Preparation of Yeast Nuclei and Spindle Pole Bodies

(Michael Rout and John Kilmartin)

I. Introduction

Spindle pole bodies (SPBs) are the sole microtubule organizing centers of budding yeast cells. SPBs are embedded in the nuclear envelope which remains intact during mitosis thus spindles are intranuclear. SPBs can be enriched six hundred fold and in high yield from Saccharomyces uvarum (Rout and Kilmartin, 1990). The procedure involves preparation of nuclei by a modification of an existing method (Rozijn and Tonino, 1964). The nuclei are then lysed and extracted to free the SPBs from the nuclear envelope, followed by two gradient steps to separate the SPBs from other nuclear components. These SPBs, which are about 10% pure, have been used to prepare mAbs and thereby identify components of the SPB and spindle (Rout and Kilmartin, 1990, 1991).

II. Materials and Instrumentation

All catalogue numbers are in brackets. Anti foam B (A5757), sorbitol (S 1876), PVP-40 (PVP-40), pepstatin (P 4265), PMSF (P 7626), digitonin (D 1407), DNase I (DN-EP), RNase A (R 5503), GTP (G 5884), bis-Tris (B 9754), EGTA (E 4378) were obtained from Sigma, Poole, Dorset, U.K. (Fax 0800 378785). Ficoll-400 (17-0400-01), Percoll (17-0891-01) were from Pharmacia, Uppsala, Sweden. Triton X-100 (30632), glucose (10117) and dimethyl sulphoxide (DMSO, 10323) were obtained from BDH, Poole, England (Fax 0202 738299) and sucrose (5503UA) from GIBCO BRL, Uxbridge, Middlesex, U. K. (Fax 0895 53159). Yeast extract (1896) was obtained from Beta Lab, East Molesey, U.K., and bactopeptone (0118-08-1) from Difco, Detroit, MI, USA. Glusulase (NEE-154) was obtained from Du Pont,Wilmington, DE, USA, Zymolyase 20T (120491) from Seikagaku, 1-5 Nihonbashi-honcho, Chuo-ku, Tokyo, Japan, and SP 299 from Novo, DK 2880 Bagsvaerd, Denmark (Mutanase is no longer commercially available). Liquid malt extract was obtained from a local health food store.

Foam stoppers (FPP6) to seal the neck of the fermentor flask were from Scientific Instruments, Loughborough, Leicestershire, U.K. *Saccharomyces uvarum* (NCYC 74) was from The National Collection of Yeast Cultures, AFRC, Colney Lane, Norwich, U. K. Centrifuge tubes, SW28 Ultraclear (344058) and 70 TI (355654), were obtained from Beckman,Palo Alto, CA, USA. The hemocytometer (AC-1) was obtained from Weber Scientific, Teddington, Middlesex, U.K., the continuous flow centrifuge (KA 1-06-525) from Westfalia, 4740 Oelde 1, Germany, the Polytron (PT 10/35 with PTA 10S probe) from Kinematica, CH-6010 Kriens, Luzern, Switzerland, the refractometer (144974) from Zeiss, 7082 Oberkochen, Germany.

III. Procedures

A. Preparation of nuclei from Saccharomyces uvarum
Solutions required for processing 36 liters of yeast cells

1. 5 liters of cold distilled water

2. 1.1M sorbitol
   To make 2 liters, dissolve 400 g sorbitol in distilled water and make up to 2 liters. Store at 4˚C.

3. *7.5% Ficoll-400 in 1.1M sorbitol*
   To make 400 ml, dissolve 30 g Ficoll-400 in 1.1 M sorbitol, it dissolves very slowly, make up to 400 ml. Store at 4˚C.

4. *8% PVP*

To make 1 liter, dissolve 80 g PVP-40, 1.57 g KH_2PO_4, 1.46 g K_2HPO_4, and 152 mg $MgCl_2 \cdot 6H_2O$ in distilled water, check the pH and adjust to 6.53, it usually needs 15 µl of conc H_3PO_4, make up to 1 liter. Store at 4˚C. Prepare 1.5 liters if also making up sucrose solutions.

