High-throughput automated refolding screening of inclusion bodies

RENAUD VINCENTELLI,[1] STÉPHANE CANAAN,[1] VALÉRIE CAMPANACCI, CHRISTEL VALENCIA,[2] DAMIEN MAURIN, FRÉDÉRIC FRASSINETTI, LORÉNA SCAPPUCINI-CALVO, YVES BOURNE, CHRISTIAN CAMBILLAU, AND CHRISTOPHE BIGNON

Architecture et Fonction des Macromolécules Biologiques, Unité Mixte de Recherche (UMR) 6098, Centre National de la Recherche Scientifique (CNRS) et Universités d'Aix-Marseille I et II, 13402 Marseille Cedex 20, France

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Abstract

One of the main stumbling blocks encountered when attempting to express foreign proteins in *Escherichia coli* is the occurrence of amorphous aggregates of misfolded proteins, called inclusion bodies (IB). Developing efficient protein native structure recovery procedures based on IB refolding is therefore an important challenge. Unfortunately, there is no "universal" refolding buffer: Experience shows that refolding buffer composition varies from one protein to another. In addition, the methods developed so far for finding a suitable refolding buffer suffer from a number of weaknesses. These include the small number of refolding formulations, which often leads to negative results, solubility assays incompatible with high-throughput, and experiment formatting not suitable for automation. To overcome these problems, it was proposed in the present study to address some of these limitations. This resulted in the first completely automated IB refolding screening procedure to be developed using a 96-well format. The 96 refolding buffers were obtained using a fractional factorial approach. The screening procedure is potentially applicable to any nonmembrane protein, and was validated with 24 proteins in the framework of two Structural Genomics projects. The tests used for this purpose included the use of quality control methods such as circular dichroism, dynamic light scattering, and crystallogenesis. Out of the 24 proteins, 17 remained soluble in at least one of the 96 refolding buffers, 15 passed large-scale purification tests, and five gave crystals.

Keywords: screening; refolding; solubility; inclusion bodies; automation; high-throughput

Reprint requests to: Stéphane Canaan or Christophe Bignon, Architecture et Fonction des Macromolécules Biologiques, UMR 6098, CNRS et Universités d'Aix-Marseille I et II, 31 chemin Joseph Aiguier, 13402 Marseille Cedex 20, France; e-mail: stephane.canaan@afmb.cnrs-mrs.fr or bignon@afmb.cnrs-mrs.fr; fax: +00-334-91-16-45-36.

[1]These authors contributed equally to this work.

[2]Present address: Institut Gilbert Laustriat, IFR85, 74 route du Rhin, BP 60024, F-67401 Illkirch Cedex, France.

Abbreviations: BAC, bacterial artificial chromosome; β-MSH, β-mercaptoethanol; BSA, bovine serum albumin; CD, circular dichroism; DLS, dynamic light scattering; DsbA, disulfide oxidoreductase; GSH, reduced glutathione; GSSG, oxidized glutathione; IB, inclusion bodies; IP, isoelectric point; MT, *Mycobacterium tuberculosis*; OD, optical density; PEG, polyethylene glycol; SEC, size exclusion chromatography; SG, structural genomics; SPINE, Structural Proteomics In Europe.

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In the context of Structural Genomics (SG) projects involving targets from *Escherichia coli* (ASG), *Mycobacterium tuberculosis* (MT), and viruses (SPINE), we have performed expression assays on ~600 genes (Sulzenbacher et al. 2002; Vincentelli et al. 2003). One of the main obstacles we and other authors have encountered when expressing recombinant proteins in *E. coli* is the relatively low soluble protein yield obtained with many of the source organisms used. In the case of eukaryotes, viruses, and *Mycobacterium tuberculosis*, most of the genes were expressed in the form of insoluble aggregates called "inclusion bodies" (IB). This obstacle to obtaining suitable targets for performing structural studies was particularly severe in the case of MT, with which 93% of our 182 tar-

gets yielded IB when proteins were expressed fused to an N-terminal His tag.

