CHAPTER 2

MATERIALS AND METHODS

I. Materials

1. Plant material

Fresh young and mature rubber-tree leaves were collected from *Hevea brasiliensis* (H.B.K) Mull. Arg. clone RRIM 600 at the Songkhla Rubber Research Center.

2. Chemicals

Vitellogenin and anti-vitellogenin were gifts from Mr. Peerapong Puengyam, Biochemistry Department, Faculty of Science, Prince of Songkla University. Leptospira infected- and non-infected human serum was provided by the Suratthani Regional Medical Sciences Center. Leptospira (deoxycholate extracted antigen; DEA Type) was a gift from Mr. Sukone Pradutkanchana, Microbiology Department, Faculty of Science, Prince of Songkla University. HMG-CoA synthase and anti-HMG-CoA synthase were prepared in our laboratory as described by Tepaya (1999). Sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) was purchased from Pierce (Rockford, IL, USA). All other of chemicals and reagents used in this study were analytical grade and were purchased from Life Technologies (Gaithersburg, MD, USA), Sigma; (St. Louis, MO, USA), Fluka (Buchs, GS,

Switzerland), Amercham Biosciences (Little Chalfont, UK), Stratagene (La Jolla, CA, USA), Promega (Madison, WI, USA), and QIAGEN (Hilden, Germany).

3. Bacterial strains

Escherichia coli strain TOP10 F^- *mcr* A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)*7697 gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G and strain XL1Blue-MRF' *Δ(mcrA)*183 Δ*(mcrCB-hsdSMR-mrr)*173 *endA*1 *supE*44 *thi-1 recA*1 *gyrA*96 *relA*1 *lac[F' proAB lacI*^{q} *Z*ΔM15 Tn10(tet^r)] (Invitrogen, Life Technologies, Carlsbad, CA, USA)were used in this study.

4. Vectors

The pCR®4 -TOPO® vector (Invitrogen Life Technologies, Carlsbad, CA, USA) (Figure 11) and the pGEM®-T Easy vector (Promega, Madison, WI, USA) (Figure 12) were used in this study.

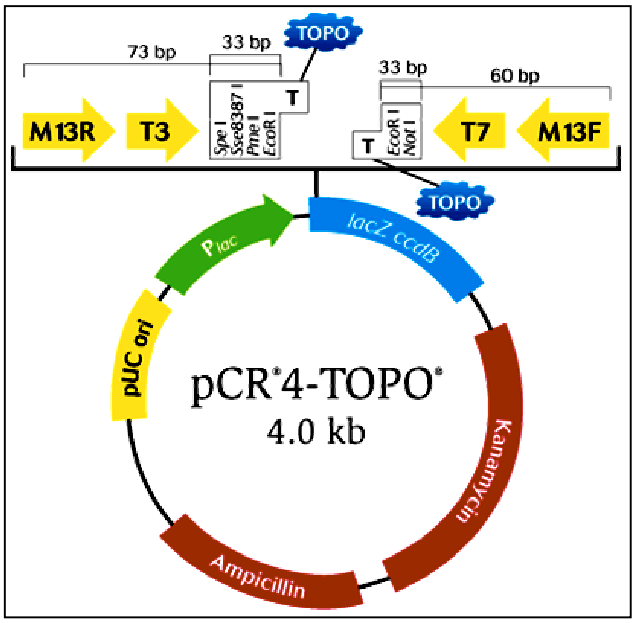

Figure 11. Map of pCR®4 - TOPO vector

(http ://kingfish.coastal.edu/biology/bio451/topotaseq_man.pdf)

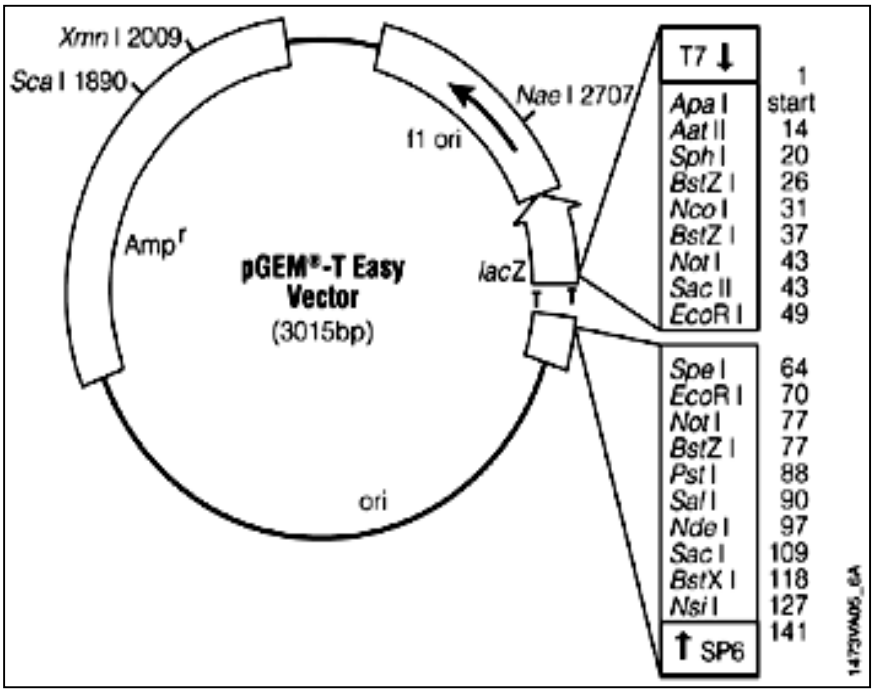

Figure 12. Maps of pGEM®-TEasy vector

(http ://www.promega.com/tbs/tm042/tm042.pdf)

5. Primers

The nucleotide primers for PCR, 5'RACE and 3'RACE as shown in Table 4 were purchased from QIAGEN, and from Invitrogen Life Technologies.

6. Enzymes

All of the enzymes were purchased from Promega, Invitrogen Life Technologies and from Sigma.

Table 4. The sequences of the primers for PCR, 5' RACE and 3' RACE.

Primer	Sequence (5' to 3')	Tm (°C)
First PCR		
FRBP1	CAYTTYCATGACTGYTT	55
RRBP1	AAAYGTRTGAGCACC	59
5' RACE		
GeneRacer™ 5′primer	GGACACTGACATGGACTGAAGGAGTA	78
GSP1 (reverse)	CGTGTGAGCACCAGATAGGGCAAC	66
3' RACE		
GeneRacer™ 3′primer	CGCTACGTAACGGCATGACAGTG	78
GSP2 (forward)	ATTCCAGGTCCTTTCGAGACCC	66

II. Methods

Section I : RBP purification and application

1. RBP purification and peroxidase activity assay

All purification steps were carried out at 4 ^{o}C, unless otherwise specified, as described by Rattanapumee (2000). *Hevea* leaves were washed and dried as quickly as possible. Leaf stems were cut off, and 100 g of leaf blade were finely cut and homogenized in 500 ml of 100 mM Tris-HCl buffer, pH 7.5, for 10 min. The homogenate was filtered through four layers of cheesecloth to remove the leaf debris and the crude extract separated as a greenish-brown supernatant (S_1, 426 ml) by centrifugation for 20 min at 11,000 *x g* using a Beckman JA-21 refrigerated superspeed centrifuge. Solid $(NH_4)_2SO_4$ was added to S_1 up to 40% saturation. The suspension was stirred for 5 hr and then centrifuged for 20 min at 11,000 *x g*, the precipitate was discarded, and the supernatant (S_2, 473 ml) was collected. Proteins in S_2 were precipitated by 80% sat. $(NH_4)_2SO_4$ overnight and then centrifuged for 30 min at 15,000 *x g*. The enzyme pellet (P_3, 7 ml) was dissolved in a minimum volume of 50 mM Tris-HCl, pH 7.5. Undissolved material was removed by centrifugation for 10 min at 1,000 *x g*.