5. *Solution P*
Dissolve 2 mg pepstatin A and 90 mg PMSF in 5 ml absolute ethanol. Store at 4˚C and discard after 3 weeks. Note that PMSF is highly poisonous.

6. *20% Triton X-100*
Dissolve 20 g Triton X-100 in distilled water and make up to 100 ml. Store at -20^{o}C, retain a small aliquot at 4˚C.

7. *Sucrose solutions for the gradient*
Prepare the sucrose solutions by weighing out sucrose and 8% PVP solution in a beaker and dissolve by stirring the beaker in a large Petri dish of hot water. When the sucrose has dissolved remove from the hot water and measure the refractive index (RI). Continue stirring and adding 8% PVP until the correct refractive index is obtained (within 0.0003). Store in 50 ml sterile tubes at -20˚C. For 2.01M sucrose, weigh out 183.3 g sucrose and add 8% PVP solution to 338 g in total, then adjust to a final RI of 1.4370. For 2.10M sucrose, weigh out 193 g sucrose and add 8% PVP to a total of 340 g (final RI 1.4420). For 2.30M sucrose, weigh out 216 g sucrose and add 8% PVP to a total of 340 g (final RI 1.4540). Note that this nomenclature differs from that used in Rout and Kilmartin, 1990, which used the apparent (rather than actual) sucrose molarities.

8. Wickerham's medium
Prepare Wickerman's medium 10X concentrated. Dissolve 108 g malt extract, 108 g yeast extract, 180 g bactopeptone and 360 g glucose in 3.6 liters distilled water and remove turbidity by centrifuging 10000 rpm for 10 min. Dilute the supernatant ten fold.

Steps

1. Two days before the nuclei preparation prepare the concentrated Wickerham's medium to grow the yeast cells. The cells can be grown in a commercial fermentor or using the simple system described below. Dispense 9 liters of diluted medium into four 10 liter glass Pyrex bottles containing a heavy magnetic stirrer. Add 2 ml of anti-foam B (use a disposable plastic pipette). Prepare a glass aeration tube by attaching a small plastic vial with 4 or 5 needle sized holes in the bottom to one end of the glass tube via a rubber stopper or thick walled rubber tubing. Attach a glass fibre filter on the inlet of the aeration tube using rubber tubing. Cut halfway into a foam stopper slightly larger than the diameter of the neck of the flask and use this to position the aeration tube and to seal the flask. Seal the stopper and the filter at the end of the aeration tube with aluminum foil, finally cover both the aeration tube and the top of the flask with a foil cap. Alternatively, simply cover the top of the flask with three or more layers of foil and tape it in place with autoclave tape; the aeration, in the form of a sterile disposable 25 ml pipette attached to the bench air supply by sterilized plastic tubing, can be added after flask autoclaving. Autoclave for 1 hr and 1 hr exhaust; or, 90 min with faster exhaust. It is important to autoclave properly, especially if the medium was not ultrafiltered, as there are spores of a remarkably heat resistant bacterium in the medium. Allow the flasks to cool at room temperature overnight.

2. Set up a starter culture from a single yeast colony in 10-20 ml medium and grow at 25˚C in a rotating incubator or small bubbler for up to 24 hr until stationary phase is reached (1.0×10^8 cells/ml). Use a hemocytometer to count the cells, use shearing or sonication to break up any clumps of cells.

3. In the afternoon of the day before the nuclei preparation set up the 10-liter flasks to grow overnight. Aim for 1×10^7 cells/ml in the morning; it is essential to have mid log phase cells to prepare good spheroplasts. It is probably pointless to proceed with the spheroplasting if the cells have overgrown beyond 50% above the optimal count. To calculate the number of starter cells to

add, first work out the number of hours the cells will grow at log phase. If the starter cells are stationary phase (1 x 10^8 cells/ml) subtract one doubling time (1.75 hr for *S. uvarum*) to allow for the lag period of growth. Divide the number of hours of log phase growth by the doubling time to give the number of divisions (d). Then the number of cells needed is the final number of cells divided by 2^d (2^d = antilog_{10} d/3.32).

