**Carter lab insect cell ZZ-tag prep (2020)**

***Buffers***

|  |  |  |
| --- | --- | --- |
| **Wash Buffer (500 ml)** | | |
| 50 mM | Hepes pH7.4 | 25 ml (1 M) |
| 100 mM | NaCl | 50 ml (1 M) |
| 1 mM | DTT | 0.5 ml (1 M) |
| 0.1 mM | ATP | 0.5 ml (0.1 M) |
| 10% | Glycerol | 50 ml (100 %) |
| 2 mM | PMSF | 5 ml (0.2 M) |
|  | *Fill to* | *500 ml* |

**Lysis buffer (50 ml)** is made from Wash Buffer + 2 mM PMSF + 1 cOmplete protease inhibitor tablet (Roche) per 50 ml. (We use the EDTA free ones, though this is not necessary for this prep because we are not using the His-tag)

|  |  |  |
| --- | --- | --- |
| **TEV Buffer (5x stock 500 ml)** | | |
| 250 mM | Tris HCl pH 7.4 | 125 ml (1 M) |
| 740 mM | K-Acetate | 36.8 g |
| 10 mM | Mg-Acetate | 5 ml (1 M) |
| 5 mM | EGTA | 12.5 ml (0.2 M) |
| 50% | Glycerol | 250 ml (100%) |
|  | *Fill to* | *500 ml* |

Add ATP (0.1 mM final) and DTT (1 mM final) just before use. Lower pH is necessary to make it compatible with the Tosoh G4000 column.

|  |  |  |  |
| --- | --- | --- | --- |
| **GF150 Buffer** | | | |
| 25 mM | Hepes pH7.4 | 6.25 ml (1 M) | 12.5 ml (1 M) |
| 150 mM | KCl | 18.75 ml (2 M) | 37.5 ml (2 M) |
| 1 mM | MgCl2 | 0.25 ml (1 M) | 0.5 ml (1 M) |
| 5 mM | DTT | 1.25 ml (1 M) | 2.5 ml (1 M) |
| 0.1 mM | ATP | 0.25 ml (0.1 M) | 0.5 ml (0.1 M) |
|  | *Fill to* | *250 ml* | *500 ml* |

Add ATP (0.1 mM final) and DTT (1 mM final) just before use. Lower pH is necessary to make it compatible with the Tosoh G4000 column.

***Insect cell expression***

-Infect 1l Sf9 cells @1-2x106/ml in Sf900II or Lonza insect-XPRESS media with 10ml P2 virus

-Expression in roller bottles for 72h (500ml per 2 L bottle)

-Harvest: centrifuge 15 min, ~2250g/3000rpm, 4 °C (JLA 12.5 rotor, 1 L Beckman bottles)

-Resuspend in ice cold PBS by swirling (~200ml total volume)

-Distribute between labelled 4x50ml tubes (i.e. 250ml equivalent pellet per tube)

-Centrifuge 15 min, ~2000g, 4 °C

-Discard supernatant and snap freeze pellets in LN2 (be careful not to close tube tightly as it might explode, I always wear safety glasses for this)

-Store at -80C

*PBS wash is not necessary since we only use serum (i.e. animal protein) free media now. However, it is still very useful to get the cells into the 50 ml tubes.*

***Protein purification***

-Thaw cell pellet (250 ml equivalent) on ice (can take quite long >1h, does not have to thaw completely - a small frozen cell clump is not a problem in the dounce). Alternatively thaw for ten minutes in a cool water bath with lysis buffer, monitoring every few minutes.

-Add lysis buffer (Wash buffer + PMSF + cOmplete tablet (total volume ~25ml)

-Can vortex and/or add stir bar to aid resuspension of cells

-Place 50ml dounce in ice bucket and move to cold room to pre-cool

-Transfer the resuspended cells into the dounce (on ice) and move plunger up and down 20-50 times (20 is sufficient, need more if there were frozen clumps)

-Move lysate to Ti70 tubes (~25ml per tube)

-Spin 45min in Ti70 rotor @70krpm, 4 °C

-During spin prepare beads. Use 5-10ml IgG beads for dynein, for BICD 1-2ml is plenty

-Pour into yellow low-pressure column (BioRad Econo-Column)

-Wash with ~50ml lysis buffer (can fill to top wait for it to flow through, can use syringe to push through last bit)

-After spin pour lysate into 50ml tube (can be a bit cloudy or viscous, this does not seem to be a problem)

-Take 20ul sample of lysate for gel

-Pour lysate on plugged column

-Put cap on and incubate on roller for 1-6h

-Let lysate flow out

-(Can catch lysate in 50ml tube and run over column multiple times, I am not sure if this really increases yield but it does not seem to harm the prep either)

-Wash beads with ~50ml lysis buffer

-Wash beads with ~50ml TEV buffer

-Resuspend beads into smaller tube for TEV cleavage (still in TEV buffer)

1ml beads 🡪 2ml total in 2ml eppendorf

2-3ml beads 🡪 5ml total in 5ml eppendorf

5ml beads 🡪 15ml total in 15ml falcon

-Add 100 µl TEV protease (4 mg/ml)

-Incubate at 4 °C on roller o/n

-Spin down beads (2 min max. speed)

-Remove sup and filter out beads (small volumes use .22um spin filter, large volumes can use column)

-Use Amicon concentrator to concentrate. Be careful not to concentrate too far as it might precipitate the protein.

-During concentration equilibrate TOSOH G4000 column GF150 buffer on AKTAmicro

-Load sample in 100ul loop and run

***Gel-filtration on AKTAmicro***

-Column is stored in water + 0.05% NaN3. If stored in 20% ethanol, first wash into water before washing into buffer.

-Filter all buffers with 0.22 µm filter before using on AKTA.

-Put tube A1 in buffer, and pumpwash.

-Before connecting column, wash through connecting tubing with buffer.

-Connect column, drop-to-drop, low flow rate, make sure pressure alarm is activated.

-Equilibrate column with buffer (0.4 ml/min, 1.5 column volumes)

-Rinse loop (100ul) with ~2ml buffer (small plastic syringe)

-Get glass Hamilton syringe (100 or 250ul)

-Load sample into loop (make sure to spin filter sample just before, use 0.22um filter. Alternatively spin for 5 min at max speed in Eppendorf centrifuge and avoid the bottom of the tube when drawing sample into syringe)

-Put 96well round bottom plate into fraction collector

-Run column at 0.3 ml/min flow rate, collect 100 µl fractions beginning at 4.5 ml.

-When done remove fractions from collector

-Move tube A1 to water + 0.05% NaN3, pumpwash

-Equilibrate column back into water + 0.05% NaN3 (0.4 ml/min, 1.5 column volumes)

-Remove column and replace caps on either end.

-Put inlet A1 into 20% ethanol, pumpwash