In-vitro Fertilization Procedural Manual

IRVINE SCIENTIFIC
2511 Daimler Street
Santa Ana, California 92705
Tel: (800) 437-5706
(949) 261-7800
Fax: (949) 261-6522
www.irvinesci.com

IrvineScientific®
Grow With Us

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Irvine Scientific Profile

MISSION STATEMENT

Irvine Scientific's mission is to set the standard for EXCELLENCE through:

- Quality Products for the Cell Culture, Diagnostics, and Biopharmaceutical Markets.
- Quality Service with respect, courtesy and loyalty to our customers.
- Quality People dedicated to complete customer satisfaction.

COMPANY BACKGROUND

Irvine Scientific was established in May 1970 in Fountain Valley, California to produce serum products for the cell culture market. In the fall of 1973, Irvine Scientific reorganized and emerged as a respected entity for cell culture products on the West Coast. In the years to follow, we extended our products, services and operations to become multi-disciplined and international in scope. By the fall of 1977, Irvine Scientific had outgrown its Fountain Valley facility and relocated to our present site in Santa Ana. In the late 1980's we further expanded and acquired the serum-free cell culture and diagnostic product lines from Hana Biologics, Inc. Most recently, Irvine Scientific has emphasized its manufacturing capabilities for custom formulations and also custom packaging with Irvine Scientific's MEDIA MANAGER line of products.

In August 1987, a Japanese-based firm, Japan Energy Company (JEC, formerly named Nippon Mining Company) acquired an 80% interest in Irvine Scientific. JEC brought new capital to Irvine Scientific and a stated goal to become a leader in the expanding bio-technical field. JEC became the sole shareholder in July 1996. Their solid support has enabled us to provide our products worldwide to manufacturers of diagnostic and pharmaceutical products, as well as the cytogenetic, reproductive and clinical laboratories.

Today, Irvine Scientific is a diversified company. We sell products in a variety of markets, including media and other related cell culture systems used in a wide variety of applications, including specialty bulk sera products (as components for the Diagnostics market), media for the Cytogenetics laboratory, as well as a complete product line for the Reproductive laboratory.

Irvine Scientific recently expanded our manufacturing facilities. In 2003 we received ISO 13485:2003 certification for our manufacturing process.

Irvine Scientific's efforts are directed toward continued growth of our products and the markets we serve. Our full line of products may be viewed on our website **www.irvinesci.com**.

ORDERING INFORMATION

Ordering Options:

Online Ordering:

- You may access our online ordering by visiting us at www.irvinesci.com.
- Orders may be placed 24 hrs a day.
- Orders may be tracked.
- Check our website for promotions and specials.
- Access contact numbers and customer support.
- Obtain copies of all product inserts and specifications.

Customer Support Line

Domestic Orders
Telephone, fax, and mail orders are accepted. Please include a purchase order number and your telephone number on all orders. Confirming purchase orders are NOT required on phone orders. If a confirming purchase order must be sent, be sure it is clearly marked - "CONFIRMATION, DO NOT DUPLICATE".
Place orders for items in this website with:
IRVINE SCIENTIFIC
Attention: Customer Service Department
2511 Daimler Street
Santa Ana, CA 92705-5588 U.S.A.
Customer Service Hours: 7:00 a.m. to 5:00 p.m. (Pacific Time)
Customer Service: 800/577-6097 (Direct Line)
Technical Service: 800/437-5706
Corporate Offices: 800/437-5706
Telephone: 949/261-7800
Facsimile: 949/261-6522 (24 hours)

International Orders
All international orders must be pre-paid prior to shipment. Payment must be made in U.S. Funds drawn on a U.S. Bank or by direct wire transfer to:

MELLON BANK, Pittsburgh, PA
Reference ABA #043000261

and indicate:
for credit to MERRILL LYNCH
Account #1011730

for further credit to Irvine Scientific Sales Co., Inc.
Account #224-04244. Swift MELNUS 3P

Please add $20 to your wire transfer amount to cover bank fees. An additional export documentation fee will be added to orders shipped outside of the United States.

GENERAL INFORMATION

Corporate Offices
Irvine Scientific
2511 Daimler Street
Santa Ana, CA 92705-5588
Phone: 800.437.5706

Customer Support
Customer Service: 800.577.6097
Technical Service: 800.437.5706
Telephone: 949.261.7800
Facsimile: 949.261.6522

Lot Reservation Policy
Samples will be provided at no charge for the purpose of testing a particular lot. Reserved lots will be held for three weeks from the date the sample is shipped. Upon completion of your testing, a purchase order for the amount reserved is required to secure product. The reserve will be automatically cancelled at the end of the three weeks unless otherwise notified.

Pricing and Terms
Irvine Scientific reserves the right to change prices without notice. Any price reduction will automatically apply to your invoice. To obtain a copy of our current price list, please fill out our online Catalogue Request Form. Invoices are due in net 30 days in U.S. dollars. A finance charge of 1.5 percent per month (annual percentage rate of 18 percent) will be charged to past due accounts.

Special Quotations
Quantity discounts are available on Standing Orders. Products can be bulk packaged in custom sized containers at specially quoted prices. Quotations can be obtained by contacting your Territory Manager.

- Shipping
- Returns
- Cancellations

Facility Tours
Irvine Scientific would like to invite our customers to visit our manufacturing facilities. We are located in Santa Ana, California, approximately one hour south of Los Angeles. If you would like to visit our site, please contact your sales representative and they can arrange a tour for you.

REDUCE • RE-USE • RECYCLE

Irvine Scientific is concerned with the environmental impact of our packaging materials and we encourage our customers to dispose of these materials responsibly. Effective re-use and recycling of these components depends on you. Please use the following guidelines to help us protect our environment for future generations:

Packaging Material	Re-use	Recycle
Glass-Type 1 Borosilicate	Not recommended for re-use	Not currently recyclable
Plastic-PETG Plastic-PET	Not recommended for re-use	Disinfected bottles can be recycled with consumer PET (soda bottles) Incineration (results in CO_2 + H_20)
Plastic-HDPE	Not recommended for re-use	Disinfected bottles can be recycled with consumer HDPE
Bubble wrap-PE	Re-use as packaging material	Contact Recycle Hot Line 800/944-8448 for the nearest collection site
Polystyrene Foam (EPS) CFC Free	Deliver to commercial packaging outlets (i.e. Mail Boxes, Etc.) for re-use	Contact Recycle Hot Line 800/944-8448 for the nearest collection site

Irvine Scientific's Global Presence

ARGENTINA AUSTRALIA AUSTRIA BAHRAIN BELGIUM BOLIVIA BRAZIL CANADA CHILE CHINA COLOMBIA COSTA RICA CYPRUS CZECH REPUBLIC DENMARK DOMINICAN REPUBLIC ECUADOR EGYPT FINLAND FRANCE GERMANY GREECE GUAM GUATEMALA GUYANA HONDURAS HONG KONG HUNGARY INDIA INDONESIA IRELAND ISRAEL ITALY JAMAICA JAPAN JORDAN KOREA KUWAIT LEBANON LUXEMBOURG MALAYSIA MEXICO NETHERLANDS NEW ZEALAND NIGERIA NORWAY PANAMA PARAGUAY PERU PHILIPPINES POLAND PORTUGAL PUERTO RICO RUSSIA SAUDI ARABIA SINGAPORE SOUTH AFRICA SPAIN SWEDEN SWITZERLAND TAIWAN THAILAND TURKEY U.A.E. UKRAINE UNITED KINGDOM UNITED STATES URUGUAY VENEZUELA

For the local distributor of Irvine Scientific products in your country,
please contact our International Customer Service Department.
Phone: 001-949-261-7800
Fax: 001-949-261-6522
www.irvinesci.com

Quality Control Program

Quality Control Program

QUALITY ASSURANCE:

Products manufactured by Irvine Scientific are classified as medical devices by the Food and Drug Administration (FDA). They are produced in compliance with the FDA's current Good Manufacturing Practices (cGMP's) and ISO 13485:2003 regulations utilizing detailed production protocols and quality control test procedures. Irvine Scientific is licensed by both Federal and State agencies and is inspected regularly for compliance. Irvine Scientific is certified to the ISO 13485:2003 International Quality Standard and is certified to the European Norm 46001 by the National Standards Authority of Ireland.

MANUFACTURING

Liquid Media

Liquid media for all applications is manufactured, filtered and bottled on site in our aseptic class 100 environment. All steps in the process undergo rigorous testing to validate sterility, repeatability and reliability. Approved filtration and filling protocols include testing for filter integrity, sterility, and product quality. Filter integrity is assessed using the bubble point test to determine the presence of holes in the filling system and is performed with every fill. Sterility is achieved by passing the media through a series of filters, the final being 0.1 µ, to achieve a Sterility Assurance Level (SAL) of 10-3. An SAL of 10-3 allows for no more than one bottle in one thousand to fail sterility testing. This level of SAL is determined by a filling validation test. A test run of three thousand bottles of media are tested using Trypticase Soy Broth. Samples are taken from each of the 3000 bottles and tested for microorganism growth. If more than three samples show the presence of any organism capable of growing in TSB, the filling process must be re-evaluated and re-validated internally.

Once a lot of media has been manufactured, the finished product testing is performed including all of the standard quality control procedures required to complete the Certificate of Analysis that accompanies every lot of media manufactured in Irvine Scientific's facility. All final product testing is standardized and performed in accordance with written Standard Operating Procedures.

Powdered Media

Bulk powered media are prepared in accordance with the corresponding original formula for the liquid when feasible. Anhydrous salts are substituted on an equimolar basis. The final powdered formulation is packed in screw-capped containers and should be stored in controlled conditions according to product labeling. Each lot of powdered media is checked for particle size, uniformity, and complete solubility. Irvine Scientific pays particular attention to maintaining rigorous quality standards to ensure thorough distribution of trace compounds in powdered media.

Chemicals

All raw material chemicals, where possible, conform to Multi-compendial standars, United States Pharmacopeia (USP) or National Formulary (NF) standards. Certificates of Analysis are obtained from manufactures or vendors for all chemicals used. Once the chemicals arrive at Irvine Scientific, samples of each lot are tested for endotoxin, identity (by Fourier Transform Infrared Spectroscopy), efficacy and toxicity as appropriate.

Human Source Raw Materials

Human Source Raw Materials used by Irvine Scientific in the manufacturing of reproductive media are of therapeutic grade and obtained from CBER licensed facilities. All human source material has been tested at the donor level with FDA licensed kits, and found to be nonreactive for the antibodies to Hepatitis B Surface Antigen (HbsAg), antibodies to Hepatitis C (HCV) and antibodies to Human Immunodeficiency Virus (HIV 1&2). Source material is also screened by questionnaire at the donor level for risk factors associated with CJD (Creutzfeldt-Jakob Disease).

Quality Control Program

Water

The water system is routinely monitored for microbial levels, endotoxin, conductivity, pH and TOC (Total Organic Carbon). The finished product water meets USP test requirements for "Water for Injection".

QUALITY CONTROL TESTS

Sterility Testing

The Sterility test is considered a biological test. Sterility testing of all media is performed in accordance with the Code of Federal Regulations (CFR) Title 21, Part 610.12 or current USP <71> recommendations. For sterility testing, random samples of every lot are filtered through 0.45 µ membranes. The membranes are incubated at 30-35°C in Fluid Thioglycollate and at 20-25°C in Soybean-Casein Digest media and observed regularly for 14 days. Any lot which shows growth over the course of the 14 days is retested. If the sample is positive a second time, the entire lot is rejected and will not be released for sale.

Mouse Embryo Testing

Mouse embryo testing, also referred to as the Mouse Embryo Assay or MEA, is performed on gamete and embryo culture media as a functional test. The test utilizes fresh, one cell mouse embryos. Aliquots of every lot are used to grow one cell mouse embryos to the blastocyst stage. Test results indicate the percentage of mouse embryos developing to fully expanded blastocyst after 96 hours in culture. Other biocompatibility testing (such as sperm survival, sperm motility recovery, or blastocyst recovery assay) is routinely performed on products where appropriate. Any lot which fails one of these tests is retested. If it again fails the lot of media is not released for sale.

Endotoxin Testing

Endotoxin is measured using the LAL gel clot or kinetic chromogenic methodology. Endotoxin testing is considered a biological test. An aliquot of media is taken at the time of manufacture for Endotoxin testing. Any products containing protein are heat treated in boiling water for 2 minutes to denature the enzymes that interfere with the enzyme cascade reaction for endotoxin detection and then tested. Any sample that is positive for endotoxin is retested. If the sample fails again, the lot of media is not released for sale.

pH Testing

The pH of each lot of media is measured at a 1X concentration in accordance with finished product specifications. Due to the constraints imposed during manufacture, 10X and powdered media may require some adjustment when diluted or dissolved for filtration. The pH readings reported on the Certificate of Analysis are indicative of the pH of the product immediately before capping and sealing and reflect the pH of the product at room temperature and under room air conditions. The user is reminded that a medium in free exchange with CO_2 in an incubator may establish a final pH different from the initial pH. Each user should determine pH of the media in their own laboratory. With pH sensitive cells, the user should always check aliquots of the final media with a device specifically designed to measure pH, such as a pH meter, as visual estimation can be deceptive. The pH of any sample is very sensitive to temperature and environmental CO_2 and should be tested in such a way as to control for these variables. Irvine Scientific recommends that the pH of a sample be tested in every incubator that is used for human embryo culture. Furthermore, we recommend that the CO_2 of each incubator be adjusted individually based on the detected pH of the sample tested in that incubator.

Osmolality

Osmolality of each lot of media is determined either by the freezing point or vapor pressure methodology. Testing is performed on precalibrated instrumentation using specific Standard Operating Procedures to ensure accuracy and consistency.

Certificate of Analysis (C of A)

A Certificate of Analysis reporting all of the QC results is provided with each shipment. Results are reported for each lot.

QUALITY CONTROL PROGRAM

Each ART laboratory should have detailed Quality Control, Quality Assurance, and Quality Improvement programs in place. A Quality Control program is a defined set of steps undertaken to ensure correct operation of laboratory equipment and processes. A Quality Assurance program is a set of steps taken to ensure that the Quality Control program is working as expected. Finally, Quality Improvement is a series of steps taken to review all aspects of the QC/QA program with the intent of improving outcomes. A QC/QA/QI program is a cooperative effort between the Laboratory Director and his/her staff. There are no nationally accepted or required steps that a laboratory must have in place in order to demonstrate that they have an appropriate AC/QA/QI program. Instead each lab may design their own program to examine those factors they believe to be most critical.

As an example of the steps involved in a QC/QA/QI program, a Laboratory Director may believe that controlling pH during all phases of the IVF program is an important step in improving pregnancy outcomes, or improving fertilization rates, or cleavage outcomes (any or all of these may be determined to be the appropriate indicator). Therefore, it would be up to the director to plan an overall program to assess and control pH. The first step to establishing would be to initially determine how to assess pH, which instrument to use to measure pH, when and where to measure it (in the incubators, in a Hoffman chamber, on the benchtop, etc.), and how often to measure it. The Director should then create a series of tools (ie., data sheets) that will help the lab staff collect the data that is necessary to assess the affect of pH. The act of measuring pH on a regular basis and in a defined manner would be the QC portion of the overall QI program.

The lab staff would begin measuring pH in the prescribed manner. At regular intervals, the Director would review the data collected and determine whether the appropriate data is being collected, and what, if anything should be changed. The Director also determines whether the pH determinations are being performed correctly, and if the pH is falling within the range that is expected or identified as being the range that the Director is interested in. If any observations are outside the expected range, then the instrumentation should be changed. For example, if the lab determines that the desired pH of culture media, in the incubator for 24 hours prior to testing, should be 7.20 – 7.25 and the measured pH as recorded on the data sheets is actually 7.35, then the pH of the media is out of range. If the CO_2 level on the incubator is turned up, the pH of the media should drop. The CO_2 levels can be adjusted until the pH falls within the accepted range. These kinds of adjustments can take several days to make. The next step would be to monitor whether the changes in the CO_2 level affected outcomes. Therefore, an outcome measure should also be determined at the start as part of the overall Quality Improvement program. Any measure that the lab chooses can be assessed. The measure should be meaningful, such as fertilization rate, early cleavage rate, number of eight cell embryos, or pregnancies to give a few examples.

In this example, setting the CO_2 level to drive the pH and monitoring pH levels on a routine basis would be the QC procedure. QA would require reviewing the QC records to ensure that pH is staying within the acceptable range and QI would be monitoring outcomes before and after to determine whether pH changes affected the outcome.

Any number of parameters can be examined in an effort to improve pregnancy rates. For example blastocyst conversion rates may be monitored in an effort to improve blastocyst culture. Embryo survival rates may be determined as an endpoint when working to improve embryo cryopreservation outcomes. Some QI programs can be conceived, executed and completed within a few days. Others may take months or even years to complete. A comprehensive program detailing multiples levels of outcomes will be necessary for a lab to continually evolve to meet the challenge of creating the best possible opportunity for a patient to achieve a pregnancy.

A lab should at the very least have a program in place to verify all aspect of incubator function including monitoring and verification of media temperature within the incubator, media pH, sterility, decontamination and cleaning, gas levels and tank replacement and humidity control. There should be a program to monitor ICSI fertilization and survival outcomes, conventional insemination fertilization rates, pregnancy outcomes, cryopreservation outcomes, and physician and scientist impact on transfer.

Embryology Protocols

OVERVIEW

General Considerations:

1. Media that is manufactured by Irvine Scientific for use in the Embryology Laboratory has been quality control tested for pH, osmolality, sterility, endotoxin, and mouse embryo development prior to its release. After receipt, each ART lab may choose to perform additional QC testing at their own discretion.

2. All media that is shipped cold should be stored in the refrigerator at the recommended temperatures until the discard date indicated on the label.

3. Media should be removed from the refrigerator and equilibrated at 37°C prior to use. The time and method required for equilibration will depend on the volume needed and the presence of bicarbonate buffer. If using a media that contains bicarbonate buffer, equilibrate for at least 4 hours in a CO_2 incubator with the lid of the container loose. If using media that does not contain a bicarbonate buffer equilibrate in either a warming block in room air or in an incubator, with the lid tightly closed, for enough time to warm the media prior to use.

4. For oocyte collection, Irvine Scientific recommends using mHTF (Cat. #90126, Modified Human Tubal Fluid), when working on the benchtop or HTF (Cat. #90125) if working in an IVF chamber.

