ABSTRACT
This chapter will discuss the various sperm preparation and selection techniques used to process sperm for use with assisted reproductive techniques: swim-down, swim-up, migration-sedimentation, density gradient centrifugation, magnetic activated cell sorting, and glass wool filtration. It will also explain the procedures used to prepare viscous semen samples as well as when to obtain and prepare semen samples using epididymal and testicular spermatozoa, assisted ejaculation, and retrograde ejaculation.

INTRODUCTION
Approximately 2–4 percent of births in developed countries involve the use of assisted reproductive techniques (ART). With ART, semen samples must first be processed before they can be used for insemination. Specifically, sperm preparation methods seek to replicate in vitro the natural process in which viable sperm are separated from other constituents of the ejaculate as they actively migrate through the cervical mucus. During processing, viable sperm cells are first separated from other constituents of the ejaculate as early as possible. If spermatozoa are not separated from seminal plasma within 30 minutes of ejaculation, the in vitro fertilization (IVF) capacity permanently diminishes. The World Health Organization (WHO) recommends separating sperm cells from the seminal plasma within one hour after ejaculation to limit damage from leukocytes and other cells present in the semen.

Various sperm separation or isolation methods exist to select sperm cells. These include swim-up methods, two-layer discontinuous gradient centrifugation, pentoxifylline wash, test-yolk buffer, sedimentation methods, polyvinylpyrrolidone (PVP) droplet swim-out, electrophoresis and fluorescence cell sorting methods. A number of these have been developed to separate viable sperm from the seminal ejaculate for use in ART such as swim-down, swim-up, migration-sedimentation, density gradient centrifugation, magnetic activated cell sorting, and glass wool filtration. This chapter will discuss these techniques—the more commonly used procedures are explained in detail. It will also explain the procedures used to prepare viscous semen samples as well as when to obtain and prepare semen samples using epididymal and testicular spermatozoa, assisted ejaculation, and retrograde ejaculation.

SIMPLE WASH METHOD
In the simple wash method, following complete liquefaction, culture medium is added to the ejaculate and centrifuged twice to remove the seminal plasma. It is essential to use lower centrifugal forces (less than 500 g) and fewer centrifugation steps to minimize the damage caused by formation of reactive oxygen species (ROS) by non-viable spermatozoa and leukocytes. Increased levels of ROS result in DNA damage in spermatozoa, decreased sperm motility, increased numbers of apoptotic spermatozoa, and decreased sperm plasma membrane integrity. Additionally, the presence of large numbers of non-viable spermatozoa in the prepared sample can inhibit capacitation—a physiological process that confers spermatozoa with the ability to fertilize an oocyte.

The simple wash technique is usually used when the semen sample has optimal parameters. This technique is often used to prepare sperm cells for intrauterine insemination because it produces very high yields of spermatozoa.

MIGRATION-BASED TECHNIQUES
Swim-Up
Swim-up is one of the most commonly used techniques for sperm preparation. Swim-up can be performed using a cell pellet or a liquefied semen sample. In conventional swim-up, a pre-washed sperm pellet obtained by a soft spin is placed in an overlaying culture medium in a conical tube (Fig. 29.1). The common steps of this method (using a cell pellet) are as follows:
- Allow specimen to liquefy completely for 15–30 minutes in a 37°C incubator before processing.
- Measure volume using a sterile 2 mL pipet.
- Transfer specimen from a plastic cup to a sterile 15 mL—conical centrifuge tube. If specimen is >3 mL, split the specimen into two aliquots.
- Gently mix the specimen with Quinn’s Sperm Wash Media (HTF) in a ratio of 1:4 using a sterile pasteur pipet.
- Centrifuge the tubes at 1600 rpm for 10 minutes.
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• Examine for sperm count and motility.
• Carefully aspirate the supernatant without disturbing the pellet and resuspend the pellet in 3 mL of fresh HTF. Transfer the resuspended sample into two 15 mL sterile round bottom tubes using a sterile serological pipette (1.5 mL in each).
• Centrifuge the tubes at 500 rpm for 5 minutes.
• Incubate the tubes at a 45° angle for 1 hour for swim-up in vertical rack in a 37°C incubator.
• After the incubation period, aspirate the entire supernatant from the round bottom tube. Use a pasteur pipet, with the tip placed just about the pellet surface.
• Pool supernatant from the two round bottom tubes into a single 15 mL conical centrifuge tube. Centrifuge the tube at 1600 rpm for 7 minutes.
• Aspirate the supernatant from the top of the meniscus using a pasteur pipet.
• Resuspend the pellet in a volume of 0.5 mL HTF using a 1 mL sterile pipet. Record the final volume.

