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► **To cite this version:**

Maria Gonzalez-Santamarta, Laurie Ceccato, Ana Sofia Carvalho, Jean-Christophe Rain, Rune Matthiesen, et al.. Isolation and Mass Spectrometry Identification of K48 and K63 Ubiquitin Proteome Using Chain-Specific Nanobodies. Manuel S. Rodriguez; Rosa Barrio. The Ubiquitin Code, 2602, Springer US, pp.125-136, 2023, Methods in Molecular Biology, 978-1-0716-2858-4. 10.1007/978-1-0716-2859-1\_9 . hal-04269347

**HAL Id: hal-04269347**

**<https://hal.science/hal-04269347v1>**

Submitted on 6 Nov 2023

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1 **Isolation and Mass Spectrometry Identification of K48 and K63 Ubiquitin**  
2 **Proteome using Chain-Specific Nanobodies.**

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5

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13

14

15 **Abstract**

16 Protein ubiquitylation is an essential mechanism regulating almost all cellular functions

17 in eukaryotes. The understanding of the role of distinct ubiquitin chains in different

18 cellular processes is essential to identify biomarkers for disease diagnosis and

19 prognosis, but also to open new therapeutic possibilities. The high complexity of

20 ubiquitin chains complicates this analysis, and multiple strategies have been developed

21 over the last decades. Here we report a protocol for the isolation and identification of

22 K48 and K63 ubiquitin chains using chain-specific nanobodies associated to mass

23 spectrometry. Different steps were optimised to increase the purification yield and

24 reduce the binding on non-specific proteins. The resulting protocol allows the

25 enrichment of ubiquitin chain specific targets from mammalian cells.

26 **Key Words:** Ubiquitin, Nanobodies, Post-translational modifications, Proteome,  
27 Isolation, Mass Spectrometry.

28 **Running head:** Ubiquitin proteome isolation using nanobodies

29

## 30 **1. Introduction**

31 Protein ubiquitylation regulates virtually all cellular processes, underlining its  
32 importance in the cell well-being, and its implications in multiple pathologies (1).  
33 Ubiquitin (Ub) attachment (AKA conjugation) occurs mainly in lysine (K) residues on  
34 the target proteins but also on any of the 8 reactive residues present on Ub itself (M1,  
35 K6, K11, K27, K29, K33, K48 or K63) (2). For this reason, Ub imposes distinct  
36 conformations to the target protein depending on the number of moieties attached to its  
37 surface, or the type of Ub chains formed. The complexity of the Ub chains increases  
38 when other post-translational modifications (PTMs) such as phosphorylation,  
39 acetylation, SUMOylation or NEDDylation act on the same Ub reactive residues. This  
40 complex language and its regulation, known as “the Ubiquitin Code”, is far from being  
41 fully understood and only some generic functions for few Ub chains have been  
42 elucidated over the years (3). For example, typical Ub chains, like K48 and K11 have  
43 been associated to proteasomal degradation, while K63 is usually linked to the  
44 regulation of signal transduction and the autophagy pathway. Less abundant atypical Ub  
45 chains such as K6, K27, K29, K33, and M1 have not been fully deciphered most likely  
46 due to the difficulty to fully characterise their functions (3, 4). The high reversibility  
47 and heterogeneity of those Ub modifications complicate their isolation, quantitation,  
48 and characterisation of the functions they regulate. A better characterisation of the  
49 molecular mechanisms underlying Ub regulated processes is essential to understand cell  
50 physiology and pathology (2).