4. Transfer these cells by sterile technique to the 1 liter flask and place in a water bath set at 25°C (the depth of water need be no more than 25 cm). If room temperature is 23°C or above, the water bath is not necessary but an appropriate correction in the cell doubling time calculations is required. Place magnetic stirrers under the bath to stir the flasks during growth, connect the aeration tube to a compressed air source and water bubbler to humidify the air, and position the aeration tube so that the bubbles of air are blown at the rotating stirring bar and thereby dispersed throughout the flask. If just using the 25 ml pipettes, and no stirring bars, then bubble air through the medium at a rate sufficient to keep the cells from settling but not too violent (as this will cool the medium). The quality of aeration can be checked by growing one culture to stationary phase, it should reach a cell count of 1 x 10^8 cells / ml.

5. Harvest the cells using a Westfalia continuous flow centrifuge or in individual centrifuge pots, spin at 4000 g for 5 min (all centrifugation steps are at 4˚C).

6. Combine the pellets in one 500 ml centrifuge pot and wash twice with cold distilled water, resuspend the pellet in 1.1M sorbitol using a thick glass rod, and spin at 5000 g for 5 min. Repeat the resuspension and pelleting in 1.1M sorbitol and finally resuspend in about one pellet volume of sorbitol for spheroplasting. Dilute this suspension 1:100 and count the cells, then adjust the suspension to 1.5 x 10^9 cells/ml.

7. Add 50 µl glusulase, 20 µl 1% zymolyase 20T, and 3 µl 5% mutanase or SP 299 per ml of suspension. The enzyme solutions should be spun or filtered beforehand to remove insoluble material. Incubate at 30°C, leaving the top of the centrifuge pot a little loose since some gas is evolved and mix carefully from time to time. This can either be done in a water bath, manually stirring the pot every 10 - 15 min, or in an air incubator with a moderate speed of stirring. The digestion, which takes 2-4 hr, can be monitored using the hemocytometer by diluting 10 µl of digesting cells in 0.2 ml of sorbitol to determine the proportion of spheroplasts and another 10 µl in 0.2 ml of water to determine the number of completely intact cells. At 1 hr in sorbitol there should be a few spheroplasts and less than 1% live intact cells in water. At about 1.5 hr the spheroplasts become very clumpy and irregular in shape. After further digestion at 2-3 hr they separate into round spheroplasts with some clumps remaining. In water there should be complete lysis with no undigested cells and less than 5 x 10^4 black cells or ghosts/ml.

8. While the cells are digesting make up the gradient tubes: 12 Beckman Ultraclear SW28 tubes or equivalent for a 36 liter prep <u>provided not more than 230 ml of cells have been digested</u>, otherwise increase the gradient tubes in proportion. Add solution P at 1:1000 to the three sucrose solutions and prepare a step gradient in each tube of 8 ml of 2.30M, 8 ml of 2.10M and 8 ml of 2.01M sucrose-PVP. Leave the tubes in an ice bucket in the cold room covered with foil.

9. While the cells are digesting calculate the approximate amount of 8% PVP needed to lyse the spheroplasts (see below), then prepare the same volume of 0.6M sucrose-PVP (205.2 g sucrose / liter 8% PVP) and enough 1.7M sucrose-PVP (581.4 g sucrose / liter 8% PVP) to resuspend the crude nuclei for the gradient (about 12 ml per tube), cool these solutions in ice. Also prepare two to four 100 ml Ficoll cushions in 250 ml clear Sorvall pots. Note that the maximum volume that can be conveniently lysed in a 250 ml pot is 150 ml, so have no more than 1.5 x 10^{11} diploid spheroplasts in each pot.

