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Establishing a Biologic Specimens Repository for Reproductive Clinical Trials: Technical Aspects

Stephen A. Krawetz[1,*], **Peter R. Casson**[2], **Michael P. Diamond**[1], **Heping Zhang**[3], **Richard S. Legro**[4], **William D. Schlaff**[5], **Christos Coutifaris**[6], **Robert G. Brzyski**[7], **Gregory M. Christman**[8], **Nanette Santoro**[5], **Esther Eisenberg**[9], and **for the Reproductive Medicine Network**

[1]Department of Obstetrics and Gynecology, Wayne State University, Detroit, MI, USA

[2]Department of Medicine and Obstetrics and Gynecology, University of Vermont, Burlington, VT, USA

[3]Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, CT, USA

[4]Department of Obstetrics and Gynecology, Pennsylvania State University, Hershey, PA, USA

[5]Department of Obstetrics and Gynecology, University of Colorado, Denver, CO, USA

[6]University of Pennsylvania, Philadelphia, PA, USA

[7]Department of Obstetrics and Gynecology, The University of Texas Health Science Center at San Antonio, TX, USA

[8]Department of Obstetrics and Gynecology, Reproductive Sciences Program, University of Michigan, Ann Arbor, MI

[9]Reproductive Sciences Branch, National Institute of Child Health and Human Development, Bethesda, MD, USA

Abstract

The individual research group or independent investigator often requires access to samples from a unique well characterized subject population. Cohorts of such samples from a well-defined comparative population are rare and limited access can impede progress. This bottleneck can be removed by accessing the samples provided by biorepositories such as the NIH/NICHD Cooperative Reproductive Medicine Network (RMN) Biorepository (detailed in the accompanying manuscript in this issue. In those cases where the individual research group or independent investigator already has access to a unique population, comparisons between well-defined groups are often sought to contextualize the data. In both cases seamless integration of data resources associated with the samples is required to ensure optimal comparisons. At the most basic level this requires standardization of sample collection and storage, as well as a de-identified data base containing demographic, clinical, and laboratory values. To facilitate such interoperability, the reagents and protocols that have been adopted by the RMN Biorepository for the collection and storage of serum, blood, saliva and sperm are described.

[*]Address correspondence to Stephen A. Krawetz, Ph.D., Charlotte B. Failing Professor of Prenatal/Fetal Therapy and Diagnosis, Associate Director, C.S. Mott Center for Human Grwoth and Development, Department of Obstetrics and Gynecology, Center for Molecular Medicine and Genetics, 275 E. Hancock, Detroit, MI, 48201. steve@compbio.med.wayne.edu.

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Keywords

biorepository; repository; Reproductive Medicine Network

RMN Biorepository Protocols for the Collection of Serum, Blood Clots, Whole Blood (DNA), Filter Paper Blood Spots, Saliva, and Sperm Samples

The Reproductive Medicine Network (RMN) is comprised of a nationwide series of clinical sites and a data coordination center (DCC). It is charged with designing, implementing, and publishing the results of high quality clinical research in reproductive medicine. Various specimens have been and continue to be collected as part of the ongoing research activities of this network. Additional aliquots of samples are often retained for additional analyses and new tests as they are introduced. Specimen collection is conducted under the auspices of each local Institutional Review Board (IRB), with attainment of participants' consent detailing whether use will be allowed for future projects that are either related to the initial project and/or other fields of medicine as outlined in the accompanying manuscript Casson et al. 2011. Because of the ongoing rapid advance of genome technologies such consent must be mindful of whether genetic information could be collected and used.

Samples derived from randomized controlled trials in well-defined populations, represent a valuable resource for future research endeavors, both inside and outside the RMN. With the increased availability and capabilities of the rapidly evolving genome-wide-technologies, the biology of the system underlying the trial results now becomes accessible. Progress is often first revealed with the introduction of a suite of biomarkers [Sistare et al. 2010]. These advances are poised to markedly change clinical practice as we know it today while moving towards personalized systems medicine.

