THAI AGRICULTURAL STANDARD

TAS 10452-2009

DIAGNOSIS OF YELLOW HEAD DISEASE IN SHRIMP

National Bureau of Agricultural Commodity and Food Standards
Ministry of Agriculture and Cooperatives
ICS 11.220 **ISBN 978-974-403-673-5**

UNOFFICAL TRANSLATION

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Published in the Royal Gazette Vol.126 Special Section 187D,
dated 28 December B.E.2552 (2009)

Technical Committee on the Elaboration of the Thai Agricultural Standard for Diagnosis of Yellow Head Disease in Shrimp

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The shrimp industry in Thailand is important and generates high income for the country. There are still problems related to epizootic diseases caused by viruses, which inflict on losses of quality and quantity outputs to shrimp farmers. The issue of diseases is also used as a barrier to strictly control the products from Thailand by importing countries.

Yellow head disease is caused by a virus bringing serious losses to the shrimp aquaculture business. The Agricultural Standards Committee deemed it necessary to establish the Thai Agricultural Standard on Diagnosis of Yellow Head Disease in Shrimp to be used as guideline for laboratory diagnosis of yellow head disease in shrimp and a reference for the certification of shrimp farms.

The standard is based on the information of the following documents:

National Bureau of Agricultural Commodity and Food Standards (ACFS). 2007. The establishment of standards for marine shrimp hatcheries and grown out farm in a protected areas that may have problems on the outbreak of white spot disease, yellow head disease and Taura syndrome. A collaborative project with the Department of Fisheries.

World Organization for Animal Health (OIE). 2009. Chapter 2.2.7. Yellow Head Disease. Manual of Diagnostic Tests for Aquatic Animals. Paris, France.

NOTIFICATION OF THE MINISTRY OF AGRICULTURE AND COOPERATIVES
SUBJECT: THAI AGRICULTURAL STANDARD:
DIAGNOSIS OF YELLOW HEAD DISEASE IN SHRIMP
UNDER THE AGRICULTURAL STANDARDS ACT B.E. 2551 (2008)

Whereas the Agricultural Standards Committee deems it necessary to establish an agricultural standard on Diagnosis of Yellow Head Disease in Shrimp as voluntary standard in accordance with the Agricultural Standards Act B.E. 2551 (2008) to promote such agricultural commodity standard to meet its quality standard and safety.

By virtue of Section 5, Section 15 and Section 16 of the Agricultural Standards Act B.E. 2551(2008), the Minister of Agriculture and Cooperatives hereby issues this Notification on Establishment of Agricultural Standard: Diagnosis of Yellow Head Disease in Shrimp (TAS 10452-2009) as voluntary standard, details of which are attached herewith.

Notified on 30 September B.E. 2552 (2009)

(Mr. Theera Wongsamut)
Minister of Agriculture and Cooperatives

THAI AGRICULTURAL STANDARD
DIAGNOSIS OF YELLOW HEAD DISEASE IN SHRIMP

1 SCOPE

The Thai Agricultural Standard establishes details for the diagnosis of yellow head disease in the laboratory using the rapid staining method, staining of haemolymph smear method, histopathological method, reverse transcription polymerase chain reaction method and *in-situ* hybridization method.

2 DEFINITIONS

For the purpose of this standard:

2.1 **Shrimp** mean invertebrate animals in the Family *Penaeidae*

2.2 **Yellow head disease (YHD**) means a disease occurring in shrimp caused by yellow head virus (YHV), characterized by yellowish discoloration of the cephalothorax caused by the underlying yellow hepatopancreas and pale body. Infected shrimp die quickly at a high rate.

2.3 **Diagnosis** means test or inspection to analyse and determine the presence of a disease.

2.4 **Larva** means newly hatched larval shrimp that will undergo metamorphosis into the stages of nauplius, zoea and mysis within approximately 8-11 days.

2.5 **Postlarva** (**PL)** means young shrimp that have the same appendages as adult shrimp, are about 5 mm long, and will grow from mysis stage to juvenile stage in about 25 days or more. The convention is to designate post larva shrimp with the abbreviation “PL” followed by a number that means the number of days since they passed from mysis to post larva stage. For example, “PL21” means shrimp that have been at the post larva stage for 21 days.

2.6 **Juvenile** means shrimp that are 2-3 cm long and are the same as adults but have not yet reached reproductive maturity.

2.7 **Adult** means fully mature shrimp that can reproduce.

2.8 **Haemolymph** means components of plasma and haemocytes in the circulatory system of shrimp.

2.9 **Presumptive test** means a fast and convenient laboratory procedure to test for a disease, such as rapid staining, haemolymph smear or histopathological tests.

2.10 **Confirmation test** means a laboratory procedure to confirm the results of a diagnosis, which is accepted to be highly specific and sensitive.

2.11 **Specificity** means the ability of a diagnosis test method to produce negative results from uninfected samples.

2.12 **Sensitivity** means the ability of a diagnosis test method to produce positive results from infected samples.

2.13 **Positive control** means a test consisting of chemicals and the standard microbes to be studied for comparison with the unknown samples when the same diagnostic procedures are performed on both.

2.14 **Negative control** means a test that does not contain the standard microbes to be studied for comparison with the unknown samples when the same diagnostic procedures are performed on both.

3 DIAGNOSIS

After receiving the results of a diagnosis test for yellow head disease in the laboratory through the rapid staining method, staining of haemolymph smear method, histopathological method, reverse transcription polymerase chain reaction method, or *in-situ* hybridization, the responsible official under the Animal Epidemic Act B.E. 2499 (1956) and its amendments should also consider the epidemiology, pathogenesis and clinical signs of the shrimp (Appendix A) to ensure the effectiveness of disease treatment and prevention.[1/]

The purposes for diagnosis of yellow head disease are to confirm if there is an outbreak, to ensure that broodstock shrimp and shrimp larvae are pathogen-free, and for disease surveillance. The different diagnostic methods have different levels of efficiency (Appendix D). The histopathological method, the rapid staining method and the staining of haemolymph smear method are used as presumptive tests but it is necessary to use the RT-PCR method to confirm the diagnosis. When certifying that broodstock shrimp or postlarval shrimp are disease-free it is necessary to use the RT-PCR method, which is sensitive and specific enough to detect latent infection that YHV is present in only small amounts.

3.1 SAMPLING

For the number of samples to be taken, refer to Table C1 (Appendix C).

3.2 RAPID STAINING TEST

3.2.1 Principle

The principle of this procedure is to detect evidence of the disease in the shrimp tissue by staining the tissue with hematoxylin and eosin (H&E) for a short time and observing it under a light microscope.

This method for diagnosis of yellow head disease can only be used on large-sized postlarval shrimp (older than PL30) that show clinical signs of the disease. The most suitable sample is gill tissue, with size no more than 0.5 cm long.

[1/] The western-blot analysis and transmission electron microscopy methods may also be used to confirm a diagnosis but they are quite limited because they cannot differentiate between YHV and Gill-associate virus (GAV) or other yellow head-related viruses that may be present in normal shrimp, either in the wild or in captivity.

