Lot-No.

Ref. K333A

MANUAL - one step

Expiry date: 1 year

STORE AT -20°C

100 Tests (Ready to use PCR kit)

Avian Influenza Virus (H5) – one step PCR

-Only for in vitro use-
-Only for veterinary use-
-Humans, only for research use-
-To be used by a technical person-

Principle and use

This amplification kit has been manufactured by *Genekam Biotechnology AG*, Germany to detect *Avian Influenza virus H5* (as well as H5N1).

This kit needs RNA which can be isolated from blood, serum, faeces, respiratory swabs, tissue, respiratory fluid and any body fluid. Kindly use good methods to isolate the RNA. Kindly take common safety laboratory precautions during working. ***Please use gloves during work. Proceed clean and carefully otherwise you may cause contamination problems. Do not touch other objects like pens, chairs etc. during Part 1.***
IMPORTANT: we added cotton or sponge in the lid of container of the kit, to avoid damage during transportation. Please remove this cotton or sponge from the lid of each container before storage.

Composition:

It contains the following **(WARNING! THAW THE TUBES SLOWLY: NEVER THAW IN HEATING BLOCK OR WITH HEAT FROM HAND):**

- Tube A (for H5) (2 tubes)
- Tube B (2 tubes)
- Tube H (for N1) (1 tube)
- Tube Y (1 tube)
- positive (+ve) control (D1) (1 tube): **it is cDNA, it should be stored at -20°C.**
- Negative (-Ve) Control (tube D2) (1 tube)
- Marker (tube E) (1 tube) (max 1000bp): 100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000bp)
- Dye (tube F) (1 tube)

Please check them before you start.

Equipment needed:

- PCR thermocycler
- Laboratory centrifuge
- UV plateform
- UV safety goggles
- microtubes (0.2ml)
- Pipette-tips with and without filter (20µl, 5µl & 1µl)
- Pipettes (quality pipettes)
- Gel Agarose chamber
- Power supply
- Paper
- Pen
- Agarose (good quality)
- Staining (Ethium Bromide)
- TAE buffer 1x

• Ice
• Vortexer

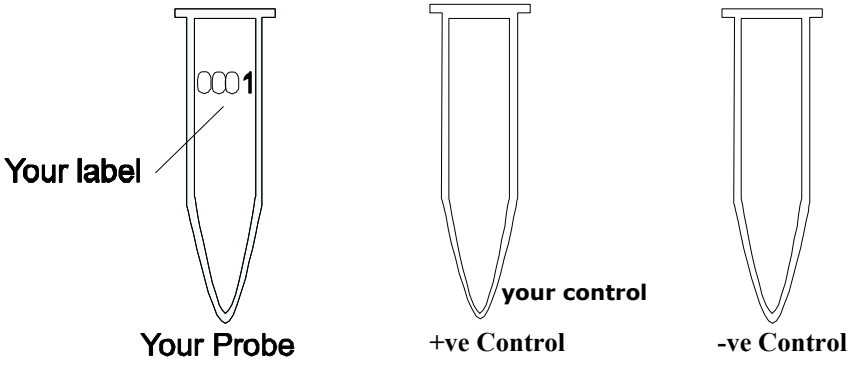
Procedure:

PART 1 – it is a one step PCR. It will identify H5 in one first step.

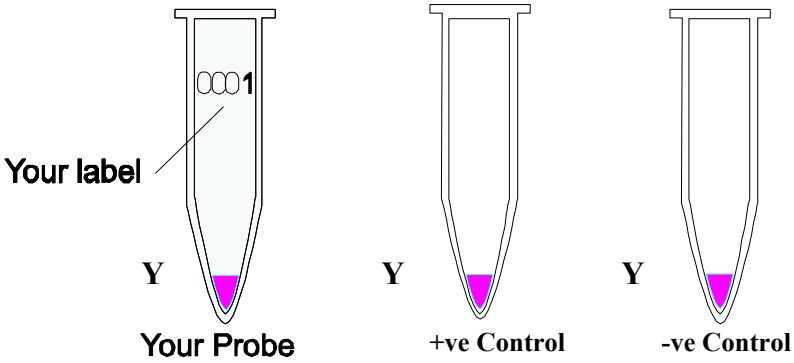
STEP A

1. Kindly thaw **one tube** each: Y, A, B, D1, D2, E, F and H. After thaw, kindly put the tubes at 4°C (as it is better). If the kit is not in use, store them at -20°C.

