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Proposed WHO International Reference Reagents for whole genome high-throughput sequencing of oral poliomyelitis vaccines

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NOTE:

This document has been prepared for the purpose of inviting comments and suggestions on the proposal(s) contained therein. Written comments on the proposal(s) MUST be received in English by **23 March 2026** and should be addressed to:

Product Standards, Specifications and Nomenclature
Department of Medicines and Health Products Policies and Standards
World Health Organization
1211 Geneva 27
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Comments may also be submitted electronically to **Dr Ivana Knezevic** at email: knezevici@who.int.

The distribution of this document is intended to provide information to a broad audience of potential stakeholders and to improve the transparency of the consultation process. Following consideration of all comments received, the proposal(s) will then be considered by the WHO Expert Committee on Biological Standardization (ECBS) prior to a final decision being made and published in the WHO Technical Report Series.

Summary

This project describes the development and evaluation of two whole-genome high-throughput sequencing (WG-HTS) candidate panels for Type 1 and Type 3 Oral Poliovirus Vaccine (OPV) to support whole-genome analysis and the investigation of MAPREC-relevant mutations using HTS-based assays. The study demonstrates that HTS is a highly sensitive and robust tool capable of distinguishing vaccine strains based on seed virus, manufacturing conditions, and cell substrates. Analysis of historical and prospective data shows that OPV manufacturers consistently maintain the genetic integrity of the poliovirus genome, particularly Single nucleotide polymorphism (SNP) profiles, over extended production periods. Comparative whole-genome SNP profiling of consecutive OPV batches from multiple manufacturers revealed manufacturer- and seed-dependent differences while maintaining strong intra-manufacturer and intra-product consistency.

The findings support the use of WG-HTS as a powerful approach for monitoring manufacturing consistency, identifying potential outliers, and providing early warning signals for process changes. While molecular variations do not necessarily indicate unacceptable product quality, deviations beyond established historical profiles warrant further investigation. The proposed path forward emphasizes building a comprehensive historical SNP database from released vaccine lots and developing validated algorithmic criteria to support objective, data-driven lot release decisions.

Based on the outcomes of this collaborative study, specific Type 1 (23/160 & 23/162) and Type 3 (23/172 & 23/174) candidates are recommended for establishment as WHO Reference Reagents to support HTS-based MAPREC assays and to validate and implement HTS for whole-genome SNP profiling of OPV vaccines. Across the candidate reference reagents, estimated SNP frequencies span 0.3% to 75.5% at multiple sites distributed throughout the genome, supporting their suitability for a range of intended assays. Collectively, these recommendations lay the foundation for incorporating WG-HTS into routine OPV lot testing, with the potential to reduce reliance on neurovirulence testing while ensuring continued product safety, quality, and consistency.

Introduction

The manufacture of live-attenuated Sabin oral poliovirus vaccines (OPV), including novel OPV strains engineered to enhance genetic stability, necessitates rigorous monitoring to ensure preservation of the attenuation phenotype and, consequently, vaccine safety. This requirement similarly applies to virus seeds used to produce inactivated poliovirus vaccines derived from Sabin strains (Sabin-IPV), where maintenance of viral genetic stability is essential to guarantee manufacturing consistency and to prevent the emergence, during production, of unfavourable mutations that may alter key biological properties such as neurovirulence, antigenicity, or immunogenicity. In accordance with these principles, the World Health Organization (WHO) recommends that the viral growth conditions employed in the production of OPV or Sabin-IPV be designed to minimize significant genetic changes that could increase viral virulence during serial propagation in the manufacturing process (1).

Since the introduction of OPV for human use, animal-based assays have been employed to evaluate viral neurovirulence (2,3,4). The monkey neurovirulence test (MNVT) remains the established gold standard for assessing the safety of OPV, while the transgenic mouse

neurovirulence test (TgmNVT), utilizing mice expressing the human poliovirus receptor, serves as a complementary/alternate assay at various stages of vaccine production (5).

Reliance on animal models—specifically nonhuman primates and transgenic mice—for neurovirulence assessment has consistently prompted the scientific community to pursue the development of alternative, quantitative methods capable of distinguishing between acceptable and non-compliant vaccine lots. In addition, the development of alternative methods is strongly encouraged in alignment with the principles of the 3Rs (Replacement, Reduction, and Refinement) governing animal research. Beyond the ethical and regulatory considerations, the existing neurovirulence assays are characterized by high cost, significant variability of results, prolonged testing timelines, and the requirement for specialized technical expertise, particularly in the precise inoculation of virus into the central nervous system and the semi-quantitative interpretation of pathological and clinical outcomes.

It is well established that the OPV is unique among human vaccines in possessing well-characterized genetic markers associated with attenuation. As part of the batch release and quality control process, production lots of live-attenuated OPV are evaluated using an *in-vitro* molecular assay—Mutant Analysis by PCR and Restriction Enzyme Cleavage (MAPREC)—to assess molecular consistency through quantification of nucleotide changes responsible for the virus reversion to virulence. This assay determines the proportion of mutants at critical nucleotide positions within the 5′ non-translated region (5′NTR), where specific point mutations are correlated with increased neurovirulence. It has been recommended by the WHO Expert Committee on Biological Standardization (ECBS) as the *in-vitro* assay of preference for lot release of OPV (1,2,3).

In the Sabin type 3 OPV component, reversion at nucleotide position 472, from the attenuated uridine (U) to the wild-type cytidine (C), is of particular concern, as elevated reversion frequencies at this site are strongly associated with enhanced neurovirulence and render vaccine lots unsuitable for use (6,7). In the type 1 OPV component, MAPREC quantifies reversion events from guanosine (G) to adenosine (A) at nucleotide 480 and from uridine (U) to cytidine (C) at nucleotide 525. For the type 2 component, reversion from adenosine (A) to guanosine (G) at nucleotide 481 is monitored. MAPREC serves as a molecular consistency test to ensure the stability of the attenuating nucleotides (8). Reversion at attenuating mutations in OPV measured by MAPREC strengthens base-pairing within domain V of the 5′NTR (IRES), restoring a more wild-type–like RNA structure and altering IRES-mediated translation.

However, the MAPREC assay is limited in its scope, as it cannot detect viral variants harbouring nucleotide substitutions at genomic sites outside the predefined loci, including those that may influence attenuation or other critical viral properties such as antigenicity. Consequently, *in-vivo* neurovirulence testing remains necessary at certain stages of OPV production. Furthermore, MAPREC assay is technically demanding, requiring the use of fluorescently labelled primers or radioisotopes in conjunction with restriction enzymes. The procedure is labour-intensive, time-consuming, and necessitates specialized equipment as well as extensive operator training. These factors collectively contribute to the high cost of implementation and present challenges to standardization and consistent assay performance. As a result, MAPREC has not contributed to a significant reduction in animal use for OPV safety testing. Consequently, only a limited number of laboratories currently perform the assay. Furthermore, the MAPREC assay is unsuitable for consistency testing of novel OPV (nOPV)

vaccines, as several targeted loci across the genome have been genetically modified and are therefore no longer applicable to this analytical approach.

Modern high throughput sequencing (HTS, also frequently referred to as next-generation sequencing, NGS) is a powerful tool for characterizing the genetic composition of viral vaccine preparations. Its ability to provide nucleotide-level resolution across the viral genome makes it particularly well suited to assessing the quality of live-attenuated viral vaccines (9,10). In 2019, the WHO ECBS recommended that a study be performed to explore the utility of HTS technology for the quality control of OPV made from Sabin strains. Study results indicated that HTS could accurately quantify 472-C mutants in monovalent bulks of OPV3 (11). A second phase of the same study showed that HTS could also accurately quantify mutations of 480-A+525-C and 481-G for OPV1 and OPV2, respectively (12). The results generated by HTS and MAPREC methods were very well correlated indicating that HTS could in principle be used as an alternative to MAPREC, providing an appropriate test format and analytical processes for establishing assay validity and pass/fail criteria were agreed with the National Regulatory Authority (NRA). HTS makes it possible to conduct whole-genome sequencing on a routine basis. Therefore, HTS has the potential to monitor the genetic stability and production consistency of OPV across the entire genome, beyond the nucleotides assessed by the MAPREC test, to identify other mutations potentially determining changes in phenotype. The degree of sequence heterogeneity expressed in terms of the number of SNPs at nucleotide positions in the genome not necessarily linked to any tangible biological properties provides a unique molecular “fingerprint” for a particular virus preparation. HTS is now recommended as an alternative test for MAPREC and suggested as a possible future replacement of the MNVT and TgMNVT for routine lot release once manufacturing consistency has been established in WHO Technical Report Series, No. 1045, 2023: Annex 2: Recommendations to assure the quality, safety and efficacy of poliomyelitis vaccines (oral, live, attenuated). It was also mentioned in the TRS that practical experience in this area is currently limited and further guidance and developmental work needs to be completed before HTS can be introduced for general regulatory purposes.

This study was conducted in accordance with World Health Organization TRS No. 1045 (2023) to develop reference reagents for whole-genome high-throughput sequencing (HTS) of Type 1 and Type 3 OPV. A Type 2 OPV standard was not pursued, reflecting cessation of OPV2 use and implementation of biological containment in line with Global Action Plan (GAPIV) for Poliovirus Containment guidance (13). We developed candidate reference reagents for Type 1 and Type 3 OPV that can be used to characterize background error (“noise”) in whole-genome SNP profiling. In addition, we developed candidates with MAPREC-relevant revertant content at high-threshold, comparable to the Type 3 MAPREC International Standard (IS; 95/542; 472-C) and the Type 1 MAPREC IS (00/418; 480-A and 525-C). The Type 3 candidate 23/174, in addition to MAPREC-specific mutations, exhibits a broad genome-wide SNP spectrum and is therefore suitable for validation of HTS-based whole-genome OPV assays. This report also provides a template HTS procedure (Appendix 1) and summarizes key troubleshooting issues encountered during the collaborative study. Appendix 2 describes a bioinformatic approach for lot-to-lot consistency testing, and Appendix 3 lists SNPs observed in manufacturing lots of vaccines with an established history of safe use in humans over multiple decades.

Aim of the study

A collaborative study was conducted during 2024-2025 with an aim to assess two candidates each for Type 1(23/160 and 23/162) and Type 3 (23/172 and 23/174) OPV for establishing as

reference reagents to be used as assay validity controls for running HTS assay on OPV bulk material. Efforts were made to mimic the SNP profile of candidate 23/162 and 23/174 for the MAPREC IS for Type 1 (00/418) and Type 3 (95/542) respectively to generate High-threshold Type1 and Type 3 assay controls for HTS platform. In addition to the MAPREC specific mutations other mutations in varying proportion spanning the whole genome were assessed. The study included a total of 25 samples tested in 5 replicates each. These 25 samples represent the candidate reference materials prepared at MHRA, historical reference vaccines available at MHRA with known MAPREC and NVT data, and vaccine bulks shared by manufacturers for the purpose of this study to be used as representative vaccine samples. The primary aim of this study is to establish reference reagents for HTS method validation and for routine day-to-day QC runs, enabling monitoring of OPV production consistency and characterization of virus bulks used for manufacture of Sabin IPV (sIPV) prior to inactivation. Overall, the study provides further scientific assessment of HTS as a potential replacement for animal neurovirulence tests (NVTs) in vaccine lot release.

Materials and methods

Proof of concept testing samples

For proof-of-concept testing, established OPV manufacturers provided consecutive production batches of OPV1, OPV2, and OPV3. Owing to cessation of OPV2 use and implementation of Global Action Plan (GAPIV) for Poliovirus Containment, the type 2 component was subsequently removed from the study. Consecutive Type 1 and Type 3 OPV batches from three manufacturers were used to evaluate an approach developed at MHRA and CBER/FDA based on prior experience: whole-genome SNP profiles from consecutive manufacturing lots produced by the same manufacturer should be highly similar. Manufacturers 1 and 3 each provided three consecutive lots of OPV1 and OPV3, whereas Manufacturer 2 provided two consecutive lots of OPV1 and OPV3. Vaccine lots were processed for RNA extraction, whole-genome poliovirus RT-PCR, and Illumina sequencing. All samples were processed in accordance with the template procedure described in Appendix 1.

Preparation and Characterization of the Type 1 HTS candidate materials (23/160 and 23/162)

MHRA developed Type 1 poliovirus candidate (23/160) from RNA transcribed from a recombinant DNA plasmid and transfected into HEp-2 (Human Epithelioma type 2) cells, with the aim of generating a virus with minimal sequence variation. As expected, this synthetic virus contains no mutations at the MAPREC-relevant nucleotide positions 480 and 525. However, as a consequence of the recombinant DNA approach, the candidate carries two fixed substitutions— silent mutations U5350C in 3A and U6187C in 3D—which serve as genetic markers. These markers enable straightforward discrimination of 23/160 from the OPV1 strains commonly used by vaccine manufacturers. Candidate 23/162 was developed in MHRA by passage of 00/428 (Type 1 reagent from NIBSC catalogue; passed both MAPREC and WHO-MNVT and tested in previous collaborative studies) on to HEp-2 cell line, incubated at 35℃ for approximately 24 hours, following which the candidate was characterized further. For 23/162 candidate, the sum of both mutations 480-A+525-C is found to be 2.43% in line with the MAPREC High Mutant Virus Reference (HMVR) for Poliovirus Type 1 (Sabin) (00/422). HMVR preparation 00/422 defines the maximum allowable percentage (2.56%) of 480-A and 525-C, helping to identify vaccines with elevated 480-A plus 525-C content (14). Additionally, levels of mutations obtained by manufacturers who have successfully implemented the MAPREC test have shown the sum of both mutations (480-A+525-C) stays less than 2.0%.

Therefore, Type 1 HTS candidates grown at MHRA was found suitable for the collaborative study. Both the virus stocks were diluted (1:50) in Minimum Essential Media (MEM) and mixed thoroughly before dispensing 800 microliter (0.800 g) of the material in sterile plastic 2 ml screw cap microtubes. A total of 1275 vials were filled for candidate 23/160 on 17th October 2023. The mean liquid fill volume of the candidates 23/160 was found to be 0.788g by liquid weight ranging from 0.79g -0.804g. The variability of the fill as indicated by the %CV was found to be 1.77%. Similarly, a total of 1271 vials were filled for candidate 23/162 on 24th April 2024. The mean liquid fill volume of the candidates 23/162 was found to be 0.783g by liquid weight ranging from 0.77g -0.80g. The variability of the fill as indicated by the %CV was found to be 1.14%. Sample A and J are blind duplicates of 23/160 (Type 1 background control candidate). Sample B & I are blind duplicates of MAPREC type1-High-threshold candidate for HTS (23/162).
A total of 1000 filled containers of 23/160 and 23/162 each is offered to WHO for establishment as Reference reagent as proposed in page 15-16 of this report. Both the candidates are stored at -80℃ at MHRA and real time stability is monitored annually.

Other Type 1 Collaborative study samples

Other type 1 samples included historical samples held at NIBSC according to their known MAPREC, MNVT and TgmNVT values. Each sample was labelled with code letters; Sample A to Sample J before being dispatched to study participants. A vaccine sample: OPV1 monovalent bulk material, sample C (23/164) was also included in the study.

Preparation and Characterization of the Type 3 HTS candidate materials (23/172 and 23/174)

MHRA has developed Type 3 poliovirus candidate (23/172) from RNA synthesized from recombinant DNA plasmid and transfected into HEp-2 (Human Epithelioma type 2) cells, with the aim of generating a virus with minimal sequence variation. This synthetic virus has no mutations observed in genomic position 472 (neurovirulent mutation tested by MAPREC), however due to the recombinant DNA technology has four fixed mutations at positions C2493U (VP1), U6061C (3D), G6304A (3D), and C7004U (3D). These four fixed mutations serve as marker for this candidate and can be easily differentiated from other common OPV3 strains used by vaccine manufacturers. Candidate 23/174 is developed at MHRA by passage of 96/572 (Type 3 Low mutant MAPREC reference virus) on to HEp-2 cell line and incubated at 35℃ for approximately 24 hours. Following which the candidate was characterized further. For Type 3 whole-genome HTS candidate SNP calling at U472C is found to be 1.04% in line with the MAPREC High Mutant Virus Reference (HMVR) for Poliovirus Type 3 (Sabin) (96/578). HMVR preparation 96/578 defines the maximum allowable percentage (1.1%) of 472-C, helping to identify vaccines with elevated 472-C content (15). Therefore, type 3 candidate grown at MHRA was found suitable for the collaborative study. Both the virus stocks were diluted (1:50) in MEM media and mixed thoroughly before dispensing 800 microliter (0.800 g) of the material in sterile plastic 2 ml screw cap microtubes. A total of 1270 vials were filled for candidate 23/172 on 26th March 2024. The mean liquid fill volume of the candidates 23/172 was found to be 0.781g by liquid weight ranging from 0.773g -0.801g. The variability of the fill as indicated by the %CV was found to be 1.48%. Similarly, a total of 1291 vials were filled for candidate 23/174 on 23rd April 2024. The mean liquid fill volume of the candidates 23/174 was found to be 0.782g by liquid weight ranging from 0.77g -0.79g. The variability of the fill as indicated by the %CV was found to be 1.02%. Sample O & Y are blind duplicates of candidate 23/172 (background control for Type 3 HTS assay). Sample P & X are blind duplicates of MAPREC type 3-High threshold candidate for HTS (23/174).

A total of 1000 filled containers of 23/172 and 23/174 each is offered to WHO for establishment as Reference reagent as proposed in page 15-16 of this report. Both the candidates are stored at -80°C at MHRA and real time stability is monitored annually.

Other Type 3 Collaborative study samples

Other type 3 samples included historical samples held at MHRA according to their known MAPREC, MNVT and TgmNVT results. Each sample was labelled with code letters; Sample O to Sample Y before being dispatched to the study participants. A vaccine sample: OPV3 monovalent bulk material, sample Q (23/176) is also included in the study. The selection of study samples for Type 3 vaccines was intended to cover a wide range of 472-C content and pass/fail outcomes to help assessing the correlation between HTS assay and NVT assay and designing a process for establishing validity criteria and pass/fail decisions for the prospective HTS test for OPV.

nOPV2 material (n=4)

Due to the increased biological risk of handling OPV2 and progress in GPEI goals it was decided to exclude OPV2 from this study. However, we received strong encouragement from the manufacturers to explore the utility of HTS assay for nOPV material. One of the study participants kindly agreed for using their vaccine drug product to test the methodology. Each sample was labelled with code letters Sample K to Sample N before being dispatched to the study participants. Sample K and L, Sample M and N are blind duplicate samples, they are prepared from drug product and therefore are treated as vaccine sample representatives. However, these samples are included in the study as a proof-of-concept that HTS can be implemented for quality control of nOPV vaccines. This study does not include preparing a reference reagent for validation of HTS assay for nOPV vaccine. Full details of the study samples are given in Table 1.

