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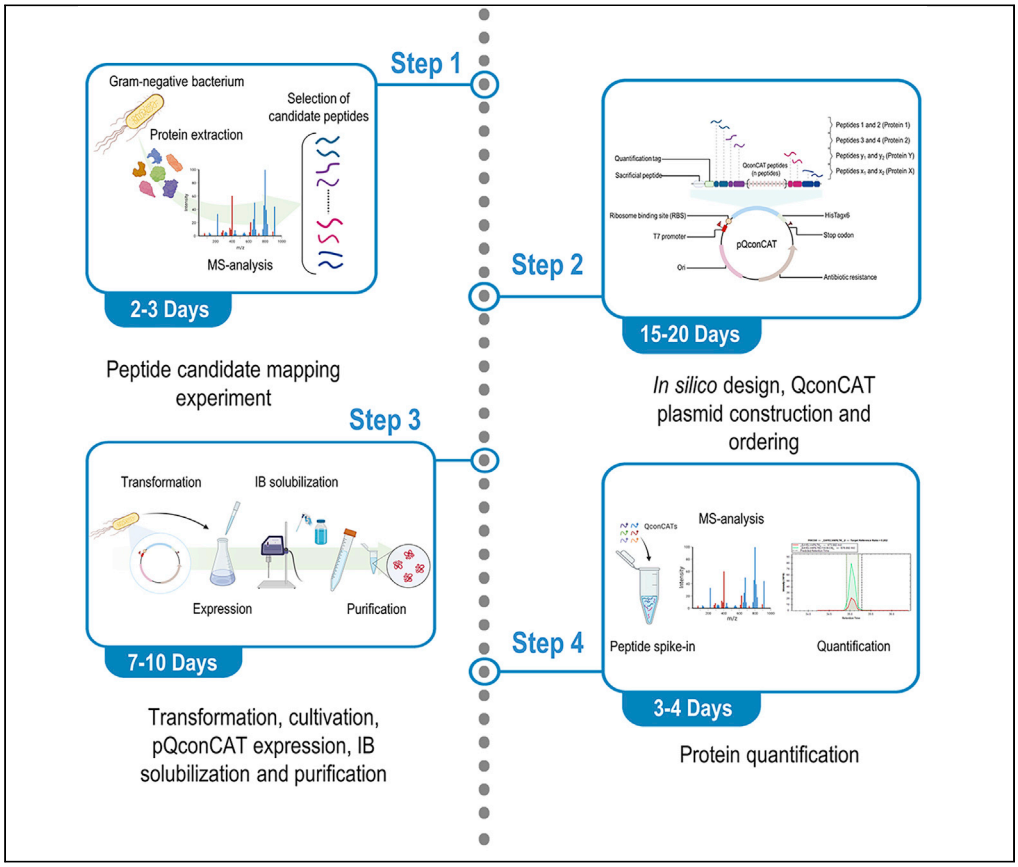
Protocol for absolute quantification of proteins in Gram-negative bacteria based on QconCAT-based labeled peptides



Mass-spectrometry-based absolute protein quantification uses labeled quantification concatamer (QconCAT) as internal standards (ISs). To calculate the amount of protein(s), the ion intensity ratio between the analyte and its cognate IS is compared in each biological sample. The present protocol describes a systematic workflow to design, produce, and purify QconCATs and to quantify soluble proteins in *Pseudomonas putida* KT2440. Our methodology enables the quantification of detectable peptide and serves as a versatile platform to produce ISs for different biological systems.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

The workflow includes the expression and purification of peptide concatamers (QconCATs)

Systematic protocol for the selection of unique peptides

Mass spectrometry was used for quantitative sample analysis

Absolute quantification of proteins using a customized Python script

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Protocol for absolute quantification of proteins in Gram-negative bacteria based on QconCAT-based labeled peptides

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SUMMARY

Mass-spectrometry-based absolute protein quantification uses labeled quantification concatamer (QconCAT) as internal standards (ISs). To calculate the amount of protein(s), the ion intensity ratio between the analyte and its cognate IS is compared in each biological sample. The present protocol describes a systematic workflow to design, produce, and purify QconCATs and to quantify soluble proteins in *Pseudomonas putida* KT2440. Our methodology enables the quantification of detectable peptide and serves as a versatile platform to produce ISs for different biological systems.

BEFORE YOU BEGIN

The protocol below describes the steps needed to conduct an integral, quantitative proteomics analysis of the model soil bacterium *Pseudomonas putida* KT2440.[1–4] This workflow (Figure 1) can be likewise applied to different Gram-negative bacteria to quantify the protein content in the bacterial biomass. Regardless of the microbial host selected, an effective method for protein extraction is required to isolate the greatest number of proteins possible[5,6]—subjected to the specific experimental conditions employed.[7,8] In the last two decades, most of the studies developed to this end have applied qualitative strategies to explore the total bacterial proteome under different conditions (often, through direct comparison to a reference sample) by calculating relative ratios or fold changes in protein content.[9–11] This methodology, however, could potentially lead to biases due to the fact that relative quantification cannot explicitly provide the actual polypeptide concentrations, given that proteins are present at contents that vary in orders of magnitude (e.g., femto-, pico-, nano- or micromoles of protein per gram/number of cells).[12] To address this challenge, the asymmetrical distribution of protein concentrations in the cell could be quantified by targeted proteomics methods, capable of accurately determining the amount of specific proteins.[13] Here, quantification can be accomplished by incorporating labeled proteotryptic peptides—adopted as internal standards—in the samples, allowing the user to calculate the absolute concentration of a selected set of proteins.

Reagent preparation

Timing: 6–8 h

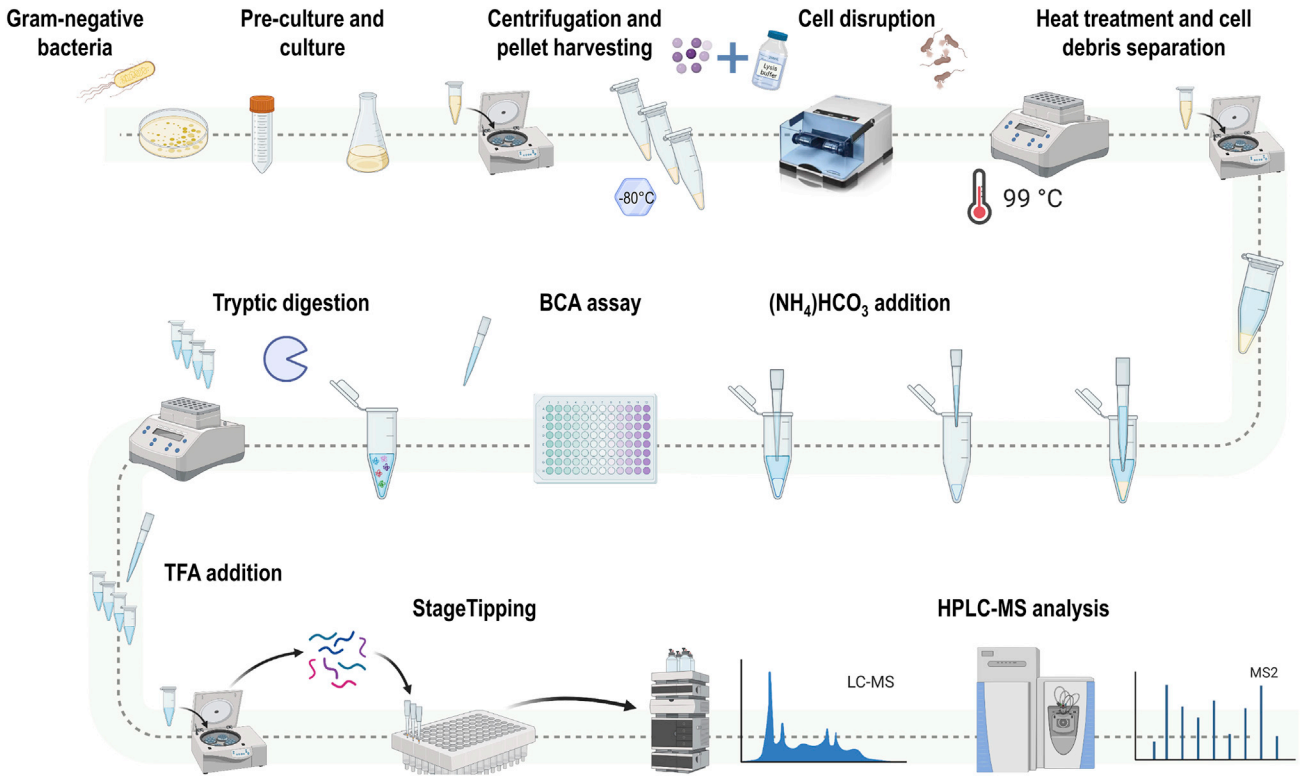

Figure 1. Workflow for the selection of candidate peptides

The scheme represents a detailed step-by-step procedure to obtain peptide candidates used in the design of the pQconCAT plasmid.