The dark brown enzyme solution was subsequently applied to a DEAE-Sephacel column (1.5 × 24 cm) equilibrated and eluted with a single step, 10 mM Tris-HCl buffer, pH 7.5, followed by 0.3 M NaCl in the same buffer with a flow rate of 16 ml/hr. Fractions of 2 ml were collected, protein contents and peroxidase activity of each fraction were monitored at A_{280} and A_{460}, respectively, using a Shimadzu 160A UV-VIS spectrophotometer. The first peak of peroxidase active fractions was pooled and concentrated by centrifugation at 1,000 *x g* in an Amicon CF-25 centriflo

membrane (25,000 MW Cut-Off). The concentrated peroxidase (2 ml) with 18.76 mg/ml protein was further purified by applying onto a Sephadex G-75 column (0.9 × 30 cm) and eluted with 10 mM Tris-HCl buffer, pH 7.5, with a flow rate of 16 ml/hr. Fractions of 2 ml were collected, protein content and peroxidase activity of each fraction were monitored as described above. The enzyme was eluted out in the first peak. Two high peroxidase activity fractions were pooled and concentrated to 1 ml by centrifugation at 1,000 *x g* in an Amicon CF-25 centriflo membrane. The partially purified enzyme was assayed for peroxidase activity and kept at -20 ^{o}C for further study. The purification procedure carried out on different batches of rubber leaves yielded similar results.

Peroxidase activity during purification was determined with *o*-dianisidine as a reducing substrate. The substrate solution which consisted of 2.79 ml of 50 mM sodium acetate buffer (pH 5.4), 0.1 ml of 25 % *o*-dianisidine in methanol and 0.1 ml of 0.1 M H_2O_2 were added to a cuvette and mixed well. Then, 10 µl of purified enzyme at the appropriate concentration was added to bring the total assay volume to 3 ml, after which the content was mixed rapidly by repeated inversion. The cuvette was put in the Shimadzu UV-VIS spectrophotometer 160A. The activity was measured by following an increase in absorbance due to the formation of reaction product at 460 nm ($\Delta\varepsilon_{460nm}$ = 1.13 × 10^4 M^{-1} cm^{-1}, Duker, 1997) against a blank containing the substrate solution and distilled water instead of enzyme solution. Peroxidase activity was reported as the change in absorbance at 460 nm in 1 min (ΔA_{460}/min) and calculated for International Unit per ml (unit/ml) using the following equation:

$$\text{Peroxidase activity (unit/ml)} = \frac{\Delta A_{460}/\text{min} \times \text{total assay volume (ml)} \times DF}{\text{volume of enzyme solution (ml)}}$$

where DF = Dilution factor

The protein in samples at various steps were determined by the Lowry method (1951) using bovine serum albumin (BSA) as the standard. The assay was performed by adding 0.1 ml of protein solution to 3.0 ml of the alkaline copper reagent containing 2% Na_2CO_3 in 0.1 M NaOH, 1% potassium sodium tartrate and 0.5% $CuSO_4$ at a ratio of 100:1:1, respectively. The solution was allowed to stand for 10 min at room temperature (about 25 ^oC, RT). Then 0.3 ml of the Folin-Ciocalteu reagent (Fluka, Switzerland) (which was freshly diluted 1:1 with distilled water) was added and mixed. The solution was allowed to stand for 30 min at RT, after which the A_{650} was measured. The standard curve was prepared using BSA at concentrations of 0-100 μg/ml.

2. Conjugation and characterization of RBP-antibody conjugate

The purified RBP prepared was conjugated with either anti-rabbit IgG or anti-human IgG using three different cross-linkers: Na-periodate, glutaraldehyde, and sulfo-SMCC [sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate] as described below.

2.1 RBP-antibody conjugate preparation by using glutaraldehyde (O'Sullivan and Marks, 1981)

The purified RBP in 200 μl solution containing 5 mg protein was added to 200 μl of 0.1 M potassium phosphate buffer, pH 6.8, containing 1.25% glutaraldehyde.

The solution was incubated at RT for 18 hr and then the excess glutaraldehyde was removed by passing through a Sephadex G-25 column (0.9 x 30 cm) equilibrated and eluted with 0.15 M NaCl at a flow rate of 8 ml/hr. Fractions of 1 ml were collected and the absorbance at 280 nm of each fraction was measured. The glutaraldehyde activated RBP in the first peak (brown fractions) were pooled and concentrated to 1 ml using Amicon CF-25 centriflo membranes. Anti-rabbit IgG (5 mg in 1 ml of 0.15 M NaCl) was added to the activated RBP, followed by 0.1 ml of 1 M carbonate bicarbonate buffer, pH 9.5, and the mixture was then incubated at 4 ^{o}C for 24 hr. Lysine (0.2 M in distilled water, 0.1 ml) was added and the mixture was left for 2 hr, the RBP-anti-rabbit IgG conjugate was then dialyzed against three changes of 1,000 ml of phosphate buffered saline (PBS: 0.1 M potassium phosphate, 0.14 M NaCl), pH 7.2, at 4 ^{o}C overnight. The RBP-anti-rabbit IgG conjugate in the dialysate was precipitated with an equal amount of sat. $(NH_4)_2SO_4$ solution at 4^{o}C. The precipitated RBP-anti-rabbit IgG conjugate was washed twice with 50 % sat. $(NH_4)_2SO_4$ solution, dissolved in a minimal volume of distilled water and dialyzed against three changes of 1,000 ml of 0.1 M PBS, pH 7.2, at 4 ^{o}C overnight. The dialyzed RBP-anti-rabbit IgG conjugate was centrifuged at 2,000 x *g* for 10 min to remove the insoluble material. The RBP-anti rabbit IgG conjugate in the supernatant was further purified, as described in section 2.6.

2.2 RBP-antibody conjugate preparation by using sulfo-SMCC (The instruction manual for sulfo-SMCC, Doc. No. 0581 from Pierce, Rockford, IL, USA.)

One milligram of sulfo-SMCC [sulfosuccinimidyl4-(N-maleimidomethyl) cyclohexane-1-carboxylate] was added to the purified RBP (5 mg in 0.1 M sodium

phosphate buffer, 0.14 M NaCl and 0.05 M EDTA, pH 7.2), mixed well for 1 hr and then left for 30 min at RT. The activated RBP was separated from free sulfo-SMCC by passing through a Sephadex G-25 column (0.9 x 30 cm) equilibrated and eluted with 0.1 M sodium phosphate buffer, pH 7.2, at a flow rate of 6 ml/hr. Fractions of 1 ml were collected. The sulfo–SMCC activated RBP peak was located by measuring the A_{280}. These fractions were then pooled and concentrated to 500 µl using Amicon CF-25 centriflo membranes.

Four milligrams of anti-rabbit was reduced by dissolving in 500 µl of 0.5 M β-mercaptoethylamine in 10 mM sodium phosphate buffer containing 0.14 M NaCl, 5 mM EDTA and incubated at 37 ^{o}C for 90 min, the solution was then loaded onto a Sephadex G-25 column (0.9 x 30 cm) equilibrated and eluted with 0.1 M sodium phosphate buffer, pH 7.2, at a flow rate of 8 ml/hr to remove excess reducing agent. Fractions of 1 ml were collected and the A_{280} in each was measured. The reduced anti-rabbit IgG in the void volume (4 fractions of the first peak) was pooled and concentrated to 500 µl using an Amicon CF-25 centriflo membrane.

