**Lot.**

# MANUAL

## Ref. K302

**Expiry time: 1 year**

**STORE AT -20°C**

100 Tests (Ready to use kit)

HUMAN PAPILLOMA VIRUS GENOTYPING

**-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 different strains: HPV 16, 18, 31, 59, 45, 33, 6, 11, 58, 52, 56, 35, 42, 43, 44, 68, 39, 51, 66 Human Papilloma Virus in nested PCR. In the first round it is HPV-specific. In the second round it will be subtype-specific.

This kit needs DNA which can be isolated from blood, tissue, HPV lesion, reproductive tract swabs, genital lesion swab, cervical tumour and any body fluid. Kindly use good methods to isolate the DNA. Kindly take common safety laboratory precautions during working.

**PRECAUTION: work very cleanly otherwise you may face the problem of cross-contamination, as it is nested PCR.**

***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) - for common
- Tube B (2 tubes)
- Tube C (1 tubes): **for 16, 18, 31, 59, 45**
- Tube H (1 tubes): **for 33, 6, 11, 58, 52, 56**
- Tube I (1 tubes): **for 35, 42, 43, 44**
- Tube J (1 tubes): **for 68, 39, 51, 66**
- Positive (+Ve) Control (tube D1) (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)
- Tube G (2 tubes)

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 and pen

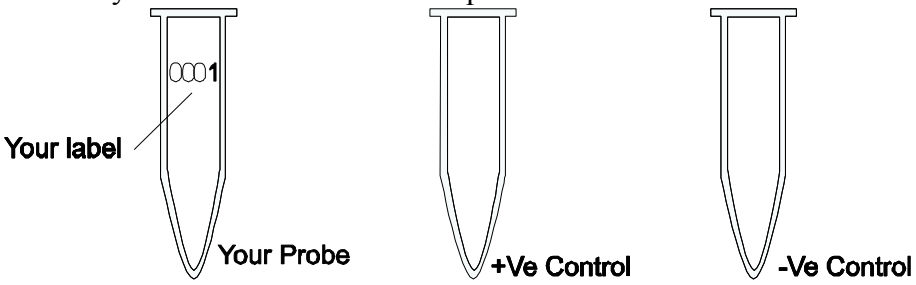
- Agarose (good quality)
- Staining (Ethium Bromide)
- TAE buffer 1x
- Ice
- Vortexer

**Procedure:** After your DNA isolation is completed. (Kindly use quality isolation method).

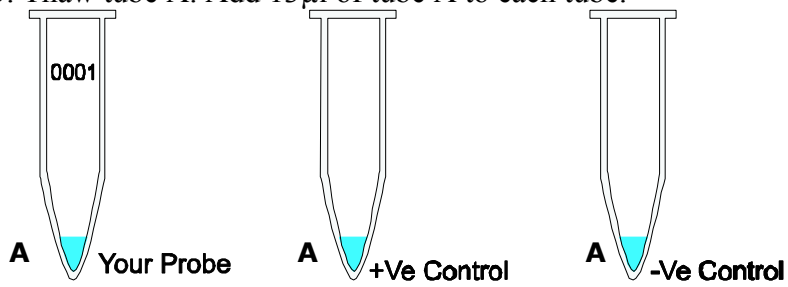
**STEP A**

1. Kindly thaw **one tube** each: A, B, C, H, I, J, D1, D2, E, F and G. 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.

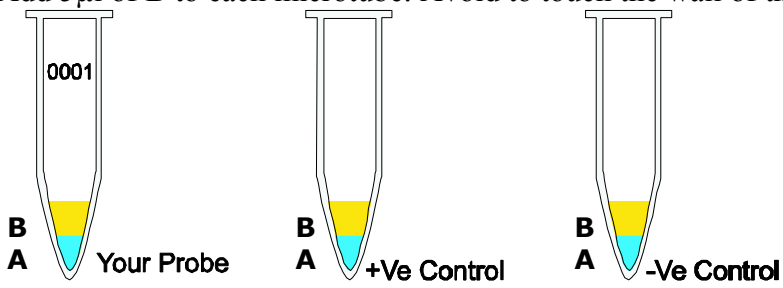
2. Mark your microtubes with a sample number and with +Ve Control and –Ve Control.




