

## Cloning and Sequencing cDNA Encoding for Rhopty-2 *Toxoplasma Gondii* Tachyzoite Local Isolate

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### Abstract

*Rhopty* protein belongs to an excretory and secretory antigens (ESAs) that play an important role during active penetration of parasite into the cell target. This protein an able *Toxoplasma gondii* to actively penetrate targeted cell, meanwhile ESAs protein stimulates intracellular vacuole modification. It is, therefore, after the parasite successfully enter the cell target then Granule (GRA) proteins are responsible for the formation of parasitophorus vacuole, which is protect the fusion with other intracellular compartments such as lysosomal vacuole. Consequently, this parasite is being able to survive and multiply at the cell target. The current study was aimed to clone and sequens cDNA encoding for ROP-2 of local isolated *T. gondii* tachyzoite through DNA recombinant technique. Total ribonucleic acid (RNA) was isolated from tachyzoites of local isolated *T. gondii* that were grown up in *Balb/c* mice. Messenger RNA was isolated from total RNA using *PolyAtract mRNA Isolation System*. Messenger RNA was used as a template for synthesis cDNA using *Riboclone cDNA Synthesis System AMV-RT*. *EcoRI* adaptor from *Riboclone EcoRI Adaptor Ligation System* was added to Complementary DNA and than ligated to pUC19. Recombinant plasmid was transformed into *E. coli* (XL1-Blue). The transformed *E. coli* XL-1 Blue were plated on LB agar containing X-Gal, IPTG and ampicillin. Recombinant clones (white colony) were picked up and grown up in the LB medium at 37°C overnight. Expression of recombinant protein was analysed by immunoblotting in order to identify cDNA recombinant wich is express ESA of *T. gondii* local isolate. Recombinant plasmid were isolated using *alkalilysis* method and were elektroforated in 1% agarose gel. The isolated DNA recombinant plasmid was cut using *Eco RI* and then sequenced through *Big Dye Terminator Mix AB1 377A Sequencer* using *M13 Forward* and *M13 Reverse primers*. The conclusion of this results showed that the recombinant clone was coding for excretory and secretory protein which has molecular weight of 54 kDa. The DNA alignments of sequence from the cloned gene showed 97% homology with gene encoding for ROP-2 of *T. gondii* RH isolate.

**Keywords:** *Toxoplasma gondii*, tachyzoite, ESA, complementary DNA, ROP2

### Backgrounds

*Toxoplasma gondii* is an intracellular parasite, infect all warm blooded animals including human. This parasite is belonging to an opportunistic pathogen and considered as a master of mind of toxoplasmosis (Dutta *et al.*, 2000). Toxoplasmosis is one of an

extensively diseases spread worldwide in mammals. However the disease prevalence is vary based on geographic and climate condition. In general, an infected mammal with good immune system does not develop any symptoms. When the immune system is adversely weakened, the symptoms are likely to be manifested such as encephalitis and ocular lesion (Yamamoto *et al.*, 2000; Martin *et al.*, 2000). The most excellent step to decrease prevention risk hit toxoplasmosis compared to medication (Wisnuwardhani,

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1990). Mohammed *et al.* (1994) suggest that in order to decrease the risk of toxoplasmosis at foetus is by vaccination before pregnancy.

The vaccine of diluted tachyzoites *T. gondii* (*live attenuated*) was reported can prevent abortus in an animal and the sheep have been already used commercially (Alexander *et al.*, 1996). The constraint of toxoplasmosis vaccines by using intact parasite among others is treatment of tachyzoites which is not peaceful expensive and also felt concerned about to become virulen strain, especially in human which is having trouble at their immune system. The progress of a toxoplasma vaccines for human being shall pursuant in the direction of usage of recombinant antigens or a synthetic peptide which perfectly given protection for hospes from all life cycle phase *T. gondii* (Prigione *et al.*, 2000).

*Toxoplasma gondii* is a Protozoan parasite, living obligate in the any type of nucleated cell (Sibley *et al.*, 1995). Tachyzoite has secretory bodies. The main immunogenic proteins of such secretory bodies are surface antigen (SAG), granulocyte antigen (GRA) and rhoptry antigen (ROP) (Pfrepper *et al.*, 2000; Nischick *et al.*, 2000; Guanjin *et al.*, 2001; Reichmann *et al.*, 2000; Martin *et al.*, 2000). Those three proteins act as a facilitator of host cell infection, invasion and parasitophorus development (Ajioka, 2001).

