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Reduction of DNA Contamination in RNA Samples for RT-PCR using Selective Precipitation by Compaction Agents

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Abstract

An important problem in measurement of mRNA levels by RT-PCR is DNA contamination, which can produce artifactually increased mRNA concentration. Current methods to eliminate contaminating DNA can compromise the integrity of the RNA, are time-consuming, or are hazardous. We present a rapid, nuclease-free, and cost-effective method of eliminating contaminating DNA in RNA samples using selective precipitation by compaction agents. Compaction agents are cationic molecules which bind to double-stranded nucleic acids, driven by electrostatic interactions and steric complimentarity. The effectiveness and DNA-selectivity of six compaction agents were investigated: trivalent spermidine, Triquat A, and Triquat 7; tetravalent spermine and Quatro-quat; and hexavalent Quatro-diquat. Effectiveness was measured initially by supernatant UV absorbance after precipitation of salmon sperm DNA. Effectiveness and selectivity were then investigated using differences in RT-PCR C_t values with synthetic mixtures of human genomic DNA and total RNA, and with total RNA isolated from cells. With 500 μM of spermidine or Triquat A, the supernatant DNA could not be detected up to 40 cycles of PCR ($C_t \geq 12.6$) while the C_t for the mRNA was increased by only 5 cycles. Therefore, spermidine and Triquat A each show strong DNA-selectivity and could be used to eliminate contaminating DNA in measurements of mRNA.

Keywords

Compaction agents; Genomic DNA contamination; RT-PCR

INTRODUCTION

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) has become a preferred method of detecting and quantifying mRNA expression due to its speed and sensitivity. One

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of the most important problems in RT-PCR is genomic DNA (gDNA) contamination in mRNA samples isolated from cell cultures and clinical specimens [1-4]. The PCR reaction cannot distinguish between the complementary DNA (cDNA) synthesized by reverse transcription, and contaminating gDNA of the same sequence, resulting in an overestimation of the amount of RNA present. The problem can sometimes be avoided by constraining primers to span intron boundaries, but this is often difficult and methods currently used to reduce DNA contamination include DNAse I digestion, gel fractionation, acid phenol:chloroform extraction, and lithium chloride precipitation.

DNAse I digestion is the method most commonly used to eliminate contaminating DNA, however, the enzyme must be deactivated or removed before the RT-PCR reaction or it may digest the newly formed cDNA [5]. Agarose (with formaldehyde) and polyacrylamide (with urea) gel electrophoresis can be used to achieve good separation between gDNA and mRNA. However, the recovery of RNA from the gels is difficult and traces of formaldehyde can inhibit reverse transcriptase. In addition, small amounts of DNA were still present in samples purified from polyacrylamide gels [1].

Acid phenol:chloroform extraction partitions the DNA into the organic phase. The RNA remains in the aqueous phase and can then be recovered by alcohol precipitation. However, this method is time consuming and is susceptible to sample loss, in addition to employing hazardous phenol. Lithium chloride is a selective precipitant of RNA with DNA being left in the supernatant, but is insufficiently effective when the level of DNA contamination is significant. In this work we present the development of a rapid, non-toxic, nuclease-free, and cost-effective method of removing contaminating DNA from RNA samples using selective precipitation by compaction agents. Compaction agents are small cationic molecules which bind in either the major or minor grooves of double stranded nucleic acid molecules, driven predominantly by electrostatic interactions and steric complimentarity [6-10]. This interaction can reduce the volume occupied by the nucleic acid by four to six orders of magnitude. *In vivo*, these agents help package DNA into cells, but can also be used to package or compact nucleic acids in many applications – including drug delivery [6, 11], enhancement of chromatographic adsorption, and recently, plasmid and RNA purification [12, 13]. Synthetic forms of compaction agents are inexpensive, non-toxic, and pose no risk of viral contamination.

Compaction agents have been shown to differ in strength and in selectivity for RNA or DNA [13]. In this study, the effectiveness and DNA-selectivity of six compaction agents was investigated. These were spermidine, Triquat A, and Triquat 7 with three positive charges; spermine and Quatro-quat with four; and Quatro-diquat with six. Effectiveness was initially measured by analyzing DNA remaining in the supernatant after precipitation of salmon sperm genomic DNA. Next, the effectiveness and DNA-selectivity of the agents was tested in eliminating contaminating DNA in synthetic mixtures of purified human gDNA and total RNA, and in RNA isolated from HepG2 human liver cells.

