MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS
STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)
COMMONWEALTH OF AUSTRALIA
Patents Act 1952-1969

CONVENTION APPLICATION FOR A PATENT

BEHRINGWERKE AKTIENGESELLSCHAFT

(1) Here insert the full name of Applicant(s) or Company and number(s) of application(s)

D-3550 Marburg, Federal Republic of Germany

(2) Here insert Title of Invention.

DETECTION OF HUMAN PAPILLOMAVIRUS DNA AND ITS EXPRESSION IN CERVICAL SMEARS

which is described in the accompanying complete specification. This application is a Convention application and is based on the application numbered (3) P38 38 269.5 for a patent or similar protection made in (4) Federal Republic of Germany on 11th November 1988

DATED this 9th day of November 1989

BEHRINGWERKE AKTIENGESELLSCHAFT

(5) Signature(s) of Applicant(s) or Company and number(s) of application(s)

D. B. Mischlewski
Registered Patent Attorney

To:

THE COMMISSIONER OF PATENTS.
In support of the Convention application made under Part XVI. of the Patents Act 1952 by BEHRINGWERKE AKTIENGESELLSCHAFT of D-3550 Marburg, Federal Republic of Germany for a patent for an invention entitled:

Detection of human papillomavirus DNA and its expression in cervical smears

We, Philipp Stein, Höhenweg 28, D-3550 Marburg
Heribert Bug, Amseleweg 7, D-3551 Niedersachsen
Federal Republic of Germany

...do solemnly and sincerely declare as follows:

1. We are authorized by BEHRINGWERKE AKTIENGESELLSCHAFT the applicant for the patent to make this declaration on its behalf.

2. The basic application(s) as defined by Section 141 of the Act was (were) made at München in the Federal Republic of Germany under No. 1-38 292 264.6

on November 11, 1988

by BEHRINGWERKE AKTIENGESELLSCHAFT

3. a) Peter Cerutti, Ch. Graminée 9, CH-1014 Lully
   b) Jeannette Whitecomb, Ch. des Tourelles 9, CH-1066 Epalinges
   c) Jacob Zijlstra, Ch. de Votres, CH-1011 Coppet
   d) Ethel-Michelle de Villiers, Fauver 14, CH-1002 Hirschberg
   a) b) c) Switzerland
d) Federal Republic of Germany

...is/are the actual inventor(s) of the invention and the facts upon which BEHRINGWERKE AKTIENGESELLSCHAFT is entitled to make the application are as follows:

The said BEHRINGWERKE AKTIENGESELLSCHAFT is the assignee of the said

Peter Cerutti, Jeannette Whitecomb, Jacob Zijlstra, Ethel-Michelle de Villiers

4. The basic application referred to in paragraph 2 of this Declaration was (were) the first application(s) made in a Convention country in respect of the invention the subject of the application.

DECLARED at Marburg, Federal Republic of Germany
this 3rd day of October, 1989

To the Commissioner of Patents

BEHRINGWERKE AKTIENGESELLSCHAFT

Prokurist
ppa. Stein

Prokurist
ppa. Bug
The invention relates to the direct detection of human papillomavirus DNA. This entailed the recently developed polymerase chain reaction (PCR; Saiki et al. (1988) Science 239, 487-491) being modified in order to improve the sensitivity and specificity. By choosing appropriate oligonucleotide primers (amplimers) and reaction temperatures, individual HPV genes from among the total cell DNA can be identified and amplified to an extent such that non-isotopic detection is possible. In addition, use of reverse transcription permits selective amplification of spliced mRNA and thus permits indication of pre-malignant or malignant conditions and/or lesions.

We have found that the polymerase chain reaction method can be simplified for the detection of HPV and that HPV can be detected in cervical smears after about 3 hours when suitable amplimers and concentrations of dimethyl sulfoxide appropriate thereto are used. The polymerase used was Taq polymerase, and temperatures of 89°C and 63°C at cycle times of 1 minute were chosen. If a cDNA synthesis step by means of reverse transcriptase is
carried out beforehand the presence of spliced mRNA to E6* can be established from the occurring DNA bands (Figure 4 and Figure 5) and the presence of pre-malignant or malignant lesions can be shown to be probable.

1. A method for the direct detection of the presence and expression of HPV by means of polymerase chain reaction (PCR),
   a) using deoxynucleotide triphosphate concentrations above 0.2 mM and amplimer concentrations above 0.5 mM
   b) with cycle times of 1 to 2 minutes
   c) at 2 temperatures of 70°C-95°C and 50°C-70°C
   d) start of the PCR above the melting temperature
   e) with direct detection of specific DNA fragments by staining after agarose gel electrophoresis.