The antigen especially SAG and ESA has a direct relationship with immunopathogenesis and cell invasion. Surface antigens is related with attachment process into membrane of infected cell. While ESA plays an important role in active penetration process and modification of parasitophorus vacuole in the cell targeted. Each of antigens is consisted of several antigen proteins in which respective protein has different functions.

Cesbron-Delauw *et al.* (1996) found a given ESA antigen with Incomplete Freud Adjuvant (IFA) was able to protect mice from

oral infection in lethal dose of *T. gondii* cyst (survival rate was 50-60%). Fischer *et al.* (1998) and Jacobs *et al.* (1999) conducted a 29 kDa dense granule antigen (GRA7) related study, while Vercammen *et al.* (2000) investigated a 23kDa dense granule antigen (GRA1), GRA7, 54kDa rhoptry antigen (ROP2) as well. ESA protein has a higher complexity compared to SAG based on protein variation as pathogenesis factor. Penetration of *Toxoplasma* into targeted cells give a response of releasing a certain ESA protein. In addition, ESA protein causes parasitophorus vacuole to be unable to fuse with other intracellular compartment such as lisosomal vacuole (intracellular vacuole modification). Consequently, *T. gondii* tachyzoites are able to survive and freely multiply in the infected cell.

The effective and efficient vaccines at the moment are required, therefore DNA recombinant technology is expected can solve internal issue of ESAs which are immunogenic enough for that purposes. DNA recombinant can also given a standing by solution of DNA vaccine or probe for toxoplasmosis.

The current study is aimed to clone and to identify cDNA encoding ESA of local isolated *T. gondii* tachyzoites through DNA recombinant technique.

## Materials and Methods

### Excretory- secretory antigens (ESA) isolation from tachyzoit of *Toxoplasma gondii*

Excretory-secretory antigens were obtained from local isolated *T. gondii*, by cultured the tachyzoites ( $10^9$  tachyzoites / ml) in RPMI 1640 medium supplemented by 10% FCS. The suspension of tachyzoites was incubated in 37°C for 3 h by continuously agitation. Following the incubation, the suspension of tachyzoites was centrifuge on 3500 rpm, 4°C, for 10

min and the obtained supernatant was ESA protein. ESA protein was identified by SDS-PAGE and stained using 0.2 % coomassie brilliant blue R-250.

### **Production of polyclonal antibody against ESA protein**

The ESA protein was used as an antigen to induce polyclonal antibody production. Five female mice (BALB/c strain), age 8-10 weeks, each was immunized by intraperitoneal routes using 5 µg of ESAs protein in the solution containing of phosphate buffered saline and IFA (incomplete freund adjuvant) in equally volume (1:1) Immunization was repeated 5 times with two weeks interval. The obtained serum (polyclonal antibody against ESA protein) was used for screening of recombinant ESA protein.

### **Total RNA Isolation**

Total RNA isolated from tachyzoites *T. gondii* local isolate using Total RNA isolation kit (Promega) as described by manufacturer. Messenger RNA was isolated from total RNA using *PolyAtract mRNA Isolation System* (Promega). The procedures consist of *annealing probe*, washing SA-PMPs, capture of annealing oligo (dT)-mRNA hybrids and mRNA elution.

### **The cDNA synthesis**

Messenger RNA was used as a template for cDNA synthesis using *Riboclone cDNA Synthesis System AMV-RT* (Promega). *EcoRI* adaptor from *Riboclone@EcoR I adaptor Ligation System* was linked to the phosphorylated cDNA and the adaptors excess was removed using *Sephacryl S-400*.

### **Ligation of cDNA into expression vector.**

One microliter pUC19 (200 ng/µl) was added with 2 µl *cDNA kinase*, 1µl ATP, 1 µl 10 X buffer T4 DNA ligase and nuclease free water until final volume 10 µl, then

incubated at 16°C, for overnight. Dephosphorilated pUC19 was ligated to previously modified cDNA.

