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Isolation of macro-nuclear DNA from the rumen ciliate *Entodinium caudatum*

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A method is described which allows isolation of macronuclear DNA from the rumen ciliate *Entodinium caudatum*. Ciliate cells were enriched from the total microbial population in the rumen of sheep with a single ciliate species present by sedimentation and size filtration. Ciliates were then lysed by homogenisation and the macronuclear component isolated on a Percoll density gradient. Macronuclei can be stored in guanidine. HCl until ready for DNA extraction. DNA which had been extracted from macronuclei fresh from the Percoll gradient, or following guanidine. HCl storage, is of sufficient quality to permit its use for PCR analysis.

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Introduction

The rumen ecosystem contains a complex microbial community involved in breakdown and digestion of the plant material ingested by the host animal. Within this community are bacteria, fungi, archaea and ciliated protozoa. The precise role of the ciliates in the rumen has long been the subject of continued debate, even though a relatively large number of genes have been identified in these organisms (Ricard et al. 2006) thereby adding extra information to the knowledge about these organisms. Two factors contribute to making genetic analysis of these organisms problematic; they cannot be grown in axenic cultures, and these cells have nuclear dimorphism.

Like all ciliates, those found in the rumen are unicellular eukaryotes which have nuclear dimorphism as one of their characteristics. In ciliates which have been studied to date, the two nuclei have different roles in the cell, and this role depends upon whether the cell is in a vegetative or meiotic state. The macronucleus (or asexual, somatic nucleus) contains all of the genes needed for vegetative growth and the micronucleus (or sexual, germ-line nucleus) is required for conjugation and meiotic reproduction.

In addition to having different cytological roles in the cell, the two types of nuclei also have different karyotypes. Previous work on ciliates has shown that although the macronucleus is bigger, it contains only a sub-set of the DNA and the genes in the micronucleus. This apparent anomaly of nomenclature is due to the macronucleus containing numerous copies of genes present as single copies in the haploid copy number of the micronuclear genome (Prescott 1994; Xu et al. 2012). Thus, although the range of micronuclear genes, and the amount of repetitive DNA represented in the macronucleus is less than that of the micronucleus, the number of copies of these genes is greater in the macronucleus.

Following conjugation between two cells, the new diploid nucleus divides without cellular division, giving a cell with identical daughter nuclei. One of them becomes the micronucleus, and the other becomes the macronucleus, with the two functioning relatively independently of each other, thereby leading to nuclear dimorphism. The processes taking place during macronuclear development are poorly understood, but the product is a nucleus with multiple copies of many genes. Some species have macronuclear chromosomes which are mega-bases long. Others possess smaller chromosomes, ranging from a gene per chromosome – mini- or nano-chromosomes (e.g. Cavalcanti et al. 2005; Swart et al. 2013) to slightly larger midi-chromosomes of around 50kb (e.g. Thomas et al. 2004).

Furthermore, it is known that in some ciliates, the sequence order within some genes in their micronuclear format requires unscrambling before being in the correct macronuclear format. The extent of this unscrambling is unknown – primarily since very few ciliate genes have been sequenced in both their micronuclear and macronuclear form; however a number of cases of this phenomenon have been described. Examples of unscrambling have been found in the α-telomere binding protein (Mitcham et al. 1992; Prescott et al. 1998), actin (DuBois and Prescott 1995; Hogan et al. 2001; Chen et al. 2015) and DNA polymerase α (Hoffman and Prescott 1996; Landweber et al. 2000). It is anticipated that further genes will be identified which are scrambled in their

micronuclear format, and these data are being maintained in database format (Cavalcanti et al. 2005). Until the true extent of macronuclear unscrambling in the various species of ciliates becomes known, it is clearly essential to ensure that when analysis of DNA from a ciliate is being undertaken, it has been derived from the nucleus of interest.

We report a method allowing separation of macronuclei from micronuclei in the rumen ciliate *Entodinium caudatum*, one of the major species found in the rumen of sheep. Examples of *E. caudatum* cells are shown in Figures 1 and 2. The two nuclei are marked, as are other cellular bodies which following disruption of the cell may be of a similar size to the micronucleus – thereby making samples free of micronuclei more difficult to identify. The method describes isolation of macronuclei free of other cellular particles, and contain DNA of sufficiently high quality to permit PCR.

