Making H3–H4 tetramers and H2A–H2B dimers

Section A: Purifying histones

1 – Expression of histones

Express His-H3 from pCZ154 (H3.2) or pR5-HisTEV-H3.3 in Rosetta (DE3) cells (Kanamycin/chloramphenicol).
Express His-H4 from pYOKO-HisTEV-H4 in Rosetta (DE3) or BL21 (DE3) cells (Ampicillin/Chloramphenicol). The H4 sequence contained in pYOKO-HisTEV-H4 is already optimised for *E. coli*, so Rosetta cells aren't necessary.
Express His-H2A from pR5-H2A (Kanamycin/chloramphenicol) in Rosetta (DE3) cells.
Express H2B from pET3a-H2B (Ampicillin/Chloramphenicol) Rosetta (DE3) cells. Note that because H2B starts with a proline after the methionine, you cannot add a TEV site to its N-terminus.
Inoculate a 1l starter culture of **1.5x TBG-M9-YE plus 0.4% glucose**, plus the appropriate antibiotics with ~12ml of o/n starter culture in the same medium. Grow at 37°C until an OD of 1-1.2 (usually in the range of ~2hrs 15min), induce expression by adding IPTG to 0.7mM, grow at 37°C for 4hrs.

Spin down (JLA 8.100, 3krpm, 12'), resuspend in ~20ml of W/L buffer (you can also use W/L without imidazole and BME), transfer to 50ml falcon tube, spin down again, remove supernatant, freeze at -80°C. Note that H3 pellets are somewhat whitish, whereas H4 pellets are more grey/greenish.

2 – Purification of inclusion bodies

Take frozen pellets, thaw in a RT water bath for ca. 15min or so. Resuspend cells in 30ml of lysis buffer using method of choice (like dounce homogeniser). Leave standing on ice for ca. 15min or so, **sonicate** (37%, 10sec on, 10sec off, 1min 40sec total time).

Spin (in **SS34** tubes – you may want to increase the volume by adding more lysis buffer) at **20,000g; 4°C; 15'**.

Discard supernatant, **rinse inclusion body pellet**. Especially for H3s, the pellet will as majority by white, with some grey-greenish stuff on top. This is general cell debris. I usually try and get rid of that by shooting 10ml or so of W/L buffer on top of the pellet without trying to disturb the underlying inclusion bodies. Discard this wash buffer with whatever of the cell debris you managed to resuspend.

Resuspend inclusion bodies: Add 5ml of W/L buffer, mix well with a spatula & by vortexing. Add another 10ml of W/L buffer, vortex. Add 15ml of W/L-T buffer, vortex.

Spin 13,000g; 4°C; 10'.

Rinse inclusion bodies twice as above with 10ml W/L.

Resuspend pellet in 5ml W/L buffer (without Triton!) as before. Add 25ml more ml of W/L buffer.

Spin 13,000 g; 4°C, 10'

(If you want you can store the inclusion bodies at this for a short time at 4°C or for a little bit longer frozen).

Resuspend pellet in 1ml DMSO as before using a spatula. This may take a little time. When done, **add 25ml of D500 buffer**.

Rotate at RT for 1-2hrs or o/n.

Spin 30,000g; 4°C; 20'

For H2B, you don't do any further purification. Instead, check that H2B is the main band by coomassie, and if that is the case, concentrate it down to ~10 ml using spin tubes.

3 – Purification from Ni-NTA (H3s, H4, H2A)

Bind supernatant to 1.5ml of Ni-NTA (equilibrated in D500) by rotating for 1.5hrs at RT.
Wash 2x in batch with ~40ml **D500**. Transfer to disposable BioRad column.
Wash on column (3x with 20ml **D500** each)
Wash on column (3x with 20ml **D1000** each)
Elute - 1x 5ml of elution buffer (incubate ca. 4min), then 2x 2.5ml.

Measure Abs_{280} of 1:10 dilution in D1000 (it is critical that your blank is elution buffer diluted 1/10 in D1000). **Determine concentration. The extinction coefficient of HisTEV-H3 is 5960, the extinction coefficient of HisTEV-H4 is 7450, the extinction coefficient for HisTEV-H2A is 5960**.

Run on a 15% PAA gel (the samples are in guanidine. Because guanidine precipitates SDS, they have to be diluted at least 1/100). Take 1µl of each eluate & mix it with 99µl 1x SDS sample buffer. Boil & load & run 15µl immediately, stain gel with coomassie.

If pure enough (I've never had them not pure enough), **add DTT to 4mM**, store at 4°C.

4 – Media & Solutions for Section A

1.5x TBG-M9-YE (1litre):

15g	Tryptone
7.5g	Yeast extract
5g	NaCl
0.15g	$MgSO_4$
1.5g	NH_4Cl
3g	KH_2PO_4
6g	Na_2HPO_4

add glucose from filtered 20% glucose stock before use. (When making glucose, pour in glucose into slightly heated, stirring water. If you pour water on top of glucose, it'll turn into a rock)

W/L (wash/lysis) buffer (~140ml/pellet)
50mM Tris-Cl (stock is pH 8 @ 22°C)
100mM NaCl
10mM imidazole
10mM BME (20µl/25ml of buffer)

Lysis buffer (~25ml/pellet)
W/L buffer
+ 1mM PMSF
+ LPC to 1x

+ 10mM BME (20µl/25ml of buffer)
+ 0.25mg/ml lysozyme
+ DNaseI to 5µg/ml final (stock is 1mg/ml in 150mM NaCl, 50% glycerol, 10mM Tris pH 7.5, stored at -20°C)

