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Original Article

# Evaluation of Different RNA Extraction Methods from Agropatch Suppressor Assay for Small Quantities of Plant Tissue and Their Application for Analysis of Gene Expression

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**Abstract**

The agroinfiltration assay provides fast and efficient way to transiently express genes into plant cells by *Agrobacterium tumefaciens*. Extraction of RNA of high quality and sufficient amounts is prerequisite for gene expression studies such as quantitative Real Time PCR (q-PCR) from infiltrated areas in agropatch suppressor assay with small quantities of plant tissue. To attain prime RNA extraction from small tissues of infiltrated *N. benthamiana* plants with Potato virus A helper component proteinase viral suppressor protein, the efficiency of three RNA extraction methods (LiCl, TRIzol reagent and commercial kit) was evaluated. The total RNA yield with LiCl method was 2.83 and 33.2-fold greater than that of TRIzol reagent and commercial kit, respectively. Also, total RNA yield using TRIzol reagent was 11.7-fold higher than that with commercial kit. The A260/A280 ratio mean for TRI reagent (1.95) and kit (1.9) extractions were within the optimum range.q-PCR revealed that the cycle threshold values of housekeeping gene, *EIF-1α* and target genes *AGO1* and *ATG6* for RNA extracted using LiCl and kit were 1.07 to 1.3 and 1.02 to 1.12 times higher than those evaluated with the TRIzol method. Overall, TRIzol method showed the most effective approach for obtaining RNA from *N. benthamiana* patches in gene expression studies.

***Keywords:*** agro infiltration; q-PCR; RNA extraction; RNA silencing; small tissue samples; TRIzol; viral suppressors of RNA silencing

## Introduction

Plant viruses are obligate intracellular parasites that recruit host machinery to accomplish their life cycle. Following viral invasion, plants can defend themselves through a variety of mechanisms including RNA silencing as a natural antiviral defense response. RNA silencing can decrease virus accumulation in the primary infected cells and limit the following virus cell-to-cell and long-distance movement (Ding and Voinnet, 2007). To counteract host RNA silencing, viruses have evolved diversified mechanisms, including expression of RNA silencing suppressors (RSS) which is one of the most common strategy in plant viruses (Ding *et al*., 2004; Roth *et al*., 2004; Voinnet, 2005; Ding and Voinnet, 2007). A large number of RSS proteins with diverse structures and functions have been identified in many plant viruses and some animal viruses (Brigneti *et al*., 1998; Kasschau and Carrington, 1998; Voinnet, 2005; Li and Ding, 2006). Potato virus A (PVA) belongs to Potyviridae (genus *Potyvirus*), as the largest virus family infecting plants (Urcuqui-Inchima *et al*., 2001). Similar to other potyviruses,the PAV genome encodes a large polyprotein subsequently cleaved by three virus-encodedproteinasesto yield up to ten mature proteins (Urcuqui-Inchima *et al*., 2001; Rajamäki *et al*., 2004). Helper component proteinase (HC-Pro) is a multifunctional protein involved in potyvirus replication, cell-to-cell movement and long-distance movement (Rojas *et al*., 1997; Sáenz *et al*., 2002), aphid transmission (Pirone and Blanc, 1996), symptom development (Redondo *et al*., 2001), viral synergism (Wang *et al*., 2002), inhibition of the endonuclease activity (Ballut *et al*., 2005), the protease activity (Sahana *et al*., 2012) of the 20S proteasome and suppression of RNA silencing-based antiviral defense in plants (Rajamäki *et al*., 2004). A variety of different assays have been established to identify and characterize viral

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suppressors of RNA silencing (VSR) (Qu and Morris, 2005). One common biological used assay is called "agropatch suppressor assay" carried out by transient expression of candidate virus-encoded gene through the co-infiltration of separate *Agrobacterium tumefaciens* (agro infiltration) harboring the VSR and a reporter gene such as green fluorescent protein (GFP) into leaves of *Nicotiana benthamiana* (Johansen and Carrington, 2001).

One major problem is associated with collecting high quality and sufficient amounts from infiltrated area in agropatch suppressor assay with small tissue in gene expression studies. Moreover, it is important for obtaining reliable gene expression data from different genomic approaches such as quantitative Real Time PCR (q-PCR), hybridization microarrays and next generation sequencing (NGS) technologies (Maciel *et al*., 2011).

