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Abstract	Protein–protein interactions (PPI) are involved in a myriad of cellular processes, and their deregulation can lead to many diseases. One such process is protein ubiquitination that requires an orchestrated action of three key enzymes to add ubiquitin moieties to substrate proteins. Importantly, this process is reversible through deubiquitinating enzymes. Both ubiquitination and deubiquitination require many PPIs that once classified can be utilized to identify small molecule inhibitors counteracting these reactions. Here, we study the protein–protein interaction between the two deubiquitinating enzymes OTUB1 and OTUD6B and report for the first time that both proteins directly interact with each other. We describe the GFP-Trap immunoprecipitation as a cell-based method to analyze the OTUD6B-OTUB1 interaction in the cellular context and the AlphaScreen (amplified luminescent proximity	

	homogeneous assay) assay as a tool to detect direct interactions and to search for PPI inhibitors.
Keywords (separated by '-')	Protein–protein interactions - GFP-trap - Immunoprecipitation - AlphaScreen - Ubiquitin - Deubiquitinase - DUB - OTUD6B - OTUB1 - Homogeneous proximity assay

Chapter 23

Studying OTUD6B-OTUB1 Protein–Protein Interaction by Low-Throughput GFP-Trap Assays and High-Throughput AlphaScreen Assays

Elisabeth Weber, Kenji Schorpp, and Kamyar Hadian

Abstract

Protein–protein interactions (PPI) are involved in a myriad of cellular processes, and their deregulation can lead to many diseases. One such process is protein ubiquitination that requires an orchestrated action of three key enzymes to add ubiquitin moieties to substrate proteins. Importantly, this process is reversible through deubiquitinating enzymes. Both ubiquitination and deubiquitination require many PPIs that once classified can be utilized to identify small molecule inhibitors counteracting these reactions. Here, we study the protein–protein interaction between the two deubiquitinating enzymes OTUB1 and OTUD6B and report for the first time that both proteins directly interact with each other. We describe the GFP-Trap immunoprecipitation as a cell-based method to analyze the OTUD6B-OTUB1 interaction in the cellular context and the AlphaScreen (amplified luminescent proximity homogeneous assay) assay as a tool to detect direct interactions and to search for PPI inhibitors.

Key words Protein–protein interactions, GFP-trap, Immunoprecipitation, AlphaScreen, Ubiquitin, Deubiquitinase, DUB, OTUD6B, OTUB1, Homogeneous proximity assay

1 Introduction

Cell-based processes use protein interactions or protein networks to positively or negatively regulate cellular pathways. Examples of these processes employing protein–protein interactions (PPIs) are receptor-mediated signaling, transcription and translation, DNA replication, cell death, cell cycle, DNA damage response, and many more. Studying protein interaction networks is key to understanding signaling pathways, development, and diseases. Thus, methodologies detecting and describing PPIs are important tools to explore biological processes. Large-scale techniques such as mass spectrometry uncover cellular protein interactions and larger networks [1]. However, clear information of the direct nature of a PPI is missing in these data sets as two proteins may interact directly or be bridged by, for example, other proteins or nucleic acids. Thus,

Anton Posch (ed.), *Proteomic Profiling: Methods and Protocols*, Methods in Molecular Biology, vol. 2261,
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biochemical assays to verify direct PPIs are needed as parallel approaches to large-scale protein network analyses. The repertoire of biochemical assays include low-throughput assays as well as high-throughput assays [2].

One cellular process that employs many PPIs is protein ubiquitination. Based on the type of protein ubiquitination different cellular events can be controlled, ranging from protein degradation to regulation of signal transduction [3, 4]. The E1 activating enzymes, the E2 conjugating enzymes and the E3 ligases work in a consecutive manner to attach ubiquitin moieties to substrate proteins [4, 5]. The whole cascade undertakes many PPIs among the involved enzymes as well as between the E3 ligases and their substrates [5, 6]. Importantly, ubiquitination is a post-translational modification that is reversible by deubiquitinating (DUB) enzymes [7, 8]. Recent years have shown that there are also protein interactions between E3 ligases and DUBs for tight regulation of cellular ubiquitination [9, 10] as well as between two DUBs [11]. Interaction of two DUBs (OTUD6B (34 kDa) and OTUB1 (31 kDa)) was demonstrated in a study using mass spectrometry analysis. However, the verification of a direct interaction between the two DUBs has not been reported [11]. Here, we use the interaction between OTUD6B and OTUB1 as a showcase to demonstrate how a low-throughput assay (GFP-Trap immunoprecipitation; Fig. 1a) and a high-throughput assay (amplified luminescent proximity homogenous assay—AlphaScreen; Fig. 2a) can be utilized to validate large-scale mass spectrometry data. This can be an essential piece in the puzzle to understand the underlying biology and to develop small molecules altering disease-relevant pathways.

