ACKNOWLEDGEMENT TO:

ISO 9001:2015

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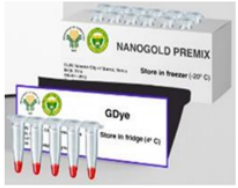
Instruction Manual

ASFV NANOGOLD BIOSENSOR TEST KIT

1. Sample collection methods
2. Sample preparation method before DNA extraction
3. DNA extraction steps
    - Fecal swab
    - Oral swab
    - Environmental surface swab
    - Water sample
4. DNA extraction steps for blood and meat samples
5. DNA amplification (applicable to all sample types)
6. Result interpretation by colorimetry

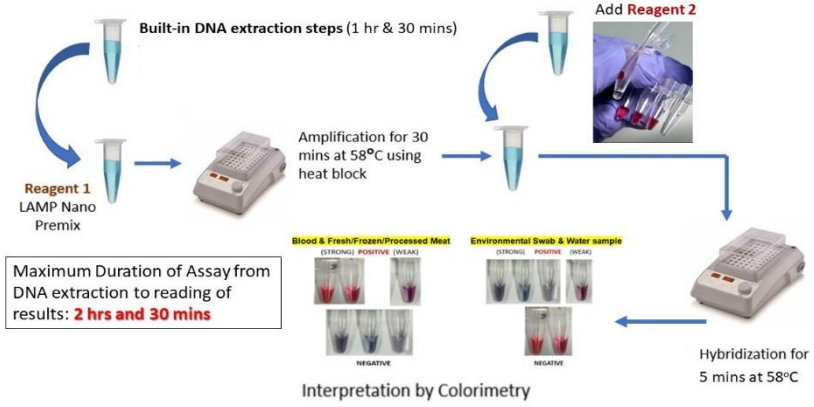
CONTENTS OF ONE (1) ASFV NANOGOLD BIOSENSOR TEST KIT



One Test Kit holds: (good for 10 samples; pooling of 3-5 samples is allowed)		
QTY	VOL	REF
10		MCT Tubes
10		Mini Droppers
100		Tuberculin Syringes with Needles
1		Rubber Floater
1	7 mL	Lysis Buffer (LB) store at room temp. inside the box
1	8 mL	Extraction Buffer (EB) store at room temp. inside the box
1	7 mL	Wash Buffer 1 (WB1) store at room temp. inside the box
1	5 mL	Wash Buffer 2 (WB2) store at room temp. inside the box
2	0.5 mL	Rehydration Buffer (RB) store at room temp. inside the box

For Amplification and Colorimetric Interpretation		
QTY	VOL	REF
2		Frozen gel packs
10	6.3 µL	Reagent 1 (PCR tube holding Nanogold Premix) ➢ Reagent 1 must be stored at (-)15oC or at freezing temperature
10	30 µL	Reagent 2 (PCR tube wrapped in foil, holding the Gdye ➢ Reagent 2 can be stored at room temp. away from sunlight or at 4oC (refrigerator)

SCHEMATIC DIAGRAM OF ASFV NANOGOLD BIOSENSOR LABORATORY WORK FLOW



Maximum Duration of Assay from DNA extraction to reading of results: **2 hrs and 30 mins**

DURATION: VIRUS REMAINS STABLE & INFECTIVE	REMARKS
18 months	Virus in serum at room temp
6 years	Virus in refrigerated blood samples (4-7°C)
1 month	Virus in blood samples kept at 37°C
Indefinitely	Virus kept at (-)70°C
Stable	pH range 4-10
Few hours to 3 days	At any pH range caveat virus is in protein medium such as serum
15 weeks	Virus in decomposed serum
Several months	Virus in bone marrow
11 days	Virus in feces, putrefaction does not inactivate the virus
15 weeks	Virus in chilled meat
3-6 months	Virus in processed ham and sausages that have not been cooked or smoked at high temp.
176 days	Virus in water during winter
15 days	Virus in urine at 4oC
Less than 15 days	Virus in feces at very warm ambient temperature
Longer survival	Virus particles in clumps protected with organic matter on surfaces. Any contaminated surface represents source of infection

CLINICAL FORMS OF ASF

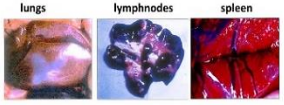
After 5-15 days exposure to infectious body secretions/excretions of a sick pig

In peracute form (before any clinical signs appear)

• Shed infective amounts of virus for 24-48 hours just before death

nose & eyes - thick whitish to blood tinge discharge
mucous membrane - congested and hemorrhagic
pregnant sows - abortion

1-7 days after clinical signs appear



hemorrhagic shock and excessive fluid in the lungs
1 to 7 days after clinical signs
– coma and death,
100% mortality

In acute forms with clinical signs enormous amount of virus is shed in all secretions and excretions, and high levels of virus present in tissues and blood.