51 Multiple approaches have been used to understand Ub chain-specific functions  
52 including the use of Ub K to R mutants and tagged Ub-like molecules (UbLs).  
53 However, the use of Ub mutants and overexpression has disadvantages, mainly because  
54 they can induce perturbations in the natural equilibrium of Ub chains and UbL  
55 molecules resulting in aberrant patterns of protein ubiquitylation complicating data  
56 interpretation (5). Furthermore, some of these techniques cannot be used to analyse  
57 samples derived from patients (6, 7). Ub-binding domains with Ub-chains preferences  
58 have been also used to explore processes regulated by protein ubiquitylation when  
59 associated to mass spectrometry (MS) (7, 8). Moreover, Ub antibodies directed against  
60 specific Ub-linkages showed some success in immunoprecipitation and  
61 immunofluorescence application. However, only few Ub-chain specific antibodies have  
62 been developed in part due to the low reactivity of this highly conserved molecule. In  
63 contrast, antibodies recognising the double glycine (“diGly”) Ub signature showed good  
64 success in MS studies even if these cannot distinguish the common signature shared  
65 with NEDD8 or ISG15 (9, 10). An important aspect that has contributed to increase our  
66 understanding of functions regulated by Ub is the huge progress in MS technology,  
67 achieved by enhancing sensitivity, accuracy and duty cycle of the machines and  
68 improving in the sample preparation methods.

69 Camelid heavy-chain antibodies, also known as VHH (variable heavy homodimers) or  
70 nanobodies, are single domain antigen-binding fragments that have been used in  
71 research, diagnostics and therapeutics (11–13). Nanobodies have a smaller size than  
72 mammalian antibodies, a single-domain nature, and can be produced as a robust entity  
73 very efficiently and with high solubility. Due to its monomeric nature, high stability,  
74 selectivity and small size, VHH have been used as sensitive detection probes in  
75 complex samples. Even if it is not the most obvious, VHHs can be generated against

76 small peptides, and can be used to purify ligands, for immunoprecipitation, interference  
77 with protein conformation/localisation, or regulate functions (*in vivo*  
78 immunomodulators) (**12, 13**).

79 Taking advantage of a full synthetic system that bypass immunogenicity and specificity  
80 problems, this scaffold has been used to develop chain-specific nanobodies (**7**). When  
81 isolated by phage display these polypeptides can be used as minibodies when cloned  
82 into vectors expressing the heavy chain of various species or as nanobodies when  
83 cloned in vectors with multiple tags (**14**).

84 Here we report the protocol for the purification of endogenous Ub chains using K63 and  
85 K48 nanobodies that can be used for identification of target proteins by MS. The  
86 protocol includes 4 sections, 1) The purification of Ub chain-specific nanobodies from  
87 bacteria; 2) The crosslinking of these nanobodies into a magnetic column (*see* **Figure**  
88 **1**); 3) Isolation of ubiquitylated proteins from mammalian cells and 4) Sample  
89 preparation for MS analysis (*see* **Figure 2**). The purified material obtained from  
90 mammalian cells can also be used for western blot (WB) analysis or  
91 immunoprecipitations with antibodies recognising proteins of interest. In this protocol,  
92 we have used mantle cell lymphoma cells, but depending on the cell type and the  
93 application of interest, this protocol can be easily adapted.

94

## 95 **2. Materials**

96 All materials have been prepared with ultrapure water, filtered and stored at 4°C. Fresh  
97 solutions should be used (*see* **Note 1**).

98

### 99 **2.1 Nanobodies purification**

- 100 1. Nanobodies used in this protocol are commercially available (Nanotag
- 101 Biotechnologies, N1910 and N1810).
- 102 2. Nanobodies can also be produced using *Escherichia coli* (WK6 strain).
- 103 3. 100 mg/ml carbenicillin LB-agarose plates
- 104 4. Terrific Broth (TB): Resuspend 47.6 g of TB powder and 8 ml of glycerol in 1 L of
- 105 distilled water and autoclave. TB media must be supplemented with 1% of glucose
- 106 when indicated.
- 107 5. IPTG (isopropyl  $\beta$ -D-1-thiogalactopyranoside): 1 M IPTG.
- 108 6. Sterile cold 1x Phosphate Buffered Saline (PBS).
- 109 7. Bacteria Lysis Buffer (BLB): 10 mM imidazole, 1 mg/ml of polymyxin B, 1 mM
- 110 phenylmethylsulfonyl fluoride (PMSF), 1.2 mg/ml lysozyme Cf, 2 mM dithiothreitol
- 111 (DTT) in 1x PBS.
- 112 8. Ni-NTA agarose-beads: Beads must be pre-washed three times during 5 minutes with
- 113 ten volumes of 1x PBS followed by centrifugation at 300 x g for 5 minutes to obtain a
- 114 50% ( $V_{\text{beads}}/V_{\text{lysate}}$ ) working reagent.
- 115 9. Nickel Column Washing Buffer (NWB): 50 mM Tris pH8, 500 mM NaCl, 10 mM
- 116 imidazole.
- 117 10. Nickel Column Elution Buffer (NEB): 50 mM Tris pH8, 500 mM NaCl, 200 mM
- 118 imidazole, 10 mM DTT.
- 119 11. Dialysis Buffer (DB): 50 mM Tris pH 8 and 500 mM NaCl.
- 120 12. Boiling Buffer (BB) 3X: 50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS,
- 121 Bromophenol Blue, 5%  $\beta$ -mercaptoethanol.
- 122 13. Ultracentrifuge filters (15 ml), nominal molecular weight cut off 3 kDa.
- 123 14. Dialysis tubing, benzoilated.
- 124 15. Prepacked gravity-flow chromatography empty plastic columns.

