Lot # XXXXX

BostonBiochem®
An R&D Systems Company

MATERIAL DATA SHEET

CHIP Ubiquitin Ligase Kit - Glow-Fold Substrate
Cat. # K-280

CHIP (Carboxy terminus of HSP70-Interacting Protein) is a U-Box ubiquitin E3 ligase that ubiquitinates and mediates the proteasomal destruction of misfolded chaperone substrates. CHIP functions in coordination with several chaperone complexes, including HSP40, HSP70, and HSP90. CHIP activity may be modulated by the deubuiquitinase Ataxin-3, which restricts the length of ubiquitin chains attached to CHIP substrates and prevents further chain extension. This kit is designed for *in vitro* ubiquitination of user-supplied substrates by CHIP or the CHIP/HSP70/HSP40 ternary complex. Ubiquitinated proteins can be used in downstream applications, or analyzed by Western blot using antibodies specific for the target protein. A Glow-Fold™ control substrate and detection antibody are included in the kit.

NOTE: Kit contains reagents sufficient for 10 x 30 µl reactions and 5 Western Blots (mini-gel format).

Reagents Provided in Kit

Component	Volume
1. 10X E1 enzyme	30 µl
2. 10X E2 enzyme (UBE2D3)	30 µl
3. 10X His_6-CHIP	30 µl
4. 10X HSP70/His_6-HSP40 Mix	30 µl
5. 10X Ubiquitin	30 µl
6. 10X Glow-Fold™ control substrate	30 µl
7. 10X Reaction Buffer	50 µl
8. 10X Mg^{2+}-ATP	30 µl
9. α-Glow-Fold™ primary antibody	50 µl
10. 5X Loading Buffer	1 ml

Storage: Store protein components at -80°C. Avoid multiple freeze/thaw cycles.
Loading Buffer may be stored at room temperature.
Mg^{2+}-ATP and α-Glow-Fold™ antibody may be stored at -20°C.

840 Memorial Drive, Cambridge, MA 02139 Phone: 617-576-2210 FAX: 617-492-3565
www.bostonbiochem.com e-mail: techsupport@bostonbiochem.com

The contents of this datasheet (unless otherwise noted) are Copyright © 2008 Boston Biochem, Inc. All rights reserved. Duplication in whole or in part is strictly prohibited without the express written consent of Boston Biochem, Inc. "Boston Biochem" is a Trademark of Boston Biochem, Inc., 840 Memorial Drive, Cambridge, MA 02139.

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
Dithiothreitol (DTT)	1 M in dH_2O (Pierce # 20290) or similar
PBST	1X PBS (Calbiochem # 524650) + 0.0 5% Tween-20 (Sigma # P1379) or similar
Non-fat milk	Dry/powdered (CARNATION® Instant Nonfat Dry Milk or similar)
Towbin Buffer	25 mM Tris Base, 192 mM glycine, 20 % methanol (pH ~ 8.3. *Do not adjust pH*)
SDS-PAGE Gels	*Criterion* 7.5% SDS-PAGE Gel (BioRad #345-0006) or similar
PVDF Membrane	*Immun-Blot* PVDF Membrane (BioRad #162-0177) or similar
HRP-α-Goat 2° Ab	(R&D Systems #HAF017) or similar
ECL Reagents	*SuperSignal West Pico* Chemiluminescent Substrate (Pierce #34080) or similar

Assay Considerations

Glow-Fold™ control protein is progressively ubiquitinated using the reagents and protocol conditions supplied in this kit. Ubiquitinated Glow-Fold™ products are visible via Western Blot after 10-15 minutes reaction time at 30°C, and continue to accumulate for up to 90 minutes (see included sample data). During the reaction, polyubiquitinated HSP70 is generated; anti-ubiquitin antibodies may detect these products in addition to polyubiquitinated substrate proteins.

