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Optimal RNAprotect® Tissue Reagent incubation and removal conditions prior to isolation of total RNA from stabilized cell samples

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Introduction

RNA is highly sensitive to degradation. Handling methods and prolonged storage of cells can greatly affect the quality of the RNA that can be later isolated. Contamination with RNases is the most significant problem, especially as they are so ubiquitous in the environment. They can degrade RNA to the point where results of downstream analyses become meaningless.

Submerging cells in RNAprotect Tissue Reagent, an RNA stabilization reagent, helps to stabilize the RNA within the cells and prevent degradation, supporting accurate downstream gene expression analyses. However, to avoid any interference from any RNAprotect Tissue Reagent components in isolation and analyses, cells must be pelleted and the reagent must be removed.

The separation of cells from excess RNAprotect Tissue Reagent via centrifugation is impeded due to the higher density of the reagent compared to standard culture medium. This means that it requires higher centrifugal forces, which might damage cells due to increased shearing forces, leading to reduced RNA yield. The aim of this study was to establish the optimal conditions for the recovery of cells from RNAprotect Tissue Reagent after RNA stabilization for maximum RNA yield and integrity.

Materials and methods

Cell culture procedure and cell stabilization

Table 1 shows the cell lines used in the experiments. They were cultured according to standard protocols in 75 cm² tissue culture flasks at 37°C and 5% CO_2 until 80% confluence was reached. Then the medium was removed and the cells were washed with phosphate-buffered saline (PBS). ▷

Table 1. Cell lines used in the experiments

Cell line	Background
Jurkat	Human T-cell leukemia cells
Ramos	Human B lymphocyte Burkitt's lymphoma cells
HeLa	Human cervix epithieloid carcinoma cells
MCF7	Human breast cancer cells

After a 10–30 s treatment with 1 ml trypsin, the cells were harvested using a cell scraper and pelleted via centrifugation (2 min, 300 *x g* at room temperature). They were then washed with PBS, diluted to 1 x 10^6 or 1 x 10^5 cells/ml with PBS, and centrifuged again. The PBS was removed and 200 µl RNAprotect Tissue Reagent was added. The tube was flicked to ensure all cells were submerged. Resuspension by vortexing is not suitable as the increased mechanical forces can damage the cells.

In parallel, cell aliquots were prepared without the addition of RNAprotect Tissue Reagent to serve as control samples. After the removal of the PBS, these aliquots were snap frozen in liquid nitrogen and stored at –90 to –65°C.

To identify the optimal conditions for removing RNAprotect Tissue Reagent after stabilization, the impact of two different incubation temperatures, centrifugation forces and times, and storage temperatures after the removal of RNAprotect Tissue Reagent were assessed (Table 2).

Table 2. Conditions applied for the incubation and removal of RNAprotect Tissue Reagent

Value	Incubation temperature	Centrifugation force	Centrifugation time	Storage
High	22°C	5000 *x g*	10 min	–30 to –15°C
Low	4°C	2500 *x g*	5 min	–90 to –65°C

RNA isolation and analysis

Total RNA was isolated using an RNeasy® Mini Kit and the protocol for purification of total RNA from animal cells using spin technology. Cell pellets (1 x 10^6 cells) were resolved by adding 350 µl Buffer RLT and following the protocol. RNA was eluted using 30 µl RNase-free water. A Nanodrop® spectrophotometer was used to determine RNA yields and the A_{260}/A_{280} ratio, and an Agilent® 2100 Bioanalyzer was used to compare RNA integrity. To check the overall quality of the stabilized RNA, the eluates were assessed using the RT^2 RNA QC PCR Array, which contains controls for RNA integrity, the presence of inhibitors of reverse transcription and PCR amplification, and the presence of genomic or other DNA contamination.

Results

RNA yield and quality

RNA yield was used as the main criteria to identify the optimal conditions for incubation with RNAprotect Tissue Reagent and its subsequent removal (Table 3). All RNA samples with a RIN value above 8.9 are considered high-quality RNA. The yields, A_{260}/A_{280} ratios, and RIN values for the RNA isolated for each cancer cell line with optimal RNAprotect Tissue Reagent incubation and removal conditions are shown in Table 4.