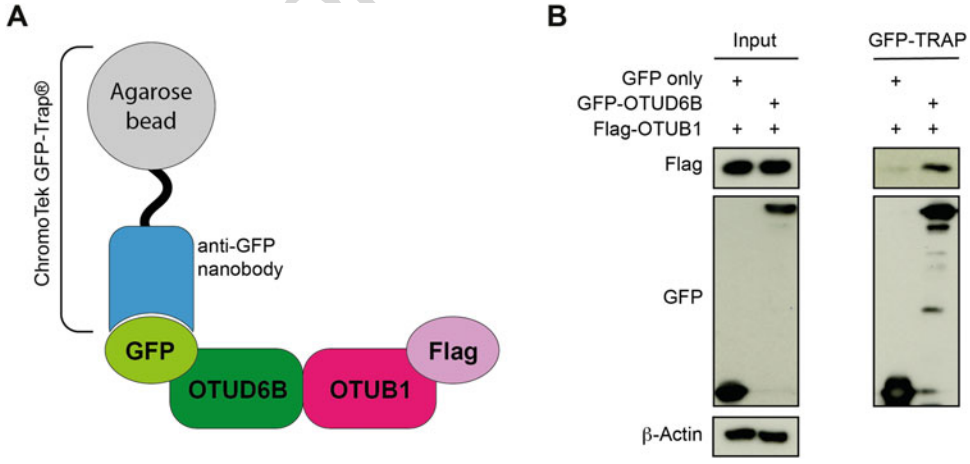

Fig. 1 (**a**) The experimental setup for a GFP-Trap immunoprecipitation assay is depicted. ChromoTek GFP-Trap® consists of an anti-GFP nanobody/V_HH coupled to agarose beads, allowing for high-affinity pulldown of GFP-fusion proteins together with its interaction partners. (**b**) Flag-OTUB1 can be coimmunoprecipitated with GFP-OTUD6B, while no Flag-OTUB1 can be detected when GFP-only is pulled down

