Methods for Detection and Identification of *Plasmodium knowlesi*: A Review Article

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Abstract

Background: *Plasmodium knowlesi* is a simian malaria parasite capable of causing malaria in human. Naturally acquired human infections with *P. knowlesi* have been detected in many South East Asian countries. As *P. knowlesi* can cause severe malaria with life threatening complications, early detection is of great importance in management of knowlesi malaria.

Objective: To provide insight into the detection methods used for diagnosis of knowlesi malaria.

Method: We performed the detailed reviews of the previous studies on knowlesi malaria with the particular emphasis on diagnostic methods, especially polymerase chain reaction-based molecular detection methods.

Result: For detection of *P. knowlesi*, blood film microscopy is not a reliable method due to the morphological similarities between *P. knowlesi* and other malaria parasites infecting human. Rapid diagnostic methods based on *P. knowlesi*-specific antibodies have not been available yet. Nested PCR has been used as the standard method for identification of *P. knowlesi* for half a decade. While requiring sophisticated equipment, real-time PCR provides rapid results and higher specificity and sensitivity, and is thus used in reference laboratories. Loop-mediated isothermal amplification is a promising method for diagnosis of knowlesi malaria in the field. These methods detect small subunit ribosomal RNA as the molecular target. However, recent studies demonstrated the use of multicopy, repetitive sequence as the molecular target in single-step PCR detection of *P. knowlesi*.

Conclusion: *P. knowlesi* is commonly mistaken for *P. malariae* by microscopy, which is responsible for some of the fatalities due to *P. knowlesi* infections because *P. knowlesi* produces more severe malaria compared to *P. malariae*. Although molecular diagnostic methods are sensitive and specific for *P. knowlesi*, these methods are not

available in the rural areas where knowlesi malaria is endemic. Therefore, in addition to these methods, a high index of suspicion would be helpful in preventing death resulting from knowlesi malaria.

Key words: Malaria, *Plasmodium knowlesi*, molecular diagnostic methods

Introduction

Although there are more than 100 *Plasmodium* species infecting a wide range of hosts including rodents, reptiles, birds, primates and other mammals, it has long been recognized that *Plasmodium* parasites are species-specific and only four species of *Plasmodium* (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale*) are responsible for causing malaria in humans.[1] *Plasmodium knowlesi* is a simian malaria parasite circulating among long-tailed macaque (*Macaca facicularis*) and pig-tailed macaque (*Macaca nemes*trina).[2] However, in 1932, Robert Knowlesi, after whom *P. knowlesi* was named, and Das Gupta demonstrated that humans could be experimentally infected with *P. knowlesi* by inoculation of infected monkey blood.[3] Recent studies have revealed that *Anopheles latens* of *Leucosphyrus* mosquitoes group is the main vector responsible for transmission of *P. knowlesi* among monkeys as well as from monkeys to humans.[2]

Even though natural *P. knowlesi* infection of human was first reported in 1965 in a US army surveyor who was stationed in jungle area of West Malaysia[4], knowlesi malaria was not considered a public heath problem until 2004 when Singh *et al.* discovered a large number of human knowlesi infections in Kapit Division of Sarawak, Malaysia.[5] Since that time, human cases of knowlesi malaria have been reported in other countries in South East Asia region including Singapore[6], Philippines[7], Vietnam[8], Thailand[9] and Indonesia[10], and even named the fifth type of human malaria. Clinically, knowlesi malaria is the first human malaria to present with daily temperature rise because *P. knowlesi* has quotidian erythrocytic cycle.[11] Thrombocytopenia is the most common laboratory finding in *P. knowlesi* infections.[12] More importantly, studies have revealed that *P. knowlesi* infections produce severe malaria with life threatening complications including hepatic dysfunction, respiratory distress, renal failure and shock, highlighting the importance of rapid diagnosis in the management of knowlesi malaria.[13,14] This review focuses on the diagnostic methods developed and/or employed so far for the diagnosis of knowlesi malaria.

