**Media and stock solutions**

Potassium salt stock solution

1.5M K2HPO4

0.35M KH2PO4

Sonication stock buffer

20 mM Tris-HCl pH 7.9

150 mM NaCl

0.2 mM EDTA

10% Glycerol

Before use add PIC and 0.1-0.5 mM TCEP

AFC buffer

30 mM Tris-HCl pH 7.9

150 mM NaCl

0.1% detergent

Before use add PIC and 0.1-0.5 mM TCEP

TEV cleavage buffer

30 mM Tris-HCl pH 7.9

150 mM NaCl

0.2 mM EDTA

0.1% detergent

Before use add PIC and 0.1-0.5 mM TCEP

Calmodulin binding buffer

30 mM Tris-HCl pH 7.9

150 mM NaCl

2 mM CaCl2

0.1% detergent

Before use add PIC and 0.1-0.5 mM TCEP

Calmodulin wash buffer

30 mM Tris-HCl pH 7.9

150 mM NaCl

2 mM CaCl2

0.1-0.5 mM TCEP

Calmodulin elution buffer

30 mM Tris-HCl pH 7.9

100 mM NaCl

10 mM EGTA

0.1-0.5 mM TCEP

TB media 1L

11 g Bio-Trypton

22 g Yaest Extract

2% Glycerol

1 ml Kanamycin (25mg/ml)

50 ml Potassium salt stock solution

Digestion buffer

50 mM NH4HCO3

1 mM CaCl2

Wetting and Equilibration solution

70% CAN in 0.1% FA

Washing solution

100% H2O in 0.1% FA

***SPA-Tagged***

***Protein Purification***

***Purification of SPA-Tagged Proteins***

1. *Culturing SPATagged E. coli Strains and Sonication*

1. Inoculate 100 μL of a SPA-tagged *E. coli* glycerol stock into

50 mL TB media solution in a 250 mL conical flask.

2. Grow the culture overnight at 32°C until late log phase. Note

that the OD 600 of the overnight culture should be ~ 5 to 6.

3. Inoculate 50 mL overnight culture into 1 L fresh TB media

Grow the culture until OD 600 ~ 2 to 3(at5-6 hours) 32ºC in 4 L flask

shaking at 200 rpm.

4. Transfer grown *E. coli* cultures from the 4 L flask to clean

centrifugation bottle.

5. Centrifuge the *E. coli* culture in a Beckman J6-HC centrifuge

at 1700 rpmfor 15 min.

6. Discard the supernatants and remove excess liquid by inverting

the bottle on paper towel. Keep the centrifugation bottle on ice.

7. Add 25 mL of sonication buffer to the centrifugation

bottle and resuspend the *E. coli* cell pellet using a clean

25 mL pipet.

8. Transfer the resuspended cultures into 50 mL polypropylene

Falcon tubes and snap freeze the Falcon tubes using liquid

nitrogen. Store it at −80°C for future use.

9. Remove the Falcon tube containing the frozen cell pellet

from the freezer and place in cold water to thaw. Than sample

is completely thawed keep it on ice.

10. Ensure that the notch of the flat-tip in the sonicator is

fastened securely. Set the sonicator (Branson Ultrasonic

Sonifier 450 analog, cat#23395) controls to Duty cycle:

“50”; Timer: “Hold” and Output control: “7.”

11. Transfer the cell sample to the stainless steel cup for sonication.

12. Place the stainless steel cup on ice in an appropriate size

box. Make sure to place the box on top of the sand at an

appropriate height so that the flat tip is submerged into the

liquid but not too close to the bottom of the container.

13. When ready, turn on the sonicator. Set timer to 5 min. Sonication

is done for 3 min followed by 2 min of cooling to

prevent overheating of the samples

14. Pour the sonicated cell lysate into pre-chilled centrifugation

tube and place it on ice.

15. Centrifuge the lysate at 16,000 rpm for 30

min using a JA-17rotor (Beckman).

16. Remove the supernatant carefully from the centrifugation

tube, transfer it to 50 mL Falcon tube, and snap freeze

with liquid nitrogen. The sonicated frozen cell

extract is stored at −80°C for future use.

