(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 September 2013 (12.09.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/132504 Al

(51) International Patent Classification:
C12Q 1/70 (2006.01)

(21) International Application Number:
PCT/IN20 12/000 160

(22) International Filing Date:
7 March 2012 (07.03.2012)

(25) Filing Language: English

(26) Publication Language: English

(71) Applicant *(for all designated States except US):* **INDIAN COUNCIL OF MEDICAL RESEARCH** [IN/IN]; V. Ramalingaswami Bhawan, Ansari Nagar, New Delhi 110 029 (IN).

(72) Inventors; and
(71) Applicants : **DESHPANDE, Jagadish Mohan** [IN/IN]; Enterovirus Research Centre, Haffkine Institute Compound, Acharya Donde Marg, parel, Mumbai 400012 (Maharastra) (IN). **NANDI, Shyam Sundar** [IN/IN]; Enterovirus Research Centre, Haffkine Institute, Compound, Acharya Donde Marg, parel, Mumbai 400 012 (IN).

(72) Inventor; and
(75) Inventor/Applicant *(for US only):* **SHARMA, Deepa kailash** [IN/IN]; Enterovirus Research Centre, Haffkine Institute Compound, Acharya Donde Marg, parel, Mumbai 400 012 (IN).

(74) Agents: **DAVAR, G., S.** et al; L. S. Davar & Co., 5/1, Kalkaji Extension, New Delhi 110 019 (IN).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available):* AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available):* ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

Published:

— *with international search report (Art. 21(3))*

WO 2013/132504 A1

(54) Title: ASSAY FOR THE DETECTION OF NUCLEOTIDE SUBSTITUTIONS IN GENOMES OF SABIN ORAL POLIOVIRUS VACCINE VIRUSES

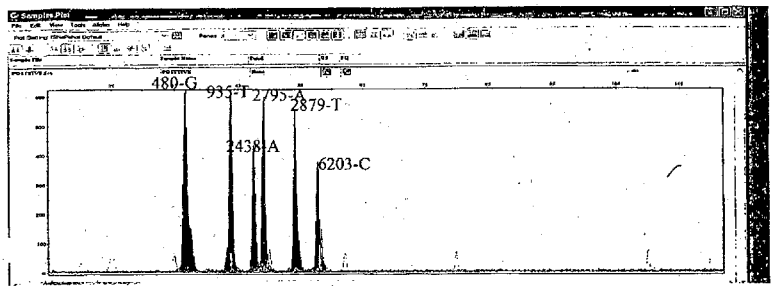

Fig-1

(57) Abstract: The present invention provides a multiplex SNP assay method and primers for the specific and simultaneous detection of mutations in the genomes of live attenuated oral poliovirus vaccine (Sabin) viruses, vaccine-like and vaccine derived polio viruses of all the three serotypes. Importantly, the inventive primers and the assay eliminate the need for costly, time-consuming and complex procedures like complete genome sequencing. This novel assay is able to test several samples in a very short time. Therefore, the assay is very simple, fast and cost-effective. A kit comprising the novel primers is also provided.

ASSAY FOR THE DETECTION OF NUCLEOTIDE SUBSTITUTIONS IN GENOMES OF SABIN ORAL POLIOVIRUS VACCINE VIRUSES

Field of invention:

The present invention broadly falls in the field of bio-technology and specifically relates to novel primers for detection of mutations at specific sites in genomes of live attenuated oral poliovirus vaccine (Sabin) viruses, vaccine-like and vaccine derived polioviruses and a kit comprising of the aforesaid primers.

Background of invention :

Dr. Albert B Sabin developed live attenuated oral poliovirus vaccine for immunization against paralytic poliomyelitis. He very carefully isolated mutated virus strains from the disease producing wild type polioviruses of all three serotypes through his meticulous laboratory work These attenuated (non-neurovirulent) poliovirus strains were used to produce live attenuated oral poliovirus vaccine used worldwide for the past 50 years. Sabin also developed a test for assessing neuro-virulence (NVT) of poliovirus in monkeys. NVT has been the standard quality control test for certification of attenuation of the oral poliovirus vaccines. Neurovirulence test in transgenic mice have recently been developed as an alternative to monkey NVT.

N.VT examines the biological character (attenuation versus virulence) of the poliovirus. Though NVT can grade the severity of the virulence it cannot determine genomic character of the virus.

In the 1980s the antigenic and molecular markers differentiating wild and Sabin OPV strains were described. Full genome sequencing, cloning and recombinant DNA technology was used to understand the genomic difference between the Sabin

attenuated and their parental wild polioviruses. These studies led to the detection of specific mutations associated with the attenuation character of Sabin vaccine polioviruses.

