Journal of Virological Methods 168 (2010) 207–211

Contents lists available at ScienceDirect

Journal of Virological Methods

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

Protocols

Long-term conservation of HCV RNA at 4 °C using a new RNA stabilizing solution

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Article history:
Received 22 September 2009
Received in revised form 13 May 2010
Accepted 20 May 2010
Available online 1 June 2010

Keywords:
RNA/DNA conservation
Long-term RNA stability
HCV RNA quantitation
HCV viral load
Competitive RT-PCR

A B S T R A C T

Protecting RNA from degradation, whilst maintaining its biological activity, is essential in molecular biology. However, RNA is very sensitive to degradation by ribonucleases, especially at temperatures above 0 °C. The stability of RNA was examined at 4 °C and −20 °C, in a new stabilizing solution consisting of a low-molarity mixture of chaotropic agents guanidinium and ammonium thiocyanate, a buffer for pH stabilization, phenol, and yeast RNA. Two substrates were tested for storage: RNA in human plasma positive for hepatitis C virus (HCV) and naked RNA (purified from HCV positive human plasma or transcribed in vitro). Stability was followed by viral load estimation, using an in-house competitive RT-PCR assay. Naked RNA purified from human plasma positive for HCV was stable at 4 °C for at least 24 months. An RNA standard transcribed in vitro was still viable after 36 months of storage at 4 °C. Human plasma dilutions positive for HCV were stable for at least 5 months in this solution when stored at 4 °C. It was concluded that the described stabilizing solution ensures long-term stability on naked RNA at 4 °C, and ideal for the storage of RNA controls and standards for molecular diagnosis, the solution may be used for preserving clinical samples prior to transport to a clinical laboratory.

1. Introduction

Most target and signal amplification assays available for molecular detection of mRNA demand well preserved RNA standards for controlling the variability of each step and reaction (Espy et al., 2006; Mulder et al., 1994; Niesters, 2002; Compton, 1991; Van Gemen et al., 1994; Pachl et al., 1995). Partially degraded standards can generate inaccurate values, however ribonucleases (RNases) are ubiquitous and even slight contamination will lead to RNA degradation.

The alternatives proposed for RNA protection (Allen et al., 1996; Heidenreich et al., 1993; Jones, 1953, 1963; Köhler et al., 1997; Rodriguez et al., 2001; Vemuri, 1995; Wiegand et al., 1975), and conservation (Kravchenko et al., 2006; Pasloske et al., 2006; Stefan et al., 2003; Vincek et al., 2003), do not guarantee its long-term stability in the presence of RNases or temperatures above 0 °C. The simplest and most traditional solution for RNA conservation has been freezing. Temperatures under −70 °C, preceded by snap freezing in liquid nitrogen, are most often used to prevent the formation of ice crystals, which can also provoke RNA damage (Mook et al., 2009). However, this method is not feasible for storing RNA for diagnostic purposes. It increases the cost of specimen storage, and repeat freeze–thaw cycles will affect the integrity of the RNA (Botling et al., 2009; Watson et al., 2007). RNA preservation at 4 °C could be easier and more cost effective for clinical laboratories than freezing.

This paper describes a simple and effective solution for long-term storage of RNA at 4 °C, which guarantees its preservation with the integrity required by amplification techniques.

2. Materials and methods

2.1. RNA stabilizing solution

The solution is made up of 0.3–0.6 mol/L ammonium thiocyanate (BDH, Poole, England); 0.6–1.0 mol/L guanidinium thiocyanate (Sigma, Saint Louis, USA); 35–40% (v/v) phenol (Merck, Darmstadt, Germany); 5–8% (v/v) glycerol (Sigma, Saint Louis, USA); 0.1–0.3 mol/L sodium acetate (Sigma, Saint Louis, USA), pH 4–5 adjusted with glacial acetic acid (Merck, Darmstadt, Germany); and 0.05–0.1 mg/mL RNA from yeast (BDH, Poole, England). Water was treated with diethylpyrocarbonate (DEPC) from Sigma, Saint Louis, USA and then sterilized for use. All reagents were of molecular biology grade.

2.2. RNA isolation from stabilized mixtures

The RNA was isolated according to a modification of the Chomczynski method (Gonzalez-Perez et al., 2007; Chomczynski, 1993).

