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Optimization of Real-time Reverse Transcriptase Polymerase Chain Reaction for Detection of Dengue Virus

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Authors' contributions

This work was carried out in collaboration among all authors. Authors KAA and RAK involved in conception and design. Authors AJN, AM and AS provided input into the study design and laboratory analysis. Authors KAA and RAK completed the final draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study was set to optimize conditions for real time reverse transcriptase polymerase chain reaction (RT-PCR) for detection of dengue virus by using rapid and simple nucleic acid extraction method.
Methodology: One step and two step real time RT-PCR were evaluated in different PCR thermocyclers. Extraction of viral RNA was done by using a simple boom method.
Results: The real time RT-PCR technique was successfully optimized using simple and rapid method for purification of nucleic acid, 'boom method'. The technique works better when performed in a two-step procedure and can works well with all range of real time PCR machines. The optimized real time RT-PCR used in the present study is a valuable and reliable technique for routine diagnosis of dengue. Further investigation on the cost effectiveness in adopting this technique for routine screening and monitoring of the dengue infection should be done.

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Keywords: Dengue virus; RT-PCR technique; boom method; RNA extraction.

ABBREVIATIONS

DENV : Dengue virus
DHS : Dengue Hemorrhagic Fever
DSS : Dengue Shock Syndrome

1. INTRODUCTION

Dengue virus (DENV) is arthropod-borne virus that is prevalent in tropical and sub-tropical regions. Main vectors for the virus are mosquitoes of the *Aedes* genus, including *Aedes egypti* and *Aedes albopictus* [1,2]. DENV belong to the family Flaviviridae, genus *Flavivirus*. The virus has a positive-sense, enveloped, single-stranded RNA genome of approximately 11 kb in length which have been categorised into four serotypes, DENV 1-4. DENV 2 reported to cause the most epidemics followed by DENV 1 [3,4,5].

In recent year's DENV have caught worldwide attention because of increase in the frequency of major epidemics [1]. Globally, 50 - 100 million DENV infections occur annually, accounting for 20,000–70,000 deaths per year. An estimated 2.5 billion people are at risk of infection. Dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) account for about 250,000 - 500,000 cases each year. Children under 5 years of age are mainly affected. In hyperendemic Asian countries, 22 - 292 per 1,000 children are infected each year [6,7,8]. Dengue epidemics have been reported in African countries since 19th century and all four DENV serotypes have been isolated [3,9]. The recent outbreak of DENV in Sub-Saharan Africa ascertain that febrile illnesses are now becoming the next threat to the population living in malaria endemic areas [10]. Early 2013, outbreak of Dengue was reported in Somalia and Kenya where majority of cases were found in the Indian Ocean Coastal town of Mombasa. Most recently, outbreak was reported in Dar es Salaam, Tanzania. In 2014 dengue outbreak, a total of 2,129 suspected case and 1,018 confirmed cases were reported, where as in 2019 outbreak about 3,000 suspected cases with 71.4% confirmed cases and 2 deaths were reported [11,12].

Many cases of DENV infections in most of the hospitals in Sub-Saharan Africa remain undiagnosed due to lack of accurate diagnostic technique. Every so often febrile illness are treated as presumptive malaria without proper laboratory diagnosis [3,13,14]. The clinical manifestation of DENV infections may be confused with those caused by Chikungunya fever and other febrile illness, but unlike Chikungunya and other febrile illness, DENV infections is associated with DHF and DSS [15].

Virus culture is often regarded as gold standard method for laboratory diagnosis of DENV but is time consuming and must be performed under biosafety level 3 conditions. Viral culture is rarely done in routine clinical diagnosis as these facilities are not widely available [1,16]. Serological diagnosis based on capture IgM and IgG ELISA are reliable can only detect IgM around 5 days from the onset of illness and in addition strong antibody cross-reactivity occurs among members of the family which may confuse interpretation of the results [1,17,18].

To date, polymerase chain reaction (PCR) methods have been suggested as appropriate laboratory diagnostic technique of DENV infections [1,14]. Real-time reverse transcriptase PCR (RT-PCR) has been developed as an accurate diagnostic technique at an early stage of infection of several arboviruses. Advantages of Real time RT-PCR over other diagnostic methods including, higher sensitivity, higher specificity and rapidity [2,16,19,20]. The aim of this study was to optimize real-time RT-PCR for detection of DENV infections by using rapid and simple nucleic acid extraction method.

