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

Jesús Ortega-Pinazo
Instituto de Investigación Biomédica de Málaga, Hospital Regional Universitario de Málaga
Pedro Jesús Serrano-Castro
Instituto de Investigación Biomédica de Málaga, Hospital Regional Universitario de Málaga
Margarita Vida-Botella
Instituto de Investigación Biomédica de Málaga, ECAI de Biología Celular
Beatriz Martínez
Instituto de Investigación Biomédica de Málaga, ECAI de Biobanco, Andalusian Pblic Health System Biobank
María Jesús Pinto-Medel
Instituto de Investigación Biomédica de Málaga, Hospital Regional Universitario de Málaga
Begoña Oliver-Martos
Instituto de Investigación Biomédica de Málaga, Hospital Regional Universitario de Málaga
Laura Leyva
Instituto de Investigación Biomédica de Málaga, Hospital Regional Universitario de Málaga
Juan Miguel Gómez-Zumaquero
Instituto de Investigación Biomédica de Málaga, ECAI de Genómica
Ana Lago-Sampedro
Instituto de Investigación Biomédica de Málaga, Hospital Regional Universitario de Málaga, CIBERDEM
Pablo Jesús Rodríguez-Bada
Instituto de Investigación Biomédica de Málaga, ECAI de Genómica
Guillermo Estivill-Torrús (guillermo.estivill@ibima.eu)
Instituto de Investigación Biomédica de Málaga, Hospital Regional Universitario de Málaga
https://orcid.org/0000-0002-7124-2678
Pedro Ferro
Instituto de Investigación Biomédica de Málaga, ECAI de Biobanco; Hospital Universitario Virgen de la Victoria, UGC de Endocrinología y Nutrición

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Research Article

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Abstract

Nucleic acids, RNA among them, are widely used in biomedicine. 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. In this study, we have compared seven different RNA extraction methods: manual (TRIzol™), semiautomated (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. These data contribute to facilitate researchers in decision-making practices and emphasize the relevance of the selection of the RNA extraction method in each experimental procedure to guarantee both quality standards and its reproducibility.

Introduction

Nucleic acids are widely used in biomedical 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 fluorimetry 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 28S, 18S, and 5S 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, at present there are no

studies comparing a substantial number of RNA extraction methods and, specially, using the so extensively studied 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 fluorimetry to determine their yield and purity. Subsequently, an assessment of integrity and a functionality study were carried out. The study included also a comparative analysis of RNAs after long-storing . Finally, a determination of cost, process time, and other related factors were 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 validity of laboratory RNA methods, according to their requirements.

Materials And Methods

2.1. Cell cultures

Human Jurkat T cells were grown in RPMI-1640 with 10% heat-inactivated fetal calf serum, 10mM HEPES, 2.5 g/l glucose, 1 mM sodium pyruvate, and 25 µg/ml gentamicin (all products from Merck Life Science SLU, Spain) at 37 ºC in a humid atmosphere of 5% CO_2. Confluent cell cultures were centrifuged to harvest cells and washed in Dulbecco phosphate-buffered saline (DPBS) without calcium or magnesium. After centrifugation, the pellet was resuspended in DPBS. Live cells were estimated by trypan blue exclusion. Aliquots of 5×10^6 cells were cryopreserved in RPMI supplemented with 40% FBS and 10% dimethyl sulfoxide (DMSO) and stored at -196 °C until RNA extraction.

2.2. Subjects

Blood samples were obtained from 30 volunteers, collected in 4 ml EDTA BD Vacutainer™ tubes (Becton Dickinson & Company, New Jersey, USA). After processing the samples were stored at -196 ºC until use, in the Biobank platform of the IBIMA, 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. The study was carried out in accordance with The Code of Ethics of the WMA (Declaration of Helsinki).

2.3. PBMC purification

The procedure was carried out as previously described by Ortega-Pinazo et al. [26]. Briefly, Lymphosep™ (Biowest, Riverside, Missouri, USA) was added to a sterile tube, blood was diluted 1:1 with physiological saline and gently deposited in these tubes. A density gradient was established by centrifugation at 616 ´g for 25 min continuously. PBMC layer was collected and transferred into a new tube. Then, two washing steps with physiological saline were performed, the first at 616 ´g for 10 min to remove the Lymphosep™ surplus and the second at 122 ´g 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. Aliquots of 3.5×10^6 cells were cryopreserved in RPMI supplemented with 40% FBS and 10% DMSO and stored at -196°C.