The most probable attenuating sites are given in the Table I below:

A molecular test i.e. mutant analysis by PCR and restriction enzyme cleavage (MAPREC) was developed to quantify mutation at a single site (the major attenuating site within 5' un-translated region) in Sabin vaccine strains and its correlation with results of NVT). This test. has been recommended by the WHO for testing quality of poliovirus vaccine. Recently, mutation detection by real time PCR has been developed. A micro-array chip capable of detecting mutations over the entire poliovirus genome has also been developed.

United States Patent 5585477 and 5691 134 relate to specific primers for detection of polioviruses in clinical samples and thus differentiating them from non-polio enteroviruses (NPEV) but do not describe the specific assay method for differentiating the polioviruses on the basis of their genetic character i.e specifically on the basis of the attenuating sites.

Complete genome sequencing and full genome micro-arrays are capable of determining the exact nucleotide (A, T, G or C) at all the known poliovirus attenuating sites but there still does not exists a rapid and convenient assay as an alternative to the aforesaid costly and complex technologies for detection of mutations at all the known attenuating sites of poliovirus I (six sites), 2 (three sites) and 3 (2 sites) in a single assay simultaneously.

Object of invention:

The object of the present application is to provide a multiplex PCR assay for

simultaneous. detection of mutations at specific sites in genomes of live attenuated oral poliovirus vaccine (Sabin) viruses, vaccine-like and vaccine derived polioviruses of the three serotypes.

Another important object of this invention is to provide novel primers for simultaneous detection of mutations at specific attenuation sites in genomes of polioviruses of the three serotypes.

Yet another object of this invention is to provide a kit comprising the aforesaid primers for use in a multiplex PCR assay for simultaneous detection of mutations at-specific sites in genomes oflive attenuated oral poliovirus vaccine (Sabin) viruses, vaccine like and vaccine derived polioviruses of the three serotypes.

Statement of invention

According to this invention there is provided an assay and primers for simultaneous detection of mutations at specific sites or SNPs in genomes of live attenuated oral poliovirus vaccine (Sabin) viruses, vaccine-like and vaccine derived ppli6viruses of the three serotypes.

Summary of invention:

The present invention relates to an assay and novel pnmers for simultaneous detection of mutations at specific sites in genomes of live attenuated oral poliovirus vaccine (Sabin) viruses, vaccine-like and vaccine derived polio viruses of the three serotypes. The invention also presents a kit comprising primers for detection of mutations at all specific sites in genomes of aforementioned viruses.

Brief Description of the invention with reference to tables/ flow chart! figures /examples etc.:

Figure 1 & 1A depicts the mutation of nucleotides at specific positions targeted for Sabin Poliovirus 1, and control- Results of the SNaP shot assay.

Figure 2 & 2A depicts the mutation of nucleotides at specific positions targeted for Sabin Poliovirus 2, and control- Results of the SNaP shot assay.

Figure 3 & 3A depicts the mutation of nucleotides at specific positions targeted for Sabin Poliovirus 3, and control - Results of the SNaP shot assay.

Figure 4 depicts 1st step multi-plex PCR result on 2% agraose gel.

Table 1 presents the most probable attenuating sites in the genomes of polio viruses.

Table 2 presents the primers used for preparing DNA amplicons of specific regions of interest.

Detailed Description of the invention with reference to figures/examples:

The present invention is described with reference to the tables! figures etc. and specific embodiments; this description is not meant to be construed in a limiting sense. Various alternate embodiments of the invention will become apparent to persons skilled in the art, upon reference to the description of the invention. It is therefore contemplated that such alternative embodiments form part of the present invention.

The invention provides an assay method; a set of specific novel primers and a kit comprising these specific primers, for detection of nucleotides at all the known attenuating sites of all the three sub-types of polio viruses i.e. poliovirus {(six sites), 2 (three sites) and 3 (2 sites) in a single assay, simultaneously.

The additional advantage of this assay is that it can be further expanded to include additional sites using the same principle. The assay is based on multiplexed reverse transcription PCR to convert poliovirus genomic RNA to cDNA and DNA amplicons. The PCR product is then used for a multiplexed SNP assay. The SNP assay product is resolved using a Genetic Analyzer to identify nucleotides at the attenuating

sites in poliovirus genome (Figure I to 3). The assay is applicable to a large numbers of samples and for testing vaccines as well as field isolates. The assay method can be converted to quantitative assay.

Details of the Assay;

1. Obtaining polio virus samples (a known serotype, e.g., Poliovirus I, 2 or 3) from various sources. **(Poliovirus Type 1, 2 and 3 were isolated from stool samples and Oral Poliovirus vaccine (Sabin) strains were obtained from NIBSc.PK).**

2. Synthesizing cDNA using antisense PCR primers **and** generation of DNA amplicons in single multiplexed reverse transcription **and** PCR reaction.

