Protein Expression Report

Date: 2015-03-02
Reported by: Dr. Michael Forchheim, PolyQuant GmbH
Order: Expression / Purification
^{15}N-labelled QconCat: Qcstel01

Aim:

The ^{15}N- labelled QconCat peptide is expressed in BL21(DE3)in 0.2l scale and purified by IMAC (Ion Metal Affinity Chromatography).

Construct:

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>Qcstel01
MAGRQSEPVYETTLADLQKFLWNWDKGTPTIQNAVVLLERVDINVYRANVQLVGTSTLLTRATVDSLPIRDLIGS
FVREEIILIAIKVGELVSLGKSVSQVVKVNYTDEVSIGYREFVTEVVGETKNASVSEAAAQESKVAQELFQKTSY
NDTHQYRVSTVVYGNDVAIAVKPRIPNTPSIPITKISVQSTLNEITIPATGNTNIRAADYGADAASGGHDNKFEV
VGQADDNSAGAVRIAVGFNYAAFKDIVTENVYFLERIHIVQKQQAGAGSAPPKGASGEDVIELQSRFGLPVDGLA
GAKAVNTAASFLAKEGDPLEIFVDRFITNILQTAQKISQTYNVPLASLAKGDGISYNVTYRANAYGHDYVPVAKL
SPLLQELKLNFNEFDLKIALDDGSIVGFSAKSSLSPALADVWRAADGSVILTVSKIIELAPQAVDKTFVSLEPNR
NTFFPTQNELVEISRQATVVMTYERGSSLGTQSYTGIIEAAGREGVNDNEEGFFSARLAAALEHHHHHH
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MW	= 56149.18
Residues	= 519
pI	= 5.0333

Best Clone Screen:

Cell growth:

The expression level of several clones (up to 6)/construct was tested in 1 ml-scale in LB-medium, supplemented with 100 µg/ml ampicillin, at 37°C and 180 rpm. Protein expression was induced at OD_{600} = 0.6 by addition of 1 mM IPTG. After induction, cultures were incubated for 4 h.
The cells were harvested by centrifugation (16600xg, 3 min, 4°C).

Cell lysis:

Cell pellets were resuspended in 150 µl of cell lysis buffer.

Cell lysis buffer:	50 mM	NaP, pH 7.4
	100 µg/ml	DNaseI
	1 mM	$MgCl_2$

200 µg/ml lysozyme
3 mM DTT
EDTA-free Protease Inhibitor Cocktail (Roche)

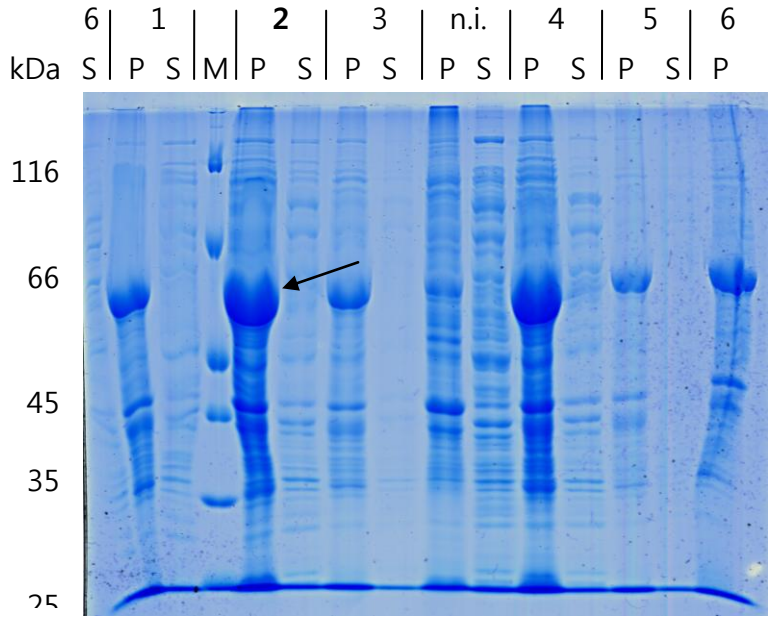
The suspension was sonicated briefly (10 sec, 40% amplitude) and then centrifuged (16600xg, 10 min, 4°C). The supernatant was transferred to a fresh vial and the remaining pellet was resuspended in 150 µl lysis buffer.

