

Product name(s):	20S/26S Proteasome ELISA Kit				
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Catalogue number:	PW0575	Batch number:		Expiry date:	6 months from receipt
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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 BIOMOL TECHNICAL SERVICES FOR ASSISTANCE IF NECESSARY.

1. Kit Description

This kit provides the means to quantify 20S proteasome concentrations in biological samples using a sandwich ELISA technique, utilizing two 20S proteasome specific antibodies for capture and detection purposes together with a highly sensitive substrate. Sample 20S proteasome levels are determined by comparison to a 20S proteasome calibration curve produced in parallel. This kit provides sufficient material for 1 × 96 well plate set-up to be run.

2. Background

Proteasomes are non-lysosomal proteolytic complexes localised primarily in the cytoplasm and in the nucleus of eukaryotic cells¹. 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 α -subunits and 7 β -subunits) arranged in a $\alpha_7\beta_7\beta_7\alpha_7$ cylindrical array. Varying catalytic subunit composition (β 1, β 1i; β 2, β 2i; β 5, β 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 activity².

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^{3,4}. Thus, protein degradation by the ubiquitin-proteasome pathway has a major regulatory function for proliferation activity and survival of both normal and malignant cells^{5,6}. The 20S proteasome has been detected in normal human blood plasma (known as circulating proteasome), possessing comparatively low specific activity and with a distinct pattern of subtypes⁷.

Proteasomes are often overexpressed in cancer cells; abnormally high expression of proteasomes having been found in human leukaemia cells⁸, renal cancer cells⁹ and in breast cancer cell lines¹⁰. In patients suffering from auto-immune diseases, malignant myelo-proliferative syndromes, multiple myeloma, acute and chronic lymphatic leukaemia, solid tumour, sepsis or trauma, the concentration of circulating proteasome has been found to be elevated, to correlate with the disease state, and may have prognostic significance⁷.

Proteasome levels have been measured by enzyme-linked immunosorbent assay (ELISA) techniques in cell lysates, serum or plasma samples^{11,12}. This approach has been used to show that proteasome concentrations in peripheral blood are elevated in patients with certain types of malignant diseases^{13,14,15}, including multiple myeloma¹¹, suggesting that circulating proteasome levels may be correlated with tumour burden^{14,15}. The link between elevated circulating proteasome levels and disease activity has also been demonstrated in patients with systemic autoimmune diseases¹⁶.

3. Suggested Uses / Applications

1. Determination of 20S proteasome levels in biological samples (cell lysates, tissue extracts, plasma, serum).
2. Comparison of 20S proteasome levels in plasma/serum samples associated with a particular disease/illness with samples from healthy controls.
3. Investigation of variation in 20S proteasome levels in abnormal cell lines/tissues.

4. Kit Components

- 1 x 96 well ELISA plate (KW0900)
- 1 vial Capture Antibody (KW8100, 25µL) - Store at -20°C
- 1 vial Detection Antibody (KW8155, 25µL) - Store at -20°C
- 1 vial HRP-labelled Goat anti-Rabbit IgG Secondary Antibody (KW0840, 10µL) Store at -20°C
- 1 vial 20S Proteasome Stock Solution (KW8720, 1mg/mL, 10µL) - DO NOT FREEZE
- 1 bottle 1x Binding Buffer (KW0845, 15mL)
- 1 bottle 10x Wash Buffer (KW0850, 75mL)
- 1 bottle 1x Blocking Buffer (KW0855, 30mL) Store at -20°C
- 1 bottle 1x ELISA Buffer (KW0860, 60mL) Store at -20°C
- 1 bottle 1x Lysis Buffer (KW0865, 20mL)
- 1 bottle 1x TMB Substrate Solution (KW0870, 12mL) - DO NOT FREEZE
- 1 bottle 1x Stop Solution (KW0875, 6mL) - Corrosive!!

NOTE: Upon receipt store the full kit at -80°C until required.

5. Other Assay Material Required

- Calibrated precision pipettes
- HPLC grade water
- Plate washer: automated or manual
- Glass or plastic tubes for diluting and aliquoting standard
- Plate rocker
- Microplate reader for recording at 450 nm.

6. Handling

- Store ELISA kit components at -80°C until required and must be defrosted completely before use.
- All kit components can be stored for up to 1 week at 4°C for convenience once defrosted.
- Avoid freeze/thaw cycles.
- Equilibrate plate and all solutions to room temperature before use except **TMB substrate solution** which can be used straight from the fridge.
- Reagents with a volume less than 100µl should be centrifuged.