### **Bacterial transformation**

Before transformation, *Escherichia coli* XL-1 Blue competent were prepared by fresh exponential phase bacterial culture (OD 600 e" 0,5) in LB medium containing 10% PEG 6.000 (w/v), 5% DMSO (v/v) and 35 mM MgCl<sub>2</sub>. Different concentration of transformed bacteria were spread on LB agar plate containing ampicillin, X-Gal and IPTG then incubated at 37°C for overnight. White bacteria colonies were picked up and grown in 5 ml LB medium containing ampicillin for overnight. The growing bacteria were centrifuge at 4,000 rpm, 10 min, 4°C and the pellet was extracted for further plasmid preparation.

### **Analysis of recombinant plasmid**

Recombinant clones were picked up, grown in the LB medium and incubated at 37°C for overnight. Plasmid was isolated using *alkalilysis methods*, digest with *EcoRI* and electrophoresis at 1% agarose gel. Recombinant protein was isolated and electrophoresis at 10% SDS-PAGE and transfer onto nitrocellulose membrane. Nitrocellulose membrane was stained with amidoblack for protein detection and for immunoblotting, nitrocellulose membrane was incubated with polyclonal antibody against ESA protein.

### **cDNA Sequencing**

The recombinant plasmid was sequenced at Molecular Biology of Eijkman Institute, Jakarta using *Big Dye Terminator Mix ABI 377A sequencer*. DNA alignment analysis was performed using NCBI Blast and Clustal W.

## Results and Discussion

### The ESA protein profile

Excretory-secretory antigens is defined as total protein that secreted and excreted from tachyzoites *T. gondii*. Those proteins are composed of the mixture of all dense granule, rhoptry and micronema proteins (Cesbron-Delauw, 2001). The ESA's profile were shown in several protein as known at 13 kDa, 14 kDa, 17 kDa, 37 kDa, 44 kDa, 54 kDa, 71 kDa, 81 kDa, 98 kDa, 113 kDa and 131 kDa (Cesbron-Delauw, 2001).

In order to define ESA's expressed from recombinant bacteria was analyzed based on molecular weight in the 12% SDS-PAGE as shown in Figure 1.

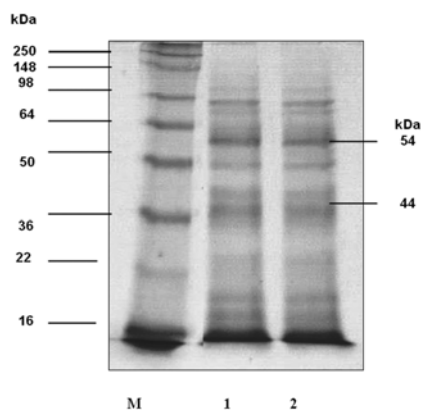


Figure 1. Profile of ESAs protein tachyzoites *Toxoplasma gondii* M: protein marker; lane 1 and 2. ESA protein

### RNA of *Toxoplasma gondii*

The total RNA of *Toxoplasma gondii* was run on 1% agarose gel was shown in Figure 2. Messenger RNA (mRNA) was isolated from 4.392 µg/µl total RNA using *PolyAtract mRNA Isolation System*. In this regard the yield of mRNA was sufficient for twice synthesis of cDNA.

### Recombinant plasmid analysis

Plasmid from recombinant clones were isolated using alkalilysis method and electrophoresis on 1% agarose gel. The result was shown in Figure 3.

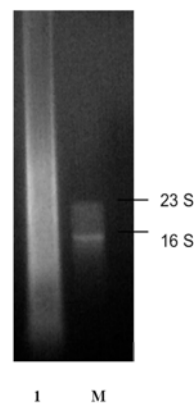


Figure 2. Electrophoresis of Total RNA *Toxoplasma gondii* local isolate M. Marker RNA ribosom, lane 1. Total RNA

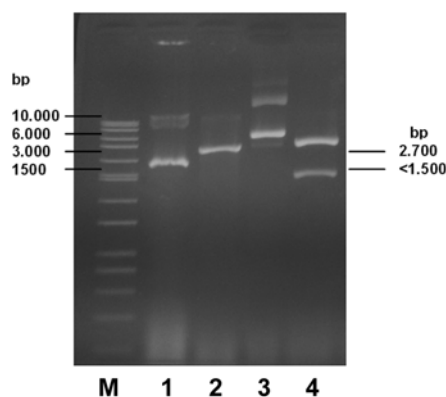


Figure 3. Electrophoresis of plasmid on 1% agarose gel M. Marker, lane 1. undigested pUC19, lane 2. pUC19 digested with *Eco R1*, lane 3. Recombinant Plasmid, lane 4. Recombinant Plasmid digested with *Eco R1*

### Recombinant protein identification

The profile of recombinant protein in line 1 was compared with the control (*E. coli* XL-1 blue) and blue colony showed different profile of ribbons.

