831	6497	15936

Table S2. *Pyrococcus furiosus* proteins, peptides and features using different sample preparation and directed MS-sequencing.

Sample Preparation (MS-Method)[1]	Proteins[2]	Peptides[3]	Features[4]
TCA (DDA)	878	5781	28535
TCA (INL)	892	6028	13876
Acetone (DDA)	938	7745	21869
Acetone (INL)	948	7958	10553

[1] The sample preparation protocol (either trichloric acid (TCA) or acetone) as well as the LC-MS method employed (data-dependent analysis (DDA) or directed inclusion list sequencing (INL)) is indicated.

[2] Accumulative number of proteins identified by ProteinProphet at a FDR below 1%.

[3] Accumulative number of peptides identified by PeptideProphet at a FDR below 1%.

[4] For DDA MS-method: Number of features detected by the SuperHirn software tool in at least 2 of the 3 DDA LC-MS runs. For INL MS-method: Number of unidentified features targeted in subsequent directed LC-MS/MS runs.

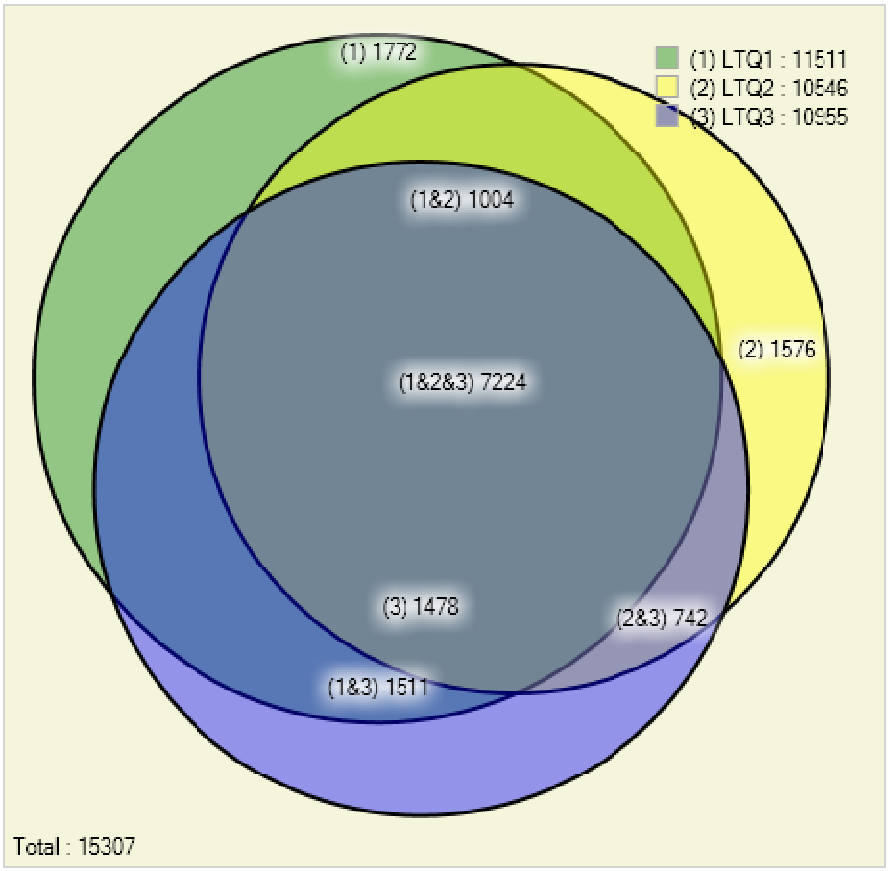

Figure S2. Comparison of the number of identified *Pyrococcus furiosus* peptides in three replicate 12-step MudPIT experiments on an LTQ mass spectrometer, at a false discovery rate of below 0.5%.

