

Lot.

**Ref. K767 A**

## **MANUAL – one step**

**Expiry date: 1 year**

**STORE AT -20°C**

100 Tests (Ready to use kit)

MYCOPLASMA MYCOIDES & AGALACTIAE  
- MULTIPLEX-

**-Only for in vitro use-**

**-Only for veterinary use-**

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

### **Principle and use:**

This amplification kit has been manufactured by *Genekam Biotechnology AG*, Germany to detect *Mycoplasma mycoides & agalactiae* cluster (in one step).

This kit needs DNA which can be isolated from blood, milk, tissue, respiratory swabs, nasal swabs, soil probes, air probes, cell cultures and any body fluid. Kindly use good methods to isolate the DNA.

***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:

- Tube A for *Mycoplasma agalactiae* (MG) (2 tubes)
- Tube C for *Mycoplasma mycoides* (MS) (2 tubes)
- Tube B (3 tubes)
- Positive (+Ve) control (tube D1) for *M. agalactiae* (1 tube)
- Positive (+Ve) control (tube D3) for *M. mycoides* (1 tube)
- Negative (-Ve) control (tube D2) (1 tube)
- Marker (tube E) (1 tube) (max 1000 bp ): 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)
- Pipettes 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

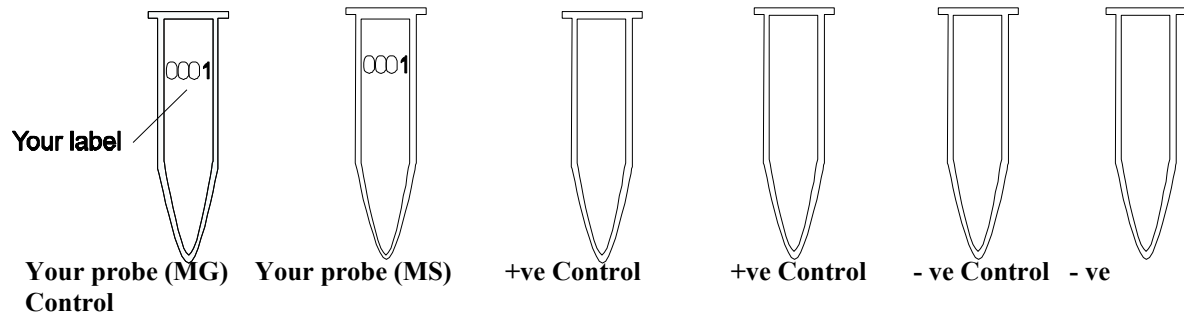
**Procedure:**

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

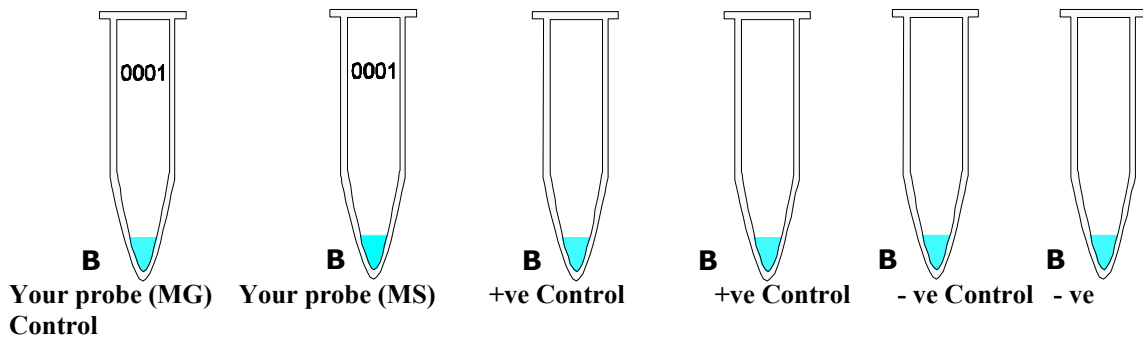
**STEP A**

1. Kindly thaw **one tube** each A, B, C, D3, D1, D2, E and F. After thawing, kindly put the tubes on 4°C (as it is better). If the kit is not in use, store them at -20°C.

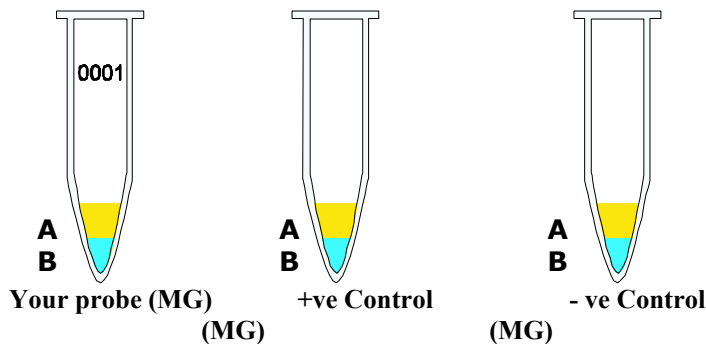
2. Mark your microtubes with a sample number and with +Ve Control and -Ve Control. You have to make two microtubes from each probe i.e. one is for MG (*Mycoplasma agalactiae*) and the other for MS (*Mycoplasma mycoides*).



