STANDARD OPERATING PROCEDURE
MUTANT ANALYSIS BY PCR AND RESTRICTION ENZYME CLEAVAGE
(MAPREC) FOR ORAL POLIOVIRUS (SABIN)
VACCINE TYPES 1, 2 OR 3

Version 5 (2012)
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### Authors  

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Part 1. Overview of the assay

1.1 **Aim**
The MAPREC assay is a molecular biological method used to determine the proportion of a single base mutation at a given point within the viral RNA. If the calculated value of the mutation at this site is greater than acceptable values (see the current WHO Recommendations to Assure the Quality, Safety and Efficacy of Live Attenuated Poliomyelitis Vaccine (oral)) characterised (1), the monovalent bulk will fail the MAPREC test.

1.2 **Introduction**
Isolates of all three serotypes of poliovirus were passaged *in vivo* and *in vitro* to produce the Sabin strains of poliovirus that make up Oral Poliovirus Vaccine (OPV). Although these attenuated vaccine strains are stable, they can revert to full or partial virulence through point mutations in the 5’ non-coding region of the viral RNA, particularly if grown at temperatures greater than the optimal 35°C. These mutations differ in each of the three types of polio vaccine (1, 2 and 3) and are well characterised (2, 3, 4).

In type 3 poliovirus vaccine, the mutation of U to C at base position 472 in the viral RNA 5’ NCR is directly related to the neurovirulence of the virus in monkeys. As the proportion of C in the viral population increases, so does the neurovirulence, such that if the C content rises above 0.9%, the vaccine will fail the standard monkey neurovirulence test (5, 6). In Type 1 and 2 OPV there are mutations within the 5’ NCR which revert rapidly when passaged in the human gut or in cell culture, in Type 1 base positions 480 G→A, 525 U→C and in Type 2 base position 481 A→G. These mutations can lead to increased neurovirulence when present in high proportions in the viral population, or possibly acting with other mutations within the viral genome. However, no correlation with virulence in monkeys has been established when these mutations are present at levels typically found in vaccine batches. Therefore, the MAPREC test for Type 1 and 2 OPV has been developed to measure the consistency of vaccine production.

1.3. **Special considerations**
MAPREC is a PCR based assay, which allows very small quantities of starting nucleic acid to be amplified to provide large amounts of DNA for quantification. The ability to amplify very small quantities of target material via the PCR reaction means that contamination with DNA or RNA, either by cross-contamination of samples, or other nucleic acids in the laboratory may present a serious problem.

One of the most important general considerations for PCR is the provision of clean areas in which to prepare reagents and reaction mixes. It is important to separate the various stages of the PCR reaction, such as RNA extraction and cDNA addition; so that there is no possibility of contamination of PCR mixes by previously amplified DNA. Staff should change laboratory coats and gloves when using the different laboratories and equipment should be dedicated to each room. Local and whole-room UV-irradiation and other decontamination measures could be used to reduce the possibility of PCR-amplified DNA contaminating reagents and samples. Other measures such as employing dUTP PCR contamination prevention protocols could also be considered.
Adequate controls should be included in the test to ensure that contamination will be detected. A cDNA control of water, which is extracted at the same time as the viral RNA, should be included as well as a PCR reaction control. The PCR control ensures that the reagents are free of DNA contamination.

If radioisotopes are used, the laboratory should comply with all national regulations for their use and disposal.

1.4. **The assay**

Each monovalent bulk is assayed individually, before the combination of the three serotypes into the final vaccine product.

Each assay will include four standard preparations:

- International Standard DNA (IS DNA)
- High Mutant Virus Reference (HMVR)
- Low Mutant Virus Reference (LMVR)
- 100% DNA control

Firstly, the RNA in each monovalent bulk is extracted, together with appropriate control materials (HMVR, LMVR, cDNA control). The extracted RNA is then reversed transcribed into cDNA using a mixture of random hexanucleotide primers and reverse transcriptase.

For each serotype, specific PCR primers are used to amplify the segment of viral cDNA containing the base to be quantified. DNA standards (IS DNA and 100% DNA control) are included along with the previously prepared cDNA. One of the primers contains the modifications required to create a unique restriction site for enzyme digestion. This primer is included at 10 times the concentration of the second PCR primer and results in the accumulation of a large amount of single stranded template. A low concentration radioisotope or fluorescently labelled primer is subsequently used to prime a second-strand DNA synthesis by a one-step DNA polymerase reaction. One half of this labelled double stranded product is then digested with the specific restriction enzyme, and the other half is used as an undigested control.

The radiolabelled/fluorescent products are separated on a polyacrylamide gel, and visualised and quantified using a suitable detector (imager). The intensity for each band in each sample is entered into a computer spreadsheet programme (which can easily be developed) or calculated manually. The mutant content of the samples is calculated. Two separate PCR reactions are generated from one cDNA preparation and are used to perform 5 individual determinations for each mutation. The MAPREC assay on monovalent bulks therefore consists of the set of 5 individual determinations.

If the MAPREC assay is used for monitoring a single harvest, fewer than 5 individual determinations may be performed, with the agreement of the national regulatory authority.
1.5. **Calculation of revertant content**

Two lanes, one with enzyme digested DNA [D] and the other with undigested DNA as a control [C], are analysed for each sample.

In each of these lanes, areas containing both the undigested full length (upper) DNA band [DU and CU] and the restriction fragment (lower) band [DL and CL] are quantified (For type 2 and type 3 MAPREC) see Figure 1.

![Image](image.png)

**Figure 1. Appearance of Types 2 and 3 MAPREC gel**

The fraction of radioisotope/fluorescence in the restriction fragment is compared to the total radioisotope/fluorescence (digested DNA + the full length DNA) for each lane:

\[
FD = \frac{DL}{DU+DL} \\
FC = \frac{CL}{CU+CL} \\
\]

FD represents the fraction of DNA molecules with the reversion plus non-specific background fluorescence.

The % of revertants = FD - FC = \([DL/[DU+DL] – CL/[CU+CL]])100.

In the Type 1 MAPREC assay, there are two digested bands which are quantified, see Figure 2.

In this case the fraction of radioisotope/fluorescence in the restriction fragment compared to the total radioisotope/fluorescence for the specific DNA is calculated for each lane:

\[
\text{The % of revertants} = FD - FC = \left[ \frac{DL1+DL2}{DU+DL1+DL2} - \frac{CL1+CL2}{CU+CL1+CL2} \right]100
\]
1.6. **Validity criteria**

Criteria 1.6.1 and 1.6.2 below apply to each determination, whilst criteria 1.6.3, to 1.6.7 apply to each set of five determinations which make up a complete assay.