1. *Anti-Flag M2 Agarose Beads*

1. Prior to use, 100 μL of anti-Flag M2 agarose beads are transferred

into a column and the beads are washed twice with 1 mL

of AFC buffer without DTT.

2. The Falcon tube containing the sonicated frozen cell extract is

thawed by placing the tube in cold water.

3. The cell extracts are incubated with 3 μL of benzonase nuclease

(Novagen cat#70746; 25 U) for 30 min at 4°C.

4. Add 10% non-ionic detergent Triton X-100 (final

concentration of Triton X-100 should be 1%) and 150 μL of

anti-flag M2 agarose beads (Sigma cat#A2220) to the Falcon

tube containing the cell extracts. Briefly mix the tube contents

by tilting the Falcon tube upside down, then rotate the Falcon

tube for 3 h at 4°C using a LabQuake shaker (Barnstead/

Thermolyne, cat#59558).

5. Centrifuge the tube at 1,700 × *g* for 6 min. Carefully remove

as much supernatant as possible, taking care not to disturb the

loose bead pellet.

6. Resuspend the pellet in the remaining supernatant and

transfer the beads into 0.8 × 4 cm Bio-Rad polypropylene

prep columns (Bio-Rad cat#732-6008). Remove the bottom

outlet plugs of the columns and allow the eluates to drain by

gravity flow.

7. Wash the columns five times with 200 μL of AFC working

buffer and twice with 200 μL of TEV cleavage buffer.

8. Close the bottom outlet of the column. Cleavage is done

in the same column by adding 200 μL of TEV cleavage

buffer and 5 μL (50 units) of TEV protease. Close the top of

the column with a cap.

9. The column containing the beads is rotated overnight at 4°C.

1. *Calmodulin-Sepharose Beads*

1. Remove the top and bottom outlet plugs of the column after

incubation with TEV protease and drain the eluates into fresh

column.

2. One hundred microliter of Calmodulin-Sepharose beads

(Amersham Biosciences cat#17-0529-0), corresponding to

200 μL of bead suspension, is transferred into a column and

washed twice with 10 mL of calmodulin binding buffer.

3. Four hundred microliters of 1X TEV cleavage buffer and 1.2

μL of 1 M CaCl 2 are added to the eluate recovered after TEV

cleavage.

4. The mixture is then transferred to the column containing the

washed Calmodulin-Sepharose beads. After closing the column,

rotate for 3 h at 4°C.

5. Remove the top and bottom plugs of the column and drain

the eluate by gravity flow.

6. The beads are washed four times with 200 μL of calmodulin

binding buffer followed by two washes with 200 μL of

calmodulin wash buffer.

7. The bound proteins are eluted in six fractions of 50 μL in a

fresh eppendorf tube using 1X calmodulin elution buffer.

1. *Detergent cleaning step*

Use a protocol from “Perfect-FOCUS”detergent cleaning kit.

1. *Sample digestion by Trypsin*

1. Dilute dry sample by adding to eppendorf 50 ul of digestion buffer .

2.Add 0.9 ul of 100 mM TCEP-HCl(tris(2-carboxyethyl)phosphine),final

Concentration of 2mM) for reduction to incubate at R/T for 45 min.

If use DTT,incubate at 37C for 30 min.

3.Add 1ul of 0.5 M IAM(Iodoacetamide,final concentration of 10 mM)

and incubate at dark for 40 min for alkylation.

4.Add 70 u/mg of Trypsin to sample and incubate at 37C 5 hours or

R/T overnight.Stop reaction by adding 1 ul of Acetic Acid to the sample.

 ***F.*** Zip-Tip sample for peptide and protein analysis.

1. Equilibrate:

 Aspirate 10 ul wetting solution into tip

 Dispence to waste

 Repeat twice

 Aspirate equilibration solution

 Dispence to waste

 Repeate twice

2. Bind and Wash:

 Aspirate and dispense 10 cycles(sample mixtures)

 Aspirate washing solution

 Dispense to waste

 Repeate twice

3. Elution:

 Dispense 10 ul of elution into clean vial(standart pipette tip) twice

 Dry the sample