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ROLE OF REAL TIME PCR (RT-PCR) IN CLINICAL DIAGNOSIS FOR COVID-19 REVIEW

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ABSTRACT

The pandemic of Coronavirus Disease 2019 (COVID19) has compelled scientists to create highly reliable diagnostic tools quickly in order to successfully and properly diagnose this pathology and thereby prevent infection transmission. Even though structural and molecular properties of the severe acute respiratory syndrome coronavirus 2 (SARSCoV2) were previously unknown, private research institutes and biomedical firms quickly developed numerous diagnostic procedures beneficial for making a correct detection of COVID19. Rapid antigen or antibody testing, immunoenzymatic serological tests, and RT-PCR based molecular assays are the most frequently used and validated procedures now available. The PCR has grown in popularity in molecular diagnostics to the point where it is still considered the gold standard for finding nucleotides from a variety of sources becoming an indispensable tool in the research lab. Because of its improved speed, sensitivity, reproducibility, and lower likelihood of carry-over contamination, real-time PCR has gained greater popularity. Currently, five different chemistries are employed to detect PCR product during real-time PCR. The self-fluorescing amplicons, DNA binding fluorophores, 5′ endonuclease, neighbouring linear and hairpin oligoprobes, and self-fluorescing amplicons are all detailed in depth. We also go through the problems that have hampered the development of multiplex real-time PCR and the importance of real-time PCR in nucleic acid quantification.

KEYWORDS: (Polymerase Chain Reaction) PCR, (Real Time-polymerase Chain Reaction (RT-PCR), (Corona Virus Disease -19) Covid-19.

INTRODUCTION

The second wave of the Coronavirus Disease 2019 (COVID19) pandemic hit Europe and the rest of the world, resulting in a rise in the number of illnesses and deaths globally, emphasising crucial challenges in the handling of this public health disaster (1-2). For these reasons, scientists are debating which diagnostic procedures are best for effectively combating the impending surge in COVID 19 infections, as well as how to differentiate between COVID19 diseases and seasonal flu. Population screening approaches have been recommended and are currently being developed in this perspective for continuous control of the COVID19 epidemiological contour and screening the immunized population; moreover, it is not yet definite which tactic is the most efficient for these vigilance programs (3-6). As a result, it is clear that its diagnostic test should be chosen based on the trial's clinical or monitoring goals, as well as the ability to repeat the test multiple times until the individuals are still no longer positive. There are three primary types of COVID19 diagnosis lab tests that meet most of these clinical and epidemiological demands – i) Molecular (Real Time-Polymerase Chain Reaction) RT−PCR swab tests; ii) serological tests; iii) rapid antigen or antibody tests.

The PCR (7-8) has become the new gold standard for identifying a wide range of templates in a variety of scientific fields, including virology. The approach employs a pair of synthetic oligonucleotides or primers, each of which hybridizes to one strand of a double-stranded DNA (dsDNA) target and spans an exponentially reproducible region. The hybridised primer serves as a substrate for a DNA polymerase (most often Taq, which is derived from the thermophilic bacteria Thermus aquaticus), which produces a complementary strain by adding deoxynucleotides sequentially.

The procedure can be broken down into three steps: I dsDNA separation at temperatures over 90°C, (ii) primer annealing at temperatures between 50 and 75°C, and (iii) optimum extension at 72–78°C. A programmable thermal cycler controls the rate of

temperature change (or ramp rate), the length of incubation at each temperature, and the number of times each set of temperatures (or cycle) is repeated. Using electronically controlled heating blocks or fan-forced warm air flows to modulate the reaction temperature, current technologies have greatly reduced ramp times. As a result, some of the gold standard cell culture, anti-genaemia, and serological assays are being replaced by PCR (9). Existing PCR and detection assay combinations (referred to as "conventional PCR" here) have been utilised to collect quantitative data with encouraging results. However, the time-consuming post-PCR processing processes required to assess the amplicon have hampered these efforts (10).

Electrophoresis of nucleic acids in the presence of ethidium bromide and visual or densitometric examination of the resultant bands after irradiation with ultraviolet light are the traditional methods for detecting amplified DNA (11). Southern blot detection of amplicon employing labelled oligonucleotide probe hybridization is also time demanding and needs additional PCR product handling procedures, increasing the danger of amplicon spreading throughout the laboratory(12).The ability of visualising amplicon identification as the amplification progressed, as opposed to traditional tests, was a welcome one (13). This method has given us a lot of information on the reaction's kinetics. It also serves as the cornerstone for kinetic or "real-time" PCR (6,14-17) . Real-time PCR has already proven to be beneficial in laboratories all over the world, thanks to the massive amounts of data generated by traditional PCR tests. The labelling of primers, probes, or amplicon with fluorogenic molecules has enabled real-time monitoring of accumulating amplicon. This chemistry has evident advantages over radiogenic oligoprobes, including the avoidance of radioactive emissions, simplicity of disposal, and a longer shelf life (18) Reduced cycle times, the elimination of post-PCR detection processes, and the use of fluorescent labels and sensitive mechanisms of detecting their outputs are all contributing to real-time PCR's enhanced speed (19-20). The incapacity to assess amplicon size while opening the system, conflict of some systems with specific fluorogenic chemicals, and the comparatively limited multiplex potential of current applications are all downsides of employing real-time PCR in contrast to classical PCR. In addition, when employed in moderate laboratories, the initial cost of real-time PCR may also be exorbitant. Fluorescence resonance energy transport among fluorogenic labels either between a fluorescence but a gloomy or 'black-hole' non-fluorescent quencher (NFQ), whereby disperses excess heat instead than fluorescence, is used in the most frequently used fluorogenic oligoprobes. Obsess is a spectroscopic phenomenon in which power is transferred between molecules with overlapping emission and spectral separated by 10–100. (21-22) Förster was the one who first proposed the mechanism for this procedure: a non-radiative causing interaction (23).