2.4.RNA extraction

Cells were thawed and washed twice with DPBS without calcium or magnesium at 122 ´g for 8 min to remove DMSO. Aliquots of 3×10^6 viable cells were prepared in DPBS as indicated in the product manuals. RNA was extracted 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 immediately analyzed. For long-term storage analysis RNA samples were stored at -26 ºC for 2 years.

- **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, DPBS was removed from samples by cold centrifugation at 122 ´g for 8 min. The supernatant was discarded, and the pellet was resuspended in 750 µl of TRIzol™ (Invitrogen, Thermo Fisher Scientific, Massachusetts, USA) and stored at -80 ºC for 48 hours. 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™:** First, DPBS was removed, and the pellet was processed and stored as previously cited. Then, samples were thawed, and RNA was manually extracted 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™:** Previously, DPBS was removed from samples by cold centrifugation at 122 ´g for 8 min, and the 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 seconds, the samples were then cold centrifuged at 12000 ´g 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 12000 ´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.

2.5. 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 (ThermoFisher 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, two standards 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 3% agarose gels stained with SYBR Safe (Invitrogen) in running buffer (1×Tris/acetic acid/ethylenediaminetetraacetic acid, pH 8.0). Gel images were analyzed using ImageQuant™ LAS 4000 (GE Healthcare, Illinois, USA).

2.6. RNA functionality

RNA functionality was evaluated by PCR amplification or real-time PCR assay. 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 -20ºC. 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 *PRKG1* and *IMPDH2* genes obtaining a PCR product of 74 and 98 bp respectively, in an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystem, California, USA) using iTaq^{TM} Universal SYBR Green Supermix (Bio-Rad). Determinations were performed in triplicate, and the C_T value was analysed. The primer sequences for real-time PCR were: forward 5'-CCACCGCCTTCGACAT- 3' and reverse 5'-CCTGCTTACTGTGGGCTCTTG- 3', for the *PRKG1* gene; and forward 5'-CCATCTCATCCCTGCGTGTCTCCGAGGACT- 3' and reverse 5'-CCTATCCCCTGTGTGCCTTGCCTCCCACGACT- 3', for the *IMPDH2* gene. PCR and real-time PCR products were assessed on a 2.5% denaturing agarose gel stained with SYBR Safe (Invitrogen).

2.7. Statistical studies

Data were presented as the mean values as mean ± 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 comparisons between the variables.

Results And Discussion

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 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 a 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 and the highest yields (Table 1). Fluorimetry confirmed the results, showing the 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.

From these findings, the best yield was obtained using QIAcube™ (automated), QIAgen™ (semi-automated), and TRIzol™ (manual) methods. In line with our results from Jurkat T cells, a previous work from Tavares et al. [28] using SK-N-MC neuroblastoma cells reported that semi-automated methods had better yield than manual ones. However, for PBMC, a yield up to 1.18 times higher was found in manual extraction methods by comparison 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 [29], used to deactivate RNases but with denaturing effects on guanidinium isothiocyanate in the lysis buffer [30]. 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 [31-33].

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 ranged from 1.77 to 2.1 for all the methods tested, with 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). Only Maxwell® showed significant differences ($P < 0.001$) when was 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). In this respect, lower A260/A230 ratios may indicate the presence of compounds absorbing at 230 nm such as proteins [31], guanidine HCL, EDTA, carbohydrates,

lipids, salts, or phenol [34]. In comparison with PBMC, the yield obtained in Jurkat T cells was about 5 times higher as corresponding to a more homogeneous matrix with fewer interfering substances than biological fluids.

The analysis of the integrity of RNA revealed the presence of both ribosomal 28S and 18S 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 28S band but not having correspondence with this. This could be due to the aforementioned elution of the RNA together with the magnetic microspheres used in this method, which might cause changes in the migratory patterns of these RNAs in agarose gels [29,33]. For 5S band only was observed for Monarch, QIAgen™, QIAcube™, and Bio-Rad methods in human Jurkat T cells, but not for any method in PBMC.

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 for C_T values in the Bio-Rad, Maxwell®, and TRIzol™ 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 were 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 results could not be completely reliable.