MATERIALS AND METHODS

Compaction agents and nucleic acids

Triquat A, Triquat 7, Quatro-quat, and Quatro-diquat were the generous gifts of Sachem, Inc. (Austin, TX). Spermidine, spermine, and salmon sperm genomic DNA were purchased from Sigma (St. Louis, MO). Human genomic DNA (pooled from multiple anonymous donors) was purchased from Promega, Inc. (Madison, WI) and human total RNA (DNA free, pooled from 10 cell lines) was from Stratagene, Inc (La Jolla, CA). Frozen HepG2 human liver cells were the generous gift of Jeremiah Whittenton of the University of Houston. Figure 1 shows the structures of the compaction agents tested.

Salmon sperm genomic DNA precipitation

Precipitation reactions were conducted in triplicate in 1.5 ml Axygen (Union City, CA) microfuge tubes with a total reaction volume of 500 uL at a salmon sperm genomic DNA concentration of 42.5 ± 0.4 ug/mL. The concentration of the compaction agents was varied between 0 and 200 mM. The NaCl concentrations investigated were 0, 50, and 100 mM. In addition, NaCl concentrations of 250 and 500 mM were investigated when using the very potent Quatro-diquat. DNA stock solutions were made in TE buffer (10 mM Tris and 1 mM ETDA, pH 8.0), while NaCl and compaction agent stock solutions were made in 10 mM Tris, pH 8.0.

Tris buffer was added first, then the NaCl stock, followed by the DNA and the compaction agent. The tube was vortexed for 5 seconds on a Scientific Industries Vortex-Genie 2 (power setting: 8) and allowed to precipitate at room temperature for 30 minutes. The tubes were then centrifuged in an Eppendorf 5145 microcentrifuge at 14,000 rpm (13,500 × *g*) for 20 minutes and the supernatant DNA concentration was measured by 260 nm absorbance in a Beckman DU 7500 spectrometer.

PCR-inhibition testing of compaction agents

Increasing concentrations of compaction agents were added directly to PCR and RT-PCR reactions using human gDNA and total RNA with primers for β-actin to test the possible inhibition of PCR by the compaction agents. The C_t (threshold cycle) values for reactions containing compaction agents were compared to reactions without.

Human genomic DNA and total RNA precipitation reactions with RT-PCR detection

The precipitation effectiveness and DNA-selectivity of the compaction agents was investigated using synthetic mixtures of purified human gDNA and total RNA. The precipitation reactions were done in 0.6 ml Axygen (Union City, CA) microfuge tubes with a total reaction volume of 50 uL. Human genomic DNA (100 ng), human total RNA (100 ng), and a mixture (100 ng of each) were used to test each compaction agent. The concentrations of the compaction agents tested were 250 uM and 500 uM for Triquat A and spermidine, and 100 uM and 200 uM for Quatro-quat and spermine. These initial test compaction agent concentrations were obtained by diluting 200-fold (same as the DNA dilution) the concentrations that caused precipitation of salmon sperm DNA. (Quatro-diquat and Triquat 7 were not further tested here since they proved to be non-selective and less-

effective, respectively, as discussed below). DNA and RNA stock solutions were made in RNAse-free TE buffer, while compaction agent stock solutions were made in RNAse-free water. The same compaction precipitation protocol was used as above and 9 uL of each supernatant was used to measure the DNA and/or RNA left over after precipitation using real-time RT-PCR with SYBR Green detection. Controls with no compaction agent were included for comparison.

RT-PCR primers: Design and sequence—Primers were designed to anneal outside of introns, in flanking exons, so as to be able to amplify both DNA and RNA targets resulting in amplicons of different lengths. A longer PCR product is obtained from the DNA template (spanning the intron) and a shorter product from the RNA template. The PCR primers used in this study target the highly expressed β-actin gene (GenBank Accession No. NM_001101) and have the following sequences: Forward/Reverse 5'-GGCCACGGCTGCTTC-3'/ 5'-GTTGGCGTACAGGTCTTTGC-3' and a predicted melting temperature of 62 °C. The amplicon lengths for DNA and RNA targets are 303 bp and 208 bp, respectively. Agarose gel electrophoresis of the PCR and RT-PCR products confirmed that amplicons of different lengths are obtained when using these primers targeting β-actin.