7. Technical Hints And Limitations

- It is recommended that all standards, controls and samples be run in triplicate.
- The Proteasome ELISA Kit is intended to be used for a single assay run only.
- Samples are likely to require dilution with **ELISA Buffer** to allow accurate 20S proteasome concentration determination within the 1.6 µg/ml 20S proteasome limit of the assay (see section 8.1 C for recommended initial sample dilutions).
- Residual wash liquid should be drained from the wells by tapping the plate forcefully on absorbent paper.
- Crystals could appear in the 10X **Wash buffer** solution due to high salt concentration in the stock solution. Solid material is readily dissolved at room temperature or at 37°C before dilution of the buffer solutions.
- Once reagents have been added to the 96-well ELISA plate, DO NOT let it DRY OUT at any time during the assay.
- The **Stop Solution** consists of dilute sulfuric acid. The Stop Solution should be handled with gloves, eye protection and protective clothing.

8. Proteasome ELISA Protocol

8.1 Preparation of reagents

A) Buffer preparation

The **10x Wash Buffer** needs to be diluted 1:10 with HPLC grade water before use (e.g. 30mL **10x Wash Buffer** + 270mL HPLC grade water). The diluted buffer can be stored at 4°C.

The **1x Blocking Buffer** and **1x ELISA Buffer** need to be thawed prior to use. Once thawed these buffers can be stored for up to 1 week at 4°C.

B) Antibody solution preparation

- **Capture antibody solution:** Dilute the desired amount of Capture Antibody (KW8100) to a concentration of 1/1000 in **Binding Buffer** (100µL required per well). 12µL in 12mL required for a full 96-well plate.
- **Detection antibody solution:** Dilute the desired amount of Detection Antibody (KW8155) to a concentration of 1/1000 in **ELISA Buffer** (100µL required per well). 12µL in 12mL required for a full 96-well plate.
- **Secondary antibody solution:** Dilute the desired amount of Goat anti-Rabbit secondary antibody (KW0840) to a concentration of 1/2500 in **ELISA Buffer** (100µL per well). 4.8µL in 12mL required for a full 96 well plate.

Note: Highly diluted antibodies are not stable and should not be stored! Prepare fresh dilutions as required.

C) Plate Preparation

- Aliquot 100µL **Capture Antibody Solution** into each ELISA plate well. Cover plate with plastic film and incubate overnight at 4°C.
- Discard the solution in the wells and add 300µL **Wash Buffer** using a multichannel pipette. Repeat for a total of 5 washes, removing all liquid between washes.
- Block plate with addition of 300µL **Blocking Buffer** to each well. Place plate on rocker and incubate for 2 hours at room temperature.
- Repeat plate washing as above.

D) 20S proteasome standard preparation

- Add 5.12µL of **20S Proteasome Stock Solution** (KW8720, 1mg/mL) to 800µL **ELISA Buffer** to give a 6.4µg/mL 20S proteasome working stock solution.
- Add 250µL of the 6.4µg/mL 20S proteasome working stock solution to 750µL **ELISA Buffer** to give a 1.6µg/mL 20S proteasome standard solution (**20S-STD**).

E) Sample preparation

Plasma/serum samples should be diluted between 1:2 and 1:50 of their original concentration and cell lysate samples between 1:100 and 1:5000 of their original concentration in **ELISA Buffer** prior to use. This dilution may require optimization to give results within the detection limit of the proteasome ELISA kit (1.6µg/mL).

Cell lysate preparation method (if required):

- Wash cells with PBS
- Lyse them in **Lysis Buffer** at a concentration of approximately 1×10^7 cells/mL.
- Vortex the lysate briefly and incubate for 15 minutes at 4°C (the lysate preparation can be stored at -20°C at this point).
- Spin at 10000 rpm for 5 minutes and transfer the supernatant to a new tube.

8.2 Assay procedure

The following information is for the preparation of full 96-well plate. If fewer samples are to be run adjust solution volumes used accordingly.

A) Perform an 'in-well' 2-fold serial dilution of the **20S-STD** solution in **ELISA Buffer**, in triplicate, to give 20S proteasome standard dilutions of 1.6 µg/mL, 0.8 µg/mL, 0.4 µg/mL, 0.2 µg/mL, 0.1 µg/mL, 0.05 µg/mL, 0.025 µg/mL and 0 µg/mL (blank).

- Add **20S-STD** Solution (200µL) to well A.
- Add 100µL **ELISA Buffer** each to wells B-H.
- Pipette 100µL from well A and mix it thoroughly with contents of well B.
- Pipette 100µL from well B and mix it thoroughly with contents of well C etc. up to well G.
- Discard the last 100µL pipetted from well G.
- Leave well H as a blank (0 µg/mL).