- 125 16. Glycerol 100%.
- 126 17. Water bath incubator (for heat-shock at 42°C).
- 127 18. Bacteria incubator (37°C).
- 128 19. Bacteria incubator with shaking (20-37°C).
- 129 20. Tip sonicator.
- 130 21. Magnetic tube holder.
- 131 22. Anti Ub antibody P4D1.

132

## 133 **2.2 Nanobody-magnetic beads crosslinking**

134 Here, we describe a BS3 (bis(sulfosuccinimidyl)suberate)-based crosslinking protocol  
135 that provides the best enrichment of ubiquitylated proteins with a low background (*see*  
136 **Note 2**).

- 137 1. Ni-NTA Magnetic Agarose Beads were pre-washed twice with cold PBS and kept at  
138 4°C until use.
- 139 2. Autoclaved sterile 1x PBS.
- 140 3. Ub chain-specific nanobodies (Nanotag).
- 141 4. Crosslinking Buffer (CB): 5 mM BS3. Prepare 1 M aliquots and store at -20°C.
- 142 5. Binding Buffer: 50 mM Tris-HCl pH 8.5, 150 mM NaCl, and 0.5% Igepal. pH  
143 adjusted in a chemical hood. Store solution at 4°C.
- 144 6. Blocking Buffer: 1.5 M Tris-HCl pH 7.5. pH adjusted in a chemical hood. Store  
145 solution at 4°C.
- 146 7. Prepare 0.5 M K<sub>2</sub>HPO<sub>4</sub> in ultrapure water and filtrate. Store at room temperature  
147 (RT).
- 148 8. Rotating wheel for microcentrifuge tubes.
- 149 9. Magnetic tube holder for microcentrifuge tubes.

150

151 **2.3 Isolation of ubiquitylated proteins from mammalian cells**

152 1. RPMI-1640 medium containing 10 % foetal bovine serum, 2 mM L-glutamine, and

153 50 µg/ml penicillin-streptomycin (*see Note 3*).

154 2. Cell lysis buffer (CLB): 20 mM Phosphate buffer pH 7.5 [(Na<sub>2</sub>HPO<sub>4</sub>, NaH<sub>2</sub>PO<sub>4</sub>), 1%

155 Igepal, 2 mM ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), 50 mM

156 sodium fluoride, 5 mM tetra-sodium pyro-phosphate, 10 mM β-glycerol-2-phosphate].

157 Filter and store at 4°C.

158 3. CLB Supplement: 1 mM PMSF, complete mini-EDTA-free protease inhibitor

159 cocktail (Roche), 10 mM N-ethylmaleimide (NEM), 10 mM iodoacetamide (IAA), 20

160 nM bortezomib. Prepared just before use (*see Note 4*). Keep supplemented CLB on ice

161 until use.

162 4. PBS-Tween 20 0.05 %.

163 5. Cold sterile PBS.

164 6. Cold centrifuge (4°C) for microcentrifuge tubes.

165 7. Rotating wheel for microcentrifuge tubes.

166 8. Magnetic rack for microcentrifuge tubes.

