

# Determining the Effects of Neddylation on Cullin-RING Ligase-Dependent Protein Ubiquitination

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As the largest family of ubiquitin (Ub) E3 ligases, cullin-RING ligases (CRLs) play crucial roles in various cellular processes, and their activities are tightly regulated by orchestrated mechanisms. Neddylation, the conjugation of a Ub-like protein NEDD8 to a target protein such as the cullin, represents a key regulatory mechanism for CRLs. Biochemical and structural studies of a few CRLs have revealed that cullin neddylation alters the CRL conformation and activates CRL-dependent protein ubiquitination. Here, using CUL2-RING ligase (CRL2) as an example, we describe our protocols for the preparation of recombinant CUL2 with or without NEDD8 conjugation, which is further used to quantitatively determine the effect of neddylation on CRL2-dependent protein ubiquitination *in vitro*. © 2022 The Authors. Current Protocols published by Wiley Periodicals LLC.

**Basic Protocol 1:** Expression and purification of CUL2•RBX1 from *Escherichia coli*

**Support Protocol:** Further purification of CUL2•RBX1 with additional chromatography on an FPLC system

**Basic Protocol 2:** Reconstitution of cullin neddylation for quantitative ubiquitination assay *in vitro*

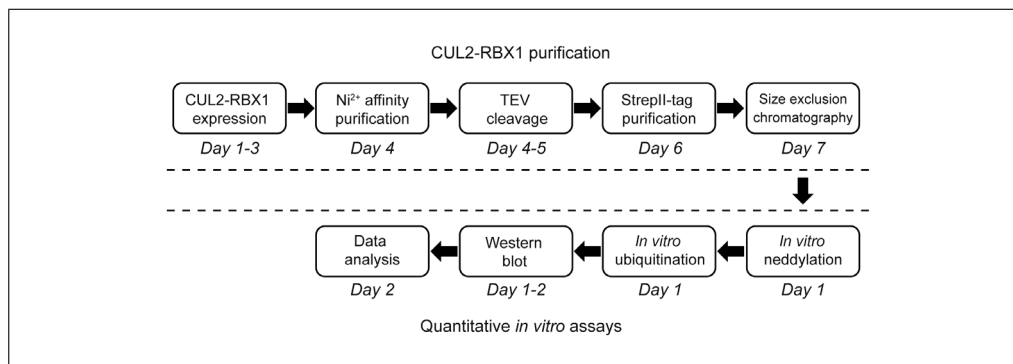
Keywords: cullin-RING ligases • *in vitro* assay • neddylation • protein purification • ubiquitination

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## INTRODUCTION

NEDD8 is a small protein that possesses high sequential and structural similarity to ubiquitin (Ub). As with protein ubiquitination, the conjugation of NEDD8 to substrates, referred to as neddylation, requires an enzyme cascade comprising NEDD8 E1 activating enzyme (NAE), NEDD8 E2 conjugating enzyme, and E3 NEDD8 ligase (Lydeard, Schulman, & Harper, 2013). Cullin (CUL) proteins are the best-known substrates for neddylation, serving as the catalytic core for cullin-RING ligases (CRLs) and mediating the ubiquitination of diverse proteins involved in various cellular pathways. A CRL becomes enzymatically active when an E2-conjugated Ub (E2~Ub) is engaged by the RING



**Figure 1** Schematic diagram summarizing the procedures included in this article for studying the effects of neddylation on CRL-dependent protein ubiquitination. For the preparation of recombinant CUL2•RBX1, CUL2 and RBX1 are co-expressed in *E. coli* and purified through Ni<sup>2+</sup> affinity chromatography, TEV cleavage for tag removal, StrepII-tag affinity chromatography, and size exclusion chromatography. To examine the effect of neddylation on CRL activity, CUL2•RBX1 with or without *in vitro* neddylation is prepared and used for substrate ubiquitination *in vitro*. The ubiquitination of substrates is monitored over time by western blot (or fluorescent scan), and the ubiquitination rate is quantitatively analyzed.

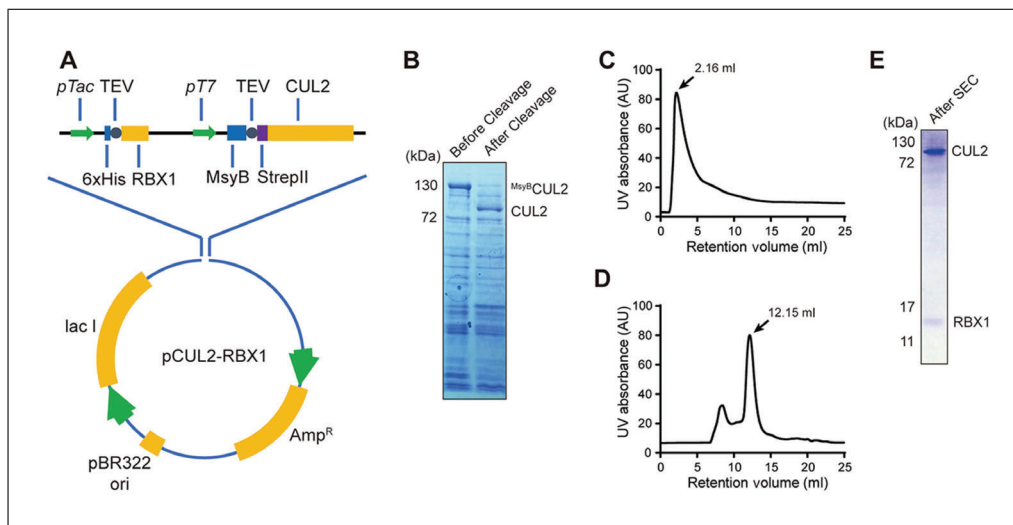
protein RBX1 (or RBX2) bound to the C-terminus of cullin and when the protein substrate is engaged by one of the interchangeable substrate receptor modules that specifically binds to the N-terminus of cullin. Biochemical studies showed that cullin neddylation promotes the activity of the CUL1-RING ligase (CRL1) (Morimoto, Nishida, Honda, & Yasuda, 2000), and two distinct effects of neddylation on CRL1 activity were revealed (Saha & Deshaies, 2008). First, neddylation enhances Ub chain initiation by bringing the substrate and E2~Ub into proximity. Second, neddylation promotes Ub chain elongation through increasing E2 recruitment. More recently, a structural study of CRL1 in complex with E2~Ub further illustrated that the conjugation of NEDD8 to CUL1 induces protein-protein interactions within the CRL1•E2~Ub complex, including the contacts between NEDD8 and E2 and between E2 and the substrate receptor. As a result, the CRL1 complex changes from an open conformation to a “locked” conformation and thereby accelerates the transfer of Ub from E2 to the substrate docked on the substrate receptor (Baek et al., 2020).

