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# MAPREC assay for quantitation of mutants in a recombinant flavivirus vaccine strain using near-infrared fluorescent dyes

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A B S T R A C T

Mutant analysis by PCR and restriction enzyme cleavage (MAPREC) is a quantitative assay of revertants in batches of live viral vaccines. The assay is highly sensitive and reliable but requires radioactive isotopes, which complicates its use in quality control laboratories. To quantify mutants in the cDNA of the West Nile (WN)/Dengue 4 chimera that was proposed as a new candidate of live vaccine against West Nile disease, alternative MAPREC protocols using non-radioactive dyes were explored. To compare the utility of different fluorescent dyes for MAPREC, the $G_{2337} \rightarrow C$ mutation that was revealed by microarray hybridization in WN/Dengue 4 chimera virus was used as a model. DNA fragments produced by restriction endonuclease digestion were visualized in polyacrylamide gels by visible-range fluorescent dyes including ethidium bromide (EtBr) and SYBR Green I as well as by near-infrared (NIR) dye SYTO 60 and NIR dyes 700 and 800. The MAPREC assay performed with SYTO 60 and SYBR Green I was more sensitive than with EtBr but less sensitive than with NIR dyes 700 or 800. The NIR dyes 700 and 800 exhibited a wide linear range that may enable the detection of 0.05% of mutants in viral stocks. The NIR-based MAPREC assay was validated by using World Health Organization (WHO) international references for poliovirus type 3 with known contents of mutants. Values of mutant content produced by the non-radioactive assay were similar to the values determined in a previous WHO international collaborative study. The modified MAPREC assay could be used as an alternative to the radioisotope-based standard protocol for quality control of live viral vaccines.

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## 1. Introduction

Populations of RNA viruses consist of large collections of genetic variants that differ from the consensus sequence at one or more sites; they are thus referred to as quasispecies (Domingo et al., 1985; Holland et al., 1982). This genetic diversity results from the low fidelity of RNA replicases and the selectively neutral nature of most mutations (Drake and Holland, 1999; Holland et al., 1982). The presence of the diverse spectrum of mutants in viral stocks helps viruses to adapt rapidly to new growth conditions, both *in vitro* and *in vivo* (Domingo et al., 1998; Eigen, 1993; Holmes and Moya, 2002; Ruiz-Jarabo et al., 2000). In addition, the presence of relative abundance of mutant virus variants in live viral vaccines can affect vaccine safety, potency, and immunogenicity (Duarte et al., 1994). For example, oral poliovirus vaccine (OPV) contains a very small amount of viral particles with higher neurovirulence. The quantitation of neurovirulent revertants in OPV was performed by mutant analysis by PCR and restriction enzyme cleavage (MAPREC) showed that the content of these revertants in vaccine batches that failed the monkey neurovirulence test was higher than in batches that passed the test (Chumakov et al., 1991). Therefore, MAPREC was approved by the WHO as a routine *in vitro* test to monitor the consistency of OPV production and is now used by vaccine manufacturers and national control authorities for lot release testing.

The presence of a small fraction of mutant virus was also demonstrated for other live viral vaccines (Amexis et al., 2001), suggesting that genetic consistency must be monitored to ensure that the accumulation of mutants does not adversely affect the safety and efficacy of the vaccine (Chumakov, 1999).

The monitoring of genetic stability is particularly important for new vaccines derived by targeted genetic manipulations and cloning. The attenuated WN/Dengue 4 chimera is a candidate vaccine against WN virus (Pletnev et al., 2002). In a recent study, this chimera was propagated in different cell lines, and its RNA genomes were screened for mutations by microarray hybridization and conventional sequencing methods. It was shown that several mutations accumulate in the passaged chimeric virus (Laassri et al., 2011). To study the importance of these mutations, the kinetics of their accumulation in each passage need to be determined. This task could be accomplished by the use of the MAPREC assay.

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The traditional MAPREC assay is sensitive, precise, and accurate but requires the use of radioactively labeled materials that complicates its practical use in quality control laboratories. Several attempts were made in the past to develop an alternative protocol using fluorescent dyes (Horie et al., 1998; Ivancic-Jelecki et al., 2006). However, these protocols did not reproduce the sensitivity and accuracy of the conventional assay, primarily because of the insufficient dynamic range of fluorescent signal. There are a large number of fluorescent dyes with different properties; however, their utility for MAPREC was not evaluated. Recently, a new generation of dyes that fluoresce in the near-infrared (NIR) spectral region (700–900 nm) was introduced. Their dynamic range of linear response is wider; therefore, these dyes could improve the performance of the MAPREC assay (Dr. Glynis Dunn, NIBSC, personal communication). In this study a number of dyes emitting in the visible and near-infrared range of the light spectrum were compared to assess their performance in the MAPREC assay for quantitation of the mutants in WN/Dengue 4 chimera strain, which was proposed as a candidate vaccine against West Nile disease.

## 2. Materials and methods

### 2.1. Viruses and DNAs

To compare the fluorescent dyes and their use for the MAPREC assay, plasmid containing the complete genome of the WN/Dengue 4 chimera virus with the $G_{2337} \rightarrow C$ mutation was generated. The prototypes of the WN/Dengue 4 chimeric virus and its plasmid were created and kindly provided to us by Dr. Alexander Pletnev from the National Institute of Health (Pletnev et al., 1992, 2002).

