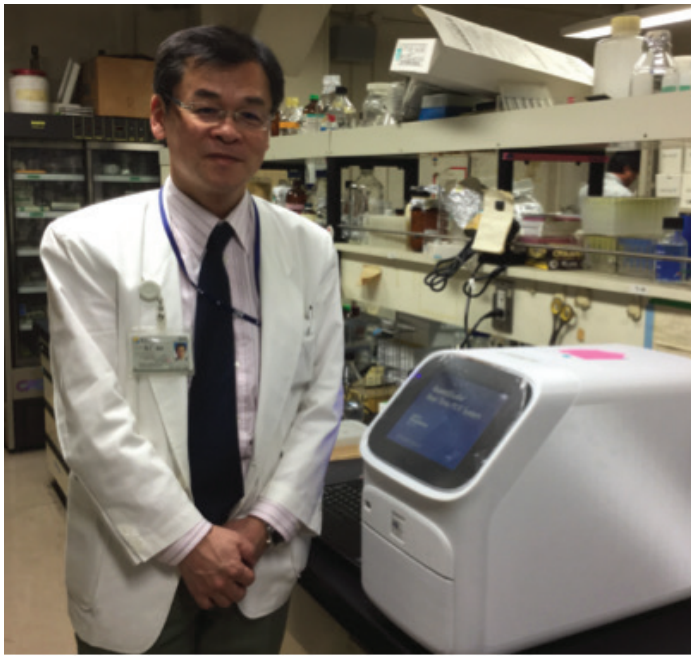
Real-time PCR technology uses multiplexing and VeriFlex functionality to differentiate between species of *Leishmania*

Customer summary

Dr. Eisei Noiri is an associate professor at the University of Tokyo Hospital involved in research focused on the prevention and diagnosis of neglected tropical diseases, especially kala-azar. This initiative is part of the Science and Technology Research Partnership for Sustainable Development (SATREPS) program funded jointly by the Japan International Cooperation Agency (JICA) and Japan Science and Technology Agency (JST). The goal of SATREPS is to address global issues, advance science and technology, and boost the capacity of developing countries for self-reliant research and development.



Abstract

Real-time PCR–based gene expression analysis requires instrument platforms that can deliver accurate and reproducible results as well as offer the flexibility for users to customize their experiments. In this study, researchers from the University of Tokyo used lyophilized reagents in experiments run on the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System to detect and differentiate between species of *Leishmania*. This genus of trypanosomatid protozoa causes kala-azar, a serious zoonotic disease that affects the liver and spleen. Genomic DNA samples purified from blood were investigated using multiplex assay experiments and Applied Biosystems™ VeriFlex™ technology to identify various species of *Leishmania*. The flexibility of the Applied Biosystems™ QuantStudio™ 5 system, VeriFlex™ Blocks, and associated reagents allowed results to be obtained with a faster turnaround time when compared to running independent thermal cycling protocols at different temperatures. The ability to quickly identify different species of *Leishmania* is valuable for researchers studying methods for the diagnosis and treatment of kala-azar.

Introduction

Kala-azar, also known as visceral leishmaniasis or black fever, is an infectious disease caused by protozoan parasites of the *Leishmania* genus [1]. Symptoms appear 1–2 months after being bitten by a sand fly carrying the parasite. The disease causes skin ulcers and swelling of the liver and spleen, and without intervention is fatal in 90% of cases. According to the World Health Organization (WHO), over 300,000 new cases of kala-azar occur annually, with 90% occurring in Sudan, Bangladesh, India, and Nepal [2].

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Project background

Among the more than 20 species of *Leishmania* that infect humans, *L. donovani* and *L. infantum* are two of the more common species that cause kala-azar. Both genome sequences are very similar, so Dr. Noiri and his team designed nested Applied Biosystems™ TaqMan™ Assays to differentiate between species. They used genomic DNA samples purified from human blood as the basis for these experiments.