### 1.6.1 The cDNA and PCR controls should be negative (no full length PCR product detected).

**Bands Appearing in the cDNA and PCR water controls**

If DNA bands are detected in either of these controls, it is important to determine whether the DNA is a true contaminant or is an aberrant amplification product often referred to as “primer dimer”.

To do this, the sample is digested with a mixture of enzymes, which will result in the complete digestion of a genuine DNA PCR product.

If DNA bands are resistant to both enzymes, they are primer–dimers and not contaminants.

If the DNA band in blank samples is digested by a mixture Mbo I and Hinf I (in case of Type 3; Bsp1286I and Afl III in case of Type 2) giving DNA fragments of appropriate size, it is a contaminant. Detection of contamination will invalidate a MAPREC determination.

Sources of contamination should be identified and eliminated.

<table>
<thead>
<tr>
<th>Poliovirus</th>
<th>Nucleotide</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>type 1</td>
<td>480-A (revertant)</td>
<td>Dde I</td>
</tr>
<tr>
<td></td>
<td>480-G (vaccine)</td>
<td>BstX I</td>
</tr>
<tr>
<td></td>
<td>525-C (revertant)</td>
<td>Nci I</td>
</tr>
<tr>
<td></td>
<td>525-U (vaccine)</td>
<td>Mro I</td>
</tr>
<tr>
<td>type 2</td>
<td>481-G (revertant)</td>
<td>Bsp1286I</td>
</tr>
<tr>
<td></td>
<td>481-A (vaccine)</td>
<td>Afl III</td>
</tr>
<tr>
<td>type 3</td>
<td>472-C (revertant)</td>
<td>Mbo I</td>
</tr>
<tr>
<td></td>
<td>472-U (vaccine)</td>
<td>Hinf I</td>
</tr>
</tbody>
</table>
1.6.2 The value for the 100% DNA control should be equal to or greater than a minimum acceptable value established within the testing laboratory.

It is important to ensure that the restriction enzyme digestion conditions are optimal and give a maximum percentage of cut DNA. However, the apparent mutant content determined in MAPREC assay for 100% DNA control is always somewhat lower than 100%. This is because the efficiency of restriction enzymes is less than 100%, even when present in a large excess. For this reason, values determined for mutant content can vary from 90 to more than 95%, and can depend on the type and source of restriction enzyme. Therefore the level of digestion for the 100% DNA should be within previously established limits, and be monitored for consistency (section 1.7).

If a particular determination falls below the historically established minimum acceptable value, the determination is deemed invalid and should be repeated. If more than one determination in a series of five falls this criterion, further experiments must be performed to identify the problem that led to incomplete digestion of the DNA. This may be due to the quality of the enzyme, which should be replaced.

1.6.3 The within assay variability for all test samples and controls, other than 100% DNA control, should be within limits established within the testing laboratory.

The within assay variability for a sample or control is measured by the standard deviation across 5 determinations of the corrected % revertants. The standard deviations should be lower than limits determined from historical data within the testing laboratory, and should be no greater than 0.3. The value 0.3 is a value based on the results of the original WHO collaborative studies, where most laboratories obtained values below this. These criteria limit the variability allowed within a complete MAPREC assay.

1.6.4 The values obtained for the IS DNA should be consistent with previously obtained values.

The % of mutation content in the International Standard (IS) DNA should be consistent with previous tests. (%480-A and 525-C for Type 1 OPV, % 481–G in for Type 2 OPV, % 472–C in for Type 3 OPV). If a particular determination for the sample falls outside the limits determined by the laboratory, it is rejected. If more than one determination in a series of five is invalidated, further experiments must be performed.

1.6.5 The ratio of the % of the duplicate DNA reference controls (A and B), should not differ significantly from each other as determined by a paired t test of % mutation values.

If the mean ratios of the IS DNA duplicates A and B, in a particular set of five valid individual determinations are significantly different, it indicates that there may be some systematic bias in the experimental procedure. The results for the whole set should be invalidated and the problems investigated and resolved.
A paired t-test of corrected % revertants may be used to assess the significance of any differences. However, the WHO collaborative studies indicated that for laboratories with very low within assay variability, the t-test approach may be too sensitive. As an alternative, limits for the mean ratio of the duplicates A and B could be established by the laboratory, based on historical data.

1.6.6 The High Mutant Virus Reference should give the expected result in the MAPREC assay.

The % of mutation content in HMVR should be consistent with previous tests within the laboratory. (%480-A and 525-C for Type 1 OPV, % 481–G in for Type 2 OPV, % 472–C in for Type 3 OPV).

1.6.7 The Low Mutant Virus Reference should give the expected result in the MAPREC assay.

The % of mutation content in the LMVR should be consistent with previous tests within the laboratory. (%480-A and 525-C for Type 1 OPV, % 481–G in for Type 2 OPV, % 472–C for Type 3 OPV).

1.7. Measures of consistency

Several of the validity criteria specify that results should be consistent with previously obtained values, or fall within previously determined limits. The method used to determine limits or to monitor consistency should follow the individual laboratory’s usual data monitoring procedures.

One possible approach is to determine a historic mean and standard deviation from previous test data, and use these to generate a control chart or limits of ±2sd or ±3sd for the particular parameter being monitored. Note that the strict application of ±2sd limits will lead to the rejection of approximately 5% of assays or determinations by chance alone, for each parameter being monitored. Depending on a laboratory’s experience with the MAPREC test, an approach using “warning limits” of ±2sd and “rejection limits” of ±3sd may be considered more appropriate.

1.8. Interpretation of MAPREC results and sample acceptability criteria

The accumulation of 472–C mutations in the 5’ NCR of type 3 OPV during vaccine production, leads to the increased neurovirulence of vaccine batches as determined by the Monkey Neurovirulence test (MNVT). When the level of 472-C mutations exceeds a certain threshold, the OPV will fail the MNVT.

For Type 1 and Type 2 OPV, the accumulation of revertants in the 5’NCR during vaccine production does not necessarily lead to increased neurovirulence in the MNVT. Therefore the MAPREC assay should be regarded as a measure of production consistency.

Results should be expressed as ratios relative to the relevant type specific International Standard for MAPREC analysis of poliovirus (Sabin). The maximum level of revertants permissible is stated in the current WHO Recommendations to
Assure the Quality, Safety and Efficacy of Live Attenuated Poliomyelitis Vaccine (oral) (1).

