Refold Master MX

Protein Refolding Kit for Histidine-Tagged Proteins

novexin

50mg His v5.1

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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).

The Novexin Protein Refolding Kit is designed for proteins with a histidine-tag and provides a simple method for refolding and purifying the target protein without the need to screen multiple refolding conditions. Therefore, a greatly reduced number of samples need to be analyzed for correct function and 3-D structure.

The kit uses Novexin'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 4x 50 ml samples to be refolded providing 4x 12.5 mg 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 but should be used within one week of initial solution preparation. Once opened Solution “e” should also be used within one week.

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Kit Contents – Ready for Use

A – Denaturation buffer (4.9 ml, x4)

110 mM Tris, 8.8 M Urea, pH 8.2

B – Refolding buffer (100, x2 ml)

50 mM Tris, pH 8.0

C – Refolding, IMAC and product collection containers (x16)

D – Protein protection agent (blue, 1100 µl, x4)

100X stock of NV10, dissolved in water

E – Redox agents (yellow, 750 µl, x4)

400 mM GSSG, 200 mM DTT, dissolved in water

F – Protection removal solution (10.5 ml)

Dissolved in water

G – Immobilised metal affinity chromatography (IMAC) column (x4)

Pre-charged Ni^{2+} affinity column in 20% ethanol

H – Syringes for resin filtration (x4)

I – Syringe loading needle

J – Equilibration and wash buffer (125 ml, x2)

50 mM Tris, pH 8.0

K – IMAC elution buffer (105 ml)

50 mM Tris, 0.5 M imidazole, pH 7.2

L – Protection removal resin (12.5 ml)

67% resin slurry in 50mM Tris, pH 7.2

M – Resin filter (x4)

Caution :

1) Solutions a, A, J, K, and L contain Tris (irritant)
2) Solutions e and E contain dithiothreitol (harmful)
3) Solution K contains imidazole (corrosive)
4) Item G contains 20% ethanol (flammable)

Notes :

1) Solution A supplied as :
   Bottle a – 15 ml, 180 mM Tris, pH 7.75
   Bottle A – 2.59 g solid urea, x4

2) Solution D supplied as :
   dry powder for water addition by user

3) Solution E supplied as :
   Tube e – 800 µl, 235 mM DTT, dissolved in water, x4
   Tube E – 186 mg oxidized glutathione, x4

Instructions for use – Solution Prep (single run, 30 min)

i) Add exactly 3 ml of the solution in bottle "a" to the solid in the bottle marked "A".

ii) Agitate "Solution A" until all the solid urea has dissolved (~ 10 min on shaker / rocker or ~ 5 min with continuous vortex mixing).

iii) Add exactly 1100 µl of ultrapure water to tube "D" and vortex mix until completely dissolved.

iv) Add 500 µl of Solution D to Solution A.

v) Add 650 µl of the solution in tube "e" to the solid in the tube marked "E".

Instructions for Use – Refolding (2 days)

1) 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 4x 500 µl is required). Incubate overnight at 4ºC (or 3 hours at 37ºC) 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, Novexin 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)

2) Add 47 ml of the refolding buffer (Solution B) to one of the refolding containers (Tube C1). If desired an alternative or proprietary refolding buffer can be substituted at this stage.

3) Optional Step – 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 C1.

4) Add 250 µl of the redox agents (Solution E) to the refolding buffer contained in Tube C1. 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).

5) Add 2.5 ml of the solubilized and denatured protein into Tube C1 and immediately vortex mix to begin the refolding process. Refolding should be performed overnight at room temperature.

6) After the overnight refold, add 2.5 ml of the protection removal solution (Solution F) to Tube C1. Vortex mix and incubate the protein solution overnight at room temperature.

7) The refolded protein is now contained in Tube C1 and can be tested for functionality or activity. Novexin recommends retaining a small sample for testing and continuing with the protein primary purification.

Protein primary purification (2 hours)

7) Resuspend the resin by inverting the IMAC column (Item G).

8) Remove the end cap and cut off the tip from the IMAC column (Item G). Remove the top plug and let the liquid drain from the tube, allowing the resin to settle at the bottom of the tube. Reapply the end cap.

9) Fill a syringe (Item H) with 30 ml of equilibration buffer (Solution J) from the storage container using the loading needle (Item I).

10) Apply 30 ml of the equilibration buffer (Solution J) to the IMAC column. Close the column by inserting the top plug and resuspend the resin by inverting the column. Let the resin settle for 10 minutes.

11) Remove the end cap and top plug and drain the liquid from the solution. Reapply the end cap.

12) Pour the refolded protein sample from Tube C1 into the IMAC column (Item G). Close the column by inserting the top plug and resuspend the resin by inverting the column. Let the resin settle for 10 minutes.

13) Remove the end cap and top plug and collect the flow through in an IMAC collection tube (Tube C2). Reapply the end cap.

14) Apply 30 ml of the wash buffer (Solution J) to the IMAC column. Close the column by inserting the top plug and resuspend the resin by inverting the column. Let the resin settle for 10 minutes.

