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Ribonucleic acid extraction: A mini-review of standard methods

Afolabi, Oluwadamilare I.

Research and Development, ARL Bio Limited, Lagos, Nigeria
Correspondence to: dare.afolabi@outlook.com; +234 (816) 976-9839

Abstract:

Different techniques have been proposed for RNA extraction, many of which have found extensive use in biological research. The introduction of these methods has greatly improved molecular diagnostics, drug discovery, and numerous other research and clinical endeavors. In this review, the working principles of the most commonly used RNA extraction methods for research and clinical applications are discussed. Current automation efforts and the quest for more efficient and cost-effective methods are highlighted.

Keywords: RNA, extraction, RNases, mini-review

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Extraction de l'acide ribonucléique: une mini-revue des méthodes standards

Afolabi, Oluwadamilare I.

Recherche et développement, ARL Bio Limited, Lagos, Nigeria
Correspondance à : dare.afolabi@outlook.com; +234 (816) 976-9839

Résumé:

Différentes techniques ont été proposées pour l'extraction de l'ARN, dont beaucoup ont été largement utilisées dans la recherche biologique. L'introduction de ces méthodes a considérablement amélioré le diagnostic moléculaire, la découverte de médicaments et de nombreux autres efforts de recherche et cliniques. Dans cette revue, les principes de fonctionnement des méthodes d'extraction d'ARN les plus couramment utilisées pour la recherche et les applications cliniques sont discutés. Les efforts d'automatisation actuels et la recherche de méthodes plus efficaces et plus rentables sont mis en évidence.

Mots clés: ARN, extraction, RNases, mini-revue

Introduction:

Initially, RNA was simply viewed as a short-lived genetic intermediate between DNA and proteins (1). Messenger RNAs (mRNAs) indeed perform this function. However, other types of RNA were, soon after, characterized as components of the cellular protein synthesis machinery. Ribosomal RNAs (rRNAs) are structural and functional components of ribosomes, while transfer RNAs (tRNAs) are adapters for amino acid delivery to ribosomes. Thomas Cech's group (2) was the first to observe RNA catalysis in the protein-independent splicing of Tetrahymena 26S rRNA. Numerous ribozymes, notably group I and II introns and ribonuclease P, have since been identified.

Many other functional classes of noncoding RNAs (ncRNAs) have been discovered. Small noncoding RNAs (sncRNAs) such as microRNAs (miRNAs), small interfering RNAs (si RNAs), small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs), Piwi-interacting RNAs (piRNAs), tRNA-derived fragments (tRFs), tRNA halves (tiRNAs), and small rDNA-derived RNAs (srRNAs), perform a host of regulatory functions, including RNA silencing, DNA methylation, histone modification, posttranscriptional RNA modification, and posttranscriptional silencing of repeat-derived transcripts (3,4). Long noncoding RNAs (lncRNAs) are distinguished from sncRNAs by their length (> 200 nucleotides) and function in chromatin remodeling, transcriptional regulation, and RNA mo-

dification and degradation (5).

There has been extensive research into RNA biogenesis, structure, function, interactions, posttranscriptional modification, localization, trafficking, turnover, pathogenesis, and prophylaxes, with many consequential discoveries. Most of the methods in molecular biology that enable these investigations require an initial extraction of RNA from tissues, cells, and other biological materials. RNA extraction typically involves obtaining a crude mixture of RNA and other biomolecules from biological samples, removing unwanted macromolecules and chemical contaminants, and concentrating so as to obtain high-quality RNA in concentrations suitable for various downstream applications.

When trying to capture the state of the transcriptome at the time of sample collection, the integrity of RNA molecules is of utmost importance. The success of gene expression analyses, including RT-qPCR and RNA-Seq, is greatly affected by RNA quality (6,7). Other RNA-centric assays are similarly affected (8, 9). Many methods have been proposed for assessing RNA yield, purity, and integrity (10-13). Given the importance of RNA quality, researchers usually adopt RNA quality thresholds for different sample types and workflows on the basis of previous performance studies and optimizations.