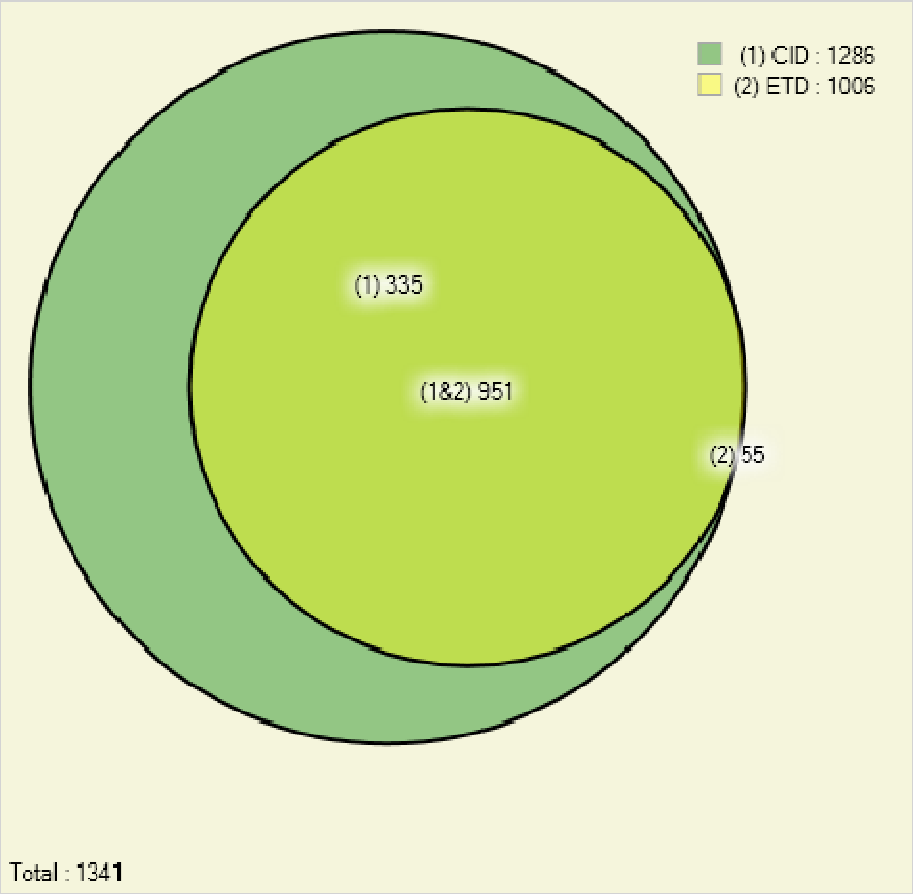

Figure S3. Comparison of the number of identified *Pyrococcus furiosus* proteins in 12-step MudPIT experiments using CID and ETD fragmentation methods on an Orbitrap-LTQ mass spectrometer, at a false discovery rate of below 0.5%. The CID fragmentation method results in a superior coverage of the *Pyrococcus furiosus* proteome.

