Comparing RNA extraction methods to face the variations in RNA quality using two human biological matrices

J. Ortega-Pinazo1,2 · M. J. Pacheco-Rodríguez1,2 · P. J. Serrano-Castro1,2 · B. Martínez1,2 · M. J. Pinto-Medel1,4 · J. M. Gómez-Zumaquero1,4 · A. Lago-Sampedro1,4 · B. García-Díaz1,2 · Guillermo Estivill-Torrús1,2 · Pedro Emilio Ferro Gallego1,5,6

Abstract

Background Nucleic acids, RNA among them, are widely used in biomedicine and Biotechnology. Because of their susceptibility to degradation by RNases, the handling and extraction process of RNA from cells and tissues require specialized personnel and standardized methods to guarantee high purity and integrity. Due to the diversity of techniques found in the market, a comparative study between different RNA extraction methods is useful to facilitate the best choice for the researcher or in research service platforms such as biobanks to see the traceability of the samples.

Methods and results In this study, we have compared seven different RNA extraction methods: manual (TRIzol™), semi-automated (QIAGEN™, Bio-Rad, Monarch®, and Canvax™), and fully automated (QIAcube™ and Maxwell®) processes, from two biological matrices: human Jurkat T cells and peripheral blood mononuclear cells (PBMC). Results showed marked differences in the RNA quality and functionality according to the method employed for RNA extraction and the matrix used.

Discussion QIAcube™ and semi-automated extraction methods were perceived as the best options because of their lower variability, good functionality, and lower cost (P < 0.001). These data contribute to facilitating researchers or research service platforms (Biobanks) in decision-making practices and emphasize the relevance of the selection of the RNA extraction method in each experimental procedure or traceability study to guarantee both quality standards and its reproducibility.

Keywords RNA · RNA extractions methods · PBMC · Jurkat T cell · PCR · Real-time PCR

Introduction

Nucleic acids are widely used in biomedical and biotechnology research and clinical practice. Specifically, ribonucleic acid (RNA), which is found in every living cell and is involved in multiple functions, due to the large existing RNA variety [1–3]. Thus mRNA, RNAi, small RNA, and microRNA (among others) have been key to improving our understanding of gene expression control for the study, diagnosis, and treatment in many different areas of...
pathology [4–8]. Because RNA degradation is critical [9, 10] it is advisable to use standardized work protocols that guarantee the quality of the material. The use of commercial or automated methods against manual protocols minimizes the exposure to degradation agents and sample handling, reducing the probability to undergo degradation [11].

Currently, there are several indicators of RNA quality. For RNA purity, spectrophotometry by evaluating the A260/ A280 and A260/A230 ratios or fluorometry using the Quant-iT™ RiBogreen® RNA can be used [12–14]. Fluorometry quantification has several advantages over spectrophotometry, because of its accuracy. However, it has some drawbacks such as giving bad quantification in samples with very low RNA concentrations, and its high cost [15]. RNA integrity can be evaluated by agarose gels to detect 28 S, 18 S, and 5 S ribosomal RNA, or by the electrophoretic-based generation using Agilent's Bioanalyzer (Agilent Technologies, Inc, Santa Clara, CA, USA), while functionality can be tested by conventional polymerase chain reaction (PCR) or real-time PCR [16]. Obtaining RNAs with optimal values for all these indicators is essential to generate reliable results.

The ideal RNA extraction method should be simple, fast, economical, reproducible, with low variability between samples, and able to maintain RNA purity and integrity. Comparative studies from different species [17–20] and matrices, including saliva, whole blood, and skin [21–25], revealed differences in the yield, quality, and functionality of RNA obtained. However, no studies are currently comparing a substantial number of RNA extraction methods, especially, using the extensively studied peripheral blood mononuclear cells (PBMC) or human Jurkat T cells.

In this study, we have compared seven RNA extraction methods including manual (TRIzol™), semiautomated (QIAGEN™, Bio-Rad, Monarch®, and Canvax™), and fully automated (QIAcube™ and Maxwell®) processes. RNA samples were evaluated by spectrophotometry and fluorometry to determine their yield and purity. Subsequently, an assessment of integrity and a functionality study were carried out. Finally, a determination of cost, process time, and other related factors was presented. This study is the first to show a comprehensive comparative study of RNA extraction and in PBMC and Jurkat T cells. Therefore, this report also contributes to helping researchers in decision-making protocols and the validity of laboratory RNA methods, according to their requirements.

