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Plasmodium vivax Duffy Binding Protein Copy Number Variation and Their Effects on Reticulocyte Invasion in Selected Parts of Ethiopia

MSc Thesis

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Plasmodium vivax Duffy Binding Protein Copy Number Variation and Their Effects on Reticulocyte Invasion in Selected Parts of Ethiopia

By: Yasin Nasir Hussen

A Thesis Submitted to Institute of Biotechnology, Addis Ababa University
in Partial Fulfillment of the Requirements for the Masters of Science
Degree in Biotechnology

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DECLARATION

I, the undersigned, declare that this thesis is my original work and has not been presented for a degree in any other University, and that all sources of information used for the thesis have been duly acknowledged.

Name of Candidate	**Signature**	**Date**
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Approval of thesis by supervisors for submission

We certify that Yasin Nasir Hussen's MSc thesis entitled "*Plasmodium vivax* Duffy binding protein copy number variation and their effects on reticulocyte invasion in selected parts of Ethiopia" has been carried out under our direct supervision and recommended the thesis to be accepted as fulfilling the requirement for the degree of Master of Science in Biotechnology.

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Approval sheet after defense

We certify that Yasin Nasir Hussen’s MSc thesis entitled “*Plasmodium vivax* Duffy binding protein copy number variation and their effects on reticulocyte invasion in selected parts of Ethiopia” is the final version. Therefore; we, as examining board, approved it as the final document to be accepted as fulfilling the requirement for the degree of Master of Science in Biotechnology.

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ABBREVIATION

ACT	Artemisinin-based Combination Treatments
CNV	Copy number variation
CSA	Central Statistical Agency
CT	Threshold cycle
DARC	Duffy Antigen Receptor for Chemokines
DBP	Duffy binding protein
DBS	Dried Blood Spots
DNA	Deoxyribonucleic Acid
DPCR	Digital Polymerase Chain Reaction
EDTA	Ethylene Diamine Tetra Acetic Acid
qPCR	Quantitative Polymerase Chain Reaction
P.v	*Plasmodium vivax*
RBP	Reticulocyte Binding Protein
RDT	Rapid Diagnostic Test
SNP	Single Nucleotide Polymorphism
SOP	Standard Operating Procedure
WHO	World Health Organization

ABSTRACT

The connection between the Duffy binding protein and the Duffy antigen receptor for chemokine is required for *Plasmodium vivax* penetration into human reticulocytes. A previous analysis of *Plasmodium vivax* samples in Ethiopia determined that Duffy binding protein duplications are more prevalent than in any other *Plasmodium vivax* location. However, its prevalence and importance in large samples remain unclear. Duffy blood group genotyping was done by amplifying the GATA1 transcription factor-binding region of DARC gene among 349 *Plasmodium vivax* isolates were determined by a real-time PCR technology. In addition, duplications of Duffy binding protein and their relationship with Duffy-negativity and parasite density were examined. Duffy binding protein duplications and Duffy-negative antigens were detected in 74% and 3.2% isolates respectively. Most of the Duffy negative participants were found in Gondar Zuria district (72.7%). To know whether Duffy binding protein amplification contributes to the takeover of Duffy-negative reticulocytes, the relationship between Duffy binding protein copy numbers and Duffy status was investigated using Fisher's exact test and post-hoc tests. Fisher's exact test reveals a significant association (***p*** = 0.000313). In addition, a post-hoc test indicates a significant association between the Duffy binding protein copy numbers (single and 2-3 copy) and the DARC status (***p*** = 0.000281). However, the number of Duffy-negative patients infected by multiple Duffy binding protein copy is small. Therefore, we cannot decide that multiple Duffy binding protein copy number increase parasite's invading ability against Duffy-negative patients. The mean parasite burden differs between Duffy negative and positive in a statistically significant way (***P*** <0.0001). Duffy-negative patients aren't resistant to *Plasmodium vivax* however; the detailed mechanisms of the infection in Duffy-negative patients remain unclear. In reference to the high rate of Duffy-binding protein duplication, further investigation should be explored by extending study site across Ethiopia by using the most sensitive molecular detection tools known as digital PCR. It is also useful to look into additional parasite ligands related to the invasion of Duffy-negative reticulocytes.

Key words: *Duffy binding protein, Duffy antigen receptor for chemokine, Duffy-negative reticulocyte, Duffy positive reticulocytes, Copy Number, Duffy binding protein amplification*

1. INTRODUCTION

1.1. Background

Malaria is one of the deadliest and most prevalent parasitic infections caused by the genus *Plasmodium* (Sato, 2021). *Plasmodium vivax* and *Plasmodium falciparum* are the principal parasites that cause malaria (Larson, 2019). *Plasmodium vivax* has the broadest geographic spread, with over 3 billion individuals residing under the transmission limit (Battle *et al.*, 2019). In 2020, *Plasmodium vivax*, which is primarily found in Asia and Latin America, was responsible for 4.5 million cases of malaria worldwide (Venkatesan, 2022). Globally, more than 85% of all *Plasmodium vivax* cases were reported in six countries, including Ethiopia (Gari *et al*., 2021). *Plasmodium vivax* was once thought to be harmless, but it has been identified as a major source of illness and death in endemic regions (Barber *et al.*, 2015; Siqueira *et al.*, 2015).

Plasmodium vivax spreads in the diverse Sub-Saharan African climate (Elgoraish *et al.*, 2019) because of its wider temperature tolerance and earlier gametocyte development than *P. falciparum* (Lo *et al.*, 2017). In Sub-Saharan Africa, 95% of confirmed malaria cases and 96% of deaths were recorded due to malaria in 2020 (Oladipo *et al.*, 2022). The parasite is endemic in East African nations like Sudan, Eritrea, and Ethiopia (Sibley, 2019).

Ethiopia is an east African country with a high prevalence of *Plasmodium vivax* malaria (Sibley, 2019), and Duffy-negative and Duffy-positive people coexist (Howes *et al.*, 2011), and the coexistence can give important insights into the epidemiology of *Plasmodium vivax*. As stated in Tsige Ketema *et al*. (2021), Ethiopia is responsible for about 12% of *Plasmodium vivax* infections and deaths worldwide. Across the country, *Plasmodium vivax* contributes to almost 40% of all infected cases followed by *plasmodium falciparum* (Hiwot S Taffese *et al*., 2018).

The connection between Duffy binding protein and Duffy antigen receptor for chemokine is required for *Plasmodium vivax* penetration into human reticulocytes (Salinas and Tolia, 2016). *Plasmodium vivax* invades humans via DARC (Duffy Antigen Receptor for Chemokine), a glycoprotein found on the outer surface of reticulocytes (Howes *et al.*, 2011).

DARC is located on both normocytes and reticulocytes; hence this ligand-receptor interaction can't govern selective entry into reticulocyte (Malleret *et al.*, 2015). When a point mutation is introduced into the DARC gene's promoter region (GATA-1 transcription factor binding), Duffy antigen expression fails (Tournamille *et al.*, 1995; Lo *et al.*, 2021). It is assumed to be the reason for the absence of the *Plasmodium vivax* burden in African countries (Allison, 2009; Howes *et al.*, 2011). Recently, a number of *Plasmodium vivax* illness studies have been conducted on Duffy-negative individuals (Gunalan *et al.*, 2018).

It has been claimed that the parasites' improved capacity to spread is due to either decreased DARC expression in Duffy-negative individuals or amplification of the Duffy binding protein (Popovici *et al*, 2020; Kepple *et al.*, 2021). According to patient samples, parasites with high Duffy binding protein copy numbers may attach to another receptor on Duffy-negative erythrocytes (Lo *et al.*, 2017). In this study, Duffy binding protein duplication and Duffy blood group status and their relationship with parasite density was determined in samples collected from different settings in Ethiopia using real-time PCR technology.

1.2. Statements of the Problem

Until recently, it was believed that *Plasmodium vivax* could not infect individuals who had Duffy-negative erythrocytes, and *Plasmodium vivax* is absent from most of African countries. Recently, a number of *Plasmodium vivax* illness studies have been conducted on Duffy-negative individuals. According to patient samples, parasites with high Duffy binding protein copy numbers may attach to another receptor on Duffy-negative reticulocytes. Ethiopia, with its diverse population of Duffy-positive and negative individuals, can give important insights into the prevalence of *Plasmodium vivax*. The national prevalence of Duffy binding protein and its impact on *Plasmodium vivax* infection among broad samples in Ethiopia remain unknown.

1.3. Significance of the study

Malaria remains a major public health problem in Ethiopia. It is known that worldwide malaria status is the sum total of each country's status, and in turn, the country's status is the sum total of its regional and local situations at each level. Therefore, information about Duffy binding protein amplification will have significant effects on knowing how the parasite interacts with Duffy negative erythrocytes and is essential to developing integrated control strategies.

1.4. Scope of the study

The study was limited to Duffy blood group genotyping and Duffy binding protein copy number variation in Adama, Batu, Gonder zuria, and Arba Minch zuria from July 2021–October 2021.

1.5. Alternative hypothesis

- There is association between Duffy binding protein duplication and Duffy-negative erythrocyte invasion.

1.6. Objective

1.6.1. General Objective:

- To assess *Plasmodium vivax* Duffy binding protein copy number variation and their effect on reticulocyte invasion in different settings of Ethiopia.

1.6.2. Specific Objectives:

- To examine Duffy status of *Plasmodium vivax* patients.
- To examine *Plasmodium vivax* Duffy binding protein duplication.
- To test association between multiple copy Duffy binding protein and Duffy-negative reticulocyte invasion.
- To test association between Duffy blood group status and parasite density.

1.7. Research Questions

- Is Duffy binding protein duplication increases parasite's invading ability in Duffy-negative patients?
- Is there parasite density difference between Duffy-positive and Duffy-negative patients?

2. LITERATURE REVIEW

2.1. *Plasmodium vivax* Epidemiology

There are five types of malaria parasites known to infect human beings, and *vivax* is one of them (Wei *et al.*, 2021). It has the broadest geographic spread, with over 3 billion individuals residing under the transmission limit (Battle *et al.*, 2019). In South America, Southeast Asia, and India, *vivax* is widely distributed. There are only a few countries in sub-Saharan Africa where there are significant infection rates (Howes *et al.*, 2015). Due to the high incidence of Duffy-negative residents in Central and West African countries, the rarity of this illness is closely connected with it (Howes *et al.*, 2011). According to meta-analysis by Kassahun Habtamu *et al*. (2022), about 8% of malaria cases are thought to be *Plasmodium vivax* (or roughly 50% if Sub-Saharan Africa is excluded). As stated in Tsige Ketema *et al*. (2021), globally, Ethiopia is responsible for about 12% of *Plasmodium vivax* infections and death. Across the country, *Plasmodium vivax* accounts for nearly 40% of malarial-infected cases (File *et al*., 2019; Anmut Assemie, 2022).

2.2. *Plasmodium vivax* life cycle

After being bitten by a female Anopheles mosquito carrying the infection, sporozoites migrate from the skin to the liver, infect liver cells, and then mature to generate an actively dividing liver schizont or dormant hypnozoite (McGregor and Krotoski, 1985). When the hypnozoite enters the bloodstream, it may stay dormant for months or years before initiating clinical relapses. Although the cause of hypnozoite activation has not been investigated, parasitic and bacterial infections may play a role (Shanks and White, 2013). When Plasmodium parasites are released into the bloodstream and infiltrate red blood cells, symptoms of malaria start to manifest. Within a red blood cell, the parasite consumes hemoglobin to get amino acids essential for production of protein. Hemoglobin digestion liberates large quantities of heme, which is toxic to the parasite. The parasite detoxifies free heme by its crystallization into inert hemozoin that remains in the parasite and offers a docking surface for continuously supplied heme (Olafson *et al*., 2017; Weissbuch & Leiserowitz, 2008). The infection of reticulocytes by *Plasmodium vivax* hepatic merozoites causes the development of complexes resembling chambers that aid in the process of blood schizogony. After four days of infection, a few of the asexual parasites go through gametocytogenesis and develop into spherical gametocytes that are sucked up by another female Anopheles mosquito along with a blood meal (Kumari and Sinha, 2023).

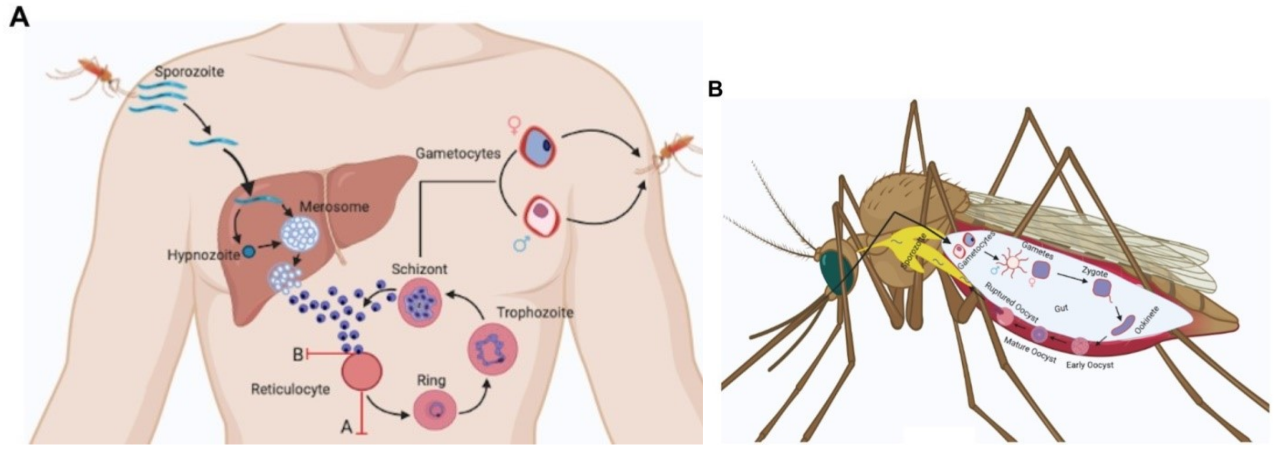


Figure 1: *Plasmodium vivax* life cycle - When *Plasmodium vivax*-infected mosquitoes bite humans, sporozoites are released into the body, migrate into the liver, infect hepatocytes, and mature to generate an actively dividing liver schizont or dormant hypnozoite. A mature schizont-containing hepatocyte bursts and releases merosomes. These merosomes travel through the bloodstream and enter erythrocytes. When the hypnozoite enters the bloodstream, it may stay dormant for months or years before initiating clinical relapses. Merozoite differentiation into erythrocytic trophozoite occurs inside the erythrocyte. When completely developed, the infected erythrocyte bursts, releasing the merozoites. These merozoites then invade fresh erythrocytes, burst, and reinvade. Among the asexual parasites, a few of them go through gametocytogenesis and develop into spherical gametocytes that are sucked up by another female Anopheles mosquito along with a blood meal, starting the life cycle's sexual stage. The gametocytes that the mosquito ingests separate into female and male gametes and unite to generate zygotes in the mosquito's midgut. The zygote develops into the parasite's invasive ookinete stage. The ookinete travels through the midgut wall, passing through epithelial cells. It then rests next to the basal lamina and changes into an oocyst, going through numerous nuclear divisions to produce many sporozoites. At maturation, the sporozoites are released into the mosquito's hemocele following the oocyst breaks, moving on to the salivary glands. The life cycle of sporozoites is completed in the salivary glands, where they spread infection to people.

