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In vitro Deneddylation Assay

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[Abstract] Nedd8 is a small ubiquitin-like protein (9 kDa) covalently attached to a conserved lysine residue of a cullin protein which is part of cullin-RING ligases (CRLs). CRLs are major E3 ligases important for protein ubiquitination in the ubiquitin-proteasome pathway (UPP). The activity of CRLs is regulated by cycles of neddylation (CulA-N8, ~98 kDa) and deneddylation (CulA ~89 kDa). The COP9 signalosome (CSN) and Deneddylase A (DenA) are capable of cleaving the isopeptide bond between Nedd8 and CullinA. In contrast to the single protein DenA, CSN is an eight subunit multiprotein complex. Protein crude extracts of different *Aspergillus nidulans csn* deletion strains were mixed with recombinant CSN subunits expressed and purified from *Escherichia coli* (*E. coli*). Western hybridization experiments using anti-CulA or anti-Nedd8 antibodies could show the ratio of neddylated vs. deneddylated CulA. Using the deneddylation assay, we could show that CsnE is the last subunit joining a 7-subunit pre-assembled CSN *in vitro* and only then CSN can perform cullin deneddylation by the metalloprotease subunit CsnE. This assay is a fast and non-expensive method, which visualizes enzyme activity for deneddylating proteins. It might be also useful for testing the activity of other isopeptidases removing post-translational modifications from substrates in *Aspergillus nidulans* (*A. nidulans*) or other organisms.

Materials and Reagents

A. Recombinant protein overexpression and protein purification
   1. 1 ml cuvettes 10 x 10 x 45 mm (Sarstedt AG, catalog number: 67.742)
   2. Chromatography columns [*e.g.* GSTrap (GE Healthcare, catalog number: 17-5130-01), HisTrap (GE Healthcare, catalog number: 17-5255-01), Superdex 200 HR 10/300 GL (GE Healthcare, catalog number: 17-5175-01), HiPrep 16/60 Sephacryl S-300 HR (GE Healthcare, catalog number: 17-1167-01)]
   3. Amicon Ultra centrifugal filters (Merck Millipore Corporation) molecular weight cut off (MWCO) depends on protein size [*e.g.* 30 kDa (Merck Millipore Corporation, MWCO, catalog number: UFC903024)]
   4. MicronSep filter paper, 0.22, 47 mm (Carl Roth GmbH + Co., catalog number: A009.1)

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5. *Escherichia coli* (*E. coli*) strains suitable for protein overexpression (*e.g.* Rosetta DE3) transformed with overexpression plasmid including protein sequence of interest with N- or C-terminal Glutathione-S-transferase- (GST-) or Histidine- (His-) tag
6. Tryptone/Peptone from Casein (Carl Roth GmbH + Co., catalog number: 8952.2) used as 1% solution
7. Yeast extract (Oxoid, catalog number: LP0021) used as 0.5 % solution
8. Sodiumchloride (NaCl) (Carl Roth GmbH + Co., catalog number: 9265.2) used as 1% solution
9. Antibiotics according to the *E. coli* strains used
10. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Carl Roth GmbH + Co., catalog number: 2316.4) used as 1 M solution, filter sterilized
11. 99.5% denatured Ethanol with 1% MEK (VWR International, catalog number: 85033.460) used as 20% solution diluted in ddH_2O, filter degassed
12. Imidazole
13. Glutathione
14. Lysogeny broth (LB) medium (see Recipes)
15. Lysis buffer (see Recipes)
16. Affinity buffer (see Recipes)
17. Elution buffer for HisTrap (see Recipes)
18. Elution buffer for GSTrap (see Recipes)
19. Size exclusion buffer (SEC) (see Recipes)

B. Preparation of protein crude extracts
1. Paper towels
2. Spores of *Aspergillus nidulans* strains
3. Miracloth (Merck Millipore Corporation, catalog number: 475855-1R)
4. Sodiumchloride (NaCl) (Carl Roth GmbH + Co., catalog number: 9265.2) used as 0.95 % solution
5. Liquid nitrogen (Air Liquid)
6. Minimal medium (see Recipes)
7. 50x AspA (see Recipes)
8. Trace elements (see Recipes)
9. B* buffer (see Recipes)

C. Deneddylation assay
1. Gel blotting paper (Schleicher & Schuell BioScience GmbH, catalog number: 10426694)
2. Blotting membrane Amersham™ Protran™ 0.45 µm NC (GE Healthcare, catalog number: 10600002)
3. Methanol (VWR International, catalog number: 20864.320)

