Lot # XXXXX

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MATERIAL DATA SHEET

E6AP/E6 Ubiquitin Ligase Kit - p53 Substrate
Cat. # K-240

E6AP (E6-Associated Protein) (**E3-230**) is a HECT domain ubiquitin E3 ligase that ubiquitinates and mediates the proteasomal destruction of substrate proteins. HECT domain ligases use an active site cysteine to accept charged ubiquitin from ubiquitin-E2 thioester complexes for subsequent transfer to substrate proteins; in this way HECT class ligases are distinct from most RING class ligases in that the latter facilitate transfer of ubiquitin from charged E2's directly to substrate proteins without an E3-ubiquitin thioester intermediate. E6 (Early protein 6) (**AP-120**) is a viral protein produced in cells infected with the Human Papillomavirus. E6 forms a complex with the host cell E6AP generating a ligase activity that polyubiquitinates tumor suppressors p53 and p73 and targets them to the 26S proteasome for degradation. As a result DNA damage and chromosomal instabilities increase, often leading to cell proliferation and cancer. The E6/E6AP complex also targets other substrates for ubiquitination, such as TERT, BAK1, FADD, and pro-CASP8—none of which appear to be substrates for E6AP in the absence of E6. This kit is designed for *in vitro* E6AP/E6-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, His_6-FLAG-p53 (**SP-452)** and detection antibody (**MAB1355**) 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 (UBE2L3)	30 µl
3. 10X His_6-E6AP	30 µl
4. 10X His_6-FLAG-p53 control substrate	30 µl
5. 10X E6 (HPV type 16)	30 µl
6. 10X ubiquitin solution	30 µl
7. 10X Reaction Buffer	50 µl
8. 10X Mg^{2+}-ATP	30 µl
9. α-p53 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-p53 antibody may be stored at -20°C.

840 Memorial Drive, Cambridge, MA 02139 Phone: 617-576-2210 FAX: 617-492-3565
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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
Dithiothreitol (DTT)	1M in dH_2O (Pierce #20290) or similar
PBST	1X PBS (Calbiochem #524650) + 0.05% 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-mouse 2° Ab	(R&D Systems #HAF007) or similar
ECL Reagents	*SuperSignal West Pico* Chemiluminescent Substrate (Pierce #34080) or similar

Assay Considerations

Using the reagents and protocol conditions supplied in this kit, approximately 50% of the p53 control substrate protein is ubiquitinated in 30 minutes at 37°C. Extending the incubation time to 90 minutes produces maximal ubiquitination of p53 (see included sample data).

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.

Recombinant HPV E6 protein (type 16, UniProt # P03126) provided in this kit contains cysteine-to-serine mutations at the following six positions: 23, 58, 87, 104, 118, and 147.

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 0.1-5 µM, final. Further information available at techsupport@bostonbiochem.com.

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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. Prepare 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 gently following addition.
        iii. 3 µl 10X His_6-FLAG-p53 protein (or substrate supplied by user)
        iv. 3 µl 10X E1 enzyme.
        v. 3 µl 10X E2 enzyme.
        vi. 3 µl 10X His_6-E6AP enzyme.
        vii. 3 µl 10X E6 protein
        viii. 3 µl 10X Mg^{2+}-ATP solution. For negative control reactions, omit ATP addition and replace with 3 µl dH_2O.
    b. At this point, reactions are ready to initiate—addition of ubiquitin in the next step will start the reaction.
    c. Add 3 µl of 10X ubiquitin solution. Mix by gently pipetting up and down 2-3 times.
    d. Spin tubes to collect contents and place reactions in 37°C water bath.
    e. After 90 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.

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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 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 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 non-fat milk per 100 ml PBST solution (PBST+5% w/v non-fat milk). 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 ml Blocking Solution overnight at 4°C with gentle rocking in a covered container or sealable bag.

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6. Antibody Staining
    a. Prepare Antibody Dilution Buffer by dissolving 0.2 grams of non-fat milk in 40 ml PBST (PBST + 0.5% w/v non-fat milk).
    b. Dilute α-p53 primary antibody by adding 10 µl antibody to 20 ml Antibody Dilution Buffer (1:2000 dilution).
    c. Decant Blocking Solution from PVDF membrane, then add the 20 ml diluted α-p53 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 #HAF007, sold separately) by adding 4 µl antibody to 20 ml Antibody Dilution Buffer (1:5000 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, p53 laddering (ubiquitination) is easily observed with film exposures of 30 seconds or less.

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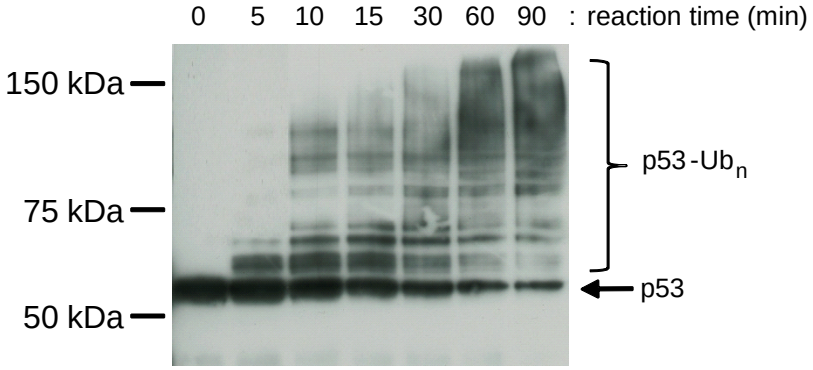
Sample Data



Ubiquitination of His_6-FLAG-p53 by the E6AP/E6 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 Loading Buffer + DTT. Western blotting with α-p53-specific antibody was performed as described in the protocol—film exposure time was 30 seconds.

Literature

References:

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

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