Materials and methods

Cell cultures

Human Jurkat T cells were grown in RPMI-1640 with 10% heat-inactivated fetal calf serum, 10 mM HEPES, 2.5 g/L glucose, 1 mM sodium pyruvate, and 25 μg/mL penicillin-streptomycin 1x (all products from Merck Life Science SLU, Spain) at 37°C in a humid atmosphere of 5% CO₂. Confluent cell cultures were centrifuged to harvest cells and washed in Dulbecco phosphate-buffered saline (DPBS) without calcium or magnesium. After centrifugation, 5 aliquots of 3.5 × 10⁶ cells from two dishes mixed in a tube were cryopreserved in RPMI supplemented with 40% FBS and 10% dimethyl sulfoxide (DMSO) and stored at -196°C until use.

Subjects

Blood samples were obtained from 50 volunteers, collected in 4 mL EDTA BD Vacutainer™ tubes (Becton Dickinson & Company, New Jersey, USA). After processing the samples to obtain PBMC from each, there were stored at -196°C until use, in the Biobank platform of the IBIMA Plataforma BIONAND, which is part of the Biobank of the Andalusian Public Health System (BBSSPA). All the enrolled volunteers gave their written informed consent obtained from the BBSSPA and approved by the Ethics Committee of Clinical Research (CEIC) from IBIMA Plataforma BIONAND. The study was carried out in accordance with The Code of Ethics of the WMA (Declaration of Helsinki).

PBMC purification

The procedure was carried out as previously described by Ortega-Pinazo et al. [26]. Briefly, all blood samples from each patient were mixed and then Lymphosep™ (Biowest, Riverside, Missouri, USA) was added to a sterile tube, and blood was diluted 1:1 with physiological saline and gently deposited in these tubes. A density gradient was established by centrifugation at 616 xg for 25 min continuously at room temperature. PBMC layer was collected and transferred into a new tube. Then, two washing steps with physiological saline at 4°C were performed, the first at 616 xg for 10 min to remove the Lymphosep™ surplus and the second at 122 xg for 10 min to remove platelets. The supernatant was discarded, and the pellet was resuspended in DPBS. Live cells were estimated by the exclusion of trypan blue (75% survival was considered optimal, if was less the sample was discarded). 7 aliquots of 3.5 × 10⁶ cells from each volunteer were cryopreserved in RPMI supplemented with 40% FBS and 10% DMSO and stored at -196°C.

RNA extraction

Jurkat T and PBMCs cells were thawed and washed twice with DPBS without calcium or magnesium at 122 xg for 8 min to remove DMSO. Then, pellets of 3 × 10⁶ viable cells...
were used for RNA extraction using seven different methods: 2 automated methods (Maxwell® and QIAcube™), 4 semiautomated methods (QIAgen™, Bio-Rad, Monarch®, and Canvax™), and 1 manual method (TRIzol™). After extraction, RNA samples were resuspended in 30 µL of sterile ultrapure water and stored at -80 ºC until use.

- **Maxwell®**: RNA was extracted from samples with the commercial Maxwell® 16 Total RNA Purification Kit (Promega, Wisconsin, USA) using the Maxwell® robot following the manufacturer’s instructions.

- **QIAcube™**: Previously, Pellet was resuspended in 700 µL of TRIzol™ (Invitrogen, Thermo Fisher Scientific, Massachusetts, USA) and stored at -80 ºC for 48 h. Then, samples were thawed, and RNA was extracted with the commercial miRNeasy mini Kit (QIAgen™, Hilden, Germany) using the QIAcube™ robot, following the manufacturer’s instructions.

- **QIAgen™**: Previously, Pellet was resuspended in 700 µL of TRIzol™ and stored at -80 ºC for 48 h. RNA was manually extracted from samples with the commercial QIAmp™ RNeasy mini Kit (QIAGEN™), following the manufacturer’s instructions.