	20S Proteasome Calibration (µg/mL)		
	1	2	3
A	1.6	1.6	1.6
B	0.8	0.8	0.8
C	0.4	0.4	0.4
D	0.2	0.2	0.2
E	0.1	0.1	0.1
F	0.05	0.05	0.05
G	0.025	0.025	0.025
H	0.0	0.0	0.0

- B) Add 100µL of each diluted sample to be assayed to the appropriate wells (triplicate) on the ELISA plate.
- C) Cover plate with plastic film and incubate, with agitation, for 1 hour at room temperature.
- D) Discard the solution in the wells and add 300µL **Wash Buffer** using a multichannel pipette. Repeat for a total of 5 washes, removing all liquid between washes.
- E) Aliquot 100µL of the **Detection antibody solution** into each ELISA plate well used.
- F) Cover plate with plastic film and incubate, with agitation, for 1 hour at room temperature.
- G) Discard the solution in the wells and add 300µL **Wash Buffer** using a multichannel pipette. Repeat for a total of 5 washes, removing all liquid between washes.
- H) Aliquot 100µL of **Secondary antibody solution** into each ELISA plate well used
- I) Cover plate with plastic film and incubate, with agitation, for 1 hour at room temperature.
- J) Discard the solution in the wells and add 300µL **Wash Buffer** using a multichannel pipette. Repeat for a total of 5 washes, removing all liquid between washes.
- K) Develop ELISA plate by adding 100µL per well **TMB substrate solution**. Cover and incubate with agitation for 10mins at room temperature.
- L) Halt colour development by adding 50µL **Stop Solution** per well, as required. Tap the side of the plate to ensure mixing/uniform colour obtained. **CAUTION – CORROSIVE SOLUTION!!**
- M) Measure absorbance immediately at 450nm using a UV-Vis spectrophotometric plate reader.

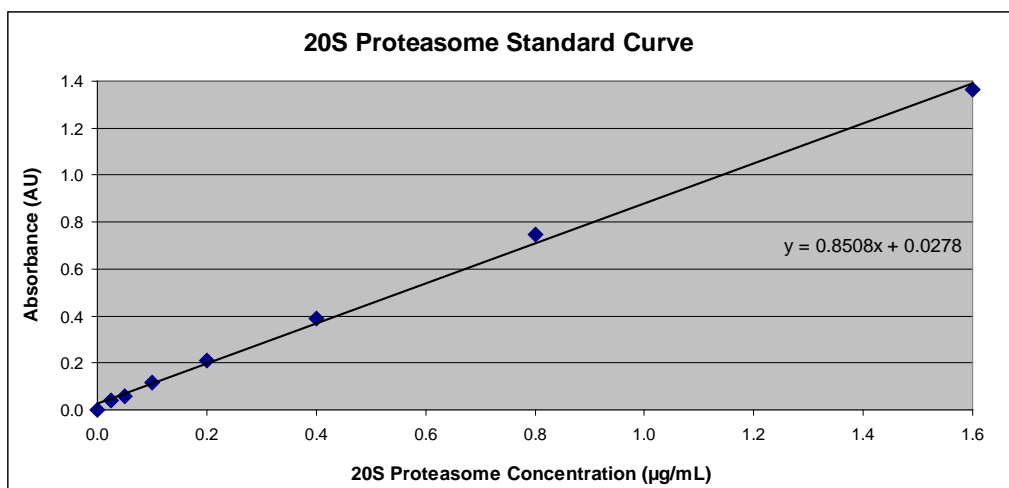
9. Calculation Of Results

- Average the triplicate readings for each standard, control and sample and subtract the average blank value (obtained with the 0 ng/mL point).
- Generate the 20S proteasome standard curve by plotting the average absorbance obtained for each standard concentration on the vertical (Y) axis vs. the corresponding 20S proteasome concentration (µg/mL) on the horizontal (X) axis (see 'Typical Results').
- Calculate results using curve-fitting statistical software. The amount of 20S proteasome in each sample is determined by interpolating for the absorbance value (Y axis) using the 20S proteasome standard curve (best-fit trendline relates concentration (x) to absorbance (y) and y-axis intercept (z) by the equation: $x = ky + z$).
- If the samples were diluted, multiply the interpolated value by the dilution factor to calculate µg/mL of 20S proteasome in the cell lysates.

10. Typical Results

Calibration Curve:

µg/mL	Triplicate Calibration			Average	Minus Blank
1.600	1.470	1.596	1.554	1.540	1.365
0.800	0.915	1.032	0.815	0.921	0.745
0.400	0.544	0.546	0.597	0.562	0.387
0.200	0.381	0.384	0.391	0.385	0.210
0.100	0.297	0.296	0.285	0.293	0.117
0.050	0.232	0.247	0.222	0.234	0.058
0.025	0.216	0.215	0.219	0.217	0.041
0.000	0.168	0.182	0.176	0.175	0.000



HeLa S100 lysate 20S proteasome determination:

Lysate	Triplicate Calibration			Average	minus blank	[Proteasome] µg/mL	× dilution factor
HEK 293T 1:200	1.377	1.408	1.403	1.396	1.221	1.402	280.4
HEK 293T 1:500	0.724	0.681	0.679	0.695	0.519	0.578	288.9
HeLa 1:500 HeLa	0.984	0.988	0.972	0.981	0.806	0.915	457.3
1:1000	0.594	0.606	0.593	0.598	0.422	0.464	463.7

11. References

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