Adapted from Kumari and Sinha 2023

2.3. Genetic diversity of *Plasmodium vivax*

Genetic variety is one of the key mechanisms by which parasites associated with malaria sustain a long-term infection. Wide genetic variation in evolutionary parasite populations increases the chances of being resistant to host immune responses that are antimalarial (Duah *et al.*, 2016). In comparison to *P. falciparum* populations, *Plasmodium vivax* populations exhibit greater genetic variation throughout their genome (Benavente *et al.*, 2017). In multiple population genetic analyses using data from the entire genome and microsatellite, *Plasmodium vivax* showed strong genomic variability (Auburn *et al.*, 2019). Moreover, investigations indicate that the genomes of *Plasmodium vivax* are more varied than the genomes of *P. falciparum* circulating in the same area (Benavente *et al.*, 2017). It has been found that isolates from varied geographical origins have SNPs and gene copy-number variation (Daniels *et al.*, 2015; Benavente *et al.*, 2017). According to Garzon-Ospina *et al.*, (2018), such characteristics are frequently found in genes that provide no functional use. This might make it possible for new, undiscovered invasion routes. This high level of antigenic diversity indicates the existence of complicated processes that have enabled this parasitic species to survive and disrupt the immune system of the host (Verma *et al.*, 2016). High antigenic variety is a major barrier for vaccine development since many important immune targets and vaccination candidates exhibit considerable variability (Chu and White, 2021).

2.4. *Plasmodium vivax's* recent adaptation on Duffy-negative erythrocytes

Until 2016, it was thought that *Plasmodium vivax* could not infect individuals who are negative for Duffy, and thus *Plasmodium vivax* is absent from most African countries. Recently, *Plasmodium vivax* illness studies have been conducted on Duffy-negative individuals (Gunalan, *et al.*, 2018). According to a meta-analysis by Wilairatana *et al.* (2022), 88.9% of Africans who were negative for Duffy had *Plasmodium vivax* infections. These findings disprove the hypothesis that erythrocytes lacking the Duffy antigen are entirely protective against *Plasmodium vivax* infection (Gunalan *et al.*, 2018). This issue raises key questions about how *Plasmodium vivax* infects Duffy-negative people's erythrocytes. It has been claimed that the parasites' improved capacity to spread is due to either decreased DARC expression in Duffy-negative individuals or amplification of the Duffy binding protein

(Popovici *et al*., 2020; Kepple *et al.*, 2021). Moreover, research from Ethiopia found that *Plasmodium vivax* patients with the Duffy-negative blood group had consistently lower asexual parasitemia (Andargie Abate *et al.*, 2022). Low parasite loads in Duffy-negative infections may indicate less effective but constantly developing attacking methods. Perhaps there is an "undetected silent reservoir," which becomes challenging for *Plasmodium vivax* malaria elimination (Baird, 2022). It spreads across a large portion of Africa and negatively affects the economy and general public health.

2.5. Effect of Duffy binding protein duplication on Duffy-negative reticulocytes

All plasmodium specious including *Plasmodium vivax* have fourteen chromosomes, Duffy binding protein is found in sixth chromosome. Sequence analysis of the Duffy binding protein in clinical isolates of *Plasmodium vivax* from patients who are negative for Duffy shows that the gene is present in two or more copies (Jessica *et al.*, 2016). In Ethiopian *Plasmodium vivax* isolates, two distinct kinds of duplicates (Malagasy and Cambodian) have been discovered (Jessica *et al.*, 2016; Lo *et al.*, 2019)**.** The Duffy binding protein locus is one of the most frequently occurring copy number variations (CNVs) in the genome (Pearson *et al.*, 2016). Recent investigations have shown that *Plasmodium vivax* can overcome the immunity of host anti-*Plasmodium vivax* Duffy binding protein by multiplying the Duffy binding protein (Popovici *et al.*, 2020). This makes it easier for Duffy-negative reticulocytes to bind to an alternate, lower-affinity receptor (Gunalan *et al.*, 2018)**.**

2.6. DARC polymorphisms and the invasion mechanism of *Plasmodium vivax*

DARC is a glycoprotein found on the outer parts of reticulocytes and it is essential for merozoites to invade RBCs (Howes *et al.*, 2011). DARC is regarded as the strongest example of positive selection in the human genome because of its substantial relationship with vivax malaria resistance. It is a glycoprotein between 35 and 50 kDa that is expressed on the surfaces of both RBCs and endothelium tissue (Höher *et al.*, 2018). The two primary alleles of the DARC gene, FY*A and FY*B, encode Duffy glycoprotein. Each allele can be received from either parent, and both the Duffy Fya and Fyb antigens produced by the genes can be expressed on the promoter region of red blood cells. One amino acid, located at position 42, distinguishes Fya from Fyb, encoding glycine in the former and aspartic acid in the latter (Golassa *et al.*, 2020). When reticulocytes are Duffy-positive, PvRBP2b attaches to TfR1 prior to Duffy binding protein interacting with the Duffy antigen to allow the merozoite to enter the cell (Popovici *et al.*, 2020). It has been shown that CD71 is a crucial receptor for reticulocyte invasion via the ligand PvRBP2b (Gruszczyk *et al.*, 2018). The parasite's preferred invasion of CD71+ reticulocytes may be due to an abundance of iron and other metabolites, which are necessary for parasite maturation (Thomson-Luque and Bautista, 2021).

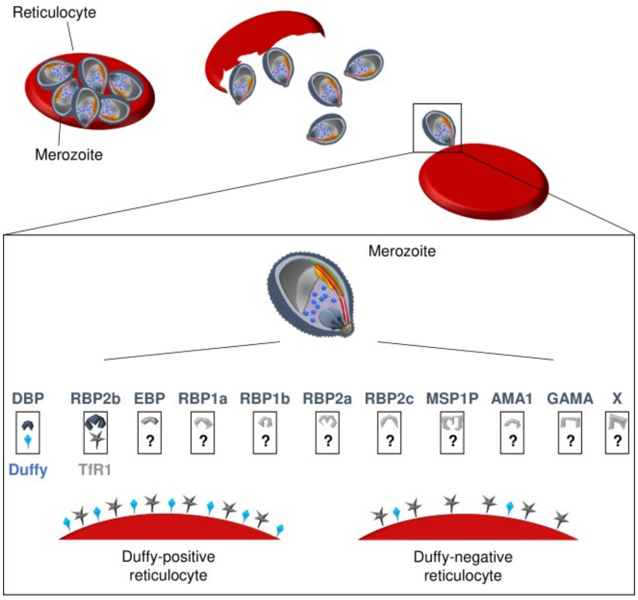


Figure 2: Interaction between *Plasmodium vivax* ligands and human reticulocyte receptors

After the erythrocytic stage terminates, the schizonts break and merozoites discharge into the circulation system, allowing the normal reticulocytes to be infected. Reticulocyte receptors and parasite ligands must interact for reticulocytes to be recognized and for the invasion process to take place. The possible receptors of ligands like PvEBP, PvRBPs, PvMSP1P, PvAMA1, or PvGAMA are now under investigation for their potential role in invasion mechanisms. When reticulocytes are Duffy-positive, PvRBP2b attaches to TfR1 prior to PvDBP interacting with the Duffy antigen to allow the merozoite to enter the cell. Duffy-negative reticulocytes' TfR1 is likely also recognized by PvRBP2b, although it is unclear what happens next during the invasion. Do parasites with many copies of the PvDBP gene possess a few Duffy molecules that allow them to enter the cell through the surface of the erythrocyte? Or do Duffy-negative reticulocyte invasion processes include different, as-yet-unidentified receptors that may be activated by ligands like PvEBP, PvMSP1P, or PvGAMA? Last but not least, the invasion process may happen through entirely unexplored pathways with unknown receptors and ligands (noted with an X). GAMA stands for glycosylphosphatidylinositol-anchored micronemal antigen, AMA1 stands for anchored micronemal antigen 1, MSP1P stands for merozoite surface protein-1 paralog, Pv is for Plasmodium vivax, RBP stands for reticulocyte binding proteins, and TfR1 stands for transferrin receptor 1.

Adapted from Popovici *et al*., 2020

2.7. Control challenges of *Plasmodium vivax*

2.7.1. Misdiagnosis of *Plasmodium vivax* infections

In co-endemic environment, *Plasmodium vivax* infections persist either alone or in combination with *P. falciparum*, making it challenging to diagnose using microscopy (Schoenherr *et al.*, 2009). In most parts of Africa, rapid diagnostic tests (RDT) are primarily designed to detect other Plasmodium species. *Plasmodium vivax* infects erythrocytes, which account for just a small proportion in circulation, commonly misdiagnosed either by accident or parasitemia is too low (Schoenherr *et al.*, 2009). Thanks to a greater capacity to utilize molecular diagnostic technologies, nowadays, nations without a history of *Plasmodium vivax* complications are starting to record a higher incidence of these illnesses (Zimmerman, 2017). As a result, standard microscopy can easily miss infections, making it difficult to estimate *Plasmodium vivax* distribution in endemic regions of African countries (Gunalan *et al.*, 2018). Furthermore, with the introduction of Plasmodium parasite whole genome sequencing, molecular examination of *Plasmodium vivax* from symptomatic data is going to show the actual frequency of infections in Duffy-positive and Duffy-negative groups (Pearson *et al.*, 2016).

2.7.2. Hypnozoite hide-and-seek

The life cycle of Plasmodium parasites is complex (Venugopal *et al.*, 2020). The liver stage of the parasite's developmental process is initiated by infected mosquito sporozoites moving to the liver and invading hepatocytes. There, they replicate and mature into schizonts that contain numerous liver merozoites (Sato, 2021). *Plasmodium vivax* has distinct biological characteristics, including an additional stage that significantly raises the disease's epidemiological and clinical complexity compared to *P. falciparum* (Adams and Mueller, 2017). Some sporozoites develop into hypnozoites, which are minute, non-replicating, un-nucleated latent forms ,but most sporozoites develop into schizonts immediately (Briquet *et al.*, 2021).

After the initial blood-stage infection, hypnozoites become active and induce subsequent relapsing infections, which are estimated to be responsible for an average of 88% of all *Plasmodium vivax* illnesses (Huber *et al.*, 2021). According to Price *et al.* (2020)**,** early relapses are significantly more common (> 80%) in tropical regions, and following relapses typically happen every 3–4 weeks. According to the data, relapses caused by hypnozoite account for the most *Plasmodium vivax*-critical infections (Noviyanti *et al.*, 2022). This dormant reservoir is one of the major challenges to malaria management because of the parasitic infection's continuous transmission and resistance to currently available antimalarial drugs (Asih *et al.*, 2018). Moreover, there are no diagnostic instruments available right now that can identify this stage (Gualdrón-López *et al.*, 2018). As a result, this characteristic encourages parasite growth all year and makes parasite management more challenging.

2.7.3. Early development of sexual blood stages (gametocytes)

According to Ngotho *et al.* (2019), the biology of gametocytes in *Plasmodium vivax* is very distinct from that of *P. falciparum*. Different gametocyte maturation times are seen in several Plasmodium species (de Jong *et al.*, 2020). Gametocytes of the *Plasmodium vivax* species quickly entered the bloodstream a few days after the asexual stage initially emerged (Koepfli *et al.*, 2021). When compared to *P. falciparum*, whose gametocytes mature just over a week, this is significantly different (Bantuchai *et al.*, 2022), since accumulation in tissues isn't a critical stage in their development (Koepfli *et al.*, 2021). Additionally, according to (Patankar *et al.*, 2018) research, *Plasmodium vivax* produces more gametocytes every cycle (up to 20% more) than *P. falciparum*. Before the onset of clinical disease, mature gametocytes of *Plasmodium vivax* are detected in the circulating blood, probably prior to the patient seeking therapy (Obaldia *et al.*, 2022).

Most gametocyte carriers showed no symptoms, which raises the possibility that latent infections may be crucial in the distribution of the parasite. This has been clearly proven in Thailand, where *Plasmodium vivax* gametocytes were present in large concentrations in patients who showed no symptoms (Nguitragool *et al.*, 2017). Therefore, *Plasmodium vivax* in those who didn't get treatment makes up a repository for human parasites. *Plasmodium vivax* gametocytes have clearly become a barrier to controlling malaria transmission (Vallejo *et al.*, 2016). *Plasmodium vivax* distribution is more probable than *P. falciparum* transmission to remain constant over time, undermining preventative efforts. Therefore, *Plasmodium vivax* preventative strategies designed for *P. falciparum* are less successful (Ome-Kaius *et al.*, 2019).

2.7.4. Drug resistance in *Plasmodium vivax*

Antimalarial medications are currently known to be ineffective against *Plasmodium vivax* (Heidari and Keshavarz, 2021). In endemic areas, *Plasmodium vivax* has been frequently found to be resistant to chloroquine (Commons *et al.*, 2018). Many alterations, including SNPs connected to antifolate and chloroquine resistance, have been discovered in *Plasmodium vivax* samples investigated in southern Thai provinces (Noisang *et al.*, 2019). Also, because *Plasmodium vivax* chloroquine resistance is so common, some nations are obligated to switch to ACTs, which has an impact on the use of primaquine as the sole anti-relapse medication (Douglas *et al.*, 2012; Ferreira *et al.*, 2021).

Resistance to chloroquine and primaquine in *Plasmodium vivax* has been extensively documented (Anmut Assemie, 2022). Despite the fact that these resistances are occasionally responsible for the inability to eradicate the hypnozoites or for therapy failure after the full term of medication and the recommended therapeutic dose have been completed (Thomas *et al.*, 2016). A number of *Plasmodium vivax* isolates have failed primaquine treatments, particularly those from the Western Pacific, South America, Southeast Asia, and specific sections of Africa (WHO, 2015). A case report from Ethiopia demonstrated that primaquine is ineffective for treating *Plasmodium vivax* malaria that has relapsed (Teklehaimanot *et al.*, 2020). Management and eradication of malaria have thus grown more difficult due to Plasmodium's drug resistance to the existing antimalarial medications.

2.7.5. In vitro cultivation of *Plasmodium vivax*

Continuous, effective in vitro cultures have been done for *P. knowles* and *P. falciparum* to understand the invasion process and parasite’s life cycle properly (Grüring *et al.*, 2014; Chua *et al.*, 2019). However, there has been no such technique for in vitro culture of *Plasmodium vivax* for a long time. In 1912, Bass & Johns reported the first effective *Plasmodium vivax* in vitro culture (Bass and Johns, 1912). But until the middle of the 1970s, there was no established detailed protocol for spreading this parasite (Trager and Jensen, 1976). Until now, many changes to the conditions for culture have been carried out, including changing the media used for culture, reticulocyte, and parasite source (Bermúdez *et al.*, 2018; Thomson-Luque *et al.*, 2019). The two main problems with *Plasmodium vivax* cultivation in vitro are rapid reticulocyte maturation (Malleret *et al.*, 2015), and the inability to invade normal reticulocytes, i.e., the proportion of pathogenic merozoites that can enter and spread infection in reticulocytes (Thomson-Luque *et al.*, 2017; Bermúdez *et al.*, 2018; Gunalan, Gunalan *et al.*, 2020). However, the biological cause of the decline in parasite invasion effectiveness is not entirely understood. The parasite, host, and environmental factors should be carefully investigated.

3. MATERIALS AND METHODS

3.1. Study area description

This investigation was done in four different settings in Ethiopia, Adama, Batu, Arba Minch zuria and Gondar zuria where *Plasmodium vivax and Plasmodium falciparum* co-occur (Figure 3).

