Lot # XXXXX

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MATERIAL DATA SHEET

HSP90/p23 Glow-Fold™ Protein Refolding Kit
Cat. # K-310

Members of the HSP90 family are essential chaperones found in all organisms from bacteria to humans. HSP90 complexes often interact with proteins in their native conformation and help to maintain/stabilize ligand-bound states. In this capacity, HSP90 plays a central role in function and turnover of many proteins involved in processes such as signal transduction, cell cycle control and apoptosis. HSP70 family members and HSP90 complexes frequently act in tandem, with the former participating in the folding of the client proteins and HSP90 stabilizing them in a way favorable for interaction with ligands. HSP90 forms complexes with an array of co-chaperones that both regulate its interaction with client proteins and stimulate its ATPase activity. By binding to different co-chaperones HSP90 acquires specificity for different families of client proteins. One such HSP90 co-chaperone is p23 (also known as Prostaglandin E Synthase 3), a protein that has passive chaperoning activity and can suppress the aggregation of denatured proteins. The p23/HSP90 complex interacts with steroid receptors and stabilizes them in a conformation able to bind hormone substrates. Many of the HSP90-client proteins are involved in tumor cell growth and HSP90 inhibitors are important as potential anticancer drugs. Inhibition of HSP90 also prevents the formation of protein aggregates in models of Parkinson disease, Huntington disease, and others.

This kit provides a functional *in vitro* HSP90α/p23 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 HSP90α/p23 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

Component	Volume
1. 10X HSP90α Solution	30 µl
2. 10X p23 Solution	30 µl
3. 10X HSP90 Reaction Buffer	50 µl
4. 10X Mg^{2+}-ATP	30 µl
5. 10X Glow-Fold™ Substrate Protein	50 µl
6. Luciferin Solution	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_2O	Sterile
Waterbaths	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 HSP90/p23 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 HSP90/p23-mediated refolding, up to 5% of the Glow-Fold activity (pre heat-shock) can be recovered, resulting in about a 5:1 signal-to-background ratio.

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 sensitive to DMSO; we recommend that the final concentration of DMSO in the reaction should not exceed 1%.

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.

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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 (≤ 30°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_2O
        ii. 2 µl 10X Reaction Buffer
        iii. 2 µl 10X Luciferase 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**.

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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 polypropylene tubes using the following volumes and order of addition:
        i. 8 µl dH_2O
        ii. 2 µl test compound in 0-10% DMSO. For both positive and negative controls, add 2 µl 0-10% DMSO only. **Note**: Test compound or DMSO is optional—add an additional 2 µl dH_2O if no compounds are tested (10 µl total).
        iii. 2 µl 10X Reaction Buffer. Mix following addition.
        iv. 2 µl 10X HSP90 Solution
        v. 2 µl 10X p23 Solution
        vi. 2 µl 10X Mg^{2+}-ATP solution. For negative control reaction, omit ATP and replace with 2 µl dH_2O.
        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).

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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 Sample Data section) to provide guidance in the anticipated results.

Refolding Reaction	Measured R.L.U. (average ± std. dev.)	% Activity
Glow-Fold™ only; no heat shock treatment	12150463 ± 277598	100
Heat shocked Glow-Fold™ + HSP90/p23; no ATP	41519 ± 1028	0.3
Heat shocked Glow-Fold™ + HSP90 only; + ATP	149683 ± 2684	1.2
Heat shocked Glow-Fold™ + p23 only; + ATP	500114 ± 50183	4.1
Heat shocked Glow-Fold™ + HSP90/p23; + ATP	754922 ± 11229	6.2

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.