2. MATERIALS AND METHODS

2.1 Clinical Samples

A total of 208 blood samples were collected from febrile children (aged 2 months to 12 years) admitted at KCMC hospital between October 2013 to April 2014.

2.2 Control Samples

Positive samples and internal extraction control obtained from National Health Laboratory Quality Assurance Training Centre (NHL-QATC) were included to validate the assay.

2.3 Extraction of Viral RNA

RNA was extracted from blood sample by using boom extraction method as described by Boom et al., [21]. In summary, 100 µl of blood samples was lysed in a 900 µl L6 lysis buffer containing

the chaotropic agent guanidine thiocyanate (Sigma-Aldrich, USA). A 50 µl of Silica (Sigma-Aldrich, USA) was then added and immediately vortexed for 5 sec followed by shaking for 10 min and centrifuged at 12,000 g for 15 sec. The silica pellet was washed twice with L2 wash buffer (120g GuSCN in 100 ml 0.1M Tris-HCL, pH 6.4), twice with 70% ethanol (Sigma-Aldrich, USA) and once with molecular grade acetone (Scharlau Chemic S.A), then dried at 56˚C in a heat block for 10 min. The RNA was eluted from the silica particles in 50 µl of diethyl pyrocarbonate (DEPC) treated water (Ambion, USA) and then vortexed briefly and incubated for 10 min at 56˚C. The sample was briefly vortexed again and centrifuged for 2 min at 12,000 g. In the final step, 35 µl of supernatant containing purified RNA was transferred to a clean eppendorf tube and then stored at -20˚C prior use.

2.4 Reverse Transcription

RNA was transcribed into cDNA by using QuantiTect-Reverse-Transcription kit (Qaigen, German) following manufacturer's protocol.

2.5 Primer and Probe Sequence

Primers and Probes were adopted from Pongsiri et al. [2] and purchased from Applied Biosystems, UK (Table 1).

2.6 Real Time RT-PCR Optimization

Assay conditions were optimized using various primer and probe concentrations, thermal conditions and different real time PCR systems. The concentrations of primers, probes and thermal profile were optimized to increase the sensitivity and specificity. The assay was optimized in a one-step and two-step real time RT-PCR.

In one-step real time RT-PCR, 10µl reaction volume contained 0.2 µl Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Invitrogen, Carlsbad CA), 5 µl of TaqMan Gene Expression master mix (Applied Biosystems, USA), 0.25 µM of dengue forward and reverse primers, 0.125 µM of dengue probe and nuclease free water. Large volume assay were performed with the AgPath-ID one step RT-PCR kit (Applied Biosystems, USA). The 25µl reaction volumes contained 12.5 µl of 2x RT-PCR Buffer, 0.1 µM of dengue forward and reverse primers, 0.05 µM of dengue probe, 1 µl of 25 RT-PCR enzyme mix, 1µl RNA sample and nuclease free water.

In two-step real time RT-PCR, 10 µl reaction volume contained 5 µl of TaqMan Gene Expression master mix (Applied Biosystems, USA), 1µl of cDNA, 0.25 µM of dengue forward and reverse primers, 0.125 µM of dengue probe and nuclease free water. The large volume, 20 µl reaction volume contained 10 µl of TaqMan Gene Expression master mix, 2 µl of cDNA, 0.25 µM of dengue forward and reverse primers, 0.125 µM of dengue probe and nuclease free water.

Thermal conditions were optimised in both one-step and two-steps real time RT-PCR. In one-step RT-PCR the thermal conditions were; reverse transcription at 50˚C for 30 min, initial denaturation 95˚C for 10 min followed by 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec. In two-step RT-PCR the thermal conditions were; UDG incubation at 50˚C for 2 min, enzyme activation 95 ˚C for 10 sec, followed by 45 cycles of 95˚C for 15 sec and 60˚C for 1 min.