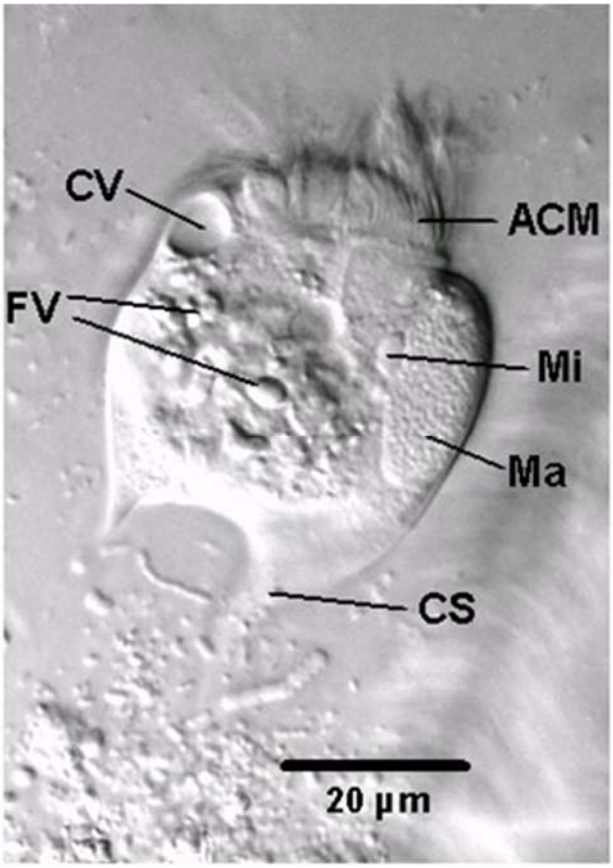

Figure 1: A typical *Entodinium caudatum* cell. The macronucleus (Ma), micronucleus (Mi), contractile vacuole (CV) food vacuoles (FV), adoral ciliary membranelles (ACM) and caudal spinum (CS). Differential interference contrast microscopy at 400 × original magnification.

Material and methods

Establishment of a clonal line

E. caudatum originated from a clonal cell line (Michalowski, unpublished), which was initiated by picking a single ciliate exhibiting typical features of *E. caudatum* and introducing it into 1 ml of "Caudatum Buffer" (5.0 g/l anhydrous K_2HPO_4, 5.5 g/l KH_2PO_4, 0.64 g/l NaCl, 90 mg/l $MgSO_4.7H_2O$ and 45 mg/l $CaCl_2$, 750mg/l anhydrous sodium acetate, 200 mg/l L-cysteine-HCl). The initiated culture was fed daily with a small quantity of food and diluted with 1 ml of fresh Caudatum Buffer. The given food consisted of powdered meadow hay (60%, v/v), wheat gluten (16%) and barley flour (24%). The daily dose of food increased continuously up to 20 mg/d, while the final volume of ciliate culture was always 40 ml.

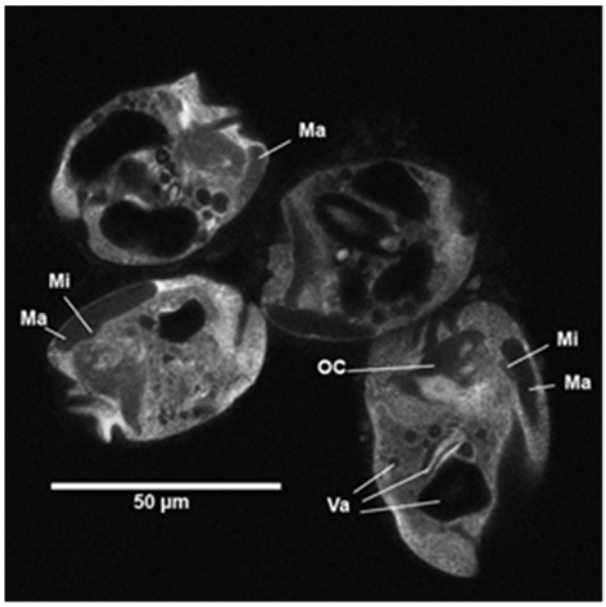

Figure 2: A single optical slice through the centre of four *Entodinium caudatum* cells after fluorescence in situ hybridisation with a Cy3-labeled Eukarya specific rRNA targeting probe (for methodological details see Fried et al. 2002). The macronucleus (Ma), micronucleus (Mi), vacuoles of different size (Va) and oral cavity (OC) are shown. Confocal laser scanning microscopy at 1000 × original magnification. Only a single optical slice of 0.8 µm thickness through the centre of the cells is shown.

