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Troubleshooting of RNA isolation methods in Papanicolaou HPV infected smears

Tea Bećirević[1], Mirza Suljagić[1], Daria Ler[1,2]
[1] Genetics and Bioengineering Department, International University of Sarajevo, Sarajevo, Bosnia and Herzegovina
[2]Molecular Diagnostics Laboratory, Eurofarm Center Sarajevo, Bosnia and Herzegovina

ABSTRACT

Cervical cancer is one of the leading causes of cancer in women, worldwide. Infection with human papillomavirus (HPV) has been accepted as the primary cause for the development of invasive cervical cancer and its precursor lesions. Despite HPV infection has been proposed as an indispensable factor for cervical cancer development, only a subset of neoplastic lesions with HPV infection persist and progress to invasive cancer. This suggests us that other molecular events are also involved in cancer progression. Aim of this study was to extract mRNA from cytobrush-collected healthy and HPV infected cervical epithelial cells and investigate various RNA extraction and purification protocols for assessment of RNA yield and quality. Taking into consideration that cervical cancer screening is based on the cytology based Papanicolaou test (Pap test), main challenge is to investigate whether the samples obtained by regular Pap testing can be used for gene expression analysis. For this purpose, a total of 68 cervical specimens were previously tested for HPV infection. Following HPV testing, samples were submitted to RNA extraction and compared to the products after additional purification step involving DNase I. Products obtained after different RNA extraction and purification methods were visualized using 2% agarose gel electrophoresis. In conclusion, DNase I based RNA purification represents a necessary step for the assurance of a high-quality extracted RNA used for gene expression analysis studies. Reliance on commercial kits for RNA extraction only, without performing additional purification step can lead to errors in drawing final conclusions and/or to false negative gene expression profiling, affecting the overall diagnostic procedure. According to obtained results, the type of sampling used in this study was not suitable for the subsequent gene expression analysis.

Keywords: HPV, Pap test, RNA extraction

Corresponding author:
Doc. Dr. Daria Ler
Molecular Diagnostics Laboratory,
Eurofarm Center Sarajevo,
Kolodvorska 5, 71000Sarajevo
Email: dariamail@gmail.com

1. Introduction

Cervical cancer screening is an essential part of women routine health and is preformed to detect abnormal cervical cells as well as early cancers. Cervical cancer screening includes two test types: cytology-based Papanicolaou (Pap) test and HPV testing. Currently, the European Guidelines recommend Pap test every 3-5 years starting at the age 22-30 [1]. Today it is well known that infection with HPV is the central cause for the development of invasive cervical cancer and its precursor lesions [2], [3], [4]. There are thirteen high-risk

(HR) HPV detected genotypes causing virtually all invasive cervical cancers (ICC's) worldwide [5], [6]. Nevertheless, the entire process of cervical carcinogenesis cannot entirely be explained by infection with HR HPV types. Differences between relatively high rates of HR HPV persistent infections and relatively low rates of HPV positive woman who actually develop carcinoma of the cervix suggest that other molecular events are also involved in cervical cancer progression. To analyze additional factors that together with HPV infection can trigger cervical cancer progression it is necessary to conduct targeted gene expression analysis study.

Differences in mRNA expression levels between individuals are thought to affect how much protein is produced from a gene, and therefore reflect gene expression. However, RNA molecule is very unstable and susceptible to degradation. The main reason of RNA genetic instability lies in the presence of 2' hydroxyl group (2'OH), making it a less stable then a deoxyribose molecule found in DNA (7). Furthermore, the large grooves on RNA molecule in comparison with relatively smaller grooves on DNA provide more docking space for RNA damaging enzymes. In order to prevent RNA degradation and make it more stable and accessible for further analysis, mRNA is synthesized into cDNA molecule. However, cDNA made from RNA that might have been, at least in part, degraded will compromise amplification process and will probably not be amplified or labeled to the same degrees as a cDNA made from an intact RNA. Therefore, determination of RNA quality is a critical initial step in any quantitative gene expression analysis workflow [8].