Participants

Six laboratories participated in the study, including four manufacturers and two National Control Laboratories. Each participating laboratory is referred to by a code number assigned at random and not necessarily representing the order of listing in Appendix 4.

Design of the study

Participants were requested to sequence the whole genome of each poliovirus study sample in five independent assays using the HTS platform and any appropriate method established at their organization. Amplicon-based PCR, random-PCR or direct RNA sequencing protocols can be used. If using PCR-based methods, PCR fragments or the entire full-length amplicon can be obtained in five independent PCR reactions from the same original extracted viral RNA. Five repeat data sets from 25 samples were received, therefore a total of 125 data sets per lab. Lab 5 returned 3 repeats for one sample, therefore for Lab 5 only 123 data sets were received. An example of amplicon-based whole-genome PCR sequencing protocol is provided in Appendix 1.

The study participants were asked to provide raw FASTQ data files for each sample and sequence determination using an agreed data transfer system, to be analysed by the collaborative study organisers. Laboratories were also encouraged to analyse whole-genome SNPs of study samples in-house if they desired using their bioinformatics pipeline/software of choice and submit the data in an Excel spreadsheet. Out of six laboratories four laboratories shared the raw FASTQ data files and performed in-house data analysis and shared Excel

spreadsheets for concordance testing of SNP profiles. Two laboratories shared only the raw FASTQ data. A total of 125 raw FASTQ data files per lab was analysed at MHRA.

HTS assays

Details of HTS methods used by the different laboratories are shown in Table 2. They include details on RNA extraction, PCR and HTS strategies. All laboratories used commercially available kits to extract the viral nucleic acid from the study samples, they included both column-based kits and automated nucleic acid extraction. Five laboratories (1, 2, 4, 5 and 6) amplified the RNA by using pan-poliovirus specific primers by one-step PCR approach (cDNA synthesis and PCR amplification carried out in the same tube as described in Appendix 1). One of the participant laboratories (Lab 3) amplified the viral RNA by two step approach, first reverse transcribing the RNA using oligo dT primers to generate cDNA and then using the cDNA to amplify using poliovirus-specific tiled PCRs. All labs successfully amplified whole genome of polioviruses in the sample. Laboratory 5 struggled to achieve uniform coverage throughout the genome with coverage dropping down to below 1000 at certain position for some samples (this is discussed in Appendix 1, troubleshooting section). All the laboratories (laboratories 1, 2, 3, 4, 5, and 6) sequenced the samples using Illumina platform and quantified the input DNA using the Qubit fluorometric system. All samples were processed with at least one RNA extraction and 5 replicate PCR from the RNA. In summary, the methods used between participating laboratories were diverse but not without their similarities. Five out of six laboratories used a one-step RT-PCR approach and only one laboratory amplified the vaccine viral RNA by a tiled two-step RT-PCR approach. All laboratories used Illumina sequencing systems. By allowing each participant to follow their own in-house methodologies for laboratory sample preparation and bioinformatics the variability introduced by different protocols could be analysed.

Bioinformatics analysis

Most participants had capacity to perform bioinformatics analyses in-house using pre-packaged software applications. Three of the participants used Geneious with custom mapping parameters. One participant used CLC Genomic Workbench application from Qiagen, another used MiSeq Reporter, IGV software. One laboratory uses its own UNIX-based Swarm software. Results included in this report to quantify and compare SNP profiles are from data analysis performed by MHRA of all raw FASTQ sequence files using prepackaged software application Geneious with custom mapping parameters. Of the six participating laboratories, four conducted in-house data analysis and provided processed SNP profile datasets in the form of Excel spreadsheets for concordance assessment. The remaining two laboratories submitted raw sequencing data (FASTQ files) comprising 125 datasets per laboratory, which were subsequently analysed at the MHRA. SNPs were determined using whole-genome sequences of Sabin 1 (GenBank ID AY184219), Sabin 3 (GenBank ID AY18422) and nOPV2 (GenBank ID MZ245455) original vaccine strains as references.

Statistical analysis

Variance components expressed as Standard Deviation (SD) and Coefficient of Variation (CV) were calculated for MAPREC specific mutation for type 1 and 3 to calculate the intra-assay, intra-lab (inter-assay) and inter-lab variabilities. Whole genome SNP profiles for candidates 23/160, 23/162, 23/172 and 23/174 were used to provide mean estimates of SNP. Dot plots were drawn to show the range of % SNP distribution along with calculated mean, SD, %CV and 95% Confidence Intervals (CI) at each position. For blind duplicate samples Type 1 (B & I), Type 3 (P & X), nOPV2 (K & L, M & N) ratios between mean estimate SNPs spanning

whole genome were calculated and the significance of the value being different from a hypothetical value of 1 was calculated. Pearson correlation coefficients and fitted linear regression lines were calculated to assess the linear correlation in reported values for single nucleotide polymorphisms for Type 1, 3 OPV and nOPV2, where values generated by MHRA pipeline and labs 4, 5 and 6 were plotted against each other. R and GraphPad Prism software packages were used for these analyses.

Results of collaborative study

Consecutive lot testing for Type 1 vaccines:

Three manufacturers provided consecutive monovalent bulk materials for a proof-of-concept testing. Manufacturer 1 provided 3 lots (lot 1 production date Jan 2020, lot 2: Feb 2020 and lot 3: March 2020). Manufacturer 2 provided 2 lots (lot 1: July 2013, lot 2: Dec 2013) and Manufacturer 3 provided 3 lots (lot 1: Feb 2021, March 2021, April 2021) of Type 1 OPV. The whole genome SNP analysis carried out at MHRA shows high consistency of SNP profile is maintained over consecutive lots of Type 1 OPV produced by these three manufacturers (Figure 1, 2 and 3).

Consecutive lot testing for Type 3 vaccines:

Three manufacturers provided consecutive monovalent vaccine bulk materials to the study coordinators for a proof-of-concept testing. Manufacturer 1 provided 3 lots (lot 1 production date November 2019, lot 2: January 2020 and lot 3: February 2020). Manufacturer 2 provided 2 lots (lot 1: March 2014, lot 2: April 2014) and Manufacturer 3 provided 3 lots (lot 1: Sept 2020, Oct 2020, Nov 2020) of Type 3 OPV. The whole genome SNP analysis was carried out at MHRA. High consistency of SNP profiles is maintained over 3 consecutive lots produced by manufacturer 1, 2 and 3. The genetic signature of the vaccines produced by manufacturer 1 shows fixed mutations at position 1699, 2493,3353,3357, 3700, 5767, 5832, 6421 and 6505 (Figure 4). The genetic signature of the vaccines produced by manufacturer 2 does not show any fixed SNP (Figure 5). The genetic signature of the vaccines produced by manufacturer 3 showed fixed SNP at position 2440, 2493 and 6760 (Figure 6). The observed manufacturer-specific differences in type 3 consensus sequences, relative to the original Sabin 3 strain, are consistent with re-derivation of the vaccine strain via distinct laboratory processes with divergent passage histories, including cloning/plaque-purification steps followed by generation of new seed lots (see Appendix 2, Ref 1 of this report).
The consecutive lot testing for Type 1 and 3 OPV supports the historical findings from MHRA and CEBR-FDA labs that comparison of whole-genome SNP profiles reveals differences between batches made from different seed lots and by different manufacturers, but there is high consistency within manufacturers and products. The SNP profiles of Type 3 vaccines are more diverse than type 1 vaccines.

Determination of 472-C content by HTS for candidate reference reagent 23/172 (background control) and 23/174 (High-threshold Type-3 MAPREC Control):

HTS estimates of 472-C content in study samples O-X obtained in each laboratory are shown in Table 3. Candidate 23/172 is included as blind duplicate (Sample O and Y), the theoretical estimate of 472-C content for candidate 23/172 was set to 0% and the reference reagent was created to act as a background control for Type 3 OPV HTS assay. Candidate 23/174 is included as blind duplicate (Sample P and X), the theoretical estimate of 472-C content for

candidate 23/174 was set to 1% and the reference reagent was created to be used as High-threshold Type-3 MAPREC control for HTS assay in line with MAPREC HMVR for Poliovirus Type 3 (Sabin) (96/578) (15). No outliers were observed. Only lab 3 gave results for sample O and Y as $\geq$0.3. Lab 3 used tiled approach for whole genome amplification of polio genome, and this is expected from the methodology (11). No significant differences between coded duplicates were noted for any lab (Table 3). Additionally, 472-C position was analysed for samples O-X by Swarm analysis (UNIX-based software). The final 472-C estimate for the candidate 23/174 is 1.04% (95% CI; 1.01-1.08) as shown in Table 4.

Intra-laboratory, between-laboratory and within-assay variability for candidate 23/172 (Sample O and Y) for whole genome SNP profile:

The intra-laboratory (between-assay, within-laboratory) and inter-laboratory variability as the SD of whole genome SNP estimates at positions 472, 2493, 6061, 6304 and 7004 from five determinations across study samples for each laboratory is shown in Table 5. The ratio of mean estimates of SNP throughout the genome at positions U472-C, C2493-U, U6061C, G6304A and C7004U estimates for duplicate samples O and Y were used to test within-assay variability which showed very good agreement with their expected value of 1 in all laboratories. The mean SNP estimates for 23/172 are as follows: U472C (0.05%), C2493U (99.93%), U6061C (99.89%), G6304A (99.82%) and C7004U (99.80%) (Figure 7).

Intra-laboratory, between-laboratory and within-assay variability for candidate 23/174 (Sample P and X) for whole genome SNP profile:

The intra-laboratory (between-assay, within-laboratory) and inter-laboratory variability was measured based on the standard deviation (SD) of SNP estimates at 15 different positions from five determination, across study samples for each laboratory is shown in Table 6 and Figure 8. The ratio of mean estimates of SNP throughout the genome at positions U472C, A769G, A1003G, U1867C, C2493U, C2950U, C3640U, A3766G, A4171G, A4872G, A4872C, A4884G, G4925A, A4935G, U5832C for duplicate samples P and X were used to test within-assay variability which showed very good agreement with their expected value of 1 in all laboratories (Figure 9). Table 7 represents the blind duplicate analysis for sample P and X where the mean ratios for the whole genome SNPs were close to 1 and the p-value obtained from paired two-tailed t-test shows no significant difference from 1. These blinded duplicate samples P and X within the panel indicate that within-assay variability is low for candidate 23/174. A similar within-assay variability threshold is measured in each MAPREC test comparing results for the synthetic DNA 0.9% 472-C WHO International Standard (IS; 95/542) for MAPREC duplicated in each MAPREC assay. Similar approach for evaluating within-assay variability validity test would be useful for routine HTS assays. The mean estimate SNP estimates for 23/174 is as follows: U472C (1.04%), A769G (1.50%), A1003G (2.22%), U1867C (1.30%), C2493U (75.56%), C2950U (6.38%), C3640U (1.88%), A3766G (1.52%), A4171G (1.45%), A4872G (12.93%), A4872C (2.90%), A4884G (2.78%), G4925A (5.08%), A4935G (6.36%), U5832C (34.00%) (Figure 8).

Determination of 480A+525C content by HTS for candidate reference reagent 23/160 (background control) and 23/162 (High-threshold Type-1 MAPREC Control):

HTS estimates of 480A+525C content in the study samples A-J obtained in each laboratory are shown in Table 8. Candidate 23/160 is included as blind duplicate (Sample A and J), the theoretical estimate of 480A+525C content for candidate 23/160 was set to 0% and the

reference reagent was created to act as a background control for Type 1 OPV HTS assay. Candidate 23/162 is included as blind duplicate (Sample B and I). The theoretical estimate of 480A+525C content for candidate 23/162 was set to 2% and the reference reagent was created to be used as High-threshold Type-1 MAPREC control for HTS assay, to be used in line with the MAPREC High Mutant Virus Reference (HMVR) for Poliovirus Type 1 (Sabin) (00/422) (14). No outliers were observed, but high variability in some cases (e.g. lab 5 sample B and G) was observed where the SD was ~0.9 (CV>35%). Only lab 5 gave results for sample A as ≥0.3. No significant coded duplicate difference noted for any lab when estimating 480A+525C content (Table 9). Additionally, 480A+525C content was analysed for sample A-J by Swarm analysis (UNIX-based software), Swarm estimates are consistently lower (~ 93% of MHRA estimates). The final 480A+525C estimate for the candidate 23/162 is 2.43% (95% CI; 2.33-2.54) as shown in Table 8.

Intra-laboratory, between-laboratory and within-assay variability for candidate 23/160 (Sample A and J) for whole genome SNP profile:

The intra-laboratory (between-assay, within-laboratory) and inter-laboratory variability as the SD of whole genome SNP estimates at positions 480, 525, 5350 and 6187 from five determinations across study samples for each laboratory is shown in Table 10 and Figure 10. The ratio of mean estimates of SNP throughout the genome at positions G480A, U525C, U5350C and U6187C estimates for duplicate samples A and J were used to test within-assay variability which showed very good agreement with their expected value of 1 in all laboratories. The blind duplicate analysis for samples A and J showed the mean ratios for the whole genome SNPs were close to 1 and the p-value obtained from paired two-tailed t-test shows no significant difference from 1. These blinded duplicate samples A and J within the panel indicate that within-assay variability is low for candidate 23/160. The mean SNP estimates for 23/160 are as follows: G480A (0.03%), U525C (0.05%), U5350C (99.93%) and U6187C (99.93%) (Table 10).

Intra-laboratory, between-laboratory and within-assay variability for candidate 23/162 (Sample B and I) for whole-genome SNP profile:

The intra-laboratory (between-assay, within-laboratory) and inter-laboratory variability as the measure of standard deviation (SD) of whole genome SNP estimates at 7 different positions from five determinations across study samples for each laboratory is shown in Table 11. The ratio of mean estimates of SNP throughout the genome at positions G480A, U525C, C661U, U691A, C1941A, U4393C and G7303A for duplicate samples B and I were used to test within-assay variability which showed very good agreement with their expected value of 1 in all laboratories except Lab 5 (Figure 11). Table 12 represents the blind duplicate analysis for sample B and I where the mean ratios for the whole genome SNPs were close to 1 and the p-value obtained from paired two-tailed t-test shows no significant difference from 1 except in Lab 5 where the p-value = 0.003 for position U691A and p value = 0.001 for position G7303A. These blinded duplicate samples B and I within the panel indicate that within-assay variability is low for candidate 23/160 for all labs except lab 5. A similar within-assay variability threshold is measured in each MAPREC test comparing results for the synthetic DNA 2% 480A+525C WHO International Standard (IS; 00/418) for MAPREC duplicated in each MAPREC assay. Similar approach for evaluating within-assay variability validity test would be useful for routine HTS assays. From the collaborative study it is observed that the range of %SNP distribution of 23/162 is conserved ranging from 1%-4% throughout the genome as compared to 23/174 where the % SNP ranged from 1%-75%. Therefore 23/174 is identified as a more

suitable candidate to be used as reference reagent when validating HTS assay for analysing whole genome SNP profiles for OPV.

Evaluation of intra-laboratory, inter-laboratory and intra-assay variability for selected mutation spanning whole genome of nOPV2 drug product:

Selected mutations (16) used for supporting lot release for novel type 2 oral poliovirus vaccine; C930U, G2528A, A2639G, C2844A, U2970C, A3053G and G3425A were evaluated in the collaborative study to understand the intra-laboratory (between-assay, within-laboratory) and inter-laboratory variability as the SD of SNP estimates for nOPV2 drug products.

Estimates at 7 different positions from five determinations across study samples (K-L & M-N) for each laboratory is shown in Table 13 & 14. The ratio of mean estimates of SNP throughout the genome at positions C930U, G2528A, A2639G, C2844A, U2970C, A3053G and G3425A estimates for duplicate samples K/L and M/N were used to test within-assay variability which showed very good agreement with their expected value of 1 in all laboratories (Figure 12 &13). Blind duplicate analysis for sample K/L and M/N where the mean ratios for the whole genome SNPs were close to 1 and the p-value obtained from paired two-tailed t-test shows no significant difference from 1. These blinded duplicate sample K/L and M/N within the panel indicate that within-assay variability is low for nOPV2 drug substance for all labs.

Concordance between SNP profiles generated by MHRA and Lab 4, 5 and 6 for all three serotypes for all positions:

This analysis shows high concordance in reported values for single nucleotide polymorphism for Type 1,3 and nOPV2 when values generated by MHRA pipeline and Lab 4, 5 and 6 (in-house pipeline) were plotted against each other as shown in Figure 14.

Stability Study

Accelerated degradation and real time degradation studies are ongoing on the proposed candidate for whole genome SNP profiling assay 23/174. Candidates 23/160, 23/162, 23/172, and 23/174 are stored at –80 °C for the real-time degradation study and will be assessed annually throughout their lifetime using whole-genome amplification to monitor RNA integrity (table 15). Accelerated degradation samples for 23/174 were stored at –20, +4, +25, and +37 °C and tested over six months. RNA integrity was evaluated using poliovirus whole-genome PCR. Table 15 presents the results of both the accelerated and real-time degradation studies.
Because the assay produces only a binary output (presence or absence of a whole-genome PCR product), the data cannot be used to generate a long-term predictive model using the Arrhenius equation. For this reason, accelerated degradation testing was performed only on 23/174.
MHRA-NIBSC has extensive experience storing polio bulks long-term at –80 °C in various formats and has seen no evidence that the presentation used for these candidates would compromise long-term stability.

Discussion

In this project, two whole-genome high-throughput sequencing (WG-HTS) candidate panels were developed for Type 1 and Type 3 Oral Poliovirus Vaccine (OPV). The panels were evaluated for its suitability in whole-genome HTS analysis and to support the investigation of MAPREC-specific mutations through HTS-based assays.

WG-HTS performed strongly as a quantitative, reproducible platform for both (i) MAPREC-equivalent measurement of key attenuation-associated mutations and (ii) broader whole-genome SNP profiling to support assessment of manufacturing consistency. Across the six participating laboratories, the study demonstrated high concordance in SNP profiles for OPV Types 1, 3 and nOPV2 with tight clustering of estimates across positions and materials. Collectively, the results indicate that WG-HTS can reliably resolve both high-frequency and low-frequency variants and can distinguish manufacturer- and seed-specific signatures while maintaining strong reproducibility between laboratories.

