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Quality affecting factors of RNA – its assessment and influence on PCR Reactions

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***Abstract*—** Good quality of RNA is the prime concern in molecular biology research works and clinical diagnosis. Different organic solvents and salt are used for RNA-isolation. Presence of different organic solvents like phenol, trizol, chloroform, isopropanol, ethanol and salt like EDTA in RNA may affect the downstream processes. The effect of these different organic solvents and EDTA on the quality /integrity of RNA and its effect on qualitative and qPCR has been evaluated in our study. We have found that the trace contamination with phenol and trizol in isolated RNA has inhibited the qPCR and its contamination in RNA cannot be identified merely by inspecting the A260:A280 and A260:A230 ratios. However, by evaluating the absorbance peak-pattern using UV absorbance between 220-340 nm, we can differentiate phenol/trizol contaminated RNA. Also, an overestimation (4-200 fold) of nucleic acid was observed for phenol and trizol-treated RNA in our study. Absorbance peak-pattern variations could only identify higher concentrations of EDTA in RNA, but for lower concentrations, it remains undetectable and higher concentrations of EDTA in RNA have shown to inhibit real-time PCR and alter the Ct value at its lower concentration. RNA contaminated with ethanol, isopropanol, chloroform cannot be distinguished even from the peak-pattern analysis and the UV-absorbance ratios. Higher concentration of alcohol decreased the PCR efficiency by huge variation in Ct values whereas lower concentrations did not show much effect. Surprisingly, the RNA integrity on agarose gel remained intact with all the organic solvents and EDTA-treated RNA.

***Keywords*—** *RNA integrity, Real time PCR, Basic Molecular Biology, Clinical Diagnostics, Transcriptomic Analysis*

I. INTRODUCTION

The isolation of an intact RNA is essential for gene expression studies. Purity and integrity of RNA are critical elements for the overall success of RNA-based analysis. For the past few decades, molecular research without the use of RNA is not imaginable. Similarly, the advancement in molecular diagnosis has made the use of RNA inevitable in molecular laboratory of clinical sectors. Till date, Trizol method is the most accepted method for RNA isolation. The resulting RNA may contain the trace of reagents which were used during the isolation. This may affect the quality of RNA and subsequently, the downstream results get affected. The ratio of absorbance at 260 nm, 280 nm, and 230 nm are generally monitored to assess the quantity and quality of RNA. The average concentration of nucleic acid (DNA/RNA) is measured usually at 260 nm, and protein concentration at 280 nm. Then other contaminants including organic solvents are estimated at 230 nm [1][2][3]. So, the ratio of 260/280 and 260/230 is evaluated to determine the purity of the nucleic acid obtained. Similarly, the integrity of RNA is assessed using agarose gel. Total RNA run on agarose gel will have sharp, clear 28S and 18S rRNA bands (eukaryotic samples); ratio of 28S:18SrRNA of about 2 indicate good quality of RNA [4].

To our knowledge, till date, no studies have shown that how the integrity of RNA and downstream experiments like PCR and RT-PCR is affected by the trace amount of organic solvents and salt used during isolation. So, in this experiment, we have deliberately introduced a few organic solvents and salt to study their effect on RNA integrity and downstream processes. Section I includes a simple introduction regarding our experiment. Section II contains the materials and methods used for our study. Section III contains our experimental results and Section IV discusses our experimental results and interprets it. In Section V, we conclude our experimental work.

II. METHODOLOGY

RNA Isolation

RNA was isolated from K562 cell line using trizol reagent (Ambion Life Technologies; Thermo Fisher Scientific) as per the manufacturer's protocol. Total RNA was dissolved using RNase and DNase-free water and was

aliquot to fresh tubes. Aliquots were used for further experiments.

To each aliquot of K562 RNA, Ethanol, Isopropanol, Chloroform, Trizol, Phenol, Ethylenediaminetetraacetic acid [EDTA] were added in 4 dilutions (Table 1). All the organic solvents and the EDTA used were of analytical grade.

Quantity, Quality and Integrity analysis

After adding the solvents and salt, the RNA was incubated at 4º C for 30 minutes. Quantity and purity was analysed by Nanodrop spectrophotometer and Integrity was analysed using 1% agarose gel electrophoresis. 1 µl of RNA sample was loaded onto the nanodrop and the reading was taken. Purity analysis using UV Absorbance 260/280 ratio and 260/230 ratio; and quantitation using absorption at 260nm was performed. 3 µl of RNA was loaded to 1% Agarose gel and by analysing the intensity of 28s RNA and 18s RNA, the integrity of all the RNA samples were evaluated.