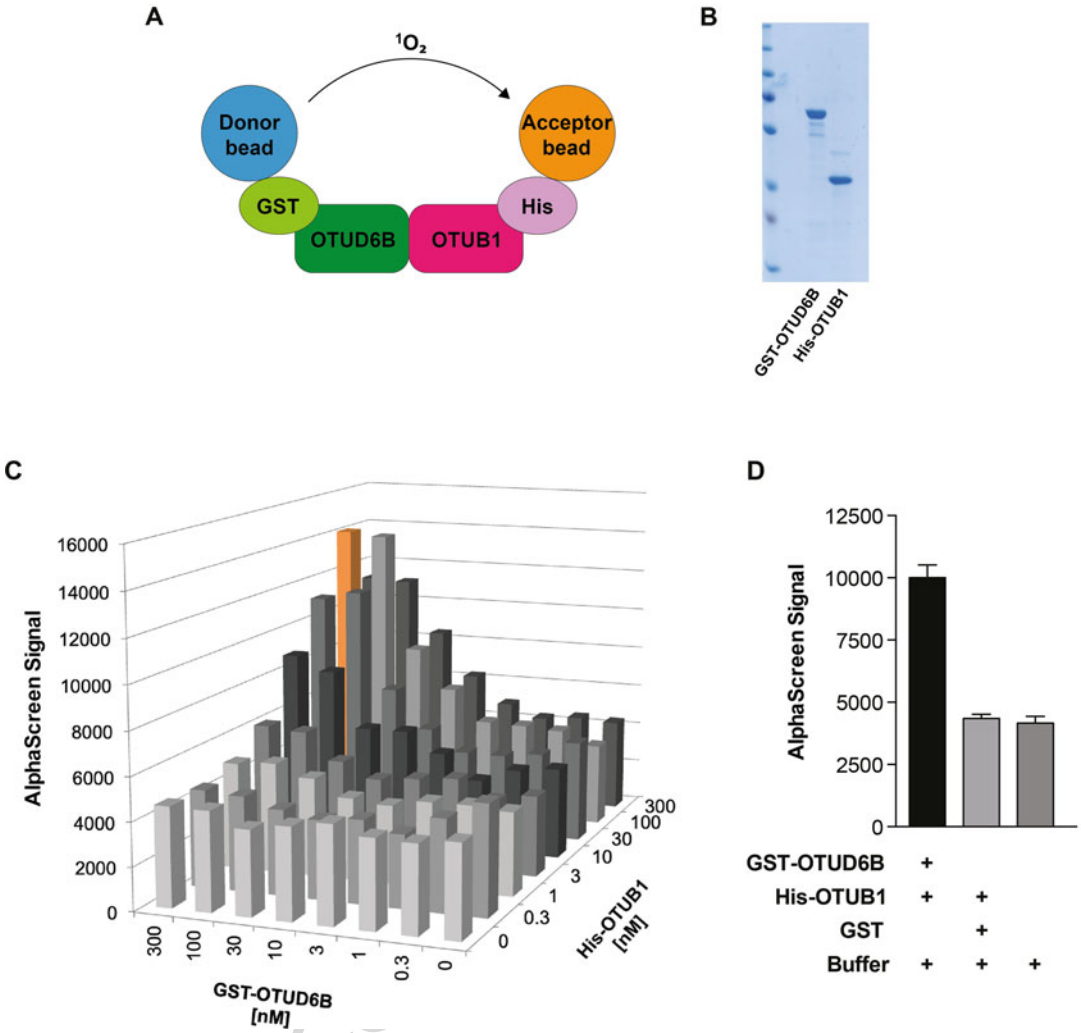

Fig. 2 (**a**) Sketch of the AlphaScreen assay setup (**b**) 2.5 μg of the purified GST-OTUD6B and His-OTUB1 proteins were loaded and separated on a 10% SDS gel and stained with Coomassie Brilliant Blue (CBB) G-250 stain. (**c**) AlphaScreen matrix titration experiment with different protein concentrations of GST-OTUD6B and His-OTUB1 in the range from 300 nM to 0.3 nM. (**d**) Based on the results in (**c**) the concentrations of 300 nM GST-OTUD6B and 100 nM His-OTUB1, respectively, were selected for an AlphaScreen assay. 300 nM GST served as a negative control. The experiment was performed as technical replicate ($n = 3$, SD)

2 Materials

2.1 Protein Expression and Purification

1. Prokaryotic expression vector pET-28a(+) (His-tagged proteins).
2. Prokaryotic expression vector pGex-4T1 (GST-tagged proteins).
3. *E. coli* strain BL21-CodonPlus (DE3) RIPL.
4. LB Agar Plate with 100 μg/mL ampicillin.
5. LB Agar Plate with 30 μg/mL kanamycin.

6. LB Medium with 100 μg/mL ampicillin and 25 μg/mL chloramphenicol.
7. LB Medium with 30 μg/mL kanamycin and 25 μg/mL chloramphenicol.
8. 1 M isopropyl-β-D-thiogalactopyranoside (IPTG) stock solution in H_2O.
9. 20% ethanol for analysis.
10. His-Lysis buffer 1: 50 mM Tris pH 8.0, 250 mM NaCl, 50 mM imidazole, 2 mM Tris(2-carboxyethyl)phosphine (TCEP), 1 mM EDTA free Protease Inhibitor Cocktail (PIC), 0.5 mg/mL lysozyme, 250 U benzonase, 2 mM $MgCl_2$ (*see* **Notes 1–3**).
11. His-Lysis buffer 2: 50 mM Tris–HCl, pH 8.0, 250 mM NaCl, 50 mM Imidazole, 2 mM TCEP, 1 mM PIC.
12. High Salt Wash buffer for His-Tag purification: 50 mM Tris–HCl, pH 8.0, 500 mM NaCl, 50 mM imidazole.
13. His-Elution buffer: 50 mM Tris–HCl, pH 8.0, 250 mM NaCl, 250 mM Imidazole.
14. His-Desalting buffer: 50 mM Tris–HCl, pH 8.0, 250 mM NaCl, 2 mM TCEP (*see* **Note 3**).
15. Nickel-Sepharose 6 Fast Flow beads.
16. GST-Lysis buffer 1: 1× PBS, 400 mM NaCl, 5 mM DTT, 1 mM PIC (EDTA free), 0.5 mg/mL lysozyme, 250 U benzonase, 2 mM $MgCl_2$.
17. GST-Lysis buffer 2: 1× PBS, 400 mM NaCl, 5 mM DTT, 1 mM PIC (EDTA free).
18. GST-Wash buffer: 1× PBS, 400 mM NaCl, 5 mM DTT (*see* **Note 3**).
19. Glutathione elution buffer: 50 mM L-glutathione reduced, 1× PBS, 400 mM NaCl, 5 mM DTT (*see* **Note 3**).
20. Glutathione-Sepharose 4 Fast Flow beads.
21. Polypropylene columns with filter (5 mL) used for gravity-flow chromatography.
22. Amicon Ultra-15 Centrifugal Filter Unit (15 mL, 3 kDa cutoff).
23. Benchtop incubator shaker with optional refrigeration.
24. BioPhotometer.
25. Ultrasonic processor with Sonotrode S2.
26. Flexible roller mixer.
27. Fast protein liquid chromatography (FPLC) system.
28. HiTrap Desalting column 5 mL.
29. NanoDrop 2000 spectrophotometer.

