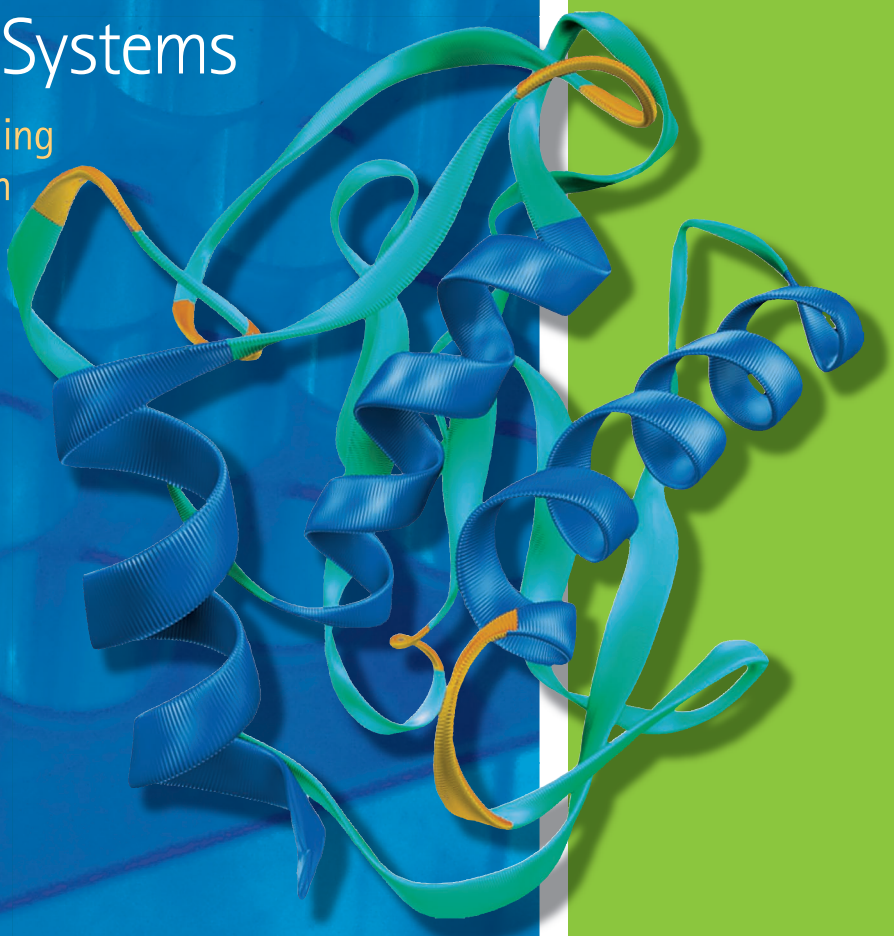
Novagen®

iFOLD™
Protein Refolding Systems

Take the guesswork out of finding the optimal refolding condition for your target protein!



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iFOLD™ Protein Refolding Systems at a Glance

Protein functional and structural studies often require a large amount of pure, correctly folded protein, which is commonly produced in *Escherichia coli* (*E. coli*) expression systems. However, production of foreign proteins in *E. coli* can result in the formation of inclusion bodies (IB) – dense, insoluble, aggregates of misfolded protein. IB also have useful attributes – they are easily purified, resistant to proteolysis, and can be solubilized with chaotropic agents. Defining conditions that promote refolding of a chemically denatured protein into its native conformation is empirical and often time consuming. Simultaneous and systematic evaluation of a large number of refolding conditions increases chances of identifying an optimal refolding condition for a given protein.

We have taken the tedium and guesswork out of finding the optimal refolding condition for your target protein! Our new iFOLD™ Protein Refolding Systems provide comprehensive refolding screens in a 96-well plate format and are based on an extensive literature review of successful refolding experiments and information contained in the REFOLD database (http://refold.med.monash.edu.au). The systems differ in the chemistry used to denature the inclusion bodies and in the refolding additives included in the 96-well plate. In addition to a 96-well plate containing 92 (System 1) or 95 (System 2) unique buffer and refolding additive combinations, both systems include the reagents needed to purify inclusion bodies and solubilize the component proteins. Significantly, all steps of the refolding screens are equally compatible with manual and high-throughput automated liquid handling.