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doi:10.1016/j.jviromet.2010.05.015

The stabilizing solution with the internal control (IC) included in its working dilution (Gonzalez-Perez et al., 2009) was used as a lysis reagent at a volume of 500 μL per each 150 μL of sample. The published protocol C (Gonzalez-Perez et al., 2007) was adapted for quantitative purposes by replacing the 10 min room temperature incubation in chloroform with 30 min at −20 °C. For naked RNA, the matrix was simulated by adding 150 μL of cryosupernatant, negative for HCV, HBV and HIV to the isolation mixture.

2.3. Competitive quantitative RT-PCR

An "in-house" competitive RT-PCR assay, using primers from the HCV 5′UTR, was used for quantitation (Gonzalez-Perez et al., 2005). The external quantitation curve, ranging from 850 IU/mL to 850,000 IU/mL, was made either from an HCV transcript (Gonzalez-Perez et al., 2009) or from HCV positive human plasma. Both were calibrated against the WHO HCV International Standard 96/790. The IC transcript, with primer binding regions identical to those of the HCV target sequence, was also amplified to control tube-to-tube variations in RT-PCR reactions. Hybridization of HCV and IC amplicons was carried out using specific probes (Gonzalez-Perez et al., 2005) and UMELOSA format standardized for quantitation (Gonzalez-Perez et al., 2003).

2.4. Data analysis: viral load (VL) estimation and RNA stability assessment

Results were normalized by calculating the ratios (R), from HCV and IC fluorescent signals. The external calibration curve was built by weighted linear regression (Montgomery, 1991), from the common logarithms of theoretical viral loads $\mathrm{Log}_{10}\,\mathrm{VL_t} - x$ values, and calculated ratios $\mathrm{Log}_{10}\,R - y$ values.

RNA stability was inferred by competitive quantitative RT-PCR (see Section 2.3), from viral load estimation of a small 5′UTR sequence of the HCV genome, by calculating Log D values i.e. the differences between the common logarithms of estimated mean viral loads and the reference viral loads (theoretical or previously estimated). Log D values within an interval of ±0.5, corresponding to the variability of the used competitive RT-PCR (reproducibility data not shown), were considered not significant from the analytical point of view, and the samples with these values classified as stable. This ±0.5 Log_{10} variation is also considered not relevant in the clinical practice (Shiffman et al., 2003; Pawlotsky, 2003).

2.5. Conservation of RNA contained in HCV positive human plasma

HCV positive human plasma was diluted in negative cryosupernatant to produce three different HCV RNA concentrations in 150 μL aliquots. These samples were mixed in polyethylene tubes with 500 μL of the stabilizing solution, already containing the IC at its competitive dilution. The mixtures were mixed gently and stored at 4 °C for 3–5 months.

RNA stability was assessed by the analysis of Log D values, calculated at 3 and 5 months of storage with respect to the reference theoretical viral loads.

2.6. Conservation of naked RNA purified from HCV positive human plasma

RNA was isolated from six 150 μL aliquots of HCV positive human plasma, and resuspended in 20 μL of DEPC treated water. The RNA aliquots were mixed, homogenized, and quantified by the competitive RT-PCR described in Section 2.3, resulting in a concentration of 1.36×10^6 IU/mL. An RNA volume of 80 μL was diluted in 24 mL of the stabilizing solution. The mixture was gently homogenized, divided into 3 mL aliquots, and added to sterile DEPC treated amber glass flasks. The flasks were stored at either 4 °C for stability evaluation, or at −20 °C as a reference for the stability assessment. Each aliquot was tested in triplicate. RNA stability was estimated at both temperatures, by analyzing the Log D values calculated at 6 and 24 months with respect to the reference Log_{10} VL values at 3 months.

2.7. Conservation of a naked RNA standard (IC) synthesized by in vitro transcription

The IC transcript was diluted in stabilizing solution, to a final concentration which was 100 times higher than its dilution of competition. The mixture was homogenized, divided into 500 μL aliquots, and stored in sterile polyethylene tubes at 4 °C. The stability of the IC was checked at least once a week by testing its activity by the competitive RT-PCR assay described previously. For this purpose, positive controls, made from dilutions of HCV positive human plasma in negative cryosupernatant were quantified. The corresponding Log D values, calculated with respect to the theoretical VLs, were taken as a measure of the quantitation accuracy achieved. This was related directly to the stability, physical integrity, and good performance of the RNA standards employed.