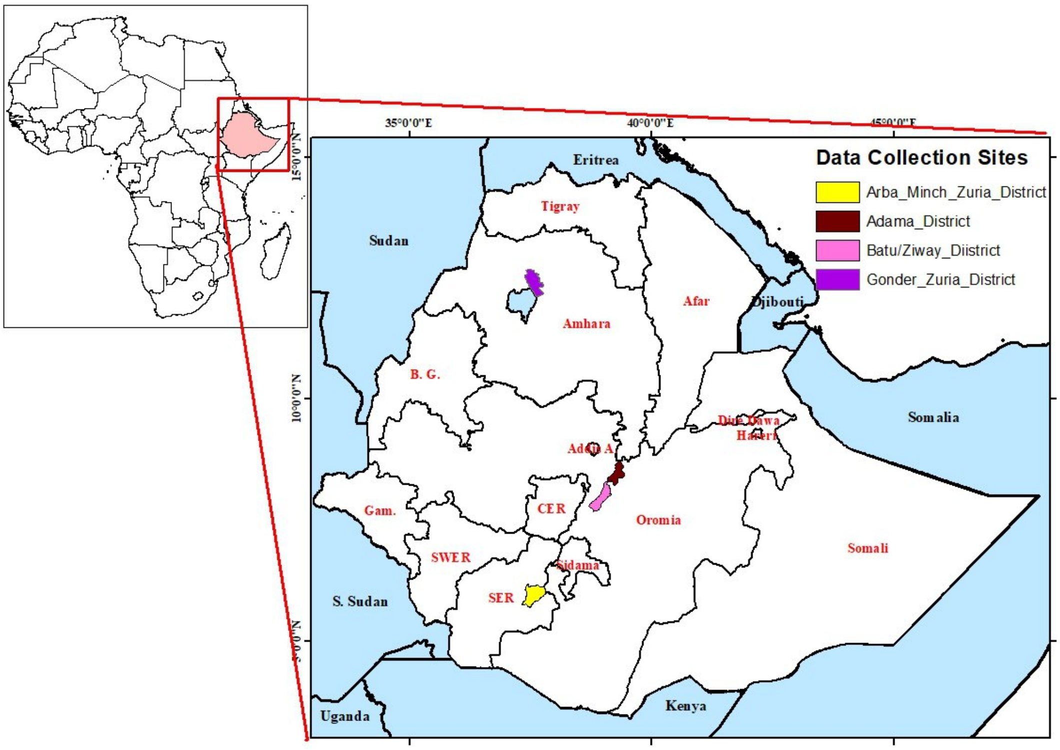


Figure 3: Location of the Study Area

Adama is a city found in the East Shewa Zone of Oromia region, Ethiopia. It is found 99 kilometers southeast of Addis Ababa at 8.54°N and 39.27°E. It is a rapidly growing, significant city in central Ethiopia near Addis, with a population of 500,000 in 2018. It is a common town affected by malaria because of its elevation of 2000 masl and a variety of factors favor breeding for mosquito. It is bounded by hills, and it floods frequently during the rainy months. The rainy season persists from the middle of June until the middle of September, with a short rainy break in March. Seasonal temperatures range from 16 to 32 degrees Celsius. *Plasmodium vivax* is the main cause of malaria in Adama (Wilson *et al.*, 2015). In contrast to the national statistics, 61% of malaria cases recorded from OPD data from 2013/14 to 2017/18 in a retrospective analysis in Adama City were *Plasmodium vivax*, and 39% were *P. falciparum*. An epidemiological change of the disease from *P. falciparum* to *Plasmodium vivax* was seen in the city from 2013/14 to 2017/18. According to a recent study conducted by Golassa and White (2017), 70% of *Plasmodium vivax* infections are thought to be the result of relapse.

Arba Minch Zuria is one of the districts of South Ethiopia region's Gamo zone is Arba Minch Zuria. The Kolla-Shele Health Center is found about 532 kilometers from Addis Ababa and roughly 27 kilometers from the town of Arba Minch. The district is home to 164,529 people in total, of whom 82,330 are males and 82,330 are women, according to the Central Statistical Agency of Ethiopia (CSA, 2007). The region is located in a tropical climate zone, with an elevation of 1200 to 3125 meters above sea level (ASL), 750 to 930 mm of yearly rainfall, and a mean annual temperature range of 16 to 37°C. *Plasmodium vivax* contributing about 40% (Endashaw Esayas *et al.*, 2020) to 50% of malaria cases that could vary between villages (Ashenafi Abossie *et al.*, 2020). The spread of malaria is notably unpredictable and very seasonal. The primary industries in the region are fishing and subsistence farming. Because of rivers like the Sille and Elgo as well as Lake Chamo, there is water accessible for mosquito breeding all year round.

Batu town is found in Oromiya Region, East Showa Zone and Judo Combolcha Adamitulu District. Is located at 7° 56' N and 38° 42' E, at an average altitude of 1657 masl, about 165 kilometers south of Addis Ababa. The town is found in the middle of Ethiopia's Rift Valley, with a total residents of 78,784 in 2018. Batu receives between 700 and 800 mm and 15–29 °C annual rainfall and annual temperature, respectively, and June through September sees a lot of rain, whereas April and May get lighter rainfall. According to malaria control program data, 52.89% of malaria infection cases in Batu were *P. vivax*.

Gondar zuria is a district, located in the central Gondar Zone of the Amhara Region. It has a latitude and longitude of 12°36′N and 37°28′E with an elevation of 2133 meters above sea level. It is located 727km far from the capital city, Addis Ababa in northwest direction. Gondar has a population size of 299,969 on an area of 192.27km2. The lowest and highest average temperature is 9.8°C and 29.7°C respectively and 1151mm average annual rainfall. Gondar, served as a landing zone for seasonal migrants during the agricultural off season, which sees a high number of clinical cases despite its high altitude of above 2000m. In the district eight health centers are found. Plasmodium infection in Gondar Zuria was 12% (30/251), of which 83.3% (25/30) were infected with *Plasmodium vivax* and 16.7% (5/30) with *P. falciparum* (Awoke Minwuyelet *et al.*, 2020).

3.2. Study Design and Period

A cross-sectional study was done in symptomatic *Plasmodium vivax* patients to investigate Duffy binding protein copy number using qPCR. The health centers in the districts are selected by the cluster random sampling technic, considering high patient flow and logistics. Samples were collected from July 2021 to October 2021.

3.3. Sample Size Determination

The sample size was calculated following the standard formula by considering 65% duplication of Duffy binding protein (Lo *et al.*, 2019), 95% of the confidence interval, and 5% of the marginal error.

$N = Z^2 * (p) * (1\text{-}p) / d^2$

N = Total Sample size

Z = Confidence level, Z value (95% is1.96)

p = Percentage of duplication, expressed as decimal (65% is 0.65)

d =Precision, confidence interval, expressed as decimal (5% is 0.05)

Therefor $$N = \frac{(1.96)^2 (0.65) (1-0.65)}{(0.05)^2} = 349$$

Since there is no study conducted for Duffy binding protein copy number variation in the study areas, equal number of samples (93, including 5% contingency) were taken from each sites.

3.4. Study Population

All patients who were diagnosed as positive for *Plasmodium vivax* and voluntary to participate in the study were considered as the study population.

3.5. Study Population Source

All inhabitants of the districts who were attending the selected health centers for malaria diagnosis during the study period were considered as study population source.

3.6. Inclusion Criteria

All *Plasmodium vivax* positive patients who are voluntary to participate in the study were included.

3.7. Sample collection and management

Each individual had thick and thin blood smears made in order to do a microscopy-based *Plasmodium vivax* screening. The samples were collected from voluntary participants that are diagnosed as positive for *Plasmodium vivax* by microscope at the health facilities. Informed written and signed consent and/or assent were obtained from all participants and/or the guardians of the children below 18 years respectively before sample collection. Basic demographic data were recorded both in red cup data entree platform and pre-tested questionnaire. After the slides were transported to the AHRI laboratory, three microscopists confirmed them for quality control.

3.8. Blood Sample collection

After obtaining consent from participants and guardians, three to four drop of finger-prick blood (each equivalent to ~50µl of blood), were collected into EDTA microtainer tubes. For each participating individual, three dried blood spots, (each of 20µL) were blotted on Whatman 3mm filter paper for further purpose and the remaining were stored in refrigerator. The DBS samples were allowed to air dry before being kept in zip-lock bags with self-indicating silica gel desiccant beads. The leftover blood in the tubes was shipped to the AHRI laboratory at Cold Chain. It was then kept frozen at -20°C until processing. All survey microscopy slides, EDTA microtainer tubes, and DBSs labeled only with survey ID.

3.9. Sample Processing

3.9.1. DNA extraction

The parasite DNA was extracted in the MNTD laboratory at Armauer Hansen Research Institute (AHRI) from whole blood by using a novel magnetic bead extraction method (MagMAX™ Multi-Sample Kit) according to the manufacturer's protocol (Tadele Emiru *et al.*, 2023). The detail SOP can be found in the appendix section (annex -3) of this thesis work.

3.9.2. *Plasmodium vivax* confirmation and quantification using qPCR

Amplification of *Plasmodium vivax* 18SrRNA gene was performed based on TaqMan protocol to investigate parasite species and density using. Since the 18SrRNA gene has both highly conserved and variable sections, it was selected as the target species identification (McCutchan *et al.*, 1995; Gardner *et al.*, 2002). *Plasmodium vivax* parasites were detected and their copies quantified using plasmid constructs by running serial dilutions (10^7 to 10^4 copies/ µL) of plasmids in duplicate per plate having the amplicon. The detail standard operating procedure (SOP) can be found in the annex section (annex-4) of this thesis work. The experiment was carried out in a total 20 µl reaction volume that contained 5 µl template DNA, 10 µl of TaqMan Fast Advanced Master Mix (2x), 0.4 µl of each primer (10µM), and a 0.22 µl probe (10µM) and 3.76 µl nuclease free water using a Bio-Rad CFX96 Real-Time PCR thermo cycler. PCR conditions were: 50 °C for 2 min, 95°C for 10 min, followed by 45 cycles at 95°C for 15 sec, and 60°C for 1 min. Just five copies of the 18S rRNA gene are present in each Plasmodium parasite (Wampfler *et al.*, 2013). In contrast, several thousand or even a million copies of 18SrRNA transcripts are typically present in each cell. To quantify parasites, 18S rRNA copy detected in each sample is converted into parasites/ µl.

3.9.3. Duffy blood group Genotyping

Using previously reported primers and probes, a qPCR-based TaqMan assay was used to analyze a point mutation in the DARC gene's GATA-1 transcription factor binding region (Lo *et al.*, 2021). The experiment was done in a total 20 µl reaction volume that contained 10µl TaqMan Fast Advanced Master Mix (2x), 2µl DNA template, 0.8µl each primer (10µM), and 0.4µl each probe (10µM) and 5.6 µl nuclease free water. PCR conditions were: 50 °C for 2 min, 95°C for 2 min, followed by 45 cycles at 95°C for 3 second, and 60°C for 30 second. Previously confirmed samples were used as positive control and nuclease free water as a negative control. When there are wild-type templates present (Duffy-positive homozygous), only wild-type probes with HEX labels hybridize with the amplicons and produce green fluorescence. When mutated templates are present (Duffy-negative), only mutant probes labeled with FAM bind to the amplicons, producing blue fluorescence, while negative controls stay dark. When heterozygous templates are present (Duffy-positive hetrozygous), amplicons are hybridized by both mutant and wild-type probes, producing blue and green fluorescence, respectively (Cheng *et al.*, 2004).

3.9.4. CNVs analysis of Duffy binding proteins

Using previously available primers (Table 1), the SYBR Green qPCR detection technique was used to determine the Duffy binding protein copy number (Popovici *et al.*, 2020). Using a CFX96 real-time PCR thermocycler, the copy counts of Duffy binding proteins were quantified in relation to one-copy β-tubulin gene. The experiment was done in a total 20 µl reaction mixture that contained 10µL GoTaq® qPCR Master Mix (2X), 0.5 µl each primer (10µM) and 2µL DNA and 7 µl nuclease free water. The following PCR conditions were used for the amplifications: 95 °C for 15 min, then 45 cycles of 95 °C for 15 s, 60 °C for 20 s, and 72 °C for 20 s. To achieve almost identical amplification efficiency, the *Plasmodium vivax*β-tubulin and Duffy binding proteins were optimized. To detect nonspecific PCR amplification, a melting curve was conducted between the temperature 65 ˚C and 95˚C with 0.5°C increments. Duffy binding protein copy numbers were estimated by using synthetic genes (*Plasmodium vivax*β-tubulin and Duffy binding protein) combined in varying ratios ranging from 1:1 to 1:6 (one copy of β-tubulin and one to six copies of Duffy binding protein). To know the perfectness of mixing diluted controls, ΔCt result must be similar with log2^(x) results, where x=1 to 6. The equation previously employed for Duffy binding protein copy number estimation was utilized to determine the Duffy binding protein copy number with a little modification (Lo *et al.*, 2019) as follow: N = 2^-ΔΔCt, where ΔΔCt = (Ct Pv*β-t*cal- Ct PvDBPcal) - (Ct pv*β-t* – CtPvDBP). The Ctpv*β-t* and CtPvDBP are threshold cycle values for the *Plasmodium vivaxβ-tubulin* and Duffy binding proteins respectively, whereas Ctcal is an average difference between Ctpv*β-tubulin* and CtPvDBP obtained for the positive control.

Table 1: Oligos used for parasite detection, DARC genotyping and CNV of DBP

PCR	Primer's name	Sequence5'-3'
SPP confirmation	Pv18S rRNA F	ACGCTTCTAGATTAATCCACATAACT
	Pv18S rRNA R	ATTTACTCAAAGTAACAAGGACTTCCAAGC
	Pv18S rRNA Probe	HEX/TTCGTATCGACTTTGTGCGCATTTTGC
Duffy genotyping	GATA-1 F	GGCCTGAGGCTTGTGCAGGCAG
	GATA-1 R	CATACTCACCCTGTGCAGACAG
	P1(MT)	FAM-CCTTGGCTCTTACCTTGGAAGCACAGG-BHQ
	P2 (WT)	HEX-CCTTGGCTCTTATCTTGGAAGCACAGG-BHQ
PvDBP CNV	CN_ PvDBP _F	AATTATAAGAGAAAACGTCGGGAAAG
	CN_ PvDBP _R	ACCAAATTCGTAAGTTCCTTCATACA
Housekeeping gene CNV	CN_β-tubulin_F	CATGTTCGTTAAGATTTCCTGGT
	CN_β-tubulin_R	GTTAGTGGTGCAAAACCAATCA
PvDBP Synthetic genes for CNV GAAAAACTGTAATTATAAGAGAAAACGTCGGGAAAGAGATTGGGACTGTAACACTAAGAAGGATGTTTGTATACCAGATCGAAGATATCAATTATGTATGAAGGAACTTACGAATTTGGTAAATAATACA		

Pv β-TubulinSynthetic genes for CNV

CAGGAGTTACATGTTCGTTAAGATTTCCTGGTCAGTTAAATTCTGATTTGAGAAA
ATTAGCTGTCAATTTAATTCCCTTCCCAAGACTCCACTTTTTTATGATTGGTTTTG
CACCACTAACAAGCAGAGG

3.10. Ethical consideration

The major project in which this thesis done was approved by the National Research Ethics (MoSHE/04/246/830/21) and the Armauer Hansen Research Institute's institutional ethics review committees (PO/46/20). An additional authorization letter was acquired for each health center from the regional health office. Prior to sample collection, informed written and signed consent were obtained. Participants become voluntary to the storage and future research of their samples as part of their consent. Throughout the trial, patient names were replaced by unique codes to maintain patient's privacy and confidentiality.

