

Refold Master

Protein Refolding Kit

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Introduction

Overexpression of proteins in transformed micro-organisms such as *E. coli* can be an efficient method of rapidly producing relatively large quantities of recombinant proteins. However, this protein often accumulates in the cell as an aggregate of inactive protein (an inclusion body).

The Expedeon Protein Refolding Master Kit provides a simple method for refolding the target protein without the need to screen multiple refolding conditions. Therefore, a greatly reduced number of samples need to be analysed for correct function and 3-D structure.

The kit uses Expedeon's technology and reagents to protect the protein during vulnerable stages in the refolding process. This provides an opportunity for the protein to refold correctly, yet greatly reduces the yield losses due to aggregation.

Denatured protein at 5 mg/ml is refolded by 20-fold dilution to a final protein concentration of 0.25 mg/ml. The kit allows 8x 10 ml samples to be refolded providing 8 x 2.5 mg protein. Alternatively a single 80 ml refold can be performed to provide 20 mg of protein.

Storage

Upon receipt store at +4°C. Discard any reagents that show discoloration or evidence of microbial contamination.

The components of Solutions "A", "D" and "E" are stable as supplied. Solutions A and E should be used within one week of initial solution preparation. Solution D should be used on the day of preparation and can be stored frozen for 3 months. Once opened Solution "e" should also be used within one week.

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Kit Contents – Ready for Use

- A – Denaturation buffer (13.5 ml)**
100 mM Tris, 8 M Urea, pH 8.2
- B – Refolding buffer (100 ml)**
50 mM Tris, pH 8.0
- C – Refolding tubes (not supplied)**
- D – Protein protection agent (blue, 4x 1.6 ml)**
25 mg/ml stock of NV10, dissolved in water
- e – Reducing agent (white, 800 µl)**
1 M DTT, dissolved in water
- E – Oxidizing agent (yellow, 290 µl)**
400 mM GSSG, dissolved in water
- F – Protection removal solution (green, 5 ml)**
Strong release agent, dissolved in water

Caution :

- 1) Solutions a, A, contain Tris (irritant)
- 2) Solution e contains dithiothreitol (harmful)

Notes :

- 1) *Solution A supplied as:*
Bottle a – 10 ml, 150 mM Tris, pH 7.75
Bottle A – 6.48 g solid urea
- 2) *Solution D supplied as:*
4 x 40 mg lyophilized powder for water addition by user
- 3) *Solution E supplied as:*
Tube E – 72 mg oxidized glutathione

Instructions for use – Solution Prep (30 min)

- i) Add exactly 9 ml of the solution in bottle “a” to the solid in the bottle marked “A”. Agitate until all the solid urea has dissolved (~ 10 min on shaker / rocker or ~ 5 min with continuous vortex mixing).
- ii) Add exactly 1.6 ml of ultrapure water to tube “D” and vortex mix until completely dissolved.
- iii) Add 250 µl ultra pure water to the solid in the tube with yellow cap marked “E”.

Instructions for Use – Refolding (2 days)

1) Solubilising the Target Protein

Use the denaturation buffer (Solution A) to solubilize the protein or inclusion body pellet. The target protein concentration is 5 mg/ml of solubilized and denatured protein (minimum of 4 ml is required). Incubate overnight at 4°C (or 3 hours at 37°C) to fully denature the protein and finally centrifuge to pellet any remaining insoluble material. Inclusion bodies typically contain significant levels of water and contaminant proteins, Expedeon recommends a starting point of 10% w/v of wet inclusion body pellet in the denaturant buffer (e.g. 100 mg inclusion body in 1.0 ml of Solution A).

After solubilization measure the total protein concentration (e.g. use Expedeon's BradfordUltra assay) and concentrate or dilute the protein to 5 mg/ml as required.

Optional step 1.1:

To enhance the solubilisation of the target protein, add 20 µl of the reducing agent (solution e) per millilitre of Solution A added to the inclusion body pellet.