5. Media should not be kept in the incubator for a prolonged period of time. Once media has been placed in the incubator it should be used within one to two days. If media has been in the incubator for more than this length of time it should be discarded and fresh media equilibrated just prior to use.

6. The pH of the bicarbonate buffered media after equilibration in a CO_2 incubator should be checked on a routine basis. Irvine Scientific recommends keeping the pH of all media that will be used to culture embryos within a range of 7.20 to 7.40. There is limited data and little agreement regarding the precise pH requirements of embryos in an *in-vitro* setting, however keeping the pH closer to 7.20 rather than 7.40 may be beneficial since there is some data to suggest that the intracellular pH of embryos is approximately 7.12. Measuring pH with the classic pH meter is difficult in an IVF setting as pH of embryo culture media changes rapidly following exposure to room air and room temperatures. Therefore, the most appropriate measurement of pH would be to determine the pH of the media in situ in the incubator. Since most laboratories do not have the kind of equipment necessary for this kind of analysis, it is recommended that pH be tested in a way that accounts for the temperature and CO_2 sensitivity of the media. One method that has been used to measure the pH of the media is a blood gas analyzer. Other methods are available, and any method is suitable as long as it takes into account temperature and pH drift.

7. Adjusting the pH of the media by the addition of acids or bases is not recommended. Instead, adjust pH by adjusting the CO_2 setting of the incubator.

8. The CO_2 levels in the incubator should be adjusted to attain the pH that is determined to be appropriate by the laboratory. Increasing the CO_2 percentage in the incubator will decrease the pH of the media, and decreasing the CO_2 levels will raise the pH. Some laboratories use reduced levels of oxygen for culture. The lower levels of O_2 in the incubator will not affect pH of the media. Each laboratory should determine the incubator setting that produces the desired pH for that incubator. It is important to understand that every incubator may be different. Therefore pH needs to be assessed in each incubator and each incubator should be individually adjusted.

9. Irvine Scientific manufactures its media to attain an osmolality of 282-290 Osmols.

10. Each laboratory needs to determine the best method to use, specify pass/fail values and rejection procedures.

11. Plasticware may be off-gassed by opening the sleeves of dishes or the packages of pipettes and allowing them to sit in room air for at least 24 hours prior to use.

12. The use of filter systems for room air and/or incubator gases is advocated, but may not be required. The decision to use filter systems is laboratory dependent and may be driven by laboratory location. Laboratories in busy metropolitan areas with high levels of pollution may choose to employ more filtration systems than laboratories located in suburban areas or those with lower levels of air pollutants.

13. Oocytes and embryos appear to be exquisitely sensitive to temperature fluctuations. Allowing the temperature of the media that contains oocytes and embryos to drift from 37°C may be detrimental to the developmental potential of the embryo. Therefore, once the oocytes leave the body, all handling and manipulations of the oocytes and embryos should be performed under tightly controlled conditions. Temperature measurements of media on the stages of microscopes, or in incubators should be monitored on a regular basis using a device that is routinely calibrated. Irvine Scientific recommends performing all micromanipulation procedures on a heated stage maintained at 37°C and keeping all dishes under CO_2 even when out of the main incubators to prevent pH drift, unless using a HEPES buffered media.

14. Culturing oocytes and embryos under oil may slow temperature, pH and osmolality fluctuations and provide a barrier against airborne dust and microbial agents.

15. Traditionally, many laboratories have pre-washed the oil used to overlay culture media. Irvine Scientific oil (Cat. # 9305, Oil for Embryo Culture) does not require washing or filtration. An aliquot of the oil may be kept in the incubator to allow it to be prewarmed before use, but is not required as a step before preparing the dishes for oocyte retrieval and embryo culture.

16. Generally it is recommended that all culture dishes and media to be used for a patient be prepared the day before and equilibrated overnight in an incubator that is gassed with CO_2. However, in the event of an emergency, a minimum of 2 to 3 hours of equilibration may suffice for media that requires CO_2 equilibration to attain the appropriate pH, especially when using microdrops of media under an oil overlay. Shorter times may be used if only temperature is of concern.

17. Media that is used for oocyte fertilization, and embryo culture should be supplemented with protein before use. Media may be purchased that is already supplemented (Cat. #9922, Complete HTF Medium with SSS; Cat. #9926, Complete P-1 with SSS; Cat. #9910, Complete P-1 with DSS; Cat. #90140, Complete ECM with SSS; Cat. #90142, Complete ECM with DSS). If the media does not have protein already added (Cat. #90125, HTF; Cat. #99242, P-1 Medium; Cat. #90138, ECM), Human Serum Albumin (Cat. #9988, HSA), Synthetic Serum Substitute (Cat. #99193, SSS) or Dextran Serum Substitute (Cat. #9301, DSS) should be added. Recommended levels are 5 mg/mL v/v of HSA, 10% v/v SSS or 10% v/v DSS as the additives for oocyte culture or sperm washing. Higher concentrations may be used for freezing specimens (up to 12% HSA, 20% SSS or 20% DSS). This higher concentration of protein may improve cryosurvival of specimens.

18. Once protein has been added to make a stock, the stock should be kept in the refrigerator and is stable for up to 4 weeks or until expiration date.

19. There is little agreement in the literature regarding universally recognized methods for performing IVF. Each laboratory should develop their own procedures and document those procedures in a Procedure Manual that is kept current and reviewed by the Labortory Director on an annual basis. The following is a general overview.

OOCYTE RETRIEVAL

General Considerations:

In most IVF programs, oocytes are collected from patients who have undergone ovarian stimulation with medications to increase the number of mature oocytes that can be collected and fertilized at one time. Occasionally patients may undergo a "natural cycle" that is relatively drug free or uses significantly reduced levels of medications. Oocyte retrieval is commonly performed by ultrasound guided visualization of the follicles within the ovary. Aspirated follicular fluid is collected in tubes and then examined under a microscope for the presence of oocytes. Oocytes are isolated from follicular fluid, rinsed and placed in culture for insemination.

Procedure:

1. Pre-warm all the surfaces and materials to 37°C that may come in contact with the oocytes.

2. All of the tubes, pipettes and dishes should be labeled with the patient's ID before proceeding with the collection. Color coding can be used to ensure patient's identification.

3. Dishes that will be used for oocytes culture should be prepared at least four hours in advance. However, there is some data showing that the equilibration rate of media to CO_2 is much faster than that to O_2. Therefore, when using a low oxygen culture environment and an oil overlay, dishes should be prepared the day before oocyte retrieval.

4. Pre-equilibrate flushing medium at 37° C in an incubator without CO_2 or in a CO_2 incubator with the tubes/bottle tightly capped if using a HEPES-buffered media (Cat. #90126, mHTF). If using an IVF Chamber with bicarbonate-buffered media (HTF, P-1, ECM, MultiBlast or the Complete Media series), the tubes/bottle should be loosely capped. The type of media selected will be determined by whether retrieved oocytes are placed in an IVF chamber or closed in room air. If the aspiration tubes are constantly maintained in a CO_2 environment then a media containing bicarbonate buffer may be used. If the tubes are transported to the laboratory and examined under room air then a HEPES buffered medium (mHTF) should be used for oocyte collection. The media used for oocyte retrieval does not require protein supplementation as follicular fluid contains significant levels of protein, but a Complete Medium containing protein may be use if determined to be advantageous by the laboratory. Heparin may be added to the media (2.5-10.0 Units/mL) that is used for follicular aspiration at the discretion of the lab.

5. Verify patient name prior to beginning an oocytes retrieval procedure.

6. Rinse the aspiration needle lumen and tubing with media (mHTF and protein) and discard the rinse media..

7. Examine follicular aspirates as quickly as possible following collection after pouring the aspirate into a pre-warmed collection dish. Identify and rinse oocytes.

8. Once the oocytes have been aspirated, located and rinsed, they should be transferred to media containing a bicarbonate buffer (HTF, P-1 or ECM).

9. Media should be supplemented with protein (5 mg/mL HAS, 10% v/v SSS or 10% v/v DSS). Complete Media which is already supplemented may be placed in a CO_2 incubator.

10. The chosen method to collect and fertilize oocytes may differ between labs. Options include, test tubes, multi-well dishes with or without an oil overlay or microdrops under oil. Each laboratory should decide which system works best in their hands.

11. Opinions vary regarding whether oocytes should be fertilized and cultured in groups or individually. Both methods have been shown to work effectively. The most commonly used methods appear to be:

a. Group culture. Group culture in 5 µL drops of media under 8-10 mL oil overlay in a dish.
b. Group culture. Group culture in 500-1000 µL of media under 300 µL of oil overlay in a multiwell dish.
c. Individual culture: Individual oocytes cultured in 10-20 µL of media under an oil overlay in a dish.

Therefore each lab should determine which system works best in their facility.

OOCYTE INSEMINATION/FERTILIZATION

General Considerations:

There is debate regarding the ideal time to inseminate oocytes after retrieval for optimal fertilization rates. Inseminating human oocytes efficiently and in a timely manner is fundamental to achieving successful fertilization. Some claim that oocytes require at least 2-5 hours in a CO_2 incubator at 37°C before performing conventional insemination. Other laboratories have documented good fertilization rates with ICSI of mature eggs when retrieval, cleaning and injection is performed immediately. Therefore, the timing of insemination is procedure dependent. Most laboratories no longer inseminate immature oocytes by ICSI since it may lead to abnormal cleavage rates and embryo fragmentation. Precise timing of the insemination should be determined and documented by each laboratory.

Notes:

1. Insemination dishes should be prepared in advance, preferably on the day prior to oocyte retrieval. If conventional insemination is used, the same dishes that were used at the conclusion of oocyte retrieval may be used. If performing ICSI, the oocytes should be stripped or cleaned to remove the surrounding cumulus cells and rinsed and maintained in fresh dishes with fresh media containing protein until injection. Following injection, oocytes should be rinsed at least three times and then placed into fresh dishes with fresh media for culture overnight.

2. It is critical to monitor and control temperature, pH and exposure to potential contaminants during all phases of insemination, regardless of insemination method.

3. However, attention should be paid to temperature, pH and exposure to potential contaminants in air during all phases of insemination, whether by conventional insemination or ICSI.

4. Following insemination by either conventional IVF or ICSI, oocytes should be placed in the incubator overnight.

FERTILIZATION CHECK

General Considerations:

If oocytes/embryos will be moved into new dishes following determination of fertilization on the morning after the retrieval, the dishes should be prepared on the afternoon of the day of oocyte retrieval and allowed to equilibrate overnight in the CO_2 incubator, particularly when using low O_2 concentrations and an oil overlay.

Procedure:

1. If conventional insemination was performed oocytes should be removed from their cumulus masses and examined for fertilization after 15-18 hours. Cumulus masses and the surrounding coronal cells may be removed by aspirating oocytes up and down into 26 gauge needles, manufactured denuding pipettes, or finely pulled pasteur pipettes. If ICSI was performed evidence of fertilization may be present as early as 12 hours post-ICSI.

2. The presence or absence of pronuclei should be assessed in the cleaned oocytes.

3. Oocytes with 2 pronuclei are normally fertilized. The two pronuclei should be touching each other and be approximately at the center of the embryo, where syngamy (the conjugation of the male and female gametes) occurs. Oocytes with single pronuclei, multiple pronuclei, or fragmented pronuclei are abnormally fertilized. Each laboratory should have a written policy documenting disposition policies for abnormally fertilized oocytes.

4. Following syngamy of the two pronuclei, the embyo begins to divide. Embryo cleavage into two cells can be observed as early as 22 hours after insemination, more commonly at 25-27 hours.

5. Normally fertilized pronuclear embryos may be cultured in groups or individually in P-1 Medium, ECM of the Complete Media series through Day 2 or 3 before transferring into the uterus or to ongoing culture. Media that is not purchased as the Complete formulation should have protein added to it (5 mg/mL HSA, 10% v/v SSS or 10% v/v DSS) at the time the dishes are prepared.

EMBRYO CULTURE AND ASSESSMENT

General Considerations:

Following fertilization determination, embryos may be transferred or cultured for several days depending on clinic policy.

Procedure:

1. Some programs choose to transfer embryos on Day 2, some on Day 3 and others delay transfer until the blastocyst stage. Each laboratory should determine their criteria for assessment of development and how to determine which embryos to transfer at a given time.

2. There appears to be no agreement among laboratories with regard to the best day to transfer or which embryos to pick for transfer. Numerous embryo evaluation systems have been reported in the literature and utilize a variety of parameters for assigning some type of value to the embryo's appearance. Such criteria may include combinations of one or more of the following: pronuclear alignment, the presence or absence of a cytoplasmic halo surrounding the pronuclei, early division at 25-27 hours, cell numbers and fragmentation rates early on Day 2, multinucleation during the multinuclear stage, cell numbers and fragmentation rates on Day 3, blastocele expansion, appearance of the inner cell mass, and trophectoderm cellularity and continuity.

3. If embryos are to be cultured to the blastocyst stage, transfer embryos from P-1 or ECM into MultiBlast Medium (Cat. #90139) supplemented with 5 mg/mL of HSA, 10% v/v SSS or 10% v/v DSS. Embryos may be transferred into this media on the afternoon of Day 2 or on the morning of Day 3. Culture dishes with MultiBlast Medium should be set up on the morning of Day 2, if the intent is to place the embryos into blastocyst culture on the afternoon of Day 2, or set up on the afternoon of Day 2 if embryos will be transferred into blastocyst media on the morning of Day 3.

4. Blastocyst culture may be performed using slightly higher pH values (pH 7.30) if so desired by the laboratory, however, the optimal *in-vitro* media pH value for each stage of embryo development has yet to be determined.

EMBRYO TRANSFER

General Considerations:

Following culture of the embryos they must be placed back into the uterus. A great deal has been written about transfer technique, and again there is little agreement as to the “proper” technique.

Procedure:

1. Most programs elect to use ultrasound guidance for transfers as observation of catheter placement with ultrasound appears to improve pregnancy rates. Patients preparing to have this procedure done will need to have full bladder.

2. Flexible, nontraumatic catheters such as the Wallace Classic Embryo Transfer Catheter (Cat. #ME1816 or ME1816N) or Wallace SureView Embryo Transfer Catheter (Cat. #CE118A or CE123A) are ideal for embryo transfers.

3. HTF with or without HEPES (Cat. #90126 or 90125) and without protein is suitable for washing out the vagina and the cervix. 10-20 mL of media should be warmed overnight by placing in an incubator in a container with the lid tightly capped if using a HEPES buffered media, or with the lid loose if using one that is buffered with bicarbonate.

4. All embryo transfer dishes should be prepared at least 4 hours prior to transfer, preferably on the afternoon of the day before transfer.

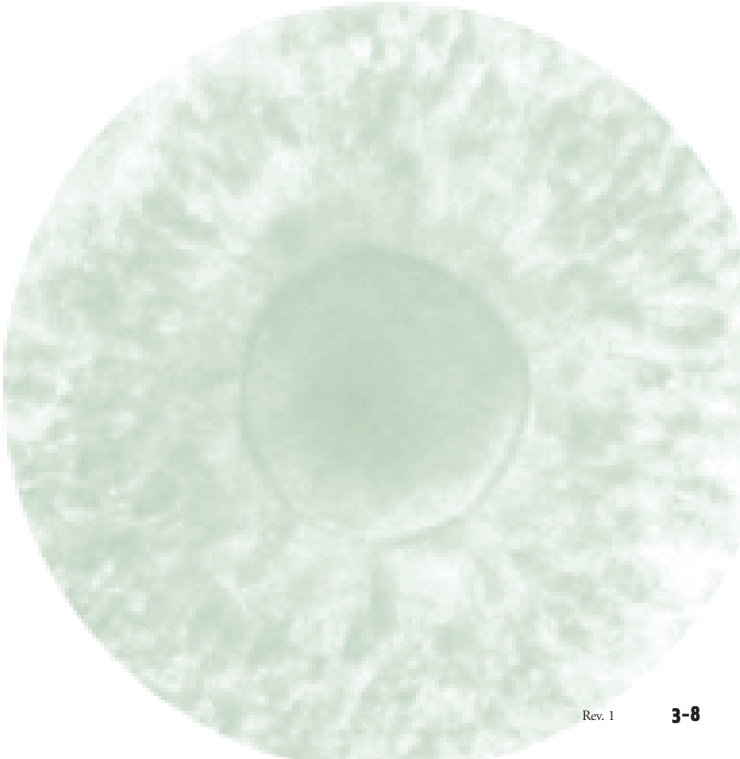
5. Transfers may be performed in Modified HTF (Cat. #90126) with protein (HSA, SSS or DSS) if embryos are loaded into the transfer catheter outside of an IVF Chamber, or in HTF (Cat. #90125) with protein (HSA, SSS or DSS) or in the Complete Media series if the catheter is loaded inside a chamber. A variety of protein concentrations have been reported in the literature, ranging from 10-50%. Each laboratory should decide which concentration is appropriate for its own use.

6. Once patients arrive in the room and in position, the cervix may be visualized by inserting a speculum into the vagina. The vagina may be cleaned using gauze or swabs, and Modified HTF. The cervix may be washed several times by flushing with media and a small catheter attached to a syringe until as much of the mucus is removed as possible.

7. A variety of devices have been utilized to load transfer catheters including AirTite syringes and gas-tight Hamilton syringes. Using standard 1 cc tuberculin syringes with the black rubber tip is not recommended as the rubber tips have been shown to be embryotoxic.

8. Catheter filling should be performed in an aseptic manner. Some programs prefer to load the catheter using a continuous column of media, other programs use small bubbles of air to isolate the embryos. Both methods appear to be satisfactory.

9. Following transfer the Embryologist should rinse the catheter well to verify that no embryos remain in the catheter. If embryos are retained, some reports in the literature suggest that the chance of achieving a pregnancy is diminished for the patients. Retained embryos at the time of transfer should be documented.



Andrology Protocols

OVERVIEW

Ejaculated semen is composed of two major components, spermatozoa and seminal plasma. In the body, during intercourse, only sperm, but not the seminal plasma component of the ejaculate, pass through the cervix to enter the uterus, and then move on up into the fallopian tubes where fertilization takes place. The seminal plasma portion of the ejaculate is filtered out and retained within the vagina and cervix.

