OVEREXPRESSION AND PURIFICATION OF HIS-TAG *E. COLI* σ^{70}

STRATEGY

Purification of His-tagged *E. coli* σ^{70} under denaturing guanidinium hydrochloride conditions using a nickel resin affinity chromotography column.

EXPERIMENTAL PROCEDURES

A/ Overexpression

1) Transform the plasmid pET21His*rpoD* and/or derivatives into competent cells BLR(DE3) carrying the T7 lysozyme on the plasmid, pLysS, using either $CaCl_2$ procedure or electroporation for greater efficiency.

2) Plate out on LB plates supplemented with 80 μg/ml ampicillin, 12.5 μg/ml tetracycline, 34 μg/ml chloramphenicol and 1% glucose. Incubate at 37°C for two nights (36 hr). Restreak if necessary.

3) From the transformations, pick a single colony and grow an overnight culture of BLR(DE3) pLysS pET21His*RpoD* (and/or derivatives) in 5 ml LB + 80 μg/ml ampicillin, 12.5 μg/ml tetracycline, 34 μg/ml chloramphenicol and 1% glucose. Incubate overnight at 37 °C with shaking.

4) The following morning, innoculate 200 ml LB + 200 μg/ml ampicillin, 12.5 μg/ml tetracycline and 34 μg/ml chloramphenicol with 3 ml of the overnight culture. Incubate at 37 °C with shaking. Harvest 1.5 ml of overnight culture in a microfuge tube and store at -20 °C for later analysis of plasmid DNA (**Miniprep 0**).

5) Once the culture has reached mid to late log (A_{650} = 0.5-0.6), collect ($500/A_{650}$) μl of culture in a microfuge tube for SDS-PAGE analysis (**Sample 0**). Harvest the sample and resuspend in 50 μl of Sample Buffer: store at -20 °C. Induce the culture with 1 mM IPTG and supplement with 100 μg/ml ampicillin (200 μl of 1 M IPTG; 500 μl of 40 mg/ml ampicillin). Incubate the culture for a further 3-4 hr at 37 °C with shaking to allow induction.

6) After induction, record the final A_{650} and collect ($500/A_{650}$) μl of culture for SDS-PAGE analysis (**Sample 1**). Harvest, resuspend and store the sample as before. Also, harvest 1.5 ml of culture in a microfuge tube and store at -20 °C for later analysis of plasmid DNA (**Miniprep 1**).

7) Harvest the culture by centrifugation at 5,000g (7,000 rpm in a 6×300 ml fixed angle rotor) for 15 min at 4 °C. Store the pellet at -70 °C until required.

8) If required, check integrity of the overexpression plasmid by performing minipreps of samples Miniprep 0 and Miniprep 1, and checking the gene inserts by restriction and sequencing. This is especially important if overexpressing toxic gene products and/or using a non-RecA$^-$ host expression strain such as BL21(DE3).

9) Check overexpression by analysing 10 μl Sample 0 and Sample 1 with 5 μl SDS 7 markers (0.2 μg/ml) on a SDS-PAGE 15% resolving gel with 6% stacker.

B/ Sample and Column preparation

1) Resuspend the cell pellet in 10 ml Lysis Buffer at 4 °C.

2) Lyse the cells by sonication. Check lysis by measuring A_{650} before and after sonication (1/50 dilution 'before' and 1/20 dilution 'after'). Sonicate the sample on ice using a microtip at 30% amplitude using 30 s bursts of 5 s 'on' and 2 s 'off'. Complete lysis of the culture should result in up to a tenfold drop in A_{650}. Cell pellets which were stored at -70 °C will already be partially or fully lysed. In these cases, sonication should be continued until the samples are no longer viscous. This is due to shearing of the genomic and plasmid DNA.

3) Centrifuge the lysate at 10,000g (11,000 rpm in a 8×50 ml fixed angle rotor) for 10 min at 4 °C.