A principal strength of the dataset is the low variability observed within and between laboratories. Variance component analysis showed low intra-assay, intra-laboratory/inter-assay and inter-laboratory variability for all samples. Across the wider set of whole-genome SNP positions, SDs were generally small relative to mean variant frequencies, supporting very good agreement between laboratories and good assay precision overall. The study demonstrated excellent concordance for blinded duplicates, providing an internal check on repeatability. Duplicate pairs for the Type 1 background control (samples A & J; candidate 23/160), Type 1 High-threshold MAPREC candidate (samples B & I; candidate 23/162), Type 3 background control (samples O & Y; candidate 23/172), and Type 3 High-threshold MAPREC candidate (samples P & X; candidate 23/174) as well as nOPV2 vaccine sample pairs K and L and M and N produced closely matched mean estimates across laboratories, confirming consistent performance across independent, blinded replicates.

Importantly, WG-HTS delivered quantitative measurements across a wide SNP frequency range, including very low frequencies in the ~0.05–0.07% range (for example, U472C in low-mutation Type 3 materials), while background control candidates returned near-zero signals, such as U472C with a mean of approximately 0.05% for candidate 23/172 and combined G480A+U525C below 0.1% for candidate 23/160. These characteristics allow estimation of run- and laboratory-specific background error rates and provide a practical basis for defining limits of detection. The ability to quantify variants at these low frequencies supports the use of WG-HTS for monitoring subtle shifts that may be relevant when applying high-threshold acceptability decisions.

Overall, the candidate reference materials appear well matched to their intended roles, with background controls exhibiting minimal target-mutation signal to quantify run- and laboratory-specific error, and High-Threshold MAPREC- candidates exhibiting target-mutation frequencies in the operational decision range to support validation, assay transfer, and ongoing performance monitoring.

Interpretation and path forward

Overall, the collaborative study supports WG-HTS as a robust tool that can complement—and, once sufficient historical data and performance criteria are established, potentially reduce reliance on—animal neurovirulence testing for OPV lot assessment. The most immediate value is the ability to quantify key MAPREC mutations with good inter-laboratory agreement, while simultaneously generating whole-genome SNP profiles that are informative for manufacturing consistency and outlier detection. A close similarity between the SNP profiles of current and historical vaccine lots serves as strong evidence of manufacturing consistency and suggests that the biological properties—such as neurovirulence—remain comparable. Variations in molecular (SNP) profiles do not necessarily indicate that a vaccine lot is unacceptable. Such

discrepancies may reflect alterations in virus growth conditions or manufacturing parameters, serving as an early warning signal that warrants further investigation.

The rrecommended approach is to continue curating manufacturer-/seed-specific historical SNP databases using lots that have passed release testing, to define expected ranges, position-specific variability, and the extent/rate of drift over time. The next is to develop and validate an algorithmic framework to support objective pass/fail decision-making based on whole-genome SNP profiling data, an example of such algorithmic framework is provided in Appendix 3.

Proposed Future Scheme for OPV Lot Testing

- A series of consistency lots of monovalent bulks of OPV should be evaluated using whole-genome high-throughput sequencing (WG-HTS) to define the acceptable range of variation in SNP profiles. These lots should also have corresponding data from established assays, such as MNVT, TgmNVT, and/or MAPREC, to ensure biological relevance.
- After the consistency of manufacture is established, whole-genome HTS can be employed as the primary tool for assessing the molecular conformity of each new OPV batch relative to historical SNP profiles.
- If the SNP profile of a new lot demonstrates consistency within predefined statistical acceptance criteria, the lot may be released without the need for neurovirulence testing (NVT).
- If the SNP profile deviates from the historical profile beyond these criteria, an investigation should be initiated, which may include performing NVT or additional analyses.
- If the investigation confirms that the deviation does not impact product quality or safety, the historical SNP database may be updated to reflect the new data.

Proposal

The following proposals are being submitted to the Expert Committee on Biological Standardization (ECBS) based on the outcomes of this collaborative study:

Type 1 OPV

- **Sample 23/160**: Proposed to be establish as a *WHO Reference Reagent (RR)* serving as a Type 1 background-noise / system-suitability control for WG-HTS. This material provides a near-zero baseline for the Type 1 MAPREC mutations (G480A and U525C) and can be used to characterize laboratory- and run-specific background error frequencies.

- **Sample 23/162**: Proposed to be established as a *WHO Reference Reagent (RR)* for High-threshold-Type1-MAPREC assay control for HTS platform, with a mean combined estimate for mutations G480A + U525C of 2.43% (95% CI: 2.33%–2.54%).

Type 3 OPV

- **Sample 23/172**: Proposed to be established as the *WHO Reference Reagent (RR)* serving as a Type 3 background-noise / system-suitability control for WG-HTS. This material provides a near-zero baseline for the Type 3 MAPREC mutation (U472C) and supports measurement of background error and limits of detection.

- **Sample 23/174**: Proposed to be established as a *WHO Reference Reagent (RR)* for High-threshold-Type 3-MAPREC assay control for HTS platform, with a mean estimate for mutation U472C of 1.04% (95% CI: 1.01%–1.08%). Additionally, 23/174 can be employed for validation and implementation of WG-HTS for whole-genome SNP profiling (variant frequencies spanning approximately 1% to 75% across genomic positions).

The collaborative study Standard Operating Procedure (SOP) is described in **Appendix 1** of this report to facilitate assay development. **Appendix 2** describes Bioinformatic approach for analysis and interpretation of HTS data and assay validity criteria. **Appendix 3** provides list of SNPs that has been seen in OPV vaccines used over decades to immunize human population. **Appendix 4** provides the list of study participants. **Appendix 5** describes proposed instruction for use of 23/160, 23/162, 23/172 and 23/174.

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Table 1. Details of collaborative study samples.

Sample	Details	Type of Poliovirus	Result		
			MAPREC	MNVT	TgmNVT
Sample A	Type 1 proposed background control (23/160)	Sabin 1	–	–	–
Sample B	Type 1 proposed High-threshold MAPREC control for HTS platform (23/162)	Sabin 1	–	–	–
Sample C	Type 1 Vaccine bulk (23/164)	Sabin 1	–	–	–
Sample D	Type 1 MAPREC Low Mutant Reference virus (00/416)	Sabin 1	Pass	–	–
Sample E	Type 1 MAPREC High Mutant Reference virus (00/422)	Sabin 1	Fail	–	–
Sample F	Historic sample used in previous collaborative studies 00/428 (SO+3)	Sabin 1	Pass	Pass	–
Sample G	Historic sample used in previous collaborative studies 00/426 (SO+3)	Sabin 1	Pass	Pass	–
Sample H	Historic sample used in previous collaborative studies 00/434	Sabin 1	Pass	–	–
Sample I (Blind Duplicate of sample B)	Type 1 proposed High-threshold MAPREC control for HTS platform (23/162)	Sabin 1	–	–	–
Sample J (Blind duplicate of sample A)	Type 1 proposed background control (23/160)	Sabin 1	–	–	–
Sample K	nOPV2-vaccine representative-lot 1a	nOPV2	–	–	–
Sample L	nOPV2-vaccine representative-lot 1b	nOPV2	–	–	–
Sample M	nOPV2-vaccine representative-lot 2a	nOPV2	–	–	–
Sample N	nOPV2-vaccine representative-lot 2b	nOPV2	–	–	–

Sample O	Type 3 proposed background control (23/172)	Sabin 3	–	–	–
Sample P	Type 3 proposed High-threshold MAPREC control for HTS platform (23/174)	Sabin 3	–	–	–
Sample Q	Type 3 Vaccine simulant (23/176)	Sabin 3	–	–	–
Sample R	Type 3 MAPREC High Mutant Reference Virus (96/578)	Sabin 3	Fail	–	–
Sample S	Type 3 MAPREC Low Mutant Reference virus (96/572)	Sabin 3	Pass	–	–
Sample T	Historic sample used in previous collaborative studies SO+3 (93/636)	Sabin 3	Fail	Fail	Fail (20/20)
Sample U	Historic sample used in previous collaborative studies RSO3(97/676)	Sabin 3	Fail	Fail	Pass
Sample V	Historic sample used in previous collaborative studies SO+3(96/568)	Sabin 3	Fail	Fail (1/2)	Fail (11/4)
Sample W	Historic sample used in previous collaborative studies RSO3(98/650)	Sabin 3	Pass	Pass	Pass
Sample X (Blind duplicate of sample P)	Type 3 proposed High-threshold MAPREC control for HTS platform (23/174)	Sabin 3	–	–	–
Sample Y (Blind Duplicate of sample O)	Type 3 proposed background control (23/172)	Sabin 3	–	–	–

Table 2. Details of HTS Laboratory methods used in the collaborative study by different labs.

Lab Name	RNA Extraction	PCR Strategy	PCR Purification	HTS Strategy	In-House Analysis Strategies
Lab 1	High Pure Viral RNA Kit (Roche), Manual Extraction	Primer name：panPV_For、panPV_Rev kit：SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity DNA Polymerase protocol：WHO Collaborative Study protocol	kit：AMPure XP, Qubit	Library prep：Nextera XT DNA Library Preparation Kit NextEra XT Index Kit HTS platform：Miseq（Illumina） Miseq Reagent Kit v2（500）	**CLC Genomics Workbench**
Lab 2	KingFisher Duo Prime automated RNA extraction platform using MagMAX™ Viral RNA Isolation Kit (Applied Biosystems)	Primer name：panPV_For、panPV_Rev kit：SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity DNA Polymerase protocol：WHO Collaborative Study protocol	kit：AMPure XP, Qubit	Library prep : Illumina DNA prep with Illumina Index Set A indexing kit. QC library : MultiQC. Sequencing : Miseq Standard V2 500 cycles.	**Use Geneious with custom mapping&SNP parameters**
Lab 3	TaKaRa MiniBEST Viral RNA/DNA Extraction Kit. Manual Extraction	Oligo dT Primer followed by tiled PCR.PrimeScriptTM II 1st Strand cDNA Sythesis Kit(Takara：Cat 6210B)	kit：AMPure XP, Qubit	Illumina , MiSeq System Instrument. Illumina Microbial Amplicon Prep (Illumina, cat#20097857) - MiSeq Reagent Micro Kit V2(300-cycles) (Illumina , cat # MS-103-1002)	**MiSeq Reporter；IGV**
Lab 4	Roche High Pure Viral RNA kit Manual Extraction	Superscript III One-Step RT-PCR System with A7-Sabin1,3 and U-S7 for Sabin 1 and 3 viruses and A7-Sabin 2 and U-S7 primers for Sabin 2	kit：AMPure XP, Qubit	MiSeq 3.0, Nextera XT Library Prep Kitv3 600 cycle kit	**Bio-informatic analysis completed by K. Chumakov**

		viruses: WHO Collaborative Study protocol			
Lab 5	QIAamp Viral RNA Minikit, Manual extraction	Superscript III One Step RT-PCR PlatinumTaq Hifi (Make: Invitrogen: 12574-035). Full length PCR using primer details provided in the CS protocol: WHO Collaborative Study protocol	kit : AMPure XP, Qubit	Library prep : Illumina DNA prep with IDT-Illumina Index Set A indexing kit. QC library : QIAxcel Advanced Sequencing : PhiX control V3 5%+library denatured 95%. Kit using Miseq Standard V2 500 cycles.	**Use Geneious with custom mapping&SNP parameters**
Lab 6	QIAamp Viral RNA Minikit, Manual extraction	Primer : A7Sab2; A7Sab1,3 ; US7 with concentration 40nM and 10nM. Reverse transcriptase : Superscript III Reverse transcriptase with 14,5 hours of cDNA process. Full length : Platinum Super Mix HiFi with 20 PCR cycles.	kit : AMPure XP, Qubit	Library prep : Illumina DNA prep with Illumina Index Set A indexing kit. Sequencing Kit: using Miseq Standard V2 500 cycles, V2 300, Micro Kit V2 300	**Use Geneious with custom mapping&SNP parameters**

Table 3. Statistical analysis for Type 3 472-C estimates from the collaborative study samples.

Analysis	Lab	T		V		U		X		P		R		W		S		Q		Y		O	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
MHRA	1	2.72	0.22	1.38	0.08	1.38	0.18	0.90	0.12	0.98	0.13	0.84	0.13	0.76	0.15	0.62	0.11	0.24	0.05	0.00	0.00	0.00	0.00
MHRA	2	2.54	0.27	1.28	0.15	1.18	0.08	0.92	0.08	0.92	0.13	0.86	0.05	0.76	0.11	0.58	0.04	0.24	0.05	0.00	0.00	0.00	0.00
MHRA	3	2.82	0.22	1.52	0.08	1.56	0.38	1.30	0.24	1.28	0.15	1.16	0.22	1.18	0.26	0.90	0.33	0.56	0.09	0.30	0.07	0.30	0.07
MHRA	4	3.18	0.41	1.50	0.26	1.46	0.18	1.34	0.17	1.20	0.07	0.78	0.18	0.86	0.17	0.68	0.34	0.30	0.12	0.00	0.00	0.00	0.00
MHRA	5	2.30	0.12	1.12	0.04	1.14	0.09	0.83	0.12	0.96	0.05	0.80	0.12	0.62	0.08	0.44	0.05	0.28	0.04	0.00	0.00	0.00	0.00
MHRA	6	2.50	0.14			1.18	0.04			0.86	0.13	0.88	0.04			0.50	0.07	0.30	0.07			0.00	0.00
MHRA	Mean	2.68		1.36		1.32		1.06		1.03		0.89		0.84		0.62		0.32		0.06		0.05	
	Intra SD	0.25		0.15		0.20		0.16		0.12		0.14		0.17		0.20		0.08					
	Inter SD	0.28		0.15		0.15		0.23		0.16		0.12		0.20		0.13		0.12					
	Total SD	0.38		0.21		0.25		0.28		0.20		0.19		0.26		0.24		0.14					
	Total CV	14.1%		15.5%		18.8%		26.4%		19.1%		21.1%		30.8%		39.3%		43.5%					
	n	6		5		6		5		6		6		5		6		6					
Swarm	Mean	2.29		1.15		1.10		0.87		0.94		0.79		0.67		0.49		0.28		0.07		0.07	
	% of MHRA	86%		84%		83%		82%		91%		90%		80%		78%		87%					

Table 4. Statistical analysis of 472-C estimates for the coded duplicate Type 3 samples P and X.

Coded duplicate (samples P & X) final analysis						
Variance components (as SD):				Variance components (as CV):		
Intra-assay		0.14		Intra-assay		13.4%
Intra-lab (inter-assay)		0.00		Intra-lab (inter-assay)		0.0%
Inter-lab		0.18		Inter-lab		17.1%
Total		0.23		Total		21.7%
Final mean estimate:		1.04				
95% LCL:		1.01				
95% UCL:		1.08				

Table 5. Blind duplicate whole genome analysis for sample O and Y.

Analysis	Lab	O										Y									
		U472C		C2493U; VP1-T6I		U6061C		G6304A		C7004U		U472C		C2493U; VP1-T6I		U6061C		G6304A		C7004U	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
MHRA	1	0.00	0.00	99.96	0.05	99.84	0.09	99.76	0.05	99.80	0.00	0.00	0.00	99.90	0.07	99.66	0.38	99.58	0.44	99.58	0.55
MHRA	2	0.00	0.00	100.00	0.00	99.94	0.05	99.92	0.04	99.90	0.00	0.00	0.00	99.98	0.04	99.92	0.04	99.92	0.08	99.90	0.00
MHRA	3	0.30	0.07	99.80	0.10	99.90	0.07	99.72	0.13	99.74	0.05	0.30	0.07	99.76	0.11	99.88	0.08	99.66	0.15	99.70	0.19
MHRA	4	0.00	0.00	99.94	0.09	99.94	0.05	99.82	0.08	99.80	0.07	0.00	0.00	99.98	0.04	99.96	0.05	99.88	0.04	99.84	0.09
MHRA	5	0.00	0.00	99.96	0.05	99.78	0.04	99.90	0.10	99.72	0.08	0.00	0.00	99.96	0.05	99.84	0.21	99.62	0.30	99.08	0.76
MHRA	6	0.00	0.00	99.94	0.05	99.94	0.05	99.82	0.04	99.82	0.04	0.00	0.00	99.96	0.05	99.94	0.05	99.84	0.05	99.82	0.04
MHRA	**Mean**	0.05		99.93		99.89		99.82		99.80		0.05		99.92		99.87		99.75		99.65	

Table 6. Summary of sample P and X estimate from whole genome profile of selected mutations.

