A miniaturized, high-throughput buffer-centric method for protein solubility screening

Adrian Svoboda[1], *Marina Molineris*[1], *Tomáš Pluskal*[1], *Teo Hebra*[1*]

[1]Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, Flemingovo náměstí 542/2, 160 00 Prague, Czech Republic

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ABSTRACT

Efficient access to soluble recombinant proteins remains a major bottleneck in biochemical and structural studies. We describe a buffer centric, fully miniaturized, 96-well plate workflow to map protein solubility across a large number of conditions in a single working day. Liquid-nitrogen-frozen *E. coli* pellets are cryogenically bead-milled with stainless-steel beads at sub-zero temperature, retaining the native intracellular milieu while ensuring uniform disruption. The resulting dry powder can therefore be extracted with any buffer of choice, enabling systematic exploration of pH, ionic strength, detergents, and chaotropes. Protein solubility is quantified by a one-microliter chemiluminescent anti-His dot-blot. Using a His-Ruby2 reporter panel, we show that dot-blot intensity can be used as a quantitative proxy of protein solubility. We also provide experimentally supported guidelines on the influence of those buffer reagents on subsequent steps of protein production, SDS-PAGE and Ni-NTA purification. This workflow is compatible with upstream genetic solubility-enhancement strategies, and enables direct transition to scale-up, the same day that soluble hits are identified. Because the entire workflow uses standard molecular-biology equipment and inexpensive consumables, it can be readily adopted or automated in any laboratory.

Introduction.

Proper protein folding and solubility are prerequisites for most biochemical and structural investigations. Low protein solubility or aggregation may impair *in vitro* catalysis assays or subsequent steps for structural biology. Although high-throughput in-vitro enzymatic screening technologies have advanced rapidly,[1,2] efficient production of soluble recombinant proteins has not kept pace.[3] It constitutes a critical bottleneck in protein function discovery and their structural characterization. Solubility depends on a broad spectrum of parameters (*e.g.* amino acid sequence,[4] host genotype, expression cassette, culture medium, lysis conditions[5]). For any given target or protein panel, the fastest route to soluble material is miniaturized, parallel expression screening. Cultivation in 24- to 96-well plates enables systematic interrogation of the relevant variables impacting protein solubility.

Within the parallelized protein extraction workflow, cell disruption is critical: the transition from the intracellular milieu to artificial extraction buffer can break protein solubility but should be compatible with 96-well plates and small liquid volumes. Mechanical and chemical lysis approaches are therefore widespread. Among standard lysis methods such as sonication and chemical lysis; cryomilling offers a gentle, low-energy alternative that preserves the native chemical environment of the cells[6] and scales seamlessly to 96-well plates and scales up culture.[7] Buffer composition is an equally powerful—yet under-utilized—lever. Buffer libraries are relatively common for membrane proteins [8], co-immunoprecipitation[6] and in crystallization[9] but are rarely used in general protein production settings, and therefore, absent from most standard solubility-screening strategies recommendation.[10] We argue that extraction buffer optimization should be implemented early in the screening procedure, and that it can be scaled to match any project scope or protein set.

Assessment of soluble expression constitutes a second bottleneck, as summarized by Baranowski *et al*.[3] Among available readouts, anti-His dot-blot analysis combines exceptional parallelizability, low cost, shareability, and compatibility with downstream experiments; it is already widely used.[11–13] Here we present a fully 96-well-plate compatible workflow that unites cryomilling, buffer-library screening, and anti-His solubility assay to assess the solubility space of recombinant proteins. The protocol builds on established common expression practices yet delivers true medium- to high-throughput performance, providing a practical route for selecting optimal conditions for subsequent functional and structural studies.