The profile of recombinant protein in the 1<sup>st</sup> line, if it is compared with the marker, showed presumably a 54kDa protein expression. The molecular weight of ribbon of recombinant protein in line 3 was 54kDa, calculated by regression equation

(attachment 8). Recombinant of *Excretory - Secretory Antigens* (ESA) with molecular weight 54kDa was identified as ROP 2 protein.

Recombinant colony lisate protein in line 1 showed different pattern of ribbons with those from blue colony and *E.coli* XL-1 Blue. The obtained protein was identified by electrophoresis in 12% polyacrilamide gel. The result is showed in Figure 5.

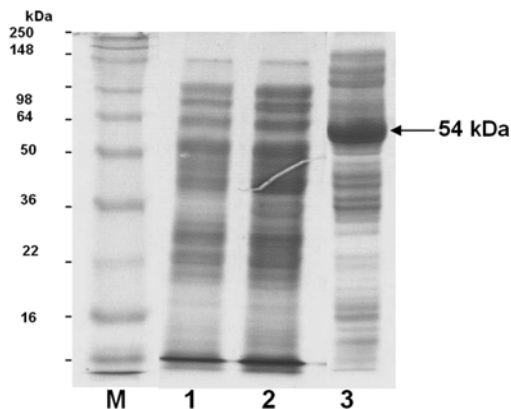


Figure 5. Profile lisat recombinant protein in 12% polyacrilamid gel M. Protein Marker, Lane 1. Recombinant Protein, Lane 2. *E.coli* XL-1 Blue Protein And Lane 3. Blue Colony.

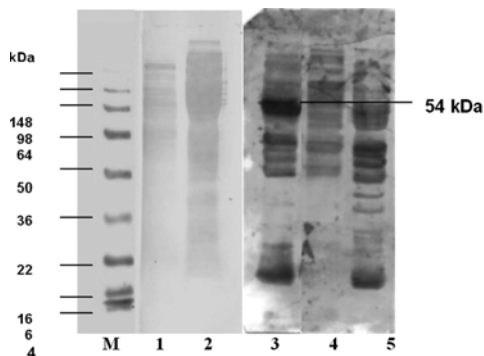


Figure 6. The *Immunoblotting* of recombinant Protein from SDS-PAGE 12% with antibody polyclonal to ESA. M. Marker, Lane 1. Lisate protein *E. coli* XL1-Blue, 2. lisate recombinant protein, Lane 3. lisate recombinant protein, Lane 4. Lisate protein *E. coli* XL1-Blue (without pUC19), Lane 5. lisate protein blue colony (pUC19 whithout insert)

The result of immunoblotting of several recombinant protein with antibody polyclonal towards ESA protein is showed in Figure 6. The importance result of the recombinant protein is able to applied for vaccine candidate or strong inducer of immune response (Vercammen *et al.*, 2000).

### DNA sequencing

Sequencing was done by the use of pUC/M13 forward sequencing primer and pUC/M13 reverse sequencing primer in the available pUC19 vector construct (Promega). Sequencing with pUC/M13 forward had resulted a 719 bp.

The sequence was analyzed with BLAST program to identify the cloned gene. Sequence that showed a significant alignment was ROP2 protein-encoding gene of *T. gondii* RH isolate. Recombinant DNA sequence alignment was showed 97% homology with ROP2 gene of the 49<sup>th</sup> base to the 561<sup>st</sup> base RH isolate (Figure 7).