Comparison of components in iFOLD Protein Refolding Systems 1 and 2

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

Buffer and additive concentrations are final, after addition of the target protein

Abbreviations: TCEP: tris(2-carboxyethyl)phosphine, GSH: reduced glutathione, GSSG: oxidized glutathione, EDTA: ethylenediaminetetraacetic acid, NDSB: non-detergent sulfobetaine, Sorbitol: D-sorbitol or D-glucitol, Trehalose: 1-O-α-D-Glucopyranosyl-α-D-glucopyranoside, PEG6000: polyethyleneglycol, average molecular weight of 6000 Da, PEG3350: polyethyleneglycol, average molecular weight of 3350 Da

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iFOLD™ Protein Refolding Systems

Brief Protocol

- Express target protein
- Harvest cells
- Lyse cells by sonication plus Lysonase™ Bioprocessing Agent
- Centrifuge to pellet inclusion bodies
- Wash inclusion bodies with Triton® X-100 (System 1) or NDSB-201 (System 2)
- Denature inclusion bodies with N-lauroylsarcosine (System 1) or guanidine-HCl or urea (System 2)
- Refold target protein by rapid dilution into iFOLD™ Protein Refolding System 1 or System 2 buffer matrix
- Assay* for correctly folded and active target protein

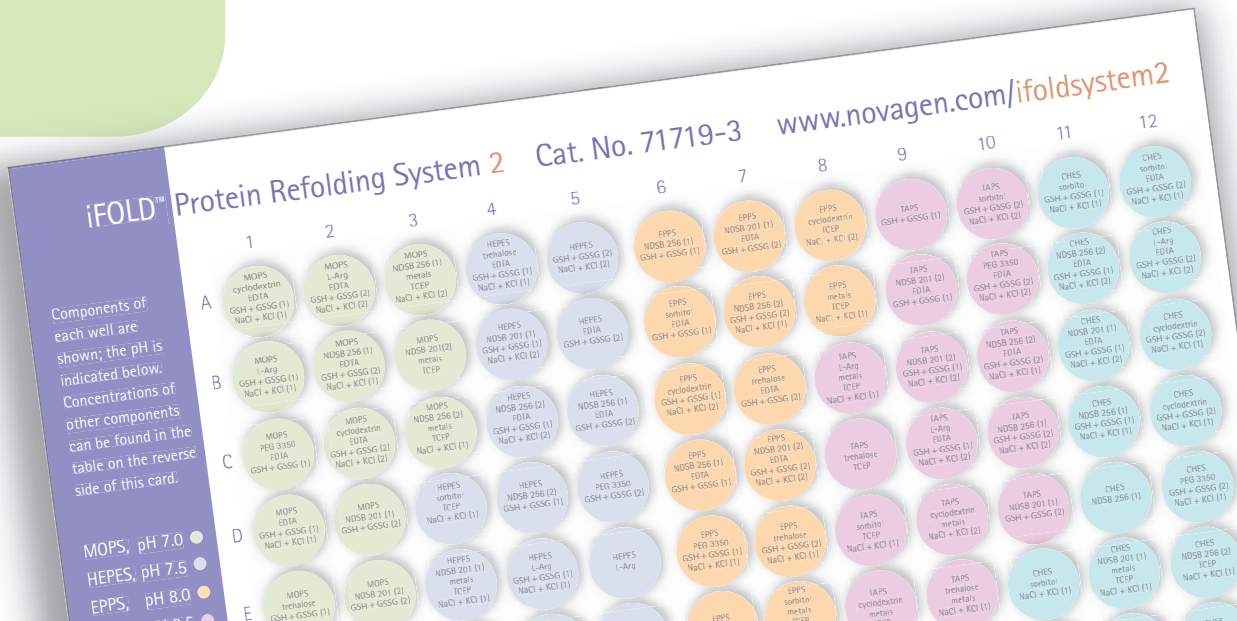
*Assay is determined by end user

iFOLD™ Protein Refolding System 1

- All reagents for inclusion body purification and pre-dispensed 96-well plate-based protein refolding matrix
- Uses N-lauroylsarcosine, a chaotropic anionic detergent, to denature the purified inclusion bodies
- 92 unique buffer and refolding additive combinations for simultaneous and systematic evaluation of protein refolding conditions
- pH range 7.0 – 8.5
- Refolding additives include salts, cyclodextrin, redox agents, chaotropes, glycols
- Refolding conditions based on extensive literature review and REFOLD database (http://refold.med.monash.edu.au)
- High-throughput compatible

iFOLD™ Protein Refolding System 2

- Entirely detergent-free system 2 contains inclusion body purification reagents and pre-dispensed 96-well plate-based protein refolding matrix
- Uses guanidine hydrochloride or urea (not included) to denature the purified inclusion bodies
- 95 unique buffer and refolding additive combinations for simultaneous and systematic evaluation of protein refolding conditions
- pH range 7.0 – 9.0
- Refolding additives include salts, redox agents, cyclodextrin, chaotropes, glycols, nondetergent sulfobetains (NDSBs)
- Refolding conditions based on extensive literature review and REFOLD database (http://refold.med.monash.edu.au)
- High-throughput compatible