Table 3. The optimal incubation and removal conditions for each tested cell line

Value	Incubation temperature	Centrifugation force	Centrifugation time	Storage
High	● MCF7, ● HeLa, ● Ramos, ● Jurkat	● HeLa, ● Ramos, ● Jurkat	● MCF7, ● Jurkat	● Ramos
Low		● MCF7	● MCF7, ● Jurkat	● MCF7, ● HeLa, ● Jurkat

Cell lines: ● MCF7 ● HeLa ● Ramos ● Jurkat

Table 4. RNA quality assessment for each cell line

Cell line	Yield (µg)	260/280	RIN
MCF7	8.90	2.05	9.2
HeLa	6.67	1.99	9.0
Ramos	2.64	2.03	8.9
Jurkat	8.28	2.03	10.0

Comparison of RNA from stabilized and nonstabilized cells

Total RNA was isolated from samples containing 1 x 10^6 or 1 x 10^5 cells that had been stabilized with RNAprotect Tissue Reagent directly snap frozen (non-stabilized samples) after collection and assessed for quality, yield and other essential parameters. The obtained mean yields, A_{260}/A_{280} ratios, and RIN values for the same cell type and number are comparable for the stabilized and nonstabilized samples, even when using 1 x 10^5 cells (Table 5). The RNA integrity of the samples is illustrated by sharp peaks in the electropherograms from an Agilent Bioanalyzer (Figure 1). ▷

Table 5. Quality assessment of total RNA isolated cancer cells stabilized with RNAprotect Tissue Reagent or not stabilized

Cell line	Sample	Cell number	Mean yield (µg)	Mean concentration (ng/µl)	Mean ratio OD 260/280	Mean RIN
MCF7	RNAprotect Tissue Reagent	10^6	11.9	848.4	1.98	9.6
		10^5	1.5	110.0	2.04	7.9
	Control	10^6	9.5	678.3	2.02	9.3
		10^5	1.2	84.2	2.03	8.7
HeLa	RNAprotect Tissue Reagent	10^6	14.7	1050.5	1.97	9.5
		10^5	0.5	33.9	1.95	8.1
	Control	10^6	14.9	1066.0	1.96	9.6
		10^5	1.3	90.2	2.00	7.8
Ramos	RNAprotect Tissue Reagent	10^6	3.7	263.7	2.07	9.1
		10^5	0.4	28.4	1.96	7.6
	Control	10^6	3.1	224.2	2.10	8.0
		10^5	0.4	28.5	2.12	7.7
Jurkat	RNAprotect Tissue Reagent	10^6	7.1	503.9	2.00	9.2
		10^5	0.6	41.3	2.09	8.7
	Control	10^6	7.5	537.4	1.95	9.4
		10^5	0.9	62.2	2.04	7.7

The RIN values for samples prepared from 1 x 10^6 cells averaged 9.2, which was slightly higher than values obtained with 1 x 10^5 cells (average RIN of 8.0). The RIN values between the corresponding RNAprotect Tissue Reagent stabilized and nonstabilized samples were highly comparable, indicating that the RNA integrity was not adversely affected by incubation with RNAprotect Tissue Reagent. Even with samples processed from the lower cell number, RNA of sufficient quality for downstream analyses was obtained.

The overall quality was assessed using the RT^2 RNA QC PCR Array (Figure 2). The mean C_T values derived from either RNAprotect Tissue Reagent stabilized or nonstabilized cells show no significant differences independent of the starting amount of cells or due to the nature of the different cancer cell type.

