

# RTS DnaK Supplement

For supplementation of *e.g.*, one hundred RTS 100 *E. coli* HY reactions

Cat. No. 03 784 843 001

5 × 180 µl

Version 1, October 2003

Store at –15 to –25°C

## 1. Introduction

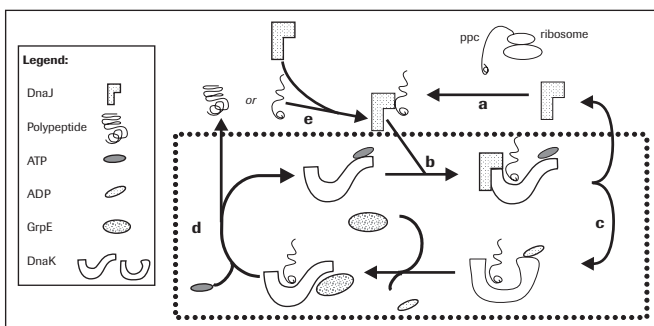
The Rapid Translation System (RTS) is a flexible and scalable tool for cell-free protein expression. It can be considered as an “open” system, meaning that the reaction conditions can easily be adapted in a protein-specific manner by adding *e.g.*, co-factors, ligands or detergents to the reaction mixture.

A very important example in this regard is the addition of chaperones in order to increase the amount of correctly folded and/or soluble recombinant protein.

DnaK (heat shock protein 70, hsp70), together with DnaJ (hsp 40) and GrpE, is one of the key chaperone systems in *E. coli*. *In vivo* it is involved in

- prevention of aggregation and refolding of misfolded proteins
- mediation of degradation of unstable proteins by proteases
- modulation of heat shock response
- protein translocation and other processes<sup>1,2,3</sup>.

For its folding activity, the DnaK protein depends on ATP/ADP-conversion and on the DnaJ and GrpE co-chaperones<sup>1,2,3,4</sup> (see Fig. 1).



**Fig. 1:** Model of protein folding mediated by the DnaK-chaperone system. The nascent polypeptide chain (ppc) is bound by the DnaJ co-chaperone (a). This complex is recognized by DnaK in its ATP-bound state (b). Subsequently, the DnaJ is released and the ATP is hydrolyzed to give the substrate-DnaK complex (c). Finally, GrpE stimulates ADP dissociation and enables DnaK to bind ATP. The binding of ATP releases the GrpE and the polypeptide (d). If the polypeptide is still not folded correctly, it may undergo another cycle (e).

Mutagenesis studies revealed which domain of DnaK interacts with the protein substrate and with the co-chaperones DnaJ or GrpE, respectively<sup>2</sup>. In addition, peptide scan experiments showed that DnaK binds to stretches of hydrophobic amino acid residues<sup>1</sup>. However, even based on these findings, it remains difficult to predict whether a given protein will be recognized as substrate for the DnaK chaperone system or not.

In contrast to the GroE chaperone system (*cf.* Cat. No. 3 263 690) the applicability of DnaK is not limited by the size of the target proteins it binds to. In addition, it can also serve as an aggregation inhibitor which binds unfolded protein or may transfer it to other chaperones like the GroE or the Hsp90 systems. A transfer of misfolded proteins to *E. coli* proteases may also occur.

## Applications

To optimize the yield and solubility of proteins expressed in the Rapid Translation System, we generally recommend to first express the protein in the RTS 100 *E. coli* HY Kit (Cat. No. 3 186 148), in the presence or absence of different chaperones and/or detergents. Once a positive effect is observed in RTS 100 *E. coli* HY batch reactions (50 µl), the same conditions can in principle be applied to CECF (continuous exchange cell-free) reactions as well (performed *e.g.* with the RTS 500 *E. coli* ProteoMaster Kit, Cat. No. 3 335 461). However, since expression rates under CECF conditions are significantly higher than in batch mode, added DnaK may come to a point in which it can not cope with the quantity of protein produced (this effect is dependant on each specific substrate protein). When using DnaK under CECF conditions, we therefore recommend to run kinetic studies by taking samples after 2, 4, and 6 hours and analyze the respective ratio of soluble and insoluble protein.

