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1 # Production and purification of human Hsp90β in
2 # *Escherichia coli*

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24 # Abstract

25 The molecular chaperone Hsp90 is an essential member of the cellular
26 proteostasis system. It plays an important role in the stabilisation and activation of
27 a large number of client proteins and is involved in fatal disease processes e.g.
28 Alzheimer disease, cancer and cystic fibrosis. This makes Hsp90 a crucial protein
29 to study. Mechanistic studies require large amounts of protein but the production
30 and purification of recombinant human Hsp90 in *E. coli* is challenging and
31 laborious. Here we identified conditions that influence Hsp90 production and
32 optimised a fast and efficient purification protocol. We found that the nutrient
33 value of the culturing medium and the length of induction had significant effect on
34 Hsp90 production in *Escherichia coli*. Our fast, single-day purification protocol
35 resulted in a stable, well-folded and pure sample that was resistant to
36 degradation in a reproducible manner. We anticipate that our results provide a
37 useful tool to produce higher amount of pure, well-folded and stable recombinant
38 human Hsp90β in *Escherichia coli* in an efficient way.

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39 # Introduction

40 The cellular proteostasis system evolved to maintain cellular health and stability
41 and to protect cells from continuously occurring stress by a tight control of protein
42 production, quality control, folding, trafficking, aggregation and degradation (1,2).
43 Chaperones are crucial elements of the proteostasis system, they prevent protein
44 misfolding and aggregation by various mechanisms and thereby contribute to
45 cellular integrity (3).

46 The molecular chaperone Hsp90 is one of the most important element of
47 the proteostasis system (4-7). It is involved in client protein folding, maturation,
48 stabilisation, activation and assembly of large protein complexes (8,9). Typically,
49 Hsp90 assists at late folding processes (10,11).

50 Hsp90 interacts with up to 10 % of the cellular proteome. Its main clients
51 include transcription factors, kinases and hormone receptors (Didier Picard. Table
52 of Hsp90 interactors. Available from:
53 https://www.picard.ch/downloads/Hsp90interactors.pdf). Moreover, it has
54 unconventional partners such as the disordered α-synuclein and Tau (5,10).
55 Hsp90 plays a crucial role in the progression of several diseases (such as cancer,
56 cystic fibrosis and Alzheimer's disease) (12-14).

57 The Hsp90 chaperone machinery is extensively studied to understand its
58 complex working mechanism and involvement with multiple fatal diseases.
59 Typically, *in vitro* biochemical and biophysical experiments require high amount of
60 recombinant protein with excellent sample purity and stability. Significant
61 overproduction of the recombinant human Hsp90β is challenging since it is a

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62 large, multi-domain protein. Moreover, Hsp90 is particularly sensitive to the
63 proteolytic cleavage of the flexible, unfolded linkers between its domains,
64 therefore a time consuming purification protocol may compromise sample quality.

65 We systematically tested several parameters to find the best conditions for
66 the overproduction of recombinant human Hsp90β. Furthermore, we developed a
67 fast and efficient purification protocol that results in pure and stable sample with
68 reproducible quality. We found that the nutrient value of the medium and the
69 length of induction time had significant effect on Hsp90 overproduction, whereas
70 the concentration of induction agent (isopropyl β-D-1-thiogalactopyranoside
71 (IPTG)), temperature and optical density (OD_{600}) at induction (within the tested
72 intervals) did not influence the process. The protein samples we purified with the
73 optimised protocol were resistant to proteolysis upon incubation at physiological
74 temperature up to one day. We showed that the folding, sedimentation and
75 molecular weight of our Hsp90 sample corresponded to earlier results.

76

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77 # Materials and Methods

78

79 ***Wild type full length Hsp90 construct***

80 pet23a+ expression vector (Novagen) was used for His_6-tagged wild type human
81 Hsp90 production in Rosetta 2 *E. coli* strain (Novagen). The sequence of wild
82 type Hsp90:

```
83 HHHHHHMPEEVHHGEEEVETFAFQAEIAQLMSLIINTFYSNKEIFLRELISNASDALDKIRYESLTDPSKLD
84 SGKELKIDIIPNPQERTLTLVDTGIGMTKADLINNLGTIAKSGTKAFMEALQAGADISMIGQFGVGFYSAYL
85 VAEKVVVITKHNDDEQYAWESSAGGSFTVRADHGEPIGRGTKVILHLKEDQTEYLEERRVKEVVKKHSQFIG
86 YPITLYLEKEREKEISDDEAEEEKGEKEEEDKDDEEKPKIEDVGSDEEDDSGKDKKKKTKKIKEKYIDQEEL
87 NKTKPIWTRNPDDITQEEYGEFYKSLTNDWEDHLAVKHFSVEGQLEFRALLFIPRRAPFDLFENKKKKNNIK
88 LYVRRVFIMDSCDELIPEYLNFIRGVVDSEDLPLNISREMLQQSKILKVIRKNIVKKCLELFSELAEDKENY
89 KKFYEAFSKNLKLGIHEDSTNRRRLSELLRYHTSQSGDEMTSLSEYVSRMKETQKSIYYITGESKEQVANSA
90 FVERVRKRGFEVVYMTEPIDEYCVQQLKEFDGKSLVSVTKEGLELPEDEEEKKKMEESKAKFENLCKLMKEI
91 LDKKVEKVTISNRLVSSPCCIVTSTYGWTANMERIMKAQALRDNSTMGYMMAKKHLEINPDHPIVETLRQKA
92 EADKNDKAVKDLVVLLFETALLSSGFSLEDPQTHSNRIYRMIKLGLGIDEDEVAAEEPNAAVPDEIPPLEGD
93 EDASRMEEVD
```

94

95 ***Protein overproduction test - cell culturing***

96 Rosetta 2 cells containing pet23a+ vector with wild type full length Hsp90 were
97 inoculated into 100 ml 2x yeast tryptone extract (2x YT; 12.8 g Bacto Tryptone
98 (Merck), 8 g Bacto Yeast Extract (Merck), 4 g NaCl (Merck) in 800 ml demi water
99 (Millipore)) medium supplemented with 34 mg/l final concentration of
100 chloramphenicol (Sigma-Aldrich) and 100 mg/l final concentration of ampicillin
101 (Sigma-Aldrich). The cells were grown over night at 37°C, shaking with 220 rpm.

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102 Next morning 100 (for low OD_{600}) or 200 (for high OD_{600}) µl culture was
103 inoculated into 4 ml lysogeny broth medium (LB; 8 g Bacto Tryptone, 4 g Bacto
104 Yeast Extract, 4 g NaCl, 20 g tymine (Sigma-Aldrich) in 800 ml demi water), 2x
105 YT or terrific broth media (TB; 12 g Bacto Tryptone, 24 g Bacto Yeast Extract, 4
106 ml glycerol in 800 ml demi water)) supplemented with 34 mg/l final concentration
107 of chloramphenicol and 100 mg/l final concentration of ampicillin. The cultures
108 were grown at 37°C, shaking with 180 rpm. The OD_{600} was monitored every 1.5
109 hours by using a 1:10 dilution of the culture in an Ultrospec 3000 pro UV/Visible
110 Spectrophotometer (GE Healthcare). The cultures were induced at low (0.6-0.9)
111 or high (1-1.3) OD_{600} with 0.1/0.25/0.5 mM IPTG (Thermo Fisher Scientific). The
112 cultures were incubated at 16°C or 18°C, the SDS-PAGE samples were taken
113 before induction and 1/3/5 day(s) after induction.
114
115 ***SDS-PAGE***

116 For the SDS-PAGE samples 0.5 ml of cell culture was taken and centrifuged for 1
117 minute by 13,300 rpm. The supernatant was discarded and the pellet was
118 resuspended in 200 µl of 1x sample buffer (0.625 M Tris (Sigma-Aldrich), 12.5 %
119 glycerol (CARL ROTH), 1 % SDS (Bio-Rad), 0.005 % Bromophenol Blue (Bio-
120 Rad), 5 mM freshly added β-mercaptoethanol (Sigma-Aldrich) 2x diluted with
121 demi water (Millipore)) and homogenised by using a syringe of a very tiny
122 diameter (BD Micro-Fine). This last step was essential for loading the samples on
123 the gel.