Summary:

Inclusion Body	400 mg
Solution A	4 ml
Solution e -- Optional	80 µl

2)

Preparation of the Refolding Mix

Add 8.950 ml of the refolding buffer (Solution B) to one of the refolding tubes (Tube C). If desired an alternative or proprietary refolding buffer can be substituted at this stage. Add 500 µl protein protection agent, solution D, to tube and mix. **If optional step 1.1 was applied then add 50 µl of solution E to the mix.**

Optional Step 2.1

If excessive protein aggregation is expected or occurred during previous experiments then additional protein protection agent can be added to the refolding buffer. If this additional protein protection is required add 500 µl of Solution D to the refolding buffer contained in Tube C.

Optional Step 2.2 – only use if step 1.1 was not used

For refolding target proteins containing cysteines, add 10 µl of the reducing agent (Solution e) and 50 µl of the oxidising agent (Solution E) to the refolding buffer contained in Tube C.

Optional Step 2.3

Metal ions or co-factors known to be important during the refolding of the target protein can also be included at this stage (e.g. divalent metal ions Ca^{2+} or Mg^{2+} , other metal ions, binding partner of native protein or enzyme substrate).

Summary:

Solution B	8.950 ml
Solution D	500 µl
	500 µl – optional
Solution e	10 µl – optional
Solution E	50 µl – optional
Cofactors, metal ions	as required
TOTAL	9.450 – 10.010 ml

3) **Protein Refolding**

Add 500 µl of the solubilized and denatured protein into Tube C and immediately vortex mix to begin the refolding process. Refolding should be performed overnight at room temperature.

4) **Using the Release Agent - Optional**

Add 500 µl of Solution F to Tube C. Vortex mix and incubate the protein solution overnight at room temperature.

In some cases the NV10 polymer may slow down the rate of refolding especially at 4°C. The addition of the protection removal solution is not always required and extended incubation of the refolding target protein at room temperature may increase yield and allow complete refolding without NV10 release.

The refolded protein is now contained in Tube C and can be tested for functionality / activity or purified. Expedeon recommends retaining a small sample for

testing and continuing with the protein primary purification.

Troubleshooting Guide**Q 1 Aggregation occurred during the refolding process, how can I prevent this?**

A 1a Perform the optional Step 2.1 and if aggregation still occurs add more than 500 µl of the Protein Protection Agent (Solution D).

A 1b Decrease the protein concentration in Step 1.

Q 2 The target protein precipitated when the Protection Removal Solution (Solution F) was added, what should I do?

A 2a Make small additions of Solution F stepwise over an 8 h period and then incubate overnight.

A 2b The protein may not be properly refolded before the release step. Consider extending the duration of Step 3 or performing at room temperature if the incubation was previously performed at 4°C.

Q 3 What methods should I use to measure protein concentration during the refolding protocol?

A 3 The three main methods are UV light absorption at 280 nm, the Bradford Assay and the BCA Assay. In all cases the values will be estimates since the starting material from the inclusion bodies will be impure. UV light absorption can be used to estimate the protein concentration when the extinction coefficient is known (extinction coefficients for denatured protein can be predicted from the protein sequence, please visit: <http://us.expasy.org/tools/protparam.html>). The BCA assay can be used to determine protein concentration when redox agents are not present. We recommend the use of Expedeon “Bradford*Ultra*” assay which is compatible with will all the solutions provided in the Refold kit.

Q 4 After following the protocol the solution does not seem to contain any soluble target protein, what could cause this?

A 4a Inclusion bodies often contain impurities that could lead to an overestimation of the concentration of the target protein. If no aggregation occurred during the refold, increase the protein concentration in Step 1.

A 4b The protein in the denaturant buffer may be disulfide-bonded oligomers or particulates. Run both reducing and non-reducing SDS-PAGE gels and look for significantly reduced levels of protein entering the non-reducing gel.