4. 2-Propanol HiPerSolv CHROMANORM (VWR International, catalog number: 20880.320)
5. Page ruler prestained protein ladder (Thermo Fisher Scientific, catalog number: 26616)
6. Acrylamid Rotiphorese® Gel 30 (37, 5:1) (Carl Roth GmbH + Co., catalog number: 3029.1)
7. Sucofin skimmed milk powder (TSI GmbH, catalog number: ST00227)
8. α-CulA, α-Nedd8 antibodies (GenScript)
9. Monoclonal α-Tubulin antibody (Sigma-Aldrich, catalog number: T0926)
10. α-Actin antibody (Novus Biologicals, catalog number: NB100-74340)
11. α-goat rabbit secondary antibody (Thermo Fisher Scientific, Invitrogen™, catalog number: G21234)
12. α-goat mouse secondary antibody (Jackson ImmunoResearch, catalog number: 115-035-003)
13. p-coumaric acid (Sigma-Aldrich**,** catalog number: C9008-5 G) used as 400 mM stock solution dissolved in DMSO (protect from light, store at -20 °C)
14. Luminol (Carl Roth GmbH + Co., catalog number: 4203.1) used as 250 mM stock solution dissolved in DMSO (protect from light, store at -20 °C)
15. 2-mercaptoethanol (Sigma-Aldrich, Fluka Chemika, catalog number: 63700)
16. Sodium dodecyl sulfate (SDS) (Carl Roth GmbH + Co., catalog number: CN30.3) used as 7%, 0.1%, 1%, 0.2% solutions
17. Bromophenol blue (Sigma-Aldrich, Riedel-de Haën, catalog number: 32768) used as 0.3% solution
18. Ammoniumpersulfate (APS) (Sigma-Aldrich, Fluka Analytical, catalog number: 09913-100 G) used as 10% solution
19. Tetramethylethylendiamine (TEMED) (Carl Roth GmbH + Co., catalog number: 2367.1)
20. Tween-20 (Carl Roth GmbH + Co., catalog number: 9127.1)
21. Tris-PUFFERAN® (Carl Roth GmbH + Co., catalog number: 4855.3) used as 250 mM solution
22. Glycine (Carl Roth GmbH + Co., catalog number: 3187.3) used as 2.5 M solution
23. 30% Hydrogen peroxide (H_2O_2) (Merck Millipore Corporation, catalog number: 822287.2500)
24. 3x SDS sample buffer (see Recipes)
25. 12% SDS gels (see Recipes)
26. 2x resolving buffer (see Recipes)
27. 2x stacking buffer (see Recipes)
28. 10x SDS running buffer (see Recipes)
29. 10x transfer buffer (see Recipes)

30. 10x TBST (Tris-Buffered Saline Tween 20) buffer (see Recipes)
31. Blocking solution (see Recipes)
32. Detection solution A (see Recipes)
33. Detection solution B (see Recipes)

D. Others (material and reagents used for more than one method)
1. Micro tube 1.5 ml (Sarstedt AG, catalog number: 72.690.001)
2. Micro tube 2.0 ml (Sarstedt AG, catalog number: 72.691)
3. Tube 115 x 28 mm, PP (Sarstedt AG, catalog number: 62.559.001)
4. 5 ml test tube
5. 2 L flasks
6. PPCO centrifuge bottles (Slaughte, Nalgene, catalog number: 525-2316)
7. Oak-Ridge centrifugation tubes, 50 ml (Carl Roth GmbH + Co., catalog number: PK03.1)
8. 300 ml baffled flasks
9. Funnels
10. Crushed ice
11. 1, 4- Dithiothreitol (DTT) (Carl Roth GmbH + Co., catalog number: 6908.2) used as 1 M solution
12. *trans*-Epoxysuccinyl-L-leucylamido(4-guanidino)butane, E-64 protease inhibitor **(**Sigma-Aldrich**,** catalog number: E3132**)** used as 1 M stock solution
13. Phenylmethylsulfonyl fluoride (PMSF) (Carl Roth GmbH + Co., catalog number: 6367.3) used as 100 mM solution in Isopropanol
14. Double destilled water (ddH_2O)
15. Millipore water
16. Sodium chloride (NaCl) (Carl Roth GmbH + Co., catalog number: 9265.2) used as 2 M solution
17. Tris- hydrochloride (Tris-HCl) (Carl Roth GmbH + Co., catalog number: 9090.3)

Note: Prepare a series of Tris-HCl solution with different concentrations and pH including 1 M solutions with pH 6.8, 7.5, 8.0 and pH 8.8; 0.75 M solution withpH 8.8; 100 mM solution with pH 8.0.

18. Ethylenediaminetetraacetic acid (EDTA) (Carl Roth GmbH + Co.) used as 0.5 mM solution
19. Nonoxinol-40 (NP-40) (Affymetrix, USB Corporation, catalog number: 19628)
20. Glycerol (Carl Roth GmbH + Co., catalog number: 3783.1)
21. Complete protease inhibitor cocktail (Roche Diagnostics, catalog number: 11873580001) (see Recipes)

Equipment

1. Biowave DNA Llife Science Spectrophotometer (Biochrom WPA, catalog number: 80-3004-70)
2. Centrifuge Sorvall RC3B Plus (Thermo Fisher Scientific)
3. Centrifuge Sorvall RC5B Plus (Thermo Fisher Scientific)
4. Rotor Sorvall SS34 (Thermo Fisher Scientific)
5. ÄKTAexplorer 10 (GE Healthcare, catalog number: 18-1300-00)
6. pH meter pH526 (Sigma-Aldrich, model: WTW)
7. Filter and degassing system (Satorius)
8. Vacuum pump (KNF NEUBERGER, catalog number: 2.03360238)
9. Sonicator Sonoplus HD2070 (Bandelin)
10. Superloop 1/16'' (GE Healthcare, catalog number: 18-1113-82)
11. Sample loop 5 ml (GE Healthcare, catalog number: 18-1140-53)
12. Mixer Mill MM 400 (Retsch)
13. Vortex Genie 2 (Scientific Industries)
14. Pipettes (1-5,000 µl) (Gilson)
15. Centrifuge Biofuge fresco (Heraeus Instruments GmbH)
16. Nanodrop Spectrophotometer (VWR International, Peqlab)
17. Mini-PROTEAN® Tetra Cell Casting Module (Bio-Rad Laboratories, catalog number: 1658008)
18. Buffer Tank and Lid (Bio-Rad Laboratories, catalog number: 1658040)
19. Mini Trans-Blot Module (Bio-Rad Laboratories, catalog number: 1703935)
20. Autoradiography casette
21. FUSION SL-4 3500.WL (Chemiluminescence System) (PEQLAB)
22. CCD camera FUSION 4 MP (VWR International, Peqlab)
23. Orbital Shaker 3015 (GFL)
24. Shaker (INFORS AG)
25. Heating block (Gebr. Liebisch GmbH & Co. KG Labortechnik)

Software

1. Fusion BIO-1D software

Procedure

Note: The preparation of buffers, solutions and SDS gels is explained in Recipes section.