3.2.2 Procedures

(1) Put 0.5 ml of HCl Davidson's fixative (see Appendix F, section F.1.2) in a 1.5 ml plastic tube. Cut off a piece of gill from the sample shrimp and immerse it in the fixative for 1 hour, or alternatively a sample taken following the method in section 3.4.2 may also be used.

(2) Pour off the HCl Davidson's fixative and cover the top of the tube with gauze then rinse in a gentle stream of tap water for 15 min (be careful not to let the sample flow out of the tube).

(3) Pour off the water and add 3 drops of hematoxylin (covering the sample) and immerse for 10 min.

(4) Pour off the stain and rinse in a gentle stream of tap water for 15 min.

(5) Pour off the water and add 3 drops of eosin (covering the sample) and immerse for 2 min or less as suitable, then pour of the stain.

(6) Add 0.5 ml 50% ethanol and immerse for 1 min, then pour off the 50% ethanol.

(7) Add 0.5 ml 70% ethanol and immerse for 1 min, then pour off the 70% ethanol.

(8) Add 0.5 ml 90% ethanol and immerse for 1 min, then pour off the 90% ethanol; repeat.

(9) Add 0.5 ml absolute ethanol and immerse for 1 min, then pour off the absolute ethanol; repeat.

(10) Add 0.5 ml xylene and immerse for 1 min, then pour off the xylene; again add 0.5 ml xylene and do not pour off but keep the sample hydrated in xylene.

(11) Using forceps, break the gill into small pieces and place on a microscope slide. Use forceps to break the gill pieces until they are broken into separate gill filament and the gill lamellae can be inspected.

(12) Add one drop Permount and cover with a cover slip. Leave for 3 to 5 min, and then observe under a light microscope at 400x.

3.2.3 Interpretation

In shrimp that are infected with YHV, the nucleus of dead cells can be seen as dark spot (pycnotic nucleus) or may be deteriorated to appear as tiny fragments (karyorrhectic nucleus). Inclusion bodies (round dots that stain dark blue) may be seen in the cytoplasm of infected cells.

3.3 STAINING OF HAEMOLYMPH SMEAR METHOD

The most suitable sample for this method is haemolymph from shrimp that show clinical signs indicative of yellow head disease taken from the same pond of moribund shrimp where yellow head disease is suspected.

3.3.1 Procedures

(1) Take a haemolymph sample from the haemocoel in the abdomen under the first pair of swimming legs (ventral sinus) or from the haemocoel under the third to last pair of walking legs (cardiac sinus) (Appendix G, Figure G.2) using a needle and syringe that is

preloaded with 10-25% formalin of a volume equal to the volume of haemolymph to be taken.

(2) Shake the syringe to let the haemolymph mix completely with the formalin. Drip a sample and smear on a microscope slide; let it dry.

(3) Stain with H&E or other haemolymph stain, such as Wright and Giemsa's stain or Dip-Quick.

(4) Examine under a light microscope at 400x.

3.3.2 Interpretation

In shrimp that are infected with YHV, the nucleus of dead cells can be seen as dark spot (pycnotic nucleus) or may be deteriorated to appear as tiny fragments (karyorrhectic nucleus) (Appendix G, Figure G.3). However, there shall be no evidence of bacterial infection in the same specimen because it may cause similar changes in haemocyte. This may cause misinterpretation.

3.4 HISTOPATHOLOGICAL METHOD

3.4.1 Principle

The principle of this method is to detect evidence of the disease in the shrimp tissue by staining fixed tissue with hematoxylin and eosin (H&E) and observing it under a light microscope.

3.4.2 Collection and storage of samples

3.4.2.1 Take live shrimp and immerse them in chilled Davidson's fixative (Appendix F, section F.1.1) of a volume that is approximately ten times the volume of the shrimp as follows:

(1) If the shrimp are nauplius to PL20 stage, they may be kept whole in Davidson's fixative.

(2) If the shrimp are PL21 stage to $\leq$ 3 g, they may be kept whole in Davidson's fixative but an incision shall be made lengthwise along the carapace to allow the fixative to reach the hepatopancreas.

(3) If the shrimp are 3-12 g, Davidson's fixative shall be injected into the shrimp's mouth, under the back of the carapace, into the hepatopancreas and the abdomen from the third to the last pair of walking legs (periopods) as well as all over the dorsal and ventral portions of the cephalothorax, using from 1 ml to 10 ml of Davidson's fixative per shrimp, depending on shrimp size. The shell shall be incised lengthwise from the sixth abdominal segment to the cephalothorax.

(4) If the shrimp are over 12 g, Davidson's fixative shall be injected thoroughly into the cephalothorax and the ventral side of the body from cephalothorax to tail, after which the shrimp shall be cut in half in cross section between the cephalothorax and abdomen.

3.4.2.2 The samples from 3.4.2.1 shall be immersed in Davidson's fixative for 24 h to 48 h, depending on the size, then transferred to 70% ethanol to extend the storage life.

3.4.3 Procedures

Samples stored as in 3.4.2 above shall be prepared for histopathological examination (Appendix E) and the tissue samples on slides shall be dyed as follows:

(1) Liquefy the paraffin in the samples by heating the slides to 60°C for 30 min, then immerse in xylene.

(2) Rehydrate the sample by immersing the slide in absolute ethanol and 95% ethanol in sequence, for 5 min each.

(3) Rinse the slide in running water for 5 min.

(4) Immerse the slide in Mayer's hematoxylin for 5-7 min.

(5) Rinse the slide in running water for 15-30 s.

(6) Immerse the slide in eosin for 30-60 s.

(7) Dehydrate the slide by immersing in 95% ethanol and absolute ethanol in sequence.

(8) Clear ethanol by immersing in xylene for 5 min.

(9) Add 1 drop Permount and cover with a cover slip.

(10) Observe under a light microscope at 400x.

3.4.4 Interpretation

In shrimp that are infected with YHV, the following abnormalities can be observed:

In the moribund shrimp, many dead cells can be observed in tissues originate from the ectoderm and mesoderm, with pycnotic nuclei or karyorrhectic nuclei can be seen (Appendix G, Figure G.4). Basophilic cytoplasmic inclusion bodies may be observed, especially in the haemocytes, lymphoid organ, gills, related subcuticular tissues, muscles, digestive tract, green gland or antennal gland, reproductive organs, nerve tracts and nerve ganglia.

The most suitable tissues to examine are the hepatopancreas, lymphoid organ, and subcuticular tissues of the stomach and gills.

3.5 REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR) METHOD

3.5.1 Principle

The principle of this method is to detect YHV RNA. The first step is to extract RNA, then transcribe the RNA to DNA or synthesize complementary DNA (cDNA), then to use specific primers to multiply the target DNA exponentially up to a detectable level, and lastly to separate the DNA bands using electrophoresis.

This method can be used to detect YHV genotype1 or YHV1 only. It cannot detect Gill-associate virus (GAV) or other strains of YHV because the genetic codes are different. It is possible to use an RT-nested PCR method to detect both YHV and GAV (see details in Appendix B).

3.5.2 Collection and storage of samples

3.5.2.1 Collecting shrimp samples

(1) Put live shrimp along with the water in which they were raised in a plastic bag or other suitable container. Samples from different ponds shall be kept separately. Clearly label the samples and transport them live to the laboratory.