Note: Sterile techniques should be used throughout specimen processing. When examining the specimen, it is important to pay particular attention to extraneous round cells, debris, and bacteria that may be present.

The medium used in this technique provides the sperm with a nourishing environment and attracts the sperm cells. The spermatozoa leave the pellet and swim into the medium. The sperm cells furthest away from the pellet are retrieved since they have the greatest probability of being motile and morphologically normal.

The swim-up method has been modified for oligozoospermic men. This modified method is called direct swim-up and involves swim-up from semen rather than swim-up from the cell pellet. Direct swim-up is the simplest and fastest method for separating sperm by migration. Round-bottom tubes are used for direct swim-up to maximize the surface area between the semen and medium. Multiple tubes with small volumes can be used to further increase this interface area and increase the number of motile sperm retrieved. With this particular procedure, incubation is performed at 34.5°C, which has been reported to result in higher motility than incubation at 37°C.

The swim-up method is simple and relatively inexpensive. Yet, it has some disadvantages:
• Centrifugation, which is performed to create a cell pellet before conventional swim-up, has been shown to generate ROS.
• The amount of motile spermatozoa retrieved is relatively low
• Only 5–10 percent of the sperm cells subjected to swim-up are retrieved
• When a concentrated cell pellet is used, some motile spermatozoa may be trapped in the middle of the pellet and thus may not travel as far as the sperm cells at the edges of the pellet.

Migration-Sedimentation

Direct swim-up from semen is used for sperm samples with average or good motility. On the other hand, migration-sedimentation is usually used for samples with low motility.
Migration-sedimentation uses the swim-up technique but also relies on the natural settling of spermatozoa due to gravity. Sperm cells migrate from a ring-shaped well into a culture medium above and then settle through the central hole of the ring. Special tubes called Tea-Jondet tubes are used for migration-sedimentation. The advantage of this technique is that it is a gentle method, and thus the amount of ROS produced is not very significant. On the other hand, the special tubes that are needed are relatively expensive.

Swim-Down

This technique relies on the natural movement of spermatozoa. A discontinuous bovine serum albumin medium is prepared. This medium becomes progressively less concentrated moving from top to bottom. The semen sample is placed onto the top of the medium, and the tube is incubated at 37°C for one hour. During migration, the most motile sperm move downward into the gradient.

Density Gradient Centrifugation

Density gradient centrifugation separates sperm cells based on their density. Thus, at the end of centrifugation, each spermatozoon is located at the gradient level that matches its density. Morphologically normal and abnormal spermatozoa have different densities. A mature morphologically normal spermatozoon has a density of at least 1.10 g/mL whereas an immature and morphologically abnormal spermatozoon has a density between 1.06 and 1.09 g/mL. As a result, the resulting interphases between seminal plasma and 45 percent, 45 percent and 90 percent containing the leukocytes, cell debris and morphologically abnormal sperm with poor motility, are discarded. The highly motile, morphologically normal, viable spermatozoa form a pellet at the bottom of the tube. Centrifugal force and time should be kept at the lowest possible values (<300 g) in order to minimize the production of ROS by leukocytes and non-viable sperm cells. Also, non-viable sperm cells and debris should be separated from viable sperm cells as soon as possible to minimize oxidative damage. Density gradients can either be continuous or discontinuous. Density gradually increases from the top of a continuous gradient to its bottom. There are clear boundaries between layers of discontinuous gradients. The latter gradient is formed when a number of layers of decreasing density are placed on top of each other. Double density gradients comprise the commonly used sperm preparation protocol for ART.