For the validation of the MAPREC assay based on NIR Dye 700, WHO international references for type 3 OPV were used as controls. The references included two synthetic DNA preparations containing 0.9% (95/542) and 100% (94/790) of $U_{472} \rightarrow C$ mutants and two viral references (96/572 and 96/578) representing oral polio vaccine lots that passed and failed the MAPREC test, respectively. The references were obtained from the National Institute for Biological Standards and Control (NIBSC) in the United Kingdom.

### 2.2. Viral RNA extraction and cDNA preparation

Viral RNA was isolated from 140 μl of virus-containing medium using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The RNA was eluted to a final volume of 60 μl in sterile, RNase-free water.

The cDNA were prepared with Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) using the manufacturer's protocol with specific reverse primers that were selected for the analysis of mutations of interest (Table 1).

### 2.3. MAPREC assay based on DNA-intercalating dyes

The region containing the $G_{2337} \rightarrow C$ mutation in the genome of WN/Dengue 4 was PCR-amplified with specific primers (Table 1). Briefly, the plasmid DNA or viral cDNA was used as a template in a total reaction volume of 50 μl containing 0.2 μM of each primer, 50 μM of each dNTP, 3 mM $MgCl_2$, and 1 U HotStarTaq DNA Polymerase (Qiagen, Valencia, CA). PCR was carried out under the following conditions: 95 °C for 15 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, and a final elongation at 72 °C for 7 min. Next, 5 μl of PCR product was digested with the appropriate restriction endonuclease (New England BioLabs, Ipswich, MA) and separated by electrophoresis in 4% agarose gels with ethidium bromide (Lonza, Rockland, ME) or in 10% native polyacrylamide gels (Invitrogen, Carlsbad, CA) and stained with SYBR Green I (Invitrogen, Carlsbad, CA) according the manufacturer's instructions. Nucleic acids in stained gels were quantified using Kodak Gel Logic 200 Imaging System and Kodak Molecular Imaging Software (Carestream Health, Inc. Rochester, NY). In the case of infrared DNA-intercalating dye SYTO 60, 10% native polyacrylamide gels were stained with 1 μM of SYTO 60 (Invitrogen, Carlsbad, CA) for 10 min at room temperature. The capture of gel images and the quantitation of stained DNA bands were carried out using Odyssey Infrared Imaging System and the Odyssey v3.0 software (LI-COR Biosciences, Lincoln, NE). Quantitation of mutants was based on the analysis of digested and non-digested DNA samples. The plasmid that did not contain mutations was used as a control. In experiments where DNA-intercalating dyes were used, the calculation of mutant content was performed by considering the intensities of both digested bands (Fig. 1A).

### 2.4. MAPREC assay using primers labeled with covalently linked infrared dyes

The region containing a mutation that was the subject of quantitative analysis was amplified using specific primers listed in Table 1. PCR was performed as described in the original MAPREC procedure (Chumakov et al., 1991) with a ten-fold excess of the reverse primer to generate single-stranded DNA (ssDNA) of negative polarity, followed by a one-step primer extension reaction with a forward primer labeled with NIR Dye 700 or 800 (Integrated DNA Technologies, Coralville, IA). The use of asymmetric PCR to generate ssDNA followed by one-cycle primer extension helped to maximize incorporation of the labeled primer. The infrared-labeled double-stranded DNA (dsDNA) product was digested and DNA fragments were separated by polyacrylamide gel electrophoresis (PAGE) in native 10% gels. Detection and quantitation of DNA was performed using the Odyssey Imaging System at 700 nm for NIR Dye 700 and 800 nm for NIR Dye 800. Quantitation was done using the formula described in Fig. 1B.

## 3. Results

### 3.1. Sensitivity of fluorescent MAPREC assay based on intercalating dyes

To evaluate the sensitivity of the MAPREC assay using SYBR Green I, EtBr, and SYTO 60, DNA samples were prepared by spiking the PCR product amplified from plasmid containing the genome of non-mutant WN/Dengue 4 recombinant with PCR product from the plasmid containing WN/Dengue 4 genome with the $G_{2337} \rightarrow C$ mutation. These samples were then digested with SfaI endonuclease and analyzed as described above. The results obtained in experiments with SYBR Green I and SYTO 60 showed that the assay was able to detect 0.5% and 0.1% of mutants, respectively (Fig. 2B and C). However, the EtBr-based assay could detect only 1% of mutants (Fig. 2A).

### 3.2. Evaluation of the linearity range for NIR Dyes 700 and 800

Because the use of the NIR Dyes 700 and 800 in Western blot showed a wide linear range, high sensitivity, and low background (see http://www.licor.com/bio/applications/odyssey_applications/quantitative_western_blot.jsp), the sensitivity and linear range in MAPREC were evaluated using PCR products labeled with these dyes. PCR products obtained by amplification of a specific segment of the WN/Dengue 4 genome using one of the primers labeled with NIR Dyes 700 or 800 were generated. Next, two-fold serial dilutions of this DNA were prepared and separated in 10% PAGE. The quantity of DNA in each lane ranged from 235 to 0.115 ng. The infrared images were captured, and the intensities of each band