

MATERIAL DATA SHEET

HSP60/HSP10 Glow-Fold™ Protein Refolding Kit Cat. # K-300

HSP60 (also known as Chaperonin 60) and HSP10 (also known as Chaperonin 10) are the eukaryotic homologues of prokaryotic GroEL and GroES chaperones, respectively. These proteins are found mainly in mitochondria, but can also be detected in cytosol and extracellular fluids including peripheral blood. The HSP60/HSP10 complex plays an essential role in the translocation and refolding of proteins from the cytosol into the mitochondrial matrix. Under physiological conditions, HSP60 creates two stacked heptameric rings that form a pair of central hydrophobic cavities. After an unfolded substrate protein enters one of cavities it is capped by a heptameric HSP10 complex, thereby trapping the unfolded protein. Structural rearrangement of the substrate-containing cavity is effected via HSP60-mediated ATP hydrolysis; this changes the lining of the cavity from hydrophobic to hydrophilic and helps promote refolding of the substrate protein. Binding of ATP to HSP60 subunits on the distal ring of the complex then causes the dissociation of the HSP10 cap complex and concomitant release of the substrate protein from the proximal cavity. If the protein is not completely folded, it can be further processed by the HSP60/HSP10 complex, or can interact with other chaperoning systems.

This kit provides a functional *in vitro* HSP60/HSP10 refolding system. Using the provided substrate protein, the kit can be used to screen for small molecules affecting the efficiency of the refolding process (such as HSP inhibitors). Alternatively, the HSP60/HSP10 complex may be used to test refolding of user-supplied proteins if a functional assay is available.

NOTE: Kit contains reagents sufficient for 15 x 20 µl reactions.

| Reagents Provided in Kit | |
|-------------------------------------|---|
| <u>Component</u> | <u>Volume</u> |
| 1. 5X HSP60 Solution | 60 µl |
| 2. 10X HSP10 Solution | 30 µl |
| 3. 10X HSP60 Reaction Buffer | 50 µl |
| 4. 10X Mg ²⁺ -ATP | 30 µl |
| 5. 10X Glow-Fold™ Substrate Protein | 50 µl |
| 6. Luciferin Reagent | 2 ml |
| Storage: | Store components at -80°C. Avoid multiple freeze/thaw cycles. |

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Reagents to be Provided by Investigator

The following reagents and materials need to be obtained by the investigator prior to using this kit.

| | |
|-------------------|---|
| dH ₂ O | Sterile |
| Water baths | One each at 30°C and 45°C |
| Reaction tubes | 0.5 ml polypropylene, microcentrifuge compatible |
| Reaction plates | 96-well half area plate, opaque white, polystyrene or preferably low protein binding (e.g. Corning #3642) |
| Plate Reader | Luminescence-capable plate reader |

Assay Considerations

In this assay, Glow-Fold™ Substrate Protein is denatured by heat shock in the presence of an HSP60/HSP10 complex and ATP. Heating the substrate in the *absence* of the chaperones leads to its aggregation and precipitation; in this state the substrate is typically not amenable to productive refolding by the heat shock protein system.

The heat shock process documented in this protocol inactivates 98-99% of input luminescence activity. After 120 minutes of HSP60/HSP10-mediated refolding, up to 30% of the Glow-Fold™ activity (pre heat-shock) can be recovered.

The protocol details a 20 µl endpoint assay with 4 µl aliquots removed for measurements taken at 0 and 60 minutes. Aliquots can be removed for analysis at other incubation times if more time points are desired. Alternative substrates may be supplied by the user—reaction times will need to be determined experimentally. Suggested concentration range for user provided substrates is 0.1-1 µM, final, depending on assay sensitivity. The assay is compatible with DMSO, but final concentration in the reaction should not exceed 5%.

Modifications to the protocol or selection of alternative reagents (particularly substrate protein) may require assay optimization by the end-user. Further information available at techsupport@bostonbiochem.com.

Recommended Assay Protocol (20 µl volume)

1. Reagent Preparation

- a. Quickly thaw all protein reagents and buffers by gently and continuously swirling tubes in a lukewarm water bath ($\leq 30^{\circ}\text{C}$). Alternatively, tubes may be thawed with a rapid back-and-forth rolling motion between palms of hands. Do not heat tubes for an extended period of time. Do not vortex or shake vigorously.
- b. When completely thawed, *gently* tap tubes to make sure components are well mixed, then briefly spin in a microcentrifuge (5 seconds) to collect contents in bottom of tubes.
- c. Immediately ice components. Entire process in steps 1a and 1b should be accomplished in approximately 5 minutes.
- d. It is ***strongly recommended*** that each reagent be divided into smaller aliquots to minimize the number of freeze-thaw cycles of kit components. Avoid multiple freeze-thaw cycles to maximize kit performance. Rapidly re-freeze any aliquoted materials in dry ice bath.