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124 15 % SDS gels were prepared (Separation buffer: 0.38 M Tris pH 8.8, 15
125 % acrylamide (National Diagnostics), 0.1 % SDS, 0.1 % APS (Sigma-Aldrich),
126 0.04 % TEMED (Sigma-Aldrich), Stacking buffer: 0.125 M Tris pH 6.8, 4 %
127 acrylamide, 0.1 % SDS, 0.075 % APS, 0.1 % TEMED) and ran in 1x Laemmli
128 buffer (0.025 M Tris base, 0.152 M glycine (SERVA Electrophoresis GmbH) , 0.1
129 % SDS, diluted from 10x stock). The gels stained with Coomassie staining
130 solution (0.2 % Coomassie Brilliant Blue (SERVA Electrophoresis GmbH), 45 %
131 methanol (Interchema Antonides-Interchema), 10 % acetic acid (Biosolve) and 55
132 % demi water and destained using destaining solution (30 % methanol, 10 %
133 acetic acid and 60 % demi water).

134 The gels were scanned in a Epson Perfection V700 Photo scanner.
135 Quantification of the lane profiles was done by using ImageJ. The lane profiles of
136 the induced samples were normalised to the lane profile of the uninduced sample
137 of the same gel using the intrinsic *E. coli* protein called EF-Tu. Subsequently, the
138 lane profiles of induced samples were aligned to the uninduced sample using the
139 first two bands in the beginning of the lanes. Finally, the uninduced lane profile
140 was deducted from the induced lane profiles and the sum of each Hsp90 peak
141 area was divided by the sum of the normalised EF-Tu peak area of the same lane
142 to be able to compare the gels with each other.

143

144 ***Overproduction and purification of wild type full length Hsp90***

145 Rosetta 2 cells containing pet23a+ vector with wild type full length Hsp90 were
146 inoculated into 200 ml 2x YT medium supplemented with 34 mg/l final

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147 concentration of chloramphenicol and 100 mg/l final concentration of ampicillin.
148 The cells were grown over night at 37°C, with shaking at 220 rpm. Next morning
149 200 ml culture was inoculated into 6x800 ml 2x YT supplemented with 34 mg/l
150 final concentration of chloramphenicol and 100 mg/l final concentration of
151 ampicillin. The cultures were grown at 37°C with shaking at 180 rpm and induced
152 with 0.5 mM IPTG at OD_{600} = 1. After induction the cells were incubated at 18°C
153 for 5 days with shaking at 180 rpm.
154 The cells were harvested in an Avanti J-26 XP centrifuge (Beckman
155 Coulter) using the JLA-8.1 rotor at 4°C at 4500 rpm for 30 minutes. The
156 supernatant was discarded and the pellet was resuspended in ice cold
157 resuspension-buffer (50 mM Na-phosphate pH 7.2 (Sigma-Aldrich), 150 mM
158 NaCl, 150 mM KCl (CARL ROTH)) and centrifuged in an MSE Harrier 18/80
159 Refrigerated Benchtop Centrifuge at 4°C at 5000 rpm for 30 minutes. The
160 supernatant was discarded and the pellet was stored at -20°C until further usage.
161 The pellet was resuspended in ice cold lysis buffer (12.5 mM Na-
162 phosphate pH 7.2. 75 mM NaCl, 5 mM β-mercaptoethanol, EDTA-free protease
163 inhibitor (1 tablet/50 ml) (Roche)). The cells were disrupted by an EmulsiFlex-C5
164 (Avestin) cell disruptor. The lysate was centrifuged in Avanti J-26 XP centrifuge
165 using JA-25.5 rotor at 21,000 rpm for 45 minutes at 4°C. The lysate was filtered
166 by 22 μm polypropylene filter (VWR) to remove the cell debris and insoluble
167 aggregates. The purification was done using an AKTA Purifier (GE Healthcare).
168 Wild type full length Hsp90 was first purified on an IMAC POROS 20MC
169 (Thermo Fischer Scientific) affinity purification column (solutions connected to

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170 pump A1 and A2: 50 mM Na-phosphate buffer pH 8.0 with 300 mM NaCl, B1:
171 demi water with 10 mM β-mercaptoethanol, B2: 1 M imidazole (Sigma-Aldrich)).
172 The eluted sample was diluted 4-fold with dilution buffer (25 mM Na-phosphate
173 buffer pH 7.2, 5 mM DTT (Sigma-Aldrich), complete protease inhibitor (1 tablet/50
174 ml) (Roche)). Next the sample was loaded on a POROS 20HQ anion exchange
175 column (Thermo Fischer Scientific) (solutions connected to pump A1 and A2: 50
176 mM Na-phosphate pH 7.2, B1: demi water with 10 mM DTT, B2: 2 M KCl). The
177 eluted sample was diluted 10-fold with dilution buffer (25 mM Na-phosphate
178 buffer pH 7.2, 5 mM DTT, complete protease inhibitor (1 tablet/100 ml)). Finally,
179 the sample was loaded on a HiTrap heparin affinity chromatography column (GE
180 Healthcare) (solutions connected to pump A1 and A2: 25 mM Na-phosphate pH
181 7.2, B1: demi water with 10 mM DTT, B2: 2 M KCl). The eluate was concentrated
182 and buffer exchanged to Hsp90 storage buffer (25 mM Na-phosphate pH 7.2, 150
183 mM NaCl, 150 mM KCl), 5 mM DTT, complete protease inhibitor (1 tablet/100
184 ml)) using a Vivaspin 20 column (50 kDa MWCO) (GE Healthcare) at 4^oC at 5000
185 rpm for 15-15 minutes until above 100 μM protein concentration. The protein
186 concentration was determined with ND-1000 program on an ND-1000
187 Spectrophotometer type NanoDrop using 57760 $M^{-1}cm^{-1}$ extinction coefficient.
188 The sample was aliquoted, frozen in liquid N_2 and stored at -80^oC. Throughout
189 the purification procedure samples were taken from the steps of the purification
190 that were run on SDS-PAGE to analyse sample purity.
191

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192 ***Protein stability***

193 10 µM concentration Hsp90 was incubated in Hsp90-buffer ((25 mM Na-
194 phosphate pH 7.2, 150 mM NaCl, 150 mM KCl, 5 mM DTT, complete protease
195 inhibitor (1 tablet/100 ml)) for 24 hours at 4°C, room temperature (~21°C) and
196 37°C. SDS-PAGE samples were taken and mixed with 2x sample buffer (1.25 M
197 Tris, 25 % glycerol, 2 % SDS, 0.01 % Bromophenol Blue, 5 mM freshly added
198 DTT) at 0/3/18/24 hours. The samples were analysed on SDS-PAGE.

199

200 ***Silver staining***

201 The Coomassie-stained and destained gels were fixed for 30 minutes in fixation
202 solution (30 % ethanol (Interchema Antonides-Interchema), 10 % acetic acid, 60
203 % demi water). The gel was washed 3-times for 20 minutes in 50 % ethanol, then
204 pre-treated for 1 minute with 0.02 % $Na_2S_2O_3$ (Scharlau Chemicals) [100x diluted
205 from stock (stock: 2 g into $Na_2S_2O_3$ 100 ml water (Milli-Q))] and quickly rinsed 4
206 times with demi water. The gels were impregnated for 20 minutes with freshly
207 prepared staining solution (2 g/l $AgNO_3$ (Merck) and 0.75 ml/l formaldehyde
208 (Calbiochem)). Next they were quickly rinsed 4 times with demi water, then
209 developed till the desired result in developer solution (60 g/l Na_2CO_3 (Sigma-
210 Aldrich), 0.5 ml/liter formaldehyde and 0.0004 % $Na_2S_2O_3$ stock). The reaction
211 was stopped in fixation solution (30 % ethanol, 10 % acetic acid, 60 % demi
212 water) for 10 minutes. The gel was stored in 1 % acetic acid solution and
213 scanned by Epson Perfection V700 Photo scanner.