Although neddylation has been considered as a general mechanism for CRL activation, to what extent neddylation promotes the ubiquitination of a specific protein by a specific CRL remains largely unexplored. Given that diverse substrate receptor modules and substrate proteins are engaged by different CRLs, a robust quantitative assay is required to help investigate and evaluate the effects of neddylation on the activity of an individual CRL toward its substrate(s).

Here, we detail our protocols for the preparation of recombinant CUL2•RBX1 and neddylated CUL2•RBX1 (Basic Protocol 1 and Support Protocol) and the subsequent quantitative assay for CRL2-dependent protein ubiquitination (Basic Protocol 2) (Diaz, Li, Wang, & Liu, 2021; Wang, Reichermeier, & Liu, 2021). The protocols enable studies on the ubiquitination of a specific protein substrate with or without CUL2 neddylation, and the rate of substrate modification under each condition can be quantified for comparison. The workflow for these protocols is summarized in Figure 1, and it can be adapted to fit analyses using a different CRL and/or substrate of interest.

### EXPRESSION AND PURIFICATION OF CUL2•RBX1 from *Escherichia coli*

The enzymatic core of a CRL is a binary protein complex containing a cullin core and a RING domain protein (RBX1 or RBX2). To co-express the recombinant CUL•RBX protein complex in *E. coli*, the coding sequences of the two protein components can be



**Figure 2** Expression and purification of CUL2•RBX1. **(A)** Schematic of the CUL2•RBX1 expression plasmid. **(B)** Representative image of the Coomassie blue–stained gel showing the CUL2 protein band before and after the TEV cleavage. **(C)** Chromatogram showing the elution profile of CUL2•RBX1 purified from the 1-ml Strep-Tactin cartridge. **(D)** Chromatogram showing the elution profile of CUL2•RBX1 purified from the Superdex 200 Increase 10/300 GL column. **(E)** Representative image of the Coomassie blue–stained gel showing the purified CUL2•RBX1 product.

incorporated either into one plasmid or separately into two plasmids. Using CUL2 as an example, this protocol describes the purification of recombinant CUL2•RBX1 complex using tandem affinity chromatography. The expression plasmid was derived from a pGEX-4T-2 vector (Cytiva, cat. no. 28-9545-50), with the  $6\times\text{His}$ -RBX1 coding sequence inserted after the Tac promoter, a T7 promoter sequence inserted after  $6\times\text{His}$ -RBX1, and the  $6\times\text{His}$ -MsyB-<sup>StrepII</sup>CUL2 coding sequence inserted after the T7 promoter. The hyper-acidic bacterial protein MsyB was fused to the N-terminus of CUL2 to improve protein solubility. A TEV protease cleavage site was inserted between the  $6\times\text{His}$  tag and RBX1 and between MsyB and <sup>StrepII</sup>CUL2. Figure 2A summarizes the structure of the CUL2•RBX1 expression plasmid.

### Materials

BL21 (DE3) chemically competent *E. coli* cells (Thermo Fisher Scientific, cat. no. C600003)

CUL2•RBX1 expression plasmid (see Fig. 2A)

LB liquid medium (sterile; see recipe; make fresh), room temperature or 37°C

LB agar plates containing 100 µg/ml ampicillin (sterile; see recipe), 37°C

100 mg/ml ampicillin stock solution (sterile; see recipe)

0.8 M IPTG stock solution (see recipe)

Phosphate-buffered saline (PBS; Sigma-Aldrich, cat. no. P4417)

Ni-NTA lysis buffer (see recipe; make fresh), 4°C

Ni-NTA agarose beads (His-Tag Purification Resin, Roche, cat. no. COHISR-RO)

Ni-NTA wash buffer (see recipe)

Ni-NTA elution buffer (see recipe)

Bradford protein assay kit (Bio-Rad, cat. no. 5000202)

4× SDS sample buffer (see recipe)

Tobacco etch virus (TEV) protease (purified from Addgene plasmid #92414)

42°C water bath

37°C microbiological incubator

Microcentrifuge (Eppendorf 5425 or equivalent)  
250-ml Erlenmeyer flask  
2.8-L Fernbach culture flasks (Thermo Fisher Scientific, cat. no. 41052800)  
Cell density meter (Biowave CO8000 or equivalent)  
1000-ml centrifuge bottles (Thermo Fisher Scientific, cat. no. 3120-1000)  
Refrigerated floor centrifuge (6-L capacity; Beckman Coulter J6-MI or equivalent)  
15- and 50-ml conical centrifuge tubes (Falcon, cat. no. 352097, or equivalent, and Corning, cat. no. 352070, or equivalent)  
Plastic beaker  
Sonicator (Fisherbrand Model 505 or equivalent)  
High-speed centrifuge tubes (Thermo Fisher Scientific, cat. no. 3139-0050)  
Refrigerated high-speed centrifuge (Beckman Avanti J30I or equivalent), 4°C  
Refrigerated benchtop centrifuge, 4°C (Eppendorf 5430 R or equivalent)  
Refrigerated incubator shaker (New Brunswick I26R or equivalent), 4°C  
Gravity flow columns (Bio-Rad, cat. no. 7321010)  
Clamp stand  
Two-way stopcock (Bio-Rad, cat. no. 7328102)

Additional reagents and equipment for SDS-PAGE (see Current Protocols article; Gallagher, 2012)

***Transform *E. coli* cells with the CUL2•RBX1 expression construct***

1. Thaw a tube of BL21 (DE3) chemically competent *E. coli* cells on ice.  
*Usually, 50 µl of competent cells is enough for one transformation.*
2. Add 0.1 to 10 ng CUL2•RBX1 expression plasmid to competent cells and mix by gently flicking tube several times.  
*The volume of plasmid should not exceed 10% of the total mixture volume.*
3. Incubate mixture on ice for 30 min.
4. Heat shock at 42°C in a water bath for 45 s.
5. Leave tube on ice for 5 min.
6. Add 900 µl LB liquid medium into mixture.  
*The LB medium can be at room temperature or prewarmed to 37°C.*
7. Place tube containing the cell mixture at 37°C in a microbiological incubator with vigorous shaking (200 to 250 rpm) for 60 min.
8. Centrifuge 2 min at 300 × *g* in a microcentrifuge to pellet cells.
9. Carefully discard supernatant and leave ~100 µl supernatant in tube.
10. Resuspend cell pellet and spread suspended cells onto an LB agar plate containing 100 µg/ml ampicillin (or other appropriate antibiotic).  
*Pre-warm the plate at 37°C before plating.*
11. Incubate plate at 37°C overnight (typically ~16 hr).
12. Check plate to make sure that a good amount of *E. coli* colonies show up on the plate.  
*The plate can be used immediately or stored for up to 2 weeks at 4°C before proceeding to the next step.*

### ***Express the recombinant protein in E. coli***

13. In a 250-ml Erlenmeyer flask, add 50 ml LB liquid medium and 50  $\mu$ l of 100 mg/ml ampicillin stock solution. Inoculate a few colonies from LB agar plate into the LB medium and grow culture overnight at 37°C with vigorous shaking (200 to 250 rpm).

