Product Manual

UBI-QAPTURE-Q® Kit

Catalog #: BML-UW8995

For the isolation and enrichment of ubiquitinylated proteins

PLEASE NOTE: All components are shipped on dry ice. Store at -80°C upon receipt. After thawing, the control lysate (BML-UW0130) should be aliquoted and stored at -80°C. All other components should be stored at 4°C.

Thank you for your attention to this matter.

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Product Manual

Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

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BACKGROUND

The covalent attachment of ubiquitin to proteins in the form of K^{48}-linked polyubiquitin chains (of at least four subunits in length) and their subsequent proteasomal degradation plays a fundamental role in the regulation of cellular function through biological events involving cell cycle, differentiation, immune response, DNA repair, chromatin structure, and apoptosis[1,2,3,4]. Attachment of polyubiquitin chains via linkage at alternative lysine residues has been implicated in a wide range of processes including DNA repair, translation, IκB kinase activation, and endocytosis[5]. In addition, the attachment of mono-ubiquitin at single or multiple sites on target proteins, or of short ubiquitin chains, has been shown to regulate the location and activity of a diverse range of cellular proteins[6]. These mechanisms presuppose the existence of recognition factors that transduce the information contained in specific ubiquitin signals into appropriate downstream consequences[7].

Ubiquitin-binding proteins generally have small (20–150 amino acid), independently folded ubiquitin-binding domains (UBDs) that can interact directly with monoubiquitin and/or polyubiquitin chains. UBDs can be found in enzymes that catalyze ubiquitinylation or deubiquitinylation, or in ubiquitin receptors that recognize and interpret signals from ubiquitin-conjugated to substrate proteins. UBDs are structurally diverse and are found in proteins that contain different structural features and that have different biological functions[8]. The presence of a UBD in a protein indicates that it can interact with ubiquitin or a ubiquitinylated protein and might be regulated by ubiquitinylation.

Raasi *et al*.[7] have proposed four empirical groups of UBDs, into one of which all currently known UBDs can be categorized. The groups are defined in terms of their selectivity (K^{48}-/K^{63}-linked), absence of selectivity, or non-binding of ubiquitin chains. Such selectivity is thought to play an important role *in vivo*, facilitating the binding of ubiquitinylated-proteins to specific interaction partners for various cellular processes.

KIT DESCRIPTION

The UBI-QAPTURE-Q® Kit is an efficient tool for the selective isolation of ubiquitinylated proteins. The Kit facilitates the isolation of both mono- and poly-ubiquitinylated proteins (independent of lysine residue chain linkage) from cell extracts and tissue lysates through use of a high-binding affinity matrix. Captured proteins can be analyzed by Western blotting using the highly sensitive ubiquitin-conjugate specific HRP-linked antibody provided, using antibodies to specific proteins of interest, or eluted from the matrix for subsequent biochemical characterization.

The UBI-QAPTURE-Q® matrix supplied with the Kit has superior binding characteristics compared to other commercially available matrices, is highly stable, exhibits minimal non-specific binding and is compatible with a wide range of lysate buffers and cell/tissue samples from a variety of species (including human, mouse, rat and yeast). Optimization of binding permits complete isolation of full range of ubiquitin-protein conjugates from a specific lysate. In contrast, other commercially available Kits permit only the capture of long polyubiquitin chain-conjugated proteins. The Kit is also supplied with a high quality ubiquitinylated protein solution for use as a positive control.

The Kit provides sufficient material for approximately 25 binding assays.

SUGGESTED USES

1. Isolation and detection of the full range of ubiquitinylated protein conjugates (mono-/multi-/poly-ubiquitin modified, lysine linkage independent) from specific cell/tissue lysates of interest, especially low abundance (regulatory) proteins involved in the ubiquitin-proteasome pathway.
2. Capture and analysis of specific ubiquitinylated protein conjugates of interest from particular cell/tissue lysates.
3. Purification/pull down of ubiquitinylated proteins from cell free *in vitro* assays; for example to isolate Ub-p53 species from ubiquitinylation assays mediated by HeLa S100 lysate (SW8750).
4. Release of free proteins in their active/native form by cleavage of ubiquitin/ubiquitin chains from the UBI-QAPTURE-Q® matrix using deubiquitinylating enzymes such as USP2 (BML-UW9850).

5. Release of ubiquitinylated proteins in their active/native form by elution from the UBI-QAPTURE-Q® matrix using, for example, high salt buffer.

Note: Protocol provided covers applications 1-3. Assay set-up can be readily modified for alternative applications by inclusion, omission or substitution of specific components.