IBs are assumed to result from illegitimate interactions between hydrophobic residues located in the core of different molecules. This process is auto-catalyzed and therefore rapidly results in the precipitation of all the recombinant proteins produced in the cell (Mukhopadhyay 1997). Methods have been designed to recover correctly folded proteins from these amorphous aggregates. These include the "dilution," "dialysis," and "solid phase" methods (De Bernardez-Clark 1998), all of which involve an initial IB solubilization step using highly concentrated solutions of chaotropic agents such as guanidinium chloride and urea. The subsequent step in all these methods consists of removing the denaturing agent and restoring the protein to its native shape from the unfolded soluble state. The pathway used to remove the chaotropic agent differs between the three methods, however, although the same result is reached in each case. With the dilution method, refolding is assumed to occur immediately upon diluting the protein in a large volume of nondenaturing buffer ("refolding buffer"), which has to be sufficiently large to both cancel out the solubilizing effect of the chaotropic agent and reduce the probability that protein interactions will occur. The dialysis method involves the use of the same initial and final buffer compositions as the dilution method, but in this case, there is no dilution to decrease the protein–protein contacts (Rudolph and Lilie 1996; Mukhopadhyay 1997). Finally, it was established that physically separating molecules from each other during the renaturation process (solid phase refolding) greatly improved the refolding yield (Stempfer et al. 1996).

Whatever the method used to replace denaturing by nondenaturing buffer (a dilution, dialysis, or solid state method), it would be easier to use a single refolding buffer. Unfortunately, experience has shown that the composition of the refolding buffer is strongly protein dependent and that simply maintaining a difference between the pH of the refolding buffer and the isoelectric point (IP) of the protein does not usually suffice to keep the protein soluble.

Hence the idea of testing several refolding buffers simultaneously. For instance, Perbio has addressed this issue with Pro-Matrix, a refolding kit consisting of nine basic buffers, which can be supplemented with additives (Qoronfleh 2004). Using a fractional factorial approach, Armstrong et al. (1999), Chen and Gouaux (1997), and Hampton Research (FoldIt) have each developed separate procedures using 16 refolding conditions.

Despite these improvements, some difficulties were still encountered in the protein solubility assays performed to monitor the refolding process. Because no solubility assay was provided with the Pro-Matrix kit, this assay had to be set up by the customer, and the methods suggested for a solubility assay in the case of the FoldIt kit (size exclusion chromatography [SEC]), as well as those used by Armstrong et al. (1999) and Chen and Gouaux (1997) (dialysis and centrifugation), were not compatible with a high-throughput or with automation, which are two of the most crucial features in SG studies.

To solve the problems associated with the above limitations, a protein solubility test based on light scattering has been devised (Trésaugues et al. 2004). In practice, the turbidity of the solution is assessed by measuring the optical density (OD) at 390 nm, before and after adding the protein. If the protein remains soluble, the absorbance remains unchanged. In the opposite case, the OD increases proportionally to the amount of precipitate produced. This procedure is much faster than SEC and can be easily automated, but the number of conditions was still limited to 12, and the proteins often precipitated in all of them. This clearly suggested that the number of conditions needed to be further increased. A method of making this quantitative jump has been experimented in microtiter plate format, using 203 refolding conditions (Sijwali et al. 2001). However, the latter study was only designed for screening different GSH:GSSG ratios.

It is worth noting that although increasing the number of refolding conditions increases the probability that a protein will meet a buffer composition favoring its solubility, it also increases the number of samples to be handled. One possible solution to this problem consists of automating the screening process. In addition, automation is required to obtain sufficiently large SG throughputs. A partially automated refolding screening procedure was recently described (Scheich et al. 2004). With this procedure, however, the automation did not include any test for assessing the solubility and only 30 refolding conditions were used.

We therefore designed a refolding strategy involving the use of 96 different buffers in microtiter plate format, based on the above mentioned idea that the probability of a protein encountering a buffer composition favoring correct folding was likely to increase with the number of buffers tested. The solubility assay used in our screening procedure is basically the same as that described by Trésaugues et al. (2004), which accounts for protein *solubility*, and not for protein *folding*. After the preparatory refolding stage, circular dichroism (CD), dynamic light scattering (DLS), and crystallogenesis quality control procedures were added to respectively assess the folding, aggregation state, and homogeneity of the protein solution. These methods were chosen because they can be applied in theory to any protein, which is a prerequisite in the field of post-Genomics, which deals mainly with proteins having an unknown function. Finally, the availability of a pipetting robot made it possible to automate the whole process in a 96-well plate format.