3. Purification of the PCR product (DNA amplicon).

4. Performing multi-plexed Single Nucleotide Polymorphism detection assay using the "novel primers" of this invention [Currently the assay for polio virus type 1 inclu,des 6 primer-pairs, type 2 includes 3 primer-pairs and type 3 includes 2 primer-pairs multiplex].

5. Resolving the SNPs by electrophoresis on a Genetic Analyzer, retrieving the data and analyzing the same using Gene Mapper software.

The novelty and inventiveness of the present invention is inherent in the novel design of primers, the convenience and specificity of the multi-plex assay for simultaneous detection of mutations at all attenuating sites and the kit comprising these specific primers. For better understanding of the invention, further details are provided as below:

Table 1: Most probable attenuating sites in the genomes of polio viruses

Poliovirus (Sabin)	Attenuating sites	Attenuating allele & Genomic positions
Poliovirus I	6	480-G, 935-T, 2438-A, 2795-A, 2879-T, 6203-C
Poliovirus 2	3	481-A, 2908-A, 2909-T
Poliovirus 3	2	472-T,2034-T

I. **Design of primers: Novel PCR primers used for detecting specific mutations**

a. Primers are one nucleotide short of the attenuating site to be investigated

b. 5'end tails of variable lengths have been designed and incorporated in the primers.

c. The tail fragments do not react (non-complementary to) with the genomic sequence targeted by the primers.

d. Primers are designed of increasing lengths (base pJirs) such that the first attenuating site is represented first and the last site last in ascending order when resolved in Genetic Analyzer.

2, **The Assay Method**

a. The assay is capable of identifying alleles at all nucleotide positions targeted i.e. detection of the nucleotide at all the attenuating sites in poliovirus I, 2 and 3 genomes is possible ...

b. Additional primers can be incorporated in one multiplexed assay if new sites should be included.

c. Different primer sets are designed for the three-poliovirus serotypes.

d. All attenuating sites can be explored in one assay.

Table 2: Primers used for preparing DNA amplicons of specific regions of interest.

Type	Reaction	Region	Primer used	Application size
Type -1	MULTIPLEX	5'NTR	PV1-2S/PV1-1A	372bp
		VP1	Y7/S1	603bp
		VP4	UG53/UC21	623bp
		3D-pol	PV1-13S/PV1-12A	334bp
Type - 2	MULTIPLEX	5NTR	PV-2321S/PV2-575A	274bp
		VP1	PV2-2830S/PV2-3018A	188bp
Type - 3	MULTIPLEX	5'NTR	PV3S-2S/PV3-2A	357bp
		VP3	PV3-5S/PV3-5A	356bp

Type - 1

1. PV128 - 5'CAT GGG ACG CTA GTT GTG AA3'
2. PVI-IA - 5'ATG AAA CCT GAG CAC CCA IT3'
3. Y7 - 5' GGG TTTGTGTCA GCC TGT AATGA3'
4.81- 5' TGG GAC GAC TAC ACA TGG CA3'
5 DG 53 - 5'TGG CTG CIT ATG GTG ACA AT 3'
6. DC21- 5'TCA'GGT AAT ITC CAC CAC CA 3'
7. PVI-J38- 5'AAG TCA TCG GGA TGC ATG IT 3'
8. PV112A - 5'CTG GCC AGC ATA GTG GTCTA3'

. Type-2

1.PV23218- 5' TGA GTC TGG ACA TTC CTC ACC3'
2.PV2-575A. 5' GTC ACC ATAAGC AGC CATGA3'

3.PV2-38308- 5' GAA CTG AGA CGC AAA CTG GA3'
4.PV2-3018A- 5'AGA GGA CGT CAG CCA CGT AD'

Type-3

I.PV3 -28 5'ACG GGA CGC TAG ITG TGA AC3'
2.PV3-2A 5' GCT CCC ATT GTG ACA CTG AA3'
3.PV3-58 5' GAG CTC GCC GAG ATA GAC AC3'
4.PV3-5A 5' AGA TGA ITG CAG GCC AAG AD'

Details of primers used for SNP assay are provided below:

SNP Primer for Type-1 (Sabin Virus)
Primer Name; PV I IF -1, Nucleotide Position-480
Sequence ID 1:
5'GACTGACTAATGCGGCTAATCCCAACCTCGG3*
Tm- 67.6, GC- 56.5%, Length -31 nt

Primer Name: PVI_ 2F-I, Nucleotide Position-935
Sequence ID 2:
5'GACTGACTGCCCATCAAGGATGTCCTGATAAAAACA3'
Tm- 66.4, GC- 42.9%, Length -36 nt/"

Primer Name: PV 1_ 3F-1, Nucleotide Position-2438
Sequence ID 3:
5'GACTGACTGACTGACTTGTAATGACTTCAGCGTGCGCTTG3'
Tm- 65.2, GC- 50%, Length -40 nt