MATERIALS SUPPLIED

1. **UBI-QAPTURE-Q® matrix, Catalog No. BML-UW0125-0500:**

   50% suspension, use 40µl per binding assay; 0.5ml settled resin provided, sufficient for approx. 25 binding assays.

2. **Control ubiquitinylated-protein lysate (Ub-lysate) Catalog No. BML-UW0130-0100:**

   5mg/ml in 50mM HEPES, pH7.6, 1mM DTT, use 25µg (5µl) per control binding assay; 500µg (100µl) provided, sufficient for 20 control binding assays.

3. **Ubiquitin-conjugate specific HRP-linked antibody solution, Catalog No. BML-PW0150-0025:**

   HRP-linked antibody, for use with ECL Western blotting detection reagents; 25µl provided. Dilution of at least 1:500-1:1000 recommended for Western blotting.

   Note: Milk should NOT be used in blocking/antibody binding solutions with BML-PW0150. Please use 1% BSA in PBS or TBS Tween instead.

Reagents require separate storage conditions.

STORAGE

All components should be stored at -80°C upon receipt. After thawing, UBI-QAPTURE-Q® matrix and antibody conjugate should be stored at 4°C. Avoid multiple freeze/thawing of lysate to ensure stability and activity.

OTHER MATERIALS NEEDED

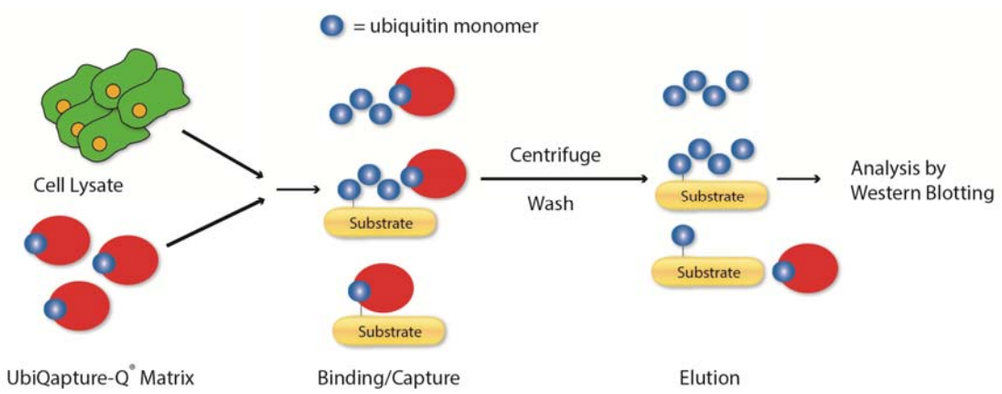
1. **5x SDS-PAGE gel loading buffer** (e.g. 0.25M Tris-HCl, pH 6.8, 15% SDS, 50% glycerol, 25% β-mercaptoethanol, 0.01% bromophenol blue).
2. **PBS Solution**; 1 × PBS
3. **HEPES Solution** (alternative to PBS as binding buffer); 50mM HEPES, pH7.5

CAPTURE / ENRICHMENT OF UBIQUITINYLATED PROTEINS

The protocol set out in this section is designed for capture of ubiquitin-protein conjugates present in the positive control lysate provided, for subsequent analysis by western blotting. These conditions should be used as a starting point for isolation of ubiquitin-protein conjugates from a specific lysate/solution but may require optimisation to ensure complete pull down/capture of ubiquitinylated proteins of particular interest.

Note: Sample lysis buffers containing components that cause protein denaturation, particularly chaotropes such as urea, should be avoided. The use of reducing agents (e.g. DTT) and detergents should be minimised if possible. High salt levels may also affect ubiquitin conjugate binding (>500mM). The control ubiquitinylated-protein lysate utilizes 50mM HEPES, pH7.5, 1mM DTT, as its lysis buffer. More complex buffers, such as RIPA, are also compatible with the UBI-QAPTURE-Q® matrix, but should be used sparingly due to the presence of detergents.

A protocol for checking lysis buffer compatibility with the UBI-QAPTURE-Q® matrix is provided at the end of this section.



A total sample protein content of ~25µg (250µg/ml concentration) is recommend for initial ubiquitin-protein conjugate capture experiments. Additional lysate material or serial dilution of lysate sam-

ples from this point may be necessary for optimization of the UBI-QAPTURE-Q® process. The binding capacity of the UBI-QAPTURE-Q® matrix is estimated to be greater than 1µg ubiquitinylated protein/20µl matrix suspension.

Note: Centrifugation of UBI-QAPTURE-Q® matrix containing solutions MUST NOT be performed at greater than 5000 *g*, to prevent damage to the UBI-QAPTURE-Q® matrix beads.

