Korean J Clin Lab Sci. 2019;51(3):309-315

https://doi.org/10.15324/kjcls.2019.51.3.309

Korean Society for Clinical Laboratory Science

Korean Journal of
CLINICAL LABORATORY SCIENCE

ORIGINAL ARTICLE

Evaluation of Commercial Complementary DNA Synthesis Kits for Detecting Human Papillomavirus

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인유두종바이러스 검출을 위한 상용화된 cDNA 합성 키트의 평가

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ARTICLE INFO

Received July 1, 2019
Revised 1st August 21, 2019
Revised 2nd August 25, 2019
Accepted August 25, 2019

Key words
Cervical cancer
Complementary DNA
Complementary DNA synthesis kit
Human papillomavirus
Real-time PCR

ABSTRACT

Cervical cancer is the fourth most common malignant neoplasm in women worldwide. Most cases of cervical cancer are caused by an infection by the human papillomavirus. Molecular diagnostic methods have emerged to detect the HPV for sensitivity, specificity, and objectivity. In particular, real-time PCR has been introduced to acquire a more sensitive target DNA or RNA. RNA extraction and complementary DNA synthesis are proceeded before performing real-time PCR targeting RNA. To identify an adequate and sensitive cDNA synthesis kit, this study evaluated the two commonly used kits for cDNA synthesis. The results show that the R^2 and efficiency (%) of the two cDNA synthesis kits were similar in the cervical cancer cell lines. On the other hand, the Takara kit compared to Invitrogen kit showed $P<0.001$ in the 10^2 and 10^3 SiHa cell count. The Takara kit compared to the Invitrogen kit showed $P<0.001$ in the 10^1 and 10^2 HeLa cell count. Furthermore, 8, 4, 2, 1, and 0.5 ml of forty exfoliated cell samples were used to compare the cDNA synthesis kits. The Takara kit compared to the Invitrogen kit showed $P<0.01$ in 8, 4, and 1 ml and $P<0.05$ in 0.5 mL. The study was performed to identify the most appropriate cDNA synthesis kit and suggests that a cDNA synthesis kit could affect the real-time PCR results.

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INTRODUCTION

Cervical cancer is the fourth common malignant neoplasm in women worldwide [1]. In accordance with World Health Organization (WHO), cervical cancer occurred with approximately 530,000 new patients and 311,000 deaths per year globally [2]. Annually, approximately 3,500 patients are diagnosed and 960 patients die due to cervical cancer in Korea [3].

Since human papillomavirus (HPV) has been considered as the main cause of cervical cancer [4, 5]. HPV test is used to screen cervical cancer [6, 7]. HPV screening test consists

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pISSN 1738-3544 eISSN 2288-1662

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of cytological diagnostic methods and molecular diagnostic methods. In cytological diagnosis, Pap smear is a procedure to test for HPV screening in women using microscopy [8, 9]. A Pap smear is initiated with collecting cells from the cervix [10]. By screening HPV, a Pap smear allows one to prevent cervical cancer [11]. Detecting these precancerous cells early with a Pap smear is a step in stopping cervical cancer. However, the cytological method is limited by the long-term procedure and the interpretation - can be subjective depending on the person [12, 13]. Therefore, molecular diagnostic methods have emerged to detect HPV for sensitivity, specificity, and objectivity [14-16].

HPV screening test is done by molecular diagnostic technology such as PCR, real-time PCR, and NASBA method using HPV DNA or RNA [17-20]. Especially, real-time PCR is more sensitive and specific molecular diagnostic method to detect live HPV in patients compared to other methods [21-24]. Real-time PCR for HPV screening test is performed after RNA extraction, complementary DNA (cDNA) synthesis, and amplification of cDNA [25-27]. Real-time PCR gene expression can depend on efficiency (%) of complementary DNA synthesis even if total RNA is extracted efficiently [28].

In this study, we selected and compared two commercially available cDNA synthesis kits for the identification of the most useful kit in HPV screening test by cervical cancer cell lines and exfoliated cell samples. Reaction efficiency (%), coefficient of variation and cycle threshold (Ct) value analysis was performed.