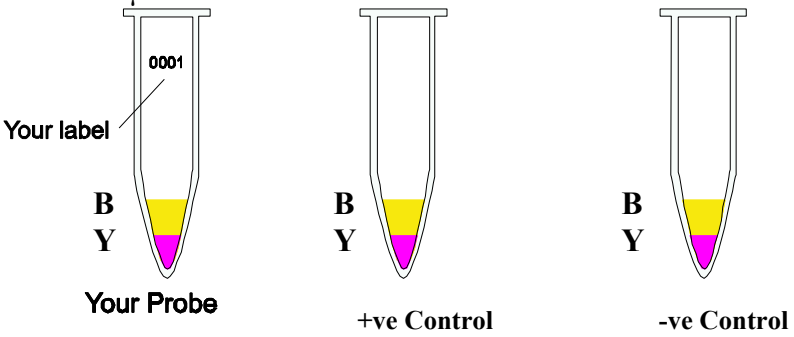
2. Mark your microtubes with a sample number, +ve Control and –ve Control.



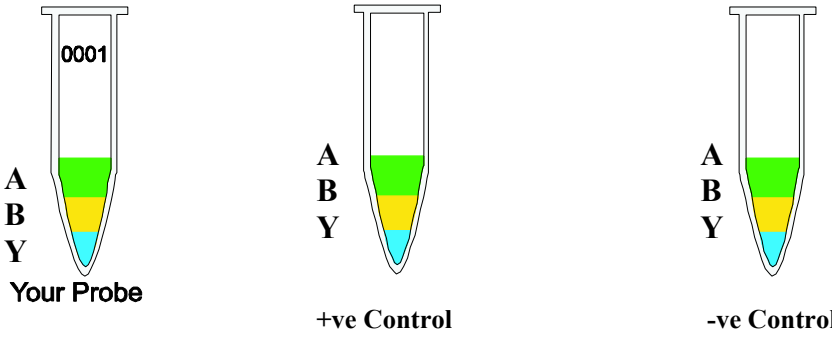
3. Add 1µl of tube Y to each tube.



4. Add 10µl of B to each micro tube. Avoid to touch the wall of the micro tubes.

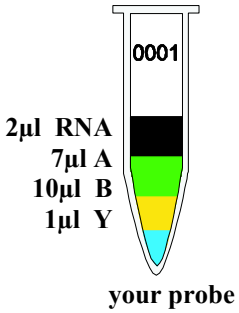


5. Add 7µl of A to each tube (avoid to touch the wall of the microtubes).

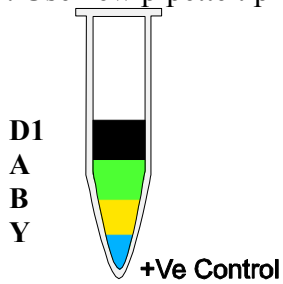


TIP: to save time and money, you can calculate how much chemicals you need to run the test. You want to run 10 test, i.e. you need 10 x 7µl of A = 70µl of A + 10 x 10µl of B = 100µl of B + 10 x 1µl of Y = 10µl of Y = 180µl in total. From this, 18µl can be added to each microtube.

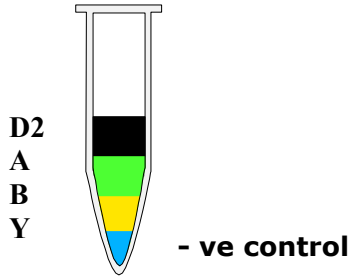
6. Add 2µl of your RNA with pipette tip with filter to each micro tube according to your label except +Ve and -Ve (Avoid touching the wall). **Use every time a new pipette tip** (for each sample)! Mix it thoroughly.



7. Use new pipette tip with filter. Add 2µl of D1. Use a new pipette tip. Mix it.

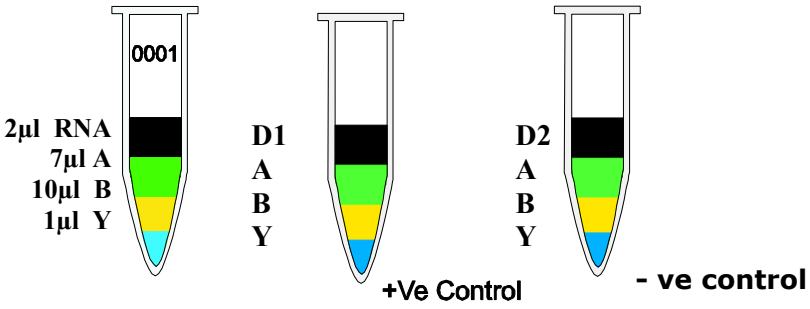


8. Use a new pipette tip. Add 2µl of –Ve (tube D2) to –Ve Control (don't touch the wall). Mix it.



9. Centrifuge all tubes for 20 sec. for 8000 rpm (this is not necessary but it is better).

10. Run the program of your thermocycler as followings:
Kindly check whether you have added everything correctly as the level of the volume of each micro tube must be almost the same.