If this occurs then add additional redox agent to the denaturant to break / shuffle any non-native disulfide bonds. Expedeon recommends adding up to 60 μ l reducing agent to the denaturation buffer (solution A). For every 10 μ l reducing agent added to solution A, 2.5 μ l oxidizing agent (solution E) must be added to the refolding buffer.

Q 5 After refolding I have high recovery of soluble non-aggregated protein but the amount of functional target protein is lower than expected, what could cause this?

A 5 The Expedeon Refolding Kit is designed to suppress aggregation and control the rate of protein refolding. The target protein may be refolding slower than expected, especially if quaternary structure is necessary for functionality or activity.

Q 6 How can I speed up the rate of protein refolding?

A 6a Ensure that Steps 3 and 4 are performed at room temperature and consider extending the duration of Step 3.

A 6b Omit the optional Step 2.1 or add less than 500 μ l of the Protein Protection Agent (Solution D) to the Refolding mix.

Q 7 Can I increase the protein concentration during the refold?

A 7 If a previous refold was successful and aggregation did not occur, the protein concentration can be increased.

Q 8 I would like to refold using a different buffer or use extra additives, is this OK?

A 8 Yes, the Expedeon Refolding Kit is designed for maximum compatibility with common components of refolding buffers. If Solution B is not used then simply substitute Solution B for the desired buffer but add the redox agents (Solution E and solution e) as described if none are present in the alternative buffer.

Q 9 Is it possible that the protein will not refold correctly even if I follow the answers to Q 4 and Q 6?

A 9 Yes. Consider adding metal ions or cofactors if these are known to be required for protein functionality or if they have been previously used for successful refolding of a similar class of protein.

Q 10 Can I use protease inhibitors with the refolding protocol and if so when should they be added?

A 10 If desired, add protease inhibitors during Step 2.

Q 11 Is it necessary to add the Protection Removal Solution?

A 11 If the quantity of refolded target protein after Step 3 is sufficient for further use then Step 4 can be omitted. Further incubation at room temperature may improve the yield without adding the protection removal solution.

Q 12 Are the purification steps necessary before protein analysis?

A 12 The protein protection agent used in the Expedeon Refolding Kit is compatible with many analytical techniques without any sample clean-up steps. However, when using higher concentration of NV10 (>2.5 mg/ml) it may be desirable to carry out the primary protein purification steps before gel-electrophoresis, Bradford analysis and ELISA since more accurate results will be obtained. A primary clean up can be performed with ion exchange chromatography (or immobilised metal affinity chromatography if the protein is histidine-tagged).

Q 13 What solutions should I use in control experiments to confirm that the protein protection agent is compatible with my analysis method?

A 13a To analyze after Step 4 make a blank with the following composition:

Solution A (no protein)	50	μl
Solution B	895	μl
Solution D	50	μl
Solution e	1	μl (if used)
Solution E	5	μl (if used)
Solution F	50	μl (if used)
<hr/>		
Total	995	– 1051μl

Q 14 What control experiments can I run to confirm kit function?

A 14 Repeat the lysozyme refolding detailed in the application note on page 21 as a positive control to confirm kit function. Since purified protein will be used, fully reduce the denatured protein and elevate the concentration to 15 mg/ml to give a final protein concentration of 0.75 mg/ml during the refold (significant aggregation of purified lysozyme will not occur without these changes). Denature the lysozyme in 8 M Urea, 32 mM DTT, 100 mM Tris, pH 8. Refold in 50 mM Tris, 5 mM oxidized glutathione, pH 8 containing 1 mg/ml NV10 and a control containing no Solution D. Analysis should be performed after Step 4 without any purification. The refolding yield when Solution D is used should be $\geq 70\%$ and higher than the negative control where D was not used.

Q 15 Can I analyze my refolded protein using column chromatography?