1.9. Critical reagents

The specific primers for PCR and International Standards detailed in the MAPREC test procedure are critical to the assay. If fresh batches of primers are obtained they should be assessed for suitability in an assay with the international standards.

1.10. Equipment

Equipment should be maintained and calibrated according to the manufacturer’s instructions or in house guidelines. Equipment records should be kept and updated as appropriate.
Part 2. MAPREC test procedures

2.1 Standards and primers used in the MAPREC assay

The reagents used in the MAPREC Standard Operating Procedures depend upon the serotype of the monovalent bulk, and the base position to be analysed.

These reagents are critical to the test and have been established by a WHO collaborative study as below (7, 8, 9):

2.1.1 Poliovirus type 1 base 480 and base 525

LMRV
HMRV
100% REVERTANT CONTROL
IS STANDARD

*two replicates of this samples should be included in each test, one labelled A, and one labelled B.

pS primer:  pS-1/445  5’ CTC CGG CCC CTG AAT GCG GCT AAT CCA AAC CTC tG  3’
Hplc purified, used at 3µg/ml

pA primer:  pA-1/526  5’ AAC ACG GAC ACC CAA AGT AGT CGG TTC CGC tCc GC  3’
Hplc purified, used at 30µg/ml

pS labeled primer:  pS-1/445  5’ CTC CGG CCC CTG AAT GCG GCT AAT CCA AAC CTC tG
3’
used at 3µg/ml

*Label: - the label should be suitable for the detector used and can be added by the manufacturer or in house for radioisotope

‡Restriction enzyme: - Dde I at 1 unit/µl and Nci I at 1 unit/µl (diluted if appropriate in the buffer supplied).

2.1.2 Poliovirus type 2 base 481

LMRV
HMRV
100% REVERTANT CONTROL
IS STANDARD

*two replicates of this samples should be included in each test, one labelled A, and one labelled B.

pS primer:  pS-2/431  5’ GCT ACA TAA GAG TCC TCC GGC CCC TGA ATG CGC CT  3’
Hplc purified, used at 3µg/ml

pA primer:  pA-2/483  5’ CGC GTT ACG ACA AGC CAG TCA CTG GTT CGC GAC CaC Gt  3’
Hplc purified, used at 30µg/ml

**pS labeled primer:**  
5’ GCT ACA TAA GAG TCC TCC GGC CCC TGA ATG CGC CT 3’  
used at 3µg/ml

*Label:* - the label should be suitable for the detector used and can be added by the manufacturer or *in house* for radioisotope

‡**Restriction enzyme:** - Bsp 1286 I at 1 unit/µl (diluted if appropriate in the buffer supplied)

### 2.1.3 Poliovirus type 3 base 472

**LMRV**  
**HMRV**

100% REVERTANT CONTROL  
IS STANDARD

*two replicates of this samples should be included in each test, one labelled A, and one labelled B.

**pA primer:**  
pA-3/484 5’ CAG GCT GGC TGC TGG GTT GCA GCT GCC TGC 3’

Hplc purified, used at 3µg/ml

**pS primer:**  
pS-3/470 5’ TGA GCT ACA TGA GAG TCC GGC CCC TGA ATG CGG CTG A 3’

Hplc purified, used at 30µg/ml

**pA labeled primer:**  
5’ CAG GCT GGC TGC TGG GTT GCA GCT GCC TGC 3’

used at 3µg/ml

*Label:* - the label should be suitable for the detector used and can be added by the manufacturer or *in house* for radioisotope

‡**Restriction enzyme:** - **Mbo I** at 1 unit/µl (If the enzyme is more concentrated, dilute to 1 unit /µl in the buffer supplied with the enzyme)

‡ Isoschizomers of the restriction enzymes may be used, however these enzymes must be validated before use.  
Examples of isoschizomers are:

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Isoschizomers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dde I</td>
<td>BstDE I, HypF3 I,</td>
</tr>
<tr>
<td>BstX I</td>
<td>-</td>
</tr>
<tr>
<td>Nci I</td>
<td>AsuC2 I, Bcn I, BpuM I</td>
</tr>
<tr>
<td>Mro I</td>
<td>Acc III, Aor13H I, BseA I, Bsp13 I, BspE 1, Kpn2 I.</td>
</tr>
<tr>
<td>Bsp 1286I</td>
<td>Mh1 I, Sdu I</td>
</tr>
<tr>
<td>Afl III</td>
<td>-</td>
</tr>
<tr>
<td>Mbo I</td>
<td>BfuC I, Bsp143 I, BssM I, BstKT I, BstMB I, Dpn II, Nde II, Sau3A I</td>
</tr>
<tr>
<td>Hinf I</td>
<td>-</td>
</tr>
</tbody>
</table>
2.2  **Detailed steps of the MAPREC assay procedure**

The RNA extraction and cDNA preparation is the same for all three types of OPV monovalent bulks.

The primers used for the PCR and labelling reactions differ for each of the three types, as do the restriction enzymes used to digest the PCR products.

### 2.2.1 Extraction of RNA

There are many procedures and kits available for the preparation of high quality RNA. The method described below requires that approximately 125µl of the original viral suspension is available for the subsequent reverse transcription reaction to cDNA.

If an alternative RNA extraction method is used, it should be ensured that it will yield sufficient RNA for the subsequent reactions.

**Equipment**
- Microfuge
- P1000, P200 Micropipettor,
- P200, P1000 Filtered Micropipettor tips
- Vortexer
- -20°C freezer
- Tube racks
- Magnetic Tube Rack
- -80°C freezer
- Micro tubes

**Materials**
- All materials are used within the manufacturer's expiry date.
- 500µl Nuclease free water
- 500µl LMVR*
- 500µl HMVR*
- 500µl each vaccine sample for testing*
- Phenol/Chloroform/Isoamyl alcohol (ratio 25:24:1 Sigma # 77617 or equivalent) or pH 7.5 Buffer saturated Phenol
- Propan-2-ol stored at -20°C
- 10% Sodium Dodecyl Sulphate (SDS)

* samples stored in at -80°C prior to testing

**Method**

2.2.1.a. Thaw 500µl of HMRV and LMVR virus reference and each test virus sample at room temperature.

2.2.1.b. Mark 3 x 1.5ml microtubes for each virus reference, sample and water, with appropriate reference numbers.