10. At the end of the digest add an equal volume of 1.1M sorbitol and pellet the spheroplasts in a swinging bucket centrifuge at 5000g for 20 min. Aspirate off the supernatant, then immediately wash the sides of the tube with sorbitol and aspirate off again. Remove as much enzyme as

possible since proteases are present. Resuspend the pellet in the same volume of sorbitol. The spheroplasts are very fragile so be very careful to avoid lysis: use a thick glass rod to partially resuspend as lumps then gently shake in ice on a rotating shaker. Use the glass rod occasionally to disperse the lumps.

11. When the cells are resuspended overlay 0.2 ml onto 0.4 ml 7.5% Ficoll in 1.1M sorbitol in a small glass test tube to check that the cells are not too dense to overlay, if the cells fall through the Ficoll dilute them further with 1.1M sorbitol until they do not. Then overlay the cells onto the 100 ml cushions of 7.5% Ficoll in 1.1M sorbitol in 250 ml clear centrifuge pots. Spin in a swinging bucket centrifuge at 5000g for 20 min.

12. Aspirate the supernatant down to the Ficoll layer, then wash the sides of the pot several times with sorbitol aspirating each time to remove as much of the enzyme containing supernatant as possible. Finally aspirate down through the Ficoll layer to the pellet and wash the sides of the tube with 8% PVP and aspirate that.

13. Add 10 ml 8% PVP, 60 µlsolution P and 12 µl20% Triton X-100 per 10^{10} spheroplasts. This should prove to be about a total of 300 ml of 8% PVP plus 1.8 ml solution P and 0.36 ml 20% Triton X-100. Use the Polytron probe at low speed first to resuspend the pellet then increase the speed setting to 3.5 to 5 (almost always keep at 3.5) and Polytron throughout the solution for 1 min, and also drag the probe across the bottom of the pot during this time to try to resuspend as much of the pellet as possible. Place the pot in ice and remove a 5 µlsample to observe by phase contrast microscopy at a magnification of about 1000X. There should be complete cell lysis, plenty of nuclei which look like small perfectly round black balls often with a slightly darker crescent region, and vacuoles which look like white balls the same size as nuclei. The presence of vacuoles is a reassuring sign that the correct quantity of Triton has been added, i.e. enough to lyse the spheroplasts without disrupting too many other membranes. Unlysed spheroplasts are highly swollen with a prominent vacuole. Check to see what proportion of nuclei are trapped in lysed but undispersed cells. Repeat the Polytron step for a further 1 min. If there is a high proportion of trapped nuclei or unlysed cells then extend the time of the Polytron treatment. Decant the lysed cells into clean centrifuge pots or a cold conical flask and add an equal volume of cold 0.6M sucrose-PVP, mix carefully and spin in a 6 x 250 ml anglehead rotor for 15 min at 10000 g. Note that this Polytron and spin step must be completed as fast as possible, since after lysis the nuclei slowly clump trapping cytoplasmic debris and thereby contaminating the gradient.

14. Retain the supernatant from the spin to check that the nuclei have pelleted, and then resuspend the nuclei in cold 1.7M sucrose-PVP (12 ml per SW28 tube) using the Polytron at low speed (speed 2 - 3; try also to resuspend any nuclei that have been daubed up the side of the tube by the spin) and load the gradient tubes with about 15 ml using either a measuring cylinder or a 10 ml pipette. Weigh the tubes and balance by overlaying with 1.0M sucrose-PVP, spin at 28000 rpm for 4 hr (Beckman SW28 rotor).

