Analytical Biochemistry 513 (2016) 21–27

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Endogenous RNase inhibitor contributes to stability of RNA in crude cell lysates: Applicability to RT-qPCR

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ARTICLE INFO

Article history:
Received 6 July 2016
Received in revised form
10 August 2016
Accepted 15 August 2016
Available online 18 August 2016

Keywords:
Cell lysate
RNA
RNase
RNase inhibitor
Reverse transcription quantitative PCR
Virus

ABSTRACT

Crude cell lysates are increasingly used as input for direct analysis by reverse transcription quantitative PCR (RT-qPCR), particularly for high-throughput applications. We previously demonstrated that a simple buffer containing a non-ionic detergent can serve as an inexpensive alternative to commercial cell-lysis reagents for the preparation of RT-qPCR-ready cell lysates; addition of an exogenous RNase inhibitor (RI) to the lysis buffer was found to be unnecessary to maintain RNA stability in cell lysates either freshly prepared or previously stored frozen at −80 °C. In the present study, we have demonstrated that the stability of RNA observed in our cell lysates is due to the presence of the endogenous RI. Furthermore, we have established the generalizability and applicability of this phenomenon by evaluating lysates prepared from cell lines commonly used in virology (A549, HeLa, MDCK, and Vero). Awareness of the mechanism underlying RNA stability may engender greater confidence in generating cell lysates for RT-qPCR without relying on addition of exogenous RI (a substantial cost-saving benefit) and encourage appropriate practices for handling and storage of samples.

1. Introduction

Crude cell lysates are increasingly used as input for reverse transcription quantitative PCR (RT-qPCR). Several commercial cell-lysis reagents for this purpose are available (such as Ambion Cells-to-C_T and Bio-Rad iScript Sample Preparation Reagent). Ease of use contributes to the appeal of these reagents, particularly for high-throughput applications in which conventional nucleic acid extraction/purification from samples would be cumbersome and rate-limiting. Recent studies suggest that crude cell lysates may offer comparable accuracy [1,2] and superior sensitivity [1] for RT-qPCR compared with purified RNA.

In order to mitigate the cost constraint imposed by commercial reagents, we developed a simple cell-lysis buffer (CL Buffer) containing a non-ionic detergent (0.25% Igepal CA-630) as an alternative and demonstrated its feasibility for generating RT-qPCR-ready cell lysates [3]. Experimental samples from adherent cells are prepared in straightforward steps by (1) washing cells, (2) briefly exposing cells to CL Buffer at room temperature, and (3) collecting the resulting cell lysates for frozen storage or immediate analysis by RT-qPCR. We found that addition of commercial RNase inhibitor (RI) to CL Buffer was not necessary to maintain RNA integrity in cell lysates either freshly prepared or previously stored frozen at −80 °C. Avoiding the requirement to add commercial RI would be a notable cost-saving benefit if it were routinely possible.

In the present study, we have established that the maintenance of RNA integrity in our cell lysates is due to the presence of endogenous RI activity. We have verified the generalizability of this phenomenon by evaluating lysates prepared from four cell lines commonly used in virology (A549, HeLa, MDCK, and Vero). In addition, we have successfully demonstrated applicability to RT-qPCR, in a manner similar to our previous studies [4,5], using lysates prepared from cells infected with influenza virus or respiratory syncytial virus (RSV). Mechanistic insights might foster confidence in data quality derived from this experimental approach and encourage appropriate procedures for handling of cell-lysate samples.

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http://dx.doi.org/10.1016/j.ab.2016.08.011

2. Materials and methods

2.1. Cells and viruses

MDCK cells (London line) [6] were obtained from J. Weir (Division of Viral Products, OVRR, CBER, FDA). A549, HeLa, and Vero cells were obtained from the American Type Culture Collection. All cells were propagated using Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (Hyclone) and 2 mM glutamine. Influenza virus A/PR/8/34 and RSV B1 were obtained from Z. Ye and J. Beeler, respectively (Division of Viral Products, OVRR, CBER, FDA). Infectivity was determined by calculating the 50% tissue culture infective dose ($TCID_{50}$) by titration using MDCK cells (for influenza virus) and Vero cells (for RSV).

2.2. Preparation of cell lysates

Cells were seeded in 10-cm culture dishes (4×10^6 cells per dish). On the following day, cells were washed twice with phosphate-buffered saline (PBS) and then exposed to CL Buffer (6 mL per dish; 10 mM Tris-HCl pH 7.4, 0.25% Igepal CA-630, 150 mM NaCl) with or without 1 mM dithiothreitol (DTT; Thermo Scientific; 20291) for 5 min at room temperature (~22 °C). The resulting lysates were collected and used immediately or stored frozen at the temperature indicated for the experiment. Cell-exposure time to CL Buffer was shortened to 2 min for some experiments to prevent non-adherence occasionally observed for one of the cell types (Vero). The soluble fraction was obtained either by (1) recovering the supernatant (UC supernatant) after subjecting the cell lysate to ultracentrifugation (208,000 × g) for 4 h at 4 °C using an SW41 rotor (modification of a protocol described by Rio et al. [7]), or (2) recovering the filtrate (Amicon filtrate) after passing the cell lysate through an Amicon Ultra filtration device (EMD Millipore; molecular weight cutoff of 100,000) by centrifugation (4000 × g) for 30 min at 4 °C. For certain experiments involving lysates, the following additions were used: RNasin Plus (Promega; final concentration of 1 unit/μL), a monoclonal antibody (Ab) specific to mammalian RI (reactive across mammalian species including dog and human; clone 3F11; TA501875; Origene; 1 μg per 200 μL lysate), and a monoclonal Ab specific to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; clone 2D9; TA802519; Origene; 1 μg per 200 μL lysate).