15) Remove the end cap and top plug and drain the liquid from the solution. Reapply the end cap.

16) Add 25 ml of the elution buffer (Solution K) to the IMAC column. Close the column by inserting the top plug and resuspend the resin by inverting the column. Let the resin settle for 10 minutes. Alternatively, to obtain a higher final protein concentration the protein can be eluted in < 25 ml by adding a reduced volume of elution buffer.

17) Remove the end cap and collect the refolded protein in a product collection tube (Tube C3).

18) The refolded and purified protein is now contained in Tube C3 and can be tested for functionality or activity.

Optional protein polishing procedure for Mass Spec

P1) Resuspend the gel in Tube L by vortex mixing. Add 3 ml of this slurry to the target protein in Tube C3 and mix for 2 min by rocking or inverting the tube.

P2) Use the loading needle (Item I) to draw the refolded target protein and resin into a syringe (Item H).

P3) Remove the loading needle (Item I) from the syringe and replace with a resin filter (Item M).

P4) Slowly press the syringe to force the refolded protein solution from the syringe into a product collection tube (Tube C4).

Note: This procedure may result in protein losses of around 10%.

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

A 1a Perform the optional Step 3 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 5 or performing at room temperature if the incubation was previously performed at 4ºC.

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, Bradford Assay and 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 at http://us.expasy.org/tools/protparam.html). Bradford assay is incompatible with Solution A, but can be used at any other stage in the protocol. BCA assay can be used to determine protein concentration when redox agents are not present (e.g. denaturation buffer if redox agents not added, after elution for IMAC column).

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 actually 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. Novexin recommends adding 10% v/v of the redox agents to the denaturation buffer (e.g. 100 µl Solution E to 1.0 ml Solution A containing the target protein) and

repeating the incubation of the denatured protein in Solution A for 1 day at 4ºC or 3 h at 37ºC.

A 4c The protein may not have bound to the IMAC column during the primary purification (the histidine tag may not be fully exposed or binding may be weak in the presence of Tris). Check the flowthrough collected in Tube C2 for a high concentration of the target protein. If this occurred then analyze the protein in the presence of the protection agent, consider using the Novexin Acidic / Basic Protein Refolding Kit or repeat the IMAC purification with the contents of Tube C2 after buffer exchange into 20 mM sodium phosphate pH 7.5.

A 4d The recovery of protein after contacting with the protection removal resin (Tube L) may be unusually low. Check for target protein at the end of Step 18 to confirm if high protein losses occurred after this stage. If so, consider omitting these optional clean-up steps (see Q 12).

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 Novexin 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 5 and 6 are performed at room temperature and consider extending the duration of Step 5.

A 6b Omit the optional Step 3 or add less than 500 µl of the Protein Protection Agent (Solution D) to the Refolding Buffer (Solution B).

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. Consider performing the optional Step 3.

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

A 8 Yes, the Novexin 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) as in Step 4 if none are present in the alternative buffer (also consider buffer compatibility with IMAC purification).

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 after Step 4.

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

A 11 If the quantity of refolded target protein after Step 5 is sufficient for further use then Step 6 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 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 optional protein polishing procedure should be used if the refolded and purified protein at the end of Step 12 provides unexpected peaks during mass spectrometry.

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 The kit is supplied with 4x ~ 100 µl of spare Protein Protection Agent (Solution D). To analyze after Step 7 make a 1 ml blank with the following composition:

Solution A	50 µl
Solution B	940 µl
Solution D	10 µl
Solution E	5 µl
Solution F	50 µl
Total	1055 µl

A 13b To analyze after Step 18 use the elution buffer (Solution K) as the blank.

A 13c To analyze after the optional polishing procedure use the elution buffer (Solution K) as the blank.

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 22 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 1.5% v/v Solution D and a control containing 0% v/v Solution D. Analysis should be performed after Step 6 without any purification. The refolding yield when Solution D is used should be ≥ 70% and higher than the negative control where D was not used.

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

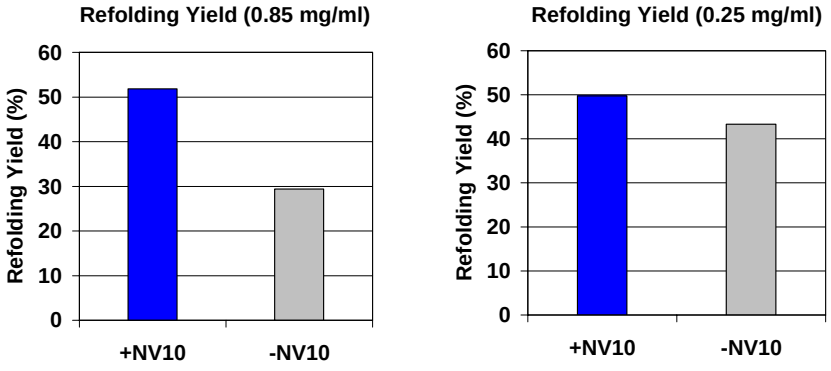
A 15 Yes, but the primary purification steps should not be omitted and it is recommended that a guard column / cartridge is employed when using RP-HPLC columns.

