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Comprehensive Review of Transcriptomics (RNAs) Workflows from Blood Specimens

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ABSTRACT

Blood is the most interesting tissue for molecular analysis in a wide range of diseases since it provides accumulative information of the whole body state. Transcriptomic analysis of the blood is one of the most favorable omics approaches for clinical and research purposes. However, blood sample preparation for RNA analyses is highly diverse encompassing specific methodology and application. In this study, we review the common sample preparation maneuvers including RNA handling techniques, sample collection and stabilization, blood cell sorting, extracellular vesicle enrichment, RNA extraction, globin/ribosomal RNA reduction, and RNA concentration and quality assessment. These techniques are the most prevalent procedures of blood sample preparation in RNA transcriptomic analyses. The methodologies, technical challenges of the current approaches, and latest advances in the field are discussed in detail.

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Transcriptomic analysis; RNA handling methods; blood; workflow

INTRODUCTION

The omics analysis of the blood could provide a myriad of accumulative information about the human body in general since it performs the most vital functions of the whole body.^[1] Although sampling from saliva, urea, or urine are less invasive and more convenient, especially when blood collection is also less favored (children, elderly, or critically ill), the blood remains a unique and inevitable biosource for omics-based clinical and research purposes in diagnosis of a wide range of diseases.^[2] Multiple biomolecules including blood cellular transcripts, extracellular vesicle (EV)-derived nucleic acids and proteins, cell-free deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), and plasma or serum-derived circulating proteins or metabolites, are main target biomolecules in many disease-associated studies.^[2] The use of omics techniques (genomics, transcriptomics, proteomics, and metabolomics) becomes increasingly popular in life science. Advances in automated systems provide powerful, selective, easy-to-operate, and high-throughput sample preparation approaches with minimal waste production.^[3] RNA analyses have played a key role in this context where use of transcript profiling is one of the most utilized molecular approaches. However, the application of transcriptional analysis for blood can be altered by poor sample preparation.^[4,5] To overcome these challenges and assure an accurate transcriptome analysis, high-quality representative RNA from whole blood or a targeted subset of blood tissue should be provided. In this study, we review the sample preparation methods for transcriptomic analysis of the blood, their methodological details, and the last advances in the field (Figure 1).

GOOD LABORATORY PRACTICE IN HANDLING RNA

Handling RNA is laborious due to the reactive hydroxyl group on the carbon number 2 of the ribose unit which makes the

RNA structure relatively unstable. In addition, ubiquitous existence of RNases which can preserve activity even after sterilization, can quickly and severely degrade purified RNA species. The minimization of RNase effects by special precautions must be considered.^[6]

Standard practices including aseptic technique applications during entire downstream processes together with using RNase-free equipment/reagents must be carefully followed for all the procedures.^[6] Use of sterile disposable gloves, frequently changing them and avoiding touching contaminated surfaces when handling RNA samples or reagents are very important cautions. Also, use of respirator masks and other personal protective equipment should be considered to reduce the risk of personnel borne contamination. Allocation of a specified area with proper ventilation for RNA work is suggested. Benchtops, glassware, pupating devices, and test tube racks must be treated with effective RNase inactivating agents. Also, benches should be wiped off with 70% ethanol to remove microorganisms prior to use. All RNA-containing samples should be stored in ice during operations because RNA is a highly temperature-sensitive molecule. Sterile, disposable RNase-free plastic wares are preferred. Non-disposable plastic ware should be treated with 0.1 M sodium hydroxide/1 mM ethylenediaminetetraacetic acid (EDTA) (2 hours, 37°C) or absolute ethanol with 1% sodium dodecyl sulfate and subsequently rinsed by diethyl pyrocarbonate (DEPC)/dimethyl pyrocarbonate (DMPC) treated water and heated to 100°C for 15 min in an autoclave. To inactivate RNases in chloroform resistant plastic ware, chloroform can also be used. Glassware should be cleaned with a detergent and rinsed properly with DEPC/DMPC treated water prior to oven-drying at 180°C for at least 4 hours. However, autoclaving alone is not adequate for removal of RNases. Polycarbonate or polystyrene materials can be treated with 3% hydrogen peroxide, followed by rinsing with

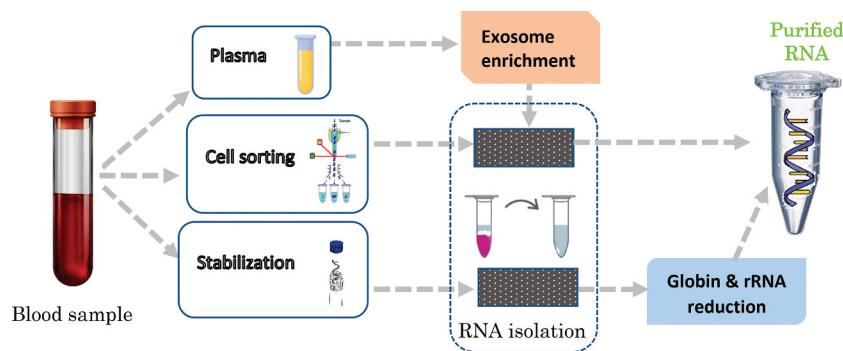


Figure 1. Common procedures for transcriptomic analysis of blood samples.

RNase-free water. Reagents used for RNA work should be kept separately. In addition, water used for buffer preparations should be RNase-free or at least treated with DEPC/DMPC overnight and then autoclaved to hydrolyze any unreacted DEPC/DMPC. In the case where buffer may contain amine groups like Tris, treatment with DEPC would not be suitable since they consume active DEPC quite quickly. In addition, reagents like RNasecureTM (Thermo Fisher, Waltham, Massachusetts, USA) can be an alternative for inactivating RNases.^[6]

SAMPLE COLLECTION AND STABILIZATION

Transcriptomic analysis of the blood is significantly affected by the sample collection conditions. RNA quality, concentration, and transcripts stability must be preserved before further downstream analysis. Traditional anticoagulant agents, such as dipotassium EDTA, lithium-heparin, and acid citrate dextrose solution can not inhibit RNase in samples.^[7–11] Therefore various pre-extraction or post-extraction sample stabilization methods may be necessary for RNA analysis of blood samples.

A simple and low-cost method for RNA preservation is freezing and storing the sample in TRIzol[®] (Thermo Fisher) which provides up to one year effective protection.^[12] However, high-quality RNA can be extracted from blood samples using a specialized collection system such as PAXgene tubes (BD, Franklin Lakes, New Jersey, USA), Tempus (Tempus, Chicago, Illinois, USA), RNeasy (Thermo Fisher), and RNeasy (GE Healthcare, Chicago, USA) stabilization reagents.^[9–11,13–16] The PAXgene and RNeasy collection tubes utilize reagents solution, which inhibits RNA degradation by RNases and blocks induction of further gene expression. In contrast, the Tempus collection tubes reagent, consist of guanidine and detergent solution which inactivate the RNases via chaotropic properties.^[15,17] RNeasy is a mix of sodium citrate, EDTA, and ammonium sulfate, which rapidly permeate cells with a high concentration of ammonium sulfate. This leads to mass precipitation of cellular proteins including RNase.^[18] The PAXgene and Tempus tubes facilitates the stabilization of the blood at room temperature for up to 72 hours with no change in the quality of RNA.^[11] Duale demonstrated that storage of samples in PAXgene and Tempus tubes at ambient temperature and transport conditions for up to seven days had little effect on RNA quality which stayed at

an acceptable range for many RNA assays.^[9] Although this result may seriously be questionable when working in warm climates and unthermostated laboratories.

Previous studies reported that the quality, purity, integrity, yield and transcript stability of RNA were stable up to six months at -20°C in short term storage, and up to six years at -80°C when using the Tempus and PAXgene tubes.^[14,19,20] However, higher RNA yields with comparable quality indicators were observed when the Tempus system was used compared to the PAXgene.^[9,11,21] In contrast, Hantzsch et al. concluded that there are no differences between Tempus and PAXgene collection tubes in messenger RNA (mRNA) expression profiling. But alterations in micro RNA (miRNA) levels were observed.^[22] Besides, significant DNA contamination and failure of stabilizing specific transcripts were also reported with the PAXgene collection system.^[14] Studies carried out for evaluation of RNeasy reagent showed that RNeasy also stabilizes the RNA in blood samples for several days at ambient temperatures and also seem to be suitable at least for up to six months storage.^[14,23] Some studies that evaluated the RNeasy stabilization reagent showing relatively higher performance compared to the PAXgene collection system.^[15,16]

The blood to RNA stabilization reagent ratio, sample mixing, and storage temperature are the most critical variables of the procedure which were investigated. The appropriate ratio is 3 volumes of blood for 6 volumes of Tempus solution or 2.5 mL blood for 6.9 mL PAXgene solution. The RNA quality and quantity will decrease if these ratios are not maintained precisely especially for adult blood samples, whereas the blood volume is less critical for cord blood samples.^[9,20,21,24] However, validation studies which collected 50 μL blood samples in 100 μL Tempus RNA stabilizing reagent or 70 μL blood in 200 μL PAXgene RNA stabilizing reagent, both demonstrated sufficient RNA quality and yield for gene expression study.^[24,25] The advantage of such validation is the small blood volume requirement, which facilitates studies on children. Besides, the integrity of the RNA extracted from Tempus collection tubes was more sensitive to fill volumes and effective inversion than to storage temperature. The integrity of RNA extracted from PAXgene collection tubes was more sensitive to effective inversion and storage temperature than to fill volumes.^[10]

The Cell-Free RNA Blood Collection Tube system (Streck, La Vista, Nebraska, USA) has RNase inhibition properties and was developed for obtaining a stable concentration of plasma

endogenous RNA. It is approved that these tubes reduce alterations in cell free RNA concentration during blood sample storage and shipping up to 3 days which facilitate further downstream analysis of cell free RNA.^[7,8] Filter paper-based solid matrices such as RNA Stabilizing Matrix (Thermo Fisher) or Fast Technology Analysis Cards (Whatman®, Millipore Sigma, Saint Louis, Missouri, USA) which are utilized for simultaneous collection and preservation of high-quality RNA at ambient temperature are highly promising to remove or reduce the limitation of conventional tube-based methods like phlebotomy, volume requirements, and RNA degradation stability during transport and storage.^[26–28]

Post extraction RNA stabilization is another important aspect of sample stabilization. Traditional approaches of RNA sample preservation are based on storing of RNA under ultra-low -80°C temperature or even -210°C in liquid nitrogen for protection from degradation. Although storage and shipment of RNA with acceptable quality can be carried out effectively via freeze based methods, power supplies and freezers are not available always and shipping under cold chains is expensive and requires special facilities.^[29] Novel technologies such as GenTegra (IntegenX), RNASTable (Biomatrica), and RNAShells (Imagene) allow the protection and storage of purified and dehydrated RNA at ambient temperature which provide more simple and cheaper approaches than traditional methods.^[28,29] These technologies are based on the principle of anhydrobiosis and imitate the mechanism of some multicellular organisms to survive in the complete absence of water.^[28] Biomatrix and IntegenX have developed a protecting RNA system where purified RNA sample mixes with the stabilization medium and subsequently dehydrates to be storable at ambient temperature.^[28] Instead, Imagene employs a little different RNA conservation system whereby RNA is encapsulated in metal shells under anhydrous and anoxic conditions to protect it from oxidation.^[28] Studies show that post-extraction RNA stabilization is adequate for short time storage at ambient

temperature which provides facilities for RNA sample shipment.^[28] However, RNASTable and RNAShells ensure a safe RNA storage for up to one year at ambient temperatures with quality equivalent to that after RNA storage at -80°C .^[29]

Commercial novel RNA stabilization methods with their principles and stabilization characteristics are summarized in Table 1. Based on the second external quality assessment for the pre-analytical phase of blood samples used for RNA based analyses, only blood collection tubes containing a cellular RNA stabilizer, provided reliable gene expression.^[30] Using a single blood collection platform and avoiding combinations of methods has been strongly recommended.

