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Isolation of ubiquitinated proteins to high purity from *in vivo* samples

i. Summary

Ubiquitination pathways are widely used within eukaryotic cells. The complexity of ubiquitin signaling gives rise to a number of problems in the study of specific pathways. One problem is that not all processes regulated by ubiquitin are shared among the different cells of an organism (e.g. neurotransmitter release is only carried out in neuronal cells). Moreover, these processes are often highly temporally dynamic. It is essential therefore to use the right system for each biological question, so that we can characterize pathways specifically in the tissue or cells of interest. However, low stoichiometry, and the unstable nature of many ubiquitin conjugates, presents a technical barrier to studying this modification *in vivo*. Here, we describe two approaches to isolate ubiquitinated proteins to high purity. The first one favours isolation of the whole mixture of ubiquitinated material from a given tissue or cell type, generating a survey of the ubiquitome landscape for a specific condition. The second one favours the isolation of just one specific protein, in order to facilitate the characterization of its ubiquitinated fraction. In both cases, highly stringent denaturing buffers are used to minimize the presence of contaminating material in the sample.

ii. Key Words

Ubiquitination, substrates, isolation, denaturing conditions.

1. Introduction

Ubiquitination of proteins is facilitated by the coordinated action of ubiquitin activating E1, conjugating E2 and ligating E3 enzymes, and can be reversed by the so-called deubiquitinating enzymes (DUBs) *(1)*. Along with these ubiquitinating enzymes, proteasomal subunits, shuttling factors and other ubiquitin binding proteins regulate the fate of the ubiquitinated substrates. Altogether, nearly one thousand proteins integrate the ubiquitin proteasome system (UPS), which in addition to being the main intracellular protein

degradation pathway, dynamically regulates the proteome by various other means too. Despite great advances in the mass spectrometry (MS) field, the identification of proteins regulated by the UPS is still a challenge, mostly because of the low levels at which ubiquitin-modified proteins are found within a cell. Historically, ubiquitin-binding domains (UBD) *(2)*, ubiquitin specific antibodies *(3*, *4)*, or epitope-tagged versions of ubiquitin *(5-7)* have been used in order to enrich the ubiquitinated protein fraction (ubiquitome). Here we present two methods that we recently developed: the first has allowed us to purify and enrich hundreds of ubiquitin conjugates *in vivo* from *Drosophila* melanogaster *(7)* and mouse *(8)* tissues, as well as from human cells *(9)*; the second has proved a valuable tool for validation of the ubiquitination of individual proteins and their mutants *(10)*, for characterization of ubiquitin linkages *(11),* as well for identification of E3 ligases responsible for their ubiquitination *(12)*.

1.1 The ^{bio}Ub purification strategy

The first successful proteomic approach ever to identify protein ubiquitination was performed in yeast *(5)* and based on His-tagged ubiquitin overexpression, which allowed for the use of denaturing buffers during the washing steps. A His-Ub transgenic mouse was reported *(13)*, although no proteomics approach was published with this model. One concern using this approach would be the presence of too many endogenous histidine-rich proteins in mammals, which bind to the nickel affinity beads, resulting in excessive background for MS approaches. On the other hand, affinity pulldowns that cannot withstand denaturing conditions result in high background that can be observed by Coomassie staining (*14)*.

In recent years, ubiquitin remnant diGly-specific monoclonal antibodies have been used for the isolation and identification of thousands of putative ubiquitination sites in a number of systems *(4, 15-19)*. However, the diGly signature is also given by other ubiquitin-like proteins such as Nedd8, which is in general less abundant than ubiquitin, but whose concentration increases dramatically after the proteasome blockade commonly employed in ubiquitome studies *(20)*. Furthermore, the diGly approach can only isolate ubiquitinated proteins after trypsin cleavage, precluding the possibility of immunoblotting to validate them or to identify the type of ubiquitin chains (mono- or poly-) formed *in vivo*. The over-reliance on one single method for the identification of the substrates could also result in a significant presence of false positives *(21)*. Even if the actual validation of this technique is based on the identification of the diGly signature by MS, 65% or more of the peptides isolated using diGly antibodies show no diGly signatures on them *(18, 19)*. One last caveat is that diGly antibodies cannot identify proteins ubiquitinated at other residues (like cysteines) in place of the canonical lysine residue *(8, 22-24)*.