## 2. Product description

The set includes 5 vials of DnaK Mix (purple cap), 180 µl each, containing a mixture of DnaK, DnaJ and GrpE as components of a bacterial lysate. In addition, 1.5 ml Energy Mix (containing ATP) is included (colorless cap).

The product can be used in the cell-free protein expression systems RTS 100 *E. coli* HY (Cat. No. 3 186 148), RTS 500 ProteoMaster *E. coli* HY (Cat. No. 3 335 461) and RTS 500 *E. coli* HY (Cat.No. 3 246 817 and 3 246 949).

## Specificity

The DnaK system (DnaK, DnaJ, GrpE) is a prokaryotic chaperone system. The system has no substrate limitation regarding the size of the proteins and can act in principle on all proteins. However, it is not possible to exactly predict to which extent a protein's folding can be improved by DnaK. Similar to *in vivo* situations (expression in intact *E. coli* cells) DnaK may also present the protein to proteases, resulting in a reduction of total protein yield.

## Stability

**DnaK Mix:** Turbid solution. Stable at –20°C. Freeze/thaw cycles up to three times do not decrease activity. After thawing stable for 4 hours at 4°C.

**Energy Mix:** Stable at –20°C. Stable for at least 20 freeze/thaw cycles. Can be aliquoted.

### 3. Application

**Note** In all protocols given below, differences between the standard (chaperone-free) procedures and procedures using DnaK Supplement are marked in **bold**.

#### 3.1 Supplementing RTS 100 *E. coli* HY reactions

##### 3.1.1 Reconstitution of reaction components

Solution*	Content	Reconstitution/preparation of working solution	For use in
1	<i>E. coli</i> Lysate Bottle 1, red cap	Reconstitute the lyophilizate with 0.36 ml of Reconstitution Buffer (bottle 5), mix carefully by rolling or gentle shaking. Do not vortex!	section 3.1.2 solution 7
2	Reaction Mix Bottle 2, green cap	Reconstitute the lyophilizate with 0.30 ml of Reconstitution Buffer (bottle 5), mix by rolling or shaking.	section 3.1.2 solution 7
3	Amino Acids Bottle 3, brown cap	Reconstitute the lyophilizate with 0.36 ml of Reconstitution Buffer (bottle 5), mix by rolling or shaking.	section 3.1.2 solution 7
4	Methionine Bottle 4, yellow cap	Reconstitute the lyophilizate with 0.33 ml of Reconstitution Buffer (bottle 5), mix by rolling or shaking.	section 3.1.2 solution 7
5	Reconstitution Buffer Bottle 5, white cap	1.6 ml Ready-to-use solution The solution is stable at 4°C but can also be stored at -20°C	solutions 1, 2, 3, 4

\* numbers refer to bottles in RTS 100 *E. coli* HY Kit

**Appearance of solutions** With the exception of the *E. coli* lysate all reconstituted lyophilizates should be clear solutions.

##### 3.1.2 Preparation of working solutions

Solution*	Content	Reconstitution/Preparation of working solution	For use in
7	Reaction Solution	Into one of the supplied reaction tubes pipet the following components: 1. 12 µl <i>E. coli</i> Lysate 2. 10 µl Reaction Mix 3. 12 µl Amino Acids 4. 1 µl Methionine 5. 5 µl Reconstitution Buffer 6. <b>5–8 µl of DnaK Mix (this kit)</b> 7. <b>1.2 µl Energy Mix (this kit)</b> 8. 0.5 µg of the circular DNA or linear template in 10 µl of water or TE-buffer • <b>Please note: the final volume will be slightly higher than in case of the standard RTS 100 <i>E. coli</i> HY protocol !</b> • A pre-mix of solutions 2 to 5 without DNA is recommended for multiple reactions run in parallel. • Mix carefully by rolling or gentle shaking. • <b>DO NOT VORTEX !</b> • Run the reaction according to the manual of the RTS 100 <i>E. coli</i> HY Kit (Cat.No. 3 186 148)	

\* numbers refer to bottles in RTS 100 *E. coli* HY Kit

**Reaction set-up** Please refer to section 3.1.4 of the manual of the RTS 100 *E. coli* HY Kit, Cat.No. 3 186 148.

### 3.2 Supplementing RTS 500 ProteoMaster *E. coli* HY or RTS 500 *E. coli* HY reactions

#### 3.2.1 Reconstitution of reaction components

**Before you start** When supplementing RTS 500 ProteoMaster or *E. coli* HY or RTS 500 *E. coli* HY reactions with chaperones, make sure the *E. coli* lysate (bottle 1 in both RTS 500 kits) is reconstituted in only 0.34 ml (instead of 0.575 ml) of reconstitution buffer. Otherwise the DnaK supplement cannot be added because of volume constraints.