167

168 **2.4 Mass Spectrometry**

169 1. Ultracentrifuge filter 0.5ml with a 30 kDa molecular weight cut-off

170 2. Urea buffer: 0.1 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 8.0

171 M Urea, pH 8.0.

172 3. 0.04 M ammonium bicarbonate buffer, pH 8.0

173 4. Iodoacetamide.

174 5. 1 M DTT.



175 6. Trypsin (sequencing grade)

176 7. C18 HPLC column.

177

### 178 **3. Methods**

#### 179 ***3.1 Purification of Ub chain-specific nanobodies***

180 Nanobodies are produced in *Escherichia coli* WK6 strain using the protocol described

181 below for production of recombinant histidinylated proteins since these small

182 polypeptides contain an N-terminal histidine (His) tag (*see Figure 1*) (*see Note 5*).

183 1. Transform bacteria with a nanobody-containing plasmid by heat shock at 42°C during  
184 2 minutes and grow them overnight for selection in 100 mg/ml carbenicillin LB-agarose  
185 plates.

186 2. The day after, pre-culture clones in 50 ml of TB containing carbenicillin at the same  
187 concentration.

188 3. Scale the culture up to 1 L in TB (supplemented with 1% of glucose) shaking at  
189 37°C, to reach a 0.7-0.8 confluence at an optical density (OD) of 600 nm.

190 4. Induce nanobody expression by the addition of 1 mM IPTG, and let bacteria grow for  
191 16 hours at 37°C or overnight (O/N) at 28°C.

192 5. Collect 1 ml of culture before and after IPTG induction to control the proper  
193 nanobody induction.

194 6. Pellet bacteria by centrifugation at 6,693 x g for 30 minutes at 4°C.

195 7. Wash pellet twice with cold PBS and resuspend in 10 ml of cold sterile PBS.

196 8. Lyse bacteria with BLB on ice by sonication at 10 μm (at least six pulses of 30  
197 seconds, 1 minute incubation on ice between each pulse).

198 9. Clarify lysate by ultracentrifugation for 1 hour at 45,000 x g at 4°C.

- 199 10. Incubate the clarified lysate with 1 ml of Ni-NTA agarose-beads, overnight, rotating  
200 at 4°C in a 50 ml tube.
- 201 11. Load agarose beads into a prepacked gravity-flow chromatography column and  
202 wash 10 times with 10 ml of NWB.
- 203 12. Elute nanobodies, 10 times with 1 ml of NEB and check the eluted protein peak by  
204 colorimetry intensity using the Bradford assay reagent.
- 205 13. Pool the most concentrated fractions and measure protein concentration by Bradford  
206 or any other method of choice.
- 207 14. Transfer the sample into benzoilated dialysis tubing and dialyse against 5 L of  
208 exchange buffer (DB) O/N at 4°C.
- 209 15. Concentrate dialysed proteins by using ultracentrifuge filter.
- 210 16. Estimate final protein concentration, using the nanodrop or any other method of  
211 your choice.
- 212 17. Supplement with 10% glycerol to store aliquoted samples at -80°C, until use.

213

### 214 ***3.2 Nickel magnetic beads crosslinking protocol***

215 Prepare nickel-magnetic beads for the affinity column and use a magnetic holder for  
216 microcentrifuge tubes to purify nanobody-interacting proteins (*see Note 6 and Note 7*).

217 To increase the number of washes and reduce the binding of non-specific proteins,  
218 crosslink nanobodies magnetic beads prior the isolation of ubiquitylated proteins (*see*  
219 **Note 8**).

- 220 1. For a large-scale purification of ubiquitylated proteins, crosslink 300-500 µg of  
221 nanobodies to 300 µl of nickel-magnetic beads in 1.5 ml microcentrifuge tubes (*see*  
222 **Note 9, Note 10 and Note 11**).