Real-time RT-PCR—A Mx3005P Real-time PCR System (Stratagene) was used for RT-PCR thermocycling and its version 2.02 software was employed for all real-time data collection and analysis. RT-PCR reactions (one-step) were conducted in 25 μl containing DNA and/or RNA template from compaction reaction supernatant (9.0 μl), 1 × Brilliant SYBR Green QRT-PCR Master Mix (Stratagene) (12.5 μl), 500 nM of each primer (1.25 μl), StrataScript RT/ RNase block enzyme mixture (0.06 μl), and 0.94 μl of nuclease-free water.

RT-PCR mixtures were incubated at 55°C for 30 min, then at 95°C for 10 min followed by 40 cycles of denaturation (95°C, 30 s), primer annealing (60°C; 1 min), and extension (72°C, 1 min). Non-template and no-RT controls were included in each experiment. Following each amplification, a melting curve was obtained to check for non-specific amplification of primer-dimers and to confirm formation of a single product. The reaction products were denatured at 95°C for 1 min, reannealed at 55°C for 30 sec, and finally the temperature was increased to 95°C at a rate of 0.01°C/sec (melting) with continuous monitoring of fluorescence.

RNA isolation from HepG2 human liver cells

The Invitrogen Micro-to-Midi Total RNA Purification System was used to isolate total RNA from frozen HepG2 liver cells. The DNAse I digestion step was omitted for selective detection of possible DNA contamination. DNA contamination was verified by RT-PCR (no-RT control) using primers for the β-actin gene with the PCR experimental conditions described above. Next, the isolated RNA (with potential DNA contamination) was diluted 50-fold with RNAse-free water and 10 μl of this solution was used in precipitation reactions with compaction agents.

Effectiveness of compaction agents

The compaction agent effectiveness with salmon sperm genomic DNA was measured by the UV absorbance of the supernatant after precipitation reactions. The effectiveness with purified human gDNA, purified human total RNA, and RNA isolated from HepG2 cells was determined by the change in C_t (threshold cycle) values obtained from RT-PCR of supernatants after compaction precipitation. The C_t value was determined using the Mx3005P Real-time PCR System software version 2.02 'amplification-based' algorithm. An adaptive-baseline correction and moving average signal optimization were used.

Samples with higher initial nucleic acid concentrations reach the PCR detection threshold earlier than those with lower initial concentrations. Therefore, a low C_t value (e.g. 10-15) indicates a higher initial nucleic acid concentration than a high C_t value (e.g. 25-35). Differences in C_t values (ΔC_t) represent approximately $2^{(\Delta Ct)}$ – fold differences in concentration; a ΔC_t of 10 implies roughly a 1000-fold difference in concentration (the empirical per-cycle increase is slightly below two-fold, as discussed below). Therefore, large differences in C_t values (e.g. 10-15) from reactions with and without compaction agents imply high precipitation effectiveness. Low differences (e.g. 2-5) in C_t values represent low precipitation effectiveness. Ideally, PCR is 100% efficient and the amplified DNA doubles each cycle. In this study, the actual efficiency was ca. 90.3% and the DNA increases only 1.8 fold each cycle. Therefore, $1.8^{(\Delta Ct)}$ was used in all calculations.

RESULTS

Salmon sperm gDNA precipitation reactions

Triquat 7 was ineffective in precipitating DNA at concentrations up to 80 mM (data not shown); further experiments will be needed to discover its precipitation range. Figure 2 illustrates the results obtained in precipitation experiments using spermidine, Triquat A, spermine, and Quatro-quat measuring supernatant UV absorbance (all data points are based on at least duplicate experiments and have an average standard deviation of <0.03 absorbance units).

In all cases, as the NaCl concentration increases from 0 mM to 100 mM, the amount of compaction agent needed to achieve precipitation increases; competition by salt counterions has previously been reported [10]. Also, increasing NaCl concentration decreases the extent of the region of nearly-complete precipitation, as well as completeness of precipitation. A redissolution of the DNA occurs at very high compaction agent concentrations (>150 mM), for all salt levels tested. The source of this phenomenon is debated, but has been suggested that it arises from charge reversal of the nucleic acids at high cation loadings [10].

At all salt levels tested, a lower concentration of spermidine is needed to achieve precipitation compared to Triquat A. In addition, Triquat A is much more sensitive to salt. In the presence of 50 mM NaCl, the precipitation range is very small, and at 100 mM NaCl, no appreciable precipitation occurs. Tetravalent spermine and Quatro-quat are much more potent than trivalent spermidine and Triquat A. For example, at 50 mM NaCl only 0.15 mM spermine is needed to precipitate DNA vs. 10 mM spermidine; Quatro-quat is comparable to

spermine at 0.2 mM. At 100 mM NaCl precipitation occurs at 0.75 mM spermine and at 1 mM Quatro-quat.