In the case of broodstock shrimp, collect only the distal section of one side of the distal pair of pleopods.

(2) If it is not possible to take live samples, put the dead samples in a plastic bag, seal it securely and pack it on ice or dry ice, then deliver it to the laboratory within 24 h. If it is not possible to send the samples to the laboratory within such time frame, freeze the specimens whole in a freezer that is -20°C or lower and send them to the laboratory as soon as possible.

(3) If it is not possible to obtain live or frozen samples, keep the samples cool in chilled 90-95% ethanol about 10 times the volume of the sample, and test within 3 days. If it is necessary to store the specimen for a longer time, then it may be fixed in fixative (Appendix F, section F.1.3). For juvenile stage shrimp or older, collect a sample of the lymphoid organ and gills. If the shrimp are smaller than 3 g, they shall be fixed whole but be tested within 7 days.

3.5.2.2 Collecting haemolymph samples

Take the sample from a haemocoel in the ventral sinus or the cardiac sinus (Appendix G, Figure G.2) using a needle and syringe that is preloaded with citrate buffer (Appendix F, section F.1.4) of a volume equal to the volume of the sample. The sample shall be refrigerated and delivered to the laboratory within 8 h for the RT-PCR test.

(1) To store the haemolymph sample in RNA fixer:

- Suction out the haemolymph sample in citrate buffer and place in a microcentrifuge tube and centrifuge at 500 *g* for 10 min at 4° C.

- Remove the supernatant, be careful not to disturb the haemocytes at the bottom of the tube.

- Add an RNA fixer such as Trizol or other RNA purification reagents to fill the bottom of the microcentrifuge tube and gently mix.

- Store the sample at -20° C to -80° C until testing time.

(2) Drop 30 µl haemolymph onto Isocode™ filter paper (or other filter paper with the same properties) and leave to air dry. Haemolymph may be stored for 3-6 months at room temperature in this manner.

3.5.3 Procedures

3.5.3.1 Extraction of RNA (the following steps shall be performed at a temperature under 4°C):

(1) Sample preparation, depending on the type of specimen, as follows:

- For large (juvenile or adult) shrimp, take 10 mg -20 mg shrimp tissue and add 150 µl Trizol. Crush and mix, then add more Trizol for a final volume of 500 µl.

- For postlarval shrimp (PL10-PL15), take 300 shrimp and add 1,000 µl Trizol. Crush to mix, then take just 150 µl of the mixture and add more Trizol for a final volume of 1,000 µl.

- If the sample is haemolymph, take 50 µl of sample and add 500 µl Trizol and mix for 20 s.

- If the sample is haemolymph stored on Isocode™ filter paper, use a paper punch to cut out discs with a diameter of 1 cm and place in a microcentrifuge tube, and then extract the RNA by adding 750 µl Trizol or TRI reagent™.

(2) Incubate the sample from (1) at 25°C for 5 min.

(3) Centrifuge at 12,000 *g* at 4° C for 10 min; pipette up the supernatant and transfer it to a new microcentrifuge tube.

(4) Add 200 µl chloroform and mix for 20 s.

(5) Incubate at 25° C for 10 min.

(6) Centrifuge at 12,000 *g* at 25° C for 10 min; pipette up the supernatant and transfer it to a new microcentrifuge tube.

(7) Add 670 µl isopropanol and mix.

(8) Incubate at 25° C for at least 10 min.

(9) Centrifuge at 12,000 *g* at 25° C for 10 min; pipette off the supernatant and discard.

(10) Rinse the pellet with 0.5 ml of 70% ethanol for at least 30 min at 25° C.

(11) Centrifuge at 12,000 *g* at 25° C for 10 min. Pipette off the supernatant and discard.

(12) Leave at room temperature for 20 min or until the pellet is dry.

(13) Add 25 µl diethylpyrocarbonate (DEPC)-treated water. Incubate at 56° C for 15 min. Mix gently and use for the next step to synthesize complementary DNA immediately or store at -70°C until ready for use.

3.5.3.2 cDNA synthesis

(1) Use RNA extracted from shrimp haemolymph or shrimp tissue with a concentration of approximately 1 µg RNA as the template RNA.

(2) Synthesize the first strand of cDNA using the specific primer 144R (see Table 1). For best results, approximately 20 µl of substrate is needed with the following composition:

- antisense primer 144R 0.75 µM

- dNTPs 1 mM (each)

- Moloney murine leukemia virus reverse transcriptase (M-MLV) 2.5 units

- $MgCl_2$ 5 mM

- PCR buffer (10 mM Tris/HCl. pH 8.3, 50 mM KCl)

(3) Insert the sample into the thermocycler for the heating stage. If the thermocycler does not have a temperature controlled cover, add 50 µl mineral oil on top of the samples in the microcentrifuge tubes to prevent evaporation.

(4) Set the temperature at 45°C for 15 min for the reverse transcription of the template RNA to produce cDNA. Then increase the temperature to 100°C for 5 min to stop the reverse transcriptase reaction and adjust the temperature of the solution down to 5°C before the next step.[2/]

[2/] The conditions are suitable for the automatic DNA thermocycler 480 (Perkin Elmer Cetus, Norwalk, CT). For other models of thermocyclers, the optimized conditions shall be determined using the positive control first.

Commercial first-strand cDNA synthesis kit may be used for every step.

Table 1. Nucleotide sequence of primers 10F and 144R

(Sections 3.5.3.2 and 3.5.3.3)

Primer	Sequence
10F	5'-CCG-CTA-ATT-TCA-AAA-ACT-ACG-3'
144R	5'-AAG-GTG-TTA-TGT-CGA-GGA-AGT-3'

Note: *Other primers, durations, temperatures, chemicals or tools may be used for diagnosis if they are proven to be at least as sensitive and specific as this one and have been published in a scientific journal.*

3.5.3.3 Multiplying target DNA

(1) Add PCR cocktail (10 mM Tris/HCl. pH 8.3, 50 mM KCl) with 2.5 U of *Taq* DNA polymerase, 2 mM $MgCl_2$ and 0.75 μM of sense primer (10F) (see Table 1) for a final volume of 100 μl to multiply the target DNA.

(2) If a random primer was used to synthesize the first strand of cDNA in 3.5.3.2, add PCR cocktail (10 mM Tris/HCl. pH 8.3, 50 mM KCl) with 2.5 U of *Taq* DNA polymerase, 2 mM $MgCl_2$ and 0.75 μM of antisense and 0.75 μM of sense primer (10F) for a final volume of 100 μl to multiply the target DNA.

(3) Insert the sample into the thermocycler for the heating stage. If the thermocycler, that are used, does not have a temperature controlled cover, add 50 μl mineral oil on top of the samples in the microcentrifuge tubes to prevent evaporation.

(4) Multiply the DNA by setting the thermocycler as follows:

Step 1 Incubate at 94° C for 30 s

Step 2 Incubate at 94° C for 30 s (for denaturing)

Incubate at 58° C for 30 s (for annealing)

Incubate at 72°C for 30 s (for extension)

Stage 2 is repeated for 40 cycles

Step 3 Incubate at 72° C for 10 min, then reduce the temperature to 4°C.

