Technical Note

Methods of RNA Purification. All Ways (Should) Lead to Rome

(RNA / mRNA / purification / automation / gene expression)

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Abstract: Ribonucleic acid (RNA) represents an important target of a wide array of laboratory analyses. Thus, RNA purification is a critical first preceding step of a number of preparative and analytical methods, important particularly in diagnostics of dozens of viral, bacterial, and parasitic diseases, diagnosis of inherited disorders, and tumours, as well as in basic research. To provide relevant and reliable results, techniques of molecular biology used for such purposes require pure and intact molecules of purified RNA. Moreover, RNA has to be purified effectively and reproducibly from various heterogeneous materials such as fresh or frozen tissues, cell lines, PCR products or long-term chemically preserved samples. Principally, methods of RNA purification can be divided into three groups. The first group of methods is based on organic phenol:chloroform extraction. The second group encompasses methods of RNA purification by means of its ability to bind specific surfaces in the presence of chaotropic salt, and the third group includes methods exploiting RNA isolation on isopycnic gradients. Although RNA can be isolated from either prokaryotic or eukaryotic organisms, this review is to give out a basic outline of methods available for eukaryotic, with emphasis on mammalian, tissues.

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Abbreviations: CsCl – caesium chloride, CsTFA – caesium trifluoroacetate, DNA – deoxyribonucleic acid, FFPE – formaldehyde/paraformaldehyde fixed, paraffin embedded, GTC – guanidinium thiocyanate, mRNA – messenger RNA, P:C – phenol: chloroform, PCR – polymerase chain reaction, PKK – protein kinase K, RNA – ribonucleic acid, RT-PCR – reverse transcriptase polymerase chain reaction, SM – silicate matrix.

Introduction

The rules of transmission of genetic information were coined in the late fifties of the past century by Crick's Central Dogma of Molecular Biology stating that the information transfer follows the way from deoxyribonucleic acid (DNA) to protein via ribonucleic acid (RNA) while the reverse way is impossible (Crick, 1970). Although this concept was partially revised, it made the basis for division of biologically active molecules into informational (DNA) and functional (proteins). In this perspective, the role of RNA seems to be more complex, since it fulfils structural, informatic, as well as regulatory functions.

Nucleic acids were discovered in 1869 by J. F. Miescher as chemical substances rich in phosphorus, sulphur and nitrogen and called "nuclein" according to their localization in the nucleus. The first novel experiments attempting to purify RNA date from 1960, when Kurland and colleagues performed the first isolation using caesium chloride (CsCl) centrifugation. In 1968, phenol:chloroform RNA isolation was introduced by Kirby. Later, Chirgwin presented purification using guanidinium thiocyanate (denaturant of plasma membrane proteins) and β-mercaptoethanol (reducing agent) followed by ethanol extraction or ultracentrifugation in caesium chloride gradient (Chirgwin et al., 1979). Since the former laborious and time-consuming protocol had many disadvantages and low reproducibility, further improvements were needed. Chomczynski and Sacchi (1987) made the method more straightforward by coupling all extraction steps into a single one, by using a reagent composed of guanidinium thiocyanate, phenol and chloroform. It reduced both the time requirements and the sample size needed, thus increasing the method throughput and minimizing the loss of RNA.

Recently, the importance of RNA analysis has increased due to the growing demands of basic and applied science as well as clinical fields. Thus, RNA purification is a critical first step of a multitude of analytical techniques including real-time quantitative polymerase chain reaction (PCR), reverse transcriptase polymerase chain reaction (RT-PCR), microarrays, Northern blot analysis, nuclease protection assays, RNA mapping, *in*

vitro translation and cDNA library construction. Consequently, there is a growing demand for commercially available kits, which are designed to purify RNA in high quality, intactness and purity with specific respect to further analytical application or scientific need. Here we review recently available methods of RNA purification from the point of view of their principle, purpose and drawbacks.