Testing was performed at Dr. Noiri's lab in Japan. The QuantStudio 5 system allowed Dr. Noiri and his team to judge the presence or absence of the intended target and distinguish two species of *Leishmania* simultaneously by using VeriFlex technology and multiplexing capabilities. Due to the geographic occurrence of kala-azar, the ability to easily share data uploaded to Thermo Fisher Cloud will allow for easier and faster collaboration between Dr. Noiri's lab in Japan and collaborators located in Bangladesh.

Materials and methods

Nested custom TaqMan Assays were designed to distinguish between two different species of *Leishmania*. Assay performance was first tested on pure strains of *L. donovani* and *L. infantum* while using a universal *Leishmania* assay as a reference. Once assay performance was determined, genomic DNA from human blood samples were run on the QuantStudio 5 system using Applied Biosystems™ TaqMan™ RNase P Assay (Cat. No. 4485714) as an endogenous reference. Genomic DNA from human blood samples were extracted using the Applied Biosystems™ DNA Extract All Reagents Kit (Cat. No. 4403319).

Results

Pure *L. donovani* and *L. infantum* strain samples were run on the QuantStudio 5 system in the plate layout shown in Figure 1. VeriFlex Blocks temperature zones were set to 65°C, 67.5°C, and 70°C for each respective strain to demonstrate nested assay performance. Experiments were run as 3-plex assays utilizing a primer/probe combination specific to *L. donovani*, a primer/probe combination specific to *L. infantum*, and a universal primer/probe combination with homology to all known strains of *Leishmania*.

Thermo Fisher Cloud

Thermo Fisher Cloud is a powerful cloud computing platform that connects scientists, instruments, and software in a collaborative, multidisciplinary environment. Data can be safely stored, analyzed, managed, and shared with colleagues. Applied Biosystems™ qPCR analysis modules are innovative cloud-based secondary data analysis solutions that bring together multiple data sets in one convenient place. This online solution makes it easier to view, store, and analyze qPCR data. Applied Biosystems qPCR analysis modules take advantage of cloud computing to provide highly versatile analysis tools that are flexible, fast, and easy to use.

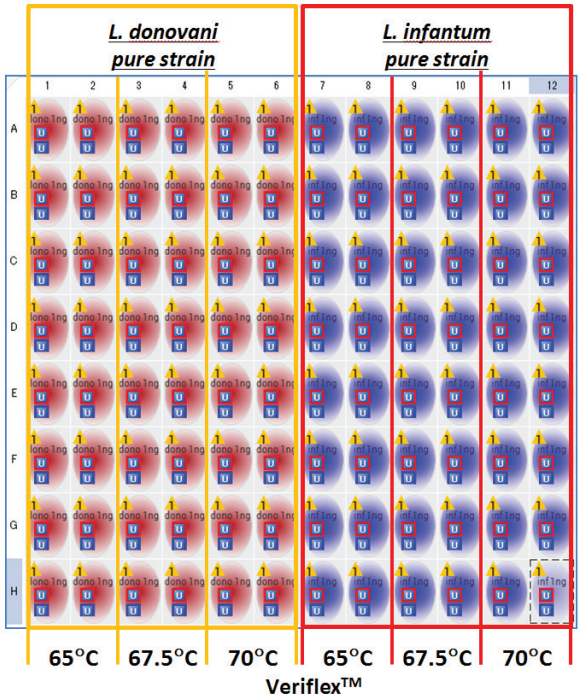


Figure 1. Combination of samples, assays, and reaction temperatures. By using the QuantStudio 5 system, it is possible to test multiple samples, assay combinations, and reaction temperatures in one experiment. The QuantStudio 5 system is equipped with VeriFlex Blocks temperature zones and 6 decoupled excitation and emission channels for multicolor detection.

As shown in Figure 2A, when running the same pure strain of *L. donovani* at different cycling temperatures, amplification of the *L. infantum* assay begins to drop off as the cycling temperature increases. By 70°C, amplification has almost fallen below the set threshold. Figure 2B shows the results of a pure strain of *L. infantum* run against the same 3 assays.