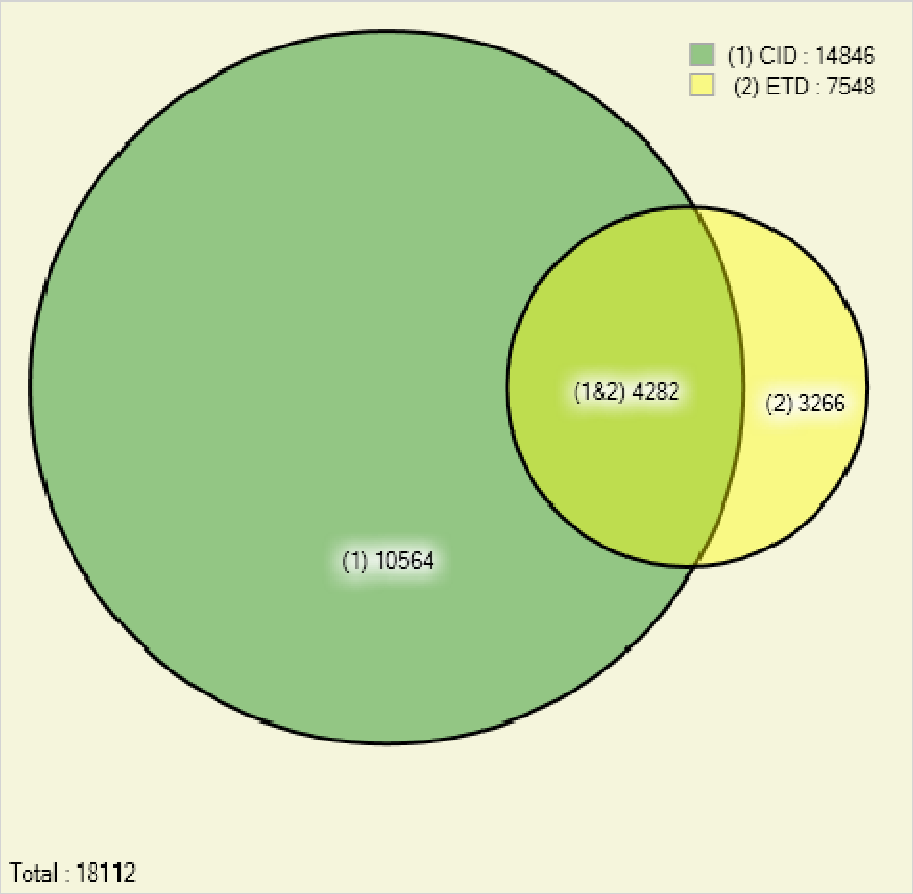

Figure S4. Comparison of the number of identified *Pyrococcus furiosus* peptides in 12-step MudPIT experiments using CID and ETD fragmentation methods on an Orbitrap-LTQ mass spectrometer, at a false discovery rate of below 0.5%. The ETD fragmentation method is complementary to the CID method, resulting in the identification of 31% (3,266) additional peptides.