Primer Name: PV1_ 4F-1, Nucleotide Position-2795
Sequence ID 4:
5'GACTGACTGACTCAGCTTCCACCAAGAATAAGGATAAGCTATTT3
Tm- 64.4, GC- 37.5%, Length -44 nt

Primer Name: PVI_5F-I, Nucleotide Position-2879

Sequence ID 5:

5'GACTGACTGACTGACTGACTTTCACCTATTCTAGATTTGATATGGAA3'

Tm- 55.7, GC- 29.6%, Length -47 nt

Primer Name: PVI_6F1, Nucleotide Position-6203

Sequence ID 6:

5'GACTGACTGACTGACTGACTACGTGGGTAACAAAATTACTGAAGTGGATGAG3

Tm 66.3, GC- 40.6-, Length -52 nt (

SNP Primers for (Poliovirus Type-2)

Primer Name: PV2_2A, Nucleotide Position-48 1

Sequence ID 7:

5'GACTGACTCGGCTAATCCTAACCACGGA3*

Tm-58.7, GC- 55%, Length- 28 nt

Primer Name: PV2_28, Nucleotide Position-2908

Sequence ID 8:

5'GACTGACTGACTTTTTGTGGTCACCTCAAACTAC3'

Tm 53.2, GC-40.9%, Length -34 nt

Primer Name: PV2_2C, Nucleotide Position-2909

Sequence ID 9:

5'GACTGACTGACTGACTTTTTGTGGTCACCTCAAACTACA3

Tm- 56, GC- 39%, Length -39 nt

SNP Primers for (Poliovirus Type-3)

Primer Name: PV3_ 3A, Nucleotide Position- 472

Sequence ID 10:

5'GACTGACTCCCCTGAATGCGGCTAAT3

Tm- 57.2, GC- 55.6 %, Length-26 nt

Primer Name: PV3_ 38, Nucleotide Position-2034

Sequence ID 11:

5'GACTGACTGACTGCTTGTCACTATCCCCAGCAT3'

Tm- 57.8, GC- 52.4%, Length -33 nt

According to one of the embodiments, the specific assay method as disclosed in this invention is capable of identifying alleles at all nucleotide positions targeted and exploring all attenuating sites in one assay and thus is useful for identifying the nucleotide at all the attenuating sites in poliovirus I, 2 and 3 genomes. Additional primers can be incorporated in one multi-plexed assay if new sites need to be included. The assay method utilizes different primer sets designed for the three-poliovirus serotypes. The assay method is applicable to a large numbers of samples and can be used for testing vaccines as well as field isolate;;. Moreover the assay method can be converted to quantitative assay and is expandable to include additional mutation sites.

Another embodiment of the invention pertains to PCR primers for detection of mutationsat -pecific sites in gehomes of live attenuated oral poliovirus vaccine (Sabin) viruses, vaccinelike and vaccine derived polioviruses of all the three serotypes. The primers are characterized in that they have variations in their lengths and are one nucleotide short of ,the attenuating site to be investigated. The primers have been designed to incorporate the 5'end tails of variable lengths,. the tail fragments being non-complementary to the genomic sequence targeted by the primers.

The primers of increasing lengths (base pairs) are designed such that the first attenuating site is represented first and the last site last in ascending order when resolved in Genetic Analyzer.

Yet another aspect of the invention relates to a kit containing the aforesaid primers, for detection of mutations (nucleotide substitutions) at specific sites in genomes of live attenuated oral poliovirus vaccine (Sabin) viruses, vaccine-like and vaccine derived polioviruses of all the three serotypes, comprising of the

aforementioned primers. The kit further comprises of deoxynucleoside triphosphates, a polymerisation enzyme, divalent cations and/or a buffer solution.

Example:

The following examples an for purpose of illustration of the invention and are not intended in any way to limit the scope of the invention

Example I:

Preparation'of DNA amplicons from three serotypes of poliovirus

Poliovirus samples were taken each serotypes (type 1,2 or 3) separately. Poliovirus, cDNA was synthesized using antisense PCR primers and DNA amplicons generation in single multiplexed reverse transcription and PCR reaction(Roche) from frozen infected cultures. PCR product (DNA amplicon) was purified by PCR purification kit (Quagcn).