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214

215 ***CD spectroscopy***

216 The protein was centrifuged on 4°C for 15 minutes at 13,300 rpm in Heraeus Pico
217 17 centrifuge (Thermo Scientific). The concentration was determined with ND-
218 1000 program on ND-1000 Spectrophotometer type NanoDrop using 57760 M^{-}
219 $^{1}cm^{-1}$ extinction coefficient. The protein was diluted to 0.1 g/l concentration with
220 CD-buffer (25 mM Na-phosphate pH 7.2 buffer, 150 mM NaF (Sigma-Aldrich)),
221 loaded into a Teflon-sealed, polarimetrically checked quartz glass cuvette with an
222 optical path length of 1 mm and a volume of 350 μl (Hellma Analytics). Far-UV
223 CD spectrum was measured with the Spectra Manager (Jasco) program on a
224 Jasco J-810 Spectropolarimeter instrument (Jasco). Experimental parameters
225 included a wavelength increment of 1 nm, a scan speed of 20 nm/min, a
226 temperature of 20°C). The data were analysed by MS Excel.

227

228 ***SEC-MALLS***

229 The protein sample was centrifuged and its concentration was measured as
230 described in the *CD spectroscopy* part. 10 μl of ~29 g/l sample was run with
231 Shimadzu on a Superdex 200 column with 0.35 ml/min speed in running buffer
232 (25 mM Na-phosphate pH 7.2, 150 mM NaCl, 5 mM freshly added DTT). The 220
233 nm absorption was detected by SPD-20A UV detector (Shimadzu), light
234 scattering by Wyatt COMET™ light scattering detector (Wyatt Technology) and
235 the refractive index by the RID-10A refractive index detector (Shimadzu). The
236 data were analysed by program Astra 6.

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237

238 ***Analytical Ultracentrifugation (AUC)***

239 The protein sample was centrifuged and its concentration was measured as
240 described in the *CD spectroscopy* part. The protein sample was diluted to 7.2 μM
241 concentration with Hsp90-buffer (25 Na-phosphate pH 7.2, 150 mM NaCl, 150
242 mM KCl, complete protease inhibitor (1 tablet/50 ml), 5 mM freshly added DTT).
243 The sample was centrifuged on 20°C for 16 hours at 42,000 rpm in a Beckman
244 XL-I ultracentrifuge using An60Ti rotor. We used absorbance detection optics for
245 our experiment. The data was analysed by SedFit (Schuck, 2000).

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246 # Results

247

248 ***Systematic testing of recombinant human Hsp90β overproduction***

249 We set out to systematically test the effect of key parameters on recombinant
250 human Hsp90β production in Rosetta 2 cells and to identify conditions resulting in
251 high protein levels.

252 We outlined our study as follows: We cultured *E. coli* cells containing
253 human Hsp90β open reading frame on a pET23a+ vector and tested media with
254 different nutrient values and different temperature of induction to modulate the
255 metabolism of *E. coli* cells. We induced the cultures with different final
256 concentration of IPTG affecting the level of T7 polymerase. We also varied the
257 length of induction and the OD_{600} value at induction, because in case of certain
258 proteins these are essential parameters for successful overproduction.

259 At given time points we took samples from the cultures and ran them on
260 SDS-gels **(Figure 1A)**. We loaded protein marker in the first, uninduced sample
261 in the second and purified Hsp90 in the last lanes of the SDS-gels. These
262 samples helped to identify the Hsp90 bands that appeared after induction. Lane
263 3-14 contained the induced samples of the different conditions tested. The most
264 abundant intrinsic *E. coli* band that we later used for quantification (EF-Tu) and
265 Hsp90 bands are marked on the right of the gels (**Figure 1A**) (15).

266

267 ***Figure 1: Experimental set up and quantification scheme for Hsp90***
268 ***overproduction.*** *(A) Schematic overview of the protein production experiment.*

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269 *E. coli cells containing human Hsp90β open reading frame were inoculated from*
270 *glycerol stock and cultured overnight at 37°C. The culture was divided into small*
271 *tubes and after induction the tubes were incubated in different conditions. Gel*
272 *samples were taken at day 1, 3 and day 5 and were run on SDS-PAGE. (B)*
273 *Schematic representation of the quantification of Hsp90 overproduction. The*
274 *intensity profiles of uninduced (orange) and induced lanes (green) were*
275 *determined and the induced lane profile were normalised to the uninduced lane*
276 *profile using the EF-Tu peak of the uninduced lane as reference (100 %) (top*
277 *panel). Subsequently, the induced lane profiles were aligned to the uninduced*
278 *lane profile using the first two left peaks (stars) of the uninduced lane profile as*
279 *reference points (middle panel). The uninduced lane profile was deducted from*
280 *the normalised and aligned induced lane profiles and the sum of difference at the*
281 *height of the Hsp90 peak was calculated (red area) (lower panel). Finally, the*
282 *Hsp90 peak area was divided by the normalised and aligned EF-Tu peak area.*
283

284 We determined the intensity profile of the uninduced (green) and induced lanes
285 (orange) (**Figure 1B,** top panel) and normalised the induced lane profile to the
286 uninduced lane profile using the EF-Tu peak of the uninduced lane as reference
287 (100 %). Subsequently, we aligned the induced lane profile (green) to the
288 uninduced lane profile (orange) using the first two left peaks (stars) of the
289 uninduced lane profile as reference points **(Figure 1B,** middle panel**)**. We
290 deducted the uninduced lane profile (orange) from the normalised and aligned
291 induced lane profile (green) and calculated the sum of difference at the height of

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292 the Hsp90 peak (red area) (**Figure 1B,** lower panel). Finally, we divided the
293 Hsp90 peak area by the area of the normalised and aligned EF-Tu peak.
294

295 ***The yield of recombinant human Hsp90β protein in E. coli using LB medium***
296 ***was insufficient for preparative purposes***

297 We tested Hsp90 overproduction in LB medium varying the length of induction,
298 the temperature, the OD_{600} at induction and the IPTG concentration to measure if
299 any of these parameters modulated the levels of the chaperone.

300 After one and three days of induction no Hsp90 band appeared in the
301 induced samples, none of the conditions led to sufficient Hsp90 overproduction
302 (**Figure 2A and B**, lane 3-14). When induced for five days an Hsp90 band was
303 apparent in several conditions (**Figure 2C**, lane 4, 5, 11 and 12). However, the
304 yield was still insufficient for preparative purposes and the production levels were
305 variable.
306

307 ***Figure 2**: **Hsp90 overproduction in LB medium lead to insufficient protein***
308 ***yield**. Hsp90 overproduction after 1 day (A), 3 days (B), 5 days (C) of induction.*
309 *Lane 1: protein marker, lane 2: uninduced sample, lane 3-8: protein production at*
310 *16°C, lane 9-14: protein production at 18°C, lane 3-5 and 9-11: induction at low*
311 *OD_{600}, lane 6-8 and 12-14: induction at high OD_{600}, lane 3, 6, 9, 12: induction with*
312 *0.1 mM IPTG, lane 4, 7, 10, 13: induction with 0.25 mM IPTG, lane 5, 8, 11, 14:*
313 *induction with 0.5 mM IPTG, lane 15: purified Hsp90 sample. EF-Tu intrinsic E.*
314 *coli protein band was used for quantification of Hsp90 overproduction.*

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315 ***2x YT medium improved the yield of Hsp90 production***

316 Since protein production in LB resulted in poor yields we hypothesised that the
317 nutrient value of the medium may be a critical parameter in case of Hsp90.
318 Therefore, we repeated the overproduction experiment in the richer 2x YT
319 medium. We varied the length of induction, the temperature, the OD_{600} at
320 induction and the IPTG concentration to determine if any these parameters affect
321 Hsp90 production in 2x YT.

322 After one and three days of induction a new band appeared at the height
323 of Hsp90 in the induced samples, suggesting that we could produce the
324 chaperone (**Figure 3A and B**). The newly appearing Hsp90 band is stronger after
325 three days of induction compared to one. Hsp90 production in 2x YT resulted in
326 higher yields than in LB within the interval of the parameters we varied.

327

328 ***Figure 3: Hsp90 overproduction was improved but still insufficient in 2x YT***
329 ***compared to LB****. Hsp90 overproduction after 1 day (A), 3 days (B), 5 days (C) of*
330 *induction. Lane 1: protein marker, lane 2: uninduced sample, lane 3-8: protein*
331 *production at 16°C, lane 9-14: protein production at 18°C, lane 3-5 and 9-11:*
332 *induction at low OD_{600}, lane 6-8 and 12-14: induction at high OD_{600}, lane 3, 6, 9,*
333 *12: induction with 0.1 mM IPTG, lane 4, 7, 10, 13: induction with 0.25 mM IPTG,*
334 *lane 5, 8, 11, 14: induction with 0.5 mM IPTG, lane 15: purified Hsp90 sample.*
335 *EF-Tu intrinsic E. coli protein band was used for quantification of Hsp90*
336 *overproduction.*

337

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338 When induced for five days, we observed significant overproduction of the
339 chaperone **(Figure 3C)** in certain cases (lane 7-9), whereas other conditions led
340 to similar results to those observed on previous gels (lane 10, 12). Hsp90
341 production levels were variable in the different conditions. We concluded that
342 Hsp90 overproduction resulted in higher yields in 2x YT after five days of
343 induction compared to shorter induction times and culturing in LB medium, but
344 the yields were still insufficient for preparative purposes.