Sample acquisition protocols along with repositories continue to be described [Ayers et al. 2007; Ennis et al. 2010; Gohagan et al. 2000; Ugolini et al. 2008]. One is referred to best practice resources of the International Society for Biological and Environmental Repositories [ISBER 2008] and the National Cancer Institute Best Practices for Biospecimen Resources [National Cancer Institute 2007]. Within this framework, the RMN established a biorepository that provides access to a source of clinically well-characterized human reproductive samples. These samples will be collected as part of three studies, which will be complemented by biorepository specimen storage as summarized in Table 1. All will have blood specimens collected including card blood spots, whole blood, serum, and the residual blood clot. Additionally, the Assessment of Multiple Intrauterine Gestations From Ovarian Stimulation (AMIGOS) trial will collect semen for biorepository storage. Anonymized specifics of the study populations and clinical characteristics can be obtained from the DCC. It is projected that in total this will include at least 10,000 serum samples, 15,000 samples of whole blood, 7,500 FTA blood spot cards, 1,000 Oragene® recovered saliva samples, and 7,500 sperm samples. A description of the methods by which the samples are being collected and stored is outlined below in this Applications Note.

Before initiating a collection

Prior to beginning any collection of human specimens for research and/or when beginning any novel research protocol, approval must be obtained from the local IRB committee [Baranzini et al. 2010; Roach et al. 2010]. Informed consent by participants must then be obtained before collecting any biological samples for research purposes and personnel must be appropriately trained in sample collection and personal biosafety. As discussed in the Casson et al. [2011] paper, it cannot be overemphasized that the informed consent must be

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robust as to withstand continued scrutiny of the IRB regarding the use of these samples as our ability to extract other information continues to grow. In addition, the shipment of biological specimens must be certified and certification programs are available through Saf-T-Pak (http://www.saftpak.com). Compliance is federally mandated.

Collection of Whole Blood

The protocols were adopted from standard clinical practice to ensure ease of implementation and compliance across collection sites. All procedures are carried out at room temperature unless otherwise noted. While the site-specific protocol may vary slightly, maintenance of cellular integrity is maintained and thus non-biological variance is assumed to be minimal. Similarly, although not definitive, there is no reason to expect that this methodology will alter the epigenetic signature in an unusual manner compared to standard clinical practice. Although the primary objective of the RMN's sample collection is to utilize whole blood for DNA extraction, samples retrieved and stored in this manner could also provide a source of proteins, RNA, and metabolites. Routine methods of cellular recovery include venipuncture, heel stick, and buccal swab. Venipuncture is typically used for adult collections compared to buccal swabs for infants and children. While heel sticks are an integral part of standard newborn screening programs, their primary use is dedicated to providing early assessment and a baseline of newborn health status immediately following birth. Logistically, buccal swab collection may be preferred as the samples are directly purposed. Accordingly, the DNA prepared from these samples should be suitable for most analyses including, SNP, genotyping, and methylation analysis as well as direct sequencing which is becoming economically feasible. FTA cards were adopted as a medium since they offered long-term stable cost effective room temperature storage in minimal space. They have even been used previously to store cervical cells [Gustavsson et al. 2009] and provide a means to immediately freeze the sample in biological time, thus preserving integrity.