A. Recombinant protein overexpression and protein purification

1. For overexpression of *Aspergillus nidulans* proteins in *Escherichia coli* (here: Rosetta DE3) inoculate 5 ml LB pre-culture, supplemented with appropriate antibiotics, for overnight incubation at 37 °C with agitation.
2. The next morning, use 1 ml of the pre-culture to inoculate 1 L LB in a 2 L flask supplemented with appropriate antibiotics. Incubate the culture at 37 °C with ~220 rpm agitation for 2 to 3 h until OD_{600} of 0.6-0.7 is reached (depends on *E. coli* strain). OD_{600} can be determined with UV Spectrophotometer by taking 1 ml aliquot of the culture measured against a LB blank sample.
3. When OD_{600} of 0.6-0.7 is reached, add 1 ml 1 M IPTG to the culture to induce the overexpression. Transfer the flask to room temperature or 16 °C for no longer than 20 h and 150-180 rpm agitation.
4. Transfer the culture to 1 L centrifugation bottles, which should be pre-cooled on ice and then centrifuge at 4,000 rpm for 30 min at 4 °C.
5. Discard the supernatant and keep the pellet on ice. Resuspend the pellet with 5-10 ml ice-cold affinity buffer.
6. Transfer the resuspended pellet to a 50 ml tube and centrifuge at 4,000 rpm, 20 min, 4 °C.
7. Discard the supernatant and store the pellet at -80 °C.
8. For protein purification thaw the pellet on ice while preparing the lysis, affinity and elution buffers. Filter-degas the buffers using the filter and degassing system with MicronSep filters.
9. Mix the pellet with 10 ml ice-cold lysis buffer per g pellet and resuspend by pipetting up and down (prevent air bubbles and keep solution on ice).
10. Put the falcon in a box or glas with ice and sonicate the sample for approximately 8-10 rounds of 30 sec with 70% power and 1 min pause between each round. Take care that no air bubbles are formed during sonication and that the sample does not become warm.
11. Transfer the lysed cells into SS34 centrifugation tubes and centrifuge at 20,000 rpm, 30 min, 4 °C.
12. During centrifugation, wash the ÄKTA pumps and column with degassed 20% ethanol solution and degassed Millipore water and equilibrate with affinity (pump A) and elution (pump B) buffers.
13. Transfer the supernatant to the superloop and connect the loop to the ÄKTA.
14. Set the parameters for the ÄKTA run (pressue limit, flow, *etc.*) according to manufacturer recommendations. Start the run by setting the flow to 0.5 ml/min and press inject. The flow through can be collected. Switch from inject to load when the superloop is empty. Then wash with affinity buffer as long as UV reaches baseline again.
15. Apply a gradient of 0- 50% elution buffer in 30 min and collect the flow through in 2 ml fractions. The protein of interest should elute during this gradient or when 100%

elution buffer is applied to the column. Afterwards the ÄKTA system and column should be washed with degassed Millipore water and degassed 20% ethanol solution.

16. The fractions containing the protein of interest can be analyzed by SDS PAGE. Therefore, take 20 µl samples and add 10 µl 3x loading dye, denature for 5 min at 95 °C and then load on a 12% SDS PAGE.
17. Combine all fractions with proper amount of the protein of interest and concentrate them if necessary (*e.g.* to decrease the volume).
18. Equilibrate the ÄKTA and size exclusion chromatography column in SEC buffer.
19. Centrifuge the protein sample at 13,000 rpm for 10 min and inject the supernatant into the sample loop.
20. Start the run with 0.35 ml/min flow and press inject. Collect the flow through and analyze peak fractions by SDS PAGE. Then combine the fractions which include the protein of interest, concentrate the solution and store at -20 °C.

B. Preparation of protein crude extracts

1. Take 100 ml autoclaved minimal media (MM) in 300 ml buffled falsks and add supplements according to the *A. nidulans* strains. Inoculate with 1 x 10^6 spores/ml medium and incubate at 37 °C for 20 h under illumination and 120-150 rpm agitation.
2. Collect the mycelium in funnels with miracloth filters. Wash the mycelium with 50-100 ml 0.96% NaCl solution and then take the miracloth filter containing the mycelium and dry it between paper towels.
3. Transfer the dry mycelium (take mycel of the size of a 1 cent coin) to a 2 ml reaction tube and put into liquid nitrogen.
4. Pre-cool the reaction tube holders of the Mixer Mill in liquid nitrogen by pouring a small amount into a Styrofoam box.
5. Add two metal balls to each tube and put the tube into the holder.
6. Close the holder and assemble them to the Mixer Mill. Set the program to 1 min and frequency to 30/sec then press start.
7. Transfer the tube holder again into the box with a little bit of liquid nitrogen to prevent thawing.
8. Take out the metal balls with a strong magnet. Then transfer the grinded mycelium to a new 2 ml tube and put the tubes again to liquid nitrogen.
9. Put all tubes on ice and open them immediately. Add 300 µl B* buffer and vortex for ~1 min.
10. Centrifuge for 30 min, 13,000 rpm, 4 °C.
11. Transfer the supernatant to a fresh 1.5 ml tube and centrifuge again for 10 min, 13,000 rpm, 4 °C. In case there is still a pellet, transfer the supernatant to a fresh 1.5 ml tube.