345

346 ***Recombinant human Hsp90β production had the best yield in the E. coli***
347 ***cells in TB medium***

348 Since the richer 2x YT medium had a positive effect on Hsp90 levels we decided
349 to test the protein production in TB medium which is higher in nutrients than 2x
350 YT. We varied the same parameters as described previously.

351 After one day of induction, a strong band appeared at the height of Hsp90
352 in several conditions (**Figure 4A**, lane 3 and 11) indicating that overproduction in
353 TB was notable. The conditions that resulted in high protein amount, however,
354 were variable. Already after three days of induction in TB medium resulted all the
355 conditions tested in a strong Hsp90 band (**Figure 4B**). We observed similar
356 Hsp90 levels with five days of induction (**Figure 4C**). Thus, TB medium resulted
357 in higher yields compared to both LB and 2x YT media, and after a shorter period
358 of time.

359

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360 ***Figure 4**: **Hsp90 overproduction lead to the highest yields in TB medium**.*
361 *Hsp90 overproduction after 1 day (A), 3 days (B), 5 days (C) of induction. Lane 1:*
362 *protein marker, lane 2: uninduced sample, lane 3-8: protein production at 16°C,*
363 *lane 9-14: protein production at 18°C, lane 3-5 and 9-11: induction at low OD_{600},*
364 *lane 6-8 and 12-14: induction at high OD_{600}, lane 3, 6, 9, 12: induction with 0.1*
365 *mM IPTG, lane 4, 7, 10, 13: induction with 0.25 mM IPTG, lane 5, 8, 11, 14:*
366 *induction with 0.5 mM IPTG, lane 15: purified Hsp90 sample. EF-Tu intrinsic E.*
367 *coli protein band was used for quantification of Hsp90 overproduction.*
368

369 ***Quantification of overproduction gels revealed that the richness of the***
370 ***medium and the length of induction are the most significant parameters for***
371 ***Hsp90***

372 To compare the protein production results of the different gels we estimated
373 Hsp90 overproduction by gel densitometry as described in Figure 1B. In LB
374 medium we observed low level of Hsp90 overproduction in each condition
375 **(Figure 5A)**. The tendency improved with the length of induction but even after
376 five days the yields were insufficient for preparative purposes in all conditions.
377

378 ***Figure 5: Quantification of Hsp90 overproduction in different media**. (A)*
379 *Summary of the quantification of Hsp90 overproduction in LB, after 1 day (white),*
380 *3 days (grey) and 5 days (black) of induction. Average of results of all conditions*
381 *(1st column group), average of results on 16°C and 18°C (2nd and 3rd column*
382 *groups), average of results at low or high OD_{600} at induction (4th and 5th column*

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383 *groups), average of results at 0.1, 0.25 and 0.5 mM IPTG concentration (6th to 8th*
384 *column groups). The length of induction is the only variable that influences Hsp90*
385 *production. (B) Summary of the quantification of Hsp90 overproduction in 2x YT.*
386 *The length of induction is the only variable that influences Hsp90 production. The*
387 *yield is higher in 2x YT compared to LB. The structure of the chart is as in (A). (C)*
388 *Summary of the quantification of Hsp90 overproduction in TB. The length of*
389 *induction is the only variable that has a significant effect on Hsp90 production.*
390 *The yield is higher in TB compared to LB and 2x YT. The structure of the chart is*
391 *as in (A).*
392

393 We noted similar trends in case of 2x YT medium, the level of protein
394 overproduction was insufficient and had similar yields after one and three days of
395 induction **(Figure 5B)**. After five days of induction in 2x YT, however, Hsp90
396 overproduction was still insignificant. Just like in LB, the other varied conditions
397 did not modulate Hsp90 levels in this medium. In 2x YT medium the yield was
398 higher in every condition compared to LB.

399 Hsp90 overproduction in TB was significantly higher than in the other two
400 media. The length of induction had a significant effect on the Hsp90 yields, and
401 here we reached good yields already after three days. The other tested
402 parameters did not influence the Hsp90 yield in TB.

403 Overall we concluded that the quantification of the gels in Figure 2, 3 and 4
404 confirmed our observations about the overproduction gels (**Figure 5A, B and C,**
405 respectively**).** We found that of the tested parameters, richness of medium used

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406 for culturing *E. coli* cells and the length of induction were the only two that had a
407 significant effect on Hsp90 overproduction and should be considered in the
408 future. Temperature, OD_{600} at induction and IPTG concentration used for
409 induction did not significantly modulate the yields of the chaperone within the
410 tested intervals.

411

412 ***Recombinant human Hsp90β purified in one day***

413 After successful protein overproduction, we set out to optimise a fast, efficient
414 and trustworthy purification protocol to ensure excellent sample quality. This was
415 necessary because like other multi-domain proteins, human Hsp90β is also prone
416 to the degradation. Its accessible, flexible regions, especially the charged linker
417 between the N-terminal domain and the middle domain is often targeted by
418 proteases, causing N-terminal degradation. Widely used purification protocols
419 include time-consuming purification steps (such as dialysis, size exclusion
420 chromatography or both) that slow down the procedure which may have
421 detrimental effects on protein quality (16-19).

422 To avoid degradation that might occur as a consequence of long
423 purification procedures and eventual freeze-thaw steps, we set out to develop a
424 condensed protocol for purification of Hsp90 in one day that results in highly pure
425 and stable sample. We purified N-terminally His_6-tagged Hsp90 protein by Ni-
426 affinity chromatography, anion exchange and heparin affinity chromatography
427 (**Figure 6A**). To ensure sample quality and avoid degradation we carried out the
428 experiment in the presence of protease inhibitors at low temperature (0-4°C). To

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429 check for protein quality throughout the purification process we ran samples of
430 the peak fractions of each column on sodium dodecyl sulfate polyacrylamide gel
431 electrophoresis (SDS-PAGE) (**Figure 6B**). We observed a gradual gain in purity
432 and loss in degradation products and impurities. After the last step the new
433 Hsp90 purification protocol resulted in a protein sample free of any significant
434 contaminations detectable by Coomassie staining. The combination of the three
435 columns was necessary for high sample purity and reproducibility.
436

437 ***Figure 6**: **The Hsp90 sample was free of degradation products and***
438 ***impurities after the three-step purification procedure**. (A) Schematic overview*
439 *of the Hsp90 purification procedure. After cell disruption and centrifugation, the*
440 *lysate was loaded first on a POROS 20MC Ni-column followed by a POROS*
441 *20HQ anion exchange column. Next, the sample was loaded on a heparin affinity*
442 *chromatography column. Finally, the eluted protein sample was concentrated,*
443 *buffer-exchanged and flash frozen. The time frame of the experiment is indicated*
444 *on the bottom of the figure. The SDS-PAGE panels on the arrows show the purity*
445 *of the samples after each purification step. (B) SDS-PAGE shows the purity of the*
446 *Hsp90 sample after each purification step. Lane 1: peak fraction sample after*
447 *POROS 20MC affinity chromatography purification step, lane 2: peak fraction*
448 *sample after POROS 20HQ anion exchange purification step, lane 3: peak*
449 *fraction sample after heparin affinity chromatography purification step.*
450

