A robust fractionation method for protein subcellular localization studies in *Escherichia coli*

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ABSTRACT

Fractionation in Gram-negative bacteria is used to identify the subcellular localization of proteins, in particular the localization of exported recombinant proteins. The process of cell fractionation can be fraught with cross-contamination issues and often lacks supporting data for fraction purity. Here, we compare three periplasm extraction and two cell disruption techniques in different combinations to investigate which process gives uncontaminated compartments from *Escherichia coli*. From these data, a robust method named PureFrac was compiled that gives pure periplasmic fractions and a superior recovery of soluble cytoplasmic proteins. The process extracts periplasm using cold osmotic shock with magnesium, prior to sonication and ultracentrifugation to separate the cytoplasm from insoluble material. This method handles cells cultivated in various conditions and allows preparation of active proteins in their respective compartments.

METHOD SUMMARY

This fractionation protocol is a robust method to isolate proteins from subcellular locations from *Escherichia coli*. The periplasmic, cytoplasmic and membrane fractions are separated without cross-contamination.

KEYWORDS:

alkaline phosphatase • cold-osmotic shock • export • fractionation • periplasm

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BioTechniques 66: 171-178 (April 2019)
10.2144/btn-2018-0135

In bacteria, proteins localize at specific sites to form an elaborate subcellular architecture [1]. Characterizing their locations further develops the understanding of their function, which can be used in various fields including medical research [2–8]. Moreover, using *Escherichia coli* as a host, recombinant proteins for both academic research and industrial applications can be directed to the periplasm for disulfide bond formation [9], ease of downstream processing [10] or toxic protein expression [11]. The subcellular location can be predicted using bioinformatic tools developed over the past decades such as SignalP [12], PSORTb [13] and SecreteP [14], but these predictions need to be validated experimentally. To do so, the cells can be fractionated to isolate the proteins in their respective subcellular compartments. The present study focuses on *E. coli*, a Gram-negative bacterium that has a different cell wall organization compared with Gram-positive bacteria [15,16], where proteins can be localized in four different compartments: outer membrane (OM), periplasm, inner membrane (IM) or cytoplasm [16].

Although published fractionation protocols are not wholly different from each other, there appears to be no standardized method to extract all compartments with associated due-diligence against cross-contamination and inconsistency [10]. Subcellular fractionation begins with extracting the periplasm, then the cytoplasm and finally the insoluble fraction that includes membranes and aggregated proteins. Further preparation to isolate the IM from the OM can also be performed but is out of the scope of this work. The periplasmic extraction is the most critical step of the process since disruption of the IM results in contamination from cytoplasmic proteins. Common methods use either an EDTA-lysozyme strategy [17,18] or cold osmotic shock [19,20], or a combination of both [21]. In the former method, EDTA is used to destabilize the OM [22], allowing the lysozyme to enter the periplasm and

hydrolyze the peptidoglycan cell wall. This process subsequently releases the periplasm and leaves the cells as spheroplasts [18]. The second method generates an osmotic shock induced by successively adding a hypertonic solution to weaken the OM, followed by a hypotonic solution to partially disrupt the OM without compromising the integrity of the IM [19].

The second and third steps in the fractionation process result in the recovery of the cytoplasmic and insoluble fraction simultaneously by cell lysis and centrifugation. The risks are poor soluble protein recovery due to incomplete lysis or the use of harsh conditions resulting in loss of soluble protein through aggregation that in turn contaminates the insoluble fraction. The most common cell-lysis techniques used are freeze–thaw cycling and sonication [10,17]. The former offers the advantage of treating several samples simultaneously whereas sonication can treat only one sample at a time. Following cell lysis the samples are centrifuged to isolate the insoluble debris such as the membrane fragments and, when present in the cell, protein inclusion bodies.

Obtaining pure fractions is especially important when studying the biology of secretion systems such as the general secretory (Sec) or the twin-arginine translocation (Tat) pathways [23]. To the best of our knowledge, no single study clearly describes, in detail, a robust method to obtain uncontaminated soluble bacterial fractions. For instance, Thein *et al.* and Tao *et al.* compared periplasmic fractions obtained by EDTA/lysozyme and cold osmotic shock and demonstrated cross-contamination in each case [17,24]. Many publications show no due-diligence to fraction purity [18,19,21,25,26]. Some do utilize controls in the form of subcompartment-specific host proteins as purity markers but are incomplete [27,28].

