INSTRUCTION MANUAL

for the use of Dot immunobinding assay kit for detection of Aleutian mink disease

1. GENERAL INFORMATION

1.1. The diagnostic kit is designed for the presence of Aleutian mink disease virus (AMDV)-specific antibodies in the blood. AMDV is known as a causative agent of Aleutian mink disease (AD).

1.2. The diagnostic kit includes a set of components including a proprietary recombinant coat protein of AMDV (ADVG strain).

1.3. One kit is sufficient for testing 2000 samples, 20 positive, 20 weakly positive, and 20 negative control samples.

2. DESCRIPTION OF KIT COMPONENTS AND SPECIFICATIONS

The kit includes:

1. **Membranes** – gridded membranes (2x100 squares each) with liners – 10 pieces in a zip-lock plastic bag,
2. **Sodium bicarbonate** – white powder – 18.5 g in a zip-lock plastic bag,
3. **Detergent** – transparent foamy liquid – 20 ml in a vial,
4. **Urea hydrogen peroxide** – tablet – 1.5 g in a zip-lock plastic bag,
5. **Blocking solution** – viscous liquid, pale-yellow to maroon in color – 20 ml in a vial,
6. **Reaction solution** – pale-yellow to maroon-colored liquid – 20 ml in a vial,
7. **Developing solution** – pale-yellow colored liquid – 25 ml in a vial,
8. **Antigen** – lyophilized powder or a liquid (colorless to pale-yellow) –0.5 ml(stock solution), 10 ml or 20 ml (working solution) in a vial,
9. **Antibodies** – lyophilized powder or a liquid (colorless to white) – 0.1ml in a vial,
10. **Conjugate** – lyophilized powder or a liquid (colorless to white) — 0.05 ml in a vial,
11. **Levamisole** – transparent liquid – 0.25 ml in a plastic test-tube
12. Control antibodies (positive - **(+) Control,** weakly positive - **(±) Control**, negative – (**-) Control**) – pale yellow to maroon-colored liquids – each, 0.05 ml in plastic test-tubes,
13. Incubation tray – 1 pc
14. Instruction manual – 1 pc

3. KIT PACKAGING

Aliquoted and closed kit components are placed in a cardboard box with dividers or slots that ensure the components stay appropriately fixed. Incubation tray and Instruction manual are included in each box.

4. PERSONAL PROTECTIVE MEASURES

The kit is biologically safe, however several immunospecific kit components (blocking solution, reaction solution, antigen, antibodies, control antibodies) include a preservative, sodium azide, which is a toxic substance, but which is not dangerous to human health at a concentration used in the kit (0.1%).

Developing solution and urea hydrogen peroxide (undiluted) may cause irritation.

Wear protective gloves to avoid contact of skin and mucosa. If kit components come into contact with skin or mucosa, the affected area must be immediately and thoroughly washed with

plenty of water. Should immunospecific components be ingested, induce vomiting and seek medical attention.

Plastic and glassware used for working with the kit must be appropriately marked.

Do not eat, drink, smoke or use cosmetics in the rooms where the kit is used.

Disposal of kit components after expiration date does not require special handling or safety measures and is done by extensive washing with tap water.

5. PROCEDURE

5.1. Blood sampling.

Blood samples are drawn from minks directly in the sheds. On the day of blood collection, do not feed minks before sampling to avoid fat in the blood. Use provided membranes (Component #1) for blood sampling. Each kit contains 10 membranes. Each membrane has a grid for 200 samples. The membranes are supplied with waterproof separation sheets attached underneath. Never remove separation sheets from the membranes before DIA processing, as this may cause cross-contamination. Fix membrane (together with separation sheet) by pins (or by lancets) on any clean and even surface. Mark the membrane at the margins using ball pen to identify samples (date of sampling or shed # or mink #). Use gloves. Do not touch the grid area with ungloved hands to avoid leaving fingerprints on the membrane. Fix mink properly to provide access to one of its paws. Puncture mink finger pad with sharp end of a disposable stainless steel blood sampling lancet*. Wait a second until a drop of blood appears. If no bleeding, massage the finger lightly. If massaging does not help, puncture another finger pad. Pick up a drop of blood using blunt end of the lancet and apply it onto the membrane. Lancets with a round blunt end are preferable to prevent membrane damage. Avoid spreading of the blood spot beyond the borders of the individual cell on the membrane. Make sure that blood samples are dry before putting the membranes together. New lancet must be used for each animal. Use alcohol-wetted cotton balls to clean and disinfect gloves periodically. Be careful. Avoid blood contamination by the disinfectant. Allow membranes with applied blood samples to air-dry. Put the set of membranes (together with separation sheets) back into zip-lock plastic bag. Send them to the lab by express mail. The DIA procedure tolerates up to three weeks of time between blood sampling and processing. However, it is strongly recommended to send membranes with samples to the lab as soon as possible. Keep in mind that intensity of positive signal starts to fade gradually after a week of storage. Before sending store locked plastic bags with membranes at +4 - +8^{o}C.