8. A method for the detection of spliced mRNA, which comprises the PCR being preceded by a cDNA synthesis step with reverse transcriptase, and amplimers which cover a splice sequence being used.

9. A combination of reagents containing amplimers as claimed in claim 8.
BEHRINGWERKE AKTIENGESELLSCHAFT

D-3550 Marburg, Federal Republic of Germany

PETER GERUTTI, JEANNETTE WHITCOMB, JACOB ZIJLSTRA and ETHEL-MICHELLE DE VILLIERS

50 QUEEN STREET, MELBOURNE, AUSTRALIA, 3000.

DETECTION OF HUMAN PAPILLOMAVIRUS DNA AND ITS EXPRESSION IN CERVICAL SMEARS

The following statement is a full description of this invention, including the best method of performing it known to us.

1.
Detection of human papillomavirus DNA and its expression in cervical smears

The invention relates to the direct detection of human papillomavirus DNA. This entailed the recently developed polymerase chain reaction (PCR; Saiki et al. (1988) Science 239, 487-491) being modified in order to improve the sensitivity and specificity. By choosing appropriate oligonucleotide primers (amplimers) and reaction temperatures, individual HPV genes from among the total cell DNA can be identified and amplified to an extent such that non-isotopic detection is possible. In addition, use of reverse transcription permits selective amplification of spliced mRNA and thus permits indication of pre-malignant or malignant conditions and/or lesions.

There is a strong correlation between the occurrence of cervical carcinomas and the detection of HPV serotypes 16, 18, 31 and 33 in cervical tissue. Admittedly, the related HPV serotypes 6 and 11 are also frequently present in the genital tract, but these are associated with benign lesions, genital warts and condylomata. The genome of HPV 16 and 18 possesses 8 open reading frames (ORF; Figure 1). L-1 and L-2 code for structural protein whereas the significance of the other ORFs is not quite as well understood. There is a strong connection between E6/E7 regions integrated into the host genome and the transformation to malignancy. It was shown for the E7 ORF that it codes for a cytoplasmic phosphoprotein present in large quantities in HPV-infected cells. The role played by this protein in transformation and maintenance of malignancy is not known. The E6/E7 regions are strongly transcribed in transformed cells. Both a transcript of the entire E6/E7 region and spliced RNAs exist. The splicing pattern which is similar in HPV 16 and 18 leads to a translation product called E6*. (Schneider-Gädicke et al. (1988) Cancer Res. 48, 2969-2974). This splicing
pattern probably correlates with the malignant potential of the HPV viruses which is displayed by HPV 16, 18, 31 and 33 but which HPV 6 and 11 do not have. The spliced mRNA transcript, which is shifted in the reading frame, for HPV 16 and HPV 18 E6* is diagrammatically shown in Figure 2 and Figure 3. An even smaller spliced mRNA of approximately 1.5 kb found for HPV 16 is shown in addition. The methods hitherto available for detection of the virus, for example in situ DNA hybridization with radiolabelled DNA probes, are difficult to carry out, time-consuming and frequently insufficiently sensitive. The polymerase chain reaction method (PCR; Saiki et al, loc cit.) used for amplification of HPV DNA was also unsuitable as a routine method.

We have found that the polymerase chain reaction method can be simplified for the detection of HPV and that HPV can be detected in cervical smears after about 3 hours when suitable amplimers and concentrations of dimethyl sulfoxide appropriate thereto are used. The polymerase used was Taq polymerase, and temperatures of 89°C and 63°C at cycle times of 1 minute were chosen. If a cDNA synthesis step by means of reverse transcriptase is carried out beforehand the presence of spliced mRNA to E6* can be established from the occurring DNA bands (Figure 4 and Figure 5) and the presence of pre-malignant or malignant lesions can be shown to be probable.

The invention thus relates to:

a) a simplified PCR method for the detection of HPV by choosing suitable conditions such as amplimer sequences, reaction temperatures (preferably 89°C and 63°C), increased concentrations of amplimers and deoxynucleotide triphosphates, short cycle times of 1 to 2 minutes, and starting the PCR at a temperature at which double-stranded DNA is still "molten" so that the specificity is considerably improved,
b) where a suitable choice of amplimers permits the simultaneous detection of several types of viruses (e.g. HPV 16, 18, 31 and 33), and DNA fragments of characteristic length are obtained for each individual virus and are quantified by densitometry after separation by agarose gel electrophoresis and staining with ethidium bromide,

c) a step for reverse transcription of mRNA preceding the amplification reactions, in order to establish the presence of E6* by way of detection of amplified spliced mRNA, the amplimers chosen being preferably those which span the splice sites when the presence of E6* is to be tested for,

d) coamplification of a single-copy gene (such as the human IL-2 receptor, β-globin or c-H-ras gene), for internal standardization and for checking that the amplification reactions have functioned satisfactorily.

