

## Video Article

# Enrichment of Pachytene Spermatocytes and Spermatids from Mouse Testes Using Standard Laboratory Equipment

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## Abstract

To characterize each step of spermatogenesis, researchers must separate different subpopulations of germ cells from testes. However, isolating discrete populations is challenging, because the adult testis contains a complex mix of germ cells from all steps of spermatogenesis along with certain populations of somatic cells. Over the past few decades, different techniques such as centrifugal elutriation, fluorescence-activated cell sorting (FACS), and STA-PUT have been successfully applied to the isolation of germ cells. A drawback is that they all require dedicated devices and specialized training. Following principles underlying the STA-PUT method, a simple protocol has been developed for the isolation of pachytene spermatocytes, round spermatids, and elongating spermatids from mouse testes. After preparing a single cell suspension of testicular cells, specific cell populations are enriched by gravity sedimentation through a discontinuous bovine serum albumin (BSA) density gradient. The cell fractions are then manually collected and microscopically analysed. This modified density gradient for round spermatids (MDR) sedimentation protocol can be widely applied, because it requires only standard laboratory equipment. Furthermore, the protocol requires minimal starting materials, reducing its cost and use of laboratory animals.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/60271/>

## Introduction

Much is still unknown about the molecular and biological events taking place during mammalian spermatogenesis, a complex process in which spermatogonial stem cells transform into highly specialized spermatozoa<sup>1,2</sup>. Spermatogenesis takes place inside the seminiferous tubules of the testis. The tubules contain a mixture of germ cells from each step of differentiation, including spermatogonial stem cells, mitotically dividing spermatogonia, meiotic spermatocytes, and postmeiotic spermatids (which undergo haploid differentiation from round spermatids to elongating spermatids, and finally to mature spermatozoa). Somatic cells of the testis include Sertoli cells that are intermingled with germ cells inside the seminiferous tubules, peritubular myoid cells that form walls of the tubules, and testosterone-producing Leydig cells in the interstitial space between tubules.

Studying molecular and biochemical processes during spermatogenesis often requires separation of distinct germ cell populations from a complex mixture of testicular cells. Many different strategies have been developed for cell enrichment. The most successful methods are STA-PUT velocity sedimentation by unit gravity<sup>3,4,5,6</sup>, centrifugal elutriation based on counterflow centrifugation<sup>7,8</sup>, and fluorescence-activated cell sorting (FACS) that separates cells according to DNA content and/or specific markers. These methods are commonly used among spermatogenesis researchers and allow for the efficient enrichment of specific germ cell types. However, a limitation of these techniques is that they require specialized, expensive hardware that require expertise.

Presented here is a simple and inexpensive protocol to isolate enriched populations of the three most abundant cell populations of mouse testes: round spermatids, pachytene spermatocytes, and elongating spermatids. This protocol is referred to as the modified density gradient for round spermatids (MDR), because it works particularly well for enriching round spermatids. The MDR method is based on the same principles as the STA-PUT velocity sedimentation, yet it requires only standard laboratory equipment. Living cells are allowed to sediment through a manually prepared discontinuous bovine serum albumin (BSA) density gradient inside a standard 50 mL tube under the earth's gravitational field. Larger cells move faster through the gradient, which separates different populations of germ cells. After sedimentation, enriched fractions of the three cell types are manually collected. The purity of these enriched cell populations is comparable to those obtained by STA-PUT and centrifugal elutriation.

In addition to covering the construction and use of the BSA gradient for velocity sedimentation, the protocol also describes a digestion method to release testicular cells from seminiferous tubules. The protocol was modified from that developed by Romrell et al.<sup>9</sup> and includes sequential

digestions with collagenase IV and trypsin. Sequential digestions combined with use of a bicarbonate buffer (i.e., the Krebs solution) have been shown to greatly enhance the separation and viability of the germ cells<sup>9</sup>.