P

Analysis	Lab	U472C		A769G		A1003G		U1867C		C2493U;VP1-T6I		C2950U		C3640U		A3766G		A4171G		A4872G;2C-E253G		A4872C;2C-E253A		A4884G;2C-D257G		G4925A;2C-D271N		A4935G;2C-Q274R		U5832C;3B-I135T	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
MHRA	1	0.98	0.13	1.40	0.14	2.04	0.15	1.24	0.09	75.82	0.97	6.28	0.27	1.72	0.15	1.38	0.13	1.32	0.08	12.66	0.42	2.68	0.27	2.58	0.19	5.24	0.33	6.18	0.22	34.04	0.38
MHRA	2	0.92	0.13	1.38	0.13	1.96	0.11	1.06	0.15	75.52	0.30	5.86	0.23	1.58	0.20	1.30	0.07	1.24	0.32	13.06	0.30	2.90	0.23	3.10	0.29	3.76	0.27	6.42	0.08	33.18	0.52
MHRA	3	1.28	0.15	1.56	0.15	2.58	0.13	1.24	0.17	74.72	0.54	6.54	0.55	2.08	0.50	2.38	0.13	1.78	0.23	13.04	0.74	2.76	0.23	2.92	0.29	5.38	0.58	6.58	0.23	33.74	0.96
MHRA	4	1.20	0.07	1.86	0.36	2.54	0.34	1.52	0.08	74.90	1.10	6.88	0.59	2.08	0.13	1.58	0.27	1.50	0.20	13.22	0.63	2.98	0.40	3.10	0.19	5.00	0.41	6.44	0.53	35.96	1.77
MHRA	5	0.96	0.05	1.32	0.08	2.16	0.19	1.24	0.17	76.20	0.35	6.56	0.24	2.14	0.28	1.30	0.12	1.50	0.16	13.44	0.51	3.08	0.40	2.64	0.11	5.28	0.45	6.40	0.40	33.94	0.32
MHRA	6	0.86	0.13	1.40	0.16	1.94	0.11	1.24	0.11	75.58	0.38	5.94	0.23	1.66	0.15	1.30	0.12	1.32	0.08	12.74	0.26	2.66	0.11	2.76	0.11	5.00	0.38	6.30	0.14	33.46	0.50
MHRA	**Mean**	1.03		1.49		2.20		1.26		75.46		6.34		1.88		1.54		1.44		13.03		2.84		2.85		4.94		6.39		34.05	
	Intra SD	0.12		0.19		0.19		0.13		0.69		0.38		0.27		0.15		0.20		0.51		0.29		0.21		0.41		0.31		0.90	
	Inter SD	0.16		0.18		0.27		0.14		0.47		0.35		0.22		0.42		0.17		0.18		0.11		0.20		0.57		0.00		0.90	
	Total SD	0.20		0.26		0.33		0.19		0.83		0.52		0.35		0.45		0.26		0.54		0.31		0.29		0.71		0.31		1.27	
	Total CV	19.1%		17.7%		15.2%		15.1%		1.1%		8.2%		18.5%		29.0%		18.3%		4.1%		11.0%		10.3%		14.3%		4.8%		3.7%	

X

Analysis	Lab	U472C		A769G		A1003G		U1867C		C2493U;VP1-T6I		C2950U		C3640U		A3766G		A4171G		A4872G;2C-E253G		A4872C;2C-E253A		A4884G;2C-D257G		G4925A;2C-D271N		A4935G;2C-Q274R		U5832C;3B-I135T	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
MHRA	1	0.90	0.12	1.46	0.11	2.16	0.19	1.16	0.13	76.06	0.36	6.32	0.38	1.72	0.04	1.28	0.16	1.26	0.15	12.42	0.33	2.62	0.29	2.72	0.13	5.30	0.24	6.44	0.27	34.08	0.98
MHRA	2	0.92	0.08	1.44	0.25	2.12	0.18	1.30	0.24	76.06	0.73	6.36	0.49	1.82	0.15	1.18	0.19	1.26	0.17	12.50	0.47	3.22	0.25	2.70	0.57	5.28	0.24	6.12	0.41	33.16	1.13
MHRA	3	1.30	0.24	1.58	0.22	2.50	0.14	1.30	0.10	75.28	0.47	6.34	0.46	1.96	0.29	2.46	0.17	1.70	0.39	13.32	0.40	3.10	0.23	2.90	0.19	5.40	0.38	6.48	0.58	33.56	0.77
MHRA	4	1.34	0.17	1.82	0.40	2.48	0.15	1.68	0.34	74.80	1.08	7.12	0.42	2.24	0.11	1.62	0.16	1.68	0.28	12.90	0.45	2.80	0.25	2.88	0.29	4.94	0.30	6.68	0.72	35.84	0.91
MHRA	5																														
MHRA	6	1.00	0.00	1.46	0.11			1.20	0.00	75.76	0.36	5.80	0.20	1.64	0.05	1.34	0.11	1.28	0.08	12.78	0.22	2.70	0.19	2.68	0.11	5.00	0.32	6.32	0.22	33.24	0.38
MHRA	**Mean**	1.09		1.55		2.32		1.33		75.59		6.39		1.88		1.58		1.44		12.78		2.89		2.78		5.18		6.41		33.98	
	Intra SD	0.15		0.23		0.16		0.19		0.64		0.39		0.15		0.16		0.23		0.41		0.25		0.31		0.29		0.48		0.84	
	Inter SD	0.21		0.12		0.22		0.17		0.43		0.39		0.20		0.49		0.18		0.28		0.27		0.07		0.14		0.07		0.92	
	Total SD	0.25		0.26		0.27		0.26		0.77		0.55		0.25		0.51		0.29		0.50		0.36		0.31		0.32		0.48		1.25	
	Total CV	23.1%		16.8%		11.8%		19.4%		1.0%		8.6%		13.5%		32.6%		20.5%		3.9%		12.6%		11.3%		6.2%		7.5%		3.7%	

Table 7. Type 3 blind duplicate P and X mean ratio analysis.

Blind duplicate analysis (P/X)	Labs					
	1	2	3	4	5	6
Mean Ratios whole genome SNPs	1.01	0.98	1.00	0.98	1.00	0.99
p-values from theoretical value One	0.52	0.37	0.81	0.26	0.97	0.30

Table 8. Summary of Type 1 480A+525C estimates for the collaborative study samples.

Analysis	Lab	E		B		I		F		D		C		G		H		A		J	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
MHRA	1	2.68	0.20	2.32	0.08	2.28	0.31	1.66	0.09	1.64	0.11	1.22	0.11	1.00	0.10	1.02	0.16	0.00	0.00	0.00	0.00
MHRA	2	2.58	0.13	2.10	0.07	2.06	0.17	1.46	0.18	1.44	0.09	1.04	0.11	0.92	0.22	0.86	0.09	0.00	0.00	0.00	0.00
MHRA	3	3.12	0.52	2.66	0.58	2.74	0.46	1.92	0.42	2.04	0.19	1.50	0.46	1.20	0.17	1.10	0.10	0.00	0.00	0.00	0.00
MHRA	4	3.06	0.38	2.94	0.35	2.62	0.45	2.00	0.51	1.62	0.11	1.36	0.27	1.12	0.18	1.10	0.12	0.00	0.00	0.00	0.00
MHRA	5	2.96	0.42	2.52	0.90	2.36	0.11	1.98	0.19	1.98	0.16	1.34	0.19	2.06	0.87	1.04	0.11	0.38	0.11	0.00	0.00
MHRA	6	2.68	0.24	2.20	0.17	2.36	0.11	1.82	0.13	1.58	0.11	1.10	0.20	0.98	0.05	1.04	0.11	0.08	0.08	0.00	0.00
MHRA	**Mean**	**2.85**		**2.46**		**2.40**		**1.81**		**1.72**		**1.26**		**1.21**		**1.03**		**0.08**		**0.00**	
	Intra SD	**0.34**		**0.48**		**0.31**		**0.30**		**0.14**		**0.25**		**0.39**		**0.12**					
	Inter SD	**0.17**		**0.22**		**0.20**		**0.16**		**0.23**		**0.13**		**0.39**		**0.07**					
	Total SD	**0.38**		**0.53**		**0.37**		**0.34**		**0.27**		**0.29**		**0.56**		**0.14**					
	Total CV	**13.4%**		**21.7%**		**15.3%**		**18.8%**		**15.6%**		**22.6%**		**45.8%**		**13.5%**					
	n	**6**		**6**		**6**		**6**		**6**		**6**		**6**		**6**					
Swarm	**Mean**	**2.65**		**2.22**		**2.22**		**1.70**		**1.62**		**1.19**		**1.13**		**0.95**		**0.17**		**0.17**	
	% of MHRA	**93.2%**		**90.5%**		**92.2%**		**93.9%**		**94.6%**		**94.3%**		**93.0%**		**93.0%**					

Table 9. Statistical analysis of 480A+525C estimates for the coded duplicate Type 1 samples B and I.

Coded duplicate (samples B & I) final analysis					
Variance components (as SD):			Variance components (as CV):		
Intra-assay		0.40	Intra-assay		16.6%
Intra-lab (inter-assay)		0.00	Intra-lab (inter-assay)		0.0%
Inter-lab		0.24	Inter-lab		9.9%
Total		0.47	Total		19.3%
Final mean estimate:		2.43			
95% LCL:		2.33			
95% UCL:		2.54			

Table 10. Whole genome analysis for sample A and J.

Analysis	Lab	A								J							
		G480A		U525C		U5350C		U6187C		G480A		U525C		U5350C		U6187C	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
MHRA	1	0.00	0.00	0.00	0.00	99.98	0.04	99.88	0.04	0.00	0.00	0.00	0.00	99.66	0.54	99.64	0.69
MHRA	2	0.00	0.00	0.00	0.00	100.00	0.00	100.00	0.00	0.00	0.00	0.00	0.00	100.00	0.00	99.98	0.04
MHRA	3	0.00	0.00	0.00	0.00	99.88	0.16	99.92	0.04	0.00	0.00	0.00	0.00	99.92	0.08	99.88	0.04
MHRA	4	0.00	0.00	0.00	0.00	99.98	0.04	99.96	0.05	0.00	0.00	0.00	0.00	99.86	0.05	99.94	0.05
MHRA	5	0.18	0.11	0.20	0.00	99.98	0.04	100.00	0.00	0.00	0.00	0.00	0.00	99.90	0.00	100.00	0.00
MHRA	6	0.00	0.00	0.08	0.08	99.98	0.04	100.00	0.00	0.00	0.00	0.00	0.00	99.98	0.04	100.00	0.00
MHRA	Mean	0.03		0.05		99.97		99.96		0.00		0.00		99.89		99.91	

Table 11. Summary of sample B and I estimates from whole genome profile of selected mutations.

B	Lab	G480A		U525C		C661U		T691A		C1941A;VP3-A59E		U4393C		G7303A	
		Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
MHRA	1	1.20	0.07	1.12	0.08	1.16	0.11	0.98	0.04	1.06	0.11	1.10	0.10	1.90	0.16
MHRA	2	1.20	0.07	0.90	0.07	1.06	0.11	1.10	0.07	1.54	0.09	1.14	0.09	1.92	0.26
MHRA	3	1.32	0.39	1.34	0.23	1.06	0.11	1.10	0.07	1.54	0.09	1.44	0.11	1.92	0.26
MHRA	4	1.62	0.37	1.22	0.13	1.70	0.16	1.46	0.17	1.30	0.19	1.26	0.23	2.30	0.32
MHRA	5	1.54	0.38	0.98	0.53	2.44	1.41	0.42	0.26	1.86	1.68	0.92	0.62	0.42	0.13
MHRA	6	1.20	0.12	1.04	0.11	1.12	0.08	0.92	0.08	1.18	0.08	1.18	0.04	2.26	0.42
MHRA	**Mean**	1.35		1.10		1.42		1.00		1.41		1.17		1.79	
	Intra SD	0.28		0.25		0.58		0.14		0.69		0.28		0.28	
	Inter SD	0.14		0.12		0.49		0.33		0.00		0.12		0.68	
	Total SD	0.31		0.28		0.76		0.36		0.69		0.30		0.74	
	Total CV	23.1%		25.0%		53.5%		36.2%		48.7%		26.0%		41.2%	

I	Lab	G480A		U525C		C661U		U691A		C1941A;VP3-A59E		U4393C		G7303A	
		Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
MHRA	1	1.24	0.18	1.04	0.18	1.22	0.31	0.98	0.13	0.96	0.15	1.00	0.23	2.42	0.78
MHRA	2	1.14	0.09	0.92	0.08	1.10	0.10	1.02	0.13	1.34	0.13	1.14	0.15	1.94	0.36
MHRA	3	1.48	0.23	1.26	0.33	1.44	0.13	1.40	0.30	0.74	0.34	1.32	0.04	2.20	0.17
MHRA	4	1.26	0.18	1.36	0.30	1.48	0.31	1.40	0.19	1.16	0.32	1.28	0.16	2.46	0.13
MHRA	5	1.28	0.08	1.08	0.04	1.38	0.11	1.26	0.05	1.18	0.08	1.36	0.05	2.00	0.07
MHRA	6	1.24	0.05	1.12	0.11	1.08	0.16	0.98	0.31	1.28	0.15	1.08	0.08	2.08	0.16
MHRA	**Mean**	1.27		1.13		1.28		1.17		1.11		1.20		2.18	
	Intra SD	0.15		0.20		0.21		0.21		0.22		0.14		0.37	
	Inter SD	0.09		0.13		0.15		0.18		0.20		0.13		0.14	
	Total SD	0.18		0.24		0.26		0.28		0.30		0.19		0.39	
	Total CV	13.8%		21.4%		19.9%		23.5%		26.7%		16.0%		18.1%	

Table 12. Type 1 blind duplicate B and I mean ratio analysis.

Blind duplicate analysis (B/I)	Labs					
	1	2	3	4	5	6
Mean Ratios whole genome SNPs	1.05	1.04	1.01	1.09	0.96	1.01
p-values from theoretical value One	0.30	0.16	0.88	0.144	0.41	0.65

Table 13. Summary of of sample K and L estimates from whole genome profile of selected mutations of nOPV2 drug substance.

K	Lab	C930U;VP4-A41V		G2528A;VP3-E234K		A2639G;VP1-S33G		C2844A;VP1-A101D		U2970C;VP1-I143T		A3053G;VP1-N171D		G3425A;VP1-E295K	
		Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
MHRA	1	2.92	0.58	17.90	1.01	3.02	0.08	5.16	0.50	1.98	0.26	22.52	0.36	29.52	1.94
MHRA	2	3.52	0.38	22.14	0.60	2.50	0.21	4.42	0.25	1.30	0.16	15.06	0.46	20.04	0.77
MHRA	3	3.34	0.55	17.50	0.56	2.18	0.34	5.20	0.86	1.96	0.09	21.02	1.19	28.66	0.80
MHRA	4	2.84	0.54	19.38	1.00	2.28	0.16	5.20	0.14	1.56	0.25	21.94	1.26	27.92	1.33
MHRA	5	3.86	0.27	16.88	0.63	2.48	0.25	4.74	0.52	2.04	0.34	19.26	1.30	26.90	1.37
MHRA	6	3.12	0.15	16.76	0.43	2.48	0.24	4.78	0.11	1.62	0.08	20.14	0.36	26.34	0.36
MHRA	**Mean**	3.27		18.43		2.49		4.92		1.74		19.99		26.56	
	Intra SD	0.44		0.74		0.23		0.48		0.22		0.93		1.21	
	Inter SD	0.33		2.02		0.27		0.24		0.28		2.66		3.35	
	Total SD	0.55		2.15		0.36		0.53		0.35		2.81		3.56	
	Total CV	16.9%		11.7%		14.3%		10.9%		20.3%		14.1%		13.4%	

L	Lab	C930U;VP4-A41V		G2528A;VP3-E234K		A2639G;VP1-S33G		C2844A;VP1-A101D		U2970C;VP1-I143T		A3053G;VP1-N171D		G3425A;VP1-E295K	
		Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
MHRA	1	3.12	0.44	17.92	1.29	2.70	0.31	5.10	0.12	1.70	0.32	23.00	1.37	28.88	0.88
MHRA	2	3.22	0.04	17.34	0.48	2.86	0.17	4.60	0.20	1.74	0.17	20.84	0.32	26.04	0.30
MHRA	3	3.14	0.38	18.66	0.82	2.32	0.31	4.66	0.47	1.90	0.42	21.54	0.96	29.50	1.44
MHRA	4	3.06	0.29	18.06	0.61	2.76	0.11	4.58	0.19	2.14	0.11	22.38	1.17	29.64	1.04
MHRA	5	3.40	0.30	17.10	2.47	2.54	0.30	4.92	0.61	1.70	0.12	19.36	2.83	25.92	3.01

MHRA	6	3.00	0.07	16.64	0.38	2.54	0.19	4.78	0.38	1.70	0.10	18.60	0.22	24.98	0.67
MHRA	**Mean**	3.16		17.62		2.62		4.77		1.81		20.95		27.49	
	Intra SD	0.29		1.24		0.24		0.37		0.24		1.43		1.50	
	Inter SD	0.05		0.48		0.16		0.12		0.14		1.59		1.96	
	Total SD	0.30		1.33		0.29		0.39		0.28		2.14		2.47	
	Total CV	9.5%		7.5%		11.1%		8.2%		15.4%		10.2%		9.0%	

Table 14. Summary of sample M and N estimates from whole genome profile of selected mutations of nOPV2 drug substance.

M	Lab	C930U;VP4-A41V		G2528A;VP3-E234K		A2639G;VP1-S33G		C2844A;VP1-A101D		U2970C;VP1-I143T		A3053G;VP1-N171D		G3425A;VP1-E295K	
		Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
MHRA	1	3.08	0.15	22.94	1.06	2.40	0.26	4.38	0.35	1.64	0.26	16.60	0.46	22.74	1.23
MHRA	2	3.52	0.38	22.14	0.60	2.50	0.21	4.42	0.25	1.30	0.16	15.06	0.46	20.04	0.77
MHRA	3	3.22	0.22	22.90	0.64	2.28	0.40	4.24	0.44	1.68	0.16	15.86	1.25	22.76	0.93
MHRA	4	2.98	0.24	24.98	0.73	2.44	0.23	3.90	0.46	1.48	0.16	16.68	0.61	21.98	1.68
MHRA	5	3.72	0.34	20.64	3.00	2.32	0.38	4.46	0.32	1.40	0.33	13.18	0.52	20.54	0.83
MHRA	6	3.14	0.18	21.78	0.47	2.24	0.18	4.06	0.13	1.44	0.09	14.48	0.33	19.74	0.34
MHRA	**Mean**	3.28		22.56		2.36		4.24		1.49		15.31		21.30	
	Intra SD	0.26		1.39		0.28		0.34		0.21		0.68		1.05	
	Inter SD	0.26		1.32		0.00		0.16		0.11		1.32		1.28	
	Total SD	0.37		1.92		0.28		0.38		0.24		1.48		1.65	
	Total CV	11.3%		8.5%		11.8%		8.9%		15.9%		9.7%		7.8%	

N	Lab	C930U;VP4-A41V		G2528A;VP3-E234K		A2639G;VP1-S33G		C2844A;VP1-A101D		U2970C;VP1-I143T		A3053G;VP1-N171D		G3425A;VP1-E295K	
		Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
MHRA	1	3.12	0.20	22.90	0.72	2.36	0.15	4.22	0.19	1.50	0.16	16.24	0.68	22.82	1.01
MHRA	2	3.52	0.20	23.44	0.77	2.34	0.40	3.72	0.37	1.40	0.21	15.52	0.73	21.94	0.90

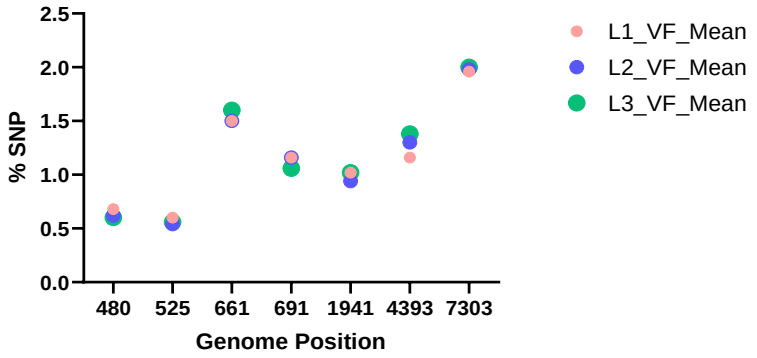
MHRA	3	3.26	0.26	23.74	0.61	2.18	0.19	4.20	0.28	1.38	0.19	16.44	0.46	22.46	0.83
MHRA	4	3.38	0.49	23.92	2.10	2.34	0.27	4.68	0.40	1.54	0.09	15.80	1.04	22.74	1.31
MHRA	5	3.48	0.39	21.02	3.41	2.36	0.35	3.68	0.48	1.74	0.21	14.70	1.85	21.18	1.03
MHRA	6	3.14	0.15	22.22	0.61	2.14	0.33	4.16	0.17	1.36	0.09	14.50	0.37	20.00	0.43
MHRA	**Mean**	3.32		22.87		2.29		4.11		1.49		15.53		21.86	
	Intra SD	0.31		1.73		0.28		0.33		0.17		0.99		0.96	
	Inter SD	0.10		0.78		0.00		0.34		0.12		0.66		1.01	
	Total SD	0.32		1.89		0.28		0.48		0.21		1.19		1.39	
	Total CV	9.7%		8.3%		12.4%		11.6%		13.9%		7.7%		6.4%	

Table 15. Accelerated and real-time degradation studies.