The present study was aimed to evaluate three different RNA extraction methods, TRIzol reagent, lithium chloride (LiCl) salt and a commercial kit (S-1010-1, Dena Zist Asia, Iran). The research describes the results of the evaluation focusing mainly on the yield and purity of RNA extracted by these methods and the performance of the q-PCR from small patches of *N. benthamiana* plants co-infiltrated with PVA HC-Pro viral suppressor and *GFP* reporter gene.

## Materials and Methods

*Plant material*

*N. benthamiana* plants were grown with a photoperiod 16 h light / 8 h dark, minimum temperature of 18 ± 4 °C and 80% humidity.

*Constructs*

The cDNA of PVA-HC-Pro ORF was cloned into a pDONRTM/Zeo vector (Life technology). Then, confirmed clones were recombined into the Gateway (Invitrogen) destination vector pGWB17 (Nakagawa *et al*., 2007) with a C-terminal myc tag and N-terminal 35S promoter. Agrobacterium strain C58C1 was used in this experiment. For expression of the GFP, the *A. tumefaciens* transformed with a binary vector p35-GFP was uesd (Germundsson *et al*., 2007).

*Agropatch assay*

*Agrobacterium* cultures harboring PVA HC-Pro, empty plasmid (EP) and GFP constructs were inoculated into 3-ml YEP broth culture and incubated at 28 °C for 20 h with shaking. One ml from these cultures was inoculated into 50-ml YEP broth cultures with the antibiotics, acetosyringone (20 µM final concentration) and 0.5 M morpholineethanesulfonic acid (MES), pH 5.6. Cultures ($OD_{600}$; 0.5) were pelleted by centrifugation at 3,500 g for 5 min and re-suspended in induction buffer containing 10 mM $MgCl_2$, 10 mM MES, pH 5.6, and 150 µM acetosyringone. Agrobacterium cultures diluted in induction buffer and were mixed with an agrobacterium culture containing GFP to infiltrate *N. benthamiana* leaves at six- to eight-week-stage.

*GFP detection*

GFP fluorescence in *N. benthamiana* leaves was checked using a hand-held long-wave UV lamp UVL-56 (UV Products). Fifty milligrams from green area of leaves indicating GFP expression observed by UV lamp was detached to extract RNA. Each assay was repeated three times.

*RNA extraction methods*

*LiCl Protocol*

Fifty milligrams of leaf tissue were ground to a fine powder in liquid nitrogen and 1.000 µl of extraction buffer (500 µl of LiCl buffer and 500 µl of phenol pH 8.0) were added. The tube was vortexed vigorously for 1 min and then placed on ice until all tubes have been completed. The tubes were then incubated for 5 min at 60 °C, followed by centrifugation for 10 min at max speed at 4 °C. The upper phase was collected in to a new tube and 600 µl of chloroform-isoamyl alcohol (24 :1; v/v) was added to the tube and centrifuged 10 min at max speed at 4 °C. The supernatant was transferred into a new micro centrifuge tube and after incubation at 65 °C for 15 min, 50 µl of 5 M NaCl and 63 µl of 40% polyethylene glycol 8.000 (w/v) was added and vortexed for 1 min. They were centrifuged for 10 min at max speed at 4 °C (the supernatant contains small RNAs and the pellet consists of large RNA). Finally, supernatant was discarded and when the pellet dried, it was re-suspended in 20 µl RNase-free water.

*TRIzol protocol*

Fifty milligrams of leaf tissue were placed in a 1.5 ml microfuge tube and finely ground in liquid nitrogen. Then, 1.5 ml of TRIzol was added to each tube and after vigorous mixing; the resulting mixture was vortexed and incubated at room temperature for 5 min and spun at 12.000 g for 10 min. Supernatant was removed to new 1.5 ml tube with 0.4 ml of chloroform and shook vigorously by hand for 15 seconds. They were kept at room temperature for 10 min and centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was transferred to a new 1.5 ml tube and an aliquot of 500 µl isopropanol was added and the resulting mixture incubated for 10 min at room temperature, followed by centrifugation at 12.000 g for 20 min at 4 °C to pellet total RNA. The pellet was washed with 1.000 µl 75% ethanol, air-dried for 5 min and ultimately, dissolved in 250 µl RNase-free water. 250 µl 4 M LiCl was added, mixed and put on ice overnight. It was centrifuged at 12.000 g for 20 min at 4 °C. The pellet contains m-RNA and r-RNA and the supernatant contains t-RNA and si-RNA and the 5S r-RNA. Finally, the pellet was washed (m-RNA) in 70% ethanol and re-dissolved in RNase free water.

