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Design, development and evaluation of commercial kit for Rodent Pathogens by Taqman Chemistry

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Abstract

Now-a-days, rapid detection and identification of rodent pathogens such as bacteria, fungi, virus etc., have become important step in the development of therapeutic management of infectious disease. Conventional microbiological diagnostic methods have made inconclusive results in early diagnosis of pathogens as well as demanding labor too. Numerous recent innovations bought us different molecular diagnostics methods aiming to automated laboratory system with rapid detection and identification of rodent pathogens. Those innovative methods targets specific nucleic acid (DNA or RNA) for the detection of microorganisms based on nucleic acid probe (TaqMan chemistry) and amplification chemistry. The present review emphasised on the application of TaqMan chemistry in the design, development and evaluation of commercial kit for rapid detection of rodent pathogens.

Key words: Pathogens, Taqman Chemistry, Design, Commercial Kits

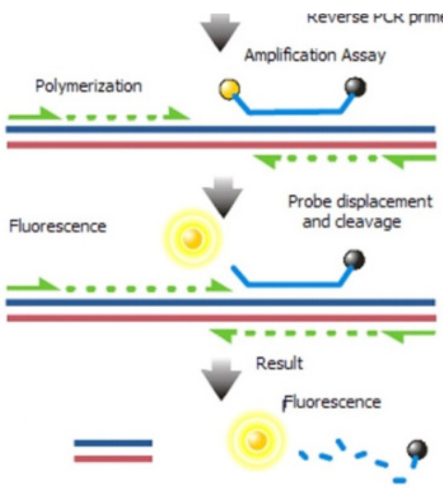
Introduction

Early and prompt laboratory diagnosis of pathogen infections is imperative in choosing appropriate antibiotic treatment (Narayanasamy, 2008; Black et al., 2002). Occasionally, early diagnosis of pathogen infections remains challenging clinically as in case of Mycoplasma pneumonia infections (Piekarska et al., 2015; Chang et al., 2013). Conventional microbiological methods rely on culture of pathogens with the specimens followed by isolation and study of their morphological characteristics under microscope, wherein sometimes derive inconclusive results, labor demanding, time consuming and cumbrous (Reta et al., 2020; Krafft et al., 2001; McFarlane et al., 1986). To overcome the disadvantages of conventional microbiological methods, recent innovations have made possible such as polymerase chain reaction (PCR) methods based on nucleic acid probe (Taqman chemistry) and amplification technology advantaging accurate detection of suspecting pathogens and gaining much more attention as a laboratory diagnostic method (Tsai et al., 2012; Krafft et al., 2001). Real time PCR based on isothermal nucleic acid amplification assays is often using now-days for accurate and efficient detection of ongoing widespread SARS-CoV-2 (Carter et al., 2020; Corman et al., 2020). The present article emphasized on the designing, development and evaluation of commercial kit for rapid identification of pathogens based on principle of Taqman Probe Chemistry.

TaqMan Probe Chemistry

TaqMan probes are hydrolysis type of probes which are intended to escalate the specificity of quantitative PCR, is reported by Kary Mullis for the first time in 1991 and the technology was subsequently developed by Hoffmann-La Roche for diagnostic assays and for research applications (Holland et al., 1991). Its principle is based on the 5′–3′ exonuclease activity of Taq polymerase to cleave a dual-labeled probe during hybridization to the complementary target sequence and fluorophore-based detection along with significant increased specificity of detection.

Figure: 1Taqman Method



TaqMan probes consist of a fluorophore covalently attached to the 5'-end of the oligonucleotide probe and a quencher molecule at the 3'-end which quenches the fluorescence emitted by the fluorophore when excited by the cycler's light source. As long as the fluorophore and the quencher are in proximity, quenching inhibits any fluorescence signals. They are designed in such a way that they anneal within a DNA region amplified by a specific set of primers. Then, it can be conjugated to a minor groove binder (MGB) moiety, dihydrocyclo pyrroloindole tripeptide (DPI3), in order to increase its binding affinity to the target sequence; MGB-conjugated probes have a higher melting temperature (Tm) due to increased stabilisation of van der Waals forces. As the Taq polymerase extends the primer and synthesizes the nascent strand (again, on a single-strand template, but in the direction opposite to that shown in the diagram, i.e. from 3' to 5' of the complementary strand), the 5' to 3' exonuclease activity of the Taq polymerase degrades the probe that has annealed to the template. Degradation of the probe releases the fluorophore from it and breaks the proximity to the quencher, thus relieving the quenching effect and allowing fluorescence of the fluorophore. Hence, fluorescence detected in the quantitative PCR thermal cycler which is directly proportional to the fluorophore released and the amount of DNA template present in the PCR (Holland et al., 1991).