2. Plate Reader Control Reaction (**optional**)

- a. Prepare the following control reaction to determine that the upper limit of Glow-Fold™ activity (untreated) in the assay does not exceed the dynamic range of the plate reader.
- b. Combine the following in a 0.5 ml reaction tube (in order):
 - i. 16 µl dH₂O
 - ii. 2 µl 10X Reaction Buffer
 - iii. 2 µl 10X Glow-Fold™ Substrate Protein
- c. Mix by pipetting or gently flicking the tube. Briefly centrifuge to collect contents in bottom of tube.
- d. Transfer 4 µl of the control mix to a 96-well half-area plate.
- e. Add 50 µl Luciferin Reagent and mix by pipetting up and down while stirring carefully with the pipette tip. Proceed *immediately* to step **2f**.
- f. Read the luminescence value for the reaction within 1-2 minutes of mixing the control reaction and Luciferin Reagent. The obtained measurement should be well within the dynamic range for the instrument. If the measurement is low, repeat the procedure using more control mix starting at step **2d** (e.g. 8-10 µl). If the measurement is high then less control mix may be used in step **2d**—this may require that the user dilute the control mix before proceeding with step **2e**.

3. Reaction Assembly

- a. Before assembling the reaction tubes, have two water baths set and pre-heated to 45°C and 30°C.
- b. Remove the required amount of Luciferin Reagent (50 µl per measurement) and bring to room temperature (21-25°C).
- c. Prepare 20 µl reactions on ice in 0.5 ml polypropylene tubes using the following volumes and order of addition:
 - i. 6 µl dH₂O
 - ii. 2 µl test compound in 0-50% DMSO. For both positive and negative controls, add 2 µl 0-50% DMSO only. **Note:** Test compound or DMSO is optional—add an additional 2 µl dH₂O if no compounds are tested (8 µl total).
 - iii. 2 µl 10X Reaction Buffer. Mix following addition.
 - iv. 4 µl 5X HSP60 Solution
 - v. 2 µl 10X HSP10 Solution
 - vi. 2 µl 10X Mg²⁺-ATP solution. For negative control reaction, omit ATP and replace with 2 µl dH₂O.
 - vii. 2 µl 10X Glow-Fold™ Substrate Protein (or substrate provided by user)
- d. Mix the contents by pipetting or gently flicking tubes. Spin briefly to collect contents in bottom of tube. **Incubate at room temperature for 15-30 minutes.**
- e. Heat the tubes for 7 minutes at 45°C, then immediately transfer to ice for 10 minutes.
- f. Once the tubes are ice-cold, spin briefly to collect contents in the bottom of tube. Mix by pipetting (or gently flicking tubes). Keep tubes on ice.
- g. Add 50 µl of Luciferin Reagent per well in an opaque white, 96-well half-area plate. One well is required for each reaction.
- h. Quickly remove 4 µl from each reaction (still on ice, step 3f) and add to the appropriate wells filled with Luciferin Reagent (step 3g). Mix by pipetting up and down and gently swirling pipette tip for 5-10 seconds. Once all samples have been loaded immediately measure luminescence. Plate should be read within 1-2 minutes of initiating this step. These measurements serve as “time = 0” points for the reactions. **Note:** reaction volume transferred to plate may vary depending on results from step 2 (optional).

4. Refolding Reaction

- a. Place tubes (from step **3f**) in 30°C water bath to initiate refolding reactions.
- b. 10 minutes prior to measuring refolding reactions (step **4c**), fill another set of wells with Luciferin Reagent as described in step **3g**. The same number of wells will be required (i.e. one per reaction).
- c. After 60 minutes (recommended reaction time), quickly remove 4 µl from each reaction and add to the appropriate wells filled with Luciferin Reagent (step **4b**). Mix by pipetting up and down and gently swirling pipette tip for 5-10 seconds. Once all samples have been loaded immediately measure luminescence. Plate should be read within 1-2 minutes of initiating this step. These measurements serve as “time = 60” points for the reactions. **Note:** reaction volume transferred to plate may vary depending on results from step **2** (optional).
- d. It should be noted that 8 µl out of the 20 µl of each reaction was used to generate two data points. More reaction time points may be taken by repeating steps **4a-4c** as necessary at desired reaction times.