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451 Certain biochemical and biophysical experiments are carried out at a higher,
452 physiological temperature (37°C) and for elevated time intervals (days or
453 months). In case of proteins that are sensitive to proteolysis, incubation at high
454 temperature for longer time can be detrimental for sample quality. To test the
455 stability of the Hsp90 sample purified by the new protocol we incubated the
456 protein at 4, 21 and 37°C and ran samples taken at 3, 18 and 24 hours on SDS-
457 PAGE. We visualised Hsp90 in the gels first with Coomassie (**Figure 7A**) and
458 subsequently, with more sensitive silver staining (**Figure 7B**).
459
460 ***Figure 7: Purified Hsp90 sample incubated on physiological temperature for***
461 ***one day remained free of degradation products****. (A) SDS-PAGE about the*
462 *stability of the purified Hsp90 sample. Protein marker (lane 1), Hsp90 incubated*
463 *for 0 hours (lane 2), Hsp90 incubated at 4°C (lane 3-5), 21°C (lane 6-8), 37°C*
464 *(lane 9-11), Hsp90 incubated for 3 hours (lane 3, 6, 9), 18 hours (lane 4, 7, 10),*
465 *24 hours (lane 5, 8, 11). (B) Silver staining about the stability of the purified*
466 *Hsp90 sample. Protein marker (lane 1), Hsp90 incubated for 0 hours (lane 2),*
467 *Hsp90 incubated at 4°C (lane 3-5), 21°C (lane 6-8), 37°C (lane 9-11), Hsp90*
468 *incubated for 3 hours (lane 3, 6, 9), 18 hours (lane 4, 7, 10), 24 hours (lane 5, 8,*
469 *11).*
470
471 No additional degradation products appeared in the incubated samples compared
472 to the starting sample within the time frame of the experiment on the Coomassie-
473 stained gels **(Figure 7A)**. Therefore we concluded that Hsp90 purified using the

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474 new purification protocol was stable if incubated at 37°C up to 24 hours. Since
475 Coomassie staining has limited sensitivity especially in the range of low molecular
476 weight, we further examined silver staining to further examine the stability of the
477 sample by silver staining **(Figure 7B)**. Here, we observed impurities and/or
478 degradation products in every sample but we did not see a systematic increase of
479 any bands upon incubation at 37°C up to 24 hours.
480 This newly optimised, reproducible purification protocol enabled us to
481 prepare a highly pure, stable and homogeneous sample within one day.
482

483 ***Hsp90 purified by our method was properly folded, had the correct***
484 ***molecular weight and sedimentation coefficient***

485 To reveal the folding status of proteins, we to analysed our Hsp90 samples with
486 circular dichroism spectroscopy (CD) that can potentially reveal the secondary
487 structure composition and folding status of the chaperone. The CD spectrum of
488 Hsp90 revealed that the chaperone is mainly composed of α-helices **(Figure 8A**).
489 We observed two minima at ~209 nm and at ~ 222 nm and a maximum at ~ 194
490 nm in the spectrum which is in agreement with the previous findings (Prodromou
491 et al, 1999).
492

493 ***Figure 8: Purified Hsp90 sample folded, sedimented correctly and its***
494 ***molecular mass was appropriate****. (A) CD spectrum of Hsp90 at 1.2 μM*
495 *monomer concentration. The overall structure of the protein typically consists of*
496 *α-helices. (B) The AUC spectrum of Hsp90 at 7.2 μM monomer concentration.*

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497 *Hsp90 dimers sediment with 5.6 S, whereas Hsp90 tetramers appear at 10.6 S.*
498 *(C) SEC-MALLS spectra of the purified Hsp90 sample at 2.2 µM monomer*
499 *concentration. The protein has the molecular weight of the dimer Hsp90 is 167.5*
500 *± 13.3 kDa. The peak maximum is at 10.07 ml. Red line: light scattering, blue*
501 *line: refractive index, green line: UV absorption at 220 nm, black dotted line: fit for*
502 *molecular mass.*
503
504 To ensure that our Hsp90 sample had the correct molecular weight and did not
505 degrade during the purification process we measured its molecular weight using
506 size exclusion chromatography - multi angle laser light scattering (SEC-MALLS)
507 (**Figure 8B**). We observed a homogeneous peak at 10.07 ml that was fitted to
508 167.5 ± 13.3 kDa. This result corroborates with the expected size of two Hsp90
509 molecules (168.2 kDa), confirming that our sample is in a dimeric state at the
510 concentration used in the experiment and also in agreement with data published
511 earlier (Lepvrier et al, 2015, Moullintraffort et al, 2010). Taken together with the
512 outcome of the stability experiments SEC-MALLS results suggested that our
513 sample contained the intact full length Hsp90 dimer without degradation.
514 We measured the sedimentation coefficient of our Hsp90 sample to
515 analyse sedimentation properties of the Hsp90 dimer. In the sedimentation profile
516 of the chaperone we observed a peak at 5.6 S that corresponded to the dimer
517 and a smaller peak at 10.6 S that was probably the tetramer molecule (dimer of
518 dimers) (**Figure 8C**). The increasing fraction above 14 S suggested that the
519 sample aggregated to some extent (but this is a typical phenomenon in case of

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520 analytical ultrancentrifugation (AUC) samples). Below 1 S we observed a small
521 peak, that could have originated from impurities or degradation products.

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522 # Discussion

523 Human Hsp90β is often produced in baculovirus system in insect Sf9 cells that is
524 expensive, requires long preparation steps and is often difficult to scale-up (20-
525 22). Our optimised recombinant human Hsp90β production protocol in *E. coli*
526 provides an attractive alternative for the baculovirus system.

527 First, we systematically tested the production of recombinant human
528 Hsp90β in *E. coli* cells to identify parameters that modulate the yield of the
529 chaperone. We showed that the length of induction and the nutrient level of the
530 medium had significant effect on Hsp90 overproduction, whereas temperature,
531 IPTG concentration and OD_{600} at induction (within the tested intervals) did not
532 significantly modify the yields of Hsp90. Induction for three days in TB medium
533 resulted in good yield of the chaperone.

534 Moreover, we purified Hsp90 using a protocol that allowed us to finish
535 within one day and resulted in well-folded, stable and pure sample. Since Hsp90
536 is extremely sensitive to proteolysis because of its flexible linkers, fast
537 preparation increases sample quality, in addition to being cheaper and time-
538 efficient. Throughout the purification procedure impurities and degradation
539 products disappeared from the sample and final product resisted further
540 degradation upon incubation on elevated temperature for long time intervals.

541 Previous Hsp90 purification protocols often contained time consuming
542 preparation steps such as dialysis, size exclusion chromatography or both that
543 may affect protein quality (16-19). Using the combination of Ni-affinity
544 chromatography, anion exchange and the Hsp90-specific heparin column allowed

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545 us to get rid of degradation products and impurities in only three purification
546 steps. The resulting sample was sufficiently pure and resistant to proteolytic
547 degradation upon incubation on physiological temperature for up to one day, and
548 showed expected biophysical properties (CD, SEC-MALLS).

549 In our AUC experiments human Hsp90 sedimented predominantly as a
550 dimer with S=5.6 and a small fraction as a tetramer with S=10.6. It is notable that
551 human Hsp90 is known to be in extended conformation (D_{max}=26 nm) in the
552 absence of nucleotide and co-chaperone p23 (10). Similarly to our results, earlier
553 findings showed that in the absence of nucleotides, consequently in its extended
554 conformation, yeast Hsp90 also had the sedimentation coefficient of 5.6 (23).
555 These results indicate that these two proteins sediment similarly when in
556 extended conformation. However, in the presence of ATP that stabilises compact
557 conformation, yeast Hsp90 sedimented with the coefficient of 6.8, suggesting that
558 structural rearrangements altered its sedimentation properties (23). In similar
559 experiments Hsp90 sedimented with the coefficient of 6.1 (24). The variations
560 between these results may be explained by the differences between the origin of
561 the samples (porcine brain Hsp90 that has different post-translational
562 modification pattern and was a mixture of α and β isoform vs Hsp90), moreover,
563 by buffer, salt and reducing agent conditions.

564 In summary, we optimised Hsp90 overproduction and a fast and efficient
565 purification protocol for the protein. The resulting sample was pure, properly
566 folded and resistant to degradation, consequently it is suitable for biochemical
567 and biophysical experiments.

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575

576 ## Author contributions

577

578 Contribution to manuscript:

579 The concept of the study originates from M. Radli and S.G.D. Rüdiger.
580 M. Radli drafted the first version of the manuscript and prepared all the figures in
581 the chapter. M. Radli and S.G.D. Rüdiger wrote the manuscript.