Effect of contaminates on downstream application analysis

The influence of different organic solvents and salt on the downstream steps was evaluated using PCR. Equal quantity of RNA (based on 260nm) was converted to cDNA using High capacity cDNA Reverse transcription kit by Applied biosystems (Thermo Fisher Scientific). PCR was performed for all sample using the 5ul of cDNA, abl (F) and abl (R) in 25µl.

Further, to evaluate the effect of chemical on the quantitative PCR, equal quantity of untreated RNA from two independent isolates were taken and the EDTA, Isopropanol, Trizol or Phenol was added in five concentration (50%, 25% 10%, 1% and 0.1%) prior to cDNA synthesis.

III. RESULTS

Quantity, Quality and Integrity analysis

NanoDrop analysis showed a single peak at 260 nm and ratio of 1.96 for A260/280 and 2.13 for A260/230 indicating good quality of isolated untreated RNA (Figure-1b& Table-2). After diluting the stock for 10 times the ratio showed a slight variation. Similarly, RNA resolved in 1% agarose gel showed two intact bands of 28s rRNA and 18s rRNA which indicates the intactness of isolated RNA (Figure-1a). Further, the integrity analysis of the RNA after adding Ethanol, Isopropanol, Chloroform, Trizol, Phenol or Ethylenediaminetetraacetic acid (EDTA) showed that RNA intactness is not affected by the organic solvents or salts (Figure-3). RNA treated with ethanol, chloroform or isopropanol showed only one single parabolic peak at 260 nm (Figure-2), however the RNA sample treated with EDTA showed peak shift to 225-240nm with an increase in peak height awfully (Figure-2). For trizol and phenol, RNA showed a broader peak that got shifted to 250-290 nm and peak height also increased drastically (Figure-2).

RT PCR analysis

Qualitative PCR:

The major intention of the study was to understand the effect of the contaminants during RNA isolation procedures on the downstream process. Therefore, initially the qualitative PCR was performed to evaluate the effect of different contaminants on PCR reaction. Results showed that except ethanol and isopropanol all other solvent used in the study showed complete or partial inhibition of the PCR reaction (Figure-4).

Quantitative PCR(qPCR):

To understand the qPCR inhibitory effect, the contaminants with increasing concentration, were used in the real time PCR method. Results showed that the EDTA, Phenol and Trizol showed complete inhibition of the PCR reaction on Real time PCR. However, in conventional PCR these organic solvents and EDTA showed only partial inhibition. Interestingly the isopropanol which had not shown any major inhibitory effect on the qualitative PCR showed great variation in *Ct* value in the Real Time PCR for its higher concentration (Figure-5).

IV. DISCUSSION

Purity and integrity of the isolated RNA is most important for reproducible and reliable results using RNA based experiments[5]. Hence, the quality and integrity analysis of RNA is performed before any downstream analysis. Apart from the scientific publication, RNA is extensively used for the molecular diagnosis. In the clinical scenario, a low-quality RNA may strongly compromise the results, which might lead to a wrong clinical interpretation. Hence, understanding the quality of isolated RNA is essentially important and how it affects the downstream processes needs to be unveiled. Isolation includes different types of chemicals other than Trizol. Hence the chance of the carryover contamination is very high. Therefore, in the present study, we have evaluated the effect of possible contaminates by deliberately introducing a trace amount of chemicals to the isolated RNA.

In the present study, we used the A260: A280; A260: A230ratios and UV absorption pattern between 220-340nm for assessing the RNA purity and agarose gel electrophoresis for the integrity of the RNA. In our experiment, we have found that the A260:A280 ratio for RNA treated with Isopropanol, Ethanol, Trizol, and Chloroform was righteous as control RNA (Table-2), whereas for higher concentration of EDTA, the A260: A280 ratio increased to about 3 and a decrease in A260: A280 value for highest concentration (10%) of phenol observed. Nanodrop spectrophotometric reading has shown a decline in A260: A280ratio for RNA with the higher concentration of phenol (10%). A ratio of absorbances at 260 and 280 nm (A260: A280) greater than 1.8 is usually considered an acceptable indicator of pure

RNA [6][1][4]. The A260: A280 value about 2.0 is considered to be typical of 'high quality' RNA without protein contamination. Significantly different 260/280 ratios may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm[7]. However, no significant difference in 260:280 observed for the RNA samples contaminated with the trace amount of phenol, Trizol or chloroform, but this trace contamination had reduced PCR reaction efficiency.

