

## Proteasome purification kit

Catalog #: BML-PW1075A

For the isolation and enrichment of proteasome complexes.

**PLEASE NOTE:**

ALL COMPONENTS ARE SHIPPED ON DRY ICE. UPON RECEIPT, THE PROTEASOME PURIFICATION MATRIX (BML-PW9005) SHOULD BE STORED AT +4°C. THE CONTROL LYSATE (BML-SW8750) SHOULD BE STORED AT -80 °C. OTHER BIOCHEMICAL COMPONENTS SHOULD BE STORED AT -20°C FOR SHORT TERM AND -80°C FOR LONG TERM.

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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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## DESCRIPTION

This kit provides a gentle and rapid means to purify proteasome complexes from biological samples using an agarose immobilized 20S subunit specific proteasome antibody, without the need for genetic modification of the expressed proteasome. Captured proteasomes are eluted under denaturing conditions prior to analysis by Western blotting or characterization by proteomic methods.

Proteasome purification matrix binds approximately 0.75 $\mu$ g 26S proteasome *per*  $\mu$ L matrix. This kit provides sufficient material for 10 x standard proteasome purification assays.

## BACKGROUND

Proteasomes are non-lysosomal proteolytic complexes localized primarily in the cytoplasm and in the nucleus of eukaryotic cells<sup>1</sup>. The 26S proteasome structure is composed of a 20S proteasome catalytic core complex and one or two 19S regulatory subcomplexes. The 20S core comprises two copies of 14 subunits (7  $\alpha$ -subunits and 7  $\beta$ -subunits) arranged in a  $\alpha_7\beta_7\beta_7\alpha_7$  cylindrical array. Varying catalytic subunit composition ( $\beta_1$ ,  $\beta_{1i}$ ;  $\beta_2$ ,  $\beta_{2i}$ ;  $\beta_5$ ,  $\beta_{5i}$ ) results in a variety of possible subtypes. The 19S regulatory subcomplexes, comprised of 6 ATPase and at least 10 non-ATPase subunits, specifically bind ubiquitylated proteins and provide the 20S core with an ATP-ubiquitin-dependent proteolytic activity<sup>2</sup>.

In addition, various proteins have been found to associate with the 19S regulator, though less tightly than the 20S core subunits. Some of them appear to promote delivery of substrates, catalyze the disassembly of ubiquitin chains, or may even regulate proteasomal activity under specific conditions. These various cofactors and activators mean that proteasomes are heterogeneous, dynamic structures, which differ in properties and probably in their specialized functions<sup>3</sup>.

The ubiquitin-proteasome system is the major non-lysosomal system for the degradation of short half-life proteins and peptides that are involved in basic cellular processes, such as cell-cycle regulation and apoptosis, transcriptional regulation, or antigen processing<sup>4,5</sup>. Thus, protein degradation by the ubiquitin-proteasome pathway has a major regulatory function for proliferation activity and survival of both normal and malignant cells<sup>6,7</sup>.

Methods for purification of proteasome complexes and their associated proteins often rely upon lengthy, multi-step purification protocols or the genetic alteration or affinity tagging of proteasomes to allow one-step purification<sup>8,9</sup>. This limits the organisms/cell lines that can be studied, may introduce artifacts compared to the wild type system and is not applicable to the study of proteasomes such as those associated with particular disease states. The Proteasome Purification Kit provides a means of rapidly and gently purifying wild type proteasome complexes from biological samples facilitating their subsequent analysis.

## SUGGESTED USES / APPLICATIONS

1. Isolation of wild type proteasome complexes from biological samples (cell lysates, tissue extracts, plasma, serum), confirmed by Western blot analysis.
2. Analysis of captured proteasome complex proteins by proteomic methods, for example to determine complex composition and subunit post-translational modification.
3. Investigation of variations in proteasome complexes in abnormal cell lines/tissues
4. Comparison of proteasome complexes isolated from biological samples associated with a particular disease/illness with samples from healthy controls.

