Refold Master

Bulk Protection and Release Reagents

Bulk v5.1

Bulk v5.1

Table of Contents

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).

Novexin's technology and reagents 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.

In the recommended protocol denatured protein at 5 mg/ml is refolded by 20-fold dilution to a final protein concentration of 0.25 mg/ml in the presence of Novexin's NV10 protein protection agent.

Storage

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

NV10 is stable as supplied but should be used within one week of initial solution preparation.

Contents (200 mg nominal protein level)

1) *NV10 protein protection agent (3 ml, x4)*

- Supplied dry in aliquots sufficient to refold 50 mg of protein following the recommended protocol.
- Resuspend aliquot in 3 ml of ultrapure water to create a 100X stock.

2) *Protection removal solution (10.5 ml, x4)*

- Supplied in ultrapure water.

Contact

For technical support email: scientist@novexin.co.uk

Novexin Ltd
Babraham Bioincubator
Babraham
Cambridge CB2 4AT

Tel: +44 (0)1223 496500
Fax: +44 (0)1223 496741

Recommended Protocol and Notes on Usage

1) The recommended protocol is to denature the target protein at 5 mg/ml and refold by 20-fold dilution into refolding buffer containing NV10. Incubate overnight at room temperature, add the protection release solution and perform a further overnight incubation.

2) The refolding buffer should contain 0.5 – 1.5X concentration of NV10 as a starting point (0.5 – 1.5% v/v of 100X NV10 stock).

3) If protein aggregation occurs increase the NV10 concentration or decrease the final protein concentration in the refold.

4) The NV10 protection agent will suppress protein aggregation and may allow refolding at final protein concentrations greater than 0.25 mg/ml. However, the NV10 may also slow down the rate of refolding especially at 4°C.

5) 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.

6) A 5% v/v addition of the protection removal solution will achieve rapid protection release. If the protein is not completely refolded aggregation may be caused by sudden protein release. If this occurs add the protection removal solution stepwise with small initial additions or consider performing analysis in the presence of NV10 without release.

7) NV10 can be added into the denaturant buffer to assist protein solubilization and then carried over into the refolding buffer without affecting functionality (e.g. add 20X concentration of NV10 to the denaturant buffer to achieve 1X final NV10 concentration in the refolding buffer).

Frequently Asked Questions

Q 1 Are any purification steps necessary before protein analysis?

A 1 The protein protection agent used in the Novexin Refolding Kit is compatible with many analytical techniques without any sample clean-up steps. However, 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. The recommended primary purification is ion-exchange chromatography, or immobilized metal affinity chromatography (IMAC) if the protein has a histidine tag. Hydrophobic interaction chromatography is recommended as a secondary protein polishing procedure if complete removal of the NV10 is required.

Q 2 What control experiments can I run to confirm kit function?

A 2 Repeat the lysozyme refolding detailed in the application note on page 12 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

glutatione, pH 8 containing 1.5X concentration of NV10 and a control without NV10. Analysis should be performed without any protein purification. The refolding yield when NV10 was used should be ≥ 70% and higher than the negative control where NV10 was not present.

Q 3 Can I analyze my refolded protein using column chromatography?

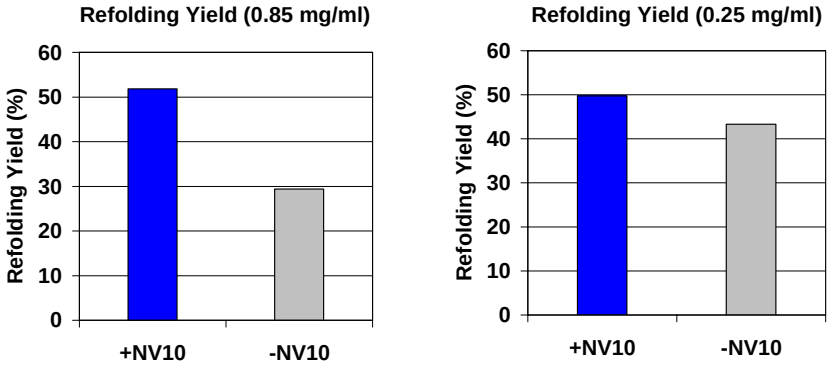
A 3 Yes, but for optimal results perform an ion-exchange or IMAC primary purification before analysis. When using RP-HPLC columns this primary purification step should not be omitted and it is recommended that a guard column / cartridge is used

Q 4 I have performed the protein purification procedures but still get unusual peaks on mass spec what should I do?

A 4 This problem can usually be solved by performing an overnight dialysis. Use the largest membrane pore-size possible that still retains the target protein (e.g. 30 kDa).

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

Additional protein protection agent (350 µl, 3.5%) was added to the refolding buffer (final NV10 concentration 4X).