To the best of our knowledge, this is the first completely automated "wide spectrum" 96-well IB refolding screening procedure to be developed based on a factorial approach. The present article describes the setup involved and con-

firms the validity of the method, based on tests carried out with proteins originating from two SG projects.

Results

Optimization of the solubility assay

The recently described solubility test, in which the turbidity of the solution is measured in terms of the light absorbance at 390 nm, involves light scattering by a protein precipitate (Trésaugues et al. 2004). As no proof was available that this wavelength was the most suitable one, we first addressed this point.

For this purpose, the absorbance of a bovine serum albumin (BSA) precipitate was scanned between 230 and 600 nm. As shown in Figure 1 (curve A), the absorbance decreased continuously from 230 to 600 nm. In addition to this regular decay, a small shoulder was present in the 280 nm region. To determine whether this feature was due to any remaining soluble proteins, the precipitate was spun down and the scanning performed again on the supernatant. Surprisingly, in this case, $OD_{230–600}$ was indistinguishable from the baseline, which means that the protein content had been entirely converted into insoluble species. These results indicate that the absorbance pattern of the protein precipitate, which is shown in Figure 1 (curve A), was entirely accounted for in terms of light scattering and not even partially in terms of the absorbance of soluble proteins.

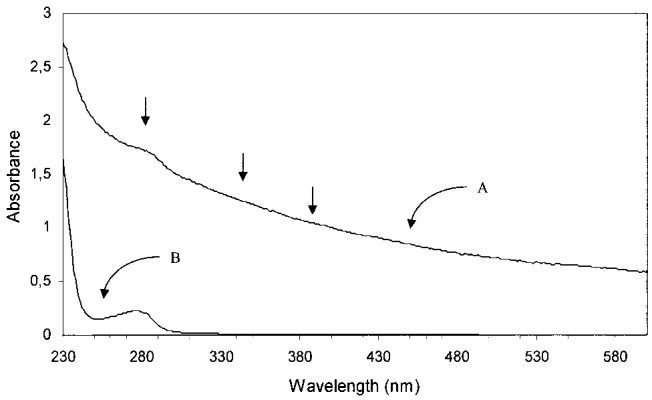

Figure 1. Absorbance spectra of precipitated and soluble forms of a protein. Twenty microliters of a 20 mg/mL BSA solution were diluted in 500 μL of either 100% isopropanol or 8 M guanidinium chloride. A chaotropic solution was used to ensure that the entire protein content was soluble. The absorbance of the resulting protein suspension (in isopropanol) or solution (in guanidinium chloride) was recorded from 230 to 600 nm, using a Varian Cary Scan 50 spectrophotometer. After subtracting the baseline (the absorbance of each solvent in the absence of protein), the absorbance intensities were plotted vs. the wavelengths. (Curve *A*) Precipitated protein in isopropanol. (Curve *B*) Soluble protein in guanidinium chloride. From *left* to *right*, three vertical arrows indicate the position of 280, 340/350, and 390 nm wavelengths, respectively.

Because the solubility assay was expected to distinguish between the absorbance due to precipitated and soluble proteins, the same experiment was performed under conditions where the proteins remained 100% soluble. In this case (Fig. 1, curve B), the absorbance profile was that of a typical protein solution, peaking at 280 nm (aromatic side chains) and at 200 nm (peptide bonds). Note that only the beginning of the peptide bonds' absorbance peak (λ max 200 nm; Stoscheck 1990) was visible between 230 and 240 nm.

In conclusion, the wavelength to be used in the solubility test should satisfy the following contradictory criteria: (1) It should be high enough above 280 nm to prevent any risk of obtaining false negative results due to the absorbance of (partially or totally) soluble proteins, at values of 280 nm and below, but (2) it should be as small as possible to provide the highest signal-to-noise ratio, according to curve A, and hence the most sensitive assay. In practice, 340- (manual procedure) and 350-nm (automated procedure) wavelengths were selected because they fulfilled these two criteria and provided better results than 390 nm.