The sample was run again at different cycling temperatures. However, the *L. infantum* assay still amplified at 70°C, identifying the sample as having higher homology to the intended target. This discrepancy in amplification between the two samples allows differentiation between the two species of *Leishmania*.

The same assays were then tested against genomic DNA from human blood samples that were known to be positive for *Leishmania*. This experiment was run as a 4-plex reaction using RNase P as an endogenous control at 67.5°C (identified in previous experiments as an assay condition that can differentiate the two species).

As shown in Figure 3, the two species of *Leishmania* can be distinguished by a difference in the amplification plots for the respective targets. This allows the infectious species in the human blood sample to be identified as *L. donovani*.

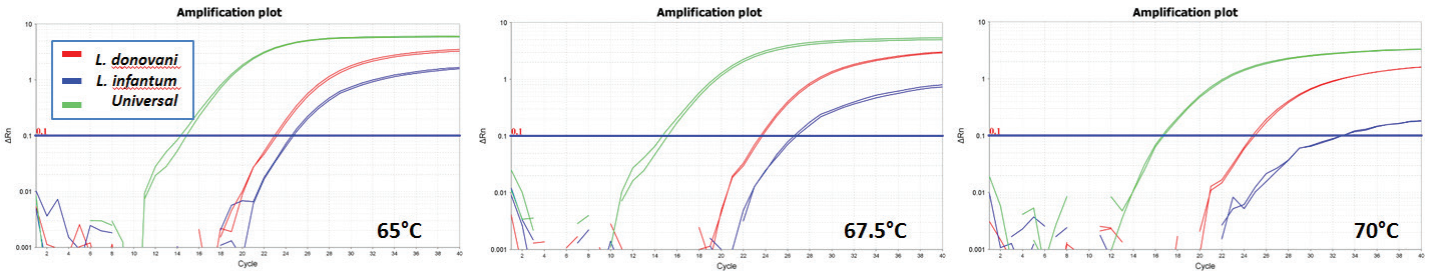
VeriFlex Blocks

VeriFlex Blocks provide 6 independent temperature zones for precise control over real-time PCR optimization. VeriFlex Blocks allow for a true linear temperature across the independently controlled thermal blocks. Each temperature zone can be set individually, allowing for better control of temperature optimization when compared to conventional gradient blocks.

Lyophilized Reagents

Applied Biosystems™ Custom TaqMan Assays are also available in lyophilized format, complete with TaqMan™ master mix. These single-use assays enable sensitive and precise real-time PCR experiments while simplifying the overall workflow. The only pipetting step required is to add sample to the reaction well. The lyophilized format extends shelf life and eliminates the need for refrigeration during shipment and storage of test kits. The lyophilized format is available for custom or catalog TaqMan Assays, and can be delivered in 8-strip tubes, 96-well plates, or 384-well plates.

