Protein Science - Purification of the Human RAF1 CR1 Protein

Purpose

This collection of protocols was developed for the purification of several constructs of RAF1 that contain the cysteine-rich domain (CRD) subdomain, as the presence of this domain was observed to lower the yield from what can be achieved with just the Ras-binding domain (RBD).

Exceptions for the following constructs/conditions are noted (see Exceptions, A, B, C):

A. WT CRAF CR1 [R717-X61-566: His6-MBP-tev-Hs. RAF1(52-192)] $^{15}N/^{13}C$

B. His-Tagged WT CRAF CR1 [R757-X57-557: MBP-tev-His6-Hs.RAF1(52-192)]

C. C95S CRAF CR1 untagged [R757-X26-566: His6-MBP-tev-Hs.RAF1(52-192) C95S]

Procedures

A. Expression

1. Transform the BL21 STAR (*rne131*) *E. coli* strain containing the DE3 lysogen and rare transfer RNAs (pRARE plasmid) with the target-expressing plasmid (R717-X61-566: His6-MBP-tev-Hs.RAF1(52-192)).
2. From a glycerol stock, inoculate 300 mL of the *E. coli* MDAG (recipe below in Recipes) seed culture (in a 2 L baffled flask) and grow overnight at 37°C with 100 µg/mL Amp (for maintenance of the expression plasmid) and 15 µg/mL Cm (for maintenance of pRARE).
3. Set up a BioFlo IV fermenter (New Brunswick) with 15 L of LB media (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract) supplemented with 300 µM ZnCl2 and 100 µg/mL Amp, 15 µg/mL Cm and inoculate with the 300 mL overnight culture, followed by growth at 37°C with the airflow set at 15 LPM and agitation maintained at 350 RPM.
4. Induce protein expression at OD_{600} of 0.5 with 0.5 mM IPTG.
5. Harvest the culture after 3 hours of induction by centrifugation using a Beckman Coulter Avanti J-20 XP with a 6 × 1 L rotor. Immediately freeze cell pellets at –80°C.

B. Purification

Unless noted otherwise, perform purification steps at room temperature (20–23°C).

1. Resuspend cells equivalent to 7.5 L of culture with 20 mM HEPES (pH 7.3), 500 mM NaCl, 5 mM TCEP, 10% (w/w) glycerol (Buffer A), using 10 mL for every 1,000 total OD units in culture/pellet. During resuspension, keep the cells on ice. Add Sigma-Aldrich protease inhibitor at a ratio of 1:200 (v/v).
2. Lyse the homogenized cells using a Microfluidizer M-110-EH (Microfluidics International Corporation, Westwood, MA) at 10,000 psi for 2 passes. Keep the lysis chamber submerged in an ice water bath for the entire procedure.
3. Add 2 mM MgCl2 and 500 units of Benzonase to the unclarified lysate and centrifuge it at 70,000 × *g* for 30 minutes at 4°C.
4. Adjust the clarified lysate to 35 mM imidazole and immediately load it onto a 20 mL IMAC column (HisPrep FF 16/10, GE Healthcare). The equilibration buffer (EB) for the column was Buffer A in 35 mM imidazole.
5. Wash the column to baseline with EB and elute proteins with a 10 column-volume (CV) gradient from 35 mM to 500 mM imidazole in Buffer A. Trigger elution collection by an A280 of > 300 mAU (usually around 115 mM imidazole) and cut off with an A280 trigger of < 300 mAU. Analyze fractions by SDS-PAGE and Coomassie staining.
6. Apply the eluted bulk pool to a HiPrep 26/10 desalting column (GE Healthcare), using Buffer A, and collect it into a container with the presence of His6-TEV protease at ~6% (v/v, using a 5 mg/mL laboratory-made stock), allowing it to digest for 10 hours at 4°C. Alternatively, you can fraction the elutions from the IMAC column without A280 monitoring, analyze them by SDS-PAGE, pool appropriate fractions, add His6-TEV protease at ~6% (v/v, using a 5 mg/mL laboratory-made stock), and allow the pool to digest for 10 hours while dialyzing (10K MWCO) to Buffer A at 4°C.

7. Load the TEV-digested sample to a fresh IMAC column equilibrated with Buffer A.
8. After loading, wash the column to baseline with Buffer A, then with a 5 CV gradient from 25–50 mM imidazole in Buffer A, followed by 3 CV gradient from 50–250 mM imidazole in Buffer A, and finally with 2 CV of 500 mM imidazole in Buffer A. The target protein is present in the shallow gradient (25–50 mM imidazole in Buffer A).
9. Analyze chromatography by SDS-PAGE gel. Concentrate pooled protein to an appropriate volume (protein concentration should not exceed 3 mg/mL) for injection onto a 26/60 Superdex S-75 (GE Healthcare) column in 20 mM HEPES (pH 7.3), 500 mM NaCl, 5 mM TCEP, 10% glycerol.
10. Concentrate pooled protein using 10K MWCO Amicon centrifugation units to an appropriate volume for dialysis into 20 mM HEPES (pH 7.3), 500 mM NaCl, 5 mM TCEP, filter with a 0.22 µM syringe filter (low protein binding), and assay for protein concentration (A280) (protein concentration should not exceed 3 mg/mL).
11. Snap freeze final proteins in liquid nitrogen in small aliquots (0.1–0.25 mL) and store at –80°C.