*This will yield a starter culture with high optical density.*

14. On the next day, add 1 ml of 100 mg/ml ampicillin stock solution into 1 L LB liquid medium in each of six 2.8-L Fernbach culture flasks and mix well by gently swirling for a few seconds.

*In this protocol, six of these culture flasks with medium are prepared, but it can be scaled up or down.*

*If the medium is sterilized by autoclaving, then the flasks should be prepared ahead of time to allow the sterilized medium to cool down and reach room temperature.*

15. Add overnight starter culture from step 13 into each 2.8-L culture flask to dilute the culture 200 times (5 ml culture per 1 L medium). Grow at 37°C with vigorous shaking (200 to 250 rpm).

16. Check optical density at 600 nm ( $OD_{600}$ ) of the culture periodically using a cell density meter to ensure that the  $OD_{600}$  does not go over 1.0.

*Measure the  $OD_{600}$  for the first time 2 hr after starting the culture. When the reading is above 0.7, check the  $OD_{600}$  every 20 to 30 min.*

17. When the  $OD_{600}$  is close to 1.0, stop shaker and cool down culture by transferring the flasks to a cold room. Then, set shaker to 16°C and allow it to cool.

18. After the culture and the shaker are cooled down, add 0.8 M IPTG stock solution to each flask to 0.2 mM to induce expression of the recombinant protein.

*The final concentration of IPTG can be modified for optimal protein yield (usually 0.1 to 1 mM).*

19. Incubate culture overnight at 16°C with vigorous shaking (200 to 250 rpm).

*The culture temperature can be adjusted, but lower temperature usually helps with protein folding and solubility.*

20. On the next day, transfer culture into 1000-ml centrifuge bottles and harvest *E. coli* cells by centrifugation for 30 min at 3000  $\times$  g using a refrigerated floor centrifuge. Discard supernatant and resuspend cells in PBS.

*The volume of PBS added should be enough to resuspend the cells and to fit the cell suspension into a 50-ml conical centrifuge tube (see the next step). Usually, 25 to 30 ml PBS is used for cells from 3 L culture and subsequently transferred to one conical tube.*

21. Transfer resuspended cells into 50-ml conical centrifuge tubes. Centrifuge 10 min at 3000  $\times$  g and discard supernatant.

*Two tubes are used for 6 L culture.*

*The pellets can be either used immediately for the next step or frozen and stored at -80°C before proceeding to the next step.*

### ***Extract CUL2•RBX1 from E. coli cells using Ni<sup>2+</sup> affinity chromatography***

22. Add cold Ni-NTA lysis buffer to tubes containing bacteria cell pellets.

*Add 25 ml buffer per pellet collected from 3 L culture.*

23. Sonicate pellets briefly to free the pellets from the bottom of the tubes.

24. Transfer pellets and lysis buffer from the two tubes into the same plastic beaker incubated in an ice-water bath.
25. Sonicate pellets in the beaker to lyse the cells thoroughly.

*The sonication conditions should be configured according to the manual for the specific sonicator. Suggested parameters are 0.5" flat tip with 50% amplitude and 1-s on time and 1-s off time for a total duration of 3 min. Run the program three times with a 5-min break between each run.*

*Sonication generates heat, so it is necessary to keep the plastic beaker in the ice-water bath with plenty of ice during this entire process.*

CAUTION: Use safety earmuffs when the sonicator is on for hearing protection.
26. Transfer equal volumes of cell lysate into two high-speed centrifuge tubes. Centrifuge 30 min at  $25,000 \times g$ ,  $4^{\circ}\text{C}$ , in a refrigerated high-speed centrifuge.

*The two tubes containing cell lysate must be carefully balanced before centrifugation. A balance can be used to determine the weight of each tube, and Ni-NTA lysis buffer can be added to the tube with lower weight to match its weight to that of the other tube.*
27. During the centrifugation period (step 26), prepare Ni-NTA agarose beads for protein extraction by first loading Ni-NTA agarose bead slurry into two 15-ml conical centrifuge tubes.

*Use 0.75 ml beads per liter of culture.*
28. Centrifuge 2 min at  $500 \times g$ ,  $4^{\circ}\text{C}$ , in a refrigerated benchtop centrifuge.
29. Discard supernatant and resuspend beads in two bed volumes of cold Ni-NTA lysis buffer.
30. Repeat steps 27 to 29 two more times. Then, transfer equal volumes of agarose bead slurry into two 50-ml conical centrifuge tubes.
31. After the centrifugation in step 26 is done, transfer supernatant into the tubes containing the Ni-NTA beads (prepared in step 30).
32. Incubate tubes at  $4^{\circ}\text{C}$  for  $\geq 2$  hr with mild agitation on a refrigerated incubator shaker.
33. Centrifuge as in step 28 and discard supernatant.
34. Resuspend beads with two bed volumes of Ni-NTA wash buffer.
35. Repeat steps 33 to 34 two more times to remove excess cell lysate from beads.
36. Set up an empty gravity flow column on a clamp stand and wash column with 10 ml Ni-NTA wash buffer.
37. Transfer all the bead slurry (from step 35) into the column. Use a two-way stopcock on bottom of the column to control the flow.
38. Wait for beads to settle and then let buffer flow out of the column.
39. Close stopcock and resuspend beads in two bed volumes of Ni-NTA elution buffer. Incubate for 10 min.
40. Open stopcock and collect eluate in a 50-ml conical centrifuge tube incubated on ice.
41. Repeat steps 39 and 40 three to four more times. Use a Bradford protein assay kit to determine if a fraction of the eluate contains a detectable amount of protein that is worth collecting.



42. Combine eluate from all collected fractions and estimate total amount (mg) of protein via the Bradford assay.
43. Mix 30  $\mu$ l eluate with 10  $\mu$ l of 4 $\times$  SDS sample buffer. Store up to 6 months at  $-20^{\circ}\text{C}$ .
44. Add TEV protease to eluate to remove the MsyB protein and the 6 $\times$ His tag. Mix by gently inverting tube several times and incubate at  $4^{\circ}\text{C}$  overnight.

*For 1 mg total protein, 20  $\mu$ g TEV is added.*

45. On the next day, mix 9  $\mu$ l eluate with 3  $\mu$ l of 4 $\times$  SDS sample buffer. Analyze this sample together with the sample from step 43 using SDS-PAGE (see Current Protocols article; Gallagher, 2012) to assess the progress of the TEV cleavage. If the cleavage is not complete, add more TEV protease and incubate longer.