PROTOCOL COLLECTION OF WHOLE BLOOD

1. Follow your institution's best practices for venipuncture.
2. Collect blood in the appropriately labeled purple top (EDTA) tubes. Sodium citrate vacutainer tubes may provide an alternative but their use by the Reproductive Medicine Network has not been standardized. Avoid using vacutainer tubes containing heparin as traces of heparin can compromise subsequent nucleic acid protocols.
3. Invert tubes 8 – 10 times to thoroughly mix. This is critical to ensure high quality DNA extraction as EDTA inhibits metal activated nucleases and clotting.
4. Place filled tubes in a test tube rack and allow the tube to stand at room temperature for 10 minutes.
5. Using a disposable Pasteur pipette, pipette out 4 × 125 μL aliquots and spot each aliquot on one of the four sections of the FTA classic collection card. Three drops for each spot. Note each drop from a Pasteur pipette is considered to contain approximately 50 μL. The likely excess volume is not a concern for this application.
6. Label the FTA classic collection card. Use a barcode if possible as it facilitates sample tracking. Let the sample air-dry 1 hour to overnight before processing further.
7. While waiting for the FTA cards to dry, aliquot the remaining whole blood into approximately equal aliquots placing each in a 4.5 mL Nunc cryovial using the disposable pipette.

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8. Label the cryovials and use a barcode if possible.
9. Store cryovials at −80°C.
10. Store FTA collection cards at −20°C in a pouch with a desiccant pack.
11. Place the appropriate associated sample information in the sample data base.

Collection of Serum and Blood Clots

Both serum and blood clots are also being recovered. On one hand, serum should provide an additional source of metabolite-enriched biomarkers. Its utility as a tool to predict preeclampsia, obstetrical complications, and Assisted Reproductive Technology outcomes is currently being explored [Gagnon et al. 2008; Grill et al. 2009; La Marca et al. 2010]. One must be careful to avoid hemolysis. This can compromise subsequent analyses. Serum samples appearing pink or red should be noted and if possible an additional specimen collected. On the other hand, blood clots that are typically discarded provide an additional source of DNA [Iovannisci et al. 2006; McCulloch et al. 2009]. The blood clot can be placed in reserve in case of catastrophic failure, or used in place of the blood samples.

PROTOCOL COLLECTION OF SERUM AND BLOOD CLOTS

1. The samples should be collected after fasting overnight, typically between 7:00 AM and 10:00 AM. Follow your institution's best practices for venipuncture.
2. Collect blood in the appropriately labeled red top (silicone-coated interior) vacutainer tube. The silicone interior promotes clotting.
3. Invert tubes 5 times to thoroughly mix and then allow tubes to stand at room temperature for at least 30 minutes, but not greater than 1 hour. This will minimize the release of cellular components into the sera [Timms et al. 2007].
4. Centrifuge at 3,000 × g for five minutes to recover the serum and note color. Samples with a pink or red hue indicate hemolysis and, if possible, an additional sample should be collected.
5. Label 1.0 mL Nunc cryovials indicating a serum sample. Use a barcode if possible as it facilitates sample tracking.
6. Using the disposable pipette, aliquot 0.5 mL of serum into each 1.0 mL cryovial until all of the serum is transferred. Store cryovial at −80°C.
7. Transfer the remaining blood clots from each red top tube into the 15 mL centrifuge tube. This provides an additional source from which DNA can be extracted. Label the centrifuge tube with a sample identifier. Use a barcode if possible.
8. Store the sample at −80°C.
9. Place the appropriate information associated with that sample in the sample data base.

Collection of Sperm Samples

Sperm are being collected as part of the evaluation of the reproductive function of the couple in addition to blood and sera. The manner in which the spermatozoa are being prepared and stored as described below has proven suitable for long-term storage. Maintaining the integrity and viability of the spermatozoa has enabled recovery of both biological functions well suited to fertilization (reviewed in [Pasqualotto et al. 2009]) and functional full-length RNAs [Goodrich et al. 2007; Ostermeier et al. 2005]. Spermatozoa prepared and stored as

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outlined below should be suitable for the current range of functional assays as well as new assays as they become available (reviewed in [Lewis et al. 2008]).