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Sample Data

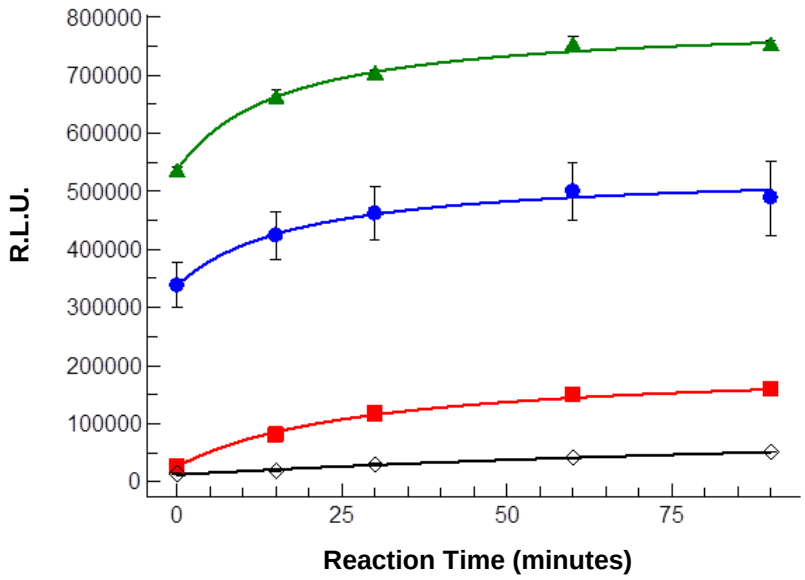

Figure 1. HSP90/p23 Mediated-Refolding of Heat Denatured Glow-Fold™ Substrate Protein

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

1. HSP90/p23 + ATP (green triangles)
2. HSP90/p23 - ATP (black diamond)
3. HSP90 + ATP (red squares)
4. P23 + ATP (blue circles)

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 µl of Luciferin Reagent. Luminescence measurements were taken using a Molecular Devices SpectraMax M5^e Platereader within 1 minute of mixing. The above graph shows averages and standard deviations from three independent experiments.

Both HSP90 and p23 provide an ATP-dependent, protective effect on the substrate during the heat denaturation process. While refolding of the substrate is evident during the 90 minute reactions, the majority of the difference between the plus-ATP and minus-ATP reactions occurs immediately after heat shock, suggesting that protection against thermal denaturation is a large component of this assay.

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Sample Data

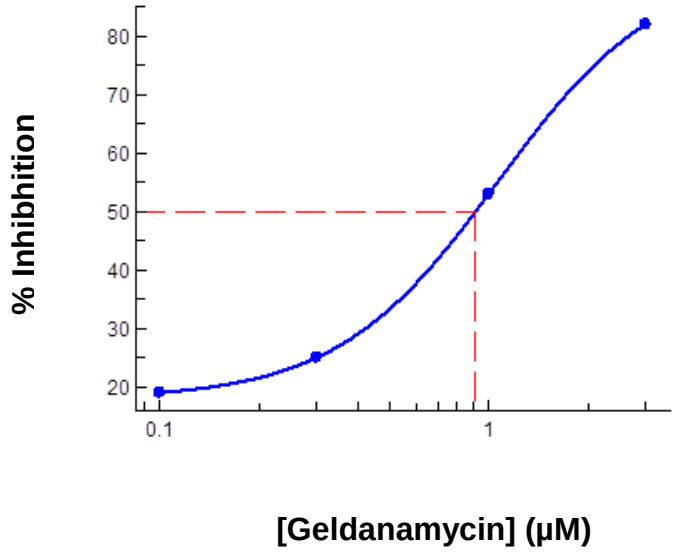

Figure 2. HSP90/p23 Mediated-Refolding of Heat Denatured Glow-Fold™ Substrate Protein—Inhibition by Geldanamycin

Test reactions were assembled using guidelines described in the protocol and contained the HSP90 inhibitor Geldanamycin; final DMSO concentration in the reactions was 1%. Controls included 1% DMSO only, or no ATP.

After 60 minutes aliquots of each reaction were added to assay wells (Costar #3693, 96 half area wells) containing 50 µl of Luciferin Reagent. Luminescence measurements were taken using a Molecular Devices SpectraMax M5^e Platereader within 1 minute of mixing. Percent Inhibition of HSP90/p23 was calculated as:

$$\% \text{ Inhibition} = 100 - 100 \times (R.L.U._{sample} - R.L.U._{\text{no ATP}}) / (R.L.U._{\text{DMSO only}} - R.L.U._{\text{no ATP}})$$

The concentration of Geldanamycin was plotted vs percent inhibiton using a non-linear 4-parameter equation (XLFit v5.3.1.3, model 205) to obtain an IC_{50} value of 0.9 µM. Samples analyzed at other incubation times yielded IC_{50} values of (0.7-1.1 µM).

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Literature

References:

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