3.11. Statistical Analysis

The data was entered into a computer, and statistical analysis was performed using R 4.3.2 (The R Foundation for Statistical Computing) software. Qualitative and quantitative data were expressed as proportion (%) and mean, respectively. To find out if the mean parasite burden differs between Duffy-negative and positive patients, an independent sample t-test was used. In addition, Fisher's exact test is observed for the associations between DARC status and Duffy binding protein copy number. Tables and figures were used to describe the study's results. Probabilities less than 5% ($P < 0.05$) was considered statistically significant.

4. RESULTS

4.1. Determination of DNA quality using the Nano-drop

Since the MagMAX™ DNA Multi-Sample Ultra Kit is designed for rapid, high-throughput isolation of high-quality genomic DNA from a variety of sample matrices, DNA quality was determined for only 50% of the samples using nano-drop 2000 Spectrophotometer. Among the samples, 3.2% (6/186) of them have less than 1.75, which is low DNA quality; it may indicate the presence of protein or other contaminants that absorb strongly at or near 280 nm. But the remaining is showing good DNA quality (between 1.75 and 2.05). For detail look the (annex 4).

4.2. *Plasmodium vivax* confirmation and quantification using qPCR

Plasmodium vivax parasites were detected and their 18SrRNA copies quantified in relation to serially diluted plasmid constructs (10^7 to 10^4 copies/ µL) (Figure 4). *Plasmodium vivax* is not detected in 1.1% (4/366) of the samples (it may be either negative for *Plasmodium* parasite or positive for *Plasmodium falciparum*, since singlex 18SrRNA real time PCR assay is done). To quantify parasites /µl, 18SrRNA copy detected in each sample is converted into parasites/ µl. The average 18SrRNA copy/ µl of 349 samples are 61380, which mean 12,276 parasites/ µl. For Duffy-positive, the mean 18S rRNA copy/ µl are 61475 (12,295 parasite/ µl), while Duffy-negatives shows 3,015 18SrRNA copy/ µl (603 parasite / µl).

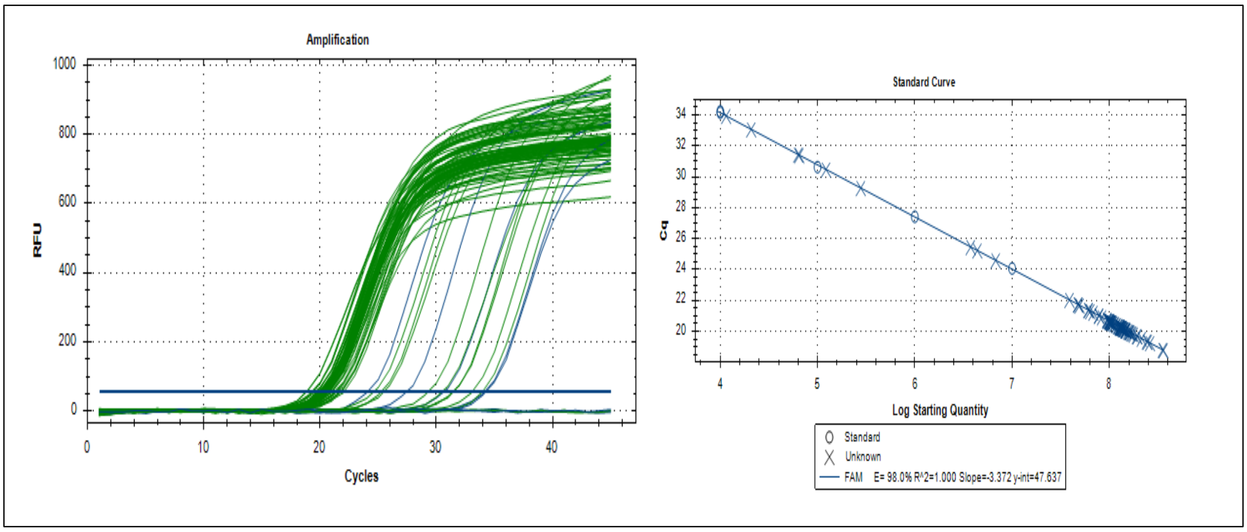


Figure 4: Amplification graph for parasite detection and confirmation

4.3. Duffy blood group Genotyping

Genotyping was carried out effectively on all 349 patients. Among these, 96.8% (338/349) of them are Duffy positive and the rest 3.2% (11/349) of sample patients are Duffy negative. Most of the participants in this study were heterozygous 81.9% (286/349) followed by homozygous 14.9% (52/349) Duffy positive. Duffy genotyping status varied between the studies sites. Most of the Duffy negative participants were detected in Gondar Zuria district 72.7% (8/11) followed Arba Minch Zuria 18.2% (2/11) and Adama 9.1% (1/11). Among Duffy-positive infections, the majority were heterozygous 81.9% (286/349) with variation between sites. All infections in Batu were heterozygous Duffy-positive. The majority of sample participants were male (58.2%); of this 97% of them is Duffy positive (Table 2).

Table 2: DARC status of patients

Variables	**Categories**	**DARC Genotyping status**			**Total participant (N=349, %)**
		Homozygous	Heterozygous	Negative	
Study site	Adama	18, 20.7	68, 78.2	1, 1.1	87,24.9
	Arba Minch	26, 29.5	60, 68.2	2, 2.3	88,25.2
	Batu	0, 0.0	88,100	0, 0.0	88,25.2
	Gondar zuria	8, 9.3	70, 81.4	8,9.3	86,24.7
sex	Male	37, 10.6	160, 45.9	6, 1.7	203, 58.2
	Female	15, 4.3	126, 36.1	5, 1.4	146, 41.8

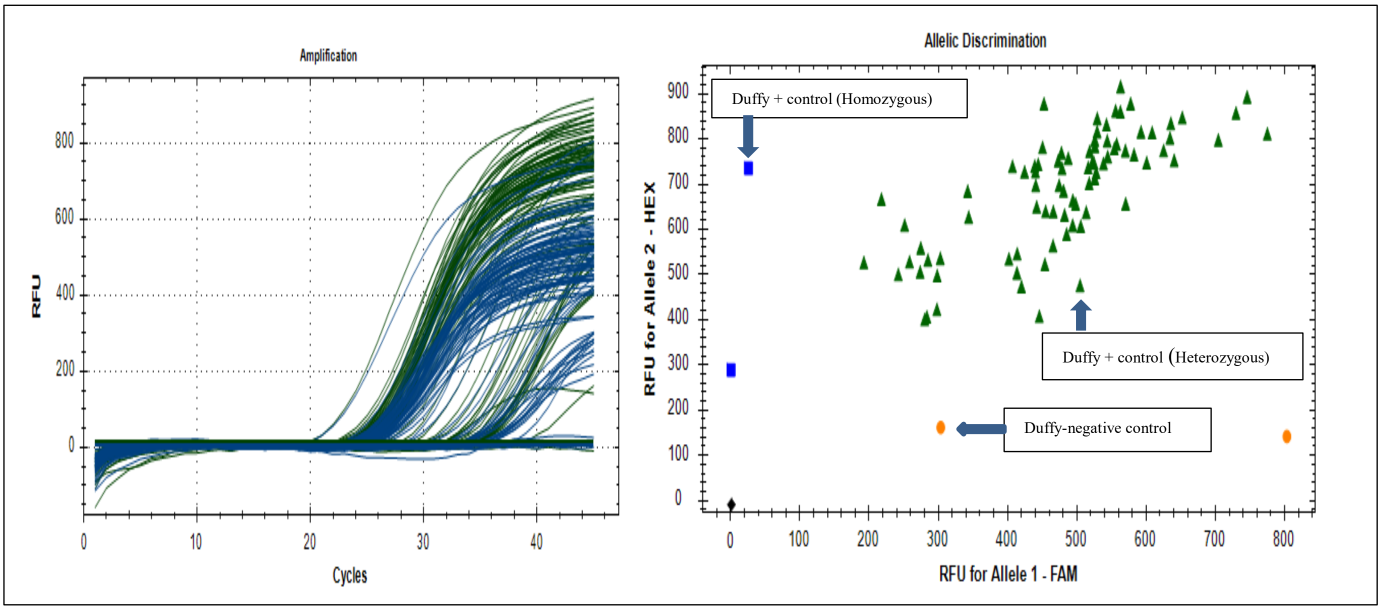
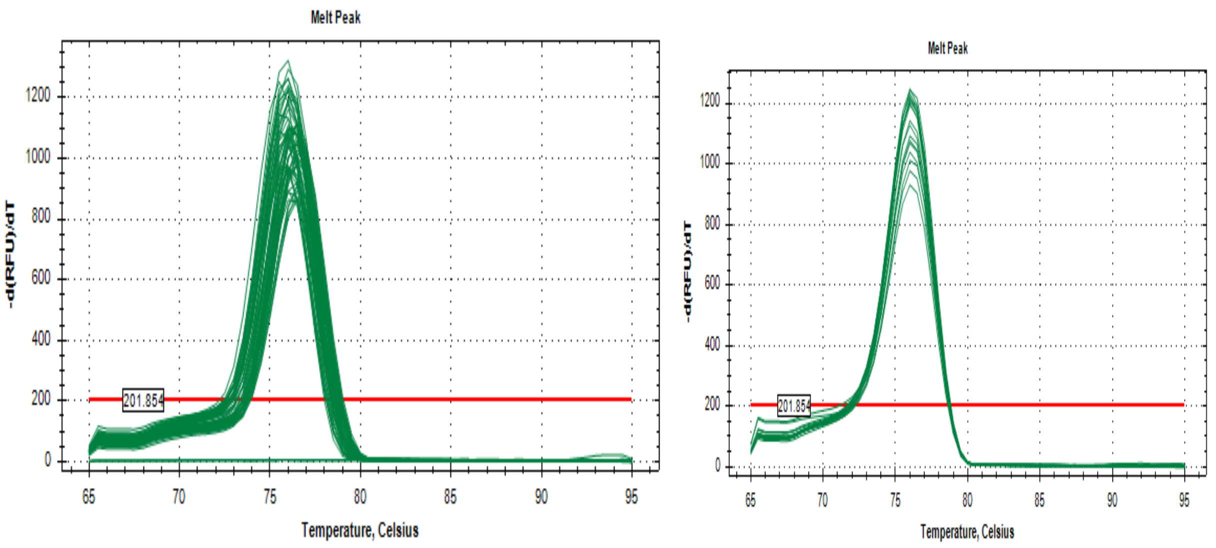


Figure 5: Amplification and Allelic discrimination of Duffy blood group genotyping

4.4. CNVs analysis of Duffy binding proteins

After amplification reaction completed, melting curve analysis was conducted between temperature 65 ˚C to 95˚C with 0.5°C increments to detect non-specific PCR amplification (Figure 6). Duffy binding protein copy number was investigated in all 349 isolates of *Plasmodium vivax*. Among these, there were 74% (259/349) Duffy binding protein copy number estimations that exceeded 0.28, which indicates a multiple Duffy binding protein copy. These samples had Duffy binding protein estimations ranging from 0.00 to 7.23, indicating the possibility of higher-order amplifications in some of the samples. These results were categorized into three groups: (1) samples that had a value ≤ 0.28 were considered to be single copies; (2) samples that had a value between 0.28 and including 1.06 were considered to be 2 to 3 copies; and (3) samples that had a value > 1.06 were considered to be more than 3 copies. According to this definition, 26% (90/349) of the samples contained a single copy, while 63% (219/349) of the samples contained two or three copies, and the rest, 11% (40/349), had >3 copies (Figure 7).



Melting curve for test samples

Melting curve for controls

Figure 6: melting curve analysis graph

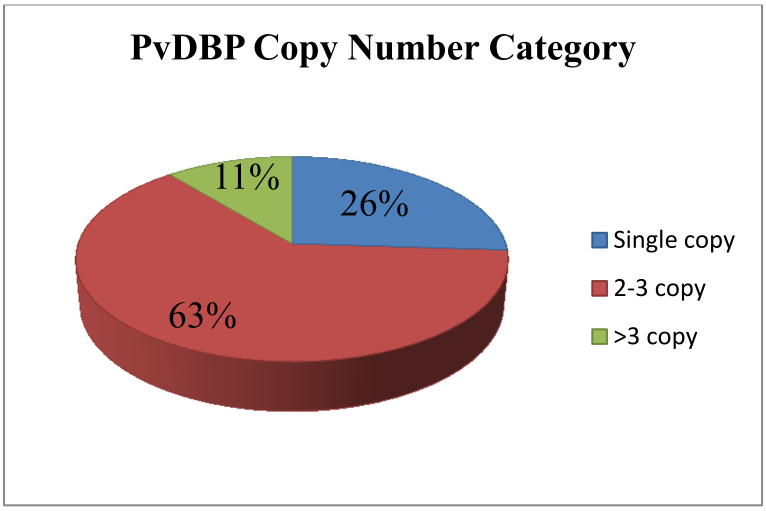


Figure 7: Pi Chart for Duffy binding protein copy number variation

4.5. Association between DBP copy number and Duffy status

To find out if the parasite's invading ability is increased by an increased Duffy binding protein copy number against Duffy-negative patients, the relationship between three different Duffy binding protein copy numbers (single copy, 2-3 copy, and >3 copy) and their preference for two categories of Duffy status (positive and negative) was investigated. The output of Fisher's exact test includes the p-value for the overall association between Duffy binding protein copy number of parasite ligands and Duffy status of human receptor. Duffy-negative belongs to the three groups of Duffy binding protein copy numbers (single copy, 2-3 copy, and >3 Duffy binding protein copy numbers); the same is true for Duffy-positive. The result reveals a substantial association between DARC status and Duffy binding protein copy number ($p = 0.000313$) (Table 3). In addition, a post-hoc test indicates a significant association between the single and 2–3 Duffy binding protein copy numbers and the DARC status ($p = 0.000281$) (Table 4). However, the number of Duffy-negative patients infected by multiple Duffy binding protein copy parasite is small. Therefore, we cannot decide that multiple Duffy binding protein copy number increase parasite's invading ability against Duffy-negative patients.

Table 3: Fisher's exact test for Duffy status & PvDBP copy number

DARC Status	Single Copy %	2-3 Copy %	>3 Copy %	Total	p-value
Duffy-Positive	82, 91.1	218, 99.5	38, 95	338, 96.8	0.000313
Duffy-Negative	8, 8.9	1, 0.5	2, 5	11, 3.2	
Total	90, 100	219, 100	40, 100	349, 100	

Table 4: Post-hoc test for Fisher's exact test

Group 1	Group 2	Total	P	P.adj
Single copy	2-3 copy	309	0. 000281	0.000843
Single copy	>3 copy	130	0.723	0.723
2-3 copy	>3 copy	259	0.0631	7.947

4.6. Association between Duffy Status and parasite load

To investigate the relation between Duffy status and parasite load among *Plasmodium vivax* patients, data were collected from both Duffy groups. An independent sample t-test was used to find out the mean difference of parasite load between Duffy-negative and Duffy-positive patients. The small p-value indicates statistically significant difference between the variables. In our case, P-vale is less than 0.0001; this suggests that the observed difference in mean of parasite load between Duffy-negative and Duffy-positive is statistically significant. In addition, mean difference in parasite load/µl indicate that parasite density in DARC-negative is lower than DARC-positive (Table 5).