- **Bio-Rad**: RNA was extracted with the commercial Aurum™ Total RNA Mini Kit (Bio-Rad, California, USA), following the manufacturer’s instructions.

- **Monarch®**: RNA was extracted with the commercial Monarch® Total RNA Miniprep Kit (New England Biolabs, Massachusetts, USA), following the manufacturer’s instructions.

- **Canvax™**: RNA was extracted with the commercial HigherPurity™ Total RNA Extraction Kit (Canvax™ Biotech S.L., Córdoba, Spain), following the manufacturer’s instructions.

- **TRIzol™**: The cell pellet was incubated with 800 µL of TRIzol™ (Invitrogen) at RT for 10 min with agitation. Then, 200 µL of chloroform was added and, after manual agitation for 15 s, the samples were then cold centrifuged at 12,000 xg for 15 min. The transparent phase was collected for RNA extraction and transferred to a new sterile tube adding 600 µL of isopropanol. After 20 min incubation at -20 ºC, samples were cold centrifuged at 12,000 ×g for 10 min. The pellet was washed once with 1 mL of ethanol 75%. After cold centrifugation at 7500 ×g for 5 min, the pellet was dried to remove the excess ethanol.

All samples were treated with Qiagen RNase-Free DNase set following the manufacturer’s instructions, and were reverse transcribed immediately. RNA surplus, were stored at -26°C for 3 years to be evaluated again.

**RNA quantification, purity, and integrity**

Absorbance at 260, 280, and 230 nm of 2 µL of each RNA sample was measured in duplicate using the NanoDrop™ 2000 (Thermo Fisher Scientific). Ultrapure water was used as blank. The concentration of RNA from 260 nm absorbance was calculated according to the Lambert-Beer law. The A260/A280 ratio (i.e., RNA/protein) was used as a purity indicator. Optimum A260/A280 ratio values or pure RNA ranged from 1.8 to 2. The A260/A230 ratio (RNA/contaminants) was used as a secondary measurement of purity, establishing the optimal, in this case, at values greater than 1.8 [27]. The concentration of RNA was also performed using a fluorometric method. Thus, Qubit™ RNA HS Assay Kit (Life Technologies, Thermo Fisher Scientific) was used following the manufacturer’s instructions. First, standard samples were assessed to generate a standard curve. Subsequently, the Qubit™ assay reagent was added to the samples and processed into the reader Qubit™ 3.0 Fluorimeter independently. The integrity of total RNA was determined by electrophoresis. Samples were separated on 2% agarose (Condalab, Madrid, Spain) denaturing gels stained with SYBR Safe (Invitrogen, Massachusetts, USA) in running buffer (1×Tris/acetate/ethylenediaminetetraacetic acid, pH 8.0). Gel images were analyzed using ImageQuant™ LAS 4000 (GE Healthcare, Illinois, USA), and bands were compared using the DNA Molecular Weight Marker IX (Roche, Basilea, Swiss).

**RNA functionality**

RNA functionality was evaluated by PCR amplification or real-time PCR assay of commonly used reference genes, available and frequently studied in our laboratory. To obtain the cDNA, an aliquot containing 1 µg of total RNA was reverse transcribed using M-MLV reverse transcriptase (Merck Life Science), 5 µM random hexamers, and 2 mM dideoxynucleotides (Roche, Basilea, Switzerland) in a total reaction volume of 20 µL, following the manufacturer’s instructions. Samples were stored at -20°C. For PCR, a fragment of 312 base pairs of the HIST1H4A gene was amplified following the protocol previously published by Ortega-Pinazo et al. [26]. For the real-time PCR assay, 25 ng of cDNA from each sample was amplified for the PRKGI and IMPDH2 genes obtaining a PCR product of 74 and 98 bp respectively and delta Ct value was compared for each extraction method, in an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystem, California, USA) using iTaq™ Universal SYBR Green Supermix (Bio-Rad). Determinations were performed in triplicate, and the Ct value was analyzed. The primer sequences for real-time PCR were: forward 5’-CCACCGCCTTCGACAT- 3’ and
reverse 5'-CCTGCTTACTGTGGCCTTCTTG- 3', for the PRKG1 gene; and forward 5'-CCATCTCATCCCTGGTCTTGGAGGACT- 3' and reverse 5'-CCATCCCTCTCCGTGGACACTTCCACGACT- 3', for the IMPDH2 gene. PCR and real-time PCR products were assessed on a 2% denaturing agarose gel stained with SYBR Safe (Invitrogen).