PROTOCOL COLLECTION OF SPERM

1. Obtain a fresh semen sample in a wide mouth sterile specimen cup via masturbation after 3 – 5 days of abstinence. Note days of abstinence and whether any specimen was lost during collection.
2. The sperm sample is kept at room temperature for 10 – 30 minutes to liquefy. Upon liquefaction record volume and proceed to determine the concentration of sperm.
3. Using a Neubauer counting chamber (others are available including Cellview, Makler, Thoma-Zeiß, and Bürker-Türk) pipette 10 μL of the evenly dispersed semen sample into one of the chambers [Ludwig and Frick 1990]. The chamber will fill by capillary action.
4. The number of sperm observed in 5 of the 25 large squares of the central square of the grid is determined. The total number of sperm per mL is calculated as the number of sperm counted × n/25 (n = number of squares counted) × 10,000 (volume of field) × dilution factor.
5. Motility is then determined using one of the many automated systems that are available.
6. The remaining liquefied sperm sample is prepared as a stock by combining with 1/3 volume Irvine Scientific test-yolk/glycerol buffer [Prins and Weidel 1986; Weidel and Prins 1987]. The resulting mixture is incubated at room temperature for 5 minutes. An additional 1/3 volume of Irvine Scientific test-yolk/glycerol buffer is added then incubated at room temperature for 5 minutes. Upon the final addition an equal volume of Irvine Scientific test-yolk/glycerol buffer will have been added.
7. The stock sample is mixed then approximately 10^7 sperm are aliquoted into separate 1.2 mL Nunc cryovials. The stock sample is mixed after each aliquot to ensure an equal number of cells in each cryovial. The vials are then labeled with a sample identifier and those containing less than 10^7 sperm noted. Use a barcode if possible.
8. The vials are then placed in a floating rack and the rack placed in a 4°C water bath for 90 minutes such that the sperm are submerged.
9. The cryovials are dried then attached to cryocanes and placed into the vapor phase of a charged liquid nitrogen dry shipper. After 60 minutes the samples are plunged and stored in liquid nitrogen.
10. Place the appropriate information associated with that sample in the sample data base.

Collection of Saliva Samples

To ensure a complete study of reproductive outcomes, samples can be collected from the newborn and DNA recovered. The use of a saliva/buccal swab is now being adopted as standard practice in neonatal screening to avoid venipuncture or heel stick. Saliva can also serve as a noninvasive method of specimen collection in children and adults. It has already proven successful in various mutation screens [Doyle et al. 2004; Pawlowski et al. 2008]. In conjunction with the other biological samples obtained above, parent of origin effects can be identified.

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PROTOCOL COLLECTION OF SALIVA

Newborns

1. Firmly grip an Oragene® swab without touching the sponge tips. Gently place one of the sponge tips inside the infant's mouth and collect as much saliva as possible by rubbing the cheeks and moving the cotton tip into the spaces between the cheeks and gums, and under the tongue
2. When the sponge tip is saturated with saliva, use the scissors to cut the sponge tip directly into the Oragene® vial.
3. Repeat above steps until there are 5 sponge tips inside the Oragene® vial. Cap and tighten securely.
4. Invert gently 5 times to mix the sample.
5. Label the Oragene® vial. Use a barcode if possible as it facilitates sample tracking. The sample is then placed in an appropriately labeled freezer-safe transparent sealable plastic bag and stored at −20°C as recommended by the manufacturer. One can consider storage of −80°C if longer periods are required prior to processing.
6. Enter the appropriate information associated with that sample into the sample data base.

Adults

1. Have subject expectorate about 2 ml of saliva into the Oragene® vial then cap and securely tighten.
2. Shake the vial gently to mix the Oragene® DNA-preserving fluid with the saliva.
3. Label the Oragene® vial. Use a barcode if possible as it facilitates sample tracking. The sample is then placed in an appropriately labeled freezer-safe transparent sealable plastic bag and store at −20°C.
4. Place the appropriate information associated with that sample into the sample data base.