Long-term storage of nucleic acids is needed, especially in the context of large medical archives or biobanks, for retrospective studies in biomedical research as well as for the development of new diagnostics or therapies. Some studies on RNA storage for long periods have been done, but these have been carried out at temperatures about -80 ºC or in blood samples preserved in Tempus tubes [35, 36]. In addition to the showed data, here we also extended the analysis on RNA obtained from Jurkat T cells after their storage at -26 ºC. The results (Table 2) show that the RNAs are viable 2 years after their storing, watching a slight decrease in yield after quantification but presenting integrity values identical to those initially obtained. Functionality studies were similar to those initially obtained for freshly extracted samples. It should be noted that the only significant differences in spectrophotometry and fluorimetry data were found for the manual method, perhaps because the reagents used for the extraction produced a partial degradation in the sample.

Finally, an important fact to consider when comparing nucleic acid extraction methods is to know the time, labour, and cost analysis for each method [34,37]. 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 less 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®. 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 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 a 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. Furthermore, extracted RNAs are also viable 2 years after freezing. 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.

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.

Data availability statement

Data sharing does not apply to this article as no datasets were generated or analyzed during the current study.

Author Disclosure Statement

The authors have no conflicts of interest to declare.

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Tables

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) and PBMC using seven different extraction methods.

kit		NANODROP ™2000			QUBIT™
		µg (mean±SD)	A260/A280 ratio	A260/A230 ratio	µg(mean±SD)
QIAgen™	HJT	27.61 ± 0.76	2.1 ± 0	1.47 ± 0.17	25.24 ± 0.22
	PBMC	4.95 ± 1.91	1.89 ± 0.11	1.10 ± 0.34	3.93 ± 1.7
Bio-Rad	HJT	8.89 ± 2.14	2.1 ±0.01	1.56 ± 0.40	8.05 ± 1.95
	PBMC	3.29 ± 1.28	2 ±0.04	1.38 ± 0.39	2.66 ± 0.79
Monarch®	HJT	17.88 ± 2.48	2.1 ± 0.02	2.15 ± 0.03	16.30 ± 1.68
	PBMC	4.02 ± 0.97	2.02 ± 0.01	2.03 ± 0.14	3.41 ± 0.87
Canvax™	HJT	4.51 ± 0.94	2.1 ± 0	1.96 ± 0.07	4.06 ± 0.53
	PBMC	--	--	--	--
Maxwell®	HJT	2.99 ±1.04	4.2±0.58	0.64 ± 0.41	2.49 ± 0.56
	PBMC	2.66 ± 0.6	6.27±1.06	1.13 ± 0.29	1.64 ± 0.45
QIAcube™	HJT	33.47 ± 3.77	2.1 ± 0.01	2.056 ± 0.41	31.02 ± 4.56
	PBMC	6.13 ± 2.98	1.98 ± 0.10	1.58 ± 0.31	4.84 ± 1.9
TRIzol™	HJT	22.27 ± 5.32	1.9 ± 0.04	0.96 ± 0.15	20.28 ± 4.17
	PBMC	7.22 ± 3.5	1.77 ± 0.12	0.54 ± 0.21	5.76 ± 2.2

Table 2. 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 and stored for 2 years.

kit	NANODROP ™2000			QUBIT™
	µg (mean±SD)	A260/A280 ratio	A260/A230 ratio	µg (mean±SD)
QIAgen™	28.32 ± 1.85	2.06 ± 0.01	1.48 ± 0.17	24,86 ± 1.34
Bio-Rad	7.25 ± 2.24	2.03 ± 0.02	1.68 ± 0,26	6,57 ± 2.5
Monarch®	16.08 ± 6.05	2.08 ± 0.007	2.15 ± 0.29	14.25 ± 5.47
Canvax™	3.70 ± 1.7	2.02 ± 0.04	1.68 ± 0.08	3.55 ± 1.01
Maxwell®	1.52 ± 0.81	4.08 ± 0.16	0.69 ± 0.51	2,1 ± 0,09
QIAcube™	29.25 ± 2.56	2.07 ± 0.001	2.07 ± 0,05	24,86 ± 2.16
TRIzol™	15.07 ± 4.73	1.91 ± 0.18	0.62 ± 0.19	12,07 ± 0,29

Table 3. Assessment of consumables cost per sample and process duration *RNA* extraction methods studied.

