

## MATERIAL DATA SHEET

### HSP70/HSP40 Glow-Fold Protein Refolding Kit Cat. # K-290

Heat shock proteins (HSPs) are a family of highly conserved stress response proteins. Heat shock proteins function primarily as molecular chaperones by facilitating the folding of other cellular proteins, preventing protein aggregation or targeting improperly folded proteins to specific degradative pathways. HSP70 function depends on its cycling between two states: ATP-bound and ADP-bound. ATP-bound HSP70 recognizes stretches of hydrophobic amino acid residues in misfolded or newly synthesized proteins and interacts with them. This initial binding event is relatively weak and reversible, and so the ATP-bound HSP70 freely binds and release peptides. However, the presence of a peptide in the binding domain weakly stimulates the latent ATPase activity of HSP70. Once ATP is hydrolyzed to ADP, the binding pocket of HSP70 is reconfigured into a tight-binding state that clamps down on protein targets and helps to prevent their aggregation. ATP hydrolysis is stimulated by HSP70 association with "J-domain" class co-chaperones such as HSP40. These co-chaperones dramatically increase the activity and the functionality of HSP70 in the presence of interacting peptides/proteins. Eventually ADP is exchanged for an ATP molecule, thus bringing the substrate-binding domain back to its low affinity state. This releases the bound protein so that it may fold correctly by itself, interact with other chaperone systems to complete folding, or bind again to HSP70.

This kit provides a functional *in vitro* HSP70/HSP40 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 HSP70/HSP40 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.

<b>Reagents Provided in Kit</b>	
<b><u>Component</u></b>	<b><u>Volume</u></b>
1. 10X HSP70 Solution	30 µl
2. 10X HSP40 Solution	30 µl
3. 10X HSP70 Reaction Buffer	50 µl
4. 10X Mg <sup>2+</sup> -ATP	30 µl
5. 10X Glow-Fold Substrate Protein	50 µl
6. Luciferin Reagent	2 x 1.5 ml
<b>Storage:</b>	Store components at -80°C. Avoid multiple freeze/thaw cycles.

### Reagents to be Provided by Investigator

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

dH <sub>2</sub> 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 HSP70/HSP40 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 HSP70/HSP40-mediated refolding, up to 40% 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](mailto: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 dH<sub>2</sub>O
  - 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 within the linear 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. 8 µl dH<sub>2</sub>O
  - 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 dH<sub>2</sub>O if no compounds are tested (10 µl total).
  - iii. 2 µl 10X Reaction Buffer. Mix following addition.
  - iv. 2 µl 10X HSP70 Solution
  - v. 2 µl 10X HSP40 Solution
  - vi. 2 µl 10X Mg<sup>2+</sup>-ATP solution. For negative control reaction, omit ATP and replace with 2 µl dH<sub>2</sub>O.
  - 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. At this point, the mix may be pre-incubated to allow interaction of test compound(s) with the HSP complex. We suggest 15-30 minutes at room temperature. If no compounds are being tested, proceed directly to step **3e**.
- 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