Seminal plasma contains a number of small factors and compounds such as prostaglandins which are capable of initiating intense uterine contractility if introduced directly into the uterine cavity during the course of an IUI. Seminal plasma also contains factors that inhibit the final capacitation and activation of the sperm and appears to contain elements that may be embryotoxic. Therefore, washing of semen is required to remove seminal plasma and it's associated capacitation inhibiting factors, and to remove dead sperm and any contaminating white cells present in the specimen prior to introducing the sperm into the uterus during IUI or exposing oocytes to the sperm during IVF or ICSI.

Determining the method of sperm preparation prior to IUI or IVF depends on the quality and source of the specimen:

A. If the specimen is normal, in terms of sperm count and motility, or if it has significant amounts of seminal debris and dead sperm then the density gradient preparation is recommended.

B. A swim-up preparation may also be done if the ejaculated specimen is normal in terms of counts and motility and if the specimen is not highly viscous or if significant agglutination is not present. Recovery of sperm from swim-up preparations is highly variable and more time intensive. Hence, swim-up preparations tend to be used less often for IUI, and more often for IVF where high numbers of sperm are not required.

C. If the specimen has low sperm concentration and low sperm motility then a single or double wash should be the method of choice.

D. If sperm has been removed directly from the testes or the epididymus, then potentially there may be very few sperm present in the sample and a simple wash will be sufficient to remove contaminating elements but retain the majority of sperm.

Each laboratory should have a procedure to determine which method of sperm preparation is appropriate for a specific patient. This procedure detail the decision making process to identify the appropriate method for processing.

General Considerations:

1. The patient should be informed ahead of time to have 2-3 days of sexual abstinence before the day of collection. He should also be informed in writing about what he will be expected to do on the day of collection. This is particularly important for patients who speak a language other than that primarily spoken by the laboratory personnel.

2. All semen specimens should be accompanied by a test request form or have some form of documentation from the physician indicating the type of test/processing that is to be done for the patient. Appropriate documentation from the physician will save significant problems later if there is a discrepancy between what test the laboratory performs and what test the physician thought was going to be performed,

3. For legal and liability purposes it is important to have a procedure in place to document the chain of custody for handling specimens in order to track each step in the specimen's movements within the laboratory from receipt of the specimen to final disposition whether via IUI or IVF. All personnel handling the specimen from receipt to insemination should be identified in writing and sign off on each step as it progresses.

4. The patient should also be expected to present appropriate identification and there should be an established protocol for verifying the female partner's association with the sperm provider.

5. All semen specimens should be collected in sterile, non-toxic containers no more than one hour before processing and be kept at room temperature or 37°C (body temperature).

6. A defined identification protocol should be followed for each patient and his partner to ensure that there is no confusion among multiple laboratory personnel regarding which sperm to use for insemination purposes.

7. The specimen should be processed in a clean work place while using non-toxic gloves, sterile non-toxic, disposable tubes, needles and pipettes. All disposable items should be used one time and then discarded in an approved biohazard waste container.

8. Laboratory personnel processing the specimen should use aseptic techniques and all specimens should be treated as potentially infectious. Therefore, Universal Precautions should be followed during the course of all handling and processing.

9. The specimen should be allowed to liquefy for 20-30 minutes before processing. If the specimen has not liquefied after 30 minutes, mix the specimen 1:2 with Sperm Washing Medium (Cat. #9983) and gently pull the mixture up and down several times using a Pasteur pipette. If the specimen remains viscous, it may be forcibly dispersed by it in and out through a 19- or 22- gauge needle attached to a 10-20 cc syringe, or equivalent. If the sperm preparation will be used to inseminate oocytes during IVF, do not use syringes with black rubber plunger tips as solutions that have come in contact with these tips have been shown to be embryotoxic.

10. Sperm Washing Medium contains HEPES buffer. It should be pre-warmed in a 37°C water bath or in a CO_2 incubator, but not exposed to the CO_2 atmosphere; therefore store all aliquots of media to be used in tubes that are kept tightly capped.

11. Sperm Washing Medium and gradient layers should be stored in the refrigerator at 2 to 8°C and should not be used beyond the expiration date printed on the label.

METHODOLOGY

A. DENSITY GRADIENT CENTRIFUGATION METHODS:

General Considerations:

ISolate® is a colloidal suspension of silica particles stabilized with covalently bound hydrophilic silane in HEPES-buffered HTF. It is designed to separate the motile sperm from the debris and seminal plasma. Since mature live sperm with an intact cell membrane have a higher density than the dead sperm or any other debris in the semen, they migrate through the ISolate layers to the bottom of the tube in a pellet form.

ISolate is available in three formulations:

1. ISolate (Cat. #99264) is a kit which provides upper and lower layer concentrations that are ready to be used without further dilution. The ISolate concentration of the upper Layer is 50% while the concentration of the lower layer is 90%.
2. ISolate Stock Solution (Cat. #99275) is a 90% density gradient medium for use in a one-step procedure or for further dilution.
3. ISolate Concentrate (Cat. #99306) is the undiluted ISolate solution (~100%) which can be diluted to any final desired concentration.

Two-Layer Discontinuous Gradient Separation Procedure:

(This is the preferred method for preparing normozoospermic specimens.)

1. Bring all of the media to 37°C.

2. If using dilutions other than the standard 50% and 90% layers, prepare appropriately.

3. Using a sterile, disposable pipette, transfer 1.5-2.0 mL of the lower layer into a sterile, disposable 15.0 mL conical centrifuge tube.

4. Using a new sterile pipette, transfer an equal volume of the upper layer on top of the Lower layer. This is done by contacting the surface of the lower layer at the side of the tube with the tip of the pipette. Carefully dispense the upper layer by spiraling the pipette tip around the circumference of the tube in an upward motion as the level of the upper layer rises.

5. Mix the liquefied semen well with a 5.0 mL or 10.0 mL volumetric pipette.

6. Gently place 1.5-2.0 mL of the liquefied semen onto the upper layer of the gradient system using a new sterile pipette. If the total volume of semen is more than 2.0 mL then use the appropriate number of tubes and distribute the final semen volume accordingly. Each tube of upper and lower layers can be used to process 1-3 mL of semen. Additional volumes require additional tubes.

7. Centrifuge for 20 minutes at 300 x g.

8. At the completion of the spin, remove the layers by inserting a clean 5 mL pipette tip just below the surface of the liquid. Hold the tip in this position during aspiration. Aspirate the layers without disturbing the sperm pellet at the bottom of the tube until approximately 0.5 mL of lower layer remains. Even if a sperm pellet is not visible, this volume should contain sperm. If the sperm pellet occupies more than 0.5 mL at the bottom of the tube, aspirate as much liquid from above the pellet as possible, but leave the pellet intact.

9. Using a new sterile pipette, add 2.0-3.0mL of Sperm Washing Medium (Cat. #9983) or Modified Sperm Washing Medium (Cat #9984) to the tube and resuspend the pellet. Centrifuge at 300 x g for 10 minutes. Remove supernatant with a clean pipette and add 2-3 mL of Sperm Washing Media again and centrifuge.

10. After the second wash, discard the supernatant and resuspend the pellet in 0.25-0.5 mL of Sperm Washing Medium if the sperm will be used for IUI or fertilization media (HTF, P-1, ECM or the Complete Media series) if the sperm will be used for IVF/ICSI. Place the tube containing the washed sperm in a warming block, or water bath if it is to be used for IUI or into a CO_2 incubator if the specimen will be used for IVF/ICSI.
    - For IUI procedures the sperm specimen should be concentrated in 0.25-0.50 mL to accommodate the volume capacity of the uterus. Tubes should be kept at 37°C until insemination by placing the tube in a CO_2 incubator with the lid tightly sealed or in a warming block or water bath.
    - For IVF procedures a final dilution of the sperm specimen in a bicarbonate-buffered media (such as HTF, P-1, ECM or the Complete Media series) can be made depending on the insemination concentration and volume that is desired.

11. Medium that does not contain protein should be supplemented and added prior to adding to the sperm. Protein supplementation products include HSA, Cat. #9988; SSS, Cat. #99193 and DSS, Cat. #9301. Prepare a 5 mg/mL of HSA or 10% v/v SSS or 10% v/v DSS in the appropriate volume of fertilization media.

One-Layer Discontinuous Gradient Separation Procedure:

(This method may also be used for normozoospermic patients or frozen specimens.)

1. Bring all of the media to 37°C.

2. If using a dilution other than 90%, prepare appropriate dilution of ISolate.

3. Using a sterile, disposable pipette, transfer 1.5-2.0 mL of the lower layer into a sterile, disposable 15.0 mL conical centrifuge tube.

4. Mix the liquefied semen well with a 5.0 mL or 10.0 mL volumetric pipette.

5. Gently place 0.5-2.0 mL of the liquefied semen onto the lower layer using a new sterile pipette. If the total volume of semen is more than 2.0 mL then use the appropriate number of tubes and distribute the final semen volume accordingly. Each tube of lower layer can be used to process up to 2 mL of semen. Additional volumes require additional tubes.

6. Centrifuge for 20 minutes at 300 x g.

7. At the completion of the spin, remove the layer by inserting a clean 5 mL pipette tip just below the surface of the liquid. Hold the tip in this position during aspiration. Aspirate the layers without disturbing the sperm pellet at the bottom of the tube until approximately 0.5 mL of lower layer remains. Even if a sperm pellet is not visible, this volume should contain sperm. If the sperm pellet occupies more than 0.5 mL at the bottom of the tube, aspirate as much liquid from above the pellet as possible, but leave the pellet intact.

8. Using a new sterile pipette, add 2.0-3.0 mL of Sperm Washing Medium (Cat. #9983) or Modified Sperm Washing Media (Cat. #9984) to the tube and resuspend the pellet. Centrifuge at 300 x g for 10 minutes. Remove the supernatant with a clean pipette, add 2-3 mL of Sperm Washing Media again and centrifuge.

9. After the second wash, discard the supernatant and resuspend the pellet in 0.25-0.5 mL of Sperm Washing Medium if the sperm will be used for IUI or fertilization media (HTF, P-1, ECM or the Complete Media series) if the sperm will be used for IVF/ICSI. Place the tube containing the washed sperm in a warming block, or water bath if it is to be used for IUI or into a CO_2 incubator if the specimen will be used for IVF /ICSI.
   - For IUI procedures the sperm specimen should be concentrated in 0.25-0.50 mL to accommodate the volume capacity of the uterus. Tubes should be kept at 37°C until insemination by placing the tube in a CO_2 incubator with the lid tightly sealed or in a warming block or water bath.
   - For IVF procedures a final dilution of the sperm specimen in a bicarbonate-buffered media (such as HTF, P-1, ECM or the Complete Media series) can be made depending on the insemination concentration and volume that is desired.

10. Medium that does not contain protein should be supplemented and added prior to adding to the sperm. Protein supplementation products include HSA, Cat. #9988; SSS, Cat. #99193 and DSS, Cat. #9301. Prepare a 5 mg/mL of HSA or 10% v/v SSS or 10% v/v DSS in the appropriate volume of fertilization media.

B. SWIM-UP PREPARATION

General Considerations:

Recommended for use with samples that have normal sperm counts and motility. Particularly appropriate for samples with elevated white cell contamination or when the lab wishes to reduce the risk of potentially generating reduced oxygen radicals by centrifugation. Recovery counts and motility are more variable than density gradient separation.

Procedure:

1. Place 1 mL of warm Sperm Washing Media (Cat. #9983) into a clean 15 mL centrifuge tube.

2. Gently layer 1 mL of semen under the media using a clean disposable 1 mL pipette.

3. Place the tube into the incubator at a 30° angle and allow the sperm to swim up into the overlying media for at least one hour.

4. After one hour, gently remove the top 200-300 µL of media and place in a clean tube.

5. Multiple aliquots of several tubes may be combined into one if multiple tubes were used. Sperm is ready to use at this point after assessment of count and motility.

C. SPERM WASHING METHOD FOR POOR SPECIMENS:

General Consideration:

Poor semen specimens are those with low sperm concentrations, low motility and/or low progression. Density gradient recovery typically averages around 25%, therefore, for specimens with extremely low initial counts density gradient centrifugation may not be appropriate. The following procedure may be used to wash sperm that has been obtained by ejaculation or by testicular biopsy or epididymal sperm aspiration.

Procedure:

1. Bring Sperm Wash Media to 37°C.

2. Testicular tissue may be minced in Sperm Washing Media, dispersed in and out through an 18-guage needle and then washed twice with 2-3 mL of Sperm Washing Media. The specimen should be centrifuged at 300 x g centrifugation between washes. The final pellet should be resuspended in fertilization media (HTF, P-1, ECM or the Complete Media series) containing protein (HSA, SSS or DSS) and the tube placed in a CO_2 incubator. Alternatively, sperm may be expelled from testicular tissue by dissecting out the seminiferous tubules from the surrounding cells then using the edge of a glass slide or the back of the bevel of a needle to apply pressure to the tubules to expel the sperm. Samples may then be washed, resuspended in a fertilization media of choice and allowed to stand in a CO_2 incubator until use, or frozen if being used at a later date.

3. Epididymal samples or ejaculates with extremely low count and/or motility may be processed by gently pipetting 1.5-2.0 mL of sample into a sterile centrifuge tube, adding 2.0-3.0 mL of Sperm Washing Medium, and gently mixing the contents by pulling the solution in and out of a 5 mL pipette. If the total volume of semen is more than 2.0 mL use the appropriate number of tubes and distribute the sperm sample volume accordingly. Centrifuge at 300 x g for 5-10 minutes. Remove supernatant and gently resuspend the pellet with 2.0-3.0 mL of Sperm Washing Medium and centrifuge for a second time for another 5-10 minutes.

4. After the final wash, discard the supernatant and resuspend the pellet in 0.25-0.5 mL Sperm Washing Medium or the appropriate medium depending on the next procedure. Place the tube containing the washed sperm in a CO_2 incubator until it is time to be used for an IUI, IVF or ICSI procedure. For HEPES buffered media such as Sperm Washing Medium the tube should be tightly capped. For media with a bicarbonate buffering system the tube should be loosely capped.

QUALITY ASSESSMENT CRITERIA

Semen analysis is performed in order to assess the specific parameters of the semen specimen. Normal semen values have been established by the World Health Organization and published in: WHO Laboratory Manual for the Examination of Human Semen and Semen-cervical Mucus Interaction. 4th ed. Cambridge University Press; 1999.

A semen specimen with the following characteristics is considered normal:

- Volume: 2.0 mL and above
- pH: 7.2-8.0. pH levels greater than this may indicate the presence of an infection. The physician requesting the test should be notified in order to address appropriate treatment for the infection. If the pH is less than 7.0 and the sperm count is low, an abnormality or potentially a blockage somewhere in the reproductive tract may be suspected and the patient should be referred for evaluation.
- Sperm concentration: ☒ 20 x 10^6 per mL
- Total sperm count: ☒ 40 x 10^6 per mL
- Motility: >50% or more with forward progression, ie, rapid and linear progressive motility or slow, sluggish linear or nonlinear motility. If 25% of the sperm have rapid linear progression, this is also considered normal.
- Progression: The rapid and linear progressive motility determines the sperm's ability to be used for IUI or IVF. The most common scale used for sperm progressive motility is the 0-4 scale with 4 rating the rigorous, rapid forward progression and 0 rating the absence of any forward progression.
- Morphology: 30% or greater with normal morphology is considered acceptable.
- White Blood Cells: Less than 1 x 10^6 per mL is normal. If greater than this number of non-sperm round cells are seen
- A differential staining should be performed to distinguish immature sperm from white cells.
- The patient should also be referred for evaluation by a urologist.

D. ANTISPERM ANTIBODY ASSESSMENT:

General considerations:

Spermatozoa are manufactured in a portion of the body that is immunologically isolated during development in most men. When the immunological barrier is breached by such events as trauma, or vasectomy, a man's immune system may begin to recognize his own sperm as being foreign, or “not self.” Part of the immune response to cells that are not recognized as “self” is the production of antibodies against the invading cells by the immune system. These antibodies then bind to the offending cell and are used by the systemic immune system to assist in the removal of “non-self” cells.

A number of different kinds of antigens that are present on the sperm may be immunogenic and stimulate the production of antibodies directed against various portions of the sperm. Depending on the antigen and the concentration of antibodies that are produced, the sperm may begin to agglutinate, or form clumps in the semen sample. Another consequence of antibody formation occurs when the antibody-bound sperm are exposed to proteins in the serum and other white cells of the immune system and result in sperm death. This cytotoxic effect can be observed by a decline in motility and vitality in a semen specimen. However, this latter is a fairly rare phenomenon. The major consequence of the presence of antibodies bound particularly to the head of the sperm is that the sperm may have a reduced capacity to bind to the zona pellucida of the oocyte, inhibiting fertilization.

Antibodies present on sperm are not always directed at sperm-specific antigens, but may also be directed against molecules that are loosely attached to the sperm.

Antibodies of several different “families” may be produced against antigens on, or associated with sperm. Antibodies of the IgG type can appear in the gential tract by transudation from the serum. Antibodies of the IgA type can be produced by cells within the mucosal lining of the tract. IgM is found occasionally but appears to have no clinical significance for fertility.

Once antibodies are bound to the sperm as they move through the male reproductive tract, particularly those of the IgA type, they can in turn bind to cervical mucins within the female partner. However, in rare instances, women may also produce their own IgG and IgA anti-sperm antibodies.

IgG and IgA on spermatozoa may be assessed directly in semen using purified antibodies covalently bound to micron-sized hydrophilic polyacrylamide beads. Antibodies directed against spermatozoa in samples that do not contain spermatozoa such as cervical mucous or serum may be indirectly assessed using antibody free sperm that is co-incubated with the sample containing the anti-sperm antibodies. The antibodies will then bind to the normally antibody-free sperm and can be detected when the sperm are in turn incubated with the polyacrylamide beads.

Procedure:

DIRECT IMMUNOBEAD ASSAY

Covalently linked beads are available for detection of IgG [Cat. #15375, Immunobead® Rabbit Anti-Human IgG (γ)], Riga [Cat. #15376, Immunobead® Rabbit Anti-Human IgA (α)] and IgM [Cat. #15377, Immunobead® Rabbit Anti-Human IgM (μ)]. These anti-antibodies have been raised in rabbits to detect the presence of specific human anti-sperm antibodies present on the surface of the sperm.

1. Immunobead reagents of any type may be reconstituted and divided into smaller volumes, then refrozen for later use. Upon receipt of the beads, reconstitute the reagent with 5 mL deionized water to produce a working stock solution of 10 mg of beads/mL of water. This rehydrated stock solution of beads is stable for 6 months when stored at 4°C or may be frozen for extended storage up to 2 years. Once the beads have been reconstituted, refrozen and then thawed, they should be used with no more than one subsequent refreeze and rethaw. Therefore it is advisable to freeze the reconstituted reagent beads in small enough volumes that repeated freezing and thawing is not necessary. Aliquot into 12 x 75 mm tubes, each with 2 mg/μl of stock solution and freeze.