It is well known that the low A260: A280 ratio indicate the protein contamination and low A260: A230 ratio indicates other contaminants. The A260: A230ratio was decreased drastically for higher concentrations of EDTA-treated RNA and the A260: A230 ratio has been reduced slightly for RNA treated with higher concentrations of phenol and trizol compared to the control RNA. It has also been shown thatA260: A230value between1.8-2.2 is an indicator for pure RNA in which the organic solvent contamination is negligible [8]. In our results, A260: A230ratio was found to be in between 1.8-2.3 for most of the RNA samples treated with the trace amount of organic solvent/salt (Table-2).

The most common method used to assess the Integrity of total RNA is to run an aliquot of an RNA sample on agarose gel stained with ethidium bromide (EtBr). For an intact eukaryote RNA, 28S rRNA band should be approximately twice as intense as the 18S rRNA band. Partially degraded RNA will have a smeared appearance, which lacks the sharp rRNA bands, or will not exhibit the 2:1 ratio. Studies have shown that fragmentation of RNA can potentially compromise results of downstream processes[9][5][4]. Interestingly, we observed that all RNA samples treated with the organic solvent or salt had similar RNA band pattern as of control RNA, which indicated that the chemicals had not influenced the RNA stabilization. However, the trace contaminates affected the PCR efficiency.

We have also found that a drastic overestimation of nucleic acid befalls with Trizol and Phenol contamination in RNA. Even the lowest concentration (0.01%) of phenol and Trizol-treated RNA showed an overestimation of RNA without affecting the ratio of A260: A280 and A260: A230 (Table-2). Hence, considering the ratio values and gel images, the amount of nucleic acid taken will be less than the actual concentration of nucleic acid. It may affect further downstream processes and even lead to wrong interpretation of results. Even the lower concentration of phenol and trizol-treated RNA showed variation in the peak pattern of nano-drop. So, a careful examination of peak pattern would help in identifying the trace contaminants of trizol and phenol in nucleic acid samples. An ideal peak-pattern for a control RNA is shown in Figure-1b(parabolic peak with an absorption maximum at 260 nm and a peak pattern ranging around 235 nm – 285nm), whereas a major Peak-shifts and alterations in peak pattern were observed for Phenol, Trizol and EDTA-treated RNA (Figure-2).The peak-pattern at 260 nm got deviated largely and shifted to 225-240 nm in range for higher concentration of EDTA. Peak pattern extend from 225 nm to 290 nm range for highest concentrations of phenol and trizol-treated RNA. But for the isopropanol, ethanol and chloroform-treated RNA, peak pattern was similar to the untreated RNA(Figure-2).

Our results from Qualitative PCR(Figure-4) showed that the isopropanol and ethanol contamination has not affected the PCR reaction efficiency. The amplification efficiency of Qualitative PCR was monitored by agarose gel and was found to be reduced for chloroform, phenol and trizol-treated RNA as a decreased band-intensity was observed in Agarose gel. The previous study by Lebuhn et al. showed that Guanidinium isothiocyanate does not affect qPCR [10]. So the inhibitory effect by trizol may be contributed by the phenol or the salt present in it. The quantitative PCR analysis of all the organic solvent and salt-treated RNA samples clearly shown inhibition. In qPCR, the quantity of PCR product in the exponential phase is proportional to the initial amount of target DNA. When fluorescence crosses the fluorescence threshold (arbitrarily chosen within the exponential phase), the cycle is termed the Threshold cycle (Ct) or 'Crossing Point' and higher the Ct, the smaller the initial amount of DNA [11]. Isopropanol-treated RNA found to alter the quantitative PCR results in Ct values (Figure-5b). It is undetectable in nanodrop spectrometer (using spectrum of A220-340). This kind of contamination may alter the results. The Real-time PCR for higher concentrations of EDTA inhibited completely, and the Ct values for other lower levels were in the range of 38 to 40 and even higher (Figure-5a).However, for the lowest dilutions of trizol, phenol-treated RNA, the quantitative PCR has shown selective inhibition for BCR-ABL and ABLin an unpredictable manner and even the Ct values altered significantly. These variations might have occurred due to the interference of phenol/trizol with the fluorochrome binding with cDNA.