Aim of this study was to extract total mRNA, from healthy and HPV infected cervical epithelial cells, form a cytobrush-collected samples and investigate two different RNA extraction and purification protocols for assessment of RNA yield and quality. In particular we wanted to explore whether the samples obtained by regular Papa testing can be used for gene expression analysis. Since Pap test represents a non-invasive and necessary type of cervical screening for all women, possibility of using this type of samples in gene expression analysis would minimize the requirement of tissue samples that can be only obtained using invasive biopsy procedure. Another advantage of using cervical cytobrush samples in gene expression analysis is that the cells from the entire surface area of the cervix are being collected, while the cervical biopsy is commonly performed to the one specific site of the cervix collecting only the cells present in that particular area of the cervical tissue. RNA extraction was performed using two different extraction methods, one was based on commercially available RNA extraction kit while other method included commonly used RNA extraction by TRIzol® Reagent.

2. Materials and Methods

2.1. Samples

A total of 68 cervical cytobrush samples, tested for HPV infection at Institute for Biomedical Diagnosis and Research 'Nalaz' in Sarajevo (B&H) were included in the study. Samples were collected in the period between February 2014 and March 2015 and stored at -20C°. Only after the consent of the Institute and patient's agreement, tested samples were implemented in this study. For the collection and transport of cervical specimens and detection of HPV, Abbott Cervi-Collect Specimen Collection Kit (Abbott, Lake Country, IL, USA) was used. HPV testing was performed using NucliSENSEasyQ HPV assay (BioMerieux S.A. Marcy l'Etoile, France). The mean age of tested woman was 34 years (range 20 – 66 years) (table 1).

Table 1. Sixty-eight (68) HPV tested samples used for RNA extraction. K: RNA extraction preformed using the GeneJET™ RNA Purification Kit (Thermo Fisher Scientific, USA), T: RNA extraction preformed using TRIzol Reagent (Invitrogen, USA).

No.	Patient code	Year of birth	HPV genotype	RNA extraction method
1	5	/	HPV HR 16, HPV HR 18, HPV HR 52, HPV HR 45, HPV LR 42	K
2	7	1990	HPV HR 52, HPV HR 56, HPV HR 31, HPV HR 73, HPV LR 84/26, HPV LR 6	T
3	8	1983	HPV HR 16	T
4	11	1986	HPV HR 52, HPV LR 43/44, HPV LR 57/71, HPV LR 84/26	T
5	12	1983	HPV HR 18, HR2	K
6	13	/	HPV HR 52	K
7	**15**	/	HPV HR 16, HPV HR 39	K
8	16	1965	HPV HR 18	K
9	18	1989	HPV HR 16, HPV HR 18	K
10	19	1986	HPV HR 52	T
11	20	1987	HPV HR 16	T
12	21	1972	HPV HR 16, HR1, HR2	T
13	22	1985	HPV HR 18, HR2	K
14	25	1983	HPV HR 52, HPV HR 66/68, HPV HR 56, HPV HR 45, HPV LR 11	T
15	26	1973	HPV HR 52, HPV LR 40/61, HPV LR 54/55	K
16	29	1980	HPV HR 52	K
17	30	/	HPV HR 16	K
18	31	1978	HPV HR 52	K
19	32	1982	HPV HR 16, HPV HR 45	K
20	34	1972	HPV HR 18, HPV HR 52, HPV HR 53	K
21	36	1987	HPV HR 52, HPV LR 57/71	K
22	37	1972	HPV HR 52, HPV LR 57/71	K
23	39	1982	HPV HR 16, HR1 HR2	K
24	40	1985	HPV HR 52, HPV LR 57/71	K
25	41	1985	HPV HR 18	K
26	42	/	HPV HR 52, HPV HR 45, HPV HR 31, HPV LR 11	K
27	43	/	HPV HR 16, HPV HR 52, HPV HR 33, HPV HR 82, HPV LR 11	K
28	44	1990	HPV HR 52, HPV LR 57/71	K
29	45	1972	HPV HR 52, HPV HR 33, HPV LR 6, HPV LR 40/61	T
30	46	/	HPV HR 16, HPV HR 39	K
31	48	/	HPV HR 18	K
32	49	/	HPV HR 16	T
33	50	1980	HPV HR 16, HPV HR 56	K
34	51	1981	HPV HR 16, HPV HR 51	K
35	52	/	HPV HR 52, HPV LR 6, HPV LR 11, HPV LR 57/71	K
36	53	1983	HPV HR 16, HR1	K
37	54	1995	HPV HR 16, HPV HR 35, HPV LR 6	K
38	55	1990	HPV HR 16	T
39	56	/	HPV HR 16	K
40	57	1970	HPV HR 16, HPV HR 51, HPV HR 56, HPV HR 59, HPV LR 49/44	K
42	58	/	HPV HR 16	K
42	59	/	HPV HR 16, HPV HR 52, HPV HR31, HPV LR 11	T
43	60	1971	HPV HR 16	T
44	61	1987	HPV HR:16,39,45,53,56,58,59,66/68, HPV LR: 6, 40/61	T
45	62	1977	HPV HR 52	T
46	63	1992	HPV HR 16	T
47	64	1978	HPV HR 16	T
48	65	1977	HPV HR 16	T
49	66	/	HPV HR 16, HPV HR 59	T
50	67	/	HPV HR 16	T
51	68	1985	HPV HR 18	T
52	69	1971	HPV HR 16, HR1, HR3	K
53	70	1965	HPV HR 16, HR1	K

