



CLONING AND EXPRESSION OF *Plasmodium falciparum* LACTATE DEHYDROGENASE (P_fLDH) IN *Escherichia coli* BL21(DE3)

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ABSTRACT

Background: Immediate and accurate diagnosis of malaria is essential for effective control of this disease. Immunochromatographic based rapid diagnostic tests (RDTs) are economical, simple to perform, and provide results in a relative short time, can be useful to assist effective management of malaria. The commercially available malaria RDT in Indonesia is still imported. Therefore, an effort to produce malaria RDT independently is necessary. One of the biomarkers used in RDTs is *Plasmodium* lactate dehydrogenase pLDH. The production and accumulation of pLDH during asexual stage or blood-stage in all human infected malaria parasites can be used to indicate parasites viability, which is correlated with the number of parasites present in the plasma of infected patients.

Objective: The aim of this research is to produce recombinant P_fLDH in *Escherichia coli* BL21(DE3).

Methods: P_fLDH gene was cloned into pET30a expression vector to obtain a 6.2 kbp recombinant plasmid pET30a-P_fLDH. *E. coli* BL21(DE3) was transformed with pET30a-P_fLDH using the heat shock method. Then, *E. coli* BL21(DE3)- pET30a-P_fLDH was cultured in LB broth containing 50 mg/mL kanamycin and was induced by 1mM IPTG at 37°C.

Results: SDS-PAGE and Western Blot analysis showed that recombinant P_fLDH was expressed with molecular mass ~30 kDa.

Conclusion: Recombinant P_fLDH is expressed in *E. coli* BL21(DE3) and can be used in further research for producing rP_fLDH as a biomarker for malaria RDT development.

Keywords : Lactate dehydrogenase, Malaria Rapid Diagnostic Test (RDT), *Plasmodium falciparum*, Recombinant protein

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INTRODUCTION

Malaria is a life threatening infectious disease, especially in tropical and subtropical areas. According to the World Malaria Report 2019, there were 1,474,636 malaria cases reported in Indonesia in 2018.[1] Malaria is caused by the protozoan parasite from the genus *Plasmodium*. There are five species of *Plasmodium* which infect humans; *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*.[2]

The causative agent of malaria are transmitted from one infected host to another by the bite of female *Anopheles* mosquitoes. The symptoms of the disease include high fever followed by chills and rigors. In endemic area, people with high fever sometimes were given antimalarial drugs without proper diagnosis, which could lead to antimalarial resistance. To avoid this, WHO recommend that proper diagnosis to all malaria suspected patients should be given before administering drugs.[2]

To accommodate this need, it is necessary to develop techniques for malaria diagnosis, which is fast, economical and accurate. One of the diagnostic test that fit those requirements is Immunochromatographic based rapid diagnostic tests (RDTs). It can detect *Plasmodium* antigens in whole blood samples, is inexpensive, simple to perform, and provide results in a short time. A biomarker that can be used for RDT development is *Plasmodium* lactate dehydrogenase. Parasite lactate dehydrogenase (pLDH) is an enzyme involved in the terminal phase of the Embden–Meyerhof pathway (glycolysis) of the malaria parasite.[3] Production and accumulation of pLDH can be used to indicate parasite viability both *in vivo* and *in vitro* [4] Moreover, pLDH was one of

the first plasmodial enzymes shown to be electrophoretically, immunologically and kinetically distinct from that of the host.[5]

Those characteristics of pLDH are suitable for biomarker. There are already several commercial malaria RDTs using pLDH, unfortunately all are imported. The aim of this research is to clone the gene encoding *Pf*LDH into the expression vector pET30a, as well as to express recombinant *Pf*LDH in *E. Coli* BL21(DE3). To confirm recombinant *Pf*LDH, SDS-PAGE and western blot are applied. This research is expected to contribute for malaria case management, surveillance, and elimination in Indonesian and as a first step to make local malaria Rapid Diagnostic Test.