BLOOD CELL SORTING METHODS

Blood consists of two essential parts: blood cells and protein-rich plasma. The blood cells include erythrocytes ($\sim 2\text{--}8\text{ }\mu\text{m}$), leukocytes ($\sim 10\text{--}15\text{ }\mu\text{m}$) and platelets ($\sim 1\text{--}3\text{ }\mu\text{m}$). Leukocytes and erythrocytes respectively comprise 0.7 and 45.0% of the whole blood volume.^[1,31] Isolation and sorting of blood components especially leukocytes and plasma is an important initial process in molecular research and clinical applications. Cell fractionation methods can isolate leukocyte populations or separate subgroups that have been largely depleted from red cells.^[1] They are separated based on differences in cell size, mass, charge, density,^[32] morphology (shape), surface protein expression and also cell contents such as proteins and nucleic acid through various process.^[33] Detail on the available methods is presented thereafter and a summary of the information is gathered in Table 2.

Sedimentation

Sedimentation is the natural settling of particles because of gravitational force when particles have a higher density than the medium. Blood cells are denser than plasma, therefore can

Table 1. Commercial novel RNA stabilization methods.

RNA stabilization methods		Principle	Stabilization period	Condition
Pre-Extraction stabilization	TRIzol/QIAzol	Solubilizing biological samples and protein denaturation	12 months	-70°C
	PAXGene	Inhibits RNA degradation and blocks induction of further gene expression	3–7 days 6 months 6 years	Ambient temperature -20°C -80°C
	Tempus	Inactivates RNases via chaotropic properties of guanidine and detergent solution	3–7 days 6 months 6 years	Ambient temperature -20°C -80°C
	RNA Later	Mass precipitation of cellular proteins including RNase via rapidly permeating high concentration of ammonium sulfate solution to the cells	4 days 6 months	Ambient temperature -80°C
	RNAgard	Inhibits RNA degradation and blocks induction of further gene expression	14 days 1 month	Ambient temperature 4°C
	Cell-free RNA Blood Collection Tube	Overall stabilization of both cell free RNA and cellular RNA via stabilizing nucleated blood cells, preventing release of cellular RNA, and inhibiting nuclease mediated degradation of cell free RNA	3 days	Ambient temperature
	RNA stabilization Matrix	Lysing blood cells and stabilizing RNA on a paper-based matrix	6 days	37°C
	FTA Filter paper-based solid matrices	Lysing cells and denaturing proteins using specific chemical denaturants and a free radical scavenger on a high-quality paper matrix while protecting nucleic acids from degradation	2 years	Ambient temperature
Post-Extraction stabilization	GenTegra RNA (IntegenX)	Mixing and dehydrating purified RNA with stabilization median	At least 1 year	Ambient temperature
	RNASTable (Biomatrica)	Mixing and dehydrating purified RNA with stabilization median	At least 1 year	Ambient temperature
	RNAShells (Imagene)	Encapsulating RNA in metal shells under anhydrous and anoxic conditions to protect RNA from oxidation	At least 1 year	Ambient temperature

Table 2. Cell sorting methods.

Method	Working Principle	Main Pros	Main Cons	Research focus
Sedimentation	Natural settling of particles because of gravitational force	Very low cost No need for high tech instruments	Labor-intensive Time-consuming Bacterial contamination risk	Finding new reagents for enhanced settling time Portable designs
Filtration	Separation based on cell size and its deforming characteristics	Low cost Efficient as pretreatment step. Low process time Closed cycle option for minimized contamination	High cell loss	New design in pore structures and materials
Centrifugation	Application of centrifugal force to separate based on density difference	Fast process Low cost	Time sensitive Low purity	New labeling reagents for selective sedimentation New designs for continuous process New materials for density, profile/gradient formation
Cell lysis	Selective lysis of unwanted cell types using chemicals	Low cost Simple and fast High throughput	Difficulty in RBC lysis loss of cells and cell subsets low purity	Finding more effective and selective lysing reagents
Immunoselection techniques				
Cell panning	Attachment of cells on surfaces coated with a specific antibody	Significant purity Low cost Easier detachment of cells from the surface Very simple	High cell loss Lack of quantitative separation	Finding antibodies for specific use
Fluorescent activated cell sorting	Attachment of antibody specifically to a cell type and subsequent fluorescence upon excitation using a laser beam	Significant throughput Very high purity High speed	Low throughput for therapeutic cells and rare cell sorting, Cell damage High equipment, operation and material cost. Need for pre-processes such as lysis of RBC and centrifugation Health risk for the user	Finding antibodies for specific use
Magnetic activated cell sorting	Cells marked with antibody-bound magnetic beads are collected selectively as they pass through a magnetic field	Less expensive comparing to FACS Simpler and faster than FACS Very high throughput High purity	Higher risk of cell damage Difficulty of separating cells from the particles	Finding antibodies for specific use
Buoyancy activated cell sorting	Attaching microbubbles to the cell by attaching antibodies and separation using flotation	Fast No need for magnets Low stress on cell wall Inexpensive No need for costly devices	Technology still in progress More elaborate comparing to FACS and MACS	Finding antibodies for specific use
Microfluidic cell sorting	Fluid behavior inside a microchannel that forms a velocity profile where the cells are exposed to different forces like drag forces and inertia	Inexpensive Minimal addition of chemicals Low process time Easy scale up	High mechanical stress Technology not established Design specific separation efficiency Heating and cell death	New designs Combination with other technologies

settle naturally over time. However, chemical agents can be added to enhance the sedimentation rate by aggregation of cells and still avoid centrifugation. A common chemical for this purpose is dextran which is a high molecular weight polysaccharide.^[34] The benefits of using dextran are low cost and no need for special equipment. The technique suffers from being labor-intensive, time-consuming and prone to bacterial contamination risk when applied in open systems. This technique is not being used so widely anymore^[34] but still being considered useful in very low-income countries since it is the least expensive method.

Researches in this field continue mainly on the application of chemicals to aggregate blood cells. Stack forming agents for aggregation of erythrocytes in rouleaux may include fibrinogen, multifunctional antibodies, polysaccharides, polyvinylpyrrolidone, or polyoxyethylene.^[35] Forced sedimentation using magnetic particles were also reported.^[35] A portable vibration-

assisted filtration device without the need for external pressure application was devised by Kim et al.^[36]

Filtration

Filtration is another widely used inexpensive technique able to separate cells based on their size and deforming characteristics. Filtration is a pretreatment step to enrich some cells of interest for further more effective purification steps.^[34] The method suffers from high cell loss proportion. With new designs, which reduce the leukocyte loss to a 5% maximum, the need for the formation of larger aggregates has been omitted by using the blood cell deformability characteristics and/or their different adhesion properties.^[34] The advantages of the method are its simplicity, low processing time, clinical effectiveness and no need for expensive devices. Besides, filtration must be applied in a fully closed system to avoid contamination and increase

the shelf life of products. Filters with new design in pore structures and materials are becoming available to improve the isolation capacity of leukocytes, erythrocytes, and to decrease cost and processing time.^[34] The goal in most filtration approaches is to separate erythrocytes from leukocytes.^[34] The LeukoLOCK® (Thermo Fisher) filters are a commercially available novel filter-based leukocyte capture method that can be used in conjunction with the RNAlater™ (Thermo Fisher) solutions to stabilize RNA in the filtered leukocyte population for months. This method was proved beneficial for transportation and recovery of RNA for use in microarray and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) applications.^[37]

Centrifugation

Centrifugation is a technique of separating substances which involves the application of centrifugal force. The popularity of centrifugation is related to its high separation capacity in a short time and low cost. However, centrifugation is a time-sensitive process in its general approach and for any given time the particles near to the settling surface will settle first even if they are smaller ones and consequently there is always a contamination problem. Low purity and cell loss are associated-problems in centrifuge as like for the sedimentation but with less severity. By proper adjustment of medium density, time, and rotation angular velocity, the low purity problem can be improved.^[32]

Blood cells differ in density and size therefore separation can be achieved by adjusting the centrifuge rotation velocity and time. Centrifugation separates whole blood into three fractions including i) packed red blood cells concentrate at the bottom, ii) cell-free plasma on top, and iii) a leukocyte and platelet-rich layer in the middle layer.^[34] High throughput, simplicity, adaptability and easy scale up for large-scale production are the centrifugation advantages.^[34]

However, to obtain the total RNA from leukocytes, peripheral blood mononuclear cells must be isolated by density-gradient centrifugation.^[38] This is performed by adding materials changing the medium density and subsequently creating a density profile/gradient in the medium allowing for density gradient centrifugation. Comparing to differential centrifugation, density gradient centrifugation can provide more efficient cell purification outputs.^[39] Density gradient centrifugations are usually employed to obtain subcellular blood fractions and to enrich for cells that could be used as sources of nucleic acids. It also ensured that mRNA carryover from reticulocytes was prevented and that the associated problems with the presence of globin transcripts were avoided.^[40] The use of Ficoll-Hypaque density gradient centrifugation has been the method of choice to achieve this enrichment. Commercial products that use this technology such as the vacutainer cell preparation tubes reduce the processing time and difficulty of working with the gradient; however, these products do not offer RNA stabilization and thus require immediate processing to collect the RNA.^[40]

Immunodensity cell separation is another approach that uses the density gradient centrifugation technique. An antibody-based labeling is implemented to label unwanted cells

and crosslink them to erythrocytes to form immuno-rosettes with much higher density comparing to mononuclear cells.^[41] During centrifugation, these heavy complexes will settle down and target cells are collected from a distinct layer above gradient.^[41]

