Efficient Protein Refolding Screening in a 96-well Format

Peter A. Leland, James L. Rane, Jennifer G. Reich, Tsetska Takova, and Anthony Grabski
Novagen, a brand of EMD Chemicals Inc. 441 Charmany Drive, Madison WI, USA 53719

Abstract

Proteomics requires large amounts of highly pure and correctly folded protein. Most commonly, this need is met using heterologous expression systems such as *Escherichia coli*. Production of foreign proteins in *E. coli* commonly results in the formation of inclusion bodies –dense, insoluble, aggregates of mis-folded protein. Inclusion bodies are typically viewed as undesirable, however, they do have positive attributes as they are easily purified, resistant to proteolysis and can be solubilized with chaotropic agents. Unfortunately, defining conditions that promote refolding of a chemically solubilized target protein into its native conformation is both empirical and difficult. The chances of identifying an optimal refolding condition are increased by simultaneously and systematically evaluating a large number of refolding conditions. To enable this type of experiment, we have developed two 96-well plate-based protein refolding screen systems. The systems differ in the chemistry used to denature the inclusion bodies and in the refolding additives included in the 96-well plate. One system uses N-lauroylsarcosine, a chaotropic anionic detergent, to denature the inclusion bodies while the second system uses either guanidine hydrochloride or urea. Protein refolding is accomplished by rapid dilution of the denatured protein into the 96-well plate that contains an array of buffers, salts, redox couples, cyclodextrin, nondetergent sulfobetains (NDSBs), as well as other refolding additives. With these refolding screening systems, we have successfully identified optimal conditions for refolding several recombinant proteins, including a green fluorescent protein fusion, phosphatase, mammalian endopeptidase, matrix metallo-protease, and viral protease. Analysis of the various target protein activities following refolding revealed clear requirements for successful refolding, both in terms of the inclusion body denaturant and in the constituents of the refolding solutions. High-yielding refolding conditions identified by the screen have successfully been scaled 10,000-fold from a 50 µg refolding reaction to 500 mg. Significantly, all steps of the refolding screens are equally compatible with manual use and high-throughput automated liquid handling.

Table 1. iFOLD™ Protein Refolding Systems Components

	iFOLD™ System 1	iFOLD™ System 2
IB wash agent	1.0% Triton X-100	0.125 M NDSB-201 (or Triton X-100)
IB denaturant	4.4% N-lauroylsarcosine	7.0 M GuHCl (or 8.0 M urea)
IB reducing agent	5 mM TCEP	10 mM TCEP
Denatured target protein concentration	1 mg/mL	5 mg/mL
Denatured target protein volume	5 mL	1 mL
Total protein per well	50 µg	50 µg
Buffer system (50 mM)	TRIS–HCl, pH 7.0, 7.5, 8.0 or 8.5	MOPS, pH 7.0 HEPES, pH 7.5 EPPS, pH 8.0 TAPS, pH 8.5 CHES, pH 9.0
Refolding additives	100 mM NaCl 250 mM NaCl 1.0 mM TCEP 3.8 mM GSH + 1.2 mM GSSG 1.0 mM EDTA 1.0 mM $CaCl_2$ + 1.0 mM $MgCl_2$ 0.5 M GuHCl 20% glycerol 0.1% PEG6000 0.5 M L-arginine 12.5 mM methyl-β-D-cyclodextrin	24 mM NaCl + 1.0 mM KCl 240 mM NaCl + 10 mM KCl 1.0 mM TCEP 9.0 mM GSH + 1.0 mM GSSG 6.0 mM GSH + 4.0 mM GSSG 1.0 mM EDTA 0.25 mM each of $CaCl_2$, $MgCl_2$, $MnCl_2$ & $ZnCl_2$ 0.5 or 1.0 M NDSB-201 0.5 or 1.0 M NDSB-256 1.5 M sorbitol 0.58 M trehalose 0.06% PEG3350 0.5 M L-arginine 10 mM methyl-β-D-cyclodextrin

Figure 1. Brief protocol

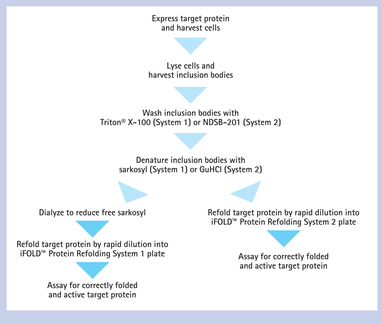


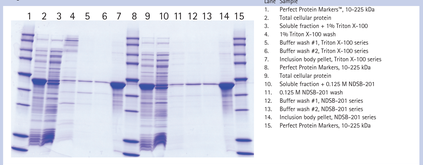
Figure 2. iFOLD Systems 1 & 2 plate layouts

Design Expert® software (Stat-Ease®, Minneapolis, MN) was used to combine reagents in a matrix with maximal diversity and a total of 92 trials (and four control wells; System 1) or a total of 95 trials (and one control well; System 2).