2. On the day of the test, thaw one tube of each of the stock bead solutions (IgG and IgA). Add the contents of the tube to 10 mL of Buffer A in a separate conical centrifuge tube. Centrifuge at 1000 x g for 10 minutes. Decant the supernatant, blot the rim, and resuspend the final pellet in 0.2 mL of Buffer B.

3. Prepare the sperm sample to be tested for the presence of antibodies. Place the semen in a conical centrifuge tube and add sufficient amounts of Buffer A to bring the final volume to 10 mL. Centrifuge at 600 x g for 10 minutes. Discard the supernatant. Wash the sperm a second time with 10 mL Buffer A, centrifuge, and discard the supernatant. Add a sufficient volume of Buffer B to bring the final concentration of sperm to <50 million motile sperm per mL. Ideally the final dilution should yield approximately 10-20 million motile sperm per mL.

4. Begin testing by adding 5 μL of washed sperm suspension to 5 μL of each washed beads, IgA and IgG, on a microscope slide. Mix well, cover with a cover slip, and leave at room temperature in a moist chamber for up to 10 minutes. Observe at 400X to 500X magnification under a phase-contrast microscope.

5. Score the percentage of motile sperm that have Immunobeads attached. Assess each type of bead separately. Count at least 100-200 motile sperm per preparation. Do not count non-moving sperm even if they appear to have beads attached. Some moving sperm may appear to have beads attached, but careful observation will show that the beads are not carried with the sperm as they move. Therefore, observe only moving sperm and record sperm as positive only if it can be observed that the sperm are carrying the beads with them as they move. Record the site of binding of the beads to the sperm (head, midpiece, tail, head and tail).

6. The test is positive if 20% or more of the motile sperm have beads attached to them, and is clinically significant if 50% or more are coated with beads. Binding of the beads to only the tail tip is not considered clinically significant.

7. Tests should also be performed using positive and negative controls alongside the sperm being analyzed. A positive control may be frozen-thawed and washed sperm from a patient who has previously tested highly positive for both IgA and IgG binding, or antibody-free sperm may be bound with antibody by co-incubating the sperm in the serum from a patient that has previously been shown to be anti-sperm antibody positive. Incubate 100 µL of washed sperm with 100 µL of antibody-positive serum and 200 µL of Buffer B in a 12 x 75 mm tube. Incubate for 1-2 hours at 37°C. At the end of the incubation time, wash the sperm with Buffer B twice and resuspend in 400 µL of Buffer B. Combine 5 µL of this sperm with 5 µL of Immunobeads of each type as described above and assess the percent of motile sperm bearing beads.

8. A negative control should also be performed. Frozen thawed sperm from a known negative donor may be used as described for the test sample. Similarly, if using co-inubation with serum to create a positive control, a negative control may be performed simultaneous by co-incubating donor sperm with a known antibody-negative serum in the same way as described for the positive control.

INDIRECT IMMUNOBEAD ASSAY:

The indirect assay is used when testing samples that do not contain sperm such as serum, follicular fluid or seminal plasma.

1. Sample Collection/Processing:
   a. Serum should be collected from the patient in question in a sterile red-top tube (no anti-coagulant), allowed to clot, and then centrifuge at 3000 rpm for 10 minutes. Transfer serum to tubes and heat inactivate complement at 56°C for one hour. Store frozen until needed for test.
   b. Seminal plasma: After routine sperm collection, the sample should be centrifuged at 600 x g for 10 minutes to remove sperm and other cells, and the supernatant collected. It should then be filtered through a 0.4 µm filter to remove any remaining sperm and debris. Seminal plasma may be tested without inactivation of complement.
   c. Follicular fluid should be centrifuged at 600 x g for 10 minutes and heat-inactivated by incubating at 56°C for one hour.

2. Fresh donor sperm that has previously been shown to be antibody-free should be washed three times with 10 mL Buffer A and centrifugation at 600 x g for 10 minutes on the day the indirect assay is to be performed. At the end of the final spin, adjust the final sperm concentration to 50 million sperm/mL in Buffer B.

3. Donor sperm that has been washed is then incubated with the test solution. If antibodies are present in the test solution they will bind to the sperm during this time. Add 100 µL of patient test fluid to 100 µL of washed donor sperm suspension and 200 µL of Buffer B in a 12 x 75 mm tube.

4. Positive and Negative controls should be run alongside the test solution:
   a. The positive control should be the serum of a patient who has previously been shown to be antibody positive with an indirect assay. Once a patient has been shown to be antibody positive, serum may be collected, heat-inactivated and then allocated into small volumes, and stored frozen until needed.
   b. The negative control should be the serum of a patient who has not tested positively for anti-sperm antibodies in the past using the indirect assay.

5. Incubate the patient test solution, the positive and the negative controls with the donor sperm for 1-2 hours at 37°C.

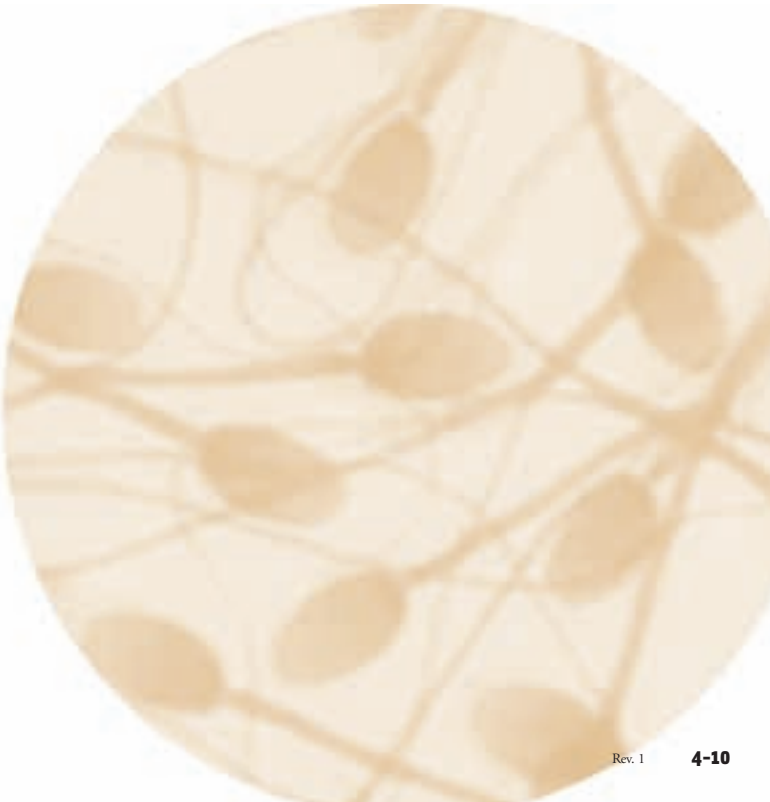
6. At the end of the incubation time, wash the mixture three times by adding one mL of Buffer B and centrifuging at 600 x g for 5 minutes. Remove supernatant and wash again. After the final wash, resuspend the sperm in 400 µL of media.

7. During the incubation period prepare the Immunobeads as described for the Direct Immunobead Assay).
   a. Thaw one tube of each of the stock bead solutions (IgG and IgA).
   b. Add the contents of the tube to 10 mL of Buffer A in a separate conical centrifuge tube.
   c. Centrifuge at 1000 x g for 10 minutes.
   d. Decant the supernatant, blot the rim, and resuspend the final pellet in 0.2 mL of Buffer B.

8. Begin testing by adding 5 µL of washed sperm suspension to 5 µL of each washed Immunobeads, IgA and IgG, on a microscope slide. Mix well, cover with a cover slip, and leave at room temperature in a moist chamber for up to 10 minutes. Observe at 400X to 500X magnification under a phase-contrast microscope.

9. Score the percentage of motile sperm that have attached Immunobeads. Assess each type of bead separately. Count at least 100-200 motile sperm per preparation. Do not count non-moving sperm even if they appear to have beads attached. Some moving sperm may appear to have beads attached, but careful observation will show that the beads are not carried with the sperm as they move. Therefore, observe only moving sperm and record sperm as positive only if it can be observed that the sperm are carrying the beads with them as they move. Record the site of binding of the beads to the sperm (head, midpiece, tail, head and tail).

10. The test is positive if 20% or more of the motile sperm have beads attached to them, and is clinically significant if 50% or more are coated with beads. Binding of the beads to only the tail tip is not considered clinically significant.



Micromanipulation Protocols

OVERVIEW

Micromanipulation media are designed for procedures including ICSI (Intra-Cytoplasmic Sperm Injection), Assisted Hatching (AH) and Embryo Biopsy. All three procedures are performed on an inverted microscope, usually with Hoffman optics, and using one of a variety of micromanipulation and microinjection devices that allow fine control of the pulled glass needles and holding pipettes that are used for these procedures. All micromanipulation procedures should be done using a heated stage that has been calibrated to maintain the temperature of the media at 37°C within the dish that is being used.

Irvine Scientific Micromanipulation Media:

1. Hyaluronidase Solution, Cat. #90101: Hyaluronidase is an enzymatic solution containing 80 IU/mL of bovine derived hyaluronidase in a HEPES buffered HTF medium supplemented with 5.0 mg/mL (or 0.5%) therapeutic grade Human Serum Albumin (HSA). It is designed for removing (denuding) cumulus and corona cells surrounding the oocytes prior to ICSI procedure.

2. PVP (Polyvinylpyrrolidone): Available in three convenient configurations:
   a. Lyophilized PVP, Cat. #99219: 10% lyophilized PVP to be reconstituted with mHTF, designed to be used with normal sperm specimens.
   b. 10% PVP Solution, Cat. #99311: Ready to use, 10% PVP reconstituted in mHTF medium, designed to be used with normal sperm specimens.
   c. 7% PVP Solution, Cat. #90119: Ready to use, 7% PVP reconstituted in mHTF medium, designed to be used with poor sperm specimens (low motility sperm).

3. Tyrode's Solution-Acidified, Cat. #99252: Ready to use solution for thinning of the zona pellucida (assisting hatching) or drilling a hole in the zona of the embryos.

4. Embryo Biopsy Medium, Cat. #90103: Embryo Biopsy Medium is a ready-to-use HEPES buffered salt solution (mHTF) with HSA (5 mg/mL) lacking Calcium and Magnesium ions and containing EDTA as a chelating agent. It is designed for use as a temporary (short term) culture medium while performing embryo biopsy on cleavage stage (6-10 cell) embryos to remove blastomeres for Preimplantation Genetic Diagnosis (PGD).

A. ICSI Procedures

General considerations:

ICSI is performed when sperm count, motility, or morphology is low such that fertilization may not occur when performing conventional insemination (incubation of oocytes and sperm in a dish). ICSI is also performed when: 1) there has been evidence of failed fertilization in a previous cycle, even when sperm parameters are within normal ranges (as outlined by the World Health Organization reference needed) or 2) when the number of available oocytes is low (to optimize the chance for fertilization). During an ICSI procedure metaphase II oocytes are prepared for injection of a sperm by removing the outer layers of cells that surround the oocyte, the cumulus oophorus and the corona. Once a clear view of the interior of the oocyte is obtained sperm may be injected into the oocytes in order to overcome a number of sperm and/or oocyte defects leading to low fertilization rates. At the time of ICSI, sperm may be placed into a solution of PVP in order to slow its movements down, although not all laboratories choose to use these solutions. Sperm is inactivated prior to insertion by rubbing the sperm at the midpiece or tail hard enough to kink the tail using a small beveled ICSI needle. The inactivated sperm is then picked up into the needle and injected into the ooctye.

Micromanipulation Protocols

OOCYTE DENUDATION

1. The ICSI procedure requires that the cells surrounding the oocytes be removed in order to visual the interior of the cell, a process called denudation, stripping, or cleaning. The time between oocyte retrieval and cumulus removal should be standardized by each laboratory. Denuding of oocytes can be achieved by either of two methods:
   - Dissection of the surrounding cumulus complex by cutting away the cells surrounding the oocyte using conventional small-bore injection needles or
   - Short term exposure to Hyaluronidase Solution (80 IU/mL) to loosen up the cumulus/corona cells. A brief, 30-second, exposure to Hyaluronidase (50 µL drops - or larger- under oil) is usually sufficient to initiate breakdown of the matrix holding the cumulus cells around the oocytes.

2. After the 30 second exposure of the oocytes to the Hyaluronidase the oocytes is aspirated into a finely pulled pipette and transferred into Hyaluronidase-free media such as HTF, mHTF, P-1 or ECM with protein (HSA, SSS or DSS). Once the oocytes are in the rinse media they may be pulled repeatedly up and down in 135-150 µm diameter pipette tips to remove the cells from around the oocytes.

3. Following cumulus/corona cell removal, the oocytes should be transferred through several rinses to remove any remnants of the Hyaluronidase Solution.

4. After all oocytes have been denuded and rinsed, the presence or absence of a polar body may be observed. Most laboratories choose to only perform ICSI on mature, Metaphase II oocytes since oocytes at earlier stages of development do not possess either mature nuclei or cytoplasm. Oocytes that are not at the MII stage may be cultured *in-vitro* until expulsion of the polar body and then injected, if the laboratory chooses.

SPERM IMMOBILIZATION

It became obvious early in the development of ICSI that fertilization rates increased when sperm were aggressively inactivated rather than injected into the oocytes while they were still moving. Although some laboratories have managed to perfect the art of catching and inactivating a sperm without the use of additives or viscous solutions, the most common method used to immobilize the sperm and slow its motility down is by suspending the sperm in Polyvinylpyrrolidone (PVP) solution.

Semen should be processed in the usual manner, either through swim-up, density gradient centrifugation or washing and the resulting motile sperm fraction should be resuspended in Sperm Washing Medium, HTF, P-1 or ECM supplemented with protein (HSA, SSS or DSS).

Sperm that has been collected by surgical means such as testicular biopsy or epididymal aspiration may be low enough in count and/or motility that very little processing should be done prior to picking up the sperm for ICSI.

INTRACYTOPLAMIC SPERM INJECTION

1. When doing ICSI, the microscope stage should be heated and main-tained at 37ºC.

2. The ICSI micropipettes along with any other micro-tools should be kept sterile.

3. Set up the scope, the microinjectors, and the micro-tools in advance, aligning the tips of the micro-tools in the center of the field of view. Raise the tools until ready to use them, or place a "holding" dish on the scope with PVP in a droplet in the center of a Falcon 1006 lid, covered with oil. Lower the injection needle into the PVP and allow it to equilibrate. The holding pipette may be equilibrated in the oil.

4. It appears as though injecting immature oocytes (those that have not completed the first round of metaphase, and extruded the first polar body), may result in embryos that are genetically abnormal, if they fertilize at all. Therefore it is advisable to only inject oocytes with a clearly visible polar body (Metaphase II). The timing of the procedure varies between laboratories throughout the world, and no standard timing has been established for ICSI following oocytes retrieval. Therefore, each laboratory should standardize the timing of the injection within its own facility. The most common time for injection appears to be around 38-40 hours post hCG administration.

5. Prepare the ICSI dish. Most laboratories have used the lid to the Falcon 1006 dishes due to their low clearance and depth. However, extended culture in these lids should not be done for any reason as they have not been treated in the same manner as the bottoms of the dishes by the manufacturer and there has been some evidence that the lids to these dishes may be embryo toxic following extended exposure. (A time should be specified regarding what is considered "extended")

6. The most common method for setting up a dish is to place a 5-10 µL drop of PVP into the center of the ICSI dish and then to surround the PVP drop with a ring of 5-10 µL droplets of HEPES-buffered media with protein. Another method is to place a 5 µL triangle of PVP in the center of the dish and then a 5-10 µL trough of media with protein above it.

7. Cover the drops with oil.

8. Place sperm into the PVP droplet. Sperm may be placed gently into the center of the drop or the outside edge of the triangle. The amount of sperm added to the PVP droplet should be determined by the count and motility of the processed specimen. The final dilution in the PVP droplet should be high enough that it is easy to visually find motile sperm around the edges of the drop or the opposite side of the triangle, but low enough that it is easy to pick up only the chosen sperm without pulling multiple sperm up into the ICSI needle.

9. Transfer oocytes into each of the droplets surrounding the sperm, or into the left side of the trough in the ICSI dish.

10. Take the dish containing the oocytes and sperm to the scope. Raise the micro-tools, remove the "holding" dish, and position the ICSI dish on the stage so that the edge of the PVP droplet is in focus.

11. At 200X magnification, focus on the sperm at the edge of the PVP drop. Search for sperm that are slightly motile at the bottom of drop or at the drop/oil interface. Assess the sperm for the quality of the motility and its morphology. Lower the ICSI needle to the top of the chosen sperm and use the tip of the pipette to swipe or "snap" the tail of the sperm in order to immobilize it. Pull the sperm up into the pipette by the tail and stabilize its movement in the injection pipette. Some laboratories choose to inject testicular sperm tail-first, rather than head-first into oocytes. Multiple sperm may be pulled up into the ICSI pipette provided sufficient space between is provided to pre-vent injecting multiple sperm into one oocytes.

12. Raise the pipette slightly off the bottom of the dish and move to the first injection droplet or the trough containing the oocyte(s). Lower the holding pipette and position the oocyte on the holding pipette so that the polar body is between 11 and 1 o'clock or 5 and 7. Make sure that the oocyte is sitting on the bottom of the dish. Bring the injection/ICSI pipette down to the edge of the oocyte and check the position of the sperm. Advance the sperm to the very tip of the needle.

13. Change magnification to 400X and focus on the oolemma. Bring the injection pipette into focus. Insert the injection pipette at the 3 o'clock position into the oocyte to approximately 3/4 of its diameter or until the membrane pops or gives indicating that it has been punctured. Aspi-rate a small portion of the cytoplasm to further ensure that the oocyte membrane has been broken. Aspiration of the cytoplasm is critical for oocyte activation.

14. Inject cytoplasm and sperm back into the oocyte. Place the sperm head slightly outside the injection path; this helps to hold the sperm in the cytoplasm and provides a better area of contact for pronuclear formation. Slowly remove the injection pipette while expelling the sperm in the oocyte.