Besides, in approaches that utilize a counterflow elutriation, the centrifugal force is balanced by the applied drag force on cells due to the counter direction flow. The centrifuge device is equipped with a conical shape chamber with varying diameters. Upon changing the rotation speed or the flow rate, cells are fractionated based on their size and to some extent by their different specific density.^[32,42] This counterflow method is commercialized and available in different scales and designs. With the development of new methods with higher resolutions, counterflow centrifugal elutriation is being less frequently used but the method is still useful in cell biology and clinical applications.^[42]

Sorting Cells Lysing Them

Removing erythrocytes from a whole blood sample through lysis using isotonic ammonium chloride solution is a common method in molecular biology. A centrifugation step follows the lysis step to abate lysates and obtains leucocytes. The isolation of leucocytes from whole blood using this technique is economical, simple and fast. There is no need for using density gradient polymeric agents like Ficoll or dextran which have shown adverse effects on leucocytes. The obtained yield for leucocytes isolation was reported as high as 73% with a purity of 78% comparing to a yield of 50% with the same 78% purity using dextran.^[43] Lysis is being widely implemented in many research works and diagnostic clinical applications where the effect of other lysing reagents must be known. Difficulty in erythrocytes lysis from some samples like acquired immune deficiency syndrome patients, newborns and patients having erythrocytes pathologies, loss of cells and cell subsets, and effects on the measurement of cell markers were reported.^[31]

The other lysis-based cell separation approach is the complement depletion method. The lysing agents involve a large number of plasma proteins form a membrane-attack complex and initiate a proteolytic cascade when activated by antibodies especially immunoglobulin (Ig)M and IgG. Targeted cell membranes are lysed via such proteolytic cascade and subsequently the lysed cells are removed through washing. This method mimics the mechanism of killing bacteria. With producing specific antibodies, the complement cascade can be used for depletion of any targeted cell population up to 95–100%.^[44]

Immunoselection-Based Techniques

Fractionation based on immunoselection methods takes the advantage of antibodies characteristics for binding specifically to specific subsets of blood cells. Purposefully selected antibody identifies markers on the cell surface and then the antibody conjugates with added fluorescent molecules or other micro-particles to isolate cells of interest. Because of the very high specificity of antibody-antigen binding, separation with high purity can be achieved in this way. The resolution is much higher

than label-free techniques. Despite its high selective characteristics, the method has been criticized for triggering changes in mRNA levels in the selected cells. Besides, there are limitations like processing time, intensive labor task, equipment cost and finding proper antibody with high specific binding characteristics for the interesting cell type.^[42] Well-known antibody-binding techniques are cell panning, Magnetic activated cell sorting (MACS),^[45] and Fluorescent activated cell sorting (FACS).^[1]

Cell Panning

In cell panning technique, a petri dish surface is coated with a specific antibody where cells having specific antigens can attach on that surface. Instead of dishes, the antibody may also be coated on microparticles. The technique provides significant purity, but limitations like high cell loss and lack of quantitative separation based on surface expression still exist. It is easier to detach cells from the surface in the cell-panning technique comparing to other antibody-binding techniques. The isolated cells are applicable for downstream analysis and as therapeutics.^[1]

Fluorescent Activated Cell Sorting

FACS is one of the most powerful methods for cell separation used in cytometry (Figure 2). A cytometer enables researchers to measure and classify cells based on multiparameter optical measurements. In this method, the cells are hydrodynamically focused through a very small microchannel and then pass an optical checkpoint one by one being illuminated by a laser

beam. At this point, scattered lights and fluorescence signals are collected and detected by multiple detectors. The decision for sorting cells is based on these signals that provide quantitative information about size and number of antibodies proportional to the cell fluorescent materials. At the end of the channel, the stream that carries the cells flows into droplets likely to contain only one cell. The formation of droplets and their size depend on the temperature of the chamber and its orifice diameter. These droplets are sorted according to the decision made in the previous step for classification and are subdivided into different groups under an electrostatic deflector system. FACS can sort up to 10,000 cells/s which is considered high but still much lower than bulk sorting methods like MACS or centrifugation.^[45] There are limitations like low throughput for therapeutic cells and rare cell sorting; and cell damage during the process, high equipment and supplies cost, operation, and need for pre-processes such as lysis of erythrocytes and centrifugation.^[46] The formation of extremely tiny aerosols may also be a problem for the user.

Magnetic Activated Cell Sorting

MACS is less expensive, simpler and faster than FACS but because of exerting a magnetic force in MACS, there is a risk of cell damage.^[45,47] In MACS (Figure 3), antibody-bound magnetic beads are used to target markers on the cell surface.^[45] These cells marked with magnetic beads are collected selectively as they pass through a magnetic field resulting from a permanent magnet. The MACS technology is a very efficient method due to the remarkable development of magnetic particle technology. These magnetic particles have been commercially available in various sizes as well as improving their surface properties. Compared to FACS, the MACS method has a higher capacity but lower purity. Another limitation is the difficulty of separating cells from the particles after the process.^[1,45] There is no need for high tech instruments in MACS.

Buoyancy-Activated Cell Sorting

BACS approach is based on attaching microbubbles to the target cell antibodies. They are then separated from the blood sample by buoyancy. These microbubbles can be made using various materials. Hsu et al. invented glass microparticles that were bonded with specific target antibodies.^[46] In the BACS method, the surface tension caused by ascending bubbles as well as the buoyancy forces are less than the cell damage limit.^[47] Biotinylated albumin microbubbles attached to avidin linkers and biotinylated antibodies have been used in the research of Liou et al to isolate subtypes of tumor cells.^[47] They have reported that the cells can be isolated in an easy and portable manner.^[47] Perfluorocarbon-filled microbubbles are widely used throughout the world in clinical work for ultrasound contrast imaging and are accepted as an intravenous injectable agent as an ultrasound contrast agent.^[48] In another study, microbubbles containing intracellular phospholipid perfluorocarbon gas used with cell adhesion antibody of anti-epithelial molecule type were used to isolate circulating tumor cells.^[49]

Microfluidic Cell Sorting Devices

This method of cell sorting is based on the fluid behavior inside a microchannel that forms a parabolic velocity profile during

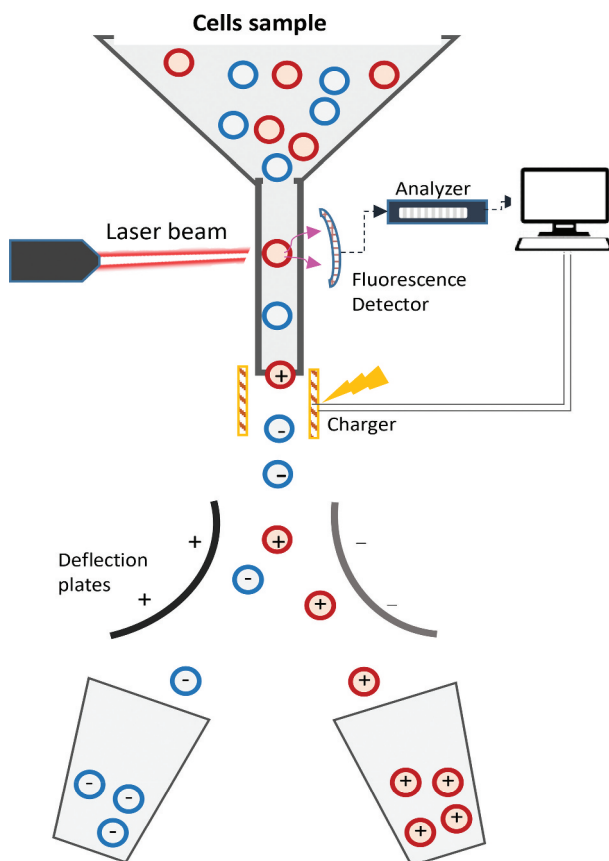


Figure 2. Fluorescent activated cell sorting system.

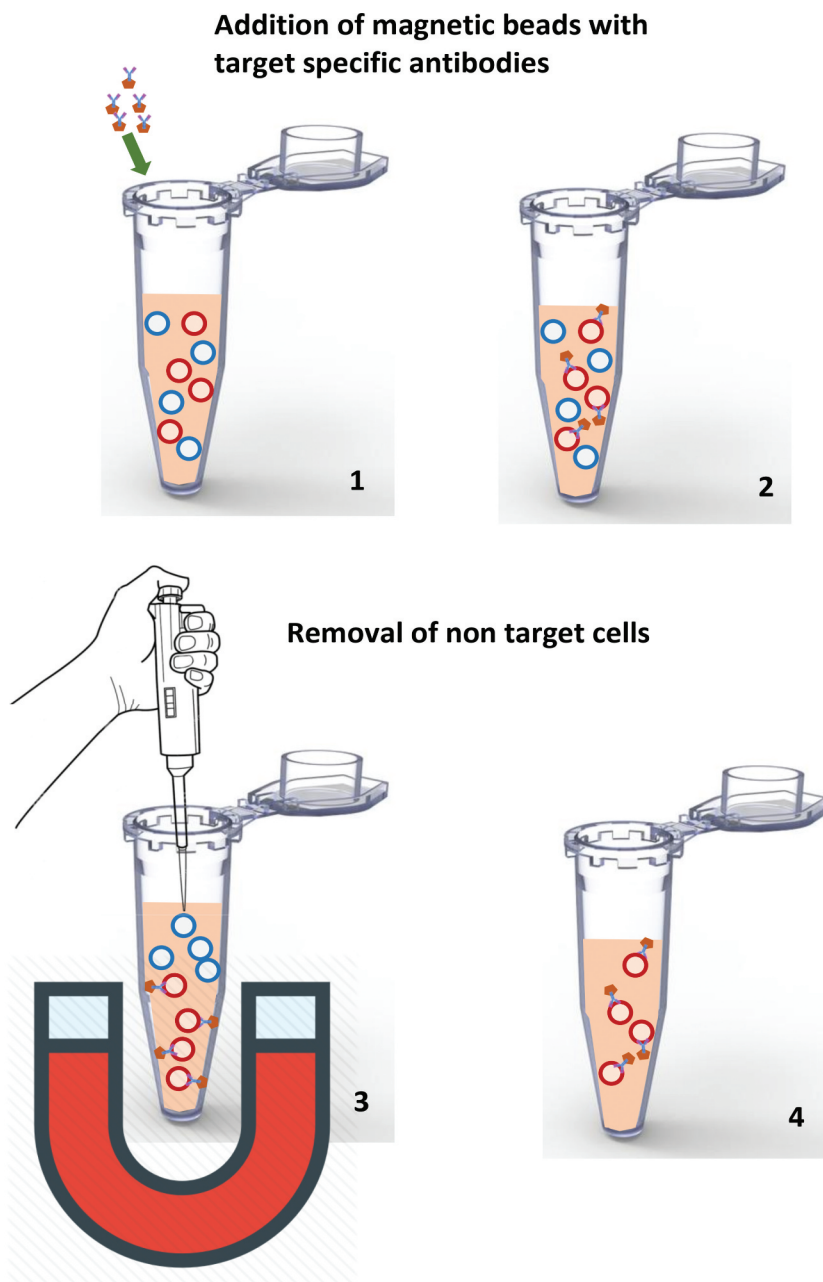


Figure 3. Magnetic activated cell sorting steps.

fluid passage and in contact with the channel walls where the cells are exposed to different forces like drag forces and inertia. The cells are positioned at different points in the velocity profile based on their size. The cells can be ultimately directed more effectively to separate streams by applying external forces to achieve cell separation. These exerted forces may be gravity, electric or magnetic field, optic source, and acoustic forces.