54	72	1985	LR HPV 54/55	K
55	76	1962	LR HPV 57/71	K
56	77	1982	LR HPV 57/71	K
57	78	1977	LR HPV 57/71	K
58	79	/	LR HPV 57/71	K
59	82	/	LR HPV 40/61	K
60	83	/	LR HPV 57/71	K
61	84	/	LR HPV 57/71	K
62	85	/	LR HPV 57/71	K
63	88	1975	HPV HR & LR neg.	K
64	89	1978	HPV HR & LR neg.	K
65	91	/	HPV HR & LR neg.	K
66	92	1967	HPV HR & LR neg.	K
67	93	1952	HPV HR & LR neg.	K
68	94	1990	HPV HR & LR neg.	K

2.2 RNA extraction

Total RNA extraction was performed using two different methods; one type of RNA extraction was accomplished using The GeneJET™ RNA Purification Kit (Thermo Fisher Scientific), the other included RNA extraction by TRIzol® Reagent following the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). RNA extraction from samples derived from HPV positive patients was conducted several months after the date of sample collection and storage at -20°C.For RNA purification step, DNase I, RNase-free kit (Thermo Fisher Scientific) was used.

To evaluate the quality of tested samples and analyze whether the storage conditions of samples could compromise RNA quality, we also used the samples obtained from healthy donors, which were processed the same day when the sample was obtained. As a positive control, the human Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) control RNA (Thermo Fisher Scientific, Waltham, MA, USA) was used. Total RNA extraction from healthy donor, using cytobrush, was performed using both extraction methods and experiments were conducted during the day of sampling.

To assess the presence and quality of extracted nucleic all extracted samples before and after the RNA purification were submitted to non-denaturing 2% agarose gel electrophoresis.

2.3 Spectrophotometry

Concentrations of isolated RNA were assessed by measuring the absorbance at 260 nm (A260) in a spectrophotometer. An absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA per ml (A260 = 1 = 40 µg/ml). In table 2 you can see the readings at 260 nm wavelength taken for thirty-three (33) samples. RNA concentrations were calculated according to the following formula:

$$c\ [ng/\mu l] = A260 \times DF \times 40$$

Dilution factor (DF) = 10 µl of RNA sample + 990 µl distilled water (1/100 dilution); A260 = absorbance of diluted sample measured in a 1 ml cuvette (RNase-free) ; Concentration of original RNA sample (c) = A260 x dilution factor x 40

3. Results

3.1 RNA extraction

Prior to RNA purification protocol, products of two different extraction protocols from 68 HPV positive samples were loaded on non-denaturing 2%agarose gel electrophoresis to examine the presence and quality of extracted nucleic acids. The basis of RNA extraction by TRIzol® Reagent is that RNA is separated from DNA with an acidic solution containing guanidinium thiocyanate, sodium acetate, phenol and chloroform, followed by centrifugation. Under acidic conditions, total RNA remains in the upper aqueous phase, while most of DNA and proteins remain either in the interphase or in the lower organic phase. Total RNA is recovered by precipitation with isopropanol, following sample centrifugation and RNA washing. Isolated RNA was resuspended in 50µl RNase free water. Figure 1. represents gel electrophoresis of isolated nucleic acids from seven (7) samples out of twenty (20) isolated using TRIzol® Reagent, samples were chosen randomly (see table 1).

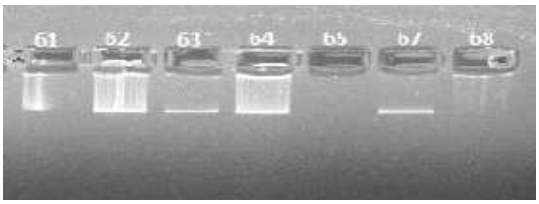

Figure 1. Gel electrophoresis of isolated nucleic acids from seven (7) randomly chosen samples (see table 1) using TRIzol® Reagent (Invitrogen, USA).