RT−PCR−based molecular tests

The gold standard procedures for making a confirmed diagnosis of COVID19 infection are RTPCR-based molecular assays (24). Since the entire sequencing of the (Severe Acute Respiratory Syndrome Coronavirus 2 (SARSCoV2) genetic code (23), researchers from various countries have started developing genomic primers or probes specific to SARSCoV2 RNA sequences in order to differentiate COVID19 infections from other pathologies of clinical features, like seasonal flu and bacterial infections (25-26). The SARSCoV2 entire genome sequence is 29,903 bp long and contains the functional components listed below. A 50-base polyA cap, an extensive reading frame 1/ab (ORF1/ab) comprising the exon for such RNA dependent RNA polymerase (RdRP), spikes protein, envelope proteins, transmembrane and nucleocapsid proteins, or a 30-base polyA tail (27). Currently, genomic regions coding for the RdRP gene, proteins composing the nucleocapsid (N gene) and spike molecules (S gene), proteins of the envelope (E gene), the membrane, and other parts of the SARSCoV2 genome are employed to design particular primers and probes.

Due to the relatively low expenses of the whole viral RNA extraction, reverse transcription, as well as amplification method, and the existence of RTPCR thermal cyclers in health facilities, research centers, and private laboratories, RTPCR-based molecular tests have been assumed the best diagnostic alternative for wide target element (28). Other benefits of RTPCR procedures over other diagnostic techniques include the procedure's time savings, ease of performance, and lack of the need for highly skilled people (29). Furthermore, a variety of RTPCR kits were based on onestep amplification procedures, in which the nasopharyngeal swab's solution is introduced into the plate as well as the machine, performs the extraction, reverse transcription, amplification, and evaluation of the specimens on its own. These processes enable quick findings while also ensuring high reproducibility and consistency of the data produced, which is less influenced by operational bias (30). Overall, due to the quickness of the procedure and the availability of instruments in public and commercial medical laboratories, RTPCR is the gold standard and the most extensively used method for making an accurate

diagnosis of COVID19 infections. Furthermore, the COVID19 infection is diagnosed with high sensitivity and specificity using the RTPCR tests now present in the market.the most common RTPCR diagnostic systems now on the market, along with a description of their primary technological features.

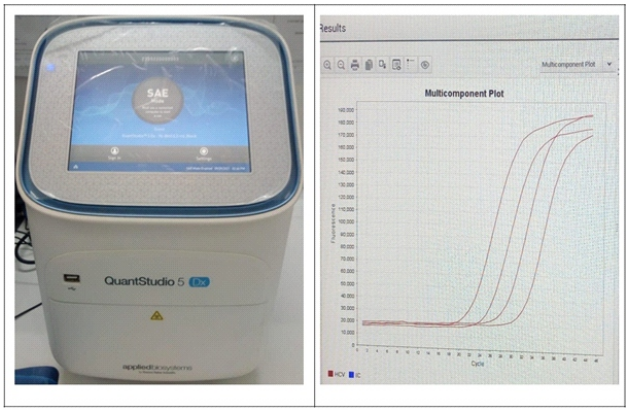

Fig. 1: RT-PCR Machine and the Multicomponent Plot

RT-PCR MOLECULAR BASED TESTS

DETECTION OF AMPLICONS

The detection methods that distinguish real-time PCR from traditional PCR tests will be discussed in the next section. There are now 5 major chemistries in use, which can be classed as viral specific genomic or non-specific real-time PCR detection systems (31). The fluorescent labels have a name linked with each of the chemistries; nevertheless, fluorescence could be used to represent these components in general discussion. That although focus in this study is on practical systems,these chemicals can also be utilised as a tag for end-point amplification detection.

