CHAPTER III

MATERIALS AND METHODS

3.1 Protein preparation

3.1.1 Vitellogenin and Vitellin preparation

Individual unfertilized eggs were homogenized in 0.5 mM EDTA in PBS. The pellet and lipid layer were eliminated after centrifugation at 10,000 g, 4°C, for 10 min. The protein content of the extract was determined by Bio-Rad Protein Assay. The results from extraction of vitellin were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis which was performed in slap gels in mini-PROTEIN II electrophoresis apparatus (Bio- Rad) to identify vitellin in the extract. Vitellin from unfertilized eggs extract and vitellogenin from serum were applied to 12% gel for SDS-PAGE according to the Bio-Rad Manual. These proteins were stained for total protein, phosphoprotein, lipoprotein and glycoprotein.

Purified VTG that extracted from serum of Greenback Mullet (*Liza subviridis*) were obtained from Center of excellent for Marine Biotechnology (CEMB), Chulalongkorn University, Thailand.

3.1.2 Zona radiata protein preparation

Individual unfertilized eggs were immersed in large 100 mM EDTA, 500 mM NaCl,pH 8.5 and were homogenized in the same buffer. The pellet of eggshell was obtained after centrifugation at 10,000 g, 4 ^{O}C, for 10 min. The eggshell pellet was washed in 500 mM NaCl for 48 hrs at 5 ^{O}C, for 6 times. Following the 500 mM NaCl wash, the eggshell was washed with distilled water for 48 hrs at 5 ^{O}C, for 6 times. The eggshell was then freeze-dried.

The dried eggshell was solubilized in 100 mM Tris/HCl, pH 8.8 containing 8M urea, 1% SDS, 300 mM Mercaptoethanol and 10 mM EDTA for 20 min at 70 ^{O}C. After the solubilized eggshell was sonicated for 60 sec at room temperature, this solubilized solution was incubated for 5 min at 70 ^{O}C again and repeat sonication. The soluble protein was dialyzed against 50% glycerol and 50mM Tris, pH 8.8 at room temperature. After dialysis, the solution was centrifuged and supernatant was collected. The protein content of the extract was determined by Bio-Rad Protein Assay. This ZRP extract was used for immunization.

3.2 Antigen preparation

Vitellin extract were adjusted to 5 mg/ml with PBS and divided for immunization in both native and denatured forms. Vitellin extract was denatured by mixing 1:1 with 2x treatment buffer (for SDS-PAGE), boiled for 1 minute and dialysed against 4 changes of distilled water for 48 h and 2 changes of PBS for 36 h. Both native and denatured vitellins were used for immunization. Similar procedure was carried out for the immunization of ZRP by replacing vitellin with ZRP.

3.3 Immunization

Four ICR mouse were injected intraperitoneally with nature and denatured vitellin (in a 1:1 ratio) or mixed with complete Freund's adjuvant at the ratio of 1:1 (v/v). At 2-week intervals, mice were subsequently injected 3 more times with nature and denatured vitellin mixed with incomplete Freund's adjuvant for the following injections. One week after the fourth injection, mouse antisera were collected and tested against vitellin extract in native and denatured forms as well as female and male serum by dot blot assays, Western blot, and immunohistochemistry. The best performing mouse was later boosted 3 days before hybridoma production. For ZRP

immunization, similar procedure was followed except using ZRP instead of vitellin for immunization.

3.4 Hybridoma production

A cell fusion procedure was adapted from the method developed by KÖhler and Milstein (1976) with modifications described by Mosmann, Bauman, and Williamson (1979). A P3X myeloma cell line was used as the fusion partner. Fusion products from 1 mouse were plated on 40 microculture plates (96 wells per plate). After identification of positive cultures by screening methods described in the next section, cells were re-cloned at least twice by the limiting dilution method (Eshhar, 1985), kept in 12% DMSO and stored in liquid nitrogen.

3.5 Screening methods

3.5.1 Primary screening by Dot blotting

Vitellin extract in both native and denatured forms were used for screening. Both vitellins (1 μl/spot) were applied to a nitrocellulose membrane and subsequently incubated in 5% Blotto for 10 min and then incubated in hybridoma conditioned media from each clone (1:20 dilution in 5% Blotto) for 3 h. After extensive washing in PBS, the membrane was incubated in horseradish peroxidase labelled goat anti-mouse IgG heavy and light chain specific (GAM-HRP; BIO-RAD) antibody at 1:1500 dilution for 3 h. The membrane was then washed for 4 times in PBS with 10 min intervals and incubated in a substrate mixture containing 0.03% diaminobenzidine (DAB), 0.006% hydrogen peroxide, 0.05% cobalt chloride in PBS (Sithigorngul et al., 2000). The intensity of the reaction with each MAb was compared by eye and scored relative to the reaction against vitellin extract. Hybridoma clones that displayed immunoreactivity against vitellin extract were confirmed for vitellogenin specificity by Western blot and immunohistochemistry before cloning and cryopreservation for

further investigation. For screening methods of ZRP, similar procedure was followed except using ZRP in the replacement of vitellin.

