

ISOLATION OF MITOCHONDRIA (*L. tarentolae*, ISOTONIC)

- Grow *L. t.* at 28°C in BHI (optimal density 80 - 120 10⁶ cells/ml; count cells during first centrifugation)
- Harvest cells in Beckman centrifuge (JA10, 5500 rpm; 10 min, 4°C)
- Combine pellets and wash it in approximately 100 ml of 1x SBG for each L of the culture (JA10, 5500 rpm; 10', 4°C)
- Resuspend cell pellet in SoTE (4x10⁹ cells/ml) and take a small sample (S1)
- Put cells in a plastic beaker into chilled Nitrogen cavitation chamber. Stir during incubation.
- Apply 55 bar for 55 min (may need to use as much as 100 bar, cell never break completely)
(pressure may drop after 5 min of incubation due to saturation of the buffer, therefore adjust to 55 bar again after 5 min)
- Carefully release pressure and pour content into chilled glass Erlenmeyer (a foamy suspension will be obtained)
- Take a small sample and compare it with S1 microscopically, estimate extent of lysis
- Spin lysate (JA20 rotor/11500 rpm; 16000 g, 10 min, 4°C)
(supernatant (S2) corresponds to cytosolic fraction, take sample if needed and flash freeze in liquid nitrogen)
- Resuspend pellet in SoTE (4x10⁹ cells/ml)
- Add MgCl₂ to 6 mM final (1/167 of total volume if stock of MgCl₂ is 1 M)
- Add DNase I to 50 µg/ml final (1/40 of 2mg/ml stock solution)
- Push lysate through G26 hypodermic needle (orange) (luer lock syringe)
- Incubate in a beaker on ice for 30 min (constantly stir during incubation).
- Add EDTA to 6 mM final (1/83 of total volume if stock of EDTA is 0.5M)
- Spin lysate (JA20 rotor/2000 rpm/484 g, 10 min, 4°C)
- Remove supernatant up the top level of the pellet (pellet will be very soft) by 10 ml pipette and keep on ice
- Resuspend each pellet in ca. 10-15 ml of SoTE each, mix vigorously by pipetting and pool
- Spin lysate (JA20 rotor/1750 rpm/330 g, 10 min, 4°C); pool both supernatants
- Spin (JA20 rotor/11500 rpm/16000 g, 10 min, 4°C), keep pellet

- r/s the pellet in 60% Percoll (6 ml of 60% Percoll / 1 liter of culture)
- layer underneath gradients (15+15 ml or 5.5+5.5 ml*) with a syringe using polyethylene tubing
(4 - 5 ml or 0.75 ml/tube)
- * gradient should be prepared earlier and frozen at - 20 °C; thaw o/n before using
- centrifuge 60 min at 24 000 rpm in SW28 (4 °C)
 or 30 min at 35 000 rpm in SW41Ti (4 °C)
- tap gradients by side puncture with #18 needle
- collect the lowest bands
- dilute with excess cold SoTE
- spin 15', 11 500 rpm, JA20
- r/s pellet in cold SoTE and recentrifuge

- resuspend in small volume of 1xSoTE and measure volume
- Spin in Eppendorf tube (2.2 ml tube) (14'000 rpm/5 min/4°C)
- Discard supernatant and resuspend pellet as concentrated as possible; measure volume
- Aliquot in Eppendorf tubes

- Flush freeze aliquots in liquid nitrogen and store at -80°C

Buffer and Material

		400 ml	200 ml	600 ml	800 ml	STOCK
<u>SBG</u>	20 mM Glucose	1.44 g	0.72 g	2.16 g	2.88 g	powder Mw 180.6
	0.15 M NaCl	12 ml	6 ml	18 ml	24 ml	5 M
	20 mM NaPi, pH 7.9	16 ml	8 ml	24 ml	32 ml	0.5 M

Prepare as 4 x SBG

Glucose (Mw 180.6) 14.4 g
 NaCl (5 M) 120 ml
 NaPi, pH 7.9 (0.5 M) 160 ml
 add H₂O up to 1000 ml

		200 ml	400 ml	STOCK
<u>SoTE</u>	0.6 M Sorbitol	50 ml	100 ml	2.4 M
	20 mM Tris-HCl, pH 7.5	4 ml	8 ml	1 M
	2 mM EDTA	0.8 ml	1.6 ml	0.5 M

Prepare as 2 x SoTE

2.4 M Sorbitol* 250 ml
 Tris-HCl, pH 7.5 (1 M) 20 ml
 EDTA, pH 8.0 (0.5 M) 4 ml
 add H₂O to 500 ml

*2.4 M Sorbitol Stock Sorbitol (MW 182.2) 437.3 g (add H₂O ad 1000 ml)
 218.6 g (add H₂O ad 500 ml)
 109.3 g (add H₂O ad 250 ml)
 Autoclave

2x SoTE		1 l	STOCK
	1.2 M sorbitol	218.6 g	powder Mw 182.2
	40 mM Tris-HCl pH 7.5	40 ml	1 M
	4 mM EDTA	8 ml	0.5 M