Note: This protocol has been used to express and purify unique peptides (i.e., peptides that are present in only one protein of a proteome of interest) from *Pseudomonas putida* (in this protocol, strain KT2440)[14,15] in *Escherichia coli* BL21(DE3) Δ*lysA* Δ*argH*. This strain is an L-lysine and L-arginine auxotroph derivative of *E. coli* BL21(DE3), commonly used for protein production,[16] and will only grow in a minimal medium when the corresponding amino acids are supplemented to the cultures.

The day before the "peptide candidate mapping experiment":

1. Prepare the following media: 2×YT and LB agar plates (with and without antibiotics as needed).
2. Make antibiotic and IPTG stock solutions.
3. Prepare lysis buffer, ammonium bicarbonate solution, resuspension buffer, Ni-resin equilibration buffer, elution buffer, phosphate buffer, SDS-PAGE buffer and [Glu1]-fibrinopeptide B solution.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
Bicinchoninic acid (BCA) assay kit	VWR	Cat#89167-792
Bacteria strains		
Escherichia coli BL21 DE3 Δ*lysA* Δ*argH*	Denmark Technical University, The Novo Nordisk Foundation Center for Biosustainability	This work
Pseudomonas putida KT2440	ATCC	Cat#47054

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Yeast extract	Sigma-Aldrich	CAS# 8013-01-2
Bacto yeast extract	Thermo Fisher	Cat#212750
Tryptone	Sigma-Aldrich	CAS# 91079-40-2
Bacto tryptone	Thermo Fisher	Cat#211705
NaCl	Sigma-Aldrich	CAS# 7647-14-5
Calcium chloride dihydrate ($CaCl_2 \cdot H_2O$)	Sigma-Aldrich	CAS# 10043-52-4
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	Sigma-Aldrich	CAS# 10034-99-8
Pyridoxine hydrochloride	Sigma-Aldrich	CAS# 58-56-0
Thiamine hydrochloride	Sigma-Aldrich	CAS# 67-03-8
Riboflavin	Sigma-Aldrich	CAS# 83-88-5
Nicotinic acid	Sigma-Aldrich	CAS# 59-67-6
Calcium D-(+)-pantothenate	Sigma-Aldrich	CAS# 137-08-6
p-Aminobenzoic acid	Sigma-Aldrich	CAS# 150-13-0
Thioctic acid	Sigma-Aldrich	CAS# 1077-28-7
Biotin	Sigma-Aldrich	CAS# 58-85-5
Folic acid	Sigma-Aldrich	CAS# 59-30-3
Vitamin B12	Sigma-Aldrich	CAS# 68-19-9
Disodium EDTA	Sigma-Aldrich	CAS# 6381-92-6
Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$)	Sigma-Aldrich	CAS# 7446-20-0
Manganese chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$)	Sigma-Aldrich	CAS# 13446-34-9
Cobalt(II) chloride hexahydrate ($CoCl_2 \cdot 6H_2O$)	Sigma-Aldrich	CAS# 7791-13-1
Copper(II) chloride dehydrate ($CuCl_2 \cdot 2H_2O$)	Sigma-Aldrich	CAS# 10125-13-0
Disodium molybdate dehydrate ($Na_2MoO_4 \cdot 2H_2O$)	Sigma-Aldrich	CAS# 10102-40-6
Iron sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$)	Sigma-Aldrich	CAS# 7782-63-0
Boric acid (H_3BO_3)	Sigma-Aldrich	CAS# 10043-35-3
Potassium iodide (KI)	Sigma-Aldrich	CAS# 7681-11-0
Disodium hydrogen phosphate (Na_2HPO_4)	Sigma-Aldrich	CAS# 7558-79-4
Potassium phosphate, monobasic (KH_2PO_4)	Sigma-Aldrich	CAS# 7778-77-0
Ammonium chloride (NH_4Cl)	Sigma-Aldrich	CAS# 12125-02-9
Agar	Sigma-Aldrich	CAS# 9002-18-0
Kanamycin monosulfate	Sigma-Aldrich	CAS# 25389-94-0
Guanidinium hydrochloride (GuHCl)	Sigma-Aldrich	CAS# 50-01-1
Tris(2-carboxyethyl)phosphine hydrochloride (TCEP)	Sigma-Aldrich	CAS# 51805-45-9
2-Chloroacetamide (CAA)	Sigma-Aldrich	CAS# 79-07-2
Tris(hydroxymethyl)aminomethane hydrochloride (Tris·HCl)	Sigma-Aldrich	CAS# 1185-53-1
Ammonium bicarbonate (NH_4HCO_3)	Sigma-Aldrich	CAS# 1066-33-7
Trifluoroacetic acid (TFA)	Sigma-Aldrich	CAS# 76-05-1
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Sigma-Aldrich	CAS# 367-93-1
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	CAS# 60-00-4
D,L-Dithiothreitol (DTT)	Sigma-Aldrich	CAS# 3483-12-3
Urea	Sigma-Aldrich	CAS# 57-13-6
Sodium phosphate dibasic heptahydrate ($Na_2HPO_4.7H_2O$)	Sigma-Aldrich	CAS# 7782-85-6
Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$)	Sigma-Aldrich	CAS# 10049-21-5

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Imidazole	Sigma-Aldrich	CAS# 288-32-4
Trypsin and LysC digestion mix	Thermo Fischer	Cat#A40007
Pierce™ Universal Nuclease for Cell Lysis	Thermo Fischer	Cat#88700
4× Laemmli sample buffer	Bio-Rad	Cat#1610747
4× 2-Mercaptoethanol	Sigma-Aldrich	CAS# 60-24-2
2-Amino-2-(hydroxymethyl)-1, 3-propanediol (Tris base)	Sigma-Aldrich	CAS# 77-86-1
Glycine	Sigma-Aldrich	CAS# 56-40-6
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	CAS# 151-21-3
Page Ruler Plus Prestained Protein Ladder, 10–250 kDa	Thermo Fischer	Cat#26619
Instant Blue staining	Abcam	ab119211
[Glu1]-Fibrinopeptide B	Sigma-Aldrich	CAS# 103213-49-6
Water for chromatography (LC-MS Grade) LiChrosolv®	Sigma-Aldrich	CAS# 7732-18-5
Deposited data		
Pseudomonas putida KT2440 soluble protein quantification	This paper	https://github.com/biosustain/QconCATquantSTAR
Recombinant DNA		
pQconCAT plasmid	This paper	N/A
Software and algorithms		
Proteome Discoverer	Thermo Fisher	https://www.thermofisher.com/us/en/home/industrial/mass-spectrometry/liquid-chromatography-mass-spectrometry-lc-ms/lc-ms-software/multi-omics-data-analysis/proteome-discoverer-software.html v2.4
Python version 3.10.5	Python Software Foundation	https://www.python.org
Other		
3 mm zirconium oxide beads	Glen Mills	Cat#7361-003000
2 mm solid-glass beads	Sigma-Aldrich	SKU 1040140500
Mixer Mill	Retsch	MM400
ThermoMixer	Eppendorf	Cat#5382000023
C18 resin	Empore	Cat#13-110-018
Dionex UltiMate 3000	Thermo Fisher	Cat#IQLAAAGABHFAPBMBFD
Orbitrap Exploris 480	Thermo Fisher	Cat#BRE725533
Thermal Mixer with 24 × 2 mL microtube block Eppendorf shaker	Thermo Fisher	Cat#13687717
250 mL Nalgene™ plastic bottle	Sigma-Aldrich	SKU B1033-4EA
Vibra-Cell sonicator	Sonic & Material Instrument	Model VCX 130
Waving shaker	VWR	Cat#10811-240
HisPur™ Ni-NTA Resin	Thermo Fisher	Cat#88222
Pierce™ Disposable Columns, 10 mL	Thermo Fisher	Cat#29924
Amicon Ultra centrifugal filters (50 mL)	Millipore	Cat#UFC901096
NanoDrop 2000 spectrophotometer	Thermo Fisher	Cat#ND-2000
PCR tube	Thermo Fisher	Cat#14-230-210
Spin microcentrifuge	VWR	Cat#521-2844
Mini-PROTEAN TGX 4%–20% resolving gel	Bio-Rad	Cat#4561096
Mini-PROTEAN tetra cell vertical gel electrophoresis system	Bio-Rad	Cat#1658004
Spatula or Knife Gel	Thermo Fisher	Cat#EI9010