*Removal of the MsyB fusion protein results in a loss of  $\sim 20$  kDa in protein size.*

## **FURTHER PURIFICATION OF CUL2•RBX1 WITH ADDITIONAL CHROMATOGRAPHY ON AN FPLC SYSTEM**

## **SUPPORT PROTOCOL**

In this protocol, the recombinant CUL2•RBX1 extracted by the Ni-NTA beads in Basic Protocol 1 is further purified by StrepII-tag affinity chromatography followed by size exclusion chromatography (SEC) on an FPLC system.

### **Materials**

Deionized water

Buffer W (see recipe)

Biotin elution buffer (see recipe; make fresh)

CUL2•RBX1 solution (see Basic Protocol 1)

SEC buffer (see recipe; make fresh)

FPLC system (ÄKTA pure 25 or equivalent)

Strep-Tactin XT 4Flow cartridge, 1 ml (IBA Lifesciences, cat. no. 2-5024-001)

Centrifugal filter units (50-kDa cutoff; Millipore, cat. no. UFC905024 or UFC805024)

Microcentrifuge (Eppendorf 5425 or equivalent),  $4^{\circ}\text{C}$

0.22- $\mu$ m syringe filters (VWR, cat. no. 28145-477)

1- and 20-ml syringes (BD, cat. no. 301025 and 301031)

Fraction collector

SEC column (Superdex 200 Increase 10/300 GL column, Cytiva, cat. no. 28990944)

0.22- $\mu$ m centrifugal filters (Millipore, cat. no. UFC30GVNB)

Syringe needles

UV spectrophotometer

Additional reagents and equipment for SDS-PAGE (see Current Protocols article; Gallagher, 2012)

**NOTE:** All buffers and water should be filtered and degassed using bottle-top filters (Thermo Fisher Scientific, cat. no. 5963320) before use on the FPLC system.

### **StrepII-tag affinity chromatography**

1. Set up FPLC system:
  - a. Wash Lines A and B with deionized water.
  - b. Insert Line A in Buffer W and Line B in biotin elution buffer.
  - c. Install 10-ml sample loop on the injection valve.

2. Prepare FPLC system:
  - a. Set pre-column pressure alarm to 0.5 MPa.
  - b. Prime Line B with  $\geq 15$  ml biotin elution buffer.
  - c. Prime Line A with  $\geq 15$  ml Buffer W.
  - d. Wash sample loop with  $\geq 20$  ml Buffer W.
  - e. Attach Strep-Tactin XT 4Flow cartridge to the FPLC system. Pay close attention to joints and make sure that no leak occurs.
  - f. Wash cartridge with 10 ml Buffer W at 1 ml/min.
3. Concentrate CUL2•RBX1 solution to  $< 10$  ml using a centrifugal filter unit (50-kDa cutoff) by centrifuging at  $5000 \times g$ ,  $4^{\circ}\text{C}$ , and checking the sample every 5 min.
4. Pass CUL2•RBX1 solution through a  $0.22\text{-}\mu\text{m}$  syringe filter attached to a 20-ml syringe to remove precipitated protein or other small particles that can clog the column.
5. Inject filtered protein sample into the sample loop.
6. Load protein sample into the Strep-Tactin cartridge at 0.5 ml/min.
 

*The UV trace will increase during this step.*
7. Wash column with Buffer W until the UV trace reaches the basal level.
8. Elute protein using 100% biotin elution buffer at 1 ml/min for  $\leq 30$  ml. Collect eluate as 1-ml fractions in the fraction collector.
 

*The UV trace will increase shortly ( $\sim 2$  ml) after the start of the elution and gradually decrease as the elution proceeds. These fractions contain mostly the target protein.*
9. Analyze fractions under the UV peak using SDS-PAGE (see Current Protocols article; Gallagher, 2012). Combine fractions containing CUL2•RBX1.

#### ***Size exclusion chromatography***

10. Set up FPLC system:
  - a. Wash Lines A and B with deionized water.
  - b. Insert Line A in SEC buffer.
  - c. Install 1-ml sample loop on the injection valve.
11. Prepare FPLC system:
  - a. Set pre-column pressure alarm to 2.8 MPa.
  - b. In “inject” mode, prime Line A with  $\geq 20$  ml SEC buffer.
 

*This will also wash the sample loop.*
12. Adjust flow rate to 0.4 ml/min and connect SEC column. Make sure that no leak occurs.
13. In “inject” mode, equilibrate column with 40 ml Buffer W.
 

*If the SEC column is filled with 20% ethanol, then the column should be washed with 40 ml water before the equilibration with Buffer W.*
14. Concentrate protein sample from step 9 to  $\sim 500$   $\mu\text{l}$  using a centrifugal filter unit (50-kDa cutoff) by centrifuging at  $5000 \times g$ ,  $4^{\circ}\text{C}$ , and checking the sample every 5 min. Filter protein sample through a  $0.22\text{-}\mu\text{m}$  centrifugal filter.
 

*This step should be done immediately before the next step.*

*If the protein solution looks cloudy, then centrifuge the sample in a 1.5-ml microcentrifuge tube to pellet the precipitate first and then filter the supernatant.*



15. Load filtered protein sample into the sample loop using a 1-ml syringe with a needle. To avoid introducing air into the sample loop, insert syringe needle into the injection port in “inject” mode and then switch to “manual load” mode and load protein solution into the sample loop.
16. Run elution program at 0.4 ml/min for 30 ml. Collect eluate as 300- $\mu$ l fractions in the fraction collector.
17. Analyze fractions under the UV peak(s) as in step 9. Pay close attention to fractions with expected retention volume (12 to 14 ml for CUL2•RBX1). Combine fractions containing the target protein and low levels of contaminants.
18. Using a UV spectrophotometer, determine concentration of the collected protein through obtaining the UV absorbance (at 280 nm) of the protein solution and the molar extinction coefficient ( $\epsilon$ ) for the target protein (see Current Protocols article; Olson, 2016).
 

*Alternatively, the protein concentration can be measured by the Bradford assay.*
19. If needed, concentrate protein stock using a centrifugal filter unit to the desired concentration.
20. Make aliquots and store up to 1 year at  $-80^{\circ}\text{C}$ .

## **RECONSTITUTION OF CULLIN NEDDYLYATION FOR QUANTITATIVE UBIQUITINATION ASSAY *IN VITRO***

**BASIC  
PROTOCOL 2**

Using CUL2•RBX1 as an example, this protocol describes the conjugation of NEDD8 to CUL2 *in vitro*. The CUL2•RBX1 protein is further incorporated into quantitative ubiquitination assays to evaluate the effect of neddylation on the activity of CUL2•RBX1.