**Statistical studies**

Data were presented as the mean values as means ± SD. Statistical analyses were performed using the R program and SPSS (v20.0). The significance between groups was determined with the Student’s and one-way ANOVA test; Only probabilities ≤ 0.05 were considered significant. The Tukey test was used to perform multiples comparison between the variables.

**Results**

Yield and quantification studies for each extraction method were initially carried out in cell cultures (Jurkat T cells) due to the homogeneity of the samples, allowing an optimal comparison between tested methods. Concerning the yield of the extraction process for each of the methods tested in human Jurkat T cells, ANOVA and Tukey analysis on data from the quantification by spectrophotometry using the NanoDrop™ system revealed significant differences (P < 0.001) for all methods tested. Pair-by-pair comparison showed statistically significant differences for all methods, except for QIAgen™ vs. QIAcube™, and Canvax™ vs. Maxwell® methods which do not substantially differ. The highest yields were obtained using TRIzol™, QIAgen™, or QIAcube™ (Table 1). The semiautomated Canvax™ and automated Maxwell® extraction methods provided worse RNA yields with respect to the rest of the methods. Similarly, quantification by fluorimetry using Qubit™ showed a significantly (P < 0.001) higher performance in the RNA samples obtained with TRIzol™, QIAgen™, or QIAcube™ methods, and substantially lower performance with Bio-Rad, Canvax™, and Maxwell®, respectively (Table 1). In all cases, the yield was within the range proposed for each method by its respective manufacturer. Six extraction methods were tested (QIAgen™, QIAcube™, Bio-Rad, Monarch®, Maxwell®, and TRIzol™) in PBMC. Only QIAcube™ and TRIzol™ methods showed statistically significant pair-by-pair differences (P < 0.001) and the highest yields (Table 1). Fluorimetry confirmed the results, showing lower performance when Maxwell® and Bio-Rad were used. All results were within the range proposed for each method by its respective manufacturer. Besides, a remarkable difference was observed depending on the matrix used for extraction. Best yields were obtained for the human Jurkat T cells, reaching up to 80% higher in QIAcube™ and QIAgen™ methods, remaining unchanged for the Maxwell® method.

Furthermore, the long-term storage of nucleic acids is needed in biomedical research to develop research lines based on previous results that allow the same samples to be studied over several years. Some studies on RNA storage for long periods have been carried out, but these have been carried out at temperatures of about − 80 °C or in blood samples preserved in Tempus tubes [28, 29]. Results obtained show that the RNAs obtained are viable 3 years after their storage at -26 °C, watching a slight decrease in yield after quantification but presenting integrity values identical to those initially obtained (Table 2). The functionality studies were similar to those obtained initially.

**Table 1** Comparative values of spectrophotometry (Nanodrop™) and fluorimetry (Qubit™) (expressed as mean ± SD), 260/280 and 260/230 ratios in RNA obtained from human Jurkat T cells (HJT) (n = 5) and PBMC (n = 50) using seven different extraction methods