41-42C°fever, huddling, rapid difficult breathing, loss of appetite

flushing to bluish purple skin with bleeding underneath

- **In the subacute forms**

  Same symptoms than in the acute form but usually less severe. 30-70% mortality after 2 and 7 weeks

- **In the chronic forms, shedding of virus cease after 30 days.**

  Low mortality, remain infected for several months to over 1 year. loss of weight, irregular fever, pneumonia-like symptoms, pericarditis, necrosis and ulcers of the skin, nervous signs

DIAGNOSTIC VALIDATION OF ASFV NANOGOLD BIOSENSOR WITH REAL-TIME PCR

Samples	Positivity Rate	% Sensitivity	% Specificity	% PPV	% NPV	Kappa Coefficient / Interpretation	% Accuracy
Surface Swab (n=40)	47.5	100.0	76.2	94.58	100.0	0.75 (substantial)	87.5
Water (n=40)	50.0	90.0	85.0	96.14	67.17	0.75 (substantial)	87.5
Fresh Meat (n=40)	82.5	100.0	85.7	96.68	100.0	0.91 (almost perfect)	97.5
Processed Meat (n=40)	82.5	97.0	85.7	96.58	87.2	0.83 (almost perfect)	95.0
Whole Blood (n=67)	80.6	96.3	77.0	94.55	83.33	0.75 (substantial)	92.5

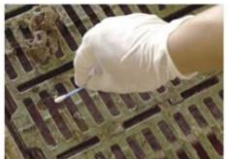
BE AWARE:

1. Pigs of all ages are affected.
2. Infective levels of virus are found only in lymph nodes, and other lymphoid tissues (spleen, bone marrow) until two months post infection.
3. **Undercooked pork, dried and smoked pork and carcass meal** derived from pigs must be regarded as potentially dangerous if fed as **swill** to pigs.
4. ASF virus is stable over a wide temperature and pH range if in a suitable protein environment.
5. As a result of its tolerance to a wide range of environmental factors, only certain disinfectants are effective in the control of ASF.

SAMPLE COLLECTION METHODS

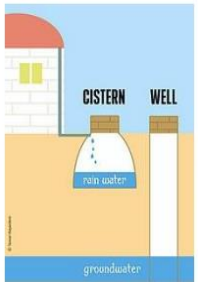
How To Collect Surface Swab Samples:

- Prepare clean cotton swabs.
- With sterile distilled water, moisten the cotton and proceed to swab representative areas in the pen. For instance, walls (where the pigs can reach), floor, nipple drinker, railings, pig ways. One swab represents one surface.
- Swab 5 representative areas per pen.
- Put the 5 swabs in one ziplock and label the origin where these came from (i.e. nursery pen, boar pen etc., & the barangay)
- Put the ziplocks in one styrobox and put gel coolants during transport.



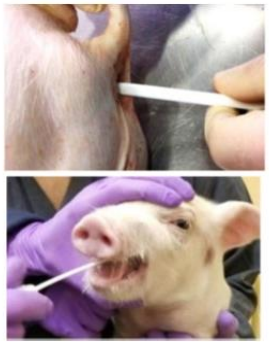

How To Collect Water Sample:

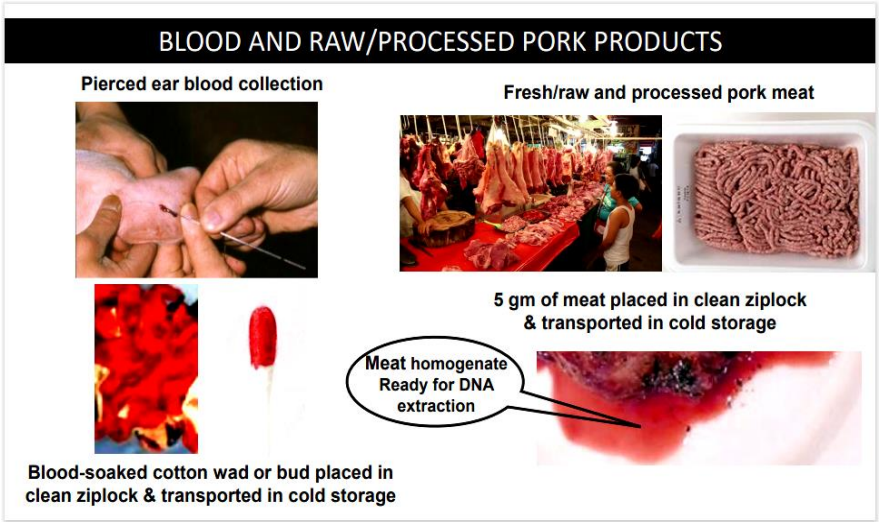
- Prepare a chemically clean plastic bottle (mineral water container).
- Clean the mouth of the bottle before using it for sample collection.
- Collect from the mainline of the farm's water source (deep well).
- Fill until half of the bottle then cover well.
- Label the plastic bottle.
- Place inside the styrobox together with the swabs. Transport with gel coolants.

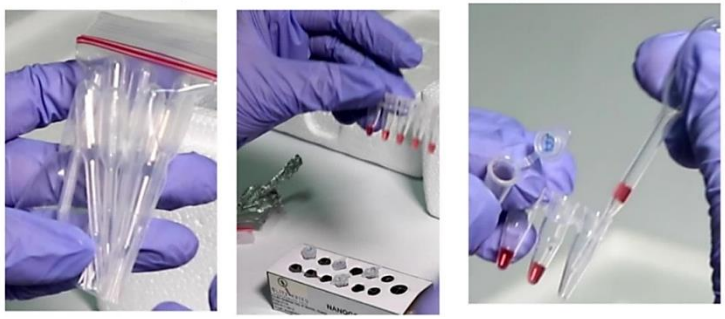

How to Collect Fecal and Oral Swabs

- Prepare clean cotton buds.
- With sterile distilled water, moisten the cotton and insert into the pig's anus with swift swirling motion.
- Choosing randomly (for surveillance), swab 5 pigs belonging to one pen and place all the swabs in one Ziplock and label the origin where these came from (i.e. nursery pen, boar pen, etc. and also the address of the farm).
- For oral swabs, prepare clean cotton buds, moisten with sterile distilled water and insert into the mouth of the animal.
- Similarly, pooling of five (5) oral swabs from 5 different pigs in the same pen can be done.
- Place the swabs in a Ziplock and label properly.



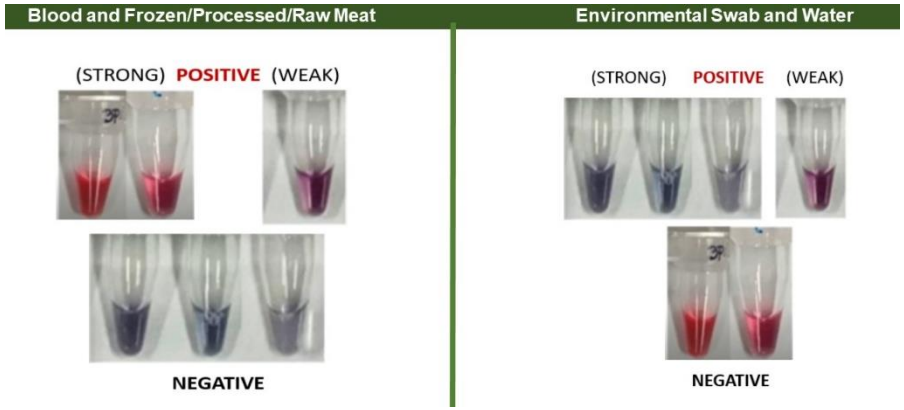


- Remove the PCR tubes from the dry bath and set aside.
- Locate the pouch containing the mini droppers, take one and aspirate the GDye liquid from one PCR tube in the tube strip.
- Carefully transfer this into one PCR tube that was taken out from the heat block.
- Repeat until all the amplified PCR tubes receive the GDye.



- **After adding Reagent 2, insert all the tubes into the heat block again for the last time for the hybridization of the probe with the gold nanoparticles to the amplified products at 58oC for 5 mins.**
- **Then interpret the test results by colorimetry. Below is the interpretation for the blood/meat and for the environmental surface swab/water samples.**

RESULT INTERPRETATION BY COLORIMETRY



DNA AMPLIFICATION (APPLICABLE TO ALL SAMPLES)

DNA AMPLIFICATION (apply to all samples)

- Preheat the dry bath at 58°C and set the time at 30 minutes.
- Locate the PCR tubes inside the styrobox. These contain the **Nanogold premixes** for amplification.
- With a syringe, aspirate 6.3 ul from the MCT and instill 1 drop into the PCR tubes containing the Nanogold premix.
- Gently tap the PCR tube to mix.