In the present study, different subcellular fractionation processes were evaluated in relation to cross-contam- ▶

Table 1. List of *Escherichia coli* K12 strains used in this study.

Strain	Phenotype	Genotype	Origin
W3110	Wildtype	F ⁻ , lambda ⁻ , IN(<i>rrnD-rrnE</i>)1, <i>rph</i> -1	ATCC 27325
MC4100	Wildtype	F ⁻ , [<i>araD</i> 139]B/r, Del(<i>argF-lac</i>)169, lambda ⁻ , e14 ⁻ , <i>flhD</i> 5301, Δ(<i>fruK-yeiR</i>)725(<i>fruA</i> 25), <i>relA</i> 1, <i>rpsL</i> 150(<i>strR</i>), <i>rbsR</i> 22, Del(<i>fimB-fimE</i>)632(::IS1), <i>deoC</i> 1	CGSC 6152
MC4100	Tat-null	F ⁻ , [<i>araD</i> 139]B/r, Del(<i>argF-lac</i>)169, lambda ⁻ , e14 ⁻ , <i>flhD</i> 5301, Δ(<i>fruK-yeiR</i>)725(<i>fruA</i> 25), <i>relA</i> 1, <i>rpsL</i> 150(<i>strR</i>), <i>rbsR</i> 22, Del(<i>fimB-fimE</i>)632(::IS1), <i>deoC</i> 1, Δ <i>tatABCDE</i>	Ref [34]

Table 2. Expression conditions.

Condition	Preculture medium	Culture medium	Growth temperature (°C)	OD ₆₀₀	Feed	Temperature PI (°C)	Harvest PI (h)
1	LB	LB	30	0.5	–	30	2
2	LB	LB	30	0.5	–	30	4
3	LB	LB	37	0.5	–	37	2
4	LB	LB	30	0.5	–	18	16
5	2x TY	2x TY	30	0.5	–	30	2
6	2x TY	2x TY	37	3.5	Yes	18	16

The details of the six conditions used in this study are listed by preculture and culture media, growth temperature after inoculation, OD₆₀₀ at inoculation as well as growth temperature PI, addition of feed at induction and harvest time PI. The feed was diluted 25 times into the culture from a stock solution of 1 M MOPS pH 7.2, 40% glycerol, 20 mM MgSO₄, 20 mM MgSO₄.
PI: Post-induction.

Table 3. List of antibodies used in this study.

Antibody	Target	Clonality	Species	Conjugation	Dilution	Supplier	Catalog number
Primary	His tag	Polyclonal	Rabbit	HRP	1/2000	Universal biologicals	A190-114P
	cMyc tag	Monoclonal	Mouse	–	1/2000	Abcam	Ab32
	LacI	Monoclonal	Mouse	–	1/2000	Abcam	AB33832
	G6PD	Polyclonal	Rabbit	–	1/2000	Assaypro	33115-05111
	MBP	Polyclonal	Rabbit	–	1/5000	Abcam	AB21144
	DsbA	Monoclonal	Mouse	–	1/1000	Abcam	AB106061
Secondary	Mouse IgG, Fcγ	Polyclonal	Goat	HRP	1/2500	Jacksonimmuno	115-036-071
	Rabbit IgG, HC+LC	Polyclonal	Goat	HRP	1/2000	Universal biologicals	A120-101P

Table 4. Fractionation methods used in this paper.

