Lot-No.

Ref. K038

MANUAL

Expiry date: 1 year

STORE AT -20°C

100 Tests (Ready to use kit)

Mycobacterium complex

- 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 *Genekam Biotechnology AG*, Germany to detect Mycobacterium tuberculosis, Mycobacterium bovis (Tuberculosis, 178bp), non tuberculoses complex (257bp) and mycobacterium avium sp. paratuberculosis (MAP) (439bp).

This kit needs DNA which can be isolated from blood, sputum, respiratory swabs, tissue and any body fluid. Kindly use good methods to isolate the DNA. Please take precautions for Mycobacterium complex is infectious to human beings.

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 (2 tubes)
- Tube B (3 tubes)
- Tube C (2 tubes)
- Positive (+Ve) Control (tube D1) for Mycobacterium tuberculosis (1 tube)
- Negative (-Ve) Control (tube D2) (1 tube)
- Positive control +ve D3 for Mycobacterium paratuberculosis (1 tube)
- Marker (tube E) (1 tube) (max 1000 bp): 100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000 bp
- 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

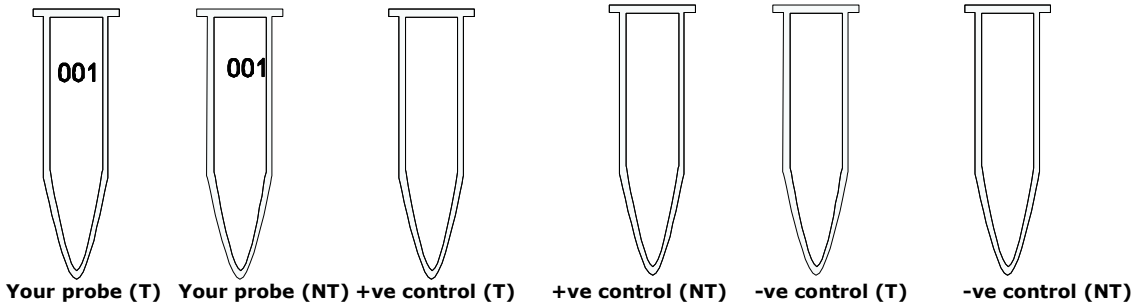
Procedure:

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

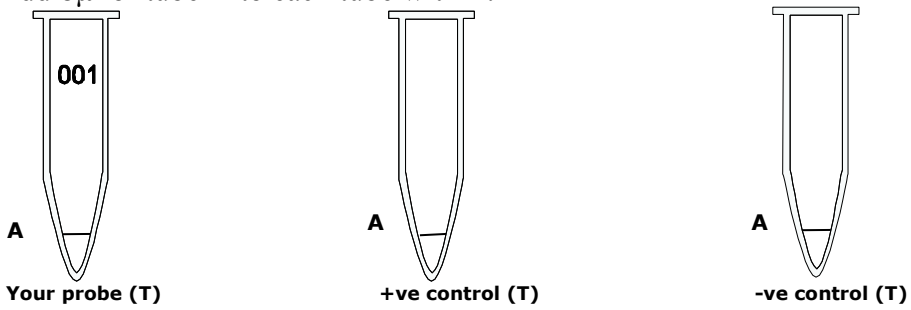
STEP A

1. Kindly thaw **one tube** each of A, B, C, D1, D2, D3, E and F. 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. You have to run probes two times. One for tuberculosis complex (T) and one for non tuberculosis complex (NT).