3. Thaw tube A. Add 13µl of tube A to each tube.

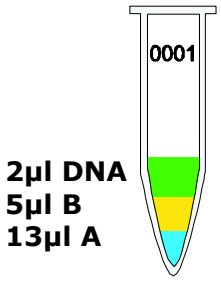



4. Add 5µl of B to each microtube. Avoid to touch the wall of the microtubes.

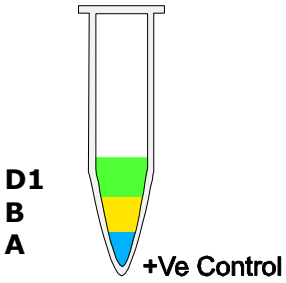



5. **TIP: you can calculate the total requirement of chemicals needed . You need 13µl A + 5µl B = 18µl per reaction. You want to run 10 reactions i.e. you need total 180µl, therefore you should mix 130µl of A + 50µl of B = 180µl from which you can take 18µl and add to each tube. This way you can save time and hardware.**

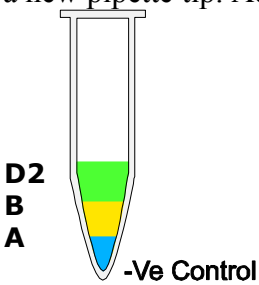
6. Add 2µl of your DNA template (DNA isolated from samples) with pipette tip with filter to each microtube according to your label except +Ve and –Ve (Avoid touching the wall). **Use every time a new pipette tip** (for each sample)! Mix it.




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.

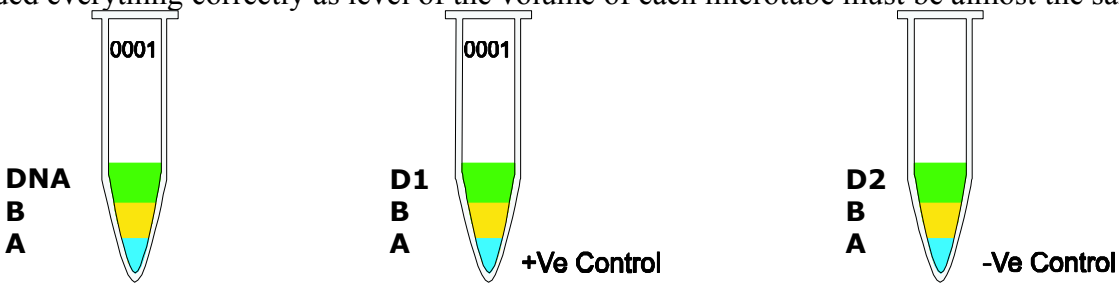



8. Use a new pipette tip. Add 2µl of –Ve (tube D2) to –Ve Control (avoid the wall). Mix it.




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 level of the volume of each microtube must be almost the same.




Now program your PCR machine as follows.

| | | | |
|---|---|---|---|
| 1. | 240 | seconds at 94°C | |
| 2. | 60 | seconds at 94°C | 40 cycles |
| | 60 | seconds at 40°C | |
| | 120 | seconds at 72°C | |
| 3. | 600 | seconds at 72°C | |