Components of the density gradient sperm separation procedure include a colloidal suspension of silica particles stabilized with covalently bonded hydrophilic silane supplied in HEPES. There are two gradients: a lower phase (90%) and an upper phase (45%). Sperm washing medium (Modified HTF with 5.0 mg/mL human albumin) is used to wash and resuspend the final pellet (Fig. 29.2).

Below are some of the main steps of the process:

- Place all components of the upper and lower phase and semen samples in an incubator at 37°C for 20 minutes.
- Transfer 2 mL of the lower phase into a sterile conical-bottom, disposable centrifuge tube.
- Layer 2 mL of the upper phase on top of the lower phase using a transfer pipet. Slowly dispense the upper phase lifting the pipet up the side of the tube as the level of the

![Figure 29.2: Density gradient centrifugation. The lower and upper gradients are carefully layered and the seminal ejaculate layered on the top. The sample is centrifuged at 1600 rpm for 20 minutes. Clear seminal plasma is retained on the uppermost part of the gradient followed by a clear separation of white blood cells, debris and other cells. The immature, abnormal sperm are seen along the gradient based on their density and motility. Highly motile normal sperm move actively to the bottom of the gradient and collected as a pellet](image-url)
upper phase rises. A distinct line separating the two layers will be observed. This two-layer gradient is stable for up to two hours.

- Measure semen volume to be loaded using a sterile 2 mL pipet. Remove a drop of semen using sterile technique for count, percent motility and presence of round cells.
- Gently place up to 3 mL of liquefied semen onto the upper phase (leaving approximately 0.1 mL in original container for a prewash analysis). If volume is greater than 3 mL, it may be necessary to split the specimen into two tubes before processing.
- Centrifuge for 20 minutes at 1600 rpm.

Note: Occasionally, samples that do not liquefy properly and remain too viscous to pass through the gradient will be encountered. Increasing the centrifugal force up to but no more than 600Xg will aid in separating the sperm in these cases.

- Using a transfer pipet, add 2 mL of HTF and resuspend pellet. Mix gently with pipet until sperm pellet is in suspension.
- Centrifuge for 7 minutes at 1600 rpm.
- Again, remove supernatant from the centrifuge tube using a transfer pipet down to the pellet.
- Resuspend the final pellet in a volume of 0.5 mL using a 1 mL sterile pipet with HTF. Record the final volume.
- The advantages and disadvantages of density gradient centrifugation are listed in Table 29.1.

### Tips to Maximize the Sperm Yield

- It is important to make sure that all components of the gradient and sperm wash medium are at room to body temperature before use. This will protect spermatozoa from "cold shock." In addition, any condensation on the media bottles will disappear, which aids in the visual detection of contamination. Any bottle whose contents appear in any way cloudy or hazy should not be used.
- Do not use the same pipet in more than one bottle of media.
- Prolonged exposure to a 5 percent CO₂ environment will alter the pH of these products, which may in turn affect their nature and performance.
- Highly viscous semen usually should be treated with 5 mg of trypsin, dissolved in 1.0 mL of sperm washing media and added to the ejaculate 5 minutes before loading on the upper gradient. This will increase the motile sperm yield without causing any measurable damage to the motile sperm.
- Avoid overloading the gradient as it causes a phenomenon called ‘rafting’. Rafting is the aggregation of desirable as well as undesirable components of the semen that will be present in the postcentrifugation pellet.
- Use the gradient within one hour after creating it—eventually the two phases over time blend into each other and a sharp interface will not exist.
- Percoll™, a colloidal suspension of silica particles coated with polyvinylpyrrolidone, was widely used by ART laboratories until it was withdrawn from the market for clinical use. Nowadays, media containing silane-coated silica particles are commonly used. Isolate™ (Irvine Scientific, Santa Ana, CA), IxaPrep™, Sperm preparation medium™ and Suprasperm™ (Origio, Medicult, Copenhagen, Denmark), SpermGrad™ (Vitrolife, San Diego, CA), SilSelect™ (Ferti Pro NV, Beerem, Belgium) and PureSperm™ (NidaCon Laboratories AB, Gothenburg, Sweden) are commonly used.