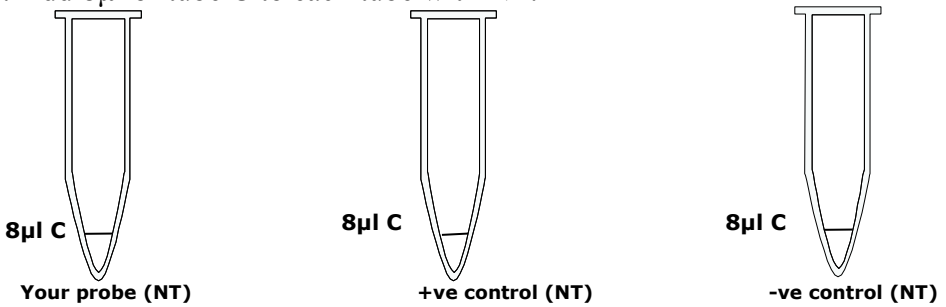


3. Add 8µl of tube A to each tube with T.



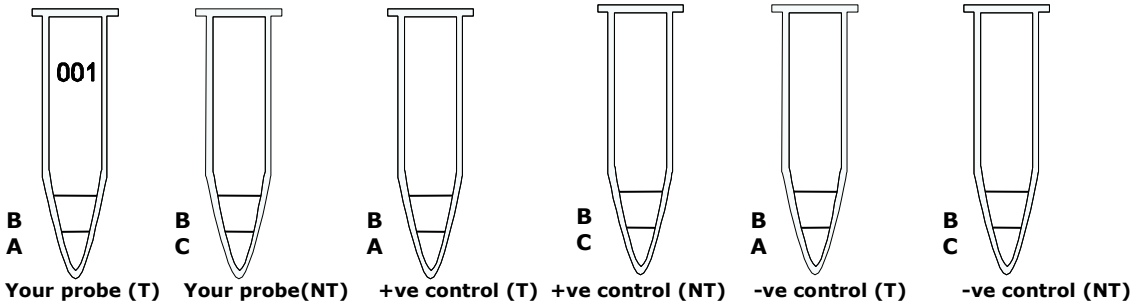
TIP: you can calculate the total requirement of chemicals needed, e.g. you need 8µl A+10µl B = 18µl per reaction. You want to run 10 reactions i.e. you need total 180µl, therefore you should mix 80µl of A + 100µl of B = 180µl from which you can take 18µl and add to each tube. This way you can save time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

4. Add 8µl of tube C to each tube with NT.

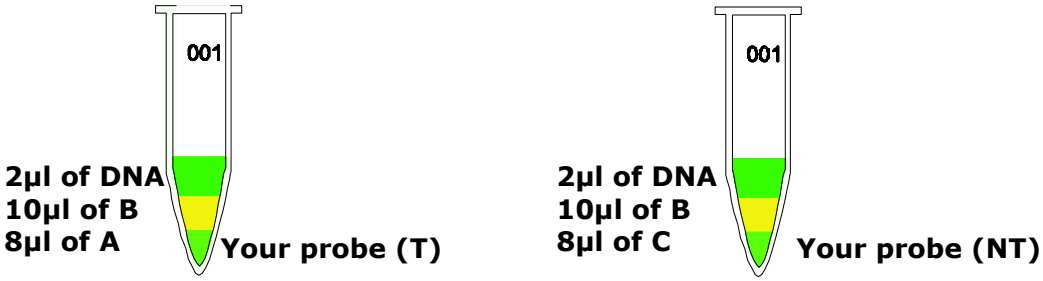


TIP: you can calculate the total requirement of chemicals needed . E.g. you need 8µl C+10µl B = 18µl per reaction. You want to run 10 reactions i.e. you need total 180µl, therefore you should mix 80µl of C + 100µl of B = 180µl from which you can take 18µl and add to each tube. This way you can save time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

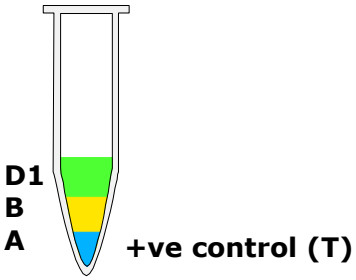
5. Add 10µl of B to each microtube. Avoid to touch the wall of the microtubes.



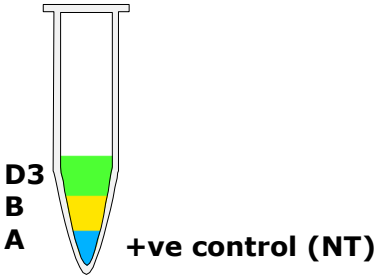
6. Add 2µl of your DNA template (DNA isolated from samples) with pipette tip with filter to each microtube according to your label except +Ve and –Ve (Avoid touching the wall). **Use everytime a new pipette tip** (for each sample)! Each probe must be added to 2 microtubes .e. you have one for tuberculosis and one for non tuberculosis. Mix it.