Accelerated Degradation Point	Temperature (°C)	Assessment of Viral genome integrity for 23/174 by performing poliovirus whole genome PCR
1 Week	-20	Detected
	4	Detected
	25	Detected
	37	Not Detected
2 Week	-20	Detected
	4	Detected
	25	Detected
1 Month	-20	Detected
	4	Detected
	25	Not Detected
3 Month	-20	Detected
	4	Detected
6 Month	-20	Detected
	4	Detected

Real-time Degradation (Ongoing)	Temperature (°C)	Assessment of Viral genome integrity for 23/160, 23/162, 23/172 and 23/174 by performing poliovirus whole genome PCR
1 Week	-80 and -20	Detected
1 Month	-80 and -20	Detected
6 Month	-80 and -20	Detected
12 Month	-80 and -20	Detected

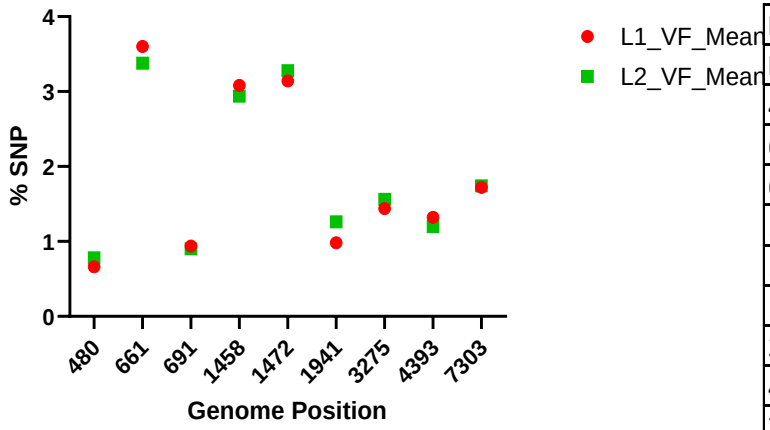
Figure 1: Consecutive lot testing of Type 1 OPV vaccines: Manufacturer 1.



Manufacturer 1_Type 1_Vaccine				Lot1	Lot2	Lot3	Between lots
Position	Change	Product	AA_Change	L1_Mean_VF%	L2_Mean_VF%	L3_Mean_VF%	SD
480	G->A			0.68	0.62	0.60	0.04
525	U->C			0.60	0.54	0.56	0.03
661	C->U			1.50	1.50	1.60	0.06
691	U->A			1.16	1.16	1.06	0.06
1941	C->A	VP3	A->E	1.02	0.94	1.02	0.05
4393	T->C	2C		1.16	1.30	1.38	0.11
7303	G->A	3D		1.96	1.98	2.00	0.02

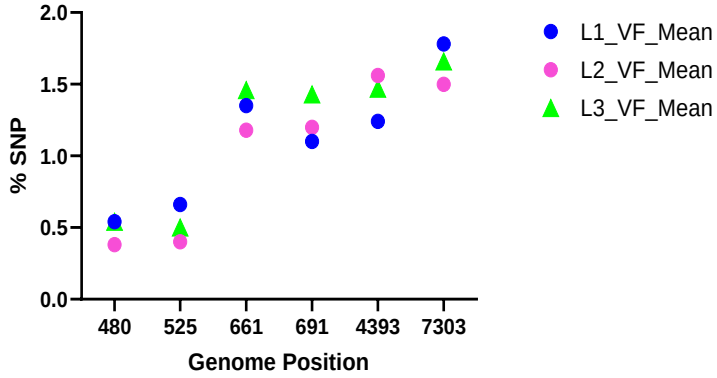
L: Lot number, VF: Variant frequency, AA Change: Amino Acid change, SD; Standard Deviation.

Figure 2: Consecutive lot testing of Type 1 OPV vaccines: Manufacturer 2.



Manufacturer 2_Type1_Vaccine				Lot1	Lot2	Between lots
Position	Change	Product	AA_Change	L1_Mean_VF%	L2_Mean_VF%	SD
480	G->A			0.66	0.78	0.08
661	C->U			3.60	3.38	0.16
691	U->A			0.94	0.90	0.03
1458	C->U	VP2	P->L	3.08	2.94	0.10
1472	U->C	VP2	C->R	3.14	3.28	0.10
1941	C->G	VP3	A->G	0.98	1.26	0.20
3275	A->G	VP1	I->V	1.44	1.56	0.08
4393	U->C	2C		1.32	1.20	0.08
7303	G->A	3D		1.72	1.74	0.01

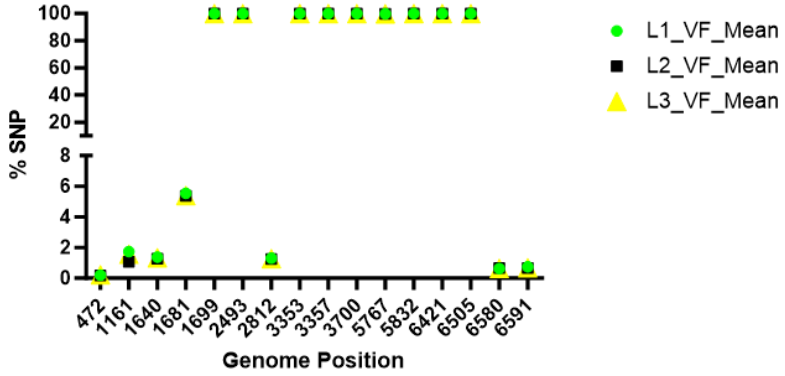
Figure 3: Consecutive lot testing of Type 1 OPV vaccines: Manufacturer 3



Manufacturer 3_Type1_Vaccine				Lot 1	Lot 2	Lot 3	Between Lots
Position	Change	Product	AA_Change	L1_Mean_VF%	L2_Mean_VF%	L3_Mean_VF%	SD
480	G -> A			0.54%	0.38%	0.54%	0.09
525	U -> C			0.66%	0.40%	0.50%	0.13
661	C -> U			1.35%	1.18%	1.46%	0.14
691	U -> A			1.10%	1.20%	1.43%	0.17
4393	U -> C	2C		1.24%	1.56%	1.47%	0.16
7303	G -> A	3D		1.78%	1.50%	1.68%	0.14

L: Lot number, VF: Variant frequency, AA Change: Amino Acid change, SD; Standard Deviation

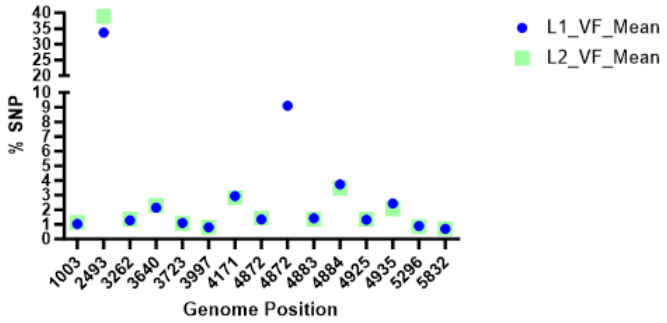
Figure 4: Consecutive lot testing of Type 3 OPV vaccines: Manufacturer 1



Manufacturer 1_Type 3_Vaccine				Vaccine Lot1	Vaccine Lot2	Vaccine Lot3	Between Lots
Position	Change	Product	AA_Change	L1_Mean_VF%	L2_Mean_VF%	L3_Mean_VF%	SD
472	U -> C			0.20%	0.18%	0.23%	0.03
1161	G -> A	VP2		1.74%	1.08%	1.56%	0.34
1640	C -> U	VP2		1.38%	1.28%	1.34%	0.05
1681	U -> C	VP2		5.54%	5.38%	5.40%	0.09
1699	G -> A	VP2		99.84%	99.84%	99.84%	0.00
2493	C -> U	VP1	T -> I	99.96%	99.86%	99.92%	0.05
2812	U -> C	VP1		1.30%	1.24%	1.28%	0.03
3353	U -> C	VP1	S -> P	99.94%	99.92%	99.92%	0.01
3357	A -> G	VP1	E -> G	99.92%	99.92%	99.86%	0.03
3700	C -> U	2A		99.92%	99.94%	99.92%	0.01
5767	U -> C	3B		99.76%	99.70%	99.74%	0.03
5832	U -> C	3B	I -> T	99.96%	99.92%	99.96%	0.02
6421	C -> U	3D		99.90%	99.90%	99.90%	0.00
6505	U -> A	3D		99.86%	99.88%	99.92%	0.03
6580	U -> C	3D		0.62%	0.66%	0.64%	0.02
6591	U-> C	3D	V -> A	0.74%	0.66%	0.68%	0.04

L: Lot number, VF: Variant frequency, AA Change: Amino Acid change, SD; Standard Deviation

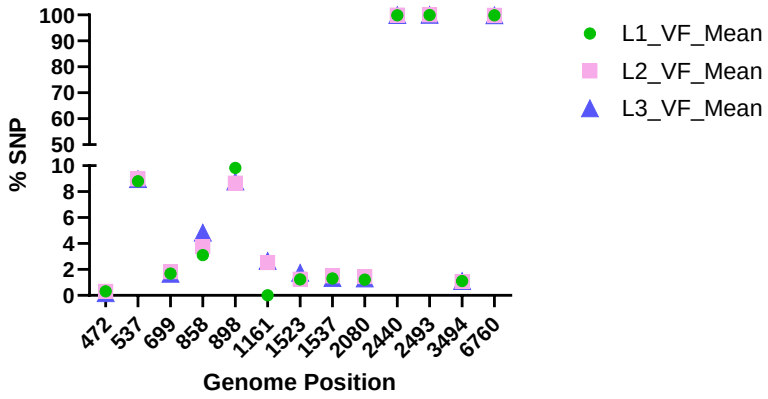
Figure 5: Consecutive lot testing of Type 3 OPV vaccines: Manufacturer 2



Manufacturer 2_Type3_Vaccine				Vaccine Lot 1	Vaccine Lot 2	Between lots
Position	Change	Product	AA_Change	VF_Mean	VF Mean	SD
1003	A -> G	VP2		1.04%	1.14%	0.07
2493	C -> U	VP1	T -> I	33.72%	38.92%	3.68
3262	C -> U	VP1		1.28%	1.34%	0.04
3640	C -> U	2A		2.14%	2.28%	0.10
3723	A -> G	2A	Q -> R	1.10%	1.06%	0.03
3997	C -> U	2B		0.80%	0.80%	0.00
4171	A -> G	2C		2.94%	2.82%	0.08
4872	A -> C	2C	E -> A	1.34%	1.44%	0.07
4872	A -> G	2C	E -> G	9.10%	11.08%	1.40
4883	G -> A	2C	D -> N	1.42%	1.34%	0.06
4884	A -> G	2C	D -> G	3.74%	3.48%	0.18
4925	G -> A	2C	D -> N	1.32%	1.34%	0.01
4935	A -> G	2C	Q -> R	2.44%	2.08%	0.25
5296	A -> G	3A		0.90%	0.84%	0.04
5832	U -> C	3B	I -> T	0.70%	0.72%	0.01

L: Lot number, VF: Variant frequency, AA Change: Amino Acid change, SD; Standard Deviation

Figure 6: Consecutive lot testing of Type 3 OPV vaccines: Manufacturer 3



Manufacturer 3_Type3_Vaccine				Lot1	Lot2	Lot3	Between lot
Position	Change	Product	AA_Change	L1_Mean_VF%	L2_Mean_VF%	L3_Mean_VF%	SD
472	U-> C			0.32%	0.32%	0.18%	0.08
537	G -> A			8.80%	8.98%	8.96%	0.10
699	C -> U			1.68%	1.82%	1.66%	0.09
858	A -> G	VP4	N -> S	3.10%	3.76%	4.84%	0.88
898	A -> G	VP4		9.82%	8.66%	8.78%	0.64
1161	G -> A	VP2		2.34	2.54%	2.66%	0.08
1523	U-> C	VP2	Y -> H	1.23%	1.25%	1.74%	0.29
1537	C -> U	VP2		1.30%	1.50%	1.34%	0.11
2080	U -> C	VP3		1.22%	1.45%	1.30%	0.12
2440	A -> U	VP3		99.88%	99.92%	99.92%	0.02
2493	C -> U	VP1	T -> I	99.98%	100.00%	100.00%	0.01
3494	U-> A	2A	L -> M	1.10%	1.07%	1.10%	0.02
6760	A -> U	3D	R -> S	99.88%	99.90%	99.88%	0.01

L: Lot number, VF: Variant frequency, AA Change: Amino Acid change, SD; Standard Deviation

Figure 7: Whole-genome profile of selected mutation of Candidate 23/172 (O&Y)

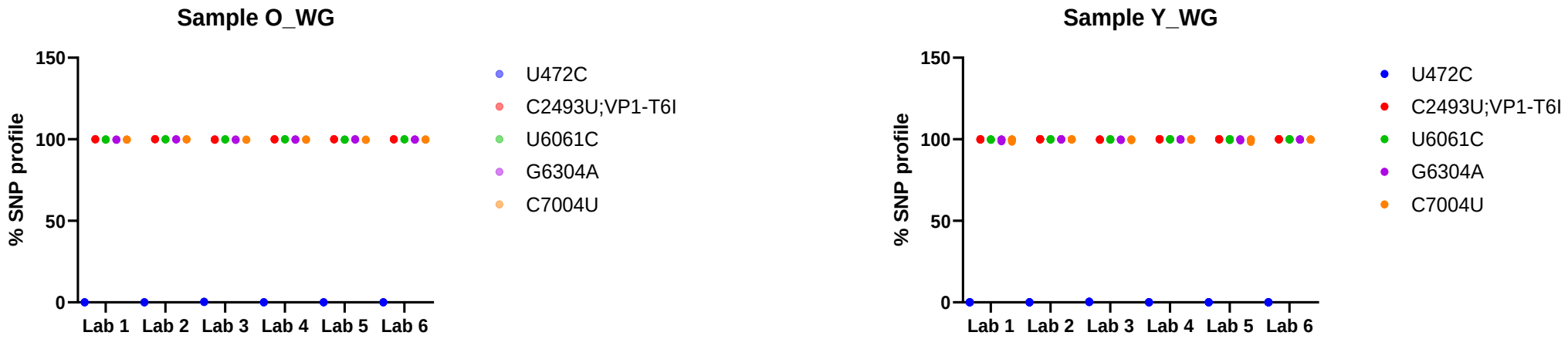
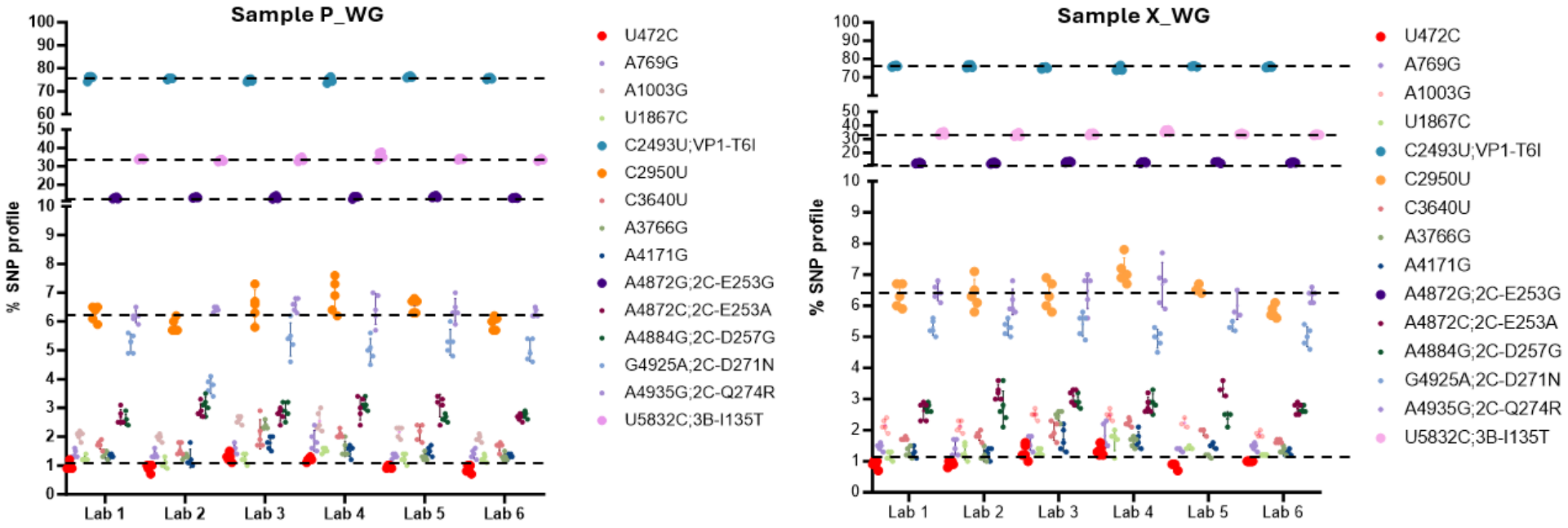


Figure 8: Whole-genome profile of selected mutation of Candidate 23/174 (P&X)



Final Estimates	C2493U	U5832C	A4872G	C2950U	A4935G	G4925A	A4872C	A4884G	A1003G	C3640U	A3766G	A769G	A4171G	U1867C	U472C
	75.56	34.00	12.93	6.38	6.36	5.08	2.90	2.78	2.22	1.88	1.52	1.50	1.45	1.30	1.04

Figure 9: Ratio between mean estimate SNPs of blind-duplicate sample 23/174 (P&X)