4) Retain the supernatant as the **soluble fraction**. Resuspend the pellet in 10 ml Triton Buffer at 4 °C. Retain as the **insoluble fraction**.

5) Estimate the protein concentration of each fraction using BioRad assay. Prepare 100 µl of /10 of each fraction and analyse 30 µl for protein diluted in 770 µl H_2O and 200 µl BioRad reagent (equivalent to 3 µl of stock fraction).

6) Analyse 10 µg of protein (as estimated from the BioRad assay) from the soluble fraction and insoluble fraction together with 10 µl Sample 1 and 5 µl SDS 7 markers (0.2 µg/µl) on SDS-PAGE 15% resolving gel with 6% stacker.

7) The majority of His-tagged σ^{70} should be present in the insoluble fraction. Estimate content of His-tagged σ^{70} in the insoluble fraction as a percentage of total protein, and from the total protein determined from BioRad assay estimate total His-tagged σ^{70}.

8) Centrifuge the insoluble fraction at 10,000g (11,000 rpm in a 8×50 ml fixed angle rotor) for 10 min at 4 °C. Resuspend the pellet in 10 ml GDHCl Buffer + 10 mM imidazole at 4 °C.

9) The QIAGEN Ni-NTA agarose has a binding capacity of 5-10 mg/ml. Prepare an appropriate amount of resin in a 10 ml BioRad polyprep column to bind the calculated total His-tagged σ^{70} present in the insoluble fraction (assuming a 100% yield in centrifugation step 8).

10) Pre-equilibrate the resin by washing in 10 column volumes of GDHCl Buffer + 10 mM imidazole at 4 °C.

C/ Protein Purification

All steps performed at 4 °C.

1) Apply desired volume of protein sample into the assembled Ni-NTA agarose BioRad polyprep column reservoir (Retain 100 µl as **Sample 2**). If necessary, top up the sample volume to 10× the column volume. Tightly seal the BioRad polyprep column and mix the sample with the resin by placing on a rocker or rotating wheel for 30 min.
2) Clamp the BioRad polyprep column to a stand and allow the resin to settle (~30 min). Break the seal on the column and loosen the top lid. Allow the protein sample to gravity flow through: collect eluate as **Sample 3**.
3) With 0.2 ml of the protein sample remaining above the resin, wash by adding 10× column volumes of GDHCl Buffer + 10 mM imidazole and allow to gravity flow through. Collect as **Sample 4**.
4) With 0.2 ml of the wash solution remaining above the resin, elute the His-tagged σ^{70} using a stepwise imidazole gradient in GDHCl Buffer. Elute with 5× column volumes for each increasing concentration of imidazole, using 20, 40, 60, 80 and 100 mM steps in GDHCl Buffer. Collect 4 fractions per step.

D/ Fraction analysis and dialysis

1) Assay fractions for protein content by analysing 20 µl of each fraction with 780 µl H_2O and 200 µl BioRad Reagent (NB. High concentrations of imidazole affect protein estimation by Bradford assay. To correct for this, assay equivalent volumes of GDHCl Buffer + appropriate [imidazole].).
2) Select fractions for further analysis of purity by SDS-PAGE (generally, fractions giving A_{595} >0.1-0.15 from the BioRad assay are chosen). Since protein samples that contain GDHCl precipitate when treated with SDS, the GDHCl is removed by TCA precipitation before analysis on SDS-PAGE.
3) Mix 50 µl of Samples 2-4 and each selected fraction with 50 µl H_2O and 100 µl (equal volume) of 10% TCA in a microfuge tube.
4) Leave on ice for 20 min. Centrifuge in a microfuge for 15 min, wash pellet with 100 µl cold ethanol and centrifuge for a further 5 min.
5) Dry the pellet and resuspend in 20 µl Sample Buffer.
6) Analyse 5 µl of TCA treated Samples 2-4 and each fraction with 5 µl SDS 7 marker (0.2 µg/µl) on SDS-PAGE 15% resolving gel with 6% stacker (use fine gel combs - 15 wells/gel)
7) Pool final selected fractions after SDS-PAGE analysis and dialyse in 500 ml Storage Buffer at 4 °C, stirring gently, overnight.
8) Change with fresh Storage Buffer at 4 °C the following morning, and change a further two times at 3 hr intervals during the day.