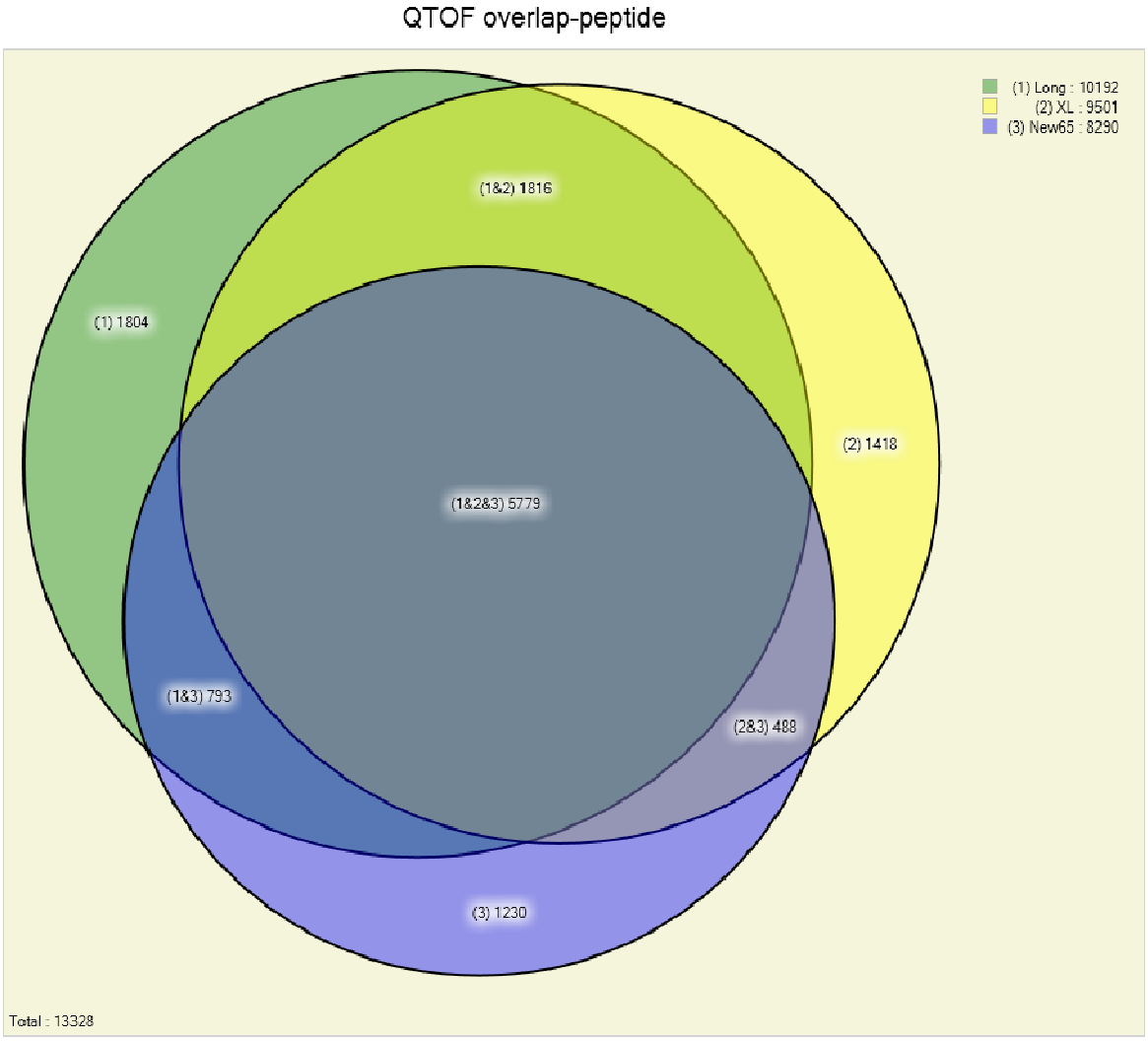

Figure S5. Comparison of the number of identified *Pyrococcus furiosus* peptides in three replicate off-line fractionation experiments on a QTOF mass spectrometer, at a false discovery rate of below 0.5%.