A



B

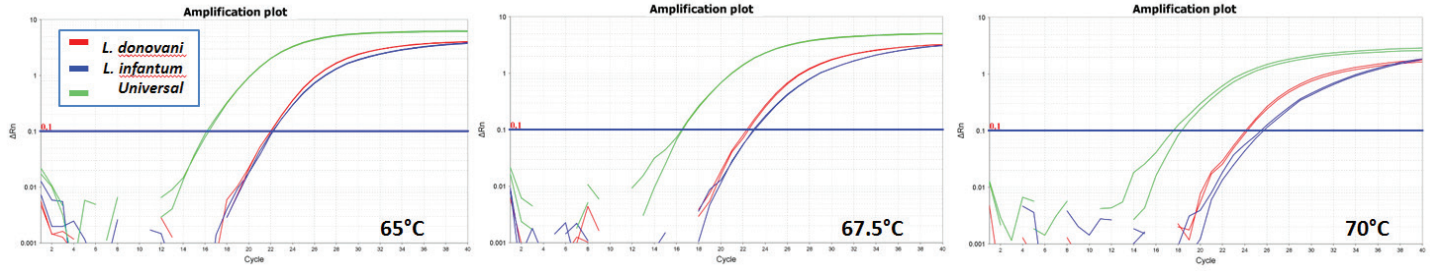


Figure 2. Differentiation between two species of *Leishmania* using a 3-plex assay. Pure strains of **(A)** *L. donovani* and **(B)** *L. infantum* were run at different reaction temperatures using VerFlex Blocks. For the *L. infantum* pure strain, the difference in amplification curves between the *L. donovani* assay and *L. infantum* assay is smaller than the *L. donovani* pure strain. This phenomenon is becomes more apparent at higher temperatures (67.5°C and 70°C).

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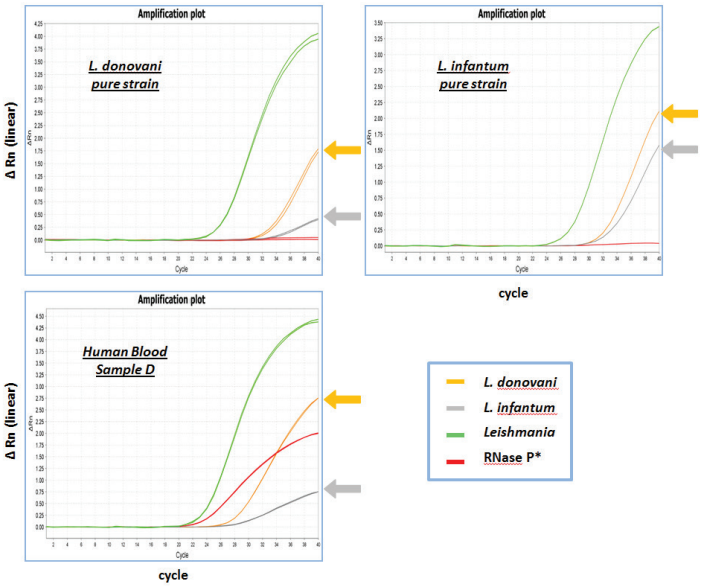

Figure 3. Differentiation between two species of *Leishmania* in a human blood sample using a 4-plex assay. *L. donovani* and *L. infantum* is differentiated by the ratio of the amplification plots of the respective assays. The large difference in amplification curves between the *L. donovani* and *L. infantum* assays in human sample D suggests that the sample contains *L. donovani*.

Conclusions

Applied Biosystems™ Custom TaqMan™ Gene Expression Assays in conjunction with the QuantStudio 5 Real-Time PCR System provide a comprehensive and affordable solution to evaluate the presence or absence of *Leishmania*, as well as the ability to distinguish between various species. The small footprint and ability to connect wirelessly through Thermo Fisher Cloud also provides greater assistance for researchers looking to access information remotely from areas where this infectious disease is most common, and more easily collaborate with researchers in other locations.

References

1. Ready PD (2014) Epidemiology of visceral leishmaniasis. *Clin Epidemiol* 6:147–154.
2. Alvar J, Velez ID, Bern C et al. (2012) Leishmaniasis worldwide and global estimates of its incidence. *PLoS One* 7:e35671.

Multiplex qPCR

Multiplex qPCR is a simple, efficient, and cost-effective solution for overcoming the challenges of limited samples and costly analysis. Successful multiplex qPCR enables the amplification of more than one target in a single reaction using different reporters with distinct fluorescent spectra, making it possible to use less sample in each experiment. The QuantStudio 5 system offers six excitation filters and six emission filters (21 different color combinations), allowing a broad range of detection chemistries and maximum multiplexing capabilities. Our Applied Biosystems™ TaqMan™ multiplex real-time PCR solution includes up to four dyes with minimal spectral overlap, a range of qPCR master mixes, and spectral calibration plates.

Assay design and ordering information is available at **thermofisher.com/multiplexqpcr**

Find out more at **thermofisher.com/quantstudio3-5**

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