Peter C. Hu
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Editors

Modern Clinical Molecular Techniques

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Preface

Clinical Molecular Diagnostics is an ever-expanding field which has come to the forefront of clinical testing with the increasing demand for molecular pathology and the ideologies of personalized medicine. It is apparent that with any rapid changes to a field, there is a learning curve. Our hope is to assist those who are either familiar or unfamiliar with Molecular Diagnostics in the areas of Infectious Disease, Oncology, Pre-/postnatal, and Human Identity Testing.

Modern Clinical Molecular Techniques is divided into six parts. Part I focuses on current clinical molecular laboratory practices including the proper handling of specimens, DNA/RNA isolation, test validation, current general molecular techniques, summary reporting, and data storage. In Part II, we begin to take a more in-depth view on some of the more advanced protocols in clinical molecular infectious disease testing ranging from specimen identification to viral load testing using current diagnostic molecular techniques. Part III is focused on clinical molecular oncology testing. In this part, current clinical molecular diagnostic techniques are showcased in areas from detecting gene rearrangements, mutation analysis, cancer microarray analysis, and minimal residual disease monitoring. Part IV shifts the focus to a different molecular discipline – the area of molecular pre-/postnatal testing. In this part, current diagnostic molecular protocols will be described in detail for a deeper understanding of current test methodologies and their uses. Part V describes current human identity testing such as parentage testing and an overview of microsatellite testing. Part VI illuminates issues surrounding the current shortage of clinical professionals in the area of diagnostic molecular testing throughout the USA. This part focuses on the clinical training aspects of both bench technologists – those from accredited programs and those with on-the-job training – and medical and laboratory directors (M.D. and Ph.D levels). The chapters conclude with a look at another fast growing trend in point-of-care testing training.

Currently, there are many books in press that focus on various aspects of the information covered in this text. However, we feel that by narrowing the focus to current diagnostic molecular testing protocols and clinical training, the reader will find it easier to not only begin creating their own diagnostic molecular testing protocols, but also to implement clinical training when issues arise. Upon reading this text, keep in mind that although many protocols on molecular testing are included, the specifics of testing change as new technologies emerge in this ever-growing, ever-changing field and will require additional updates and continual education.

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Part I
Current Molecular Laboratory Methods

Chapter 1
Specimen Collection, Handling, and Processing

Lindsy Hengesbach and John A. Gerlach

Keywords Collection • Contamination • Identification • Inhibiting substance • Nucleic acid • Pre-analytic • Safety • Sample • Sample integrity • Sample shipping

1.1 Introduction

Specimen collection handling and processing are key to the pre-analytic phase of clinical laboratory testing. In computer jargon there is an acronym "GIGO" that stands for garbage in equals garbage out. It is also applicable to clinical laboratory testing. If the proper sample is not collected from the individual to be tested and if the sample is not handled and transported to the laboratory in an efficient and efficacious manner, there will not be usable diagnostic information provided. This chapter deals with the pre-analytic process of sample collection, handling and processing for diagnostic molecular testing.

1.2 The Collection and Processing of Specimens in the Clinical Molecular Laboratory

1.2.1 Introduction to the Pre-analytical Phase of Testing

Pre-analytical testing in a clinical laboratory encompasses all of the aspects involved in procuring and preparing the specimen before analytical testing takes place. This includes maintaining the integrity and confidentiality of the patient's specimen; the proper collection of numerous types of specimens, all with different requirements; the transport of the specimen to and from the laboratory; the storage of these specimens while waiting for testing; and the processing of these specimens so they are ready for testing protocols. All of this must be accomplished, as well as maintaining a safe work environment by following Biosafety Level 2 precautions throughout the entire process. Often the complexity of the pre-analytical phase of testing is underrated. The pre-analytical phase of testing is one of the most critical because the majority of laboratory errors leading to misdiagnosis or mistreatment of a patient occur in the pre-analytical phase of testing [5, 12, 22].

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1.2.2 Specimen Identification

- It is the responsibility of the laboratory to "establish and follow" set guidelines and written protocols for maintaining the identity of the patient's specimen throughout the entire testing process including the pre-analytical phase of testing [12].
- It is the laboratory's responsibility to maintain the security of a patient's identification by following the Human Insurance Portability and Accountability ACT (HIPAA) guidelines throughout the entire testing process [13].
- At a minimum, a patient's sample must have a label containing a specimen identification number, the date of collection, the time of collection, and the specimen source. It is highly recommended that the name of the individual collecting the specimen is present as well [6, 7].