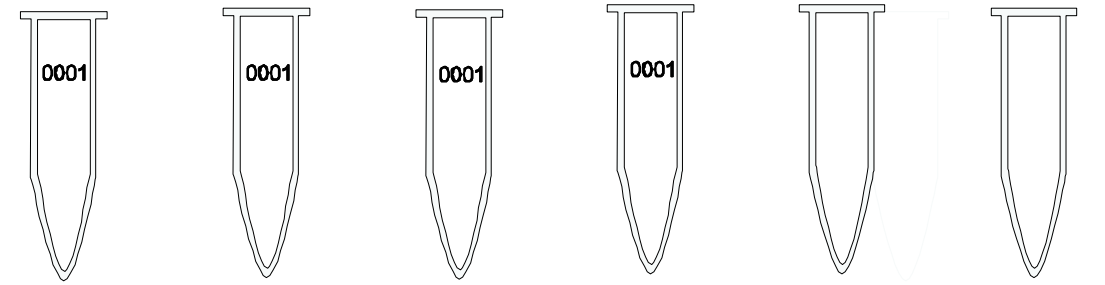
Before you start the PCR program, kindly check whether tubes are closed properly. **Microtubes must be in contact with metal block** (very important!). There should be no air or lose contact with metal block of thermocycler.

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

To see HPV-16/18, you can go directly to step gel electrophoreses (STEP C). Meanwhile you can go to step B for detecting genotype.

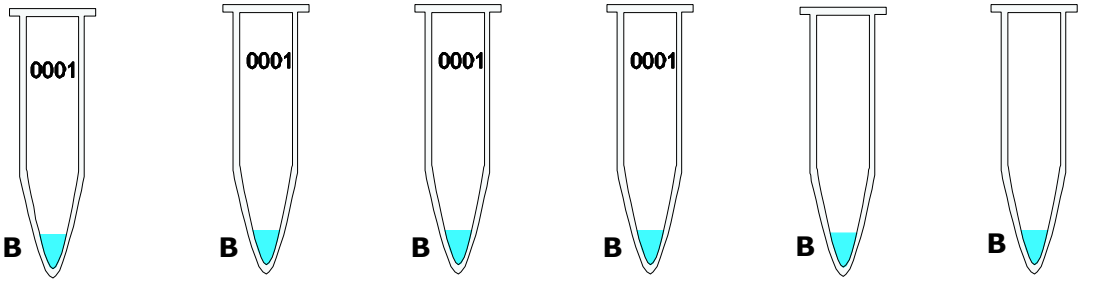
**STEP B (to avoid cross contamination, please work very, very cleanly !):**

1. Mark your microtubes with a sample number and with +Ve Control and –Ve Control. **You have to make 4 sample tubes for each probe. Each tube should be marked C, H, I and J.**



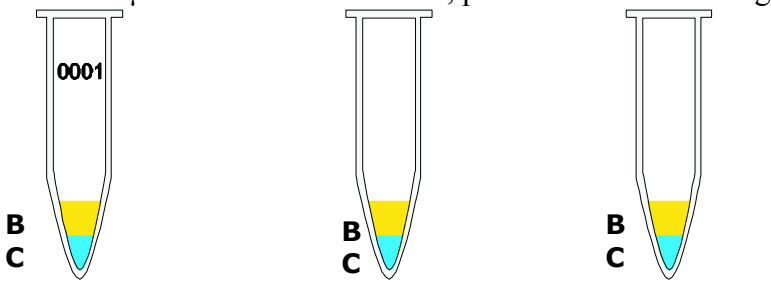

**your probe (C)** | **your probe (H)** | **your probe (I)** | **your probe (J)** | **++ve control (C)** | **--ve control (C)**

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



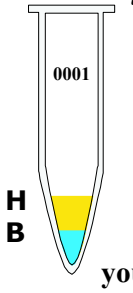

**your probe (C)** | **your probe (H)** | **your probe (I)** | **your probe (J)** | **++ve control (C)** | **--ve control (C)**

3.1 Add 13µl of tube C to each tube, positive control and negative control labelled as C.



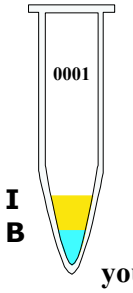

**your probe (C)** **++ve control (C)** **--ve control (C)**

3.2 Add 13µl of tube H to each tube marked with H.



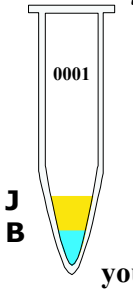

**your probe (H)**

3.3 Add 13µl of tube I to each tube marked with I.



**your probe (I)**

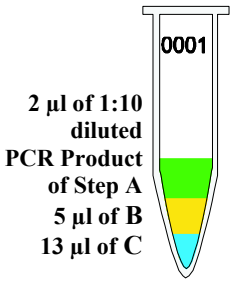
3.2 Add 13µl of tube J to each tube marked with J.