Table 5: t-test analysis of DARC status and parasite load

DARC status	Mean of 18SrRNA copy/µl	Mean of parasite load/µl	t-value	DF	P-value
Negative	3,015	603	-9.6	207.51	< 0.0001
positive	61,475	12,295			

5. DISCUSSION

Plasmodium vivax causes nearly as many malaria cases in Ethiopia as *P. falciparum*; however, research into *P. falciparum* is significantly faster than *Plasmodium vivax*. In reference to *P. falciparum*, very few *Plasmodium vivax* genomes have been studied and made publicly accessible. Knowledge about the epidemiology and detail mechanism of infection for *Plasmodium vivax* is still limited. Therefore, filling the informational gap on *Plasmodium vivax* is crucial to addressing the WHO's 2030 target of eradicating malaria.

In this study, real-time PCR method was used to assess the parasite load, copy number for Duffy binding protein, and Duffy blood group genotyping. This method has been used in many studies and is an efficient method that saves time and money for analyzing a lot of samples. Duffy binding protein copy number estimation may be affected by variations in the parasite DNA content of the samples. Since 18S rRNA quantification came before the Duffy binding protein qPCR experiment, the parasite DNA present in each reaction was determined. To calibrate and determine the Duffy binding protein amplification for each sample, one-copy housekeeping gene known as *Plasmodium vivax beta*-tubulin was used as an internal standard.

Duffy blood group genotyping were successfully performed on all the 349 patients. *Plasmodium vivax*-infected Duffy-negative patients are discovered in the stud. Among all samples, 96.8% of sample patients are Duffy-positive and the rest 3.2% are Duffy- negative; which are detected from Adama, Arbaminch, and Gondar zuria. The majority of sample participants were female (58.2%); of this 97.4% of them are Duffy-positive. High number of Duffy- negativity was seen in Gondar, which is 73% (8/11). Human movement from neighboring country and Genetic background of patients may be the reason for High distribution of Duffy-negativity in Gondar. In Batu, no Duffy- negative patients detected. This study confirms the coexistence of positive and negative populations for Duffy in Ethiopia (Howes *et al.*, 2011). According to a recent study done in Ethiopia, 10.5%–11.9% of individuals with *Plasmodium vivax* infection had Duffy-negative results (Lo *et al.*, 2021). In addition, research done in Adama and its surrounding indicate that 2.9% (4/138) *Plasmodium vivax* infected individuals are Duffy-negatives (Andargie Abate *et al.*, 2022). The most recent study in Ethiopia shows that 4.4% (16/361) *Plasmodium vivax* infected individuals are Duffy-negatives (Abnet Abebe *et al.*, 2023). This study suggests that the proportion of *Plasmodium vivax*-infected Duffy-negative patients (3.2%) is relatively lower than previous research conducted in Ethiopia (10.5%–11.9% and 4.4%) (Lo *et al.*, 2021; Abnet Abebe *et al.*, 2023); but higher than studies conducted in Adama city administration (Andargie Abate *et al.*, 2022) which may be indicating Duffy negativity adaptation.

Duffy binding protein copy number variation analysis was done by SYBR green assay using real time PCR. In this assay, it was difficult to distinguish fluorescent signal of target gene from control gene in a single well of PCR plate. Therefore, we are obligated to set up parallel reaction mixture containing different primers in separate wells. To detect nonspecific PCR amplification, a melting curve analysis was conducted between 65 ˚C – 95˚C with 0.5°C increments. Previous *Plasmodium vivax* sample analysis demonstrated that Duffy binding protein infections with multiple copies are more common in Ethiopia than in any other *Plasmodium vivax* site (Auburn *et al.*, 2019). In a recent small-scale study using just 25 Ethiopian *Plasmodium vivax* samples, 56% of the samples showed duplication of *Plasmodium vivax* Duffy-binding protein (Hostetler *et al*., 2016). This indicates that Ethiopia is more susceptible than any other country in the world to having several copies of *Plasmodium vivax* Duffy-binding protein infections. According to recent study conducted in southern Ethiopia, 79% (19/24) *Plasmodium vivax* isolates containing multiple copies of *Plasmodium vivax* Duffy binding protein (Auburn *et al.*, 2019). In addition, study conducted in Jimma Ethiopia shows 65.5% (95/145) *Plasmodium vivax* samples containing multiple copies of *Plasmodium vivax* Duffy binding protein (Lo *et al.*, 2019). Increasing to 349 in this study, 74% (259/349) *Plasmodium vivax* isolates containing multiple copies of *Plasmodium vivax* Duffy binding protein; which is consistent with previous studies in Ethiopia (Auburn *et al.*, 2019; Lo *et al.*, 2019). As far as we understand, this is the only report of a large number of *Plasmodium vivax* isolates compared to earlier Ethiopian research. These findings demonstrate that Duffy binding protein amplification is a prevalent occurrence in Ethiopia. High rate of Duffy-binding protein duplication may become obstacle for national malaria elimination in Ethiopia.

To find out if the parasite's invading ability is increased by an increased Duffy binding protein copy number against Duffy-negative patients, the relationship between Duffy binding protein copy numbers and Duffy status was investigated using Fisher's exact test. The output of Fisher's exact test includes the p-value for the overall association between Duffy binding protein copy number and DARC status. The results reveal that there is a significant association between Duffy binding protein copy number and Duffy status. In addition, the post-hoc test indicates that there is a significant relationship between the Duffy binding protein copy number (single and 2-3 copy numbers) and the DARC status. However, the number of Duffy-negative patients infected by multiple Duffy binding protein copy parasite is small. Therefore, it is difficult to decide that multiple Duffy binding protein copy number increase parasite's invading ability against Duffy-negative patients.

Asexual parasites were observed under a microscope in a small number of Duffy-negative people. In comparison to the Duffy-negative patient's parasite load which was microscopically undetected, those detected displayed increased parasite load after qPCR confirmation. The majorities of the *Plasmodium vivax*-infected Duffy-negative people were submicroscopic and had a fever when samples were taken. Just five copies of the 18S rRNA gene are present in each Plasmodium parasite (Wampfler *et al.*, 2013). In contrast, several thousand or even a million copies of 18S rRNA transcripts are typically present in each cell. To quantify parasites, 18S rRNA copy detected in each samples is converted into parasites/μl. Research from Ethiopia found that *Plasmodium vivax*-infected patients with the Duffy-negative genotype consistently demonstrated a low level of parasite load (Lo *et al.*, 2021; Andargie Abate *et al.*, 2022). The results of this investigation also indicate that parasite burden is significantly lower in Duffy-negative patients than in Duffy-positive ones, which is consistent with previous studies (Lo *et al.*, 2021; Andargie Abate *et al.*, 2022). Peripheral blood *Plasmodium vivax* blood stages may not accurately reflect the entire parasite biomass retained by the host, as parasites also seem to "sequester" in the bone marrow (Obaldia N *et al.*, 2018). Low parasite loads in Duffy-negative infections may indicate a less effective but constantly developing attack method, resulting in a more adverse influence on public health in Ethiopia in the coming years. In addition, low parasite loads in Duffy-negative patients may represent an undetected reservoir. It is essential to keep in mind that the widespread occurrence of *Plasmodium vivax* among Duffy-negatives may be related to the use of a higher-sensitivity tool known as real time PCR. Most of the time, *Plasmodium vivax* was missed in Duffy-negative individuals, where malaria is commonly diagnosed under a microscope (Lo *et al.*, 2021). It was discovered that *Plasmodium vivax* could survive in patients that were negative for Duffy without producing any symptoms (Niang *et al.*, 2018), but may be transferred between hosts (Commons *et al.*, 2019).

The limitation of this work is that the number of Duffy-negative patients infected by multiple Duffy binding protein copy parasite is small. Therefore, it is difficult to decide that multiple Duffy binding protein copy number increase parasite's invading ability against Duffy-negative patients. Using a qPCR method, we found that most of patients have numerous Duffy binding protein copy numbers. Investigating large number of samples by using Droplet PCR and sequencing techniques may be able to change this issue.

6. Conclusion and Recommendations

To conclude, a high number of *Plasmodium vivax* isolates showed multiple Duffy binding protein copies. Therefore, Duffy binding protein duplication is a prevalent occurrence in Ethiopia. Duffy-negative People do not resist *Plasmodium vivax.* Low parasite load in Duffy-negative patients may indicate a less effective but constantly developing invading technique. This low level of parasite load in Duffy-negative patients may represent the presence of an undetected reservoir in the human host. Therefore, it may challenge control and eradication plan, affecting the economy and general public health in Ethiopia unless focused on the following recommendations.

- In reference to the high rate of Duffy-binding protein duplication, further investigation should be extended to different regions of Ethiopia by using droplet digital PCR assay.
- It is also important to look into other ligand-receptors associated with Duffy-negative reticulocyte invasion.

7. REFERENCE

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Appendix 1: Questionnaire and Consent Form

Questionnaire: Socio Demographic Information and Survey Case Report Form

A. Participant Information

1. Patient ID:____________________ 2. Health Center:__________________ 3. Date of Visit:…/……/…..

4. Age (Years):________ 5. Sex☐.Male ☐ Female 6. Kebele:……………

B. Microscopy Examination

Field health facility

7. status: ☐Positive ☐ Negative ☐Not Done

8. Species ☐*P.falciparum* ☐*P.vivax* ☐Mixed Species

9. Parasite count (parasite per microliter)........... 10. Name of microscopist...............

Confirmation at AHRI

11. status: ☐Positive ☐ Negative ☐Not Done

12. Species ☐*P.falciparum* ☐*P.vivax* ☐Mixed Species

13. Parasite count (parasite per microliter)........... 14. Name of microscopist...............

15. If positive to question 7 above, is treatment provided? ☐Yes ☐No

C. Sample Type Collected

16. Is EDTA sample collected? ☐Yes ☐No

17. Is DBS prepared? ☐Yes ☐No

18. Is consent for long term storage of blood samples taken? ☐Yes ☐No

E. Previous malaria infection and travel history

19. In the past 2 weeks, have you had a test for malaria? ☐Yes ☐No

20. If yes, what was the result if the test? ☐Positive ☐Negative ☐ Don't know

21. In the past 2 weeks, have you taken any medicine for malaria? ☐Yes ☐No

22. If yes, what was the anti-malarial medicine taken?

☐ACT ☐AL ☐Artesunate ☐Fansidar(SP)

☐Quinine ☐Panadol ☐Other ☐Unknown

23. Have you travel to another locality of the country in the past 30 days? ☐Yes ☐No

24. If yes, where did you travel to the past 30 days?

Country........... Region............. District...... City/Village....... .....Don't Remember

25. If yes, for how long you travelled in the past 30 days?

No of days……… from...../....../........ To...../...../......

Name and signature of data collector...

Consent Form

Certificate of consent for Adults (>18Years)

Survey ID:___

I have been invited to participate in a study that aims to better understand if the malaria parasite is changing over time and affecting how well rapid diagnostic tests are working in Ethiopia. I have read the above information, or it has been read to me. I have had the opportunity to ask questions, and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate in this study.

Name of participant:_Signature:____Date:__(dd/mm/yyyy)

Long-term storage and future studies: I agree to allow the study team to store my blood sample for future studies on malaria. I understand that I can change my mind to not have my blood sample stored and used for future research.	If you agree, circle “YES,” if you Do not agree, circle„ NO‟.	
	YES	NO
Name:_	Signature	Date / /

Witness’ signature: A witness’ signature and the patient’s thumbprint are required only if the patient is illiterate. In this case, a literate witness must sign. If possible, this person should be selected by the participant and should have no connection with the study team.

I have witnessed the accurate reading of the consent form to the potential participant, who has had the opportunity to ask questions. I confirm that the participant has given consent freely.

Thumb print of participant ☐

Name of witness:____Signature:____Date:__(dd/mmm/yyyy)

Investigator’s signature:

I have accurately read or witnessed the accurate reading of the consent form to the potential participant, who has had the opportunity to ask questions. I confirm that the participant has given consent freely. Name of Investigator:__Signature:___Date:__(dd/mm/yyyy)

Certificate of consent from the child`s family/guardian (<12years)

Survey ID:___

I have been invited for my child`s participation in a study that aims to better understand if the malaria parasite is changing over time and affecting how well rapid diagnostic tests are working in Ethiopia. I have read the above information, or it has been read to me. I have had the opportunity to ask questions, and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to my child to participate in this study.

Name of participant`s parent:_Signature:____Date:__(dd/mmm/yyyy)

<table>
<tr><td rowspan="3">Long-term storage and future studies:
I agree to allow the study team to store my child’s blood sample for future studies on malaria. I understand that I can change my mind to not have my child`s blood sample stored and used for future research.
Name:_</td><td colspan="2">If you agree ,circle “YES, ”if You do not agree, circle„ NO“.</td></tr>
<tr><td>YES</td><td>NO</td></tr>
<tr><td>Signature</td><td>Date__/_____/</td></tr>
</table>

Witness’ signature: A witness’ signature and the participant’s thumbprint are required only if the participant is illiterate. In this case, a literate witness must sign. If possible, this person should be selected by the participant and should have no connection with the study team.

I have witnessed the accurate reading of the consent form to the potential participant, who has had the opportunity to ask questions. I confirm that the participant has given consent freely.

Thumb print of participant

Name of witness_____Signature_____Date:__

Long-term storage and future studies: I have witnessed the accurate reading of the request for long term storage of	If you agree, circle “YES,” if

samples, for future studies on malaria. I understand that the participant family can change his/her mind to not have the blood sample stored and used for future research. The potential participant, who has had the opportunity to ask questions,. I confirm that the Participant agrees:	you do not Agree, circle,, NO‟.	
	YES	NO

Investigator’s signature:

I have accurately read or witnessed the accurate reading of the consent form to the potential participant, who has had the opportunity to ask questions. I confirm that the participant has given consent freely.

Name of Investigator:_Signature:____Date:__(dd/mm/yyyy)

Certificate of consent from the child`s family/guardian (≥12<18years)

Survey ID:___

I have been invited for my child to participate in a study that aims to better understand if the malaria parasite is changing over time and affecting how well rapid diagnostic tests are working in Ethiopia. I have read the above information, or it has been read to me. I have had the opportunity to ask questions, and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to my child to participate in this study.

Participant`s parent name:___Signature:____Date:__

Long-term storage and future studies: I agree to allow the study team to store my child’s blood sample for future studies on malaria. I understand that I can change my mind to not have my child`s samples to read and used for future research.	If you agree, circle “YES” ,if you do not agree, circle„ NO‟.	
	YES	NO
Name:_	Signature	Date /_____/_____

Witness’ signature: A witness‟ signature and the patient’s thumbprint are required only if the patient is illiterate. In this case, a literate witness must sign. If possible, this person should be selected by the participant and should have no connection with the study team.

I have witnessed the accurate reading of the consent form to the potential participant, who has had the opportunity to ask questions. I confirm that the participant has given consent freely.

Thumb print of participant

Witness name:Signature:____Date:__

Long-term storage and future studies: I have witnessed the accurate reading of the request for long term storage of samples, for future studies on malaria. I understand that the participant can change his/her mind to not have the blood sample stored and used for future research. The potential participant, who has had the opportunity to ask questions,. I confirm that the participant agrees:	If you agree, circle “YES, ”if you do not agree, circle„ NO‟.	
	YES	NO

Investigator’s signature:

I have accurately read or witnessed the accurate reading of the consent form to the potential participant, who has had the opportunity to ask questions. I confirm that the participant has given consent freely.

