Ref. K002

100 Tests (Ready to use kit)

MANUAL

STORE AT -20°C

MYCOPLASMA BOVIS

- Only for in vitro use-
-Only for veterinary use-
-To be used by technical person-

Principle and use

This amplification kit has been manufactured by *Genekam Biotechnology AG*, Germany to detect Mycoplasma bovis in nested PCR. This kit needs DNA which can be isolated from blood, respiratory swabs, lung tissue, cultures, milk of mastitis cows, synovial samples, bronchial as well as lung lavage fluid, milk, feaces, tissue and any body fluid. Kindly use good methods to isolate the DNA. Kindly take common safety laboratory precautions during working. This should be used by a technical person. The sensitivity of this kit is around 10 CFU/ml however it indicates only +ve or –ve results.

Detection limit: 10 CFU/ml milk

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)
- Positive (+Ve) Control (tube D1) (1 tube)
- 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 and pen
- Agarose (good quality)
- Staining (Ethium Bromide)
- TAE buffer 1x
- Ice
- Vortexer

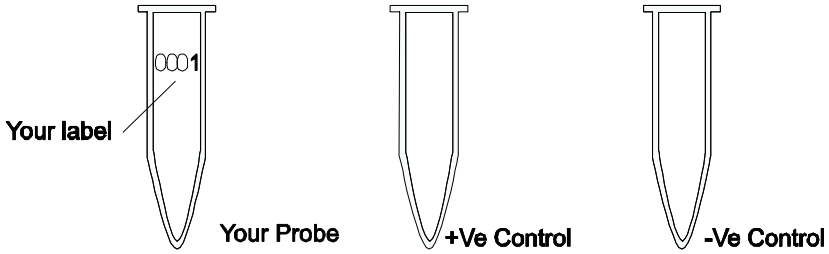
Procedure:

After your DNA isolation is completed. (Kindly use good quality isolation method).

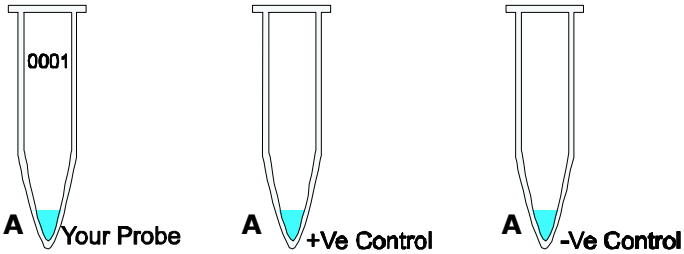
STEP A

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

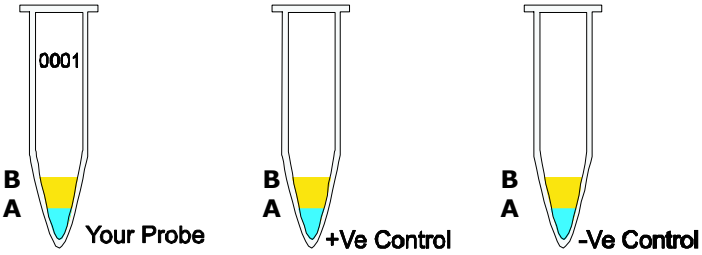
2. Mark your microtubes with a sample number and with +Ve Control and –Ve Control.



3. Thaw tube A. Add 8µl of tube A to each tube.

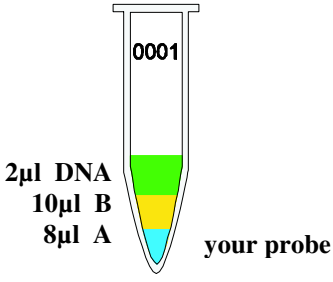


4. Add 10µl of B to each microtube. Avoid to touch the wall of the microtubes.

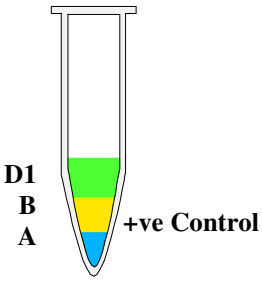


5. **Tip: You can calculate your complete requirement i.e. you need 8 ul of A and 10 ul of B. In case want to run 10 reactions, you can add 100 µl of B + 8 µl of A = 180 µl together. From here you can take 18µl each. In this way, one can save the costs.**

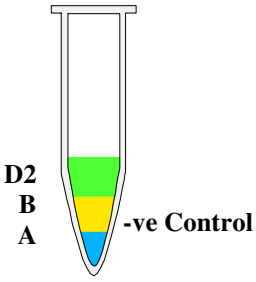
6. Add 2µl of your DNA template (DNA isolated from samples) with pipette tip with filter to each microtube according to your label except +Ve and –Ve (Avoid touching the wall). **Use everytime a new pipette tip** (for each sample)! Mix it.



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.