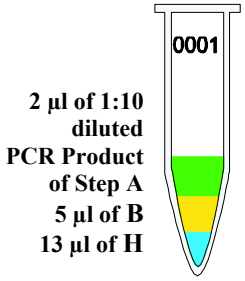
**your probe (J)**

4. **TIP: you can calculate the total requirement of chemicals needed . You need 13µl C + 5µl B = 18µl per reaction. You want to run 10 reactions i.e. you need total 180µl, therefore you should mix 130µl of C + 50µl of B = 180µl from which you can take 18µl and add to each tube. This way you can save time and hardware. Similarly it can be done with other tubes.**

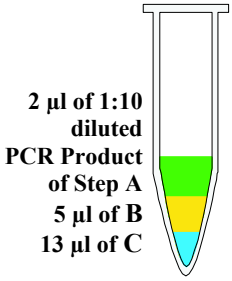
5. **Kindly dilute your PCR product of Step A** 1:10 (1µl of PCR product with 9µl of G). Now take 2 µl of this diluted PCR product and add each new microtube according to labelling plan (avoid touching the wall). (Same number or label name as your microtubes of step A.) **Use every time a new pipette tip with filter** (For each sample)! Mix it.
**You have to add your sample to all tubes marked C, H, I, J, positive control and negative control.**



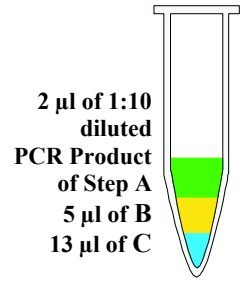
**your probe (C)**



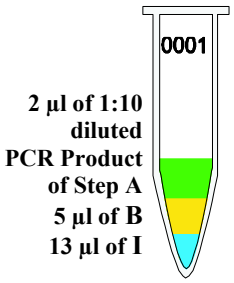
**your probe (H)**



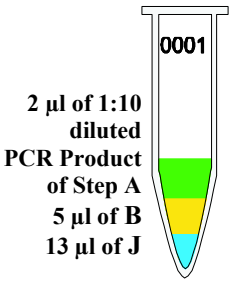
**++ve control (C)**



**--ve control (C)**



**your probe (I)**



**your probe (J)**

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

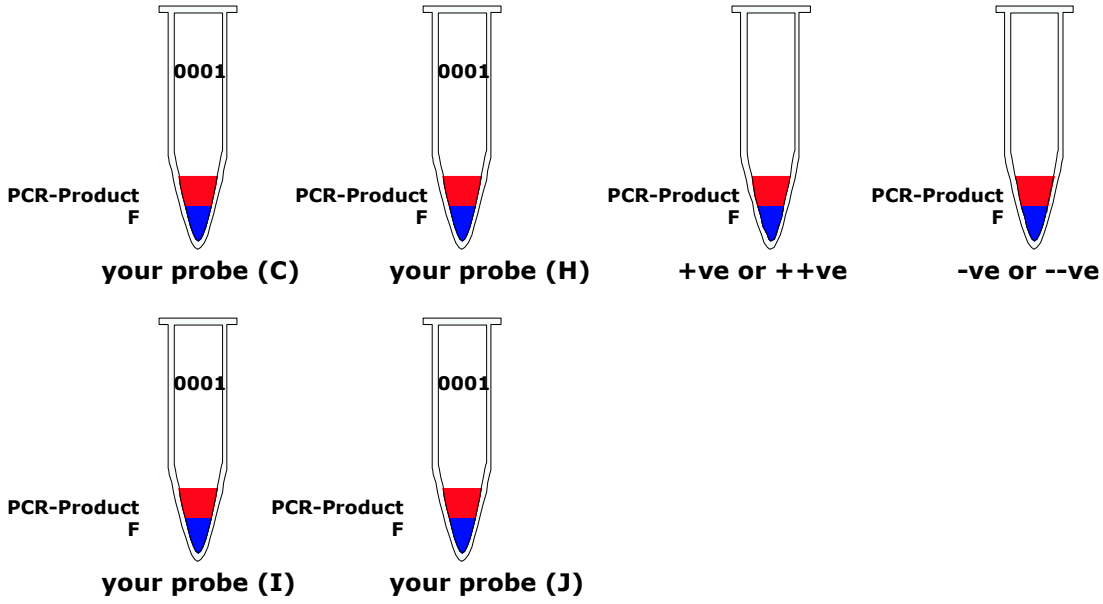
7. Kindly check whether you have added everything correctly. The level of volume should be the same in each tube. Program the thermocycler