1. Principles of RNA purification methods

1.1 General requirements

Irrespective of the principle of RNA isolation used, individual purification protocols have a number of common requirements – steps exploited by all of them. Firstly, as a general rule, biological material has to be handled consciously and safely. Proper storage of samples as e.g. bioptic material in liquid nitrogen or at -70 °C or in the appropriate preservative agent (e.g. "RNA Later" from Qiagen, Valencia, CA) is critical. When samples are collected from sacrificed animals or cadavers, it is important to limit the period between death and sample collection for the best yield of high-quality RNA. Before separation, samples have to be mechanically homogenized or enzymatically digested. Due to the high risk of RNA degradation during the procedure, all typical precautions against RNase contamination should be rigorously observed, i.e. gloves should be worn at all times and changed frequently to avoid introduction of the "finger RNases". Bags containing tubes and solution bottles should be closed when they are not in use to avoid contamination with dust. In parallel, endogenous RNases naturally occurring in the tissues have to be inactivated by inhibitors or by chemical treatment as quickly as possible after sample collection. The most common reagent for such a purpose is guanidinium thiocyanate, a strong chaotropic denaturant. RNA degradation can also be minimized by reducing the sample size (tissues or cells).

Further steps of RNA isolation are based on the different solubility of constituents of the homogenized material. In that point, particular types of the RNA purification strategies can be divided into three groups. Recovered RNA is then gained either from the solution using specific separation as described later, or by alcohol precipitation.

For troubleshooting RNA degradation problems, a radio-labelled synthetic RNA transcript "reporter system" may be used. If intact ^{32}P labelled RNA is recovered in the final step, then the protocol works optimally in degrading RNases.

The expected yields of RNA vary widely between tissues. Tissues such as liver or kidney typically have a higher RNA content and yields compared to tissues such as muscle, lung or brain.

As mentioned above, there are three basic approaches used to isolate cellular RNA: (i) procedures relying on the different solubility of cellular components in organic solvents, such as phenol, ethanol or isopropanol; (ii) methods based on RNA adsorption to specific surfaces in the presence of chaotropic salts; (iii) protocols exploiting RNA separation on isopycnic gradient centrifugation.

1.2 Phenol:chloroform method

This procedure is based on the sample lysis in cationic detergent guanidinium thiocyanate (GTC), followed by organic extractions and alcohol precipitation. Moreover, GTC is effective at inactivating endogenous ribonucleases. For better removal of DNA from the aqueous phase, addition of low-pH phenol is recommended, and thus it became part of home-made mixtures such as original "Solution D" (Chomczynski and Sacchi, 2006) as well as commercially available reagents like TRIZOL (Invitrogen, Carlsbad, CA) or TRI (Sigma-Aldrich, St. Louis, MO), with pH of about 4 (both based on U.S. patent 5346994). Chloroform is a purely organic solvent, unable to mix with the cell lysate. The solution is properly interspersed, and then the centrifugation enables effective separation of the "upper phase" and "lower phase" containing the cell lysate and chloroform, respectively. At the interface, a white ring of precipitated proteins appears. The "upper phase", which contains nucleic acids, is transferred into a new sterile test tube, where it undergoes the reaction with isopropanol resulting in precipitation of RNA. After further centrifugation, a white pellet representing total RNA is recovered. This characteristic white colouring is due to the presence of residual salts precipitating together with RNA. Such salts are removed by washing the pellet using ethanol (concentration varies from 75–90 % according to the manual instructions).

Pure RNA is then dissolved in RNase-free water or in an appropriate buffer. Kits based on the above-mentioned principle have recently been provided by Ambion [A9] (Karanowszka and Ryden-Aulin, 2004) or Stratagene [S4]. One of the main drawbacks of this principle may be the incomplete dissociation of nucleoproteins from the RNA. To avoid this, the "upper phase" after isopropanol addition should be kept at room temperature for a few minutes before the consequent centrifugation. Cross-contamination of both "upper" and "lower" phases represents another possible separation obstacle. Carryover when transferring the aqueous phase to the fresh tube has to be avoided. Irrespective of these technicalities, the phenol:chloroform method represents the recent gold standard, namely for routine "in-lab" individually designed protocols.