Method	Periplasmic extraction	Cell disruption
Method 1	Cold osmotic shock	Freeze–thaw cycling
Method 2	Cold osmotic shock with MgCl ₂	Freeze–thaw cycling
Method 3	EDTA/lysozyme/cold osmotic shock	Sonication
Method 4	EDTA/lysozyme/cold osmotic shock	Freeze–thaw cycling
PureFrac	Cold osmotic shock with MgCl ₂	Sonication

► ination in order to devise an optimal complete method. Alkaline phosphatase (PhoA) variants were used as reporter proteins to demonstrate either cytoplasmic

or periplasmic cellular localization. The former lacks a signal peptide whereas in the latter, PhoA was fused to either a Sec-specific [28,29] or Tat-specific signal

peptide [19,21,26,28,30] for export. PhoA is naturally exported by the Sec pathway [28], but has also been shown to export into the periplasm as a fully folded protein via the

Tat pathway when fused to a TorA signal peptide [26,28]. As PhoA contains two disulfide bonds required for activity, for cytoplasmic expression and Tat export where correct folding is a requisite [26,28], the CyDisCo technology (human protein disulfide isomerase PDI and sulfhydryl oxidase from *Saccharomyces cerevisiae* Erv1p) was utilized [26,29,31–33].

As a negative control that is defective in periplasmic export via Tat, a MC4100 Tat-null mutant [34] was used to test the robustness of the technique. This mutant is further characterized with a phenotype distinct from wildtype (WT), as being prone to lysis, bearing a filamentous morphology and leaky periplasm and thereby a compromised IM [6,35,36].

MATERIALS & METHODS

All chemicals, enzymes and protein reagents were supplied by Sigma-Aldrich unless otherwise stated, and all *de novo* gene synthesis was performed by ATUM Inc.

Strain & plasmid construction

The strains *E. coli* K12 W3110 WT, MC4100 WT and MC4100 Tat-null were used for gene expression studies (Table 1). All cloning and plasmid amplification was performed using TOP10 chemically competent cells (Thermo-Scientific) according to the manufacturer's recommendations. Gene expression plasmids were constructed using the Golden Gate technology [37]. Initially, the vector pDPH340 [38] was modified by PCR mutagenesis to remove the *TypellS* restriction site, *Bsal* in the vector backbone; subsequently *Bsal* sites were strategically inserted at specific points downstream from the IPTG-inducible *tac* promoter, to accept either one or two compatible-gene cassettes for in-frame single or polycistronic gene expression.

Protein expression

Chemically competent cells [39] were transformed with the appropriate plasmid, as outlined in Sambrook and Russel [40], plated onto Lennox agar [40] supplemented with tetracycline (10 µg/ml) and incubated for 16 h at 37°C. A preculture was prepared by inoculating 10 ml of selective Lennox medium (LB) [40] or 2xTY [40] with a single colony from a fresh plate, and incubating

for 16 h at 37°C with shaking at 220 rpm. Subsequently, shake flasks containing 25 ml of selective media and supplemented with cofactors, MgSO₄ and ZnSO₄ at a final concentration of 0.5 mM, were inoculated with the preculture at a starting OD₆₀₀ of ~0.05. Flasks were incubated at 30 or 37°C with shaking at 220 rpm and the OD₆₀₀ followed with time. When the OD₆₀₀ reached 0.5, gene expression was induced with a final concentration of 100 µM IPTG and cultures were incubated at the specific conditions shown in Table 2.

Fractionation methods

Following protein expression, a volume of culture equivalent to an OD₆₀₀ of 8 was transferred to a 50-ml tube and centrifuged for 15 min at 4816 x g at 4°C. The supernatant was carefully discarded with a pipette and the resulting cell pellet was incubated on ice.

Throughout the process, samples and solutions were incubated on ice and all centrifugations were performed for 3 min at 20,817 g at 4°C unless otherwise stated. PBS, water and buffers were freshly supplemented with 100 mM of N-Ethylmaleimide (NEM) to trap the thiol-disulfide status during sample processing and with the cOmplete™ Protease Inhibitor Cocktail (Roche) at the recommended dose. To minimize cross-contamination of fractions, all steps requiring the handling, transfer or removal of small volumes of liquid were undertaken using single channel pipettes without disturbing the pellet. Prepared fractions were stored at -20°C until required. The methods are summarized in the Table 4.

Method 1

The periplasm was extracted by cold osmotic shock and the cells were lysed by freeze–thaw cycling as described previously [20].