	QIAgen™	Bio-Rad	Monarch®	Canvax™	QIAcube™	Maxwell®	TRIzol™
Cost per sample (USD)	7.0	5.91	4.82	5.25	7.0	10.18	4.43
ocess duration (10 samples)	70 min	60 min	90 min	45 min	70 min	45 min	100 min
Overnight incubation	Yes	No	No	No	Yes	No	Yes
aximum capacity (samples)	24	24	24	24	12	16	24

Figures

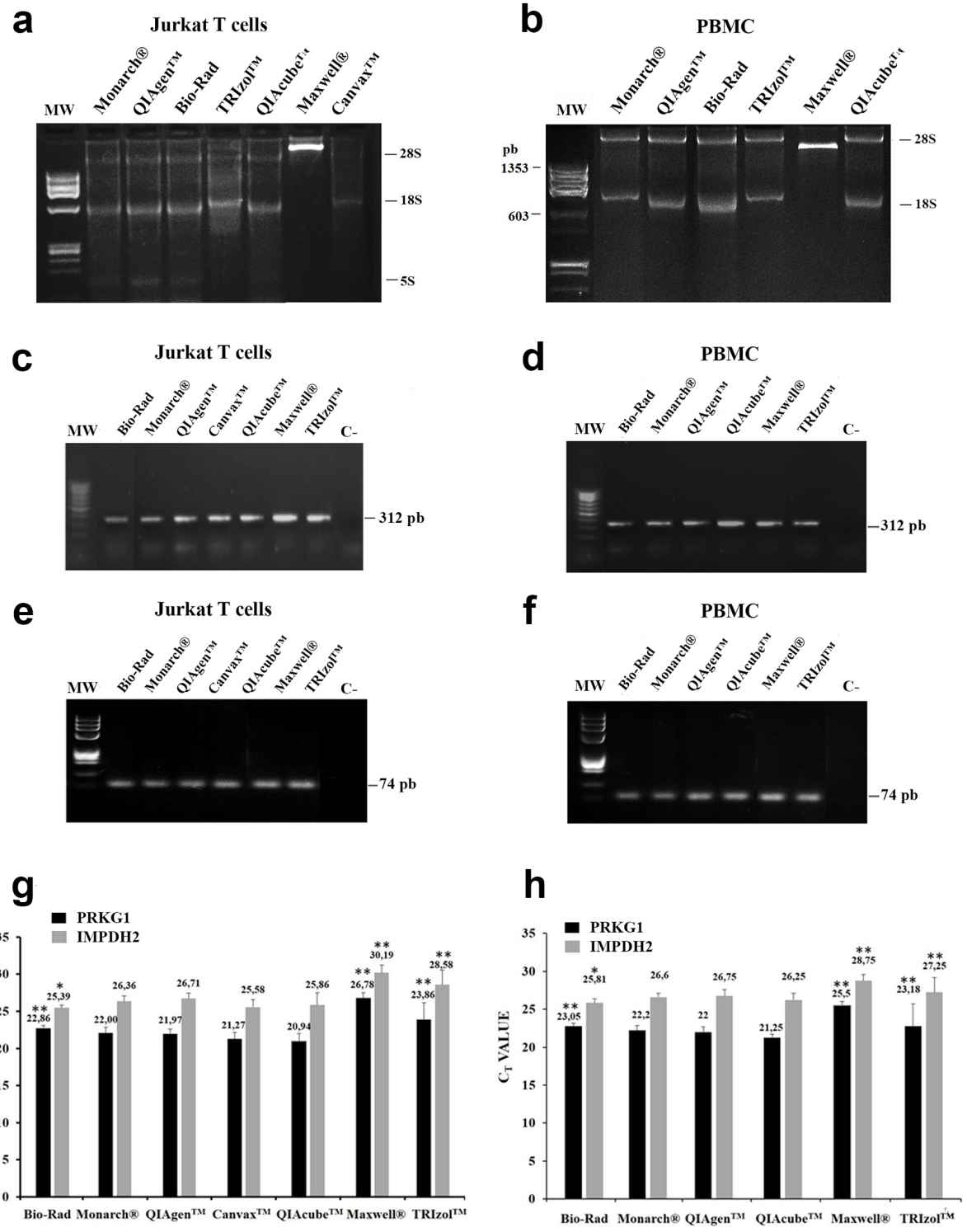

Figure 1

RNA analysis in human Jurkat T cells and PBMC. (a, b) RNA integrity in samples processed with different extraction methods in human Jurkat T cells (a) and PBMC (b) in 3% 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 CT value and standard deviation for each type of source were calculated. * $0.01 < p \leq 0.05$ and ** $0.001 < p \leq 0.01$