#### MAGNETIC ACTIVATED CELL SORTING

Magnetic activated cell sorting (MACS) separates apoptotic spermatozoa from non-apoptotic spermatozoa. During apoptosis (programmed cell death), phosphatidyl serine residues are translocated from the inner membrane of the spermatozoa to the outside. Annexin V has a strong affinity for phosphatidyl serine but cannot pass through the intact sperm membrane. Colloidal superparamagnetic beads (~50 nm in diameter) are conjugated to highly specific antibodies to annexin V and used to separate dead and apoptotic spermatozoa by MACS. Annexin V binding to spermatozoa indicates compromised sperm membrane integrity.

A 100 µL sperm sample is mixed with 100 µL of MACS microbeads and incubated at room temperature for 15 minutes. The

### Table 29.1: Advantages and disadvantages of density gradient centrifugation

<table>
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<tr>
<th>Advantages of density gradient centrifugation</th>
<th>Disadvantages of density gradient centrifugation</th>
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<tbody>
<tr>
<td>Density gradient centrifugation requires maximally a thirty-minute centrifugation. It takes less time than the swim-up technique which requires one-hour incubation.</td>
<td>Production of good interphases between layers can take some time.</td>
</tr>
<tr>
<td>Density gradient centrifugation is relatively easy to perform under sterile conditions.</td>
<td>There is a risk of contamination with endotoxins.</td>
</tr>
<tr>
<td>Spermatozoa from oligozoospermic semen can be effectively separated with density gradient centrifugation.⁹</td>
<td>Some scientists have claimed that density gradient centrifugation negatively affects sperm DNA integrity. For instance, Zini et al.¹⁰ found that spermatozoa recovered after density gradient centrifugation possess lower DNA integrity than spermatozoa recovered after swim-up.</td>
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<tr>
<td>Density gradient centrifugation eliminates the majority of leukocytes in the ejaculate.</td>
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mixture is loaded on top of the separation column which is placed in the magnetic field [0.5 Tesla (T) between the poles of the magnet and 1.5 T within the iron globes of the column]; 1 Tesla = 10,000 gauss (Figs 29.3A and B). The column is rinsed with buffer. All the unlabeled (annexin V-negative) non-apoptotic spermatozoa pass through the column (Fig. 29.3C). The annexin V-positive (apoptotic) fraction is retained in the column. The column is removed from the magnetic field, and annexin V-positive fraction is eluted using the annexin V-binding buffer.5

Spermatozoa prepared by density gradient centrifugation followed by MACS have a higher percentage of motility, a higher percentage viability, and a lower expression of apoptotic markers than spermatozoa prepared by density gradient centrifugation alone.14 Annexin V-negative spermatozoa have a higher motility, lower caspase activation, lower membrane mitochondrial potential disruption, lower amounts of DNA damage, and higher oocyte penetration capacity than annexin V-positive spermatozoa.15 Magnetic activated cell sorting improves the acrosome reaction in couples with unexplained fertility.16 Annexin V-negative sperm cells show significantly higher motility and survival rates following cryopreservation than annexin V-positive sperm cells.17 Dirican et al.18 reported that spermatozoa selected by MACS were associated with higher cleavage and pregnancy rates than spermatozoa selected by density gradient centrifugation in oligoasthenozoospermic cases.

The advantages and disadvantages of magnetic activated cell sorting are outlined in Table 29.2.

**GLASS WOOL FILTRATION**

Glass wool filtration separates motile sperm cells from other contents of semen by filtration through densely packed glass wool fibers.9 The filtration separates out immotile sperm cells, leukocytes and debris. Henkel et al.19 reported that glass wool filtration eliminates 87.5 percent of leukocytes in semen. This is important since leukocytes are the main source of ROS in semen. After filtration, the semen is centrifuged to remove seminal plasma from viable sperm cells. The fact that centrifugation is carried out without leukocytes and non-viable spermatozoa are important since the absence of these populations limits the production of ROS. The advantages and disadvantages of this method are shown in Table 29.3.

