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Protocols

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

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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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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 $\mathrm{VL_t}$.