10-20-30% Opti-prep gradient:

cells on filter or TC-plates. I usually take 3-4x dense 150mm plates ($2x10^7$ cells per plate) or 6-9x 10cm filter plates per experiment

next step optional (I found that the separation with cross-linking is better then without):

1. DSP cross-linking: DSP: stock 20mg/ml in DMSO (make always fresh).
- in cold room, rinse plate 3x w/ Ringer (3-4ml ea)
- use DSP @ 200µg/ml that is 1:100 in Ringer solution for 20min. @ RT
- quench X-linking on ice (or cold room) with 5 washes in TBS+50mM NH_4CL

Ringer Saline:

	500 ml
10 mM Hepes, pH=7.4	5ml (1M Hepes, pH=7.4)
154 mM NaCl	15.4 ml 5M NaCl
7.2 mM KCl	3.6 ml 1M KCl
1.8 mM $CaCl_2$	0.9 ml 1M $CaCl_2$

2. Prepare post-nuclear supernatant:
- in cold room, rinse plate 3x w/ Ringer (3-4ml ea)
- scrape cells in homo-buffer I (I divide up the 9mls of buffer between the plates I have for each experiment)
  - make fresh for each experiment:
  - 8µl L/A/P (5mg/ml Leupeptin, 5mg/ml Antipain, 5mg/ml Pepstatin in DMSO)
  - 90µl Sucrose (2.5M stock)
  - 30µl Pefabloc (0.1M stock)
  - 900µl homo-buffer 10x
  - 7.97 ml ddH_2O

  Homo-buffer (10x):

200 mM Hepes-KOH, pH=7.2
900 mM K-Acetate
20 mM Mg-Acetate

- pool cells in 15ml conical tube (use polypropylene tubes)
- Pellet cells by centrifugation @ 1000rpm (=228xg), 4°C, 10 min.
- resuspend in 2ml total volume homogenisation buffer II:
  - make fresh for each experiment:
  - 8µl L/A/P (5mg/ml Leupeptin, 5mg/ml Antipain, 5mg/ml Pepstatin in DMSO)
  - 8µl Aprotinin
  - 30µl Sucrose (2.5M stock)
  - 30µl Pefabloc (0.1M stock)
  - 300µl homo-buffer 10x

- 2.63 ml ddH_2O

- Homogenize cells in ball bearing homogenizer, fitted with 0.3747’’ stainless steel ball-bearing; pass cells back & forth ~ 10 times; flush homogenizer w/ 1 ml homo-buffer II; centrifuge homogenate @2000rpm (=930xg) for 10 min. to pellet nuclei and unbroken cells.

3. Fractionate samples (Pellet) by centrifugation in 3-phase 10-20-30% Opti-Prep Gradient (Isopycnic gradient).

percentage	Opti-prep	Homo-buffer	PNS
10%	1.25ml	3.25ml	-
20%	2ml	2.7ml	-
30%	2.95ml	-	2.25ml

After you prepare solutions, measure density with refractometer and compare reading with supposed reading for 10, 20 or 30 % solution, respectively. Use table that comes with opti-prep (page 2, table upper left corner – with sucrose). Adjust solution accordingly until you get correct reading.

4. overlay and spin gradients in Vti65.1rotor @ 61,000rpm (=350,000xg) for 3hrs 5min (decel=5).
5. Collect fractions at 0.5ml from top of gradients.