3. Results

3.1. Conservation of RNA contained in HCV positive human plasma

Dilutions of human plasma positive for HCV were stable in the stabilizing solution at 4 °C for at least 5 months (Table 1). Their Log D values at 3 and 5 months were within the interval of variability allowed and were not considered significant.

3.2. Conservation of naked RNA purified from HCV positive human plasma

Naked RNA purified from HCV positive human plasma was stable for at least 24 months, when stored as described in the stabilizing solution at 4 °C (Table 2). At this temperature the averaged values of Log D, calculated from the corresponding modular values, were not significant and were equal to 0.16, at 6 and 24 months. However, for samples stored at −20 °C the results were more heterogeneous. The corresponding averaged values of Log D, similarly calculated, were 0.61 and 0.14, at 6 and 24 months, respectively.

Table 1
Stability of dilutions of HCV positive human plasma in stabilizing solution stored at 4 °C.

$\mathrm{VL_t}$ (IU/mL)	$\mathrm{VL_m}$ (IU/mL)	Log D
VL estimations at 3 months		
8.50×10^4	1.69×10^5	0.30
8.50×10^5	7.99×10^5	−0.03
VL estimations at 5 months		
8.50×10^2	4.97×10^2	−0.23
8.50×10^4	5.86×10^4	−0.16
8.50×10^5	8.05×10^5	−0.02

VL: viral load, $\mathrm{VL_t}$: theoretical viral load, $\mathrm{VL_m}$: mean VL from two estimates of the same replicate, Log D: difference between the common logarithms of $\mathrm{VL_m}$ and $\mathrm{VL_t}$.

Table 2
Stability of naked RNA purified from human plasma positive for HCV, in stabilizing solution at 4 °C and −20 °C.

T	VL estimations at 3 months			VL estimations at 6 months			VL estimations at 24 months		
	Replicate's[a] VL_m (IU/mL)	VL (IU/mL)	Reference Log_{10} VL	Replicate's[a] VL_m (IU/mL)	VL (IU/mL)	Log *D*[b] 6/3 months	Replicate's[a] VL_m (IU/mL)	VL (IU/mL)	Log *D*[b] 24/3 months
4 °C	2.67×10^4	2.91×10^4	**4.46**	2.39×10^4	2.09×10^4	−0.09	3.67×10^4	3.75×10^4	0.10
	1.61×10^4			2.45×10^4		−0.07	3.83×10^4		0.12
	4.45×10^4			1.42×10^4		−0.31	5.16×10^4		0.25
−20 °C	1.13×10^5	8.68×10^4	**4.94**	2.62×10^4	2.16×10^4	*−0.52*	6.66×10^4	9.46×10^4	−0.11
	1.18×10^5			2.16×10^4		*−0.60*	1.23×10^5		0.15
	2.97×10^4			1.70×10^4		*−0.71*	1.24×10^5		0.15

T: storage temperature, VL_m: mean VL from two estimates of the same replicate, VL: averages of VL_m values.
[a] Replicates correspond to different RNA isolations, amplifications and fluorimetric detections from the same RNA aliquot.
[b] The difference in common logarithms of VL_m values at 6 and 24 months, with respect to the corresponding reference Log_{10} VL at 3 months (in bold).

3.3. Conservation of a naked RNA standard (IC) synthesized by in vitro transcription

Table 3 shows data of an IC test for stability assessment. The positive controls for this assay were quantified using an IC preserved in the stabilizing solution, at 4 °C for 21 months. The parameters of the calibration curve were: coefficient of determination $R^2 = 0.9999$, slope $S = 0.64$, and intercept $a = -3.03$. Both positive controls were accurately quantified. Their Log *D* values were also not significant (see Table 3).

When stored as described the IC transcript was stable for at least 36 months (data not shown).

4. Discussion

4.1. Conservation of RNA contained in HCV positive human plasma

The 5-month stability achieved for plasma in the new stabilizing solution at 4 °C, indicates that this mixture would be useful for storing clinical samples until they can be transported to laboratories for testing. This would be particularly useful considering that the proposed solution is compatible with a variety of RNA isolation procedures: phenolic extraction (Sambrook et al., 1989), one step RNA/DNA isolation (Chomczynski, 1993), and silica isolation (Boom et al., 1990; Gonzalez-Perez et al., 2007).