12. Measure the protein concentration with Nanodrop. To prevent protein aggregate formation, do not store the crude extracts longer than 1 week at -20 °C (to perform the deneddylation assay at the same or next day would be optimal).

C. Deneddylation assay

1. Protein crude extracts of *csn* deletion and wild type strains serve as neddylated and deneddylated cullin source. Recombinant *A. nidulans* CSN proteins are used to complement the deletions. If necessary, thaw all protein solutions, centrifuge at 13,000 rpm for 10 min at 4 °C (do not freeze and thaw protein solutions more than 2-3 times as proteins form aggregates). Transfer the supernatant to a new tube. Re-measure the concentration using Nanodrop.
2. For each reaction, mix 80 µg of the protein crude extracts with 8 µg recombinant protein, 100 mM DTT and B* buffer to a final volume of 30 µl in a 1.5 ml reaction tube (Table 1). As a control, prepare the same reactions without recombinant protein. Incubate at 37 °C for 30 min and 120 rpm agitation.

Table 1. Pipetting scheme (example)

	Wild type	ΔstrainA	ΔstrainA + proteinA
80 µg crude extract	x µl	x µl	x µl
8 µg rec. protein	-	-	x µl
100 mM DTT	1 µl	1 µl	1 µl
B* buffer to 30 µl	x µl	x µl	x µl
Final volume	30 µl	30 µl	30 µl
3x sample buffer	15 µl	15 µl	15 µl

3. Add 15 µl 3x SDS sample buffer to each reaction, mix, denature at 95 °C for 5 min and then cool down on ice for 1 min. Flash spin the samples for 3-5 sec in a table centrifuge.
4. Prepare 12% SDS gels.
5. Load 10 µl of each sample on a gel and use 3.5 µl of Page Ruler Prestained Protein Ladder as size standard. Use 1x running buffer to run the electrophoresis. Apply 120 V until the proteins reach the resolving gel and then increase the voltage to 150-180 V until the 25 kDa band of the page ruler ran out of the gel.
6. Stop the migration and carefully transfer the gel to the western blotting apparatus. Assemble the western blot according to the manufacturer recommendations and as represented in Figure 1. Close the cassette with the colorless site and transfer it into the Mini trans-blot central core after it was placed into the electrophoresis chamber filled with transfer buffer. The proteins can be blotted either for at least 1 h at 100 V and appropriate cooling of the buffer or overnight at 35 V.

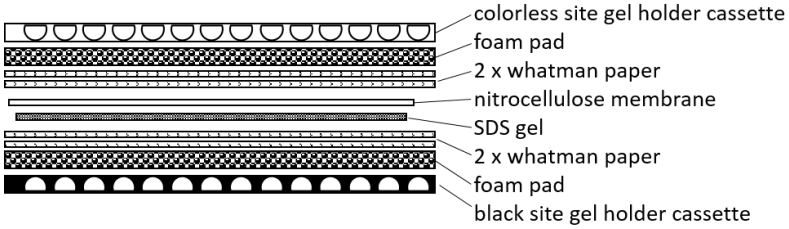

Figure 1. Western blot assembly. Schematic representation of the order of blot assembly for western hybridization.

7. Disassemble the blot and cover the membrane with blocking solution for 1 h at room temperature and gentle agitation.
8. Discard the blocking solution and separate the membrane into two pieces at the 70 kDa page ruler band (Figure 2). Add the primary antibody α-Nedd8 or α-CulA (dilution in blocking solution 1:1,000) to the upper membrane. As loading control the primary antibody α-Tub or α-Act (dilution in blocking solution 1:1,000) can be added to the lower membrane part. Incubate for 1 h at room temperature and gentle agitation.

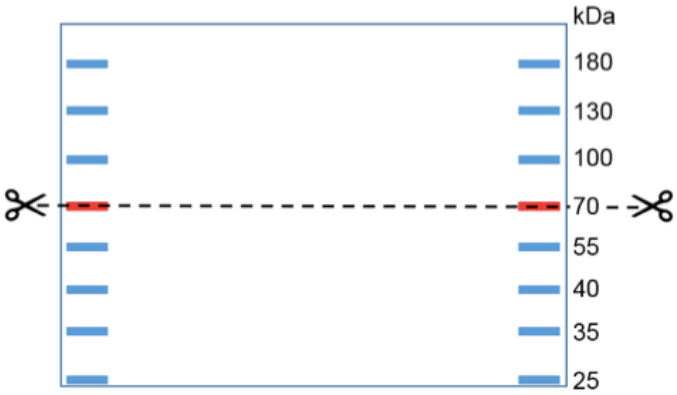

Figure 2. Western blot membrane. The dashed line indicates where the membrane needs to be cut in order to develop the loading control (Actin or Tubulin ~40 kDa) simultaneously with the CulA or Nedd8 antibody (signal ~100 kDa).

9. Remove the primary antibodies and wash the membranes 3 x 10 min with 1x TBST at room temperature and gentle agitation.
10. Add the secondary antibody α-goat rabbit to the upper membrane and α-goat mouse (both 1:1,000 in blocking solution) to the lower membrane for 1 h at room temperature and gentle agitation.
11. Remove the secondary antibodies and wash the membranes 3 x 10 min with 1x TBST buffer at room temperature and gentle agitation.

