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 NH₄Cl

Ringer Saline:

<table>
<thead>
<tr>
<th>500 ml</th>
<th>500 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM Heps, pH=7.4</td>
<td>5ml (1M Heps, pH=7.4)</td>
</tr>
<tr>
<td>154 mM NaCl</td>
<td>15.4 ml 5M NaCl</td>
</tr>
<tr>
<td>7.2 mM KCl</td>
<td>3.6 ml 1M KCl</td>
</tr>
<tr>
<td>1.8 mM CaCl₂</td>
<td>0.9 ml 1M CaCl₂</td>
</tr>
</tbody>
</table>

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 ddH₂O

   Homo-buffer (10x):  
   - 200 mM Heps-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 ddH₂O

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).

<table>
<thead>
<tr>
<th>percentage</th>
<th>Opti-prep</th>
<th>Homo-buffer</th>
<th>PNS</th>
</tr>
</thead>
<tbody>
<tr>
<td>10%</td>
<td>1.25ml</td>
<td>3.25ml</td>
<td>-</td>
</tr>
<tr>
<td>20%</td>
<td>2ml</td>
<td>2.7ml</td>
<td>-</td>
</tr>
<tr>
<td>30%</td>
<td>2.95ml</td>
<td>-</td>
<td>2.25ml</td>
</tr>
</tbody>
</table>

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 Vti65.1 rotor @ 61,000rpm (=350,000xg) for 3hrs 5min (decel=5).

5. Collect fractions at 0.5ml from top of gradients.