The microfluidic technology can be divided into two categories based on the separation strategy. In one approach, a micro-scale fluid flow is used to guide cells to different streams. In the second approach, micro-barriers form a network that performs the filtration process.^[50–52] In the first approach the size of the cell is the determining factor but in the filtration approaches both the size and deformability are considered as effective factors.^[51] Lately, dielectrophoresis

based approaches have also been introduced and applied for polarizable particles when exposed to a non-uniform electrical field.^[52,53] Microfiltration-based designs face the problem of changes in hydrodynamic resistance due to cell aggregation in microstructures.^[50] McFaul proposed a microfluidic structural ratchet mechanism able to separate cells based on size and deformability using an oscillatory flow.^[50] Another design with the same working principles was reported by Pang.^[51] Choi proposed a microfluidic device with slanted fences and filtration obstacles for the separation of erythrocytes from leukocytes. A 210-fold enrichment in leukocytes was achieved in 0.3 s.^[54]

The designed devices must allow large magnetic or electric fields to be applied over a wide range; it must be possible to connect multiple microfluidic devices simultaneously for

separation and analysis purposes with a higher workload. Low process time and low cost and no need for the experienced user and technologically sensitive equipment are other advantages of microfluidic devices. In such devices, the applied shear stress may damage the cells and therefore it must be considered in every design.^[47] Two major problems when the electric field is applied are induced membrane stress and Joule heating. Both increase the chance of cell death by heat and/or stress on the cell membrane.^[33] However common laboratory practice often includes fractionation of whole blood components before RNA extraction.^[55] The process allows for removal of red blood cells from whole blood samples which facilitates the study of more homogeneous cell populations. Depending on the selected fractionation method, partial or complete removal of reticulocytes may be achieved.^[55]

Extracellular Vesicle Enrichment

Extracellular vesicle (EV) derived RNA in plasma is another robust information source. However, exosome isolation with high yields, purity and quantities from blood is a vital step for the accurate detection of exosomal RNAs but remains still technically challenging.^[56–58] The major challenges of EVs isolation from plasma involve aggregation of vesicles, significant contamination with soluble plasma proteins, and co-isolation with EV-sized lipoproteins.^[57] EV isolation is also related to high plasma viscosity, high lipid, and protein content and the presence of platelets.^[59] These could hinder functional and analytical studies on EVs since certain proteins and, particularly lipoproteins may carry microRNA.^[57] Many methods, such as ultracentrifugation, ultrafiltration, polymeric precipitation, immunoaffinity isolation, size exclusion chromatography (SEC), and microfluidic techniques have been implemented to isolate entire populations of EVs from blood samples (Figure 4).^[56,58,60–65]

Ultracentrifugation

Ultracentrifugation is the most common exosomes isolation approach for extracellular vesicles especially from plasma/serum.^[56,57,60,66,67] The complete separation of exosomes can not be attained by ultracentrifugation alone, due to the co-sedimentation of vesicles and other macromolecules and impurities.^[60,68] Further purification can be carried out by multiple centrifugation using discontinuous ultracentrifugation or discontinuous density gradient ultracentrifugation like flotation in a sucrose cushion, or by continuous density gradient using solutions of iodixanol, which separates vesicles based on density and allows improvement of EV purity and classification of EVs (Figure 4(b)).^[57,60,66,68,69] Ultracentrifugation followed by differential gradient centrifugation which separates vesicles based on density is considered as the gold standard for exosome enrichment from EVs.^[64,68,69] Ultracentrifugation-based methods have several limitations such as deforming vesicles due to the high centrifugal forces, inefficient and aggregation of EVs that affect downstream analyses. It is time-consuming, expensive, and laborious requiring trained personnel. At the same time, ultracentrifugation has a highly variable yield due to operator change and existing washing and pelleting steps. Also,

the technique may lead to co-precipitate lipoproteins, proteins and other contaminants like especially albumin, the most abundant proteins in blood. Additionally, this method requires the use of an ultracentrifuge device that is not always available.^[56–60,62,64,66–68] Although density gradient ultracentrifugation which uses solutions of iodixanol, isolate exosomes with high purity from blood plasma, it has low yield due to the multiple-step protocols.^[57,69]

Precipitation-Based Protocols

Precipitation-based isolation relies on the reduction of EV solubility in a sedimentary material such as polyethylene glycol (PEG) polymers (Figure 4(e)). Polymer-based exosome isolation is simple, cheap, easy to implement and a high recovery efficiency method.^[56,61,64,70] Limitations of using these polymer-based methods are long incubation times, low specificity due to nonspecific precipitation of all soluble particles and proteins, contamination with residual PEG polymer which is hard to remove.^[56,60–62,64]

In recent years a rapid and simple method of EV isolation from the blood sample, using a PEG/dextran aqueous two-phase system provides a 70% EV recovery efficiency in 15 min which can remarkably increase the applicability of the polymer-based isolation method. Shin et al. demonstrated that the aqueous two-phase system EV isolation method does not have adverse effects on isolated EV total RNA contents and even has a higher yield than ultracentrifugation. However, the small RNA profile differs slightly between the aqueous two-phase system and ultracentrifugation method.^[71]

Immunoaffinity Isolation

Immunoaffinity isolation is based on using bead coupled with a specific antibody that recognizes an EV-specific protein to capture and enrich a specific subpopulation of EVs (Figure 4(g)).^[56,60] This method has high specificity but may need high amounts of antibodies which are costly and further ultracentrifugation steps to recover exosomes. In addition, it may extract larger vesicles more than other exosomes and also it is hard to detach the EVs from beads.^[61,62,72] Immunoaffinity-based capture techniques are considered in various studies especially when tumor-derived EV enrichments were sought.^[61,62,65,70,72,73]

Ultrafiltration

Ultrafiltration utilizes semipermeable membranes with defined pore size or molecular weight cutoffs (dotted line in Figure 4(c)) to purify and concentrate the vesicles. During centrifugation, exosomes are held on the filter, smaller impurities and other proteins pass through the membrane.^[60,64] However, it does not allow fractions of different EV types or guarantee protein separation.^[60] It is a fast, easy and valid method to reduce sample volume with minimal EV interference. But the technique has a limited capacity to purify exosomes due to the retention of high levels of protein complexes/aggregates and adhesion of exosomes to filters.^[62] Diaz reported that ultrafiltration combined with size exclusion chromatography resulted in high yield and optimal vesicle enrichment and soluble protein removal.^[64]

Size Exclusion Chromatography

Exosome isolation by the size exclusion chromatography (SEC) method (Figure 4(d)) is based on transferring the mobile phase containing the sample through a porous polymer packed in the SEC column able to sort particles by size.

EVs are bigger in size than the polymer pores and pass the column faster comparing to smaller particles such as proteins that get entangled in polymer pores so that their passing out of the column takes longer time than vesicles. [56,60,64]

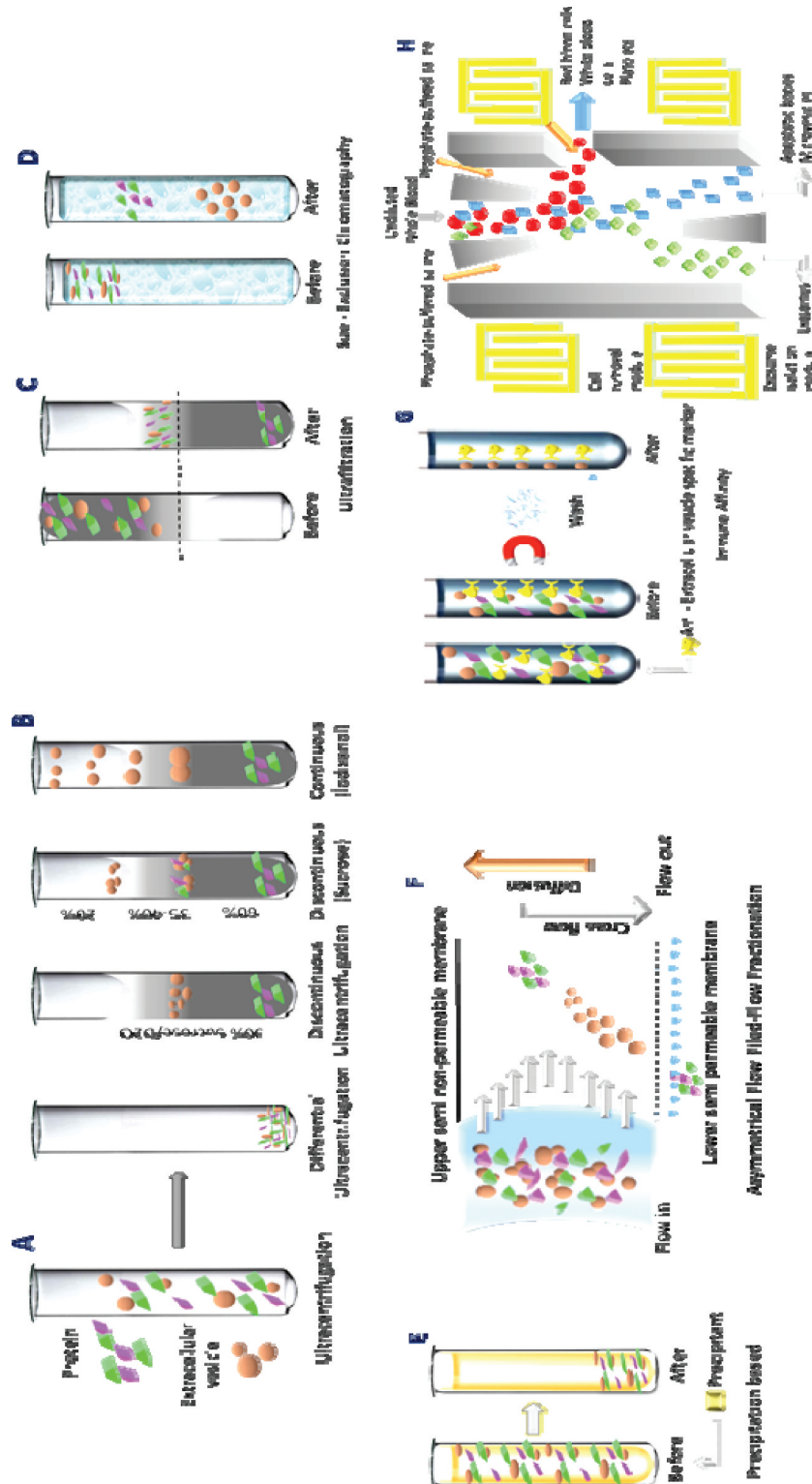


Figure 4. Techniques to isolate extracellular vesicles (EVs) from blood samples. (a): Ultracentrifugation (b): Discontinuous ultracentrifugation with a sucrose step density gradient or continuous with iodixanol solutions. (c): Ultrafiltration. (d): Size exclusion chromatography. (e): Precipitation-based isolation adding PEG that reduces EV solubility. (f): Asymmetrical flow filed-flow fractionation. (g): Immunoaffinity isolation using an EV specific antibody coupled to beads. (h): Acoustofluidic integrates acoustics and microfluidics to separate particles in an acoustic field.