1.2.3 Laboratory Safety/Standard Precautions

Working in a biomedical laboratory poses an inherent risk of exposure to unknown pathogens. Each laboratory is responsible for issuing and enforcing operational procedures that follow recommendations put forth in the OSHA Bloodborne Pathogen Standard to prevent the occupational transmission of human immunodeficiency virus, hepatitis B virus, and other bloodborne pathogens [6, 27]. The appropriate safety precautions can significantly reduce the risk of exposure of mucous membranes and non-intact skin to potentially infectious agents. It is common to follow Biosafety Level 2 practices in a molecular diagnostics laboratory because the majority of specimens submitted for testing are human derived and therefore potentially infectious. Potentially infectious materials must be kept in a durable, leakproof container during collection handling, storage, and transport to the laboratory. Personal protective equipment including gloves, gowns, and face or eye protection must be utilized when in contact with human-derived specimens. This equipment should be removed before leaving designated areas. Molecular labs typically designate equipment to specific areas of the lab to prevent the contamination of specimens with spurious or amplified nucleic acids (DNA/RNA) [5]. Engineering controls such as a biological safety cabinet should be utilized to minimize exposure to aerosols and as a method for limiting exposure of the sample to contaminating DNA from the environment/other specimens [18].

1.2.4 Sample Acceptability

- Source: When an assay is validated by a laboratory, one of the criteria of the validation process is to use the range of samples that are expected to be received. When a laboratory receives a sample that is outside of those used in validation, it must either revalidate or reject the sample; therefore, the source of the sample is an important criterion.
- Volume: The sample volume must be sufficient to allow recovery of enough nucleic acid material to perform the assay. It is best to have excess, but it should be a minimal excess.
- Shipping: The sample should be shipped appropriately to the laboratory, arrive in a timely fashion without being overexposed to environmental influence, and should be protected from freezing and overheating.
- Storage: Once received, the specimen must be processed and stored properly in accordance with the laboratory's validated protocol.

1.2.5 Specimen Collection

When collecting a specimen for molecular testing, it is critical to protect the specimen from contamination from exfoliated skin cells as well as to protect the individual collecting the specimen from potentially infectious materials and infectious agents. Gloves should be worn throughout the entire collection process and gloves should be changed frequently. There are numerous specimens from which nucleic acid can be obtained.

- Whole blood, plasma, and serum are common sources of samples for nucleic acid testing. Whole blood often yields a very high quality and ample quantity of nucleic acid for testing. The impact of anticoagulants on downstream analysis must be considered before collecting whole blood. The most common anticoagulants are ethylenediaminetetraacetic acid (EDTA) or acid citrate dextrose (ACD) [1]. Both chelate calcium as their mode of action. ACD is useful when prolonged shipping may be involved as it will help maintain the integrity of the nucleated cells. The DNA in plasma or serum is most likely from dead cells. It is a good source but it may not be of high molecular size [10]. On another note though, the RNA that is collected from plasma or serum may actually be the material targeted as it may be of viral origin [28].
- Dried blood spots are commonplace in newborn screening scenarios as well as studies that require shipping across large distances or long-term storage. There are several manufactures that supply the filter papers used and the basic premise is to collect a sample, most often capillary blood from a lancet poke (finger or heel) that is allowed to saturate a filter paper. Often there is a circle printed on the filter to demark the appropriate volume of sample needed. Blood is allowed to seep onto/into the filter until the circle area is filled. The sample is allowed to dry and then it is shipped or archived. If archived, it is usually vacuum-sealed with a desiccant [19, 26, 30].
- Anatomic samples, either fresh, frozen, fixed, and/or archived, are very useful in molecular diagnostic testing. Isolation methods, as discussed later, will need to take into account if there have been fixatives used. Cytological samples would fall within this group also. These are samples collected from body sites by a lavage/washing/flushing process. Cells are dislodged or washed away by the process and collected into a container that has preservative or transport medium. How the sample is collected needs to ensure that there is not contamination from the environment. The container and any solutions or preservatives used must follow the guidelines of how the test was validated [2, 9].
- Body fluids can be a source of nucleic acid material for testing as well as the nucleated cells that are present. It may be important that the collection and transport maintains the integrity of the cells present as both the fluid and the cellular nucleic acid materials may need to be tested separately. Again the caution here is to follow the collection procedure as it was validated during assay development [16].
- Swabs are a common collection method for nucleic acid testing, and there are mainly two paradigms. The first is a cheek swab, and it is useful when the samples are not being collected at the institution but are collected off-site at clinics and possibly by the client. There are many types of fibers available, and some even have course bristles. The concept is to brush or swipe the inside of the cheek to remove cells containing nucleic acids. Care needs to be taken in how hard the cheek is "scrubbed" as bleeding may ensue at which point there will be erythrocytes that may lead to complication during the isolation process. It is also a good idea to have the client rinse prior to the process to remove debris that may contribute to sample mass collected. A second approach is a swab of a lesion for submission, most often to identify a pathogen's nucleic acid. Viral, bacterial, and fungal targets are commonly found using a swab from a lesion [11, 20, 23, 29].
- Saliva is another sample chosen when it is deemed best to avoid venipuncture or direct blood handling. A container is provided with a mark denoting the volume necessary to collect and the client is directed to "expectorate into the container." It is the cells that are present in the saliva, the same that you would collect from the cheek swab, which will be source of the nucleic acids isolated [7, 14].