2.2 Immunoprecipitation Using GFP-Traps

1. CO_2 incubator.
2. HEK293T mammalian cell line (ATCC: CRL-2316).
3. Dulbecco's Modified Eagle's Medium (DMEM): high Glucose, + L-Glutamine.
4. Fetal bovine serum (FBS).
5. Penicillin-Streptomycin (Pen-Strep) solution.
6. Opti-MEM reduced serum medium.
7. DNA transfection reagent (e.g., X-tremeGENE HP).
8. Mammalian expression vectors: ~~pEF2× Flag~~, pEGFP-C1.
9. 1× PBS.

Please change to: pEF4-2xFlag

10. GFP-Trap Agarose for immunoprecipitation (ChromoTek).
11. RIPA Trap Lysis buffer: 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.1% SDS, 1% Triton X-100, 2.5 mM $MgCl_2$, 1% deoxycholate, 1 mM PIC (EDTA free).
12. GFP-Trap wash/dilution buffer: 10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA.
13. Benzonase (≥250 U/μL).
14. 1 mL syringe with Needle 26 G.
15. Rotator, end-over-end.
16. 4× Laemmli protein gel loading buffer, reducing conditions.
17. Electrophoresis apparatus.
18. Semidry blotter and blotting membrane.
19. Primary antibodies: mouse anti-GFP B-2 (Santa Cruz, #sc-9996); mouse anti-Flag M2 (Sigma, # F1804); mouse anti--β-Actin 8H10D10 (Cell Signaling, #3700S).
20. Secondary antibodies: donkey anti-mouse HRP.
21. Hyperfilm ECL.
22. Automatic film processor.

2.3 "AlphaScreen" Homogeneous Proximity Assay

1. AlphaScreen Glutathione donor beads.
2. AlphaScreen Histidine (Nickel Chelate) Detection Kit containing Streptavidin Donor beads and nickel chelate (Ni-NTA) AlphaScreen Acceptor beads.
3. AlphaScreen buffer: 1× PBS, 0.5% BSA, 0.01% Tween 20 (*see* **Note 4**).
4. 384-well OptiPlate, white (PerkinElmer).
5. Envision multilabel plate reader including an AlphaScreen module with a filter set for excitation at 680 nm and emission at 520–620 nm.

3 Methods

DNA fragments encoding human full length OTUB1 and OTUD6B were amplified from plasmids by PCR using specific primer pairs in standard PCR protocols and were subsequently subcloned (*see* **Note 5**).