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**Figures 29.3A to C:** Magnetic activated cell sorting: (A) The Octet magnetic collection device can be used for loading up to a maximum of 8 samples. The tubes are placed between each open slot surrounded by the magnetic field. (B) The apoptotic and the nonapoptotic cells are labeled with the annexin V antibody beads (magnetic). These attach to the outer surface of the sperm that are apoptotic. Annexin V beads (magnetic) do not bind to the sperm that are non apoptotic and have intact membranes. (C) Apoptotic sperm with annexin V beads (magnetic) do not bind to the sperm that are non apoptotic and have intact membranes. The apoptotic and the nonapoptotic cells are labeled with the annexin V antibody beads (magnetic). These attach to the outer surface of the sperm that are apoptotic. Annexin V beads (magnetic) do not bind to the sperm that are non apoptotic and have intact membranes. (C) Apoptotic sperm with annexin V beads (magnetic) are retained in the column while the nonapoptotic sperm are eluted out and collected in a tube below the collection device.
### REDUCTION OF SEMEN VISCOSITY

Human semen normally liquefies within 5-20 minutes after ejaculation. However, some ejaculates fail to liquefy and some are viscous by nature. Semen viscosity is a problem since it can reduce sperm motility. To reduce viscosity, the semen can be mixed with a medium. Liquefaction achieved by this method might not be adequate for highly viscous samples. Forcing the viscous semen through a needle with a narrow gauge is another option. However, this technique damages the sperm cells. A commonly used viscosity treatment system involves enzymatic liquefaction using trypsin (5 mg). These can also be obtained prepackaged in 5 mg vials (VTS; Vitrolife, San Diego, CA). If the sample fails to completely liquefy following 20 minutes of incubation at 37°C, trypsin is added directly to the semen specimen. The specimen is then swirled and incubated for an additional 10 minutes. This results in complete liquefaction of the sample.

### WHEN TO USE A PARTICULAR SPERM PREPARATION TECHNIQUE

The choice of sperm preparation method depends on the characteristics of the semen sample. When sperm parameters such as concentration and motility are within the normal ranges, the direct swim-up technique is preferred. For significantly oligozoospermic, teratozoospermic and asthenozoospermic samples, density gradient centrifugation is preferred since density gradient centrifugation leads to a higher recovery of motile sperm cells than the swim-up technique. Also, density gradient centrifugation can be modified to address the issues of each individual specimen, and it is the method of choice for sperm preparation in the majority of ART and andrology laboratories. Glass wool filtration is also effective for the separation of sperm cells from semen with suboptimal parameters.

### SPERM SELECTION BASED ON MEMBRANE CHARGE

Mature sperm possess an electric charge of -16 to -20 mV called zeta potential (electrokinetic potential). In this method, washed sperm (0.1 mL) is pipetted into the tube and diluted with 5 mL of serum-free HEPES-HTF medium. The positive charged (+2 up to +4 kV at 1 inch) on the tube is maintained by placing the tube inside a latex glove up to the cap and by grasping the cap, the tube is rotated two or three turns and rapidly pulled out. The electrostatic charge is verified using electrostatic voltimeters.
To allow adherence of the charged sperm to the wall of the centrifuge tube, each tube is kept at room temperature (22°C) for 1 minute. The tube is held by the cap to avoid grounding the tube. After 1 minute, the tube(s) are centrifuged at 300 g for 5 minutes and each tube is simply inverted to remove the nonadhering sperm and other cell types and excess liquid is blotted off at the mouth of each tube. To detach the charged adhering sperm, serum supplemented HEPES-HTF medium (0.2 mL) is pipetted into each tube allowing the medium to trickle down the side of the tube. The collected medium at the bottom of each tube is repipetted and used to rinse the wall of the same tube several times to increase the number of recovered sperm.

- The zeta method can be carried out immediately as sperm cells lose the charge with the onset of capacitation.
- To maximize the charge, a new centrifuge tube must be used.
- The use of culture medium with a higher percentage of serum or discharging the tube may improve recovery of detached sperm in low sperm concentration situations.

The zeta method of sperm processing is simple to perform, inexpensive, and permits rapid recovery of sperm with improved sperm parameters, particularly strict normal morphology, DNA normal integrity, and aniline blue maturity. These parameters are associated with improved fertilization and pregnancy after intracytoplasmic sperm injection (ICSI). Sperm progressive motility and hyperactivation (predictive of successful pregnancies after ART procedures) is improved in this method, suggesting that the brief exposure to the serum-free condition or the manipulation from the attaching—detaching process triggers sperm metabolic activity without causing premature acrosome reactions. To maximize the isolation of motile sperm, it is recommended that the sperm are preprocessed on density gradient.