1.3 Adsorption methods

Adsorption methods are based on the ability of RNA to create a linkage to specific surfaces in the presence of chaotropic salts (GTC). Adsorbing "RNA-beads" or alternatively "coated test tubes" can be made of magnetic [A2, A3] (Ovcharenko, 2005), silica [A5, A7, C1] (Huang et al., 2002; Duchaine et al., 2006), polystyrene-latex materials [Q8] (Iwami et al., 1996; Dudas et al.,

Table 1. Representative commercially available RNA purification kits

	Company/kit	principle	downstream application[8]	sample material[8]	note[8]
	Amersham Biosciences				
AB1	illustra Cesium Trifluoroacetate (CsTFA) Solution	Isopycnic C-fug	universal	eukaryotic	also for DNA purification
AB2	illustra RNAspin 96 Kit	C-fug	universal	eukaryotic	96-well format
AB3	illustra QuickPrep Micro mRNA Purification Kit	CC	PCR, Northern blot hybridization and *in vitro* translation	eukaryotic	mRNA separation, single-cell application
AB4	illustra RNAspin Midi Kit	CC	requiring high yields	eukaryotic	
AB5	illustra RNAspin Mini Kit	CC	sensitive applications	eukaryotic	suitable for small amounts of sample
AB6	illustra QuickPrep™ mRNA Purification Kit	CC	PCR, Northern blot hybridization and *in vitro* translation	eukaryotic	mRNA separation
AB7	illustra mRNA Purification Kit	CC	PCR, Northern blot hybridization and *in vitro* translation	eukaryotic	mRNA separation
	Ambion				
A1	MaqMAX™ 96 for Microarrays Total RNA Isolation Kit	MB, auto	microarrays	mammalian	
A2	MaqMAX™ 96 for Total RNA Isolation Kit	MB	universal	mammalian, plant	96-well format, suitable for small amounts of sample
A3	MELT™ Total Nucleic Acid Isolation Kit	MB	universal	mammalian	also for DNA purification alternative tissue disruption
A4	PARIS™ (Protein And RNA Isolation System)	GFF	blot hybridization, *in vitro* translation, cDNA synthesis, RT-PCR	mammalian	also for protein purification, cytoplasmic and nuclear fractions sub-separation
A5	mirVana™ PARIS™	SM	universal	mammalian	also for protein purification, cytoplasmic and nuclear fractions sub-separation, specific separation of miRNA
A6	RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE Tissues	GFF	RT-PCR, PCR, mutation screening, microarrays	FFPE samples	also for DNA purification
A7	mirVana™ miRNA Isolation Kit	SM	universal	mammalian	specific separation of miRNA
A8	RNAqueous®-96 Automated	GFF, auto	universal	mammalian	96-well format, small amounts of sample
A9	ToTALLY RNA™ Kit	P:C	universal	mammalian, plant, bacteria	
A10	LeukoLOCK™ Total RNA Isolation System	MB	RT-PCR, microarrays	leukocytes	
A11	MaqMAX™ -96 Blood RNAIsolation System	MB, auto	universal	blood	separation of viral RNA
A12	RiboPure™ Kit	GFF	sensitive applications	eukaryotic	
A13	Plant RNA Isolation Aid	GFF	universal	plant	
A14	MICROBEnrich™ Kit	GFF	special kits for bacterial species	bacteria	selective removal of non-bacterial RNA
A15	MaqMAX™ Viral RNA Isolation Kits	MB	RT-PCR	viral RNA	
A16	Poly(A)Purist™ mRNA purification Kits	MB	universal	total RNA	specific for mRNA
	Promega				
P1	SV Total RNA Isolation System (10, 50, 250 preps)	C-fug	RT-PCR, Northern blot	mammalian	