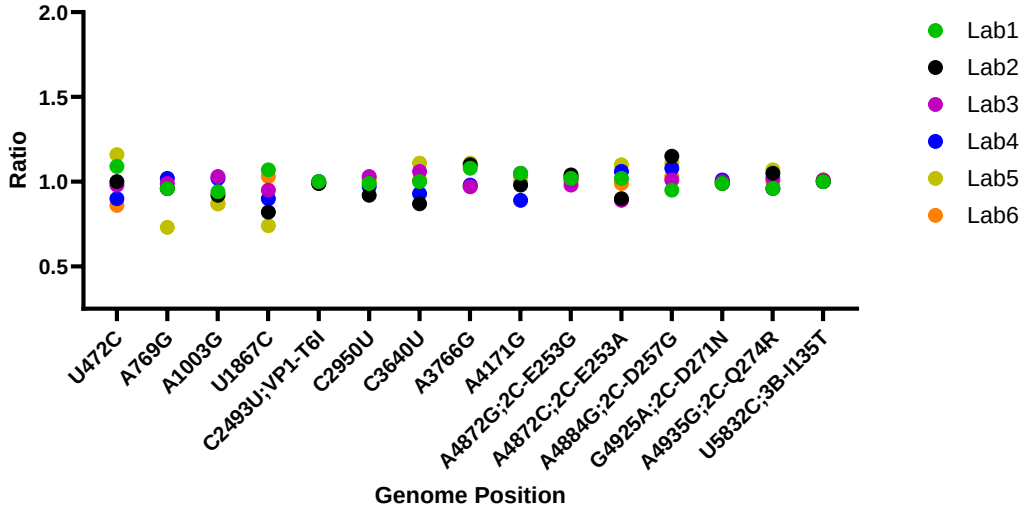


Figure 10: Whole-genome profile of selected mutation of Candidate 23/160 (A&J)

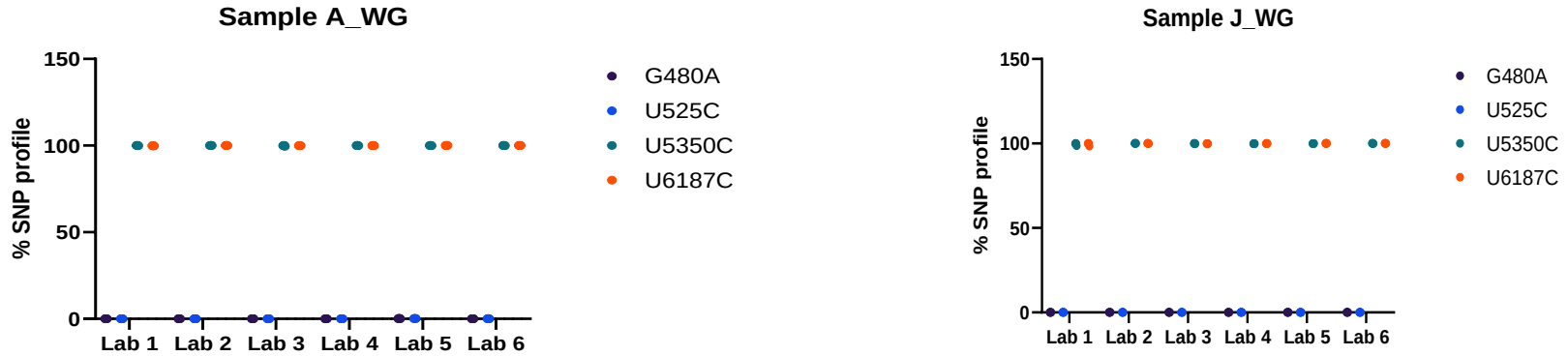


Figure 11: Ratio between mean estimate SNPs of blind-duplicate sample 23/162 (B&I)

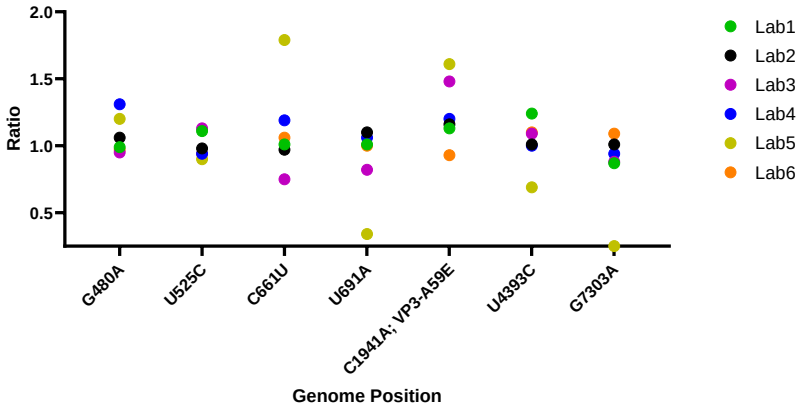


Figure 12: Ratio between mean estimate SNPs of blind-duplicate sample K and L:

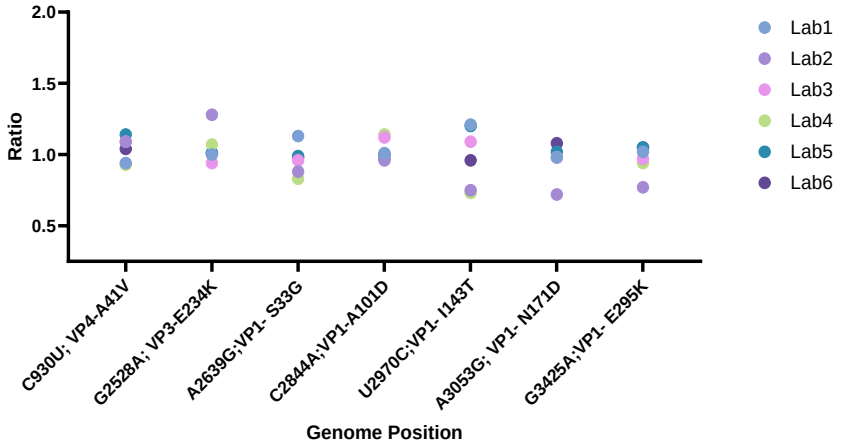


Figure 13: Ratio between mean estimate SNPs of blind-duplicate sample M and N

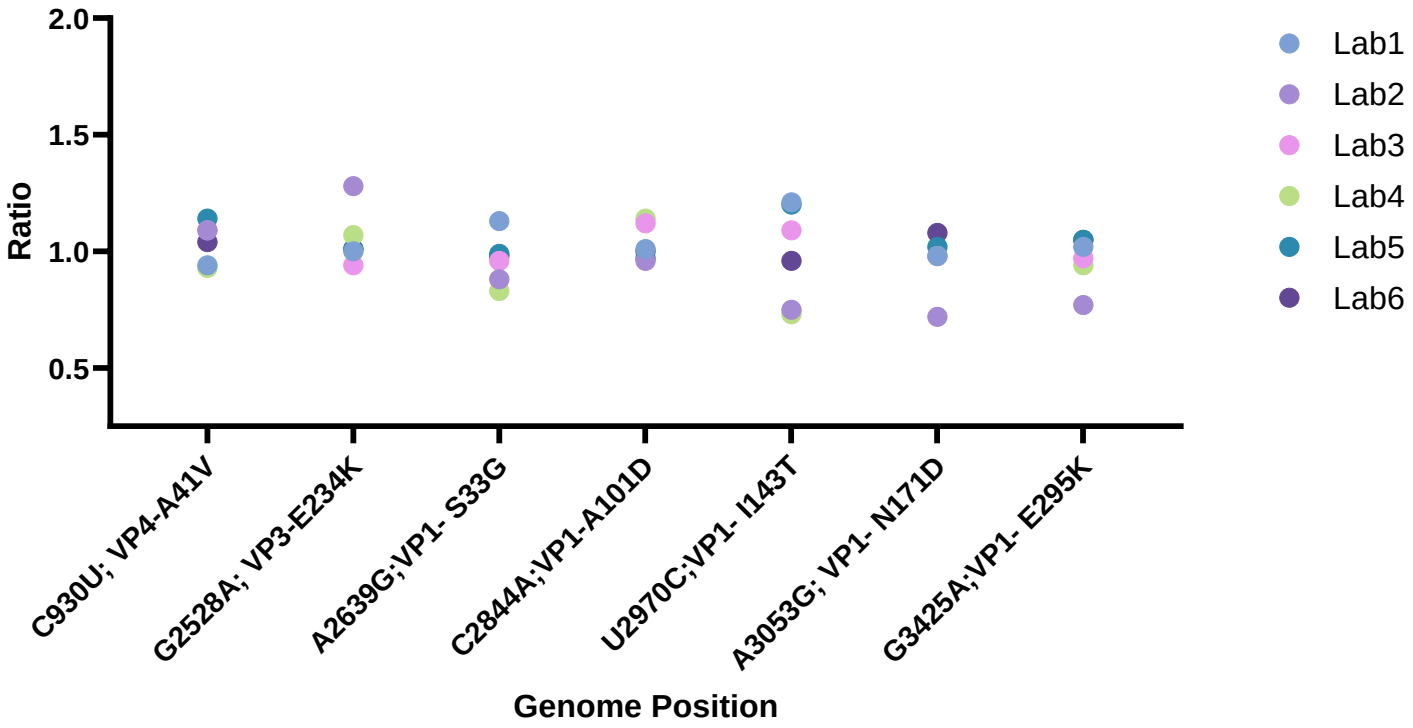
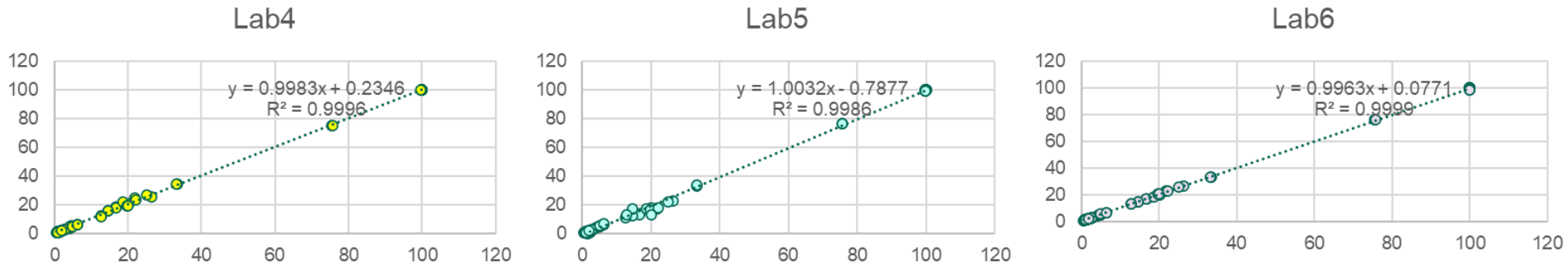


Figure 14: Concordance graphs for all data points from labs 4, 5 and 6 versus MHRA analysis. SNP profile obtained from MHRA analysis is plotted on X-axis and that from the individual labs is plotted on Y-axis.



Appendix 1

RNA extraction, whole genome amplification and indexed library preparation from oral poliovirus vaccines

1. Purpose

This Standard Operating Procedure (SOP) provides an example workflow for extracting RNA from vaccine lots and bulks using commercially available kits, followed by the preparation of indexed libraries to perform high-throughput sequencing (HTS) of poliovirus vaccines.

This SOP is intended as a representative guide only and is not restricted to the specific reagents, kits, or sequencing systems described herein. It has been developed to provide laboratories with a starting framework for planning and implementing HTS capabilities within their facilities. Laboratories may adapt, modify, or optimize this SOP as necessary to suit their specific requirements, available resources, and applicable regulatory considerations.

2. Responsibilities

- Live type 2 vaccine candidates shall be handled at Containment Level 3 (CL3) in accordance with the GAPIV guidelines and the requirements of the local biosafety committee.
- Type 1 and Type 3 vaccine candidates may be handled at Containment Level 2 (CL2) following approval from the local biosafety committee.

3.1 Equipment, Materials, and reagents Needed

- Pipettes and tips (sizes:10, 20, 200, 1000 µl)
- Centrifuge (Eppendorf, San Diego, CA): 5417R
- Thermal Cycler (Eppendorf: Mastercycler Nexus gradient)
- Lo-Bind microcentrifuge tubes (volumes: 200, 500, 1500 µl)
- Nuclease Free water (Promega: P119C)
- RT-PCR grade Water (Invitrogen: AM9935)
- Roche High Pure Viral RNA Kit (Product No: 11858882001)
- Ethanol BioUltra, for molecular biology, ≥99.8% Sigma (Cat: 51976)
- Qubit 4 Fluorometer (Thermofisher Scientific) Broad Range and High Sensitivity reagents (Q32850)
- Qubit® Assay Tubes (Q32856)
- SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity DNA Polymerase (12574035)
- Specific primers for full-length amplification of poliovirus (See Section: 4.4)
- Agencourt AMPure XP Reagent for PCR Purification (cat# A63880 / A63881 / A63882)
- 96-Well Microtiter Plate Magnetic Separation Rack (Sigma: AM10027)

- 0.8 ml U-bottom 96-well Plates (Thermofisher, cat # 12194162)
- Electrophoresis Chamber
- Gels for electrophoresis:
  1% Agarose (Bioline: BIO-41027), 1X TBE Buffer (In-house) + Gel Red (Biotium, cat# 41003)
- DNA Ladder:1 KB (Promega Cat # G571A), Loading Dye (Qiagen cat# 239901)

3.1.1 Recommended/example equipment, materials and reagents

3.1.1.1 Example library preparation and index kits

- Illumina DNA Prep, (M) Tagmentation (IPB) (24 samples Illumina, cat # 20060060) (96 samples Illumina, cat # 20060059)
- Illumina® DNA/RNA UD Indexes Set A, Tagmentation (96 Indexes, 96 Samples) (Illumina, cat # 20091654) *Note: Sets B, C, and D are also compatible along with Nextera™ DNA CD Indexes (96 Indexes, 96 Samples) (Illumina, cat # 20018708)*

OR

- Nextera XT DNA Library Preparation Kit (24 samples, Illumina cat # FC-131-1024) (96 samples, Illumina cat # FC-131-1096) *Note: Indexes provided above should also be compatible with this kit.*

3.1.1.2 Example automated electrophoresis platforms and kits

- 2100 Bioanalyzer Instrument (Agilent Technologies Inc.)
- **High Sensitivity DNA Kit (**Agilent Technologies**,** cat #5067-4626)

OR

- 4200 TapeStation Bioanalyzer Instrument (Agilent Technologies Inc., G2991BA) or 4150 Instrument (Agilent Technologies Inc., G2992AA)
- DNA ScreenTape Analysis: D5000 ScreenTape (Agilent Technologies, 5067-5588) and D5000 Reagents (Agilent Technologies, 5067-5589)

OR

- High Sensitivity DNA ScreenTape Analysis: High Sensitivity D5000 ScreenTape (Agilent Technologies, 5067-5592) and High Sensitivity D5000 Reagents (Agilent Technologies, 5067-5593)

3.1.1.3 Example sequencing platform, kits and reagents

- Illumina MiSeq Sequencing System (Illumina)
- MiSeq Reagent Kit v2 (500-cycles) (Illumina, cat # MS-102-2003)
- Illumina PhiX Control (Illumina, cat # FC-110-3001)

Note: Depending on library preparation kit used, you may need additional non-Illumina reagents; see below:

- 1 N NaOH, molecular biology grade (General lab supplier)

3.2. Extraction of viral RNA using Roche High Pure Viral RNA Kit

- Thaw poliovirus vaccine tubes on ice. Once thawed gently vortex, centrifuge and place them back on ice.
- Add 400 µl of binding buffer supplemented with polyA and proteinase K to each 1.5ml microcentrifuge tube (MCT).
- Add 200 µl of thawed poliovirus vaccine into individual 1.5 ml MCT and incubate the mixture at room temperature for 10 mins. Gently vortex and briefly centrifuge.
- To transfer the sample to a High Pure Filter Tube assembly:
- insert one High Pure Filter column in one Collection Tube.
- Pipette entire sample mixture into the upper reservoir of the Filter Tube
- Centrifuge the tube assembly for 1 min at 8,000 × g.
- Remove the Filter Tube from the Collection Tube, discard the flowthrough liquid, and the Collection Tube. Insert the Filter Tube into a new Collection Tube.
- Add 500 µl Inhibitor Removal Buffer to the upper reservoir of the Filter Tube assembly and centrifuge 1 min at 8,000 × g.
- Discard flowthrough and place the Filter Tube on a new Collection Tube.
- Add 450 µl Wash Buffer to the upper reservoir of the Filter Tube.
- Centrifuge 1 min at 8,000 × g and discard the flowthrough and place the Filter Tube on a new Collection Tube.
- Repeat wash step twice.
- Place the Filter tube onto a new collection tube and centrifuge at maximum speed for 1 min (approximately 13,000 × g) to remove any residual Wash Buffer.
- Discard the Collection Tube and insert the Filter Tube into a clean, sterile 1.5 ml MCT.
- Add 50µL Elution Buffer to the upper reservoir of the Filter Tube, wait for 5 minutes.
- Centrifuge the tube assembly for 1 min at 8,000 × g.
- Discard the filter tube, properly label the MCT place it on ice for downstream processing/ transfer to -80°C for future use.

- Other RNA extraction kits commonly used in labs: QIAamp Viral RNA Kits for RNA Extraction (Catalogue number: 52904), MagMAX™ Viral RNA Isolation Kit (Catalogue number: AM1939)

3.3. Preparation of Poliovirus whole genome PCR products using Pan-Poliovirus primers and Superscript III One-Step RT-PCR System

Prepare a Master mix in a 1.5 ml lobind MCT tube using reaction volumes as detailed below:

(For primer details refer to table 1)

	1 Reaction (µL)	Reactions
2x Master Mix	12.5	µL
SSIII Platinum Taq mix (HiFi)	1	µL
Reverse Primer (10 mM)	1	µL

Nuclease free Water	4.5	µL
Total volume	19	

- Briefly vortex and centrifuge down. Aliquot 19 µL of master mix into each 0.2 ml PCR tube and add 5 µL of eluted RNA

- Add 19 µL of master mix to an additional PCR tube with 5 µL nuclease free water to use as a negative control

- Incubate at 50 °C for 30 minutes in Thermocycler.

- Add 1 µL of the forward primer to the individual tubes.

- Amplify using the following cycling conditions:

CYCLE	STEP	TEMP (°C)	TIME
1	Initial Denaturation	94	2 minutes
30	Denaturation	94	15 seconds
	Annealing	55	30 seconds
	Extension	68	8 minutes
1	Final Extension	68	5 minutes
-	Hold	10	-

3.4. Gel Identification

- 1% 1x TBE Agarose gel containing 1xGelRed
- Load 2 µl PCR product+ 2 µl loading dye
- Expected image: **Specific band around 7Kb (Visualization of the 7 Kb whole genome product is an important Quality control step. Absence of full-length product, appearance of multiple non-specific bands, appearance of smear suggests bad quality RNA this can affect the final outcome (quantitation of single nucleotide polymorphism (SNP) profiles).**

3.5. Purification of PCR products

- Use x1 volume of Agencourt AMPure XP Reagent [beads] (Beckman Coulter, Inc) to the PCR reaction mix for the isolation of DNA from a total PCR mix and follow the manufacturer's protocol.
- Elute the extracted DNA in a final volume 50 µl of Nuclease free water and save it in -20°C for further use.