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

## MATERIALS SUPPLIED

1. **Proteasome purification matrix**, Catalog No. BML-PW9005-0250  
50% suspension, use 50 $\mu$ L per binding assay (25 $\mu$ L settled resin).  
250 $\mu$ L settled resin provided, sufficient for 10 binding assays.
2. **HeLa S100 fraction (Control lysate)**, Catalog No. BML-SW8750-0100  
5mg/mL in 50mM HEPES, pH7.4, containing 1mM DTT, use up to 250 $\mu$ g (50 $\mu$ L) per control binding assay.  
100 $\mu$ L (500 $\mu$ g) provided, sufficient for  $\geq$  2 control binding assays.
3. **Proteasome 20S  $\beta$ 5, pAb**, Catalog No. BML-PW8895-0010  
Proteasome 20S  $\beta$ 5 subunit, Rabbit polyclonal antibody.  
10 $\mu$ L provided. Dilution of at least 1:1000 recommended for Western blotting.



Reagents require separate storage conditions.

## STORAGE

Proteasome Purification Matrix (BML-PW9005) should be stored at 4°C upon receipt. The Control lysate (BML-SW8750) should be stored at -80 °C. Other biochemical components should be stored at -20 °C for short term and -80°C for long term. Avoid multiple freeze/thawing. Aliquot upon initial thawing.

## 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, 0.05% bromophenol blue). (Other equivalent SDS-PAGE loading buffer can also be used by supplementing reducing reagent as needed.)
2. **Binding Buffer** (25mM HEPES, pH 7.4, 10% glycerol, 5mM MgCl<sub>2</sub>, 1mM ATP, 1mM DTT)  
*Note: glycerol and Mg<sup>2+</sup>-ATP are required to prevent dissociation of 26S complex.*
3. **(Suggested) Lysis Buffer** (25mM HEPES, pH 7.4, 10% glycerol, 5mM MgCl<sub>2</sub>, 1mM ATP, 1mM DTT).

## CAPTURE / ENRICHMENT OF PROTEASOME COMPLEXES

The protocol set out in this section is designed for capture of proteasome complexes present in the positive control lysate provided followed by elution under denaturing conditions for analysis by Western blotting, as described, or characterization by proteomic methods.

These conditions should be used as a starting point for isolation of proteasome complexes from a specific lysate/solution and may require optimization/scale up to ensure efficient pull down/capture of proteasome complexes for analysis by particular methods of interest.

A total sample protein content of ~100 $\mu$ g (max. volume: 100 $\mu$ L) together with 25 $\mu$ L proteasome purification matrix (settled resin) is recommended for initial proteasome purification experiments, though proteasome purification from up to 250 $\mu$ g control lysate has been successfully performed using the method described.

The binding capacity is estimated to be approximately 0.75 $\mu$ g 26S proteasome complex *per*  $\mu$ L proteasome purification matrix.

### Technical Tips:

#### General

- Keep all components on ice throughout.
- Use wide bore pipette tips for aliquoting the proteasome purification matrix.
- DO NOT centrifuge proteasome purification matrix containing solutions at greater than 5000 $xg$  to prevent damage to the matrix.

#### Lysate Sample preparation

- Prepare samples under native conditions – required for capture by the Proteasome purification matrix to occur.
- 25mM HEPES, pH 7.4, 10% glycerol, 5mM MgCl<sub>2</sub>, 1mM ATP, 1mM DTT buffer is recommended for lysate preparation.
- Alternative buffers may be used if preferred but MUST include glycerol and Mg<sup>2+</sup>-ATP to prevent 26S proteasome complex dissociation.
- 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. High salt levels may affect proteasome binding (>500mM).



### Assay optimization suggestions

- Adjust the ratio of total protein content to proteasome purification matrix used.
- To increase the total amount of proteasome isolated purifications can be scaled up by using a greater amount of sample and proteasome purification Matrix while maintaining their relative proportions.