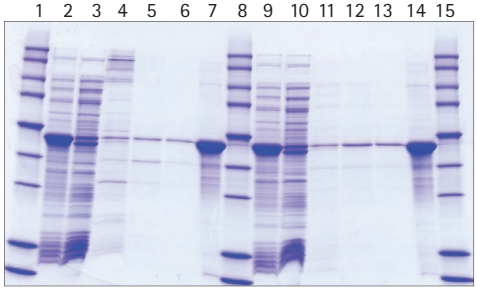
Preparation of Inclusion Bodies

Typically, inclusion bodies (IB) are highly enriched for the target protein but contain some contaminating biomolecules (nucleic acids, phospholipids, lipopolysaccharides, other proteins). Usually these contaminants are reduced by washing the IB pellet with a detergent, such as Triton® X-100 or sodium deoxycholate. The iFOLD™ System 1 uses 1.0% Triton X-100 for IB washing step.

When refolded proteins are used in downstream applications that can not tolerate even trace amounts of detergent, we recommend the iFOLD System 2, which uses a non-detergent sulfobetaine, NDSB-201, to wash the IB pellet. NDSB-201 is a 201 Da zwitterionic compound that does not form micelles and is easily removed by dialysis.

Comparison of a thioredoxin-GFP fusion protein from inclusion bodies washed with NDSB-201 or Triton® X-100

The trx-GFP fusion protein was expressed in *E. coli* strain BL21(DE3) using Overnight Express™ Instant TB medium. Cells were lysed by sonication and IB were processed according to the iFOLD 2 protocol, using 1.0% Triton X-100 (lanes 2-7) or 0.125 M NDSB-201 (lanes 9-14) for the IB washes. Samples from each step were assayed by SDS-PAGE (10-20% gradient gel) and detected with Coomassie blue.

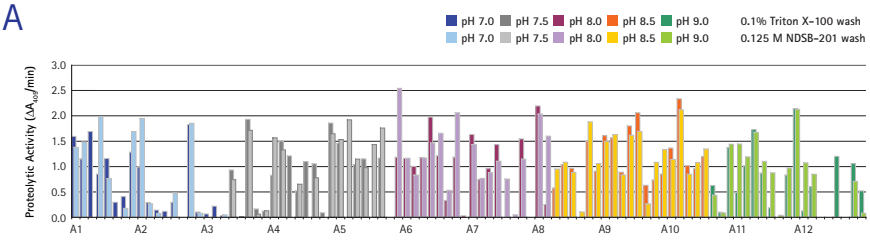


Lane	Sample
1.	Perfect Protein™ Markers, 10-225 kDa
2.	Total cellular protein
3.	Soluble fraction after lysis, supplemented with 1% Triton X-100
4.	Supernatant from 1% Triton X-100 wash
5.	Supernatant from buffer wash #1, Triton X-100 series
6.	Supernatant from buffer wash #2, Triton X-100 series
7.	1% Triton X-100 washed inclusion body pellet
8.	Perfect Protein Markers, 10-225 kDa
9.	Total cellular protein
10.	Soluble fraction after lysis, supplemented with 0.125 M NDSB-201
11.	Supernatant from 0.125 M NDSB-201 wash
12.	Supernatant from buffer wash #1, NDSB-201 serires
13.	Supernatant from buffer wash #2, NDSB-201 serires
14.	0.125 M NDSB-201 washed inclusion body pellet
15.	Perfect Protein Markers, 10-225 kDa