FLUOROPHORES THAT ATTACH TO DNA

The DNA-binding luciferase molecule is the foundation of sequencing non-specific detection approaches. The earliest and most basic real-time PCR methods are included in this category. When dsDNA is subjected to a sufficient spectrum of light, ethidium bromide (32), YO-PRO-1 (33-34), and SYBR® green 1 (35) all glow. This method requires less specialised expertise than the development of fluorescent oligoprobes, is less inexpensive, and does not profit from changes in the template sequence, that can prevent oligoprobe hybridization (36). The synthesis of primer-dimers (37) is widespread, and it is tightly linked to the PCR entering the plateau phase , along with the formation of particular products (38-39). When a DNA-binding fluorophore is used with an amplicon or other non-specific amplified products, the findings can be difficult to interpret. After the extended step for which fluorescence data is gathered, a short, high thermal incubation is added to reduce the impact of these compounds to the fluorophore (40).

LINEAR OLIGOPROBES

The use of a set of two fluorogenic hybridisation oligoprobes had first been defined in the late 1980s (41-42). and they've since become the preferred method for the Light Cycler TM (Roche Molecular Biochemicals, Germany), a capillary-based microvolume fluorimeter as well as thermocycler with quick temperature control (43).

The upwards oligoprobe is frequently labelled with a 3′ provider fluorophore (FITC), whereas the downstream probe is generally labelled with both a LightCycler Red 640 or Red 705 recipient fluorophore at the 5′ endpoint, so that when two oligoprobes are hybridised, the result is a positive signal. The two probes are still within 10 nanometers of each other, earning them the nickname "kissing" probes. The optically transparent plastic and glass combination capillaries serve as cuvettes for fluorescent investigation while also permitting quick heat transfer. Three photodetectors diodes with varied wavelength filters watch fluorescence as capillaries revolve through a florescent light diode. The temp is changed by quickly heating and cooling air with a heat source and fan that produces 20°C/s ramp rates, allowing polymerase life to be prolonged (44) The chiral cyanine fluorescence thiazole orange is coupled to a light-up probes, which would be a peptide nucleic acid (45). The fluorescence becomes highly luminous when hybridised with only a nucleic acid target, or as a duplex or triplex, based on the oligoprobe's sequencing. These probes do not interact with PCR, do not require isomerization, are responsive to single nucleotide dissimilarities, enables fluorophore melting analysis, and since only one reporter is used, a real - time monitoring of fluorescence rather than a change in fluorophores among two fluorophores can be formed (45-46). However, during later cycles with these markers, non-specific fluorescence has been reported (47).

NUCLEASE OLIGOPROBES

Homogeneous experiments were rare in the late 1980s, but significant developments in thermocycler equipment and also the chemistry of nucleic acid modification have made them routine since then. The effectiveness of these assays is dependent on a signal altering in a timely and detectable way when a probe is hybridised to its target (48). The time needed for an oligoprobe to hybridise to its target is greatly reduced when a surplus is used, especially when the quantity of that targets has been raised by PCR or another amplifying procedure (48). Holland et al. published a

paper in 1991 that laid the groundwork for homogenous PCR with fluorogenic oligoprobes. The impact of Taq DNA polymerase's 5′3′ restriction enzymes activity on particular oligoprobe/target DNA duplexes was used to detect Amplicon. Liquid chromatography was utilised to evaluate the radiolabelled molecule, and the occurrence or lack of hydrolysis was employed as a marker of duplex formation. These oligoprobes had a 3′ phosphate group, which prevented the polymerase from extending them, but had no effect on the amplicon's output. The minor groove binding (MGB) oligoprobes are still the product of a recent improvement to a nuclease oligoprobe. This chemistry contains a ligand that maintains the oligoprobe-target duplexes by wrapping into the minor groove of the dsDNA and replacing the usual TAMRA quencher with an NFQ (49). This enables the use of oligoprobes with very short lengths (14 nucleotide), which are perfect for finding single nucleotide mutations (SNPs). Dual-labeled oligonucleotide sequencing have also been used to provide the signalling component of the DzyNA–PCR method (50).

OLIGOPROBES WITH HAIRPINS

The first hairpin oligoprobes were Molecular signals, which are a variant of the dual-labelled nuclease oligoprobe. The fluorescence - based labels on the hairpin oligoprobe were named fluorophore and quencher, but they are located at the oligoprobe's termini. The labels are kept in close proximity by distal stem sections of homologous complementary base, which are purposefully intended to generate a hairpin structure, resulting in quenching by FRET or straight transfer of energy via a collisional process (51). The oligoprobe will hybridize and shift into an open sources in the context of a complementary sequence engineered to occur within the boundaries of a primer binding sites. The fluorophore has become spatially separated from the action of the quencher, and fluorescence emissions are monitored throughout each cycle (52).

A wavelength-shifting hairpin probe, that employs a second, catching fluorophore, is a recent advancement in this chemistry. Electron density from a blue light source is passed through the harvester and released as fluorescent radiation in the far-red frequencies. The energy is subsequently transferred to a receptive 'emitter' fluorophore, which emits light at specific wavelengths. Using presently offered technologies, this could lead to enhanced multiplex real-time PCR or SNP detection (53). Because the exact hybridisation of the tip is required for these oligoprobes to work, precise design is essential.