2.2.1.c. Take 450µl of each virus and nuclease free water and place in one of the appropriately marked tubes.
2.2.1.d. Add 50µl of 10% SDS to each of these tubes.

2.2.1.e. Add 500µl of phenol to each tube, cap, vortex and centrifuge at high speed for 5 minutes at room temperature. Remove the upper aqueous phase to a fresh tube.

2.2.1.f. Repeat step 2.2.1.e.

2.2.1.g. Add 500µl of chloroform to each tube, cap, vortex and centrifuge at high speed for 5 minutes at room temperature. Remove the upper aqueous phase to a fresh tube.

2.2.1.h. Add 1ml of Propan-2-ol (stored at –20°C) to each supernatant, mix thoroughly and place at -20°C overnight.

2.2.1.i. Remove the RNA/Propan-2-ol suspensions from the freezer and mix thoroughly using a vortexer.

2.2.1.j. Remove 300µl of each sample to a fresh, labelled 1.5ml microtube. Close and centrifuge for 15 minutes at approximately 14,000 rpm (20,000rcf) at +4°C.

2.2.1.k. Carefully remove and discard the supernatant. Add 300µl of 70% ethanol and wash each pellet in the ethanol, very gently.

2.2.1.l. Centrifuge at approximately 14,000 rpm (20,000rcf) at +4°C for five minutes, remove the supernatant with a micropipette, and discard.

2.2.1.m. Dry the RNA pellet.

### 2.2.2 Preparation of cDNA

There are a number of kits available which can be used to prepare cDNA. The method below describes a procedure based on the purchase of individual components and a dried RNA pellet. If the RNA has been eluted into a buffer, then vary the amount of water used to make up the mix.

**Equipment**

- Vortex mixer
- P20, P200, P1000 Micropipettor
- P20, P200, P1000 Micropipettor filtered tips
- -20°C freezer
- 1.5ml microtubes
- Tube rack
- Micro centrifuge
- Water bath set at 37°C
- Refrigerated micro centrifuge
- Vortex mixer
- 0.75ml microtubes
- Tube rack
- Heating block set at 94°C

**Materials**
Nuclease free water  
MMLV reverse transcriptase (RTase) (200 units/µl)  
5 x MMLV reverse transcriptase buffer  
10mM dNTP’s  
Random Primer (50µg/ml)  
0.1M DTT (Dithiothreitol)  
RNA preparations from:  
  LMVR- Passed virus Reference  
  HMVR- Failed virus Reference  
  Poliovirus vaccine test samples  
  Nuclease free water (cDNA control)

**Method**

2.2.2.a. Prepare sufficient cDNA mixture for the required number of reactions as set out in the table below. The number of reactions prepared will include those required for LMVR, HMVR, water plus the number of samples to be tested, with extra mix to allow for the viscosity of the mix. Add all the components, except MMLV RTase, in any order, mix thoroughly and spin in microfuge, briefly.

2.2.2.b. Label the tube appropriately and store at +4°C (or on ice) until required.

2.2.2.c. The appropriate amount of MMLV Reverse Transcriptase is added immediately before use.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>For 1 test sample</th>
<th>For 2 test samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x RTase Buffer</td>
<td>20.0 µl</td>
<td>24.0 µl</td>
</tr>
<tr>
<td>10mM dNTP</td>
<td>5.0 µl</td>
<td>6.0 µl</td>
</tr>
<tr>
<td>DTT</td>
<td>2.5 µl</td>
<td>3.0 µl</td>
</tr>
<tr>
<td>Random Primer (50µg/ml)</td>
<td>2.5 µl</td>
<td>3.0 µl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>65.0 µl</td>
<td>78.0 µl</td>
</tr>
<tr>
<td>*MMLV RTase (200 units/µl)</td>
<td>5.0 µl</td>
<td>6.0 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100.0 µl</strong></td>
<td><strong>120.0 µl</strong></td>
</tr>
</tbody>
</table>

*Add immediately before use

2.2.2.d Add 20.0µl of the cDNA mix to the appropriate RNA pellet and mix thoroughly. Dissolve the RNA completely in the cDNA mix, taking care not to cross-contaminate the samples.

2.2.2.e Incubate the samples in the water bath set at 37°C for 1 hour.

2.2.2.f Inactivate the RTase by heating in the heating block set at 94°C for approximately five minutes. (The reverse transcriptase will be inactivated at temperatures above 75°C).
2.2.2.g Briefly centrifuge each tube, and store in -20°C freezer. The cDNA may be stored for up to one year.

2.2.3. Preparation of reaction mixtures for the determination of optimal cDNA concentrations
Steps 2.2.3.a-d and 2.2.3.e-m are performed in separate rooms or areas using different equipment and materials

Equipment in Area 1 for steps 2.2.3.a-d
Vortex mixer
P20, P200, P1000 Micropipettor
P20, P200, P1000 Micropipettor filtered tips
-20°C trend monitored freezer
0.75ml microtubes
Tube rack

Materials in Area 1 for steps 2.2.3.a-d
PCR Primers:

<table>
<thead>
<tr>
<th>Type</th>
<th>pS primer</th>
<th>pA primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pS-1/445 (3µg/ml)</td>
<td>pA-1/526 (30µg/ml)</td>
</tr>
<tr>
<td>2</td>
<td>pS-2/431 (3µg/ml)</td>
<td>pA-2/483 (30µg/ml)</td>
</tr>
<tr>
<td>3</td>
<td>pS-3/470 (30µg/ml)</td>
<td>pA-3/484 (3µg/ml)</td>
</tr>
</tbody>
</table>

Nuclease free H₂O
Components for PCR Mix:
100mM dATP
100mM dGTP
100mM dCTP
100mM dTTP
H₂O
Taq DNA polymerase (5units/µl)
Or use a PCR Master mix: 2 x PCR Master Mix (eg. Reddy-mix Thermo-Fisher Scientific AB-0575/DC/LC/B or equivalent)

Method for steps 2.2.3.a-d
Prepare the PCR mix for one sample and references as follows:

From separate components:
Take: 10µl 100mM dATP,
10µl 100mM dGTP,
10µl 100mMdCTP
10µl 100mM dTTP
and place into a 1.5ml microtube with 960µl H₂O to give 1mM dNTP mix.