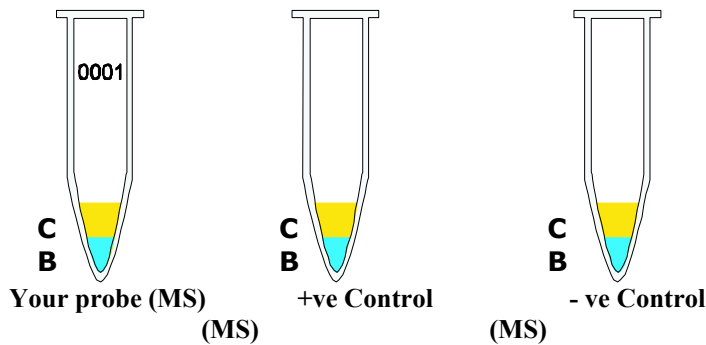
3. Add 10µl of B to each microtube. Avoid to touch the wall of the microtubes?



4. Add 8µl of tube A to each tube with MG, positive control and negative control. These tubes will identify only *Mycoplasma agalactiae*.



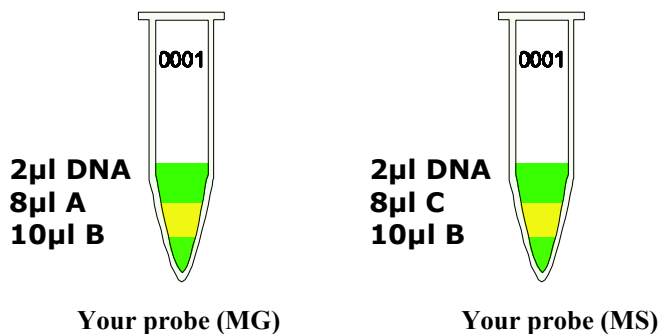
4a. Please add 8µl of tube C to each tube for probe MS. These tubes will only identify *Mycoplasma mycoides*.



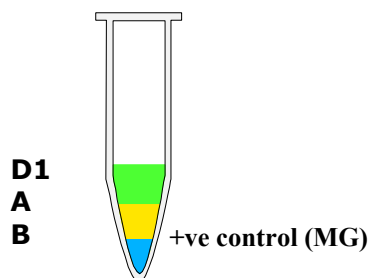
**TIP: you can calculate the total requirement of chemicals needed . You need 8µl A + 10µl B = 18µl per reaction. You want to run 10 reactions i.e. you need total 180µl, therefore you should mix 80µl of A + 100µl of B = 180µl from which you can take 18µl and add to each tube. This way you can save time and hardware. This is for probes with *MG* , positive control and negative control.**

**TIP: you can calculate the total requirement of chemicals needed . You need 8µl C + 10µl B = 18µl per reaction. You want to run 10 reactions i.e. you need total 180µl, therefore you should mix 80µl of C + 100µl of B = 180µl from which you can take 18µl and add to each tube. This way you can save time and hardware. This is for probes with *Mycoplasma mycoides* positive and negative control.**

5. 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 thoroughly. **You have to add isolated DNA to each tube with sign “your probe” for *MG* and *MS*.**



6a. 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. This is only for *Mycoplasma agalactiae*.

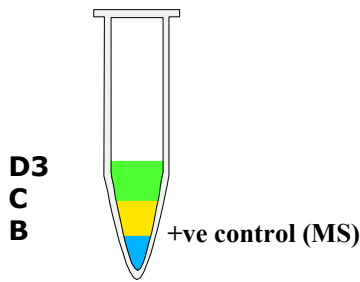


MYCOPLASMA MYCOIDES & AGALACTAE - MULTIPLEX

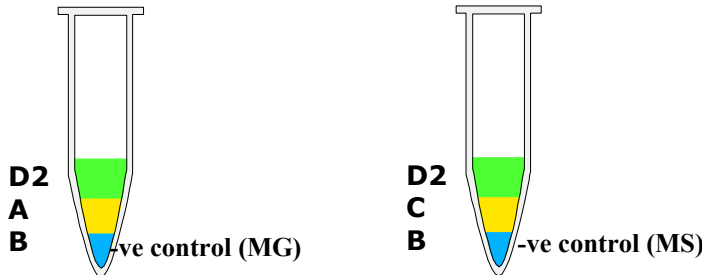
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6b. Use new pipette tip with filter. Add 2µl of +ve (tube D3) to +ve Control (MS) (avoid to touch the wall). Use a new pipette tip. Mix it. This is only for Mycoplasma mycoides.