The activated RBP was mixed with the reduced anti-rabbit IgG, incubated at RT for 30 min and left at 4 ^{o}C overnight. The RBP-anti IgG conjugate formed was purified as described in section 2.6.

2.3 RBP-antibody conjugate preparation using sodium-periodate ($NaIO_4$) (O'Sullivan and Marks, 1981)

Purified RBP (5 mg protein in 200 µl) was dissolved in 1.0 ml of 0.3 M sodium bicarbonate, pH 8.1, (freshly prepared), then 0.1 ml of 1 % fluorodinitrobenzene (in absolute ethanol) was added and mixed gently for 1 hr at RT. One ml of 0.06 M $NaIO_4$ in distilled water was added and mixed gently for 30 min,

then 1.0 ml of 0.06 M ethylene glycol in distilled water was added, and the solution was then mixed well for another hr at RT. The mixture was dialyzed against three changes of 1 liter of 0.01 M sodium carbonate buffer, pH 9.5, at 4^{o}C overnight. The solution was centrifuged at 2,000 *x g* at 4^{o}C for 10 min to remove the insoluble material. Anti-rabbit IgG or anti-human IgG (5 mg) was then added to the supernatant, mixed gently and incubated for 3 hr at RT followed by the addition of sodium borohydride ($NaBH_4$, 5 mg), gentle mixing and incubation at 4^{o}C for at least 3 hr or overnight. The RBP-antibody conjugate formed was dialyzed against three changes of 1,000 ml of 50 mM Tris-HCl, pH 7.5, at 4^{o}C overnight. The dialyzed RBP-antibody conjugate was centrifuged at 2,000 *x g* for 10 min to remove the precipitate and the supernatant containing RBP-antibody conjugate was further purified.

2.4 Determination of the optimal concentration of $NaIO_4$ for the formation of RBP-antibody conjugate

In order to establish the optimal concentration of $NaIO_4$ for the formation of RBP-antibody conjugate, halfscale of RBP-anti-rabbit IgG conjugate preparation were done by the periodate oxidation method with three concentrations of $NaIO_4$: 0.04, 0.06, and 0.08 M. The RBP-anti-rabbit IgG conjugate prepared was purified using a Sephadex G-200 column (1.6 x 30 cm) with 10 mM Tris-HCl, pH 7.5, and a flow rate of 6 ml/hr. The optimal concentration of $NaIO_4$ for RBP-antibody conjugate was determined as that yielding the highest retention of peroxidase activity in the conjugate.

2.5 RBP-antibody conjugate preparation by modified periodate oxidation method

The RBP-anti-rabbit IgG conjugate, prepared using periodate as described above, was separated from other compounds by adding an equal volume of sat. $(NH_4)_2SO_4$ solution at 4 ^oC and centrifuging at 2,000 x *g* for 10 min. The RBP-antibody conjugate was washed twice with 50% sat. $(NH_4)_2SO_4$ solution and recentrifuged. The precipitate was dissolved in 1.5 ml of 50 mM Tris-HCl, pH 7.5, and desalted using a Sephadex G-25 column (1.3 x 5 cm). Fractions of 1 ml were collected and their absorbances at the A_{280} were measured. Protein in the first peak was pooled, concentrated to 1.5 ml using an Amicon CF-25 Centriflo membrane and further purified.

2.6 Purification of RBP-antibody conjugate

Both RBP-anti-rabbit IgG conjugate and RBP-anti-human IgG conjugate obtained from various methods were further purified using a Sephadex G-200 column (1.3 x 65 cm) equilibrated and eluted with an appropriate buffer according to the method of conjugation. Collected fractions of 1 ml were monitored for protein using the A_{280}, the A_{403} for heme group, and the A_{460} for peroxidase activity using *o*-dianisidine as an enzyme substrate. Fractions with highest peroxidase activity accompanied with the absorbance at 280 and 403 nm were pooled and concentrated to 1 ml using Amicon CF-25 Centriflo membrane.

The efficiency of each conjugation method was compared on the basis of the absorbance at 403 nm and the retained peroxidase activity of RBP-antibody conjugate.

2.7 Molecular weight determination of RBP-antibody conjugate

The molecular weight (MW) of the RBP-anti-rabbit IgG conjugate prepared by periodate oxidation was determined by gel filtration chromatography using high MW proteins (ferritin: MW = 460,000; catalase: MW = 232,000; BSA: MW = 67,000; chymotrypsinogen A: MW = 25,000 dalton) as molecular weight standards. RBP-anti-rabbit IgG conjugate, RBP, anti-rabbit-IgG and standard markers (1.5 ml containing 2.5 mg/ml protein) were separately applied onto a Sephadex G-200 column (1.3 x 65 cm), equilibrated and eluted with 50 mM Tris-HCl buffer, pH 7.5, at a flow rate of 6 ml/hr. Fractions of 1 ml were collected, A_{280} and elution volume (V_e) were measured. Blue dextran (MW = 2,000,000) and $K_2Cr_2O_7$ were also applied to the column under the same eluting condition as above. Absorbance at 620 and 480 nm were measured for void volume (V_o) and total volume (V_t) calculation, respectively. The MW of the conjugate was estimated from a plot of log MW vs K_{av} (distribution coefficient) calculated from V_e of markers using the following equation.

$$K_{av} = (V_e - V_o)/(V_t - V_o)$$

2.8 Antigen-antibody interaction of RBP-antibody conjugate

The ability of the RBP-anti-rabbit IgG conjugate as antibody to react with antigen, rabbit IgG, was tested by the Ouchterlony double diffusion method as described by Ouchterlony (1956). A microscope slide was coated with 3 ml of 0.3 % agarose in 0.85 M NaCl. A coated slide was allowed to stand at RT for 15 min, and then it was completely dried at 60 ^{o}C. The slide was then coated with 5 ml of 1.5% agarose in 0.9 M NaCl, allowed to stand at RT for 15 min and incubated in a

refrigerator for 5 min. A pasture pipette was used to cut the gel for a line of three circular sample wells (diameter 5 mm). Rabbit serum was placed in the central well and pooled RBP-anti-rabbit IgG conjugate 10 μl in the other wells. The slide was incubated in a moist box at RT overnight or until the precipitation line occurred, then the gel was stained with either 0.1% Coomassie Brillian Blue R (in 1 : 4.5 : 4.5 acetic acid : absolute ethanol : H_2O) or with peroxidase activity assay solution (0.05 % *o*-dianisidine, 16.8 % methanol, 50 mM sodium acetate buffer, pH 5.4, and 0.1 M H_2O_2). After the colour of the precipitation line developed, the gel was washed with distilled water and then photographed.

2.9 Determination of RBP-antibody conjugate titer

In order to establish an optimal concentration of the RBP-anti-rabbit IgG conjugate as secondary antibody in an enzyme immunoassay, grids of squares (1 x 1 cm) were drawn on a nitrocellulose membrane. The membrane was washed with distilled water for 5 min in a small shaking container, then it was removed and dried at RT. A dot of 5 μl of rabbit serum was placed on the center of each of the grid squares and dried at RT. The membrane was washed three times for 5 min each with Tris buffer saline (TBS), pH 7.5, containing 0.025 M Tris, 0.5 M NaCl and 0.02 % sodium azide (NaN_3), then blocked with 3 % BSA in TBS containing 0.05 % Tween 20 (TTBS) at RT for 2 hr and incubated at 4 ^{o}C overnight. The pooled RBP-anti-rabbit IgG conjugate was diluted by serial dilution (1:25, 1:50, 1:100, 1:200, 1:400, 1:800) and 5 μl of these solutions were placed at the center of the grid squares (overlaying on top of the rabbit serum) and dried at RT. The membrane was blocked with 3% BSA in TTBS at RT for 30 min, followed by three washings of 5 min each with TBS, then stained with peroxidase activity assay solution (0.05% *o*-dianisidine,

16.8% methanol, 50 mM sodium acetate buffer, pH 5.4, and 0.1 M H_2O_2). The reaction was stopped with 0.1 M phosphate buffered saline (PBS) containing 50 mM EDTA, pH 7.2.

