Efficient Removal of Transfected Plasmid DNA from Total Cellular RNA Prepared with Agilent Total RNA Isolation Mini Kit

Application

Gene Expression

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Abstract

Quantitative, real time, reverse transcription-polymerase chain reaction (qRT-PCR) is a powerful tool for gene expression analysis. The quality and purity of RNA samples are critical parameters for reliably obtaining accurate and reproducible results from these assays. When working with transiently transfected cells, the presence of residual input plasmid DNA may interfere and obscure true expression levels. Consequently, removal of plasmid DNA from RNA preparations isolated post-transfection can help to provide more precise estimates of gene expression levels. In this study we explored the use of the Agilent Total RNA Isolation Mini Kit to remove plasmid DNA as well as to isolate RNA from transfected cell lysates. Using a direct TaqMan® PCR assay to detect beta-galactosidase (βgal) sequences, we found that the Agilent kit was able to remove more input plasmid DNA (pUC19, pβgal-Basic) than a competitor's silica-based kit. RNA isolated from HeLa cell lysates spiked with plasmid DNA contained <0.001% of the input plasmid DNA. In addition, a new method was devised that incorporates organic extraction and in-solution DNase treatment steps into the standard RNA isolation protocol. For comparison, an analogous method was adapted for use with the leading silica-based RNA isolation kit. Using these two methods, RNA was isolated from cultures of various cell lines transfected by several transfection techniques. We performed real time RT-PCR assays to analyze the amount of DNA present in transfected cells and found that the change in cycle-threshold values (ΔCt) was consistently better when using the Agilent method.

Introduction

Isolation of RNA is often the critical first step for gene expression analyses in both clinical and research applications. With recent advances of diagnostic tools for gene expression such as microarrays and quantitative amplification technologies including reverse transcription-polymerase chain reaction (RT-PCR), there is an increasing demand for highly purified, intact cellular RNA. There are a number of systems commercially available that are relatively simple and efficient, and yield intact total RNA with low levels of protein and other contaminants. However, these methods are rather inefficient at removing DNA molecules. For applications in which genomic DNA contamination is a primary consideration, scientists may be able to engineer their experimental design to avoid amplifying intronless sequences. However, for gene regulation and expression

experiments dependent on transient transfection of plasmid DNA, the presence of residual input DNA in RNA preparations could easily confound the analysis of expression levels.

The Agilent Total RNA Isolation Mini Kit (p/n 5185-6000) contains a unique prefiltration column which is capable of removing the majority of DNA species from a tissue or cell homogenate.

For the application described here, the kit protocol was modified to optimize the removal of plasmid DNA by the addition of an organic extraction step and an in-solution DNase treatment step. A similar method was also devised for use with a leading silica-based kit for comparative studies. Results from quantitative polymerase chain reaction (qPCR) and qRT-PCR assays demonstrate that the Agilent kit provides superior performance for removing unwanted DNA in RNA preparations isolated from transfected cells.

Materials and Methods

Plasmid-Spiked Experiments

For experiments in which cell lysates were spiked with plasmid DNA, 100 ng of plasmid was added to 1×10^6 HeLa cells in 350 µL of lysis solution. Each of three RNA isolation methods was done in triplicate for each of two plasmids.

- Method 1 was the standard Agilent kit protocol.
- Method 2 was the standard silica-based kit protocol.
- Method 3 was the standard silica-based protocol plus the manufacturers recommended on-column DNase treatment.

Each of the three methods was tested with two plasmids of different sizes that contain β-galactosidase coding sequence:

Plasmid-1) 2.9 Kb pUC19
(Invitrogen, cat. # 15364-011)

Plasmid-2) 7.5 Kb p gal-Basic
(BD-Clontech, cat. # PT2109-5)

The presence of plasmid DNA in the RNA preparations was determined by a TaqMan real time qPCR assay using a set of primers and a fluorescent probe specific for βgal. RNA samples were added directly to the PCR mix, with no reverse transcription step performed, to ensure that all detected signals were from the amplification of residual plasmid DNA. No-template control (NTC) reactions included use of nuclease-free H_2O instead of RNA sample. The βgal primer/probe set was designed to amplify a 79-base pair amplicon. The sequences of the oligonucleotides were as follows:

