

UBI-QAPTURE-Q[®] Kit

Catalog number: BML-UW8995A

For the isolation and enrichment of ubiquitinated proteins

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Product Manual

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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 K⁴⁸-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 endocytosis⁵. 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 proteins⁶. These mechanisms presuppose the existence of recognition factors that transduce the information contained in specific ubiquitin signals into appropriate downstream consequences⁷.

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 functions⁸. 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 (K⁴⁸-/K⁶³-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 ubiquitinated proteins. The Kit facilitates the isolation of both mono- and poly-ubiquitinated 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 ubiquitinated 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 ubiquitinated 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 ubiquitinated protein conjugates of interest from particular cell/tissue lysates.
3. Purification/pull down of ubiquitinated proteins from cell free *in vitro* assays; for example to isolate Ub-p53 species from ubiquitination assays mediated by HeLa S100 lysate (Prod. No. BML-SW8750).
4. Release of free proteins in their active/native form by cleavage of ubiquitin/ubiquitin chains from the UBI-QAPTURE-Q[®] matrix using deubiquitinating enzymes such as USP2 (Prod. No. BML-UW9850).

5. Release of ubiquitylated 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 40 μ L per binding assay; 0.5 mL settled resin provided, sufficient for approx. 25 binding assays.
2. **Control ubiquitylated-protein lysate (Ub-lysate) Catalog No. BML-UW0130-0100:**
5 mg/mL in 50 mM HEPES, pH7.6, 1 mM DTT, use 25 μ g (5 μ L) per control binding assay; 500 μ g (100 μ L) provided, sufficient for 20 control binding assays.
3. **Mono- and polyubiquitylated conjugates mAb (UBCJ2) (HRP conjugate) (Ubiquitin-conjugate specific HRP-linked antibody solution) Catalog No. ENZ-ABS840HRP-0025:**
HRP-linked antibody, for use with ECL Western blotting detection reagents; 25 μ L 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 ENZ-ABS840HRP. Please use 1 - 5% 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

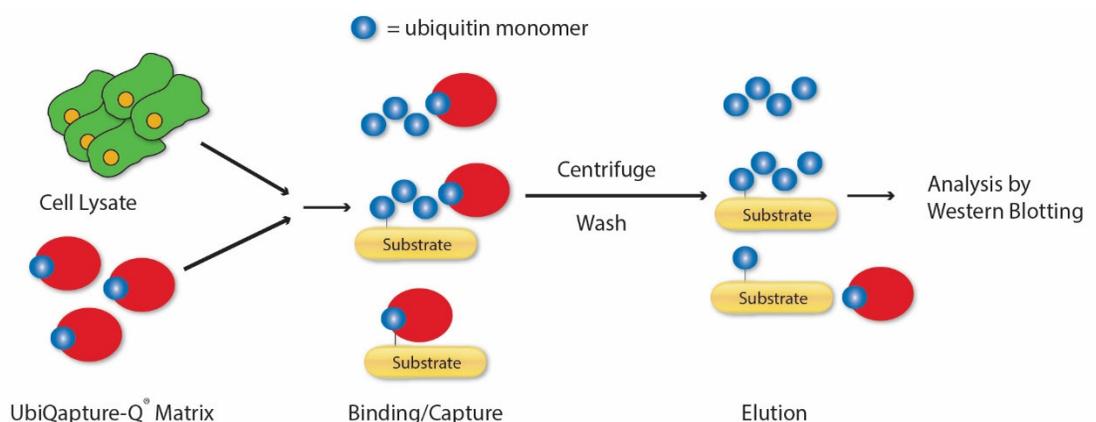
1. **5x SDS-PAGE gel loading buffer** (e.g. 0.25M Tris-HCl, pH 6.8, 15% SDS, 50% glycerol, 25% β -mercaptoethanol, 0.01% bromophenol blue).
2. **PBS Solution**; 1 x PBS
3. **HEPES Solution** (alternative to PBS as binding buffer); 50mM HEPES, pH 7.5

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 optimization to ensure complete pull down/capture of ubiquitinated 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 minimized if possible. High salt levels may also affect ubiquitin conjugate binding (>500mM). The control ubiquitinated-protein lysate utilizes 50mM HEPES, pH 7.5, 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 ~25 µg (250 µg/mL concentration) is recommended for initial ubiquitin-protein conjugate capture experiments. Additional lysate material or serial dilution of lysate samples 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 1 µg ubiquitinated protein/20 µL matrix suspension.

Note: Centrifugation of UBI-QAPTURE-Q[®] matrix containing solutions MUST NOT be performed at greater than 5000 x 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 ubiquitinated-protein lysate solution as follows:

1. Thaw the Control ubiquitinated-protein lysate solution at 4°C and divide into 5-10 µL 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 5 µL of the Control ubiquitinated-protein lysate with 95 µL of PBS at 4°C in a clean 1.5 mL microcentrifuge tube (100 µL final volume) to give the **control binding solution**. This diluted sample contains 25 µg total protein at a concentration of 250 µg/mL.
4. Using 25 µg total protein content as a starting point prepare lysate samples for ubiquitin enrichment, in clean 1.5 mL microcentrifuge tubes, by dilution of stock lysate solution in appropriate volumes of PBS, to give a final diluted **sample binding solution** volume of 100 µL (protein concentration 250 µg/mL).

To allow comparison between captured ubiquitinated species and original lysate samples (Starting Material) please retain an equivalent amount of each sample (e.g. 5 µL Control ubiquitinated-protein lysate diluted to 100 µL final volume).