AMPLICON THAT SELF-FLUORESCES

The notion of a self-priming amplicon is related to that of a hairpin oligoprobe, only the label is permanently incorporated into the Amplified product. Sunrise primers (now commercially known as Amplifluor TM hairpin primers) and scorpion primers have already been described (31'54). A 5′ fluorophore and a DABCYL NFQ make up the sunrise primer. When the dawn primer is closed, complimentary sequence lengths separate the labels, forming a stem. A destination primer sequence is located at the 3′ terminus. . The dawn primer's sequence is designed to be repeated by the emerging complementary strand, which destabilises the stem, keeps the two fluorophores apart by 20 nt (54), and allows the fluorophore to release its excitation energy for observing (54). Due to replication of the sunrise primer sequencing during primer-dimer synthesis, this system may exhibit non-specific fluorescence. Except for a neighbouring hexethylene glycol molecule that prevents replication of a scorpion's signalling section, the scorpion primer is nearly identical in composition. In addition to the structural differences, scorpion primers have a slightly different function except that the oligonucleotide's 5′ region is optimized to fuse to a complementary area within the amplicon. As with hairpin probes, such hybridisation pulls the labels apart, fracturing the hairpin and allowing emission (31).

QUANTITY OF VIRUSES

To date, the vast majority of diagnosing PCR assays were described in a qualitative, or "yes/no" format. True quantification of target DNA molecules has moved from the pure research facility to the diagnostic lab thanks to the introduction of real-time PCR.

PCR can be used to decide the quantity of template in two directions: relative quantification and absolute quantitation. Changes in the quantity of a target sequence related to its quantity in an associated matrix are described by relative quantitation. The exact quantity of nucleic acid targets contained in the sample in proportion to a certain unit is known as exact quantitation (55). In most cases, relative quantitation gives adequate information and is easier to implement. Absolute quantitation, on the other hand, is important for monitoring the progression of an infection because it allows scientists and clinicians to communicate data in units that are understood by both scientists and doctors and can be shared across platforms. When there aren't enough sequential specimens to show variations in virus loads, there's no suitable standardized reference reagent, and or viral load is utilized to distinguish active from chronic infection, absolute quantification may be required.

Although the ultimate values of absolute standard curves, relative standard curves, and CT values are all comparable (56). the general consensus is that an internal control, as well as copies of each sample, are required for accurate PCR quantification (38-39). However, real-time PCR software that can compute an unknown's quantity by comparing signal produced by an amplified target and now an internal control is still in its infancy. Hopefully, this situation will be rectified in future commercial editions (57).

Because of its large dynamic range, which can accept at least eight log10 copies of nucleic acid template (58-68). Real-time PCR offers considerable advances in viral load quantification. In correlation to conventional viral culture, traditional single, and nested PCR, real-time PCR is a desirable alternative to conventional PCR for the research of viral load along with its low inter-assay and intra-assay fluctuation (60,66,69) and equal or higher analytical sensitivity (60,70-75). It has been observed that real-time PCR is at least as accurate as a Southern blot (71). Although, because using software to generate optimised primers or oligoprobes is now more frequent, these data could be an underestimate due to such smaller targets, that amplify more effectively, by use of different or superior primers during real-time assays.

REAL-TIME MULTIPLEX PCR

Multiplexing (amplification of numerous templates in a pcr reaction utilizing multiple primers) is a helpful use of traditional PCR (77). However, the transition to real-time PCR has muddled the terminology. The employment of numerous fluorogenic oligoprobes for the discriminating of multiple amplicons is more frequently referred to as multiplex real-time PCR. Due to the general limited number of fluorophores present (14) and the typical use of a homogenous energizing light source, the transference of this technology has proven difficult.

VIROLOGY APPLICATIONS

Real-time PCR has proved tremendously effective in the research of infectious illness viral agents and in the clarification of disputed infectious disease states. When compared to traditional approaches, most of the assays published in the literature allow for a higher frequency of viral detection, making real-time PCR appealing to many areas of virology.

Of course, real-time PCR is becoming increasingly beneficial for general virological studies, however these applications are becoming increasingly difficult to analyses due to their nature as a technique rather than the emphasis of published studies. Such research has looked at the involvement of viruses in a variety of disorders by merely confirming the existence or lack of the virus (78-79) or, in the coming, monitoring the levels of essential gene activity (80) as a result of altered growing circumstances Real-time PCR could be used to track changes in viral entrance or replication induced by target tissue alteration, as well as linkages between pathogenicity and cellular gene expression (81-83).

Real-time PCR has improved the speed and scope of determining viral strain and titre differences in individuals with several syndromes caused by the same virus (76). Real-time PCR could also consistently find out the amount of two nucleotide objectives within a single reaction, which has enhanced the speed and range of epidemiological research (70,84). New chemistries have improved the discriminating of various viral genotypes inside a single vial (85), contributing to morbidity and mortality assays for virus identification.

This technology is now a must-have tool for thoroughly evaluating viral gene therapy vectors before they are used in clinical trials. These studies, which test the bioavailability, function, and quality of these therapeutic formulations, have most typically used nuclease oligoprobes (86-91).