Make a PCR mix containing the following components:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>1 sample + references</th>
<th>2 samples + references</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 x PCR Buffer</td>
<td>168.0 µl</td>
<td>214.0 µl</td>
</tr>
</tbody>
</table>
1mM dNTP 168.0 µl 214.0 µl
Antisense primer (pA) 168.0 µl 214.0 µl
Sense primer (pS) 168.0 µl 214.0 µl
Taq DNA polymerase 5 units/µl 6.7 µl 8.6 µl
H₂O 833.3 µl 1061.4 µl
TOTAL 1512.0 µl 1926.0 µl

Where each additional sample would require:
46.00 µl 10x PCR buffer
46.00 µl 1mM dNTPs
46.00 µl antisense primer (pA)
46.00 µl sense primer (pS)
1.84 µl Taq DNA polymerase 5u/µl
228.16 µl H₂O
414.00 µl Total

Using the 2x PCR Master mix:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>1 sample + references</th>
<th>2 samples + references</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 x PCR Master mix</td>
<td>756.0 µl</td>
<td>963.0 µl</td>
</tr>
<tr>
<td>pA</td>
<td>168.0 µl</td>
<td>214.0 µl</td>
</tr>
<tr>
<td>pS</td>
<td>168.0 µl</td>
<td>214.0 µl</td>
</tr>
<tr>
<td>H₂O</td>
<td>420.0 µl</td>
<td>535.0 µl</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>1512.0 µl</strong></td>
<td><strong>1926.0 µl</strong></td>
</tr>
</tbody>
</table>

Each additional sample would require:
207.0 µl 2x PCR Master Mix
46.0 µl pA
46.0 µl pS
115.0 µl H₂O
414.0 µl Total

Keep on ice and use the mix within 2 hours.

2.2.3.a. Label 8 x 0.75ml microtubes for each test sample cDNA, HMVR cDNA and LMVR cDNA, 1-8.

2.2.3.b. To tube ‘1’ in each series add 56.25 µl* of PCR mix.
*If the limit of accuracy of the micropipettor does not allow for this measurement, pipette 56.2 µl.

2.2.3.c. Add 50.0 µl of PCR mix to all the other tubes labelled ‘2-8’.

2.2.3.d. Dispense 45 µl of PCR mix into each of 5 control tubes labelled appropriately:
100% DNA Control  For Type 1, Type 2 or Type 3
*IS DNA (A)  For Type 1, Type 2 or Type 3
*IS DNA (B)  For Type 1, Type 2 or Type 3
cDNA control  For Type 1, Type 2 or Type 3
PCR control  For Type 1, Type 2 or Type 3

**Equipment in Area 2 for steps 2.2.3.e-m**

- Vortex mixer
- P20, P200, P1000 Micropipettor
- P20, P200, P1000 Micropipettor filtered tips
- -20°C trend monitored freezer
- 0.75ml microtubes
- Tube rack
- Thermal Cycler

**Materials in Area 2 for steps 2.2.3.e-m**

- cDNA preparations (see section 2.2.2)
- PCR mix (see section 2.2.3.a)
- 100% (DNA)*
- DNA IS (A) (DNA)*
- DNA IS (B) (DNA)*  *see below
- Mineral oil

2.2.3.e. Vortex the vaccine test sample, LMVR, HMVR and control cDNA preparations and then microfuge them briefly.

2.2.3.f. For each cDNA preparation:

   Add 6.25μl of the cDNA to tube number ‘1’. Discard the tip. Mix using a fresh tip and transfer 12.5μl to tube ‘2’. Discard the tip. Mix, then transfer 12.5μl to tube ‘3’, proceed in this manner until the last tube. After mixing, discard 12.5μl.

2.2.3.g. Add 5.0μl of cDNA control to the cDNA control tube.

2.2.3.h. Add 5.0μl of H₂O to the PCR control tube.

2.2.3.i. Add 5μl of each reference samples to the appropriately labelled PCR reaction tube.*

2.2.3.j. Add 50μl of mineral oil to all the tubes, including controls: 100%DNA control, IS DNA controls A and B, cDNA control and PCR control.

   *this is not necessary if the thermal cycler has a heated lid.

**To prepare International Standard (IS) DNA A and B and 100% standard DNA.**

Resuspend freeze dried International Standard (IS) DNA, (A and B) control in 100μl H₂O. Transfer the solution into appropriately marked 1.5ml microtubes.

Resuspend freeze dried reference DNA sample, 100% control in 100μl H₂O and transfer the solution into a marked 1.5ml microtube.
Reconstituted reference material is stored at -20°C after use, until required for the second PCR. This material may be stored for up to one year.

2.2.3.1. Incubate the samples as follows:-

- 94°C for 10 minutes
- 94°C for 30 seconds
- 55°C for 15 seconds
- 65°C for 3 minutes

40 cycles

* or other suitable cycling programme

Temperatures and times may be varied depending on the thermal cycler used and are as indicated on the cycler.

2.2.3.m. After incubation is complete, the samples are stored in the -20°C freezer for up to one year.

2.2.4 MAPREC labelling primer preparation

The primer that was used at the lowest concentration for the PCR, should be used for labelling. Either use a commercially prepared labelled primer or add a radiolabel using polynucleotide kinase.

**Equipment**

P10, P20, P200 Micropipetor and filtered tips
-20°C freezer
0.75ml microtubes
Tube racks

**Materials** [All materials are used within the manufacturers’ expiry date.]

<table>
<thead>
<tr>
<th>Type</th>
<th>Primers for radiolabelling</th>
<th>Fluorescent primer</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pS-1/445</td>
<td>pS-1/445</td>
<td>used at 3µg/ml</td>
</tr>
<tr>
<td>2</td>
<td>pS-2/431</td>
<td>pS-2/431</td>
<td>used at 3µg/ml</td>
</tr>
<tr>
<td>3</td>
<td>pA-3/484</td>
<td>pA-3/484</td>
<td>used at 3µg/ml</td>
</tr>
</tbody>
</table>

Nuclease free water
Primer (3 µg/ml)
Gamma [³²P] ATP, 10 µCi/µl
10x PNK buffer
T4 PNK, 1 u/µl

For radiolabelling

**Method**

2.2.4.a. Making the radiolabelled primer:

Add the following components together in a 0.75 ml microtube:-
2.2.4.b. For the fluorescently labelled primers:
Add the following components together in a 0.75 ml microtube:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labelled primer (3 µg/ml)</td>
<td>17.6 µl</td>
</tr>
<tr>
<td>gamma [³²P] ATP, 10 µCi/µl</td>
<td>4.0 µl</td>
</tr>
<tr>
<td>10x PNK buffer</td>
<td>2.7 µl</td>
</tr>
<tr>
<td>T4 PNK, 1 u/µl</td>
<td>2.7 µl</td>
</tr>
<tr>
<td>Total</td>
<td>27.00 µl</td>
</tr>
</tbody>
</table>

Incubate at 37°C for 30 minutes.
Then add:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10mM dNTP Mix</td>
<td>50.5 µl</td>
</tr>
<tr>
<td>Taq DNA Polymerase</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>Total</td>
<td>80.0 µl</td>
</tr>
</tbody>
</table>

2.2.4.c. Place the mix on ice and use within 5 hours. This is the labelled primer mix.