It is considered that RNA integrity and purity determine the efficiency of RT reaction and yield of PCR [12]. However, different studies have also shown that the intact RNA does not assure good results because RNA sample may contain inhibitors that can reduce reaction efficiency [13][14]. Studies have already proven that regarding the RNA-integrity, that sub-optimal quality of RNA can also give meaningful results indicating that partially degraded RNA may not affect the efficiency of PCR[15]. Hence, currently, the ratio of 260/280 and 260/ 230 is used extensively for the quality analysis of the RNA. PCR efficiency could also be influenced by various other factors like annealing temperature, the primer length or by the length of the amplified product [16]. However, all these are experimentally controlled factors. Here we have shown that trace contaminate had not affected the absorbance ratio, but the PCR efficiency was affected drastically. So, minor contamination with phenol or trizol or other contaminants

can lead to misleading results of clinical samples which may have far-reaching impact. Hence, more importance has to be given for evaluating the purity of an isolated RNA.

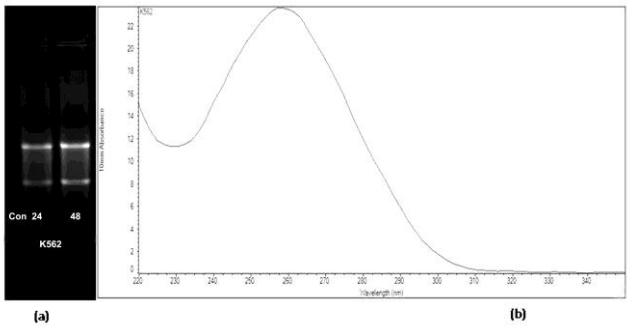

Figure 1: (a) K562 RNA showing two distinct intact bands of 28s rRNA and 18s rRNA on 1% Agarose gel**; (b)** Absorbance spectral graph of K562 RNA. Ideal peak for a good quality RNA at 260 nm visible.