**Important note** It is strongly recommended to centrifuge the reconstituted lysate in order to optimize the folding conditions for highest yields of soluble protein.  
Setting the reaction temperature to 25°C instead of 30°C is necessary to give the chaperones more time to fold the synthesized protein.

Solution*	Content	Reconstitution/Preparation of working solution	For use in
1	<i>E. coli</i> Lysate Bottle 1, red cap	Reconstitute the lyophilizate with <b>0.340</b> ml of Reconstitution Buffer (bottle 6), mix carefully by rolling or gentle shaking. Do not vortex! <b>Important note:</b> Centrifuge the reconstituted lysate at 14 000 rpm for 10 min. Separate the supernatant from the pellet and discard the pellet.	• section 3.2.2 • solution 8
2	Reaction Mix Bottle 2, green cap	Reconstitute the lyophilizate with 0.25 ml of Reconstitution Buffer (bottle 6), mix by rolling or shaking.	• section 3.2.2 • solution 8
3	Feeding Mix Bottle 3, blue cap	Reconstitute the lyophilizate with 8.1 ml of Reconstitution Buffer (bottle 6), mix by rolling or shaking.	• section 3.2.2 • solution 7
4	Amino Acid Mix without Methionine Bottle 4, brown cap	Reconstitute the lyophilizate with 3 ml of Reconstitution Buffer (bottle 6), mix by rolling.	• section 3.2.2 • solution 7 and 8
5	Methionine Bottle 5, yellow cap	Reconstitute the lyophilizate with 1.8 ml of Reconstitution Buffer (bottle 6), mix by rolling or shaking.	• section 3.2.2 • solution 7 and 8
6	Reconstitution Buffer Bottle 6, white cap	• Ready-to-use solution • The solution is stable at 4°C but can also be stored at -20°C	solution 1, 2, 3, 4, 5

\* numbers refer to bottles in the RTS 500 kits

**Appearance of solutions** With the exception of the *E. coli* lysate all lyophilizates should be clear solutions after reconstitution.

### 3.2.2 Preparation of working solutions

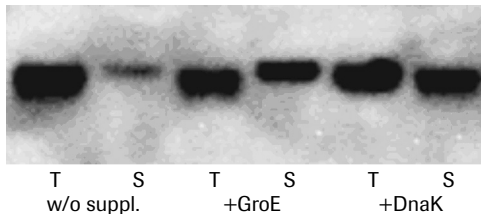
Solution	Content	Reconstitution/Preparation of working solution	For use in
7	Feeding Solution	Add 2.65 ml of the reconstituted Amino Acid Mix without Methionine (solution 4) and 0.3 ml of reconstituted Methionine (solution 5) to solution 3. Finally add <b>66 µl Energy Mix</b> and mix by rolling or shaking. Total volume now will be 11 ml.	section 3.1.4 of the manual of Cat. No. 3 335 461, 3 246 817 or 3 246 949
8	Reaction Solution	To the content of solution 1 add 0.225 ml of the reconstituted Reaction Mix (solution 2), 0.27 ml of the reconstituted Amino Acid Mix without Methionine (solution 4) and 30 µl of reconstituted Methionine (solution 5). Add <b>180 µl of DnaK Mix (this kit) and 6 µl Energy Mix (this kit)</b> . Finally, add 10–15 µg of DNA template in a maximum volume of 50 µl. Mix carefully by rolling or gentle shaking. Total volume now will be 1.1 ml. Do not vortex!	section 3.1.4 of the manual of Cat. No. 3 335 461, 3 246 817 or 3 246 949

**Reaction set-up** Please refer to section 3.1.4 of the manual of Cat. Nos. 3 335 461 or 3 246 817 (2 reactions pack size) or 3 246 949 (5 reactions pack size).  
**Important note:** We recommend to run DnaK-supplemented CECF reactions at 25°C instead of 30°C, in order to give the chaperones more time for folding the synthesized protein.