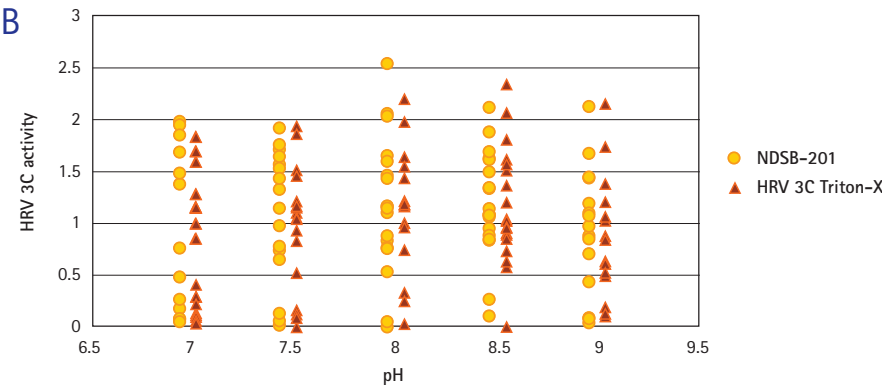
NDSB-201 comparable to Triton X-100

Refolded HRV 3C protease activity from IB washed with NDSB-201 or Triton X-100

A



B



HRV 3C IB were washed with NDSB-201 or Triton X-100, using the iFOLD System 2 protocol. Washed inclusion bodies were denatured with 7.0 M GuHCl and diluted into the iFOLD Protein Refolding Plate 2. After refolding for 20 h at 22°C, reactions were dialyzed in D-Tube96™ Dialyzers against2 x 4.0 L buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5.0 mM DTT, and 0.03% Brij®-35) overnight at 10°C. Enzymatic activity of the refolded and dialyzed proteins was quantified by measuring cleavage of a peptide substrate (Glu-Ala-Leu-Phe-Gln-pNa: Bachem L-2050). **A**. Graph of HRV 3C activity for individual wells from the refolding screen. **B.** Data grouped by refolding buffer pH.

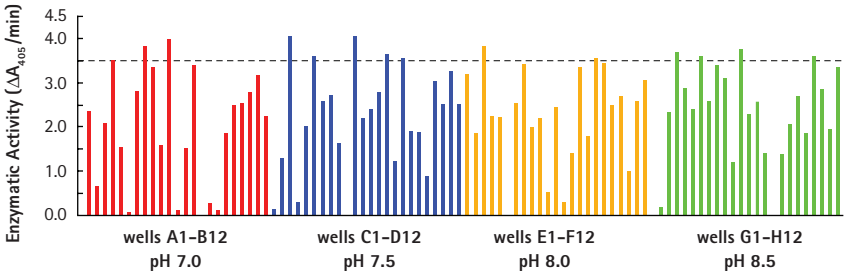
NDSB-201 comparable to Triton X-100

For more information or to place an order, contact your local office (see back cover).

Denaturing Inclusion Bodies, System 1

With the iFOLD™ Protein Refolding System 1, the purified inclusion bodies are denatured by addition of TCEP and N-lauroylsarcosine and refolded by rapid dilution into the iFOLD Protein Refolding Plate 1.

Refolded HRV 3C protease activity from IB denatured with N-lauroylsarcosine



Enzymatic activity of HRV 3C protease (22 kDa) refolded at 22°C was determined by measuring ΔA_{405}/min, indicating cleavage of a *p*-nitroanilide-labeled peptide substrate (Wang 1997).

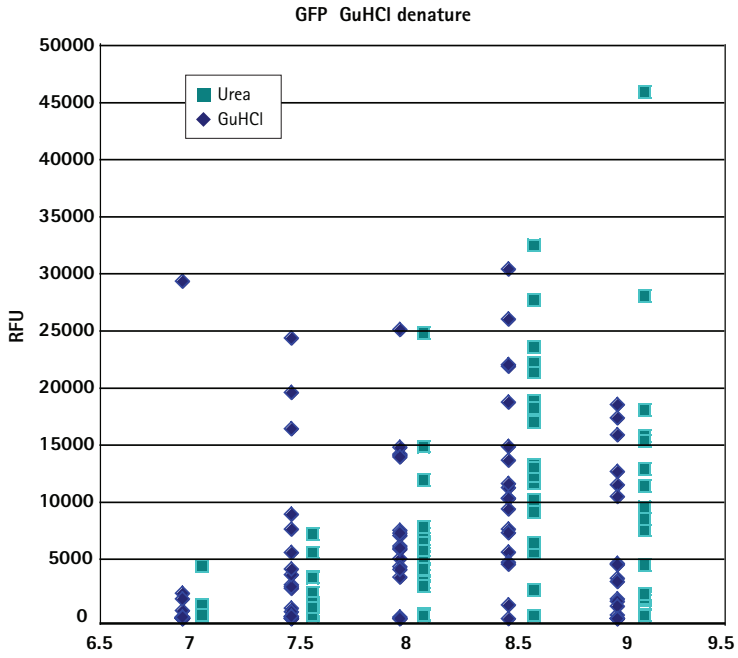
Reference

Wang, Q. et al. 1997. *Anal. Biochem.* **252**, 238.