In addition to the protocols described above, several reviews [Holland et al. 2005; Schrohl et al. 2008; Vaught 2006] are available that describe tissue isolation, preparation, and storage. As with the above, all emphasize the need for standardization to yield samples of uniform high-quality. The isolation and storage conditions of the RMN samples are summarized below in Table 2. When stored in the appropriate manner, samples can remain stable for a considerable period [Kaaks et al. 2000]. Cryovials with rubber gaskets are essential for any long–term storage to prevent sample incursion of any form to ensure integrity while maintaining fidelity.

Consideration must also be given to establishing the appropriate size of the aliquot for each sample. This primarily reflects how their downstream use is envisioned. For example, depending on the specific application, i.e., a Polymerase Chain Reaction base diagnostic–assay, each blood spot can contain sufficient material for at least 100 if not greater assays. As of today an FTA blood spot would not provide sufficient sample for an epigenome study that must rely on the use of the whole blood or other similar large sample. However, with the advances in technology of sample preparation and analysis approaching the single cell level, this may no longer present a limitation. These innovations will spur reevaluation to reduce the amount of sample made available, thereby extending the life of this resource. If viability can be assured, then prior to shipment the sample would be re-aliquoted such that

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each reduced aliquot would be sufficient for single use. In this manner the residual sample would be retained by the Repository for future use.

Conclusion

The growth and expansion of biorepositories since the late 1940s [Strong 2000] attests to the utility of these resources. Since the early 1980s various neonatal screening programs [Norgaard-Pedersen and Simonsen 1999] have created these resources providing one of the pillars of personalized medicine. For example, ELEMENT (Early Life Exposures in Mexico to Environmental Toxicants) [Pilsner et al. 2009] was one of the first studies to suggest that the maternal lead burden could modify the fetal epigenome. The spectrum of resources provided by the RMN biorepository provides a unique platform to assess developmental outcomes from conception to birth. By making the scientific community aware of the existence of the RMN repository and providing the collection and storage protocols, investigators should have sufficient information to collect samples of interest that can be directly compared to RMN cohort. In addition, the investigator may select and then request the most appropriately paired sample specimens from the RNM biorepository for inclusion in the specific study of interest.

Abbreviations

RMN	Reproductive Medicine Network
DCC	data coordination center
IRB	Institutional Review Board

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Table 1

RMN studies, their primary hypothesis, and biorepository specimens to be collected.

Title	Primary Hypothesis	Biorepository Specimens
Pregnancy in Polycystic Ovary Syndrome II (PPCOS II)	Ovulation induction with an aromatase inhibitor is more likely to result in live birth then ovulation induction with a selective estrogen modulator in infertile women with polycystic ovary syndrome.	Blood Spot Cards Blood Clot Whole Blood Serum
Assessment of Multiple Intrauterine Gestations From Ovarian Stimulation (AMIGOS)	In infertile ovulatory women undergoing ovarian stimulation and intrauterine insemination, the use of aromatase inhibitors will stimulate the ovaries sufficiently to produce no reduction in the rate of pregnancy, while significantly reducing the numbers of multiple gestation pregnancies that result from stimulation with clomiphene citrate or follicle stimulating hormone.	Blood Spot Cards Blood Clot Whole Blood Serum Semen
RMN Pregnancy Registry	A registry of babies born as a result of clinical trials of the RMN.	Additional blood spots of baby, Saliva sample of father

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Table 2

Samples Collection and Storage Parameters.

Sample	Collection	Storage	Storage Temperature
Whole blood	Purple top (EDTA) vacutainer	Cryovial	−80°C
Whole blood	Purple top (EDTA) vacutainer	FTA collection card	−20°C
Blood Clot	Red top (silica) vacutainer	Cryovial	−80°C
Serum	Red top (silica) vacutainer	Cryovial	−80°C
Saliva	Swab / Oragene® vial	Oragene® vial	−20°C
Sperm	Cup	Cryovial – IVF freezing media	−190°C