*Commercial kit protocol*

Fifty milligrams of leaf tissue were ground to a fine powder in liquid nitrogen and the powder was then placed in 2-ml micro tubes containing 1 ml DR1 buffer. Then, tissue lysate was homogenized in DR1 buffer for 15 seconds and incubated at room temperature for 5 minutes. The tubes were centrifuged at 10,000 rpm for 10 minutes at 4 °C and the supernatant was then transferred to a fresh microfuge (1.5 ml) with 200 µl chloroform and mixed gently for 1 min. After centrifugation for 10 min at 12.000 rpm, the top aqueous phase was transferred to a new tube

and equal to half of the volume of the aqueous phase was addedfrom 100% ethanol. 700 µl DR2 was added to the spin column, centrifuged at 13.000 rpm for 1 minute at room temperature. Without adding any solution, the tubes centrifuged one more time at 13.000 rpm for 3 minutes at room temperature and the column was transferred onto a new 1.5 ml microfuge tube and 50 µl DR3 buffer was added to the center of the column and incubated at room temperature for 2 minutes. They were centrifuged at 10,000 rpm for 2 minutes at room temperature and the eluted solution was returned from the previous step onto the center of spin column and the spin column was mounted on the microfuge tube for 2 minutes at room temperature. Finally, the tube was centrifuged at 13,000 rpm for 2 minutes.

*Quantification and RNA quality estimation*

The samples were quantified and the absorbance ratios at wavelengths of 230, 260 and 280 nm were measured using a ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, U.S.A.). Then the purity was detected according to the as the ratio of the absorbance at 260 nm and 280 nm (A260 / A280) along with the ratio of the absorbance at 260 nm and 230 nm (A260 / A230). The value of $OD_{260/230}$ was used as reference value. Agarose gel electrophoresis was used to assay the RNA for intact 28S and 18S rRNA. The integrity of total RNA was verified by analyzing approximately 3 µg of RNA on 1.0% (w/v) agarose gel, 75 mA for 20 min, electrophoresed, stained with ethidium bromide (EtBr) and visualized under UV light to assess the integrity of total RNA bands.

*Synthesis of complementary DNA (cDNA)*

RNA samples were treated with DNase I (Invitrogen) following the manufacturer's protocol. 1 µg of total RNA was used to synthesize cDNA via reverse transcription according to the first strand cDNA synthesis kit protocol (Parstous Biotechnology, Iran). cDNA was synthesized using 1 µl of reverse primer (10 pmol), 4 µl of 5x reverse transcriptase buffer, 1 µl of M-MuLV RT (100 U $\mu L^{-1}$) and 9 µl of DEPC-treated water and 2 µl of 10 mM dNTP mix were mixed in Eppendorf tubes, the mixture was incubated at 42 °C for 1 hour, and then 10 min at 70 °C. The synthesized cDNA was stored at -20 °C until used.

*qPCR*

The *AGO1*, *EIF-1α* and *ATG6* expression was analyzed by q-PCR using a lineGeneK thermal cycler (Exicycler TM96) apparatus according to the manufacturer's recommendations. The q-PCR primers were designed for the reference gene, i.e. *EIF-1α*, *AGO1* and *autophagy-related 6 gene* (*ATG6*) (Table 1). The q-PCR analyses were performed in 20 µl volume containing cDNA (250 ng) template, 10 µM of each primer (0.6 µl), qPCR SYBR® Green master with low ROX (Jena Bioscience, Germany) (10 µl) and sterile water (fill up to 20 µl). The genes were analyzed using the following profile: 95 °C for 2 min, then 40 cycles of 95 °C for 15 sec, 60 °C for 1 min. Data were expressed as cycle threshold (Ct) values. Three biological replicates were used and three technical replicates were performed for each biological replicate.

*SDS-PAGE for GFP protein*

The total proteins extracted from the infiltrated *N. benthamiana* patches with agrobacterium cultures harboring HC-Pro or EP mixed with GFP culture were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% polyacrylamide gel (Sambrook and Russell, 2001). Following electrophoresis, the gel was stained using Coomassie brilliant blue.

*Statistical analysis*

The data were analyzed statistically using SAS 9.4 software and means were separated by Duncan's multiple range test with probability at 5% level of significance to estimate the differences among the means of treatments (Duncan, 1951).