### ***Materials***

Distilled water  
 4 $\times$  SDS sample buffer (see recipe)  
 Anti-FLAG antibody (Sigma-Aldrich, cat. no. F1804; 1:1000 dilution)  
 Anti-VHL antibody (Cell Signaling Technology, cat. no. 68547S; 1:1000 dilution)  
 Anti-CUL2 antibody (Thermo Fisher, cat. no. 51–1800; 1:1000 dilution)

1.5-ml microcentrifuge tubes  
 100 $^{\circ}\text{C}$  heat block  
 ImageJ or equivalent software

Additional reagents and equipment for assembling neddylation reaction (see Table 1), “substrate + substrate receptor mixture” (see step 3 annotation), and “Ub mixture” (see Table 2), for SDS-PAGE (see Current Protocols article; Gallagher, 2012), and for western blot (see Current Protocols article; Gallagher, Winston, Fuller, & Hurrell, 2011)

### ***Reconstitute CUL2 neddylation in vitro***

1. neddylation reaction by mixing all reagents at the concentrations listed in Table 1. Add distilled water to adjust to desired final volume before adding the protein stocks.

*Determine the neddylation reaction volume based on the number of time points desired. Usually  $\geq 5 \mu\text{l}$  is required for one time point.*

*For reactions with unneddylated CUL2 and mock control, NEDD8 or CUL2•RBX1 can be respectively omitted.*

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**Table 1** Composition of the Neddylation Reaction Mixture

Component	Concentration
Reaction buffer (see recipe for 10×)	1×
DTT (see recipe for 1 M)	2 mM
ATP (see recipe for 200 mM)	2 mM
NEDD8 (R&D Systems, cat. no. UL-812-500)	2 μM
NAE (R&D Systems, cat. no. E-313-025)	0.25 μM
UBC12 (R&D Systems, cat. no. E2-656-100)	3 μM
CUL2•RBX1 (see Basic Protocol 1 and Support Protocol)	0.2 μM

**Table 2** Composition of the “Ub Mixture”

Component	Concentration
Reaction buffer (see recipe for 10×)	2×
DTT (see recipe for 1 M)	4 mM
ATP (see recipe for 200 mM)	4 mM
Ub (R&D Systems, cat. no. U-100H-10M)	120 μM
UBE1 (R&D Systems, cat. no. E-305-025)	2 μM
CDC34 (R&D Systems, cat. no. E2-610-100)	0.8 μM

- Incubate reaction mixture at room temperature for 1 hr.

*This reaction condition should drive almost all CUL2 to neddylated CUL2 (CUL2<sup>N8</sup>). It can be scaled up to generate a large amount of CUL2<sup>N8</sup>•RBX1, which can be further purified using StrepII-tag affinity chromatography (see Support Protocol).*

#### **Evaluate the effect of cullin neddylation on substrate ubiquitination in vitro**

- Prepare “substrate + substrate receptor mixture” at concentrations four times the final concentration in the ubiquitination reaction (step 5).

*In this protocol, 0.1 μM substrate and 0.1 μM VHL•EloB•EloC (substrate receptor module) are used for the reaction, so 0.4 μM of each component is included in the mixture. The ratio of substrate to substrate receptor can be changed when a different experimental condition is desired.*

*Either recombinant protein or chemically synthesized degron peptide can be used as the substrate. Substrate protein can be purified from appropriate human cell lines following a previously published Current Protocols article (Adelmant, Garg, Tavares, Card, & Marto, 2019).*

*The final volume of this solution should be at least half that of the neddylation reaction mixture.*

- Prepare “Ub mixture” by mixing all reagents at the concentrations listed in Table 2. Add distilled water to adjust to desired final volume before adding the protein stocks.

*The volume of the “Ub mixture” should be the same as that of the “substrate + substrate receptor” mixture (step 3).*

*This mixture should be prepared immediately before the next step.*

- Initiate ubiquitination reaction by mixing the solutions as described in Table 3 in a 1.5-ml microcentrifuge tube and incubating at room temperature.

*The final concentrations of all components in the ubiquitination reaction are shown in Table 4.*

*A different reaction temperature can be used to adjust the rate of substrate ubiquitination.*

**Table 3** Composition of the Ubiquitination Reaction Mixture

Volume	Component
60 $\mu$ l	CUL2 <sup>N8</sup> •RBX1 or CUL2•RBX1 or mock control (step 2)
30 $\mu$ l	Substrate + substrate receptor mixture (step 3)
30 $\mu$ l	Ub mixture (step 4)

**Table 4** Final Concentrations of All Components in the Ubiquitination Reaction

Amount	Component
1 $\times$	Reaction buffer
2 mM	DTT
2 mM	ATP
0.5 $\mu$ M	Ub E1
0.2 $\mu$ M	Ub E2 (Cdc34)
30 $\mu$ M	Ub
0.1 $\mu$ M	CUL2 <sup>N8</sup> •RBX1 (or CUL2•RBX1 or mock control)
0.1 $\mu$ M	VHL•EloB•EloC
0.1 $\mu$ M	Substrate

6. At each desired time point after the start of the reaction, withdraw 18  $\mu$ l reaction mixture and mix with 6  $\mu$ l of 4 $\times$  SDS sample buffer to stop reaction. When samples from all time points have been collected, incubate them at 100°C for 5 min in a heat block.

*For the first experiment, we recommend trying a wide range of time points, such as 1, 5, 15, 30, 60, and 120 min. Based on the results from the first trial, time points should be adjusted in follow-up experiments, with more frequent time points in the fast-changing phase and less frequent ones in the slow-changing phase.*

7. Run all samples on an SDS-PAGE gel. Then, detect unmodified and ubiquitinated substrates by western blot (see Current Protocols article; Gallagher, Winston, Fuller, & Hurrell, 2011) with anti-FLAG antibody, anti-VHL antibody, and anti-CUL2 antibody.

*Gradient SDS-PAGE gels (precast 4% to 20% Tris-glycine gradient gels, Thermo Fisher Scientific, cat. no. XP04205BOX) are recommended for better separation of CUL2<sup>N8</sup> vs. CUL2 and unmodified vs. ubiquitinated substrates.*

*If the substrate contains an epitope tag (e.g., FLAG tag), then an antibody for the epitope tag is recommended for western blot. When antibodies against the substrate itself need to be used, then make sure that the region of the substrate protein recognized by the antibody is not ubiquitinated. As a loading control, VHL (or the applicable substrate receptor protein) on the same gel as in step 7 should be analyzed by western blot. In addition, CUL2 should be analyzed to assess the level of cullin neddylation.*

*If the substrate is fluorescently labeled, then image the SDS-PAGE gel on a fluorescent scanner (instead of western blot).*

8. Quantify intensity of the unmodified substrate bands from all time points using ImageJ or equivalent software (Davarinejad, 2015). Also quantify intensity of the VHL (loading control) bands for normalization purposes.