(5) Pipette the DNA solution out from under the mineral oil and deposit in a 0.5 ml microcentrifuge tube.

3.5.3.4 Electrophoresis for DNA separation

(1) Combine loading dye with DNA solution and place 10 μl -15 μl in a well of agarose gel, concentration of 2%.

(2) Use a 100-bp DNA ladder as the marker.

(3) Apply electric current to separate the DNA in 0.5X TBE solution (Tris, boric acid, EDTA).

(4) Stain with ethidium bromide (0.5 μg/ml) or other dye.

(5) Read with a UV transilluminator. The DNA band that stained with ethidium bromide will appear luminescent on the agarose gel.

3.5.4 Interpretation

- a 135 bp DNA band means a positive reading compared to the positive control.

- no 135 bp DNA band means a negative reading compared to the negative control.

3.5.5 Precautions:

To prevent contamination of DNA and RNase, the materials, equipment and work space shall be kept separately when extracting DNA and mixing the PCR cocktail. Filtered tips should be used for mixing the PCR cocktail and preparing the template RNA.

3.6 *In-situ* HYBRIDIZATION

3.6.1 Principle

The principle of this method is to detect YHV RNA or DNA in the shrimp tissue by using a specific probe to bind with the target RNA or DNA.

This method can be used to detect both YHV and GAV using a DIG (digoxigenin)-labelled cDNA probe produced by the PCR labeling method with the primers shown in Table 2.

Table 2 Nucleotide sequence of primers YHV1051F and YHV1051R

(Section 3.6)

Primer	Sequence
YHV1051F	5'-ACA-TCT-GTC-CAG-AAG-GCG-TC-3'
YHV1051R	5'-GGG-GGT-GTA-GAG-GGA-GAG-AG-3'

3.6.2 Collection and storage of samples[3/]

As specified in the histopathological method (Section 3.4.2).

3.6.3 Procedure

Prepare samples using the histopathological method (Appendix E). Slice the paraffin-embedded samples with a microtome and place on pre-treated slides (Appendix F, section F.3.1) or positively charged slides, then incubate at 60°C for 30 min to partially melt the paraffin and proceed as follows:

3.6.3.1 Deparaffinization

Immerse tissue in xylene twice, 5 min each time.

3.6.3.2 Rehydration

(1) Immerse tissue in absolute ethanol twice, 5 min each time.

(2) Immerse tissue in 95% ethanol twice, 5 min each time.

(3) Immerse tissue in 70% ethanol twice, 5 min each time.

(4) Immerse tissue in 50% ethanol twice, 5 min each time.

(5) Immerse tissue in distilled water for 5 min.

[3/] For the *in situ* hybridization method the shrimp tissue shall be processed directly after it has been soaked in Davidson's fixative for 24 h. If it is not possible to proceed with the diagnosis at once, then RNA friendly fixative (R-F fixative) shall be used instead (Appendix F, section F.3.2). The samples can be soaked for 24 h - 48 h in R-F fixative then transferred to 70% ethanol, after which it can be stored for up to 2 weeks.

3.6.3.3 Pre-hybridization treatment

(1) Drop proteinase K (100 μg/ml in 50 mM Tris/HCl pH 7.4, 10 mM NaCl, 1 mM EDTA) to completely cover the tissue and incubate at 37° C for 15 min (the most suitable concentration of proteinase K and the most suitable length of time for denaturation shall be test first).

(2) Drop formaldehyde 0.4% to completely submerse the tissue and leave for 5 min.

(3) Immerse in 2x standard saline citrate (SSC) (NaCl 0.3 M, sodium citrate 30 mM, pH 7.0) twice for 2 min each time.

(4) Drop 500 μl hybridization buffer (4x SCC, 50% formamide, 1x Denhardt's, 0.25 mg/ml yeast RNA, 0.5 mg/ml sheared salmon sperm DNA, 5% dextran sulfate) on the tissue and wait for the buffer to dissipate throughout the tissue, then incubate at 42° C for 30 min.

3.6.3.4 Hybridization

Dilute the DIG-labelled cDNA probe with hybridization buffer to a concentration of 20 ng/ml–40 ng/ml; heat in boiling water for 5 min then place on ice for at least 5 min. Drop 250 μl of the hybridization buffer; gently place the slide with the tissue sample on it down in contact with the buffer; wait for the buffer to spread throughout the tissue, then incubate at 42°C in a moisture retention chamber for 12h -16 h.

3.6.3.5 Washing and non-specific blocking

(1) Remove the cover slip and immerse the sample tissue in SSC 2x at room temperature for 30 min.

(2) Immerse in SSC 1x at 37° C twice for 5 min each time.

(3) Immerse in SSC 0.5x at 37° C twice for 5 min each time.

3.6.3.6 Detection

(1) Dilute sheep anti-digoxigenin-alkaline phosphatase congjugate (Boehringer Mannheim, Germany) with 3% bovine serum albumin (BSA) to a concentration of 1:300. Drop to cover the tissue; incubate at 37°C for 30 min.

(2) Immerse in 0.1 M Tris/HCl pH 7.5, 0.15 M NaCl at room temperature twice for 10 min each time.

(3) Add 0.1 M Tris/HCl pH 9.5, 0.15 M NaCl

(4) Add one drop nitroblue tetrazolium; NBT/5-bromo-4-chloro-3-indolyl phosphate (BCIP) to completely cover the tissue and leave at room temperature for 1 h -2 h.

(5) Add a drop of 0.5% Bismark brown Y stain to completely cover the tissue for 5 min.

(6) Immerse tissue in 50% ethanol twice, 5 min each time.

(7) Immerse tissue in 70% ethanol twice, 5 min each time.

(8) Immerse tissue in 95% ethanol twice, 5 min each time.

(9) Immerse tissue in absolute ethanol twice, 5 min each time.

(10) Immerse tissue in xylene twice, for 5 min each time.

(11) Cover with a cover slip and examine under a light microscope.

3.6.4 Interpretation

YHV-infected tissues will be stained dark blue to black compared to normal tissue, which will be stained brown.

APPENDIX A

EPIDEMIOLOGY, PATHOGENESIS AND CLINICAL SIGNS OF YELLOW HEAD DISEASE

A1. EPIDEMIOLOGY

Yellow head disease is very common in the genus *Penaeus*. It is caused by YHV, which is a virus in the order Nidovirales, family Roniviridae, genus *Okavirus*. It is related to viruses in the families Coronaviridae and Arteriviridae. It is a single-stranded RNA virus. The study using transmission electron microscopy shows its rod-shaped particles of 150-200 nm in length and 40-60 nm in diameter with an envelope. It can be found in the cytoplasm of infected cells and in intracellular spaces.

Yellow head disease was first reported in Thailand in 1990 when it began to spread and caused serious damage in several provinces. Most outbreaks have been reported in Asia but the disease has also been found in America and Australia. Natural infection of YHV has been detected in *Penaeus monodon, Marsupenaeus japonicus, Fenneropenaeus merguiensis, Litopenaeus vannamei, Litopenaeus stylirostris, Farfantepenaeus aztecus* and *Euphasia superba.* Laboratory trials have confirmed that YHV can cause infections in *Litopenaeus vannamei, Litopenaeus stylirostris, Farfantepenaeus aztecus* and *Farfantepenaeus duorarum* with high mortality. YHV can infect almost every variety of shrimp that is raised commercially and can cause death. Six genotypes of viruses in the yellow head complex have now been identified. The genotype that causes yellow head disease is YHV1. The other genotypes, such as YHV3, YHV4, YHV5 and YHV6 are less virulent and can occur in normal shrimp without causing any clinical signs or damage.