P2	PureYield™ RNA MidiPrep System	C-fug	universal	mammalian, bacteria, yeast, plants, blood	
P3	PolyATract® System	MB	universal	eukaryotic	specific for mRNA
	Qiagen				
Q1	PAXgene Blood RNA Kit (96)	Spin columns	universal	blood cells	
Q2	EZ1 RNA Cell Mini Kit	Auto	universal	cell suspensions, blood cells	
Q3	MagAttract Direct mRNA M48 Kit	Auto	universal	cell suspensions, blood cells	specific for mRNA
Q4	QIAamp MiniElute Virus Spin Kit	Auto	universal	cell-free body fluids	separation of viral RNA
Q5	RNeasy kit (mini, midi, maxi)	Spin columns	universal	mammalian, yeast	
Q6	RNeasy FFPE kit	PKK	universal	FFPE	
Q7	QIAamp Viral RNA	SM	universal	cell-free body fluids	separation of viral RNA
Q8	Oligotex mRNA kit (mini, midi, maxi)	P-L beads	universal	eukaryotic	specific for mRNA
Q9	AllPrep RNA/Protein Kit	Spin columns	universal	cell suspensions	also suitable for protein separation
Q10	AllPrep DNA/RNA/Protein Mini Kit	Spin columns	universal	mammalian	DNA and protein separation
Q11	RNeasy Lipid Tissue Kit	Spin columns	universal	lipid tissues	
Q12	RNeasy Fibrous Tissue Kit	Spin columns	universal	fibrous tissues	
	Stratagene				
S1	Absolutely mRNA™ Purification Kit	MB	RT-PCR, PCR, microarrays, cloning	eukaryotic	specific for mRNA
S2	Absolutely RNA ® FFPE Kit	PKK	QRT-PCR	FFPE	also suitable for DNA separation
S3	Absolutely RNA ® Purification Kit	SM	RT-PCR, PCR, microarrays, Northern blot	eukaryotic	
S4	miRACLE™ miRNA Isolation Kit	P:C	universal	eukaryotic	specific for miRNA
S5	SideStep™ Lysis and Stabilisation Products	skip RNA purification	universal	eukaryotic	suitable for microdissected cells
	Zymo Research				
ZR1	PinPoint Slide RNA Isolation System I / II	RNA extraction	RT-PCR	tissue mounted on glass slides	
ZR2	ZR Whole-Blood Total RNA Kit™	Spin column	universal	blood cells	
ZR3	ZR Viral RNA Kit™	Spin column	RT-PCR	eukaryotic	separation of viral RNA
ZR4	ZR Urine RNA Kit™	Spin column	RT-PCR	urine	

Table 1 legend:
The listed products are dominantly dedicated for RNA purification from mammalian cells, although some of them also declare suitability for processing of non-mammalian material. Abbreviations used in the table: c-fug: centrifugation, CC: cellulose columns, auto: automation, SM: silicate matrix, P:C: phenol:chloroform, GFF: glass fibre filter, P-L beads: polystyrene-latex beads, MB: magnetic beads, PKK: protein kinase K, FFPE: formaldehyde/paraformaldehyde fixed, paraffin embedded
[8] As declared by producer.

2005), cellulose matrices [AB3-AB7], or glass fibres [A12] (Fenton et al., 2004). The target sample is lysed in GTC, then exposed to the particular adsorbing material, and vigorously shaken, to facilitate binding. The contaminants are then eased off by centrifugation. After removal of the supernatant, the beads with adsorbed RNA are washed again. Then, RNA is released using a specific chaotropic salt-free buffer. Following spinning, beads devoid of RNA are sedimented while the RNA remains in the supernatant.

Since the basis of these procedures is to immobilize RNA on RNA-binding beads, any loss of beads during the procedure will result in loss of RNA and thus has to be carefully avoided. In general, the RNA binding beads will disperse more easily when the temperature of the mixture is warmer. Beads have to be fully re-suspended before consecutive steps; otherwise, formation of aggregates will negatively influence RNA recovery. Beads have therefore to be shaken thoroughly and their overdrying must be avoided. If the beads are inadvertently over-dried, shaking time should be extended in order to rehydrate the beads. Aspiration of binding beads when removing supernatant from the captured beads should be prevented. Thus, the aspiration speed is to be reduced and use of long flexible pipette tips is recommended.

The quickness and easiness of this method is the reason why most commercially available kits are based on it. Individual kits are also optimized for specific sample material and quantity.

1.4 Isopycnic gradient method

Despite the fact that this principle was the first used for RNA isolation, it is just rarely exploited in commercially marketed kits. As an example, Amersham Biosciences (Piscataway, NJ) can be mentioned [AB1].

The first step – dissolution of the sample of experimental material using GTC – is common with previously described principles. The following equilibrium gradient centrifugation is run on CsCl or CsTFA (caesium trifluoroacetate), which are both soluble in ethanol. Hence, the RNA captured in the appropriate density fraction is then precipitated by ethanol. Both salts (chloride, trifluoroacetate) are strong chaotropic agents effectively suppressing RNases. Analogous procedures can be seized for the isolation of DNA.

Equilibrium gradient centrifugation is seen to be a method of choice for isolating RNA free of proteins, DNA, polysaccharides and other cellular components (Glisin et al., 1974).