Requirements for designing and development of Commercial Diagnostic Kit based on Taqman Chemistry

1. Primers and Probes

Firstly, the primers and probes were designed from configurations of desired pathogens to be detected and also on the basis of the suggestions provided for real-time PCR. The rDNA sequences should be obtained from the nucleotide sequence from National Center for Biotechnology Information (NCBI) database using programmer Perkin-Elmer, Applied Biosystems, USA. Initially, the ability of the primers and probe to identify desired pathogen sequences should be assessed by inputting into the program using default settings and the optimal primer and probe sequences can be obtained. TaqMan probes should preferentially have melting temperatures 10°C higher than the primers. The probes are labeled with a fluorescent reporter dye on the 5′ end and a minor groove binder-nonfluorescent quencher dye on the 3′ end. The presence of 5′-end guanosine residue and long sequences of identical nucleotides should be avoided.

Two sets of primers and probes should be designed based on the genome of desired pathogen to be diagnosed using Primer Express software. A set of principles will be applied while design of primers and probes such as primer and probe length, their melting temperature, nucleotide bases G or C content. For example, to diagnose SARS-CoV-2, the principles are primer length of 18–25 bp; primer melting temperature (Tm) of 55–60^{0}C; primer G + C content of 40%–60%; probe length of 20–30 bp; probe melting temperature (Tm) of 60–65^{0}C; probe G + C content of 40%–60% (Liu et al., 2020; Corman et al., 2020; Hughes et al., 2004; Smythe et al., 2004).

2. Plasmid as standard reference molecule

Two standard plasmids as molecular references are fabricated by using PCR 4-topoisomerase I vector and used to determine the detection limit of real-time PCR. Polymerase chain reaction amplicons from positions of interest on gene will obtain using primer pair from genomic deoxyribonucleic acid (DNA) of the Shope strain as a template. The PCR products are then ligated into PCR 4 topoisomerase vector by Taq-amplified (TA) Cloning Kit. The cloned DNA is prepared with Qiagen Plasmid Mini Kit, then confirmed by sequencing and its concentration determined by spectrophotometry. Copy numbers of each standard plasmid are determined by calculating the molecular weight of each cloned plasmid (Ma et al., 2008).

3. Preparation of DNA

Pathogen of interest DNA is extracted from samples including culture fluid, vaccine, nasal swab sample, blood sample or tissue homogenate according to commercially available kits. Pathogenic DNA is eluted in 200 µl nuclease-free water which will be used as template for PCR.

4. PCR conditions and assay

The TaqMan reactions will be performed in reaction volume preferably 25µl comprised of TaqMan master mix, TaqMan primer (at different concentrations), probe (at different concentrations), nuclease free water and cDNA. All TaqMan

PCRs will be performed as uniplex reactions with one set of TaqMan primers and probe per well and reactions will be carried out in 96-well reaction plates sealed with optical caps. Then, the plates can transferred to an ABI Prism 7700 sequence detection system, and DNA will be amplified using program cycle of 1 cycle each of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles each of 95°C for 15 s and 60°C for 1 min.

TaqMan runs with experimental test samples with at least four replicates each of a known positive control, negative control cDNA and nuclease free water. Each test sample will run in triplicate with each of specific TaqMan primer and probe along with positive control cDNA, negative control cDNA and nuclease-free water using β-actin as an endogenous control of standard mRNA. A number of sample replicates can run on the same plate, thus variability within the run and in between the runs can measure. Standard curves were not generated for quantification experiments as all total RNA levels were within the linear and equal amplification range of the assay and thus applicable to quantification through normalization with β-actin mRNA (Mizusawa et al., 2017).

For each PCR, a threshold cycle number (Ct) is obtained corresponding to the PCR cycle number during which the fluorescence of the reaction rose above a threshold value statistically determined by the computer software. The Ct values are inversely proportional to the $\log_{10}$ of the amount of template in the PCR. A difference of 1 Ct corresponds to a twofold difference in template amounts. A Ct value less than the mean plus two standard deviations of the negative control wells was considered positive. A Ct value of 40 corresponds to no amplification. The levels of β-actin is achieved by subtracting the highest mean β-actin Ct value from the mean β-actin Ct of each sample. This difference was subtracted from the mean Ct value obtained from the specific TaqMan PCR. This provided a method to account for differences in the levels of viral RNA due to sample heterogeneity. Data were adjusted in sets according to tissue type because the suitability of endogenous controls can be tissue specific (Feng et al., 2009; Smith, 2002).