- 223 2. Adjust the volume to the maximal tube capacity with Binding Buffer and incubate for  
224 1-2 hours at 4°C using a rotating wheel (*see Note 12*).
- 225 3. After incubation, let the sample in the magnetic rack/holder for at least 5 minutes  
226 until magnetic beads are all attached to the magnet.
- 227 4. Keep the samples in the magnetic rack and remove the supernatant using aspiration or  
228 a 1 ml pipette.
- 229 5. Wash beads twice with cold sterile PBS and let beads attach to the magnet for every  
230 wash.
- 231 6. Incubate beads with 5 mM BS3 (CB) in the rotating wheel at RT for 1h.
- 232 7. Wash magnetic beads once with PBS, to remove the remaining BS3 solution.
- 233 8. Incubate beads with Blocking Buffer at RT for 15 minutes while rotating.
- 234 9. Wash beads with PBS to remove the remaining Blocking Buffer.
- 235 10. Wash once with 0.5 M K<sub>2</sub>HPO<sub>4</sub> to remove non-specific binding to the Nickel beads.
- 236 11. Wash three times with PBS to eliminate K<sub>2</sub>HPO<sub>4</sub> residues.
- 237 12. Wash and equilibrate crosslinked nanobody-nickel magnetic beads in Binding  
238 Buffer. Beads can be stored at 4°C for a few hours until the lysis of eukaryote cells is  
239 performed.

240

### 241 ***3.3 Isolation of ubiquitylated proteins from mammalian cells***

242 The following protocol is implemented using 40 million Mantle Cell Lymphoma (MCL)  
243 cells for a large-scale purification to be analysed by MS (*see Note 10*). To evaluate the  
244 capture efficiency and background, silver staining and WB against Ub antibodies are  
245 performed with all fractions before sending samples to MS analysis.

246 1. Prepare lysates using  $40 \times 10^6$  cells (or 2 mg of total protein) per experimental point.  
247 Pellet cells by 5 minutes centrifugation  $300 \times g$  at RT and wash twice with cold PBS.  
248 Keep cell pellets on ice until use.

249 2. Lyse cells pellets with 500  $\mu$ l of CLB supplemented with protease inhibitors (*see*  
250 **Note 4**) during 10 minutes. Disrupt cell pellets by gentle pipetting or intermittent soft  
251 shaking. All the procedure should be done on ice or at  $4^\circ\text{C}$  (*see* **Note 13**).

252 3. Clarify samples by centrifuging at  $48,000 \times g$  during 20 minutes at  $4^\circ\text{C}$ .

253 4. Save 1/20 of clarified supernatant (50  $\mu$ l) to control the input fraction. Mix the input  
254 fraction with an equal volume of BB and store at  $-20^\circ\text{C}$ .

255 5. Incubate supernatants with the crosslinked magnetic beads for 2 hours at  $4^\circ\text{C}$  in a  
256 rotating wheel (*see* **Note 14**).

257 6. After the binding step, use a magnetic holder to separate and collect the flow-through  
258 fraction (FT). Mix 1/20 (50  $\mu$ l) of FT with equal volume of BB and save to control the  
259 efficiency of the capture.

260 7. Remove supernatants as much as possible using a vacuum aspiration system or 1 ml  
261 pipette.

262 8. To eliminate unspecific proteins bound to nanobodies and with the help of the  
263 magnetic holder, wash beads 4-6 times with 1 ml of PBS-Tween 0.05% (*see* **Note 15**  
264 *and* **Note 16**).

265 8. Perform a final wash with 1 ml of cold PBS to transfer the sample to a new 1.5 ml  
266 tube.

267 9. Dry beads using the magnetic holder and a vacuum aspiration system.

268 10. Resuspend beads in 100  $\mu$ l of 1x BB and keep at RT until use.

269 11. Just before WB analysis, boil samples for 45 minutes at  $95^\circ\text{C}$  (*see* **Note 17 and**  
270 **Note 18**) with strong agitation.