- Forward primer:
  5'...CGATCTGTCTATTTCGTTCATCCA...3'
- Reverse primer:
  5'...AGATGGTAAGCCCTCCCGTATC...3'
- TaqMan probe:
  5'...6-FAM-AGTTGCCTGACTCCCCGTCGTTAGA-BHQ-1...3'

Transient Transfection Experiments

For transient transfection experiments, HepG2, MCF-7, and Ishikawa cell lines were transfected by one or more of the following methods:

- Calcium phosphate-DNA co-precipitation (as described in Reference 1)
- Lipofectamine 2000 (Invitrogen cat. # 11668-027, following manufacturers protocol)
- FuGENE 6 (Roche cat. # 1 814 443, following manufacturers protocol)

After incubation at 37 °C/5% CO_2 for 36–72 hours, the cells were harvested for preparation of total RNA. Cell preparation steps are outlined below

1. Cells were washed twice with 1× PBS, lysed according to the Agilent protocol, and the lysates passed through Agilent prefiltration columns.
2. The flow-through volumes were extracted once with an equal volume of phenol-chloroform (1:1) and the aqueous phase transferred to new tubes.
3. An equal volume of isopropanol was added, mixed, and the mixture incubated for 10 minutes at room temperature, and then centrifuged for 12 minutes at 12,000 rpm.
4. The pellet was washed two times with 70% ethanol, and then air dried in a hood for 1 hour.
5. The RNA was dissolved in 100 µL of nuclease-free H_2O and incubated for 20 minutes at 70 °C with vortexing every 5 minutes.
6. The sample was digested with 20 units of DNase1 (RNase-free, Promega) in 300 µL of digestion buffer for 2 hours at 37 °C. (For >60 µg of RNA, increase the enzyme and buffer amounts accordingly.)

7. One-tenth volume of 3M sodium acetate (pH 5.2) and two volumes of 100% ethanol were added. The tubes were then incubated at –20 °C for 30 minutes to precipitate the RNA.
8. Tubes were spun at 12,000 rpm for 15 minutes at 4 °C to pelletize the RNA.
9. The supernatants were removed and the pellets allowed to sit for 5 minutes at room temperature.
10. Lysis solution (300 µL) was added and the tubes vortexed twice to dissolve the pellets.
11. RNA was then purified using Agilent isolation columns and protocol.

RNA samples were examined for presence of plasmid DNA by TaqMan real time RT-PCR assays with and without a reverse transcription step. Gene specific primers and probe sets were designed to amplify sequences from experimental plasmids 1 and 2. Cycle threshold (Ct) values were determined for ± RT reactions as well as for no-template controls using H_2O. RNA samples isolated as described above were also compared to RNA isolated using a similar method modified for use with a silica-based spin column system. Levels of plasmid DNA contamination were measured using the TaqMan assay outlined above.

Results

Plasmid DNA Spiking Experiments

In these experiments, a TaqMan direct PCR assay was used to measure the amount of plasmid DNA remaining in total RNA samples isolated using either the Agilent method or a silica-based method. Data from these tests show that the Agilent method is able to remove better than 99.99% of the input DNA. This is 30–100 times more DNA removed than with the silica-based method. As shown in the amplification plots in Figure 1, the fluorescent signal indicating amplification of βgal sequences comes up at a much later cycle in the Agilent preparations than with the silica-based method. The addition of a DNase treatment step provides some benefit to the silica-based method; however, the improvement after DNase treatment using the silica-based method is not sufficient to match the Agilent kit's performance without DNase.