E/ Determination of protein concentration by Biorad assay

1) Make up 10 mg/ml BSA stock in sterile water and prepare the following dilutions;

   20 µl 10 mg/ml BSA + 180 µl H_2O - /10 1 mg/ml

   25 µl 1 mg/ml BSA + 975 µl H_2O - /40 0.025 mg/ml

2) Prepare a BSA standard curve from the 0.025 mg/ml BSA stock as below. Add the BioRad protein assay solution last to each tube and incubate at room temperature for 5 min.

µg BSA	µl of 0.025 mg/ml BSA	µl H_2O	BioRad Reagent	A_{595}
0	-	800	200	(blank)
1.25	50	750	200	
2.5	100	700	200	
5.0	200	600	200	
10.0	400	400	200	

3) Prepare a 1/100 dilution of the His-σ^{70} preps. as follows;

   20 µl stock + 180 µl H_2O - /10

   150 µl /10 + 1350 µl H_2O- /10

   Set up the assays as below;

Protein solution	µl H_2O	BioRad Reagent	A_{595}
1/ 800 µl /100	-	200	
2/ 400 µl /100	400	200	

   If the preps. are too dilute then prepare a third assay as follows;

3/ 40 µl stock	760	200	

4) From the g/l concentration of the His-σ^{70} stocks, the Molar concentration can be calculated given that the Molecular weight of σ^{70} is 72 kDa.

F/ SDS-PAGE analysis of His-σ^{70} samples by comparison with BSA

Preparation of the samples; (Boil all for 2 min before loading).

1) SDS-7 markers (0.2 µg/µl). Load 5 µl

2) BSA Prepare 0.2 µg/µl BSA stock in sample buffer. Load 2.5 µl, 5 µl and 10 µl (0.5 µg, 1.0 µg, 2.0 µg).

3) His-σ^{70} samples. Using the protein concentrations estimated from the BioRad assays, prepare either 0.2 µg/µl or 0.1 µg/µl stocks in Sample Buffer and load 0.5 µg, 1.0 µg and 2.0 µg.

4) If possible, load some neat His-σ^{70} sample (e.g. 10 µl + 5 µl Sample Buffer) to check the purity of your prep.

SOLUTIONS AND REAGENTS

Autoclave all solutions. DTT should only be added to solutions after autoclaving.

1 M Tris.HCl (pH 7.6 @ 4 °C)

Stock	Volume/g
M_r=121.1	12.1 g
(pH to 7 @ 25 °C using conc^d. HCl)	
Total Volume	100 ml

5 M NaOH

Stock	Volume/g
M_r=58.44	29.22 g
Total Volume	100 ml

1 M $MgCl_2$

Stock	Volume/g
M_r=203.30 (.2H_2O)	20.33 g
Total Volume	100 ml

0.5 M EDTA

Stock	Volume/g
M_r=372.24 (.2H_2O)	18.61 g
(pH to 8 with conc^d NaOH)	
Total Volume	100 ml

1 M IPTG

(Do not autoclave: store at -20 °C)

Stock	Volume/g
M_r=238.3	1.192 g
Total Volume	5 ml

1 M DTT

(Do not autoclave: store at -20 °C)