Microscopy

In areas of high endemicity, microscopic examination of the peripheral blood is the gold standard for diagnosis of malaria.[15] The species identification of malaria parasites is achieved by observation of the morphological differences between the species of

malaria parasite. It requires expertise and is not useful for screening a large number of samples in short time. The minimum level of detection is 10 - 30 parasites per microliter of blood.[15] In the case of *P. knowlesi*, early blood forms are indistinguishable from those of *P. falciparum* and late blood forms are similar with *P. malariae*, making it impossible to distinguish *P. knowlesi* from other human malaria parasites by microscopy. Singh *et al.* demonstrated that 116 of 126 knowlesi malaria cases (92%) were misdiagnosed as *P. malariae* infections based on microscopy result.[5]

Rapid diagnostic test

Rapid diagnostic tests are useful for making diagnosis in the field because they can give the diagnosis within very short time without requiring any laboratory facility. Although knowlesi-specific rapid diagnostic test has not been developed, McCutchan *et al.* described a method to detect *P. knowlesi* using a battery of monoclonal antibodies against lactate dehydrogenase of the *Plasmodium* parasites. *P. knowlesi* showed reaction with *P. falciparum* and *P. vivax*-specific antibodies but not with *P. ovale* and *P. malariae*-specific antibodies.[16] Although this method allows the differentiation of knowlesi malaria from *P. malariae* infection, it is not reliable in the case of mixed infection with *P. falciparum* and *P. vivax*. This method should only be used in conjunction with microscopy to exclude the possibility of mixed infection. In addition, Kawai *et al.* reported that commercially available rapid diagnostic tests for malaria such as OptiMAL and Entebe Malaria Cassette could incorrectly identify knowlesi malaria as *P. falciparum* or *P. vivax* infection. Given the fact that *P. knowlesi* could infect humans and cause life-threatening infections, care must be taken when using these tests for malaria diagnosis in *P. knowlesi*-endemic areas.[17] Rapid diagnostic test based on *P. knowlesi*-specific antibody would be a valuable tool in endemic areas where knowlesi malaria poses significant threat.[16]

Nested PCR assays

Nested PCR assays have been used for specific detection of *P. knowlesi.* In 2004, Singh *et al.* developed a nested PCR assay for the detection and species differentiation of human malaria parasites including *P. knowlesi*. This assay targets the small subunit ribosomal RNA (SSU rRNA) and involves the amplification of DNA in each blood sample with *Plasmodium*-specific primers and subsequent amplification of the resulting amplicon with species-specific primers (Figure 1).[5] *P. knowlesi*-specific primers Pmk8 and Pmkr9 were designed based on SSU rRNA gene sequences of *P. knowlesi* isolates in Kapit Division of Sarawak, Malaysia. These isolates, though originally misidentified as *P. malariae* by microscopy, were found to be *P. knowlesi* by sequencing analysis of SSU rRNA gene. The lower limit of detection for this assay is 6 parasites per microliter of blood.[5] Screening with this nested PCR has led to the discovery of a large number of human cases of knowlesi malaria, highlighting zoonotic transmission of *P. knowlesi* from monkeys to humans and the public health significance of knowlesi malaria.[5]

Imwong *et al.* reported that this knowlesi-specific primer set designed by Singh et al cross-reacted with *P. vivax* yielding false-positive results and designed two new primer sets PkF1060-PkR1550 and PkF1040-PkR1550 targeting SSU rRNA of *Plasmodium* parasites.[18] These primers were demonstrated to be specific to *P. knowlesi* without producing amplicon with any other parasite species. The nested PCR assays with these primers could detect 1 to 10 parasite genomes.

In 2009, Putaporntip *et al.* carried out the species identification of the *Plasmodium* parasites causing malaria in Thailand using their own nested PCR assay. The primers PK18SF and PK18SR were used for detection *P. knowlesi*.[9] The *P. knowlesi*-specific primers used in above mentioned nested PCR assays are mentioned in Table 1.

Real-time PCR assays

Two real-time PCR assays have been described for detection of *P. knowlesi*. Both are modified versions of the assays previously developed for the detection of malaria parasites infecting humans and target the gene coding for the 18S ribosomal RNA subunit of *Plasmodium* species.