Gill-associate virus (GAV) is a virus with similar genetic structure to YHV. It was first reported in black tiger shrimp in Australia. Shrimp infected with GAV may show severe acute clinical signs, chronic clinical signs or no clinical signs at all. There is a high incidence of chronic GAV infections in black tiger shrimp living in the wild of the eastern coast of Australia. There is evidence that the disease can be vertically transmitted from brooder shrimp to their offspring during egg laying. Acute GAV infections result in high mortality rates. The external clinical signs and distribution of the virus in shrimp tissue differ from YHV but genetic studies show that GAV is genetically very similar to YHV and can be classified in the same group as YHV2.

Because yellow head disease has caused serious damage and affects international trade, the World Organization for Animal Health (Office International des Epizooties; OIE) puts it on the notifiable list of OIE aquatic animal disease. Thai authorities included it in a 2005 ministerial regulation under the Animal Epidemic Act B.E. 2499 (1956).

A2. PATHOGENESIS

Shrimp can get infected with YHV from the late post-larval stage onwards but clinical signs are most commonly observed and the mortality rate is the highest during the early to late juvenile stages. In earthen ponds, shrimp can get infected from other shrimp when the virus is

transmitted through the water or when they ingest tissues that are contaminated by dead shrimp. Crustaceans and other animals may also act as disease vectors.

Histopathological studies have revealed that YHV causes septicemia and destroys tissues that originate from the ectoderm and mesoderm. Once infected, the virus quickly spreads to organs such as the lymphoid, gills, cuticular epithelium, digestive tract, heart, antennal gland, haemolymph generating tissue, midgut cecum, myoepithelial cells of the hepatopancreas and endocardium. Necrotic cells can be observed in the lymphoid organ, gills and haemocytes. YHV can also be detected in the haemolymph of shrimp with early stage infections that have not yet begun to show advanced clinical signs.

A3. CLINICAL SIGNS

Clinical signs of yellow head disease are usually noticeable in juvenile to subadult shrimp. At first, they will have high feeding activity for several days, then they will abruptly stop eating 2-4 days after some of the shrimp have begun to show clinical signs or die. Large numbers of shrimp may be seen floating near the edges of the pond. The most noticeable external abnormalities are paleness, swelling of the cephalothorax, and a yellowish discoloration due to the hepatopancreas turning yellow. The hepatopancreas will be softer than normal. Usually, all the shrimp in the pond start to show clinical signs and die within only 2 or 3 days.

APPENDIX B

DIAGNOSIS BY REVERSE TRANSCRIPTION-NESTED POLYMERASE CHAIN REACTION (RT-NESTED PCR)

(Section 3.5.1)

The principle of this method is to detect YHV RNA by extracting the RNA, converting the RNA to DNA (synthesize cDNA), multiplying the target DNA exponentially up to a detectable level using specific primers, and separating the DNA by electrophoresis. It differs from the ordinary RT-PCR method because there is more than one step for multiplying the DNA and primers with high levels of specificity are used. In this appendix, two methods of RT-nested PCR diagnosis using different primers are described.

B1. Method 1

This method can be used to detect both YHV and GAV in the first round of DNA multiplication and to differentiate between YHV and GAV in the second round. It is useful for identifying YHV or GAV in the case of an outbreak or for screening shrimp to see if they are carriers. This method can also be used to detect YHV3, which is sometimes found in healthy black tiger shrimp in Asia. It cannot be used to detect YHV4, which has been found in healthy shrimp in India. This RT-nested PCR technique uses the following specific primers listed in Table B1:

Table B1. Nucleotide sequence of primers GY1, GY2, GY4, GY5, Y3 and G6

(Section B.1)

Primer	Sequence
GY1	5’-GAC-ATC-ACT-CCA-GAC-AAC-ATC-TG-3’
GY2	5’-CAT-CTG-TCC-AGA-AGG-CGT-CTA-TGA-3’
GY4	5’-GTG-AAG-TCC-ATG-TGT-GTG-AGA-CG-3’
GY5	5’-GAG-CTG-GAA-TTC-AGT-GAG-AGA-ACA-3’
Y3	5’-ACG-CTC-TGT-GAC-AAG-CAT-GAA-GTT-3’
G6	5’-GTA-GTA-GAG-ACG-AGT-GAC-ACC-TAT-3’

B1.1 Extraction of target RNA

Use the same procedure as the RNA extraction in the RT-PCR method (Section 3.5.3.1)

B1.2 cDNA synthesis

(1) Take 0.1 µg -1.0 µg of the RNA extracted from the shrimp tissue or haemolymph and combine with 0.7 µl of primer GY5 at a concentration of 50 pmol/µl then adjust to a final volume of 6 µl by adding DEPC-treated water. Incubate at 70° C for 10 min, then chill on ice.

(2) Create complementary DNA by adding:

- 2 µl of Superscript II buffer 5x (250 mM Tris/HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$);

- 1 µl of 100 mM dithiothreitol (DTT)

- 0.5 µl of 10 mM dNTP

(3) Incubate at 42° C for 2 min, then add 0.5 µl of 200 U/ µl reverse transcriptase

(4) Incubate at 42° C for 1 hour, warm to 70° C for 10 min, then chill in ice until use.

B1.3 Target DNA multiplication

First round

(1) Prepare 50 µl PCR cocktail in a 0.5 ml tube, consisting of:

- 1x *Taq* buffer (10mM Tris/HCl pH 8.3, 50 mM KCl, 0.1% Triton X-100)

- 1.5 mM $MgCl_2$

- 35 pmol each of primers GY1 and GY4

- 200 µmol each dNTP

- 2.5 U *Taq* polymerase

(2) For the heating stage, if the thermocycler does not have a temperature controlled cover, add 50 µl mineral oil on top of the samples in the microcentrifuge tubes to prevent evaporation. Heat the PCR cocktail at 85 °C for 2 min - 3 min before adding 1µl of the sample cDNA from B1.2.

(3) Set the thermocycler for the following steps:

Step 1	Incubate at 95° C for 30 s
Step 2	Incubate at 95° C for 30 s (denaturation)
	Incubate at 66° C for 30 s (annealing)
	Incubate at 72° C for 45 s (extension)
	Repeat step 2 for a total of 35 times
Step 3	Incubate at 72° C for 7 min, then store at 4° C

Second round

(1) Prepare 50 µl PCR cocktail in a 0.5 ml tube, consisting of:

- 1x *Taq* buffer (10mM Tris/HCl pH 8.3, 50 mM KCl, 0.1% Triton X-100)

- 1.5 mM $MgCl_2$

- 35 pmol each of primers GY2, Y3 and G6

- 200 µmol each dNTP

- 2.5 U *Taq* polymerase

(2) Set the thermocycler for the following steps:

Step 1	Incubate at 95° C for 30 s
Step 2	Incubate at 95° C for 30 s (denaturation)
	Incubate at 66° C for 30 s (annealing)
	Incubate at 72° C for 30 s (extension)
	Repeat step 2 for a total of 35 times
Step 3	Incubate at 72° C for 7 min, then store at 4° C

(3) Pipette up the DNA solution from under the mineral oil and place in a 0.5 ml microcentrifuge tube.