Electrophoresis:

15 µl of the supernatant and the resuspended pellets were mixed with 5 µl 4xSDS-Loading Dye and incubated at 95°C for 10 min. 15 µl sample/lane were loaded onto a 12.5% SDS-Polyacrylamide-gel.
Electrophoresis was performed at 180 V for 65 min. The gel was stained with Coomassie Blue.

Abbreviations used:

M: Marker
P: Pellet
SN: Supernatant
-: uninduced control
1-5: clone number



Clone 2 showed the higest levels of induction and was stored as glycerol stock and used for test expression. The QconCAT was detected in the insoluble fraction.

Test Expression:

Clone 2 was inoculated in 300 ml LB + Amp and grown to OD_{600} = 0.6. Expression was induced by addition of 1 mM IPTG (final concentration) and the culture was incubated at 37°C for 4-5 hours.

The pellet was collected by centrifugation for 10 minutes at 6000xg. After removing the medium, the pellet was stored at -70°C until use.

The pellet was resuspended in 5 ml cell lysis buffer.

Cell lysis buffer:	50mM	NaP, pH 7.4
	100µg/ml	DNaseI
	1mM	$MgCl_2$
	200µg/ml	lysozyme
	3mM	DTT
	1x EDTA-free Protease Inhibitor Cocktail (Roche)	

Cells were lysed by sonication (amplitude = 40%, 4 x 15sec, on ice).
The cell lysate was centrifuged (4°C, 16000xg, 10min) and the soluble fraction was removed.
The remaining pellet representing the insoluble fraction of the cell lysate was suspended in 1 ml solubilisation buffer.

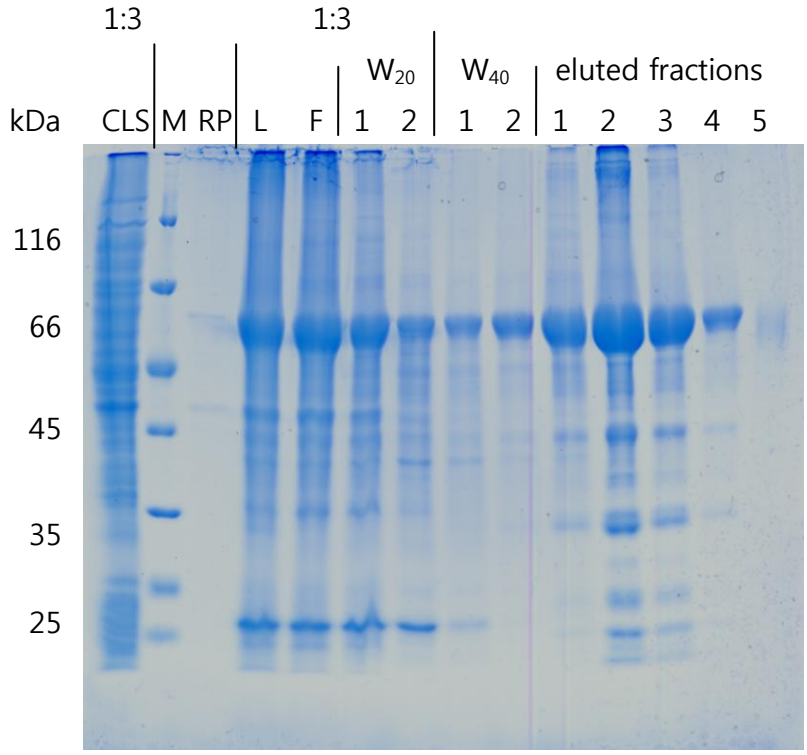
Solubilisation/binding buffer:	50 mM	NaP, pH 7.4
	6 M	GdnHCl
	300 mM	NaCl
	20 mM	Imidazole

The suspension was incubated on ice for 1-2 hours.

In the meantime, Ni-NTA colums (approx. 1 ml beads) were prepared by washing several times with binding buffer.
The lysate was applied and allowed to pass the column by gravity flow. The flow-through was collected. After two washes with binding buffer (W1 & W2), the column was washed with binding buffer supplemented with 40 mM Imidazole (W3 & W4). The bound protein was eluted with elution buffer (binding buffer with 500 mM Imidazole; 5x1000 µl).
Samples of the intermediate steps and the eluted fractions were dialyzed against 0.1% FA and analyzed via 12.5% SDS-PAGE and Coomassie staining.