15. If using the "trough" method, move the oocyte to the other end of the trough and then return to the uninjected oocytes and continue as described in the previous steps until all oocytes are injected. If using drops, release the oocytes in its drop and move the dish to the next drop.

16. When finished injecting, move all oocytes through three rinses of cul-ture medium and place them into a drops of media covered with oil in a culture dish. Oocytes may be cultured in groups or individually. Dishes for culture should have been made the previous day to allow for equilibration, especially when using low oxygen incubators as it appears that oxygen diffusion through oil occurs much more slowly than CO_2 diffusion. If using group culture, then drops may be in the 20-50 µL range. When using single embryo culture, drop size may be less, in the 10-20 µL range.

17. Check for two pronuclei at 16-20 hours post injection, as the evidence of fertilization. Separate out the fertilized from unfertilized zygotes and culture.

B. ASSISTED HATCHING

General Considerations:

Assisted Hatching is used as a method to assist the embryo in its final expul-sion from the zona as it begins to implant, or to open the zona sufficiently at the time of embryo biopsy for Preimplantation Genetic Diagnosis (PGD). Several methods may be used to put a hole in the zona such as etching or dissolving a hole in the zona with acidified Tyrode's solution, using a spike or PZD needle to rub a hole in the zona, or using a laser to remove portions of the zona.

Performing zona removal in an effort to improve implantation rates is still somewhat controversial and the outcomes reported in the literature seemed to be mixed. However, when a benefit is shown, it seems to be when using embryos from older women and/or frozen/thawed embryos. However some programs routinely perform assisted hatching on all embryos prior to transfer. Therefore, criteria for determining when, and which, embryos to hatch should be developed by each individual laboratory.

The technique for performing assisted hatching for implantation improvement and opening a hole in the zona for embryo biopsy is similar, except for the final size of the opening. When doing assisted hatching to improve implantation, the size of the hole should be approximately one-quarter to one-third the size of a blastomere. When doing assisted hatching for embryo biopsy the size of the hole will have to be large enough to allow introduction of the biopsy tool or approximately the size of blastomere. Therefore the procedure should be modified to accommodate the conditions for its use.

Procedure for assisted hatching for implantation improvement using Acid Tyrode's Solution:

1. Set up the micromanipulation microscope with a holding pipette on the left and the AH pipette on the right. Align the microtools in the center of the field of view.

2. Remove the Acidifed Tyrode's Solution from the refrigerator and allow it to come to room temperature while setting up the scope.

3. AH should be done at 37°C.

4. Place 10 µL of Acidified Tyrode's solution in the center of low-walled dish, cover with oil and place it on the stage of the microscope. Lower the hatching microtool into the Tyrode's solutions, apply suction, and allow the microtool to fill and equilibrate for a few minutes before using.

5. While waiting for the tool to equilibrate, place several 10 µL drops of Modified HTF with protein (HSA, SSS or DSS) in the dish and cover with oil.

6. Add one embryo to the first drop. It is not advisable to let an embryo sit in a droplet with acid Tyrode's for an extended period of time. Therefore once the embryo has been hatched it should be transferred out of that drop and rinsed immediately. Then the next embryo may be placed in the second drop, hatched, removed, rinsed and then the third embryo hatched (if multiple embryos are being hatched and transferred).

7. Take the dish to the micromanipulation scope. Raise the hatching microtool while applying a slight amount of pressure to prevent oil from entering the tool as it is raised through the oil. Locate the drop with the embryo and pull the embryo onto the holding pipette. Orient the embryo on the holding pipette so that the area at 3 o'clock, where the hatching will be done, falls between two blastomeres or is adja-cent to an area of fragmentation in the embryo.

8. Lower the AH microtool while applying a slight amount of pressure to prevent oil from entering the AH pipette. Align the AH pipette with the space between the blastomeres or next to the area of fragmentation.

9. Begin to expel the solution onto the zona. The outermost layer of the zona usually dissipates most easily. While expelling the solution, move the tip of the pipette up and down slightly to assist the removal of the zona and move in through the zona as it dissolves. Move more carefully as the zona thins to avoid blowing large amounts of acidified Tyrode's into the interior of the embryo once the integrity of the innermost layer of the zona is breached. The hole will look like a triangle with the widest part of the triangle at the outermost edge of the zona and the point being at the interior of the zona.

10. Some embryologists choose to remove fragments from the interior of the embryo at the time of hatching. This requires a steady hand and patience to prevent sucking up intact blastomeres as well as fragments, but can be done at this time if the laboratory chooses.

11. Once the zona has been breached, and the fragments have been removed, pull the pipette out. Aspirate the immediate area adjacent to the hole in an effort to reduce continued exposure of the embryo to the acidified Tyrode's.

12. Remove the dish from the field of view, and return the dish with the drop of acid typrodes in it. Lower the hatching pipette into the Tyrode's and let it re-equilibrate.

13. Transfer the embryo from the hatching drop through at least three rinses in media with protein. Place it in the dish that will be used for transfer.

14. Repeat with the remaining embryos.

Procedure for assisted hatching for implantation improvement using Partial Zona Dissection:

1. The use of Acidifed Tyrodes Solution is not necessary when using PZD.
2. Place the holding pipette on the left. Align the microtools in the center of the field of view then raise both tools.
3. Prepare a dish for PZD using Modified HTF plus 5 mg/mL HSA or 10% v/v SSS or 10% v/v DSS. Place 2-4 x 10 µL drops in a circle and cover with oil.
4. Transfer one embryo into the first drop. Place the dish onto the micromanipulation microscope stage and lower the holding pipette.
5. Suck the embryo onto the holding pipette, aligning the embryo such that the biggest space within the embryo or the widest distance between blastomeres is oriented at the top of the embryo.
6. Insert the PZD needle from right to left across the top of the embryo, entering at about the one o'clock position and exiting at the 11 o'clock position without entering any blastomeres. The embryo will now be "impaled" on the PZD needle, with the tip of the PZD needle exiting from the zona on the left and the wider back end of the PZD needle entering the zona from the right.
7. Release the pressure on the holding pipette. Lower the PZD needle to the bottom half of the field of view. Move the holding pipette up to the top of the embryo.
8. Rub the holding pipette from left to right across the top of the zona, "squeezing" the zona between the PZD needle and the holding pipette. Rub the zona until the PZD needle has created a slit in the zona and the embryo falls off of the PZD needle.
9. Occasionally the zona will be sticky enough that the embryo does not actually "fall" off of the PZD, but may be rolled off with the holding pipette once the slit has been made.
10. Transfer the embryo to the dish where it will be held until transfer.
11. Repeat with the remaining embryos.

Assisted Hatching using a Laser

The zona may be hatched using a series of "holes" created by a laser also. However there appears to be little agreement among embryologists as to how many holes to place in the zona, whether to simply thin the zona or to actually breach the zona, what pattern to use when making the holes (ie., multiple holes along the inside of the zona, but only one smaller opening on the outside, multiple holes along the outside of the zona, but only one on the inside, or to created one aligned set of holes breaching the entire thickness of the zona).

Therefore, the setup and use of laser hatching should be determined by each laboratory.

However, the following is one option for laser hatching using a Research Instruments Laser. This protocol is used with the permission of Joe Conaghan, PhD., Director, Fertility Laboratories, Pacific Fertility Center, San Francisco, CA, USA.

1. Set up the inverted microscope; turn it on and check the temperature of the stage. If not already on, turn on the computer to which the laser and microscope camera are attached. Double click on the "coronus" icon on the desktop to activate the laser software.

2. Activate the laser by pressing any button on the hand control (Figure 1). Ignite the pilot light by holding down the circular red button in the cen-ter of the control until it beeps. Rotate the laser lens into position on the microscope and place an empty petri dish in the light path. Focus the microscope until the pilot light can be seen in focus on the surface of the dish. Now check that the pilot light is aligned with the laser target on the computer screen.

Figure 1: Hand control for the laser.

3. If the pilot and target are not aligned, first check that none of the microscope controls have been adjusted to alter the light path. In particular, if the control to send light to the camera has been moved even slightly, it could alter the position of the pilot light. In the rare event that the pilot and target do not line up and the microscope has not been adjusted, double click on the target with the computer mouse. This unlocks the target and it can then be picked up and moved with the mouse. Once aligned with the pilot (Figure 2), the target is locked by double clicking on it again. Turn off the pilot light using the same button as before. The laser has 3 settings and these are selected by going back and forth with the arrows on the control unit. Select the largest of the 3 settings. The laser is now ready to use.

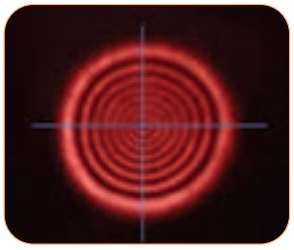


Figure 2: Make sure that the target is aligned with the pilot light. This must be checked every time the laser is used.

4. Take the plate containing the patient's embryos out of the incubator and move the embryos to be transferred to the central well using the Drummond pipette.

5. Place the dish on the stage of the inverted scope and locate these embryos under low power (4x lens). Rotate the laser lens into position and focus on the first embryo.

6. The laser target should be placed over the zona in a place that has no blastomere directly underneath. The safety circle around the target indicates the area through which heat from the laser will be dispersed. Blastomeres should be outside this circle (see Figure 3).

Figure 3: Position the target on the zona making sure that the safety circle does not overlap a blastomere

7. If it is not possible to place the target over an area of zona without the safety circle overlapping a blastomere, consider the following alternatives:
   a. Change to a lower power setting on the laser and make 2 or 3 small holes in the zona instead of one.
   b. Using any laser setting, thin the zona as much as can be achieved without going to close to a blastomere. Thinning should be done by using one, two or at most 3 pulses from the laser, and the pulses should overlap.
   c. Discuss the issue with the physician and explain that it is not possible to hatch a particular embryo without risk of damage.

8. Hatch each embryo in turn by breaching the zona with a single pulse from the laser. If the zona is particularly thick, 2 pulses might be needed to make a hole (one hitting the inner zona, and one outside the first hole on the outer zona).

9. Hatching of each embryo in turn should be accomplished quickly and efficiently. Find a target site on the zona, fire the laser and move on immediately to the next embryo.

10. After all embryos have been hatched, they can be moved to the transfer dish.

C. Embryo Biopsy

General Considerations:

1. Embryo biopsy is the procedure where one or two blastomeres are removed from a 6-8 cell embryo for Preimplantation Genetic Diagnosis (PGD). The procedure is performed just before compaction of the blastomeres.

2. Patients having In Vitro Fertilization and a known risk for a genetic defect, are eligible for this procedure. It is strongly recommended that ICSI fertilization is used for patients requesting embryo biopsy, as this prevents possible contamination from sperm attaching to the blastomere during biopsy.

3. The embryos to be biopsied should be at day 3 of development (72 hours from oocyte retrieval) and have 5 or more cells. Embryos with fewer cells or those that have not cleaved for 24 hours may not give reliable results, and the viability of the embryos may be adversely impacted following the biopsy.

4. The requirement is to get a cell containing a nucleus from each embryo. If the first biopsied cell has an abnormal, fragmented, or multiple nucleus, or no visible nucleus, the embryo should be biopsied again until a cell with a normal nuclear number/morphology is obtained. In some instances, this may adversely impact the viability of the embryo if too many blastomeres are removed.

5. The cell should be biopsied using a biopsy pipette with an inner diameter of not less than 30 µm.

6. Stability of specimen: The biopsied cell should be kept at room temperature and should be placed in lysis buffer in a PCR tube or lysed and fixed on a glass slide for transport to the genetics laboratory within one hour after the procedure. Occasionally, cells will be seen to lyse or die following biopsy and although these can be sent to the genetics laboratory, they may not yield a result. If the cell lyses prematurely, the embryo should be re-biopsied.

7. Unacceptable specimen: UNLABELED OR IMPROPERLY LABELED SPECIMENS ARE UNACCEPTABLE AS THEY CANNOT BE USED FOR A GENETIC DIAGNOSIS. IT IS IMPORTANT THAT THE BIOPSIED CELL SPECIMEN IS LABELLED CORRECTLY WITH THE SAME IDENTIFICATION AS THE EMBRYO FROM WHICH IT WAS OBTAINED. Cells without nuclei, or those with nuclear abnormalities (multiple nuclei, fragmented nuclei) cannot be considered reliable for the diagnosis.

8. Compromising conditions: If the cell is lysed or dead, or if the nucleus is missing or abnormal, the analysis may not succeed. If any of these scenarios arise, consultation with the individual placing the cells in the PCR tubes or fixing and analyzing the nuclei should be undertaken as soon as they have had a chance to examine the cell. Such conditions would require the embryo to be re-biopsied to obtain an acceptable cell for analysis.

9. Timing considerations: The embryos should be biopsied as early as possible on the morning of Day 3, to minimize complications arising from embryo compaction.

10. Special equipment: For the purpose of the biopsy, a laser can be used to allow the embryologist to work quickly on the embryos. Specifically, the laser allows a hole to be made in the zona pellucida without the need for a double tool holder, acid Tyrodes and a hatching pipette.

11. Embryos should be viewed as biohazardous, and the appropriate precautions taken.

EMBRYO BIOPSY PROCEDURE

1. Prepare the embryo biopsy dishes with 4 x 25 µL drops of Embryo Biopsy Medium and overlay with oil. The dishes should be clearly labeled with the patient's name and the type of medium in the dish. Locate the drops centrally in the dish and place in the non gassed 37°C incubator to warm up for at least 1 hour. The dishes can also be made the day prior to the biopsy procedure.

2. Set up the microscope with the biopsy pipette (diameter 35 µm) on the right manipulator. Attach a holding pipette on the other manipulator such that it opposes the biopsy pipette exactly. Align the pipettes such that they will both have good access to any embryo placed on the stage. The pipettes should also be checked for range of motion (i.e. don't have them at the end of their range while working on an embryo).

3. Prepare the paperwork for the case.

4. Score the embryos in the normal way. It is usual to do the biopsies starting with the embryo with the fewest intact cells, working through to the best embryos last. Also, it is often a good idea to biopsy an abnormal or degenerating embryo first to ensure that the biopsy can be performed easily. This allows for problems to be identified and solved before beginning work on the viable embryos.

5. Set up the inverted microscope; turn it on and check the temperature of the stage. If not already on, turn on the computer to which the laser and microscope camera are attached. Double click on the “coronus” icon on the desktop to activate the laser software.

6. Activate the laser by pressing any button on the hand control (Figure 1). Ignite the pilot light by holding down the circular red button in the center of the control until it beeps. Rotate the laser lens into position on the microscope and place an empty petri dish in the light path. Focus the microscope until the pilot light can be seen in focus on the surface of the dish. Now check that the pilot light is aligned with the laser target on the computer screen.

Figure 1: Hand control for the laser.

7. If the pilot and target are not aligned, first check that none of the microscope controls have been adjusted to alter the light path. In particular, if the control to send light to the camera has been moved even slightly, it could alter the position of the pilot light. In the rare event that the pilot and target do not line up and the microscope has not been adjusted, double click on the target with the computer mouse. This unlocks the target and it can then be picked up and moved with the mouse. Once aligned with the pilot (see Figure 2), the target is locked by double clicking on it again. Turn off the pilot light using the same button as before. The laser has 3 settings and these are selected by going back and forth with the arrows on the control unit. Select the largest of the 3 settings. The laser is now ready to use.

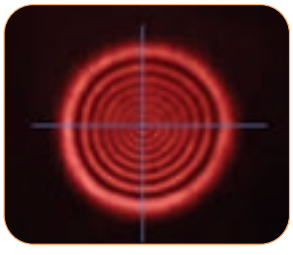


Figure 2: Make sure that the target is aligned with the pilot light. This must be checked every time the laser is used.

8. For molecular genetics cases have PCR tubes each with 5 µL of lysis buffer ready to receive the cells. Each embryo will require a tube for the cell and a tube for a media blank. It is also useful to have a number of extra tubes ready in case any embryo needs to be biopsied twice or in case a tube is dropped. For cytogenetics cases, the hypotonic and fixative solutions should be freshly made and glass slides ready for the fixing procedure.

9. Take the plate containing the patient's embryos out of the incubator and move the first embryos to be biopsied to the 1st drop of the 1st biopsy dish (containing the Embryo Biopsy Medium) using a Drummond pipette with a sterile 170 µm tip.

10. Once the above steps have been completed, the biopsy can begin. Two embryologists should be involved from this point forward; one to do the biopsies and one to move embryos and blastomeres around. The first (and worst) embryo should now be in the dish and placed on the microscope. Lower the microtools into the oil and pick up the embryo on the holding pipette. Make a hole in the zona that will be big enough for the biopsy pipette to squeeze through (almost the size of an 8-cell blastomere). Place the dish on the stage of the inverted scope and locate the embryo under low power (4x lens). Rotate the laser lens into position and focus on the first embryo.

11. The laser target should be placed over the zona (the target is fixed so this is accomplished by moving the microscope stage) in a place that has no blastomere directly underneath. The safety circle around the target indicates the area through which heat from the laser will be dispersed. Blastomeres should be outside this circle (see Figures 3 and 4).

Figure 3: Position the target on the zona making sure that the safety circle does not overlap a blastomere

12. An alternative method for making a hole is to line up the embryo with a blastomere directly under the area where the hole is to be made. In this case, the blastomere will receive some heat from the laser, but that particular blastomere is the one to be biopsied, so no harm will come to the embryo. This method best allows the operator to get a cell with a nucleus at the first attempt since a cell with a clear nucleus can be targeted before ever making a breach in the zona.

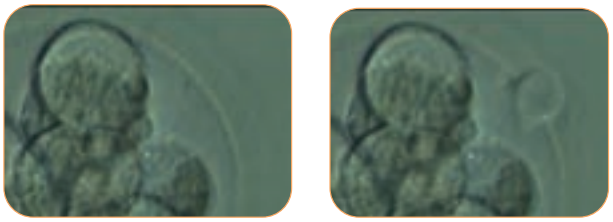


Figure 4. Embryo before and after 1st laser pulse.

13. Once a hole has been made, slip the biopsy pipette through and very gently aspirate a single blastomere (Figure 5). Target a blastomere that is accessible (close to the hole) and one in which a nucleus can be seen. Avoid pushing the pipette too far into the opening or up against other blastomeres. The blastomere does not necessarily have to be aspirated completely into the pipette. Often, having just 1/3 of or 1/2 of the blastomere in the pipette gives enough of a hold to drag the blastomere out of the embryo (see Figure 6).

