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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 β -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% β -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 K₂HPO₄ 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 [(Na₂HPO₄, NaH₂PO₄), 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 K₂HPO₄ to remove non-specific binding to the Nickel beads.
- 236 11. Wash three times with PBS to eliminate K₂HPO₄ 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 40×10^6 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 μ 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°C (*see* **Note 13**).

252 3. Clarify samples by centrifuging at $48,000 \times g$ during 20 minutes at 4°C .

253 4. Save 1/20 of clarified supernatant (50 μ l) to control the input fraction. Mix the input
254 fraction with an equal volume of BB and store at -20°C .

255 5. Incubate supernatants with the crosslinked magnetic beads for 2 hours at 4°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 μ 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 μ l of 1x BB and keep at RT until use.

269 11. Just before WB analysis, boil samples for 45 minutes at 95°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

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353

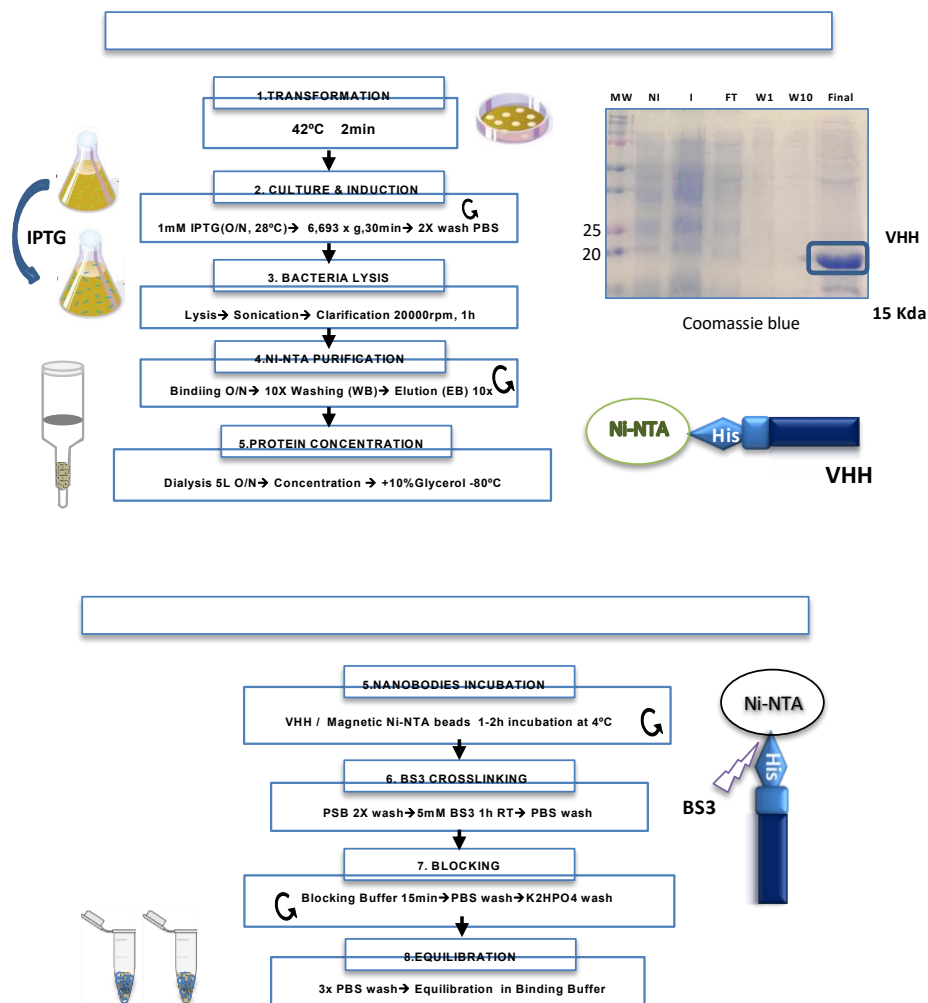
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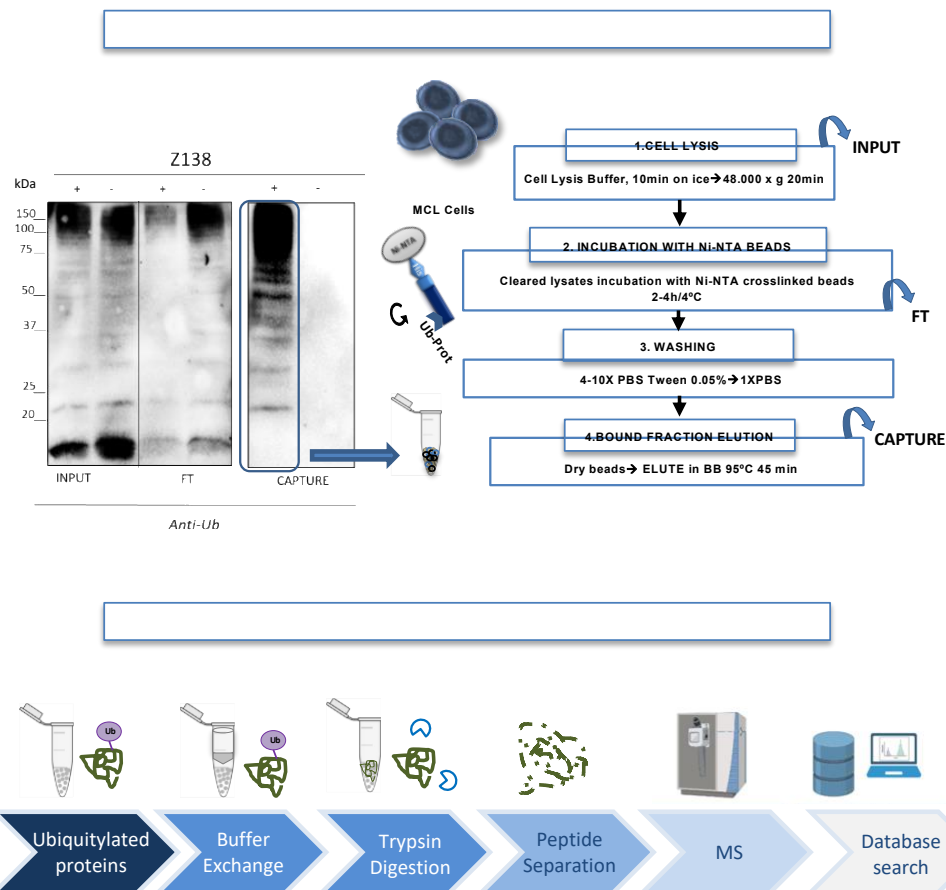
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398

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.