Protein aggregates, including albumin, larger vesicles, and very-low-density lipoproteins are often co-isolated with exosomes if only SEC is used.^[59,64] However, the method is favorable for a rapid (~20 min) EV isolation from small volumes of serum/plasma (from 0.5 μ L up to 2 mL). Therefore, for an ideal exosome derived RNA analysis, SEC is routinely preferred and used in combination with other methods.^[56,62,67] Previously, a series of successful isolation of exosomes from blood plasma through the use of ultrafiltration before SEC, a combination of ultracentrifugation and SEC methods has been employed.^[56,62,68,71]

Also, various combinations of differential centrifugation followed by microfiltration and a final ultracentrifugation,^[63,72] density gradient ultracentrifugation followed by bind-elute chromatography,^[57] ultrafiltration and centrifugation in sucrose-deuterium oxide cushions^[72] were developed. A pre-treatment of serum or plasma samples with proteinase K, to reduce the amount of albumin and apolipoproteins was also reported.^[64] Other methods, including nanoscale lateral displacement arrays, nanostructure-based filtration, nanoplasmonic chip, magneto electrochemical sensor, and dialysis membrane filtration, field-flow fractionation (Figure 4(f)) and flow cytometry have been implemented.^[60]

Microfluidics Technology

Microfluidic technology and chip-style biosensors in terms of EV separation present a series of efficient and promising methods. Microfluidic devices utilize innovative sorting mechanisms such as acoustics, electrophoretic or electromagnetics, with minimal sample volumes and reagent consumption.^[60] In a recent study, the development of an acoustofluidics based integrated platform to separate the exosomes directly from whole blood was reported.^[69] Figure 4(h) shows that two modules for microscale cell-removal and exosome-isolation with blood cell removal rate of over 99% were associated.^[69] However, various microfluidic modules for EV separations have also been developed to isolate exosomes, based on immunoaffinity or SEC principles.^[69,72,73] The enrichment of tumor-derived EVs is still a considerable technical challenge in cancer studies since the majority of EVs in circulation are not of tumor origin. In an study, a new platform capable of highly efficient and reproducible immunoaffinity capture of tumor-derived EVs from blood plasma samples has been developed.^[65]

Currently, the most commonly applied methods for EV isolation from blood plasma are based on differential ultracentrifugation, size exclusion chromatography, filtration, or their combination^[57,67] but standardized methods for the isolation of blood plasma-derived exosomes are still missing.^[58–60,64,67,68]

RNA EXTRACTION

RNA extraction is the chore of transcriptomic analyses and its principle has vital necessities in all methods.^[74] First of all, biological samples are highly perishable implying conscious and safe handling with appropriate storage and inactivation of endogenous RNases by inhibitors or by chemical stabilization.^[74] Lysis or cellular disruption to isolate the nucleic acids

is another fundamental step. Various approaches have been developed for cell disruption and RNA extraction from blood samples.^[75] Generally, the cell lysis methods are categorized as mechanical and non-mechanical techniques.^[74,75] Mechanical lysis is the most popular and commercially available method with high throughput and high efficiency. A shear force causes the cell membrane break down. Among the different types of mechanical lysis techniques, bead beating using beads made of glass, zirconium oxide, tungsten carbide, stainless steel, and ceramic are mainly used for RNA extraction in laboratory scale.^[75] However, high cost and sample heating due to the collision between beads and cells and subsequent RNA degradation are the drawbacks of the method.^[75] Among non-mechanical methods, chemical lysis using chaotropic agents like guanidinium isothiocyanate, guanidinium chloride, urea and EDTA are widely used for cell lysis and especially for blood samples.^[75,76] Finally, there are various commercial or in-house basic approaches for RNA purification from lysate.^[74] The basic approaches for RNA isolation from blood samples, their principle, and main pros and cons are summarized in Table 3.

Isopycnic Gradient Method

Gradient centrifugation with added cesium chloride or trifluoroacetate right after dissolving sample by guanidinium thiocyanate (GTC) was the first developed procedure for RNA isolation. The captured RNA in appropriate density is then precipitated by ethanol. CsCl gradient ultracentrifugation have been used in some studies for high sensitive RNA detection in blood samples.^[74] This approach is now seldom used for high sensitive detection of RNA from blood samples because of its time-consuming procedure and aftermath disadvantages.

Guanidinium Thiocyanate/Phenol/Chloroform Single-Step Method

The cell lysis and inactivating ribonucleases in GTC, followed by an extraction step using organic solvents like phenol-chloroform for phase separation and alcohol precipitation using isopropanol is the most prevalent simultaneous extraction of RNA, DNA, and protein from the blood.^[74,76–79] TRIzol or TRI Reagent and similar solutions like QIAzol are monophasic GTC solutions with phenol able to dissolve biological samples and denature proteins. After solubilization, chloroform is added to induce a phase separation.^[74] Under acidic conditions, the total RNA concentrates in the upper aqueous phase, while proteins and most DNA molecules remain in the lower organic phase and interphase.^[77–80] Isopropanol is added to precipitate a pellet containing RNA possibly contaminated by residual salts. The pellet is washed with ethanol to recover pure RNA.^[74,79]

Trizol/TRI-Reagent based isolation is an effective and advantageous method for isolating miRNA and even other small RNAs such as piwi-associated RNAs and small interfering RNAs.^[81] Cost, incomplete separation of nucleoproteins from the RNA, probability of cross-contamination between upper aqueous and lower organic phases, residual phenol, chloroform, salts, and genomic DNA contamination are the

Table 3. RNA extraction methods.

Methods	Principle	Main Pros	Main Cons
Isopycnic gradient	Gradient centrifugation on the presence of CsCl or CsTFA right after dissolving sample by GTC	High sensitive detection of RNA from blood samples	Time-consuming Laborious protocol
Guanidinium thiocyanate-phenol-chloroform	Organic extractions using phenol-chloroform for phase separation, and alcohol precipitation after cell lysis via guanidinium thiocyanate (GTC).	Simultaneous extraction of RNA, DNA, and protein Effective and advantageous method for isolating miRNA and other small RNAs	High cost Probability of residual phenol, chloroform, salts, and genomic DNA contamination Not preferred for low volume of blood sample
Solid-phase Nucleic Acid Extraction	Binding RNA to the particular surfaces under the optimal concentration of chaotropic salts and certain pHs	Relatively rapid procedure Providing highly pure RNA Poly A selection when especially mRNA isolation is interested	High cost Probability of losing a part of RNA specially small RNAs like miRNA from lysate during different steps
Skip RNA purification	Optimizing the lysis buffer for direct reverse transcription from the lysate using mild nonionic detergents	Highly desirable when downstream application is qRT-PCR	Required highly optimized conditions. It is not preferred for downstream applications except qRT-PCR
Micro RNA isolation	Precipitating miRNA from total RNA after small RNA enrichment	Highly interested for using miRNA as biomarker in clinical studies	High cost, laborious protocol health risks due to the use of hazardous chemicals, low concentrations, sample contamination Require complicate procedures and specific approach to isolate RNA
Chip-based isolation approaches	Miniaturizing the whole or a part of the process on a micro-scale device	Significantly convenient, rapid, low cost and sensitive procedure Well suited for low-level sample size	-

main limitations of this GTC method.^[74,81–83] To prevent of DNA contamination, several simple modifications have been reported. It has been debated whether it is necessary to apply the additional DNase treatment step through column-based RNA purification or not.^[82,83] Although the TRI reagent is not preferred because of unsuccessful results with low blood volumes, a study reported acceptable RNA quality and yield obtained with 2 μ L of blood using 1:1 TRI reagent blood ratios.^[84]

RNAzol RT (MRC, Cincinnati, Ohio, USA) is an alternative single step chloroform-free approach, where phase separation is induced upon guanidine and phenol interaction with cellular components. The homogenized lysate is supplemented with Rnase-free water inducing DNA, proteins, and polysaccharides precipitation while RNA remains soluble. After centrifugation and removal of precipitates, RNA with high purity and quality can be isolated from the supernatant by alcohol precipitation. The RNAzol method does not require DNase treatment step to remove DNA contamination. Two protocols for total RNA isolation are possible. They are size-based for separation of RNA longer than 200 bases (rRNA and mRNA) and shorter than 200 bases (ribosomal RNA, transfer RNA, and microRNA).^[80]

Solid-phase Nucleic Acid Extraction Based Methods

Solid-phase nucleic acid extraction methods are based on the RNA capability to bind to the particular surfaces under the optimal concentration of chaotropic salts at certain pHs.^[74,79] The sample is lysed in GTC, and then exposed to specifically adsorbing material such as silica matrices, glass particles, magnetic beads, polystyrene latex materials or cellulose matrices.^[74,79,85] The RNA will be retained in the matrices under the specific pH and salt concentration via hydrogen-bonding interaction, ionic exchange or affinity and size exclusion

mechanisms.^[79] Other contaminants are removed in the washing step and RNA is finally released using a salt-free buffer.^[74,79,85] A part of RNA, especially small RNAs may be lost from lysate either degraded or washed out specially when alcohol precipitation is used.^[74,85] In contrast, adsorption methods have significant advantages being fast and avoiding any alcohol precipitation.^[74,85] The guanidinium-phenol-chloroform RNA extraction over silica-based columns is the best specifically when reduction of RNA contamination and loss is desired. The columns give also a good RNA yield of high quality.^[83] Such an approach was successfully used for the co-extraction of RNA and genomic DNA from peripheral blood mononuclear cells.^[78,86] The column-based method has the advantage of providing highly pure RNA, however, the reagent cost especially when the number of samples is large may become a big issue while miRNA isolation may be still challenging.^[83,86] In procedures which are based on immobilizing RNA onto beads, any loss of beads leads to the loss of RNA and therefore should be prevented.^[79]

mRNA selective isolation is an alternative solid-based method that can be carried out based on the complementary base pairing of polyadenylate “d(A)” tail at the 3' terminus of mRNA with synthetic oligo “d(T)” chains which covalently bound to special matrices or beads. After oligo dT-dA base pairing under high buffer salt concentration, the obtained solids can be separated via centrifugal sedimentation, magnetic force or filtration.^[74,85,87,88] Then, non-polyadenylated RNAs and other impurities are flushed out of the solid via washing. Then a low salt concentration buffer is used to dissociate the duplex structure and release the mRNA from solid.^[79] Previous studies discussed that an additional DNase treatment reduced DNA contamination and increased mRNA purity. Also, the sensitivity of low-level transcripts quantification was also better decreasing the clumping of sedimented beads that facilitates bead resuspension.^[88]