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

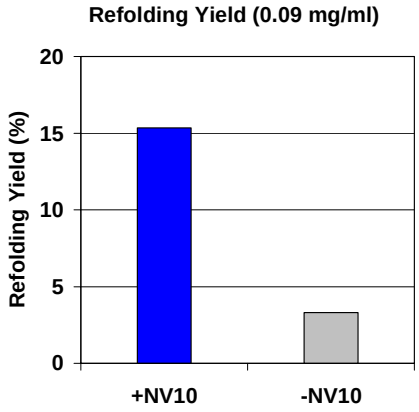
- The NV10 protectant is able to increase yields for proteins where no aggregation is visible by eye, by preventing the formation of low molecular weight (soluble) aggregates.

- 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.

Notes:

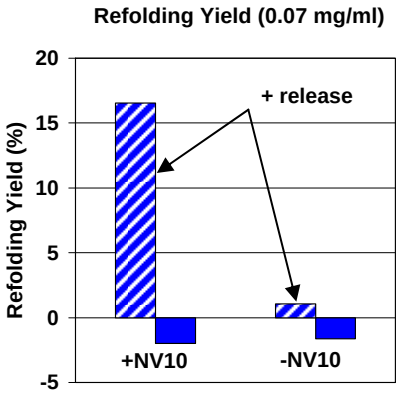
1) Carbonic anhydrase was obtained in a purified form from Sigma (Product # C-3934)
2) Carbonic anhydrase is a 29 kDa monomeric protein with a pI of 5.9 that contains no disulfide bonds in the native structure.
3) Final protein concentrations of 0.85 mg/ml and 0.25 mg/ml correspond to denatured protein solutions at 17 and 5 mg/ml respectively.
4) Denatured protein contained 10X concentration of NV10.
5) 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.
6) Activity was determined without carrying out the purification steps, since the protectant did not interfere with the activity assay.

Citrate Synthase (purified, dimeric protein) was denatured and refolded at final concentrations of 0.09 mg/ml and 0.07 mg/ml. In example 1, citrate synthase (0.09 mg/ml) was denatured in 8 M Gn-HCl, 40 mM DTT, 10X NV10, 100 mM Tris-HCl, pH 7.6, 0.75 mM EDTA and refolded by 20-fold batch dilution. In example 2, citrate synthase (0.07 mg/ml) was denatured in 8 M urea, 32 mM DTT, 10X NV10, 100 mM Tris, pH 8.2 and refolded by 20-fold dilution. Extra protein protection agent (100 µl, 1%) was added to the refolding buffer and subsequently released by the addition of 200 µl (2%) protection removal solution.



- Example 1 – The Novexin 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 (final NV10 concentration 0.5X).



- Example 2 – Extra protein protection agent can be added to the refolding buffer provided that the protein protection agent is released by addition of the protection removal solution (final NV10 concentration was 1.5X).

- NV10 concentrations greater than 3X 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.

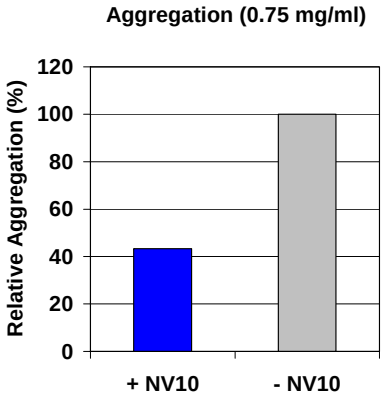
2) Active citrate synthase (from porcine heart) is a homo-dimeric protein composed of two identical subunits, each with a molecular mass of 49 kDa.

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 without carrying out the purification steps, since the protectant did not interfere with the activity assay.

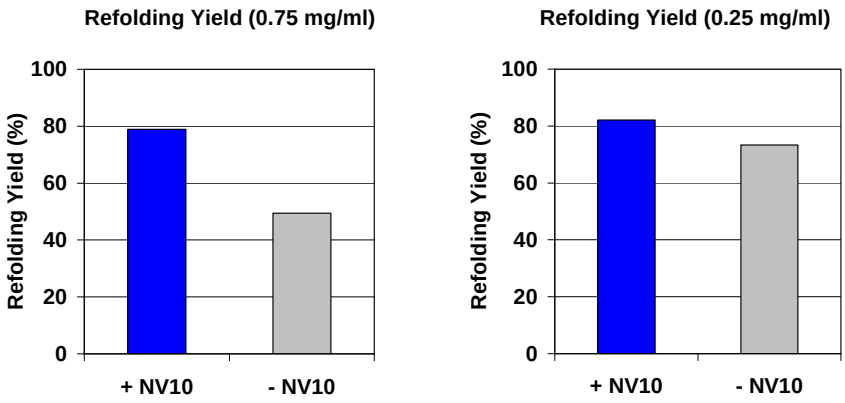
Lysozyme (purified) was denatured and refolded at final concentrations of 0.75 and 0.25 mg/ml using the Novexin Refold Master. High protein concentrations and full reduction were used to more closely resemble refolding following inclusion body solubilization where the target protein is impure and aggregation is likely to be a significant problem.

Additional protein protection agent (100 µl, 1%) was added to the refolding buffer to give a final NV10 concentration of 1.5X.



- 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 4X 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, 10X NV10, 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 µl 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 µl of each diluted sample was used in the assay.
9) Activity was determined without carrying out the purification steps, since the protectant did not interfere with the activity assay.