Material and methods

Growth Media, Strains and Plasmids

Chemicals used for media and buffer preparation were purchased from either Sigma-Aldrich, Duchefa Biochemie (Haarlem, Netherlands), Lach:ner (Neratovice, Czech Republic), or Penta Chemicals (Prague, Czech Republic). ZYM-5052 medium was prepared according to Studier *et al.*[14] with ZY base: tryptone 1 % (v/w) yeast extract 0.5 % (v/w); M base (50 ×): 1.25 M Na_2HPO_4, 1.25 M KH_2PO_4, 2.5 M NH_4Cl, 0.25 M Na_2SO_4; 5052 base (50 ×): glycerol 25 % (v/w), glucose 2.5 % (v/w), α-lactose 10 % (v/w); 1 M $MgSO_4$; trace elements (1000 ×): 50 mM $FeCl_3$; 20 mM $CaCl_2$; 10 mM $MnCl_2$; 10 mM $ZnSO_4$; 2 mM $CoCl_2$, 2 mM $CuCl_2$; 2 mM $NiCl_2$; 2 mM Na_2MoO_4; 2 mM Na_2SeO_3; 2 mM H_3BO_3.

Plasmids were generated using Golden Gate Assembly in a 10 µL reaction volume–T4 ligase buffer, 1 µL; T4 ligase, 0.5 µL (M0202L, New England Biolabs; BsaI-HFv2, 0.5 µL (R3733L, New England Biolabs); pYTK034 (Ruby2), 0.5 µL (20 fmol); p3Xpress_Eco (MBP and Trx tag, in house backbones) and ddH2O, up to 10 µL. Reactions were conducted in a ProFlex 3 × 32-well PCR system thermocycler (Applied Biosystem). Thermocycling conditions used the following program: for 25 cycles, 37 °C for 5 min, 16 °C for 5 min, then 60 °C for 30 min, and 80 °C for 10 min. Plasmid maps are available as supplementary material.

DH10 electrocompetent *E. coli* cells were used for all molecular cloning experiments. Transformed cells were selected on lysogeny broth (LB) with kanamycin.
BL21(DE3) electrocompetent *E. coli* cells were used for expression of proteins. Transformed cells were selected on lysogeny broth (LB) with kanamycin.

Protein extraction

Culture (1 mL) of E. coli culture was centrifuged 10 min, 5000 *g*, 4 °C and the supernatant was removed. Then cell pellets were flash-frozen by immersion in liquid nitrogen. A 5-mm stainless-bead was added and frozen samples were mechanically lysed using QIAGEN TissueLyser II**,** 25 Hz, 30 s then were incubated on ice for 1 minute. Then 200 µL of extraction buffer were added to each sample and samples were resuspended by vortex, 5 s, and 3 cycles of bath sonication 5 s, cool down on ice 15 s. Sample extraction can be Carried out from 5 min to 2 d. To recover only soluble protein samples, supernatant was transferred after centrifugation 20 min, 18 000 *g*, 4 °C.

Protein Purification

Resin slurry (20 µL) of Ni-NTA Agarose (QIAGEN) was washed three times by centrifugation 5 min, 700 *g*, 25 °C and resuspended in 30 µL of extraction buffer. 90 µL of clarified lysate, normalized by fluorescence, was added to the resin and gently mixed for 7 min at 4 °C. Samples were centrifuged for 5 min, 700 *g*, 4 °C and supernatant was removed and pellet was resuspended in 300 µL of extraction buffer and were centrifuged for 5 min, 700 *g*, 4 °C. Supernatant was removed and proteins were recovered from resin by addition of 20 µL of extraction buffer with 500 mM imidazole, gentle mixing for 3 min at 4 °C and centrifuged for 5 min, 700 *g*, 4 °C.

SDS PAGE

For SDS-PAGE analysis, protein-containing solutions were denatured at 95 °C for 5 min and mixed with 2 × SDS loading buffer.

Samples were loaded to a 10 % acrylamide gel prepared with TGX FastCast acrylamide kit (Bio-Rad) and the electrophoresis was run for 40 min, 150 V. After electrophoresis, gel was washed with distilled water, covered with coomassie blue, microwaved 20 sec, washed in PBS for 30 min at room temperature and was unstained 2 h with MeOH/H_2O/Acetic Acid (50:40:10) destaining solution at room temperature.