W/L-T buffer (15ml/pellet)
W/L buffer
+1% Triton X-100

D500 buffer (~180ml/pellet)
6M Guandidine HCl
500mM NaCl
50mM Tris-Cl (stock is pH 8 @ 22°C)
5mM BME (10µl/25ml of buffer)
7.5mM imidazole
(May need a little bit of heating up to bring everything into solution. pH ends up at ca. 7.9 @ 22C)

D1000 buffer (~60ml/pellet)
Like D500, but 1M NaCl
(Right at the solubility limit. Heat up & stir to bring into solution, add water stepwise until everything is dissolved)

Elution buffer (~20ml/pellet)
D1000 but made with a Tris stock that is pH 7.5 @ 22°C
+ 300mM imidazole
(Again – right at the solubility limit. Check notes for D1000). pH ends up at ca. 7.4 @ 22C

Section B: Refolding into H3/H4 tetramers and H2A/B into dimers, tag removal & gel filtration

1 – Refolding by dialysis

If you have enough of each histone, make a mix of a total of 4.5ml at a concentration of **45µM of both the H3 and the H4 or the H2A and the H2B** that you want to use. Most likely the stocks will be much higher concentration than that, so add the appropriate volume of each & **bring volume up to 4.5ml with D500** buffer (from the previous section). Measure Abs_{280} of 1:4 dilution.

Transfer the mix to **snake skin dialysis membrane** (double clip each end) & **dialyse into 4l DiaB1 at 4°C o/n**. Prepare 4l each of DiaB2 & DiaB3 as well & leave at 4°C for use on the next day.

The next day, in almost all cases, you will see that the dialysis solution has become cloudy. This is due to some of the histones precipitating during the dialysis. The more your error is in determining the concentration of the histones you use (ie, the stronger the imbalance between H3 & H4 is), the more precipitate will you see. Recover the solution from the tubing & transfer it to a 15ml falcon tube. **Spin in clinical centrifuge (4,500g, 4°C, 10')**. **Remove & save sup** – this contains your refolded tetramer. If you want you can dissolve the pellet in 500µl 1x SDS sample buffer and see what's in it, but this is not necessary…

Take a protein gel sample of the sup (10µl plus 6µl 3x SDS sample buffer plus BME or 3.5µl 4x SDS sample buffer plus BME) & also **measure Abs_{280} of the sup** (dilute 1:4 in D500).

Move the sup to fresh dialysis tubing & **dialyse 3-4hrs against DiaB2 at 4°C.** Move the tube (most likely there won't be any precipitate this time). **Move tubing into DiaB3, dialyse 3-4hrs at 4°C**.

Remove from tubing, transfer to 15ml falcon tube, **spin** 4,500g; 4°C; 10min. Save supernatant. Take **protein gel sample** as before & measure Abs_{280} as before.

2 – Removal of the His tag

Add 50µl of TEV protease (2mg/ml stock) to each refolding reaction. **Incubate at 16°C for ca. 16hrs** (in the frog incubator). **Take protein gel sample** as before. Run all samples of refolding/cutting on a 15% gel. **If cutting is sufficient, concentrate sample to ~1ml** with vivaspin devices. Filter through small 20µm filter & load onto HiLoad 16/60 Superdex 75 prep grade column (see below).

3 – Gel filtration

H3/H4 tetramers are purified on a HiLoad 16/60 Superdex 75 prep- grade column (GE Healthcare), and H2A/B dimers are purified on a Superdex 200 10/300 GL column (GE Healthcare). Below, only the procedure for the S75 column is described. The S200 procedure is similar, but with smaller volumes.

You should start equilibrating the column the same day as you're doing your dialysis into DiaB2 & DiaB3. Make 500ml of GF running buffer. Transfer S75 into **water** (180ml, at a running speed of 0.5-1ml/min). Then transfer into running buffer.

Load your cleaved tetramer onto the loop & run the S75 GF program. You usually empty the loop with 2ml, and elute with 1.5 column volumes at a speed of 0.5ml/min.

Your tetramers should come out at ca. 50mls. TEV should elute later (ca. 65-70ml).

Run peak fractions on a gel (10µl plus 6µl 3x SDS sample buffer plus BME, or equivalent)**, combine appropriate fractions that look good. Measure Abs_{280} of undiluted sample.** Most likely, you won't get a meaningful reading because the concentration is probably too low…

Store at 4°C.

4 – Solutions for Section B

Dialysis buffer 1 (DiaB1)
20mM MOPS
500mM NaCl
1mM EDTA
10% glycerol
set pH to 7 @ 22°C
5mM BME (10µl/25ml, 1.6ml/4l)

Dialysis buffer 2 (DiaB2)
20mM MOPS
500mM NaCl
1mM EDTA
5% glycerol
set pH to 7 @ 22°C
4mM DTT

Dialysis buffer 3 (DiaB3)
20mM MOPS
500mM NaCl
1mM EDTA
2.5% glycerol
set pH to 7 @ 22°C
4mM DTT

GF running buffer
As DiaB3 but 2% glycerol, 0.5mM EDTA, 1mM TCEP instead of DTT. Make sure you set pH afterwards – TCEP is very acidic. I usually use un-pH'ed Tris, the buffer will then (after adding everything, including the TCEP) be just above the pH you want. Use HCl to set pH)