Furthermore, its use of real-time PCR as a method to reveal linkages between novel viral sequences and clinical symptoms and signs has aided the study of new viruses (73,75,92-93). Commercial interests have found real-time PCR's speed and quality valuable for detecting microbial contamination of humongous reagent preparations made through eukaryotic expression systems (94-95).

CONCLUSION

As our knowledge of real-time PCR has grown, so has the amplified hardware and fluorescence - based detection chemicals, and this review attempts to bring the scientist up to date on the current state of the art. In order to focus on the several areas in which the implementation of real-time PCR has offered methodologic advantages and better health outcomes, we discuss the background, benefits, and restrictions of real-time PCR and review the literature as it pertains to virus isolation in the regular and research laboratory. Furthermore, the technology presented has been used in other fields of microbiology, including gene expression research and genetic disease research.

REFERENCES

1. Bontempi E. The europe second wave of COVID-19 infection and the Italy "strange" situation. Environ Res. 2021; 193:110476.
2. Cacciapaglia G., Cot C. Sannino F. Second wave COVID-19 pandemics in Europe: a temporal playbook. Sci Rep. 2020; 10, 15514.

3. García-Basteiro AL, Chaccour C, Guinovart C, et al. Monitoring the COVID-19 epidemic in the context of widespread local transmission. Lancet Respir Med. 2020; 8(5): 440-442.
4. Wu AHB. Screening the General Population for SARS-CoV-2 Virus and COVID-19 Antibodies: A Counterargument. J Appl Lab Med. 2020; 5(5): 1107-1110.
5. Rostami A, Sepidarkish M, Leeflang MMG, et al. SARS-CoV-2 seroprevalence worldwide: a systematic review and meta-analysis. Clin Microbiol Infect. 2021; 27(3): 331-340.
6. Wells PM, Doores KJ, Couvreur S, et al. Estimates of the rate of infection and asymptomatic COVID-19 disease in a population sample from SE England. J Infect. 2020; 81(6): 931-936.
7. Freymuth F, Eugene G, Vabret A, et al. Detection of respiratory syncytial virus by reverse transcription-PCR and hybridization with a DNA enzyme immunoassay. J Clin Microbiol. 1995; 33(12): 3352-3355.
8. Mullis KB, Faloona FA. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods Enzymol. 1987; 155: 335-350.
9. Niubo J., Perez J.L., Carvajal A., et al. Effect of delayed processing of blood samples on performance of cytomegalovirus antigenemia assay. J. Clin. Microbiol. 1994; 32: 1119-1120.
10. Guatelli JC, Gingeras TR, Richman DD. Nucleic acid amplification in vitro: detection of sequences with low copy numbers and application to diagnosis of human immunodeficiency virus type 1 infection. Clin Microbiol Rev. 1989; 2(2): 217-226.
11. Kidd IM, Clark DA, Emery VC. A non-radioisotopic quantitative competitive polymerase chain reaction method: application in measurement of human herpesvirus 7 load. J Virol Methods. 2000; 87(1-2): 177-181.
12. Holland PM, Abramson RD, Watson R, et al. Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. Proc Natl Acad Sci U S A. 1991; 88(16): 7276-7280.
13. Lomeli H, Tyagi S, Pritchard CG, et al. Quantitative assays based on the use of replicatable hybridization probes. Clin Chem. 1989 ; 35(9):1826-1831.
14. Lee LG, Connell CR, Bloch W. Allelic discrimination by nick-translation PCR with fluorogenic probes. Nucleic Acids Res. 1993;21(16):3761-3766..1093/nar/21.16.3761.
15. Livak KJ, Flood SJ, Marmaro J, et al. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl. 1995; 4(6): 357-362.
16. Heid C.A., Stevens J., Livak K.J., et al. Real time quantitative PCR. Genome Res. 1996; 6: 986-994.
17. Gibson UE, Heid CA, Williams PM. A novel method for real time quantitative RT-PCR. Genome Res. 1996; 6(10): 995-1001.
18. Matthews JA, Kricka LJ. Analytical strategies for the use of DNA probes. Anal Biochem. 1988; 169(1): 1-25.
19. Wittwer CT, Fillmore GC, Garling DJ. Minimizing the time required for DNA amplification by efficient heat transfer to small samples. Anal Biochem. 1990; 186(2): 328-331.
20. Wittwer CT, Ririe KM, Andrew RV, et al. The Light Cycler: a microvolume multisample fluorimeter with rapid temperature control. Biotechniques. 1997; 22(1): 176-181.
21. Stryer L, Haugland RP. Energy transfer: a spectroscopic ruler. Proc Natl Acad Sci U S A. 1967; 58(2): 719-726.
22. Clegg R.M. Fluorescence resonance energy transfer and nucleic acids. Methods Enzymol. 1992; 211: 353-388.
23. Förster T. Zwischenmolekulare energiewanderung und fluoreszenz. Ann. Phys. 1948; 6: 55-75.
24. Mahendiratta S, Batra G, Sarma P, et al. Molecular diagnosis of COVID-19 in different biologic matrix, their diagnostic validity and clinical relevance: A systematic review. Life Sci. 2020; 258: 118207.
25. Da Silva SJR, Silva CTAD, Guarines KM, et al. Clinical and Laboratory Diagnosis of SARS-CoV-2, the Virus Causing COVID-19. ACS Infect Dis. 2020; 6: 2319-2336.
26. Li D, Zhang J and Li J. Primer design for quantitative real-time PCR for the emerging Coronavirus SARS-CoV-2. Theranostics. 2020; 10: 7150-7162.
27. Chan JF, Kok KH, Zhu Z, et al. Genomic characterization of the 2019 novel human-pathogenic coronavirus isolated from a patient with atypical pneumonia after visiting Wuhan. Emerg Microbes Infect. 2020; 9: 221-236.
28. Dorlass EG, Monteiro CO, Viana AO, et al: Lower cost alternatives for molecular diagnosis