MATERIALS AND METHODS

1. Cell line culture and clinical samples

Cervical cancer cell lines (HeLa and SiHa) were purchased from American Type Culture Collection ATCC (Manassas, USA) and Korean Cell Line Bank (Seoul, Korea). SiHa and HeLa cell line were cultured in Dulbecco's modified Eagle's Medium (DMEM; Gibco, Carlsbad, USA), with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) and 1% streptomycin-penicillin (Gibco, Carlsbad, CA, USA). All cell lines were incubated at 37°C in humidified 5% CO_2 atmosphere. Forty exfoliated cell samples were collected from cervical cancer patients and healthy subjects at Wonju Severance Christian Hospital, Wonju, Korea, from January 2010 to December 2014. All subjects provided clinical information and this study was approved by the Institutional Ethics Committee of Yonsei University Wonju College of Medicine (Approval No. YWMR-12-4-010).

2. Total RNA extraction

To extract RNA in cervical cancer cell lines, 1 mL of Isol-RNA Lysis Reagent (5Prime, Hamburg, Germany) was added to the cell pellet. Cells were lysed by vortexing or repeated pipetting and left to stand at room temperature for 5 min. Subsequently, 200 μL of chloroform was added and the mixture was shaken vigorously and incubated at room temperature for 3 min before centrifugation at 12,000 ×g for 15 min. The resulting aqueous layer was transferred to a new tube and an equal volume of isopropanol was added and mixed by inverting the tube. After incubation for 10 min at 25°C and centrifugation at 12,000 ×g for 10 min, 1 mL of 75% ethanol was added to the supernatant and mixed by inverting the tube. Finally, the mixture was centrifuged at 7,500 ×g for 5 min and the supernatant was removed. The RNA pellet was dried and eluted in 25 μL of diethylpyrocarbonate-treated water (Intron Biotechnology, Seoul, Korea). The purity and concentration of total RNA were determined by measuring the absorbance at 260 nm and 280 nm using an Infinite 200 spectrophotometer (Tecan, Vienna, Austria). All steps in the preparation and handling of total RNA were conducted in a laminar flow hood under RNase-free conditions. The isolated total RNA was stored at −70°C until use.

3. cDNA synthesis

By using MMLV Reverse Transcriptase kits (Invitrogen, Carlsbad, CA, USA) and random hexamers (Invitrogen, Carlsbad, CA, USA), complementary DNA (cDNA) was synthesized according to the manufacturer's recom-

mendation. In short, 5 μL of total RNA was added to a mixture containing 1 μL of 10 mM dNTP mix at neutral pH, 1 μL of 0.25 μg/μL random hexamers, and 6 μL of DEPC-treated water. The PCR mixtures were incubated at 65°C for 5 min and chilled on ice. After adding a mixture of 4 μL of First-strand Buffer (5×), 2 μL of 0.1 M dithiothreitol (DTT), and 1 μL of MMLV reverse transcriptase (at room temperature), cDNA synthesis was performed at 25°C for 10 min, 37°C for 50 min and 70°C for 15 min. The cDNA was stored at −70°C until used. And By using PrimeScript RT Master Mix kits (Takara, Japan), cDNA was synthesized according to the manufacturer's recommendation. In short, 5 uL of total RNA was added to a mixture containing 5 uL of 5× Master mix and 11 uL of DEPC-treated water. cDNA synthesis was performed at 37°C for 15 min, and 85°C for 5 s.

4. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) for detecting HPV types

The assay consists of three different sets of HR-HPV and detects 16 HR-HPV genotypes in three tubes (group I: HPV 16 [FAM], 33, 58 [HEX], and 31, 35 [Cy5]; group II: HPV 18 [FAM], 39, 68 [HEX], and 45~51 [Cy5]; group III: HPV 53, 56, 66 [FAM], 59, 69 [HEX], and 52 [Cy5]), by incorporating specific TaqMan probes labeled with different fluorophores. RT-qPCR were performed using 10 μL of 2× Thunderbird probe qPCR mix (Toyobo, Osaka, Japan), 5 μL of primer and TaqMan probe mixture, 2 μL of template cDNA, and distilled water to a final volume of 20 μL per sample. No-template controls as negative controls were included in each run and contained sterile distilled water rather than template DNA. The PCR cycle was run as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 3 s, and 55°C for 30 s. mRNA levels were quantified by determining the cycle threshold (CT), which is defined as the number of PCR cycles required for fluorescence to exceed a value significantly higher than that of the background fluorescence. For internal control, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used.