Abbreviations used:

M:	Marker
CLS:	cell lysis supernatant
RP:	remaining pellet after solubilisation
L:	lysate (solubilised inclusion bodies)
F:	flow through
W:	wash
E:	eluate

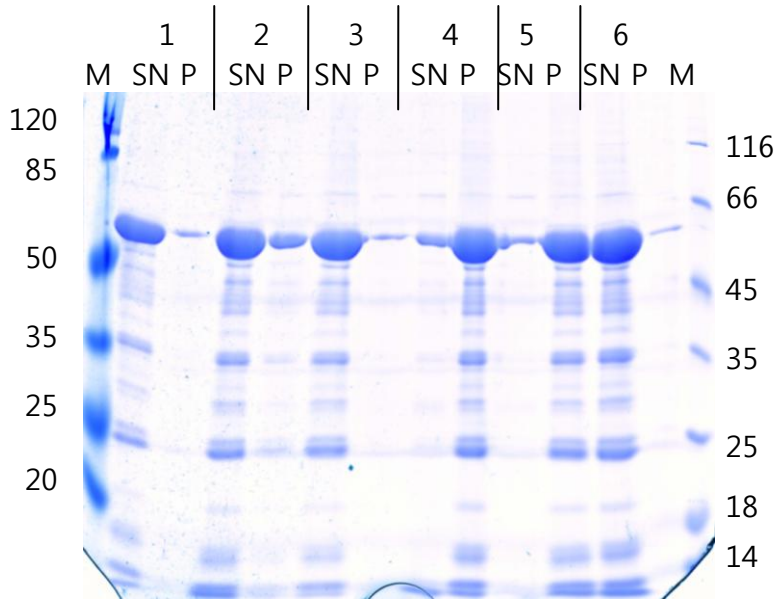


The fractions containing the highest amount of protein (2&3) were united and used for determining the appropriate solubilisation and storage buffer.

Buffer screen:

To determine the appropriate solubilisation and storage buffer, an aliquot of the purified protein was dialyzed against the buffers listed below.

1: 0.1% FA
2: 0.1% TFA
3: 0.5% HAc
4: 50 mM NH_4HCO_3 pH 9.0
5: PBS
6: 0.1% SDS, 125 mM Tris pH 6.8



Solubility of the QconCAT: 0.1% SDS, 125 mM Tris pH 6.8 > 0.5% HAc > 0.1% FA > 0.1 % TFA >>> 50 mM NH_4HCO_3 pH 9.0 > PBS. 0.5% HAc was chosen as detergent- free storage buffer.

Purification of ^{15}N-labeled QconCAT:

Medium:

Minimalmedium containing ^{15}N:

Na_2HPO_4	48 mM
KH_2PO_4	22 mM
NaCl	8.5 mM
$^{15}NH_4Cl$	19 mM
$MgSO_4$	1 mM
$CaCl_2$	0.1 mM
Glucose	0.2 % (wt/vol)
Ampicillin	100 µg/ml

Cells: BL21(DE3)- pET21a-QcStelder clone 2

Growth conditions:

Cells were grown in 0.2 l Medium at 37°C until OD_{600} reached 0.5-0-6. Protein expression was induced by addition of 1 mM IPTG. Protein was expressed for 4-5 h at 37°C. Cells were harvested by centrifugation at 6000 x g for 15 min.
The cell pellet was stored at –70°C.

Lysis and IMAC:

All buffers for cell lysis and purification have to be pre-chilled at 4°C.

Buffer:

Lysis buffer:	50 mM	NaP, pH 7.4
	100 µg/ml	DNaseI
	1 mM	MgCl2
	200 µg/ml	Lysozyme
	3 mM	DTT
	1 x EDTA free Protease Inhibitor	
Binding buffer:	50 mM	NaP, pH 7.4
	6 M	GdnHCl
	500 mM	NaCl
	40 mM	Imidazole
	1 mM	DTT
Elution buffer:	50 mM	NaP, pH 7.4

6 M	GdnHCl
500 mM	Imidazole
1 mM	DTT

Protocol:

The cell pellet was suspended in 5 ml lysis buffer.
Cells were disrupted by sonication (5 x 15 sec, 40% amplitude) on ice. The cell lysate was centrifuged (16000 x g, 10min, 4°C).

2 ml of Ni-Sepharose 6 FF-slurry (GE-Healthcare) were equilibrated in binding buffer.