2.2.5 Labelling of PCR bands of cDNA optimisation

**Equipment**
- P10, P200 Micropipettor Pipettes
- P10, P200 Micropipettor filtered tips
- Heating block
- Microfuge
- High voltage power pack
- Electrophoresis tank
- 0.75ml microtubes
- Tube racks

**Materials**
- PCR products from the dilutions of cDNA for each virus sample as described in section 3.
- PCR products from the standard references and PCR + cDNA control samples prepared as described in section 3.
- Labelled primer mix, prepared as described in section 4.
- Loading buffer: 10x Bromophenol blue or equivalent (eg 10x Orange Gel Loading dye (Licor # 927-10100))
- 10% polyacrylamide gel(s), prepared as described in Annex 1
- 0.75ml microtubes
- Gel loading tips
- Blue/Orange Loading dye for gel tracking (Promega # G1881)

**Method**
2.2.5.a Label 0.75ml microtubes appropriately and add 5µl of each PCR product to the tube.

2.2.5.b Add 1µl of labelled primer mix to each PCR product, microfuge briefly and incubate at 72°C (as indicated on the machine) for 10 minutes.

2.2.5.c Microfuge all the tubes, then add 2µl of loading dye mix to each tube. Microfuge all the tubes briefly.

2.2.5.d The samples are loaded onto a 10% polyacrylamide gel, leaving an empty well between each sample set, and between each standard and control. (See Figure 3.)

2.2.5.e Run the gel at a constant 40 watts until the orange dye front has travelled at least 6 cm.

2.2.5.f Remove the gel from the electrophoresis tank. Rinse the glass plates briefly in water to remove buffer. Visualise the bands using a suitable detection system.

Figure 3. Example of a calibration gel (Type 3)

<table>
<thead>
<tr>
<th></th>
<th>LMVR</th>
<th>HMVR</th>
<th>Vaccine</th>
<th>cDNA control</th>
<th>PCR control</th>
<th>IS DNA (A)</th>
<th>IS DNA (B)</th>
<th>100% DNA control</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR Product</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Excess Primer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.2.6 **Assessment of Optimisation**

I. There should be no primer dimers.

II. There should be PCR product in the 1 in 125 dilution of each cDNA titration for the test sample, LMVR and HMVR.
   ie. There should be PCR products in the first 4 wells of each dilution set.

III. There should be PCR product in the DNA controls IS A, IS B and the 100% DNA.

IV. The cDNA and PCR controls should be negative

2.2.7 **Quantification of base mutation**
**Equipment**

P10, P200 Micropipettor Pipettes
P10, P200 Micropipettor filtered tips
Heating block
Microfuge
High voltage power pack
Electrophoresis tank
0.75ml microtubes
Tube racks

**Materials**

PCR products from the lowest appropriate dilutions of cDNA for each virus sample as described in section 2.

PCR products from the standard references and PCR + cDNA control samples prepared as described in section 2

Labelled primer mix, prepared as described in section 4

Restriction enzyme:

<table>
<thead>
<tr>
<th>Polio Type</th>
<th>Type 1</th>
<th>Type 2</th>
<th>Type 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Restriction enzyme (1u/µl)</td>
<td>Ddel and Nci I</td>
<td>Bsp1286</td>
<td>Mbo I</td>
</tr>
</tbody>
</table>

*Note: restriction enzymes that were isolated from different microorganisms, but have the same substrate specificity (isoschizomers) can also be used after appropriate validation as a replacement for the above enzymes.*

Proteinase K (10mg/ml)

10x Loading dye (eg Bromophenol Blue or Orange Gel Loading dye Licor # 927-10100)

10% polyacrylamide gel, prepared as described in Annex 1

0.75ml microtubes

Blue/Orange Loading dye mix (Promega G1881) – optional tracking dye *

**Method**

2.2.7.a. Label 8 x 0.75ml microtubes appropriately and add 10µl of PCR product to the tube.

2.2.7.b. Add 2µl of labelled primer mix to each PCR product, microfuge briefly and incubate at 72°C (as indicated on the machine) for 10 minutes.

2.2.7.c. Microfuge tubes. Label two sets of 0.75µl tubes for each labelled product and put 6µl of the labelled product into each tube.

2.2.7.d. Add 1µl of restriction enzyme to one tube of each pair (For type 1 add 1µl of both restriction enzymes). Cap all tubes and microfuge briefly.

2.2.7.e. Incubate all tubes at 37°C (as indicated on the machine) on the heating block for 1 hour.

*In some instances, the enzymes will remain bound to the PCR product. To remove these enzymes it may necessary to digest the enzyme/PCR mix with Proteinase K: Microfuge all tubes. Add 1µl of Proteinase K (10mg/ml) to each tube and incubate at 55°C for 30 minutes.*

2.2.7.f. Microfuge all the tubes, then add 2µl of loading dye mix to each tube. Microfuge all the tubes briefly.
2.2.7.g. The samples are loaded onto a 10% polyacrylamide gel, leaving an empty well between each sample, and with digested and undigested samples adjacent to each other. See Annex 1 for an example of a gel loading scheme.

2.2.7.h. The gels are run at a constant 40 watts, in 1 x TBE buffer, until the orange dye front has migrated about 15-16 cm (approximately 40 minutes to an hour).

2.2.7.i. The gel plates removed from the electrophoresis tank and rinsed briefly in tap water, then patted dry. The gels are identified by the blue/orange tracking dye and each gel is analysed using a suitable detection system.

2.2.7.j. All waste gel and electrophoresis buffer is discarded according to local regulations. Quantify the bands using the instructions provided by the manufacturer of the detector.

---

**Figure 4. Appearance of digested PCR Fragments (Type 2 and Type 3)**

<table>
<thead>
<tr>
<th></th>
<th>LMVR</th>
<th>HMVR</th>
<th>Sample</th>
<th>cDNA Ctl</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
</tbody>
</table>

- Upper Full length PCR Product (U)
- Lower Digested Band (L)
- Primer
Calculation of % base content.