12. For signal detection prepare the detection solutions freshly. Then mix detection solution A with detection solution B on the membrane for 2 min at room temperature and gentle agitation. Subsequently place the membrane into a plastic foil and transport it via an autoradiography cassette to the Fusion imaging system (important: switch on the PC and Fusion imaging system before you are ready to use, because the camera needs approximately 10 min for cooling down).
13. Click on 'start preview', place the membrane into the apparatus, click 'Set-up' → 'Sensitivity' → 'Super Sensitivity' → 'Auto Exposure' (takes 1-4 min to detect appropriate signals). Save the image as TIF file (also automatically saved as STB file).
14. Analyze signals with Fusion BIO-1D software as followed:
    a. Click 'Open an Image' and then choose the image. Click 'Optical Density'.
    b. *A- Lane definition:* click *'New Lane'*, a box with green lines appears (Figure 3). Drag around the signals to define the area to be analyzed. click '*Select all'* → '*Next >>'*.
    c. *B- Background subtraction:* click '*Do rolling ball'* → '*Next >>'*.
    d. *C- Spot separation:* click '*Add separation'* or use given separations and place the brackets around band boundaries by dragging the cursor (Figure 4). Click '*Next >>'*.
    e. Untick '*Concentration', 'Height', Area'* and *'Molecular Weight'* (only '*Volume'* has a tick) (Figure 5). Press the *'Excel'* button. An Excel window opens with the volume data representing the pixel intensity inside a defined boundary (Figure 6). Further quantify signals considering wild type signal and loading controls.

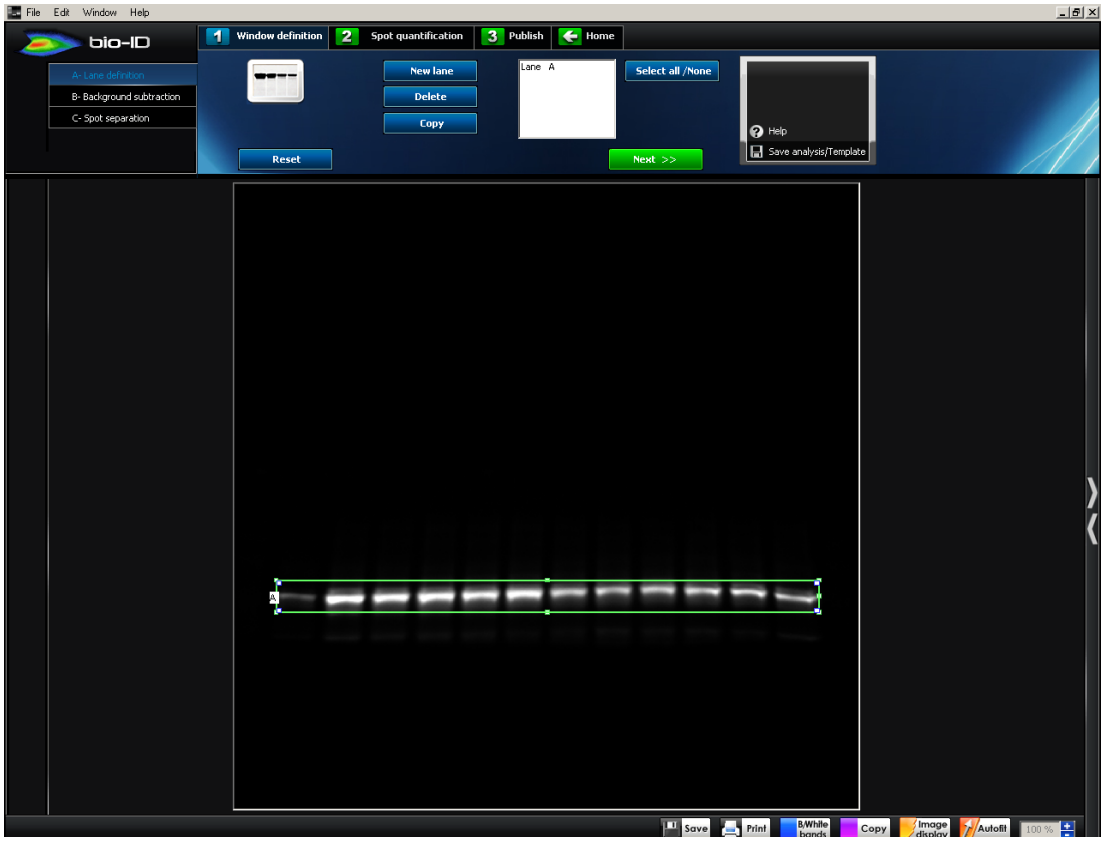

Figure 3. BIO-1D, A-Lane definition. Screenshot of BIO-1D software showing a green lined box that can be dragged to define the analysis area.