3.1 Protein Expression and Purification Protocols

3.1.1 Recombinant Production and Purification of His-OTUB1

1. Transformation of *E. coli* BL21-DE3-RIPL with pET-28a(+)-OTUB1 using standard procedure and plating on kanamycin plates.
2. Preculture #1: inoculate a single transformed clone in 5 mL of LB medium with matched antibiotics and incubate O/N at 37 °C and 180 rpm.
3. Preculture #2: next day, inoculate 5 mL of preculture #1 in 50 mL of LB medium with matched antibiotics and incubate O/N at 37 °C and 180 rpm.
4. Next day, inoculate 50 mL of preculture #2 in 500 mL of LB medium with matched antibiotics and incubate at 37 °C and 180 rpm.
5. Measure OD_{600} and incubate up to $OD_{600} = 0.6$; then add another 500 mL of LB medium with matched antibiotics and incubate at 37 °C and 180 rpm.
6. At $OD_{600} = 0.6–0.8$ switch to 18 °C, allow the culture to cool down for 20 min and induce protein expression with ~~final~~ IPTG to a final concentration of 1 mM.
7. Incubate overnight at 18 °C and 150 rpm.
8. Next day, centrifuge culture for 20 min, 4 °C, 2500 × *g*.
9. Discard supernatant and use pellet for protein purification.
10. Resuspend pellet in 10 mL His-Lysis buffer 1 (*see* **Notes 6** and 7).
11. Incubate for 20 min at RT and then incubate on ice.
12. Sonicate lysate ten times on ice (20 impulses; 30 s resting time in between each sonication cycle) (*see* **Note 8**).
13. Transfer total lysate into prechilled centrifugation tubes; centrifugation at 20,000 × *g*, 50 min, 4 °C.
14. Prepare 3 mL Nickel-Sepharose slurry: Wash Sepharose in His-Lysis buffer 2, then spin down for 2 min at 4 °C and 200 × *g*. Repeat this step three times.
15. Transfer lysate supernatant into 50 mL reaction tube, fill up with His-Lysis buffer 2 to total 30 mL and add 3 mL prewashed Nickel-Sepharose slurry.
16. Incubate for 2 h at 4 °C with constant inverting.

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... 200 x g. Discard excess supernatant. Repeat...

17. Pour bead–lysate mix into propylene column with filter. Allow Sepharose to settle down by gravity flow.
18. Wash with 100 mL High Salt Wash buffer for His-Tag purification.
19. Let resin run (almost) dry.
20. Elute in five steps with His-Elution buffer (1 mL each) into 15 mL Concentrator tubes.
21. Concentrate to 1 mL total.
22. Prepare HiTrap Desalting 5 mL column by washing with EtOH, H_2O, and His-Desalting buffer (3 column volumes each = 15 mL) on a chromatography system.
23. Desalt proteins on a chromatography system in His-Desalting buffer.
24. Collect main peak fraction and measure protein concentration and DNA/RNA contamination.
25. Aliquot and snap-freeze protein samples in liquid nitrogen and store at −80 °C (*see* **Note 9**).

3.1.2 Recombinant Production and Purification of GST-OTUD6B

1. Transformation of *E. coli* BL21-DE3-RIPL with pGex-4T1-OTUD6B using standard procedure and plating on ampicillin plates.
2. Preculture #1: inoculate a single transformed clone in 5 mL of LB medium with matched antibiotics and incubate O/N at 37 °C and 180 rpm.
3. Preculture #2: next day, inoculate 5 mL preculture #1 in 50 mL of LB medium with matched antibiotics and incubate O/N at 37 °C and 180 rpm.
4. Next day, inoculate 50 mL of preculture #2 in 500 mL of LB medium with matched antibiotics and incubate at 37 °C and 180 rpm.
5. Measure OD_{600} and incubate up to $OD_{600} = 0.6$; then add another 500 mL of LB medium with matched antibiotics and incubate at 37 °C and 180 rpm.
6. At $OD_{600} = 0.6$–0.8 switch to 18 °C, allow the culture to cool down for 20 min and induce protein expression with final 1 mM IPTG.
7. Incubate O/N at 18 °C and 150 rpm.
8. Next day, centrifuge for 20 min, 4 °C, 2500 × *g*.
9. Discard supernatant and use pellet for protein purification.
10. Resuspend pellet in 10 mL GST-Lysis buffer 1 (*see* **Notes 6** and 7).
11. Incubate for 20 min at RT and then incubate on ice.

12. Sonicate lysate ten times on ice (20 impulses; 30 s resting time in between each sonication cycle) (*see* **Note 8**).
13. Transfer total lysate into prechilled centrifugation tubes; centrifugation at 20,000 × *g*, 50 min, 4 °C.
14. Prepare 3 mL glutathione–Sepharose slurry: Wash Sepharose in GST-Lysis buffer 2, then spin down for 2 min at 4 °C and 200 × *g*. Repeat this step three times.
15. Transfer lysate supernatant into 50 mL reaction tube, fill up with GST-Lysis buffer 2 to total 30 mL and add 3 mL prewashed glutathione–Sepharose slurry.
16. Incubate for 2 h at 4 °C with constant inverting.
17. Pour bead–lysate mix into propylene-column with filter. Allow Sepharose to settle down by gravity flow.
18. Wash with 100 mL GST-Wash buffer.
19. Let resin run (almost) dry.
20. Elute in five steps in Glutathione Elution Buffer (1 mL each) into 15 mL Concentrator tubes.
21. Concentrate to 1 mL total.
22. Prepare HiTrap Desalting 5 mL column by washing in EtOH, H_2O and GST-Wash buffer (3 column volumes each = 15 mL) on a chromatography system.
23. Desalt proteins on chromatography system in GST-Wash buffer.
24. Collect main peak fraction and measure protein concentration and DNA/RNA contamination.
25. Aliquot and snap-freeze protein samples in liquid nitrogen and store at −80 °C (*see* **Note 9**).