We developed the ^{bio}Ub strategy *(7-9, 25)* based on a short biotinylable motif *(26)* that is expressed N-terminally to each of several ubiquitin moieties expressed in tandem. This precursor polypeptide, which also contains the bacterial BirA enzyme, is digested by the deubiquitinating activity of endogenous DUBs, therefore allowing the BirA enzyme to

specifically biotinylate all its target ubiquitins before they are attached to their substrates. Thanks to the strong affinity of the avidin-biotin interaction, very stringent washes can be applied to biotinylated material purified on avidin resins, resulting in a high enrichment of the ubiquitinated fraction in the eluted sample (Figure 1). Furthermore, the stringent isolation and washing procedures prevent any protease and DUB activity and help to maximize the yield of purified ubiquitinated material. Additionally, the use of specific expression systems, such as the GAL4/UAS in *Drosophila* or tissue-specific/Tetracycline-regulated promoters in mice, can direct the biotinylated ubiquitin (^{bio}Ub) to certain cell populations and/or stages during development, allowing the isolation of ubiquitinated material in a tissue- and time-specific manner. We have so far identified - by MS - over 4,000 ubiquitinated proteins isolated using this strategy in various systems *(7, 8, 10 and unpublished results)*.

1.2 The GFP-pulldown strategy

In vitro strategies are routinely used to test ubiquitin substrates. *In vivo* validation of protein ubiquitination is more complicated: a number of pulldown approaches have been developed to test for bait/target protein ubiquitination. In most cases, however, these purifications are not carried out under denaturing conditions, leaving open the possibility that the detected ubiquitin signal arises from an interacting protein.

Single chain anti-GFP beads (Chromotek GmbH) are usually washed with non-denaturing buffers to achieve pulldowns in which protein interactions are preserved. We found, however, that these beads also allow for extremely stringent washes, giving a sensitive and quantifiable assay for ubiquitination *(10, 12)*. Since ubiquitination is a covalent conjugation, which can resist highly denaturing conditions, the usage of stringent washes allows for the elimination of all non-covalently bound interactions. We describe here a protocol that takes therefore full advantage of the potential of GFP nanobody technology. Capture of the GFP-tagged proteins is performed under non-denaturing conditions, with the buffer being supplemented with NEM to block deubiquitination, or post-lysis ubiquitination of substrates. Once the GFP-tagged proteins are bound to beads, highly stringent washes are applied. If improved detection of the ubiquitinated material is required, cells can be co-transfected with FLAG-tagged ubiquitin, or ubiquitin labelled with any other small tag (biotin, HA, His). Immunoblotting to reveal the ubiquitinated fraction of the purified GFP-tagged proteins can then be performed with antibodies to the tag carried on ubiquitin (Figure 2). Observation of slower migrating ubiquitin-loaded species confirms ubiquitination of candidate substrates. Characterization of the ubiquitinated fraction can also be carried out by immunoblotting with antibodies to specific ubiquitin chain linkages (*11*). The key advantage of this protocol is its simplicity and accessibility to any researcher, as well as the fact that the ubiquitin signal is not distorted by co-purifying species that would otherwise prevent a reproducible quantification of *in vivo* ubiquitination.

This strategy can be used to confirm and characterize the ubiquitination of a given substrate, but can also be applied to other post-translational modifications, such as SUMOylation. The approach can be used to validate E3 ligases / DUBs involved in modifying a given substrate and to test if a candidate site is indeed so modified in the system of interest.

As compared to *in vitro* (from reconstituted components) or *ex-vivo* (using *Xenopus* egg extracts, for example) approaches, this strategy can be used to monitor ubiquitination as it happens within the cells, from *in vivo*-isolated material (cells, flies, mice). It works best with co-transfection of tagged ubiquitin (since antibodies against tags tend to be more sensitive than those against ubiquitin) together with the GFP-tagged protein of interest, but we have also successfully used this assay to look at endogenous ubiquitin chains *(11)*. We believe this *in vivo* ubiquitination assay will also facilitate the identification of both ubiquitination sites and ubiquitin chain linkages by MS on a specific substrate, thanks to the purity of the sample.