1.5 Special methods

1.5.1 mRNA

Messenger RNA (mRNA) represents only 1–5 % of the cellular RNA. Expected yields of mRNA vary widely among tissues and cells, depending on their type, actual functional state and metabolic activity. Although the purification of the total RNA typically precedes isolation of mRNA, methods isolating specifically mRNA directly from the sample are available [AB6]. Techniques of mRNA purification from the total RNA utilize the unique chemical structure of mRNA, as well as the potential of complementary base pairing. Eukaryotic mRNA features the polyadenylate "d(A)" tail (sequence at 3' terminus), which is effectively used for mRNA trapping. Since synthetic oligo "d(T)" chains can be covalently bound to special matrices or beads, mRNA can be easily captured and selectively fastened to a particular surface. The resulting mRNA can be obtained by chromatographic methods, or by magnetic field depending on the sort of adsorbing strategy (analogical to the adsorption methods, see paragraph 1.2.). Commercially available kits are vended by most suppliers, differing slightly in specific materials used for mRNA adsorption; the oligo d(T) sequence can be bound to cellulose matrix (Kirikoshi et al., 2001), magnetic beads [A16, P3] (Ito et al., 2003), polystyrene-latex particles [Q8] (Iwami et al., 1996; Dudas et al., 2005) or glass filter [A12] (Fenton et al., 2004). Residual salt contamination may inhibit mRNA purification substantially. Thus, in case of mRNA separation from the total cellular RNA, salt remnants have to be removed thoroughly by washing the RNA pellet with 70% ethanol.

As an example, Poly(A)Purist™ mRNA purification Kits by Ambion (Applied Biosystems, Foster City, CA) claim elimination of unspecific binding of rRNA (ribosomal RNA) to the adsorbing matrix or to purified mRNA [A16] (Ito et al., 2003).

1.5.2 Cell-free mRNA

The cell-free nucleic acids, also called circulating nucleic acids, are present in the plasma, serum or in other biological fluids such as urine, bronchial lavage and amniotic fluid. As a promising diagnostic target of the future, the importance of cell-free mRNA analysis is dramatically rising since it might represent an extremely potent biological marker of various tumours (Goebel et al., 2005), viral infections (Buisson et al., 2000) and in prenatal diagnostics (Wong et al., 2005). For all purposes, extremely sensitive methods, capable of detecting low numbers of circulating nucleic acid copies, are needed. The QIAamp Viral RNA kit (Qiagen) combines the selective binding properties of a silica gel-based membrane with the high-speed micro spin or vacuum technology and is ideally suited for simultaneous processing of multiple samples.

1.5.3 Small RNA molecules

Traditionally, RNA purification relies on organic extraction followed by alcohol precipitation. Unfortunately, this procedure removes the majority of small RNAs, such as miRNAs (micro RNAs), from the whole RNA. To overcome this challenge and to offer better chance for specific work with small RNAs, appropriate kits were developed. For example, miRACLE™ miRNA Isolation Kit from Stratagene (La Jolla, CA) [S4] employs organic extraction followed by small RNAs' binding on a silica fibre matrix. MirVana™ miRNA Isolation

Kit (Ambion) [A7] provides a unique tool permitting purification of native RNAs, including small RNAs such as miRNA and siRNA (small interfering RNA), in parallel with the native protein. The kit employs organic extraction followed by immobilization of RNA on glass-fibre filters to purify either total RNA, or RNA enriched for small species.

It is important to perceive that specific miRNAs have a characteristic tissue distribution, and some tissues are devoid of detectable levels of them at all.

1.5.4 Skip RNA purification

Reverse transcriptase polymerase chain reaction (RT-PCR) probably represents the most common technique of purified RNA downstream application. Considering that, methods skipping RNA isolation directly providing coding DNA from the sample are offered. The main disadvantage of pioneering column-based affinity procedures, designed to direct cDNA isolation, is their multi-step concept. Consequently, there is a significant danger that RNA or DNA will be lost at each step, harming consequent downstream quantification, namely when working with small cell numbers. Additionally, even when using a large number of cells, low-abundance messages can be lost during the purification process. To avoid that, Stratagene provides SideStep™ Lysis and Stabilisation Products kit [S5].

It uses a single-tube, solution-based format instead of a classical column-based format. This makes the kit an ideal choice to analyse rare or precious samples, particularly from laser micro-dissected cells from fresh tissues for subsequent analysis.

1.5.5 Simultaneous purification

Methods for simultaneous co-purification of protein and RNA from one sample are suitable for various downstream applications, since it eliminates imminent bias given by inter-sample variability (Correnti et al., 2005). For such purpose, Ambion provides PARIS kit [A4].