DNA AMPLIFICATION (apply to all samples)

- Label the PCR tube caps.
- Insert in the dry bath for a 30 min. amplification.
- Locate the PCR tube strips wrapped in foil that contain the synthesized gold nanoparticles **(GDye)**.

SAMPLE PREPARATION BEFORE DNA EXTRACTION

SAMPLE PREPARATION - Fecal, oral and surface swabs

- Put 1ml of sterile distilled water into the ziplock.
- Press well the swabs between the walls of the plastic Ziplock until the dirt from the buds are squeezed out into the water.

- Get 1 syringe provided in the package, aspirate 0.1 ml of the liquid and transfer into the microcentrifuge tube (found inside the box).
- Repeat this using a new syringe for each sample.
- Label the tube properly.

SAMPLE PREPARATION - Water sample

- Get 1 syringe provided by the package, aspirate 0.1 ml of the surface water and transfer into the microcentrifuge tube (found inside the box).
- Do this using a new syringe for the other samples.
- Label the tube properly.

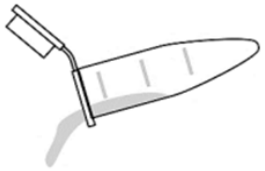
DNA EXTRACTION STEPS FOR FECAL, ORAL, SURFACE SWABS AND WATER SAMPLES

DNA Extraction

Lysis Phase

- Locate the lysis buffer (LB) inside the kit and shake to mix well.
- Aspirate 1.0 mL of **LB** and transfer into the sample's microcentrifuge tube, mix thoroughly.

- After adding the **LB** to all samples, mix well by shaking the microcentrifuge tubes.
- Centrifuge **at 6,000 rpm** for **3 minutes** or **4,500 rpm** for **5 minutes**.
- Remove the supernate by pouring or by aspirating*.



6. With a fresh tuberculin syringe, aspirate 700 ul or 0.7 ml of Extraction Buffer and add into the MCT.
7. The MCT is incubated for 30 mins at room temperature.
8. Meanwhile, boil water in a kettle and transfer into a pan.
9. The MCT is inserted into rubber floater and allowed to float in boiled water or at 95ºC for 5 mins. Cover the pan to sustain the temperature for 5 mins.

10. With a fresh tuberculin syringe, aspirate 700 ul or 0.7 ml of Wash Buffer 1 and add into the MCT after 5 mins of boiling.
11. Incubate at room temperature for another 30 mins to precipitate the DNA.
12. Centrifuge the MCT at 14,500 rpm for 15 mins.
13. The supernate is removed by aspiration or by decanting the MCT.

14. With a fresh tuberculin syringe, aspirate 500 ul or 0.5 ml of Wash Buffer 2 and add into the MCT. Centrifuge at 11,500 for 5 mins.
15. The supernate is removed by aspiration or by decanting the MCT and this is air dried with an open lid for about 15 mins.
16. Finally, the pellet is rehydrated with 30 to 40 ul or approximately 3 drops of Rehydration Buffer using a fresh tuberculin syringe.

DNA EXTRACTION STEPS FOR BLOOD AND MEAT SAMPLES

1. If the blood is collected with a tuberculin syringe from the ear vein, instill 20 drops into a 1.5 ml microcentrifuge (MCT) provided in the kit. If the blood is soaked in a sterile cotton bud, extract the blood by putting the cotton bud in a clean container with 10 ml sterile distilled water. Squeeze the cotton and aspirate the extracted blood in the water with a tuberculin syringe. Instill 50 ul or 0.05 ml or 20 drops into a 1.5 ml MCT provided in the kit.

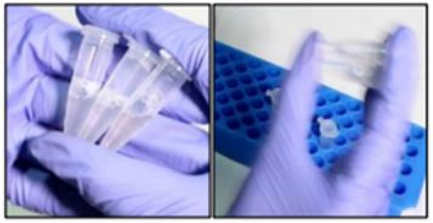
2. For either raw or processed meat, cut into small pieces and mince with sterile scissors. Extract the homogenate or meat juice from the raw meat and aspirate using a tuberculin syringe. Instill 0.05 ml or 20 drops into the MCT in the kit. On the other hand, get 50 mg of the processed meat and place into the MCT.