2. Materials

2.1 The ^{bio}Ub purification strategy

1. Cell lines, flies or mice expressing either the precursor carrying the tandem modified-ubiquitin molecules plus BirA, or BirA alone as control population.
2. Two dounce tissue grinders 7 mL (Jencons).
3. High-capacity NeutrAvidin-agarose beads (ThermoScientific).
4. PD10 desalting columns (GE Healthcare).
5. N-ethylmaleimide (NEM, Sigma).
6. Protease inhibitor cocktail (Roche Applied Science) at 25X concentration: 1 tablet in 2 mL of Lysis/Binding buffer.
7. Lysis buffer: 8 M Urea, 1% Sodium Dodecyl Sulfate (SDS), 50 mM NEM in Phosphate Buffered Saline (PBS) (see **Note 1**).
8. Binding buffer: 3 M Urea, 1 M Sodium Chloride (NaCl), 0.25% SDS, 50 mM NEM in PBS.
9. Dilution buffer: 1.43 M NaCl, 1X Protease inhibitor cocktail, 50 mM NEM in PBS (see **Note 2**).
10. Washing buffer 1 (WB1): 8 M Urea, 0.25% SDS in PBS.
11. WB2: 6 M Guanidine Hydrochloride (GdnHCl) in PBS.
12. WB3: 6.4 M Urea, 1 M NaCl, 0.2 % SDS in PBS.
13. WB4: 4 M Urea, 1 M NaCl, 10% Isopropanol, 10% Ethanol, 0.2% SDS in PBS.
14. WB5: 8 M Urea, 1% SDS in PBS.
15. WB6: 2% SDS in PBS.

16. Elution buffer (4X Laemmli SDS loading buffer): 200 mM Tris-HCl, pH 6.8, 8% SDS, 40% Glycerol, 0.8 mg/mL Bromophenol blue, with the addition of 100 mM Dithiothreitol (DTT) prior to use.
17. Mini-column clarifying filters (Sartorious).

2.2 The GFP-pulldown strategy

1. Cells or tissue expressing a GFP-tagged candidate ubiquitinated protein (**Note 3**).
2. Lysis buffer: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Triton-X100, 1X Protease Inhibitor cocktail (Roche Applied Science), 50 mM NEM.
3. GFP Trap-A or GFP Trap-MA beads suspension (Chromotek GmbH).
4. Dilution buffer: 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1X Protease Inhibitor cocktail, 50 mM NEM.
5. Stringent wash buffer: 8 M Urea, 1% SDS in PBS.
6. Non-denaturing wash buffer: 10 mM Tris-HCl pH 7.6, 1 M NaCl, 0.5 mM EDTA, 1% (v/v) Triton-X100.
7. 1% SDS in 1X PBS
8. Elution buffer (4X Laemmli SDS buffer): 200 mM Tris-HCl pH 6.8, 8% SDS, 40% Glycerol, 0.8 mg/mL Bromophenol blue, with the addition of 100 mM DTT prior to use.

3. Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 The ^{bio}Ub purification strategy

1. Prewash about 0.2 mL of NeutrAvidin-agarose beads suspension by resuspending in binding buffer and centrifuging 1 min at 1000 rpm. Discard the supernatant and keep beads for later (See **Note 4**).
2. Collect ^{bio}Ub tissues in 2.5 mL of Lysis buffer + 400 µL of 25X Protease Inhibitor cocktail (prepared in Lysis Buffer) (see **Note 5**).
3. Crush tissues using a 7 mL Dounce tissue homogenizer (see **Note 6).**
4. Centrifuge 1 min at 14,000 rpm and discard the pellet.
5. Centrifuge the supernatant for 5 min at 14,000 rpm at 4ºC. Repeat this step if needed.
6. Apply supernatant to a PD10 column previously equilibrated with 25 mL of binding buffer (see **Note 7**).
7. Collect eluate extract (3.5 mL) into 250 µL of 25X Protease Inhibitor cocktail (diluted in binding buffer). Keep 1% of the extract as input for immunoblot analysis.