During MDR enrichment, cells spend around 4 h together outside the environment of the seminiferous tubules and are not subjected to stressful mechanical forces, which allows for the collection of highly viable cellular fractions for downstream analysis. In addition, similar to the centrifugal elutriation and STA-PUT, the MDR protocol does not require any chemical treatment or labelling of cells, which also helps to maintain their viability. Importantly, as little as two adult mouse testes are sufficient for the MDR isolation and therefore, one adult mouse provides enough enriched cells for both RNA and protein analysis. Standard STA-PUT protocol recommends use of as many as 12 adult mice for cell isolation<sup>6</sup>; although, based on prior experience, it is known that successful isolation can be done from three to four adult mice. The lowest amount of starting material reported to be sufficient for centrifugal elutriation is six mouse testes (three mice)<sup>8</sup>. Therefore, besides eliminating the need for expensive specialized equipment, the MDR protocol reduces the number of laboratory animals required.

## Protocol

The maintenance of laboratory mice and all experiments were performed in accordance with the relevant guidelines and regulations for the care and use of laboratory animals.

### 1. Equipment and Reagent Set-up

1. Set the water bath to 37 °C.
2. Set the cell culture incubator to 34 °C, 5% CO<sub>2</sub>, 95% humidity. Put the tube rotator inside the incubator.  
**NOTE:** Incubators require a long time to change internal temperature. If an incubator constantly set at 34 °C is not available, set one up 1 day before the experiment.
3. Prepare and label the appropriate amount of microscopy glass slides. Draw a ring of ~1 cm in diameter with a grease pen and let the grease dry.
4. Prepare 1x Krebs buffer, pH 7.8 (**Table 1**). Put two conical tubes with 50 mL of 1x Krebs to 34 °C to pre-warm for steps 2.5–2.8. Store the rest of the 1x Krebs at 4 °C or on ice.
5. Prepare BSA solutions. First prepare 25 mL of 10% (w/v) BSA solution in Krebs by dissolving 2.5 g of BSA in 1x Krebs buffer to a final volume of 25 mL. Dilute the 10% BSA solution with 1x Krebs buffer to obtain different BSA concentrations (**Table 2**). Keep all BSA solutions at 4 °C.  
**NOTE:** Prepare solutions the same day of the procedure and store at 4 °C until use.
6. Prepare the digestion enzymes by weighing the correct amount of trypsin and collagenase to 50 mL conical tubes (**Table 3**).

### 2. Animal Dissection and Preparation of Germ Cell Suspension

**NOTE:** This takes approximately 1 h to complete.

1. Sacrifice an adult male mouse (aged 7 weeks or older, testis weighing 80–120 mg depending on the strain and age) via cervical dislocation or CO<sub>2</sub> asphyxiation.
2. Spray the ventral abdomen of the mouse with 70% ethanol. Open the abdominopelvic cavity using scissors, making a V-shaped opening.
3. Pulling on the epididymal fat pad with forceps, locate the testes and remove them with scissors. Avoid disturbing the tunica albuginea. Place the testes on a 6 cm Petri dish containing 1x Krebs.
4. Decapsulate the testes and discard the tunica albuginea. Slightly disperse the seminiferous tubules by gently teasing them apart with forceps.
5. Use forceps to transfer the seminiferous tubules into a 50 mL conical tube containing 2 mL of freshly prepared collagenase solution (**Table 3**).
6. Incubate the tubules in the 37 °C water bath for 3 min. Agitate gently by rocking the tube.  
**NOTE:** Freely floating tubules should occur within 3 min due to the removal of interstitial cells. The physiological temperature for testicular cells is 34 °C; therefore, long digestions are usually done at this temperature. However, the short 3 min digestion can be conducted at 37 °C (recommended temperature by the manufacturer). Note that if 34 °C is used, the digestion time should be re-optimized.
7. Add at least 40 mL of warm 1x Krebs and allow the tubules to sediment (~1 min) at room temperature (RT). Remove the supernatant and repeat 1x.
8. Add 25 mL of freshly prepared trypsin solution (**Table 3**), place the tube on the tube rotator inside the 34 °C incubator, and incubate for 15–20 min (~15 rpm). Sporadically check the status of the tubules. Once the solution becomes cloudy and only small pieces of tubules remain, place the tube on ice and immediately proceed to the next step.  
**NOTE:** To avoid overdigestion and cell lysis, move swiftly to the following washing steps. Some protocols include the deactivation of trypsin by fetal bovine serum (FBS). In this protocol, FBS treatment is omitted, and instead, trypsin is removed by immediate centrifugation and subsequent washes with cold 1x Krebs.
9. Filter the solution through a 40 µm cell strainer into a new 50 mL conical tube on ice.
10. Centrifuge 600 x g for 5 min at 4 °C to pellet the cells.  
**NOTE:** Centrifugation with too strong of a force may harm the cells.
11. Remove supernatant by carefully pouring it out.
12. Tap the cell pellet to resuspend the cells in the remainder of 1x Krebs.
13. Add at least 40 mL of cold 1x Krebs to the resuspended cells.
14. Repeat steps 2.10 and 2.11.
15. Tap the tube with the cell pellet to resuspend the cells. Add 1 mL of 0.5% BSA in 1x Krebs and with a cut pipette tip resuspend the cells by pipetting the solution up and down. Avoid making bubbles.
16. Finally, add 1–3 mL of 0.5% BSA in 1x Krebs so that the final volume is ~3 mL. Filtrate the germ cell suspension through a 40 µm cell strainer and proceed immediately to loading the cells on the BSA gradient.

