UBPBio
12635 E. Montview Blvd. Ste 100
Aurora, CO 80045, USA
Fax: 1-800-682-6801
Email: info@ubpbio.com
www.ubpbio.com

Rapid 26S Proteasome Purification Kit-S (Cat. # J4310)

This kit is sufficient for purification of the 26S proteasome from 50 mg or 5 X 10 mg cell or tissue extracts at a concentration of 5 mg/ml. Usually, approximately 1% of total cellular protein is the 26S proteasome.

Description

The Rapid 26S Proteasome Purification Kit is designed to rapidly purify the endogenous 26S proteasome from cell or tissue extracts within 8 hours according to a published method (reference 1) with modifications. This approach uses the N-terminal ubiquitin-like (Ubl) domain of human RAD23B as the affinity bait, which allows the rapid and gentle isolation of endogenous 26S proteasomes. The bound 26S proteasome is subsequently eluted using the C-terminal two ubiquitin-interacting motifs (UIM) of human S5a that bind Ubl.

Components

• GST-Ubl (4 mg/ml)	0.6 ml
• GST (4 mg/ml)	0.5 ml
• 6xHis-UIM (4 mg/ml)	0.2 ml
• 20X Lysis Buffer	1.2 ml
• NaCl (1M)	0.5 ml
• ATP (500mM)	0.1 ml
• SUC-LLVY-AMC (50 mM)	10 µl
• Glutathione Agarose Resin (50% slurry)	1.5 ml
• Nickel *XPure* Agarose Resin (50% slurry)	1.0 ml

20X Lysis Buffer

- 800 mM Tris, pH 7.6 at 4°C
- 800 mM NaCl
- 40 mM βME
- 100 mM $MgCl_2$

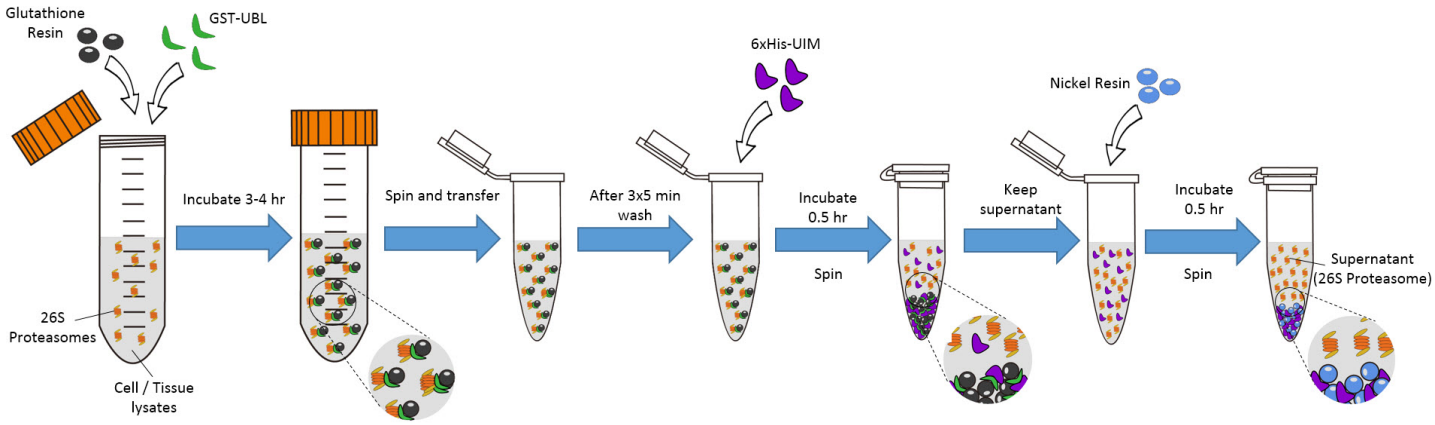
Additional reagents required (not included in the kit)

- Glycerol

UBPBio
12635 E. Montview Blvd. Ste 100
Aurora, CO 80045, USA
Fax: 1-800-682-6801
Email: info@ubpbio.com
www.ubpbio.com

Protocol

The following protocol has been used to test the kit for purification of the 26S proteasome from 50 mg HEK 293T whole cell lysates, optimizing the purification conditions may be necessary for your own experiments.