In case (c) amplimers of 14 - 20 nucleotides can be chosen which span the splice site approximately symmetrically, if only amplification products of the reverse transcriptase reaction are to be obtained. The amplimer ACAGAGGTGC, the arrow marking the splice site, is an example. However, the E6* RNA is also detected by its different fragment length, as described in the examples with the amplimer pairs 24/26 and 21/22 for HPV 18 and HPV 16, respectively. The method described above using the example of E6* of HPV 18 and HPV 16 is generally applicable for the detection of spliced mRNA by choice of suitable amplimers.

The invention is further described in the following examples and patent claims.
Example 1: Removal of clinical specimens for the detection of HPV DNA and/or for the detection of E6* RNA

After application of the cervical smear for the histological examination ("pap smear") the usual wooden spatula containing the residual material was immersed in 20 ml of ice-cold Earle’s BSS glucose (0.2 g/l CaCl₂, 0.4 g/l KCl, 0.2 g/l MgSO₄, 7H₂O, 6.8 g/l NaCl, 2.2 g/l NaHCO₃, 0.14 g/l NaH₂PO₄, H₂O, 1 g/l glucose). After a maximum of 5 hours on ice the sample was shaken manually and the spatula removed. The sample was centrifuged at 4°C/100 x g, during which the mucus floats to the top while the cells are deposited at the bottom of the tube. The supernatant was decanted and the cell residue washed once with phosphate-buffered saline. The cell residue was frozen at -70°C and kept in the frozen state until processed further.

Example 2: Reverse transcription

A reaction mixture having a total volume of 5 µl has the following composition:

- Buffer A (10 x) 0.5 µl
- 10 x BSA 0.5 µl
- Nucleoside triphosphates (NTPs, 5 mM) 1.0 µl
- RNasin 0.25 µl
- Dithiothreitol 0.25 µl
- Amplimer (primer) 0.5 µl
- Reverse transcriptase 0.5 µl
- H₂O 0.5 µl
- Cell sample 0.1 µl

Buffer A (10 x) has the following composition:

- 500 mM Tris HCl pH 8.3
- 70 mM MgCl₂
500 mM KCl
100 mM β-mercaptoethanol

10 x BSA contains 1.70 mg/ml BSA in H₂O
RNasin (promega) contains 40 U/µl
Dithiothreitol is 20 mM
Amplimers are contained in H₂O in a concentration of 400 µg/ml. Reverse transcriptase (Boehringer Mannheim) contains 20 - 25 U/µl.

The reaction mixture, without addition of the cell sample, was mixed and 4 µl portions were pipetted into Eppendorf tubes; 1 µl portions of the cell sample to be tested were then added. A 200 µl layer of liquid paraffin was then placed on top, the mixture was sonicated at setting 2 with a B15 Bronson sonicator for 15 seconds, and the emulsion was separated in an Eppendorf bench centrifuge at room temperature. Finally this was followed by incubation at 42°C for 10 minutes with the reaction subsequently stopped by heating to 89°C.

Example 3: Amplification

A reaction mixture is formed of 20 µl of amplification mixture and 5 µl of reaction mixture from the reverse transcription reaction or appropriately buffered cell sample, the amplification mixture having the following composition:

- Buffer B (10 x) 2 µl
- 10 x BSA 2 µl
- Deoxyribonucleotide triphosphate (dNTPs, 25 mM) 1 µl
- Amplimer 0.5 µl
- Taq polymerase 0.4 µl
- H₂O 14.3 µl
Buffer B (10 x) has the following composition

70 mM MgCl₂
500 mM KCl
100 mM β-mercaptoethanol

The Taq polymerase (Biore) has 5 U/μl (for other constituents see Example 2).

20 μl of amplification mixture equilibrated at 89°C was then added to 5 μl of sample which was also incubated at 89°C. The DNA amplification was then carried out in 1-minute cycle steps at 89°C/63°C, 25 to 40 cycles as a rule being sufficient for detection of HPV DNA or mRNA in up to at least 20 cells.

**Example 4a:** Selection of amplimers for HPV 18

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<th>ATTAAATCTT TAACAAATT TAGTATATAA AAAAAACGAGT AAGCAGAAAAC</th>
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</tr>
<tr>
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<tr>
<td>151</td>
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<td>ATAAAATGTTAT CAAATTTTAT TCAGAATAA GAAATTTAACGATTTTA</td>
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<td>GTGTATACGG CTGTTTATG GCAAGACCTA TGTAGACCA AAAAAAGAGGA</td>
</tr>
</tbody>
</table>

Amplimer positions are underlined.