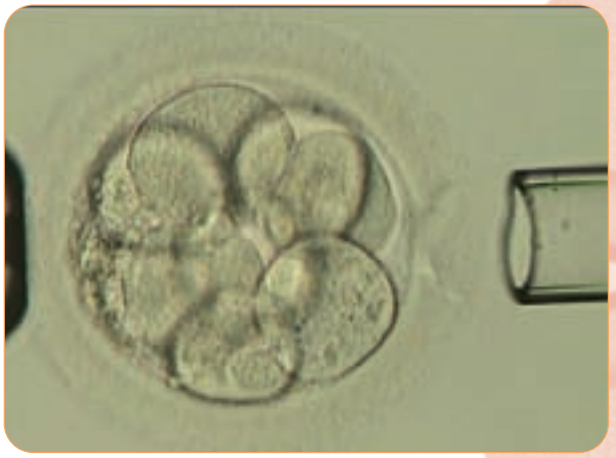


Figure 5. A triangular shaped hole made with 3 laser pulses is often useful for guiding the biopsy pipette into the embryo. The first and central pulse is the only one to breach the inner zona. Two additional pulses, flank the first hole and breach only the outer zona.

14. Drop the aspirated blastomere next to the embryo and examine. If the blastomere has a clear nucleus and is not lysed, it can be given to the embryologist preparing the slides/tubes for the genetics lab. If not, repeat step 13.

15. Continue to biopsy the embryos until each one has resulted in a blastomere with nucleus that is acceptable to the embryologist preparing the cells for analysis. In some cases, this individual may ask you to re-biopsy an embryo if they are not happy with what they received. For example, the individual fixing the cells in a cytogenetics case may fail to get the nucleus fixed onto a slide and ask you for another cell from that embryo.

16. In cytogenetics cases, 5-10 blastomeres will be fixed and numbered on individ-ual glass slides. In molecular genetics cases the PCR tubes are numbered to receive the cells and loaded with 5µL lysis buffer (tubes and buffer are supplied by Dr. Hughes in advance of the case). Each blastomere will need one tube and it is usual to create a blank tube (buffer without a cell) to be tested in parallel with the blastomere. Label the tubes 1, 1B, 2, 2B, 3, 3B…and so on, accounting for the number of embryos to be biopsied and also allow for extra blastomeres. A patient with 12 embryos therefore, might have 12 blastomere tubes, 12 blank tubes and 4-6 extra tubes, all labeled and containing buffer.

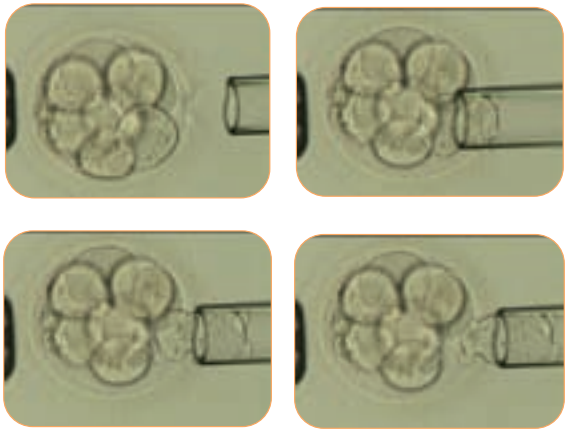


Figure 6. Insert the biopsy pipette through the zona hole and gently aspirate a blastomere either fully or partially into the pipette. Drag the blastomere out of the embryo.

17. For cytogenetic (aneuploidy) cases, skip to step 24.

18. The blastomere should be washed twice in wash buffer and placed in the tube of lysis buffer. The embryologist performing this part of the procedure should be careful to observe the cell clearly going into the buffer. If the cell is not clearly seen going into the tube, or if the cell is observed to have an abnormal number of nuclei, a second cell should be biposied from the embryo.

19. Using the medium remaining in the pipette after depositing the blas-tomere, open the blank tube and deposit about the same volume of medium as the cell-containing tube received. Both tubes should then be tightly capped and placed in the freezer.

20. Complete the paperwork and tracking documentation for this embryo and blastomere.

21. Repeat steps 10 through 20 until all the embryos have been biopsied.

22. To conclude the procedure, the frozen tubes should be packaged up with ice blocks to keep them cool, and sent to the appropriate lab for analysis.

23. For molecular genetics cases, skip to step 25.

24. Place the biopsied cell into mHTF with protein and pass to the embryologist to do the fixing.

25. The biopsied embryo should be washed twice in mHTF and then placed back in culture medium. It is important that each embryo should spend no more than 5 minutes in the biopsy medium. The embryo should be placed in culture in such a way as there is no possibility of having it confused with subsequent biopsied embryos

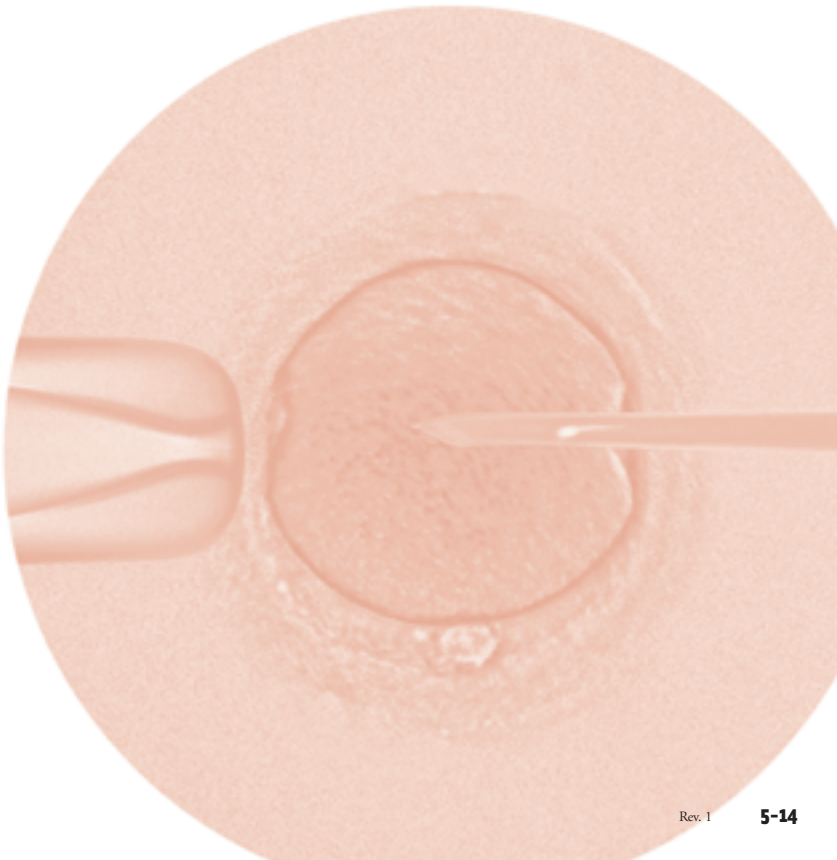
26. Continue to culture the embryos until the results of the biopsy are available, usually 2 more days. Embryo transfer is performed in the normal way.

27. The embryo transfer will happen on Day 5, 2 days after the biopsy.

References

Mark Hughes, 2000, PGD and biopsy protocol.

Trounson, A.O. and Gardner, D.K., 2000, Handbook of *in-vitro* fertilization, CRC Press, New York.



Cryopreservation Protocols

General Considerations:

There are many protocols that have been introduced for different cryopreserva-tion applications including sperm freezing and thawing, slow freezing/rapid thawing for embryos and blastocysts, and vitrification for gametes, and embryos. The ultimate goals of cryopreservation, regardless of cell type, are to reduce intracellular ice-crystal formation, minimize solute effects and toxicity of cryoprotectants and curtail osmotic shock. Cryoprotectants, both permeating and non-permeating, are used to reduce Ice crystal formation by dehydrating the cells before the cooling process. Any potential toxic effects of the cryoprotectants may in turn be minimized by limiting the exposure time or reducing the concentration of cryoprotectants used during the freezing and thawing process. The choice of cryoprotectant and freezing protocol depends on the developmental stage of the gamete or embryo.

All cryopreservation media utilize: a buffered basal medium (typically with HEPES buffer), permeating and non-permeating cryoprotectants, and macro-molecules such as Plasmanate, Human Serum Albumin (HSA), or Serum Substitute Supplement (SSS). The most common permeating cryoprotectants (those that enter the cell) are DMSO, glycerol, ethylene glycol and propanediol. Sucrose is the most commonly utilized non-permeating cryoprotectant which serves to increase the extracellular concentration of solute and regulate the rate and extent of dehydration and rehydration during cooling and warming, respectively.

Irvine Scientific manufactures several types of cryopreservation media and kits for use with sperm, oocytes and embryos:

1) Sperm Freezing Medium - Test Yolk Buffer (TYB) with glycerol & gentamicin (Cat. #90128)
2) Sperm Refrigeration Medium - Test Yolk Buffer (TYB) with gentamicin (Cat. #90129)
3) Sperm Maintenance Medium (Cat. #99276)
4) Embryo Freeze & Thaw Media (Cat. #90116 & 90124)
5) Blastocyst Freeze & Thaw Media (Cat. #90108 & 90110)
6) Vitrification Freeze & Thaw Kits (Cat. #90133 & 90137)

SPERM CRYOPRESERVATION

Sperm freezing offers patients the opportunity to preserve sperm for later use, or for sperm donors to provide specimens for use by others through sperm banks. Patients undergoing cancer therapies or having prostate or testicular surgery may bank their sperm prior to treatment, for subsequent use at a later date. Freezing also allows stored sperm to be used for repeated inseminations via intrauterine insemination (IUI) or *in-vitro* fertilization (by conventional IVF or ICSI). This allows couples to continue therapy for infertility and have sperm available for insemination, even when the male partner may be unable to produce a semen specimen due to surgery, illness or absence. Cryopreservation of sperm has also been used to accumulate multiple samples from a patient with low counts and then combine them to increase the volume and concentration of viable sperm for insemination.

Irvine Scientific manufactures several types of media for sperm cryopreservation, transport and storage.

Cryopreservation Protocols

IRVINE SCIENTIFIC SPERM CRYOPRESERVATION MEDIUM:

Media containing TEST- yolk buffer (TYB) have been shown to be effective in preserving sperm for short term storage when refrigerated, and for long term storage with the addition of glycerol as a cryoprotectant when frozen.

For long-term storage:

- Freezing Medium-Test Yolk Buffer (TYB) with Glycerol & Gentamicin (Cat. #90128) for long term freezing and storage of human sperm. This medium contains 12% (v/v) glycerol and 20% (v/v) heat inactivated egg yolk (inactivated at 56°C for 30 minutes) as well as 10 µg/mL gentamicin sulfate.

- Sperm Maintenance Medium (Cat. #99176) for long term cryopreservation of human sperm. This medium contains a higher concentration of glycerol than Sperm Freezing Medium, with 28% (v/v) glycerol and has 20 mg/mL of Human Serum Albumin also added, but does not contain egg yolk or antibiotics. It may be used in place of Sperm Freezing Medium-TYB. The formulation of Sperm Maintenance Medium with Glycerol is a modified version of Human Sperm Preservation Medium (HSPM) that was first described by Mahadevan and Trounson in 1983. The major formula-tion changes included lowering the osmolarity, stabilizing the buffer system and increasing the albumin and glycerol concentrations.

For short-term storage and transport:

- Sperm Refrigeration Medium-Test Yolk Buffer (TYB) with Gentamicin (Cat. #90129) for short term storage of human sperm at 2 to 5°C before IUI, IVF or ICSI. Sperm may be stored in this medium for a few hours up to 4 days (96 hr) if maintained at 2 to 5°C. It contains 20% (v/v) egg yolk (heat inactivated at 56°C for 30 minutes) and gentamicin sulfate, but does not contain glycerol. This medium can also be used to capacitate sperm during a 2 hour incubation at room temperature.

SEMEN COLLECTION:

General Considerations:

- Semen should be collected by masturbation following two to three days of abstinence. Collection instructions should be given to each patient prior to collection of the specimen. At the time of collection the laboratory should verify that the patient understands the instructions.
- Patient identification at the time of collection should be carefully verified and documented. If the patient has a partner she should also be identified.
- Chain of custody documentation should be maintained in order to trace all steps and individuals handling the sperm during processing.
- Patients should be assigned a unique accession number at the time of collection and that number should be used to identify all tubes and vials used during processing and for storage.
- After collection, the sample should be allowed to liquefy at room temperature or 37°C for 15-30 minutes and a semen analysis should be performed and documented, to include: the sperm counts, motility and morphology.
- Prior to mixing with liquefied semen, one vial of the sperm cryopreservation medium should be brought to room temperature.
- It is important to add the cryopreservation medium to the semen specimen drop-wise over a 30-second time period, and mix it thoroughly after each drop is added to allow for adequate equilibration.

* Note: Sperm counts, motility and morphology should be determined according to standard lab procedures established at each facility.

Sperm Freezing Medium (Cat #90128):

General Considerations:

- Store Freezing Medium-TYB at -10°C or colder.
- Do not expose medium to repeated freeze-thaw cycles. If smaller aliquots are desired, thaw the product, aliquot working volumes into sterile labeled containers, and freeze until needed.
- When stored as directed, Freezing Medium-TYB is stable until the expira-tion date shown on the bottle label.

Freezing Procedure:

1. Take a 5.0mL vial of Freezing Medium-TYB out of the freezer and allow it to come to room temperature (20-25°C).

2. After collection and liquefaction, the semen specimen may be used as a raw specimen or it can be processed with a density gradient and washed with Sperm Washing Medium (Cat. #9983) prior to mixing with freezing medium.

3. Add a comparable volume of freezing medium-TYB to the semen/processed specimen to make a 1:1 (v/v) dilution. Add the freezing medium drop wise over a 30-second period. Mix it thoroughly after each drop is added so it equilibrates adequately.

4. After the entire volume of freezing medium has been added to the semen specimen, allow the mixture to equilibrate for 3 minutes.

5. Transfer/aspirate the final mixture into the cryostraws or cryovials, seal and start the freezing process.

6. The freezing process from room temperature (20-25°C) to -80°C can be accomplished either by a programmable freezing system or by manually assisted vapor phase cooling.
   - For programmagle freezing systems, use according to the manufacturers instruction manual.
   - For the manually assisted vapor phase cooling method, the cryostraws/ cryovials should be attached to the cryocane, and placed in the refrigerator (2-5°C) for one hour, then exposed to liquid nitrogen vapor for 1.5-2.0 hours by either suspending them in the liquid nitrogen stor-age tank above the liquid level or by placing them in the vapor phase in a small temporary liquid nitrogen dewar.

7. The final step should be to transfer the cryostraws/cryovials quickly onto a labeled cane and then into the liquid nitrogen tank for storage.

Sperm Maintenance Medium (Cat #99176):

General considerations:

- Store Sperm Maintenance Medium with Glycerol at -10°C or colder.
- Do not expose medium to repeated freeze-thaw cycles. If smaller aliquots are desired, thaw the product, aliquot working volumes into sterile labeled containers, and freeze until needed.
- When stored as directed, Sperm Maintenance Medium is stable until the expiration date shown on the bottle label.
- The medium does not contain antibiotics. For procedures requiring antibiotics, Gentamicin Sulfate, or Penicillin G and Streptomycin Sulfate may be optionally added prior to use. In all cases, antibiotic usage should be determined by appropriate medical personnel to ensure that the patient is not sensitized to these antibiotics.

Freezing Procedure:

1. Sperm should be collected by masturbation following 2-3 days of abstinence and the sample allowed to liquefy at room temperature or 37°C for 15-30 minutes. Refer to section above: Semen Collection, General Considerations.

2. One vial of a previously prepared aliquot of Sperm Maintenance Medium with Glycerol should be thawed and brought to room temperature or 37°C. If antibiotics are desired they may be added at this step.

3. The liquefied semen sample is transferred to a sterile 15 mL conical centrifuge tube. The specimen volume is determined and an appropriate volume of thawed Sperm Maintenance Medium is added drop-wise until a 3:1 sample to medium ratio is reached. For example, for each 1 mL of specimen add 0.33 mL of medium.

4. Aliquot the sample-medium mixture into labelled cryotubes or straws. To allow for expansion, do not overfill cryotubes.

5. Freeze specimens either directly or after optional slow cooling step (see below) using a programmable freezer or vapor freezing procedure.

6. Optional directions for slow cooling prior to freezing: Attach the filled cryovials to an aluminum cane. Place the cane(s), sample end down, into an ambient temperature (20-25°C) water bath and then place the water bath in a refrigerator (2 to 8°C) for 60 to 90 minutes before freezing.

7. Once the initial pre-freezing has been performed the canes may be frozen using either a programmable freezing system or by manually assisted vapor phase freezing by suspending the canes in liquid nitrogen vapor.

8. Store in appropriately identified locations.

Sperm Refrigeration Medium (Cat. #90129):

General Considerations:

- Store the unopened bottles at -10°C or colder.
- Do not expose medium to repeated freeze thaw cycles. If smaller aliquots are desired, thaw the product, aliquot working volumes into sterile labeled containers and freeze until time of use.
- Refrigeration Medium is stable until the expiration date shown on the bottle or vial label when stored as directed.

Procedure:

1. Semen should be collected by masturbation following 2-3 days of abstinence and the sample allowed to liquefy at room temperature for 37°C for 15-30 minutes. Refer to section above: Semen Collection, General Considerations.

2. One vial of medium can be used per patient. Thaw one vial and bring to room temperature.

3. After the specimen has liquefied, transferred it to a sterile, 15 mL conical centrifuge tube. Determine the volume and add the refrigeration medium dropwise until a 1:1 sample:medium ratio is achieved. Note: Samples displaying high viscosity may require the additional step of passage through an 18 gauge needle or repeated pipetting to ensure thorough mixing.

4. Place the sample-medium mixture into a beaker or other suitable container of 37°C water.

5. Place the container into the refrigerator at 2°C to 5°C and store for a maximum of 96 hours to allow a slow cooling of the mixture (0.5°C/minute).

Thawing of Frozen Sperm

General Considerations:

- For raw semen that was cryopreserved, the specimen should be washed using a density gradient.

- For sperm that was processed using density gradient centrifugation prior to adding it to the Sperm Freezing Medium or Sperm Maintenance Medium, the specimen may be thawed and then simply washed.

Thawing Procedure (for raw semen):

1. Remove frozen specimen(s) from storage tank thaw as follows:
   - For cryovials, allow the vial to warm for at least 15 minutes to room temperature.
   - For cryostraws, allow the straws to thaw for 2 minutes in room air.