- Other sources: These may be pathogen cultures or possibly parasites that have been harvested. The forensics laboratories can fill volumes on what they receive to isolate nucleic acids for analysis. The clinical laboratory often pales in vast diversity of the forensics laboratory but there can be surprises. The caution here is to note that the assay being requested was validated for a particular range of samples and anything outside of that list needs to be thoroughly documented throughout the assay process and any variation needs to be included in the interpretation of the assay. Just because you can isolate nucleic acids from the material (most likely it is probable) does not mean the material will be of sufficient quantity and/or quality to provide a meaningful result. Caution needs to be taken when unusual circumstances are presented to the laboratory.

Special circumstances and/or materials and supplies need to be considered when the goal is to isolate RNA. There are special tubes and timeframes that need to be adhered to with viral load testing [8, 15]. Equipment and supplies need to be RNase-free. These should be addressed in the assay protocol, and these stipulations in the protocol must be adhered to for the sample collected to deliver a meaningful diagnostic value [31].

The integrity of a sample my suffer because inhibiting substances are often co-collected with the patient specimen [3, 4]. Materials such as the anticoagulant utilized (heparin), the presence of hemolysis (free hemoglobin), high levels of lipids or carbohydrates, and even the pharmaceutical agents (remember the samples are being collected from physically ill individuals that are being medicated) present at the time of collection may be co-isolated with the nucleic acids and impact the usefulness of the sample [4, 21]. When possible and there is a priori knowledge, avoid these circumstances. Often inhibiting substances are not detectable and can lead to false negative results. A listing of inhibiting substances and conditions is often a useful document to help guide specimen collection. These lists may be generic for all assays or specific for a particular assay. All individuals involved in sample collection need to be armed with this information to help preserve the integrity of the pre-analytic portion of laboratory testing.

1.2.6 Sample Transport

Once the sample is collected in accordance with assay protocol, it needs to make its way to the laboratory for testing. This requires transport. It may be as simple as moving within an institution by hand or as complex as preparing a sample for international shipping. At all times, there are two main concerns: maintaining the integrity of the sample in terms of keeping it from being contaminated as well as the physical extremes it may encounter. Once again the protocol for the assay needs to be followed, and the assay needs to be validated with samples that have endured the "usual" transportation methods used. The prevention of freezing and overheating during shipping as well as breakage are the most obvious perils.

The second major concern with shipping is protecting the "transporter" and the environment from the sample. At the very least, the samples being discussed are biological in nature and therefore, the practice of universal precautions should be applied as the process is designed. It is also probable that the material itself may be infectious, warranting different, much more stringent regimen of containment and labeling. Below is a minimal set of guidelines. It would be imprudent to go into extreme detail on shipping and transport regulations as the guidelines and regulations change quite regularly and the best practice is to continue to monitor shipping vendor sites and federal sites for updates. The federal sources would be the Centers for Disease Control and Prevention, the Department of Transportation, the Federal Aviation Administration, as well as Homeland Security [25].