The first one described by Babady *et al.* involves amplification of the 18S ribosomal RNA with primers PF1 and PF2 and employs two sets of fluorescent resonance energy transfer (FRET) probes, PF3 and PF4 for detection of genus-specific sequence in the amplicon and PK1 and PK2 for detection of *Plasmodium knowlesi*-specific sequence (Figure 2), which are distinguished by excitation at different wavelengths, 640 nm and 705 nm, respectively. However, this assay has not been validated using clinical samples.[19]

Divis *et al.* developed another *P. knowlesi*-specific real-time PCR assay using Taqman hydrolysis probe. This assay consists of two reactions - the screening reaction for detection of *Plasmodium* DNA and the specific reaction for detection of *P. knowlesi*-specific DNA. The same set of primers Plasmo 1 and Plasmo 2 was used for both reactions but different probes were used: *Plasmodium* screening probe Plasprobe and *P. knowlesi*-specific Pk probe (Figure 3). The 5' ends of these probes were labeled with the fluorophore and 3' ends with the quencher. Plasprobe recognizes the conserved DNA sequence within 18S ribosomal RNA genes in all four species of human malaria parasite as well as *P. knowlesi*. The Pk probe was designed to bind to *P. knowlesi*-specific 30 base pair sequence within 18S ribosomal RNA gene.[20] This real time PCR-assay was found to detect *P. knowlesi* DNA even if the parasitaemia level is as low as 3 parasites per microliter of blood. The sequences of primers and probes used in these assays were shown in Table 2.

Loop-mediated isothermal amplification (LAMP) assays

Two LAMP assays have been developed for detection of *P. knowlesi* infection. These assays used the different targets: the first one targeting species-specific β tubulin gene [21]

and the second one targeting apical membrane antigen-1 (AMA-1) gene of *P. knowlesi*[22]. Both assays use six sets of primers that specifically bind to eight regions of the target sequence. In both assays, the amplicon was detected by assessment of turbidity, the ladder pattern of amplified products on gel electrophoresis or direct visualization of amplified products in the presence of fluorescent nucleic acid stain. Both assays can detect *P. knowlesi* whole blood even if parasitaemia level is 0.01%.[21,22] The advantage of these assays is that detection of knowlesi malaria was accomplished under isothermal condition within one hour without requiring expensive thermal cyclers. The LAMP assay targeting the β tubulin genes was not tested against clinical samples.[21]

Single-step PCR assay

Although ribosomal RNA gene is the target in PCR-based detection of *Plasmodium* species infecting humans including *P. knowlesi*, this target occur in only 4 - 8 copies per genome.[23] As the low copy number of the target limits the sensitivity of the PCR assay, Demas *et al.* performed the bioinformatics analysis of whole genome sequence of *P. falciprum* and *P. vivax* to identify the new multicopy target DNA sequences for PCR-based detection of malaria parasites.[23] As the result, one putative diagnostic high-copy number target was identified in each species: Pfr364 occurring in 41 copies in *P. falciparum* and Pvr47 occurring in 14 copies in *P. vivax*.[23] Using a similar approach, Lucchi *et al.* identified four such multicopy genomic sequences in *P. knowlesi* genome. Fourteen sets of primers were designed to amplify these target sequences from *P. knowlesi* genome. Only three sets of primers, namely Pkr140-3, Pkr140-4 and Pkr140-5, produce the amplicon and all of these primers recognize the Pkr140 repeat sequence which occurs in 7 copies in *P. knowlesi* genome. However, two of these three primers sets Pkr140-3, Pkr140-4 are not specific for *P. knowlesi* and yielded non-specific amplicon when tested with simian malaria parasites.[24] The remaining primer set Pkr150-5 is 100% specific for *P. knowlesi* and does not show cross-reactivity with non-knowlesi human and simian malaria parasites. The sequences of these primers are shown in Table 3. Furthermore, non-nested PCR assay using this new primer set could detect the parasitaemia level as low as one parasite per microliter of blood[24], indicating that the limit of detection for this assay is lower than reported for previous nested PCR assay.[5]

In contrast to the previously identified repeat sequence target in *P. falciparum* and *P. vivax* that are confined to the chromosome end[23], Pkr140 repeat sequence targeted by this new primer set is distributed throughout the entire length of chromosomes.[24] The chromosome ends are rich in multicopy, species-specific genes and are therefore the good place to search for new diagnostic targets.

