

Lot-No.

Ref. FR006

## MANUAL – Real time one step

Expiry date: 1 year

**STORE AT -20°C**

100 Tests (Ready to use kit)

AVIAN INFLUENZA VIRUS H5N1 – one step (Real time)

**-Only for in vitro use-**

**-Only for research use-**

**-To be used by a technical person-**

### Principle and use:

This amplification kit has been manufactured by *Microboss Hightech GmbH*, Germany to detect *avian influenza virus H5N1* in real time PCR.

Real time PCR is based on fluorogenic dyes. In our kit we use 2 dyes, they are 6-Carboxy tetramethyl rhodamine (reporter) and Carboxy-fluorescein (quencher). Up to 36 Ct should be taken positive. Value between 36-40 Ct should be taken as marginal positive (doubtful).

This kit needs DNA which can be isolated from nasal swabs, blood, urine, respiratory swabs, lung tissue, faecal samples, blood serum, tissue and any body fluid. Kindly use good methods to isolate the DNA.

Safety precautions should be taken as avian influenza virus is infectious for human beings and animals. Always clean your hands before the test use and clean the hands after the test. Wash your face after the test, if possible. Disinfect your working place.

***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 (2 tubes)
- Tube B (2 tubes)
- Tube C (1 tube)
- Tube Y (1 tube)
- Positive (+Ve) Control (tube D1) (1 tube). **This tube must be stored at -20°C. The user can also make his own positive control, if this positive control is not functioning or the quantity is not sufficient.**
- Negative (-Ve) Control (tube D2) (1 tube)

Please check them before you start. **Please store them at -20°C and dark.**

### Equipment needed:

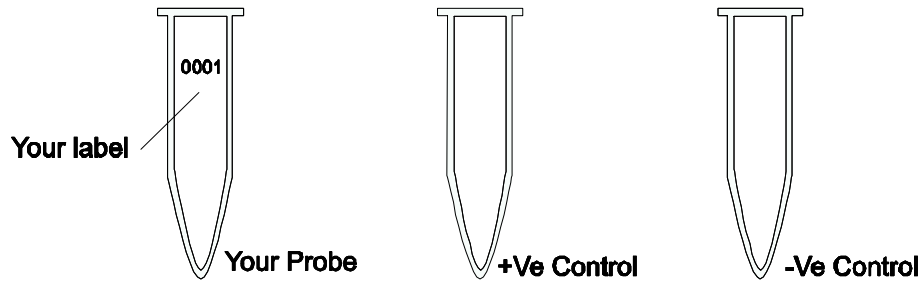
- Laboratory centrifuge
- microtubes (0.2ml)
- Pipette-tips with and without filter (20µl, 5µl & 1µl)
- Pipettes (quality pipettes)
- Paper
- Pen
- Vortexer
- 96 well microplates for PCR
- Real time machine

**Procedure:**

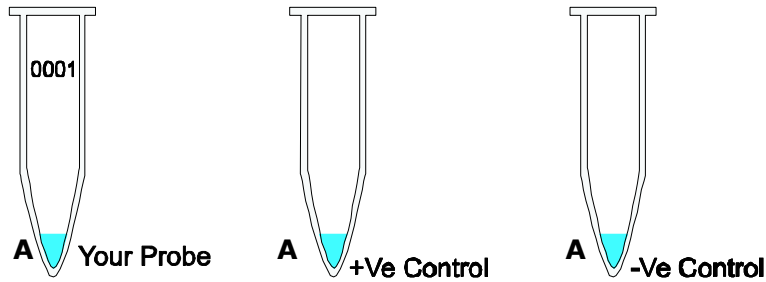
After your RNA isolation is completed. (Kindly use good quality isolation method).  
 Please go to PCR step

**STEP A**

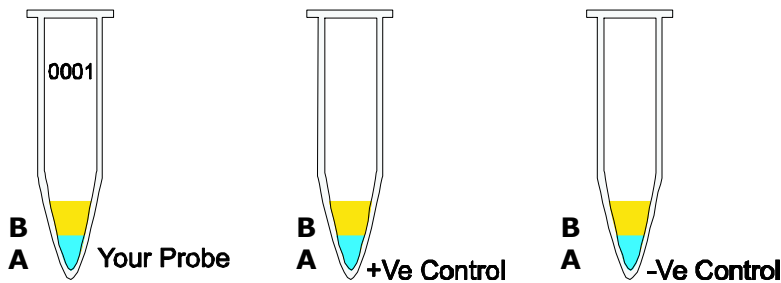
1. Kindly thaw **one tube** each: A, B, C, Y, D1 and D2. After thaw, kindly put the tubes on 4°C (as it is better). If the kit is not in use, store them at -20°C. Kindly keep tubes away from sunlight. Avoid frequent thawing and freezing of chemicals.
2. Mark your microtubes with a sample number and with +Ve Control and -Ve Control.