Selection of 96 refolding conditions

The chemicals listed in Table 1, which were used to prepare the refolding mixes presented in Figure 2, were selected on the basis of the following criteria:

1. A 4 pH to 9 pH range was chosen because the proteins to be screened had various IPs and were likely to denature below or above these values.
2. Various ionic strengths (none; 100 mM NaCl or KCl; and 200 mM NaCl) were used because the solubility can increase (salting in) or decrease (salting out) with the salt concentration from one protein to another.
3. With the dilution method used, refolding was allowed to proceed for a very short time. Amphiphilic components (glycerol, PEG) were introduced to prevent the hydrophobic residues of different molecules still accessible at intermediate refolding stages from interacting with each other. In addition, glycerol and PEG were already provided in other refolding kits (Trésaugues et al. 2004) and were compatible with crystallogenesis. Glucose and arginine were used for the same reason, although Arg had to be removed before the crystallogenesis trials (see below).
4. Solubilizing reagents in the NDSB series were selected because they have been successfully used in protein crystallogenesis (Karaveg et al. 2003) and refolding experiments (Vuillard et al. 1998; Expert-Bezancon et al. 2003).
5. Proteins bearing odd numbers of cystein can form unnatural intermolecular disulfide bonds, which is a pos-

Table 1. *Chemicals used to make the 80 first refolding buffers*

Buffer (50 mM)	Ionic strength	Amphiphilic	Detergent (100 mM)	Reducing agent (10 mM)	Additive
NaAc, pH 4	NaCl 100 mM	Glycerol 20% (v/v)	NDSB 195	β-MSH	Arginine 800 mM
MES, pH 5	NaCl 200 mM	PEG 4000 0.05% (w/v)	NDSB 201		Glucose 500 mM
MES, pH 6	KCl 100 mM	PEG 400 0.05% (w/v)	NDSB 256		Cocktail[a]
TRIS, pH 7					EDTA 1 mM
TRIS, pH 8					
CHES, pH 9					

The concentrations indicated are those used before adding the protein.
[a] Consisted of 50 μM of each of the following: NADH, thiamine HCl, biotine, $CaCl_2$, $MgCl_2$, $CuSO_4$, $ZnCl_2$, $CoSO_4$, ADP, and $NiCl_2$.

sible cause of precipitation during the refolding process. Ten millimolar of β-MSH were introduced to prevent this mispairing.

6. The "cocktail" contained potential cofactors that might be required during the refolding process in the case of some proteins, whereas some other proteins tend to precipitate in the presence of divalent cations, hence the presence of EDTA.
7. The chaotrops (urea and guanidinium chloride) present in the commercial kits were discarded because they were liable to damage the robot's pipetting valves.

It was necessary to use a fractional factorial approach on the first 80 wells, because the combination of 20 chemicals would have resulted in too many experimental points (the full factorial design would have been 2560 combinations).