Now program your PCR machine as follows.

1. 3000 seconds at 42°C
2. 600 seconds at 48°C
3. 600 seconds at 70°C
4. 300 seconds at 95°C
5. A. 30 seconds at 94°C
   B. 30 seconds at 55°C
   C. 30 seconds at 72°C
   } 40 cycles
6. 300 seconds at 72°C

Before you start the PCR program, kindly check whether tubes are closed properly. **Microtubes must be in contact with metal block** (very important!). There should be no air or lose contact with metal block of thermocycler.

11. After step 10 is finished take out the microtubes and centrifuge for a while.

Now go to STEP B.

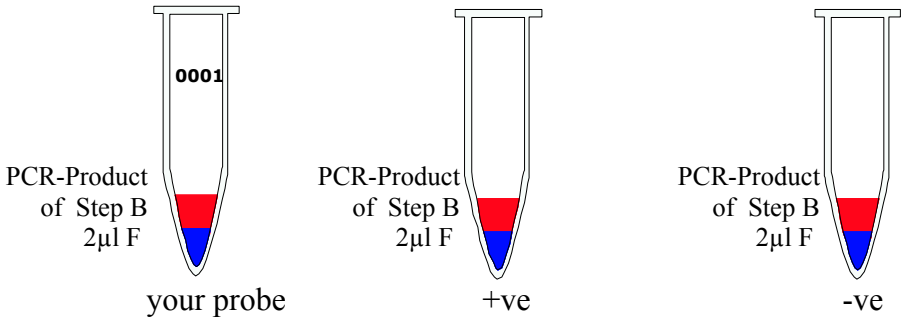
STEP B

1. Prepare the gel Agarose 1.5% or 2% in TAE (1x) buffer.

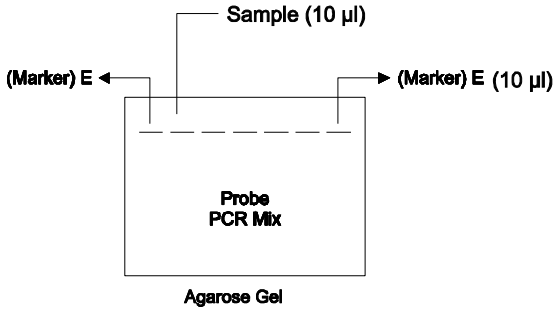
2. Let the Gel dry and add this TAE (1x) buffer in gel chamber.

3. Take the tube E (marker). Make ready to use for gel electrophoresis.

4. After the PCR step is finished, now you can prepare for gel Agarose electrophoresis. Take 2µl of Dye (tube F) and add to each micro tube (with the same number as your PCR microtubes including +Ve, -Ve controls).



5. Add 10µl of marker (tube E: 100bp) to first and last lane of electrophoresis. (Kindly make lane plan on paper according to your probes in order to identify later and see the results).



6. Add 10µl of mix of step 4 to each lane of gel Agarose (between first and last lane). Change the pipette tip for each lane.

7. Run the gel for **55 min**. at **120 Volt**. It may vary

8. Make staining solution ready.

9. Put the gel for 5-30 minutes staining solution (0.5µg/ml).

10. View the gel under UV light. UV light is dangerous for your eyes. Use UV goggles.
11. You must find the bands in positive control and no band in negative control.
    You will see band 219bp in positive control and positive samples for H5.
    Genesequencing is highly recommended to subtype N1.

STEP C: detection of N1

It can be performed separately as in this step, you will test for N1 for all probes which are positive for H5. This step is similar to Step A, where instead of A you will use H. All other conditions will be the same as in Step A. You will have to go Step B (electrophoresis). You will find the band at 616bp if it is positive for N1. If you don't find any band, but you have H5 positive, please go genesequencing to be sure for the subtype of H5.

Tip: Gene sequencing is highly recommended to reconfirm the results, as this is the recommendation of WHO to make the best results.

If you should find any mistakes, please let us know. Thank you.

Suggestion: **This manual has been written specifically for beginners, hence persons with experience in PCR must use their experience to keep each step as small as possible e.g. you should calculate the amount of the needed chemicals, before starting with testing.** **Last update: 02-02-2012** **v1.7**	**Genekam Biotechnology AG** **Dammstr. 31-33** **47119 Duisburg** **Germany** **Tel. (+49) 203 / 555858-31,-32,-33** **Fax (+49) 203 / 35 82 99** **anfrage@genekam.de** **http://www.genekam.de**