MATERIALS AND EQUIPMENT

LB agar plates containing kanamycin (or any other selected antibiotic)		
Reagent	Final concentration	Amount
Yeast extract	5 g/L	5 g
Tryptone	10 g/L	10 g
NaCl	10 g/L	10 g
Agar	15 g/L	15 g
Kanamycin (1,000×)	50 μg/mL (1×)	1 mL
Deionized water	Not applicable (N/A)	Up to 1 L
Total	**N/A**	**1 L**

Note: Autoclave LB agar medium at 15 psi, 121°C–124°C for 20 min and cool down to 45°C–50°C before adding kanamycin or the selected antibiotic. These can be stored at 4°C for ~1 month.

2×YT broth		
Reagent	Final concentration	Amount
Yeast extract	10 g/L	10 g
Tryptone	16 g/L	16 g
NaCl	5 g/L	5 g
Deionized water	N/A	Up to 1 L
Total	**N/A**	**1 L**

Note: This medium can be autoclaved (at 15 psi, 121°C–124°C for 20 min) and stored at 23°C for ~6 months.

M9 medium glucose		
Reagent	Final concentration	Amount
Calcium chloride ($CaCl_2$) solution	0.1 mM	0.1 mL
Magnesium sulfate ($MgSO_4$) solution	2 mM	1 mL
Wolfe's vitamin solution (1,000×)	1×	1 mL
Trace element solution (2,000×)	1×	0.5 mL
M9 medium salts (10×)	1×	100 mL
Glucose monohydrate	40 mM	7.2 g
Double deionized water	N/A	Up to 1 L
Total	**N/A**	**1 L**

Note: This medium can be stored at 23°C for ~6 months.

Calcium chloride solution		
Reagent	Final concentration	Amount
Calcium chloride ($CaCl_2$) dihydrate	1 M	147.01 g
Double deionized water	N/A	Up to 1 L
Total	**N/A**	**1 L**

Note: This medium can be stored at 23°C for ~12 months.

Magnesium sulfate solution		
Reagent	Final concentration	Amount
Magnesium sulfate ($MgSO_4$) heptahydrate	2 M	240.73 g
Double deionized water	N/A	Up to 1 L
Total	**N/A**	**1 L**

Note: This medium can be stored at 23°C for ~12 months.

Wolfe's vitamin solution (1,000×)		
Reagent	Final concentration	Amount
Pyridoxine hydrochloride	10.0 mg/L	10.0
Thiamine hydrochloride	5.0 mg/L	5.0
Riboflavin	5.0 mg/L	5.0
Nicotinic acid	5.0 mg/L	5.0
Calcium D-(+)-pantothenate	5.0 mg/L	5.0
p-Aminobenzoic acid	5.0 mg/L	5.0
Thioctic (α-lipoic) acid	5.0 mg/L	5.0
Biotin	2.0 mg/L	2.0
Folic Acid	2.0 mg/L	2.0
Vitamin B12	1.0 mg/L	1.0
Double deionized water	N/A	Up to 1 L
Total	**N/A**	**1 L**

Note: First, dissolve all compounds in 500 mL MQ water. Add double deionized water to a final volume of 1 L. Filter sterilize. Store the sterile solution at 4°C in the dark for ~12 months.

Trace element solution (2,000×)		
Reagent	Final concentration	Amount
Disodium EDTA	15 g/L	15
Zinc sulfate ($ZnSO_4$) heptahydrate	4.5 g/L	4.5
Manganese chloride ($MnSO_4$) tetrahydrate	0.7 g/L	0.7
Cobalt(II) chloride ($CoCl_2$) hexadrate	0.3 g/L	0.3
Copper(II) chloride ($CuCl_2$) dihydrate	0.2 g/L	0.2
Disodium molybdate (Na_2MoO_4) dihydrate	0.4 g/L	0.4
Calcium chloride ($CaCl_2$) dihydrate	4.5 g/L	4.5
Iron(II) sulfate ($FeSO_4$) heptahydrate	3.0 g/L	3.0
Boric acid (H_3BO_3)	1.0 g/L	1.0
Potassium iodide (KI)	0.1 g/L	0.1
Double deionized water	N/A	Up to 1 L
Total	**N/A**	**1 L**

Note: First, dissolve EDTA and $ZnSO_4 \cdot 7H_2O$ in 0.75 L of double deionized water and set the pH to 6.0 with 1 M NaOH. Whilst maintaining the pH at 6.0, dissolve in the other compounds one by one. When ready, set the pH to 4.0 with 1 M HCl and adjust the volume to 1.0 L. Sterilize by autoclaving (15 psi, 121°C–124°C for 20 min). Store the sterile solution at 4°C in the dark for ~12 months.

M9 medium salts (10×)		
Reagent	Final concentration	Amount
Disodium hydrogen phosphate (Na_2HPO_4)	68 g/L	10 g

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
Potassium phosphate monobasic (KH_2PO_4)	30 g/L	16 g
Sodium chloride (NaCl)	5 g/L	5 g
Ammonium chloride (NH_4Cl)	10 g/L	10 g
Double deionized water	N/A	Up to 1 L
Total	**N/A**	**1 L**

Note: This medium can be stored at 23°C for ~12 months.

Buffer A – Inclusion bodies solubilization		
Reagent	Final concentration	Amount
Tris·HCl (pH = 8.0)	20 mM	242.3 mg
EDTA	1 mM	29.2 mg
DTT	5 mM	77.1 mg
Double deionized water	N/A	Up to 100 mL
Total	**N/A**	**100 mL**

⚠ CRITICAL: D,L-Dithiothreitol (DTT) is a reducing agent that can cause skin, eye and respiratory irritation. It is highly recommended to wear appropriate protective equipment and work under the chemical hood.

Note: Buffer A can be stored at 4°C for ~12 months.

Buffer B – Inclusion bodies solubilization		
Reagent	Final concentration	Amount
Tris·HCl (pH = 8.0)	20 mM	242.3 mg
EDTA	1 mM	29.2 mg
DTT	5 mM	77.1 mg
Urea	8 M	48.05 g
Double deionized water	N/A	Up to 100 mL
Total	**N/A**	**100 mL**

⚠ CRITICAL: Urea can cause irritation to the skin, eyes, and respiratory tract. Urea is harmful if swallowed or inhaled.

Note: Buffer B can be stored at 4°C for ~12 months.

Phosphate buffer (pH = 7.4)		
Reagent	Final concentration	Amount
Sodium phosphate dibasic heptahydrate ($Na_2HPO_4 \cdot 7H_2O$)	1 M	20.21 g
Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$)	1 M	3.39 g
Double deionized water	N/A	Up to 100 mL
Total	**N/A**	**100 mL**

Note: Phosphate buffer can be stored for at least 2 years at 23°C.

Resuspension buffer		
Reagent	Final concentration	Amount
1 M phosphate buffer (pH = 7.4)	20 mM	2 mL
NaCl	300 mM	1.75 g
Imidazole	20 mM	0.136 g
Double deionized water	N/A	Up to 100 mL
Total	**N/A**	**100 mL**

Note: Resuspension buffer can be stored for at least 2 years at 4°C protected from light.