| Kit          | Mean µg ± SD | A260/A280 ratio | A260/A230 ratio | Mean µg ± SD |
|--------------|--------------|-----------------|----------------|--------------|
| QIAgen™      | HJT          | 27.61 ± 0.76    | 2.1 ± 0        | 1.47 ± 0.17  |
|              | PBMC         | 4.95 ± 1.91     | 1.89 ± 0.11    | 1.10 ± 0.34  |
| Bio-Rad      | HJT          | 8.89 ± 2.14     | 2.1 ± 0.11     | 1.56 ± 0.40  |
|              | PBMC         | 3.29 ± 1.28     | 2.04 ± 0.04    | 1.38 ± 0.39  |
| Monarch®     | HJT          | 17.88 ± 2.48    | 2.1 ± 0.02     | 2.15 ± 0.03  |
|              | PBMC         | 4.02 ± 0.97     | 2.02 ± 0.01    | 2.03 ± 0.14  |
| Canvax™      | HJT          | 4.51 ± 0.94     | 2.1 ± 0        | 1.96 ± 0.07  |
|              | PBMC         | -                | -              | -            |
| Maxwell®     | HJT          | 2.99 ± 1.04     | 4.2 ± 0.58     | 0.64 ± 0.41  |
|              | PBMC         | 2.66 ± 0.60     | 6.27 ± 1.06    | 1.13 ± 0.29  |
| QIAcube™     | HJT          | 33.47 ± 3.77    | 2.1 ± 0.01     | 2.05 ± 0.03  |
|              | PBMC         | 6.13 ± 2.98     | 1.98 ± 0.10    | 1.58 ± 0.31  |
| TRIzol™      | HJT          | 22.27 ± 5.32    | 1.9 ± 0.04     | 0.96 ± 0.15  |
|              | PBMC         | 7.22 ± 3.5      | 1.77 ± 0.12    | 0.54 ± 0.21  |
To evaluate the purity of RNA, A260/A280 (DNA/protein) and A260/A230 (DNA/contaminants) absorbance ratios were determined. Accordingly, results showed acceptable A260/A280 ratio values ranging from 1.77 to 2.1 for all the methods tested, with the exception of Maxwell®, which showed an A260/A280 ratio of 4.2 and 6.27 in human Jurkat T cells and PBMC, respectively (Table 1), although ratio values between 1.6 and 2 were obtained in positive controls used for this method corresponding to plasma and Human Umbilical Vein Endothelial Cells (HUVECs) samples. Only Maxwell® showed significant differences (P < 0.001) when compared with the rest of the methods. The lower A260/A230 ratios were obtained with Maxwell® and TRIZOl™ methods for human Jurkat T cells, and only using TRIZol™ in PBMC. Best results, among 1.96–2.15, were obtained for QIAcube™, Monarch®, and Canvax™ methods (Table 1).

The analysis of the integrity of RNA revealed the presence of both ribosomal 28 S and 18 S bands, showing the typical 2:1 proportion of intensity, in all RNA samples either from human Jurkat T cells (Fig. 1a) or from PBMC (Fig. 1b), irrespective of the method of extraction, as usually reported [20, 21], excepting for Maxwell®. In this case, a single very intense band was observed in both biological matrices (Fig. 1), located close to the 28 S band but not having correspondence with this.

To determine the functionality of RNA samples PCR and real-time PCR were performed in genes whose expression level was similar in both human Jurkat T cells and PBMC. All samples were positive for the genes studied, and an amplification band of the desired size was obtained regardless of the type of extraction method and the matrix evaluated (Fig. 1c, d). Multiple comparisons showed significant differences in C_T values in the Bio-Rad (P < 0.05), Maxwell® (P < 0.01), and TRIZol™ (P < 0.01) methods (Fig. 1g, h). All RNA obtained in our study was shown as functional by conventional PCR and real-time PCR, as observed in related studies [19, 23]. However, statistically significant differences were found in the results of real-time PCR for Bio-Rad, Maxwell®, and TRIZol™ methods when compared with the rest of the methods tested in both matrices. These results suggest these methods could compromise the functionality of the RNAs obtained, so the results could not be completely reliable.

Finally, an important fact to consider when comparing nucleic acid extraction methods is to know the time, labour, and cost analysis for each method [30, 31]. The feasibility of each method in terms of time and costs per sample is reported in Table 3. The fastest extraction methods were the Canvax™ and Maxwell®, while the most time-consuming were the QIAgen™, QIAcube™, and manual, which required overnight incubation. On the other hand, the manual was the cheapest one, followed by the Monarch® and Canvax™, two semiautomated methods requiring lower manipulation by specialized personnel than the manual method. Additionally, manual or semiautomated methods can process 24 samples, approximately twice those of automated methods QIAcube™ and Maxwell®.