Periplasmic extraction. The cell pellet was washed with 850 µl PBS at pH 7.4 (NaCl 137 mM, KCl 2.7 mM, Na₂HPO₄ 24.2 mM, KH₂PO₄ 5.2 mM), centrifuged and the supernatant was carefully discarded. The cell pellet was then resuspended in 900 µl of spheroplast buffer (0.1 M Tris pH 8.0, 500 mM sucrose, 0.5 mM EDTA pH 8.0), incubated for 5 min, centrifuged and the supernatant carefully discarded. The pellet was then resuspended in 400 µl of

hypotonic solution (distilled water) and the sample was incubated for 15 s on ice before adding 20 µl MgSO₄ (20 mM). After centrifugation, the resulting periplasmic fraction was carefully transferred to a new tube and the subsequent pellet incubated on ice or stored at -20°C.

Cell disruption. The resulting pellet was gently resuspended in 900 µl of spheroplast buffer before adding lysozyme (235 µg/ml working concentration) and gently mixed. The suspension was diluted with 900 µl distilled water and incubated for 5 min. After centrifugation, the supernatant was carefully discarded and the pellet was resuspended in 1.2 ml Tris pH 8.0 (10 mM). For cell lysis, the suspension was rapidly freeze–thaw cycled four times in liquid nitrogen for 2 min and in a 37°C water bath for 3 min. After addition of 10 U of benzonase to degrade DNA and 20 µl 1 M MgSO₄, the sample was incubated for 10 min, prior to centrifugation for 25 min at 20,817 x g. The resulting supernatant was carefully transferred to a fresh tube as the cytoplasmic fraction and stored at -20°C. The pellet was resuspended in 1 ml Tris pH 8.0 (10 mM) and stored as the insoluble fraction at -20°C.

Method 2

Periplasmic extraction. By cold osmotic shock as described in method 1 except 1 mM MgCl₂ was used instead of water as the hypotonic solution.

Cell disruption. By freeze–thaw cycling as described in method 1.

Method 3

The periplasm was extracted by EDTA/lysozyme/cold osmotic shock and the cells were lysed by sonication as described ► previously [30].

Periplasmic extraction. The cell pellet was washed in 850 µl PBS, resuspended in 500 µl Buffer 1 (100 mM Tris-acetate, 500 mM sucrose, 5 mM EDTA pH 8.0) and diluted with 500 µl distilled water before adding 40 µl lysozyme (2 mg/ml). After a 5-min incubation followed by the addition of 20 µl MgSO₄ (1 M), the cells were centrifuged and the supernatant was transferred into a fresh tube as the periplasmic fraction.

Cell disruption. The resulting pellet was resuspended in 1 ml Buffer 2 (50 mM Tris-acetate pH 8.2, 250 mM sucrose, 10 mM MgSO₄, 1 U/ml benzonase), centri- ►

once resuspended in 1 ml Buffer 3, was reserved as the insoluble fraction.

Method 4

Periplasmic extraction. By EDTA/lysozyme/cold osmotic shock as described in method 3.

Cell disruption. By freeze-thaw cycling as described in method 1.

Western blotting

Samples were loaded onto 4–20% Novex Tris-glycine gels (ThermoScientific) according to the manufacturer's instructions with the molecular weight markers MagicMark XP (ThermoScientific) and PageRuler Plus Prestained Protein Ladder (ThermoScientific) before separation by electrophoresis at 100 V for 100 min in Novex (1X) Tris-glycine buffer (ThermoScientific). The proteins were dry transferred onto a PVDF membrane using the iBlot system (ThermoScientific) according to the manufacturer's recommendations. Immunostaining was carried out according to Sambrook and Russel [40] using antibodies as listed in the Table 3. Detection was performed using Pierce™ ECL Plus Western Blotting Substrate (Life Technologies) and imaged by the ImageQuant LAS 4000 device (GE Healthcare).