⚠ CRITICAL: Imidazole may form combustible dust concentrations in the air. It is harmful if swallowed and can cause severe skin burns and eye damage; it may also cause respiratory irritation. Personal protective equipment (e.g., goggles, laboratory coat and gloves) must be used when handling this compound.

Equilibration buffer Ni-resin		
Reagent	Final concentration	Amount
1 M phosphate buffer (pH = 7.4)	20 mM	2 mL
NaCl	300 mM	1.75 g
Imidazole	20 mM	0.136 g
Urea	8 M	48.05 g
Double deionized water	N/A	Up to 100 mL
Total	**N/A**	**100 mL**

Note: Equilibration buffer Ni-resin can be stored for at least 2 years at 4°C protected from light.

Elution buffer Ni-resin		
Reagent	Final concentration	Amount
1 M phosphate buffer (pH = 7.4)	20 mM	1 mL
NaCl	300 mM	0.825 g
Imidazole	500 mM	1.7 g
Urea	8 M	24.02 g
Double deionized water	N/A	Up to 50 mL
Total	**N/A**	**50 mL**

Note: Elution buffer Ni-resin can be stored for at least 2 years at 4°C protected from light.

Sample exchange buffer		
Reagent	Final concentration	Amount
1 M phosphate buffer (pH = 7.4)	20 mM	2 mL
Double deionized water	N/A	Up to 1 L
Total	**N/A**	**1 L**

Note: Sample exchange buffer can be stored for at least 2 years at 23°C.

SDS-PAGE buffer 10×		
Reagent	Final concentration	Amount
Tris base	30.3 g/L	30.3 g
Glycine	144.4 g/L	144.4 g
SDS	10 g/L	10 g
Double deionized water	N/A	Up to 1 L
Total	**N/A**	**1 L**

Note: SDS is a detergent, do not shake the mixture to avoid formation of foam.

⚠ CRITICAL: Sodium dodecyl sulfate (SDS) can cause skin, eye, and respiratory irritation. Avoid inhalation of dusts, substance contact, and keep away from heat and sources of ignition. Personal protective equipment (e.g., goggles, laboratory coat and gloves) must be used when handling this compound.

Note: This medium can be stored at 23°C for ~12 months.

[Glu1]-Fibrinopeptide B solution		
Reagent	Final concentration	Amount
[Glu1]-Fibrinopeptide (EGVNDNEEGFFSAR)	0.1 μg/μL	100 μg
LC-MS grade water	N/A	Up to 1 mL
Total	**N/A**	**1 mL**

Note: [Glu1]-Fibrinopeptide B solution must be stored at –20°C. Storage time ~2 years.

Ammonium bicarbonate solution		
Reagent	Final concentration	Amount
Ammonium bicarbonate (NH_4HCO_3)	50 mM	198 mg
LC-MS grade water	N/A	Up to 50 mL
Total	**N/A**	**50 mL**

Note: The ammonium bicarbonate solution can be stored for at least 2 years at 23°C.

Tris·HCl buffer (pH = 8.5)		
Reagent	Final concentration	Amount
Tris·HCl	1 M	7.88 g
LC-MS grade water	N/A	Up to 50 mL
Total	**N/A**	**50 mL**

Note: Adjust the pH of the solution to 8.5 with 5 M NaOH. The solution can be stored at 23°C for at least 2 years.

Lysis buffer		
Reagent	Final concentration	Amount
Gu·HCl	6 M	28.66 g

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Reagent	Final concentration	Amount
TCEP	5 mM	71.7 mg
CAA	10 mM	46.7 mg
Tris·HCl (pH = 8.5)	100 mM	5 mL
LC-MS grade water	N/A	Up to 50 mL
Total	**N/A**	**50 mL**

⚠ CRITICAL: Guanidinium hydrochloride (Gu·HCl) is harmful if swallowed. It can also cause eye and skin irritation. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) is corrosive and can cause severe skin and eye burns. 2-Chloroacetamide (CAA) is toxic upon ingestion and may cause an allergic skin reaction. Use appropriate protective personal equipment (e.g., goggles, laboratory coat and gloves) when working with these substances.

Note: This medium can be stored at 4°C for ~6 months.

Alternatives: Chemical compounds produced by manufacturers other than the ones specified in the key resources table are adequate substitutes, where and whenever are of highest purity, sequence grade, and LC-MS grade. The enzymes used in this protocol (Trypsin/LysC digestion mix and Pierce™ Universal Nuclease for Cell Lysis) should be purchased using the same vendor to ensure workflow reproducibility.

HPLC-MS and data analysis settings

For sample analysis, utilize a Dionex UltiMate 3000 coupled to an Orbitrap Exploris 480. Capture the sample on a pre-column (μ-precolumn C18 PepMap 100, 5 μm, 100 Å) at a flow rate of 10 μL/min. Separate the peptides on a 15 cm C18 easy spray column (PepMap RSLC C18 2 μm, 100 Å, 150 μm × 15 cm) at a flow rate of 1.2 μL/min, with an applied gradient from 4% (v/v) acetonitrile in water with 0.1%–76% (v/v) formic acid over a total of 60 min. Operate the instrument in data-dependent mode using the following settings: MS-level scans performed with resolution set to 120,000; AGC target of 3.0×10^6; maximum injection time of 50 ms; intensity threshold of 5.0×10^3; and dynamic exclusion at 25 s. Perform data-dependent MS2 selection in Top 20 Speed mode with HCD collision energy set to 28% (AGC target 1.0×10^4; maximum injection time of 22 ms; and isolation window 1.2 m/*z* with a resolution of 30,000).

For analysis of the .RAW files, run Proteome Discoverer v2.4 (Thermo Fisher Scientific, Waltham, MA, USA) with the following settings. Dynamic modifications: carbamidomethyl of cysteine residues and oxidation of methionine residues; label ^{13}C(6) in arginine and lysine residues; precursor mass tolerance set to 10 ppm; fragment mass tolerance at 0.02 Da; trypsin (full) as digestion enzyme, allowing for a maximum of two missed cleavages; minimum peptide length of 6; maximum peptide length of 144; and the false-discovery rate (FDR) set at 0.1%.

STEP-BY-STEP METHOD DETAILS

The first part of the protocol involves the selection of several unique peptides used in the design of the quantification concatamers (QconCATs) for absolute protein determination. QconCATs are implemented to precisely quantify the content of desired proteins not only in bacteria but also in any biological system by slightly adjusting the protein extraction procedure. The presented protocol includes all the necessary steps—from the *in silico* design to the calculation of the *in vivo* protein concentrations—to carry out an end-to-end workflow in Gram-negative bacteria (e.g., *P. putida* or *E. coli*).

Peptide candidate mapping experiment

Timing: 2–3 days

1. Grow the pre-culture of the selected Gram-negative bacterial strain for ~16 h at its optimal growth condition in a 50-mL Falcon tube containing 10 mL of 2×YT medium. Use colonies from a freshly-inoculated LB agar plate.

   Note: *E. coli* should be grown at 37°C and *P. putida* cultures are incubated at 30°C.

2. Re-inoculate a 250-mL Erlenmeyer flask containing 50 mL of 2×YT medium by diluting the pre-culture 100× (500 µL).
3. When the culture reaches mid-exponential phase (roughly equal to $0.5 \times OD_{600}^{max}$, i.e., half the maximum optical density measured at 600 nm) or ~0.4–0.5 mg of total cell dry weight.
4. Harvest 1 mL of the suspension by centrifugation at 10,000 g for 10 min at 4°C.
5. Remove the supernatant and freeze the cell pellets at –80°C until sample preparation.
6. Thaw the cell pellets on ice. Add two 3-mm zirconium oxide beads to each sample, and 100 µL of lysis buffer (6 M Gu·HCl [guanidinium hydrochloride], 5 mM TCEP [tris(2-carboxyethyl)phosphine], 10 mM CAA [2-chloroacetamide] and 100 mM Tris·HCl, pH = 8.5) to the samples.
7. Disrupt the cell suspension in a Mixer Mill set at 25 Hz for 5 min at ~20°C. Heat the sample for 10 min in a ThermoMixer at 99°C and 1,800 rpm.
8. Remove the cell debris by centrifugation at 15,000 *g* for 10 min at 23°C and collect 50 µL of supernatant into a new Eppendorf tube. Dilute the cell extract with 50 µL of 50 mM $(NH_4)HCO_3$ (ammonium bicarbonate).
9. Quantify protein concentration *via* the bicinchoninic acid (BCA) assay[4,17] and take 20 µg of protein for tryptic digestion into a new Eppendorf tube.
10. Dilute the tryptic digestion to reach a total volume of 100 µL by using the appropriate amount of 50 mM $(NH_4)HCO_3$ solution.