15. The nuclei mainly band at the 2.10/2.30M layer. Unload by aspirating down from the top, wiping the top part of the tube with tissue as you go down. If the wad of material at the top is particularly thick, scoop it out with the end of the Pasteur pipette. Stop at the 2.01/2.10M interface for the first tube only and remove 5 µl (wipe the sides of the tip to remove sucrose) to check by phase contrast microscopy if there are significant quantities of nuclei there (usually there are a few but they are too contaminated with debris to be of much use), and also check the 2.10M layer. Aspirate down to just above the nuclei band in the 2.10/2.30M layer then quickly wipe the sides of the tube with a rolled up tissue, paying particular attention to that part where the main band was at the sample to 2.01M interface. Try to get as close to the nuclei as possible as osmiophilic membranes (probably from the plasma membrane) are concentrated in the 2.10M layer. Ring the nuclei layer with the sealed off Pasteur pipette to separate it from the walls of the tube, then suck off with a 10 ml pipette, going down partially into the 2.30M layer to remove as much nuclei as possible. Do not get too close to the pellet containing empty cell walls. Store the nuclei at -70^{o}C; they seem to be very stable to storage at this temperature.

B. Preparation of Spindle Pole Bodies

Solutions

1. *0.9M sucrose-PVP*
   Dissolve 30.8 g sucrose in 8% PVP and make up to 100 ml. Store at -20˚C.

2. *0.01M bis-Tris-HCl pH 6.5, 0.1mM $MgCl_2$ (bt buffer)*
   Prepare a 0.1 M stock of bis-Tris, dissolve 20.9 g bis-Tris in distilled water, adjust the pH to 6.5 with conc HCl and make up to 100 ml. Store at -20˚C. To prepare 100 ml bt buffer add 10 ml 0.1M bis-Tris-HCl pH 6.5 and 10 µl 1 M $MgCl_2$ (dissolve 0.2 g $MgCl_2$ in a total of 1 ml water) to 90 ml of distilled water.

3. Sucrose solutions in bt buffer
   Prepare stock 2.50M sucrose in bt buffer (214 g/250 ml) as described above for the sucrose-PVP solutions, the refractive index should be 1.4533. Prepare 1.75M, 2.00M and 2.25M sucrose-bt solutions by dilution of the stock 2.50M solution with bt buffer, the final refractive indices should be 1.4174, 1.4295 and 1.4414 respectively. Add a 1:1000 dilution of solution P just before pouring the gradient. Store at -20˚C.

4. Nuclear lysis solution
   For 10 ml add 0.2g digitonin and 0.15 ml Triton X-100 to 2 ml DMSO and about 1 ml distilled water (wear gloves since digitonin is toxic). Microwave to almost boiling then stir to dissolve the digitonin. Make up to 10 ml in a measuring cylinder by slowly adding distilled water down the side of the cylinder without mixing, add 1 µl 1M MgCl2. Place the solution in the cold room and mix immediately before use, it should be a clear solution.

5. 2% DNase I
   Dissolve 5 mg of DNase I in 0.25 ml 0.25M sucrose, 0.05M triethanolamine pH 7.4, 0.25M KCl, 5mM MgCl2. Store at -20˚C.

6. *RNase A*
   Dissolve 2 mg RNase A in 1 ml of distilled water.

7. *DMSO buffer*
   Prepare this immediately before use. Prepare stocks of 0.1M GTP (dissolve 55 mg GTP in 1ml water, store at -20˚C), 0.1M EGTA (dissolve 3.8 g EGTA in 100 ml water and adjust the pH to 7.0 with 10N NaOH), 1.0M DTT (dissolve 154 mg DTT in 1 ml water, store at -20˚C). For 300 ml of DMSO buffer use 30 ml 0.1M bis-Tris-HCl pH 6.5, 30 µl 1.0M $MgCl_2$, 300 µl 0.1M GTP, 300 µl 0.1M EGTA pH 7.0, 30 µl 1.0M DTT, 300 µl solution P, 60 ml DMSO, make up to 300 ml with distilled water and cool in ice.

8. *bt-DMSO buffer.*
   For 100 ml use 10 ml 0.1M bis-Tris-HCl pH 6.5, 10 µl 1.0M $MgCl_2$, 100 µl solution P, 20 ml DMSO and make up to 100 ml with distilled water. Cool in ice.