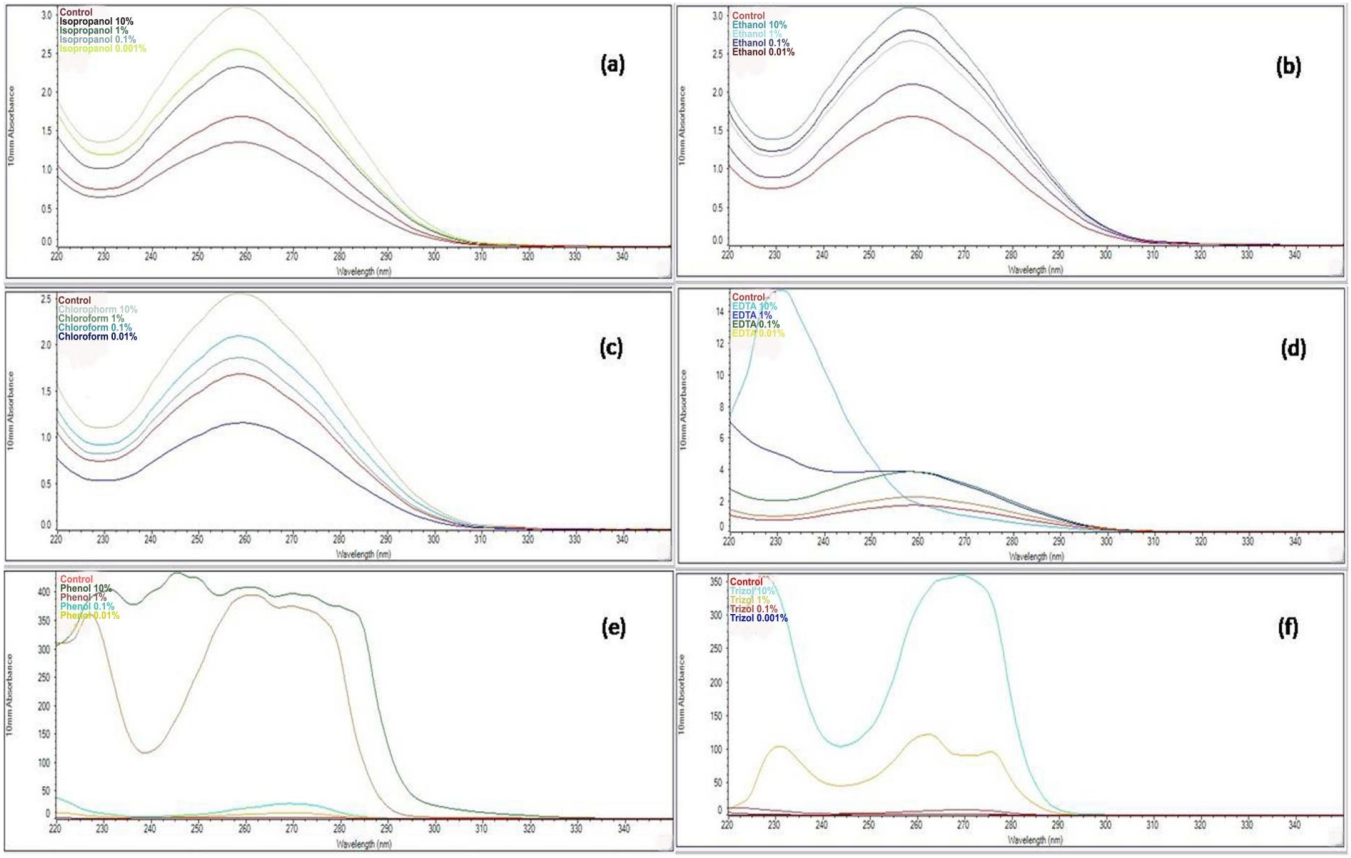

Figure 2: Absorbance spectral graph of different dilutions of **(a)** Isopropanol, **(b)** Ethanol, **(c)** Chloroform, **(d)** EDTA, **(e)** Phenol and **(f)** Trizol-treated RNA with respect to control K562 RNA.

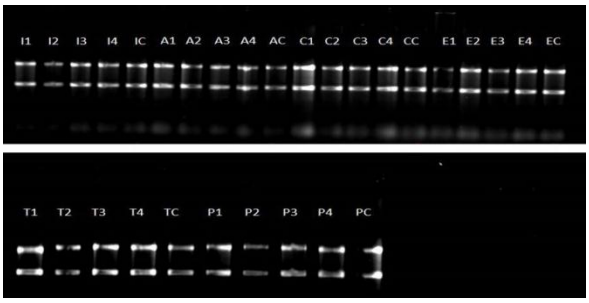

Figure 3: The RNA integrity of solvent/salt treated k562RNA checked by running samples on 1% Agarose gel. I1- I4: Isopropanol dilutions; A1-A4: Ethanol dilutions; C1-C4: Chloroform dilutions; E1-E4: EDTA dilutions; T1-T4: Trizol dilutions; P1-P4: Phenol dilutions; PC: Positive control.

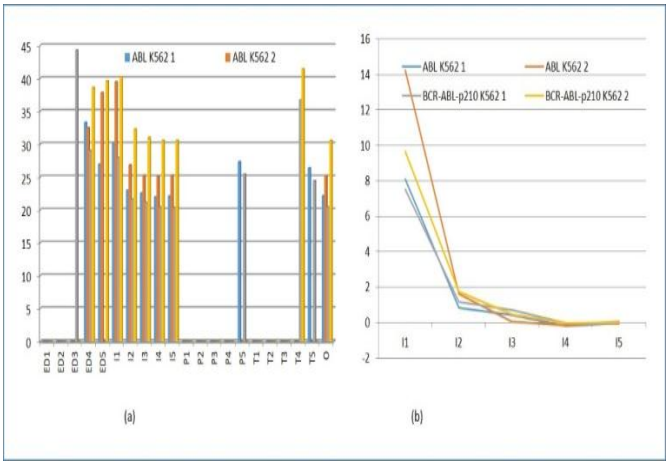

Figure5: **(a)***Ct* value of EDTA, Isopropanol, Phenol, Trizol-treated RNA and untreated RNA (O). EDTA (ED1-ED5), Isopropanol (I1-I5), Phenol (P1-P5), Trizol (T1-T5); **(b)** Difference in the *Ct* value of *ABL* and *BCR-ABL* in isopropanol treated RNA compared to untreated RNA.