    Note: The expected protein concentration might vary according to the organism under study, carbon source and culture conditions. For a typical bacterial culture at OD_{600} = 1.0, the estimated protein concentration is ~2 µg/µL.

11. Add 20 µL of 0.1 µg/µL trypsin and LysC digestion mix (ratio 1:1). Initiate the tryptic digestion at constant shaking (400 rpm) for 8 h and 37°C.

    Note: Ensure at least a 4× dilution to avoid interferences between the Gu·HCl present in the lysis buffer and the digestion enzymes.

12. Add 10 µL of 10% (w/v) trifluoroacetic acid (TFA) to stop the tryptic digestion. Vortex and centrifuge the samples at 15,000 *g* for 15 min at 23°C (See problem 3 in troubleshooting).
13. Perform StageTipping, using C18 resin (solid phase extraction disk) to desalt the samples prior to HPLC-MS analysis. For a detailed explanation on StegeTipping desalting process, please refer to Rappsilber et al.[18] for further details.
14. HPLC-MS analysis of the samples is performed using a Dionex UltiMate 3000 coupled to an Orbitrap Exploris 480 operated using data-dependent acquisition. The data analysis of the .RAW files is executed using Proteome Discoverer. Further details on HPLC-MS and data analysis can be found in the materials and equipment section; please also refer to Wirth et al.[19] and Kozaeva et al.[20] for details.

The experimental workflow to build the peptide list is shown in Figure 2.

Note: Only consider unique peptides for quantification. Assign the detected peptides to their functions using a protein database consisting of the reference Gram-negative bacteria proteome. If necessary, supplement heterologously expressed proteins by adding those proteins and sequences to the FASTA file obtained from the database.

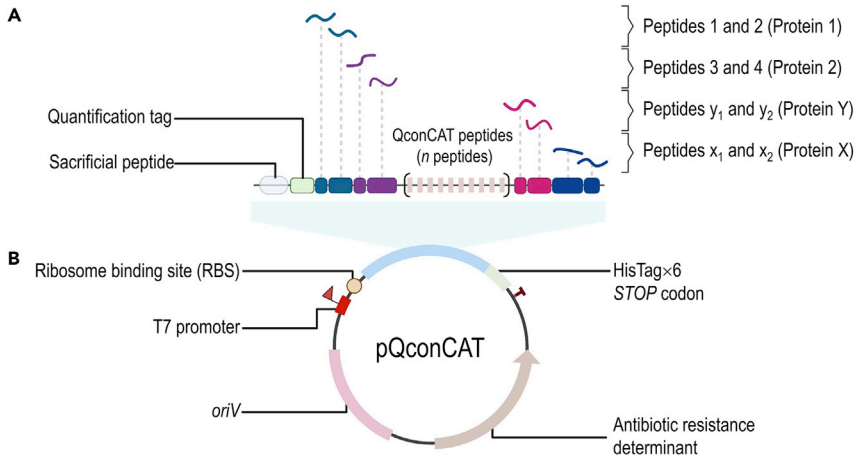

Figure 2. Standard design of the inducible pQconCAT plasmid to produce labeled peptides for absolute protein quantification

(A and B) The plasmid encompasses different parts that allow the production of the desired protein: (A) concatenated peptides (*n* peptides) selected in "peptide candidate mapping experiment" and (B) a T7 RNA polymerase promoter region, ribosome binding site (RBS), quantification tag (e.g., [Glu1]-fibrinopeptide B), sacrificial peptide, hexahistidine purification tag (HisTag×6), stop codon, antibiotic resistance determinant and origin of vegetative replication (*oriV*). This design allows for the production of an equimolar (1:1) amount of QconCAT peptides, which can be added in known quantities to the sample. Then, the amount of each represented peptide standard can be calculated based on the [Glu1]-fibrinopeptide B, serving as a reference to accurately quantify proteins.

15. From the peptide candidate list obtained, select the peptides using the following criteria:
    a. Mass peptide: select peptides ranging 350–5,000 Da in mass.

Avoid proline (P) residues on the carboxyl side of the cleavage site (RP or KP) as well as acidic residues—aspartate (D) and glutamate (E)—on either side of the cleavage site due to the lower rate of hydrolysis.

16. Pick two or more proteotyptic peptides per protein with the highest fragment intensity. Please refer to Calderón-Celis et al.[21] for details.

In silico QconCAT plasmid design (pQconCAT)

Timing: 15–20 days

17. *In silico* design of pQconCAT vectors.
    a. The plasmid design should contain the following structure:
        i. T7 RNA polymerase promoter region;
        ii. Ribosome binding site;
        iii. Quantification tag (e.g., [Glu1]-fibrinopeptide B);
        iv. Sacrificial peptide;
        v. Concatenated peptides (*n* peptides) selected in "peptide candidate mapping experiment";
        vi. Hexahistidine purification tag (HisTag×6);
        vii. *STOP* codon;
        viii. Antibiotic resistance determinant;
        ix. Origin of vegetative replication (*oriV*).

        The general structure of plasmid pQconCAT is depicted in Figure 2.

Note: Optimize (optional) the QconCAT gene for codon usage in *E. coli* to facilitate production of the concatamers.

b. Order the plasmid previously designed (typical synthesis time: 15 days).

QconCAT transformation and expression

Timing: 7–10 days

18. Competent cells for pQconCAT transformation.
    a. Grow for ~16 h a pre-culture of *E. coli* BL21(DE3) Δ*lysA* Δ*argH* in 5 mL of LB medium at 37°C in a shaker at 250 rpm.
    b. Inoculate 50 mL of fresh LB medium, placed in a 250-mL Erlenmeyer flask, with 0.5 mL of the pre-culture to yield an OD_{600} ~0.05.
    c. Grow cells at 37°C in a shaker at 250 rpm until the culture reaches OD_{600} ~0.5 (~2–3 h).
    d. Place the Erlenmeyer flask to ice to prevent further bacterial growth.

    Note: From now on, steps e-k should be performed at 4°C, maintaining cells on an ice bath and using chilled tubes.

    e. Transfer the cells to a pre-chilled 50-mL Falcon conical tube.
    f. Harvest the cells in a pre-cooled centrifuge at 4°C for 10 min and 4,500 *g*. Discard the supernatant.
    g. Resuspend the pellet in 1-mL chilled, filter-sterile 10% (v/v) glycerol solution and transfer to a chilled 1.5-mL Eppendorf tube.
    h. Centrifuge for 1 min at 11,000 *g* and 4°C. Discard supernatant.
    i. Repeat steps g and h at least twice.

    Note: The pellet will become softer with every wash round. Be careful when removing the supernatant.

    j. Resuspend the cell pellet in 10% (v/v) glycerol to 1:100 volume of initial culture (i.e., 500 µL). Culture volumes can be scaled down accordingly if only a few transformations will be performed.
    k. Transfer 30–50 µL aliquots to chilled 1.5-mL Eppendorf tubes.

    Note: Cells can be flash-frozen and maintained at –80°C until transformation or used directly for electroporation.

19. pQconCAT transformation.
    a. Take competent cells *E. coli* BL21(DE3) Δ*lysA* Δ*argH* from –80°C and place them on ice.
    b. Prepare the plasmid suspension by diluting it in the tube to a concentration of 100 ng/µL using MQ water.
    c. Add ~25–50 ng of the plasmid into the cell tubes and vortex quickly one time.
    d. Put the cells and the plasmid on ice for 30 min.
    e. Place the tube into the water bath at 42°C for 60 s. After that, place them back on ice for 2 min.
    f. Add 1 mL of LB medium (without antibiotics), transfer the cells into a 2-mL Eppendorf tube and incubate the bacterial suspension for 1 h at 37°C in a Thermal Mixer with a 24 × 2 mL microtube block Eppendorf shaker at 800 rpm.
    g. Plate 50 µL of the cell suspension on LB agar plates (with the corresponding antibiotic) using pre-sterilized 2-mm glass beads.