Note: HEPES can be used as an alternative to PBS as the binding buffer.

Before starting

Prepare the Control ubiquitinylated-protein lysate solution as follows:

1. Thaw the Control ubiquitinylated-protein lysate solution at 4°C and divide into 5-10µl aliquots.
2. Freeze all aliquots, except those to be used immediately, and store at –80°C.

UBI-QAPTURE-Q® protocol

Keep all components on ice throughout.

3. Dilute 5µl of the Control ubiquitinylated-protein lysate with 95µl of PBS at 4°C in a clean 1.5ml eppendorf tube (100µl final volume) to give the **control binding solution**. This diluted sample contains 25µg total protein at a concentration of 250µg/ml.
4. Using 25µg total protein content as a starting point prepare lysate samples for ubiquitin enrichment, in clean 1.5ml eppendorf tubes, by dilution of stock lysate solution in appropriate volumes of PBS, to give a final diluted **sample binding solution** volume of 100µl (protein concentration 250µg/ml).

   To allow comparison between captured ubiquitinylated species and original lysate samples (Starting Material) please retain an equivalent amount of each sample (e.g. 5µl Control ubiquitinylated-protein lysate diluted to 100µl final volume).
5. Prepare ‘Starting Material’ samples for subsequent analysis by addition of 25µl 5× SDS-PAGE gel loading buffer to each 100µl ‘Starting Material’ lysate solution, followed by heating to 95°C for 10 minutes. Store at -20°C until required.
6. Resuspend the UBI-QAPTURE-Q® matrix by gently inverting the tube several times.

7. Using a wide-bore pipette tip, aliquot 40µl of matrix suspension to a fresh tube for each of the samples to be analyzed. Add 200µl PBS to each tube, mix gently by inversion, centrifuge for 10 seconds at 5000 g and carefully remove the buffer, so as not to disturb the matrix pellet, by aspiration using a long, thin (gel-loading) pipette tip.
8. Add **sample/control binding solution** (100µl) to tube containing washed UBI-QAPTURE-Q® matrix and resuspend gently by inversion.
9. Allow ubiquitinylated protein conjugates to bind to the affinity matrix at 4°C on a horizontal rotor mixer for a minimum of two hours.

   Note: Binding times of four hours should be sufficient for capture of most ubiquitinylated proteins though overnight binding may be required in some cases.

10. Centrifuge samples for 15-30 seconds at a speed of 5000 *g* to collect the UBI-QAPTURE-Q® matrix.
11. Carefully aspirate the supernatant, so as not to disturb the matrix pellet, using a long, thin (gel-loading) pipette tip to a fresh tube. Label as 'Unbound Fraction' (approximately 100µl).

    Prepare 'Unbound Fraction' samples for subsequent analysis by addition of 25µl 5× SDS-PAGE gel loading buffer followed by heating to 95°C for 10 minutes. Store at –20°C until required.

12. Wash matrix with 200µl PBS. Mix by gently inverting tube.
13. Centrifuge samples at for 30 seconds at a speed of 5000 g to collect the UBI-QAPTURE-Q® matrix.
14. Carefully remove the supernatant, as described previously. Can be stored for analysis if required (Wash Fraction).
15. Repeat Wash once more (**steps 13-15**).
16. Elute ubiquitin-protein conjugates by addition of 100µl PBS and 25µl 5 × SDS-PAGE gel loading buffer to each matrix containing sample.
17. Quench by mixing at room temperature for 5 minutes, followed by heating to 95°C for 10 minutes. Label as 'Elution Fraction'.

    Elution Fraction should then be clarified by centrifugation prior to analysis.

    Proceed directly to "Analysis by Western Blotting" or store at –20°C until ready.

Note: If active/native protein elution is required, experiments should be repeated once optimized conditions are established. Simply follow the UBI-QAPTURE-Q® Kit protocol to **Step 16** (matrix washing) and elute native proteins by user-preferred method.

Lysis Buffer Compatibility

It is possible that some lysis buffers may interfere with the binding of poly-Ub modified proteins to the UBI-QAPTURE-Q® matrix. The ubiquitin:matrix binding interaction depends upon a degree of structural recognition so buffer components that cause denaturation or changes to protein structure can be a problem. The presence of chaotropes such as urea should be avoided and the use of reducing agents (e.g. DTT) and detergents should be minimized if possible. High salt levels may also affect binding (>500mM).