‡6* splice sequences are dotted and underlined.
Amplimer sequences:

No. 24 (position 167 - 186)
AGT GAA TTC TTC GAA CAC TTC ACT GCA AGA CA

No. 26 (position 667 - 686)
AGT GAA TTC GCG CGC TTA ATT GCT CGT GAC AT

No. 25 (position 647 - 666)
AGT GAA TTC TCT AGA AGG TCA ACC GGA ATT TC

The 4 "leader" triplets are EcoRI-cleavable oligonucleotide linkers.

An intron is present between position 236 and 417 (182 bp), so that a band of 544 bp is detectable on amplification of DNA with the No. 24/No. 26 amplimer pair.

On amplification of RNA, that is after a preceding step with reverse transcription of the spliced mRNA, an additional band of 362 bp is detected.
Example 4b: Selection of amplimers for HPV 16

Amplimer positions are underlined.
E6* splice sequences are dotted and underlined.

Amplimer sequencos:

No. 21 (position 198 - 207)
AGT GAA TTC AGT ACT GCA AGG AAC AGT TAC TG

No. 22 (position 601 - 620)
AGT GAA TTC AAC GTC TGT GTC TGC AAA TC

No. 23 (position 658 - 677)
AGT GAA TTC AGA TCT ATT TGA TCC TCC TCC TCC TC

The 4 "leader" triplets are each EcoRI-cleavable oligonucleotide linkers.
The introns are present at about position 225 to 410 (186 bp) and 254 to 524 (270 bp). A band of 447 bp is detectable on amplification of DNA with the No. 21/No. 22 amplimer pair, and additional bands of about 261 and 177 bp are detected on amplification of RNA, that is after a preceding step with reverse transcription of spliced mRNA.
Legend to Figure 1: Open reading frames of HPV 18 (HPV 16 has similar structure).

Legend to Figure 2: Graphic representation of the E6/E7 region of HPV 18
a) DNA organization
b) E6/E7 mRNA of 3.4 kb
c) spliced mRNA for E6* of 1.6 kb.

Legend to Figure 3: Graphic representation of the E6/E7 region of HPV 16
a) DNA organization
b) E6/E7 mRNA of 4.5 kb
c) spliced mRNA for E6* of 2.3 kb
d) smaller spliced mRNA of about 1.5 kb.

Legend to Figure 4: Amplimer positions and splice sites in amplified HPV 18 DNA segments
a) E6/E7 portion of the HPV 18 genome
b) position of the amplimers 24, and 26
c) size of the amplified DNA fragment or o. unprocessed mRNA
d) Size of the amplified cDNA of the spliced E6* RNA.

Legend to Figure 5: Amplimer positions and splice sites in amplified segments of HPV 16 DNA
a) E6/E7 portion of HPV 16 genome
b) position of amplimers 21, 2 and 23
c) size of the amplified DNA fragment or of the unprocessed mRNA
d) size of the amplified cDNA of the spliced E6* RNA
THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for the direct detection of the presence and expression of HPV by means of polymerase chain reaction (PCR),
   a) using deoxynucleotide triphosphate concentrations above 0.2 mM and amplimer concentrations above 0.5 mM
   b) with cycle times of 1 to 2 minutes
   c) at 2 temperatures of 70°C-95°C and 50°C-70°C
   d) start of the PCR above the melting temperature
   e) with direct detection of specific DNA fragments by staining after agarose gel electrophoresis.

2. The method as claimed in claim 1, wherein temperatures of 89°C and 63°C are chosen.

3. The method as claimed in claim 1 or 2, wherein nucleic acid liberation is brought about by sonication of the cell sample.

4. The method as claimed in claim 1 or 2, wherein the PCR is carried out with addition of up to 20% v/v dimethyl sulfoxide.

5. The method as claimed in claim 1, 2 or 3, wherein the PCR is preceded by a reaction with reverse transcriptase.

6. The method as claimed in claim 5, wherein amplimers which cover a splice sequence are used.

7. A combination of reagents containing amplimers as claimed in claim 6.

8. A method for the detection of spliced mRNA, which comprises the PCR being preceded by a cDNA synthesis step with reverse transcriptase, and amplimers which cover a splice sequence being used.

9. A combination of reagents containing amplimers as claimed in claim 8.

DATED this 9th day of November 1989.

BEHRINGWERKE AKTIENGESELLSCHAFT

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FIG. 4

a) E6  

b) 24  

c)  

d) E6* 

FIG. 5

a) E6  

b) 21  

c)  

d) E6* 

e)  

*
END