The kit protocol is designed for reaction termination with SDS-PAGE Sample Buffer; therefore, proteins are denatured and typically not suitable for further enzymatic manipulation. If ubiquitinated protein is to be utilized in further reactions prior to SDS-PAGE analysis, reactions may be terminated by the addition of EDTA (10 mM final) plus DTT or βME (5-10 mM final) if compatible with downstream experimental protocols.

SDS-PAGE gels, PVDF membrane, blocking reagent, antibodies and protocols provided or referenced in this kit have been chosen following careful testing at Boston Biochem. Modifications to the protocol or selection of alternative reagents (particularly substrate protein) may require assay optimization by the end-user. Suggested concentration range for user provided substrates is 0.5-5 µM, final. Further information available at techsupport@bostonbiochem.com.

840 Memorial Drive, Cambridge, MA 02139 Phone: 617-576-2210 FAX: 617-492-3565
www.bostonbiochem.com e-mail: techsupport@bostonbiochem.com

The contents of this datasheet (unless otherwise noted) are Copyright © 2008 Boston Biochem, Inc. All rights reserved. Duplication in whole or in part is strictly prohibited without the express written consent of Boston Biochem, Inc. "Boston Biochem" is a Trademark of Boston Biochem, Inc., 840 Memorial Drive, Cambridge, MA 02139.

BostonBiochem®
An R&D Systems Company

Recommended Assay Protocol (30 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 (SDS-PAGE Sample Buffer may be inverted to mix), then briefly spin in a microcentrifuge (5 seconds) to collect contents in bottom of tubes.
   c. Immediately ice components. (SDS-PAGE Sample Buffer may be kept at room temperature.) Entire process from steps 1a-1c 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. SDS-PAGE Sample Buffer may be stored at room temperature.

2. Reaction Assembly
   a. Before assembling the reaction tubes have ready 43°C and 30°C water baths.
   b. Prepare 30 µl reactions on ice in 0.5 or 1.5 ml polypropylene tubes using the following volumes and order of addition:
      i. 6 µl dH_2O.
      ii. 3 µl 10X Reaction Buffer. Mix following addition.
      iii. 3 µl 10X Mg^{2+}-ATP solution. For negative control reactions, omit ATP addition and replace with 3 µl dH_2O.
      iv. 3 µl 10X HSP70/HSP40 Mix.
      v. 3 µl 10X Glow-Fold™ substrate (or substrate provided by user)
   c. Mix the contents by pipetting or gently flicking tubes. Spin briefly to collect contents in bottom of tube.
   d. Heat the tubes for 7 minutes at 43°C, then immediately transfer to ice for 10 minutes.
   e. Once the tubes are ice-cold, spin briefly to collect contents in the bottom of tube, then add the following reagents (again, on ice):
      i. 3 µl 10X E1 enzyme
      ii. 3 µl 10X E2 enzyme
      iii. 3 µl 10X CHIP

840 Memorial Drive, Cambridge, MA 02139 Phone: 617-576-2210 FAX: 617-492-3565
www.bostonbiochem.com e-mail: techsupport@bostonbiochem.com

The contents of this datasheet (unless otherwise noted) are Copyright © 2008 Boston Biochem, Inc. All rights reserved. Duplication in whole or in part is strictly prohibited without the express written consent of Boston Biochem, Inc. "Boston Biochem" is a Trademark of Boston Biochem, Inc., 840 Memorial Drive, Cambridge, MA 02139.

f. At this point, reactions are ready to initiate—addition of ubiquitin in the next step will start the reaction.
g. Add 3 µl of 10X ubiquitin solution. Mix by gently pipetting up and down 2-3 times. Spin tubes to collect contents and place reactions in 37°C water bath.
h. After 60 minutes terminate reactions with addition of 8 µl 5X Loading Buffer (SDS-PAGE sample buffer) and 2 µl 1M DTT. Heat reactions to 90°C for 5 minutes.