5.2. Spotting control antibodies

Control antibodies (positive, weakly positive, and negative — kit component №12) are spotted onto the membrane using a glass capillary on the free area in the center of the membrane as two spots per control. This is done either following the blood sample spotting, or 0.5-1 hrs before membrane processing.

5.3. Reagent preparation

5.3.1. Preparation of the washing solution.

Dissolve the content of the plastic bag (kit component №2) in 2 liters of distilled water. Then, add the Detergent (kit component №3) and mix thoroughly.

5.3.2. Preparation of the hydrogen peroxide solution

Dissolve the tablet (kit component №4) in 30 mls of the washing solution shortly before use.

5.3.3. Preparation of the blocking solution

Place the vial with the blocking solution (kit component №5) into a water bath set at +45-50°C shortly before use.

5.3.4. Preparation of the antigen working solution

Shortly before use, bring the content of the vial with the antigen (kit component №8) to 20 ml with a room-temperature washing solution (no heating!); this may cause the solution to become temporarily opaque.

5.3.5 Preparation of the antibody working solution

Prior to use, transfer the entire content of the Antibody vial (kit component №9) into Reaction solution (kit component №6), let dissolve by incubating at +37°C. The active component of the Antibody vial is found in the insoluble pellet. Alternatively, prepare the antibody working solution and let stay for 1 hour at room temperature.

5.3.6. Preparation of the conjugate working solution

Shortly before use, dissolve the content of the Conjugate vial (kit component №10) in 20 ml of the washing solution by incubating at +37°C. Alternatively, the solution can be prepared 1 hour before the use and left at room temperature.

5.3.7. Preparation of the developer

Heat the Developing solution vial (kit component №7) to +37°C and add Levamisole (component № 11). Developer is prepared immediately before use.

5.4. **ELISA procedure**

Adhere strictly to the indicated temperature regimes to obtain high-quality reactions. For the membranes to be uniformly wetted by the reagents used (except for the very first one), the stacks of membranes are placed into incubation tray(s) and then are layered onto the solution (active shaking) one-by-one. It is preferable that a thermoshaker (10 mm orbit) is used for this purpose. Should such thermoshaker be unavailable, a combination of a thermostate and a shaker (min orbir 10 mm) can be used.

5.4.1. Soaking the membranes (to be performed at +18-37 °C)

Pour 40 ml of washing solution into a tray. Remove the separation sheet. Load the membrane, sample side facing up, horizontally into the washing solution. When the membrane has soaked completely, let it sink by slightly shaking the tray. This way, all ten membranes are transferred into the tray one-by-one. Next, the washing solution is poured off, and replaced with 30-40 ml of fresh washing solution. Let the tray rock for 3-5 minutes and replace the solution with a new portion. Keep changing the washing solution every 3-5 minutes until no longer becomes blood-colored (4-6 changes). Whenever fibrin clots remain stuck on the memrane, gently remove the clots with a cotton swab or a sponge. Make sure no clots remain attached to the back side of the membrane and that the entire stage takes at least 20 minutes.

5.4.2. Membrane destaining (to be performed +18-40° C)

Pour off the washing solution from the tray and add the hydrogen peroxide solution (step 5.3.2.). Keep the tray for 5 minutes. Replace the hydrogen peroxide solution with 30-40 ml washing solution under constant agitation. Repeat the washes (3-5 minutes each) two more times. Total washing time must be at least 15 minutes.

5.4.3. Blocking the membrane (to be performed at solution temperature +45°-50°C)

Discard the washing solution from the tray and add the blocking solution (step 5.3.3.). Proceed with the membrane re-stacking procedure as follows: hold the stack of membranes vertically using blunt-end forceps against the wall of the tray, then place the membranes one by one back into blocking solution, shake the tray slightly to ensure the solution has evenly wetted each membrane. Incubate at +45°C solution temperature (never exceed +50°C) for 30 minutes with constant rocking. Wash the membrane 3-4 times with a washing solution: 30-40 ml, 3-5 min per wash, under constant agitation. Total washing time must be at least 15 minutes.