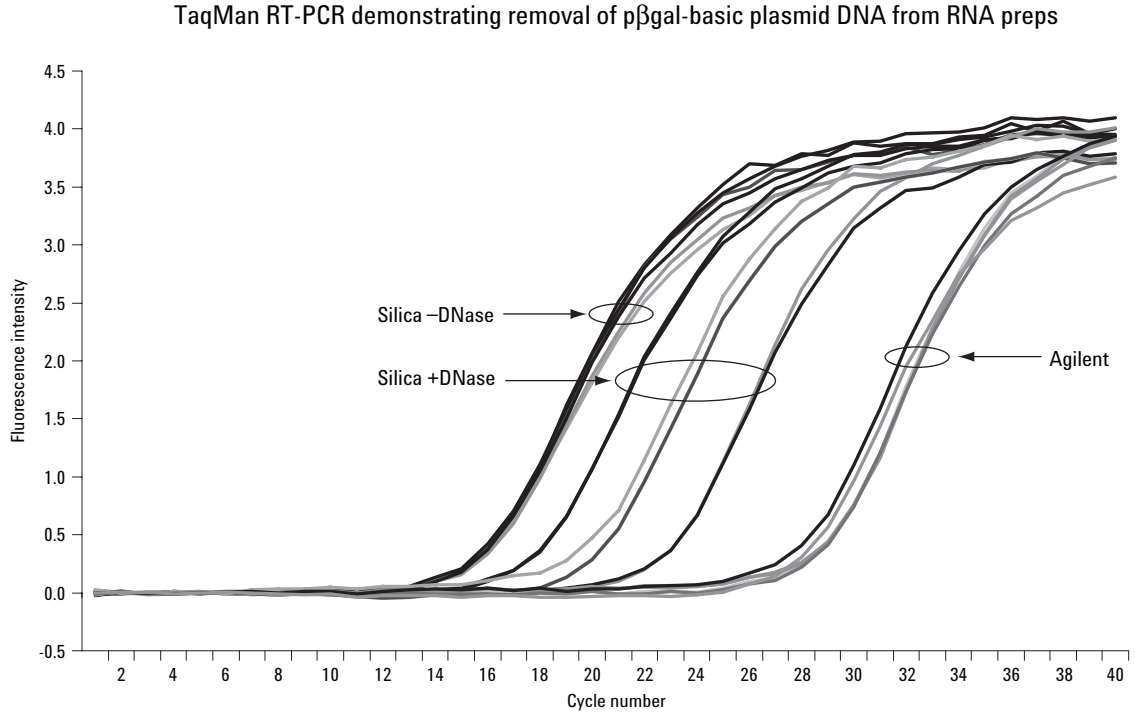


Figure 1. Total RNA samples were isolated from HeLa cell lysates spiked with pßgal-Basic plasmid DNA. The RNA was isolated using Agilent's method with or without DNase treatment, or the silica-based method with or without DNase treatment. The presence of DNA was measured by TaqMan direct PCR (no RT step) assay using a ßgal specific primer/probe set.

As Figure 2 shows, the results are similar when a smaller plasmid, (2.9 Kb pUC19 versus the 7.5 Kb pβgal-Basic) is used as the input DNA. In this case, the DNase treatment resulted in significant improvement for the silica-based method. However, again, the Agilent kit minus DNase treatment outperformed the silica-based kit plus DNase treatment.

Transient Transfection Experiments

HepG2, MCF-7, and Ishikawa cell lines were transfected with Plasmid-1 or Plasmid-2 (both ≅9 Kb) using $CaPO_4$, Lipofectamine 2000, or FuGENE 6 according to published methods. RNA isolated from these cells was then used in qPCR assays plus or minus the RT step. Ct values from +RT and –RT reactions as well as NTC were measured and are shown in Table 1. Regardless of plasmid, cell line, or transfection method used, a significant difference is seen between the +RT and –RT values. This provides a large signal to noise ratio within which to compute true gene expression levels. In experiments that included NTC reactions, the Ct values for –RT and NTC samples are very close indicating only minute trace amounts of DNA remain in the RNA samples.