2.10 Stability of RBP-antibody conjugate

The stability of the RBP-anti-rabbit-IgG conjugate and RBP-anti-human IgG conjugate were checked based on their immunological activity and peroxidase activity after conjugation, after being kept for 2, 4, 6 and 8 months at -20 ^{o}C.

The immunological activity of each was tested in a simple direct test against either rabbit IgG or human IgG in a microtiter plate. Each well of the microtiter plate was coated with 100 µl of serial dilutions of rabbit or human serum (0, 20, 40 60, 80, 100 µg/ml in coating buffer containing 0.1 M sodium carbonate bicarbonate buffer and 0.02% NaN_3, pH 9.5) and incubated at 4 ^{o}C overnight. The plate was washed five times with 0.2 ml of PBS containing 0.05% Tween 20. Non-specific binding sites on the plate were blocked by adding 0.2 ml of blocking buffer containing 3% BSA in 0.1 M PBS, pH 7.4, at RT for 1 hr. The plate was washed five times as above. The RBP-anti-rabbit IgG (100 µl, dilution 1:800) or RBP-anti-human IgG conjugate (100 µl, dilution 1:1,200) kept for 2, 4, 6 and 8 months at -20 ^{o}C were dissolved in assay buffer containing 1% BSA and 0.05% Tween 20 in 0.1 M PBS, pH 7.4, and then added to wells in the plate and incubated at RT for 2 hr. The plate was washed five times. The reactions between rabbit IgG and anti-rabbit IgG or human IgG and anti-human IgG were monitored by peroxidase activity. Peroxidase substrate solution containing 0.4 mg/ml *o*-phenylenediamine dihydrochloride (OPD), 0.012 % H_2O_2 in 50 mM citric acid phosphate buffer, pH 5.0 (150 µl), was then added and incubated in the dark condition at RT for 15 min. After the reaction was stopped by adding 50 µl

of 2 M H_2SO_4, the plate was immediately read at 492 nm using a Bio-Tek Elx808 microtiter plate reader.

The peroxidase activity of both conjugates kept for 2, 4, 6 and 8 months were also examined by using *o*-dianisidine as an enzyme substrate at 460 nm, as described in method 1.

3. Application of RBP-antibody conjugate

The prepared RBP-antibody conjugate was used as a secondary antibody in detecting or determining the amount of samples of interest, for example, HMG-CoA synthase, vitellogenin and infection of leptospira, in samples using western blot or ELISA techniques. These techniques required preparation of HMG-CoA synthase, vitellogenin and leptospira, as well as rabbit anti-HMG-CoA synthase, anti-vitellogenin and anti-leptospira.

3.1 Preparation of HMG-CoA synthase and rabbit anti-HMG-CoA synthase

HMG-CoA synthase was prepared from C-serum of rubber latex as described by Tepaya (1999). Fresh latex was collected from *Hevea* trees, clone RRIM 600, growing at the Songkhla Rubber Research Center, Hat Yai. It was kept on ice before centrifugation using Beckman L8-70M Ultracentrifuge at 49,000 x *g* for 30 min. Four fractions were obtained: the top layer (rubber), the Frey-Wyssling particles, the C-serum, and the bottom fractions. The C-serum was removed with the aid of a syringe and kept at -20 ^{o}C for further study. The C-serum (18 ml) was mixed with 10 g of CM-Cellulose ion exchange resin in 0.1 M Tris-HCl, pH 7.5, and left for 45 min on ice. The resin was then separated from the supernatant by centrifugation at 2,000 x *g*,

4 ^{o}C for 10 min. To the supernatant once collected, 3 ml of glycerol was added. The mixture was concentrated by using CM-cellulose. The concentrated enzyme (3 ml) was further purified by passing through a Sephadex G-75 column (1.3 x 16 cm), equilibrated and eluted with 0.1 M Tris-HCl, pH 8.2, with a flow rate of 18 ml/hr. Fractions of 2 ml were collected. These fractions were monitored for protein at 280 nm and for HMG-CoA synthase activity as described by Miziorko (1985).

Fractions with high specific activity of the HMG-CoA synthase were pooled and concentrated, purified using non-denaturing preparative polyacrylamide gel electrophoresis with a 7-15 % gradient gel. The electrophoresis was done with a running buffer containing 0.025 M Tris-HCl and 0.192 M glycine, pH 8.3. Low molecular weight proteins (phosphorylase b; MW = 94,000, BSA; MW = 67,000, ovalbumin; MW = 43,000, carbonic anhydrase; MW = 30,000; soybean trypsin inhibitor; 20,000; α-lactalbumin; MW = 14,000 dalton) were used as molecular weight standards. At the end of the separation, the gel was cut horizontally into small slices. The slice with HMG-CoA synthase was extracted with 50 µl of 0.1 M Tris-HCl, pH 7.5, using a Wheaton homogenizer. The extracted enzyme was separated from the gel by centrifugation at 2,000 *x g*, 4 ^{o}C for 10 min., pooled and concentrated to 150 µl (containing 200 µg protein), mixed vigorously with 200 µl of the Complete Freud's Adjuvant and 400 µl of 0.85% NaCl to obtain a homogeneous HMG-CoA synthase/adjuvant solution.

Five milliliters of rabbit blood was taken from the large vein of a six week-old, male rabbit (1.5 kg body weight) ear. The blood was left at RT for 2 hr, then centrifuged at 2,000 *x g*, 4 ^{o}C for 15 min. After centrifugation, the rabbit serum containing rabbit IgG was collected and kept at -20 ^{o}C for further use.

Anti-HMG-CoA synthase in rabbit was raised by injection of HMG-CoA synthase/adjuvant solution into the rabbit. Three consecutively subcutaneous injections of 200 μg of HMG-CoA synthase were done every 3 weeks, first on the upper back, then on the left thigh and finally on the right thigh. Seven days after the last injection, blood (2 ml) was collected from the rabbit. Rabbit serum was collected as described above for preimmune serum and checked for anti-HMG-CoA synthase using the Ouchterlony double diffusion, as described in method 2.8.

The C-serum of rubber latex containing HMG-CoA synthase and rabbit serum containing anti-HMG-CoA synthase were kept for further use as the specific antigen and antibody, respectively.

3.2 Vitellogenin and rabbit anti-vitellogenin

Vitellogenin in mullet plasma and rabbit anti-vitellogenin were gifts, prepared and given by Mr. Peerapong Puengyam (Puengyam, 2002).

3.3 Leptospira and anti-leptospira

Leptospira DEA type extracted from *Leptospira interrogans* serovar Bataviae were gifts, prepared and given by Mr. Sukone Pradutkanchana (Pradutkanchana, 2003).

Human serum with anti-leptospira were provided by the Suratthani Regional Medical Sciences Center.