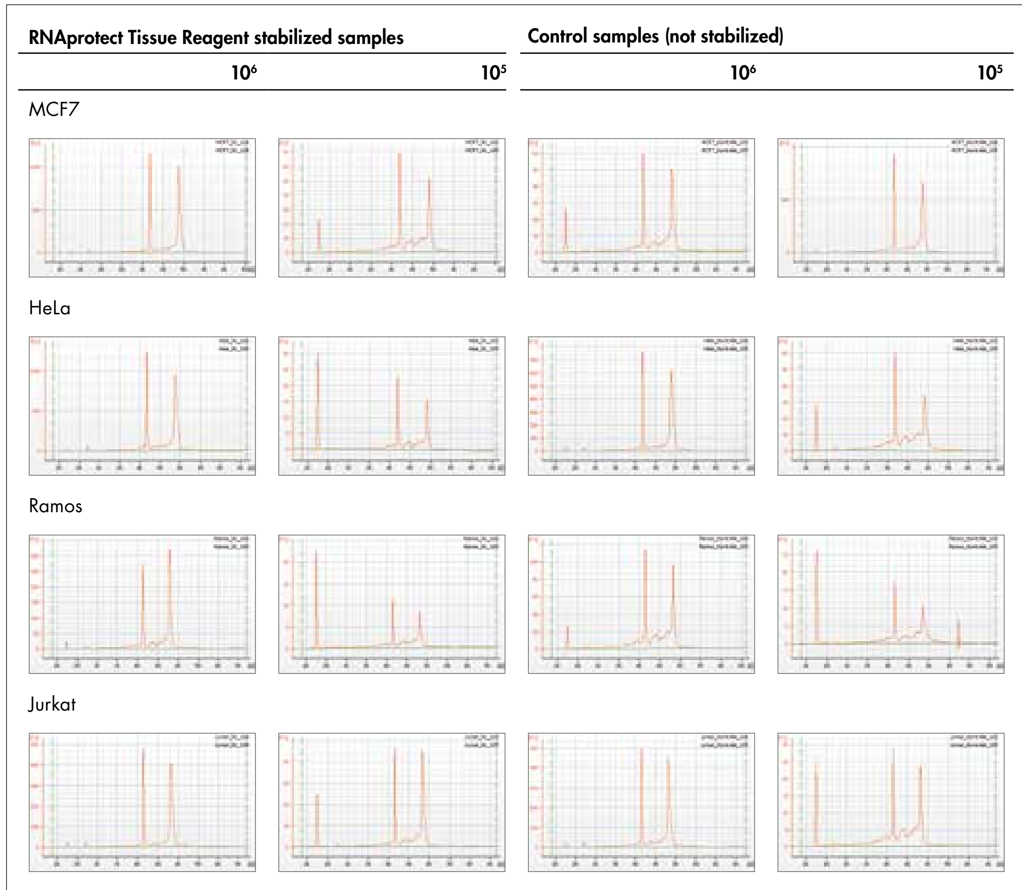

Figure 1. Electropherograms for high-quality RNA from four cancer cell lines. RNA was isolated from RNAprotect Tissue Reagent stabilized and nonstabilized samples from different cancer cell lines. Analysis of 1 µl samples of each eluate on an Agilent Bioanalyzer revealed high-quality RNA from all four cancer cell lines.

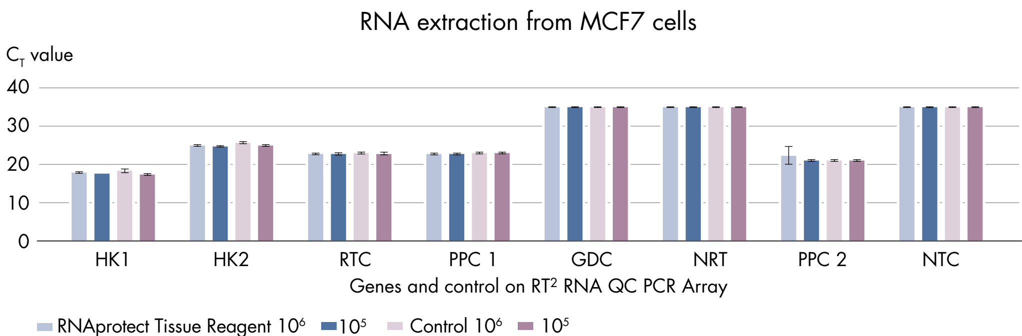

Figure 2. Overall RNA quality of RNAprotect stabilized and nonstabilized cells were comparable. The RNA analyses were performed using the RT^2 RNA QC PCR Array. This shows the mean C_T values for the RNA eluates isolated from either RNAprotect Tissue Reagent stabilized or nonstabilized cells with a starting amount of 1×10^6 or 1×10^5 cells. The mean C_T values for stabilized or nonstabilized samples are comparable with no significant differences. The levels for the ACTB (HK1) and HPRT1 (HK2) housekeeping genes do not vary significantly between samples. There is no indication of inhibition of reverse transcription (NRT) or PCR (PPC1) or contamination with genomic DNA (GDC). The cutoff values for PPC1, GDC, NRT, and NTC are $\geq C_T$ 35. ▷

Conclusion

The optimal conditions for incubation of cultured cells in RNAprotect Tissue Reagent and subsequent removal of the stabilization reagent were established. Incubation at 22°C applied to all cell lines studied. Centrifugation at 5000 *x g* or 2500 *x g* for 5 or 10 minutes was optimal depending on the cell type. The conditions meet the criteria for a generic applicable procedure for the recovery of cultured cells from RNAprotect Tissue Reagent. High-quality RNA could be isolated from such stabilized cells without any loss in RNA yield or integrity compared to RNA isolated from cells snap frozen directly after culture and harvesting. RNAprotect technology allows large numbers of samples to be easily processed and replaces inconvenient, dangerous and equipment-intensive methods, such as snap-freezing of samples in liquid nitrogen, storage at –90 to –65°C, cutting and weighing on dry ice or immediate processing of harvested samples.

Ordering Information

Product	Contents	Cat. no.
RNAprotect Tissue Reagent (50 ml)	50 ml RNAprotect Tissue Reagent for stabilization of RNA in 25 x 200 mg tissue samples	76104
RNAprotect Tissue Reagent (250 ml)	250 ml RNAprotect Tissue Reagent for stabilization of RNA in 125 x 200 mg tissue samples	76106
RNeasy Mini Kit (50)	50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	74104
RT^2 RNA QC PCR Array	RT^2 Profiler QC Array	Varies

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services or your local distributor.

For more information on best practices for RNA isolation, visit **www.qiagen.com/RNA**.

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