   Most laboratories use vials when freezing raw specimens and straw when routinely freezing processed specimens.

2. While waiting for the specimen to thaw, label a 15 mL Falcon tube with the patient name.

3. Layer 1.5-2.0 mL of ISolate lower layer and then and equal volume of ISolate upper layer into the tube using a sterile pipette, according to product insert instructions. Cap tube and place in warming block while specimen thaws.

4. When the specimen has thawed, use a 1 cc pipette to layer the specimen from the cryovial onto the prepared two-layer ISolate gradient.

5. Centrifuge tube at 300 x g for 20 minutes.

6. Then remove the supernatant with a clean pipet, leaving about 500 µL of medium without disturbing to pellet.

7. Using another sterile pipette, add 2 mL of Sperm Washing Medium (Cat. #9983).

8. Centrifuge the tube at 300 x g for 10 minutes.

9. Then remove the supernatant with a clean pipet, leaving about 500 µL of medium and the sperm pellet. Discard supernatant and resuspend the pellet in 2 mL of Sperm Washing Medium.

10. Using a sterile pipette tip perform count and motility on specimen and record values.

11. Place the specimen in a warming block or in a 37°C incubator with the lid tightly closed until time for insemination.

Thawing Procedure (for processed sperm) :

1. Remove the frozen specimen from storage tank thaw as follows:
   - For cryovials, allow the vial to warm for at least 15 minutes to room temperature.
   - For cryostraws, allow the straws to thaw for 2 minutes in room air.

2. Label a 15mL Falcon tube with patient's name. After the specimen has thawed, use a sterile 1 cc pipette to transfer the specimen from the cryovial to the Falcon tube, or cut the cryostraw and allow contents to drain into the Falcon tube.

3. Using another sterile pipette, add 3 mL of Sperm Washing Medium.

4. Centrifuge the tube at 300 x g for 10 minutes.

5. Then remove the supernatant with a clean pipette, leaving about 500 µL of media and the sperm pellet. Discard supernatant and resuspend the pellet in 3 mL of Sperm Washing Medium.

6. Using a sterile pipette tip perform count and motility on specimen and record values.

7. Place the specimen in a warming block or in a 37°C incubator with the lid tightly closed until time for insemination.

EMBRYO CRYOPRESERVATION

Successful embryo cryopreservation is an important tool for the IVF laboratories in order to maximize a patient's IVF cycle. The storage of excess embryos for future transfer allows multiple transfers from one stimulation cycle, thereby reducing the monetary cost to the patient and reducing the potential for complications associated with repeated drug stimulation cycles. The embryo cryopreservation procedure includes exposure of the embryos to cryoprotectants, cooling to sub-zero temperatures, storage in liquid nitrogen tanks, and the later thawing procedures. The resulting pregnancy rates from the transfer of cryopreserved embryos has often been shown to be comparable to that of fresh transfers.

IRVINE SCIENTIFIC CROPRESERVATION MEDIA FOR EMBRYOS:

Irvine Scientific's Cryopreservation Media has been specially formulated for the cryopreservation of human cleavage-stage embryos using a two-step freezing protocol and three-step thawing protocol. Basal medium consists of Modified HTF (with HEPES), 12 mg/mL Human Serum Albumin (HSA) and 10 µg/mL Gentamicin Sulfate.

Embryo Freeze Media Kit (Cat. #90116):

Includes:

- Embryo Freeze Medium - F1 is the 1st step freeze medium contains 1.5 M Propanediol
- Embryo Freeze Medium - F2 is the 2nd step freeze medium contains 1.5 M Propanediol, and 0.1 M sucrose.

Embryo Thaw Media Kit (Cat. #90124):

This kit allows stepwise dilution of the permeating cryoprotectant propanediol and utilizes a sucrose solution that is twice that of the Freeze Media in order to prevent rapid re-expansion of the embryo prior to removal of the propanediol.

- Embryo Thaw Medium - T1 is the 1st step thawing medium and contains 1.0 M Propanediol plus 0.2 M sucrose.
- Embryo Thaw Medium - T2, the 2nd step thaw medium, contains 0.5 M Propanediol and 0.2 M sucrose.
- Embryo Thaw Medium - T3, the 3rd step thaw medium, contains 0.2 M sucrose.

Pronuclear and Multicell Cryopreservation

Critical Points:

- The procedures should be performed at room temperature atmosphere and does not require the use of an oil overlay. However, if a lab chooses to perform the procedures under oil there is no evidence to suggest that this is harmful.
- The success of embryo cryopreservation depends on the accuracy of the freezing and thawing rates as well as on the quality of the embryos chosen for cryopreservation. Only morphologically normal embryos should be cryopreserved.
- Other factors affecting the success of the embryo cryopreservation are the type of cryoprotectant used, the method of cryopreservation used and the developmental stage of the embryos.
- This kit may be used for embryonic stages ranging from the pronuclear through the multicell stage. Pronucleate embryos/zygotes should be frozen 24-28 hours after insemination, prior to syngamy or fusion of the pronuclei. Multicell cryopreservation may be performed on Day 2 or 3.
- CAP inspection in the United States requires redundancy in the main-tanence of cryo information for a patient. In addition, a program must document that they are capable of satisfactorily cryopreserving embryos. Therefore each laboratory should have a QC/QA program that monitors outcomes for freezing embryos to determine whether the program is successful.
- All media should be brought to room temperature before using.
- Verify patient name and identify prior to thawing. Verify number of em-bryos to be thawed.
- Thawing timing will depend on stage of embryos frozen.

Procedure for Freezing Embryos through Day 3:

1. Prepare and label sterile, non-toxic straws. Labeling may be performed by writing on the straws themselves, adding colored plugs to the tops of the straws, or by applying a label to the straw, the plug or a larger straw that is attached to the end of the cryostraw that will contain the embryos.

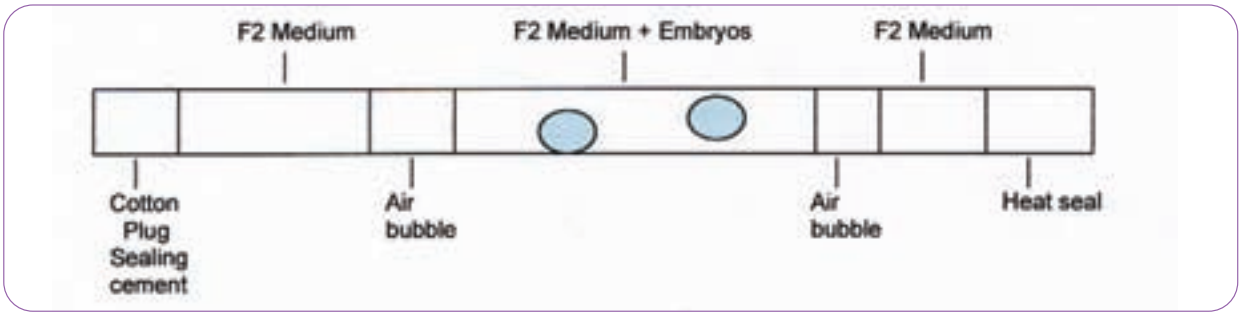
2. Bring the freezing media to room temperature (20-25°C) and aliquot 1mL of each medium (F1 and F2) into pre-labeled independent culture dishes or wells.

3. Identify the morphologically normal embryos to be frozen.

4. Transfer embryos to be frozen (recommend 2 embryos/ straw) to the F1 medium for 10 minutes. Move the embryos about two or three times to rinse them. The embryos will initially shrink and then return to the original size after equilibration.

5. Transfer the embryos to F2 Medium. Allow pronucleate embryos to equilibrate for 2-3 minutes before loading. Load multicellular embros immediately.

6. Load embryos into the pre-rinsed straws shown as following:



7. Place the straws into the freezing chamber and start the freezing program.

8. Cool straws from room temperature to -6°C. Alternatively, the freezing chamber may be pre-cooled and held at -6°C until the straws are ready. Once the straws are placed in the -6°C chamber, they may be held for 5 minutes and then seeded.

9. Manually induce ice nucleation (seed) at -6°C by touching each straw near cotton plug with cold forceps or with a cotton swab that has been chilled by placing it in liquid nitrogen. Hold the embryos at -6°C for a total of 10-15 minutes.
NOTE: When seeding, do not touch the area of the straw that contains the embryos. The formation of ice crystals could damage the embryos.

10. Allow the programmable freezing system to continue the freezing process to -35°C.

11. Remove straws from the freezing unit and plunge into liquid nitrogen, then store in the liquid phase of liquid nitrogen for long term storage.

Procedure for Thawing Early Cleavage Embryos:

1. Remove the straw from the liquid nitrogen tank and hold in ambient tem-perature (20-25°C in air) for 30-40 seconds.

2. Then immerse the straw into a 30-35°C-water bath for 40-50 seconds (until all the ice has been melted).

3. Remove straw from water bath and wipe it gently with a sterile wipe.

4. Cut the plug end first with a pair of sterile scissors and then attach it to a 1mL syringe or other expulsion device.

5. Bring the straw with syringe attached to the top of a sterile dish and then cut the heat sealed end.

6. Gently expel the contents of the straw as a single drop into the sterile dish and quickly locate the embryos.

7. An alternative to this method is to cut the sealed plug at the division between the two plugs. Cut the heat sealed end, insert a red plastic plunger and depress the innermost portion of the plug to the end. The contents should be expelled onto the dry surface of a Falcon dish.

8. Locate the embryos in the drop, pick up them up in a minimal amount of solution with a pulled sterile Pasteur Pipette, or a sterile Stripper tip, and transfer them to 2-3 mL of Embryo Thaw Medium - T1. Incubate the embryos in T1 for 5 minutes.

9. Move the embryos in a minimal amount of medium into 2-3 mL of the Embryo Thaw Medium - T2 and incubate for 5 minutes.

10. Transfer the embryos in a minimal amount of medium into 2-3 mL of the Embryo Thaw Medium - T3 for 10 minutes.

11. Wash the embryos by transferring them in a minimal amount medium into 2-3mL of mHTF Medium supplemented with 12mg/mL HSA for 10 minutes at ambient temperature.

12. Finally, transfer the embryos to fresh culture medium (HTF, P-1 or ECM supplemented with 5 mg/mL of HSA, 10% v/v SSS or 10% v/v DSS). Use HTF or MultiBlast Medium supplemented with protein instead if the embryos were cryopreserved on Day 3). The embryos at this step could be further cultured to blastocyst stage or transferred to the patient's uterus after thawing.

BLASTOCYST CRYOPRESERVATION

The ability to reliably freeze and thaw blastocysts has become increasingly important in recent years. As more programs are trying to reduce the twin rate by taking more and more patients and their embryos on to the blastocyst stage, it has become critical that a laboratory have a healthy blastocyst freezing program. The blastocyst cryopreservation requires different cryopreservation media and procedures as compared to freezing at the early cleavage stages.

IRVINE SCIENTIFIC BLASTOCYST FREEZING MEDIA:

Media specially formulated for the cryopreservation and thawing of human blastocysts uses a two-step freezing and two-step thawing protocol. Basal medium consists of Modified HTF (with HEPES), 12 mg/mL Human Serum Albumin (HSA) and 10 µg/mL Gentanicin Sulfate.

Blastocyst Freeze Media Kit (Cat. #90108):

Includes:

- Blastocyst Freeze Medium - F1 is the 1st step freeze medium contains 5% glycerol
- Blastocyst Freeze Medium - F2 is the 2nd step freeze medium contains 9% glycerol and 0.2 M Sucrose.

Blastocyst Media Thaw Kit (Cat. #90110):

Includes:

- Blastocyst Thaw Medium - T1 is the 1st step thaw medium contains 0.5 M Sucrose.
- Blastocyst Thaw Medium - T2 is the 2nd step thaw medium contains 0.2 M Sucrose.

Blastocyst Freezing

Critical Points:

- The procedures should be performed in ambient atmosphere.
- The success of blastocyst cryopreservation depends on the accuracy of the freezing and thawing rates as well as the high quality of the blastocyst chosen for cryopreservation. Only high quality of the blastocyst should undergo cryopreservation.
- All media should be brought to room temperature before using.
- Verify patient name and identify prior to thawing. Verify number of em-bryos to be thawed.
- Some programs have reported improved survival rates when culturing blastocysts in media that contains 20% SSS or at least 12 mgs/mL after thawing and before transfer.

- Some programs have also reported that artificially collapsing the blastocoel cavity of the embryo before freezing also improves survival of the embryo, Collapsing the cavity may be done by using a PZD needle and inserting it from right to left through the blastocyst in an area removed from the inner cell mass. Another method that has been employed is to pull the blastocyst up and down in decreasing sizes of stripper tips during the incubation in the first freezing media solution.

Procedure for Freezing Blastocysts:

1. Prepare and label sterile, non-toxic straws for freezing by rinsing them with the Blastocyst Freeze Medium - F2.
2. Bring the freezing media to room temperature (20-25°C) and aliquot 1mL of each medium (F1 and F2) into pre-labeled independent culture dishes or wells. Some programs have reported that they begin blastocyst freezing with the first solution at 37°C; once the blasts have been placed in this solution the temperature is then allowed to come to room temperature. This may expedite absorption of the glycerol into the expanded blastocoel.
3. Chose the fully expanded, high quality blastocysts for freezing.
4. Transfer blastocysts to be frozen (recommend 2 blastocysts/straw) in minimal volume of culture medium into the F1 medium for 10 minutes. The blastocysts will initially shrink and then return to the normal size af-ter equilibration, unless the blastocoel has been artificially collapsed. In this case re-expansion should not be observed.
5. Transfer blastocysts to the Blastocyst Freeze Medium - F2 for 10 minutes.
6. Load blastocysts into the pre-rinsed straws by following the diagram shown in the embryo freezing procedure.
7. Place the straws into the freezing chamber and start the freezing program.
8. Cool straws from room temperature to -6°C, or begin the freezing program by loading the straws into a chamber that has been pre-cooled to -6°C.
9. Manually induce ice nucleation (seed) at -6°C by touching each straw near cotton plug with cold forceps. Hold them at -6°C for a total of 10-15 minutes.
10. Allow the programmable freezing system to continue the freezing process to -35°C.
11. Remove straws and plunge into liquid nitrogen, then store in the liquid phase of liquid nitrogen for long term storage.

Procedure for Thawing Blastocysts:

1. Remove the straw from the liquid nitrogen tank and thaw them in ambient temperature (20-25°C in air) for 30 seconds.
2. Immerse straw in a 30-35°C water bath for 45 seconds (until all the ice has been melted).
3. Remove straw from water bath and wipe it gently with a sterile wipe.
4. Cut the plug end first with a pair of sterile scissors and then attach it to a 1mL syringe. Or cut the plug half way up between the two sections of the plug and insert the red plastic plunger.
5. Bring the straw to the top of a sterile dish and cut the heat sealed end.

6. Gently expel the contents of the straw as a single drop into a sterile dish and quickly locate the blastocysts.

7. Pick the blastocysts up in a minimal amount of solution and transfer them into 2-3 mL of Blastocyst Thaw Medium T1 for 10 minutes.

8. Transfer the embryos in a minimal amount of T1 into 2-3 mL of the Blastocyst Thaw Medium T2 for another 10 minutes.

9. Wash the blastocysts by transferring them in a minimal amount of medium into 2-3 mL of Modified HTF Medium supplemented with 12mg/mL HSA for 10 minutes at ambient temperature.

10. Transfer into a dish with culture media and 12 mg/mL HAS or 20% SSS until transfer.

VITRIFICATION OF OOCYTES/ EMBRYOS/ BLASTOCYSTS

Vitrification is an ultra-rapid freezing method in which human oocytes, early cleavage embryos or blastocysts suspended in a high concentration of cryoprotectant solution are plunged directly into liquid nitrogen. The thawing protocol is also an ultra-rapid thawing method by removing the frozen specimens from liquid nitrogen and plunging directly into 37°C waterbath.

FREEZING OF OOCYTES AND EMBRYOS:

IRVINE SCIENTIFIC VITRIFICATION FREEZE KIT (Cat. #90133):

The Vitrification Freeze Kit is intended for use in assisted reproductive procedures for vitrification of human oocytes (MII) and embryos (2PN to blastocyst). The CryoTip (the specimen carrier device) is designed to be a closed system to prevent potential viral contamination of the oocytes/embryos from liquid nitrogen as both ends of the device are securely sealed before plunging into liquid nitrogen. The Kit includes:

- Equilibration Solution-ES: a HEPES buffered solution of Medium-199 containing 7.5% (v/v) of each DMSO and ethylene glycol and 20% (v/v) Serum Substitute Supplement. This solution is packaged in the tubes with the white labels and tops.
- Vitrification Solution-VS: a HEPES buffered solution of Medium-199 containing 15% (v/v) of each DMSO and ethylene glycol, 20% (v/v) Serum Substitute Supplement and 0.5 M sucrose. This solution is packaged in the tubes with the blue labels and tops.
- CryoTips: with metal cover sleeve (stores up to 2 specimens)
- Connectors: 2 connectors are provided that will allow attachment of the Cryotip to a number of aspiration tools.

Using the Vitrification Freeze Media Kit:

Critical Points:

- The ES and VS solutions are to be used in sequence for the step-wise microdrop vitrification protocol.
- Incubation time in ES and VS solutions should be monitored closely. The high concentration of the cryoprotectants will cause toxicity to the specimens if the incubation time is extended for too long.
- The kits are designed to be used at room temperature and without oil.
- Recommended drop size is 20 µL.

- It is strongly recommended to practice the sealing and expelling processes before starting the vitrification protocol.
- Great caution should be exercised when handling liquid nitrogen during the vitrification process.
- A variety of suction devices have proven effective at loading the CryoTip. Each laboratory may find that they are comfortable with their own version of a suction device. Some alternatives include: 250 µL Hamilton gas tight syringes, 1 cc Airtight syringes, Eppendorf or Gilson repipettors, or the "Mushroom" microinjectors.
- Before using a CryoTip, slide the metal sleeve up and down to make sure it moves freely along the CryoTip.
- Once embryos or oocytes are vitrified they cannot be warmed without immediate thawing of the CryoTip. Therefore, care should be taken to ensure that the tips are never exposed to ambient air temperatures until the time of thawing.

Procedure for Vitrification of Embryos (2PN to Blastocyst):

1. Bring one vial of each ES and VS to room temperature (20-25°C) for at least 30 minutes prior to vitrification of specimens.