- Samples should be in a double container (even if it is only double bagging), so that if the integrity of the first container is compromised, the second will still provide the protection intended.

- Liquid sample should have an absorbent material sufficient to soak up the material if the holding container leaks or is broken.
- The Centers for Disease Control and Prevention provides a list of highly infectious agents as well as a list of select agents. These need special permits and handling.
- Material transfer agreements may also be necessary by the shipping or receiving institution and may often be on a case-by-case basis.

1.3 Sample Accessioning or Processing

This is one of the crucial steps in the pre-analytic phase of a laboratory operation. It is here, where the sample is received, the test has been ordered and the integrity and the appropriateness of the sample is reviewed and logged. This process is where the decision on whether to proceed, reject, or document variation of conditions outside of those prescribed by the procedure or laboratory policies is made. Policies and guidelines, as stated throughout this section, are a necessity.

- Sample receipt involves the un-packaging of the sample and an assessment on whether to proceed with the test ordered or to notify the source that the sample does not meet criteria for acceptance. Regardless of whether accepted or not, the sample and test ordered need to be logged into the system for subsequent tracking.
- Decisions on sample integrity and appropriateness may result in sample rejection. This may be because the sample is
  - From an inappropriate source
  - Of insufficient quantity
  - Collected in the wrong anticoagulant
  - Displaying evidence of hemolysis
  - Showing evidence of extreme environmental exposure during shipping or storage
  - Misidentified or has no identification
- If a sample is rejected, it needs to be logged into the normal system and the rejection noted. There also needs to be documentation that the source is notified of the rejection and the reason, as well as how the rejected sample is discarded.
- Aliquots of the original sample may need to be prepared. This requires a written protocol for staff to follow. It is important here, as well as during collection and shipping, that the integrity of the sample be maintained to prevent contamination, and there needs to be safeguards for the individual preparing the aliquots. An easy way to achieve both goals is to perform this function in a biosafety cabinet using sterile supplies. It is important to note that labeling the aliquots should be the primary focus to help maintain the sample identity throughout the testing and storage process.
- Specimen storage will again be determined by the assay requirements as validated during the assay development. As a general set of guidelines:
  - DNA can be stored short term at 4°C and long term at −20°C or colder.
  - RNA is best stored −20°C or colder.
  - Samples pre-isolation – this will be variable and the best reference will be the protocol for the particular assay.

With storage of samples used in molecular diagnostic testing, a concern for ethical or legal issues arises that are not quite as evident with other sample biobanking. Genetic testing falls under continued public and legal scrutiny because of the ability to stratify people based on inherited traits and the potential to use that information for discriminatory purposes. This is a concern and can be more an emotional issue than a logical concern. It does put an additional onus on a molecular diagnostic

laboratory to thoroughly define the need to maintain a sample after an assay is completed and also to have clinically justifiable reasons for the retention and subsequent use of banked samples. It would be judicious for a laboratory involved in biobanking and one that uses banked samples to seek approval of an Institutional Review Board of the guidelines and circumstances [24].

The process of sample collection really encompasses more than a needlestick, skin scrape, or placing a piece of tissue into a container. Sample collection is at the heart of the pre-analytic phase of molecular diagnostic testing, and it is here where adherence to the protocols and policies of the laboratory ensures the provision of useful diagnostic information.

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Chapter 2
DNA/RNA Isolation and Quantitation

Jing Wang

Keywords DNA • RNA • miRNA • Extraction • Quantification

2.1 Introduction

2.1.1 DNA Extraction

Nucleic acids are very large biological molecules essential for life. DNA (deoxyribonucleic acid) is found mainly in the nucleus of the cell, and RNA (ribonucleic acid) is found mainly in the cytoplasm of the cell although it is usually synthesized in the nucleus. DNA contains the genetic codes to make RNA, and the RNA in turn then contains the codes for the primary sequence of amino acids to make proteins. Nucleic acid preparation is the important initial step for molecular diagnosis. There are varieties of methods available for nucleic acid preparation [1, 2]. Strategies for DNA extraction and DNA purification depend on the sample volume, the major sample type, and the downstream applications.