3.4 Detecting HMG-CoA synthase with RBP-antibody conjugate using dot blots.

Grids of squares (1 x1 cm) were drawn on nitrocellulose membrane, washed with distilled water for 5 min in a shaker, and dried at RT. A dot of 5 μl of C-serum of rubber latex (1.13 mg/ml protein) was placed on the center of one of the grid

squares and left at RT until dried. The membrane was washed three times for 5 min each with TBS then blocked with 3% BSA in TTBS and incubated at 4°C overnight. The membrane was removed and placed on a sheet of parafilm. Dots of 1 µl of rabbit-anti-HMG-CoA synthase (17.1 mg/ml protein, dilution 1:1,000) were placed on the center of one of the grid squares and dried at RT. The membrane was washed three times for 5 min each with TBS, pH 7.5, blocked with 3% BSA in TTBS for 30 min, then washed three times 5 min each with TBS, pH 7.5. A dot of 3 µl of selected RBP-anti-rabbit IgG conjugate fractions No. 45, 49, 53, 57, 61, 67, 69, 81, 85, 87, 105 and 107 from the Sephadex G-200 column was placed on the center of the grid squares on top of the rabbit serum and dried at RT. The membrane was blocked with 3% BSA in TTBS at RT for 2 hr, and washed three times for 5 min each with TBS and stained with peroxidase activity assay solution (method 2.9). The reaction was stopped by adding distilled water.

3.5 HMG-CoA synthase and vitellogenin determination by Western blot.

Proteins of interest (HMG-CoA synthase in C-serum, 11 µg or vitellogenin in mullet plasma, 23 µg) were separated by SDS-PAGE on a 6 to 18% gradient gel according to the method of Laemmli (1970). The proteins separated on the gel were then electrophoretically transferred to a nitrocellulose membrane using a tank transfer system at 100 volt (450 mA) for 100 min with cooling. The gel was stained with 0.2% Coomassie Brilliant Blue R250 to verify a complete transfer of protein to the membrane. Proteins in the membranes were stained with 0.2% Ponceau-S in 10% acetic acid for 10 min. The background was removed by washing with distilled water, and the membrane was then photographed. After destaining, the membrane was

immersed in blocking solution [3% BSA in TTBS (method 2.9)] with shaking at RT for 3 hr, then kept at 4^{o}C overnight. The membrane was then incubated with primary antibody rabbit anti-HMG-CoA synthase 1:1,000 or anti -vitellogenin 1:300 in 1% BSA in TTBS with shaking at RT for 2 hr. The blots were washed with four changes of TTBS for 15 min each followed by two changes of TBS for 10 min to remove non-specific binding proteins. The membranes were then incubated with 1:200 RBP-anti-rabbit IgG conjugate in 1% BSA in TTBS, which acts as a secondary antibody directed against primary (rabbit) antibody, at RT for 1 hr, then washed as described above. A commercial HRP-anti-rabbit IgG conjugate at 1:3,000 dilution in the same buffer was also used as a positive control. The proteins of interest were identified by peroxidase activity staining (0.05% *o*-dianisidine, 16.8% methanol, 50 mM sodium acetate buffer, pH 5.4, and 0.1 M H_2O_2). The reaction was stopped by adding distilled water.

3.6 Determination of leptospira with RBP-antibody conjugate using enzyme-linked-immunosorbent assay (ELISA)

The prepared RBP-anti-human IgG conjugate was checked for the ability to act as a secondary antibody in detecting the presence of antibody to leptospira in human serum.

Each well of the microtiter plate was coated with 100 µl of the Leptospira DEA Type dissolved in coating buffer containing 0.1 M sodium carbonate bicarbonate buffer and 0.02% NaN_3, pH 9.5) and incubated at 4^{o}C overnight. The

plate was washed five times with 0.2 ml of PBS containing 0.05% tween 20. Non-specific binding sites on the plate were blocked by adding 0.2 ml of blocking buffer containing 3% BSA in 0.1 M PBS, pH 7.4, at RT for 1 hr. The plate was washed five times with 0.2 ml PBS. Human serum, positive and negative control (100 µl, dilution 1:100) in assay buffer containing 1 % BSA, 0.05% Tween 20 in 0.1 M PBS, pH 7.4, were separately added to wells in the plate and incubated at RT for 2 hr. The plate was washed five times with PBS, then 100 µl of RBP-anti-human IgG conjugate (dilution 1:1,200) or HRP-anti-human IgG conjugate (dilution 1:12,000) in assay buffer was added and incubated at RT for 2 hr. Peroxidase activity assay solution (as described in 2.10) was then added and incubated in the dark condition at RT for 15 min. The reaction was stopped by adding 50 µl of 2 M H_2SO_4. The color formed in each well was immediately read at 492 nm using the Bio-Tek Elx808 microtiter plate reader. The optimal dilutions of human serum and RBP-anti-human IgG conjugate were checked previously by chequerboard titration.

3.7 Application of purified RBP in cholesterol determination

To check whether the purified RBP can be used for cholesterol determination instead of HRP, RBP was used and compared with the assay using HRP.

Reagents used for cholesterol determination according to the instruction manual of the kit from CPT Diagnostic (Barcelona, Spain) were as follows: >0.2 U/ml cholesterol esterase, >0.1 U/ml cholesterol oxidase, RBP or HRP >0.8 U/ml, 0.5 mM sodium cholate, 28 mM phenol and 4-aminoantipyrine 0.5 mM in 35 mM PIPES buffer, pH 7.0, kept at 2-8 ^{o}C. Cholesterol in samples was determined by mixing 10 µ l of human serum and 1.0 ml of reagent prepared by using either RBP or HRP,

incubated at RT for 10 min. The absorbance of quinoneimine, the reaction product, at 500 nm was measured (Allain *et al*., 1974). Cholesterol concentration in serum was determined by comparing its A_{500} value it to the linear plot of A_{500} vs. standard cholesterol concentration (mg/dl). The results were multiplied by 0.0259 to obtained cholesterol in mmol/l for International Units (1,000 mg/dl = 25.9 mmol/l).

4. Amino acid sequence analysis of purified RBP

4.1 RBP further purification

The purified RBP from the Sephadex G-75 column (500 µl/tube) containing 3.2 mg/ml protein was dissolved in 0.5 ml of TC buffer containing 10 mM Tris-HCl, pH 8.0, 140 mM NaCl and 10 mM $CaCl_2$ (TC buffer), then further purified by passing through a Con-A agarose column (2 x 10 cm) equilibrated with TC buffer. Fractions of 60 drops/tube were collected. The enzyme eluted by TC buffer was collected in fractions No. 1-21. The buffer was changed to TE buffer containing 10 mM Tris-HCl, pH 8, 140 mM NaCl and 10 mM EDTA at fractions No. 22-45. After that the elution buffer was changed to TE buffer containing 0.1 M methyl-mannoside.

Protein contents of each fraction was monitored as described in method 1. Peroxidase activity was monitored and reported as the change at 460 nm/min per tube of assay using bentidin as a peroxidase substrate. One ml of the solution reaction consists of 0.6 M bentidin, 30 mM Tris-HCl, pH 7.4, 3 mM $CaCl_2$, 5 mM H_2O_2, and 20 µl of enzyme solution. The reaction was started by addition of H_2O_2 and left for 10 min at RT (23-25^{o}C), after which the absorbance at 460 nm was measured using the solution reaction without the enzyme, as a blank.

Fractions with high peroxidase activity were analyzed by 15% gel native-PAGE. The gels were first stained with peroxidase activity staining solution, which

consists of 0.6 mM bentidin, 30 mM Tris-HCl, pH 7.4, 3 mM $CaCl_2$ and 5 mM H_2O_2. These gels were then stained with 0.02% Coomassie Brilliant Blue R-250.

4.2 Amino acid sequence analysis

RBP purified by Con-A agarose chromatography in each fraction was first treated with 10% cyanogen bromide (CNBr) and separated by HPLC and then the peptide amino acid sequences were analysed with an Applied Biosystems 492 Protein Sequencer. Each fraction was dialyzed against Milli-Q water and dried by centrifugal concentrator at 35 ^{o}C, then digested with 1.5 µl of lysyl endopeptidase (LEP) in 0.1 M Tris-HCl, pH 9.0, and 7 µl of 8 M urea, separated by HPLC and then their amino acid sequences were analysed by the protein sequencer again, since only a few pure peptides were obtained after CNBr treatment.