5. Prepare 'Starting Material' samples for subsequent analysis by addition of 25 µL 5× SDS-PAGE gel loading buffer to each 100 µL '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 40 μ L of matrix suspension to a fresh tube for each of the samples to be analyzed. Add 200 μ L 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** (100 μ L) to tube containing washed UBI-QAPTURE-Q[®] matrix and resuspend gently by inversion.
9. Allow ubiquitinated 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 ubiquitinated 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 100 μ L).

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

12. Wash matrix with 200 μ L PBS. Mix by gently inverting tube.
13. Centrifuge samples for 30 seconds at a speed of 5000 *g* to collect the UBI-QAPTURE-Q[®] matrix.
14. Carefully remove the supernatant, as described previously. (Optional) supernatant 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 100 μ L PBS and 25 μ L 5 \times 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 ($>500\text{ mM}$).

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 5 μL control lysate (BML-UW0130) in 95 μL 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 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA dilute the control lysate in PBS containing 1 mM Tris-HCl, pH 7.5, 5 mM NaCl, 0.25 mM 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 ubiquitinated protein binding ability, using the standard UBI-QAPTURE-Q[®] protocol detailed previously.
- Analyze both binding assays using the protocol detailed in the “Analysis by Western Blotting” section. If the proposed lysis buffer exhibits considerably lower ubiquitinated 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 ubiquitinated proteins captured under specific conditions), equivalent amounts of material must be analyzed. 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 nitrocellulose or 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 - 5% 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 precast protein gel (e.g. Criterion™, 4-15% Linear Gradient, Bio-Rad, 345-0029).

Pre-stained SDS-PAGE molecular weight markers

(e.g. SeeBlue™ Plus 2, Pre-stained Protein Standards, ThermoFisher Scientific, LC5925).

Nitrocellulose Blotting Membrane

(e.g. Amersham™ Protran™ Membrane (0.45µm), GE Healthcare Life science, 10600002).

PVDF membrane

(e.g. Immobilon-P PVDF Membrane (0.45µm), 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. BioFX[®] TMB Enhanced One Component HRP Membrane Substrate, SURMODICS, ESPM-1000, or ECL Reagent, Clarity[™] Western ECL substrate, Bio-Rad, 1705060).

PBS solution

1 x PBS (e.g. 10X PBS Solution (Teknova, P0495), dilute with diH₂O to 1X PBS).

PBS-T solution

1 x 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 20 μ L 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 nitrocellulose or 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 x 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 x 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 x 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 x 10mins with PBS-T on a rocking platform.

Analysis

14. Prepare western blotting detection reagent according to the manufacturer's instructions.
15. Incubate membrane with ECL reagent according to the manufacturer's recommendation.
16. Detect emitted signal by luminography or CCD imaging instrument. Alternatively, if colorimetric substrate is used, stain membrane according to manufacturer's instruction and take image with appropriate scanner.

EXAMPLE RESULTS FOR WESTERN BLOTTING

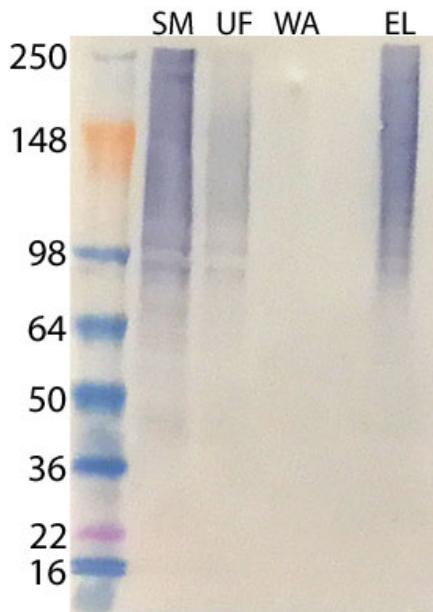


Figure: Western blot analysis of ubiquitin enrichment of lysate derived ubiquitinated proteins.

Ubiquitin enrichment experiment set-up and run as described in “Capture/enrichment of ubiquitinated 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 (ENZ-ABS840HRP) at a dilution of 1:1000 dilution.

Capture of Ub-protein conjugates from Control ubiquitinated-protein lysate (Prod. No. BML-UW0130).

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

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

REFERENCES

1. Hershko, A. and Ciechanover, A. The ubiquitin system. *Annu.Rev.Biochem.* 67, 425-479 (1998)
2. Haas, A.L. and Siepmann, T. J. Pathways of ubiquitin conjugation. *FASEB J.* 11, 1257-1268 (1997)
3. Pickart, C.M. Mechanisms underlying ubiquitination. *Annu.Rev.Biochem.* 70, 503-533 (2001)
4. Strous, G.J. and Govers, R. The ubiquitin-proteasome system and endocytosis. *J.Cell Sci.* 112 (Pt 10), 1417-1423 (1999)
5. Pickart, C.M. and Fushman, D. Polyubiquitin chains: polymeric protein signals. *Curr.Opin.Chem.Biol.* 8, 610-616 (2004)
6. Hicke, L. Protein regulation by monoubiquitin. *Nat.Rev.Mol.Cell Biol.* 2, 195-201 (2001)
7. Raasi, S., Varadan, R., Fushman, D., and Pickart, C. M. Diverse polyubiquitin interaction properties of ubiquitin-associated domains. *Nat.Struct.Mol.Biol.* 12, 708-714 (2005)
8. Hicke, L., Schubert, H. L., and Hill, C. P. Ubiquitin-binding domains. *Nat.Rev.Mol.Cell Biol.* 6, 610-621 (2005)



Product Manual

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