8. Incubate extract with the prewashed NeutrAvidin agarose beads for 40 min at room temperature and 2 h at 4ºC (see **Note 8**).
9. Spin down the beads (2 min at 1000 rpm) and keep the supernatant as 'unbound fraction' for immunoblot analysis.
10. Wash beads in 15 mL tubes with washing buffers (WB, about 12 mL each time). Incubate 5 min with each WB and gentle rolling, then spin down the beads (2 min at 1000 rpm) and discard the used buffer. The number of washes and the order in which buffers are applied is as follows: 3 times with WB1, 3 times with WB2, 1 time with WB3, 3 times with WB4, 1 time with WB1, 1 time with WB5 and 3 times with WB 6.
11. Boil beads in 100 µL elution buffer (see **Note 9**) for 10 min.
12. Centrifuge boiled beads in a mini-column filter for 2 min at 14,000 rpm to recover the ubiquitin conjugates.
13. Eluted samples are typically processed for SDS gel electrophoresis and immunoblotting, or for LC-MS/MS analysis (see **Note 10**).

3.2 The GFP-pulldown strategy

We have successfully applied this basic protocol to insect cells and to mammalian cells, with different optimizations achieved where indicated. Users should optimize conditions for pulldown of their preferred GFP-tagged protein.

1. Prewash 5 - 15 µL per sample of GFP Trap-A or GFP Trap–MA beads suspension by collecting the beads and resuspending them in Dilution buffer. GFP Trap-A beads are collected by centrifuging 2 min at 2700 rpm and GFP Trap-MA beads on a magnetic stand. Repeat once or twice.
2. Wash cells (or tissues) once in PBS and harvest using Lysis buffer (see **Note 11**). We typically resuspend 3 x 10^6 harvested mammalian cells in 100 µL ice-cold Lysis buffer and incubate on ice for 30 min with regular mixing of tube contents (see **Note 12**), or else scrape insect cells from 6-well plates straight into 300-500 µL of Lysis buffer per well (typically, 1 x 10^6 cell/mL).
3. Centrifuge lysate for 5 min at 12,500 rpm in a cold room. Collect the supernatant, which can be diluted to 0.1% Triton X-100 (using Dilution buffer) to improve binding to the beads. Remove 25 µL sample for immunoblotting as 'input fraction'. Mix lysate with previously washed GFP Trap beads and incubate at RT for 2h with gentle rolling.
4. Collect beads and remove supernatant, keeping 25 µL sample as 'unbound fraction' for immunoblotting.
5. Wash beads once with 1 mL of ice-cold Dilution buffer.
6. Resuspend beads in 1 mL of Stringent wash buffer and incubate them for 1 - 5 min with gentle rolling before collecting beads and discarding supernatant.
7. Wash beads three times with 1 mL of non-denaturing wash buffer, incubating for 5 min with gentle rolling each time.

8. Wash beads once with 1 mL SDS buffer, incubating for 5 min with gentle rolling.
9. Elute bound proteins from beads, by boiling at 95ºC for 10 min with 5 - 20 µL of elution buffer.

4. Notes

1. As solubilized urea is in equilibrium with ammonium cyanate that leads to carbamylation of amine groups in proteins, a reaction accelerated by heating, we generally use fresh urea solutions. In the case of WB3 and binding buffer, if SDS precipitates and needs to be re-dissolved, we warm up the buffer to 35 ºC for no longer than 15 min.

2. Dilution buffer is only needed when ^{bio}Ub pulldown is performed from cells, to adjust the composition of cell lysates which are usually too dense to pass through the PD10 column. In this experiment, lysates are diluted with the volume of Dilution buffer required to adjust Urea concentration to 3 M (as in Binding buffer).

3. We have also successfully used this protocol with Venus- and YFP- tagged candidate proteins.

4. The bead volume needs to be optimized according to the amount of tissue/cells used and to the level of ^{bio}Ub expression. We usually titrate the bead volume, immunoblotting the unbound fraction to find the minimum bead volume that does not compromise the efficiency of pulldown (Figure 1A). Similarly, the quantity of tissue/cells needs to be adjusted, as beads have a limited binding capacity. For instance, for 1 g of *Drosophila* embryos expressing ^{bio}Ub in the nervous system we typically use 0.1 mL of beads. In the case of mice, however, 0.3 mg of liver is enough to saturate a similar quantity of beads. In the case of mammalian cells we have used 1 mL of beads for 3 x 10^8 cells *(9)*.

5. For 3 x 10^8 mammalian cells we have used 10 mL of Lysis buffer.

6. For cells, the lysate should be syringed 5X through a 22G needle (to shear DNA) at this step, then diluted (as described in Note 2 and 7) before skipping to Step 8 of the protocol.