Section II : Molecular cloning and sequencing of cDNA encoding RBP

1. PCR for *rbp* cDNA

1.1. Total RNA extraction

Total RNA from rubber leaves was extracted by a modified method described by Suwanmanee *et al*., 2004. Five gram of leaves was ground to powder in liquid N_2 with 0.25% (w/w) polyvinylpyrolidone and transferred into a tube containing 10 ml of extraction buffer and 10 ml of the mixture of phenol:chloroform: isoamyl alcohol (25:24:1 v/v) and vigorously shaken at RT for 5 min. The homogenate was centrifuged at 7,000 *x g* at 4^{o}C for 30 min. Then, the supernatant was deproteinized with an equal volume of phenol:chloroform:isoamyl alcohol and followed by repeated extraction with chloroform:isoamyl alcohol (24:1 v/v). The nucleic acid in the supernatant was precipitated with 3 M sodium acetate, pH 5.2, and 2.5 volume of ethanol at -20 ^{o}C for 1 hr and centrifuged at 11,000 *x g*, 4^{o}C for 30 min. The pellet

was washed twice with 70% ethanol, and then resuspended in DEPC treated water. Then 8 M LiCl was added to a final concentration of 1.8 M, and the tube was incubated at 4^{o}C overnight. The RNA was precipitated by centrifuging at 11,000 *x g*, 4^{o}C for 30 min and washed one time with 1.8 M LiCl, then washed twice with 70% ethanol. The total RNA was air dried for 2 min, dissolved in DEPC treated water and stored at -20^{o}C until used. The total RNA concentration was determined by a spectrophotometer using the A_{260} (1 A_{260} RNA = 40 µg/ml) and the purity of the RNA was judged by the A_{260}/A_{280} ratio (pure RNA solution has a ratio of 1.6-1.8). The pattern of intact RNA was visualized by agarose gel electrophoresis under UV light (method 1.3, section II).

1.2 Random amplification of cDNA by two-step RT-PCR

Firstly, first strand cDNA was synthesized as follows. The extracted total RNA (2 µg) was applied into a 1.5 ml microcentrifuge tube containing 1 µg of oligo dT primer (Promega), mixed briefly, incubating at 70^{o}C for 5 min, then quickly chilled on ice for 5 min. The intact RNA was added with 5 µl of AMV reverse transcriptase 5x reaction buffer, 2.5 µl of 10 mM dNTP mix, 5 µl of RNase Ribonuclease inhibitor, 3 µl of AMV reverse transcriptase and 9.5 µl of RNase-free water, then mixed carefully by pipetting and incubated at 37^{o}C for 1 hr. Secondly, the synthesized first strand cDNA was used as cDNA template to amplify second strain cDNA using degenerate primers, FRBP1 and RRBP1 (Table 4). The PCR reaction composed of 2 µl of 10 mM dNTP mix, 2.5 µl of 10x PCR buffer, 2 µl of 2.5 mM $MgCl_2$, 2.5 µl of reverse primer (RRBP1), 2.5 µl of forward primer (FRBP1), 1 µl of cDNA template, 0.5 µl of *Taq* DNA polymerase and 12.5 µl of RNase free water to a

total volume of 25 µl. The amplification was performed using the Eppendorf thermo cycler, USA with the cycling parameters as follows: initial denaturation at 95°C for 5 min, annealing at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 35 cycles, and final extension at 72°C for 1 min. Five microliters of PCR product was examined for the expected DNA fragment by 1.5% agarose gel electrophoresis, then the rest of PCR product was purified by QIAquick PCR Purification Kit (QIAGEN) according to the manufacture's instruction.

1.3 Agarose gel electrophoresis

Agarose gel electrophoresis was used to visualize the extracted DNA of interest. Agarose 1.5% (w/v) was melted in 0.5x TAE buffer (20 mM Tris-acetate and 0.5 mM EDTA), then 2 µl of 10 mg/ml ethidium bromide (EtBr) solution was added into the melted gel before pouring it on the plastic tray with a comb placed in the gel. After the gel was completely set, the comb was removed and the gel was placed in an electrophoresis chamber containing 0.5x TAE buffer. The DNA samples (10 µl) were mixed with 1.5 µl of 6x loading buffer [0.25% (w/v) Bromphenol Blue, 4% (w/v) sucrose] and loaded into the slots of the gel. Gel electrophoresis was carried out at a constant 50-100 V for 30-60 min. The expected DNA fragment was visualized on either a gel documentation system (VisiDoc-It system) or on an ultraviolet (UV) light box.

1.4 Purification of DNA fragment using the QIAquick PCR Purification Kit (QIAGEN)

The expected DNA fragment was purified from the PCR product. Five volumes of PB buffer containing 96-100% ethanol was added to one volume of the PCR reaction and mixed by pipetting. The mixture was applied into a QIAquick

column placed in a 2 ml collection tube, centrifuged for 1 min at 11,000 *x g* and the flow-through was discarded. The DNA bound to the silica membrane in the column was washed by adding 0.75 ml of ethanol-EDTA-containing washing buffer (PE) and centrifuging to discard the flow-through. The QIAquick column was placed into a clean 1.5 ml microcentrifuge tube and the DNA fragment was eluted with 30 ml of eluting buffer (EB) (10 mM Tris-HCl, pH 8.5) by centrifugation at 11,000 *x g* for 1 min.

1.5 Ligation of DNA fragment into pGEM®-T Easy vector (Promega)

The purified cDNA fragment was ligated with pGEM-TEasy vector (Figure 12) according to the manufacture's instruction. Ten microliters of the reaction mixture containing 5 µl of the 2x rapid ligation buffer, 1 µl of pGEM-T Easy vector, 1 µl of T4 DNA ligase, and 3 µl of purified PCR product was mixed carefully by pipetting and incubated at 4 ^{o}C overnight, then the ligated plasmids were further transformed into XL-1Blue competent cells.

1.6 Transformation of plasmid into *E. coli* XL1-Blue MRF′ competent cells

Frozen competent cells 200 µl in a microcentrifuge tube were taken from -80^{o}C, thawed on ice and mixed gently with 0.1-1 µg plasmid DNA from method 1.5, then placed on ice for 30 min. The cells were heat-shocked at 42^{o}C for 90 sec, then immediately chilled on ice for 5 min before mixing with 800 µl of warm (50-55 ^{o}C) LB medium. The tube was incubated at 37^{o}C for 2 hr with moderate shaking. After incubation, 100-200 µl of cell culture was spreaded on a selective LB medium plus 100 µg ampicillin (LBA) plate which was previously spreaded with 100 µl of 100

mM IPTG in water and 20 µl of 40 µg/ml X-gal in dimethylformamide (DMF), and then incubated at 37°C overnight. Single white colonies developed under these selective conditions were selected.