of COVID-19: Conventional RT-PCR and SYBR Green-based RT-qPCR. Braz J Microbiol. 2020; 51: 1117-1123.

29. Neilan AM, Losina E, Bangs AC, et al: Clinical Impact, Costs, and Cost-Effectiveness of Expanded SARS-CoV-2 Testing in Massachusetts. Clin Infect Dis. 2020; 15: 22-26.
30. Smyrlaki I, Ekman M, Lentini A, et al: Massive and rapid COVID-19 testing is feasible by extraction-free SARS-CoV-2 RT-PCR. Nat Commun. 2020; 11: 4812.
31 Whitcombe D., Theaker J., Guy S. et al. Detection of PCR products using self-probing amplicons and fluorescence. Nat Biotechnol. 1999; 17: 804-807.
32. Higuchi R, Dollinger G, Walsh PS, et al. Simultaneous amplification and detection of specific DNA sequences. Biotechnology (N Y). 1992; 10(4): 413-417.
33. Ishiguro T, Saitoh J, Yawata H, et al. Homogeneous quantitative assay of hepatitis C virus RNA by polymerase chain reaction in the presence of a fluorescent intercalater. Anal Biochem. 1995; 229(2): 207-213.
34. Tseng SY, Macool D, Elliott V, et al. An homogeneous fluorescence polymerase chain reaction assay to identify Salmonella. Anal Biochem. 1997; 245(2): 207-212.
35. Morrison TB, Weis JJ, Wittwer CT. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. Biotechniques. 1998; 24(6): 954-958.
36. Komurian-Pradel F, Paranhos-Baccalà G, Sodoyer M, et al. Quantitation of HCV RNA using real-time PCR and fluorimetry. J Virol Methods. 2001;95(1-2):111-119.
37. Chou Q, Russell M, Birch DE, et al. Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications. Nucleic Acids Res. 1992; 20(7): 1717-1723.
38. Halford WP. The essential prerequisites for quantitative RT-PCR. Nat Biotechnol. 1999; 17(9): 835.
39. Halford WP, Falco VC, Gebhardt BM, et al. The inherent quantitative capacity of the reverse transcription-polymerase chain reaction. Anal Biochem. 1999; 266(2): 181-191.
40. Wittwer C. Rapid Cycle Real-Time PCR: Methods and Applications. In: Meuer S., Wittwer C., Nakagawara KI. (eds) Rapid Cycle Real-Time PCR. Berlin: Springer; 2001.
41. Heller M.J. and Morrison L.E. In Kingsbury, D.T. and Falkow,S. (eds), Rapid Detection and Identification of Infectious Agents. New York: Academic Press; 1985.
42. Cardullo RA, Agrawal S, Flores C, et al. Detection of nucleic acid hybridization by nonradiative fluorescence resonance energy transfer. Proc Natl Acad Sci U S A. 1988; 85(23): 8790-8794.
43. Wittwer CT, Herrmann MG, Moss AA, et al. Continuous fluorescence monitoring of rapid cycle DNA amplification. Biotechniques. 1997; 22(1): 130-131.
44. Weis JH, Tan SS, Martin BK, et al. Detection of rare mRNAs via quantitative RT-PCR. Trends Genet. 1992; 8(8): 263-264.
45. Svanvik N., Westman G., Wang D. et al. Light-up probes: thiazole orange-conjugated peptide nucleic acid for the detection of target nucleic acid in homogensous solution. Anal. Biochem. 2001; 281: 26-35.
46. Isacsson J, Cao H, Ohlsson L, et al. Rapid and specific detection of PCR products using light-up probes. Mol Cell Probes. 2000;14(5): 321-328.
47. Svanvik N, Ståhlberg A, Sehlstedt U, et al. Detection of PCR products in real time using light-up probes. Anal Biochem. 2000; 287(1): 179-182.
48. Morrison LE, Halder TC, Stols LM. Solution-phase detection of polynucleotides using interacting fluorescent labels and competitive hybridization. Anal Biochem. 1989; 183(2): 231-244.
49. Kutyavin IV, Afonina IA, Mills A, et al. 3'-minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. Nucleic Acids Res. 2000; 28(2): 655-661.
50. Todd A.V., Fuery C.J., Impey H.L., et al. DzyNA–PCR: use of DNAzymes to detect and quantify nucleic acid sequences in a real-time fluorescent format. Clin. Chem. 2000; 46: 625-630.
51. Tyagi S, Bratu DP, Kramer FR. Multicolor molecular beacons for allele discrimination. Nat Biotechnol. 1998;16(1): 49-53.
52. Tyagi S, Kramer FR. Molecular beacons: probes that fluoresce upon hybridization. Nat Biotechnol. 1996; 14(3): 303-308.
53. Tyagi S, Marras SA, Kramer FR. Wavelength-shifting molecular beacons. Nat Biotechnol. 2000; 18(11): 1191-1196.
54. Nazarenko IA, Bhatnagar SK, Hohman RJ. A closed tube format for amplification and