7. PD10 columns are used to eliminate free biotin, but also as a buffer exchange step. We equilibrate the column with Binding buffer, so the sample is exchanged into Binding buffer ready to incubate with the beads. In the case of cells do not use PD10

columns; instead, add 17.5 mL of Dilution buffer to 10 mL cell lysate and mix directly with 1 mL of NeutrAvidin beads.

8. Incubation has been optimized to the minimum time required for proper binding of biotinylated material to the beads. However, incubation can be alternatively performed overnight at 4 ºC, although this might increase non-specific binding.

9. The volume of elution buffer can be reduced if more concentrated sample is required.

10. We recommend to always run 10% of the eluate in a separate gel for Silver staining. The observation of a significant difference in total material purified from cells expressing ^{bio}Ub versus material purified from control BirA cells, with similar levels of the endogenous biotinylated proteins (see Figure 2), allow us to confirm that the whole process was performed correctly. If samples are intended for MS analysis, performing the pulldown in triplicate is recommended.

11. The number of cells and volume of beads to use should be determined empirically based on the size, expression level and ubiquitination level of the substrate. In our experience of purifying GFP-tagged proteins from mammalian cells to look for a ≤ 1% polyubiquitinated fraction, 5 µL GFP Trap slurry and 3 x 10^6 cells is required to generate a robustly quantitative signal from one immunoblot. In our experience of detecting mono- or multi- ubiquitinated fractions of GFP-tagged proteins from insect cells, 5 x 10^5 insect cells and 15 µL GFP Trap slurry is required. We have found GFP Trap-A and –MA beads to have a similar binding capacity for GFP-tagged proteins.

12. To extract nuclear proteins, increase Triton-X100 concentration to 1%.

5. References

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Figure legends

Figure 1. Isolation of ubiquitin conjugates using the ^{bio}Ub pulldown. A) Titration of bead volumes (see Note 4): Lysates from ^{bio}Ub-expressing mammalian cells were prepared as described from 3.1.1 through 3.1.7 and incubated with the volumes of bead suspension indicated per 100ul of lysate. 10ul of each unbound fraction was tested by immunoblot with antibody against biotin to reveal the volume of bead suspension required for efficient pulldown at full bead capacity (between 5 and 10 ul for high-capacity NeutrAvidin-agarose (Thermoscientific) and 100ul for streptavidin-Dynabeads (Life Technologies)). The prominent band at 25kDa is mono-ubiquitinated histone H2A. **B)** Monitoring of ^{bio}Ub pulldown protocol: Lysates from ^{bio}Ub-expressing *Drosophila melanogaster* embryos (^{bio}Ub) were subjected to the biotin pulldown as described in section 3.1. Anti-biotin western immunoblotting to those pulldown experiments reveal a yield of approximately 25%. Flies overexpressing only BirA were used as the control sample for the pulldown (BirA) **C)** Eluates from ^{bio}Ub pulldown protocol: Liver lysates from ^{bio}Ub-expressing mice (^{bio}Ub) were prepared as described in section 3.1. Silver staining from three independent pulldown experiments confirmed that the purification of ubiquitinated conjugates is specific to the ^{bio}Ub sample: only a few endogenously biotinylated proteins are purified from the control (BirA) mouse liver. This silver stained gel is presented here with the permission of Dr. Benoit Lectez.

Figure 2. Testing the *in vivo* ubiquitination of lysine mutants using the GFP pulldown. A) *Drosophila* BG2 cells were transfected with a GFP-tagged ubiquitin substrate (wild type: WT, single lysine mutants K71R and K78R, and double lysine mutant: DM) as well as with FLAG-tagged ubiquitin. Anti-GFP (green) and anti-FLAG (red) antibodies were used to monitor the transfection levels in the whole cell extract. Both GFP-tagged protein and FLAG-tagged ubiquitin transfection efficiency were similar among samples **B)** Lysates from *Drosophila* BG2 *cells* were subjected to the GFP pulldown as described in section 3.2. Anti-GFP and anti-FLAG antibodies were used to, respectively, verify that similar amounts of GFP-tagged substrate was purified for all constructs and to monitor the level of ubiquitination for each construct. While GFP levels were similar among different constructs, ubiquitination was clearly reduced in those proteins were Lysines were mutated, especially in the double lysine mutant (DM).