### Discussion

It is fundamental to produce reliable results in biomedical research to have a good knowledge of all extraction protocols to obtain high-quality RNA. For RNA extraction it is possible to find manual, automated, or semi-automated methods. Because many of them present disadvantages, a thorough study of their profitability is necessary. Some studies have been conducted in other biologic species or in other human matrices, but, to the best of our knowledge, no approximation with the kits presented in this study has been carried out thus far. In this study, six extraction methods were tested and exhibited detectable differences also depending on the matrix used for extraction. RNAs were shown as viable after 3 years at -26 °C, and only slight significant differences were found when the manual method was chosen, perhaps because the reagents used in the extraction produced a partial degradation in the sample.

From our findings, the best yield was obtained for QIAcube™ (automated), QIAgen™ (semi-automated), and TRIZol™ (manual) methods. In line with our results from

### Table 2

| Method     | 2019  | 2021  |
|------------|-------|-------|
|            | Nanodrop™2000 | A260/A280 ratio | A260/A230 ratio | Qubit™ | Nanodrop™2000 | A260/A280 ratio | A260/A230 ratio | Qubit™ |
| QIAgen™    | 27.61 ± 0.76 | 2.1 ± 0.004 | 1.5 ± 0.2 | 25.24 ± 0.22 | 28.32 ± 1.85 | 2.06 ± 0.008 | 1.48 ± 0.17 | 24.86 ± 1.34 |
| Bio-Rad    | 8.89 ± 2.14 | 2.1 ± 0.01 | 1.6 ± 0.4 | 8.05 ± 1.95 | 7.25 ± 2.24 | 2.03 ± 0.01 | 1.53 ± 0.39 | 6.57 ± 2.5 |
| Monarch®   | 17.88 ± 2.48 | 2.15 ± 0.03 | 2.1 ± 0.2 | 16.30 ± 1.68 | 16.08 ± 6.05 | 2.08 ± 0.007 | 2.1 ± 0.008 | 14.25 ± 5.47 |
| Canvax™    | 4.51 ± 0.94 | 2.1 ± 0.007 | 2 ± 0.05 | 4.06 ± 0.53 | 3.70 ± 1.7 | 2.02 ± 0.04 | 1.57 ± 0.22 | 3.55 ± 1.01 |
| Maxwell®   | 2.99 ± 1.04 | 4.1 ± 0.57 | 0.5 ± 0.33 | 2.49 ± 0.56 | 1.52 ± 0.81 | 3.79 ± 0.59 | 0.48 ± 0.31 | 2.1 ± 0.09 |
| QIAcube™   | 33.47 ± 3.77 | 2.1 ± 0.005 | 2.1 ± 0.04 | 31.02 ± 4.56 | 29.25 ± 2.56 | 2.07 ± 0.002 | 2.1 ± 0.005 | 24.86 ± 2.16 |
| TRIZol™    | 22.27 ± 5.32 | 1.9 ± 0.04 | 0.9 ± 0.09 | 20.28 ± 4.17 | 15.07 ± 4.73 | 1.91 ± 0.15 | 0.62 ± 0.19 | 12.07 ± 0.29 |
Fig. 1 RNA analysis in human Jurkat T cells (n=9) and PBMC (n=50). (a, b) RNA integrity in samples processed with different extraction methods in human Jurkat T cells (a) and PBMC (b) in 2% agarose gels. MW: Molecular weight marker (0.072–1.35 kbp). (c-f) Assessment of functionality of RNA obtained in human Jurkat T cells and PBMC; HIST1H4A gene fragment (312 bp) amplified from cDNA in 2% agarose gels by conventional PCR (c, d); PRKG1 fragment gene (74 bp) amplified from cDNA in 2% agarose gels by real-time PCR (e, f); MW: Molecular weight marker (0.072–1.35 kbp), C-: Negative control. (g, h) RNA performance for real-time PCR assay in human Jurkat T cells and PBMC for PRKG1 (black) and IMPDH2 (gray) genes. The average $C_T$ value and standard deviation for each type of source were calculated. * $0.01 < p \leq 0.05$ and ** $0.001 < p \leq 0.01$
Jurkat T cells, previous work from Tavares et al. [32] using SK-N-MC neuroblastoma cells reported that semi-automated methods had better yield than manual ones. However, for PBMC, a yield of up to 1.18 times higher was found in manual extraction methods when compared with commercial kits [18, 21], in agreement with our study. The decrease in the yield of some semi-automated methods, such as Bio-Rad and Canvax™, may be due to the use of β-mercaptoethanol, used to deactivate RNases but with denaturing effects on guanidinium isothiocyanate in the lysis buffer [33]. Additionally, the poorer results in Canvax™ and Maxwell® could be attributable to the probable contamination in the eluted RNA such as the remains of the magnetic microspheres, interfering in the spectrophotometric quantification, and even in subsequent applications of the RNA samples [34-36]. Similarly, the evaluation of RNA purity pointed out the QIAcube™ method as the best option in accordance with Sharma P et al. [37] in pediatric PBMCs using this method. It is interesting to note that lower A260/A230 ratios may indicate the presence of compounds absorbing at 230 nm such as proteins [35], guanidine HCL, EDTA, carbohydrates, lipids, salts, or phenol [30]. Compared with PBMC, the yield obtained in Jurkat T cells was about 5 times higher as would befit to a more homogeneous matrix with fewer interfering substances than biological fluids. In line with the RNA quality analysis, the study of the integrity is not affected by the method of extraction used [20, 21]. However, caution is recommended for the elution process given the unusual band revealed when the Maxwell® method was used. This could be due to the aforementioned elution of the RNA together with the magnetic microspheres used in this method, which could cause changes in the migratory patterns of these RNAs in agarose gels [29, 36].