The method is based on differential lysis of plasma and nuclear cell membranes by non-ionic detergents. Cells are either disrupted directly, or alternatively, they may be first separated into nuclear and cytoplasmic fractions. In parallel, the resulting nuclear and cytoplasmic native protein fractions can be used directly for applications such as enzymatic assays, immunoprecipitation, gel shift assays, two-dimensional gel electrophoresis, and Western blotting. To prevent protein degradation or modification, protease inhibitors, phosphatase inhibitors and anti-oxidants are indispensable components of the reaction mixtures. These reagents should not adversely affect subsequent RNA isolation from samples. On the other hand, risk of cross-contamination between the nuclear and cytoplasmic fractions should be considered here. It could be caused by improper collecting of fractions, incomplete lysis of the plasma membrane causing just partial recovery of the cytoplasm and consequent contamination of the nuclear fraction with cytoplasmic components.

The most recent advanced simultaneous purification kits enable parallel isolation of both DNA-RNA [Q9] and even DNA-RNA-protein [Q10] within one purification process. Such co-purification is advantageous since it is less time-consuming and from the analytical point of view, it allows direct correlations of genetic, genomic and proteomic data.

1.5.6 Microarrays

Microarray technologies recently used to detect biological macromolecules such as DNA, RNA and proteins represent high-throughput multiplex methods with a growing application potential in molecular biology and medicine. In case of nucleic acid microarrays, they consist of an arrayed series of thousands of microscopic spots of oligonucleotides each containing picomoles of a specific nucleic acid sequence of a part of a gene or other nucleic acid element serving as probes to hybridize with cDNA or cRNA from the analysed sample. Probe-target hybridization is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labelled targets to determine the relative abundance of a given molecule in the analysed sample.

In standard microarrays, the probes are attached to a solid surface by a covalent bond. The solid surface can be glass or a silicon chip (e.g. Affymetrix, Santa Clara, CA; Yun-Shien et al., 2009) or microscopic beads (e.g. Illumina, San Diego, CA). Although there are many comparative inter-laboratory studies analysing reproducibility, accuracy, trueness and precision available (Duewer, 2009; Scott et al., 2009), there is still evidence of deep sequencing-based expression analysis being more robust than the microarray platforms (‘t Hoen et al., 2008). In light of that, among other strictly defined conditions of microarray processing, high quality of analysed sample preparation is one of the key requirements. Some producers of commercial kits (e.g. [P1]) explicitly declare the suitability of their product for such purpose.

2. Resulting RNA purity and integrity check

The methods of RNA recovery have to be optimized in order to protect RNA during the entire process of its isolation. Thus, an ideal RNA sample has to be of satisfactory intactness, purity, and to remain in native state. Basic demands for the method of choice depend on sample source, quantity, type of downstream RNA application, laboratory equipment available (and sometimes financial resources).

As for purity, all available kits designated for RNA isolation are declared to purify RNA according to its further downstream applications. Each analysis is precluded by spectrophotometric quantification of RNA, additionally serving as a check of purity. The method itself is based on scaling the purified RNA sample ab-

sorbance at the wavelengths of 260 and 280 nm. The ratio of A260/A280 ≥ 1.80 indicates satisfactory purity of the isolated RNA. A lower value of the ratio indicates presence of contaminants, such as proteins and DNA, or low pH of RNA-containing solution. RNA sample with the absorbance ratio outside the recommended values may still function well for RT-PCR or other downstream applications; however, it has to be checked individually. There are also available analysers to quantify samples and at the same time to check the integrity of RNA samples (e.g. Bio-Rad Experion, Hercules, CA, or Agilent Bioanalyzer 2100, Santa Clara, CA). The integrity seems to be even more important for obtaining meaningful gene expression data than RNA quantity itself.

3. Starting material for RNA purification

To reach maximum recovery of high-quality RNA without contaminants, the nature of the source material has to be considered in selection of the particular RNA purification method.

In general, most of commercially available kits are designed for a wide spectra of various entering materials, i.e. fresh or frozen tissues, cell cultures, yeast, bacteria, etc. However, there are of course some limitations of particular kits, namely for the purpose of long-term chemically embedded samples and RNA purification from electrophoresis gels, or PCR products.