### 3. Separation of Germ Cells Through the Discontinuous BSA Gradient

**NOTE:** This section takes approximately 2 h to complete. Start preparing the discontinuous BSA gradient during the wash steps (steps 2.10–2.14) to load the cells as soon as the pretreatment is ready.

1. Accommodate a 50 mL tube vertically on ice so that it is possible to see one side of the tube. Alternatively, run the protocol in a cold room at 4 °C, in which case no ice is needed.
2. Cut the tip of a 10 mL serological pipette about 5–10 mm from the tip to obtain a larger aperture (**Figure 1A**), and mount it on the pipette controller (**Figure 1C**).  
**NOTE:** This will decrease the velocity during pipetting, which facilitates stacking of the different BSA solutions. Alternatively, use a 1 mL mechanical pipette with a smooth piston and cut the pipette tips with an aperture of about 3 mm in diameter (**Figure 1B**).
3. Start by pipetting 5 mL of 5% BSA solution to the bottom of the 50 mL tube.
4. Slowly pipet 5 mL of 4% BSA solution on top of the 5% solution. Start by gently touching the surface of the 5% solution with the cut tip of the pipette and layer the two solutions while carefully maintaining contact with the surface, ensuring to not allow the pipet tip to become immersed.  
**NOTE:** A clear line should be visible between the two layers at the end of this step.
5. Repeat step 3.4 with the other BSA solutions obtain a discontinuous gradient from 5% up to 1% (**Figure 1D**).
6. Carefully load the single-cell suspension on top of the gradient without disturbing. Let the cells sediment through the gradient for 1.5 h at 4 °C or on ice.

### 4. Collection of Enriched Germ Cell Fractions

**NOTE:** This section takes approximately 30 min to complete.

1. Mount a 1 mL pipette tip onto a 1 mL mechanical pipette and cut the tip so that the aperture is ~3 mm in diameter (**Figure 1B**).
2. Carefully collect ~1 mL fractions in separate 1.5 mL tubes, starting from the top of the BSA gradient, and store them on ice. Number the tubes in the same order as the fractions will be collected.  
**NOTE:** At this point, there should be a series of ~28 tubes containing cell suspensions. Use a 1 mL mechanical pipette with a smooth piston and cut the pipette tips to minimize the risk of disturbing the BSA gradient.
3. Centrifuge the germ cell fractions at 600 x g for 10 min at 4 °C.
4. Careful not to disturb the pellet, discard most of the supernatant and resuspend the cell pellet by flicking the tube. Add 1 mL of ice-cold 1x Krebs buffer to each tube and repeat centrifugation.  
**NOTE:** Repeat the washing step if BSA interferes with downstream analysis.
5. Discard most of the supernatant but leave ~100 µL after the final wash. Resuspend the cell pellet carefully.  
**NOTE:** Resuspending the cells carefully ensures that the sample taken from this solution represents all cells of the given fraction. Keep the cell suspensions on ice while preparing the slides.