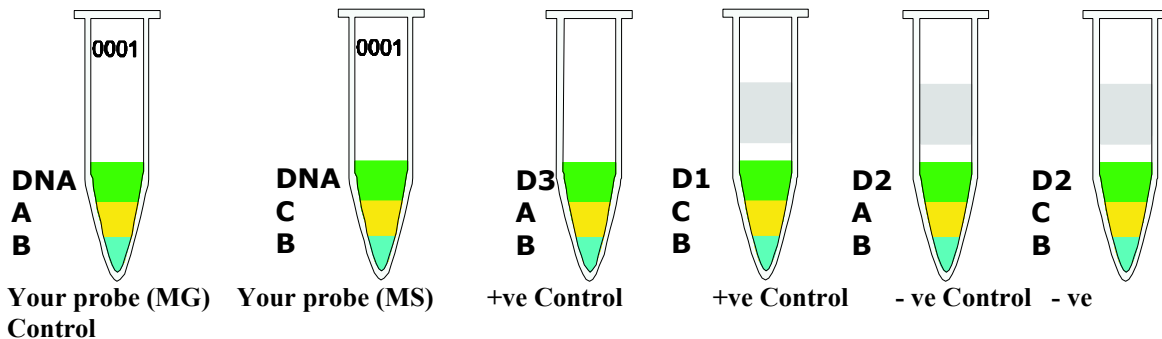
7. Use a new pipette tip. Add 2µl of -Ve (Tube D2) to -Ve Control (avoid the wall). Mix it. Add to tube with MG as well as MS.



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

9. 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 almost the same.



Now program your PCR machine as follows:

1. 300 seconds at 95°C
  2. 60 seconds at 94°C
  - 60 seconds at 46°C
  - 120 seconds at 72°C
  3. 600 seconds at 72°C
- } 30 cycles

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

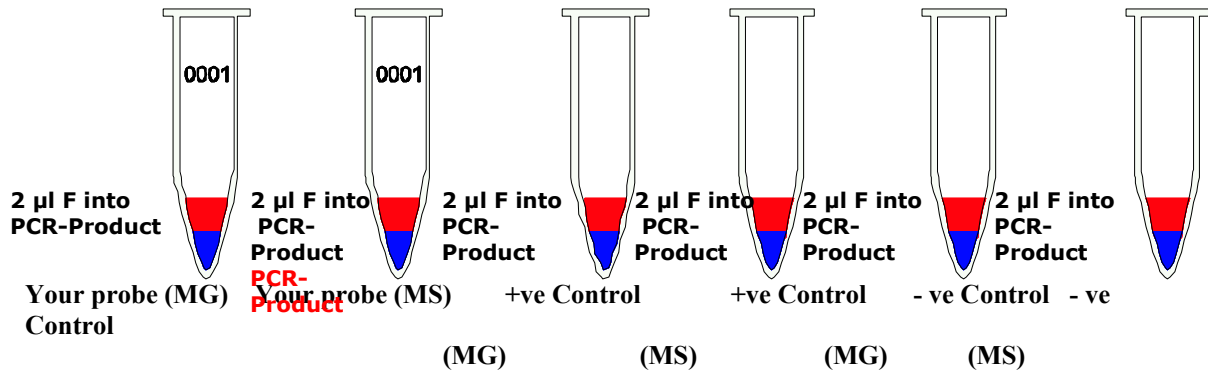
**Microtubes must be in contact with metal block** (very important!). There should be no air or lose contact with metal block of thermocycler. Run PCR now.

10. After step 9 is finished take out the microtubes.

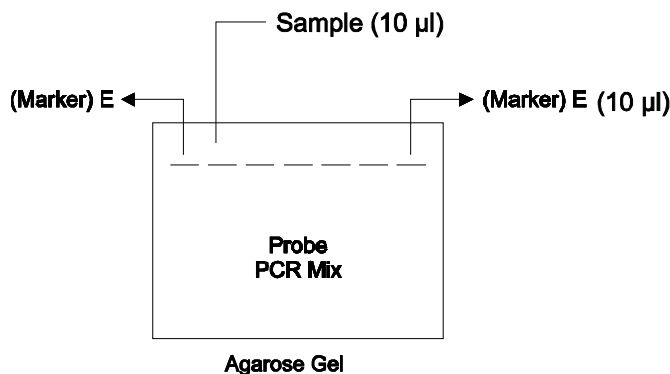
To see Mycoplasma agalactiae and Mycoplasma mycoides DNA, you can go directly to step gel electrophoresis (STEP B).

**STEP B**

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 of dye (tube F) and add to each microtube (with the same number as your PCR microtubes including +Ve & -Ve Controls) containing PCR product.



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 **60min.** at **100 Volt.**
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 +Ve Controls, positive samples and no bands in -Ve controls.  
**375 bp** for positive samples of Mycoplasma agalactiae and +ve control.  
**259 bp** (257 up to 260 bp) in positive samples for Mycolasma mycoides and +ve control.

**Recommendation: gene sequencing is highly recommended to reconfirm the positive results.**

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

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**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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