RNA extraction is complicated by the molecule's short half-life and susceptibility to ribonuclease (RNase) degradation. Unlike DNA, RNA nucleosides contain a ribose sugar with the typical 2′-hydroxyl group, making RNA more susceptible to spontaneous hydrolysis. RNases are a considerable problem in RNA extraction and manipulation. *In vivo*, RNases contribute to RNA biogenesis and homeostasis and serve as the body's first line of defense against non-self RNA, including RNA viruses (14). However, once released from cells and protection of accessory proteins, RNA is exposed to RNases simultaneously released from the sample. Additionally, due to their high stability, RNases accumulate in the environment and are readily introduced into *in vitro* preparations, contributing to RNA degradation.

There are multiple proven strategies for minimizing RNA degradation. Lowering the temperature slows down self-hydrolysis and RNase activity, reducing RNA degradation. Stable low temperatures are maintained by flash-freezing biological samples in liquid nitrogen immediately after harvesting, prechilling extraction buffers, conducting extraction and downstream manipulations on ice, and using refrigerated centrifuges where applicable. RNA in biological samples stored at -80°C remains stable for extended periods (15,16).

Additionally, following extraction, RNA samples are aliquoted before freezing at -80°C to avoid freeze-thawing. As an alternative to flash-freezing, biological samples are homogenized in chaotropic-based cell lysis solutions or permeated with RNA stabilization solutions before storage. RNA degradation is further prevented by: maintaining an RNase-free workspace using RNase-free filter pipette tips, tubes and reagents; using RNase-decontaminated equipment; and wearing appropriate personal protective equipment (PPE) to avoid introducing RNases from skin and saliva.

Methods of RNA extraction:

Phenol-chloroform extraction

Ingle and Burns (17) reported the effectiveness of phenol:chloroform:isoamyl alcohol (PCI; 25:24:1; v/v) extraction of total nucleic acids from aqueous lysed biological samples. When an emulsion is formed by mixing an aqueous biological lysate with phenol and chloroform (both organic solvents) and allowed to settle, proteins contained in the lysate are permanently denatured and preferentially displaced from the aqueous lysate into the organic solvents, leaving polar nucleic acids in the aqueous phase. When centrifuged, the heavier organic phase containing proteins separates to the bottom of the tube, displacing the aqueous phase containing nucleic acids to the top, where it can easily be aspirated for subsequent alcohol precipitation.

Chomczynski and Sacchi's (18) modified method that uses an acid-guanidinium thiocyanate-phenol-chloroform mixture, has become widely used for isolating total RNA from biological samples of different sources (19-21). The increased acidity causes DNA to separate into the lower organic phase and interphase, leaving RNA in the aqueous phase. The DNA can be separately recovered from the organic phase by precipitation with ethanol or isopropanol. Additionally, guanidinium isothiocyanate, a chaotropic salt, lyses cells and inactivates RNases, removing the need for prior lysis. The method provides a pure preparation of undegraded total RNA in high yield and can be completed within 4 hours.

Great care is required when aspirating the aqueous phase, as disturbing the interphase or organic phase will result in organic and DNA contamination. In addition to careful aspiration, different strategies have been proposed to reduce contamination. For example, phase lock gels eliminate interphase protein and DNA contamination, ensuring faster results with improved aqueous phase recoveries (22). More commonly, methods employ additional chloroform extractions of the aqueous phase and additional ethanol washes of the alcohol precipitate (23,24).

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Column-based extraction:

Chaotropic agents disrupt the hydrogen bonding network between water molecules, interfering with the noncovalent forces required for macromolecular structure and supramolecular assembly. Through this means, chaotropes permanently denature proteins, dissolve lipids, and lyse cells. Conversely, chaotropic agents destroy the native hydration shells that maintain the solubility of nucleic acids, making them less soluble in water. Eliminating hydration shells permits positively charged ions to form salt bridges between the negatively charged phosphate backbone of nucleic acids and negatively charged hydroxyl groups on the surface of solid matrices such as silica and cellulose.