Two lanes, one with enzyme–digested DNA (D) and the other with undigested control (C), are analyzed for each sample. In each of these lanes, areas containing both the full–length (upper) DNA band (DU and CU) and the restriction fragment (lower) band (DL and CL) are quantified. (see Figures 4 and 5).

The fraction of radioactivity/fluorescence in the restriction fragment compared to the total radioactivity/fluorescence in the specific DNA is calculated for each lane:

\[
\begin{align*}
\text{FD} &= \frac{(DL1+DL2)}{(DU+DL1+DL2)} \quad \text{or} \quad \frac{DL}{(DU+DL)} \\
\text{FC} &= \frac{(CL1+CL2)}{(CU+CL1+CL2)} \quad \text{or} \quad \frac{CL}{(CU+CL)}
\end{align*}
\]

FD represents the fraction of DNA molecules with reversion plus some nonspecific background. FC represents nonspecific background caused by uniform smearing of radioactive/fluorescent materials along the electrophoresis track, as well as artifactual DNA products that may have formed. Therefore:

\[
\% \text{ Revertants} = \frac{\text{FD} - \text{FC}}{(DU+DL)/(DU+DL1+DL2) - (CU+CL1+CL2)/(CU+CL+CL2)} \times 100
\]

It is recommended that computer worksheets be used (e.g. Microsoft Excel or Lotus 1–2–3) to perform these calculations, as well as further statistical treatment, validation of the test, and for making pass/fail decisions.

Validation criteria for each individual determination:

I. No contamination in water blank or mock cDNA controls should be detected, see Overview 1.6.1
II. There should be no primer dimer, but see Overview 1.6.1.

III. Digestion of the 100% DNA sample should be within previously established limits. eg 90% or above, see Overview 1.6.2.

IV. The % of mutant content in International Standard (IS) DNA is consistent with previous tests. See Overview 1.6.4.

A complete MAPREC test consists of 5 quantitative determinations following the steps outlined in sections 2.2.4 to 2.2.8.

The 5 determinations should be made using at least 2 separate PCR amplifications. Additional PCR reactions are performed as described in section 2.2.8 below. Once the five determinations have been completed the Mean and Standard Deviations for each vaccine is calculated.
2.2.8 Additional PCR reactions for the quantification of the base mutation.

**Equipment**
- P10, P20, P200, P1000 Micropipettor
- P10, P20, P200, P1000 Micropipettor filtered tips
- 1.5ml and 0.75ml microtubes
- -20°C freezer
- Microfuge
- Thermal cycler

**Materials**
All materials are used within the manufacturer’s expiry dates:
- 2 x PCR Master mix (eg. Reddy-Mix Thermo-Fisher Scientific AB-0575/DC/LC/B)
- PCR primers:
  - **Type**
  - **pS primer**
  - **pA primer**
  - 1  
    - pS-1/445 (3µg/ml)
    - pA-1/526 (30µg/ml)
  - 2  
    - pS-2/431 (3µg/ml)
    - pA-2/483 (30µg/ml)
  - 3  
    - pS-3/470 (30µg/ml)
    - pA-3/484 (3µg/ml)

  - Nuclease free water
  - 100% DNA control
  - IS-DNA A
  - IS-DNA B

  cDNA’s from section 2.2.2:
  - Control
  - Test sample
  - LMVR
  - HMVR
  - H₂O
  - Mineral Oil

**Method**
2.2.8.a Prepare the PCR mix as follows: For 3 cDNA’s and 5 controls:
- 2x PCR Master mix 225.00µl
- pA (30µg/ml for type 1 and 2, 3µg/ml for type 3) 45.00µl
- pS (3µg/ml for type 1 and 2, 30µg/ml for type 3) 45.00µl
- H₂O 90.00µl
- 405.00µl

2.2.8.b Transfer 45µl of PCR mix into each of 8 tubes labelled as follows:- cDNA control, LMVR, HMVR, test sample, IS DNA- A, IS DNA- B, PCR control.

2.2.8.c Thaw all reagents and microfuge briefly.

2.2.8.d Add 5µl of DNA/cDNA to each appropriately labelled tube. Add 5µl of water to the PCR control. Add 50µl of mineral oil to each tube. Close all tubes and microfuge briefly.

2.2.8.e Place all the samples in the PCR thermal cycler and incubate as follows*.

- 94°C for 10 minutes
- 94°C for 30 seconds
- 55°C for 15 seconds
- 65°C for 3 minutes

  40 cycles

Temperature and times are as indicated on the machine
* or other suitable cycling programme

2.2.8.f. After incubation is complete, store the PCR products at -20°C for up to 8 weeks.

**Validation criteria for a set of five individual determinations:**

I. The ratio of the IS DNA duplicates should not be significantly different from 1.0.

II. The mean ratio of the IS DNA, for the five determinations should be consistent with previous results, see Overview 1.6.4.

III. The LMVR should have a mutant content less than the concurrently tested IS.

IV. The HMVR should have a mutant content higher than the concurrently tested IS.
Annex 1. Preparation of 10% Polyacrylamide gel

Gel thickness and well spacing will affect the running conditions and migration of the PCR fragments. If alternative gel systems are used the parameters described below may not be applicable.

**Equipment**
A suitable gel electrophoresis system (eg Hoefer™ SE 600 Chroma System using 0.75mm spacers and combs)
0.75mm x 0.5mm x 15 wells for the quantitation gel
Or 0.75mm x 0.4mm x 20 wells for the cDNA optimisation gel
Prepare the 10% polyacrylamide gel according to the manufacturer’s instructions.
100ml measuring cylinder
P200 Micropipettor
P200 Micropipettor tips
Gloves
50ml syringe

**Materials**
Ethanol
Siliconising solution
10 x TBE Buffer [eg Sigma]
40% Stock solution of 19:1 Acrylamide: Bis-acrylamide [eg Sigma]
10% Ammonium persulphate [APS]
TEMED [eg Sigma]

**Method**
Wear gloves throughout.

a. Wash the glass plates thoroughly in hot water, and dry.

b. Apply 2ml of siliconising solution to each plate and spread over the surface of the plate with a tissue, until the solution has dried. Remove excess siliconising solution by applying ethanol to each plate from a wash bottle and polishing the plate with a tissue (prevents gel sticking to glass surface).