Conclusion

Knowlesi malaria is a life-threatening disease and early diagnosis is essential. Although conventional microscopy is the routinely used method for diagnosis of malaria, it has

many limitations as described above and, more importantly, not reliable for diagnosis of knowlesi malaria because of the morphological similarity of *P. knowlesi* with other malaria parasites infecting humans. PCR-based detection methods targeting specific rRNA genes and multicopy repeat sequences are more sensitive, specific and reliable than conventional microscopy for diagnosis of malaria. The use of these methods is impractical for the application in the field and is limited to reference laboratories because these assays require sophisticated equipment and trained personnel. In contrast, detection methods based on LAMP are simple and easy to perform and would be useful in places where expensive thermal cyclers are not available. Rapid diagnostic test specific for *P. knowlesi* is still lacking but such test would be of great use for the diagnosis of knowlesi malaria in remote areas, the area most likely to be affected by knowlesi malaria.

Further recommendation

Based on detailed reviews of the malaria deaths in 2010-2011 period, Rajahram *et al*. reported that deaths resulting from knowlesi malaria were attributable to misidentification of *P. knowlesi* as *P. malariae* on blood film examination by microscopy. Patients diagnosed as having *P. malariae* infection are treated with oral antimalarial therapy and less likely to receive parenteral antimalarial therapy because *P. malariae* infection have relatively benign course.[25] This report highlights the importance of molecular diagnostic methods in the diagnosis of *P. knowlesi* infections and the need to develop molecular diagnostic tests that can be applied in the field. In addition, the health care providers including clinicians should be educated to expect severe illnesses in non-falciparum malaria especially in *P. knowlesi* endemic areas and initiate parenteral antimalarial therapy as early as possible in suspected cases of knowlesi infections. A high index of suspicion, combined with molecular diagnostic methods, is the key to preventing fatalities due to *P. knowlesi* infections.

List of abbreviations

PCR: Polymerase chain reaction; SSU rRNA: Small subunit ribosomal RNA; FRET: Fluorescent energy transfer; LAMP: Loop-mediated isothermal amplification; AMA-1: Apical membrane antigen-1.

Conflict of Interest: The authors have no conflict of interest to declare.

References

1. Garnham PCC. Malaria Parasites and other Haemosporidia, Blackwell Scientific Publications. 1966.

2. Antinori S, Galimberti L, Milazzo L, Corbellino M. *Plasmodium knowlesi*: the emerging zoonotic malaria parasite. *Acta Trop.* 2013;125:191-201. doi: 10.1016/j.actatropica.2012.10.008.

3. Knowles R, Das Gupta BM. A study of monkey-malaria and its experimental transmission to man. *Indian Medical Gazette* 1932;67: 301-320.

4. Chin W, Contacos PG, Coatney GR, Kimball HR. A naturally acquired quotidian-type malaria in man transferable to monkeys. *Science* 1965;149:865.

5. Singh B, Kim Sung L, Matusop A, et al. A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. *Lancet* 200;363:1017-1024. doi:10.1016/S0140-6736(04)15836-4.

6. Ng OT, Ooi EE, Lee CC, et al. Naturally acquired human *Plasmodium knowlesi* infection, Singapore. *Emerg Infect Dis.* 2008;14:814-866. doi: 10.3201/eid1405.070863.

7. Luchavez J, Espino F, Curameng P, et al. Human infections with *Plasmodium knowlesi*, the Philippines. *Emerg Infect Dis.* 2008;14:811-813. doi:10.3201/eid1405.071407

8. Van den Eede P, Van HN, Van Overmeir C, et al. Human *Plasmodium knowlesi* infections in young children in central Vietnam. *Malar J.* 2009;8:249. doi: 10.1186/1475-2875-8-249.

9. Putaporntip C, Hongsrimuang T, Seethamchai S, et al. Differential prevalence of Plasmodium infections and cryptic *Plasmodium knowlesi* malaria in humans in Thailand. *J Infect Dis.* 2009;199:1143-1150. doi: 10.1086/597414.

10. Figtree M, Lee R, Bain L, et al. *Plasmodium knowlesi* in human, Indonesian Borneo. *Emerg Infect Dis.* 2010;16:672-674. doi: 10.3201/eid1604.091624.

11. Cox-Singh J, Singh B. Knowlesi malaria: newly emergent and of public health importance? *Trends Parasitol.* 2008;24:406-410. doi: 10.1016/j.pt.2008.06.001.

12. Daneshvar C, Davis TME, Cox-Singh J, et al. Clinical and laboratory features of human *Plasmodium knowlesi* infection. *Clin Infect Dis.* 2009;49:852-860. doi: 10.1086/605439.