Steps

1. Pellet the nuclei by first decreasing the sucrose concentration from about 2.4M to 2.1M (refractive index 1.434) by addition of 0.9M sucrose-PVP. Mix very thoroughly by shaking. Calculate the total number of OD's of nuclei by measuring the OD at 260 nm of 10 µl nuclei in 1 ml of 1% SDS (10^{10} Nuclei is about 100 OD_{260nm}). Decant the nuclei into Beckman 70 TI tubes or equivalent. Six tubes are usually enough for nuclei from $3x10^{11}$ spheroplasts. Mix the tubes thoroughly again; any unmixed sucrose will prevent proper pelleting of the nuclei. Spin the 70 TI tubes in a 70 TI rotor, 40,000 rpm for 1 hr. After spinning, immediately remove the supernatant by

aspiration, being careful not to disturb the pellet. These pellets can be stored in the tubes at -70^{o}C until needed.

2. Prepare the gradients in Beckman SW28 Ultraclear tubes. Four tubes are usually enough. Each tube contains 2.5 ml 2.50M sucrose-bt, 7.5 ml 2.25M sucrose-bt, 5.0 ml 2.00M sucrose-bt and 5.0 ml 1.75M sucrose-bt. Place tubes in ice.

3. To lyse the nuclei thaw the 70 TI tubes containing the nuclei pellets (if frozen), aspirate any residual sucrose-PVP, and place in ice. Add 1.0 ml nuclear lysis solution, 10 µl solution P and 1 µl 2% DNase I for each 100 OD_{260nm}'s in the nuclear pellets and resuspend at 4^{o}C by whirlmixing vigorously until about a minute after the last traces of pellet have disappeared. The suspension will froth.

3. After resuspension, warm the tubes in the hand, stand them for 5 min at room temperature and then add 2.50 M sucrose bt buffer (at room temperature) equal to the volume of nuclear lysate to each tube. The nuclear lysate must be properly warmed up before incubation at room temperature. If the lysate has not reached room temperature then the DNA digestion may not be complete at the end of the incubation. Shake well and spin in a 70 TI rotor, 6000 rpm for 6 min. Do not resuspend any pellet that may form at this stage. Distribute the supernatant equally on top of the SW28 gradients (check on a balance); each gradient tube should be loaded to within 5 mm of its top, and overlayered if necessary with 1.0M sucrose-bt. Spin tubes in an SW28 rotor at 28000 rpm for 6 hr.

5. Remove the gradient layers from the top of the tube using a Pasteur pipette, taking special care when removing material from around the sides of the tubes or at the gradient interfaces. Take off the top layer (sample layer) to within 5 mm of the first interface (sample/1.75M), then save each interface fraction separately taking from 5 mm above one interface through to 5 mm above the next. The final interface (2.25 M / 2.50 M) and pellet are resuspended with a sealed Pasteur pipette and whirlmixed before removing. The spindle pole bodies should be mainly (~70%) in the 2.00M / 2.25M fraction. It is usual to assay the SPB count and protein concentration protein assay (Bradford's) of all the fractions taken. Store these fractions at -20°C.

6. SPBs can be further enriched on a Percoll gradient. For each SW28 tube add 20 µl of 2mg/ml RNase A to 2 ml of the 2.00M/2.25M sucrose SPBs and incubate for 15 min at room temperature. Then add in turn, mixing at each step, 7 ml 2.50M sucrose-bt, 1 ml Percoll and 4 ml cold DMSO buffer. Cool to 4˚C and place in the SW28 tube, gently overlaying with cold DMSO buffer. Spin at 28000 rpm for 6 hr.

7. After the spin the SPBs should be visible as a faint band (viewed against a black background) about 1 cm into the gradient. Mark its position on each tube and then collect the gradient solution above it to within 2-3 mm of this band (~ 3.5 ml), using a Pasteur pipette. This is fraction 1. Collect the next layer (fraction 2), right through the SPB band to about 17 mm from the bottom of the tube (~ 4.5 ml). This fraction contains 60-70% of the SPBs originally loaded on the gradient. Collect the next 3.5 ml (fraction 3), then whirlmix the final 2.0 ml and save (fraction 4). Store the fractions at -20°C.