Table 1. Primer and probe sequence used in the assay

Name	Primer/Probe sequence	Region, Position	Length (kb)	Mol. Weight	Dye	DOI
DenvF	GAC TAG YGG TTA GAG GAG ACC	3'UTR, 10520-10541	21	6528		10.1016/S1995–7645(12)60055–8
DenvR	GHR GAG ACA GCA GGA TCT CTG	3'UTR, 10674-10694	21	6501		
DenProb	AAG GAC TAG MGG TTA GWG GAG ACC C	3'UTR, 10610-10634	25	9430	6-FAM MGBNFQ	

Assay conditions were also optimised in different real time PCR systems namely Stratagene® Mx3000P™and Applied Biosystems® ViiA™7 real time PCR system. Positive controls and no template control were included in our assay for each run, and a run was validated if the no template control did not exhibit fluorescent signal that cross the threshold line. A positive result was considered when the fluorescent signal crossed the threshold (i.e. exceeds background level).

3. RESULTS

3.1 Optimization of Real Time RT-PCR

Various primer and probe concentrations, thermal conditions and real time RT-PCR systems were evaluated. Primer concentrations ranging from 5 µM to 10 µM and probe concentrations ranging from 3 µM to 10 µM were evaluated; final concentrations of 0.25 µM of each primer and 0.125 µM of probe were found to be the most sensitive in the assay.

Thermal profile efficiencies were compared, with annealing temperatures ranging from 55°C to 60°C. Annealing temperature was maintained at 60°C which was found to be the best and thus had the best specificity for the assay.

TaqMan Gene Expression master mix kit (Applied Biosystems, USA) and the AgPath-ID one step RT-PCR kit (Applied Biosystems, USA) were also compared in the assay. TaqMan Gene Expression master mix kit (Applied Biosystems, USA) was found to be most efficient in both one-step and two step-methods. The 10µl reaction volume containing 5 µl of TaqMan Gene Expression master mix, 1 µl of cDNA, 0.25 µM of each dengue forward and reverse primers, 0.125 µM of dengue probe and nuclease free water was found to be efficiency in our assay.

Reaction efficiencies were similar in both one-step and two-step RT-PCR methods but the sensitivity of the two-step RT-PCR method was slightly better than one-step RT-PCR method. There was a roughly 3-cycle difference between one-step and two step method which indicates 8 fold change in detection. The calculation is done by the formula $2^{\Delta CT}$ where 2 is used as an assumption of doubling in every cycle.

The technique was successfully optimized using two different PCR thermocyclers namely, Stratagene® Mx3000P™and Applied Biosystems® ViiA™7. Figs. 1 and 2 shows all positive controls can be amplified with both real time PCR systems but with different fluorescence detections efficient.