3. SDS-PAGE
   a. Assemble SDS-PAGE gel according to manufacturer's instructions. (We utilize the BioRad *Criterion* gel unit with Tris buffering system.)
   b. Load 5 µl of terminated reaction per well (*Criterion* Precast 7.5% Tris-HCl, 1.0 mm thickness, 18 well comb, 30 µl/well capacity). Volume loaded per well will depend on sensitivity of every particular substrate primary antibody, and your choice of gel. The volume recommended above works well for the control substrate and antibody provided with the kit.
   c. Run gel until dye-front just reaches bottom of gel (approximately 1 hour at 180V using the *Criterion* gels referenced in step 3b—adjust run times and voltage accordingly for your system).
   d. Carefully disassemble gel and prepare for electro-transfer to blotting membrane.

4. Gel Transfer
   a. Soak gel in 50-100 ml of Towbin Buffer at room temperature for 20 minutes with gentle rocking. Some gels tend to float on top of the buffer—this should be minimized with gentle agitation.
   b. Prepare PVDF membrane by wetting in a small amount of 100% methanol for 1-2 minutes. When membrane is completely wetted, transfer to 20-30 ml of Towbin Buffer for 5 minutes at room temperature with gentle rocking.
   c. Assemble transfer "sandwich" using blotting paper, gel, PVDF membrane, and any other components for electroblotting according to system manufacturer's suggested protocol. (We utilize the BioRad *Trans▪Blot SD* Semi Dry Transfer Cell)
   d. Electroblot gel contents to PVDF at 15V (constant) for 45 minutes at room temperature (settings will depend on your system).
   e. Disassemble gel sandwich and prepare for Blocking/Antibody Detection.

840 Memorial Drive, Cambridge, MA 02139 Phone: 617-576-2210 FAX: 617-492-3565
www.bostonbiochem.com e-mail: techsupport@bostonbiochem.com

The contents of this datasheet (unless otherwise noted) are Copyright © 2008 Boston Biochem, Inc. All rights reserved. Duplication in whole or in part is strictly prohibited without the express written consent of Boston Biochem, Inc. "Boston Biochem" is a Trademark of Boston Biochem, Inc., 840 Memorial Drive, Cambridge, MA 02139.

5. Membrane Blocking
    a. Prepare Blocking Solution by dissolving 3 grams of dry, non-fat milk per 100 ml PBST solution. The Blocking Solution may be stored at 4°C for up to 5 days, but we suggest preparing it fresh when needed.
    b. Soak PVDF membrane in 50-100 ml Blocking Solution overnight at 4°C with gentle rocking in a covered container or sealable bag.

6. Antibody Staining
    a. Prepare Antibody Dilution Buffer by dissolving 0.2 grams of solid, non-fat milk in 40 ml PBST (PBST + 0.5% w/v non-fat milk).
    b. Dilute α-Glow-Fold™ primary antibody by adding 10 µl antibody to 20 ml Antibody Dilution Buffer (1:2000 dilution). If using a primary antibody against a different substrate, dilute as appropriate.
    c. Decant Blocking Solution from PVDF membrane. Add the diluted primary antibody solution. There is no need to rinse the PVDF membrane prior to adding antibody as long as the Blocking Solution is completely removed.
    d. Incubate membrane in primary antibody solution for 60 minutes at room temperature with rocking or shaking. Make certain that the entire PVDF membrane surface is exposed to antibody solution uniformly.
    e. Decant primary antibody solution.
    f. Wash membrane with 50-100 ml PBST for 15 minutes at room temperature using rocking or shaking. Decant wash solution.
    g. Repeat step 6f twice more, for three washes total.
    h. Dilute HRP-labeled secondary antibody (R&D Systems #HAF017, sold separately) by adding 4 µl antibody to 20 ml Antibody Dilution Buffer (1:1000 dilution). Add diluted antibody to membrane. If a different secondary antibody is utilized, follow manufacturer's guide for appropriate dilution.
    i. Incubate membrane in secondary antibody solution for 60 minutes at room temperature with rocking or shaking. Make certain that the entire PVDF membrane surface is exposed to antibody solution uniformly.
    j. Wash membrane with 50-100 ml PBST for 15 minutes at room temperature using rocking or shaking. Decant wash solution.
    k. Repeat step 6j twice more, for three washes total.
    l. Membrane is now ready for ECL detection.