Sequencing of plasmid recombinant was done with pUC/M13 forward sequencing primer and pUC/M13 reverse sequencing primer (available at pUC 19 vector construct/Promega). The analysis of the DNA sequence obtained from the BLAST program revealed a significant degree with gene encoding *rop2 T. gondii* RH isolate. Sequence alignment sequenced using *pUC/M13 forward* showed 99% homolog from base 49 until 561. Alignment sequence which sequenced using *pUC/M13 reverse* showed 95% the differences were lied on base 967 and 1628. The difference bases at 1330 did not change the amino acid; (GTT to GTC: Valine ), difference at 1500 did not change the amino acid; (TGC to TGT: Cysteine), and difference at 1592 lead to alter amino acid glutamic acid (GAA) to alanic acid (GCA). Transformation of glutamic acid into alanic acid, in the chemical structure, is not significantly different. It is, therefore, no change on

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Query 124  GCCAATTTGTGGTATGTGGCAACCGTCTGCATTGAATCCGCCGTAGTCAACACGAGATGC 183
          |||
Sbjct 49   GCCAATTTGTGGTATGTGGCAACCGTCTGCATTGAATCCGCCGTAGTCAACACGAGATGC 108
CDS ROP2 p          M L A T V C I E S A V V N T R C
Query 184  AGCTGCTCACGCTGGAATCATTGATTCTTCCCGCTTTGTGGGAGGCACGCGTATGTAGG 243
          |||
Sbjct 109  AGCTGCTCACGCTGGAATCATTGATTCTTCCCGCTTTGTGGGAGGCACGCGTATGTAGG 168
CDS ROP2 p          S C S S R W K S L I L P A L W E A R V C R
Query 244  GTAAAGACATCTAGCAAAGCAACTCCACGAGTATCGGTGGCCCCA-CGCAAGTGACGGAG 302
          |||
Sbjct 169  GTAAAGACATCTAGCAAAGCAACTCCACGAGTATCGGTGGCCCCAGCGCAAGTGACGGAG 228
CDS ROP2 p          V K T S S K A T P R V S V A P A Q V T E
Query 303  CGCCTACAGAGACGAACATCCGACCCACGCACCGACTGTCCTTCCATCGTAGTTGACGTT 362
          |||
Sbjct 229  CGCCTACAGAGACGAACATCCGACCCACGCACCGACTGTCCTTCCATCGTAGTTGACGTT 288
CDS ROP2          R L Q R R T S D P R T D C P S I V V D V
Query 363  TTGAGCCATGCTGCAAAACTGCACGCAGTAGTGAGGAGAATGGTATCCACGTCATCTTGT 422
          |||
Sbjct 289  TTGAGCCATGCTGCAAAACTGCACGCAGTAGTGAGGAGAATGGTATCCACGTCATCTTGT 348
CDS ROP2 p          L S H A A K L H A V V R R M V S T S S C
Query 423  TTTTCCGCGTGTATTTTCAGAAGATTCTCGCGAGAAGTGTGGATGCCCTTGTACATGGTA 482
          |||
Sbjct 349  TTTTCCGCGTGTATTTTCAGAAGATTCTCGCGAGAAGTGTGGATGCCCTTGTACATGGTA 408
CDS ROP2 p          F S A C I S E D S R G N C G C P C H N V
Query 483  ACGGTTGTTTTGTGTGTTACACATCTGCAACATGGAAAAGT-GCGTCGGTCAGATCATC 541
          |||
Sbjct 409  ACGGTTGTTTTGTGTGTTACACATCTGCAACATGGAAAAGTGTGCGTCGGTCAGATCATC 468
CDS ROP2          T V V L C V T H L Q H G K L A A S V R S
Query 542  GTCGTGCTTATCTGGCTAGCTGCCGATTCTTTGTTTCGGCACTTGGCCACGTACAGCA 601
          |||
Sbjct 469  GTCGTGCTTATCTGGCTAGCTGCCGATTCTTTGTTTCGGCACTTGGCCACGTACAGCA 528
CDS ROP2          V V S Y L A S C R I L C F G T W P R T A
Query 602  AGGCGCTGGCGTTGTGCGGCCTCGCCACTGGCA 634
          |||
Sbjct 529  AGGCGCTGGCGTTGTGCGGCCTCGCCACTGGCA 561
CDS ROP2 p          R R W R C A A S P L A
    
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Figure 7. Alignment DNA recombinant (The result of sequence with M13 forward primer) and gene encoding ROP2 RH isolate.

protein structure as well as no influence on epitope of ESA protein.

**Conclusion**

Complementary DNA from *T. gondii* local isolate was successfully cloned into pUC19 vector and transformed to XL-1 Blue *E.coli*. The correspondence sequence of cDNA was encoding of a 54-kDa protein. Alignment analysis of the sequenced cDNA was shown 97% homology with the *rop2* gene of *T. gondii* RH strain.