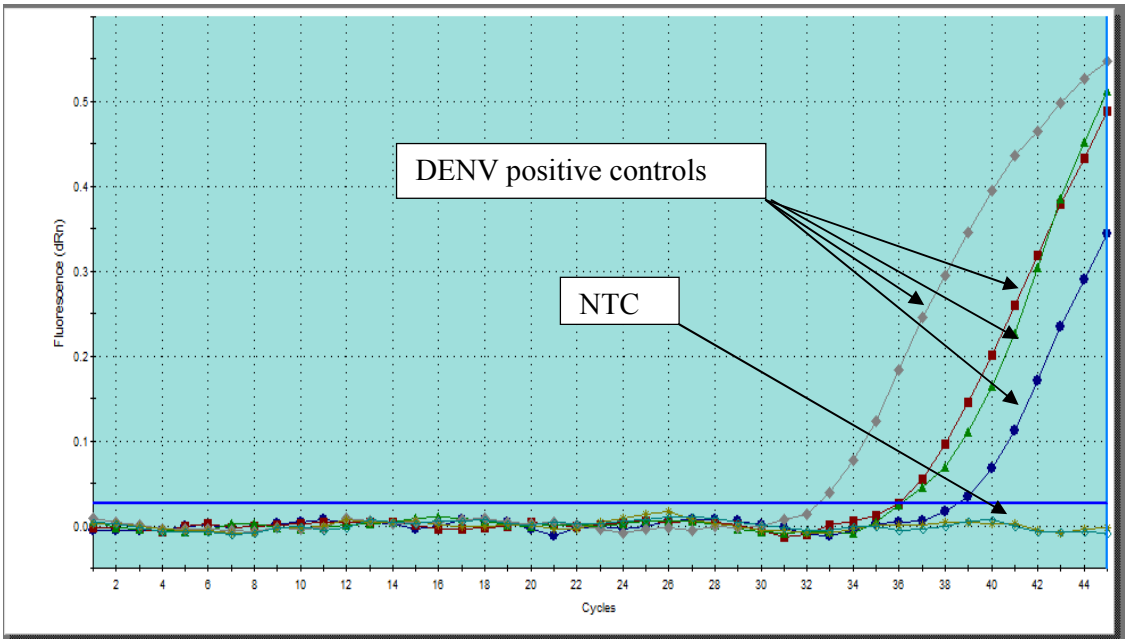

Fig. 1. Amplification of dengue controls using Stratagene Mx3000P
DENV=dengue virus positive control; NTC=no template control

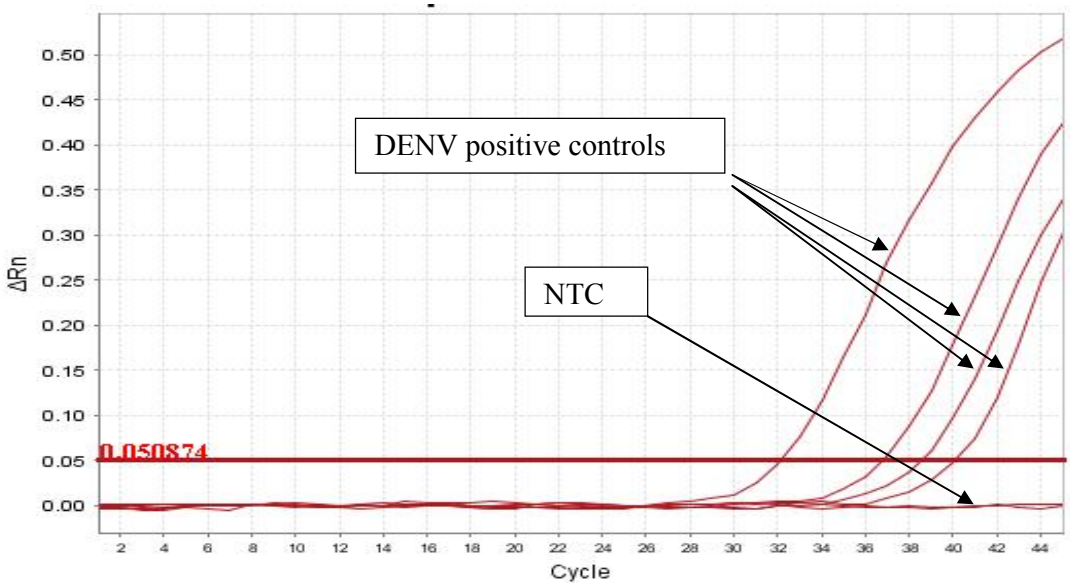

Fig. 2. Amplification of dengue controls using Applied Biosystems ViiA7
DENV=dengue virus positive control; NTC=no template control

3.2 Extraction of Viral RNA

Nucleic acid were purified from blood samples in less than 1 hour. Nucleic acid concentration and purity were assessed and quantified by NanoDrop UV Visible Spectrophotometer.

3.3 Validation of Dengue Diagnostic Real Time PCR

Validation and determination of performance of our assay were performed on control samples from National Health Laboratory Quality Assurance Training Centre (NHL-QATC), Dar es Salaam. All four positive control samples were tested positive for DENV by the real time RT-PCR assay. The 100% correlation of the results demonstrated 100% sensitivity of the real time RT-PCR assay.

3.4 Clinical Samples

PCR screening of the febrile children revealed that none of them were positive for DENV. In the present study, the prevalence of DENV infections among febrile children admitted at KCMC hospital Paediatric Department was found to be 0.0%.

4. DISCUSSION

We report optimization of real time RT-PCR assay for detection of DENV nucleic acid. The real time RT-PCR assay optimised has been shown to be sensitive enough to detect all of the four serotypes of DENV. The assay was rapid and accurate that can be performed in four hours including nucleic acid extraction. The rapidity and accurateness of the real time RT-PCR is essential in facilitating management and initiation of suitable therapy.