3.5.2 Secondary screening

3.5.2.1 Dot blotting

Selected antigens, vitellin extract in native and denatured forms and female and male serum, were used for the second step of VTG screening and ZRP, denatured vitellin, female and male serum were used for the second step of ZRP screening. All selected antigens (1 µl/spot) were applied to a nitrocellulose membrane and subsequently incubated in 5% Blotto for 10 min and then incubated in hybridoma conditioned media from each clone (1:20 dilution in 5% Blotto) for 3 h. After extensive washing in PBS, the membrane was incubated in horseradish peroxidase labelled goat anti-mouse IgG heavy and light chain specific (GAM-HRP; BIO-RAD) antibody at 1:1500 dilution for 3 h. The membrane was then washed for 4 times in PBS with 10 min intervals and incubated in a substrate mixture containing 0.03% diaminobenzidine (DAB), 0.006% hydrogen peroxide, 0.05% cobalt chloride in PBS (Sithigorngul et al., 2000). The intensity of the reaction with each MAb was compared by eye and scored relative to the reaction against vitellin extract in native and denatured froms and female and male serum. Hybridoma clones that displayed immunoreactivity against vitellin extract and female serum (vitellogenin), but not against male serum (control), were confirmed for vitellogenin specificity by Western blot and immunohistochemistry before cloning and cryopreservation for further investigation.

3.5.2.2 Western blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in slap gels in mini-PROTEIN II electrophoresis apparatus (Bio-Rad) to identify vitellin and ZRP in the extract. Vitellin and ZRP from unfertilized eggs extract or serum were applied to 12% gel for SDS-PAGE according to the Bio-Rad Manual. Proteins in the gel were transferred to nitrocellulose sheet using Transblot apparatus (Bio-Rad) at 50 V for 3 h. The nitrocellulose membrane was incubated in 5% Blotto for 10 min, treated with 1:200 hybridoma conditioned media for 3 h and then processed as described above in dot-blotting. Low and high molecular standard markers (BIO-RAD) were used as standards.

3.5.2.3 Immunohistochemistry

Cephalothoraces of Greenback Mullet (*Liza subviridis*) oocyte were cut and fixed in Davidson's fixative solution for 24 h then processed for paraffin sectioning. Serial section (8 μm thickness) were prepared and permeabilized with 1% Triton X-100 in PBS for 30 min at 37 °C and were washed three times in PBS with 5 min intervals, then blocked with $P1^{+}$(10% fetal bovine serum in PBS with 0.1% merthiolate) and stored in a moist chamber at 4°C until use.

The section were processed for indirect immunoperoxidase staining using various MAbs and GAM-HRP diluted to 1:1000 with 10% calf serum in PBS. Peroxidase activity was revealed by incubation with 0.03% DAB and 0.006% H_2O_2 in PBS. Preparations were counter-stained with hematoxylin and eosin Y (H&E), dehydrated in a graded ethanol series, cleared in xylene and mounted in permount (Sithigorngul et al. 2000; 2002). Positive reaction was visualized as brown coloration against the pink and purple of H&E.

3.6 Monoclonal antibodies characterization

3.6.1 Polyacrylamide gel electrophoresis and immunoblotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in slap gels in mini-PROTEIN II electrophoresis apparatus (Bio- Rad) to identify vitellin or ZRP in the extract and vitellogenin or ZRP in serum. Unfertilized eggs extract or serum was applied to 12% gel for SDS-PAGE according to the Bio-Rad Manual. Parts of the gel were cut off and visualized by staining with 0.1% Coomassie brilliant R250 for total proteins, with Sudan black B for lipoproteins, with phosphoprotein staining for phosphoprotein, with periodic acid Schiff reagent (PAS) for glycoproteins. Proteins in the gel were transferred to nitrocellulose sheet using Transblot apparatus (Bio-Rad) at 50 V for 3 h. The nitrocellulose sheet was then separated from the gel and quenched in 5% blotto, cut into strips and assayed for antibody binding as described for the dot blotting assay.

3.6.2 Isotype and subisotype determination

Isotype and subisotype of mouse immunoglobulin produced by hybridomas were determined by sandwich ELISA using Zymed's Mouse MonoAb ID Kit (HRP).

3.6.3 Epitope of antibody determination

The epitope of MAbs were determined using indirected ELISA. Fifty microliter of vitellin extract was added to the microtiterplate wells (10 µg/well). After the plates were incubated in humidified box at 4° C overnight, the solution was decant and the plates were slapped onto paper tower until dry. Immediately all wells were washed 4 times with 150 µl of 0.5% skim milk, for 10 min. Then, 100 µl of diluted MAbs (1:20) were added into the well of each row and column and left with rocking

at 4° C overnight (Table 3.1). After the plates were incubated in humidified box at 4° C overnight, the solution was decant and plates were slapped onto paper tower until dried. Immediately all wells were washed 4 times with 150 µl of 0.5% skim milk, for 10 min. Next, 100 µl of diluted HRP-GAM were added to each well and incubated for 7 h. Washing step were repeated 4 times with 150 µl of PBS, for 10 min. To develop the reaction, 100 µl of OPD working substrate solution (0.1M Sodium citrate, 0.002 %H_2O_2) were added to each well and incubated for 5 min before the reaction was stopped by adding 100 µl of 1N H_2SO_4 per well. The OD was measured at 490 nm by an ELISA reader