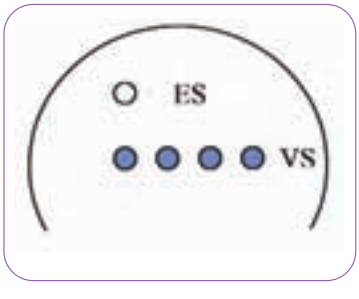
2. Fill the liquid nitrogen reservoir with liquid nitrogen (at least 4 inches in depth) and place it close to the microscope. Attach a 4.5 mL cryotube (uncapped) or a cryogoblet to the bottom clamp of a cryocane and submerge in the liquid nitrogen in preparation for storage of the vitrified specimens.

3. Determine the number of embryos to be vitrified.

4. NOTE: 1-2 embryos may be loaded into each CryoTip.

5. Label each sterile culture dish and CyroTip with the patient's ID. One-half cc cryostraws may also be labeled and used as a handle for the CryoTips as the larger cryostraw will slide easily down over the wider section of the CryoTip and over the pinched upper end of the metal sleeve once it has been pulled down to cover the tip of the CryoTip. Additional labels can then be attached to the 1/2 cc cryostraws, or colored plugs may be added to the opposite, open end.

6. Mix one vial of ES content and one vial of VS content by gently inverting the vial twice. Then dispense a series of five 20 µL micro-drops on a dish as follows:
   - One x 20 µL drop of ES (white topped tube; see standard vitrification protocol diagram for embryos)
   - Four x 20 µL drops of VS (blue topped tube).



7. Remove the culture dishes with the embryos from the incubator and check the growth and quality of the embryos under the microscope. Select the best embryos for vitrification.

8. Draw up some ES solution from the ES drop with the transfer pipette. Then move the embryos (no more than the number to be placed in one CryoTip at a time) with a minimal volume of medium from the culture dish to the top of the drop of the ES and start the timer. Watch the embryos. Once they have shrunken and then re-expanded they may be moved to the VS drops. Pronuclear embryos will take less time to shrink (probably less than 5 minutes, although this is patient and embryo dependent) and re-expand than blastocysts (10-15 minutes). Blastocysts may not fully re-expand.

9. During the equilibration time, prepare the CryoTip: carefully attach the larger end of the tip to the connector on the suction device, then slide the metal cover sleeve toward the larger end to expose the fine tip end. Avoid touching or bending the fine tip end while handling the CryoTip.

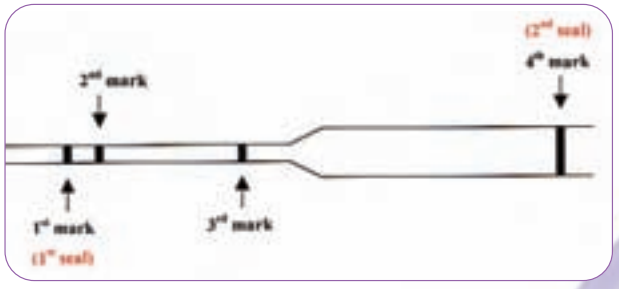
The following steps should be completed in 90 seconds; exposure of the embryos to VS should be limited to prevent cytotoxicity.

10. After the equilibration of embryos is complete, draw up some ES into the transfer pipette and transfer the specimen with minimal volume from the drop of ES into the bottom of the first drop of VS (VS1) for 5 seconds. The embryos will rise to the surface of the drop immediately. This step should be observed under a magnification level that allows the observer to follow this movement of the embryos to the top of the drop.

11. Quickly transfer the specimen from the first drop of VS to the bottom of the second drop of VS (VS2) for 5 seconds and follow their rise to the top of the drop.

12. Then transfer the specimen to the bottom of the third drop of VS (VS3) for 10 seconds.

13. Finally transfer the embryos to the fourth drop of VS (VS4). Immediately Load and heat seal the CryoTip as follows:
    - While observing under the microscope, carefully aspirate a small volume of VS to the 1st mark on the CryoTip.
    - Continue observation under the microscope and gently aspirate the specimen with VS to the 2nd mark on the CryoTip.
    - Now observe the CryoTip directly from the side and aspirate more VS to the 3rd mark.



    - Heat seal the CryoTip on the 1st mark with the heat sealer set on 2-3, carefully slide the metal cover sleeve toward the fine tip end to cover the fine tip completely, remove the aspiration syringe and then heat seal at the other (thick) end of the CryoTip above the 4th mark with the heat sealer set at 5-6.

14. Attach the 1/2 cc cryostraw at this time, if using it. Hold the covered CryoTip with tweezers and immerse it, metal end first, directly into liquid nitrogen.

15. Place the covered tip into the submerged liquid nitrogen filled 3.6 mL cryotube or goblet on the cryocane.

16. Repeat these steps for remaining embryos to be vitrified.

17. Move the liquid nitrogen reservoir close to the liquid nitrogen tank and transfer the cryocane and its contents into the liquid nitrogen tank for long term storage.

Procedure for Vitrification of Oocytes:

1. After warming the vials of ES and VS to room temperature, set up the vitrification dish as shown in Figure 1. Add one drop of culture medium (CM; HEPES buffered media containing 20% SSS or 12 mg/mL HSA) and three drops of ES, (20 µL for each). Place the CM drop and the first two drops of the ES solution (solutions from the white top tubes) in close proximity to each other. The third ES drop may be placed a further distance away from the group.

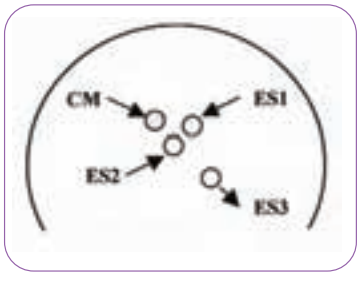


FIGURE 1

2. Transfer the oocytes from main culture dish into the 20 µL drop of culture medium (CM). Connect the drop of ES1 to the CM drop (See Figure 2, arrow 1), moving from the ES1 drop toward the CM drop, with the tip of the transfer pipette and allow gradual mixing of the two solutions to occur for 2 minutes. Then connect the drop of ES2 (arrow 2) to the merged drop of CM and ES1 (arrow 2). Allow gradual mixing of the 3 solutions to occur for 2 more minutes. Then transfer the oocytes from the combined drops to the bottom of the ES3 drop for 3 minutes using a pulled Pasteur Pipette or a stripper tip of the appropriate size.

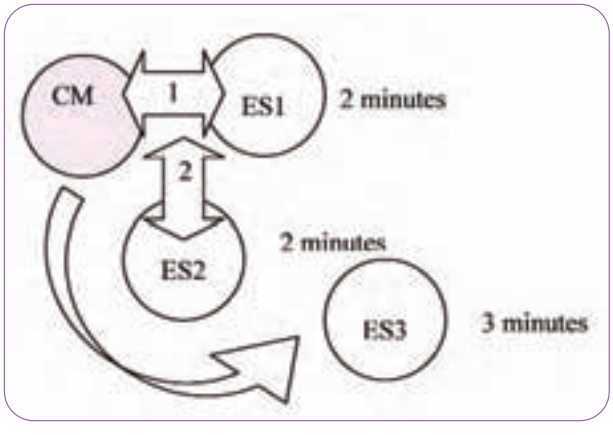


FIGURE 2

3. During the 3 minutes waiting time set up 4 drops of VS solution as shown on Figure 3 below:

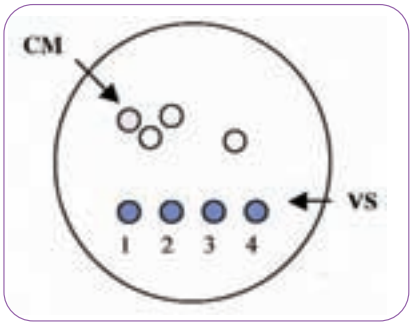


FIGURE 3

4. Transfer the oocyte to the BOTTOM of the first drop of VS (VS1) for 5 seconds, counting mentally. After 5 seconds transfer the oocytes to the second drop where it will remain for 5 seconds, then to the third drop for 10 seconds and finally into the last drop. As with embryos, oocytes will also float to the top of the drop. Therefore observe the moving of the oocytes through the drops from a low enough power to be able to locate the oocytes at the top of the drop after placing them at the bottom. Once the oocytes are in the final drop they should immediately be loaded into the CryoTip, the tip sealed and then plunged into the liquid nitrogen. Exposure to VS, loading tip and plunging in LN2 should be completed within 90 seconds.

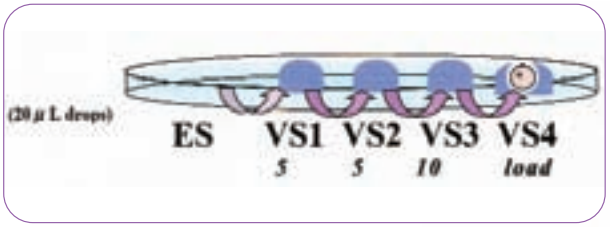
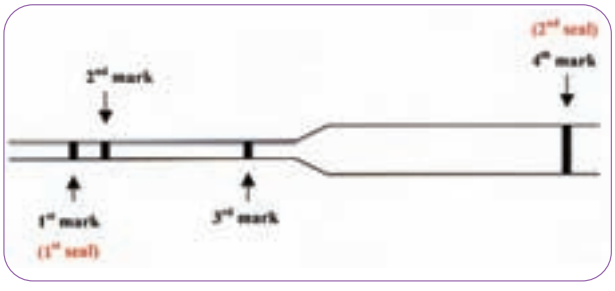


FIGURE 4

5. Load and heat seal the CryoTip as follows:
   - While observing under the microscope, carefully aspirate a small volume of VS to the 1st mark on the CryoTip.
   - Continue observation under the microscope and gently aspirate the specimen with VS to the 2nd mark on the CryoTip.
   - Now observe the CryoTip directly from the side and aspirate more VS to the 3rd mark.



   - Heat seal the CryoTip on the 1st mark with the heat sealer set on 2-3, carefully slide the metal cover sleeve toward the fine tip end to cover the fine tip completely, remove the aspiration syringe and then heat seal at the other (thick) end of the CryoTip above the 4th mark with the heat sealer set at 5-6.

6. Attach the 1/2 cc cryostraw at this time, if using it. Hold the covered CryoTip with tweezers and immerse it, metal end first, directly into liquid nitrogen.

7. Place the covered tip into the submerged liquid nitrogen filled 3.6 mL cryotube or goblet on the cryocane.

8. Repeat these steps for remaining oocytess to be vitrified.

9. Move the liquid nitrogen reservoir close to the liquid nitrogen tank and transfer the cryocane and its contents into the liquid nitrogen tank for long term storage.

Warming Vitrified Oocytes and Embryos

IRVINE SCIENTIFIC VITRIFICATION THAW MEDIA KIT (Cat # 90137)

Vitrification Thaw Media Kit is intended for use in assisted reproductive procedures for thawing and recovery of human oocytes (MII) and embryos (2PN to blastocyst) that have been vitrified using Irvine Scientific's Vitrification Freeze Media Kit. The product includes:

- Thawing Solution-TS is a HEPES buffered solution of Medium-199 containg 1.0 M sucrose and 20% (v/v) Serum Substitute Supplement. This solution is packaged in the tubes with the yellow labels and tops.
- Dilution Solution-DS is a HEPES buffered solution of Medium-199 containg 0.5 M sucrose and 20% (v/v) Serum Substitute Supplement. This solution is packaged in the tubes with the orange labels and tops.
- Washing Solution-WS is a HEPES buffered solution of Medium-199 containg 20% (v/v) Serum Substitute Supplement. This solution is packaged in the tubes with the red labels and tops.

Using the Vitrification Thaw Media Kit

Critical Points:

- Great caution should be exercised when handling liquid nitrogen during the vitrification process.
- The CryoTip is very thin and the solution in it will thaw within 1-3 seconds. Therefore, do not expose the tip to room air until ready to thaw. This means that all transport or transfer of the embryos from one vessel to another should be done under liquid nitrogen.
- A variety of pressure devices may be used to expel the embryos from the CryoTip. These include, but are not limited to, 250 µL Gas-tight Hamilton syringes, 1cc Airtight syringes, Eppendorf or Gilson re-pipettors, or "Mushroom" microinjectors. Each lab should determine which device works best for them.
- It is very important to cut the thin end (where embryos located) on top of the dish since the pressure inside the tip after thawing will push out the contents after cutting. Cutting the tip should be done using very fine scissors and a quick cutting motion. Check the blades of the scissors after cutting to make sure that no fluid from the tip has accumulated on the blades. If no oocytes/embryos are found in the expelled solution, then the fluid on the blade may contain the oocytes/embryos and should be washed off and collected in a small volume of TS.
- Bubbles are detrimental to fragile thawed embryos. Care should be taken to avoid the formation of bubbles while pushing out the contents from the tip.
- All drops are 20 µL and thawing should be done with drops at room temperature and without an oil overlay.
- Allow 3 hours for oocytes recovery after thawing before performing ICSI to inseminate the oocytes.

Procedure for Thawing of Oocytes and Embryos (2PN to Blastocyst):

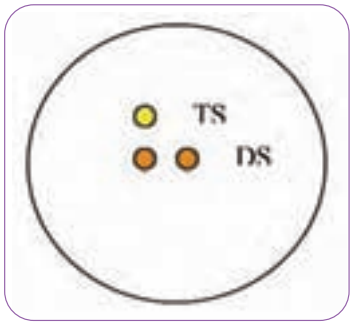
1. Bring one vial each of Thaw Solution (TS; yellow top), Dilution Solution (DS; orange top) and Washing Solution (WS; red top) to room temperature (20-27ºC) for at least 30 minutes prior to thawing the vitrified embryos.

2. Fill the liquid nitrogen reservoir with liquid nitrogen (~80% full), and place it close to the liquid nitrogen tank.

3. Remove the canes containing the cryovials with the vitrified specimens from the liquid nitrogen tank and transfer them into the liquid nitrogen reservoir and then place it close to the microscope for rapid manipulation.

4. Label a sterile culture dish with necessary information and prepare the dish with the thaw media.

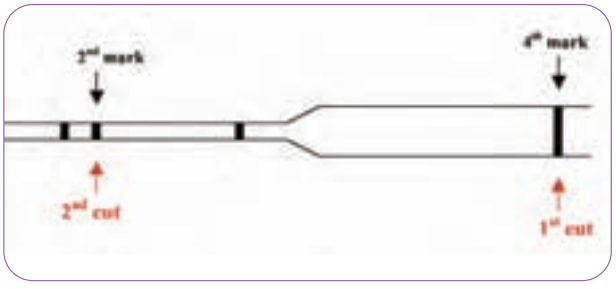
5. Mix each vial of TS, DS and WS by gently inverting twice, then aseptically dispense a sequence of 3 micro drops on a sterile culture dish as follows (See procedural diagram for thawing protocol).
   - One-20 µL drop of TS and
   - Two-20 µL drops (40 µL) of DS and place the dish under the microscope



6. Place the 37ºC water bath close to the microscope. Have a transfer pipette, a pair of scissors, syringe with connector, timer and sterile wipes ready.

7. Using tweezers retrieve the covered CryoTip from the cane in liquid nitrogen reservoir, quickly immerse it into the 37ºC water bath and swirl gently for 3 seconds to thaw.

8. Gently wipe the CryoTip with a sterile wipe and cut the seal on the thick end on the 4th mark on the tip. Attach the thick end to the connector and the syringe, or other expulsion device. Slide the metal cover sleeve toward the thick end to expose the fine tip. Gently wipe the fine tip with a sterile wipe. With the fine tip positioned over the thawing dish, cut the seal on the 2nd mark of the fine tip end and dispense the contents of the CryoTip as a small drop with the embryos onto a dry region of the dish near, but not in, the TS drop. Note: avoid bubbles while dispensing the contents. Oocytes/embryos should immediately be visible in this drop.



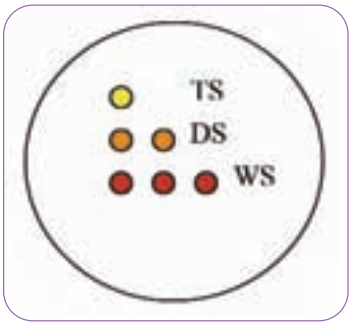
The following steps should be done using a timer. After each embryo transfer, blow out the contents of the transfer pipette prior to the next manipulation. Avoid creating bubbles during the transfers.

9. Using the CryoTip, pull up an equivalent volume (~1 µL) of TS, and place it immediately adjacent to the already expelled contents of the CryoTip that contains the oocytes or embryos (without touching the drop). Then connect the two drops with the pipette tip and wait for one minute. If no oocytes/embryos are visible in this time, check the blades of the scissors. If no solution could be expelled from the tip then the tip may not have been cut far enough back to completely remove the seal. Examine the tip under the scope to make sure that the seal has been cut away.

10. Draw up some TS into the transfer pipette and transfer the oocytes or embryos with minimal volume into the bottom of the drop of TS (20 µL drop) for one minute. Transferring the oocytes or embryos from this point on may be done using a pulled Pasteur Pipette, or a stripper tip of the appropriate size. Note: The cells will shrink and float to the top of the drop.

11. Draw up some DS into the transfer pipette and transfer the oocytes or embryos from the drop of TS with minimal volume, to the bottom of the first drop of DS (DS1) for two minutes.

12. Then transfer them to the bottom of the second drop of DS (DS2) for two minutes.
    Note: During exposure to DS the cells will remain shrunken. During this two minutes waiting period, dispense a sequence of 3 micro drops (20 µL each) of WS on the same dish.



13. Transfer the oocytes/embryos to the bottom of the first drop of WS (WS1) for three minutes.
    Note: They should re-expand to the original size within three minutes in WS.

14. Then transfer the oocyes/embryos to the top of the second drop of WS (WS2) for three minutes.

15. Transfer them to the top of the third drop of WS (WS3) for three minutes.

16. Finally, transfer them to a prepared dish containing the appropriate culture medium. Incubate the oocytes for 3-4 hours to let them recover prior to injection. Embryos may also be held prior transfer or further culture procedures after warming.

Note: This same protocol is applicable for use with oocytes or embryos. However, when using oocytes they must be allowed to recover for at least 3 hours prior to insemination to allow for reformation of the spindle apparatus. Insemination must be performed by ICSI as zona hardening due to release of the cortical granules occurs during vitrification just as it does during slow cooling procedures.

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