Quatro-diquat, with six positive charges, is overall the most powerful compaction agent investigated here. At the very low level of 0.05 mM, almost complete precipitation was observed in both 50 mM NaCl and 100 mM NaCl. In the presence of 250 mM NaCl, a concentration of only 1 mM Quatro-diquat was needed for precipitation. However, in the presence of 500 mM NaCl, no precipitation occurred up to 5 mM Quatro-diquat (at which UV absorbance became significant). While Quatro-diquat was a potent precipitant, it proved not to be sufficiently selective for DNA. In all of the Quatro-diquat concentrations tested (0.005 mM – 5 mM), gel electrophoresis analysis of the precipitate and supernatant showed significant amounts of both DNA and RNA in each phase (results not shown).

PCR-inhibition testing of compaction agents

Increasing concentrations of spermidine, Triquat A, spermine, and Quatro-quat were added directly to PCR and RT-PCR reactions using human gDNA and total RNA and primers for β-actin to test the possible inhibition of PCR by the compaction agents. There was no significant change in C_t values between reactions with no compaction agent added and those including spermidine or Triquat A at concentrations up to 250 μM and spermine or Quatro-quat at concentrations up to 36 μM (results not shown). However, at a Quatro-quat concentration of 72 μM, the C_t value increased by 7 cycles. It is expected that high concentrations of compaction agent directly added to the PCR reaction will compact the nucleic acid and interfere with the reaction. However, as a substantial fraction of any added compaction agent is expected to be removed with the precipitated DNA, these results suggested (as confirmed below) that these agents are compatible with PCR at concentrations high enough to be used to remove genomic DNA.

Purified human gDNA and total RNA precipitation reactions with RT-PCR detection

Spermidine, Triquat A, spermine, and Quatro-quat were tested in compaction reactions with purified human gDNA (100 ng), total RNA (100 ng), and synthetic mixtures (100 ng of each). The reaction supernatants were then used in RT-PCR reactions with and without RT enzyme and primers for β-actin.

β–actin primers: No compaction agent—Figure 3 shows the amplification and melting curves obtained from RT-PCR reactions with primers for β-actin using purified human gDNA (100 ng), total RNA (100 ng), and an equal mass mixture (100 ng each). Successful amplification was obtained with all three templates and the melting curves show a single peak for each reaction corresponding to a single amplicon. As seen in Figure 3,, the total RNA and mixture are detected earlier (C_t = 17.0 and 16.8 respectively) than the genomic DNA (C_t = 21.9). This occurs since the β-actin gene is highly expressed and in the same mass of RNA and DNA, there are many more transcripts of the gene in the RNA than there are copies of the gene in the DNA. Therefore, the equal-mass mixture is dominated by the RNA present and is detected similarly to the RNA alone. Similar results were obtained using primers for the less highly-expressed IL-5 gene (results not shown).

β–actin: All compaction agents—Figure 4 shows the amplification curves obtained from RT-PCR with β-actin primers using a synthetic equal-mass mixture of purified gDNA and total RNA, without and with each compaction agent. Spermidine and Triquat A results are shown using 500 μM (250 μM was ineffective at removing DNA). Results for spermine and Quatro-quat are shown using 100 μM (200 μM precipitated all nucleic acid from the supernatants). When 500 μM spermidine was used in the precipitation reactions, the no-RT sample (DNA) was detected 9.6 cycles later than that without spermidine. The RT sample (dominated by RNA) was detected 2.9 cycles later than the RT sample without spermidine. This indicates that the supernatant DNA amount was decreased by a factor of ca. $1.8^{9.6}$ or 280-fold. The RNA-dominated mixture signal was only decreased by a factor of ca. $1.8^{2.9}$ or 5.5-fold (or RT-PCR is slightly inhibited). Therefore, spermidine is shown to be highly selective for DNA and effectively decreases the DNA amount when used with a synthetic mixture of RNA and DNA.