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Query 59 CGGTACGGATCCTGGAGACGTCGTCATATGGGAGTTNTTCAATCGTATAACCGGAAAACAA 119
          |||
Sbjct 1628 CGGTACGGATCCTGGAGACGTCGTCATATGGGAGCTGTTCAATCGTATAACCGGAAA-CAA 1568
CDS ROP2 p          M G ↓ X Q S Y T G K Q
                    1592
Query 120 GCGTATGGAATGAGAACGAACGCGNCTGTGCGAACGCCAACCATCTTCTAGTGTCACAG 180
          |||
Sbjct 1567 GCGTATGGAATGAGAACGAACGCGTCTGTGCGAACGCCAACCATCT-AGTGTCACAG 1507
CDS ROP2 p A Y G M R T N A X C R T P T I F C P Q
Query 181 CATTGTTGGCGTAATGAGCAGAGCTTCCGCGTGGAGTCGGAGCTGGGCGAGCGTCCAAGG 241
          |||
Sbjct 1506 CATTGCTGGCGTAATGAGCAGAGCTTCCGCGTGGAGTCGGAGCTGGGCGAGCGTCCAAGG 1446
CDS ROP2 p H ↓ W R N E Q S F R V E S E L G E R P R
                    1500
Query 242 ACGCTAGTCAGAGGCCAGTGCTCCGCGACGACGGCTCGTATATCTGTCTCGAGGCGACC 302
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Sbjct 1445 ACGCTAGTCAGAGGCCAGTGCTCCGCGACGACGGCTCGTATATCTGTCTCGAGGCGACC 1358
CDS ROP2 p T L V R G P V L R D D G S Y I C L E A T
Query 303 GACCAGGAGACAGGAGCAATCCCACCTTGAGGTGCACGTTCCATATTTACGGAACGGCCG 363
          |||
Sbjct 1357 GACCAGGAGACAGGAGAA-CCACTTGAGGTGCACGTTCCATATTTACGGAACGGCCG 1297
CDS ROP2 p D Q E T G ↓ P L E V H V P Y F T E R P
                    1330
Query 364 CCTTCCCAACGCGATCAAGCAGTTGAGCGAGCAGGTGCTGCGCCTACGCTTGCTACGAGG 424
          |||
Sbjct 1296 CCTT-CCAACGCGATCAAGCAGTTGAGCGAGCAGGTGCTGCGCCTACGCTTGCTACGAGG 1236
CDS ROP2 p P S Q R D Q A V E R A G A A P T L A T R
Query 425 CATCAAAAACAGAGGCAAGCCAAAGGGGCGTATCTCAGATTTATATTTCCCATCGATTT 485
          |||
Sbjct 1235 CATCAAAAACAGAGGCAAGCCAA-GGCGTATCTCAGATTTATATTTCCCATCGATTT 1175
CDS ROP2 p H Q K P E A S Q R G V S Q I Y I P H R F
Query 486 GGTGAAGGACCCAAAG-GGAAGATGATCCGGGTTGCGCTTAGATGAGAGGGATATGTG 546
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Sbjct 1176 GGTGAAGGACCCAAAGAAAAGGAAGATGATCCGGGTTGCGCTTAGATGAGAGGGATATGTG 1116
CDS ROP2 p G E G P K G K M I R V R L D E R D M
Query 547 GGTCTTGAGCAGATTCTTTCTGTATCCCGAATGCAGAGTACCCTTCATATTCCTGGAGA 607
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Sbjct 1115 GGTCTTGAGCAGATTCTTTCTGTATCCCGAATGCAGAGTACCCTTCATATTCCTGGAGA 1055
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Sbjct 1054 CGT-CCTACTGAGTCATTCCTC-AACACACAAGTCCCTCGTGCACCACGCTCGGTTG 994
CDS ROP2 p R P P T E S F L X X X K S L V H H A R L
Query 669 CAGCTCACGCTTCAGCTCATAAGGTTGG 695
          |||
Sbjct 993 CAGCTCACGCTTCAGCTCATAAGGTTGG 967
CDS ROP2 p Q L T L Q L I R L
    
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Figure 8. Alignment DNA recombinant (The result of sekuensing with *pUC/M13 reverse primer*) and gene encoding ROP2 RH isolate.

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