detection of DNA based on energy transfer. Nucleic Acids Res. 1997; 25(12): 2516-2521.

55. Freeman WM, Walker SJ, Vrana KE. Quantitative RT-PCR: pitfalls and potential. Biotechniques. 1999;26(1):112-122.
56. Johnson MR, Wang K, Smith JB, et al. Quantitation of dihydropyrimidine dehydrogenase expression by real-time reverse transcription polymerase chain reaction. Anal Biochem. 2000; 278(2): 175-184.
57. Kleiber J, Walter T, Haberhausen G, et al. Performance characteristics of a quantitative, homogeneous TaqMan RT-PCR test for HCV RNA. J Mol Diagn. 2000 Aug;2(3):158-66.
58. Ishiguro T, Saitoh J, Yawata H, et al. Homogeneous quantitative assay of hepatitis C virus RNA by polymerase chain reaction in the presence of a fluorescent intercalater. Anal Biochem. 1995; 229(2): 207-213.
59. Brechtbuehl K, Whalley SA, Dusheiko GM, et al. A rapid real-time quantitative polymerase chain reaction for hepatitis B virus. J Virol Methods. 2001; 93(1-2): 105-113.
60. Locatelli G, Santoro F, Veglia F, et al. Real-time quantitative PCR for human herpesvirus 6 DNA. J Clin Microbiol. 2000; 38(11): 4042-4048.
61. Kimura H, Morita M, Yabuta Y, et al. Quantitative analysis of Epstein-Barr virus load by using a real-time PCR assay. J Clin Microbiol. 1999; 37(1): 132-136.
62. Najioullah F, Thouvenot D, Lina B. Development of a real-time PCR procedure including an internal control for the measurement of HCMV viral load. J Virol Methods. 2001; 92(1): 55-64.
63. Ryncarz AJ, Goddard J, Wald A, et al. Development of a high-throughput quantitative assay for detecting herpes simplex virus DNA in clinical samples. J Clin Microbiol. 1999; 37(6): 1941-1947.
64. Monpoeho S, Dehée A, Mignotte B, et al. Quantification of enterovirus RNA in sludge samples using single tube real-time RT-PCR. Biotechniques. 2000; 29(1): 88-93.
65. Alexandersen S, Oleksiewicz MB, Donaldson AI. The early pathogenesis of foot-and-mouth disease in pigs infected by contact: a quantitative time-course study using TaqMan RT-PCR. J Gen Virol. 2001; 82(Pt 4): 747-755.
66. Abe A, Inoue K, Tanaka T, et al. Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. J Clin Microbiol. 1999; 37(9): 2899-903.
67. Gruber F, Falkner FG, Dorner F, et al. Quantitation of viral DNA by real-time PCR applying duplex amplification, internal standardization, and two-color fluorescence detection. Appl Environ Microbiol. 2001; 67(6): 2837-2839.
68. Moody A, Sellers S, Bumstead N. Measuring infectious bursal disease virus RNA in blood by multiplex real-time quantitative RT-PCR. J Virol Methods. 2000; 85(1-2): 55-64.
69. Schutten M, van den Hoogen B, van der Ende ME, et al. Development of a real-time quantitative RT-PCR for the detection of HIV-2 RNA in plasma. J Virol Methods. 2000; 88(1): 81-87.
70. Kearns AM, Turner AJ, Taylor CE, et al. LightCycler-based quantitative PCR for rapid detection of human herpesvirus 6 DNA in clinical material. J Clin Microbiol. 2001; 39(8): 3020-3021.
71. Capone RB, Pai SI, Koch WM, et al. Detection and quantitation of human papillomavirus (HPV) DNA in the sera of patients with HPV-associated head and neck squamous cell carcinoma. Clin Cancer Res. 2000; 6(11): 4171-4175.
72. Leutenegger CM, Klein D, Hofmann-Lehmann R, et al. Rapid feline immunodeficiency virus provirus quantitation by polymerase chain reaction using the TaqMan fluorogenic real-time detection system. J Virol Methods. 1999; 78(1-2): 105-116.
73. Smith IL, Halpin K, Warrilow D, et al. Development of a fluorogenic RT-PCR assay (TaqMan) for the detection of Hendra virus. J Virol Methods. 2001; 98(1): 33-40.
74. Van Elden LJ, Nijhuis M, Schipper P, et al. Simultaneous detection of influenza viruses A and B using real-time quantitative PCR. J Clin Microbiol. 2001; 39(1): 196-200.
75. Lanciotti RS, Kerst AJ, Nasci RS, et al. Rapid detection of west nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. J Clin Microbiol. 2000; 38(11): 4066-4071.
76. Furuta Y, Ohtani F, Sawa H, et al. Quantitation of varicella-zoster virus DNA in patients with Ramsay Hunt syndrome and zoster sine herpete. J Clin Microbiol. 2001; 39(8): 2856-2859.
77. Chamberlain JS, Gibbs RA, Ranier JE, et al. Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic Acids Res. 1988; 16(23): 11141-11156.
78. Kato T, Mizokami M, Mukaide M, et al.