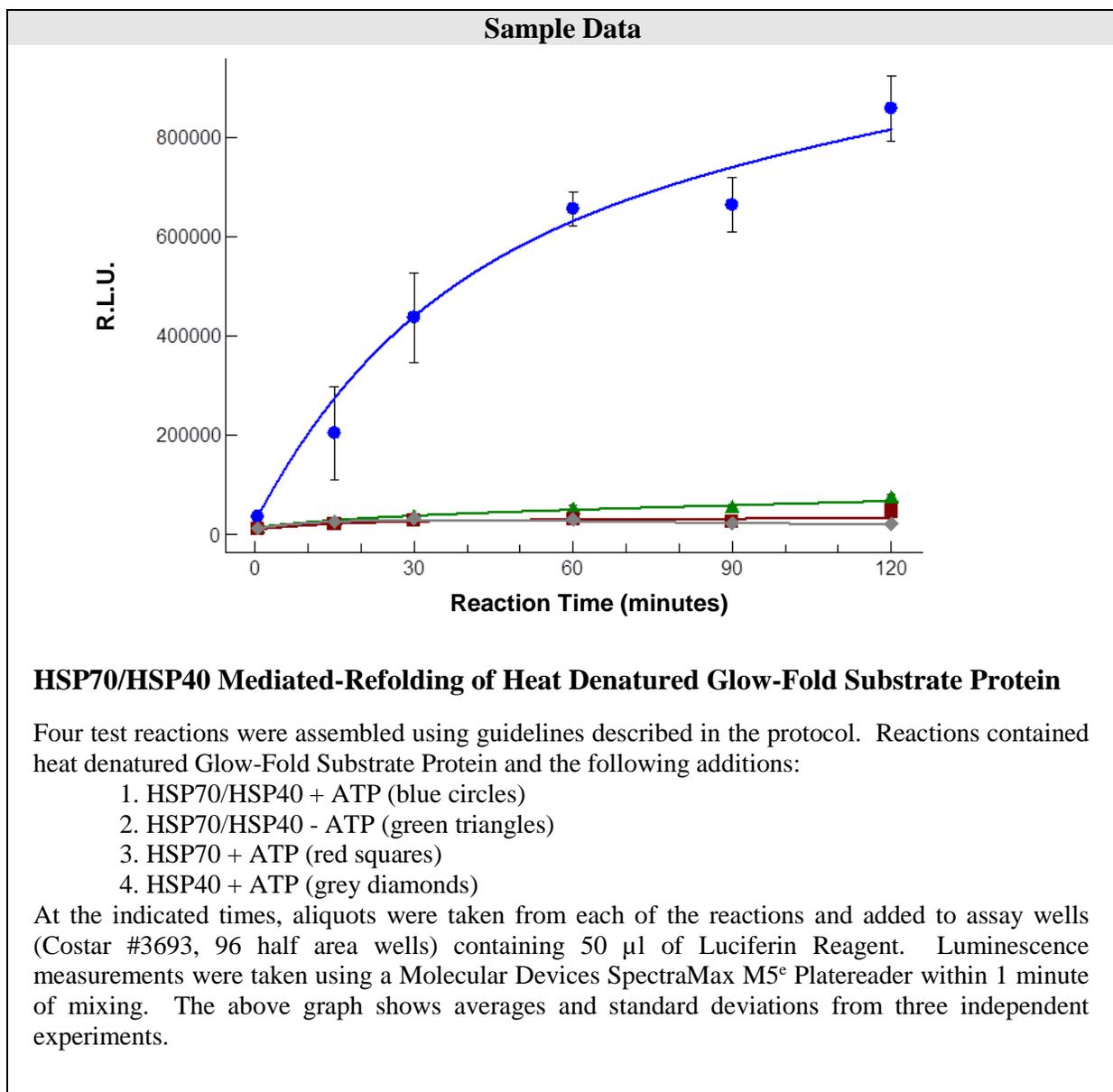
- a. Place tubes (from step 3f) in 30°C water bath to initiate refolding reactions.
- b. 10 minutes prior to measuring refolding reactions (step 4c), fill another set of wells with Luciferin Reagent as described in step 3g. The same number of wells will be required (i.e. one per reaction).
- c. After 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 therefore 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	1742769 ± 148387	100
Heat shocked Glow-Fold + HSP70/HSP40; no ATP	54139 ± 4481	3
Heat shocked Glow-Fold + HSP70 only; + ATP	30611 ± 2914	2
Heat shocked Glow-Fold + HSP40 only; + ATP	30861 ± 2382	2
Heat shocked Glow-Fold + HSP70/HSP40; + ATP	656344 ± 34847	38

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.



### Literature

- References:** Hartl F.U. & Hayer-Hartl M. (2009) *Nat. Struc. Mol. Biol.* **16**: 574-581  
Kampinga H. H. & Craig E.A. (2010) *Nat. Rev. Mol. Cell Biol.* **11**: 579-592

For help with this kit, e-mail: [techsupport@bostonbiochem.com](mailto:techsupport@bostonbiochem.com)

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