RESULTS & DISCUSSION

Identification of a robust fractionation method

For cytoplasmic expression, the leaderless PhoA (48.3 kDa), Erv1p (22.9 kDa) and PDI (56.7 kDa) were co-expressed from an IPTG-inducible polycistronic construct controlled by the tac promoter and where LacI (38.7 kDa) was used as the repressor. PhoA and the CyDisCo components were tagged with C-terminal His and cMyc tags, respectively, to aid identification in Western blots. All four proteins therefore served as cytoplasmic markers. The endogenous proteins, the cytoplasmic glucose-6-phosphate dehydrogenase (G6PD, 55.7 kDa) and two periplasmic proteins, maltose-binding protein (MBP, 43.4 kDa) and protein disulfide isomerase DsbA (23.1 kDa) [41–43], were also used as native host markers to validate fraction purity.

Three periplasmic extraction methods, cold-osmotic shock, cold-osmotic shock with $MgCl_2$ and EDTA/lysozyme/cold-osmotic [30]; and two cell disruption

Figure 1. Evaluation of periplasmic and cytoplasmic extraction methods. W3110 WT and MC4100 Tat-null strains co-expressed cytoplasmic His-tagged PhoA and C-terminally cMyc-tagged Erv1p and PDI proteins. The cells were fractionated according to methods 1 (cold osmotic shock and freeze-thaw cycling), 2 (cold osmotic shock with magnesium and freeze-thaw cycling), 3 (EDTA/lysozyme/cold osmotic shock and freeze-thaw cycling) and 4 (EDTA/lysozyme/cold osmotic shock and freeze-thaw cycling). The fractions are represented as cytoplasmic (C), insoluble (I) and periplasmic (P). Western blots show from top to bottom, the detection of PhoA, PDI, Erv1p, G6PD, LacI, MBP and DsbA proteins as detected by anti-His, anti-cMyc, anti-cMyc, anti-G6PD, anti-LacI, anti-MBP and anti-DsbA antibodies, respectively. Molecular weight markers are indicated on the right in kDa. WT: Wildtype.

► fuge for 5 min, and the supernatant was carefully discarded. The pellet was resuspended in 750 ml Buffer 3 (Tris-acetate pH 8.2, 2.5 mM EDTA pH 8.0) and the suspension was sonicated (5 cycles

of 10 s at amplitude 8 μ m) on ice using a Soniprep150 (MSE). After ultracentrifugation at 186,000 g for 30 min, the supernatant was transferred to a new tube as the cytoplasmic fraction and the pellet,

techniques, sonication [30] and freeze–thaw cycles, were compared in combinations to assess fraction purity (Table 4). Protein localization and cross-contamination was monitored by Western blot of overexpressed proteins and selected markers.

Cytoplasmic PhoA, Erv1p and PDI were co-expressed in W3110 WT and MC4100 tat-null strains as outlined in Table 2 (condition 4) and the cells were fractionated. As shown in Figure 1, the periplasmic fraction isolated by cold-osmotic shock (method 1) presented minor bands with PDI and LacI and, to a lesser extent, PhoA and G6PD in both strains indicating some cross-contamination from the cytoplasm. The markers MBP and DsbA were located in the periplasmic fractions as anticipated, confirming a successful extraction of the periplasm. However, cytoplasmic contamination can be completely avoided by the use of MgCl₂ during the cold-osmotic shock (method 2) while the periplasmic markers are still extracted effectively (Figure 1). The addition of MgCl₂ to the hypotonic solution has previously been suggested [44] because magnesium plays a major role in maintaining the stability of the OM [22,45]. It therefore appears that the osmotic shock step with Mg used in this study resulted in the disruption of only the OM without affecting the integrity of the IM. However, minor bands of periplasmic MBP were still detected in the cytoplasmic and insoluble fractions (Figure 1), presumably from lysis of a small proportion of intact cells that escaped processing in the periplasmic extraction step.

By contrast, when using the EDTA/lysozyme/cold-osmotic shock (method 3 and 4), an incomplete periplasmic extraction was detected for both strains. DsbA was not detected and MBP was only partially detected in the periplasmic fraction, both suggestive of ineffective periplasmic extraction. Moreover, with this method, all cytoplasmic markers were detected in the periplasmic fractions of the Tat-null strain, suggesting that this method can be sub-optimal for some strains. The cytoplasmic contamination can be explained by a disrupted IM because of the destabilized cell wall; phospholipids permeabilization of the OM due to cation chelation by the EDTA [22] and PG digestion by the lysozyme. Overall, due to cross-contamination, the EDTA/

lysozyme/cold-osmotic shock technique can lead to false-negative and false-positive conclusions and therefore is not fit for this purpose.