Note: The antibiotic to be added to the plates depends on the selection marker in the plasmid used for the protein expression. Prepare a 1,000× antibiotic stock solution and dilute to 1×.

h. Centrifuge the tube containing the remaining cells at 10,000 *g* for 5 min and discard most of the medium (~900 µL). Resuspend the cells in the remainder liquid.
i. Plate the cell suspension on LB agar plates (with the corresponding antibiotic) using 2-mm glass beads and incubate the plates for ~16 h in an incubator at 37°C.
j. Pick the colonies, confirm presence of the plasmid by mini-prep and sequencing, and prepare glycerol stocks of the selected clones.

20. QconCAT expression.
    a. Inoculate pre-cultures in 50-mL Falcon tubes containing 10 mL of M9 minimal medium with 40 mM glucose, 0.1 mg/mL U^{13}C-L-lysine, 0.1 mg/mL U^{13}C-L-arginine + the selected antibiotic (1× final concentration, see above) at 37°C at 200 rpm for ~16 h (see problem 1 in troubleshooting).
    b. Dilute the pre-culture 1:100 in 100 mL of the same medium described in 3.a. placed in 500-mL Erlenmeyer flask, and incubate the culture at 37°C.
    c. When the culture reaches OD_{600} ~ 0.5–0.6, cool down the flask (in ice or cold room) until it reaches approximately ~23°C.
    d. Induce the expression of the construct borne by plasmid pQconCAT by adding IPTG to a final concentration of 1 mM and incubate for 24 h at 30°C and 200 rpm (see problem 2 in troubleshooting).

Note: The concatamer polypeptide tends to form inclusion bodies (IBs) when using this IPTG concentration to induce the expression of the construct. It is possible to optimize the IPTG concentration for different polypeptides if needed. In case that no information is available, a low IPTG concentration (e.g., 0.2 mM) is a good starting point. Please refer to Lozano Terol et al.[22] for further information.

    e. Centrifuge the culture at 3,000 *g* and 4°C for 20 min in a 250-mL Nalgene™ plastic bottle.
    f. Freeze the bacterial pellets at –20°C for 1 h to weaken the cell membrane.

Pause point: It is possible to store the pellets at –20°C gor ~16 h and continue with the rest of the procedure the next day.

21. Cell disruption, inclusion bodies solubilization and pQconCAT purification.
    a. Cell disruption:
        i. Thaw the frozen pellets and resuspend the biomass in 25 mL of resuspension buffer by vortexing. Pour the suspended pellet solution in a 50-mL Falcon tube.
        ii. Sonicate the samples using a Vibra-Cell sonicator at 65% intensity for 10 min with ON-OFF cycles of 30 s.

Note: The samples should be maintained at 4°C during the sonication.

        iii. Incubate the lysate with 2 µL of Pierce™ Universal Nuclease for Cell Lysis for at least 30 min at 23°C to digest DNA and reduce the viscosity of the sample.
        iv. Centrifuge lysates for 20 min at 16,000 *g* in a cooled centrifuge (4°C).

⚠ CRITICAL: At this point, the soluble proteins should be present in the supernatant. However, because of the nature of the polypeptide, the QconCAT will be typically recovered from IBs. These IBs will be present in the pellet (insoluble fraction) of the sample, with a negligible amount of QconCATs in the supernatant.

        v. Recover the pellet and use it in the next step for IB solubilization.

Note: The supernatant should be stored at 4°C in case that no protein is detected in the IB fraction (pellet); if this is the case, filter the supernatant using a 0.45 μm and then a 0.22 μm membrane. Continue to step 5.a. if the protein of interest is in the soluble fraction.

Pause point: The pellet can be stored at –20°C in case you need to pause the process.

⚠ CRITICAL: The solubilization buffers A and B must be prepared immediately before this step.

b. IBs solubilization:
   i. Resuspend the pellet in 2 mL of buffer A by pipetting up and down until the protein pellet is completely dissolved. Place the dissolved protein in a new 2-mL Eppendorf tube.
   ii. Centrifuge for 60 min at 4°C and 15,000 *g*. Remove the supernatant and proceed with pellet solubilization.
   iii. Solubilize the protein by adding 5–10 mL of buffer B.

Note: Do not add the whole volume of buffer B directly to the pellet; rather add 1-mL aliquots at a time to gradually dissolve the materials. Repeat this procedure until dissolving the whole insoluble fraction. Do not use more than 10 mL of the solubilization solution. Place the solution in a 15-mL Falcon tube.

   iv. Stir gently for 1 h at 4°C in a waving shaker with an inclination of 30°C.

Note: In case the pellet is not completely dissolved, it is recommended to leave the sample for ~16 h at the conditions mentioned above to complete the solubilization of the polypeptide.

   v. Centrifuge for 15 min at 4°C and 15,000 *g* to eliminate insoluble debris. Store the supernatant containing the solubilized protein. Continue to the purification step.

c. QconCAT purification:
   i. Assemble the purification Pierce™ Disposable Column by placing a filter disc in the bottom, cap the column tip and add 2 mL of HisPur™ Ni-NTA resin.

Note: Mix the resin vigorously to form a homogenous solution before adding it to the column.

   ii. Allow the resin to drain without becoming completely dry by removing the cap. Once the resin is drained, recap the column.

Note: To drain buffers/solution, remove the cap and recap once the column is drained. Place several 50-mL Falcon tubes under the column during the different intermediate steps in order to collect the fractions.

   iii. To wash and equilibrate the column, add 2 mL of equilibration buffer (no need to be gentle with the resin) and let the resin drain. Place the cap on the tip.
   iv. Load the solution containing the QconCATs on top of the resin. Collect the flow-through and repeat this step twice to increase protein recovery.

Note: It is important to keep the fraction containing the QconCATs at 4°C.

   v. Wash the resin twice with 10 mL of equilibration buffer Ni-resin.
   vi. Elute twice with 2 mL of elution buffer.
   vii. Load the eluent in a 50-mL Amicon Ultra centrifugal filters (with a cut-off of at least 10 kDa below the size of the polypeptide of interest) and proceed to exchange the buffer.

viii. Centrifuge at 4,000–7,000 *g* for 10 min at 4°C and refill the column with 4 mL of sample exchange buffer (see problem 4 in troubleshooting).
ix. Repeat the step above 3–4 times to properly exchange the buffer. Then, recover the purified proteins in a final volume of 1 mL of the buffer by pipetting up and down. Centrifuge the sample again in case that more than 1 mL is obtained.
x. Measure the protein concentration by using NanoDrop 2000 spectrophotometer. Absorbance ratio at 260 nm over 280 nm (A_{260}/A_{280}) should be around 0.6.
The schematic representation of pQconCAT transformation, pQconCAT expression, cell disruption, and inclusion bodies solubilization is presented in Figure 3. The QconCAT purification workflow is illustrated in Figure 4.

22. SDS-PAGE of purified proteins.
a. Prepare your protein samples in a 0.2-mL PCR tube by mixing the components listed in Table 1:

Note: Load ~0.2–1 μg of the purified protein in the polyacrylamide gel to get clear bands. Bigger protein amounts can lead to streaked bands, while lower a mass below 0.2 μg can result in the absence of noticeable bands in the gel.

⚠ CRITICAL: 4× 2-Mercaptoethanol is a 600 mM solution (in water); work in the chemical fume hood when preparing and handling this solution.

b. Incubate the samples at 95°C for 5 min and spin them down in a spin microcentrifuge.
c. Take a Mini-PROTEAN TGX 4%–20% resolving gel and remove the comb carefully to avoid damaging the wells. Remove the green tape at the bottom of the gel and insert it in a Mini-PROTEAN tetra cell vertical gel electrophoresis system.
d. Place the cell in the suitable position of the tank buffer. Use the 1× SDS-PAGE buffer to fill the cell volume completely and the rest of the buffer tank up to the appropriate level mark.
e. Load 10 μL of the suspension sample. Include at least one well with 2–3 μL of Page Ruler Plus Prestained Protein Ladder, 10–250 kDa.
f. Run the SDS-PAGE at 200 V for approximately 25 min.