3.7 Determination of vitellogenin and Zona radiata protein in experiment fish

3.7.1 Experiment fish

Juveniles of Greenback Mullet (*Liza subviridis*) were held in 4 experiment tanks, each tank contain 15 fish. After acclimatizing for 1 week, fish were injected with 0, 2.5, 5, and 50 mg of estradiol (E2) /kg body weight. After 2 week of E2 injection, fish were anesthetized in 150 ppm of MS-222 and blood was collected from caudal vein. Blood samples were centrifuged at 5000 rpm for 5 min, plasma were collected and frozen at -20°C.

3.7.2 Polyacrylamide gel electrophoresis and immunoblotting

The procedures of Polyacrylamide gel electrophoresis and immunoblotting were processed in the same manner as described above.

3.7.3 Competitive ELISA

VTG and ZRP content in serum of experiment fish were determined by Indirect competitive ELISA. For the method of VTG, the ovarian extract (5 μg/well protein) was plates (NUNC). Blotto (5% or 0.5% nonfat dry milk in PBS, washed as blocking solution, antibody diluent, sample diluent, and washing solution). VTG antibodies was performed using combination of 4 monoclonal antibodies (VTG-64, 90, 486 and 496) at the dilution of 1:800. For the method of ZRP, the ZRP extract (2 μg/well protein) was plates and ZRP antibodies was performed using combination of 3 monoclonal antibodies (ZRP-9, 68 and 102) at the dilution of 1:1000.

The range of VTG and ZRP concentration that could be measured by these monoclonal antibodies was determined by Competitive ELISA using the lowest concentration of each monoclonal antibody that gave maximal absorbance mixed with various dilutions of VTG and ZRP or blood samples. The intra-assay and inter-assay variations were tested using 2 serum sample at a low concentration and a high concentration.