Dot blot

For dot blot analysis, 1 µL of sample was transferred onto PVDF membrane and was dried for 30 minutes. The PVDF membrane was incubated 1 h with PBS, 5 % milk then with PBS, 5 % milk, Anti-polyHistidine−Peroxidase antibody for 1 h. The membrane was washed 3 times with 1 × PBS for 5 min and was visualized using 1 mL of 3,3′,5,5′-Tetramethylbenzidine for 5 min.

Fluorescence measurement

Fluorescence was measured using Spark® Multimode Microplate Reader (Tecan) with following parameters: excitation wavelength: 559 nm, excitation bandwidth: 15 nm**,** emission wavelength: 600 nm, emission bandwidth: 15 nm**,** Number of flashes: 30**,** Integration time: 40 µs, Gain optimal

Data analysis

Images were collected in Azure 500 NIR fluorescent imager using orange tray and Images were processed (cropped and contrast enhancing) up using ImageJ software. Raw data from fluorescence were processed using Rstudio and figures were generated using ggplot2 package and Adobe illustrator.

Results

The workflow described here enables rapid, systematic screening of protein-solubility space and accommodates every common optimization parameter—host organism and strain, expression vector, fusion tag, growth medium, and chemical supplementation. Cells are cryomilled as nitrogen-frozen pellets, bead-beaten at sub-zero temperature, extracted with user-defined buffer libraries, and evaluated for soluble expression by an anti-His dot-blot assay.

Sample preparation workflow

We recommend 96-well deep-well plates for comprehensive solubility screening when many constructs or strains are tested in parallel (Fig 1.). For a single protein against multiple buffers, conventional baffled flasks suffice. Cultures are inoculated from fresh plates into ZYM-5052 auto-induction medium.[14] After a 2 h incubation at 37 °C, the cultures are shifted to 18 °C for 14–18 h. Although auto-induction usually yields less protein yield than optimized IPTG induction, its gentle transcriptional activation and hand-off handling makes it ideal for high-throughput campaigns. After protein production, cells are harvested at 5 000 *g*, 10 min and the supernatant is discarded. Then, the pellet is plunged into liquid nitrogen. One stainless-steel bead is added per sample and pellets are disrupted in a TissueLyser (30 s, 25 Hz). Following mechanical lysis, samples are warmed on ice for 1 min, extraction buffers are dispensed, in each sample and suspensions are homogenized by five cycles of pulse sonication on a water bath (5 s on / 15 s on ice). Lysates may be clarified immediately (30 min, 4 °C), but we found out that a prolonged hold at 4 °C routinely increases the extraction yield.

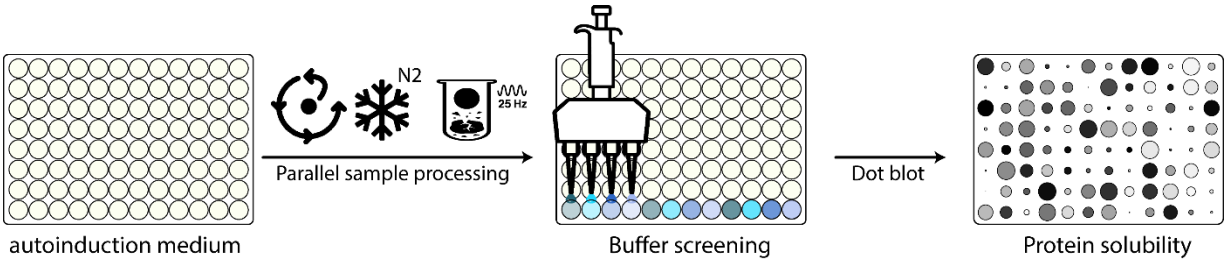

Figure 1. Workflow for quick assessment of protein solubility. Host cells for protein production are cultivated in 96-well plates overnight in auto-induction medium before being centrifuged, the supernatant removed, the pellets frozen in liquid nitrogen, and then mechanically lysed at 25 Hz with stainless-steel beads. Lysates are subsequently extracted with a set of buffers, and protein solubility is assessed by dot-blot.