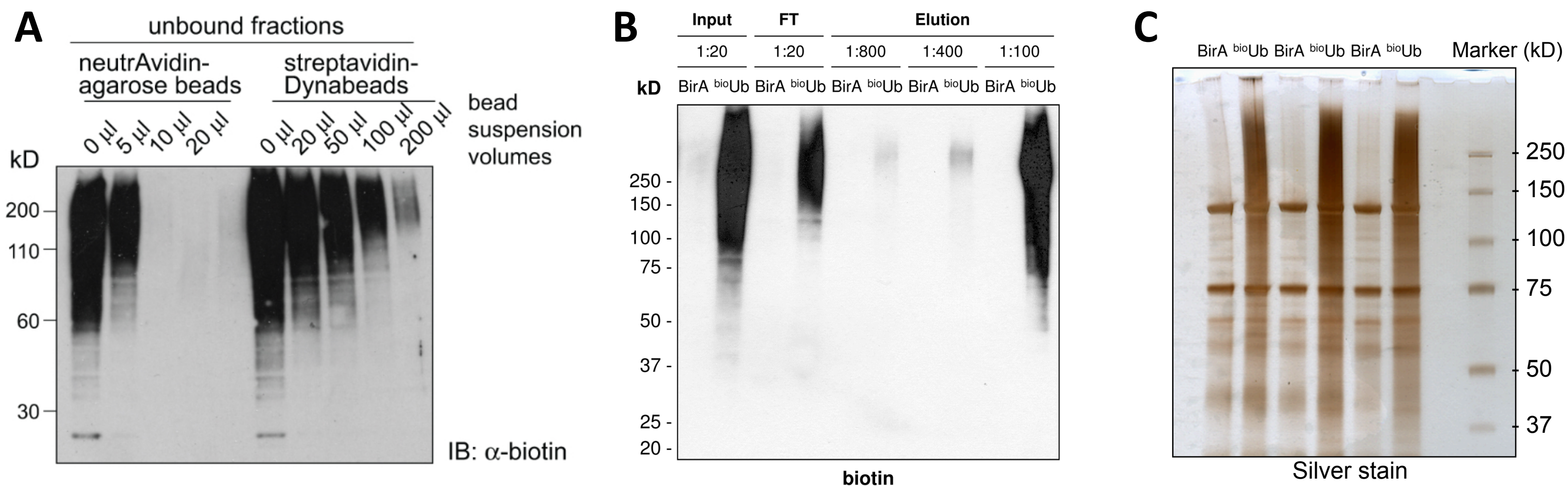

Figure 1

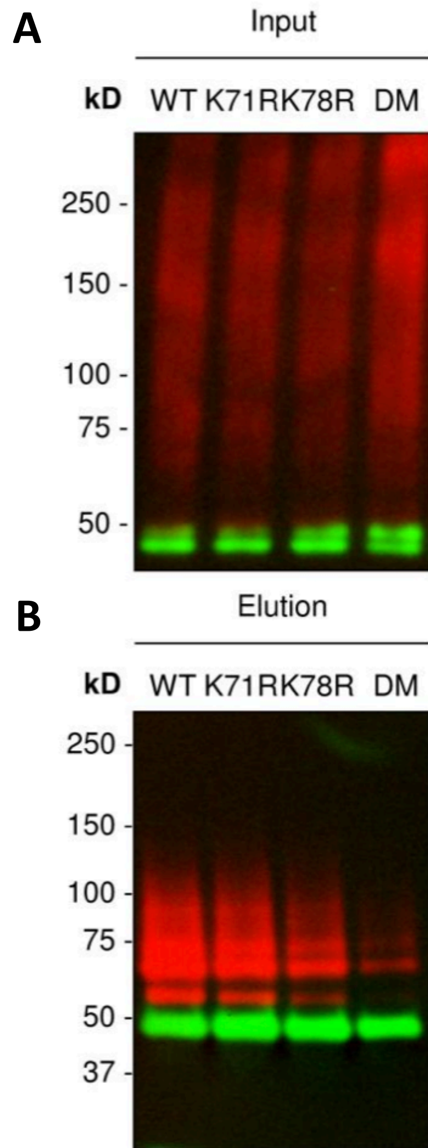

Figure 2