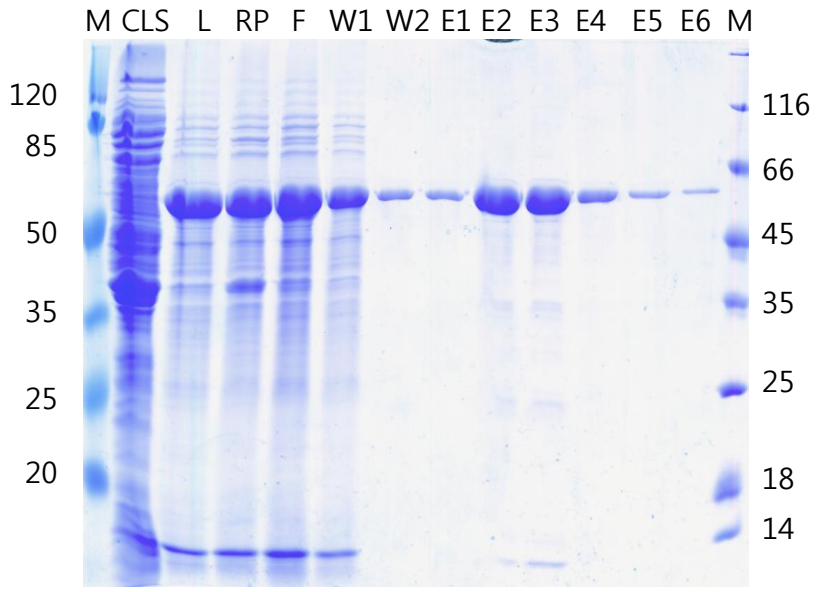
After centrifugation the soluble fraction of the cell lysate was removed. The insoluble material was suspended in 8 ml binding buffer. The samples were kept on ice for 2 h to ensure proper solubilisation of the inclusion bodies.

After centrifugation (16000xg, 10 min, 4°C) the supernatant was loaded onto the equilibrated resin and allowed to pass the column by gravity flow. The column was washed 2 times (wash 1 = 4 ml, wash 2 = 8 ml) with binding buffer to remove unbound material. Target proteins were eluted with elution buffer in 6 fractions of 1 ml.
Samples for SDS-PAGE were dialysed against 0,5% HAc.

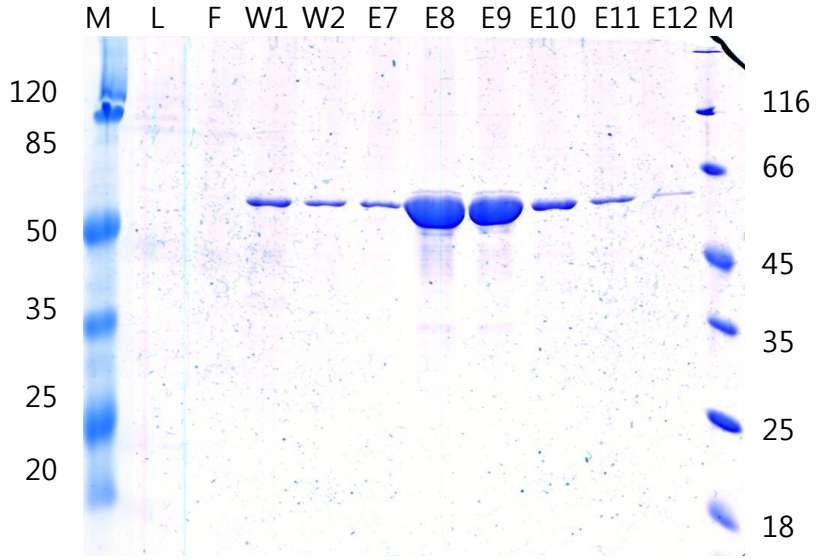
Electrophoresis (SDS-PAGE):
15 µl of each sample were mixed with 5 µl 4XSDS-Loading Dye and incubated at 95°C for 10 min.
Electrophoresis was performed at 180 V for 90 min. The gel was stained with Coomassie Blue.

Abbreviations:

CLS	cell lysate supernatant
RP	remaining pellet after solubilisation of inclusion bodies
L	load
F	flowthrough
W	wash
E	eluate



Due to the high protein content, the flow-through fraction was re-purified by diluting with binding buffer w/o imidazole to 20 mM imidazole and re-applying to the Ni-sepharose. After 2 washes with binding buffer, proteins were eluted and samples for western blot analysis were prepared.



Fractions 2, 3, 8 & 9 were pooled and dialysed against 0.5% HAc. Protein concentration of the pooled samples was determined via Bradford-assay.

Summary:

All six constructs could be over-expressed ^{15}N-labelled at a high level.
The QconCATs were purified by Ion Metal Affinity Chromatography.

The produced material will be used for LC-MS-optimization.