In contrast to TRIzol® Reagent, the GeneJET™ RNA Purification Kit utilizes a silica-based membrane technology in the form of a convenient spin column, eliminating the need for tedious cesium chloride gradients, alcohol precipitation or toxic phenol-chloroform extractions. Isolated RNA was eluted under low ionic strength conditions with 200µl of nuclease-free water. Figure 2. represents gel electrophoresis of isolated nucleic acids from sixteen (16) randomly chosen samples, out of total (48) isolated with the GeneJET™ RNA Purification Kit (see table 1.).

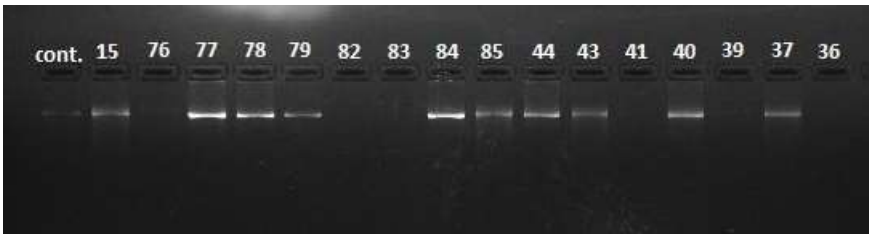

Figure 2. Gel electrophoresis of isolated nucleic acids from sixteen (16) randomly chosen samples (see table 1.) using GeneJET™ RNA Purification Kit (Thermo Fisher Scientific, USA)

Following RNA extraction by both protocols, RNA extracts were submitted to RNA purification, treating with DNase I, RNase-free solution. Following RNA purification protocol, samples loaded on to2% agarose gel, did not show any detectible bands. Figure3.shows results of three randomly chosen samples before and after RNA purification step.

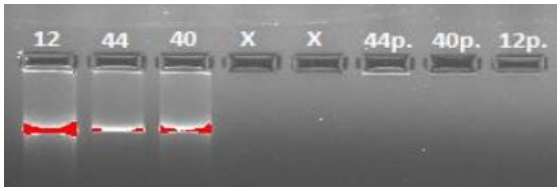

Figure 3. Samples before (sample codes: 12, 44 and 40) and after RNA purification (44p, 40p, and 12p).

Similar results were observed in samples from healthy patients (HD) that were submitted to RNA extraction during the same day when the sample was obtained. Extraction products following both extraction methods were successfully confirmed on 2% agarose gel electrophoresis, however after the RNA purification protocol no visible bands were detected (Fig. 4).

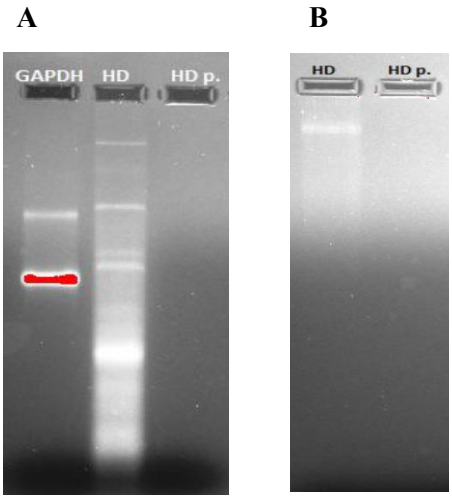

Figure 4. RNA extraction from, healthy donors (HD), using A) TRIzol® Reagent B) GeneJET™ RNA Purification Kit. No visible bands were detected after the sample purification (HD p.)

3.2 Spectrophotometry

Following RNA extraction, concentrations of isolated RNA were calculated by measuring absorbances at 260 nm (A260) in a spectrophotometer. According to the obtained RNA concentrations we could conclude that

there was no significant difference in quantitative RNA extraction yiel between the GeneJET™ RNA Purification Kit and TRIzol® Reagent used in this study (table 2).

Table 2. Spectrophotometry analysis of thirty-three (33) extracted samples. K: RNA extraction preformed using the GeneJET™ RNA Purification Kit (Thermo Fisher Scientific, USA), T: RNA extraction preformed using TRIzol Reagent (Invitrogen, USA).