Dot-blot assay for rapid verification of protein solubility

Soluble expression of the target protein is assessed by a one-microlitre dot-blot assay. Clarified lysate (or buffer control) is spotted onto an 86 × 126 mm PVDF membrane through the grid of a standard 96-well 10 µL pipette-tip rack, which guarantees uniform spacing and reproducible spot geometry for up to 96 samples per membrane. After blocking, the membrane is incubated with an anti-His antibody directly conjugated to horseradish peroxidase, and chemiluminescent signals are recorded. The procedure follows the conventional dot-blot workflow and provides a semiquantitative “soluble / insoluble” read-out within two hours (Fig. 2).

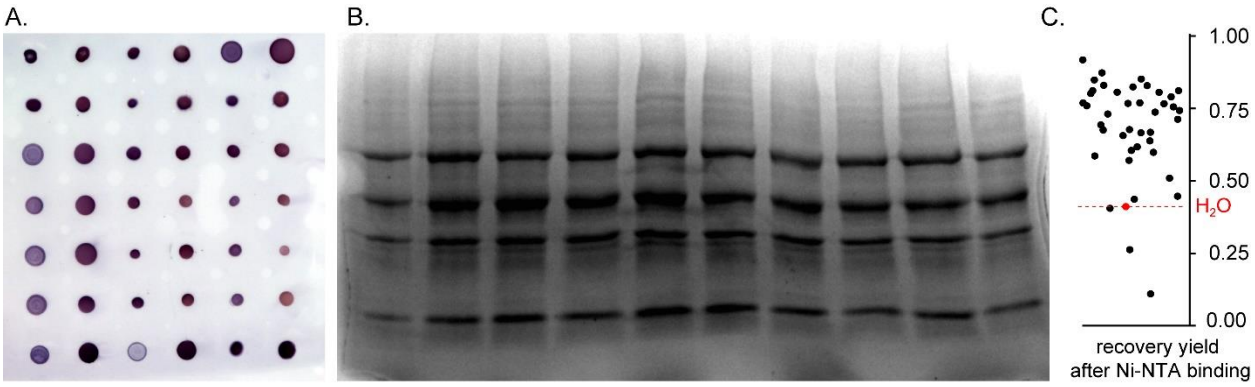

Figure 2. Illustration of the influence of buffer composition for protein solubility after extraction (A), SDS-PAGE migration and intensity (B) and Ni-NTA purification yield based on fluorescence measurement (C).

Buffer compatibility with downstream analyses

The buffers used in this study were selected based on variation of commonly employed buffers for proteins extraction (i.e. HEPES, Tris-HCl and Ammonium acetate), modified with different concentrations of salt and additives. They cover a decent range of the classical buffer space used in common biochemistry assay but can be extended or modified as needed. It is worth mentioning that the addition of detergent does not lead to any foaming during the extraction step.

Since solubility screening is only the first step toward protein characterization, we evaluated whether a subset of our extraction buffers interfered with the two most common downstream assays after protein expression: (i) SDS-PAGE with Coomassie staining (Fig. 2B, S1-S9) and (ii) immobilized-metal affinity chromatography on Ni-NTA resin (Fig. 2C). Overnight, *E. coli* cultures grown in auto-induction medium at 18 °C were pelleted, resuspended, and boiled directly in their respective extraction buffers. The lysates were analyzed by SDS-PAGE and visualized with standard Coomassie staining. In parallel, *E. coli* extracts from our solubility screen containing HIS_8-Ruby2 were subjected to Ni-NTA purification to assess recovery of His-tagged protein under native conditions.