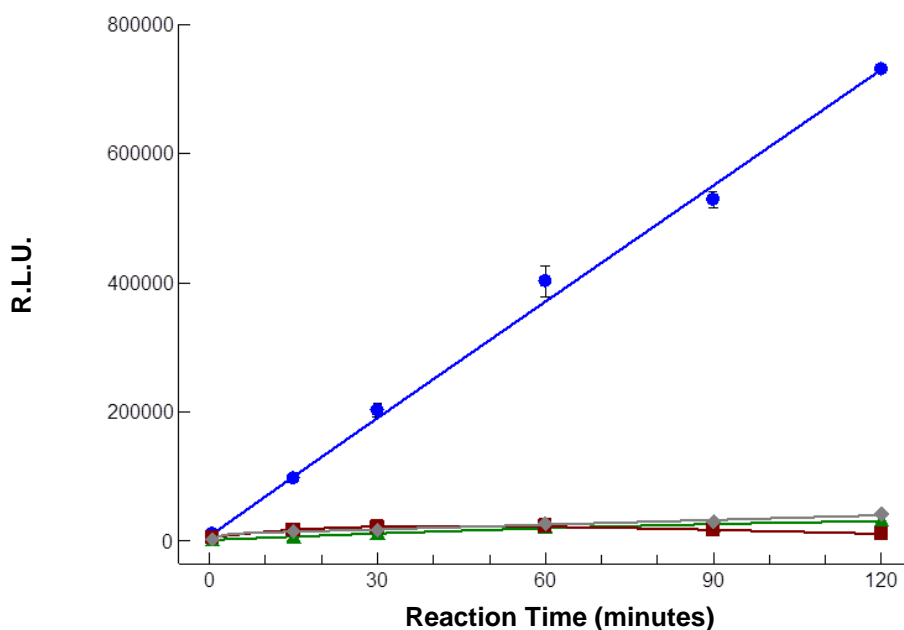
5. Data Analysis

Data analysis is highly dependent on the experimental goals of the researcher and exceeds the scope of this protocol. Selected data from test reactions is provided below (and in the Sample Data section) to provide guidance in the anticipated results.

| Refolding Reaction | Measured R.L.U. (average ± std. dev.) | Percent Activity |
|---|--|------------------|
| Glow-Fold™ only; no heat shock treatment | 2357135 ± 213715 | 100 |
| Heat shocked Glow-Fold™ + HSP60/HSP10; no ATP | 21349 ± 5118 | 1 |
| Heat shocked Glow-Fold™ + HSP60 only; + ATP | 27171 ± 3208 | 1 |
| Heat shocked Glow-Fold™ + HSP10 only; + ATP | 23621 ± 2335 | 1 |
| Heat shocked Glow-Fold™ + HSP60/HSP10; + ATP | 402517 ± 23967 | 17 |

Refolding Reactions were conducted at 30°C for 60 minutes with the indicated components. The average luminescence measured from three independent experiments is given, and the Percent Activity is given relative to the luminescence measured for the untreated (no heat shock, no refolding) Glow-Fold™. The Relative Luminescence Unit (R.L.U.) values for the untreated Glow-Fold™ (no heat shock, no refolding) reactions were measured at time = 0. All other reported R.L.U. values were obtained from the 60 minute time point.

Sample Data



HSP60/HSP10 Mediated-Refolding of Heat Denatured Glow-Fold™ Substrate Protein

Four test reactions were assembled using guidelines described in the protocol. Reactions contained heat denatured Glow-Fold™ Substrate Protein and the following additions:

1. HSP60/HSP10 + ATP (blue circles)
2. HSP60/HSP10 - ATP (green triangles)
3. HSP60 + ATP (red squares)
4. HSP10 + ATP (grey diamonds)

At the indicated times, aliquots were taken from each of the reactions and added to assay wells (Costar #3693, 96 half area wells) containing 50 μ l of Luciferin Reagent. Luminescence measurements were taken using a Molecular Devices SpectraMax M5^c Platereader within 1 minute of mixing. The above graph shows averages and standard deviations from three independent experiments.

Literature

- References:** Freeman B.C. & Yamamoto K.R. (2002) *Science* **296**: 2232-2235
 Forsythe H.L., *et al.* (2001) *J. Biol. Chem.* **276**: 15571-15574
 Patwardhan C.A., *et al.* (2013) *J. Biol. Chem.* **288**: 7313-7325

For help with this kit, e-mail: techsupport@bostonbiochem.com

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