The *in vitro* culture only allows a relatively low biomass of cells to be established. Following establishment of the line in vitro, the ciliate culture was transferred to the rumen of a sheep fitted with a rumen cannula in order that the biomass could be increased. This cell line was maintained as the only ciliate species in the rumen of this sheep.

Macronuclear extraction

The method used for cellular extraction is summarised in Figure 3. It is described in more detail below.

Whole ciliate cells were extracted as described previously (Williams and Coleman 1992). 500 ml of rumen fluid was withdrawn 2 hours after feeding. Large pieces of plant particulate matter were removed by filtering through a double layer of muslin (pore size > 1 mm). One volume of Coleman's Salts Solution D (6.4 g/l K_2HPO_4, 5.5 g/l anhydrous KH_2PO_4, 0.64 g/l NaCl, 90 mg/l $MgSO_4 \cdot 7\ H_2O$ and 51 mg/l $CaCl_2$) pre-warmed to 39 °C and maintained anaerobic by bubbling with CO_2, was added. Glucose was added to a final concentration of 50 mM. Cells were added to a separation funnel and separated by gravity for 20 - 30 minutes at 39 °C. The larger plant fragments, which had passed through the filter, sedimented to the bottom of the funnel, and partially fermented plant material floated on the surface. Both of these were discarded and the central phase collected. At collection, care was taken to remove only areas with maximal ciliate purity, rather than based on maximal concentration, meaning that ciliates outwith the central area were sacrificed in an effort to minimise contaminating plant material. This was sequentially passed through decreasing pore sized nylon cloths; 100 µm, 60 µm, 30 µm and 20 µm, and washed

with 250 ml of Coleman's Salts Solution D at each step. Material on top of the 20 µm membrane was collected by resuspension in Coleman's Salts Solution D.

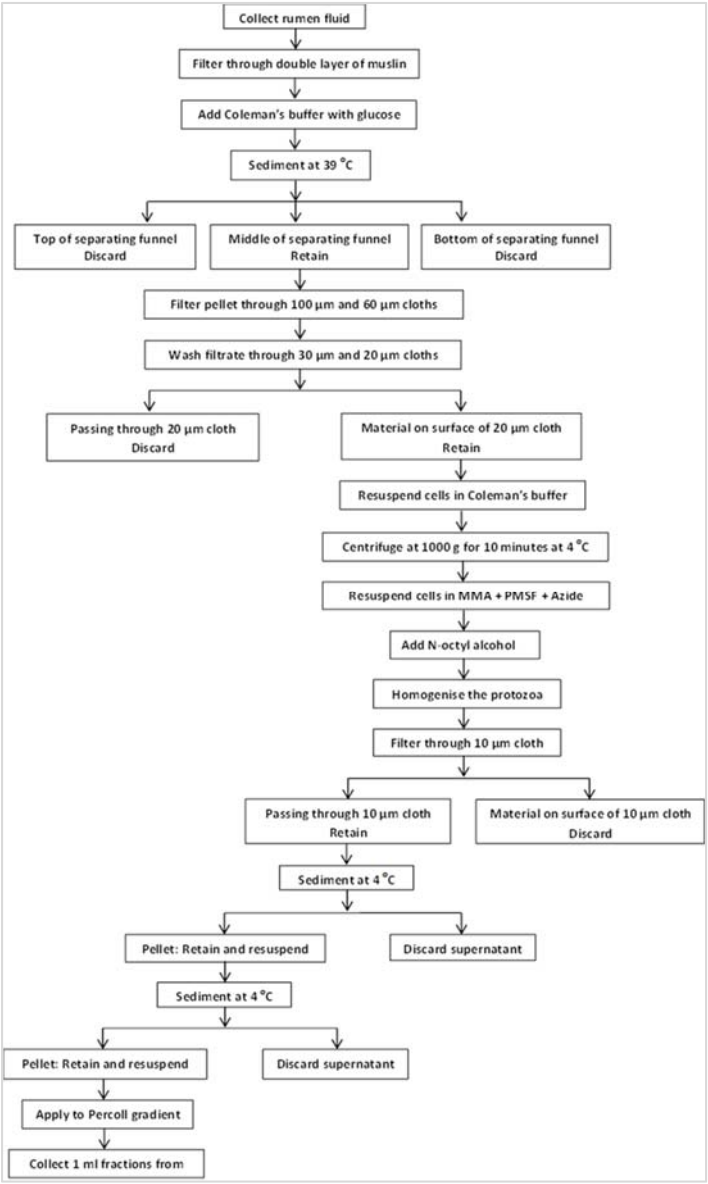

Figure 3: A schematic representation of the macronuclear isolation process.