Most tested buffers proved fully compatible with both SDS-PAGE/Coomassie detection and Ni-NTA purification (Table 1). A concise summary of the few adverse interactions observed is provided in Table 1, to guide buffer choice or indicate when a rapid buffer exchange is recommended before further analytical or preparative steps.

Discussion

Our results demonstrate that a miniaturized high-throughput workflow – combining cultivation in 1 mL, cryomilling, buffer library screening, and anti-His dot-blot detection – is highly efficient for assessing and optimizing recombinant protein solubility. This integrated approach directly tackles the persistent bottleneck of obtaining soluble protein for downstream studies. Moreover, it integrates seamlessly with other suggested parameters to increase protein solubility.[3,15] A key

innovation is the use of cryomilling for cell lysis. Cryogenic grinding of frozen cell pellets provided efficient and uniform disruption without the heat or shear stresses that can denature proteins during conventional lysis.[16] This ensured that proteins were not prematurely aggregated by the lysis process.

Buffer Code	Buffer composition			SDS-PAGE	Purification yield, n = 2*	
	Buffer	Salt	Additive			
A1	Tris-HCl 50 mM; pH 8.5	50 mM NaCl	10 % glycerol	+++	**0.26**	**0.60**
A3	Tris-HCl 50 mM; pH 8.5	50 mM NaCl	5 % ethanol	+++	**0.41**	**0.44**
A4	Tris-HCl 50 mM; pH 8.5	50 mM NaCl	1 % triton X10	+++	**0.58**	**0.67**
A5	Tris-HCl 50 mM; pH 8.5	50 mM NaCl	100 mM Urea	+++	**0.51**	**0.57**
B2	HEPES 50 mM; pH 7.0	50 mM NaCl	5 % DMSO	+++	**0.67**	**0.68**
B3	HEPES 50 mM; pH 7.0	50 mM NaCl	5 % EtOH	+++	**0.62**	**0.69**
B4	HEPES 50 mM; pH 7.0	50 mM NaCl	1 % triton X10	+++	**0.76**	**0.68**
B5	HEPES 50 mM; pH 7.0	50 mM NaCl	100 mM Urea	+++	**0.81**	**0.64**
C	Tris-HCl 50 mM; pH 8.5	300 mM NaCl	10 % glycerol	+++	**0.45**	**0.66**
G4	Tris-HCl 20 mM, pH 8.0	400 mM NaCl	1 % triton X10	+	**0.71**	**0.76**
H2	Tris-HCl 20 mM, pH 8.0	0.4 M NH4Ac		+	**0.77**	**0.85**
H6	Tris-HCl 20 mM, pH 8.0	2.0 M NH4Ac		+	**0.74**	**0.82**
J2	Tris-HCl 20 mM, pH 8.0	0.4 M NH4Ac	1 % triton X10	+	**0.81**	**0.74**
K1		2.0 M NH4Ac		-	**0.81**	**0.83**
K5		1.5 M NH4Ac		+++	**0.73**	**0.77**
P2	HEPES 50 mM; pH 7.4	200 mM NaCl	1 % triton X10	+	**0.91**	**0.83**
R2	HEPES 50 mM; pH 7.4	0.4 M NH4Ac		+	**0.77**	**0.85**
R6	HEPES 50 mM; pH 7.4	2.0 M NH4Ac		+	**0.61**	**0.80**
T2	HEPES 50 mM; pH 7.4	0.4 M NH4Ac	1 % triton X10	+	**0.87**	**0.81**
T6	HEPES 50 mM; pH 7.4	2.0 M NH4Ac	1 % triton X10	+	**0.76**	**0.79**
W	mQ water			+++	**0.11**	**0.41**

Table 1. Effect of the buffer composition on SDS PAGE and Ni-NTA purification. SDS-PAGE: +++: sharp strong signal, +: peaks are spread or signal fainter, -: very faint signal of smear. W corresponds to the use of mQ water as a control. * yield is calculated based on the Ruby2 fluorescence of purified of His8_Trx_Ruby2 and His_MBP_Ruby2.