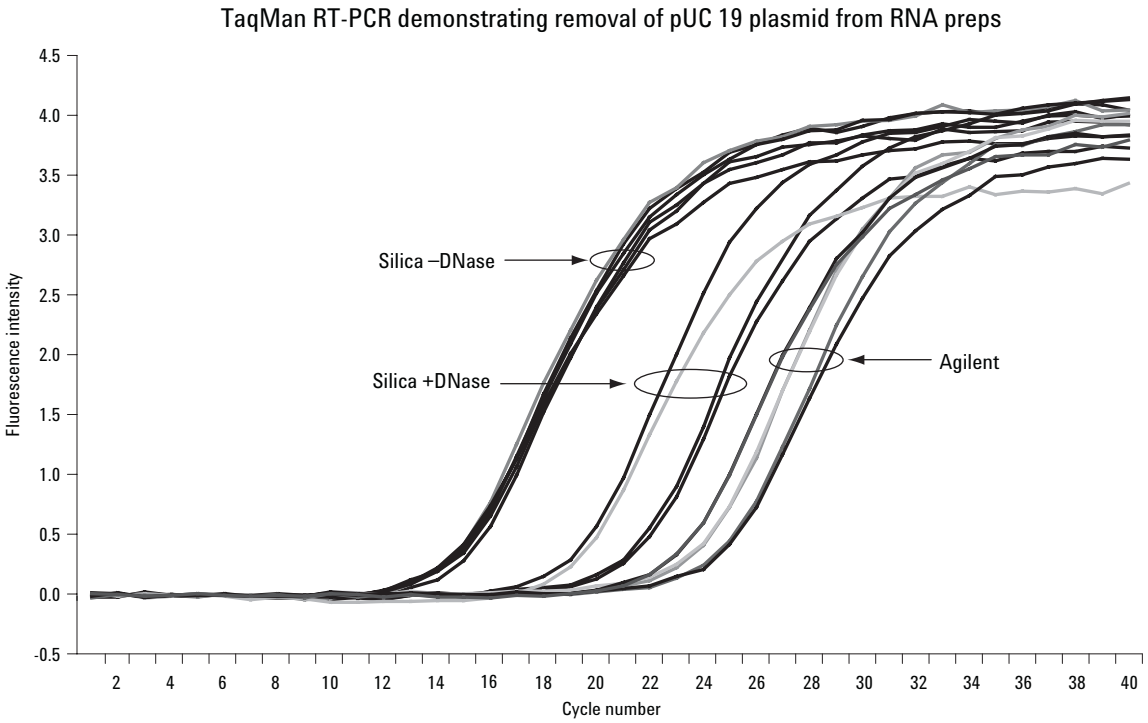


Figure 2. Total RNA samples were isolated from HeLa cell lysates spiked with pUC19 plasmid DNA. The RNA was isolated using the Agilent method with or without DNase treatment, or the silica-based method with or without DNase treatment. The presence of DNA was measured by TaqMan direct PCR (no RT step) assay using a ßgal specific primer/probe set.

Table 1. Cycle threshold (Ct) Values from TaqMan Assays of RNA Samples Isolated from Transiently Transfected Cells

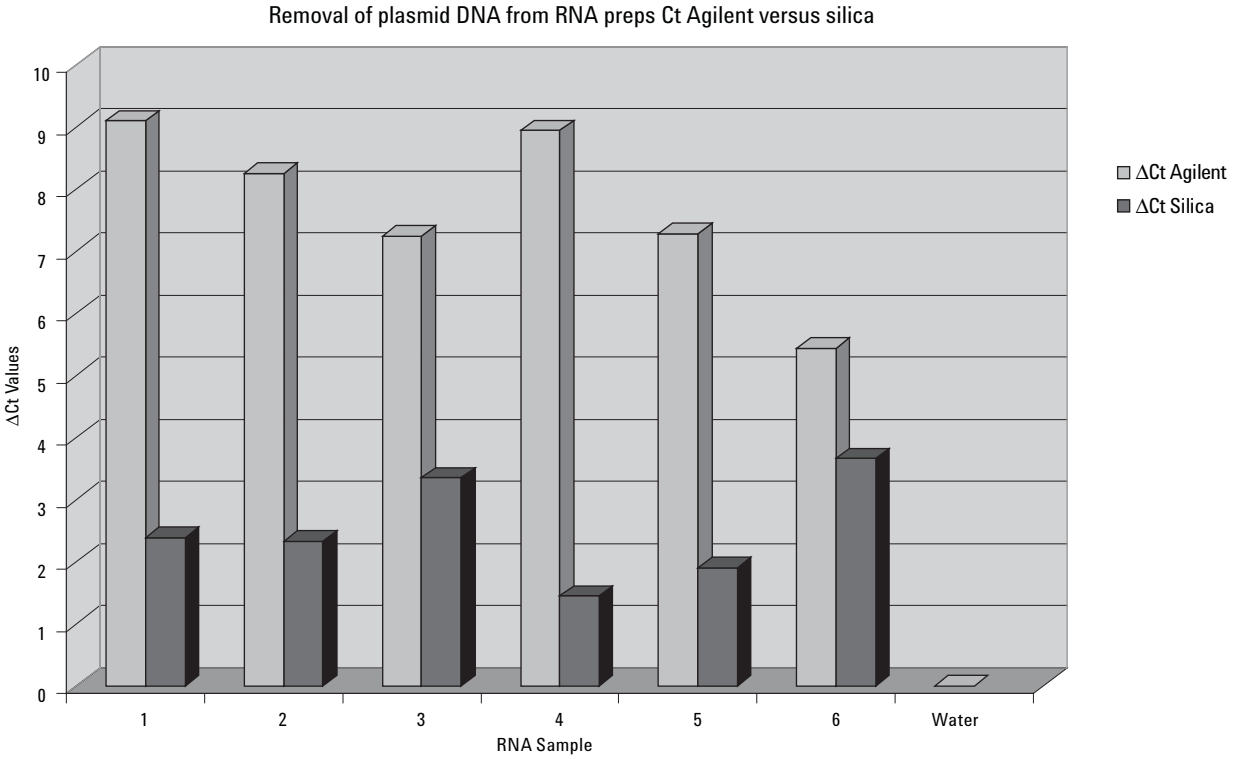
Plasmid number	Transfection method	Cell line	Ct, +RT*	Ct, –RT*	Ct, NTC**
1	CaPO4 precipitation	HepG2	23.1±1.2	38.2±1.7	ND
1	Lipofectamine 2000	HepG2	26.2±1.1	37.7±2.5	ND
2	FuGene 6	MCF-7	27.1±0.5	34.8±1.3	34.8
2	FuGene 6	Ishikawa	21.5±1.0	31.6±2.8	32.9