Skipping RNA Purification

Direct quantification of miRNAs/mRNAs from lysates is highly desirable especially when the downstream application is quantitative real time-polymerase chain reaction (qRT-PCR).^[74,89] Several commercial and in-house methods are reported in previous studies.^[89] These methods use a mild nonionic detergent like NP-40 and Triton X-100 in the presence of RNase inhibitors and DNase to optimize the lysis buffer for direct reverse transcription from the lysate.^[89] They benefit from thermal lysis for a small amount of whole blood (2–20 μ L).^[90] Recent studies presented the Direct S-Poly(T) Plus assay for quantification of circulating microRNAs without RNA extraction from plasma/serum.^[91] The *Thermus thermophilus* (Tth) polymerase method is resistant to various prevalent PCR inhibitors and displays reverse transcriptase activity in the presence of manganese ions.^[92] Moreover, a method based on the capture of mRNA directly from lysate, was recently developed using a stainless steel needle amino-linked to dT oligonucleotides. Then, the needle with attached mRNA is transferred in a microchip device for qRT-PCR.^[85]

Micro RNA Isolation

Cellular micro RNA, RNAs encapsulated into exosomes, and cell-free RNAs are highly stable in biological fluids due to co-existence in complexes with proteins and lipoproteins. Therefore, they can be considered as a highly interesting biomarker in many clinical studies. miRNAs need to be efficiently isolated. Otherwise, it can impact on all downstream analyses and even may lead to controversial results. Besides, studies on these subsets of RNAs require specific tasks such as small RNA enrichment, exosomes enrichment and specific tasks to isolate RNA.^[86,93,94]

The acid phenol-chloroform extraction method greatly eliminates isolation concerns. After purification of the total RNA, the large RNAs precipitate using a precipitant solution containing potassium acetate, PEG, sodium acetate, or lithium chloride. The micro RNA recovery is done from the small RNA enriched supernatant subsequently via alcohol precipitation. However, it should be noted that it is a costly and laborious protocol with health risks due to the use of hazardous chemicals.^[94]

An alternative method to reduce the exposure level with hazardous chemicals is a phenol-free protocol.^[93,94] In this alternative protocol, phenol is replaced by 2-mercaptoethanol as denaturant agent in lysis buffer and demolition of RNA-containing complexes in a single- aqueous/organic phase to release miRNA. The chaotropic salt guanidine thiocyanate associated to an ethanol:chloroform mix facilitates the binding of miRNA to a solid-phase carrier such as silica spin column (OPS Diagnostics, Lebanon, New Jersey, USA) under optimal pH.^[94] Another phenol-free protocol proposed an initial octanoic acid treatment before applying the silica spin column for purification of miRNA from plasma. Octanoic acid can form insoluble complexes with most plasma proteins. Since GTC alone is not adequate for the destruction of the miRNA-protein complexes, a compound with protein affinity such as octanoic acid, prevents hydrophobic and destroys vesicular membranes

and separates protein and lipoprotein complexes from miRNAs. However, a combination of denaturing/precipitating agents like GTC and octanoic acid provides a rapid and high yield miRNA isolation from plasma.^[93] Other phenol-free method for microRNA isolation from human blood plasma were reported, such as a treatment with lysis buffer in which β -mercaptoethanol was replaced by dithiothreitol and a linear polyacrylamide, poly(A)RNA was added with transfer RNA as coprecipitator to improve the yield of microRNA isolation.^[95]

Automated systems based on organic extraction principles also provide convenient purification of extracellular RNAs. However several challenges such as low concentrations, sample contamination, and presence of inhibitors must be overcome before circulating miRNAs can be used for diagnosis and monitoring.^[96] Indeed, the detection of miRNA in blood with femtomolar sensitivity still remains a challenge.^[97]

Chip-based Isolation Approaches

Conventional processes are expensive and time-consuming due to different sample handling steps such as filtration, centrifugation, distillation, dilution, and extraction. To simplify the conventional method, several methods have been developed based on solid-phase being faster and more cost-effective ways. However, these methods still need manual operations and have some limitations.^[98] An automated system for nucleic acid extraction from whole blood, saliva, and serum/plasma, benefiting from solid-phase extraction with magnetic bead and spin column, provided very reliable and reproducible results quickly and avoiding contamination. However, this Versa NAP system (Aurora, Vancouver, British Columbia, Canada) was designed for medium to large laboratories to reduce working time and labor costs as well increase worker safety and the laboratory efficiency. Nonetheless, the liquid handling and manual pre-processing stages by expert staff is still required being the main obstacle.

Microfluidic devices are miniaturized platforms performing separation processes in micro-scale and developing more convenient, rapid, low cost and sensitive procedures suited for low-volume samples. These microfluidic devices overcome the drawbacks and incompatibilities of the traditional and even automated methods.^[75,98,99] The microfluidic lysis technology utilizes mechanical, thermal, chemical, optical, acoustic or electrical approaches.^[75,98] These devices commonly utilize the shear forces applied by bead beating or friction on nanoscale barbs to disrupt the cell mechanically.^[75,98,100] The high surface to volume ratio feature in microfluidic devices leads to dissipate high heat quickly and disrupting the cell membranes effectively without damaging the nucleic acids. Therefore, thermal lysis provides an appealing approach due to the easy integration of PCR modules to microfluidic devices.^[75,98] However, chemical lysis chips have a number of halting sides. Such devices require a complex structure for injection channels with micro-mixers. Also, it takes long time for reagents delivery. In addition, since separating reagents from target molecules is not easy, interferences with the downstream assay may occur. Difficulties regarding the reagent storage for a long time is another limiting problem.^[75,98] Application of high energy

sound waves, strong electric fields, lasers, and optically induced dielectrophoresis techniques could potentially damage the cell membrane.^[75]

Most microfluidic systems are conceived for DNA sample preparation mainly from infectious agents in biofluids.^[98,99] RNA targeted systems are relatively rare due to the instability and complexity of the different RNA types with delicate sample preparation.^[98] In fact, RNA isolation in microfluidic systems is becoming increasingly important, especially since the microfluidic approach presents some critical solutions for RNA isolation challenges.^[98] Few single module devices for sample preparation from blood cells or only for amplification of captured mRNA in microchips are available.^[85,100] Additionally, multifunctional devices are also designed. A digital microfluidics technique for extraction and purification of high-quality RNA from low volumes of human whole blood samples was developed in 2014.^[101] Another development of a rapid RNA micro extractor using lateral magnetophoresis generated by a ferromagnetic wire array inlaid on a glass substrate and magnetic oligo-dT covered beads was presented for direct isolation of RNA from peripheral blood lysate.^[102] An integrated module was introduced for RNA extraction and reverse transcription able to extract RNA from peripheral blood lysate in 1 min. The module works with magnetic oligo-dT covered beads and subsequently complementary cDNA synthesis in microchambers, which are monolithically integrated with the RNA extraction compartment.^[103] The last developed devices provide integrated modules for RNA extraction, reverse transcription to cDNA, amplification, and detection of mRNA or even miRNA from blood samples.^[104,105]

GLOBIN AND RIBOSOMAL RNA REDUCTION

The solid phase of blood is composed of a heterogeneous population of erythrocytes (95%), platelets (5%) and white blood cells (< 1%). Although white blood cells are in the minority, they are the most informative cells for the transcriptomic analysis.^[106–108] Due to the blood natural structure, the detection of gene expression profile from only a subset of cell type is difficult.^[107] However, capturing the most accurate transcriptome profile at the time of blood collection is required to minimize extra processing steps.^[109] Along with the opportunities provided in using whole-blood RNA for expression profiling, there are also several challenges such as low proportion of leukocytes and a high amount of cytoplasmic and mitochondrial rRNA and α and β globin mRNA (gmRNA) transcripts which can constitute up to 80% of the total RNA.^[106–108,110–114] As a result, the presence of a high abundance of gmRNA and rRNA in samples complicates the transcriptome studies of whole blood RNA and affects accuracy, sensitivity, reproducibility, and cost of downstream analyses.^[106] The biological significance of less abundant transcripts can be concealed.^[106,107,109–111,114,115] To overcome such problems, gmRNA and rRNA levels must be reduced. This is achieved either by fractionating blood cells to reduce the red blood cell population and particularly reticulocytes which are the primary source of globin transcripts, or by depleting these transcripts from total RNA.^[40] Several depletion methods based on enzymatic reactions, blocking reverse transcription, capturing

unwanted transcripts, poly-A selection, and post-library construction were successfully developed.

Enzymatic Procedures

To deplete selectively rRNA and globin transcripts from whole blood, commercial protocols were initially developed using enzymatic procedures. These procedures included the hybridization of antisense DNA oligo to the rRNAs and various globin transcripts in blood followed by digestion of the RNAs: DNAs hybrid with RNase H.^[40,114] In one of the first protocols suggested by Affymetrix (Santa Clara, California, USA), antisense DNA oligos targeted $\alpha 1$, $\alpha 2$, and β globin transcripts poly-A tails. Globin reduction oligos are specific to sequences just close to the Poly-A tail region of gmRNA. After hybridization of DNA oligos with gmRNAs, the formed hybrid are digested by RNase H to remove these transcripts and prevent its intervention in the following oligo (dT)-primed reverse transcription reaction during the labeling assay.^[116] The NEBNext® Globin & rRNA Depletion Kit (Biolabs GmbH, Frankfurt, Germany), Kapa RiboErase (Roche, Indianapolis, USA), and Takara/Clontech RiboGone (Takara, Kusatsu, Shiga, Japan) also utilize the RNase H-based RNA depletion workflow to deplete gmRNA, cytoplasmic rRNA and mitochondrial rRNA.^[117,118] The reverse transcriptase-mediated ribosomal RNA depletion strategy (Figure 5) was proposed recently. It uses rRNA-specific DNA probes to produce RNA/DNA hybrids fragments from rRNA transcripts followed by exonuclease I, RNase H and Dnase I treatment and ethanol precipitation of purified RNA.^[114]