1.7 Analysis of the positive clones from transformation

The colonies carrying plasmid with insert, namely the positive clones, were identified. Five to ten single white colonies were picked individually and transferred to a new LBA plate and incubated at 37°C overnight. At the same time, these selected colonies were also transferred individually to a new 1.5 ml microcentrifuge tube containing 0.5 ml of liquid LBA and incubated at 37°C for 4 hr with horizontal shaking. After incubation, the cell suspension was precipitated by centrifugation at 5,000 *x g* for 5 min. The supernatant was removed by pipette and the precipitated cells in the tube were mixed with 5 µl of extraction solution, then 10 µl of phenol:chloroform: isoamyl alcohol (25:24:1) was added. The whole set was mixed vigorously by vortex and the supernatant was separated from the cell lysate by centrifugal precipitation at 11,000 *x g* for 5 min and then transferred to a clean microcentrifuge tube. The supernatant was added with 30 µl of cool ethanol and incubated at -20°C for 20 min. After centrifugation at 11,000 *x g* for 5 min, the pellet was collected in a tube and dried at 37°C for 2 min. To the dried, cleaned plasmid, 5 µl of the restriction enzyme solution containing 1 µl of *Eco*R I buffer, 1µl of RNase A, 1 µl of *Eco*R I and 2 µl of RNase-free water prepared just before use was added. The contents of the tube was mixed by tapping and spun down, then incubated at 37°C for 2 hr. The inserted plasmid was checked by agarose gel electrophoresis using a non-inserted plasmid (single blue colony), which showed a smaller MW than inserted plasmid as a control. The positive clones were picked from the new overnight culture

plate and grown further in 3.2 ml of liquid medium at 37^{o}C overnight with moderate shaking at 200 rpm. The plasmid was isolated to verify the nucleotide sequence of the inserted DNA.

1.8 Isolation of plasmid DNA using the QIAprep Spin Miniprep Kit (QIAGEN).

Plasmid DNA was purified from XL1-Blue MRF´ cells by the alkaline lysis method, as from QIAprep Spin Miniprep Kit (QIAGEN). Overnight cell cultures were incubated at 4^{o}C for 30 min before precipitating by centrifugation at 5,000 *x g* for 5 min. The supernatant was discarded by pipetting and the pellet was resuspended with 250 µl of ribonuclease (RNase)-containing buffer (P1) and transferred to a 1.5 ml microcentrifuge tube. Then, 250 µl of sodium hydroxide-containing buffer (P2) was added, and the tube was mixed gently by inversion 4-6 times before adding and immediately mixing in 350 µl of acetic acid-guanidine hydrochloride-containing buffer (N3). The supernatant was collected after precipitation at 11,000 *x g* for 10 min and applied to a QIA spin column placed in a 1.5 ml microcentrifuge tube. The column was centrifuged at 11,000 *x g* for 30-60 sec to discard the flow-through. The plasmid DNA trapped in the column was washed 2 times, first, with 0.5 ml of isopropanol-guanidine hydrochloride-containing buffer (PB), second, with 0.75 ml of ethanol-containing buffer (PE) followed by centrifugation after each wash. The residual washing buffer was removed by an additional centrifugation for 1 min, then the column was removed to a new microcentrifuge tube and 30 µl of 10 mM Tris-HCl buffer, pH 8.5, containing EDTA (EB) was added to the column center . After 1 min the column was centrifuged for 1 min. The plasmid DNA in the flow-through was collected and diluted 1:70 with sterile water. DNA concentration was determined

by a spectrophotometer using the A_{260} (1 A_{260} DNA = 50 µg/ml) and the purity of the isolated DNA was judged by the A_{260}/A_{280} ratio (pure DNA solution has a ratio of 1.9-2.0).

1.9 Automate DNA sequencing

DNA fragments containing the sequence of interest were sequenced by the dideoxynucleotide chain termination method (Sanger *et al*., 1977) using the ABI PRISM™ BigDye terminator V 1.1 cycle sequencing kit (ABI). The PCR cocktail contained 2 µl of Big Dye Terminator ready reaction mix, 1 µl of Big Dye sequencing buffer, 4 µl of 150-300 ng DNA template, 1µl (1.6 pmole) of an apropiate primer, and sterile water up to 10 µl of total volume. The amplification reaction was carried out as follows: initial denaturation at 96°C for 1 min, 25 cycles of denaturation, annealing and extension at 96°C for 10 sec, 66°C for 5 sec and 60°C for 4 min, respectively, and final extension at 60°C for 7 min. The amplification product was purified by ethanol precipitation in which the 10 µl of PCR product was applied into a 1.5 ml microcentrifuge tube containing 2.5 µl of 125 mM EDTA and 30 µl of absolute ethanol, mixed briefly and left for 15 min at RT. The DNA pellet was precipitated by centrifugal fractionation at 11,000 *x g*, 4 °C for 20 min followed by 75% isopropanol precipitation and then centrifugation again for 5 min. Then, the supernatant was immediately removed by pipetting. The DNA pellet was dried at 90°C for 1 min and 4 µl of loading dye was added. The dyed-labeled DNA was analyzed on an Applied Biosystems 377 sequencer (Applied Biosystems, CA, USA).

2. Generating the full-length cDNA encoding RBP

Generating of the 5′ and 3′ cDNA ends for RBP was performed as in the GeneRacer™ Kit (RLM-RACE) protocol (Invitrogen) as diagrammed in Figure 13.

2.1 5′ RNA dephosphorylation

Five micrograms of total RNA prepared by method 1.1 was treated with calf intestinal phosphate (CIP) to remove the 5′ phosphate at 50°C for 1 hr. The treated RNA was extracted with phenol and precipitated with ethanol. Then, 90 µl of RNase free water and 100 µl of phenol:chloroform (1:1) were added, mixed vigorously, and then centrifuged at 11,000 *x g*, RT for 5 min. The supernatant was collected and applied to a new 1.5 ml microcentrifuge tube containing 2 µl of 10 mg/ml mussel glycogen, and 10 µl of 3 M sodium acetate, pH 5.2. The contents were mixed by tapping the tube and then 220 µl of 95% ethanol was added. The tube was placed in dry ice for 10 min before centrifugation at 11,000 *x g*, 4°C for 20 min. The collected pellet was washed with 500 µl of 70% ethanol, followed by centrifugation at 11,000 *x g*, 4°C for 2 min. The supernatant was discarded and the clear pellet of dephosphorylated RNA was carefully collected by pipette, then dried at RT and resuspended with 7 µl of RNase-free water.

2.2 Removing 5′ cap structure

Dephosphorylated RNA was incubated with 1 µl of 10x TAP buffer, 1 µl of RNaseOut™, 1 µl of TAP (tobacco acid pyrophosphatase) at 37°C for 1 hr and phenol extraction and ethanol precipitation was performed as described in method 2.1.