System 1

System 2

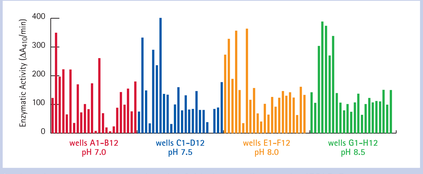
Figure 3. Preparation of thioredoxin-GFP (trx-GFP) inclusion bodies using iFOLD Systems 1 and 2



trx-GFP was expressed in *E. coli* strain BL21-DE3 using Overnight Express™ Instant TB medium. Cells were lysed by sonication and inclusion bodies prepared according to the iFOLD System 1 protocol (1% Triton X-100 wash; lanes 2–7) or to the iFOLD System 2 protocol (0.125 M NDSB-201 wash; lanes 9–14).

The wash protocols of Systems 1 and 2 yield inclusion body pellets of comparable purity (compare lanes 7 and 14). Use of NDSB-201 as a wash agent in System 2 allows for a refolding experiment that is free of detergent.

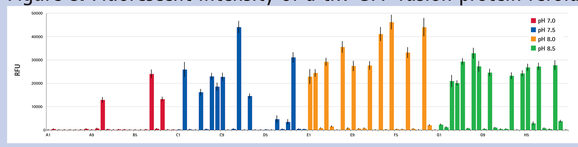
Figure 4. Catalytic activity of λ phosphatase refolded with iFOLD System 1



λ phosphatase was prepared and refolded according to the iFOLD System 1 protocol (Figure 1). Following 20 h at 22°C, catalytic activity was measured by recording cleavage of 4-nitrophenyl phosphate substrate at 30°C.

Review of the λ phosphatase refolding screen revealed that 13 of the 14 conditions with the greatest enzymatic activity contained TCEP or the glutathione redox buffer. λ phosphatase contains a surface exposed Cys that can cause aggregation and precipitation during refolding.

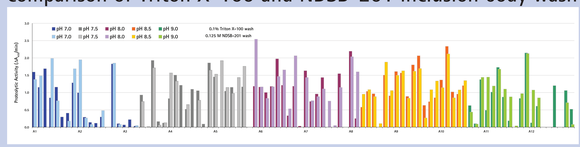
Figure 5. Fluorescent intensity of a trx-GFP fusion protein refolded with iFOLD System 1.



trx-GFP was prepared and refolded according to the iFOLD System 1 protocol (Figures 1 & 2). Following 24 h at 22°C, refolding trials were diluted 1:4 with 50 mM Tris-Cl, pH 8.0, and the relative fluorescent intensity for each trial was recorded (390 nm excitation, 510 nm emission). Data represent the average ± standard error of the mean for 11 experiments.

Review of the trx-GFP refolding screen revealed that each of the 10 brightest wells contained methyl-β-D-cyclodextrin. The cyclodextrin acts as a detergent trap, effectively removing sarkosyl from the refolding solution. Thus, residual sarkosyl appears to be detrimental to refolding of trx-GFP.

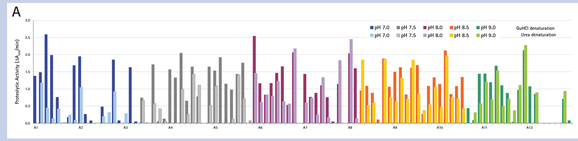
Figure 6. Enzymatic activity of HRV 3C protease after refolding with iFOLD System 2; comparison of Triton X-100 and NDSB-201 inclusion body wash protocols

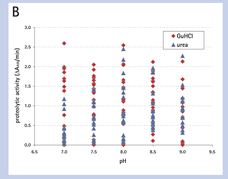


HRV 3C inclusion bodies were washed with 1% Triton X-100 or 0.125 M NDSB-201, denatured with guanidine-HCl and diluted into the iFOLD System 2 refolding matrix. Following 24 h at 22°C, refolding trials were dialyzed overnight against 8.0 L of 20 mM Tris-Cl, pH 7.0, containing 150 mM NaCl, 5.0 mM DTT, and 0.03% Brij-35 using the D-Tube96™ Dialyzer (EMD). Enzymatic activity of the refolded and dialyzed samples was quantified by measuring cleavage of a peptide substrate (H-Glu-Ala-Leu-Phe-Gln-pNA; Bachem L-2050).