Blocking Oligonucleotides

The current developments are generally related to blocking oligonucleotides which especially have been utilized in gmRNA reverse transcription inhibition.^[108] A possible non-enzymatic approach for gmRNA reduction is based on using peptide nucleic acid (PNA) oligonucleotides. PNAs can bind to the targeted mRNA and lead to stop the reverse transcription half-way during the first-strand cDNA synthesis due to PNA-mRNA double-strand complexes (Figure 6). Therefore, PNA oligomers can be effectively used in blocking gmRNA during the process of reverse transcription.^[108] Affymetrix (ThermoFisher) and PreAnalytiX (Qiagen GmbH, Hilden, Germany) have developed approaches utilizing globin 3' specific PNA oligomers as blocking molecules to inhibit reverse transcription in cDNA synthesis during T7 RNA polymerase-based linear amplification.^[40,106,115] Blocking gmRNA with PNA oligomers provides high performance with precise and reproducible results. However, the approach requires a high amount of RNA input and is one of the most time-consuming methods.^[109]

Another recently introduced method is GlobinLock® (GlobinClear, ThermoFisher) which uses several gmRNA-specific single-strand DNAs which are either locked or Z oligonucleotide where their 3' site is modified. These are added in RNA initial denaturation buffer and bind to gmRNA molecules at 3' site where specifically block the binding site of oligo-T primer to prevent globin cDNA synthesis

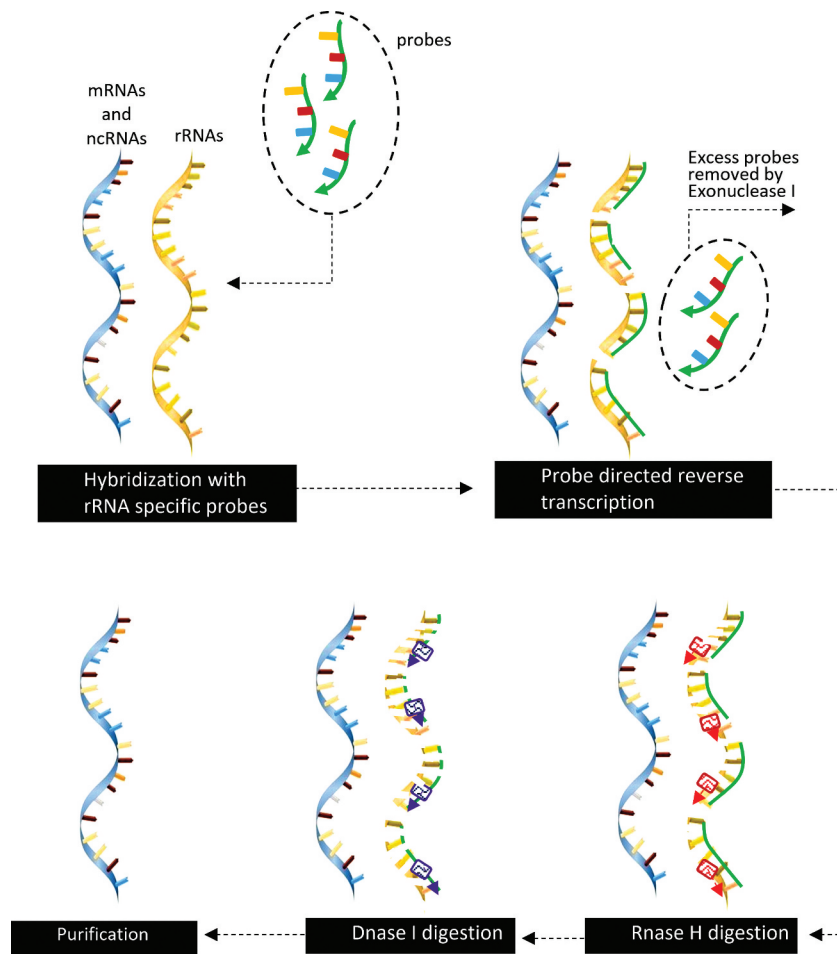


Figure 5. Reverse transcriptase-mediated ribosomal RNA depletion (RTR2D).

priming.^[108] Methods that benefit from specific oligos that could bind to the first-strand cDNA of gmRNA and prevent the generation of amplifiable library fragments from globin mRNAs during second-strand synthesis are also considered in some commercial methods.

Capture gmRNA

Abundant and unwanted RNA transcripts such as rRNA and gmRNA can be captured by hybridization to complementary biotinylated oligo probes, followed by extraction with streptavidin-coated magnetic beads. An alternative strategy provided by Ambion is the GLOBINclear kit, which is a non-enzymatic gmRNA reduction technology that depletes >95% of α and β gmRNA derived from whole blood total RNA samples prior to amplification.^[40,107,108,119] The method utilizes long biotinylated capture oligonucleotides which specifically bind to the gmRNA and resulting hybrids are then captured by streptavidin-coupled magnetic beads (Figure 7). Further purification and enrichment by clean-up procedures are required to apply on globin reduced RNA. This method results in a dramatic reduction of the globin gene transcripts from whole blood RNA. Thus, globin transcript reduction prevents the loss of significant information caused by the distorted transcript composition in human whole blood.^[40,107–109,115] However, the Ambion GLOBINclear Protocol achieved similar results with

the PNA method in terms of sensitivity and reproducibility and also require a relatively high amount of sample a big drawback.^[107,109] RiboPOOLS developed by siTOOLS Biotech, Illumina's Globin-Zero and RiboZero, Lexogen RiboCop, Qiagen GeneRead rRNA depletion, and The NEXTFLEX RiboNaut rRNA depletion kits are other commercially available approaches which efficiently removes rRNA or gmRNA through this route.^[112,113,117,120,121]

The capture oligo method had slightly better transcript detection sensitivity compared to other methods. However, larger amounts of initial high-quality RNA are required to obtain sufficient cRNA amounts, and these procedures were more complex and time-consuming than the inhibitory method.^[115]

Poly-A Selection

The most common method of rRNA depletion is poly-A selection, which relies on the use of oligo (dT) primers attached to a solid support (e.g. magnetic beads) to isolate polyadenylated RNA transcripts. Losses of non-polyadenylated transcripts such as some protein-coding RNAs (histones) which lack polyA tails, and some long non-coding RNAs and microRNAs and small nucleolar RNAs during poly-A selection method are the main drawbacks which cause the failure of the Poly-A method in covering a significant portion of the transcriptomic information.

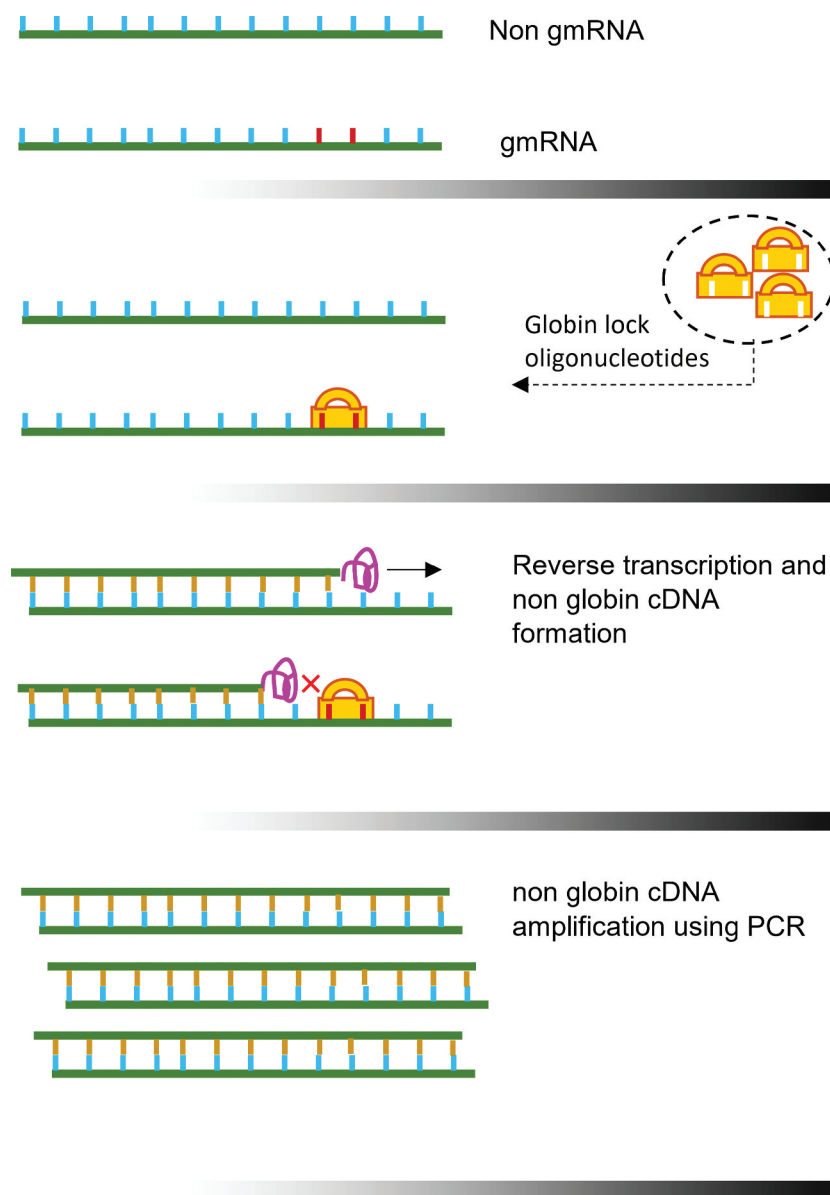


Figure 6. Schematic overview of the globin block modules for globin depletion.

However, the Poly-A selection might be the most efficient method when only focused on protein-coding genes.^[112,113,117,120–122]

Post Library Construction Target Depletion

Methods based on post-library construction target depletion have been developed by NuGEN that reduce the impact of abundant RNA transcripts without manipulation of starting RNA. In a common procedure, after producing dscDNA using random and oligo(dT) of DNA/RNA chimerical primers, single-strand cDNA amplification from the double-strand cDNA template has been performed in a cycle with a single primer linear isothermal amplification (SPIA Amplification) and subsequently purification of SPIA-cDNA.^[123]

Also, NuGEN developed an enrichment method for non-rRNA and non-globin sequences in next-generation sequence libraries using NuGEN Insert Dependent

Adaptor-Cleavage (InDA-C) technology for targeted removal of picked library sequences. This led to a significant reduction of reading sequences derived from rRNA and gmRNA. The streamlined workflow consists of using a mixture of random and poly(T) primers to produce dscDNA, fragmentation of dscDNA, end-repair and adaptor ligation, strand selection via nucleotide analog-targeted degradation, InDA-C-mediated adaptor cleavage and PCR amplification to produce the final library. Post library construction target depletion maximizes informative reads without manipulation of starting RNA. Takara/Clontech SMARTer Pico kit also follows a similar approach using low input samples that targets the ribosomal RNA sequences after conversion to cDNA and library preparation using the ZapR enzyme.^[117]

An alternative method to deplete rRNA involves the use of “not so random” hexamer/heptamer primers with a decreased affinity for rRNA during first-strand cDNA synthesis. This is