3. With a fresh tuberculin syringe, aspirate 1000 ul or 1 ml of Lysis Buffer. Add this volume into the MCT holding the sample.
4. The MCT is incubated at room temperature for 30 mins followed by centrifugation at 7500 rpm for 5 mins to isolate the pellet.
5. The supernate is removed by aspiration or by decanting the MCT.

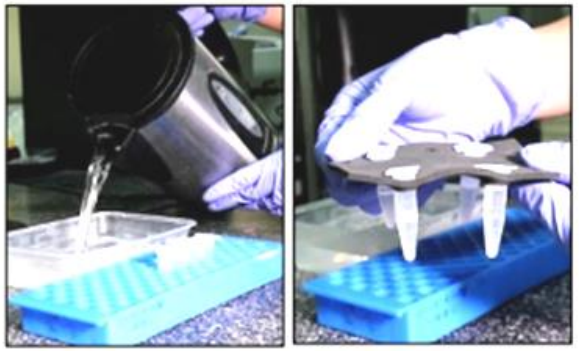
Extraction Phase

- Locate the Extraction Buffer **(EB)** inside the kit and shake to mix well.
- With a 1 ml syringe, aspirate 0.7 ml of **EB** and transfer into each microcentrifuge tube that contains the sample.

- Mix well by shaking the tubes.



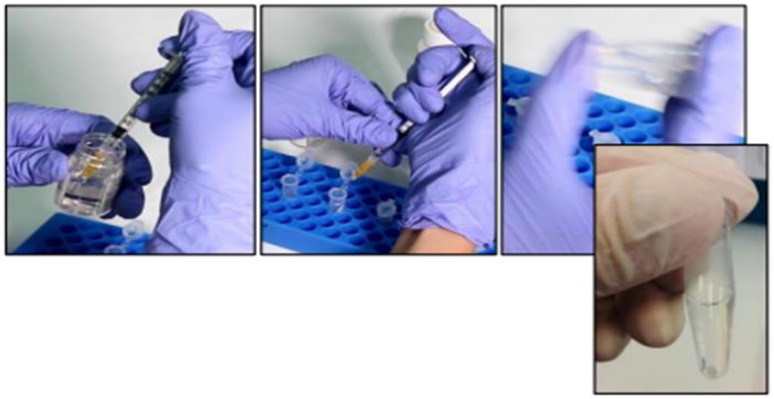
- Meanwhile, boil water separately.
- Locate the rubber float inside the box and insert the microcentrifuge tubes.



- When the water is already boiling, pour into a container and place the float carrying the tubes on the water.
- Cover the container with aluminum foil. Incubate for 5 minutes.
- Afterwards, take out the float, remove the tubes and set them aside.

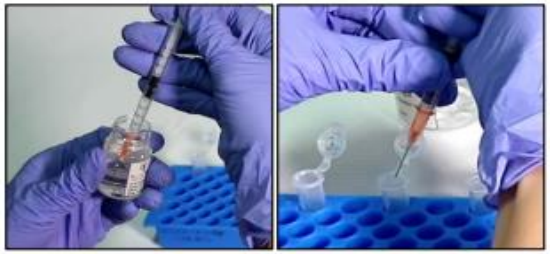
Wash Buffer 1 Phase

- Locate the wash buffer 1 **(WB1)** inside the box and shake to mix well.
- With a 1 ml syringe, aspirate 0.7 ml of **WB1** and transfer to the microcentrifuge tube.
- Mix by inverting and observe for precipitate formation. Allow to stand for **30 minutes** either at room temperature or in the freezer.
- Centrifuge at **14,500 rpm** for **15 minutes**.



Wash Buffer 2 Phase

- Remove the supernate by pouring or by aspirating.
- Locate the wash buffer 2 **(WB2)** inside the kit.
- With a 1 ml syringe, aspirate 0.5 mL of **WB2** and transfer to the microcentrifuge tube.
- Centrifuge again at **11,500 rpm** for **5 minutes**.



Rehydration Buffer Phase

- Remove the supernate using another syringe. Avoid touching the pellet.
- Air dry the microcentrifuge tubes by inverting them with open lids on a dry towel for 15 minutes. Examine for DNA pellet.
- Locate the rehydration buffer (RB) and with av 1 ml syringe, aspirate the content and instill 3 drops to each tube.
- Proceed to amplification or store the rehydrated samples in the freezer until use.