### 5. Analysis of Cell Fractions

**NOTE:** Analysis takes 2 h to complete.

1. One at a time, pipet 20 µL of 4% paraformaldehyde (PFA) inside each grease pen ring on a numbered microscopy slide.
2. Immediately add 2 µL of the resuspended cell suspension from the corresponding fraction. Repeat for each fraction.  
**NOTE:** While preparing slides, keep all cell suspensions on ice.
3. Dry the slides at RT for 1 h to overnight (O/N).  
**NOTE:** During this step, after taking a sample from each fraction, the cells can be processed for downstream analyses or storage (section 6).
4. Rinse the slides once with 1x PBS and mount with mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (**Table of Materials**).
5. Analyze the slides under a fluorescence microscope to estimate which particular germ cell type is enriched in each fraction.  
**NOTE:** Different cell types can be recognized by their characteristic nuclear morphology visualized by DAPI staining. **Figure 2A** shows representative fractions enriched in elongating spermatids, round spermatids and pachytene spermatocytes. The round spermatid nucleus is small (6–7 µm in diameter) and round (**Figure 2A**). Mouse round spermatid nuclei are also characterized by a single, round heterochromatin structure called the chromocenter. Nuclei of elongating spermatids have a typical elongated shape and reduced nuclear size due to chromatin condensation (**Figure 2A**). Pachytene spermatocyte nuclei are much larger (diameter more than 10 µm) and more irregular in shape, with areas of densely packed chromatin distributed throughout (**Figure 2A**).
6. **If necessary, perform immunostaining in addition to the DAPI staining to support the recognition of the different cell types.**
  1. In this case, spread 2 µL of cell suspension with 20 µL of a fixing solution (4% PFA and 0.1% nonionic surfactant in PBS) inside a grease pen ring. After drying the slides, postfix the cells with 4% PFA for 10 min at RT.
  2. Rinse with PBS, then permeabilize with 0.1% nonionic surfactant in PBS for 5 min. Rinse with PBS and deactivate any remaining PFA by incubating slides in 100 mM NH<sub>4</sub>Cl for 5 min.
  3. Rinse with PBS and proceed to a standard immunofluorescence protocol consisting of blocking and incubations with specific primary antibodies and fluorochrome-labeled secondary antibodies. Mount the slides with antifade mountant with DAPI (**Table of Materials**) and allow them to set for 24 h.  
**NOTE:** Examples of useful markers for primary antibody incubations include peanut agglutinin (PNA; 1:2,000 in blocking solution) that stains the acrosome in round and elongating spermatids, anti-DDX4 antibody (1:200 in blocking solution) that labels a single cytoplasmic granule in round spermatids, and anti-γH2AX antibody (1:100 in blocking solution) that recognizes sex bodies in pachytene spermatocytes. See the representative results and **Figure 2C,D** for examples of immunofluorescence analysis of the fractions.

## 6. Processing the Remaining Samples for Storage

**NOTE:** Section 6 takes approximately 20 min to complete.

- Once a sample from each fraction has been taken for a microscopy slide, add 1 mL of ice-cold 1x Krebs to each fraction and centrifuge the cells down at 600–13,000  $\times g$  for 10 min at 4 °C.  
**NOTE:** A low-speed centrifugation results in a loose pellet, which makes it difficult to completely remove 1x Krebs, while a high-speed centrifugation may harm the cells. Choose the centrifugation speed according to specific requirements of the downstream protocol.
- Remove and discard the supernatant and continue with preferred downstream analysis.  
**NOTE:** At this point, the cells can be snap-frozen in liquid nitrogen and stored at -70 °C. Once familiar with this technique, it is possible to continue with the downstream protocol of preference directly, but it is always advisable to take a sample and ensure the purity of each fraction. Total RNA can be extracted, quantified, and analysed by methods such as reverse transcription polymerase chain reaction (PCR) and RNA sequencing. Total protein extracts can also be prepared from which immunoprecipitation or western blotting analysis can be performed (**Figure 3**).