9. Fit normalized intensity of the unmodified substrate bands with a one-phase exponential decay model.

*A detailed guide on curve fitting has been provided previously (Motulsky & Christopoulos, 2004).*

## **REAGENTS AND SOLUTIONS**

### ***Ampicillin stock solution, 100 mg/ml***

Dissolve 10 g ampicillin (Fisher Scientific, cat. no. BP1760-25) in 100 ml ultrapure H<sub>2</sub>O  
Sterilize by filtering through 0.22- $\mu$ m syringe filter (VWR, cat. no. 28145-477)  
Aliquot and store  $\leq$ 6 months at  $-20^{\circ}\text{C}$

### ***ATP stock solution, 200 mM***

Dissolve 1.1 g adenosine 5'-triphosphate disodium salt hydrate (Chem-Impex International, cat. no. 00015) in 7 ml ultrapure H<sub>2</sub>O  
Adjust pH to 7.0 by adding 1 M KOH  
Ultrapure H<sub>2</sub>O to 10 ml  
Aliquot and store  $\leq$ 3 months at  $-80^{\circ}\text{C}$

### ***Biotin elution buffer***

100 mM Tris-HCl, pH 8.0  
300 mM NaCl  
5% (v/v) glycerol  
50 mM biotin (Enzo, cat. no. ALX-460-002)  
Adjust pH to 8.0 by adding 6 N HCl  
Prepare fresh immediately before use and store at  $4^{\circ}\text{C}$   
Filter and degas by vacuum before use

### ***Buffer W***

100 mM Tris-HCl, pH 8.0  
150 mM NaCl  
Store  $\leq$ 1 month at  $4^{\circ}\text{C}$   
Filter and degas by vacuum before use

### ***DTT stock solution, 1 M***

Dissolve 1.54 g dithiothreitol (DTT; Fisher Scientific, cat. no. BP172-25) in 10 ml ultrapure H<sub>2</sub>O  
Aliquot and store  $\leq$ 3 months at  $-20^{\circ}\text{C}$

### ***IPTG stock solution, 0.8 M***

Dissolve 19 g isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG; Sigma-Aldrich, cat. no. I6758) in 100 ml ultrapure H<sub>2</sub>O  
Aliquot and store  $\leq$ 1 year at  $-20^{\circ}\text{C}$

### ***LB agar plates containing 100 $\mu\text{g/ml}$ ampicillin***

Add 15 g agar (BD, cat. no. 214010) to 1 L LB liquid medium (see recipe)  
Sterilize by autoclaving  
Cool to  $\sim 50^{\circ}\text{C}$   
Add 1 ml of 100 mg/ml ampicillin stock solution (see recipe) and mix well  
Pour into sterile 10-cm Petri dishes (Fisher Scientific, cat. no. FB0875712)  
Wait until agar is solidified and cool  
Store  $\leq$ 1 month at  $4^{\circ}\text{C}$

### ***LB liquid medium***

Add one LB broth pre-buffered capsule (Fisher Scientific, cat. no. BP97315)  
per 1 L ultrapure H<sub>2</sub>O  
Sterilize by autoclaving  
Prepare fresh immediately before use

### ***Ni-NTA lysis buffer***

Dissolve one tablet of EDTA-free Protease Inhibitor Cocktail (Roche, cat. no. COEDTAF-RO) in 50 ml Ni-NTA wash buffer (see recipe)  
Prepare fresh immediately before use and keep at 4°C

### ***Ni-NTA elution buffer***

50 mM HEPES, pH 7.5  
150 mM NaCl  
250 mM imidazole (Sigma-Aldrich, cat. no. 56750)  
Store ≤3 months at 4°C

### ***Ni-NTA wash buffer***

30 mM Tris·HCl, pH 7.5  
200 mM NaCl (Sigma-Aldrich, cat. no. S7653)  
10% (v/v) glycerol (Sigma-Aldrich, cat. no. G9012)  
10 mM imidazole (Sigma-Aldrich, cat. no. 56750)  
Adjust pH to 7.5 by adding 6 N NaOH  
Store ≤3 months at 4°C  
Immediately before use, add 5 mM β-mercaptoethanol (Sigma-Aldrich, cat. no. M6250)

### ***SDS sample buffer, 4×***

240 mM Tris·HCl, pH 6.8  
8% (w/v) SDS (Sigma-Aldrich, cat. no. 74255)  
40% (v/v) glycerol  
0.08% (w/v) bromophenol blue (Sigma-Aldrich, cat. no. B5525)  
Aliquot and store ≤6 months at −20°C  
Immediately before use, add 0.5 M β-mercaptoethanol (Sigma-Aldrich, cat. no. M6250)

### ***SEC buffer***

30 mM Tris·HCl, pH 7.5  
100 mM NaCl  
10% (v/v) glycerol  
1 mM DTT (from 1 M stock; see recipe)  
Prepare fresh immediately before use and store at 4°C  
Filter and degas by vacuum before use

### ***Reaction buffer, 10×***

300 mM Tris·HCl, pH 7.5  
50 mM MgCl<sub>2</sub> (Sigma-Aldrich, cat. no. M8266)  
Store ≤3 months at 4°C

## **COMMENTARY**

### **Background Information**

NEDD8 is a highly conserved Ub-like protein in eukaryotic species. Structurally, NEDD8 is composed of a globular core and a C-terminal tail. NEDD8 is initially trans-

lated as a precursor containing a tail with the “GGLRQ” sequence. This tail is then proteolytically cleaved by Ub C-terminal hydrolase L3 (UCHL3) or deneddylase 1 (DEN1) to expose the Gly residues that are vital to the