271 12. Use a magnetic holder to collect the captured proteins of interest and eliminate  
272 beads. Keep the captured fraction at -80°C until MS analysis.  
273 13. Analyse the input, flow-through and captured fractions using an SDS  
274 polyacrylamide gel electrophoresis (SDS-PAGE) and stain the gel using Coomassie  
275 blue or silver staining. All fractions can be analysed by WB against anti-Ub antibodies.  
276

### 277 ***3.4 Sample preparation for Mass Spectrometry analysis***

278 1. Load captured proteins obtained in section 3.3 (step 10) into ultracentrifuge filters  
279 with 30 kDa cut off and spin 25 minutes at 14,000 x *g* (*see Note 19*).  
280 2. Wash proteins in the retentate with 0.2 ml of Urea Buffer, six times by spinning 25  
281 minutes at 14,000 x *g*.  
282 3. Incubate proteins in the retentate with 0.1 ml of 0.05 M iodoacetamide for 20  
283 minutes.  
284 4. Spin proteins in the retentate for 35 minutes at 14,000 x *g*.  
285 5. Wash proteins in the retentate with 0.1 ml of Urea Buffer, four times by spinning 25  
286 minutes at 14,000 x *g*.  
287 6. Wash proteins in the retentate with 0.1 ml of 0.04 M ammonium bicarbonate, pH 8.0,  
288 four times by spinning 25 minutes at 14,000 x *g*.  
289 7. Incubate proteins in the retentate with 40 µl 0.01 µg/µl of trypsin overnight at 37°C.  
290 8. Collect digested proteins by adding 40 µl of 0.04 M ammonium bicarbonate, pH 8.0,  
291 two times by spinning 25 minutes at 14,000 x *g*.  
292 9. Desalt peptides using reverse phase C18 HPLC column and then store them at -20°C,  
293 before MS analysis.

294

## 295 **4. Notes**

- 296 1. Solutions should not be stored for more than one week before their use.
- 297 2. Other crosslinking procedures can be tested to optimise the recovery of the captured  
298 ubiquitylated proteins, including dimethyl pimelimidate-based protocols.
- 299 3. This medium was used to grow mantle cell lymphoma cell lines (MCL) but medium  
300 can be adapted to other cell lines.
- 301 4. Alternative inhibitors such as MG132 instead of bortezomib, PR619 instead of  
302 chemical deubiquitinase inhibitors such as iodoacetamide or N-ethylmaleimide can be  
303 used to avoid losing ubiquitylated proteins during the lysis step.
- 304 5. The His tag or a Myc tag also included in the nanobody can be used to analyse the  
305 integrity and the quality of purification of these tools.
- 306 6. In order to reduce contaminants during the isolation of ubiquitylated proteins from  
307 mammalian cells, affinity chromatography is performed within a clean area using gloves  
308 during all the procedure.
- 309 7. Affinity columns can be prepared using other supports such as nickel-agarose or  
310 nickel-sepharose beads.
- 311 8. Magnetic beads also avoid the use of centrifugation steps that could precipitate  
312 unspecific high molecular weight protein complexes
- 313 9. Excess of Nickel beads should be prepared since some could be lost during the  
314 washing steps of the crosslinking protocol.
- 315 10. Different cells provide distinct total protein concentration per million of cells. For  
316 this reason, 500 µg of total protein is a better reference to set up a protocol before  
317 scaling up to 2 mg of total protein per point.
- 318 11. The number of beads and nanobodies required to capture the ubiquitylated fraction  
319 from a given sample must be optimised to avoid saturation of the beads or nanobodies.

320 This situation could make difficult the identification of physiological differences  
321 between samples.

322 12. If aggregates are observed during the nanobody/beads binding step, the  
323 concentration of detergent should be increased in the Binding Buffer.

324 13. Cell lysis should be done on ice to prevent enzymatic activities that could remove  
325 Ub or degrade modified proteins. The time between the lysis and the incubation with the  
326 crosslinked nanobodies should be as short as possible.

327 14. Binding time can be adjusted: increase it if ubiquitylated proteins are highly  
328 abundant or decrease it in the opposite situation. During the binding step, keep tubes  
329 safely close to avoid spills.

330 15. The use of crosslinked nanobodies allows to increase the number of washes or the  
331 use of stringent washing conditions if required. Detergent can be added to increase the  
332 stringency.

333 16. The different washing steps were optimised to reduce the binding of unspecific  
334 proteins compared to a negative control (magnetic beads only).

335 17. Do not freeze magnetic beads bound to captured ubiquitylated proteins prior the  
336 final heating step, since this could damage the magnetic properties of some beads.