When 500 μM of Triquat A was used in the precipitation reactions, the no-RT sample (DNA) was detected 13.1 cycles later than the untreated no-RT sample. The RT sample (dominated by RNA) was detected 3.1 cycles later than the RT sample without Triquat A. Therefore, the supernatant DNA amount was decreased by a factor of ca. $1.8^{13.1}$ or 2200-fold. The RNA-dominated mixture signal was only decreased by a factor of ca. $1.8^{3.1}$ or 6.2-fold. Triquat A, like spermidine, is also shown to be highly selective for DNA removal.

When 100 μM of spermine was used in the precipitation reactions, the no-RT sample (DNA) was detected 7.1 cycles later (*ca.* 65-fold DNA reduction) than the no-RT sample without spermine. The RT sample (dominated by RNA) was detected 7.8 cycles later than the RT sample without spermine implying a ca. 98-fold reduction of RNA. Therefore, spermine shows relatively low selectivity for either DNA or RNA.

When 100 μM of Quatro-quat was used in the precipitation reactions, the no-RT sample (DNA) was not detected (≥ 15.9 cycles later than the no-RT sample without Quatro-quat). The RT sample (dominated by RNA) was detected 12.0 cycles later than the RT sample without Quatro-quat. Quatro-quat proves to be the strongest precipitant since it completely eliminates the DNA and even decreases the RT signal by 12.0 cycles (*ca.* 1200-fold). It is unclear as to how selective Quatro-quat is for DNA since the DNA could not be detected and therefore cannot be compared to the RNA- dominated signal. However, since Quatro-quat contains four positive charges (like spermine), it is anticipated that it will have low selectivity for DNA.

RNA isolation from HepG2 human liver cells and precipitation reactions with RT-PCR detection

The more selective compaction agents, spermidine (500 μM) and Triquat A (500 μM) were tested in compaction reactions with DNA-contaminated total RNA isolated from HepG2 liver cells. Figure 5 shows the amplification curves obtained from RT-PCR using primers targeting β-actin and total RNA isolated from HepG2 cells without and with spermidine and Triquat A compaction. When 500 μM of spermidine was used in the precipitation reactions, the RT sample (dominated by RNA) was detected 5.3 cycles later than the RT sample without spermidine. The no-RT sample (DNA) was not detected within 12.6 cycles of the

untreated no-RT sample. This indicates that the supernatant DNA amount was decreased by at least a factor of *ca.* 1600 and the RNA-dominated signal was decreased by a factor of *ca.* 26. The DNA remaining was not detectable in 40 cycles of PCR and therefore, spermidine is selective for DNA and could be used to eliminate DNA contamination from RNA samples.

When 500 μM of Triquat A was used in the precipitation reactions, the no-RT sample (DNA) was not detected within 12.6 cycles of the no-RT sample without Triquat A. The RT sample (dominated by RNA) was detected 5.1 cycles later than the RT sample without Triquat A. This indicates that the supernatant DNA amount was decreased by a factor of ≥ 1600 while the RNA-dominated signal was decreased by a factor of ca. 20. The DNA present was not detectable in 40 cycles of PCR and therefore, Triquat A is selective for DNA and could be used to eliminate DNA contamination from RNA samples.

DISCUSSION

We tested the use of selective precipitation by compaction agents to remove DNA contamination from RNA samples since this method proves to be fast, non-toxic, nuclease-free, and inexpensive. The effectiveness and DNA selectivity of six compaction agents, with different charges, were investigated, and it was found that effectiveness of compaction increased and DNA selectivity decreased with charge. Hexavalent Quatro-diquat was the strongest precipitant but showed no selectivity for DNA. Spermidine and Triquat A were more selective compaction agents and when applying 500 μM to an RNA isolate, the supernatant contaminating DNA was undetectable in 40 cycles of PCR. Some challenges remain to be addressed, however. When starting RNA amounts are unusually low, an excess of compaction agent could result in a loss of the entire sample. When DNA contamination is unusually high, the amount of compaction agent could be insufficient. The development of a small number of general protocols for classes of samples (e.g., blood cells, or a standardized number of cultured mammalian cells) would address this issue.

In conclusion, selective precipitation of DNA by compaction agents, particularly spermidine and Triquat A, shows promise as a non-toxic, nuclease-free, and cost-effective method of eliminating contaminating DNA in RT-PCR samples.