*Average and standard deviation of six samples

**No Template Control = H_2O

ND Not done

The performance of the modified Agilent method of removing DNA from RNA preparations was compared to that of a similar method employing a silica-based spin column kit. The two methods were used to isolate RNA from MCF-7 cells transfected with Plasmid-2 using the FuGENE 6 kit. As shown in Figure 3, the ΔCt values (that is, the difference between the Ct values for +RT and –RT reactions) are greater for all 6 RNA samples isolated using the modified Agilent method. Furthermore, as seen in the table accompanying Figure 3, Ct values for the +RT reactions are lower when RNA was isolated using the modified Agilent method. This suggests that not only the yield, but possibly the quality, of the RNA is better when using the Agilent method.



Sample	Ct –RT	Ct +RT	ΔCt Agilent	Sample	Ct –RT	Ct +RT	ΔCt Silica
1	35.55	26.45	9.10	1	30.61	28.22	2.39
2	34.32	26.07	8.25	2	30.47	28.14	2.33
3	34.28	27.04	7.24	3	31.17	27.82	3.35
4	35.11	26.17	8.94	4	30.83	29.39	1.44
5	34.49	27.21	7.28	5	31.45	29.55	1.90
6	34.89	29.45	5.44	6	33.20	29.54	3.66
Water	34.80	35.10	–0.30				

Figure 3. MCF-7 cells were transfected with test plasmid-2 following the protocol for FuGene 6. RNA was isolated from cells 12–36 hours post-transfection by either the modified Agilent method or modified silica-based method. RNA samples were used as templates for TaqMan real time RT-PCR assays with and without the reverse transcription step. Bars indicate the change in Ct value between –RT and +RT reactions.

Conclusion

The Agilent Total RNA Isolation Mini kit is able to remove significant amounts of plasmid DNA from tissue culture cell lysates. This attribute of the kit was not limited by the size of the plasmid. Furthermore, using a modified protocol, the kit removed plasmid DNA from transiently transfected cell extracts, regardless of cell line or transfection method. This application of the Agilent RNA isolation kit is extremely beneficial to researchers whose gene expression analyses are dependent on DNA transfection. Removal of plasmid DNA with this method is particularly useful for applications involving poorly expressed or low abundance messenger RNAs.

Reference

1. S. Mattick, K. Glenn, G. de Haan, and D.J. Shapiro, “Analysis of ligand dependence and hormone response element synergy in transcription by the estrogen receptor”, (1997) *J Steroid Biochem Mol Biol.*, **60** (5–6):285–294.

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