Schematic of 26S proteasome purification

All purification steps are operated at 4°C!

[Buffer Preparation]

- To make 15 ml 1X Lysis Buffer, mix the following components in a 15 ml conical tube.

20X Lysis Buffer	750 µl
500mM ATP	60 µl
Glycerol	1.5 ml
ddH_2O	12.69 ml

- To make 5 ml 1X Wash Buffer, mix the following components in a 15 ml conical tube.

20X Lysis Buffer	250 µl
500mM ATP	20 µl
1M NaCl	200 µl
Glycerol	500 µl
ddH_2O	4.03ml

[Prepare and Lyse cells]

1. Eight dishes (150 mm) of HEK 293 cells were grown in DMEM supplemented with 10% fetal bovine serum to approximately 95% confluence. Cells were harvested and kept in a 50 ml conical tube. Cells can be frozen in -80 °C freezer for future use.
2. Resuspend the cell pellet in 12 ml cell lysis buffer (we usually do not add any proteasome inhibitors in the cell lysis buffer, but you can add inhibitors as necessary for your purpose). Briefly sonicate cells using a 550 Sonic

Dismembrator (Fisher Scientific). Settings: power output: 2, 20 seconds/time for three times, rest 2 min between sonications.

3. Ultracentrifuge the cell lysates using a 70.1TI rotor (Beckman) at 36,000 rpm for 45min.
4. Carefully transfer the supernatant (avoid the lipids on the top) to a new 15 ml conical tube. We use a 10 ml syringe with a 21 gauge needle to take the supernatant. Repeat step 3 to further remove lipids if necessary.
5. Measure the cell lysate concentration using the Bradford assay. We obtained a total of 48 mg cell extracts.

[Bind the 26S proteasome to GST-Ubl]

6. Mix the supernatant in step 4 with 0.2 ml pre-washed glutathione agarose resin (net resin volume) and 0.5 ml GST-UBL (Figure A), mildly rotate the mixtures for 3 hours using a LABQUAKE rotator. The GST-Ubl concentration in the supernatant is 0.17 mg/ml. We recommend the final GST-Ubl concentration is 0.1-0.2 mg/ml in the reaction. If a control pulldown is necessary, please perform one pulldown reaction adding 0.5 ml supplied GST.

[Wash Glutathione Sepharose 4B resin]

7. After incubation, centrifuge the mixtures using an IEC Centra CL3R centrifuge at 1000 X*g* for 5 min, discard the supernatant.
8. Add 0.5 ml 1X Wash buffer to the conical tube, transfer glutathione resins to a 1.7 ml microcentrifuge tube (try to transfer all resins, multiple rinses and transfers are usually necessary).
9. Rotate the mixtures in step 8 for 3 min using a LABQUAKE rotator, then centrifuge using a desktop centrifuge at 1000 X*g* for 3 min, discard the supernatant.
10. Repeat the washing step two more times using 1.5 ml washing buffer per time. Discard the supernatant after centrifugation.

[Elute the 26S proteasome]

11. Resuspend the washed glutathione agarose resins from step 10 into 100 µl cell lysis buffer, add 100 µl 6xHis-UIM (Figure B). Mildly rotate or rock the mixtures for 30 min. The 6XHis-UIM concentration is 2 mg/ml in the mixtures (keep the elution volume small is necessary; otherwise the final concentration of the 26S proteasome is low).
    Note: UIM binds GST-UBL, which causes the release of the 26S proteasome bound on GST-UBL.
12. Centrifuge the mixtures in step 11 using a desktop centrifuge at 1000 X*g* for 3 min. Carefully transfer the supernatant containing the 26S proteasome into a new 0.65 ml microcentrifuge tube. Rinse the glutathione agarose resins with 60 µl lysis buffer and repeat the centrifugation step, combine the supernatant with the first supernatant.