Name of Investigator:_Signature:____Date:__(dd/mm/yyyy).

Appendix 2: Amharic questioners

የተሳታፊዎች መረጃ እና የስምምነት መግለጫ ቅጽ

I. የተሳታፊዎች መረጃ ቅፅ

እኔ በአዲስ አበባ ዩኒቨርሲቲ በ ባዮቴክኖሎጂ ት/ት ተቋም የማስተርስ ተማሪ ስሆን የመመራቂያ ጽሁፌን በተለያየ ኢትዮጵያ ክፍል በሚገኙ ወባ በያዘአቸዉ ሰዎች በ ደም ናሙና ፒ.ቪ.ቫክስ ደፊ ባይንዲንግ ፕሮቲን ቅጂ ቁጥር ከ ደፊ ሁኔታ ጋር ያለውን ግንኙነት ለመዳሰስ አቅጄለሁ፡፡በጥናቱ በመሳተፍዎ ከልብ እያመሰገንኩ ከመወሰንዎ በፊት ይህን ቅፅ ሲነበብልዎ በትክክል እንድታዳምጡ፤ እንዲሁም ግልጽ ያልሆነልዎትን ጥያቄ በሙሉ በነፃነት እንድትጠይቁ ስል በትህትና እጠይቃለሁ::

አጠቃላይ መረጃ፡-

ወባ በፕላዝሞዲየም ዝርያ የሚመጣ ጥገኛ በሽታ ነው። የዓለም ጤና ድርጅት የአፍሪካ አህጉር ከ2000-2019 ዓ.ም ባለው ጊዜ ውስጥ 215 ሚሊዮን የሚገመቱ የወባ ተጠቂዎች ሲኖሩ ሀገራችንም ከፍተኛ ቁጥር ትይዛለች ። አምስት ጥገኛ ዝርያዎች በሰዎች ላይ የወባ በሽታ ያስከትላሉ፣ በኢትዮጵያ ከሚገኙ 109 ሚሊዮን ነዋሪዎች መካከል 2.4 ሚሊዮን ግምት እና 962,000 የተረጋገጠ የወባ በሽታ በ2018 ተመዝግቧል። በሽታውን ኣምጪ ተህዋሲያኖች ውስጥ አብዛኛዎቹ በፒ.ፊልሲፋርም እና ከዚያም በፒ.ቪ.ቫክስ የተከሰቱ ናቸው።

የጥናቱ ዋና አላማ፡- በተለያየ የኢትዮጵያ ክፍል በሚገኙ ወባ በያዛቸዉ ሰዎች ደም ናሙና ላይ በፒ.ቪ.ቫክስ ደፊ ባይንዲንግ ፕሮቲን ቅጂ ቁጥር ከ ደፊ ሁኔታ (ደፊያለው ወይም የሌለው) ጋር ያለውን ግንኙነት ለመዳሰስ አቅጄለሁ።

ጥናቱ ለተሳታፊዎች ያለው ጠቀሜታ፡

በጥናቱ ሚሳተፉ ፍቃደኛ ተሳታፊዎች ምንም አይነት የገንዘብ ክፍያ የለም፣ነገር ግን በምርመራው ውጤት መሰረት የመታከም እድሉ ይኖርዎታል፡፡ በተጨማሪም የጥናቱ ውጤት በሽታውን ለመቆጣጠር ተጨማሪ ግብአት ይሆናል፡፡በተዘዋዋሪ መንገድ ሌላ ህመምተኛ እንዲሁም ህብረተሰቡን የመጥቀም እድል ያስገኛል፡፡

በጥናቱ ተሳታፊዎች ላይ ያለው ጉዳትና ተዛማጅ ችግር

በዚህ ጥናት በመሳተፍወ ሊደርስብዎ የሚችል አንድም ጉዳት አይኖርም ነገር ግን ለጥናቱ የደም ናሙና የሚያስፈልግ ሲሆን ናሙና በሚሰጡበት ጊዜ ቀለል ያለ ህመም ሊሰምዎ ይችላል።

የመረጃ ሚስጥራዊ አጠባበቅ

ከእርስዎ ማህበራዊ መረጃ ጋር ተያይዞ የተገኘ እና ከእርስዎ ጋር ሊታወቅ የሚችል ማንኛውም መረጃ ሚስጥራዊ ሆኖ ይቆያል። በማንኛውም ጊዜ ጥናቱን የማቆም ወይም የመሰረዝ መብት አለዎት። ስለእርስዎ የተሰበሰበ መረጃ ቁጥሮችን በመጠቀም ኮድ ይደረጋል። የተሰበሰበ መረጃ ከጥናቱ ዋና ተመራማሪ ዉጪ ለሌሎች ሰዎች አይገለጽም ወይም በዚህ ጥናት ውስጥ በማንኛውም ዘገባ ላይ በቀጥታ አይታይም።

ጥናቱን የሚያካሂደው ሰው ማረጋገጫ

ለዚህ ጥናት ሃላፊነቱን ለመውሰድና ማናቸውንም ጥናቱ የሚመለከቱ ጉዳዮችን ክትትል ለማድረግና ለሚመለከተው አካል መግለጫ ለመስጠት በፊርማዬ አረጋግጣለሁ፡፡

ስም፡ ያሲን ናስር ሁሴን

(አዲስ አበባ ዩኒቨርሲቲ፣ ባዮቴክኖሎጂ ማስተርስ ተማሪ).

ፊርማ ------------------------------ ቀን --------------------------

ማንኛውንም ጥያቄ መጠየቅ ለሚሹ የሚቀጥለውን አድራሻዬን መጠቀም ይችላሉ፡፡

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II የስምምነት መግለጫ ቅጽ

መለያ ቁጥር __________________

እኔ ከዚህ በታች ስሜ የተጠቀሰውና የፈረምኩት ግለሰብ ፒ.ቪ.ቫክስ ደፊ ባይንዲንግ ፕሮቲን ቅጂ ቁጥር ከ ደፊ ሁኔታ ጋር ያለውን ግንኙነት ስለማወቅ በሚደረገው ጥናት እና የጥናቱ አላማ የተነገረኝና ግልጽ የተደረገልኝ ሲሆን እንዲሁም ከኔ የተወሰዱት መረጃዎች በሙሉ በሚሰጥር የሚጠበቁ መሆናቸው ተነግሮኛል፡፡ በተጨማሪም በማንኛውምሁኔታና ጊዜ ከጥናቱ ያለመሳትፍ መብት ተረጋግጦልኛል፡፡ ስለዚህ በጥናቱ ለመሳተፍም ሆነ አስፈላጊውን ናሙናና መረጃ ለመስጠት ተስማምቻለሁ፡፡

ስም____

ፊርማ_______________________________________

ቀን____

III. የወላጆች ወይም የአሳዳጊዎች የስምምነት መግለጫ ቅጽ

መለያ ቁጥር __________

እኔ ከዚህ በታች ስሜ የተጠቀሰውና የፈረምኩት ግለሰብ ፒ.ቪ.ቫክስ ደፌ ባይንዲንግ ፕሮቲን ቅጂ ቁጥር ከ ደፌ ሁኔታ ጋር ያለውን ግንኙነት ስለማወቅ በሚደረገው ጥናት እና የጥናቱ አላማ የተነገረኝና ግልጽየተደረገልኝ ሲሆን እንዲሁም ከኔልጅ የተወሰዱት መረጃዎች በሙሉ በሚሰጥር የሚጠበቁ መሆናቸው ተነግሮኛል፡፡ በተጨማሪም በማንኛውም ሁኔታና ጊዜ ከጥናቱ ልጄን ያለመሳትፍ እና የማቋረጥ መብት እንዳለኝ ተረጋግጦኛል፡፡ ስለዚህ በጥናቱ ልጀን ለመሳተፍም ሆነ አስፈላጊውን ናሙናና መረጃ ለመስጠት ተስማምቻለሁ፡፡

ስም____________________________________

ፊርማ____________________________________

ቀን____________________________________

IV. አማርኛ ቃለ መጠይቅ

ሰላም

እኔ ያሲን ናስር እባላለው በአዲስ አበባ ዩኒቨርሲቲ በባዮቴክኖሎጂ ት/ት ተቋም ባዮቴክኖሎጂ የሁለተኛ ድግሪ ተማሪ ስሆን በ ኢትዮጵያ በሚገኙ ወባ በያዘአቸዉ ሰዎች ላይ ፒ.ቪ.ቫክስ ደፊ ባይንዲንግ ፕሮቲን ቅጂ ቁጥር ከ ደፊ ሁኔታ ጋር ያለውን ግንኙነት ለመዳሰስ ለአካባቢው ህብረተሰብ ግንዛቤ መፍጠርና ለሚመለከተዉ አካል ተገቢውን የመከላከያ ዘዴ እንዲወስዱ ማድረግ ነው። ስለሆነም እኔ እርስዎን ለጥናቱ ጠቃሚ መረጃዎችን እንደሚሰጡኝ በማመን ለዚህ ጥናት ተሳታፊ እንዲሆኑ መርጬዎታለሁ። ስለዚህ ፈቃደኛ ከሆኑ የተወሰኑ ጥያቄዎች ንእንዲመልሱልኝ እና የደም ናሙና እንዲሰጡኝ እጠይቃዎታለሁ። እርስዎ የሚሰጡኝ ማንኛዉም መረጃ ሚስጥሩ የተጠበቀ ነው። የእርስዎ የደም ናሙና የላብራቶሪ ዉጤት ወባን የሚያመለክት ከሆነ አስፈላጊውን መዳሃኒት እነዲሰጥዎት እናደርጋለን።

	መለያ ኮድ-------------	ቀን……………………..
ተ. ቁ	የማህበራዊ እና በህሪነክ ጉዳዮች ቃለመ ጠይቅ	
1	ፆታ	ሀ. ወንድ ለ. ሴት
2	እድሜ	________________
3	የላብላቶሪ ውጤት	…………………………..

የመረጃ ሰብሳቢው ስም ___________ ፊርማ ___________________

የሱፐርቫይዘር ስም _____________ ፊርማ ___________________

መረጃው የተሰበሰበበት ________ ቀን___________________

Annex 3: Standard Operating Procedure for Genomic DNA Extraction from Whole Blood Using Magmax Multisample Kit on Kingfisher Flex Instrument

Purpose: To describe the primary standard operating procedure (SOP) for extraction of high yields of purified genomic DNA from whole blood using MagMax DNA multi-sample kit that is free from PCR inhibitors.

Scope: For use by MNTD laboratory staff members genomic DNA from whole blood using automated when performing DNA extractions to provide pure extractor as stated in this SOP.

Abbreviations

- ✓ **DNA**- Deoxyribonucleic Acid
- ✓ **EDTA**- Ethylenediaminetetraacetic Acid
- ✓ **MNTD**- Malaria and Neglected Tropical Disease
- ✓ **PCR**- Polymerase Chain Reaction
- ✓ **PK**- Proteinase K
- ✓ **Rnase**- Ribonuclease
- ✓ **SOP**- Standard Operating Procedure
- ✓ **QC**- Quality Control

Responsibility

Perform purified DNA extraction and quality control from pure DNA isolates for PCR and sequencing using this SOP and Communicate with Dr. Fitsum Girma (fitsum.girma@ahri.gov.et) and Lina Alemayehu (linaalemayehu1@gmail.com) for assistance with this SOP, suspected reagent issues and/or suspected instrument issues.

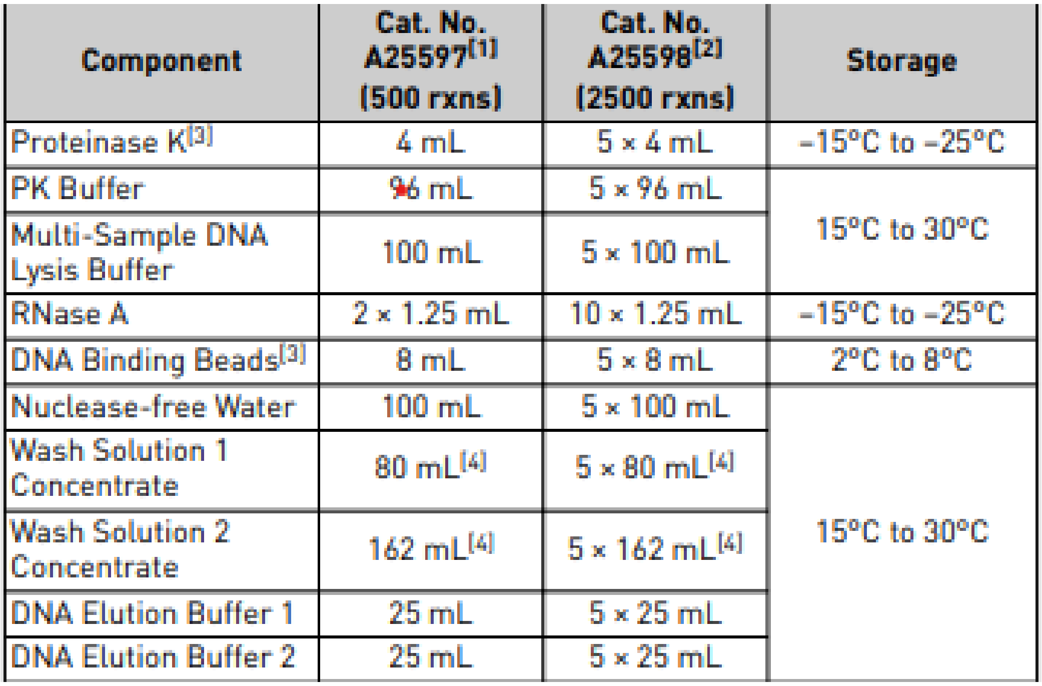
Definition

DNA extraction is a method to purify DNA by using physical and/or chemical methods from a sample separating DNA from cell membranes, proteins, and other cellular components. The use of DNA isolation technique should lead to efficient extraction with good quantity and quality of DNA, which is pure and is devoid of contaminants, such as RNA and proteins. DNA extraction involves lysing the cells and solubilizing DNA, which is followed by chemical or enzymatic methods to remove macromolecules, lipids, RNA, or proteins.

Principle

The MagMAX™ DNA Multi-Sample Ultra Kit is designed for rapid, high-throughput isolation of high-quality genomic DNA from a variety of sample matrices. The kit uses MagMAX™ magnetic bead technology, ensuring reproducible recovery of PCR-ready DNA suitable for a broad range of applications, such as SNP genotyping and copy number experiments. This protocol describes isolation of DNA from mammalian whole blood samples, optimized for use with the KingFisher™ Flex Magnetic Particle Processor (96-well deep well setting). The typical DNA yield obtained from 50 µL of whole blood is 1.5–4 µg at a concentration.