Cells from the membrane surface were centrifuged at 1,000g for 10 minutes at 4 °C in an ALC PK121R A-M10 rotor. The pellet was resuspended in 8 ml of MMA (2% [w/v] Gum Arabic (Sigma), 2% [w/v] sucrose, 2 mM $MgCl_2$, 100 mM PMSF, 0.1% [w/v] sodium azide, 5 mM Tris. HCl, 0.5 mM EDTA, pH 6.75) and 50 µl of 0.63% [w/v] N-octyl alcohol was added (Allen et al. 1983; Allen 1999). Cells were disrupted with a Potter homogeniser attached to a Draper drill for 4 x 30 seconds set at low speed (2,300 rpm), with the tube being placed on ice for 15 seconds between homogenisation periods. This was followed by a final homogenisation step for 15 seconds. The typical products following homogenisation are seen in Figure 4. After the fifth homogenisation step, disrupted cells were passed through a 10 µm nylon cloth and the first 100 ml of the filtrate collected – collecting larger volumes of filtrate presented a risk of intact cells being washed through. Further homogenisation steps resulted in more intact ciliate cells bursting, but also resulted in freed macronuclei being ruptured – since the macronuclear membrane is less robust than the skeletal plates of the intact ciliate. Thus it was decided that additional homogenisation was counterproductive.

Homogenate products were left to sit at 4 °C for 5 hrs. Macronuclei settled at the bottom of the tube, whilst many smaller components remained in suspension. The upper phase was drawn off, and the pelleted material was re-suspended in 10 ml of MMA. At this point, cells could be incubated overnight at 4 °C with no noticeable loss of macronuclei. If an overnight incubation was used, the macronuclear suspension was made homogeneous by inverting the tube.

This was also allowed to sit at 4 °C for 5 hrs before the upper phase was drawn off. The material at the bottom of the tube was then re-suspended in 3 ml of MMA. The volume for centrifugation was made up to 23 ml by addition of a 1:1 mixture of Percoll and 2 x MMA. This material was then centrifuged. Two different centrifugation approaches were tried to establish Percoll gradients, one with a fixed head rotor, and the other with a swing-out rotor:

Rotor 1 – fixed angle

The tube was centrifuged at 60,000 g (maximum value) for 45 minutes at 4 °C using a 55.2 Ti rotor on a Beckman L-100XP ultracentrifuge, with the brake switched off. Areas of turbidity could be seen in the tube following centrifugation, however none of these corresponded to an area of solely macronuclei. 1 ml fractions were drawn off – with numbering starting from the top of the tube. Each fraction was then assessed for numbers of macronuclei and smaller contaminating particles by microscopic analysis. Macronuclei were identified on the basis of their size, with macronuclei typically being 10 µm or greater in length. During the development of the methodology, macronuclear identification was facilitated by staining with 1 mg/ml methyl green.

Rotor 2 – swing-out rotor

The method above was repeated using a SW28 rotor (swing out rotor).

Extraction of DNA

Those fractions containing only macronuclei (as assessed using a microscope with 1050 x magnification) were diluted with 14 ml of PBS and centrifuged at 1,000 g for 10 minutes at 4 °C on the ALC PK121R centrifuge. The supernatant was removed, and the pellet resuspended in 1 ml PBS. Centrifugation was repeated. The supernatant was again decanted and the pellet resuspended in 1 ml sterile ultrapure water. Centrifugation was repeated again, and the pellet resuspended in 20 µl ultra-pure water for immediate PCR, or 4 M Guanidine. HCl for future genetic analysis.

PCR conditions

2 µl of the aqueous suspension of macronuclei was directly to the PCR buffer. The PCR buffer comprised 0.2 µM of each primer [Forward primer = ATC ATT GAT AAC GGT ACT GG and reverse Primer = AGT AAG GTC TCT TCC GGA TAG]; 200 M of each dNTP; 4 mM $MgCl_2$; 10 mM Tris. HCl [pH 8.3]; 1 mM EDTA; with the following PCR programme: 94 °C for 5 min, followed by 30 cycles of [94 °C for 1 min, 55 °C for 45 sec, 72 °C for 1 min] and a final cycle of 94 °C for 1 min, 55 °C for 45 sec, 72 °C for 10 mins. This should result in amplicons

of around 500 bp of the ciliate's actin gene. Following PCR, the success of the reaction was checked by electrophoresis on a 1% [w/v] TBE agarose gel (Maniatis et al. 1982).