Extracted DNA has been widely used for clinical analysis such as genotyping, diagnosis of infectious diseases, transplant engraftment assessment, and other tests such as forensic and ancestry testing. Different extraction methodologies have been implemented and evaluated. The basic steps for DNA extraction include (1) cell or tissue lysis using detergents, proteinase K, and/or RNase; (2) protein removal, avoiding RNA and chemical contamination; and (3) DNA purification. The conventional manual DNA extraction procedures involve phenol-chloroform purification and precipitation using ethanol or isopropanol. Although the manual DNA extraction procedures can easily handle large sample volume, for example, 5–10 ml of blood, or different types of specimen, and yield high quality and quantity of DNA with low cost. However, the sample processing procedure is very time consuming. The automated DNA extraction provides the high throughput of sample handling and shorter processing time [3, 4]. It typically utilizes magnetic beads technology and has the advantage of standardized sample treatment and avoidance of error during sample handling and contamination due to intermediate

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process. However, the quality and quantity of DNA extracted by automated methods may not be as good as by manual methods [5, 6] and, therefore, may not be optimal for some molecular tests such as Southern blotting and microarray testing.

The DNA extraction protocol presented here is adapted from Gentra Puregene DNA extraction molecular weight nuclear and mitochondrial DNA from variety of sample sources, including whole blood, frozen solid tissue, cultured cell, and paraffin blocks.

2.1.1.1 Reagents and Equipments

Reagents and Consumables Required

Supplied by Gentra Puregene DNA extraction kit (QIAGEN Inc., Valencia, CA):

- Red cell lysis solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA, pH 8.0)
- Cell lysis solution
- RNase A solution (4 mg/ml)
- DNA hydration solution or 1× TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA)
- Protein precipitation solution
- Glycogen solution (20 mg/ml)
- Proteinase K (20 mg/ml)
- 100% Isopropanol
- 70% Ethanol

For cultured cells:

- 1× PBS solution (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4)
- Phenol-chloroform
- Chloroform

For paraffin block:

- Xylene or Hemo-De (Nontoxic alternative/Scientific Safety Solvents)
- 100% Ethanol
- Microcentrifuge tube pestle

Consumables:

- 1-ml Microcentrifuge tubes
- 15-ml Centrifuge tubes
- Pipetman (2 µl, 100 µl, 1 ml) and tips

Equipment:

- Microcentrifuge (Eppendorf Centrifuge 5415D)
- Bench Top Centrifuge (Beckman Coulter Allegra™ X-22 or equivalent)
- Vortex mixer

Note:

- Wear gloves and a laboratory coat throughout the procedure.
- Be careful and avoid cross-contamination of samples and reagents.
- Make sure reagent tubs, tubes, and plates are clearly labeled and clean. Dispose of all used materials and media as biohazard waste.

2.1.1.2 Protocol

Cell Lysis

DNA Extraction from Whole Blood

Note: This protocol is designed for extraction of 3 ml whole blood with expected yield of 50–150 μg of DNA. For best results, blood must not exceed 6 days before processing.

Control: A blank control is extracted along with each batch's blood samples. 3 ml of sterile water is replaced with the 3 ml of blood. After the extraction process, the blank is quantified and amplified in a PCR reaction to check for any contaminations in the extraction reagents:

1. Wear latex gloves when working under the biological safety hood and mix the tube of whole blood by inverting the tube six times.
2. Pour 3 ml of blood into a labeled 15-ml conical centrifuge tube.
3. Add 9 ml red blood cell lysis solution to the 3 ml of blood. Invert ten times to mix and incubate 5 min at room temperature; invert at least once during incubation.
4. Centrifuge for 5 min at 2,000× g (on the Beckman Coulter Allegra™ X-22). Remove supernatant, leaving the visible white cell pellet in the tube.
5. Resuspend the pellet in the residual supernatant by tapping tube; this greatly facilitates cell lysis in step 6 below.
6. Add 3 ml of cell lysis solution to the resuspended pellet. Incubate at 37°C for 2 h. Additional incubation at room temperature overnight can be added to ensure the solution is homogenous. This action can be further facilitated by placing the tube on a shaker. Samples are stable in cell lysis solution for at least 18 months at room temperature.