For the isolation of cytoplasmic fractions, two methodologies, freeze–thaw cycling and sonication, were compared. Generally, sonication (method 3) appeared to be superior with notably higher recovery of soluble cytoplasmic proteins as indicated by PDI, Erv1p, G6PD and LacI (Figure 1). The proteins detected in the insoluble fractions most likely came from unlysed cells or prefractionation aggregates. Benzoniase was essential to liquify the lysate by DNA degradation with freeze–thaw cycling but was not required for sonication.

In conclusion, cold-osmotic shock with MgCl₂ appears to be the superior method for periplasmic extraction while sonication gave the optimal results for cytoplasmic recovery. These two techniques were subsequently tested together in a single new method.

Validation of the composite method

The optimal method, consisting of cold-osmotic shock with MgCl₂ and sonication, named PureFrac, is described in Table 4 and Supplementary Materials. To assess the extraction of a recombinant secreted protein from periplasm using PureFrac, the

periplasmic PhoA (fused to a TorA-signal peptide) was co-expressed with PDI and Erv1p in WT and Tat-null strains. Such cells were cultivated in varying settings (Table 2) that are commonly used to overexpress proteins in the literature [18,19,25,26,32,33,38] to test whether growth conditions affect cross-contamination propensity.

As shown in Figure 2A, PhoA was only observed in the periplasmic fractions of WT cells indicating exported protein but to varying degrees, this being dependent on the growth parameters used. Culture condition 2 gave the optimal level of exported PhoA. As expected, PhoA was not observed in the periplasm of the Tat-null strain confirming Tat specific export [26] and demonstrating fraction purity. Discounting proteins present in the insoluble fraction, the cytoplasmic markers PDI, Erv1p, LacI, and the periplasmic marker MBP were all observed in their predicted fractions for both strains (Figure 2B).

To further validate the utility of PureFrac, PhoA was expressed in the cytoplasm (no signal peptide) and periplasm via both Sec and Tat export pathways (OmpA and TorA signal peptides, respectively). The cytoplasmic and Tat-exported PhoA were co-expressed with PDI and Erv1p. The MC4100 WT and Tat-null strains were cultivated according to condition 2 (Table 2), ►

Figure 2. Evaluation of optimized subcellular fractionation method. W3110 WT and MC4100 Tat-null strains co-expressing periplasmic (Tat pathway specific) His-tagged TorA_{sp}-PhoA and C-terminally cMyc-tagged Erv1p and PDI were grown in different conditions: induced in LB (1) for 2 h at 30°C or (2) for 4 h at 30°C, (3) for 2 h at 37°C, (4) for 16 h at 18°C or (5) induced in 2x TY for 2 h at 30°C or (6) induced in fed-batch culture for 16 h at 18°C. The cells were then fractionated using PureFrac method into the cytoplasmic (C), insoluble (I) and periplasmic (P) fractions. (A) Western blots of PhoA-His detected by anti-His antibodies. (B) Western blot showing Erv1p, PDI, LacI and MBP proteins detected by anti-cMyc, anti-cMyc, anti-LacI and anti-MBP antibodies respectively. Molecular weight markers are indicated in kDa on the right of each blot. WT: Wildtype.

Figure 3. Validation of PureFrac. An empty vector (1), cytoplasmic His-tagged PhoA (2), periplasmic (Sec pathway) $OmpA_{sp}$ -PhoA (3) and periplasmic (Tat pathway) $TorA_{sp}$ -PhoA co-expressed with cMyc-tagged PDI and Erv1p (4) were expressed in MC4100 WT and Tat-null strains. The cells were then fractionated using PureFrac method into the cytoplasmic (C), insoluble (I) and periplasmic (P) fractions. Western blots show PhoA-His, Erv1p, PDI, LacI and MBP proteins detected by anti-His, anti-cMyc, anti-cMyc, anti-LacI and anti-MBP antibodies, respectively. Molecular weight markers are indicated on the right in kDa. WT: Wild type.