5. Data calculation and statistical analysis

The qPCR R^2 and efficiency (%) was calculated by qPCR Efficiency Calculator Program (ThermoFisher Scientific, CA, USA). Statistical analysis was conducted using GraphPad Prism software (Version 5.02, La Jolla, CA, USA). Two way-ANOVA tests were used to determine the statistical significance in cDNA synthesis kits. For all tests, $P<0.05$ was considered statistically significant. The differences were considered statistically significant when $^{*}P<0.05$, $^{**}P<0.01$, or $^{***}P<0.001$.

RESULTS

1. Reaction efficiency (%), R^2 and limit of detection in SiHa cell line

To compare the cDNA synthesis efficiency (%) of two commercial kits, SiHa cell line was diluted from 10^6 to 10^0 and then SiHa cell line cDNA was synthesized by two RT-PCR kits and used for amplification of real-time PCR targeting HPV 16 and GAPDH. R^2 of the two cDNA synthesis kits was 0.99 and 0.99, respectively (Table 1). And efficiency (%) of the two cDNA synthesis kits was 107.71 and 103.98 respectively (Table 1). And limit of detection was 10 and 10^2 cells in Takara and Invitrogen kits (Table 1). Takara kit compared to Invitrogen kit showed $P<0.001$ in 10^2 and 10^3 cell count (Figure 1). 95% confidence interval were "−8.004 to 0.004441" "−12.00 to −3.996" "−14.80 to −6.796" "−5.404 to 2.604" "−5.104 to 2.904" "−5.104 to 2.904" from 10^1 to 10^6. GAPDH Ct value range was between 25~30.

Table 1. R^2, efficiency (%) and limit of detection in SiHa cell line according to cDNA synthesis kits

	Invitrogen	Takara
R^2	0.99	0.99
Efficiency (%)	107.71	103.98
Limit of detection (Cells)	10^2	10

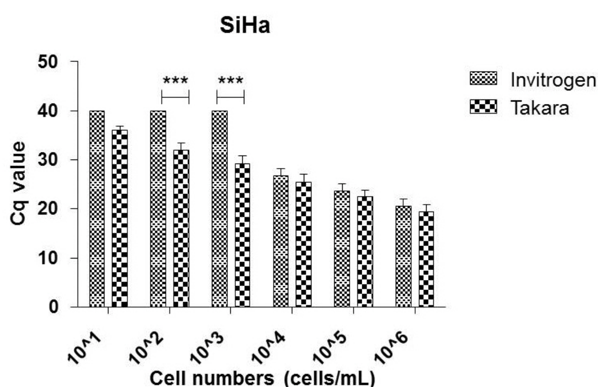

Figure 1. Ct values comparison between Invitrogen and Takara kit in SiHa cell line. Takara kit compared to Invitrogen kit showed $P<0.001$ in 10^3 and 10^2 cell count (bar represents mean and standard deviation).

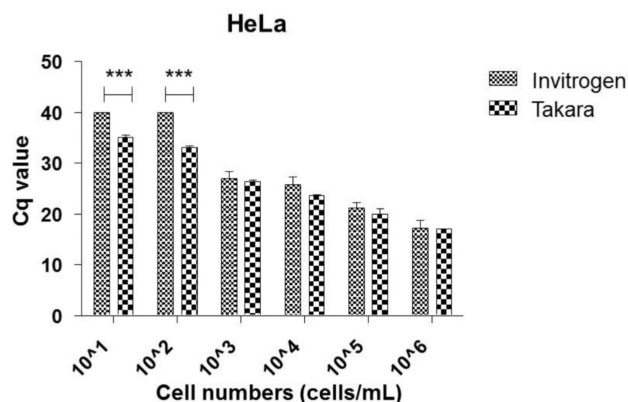

Figure 2. Ct values comparison between Invitrogen and Takara kit in HeLa cell line. Takara kit compared to Invitrogen kit showed $P<0.001$ in 10^2 and 10^1 cell count (bar represents mean and standard deviation).