Note: Recycle the buffer outside the electrophoresis cell as long as the dye inside the precast cell has not escaped through the bottom of the gel.

g. Open the precast gel using a spatula or gel knife, wash it with distilled water and incubate it in ~15–20 mL of Instant Blue staining (enough volume to cover the gel) with gently shaking at 23°C. Bands will be visible after 15 min and completely stained after 1 h.
h. Wash the gel to remove the dye by washing it with 30 mL of distilled water for 5 min. Repeat this step three times.

Quantification of the QconCAT

Timing: 2 days

23. For each purified QconCAT from step 21.c.x, add 10 μL to a new Eppendorf tube. Next, add 60 μL of 0.1 μg/μL quantification tag peptide [Glu1]-fibrinopeptide B as internal standard.
24. Perform tryptic digestion, StageTipping, HPLC-MS and data analysis as described in steps 6–14 of Section "peptide candidate mapping experiment".
25. Using the sequence of the quantification tag peptide and the Proteome Discoverer results file containing the "Peptide Groups", the abundance of the spiked-in quantification tag (light peptide) and the abundance of the ^{13}C-labeled quantification tag from the QconCAT (heavy peptide) can be determined. Next, the concentration of each QconCAT is calculated as shown in Equation 1:

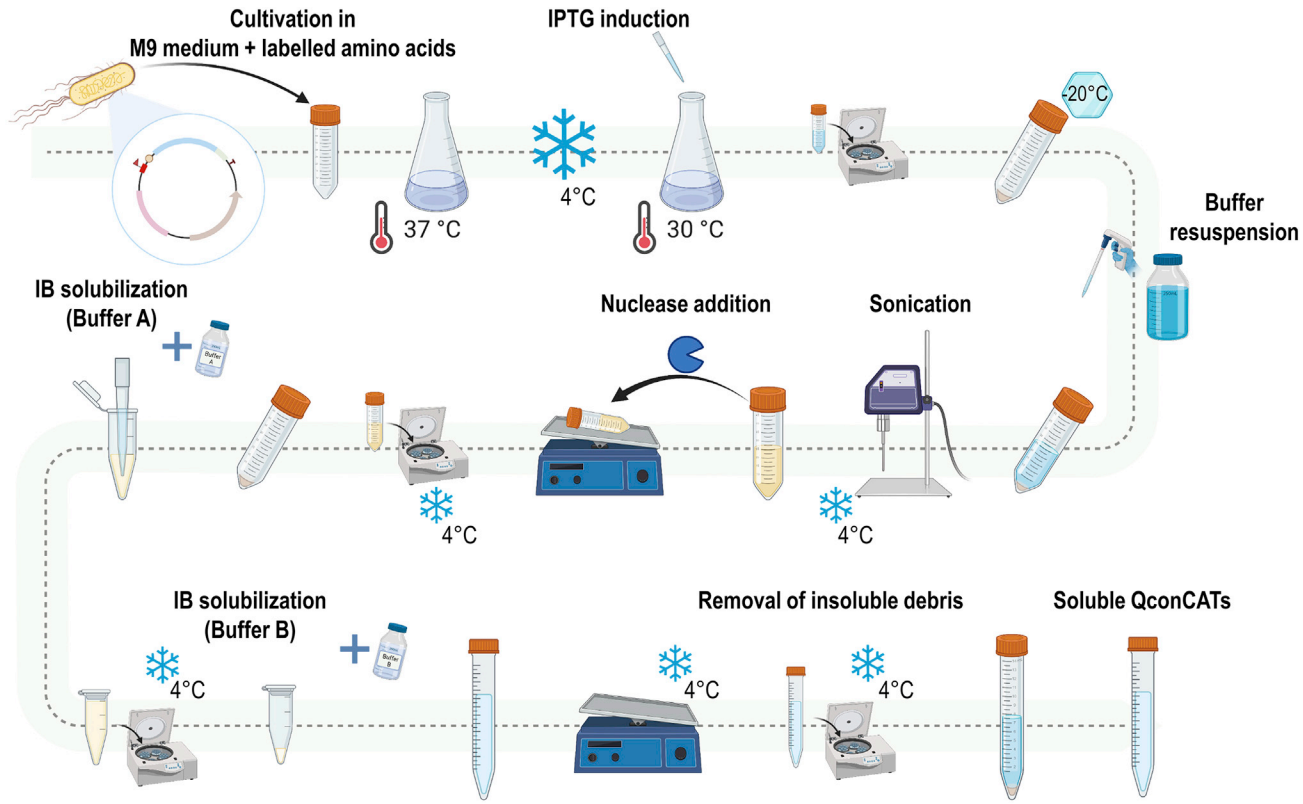

Figure 3. Cultivation procedures and expression of pQconCAT and inclusion bodies (IB) solubilization
The workflow lists the most important steps to obtain the final protein before the purification.

$$c_{QconCAT}\left(\frac{\mu g}{\mu L}\right) = c_{Qtag,light}\left(\frac{\mu g}{\mu L}\right) \cdot \frac{A_{Qtag,heavy}}{A_{Qtag,light}} \cdot \frac{MW_{QconCAT}\left(\frac{g}{mol}\right)}{MW_{Qtag,light}\left(\frac{g}{mol}\right)} \quad \text{(Equation 1)}$$

where $c_{QconCAT}$ is the concentration of the QconCAT, $c_{Qtag,light}$ is the concentration of the quantification tag peptide solution (i.e., 0.1 µg/µL in this example), $A_{Qtag,light}$ is the abundance of the spiked-in quantification tag (light peptide), $A_{Qtag,heavy}$ is the abundance of the ^{13}C-labeled quantification tag from the QconCAT (heavy peptide), $MW_{QconCAT}$ is the molecular weight of the QconCAT protein, and $MW_{Qtag,light}$ is the molecular weight of the spiked-in quantification tag, assuming equimolar concentrations of the ^{13}C-labeled quantification tag (heavy peptide) and the QconCAT protein (see problem 5 in troubleshooting).

Quantification of samples and expected results

Timing: 2 days

26. A biological sample from any experimental condition can be used for protein quantification using the QconCAT standards. Ensure that the samples (cell pellets, as described in step 1 of Section "peptide candidate mapping experiment") correspond to a total OD_{600} ranging from 1 to 2.
27. Perform cell lysis and total protein quantification following steps 6–9 of Section "peptide candidate mapping experiment".
28. Add an amount of each QconCAT protein to the samples that corresponds to approximately the mass of the endogenous proteins per 20 µg of total protein. These are the spiked-in concentrations of the QconCATs, which are required for quantification.