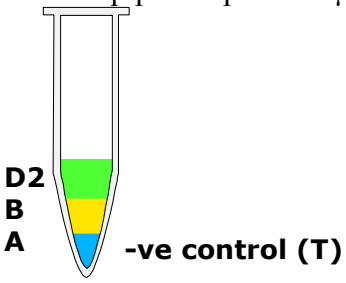
7. Use new pipette tip with filter. Add 2µl of +Ve (tube D1) to +Ve Control (avoid to touch the wall). Use a new pipette tip. Mix it. It is for tuberculosis complex.



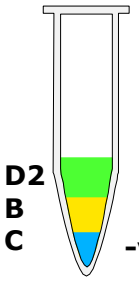
7a. Add 2µl of D3 to +ve control (NT). D3 is positive control for mycobacterium avium sp. Paratuberculosis.



8. Use a new pipette tip. Add 2µl of –Ve (Tube D2) to –Ve Control (avoid the wall). Mix it.



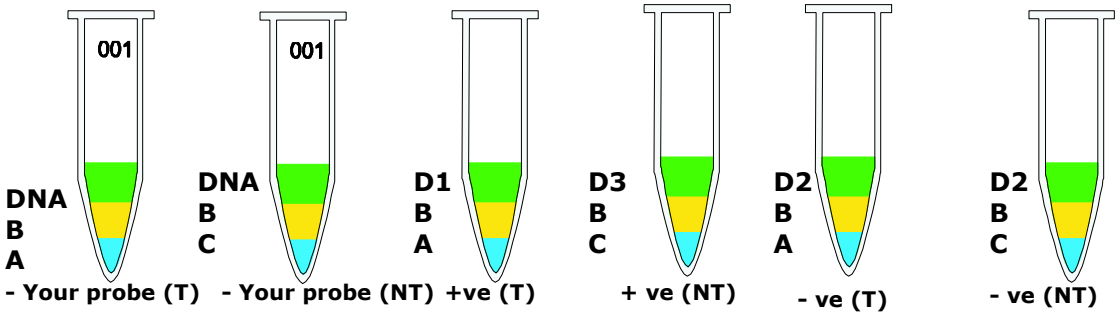
8a. Use a new pipette tip. Add 2µl of D2 to –ve control (NT). D2 is negative control control for non tuberculosis.



-ve control (NT)

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 microtube must be almost the same.



Now program your PCR machine as follows.

-> See temperature page at the end of this document !

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 the program now.

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

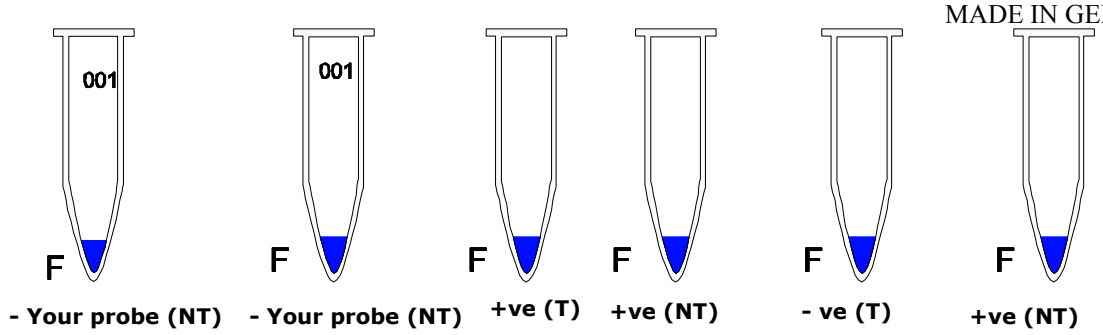
You go directly to step gel electrophoresis (STEP B).

STEP B:
1. Prepare the gel Agarose 2% in TAE (1x) buffer.

