SOLUTIONS FOR SPERM NUCLEI PREP

30mls 2XNPB:

final [ ] in 1X soln:

10mls	1.5M	sucrose (-20ºC stock)	250mM
900µl	1M	HEPES (pH with KOH so 7.7 at 15mM; store at -20ºC)	15mM
120µl	0.5M	EDTA	1mM
3.0ml	10mM	spermidine (-20ºC stock)	0.5mM
1.2ml	10mM	spermine (-20ºC stock)	0.2mM
608µl	0.1M	DTT (-20ºC stock)	1mM
14.78ml		dH20	

20mls	1XNPB	for testis washes
20mls	1XNPB	spins and lysolecithin treatment

(8, 8, 2, 1)

10mg/ml lysolecithin from dry stock
10% BSA (-20ºC stock or make up fresh)

10mls 1XNPB +3% BSA
(with inhibitors):

5mls 2XNPB, 2mls dH20, 3mls 10%BSA, add:
10µl leupeptin (10mg/ml stock in DMSO to 10µg/ml final)
10µl PMSF (0.3M in EtOH to 0.3mM final)

5mls 1XNPB +0.3%BSA
(without inhibitors):

2.5mls 2XNPB, 2.2mls dH2O, 0.3mls 10% BSA

1ml storage buffer:

500µl 2XNPB
300µl glycerol
170µl dH2O
30µl 10% BSA

Sperm dilution buffer (20mls; store at -20ºC in 0.5 ml aliquots):

3.34mls	1.5M	sucrose (-20ºC stock)	150mM
1.5mls	1M	KCl	
1.0ml	10mM	spermidine (-20ºC stock)	
400µl	10mM	spermine (-20ºC stock)	
~2P0µl	0.1N	NaOH (should bring to pH 7.3-7.5)	
13.68ml		dH20	

Also needed:
10 mg/ml Hoechst 33342 stock (Sigma B-2261; stored in water in a light-tight vessel at -20ºC; or DAPI, see Murray chapter in Meth. in Cell Biol. 36

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(as Murray, Methods in Cell Biology 36, pp. 581 except that the protease inhibitors leupeptin and phenylmethylsulfonyl fluoride are only added to NPB until the wash/spin immediately following the lysolecithin treatment and are omitted during later steps).

All solutions should be prepared and placed on ice prior to beginning the prep, as shown on the previous page.

1) Remove testes from 1-2 male Xenopus laevis. We generally use 2 males to prepare a large number of aliquots of nuclei that can be frozen at -80 and used for approximately 6 months. Rinse testes 3 times in cold 1XMMR and 2 times in 1XNPB in petri dishes, removing fat body and visible blood vessels as possible without damaging testes.

2) Transfer testes to a dry petri dish and macerate testes with forceps until they look like a slurry with no visible chunks of testis. Maceration should be be done very thoroughly to obtain high yields of nuclei.

3) Add 2mls NPB to the macerate and gently pipette up and down through a fire-polished truncated pasteur pipette with an opening of about 3mm in diameter

3) squirt macerate through about 4 thicknesses of cheesecloth on a funnel, collecting into a 15 ml round bottom tube (Falcon 2059 from Fisher); Rinse dish with 8mls of NPB and put this rinse through the cheesecloth, collecting it in the tube.

4) spin down at 3000 rpm for 10 min. at 4ºC; in Beckman swinging bucket rotor (or Sorvall SA-6)

Decant supernatant; add 8ml NPB to this tube and pipette up and down to resuspend pellet-spin down again as above and decant supernatant

5) resuspend pellet in 1ml NPB (use a clipped pipette tip), warm to room temperature and add 50µl of 10mg/ml lysolecithin, mix by gentle rocking, and incubate for 5 min. at room temperature.

6) Add 10 ml cold NPB +3%BSA (with protease inhibitors) to the tube, mix gently by pipetting up and down, and spin down for 10 min, 3000 rpm. Decant supernatant. The lysolecithin-treated pellet should look slightly more translucent (less opaque white) than it did prior to lysolecithin treatment).

7) Decant supernatant and resuspend pellet in 5 ml cold NPB +0.3%BSA (no inhibitors) , mix gently by pipetting and and spin down for 10 min, 3000rpm

8) Decant supernatant and resuspend pellet in 500µl NPB (no inhibitors), +glycerol. This is now your nuclei stock. Store on ice while you check the yield of nuclei obtained as described below.

9) To check the yield of nuclei:

To 98 μl of SDB, add 1 μl of the nuclear stock and add 1μl of 1:100 dilution of Hoechst stock. Mix the nuclear stock very well using a clipped (large-opening) pipette tip just before removing the 1 μl. Mix the diluted SDB/Hoechst/nuclei very well and allow a small amount to flow into the chamber of a hemocytometer by capillary action. Count nuclei in a square of the hemocytometer under a compound microscope (I usually visualize with Hoechst and brightfield combined). From one male, you should obtain counts of at least 100-125 (X10^4 cells/ml) for a 1:100 dilution of our 0.5 ml suspension. From two males the count should be at least 200-250.

> (prep yields >1X10^7 sperm). So, for a 1:100 dilution, 750-1250 sperm/μl or 0.75-1.25 sperm/nl. Since we assume a 5-15 nl injection volume during transplantation with large 60-75μm needle tips, we dilute 1:5-1:10 additionally (1:500-1:1000 total)

Right mixing the nuclear stock very well (using a clipped, large orifice pipette tip), prepare 50μl aliquots and freeze at -80ºC. Aliquots can be stored for at least 6 months without too much loss of developmental capacity and thawed on day of use for transgenesis.