Vogelstein and Gillespie (25) first reported the near-quantitative binding of DNA to flint and borosilicate glass particles and silica powder under chaotropic salt concentrations and efficient DNA recovery in any convenient buffer. Silica binding and recovery of nucleic acids have since been incorporated into many centrifugation column-based workflows as one of the most common methods for nucleic acid extraction. Plant-derived cellulose binding materials have also been described for nucleic acid extraction (26,27). Solid-phase RNA extraction is especially appealing because it avoids the dangerous organic solvents used in liquid-liquid methods.

Spin-column workflows typically start with a lysis step in which suitable detergents, lytic enzymes and chaotropes lyse biological samples. Next, using centrifugation or vacuum suction, the lysate is passed through the binding material under chaotropic conditions. The bound nucleic acids are washed to remove unwanted biomolecules and chemical contaminants. For pure RNA, a single DNase treatment can be performed between multiple washes. Additionally, pH, ionic strength, and alcohol-to-sample ratios are adjusted to retain or exclude different types and sizes of nucleic acids (28-30). Finally, purified RNA is eluted from the binding material using low-ionic buffers.

Magnetic beads extraction:

Professor John Ugelstad of the Norwegian Institute of Technology revolutionized the separation of biological materials when he succeeded in producing monosized monodispersed magnetizable microspheres under laboratory conditions (31-34). Magnetic beads, usually magnetite (Fe_3O_4) particles coated according to the application, exhibit super paramagnetism, which means that they are strongly reversibly magnetized in the presence of an external magnetic field. This allows magnetic beads to be separated in suspension, along with any molecules that are bound to their coating. Since the magnetism is completely reversible, once the magnetic field is removed, magnetic beads can be easily resuspended in binding, wash, or elusion buffers.

A wide range of surface coatings are available for magnetic beads to suit different applications. Negatively charged carboxyl and silica coatings, both of which reversibly bind nucleic acids, are widely used for nonspecific nucleic acid purification (35-37). However, silica-coated magnetic beads are favored when sample amounts are low. By varying pH, ionic strength, alcohol concentration, and other buffer conditions, magnetic beads can be made to preferentially retain or exclude different types and sizes of nucleic acids. To obtain pure RNA, a single DNase treatment is often performed between washes. Along with their use in sample lysis and RNA binding, detergents, chaotropes, and RNase inhibitors prevent RNA degradation during extraction.

Oligo(dT) coating covalently hybridizes with the poly(A) tail present in most eukaryotic mRNA, enabling mRNA isolation from biological samples (38,39). RNA containing known sequences are also enriched or depleted from RNA pools in this way (40,41). Alternatively, streptavidin- and other avidin-coated beads form a very strong, highly specific, reversible bond with biotin, enabling the isolation of biotin-labeled targets, including RNA and RNA-DNA hybrids (40,42,43).

Conclusion:

Since the first organic isolation of total nucleic acid by Ingle and Burns (17) and the subsequent total RNA extraction by Chomczynski and Sacchi (18), many techniques for RNA purification have been developed. From column-based extraction of total RNA, small RNA, and RNA clean-up to bead-based methods, RNA extraction has played a fundamental role in various scientific and medical applications. Most biological endeavors, including gene expression analyses, transcriptomics, drug development, functional genomics, molecular diagnostics, cancer research, developmental biology, environmental monitoring, and forensics, are predicated on our ability to obtain good-quality RNA from different sources.

Not much can be done to remedy degraded RNA. However, low-yield and contaminated samples can be concentrated and cleaned up with the same methods used for extracting RNA from crude samples. Furthermore, automated RNA extraction systems have been developed due to modern advancements in engineering. Automation is appealing because it can increase throughput, reproducibility, quality, safety, and labor savings. Solid-phase

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reversible immobilization (SPRI) bead-based RNA extraction is ideal for automation due to its yield, reproducibility, and ease of manipulation with magnetic fields. However, numerous organic and column-based liquid handling platforms for RNA extraction are also available. Existing technologies are constantly being improved, but additional discoveries may be required to further lower costs, improve throughput, enable single-cell extraction, and much more.

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Conflict of interest:

The author declares that the review was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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