### Representative Results

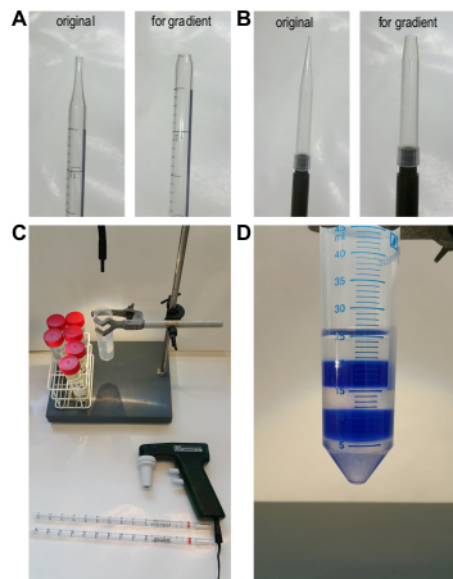
Sufficient enrichment of a germ cell type is usually considered to be above 80%<sup>6</sup>. The MDR protocol works particularly well for enriching round spermatids. A high number of >90% pure round spermatids can routinely be obtained using this technique. Optimal fractions of pachytene spermatocytes and elongating spermatids are enriched to ~75% and ~80%, respectively. Elongating spermatids tend to stay on top of the gradient and are collected with the first fraction. In the example shown, fraction 1 contained ~80% of elongating spermatids (**Figure 2B**). Most elongating spermatids obtained with this technique have condensed nuclei, while non-condensed early elongating spermatids are scarce (**Figure 2A**). The following fractions contain enriched round spermatids. In the example, the enrichment of round spermatids was more than 90% in fractions 2, 3, and 4, and enrichment above 80% was seen altogether in seven fractions (2–8) (**Figure 2B**). Due to their large size, pachytene spermatocytes sediment faster and are collected last. In the purification example, enrichment was around 75% in fractions 14 and 15 (**Figure 2B**).

While nuclear morphology, visualized by DAPI staining, usually suffices for recognition of the cells, immunofluorescence analysis can be performed to support the analysis. PNA stains the developing acrosomes of round and elongating spermatids, and the acrosomal appearance can be exploited to further categorize round spermatids into steps 1–8 of differentiation<sup>10</sup>. Distinguishing PNA-stained elongating and round spermatids relies on the differences in their nuclear shape (**Figure 2C**, left panel). Anti-DDX4 antibody is a useful marker for the round spermatid fractions since it visualizes one single DDX4-positive perinuclear granule, the chromatoid body (CB), in the cytoplasm of each round spermatid. This CB-specific staining is easy to distinguish from more widely distributed cytoplasmic staining in spermatocytes (**Figure 2C**, middle panel). Pachytene spermatocytes can be recognized by anti- $\gamma$ H2AX antibody that labels the nuclear sex body specifically appearing at the pachytene phase of the first meiotic division (**Figure 2C**, right panel). In this purification, PNA staining revealed that MDR enriched round spermatid fractions contained a mixture of round spermatids at various steps of differentiation, all containing their signature structure, the DDX4-positive CB (**Figure 2D**, RS fraction). Anti- $\gamma$ H2AX further validated the enrichment pachytene spermatocytes in fraction 16 (**Figure 2D**, PSpc fraction).

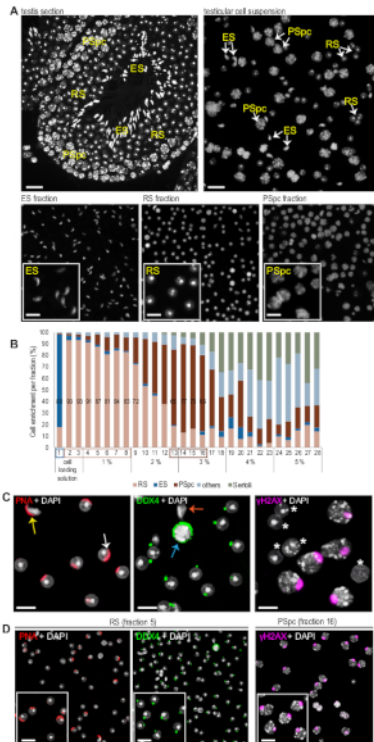
The cell counting revealed that the number of cells in the round spermatid and pachytene spermatocyte fractions is adequate for various downstream analyses. Round spermatid fractions (5–8) each contained around  $2.5 \times 10^6$  cells. Therefore, by pooling these fractions, it is possible to obtain more than 10 million cells. Pachytene spermatocyte fractions (14 and 15) usually contained somewhat fewer cells. In this isolation, around  $1.5\text{--}2.0 \times 10^6$  cells per fraction were counted. The first fraction contained  $0.75 \times 10^6$  elongating spermatids.