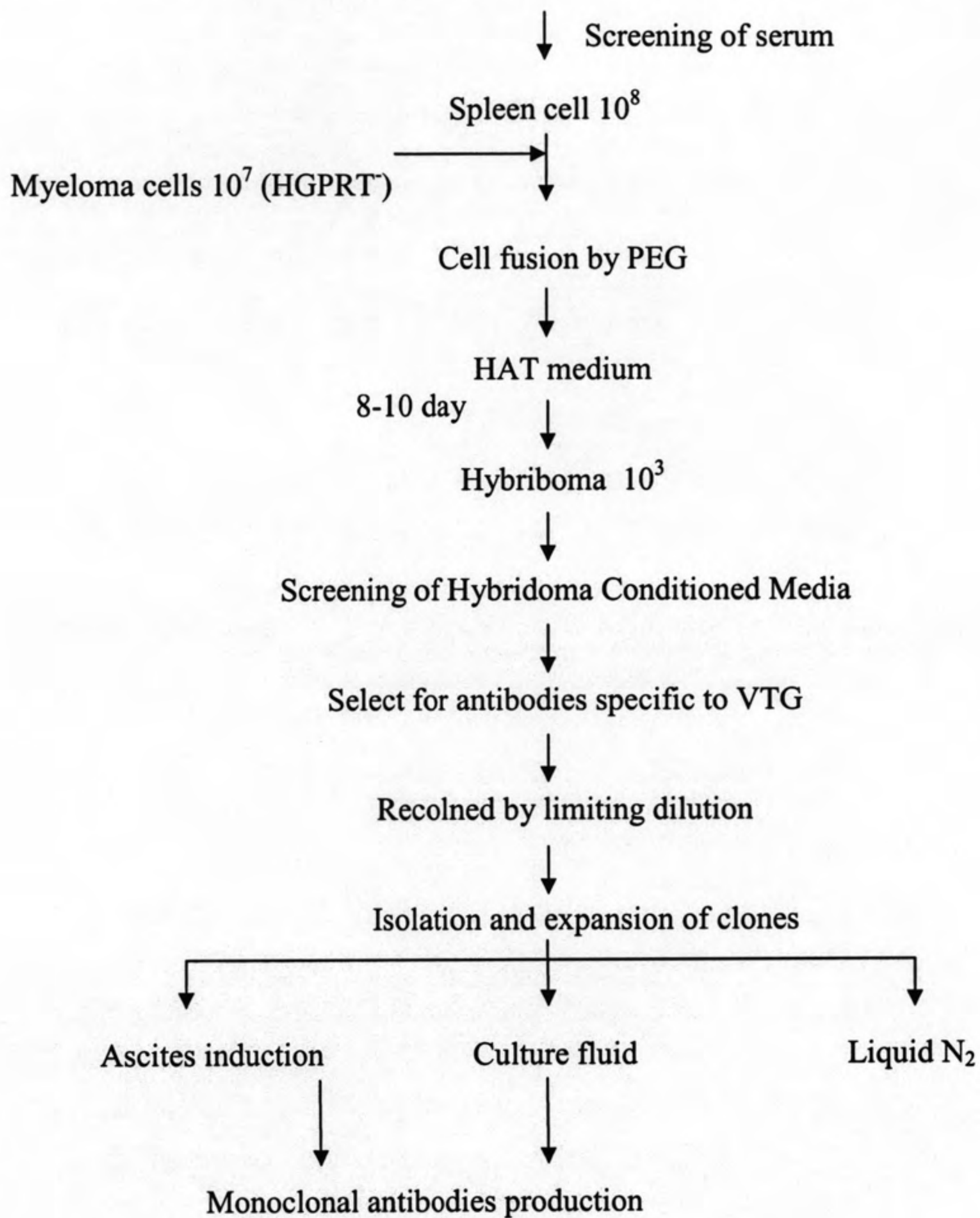


Figure 3.1 Diagram of hybridoma production (Sithigorngul et al., 2000).

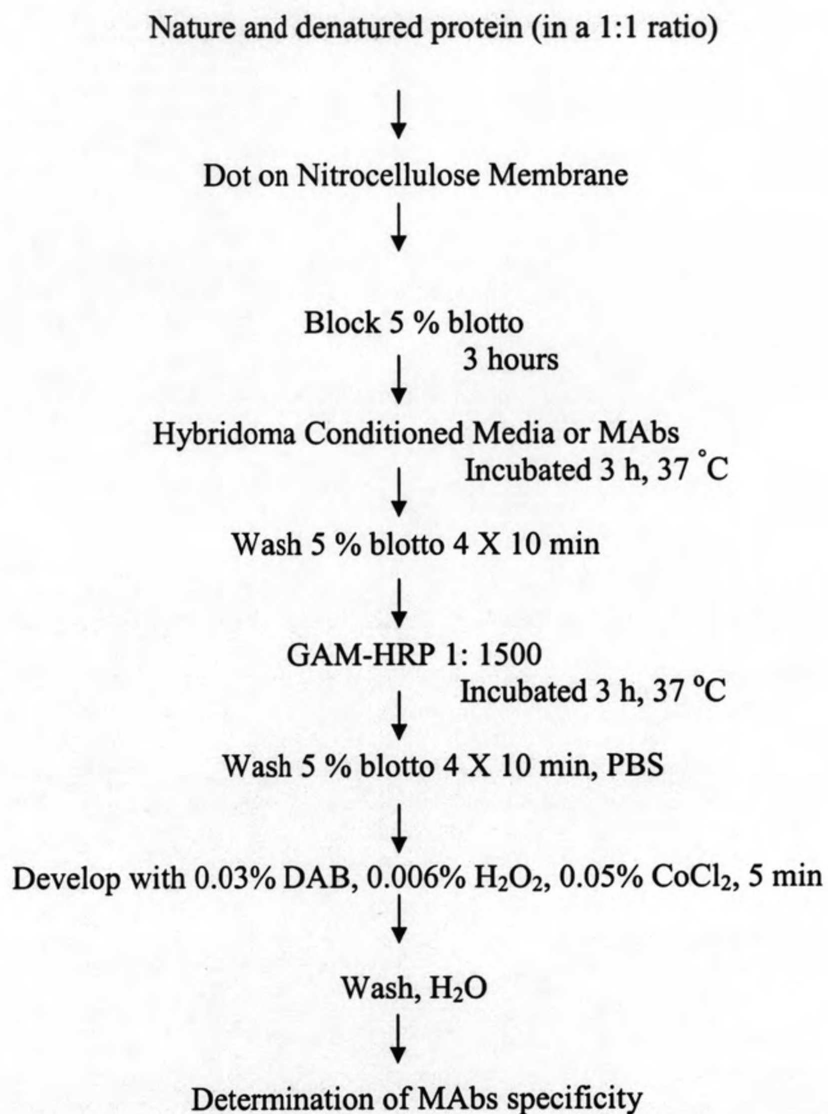


Figure 3.2 Diagram of dot blotting for screening method and characterization of monoclonal antibodies (Sithigorngul et al., 2000).