In the 16 remaining microplate wells, mini chaperones (a soluble form of GroEL; Altamirano et al. 1997) and redox components (GSSH, GSSG, DsbA) were combined, because the disulfide bond formation/reduction during the folding process itself has been found to be crucial (Wei et al. 1999). Details of each of the refolding conditions are given in Figure 2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	pH 4, β-MSH, Arg	pH 4, KCl, β-MSH, NDSB256	pH 5, NaCl 200 mM	pH 5, KCl, glucose	pH 6, KCl	pH 7	pH 7, NDSB201, Arg	pH 8, KCl, β-MSH, NDSB201, Arg	pH 8, β-MSH	pH 9, glycerol, NDSB201	* GSH 5 mM	* GSH 5 mM, GSSG 5 mM, DsbA 10 mM
B	pH 4, β-MSH, NDSB256	pH 4, β-MSH, NDSB201, glucose	pH 5, NDSB256	pH 5, cocktail	pH 6, NDSB256, glucose	pH 7, KCl	pH 7, PEG 4000, β-MSH	pH 8, glucose	pH 8, NDSB201	pH 9, NaCl 100 mM, glucose	* GSH 5 mM, GSSG 2 mM	* GSH 2 mM, GSSG 5mM, DsbA 10 mM
C	pH 4, NaCl 100 mM, β-MSH	pH 4, KCl, NDSB195	pH 5, EDTA, β-MSH, NDSB201	pH 6, PEG 400, β-MSH, NDSB201	pH 6, glycerol, β-MSH	pH 7, NaCl 200 mM, NDSB201	pH 7, NDSB195, glucose	pH 8	pH 9, PEG 4000, β-MSH, glucose	pH 9, β-MSH, NDSB195, Arg	* GSH 5 mM, GSSG 5 mM	* GSSG 5mM, DsbA 10mM
D	pH 4, glycerol	pH 4, PEG 4000, glucose	pH 5, β-MSH, glycerol	pH 6, β-MSH, NDSB195, glucose	pH 6, EDTA	pH 7, glycerol, β-MSH	pH 7, EDTA, β-MSH, NDSB195	pH 8, PEG 4000, NDSB195	pH 9, KCl, β-MSH, NDSB201, glucose	pH 9, PEG 400	* GSH 2 mM, GSSG 5 mM	* GSSG 10mM, DsbA 10mM
E	pH 4, PEG400, glucose	pH 5, EDTA, Arg	pH 5, PEG 400, β-MSH, Arg	pH 6, glycerol, NDSB256, Arg	pH 6, NaCl 100 mM, β-MSH, NDSB195	pH 7, PEG 4000, NDSB256, Arg	pH 7, cocktail	pH 8, β-MSH, glucose	pH 9, β-MSH	pH 9	* GSSG 5 mM	* dbGroeL 10mM
F	pH 4, EDTA	pH 5, NaCl 100 mM, Arg	pH 5, β-MSH, NDSB256	pH 6, NaCl 200 mM, β-MSH, glucose	pH 6	pH 7, NaCl 100 mM, NDSB201	pH 8, Arg	pH 8, NaCl 100 mM, β-MSH, NDSB256	pH 9, NaCl 100 mM, NDSB256	pH 9, EDTA, β-MSH, Arg	* GSSG 10 mM	* dbGroeL 10 mM, DsbA 10mM
G	pH 4, NDSB201	pH 5, PEG 4000, β-MSH, NDSB201	pH 5, glycerol, β-MSH, NDSB195	pH 6, β-MSH NDSB201, Arg	pH 6, PEG 4000	pH 7, β-MSH, Arg	pH 8, EDTA, NDSB256, glucose	pH 8, NaCl 200 mM, β-MSH, glucose	pH 9, β-MSH, NDSB195	pH 9, NDSB195, Arg	* GSH 5 mM, DsbA 10 mM	* GSSG 10mM, dbGroeL 10mM, DsbA 10mM
H	pH 4, NaCl 200 mM, β-MSH, NDSB195, Arg	pH 5, NaCl 200 mM, NDSB195	pH 5, β-MSH	pH 6, PEG 400, NDSB256	pH 7, PEG 400, β-MSH, NDSB256, glucose	pH 7, β-MSH	pH 8, glycerol, β-MSH	pH 8, PEG 400, NDSB195	pH 9, NaCl 200 mM, β-MSH, NDSB256	pH 9, cocktail	* GSH 5 mM, GSSG 2 mM, DsbA 10 mM	*

Figure 2. Detailed composition of each well in the refolding plate. (*) Tris (pH 8), NaCl 150 mM, EDTA. For details, see Table 1.

Testing of 96 refolding conditions

The 96-well screening procedure was tested on a panel of 24 proteins from two SG projects: MT (18 targets) and SPINE (6 targets). The results obtained are given in Table 2. Eleven out of the 18 MT targets (61%) and all the SPINE targets subjected to screening remained soluble under at least one of the 96 refolding conditions. In addition, except for MT target Rv1373 (buffer 57), all the responsive targets remained soluble in many buffers, which made it possible to choose the most suitable one(s) for the downstream steps such as crystallogenesis. In addition, the pH was not found to be a decisive parameter, because most of the targets remained soluble in a wide pH range, except Rv1525, Rv1515c, Rv0323c, and Rv2045, which remained soluble only at pH 4. Generally speaking, no particular buffer composition (pH, ionic strength, etc.) peaked more than the others, which suggests that the solution was always protein specific. The solubility yield at the production stage also appeared to be very high: 10 out of the 11 responsive MT targets (91%), and five out of the six responsive SPINE targets (83%) succeeded in passing the large-scale refolding and the first concentration steps. Only one SPINE (63) and two MT (Rv0323c and Rv1515c) targets were lost during the second concentration step following the gel filtration. In these particular cases, CD was nonetheless performed, but on protein solutions with concentrations too low for crystallogenesis.