582

583 Contribution to experiments:

584 M. Radli tested Hsp90 production, ran the SDS-PAGE gels and quantified Hsp90
585 overproduction.
586 M. Radli optimised the purification of Hsp90 and purified all the protein used for
587 the experiments in this chapter.
588 M. Radli tested the stability of Hsp90 on different temperatures.
589 M. Radli performed and analysed the analytical ultracentrifugation (AUC), SEC-
590 MALLS and circular dichroism (CD) experiments.

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591 D.B. Veprintsev supervised the AUC experiments, helped analyzing the data and
592 interpreting the results.
593 S.G.D. Rüdiger supervised the experiments.

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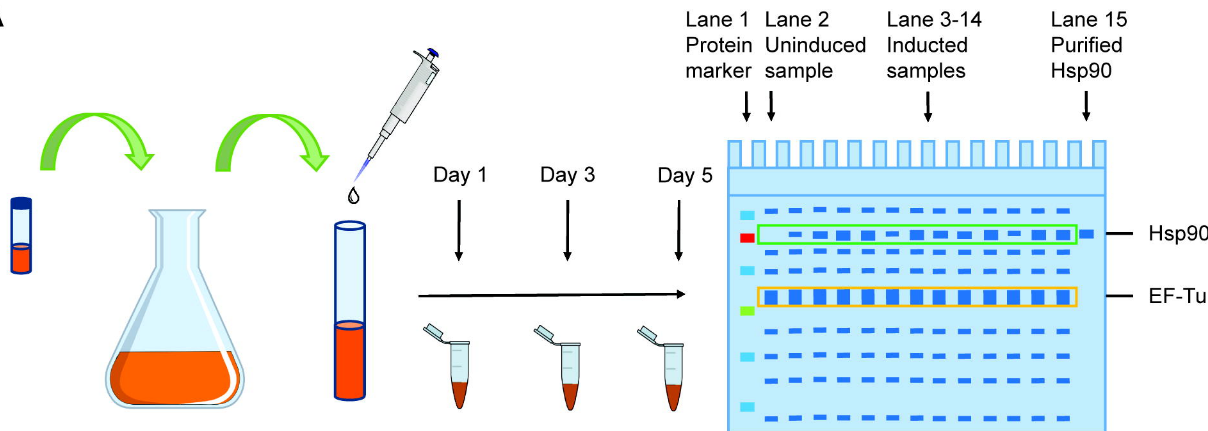
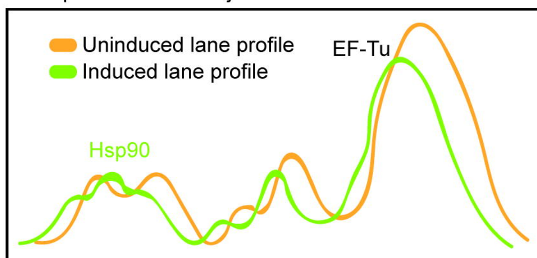
A



B

Lane profiles before adjustments



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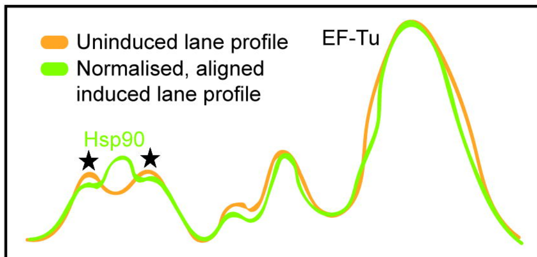
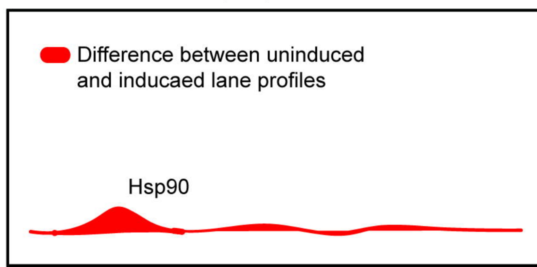


Surface area of the Hsp90 peak



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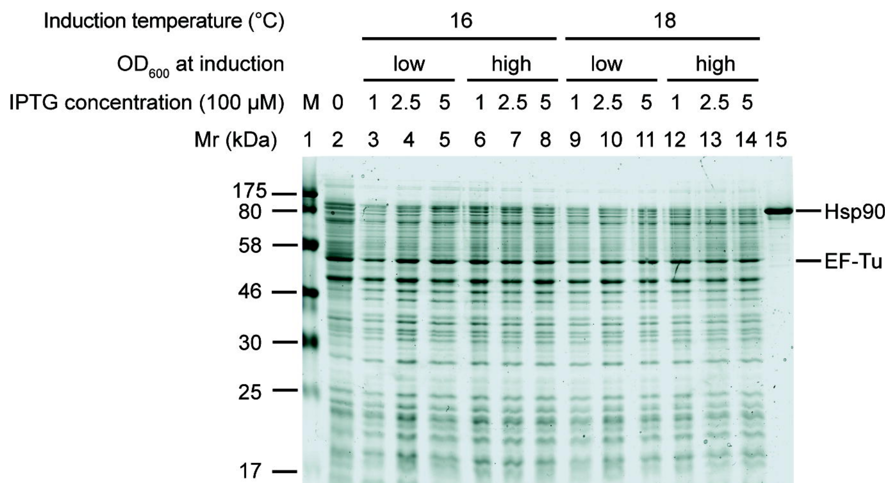

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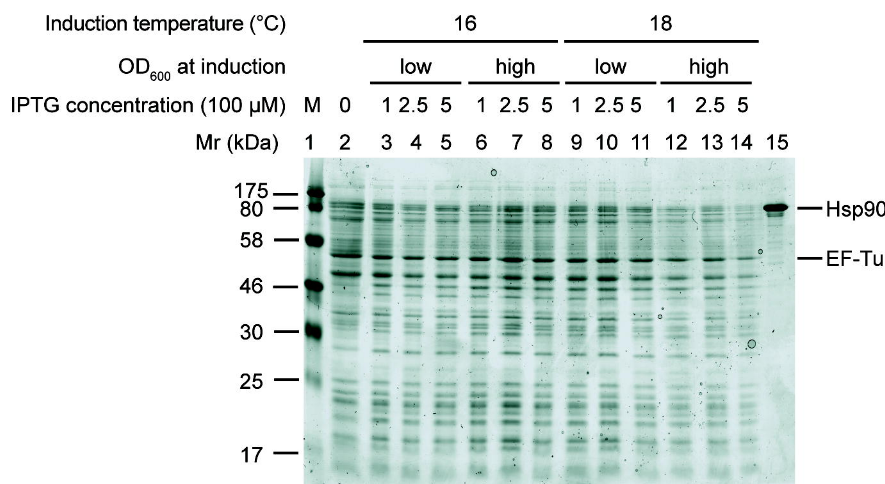
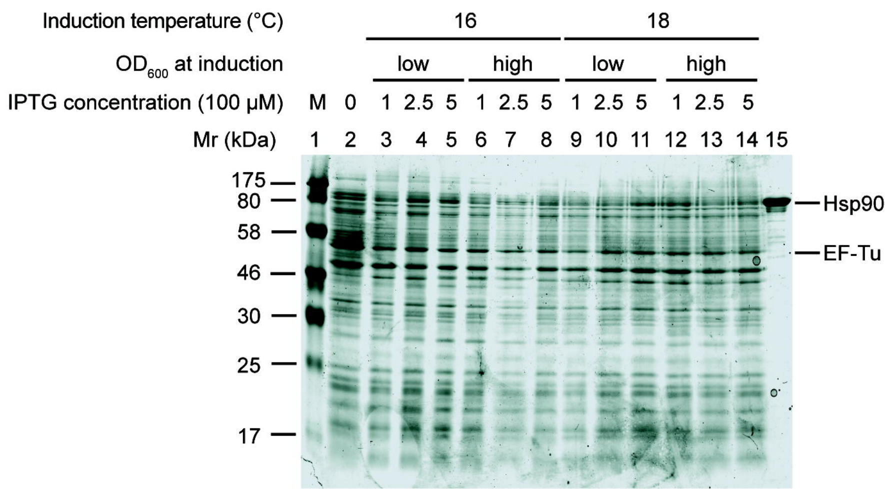