## 4. Example

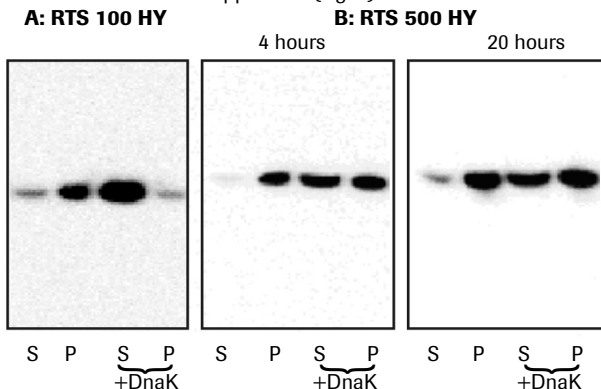
### 4.1 Expression of protein kinase C

Using the standard workflow for cell-free protein expression in the RTS *E. coli* system (see [www.proteinexpression.com](http://www.proteinexpression.com)), it was found that the solubility of protein kinase C (44 kDa) could be significantly increased by the addition of DnaK Supplement (Fig. 2).



**Fig. 2:** Expression of protein kinase C from a PCR-generated linear expression template, using chaperone additives (analysis by anti-His<sub>6</sub> Western blot).  
 T: Total reaction  
 S: Soluble fraction (supernatant)

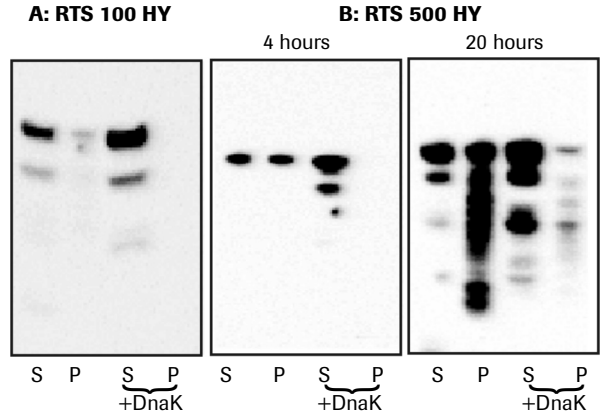
After cloning into an RTS pIVEX plasmid, the protein was expressed using the RTS 100 *E. coli* HY Kit (batch mode, 30°C) and the RTS 500 ProteoMaster *E. coli* HY kit (CECF mode, 25°C). In both cases, the yield of soluble protein was enhanced significantly by the addition of DnaK Supplement (Fig. 3).



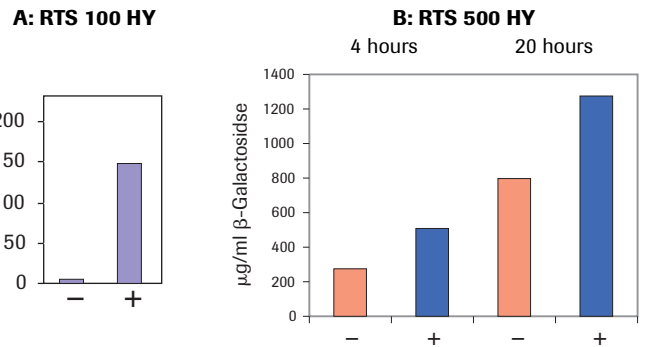
**Fig. 3:** Western blot analysis (anti-His<sub>6</sub>) of the expression of protein kinase C in RTS 100 *E. coli* HY (A) and RTS 500 *E. coli* HY (B) after 4 and 20 hrs without (first two lanes on each gel) and with

### 4.2 Expression of β-galactosidase

β-Galactosidase (116 kD) was expressed using the RTS 100 *E. coli* HY Kit (batch mode, 30°C) and using the RTS 500 ProteoMaster *E. coli* HY Kit (CECF mode, 25°C). In both cases, the yield of soluble protein (Fig. 4) as well as the corresponding enzymatic activity (Fig. 5) was enhanced significantly when DnaK Supplement was added to the expression reaction.