**Results and Discussion**

Infiltrated *N. benthamiana* leaves with a mixture of *A. tumefaciens* cultures containing pBinGFP plus either pBin PVA-HC-Pro or pBin were checked using a hand-held long-wave UV lamp at 5 dpi. Infiltrated leaves with a mixture of suspensions containing GFP and PVA-HC-Pro constructs showed GFP expression (Fig. 1). Also, SDS-PAGE analysis indicated that GFP with 27 KD size was expressed in *N. benthamiana* in the presence of PVA-HC-Pro at 5 dpi (Fig. 2).

There were compared three different methods to isolate RNAs from fifty mg of fresh *N. benthamiana* patches co-infiltrated with PVA HC-Pro or EP plus GFP construct aiming to identify which method is more efficient. In this assay, RNA quality was assessed based on fourcriteria: A260 / 280, A260 / 230, appearance on agarose gel and q-PCR assay.

Considerable differences in quantity and quality of RNA extracted by each of the three methods were identified. As the first criteria for assessing the RNA purity, A260 / A280 ratio mean was recorded as 1.95 with TRIzol reagent, 1.74 with LiCl method, and 1.9 with DenaZist kit. However, typical values of 1.8-2.1 for A260 / A280 ratio and 2.0-2.2, for the A260 / A230 ratio are generally accepted for purity of RNA (Alemzadeh *et al*., 2005; Winfrey *et al*., 1997). The A260 / A280 ratios for TRI reagent and Dena Zist kit extractions were within the optimum range, however the ratio for LiCl method was lower, implying protein contamination with this method. Also, RNA extracted by TRIzol reagent constantly showed the highest A260 : A280 ratio. A260 / A230 ratio mean was also determined as the other criteria of RNA purity, for purified RNA obtained with TRI reagent (2.06), LiClmethod (1.84) and DenaZist kit (1.98). The value of A260 / A230 ratios for RNA purified with LiCl method was lower than those of TRI reagent and DenaZist kit extractions.

The lower A260 / A230 values may be due to the high salt content of elution buffer and phenol used in extraction methods (Loulakakis *et al*., 1996; Schultz *et al*., 1994). Hence, LiCl method requires an additional clean-up step (phenol or chloroform-phenol extractions in the presence of about 0.3 M sodium acetate) to improve its purity. Except

LiCl method, obtained ratios for other methods were close to the threshold number of 2 and were considered satisfactory.

Visualization of the major ribosomal RNA (18S or 28S rRNA) bands and any degradation products, can provide a general information about RNA integrity. Generally, intact 28S: 18S rRNA bands and a 28S : 18S rRNA intensity ratio of 2 : 1, are indicative of RNA integrity. Therefore, in the present study electrophoresis was performed to further validate the quality (integrity) of RNA extracted using the three different methods. Total RNA from commercial kit and large RNA extracted by TRIzol and LiCl methods were run on 1.0% agarose gel electrophoresis (Fig. 3) showed the presence of intact and clearly visible 28S and 18S rRNA bands, indicative of good quality RNA, extracted by all of the three methods.

However, TRIzol method produced more distinct 28S and 18S rRNA bands in agarose gel, than those of the other two methods indicating the ability of this reagent for effective inhibition of RNase activity and maintaining the integrity of the RNA species (Zhu *et al*., 2012). Table 2 shows the yields of total RNAs extracted from co-infiltrated patches of *N. benthamiana* plants with PVA HC-Pro viral suppressor and *GFP* reporter gene.

There were large differences in the amount of RNA extracted from 0.05 gram of leaf-tissue, among the three protocols. LiCl method produced the highest yield of RNA (3,782 ng/µL) (Table 2).

Also, the TRIzol method yielded good RNA quantity (1.336 ng/µL) (Table 2). However, the yield of total RNA extracted with DenaZist kit exhibited the lowest mean value (113.9 ng/µL).

The quality of RNA samples with the three methods was assessed for use in q-PCR assay. For this purpose, the expression patterns of house keeping gene, *EIF-1α* and target genes *AGO1* and *ATG6* were analyzed and presented as cycle threshold (Ct) values.