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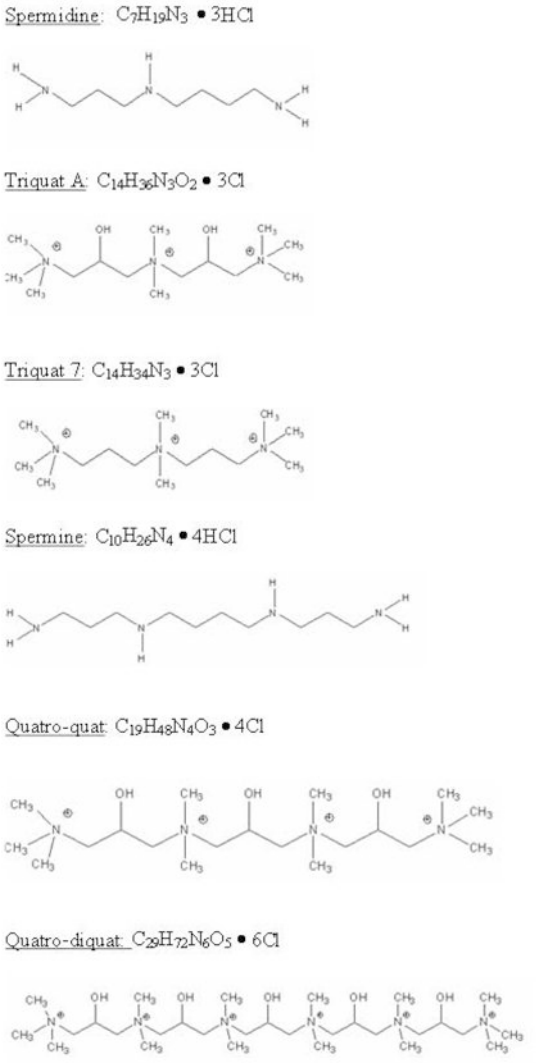

FIG.1.
Chemical formulas and structures of compaction agents.

