

cGAMP reporter expression and purification

1 - Expression

Transform pCZ270 and/or pCZ271 plasmids into Rosetta (DE3) pLysS chemically competent cells. Grow 20 ml over night cultures with Carbenicillin (Carb; 50 µg/ml)/Chloramphenicol (Cam; 34 µg/ml) in Terrific Broth. [Note: You can also go straight from a glycerol stock of these cells]. Next day, inoculate 1 l of TB with Carb/Cam/glucose (0.4%, added after autoclaving from sterile filtered 20% stock) with 10 ml of the over night culture. Grow until the OD600 reaches ~0.8. Then add IPTG to 0.25 mM and shake at 18 °C for 6 h. Harvest cells by centrifugation (3000 rpm, 15 min, 4 °C), wash once in PBS, freeze at -80 °C until use. Don't forget to take samples before and after induction for gel electrophoresis (spin down the equivalent of 500 µl of an OD600 of 0.8 and resuspend/boil in 100 µl 1x SDS sample buffer + reducing agent. Spin for 3 min at 15000 g).

2 – Lysis and binding to Ni-NTA

Resuspend cells in Lysis buffer [50 mM Tris-Cl pH = 7.5 @ 4C, 100 mM NaCl, 20 mM Imidazole, 1 mM TCEP (from pH 7 stock), 1x protease inhibitors, 0.25 mg/ml lysozyme]. After 20 min incubation on ice, lyse by sonication (We use a 3 min total sonication time, 10 sec on; 10 sec off, 30% amplitude). Centrifuge lysate (40000 rpm, 45Ti rotor, 4 °C). In the meanwhile, equilibrate 1 ml HisPur Ni-NTA Resin (Thermo) in wash buffer (500 g, 5 min, 4 °C spins). After the spin, move supernatant to fresh tube, and take samples of both the supernatant and the pellet (for the supernatant, add 10 µl to 90 µl 1x SDS sample buffer, for the pellet, put 100 µl 1x SDS sample buffer into a tube, then poke a pipette tip into the pellet and transfer whatever is stuck to it to the tube with sample buffer and pipet up and down a few times).

Add supernatant to resin, and incubate with rotation at 4 °C for 1 h. Spin down (500 g, 5 min, 4 °C). Recover supernatant and take a sample (10 µl + 90 µl 1x SDS sample buffer. Call this "Flow-through"). Wash the resin with bound proteins three times in batch, then transfer to a column. In the cold room, wash 4 x with 20 ml of wash buffer.

3 – ATP/MgCl₂ wash to remove chaperones

Remove column to room temperature and wash twice with 20 ml of (room temperature) wash buffer containing 5 mM ATP and 20 mM MgCl₂.

4 – Elution from Ni-NTA

Move column back to cold room, wash twice with 20 ml of wash buffer. Almost drain the column and close the tap. Add 3 ml of Elution buffer [50 mM Tris-Cl pH = 7.5, 100 mM NaCl, 300 mM Imidazole, 1 mM TCEP (from pH 7 stock)] and incubate for 2 min. Repeat 3 more times (last two times with 2 ml of elution buffer). After that, top up the resin with a few ml of wash buffer and keep until you know that your protein came off. Run +/- IPTG protein samples, pellet, Sup, FT and elution samples on a gel. For elution samples, add 10 µl + 90 µl 1x SDS sample buffer samples. Also take a beads sample: Add 90 µl of 1x SDS sample buffer to a tube and add 10 µl of the beads/wash buffer slurry; boil and spin.

Pool all elution fractions that contain protein.

5 – Purification on MonoQ anion exchange column

Even with all the washes and the ATP/MgCl₂ wash, the eluate probably won't be clean, and will probably still have some potential HSP70 contaminant. To purify it further, we use an AKTA FPLC. Please follow local guidance on how to use this type of equipment.

Take a HiTrap FF Q column, and insert into an AKTA FPLC. Wash with 5 CV (column volumes) of water, 5 CV of low salt buffer, 5 CV of high salt buffer, re-equilibrate with 5 CV of low salt buffer.

Filter the pooled eluates through a 0.2 μm syringe filter, take a sample (10 μl + 6 μl 3x SDS sample buffer with reducing agent), and load onto the column. Make sure you save the flow-through, as this will contain most of your protein!

Elute bound proteins with a gradient to 100% high salt buffer over 17 CV. Wash column with 5 CV high salt, and then re-equilibrate with 5 CV of low salt buffer. [The column can be reused – wash with 5 CV of water, then transfer into 20% EtOH with 5 CV] Load fractions with substantial UV absorbance on a gel together with your input and FT samples (as before, 10 μl sample with 6 μl 3x SDS sample buffer).

Pool all fractions that look good.

6 – Purification on S200 gel filtration

Spin pooled fractions to get rid of any potential precipitates (4,500 g, 4 °C, 20 min), and concentrate to about 650 μl with a Vivaspin filtration device (first equilibrate the membrane with GF buffer by adding a few ml and spinning at 4,000 g for 5 min. Discard the equilibration buffer from both tubes). At the same time, equilibrate the S200 column with GF buffer. Again, follow local guidance on AKTA FPLC usage.

Spin the concentrated protein (16,000 g, 4 °C, 15 min) and transfer to a second tube. If you see a precipitate, spin again until you don't see one anymore. Take input sample (10 μl + 6 μl 3x SDS sample buffer). Load onto column and take samples of UV peak fractions. Pool best fractions and concentrate to ~300 μM (Abs₂₈₀ = 66,700). Then mix with equal volume of GF buffer containing ~96% glycerol. Store at -20 °C.

Buffers

Ni-NTA Wash buffer

50 mM Tris-Cl pH = 7.5 @ 4C
100 mM NaCl
20 mM Imidazole
1 mM TCEP (from pH 7 stock)

Lysis buffer

As wash buffer but with freshly added

Ni-NTA Elution buffer

50 mM Tris-Cl pH = 7.5 @ 4C
100 mM NaCl
300 mM Imidazole
1 mM TCEP (from pH 7 stock)

MonoQ low salt buffer

50 mM Tris-Cl pH = 7.3 @ 4C
80 mM NaCl
1 mM DTT

MonoQ high salt buffer

50 mM Tris-Cl pH = 7.3 @ 4C
1 M NaCl
1 mM DTT

GF buffer

40 mM Tris pH 7.5 @ 4C
100 mM NaCl
1 mM MgCl₂
0.5 mM TCEP (from pH 7 stock)