HRV 3C inclusion bodies refolded with comparable efficiency when washed with NDSB-201 or TRITON X-100.

Figure 7. Enzymatic activity of HRV 3C protease after refolding with iFOLD System 2; comparison of GuHCl and urea denaturation protocols

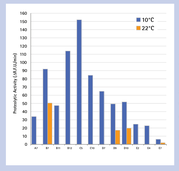




HRV 3C inclusion bodies were washed with 0.125 M NDSB-201, denatured with 7.0 M GuHCl or 8.0 M urea, and diluted into the iFOLD System 2 refolding matrix. Following 24 h at 22°C, refolding trials were dialyzed overnight against 8.0 L of 20 mM Tris-Cl, pH 7.0, containing 150 mM NaCl, 5.0 mM DTT, and 0.03% Brij-35 using the D-Tube96 Dialyzer. Enzymatic activity of the refolded and dialyzed samples was quantified by measuring cleavage of a peptide substrate (Glu-Ala-Leu-Phe-Gln-pNA; Bachem L-2050). Figure 7A shows individual refolding wells; Figure 7B shows refolding wells grouped by pH.

Figure 7B illustrates that at pH 7.0 and 7.5, proteins denatured with GuHCl refold with greater efficiency than do proteins denatured with urea.

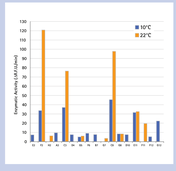
Figure 8. Enzymatic activity of enterokinase (EK) after refolding with iFOLD System 2



EK inclusion bodies were washed with 1% Triton X-100, denatured with 7.0 M GuHCl, and diluted into the iFOLD System 2 refolding matrix equilibrated to 10 or 22°C. Following 24 h at 10 or 22°C, refolding trials were dialyzed overnight against 8.0 L of 20 mM Tris-Cl, pH 7.5, containing 150 mM NaCl using the D-Tube96 Dialyzer. Enzymatic activity of the refolded and dialyzed samples was quantified by measuring cleavage of a peptide substrate (Gly-Asp-Asp-Asp-Asp-Lys-β-naphthylamide ; Sigma G5261).

Unlike most targets tested with the iFOLD Systems, EK refolding was more efficient at a reduced temperature. All 12 conditions with detectable proteolytic activity contained a glutathione redox buffer. EK includes four disulfide bonds and one cysteine residue.

Figure 9. Enzymatic activity of matrix metalloprotease12 (MMP12) after refolding with iFOLD System 2



MMP12 inclusion bodies were washed with 1% Trition X-100, denatured with 7.0 M GuHCl and diluted into the iFOLD System 2 refolding matrix equilibrated to 10 or 22°C. Following 24 h at 10 or 22°C, refolding trials were dialyzed overnight against 8.0 L of 50 mM Tris-Cl, pH 7.5, containing 150 mM NaCl, 2.0 mM $CaCl_2$, 1.0 µM $ZnCl_2$ and 0.03% Brij-35 using the D-Tube96 Dialyzer. Enzymatic activity of the refolded and dialyzed fractions was quantified by measuring cleavage of BODIPY®-labeled elastin (DQ-Elastin™, Molecular Probes E-12056).

Analysis of results from the refolding screen reveals clear requirements for successful refolding of MMP-12. Three of the four wells with greatest enzymatic activity contain divalent metals. Additionally, the four wells with the greatest activity contain the refolding additives NDSB-256 or L-Arg.

Conclusions

The iFOLD Protein Refolding Systems are simultaneous and systematic screens of refolding conditions for target proteins expressed as bacterial inclusion bodies. The kits contain reagents to purify and denature the inclusion bodies and a 96-well refolding plate.

The iFOLD System 2 protocol is flexible, accommodating a choice of inclusion body washing agents (NBSD-201 or Triton X-100) and a choice of inclusion body denaturation agents (guanidine-HCl or urea). When NDSB-201 is used as an IB wash agent, the iFOLD System 2 is free of detergent.

Refolding screens using the iFOLD Systems commonly identify a singular, superior refolding condition. In experiments where no single condition predominates, analysis of results reveals clear commonalities among successful refolding reactions.

The literature that accompanies the iFOLD Systems details the composition of all refolding wells and includes a vendor list for the source materials, facilitating design and assembly of large-scale refolding experiments.

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