

**Renaturation Basic Kit for Proteins**

Product Number 96827

Store at 2-8 °C

**TECHNICAL BULLETIN****Application**

The Renaturation Basic Kit for Proteins is a rapid empirical screening method used to determine the best conditions for the renaturation of proteins which have been solubilized from inclusion bodies. It is known that physical parameters, such as pH, redox potential, ionic strength, low-molecular weight additives and temperature, may have an effect on the efficiency of *in vitro* renaturation processes. 1-4 Screening with this kit is an easy method to find renaturation conditions in a defined pH range of 6.5 to 8.5, defined redox potential and ionic strength conditions, as well as the effect of one sulfobetaine, namely 3-(1-1-pyridinio)-1-propanesulfonate. Other parameters, e.g., concentration of the solubilized protein, temperature and additional additives, can easily be added to the basic screening conditions of this kit.

Overexpression of proteins in prokaryotes often leads to the production of insoluble aggregates of misfolded proteins in inclusion bodies. Often considered a nuisance, the formation of inclusion bodies has the advantage of a high enrichment of the desired protein at an early stage of purification. Furthermore, the recombinant protein is protected in inclusion bodies against proteolysis by intracellular proteases. These inclusion bodies can easily be purified and may be the best method for the production of proteins that are lethal to the host cells. However, the solubilization of the expressed protein can only be obtained using strongly denaturing conditions. The major task is to achieve an efficient *in vitro* renaturation to the properly folded protein.

**Formulation/Storage/Stability**

All reagents are formulated using high purity reagents, mostly Fluka Microselect, and ultrapure water. Microselect chemicals have been used successfully for different crystallization methods and other applications that are highly sensitive to impurities. All solutions are sterile filtered using 0.22 micron filters and are available separately as 100

ml bottles. Larger quantities are available on request.

Reagents are stable at room temperature if the bottles aren't opened. To enhance reagent stability, it is strongly recommended that kit reagents be stored at 2-8 °C or -20 °C. Do not expose the reagents to ultraviolet light.

If the samples contain phosphate, borate, or carbonate buffers, inorganic crystals may form when using those reagents containing divalent cations, e.g., magnesium, calcium, or zinc. To avoid false positives, use phosphate, borate, or carbonate buffers at concentrations of 10 mM or less, or replace the phosphate, borate, or carbonate buffers with a more soluble buffer that does not complex with divalent cations.

**Sample Preparation**

Since inclusion body proteins do not readily disintegrate under physiological conditions, the solubilization requires rather strong denaturants such as 6 M guanidine hydrochloride (Microselect Quality) or 6 – 8 M urea (Fluka 51457, Microselect). Guanidine hydrochloride is usually preferred over urea because it is a rather strong chaotropic agent that may solubilize extremely sturdy inclusion bodies, and because urea solutions may contain isocyanate leading to carbamylation of the free amino groups of the polypeptide. If urea is used for inclusion body solubilization, scavengers containing free amino groups should be included in the solubilization cocktail. In the case of proteins containing cysteine, the isolated inclusion bodies usually contain some interchain disulfide bonds which reduce the solubility. Addition of reducing agents, like low-molecular weight thiol reagents, in combination with chaotropic agents allows reduction of the interchain disulfide bonds.

The degree of purification of the proteins depends on the intended use. Because inclusion bodies collected after cell disruption are usually relatively homogeneous, the proteins can be renatured directly after solubilization without further purification. Rigorous purification of the inclusion body protein may not be required for efficient

refolding. If the influence of other proteins like chaperones or other folding enhancers is of interest, or if spectroscopic techniques are used to monitor *in vitro* folding, purification of the solubilized inclusion body protein may be necessary.

The solubilization of the Inclusion bodies is a prerequisite for the application of this renaturation kit and is usually achieved by using 6M guanidine hydrochloride solution. The solubilization can itself be optimized, but as a starting point the following procedure can be useful.

1. The pellet from 1L bacterial suspension is resuspended in 20 ml 50 mM HEPES-NaOH, pH 7.5, 0.5 M NaCl (Fluka 71378), 1 mM PMSF (Fluka 78830), 5 mM DTT (Fluka 43817) and 0.35 mg/ml lysozyme (Fluka 62970), then incubated for 30 min at 20 °C.
2. 200 µl Triton X-100 (Fluka 93418) is added and then sonicated until the solution clears.
3. The extract is treated with 20 mg/ml DNase I (Fluka 31132) at 37 °C and the inclusion bodies are centrifuged at 30'000 g for 30 min at 4 °C.
4. The pellet is washed twice with TBS (Fluka 93312) or PBS (Fluka 79378) with 1% Triton X-100 and centrifuged at 30'000 g for 30 min at 4 °C.
5. The pellet (inclusion bodies) is solubilized in 2 ml 50 mM HEPES-NaOH, pH 7.5, 6 M guanidine hydrochloride, 25 mM DTT and incubated for 1 hr at 4 °C.
6. Insoluble material is removed by centrifugation at 100'000 g for 10 min. It is important to remove existing aggregates that can act as nuclei to start aggregation during renaturation.
7. Determine the protein concentration and adjust to 1 mg/ml using 50 mM HEPES-NaOH, pH 7.5, 6M guanidine hydrochloride, 25 mM DTT and proceed directly to renaturation.