In this refolding experiment, the iFOLD System 1 revealed that presence of residual detergent is essential for refolding HRV 3C protease. None of the 14 wells with the highest enzymatic activity contain cyclodextrin, which acts as a detergent trap.

Denaturing Inclusion Bodies, System 2

With the iFOLD Protein Refolding System 2, the purified inclusion bodies are denatured with guanidine-HCl or urea (not included) and refolded by rapid dilution into the iFOLD System 2 96-well buffer matrix.



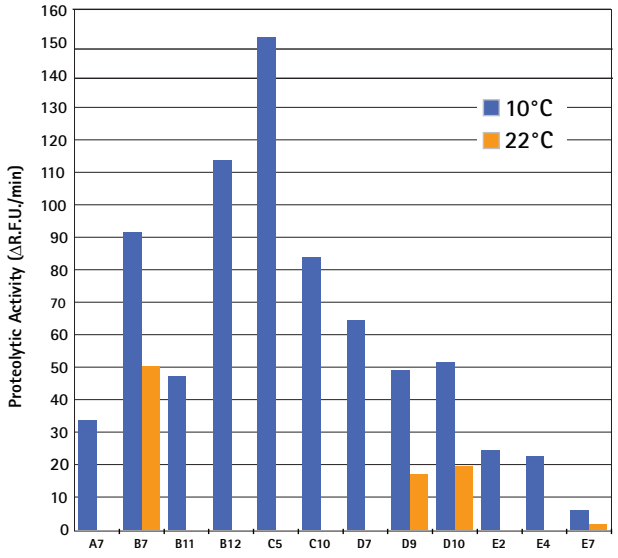
Comparison of GFP-Trx fluorescence after refolding from IB denatured with GuHCl or urea

Thioredoxin-GFP inclusion bodies were isolated and washed with 0.125 M NDSB-201 according to the iFOLD System 2 protocol, denatured with 7.0 M guanidine-HCl or 8.0 M urea, and diluted into the iFOLD System 2 refolding matrix. Following a 24 h incubation at 22°C, refolding reactions were diluted 1:4 with 50 mM Tris-Cl, pH 8.0, and the relative fluorescent intensity recorded (390 nm excitation, 510 nm emission).

Protein Refolding Temperature

Following addition of the target protein, the refolding plate is incubated at 4-22°C for approximately 20 h. iFOLD™ System 2 buffers maintain a relatively constant pH between 4 and 22°C, facilitating refolding experiments at different temperatures to accommodate the requirements of different target proteins. For many proteins, refolding at 22°C results in a greater fraction of active protein than refolding at lower temperatures. If the target protein isthermally unstable or particularly difficult to refold, refolding can be done at lower temperatures (4-10°C)

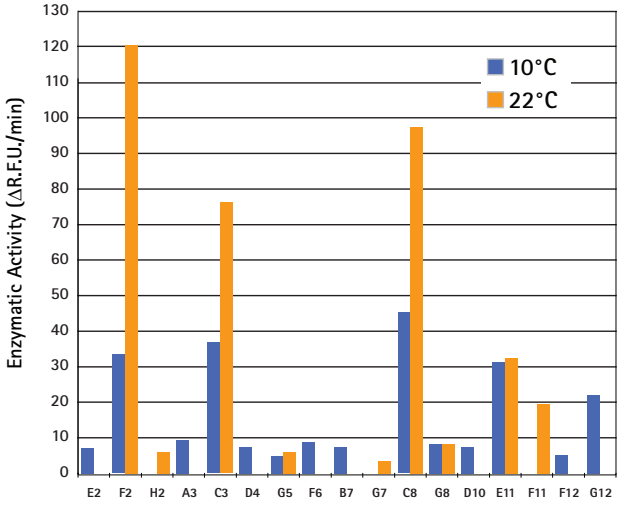
Comparison of recombinant enterokinase (rEK) activity after refolding at 10°C and 22°C