337 18. Eluted magnetic beads can be resuspended in Boiling Buffer to test if some  
338 ubiquitylated proteins are still attached. In this case, another boiling step is required to  
339 recover those proteins.

340 19. If the protein sample volume exceeds 0.5 ml, perform step 1 of section 3.4 the  
341 number of times necessary to load the entire sample volume to the ultracentrifuge filter.  
342  
343

344 **Acknowledgements:**

345 We thank all members of the UbiCARE lab, Clémence Coutelle-Rebut for the  
346 proofreading of this manuscript. MGS is a fellow from the UbiCODE project funded by  
347 the EU's Horizon 2020 research and innovation program under the Marie Skłodowska-  
348 Curie grant agreement (No 765445) and the Association contre le Cancer (ARC). We  
349 acknowledge the COST Action CA20113 `PROTEOCURE` supported by COST  
350 (European Cooperation in Science and Technology). MSR was also funded by  
351 CONACyT-SRE (Mexico) grant 0280365 and the REPERE and prématuration  
352 (Ubipièges) programs of Occitanie.

353

354

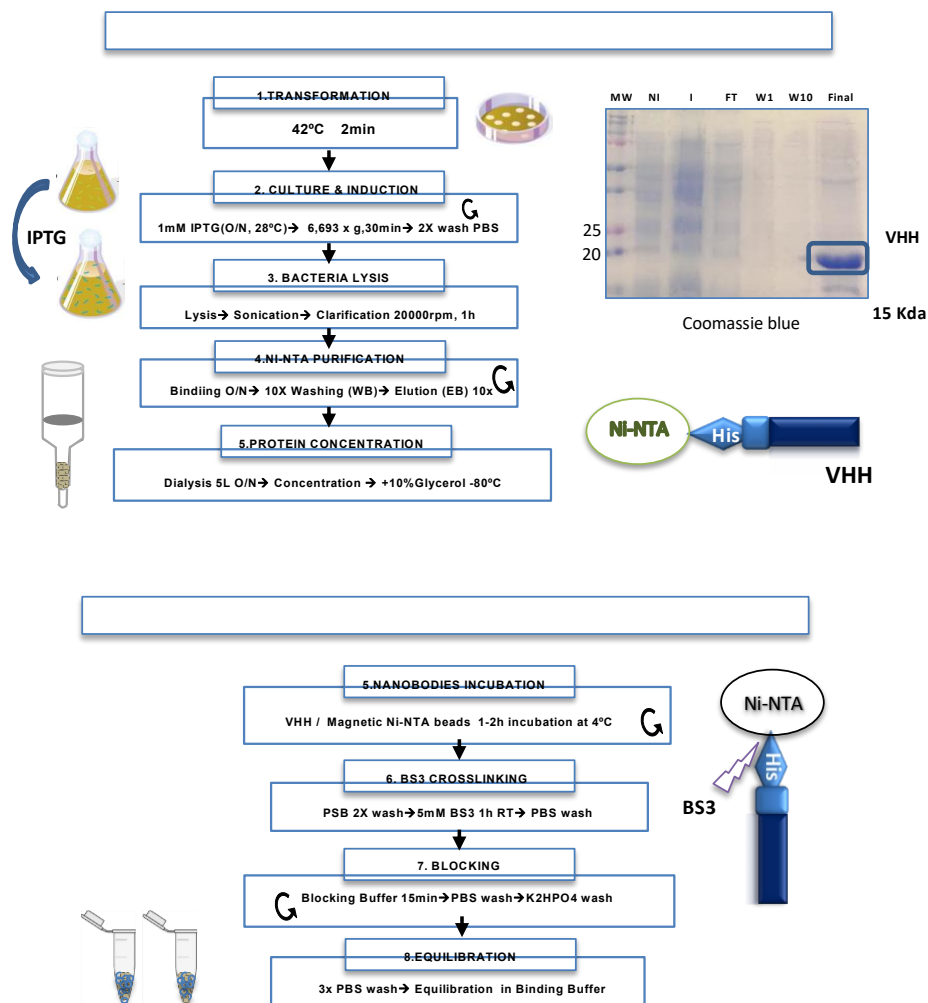
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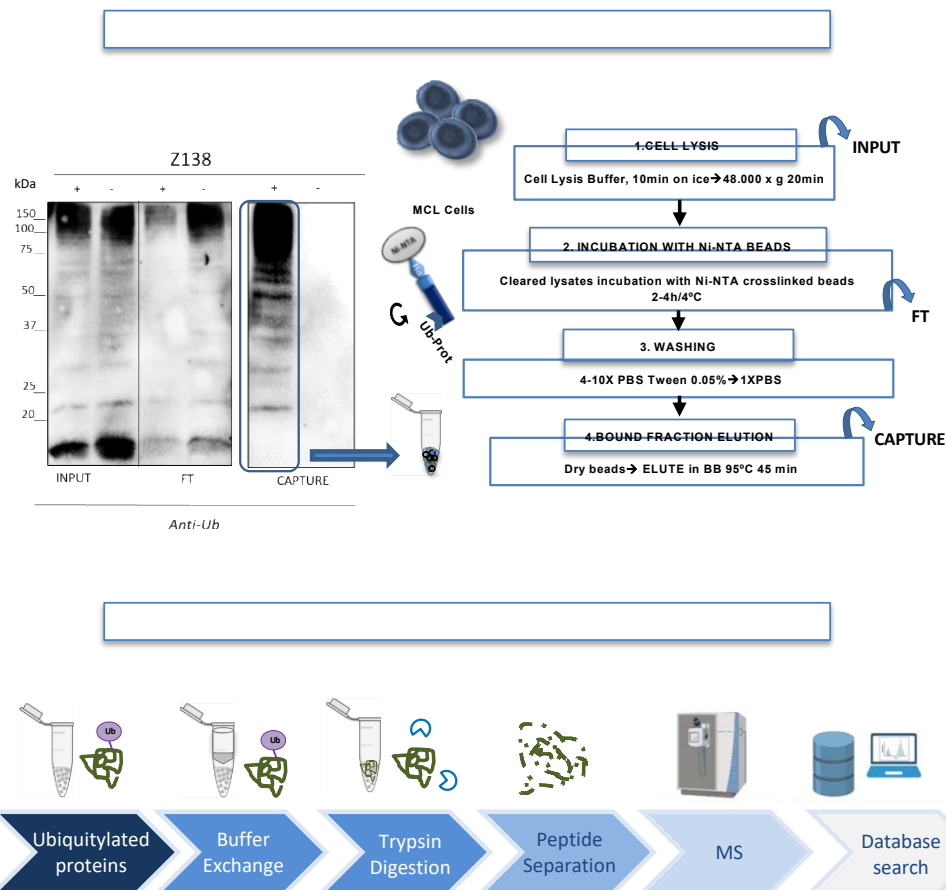
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399 **Figure captions**