RT-PCR- For RT-PCR analysis, frozen infected cultures (lfll) were taken directly and 67 mM Tris/HCI (pH 8.8), 17 mM ammonium sulfate, 6 mM EDTA, 2 mM MgCh, 200 Mm each dNTP, I mM dithiothreitol, I mM each primer, 10 U placental RNase inhibitor (Roche), 3 U avian myeloblastosis virus (AMV) reverse transcriptase (Roche) and 5U Taq DNA polymerase (Roche Applied Science) in a total volume of 50 ill. The reactions were incubated at 50°C for 30 min followed by 94°C for 3 min. ~Th.-.ermocycling was performetl for 35. cycles at 94°C for 30 s, 42°C for 30 sand 72 °c for 30 s in a 9700-model thermbcYcler (Applied Biosystems). Thermocycling was followed by incubation at 72 °c for 5 min. The reaction products were analysed by electrophoresis in a 10% polyacrylamidelTBE gel and stained with 0.5 fig ethidium bromide mrl. DNA was purified by QIA quick PCR Purification kit (Qiagen).

Example 2:

Single Nucleotide Polymorphism Assay (SNP Assay) for identifying mutations

10 SNP reactions were carried out in a 10 III final volume containing SNaPshot multiplex ready reaction mix (51 1l),primer mix (31 11), (final concentrations, 0.15- 0.6 Ilmolll), and templates (2 111) consisting of the multiplex PCR products described above, which had been purified with the QIAquick PCR Purification Kit (Qiagcn). Multiplex PCR products amplified from viral RNA were pooled and purified on the same column before the SNaPshot reaction. The cycling program included 25 cycles of 96°C for lOs, 50°C for 5 s, and 60°C for 30 s. Extension products were purified by a IS-min incubation with I U of Shrimp Alkaline Phosphatase (Fermentas) at 37°C and a subsequent IS-min incubation at 75°C to inactivate the enzyme. The purified products (0.5 111) were mixed with 9 III of formamide and 0.5 III of GeneScan- 120 LIZ Size Standard (Applied Biosystems) and separated by capillary electrophoresis (ABI PRISM 3130 Genetic Analyzer; Applied Biosystems). The results were analyzed with GeneMapper 3.0 software (Applied Biosystems).

SNP primers were designed in such a way that each primer is one nucleotide short of the attenuating site to be investigated and synthesized from Sigma. Primers are designed of increasing lengths (base pairs) such that the first attenuating site is represented first and the last site last. Multiplexed Single Nucleotide Polymorphism detection assay was done by the SnaPshot kit (Applied Biosystem). SNPs are resolved by capillary electrophoresis on a Genetic Analyzer 3130 (Applied Biosystem) and data retrieved and analyzed by Gene Mapper software. ,

Advantages of the invention

1. All attenuating sites explored in one assay and detection of the nucleotide at all the

attenuating sites in poliovirus 1,2 and 3 genomes is possible.

2. The assay method is capable of detecting alleles (mixed bases) at all the sites investigated.

3. The assay method IS capable of identifying alleles at all nucleotide positions targeted.

4. The assay comprises of multiplexed reactions.

5. The assay is capability of handling large numbers of samples.

6. .The assay is useful for vaccine testing as well as field isolates testing.

7. The assay can be converted to quantitative assay.

8. The assay is expandable to include additional sites

We Claim:

1. An assay for simultaneous detection of mutations at specific sites or SNPs In genomes of polioviruses of three serotypes, comprising the following steps:

a. obtaining polio virus samples from various sources,

b. generating DNA amplicons of the poliovirus of step a by synthesizing cDNA using antisense PCR primers,

c. purifying the DNA amplicon of step b,

d. performing multi-plexed Single Nucleotide Polymorphism detection assay using the PCR primers of SEQ IDs I-II,

e. resolving the SNPs by electrophoresis,

f. retrieving the data and analyzing the same using Gene Mapper software.

2. The assay for simultaneous detection of mutations at specific sites or SNPs in genomes of polioviruses of three serotypes as claimed in claim I, wherein the virus is selected from Poliovirus Type-I (Sabin Virus), Poliovirus Type-2 and Poliovirus Type-3 .

3. The assay for simultaneous detection of mutations at specific sites or SNPs in genomes of polio viruses of three serotypes as claimed in claim I, wherein the primer seq,uences IDs I to 6 detect SNP in Poliovirus Type-I(Sabin Virus), primer sequences IDs 7 to 9 detect SNP in Poliovirus Type-2 and primer sequences IDs 10 to II are for detection of SWP in Poliovirus Type-3.

4. The assay for simultaneous detection of mutations at specific sites or SNPs in genomes of polioviruses of three serotypes as claimed in claim I, wherein the

primers detect SNP in live attenuated oral poliovirus vaccine (Sabin) viruses, vaccine-like and vaccine derived polioviruses of three serotypes.

5. The assay for simultaneous detection of mutations at specific sites or SNPs in genomes of polioviruses of three serotypes as claimed in claim I, wherein the primers have variations in their lengths.

6. The assay for simultaneous detection of mutations at specific sites or SNPs in genomes of polioviruses of three serotypes as claimed in claim 5, wherein the variation in primer is one nucleotide short of the attenuating site to be investigated.