Previously prepared rEK IB were denatured with GuHCl according to the iFOLD System 2 protocol. Denatured IB were diluted into an iFOLD Protein Refolding Plate 2 equilibrated at 10°C or 22°C. Following a 20-h incubation, refolding reactions were dialyzed in D-Tube96™ Dialyzers against 2 × 4.0 L buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl) overnight at 10°C. Enzymatic activity of refolded and dialyzed reactions was quantified by measuring cleavage of a peptide substrate (Gly-Asp-Asp-Asp-Asp-Lys-β-naphthylamide; Sigma G5261).

rEK, a protease with 9 cysteines (8 participating in disulfide bonds), was refolded using iFOLD System 2 at 10°C or 22°C. Successful refolding was determined by a protease activity assay, which indicated that refolding at 10°C was significantly better than at 22°C. Each of the 12 refolding wells with detectable proteolytic activity included glutathione redox buffers.

Comparison of MMP-12 refolding at 10°C and 22°C



Previously prepared MMP-12 IB were denatured with GuHCl according to the iFOLD System 2 protocol. Denatured IB were diluted into an iFOLD Protein Refolding Plate 2 equilibrated at 10°C or 22°C. Following a 20-h incubation, refolding reactions were dialyzed in D-Tube96™ Dialyzers against 2 x 4.0 L buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2.0 mM $CaCl_2$, 1 µM $ZnCl_2$, and 0.03% Brij®-35) overnight at 10°C. Enzymatic activity of refolded and dialyzed reactions was quantified by measuring cleavage of a BODIPY®-labeled elastin (Invitrogen, E 12056).

In contrast to rEK, MMP-12 refolding was significantly better at 22°C. The refolding screen also revealed that divalent metals (wells C3, C8, and F2) are required for successful MMP-12 refolding.

For more information or to place an order, contact your local office (see back cover).

Measuring Refolding

Although no universal, accessible, high-throughput method is available to monitor protein refolding, sample absorbance at 340 nm (A_{340}) provides an initial screen for refolding efficiency (Vincentelli 2004). Low A_{340} values indicate soluble proteins. Light scattering instruments can provide a more accurate measure of protein precipitation. However, as a soluble protein does not always correlate with a correctly folded and active protein, we recommend to use an activity assay specific for the target protein.

Reference

Vincentelli, R. et al. 2004. *Protein Science.* **13**, 2782-2792.

Product	Cat. No.	Size	Price
iFOLD™ Protein Refolding System 2	71719-3	1 system	

Components:

- 30 mL 10X IB•Prep™ Buffer
- 100 µL Lysonase™ Bioprocessing Reagent
- 0.5 mL 1.0 M TCEP
- 10 mL 1.5 M NDSB-201
- 10 mL iFOLD System 2 Denaturation Buffer (50 mM Tris-HCl, 0.2 M NaCl, 2.0 mM EDTA, 7.0 M GuHCl, pH 8.0)
- 1 iFOLD Protein Refolding Plate 2
- 2 Aluminum plate sealers

iFOLD™ Protein Refolding System 1	71552-3	1 system	

Components:

- 30 ml 10X IB-Prep™ Buffer
- 0.5 ml 1M TCEP
- 1.5 ml Triton® X-100
- 0.1 ml Lysonase™ Bioprocessing Reagent
- 10 ml 30% N-Lauroylsarcosine
- 50 ml 50X iFOLD Dialysis Buffer
- 1 iFOLD Protein Refolding Plate 1
- 2 Aluminum Plate Sealers

D-Tube 96™ and D-Tube Dialyzers

D-Tube96™ Dialyzers allow convenient, high throughput dialysis of 96 samples simultaneously. The device features the advantages of the D-Tube™ Dialyzer Mini in a 96-tube format. D-Tube Dialyzers are easy to handle tubes with dual membranes providing a large surface area for fast, efficient dialysis. The membrane is ultra clean, EDTA-treated, regenerated cellulose that is sulfur- and heavy metal-free. After screening for optimal refolding conditions with the iFOLD™ Protein Refolding Systems, D-Tube96 Dialyzers provide convenient buffer exchange for the 96 protein samples into a physiologically relevant buffer.

Individual D-Tube Dialyzers are available with molecular weight cut-offs (MWCO) from 3.5 to 14 kDa and are designed with four volume capacities: Mini (10-250 µl), Midi (50-800 µl), Maxi (100-3000 µl), and Mega (3-20 ml). Each kit contains 10 D-Tubes and one floating rack.