conjugation of NEDD8 to its substrates (Wada, Kito, Caskey, Yeh, & Kamitani, 1998; Wu et al., 2003). The processed NEDD8 is covalently conjugated to substrate proteins through concerted actions of the NEDD8 E1, E2, and E3. As the first step, NEDD8 forms a thioester bond with NAE (E1) via its C-terminal Gly. The human NAE complex contains two subunits, amyloid- $\beta$  precursor protein binding protein 1 (APPBP1) and Ub-activating enzyme 3 (UBA3). NEDD8 binds the heterodimeric NAE complex with extensive contacts (Walden et al., 2003). In particular, a conserved adenylation domain on UBA3 and a catalytic cysteine domain on APPBP1 are crucial for the bipartite interface. The NAE-mediated activation of NEDD8 is an ATP-dependent process. First, NAE catalyzes the formation of the NEDD8-AMP intermediate that binds the adenylation domain of UBA3. The intermediate is then transferred to the catalytic Cys, generating a high-energy thioester bond. Following the activation, the thioester-linked NEDD8 is transferred to a conserved Cys on the NEDD8 E2 via a transthioylation reaction, forming the E2~NEDD8. UBC12 (also known as UBE2M) and UBE2F are the two known NEDD8 E2s that exhibit distinct substrate specificities. UBC12 cooperates with RBX1 to neddylation CUL1/2/3/4, whereas UBE2F recognizes RBX2 for the neddylation of CUL5 (Huang et al., 2009). At the last step, E2~NEDD8 interacts with the NEDD8 E3 to transfer the NEDD8 to the protein substrate. Evidence from biochemical and structural studies has suggested a dual E3 mechanism for cullin neddylation (Kurz et al., 2008). The RING domain protein RBX1 (or RBX2) functions as the main E3 that binds and activates the E2~NEDD8. In addition, through binding both the cullin protein and the acetylated N-termini of NEDD8 E2s, defective-in-cullin neddylation (DCN)-like (DCNL) proteins serve as co-E3s that shape the flexible RBX-bound E2~NEDD8 to a catalytically favorable geometry (Monda et al., 2013). Taken together, the biochemical mechanisms of cullin neddylation indicate that an *in vitro* cullin neddylation reaction minimally requires NEDD8, NAE, UBC12/UBE2F, CUL•RBX1, and ATP. The neddylation co-E3 such as DCNL1 can be incorporated to accelerate the reaction or to study the kinetics of cullin neddylation.

The family of cullin proteins—including the canonical CUL1, CUL2, CUL3, CUL4a, CUL4b, and CUL5 and the non-canonical CUL7 and CUL9—are the best characterized

neddylation substrates. Each type of cullin engages a unique adaptor (with the exception of CUL3) and a set of substrate receptor proteins through its N-terminus, forming a subfamily of CRLs (Petroski & Deshaies, 2005). Given that different CRLs comprise different subunits, it is not hard to imagine that structural differences exist across different CRL subfamilies and even within the same subfamily. Moreover, the same CRL can bind distinct substrates for ubiquitination, which adds another layer of conformational diversity. Therefore, although neddylation has been considered as a general mechanism for CRL activation, it remains important to quantitatively define the effects of neddylation on the ubiquitination activity of a specific CRL toward a given substrate.

The *in vitro* assays described here provide advantages over cell-based assays for studying protein neddylation and ubiquitination. Although the rate of protein ubiquitination in cells can be estimated by monitoring the remaining level of the target protein in a cycloheximide assay, the result can be affected by various factors other than ubiquitination or neddylation. In cells, the assembly of CRLs is dynamically regulated by multiple players to achieve timely and efficient ubiquitination of a changing pool of CRL substrates (Liu et al., 2018; Pierce et al., 2013; Reitsma et al., 2017; Wang, Deshaies, & Liu, 2020). After ubiquitination and before proteasome-dependent degradation, substrates can be reverted to the unmodified form by deubiquitinating enzymes (Reyes-Turcu, Ventii, & Wilkinson, 2009), resulting in a lower apparent rate of ubiquitination. Further, the CRL substrate may be ubiquitinated and/or degraded via CRL-independent pathways, making cell-based analyses nonspecific to the CRL of interest (Wang et al., 2021). Due to the complexity of the cellular environment, it is challenging to establish a cell-based assay to directly compare the activity of a specific CRL with and without cullin neddylation. Instead, reconstitution of the cullin neddylation and ubiquitination reactions *in vitro* allows the protein components and their concentrations to be precisely controlled for quantitative analyses. Thus, these *in vitro* assays provide important tools for studying cullin neddylation and CRL-dependent protein ubiquitination.

It is noteworthy that quite a few proteins besides cullins have been identified as neddylation substrates (Enchev, Schulman, & Peter, 2015). The complete neddylation enzymatic cascade for these substrates remains unclear,



**Table 5** Troubleshooting Guide for Basic Protocol 1

Problem	Possible cause	Solution
Poor expression in bacteria	Incorrect construct	Confirm the sequence of the construct
	Incorrect bacterial strain used for transformation or the transformants are too old	Use the BL21 (DE3) strain to express genes driven by the T7 promoter. Use freshly transformed cells for expression.
	Inefficient induction	Use a new aliquot of IPTG stock solution or increase the IPTG concentration for induction
Poor protein recovery	Protein precipitation	Increase the NaCl concentration in the lysis buffer (e.g., 500 mM) to help with protein solubility. Make sure $\beta$ -mercaptoethanol is freshly added to the lysis and wash buffers. Include 5-10% glycerol in all buffers to help with protein solubility and stability. Keep the protein solution on ice or at 4°C as much as possible.
	Insufficient binding to the Ni-NTA beads	Check the extraction buffer. If Ni-NTA beads from a different vendor are used, then increase the Tris-HCl concentration to 50-100 mM and adjust the pH to 8.0.
	Inefficient elution	Freshly prepare the elution buffer. Make sure the imidazole concentration is correct.
Inefficient TEV cleavage after the first incubation period	Low TEV activity	Repeat the TEV cleavage and incubate the protein solution at 16°C

which hinders the development of corresponding neddylation assays *in vitro*. Thus, the important roles of neddylation in biological processes need to continue to be revealed through constant endeavors to fill knowledge gaps.

## Critical Parameters

### *Strategy for expressing recombinant CUL•RBX1 in E. coli*

Cullin proteins are routinely co-expressed with RBX1 (or RBX2 for CUL5), and the two subunits form a stable complex in *E. coli*. The MsyB protein fused to the N-terminus of CUL2 was designed to improve the solubility and folding of CUL2, and the same strategy can be used to help express other proteins with low solubility in bacterial cells. As an alternative strategy, “Split-n-Coexpress” can be used to co-express RBX1 with CUL that is split into two halves. This approach has been reported for CUL1•RBX1 (Li, Pavletich, Schulman, & Zheng, 2005), CUL2•RBX1 (Diaz et al., 2021), and CUL3•RBX1 (Zhuang et al., 2009).

### *Use of DCNL proteins*

The activity of DCNLs relies on the N-terminal acetylation of the NEDD8 E2 enzymes (see Background Information above). Therefore, DCNLs should be used in a pair

with the NEDD8 E2 expressed and purified from the baculovirus expression system.

### *Purification of full-length CRL substrate*

Although chemically synthesized degron peptides are frequently used as the substrate in *in vitro* studies, it may be necessary to use full-length substrates for the ubiquitination assay. When enriching the epitope-tagged substrate protein from mammalian cells via affinity purification, the substrate receptor and/or Ub ligase co-immunoprecipitated with the substrate should be minimized. Using quantitative western blot, the concentration of the purified substrate can be determined, provided that a recombinant protein with the same epitope tag and a known concentration is included as the standard for quantification.