As shown in **Figure 3A**, the total RNA amount obtained from the majority of fractions ranged from 0.5–2.5  $\mu$ g, which is sufficient for downstream RNA analyses such as reverse transcription PCR, RNA sequencing, or visualizing RNA on a gel. The amount of protein obtained from each fraction typically ranges from 20–140  $\mu$ g (**Figure 3B**), which is sufficient for several western blots. Whole cell lysates were prepared from collected fractions, and western blot analysis was performed using antibodies detecting DDX4, PIWIL1, and PIWIL2, which are all highly expressed in pachytene spermatocytes and round spermatids as well as the ubiquitously expressed glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

In this protocol, 10% of the protein lysates derived from single fractions was sufficient to clearly detect all these proteins on a standard western blot setting (**Figure 3C**). The amount of protein in one fraction was also shown to be sufficient for immunoprecipitation using an antibody against PIWIL1, as well as for the detection of co-immunoprecipitated PIWIL2 (**Figure 3D**). Furthermore, this protocol has been successfully used to obtain enriched fractions of pachytene spermatocytes and round spermatids from control and genetically modified mice for downstream applications such as quantitative reverse transcription PCR<sup>11</sup> and high throughput RNA sequencing<sup>12</sup>.

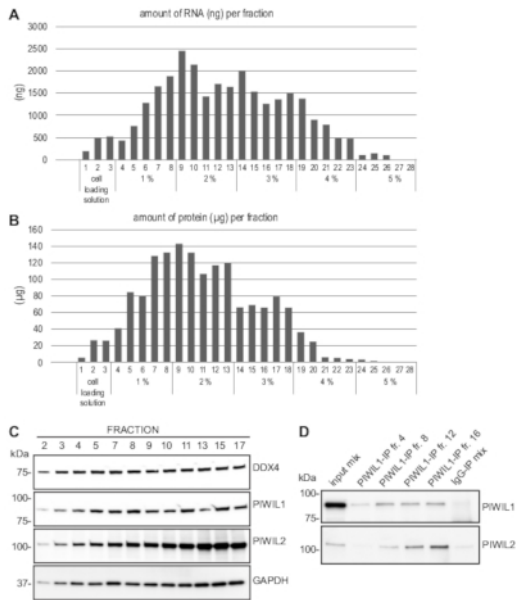


**Figure 1: Preparation of a discontinuous BSA gradient.** (A) A 5 mL serological pipette tip for preparation of the gradient. (B) A 1 mL pipette tip for preparation of the gradient and collection of the germ cell fractions after sedimentation. (C) The equipment needed for preparation of the gradient. (D) Lateral view of the discontinuous BSA density gradient from the 5% (bottom) to the 1% (top) BSA solution; the 2% and 4% BSA solutions are blue in color for better visualization. [Please click here to view a larger version of this figure.](#)