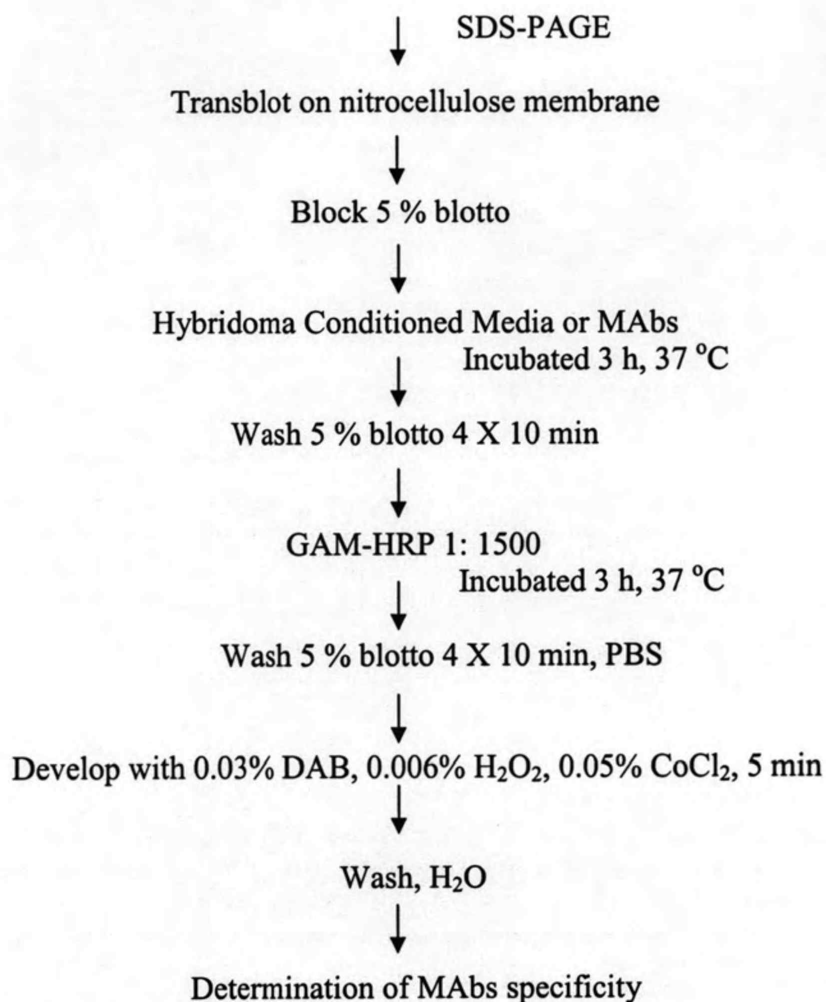


Figure 3.3 Diagram of Western blot analysis for screening method and characterization of monoclonal antibodies (Sithigorngul et al., 2000).

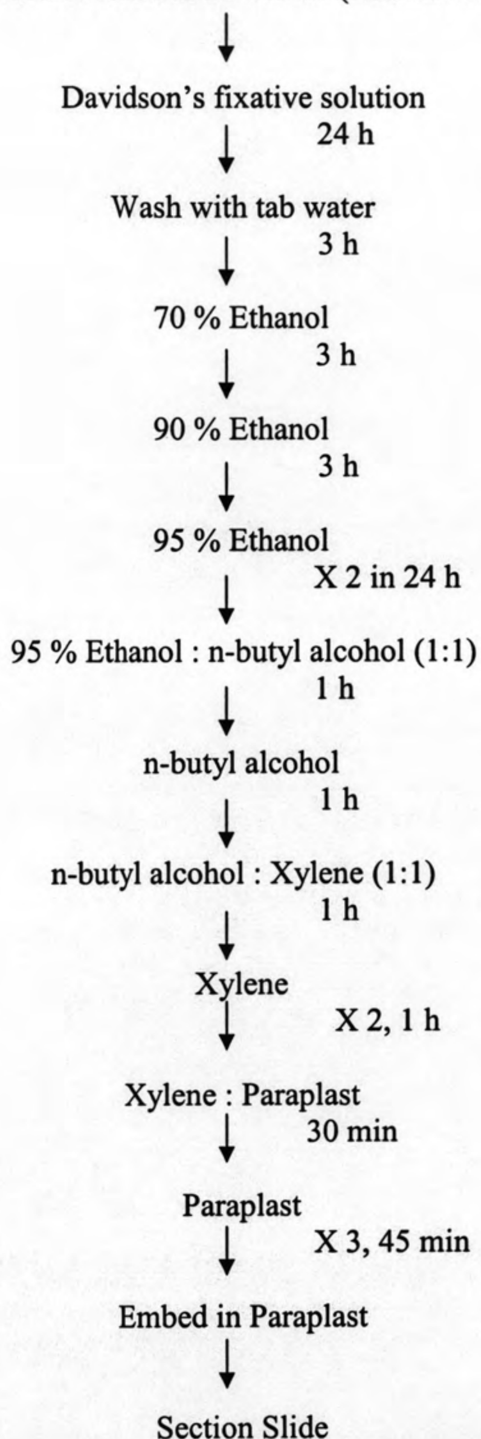


Figure 3.4 Diagram of Immunohistochemistry for characterization of monoclonal antibodies (Sithigorngul et al., 2000).