As shown in Figs. 4 and 5, the Ct values obtained from TRIzol, LiCl and kit methods were 23.09 ± 1.83, 30 ± 2.2 and 23.48 ± 3.3 for EIF-1α, 33.91 ± 2.12, 36.26 ± 1.7 and 35.4 ± 0.85 for AGO1, 26.51 ± 1.08, 29.38 ± 2.51 and 28.47 ± 1.06 for ATG6, and 23.47 ± 1.92, 27.13 ± 1.34 and 26.17 ± 1.28 for EIF-1α in ATG6 experiment, respectively. The Ct values of housekeeping gene, *EIF-1α* and target genes *AGO1* and *ATG6* for RNA extracted using LiCl and kit were 1.07 to 1.3 and 1.07 to 1.12 times higher than those evaluated with the TRIzol method, clearly indicating that RNA extracted with TRI reagent is of higher quality.

In current study, the data obtained by q-PCR revealed lower Ct values of the housekeeping and target genes, (*EIF-1α*, *AGO1* and *ATG6*) for RNA extracted using TRI reagent, compared to those of LiCl and DenaZist methods (Fig. 4 and 5). The extracted RNA samples need sufficient RNA for use in gene expression analysis such as q-PCR.

These results indicated that RNA isolated by our protocols was suitable for q-PCR with no interference in PCR amplification. It allows to study plant genes expression in the green area (indicating GFP expression in the presence of VSR) in response to viral suppressors.

Table 1. Details of specific primers used in this study

| Gene name | Primer name | Sequence from 5' to 3' | Expected product size (bp) |
|---|---|---|---|
| AG01 | AG01-F | GCCATGGGGCACCTTCTG | 132 |
| | AG01-R | GAGACGAGGAACCAGCCTC | |
| ATG6 | ATG6-F | ACCTGCGTAAAGGAGTTTGCTTC | 164 |
| | ATG6-R | AGAGCTTTGGTCCAACTTTCCTGC | |
| EIFI-α | EIFI-α-F | AGCTTTACCTCCCAAGTCATC | 135 |
| | EIFI-α-R | AGAACGCCTGTCAATCTTGG | |

F = Forward strand, R = Reverse strand

Table 2. Purity and yield* analysis of total RNA from *N. benthamiana* extracted by three different methods

| Method | Average 260/280 | Average 260/230 | Large RNA yield (ng/µL) |
|---|---|---|---|
| TRIzol | 1.95 (0.08)[a] | 2.06 (0.09)[a] | 1336[a] |
| LiCl | 1.74 (0.04)[b] | 1.84 (0.08)[b] | 3782[b] |
| Kit | 1.90 (0.09)[a] | 1.98 (0.06)[c] | 113.9[c] |

Yields and spectrophotometric A260 / A280 and A260 / A230 ratios of total RNA extracted from *N. benthamiana* plants expressing PVA HC-Pro viral suppressor with different extraction methods. 0.05 g of tissue was used for each RNA extraction method and quantified in a Nanodrop spectrophotometer. Values are spectrophotometric A260 / A280 and A260 / A230 means (standard deviation (SD)) from a total of nine plants (three plants in three replicates). Means followed by the same letters are not significantly different from each other at ($P = 0.05$) determined by Duncan's Multiple Range Test (DMRT).

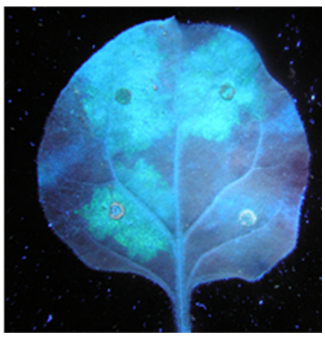

Fig. 1. Leaves of *N. benthamiana* infiltrated with a mixture of *A. tumefaciens* cells harboring pBinGFP plus either pBin PVA-HC-Pro or pBin (right side, down). Detection of GFP fluorescence in detached *N. benthamiana* leaves was performed using a hand-held long-wave UV lamp UVL-56 (UV Products) at 5 dpi