Q 16 I have performed the optional protein polishing procedure but still get unusual peaks on mass spec what should I do?

A 16 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 in Step 3 (final NV10 concentration 4X).



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

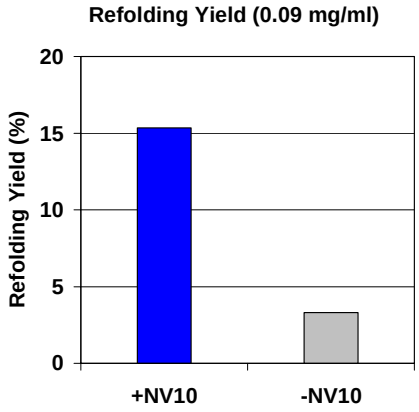
- The NV10 protectant is able to increase yields for proteins where no aggregation is visible by eye, by preventing the formation of low molecular weight (soluble) aggregates.

- 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:

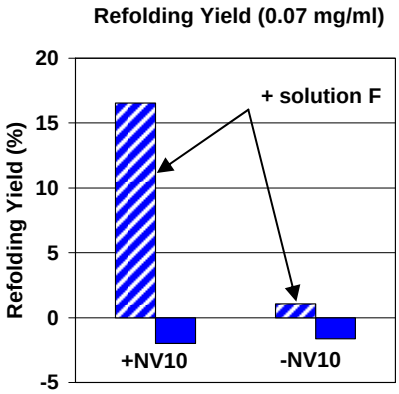
1) Carbonic anhydrase was obtained in a purified form from Sigma (Product # C-3934)
2) Carbonic anhydrase is a 29 kDa monomeric protein with a pI of 5.9 that contains no disulfide bonds in the native structure.
3) Final protein concentrations of 0.85 mg/ml and 0.25 mg/ml correspond to denatured protein solutions at 17 and 5 mg/ml respectively.
4) 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 7, 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 using the Novexin Refold Master. In example 1, citrate synthase (0.09 mg/ml) was denatured in 8 M guanidine-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 Solution A and refolded by 20-fold batch dilution. Extra protein protection agent (100 µl, 1%) was added to the refolding buffer in Step 3 and subsequently released by the addition of 200 µl (2%) Solution F (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 F (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 the addition of solution F (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

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Notes:

1) Citrate synthase was obtained from Sigma (Product # C-3260) in a purified form as a suspension in ammonium sulfate.

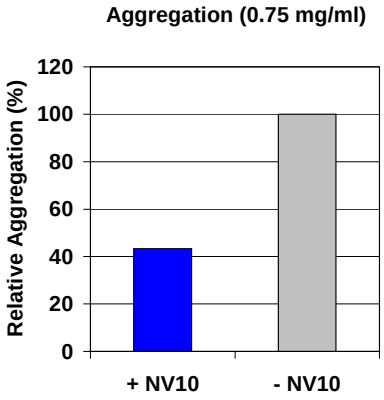
2) Active citrate synthase (from porcine heart) is a homo-dimeric protein composed of two identical subunits, each with a molecular mass of 49 kDa.

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 at the end of Step 7, without carrying out the purification steps, since the protectant did not interfere with the activity assay.

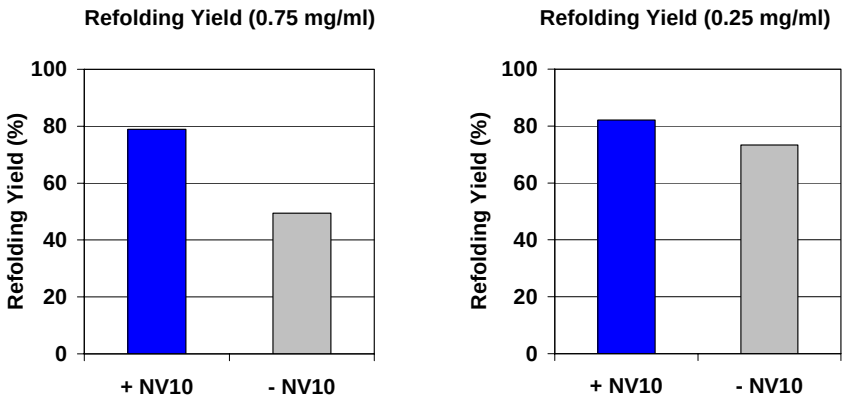
Lysozyme (purified) was denatured and refolded at final concentrations of 0.75 and 0.25 mg/ml using the Novexin Refold Master. High protein concentrations and full reduction were used to more closely resemble refolding following inclusion body solubilization where the target protein is impure and aggregation 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, 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 at the end of Step 7, without carrying out the purification steps, since the protectant did not interfere with the activity assay.