4.2. Conservation of naked RNA purified from HCV positive human plasma

After 6 months of storage at −20 °C, the Log *D* values for the three replicates tested had decreased outside of the allowed range (see values in bold and italics in Table 2). This suggests that although all aliquots were kept under "identical conditions", they probably froze unevenly and/or thawed unevenly before testing, causing variation in their integrity. These results agree with a report by Avison (2006) that RNA is less stable after defrosting than if the sample had not been frozen at all.

These results, together with the advantages of storage at 4 °C discussed in Section 4.4, support the conclusion that this temperature is more appropriate for RNA storage in the proposed buffer than at −20 °C.

4.3. Conservation of a naked RNA standard (IC) synthesized by in vitro transcription

The accuracy achieved when quantifying these positive controls shows that the IC is stable and performs well when stored in the stabilizing buffer at 4 °C. This result agrees with those seen for naked RNA purified from HCV positive human plasma, and leads us to conclude that the described stabilizing solution confers long-term

Table 3
Quantitation of positive controls for HCV, employing an IC preserved in stabilizing solution at 4 °C for 21 months.

VL estimations, IC 21 months			
VL_t (IU/mL)	VL_e (IU/mL)	VL_m (IU/mL)	Log *D*
Positive controls for HCV			
8.50×10^3	1.47×10^4 1.52×10^4	1.50×10^4	0.25
8.50×10^4	6.64×10^4 5.36×10^4	6.00×10^4	−0.15

IC: internal control, VL: viral load, VL_t: theoretical viral load, VL_e: VL estimates from different fluorimetric detections of the same replicate, Log *D*: difference between the common logarithms of VL_m and VL_t.

stability on naked RNA at 4 °C. The 36-month stability, achieved for the IC at 4 °C, exceeds those reported for Armored packed RNA at this temperature namely 11 months (Pasloske, 1999), or 6 months when the particles are stored in human plasma (Pasloske et al., 1998), or even as low as 38 days (Pasloske et al., 2006). The achieved resistance to RNases, makes the proposed stabilizing solution ideal for storing standards and controls of viral diagnostic assays with compatible RNA isolation procedures at 4 °C.

4.4. Overall discussion

Although RNA protection in lysates of guanidinium isothiocyanate has been previously reported, those studies used storage temperatures of −20 °C and −80 °C (Gilleland and Hockett, 1998). Guanidinium thiocyanate, another chaotropic agent frequently used to inhibit RNases (Birnboim, 2003; Chirgwin et al., 1979; Chomczynski and Sacchi, 1987; Sambrook et al., 1989), has been also used for RNA stabilization, but similarly, at temperatures below zero and at high molarities (4–6 mol/L).

In this study however, it was found that RNA, naked-purified or contained in its natural source, is stable at 4 °C in a low-molarity mixture of chaotropic agents guanidinium and ammonium thiocyanate, containing a buffer for pH stabilization at 4–6, phenol, and yeast RNA.

The demonstrated stability of RNA in the proposed solution may be explained by the presence of yeast RNA, as indicated by previous reports that nucleic acids are protected from nuclease degradation by interacting directly with RNA (Köhler et al., 1997; Wiegand et al., 1975). The long-term stability achieved for naked RNA in the proposed stabilizing solution at 4 °C is novel and has not been reported previously for any other combination of reagents.

Conservation of RNA at 4 °C is very appropriate and advantageous for molecular diagnosis. It is cheaper than freezing, and enables the reuse of the same RNA aliquot, avoiding the fragmentation of the material into smaller volumes, which is laborious, time-consuming, and expensive when dealing with large amounts of material. The possibility of reuse should also be considered an advantage with respect to some commercial assays, whose RNA standards have to be discarded after use to avoid contamination with RNases.

Although the examples shown are only related to RNA conservation, the proposed mixture could also be used to stabilize DNA for long-term storage.

Conflicts of interest

We state that there are not conflicts of interest. We do not have any financial or personal relationships with other people which could inappropriately influence this work.

Acknowledgements

The authors are very grateful to Dr. Collette Bromhead (Head of Department, Molecular Biology, Wellington, New Zealand) for her critical review of the English manuscript and her helpful suggestions and comments on the work. The authors also thank Professor Senia Bermúdez Velázquez from Centro de InmunoEnsayo, Cuba, for revising the English manuscript.

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