C



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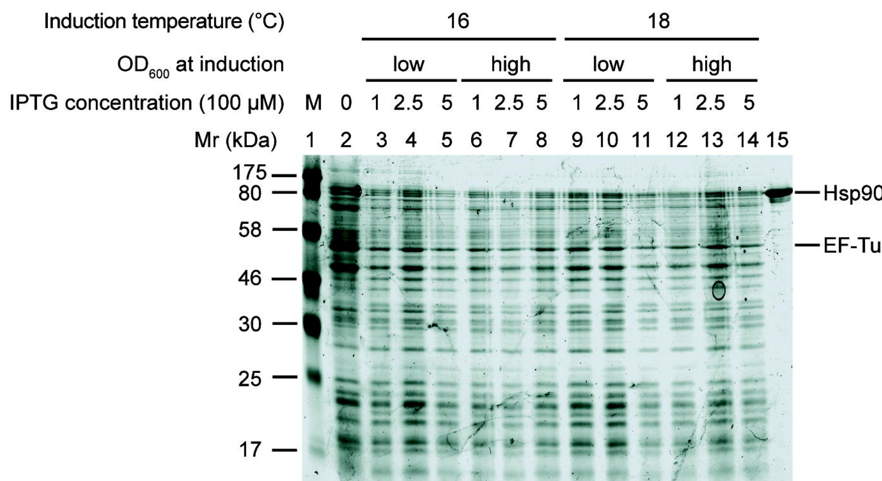

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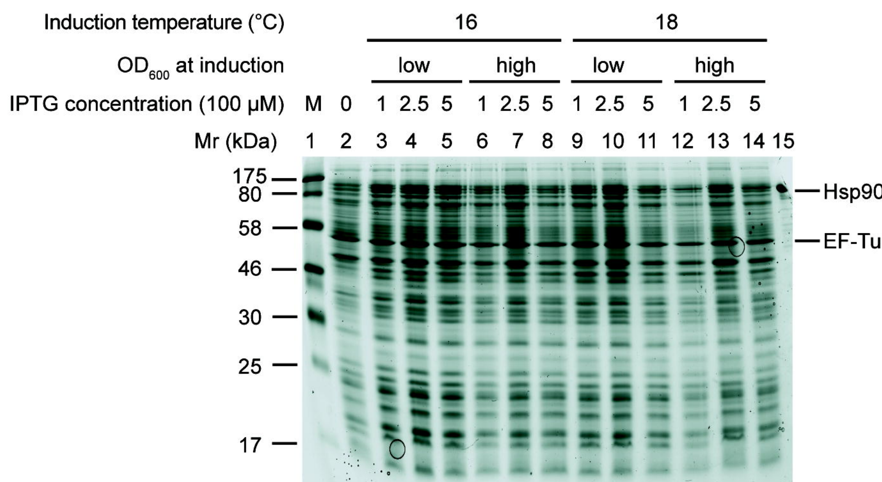

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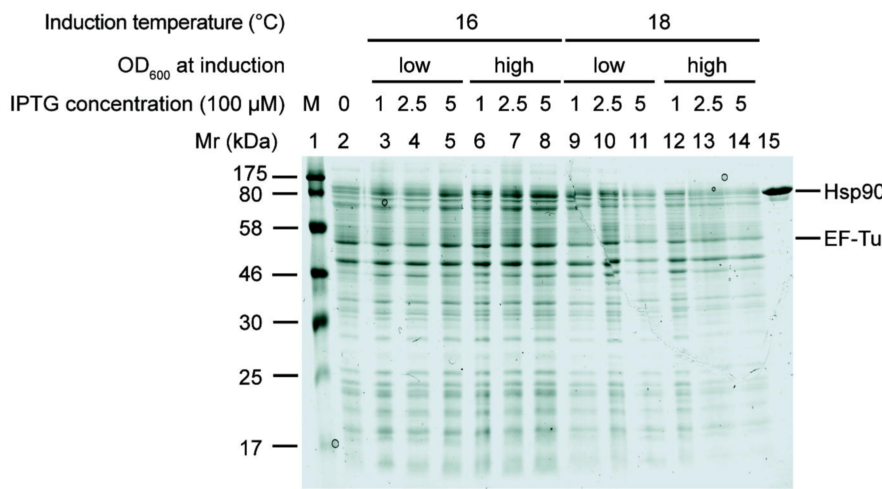

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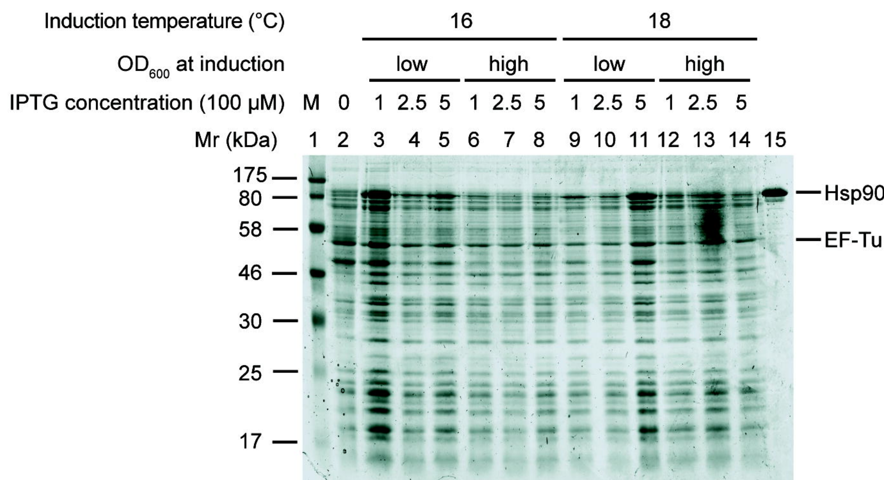

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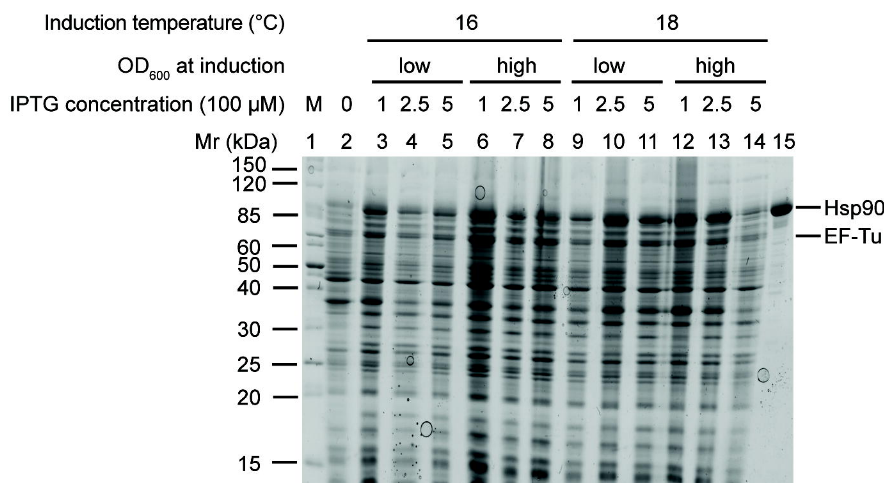