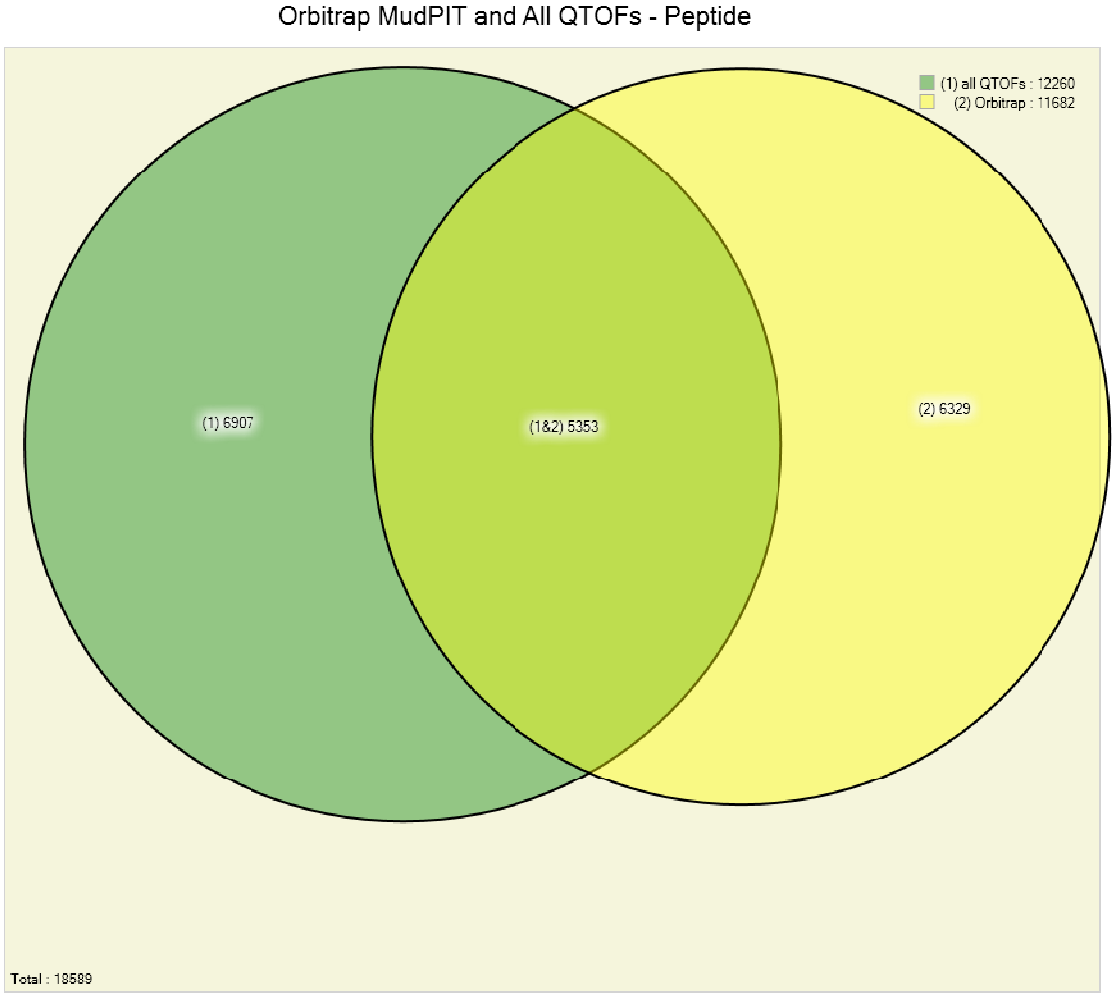

Figure S6. Comparison of the total number of identified *Pyrococcus furiosus* peptides in protein fractionation experiments on a QTOF mass spectrometer and a 12-step MudPIT experiment on an Orbitrap-LTQ mass spectrometer, at a false discovery rate of below 0.5%.

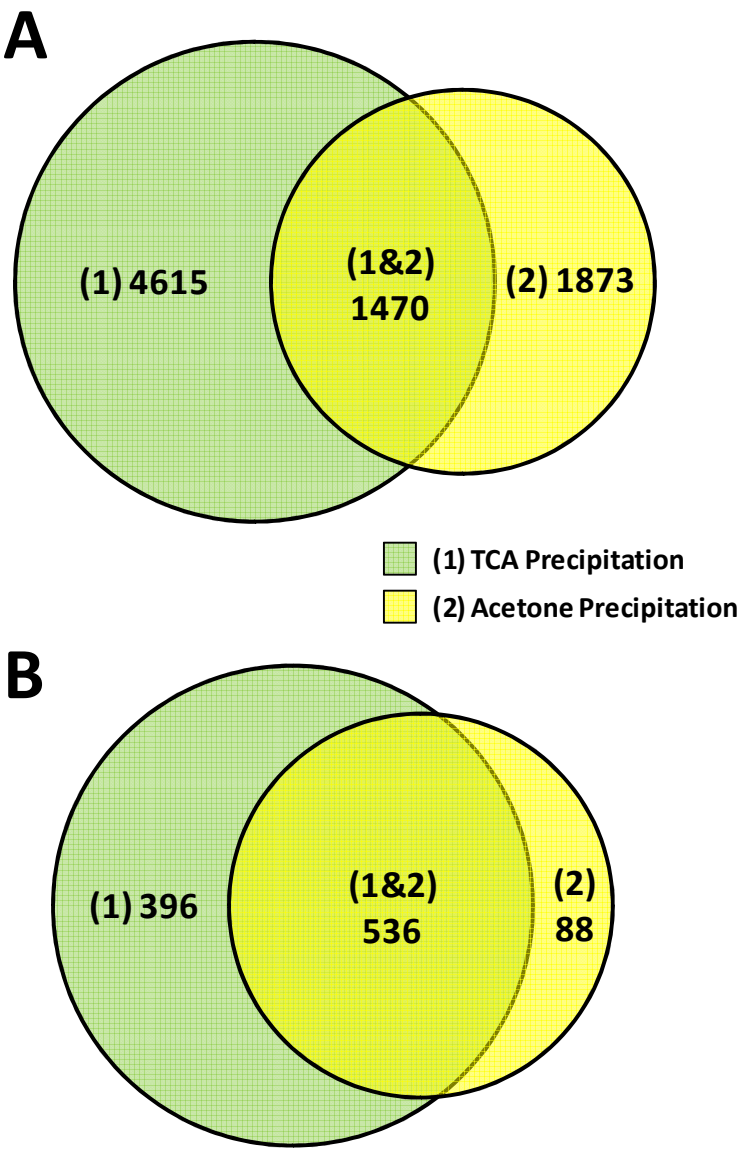

Figure S7. Comparison of the number of identified *Pyrococcus furiosus* proteins and peptides applying two different protein precipitation protocols. (A) Number of unique and common peptide identifications obtained from the TCA (1) and acetone (2) precipitated sample. (B) Like (A) for protein identifications.