Table 2. R^2, efficiency (%) and limit of detection in HeLa cell line according to cDNA synthesis kits

	Invitrogen	Takara
R^2	0.95	0.99
Efficiency (%)	96.84	107.23
Limit of detection (Cells)	10^3	10

2. Reaction efficiency (%), R^2 and limit of detection in HeLa cell line

To compare the cDNA synthesis efficiency (%) of two commercial kits, HeLa cell line was serially diluted 10-fold from 10^6 to 10^0. Then HeLa cell line cDNA was synthesized by two RT-PCR kits and used for amplification of real-time PCR targeting GAPDH and HPV 18. R^2 of the two cDNA synthesis kits was 0.95 and 0.99, respectively (Table 2). And efficiency (%) of the two cDNA synthesis kits was 96.84 and 107.23 respectively (Table 2). And limit of detection was 10 and 10^3 cells in Takara and Invitrogen kits (Table 2). Takara kit compared to Invitrogen kit showed $P<0.001$ in 10^1 and 10^2 cell count (Figure 2). 95% confidence interval were "−7.790 to −2.210" "−9.790 to −4.210" "−3.390 to 2.190" "−4.890 to 0.6903" "−3.990 to 1.590" "−2.890 to 2.690" from 101 to 106. GAPDH Ct value range was between 25~30.

3. Ct value analysis in exfoliated cell samples

8, 4, 2 and 1 ml of forty exfoliated cell samples were

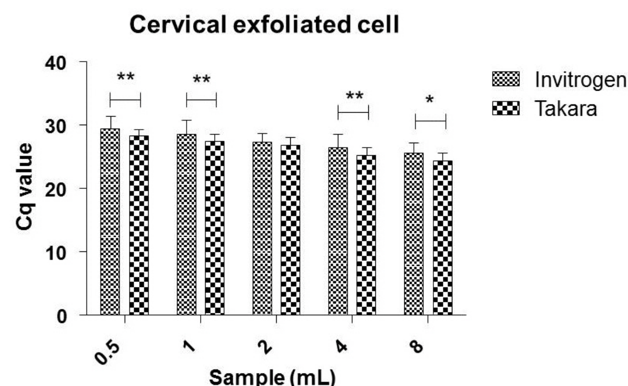

Figure 3. Ct values comparison between Invitrogen and Takara kit in clinical samples. Takara kit compared to Invitrogen kit showed $P<0.01$ in 8, 4, 1 mL and $P<0.05$ in 0.5 mL (bar represents mean and standard deviation).

used to compare the cDNA synthesis efficiency (%) of two commercial kits. Takara kit compared to Invitrogen kit showed $P<0.05$ in 0.5 ml and $P<0.01$ in 8, 4, 1 mL (Figure 3) representing that Takara kit has capacity to detect more HPV nucleic acid. 95% confidence interval were "−1.902 to −0.09788" "−2.002 to −0.1979" "−1.402 to 0.4021" "−2.102 to −0.2979" "−2.102 to −0.2979" according to 1,2,4 and 8 ml of clinical samples. GAPDH Ct value range was between 25~30.

DISCUSSION

The HPV-induced carcinogenesis has the pattern that expression of the E6 and E7 gene of HPV is maintained,

while other viral DNA is deleted or disturbed [36]. Detection of HPV oncogene activity through the detection of mRNA transcripts may therefore be a better indicator of HPV infection associated with increased risk of progression to neoplasia than detection of HPV DNA [37].

Recently, real-time PCR has become the most powerful detection system for nucleic acids of bacteria, virus, and human and is used to diagnose cervical cancer in several hospital especially [29-31]. Diagnostic lab system for introducing real-time PCR are now confronted with defining the most adequate kit for their real-time PCR applications. Concerns have been raised that real-time PCR components and condition can affect on sensitivity and specificity of results for real-time PCR platform [32]. Real-time PCR performance needs to be verified, for many parameters can influence the results of real-time PCR amplification. Therefore, it is essential that laboratories employing real-time PCR platform apply strict laboratory quality control standards.

