

MATERIAL DATA SHEET

XIAP Ubiquitin Ligase Kit - Diablo Substrate Cat. # K-250

The RING-finger Ubiquitin E3 ligase X-Linked Inhibitor of Apoptosis Protein (XIAP, also known as API3, BIRC4, IAP3) is an important regulator of cell survival/death. The BIR-domains of XIAP have been reported to directly bind and inhibit Caspase 3 and Caspase7, and also function to inhibit Caspase 9. XIAP ubiquitinates numerous proteins in apoptosis and/or NFκB pathways including Diablo (Direct IAP-binding protein with low pI, also known as Second Mitochondrial-Derived Activator of Caspases, or “SMAC”), an apoptosis promoting protein. Certain isoforms of Diablo bind to and activate the autoubiquitination of XIAP, resulting in the proteasomal degradation of the ligase. This kit is designed for *in vitro* XIAP-mediated ubiquitination of user-supplied substrates. Ubiquitinated proteins can be used in downstream applications, or analyzed by Western blot using antibodies specific for the target protein. A control substrate, His₆-Diablo (**789-SM**), and detection antibody (**AF789**) 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	
<u>Component</u>	<u>Volume</u>
1. 10X E1 enzyme	30 μl
2. 10X E2 enzyme (UBE2D2)	30 μl
3. 10X His ₆ -XIAP	30 μl
4. 10X His ₆ -Diablo	30 μl
5. 10X ubiquitin solution	30 μl
6. 10X Reaction Buffer	50 μl
7. 10X Mg ²⁺ -ATP	30 μl
8. α-Diablo primary antibody	100 μl
9. 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-Diablo antibody may be stored at -20°C.

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
Dithiothreitol (DTT)	1M in dH ₂ O (Pierce #20290) or similar
PBST	1X PBS (Calbiochem #524650) + 0.05% Tween-20 (Sigma #P1379) or similar
5% BSA in PBST	OmniPur BSA, Fraction V (Calbiochem #9049-46-8) or similar
Towbin Buffer	25 mM Tris Base, 192 mM glycine, 20% methanol (pH ~ 8.3. <i>Do not adjust pH</i>)
SDS-PAGE Gels	<i>Criterion</i> 10-20% SDS-PAGE Gel (BioRad # 345-0043) or similar
PVDF Membrane	<i>Immun-Blot</i> PVDF Membrane (BioRad #162-0177) or similar
HRP- α -rabbit 2° Ab	(R&D Systems #HAF008) or similar
ECL Reagents	<i>SuperSignal West Pico</i> Chemiluminescent Substrate (Pierce #34080) or similar

Assay Considerations

Diablo is rapidly ubiquitinated using the reagents and protocol conditions supplied in this kit. Reactions are complete within 60 minutes at 37°C, resulting in ubiquitination of approximately 50% of the Diablo substrate. During the reaction XIAP becomes highly autoubiquitinated. Western Blot protocols using α -ubiquitin antibodies will detect high molecular weight, polyubiquitinated XIAP conjugates 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 Diablo (or XIAP) is to be utilized in further reactions prior to SDS-PAGE analysis, ubiquitination reactions may be terminated by the addition of EDTA (10 mM final) plus DTT or β ME (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 may require assay optimization by the end-user. Further information is available via techsupport@bostonbiochem.com.

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 ($\leq 30^{\circ}\text{C}$). 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 (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. Prepare reactions on ice in 0.5 or 1.5 ml polypropylene tubes using the following volumes and order of addition:
 - i. 9 µl dH₂O.
 - ii. 3 µl 10X Reaction Buffer. Mix gently following addition.
 - iii. 3 µl 10X Diablo substrate protein.
 - iv. 3 µl 10X E1 enzyme.
 - v. 3 µl 10X E2 enzyme.
 - vi. 3 µl 10X XIAP E3 enzyme.
 - vii. 3 µl 10X Ubiquitin solution.
- b. Pre-incubate tubes for 30 minutes at 21°C without Mg²⁺-ATP— addition of Mg²⁺-ATP in the next step will start the reaction. ***For best results, do not omit this pre-incubation step.***
- c. Add 3 µl of 10X Mg²⁺-ATP solution. Mix by gently pipetting up and down 2-3 times. For negative control reactions, omit ATP addition and replace with 3 µl dH₂O.
- d. Spin tubes to collect contents and place reactions in 37°C water bath.
- e. After 60 minutes, terminate reactions with addition of 8 µl 5X Loading Buffer (SDS-PAGE sample buffer) and 1 µl 1M DTT. Heat reactions to 90°C for 5 minutes.