400 **Figure 1.** Purification and preparation of nanobody-affinity column. A) Production of  
 401 chain-specific nanobodies. Purification steps are illustrated. A fraction of each step,  
 402 including a not induced (NI), induced (I), Flow-through (FT), Washing 1 (W1) and 10  
 403 (W10) and final concentrated nanobody preparation, was analysed by Coomassie blue.

404 B) Crosslinking of nanobodies to nickel magnetic beads. Purified nanobodies were  
 405 crosslinked using BS3. After crosslinking, the affinity column was stored at 4°C and  
 406 equilibrated in binding buffer before use. VHH (variable heavy homodimers) or  
 407 nanobodies. Molecular weight (MW).



408  
 409 **Figure 2.** Isolation of ubiquitylated proteins and sample preparation for MS analysis. A)  
 410 Cleared cell lysates were incubated with the nanobody affinity column and a control  
 411 column without nanobody. Washes were repeated until the background was absent from  
 412 the control column. A sample of each purification step including Input, flow through  
 413 (FT) and captured fractions were analysed by WB with a Ub antibody. B) Sample  
 414 preparation for MS analysis. Eluted ubiquitylated proteins were submitted to a buffer  
 415 exchange before trypsin digestion. Peptides were analysed by MS and data were  
 416 submitted to database search.