B1.4 Electrophoresis for DNA separation

Same as for the RT-PCR method (Section 3.5.3.4)

B1.5 Interpretation

(1) a 277 bp DNA band and a 794 bp band or no 794 bp band means a positive reading for YHV compared to the positive control.

(2) a 406 bp DNA band and a 794 bp band or no 794 bp band means a positive reading for GAV compared to the positive control.

(3) a 277 bp band and a 406 bp band and a 794 bp band or no 794 bp band means a positive reading for both YHV and GAV compared to the positive control.

(4) no DNA bands of any size means a negative reading compared to the negative control.

B2 Method 2

This method can be used to detect all 6 genotypes of YHV but it cannot differentiate between them. It may be used for general disease screening of shrimp. This RT-nested PCR technique uses the following specific primers listed in Table B2:

Table B2. Nucleotide sequence of primers YC-F1a, YC-F1b, YC-R1a, YC-R1b, YC-F2a YC-F2b, YC-R2a, and YC-R2b

(Section B2)

Primer	Sequence
YC-F1a	5’-ATC-GTC-GTC-AGC-TAC-CGC-AAT-ACT-GC-3’
YC-F1b	5’-ATC-GTC-GTC-AGY-TAY-CGT-AAC-ACC-GC-3’
YC-R1a	5’-TCT-TCR-CGT-GTG-AAC-ACY-TTC-TTR-GC-3’
YC-R1b	5’-TCT-GCG-TGG-GTG-AAC-ACC-TTC-TTG-GC-3’
YC-F2a	5’-CGC-TTC-CAA-TGT-ATC-TGY-ATG-CAC-CA-3’
YC-F2b	5’-CGC-TTY-CAR-TGT-ATC-TGC-ATG-CAC-CA-3’
YC-R2a	5’-RTC-DGT-GTA-CAT-GTT-TGA-GAG-TTT-GTT-3’
YC-R2b	5’-GTC-AGT-GTA-CAT-ATT-GGA-GAG-TTT-RTT-3’

***Note**: Mixed base codes: R=A/G, Y=C/T, D=A/G/T*

B2.1 Extraction of target RNA

Use the same procedure as the RNA extraction in the RT-PCR method (Section 3.5.3.1)

B2.2 cDNA synthesis

(1) Take 2 µg of the RNA extracted from the shrimp tissue or haemolymph and combine with 50 ng random hexamer primers and 1 µl of 10 nM dNTP then adjust to a final volume of 14 µl by adding DEPC-treated water. Incubate at 65°C for 5 min, then chill on ice.

(2) Create complementary DNA by adding:

- 4 µl of Superscript III buffer 5x

- 1 µl of 100 mM dithiothreitol (DTT)

- 1 µl of RNaseOUT^{TM} (40 U/µl)

- 1 µl of reverse transcriptase (200 U/µl)

(3) Incubate at 25°C for 5 min, then increase temperature to 42°C for 55 min

(4) Heat to 70° C for 15 min to stop the reaction of the reverse transcriptase, and then chill in ice until use.

B2.3 Target DNA multiplication

First cycle

(1) Prepare 25 µl PCR cocktail in a 0.5 ml tube, consisting of:

- 1x *Taq* buffer (10mM Tris/HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100)

- 25 mM $MgCl_2$ (1.5 µl)

- 25 µM each of primers YC-F1a, YC-F1b, YC-R1a and YC-R1b (0.35 µl)

- 10mM dNTP (0.5 µl)

- 0.25 µl *Taq* polymerase (5 U/ µl)

(2) Add 1µl of the sample cDNA from B2.2 to the solution. For the heating stage, if the thermocycler does not have a temperature controlled cover, add 50 µl mineral oil on top of the samples in the microcentrifuge tubes to prevent evaporation.

(3) Multiply target DNA in the thermocycler by setting the following steps:

Step 1	Incubate at 95° C for 1 min
Step 2	Incubate at 95° C for 30 s (denaturation)
	Incubate at 60° C for 30 s (annealing)
	Incubate at 72° C for 40 s (extension)
	Repeat step 2 for a total of 35 times
Step 3	Incubate at 72° C for 7 min, then store at 4° C

Second cycle

(1) Prepare 25 µl PCR cocktail in a 0.5 ml tube, consisting of:

- 1x *Taq* buffer (10mM Tris/HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100)

- 25 mM $MgCl_2$ (1.5 µl)

- 25 µM each of primers YC-F2a, YC-F2b, YC-R2a and YC-R2b (0.35 µl)

- 10mM dNTP (0.5 µl)

- 0.25 µl *Taq* polymerase (5 U/ µl)

- PCR product from the first cycle (1 µl)

(2) Set the thermocycler for the following steps:

Step 1	Incubate at 95° C for 1 min
Step 2	Incubate at 95° C for 30 s (denaturation)

Incubate at 60° C for 30 s (annealing)

Incubate at 72° C for 30 s (extension)

Repeat step 2 for a total of 35 times

Step 3 Incubate at 72° C for 7 min, then reduce temperature to 4° C

(3) Pipette up the DNA solution from under the mineral oil and place in a 0.5 ml microcentrifuge tube.

B2.4 Electrophoresis for DNA separation

Use the same procedure as the RT-PCR method (Section 3.5.3.4)

B1.5 Interpretation

(1) a 147 bp DNA band and a 359 bp band or no 359 bp band means a positive reading for YHV strains compared to the positive control.

(2) no DNA bands of any size means a negative reading compared to the negative control.

APPENDIX C

SAMPLING

(Section 3.1)

Table C1. Sampling guide

Population (number of shrimp)	Number of samples per expected disease prevalence			
	1%	**2%**	**5%**	**10%**
50	50	50	35	20
100	96	75	45	23
250	194	110	50	25
500	225	130	55	26
1,000	258	140	55	27
1,500	271	140	55	27
2,000	277	145	60	27
4,000	288	145	60	27
10,000	299	145	60	27
≥100,000	299	150	60	30

Source: The establishment of standards for marine shrimp hatcheries and grown out farm in a protected areas that may have problems on the outbreak of white spot disease, yellow head disease and Taura syndrome. A collaborative project with the Department of Fisheries, 2007

Note: *The sample size depends on the maximum expected prevalence of the disease at a confidence level of 95% and the population of shrimp in the pond.*

(1) For PL shrimp the sample size shall be based on the expected disease prevalence of 1% or 2%. Live shrimp shall be taken from 5 different spots around the pond. For testing broodstock shrimp, all of the shrimp shall be tested or the number of samples shall be based on the expected prevalence of 5%. For juvenile shrimp, the expected prevalence may be 2%-10%.

(2) Interpretation: for example, if the expected prevalence is 2%,

- Positive diagnosis means 2% or more of the samples are infected

- Negative diagnosis means less than 2% of the samples are infected or none of them are infected.