The anti-His dot-blot is a rapid, convenient, and sensitive read-out for soluble expression. We reliably detected the target His-tagged protein directly from clarified lysates by spotting 1 µL samples onto a membrane, yielding a semiquantitative "soluble/insoluble" signal within ~2 h. This speed and throughput far exceeded what is feasible with traditional SDS-PAGE or western blotting for dozens of samples. The dot-blot signal provided an immediate ranking of buffer conditions to confirm soluble yield, which helped final buffer selection. However, dot-blot intensity was not a direct proxy for activity: it showed only a weak positive correlation ($R^2 = 0.38$) with fluorescence rank (Sup Fig. 10). In general, intense dots were associated with strong fluorescence, whereas weak dots were accompanied by low signal.

Not unexpectedly, the extraction buffer composition strongly affects both yield and post-lysis solubility—even for the highly soluble reporter Ruby2. This underscores the importance of the extraction buffer effect for proteins that are harder to express and extract at a yield high enough to proceed with further experiments such as crystallography. We found that certain additives had pronounced effects on SDS-PAGE and Ni-NTA purification. For example, buffers containing lower amounts of salt tend to provide a brighter signal in Coomassie blue mediated protein staining. However, for purification of the HIS-tagged protein, we observed the opposite effect: a higher amount of salt favors the binding of the proteins to the Ni-NTA resin, increasing the protein purification yield. These observations highlight the importance of empirically screening a broad buffer space: each protein and subsequent experiment responds differently to buffer constituents, and there is no single "best" buffer for all targets and applications.

An additional strength of our strategy is its throughput and practicality for real-world protein expression optimization. The total sample requirement for each condition is small (1 mL of culture, 1 µL of extraction buffer), making the approach cost-effective. Importantly, we verified that the vast majority of the buffers in our library were compatible with standard downstream analyses. This means that hits from the screen can be translated to preparative scale with minimal adjustments. By uniting upstream lysis through cryomilling with a downstream-friendly buffer screen, our workflow provides a streamlined path from expression to soluble protein ready for purification.

Conclusion

We developed a high-throughput, small-volume method for screening recombinant protein solubility that integrates cryomilling-based cell lysis, a diverse set of extraction buffers, and a rapid dot-blot assay. This 96-well format workflow efficiently identifies optimal buffer conditions and maximizes the soluble yield of a target His-tagged protein. This method is effective as almost all tested buffer conditions are immediately compatible with standard purification and analysis steps such as Ni-NTA purification and SDS-PAGE analyses, demonstrating a clear translation from screening leads to scalable protein production. The practical benefits (speed, low cost, and the ability to pinpoint favorable conditions early) make this integrated method a valuable tool for any workflow aiming to produce challenging recombinant proteins.

ASSOCIATED CONTENT

The following files are available free of charge.
Supporting tables and figures (.pdf)
Raw data and raw imaged (.zip)
Detailed protocol and material (.zip)

AUTHOR INFORMATION

Corresponding Author

* Téo Hebra - Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, 160 00 Prague, Czech Republic; Orcid https://orcid.org/0000-0002-5878-259X Email: teo.hebra@uochb.cas.cz

Authors

Adrian Svoboda- Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, 160 00 Prague, Czech Republic

Marina Molineris- Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, 160 00 Prague, Czech Republic

Tomáš Pluskal - Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, 160 00 Prague, Czech Republic; Orcid https://orcid.org/0000-0002-6940-3006;

Author Contributions

A.S.: Investigation and validation. M.M.: Investigation. T.P.: Resources, funding, project administration, writing, and revisions. T.H.: Conceptualization, methodology, investigation, supervision, validation, writing, and revisions.

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ABBREVIATIONS

SDS-PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis, Ni-NTA: nickel Nitriloacetic acid; PVDF : Polyvinylidene fluoride or polyvinylidene difluoride

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