13. Cox-Singh J, Davis TME, Lee KS, et al. *Plasmodium knowlesi* malaria in humans is widely distributed and potentially life threatening. *Clin Infect Dis.* 2008;46:165-171. doi: 10.1086/524888.

14. William T, Menon J, Rajahram G, et al. Severe *Plasmodium knowlesi* malaria in a tertiary care hospital, Sabah, Malaysia. *Emerg Infect Dis.* 2011;17:1248-1255. doi: 10.3201/eid1707.101017.

15. Gilles HM. Third edition. Diagnostic methods in malaria. Gilles HM, Warrell DA, ed. Bruce-Chwatt's Essential Malariology. London: Edward Arnold; 1993.

16. McCutchan TF, Piper RC, Makler MT. Use of malaria rapid diagnostic test to identify *Plasmodium knowlesi* infection. *Emerg Infect Dis.* 2008;14:1750-1752. doi: 10.3201/eid1411.080480.

17. Kawai S, Hirai M, Haruki K, Tanabe K, Chigusa Y. Cross-reactivity in rapid diagnostic tests between human malaria and zoonotic simian malaria parasite *Plasmodium knowlesi* infections. *Parasitol Int.* 2009;58:300–302. doi: 10.1016/j.parint.2009.06.004.

18. Imwong M, Tanomsing N, Pukrittayakamee S, Day NP, White NJ, Snounou G. Spurious amplification of a *Plasmodium vivax* small-subunit RNA gene by use of primers currently used to detect *P. knowlesi*. *J Clin Microbiol.* 2009;47: 4173-4175. doi: 10.1128/JCM.00811-09..

19. Babady NE, Sloan LM, Rosenblatt JE, Pritt BS. Detection of *Plasmodium knowlesi* by real-time polymerase chain reaction. *Am J Trop Med Hyg.* 2009;81:516-518.

20. Divis PCS, Shokoples SE, Singh B, Yanow SK. A TaqMan real-time PCR assay for the detection and quantitation of *Plasmodium knowlesi*. *Malar J.* 2010;9:344. doi: 10.1186/1475-2875-9-344.

21. Iseki H, Kawai S, Takahashi N, et al. Evaluation of a loop-mediated isothermal amplification method as a tool for diagnosis of infection by the zoonotic simian malaria parasite *Plasmodium knowlesi*. *J Clin Microbiol.* 2010;48:2509-2514. doi: 10.1128/JCM.00331-10.

22. Lau YL, Fong MY, Mahmud R, et al. Specific, sensitive and rapid detection of human *Plasmodium knowlesi* infection by loop-mediated isothermal amplification (LAMP) in blood samples. *Malar J.* 2011;10:197. doi: 10.1186/1475-2875-10-197.

23. Demas A, Oberstaller J, DeBarry J, et al. Applied genomics: data mining reveals species-specific malaria diagnostic targets more sensitive than 18S rRNA. *J Clin Microbiol*. 2011;49:2411-2418. doi: 10.1128/JCM.02603-10. Epub 2011 Apr 27.

24. Lucchi NW, Poorak M, Oberstaller J, et al. A new single-step PCR assay for the detection of the zoonotic malaria parasite *Plasmodium knowlesi*. *PLoS One* 2012;7:e31848. doi: 10.1371/journal.pone.0031848.

25. Rajahram GS, Barber BE, William T, Menon J, Anstey NM, Yeo TW. Deaths due to *Plasmodium knowlesi* malaria in Sabah, Malaysia: association with reporting as *Plasmodium malariae* and delayed parenteral artesunate. *Malar J.* 2012;11:284. doi: 10.1186/1475-2875-11-284.

Table 1: Primers used in nested PCR for detection of *P. knowlesi*

Primers	Primer sequence	Annealing temperature	References
***Plasmodium*-specific primers**			
rPLU1 rPLU5	5'-TCAAAGATTAAGCCATGCAAGTGA-3' 5'-CCTGTTGTTGCCTTAAACTCC-3'	55°C	Singh *et al.*, 1999
M18SF0 M18SR0	5'-CCATTAATCAAGAACGAAAGTTAAGG-3' 5'-CAAGGAAGTTTAAGGCAACAACA-3'	60°C	Putaporntip *et al.*, 2009
***P. knowlesi*-specific primers**			
Pmk8 Pmkr9	5'-GTTAGCGAGAGCCACAAAAAAGCGAA-3' 5'-ACTCAAAGTAACAAAATCTTC CGTA-3'	60°C	Singh *et al.*, 2004
PkF1140 PkF1160 PkR1150	5'-GATTCATCTATTAAAAATTGCTTC-3' 5'-GATGCCTCCGCGTATCGAC-3' 5'-TCTTTTCTCTCCGGAGATTAGAACTC-3'	50/55°C	Imwong *et al.*, 2009
PK18SF PK18SR	5'-GAGTTTTTCTTTTCTCTCCGGAG-3' 5'-ACGTTAAATGTGATTCCTTTCCC-3'	60°C	Putaporntip *et al.*, 2009