## Troubleshooting

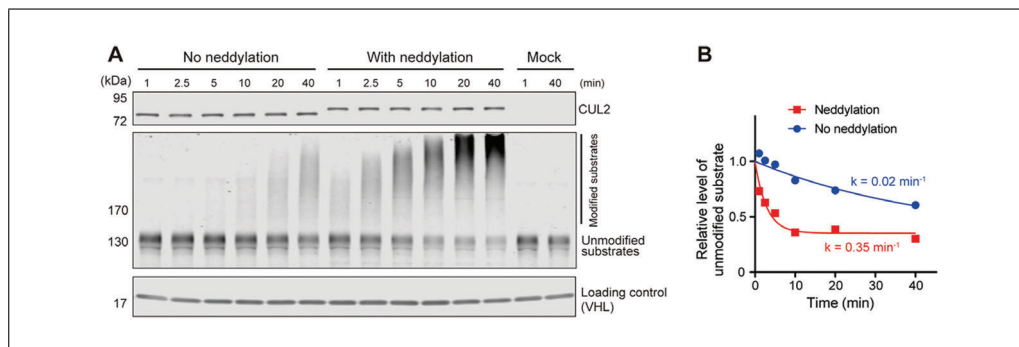
Please refer to Tables 5 and 6 for troubleshooting guidance for Basic Protocols 1 and 2, respectively.

## Understanding Results

In Basic Protocol 1 and the Support Protocol, the CUL2•RBX1 is purified sequentially by Ni-NTA affinity chromatography, removal of fusion proteins and tags by TEV cleavage, Strep II-tag affinity purification, and SEC. After the TEV cleavage, the MsyB protein fused to the N-terminus of CUL2 is

**Table 6** Troubleshooting Guide for Basic Protocol 2

Problem	Possible cause	Solution
Low neddylation or ubiquitination efficiency	Reaction components have become inactive; for example, proteins are aggregated or denatured or the ATP has broken down	Use new aliquots of protein stocks or freshly prepared ATP stock solution
	Insufficient reaction time	Incubate for a longer time
	Improper reaction temperature	Perform the reactions at or above room temperature (22–37°C)
The unmodified substrates disappear much faster than the appearance of the ubiquitinated substrates	Ubiquitinated substrates located in the top portion of the SDS-PAGE gel have been removed	Gradient SDS-PAGE gels (e.g., 4–20%) are recommended for sample fractionation, and the whole gel should be preserved for downstream analyses
	Unmodified substrates are cleaved by proteases present in the reaction mixture, which is more likely to occur when substrate proteins are immunoprecipitated from human cells	Include protease inhibitor in the reaction mixture. The protease inhibitor cocktail used in the Ni-NTA lysis buffer can also be used here, and the final concentration can be doubled.
The unmodified substrates disappear over time in the mock control group	The reaction mixture is contaminated with proteases (see above)	Add protease inhibitor to the reaction mixture (see above)



**Figure 3** Quantitative analysis of the effect of neddylation on CUL2-dependent protein ubiquitination. **(A)** CUL2•RBX1 with or without neddylation was used for time course–dependent protein ubiquitination *in vitro*. Mock samples with no CUL2•RBX1 were included as the negative control. Samples were analyzed by western blot with antibodies against CUL2, the FLAG epitope (for the substrate), and VHL (the substrate receptor). **(B)** Relative levels of unmodified substrates versus time were plotted and fit with single exponential curves.

removed, and a shift of the CUL2 band is expected on the SDS-PAGE gel (Fig. 2B). After the TEV cleavage is complete, CUL2•RBX1 is bound to a 1-ml Strep-Tactin cartridge on an ÄKTA FPLC system. Eluting CUL2•RBX1 from the cartridge using biotin elution buffer is very efficient, with the eluted protein peak lasting from ~2 ml to ~15 ml (Fig. 2C). It is very common for the elution peak to tail, and eluate in the tail region usually contains the target protein and should be collected. At this point, the CUL2•RBX1 should look very pure on the SDS-PAGE gel. Lastly, SEC is used to further separate the correctly folded CUL2•RBX1 from the aggregated population

and to change the buffer for better protein storage. The retention volume of CUL2•RBX1 on the Superdex 200 Increase 10/300 GL column is ~12 ml (Fig. 2D and E). Aggregated CUL2•RBX1 has a retention volume of ~8 ml (void volume) and should not be collected. In terms of the protein yield, ~0.2 mg CUL2•RBX1 per liter of bacterial culture can be expected.

In Basic Protocol 2, CUL2 is conjugated with NEDD8 *in vitro*. Almost all CUL2 is converted to the neddylated form, and the CUL2 band is expected to shift upward in the western blot (Fig. 3A, upper blot). The neddylated CUL2•RBX1 is then used for time

course-dependent substrate ubiquitination. Of note, a mock (negative) control group where no CUL2•RBX1 is added should be included and is expected to show no change in the level of unmodified substrate over time (Fig. 3A, last two lanes of the middle blot). Only with correct results obtained from the negative control group can the disappearance of the unmodified substrates in the presence of CUL2•RBX1 be interpreted as a result of CRL2-dependent ubiquitination (Fig. 3A, middle blot). To compare the rates at which substrates are ubiquitinated when different forms of CUL2•RBX1 are supplied, the remaining amounts of unmodified substrates at different time points should be fit with a one-phase exponential decay model. Intensities of the unmodified substrate bands are used for this quantification, and the values are normalized to account for variations in sample loading volumes. Because the same “substrate + substrate receptor” master mix is used for all samples, intensities of the substrate receptor bands are used for normalization (Fig. 3A, bottom blot). Finally, the normalized levels of unmodified substrates are used for regression analysis, and rates for substrate ubiquitination are quantitatively compared (Fig. 3B).

### Time Considerations

Basic Protocol 1 (followed by the Support Protocol) can be completed in 7 days: plasmid transformation (~2 hr) on Day 1, growth medium and starter culture preparation (~2 hr) on Day 2, protein expression in *E. coli* (~7 hr) on Day 3, bacterial cell collection (~2 hr) on Day 4, Ni-NTA affinity purification (~6 hr) on Day 4 (or another day), TEV cleavage and SDS-PAGE analysis (~2 hr) on Day 5, StrepII-tag affinity purification and SDS-PAGE analysis (~5 hr) on Day 6, and SEC (~3 hr) on Day 7.

Basic Protocol 2 can be completed in 2 days: *in vitro* neddylation assay (2 hr) and *in vitro* ubiquitination assay (2 hr) on Day 1, sample analysis by SDS-PAGE (2 hr) on Day 1, and western blot on Days 1 and 2.

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### Author Contributions

**Kankan Wang:** Data curation, Formal analysis, Writing — original draft; **Xing Liu:**

Funding acquisition, Supervision, Writing — review and editing.

### Conflict of Interest

The authors declare that they have no conflict of interest.

### Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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