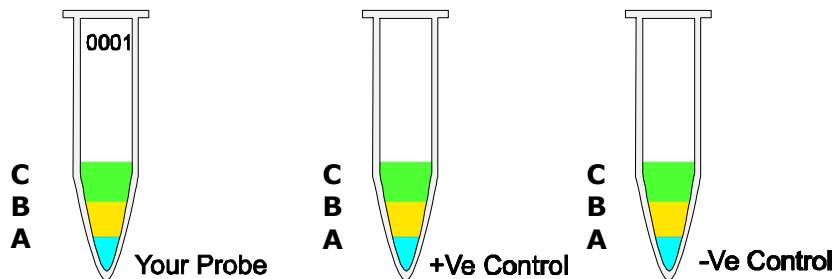
3. Thaw tube A. Add 6µl of tube A to each tube. One can also use 96 micro well plate.



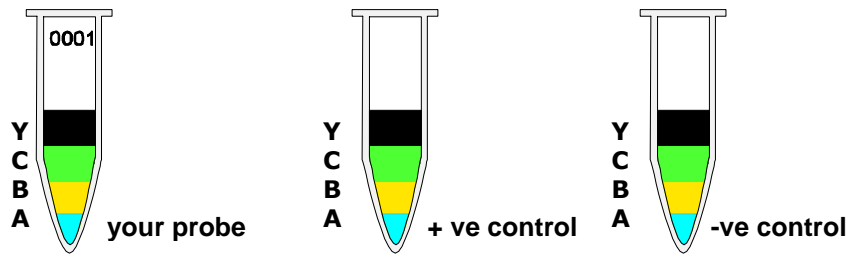
4. Add 10µl of B to each microtube. Avoid to touch the wall of the microtubes.



5. Add 1µl of C to each tube.

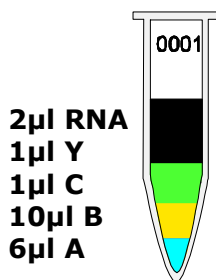


6. Add 1µl of Y to each tube.

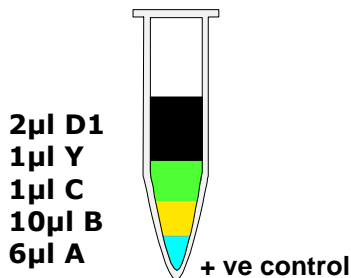


**TIP: you can mix 6µl of A + 10µl of B + 1µl of C + 1µl of Y together in one tube (it will be a total volume of 180µl for 10 reactions). From this one can take 18µl and distribute in each tube. In this way one can save the hardware and time.**

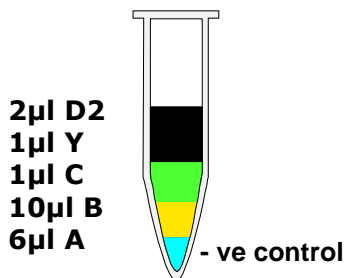
6. Add 2µl of your RNA template (RNA isolated from samples) with pipette tip with filter to each microtube according to your label except +Ve and –Ve (Avoid touching the wall). Use **everytime a new pipette tip** (for each sample)! Mix it.



7. Use new pipette tip with filter. Add 2µl of +Ve (tube D1) to +Ve Control (avoid to touch the wall). Use a new pipette tip. Mix it.

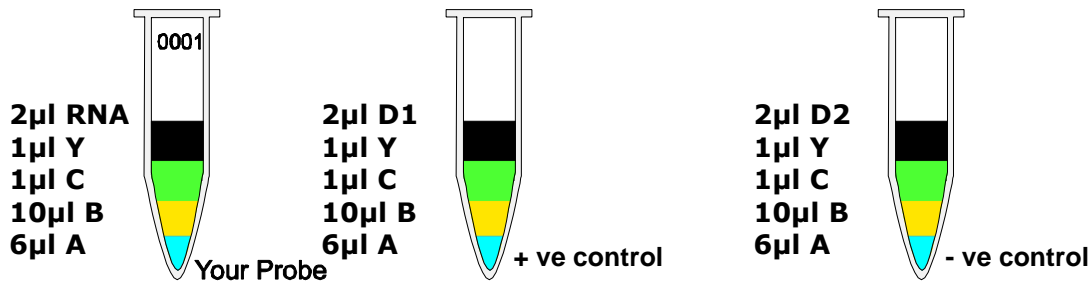


8. Use a new pipette tip. Add 2µl of –Ve (D2) to –Ve Control (don't touch the wall). Mix it.



9. Centrifuge all tubes for 20 sec. for 8000 rpm (not necessary but better). Run PCR now.

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



You must use quencher and reporter dye to setup your software (see FAQ) and run the following program:

1. 60 minutes at 42°C  
10 minutes at 70°C
2. 15 seconds at 95°C } x 40 cycles  
60 seconds at 60°C }

Before you start the PCR program, kindly check whether tubes are closed properly.

**Microtubes must be in contact with metal block** (important!). There should be no air or lose contact with metal block of thermocycler. Run your PCR now.

11. After step 10 is finished take out the microtubes.

### STEP B

Once the program will be finished one can see the graphics. The negative control should run along with the bottom and positive control must give a curve in the software graphics. Use your software to analyse the results.

**Recommendation: the results should be reconfirmed by gene sequencing.**

**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.

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#### FAQ:

- 1) Q: I cannot find quencher and reporter dye in my software:  
A: Many software has got the words: FAM (as reporter) and TAM (as quencher). Therefore select both in your software.  
If your machines has only one word (for some machines only use the word FAM) you should select this one.