No.	Sample code	RNA extraction method	A260	Concentration
1	11	T	0.27	1080 ng/µl
2	12	K	0.24	960 ng/µl
3	13	K	0.25	1000 ng/µl
4	16	K	0.25	1000 ng/µl
5	18	K	0.26	1040 ng/µl
6	22	K	0.24	960 ng/µl
7	26	K	0.25	1000 ng/µl
8	29	K	0.25	1000 ng/µl
9	30	K	0.24	960 ng/µl
10	31	K	0.25	1000 ng/µl
11	32	T	0.27	1080 ng/µl
12	37	K	0.25	1000 ng/µl
13	40	K	0.25	1000 ng/µl
14	43	K	0.25	1000 ng/µl
15	44	K	0.25	1000 ng/µl
16	48	K	0.26	1040 ng/µl
17	50	K	0.28	1120 ng/µl
18	53	K	0.27	1080 ng/µl
19	57	K	0.26	1040 ng/µl
20	59	T	0.27	1080 ng/µl
21	63	T	0.39	1560 ng/µl
22	67	T	0.30	1200 ng/µl
23	70	K	0.26	1040 ng/µl
24	72	K	0.27	1114 ng/µl
25	76	K	0.25	1000 ng/µl
26	77	K	0.28	1133 ng/µl
27	78	K	0.25	1000 ng/µl
28	79	K	0.26	1040 ng/µl
29	84	K	0.26	1040 ng/µl
30	85	K	0.23	940 ng/µl
31	88	K	0.26	1040 ng/µl
32	89	K	0.26	940 ng/µl
33	91	K	0.24	960 ng/µl

4. Discussion

Up to date only HPV based biomarkers for cervical cancer have been proposed. Integration of viral DNA into the host cellular genome and constitutive expression of the oncoproteins E6 and E7 represent two major events connecting HR HPV infection with the development of the cervical cancer [10, 11]. However, differences between relatively high rates of HR HPV persistent infections and relatively low rates of HPV

positive woman who actually develop carcinoma of the cervix suggest that the development of cervical cancer is a multistep process that cannot entirely be explained by infection with HR HPV types [12]. Additional factors such as altered oncogenic kinase signaling, activation of cellular proto oncogenes or chromosomal alternations may also contribute to the development of cervical cancer [13]. Clearly in order to investigate molecular events included in the progressions of cervical cancer in this particular case, mRNA obtained from total RNA extraction is necessary. Differences in mRNA levels between individuals are thought to affect how much protein is produced from a gene, and therefore reflect gene expression. However, both total RNA and mRNA molecules are very unstable and rapidly decomposed.

In this study we have experienced that total RNA extraction from cytobrush-collected cervical epithelial cells couldn't be accomplished with either of the two used techniques. A possible cause of RNA degradation could be relatively longer storage time (more than one month at -20°C.) of samples before RNA extraction. Nevertheless, after conducting the same extraction protocol on the fresh samples obtained during the same day, no visible RNA product could be observed following RNA purification step. These results clearly indicated that time and storage conditions are not the only factors that have contributed to RNA degradation in tested samples. Bearing in mind how highly susceptible for degradation the RNA molecule is, the sampling procedure can eventually compromise RNA molecules in epidermal area. Presented data demonstrate that the products of RNA extraction, using two different extraction methods are visible on the gel, however no bends could be seen after involving additional purification step. These results clearly indicate that RNA extraction products were contaminated with residual genomic DNA and degraded by DNAaseI in a subsequent purification step.

4.1. Conclusion

Results obtained by this study undoubtedly indicate the importance of the RNA purification step in all gene expression analysis studies, after using cytobrush sampling methodology. Since the RNA molecule is highly unstable and it has relatively low integrity in comparison with DNA molecule, most methods for RNA isolation will also extract traces of genomic DNA. If no appropriate measurements for RNA quality assurance have been undertaken by using DNase I in a purification step, these traces of DNA molecule can be mistakenly be seen as RNA extracts using simple agarose gel electrophoresis or UV absorbance ratios only. In addition, RNA degradation can occur very easily during any time of processing, particularly during the extraction process but also during the sample storage or post extraction preparation, leading to the complete loss of RNA. Usage of available RNA extraction commercial kits only, without a downstream assessment of an RNA quality, can lead to serious errors in gene amplification. This is especially important in clinical testing where both, the RNA yield and purity can affect assay sensitivity [14].

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