5.4.4. Antigen binding (to be performed at temperatures below +37°C)

Discard the washing solution and add the antigen working solution (step 5.3.4.). Proceed with the membrane re-stacking procedure as described above. Incubate at +30°-+37°C for 1 hour with constant rocking. Wash the membrane 3-4 times with a washing solution: 30-40 ml, 3-5 min per wash, under constant agitation. Total washing time must be at least 15 minutes.

5.4.5. Antibody binding (to be performed at +37°C)

Replace the washing solution with the antibody working solution (step 5.3.5.). Proceed with the membrane re-stacking procedure as described above. Incubate at +30°-+37°C for 1 hour with constant rocking. Wash the membrane 3-4 times with a washing solution: 30-40 ml, 3-5 min per wash, under constant agitation. Total washing time must be at least 15 minutes.

5.4.6. Conjugate binding (to be performed at +37°C)

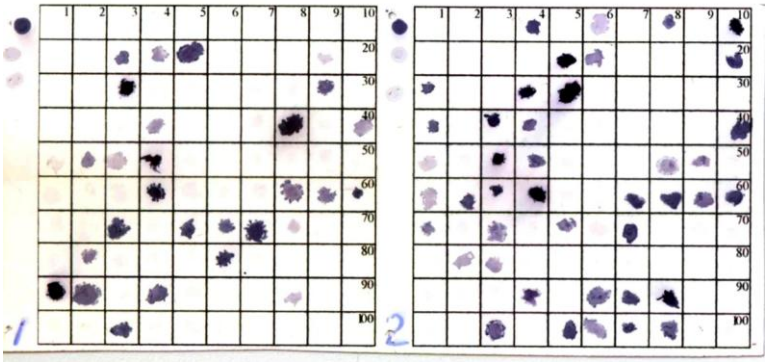
Replace the washing solution with the conjugate working solution (step 5.3.6.). Proceed with the membrane re-stacking procedure as described above. Incubate at +30°-+37°C for 1 hour with constant rocking. Wash the membrane 8 times with a washing solution: 40 ml, 5 min per wash, under constant agitation.

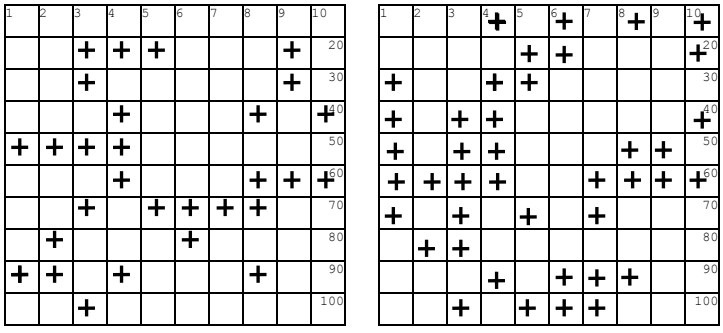
5.4.7. Developing the membranes

Pour off the washing solution from the tray and add the developer (step 5.3.7) preheated to +37°C with vigorous shacking. Quickly re-stack the membranes, cover them with a light-proof cover or foil. Monitor the developing reaction visually. When positive controls turn violet-black and the weakly positive control spot become clearly visible (5-20 minutes at +37°C), discard the developer and add 40-50 ml of distilled water. Rock for 2-3 minutes, and wash 2-3 times with plenty of tap water (total 1-2 liters). Transfer the membranes onto the filter paper.

5.5. **Interpreting the reaction results**

Wet membranes are analyzed under transmitted light (the membranes are held against a table-top lamp (>75 Watt)). The reaction spots are matched against the controls. Presence of a light-purple to black spot (corresponding to the weakly positive and positive controls, respectively) with a clear border should be interpreted as a positive result. The animal that produced such signal is considered infected with AMDV.





Weak dots with diffuse borders, small stained dots, star-shaped spots, etc, indicate that some technical issues have occurred during blood drawing, sample spotting, membrane storage or reaction set-up.

If the signal observed is difficult to interpet, it is advised that the same animal is re-sampled and tested using CIOEP or ELISA. It must be stated, that in rare instances weakly positive reaction signals may be observed in healthy animals 2-3 weeks following vaccination. For this reason, the animals should not be screened using this test sooner than 3 weeks post-vaccination

Animals that reproducibly show positive reactions are considered infected and sanitary measures specified in the farm instructions are performed.

6. STORAGE CONDITIONS AND EXPIRATION DATE

The kits must be stored in a dark dry place at +2°-+8°C. Developing solution (kit component № 7) is light-sensitive, and so it should never be left open under light.

The kit shelf life is 6 months since the date of manufacture, under indicated storage conditions.

Instruction manual was developed by the «Imgen+» Ltd