Lastly, time-consuming and cost analysis for each method allowed us to visualize the complete scenery, as a no less important aspect. The analysis performed in this study showed that automated methods were quite expensive and had a low capacity to work with several samples simultaneously, while the manual method (TRIzol™) extended the protocol by more than one day, increasing the risk of degradation. Therefore, semi-automated methods could be more advisable because they have a more affordable price, a greater capacity than other methods, and less execution time.

**Conclusion**

In summary, our data revealed differences attributable to the method chosen. Given our results, QIAcube™ and semi-automated extraction methods were perceived as the best options because of their lower variability, good functionality, and lower cost. Noteworthy Monarch® appeared as the second-best option because it showed quality indicators closer to expected, which guarantees reliable results. Despite larger studies with a greater number of methods and matrices would be advisable, the variety of methods compared in this study emphasize the relevance of the choice of an optimal RNA extraction method in biomedical and nucleic acid research.

In addition, RNA stored for 3 years at -26°C showed that they maintained their purity and functionality, which would be very useful for optimizing sample storage in research platforms such as biobanks.

**Acknowledgements and funding information** We gratefully acknowledge IBIMA Plataforma BIONAND’s joint support structures for research (ECAI) of General Services, Genomic, and Biobank, as well as technical assistance. Likewise, we are obliged to the Biobank of the Andalusian Public Health System (SSPA Biobank, Andalusian Regional Ministry of Health and Families).

This work was supported by grants from the Spanish Ministry of Science, Innovation, and Andalusian Regional Ministry of Health and Consumption, co-funded by the European Regional Development Fund (ERDF, EU), (PI16/01510 and PECOVID-0099-2020, to GET). GET is under a contract of the “Nicholas Monardes” programme from the Andalusian Health Service, Andalusian Regional Ministry of Health and Consumption. BGD is under a “Miguel Servet” contract from the Health Institute Carlos III, Ministry of Science and Innovation, Spain.

**Data Availability** Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

**Declarations**

**Ethical approval and consent to participate** All applicable international, national, and/or institutional guidelines were followed. All the enrolled volunteers gave their written informed consent obtained from the SSPA Biobank and approved by the Ethics Committee of Clinical Research (CEIC) from IBIMA. The study was carried out in accordance with The Code of Ethics of the WMA (Declaration of Helsinki).

**Consent for publication** All authors have directly participated in the planning, execution, or analysis of this study and approved the contents of the manuscript and its submission.
Author Disclosure Statement  The authors have no conflicts of interest to declare.