Stock	Volume/g
M_r=154.2	154 mg
Total Volume	1 ml

Lysis Buffer
10 mM Tris.HCl (pH 7.6 @ 4 °C)
1 mM EDTA

Stock	Volume/g
1 M Tris.HCl (pH 7.6)	1 ml
0.5 M EDTA	200 µl
Total Volume	100 ml

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Triton Buffer

	Stock	Volume/g
50 mM Tris.HCl (pH 7.6 @ 4 °C)	1 M Tris.HCl (pH 7.6)	5 ml
500 mM NaCl	5 M NaCl	10 ml
0.5% Triton	Triton	0.5 ml
	Total Volume	100 ml

GDHCl Buffer + 10 mM I

	Stock	Volume/g
6 M GDHCl	M_r=95.53	172 g
10 mM Tris.HCl (pH 7.6 @ 4 °C)	1 M Tris.HCl (pH 7.6)	3 ml
500 mM NaCl	5 M NaCl	30 ml
10 mM imidazole (I)	M_r=68.08	204 mg
(Do not autoclave)	Total Volume	300 ml

GDHCl Buffer + 200 mM I

	Stock	Volume/g
GDHCl Buffer + 10 mM I	GDHCl Buffer + 10 mM I	40 ml
200 mM imidazole (final)	M_r=68.08	517 mg
	Total Volume	40 ml

GDHCl Buffer + stepwise gradient of imidazole

GDHCl + [imidazole]	GDHCl + 10 mM I	GDCHl + 200 mM I
20 mM	18.95 ml	1.05 ml
40 mM	16.84 ml	3.16 ml
60 mM	14.74 ml	5.26 ml
80 mM	12.63 ml	7.37 ml
100 mM	10.53 ml	9.47 ml

Storage Buffer

	Stock	Volume/g
10 mM Tris.HCl (pH 7.6 @ 4 °C)	1 M Tris.HCl (pH 7.6)	20 ml
10 mM $MgCl_2$	1 M $MgCl_2$	20 ml
0.1 mM EDTA	0.5 M EDTA	4 ml
50% w/v glycerol	1 g = 0.8 ml	800 ml
0.1 M KCl	M_r=74.55	14.91 g
0.1 mM DTT (after autoclaving!)	1 M DTT	200 µl
	Total Volume	2,000 ml

Calculation for imidazole stepwise gradient

GDHCl + [imidazole]	No. of mmol imidazole	GDHCl + 10 mM I (0.01 mmol/ml I)	GDCHl + 200 mM I (0.2 mmol/ml I)
20 mM	0.4	18.95 ml	1.05 ml
40 mM	0.8	16.84 ml	3.16 ml
60 mM	1.2	14.74 ml	5.26 ml
80 mM	1.6	12.63 ml	7.37 ml
100 mM	2.0	10.53 ml	9.47 ml

If we let the No. of mmol imidazole be a
the volume of GDHCl + 10 mM I added be x
and the volume of GDHCl + 200 mM I added be y,

then a given quantity of imidazole, a, is made up with x ml of GDHCl + 10 mM I (at 0.01 mmol/ml) and y ml of GDHCl + 200 mM I (at 0.2 mmol/ml). This is represented by the equation;

$$0.01x + 0.2y = a \qquad \text{(Equation 1)}$$

Also, since the final volume of each solution is 20 ml,

then; $x + y = 20$

Rearranging to give;

$$y = 20 - x \qquad \text{(Equation 2)}$$

Substitute $y = 20 - x$ into equation 1 to give;

$$x = (4 - a)/0.19 \qquad \text{(Equation 3)}$$

Therefore, if 2 mmol imidazole is required (i.e. 20 ml of a 100 mM solution), then $a = 2$, and by substituting into equation 3;

$$x = (4 - 2)/0.19$$

$$x = 10.53$$

and by substituting $x = 10.53$ into equation 2;

$$y = 20 - 10.53$$

$$y = 9.47$$

Thus, for 20 ml of 100 mM imidazole in GDHCl Buffer, you require 10.53 ml of 10 mM imidazole and 9.47 ml of 200 ml imidazole in GDHCl Buffer.