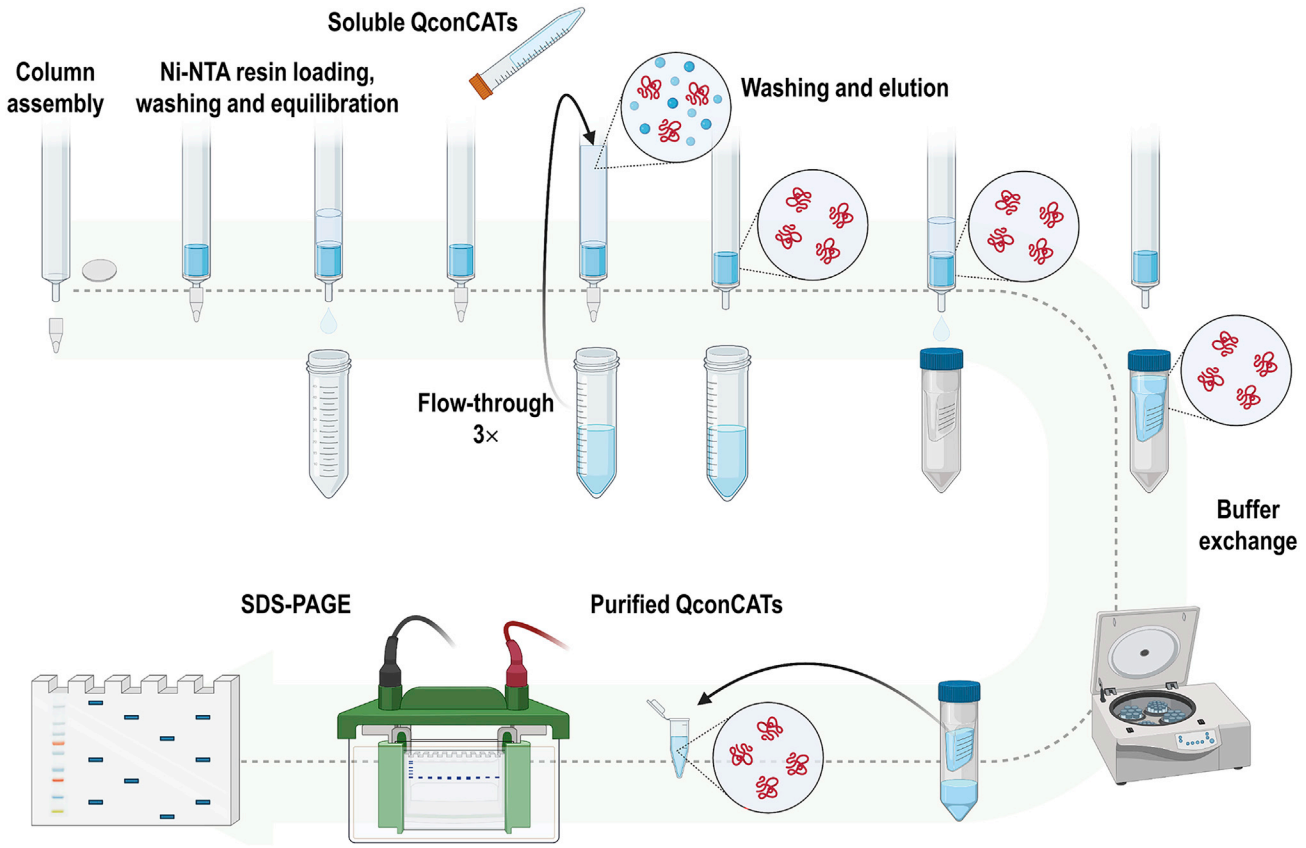

Figure 4. QconCAT purification using HisPur™ Ni-NTA Resin

Systematic procedure for QconCAT purification based on the HisTag technology.

Note: The endogenous protein quantity can be estimated from previous studies.[23] Usually, 200 fmol/μg total protein is a reasonable start point; this means that if 20 μg of protein are taken for the digestion, 4,000 fmol will be present in the sample. Hence, the QconCAT amount to spike-in would be around 4,000 fmol.

29. Perform tryptic digestion, StageTipping, HPLC-MS and data analysis as described in steps 6–8 of Section "peptide candidate mapping experiment".
30. Using the Proteome Discoverer results file containing the "Peptide Groups", the abundance of each QconCAT peptide (heavy peptide) and of each corresponding endogenous peptide (light peptide) can be determined. The concentration of the endogenous peptides, for which a QconCAT protein was constructed, are calculated as shown in Equation 2:

$$c_{pep,end} = \frac{A_{pep,end}}{A_{pep,QconCAT}} \cdot c_{pep,QconCAT} = c_{prot,end} \text{ or } c_{prot,end} = \frac{\sum c_{pep,end}}{\#\ end\ pep} \quad \text{(Equation 2)}$$

where $c_{pep,end}$ is the concentration of the endogenous peptide, $A_{pep,end}$ is the abundance of the endogenous peptide (light peptide), $A_{pep,QconCAT}$ is the abundance of the ^{13}C-labeled QconCAT peptide (heavy peptide), $c_{pep,QconCAT}$ is the concentration of the QconCAT peptide, $c_{prot,end}$ is the concentration of the corresponding endogenous protein, and $\#\ end\ pep$ is the number of endogenous peptides for which a ^{13}C-labeled QconCAT peptide was constructed, for a particular endogenous protein. The concentration of the endogenous peptide will have the same unit as the concentration of the QconCAT peptide, e.g., fmol/μg total protein. The concentration of the endogenous protein is either the same as the molar concentration of the corresponding

Table 1. Composition of protein samples for SDS-PAGE analysis

Component	Volume [μL]
Protein sample	Up to 6
4× Laemmli sample buffer	3
4× 2-Mercaptoethanol	3
Water	As needed
Final volume	12

endogenous peptide (due to equimolar concentrations of peptide and protein), or an average of the endogenous peptide concentrations when multiple peptides per endogenous protein where present in the QconCAT protein standard.

Please refer to the following repository https://github.com/biosustain/QconCATquantSTAR to calculate the peptide concentrations. Use the Python script (QconCATquantSTAR.py) to calculate those concentrations in the samples. See the *Pseudomonas putida* dataset example in the repository for a detailed outcome.

EXPECTED OUTCOMES

This protocol enables the production of labeled peptide concatamers to be used as internal standard. Following this protocol, it is possible to obtain pure QconCATs for absolute quantification of proteins in Gram-negative bacteria. Refer to Figure 5 to see the expected outcomes of the SDS-PAGE analysis of purified QconCATs.

LIMITATIONS

The main limitation is the unknown nature of the QconCAT produced in a biological (bacterial) system. As it is a construction of several peptides (chimeric protein), it is almost impossible to predict if the protein of interest will be produced in the form of soluble or insoluble protein (Inclusion body). Another limitation is that it is sometimes impossible to select two or three unique or signature peptides for each protein of interest.

TROUBLESHOOTING

Problem 1

Slow growth of the strain in minimal medium containing the labeled amino acids after 24 h (step 3.d).

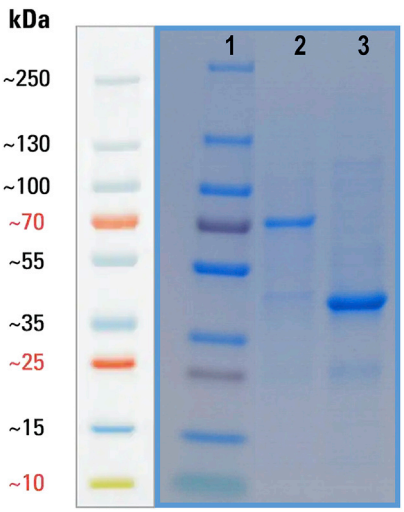

Figure 5. SDS-PAGE analysis of QconCATs

Line 1 corresponds to the Page Ruler Plus Prestained Protein Ladder, 10–250 kDa. Line 2 and 3 shows two examples of purified QconCAT proteins produced by applying this protocol.

Potential solution

Leave the culture for another 24 h and track the changes in OD_{600} of the culture; it should reach maximum OD_{600} after 48 h.

Problem 2

Poor expression of the QconCAT plasmid. This problem can be caused by (i) protein instability and/or (ii) protein toxicity.

Potential solution

In the case of (i), vary expression conditions such as temperature, aeration and induction time. For protein toxicity issues (ii), test different *E. coli* strains [e.g., BL21(DE3) pLysS or BL21(DE3) pLysE].[24]

Problem 3

Incomplete QconCAT digestion.

Potential solution

Increase amount of trypsin and modify digestion conditions in order to ensure complete proteolysis.

Problem 4

Filter blocked due to insoluble debris (step 21.c.viii.).

Potential solution

Resuspend the solution inside the filter and transfer it to a 15-mL Falcon tube. Centrifuge at 5,000 *g* for 10 min and repeat the buffer exchange from step 21.c.vii.

Problem 5

GluFib (or other tag) are not detected or identified in the analysis.

Potential solution

Repeat the analysis adding more QconCAT in the sample.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Pablo Iván Nikel (pabnik@biosustain.dtu.dk).

Materials availability

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Pablo Iván Nikel (pabnik@biosustain.dtu.dk).

Data and code availability

The published article includes figures, code and datasets generated with this protocol. The code and datasets are available on GitHub (https://github.com/biosustain/QconCATquantSTAR) and also on Zenodo (https://doi.org/10.5281/zenodo.7330244).

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AUTHOR CONTRIBUTIONS

N.G. developed, executed, optimized the protocol, and drafted the manuscript. S.K.T.P. performed sample preparation and data analysis and drafted the manuscript. M.F. executed and optimized the methodology. T.W. performed data analysis and optimized the protocol. L.K.N. and P.I.N. provided supervision and funding and contextualized and finalized the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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