Solvent/salt	Dilutions	Concentration
Isopropanol(I), Ethanol(A), Phenol(P), chloroform(C) and Trizol (T)	1	10%
	2	1%
	3	0.1 %
	4	0.01%
EDTA (E)	1	50 mM
	2	5 mM
	3	0.5 mM
	4	0.05 mM

Table 1: The different dilutions of Isopropanol, Ethanol, phenol, chloroform and Trizol and EDTA used for the

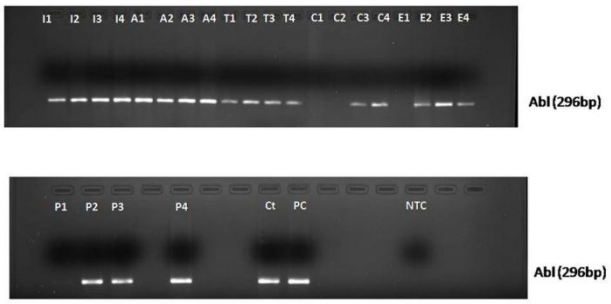

Figure 4: PCR for *ABL* using the solvent/salt treated samples run on 2% Agarose gel. I1- I4: Isopropanol dilutions; A1-A4: Ethanol dilutions; C1-C4: Chloroform dilutions; E1-E4: EDTA dilutions; T1-T4: Trizol dilutions; P1-P4: Phenol dilutions; Ct: control NTC: Non template control.

Sl no	sample	Nucleic acid (ng/µl)	260/280	260/230
1	K562 RNA	670.7	1.96	2.13
2	K562 control	67.1	1.82	2.27
3	I1	53.8	1.93	2.11
4	I2	92.7	1.85	2.3
5	I3	123.6	1.82	2.29
6	I4	101.5	1.92	2.14
7	A1	123.7	1.89	2.24
8	A2	106.3	1.86	2.28
9	A3	111.8	1.84	2.27
10	A4	83.8	1.76	2.37
11	C1	74.1	1.84	2.27
12	C2	102	1.77	2.32
13	C3	83.4	1.78	2.28
14	C4	46.1	1.86	2.19
15	P1	16351.8	1.09	1.02
16	P2	15757.7	1.37	1.26
17	P3	730	1.72	2.04
18	P4	294.5	1.82	2.08
19	T1	12459.5	1.84	0.91
20	T2	4674	2.25	1.14
21	T3	246	1.75	0.86
22	T4	73.5	1.72	1.26
23	E1	71.2	3.09	0.12
24	E2	151.3	2.13	0.76
25	E3	151.8	1.99	1.92
26	E4	88.2	1.8	2.22

V. CONCLUSION

Evaluating the purity of isolated RNA is still a matter of concern with the current molecular biology techniques. Sometimes a good ratio of Absorbance A_{260}:A_{280}, A_{260}:A_{230}value cannot really show the extent of contamination in a nucleic acid sample as seen from our study. The solvents like ethanol, isopropanol and chloroform-contaminated samples cannot be easily identified using current purity-quality-integrity analysis methods. The only way to eliminate such solvent contamination is to properly air-dry the RNA samples. If one has followed a thorough and careful isolation of RNA, a good-quality RNA could be obtained. The nucleic acid quantitation is necessary to determine the amount of RNA required for further downstream processes. Bizarre overestimation of nucleic

acid may happen due to the phenol or Trizol contamination even at trace levels. This may lead to take less RNA than true quantity of RNA. A parabolic peak with an absorption maximum at 260 nm is a good indication of pure isolated nucleic acid. An interrupted peak-pattern indicates sample contamination with organic solvents like phenol, trizol and salts like EDTA used during isolation. A careful isolation could possibly minimize the contamination rate with these solvents. Thus, A_{260}:A_{280} ratio and A_{260}:A_{230} ratio alone cannot be taken as a gold standard for analysing purity of RNA. Simultaneously, the peak at 260 nm should also be evaluated to check for salt, phenol or trizol contamination.