## Procedure

The following describes one way of using the Renaturation Basic Kit for Proteins.

**Note:** It is important to mix the samples quickly and thoroughly.

Prepare plates with 24 wells.

1. Using a clean pipet tip for each reagent, pipet 900 µl of cold reagent 1 into the first well, A1.
2. Add 100 µl of the solubilized protein sample into the well. Mix the solubilized protein as quickly as possible by aspirating and dispensing. The final protein concentration should not exceed 0.1 mg/ml. Keep the tip in the drop during mixing to avoid foaming.
3. Repeat 1. and 2. using the remaining reagents.
4. Seal the entire plate with clear sealing tape.
5. The screening can be performed in duplicate at different temperatures if sample quantities permit. Incubate and store the plates in a place with stable temperature.
6. Determine yields of renaturation after 1 hr and 24 hr of renaturation by using a reliable, fast and easy assay to monitor native structure formation: enzymatic activity, HPLC, spectroscopy, ligand binding, ELISA or bioassays. Measuring the solution turbidity is a good indication of protein aggregation.
7. Compare the observations between the sample in the different reagents, and at the different incubation temperatures.

The suggested reagents (substances, concentration) are starting points and have to be optimized in order to obtain highest renaturation yields.

## Interpreting Results

In order to interpret the renaturation yield, a quantitative measure of the function of the properly folded pure protein, e.g. maximum specific enzyme activity is needed.

$$\text{renaturation yield} = \frac{\text{properly folded protein}}{\text{solubilized protein in chaotrope}}$$

This kit allows a quick screen to determine whether the protein of interest can be renatured. After successful renaturation from inclusion bodies, the kit gives some insight into which of the factors are most important and this information can be used to refine the renaturation conditions. Key parameters are protein concentration, residual guanidine concentration and temperature.

If the results of the screen indicates that the renaturation from inclusion bodies is not feasible, additional auxiliary reagents should be screened which favor the formation of the native fold and minimize the aggregation of folding intermediates. A

broad range of such reagents, e.g., arginine, PEG, sulfobetaines, detergents and chaotropes, are available from Fluka (see catalog). It is recommended that reagents with the highest purity are used to minimize aggregation and to increase the yield of the properly folded protein.

## References

1. R. Rudolph & H. Lilie, *The FASEB Journal*, 10, 49 (1996)
2. L. Vuillard, T. Rabilloud & M.E. Goldberg, *Eur. J. Biochem.* 256, 128 (1998)
3. P.H. Bessette, F. Aslund, J. Beckwith & G. Georgiou, *PNAS* 96, 13703 (1999)
4. N. Armstrong, A. DeLencastre & E. Gouaux, *Protein Science* 8,1475 (1999)

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Sample: \_\_\_\_\_

Date: \_\_\_\_\_

Sample Concentration (protein): \_\_\_\_\_

Start Time: \_\_\_\_\_

Sample Puffer: \_\_\_\_\_

Temperature: \_\_\_\_\_

Volume: Total \_\_\_\_\_ ul Sample \_\_\_\_\_ ul Reagent \_\_\_\_\_ ul other possible Additives \_\_\_\_\_ ul