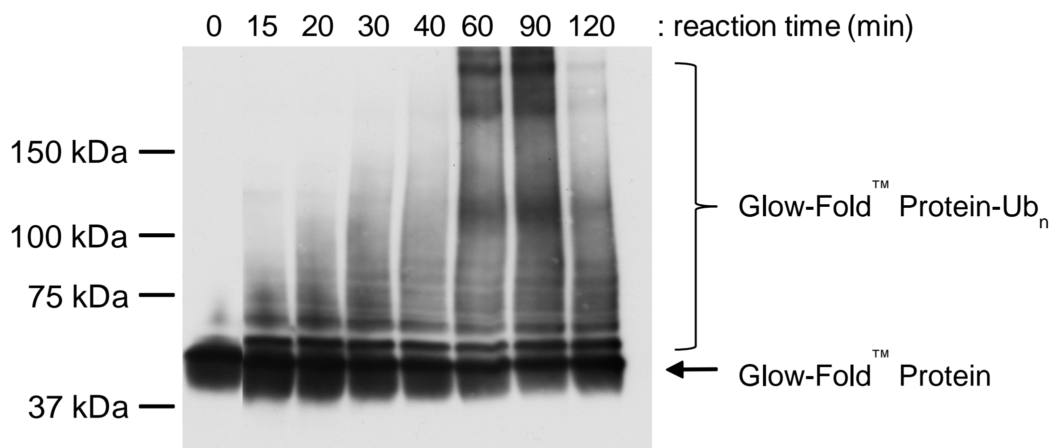
840 Memorial Drive, Cambridge, MA 02139 Phone: 617-576-2210 FAX: 617-492-3565
www.bostonbiochem.com e-mail: techsupport@bostonbiochem.com

The contents of this datasheet (unless otherwise noted) are Copyright © 2008 Boston Biochem, Inc. All rights reserved. Duplication in whole or in part is strictly prohibited without the express written consent of Boston Biochem, Inc. "Boston Biochem" is a Trademark of Boston Biochem, Inc., 840 Memorial Drive, Cambridge, MA 02139.

7. ECL Detection

Chemiluminescence reagents for detecting reaction products are available from a number of sources. We recommend using *SuperSignal West Pico* Chemiluminescent Substrate from Pierce. Typical film exposure times range from 5 seconds to 2 minutes. Using the protocol listed above, Glow-Fold™ laddering (ubiquitination) is easily observed with film exposures of 30 seconds or less.

Sample Data



Ubiquitination of Glow-Fold™ control substrate by the CHIP E3 ligase

Reaction contained all components as described in the protocol, and was initiated by the addition of ubiquitin. At indicated time, an aliquot of the reaction was removed and terminated with SDS-PAGE Sample Buffer + DTT. Western blotting with α-Glow-Fold™-specific polyclonal antibody was performed as described in the protocol—film exposure time was 10 seconds.

Literature

References:

Connell P., *et al.* (2001) Nat. Cell Bio. **3**: 93-96
Qian S.B., *et al.* (2006) Nature **440**: 551-555
Scaglione K.M., *et al.* (2011) Mol. Cell **43**: 599-612

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

For Laboratory Research Use Only, Not For Use in Humans

Rev: 06/17/13

840 Memorial Drive, Cambridge, MA 02139 Phone: 617-576-2210 FAX: 617-492-3565
www.bostonbiochem.com e-mail: techsupport@bostonbiochem.com

The contents of this datasheet (unless otherwise noted) are Copyright © 2008 Boston Biochem, Inc. All rights reserved. Duplication in whole or in part is strictly prohibited without the express written consent of Boston Biochem, Inc. "Boston Biochem" is a Trademark of Boston Biochem, Inc., 840 Memorial Drive, Cambridge, MA 02139.