APPENDIX D

COMPARISON OF ACCURACY OF DIAGNOSIS TECHNIQUES

(Section 3)

Table B.1 Comparison of the accuracy of different methods to diagnose yellow head disease

Diagnosis method	**Screening**				**Presumptive**	**Confirmation**
	larva	**PL**	**juvenile**	**adult**		
Gross signs	-	-	+	+	+	-
Histopathology	-	-	+	+	+++	-
in situ hybridization	-	-	+	+	++	+++
PCR	+++	+++	+++	+++	+++	+++

Source: Adapted from the Manual of Diagnostic Tests for Aquatic Animals (OIE, 2009)

- \- = no test available or unsuitable
- \+ = for limited use
- ++ = standard test, commonly used, with good efficiency and specificity
- +++ = recommended test with high specificity and high sensitivity

APPENDIX E

SAMPLE PREPARATAION FOR HISTOPATHOLOGICAL DIAGNOSIS

(Sections 3.4.3 and 3.6.3)

E1. Embedding

(1) Take the tissue sample from Section 3.4.2 and immerse in the following solutions in sequence:

- 70% ethanol in the first bottle for 1 h
- 70% ethanol in the second bottle for 1 h
- 80% ethanol in the first bottle for 1 h
- 80% ethanol in the second bottle for 1 h
- 95% ethanol in the first bottle for 1 h
- 95% ethanol in the second bottle for 1 h
- 100% ethanol in the first bottle for 1 h
- 100% ethanol in the second bottle for 1 h
- xylene in the first bottle for 1.5 h
- xylene in the second bottle for 1.5 h

(2) Take the sample tissue from (1) above and immerse in warm liquid paraffin in the first bottle for 2 h then immerse in warm liquid paraffin in the second bottle for 2 h

(3) Place the sample in an embedding mold filled with liquid paraffin. Cover with the block and pour in liquid paraffin to fill the block. Place on a cool tray until the paraffin solidifies, then keep at room temperature.

(4) Use a microtome to section the tissue embedded in paraffin to a thickness of 4 μm -6 μm and place on microscope slides.

APPENDIX F
CHEMICAL SOLUTION PREPARATION

(Section 3)

F.1 Solutions preparation for sample collection

F.1.1 Davidson's fixative: 1,000 ml

95% ethanol	330	ml
100% formalin (37-39% formaldehyde)	220	ml
Glacial acetic acid	115	ml
Distilled water	335	ml

F.1.2 HCl Davidson's fixative: 1,000 ml

95% ethanol	330	ml
100% formalin (37-39% formaldehyde)	220	ml
HCl	115	ml
Distilled water	335	ml

F.1.3 Tissue fixative: 1,000 ml

95% ethanol	797.5	ml
Glycerol	200	ml
ß-mercaptoethanol	2.5	ml

F.1.4 Haemolymph fixative: 1,000 ml

NaCl	26.3	g
Trisodium citrate	8.8	g
Citric acid	5.5	g
EDTA	3.7	g

(1) add 700 ml water then adjust to pH 7.0

(2) autoclave

(3) cool to room temperature, add 100 ml 1 M glucose (36 g glucose dissolved in 200 ml distilled water)

(4) add water to 1,000 ml

F.2 Solutions for RT-PCR

F.2.1 5x EZ buffer, pH 8.2

25 mM bicine
57.5 mM potassium acetate
Glycerol 40% (w/v)

F.2.2 5x TBE

Tris base	54	g
Boric acid	27.5	g
0.5 M EDTA (pH 8)	20	ml

F.3 Equipment and solutions for *in situ* hybridization

F.3.1 Pre-treated slides

(1) Wash slides in 1 M HCl for 20 min, then rinse in tap water for 30 min

(2) Dip in distilled water then leave to air dry

(3) Dip in 2% organosilane (gamma-aminopropyltriethoxy silane) in acetone; leave at air temperature for 30 min

(4) Rinse in tap water for 30 min, then dip in distilled water 3 times, changing the water each time

(5) Incubate at 100° C for 1 h

(6) Store at room temperature in a dust-free place.

F.3.2 RNA friendly fixative (R-F fixative): 1,000 ml

100% formalin (37-39% formaldehyde)	349	ml
95% ethanol	407	ml
Distilled water	222	ml
Ammonium hydroxide (28-30% as NH_3)	22	ml

APPENDIX G

YELLOW HEAD DISEASE DIAGNOSIS ILLUSTRATIONS

(Section 3)

(G.1.1)

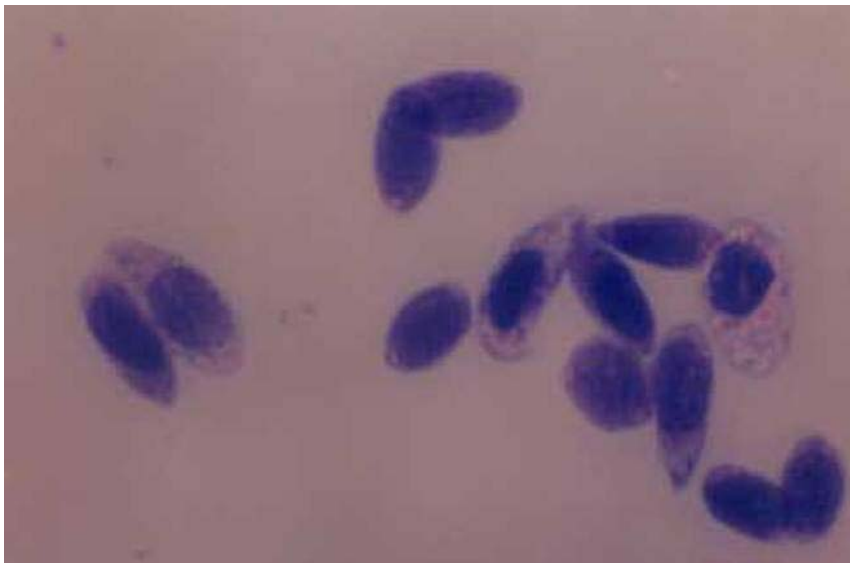
(G.1.2)

Figure G.1 Clinical signs of yellow head disease. In advanced stages the shrimp body is pale and the head and abdomen become yellow because the hepatopancreas turn yellow.

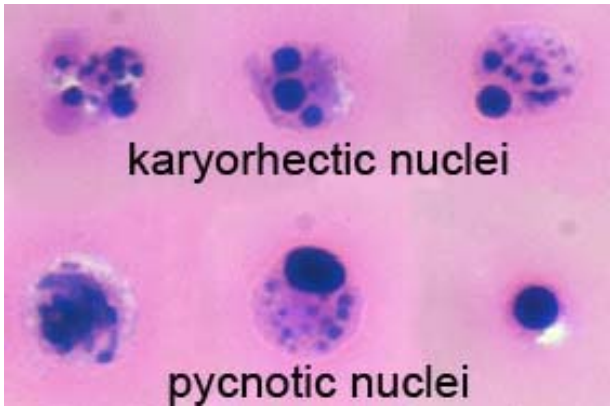
Sources: - Center of Excellence for Shrimp Molecular Biology and Biotechnology, Mahidol University (Figure G.1.1)
- Shrimp Culture Research Center, Charoen Pokphand Food (Public) Company Limited (Figure G.1.2)

Figure G. 2 Method for collecting haemolymph from the haemocoel in the abdomen under the first pair of swimming legs.