3.2 GFP-Trap Immunoprecipitation

3.2.1 Seeding of Cells and Transfection

1. Seed HEK293T cells in 2 × 10 cm cell culture dishes with a density of 2 × 10^6 cells in 10 mL DMEM medium + 10% FBS + 1% Pen-Strep. One dish serves as a GFP-control sample, the other as the actual PPI samples (*see* **Note 10**). Allow cells to attach overnight by culturing cells in an incubator at 37 °C, 5% CO_2 in a H_2O saturated atmosphere.
2. The next day, perform cell transfection with the following plasmids: pEGFP-C1-OTUD6B + pEF4-2×Flag-OTUB1 and as a control pEGFP-C1-Empty + pEF4-2×Flag-OTUB1.
3. Prepare a transfection mix for the control sample: combine 500 µL Opti-MEM, 1 µg ~~pEGFPC1-Empty~~, 2 µg ~~pEF2 × Flag-OTUB1~~ and briefly vortex the solution (*see* **Note 11**).
4. Add 9 µL DNA transfection reagent X-tremeGene HP and briefly vortex (*see* **Note 12**).
7. Incubate for 10–20 min at room temperature.

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... 200 x g. Discard excess supernatant. Repeat...

please change to: pEE4-2xFlag-OTUB1

please change to: pEGFP-C1-Empty

please change to:
pEE4-2xFlag-OTUB1

8. Prepare a transfection mix for the Heterodimer sample: combine 500 µL Opti-MEM, 2 µg ~~pEGFPC1-OTUD6B~~, 2 µg ~~pEF2×-Flag-OTUB1~~ and briefly vortex (*see* **Note 11**).
9. Add 12 µL DNA transfection reagent X-tremeGene HP and briefly vortex (*see* **Note 12**).
10. Incubate for 10–20 min at room temperatu
11. Add transfection mix dropwise on cells and incubate for 48 h at 37 °C, 5% CO_2.

please change to:
pEGFP-C1-OTUD6B

3.2.2 Harvesting the Cells

1. Aspirate medium and wash cells carefully with ice-cold 1× PBS.
2. Aspirate PBS completely.
3. Lyse the cells with 200 µL ice-cold RIPA-Trap lysis buffer and scrape cells from dish.
4. Transfer into precooled 1.5 mL reaction tube.
5. Add 125 U benzonase to each sample (*see* **Note 6**).
6. Lyse each sample ten times with a 26G needle and 1 mL syringe.
7. Incubate for 20 min at 4 °C and rotate end-over-end.
8. Centrifuge samples at 20,000 × *g* for 10 min at 4 °C to clear cell lysates from debris.
9. Transfer supernatant into new precooled 1.5 mL reaction tubes.
10. Add 300 µL GFP-Trap Wash/Dilution buffer to each sample to achieve a final volume of 500 µL per sample.
11. Transfer ~~60~~ µL of total cell lysate to 20 µL 4× protein loading buffer. please change to: 50 µl
12. Boil the sample for 5 min at 95 °C. Store the sample at −20 °C until performance of SDS-PAGE.

3.2.3 Bead Equilibration of GFP-Trap-Agarose

1. ~~Equilibrate GFP-Trap Agarose beads with 15 µL bead slurry and 500 µL GFP-Trap Wash/Dilution buffer.~~
2. Mix the solution by inverting.
3. Centrifuge beads at 2500 × *g* for 2 min at 4 °C.
4. Discard excess supernatant (do not dry beads completely).
5. Repeat the washing step twice.

3.2.4 GFP-Trap (Binding, Washing and Elution)

1. Add 15 µL equilibrated GFP-Trap Agarose beads to each cell lysate to bind proteins.
2. Rotate end-over-end for 1.5 h at 4 ° C.
3. Centrifuge at 2500 × *g* for 2 min at 4 °C and discard supernatant.