7. The assay as claimed in any of the aforesaid claims, wherein the primers have been designed to incorporate the 5'end tails of variable lengths.

8. The assay as claimed in any of the aforesaid claims, wherein the primers are designed with increasing lengths such that the first attenuating site is represented first and the last site last in ascending order when resolved in Genetic Analyzer.

9. The assay as claimed in claim I, wherein additional primers can be incorporated in one multiplexed assay if new sites are to be included.

10. A kit for detection of mutations at specific sites or SNPs in genomes of live attenuated oral poliovirus vaccine (Sabin) viruses, vaccine-like and vaccine derived polioviruses of all the three serotypes, wherein the kit comprises of the primers of Sequence IDs I to II.

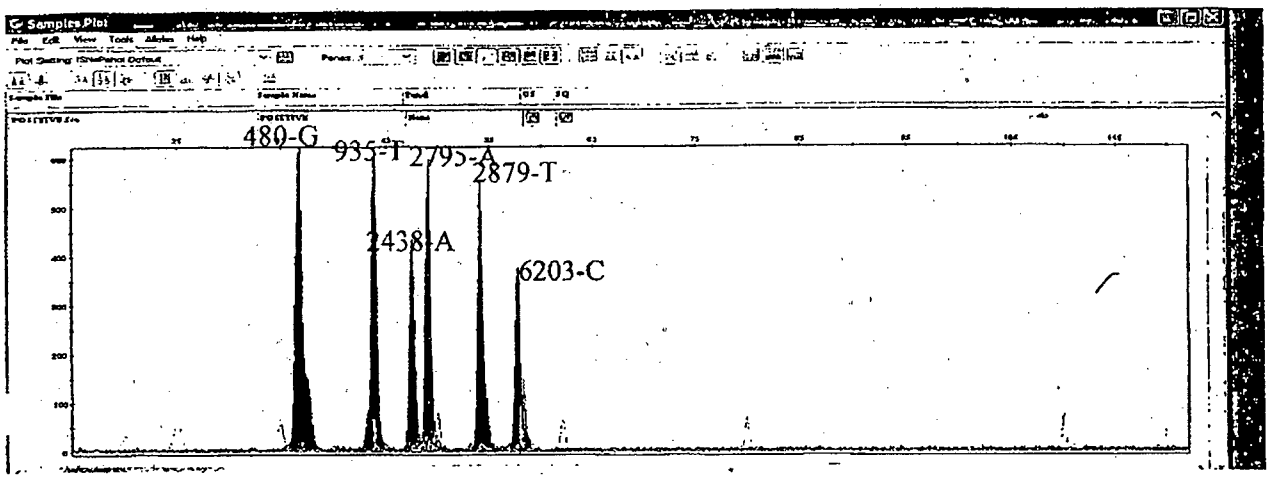

Fig-1

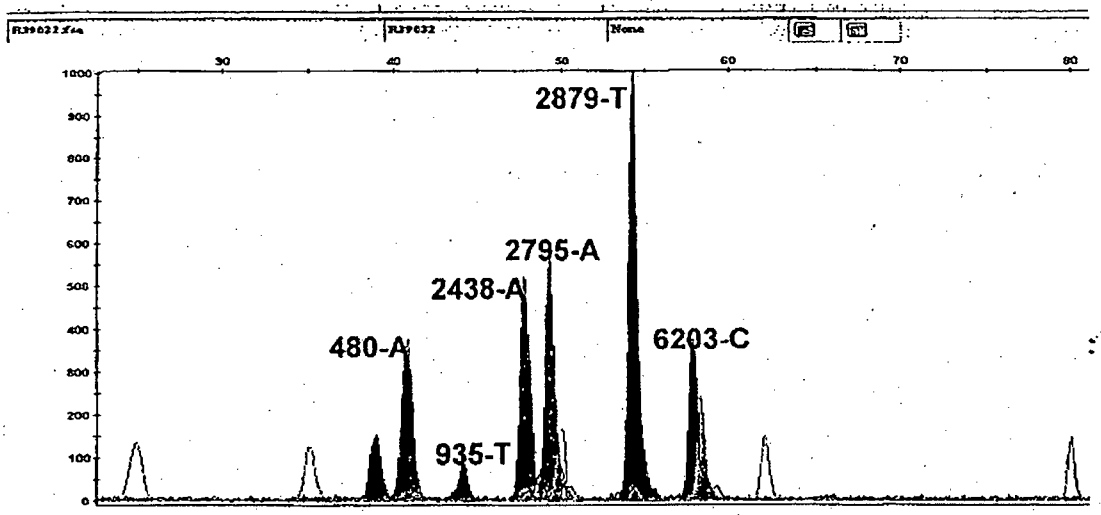

Fig-1A

(C M Gaind)
of L.S.DAVAR & CO.,
APPLICANT'S AGENT.