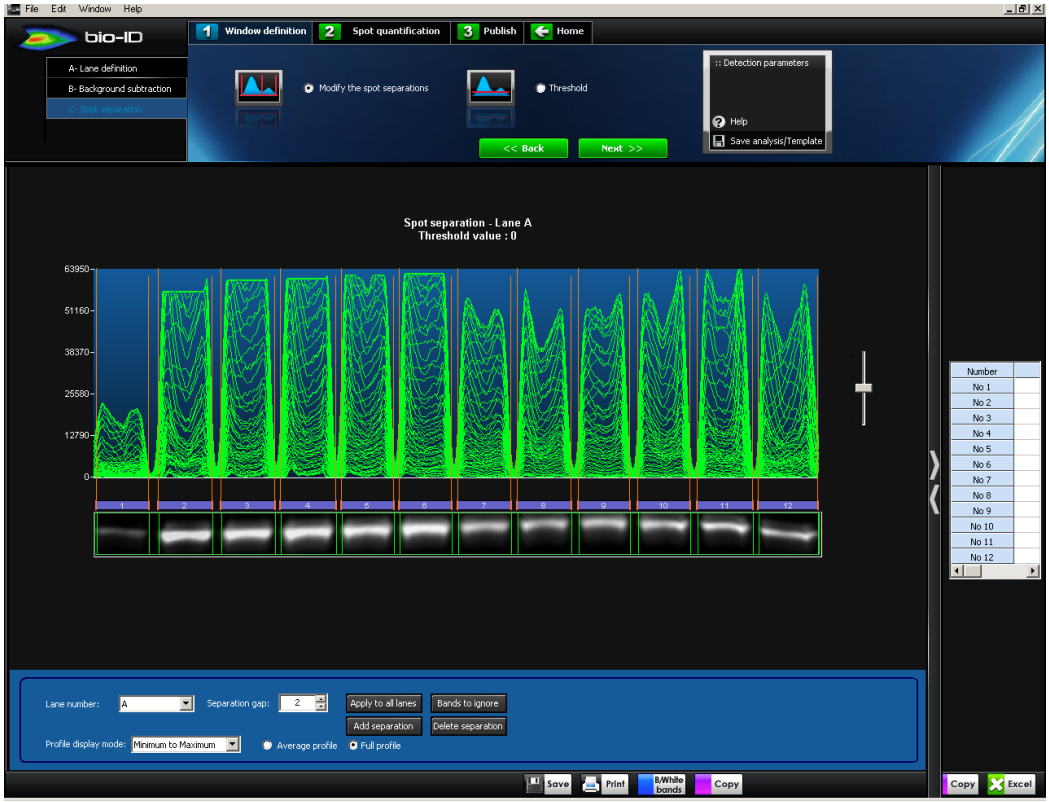

Figure 4. BIO-1D, C-Spot separation. Screenshot of BIO-1D software displaying the separation of the band boundaries by placing brackets.

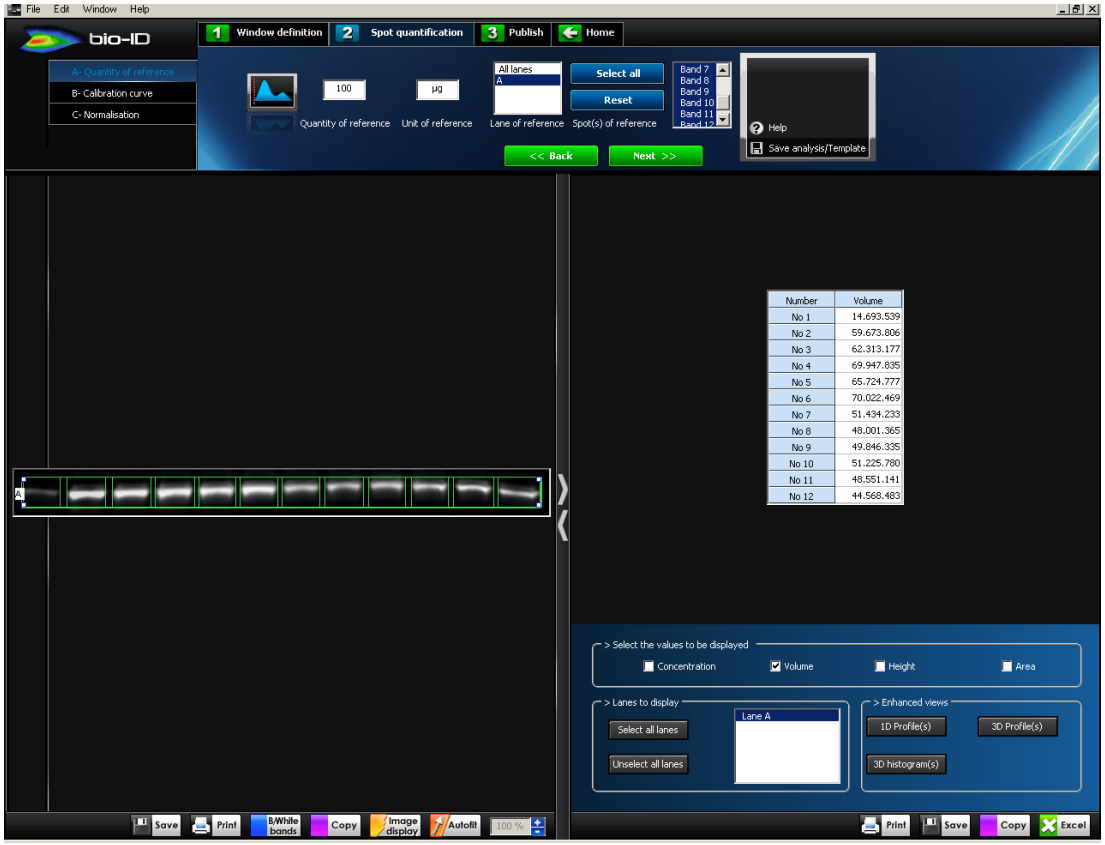

Figure 5. BIO-1D, Data export to Excel. Screenshot of BIO-1D software displaying the step where the value of the Volume has to be selected to be exported to Excel.