Please change to:
Equilibrate 15 µl slurry of GFP-Trap Agarose beads with 500 µl GFP-Trap Wash/Dilution buffer per sample

4. Wash beads by adding 500 µL GFP-Trap Wash/dilution buffer per sample and mix by inverting.
5. Centrifuge at 2500 × *g* for 2 min at 4 °C.
6. Completely discard supernatant and repeat washing step twice. After final wash, completely aspirate supernatant from beads. Be careful not to aspirate beads (*see* **Note 13**).
7. Elute immunocomplexes from GFP-Trap Agarose beads by addition of 50 µL of 2× protein loading buffer.
8. Boil samples at 95 °C for 5 min.
9. Centrifuge at 20,000 × *g* for 3 min.
10. Perform SDS-PAGE and Western blot [12, 13]. Data analysis (*see* Fig. 1b) revealed that there is an interaction between OTUD6B and OTUB1, thereby confirming previously published large-scale mass spectrometry data [11].

3.3 "AlphaScreen" Homogeneous Proximity Assay

In order to identify the optimal protein concentration for the PPI (robust signal with minimal protein concentration), a matrix titration experiments needs to be performed in accordance with the manufacturer's protocol (PerkinElmer) (*see* **Note 14**).

3.3.1 AlphaScreen Matrix Titration Experiment

1. Perform all dilution steps in AlphaScreen buffer.
2. Prepare 200 µL of 4× working solution (1.2 µM) of each protein in the AlphaScreen buffer (*see* **Note 15**).
3. Subsequently, prepare a serial dilution of the 1.2 µM stock (400, 120, 40, 12, 4, 1.2 nM).
4. The assay is done in white 384-well microtiter plates with a final volume of 40 µL per well (*see* **Note 16**).
5. Add 10 µL of 4× concentrated GST-OTUD6B (1.2 µM; 400 nM; 120 nM; 40 nM; 12 nM; 4 nM; 1.2 nM; 0 nM) into a microtiter plate (each 8-times column-wise).
6. Add 10 µL of 4× concentrated His-OTUB1 (1.2 µM, 400 nM, 120 nM, 40 nM, 12 nM, 4 nM, 1.2 nM, and 0 nM) into the microtiter plate (each 8-times row-wise) (*see* Fig. 2c).
7. Incubate for 1 h at room temperature.
8. Prepare 1.5 mL 2× working solution with AlphaScreen glutathione donor beads (40 µg/mL) and AlphaScreen Ni^{2+}-NTA acceptor beads (40 µg/mL) (*see* **Note 17**).
9. Immediately after preparation, add 20 µL of the AlphaScreen bead mix to each well. Final AlphaScreen bead concentration is 20 µg/mL (*see* **Note 15**).
10. Incubate for 1 h at room temperature in the dark or in dimmed light (*see* **Note 18**).
11. Analyze the assay using a multilabel plate reader (settings: laser excitation at 680 nm, emission at 520–620 nm). Based on this result, we conclude that there is a direct protein–protein

interaction between OTUD6B and OTUB1. The optimal concentration for AlphaScreen appears to be 300 nM of GST-OTUD6B and 100 nM His-OTUB1.

3.3.2 AlphaScreen PPI Assay at Defined Concentration

1. Chose the optimal concentration for the protein pair from the matrix titration experiment (*see* Subheading 3.3.1). Here, the combination of 300 nM GST-OTUD6B with 100 nM His-OTUB1 results in a robust AlphaScreen signal (Fig. 2c), while the proteins alone without their corresponding binding partner show a markedly reduced signal (background).
2. Include a tag alone control (GST-alone in this case) (Fig. 2d) when performing the assay (*see* **Note 19**).
3. Perform the assay in white 384-well microtiter plates with a final volume of 40 µL per well (*see* **Note 16**).
4. Add 10 µL of 4× concentrated GST-tagged proteins (GST-OTUD6B or GST) (each 1.2 µM) into a microtiter plate.
5. Add 10 µL of 4× concentrated His-OTUB1 (400 nM) into the same wells of the microtiter plate.
6. OPTIONAL: add an inhibitory compound dissolved in DMSO at the desired concentration using a 1:100 dilution (e.g., final 10 µM = 0.2 µL of 2 mM solution) (*see* **Notes 20** and **21**).
7. Incubate for 1 h at room temperature.
8. Add 20 µL of a freshly prepared 2× solution with AlphaScreen glutathione donor beads (40 µg/mL) and AlphaScreen Ni^{2+}-NTA acceptor beads (40 µg/mL) (*see* **Notes 15** and **17**).
9. Incubate for 1 h at room temperature in the dark or in dimmed light (*see* **Note 18**).
10. Analyze the assay using a multilabel plate reader (settings: laser excitation at 680 nm, emission at 520–620 nm).