Table 2: Primers and probes used in real time-PCR for detection of *P. knowlesi*

Primers	Sequence	References
Plasmo 1	5'-GTTAAGGGAGTGAAGACGATCAGA-3'	Divis *et al.*, 2010
Plasmo 2	5'-TTATGAGAAATCAAAGTCTTTGGGTT-3'	
Plasprobe	5'-ACCGTCGTAATCTTAACCATAAACTATGCC GACTAG-3'	
Pk probe	5'-CTCTCCGGAGATTAGAACTCT TAGATTGCT-3'	
PF1	5'-CATTYGTATTCAGATGTC-3'	Babady *et al*., 2009
PF2	5'-TTCTTTTAACTTTCTCGC-3'	
PF3	5'-GATACCGTCGTAATCTTAACCATAACCTAT-3' Fluorescein	
PF4	LC RED640 5'-GACTAGGTGTTGGATGAAAGTG-3'	
PK1	5'-CCGGAGATTAGAACTCTTAGATTG-3' Fluorescein	
PK2	LC-RED705 5'-TCCTTCAGTGC CTTATGAGAAA-3'	

Table 3: Primers used in repetitive sequence-based PCR assay for detection of *P. knowlesi*

Primers	Sequence	Annealing temperature	References
Pkr140-5F	5'-CAGAGATCCGTTCTCATGATTTCCATGG-3'	57ºC	Lucchi *et al*., 2012
Pkr140-5R	5'-CTRAACACCTCATGTCGTGGTAG-3'		

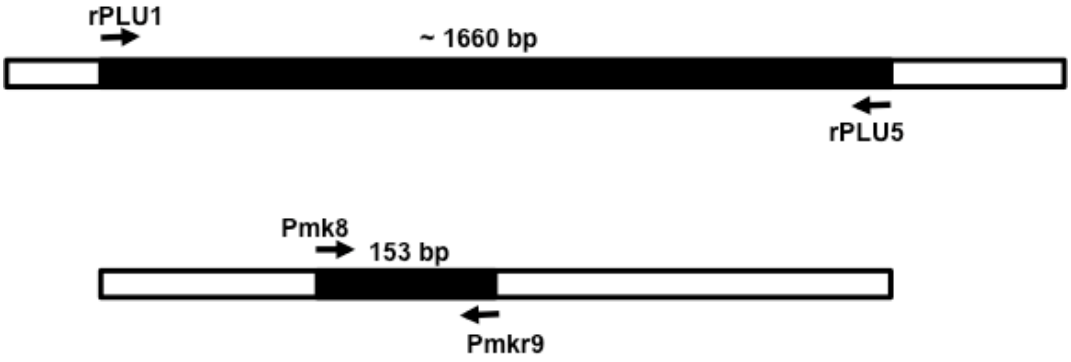

Figure 1: Schematic representation of nested PCR for detection of *P. knowlesi*. The number indicates expected size of the amplicons. Arrows indicates the binding site of the primers. The amplicon obtained in first PCR amplification with genus-specific primers rPLU1 and rPLU5 is used as the template for second PCR amplification with *P. knowlesi*-specific primers Pmk8 and Pmkr9.