While the reaction efficiencies were similar there was a difference in sensitivity of detection between one-step and two-step real time RT-PCR methods. The lower CT value expressed in two-step method was roughly 3-cycle differences (8-fold difference in detection levels) indicating two-step method was more sensitive than one-step method. Since the same PCR conditions was used, the difference was probably due to reverse transcriptase reaction conditions and different enzymes used which were likely correlated to reduced sensitivity of detection in one-step real time RT-PCR method.

The assay was sensitive enough to be used with a simple RNA extraction method such as the boom method. Boom extraction method proved to work effectively by produced purified viral RNA reliable for amplification. Boom extraction method seem compatible for accurate real time RT-PCR and can be used at any setting. To our knowledge this is the first study to investigate on boom extraction method in extraction of DENV genome in East Africa. Investigation on

extraction methods is important information in selecting the proper method of genomic extraction.

Our study demonstrated that none of the study participants had tested positive for DENV infection by real time RT-PCR. The 0% prevalence of DENV infection revealed by the present study could be explained by absence or very minimal circulation of DENV in Northern Tanzania as results of climate factors which influences dengue ecology. Community behaviour factors such as environmental management could also count for the reported prevalence. To date there is no any epidemiological data that indicates outbreak of DENV infections in Northern Tanzania. In spite of the fact that we did not had any children tested positive by the real time RT-PCR, our findings support that other arboviruses are circulating in Northern Tanzania as most of the study participants presented with signs and symptoms that are closely related to arboviral infections. As arboviral infections are common cause of febrile illness and routinely misdiagnosed as malaria, diagnosis of arboviral could be tested by rapid and accurate diagnostic methods such as real time PCR so as to understand the contribution of these infections to febrile illness.

5. CONCLUSION

The optimized technique utilize a simple RNA extraction method such as the boom method and can works well with all range of real time PCR machines. Following the recent outbreak of dengue in Sub-Saharan Africa, further investigation on the cost effectiveness in adopting this technique for routine screening and monitoring of the infection should be done.

CONSENT AND ETHICAL APPROVAL

Ethical approval for this study was granted and approved by the Kilimanjaro Christian Medical University College Ethics and Research Committee (CRERC) with a certificate No. 612. Written informed consent was obtained from parents or legal guardian of the children before inclusion in the study.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Lai YL, Chung YK, Tan HC, Yap HF, Yap G, Ooi EE, Ng LC. Cost-effective real-time reverse transcriptase PCR (RT-PCR) to screen for Dengue virus followed by rapid single-tube multiplex RT-PCR for serotyping of the virus. J Clin Microbiol. 2007;45:935-941.
2. Pongsiri P, Praianantathavorn K, Theamboonlers A, Payungporn S, Poovorawan Y. Multiplex real-time RT-PCR for detecting chikungunya virus and dengue virus. Asian Pac J Trop Med 2012;5;342-346.
3. Amarasinghe A, Kuritsky JN, Letson GW, Margolis HS. Dengue virus infection in Africa. Emerg Infect Dis. 2011;17(8):1349-1354.
4. Dussart P, Baril L, Petit L, Beniguel L, Quang LC, Ly S, Azevedo Rdo S, Meynard JB, Vong S, Chartier L, Diop A, Sivuth O, Duong V, Thang CM, Jacobs M, Sakuntabhai A, Nunes MR, Huong VT, Buchy P, Vasconcelos PF. Clinical and virological study of dengue cases and the members of their households: The multinational DENFRAME Project. PLoS Negl Trop Dis. 2012;6:e1482.
5. Tang KF, Ooi EE. Diagnosis of dengue: An update. Expert Rev Anti Infect Ther. 2012;10:895-907.
6. Johnson BW, Russell BJ, Lanciotti RS. Serotype-specific detection of dengue viruses in a fourplex real-time reverse transcriptase PCR assay. J Clin Microbiol. 2005;43:4977-4983.
7. Deen JL, Harris E, Wills B, Balmaseda A, Hammond SN, Rocha C, Dung NM, Hung NT, Hien TT, Farrar JJ. The WHO dengue classification and case definitions: Time for a reassessment. Lancet. 2006;368:170-173.
8. Vairo F, Nicastri E, Meschi S, Schepisi MS, Paglia MG, Bevilacqua N, Mangi S, Sciarrone MR, Chiappini R, Mohamed J, Racalbuto V, Di Caro A, Capobianchi MR, Ippolito G. Seroprevalence of dengue infection: A cross-sectional survey in mainland Tanzania and on Pemba Island, Zanzibar. Int J Infect Dis. 2012;16:e44-46.