Control lysis buffer compatibility binding assay

In order to check that a particular lysis buffer is compatible with the UBI-QAPTURE-Q® Kit run the following additional control binding experiment:

- Dilute 5µl control lysate (BML-UW0130) in 95µl PBS containing equivalent amounts of lysis buffer components to those that would be present in the proposed sample binding solution after 20x dilution in PBS.

  i.e., If proposed lysis buffer contains 20mM Tris-Cl, pH 7.5, 100mM NaCl, 5mM EDTA dilute the control lysate in PBS containing 1mM Tris-Cl, pH7.5, 5mM NaCl, 0.25mM EDTA to give the lysis buffer compatibility test binding solution.

- • Run the lysis buffer compatibility test binding solution alongside the standard control binding solution (lysate + PBS only), to compare their ubiquitinylated protein binding ability, using the standard UBI-QAPTURE-Q® protocol detailed previously.
- • Analyse both binding assays using the protocol detailed in the “Analysis by Western Blotting” section. If the proposed lysis buffer exhibits considerably lower ubiquitinylated protein binding capability its composition may need to be modified or, if possible, an alternative lysis buffer used.

ANALYSIS BY WESTERN BLOTTING

Wash Buffer

Note: If direct comparison of Starting Material, Unbound Fraction, (Wash Fraction) and Elution Fraction is required (for example to assess the proportion of lysate ubiquitinylated proteins captured under specific conditions), equivalent amounts of material must be analysed. Hence, following on from the capture / enrichment protocol (section 6), equal volumes of Starting Material, Unbound Fraction and Elution Fraction should be compared by SDS-PAGE/western blotting.

Summary of analysis steps

1. Separate proteins by SDS-PAGE.
2. Western transfer to PVDF membrane.
3. Block membrane with BSA/PBS-T solution.
4. Probe blot with either: a) ubiquitin-conjugate specific HRP-linked antibody supplied or b) appropriate target protein specific primary antibody in conjunction with a suitable secondary antibody.
5. Develop with western blotting detection reagents.

Note: Do NOT use milk in blocking/antibody binding solutions. Please use 1% BSA in PBS or TBS Tween instead.

Materials required

Suggested products/suppliers shown.

SDS-PAGE gels
User prepared (10% standard / 4-15% linear gradient) or pre-formed. (e.g. ReadyGel, 4-15% Linear Gradient, BioRad, Product No. 161-1104).

Pre-stained SDS-PAGE molecular weight markers
(e.g. See Blue Plus 2, pre-stained SDS-PAGE markers, Invitrogen, LC5925).

PVDF membrane
(e.g. Immobilon-P PVDF Membrane (0.45µm, 26.5cm (w)), Millipore, IPVH00010).

Target protein specific primary antibody (if required).

Appropriate secondary antibody-HRP conjugate (if required)
For use with chosen target protein specific primary antibody.

Western blotting detection reagents
(e.g. ECL Reagent, Amersham, RPN2209).

PBS solution
1 × PBS.

PBS-T solution
1 × PBS containing 0.2% Tween 20 (e.g. Sigma, P1379).

BSA/PBS-T blocking solution
PBS-T containing 1% bovine serum albumin (BSA) (e.g. Albumin [bovine serum], Sigma, A7906).

EXAMPLE PROCEDURE FOR WESTERN BLOTTING

Note: This protocol has been optimized using the materials indicated above. Using materials other than those listed may require additional optimization.

1. Apply 20µl of each UBI-QAPTURE-Q® assay fraction to the gel (equivalent amount of Starting Material, Unbound Fraction and Elution Fraction allows direct comparison), alongside selected molecular weight markers, electrophorese, and transfer protein to PVDF membrane according to standard procedures.

Note: Drying PVDF membrane prior to blocking, as per most Manufacturers' instructions, considerably enhances results.

2. Remove membrane from the transfer unit and block with BSA/PBS-T blocking buffer for 1 hour at room temperature on a rocking platform, or overnight at 4°C.
3. Wash membrane for 3 × 10mins with PBS-T on a rocking platform at room temperature.

Ubiquitin-conjugate detection

4. Dilute supplied ubiquitin-conjugate specific HRP-linked antibody 1:500 or 1:1000 in BSA/PBS-T.
5. Incubate membrane with ubiquitin-conjugate specific HRP-linked antibody solution for 1 hour at room temperature on a rocking platform, or overnight at 4°C.
6. Wash membrane for 6 × 10mins with PBS-T on a rocking platform.
7. Proceed to step 14.