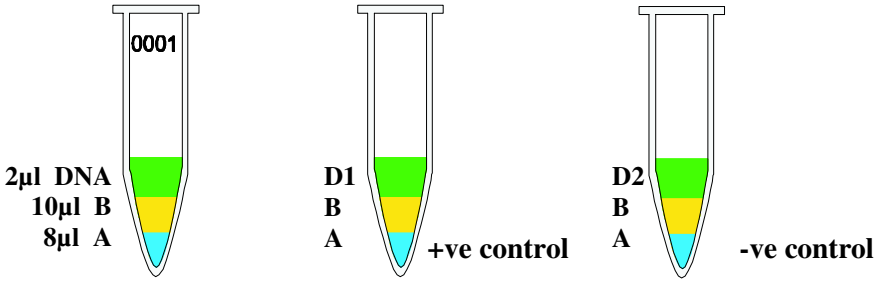


8. Use a new pipette tip. Add 2µl of –Ve (D2) to –Ve Control (don't touch the wall). Mix it.



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

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



Now program your PCR machine as follows.

1. 690 seconds at 94°C
2. 30 seconds at 94°C
   60 seconds at 48°C
   150 seconds at 72°C
   } 35 cycles
3. 300 seconds at 72°C

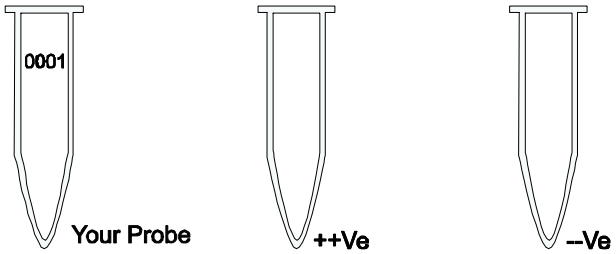
Before you start the PCR program, kindly check whether tubes are closed properly.
Microtubes must be in contact with metal block (very important!). There should be no air or lose contact with metal block of thermocycler.

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

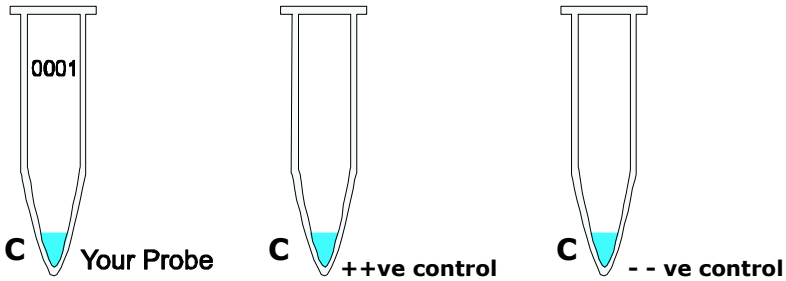
To see Mycoplasma bovis, you can go directly to step gel electrophoreses (STEP C).
Meanwhile you can go to step B (This step is for Mycoplasma bovis).

STEP B

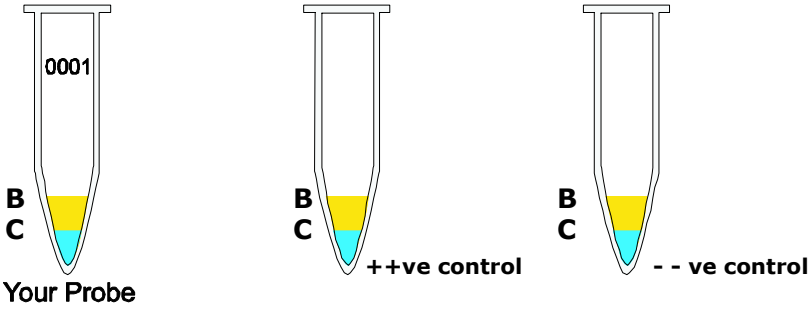
1. Mark your microtubes with a sample number and with +Ve Control and –Ve Control.



2. Add 5µl of tube C to each tube.



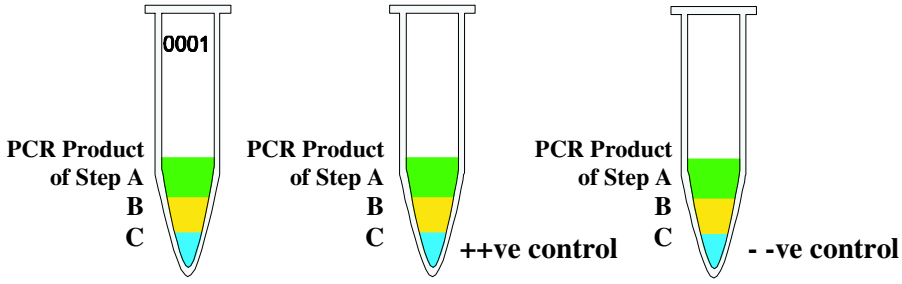
3. Add 5µl of B to each microtube. (Avoid to touch the walls of the microtubes).