MATERIAL AND METHODS

Isolation of *Plasmodium falciparum* Genomic DNA and Amplification of the *P. falciparum* LDH Gene

Genomic DNA was obtained from *P. falciparum* infected blood samples kindly provided by Balai Penelitian dan Pengembangan Kesehatan, Kementerian Kesehatan Jayapura, Indonesia (Ethical Clearance Nr 445/658/RSUD/2018). Genomic DNA was isolated from the blood samples according to the procedure described in Zymo Research Kit for Genomic DNA Isolation from Blood Samples. The *Pf*LDH gene was amplified by the Polymerase Chain Reaction (PCR) method using genomic DNA of *P. falciparum* as template. A pair of oligonucleotide primers were designed to amplify *Pf*LDH gene based on sequence of *P. falciparum* 3D7 *L-lactate dehydrogenase*, Pf3D7_1324900. The forward primer was *Pf*LDH-F (*Nco*I), ccatggATGGCACCAAAAAGCAAAAA and reverse primer was *Pf*LDH-R (*Xho*I), ctcgagAGCTAATGCCTTCATTCTCT.

Recombinant Plasmid Construction

The amplified *PfLDH* gene was cloned into pGEM-T easy resulted in recombinant plasmid, pGEM-T-*PfLDH* and was sequenced by Macrogen Inc. (Korea). Thereafter, pGEM-T-*PfLDH* and expression vector pET30a were digested with *NcoI* and *XhoI*. The digested *PfLDH* fragment was then ligated to the digested pET-30a using T4-DNA Ligase to produce recombinant plasmid, designated pET30a-*PfLDH*, where *PfLDH* gene was tagged with 6xHis at C terminal site. The pET30a-*PfLDH* was then sequenced by Macrogen Inc (Korea).

Transformation of *Escherichia coli* BL21 (DE3) and Recombinant *PfLDH* Expression

Fresh *E. coli* BL21 (DE3) competent cells were made using CaCl₂ treatment method[6] and transformation of *E. coli* BL21(DE3) was performed by heat shock method. The screening of the resultant colonies was carried out by direct PCR colonies methods using insert specific primers. Expression of r*PfLDH* was started by making overnight culture of positive colony. This culture was then transferred (1% v/v) into LB broth containing 50 g/mL kanamycin and incubated in 37°C, 150 rpm for about 2 hours until OD₆₀₀ reached 0.6. Bacterial culture was induced by adding 1mM IPTG and further incubated in 37°C, 150 rpm for 3 hours. Centrifugation at 2,800 g, 4 °C was performed to harvest the culture.

Western Blot

The cell culture pellet was dissolved in sample buffer and boiled for 10 minutes before loading. SDS-PAGE was conducted as described by Schägger.[7] The gel was stained by Coomassie blue staining, except the gels for further analysis with Western blot. Protein from the SDS-PAGE gel was

transferred to nitrocellulose membrane using the eBlot Protein Transfer System (GenScript, USA). The membrane was blocked by 5% (w/v) skimmed milk in TBST over night at room temperature, then washed three times with 10 mL TBST. The membrane was further incubated with Mouse 6x his antibody for one hour at room temperature, then washed three times with 10 mL TBST. The membrane was then incubated with anti-mouse-HRP-conjugates antibody for 1 hour at room temperature, washed three times with 10 mL TBST, then 5 mL ChromoSensor™ One solution TMB substrate (GenScript, USA) was added, shaken at low speed at room temperature until color formed.

RESULTS

The Recombinant Plasmid Construction and Transformation of *Escherichia coli* BL21 (DE3)

The *PfLDH* gene was amplified as a 961 bp DNA fragment (**Figure 1**). As positive control, specific primers for amplification of IEBA175 RIII-V (1502 bp) were used to confirm that the genomic DNA used is still in good condition. The amplified 951 bp *PfLDH* gene was first cloned into pGEMT easy cloning vector resulting in pGEMT-*PfLDH*. Plasmid DNA was isolated and verified by sequencing. Sequence comparison with other available *PfLDH* sequences in GenBank confirmed its identity (100%).