### Proteasome purification protocol

1. Aliquot 20 $\mu$ L of the Control Lysate (HeLa S100 fraction) into a clean 0.5mL screw capped tube and dilute with 80 $\mu$ L Binding Buffer to give the control binding solution (100 $\mu$ g total protein at 1mg/mL).
2. Using 100 $\mu$ g total protein content as a starting point prepare lysate samples for proteasome purification, in clean 0.5mL screw capped tubes, using either neat lysate (containing glycerol/Mg-ATP) or stock lysate solution diluted in Binding Buffer, to give a final sample binding solution volume of 100 $\mu$ L (protein concentration 1mg/mL).
3. To allow Western blot comparison between captured proteasome and original lysate samples (Starting Material) retain an equivalent amount of each sample (e.g. 20 $\mu$ L Control Lysate diluted to 100 $\mu$ L final volume, as described in Step 1).
  - Prepare 'Starting Material' samples for subsequent Western blot analysis by addition of 25 $\mu$ L 5X SDS-PAGE gel loading buffer to each 100 $\mu$ L 'Starting Material' lysate solution, followed by heating to 95°C for 10 minutes. Store at -20°C until required.
4. Re-suspend the proteasome purification matrix (BML-PW9005) by gently inverting the tube several times.
5. Aliquot 50 $\mu$ L of the matrix suspension to a fresh tube for each of the samples/control to be analyzed.
  - Add 200 $\mu$ L Binding Buffer to each tube, mix by inversion, centrifuge for 30 seconds at 5000xg and carefully remove the buffer.
6. Wash matrix with a further 200 $\mu$ L Binding Buffer, centrifuge and remove buffer.
7. Add sample/control binding solutions (100 $\mu$ L) to tubes containing the washed proteasome purification matrix and re-suspend by inversion.
8. Incubate proteasome binding solutions for 4 hours at 4°C on a horizontal rotor mixer. Binding assays can be run overnight at 4°C if required.
9. Centrifuge samples for 30 seconds at a speed of 5000xg to collect the proteasome purification matrix.
10. Carefully remove the unbound supernatant to a fresh tube.

- To analyze by Western blotting, label as 'Unbound Fraction' (approximately 100 $\mu$ L), add 25 $\mu$ L 5X SDS-PAGE gel loading buffer and heat to 95 $^{\circ}$ C for 10 minutes. Store at -20 $^{\circ}$ C until required.
11. Wash matrix with 200 $\mu$ L Binding Buffer. Mix by gently inverting tube.
  12. Centrifuge samples for 30 seconds at a speed of 5000xg to collect the proteasome purification matrix.
  13. Carefully remove the supernatant and discard.
  14. Repeat wash step twice more (steps 11-13).
  15. Elute proteasome complexes by addition of 100 $\mu$ L Binding Buffer and 25 $\mu$ L 5X SDS-PAGE gel loading buffer to each sample followed by heating to 95 $^{\circ}$ 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 analyze by alternative methods.
    - Samples can be stored at -20 $^{\circ}$ C if required.

## Alternative Elution Method

If Elution Fraction samples are to be analyzed by methods not requiring separation by SDS-PAGE (e.g. LC-MS) appropriate buffers including a denaturant, such as 8M urea, can be used as an alternative to the elution buffer used in Step 15, followed by mixing/incubation at room temperature and clarification of Elution Fraction by centrifugation.

## ANALYSIS BY WESTERN BLOTTING

Method describes analysis of eluted proteasome samples by SDS-PAGE/western blotting to confirm successful purification and/or presence of specific proteasome subunits/associated proteins of interest.

### 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) proteasome 20S  $\beta$ 5 subunit antibody supplied or b) proteasome subunit/associated protein specific primary antibody in conjunction with suitable secondary antibodies.
5. Develop with Western blotting detection reagents.

## Materials required

1. SDS-PAGE gels - User prepared (12% standard / 4-15% linear gradient)
2. Pre-stained SDS-PAGE molecular weight markers (e.g. See Blue Plus 2)
3. Nitrocellulose or PVDF membrane
4. Anti-rabbit IgG secondary antibody (AP or HRP-linked) – for use with proteasome 20S  $\beta$ 5 subunit antibody provided (e.g. Goat anti-rabbit IgG, pAb (HRP conjugate), Prod. No. ADI-SAB-300 or Goat anti-rabbit IgG, polyclonal antibody (AP conjugate), Prod. No. ADI-SAB-301)
5. Specific proteasome subunit/associated protein specific primary antibody (user supplied) and appropriate secondary antibody-HRP conjugate (if required).
6. Western blotting detection reagents (e.g. ECL Reagent).
7. PBS solution 1X PBS.
8. PBS-T solution 1X PBS containing 0.2% Tween 20.
9. BSA/PBS-T blocking solution PBS-T containing 4% bovine serum albumin (BSA)