2.3. Western blot analysis

Cell lysates were prepared using a non-ionic detergent lysis buffer (50 mM Tris-HCl pH 8.0, 1% Igepal CA-630, and 150 mM NaCl) supplemented with a protease inhibitor cocktail (P8340; Sigma; 1:100 dilution). Protein concentration was determined using the Pierce BCA kit (Thermo Fisher Scientific).

Cell lysates (12 μg per lane) were applied to NuPAGE Bis-Tris 4–12% gels (Life Technologies), and electrophoresis was performed under reducing conditions using MOPS SDS Running Buffer (Life Technologies). Proteins were transferred to PVDF membranes. After blocking with 5% non-fat milk, PVDF membranes were incubated with primary Ab against mammalian RI (TA501875; Origene; 1:500) or β-actin (3700; Cell Signaling Technology; 1:1000). Following incubation with an HRP-conjugated secondary Ab against mouse IgG, protein bands were visualized by chemiluminescence using the Amersham ECL system (GE Healthcare Life Sciences).

2.4. Microfluidics-based analysis of RNA

Total RNA was purified from cell lysates (~200 μL) using the RNeasy Mini kit (Qiagen) according to the "cleanup" protocol supplied with the kit. RNA was eluted in 30 μL of nuclease-free water and stored frozen at −80 °C until assessment. Samples (1 μL) were subjected to microfluidics-based Experion RNA StdSens electrophoresis (Bio-Rad). RNA Quality Indicator (RQI) values were calculated in an automated and unbiased manner (by an algorithm described in Bio-Rad Technical Note 5761) using the Experion software version 3.2. According to the default Experion setting, RQIs between 7.0 and 10.0 indicate RNA of acceptable quality for most downstream applications.

2.5. RT-qPCR analysis

A549, HeLa, MDCK, and Vero cells were seeded in 96-well culture plates (15,000 cells per well). On the following day, cells were infected with a dilution series (3-fold) of either influenza virus strain A/PR/8/34 or RSV strain B1 ($TCID_{50}$ per well ranging from 100 to 8100). Infected cells (at 6 h post-infection for influenza virus and 24 h post-infection for RSV) were washed twice with PBS (100 μL per well) and then exposed to CL Buffer containing 1 mM DTT (100 μL per well) for 2 min at room temperature (~22 °C). The resulting lysates were subjected to RT-qPCR analysis.

RT-qPCR experimental design was facilitated by awareness of the MIQE guidelines [8]. RT-qPCR was performed as described in earlier studies [4,5]. For RT-qPCR targeting the influenza virus A matrix-gene transcript, the following PCR primers were used: AAGACCAATCCTGTCACCTCTGA and CAAAGCGTCTACGCTGCAGTCC. Each reaction for influenza virus contained: 1 μL of cell lysate, 1X iScript One-Step SYBR Green RT-PCR Supermix (170–8893; Bio-Rad), 600 nM of each primer (synthesized at the Facility for Biotechnology Resources; CBER, FDA; Silver Spring, MD), 0.2 μL iScript reverse transcriptase, and nuclease-free water to 10 μL. A CFX96 real-time PCR instrument (Bio-Rad) was used with the following protocol: 50 °C for 10 min (1X), 95 °C for 5 min (1X), 95 °C for 10 s/61 °C for 15 s/72 °C for 30 s (40X; data collection occurred after the 72 °C step). For RT-qPCR targeting the RSV N gene transcript (subgroup B), the following PCR primers were used: CTGTCATCCAGCAAATACACTATTCA and GCACATCATAATTGGGAGTGTCA. Each reaction for RSV contained: 1 μL of cell lysate, 1X iScript One-Step SYBR Green RT-PCR Supermix, 300 nM of each primer, 0.2 μL iScript reverse transcriptase, and nuclease-free water to 10 μL. The following thermocycling protocol was used: 50 °C for 10 min (1X), 95 °C for 5 min (1X), 95 °C for 10 s/64 °C for 30 s (40X; data collection occurred after the 64 °C step). Total RNA standards purified from cells infected with influenza virus or RSV were used as described previously [4,5]. For each RT-qPCR run, a 10-fold dilution series of RNA standard (using a cell lysate prepared from uninfected cells as the diluent) was assessed to verify RT-qPCR performance and to facilitate quantification. In addition, each RT-qPCR run included negative controls (lysate from uninfected cells as input) and no-reverse transcription controls (initial dilution of RNA standard described above as input); these controls typically result in no amplification, or occasionally, low-level non-specific amplifications (indicated by melt-curve analysis) with quantification cycle (C_q) values > 36.

3. Results

3.1. Impact of DTT on RNA stability of cell lysates following frozen storage at −20 °C and stress at 37 °C

We previously demonstrated the feasibility of using our CL Buffer (10 mM Tris-HCl pH 7.4, 0.25% Igepal CA-630, and 150 mM NaCl) for generating RT-qPCR-ready cell lysates using MDCK cells [3]. We hypothesized that the stability of RNA observed in cell