Thereafter, *PfLDH* gene was inserted in-frame with initiator codon by cloning site *NcoI* and *XhoI* and C-terminal 6x His tag encoding sequence of bacterial expression vector pET30a under the control of inducible phage T7 promotor. The expression vector pET30a-*PfLDH* is depicted in **Figure 2A**. The construct is predicted to encode recombinant *PfLDH* with molecular weight of approximately 35.2 kDa.

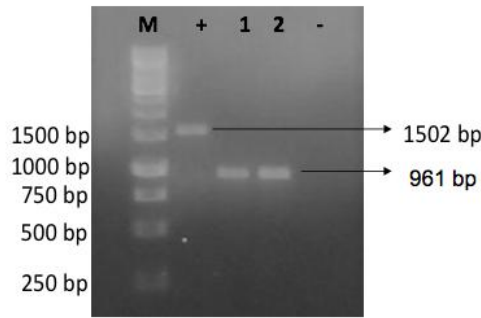


Figure 1. Amplification of *Plasmodium falciparum* Lactate Dehydrogenase (*PflDH*) was shown in lane 1 and 2 as DNA fragment (960 bp). Control positive (+) and control negative (-) was shown as well.

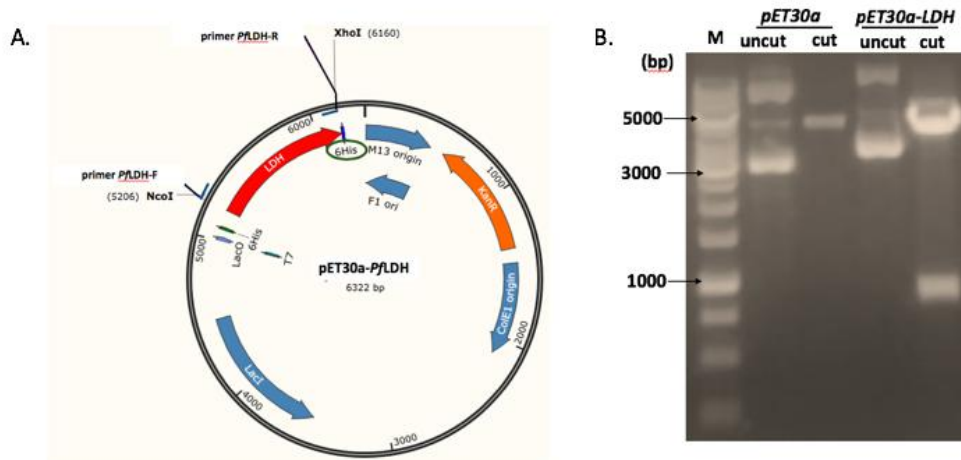


Figure 2. (A) Schematic representation of recombinant plasmid pET30a-*PflDH* construct carrying *PflDH* gene with 6x His tag at its C-terminus. The figure was created using Snapgene Software[11] (B) Restriction enzyme analysis of pET30a-*PflDH* recombinant plasmid using *NcoI* and *XhoI* enzymes with its uncut control.