References

1. Cooper TA, Wan L, Dreyfuss G (2009) RNA and disease. Cell 136:777–793. https://doi.org/10.1016/j.cell.2009.02.011
2. Zhang C (2009) Novel functions for small RNA molecules. Curr Opin Mol Ther 11:641–651
3. Liu L, Wang J, Khanabadi R, Kalionis B, Tai X, Xia S (2017) Circular RNAs: isolation, characterization and their potential role in diseases. RNA Biol 14:1715–1721. https://doi.org/10.1080/15476286.2017.1367886
4. Hawkins PG, Morris KV (2008) RNA and transcriptional modulation of gene expression. Cell Cycle. https://doi.org/10.4161/cc.7.5.5522. 7.602–607
5. Mihailescu R (2015) Gene expression regulation: lessons from noncoding RNAs. RNA. https://doi.org/10.1261/rna.058015.115. 21.695–696
6. Bai JP, Alekseyenki AV, Statnikov A, Wang IM, Wong PH (2013) Evaluation of four viroid RNA extraction methods for the molecular detection of classical swine fever by real-time and conventional reverse transcription-PCR. J Vet Diagn Invest 17:574–578. https://doi.org/10.1177/104063870105000609
7. Liu L, Wang J, Khanabadi R, Kalionis B, Tai X, Xia S (2017) Circular RNAs: isolation, characterization and their potential role in diseases. RNA Biol 14:1715–1721. https://doi.org/10.1080/15476286.2017.1367886
8. Hawkins PG, Morris KV (2008) RNA and transcriptional modulation of gene expression. Cell Cycle. https://doi.org/10.4161/cc.7.5.5522. 7.602–607
9. Mihailescu R (2015) Gene expression regulation: lessons from noncoding RNAs. RNA. https://doi.org/10.1261/rna.058015.115. 21.695–696
10. Bai JP, Alekseyenki AV, Statnikov A, Wang IM, Wong PH (2013) Evaluation of four viroid RNA extraction methods for the molecular detection of classical swine fever by real-time and conventional reverse transcription-PCR. J Vet Diagn Invest 17:574–578. https://doi.org/10.1177/104063870105000609
and long-term DNA banking using blood samples. PLoS ONE e0115960. https://doi.org/10.1371/journal.pone.0115960
31. Chacon-Cortes D, Haupt LM, Lea RA, Griffiths LR (2012) Comparison of genomic DNA extractions techniques from whole blood samples: a time, cost and quality evaluation study. Mol Biol Rep 39:5961–5962. https://doi.org/10.1007/s11033-011-1408-8
32. Tavares L, Alves PM, Ferreira RB, Santos CN (2011) Comparison of different methods for DNA-free RNA isolation from SK-N-MC neuroblastoma. BMC Res Notes 4:3. https://doi.org/10.1186/1756-0500-4-3
33. Mommaerts K, Sanchez I, Betsou F, Mathieson W (2015) Replacing beta-mercaptoethanol in RNA extractions. Anal Biochem 479:51–53. https://doi.org/10.1016/j.ab.2015.03.027
34. Stulnig TM, Amberger A (1994) Exposing contaminating phenol in nucleic acid preparations. Biotechniques 16:402–404
35. Liu PF, Avramova LV, Park C (2009) Revisiting absorbance at 230 nm as a protein unfolding probe. Anal Biochem 389:165–170. https://doi.org/10.1016/j.ab.2009.03.028
36. Martín-Núñez GM, Gómez-Zumaquero JM, Soriguier F, Morcillo M (2012) High resolution melting curve analysis of DNA samples isolated by different DNA extraction methods. Clin Chim Acta 413:331–333. https://doi.org/10.1016/j.cca.2011.09.014
37. Sharma P, Singh M, Singh A, Bhardwaj D, Bhatia P (2022) Experience of quantity and quality of DNA and RNA extraction from limited pediatric blood samples: a comparative analysis of automated and manual kit-based method. Indian J Pathol Microbiol 65(1):195–110. https://doi.org/10.4103/IJPM.IJPM_946_20

Publisher’s Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor (e.g. a society or other partner) holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.