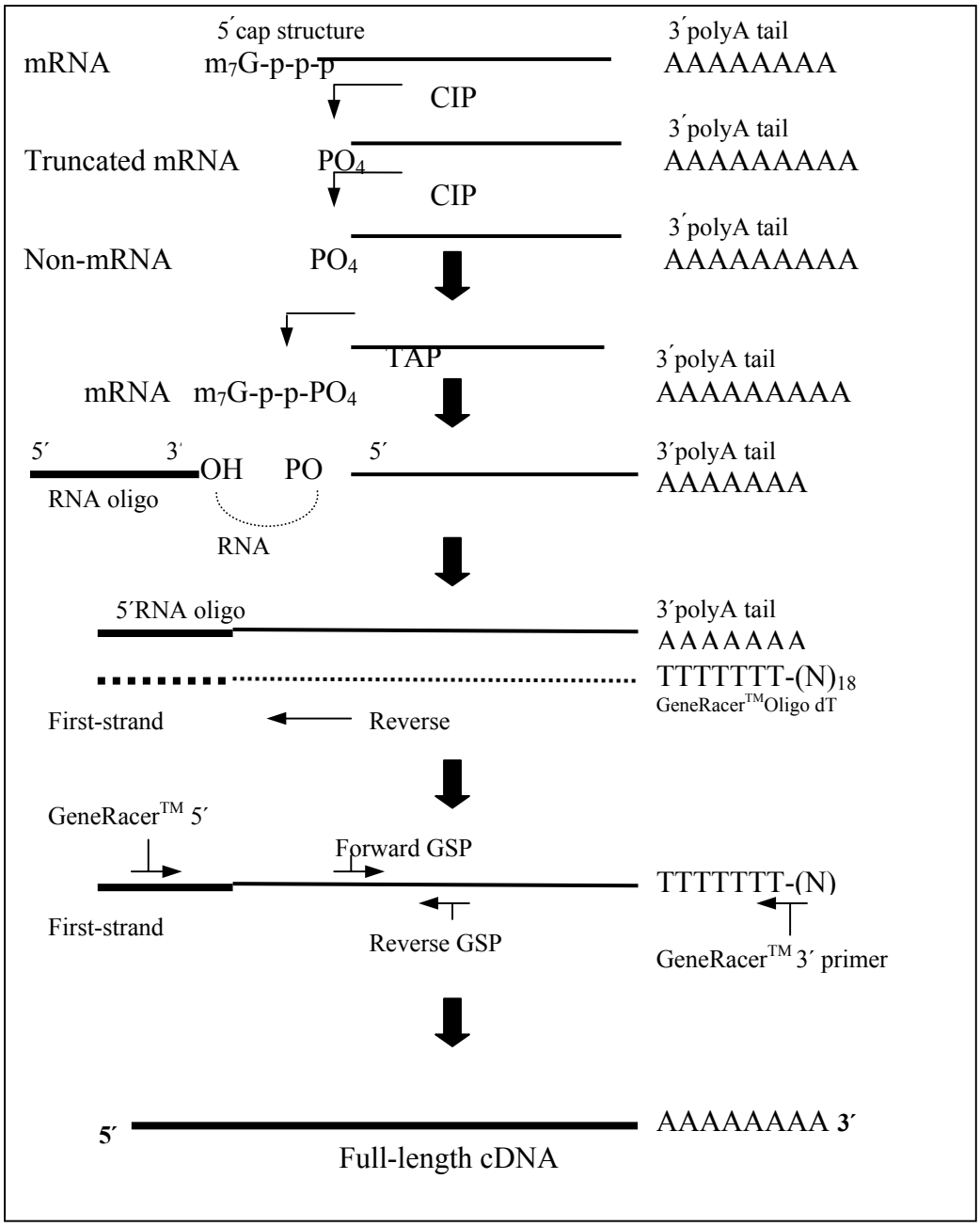

Figure 13. 5′ and 3′ Rapid Amplification cDNA Ends (RACE) diagram.

2.3 Ligation of 5′ end mRNA by GeneRacer™ RNA (Oligo) dT primer

The full-length mRNA was ligated to the Gene Racer RNA oligo sequence at the 5′ end. Firstly, 7 µl of decapped RNA was incubated in the tube of lyophilized GeneRacer™ RNA Oligo at 65 °C for 5 min to remove RNA secondary structure. Secondly, the tube was placed on ice for 2 min, after which four solutions were added: 1 µl of 10x ligase buffer, 1 µl of 10 mM ATP, 1µl of RNaseOut™, and 1 µl of T4 RNA ligase, followed by incubation at 37 °C for 1 hr. The ligated RNA was purified by phenol extraction and ethanol precipitation as described in method 2.1.

2.4 First strand cDNA synthesis

The ligated mRNA was used as template to synthesize the first strand cDNA. The template (10 µl) was incubated together with 1µl of GeneRacer™ RNA oligo dT primer and 1 µl of 10 mM dNTP, and mixed at 65 °C for 5 min to remove the secondary structure of RNA. The tube was placed on ice and four solutions were then added: 4 µl of 5x first strand buffer, 2 µl of 0.1 M DTT, 1 µl of RNaseOut, and 1 µl of Superscript™ III reverse transcriptase. The subsequent incubation at 42 °C for 50 min was followed by denaturation of the reverse-transcriptase at 70 °C for 15 min, and then 1 µl of RNase H was added to digest the RNA. The GeneRacer Oligo (dT)-primed cDNA was used as template to amplify the 5′ and 3′ ends of the cDNA by PCR.

2.5 Amplification of 5′and 3′end of cDNA (5′ and 3′ RACE) by using gene specific primer (GSP)

5′ or 3′ RACE PCR reaction were performed with either 3 µl of GeneRacer™ 5′ primer and 1 µl of GSP1 (Table 4), or 3 µl of GeneRacer™ 3′ primer and 1 µl of

GSP2 (Table 4). The primers were separately mixed with 1 µl of cDNA template, 5 µl of 10x High Fidelity PCR buffer, 2.5 µl of 10 mM dNTP mix solution, 0.5 µl of Platinum *Taq* DNA polymerase (Invitrogen), 2 µl of 50 mM $MgSO_4$ and 36.5 µl of RNase free water. The following program was executed in the PCR thermo cycler: 94°C for 2 min, 5 cycles of 94°C for 30 sec and 72°C for 2 min, 5 cycles of 94°C for 30 sec, and 70°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 66°C for 30 sec, and 72°C for 2 min and finally 72°C for 10 min. The PCR product was analyzed in 1.5% agarose gel electrophoresis (method 1.3) and purified using either the QIAquick PCR purification kit (QIAGEN) (method 1.4) or QIAquick gel extraction Kit (QIAGEN).

2.6 Purification of DNA fragments using the QIAquick Gel Extraction Kit (Qiagen)

The expected DNA fragment was excised from the agarose gel in a small piece and weighed. The excised gel was transferred into a 1.5 ml microcentrifuge tube and dissolved with 3 volumes of QG buffer (Guanidine thiocyanate, pH ≤ 7.5) to one volume of gel. The tube was incubated at 50 °C for 10 min or until the gel was completely melted, with mixing by vortex 2-3 times during the incubation. One gel volume of isopropanol was added to the mixture. The DNA fragment was bound to a silica membrane by applying the mixture into a QIAquick column placed in a 2 ml collection tube and centrifuging it for 1 min at 11,000 *x g*. The residual agarose gel was removed from the DNA fragment by adding washing buffer (ethanol-containing PE buffer) and centrifuging for 1 min at 11,000 *x g*. The QIAquick column was placed into a clean 1.5 ml microcentrifuge tube and the DNA fragment was eluted

with 30 ml of EB buffer (10 mM Tris-HCl, pH 8.5) by centrifugation at 11,000 *x g* for 1 min.

2.7 Ligation of DNA fragments into the pCR®4 -TOPO® vector (Invitrogen Life Technologies, USA).

The purified cDNA fragment was ligated with the pCR®4 -TOPO® vector (Figure 11) according to the manufacture's instruction. Five microliters of the reaction mixture containing 4 µl of fresh PCR product, 1 µl of salt solution, and 1 µl of TOPO® vector was mixed gently and incubated at 22-23°C for 30 min and placed on ice, then the ligated plasmids were further transformed into TOP10 chemically competent cells.

2.8 Transformation of plasmid into *E. coli* TOP10 chemically competent cells (Invitrogen)

Frozen competent cells 50 µl in a microcentrifuge tube were taken from -80 °C, thawed on ice and mixed gently with 2 µl of the plasmid DNA cloning reaction from method 1.6, then placed on ice for 30 min. The transformed cells were heat-shocked at 42 °C for 30 seconds without shaking, then immediately chilled on ice for 5 min before adding 250 µl of room temperature S.O.C. medium. The tube was incubated at 37 °C for 1 hr with horizontally shaking at 200 rpm. After incubation, 100µl of cell culture was spread on a prewarmed selective LB medium plus 100 µ g/ml ampicillin (LBA) in plate, which was previously spread with 100 µl of 100 mM IPTG in water and 20 µl of 40 µg/ml X-gal in dimethylformamide (DMF), and then incubated at 37°C overnight.

2.9 Analysis of the positive clones from transformation

The positive clone of interest was identified as described in method 1.7, and then the plasmid DNA was purified from overnight culture TOP10 chemically competent cells as described in method 1.8. After that DNA fragments containing the sequence of interest were sequenced as described in method 1.9.