Features

- Efficient dialysis – dual membrane surface
- D-Tube96 Dialyzers are ideal for sample volume from 10-250 µl per well/tube
- D-Tube Dialyzers are ideal for sample volume from 10 µl to 20 ml per well/tube
- Convenient – use single or multichannel pipet or robotic dispensing
- High sample recovery, >97%
- Protease-, RNase-, and DNase-free
- Versatile – dialyze proteins, oligonucleotides, RNA, or DNA

Product	Cat. No.	Size	Price
D-Tube™ Dialyzer Mini, MWCO 6-8 kDa	71504-3	1 kit	
D-Tube™ Dialyzer Mini, MWCO 12-14 kDa	71505-3	1 kit	
D-Tube™ Dialyzer Midi, MWCO 3.5 kDa	71506-3	1 kit	
D-Tube™ Dialyzer Midi, MWCO 6-8 kDa	71507-3	1 kit	
D-Tube™ Dialyzer Maxi, MWCO 3.5 kDa	71508-3	1 kit	
D-Tube™ Dialyzer Maxi, MWCO 6-8 kDa	71509-3	1 kit	
D-Tube™ Dialyzer Maxi, MWCO 12-14 kDa	71510-3	1 kit	
D-Tube™ Dialyzer Mega, 10 ml, MWCO 3.5 kDa	71739-3	1 kit	
D-Tube™ Dialyzer Mega, 10 ml, MWCO 6-8 kDa	71740-3	1 kit	
D-Tube™ Dialyzer Mega, 10 ml, MWCO 12-14 kDa	71741-3	1 kit	
D-Tube™ Dialyzer Mega, 15 ml, MWCO 3.5 kDa	71742-3	1 kit	
D-Tube™ Dialyzer Mega, 15 ml, MWCO 6-8 kDa	71743-3	1 kit	
D-Tube™ Dialyzer Mega, 15 ml, MWCO 12-14 kDa	71744-3	1 kit	
D-Tube™ Dialyzer Mega, 20 ml, MWCO 3.5 kDa	71745-3	1 kit	
D-Tube™ Dialyzer Mega, 20 ml, MWCO 6-8 kDa	71746-3	1 kit	
D-Tube™ Dialyzer Mega, 20 ml, MWCO 12-14 kDa	71747-3	1 kit	
D-Tube96™ Dialyzer, MWCO 6–8 kDa	71712-3	1 kit	
D-Tube96™ Dialyzer, MWCO 12–14 kDa	71713-3	1 kit	

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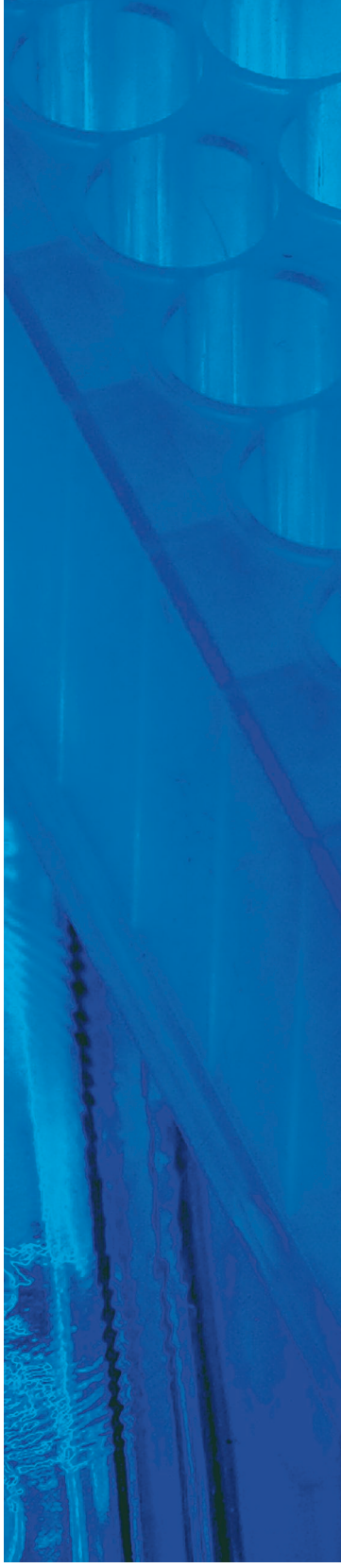

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