3.6. DNA Concentration measurement

Use 2µl of DNA to measure the concentration with Qubit 4.0 Fluorometer (Invitrogen by Life Technologies) and Qubit dsDNA HS Assay Kit (Invitrogen™by Thermo Scientific, cat #Q32851) or Qubit dsDNA BR Assay Kit (Invitrogen™by Thermo Scientific, cat #Q32850). Follow the manufacturer's protocol.

3.7. Example Sample preparation for deep sequencing

If using Nextera XT library preparation kit, follow manufacturer’s protocol https://support-docs.illumina.com/LP/NexteraXTRef/Content/LP/FrontPages/NexteraXT.htm

For comprehensive list of documents related to Nextera XT (checklist, consumables and equipment list, index adapters pooling guide, etc.):
https://emea.support.illumina.com/sequencing/sequencing_kits/nextera_xt_dna_kit/documentation.html
If using Illumina DNA Prep kit, follow manufacturer's protocol (link includes guidance for index adapter sequences and index adapter pooling guide):
https://emea.support.illumina.com/sequencing/sequencing_kits/illumina-dna-prep/documentation.html
Additional documents for Illumina DNA Prep kit can be found here:
https://emea.support.illumina.com/sequencing/sequencing_kits/illumina-dna-prep.html

Assess the library quality. This step is optional and can be done before step Library Normalisation (if using Illumina Nextera XT workflow). Run 1 µl of DNA in the Agilent Bioanalyzer® 2100 (Agilent Technologies, Inc.)) OR in the 4200 TapeStation Bioanalyzer (OR equivalent automated electrophoresis platform).

3.8. Deep sequencing

Illumina sequencing libraries can be sequenced on Illumina platforms. For example, when sequencing libraries on an Illumina MiSeq system with Illumina MiSeq reagents. Refer to instrument documentation and denature dilute guides (based on your system) for details.
Example - MiSeq instrument guide:
https://emea.support.illumina.com/sequencing/sequencing_instruments/miseq/documentation.html
Example – Denature and Dilute protocol:
https://emea.support.illumina.com/downloads/denature-and-dilute-protocol-generator.html

Table 1: Details of Whole genome primers that can be used:

Virus strain	Primer name	Sequence 5'→3'
Pan-polio	panPV_For	AGAGGCCCACGTGGCGGCTAG
Pan-polio	panPV_Rev	CCGAATTAAAGAAAATTTACCCCTACA
Sabin 1 and 3	A7-Sabin 1,3	ACGCGTTAATACGACTCACTATAGGCCTCCGAATTAAAGAAAAATT
Sabin 2	A7-Sabin2	ACGCGTTAATACGACTCACTATAGGCCCCGAATTAAAGAAAAATTT
Pan-polio	U-S7	ACCGGACGATTTAGGTGACACTATAGTTAAAACAGCTCTGGGGTTG

Primer named panPV_For and panPV_Rev amplifies all 3 serotypes of polioviruses and can be used in single reaction.
Primer named A7-Sabin 1,3 should be used as reverse primers to amplify Poliovirus Type 1 and 3 and A7-Sabin2 should be used as reverse primers to amplify Poliovirus Type 2. For both this reaction U-S7 can be used as forward primer to amplify all three serotypes of Type 1, 2 and 3.

3.9 Troubleshooting tips and recommendations:

- RNA extraction is a very important step in the protocol as this protocol requires intact Poliovirus RNA (7500 bp).
- Poor PCR products, double bands and faint bands affects the downstream processing leading to differences in variant calling.
- If one of the repeats shows huge background, consider discarding it from the analysis and performing a new repeat
- For example, library preparation kits provided; both Nextera XT and Illumina DNA Prep kits share the same principle of enzymatic fragmentation and partial adapter addition using a transposome enzyme. The enzyme is bead-bound in Illumina DNA Prep which allows for only a fixed amount of input DNA to be processed through the preparation. Beads are saturated with >100 ng DNA input and results in "normalised" libraries.
- When sequencing at longer read lengths (above 150 bp paired-end reads), increased adapter contents in reads may be observed. Adjusting the library fragment size via e.g. adjusting the 2-step size selection and clean-up stage of the preparation may prove beneficial.
- Different sequencing platforms and kit chemistries may generate specific errors. Understanding your platform's error profiles is key in the event of unusual results or possible observance of potential artifacts. We recommend you contact your sequencing/bioinformatics team to review the sequencing run and data quality to verify the source of the issue. Alternatively, contact the manufacturer technical support.
- Bioinformatic analysis have shown the ends of the segment of the generated data while performing an alignment can have poor quality, setting up a stringent trimming parameter and overlapping parameters for generating contigs can help avoid artifacts.
- In the final output table always check the query coverage and set a minimum range for your assay for e.g. a minimum of 3000 coverage per nucleotide.
- Depending on variant callers used (and parameters applied), you may detect spurious SNPs. Verify whether this is a legitimate variant call by observing strand bias, Q score, Coverage, etc. We recommend not to consider SNP with more than 95% stand bias and less than 30 Q-Score.

References:

1. Report on the WHO collaborative study to investigate the utility of next generation sequencing (NGS) as a molecular test of virus stocks used in the manufacture of Poliovirus vaccine (Oral). WHO/BS/2019.2359, October 2019.

2. Report on the WHO collaborative study to investigate the utility of next generation sequencing (NGS) as a molecular test of virus stocks used in the manufacture of Type 1 and 2 Poliovirus vaccine (Oral). WHO/BS/2022.2438, October 2022.
3. Recommendations to assure the quality, safety and efficacy of poliomyelitis vaccines (oral, live, attenuated) Replacement of Annex 2 of WHO Technical Report Series, No. 980. The direct link to the document is: https://cdn.who.int/media/docs/default-source/biologicals/annex-2---recommenations-polio-vaccines-who_trs_1045_web.pdf?sfvrsn=e47991d8_1&download=true

Appendix 2

Bioinformatic analysis of High-Throughput Sequencing (HTS) data for quality control of monovalent bulks of Oral Polio Vaccine (OPV)

Purpose

Traditionally lot release of OPV included *in vivo* test for neurovirulence in monkeys (MNVT) or transgenic mice susceptible to poliovirus (Tg-mice), and *in vitro* mutant analysis by PCR and restriction enzyme cleavage (MAPREC). This latter test determines the content of mutations that emerge during virus growth under inappropriate conditions and increase neurovirulence in monkeys and Tg-mice. These mutations are located in domain V of the internal ribosome entry site (IRES) in the 5'-untranslated regions (UTR) of all three types of Sabin poliovirus. They are: 480-G->A and 525-U->C in Sabin 1 virus, 481-A->G in Sabin 2, and 472-U->C in Sabin 3. These mutations are present in small quantities in all batches of OPV, and MAPREC sets the limit for the maximum acceptable content of these mutants. Therefore, MAPREC tests for the presence of critical but limited number of genetic changes that adversely affect vaccine quality.

MNVT and TgmNVT determine the overall neurovirulence of vaccine virus which is a critical quality characteristic. It ensures that no loss of attenuated phenotype took place during virus growth by accumulating sequence heterogeneities in genomic sites other than those tested by MAPREC.

The advancement of molecular technology and the advent of HTS (otherwise known as massively parallel or next-generation sequencing, NGS) enabled sensitive and accurate quantification of all sequence heterogeneities in the entire viral genome. Several international collaborative studies demonstrated the utility of HTS technology as an alternative to both MAPREC and animal NVT. Therefore, the WHO Expert Committee on Biological Standardization (ECBS) approved it as adequate alternate for MAPREC and *in-vivo* neurovirulence tests.

This SOP explains how to analyze HTS data starting from FASTQ files generated by Illumina instruments and to make decisions about acceptability of monovalent OPV bulks to be used for formulation of the final vaccine product and its release for immunization programs.

The analysis includes two aspects. The first one is a limit test on the content of critical de-attenuating mutations in domain V of the 5'-UTR. This aspect serves as a replacement of MAPREC. The second aspect is the analysis of whole-genome single-nucleotide polymorphisms (SNP) to ensure that no unexpected genetic changes took place during virus growth that could adversely affect vaccine quality, including not only animal neurovirulence, but potentially also immunogenicity. This is a consistency test that compares whole-genome SNP profiles of a new vaccine batch with the established historical profile of SNPs found in previously released vaccine batches. If the new batch conforms to the historical profile, then it can be released without performing the animal NVT. Alternatively, troubleshooting is performed to establish and eliminate the reason for the non-conformity. If the conclusion of the troubleshooting (that may include conducting animal NVT) is

that the batch is acceptable, the historical database is updated to include data for the newly released vaccine batch.

Because HTS is a rapidly evolving technology, the SOP does not describe a single solution for each step. Instead, it provides a guidance for the proper selection of the methods, algorithms, software and protocols to generate quality data and to make reliable regulatory decisions.

Applicability

The procedures described in this document are used by relevant quality control laboratories at vaccine manufacturing / regulatory authority establishments.

Responsibilities

Upstream processing of poliovirus stocks including preparation of Illumina DNA libraries and generation of nucleotide sequences by Illumina equipment is performed by staff trained in these procedures based on relevant SOPs, an example of such SOP is provided in appendix 1. Bioinformatic procedures descried in this document are performed by staff proficient in using computers and relevant software and having basic statistical training.

Equipment and software

- Desktop computers, computer clusters, or cloud-based systems can be used, depending on the software requirements.
- Operating systems can include UNIX/Linux, MacOS, Windows, and potentially others.
- Different software packages for analysis of raw HTS data (FASTQ files) can be used. It can be either commercial software packages such as Geneious, DRAGEN, CLC Genomic Workbench, or in-house custom software. The required functionality must include quality control and filtering / trimming of short sequence reads, their alignment to reference genomes of vaccine poliovirus, and variant calling to generate full-genome single-nucleotide polymorphism (SNP) profiles. The profiles must contain quantitative information about the content of all possible SNPs in the entire genome separately for forward and reverse read orientation, the depth of sequencing coverage, and the average HTS data quality (PHRED scores).
- Microsoft Office software package that includes MS Excel
- Comparison of SNP profiles can be done using Excel or other statistical programs, as well as custom software / scripts implementing procedures described below.

Upstream quality control and filtering of raw HTS data

Raw HTS data may contain artifactual data that needs to be eliminated from the dataset. The source of artifactual results can be in either wet-lab steps (low virus titer, RNA isolation, cDNA preparation and PCR amplification, Illumina library preparation, etc.). Therefore, before executing the HTS step it is important to analyze the quality (size distribution) of PCR products and Illumina libraries using gel electrophoresis or other appropriate methods. PCR products should contain only one band corresponding to the full-genome double-stranded DNA (ca. 7500 base pairs). If a significant amount

of shorter DNA fragments is detected, the troubleshooting is conducted. The change of the source of reagents and enzymes can be considered along with the optimization of the wet-lab procedures. A potential remedy for the presence of shorter bands in the PCR product is size-selection by gel electrophoresis or other appropriate methods.

Bioinformatic quality control is performed at both upstream and downstream stages, i.e. before the alignment of sequence reads to the reference genome template, and after. Below are some aspects to be considered at the upstream stage:

- Illumina adapters must be removed from sequence reads.
- The target length of sequence reads must be between 100 and 300 nucleotides. Abnormally short sequence reads (e.g. less than 30) should be discarded.
- Base-calling quality: individual datapoints with PHRED scores below 30 (99.9% accuracy) should be disregarded.
- Entire sequence reads that contain disproportionately large number of low-PHRED bases should be discarded
- Datapoints at the ends of short sequence reads tend to be unreliable. Therefore, the ends must be trimmed and disregarded. As a rule of thumb 5-nucleotide-long trimming is sufficient.

Sequence read alignment on the reference sequence and variant calling

Reference sequences are those of Sabin 1, Sabin 2, and Sabin 3 attenuated poliovirus vaccine strains. The accession numbers in GenBank are AY184219, AY184220, and AY184221, respectively. The latter sequence of Sabin 3 virus represents the consensus sequence of rederived Sabin Original (Pfizer or RSO) seed virus. Manufacturers producing vaccine from alternative seed viruses (e.g. for Japanese and Chinese type 3 seed viruses) may use appropriate consensus sequences.

Alignment of sequence reads on the reference template can be done by various algorithms, including Smith-Waterman, Burrows-Wheeler aligner, Bowtie2, and other similar algorithms.

Variant calling is performed after alignment by counting the number times of each nucleotide was detected at each genomic position, separately for forward and reverse reads.

Downstream quality control

This is the last step in generating whole-genome SNP profiles. It includes the following steps:

- Genomic position in which coverage is low (e.g. less than 3000 reads per nucleotide) are disregarded. This regularly happens at both ends of the genome, e.g. within the first and last 50 nucleotides from each end. However, low coverage in the middle of genome calls for troubleshooting and such dataset should be invalidated.
- Uniformity of sequencing coverage. As mentioned, coverage at the ends of the genome usually declines. However, starting about 100 nucleotides from each end it is typically uniform, but not fully identical. Significant variations and abrupt changes in the coverage indicate low quality of full-genome PCR amplification and/or Illumina DNA library. Severe non-uniformity of sequencing coverage calls for troubleshooting of PCR and DNA library preparation steps.

- Polarity bias, i.e. disparity between the SNP frequency in forward and reverse read orientation may indicate artifactual variant calling. In most cases it is reasonable to set the polarity bias threshold at 1:10.
- Insertions and deletions of significant length. They are very rare in vaccine preparations since they render virus unviable. Their length over one base-long is likely a result of improper alignment or PCR artifacts, requiring such sequence reads to be disregarded.

SNP profile files structure

SNP profiles are text tables in tab-delimited or comma-separated values (CSV) format. Each row of the table represents individual position in the virus genome, and columns contain the following information:

- Genomic position
- The number of A, C, G, T, insertions, and deletions in forward orientation
- The number of A, C, G, T, insertions, and deletions in reverse orientation
- Number of reads in forward orientation
- Number of reads in reverse orientation
- Average PHRED score for A, C, G, and T (optional)

Quantitative analysis of 5'-UTR mutations linked to de-attenuation

This step of the analysis has the same objective as MAPREC test: to identify monovalent OPV bulks that contain SNPs linked to the increased neurovirulence in monkeys and Tg-mice. Samples of each bulk are independently sequenced five times, so the complete dataset for each sample consists of five SNP profiles. The comparison is performed relative to the reference vaccine sample sequenced five times in the same experiment. The reference was selected so that the content of neurovirulent SNPs was at the maximum acceptable level, thus defining the cutoff level.

The statistical significance of the difference between the content SNPs corresponding to the 5'-UTR neurovirulent mutations in the reference and the test vaccines is calculated using Student t-test at 95% confidence. If the SNP content in the test monovalent bulk exceeds that in the reference preparation by a statistically significant margin, the test bulk is considered unacceptable, and appropriate troubleshooting that may include animal neurovirulence testing is performed to identify the problem. The lot can be released if it passes the MNVT or TgmNVT during the troubleshooting.

Monitoring genetic consistency using whole-genome SNP profiles

The second phase of the analysis is performed on monovalent bulks that passed the first phase (have the content of neurovirulent SNPs in the acceptable range). The objective of this phase is to identify all SNPs (if any) with the content significantly exceeding the content observed in previously released monovalent bulks (outliers). The historical database of previously released lots is compiled during the establishment phase of vaccine manufacturing process (at the beginning of manufacture or after a significant manufacturing process change, e.g. new seed virus lot) and is continuously updated to include information about the newly manufactured monovalent bulks that were deemed acceptable.

Comparing different whole-genome SNP profiles

Each sample from a monovalent vaccine bulk is sequenced five times, so the dataset for each monovalent bulk consists of five SNP profiles. The calculations described below are performed only

for sequence polymorphisms, while the content of consensus nucleotide is not considered. If the average content of an SNP is below 1% in both datasets, it is deemed to be a background noise and is disregarded.

To identify the difference between sets of SNP profiles, each SNP is analyzed by Student t-test at 95% confidence, and a list of SNPs that demonstrate statistically significant difference in test bulk vaccine vs. the historical database of acceptable bulks is complied. Typically, for established manufacturers, such lists are very short and contain SNPs at just a few genomic positions. If no such SNPs are found, or the content of SNPs in test monovalent bulk is lower than in historical profile, the vaccine bulk can be released. Alternatively, the next step is performed to determine whether the statistical significance of SNP content translates to biological significance.

Testing biological significance of sequence polymorphisms

Every batch of OPV, especially OPV3 contains sequence heterogeneities made of neutral mutations that do not affect virus biological characteristics and vaccine quality. Analysis of multiple vaccine samples produced and successfully released by different manufacturers over several decades revealed a list of nucleotide changes that are often present in monovalent bulks without affecting their quality. If the mutations identified in the test sample are present in this list, it indicates that the monovalent bulk is acceptable and can be released. If the SNP in test sample is not in the list of acceptable sequence heterogeneities, then an expert assessment should be conducted based on the current knowledge of molecular biology of poliovirus. For example, silent mutations are very unlikely to affect biological properties of the virus, unless they are located in RNA structures known to perform important functions in RNA replication and translation. Examples of such structures are cre element in 2C region, IRES element, complementary regions in the 5’ and 3’ regions, genomic regions coding for protective epitopes. If the evaluation suggests that the heterogeneity can adversely affect vaccine quality, a wet-lab evaluation or animal testing could be recommended.

Conclusion

HTS analysis of monovalent OPV bulks can confirm the absence of neurovirulent mutations and molecular consistency of whole-genome profiles of SNPs. The method can identify small variations in SNP content with sensitivity far exceeding that of MNVT and TgmNVT, and therefore serves as a suitable replacement for both MAPREC and animal neurovirulence testing.

Appendix 3

a) List of SNPs that are present in manufacturing lot of Oral Polio vaccines type 1 (OPV1) used to vaccinate human population over decades.