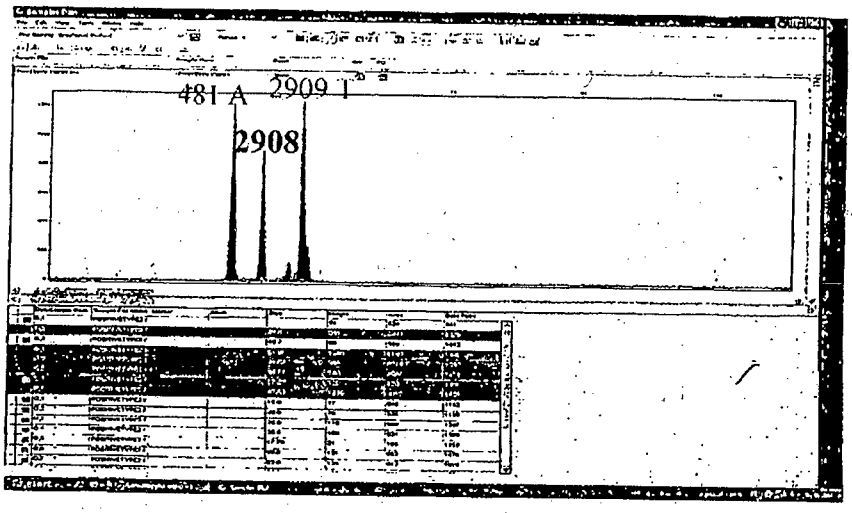

Fig-2

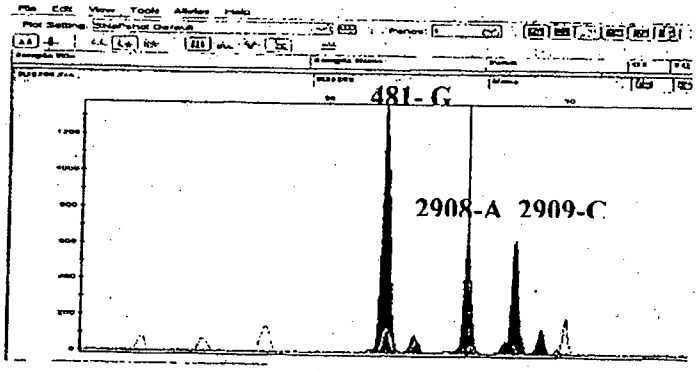

Fig-2A

(C M Gaind)
of L.S.DAVAR & CO.,
APPLICANT'S AGENT.

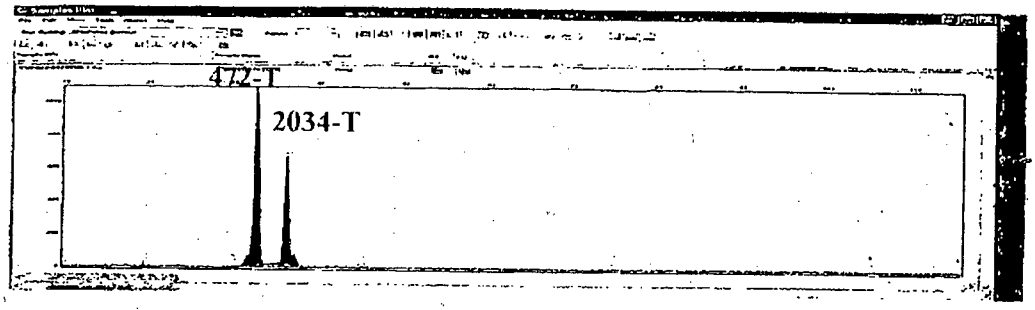

Fig-3

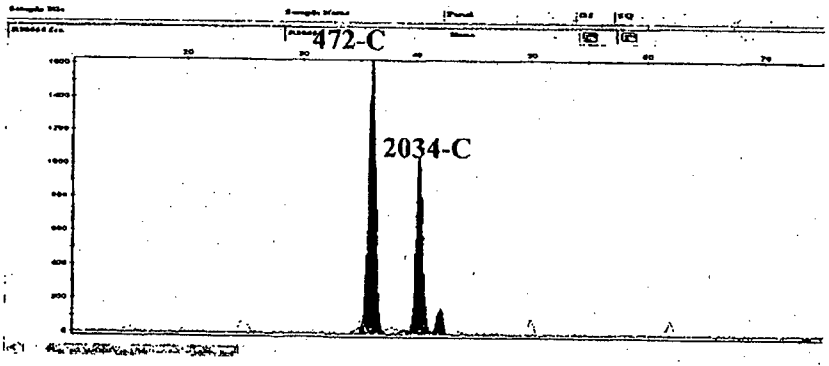

Fig-3A

(C M Gaind)
of L.S.DAVAR & CO.,
APPLICANT'S AGENT.