**EXAMPLE PROCEDURE FOR WESTERN BLOTTING**

1. Apply equivalent amounts of 'Starting Material', 'Unbound Fraction' and 'Elution Fraction' sample(s) to the SDS-PAGE gel alongside selected molecular weight markers, electrophorese, and transfer protein to nitrocellulose or PVDF membrane according to standard procedures.
2. Remove membrane from the transfer unit and block with BSA/PBS-T blocking buffer for 1 hour at room temperature on a rotor mixer.

**Note:** Drying PVDF membrane prior to blocking, as per Manufacturers' instructions, may considerably enhance results.

3. Wash membrane for 3 x 10mins with PBS-T on a rocking platform at room temperature..
4. Dilute supplied proteasome 20S  $\beta$ 5 subunit antibody provided 1:1000 in BSA/PBS-T.
5. Incubate membrane with proteasome 20S  $\beta$ 5 subunit antibody solution for 1 hour at room temperature or overnight at 4°C on a rotor mixer.
6. Wash membrane for 3 x 10mins with PBS-T on a rocking platform.
7. Dilute appropriate anti-rabbit IgG secondary antibody (HRP-linked) according to the manufacturer's instructions (e.g. 1:5000 in BSA/PBS-T).
8. Incubate membrane with secondary antibody solution for 1 hour at room temperature on a rocking platform, or as specified by the manufacturer.
9. Wash membrane for 3 x 10mins with PBS-T on a rocking platform.
10. Proceed to **step 17**.

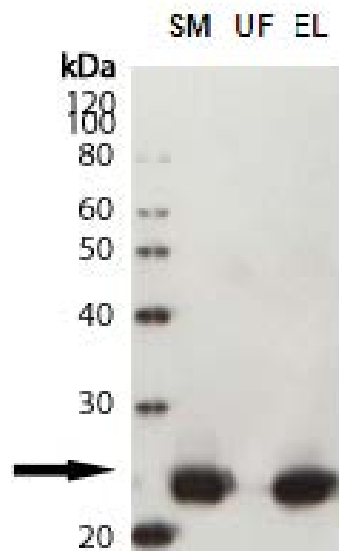
**Specific proteasome subunit/associated protein detection (if required)**

11. Dilute appropriate protein specific primary antibody according to manufacturer's instructions.
12. Incubate membrane with protein specific primary antibody solution overnight at 4°C on a rotor mixer.
13. Wash membrane for 3 x 10mins with PBS-T on a rocking platform.
14. Dilute appropriate secondary antibody according to the manufacturer's instructions.
15. Incubate membrane with secondary antibody solution for 1 hour at room temperature on a rocking platform, or as specified by the manufacturer.
16. Wash membrane for 3 x 10mins with PBS-T on a rocking platform.

**Analysis**

17. Prepare Western blotting detection reagent according to the manufacturer's instructions.
18. Incubate membrane with ECL reagent for 1 minute.
19. Detect emitted signal by luminography or CCD imaging instrument.

## EXAMPLE RESULTS FOR WESTERN BLOTTING



**Figure:** Western blot analysis of proteasome purification from control lysate.

Proteasome purification assay set-up and run as described in “Capture / enrichment of proteasome complexes”. The presence of purified proteasome in Starting Material, Unbound Fraction and Elution Fraction was detected by Western blotting as described in “Analysis by Western blotting”, using the provided proteasome 20S  $\beta$ 5 subunit antibody at a dilution of 1:1000.

Capture of proteasome from Control lysate (Prod. No. BML-SW8750).

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

Results demonstrate purification of 20S proteasome complex from the control cell lysate using the proteasome purification matrix provided with the proteasome purification kit detected using the supplied proteasome 20S  $\beta$ 5 subunit antibody (Prod. No. BML-PW8895).

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# Product Manual

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