4 Notes

1. His-OTUB1: All buffers used for His-tag purification need to be without EDTA. This includes buffers for lysing cells as well as purification buffers. Protease inhibitor supplements should be without EDTA.
2. TCEP is the preferred reducing agent for His-Purification (Ni-Sepharose) as it does not reduce nickel. In contrast, DTT reduces Nickel.
3. Buffers containing DTT (e.g., GST-Lysis Buffer for purification of GST-tagged OTUD6B) and buffers with TCEP (for purification of His-tagged proteins) should be prepared fresh every time; TCEP is generally slightly more stable than DTT.

4. Use protease free, fatty acid free, essentially globulin free Bovine Serum Albumin (BSA) with purity ≥98%.
5. Vectors for GFP-Trap immunoprecipitation: pEGFP-C1 (N-terminal GFP-Tag): **GFP-OTUD6B** = pEGFP-C1-OTUD6B; pEF4-2×Flag (N-terminal Flag-Tag): **Flag-OTUB1** = pEF4-2×Flag-OTUB1. Vectors for AlphaScreen assay: pGex-4T1 (N-terminal GST-Tag): **GST-OTUD6B** = pGex-4T1-OTUD6B; pET-28a(+) (N-terminal His_6-Tag): **His-OTUB1** = pET-28a(+)-OTUB1.
6. Addition of benzonase helps to reduce DNA and RNA impurification.
7. Mg^{2+} (1–2 mM) is required for benzonase enzyme activity.
8. During the process of sonication, be careful that the lysate does not heat up too much. This process needs to be done on ice and with intervals of sonication and chilling on ice to guarantee optimal protein quality.
9. Aliquot purified proteins and snap-freeze in liquid nitrogen prior to long-term storage at −80 °C. Prepare small aliquots to avoid multiple freeze and thaw cycles of recombinant proteins.
10. A nonbinding Tag-alone control should always be included to identify unspecific binding effects: GFP-alone.
11. In order to prepare transfection complexes using XtremeGene HP it is crucial to prepare the complexes in serum-free media (e.g., Opti-MEM), as serum can inhibit the formation of these complexes and thereby reduce transfection efficiency.
12. To achieve low cytotoxicity and high transfection efficiency it is often helpful to test the appropriate µg ~~DNA–µL~~ transfection reagent ratio. Here, 1 µg DNA:3 µL XtremeGene HP works best.

    Please change to "… DNA:µL …"
13. To completely dry the GFP-Trap Agarose beads after the final wash, it is crucial not to accidentally lose the beads. We therefore recommend using tips with flattened head that allow complete removal of buffer (e.g., Corning CLS4185-400EA).
14. When working with the AlphaScreen technology, the hook effect needs to be considered. This effect describes the fact that too much of the proteins can reduce the signal.
15. Both the protein solutions and the AlphaScreen bead mix must be freshly prepared on ice and used immediately to avoid increased background signals.
16. When pipetting the different solutions into the 384-well plate, try to avoid air bubbles. Reverse pipetting can help here.
17. There are beads covered with Glutathione or an anti-GST antibody to detect the GST-tagged protein. Similarly, there are beads coated with Ni^{2+}-NTA or an anti-His-Tag antibody

to detect His-tagged proteins. Researchers can consider all variants for their research. In some cases, one variant might work superior to the other.

18. AlphaScreen assays should always be carried out in the dark/in dimmed light surrounding to ensure no bleaching of the beads due to photosensitivity.
19. A nonbinding Tag-alone control should always be included to identify unspecific binding effects: GST-alone.
20. When using the AlphaScreen technology for testing of inhibitory compounds or high-throughput screening of small molecule libraries, it is important to be aware that many compounds interfere with the underlying chemistry. These compounds will generate false positive hits that need to be eliminated from the hit list. Chemoinformatics filters can help identify these frequent hitters [14–16].
21. The DMSO concentration of dissolved compounds should be kept below 1%, ideally below 0.5% as DMSO can affect the proteins as well as the AlphaScreen technology.

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