► which gave optimal Tat export (Figure 2), and subsequently fractionated according to the PureFrac protocol. In Figure 3, cytoplasmic PhoA was not detected in the periplasm as anticipated, consistent with previous observations (Figure 1). As predicted, PhoA corresponding to Sec-specific export ($OmpA_{sp}$ -PhoA) was secreted into the periplasm in both strains whereas Tat-specific export of PhoA ($TorA_{sp}$ -PhoA) was only observed in the WT strain. All cytoplasmic (PDI, Erv1p, LacI) and periplasmic (MBP) markers were observed in their expected subcellular fractions, further supporting the robustness of the fractionation method. Furthermore, this protocol is not so aggressive as to denature proteins, as demonstrated by active PhoA in periplasmic extracts of Sec and Tat (data not shown).

We have demonstrated the robustness of the PureFrac method to isolate pure fractions and we show that markers must always be utilized as controls to highlight any cross-contamination artefacts. However, some steps may be further optimized, for example solving the issue of unlysed cells or prefractionation aggregates. Also, the insoluble preparation step calls for a 186,000 g ultracentrifugation step, which could be mitigated to a lower speed of 30,000 g with a wash step to remove all potential cytoplasmic contaminating material [46,47]. In our hands, comparable fractionation purity was obtained in export localization studies with human growth hormone, superfolder GFP, β -galactosidase, antibody fragments and PaoA (data not shown). We believe that reliable conclusions can be drawn from observations made using this PureFrac technique and specific controls.

FUTURE PERSPECTIVE

The present study highlights the strengths and weaknesses of the main subcellular fractionation steps from current published protocols. To the best of our knowledge, a comparison of this scale has never been published that identifies the steps that are fraught with cross-contamination and inevitably lead to false data and lack of reproducibility. With this in mind, we have identified processing steps that can generate accurate data and in turn, compiled an effective fractionation protocol

which we hope researchers would recognize for its superior properties and increasingly implement as their 'go to' method. Irrespective of which protocol a researcher uses, we have emphasized the importance of using specific markers as controls, to help identify potential risks at each step.

As PureFrac was developed with a Gram-negative bacterium, we have demonstrated the versatility of this method by application to a strain with a compromised envelope. It is therefore conceivable that this protocol could be applied to other *E. coli* mutants or other Gram-negative bacterial species, thus expanding its value.

AUTHOR CONTRIBUTIONS

GM conceived, designed, performed all the experiments and wrote the paper. The study was supervised by DPH and ED who provided guidance and advice by assisting experiment design, data interpretation, manuscript evaluation and edition.

ACKNOWLEDGMENTS

The authors would like to thank Colin Robinson for kindly sharing the MC4100 Tat-null strain and members of his team for sharing of periplasmic expression methods.

FINANCIAL & COMPETING INTERESTS DISCLOSURE

The research leading to these results has received funding from the People Programme (Marie Skłodowska-Curie Actions) of the European Union's Horizon 2020 Programme under REA grant agreement no. 642836. This material reflects only the authors' views and the Union is not liable for any use that may be made of the information contained therein. DPH holds UCB shares. The authors

have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

SUPPLEMENTARY DATA

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2018-0135

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EXECUTIVE SUMMARY

- This article describes a robust sub-cellular fractionation method to investigate protein localization in *Escherichia coli*.
- The method uses cold-osmotic shock with MgCl₂ to extract the periplasm and sonication for cell disruption followed by ultracentrifugation to separate the cytoplasm from the membranes and inclusion bodies.
- This method is robust enough to obtain pure cytoplasmic and periplasmic

fractions in various expression conditions and a mutant strain with a compromised cell envelope.

- Subcellular protein localization can be achieved using this technique to assess the secretion of endogenous and recombinant proteins.
- Periplasmic fractions extracted by the EDTA/lysozyme/cold-osmotic shock method were contaminated by cytoplasmic

proteins and do not contain all periplasmic proteins tested.

- Periplasmic fractions extracted by the cold-osmotic shock method were contaminated by cytoplasmic proteins.
- More soluble cytoplasmic proteins can be recovered using sonication than freeze–thaw cycles.

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