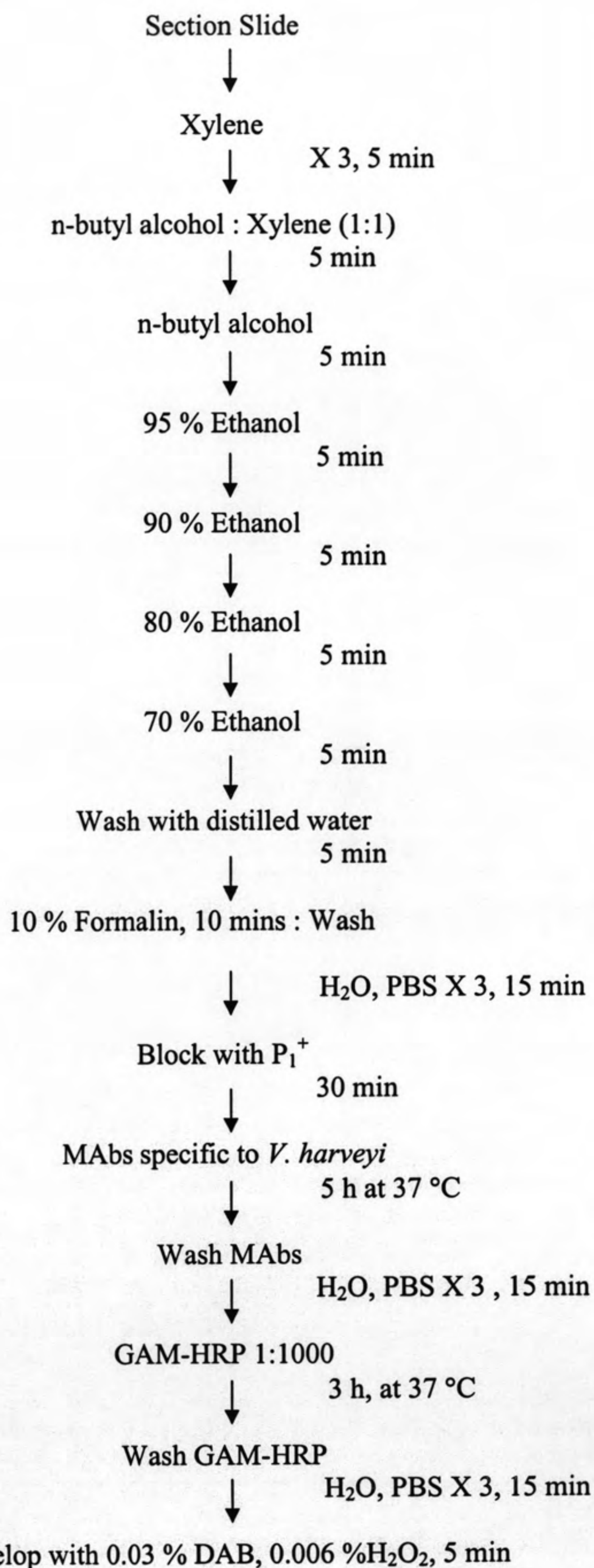
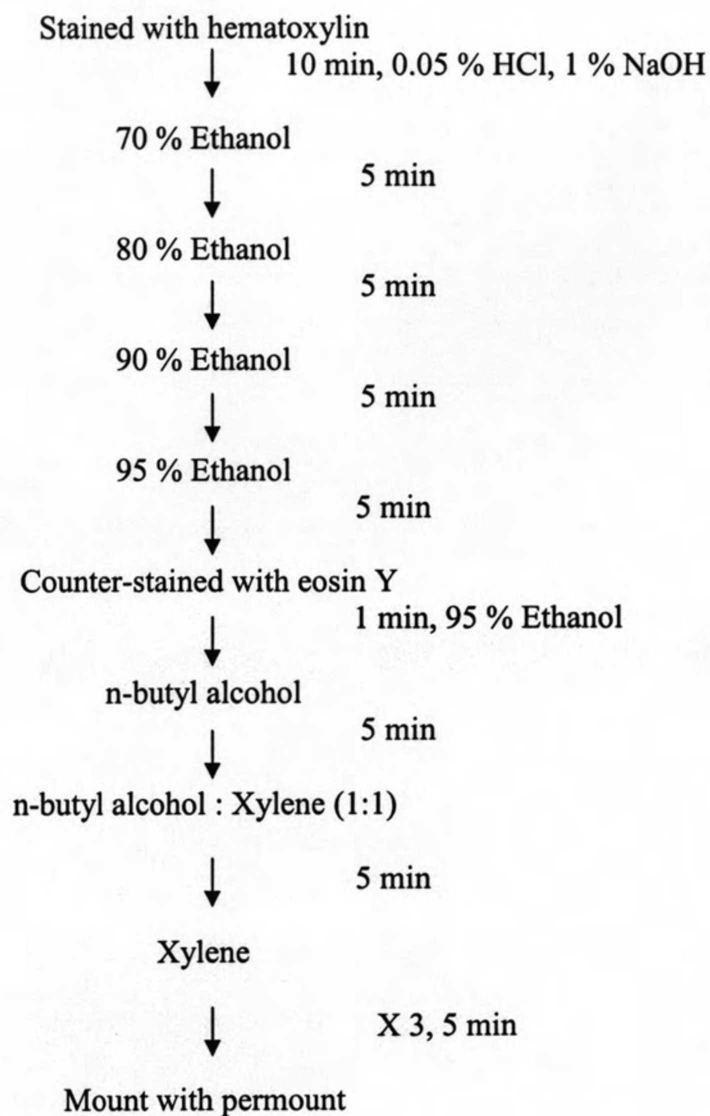


Figure 3.4 (Cont.)