A 15 Yes, but a primary ion exchange (or immobilised metal affinity) purification is recommended. The use of a guard column / cartridge is also recommended when using RP-HPLC columns.

Q 16 Can I analyze my refolded protein using Mass Spectrometry?

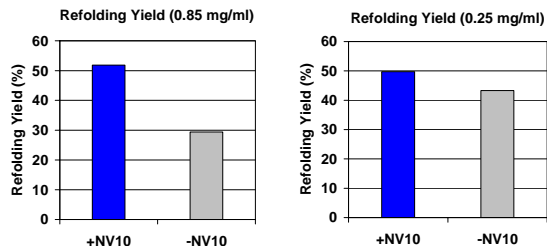
A 16 Yes, but the sample must be purified. Use standard C4 zip tip for sample clean up.

Application Note – Refolding of Carbonic Anhydrase

for latest case-studies see www.expedeon.com

Carbonic Anhydrase (purified) was denatured and then refolded at final concentrations of 0.85 and 0.25 mg/ml using the Expedeon Refold Master. The example below demonstrates that even when aggregation is not visible by eye the Expedeon NV10 protein protection agent allows refolding of proteins at higher concentrations, with higher yields, than standard dilution refolding.

Additional protein protection agent was added to the refolding buffer in Step 3 (final NV10 concentration 2.5 mg/ml).



- The Expedeon Refolding Kit allows the protein concentration to be increased without sacrificing refolding yields.

- The NV10 protectant is able to increase yields for proteins where no aggregation is visible by eye, by preventing the formation of low molecular weight (soluble) aggregates.
 - The NV10 protectant can be added to a different refolding buffer from the one specified in the Novexin Refolding Kit to provide protein protection in the user's first choice of buffer.
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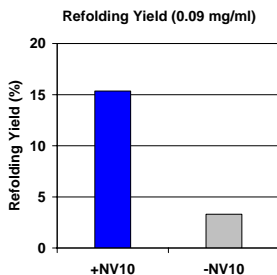
Notes:

- 1) Carbonic anhydrase was obtained in a purified form from Sigma (Product # C-3934)
- 2) Carbonic anhydrase is a 29 kDa monomeric protein with a pI of 5.9 that contains no disulfide bonds in the native structure.
- 3) Final protein concentrations of 0.85 mg/ml and 0.25 mg/ml correspond to denatured protein solutions at 17 and 5 mg/ml respectively.
- 4) Carbonic anhydrase activity was determined from the hydrolysis rate of a 10 mM solution of para-nitrophenol acetate in 50 mM Tris pH 7.8, measured by an increase in absorption at 405 nm.
- 5) Activity was determined at the end of Step 4, without carrying out purification steps, since the protectant did not interfere with the activity assay.

Application Note – Refolding of Citrate Synthase

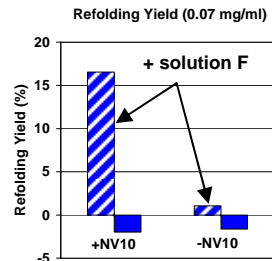
for latest case-studies see www.expedeon.com

Citrate Synthase (purified, dimeric protein) was denatured and refolded at final concentrations of 0.09 mg/ml and 0.07 mg/ml using the Expedeon Refold Kit. In example 1, citrate synthase (0.09 mg/ml) was denatured in 8 M guanidine-HCl, 40 mM DTT, 100 mM Tris-HCl, pH 7.6, 0.75 mM EDTA and refolded by 20-fold batch dilution in buffer containing 0.3mg/ml NV10. In example 2, citrate synthase (0.07 mg/ml) was denatured in Solution A and refolded by 20-fold batch dilution in buffer containing 1 mg/ml NV10 and subsequently released by the addition of Solution F (protection removal solution).



Example 1 – The Expedeon Refolding Kits are suitable for refolding proteins with quaternary structure.