DNA Extraction from Solid Tissue

Note: This protocol is designed for 25–50 mg of solid tissue extraction; expect to yield 50–250 μg of DNA.

1. Dissect tissue sample quickly on dry ice. Work very quickly and keep tissue on dry ice at all times. Fresh tissue may also be used. Freeze the remaining tissue at −80°C or lower.
2. Coarsely ground 25–50 mg tissue with a surgical blade. Transfer the grounded tissue to a 15-ml centrifuge tube containing 3 ml cell lysis solution**.** Add 15 μl of proteinase K solution (20 mg/ml) to the lysate. Mix by inverting 25 times. Incubate at 55°C for 3 h or until tissue has completely lysed. The sample can be incubated at 37°C overnight for maximum yield.

DNA Extraction from Cultured Cells

Note: This protocol is designed for extraction of DNA from $1–2 \times 10^7$ monolayer cultured cells; expect to yield 50–100 μg of DNA.

1. Pipette out the culture medium. Wash cells with 5 ml PBS at room temperature, then remove all PBS solution.
2. Detach cells by trypsinization or use a cell scraper. Transfer the cell to a 15-ml centrifuge tube.
3. Add 5 ml PBS to the harvested cells. Invert the tube two times.
4. Centrifuge 500× g for 3 min to pellet the cells.
5. Carefully discard the supernatant, leaving approximately 200 μl liquid.
6. Vortex the tube vigorously to suspend the cells. Add 3 ml cell lysis solution to the suspended cells. Vortex on high speed for 10 s.

7. Incubate at 37°C for 2 h. Additional incubation at room temperature overnight can be added to ensure the solution is homogeneous. This action can be further facilitated by placing the tube on a shaker. Samples are stable in cell lysis solution for at least 18 months at room temperature.

DNA Extraction from Paraffin Block

Sample Deparaffinization

1. Place 5–10 mg of finely minced tissue in a 1.5-ml tube. Add 300 µl Xylene or Hemo-De and incubate 5 min with constant mixing at room temperature.
2. Centrifuge at 13,000–16,000× g (Eppendorf Centrifuge 5415D) for 1–3 min to pellet the tissue. Discard the Xylene or Hemo-De.
3. Repeat steps 1 and 2 twice (for a total of three washes).
4. Add 300 µl of 100% ethanol to the tube and incubate 5 min with constant mixing at room temperature.
5. Centrifuge at 13,000–16,000× g for 3 min to pellet the tissue. Discard the ethanol.
6. Repeat steps 4 and 5 (for a total of two ethanol washes).

Cell Lysis

1. Add 300 µl cell lysis solution and mix by using 30–50 strokes with a microcentrifuge tube pestle.
2. Incubate lysate at 65°C for 15–60 min.
3. If maximum yield is required, 1.5 µl proteinase K solution (20 mg/ml) may be added to the lysate. Mix by inverting 25 times and incubate at 55°C until tissue particulates have dissolved (3 h to overnight). If possible, invert tube periodically during the incubation.

RNase Treatment (Optional)

4. Add 1.5 µl RNase A solution (4 mg/ml) to the cell lysate.
5. Mix the sample by inverting the tube 25 times and incubate at 37°C for 15–60 min.

Protein Precipitation:

1. After cell lysis steps described above for different types, cool sample to room temperature.
2. Add 1 ml of protein precipitation solution to the cell lysate. For paraffin block DNA extraction, add 100 µl protein precipitation solution to the cell lysate.
3. Vortex vigorously at high speed for 20 s to mix the protein precipitation solution uniformly with the cell lysate.
4. Centrifuge at 13,000–16,000× g for 5 min. The precipitated proteins will form a tight pellet. If the protein pellet is not visible, repeat step 3 followed by incubation on ice for 5 min, then repeat step 4.

DNA Precipitation:

1. Pour the supernatant from protein precipitation protocol step 4 containing the DNA into a labeled 15-ml conical centrifuge tube containing 3 ml of isopropanol. If DNA yield is expected to be below 0.1 µg, add 3.0 µl glycogen solution (20 mg/ml) to the isopropanol.