Discussion:

Since the beginning of RT PCR, some of the measuring methods are used to compute data and each one optimized for an exceptional goal. In qRT-PCR two methods have more popularity, TaqMan and SYBR Green. Commonly, working with the SYBR Green method is inexpensive and easier than TaqMan. In SYBR Green no need to probe design and synthesis, nevertheless in many cases scientists prefer TaqMan method (Orlando et al., 1998; Valasek and Repa 2005). Influence of TaqMan method due to its exclusive design based on oligonucleotide double labeled probe and the exonuclease activity of Taq polymerase enzyme, whereas SYBR Green design based on binding of florescent dye to dsDNA. Obviously in SYBR Green method, any non-specific product like primer-dimer can make false positive results and this incorrect and shifted data can finally lead to decrease the performances (Wilhelm et al., 2003). But The analysis of TaqMan based PCR exploits the 5' -3' nuclease activity of the Taq polymerase to notice and measure specific PCR products as the reaction proceeds. The internal target specific TaqMan probe is attached with a receptor fluorochrome (e.g., FAM, VIC or JOE) and a quencher fluorochrome (e.g., TAMRA). As long as these two fluorochromes are in each others close vicinity, the fluorescence emitted by the reporter fluorochrome is absorbed by the quencher fluorochrome. However, upon amplification of the target sequence the TaqMan probe is degraded by the *Taq* polymerase, resulting in the separation of the reporter and quencher fluorochrome. As a result, the fluorescence signal of the reporter and quencher fluorochrome will become detectable and further increases during the consecutive PCR cycles because of the progressive accumulation of free reporter fluorochromes. Major advantage of TaqMan Chemistry is Exact hybridization between probe and target is essential to produce fluorescent signal, probes can be labeled with various, distinguishable reporter dyes, which permits amplification of two different sequences in one reaction tube, post-PCR processing is eradicated, which reduces assay labor and material costs.

Conclusion

TaqMan is one of the new emerging technologies adopted by industries and laboratories for diagnosing different pathogens in a more accurate and reliable manner. Many diagnostic kits have been developed based on the working principles of TaqMan nuclear probe chemistry and amplification which is a crucial step in the therapeutic management of diseases clinically. TaqMan method uses dual-labeled probes for detection of the accumulated DNA. Real-time systems for PCR were improved by the introduction of fluorogenic-labeled probes that use the 5´nuclease activity of Taq DNA polymerase. The availability of these fluorogenic probes enabled the development of a real-time method for detecting only specific amplification products. The development of fluorogenic labeled probes also made it possible to eliminate post-PCR processing for the analysis of probe degradation.

Declaration of Competing Interest:

The authors declare no conflict of interest.

References

1. Chang HY, Chang LY, Shao PL, Lee PI, Chen JM, Lee CY, Lu CY, Huang LM (2013). Comparison of real-time polymerase chain reaction and serological tests for the confirmation of Mycoplasma pneumoniae infection in children with clinical diagnosis of atypical pneumonia, *Journal of Microbiology, Immunology and Infection*. http://dx.doi.org/10.1016/j.jmii.2013.03.015

2. Piekarska K, Rastawicki W, Michalak A, Rzeczkowska M, Kamińska T, Wojtas G, Paradowska-Stankiewicz I. Wady i zalety (2015). mikrobiologicznych metod diagnostycznych stosowanych w rozpoznaniu zakażenia wywołanego przez Mycoplasma pneumoniae na przykładzie wybranej sytuacji klinicznej [Advantages and disadvantages of microbiological methods used in the diagnosis of Mycoplasma pneumoniae infections in selected clinical situation]. *Med Dosw Mikrobio*l. 67(1): 39-46.

3. Reta DH, Tessema TS, Ashenef AS, Desta AF, Labisso WL, Gizaw ST, Abay SM, Melka DS, Reta FA (2020). Molecular and Immunological Diagnostic Techniques of Medical Viruses, *International Journal of Microbiology*, 2020: 883272. https://doi.org/10.1155/2020/8832728

4. Narayanasamy P. (2008) Molecular Techniques for Detection of Microbial Pathogens. In: Molecular Biology in Plant Pathogenesis and Disease Management. Springer, Dordrecht. https://doi.org/10.1007/978-1-4020-8243-6_2.