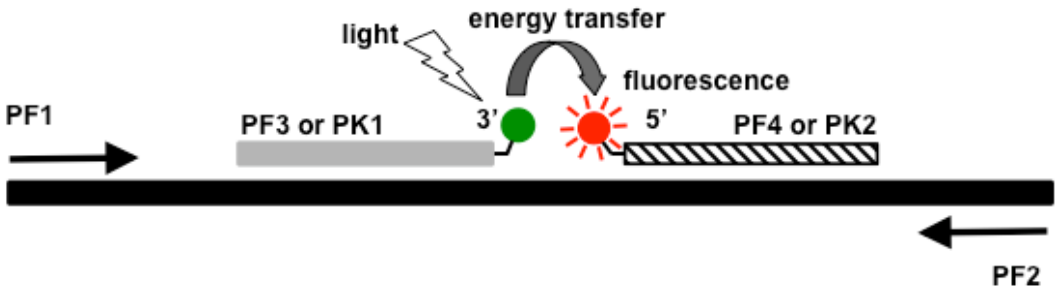

Figure 2: Mechanism of FRET probes. FRET probes labeled with fluorescein and LC-RED (PF3 and PF4) or (PK1 and PK2) bind to the amplicon in close proximity to each other. Excitation is transferred from donor to acceptor fluorophore and fluorescence is emitted from the acceptor fluorophore.

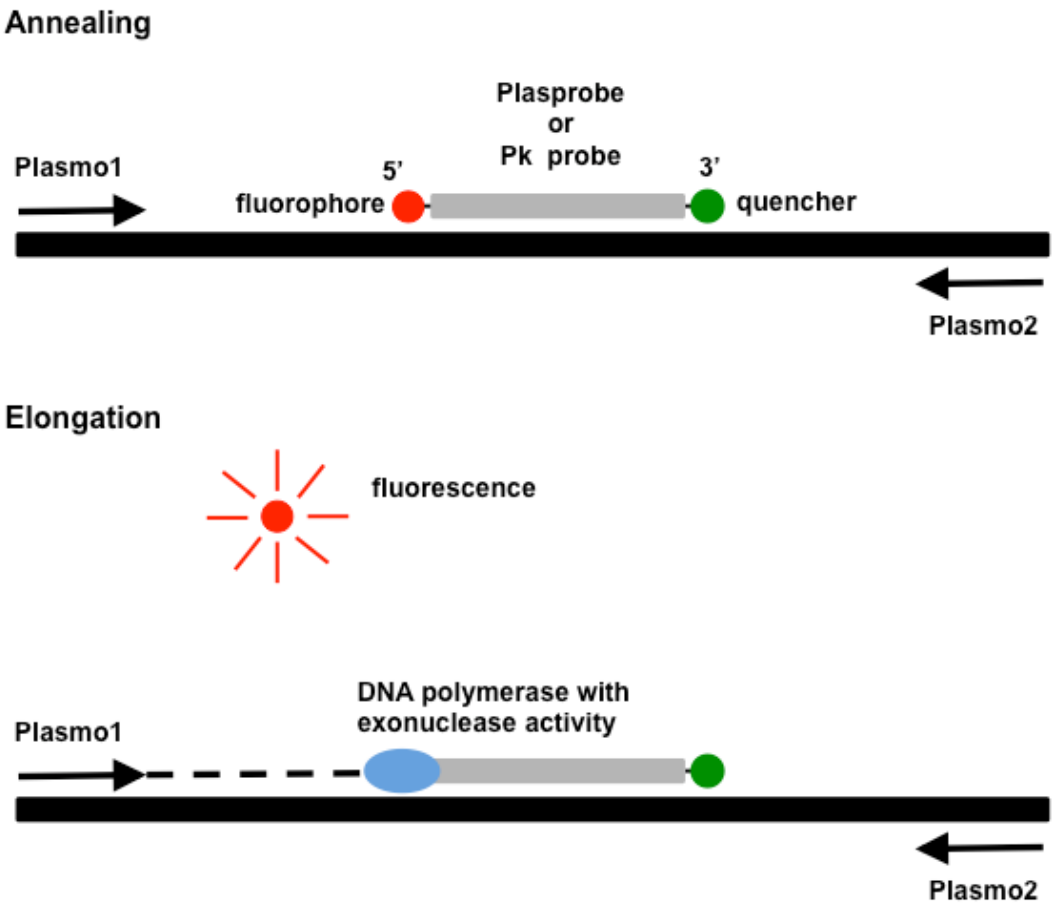

Figure 3: Mechanism of action of TaqMan hydrolysis probe. Quencher at the 3' end of the probe reduces the fluorescence of the fluorophore in intact probe. Fluorescence is emitted when Plasprobe or Pk probe, bound to the target sequence, is digested by exonuclease activity of the Taq DNA polymerase during elongation phase.