Validity of the refolding screening procedure

Protein *solubility* and *folding* superimpose satisfactorily, but the overlap is not always 100%. We therefore tried to assess

Table 2. *(A) MT and SPINE targets remaining soluble in at least one refolding buffer and (B) summary of positive targets at each step*

A	Target	MW	Organism	Soluble in buffer [a]	Purification	IP	pH	CD	DLS	Crystal
	Rv2391	66	MT	39, 54, 57	57 (–Arg)	6.31	8	nd	nd	No
	Rv2392	30	MT	39, 49, 55, 56, 59, 61, 63, 64, 66	59	5.87	8	Ok	nd	Yes
	Rv1399c	36	MT	41, 44, 48, 49, 56, 59, 65, 66	41	4.38	7	Ok	M	Yes
	Rv1208	37	MT	41, 43, 48, 54, 56, 59, 63, 65, 66, 68, 69, 70, 74, 80	74	4.75	9	Ok	A	Yes
	Rv1373	40	MT	57	57 (–Arg)	6.36	8	nd	A	No
	Rv1564c	84	MT	41, 43, 44, 49, 56, 57, 59, 63, 66	41	4.95	7	Ok	D	No
	Rv1523	40	MT	4, 7, 10, 11, 12	4 (–glyc)	8.06	4	Ok	nd	No
	Rv1515c	36	MT	4, 5, 7, 10, 11, 12	4 (–glyc)	6.79	4	Ok	nd	No[b]
	Rv0323c	27	MT	2, 3, 4, 5, 9, 10, 11, 12	4 (–glyc)	5.81	4	Ok	nd	No[b]
	Rv2045c	59	MT	3, 4, 5, 6, 7, 10, 11, 12	4	7.67	4	Ok	nd	No
	Rv3487c	29	MT	2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, 15, 18, 19, 20, 21, 22, 23, 24, 29, 45, 47, 49, 54, 57, 75	nd	8.85		nd	nd	nd
	SPINE 5	23	Sendai	10, 58, 59, 67, 73, 76	69	5.06	9	Ok	T	Yes
	SPINE 10	23	Measles	1, 2, 3, 4, 5, 6, 9, 10, 11, 12, 16, 17, 22, 24, 26, 32, 49, 54, 75, 78, 79	6	8.99	4	Ok	A	No
	SPINE 21	52	SFV	1, 2, 3, 4, 6, 7, 8, 9, 11, 12, 13, 21, 29, 31, 45, 49, 75, 78	4, 6	8.80	4	nd	A	No
	SPINE 22	53	SFV	2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 31, 45, 49, 57, 78, 79	nd	9.03		nd	nd	nd
	SPINE 23[c]	23	Human	All except 5, 6, 16, 17, 26, 42, 53, 61, 65, 76	33	8.68	6	Ok	D	Yes
	SPINE 63	23	HIV	1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 25, 26, 27, 28, 29, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 47, 48, 49, 51, 52, 54, 55, 57, 58, 66, 78	19	9.9	5	Ok	H	nd[b]

B	Target number	Responsive targets	Large-scale purification	CD OK	DLS OK	Crystal
MT	18	11	10	8	2	3
SPINE	6	6	5	4	3	2

(Target) The Rv nomenclature used was that of the MT genome (Cole et al., 1998; Camus et al. 2002). (MW) theoretical molecular weight (kDa). (IP) isoelectric point (taking into account the His tag when present). (pH) pH of the mix used for large-scale purification. (CD) ok, the protein fulfilled the criteria defined in Materials and Methods. (DLS) Only the main (>95%) population (M, D, etc.) was included in the table. (M) monomeric; (D) dimeric; (T) tetrameric; (H) Hexameric; (A) Aggregates (see Materials and Methods for details).

[a] The numbers refer to the buffers listed in Fig. 2 (1 = 1A, 2 = 1B … 9 = 2A, etc.). (–Arg), (–glyc) protein purification was performed using the buffer indicated devoid of arginine or glycerol, respectively.

[b] Lost during gel filtration or after the last concentration step.

[c] This target was not refolded from IB, but from a Ni eluate that precipitated just after elution.

(Target number) Number of targets subjected to refolding screening. (Responsive targets) Number of targets subjected to refolding screening that remained soluble in at least one refolding buffer. (DLS OK) DLS was taken to be satisfactory when the criteria defined in Materials and Methods were fulfilled.

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