Specific target protein detection (if required)

8. Dilute appropriate target protein specific primary antibody according to manufacturer's instructions (e.g. p53 monoclonal antibody, PAb1801, at 1:1000).
9. Incubate membrane with target protein specific primary antibody solution for 1 hour at room temperature on a rocking platform, or overnight at 4°C.
10. Wash membrane for 3 × 10mins with PBS-T on a rocking platform.
11. Dilute appropriate secondary antibody according to the manufacturer's instructions (e.g. Sigma Anti-Mouse Polyvalent Immunoglobulins (G, A, M)-Peroxidase antibody (A0412) diluted 1:5000 in BSA/PBS-T).
12. Incubate membrane with secondary antibody solution for 1 hour at room temperature on a rocking platform, or as specified by the manufacturer.
13. Wash membrane for 6 × 10mins with PBS-T on a rocking platform.

Analysis

14. Prepare western blotting detection reagent according to the manufacturer's instructions (e.g. ECL reagent: Mix equal amounts of Reagent A and B and allow to stand for 1 minute).
15. Incubate membrane with ECL reagent for 1 minute.
16. Detect emitted signal by luminography or CCD imaging instrument.

EXAMPLE RESULTS FOR WESTERN BLOTTING

Figure: Western blot analysis of ubiquitin enrichment of lysate derived ubiquitinylated proteins.

Ubiquitin enrichment experiment set-up and run as described in “Capture/enrichment of ubiquitinylated proteins”. Ubiquitin-protein conjugates present in starting material, unbound fraction and elution fraction were detected by western blotting as described in “Analysis by western blotting”, using the provided ubiquitin-conjugate specific HRP-linked antibody (BML-PW0150) at a dilution of 1:1000 dilution.

Capture of Ub-protein conjugates from Control ubiquitinylated-protein lysate (Cat. #BML-UW0130).

Key: **SM** = Starting Material, **UF** = Unbound Fraction and **EL** = Elution Fraction.

Results demonstrate capture/enrichment and subsequent detection of ubiquitinylated proteins from different sources using the UBI-QAPTURE-Q® matrix provided with the UBI-QAPTURE-Q® Kit.

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ALSO AVAILABLE FROM ENZO LIFE SCIENCES

Description	Catalog #	Quantity
Ubiquitin Binding Entities (UBEs)		
p62-derived UBA domain, agarose conjugate	BML-UW9010	0.5ml
hHR23B-derived UBA2 domain, agarose conjugate	BML-UW9440	0.5ml
NBR1-derived UBA domain, agarose conjugate	BML-UW9445	0.5ml
NUB1/NUB1L UBA domain, agarose conjugate	BML-UW9700	0.5ml
UQ1 UBA Domain, agarose conjugate	BML-UW9830	0.5ml
Dsk2 UBA-agarose conjugate	BML-UW9835	0.5ml
19S Subunit S5a (Rpn10), agarose conjugate	BML-UW8635	0.5ml
19S Subunit S5a (Rpn10) UIM domain, agarose conjugate	BML-UW9820	0.5ml
VPS9-derived CUE domain, agarose conjugate	BML-UW9450	0.5ml
UBE Sampler Kit (containing all the above conjugates)	BML-UW0120	9×100µl
Cell Fractions		
HeLa S100 Fraction	BML-SW8750	1mg
Fraction I (FrI, *HeLa*)	BML-HW8600	1mg
Fraction II (FrII, *HeLa*)	BML-HW8605	1mg
Ubiquitin derivatives and mutants		
Ubiquitin	BML-UW8795	5mg
Methylated ubiquitin	BML-UW8555	1mg
Biotinylated ubiquitin	BML-UW8705	100µg
[K^{48}R]Ubiquitin	BML-UW8615	1mg
Inhibitors		
Ubiquitin aldehyde	BML-UW8450	50µg
Epoxomicin	BML-PI127	100µg
Proteasome inhibitor pack	BML-PW9900	1 Set
Antibodies		
Mono- and polyubiquitinylated conjugates, monoclonal antibody (clone FK2)	BML-PW8810	0.5mg
Polyubiquitinylated conjugates, monoclonal antibody (clone FK1)	BML-PW8805	0.5mg
Ubiquitin-protein conjugates, polyclonal antibody	BML-UG9510	25/100µl
Anti-Polyubiquitinylated Conjugates, Horseradish Peroxidase Conjugate (FK1HTM)	BML-PW0145	25/100µl
Anti-Mono- and Polyubiquitinylated Conjugates, Horseradish Peroxidase Conjugate (FK2HTM)	BML-PW0150	25/100µl
p53, mouse monoclonal antibody (PAb1801)	BML-PW1085	25/100µl
Activating Solutions		
ATP-(Energy) regeneration solution	BML-EW9810	100µl
Mg^{2+}/ATP activating solution	BML-EW9805	100µl

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