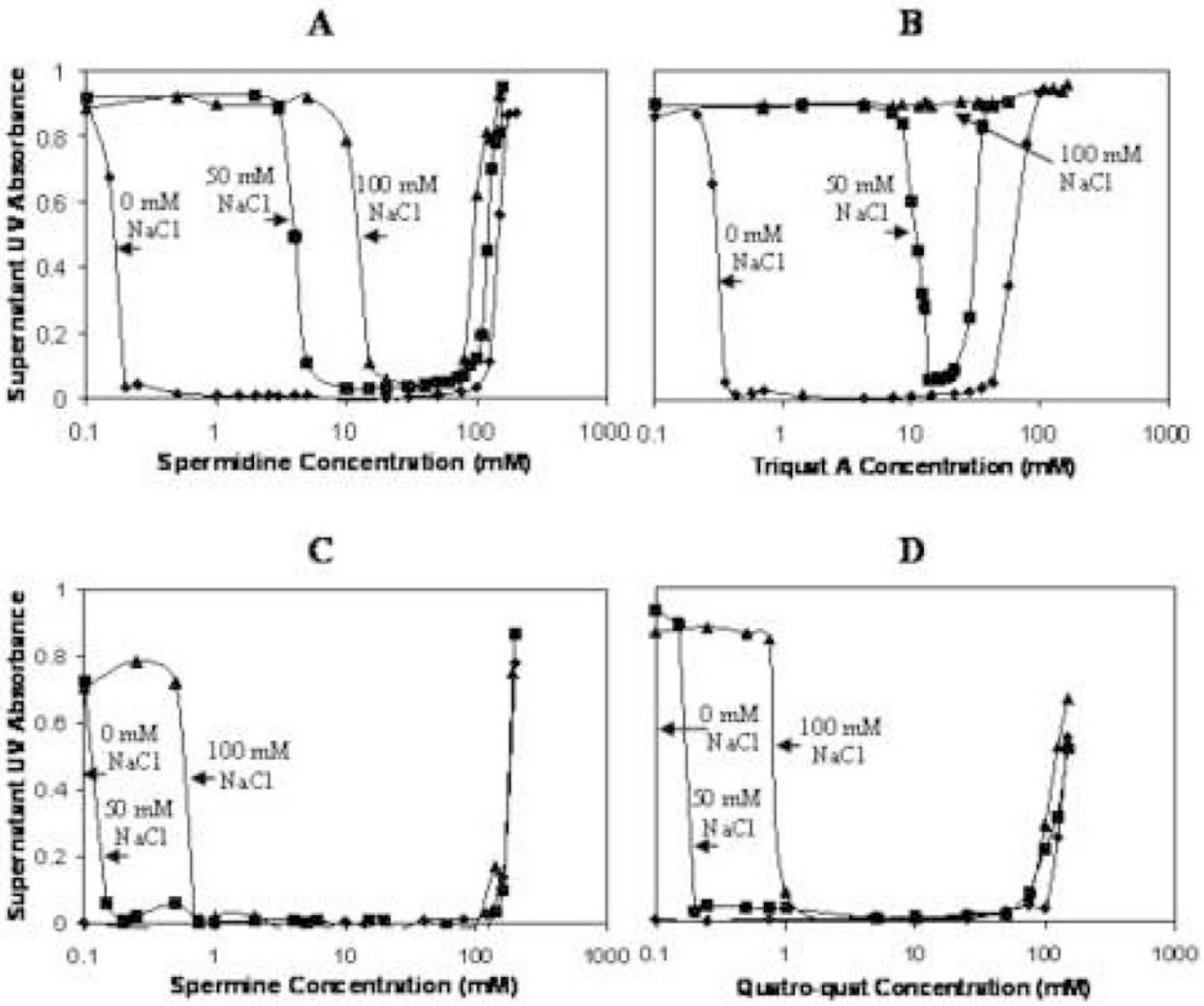

FIG 2.

UV-monitored salmon sperm genomic DNA (21.3 ± 0.2 μg) precipitation using (A) spermidine; (B) Triquat A; (C) spermine; and (D) Quatro-quat in the presence of 0 mM NaCl (◆), 50 mM NaCl (■), and 100 mM NaCl (▲).

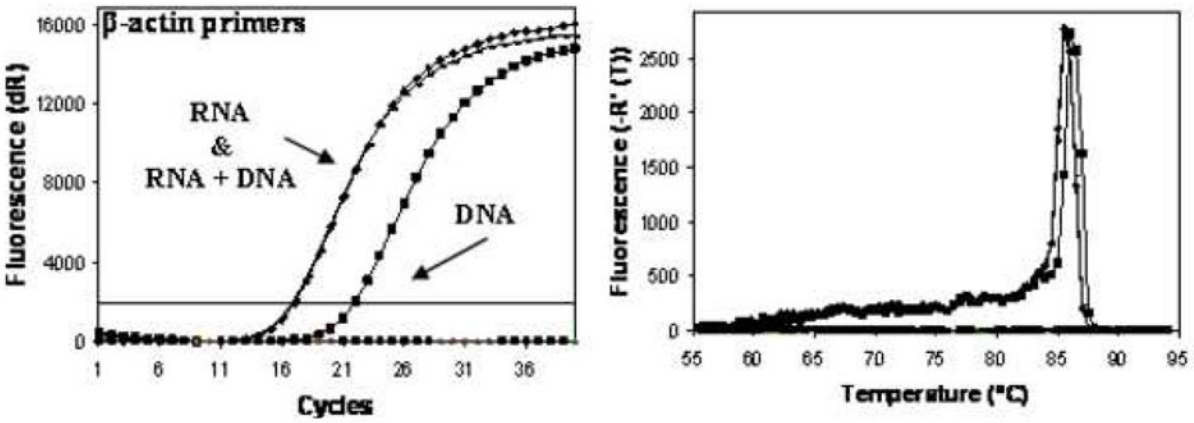

FIG 3.

RT-PCR amplification curve and melting curve obtained using 100 ng of human gDNA, 100 ng of total RNA, and an equal mass mix with β-actin primers. The non-template control and no-RT control overlap with the X –axis. The horizontal line is the amplification threshold at 1898 fluorescence units.