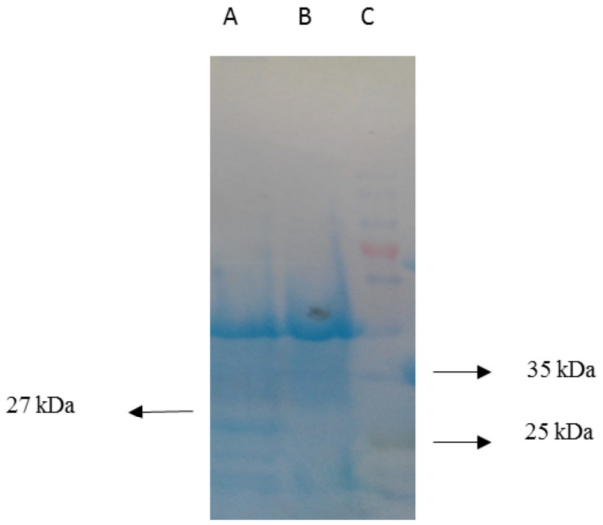


Fig. 2. SDS-PAGE analysis of the expressed GFP in *N. benthamiana*. Total protein samples from pre-infiltrated leaves with PVA HC-Pro and EP (lanes A and B). Lane C, protein size marker. The presence of a band of approximately 27 kDa in the infiltrated leaves with PVA HC-Pro sample but not in EP sample confirmed the expression of GFP in the presence of PVA HC-Pro

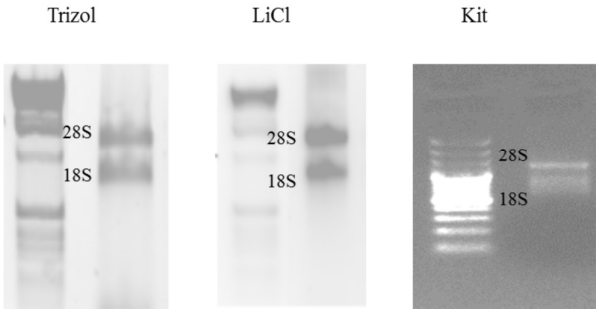


Fig. 3. Comparison of total RNA isolated from *N. benthamiana* plants expressing PVA HC-Pro viral suppressor with different extraction methods. Total RNA was extracted with three different RNA extraction methods. 0.05 g of tissue was used for each sample and 3 µg volume of each sample was loaded on a 1% agarose gel (gel stained with ethidium bromide)

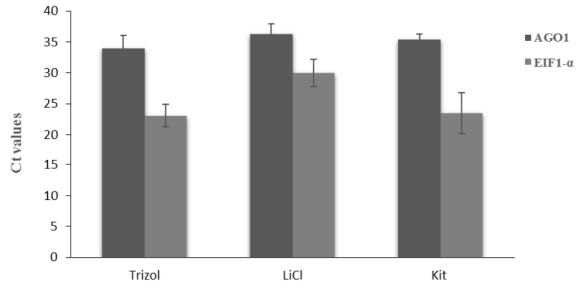


Fig. 4. Graph of cycle threshold (Ct) values of samples tested by the real-time q-PCRfor *AGO1* in *N. benthamiana* leaf samples harvested at 5 dpi and extracted using either theTRIzol, LiCl or commercial kit methods. Total RNA was extracted using either theTRIzol, LiCl or commercial kit methods. Data were expressed as cycle threshold (Ct) values. Then, Ct values of housekeeping gene *EIF1-α* and target gene *AGO1* was compared using three different RNA extraction methods by SYBR green real time q-PCR, each consisting of three biological and technical replicates. Error bars represent the standard deviation of the mean

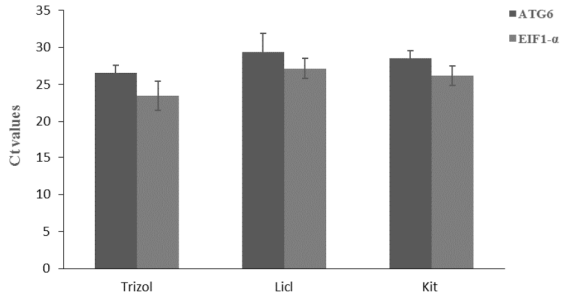


Fig. 5. Graph of cycle threshold (Ct) values of samples tested by the real-time q-PCRfor *ATG6* in *N. benthamiana* leaf samples harvested at 5 dpi and extracted using either theTRIzol, LiCl or commercial kit methods. Total RNA was extracted using either theTRIzol, LiCl or commercial kit methods. Data were expressed as cycle threshold (Ct) values. Then, Ct values of housekeeping gene *EIF1-α* and target gene *ATG6* was compared using three different RNA extraction methods by SYBR green real time q-PCR, each consisting of three biological and technical replicates. Error bars represent the standard deviation of the mean

## Conclusions

Although leaf patches of *N. benthamiana* were used, TRI reagent method can be particularly useful for anyone working with small leaf samples that are not amenable to multiple independent extractions. Overall, based on high total RNA yield, optimum absorbance ratios and q-PCR results, the TRIzol method was selected as the best for further studies.

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