| | | | |
|---|---|---|---|
| 1. | 240 | seconds at 94°C | |
| 2. | 30 | seconds at 94°C | 35 cycles |
| | 30 | seconds at 56°C | |
| | 45 | seconds at 72°C | |
| 3. | 240 | seconds at 72°C | |

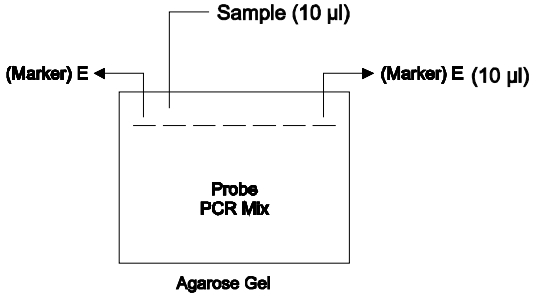
After the program is over you can go to **STEP C**.

**STEP C:**

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 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 **50 min**. at **120 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 positive control and no band in negative control.

    1st round: **630 bp** in +ve control as well as in positive samples **(HPV-specific).as it can also vary in size.**
    2nd round: **See results on extra page.**

Sometimes you get only band in 2nd round, it is positive.

**Recommendation: genesequencing is highly recommended to reconfirm the positive results.** The purpose of this kit is to screen the samples quickly and economically. Kindly note that in multiplex assay, one band or target per reaction can be skipped, one in case of positive samples, but the results are accurate in case of negative samples. Therefore it is highly recommended that positive samples should be checked indivually for each target for positive samples in case one needs very accurate results. To do indivual testing contact Genekam Biotechnology AG. Biggest advantage of this multiplex assay is too detect the samples, where more than one kind of HPV strains occurs.

**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: 10-01-2012**
**v1.0**

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## FAQ:

Q: I do not have any band in the first round (StepA). Should I go for the second round (step B) ?
A: Yes, you should go, as there may be a band in the second round in some samples.

Q: How can I avoid cross-contamination ?
A 1) work very cleanly.
2) Clean your PCR place daily.
3) Do different steps at different places.

**RESULTS of the second round:**

1. Group C

| | |
|---|---|
| HPV 16 | 457bp |
| HPH 18 | 322bp |
| HPV 31 | 263bp |
| HPV 59 | 215bp |
| HPV45 | 151bp |

2. Group H

| | |
|---|---|
| HPV 33 | 398bp |
| HPV 6/11 | 334bp |
| HPV 58 | 274bp |
| HPV 52 | 229bp |
| HPV 56 | 181bp |

3. Group I

| | |
|---|---|
| HPV 35 | 358bp |
| HPV 42 | 277bp |
| HPV 43 | 219bp |
| HPV 44 | 163bp |

4. Group

| | |
|---|---|
| HPV 68 | 333bp |
| HPV 39 | 280bp |
| HPV 51 | |
| HPV 66 | 172bp |