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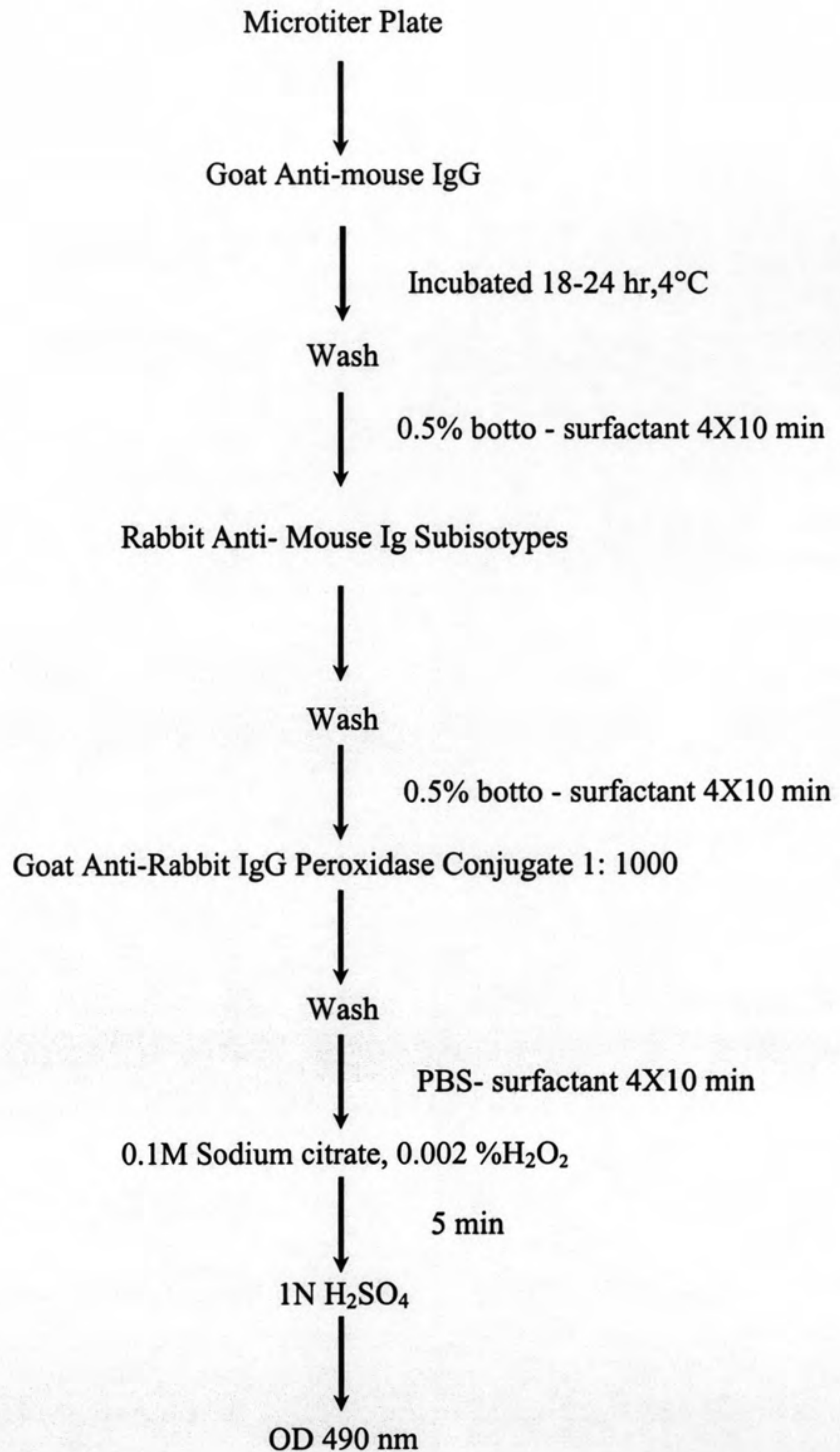

Figure 3.5 Diagram of sandwich ELISA for determination of antibody isotype and subisotype (Longyant et al., 1999)

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Table 3.1 VTG-MAb mixtures for determination of MAb epitope

MAbs	1 VTG90	2 VTG496	3 VTG64	4 VTG149	5 VTG313	6 VTG144	7 VTG483	8 VTG183	9 VTG231	10 VTG530	11 VTG313
1 VTG90	1+1	1+2	1+3	1+4	1+5	1+6	1+7	1+8	1+9	1+10	1+11
2 VTG496		2+2	2+3	2+4	2+5	2+6	2+7	2+8	2+9	2+10	2+11
3 VTG64			3+3	3+4	3+5	3+6	3+7	3+8	3+9	3+10	3+11
4 VTG149				4+4	4+5	4+6	4+7	4+8	4+9	4+10	4+11
5 VTG313					5+5	5+6	5+7	5+8	5+9	5+10	5+11
6 VTG144						6+6	6+7	6+8	6+9	6+10	6+11
7 VTG483							7+7	7+8	7+9	7+10	7+11
8 VTG183								8+8	8+9	8+10	8+11
9 VTG231									9+9	9+10	9+11
10 VTG530										10+10	10+11
11 VTG313											11+11