Development of a TT virus DNA quantification system using real-time detection PCR. J Clin Microbiol. 2000; 38(1): 94-98.

79. Iriyama M, Kimura H, Nishikawa K, et al. The prevalence of TT virus (TTV) infection and its relationship to hepatitis in children. Med Microbiol Immunol. 1999; 188(2): 83-89.
80. Liu J, Feldman P, Chung TD. Real-time monitoring in vitro transcription using molecular beacons. Anal Biochem. 2002; 300(1): 40-45.
81. Kennedy MM, O'Leary JJ, Oates JL, et al. Human herpes virus 8 (HHV-8) in Kaposi's sarcoma: lack of association with Bcl-2 and p53 protein expression. Mol Pathol. 1998; 51(3): 155-159.
82. Kennedy MM, Biddolph S, Lucas SB, et al. CD40 upregulation is independent of HHV-8 in the pathogenesis of Kaposi's sarcoma. Mol Pathol. 1999;52(1):32-36.
83. Kennedy MM, Biddolph S, Lucas SB, et al. Cyclin D1 expression and HHV8 in Kaposi sarcoma. J Clin Pathol. 1999; 52(8): 569-573.
84. Zerr DM, Huang ML, Corey L, et al. Sensitive method for detection of human herpesviruses 6 and 7 in saliva collected in field studies. J Clin Microbiol. 2000; 38(5): 1981-1983.
85. Jordens JZ, Lanham S, Pickett MA, et al. Amplification with molecular beacon primers and reverse line blotting for the detection and typing of human papillomaviruses. J Virol Methods. 2000; 89(1-2): 29-37.
86. Josefsson AM, Magnusson PK, Ylitalo N, et al. Viral load of human papilloma virus 16 as a determinant for development of cervical carcinoma in situ: a nested case-control study. Lancet. 2000; 355(9222): 2189-2193.
87. Choo CK, Ling MT, Suen CK, et al. Retrovirus-mediated delivery of HPV16 E7 antisense RNA inhibited tumorigenicity of CaSki cells. Gynecol Oncol. 2000; 78(3 Pt 1): 293-301.
88. Gerard CJ, Arboleda MJ, Solar G, et al. A rapid and quantitative assay to estimate gene transfer into retrovirally transduced hematopoietic stem/progenitor cells using a 96-well format PCR and fluorescent detection system universal for MMLV-based proviruses. Hum Gene Ther. 1996; 7(3): 343-354.
89. Hackett NR, El Sawy T, Lee LY, et al. Use of quantitative TaqMan real-time PCR to track the time-dependent distribution of gene transfer vectors in vivo. Mol Ther. 2000; 2(6): 649-656.
90. Sanburn N, Cornetta K. Rapid titer determination using quantitative real-time PCR. Gene Ther. 1999; 6(7): 1340-1345.
91. Scherr M, Battmer K, Blömer U, et al. Quantitative determination of lentiviral vector particle numbers by real-time PCR. Biotechniques. 2001; 31(3): 520-524.
92. Lanciotti RS, Kerst AJ. Nucleic acid sequence-based amplification assays for rapid detection of West Nile and St. Louis encephalitis viruses. J Clin Microbiol. 2001; 39(12): 4506-4513.
93. Gibb TR, Norwood DA Jr, Woollen N, et al. Development and evaluation of a fluorogenic 5' nuclease assay to detect and differentiate between Ebola virus subtypes Zaire and Sudan. J Clin Microbiol. 2001; 39(11): 4125-4130.
94. Brorson K., Swann P.G., Lizzio E., et al. Use of a quantitative product-enhanced reverse transcriptase assay to monitor retrovirus levels in mAb cell-culture and downstream processing. Biotechnol. Prog. 2001; 17: 188-196.
95. De Wit C, Fautz C, Xu Y. Real-time quantitative PCR for retrovirus-like particle quantification in CHO cell culture. Biologicals. 2000; 28(3): 137-148.

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