- NV10 can be added to the denaturant buffer to minimise aggregation and improve the refolding yield without addition of the release solution F (final NV10 concentration 0.3 mg/ml).



Example 2 – Extra protein protection agent can be added to the refolding buffer provided that the protein protection agent is released by the addition of solution F (final NV10 concentration was 1 mg/ml).

- NV10 concentrations greater than 2 mg/ml did not allow dimerization and recovery of protein activity in the experimental time scale

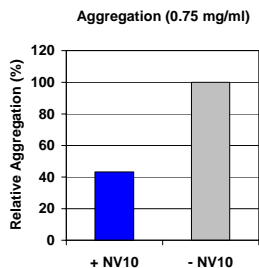
Notes:

- 1) Citrate synthase was obtained from Sigma (Product # C-3260) in a purified form as a suspension in ammonium sulfate.
- 2) Active citrate synthase (from porcine heart) is a homodimeric protein composed of two identical subunits, each with a molecular mass of 49 kDa.
- 3) Citrate synthase activity was determined from the rate of formation of citrate from acetyl-CoA and oxaloacetate. The concomitant release of CoA-SH was then detected by reaction with 5,5'-Dithiobis (2-nitro-benzoic acid), causing an increase in absorbance at 405 nm.
- 4) Activity was determined at the end of Step 4, without carrying out purification steps, since the protectant did not interfere with the activity assay.

Application Note – Refolding of Lysozyme

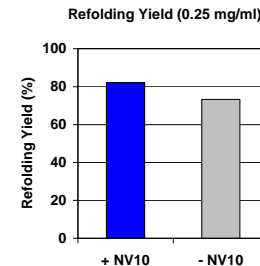
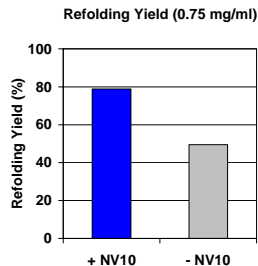
for latest case-studies see www.expedeon.com

Lysozyme (purified) was denatured and refolded at final concentrations of 0.75 and 0.25 mg/ml using the Expedeon Refold Kit. High protein concentrations and full reduction were used to more closely resemble refolding following inclusion body solubilization where the target protein is impure and aggregation is likely to be a significant problem. NV10 was used as protein protection agent in the refolding buffer to a final concentration of 1 mg/ml.



The NV10 protectant is able to suppress protein aggregation and maintain the lysozyme in a soluble form with full refolding potential.

- The high refolding yield obtained at high protein concentration (0.75 mg/ml) demonstrates that the protected protein can be recovered in a fully active form.



- Yields of ~ 90% were achieved with a 2.5 mg/ml final NV10 concentration.

Notes:

- 1) Lysozyme was obtained in a purified form from Fluka (Product # 62971).
- 2) Lysozyme is a 14 kDa protein with a pI of 10.8 and four disulfide bonds in the native structure.
- 3) Final protein concentrations of 0.75 mg/ml and 0.25 mg/ml correspond to denatured protein solutions at 15 mg/ml and 5 mg/ml respectively.
- 4) Denaturation buffer: 8 M Urea, 32 mM DTT, 100 mM Tris, pH 8.2.
- 5) Refolding buffer: 50 mM Tris, pH 8, 5 mM oxidized glutathione.
- 6) Increase in solution turbidity at 492 nm was used to indicate protein aggregation.
- 7) Lysozyme activity was determined from the lysis rate of 200 μ l of a 1.5 mg/ml solution of *Micrococcus lysodeikticus*

in 67 mM sodium phosphate buffer at pH 6.25 (performed at room temperature).

- 8) 0.75 mg/ml lysozyme samples were diluted 3x to 0.25 mg/ml max final concentration and 20 μ l of each diluted sample was used in the assay.
- 9) Activity was determined at the end of Step 4, without carrying out purification steps, since the protectant did not interfere with the activity