Table 3.2 ZRP-MAb mixtures for determination of MAb epitope

MAbs	1 ZRP9	2 ZRP30	3 ZRP72	4 ZRP109	5 ZRP21	6 ZRP124	7 ZRP68	8 ZRP110	9 ZRP125	10 ZRP102	11 ZRP113
1 ZRP9	1+1	1+2	1+3	1+4	1+5	1+6	1+7	1+8	1+9	1+10	1+11
2 ZRP30		2+2	2+3	2+4	2+5	2+6	2+7	2+8	2+9	2+10	2+11
3 ZRP72			3+3	3+4	3+5	3+6	3+7	3+8	3+9	3+10	3+11
4 ZRP109				4+4	4+5	4+6	4+7	4+8	4+9	4+10	4+11
5 ZRP21					5+5	5+6	5+7	5+8	5+9	5+10	5+11
6 ZRP124						6+6	6+7	6+8	6+9	6+10	6+11
7 ZRP68							7+7	7+8	7+9	7+10	7+11
8 ZRP110								8+8	8+9	8+10	8+11
9 ZRP125									9+9	9+10	9+11
10 ZRP102										10+10	10+11
11 ZRP113											11+11

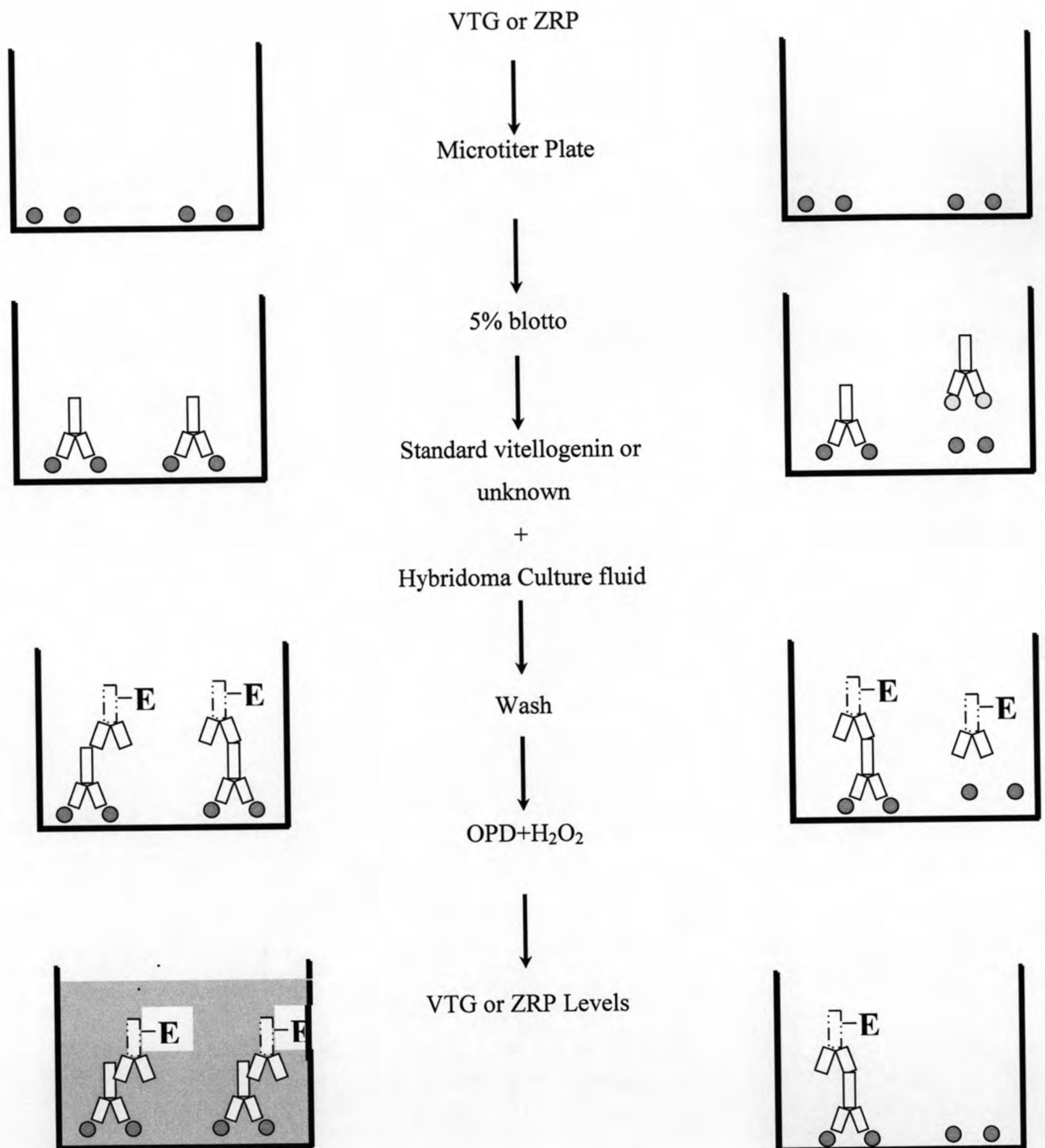

Figure 3.6 Diagram of competitive ELISA for determination of VTG or ZRP levels (Longyant et al., 1999)