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Sheethel Kunnath Poovakka & Durga Ramadas equally contributed for the paper

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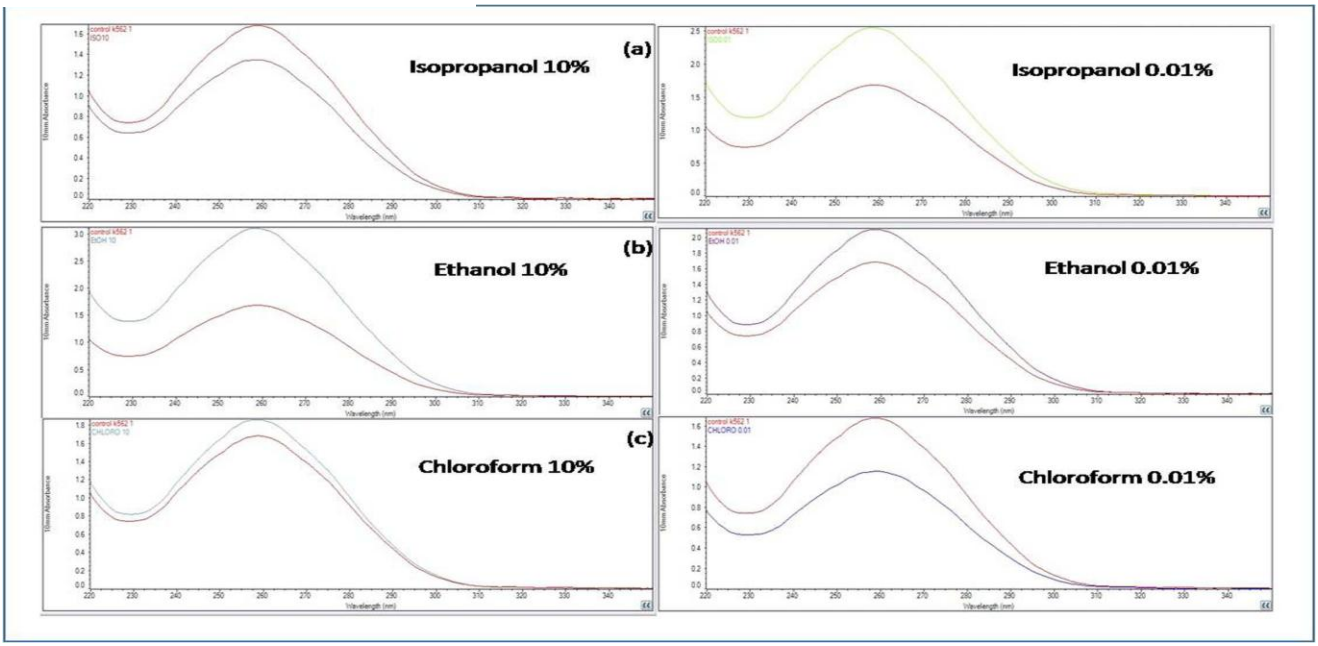
Ms. Durga Ramadas Nayanar, currently pursuing her B Tech Chemical Engineering degree from Amrita School of Engineering, Amrita Vishwa Vidyapeetham, Coimbatore, India. She has been in the top five positions in her batch. She is interested in developing a simple and novel technique of reactive extraction which

can lead to higher reaction rates and microchannel reactors. She has done her project work from IIT Mumbai.