Extra steps for paraffin block and cultured cell extraction:

(a) Pour the supernatant containing the DNA into a clean 15-ml tube and add equal volume of phenol-chloroform. Mix by rocking gently back and forth for 15 s.
(b) Centrifuge at 2,000× g for 5 min using the centrifuge.
(c) Carefully transfer the upper phase of supernatant containing the DNA into a clean 15-ml tube and add equal volume of chloroform. Mix by rocking gently back and forth for 15 s. Save everything until DNA is recovered.

(d) Centrifuge at 2,000× g for 5 min.
(e) Carefully transfer the supernatant containing the DNA (upper phase) into a labeled 15-ml tube containing 6 ml of isopropanol.

2. Mix the tube by inverting gently 50 times.
3. Centrifuge at 2,000× g for 5 min. The DNA will be visible as a small white pellet.
4. Pour off supernatant and transfer the DNA pellet and remaining liquid to a 1.5-ml centrifuge tube.
5. Add 500 µl 70% ethanol and invert tube several times to wash the DNA pellet.
6. Centrifuge at 13,000–16,000× g for 1 min. Carefully pour off the ethanol. Pellet may be loose, so pour slowly and watch out for the pellet.
7. Invert and drain the tube on clean absorbent paper and allow to air-dry for 10–15 min.
8. Estimate the size of the pellet and add 200–400 µl DNA hydration solution.
9. Allow DNA to rehydrate overnight with gentle rocking at 37°C or 30 min at 65°C.
10. Measure the DNA concentration by using the NanoDrop spectrophotometer. Refrigerate DNA at 4°C.

2.1.2 RNA Extraction

There are two main methods for RNA extraction: phenol-based extraction and silica matrix or glass fiber filter (GFF)-based binding. Phenol-based reagents, such as TRIzol, contain a combination of denaturants and RNase inhibitors for cell and tissue disruption and subsequent separation of RNA from contaminants. Phenol-based isolation procedures can recover RNA species in the 10–200 nucleotide range, including miRNAs, 5S rRNA, 5.8S rRNA, and U1 snRNA. If a sample of "total" RNA was purified by the silica matrix column or GFF procedure, it will be significantly depleted in small RNAs. Extraction procedures using TRIzol Reagent are the recommended methods for isolating total RNA from biological samples that will contain miRNAs/siRNAs. TRIzol Reagent is a monophasic solution of phenol and guanidine isothiocyanate. During sample homogenization or lysis, TRIzol Reagent maintains the integrity of the RNA while disrupting cells and dissolving cell components [7].

This protocol utilizes the powerful TRIzol Reagent extraction method which allows the rapid isolation of RNA. High yields of pure, undegraded total RNA can be recovered from even small quantities of tissue or cells.

2.1.2.1 Reagents and Equipments

Reagents and Consumables:

- RNA*later* (Ambion Inc., Austin, TX)
- RNase*ZAP* (Ambion Inc., Austin, TX)
- TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA)
- RNase Away surface decontaminant (Fisher Scientific, Hanover Park, IL)
- Red cell lysis solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA, pH 8.0)
- Ultrapure DNase/RNase-Free Distilled Water
- Chloroform
- 100% Isopropanol
- 70% Ethanol (keep cold at 4°C)
- High salt buffer (0.8 M NaCitrate/1.2 M NaCl)
- 1× PBS solution (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM)

Accessories Required:

- Refrigerated centrifuge
- Microcentrifuge
- Micropipettors
- Vortex mixer
- Glass-Teflon or power homogenizer

2.1.2.2 Protocol

Note: Always wear lab coat, gloves, and eye protection. Avoid contact with skin or clothing. Operate in a biochemical safety hood. Avoid breathing vapor. Use RNase*ZAP* to clean all tools, pipettes, bench, and scale area before starting.

Cell Lysis

RNA extraction from whole blood:

Note: This protocol is designed to extract whole blood samples of 5–8 ml and expect to yield approximately 30 μg of RNA.