No.	Fluka No.	Reagent Name	Time & Renaturation yield:	Time & Renaturation yield:	Time & Renaturation yield:
1.	72903	MES (pH 6.5) 0.05M, Na-chloride 0.1M			
2.	83047	MES (pH 6.5) 0.05M, Na-chloride 0.1M, PPS 1M			
3.	82953	MES (pH 6.5) 0.05M, Na-chloride 0.2M			
4.	72905	MES (pH 6.5) 0.05M, Na-chloride 0.2M, PPS 1M			
5.	73021	MES (pH 6.5) 0.05M, DTT 0.001M			
6.	71023	MES (pH 6.5) 0.05M, DTT 0.001M, PPS 1M			
7.	93038	MES (pH 6.5) 0.05M, Na-chloride 0.1M, DTT 0.001M			
8.	85312	MES (pH 6.5) 0.05M, Na-chloride 0.1M, DTT 0.001M, PPS 1M			
9.	83809	MES (pH 6.5) 0.05M, Na-chloride 0.2M, DTT 0.001M			
10.	73194	MES (pH 6.5) 0.05M, Na-chloride 0.2M, DTT 0.001M, PPS 1M			
11.	72476	MES (pH 6.5) 0.05M, L-GSH red. 0.005M, L-GSH ox. 0.001M			
12.	77809	MES (pH 6.5) 0.05M, L-GSH red. 0.005M, L-GSH ox. 0.001M, PPS 1M			
13.	72914	MES (pH 6.5) 0.05M, Na-chloride 0.1M, L-GSH red. 0.005M, L-GSH ox. 0.001M			
14.	78569	MES (pH 6.5) 0.05M, Na-chloride 0.1M, L-GSH red. 0.005M, L-GSH ox. 0.001M, PPS 1M			
15.	78582	MES (pH 6.5) 0.05M, Na-chloride 0.2M, L-GSH red. 0.005M, L-GSH ox. 0.001M			
16.	94718	MES (pH 6.5) 0.05M, Na-chloride 0.2M, L-GSH red. 0.005M, L-GSH ox. 0.001M, PPS 1M			
17.	94717	HEPES Na-salt (pH 7.5) 0.05M			
18.	78593	HEPES Na-salt (pH 7.5) 0.05M, PPS 1M			
19.	87062	HEPES Na-salt (pH 7.5) 0.05M, Na-chloride 0.1M			
20.	78579	HEPES Na-salt (pH 7.5) 0.05M, Na-chloride 0.1M, PPS 1M			
21.	87605	HEPES Na-salt (pH 7.5) 0.05M, Na-chloride 0.2M			
22.	72906	HEPES Na-salt (pH 7.5) 0.05M, Na-chloride 0.2M, PPS 1M			
23.	78152	HEPES Na-salt (pH 7.5) 0.05M, DTT 0.001M			
24.	72824	HEPES Na-salt (pH 7.5) 0.05M, DTT 0.001M, PPS 1M			
25.	72588	HEPES Na-salt (pH 7.5) 0.05M, Na-chloride 0.1M, DTT 0.001M			
26.	76983	HEPES Na-salt (pH 7.5) 0.05M, Na-chloride 0.1M, DTT 0.001M, PPS 1M			
27.	73193	HEPES Na-salt (pH 7.5) 0.05M, Na-chloride 0.2M, DTT 0.001M			
28.	80293	HEPES Na-salt (pH 7.5) 0.05M, Na-chloride 0.2M, DTT 0.001M, PPS 1M			
29.	80292	HEPES Na-salt (pH 7.5) 0.05M, L-GSH red. 0.005M, L-GSH ox. 0.001M			
30.	76951	HEPES Na-salt (pH 7.5) 0.05M, L-GSH red. 0.005M, L-GSH ox. 0.001M, PPS 1M			
31.	88503	HEPES Na-salt (pH 7.5) 0.05M, 0.1M Na-chloride, L-GSH red. 0.005M, L-GSH ox. 0.001M			
32.	83068	HEPES Na-salt (pH 7.5) 0.05M, Na-chloride 0.1M, L-GSH red. 0.005M, L-GSH ox. 0.001M, PPS 1M			
33.	83796	HEPES Na-salt (pH 7.5) 0.05M, Na-chloride 0.2M, L-GSH red. 0.005M, L-GSH ox. 0.001M			
34.	80409	HEPES Na-salt (pH 7.5) 0.05M, Na-chloride 0.2M, L-GSH red. 0.005M, L-GSH ox. 0.001M, PPS 1M			
35.	75837	TRIS-HCl (pH 8.5) 0.05M, Na-chloride 0.1M			
36.	91810	TRIS-HCl (pH 8.5) 0.05M, Na-chloride 0.1M, PPS 1M			
37.	82959	TRIS-HCl (pH 8.5) 0.05M, Na-chloride 0.2M			
38.	83943	TRIS-HCl (pH 8.5) 0.05M, Na-chloride 0.2M, PPS 1M			
39.	82305	TRIS-HCl (pH 8.5) 0.05M, DTT 0.001M			
40.	70603	TRIS-HCl (pH 8.5) 0.05M, DTT 0.001M, PPS 1M			
41.	75812	TRIS-HCl (pH 8.5) 0.05M, Na-chloride 0.1M, DTT 0.001M			
42.	81220	TRIS-HCl (pH 8.5) 0.05M, Na-chloride 0.1M, DTT 0.001M, PPS 1M			
43.	72259	TRIS-HCl (pH 8.5) 0.05M, Na-chloride 0.2M, DTT 0.001M			
44.	72991	TRIS-HCl (pH 8.5) 0.05M, Na-chloride 0.2M, DTT 0.001M, PPS 1M			
45.	78217	TRIS-HCl (pH 8.5) 0.05M, L-GSH red. 0.005M, L-GSH ox. 0.001M			
46.	95739	TRIS-HCl (pH 8.5) 0.05M, L-GSH red. 0.005M, L-GSH ox. 0.001M, PPS 1M			
47.	82972	TRIS-HCl (pH 8.5) 0.05M, Na-chloride 0.1M, L-GSH red. 0.005M, L-GSH ox. 0.001M			
48.	72403	TRIS-HCl (pH 8.5) 0.05M, Na-chloride 0.1M, L-GSH red. 0.005M, L-GSH ox. 0.001M, PPS 1M			
49.	85323	TRIS-HCl (pH 8.5) 0.05M, Na-chloride 0.2M, L-GSH red. 0.005M, L-GSH ox. 0.001M			
50.	93039	TRIS-HCl (pH 8.5) 0.05M, Na-chloride 0.2M, L-GSH red. 0.005M, L-GSH ox. 0.001M, PPS 1M			