8. To pellet the SPB fractions add 3.5 volumes of cold bt-DMSO to each, mix well by shaking and portion between Beckman 70 TI tubes (about three-quarters full in each). Centrifuge in a 70 TI rotor, 40,000 rpm for 1 hr. The SPBs pellet as a faint translucent layer on top of a transparent Percoll pellet, and after 5 min on ice this layer slides off the Percoll pellet to the bottom of the tube. Carefully aspirate off the supernatant and recover the delicate SPB layer in about 0.2 ml of liquid per tube. The presence of SPBs can be assayed by Coomassie staining of SDS gradient gels. A comparison of fractions 1-4 should show the enrichment of the tubulin (55 kD) and 110 kD bands associated with the SPBs in fraction 2. Alternatively immuno-blotting with anti-SPB mAbs (Rout and Kilmartin 1990, 1991) could also be used to detect the presence of SPBs. A fast and quantitative assay for SPBs is by dark field microscopy (Rout and Kilmartin, 1990).

IV. Comments and Modifications

This procedure for spindle pole enrichment has also been applied to *S. cerevisiae* strains. The extent of enrichment is not as good because these strains do not spheroplast as well as *S. uvarum*, leading to contamination of the nuclei band with cells. In addition these strains do not appear to disperse their cellular contents during lysis as well as *S. uvarum* leading to further contamination of the nuclei layer with large aggregated cytoplasmic masses. The lower quality of the nuclei leads to a corresponding decrease in the quality of the SPBs. However, a number of modifications can be incorporated so that this procedure can be applied successfully to *Saccharomyces cerevisiae* strains, if necessary on a much smaller (and so more convenient) scale, allowing different strains to be processed in parallel. The quality of these nuclei can be nearly as high as those from *S. uvarum*. They may be prepared on such a small scale by the following modifications to the numbered steps in the original protocol. A large scale *S. cerevisiae* nuclei preparation can also be made by scaling up these same modifications as appropriate:

1-4. Set up an overnight starter culture of the *S. cerevisiae* strain(s) you wish to use in 5 ml of autoclaved YPD medium (1% yeast extract, 2% bactopeptone, 2% glucose) in a rotating incubator at 30°C. Prepare 1-2 liters of autoclaved Wickerham's on the same day in 1-liter culture flasks. On the following day, pour the starter culture into 50 ml of Wickerham's and grow during the day in a rotating incubator at 30°C. In the afternoon / evening, set up the 1-liter cultures to grow overnight at 30°C in a rotating incubator, aiming for between 2 x 10^7 cells/ml (if diploid) to 5 x 10^7 cells/ml (if haploid). The only additional solution to be prepared is 0.3 M sucrose-PVP, made by mixing equal volumes of the stock PVP solution and 0.6 M sucrose-PVP.

5. Harvest the cells in the appropriate number of 500 ml centrifuge pots (3000 *g* for 5 min).

5a. Resuspend the cells in distilled water to an approximate volume of 50 ml. Place the suspension in a 50 ml graduated Falcon tube and centrifuge in a swing-out rotor at ~1,500 *g* for 5 min.

5b. Resuspend the cell pellet in ~50 ml 100 mM Tris-HCl, 10 mM DTT pH 9.4 and incubate with occasional swirling for 10 min at 30°C. Then centrifuge again as in step 5a.

5c. Repeat step 5a.

6. Resuspend the cell pellet with ~40 ml of 1.1 M sorbitol, centrifuge again as in step 5a. Note the volume of the packed cell pellet and resuspend it (by vigorous shaking of the sealed tube) with an equal volume of 1.1 M sorbitol.