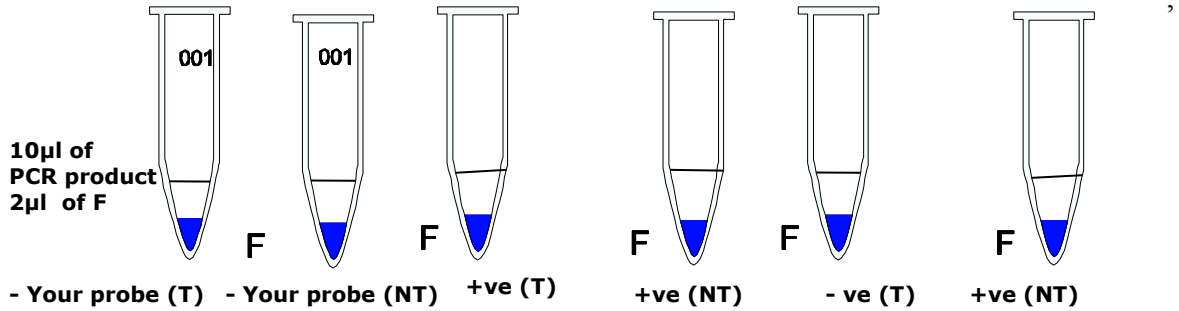
2. Let the gel dry and add this TAE (1x) buffer in gel chamber.

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

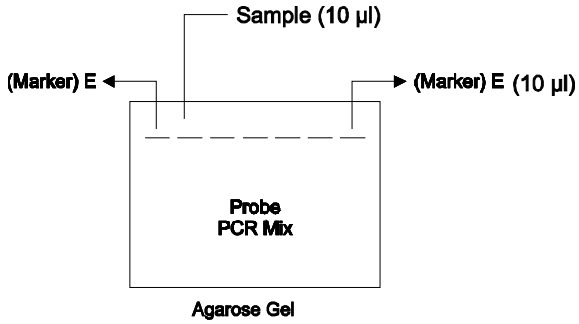
4. After the PCR step is finished, now you can prepare for gel Agarose electrophoresis. Take 2µl of dye (tube F) and add to new microtubes (with the same number as your PCR microtubes including +Ve & -Ve Controls and mark them).



5. Take 10µl of your PCR product and add to your tube containing 2µl of dye. Mix thoroughly. (Change the pipette tip every time. Check the mark of each microtube in order to avoid mistakes).



6. 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).



7. Add 10µl of mix of step 5 to each lane of gel Agarose (between first and last lane). Change the pipette tip for each lane.

8. Run the gel for **60 min**. at **100 Volt**.

9. Make staining solution ready.

10. Put the gel for 10-30 minutes staining solution (0.5µg/ml).

11. View the gel under UV light. UV light is dangerous for your eyes. Use UV goggles.

12. - **178 bp** tuberculosis positive in tubes with (T).
    - **257 bp** non tuberculosis positive in tubes with (NT).
    - **439 bp** Mycobacterium avium sp. Paratuberculosis (MAP) – it will be found only in tubes with (NT).

Hint: 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.** **Last update: 16-12-2011** **v1.4**	**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**

TEMPERATURE PAGE:

Program your PCR machine as follows. Increase the time one second in each cycle in Step A and C till you reach 59 seconds. That is 30 seconds.

1. 180 seconds at 96°C

2. A. **30** seconds at 96°C
   B. 45 seconds at 58°C
   C. **30** seconds at 72°C (1 cycle)

3. A. **31** seconds at 96°C
   B. 45 seconds at 58°C
   C. **31** seconds at 72°C (1 cycle)

4. A. **32** seconds at 96°C
   B. 45 seconds at 58°C
   C. **32** seconds at 72°C (1 cycle)

5. A. **33** seconds at 96°C
   B. 45 seconds at 58°C
   C. **33** seconds at 72°C (1 cycle)

   go on until you reach **59 seconds** for Step A and C.
   .
   .
   .

30. A. **59** seconds at 96°C
    B. 45 seconds at 58°C
    C. **59** seconds at 72°C (1 cycle)

31. 900 seconds at 72°C