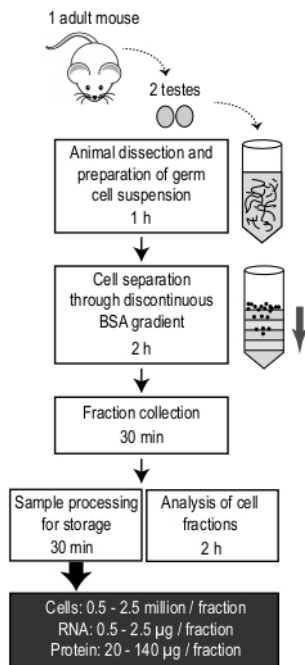


**Figure 2: Representative images of the collected cell fractions and the enrichment analysis.** (A) DAPI-stained testicular cells. The upper row shows testicular cells in the intact seminiferous epithelium of a PFA-fixed, paraffin-embedded testis section (left) or in suspension (right). The lower row shows fractions of enriched elongating spermatids (ES), round spermatids (RS), or pachytene spermatocytes (PSpC). Scale bars = 20  $\mu$ m for the upper row and 10  $\mu$ m for insets of the lower row. (B) Relative quantification of the different germ cell types in each fraction. Cells were manually counted using the ImageJ software and classified into RS, ES, PSpC, Sertoli cells, and other cells. The fraction numbers and respective percentages of BSA in the gradient are indicated on the x-axis. (C) Immunofluorescence analysis of testicular cells. Left panel: rhodamine-labelled PNA stains the acrosome in both ES (yellow arrow) and RS (white arrow). Middle panel: DDX4 antibody stains a single perinuclear granule in RS, which is easy to distinguish from the more diffuse cytoplasmic signal in spermatocytes (blue arrow). No DDX4 signal is detected in ES (orange arrow). Right panel:  $\gamma$ H2AX antibody recognizes the characteristic nuclear sex body present only in pachytene spermatocytes ( $\gamma$ H2AX-negative cells marked by a white asterisk). Scale bars = 10  $\mu$ m. (D) MDR enriched cell fractions were labelled with PNA (RS fraction), anti-DDX4 (RS fraction), and anti- $\gamma$ H2AX (PSpC fraction) to further validate the cell enrichment in each fraction. Scale bars = 10  $\mu$ m. [Please click here to view a larger version of this figure.](#)





**Figure 3: Downstream analyses after MDR cell enrichment.** (A) RNA was extracted from each fraction after a representative MDR cell enrichment and quantified. The fraction numbers and respective percentages of BSA in the gradient are indicated on the x-axis. (B) Proteins were extracted from each fraction by lysing the cell pellet in radioimmunoprecipitation assay (RIPA) buffer then quantified. (C) Whole cell protein extracts were prepared and analysed by western blotting using antibodies against DDX4, PIWIL1, PIWIL2, and GAPDH. 10% of the lysate was loaded from each indicated fraction. (D) Immunoprecipitation was performed from indicated fractions using anti-PIWIL1 followed by western blotting with anti-PIWIL1 and anti-PIWIL2 antibodies. The input sample includes a mixture of protein lysates from different fractions, and the control immunoprecipitation (IP) using rabbit IgG was also performed from a mixed lysate. [Please click here to view a larger version of this figure.](#)



**Figure 4: Schematic representation of MDR protocol and time required for completing each step.** Starting material is composed of two testes from an adult mouse. The average number of cells and the amount of RNA and protein obtained from one fraction are indicated. [Please click here to view a larger version of this figure.](#)

Buffer	Reagents	Preparation	Storage
Krebs buffer (10x)	3.26 g KH <sub>2</sub> PO <sub>4</sub>	Bring to 2 L with H <sub>2</sub> O, filter 0.22 μm and autoclave.	Can be stored at 4 °C for several months
	139.5 g NaCl		
	5.89 g MgSO <sub>4</sub> ·7H <sub>2</sub> O		
	50 g dextrose		
	3.78 g CaCl <sub>2</sub> ·2H <sub>2</sub> O		
	7.12 g KCl		
Krebs buffer (1x)	4.24 g NaHCO <sub>3</sub>	Dissolve NaHCO <sub>3</sub> to 100 mL of H <sub>2</sub> O, add 200 mL of 10x Krebs buffer, and bring to 2 L with H <sub>2</sub> O.	To be prepared fresh
	200 mL 10x Krebs buffer		

**Table 1: Preparation of Krebs buffer.**

BSA concentration (w/v)	10% BSA solution	1x Krebs buffer
0.50%	0.5 mL	9.5 mL
1%	1 mL	9 mL
2%	2 mL	8 mL
3%	3 mL	7 mL
4%	4 mL	6 mL
5%	5 mL	5 mL

**Table 2: Preparation of BSA solutions.**

Digestion solution	Working concentration	Reagents	Preparation
Collagenase	1 mg/mL	Collagenase IV	Weigh 2 mg of collagenase IV to a 50 mL conical tube, and add 2 mL of warm 1x Krebs buffer right before the digestion (step 2.3).
Trypsin	0.6 mg/mL	Trypsin	Weigh 15 mg of trypsin to a 50 mL conical tube, and add 25 mL of warm 1x KREBS buffer and 40 μL of DNase I right before the digestion (step 2.6).
	>3.2 ku/mL	DNase I	

**Table 3: Preparation of the digestion enzymes.**

## Discussion

Presented here is a simple and inexpensive protocol to isolate enriched populations of round spermatids, pachytene spermatocytes, and elongating spermatids using standard laboratory equipment (overview of the protocol shown in **Figure 4**). Though no expertise or expensive machinery is required, there are some critical steps that must be considered during tissue digestion, construction of the gradient, and loading of the cell suspension onto the gradient.