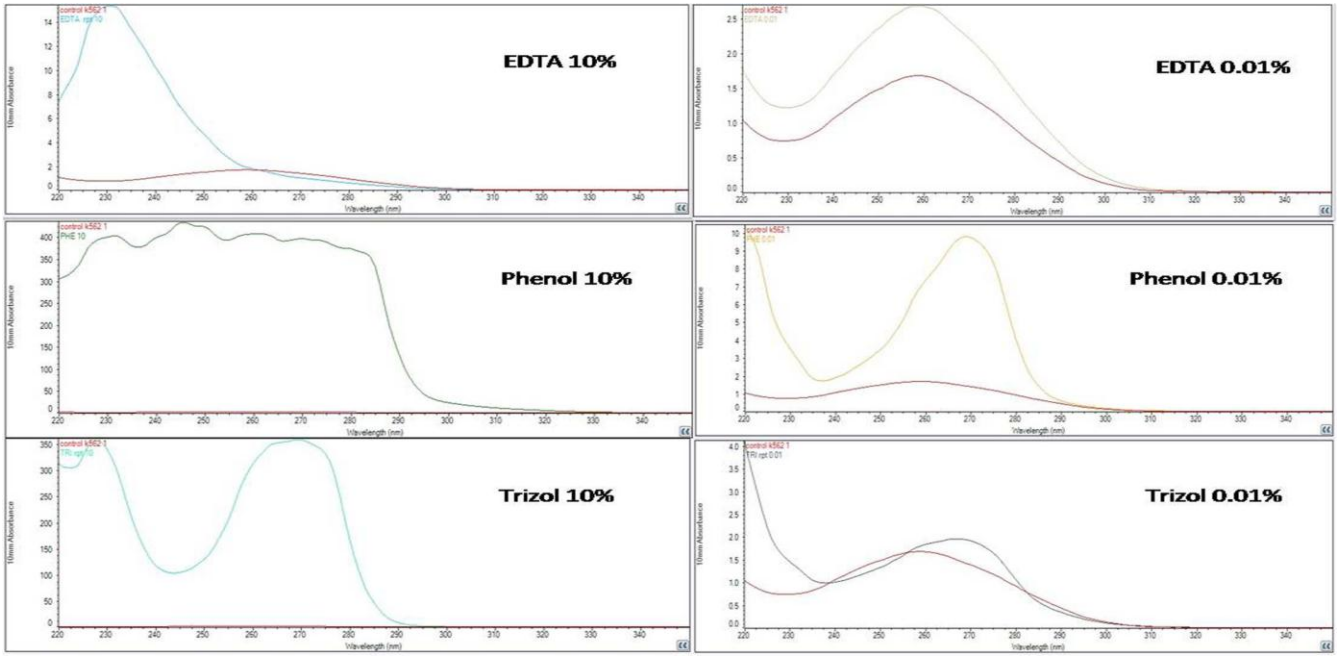
Ms. Sheethel Kunnath Poovakka completed her M.Sc degree in Biotechnology from Amrita School of Biotechnology. Currently, working as a Project Fellow at Division of Genetics and Cytogenetics, Malabar Cancer center, Thalassery. Her research interests are cancer biology and cancer genetics.

Dr. Deepak Roshan V G completed his Masters in Biotechnology from Bharathiar University and PhD degree in cancer biology from Kerala University. He is currently working as Assistant Professor in Division of Genetics at Malabar cancer center, Thalassery. Research interest is cancer biology – genetics, epigenetics, and cell signaling

Supplementary figure and tables



Supplementary Figure 1: (a) Control RNA vs Isopropanol treated RNA- 10% and 0.01%; (b) Control RNA vs Ethanol treated RNA- 10% and 0.01%; (c) Control RNA vs Chloroform treated RNA- 10% and 0.01%.



Supplementary Figure 2: (a) Control RNA vs EDTA treated RNA-10% and 0.01%; (b) Control RNA vs Phenol treated RNA- 10% and 0.01%; (c) Control RNA vs Trizol treated RNA- 10% and 0.01%.

Primer Sequence

Primer sequences used for qualitative PCR and qPCR

Primers used for qualitative PCR

Primers	Primer sequence (5' to 3')
ABL– Forward	TTCAGCGGCCAGTAGCATCTGACTT
ABL – Reverse	CCAGGAGTGTTTCTCCAGACTG

Primers used for qPCR

Primers	Primer sequence (5' to 3')
BCR-ABLMbcr/mbcr-ABL-Pr	FAM-CCCTTCAGCGGCCAGTAGCATCTGA-TAMRA
BCR-ABLMbcr/mbcr-ABL-R	CACTCAGACCCTGAGGCTCAA
BCR-ABLMbcr/mbcr-ABL-F	TCCGCTGACCATCAAYAAGGA
CONTROL-ABL-F	TGGAGATAACACTCTAAGCATAACTAAAGGT
CONTROL-ABL-Pr	FAM-CCATTTTTGGTTTGGGCTTCACACCATT- TAMRA
CONTROL-ABL-R	GATGTAGTTGCTTGGGACCCA

PCR condition for Real Time PCR

Temperature (ºC)	Time (Min)	No of Cycles
50	2	1
95	10	1
95	0.15	45
60	1	

PCR condition for qualitative PCR:

Temperature (ºC)	Time (Minutes)	No of Cycles
94	5	1
94	1	35
62	1	
72	2	
72	10	1