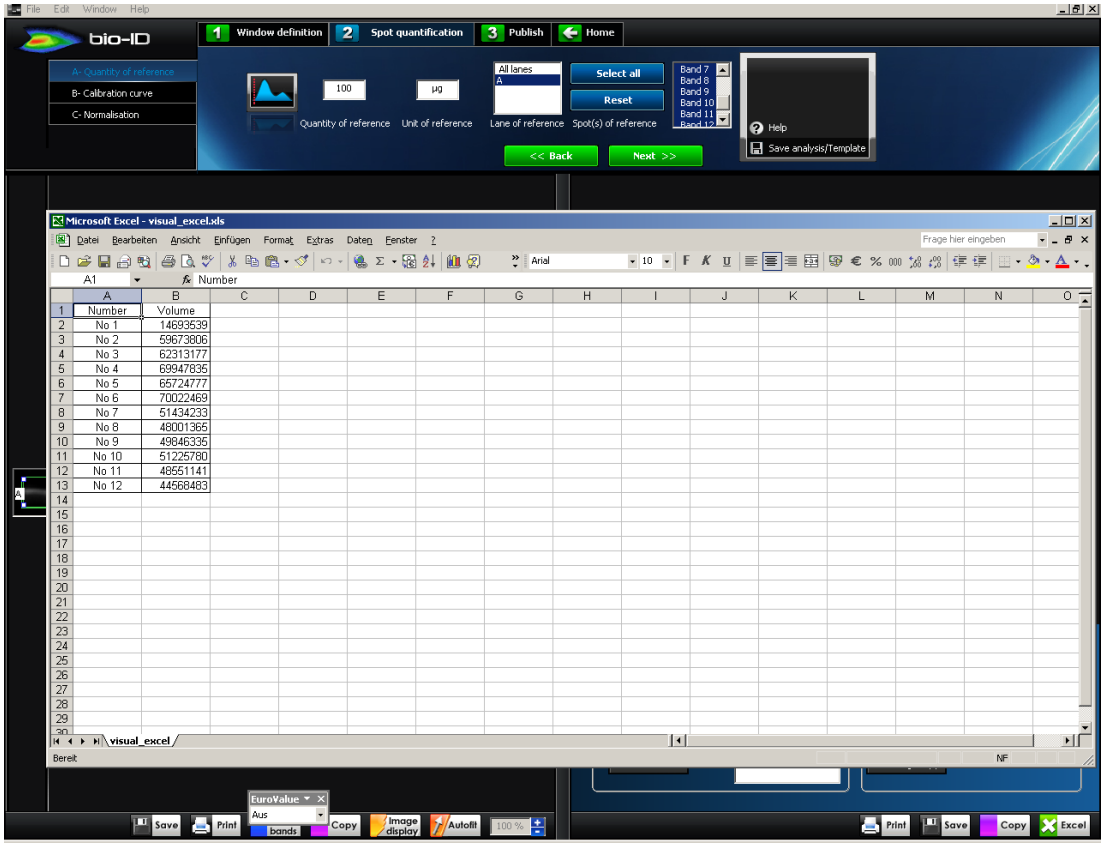

Figure 6. BIO-1D, Data in Excel. Screenshot of Excel table with values of pixel intensity inside a defined boundary.

Representative Data

Figure 7 shows a typical result of an *in vitro* deneddylation assay similar to those shown in Beckmann *et al.* (2015). Usually most cullins are deneddylated in wild type. This is visible in the western hybridization and quantification of crude extracts from wild type (wt) where the Nedd8 antibody gives a weak signal at ~100 kDa, with CulA hybridization a strong signal for deneddylated CulA (CulA, **) and a slight signal for neddylated CulA (CulA-Nedd8, *) (Figure 7). In contrast, crude extracts of *csn* deletion strains show increased neddylated CulA amounts, which cannot be complemented by the addition of the respective recombinant CSN subunit to the deletion strain. The only exception where CulA is mostly deneddylated like in wild type is when recombinant CsnE is added to *csnE* deletion crude extract. Only then the CSN seems to be active.

Signals were quantified relative to the loading control and for Nedd8 relative to wild type. For CulA signals the ratio of neddylated CulA to non-neddylated CulA were calculated.

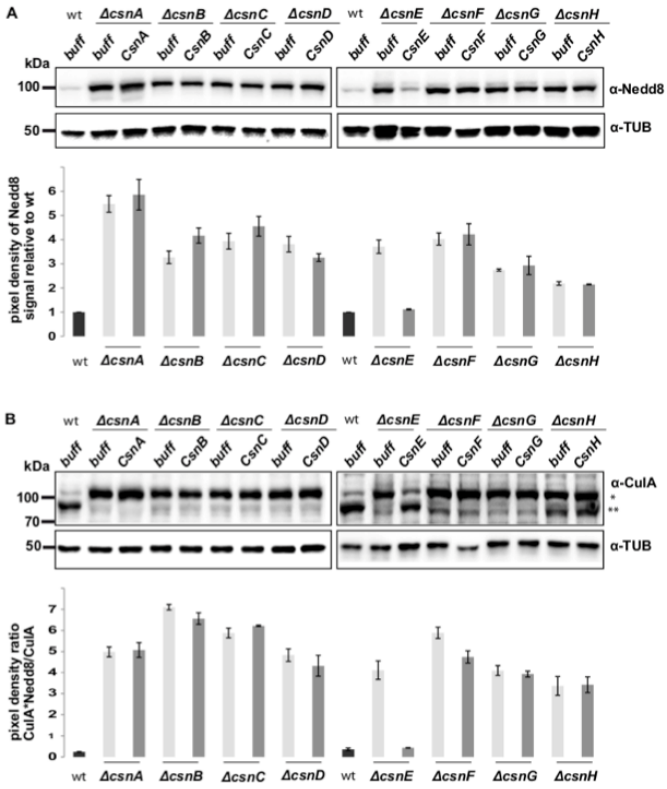

Figure 7. An example for *in vitro* deneddylation assay: CsnE can restore deneddylation activity of CSN. Western hybridization of *A. nidulans* crude extracts incubated with and without recombinant CSN proteins. A. The membrane was incubated with α-Nedd8 antibody resulting in a weak signal for wt and ΔcsnE + CsnE and strong signals for all other *csn* deletion strains with or without recombinant protein at ~100 kDa. α-Tub antibody was used as loading control. B. Detection with α-CulA underlined the result from (A) where the ratio of neddylated CulA (*) to deneddylated CulA (**) in Δ*csnE* + recombinant CsnE was similar to the wild type and inverted in comparison to all other samples. Results were quantified using BIO1D software (Peqlab).