Exceptions

A. WT CRAF CR1 [R717-X61-566: His6-MBP-tev-Hs. RAF1(52-192)] $^{15}N/^{13}C$

1. Inoculate a seed culture from a glycerol stock and grow it in 50 mL of MDAG 135, 100 µg/mL Amp, 15 µg/mL Cm in a 250 mL baffled shake flask for 16 hours at 37°C until mid-log-phase growth.
2. Remove 40 mL of the overnight seed culture (1:50 of production volume) and centrifuge at 3,000 × *g* for 10 minutes at 25°C. Resuspend the pellet with 40 mL of Mod M9 medium, +100 µg/mL Amp, 15 µg/mL Cm.
3. Use the suspension to inoculate 2 L of Mod M9, 100 µg/mL Amp, 15 µg/mL Cm in a 3 L Bioflow 110 bioreactor (Eppendorf/NBS). Grow the culture at 37°C with the airflow set at 2.0 LPM and agitation maintained at 481 RPM. When the OD_{600} reaches 0.4–0.6 (~4 hours), add $ZnCl_2$ to a final concentration of 300 µM and IPTG to a final concentration of 500 µM. Note that the timing of the addition of the $ZnCl_2$ is not critical; it can be incorporated into the starting medium.

The length of induction, cell harvest, and purification are the same as outlined above for expression in the standard medium.

B. His-Tagged WT CRAF CR1

Note that, given the recent identification of a problem with this protein in binding to KRAS, we have focused on the Avi-tagged version. Regardless, we have expressed this protein with two different approaches. More commonly in *E. coli* with R757-X57-596: MBP-tev-His6-Hs. RAF1(52-192), but also more recently, we are converting most, if not all, of our RAF1(52-192) expression to *Vibrio natriegens* with R757-X57-557: MBP-tev-His6-Hs.RAF1(52-192). The difference between these two expression plasmids (R757-X57-596 vs. R757-X57-557) is in the promoter, and while this doesn't seem to be critical, we have yet to do a good side-by-side comparison. RAF-CRD proteins from *Vibrio* are generally higher in quality and quantity for RAF constructs (including BRAF). However, we will only address the *E. coli* protocols here. The *E. coli* expression protocol is the same as for WT RAF CR1 (R717-X61-566: His6-MBP-tev-Hs. RAF1(52-192)) above. The purification begins with the same buffer and lysis as well and diverges after TEV IMAC.

1. Amend the eluted bulk IMAC1 pool with Strep-TEV protease at ~6% (v/v, using a 5 mg/mL laboratory-made stock) and allow it to digest for 10 hours while dialyzing (10K MWCO) to Buffer A at 4°C.
2. Remove the TEV-digested sample from dialysis and apply it to the IMAC column. Wash the column to baseline (~2.0 CV) and elute proteins as follows: a gradient of 0–50 mM imidazole in Buffer A for 3 CV, a gradient of 50–500 mM imidazole in Buffer A for 4 CV, and continued elution at 500 mM imidazole in Buffer A for 2 CV. Collect 14 mL fractions through all elution steps. Cleaved MBP can be found in the flow through, Strep-TEV, additional MBP are found in the 0–50 mM imidazole gradient, and His-tagged target protein elutes at a high imidazole concentration.
3. Analyze the chromatography by SDS-PAGE gel.

Subsequent sample handling (pooling, concentrating, SEC, desalting, final sample prep) is the same as for the untagged protein.

C. C95S CRAF CR1 untagged [R757-X26-566: His6-MBP-tev-Hs.RAF1(52-192) C95S]

Both expression and purification of this mutant are the same as for the WT RAF1(52-192).

Recipes

A. MDAG 135 media

- 25 mM Na_2HPO_4
- 25 mM KH_2PO_4
- 50 mM NH_4Cl
- 5 mM Na_2SO_4
- 2 mM $MgSO_4$
- 50 µM $FeCl_3$
- 20 µM $CaCl_2$
- 10 µM $MnCl_2$-$4H_2O$
- 10 µM $ZnSO_4$-$7H_2O$
- 2 µM $CoCl_2$-$6H_2O$
- 2 µM $CuCl_2$-$2H_2O$
- 2 µM $NiCl_2$-$6H_2O$
- 2 µM Na_2MoO_4-$2H_2O$
- 2 µM Na_2SeO_3-$5H_2O$
- 2 µM H_3BO_3
- 19.4 mM glucose
- 7.5 mM aspartate
- 200 µg/mL each of 18 amino acids E, D, K, R, H, A, P, G, T, S, Q, N, V, L, I, F, W, M)

B. Mod M9 medium (for ^{13}C and ^{15}N incorporation)

- 33.5 mM Na_2HPO_4
- 44.1 mM KH_2PO_4
- 17.1 mM NaCl
- 2 g/L ^{13}C-glucose
- 1 g/L ^{15}N NH_4Cl
- 2 mM $MgSO_4$
- 100 µM $CaCl_2$
- 4 µM $ZnSO_4$
- 1 µM $MnSO_4$
- µM H_3BO_3
- 0.7 µM $CuSO_4$