5. Krafft, Amy E.; Kulesh, David A. (2001). Applying Molecular Biological Techniques to Detecting Biological Agents. *Clinics in Laboratory Medicine,* 21(3): 631–660.

6. Tsai Y-L, Wang H-TT, Chang H-FG, Tsai C-F, Lin C-K, Teng P-H, et al. (2012) Development of TaqMan Probe-Based Insulated Isothermal PCR (iiPCR) for Sensitive and Specific On-Site Pathogen Detection. PLoS ONE 7(9): e45278. https://doi.org/10.1371/journal.pone.0045278

7. Holland, P. M.; Abramson, R. D.; Watson, R.; Gelfand, D. H. (1991). Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. *Proceedings of the National Academy of Sciences of the United States of America*. 88 (16): 7276–7280.

8. Carter LJ, Garner LV, Smoot JW, Li Y, Zhou Q, Saveson CJ, Sasso JM, Gregg AC, Soares DJ, Beskid TR, Jervey SR, Cynthia Liu C (2020). Assay Techniques and Test Development for COVID-19 Diagnosis. *ACS Central Science* 6 (5): 591-605.

9. Liu Y, Wang Y, Wang X, Xiao Y, Chen L, Guo L, Li J, Ren L, Wang J (2020). Development of two TaqMan real-time reverse transcription-PCR assays for the detection of severe acute respiratory syndrome coronavirus-2. *Biosaf Health*. 2(4): 232-237. doi: 10.1016/j.bsheal.2020.07.009.

10. Hughes GJ, Smith JS, Hanlon CA, Rupprecht CE (2004). Evaluation of a TaqMan PCR assay to detect rabies virus RNA: influence of sequence variation and application to quantification of viral loads. *J Clin Microbiol*. 42(1): 299-306. doi: 10.1128/JCM.42.1.299-306.2004.

11. Smythe, L.D., Smith, I.L., Smith, G.A, Dohnt MF, Symonds ML, Barnett LJ, McKay DB (2002). A quantitative PCR (TaqMan) assay for pathogenic Leptospira spp. BMC Infect Dis. 2, 13. https://doi.org/10.1186/1471-2334-2-13.

12. Ma W, Lager KM, Richt JA, Stoffregen WC, Zhou F, Yoon KJ (2008). Development of Real-Time Polymerase Chain Reaction Assays for Rapid Detection and Differentiation of Wild-Type Pseudorabies and Gene-Deleted Vaccine Viruses. *J Veterinary Diagnostic Investigation*. 2008; 20(4). https://doi.org/10.1177/104063870802000405.

13. Black E. M., J. P. LowingsJ. Smith P. R. Heaton and. McElhinney LM (2002). A rapid RT-PCR method to differentiate six established genotypes of rabies and rabies-related viruses using TaqMan technology. *J. Virol. Methods*. 105: 25-35.

14. Corman V.M., Landt O., Kaiser M (2020). Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Eurosurveillance*. 25(3): 2000045. doi: 10.2807/1560-7917.ES.2020.25.3.2000045.

15. McFarlane, RG, Thawley, DG, Solorzano, RF (1986). Detection of latent pseudorabies virus in porcine tissue, using a DNA hybridization dot-blot assay. *Am J Vet Res*. 47: 2329–2336.

16. Mizusawa M, Miller H, Green R, Lee R, Durante M, Perkins R, Hewitt C, Simner PJ, Carroll KC, Hayden RT, Zhang SX. (2017). Can multidrug-resistant Candida auris be reliably identified in clinical microbiology laboratories? *J Clin Microbiol*. 55: 638–640.

17. Feng Y, Feng Y, Bai J, Xia X, Zhao W (2009). Development of Real-Time PCR Assays for the Quantitative Detection of CD81 Receptor Gene of Hepatitis C Virus in Tupaia Belangeri, *2nd International Conference on Biomedical Engineering and Informatics*, pp. 1-5.

18. Smith, J. S. (2002). Molecular epidemiology, p. 79-111. In A. C. Jackson and W. H. Wunner (ed.), Rabies. Academic Press, New York, N.Y

19. Valasek M. A. and Repa J. J. (2005). The power of real-time PCR. *Advances in Physiology Education*, 29(3): 151-159.

20. Orlando C. Pinzani, P. and Pazzagli M. (1998). Developments in quantitative PCR. *Clin Chem Lam Med*. 36,(5): 255-269.

21. Wilhelm, J., Pingoud, A., and Hahn, M. (2003). Real time PCR based method for the estimation of genome sizes. *Nucleic Acids Research,* 31(10), e56-e56.