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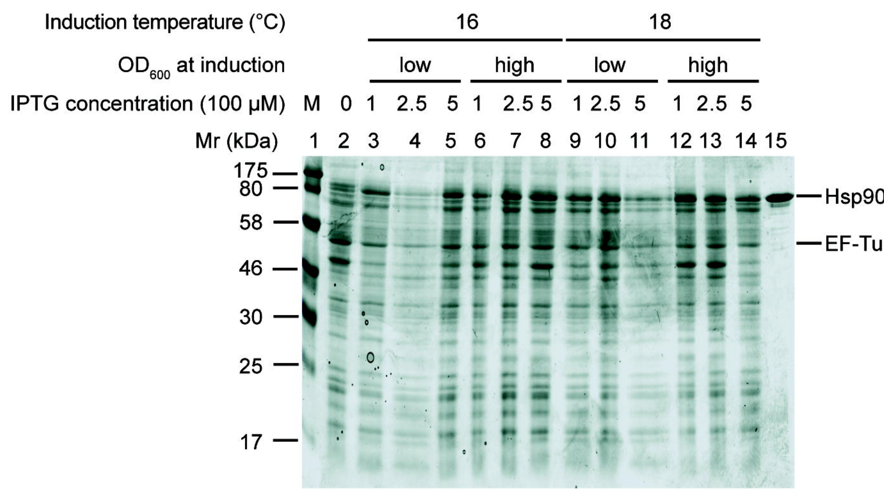
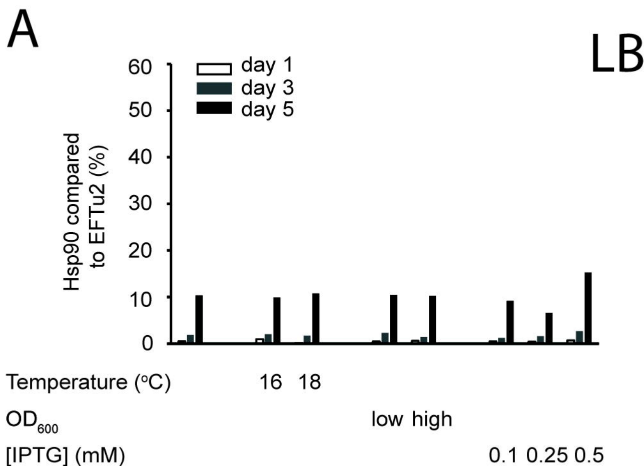
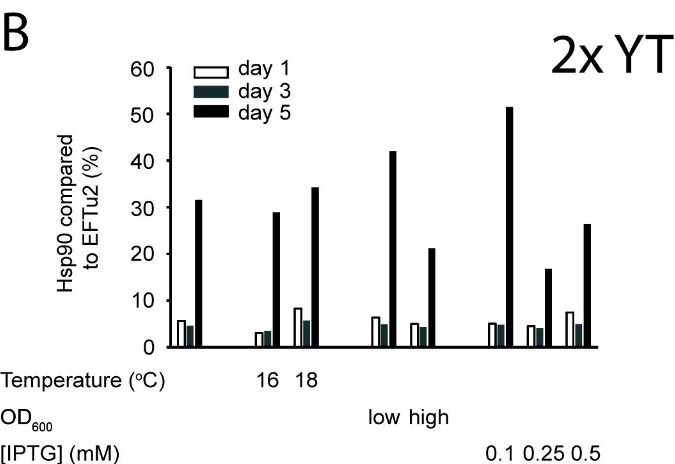
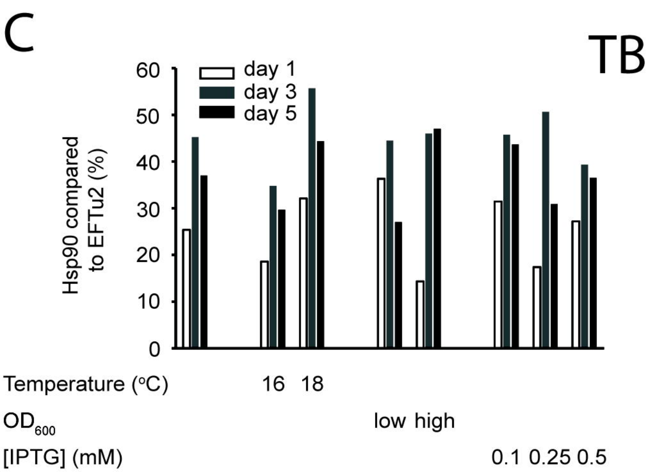



A
LB
60
50
40
30
20
10
0
day 1
day 3
day 5
Hsp90 compared to EFTu2 (%)
Temperature (°C)
16 18
OD600
low high
[IPTG] (mM)
0.1 0.25 0.5


B
2x YT
60
50
40
30
20
10
0
day 1
day 3
day 5
Hsp90 compared to EFTu2 (%)
Temperature (°C)
16 18
OD600
low high
[IPTG] (mM)
0.1 0.25 0.5


C
TB
60
50
40
30
20
10
0
day 1
day 3
day 5
Hsp90 compared to EFTu2 (%)
Temperature (°C)
16 18
OD600
low high
[IPTG] (mM)
0.1 0.25 0.5

A

20MC 20HQ Heparin

Cell lysis, centrifugation

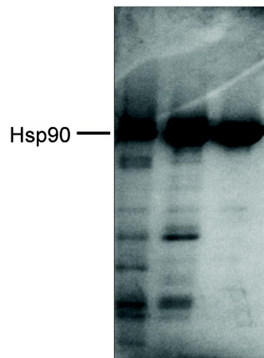
Concentration

90’ 60’ 30’ 120’ 120’

B

After column 20MC 20HQ Heparin

Hsp90



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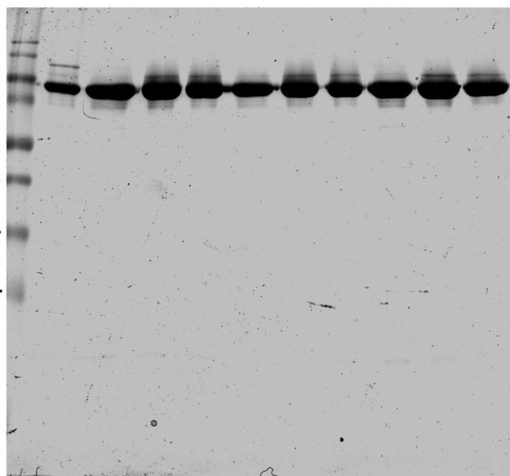

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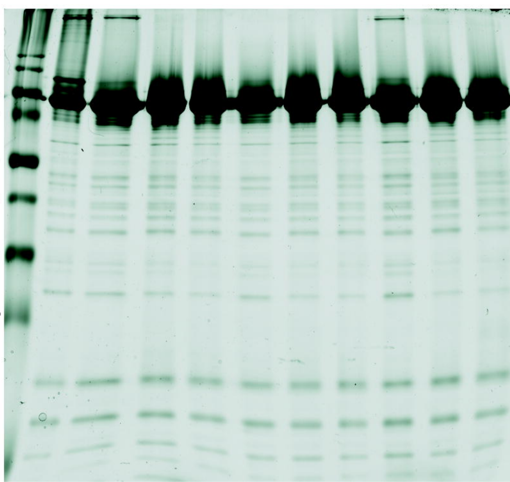
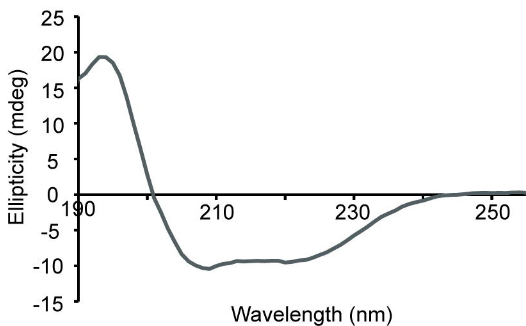
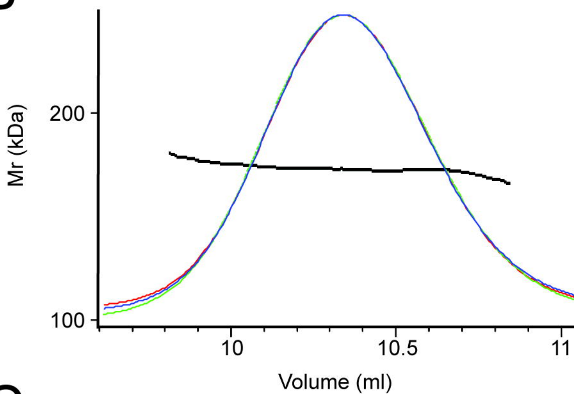
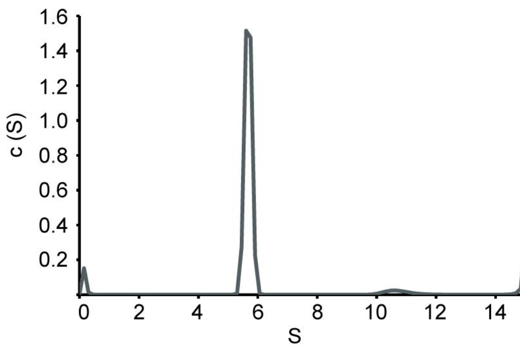



A
25
20
15
10
5
0
-5
-10
-15
190
210
230
250
Ellipticity (mdeg)
Wavelength (nm)


B
200
100
Mr (kDa)
10
10.5
11
Volume (ml)


C
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
c (S)
0
2
4
6
8
10
12
14
S