Two-step real-time PCR has flexibility using random primer and oligo dT and is used for the amplification of different targets in small amount of same sample [33]. In the study, we selected and evaluated the two-step real-time PCR kits for HPV is divided into various low-risk and high-risk groups which exist in small amount of clinical samples.

Especially, the reverse transcription reaction before acquiring real-time PCR results is a key step in real-time PCR. RT enzymes, which are the most important for real-time RT-PCR assay, are critical for synthesizing cDNA resulting in real-time PCR data analysis [34]. For many quantitative results, the MMLV (Moloney Murine Leukemia Virus) reverse transcriptase is used. The enzyme of capacity is greater than any other commercial reverse transcriptase [35]. In the study, two commercial kits used RT originated from MMLV using cervical cancer cell lines and clinical samples.

The purpose of the study was for evaluating the efficiency (%) of cDNA synthesis kits. For that study, HPV was selected to generate HPV RNA transcript. We selected two different commercial cDNA synthesis kits for detecting HPV and evaluated the results using efficiency and R^2 as shown in a paper [28]. Results showed that efficiency (%) was between 90%~110%, R^2 was above 0.96. Results demonstrated that there was no difference in reaction efficiency (%) and R^2 values between two methods in cervical cancer cell line which has HPV 16 and 18 for causing cervical cancer. Cervical cancer cell lines except other cell lines were used to define the diagnostic method for cervical cancer. However, considering limit of detection, Takara kit compared to Invitrogen kit showed statistically significant that Takara kit is more sensitive method for detecting HPV load in same quantity. It is assumed that Takara kit has more primers binding to RNA such as oligo dT and random hexamer with RNase inhibitor. Actually, random primer can be used for shortness of gene sequence or sequence region which has difficulty to be elongated by binding to mRNA, rRNA and tRNA. Oligo dT has advantage that full-length cDNA can be synthesized and small amount of target mRNA can be amplified by several elongation steps by binding to same mRNA molecule. In conclusion, these results suggest quality of real-time PCR data analysis and experiment can be elevated according to cDNA synthesis kit strongly. Further studies need to be performed on a variety of clinical samples, more cDNA synthesis kits and various cancers.

요 약

자궁경부암은 전 세계적으로 네번째를 차지하는 여성암이다. 자궁경부암의 대부분 원인은 인유두종 바이러스의 감염이다. 인유두종 바이러스를 검출하기 위해 다양한 분자진단학적 방법들이 고안되었다. 분자진단학적 방법 중의 real-time PCR은 목표 DNA 또는 RNA의 정량과 민감도 향상을 목표로 도입되었다. 특히, real-time PCR 과정은 수행 전에 RNA 추출 및 상보적인 DNA 합성 과정이 필요하다. 따라서 본 연구에서는 민감하고 적합한 상보적인 DNA 합성 키트를 알아보기 위해서 상보적인 DNA 합성에 이용되는 두 개의 상용화된 키트를 평가하였다. 자궁경부암 세포주에서 두개의 상보적인 DNA 합성 키트의 R^2과 효율성을 비교한 결과 차이가 없었다. 그러나 Invitrogen 키트보다 Takara 키트가 10^2 및 10^3 SiHa 세포주에서 $P<0.001$를

나타내었고 10^1 및 10^2 HeLa 세포주에서도 $P<0.001$를 나타내었다. 이를 통해 Takara 키트가 Invitrogen키트보다 민감도가 높음을 알 수 있었다. 또한 40개의 탈락세포검체의 8, 4, 2, 1 mL을 이용하여 상보적인 DNA 합성 키트를 비교한 결과 Invitrogen 키트보다 Takara 키트가 8, 4, 1 mL에서 $P<0.01$ 및 0.5 mL에서 $P<0.05$을 나타내어 임상 검체를 이용하였을 때에도 Takara 키트가 Invitrogen 키트보다 민감도가 높음을 알 수 있었다. 본 연구는 적합한 상보적인 DNA 합성 키트를 확인하기 위해 수행되었으며, 상보적인 DNA 합성 키트가 real-time PCR 결과 다양성에 영향을 미친다는 것을 시사하였다.

Acknowledgements: This research was supported by Hyejeon College grant.

Conflict of interest: None

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