[Remove 6xHis-UIM]

13. Add 40 µl (net resin) pre-washed Ni XPure agarose resins into the supernatant in step 12, mildly rotate or rock the mixture for 20 min. Centrifuge using a desktop centrifuge at 1000 X*g* for 4 min, transfer the supernatant to a new 0.65 ml microcentrifuge tube.
14. Add 20 µl pre-washed Ni XPure agarose resins to the supernatant in step 13, mildly rotate or rock the mixture for 10 min. Centrifuge the mixtures using a desktop centrifuge at 1000 X*g* for 4 min, transfer the supernatant to

a new 0.65 mirocentrifuge tube. The supernatant containes the purified 26S proteasomes. The final volume was 280 µl.

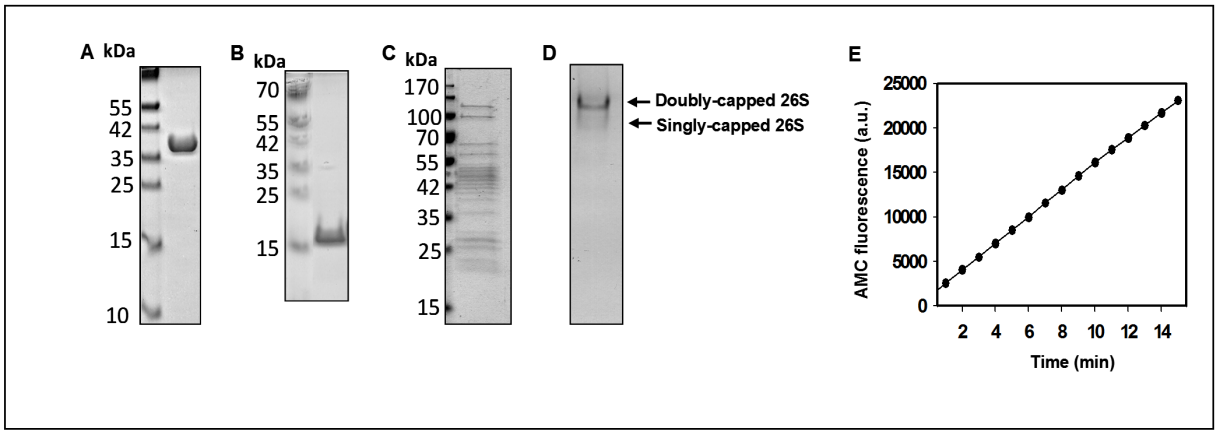
Note: Ni XPure agarose resin binds 6XHis-UIM and thus removes it from the 26S proteasome.

[Quantification]

15. Measure the proteasome concentration using the Bradford assay. The concentration was 0.16 mg/ml. The yield was 45 µg.
16. Resolve 3 µg of the purified 26S proteasome in an 11% Tris-glycine SDS-PAGE, stained with Coomassie (Figure C).
17. Resolve 3 µg of the purified 26S proteasome in a 4% native-PAGE, stained with Coomassie (Figure D). For Native-PAGE assay, please refer to reference 2.
18. Assay the 26S proteasome's peptidase activity using the provided SUC-LLVY-AMC as the fluorogenic substrate (Figure E). For Peptidase activity assay, please refer to reference 3.

[References]

1. Besche HC, Haas W, Gygi SP, Goldberg AL. Biochemistry. 2009; 48:2538-49.
2. Elsasser S, Schmidt M, Finley D. Methods Enzymol. 2005; 398:353-63.
3. Kisselev AF, Goldberg AL. Methods Enzymol. 2005; 398:364-78.



A. Coomassie stained SDS-PAGE of 5 µg purified GST-Ubl.
B. Coomassie stained SDS-PAGE of 5 µg purified 6xHis-UIM.
C. Coomassie stained SDS-PAGE of 3 µg purified human 26S proteasomes.
D. Coomassie stained native-PAGE of 3 µg purified human 26S proteasomes.
E. Solution peptidase assay of the purified human 26S proteasome. 150 µl reaction mixtures contained 10 nM purified human 26S proteasome in 50 µM SUC-LLVY-AMC in 20 mM Tris, pH 7.1 at 37 0C, 2 mM βME. The released AMC fluorescence was measured using a plate reader with excitation and emission filters at 360/40 and 460/30 nm, respectively. This assay was used to monitor the chymotrypsin-like activity of the 26S proteasome.