Source: Shrimp Culture Research Center, Charoen Pokphand Food (Public) Company Limited



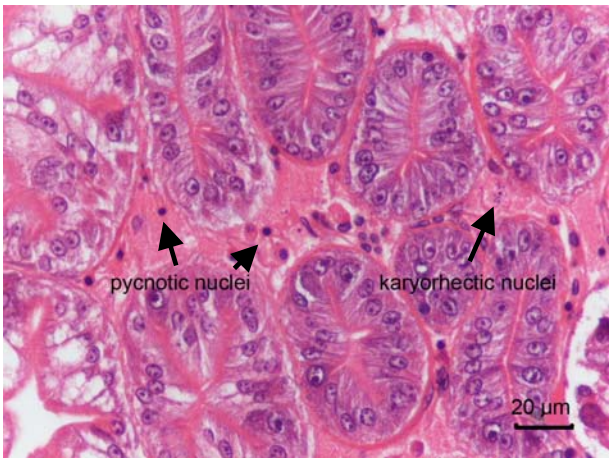
(G.3.1) normal nucleus



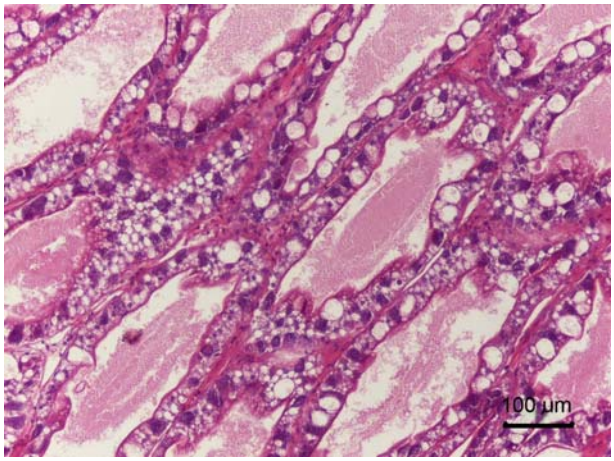
(G.3.2) pycnotic nuclei and karyorhectic nuclei of infected cells

Figure G.3 Histopathological characteristics of haemocytes of shrimp with yellow head disease detected using the staining of haemolymph smear method

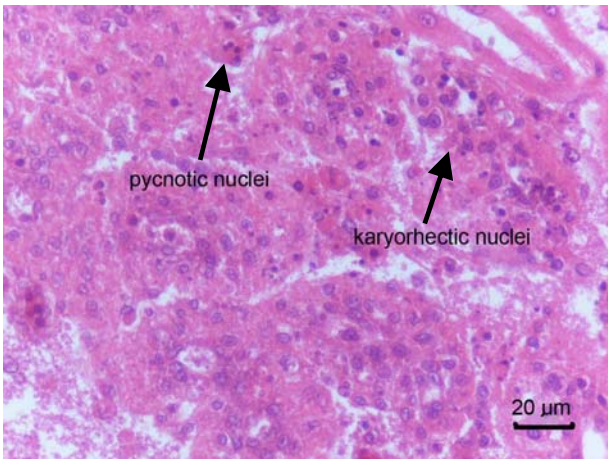
Sources: - Shrimp Culture Research Center, Charoen Pokphand Food (Public) Company Limited (Figure G.3.1)
- Center of Excellence for Shrimp Molecular Biology and Biotechnology, Mahidol University (Figure G.3.2)



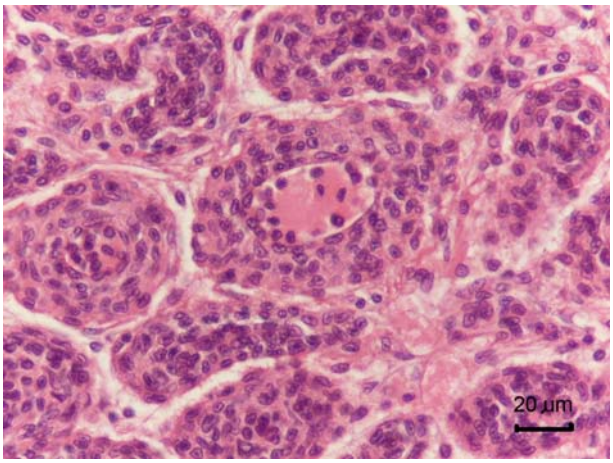
G.4.1 YHV infected hepatopancreas



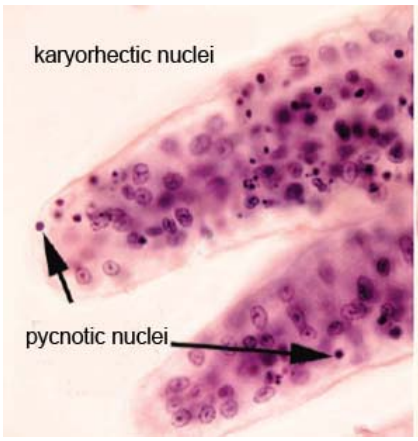
G.4.2 normal hepatopancreas



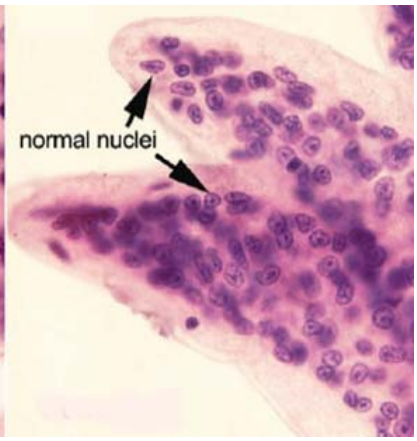
G.4.3 YHV infected lymphoid organ



G.4.4 normal lymphoid organ



G.4.5 YHV infected gills



G.4.6 normal gills

Figure G.4 Histopathological characteristics of yellow head disease in various tissues showing pycnotic nuclei and karyorhectic nuclei of infected cells compared to normal tissue.

Sources: - Shrimp Culture Research Center, Charoen Pokphand Food (Public) Company Limited (Figures G.4.1, G.4.2, G.4.3 and G.4.4)
- Center of Excellence for Shrimp Molecular Biology and Biotechnology, Mahidol University (Figure G.4.5 and G.4.6)

APPENDIX H
UNITS

The units and symbols used in this standard and the units recognized by the International System of Units *(le Système International d' Unités*) or SI are as follows:

Measurement	Units	Symbols
Mass	gram	g
	milligram	mg
	microgram	µg
	picogram	pg
Volume	milliliter	ml
	microliter	µl
Length	centimeter	cm
	millimeter	mm
	micrometer	µm
Time	hour	h
	minute	min
	second	s
Chemical quantity	mole	mol
Temperature	degree Celsius	^{o}C
Chemical concentration	milligram per milliliter	mg/ml
	microgram per milliliter	µg/ml
	nanogram per milliliter	ng/ml
	picomole per microliter	pmol/µl
Centrifugation	gravity	*g*