1. Transfer 5–8 ml of whole blood into a 50-ml polypropylene conical centrifuge tube. Bring volume to 50 ml with red cell lysis solution.
2. Invert the tube ten times to mix. Let stand at room temperature for 5 min.
3. Centrifuge for 5 min at 2,000× g. Remove supernatant, leaving the visible white cell pellet in the tube.
4. Gently resuspend the pellet in 1 ml of red cell lysis solution and transfer to a 1.5-ml microcentrifuge tube. Let it stand for 2–5 min.
5. Pellet cells by centrifuging at 13,000× g for 2 min at room temperature. Carefully aspirate the supernatant.
6. If the resulting pellet is still red, repeat the RBC lysis (steps 4–5) as needed.
7. Resuspend the pellet in 1 ml of sterile PBS.
8. Pellet cells by centrifuging at 13,000× g for 2 min.
9. Carefully aspirate the supernatant.
10. Add 1 ml of TRIzol solution to each tube and resuspend the cells.

RNA extraction from solid tissues:

1. Zero the balance with about 2 ml RNA*later* in a weigh boat.
2. Place 50–100 mg sample in the weigh boat with RNA*later* and record the amount of tissue on the extraction log.
3. Store excess tissue at −80°C.
4. Homogenize tissue samples in 1 ml of TRIzol Reagent per 50–100 mg of tissue using a glass-Teflon or power homogenizer. The sample volume should not exceed 10% of the volume of TRIzol Reagent used for the homogenization.

RNA extraction from cells grown in monolayer:

1. Rinse cell monolayer with ice cold PBS once.
2. Lyse cells directly in a culture dish by adding 1 ml of TRIzol Reagent per 3.5-cm-diameter dish and scraping with cell scraper.
3. Pass the cell lysate several times through a pipette. Vortex thoroughly.

Note: The amount of TRIzol Reagent added is based on the area of the culture dish (1 ml per 10 cm^2) and not on the number of cells present. An insufficient amount of TRIzol Reagent may result in DNA contamination of the isolated RNA.

RNA Precipitation:

1. Incubate the homogenized sample for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes.
2. Centrifuge 13,000× g for 15 min at 4°C to remove cell debris. Transfer supernatant to a new 1.5-ml tube. Discard the pellet, which is cellular debris and DNA.

Note: If there is an oily/fat layer on top, remove fatty layer with a pipetman then pour supernatant into new tube. If the pellet moves, remove liquid with a pipette.

3. Add 0.2 ml chloroform per 1 ml TRIzol to each tube and close tubes. Vortex 15 s to mix.
4. Incubate samples at room temperature for 10 min. Centrifuge 13,000× g for 15 min at 4°C. Transfer *aqueous phase* (top layer) into a new tube.

Note: The RNA isolation can be stopped at this step. The collected *aqueous phase* can be stored at −80°C or immediately enriched for small RNAs (see Sect. 2.3).

5. Add 0.25 ml high salt buffer per 1 ml of TRIzol to each sample. Add 0.25 ml isopropanol per 1 ml TRIzol to each sample. Mix well by gently inverting tubes three to four times.
6. Incubate at room temperature for 20–30 min. Centrifuge 13,000 rpm for 15 min at 4°C.
7. Pour liquid (high salt/isopropanol) into a liquid waste container leaving the pellet behind. Tap tube on a paper towel to get as much liquid out as possible and place tube back on ice.
8. Add 1 ml cold 70% ethanol to each sample. Vortex pellet gently to remove residual high salt/isopropanol. Centrifuge 13,000× g for 1 min at 4°C.
9. Air-dry pellet for 10–30 min on ice with KimWipe on top of the open tubes. If pellets are thick, drying time may increase. Make sure the pellet is completely dry as residual ethanol can inhibit downstream applications.
10. Dissolve pellets using Ultrapure DNase/RNase-Free Distilled Water on ice. Amount of water depends on size of the pellet.

Note:

- Collect TRIzol and RNase Away into the phenol waste container in the hood.
- RNA*later* waste can go down the sink while running the water for 10 min.
- Make sure bench paper in the hood is clean with no spilled TRIzol. If not, dispose of it immediately and replace with new bench paper.
- For microarray experiments, total RNA isolated by TRIzol method needs to be further cleaned using RNeasy cleanup protocol.

2.1.3 miRNA Enrichment

In the past 10 years, research interest in microRNAs (miRNAs) has rapidly expanded. miRNAs are small (~22 nucleotides) noncoding RNAs expressed by virtually all eukaryotes. miRNAs are post-transcriptional regulators that bind to complementary sequences on target messenger RNA transcripts (mRNAs). They have been shown to regulate target gene expression in various organisms.