9. Were F. The dengue situation in Africa. PaediatrInt Child Health. 2012;32 Suppl:118-21.
10. Hertz JT, Munishi OM, Ooi EE, Howe S, Lim WY, Chow A, Morrissey AB, Bartlett JA, Onyango JJ, Maro VP, Kinabo GD, Saganda W, Gubler DJ, Crump JA. Chikungunya and dengue fever among hospitalized febrile patients in northern Tanzania. Am J Trop Med Hyg. 2012;86:171-177.
11. Okada K, Morita R, Egawa K, Hirai Y, Kaida A, Shirano M, Kubo H, Goto T, Yamamoto SP. Dengue Virus type 1 infection in traveler returning from Tanzania to Japan. Emerg Infect Dis. 2019; 25(9):1782-1784.
12. Ward T, Samuel M, Maoz D, Runge-Ranzinger S, Boyce R, Toledo J, Velayudhan R, Horstick O. Dengue data and surveillance in Tanzania: A systematic literature review. Trop Med Int Health. 2017;22:960–70.
13. Sutherland LJ, Cash AA, Huang YJ, Sang RC, Malhotra I, Moormann AM, King CL, Weaver SC, King CH, La Beaud AD. Serologic evidence of arboviral infections among humans in Kenya. Am J Trop Med Hyg. 2011;85:158-161.
14. Taraphdar D, Sarkar A, Chatterjee S. Mass scale screening of common arboviral infections by an affordable, cost effective RT-PCR method. Asian Pac J Trop Biomed. 2012;2:97-101.
15. Lee VJ, Chow A, Zheng X, Carrasco LR, Cook AR, Lye DC, Ng LC, Leo YS. Simple clinical and laboratory predictors of chikungunya versus dengue infections in adults. PLoS Negl Trop Dis. 2012; 6:e1786.
16. Laurent P, Le Roux K, Grivard P, Bertil G, Naze F, Picard M, Staikowsky F, Barau G, Schuffenecker I, Michault A. Development of a sensitive real-time reverse transcriptase PCR assay with an internal control to detect and quantify chikungunya virus. Clin Chem. 2007;53:1408-1414.
17. Dos Santos HW, Poloni TR, Souza KP, Muller VD, Tremeschin F, Nali LC, Fantinatti LR, Amarilla AA, Castro HL, Nunes MR, Casseb SM, Vasconcelos PF, Badra SJ, Figueiredo LT, Aquino VH. A simple one-step real-time RT-PCR for diagnosis of dengue virus infection. J Med Virol. 2008;80:1426-1433.
18. Simmons M, Myers T, Guevara C, Jungkind D, Williams M, Houng H. Development and validation of a quantitative, one-step, multiplex, real-time reverse transcriptase PCR assay for detection of dengue and chikungunya viruses. J Clin Microbiol. 2016;54(7).
19. Shu PY, Chang SF, Kuo YC, Yueh YY, Chien LJ, Sue CL, Lin TH, Huang JH. Development of group- and serotype-specific one-step SYBR green I-based real-time reverse transcription-PCR assay for dengue virus. J Clin Microbiol. 2003;41:2408-2416.
20. Muna M, Baeb J, Kima J, Kima S, Leeb I, Kimb J, Parkb M, Parkb MS, Nama Y. One-step multiplex real-time RT-PCR for detection and typing of dengue virus. Molecular and Cellular Probes. 2019;43:86–91.
21. Boom R, Sol CJ, Salimans MM, Jansen CL, Wetheim-van DPM, Van Der Noordaa J. Rapid and simple method for purification of nucleic acid. J Clin Microbiol. 1990; 28(3):495-503.

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