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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 13 μ l of terminated reaction per well (*Criterion* Precast 10-20% Tris-HCl, 1.0 mm thickness, 18 well comb, 30 μ l/well capacity). Volume loaded per well will depend on your choice of gel.
- c. Run gel until dye-front just reaches bottom of gel (approximately 1 hour at 180V using the *Criterion* gels referenced in 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.

5. Membrane Blocking

- a. Prepare Blocking Solution by dissolving 5 grams solid, BSA powder into 100 ml PBST solution (PBST+5% w/v BSA). The Blocking Solution may be filter sterilized and stored at 4°C for up to 5 days, but we suggest preparing it fresh when needed.
- b. Soak PVDF membrane in 50-100 mls Blocking Solution overnight at 4°C with gentle rocking in a covered container or sealable bag.

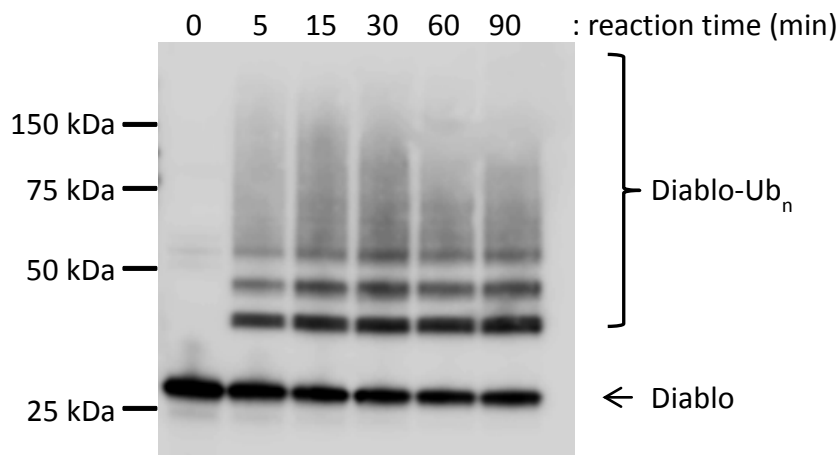
6. Antibody Staining

- a. Prepare Antibody Dilution Buffer by dissolving 0.2 grams of solid, BSA powder in 40 ml PBST (PBST + 0.5% w/v BSA).
- b. Dilute α -Diablo primary antibody by adding 20 μ l antibody to 20 ml Antibody Dilution Buffer (1:1000 dilution).
- c. Decant Blocking Solution from PVDF membrane, and then add the 20 ml diluted α -Diablo 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 #HAF008, sold separately) by adding 2 μ l antibody to 20 ml Antibody Dilution Buffer (1:10,000 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.

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, Diablo laddering (ubiquitination) is easily observed with film exposures of 1 minute or less.

Sample Data



Ubiquitination of Diablo by the XIAP E3 ligase

Reaction contained all components as described in the protocol, and was initiated after 30 minutes pre-incubation at 21°C by the addition of Mg²⁺ATP. Incubation was then run at 37°C. At indicated times, an aliquot of the reaction was removed and terminated with SDS-PAGE Sample Buffer + DTT. "0" timepoint was obtained from reaction prior to the addition of Mg²⁺-ATP. Western blotting with α-Diablo-specific polyclonal antibody was performed as described in the protocol— film exposure time was 30 seconds.

Literature

- References:**
- MacFarlane M., *et al.* (2002) *J.Biol.Chem* **277**: 36611-36616
 - Flanagan L., *et al.* (2010) *Cell Death and Dis.* **1**: e49
 - Martinez-Ruiz G., *et al.* (2008) *J Exp Clin Cancer Res.* **27**: 48
 - Eckelman B.P., *et al.* (2006) *EMBO Rep.* **7**: 988-94

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

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