c. Place the plastic spacers along each short side of the plain glass plate and cover with eared plate, siliconised sides facing inwards. Remove gloves and tape both sides and the bottom of the gel together, with electrophoresis tape, or clamp.

d. Put on gloves and pour 25 ml of 40% acrylamide into a 100 ml measuring cylinder and add 75 ml of 1 x TBE. Add 1.2 ml of 10% APS to the solution.

e. Pour 50 ml of the solution into a 50 ml syringe and add 50 µl of TEMED. Mix thoroughly and pour between the glass plates immediately.

f. Place the comb into the top of the gel, centrally, and clamp the top of the plate with bulldog clips if required. Leave the gel to set, approximately 10 minutes.

g. Pour 1 x TBE buffer into the bottom buffer chamber of the electrophoresis tank. Remove the electrophoresis tape from the bottom of the gel plates.
h. Place the gel/plates into the electrophoresis tank, eared plates facing the perspex. Remove the bulldog clips and reuse to clip the plates to the tank along the side spacers.

i. Pour 1 x TBE buffer into the top buffer chamber of the electrophoresis tank, and remove the comb. Rinse out the wells in the gel with buffer using a liquipette.

j. The gels can be kept for up to 1 week in buffer. Any remaining solution should be stored at +4°C for up to one week.

**Gel Loading Schemes**

**Diagram of Loading for a Calibration Gel**

![Diagram of Loading for a Calibration Gel]

**Quantification Gels:**

Load the samples onto the gel(s) in the following order:

1. LMVR control  
2. LMVR virus control  
3. HMVR virus control  
4. Test sample  
5. cDNA control  
6. IS DNA control A  
7. IS DNA control B  
8. PCR control  
9. 100% DNA control

Digested

undigested

Digested

undigested

Digested

undigested

Digested

undigested

Digested

Undigested
Diagram of a Gel Loading Scheme for Single Sample – which may use one or two gels*

*Tracking dye - If more than one gel is used, identify the gels by loading:
one extra lane of blue/orange loading dye mix for gel 1, and
two extra lanes of blue/orange loading dye mix for gel 2.
Annex 2. PCR fragments generated during MAPREC assay

Type 1 MAPREC primers

pS1- 445  5’ CTC CGG CCC CTG AAT GCG GCT AAT CCa AAC CTC tG  3’ (35 bp)
pA1- 526  5’ AAC ACG GAC ACC CAA AGT AGT CGG TTC CGC TeC GG  3’ (35 bp)

Type 1 MAPREC fragments

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Fragments generated</th>
<th>Base Pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dde I</td>
<td>*CTCCGGCCCCCTGAATGCGGCTAATCCCAACCTCTCTAGCAGGTGGTCGTCACAAACC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C’TNAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CTCCGGCCCCCTGAATGCGGCTAATCCCAACCTCTCTCTAGCAGGTGGTCGTCACAAACC</td>
<td></td>
</tr>
<tr>
<td>Nci I</td>
<td>AGTGAATTGGCCTGTGCAGCGACAGCGGAACCAGCATCTTTTGAGGTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CC’CGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AGTGAATTGGCCTGTGCAGCGACAGCGGAACCAGCATCTTTTGAGGTG</td>
<td></td>
</tr>
</tbody>
</table>

Fragments generated: Base Pairs

Full length = 115
pS + Nci I = 82
pS + Dde I = 33

Type 2 MAPREC primers

pS-2/431  5’ GCT ACA TAA GAG TCC TCC GGC CCC TGA ATG CGe CT  3’
pA-2/483  5’ CGC GTT ACG ACA AGC CAG TCA CTG GTT CGC GAC CaC Gt  3’

Type 2 MAPREC Fragments

*GCTACATAAG AGTCTCTCGG CCCCTGAATG CGCCTAATCC TAACCACGGA
GCTACATAAG AGTCTCTCGG CCCCTGAATG CGCCTAATCC TAACCACGGA
Bsp 1286I
GD GCH’C
GCA GGCGGTC GCGAACAGT GACTGGCTTG TCGTAACCG
ACA CGTGTC GCGAACAGT GACTGGCTTG TCGTAACCG

Fragments generated: Base Pairs
Full length = 90

pS + Bsp 1286I = 53

**Type 3 MAPREC primers**

pS-3/470  5' TGA GCT ACA TGA GAG TgC TCC GGC CCC TGA ATG CGG CTG A 3'

pA-3/484  5' CAG GCT GGC TGC TGG GTT GCA GCT GCC TGC 3'

**Type 3 MAPREC Fragments**

MboI

GA TC

TGAGCTACAT GAGAGTgCTC_ CGGCCCCTGA ATGCGGCTgA TcCTAACCAT

TGAGCTACAT GAGAGTcCTC_ CGGCCCCTGA ATGCGGCTAATTCTAACCAT

GGAGCAGGCA_ GCTGCAACCC AGCAGCCAGC CTG *

GGAGCAGGCA_ GCTGCAACCC AGCAGCCAGC CTG

**Fragments generated:**

<table>
<thead>
<tr>
<th></th>
<th>Base Pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full length</td>
<td>83</td>
</tr>
<tr>
<td>pA + Mbo I</td>
<td>43</td>
</tr>
</tbody>
</table>
**Authors**

The first version of the protocol for MAPREC was written by Dr Konstantin Chumakov, Centre for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland, USA and Dr David Wood, National Institute of Biological Standards and Control, South Mimms, UK. The subsequent versions were prepared by Ms Glynis Dunn, National Institute of Biological Standards and Control, South Mimms, UK; Mr Alan Heath, National Institute of Biological Standards and Control, South Mimms, UK and Dr Konstantin Chumakov, Centre for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland, USA, following WHO public consultation during Nov 2011-Feb 2012, with input from Dr Laetitia Agostini Bigger for coordinating compiled comments from IFPMA Vaccines Committee IFPMA, Geneva, Switzerland, Dr Iin Susanti Budiharto, Bio Farma, Bandung, Indonesia; Dr Morag Ferguson, Horning, UK; Ms Jackie O’Brien, National Institute of Biological Standards and Control, South Mimms, UK; Dr Lucia Fiore Istituto Superiore di Sanità, Roma, Italy; Dr Anna Laura Salvati, Istituto Superiore di Sanità, Roma, Italy and Dr TieQun Zhou, Quality, Safety and Standards (QSS)/Immunization, Vaccines and Biologicals (IVB)/Family, Women's and Children's Health (FWC), World Health Organization (WHO), Geneva, Switzerland.

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