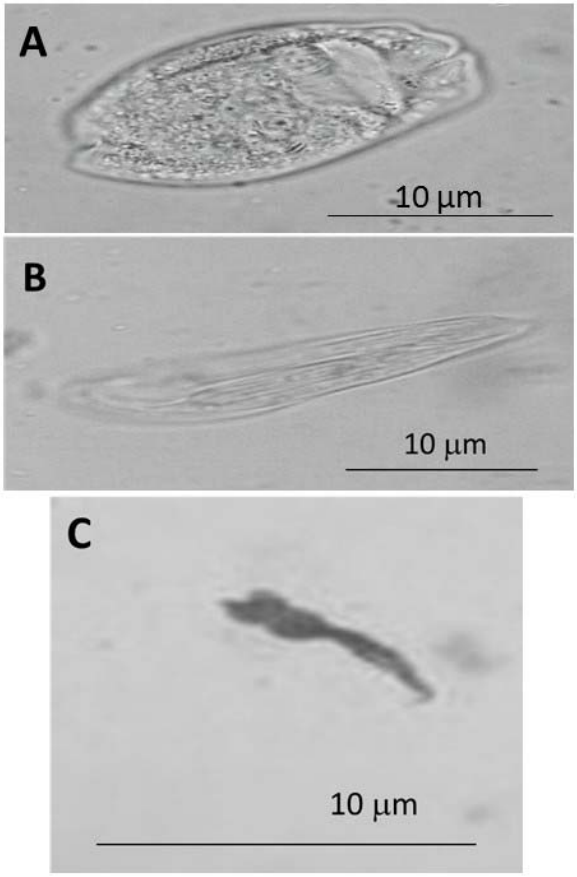

Figure 4: Products following homogenisation of ciliate cells – (A) an intact cell, (B) a ghost cell which no longer has a macronucleus present and (C) a macronucleus which has been freed from the cell. Material was stained with 1 mg/ml methyl green before viewing.

Results and discussion

In the lower fractions of the tube for both ultracentrifugation methods, there was an area where macronuclei were free of contaminating smaller particles. No attempt was made to identify these contaminants, as they could include; methanogens, bacteria ingested by ciliates prior to isolation, bacteria loosely adhered to the ciliate's surface, and sub-cellular components of the ciliates (e.g. the micronucleus). However, even when the sample appeared free of small contaminants before homogenisation, approximately 1000 small particles were released per original ciliate cell.

With the 55.2 Ti rotor (fixed angle rotor) the position of the fragment(s) containing only macronuclei varied, but lay between fractions 17 and 19 on the Percoll gradient. This variability meant that all fractions around this area (fractions 16 - 20) had to be checked for macronuclei and potential contaminants. At times most macronuclei were found in a fraction with small contaminants, meaning that they could not be used in subsequent analysis. The number of macronuclei in the 1 ml fractions which contained only macronuclei was typically of the order of around 10% of the isolated macronuclei put onto the gradient, giving around 104 to 105 macronuclei.

With the SW28 rotor (spin-out rotor) the fractions with only macronuclei were fractions 22 and 23 (i.e. lower than the position above) on the Percoll gradient. These fractions corresponded to areas where the Percoll gradient had a solution density in the range 1.14 – 1.20 (the density of the solution being calculated using an Anton Paar Digital Density Meter DMA 40). Around 50% of the isolated macronuclei put onto the gradient were located in this fraction. Due to the spinout rotor giving the higher yield of macronuclei which were free of small particles, this method of ultracentrifugation was selected as the method of choice.

Although by light microscopy these samples were counted as pure macronuclear preps, it should be noted that small bacteria may elude identification by microscopy and may be present in the fractions counted as being pure macronuclei. However, it is important to realise that the purpose of the separation procedure was to purify macronuclei away from the micronuclei. This objective has been achieved.

When PCR was performed on macronuclear preparations, amplicons of the predicted correct molecular weight were observed on agarose gels. Nevertheless, amplification of a product of the correct size does not demonstrate that the correct product has been amplified. To check that the products were those intended, the amplicon was cloned and the sequence determined. When this sequence was compared with other sequences, it confirmed that the PCR product was an actin gene of ciliate origin.