3.1 Sample material

Some kits are claimed for "trouble" materials: for high-lipid content materials such as brain or adipose tissues [Q11] (Bacharova et al., 2005), for fibrous tissues such as heart or aorta [Q12] (Leoni et al., 2005). Unfortunately, the rationale behind the product specificity is often not specified by the provider. Other specialized kits are designated for RNA isolation from white blood cells. A number of enzyme inhibitors naturally present in blood as well as common anticoagulants such as heparin or EDTA, used during blood collection, interfere with downstream RNA analysis. Therefore, kits offered by Zymo Research (Orange, CA) [ZR2], Ambion [A10] or Qiagen [Q10] are designed to sub-separate cells of interest from the whole blood before the RNA purification step.

3.2 Sample storage

Although the ideal source for analytical purposes is represented by fresh or deeply frozen material, RNA purification from long-term chemically preserved samples, such as formaldehyde/paraformaldehyde-fixed, paraffin-embedded (FFPE) samples, may be an indispensable source for retrospective studies at both genomic and gene expression levels (Ravo et al., 2008; Schoebesberger et al., 2008; Siebolts et al., 2008). Unfortunately, nucleic acids in such samples are both trapped and modified by extensive protein-protein and protein-nucleic acid cross-links. High temperature required for paraffin infiltration during standard embedding processes accelerates chemical reactions modifying RNA. Moreover, the above-mentioned chemical modifications typically lead to RNA fragmentation during further prolonged storage, compromising RNA quality for further molecular analysis.

However, Masuda et al. (1999) have found that the fixation process itself does not necessarily lead to complete RNA fragmentation. The RNA purification in such cases is preceded by elimination of preservant and by the protease digestion step. FFPE samples are deparaffinized using a series of xylene and ethanol washes. Next, they are subjected to rigorous protease digestion with an incubation time tailored for recovery of RNA. Of course, RNA fragmentation that has already occurred in FFPE samples prior to the nucleic acid isolation cannot be rescued. However, the protease digestion conditions of the RecoverAll™ Total Nucleic Acid Isolation Kit (Applied Biosystems) [A6] are designed to release, in a short period of time (30 min), a maximal amount of RNA fragments of all sizes, including miRNA. Stratagene [S2] and Qiagen [Q6] produce other kits of such type (Werner et al., 2005). Nevertheless, one has to be aware that some long-term stored samples contain RNA in a form that renders it unsuitable for further analysis.

3.3 Sample quantity

Before starting RNA purification, it is therefore important to determine the amount of sample material available as well as to decide the amount and concentration of finally purified RNA that is required. In general, the amount of starting material varies around 5 mg (micro kits), 40 mg (mini kits), 200 mg (midi kits) and 1000 mg. A larger sample size of the starting material significantly slows down digestion reaction, compromising RNA integrity. Rapid tissue digestion is important for high yields of pure RNA. Thus, a larger surface area exposed to the digestion reagent, e.g. cutting of starting material into smaller fragments, improves RNA yield and quality.

4. Automation

The number of samples that can be processed per run varies, depending on the specific type of kit as well as on the purification principle used. In general terms, it is possible to find protocols suitable for small laboratories with throughput ranking in tens of samples as well as for clinical laboratories requiring fully automatic process protocols with even ten times higher capacity. In parallel, kits offering high-throughput processing also ensure, as added value, high quality and effective purification since the human impact is constricted to a minimum.

Therefore, less contaminants or inequalities concerning time, volumes of chemicals or improper handling are avoided. Automation therefore increases the efficiency, accuracy and velocity of RNA purification. Qiagen kits [Q2 and Q3] are designed for the company platform technology BioRobot systems, processing up to 48 samples per run (Vandenberg et al., 2002; Kishore et al.,

2006). Other automatic protocols are offered by Promega (Madison, WI) [P1] (Fowler et al., 2004), offering 96 samples per run; Ambion [A8] and Stratagene [S1] designed kits for up to 96 samples per run. Qiagen provides the "QIAcube" robotic system, designed for application of the company purification kits [e.g. .Q5, Q6].

Conclusion

Precipitous development of molecular biology is accompanied by growing demands for methodological and analytical techniques and their further goal-directed improvements. A wide spectrum of RNA purification approaches might serve as a good example of that trend. Although basically only three principles of RNA purification have been exploited so far, there are a large number of their modifications, suitable for each particular task and fitting specific conditions.

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