The zeta method does not require the use of expensive electrophoresis equipment, Tris buffers, extreme pH environments, and UV irradiation. A limitation of the zeta method is the low recovery of processed sperm, and thereby limiting its usefulness especially in oligozoospermic patients. This method is not be useful for testicular or epididymal sperm aspirates as they lack sufficient net electrical charge on the sperm membrane surface.

## SPERM PREPARATION FOR ART

Density gradient centrifugation is usually used to prepare sperm cells for standard in vitro fertilization (IVF). If the semen sample is poor in terms of motility and concentration, intracytoplasmic sperm injection (ICSI) should be considered.13

The sperm sample can be prepared for ICSI with density gradient centrifugation or swim-up. Oligozoospermic samples can be prepared using the swim-up technique if there are some viable spermatozoa with forward motility.13 If needed, sperm cells in oligozoospermic samples can be concentrated with a wash and re-suspension. These concentrated samples can be used directly or be subjected to density gradient centrifugation or swim-up before being used. Density gradient centrifugation is a better option than swim-up for significantly oligozoospermic and significantly asthenozoospermic samples as well as for samples with high quantities of debris.13 The hypo-osmotic swelling test is also an efficient method for selecting sperm for ICSI.20

### Preparation of Epididymal and Testicular Spermatozoa

In case of epididymal obstruction or complete azoospermia, spermatozoa can be obtained from the epididymis or the testicular tissue, both require special preparation. Usually, large numbers of sperm cells can be collected from the epididymis.4 Sperm samples obtained from the epididymis do not contain a significant amount of non-germ cells such as red blood cells. If sufficient numbers of epididymal sperm cells are collected, density gradient centrifugation can be used to prepare the spermatozoa for ART. On the other hand, the simple wash technique will be used if the number of spermatozoa aspirated is low.4

Spermatozoa can be retrieved from the testes by open biopsy or by percutaneous needle biopsy.4 Testicular samples contain large numbers of non-germ cells such as red blood cells. Spermatozoa need to be separated from these non-germ cells. Also, the elongated spermatids, which are bound to the seminiferous tubules, must be freed. Sperm cells collected from the testes are used in ICSI because low numbers of spermatozoa with poor motility are generally aspirated. Pentoxifylline is occasionally used to increase the motility of epididymal and testicular spermatozoa before ICSI.9

### Preparation of Assisted Ejaculation Samples

Direct penile vibratory stimulation or indirect rectal stimulation is used to retrieve semen from men who have disturbed ejaculation or who cannot ejaculate due to health issues such as spinal cord injury.4 Patients with spinal cord injury often have ejaculates with a high sperm concentration and low sperm motility.5 These ejaculates are also contaminated with red blood cells and white blood cells.4 Ejaculates obtained by electroejaculation are most effectively prepared with density gradient centrifugation.4

It has been reported that semen obtained by vibratory stimulation is of better quality than semen obtained by electroejaculation for men with spinal cord injuries.21

### Preparation of Retrograde Ejaculation Samples

Retrograde ejaculation occurs when semen is directed into the urinary bladder during ejaculation. If there is an inadequate number of spermatozoa in the ejaculate, sperm cells in the urine need to be retrieved. At the laboratory, the patient is first asked to urinate without entirely emptying his bladder.4 Then, he is asked to ejaculate and urinate again into another specimen cup containing 5–6 mL of culture medium, which alkalinizes the urine.4 The urine sample volume is noted and analyzed after centrifugation.4 The concentrated retrograde specimen and the antegrade specimen are usually prepared with density gradient centrifugation.4

The Liverpool solution given orally to alkalinize urine have been recently described and was demonstrated to be associated with improved sperm motility.22
CONCLUSION

In summary, a number of sperm preparation methods are available to process sperm for use in ART. Each infertile couple must be carefully examined to determine the best sperm preparation method. Future research should seek to improve the efficacy and the safety of the sperm preparation techniques.

REFERENCES