Reagents and Supplies



Component	Cat. No. A25597[1] (500 rxns)	Cat. No. A25598[2] (2500 rxns)	Storage
Proteinase K[3]	4 mL	5 × 4 mL	–15°C to –25°C
PK Buffer	96 mL	5 × 96 mL	15°C to 30°C
Multi-Sample DNA Lysis Buffer	100 mL	5 × 100 mL	
RNase A	2 × 1.25 mL	10 × 1.25 mL	–15°C to –25°C
DNA Binding Beads[3]	8 mL	5 × 8 mL	2°C to 8°C
Nuclease-free Water	100 mL	5 × 100 mL	15°C to 30°C
Wash Solution 1 Concentrate	80 mL[4]	5 × 80 mL[4]	
Wash Solution 2 Concentrate	162 mL[4]	5 × 162 mL[4]	
DNA Elution Buffer 1	25 mL	5 × 25 mL	
DNA Elution Buffer 2	25 mL	5 × 25 mL	

Required reagents which is not supplied

- Absolute Ethanol
- Isopropanol 100 %(molecular grade or higher)

Supplies
• Micro Amp clears adhesive film and Paraffin film • Autoclaved Microfuge tube (2.0mL)

Equipment

- Kingfisher Flex magnetic particle processor with 96 deep well head and 96 well heat blocks.
- Kingfisher Flex 96 Deep well plates
- Kingfisher Flex 96 Tip comb and plate
- Adjustable micropipettes
- Micro-plate shaker
- Incubator
- Vortex mixer

Sample and container type

Collect blood samples using proper venepuncture collection and handling procedures in EDTA anticoagulant tubes. Invert the tube to ensure thorough mixing. Store the samples between −20°C and −80°C.

- ❖ It’s recommended to store the samples in smaller volumes to prevent multiple freeze/thaw cycles.

Environmental and safety controls

- Do not under any circumstances use formaldehyde.
- Do not spill any alkaline onto any instrument surfaces to avoid damage of the instrument. If needed, use suitable protection covering.
- Kingfisher Flex instrument contains very strong permanent magnets. People wearing a pacemaker or metallic prostheses should not use this product. A pacemaker or prostheses may be affected or damaged if it comes in close contact with a strong magnetic field.

Procedure

- ✓ Procedure guidelines

- If the whole blood is frozen prior to use, thaw the sample at 25–37°C in a water bath until it is completely liquid, and place on ice until needed.

-Perform all steps at room temperature (20–25°C) unless otherwise noted.

- When mixing samples by pipetting up and down, avoid creating bubbles.

- Cover the plate during the incubation and shaking steps to prevent spill-over and cross-contamination. The same MicroAmp™ Clear Adhesive Film can be used throughout the procedure, unless it becomes contaminated.

- If you use a plate shaker other than the recommended shaker, verify that: – The plate fits securely on your plate shaker.

- ✓ Before first use of the kit

  - Prepare the Wash Solutions from the concentrates:

– Add 280 mL of isopropanol to MagMAX™ Wash Solution 1 Concentrate mix, and store at room temperature.

– Add 640 mL of ethanol to MagMAX™ Wash Solution 2 Concentrate mix, and store at room temperature.

Note: Before each use of the kit Pre heat the incubator to 65°C.

- ✓ Perform DNA extraction and elution

Digest samples with proteinase K

A. Prepare sufficient PK Mix according to the following table, then invert several times to thoroughly mix components. IMPORTANT! Prepare the PK Mix just before use.

Note: Do not place the PK Buffer or the PK Mix on ice, to avoid precipitation.

Component	Volume per well
Proteinase k	8ul
PK Buffer	192ul
Total PK Mix	200ul

B. Add 200 µl PK Mix to each sample well of a deep-well plate (PK Plate).

C. Transfer 50 µl of whole blood to the appropriate well containing PK Mix.

Note: Invert the tube containing the blood sample before pipetting to ensure homogenous mixing.

D. Seal the plate with a clear adhesive film, and then shake the sealed plate for 5 minutes at 550 rpm.

E. Incubate for 10 minutes at 65°C.

Note: Arrange plates in the incubator to allow adequate flow around the plate wells, to ensure that samples quickly reach and maintain the incubation temperature

- ✓ Set up the processing plate

  A. While the samples are incubating, set up the Wash, Elution, and Tip Comb Plates outside the instrument as described in the following table.

Plate ID	Plate Position	Plate Type	Reagent	Volume per well
Wash Plate 1	2	Deep well	Wash solution 1	150 µl
Wash plate 2	3	Deep well	Wash solution 2	150 µl
Wash plate 3	4	Deep well	Wash solution 2	150 µl
Elution plate	5	Standard	DNA elution buffer 1	50 µl
Tip comb	6	Deep well	Use the pre-assembled KingFisher™ Flex 96 Deep Well Tip Comb and plate.	

Note: (Optional) to prevent evaporation and contamination, cover the prepared processing plates with paraffin film until they are loaded into the instrument.

- ✓ Add Bead/RNase A Mix, Multi-Sample DNA Lysis Buffer, and isopropanol

A. Prepare sufficient Bead/RNase A Mix according to the following table.

Note: Prepare the Bead/RNase A Mix no more than 20 minutes before use. Prolonged storage at room temperature can reduce its efficiency

Vortex the DNA Binding Beads at moderate speed to form a uniform suspension before preparing the Bead/RNase A Mix.

Component	Volume per well
DNA binding mix	16ul
Rnase A	5ul
Nuclease free water	19ul
Total Bead/Rnase A mix	40ul

B.Vortex the Bead/RNase A Mix at moderate speed to ensure thorough mixing, then add 40 µL to each sample.

Note: If you see that the beads in the Bead/RNase A Mix are settling, vortex the mix again briefly before continuing to pipette.

D. Seal the PK plate with the clear adhesive film, and then shake for 5 minutes at 900-950 rpm for 5minutes.

E. Add 200 µl of Multi-Sample DNA Lysis buffer to each sample

F. Add 240 µl of isopropanol to each sample, then proceed immediately to DNA isolation.

- ✓ Process samples on the instrument

A. Select the program A25597 blood_ buccal on the instrument.

B. Start the run, remove the temporary paraffin plate seals (if present), and load the prepared processing plates to their positions when prompted by the instrument.

C. Load the PK plate (containing lysate, Bead/RNase A Mix, and isopropanol) at position 1 when prompted by the instrument.

D. When prompted by the instrument (approximately 28-30 minutes after initial start):

1. Remove the Elution Plate from the instrument.

2. Add 50 µL of DNA Elution buffer 2 to each sample well.

Note: Add DNA Elution buffer 2 immediately after the prompt, to prevent excessive drying of any beads that are still captured on the Tip Comb.

3. Load the Elution Plate back onto the instrument, and press Start.

E. At the end of the run (approximately 38 minutes after initial start), remove the Elution Plate from the instrument and seal immediately with a new MicroAmp™ Clear Adhesive Film.

- If precipitated DNA is visible, pipet up and down 5–10 times before sealing the plate, to ensure complete re-suspension.

- (Optional) Eluates can be transferred to a storage plate after collection.

- If excess bead residue is seen in the wells, place the Elution Plate on the 24-Well Magnetic Separator to capture any residue prior to downstream use of the DNA.

Note: Do not allow the purified samples to sit uncovered at room temperature for more than 10 minutes, to prevent evaporation and contamination.

The purified samples are ready for immediate use. Alternatively, store the covered Elution Plate:

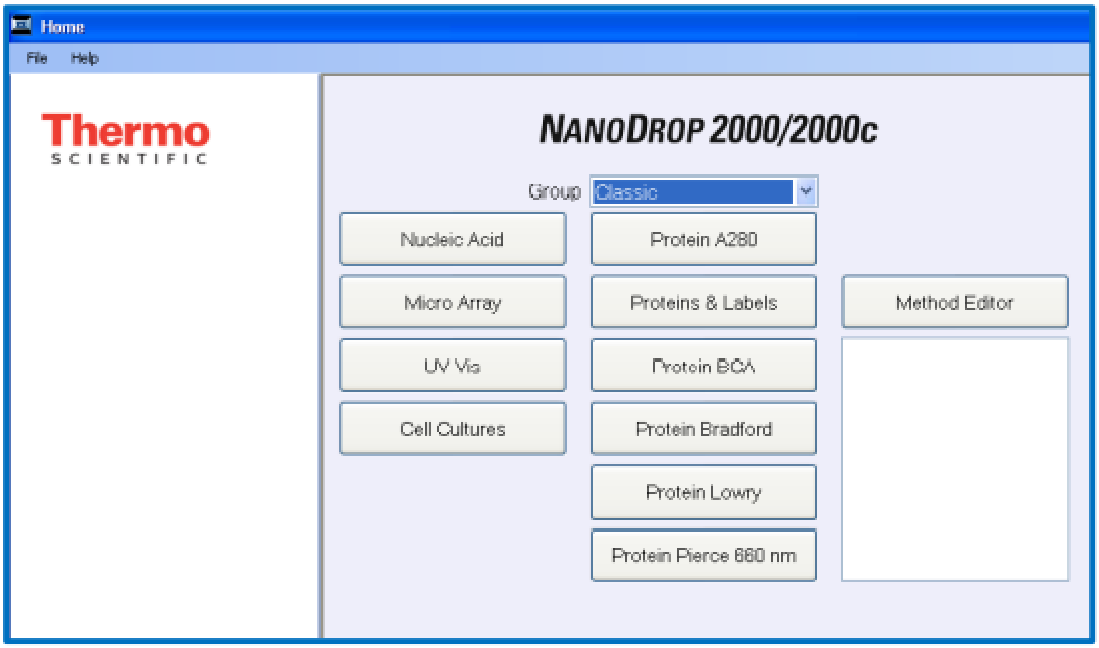
- At 2–8°C for up to 24 hours.
- At –20°C or –80°C for long-term storage.

Quality Control

Determination of DNA quality using the Nanodrop 2000 Spectrophotometer

A. Select Nanodrop 2000 icon on the desktop of the Nanodrop workstation.

B. Select appropriate application from the screen, in this case “nucleic acid”, and follow prompts to begin initialization.



Group Classic

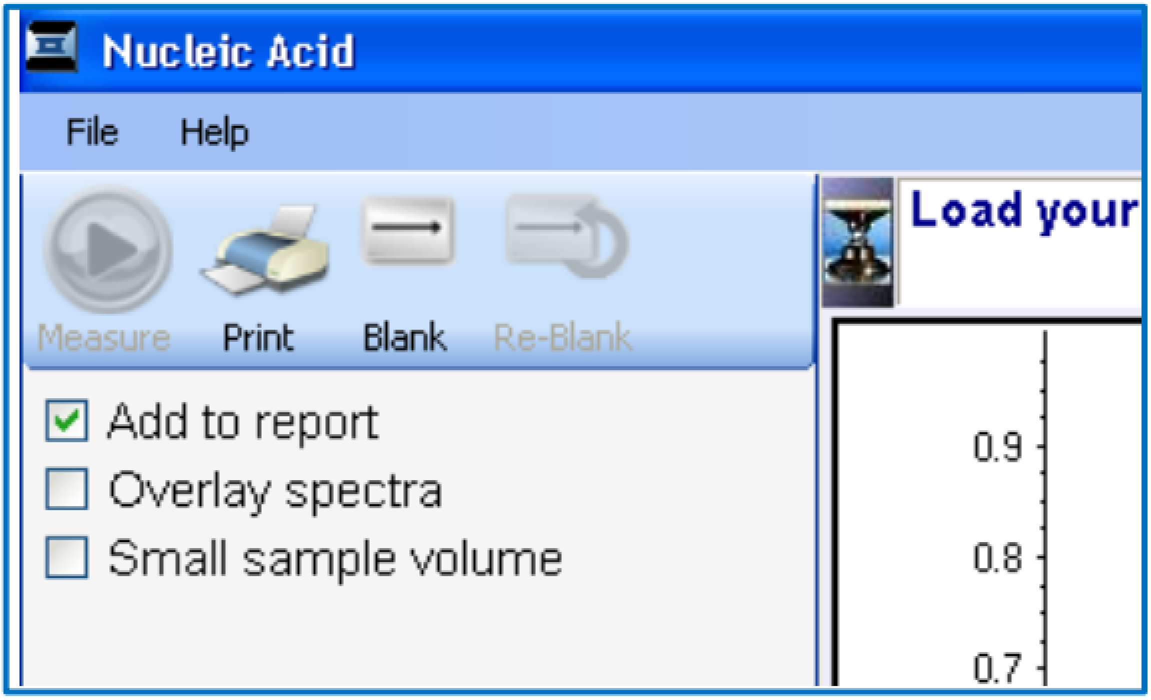
When asked if you would like to open up your last workbook, choose “yes” if you would like to append results to previously saved data, or choose “no” if you would like to begin a new workbook.

Note: The arm of the Nanodrop must be lowered during initialization.

C. Lift the arm of the Nanodrop and use a lint-free laboratory wipe dampened with distilled H2O to wipe down the top and bottom pedestals.

D. Pipette 1-2 µl of blanking solution (e.g. 10 mM Tris-HCl pH 8.0) onto the bottom pedestal and lower the arm after loading. Ensure that "Add to report" is selected in the upper left quadrant of the screen so that measurements are saved, and click the "Blank" button on the screen (upper left).

Note: Ensure that there are no bubbles in the sample on the stand so that an accurate read is obtained.



☑ Add to report
☐ Overlay spectra
☐ Small sample volume

E. After the instrument has read the blank, lift the arm and wipe both pedestal and arm with a dry laboratory wipe to ready the Nanodrop for measuring samples

F. Pipette 1-2 µl of undiluted DNA sample onto the bottom pedestal and lower the arm.

G. Enter the sample name in the "Sample ID" field (upper right) and click "Measure" (upper left). When the measurement has been taken, results will populate the screen.

H. A prompt will appear after the first sample is measured: follow the prompt to save the workbook. The workbook must be saved before continuing.

I. Wipe both pedestals clean with a lint-free wipe dampened with deionized water and repeat steps F-H for all samples.

J. After all samples have been measured, click on “Reports” icon in the lower left quadrant of the screen to print and/or export data if desired.

K. Record the data by either electronic export as described below or by manual recording.

- Click on the “Reports” icon in the lower left quadrant of the screen.
- Select “Export”.
- Save the NanoDrop workbook to the appropriate network space and send to your email address.
- Transfer the data into the appropriate log sheet.
- Readings for 260/280 fields should be between 1.75 and 2.05 (this includes a 0.05 or 3% error rate) for the extracted DNA to be accepted as ‘pure’ and suitable for WGS. If the ratio is lower than 1.75, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

Calculation: N/A

Result Reporting and Interpretation:

After successful extraction of DNA from the whole blood using the MagMax Multi sample kit it is expected to gain 1.5-4 µg of DNA.

Appendix 4: Procedure for Detection and Quantification of Malaria Parasites using Real-time qPCR (Bio-Rad CFX96 Deep Well Touch Real-Time System)

Abbreviations:

MM: Master Mix
µL: Microliter
NTC: Non-template control
N/A: Not Applicable

Pf: *Plasmodium falciparum*

Pv: *Plasmodium vivax*

qPCR: Real-Time Polymerase Chain Reaction.

SOP: Standard Operating Procedure

Principle:

Real-Time Polymerase Chain Reaction (qPCR) is a technique that monitors the progress of PCR reaction in real-time. It is based on the detection of the fluorescence produced by a reporter molecule. There are many different fluorescence markers (fluorophore-containing DNA probes) used in qPCR but one of the most common is TaqMan probe. It is a hydrolysis probe which bear a reporter dye, often fluorescein (FAM) at its 5’ end and a quencher tetramethylrhodamine (TAMRA), attached to the 3’ end of the oligonucleotide. As the taq-polymerase start to synthesize new DNA strand in the extension stage, it causes degradation of the probe by 5’ end nuclease activity and the fluorescein is separated from the quencher as a result of which fluorescence signal is generated. As this procedure continues, in each cycle the number of signal molecule increases, causing the increase in fluorescence which is positively related with the amplification of the target.