4. Tip: Kindly make the calculation how much chemicals are needed. 5µl of C + 5 µl of B. To do 10 probes, please mix 50 µl of C and 50 µl of B = 100 µl. Take our 10 µl for each sample.

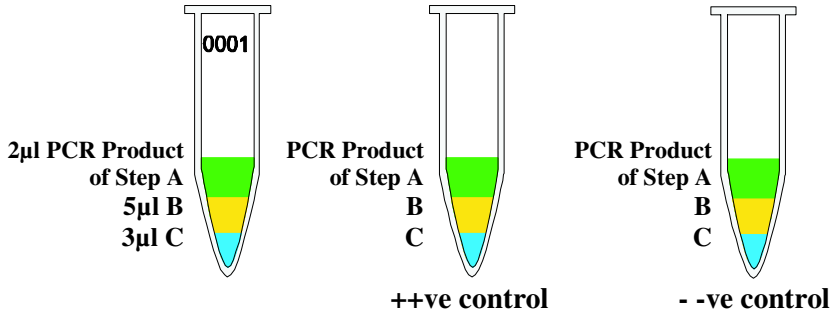
5. **Kindly dilute your PCR product of Step A.** Kindly just dip the pipette tip (please use only 10ul pipette tips) in the PCR tube to pick up PCR product of first step of nested PCR and add this to each new microtube according to labelling plan (avoid touching the wall). (Same number or label name as your microtubes of step A.) **Use every time a new pipette tip with filter** (For each sample)! Mix it.

Explanation: Once the user will dip pipette tip in PCR tube containing PCR product of first part of nested reaction, it will pick up some PCR product, which is sufficient to run the 2nd stage of nested PCR. Do not use 0.5µl or 1µl PCR product as it is too much for PCR reaction and the results may be not be correct. If you do not want to use the dipping method, you have to dilute PCR product 1:100 (1µl of PCR product and 99µl of molecular water) and you have to use 2µl of this diluted PCR product.



6. Centrifuge each microtube for 20 sec. for 780 rpm (this is not necessary but it is better).

7. Run the program of your thermocycler as followings: Kindly check, whether you have added everything correctly. The level of volume should be the same in each tube.



Program of thermocycler: Run this program

1. 690 seconds at 94°C
2. 45 seconds at 94°C
   60 seconds at 54°C
   120 seconds at 72°C
   } 30 cycles
3. 300 seconds at 72°C

After the program is over you can go to **STEP C**.

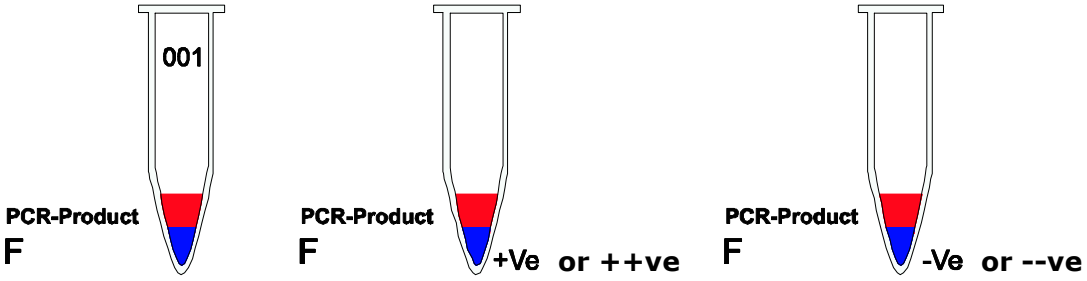
STEP C

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

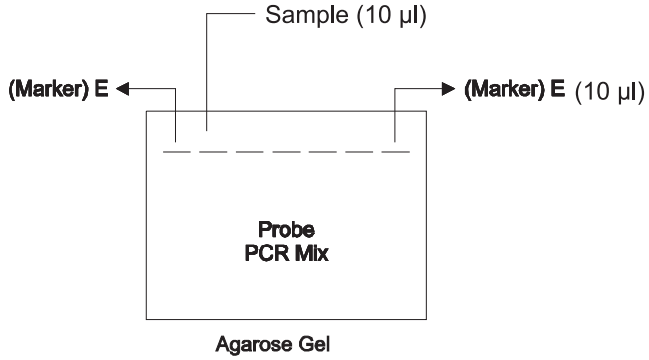
2. Let the Gel dry and add this TAE (1x) buffer in gel chamber.

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 or 1 ul of Dye (tube F) and add to each microtube (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 **60 min**. at **110 Volt**. It may vary.

8. Make staining solution ready.

9. Put the gel for 5-15 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.
    In the first step: 1911 bp in positive samples i.e. it Mycoplasma bovis positive
    In the 2nd step: 442 bp band in positive samples i.e. it is Mycoplasma bovis positive

Both bands 1911 in Step A and 442 in Step B indicate also *Mycoplasma bovis* positive.

It may be possible that there is no band in the first step, but there is a band in the second round. In that case it should be considered positive.

Recommendation: genesequencing is highly recommended to reconfirm the positive 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.

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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