Notes

Users of this protocol should have experience in or collaborative groups for protein purification using the ÄKTA system.

For reliable data, at least three biological repetitions should be performed. This is also important for calculating error bars. Mean values and standard deviation were calculated using Excel with the formula below:

$$\sigma = \sqrt{\frac{\Sigma_{i=1}^{n} (x_i - \bar{x})^2}{(n-1)}}$$

With σ as standard deviation, x_i representing each value of the dataset, $\bar{x}$ arithmetic mean value, n total number of data points, Σ the sum of $(x_i - \bar{x})^2$ of all data points.

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Recipes

1. Lysogeny broth (LB) medium
   1% (w/v) Peptone/Tryptone
   0.5% (w/v) yeast extract
   1% (w/v) NaCl
   Autoclave and supplement with antiobiotics (1:1,000) before usage.
2. Lysis buffer
   500 mM NaCl
   50 mM Tris (pH 8.0)
   Add just before use: 1 mM PMSF
3. Affinity buffer
   500 mM NaCl
   50 mM Tris (pH 8.0)
   5% (w/v) Imidazole in case of HisTrap columns
4. Elution buffer for HisTrap
   500 NaCl
   50 mM Tris (pH 8.0)
   500 mM Imidazole
5. Elution buffer for GSTrap
   500 NaCl
   50 mM Tris (pH 8.0)
   20 mM Glutathione
6. Size exclusion buffer (SEC)
   120 mM NaCl
   20 mM Tris pH 8.0
   1 mM DTT
   1 mM EDTA
   5% (v/v) Glycerol
7. Minimal medium
   1% (w/v) Glucose
   1x AspA
   2 mM $MgSO_4$
   1x trace elements
8. 50x AspA
   3.5 M $NaNO_3$
   350 mM KCl
   560 mM KH_2PO_4
   Adjust to pH 5.5 with KOH (~30 mL 5 M KOH needed!), autoclave
9. Trace elements

18 µM $FeSO_4$

174 µM EDTA

76 µM $ZnSO_4$

178 µM H_3BO_3

25 µM $MnCl_2$

7.1 µM $CoCl_2$

6.4 µM $CuSO_4$

6.2 µM Na_2MoO_4

Sterile filter

10. B* buffer

    300 mM NaCl

    10 mM Tris-HCl (pH 7.5)

    0.5 mM EDTA

    0.2% (v/v) NP-40

    10% (v/v) Glycerol

    Add just before use:

    1 mM PMSF

    2 mM DTT

    5 mM E64

    100 µl/10 ml Complete Protease Inhibitor Cocktail

11. 3x SDS sample buffer

    250 mM Tris-HCl (pH 6.8)

    15% 2-mercaptoethanol

    30% Glycerol

    7% SDS

    0.3% Bromophenol blue

12. 12% SDS gels

    for 2 gels mix the following solutions in a 50 ml reaction tube:

    Resolving gel:

    5 ml 2x resolving buffer

    4 ml 30% acrylamide

    1 ml H_2O

    150 µl 10% APS

    15 µl TEMED

    a. Invert the solution and then fill between the glas plates.
    b. Add ~1 ml 100% isopropanol on top of the gel to prevent air bubbles.
    c. Let the gels dry ~10-20 min.
    d. Then remove the isopropanol carefully.
    e. Use whatman paper to remove remaining drops.
    f. Then mix all components for the stacking gel in a 50 ml reaction tube.

Stacking gel:

2 ml 2x stacking buffer

0.5 ml 30% acrylamide

1.5 ml H_2O

60 µl 10% APS

7.5 µl TEMED

a. Invert the tube and then fill the stacking solution onto the resolving gel up to the top.
b. Then place the comps carefully into the stacking gel.
c. Let the gels dry for another 10-20 min.

13. 2x resolving buffer

    0.75 M Tris-HCl (pH 8.8)

    0.1% (w/v) SDS

14. 2x stacking buffer

    0.25 M Tris-HCl (pH 6.8)

    0.1% (w/v) SDS

15. 10x SDS running buffer

    250 mM Tris-base

    2.5 M Glycine

    1% (w/v) SDS

    Dilute the buffer to 1x working solution with ddH_2O

16. 10 x transfer buffer

    250 mM Tris-base

    1.92 M glycine

    0.2% (w/v) SDS

    Dilute the buffer to 1x working solution with ddH_2O

17. 10x TBST (Tris-Buffered Saline Tween 20)

    100 mM Tris-HCl (pH 8.0)

    1.5 M NaCl

    0.5% (v/v) Tween-20

    Dilute the buffer to 1 x working solution with ddH_2O

18. Blocking solution

    5 % milk powder dissolved in 1x TBST buffer

19. Detection solution A (prepare fresh just before use)

    9 ml ddH_2O

    1 ml Tris-HCl (pH 8.5)

    100 µl 250 mM Luminol (dissolve stock solution in DMSO, protect from light, store at -20 °C)

    44 µl 400 mM p-coumaric acid (dissolve stock solution in DMSO, protect from light, store at -20 °C)

20. Detection solution B (prepare fresh just before use)
    9 ml ddH_2O
    1 ml 1 M Tris-HCl (pH 8.5)
    6.15 µl 30% H_2O_2
21. Complete Protease Inhibitor Cocktail
    Dissolve 1 ½ tablets in 1 ml B*buffer

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