**Fig. 4:** Western blot analysis (anti-His<sub>6</sub>) of the expression of β-galactosidase (N-term. His<sub>6</sub>-tag) in RTS 100 *E. coli* HY (A) and RTS 500 *E. coli* HY (B; after 4 and 20 h) with and without (first two lanes on each gel) and with DnaK-supplement. S: soluble fraction P: pellet



**Fig. 5:** Activity of β-galactosidase (N-term. His<sub>6</sub>-tag) after expression in RTS 100 *E. coli* HY (A) and RTS 500 *E. coli* HY (B; after 4 and 20 h) with and without DnaK-supplement. - : without DnaK, + : with DnaK

## 5. References

- 1 See "Molecular Chaperons and Folding Catalysts", Harwood Academic Publishers, 1999 (ed. Bernd Bukau): Buchberger, A., Reinstein, J., Bukau, B. (pp. 609-637).
- 2 Guidebook to Molecular Chaperones and Protein-Folding Catalysts, Oxford University Press, 1997 (edited by Mary-Jane Gething): Buchberger, A.; Bukau, B (pp. 22-24); A. Wawrynaw, A.; Zyllicz, M. (pp. 95-97).
- 3 Walter, S.; Buchner, J., *Angew Chem Int Ed Engl.* (2002); **41**(7), 1098-113.
- 4 Schmid, D.; Baici, A., Gehring, H., Christen, P. (1994) *Science* **263**, 971-73.

## 7. Related products

Product	Pack Size	Cat. No.
<b>Rational Gene Design</b>		
ProteoExpert RTS <i>E. coli</i> HY	1 access number	3 115 569
	5 access numbers	3 115 577
	25 access numbers	3 115 585
<b>Linear Template Generation by PCR</b>		
RTS <i>E. coli</i> Linear Template Generation Set, His <sub>6</sub> -Tag	96 reactions	3 186 237
RTS <i>E. coli</i> Linear Template Generation Set, HA-Tag	96 reactions	3 315 860
RTS <i>E. coli</i> Linear Template Generation Set, MBP fusion	96 reactions	3 358 828
<b>Rapid Expression Screening and Optimization</b>		
RTS 100 <i>E. coli</i> HY Kit <sup>3</sup>	24 reactions	3 186 148
	96 reactions	3 186 156
<b>Preparative-Scale Expression</b>		
RTS 500 ProteoMaster <i>E. coli</i> HY Kit <sup>1,3,4,5</sup>	5 reactions	3 335 461
RTS 500 <i>E. coli</i> HY Kit <sup>2,3,4,5</sup>	2 reactions	3 246 817
	5 reactions	3 246 949
RTS 9000 <i>E. coli</i> HY Kit <sup>3,4,5</sup>	1 reaction	3 290 395
	3 reactions	3 290 468
<b>AviTag Biotinylation Reagents</b>		
RTS AviTag <i>E. coli</i> Biotinylation Kit, Linear Template	For 96 reactions (RTS 100)	3 521 818
RTS AviTag <i>E. coli</i> Biotinylation Kit, Plasmid	For 96 reactions (RTS 100) or 5 reactions (RTS 500)	3 514 919
RTS AviTag Biotinylation Kit	For 96 reactions (RTS 100) or 5 reactions (RTS 500)	3 514 935
<b>Instrumentation</b>		
RTS ProteoMaster Instrument	1 instrument	3 265 650
<b>Vectors</b>		
RTS pIVEX His <sub>6</sub> -Tag 2 <sup>nd</sup> Generation Vector Set	2 vectors, 10 µg each	3 269 019
RTS pIVEX HA-tag Vector Set	2 vectors, 10 µg each	3 268 993
RTS pIVEX MBP Fusion Vector <sup>6</sup>	1 vector, 10 µg	3 268 985
RTS pIVEX GST Fusion Vector <sup>7</sup>	1 vector, 10 µg	3 268 969
<b>Other Reagents</b>		
RTS GroE Supplement	for five RTS 500 reactions	3 263 690
RTS Amino Acid Sampler	for five RTS 500 reactions	3 262 154

<sup>1</sup> For use with the RTS ProteoMaster Instrument only

<sup>2</sup> For use with the RTS 500 Instrument

<sup>3</sup> For Research Use Only. Proteins expressed using the RTS, and data derived therefrom that would enable the expression of such proteins (collectively, "Expressed Proteins"), may be used only for the internal research of the purchaser of this system. Expressed Proteins may not be sold or transferred to any third party without the written consent of Roche Diagnostics.

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