In general, Bio-Rad’s CFX Dx real-time PCR amplification systems allow PCR quantification with standard curve, gene expression analysis, allelic discrimination, and end-point analysis.

Reagents and Supplies:

Reagents
70% alcohol
DNA away solution
PCR master mix reagents (TaqMan MM 2x buffer, 18S forward primers, 18S reverse primers and probes)
PCR water
Pv18S serial dilutions (plasmids)

Supplies and materials
• M-Tork • Fine tip markers • Gloves • Pipette tips (10µL, 20µL, 100µL, 200µL, 1000/1250µL) • PCR plate • PCR tubes • Plate sealer (Microseal® ‘B’ PCR Plate Sealing Film, Bio-Rad) • 2 mL Eppendorf tubes

Equipment:

- Rack for holding the Eppendorf and PCR tubes
- Box (Bowel) to remove used tips
- Dispenser
- Pipettes (10µL, 20µL, 100µL, 200µL, 1000µL)
- Biosafety cabinet

- PCR plate spinner
- Tube spinner
- Ice maker machine
- CFX96 Touch™ Real-Time PCR Detection System

Procedure:

Step	Action
1.	Take the master mix box to the master mix room.
2.	Turn on the master mix safety hood and also put on the light.
3.	Clean the master mix cabinet by using 70% ethanol followed by wiping away with M-tork.
4.	Also clean all pipettes, tip boxes and rack by 70% ethanol. Use "DNA away" to clean pipettes in the MM room if we use them in other rooms.
5.	Take out the master mix reagents from the fridge except the probes, then leave them and put on the rack to thaw for some time. **Note:** Withdraw the probes from the fridge after the other components are mixed.
6.	In between MM preparation activities, take out the extracted samples to be tested and serial dilutions/plasmids from -20^{o}C and put them on the sample room to thaw.
7.	Ready a 2 mL Eppendorf tube to mix all master mix components.
8.	According to their order, add TaqMan MM 2x (buffer), *Plasmodium vivax* FW and RV primers, nuclease free water (PCR-water, or MQ, or ddH2O) into the labeled tube. **Note:** While adding the components, mix them using the pipette by sucking up and down manner. Also, care has to be taken to not create bubbles. Switch off the light when we use the probe as its light sensitive.
9.	Return all the primer and probe aliquots to the fridge.
10.	Dispense the prepared MM (15µL in each well) into the PCR plate wells considering the duplicate plasmids, negative controls (MQ/NTCs) and number of target samples (unknown).
11.	Switch off the light in the sample room as our mix contains probe that is light sensitive.
12.	Dispense 5µL of the duplicate plasmids (standards), samples and MQs into the dispensed master-mix as of the plate layout.
13.	After pipetting our PCR samples align the adhesive film (the Bio-Rad microseal® 'B' seals) to the plate so that all wells are covered tightly.
14.	Spin the plate in the PCR spinner to remove all bubbles.

15. Turn on the computer and then turn on the BIO-RAD by the switch at the back of the machine.

16. On the desktop of the computer, double click on the icon “Bio-Rad CFX Maestro” software.

17. Click on the “User-defined” run type button in the main software toolbar.

18. From the window that appears click on “Select Existing”, on Local Disc D then select “18S TaqMan”.

19. Check /adjust the real time PCR machine thermal cycler conditions on the Protocol window as follows:

Thermo cycler conditions		
Temp	**Time**	**Cycle**
50^{o}C	2:00	1
95^{o}C	10:00	1
95^{o}C	0:15	45X
60^{o}C	1:00	

20. Click on “Next” and in the window that appears click on “Edit selected”. Insert all the information and indicate which wells are containing the standards, non-templates and unknown samples followed by selecting the fluorophores,

21. Click on the “Open lid” button located on the software’s “Start Run” tab to open the motorized lid.

22. Load samples in the “block” of the CFX96 Deep Well system, and then click on “Close lid” to close the motorized lid.

23. Select our target gene as “18S”.

24. Start the reaction by clicking on “Start Run”, after filling all the required information for the reaction.

25. Once cycling is completed, check our results by adjusting the baseline thresholds.

26. Export the result to excel and save the excel workbook.

27. Remove the samples from the block and close the system.

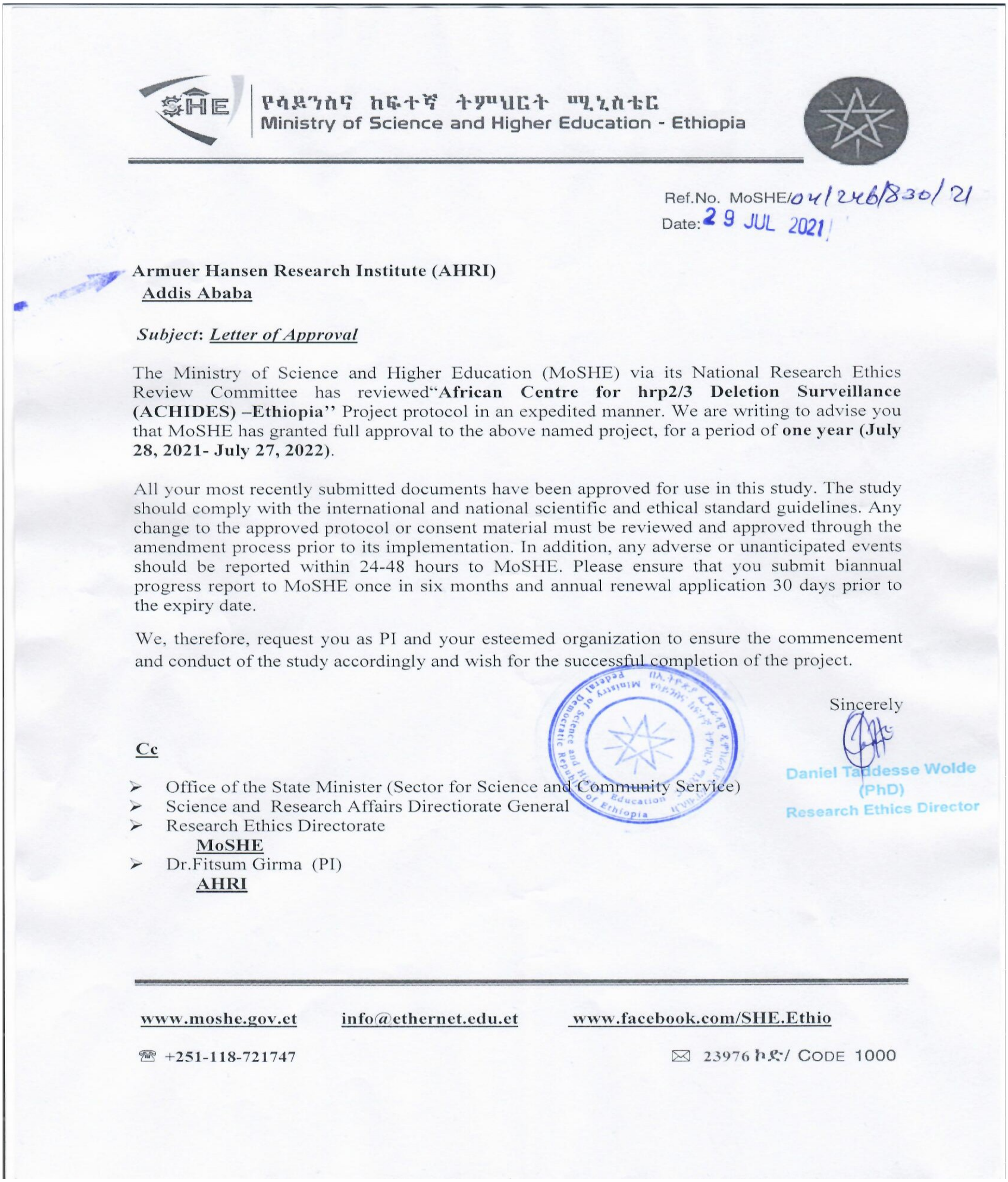
28. Press the power switch on the back of the C1000 thermal cycler to power it down.

Result Reporting and Interpretation:

A successful real-time PCR experiment will have the following characteristics:

- Curves are all S-shaped
- Dilution series has expected spacing
- Replicates are tightly clustered
- Baselines are relatively flat
- Plateau height doesn’t matter
- Curves are smooth
- Melt curve has one peak per product
- No signal for NTC (non-template control).

In general, slopes between -3.1 and -3.6, giving PCR efficiencies between 90 and 110%, are typically acceptable.



SHE
የሳይንስና ከፍተኛ ትምህርት ሚኒስቴር
Ministry of Science and Higher Education - Ethiopia

Ref.No. MoSHE/04/246/830/21
Date: 29 JUL 2021

Armuer Hansen Research Institute (AHRI)
Addis Ababa

Subject*: *Letter of Approval

The Ministry of Science and Higher Education (MoSHE) via its National Research Ethics Review Committee has reviewed"**African Centre for hrp2/3 Deletion Surveillance (ACHIDES) –Ethiopia''** Project protocol in an expedited manner. We are writing to advise you that MoSHE has granted full approval to the above named project, for a period of **one year (July 28, 2021- July 27, 2022)**.

All your most recently submitted documents have been approved for use in this study. The study should comply with the international and national scientific and ethical standard guidelines. Any change to the approved protocol or consent material must be reviewed and approved through the amendment process prior to its implementation. In addition, any adverse or unanticipated events should be reported within 24-48 hours to MoSHE. Please ensure that you submit biannual progress report to MoSHE once in six months and annual renewal application 30 days prior to the expiry date.

We, therefore, request you as PI and your esteemed organization to ensure the commencement and conduct of the study accordingly and wish for the successful completion of the project.

Sincerely

Daniel Taddesse Wolde
(PhD)
Research Ethics Director

Cc

- Office of the State Minister (Sector for Science and Community Service)
- Science and Research Affairs Directorate General
- Research Ethics Directorate
  MoSHE
- Dr.Fitsum Girma (PI)
  AHRI

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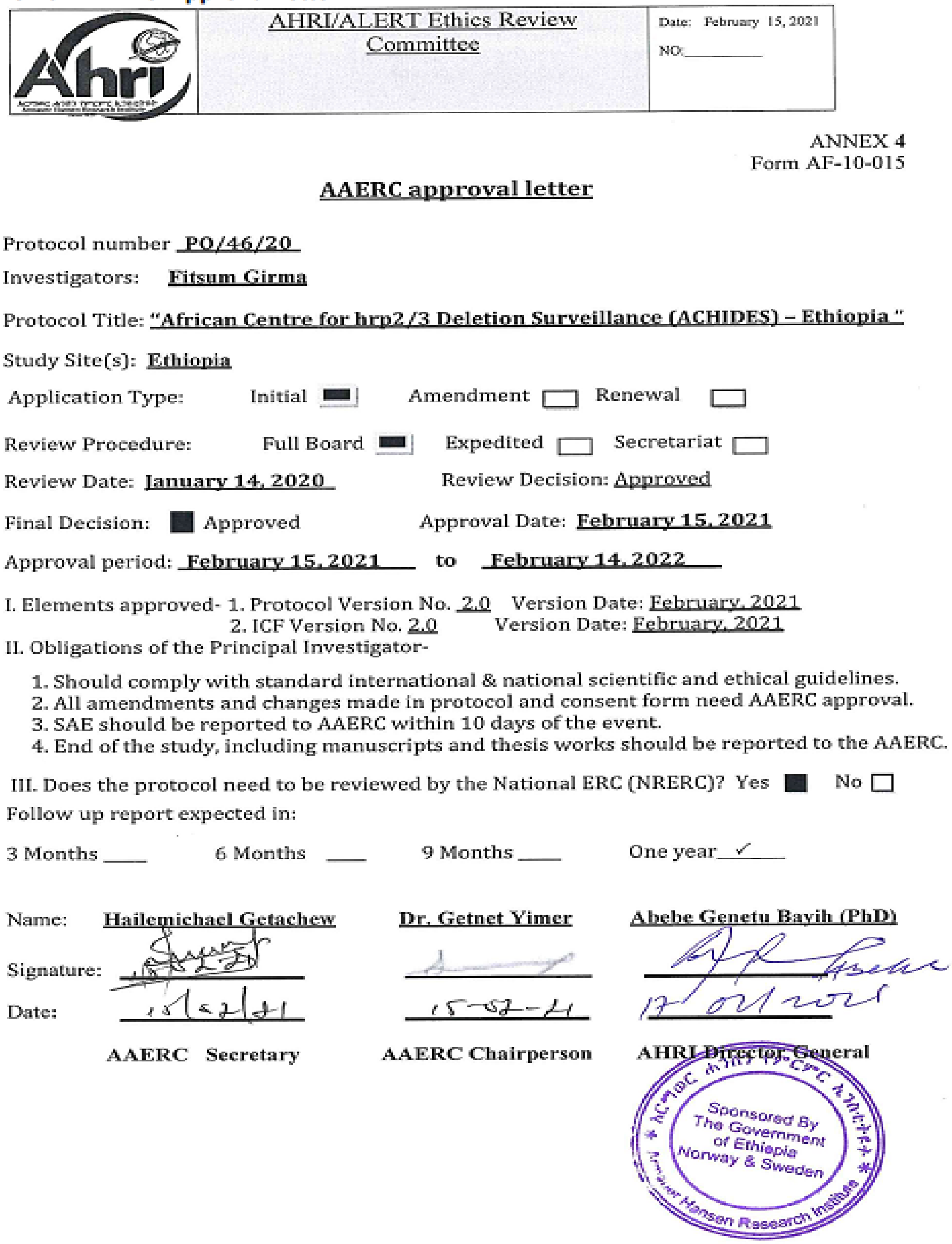
8.10. AAERC Approval letter



	AHRI/ALERT Ethics Review Committee	Date: February 15, 2021 NO:__________

ANNEX 4
Form AF-10-015

AAERC approval letter

Protocol number **PO/46/20**

Investigators: **Fitsum Girma**

Protocol Title: **"African Centre for hrp2/3 Deletion Surveillance (ACHIDES) – Ethiopia "**

Study Site(s): **Ethiopia**

Application Type: Initial ■ Amendment ☐ Renewal ☐

Review Procedure: Full Board ■ Expedited ☐ Secretariat ☐

Review Date: **January 14, 2020** Review Decision: Approved

Final Decision: ■ Approved Approval Date: **February 15, 2021**

Approval period: **February 15, 2021** to **February 14, 2022**

I. Elements approved- 1. Protocol Version No. 2.0 Version Date: February, 2021
2. ICF Version No. 2.0 Version Date: February, 2021

II. Obligations of the Principal Investigator-

1. Should comply with standard international & national scientific and ethical guidelines.
2. All amendments and changes made in protocol and consent form need AAERC approval.
3. SAE should be reported to AAERC within 10 days of the event.
4. End of the study, including manuscripts and thesis works should be reported to the AAERC.

III. Does the protocol need to be reviewed by the National ERC (NRERC)? Yes ■ No ☐

Follow up report expected in:

3 Months ____ 6 Months ____ 9 Months ____ One year ✓

Name:	**Hailemichael Getachew**	**Dr. Getnet Yimer**	**Abebe Genetu Bayih (PhD)**
Signature:			
Date:	15/02/21	15-02-21	17 02/2021
	AAERC Secretary	**AAERC Chairperson**	**AHRI Director General**