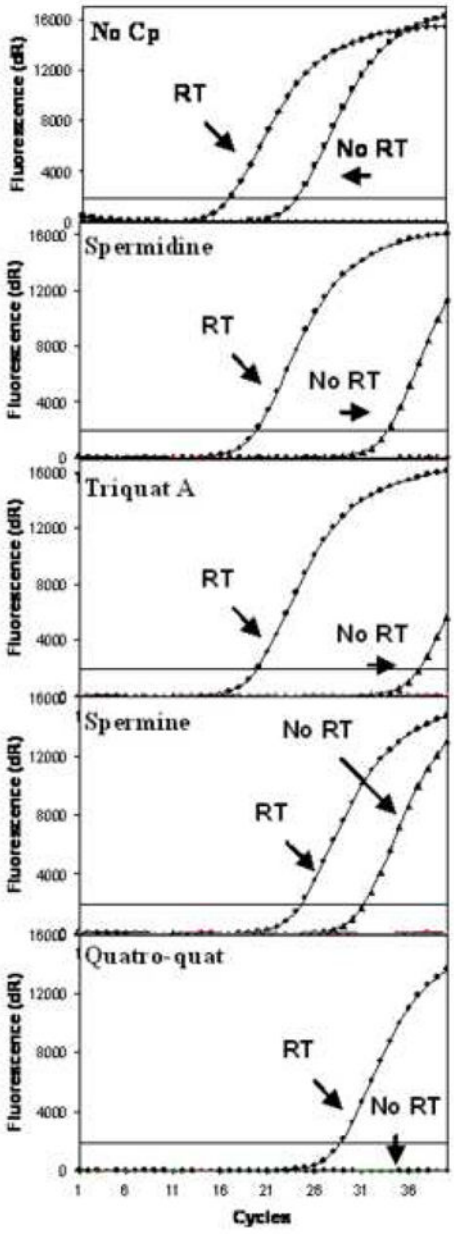

FIG 4.

RT-PCR amplification curves obtained using a mixture of 100 ng of human gDNA and 100 ng of total RNA with RT and no-RT with β-actin primers. (A) No compaction agent is used. (B) Spermidine at 500 μM (C) Triquat A at 500 μM (D) spermine at 100 μM (E) Quatro-quat at 100 μM. The horizontal line is the amplification threshold at 1898 fluorescence units

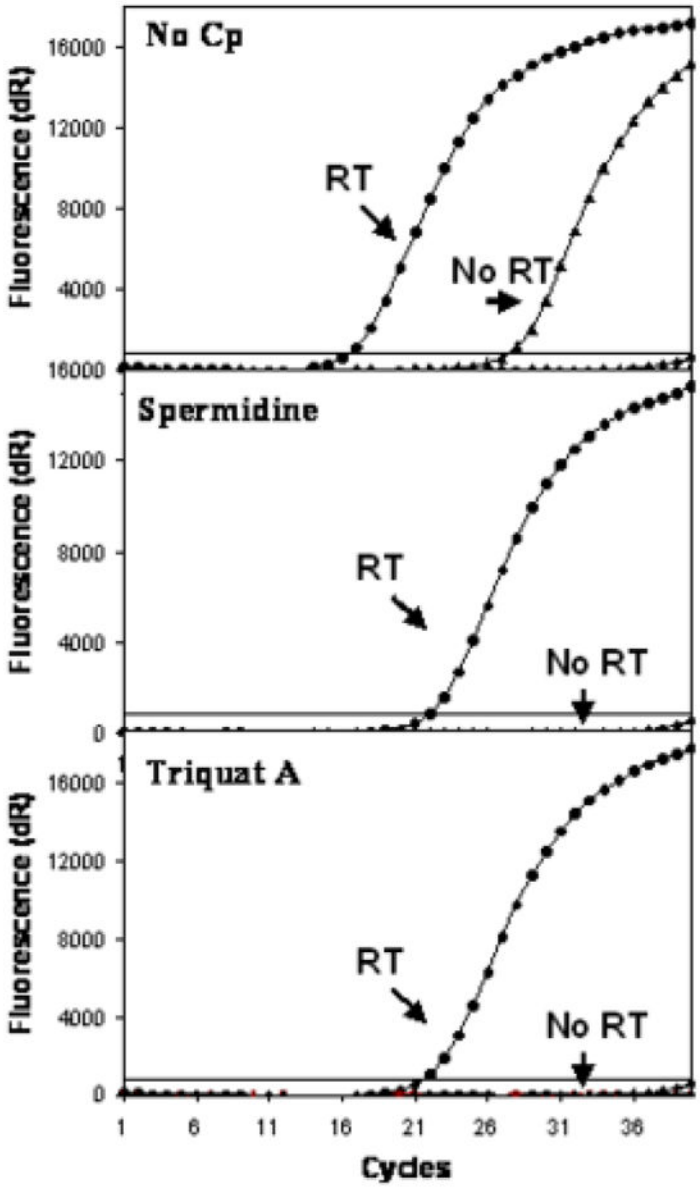

FIG 5.

RT-PCR amplification curves obtained using a total RNA isolated from HepG2 human liver cells with RT and no-RT with β-actin primers. (A) No compaction agent is used. (B) Spermidine at 500 μM. (C) Triquat A at 500 μM. The horizontal line is the amplification threshold at 831 fluorescence units.