Germ cells are released from seminiferous tubules by two consecutive enzymatic digestions. The first digestion with collagenase IV separates seminiferous tubules by removing interstitial cells. Prolonged digestion time may damage the tubules and lead to loss of spermatids, as (if released from the tubules during this step) they will be discarded in the following steps. The second digestion step with trypsin releases germ cells from seminiferous tubules. There may be occasional cell lysis and typically some clumps form due to released genomic DNA. Exceeding the suggested duration or temperature of the digestion is not advised, as this can lead to poorer viability, increased cell lysis, and clumping. If mild clumping does occur, the clumps can be ignored. However, in cases of significant clumping and loss of cells, trypsin digestion time or concentration should be reduced. It should also be noted that the enzymatic activity of trypsin may vary between batches and during prolonged times of storage. The amount of DNase I during trypsin digestion can also be increased to remove excess clumps, but this should be considered a secondary solution. It is important to obtain a homogenous single cell suspension at the end of pre-treatment, since clumped cells will sediment faster, contaminating the fractions and disrupting the gradient.

Building the gradient may require some practice. If there is discomfort using a 5 mL pipette tip with a pipette controller, it is recommended to use a normal 1 mL mechanical pipette with a smooth piston then cut the pipette tips to an aperture of ~3 mm in diameter (**Figure 1B**). A wider aperture and smooth loading of the BSA solutions will decrease the risk of mixing the gradient. When properly prepared, it is possible to see the boundaries between adjacent BSA solutions due to their different refractive indices. The gradient should be produced directly prior to use. It should also be noted that any small shaking or vibration may disturb the gradient, so the gradient should be set in an environment where it will not be disturbed.



Loading of the cell suspension onto the gradient must be done very carefully. After loading, the cell suspension should stay on top of the gradient, from which the cells will slowly start to sediment through the first layer. If large groups of cells are seen moving fast through the gradient, the cells were likely not resuspended carefully or there is excess clumping. If the cells do not stay on the top of the discontinuous BSA gradient upon loading but immediately sink between the 1% and 2% BSA layers (step 3.6), the cell suspension is likely too dense. This protocol has been optimized using two testes of an adult mouse (80-120 mg/testis) as starting material; although, successful isolations using reduced amounts of starting material has been performed. To upscale the protocol and obtain more enriched germ cells from higher numbers of testes, more 50 mL tubes with the gradient should be introduced.

The protocol was initially developed and optimized to enrich haploid round spermatids from adult mouse testes, and the purity of round spermatid fractions is expected to be more than 90%. In addition to highly pure round spermatid fractions, satisfactory results for the enrichment of pachytene spermatocytes and elongating spermatids were obtained. It should be noted that erythrocytes may contaminate the elongating spermatid fractions, and further steps to eliminate them should be taken if their presence is expected to interfere with downstream analyses. We have not been able to enrich other cell types such as premeiotic or early meiotic cells (prior to pachytene phase) from adult mice using the MDR protocol.

Additionally, STA-PUT sedimentation has been successfully used to obtain enriched fractions of spermatogonia or preleptotene, leptotene, and zygotene spermatocytes using juvenile testes collected at given timepoints after birth<sup>13</sup>. This approach takes advantage of the appearance of these cell types during the first wave of spermatogenesis. The same approach can likely be applied to MDR enrichment, but it has not yet been tested in practice. Another method that is a good option for the purification of premeiotic and meiotic cells at specific stages of differentiation is FACS, which has the important advantage of allowing the isolation of specific cells types based on the presence specific markers<sup>14,15,16,17</sup>.

Overall, the MDR velocity sedimentation serves as a useful method for germ cell enrichment. While this method is not superior to other well-established methods in terms of the purity or quantity of enriched cells, its clear advantages are its simplicity and low set-up costs. This, together with the low amount of required starting materials, render this protocol a great option for researchers in the spermatogenesis field and those in other fields who may not wish to invest in specialized hardware or large groups of animals.

## Disclosures

The authors have nothing to disclose.

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