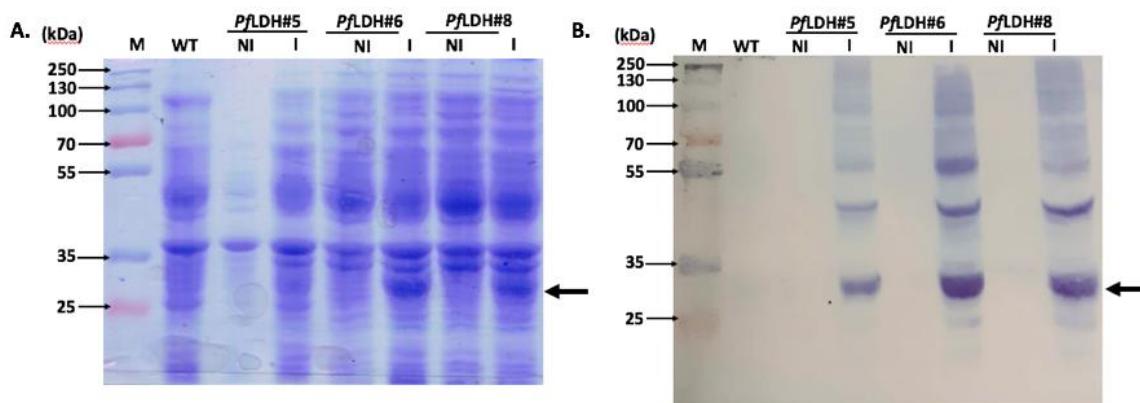


Figure 3. (A) SDS PAGE analysis of r*PflDH* expressed in *E. coli* BL21(DE3) and (B) Western Blot with anti-His antibodies. WT indicates wild type *E. coli* BL21(DE3) whole cell lysate as negative control, NI indicates uninduced whole cell lysate; and I indicates induced whole cell lysate. Three positive colonies were analysed namely *PflDH*#5, *PflDH*#6 and *PflDH*#8. The arrows at the right indicate the position of r*PflDH* (34 kDa). The positions of molecular weight markers (M) are indicated on the left of the gel.

The recombinant protein was supposed to carry 6 additional histidin residues encoded by pET30a at its C-terminus. This vector was then transformed into the *E. coli* BL21(DE3) and transformants were selected in the presence of kanamycin. Ten randomly picked colonies were then confirmed using PCR. From the PCR positive colony, recombinant plasmid pET30a-*PfLDH* were isolated and were verified using restriction enzyme analysis (**Figure 2B**).

Recombinant *PfLDH* Expression in *Escherichia coli* BL21 (DE3)

Cultures of three positive clones, designated clone 5, 6 and 8, which were previously selected on the basis of PCR screening, were subjected to IPTG induction at 37 °C to identify clones which expressed the predicted 35.2 kDa *rPfLDH*. SDS-PAGE analysis results shown in **Figure 3A** represent the induction profile of those clones. From the figure it is indicated that IPTG induction of *PfLDH* gene expression was accompanied by the appearance of approximately a 33.4 kDa band. In contrast to induced transformed cells, untransformed cells (WT) cells failed to express the 33.4 kDa protein after induction with IPTG. As expected, uninduced transformed cells also failed to express the 33.4 kDa protein. To confirm that the 33.4 kDa protein is indeed *PfLDH*, western blot with anti-His antibodies was performed. From the three clones identified, clone 6 showed the highest level of protein expression. This clone will be used for further research.

Western Blot

The molecular mass of *rPfLDH* in crude extract and also the identity of *rPfLDH* was confirmed by Western blot analysis using anti-His antibodies. Although unspecific binding occurred, there appeared a single band of protein around ~30 kDa in samples that were

derived from positive clones 5, 9 and 17 (**Figure 3B**). The same band of protein did not appear in the sample that was derived from non-induced and WT.

DISCUSSION

PfLDH is widely used as a biomarker for the identification of *P. falciparum* in Immunochromatography based Rapid Diagnostic Test. [8,9] In this research, recombinant *PfLDH* was produced in three positive clones of *E. coli* BL21(DE3)- pET30a-*PfLDH*. [5,6,8] In SDS-PAGE analysis, *rPfLDH* is predicted as an approximately 30kDa protein (**Figure 3A**). This prediction is also supported by western blot analysis, where induced cells showed reactivity with anti-His antibodies at a protein fragment with molecular weight approximately 30kDa. Whereas, the estimated molecular weight of *rPfLDH* is 35.4 kDa. Previous study [10] showed that there is possibility the sequence starts at an internal methionine residue (19th residue of *PfLDH*). *rPfLDH* expressed from 19th residue will have a molecular weight of 33.4 kDa, which is detected by SDS-PAGE and Western Blot. From both analyses, it could be concluded that *rPfLDH* produced in this study is in uncomplete/truncated form. However, the expressed protein still has His-tag for purification purpose.

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