7. Add 0.1 volumes glusulase and 0.01 volumes 1% zymolyase 20T / 1% mutanase, and 1:500 dilution of 1.0 M DTT solution.. Incubate at 30°C as normal.

8. Usually one gradient tube is adequate for 2 x 10^{10} diploid cells or 5 x 10^{10} haploid cells.

9. For each strain, prepare one 15 ml Ficoll cushion in a 50 ml round-bottomed polypropylene centrifuge tube, and chill on ice.

10. At the end of the digest, add an equal volume of ice-cold 1.1 M sorbitol and pellet the spheroplasts in a swing-out rotor at 1,500 *g* for 10 min at 4°C. Using a Pasteur pipette, gently resuspend the spheroplasts with 20 ml of ice-cold 1.1 M sorbitol and pellet again in a swing-out rotor at 1,500 *g* for 5 min at 4°C.

11. Gently resuspend the spheroplasts to a total of 20 ml with ice-cold 1.1 M sorbitol, overlay onto the ficoll cushion and centrifuge in a swing-out rotor at 10,500 *g* for 15 min at 4°C. At this stage, prepare 20 ml 8% PVP solution, 100 µl 1 M DTT, 200 µl Solution P, 50 µl 10% Triton X-100; and 10 ml 0.3 M sucrose-PVP containing 100 µl Solution P for each spheroplast pellet and cool on ice.

12. Aspirate and rinse the tube and pellet as in the original protocol.

13. To each spheroplast pellet add the 20 ml of ice-cold PVP solution and Polytron to resuspend at a setting of 3.5 for 1 min. Allow the suspension to sit on ice for 1 min, during which time the degree of spheroplast lysis should be monitored as usual. Repeat the Polytron and monitoring steps until lysis is complete. Then, gently underlay each tube with 10 ml of the 0.3 M sucrose-PVP, and centrifuge in a swing-out rotor at 16,500 *g* for 20 min at 4°C.

14. Each crude nuclei pellet is resuspended with 6 ml of cold 1.7 M sucrose-PVP containing 60 µl Solution P, using the Polytron on setting 2.5 for two 30 sec bursts separated by 1 min on ice. Any nuclei adhering to the tube bottom can be dislodged by scraping with a sealed Pasteur pipette during the 1 min rest. The nuclei resuspension is then adjusted to a refractive index of 1.425 with 2.30 M sucrose-PVP, making a total volume of ~12 ml; this is usually sufficient to fill one gradient tube, but if necessary the overlayer can be added. The gradient tubes are centrifuged as usual, except in two cases: (i) the strain is haploid, or (ii) nuclear fragmentation was observed during spheroplast lysis, in which case the centrifuge run time should be increased to 8 hr.

15. Unloading and storage of the nuclei are as previously described.

V. Pitfalls

1. The quality of the spheroplast preparation largely determines the quality of the subsequent nuclei preparation. The amount of glusulase added seems to be crucial: the amount given for this protocol is a minimum for reasons of expense; thus any problems to do with spheroplast quality can probably be cured by addition of more glusulase.

2. The nuclei sucrose gradient is very sensitive to the presence of excess amounts of empty partially digested cell walls. These appear to aggregate at the nuclei band (2.1M/2.3M sucrose-PVP) forming a solid mass, making it impossible to unload the nuclei. It is important to adhere to the loading limits suggested in the nuclei protocol.

3. The type of digitonin seems to be important in successful nuclear extraction; always use a water soluble type.

4. One should note that the procedures for preparing the SPB fraction described in this chapter do not work well when nuclei prepared using techniques other than the one described are used as a starting material. This may be related to the fact that the conditions used to pack the nuclear pellet cannot be varied much. If the pellet is too loose, the nuclei resuspend without lysing properly; whilst if too firm, the vortexing will not resuspend them at all. Also, variations in available equipment may necessitate some experimentation to determine the correct lysis conditions for each laboratory. *These points cannot be stressed too hard because they have been the source of much frustration for people trying to reproduce this technique.*

References

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