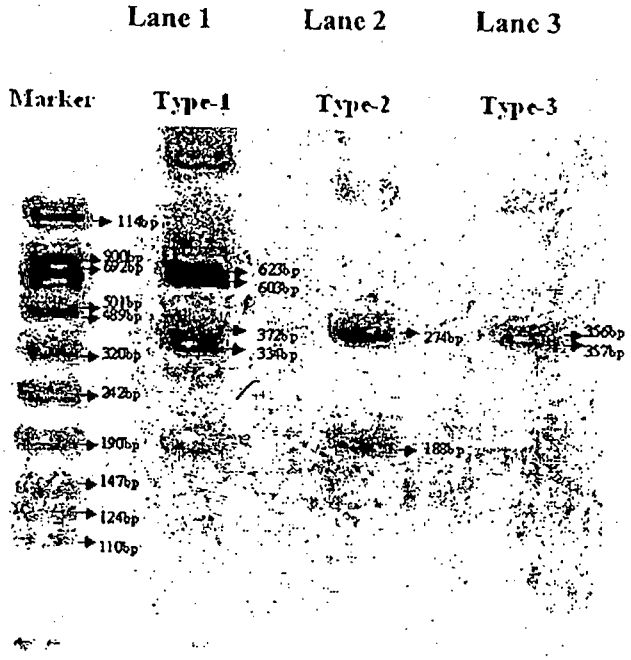

Figure 4

(C M Gaind)
of L.S.DAVAR & CO.,
APPLICANT'S AGENT.

INTERNATIONAL SEARCH REPORT

International application No
PCT/IN2012/000160

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/70
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal , WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	"ABI Prism SNaPshot Multiplex Kit", , 1 January 2010 (2010-01-01) , XP55041340, Retrieved from the Internet: URL: http://tools.invitrogen.com/content/sfs/manuals/cms_041203.pdf [retrieved on 2012-10-17] the whole document pages 9, 29	1-10

-/- .

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" documentwhich may throw doubts on priority claim(s) orwhich is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 October 2012	02/11/2012

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Quirin, Katharina

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CARITA SAVOLAINEN-KOPRA ET AL: "Mechanisms of genetic variation in poliovirus", REVIEWS IN MEDICAL VIROLOGY, vol. 20, no. 6, 15 October 2010 (2010-10-15), pages 358-371, XP55041761, ISSN: 1052-9276, DOI: 10.1002/rmv.663 the whole document table 2	1-10
A	D. J. WOOD ET AL: "Laboratory Tests for Live Attenuated Poliovirus Vaccines", BIOLOGICALS, vol. 25, 1 January 1997 (1997-01-01), pages 3-15, XP55041623, the whole document pages 6-7, 9	1-10
A	S. GUILLOT ET AL: "Natural Genetic Exchanges between Vaccine and Wild Poliovirus Strains in Humans", JOURNAL OF VIROLOGY, vol. 74, no. 18, 15 September 2000 (2000-09-15), pages 8434-8443, XP55041508, ISSN: 0022-538X, DOI: 10.1128/JVI.74.18.8434-8443.2000 the whole document table 1	1-10
A	US 5 728 519 A (LEVENBOOK INESSA S [US] ET AL) 17 March 1998 (1998-03-17) the whole document sequences 21,24,25,38,58	1-10
A	WO 95/14107 A1 (SMITHKLINE BEECHAM BIOLOG [BE]; COLAU BRIGITTE DESIREE ALBERTE [BE]; M) 26 May 1995 (1995-05-26) the whole document sequence 7	1-10
A	A. NEVEROV ET AL: "Massively parallel sequencing for monitoring genetic consistency and quality control of live viral vaccines", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 107, no. 46, 16 November 2010 (2010-11-16), pages 20063-20068, XP55041954, ISSN: 0027-8424, DOI: 10.1073/pnas.1012537107 the whole document	1-10

-/--

1

INTERNATIONAL SEARCH REPORT

International application No
PCT/IN2012/000160

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	M. LAASSRI ET AL: "Genomic Analysis of Vaccine-Derived Poliovirus Strains in Stool Specimens by Combination of Full-Length PCR and Oligonucleotide Microarray Hybridization", JOURNAL OF CLINICAL MICROBIOLOGY, vol. 43, no. 6, 1 June 2005 (2005-06-01), pages 2886-2894, XP55041992, ISSN: 0095-1137, DOI: 10.1128/JCM.43.6.2886-2894.2005 the whole document ----	1-10

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IN2012/000160

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5728519 A	17-03-1998	NONE	
WO 9514107 A1	26-05-1995	NONE	