The method presented here relies on a number of different approaches to purification of the macronuclei; filtration, sedimentation under gravity and gradient centrifugation. As such the approach is something of a hybrid method. However, in our hands no one approach to isolation successfully produced clean macronuclear preparations free of contaminating particles which could be identified using a microscope. Attempts to apply the sample to the Percoll gradient immediately after homogenisation failed to yield a pure sample. Likewise, applying the sample to another round of filtration (e.g. use of a 5 µm membrane) after homogenisation also proved unsuccessful, as did relying solely on gravity. In the case of further filtration, the use of a 5µm membrane proved unsuccessful on two counts. Firstly, the filter was prone to blockage. This was true for samples (i) allowed to filter under gravity, (ii) where filtration was accelerated by suction from below, and (iii) where a positive pressure was exerted by blowing CO_2 from above. Furthermore, where samples were split into smaller volumes and done as a series of filtrations, the plasticity of the macronuclear membrane allowed the macronucleus to pass through the 5 µm pores. In addition, following the homogenisation step, the use of filtration with the 10 µm mesh removed ciliate cells which had not yet been burst open. Likewise this step removed the remaining 'ghost' cells – cells which have been burst open, their macronucleus released, but the rest of the cell has remained together. The two rounds of sedimentation under gravity resulted in many of the smaller products of homogenisation remaining in suspension and not sedimenting out. Typically this resulted in an increase in the macronucleus: small component ratio being enriched around 100 – 1000 fold. This enrichment step appears necessary prior to applying the solution to the Percoll gradient. Finally, the Percoll gradient resulted in fractions which could be isolated where the only components visible with a light microscope were macronuclei. As already mentioned, this did not preclude the possibility that small bacterial cells are

also associated with this fraction. However, anything which was too small to be undetected by use of a light microscope was going to be too small to be a micronucleus.

A number of the steps resulted in significant levels of macronuclear loss. These are summarised in Table 1. However, despite losses occurring, these steps were found necessary to ensure purity of macronuclei which were free of micronuclei.

In conclusion, the method described here can successfully lead to micronucleus-free samples of macronuclei. Furthermore, the DNA obtained was sufficiently long, and also sufficiently clear of PCR inhibitors to allow PCR analysis to be performed on a macronuclear sample, without the worry of amplifying micronuclear contaminating DNA. In turn, this will hopefully allow a better understanding of the ciliate genes, and in turn the role of the ciliates in the rumen ecosystem.

Table 1: Summary of losses of macronuclei at each step in the protocol. Macronuclear numbers were counted using a haemocytometer viewed at 105 x magnification

Step	Macronuclei left	Percentage lost	Comments
Collect rumen fluid	5.0×10^8 as ciliates	-	
Separation funnel	2.5×10^8 as ciliates	50%	Ciliates which are in the fraction with plant debris are excluded in the interests of ciliate purity
Filtrate from 100 µm, 60 µm, and 30 µm mesh	8.6×10^7 as ciliates	65%	Many of the ciliates are retained on the surface of the meshes (particularly the 30 µm mesh). However, these meshes should remove any remaining plant cells.
Fraction on surface of 20 µm mesh	6.0×10^7 as ciliates	30%	Some ciliates become trapped in the membrane, and others are able to pass through even a 20 µm mesh
1000 g centrifugation	4.5×10^7 as ciliates	25%	Some cells do not reach the pellet, but centrifuging at higher speed or for longer time increased the risk of non-ciliate contamination
Homogenisation	6.0×10^6	87%	This is the largest area of loss of material. However, although additional rounds of homogenisation released more macronuclei, in turn this led to those already isolated being broken – macronuclei being less robust than intact ciliates and easier to break
Filtrate from 10 µm mesh	3.0×10^6	50%	Some of the free macronuclei are trapped on the 10 µm mesh. However, this step is necessary to remove intact ciliate cells
First gravity sedimentation	1.5×10^6	50%	Although around half of the macronuclei were lost during sedimentation, the ratio of macronuclei: small particles was enhanced over 25 fold
Second gravity sedimentation	1.0×10^6	33%	Although macronuclei were lost during sedimentation, the ratio of macronuclei: small particles was enhanced a further 3-4 fold
Percoll gradient	5.0×10^5	50%	Although around half of the macronuclei were lost during centrifugation, they were associated with a fraction where there was a low concentration of contaminating small particles

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