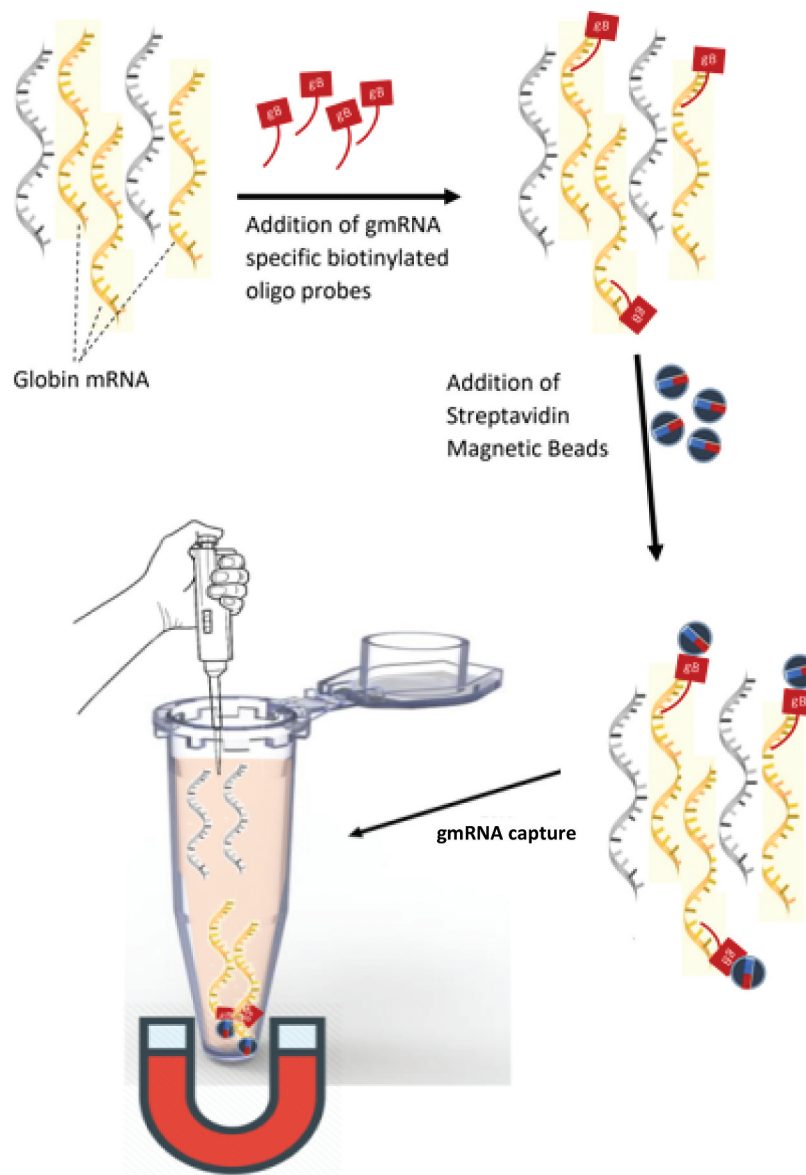


Figure 7. Globin mRNA capture through biotin/streptavidin binding, the specificity of nucleic acid hybridization, and use of magnetic bead separations to deplete globin mRNA.

employed by the Ovation RNA-Seq kits from NuGen. The Nugen kit also incorporated an RNase-mediated degradation of unwanted transcripts during the final library construction steps. This indicates that targeted amplification alone cannot remove rRNA. The method however works with low input amounts and degraded samples.^[112,113,117,120–122]

The employed rRNA and gmRNA depletion protocol removed approximately 90% of the unwanted transcripts, improved the sensitivity and reproducibility of high-throughput mRNA expression analysis allowed for reliable detection of thousands of additional transcripts and represent the true biological variation.^[111,119,120] The combination of depletion protocols with whole-blood RNA stabilization represents a robust clinical research methodology for the discovery of biomarkers from blood. Nevertheless, prior studies show that globin reduction procedures resulted in a significant reduction in RNA yields down to 5–9%, and slightly lower RNA Integrity Number (RIN) value indicating a minor

reduction in RNA quality.^[106,111] The selection of a protocol for expression profiling studies in blood depends on several factors, including implementation requirements of the method and study design.^[109] However, rRNA and gmRNA reduction may not be always beneficial or necessary in the study of certain diseases.

RNA CONCENTRATION AND QUALITY ASSESSMENT

For an efficient transcriptomic analysis, determining the RNA quality is the primary step. This first step is crucial to ensure that long-term and costly analyses do not yield meaningless results. Several methods have been tried to be standard in the evaluation of RNA quality. Each has different advantages and disadvantages. The application of these preliminary quality determination methods is especially important to be aware of the degraded RNA and to complete modern molecular and biological methods.

Fluorescent Dye-based Measurements

In this method, fluorescent dyes are bound to nucleotides such as dsDNA, RNA and ssDNA for ultrasensitive detection. The fluorescence light is proportional to the nucleotide concentration. High sensitivity and low-level samples requirement are the main advantages of the method. Easy inoculation of samples, low incubation times and short processing times are other important advantages of dye-based fluorescent measurements. However, samples with high nucleic acid content must be diluted before dye-based methods measurement because only a small amount of sample is required for the measurement. Also using RNA-specific dye is required when RNA concentration measurement are considered. Besides, fluorescent dyes are often chemicals that fall under the potential hazard class. Therefore, caution should be exercised when performing these analyses.^[124]

Absorbance Measurements

As any chemical, nucleic acids have an absorption spectrum. In absorbance measurements, the Beer–Lambert law relates the amount of absorbed light to the nucleic acid concentration usually measured at 3 basic UV wavelengths: 230 nm, the preferred 260 nm, and 280 nm. DNA and RNA concentrations are determined by calculating the conversion factor and quenching coefficients for each nucleic acid. The 280 nm wavelength is mostly used to identify proteins and 230 nm is used to identify different contaminants found only in some samples.^[124] In terms of determining the purity of RNAs, the ratio RNA absorbance to protein absorbance, A260/A280, is used.^[124] A ratio of ~1.8 and ~2.0 is generally accepted as “pure” for DNA and RNA, respectively. The second indicator is the A260/A230 ratio values in contrast with the A260/A280 ratio values. Expected A260/A230 values are usually in the 2.0–2.2 range. A significantly lower ratio of A260/A280 and A260/A230 than accepted values could indicate the existence of various contaminants such as proteins, TRIzol reagent, guanidine hydrochloride, guanidine thiocyanate, EDTA, carbohydrates, or phenol. All absorb at or near 280 and 230 nm.^[125] However, a A260/A280 absorbance ratio of 1.7–2.0 is acceptable for RNA downstream analysis. Although there is no consensus on the acceptable lower limit of the A260/A230 ratio, it must be approximately 2 or slightly above that.^[125] Devices such as NanoDrop (Thermo Scientific) and NanoVue (GE Healthcare) are capable of determining the RNA quality, using small sample volumes (0.5–2 µL) with a wide detection range in a short time. However, the main limitation of such methods is the absorbance of all types of nucleic acids (dsDNA, RNA, and ssDNA) at the same 260 nm wavelength. Therefore, it is difficult to distinguish between nucleic acids. Also absorbance does not provide information about RNA intactness.^[40,124]

In addition, one should consider that in-vitro hemolysis of the samples has a substantial impact on the plasma/serum endogenous RNA, especially where miRNA profiling is sought.^[126] Only non-hemolysed samples could be used for biomarker discovery. Serum/plasma endogenous miRNA profile can be impaired with erythrocyte-specific miRNA. Hemolysis could increase the circulating miRNA level up to 50 folds.^[5]

Therefore, the determination of hemolysed samples before the study is very important doing spectrophotometric measurement of oxyhemoglobin at visible light between 350 and 650 nm wavelengths. An optical density of free hemoglobin exceeding 0.22 g/liter in the plasma at 414 nm indicates sample hemolysis.^[126] There are other introduced methods to determine the erythrocyte specific miRNA which requires isolation and real-time PCR and may not be suitable when sample volume is limited.^[5]

RNA Integrity Measurement

In cases where it must be confirmed that the RNA structure is intact, RNA integrity measurements can be implemented. The method includes analysis of ribosomal 18s and 28s subunit to determine RNA degradation using denaturing agarose electrophoresis or microcapillary electrophoresis on an Agilent 2100 (Agilent, Santa Clara, California, USA) bioanalyzer to measure the RNA integrity number (RIN) through quantification and qualitative size calculations. This bio-analytical device is based on a combination of microfluidic chips, electrophoretic size separation in gel-filled channels and laser-induced fluorescence detection on a miniaturized scale. RNA molecules are stained and detected using laser-induced fluorescence. Data are available as electropherograms. The RIN scale is shown in numbers from 0 to 10. The number 10 stands for intact RNA. Generally, RIN scores of 7 or higher predict satisfactory results in downstream analyses. However, bioanalyzer reagents and chips are expensive and unable to identify protein and DNA contamination.^[40]

Agarose and Acrylamide Gel Electrophoresis Measurements

A qualitative interpretation of the RNA quality and concentration can be achieved by comparing RNA bands with known standards of fluorescent intensity in agarose or acrylamide gel electrophoresis. However, quantification of the quality of RNA requires an instrument capable of determining the density of the gel from the image and also an advanced computer to perform calculations. Moreover, since this information does not lead to a firm conclusion and also for the method costs, sensitivity to the amount of RNA, existing inhibitory agents, and use of highly toxic chemical dyes, this method is not desirable.

CONCLUSION

RNA molecules are extremely sensitive to pollutants and unstable, therefore RNA samples preparation should be handled with great care. The complex structure of blood demands various preanalytical treatments to obtain high-quality RNA samples. The main preanalytical steps are blood sample stabilization, cell sorting, exosome enrichment, RNA extraction and gmRNA/rRNA reduction when working. The selected methods and techniques depend on the final analyses of interest. Stabilization of RNA and inactivation of RNase using stabilization reagents have crucial importance for reliable results even when fresh samples are

used. The technology for cell sorting has dramatically improved recently. High-affinity particles or antibodies make it possible to obtain cell populations with very high purity. In terms of extracellular vesicle isolation, methods like ultracentrifugation, size exclusion chromatography, filtration and/or their combination are the classical, effective and applicable ones. RNA extraction is the fundamental step and backbone of all transcriptomic analyses. Although acid phenol-chloroform is considered as the standard total RNA extraction method, several limitations exist which can be improved by using a solid-phase separation method. Although modifications of the acid phenol-chloroform method were improving small RNA isolation, there is no established standard method yet. Advances in microfluidic technology are very promising wherein almost all procedures, even mRNA and small RNA detection, can be done at the μL level. Although obtaining a specific subset of blood sample is a common target for transcriptomic profiling and biomarker discovery, analyses of whole blood provide the most accurate transcriptome profile and accordingly, the reduction of gmRNA and rRNA should be considered as well. Finally, before any analysis, RNA quality and integrity measurement are necessary procedures for a successful and reliable analysis.

Disclosure statement

No potential conflict of interest was reported by the authors.

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