Nucleotide	Reference base	Mutation	Gene	Amino acid	Reference amino acid	Mutant amino acid	Max SNP% observed in good vaccines
661	C	U	non-coding				2.66
691	U	A	non-coding				1.18
1458	C	U	protein VP2	170	Pro	Leu	2.11
1472	U	C	protein VP2	175	Cys	Arg	3.23
1941	C	G	protein VP3	59	Ala	Gly	1.83
2779	U	A	protein VP1	100	Asn	Lys	4.91
3275	A	G	protein VP1	266	Ile	Val	1.20
4393	U	C	protein 2C				1.19
7303	G	A	protein 2D				2.01

This list is not intended to be exhaustive; it is derived from the results of this collaborative study and supported by historical data obtained from CBER-FDA and MHRA laboratories. This includes changes found from different OPV1 vaccines, made by different manufacturers.

b) List of SNPs that are present in manufacturing lot of Oral Polio vaccines type 3 (OPV3) used to vaccinate human population over decades.

Nucleotide	Reference base	Mutation	Gene	Amino acid	Reference amino acid	Mutant amino acid	Max observed SNP% in good vaccines
537	G	A	non-coding				14.05
699	C	U	non-coding				1.67
713	A	G	non-coding				2.01
858	A	G	protein VP4	39	Asn	Ser	2.78
898	A	G	protein VP4				14.34
1003	A	G	protein VP2				1.14
1161	G	A	protein VP3				2.66

1537	C	U	protein VP2				1.50
1640	C	U	protein VP2				1.33
1681	U	C	protein VP2				5.64
1699	G	A	protein VP2				99.86
2440	A	U	protein VP3				99.88
2493	C	U	protein VP1	6	Thr	Ile	99.95
2696	A	G	protein VP1	74	Thr	Ala	7.97
2702	G	C	protein VP1	76	Glu	Gln	2.08
2703	A	G	protein VP1	76	Glu	Gly	3.34
2812	U	C	protein VP1				1.20
3262	C	U	protein VP1				2.03
3353	U	C	protein VP1	293	Ser	Pro	99.93
3357	A	G	protein VP1	294	Glu	Gly	99.95
3640	C	U	protein 2A				2.87
3700	C	U	protein 2A				99.92
3723	A	G	protein 2A	116	Gln	Arg	7.47
3956	A	G	protein 2B	45	Ile	Val	92.76
4054	G	A	protein 2B				4.30
4171	A	G	protein 2C				4.43
4872	A	G	protein 2C	253	Glu	Gly	10.00
4883	G	A	protein 2C	257	Asp	Asn	2.93
4884	A	G	protein 2C	257	Asp	Gly	3.79
4925	G	A	protein 2C	271	Asp	Asn	1.35
4935	A	G	protein 2C	274	Gln	Arg	2.45
5296	A	G	protein 3A				1.62
5473	U	C	protein 3C				6.78
5476	U	C	protein 3C				6.34
5767	U	C	protein 3C				99.90
5832	U	C	protein 3C	135	Ile	Thr	99.93
6001	A	G	protein 3D				4.97
6178	C	U	protein 3D				1.04
6421	C	U	protein 3D				99.94
6421	A	G	protein 3D				3.1
6505	U	A	protein 3D				99.94
6760	A	U	protein 3D	261	Arg	Ser	99.89

This list is not intended to be exhaustive; it is derived from the results of this collaborative study and supported by historical data obtained from CBER-FDA and MHRA laboratories. This includes changes found from different OPV3 vaccines, made by different manufacturers.

Appendix 4

Collaborative study participants (In alphabetical order by country)

Name	Laboratory	Country
Jie Song	IMBCAMS	China
Yifan Liu	Kunming	China
Panduranga Pavuluri	Bio E	India
Umakantha Madala	Bio E	India
Yuvraj Jadhav	Bio E	India
Ramurthy Gudla	Bio E	India
Saikumar Ale	Bio E	India
Muhammad Luthfi Nugraha	PT Bio Farma	Indonesia
Istianti Nurisa	PT Bio Farma	Indonesia
Vinca Medica	PT Bio Farma	Indonesia
Gemi Utami Pertiwi	PT Bio Farma	Indonesia
Iin Susanti	PT Bio Farma	Indonesia
Ochiai Susumu	Biken	Japan
Minami Koji	Biken	Japan
Mariët Zekveld	Bilthoven Bio	Netherlands
Sarah Carlyle	MHRA	UK
Andrew Macadam	MHRA	UK
Marilyn Quinlan	MHRA	UK
Yemisi Adedeji	MHRA	UK
Edward Mee	MHRA	UK
James Condron	MHRA	UK
Amy Rosenfeld	CBER-FDA	USA
Andrew R Hoffmann	Tulane University	USA
Lori A Rowe	Tulane University	USA
Nicholas J Maness	Tulane University	USA
Afsar Mir	PATH	USA

Appendix 5

Proposed Instruction for Use

WHO Reference Reagent
NIBSC code: 23/160
Instructions for use

1. INTENDED USE

23/160 is proposed to be establish as a WHO Reference Reagent (RR) serving as a Type 1 background-noise / system-suitability control for High throughput sequencing assays. This material provides a near-zero baseline for the Type 1 MAPREC mutations (G480A and U525C) and can be used to characterize laboratory- and run-specific background error frequencies.

2. CAUTION

This preparation is not for administration to humans or animals in the human food chain.

Biological Agent- Identification: The material contains liquid frozen, poliovirus type 1 (Sabin) material. Material is infectious in nature and must be handled inside a Microbiological safety cabinet.

Biological Agent- Controls: This preparation should be regarded as potentially hazardous to health. This material may be handled at Containment Level 2 (CL2) following approval from the local biosafety committee.

3. UNITAGE

Based on a WHO collaborative study, the single nucleotide polymorphism (SNP) estimates for 23/160 are as follows: G480A (0.03%), U525C (0.05%), U5350C (99.93%) and U6187C (99.93%) where; Sabin 1 sequence (GenBank ID AY184219) is used as the reference for contig assembly.

4. CONTENTS

Country of origin of biological material: United Kingdom.
Each vial contains approximately 0.8 ml of poliovirus type 1 (Sabin) grown in serum free medium.

5. STORAGE

The material should arrive frozen.
Unopened vials should be stored at -70℃ or below.

6. DIRECTIONS FOR OPENING

Vials have a screw cap. The cap should be removed by turning anticlockwise. Care should be taken on removal of cap to prevent loss of the content.

7. USE OF MATERIAL

This material provides a near-zero baseline for the Type 1 MAPREC mutations (G480A and U525C) and can be used to characterize background error frequencies. Appendix 1 of this report provides an example of intended use of this material using Illumina systems.

8. STABILITY

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label.

9. REFERENCES

1. Report on the WHO collaborative study to investigate the utility of next generation sequencing (NGS) as a molecular test of virus stocks used in the manufacture of Poliovirus vaccine (Oral). WHO/BS/2019.2359, October 2019.
2. Report on the WHO collaborative study to investigate the utility of next generation sequencing (NGS) as a molecular test of virus stocks used in the manufacture of Type 1 and 2 Poliovirus vaccine (Oral). WHO/BS/2022.2438, October 2022.
3. Recommendations to assure the quality, safety and efficacy of poliomyelitis vaccines (oral, live, attenuated) Replacement of Annex 2 of WHO Technical Report Series, No. 980. The direct link to the document is: https://cdn.who.int/media/docs/default-source/biologicals/annex-2---recommenations-polio-vaccines-who_trs_1045_web.pdf?sfvrsn=e47991d8_1&download=true
4.WHO/BS/2026.2505

10. ACKNOWLEDGEMENTS

We gratefully acknowledge the important contributions of the collaborative study participants. The project has been funded by Program for Appropriate Technology in Health (PATH).

11. FURTHER INFORMATION

Further information can be obtained as follows;
This material: enquiries@nibsc.orgWHO Biological Standards: http://www.who.int/biologicals/en/JCTLM Higher order reference materials:http://www.bipm.org/en/committees/jc/jctlm/Derivation of International Units:http://www.nibsc.org/standardisation/international_standards.aspxOrdering standards from NIBSC:http://www.nibsc.org/products/ordering.aspxNIBSC Terms & Conditions:http://www.nibsc.org/terms_and_conditions.aspx

12. CUSTOMER FEEDBACK

Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to enquiries@nibsc.org

13. CITATION

In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC code number, and the name and address of NIBSC are cited and cited correctly.

14. MATERIAL SAFETY SHEET

Classification in accordance with Directive 2000/54/EC, Regulation (EC) No 1272/2008: Not applicable or not classified

Physical and Chemical properties	
Physical appearance: Liquid	Corrosive: No
Stable: Yes	Oxidising: No
Hygroscopic: No	Irritant: No
Flammable: No	Handling: See caution, Section 2
Other (specify):	
Toxicological properties	
Effects of inhalation: Not established, avoid inhalation	
Effects of ingestion: Not established, avoid ingestion	
Effects of skin absorption: Not established, avoid contact with skin	
Suggested First Aid	
Inhalation: Seek medical advice	
Ingestion: Seek medical advice	
Contact with eyes: Wash with copious amounts of water. Seek medical advice	
Contact with skin: Wash thoroughly with water.	
Action on Spillage and Method of Disposal	
Spillage of vial contents should be taken up with absorbent material wetted with an appropriate disinfectant, such as hypochlorite. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.	

15. LIABILITY AND LOSS

In the event that this document is translated into another language, the English language version shall prevail in the event of any inconsistencies between the documents.

Unless expressly stated otherwise by NIBSC, NIBSC's Standard Terms and Conditions for the Supply of Materials (available at http://www.nibsc.org/About_Us/Terms_and_Conditions.aspx or upon request by the Recipient) ("Conditions") apply to the exclusion of all other terms and are hereby incorporated into this document by reference. The Recipient's attention is drawn in particular to the provisions of clause 11 of the Conditions.

16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 0.8 g
Toxicity Statement: Toxicity not assessed
Veterinary certificate or other statement if applicable. **Attached: Not Applicable**

17. CERTIFICATE OF ANALYSIS

NIBSC does not provide a Certificate of Analysis for WHO Biological Reference Materials because they are internationally recognised primary reference materials fully described in the instructions for use. The reference materials are established

according to the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards http://www.who.int/bloodproducts/publications/TRS932Annex2_Inter_biolefstandardsrev2004.pdf (revised 2004). They are officially endorsed by the WHO Expert Committee on Biological Standardization (ECBS) based on the report of the international collaborative study which established their suitability for the intended use.

WHO Reference Reagent
NIBSC code: 23/162
Instructions for use

1. INTENDED USE

23/162 is proposed to be established as a WHO Reference Reagent (RR) for High-threshold-Type1-MAPREC assay control for High throughput sequencing platform, with a mean combined estimate for mutations G480A + U525C of 2.43% (95% CI: 2.33%–2.54%).

2. CAUTION

This preparation is not for administration to humans or animals in the human food chain.

Biological Agent- Identification: The material contains liquid frozen, poliovirus type 1 (Sabin) material. Material is infectious in nature and must be handled inside a Microbiological safety cabinet.

Biological Agent- Controls: This preparation should be regarded as potentially hazardous to health. This material may be handled at Containment Level 2 (CL2) following approval from the local biosafety committee.

3. UNITAGE

Based on a WHO collaborative study the combined estimate for mutations G480A + U525C was found to be 2.43% (95% CI: 2.33%–2.54%), where; Sabin 1 sequence (GenBank ID AY184219) is used as the reference for contig assembly.

4. CONTENTS

Country of origin of biological material: United Kingdom.
Each vial contains approximately 0.8 ml of poliovirus type 1 (Sabin) grown in serum free medium.

5. STORAGE

The material should arrive frozen.
Unopened vials should be stored at -70℃ or below.

11. DIRECTIONS FOR OPENING

Vials have a screw cap. The cap should be removed by turning anticlockwise. Care should be taken on removal of cap to prevent loss of the content.

12. USE OF MATERIAL

This material can be used as a high threshold control for the Type 1 MAPREC mutations (G480A and U525C). Appendix 1 of this report provides an example of intended use of this material using Illumina systems.

13. STABILITY

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label.

14. REFERENCES

1. Report on the WHO collaborative study to investigate the utility of next generation sequencing (NGS) as a molecular test of virus stocks used in the manufacture of Poliovirus vaccine (Oral). WHO/BS/2019.2359, October 2019.
2. Report on the WHO collaborative study to investigate the utility of next generation sequencing (NGS) as a molecular test of virus stocks used in the manufacture of Type 1 and 2 Poliovirus vaccine (Oral). WHO/BS/2022.2438, October 2022.
3. Recommendations to assure the quality, safety and efficacy of poliomyelitis vaccines (oral, live, attenuated) Replacement of Annex 2 of WHO Technical Report Series, No. 980. The direct link to the document is: https://cdn.who.int/media/docs/default-source/biologicals/annex-2---recommenations-polio-vaccines-who_trs_1045_web.pdf?sfvrsn=e47991d8_1&download=true
4.WHO/BS/2026.2505

15. **ACKNOWLEDGEMENTS**

We gratefully acknowledge the important contributions of the collaborative study participants. The project has been funded by Program for Appropriate Technology in Health (PATH).

11. FURTHER INFORMATION

Further information can be obtained as follows;
This material: enquiries@nibsc.orgWHO Biological Standards: http://www.who.int/biologicals/en/JCTLM Higher order reference materials:http://www.bipm.org/en/committees/jc/jctlm/Derivation of International Units:http://www.nibsc.org/standardisation/international_standards.aspxOrdering standards from NIBSC:http://www.nibsc.org/products/ordering.aspxNIBSC Terms & Conditions:http://www.nibsc.org/terms_and_conditions.aspx

12. CUSTOMER FEEDBACK

Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to enquiries@nibsc.org

13. CITATION

In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC code number, and the name and address of NIBSC are cited and cited correctly.

14. MATERIAL SAFETY SHEET

Classification in accordance with Directive 2000/54/EC, Regulation (EC) No 1272/2008: Not applicable or not classified

Physical and Chemical properties	
Physical appearance: Liquid	Corrosive: No
Stable: Yes	Oxidising: No
Hygroscopic: No	Irritant: No
Flammable: No	Handling: See caution, Section 2
Other (specify):	
Toxicological properties	
Effects of inhalation: Not established, avoid inhalation	
Effects of ingestion: Not established, avoid ingestion	
Effects of skin absorption: Not established, avoid contact with skin	
Suggested First Aid	
Inhalation: Seek medical advice	
Ingestion: Seek medical advice	
Contact with eyes: Wash with copious amounts of water. Seek medical advice	
Contact with skin: Wash thoroughly with water.	
Action on Spillage and Method of Disposal	
Spillage of vial contents should be taken up with absorbent material wetted with an appropriate disinfectant, such as hypochlorite. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.	

15. LIABILITY AND LOSS

In the event that this document is translated into another language, the English language version shall prevail in the event of any inconsistencies between the documents.

Unless expressly stated otherwise by NIBSC, NIBSC's Standard Terms and Conditions for the Supply of Materials (available at http://www.nibsc.org/About_Us/Terms_and_Conditions.aspx or upon request by the Recipient) ("Conditions") apply to the exclusion of all other terms and are hereby incorporated into this document by reference. The Recipient's attention is drawn in particular to the provisions of clause 11 of the Conditions.

16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 0.8 g
Toxicity Statement: Toxicity not assessed
Veterinary certificate or other statement if applicable. **Attached: Not Applicable**

17. CERTIFICATE OF ANALYSIS

NIBSC does not provide a Certificate of Analysis for WHO Biological Reference Materials because they are internationally recognised primary reference materials fully described in the instructions for use. The reference materials are established according to the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards

http://www.who.int/bloodproducts/publications/TRS932Annex2_Inter_biolefstandardsrev2004.pdf (revised 2004). They are officially endorsed by the WHO Expert Committee on Biological Standardization (ECBS) based on the report of the international collaborative study which established their suitability for the intended use.

WHO Reference Reagent
NIBSC code: 23/172
Instructions for use

1. INTENDED USE

23/172 is proposed to be establish as a WHO Reference Reagent (RR) serving as a Type 3 background-noise / system-suitability control for High throughput sequencing assays. This material provides a near-zero baseline for the Type 3 MAPREC mutations (U472C) and can be used to characterize laboratory- and run-specific background error frequencies.

2. CAUTION

This preparation is not for administration to humans or animals in the human food chain.

Biological Agent- Identification: The material contains liquid frozen, poliovirus type 3 (Sabin) material. Material is infectious in nature and must be handled inside a Microbiological safety cabinet.

Biological Agent- Controls: This preparation should be regarded as potentially hazardous to health. This material may be handled at Containment Level 2 (CL2) following approval from the local biosafety committee.

3. UNITAGE

Based on a WHO collaborative study, the single nucleotide polymorphism (SNP) estimates for 23/172 are as follows: U472C (0.05%), C2493U (99.93%), U6061C (99.89%), G6304A (99.82%) and C7004U (99.80%) where; Sabin 3 sequence (GenBank ID AY184221) is used as the reference for contig assembly.

4. CONTENTS

Country of origin of biological material: United Kingdom.
Each vial contains approximately 0.8 ml of poliovirus type 3 (Sabin) grown in serum free medium.

5. STORAGE

The material should arrive frozen.
Unopened vials should be stored at -70℃ or below.

16. DIRECTIONS FOR OPENING

Vials have a screw cap. The cap should be removed by turning anticlockwise. Care should be taken on removal of cap to prevent loss of the content.

17. USE OF MATERIAL

This material provides a near-zero baseline for the Type 3 MAPREC mutations (U472C) and can be used to characterize background error frequencies. Appendix 1 of this report provides an example of intended use of this material using Illumina systems.

18. STABILITY

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label.

19. REFERENCES

1. Report on the WHO collaborative study to investigate the utility of next generation sequencing (NGS) as a molecular test of virus stocks used in the manufacture of Poliovirus vaccine (Oral). WHO/BS/2019.2359, October 2019.
2. Report on the WHO collaborative study to investigate the utility of next generation sequencing (NGS) as a molecular test of virus stocks used in the manufacture of Type 1 and 2 Poliovirus vaccine (Oral). WHO/BS/2022.2438, October 2022.
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4.WHO/BS/2026.2505

20. **ACKNOWLEDGEMENTS**

We gratefully acknowledge the important contributions of the collaborative study participants. The project has been funded by Program for Appropriate Technology in Health (PATH).

11. FURTHER INFORMATION

Further information can be obtained as follows;
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12. CUSTOMER FEEDBACK

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13. CITATION

In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC code number, and the name and address of NIBSC are cited and cited correctly.

14. MATERIAL SAFETY SHEET

Classification in accordance with Directive 2000/54/EC, Regulation (EC) No 1272/2008: Not applicable or not classified

Physical and Chemical properties	
Physical appearance: Liquid	Corrosive: No
Stable: Yes	Oxidising: No
Hygroscopic: No	Irritant: No
Flammable: No	Handling: See caution, Section 2
Other (specify):	
Toxicological properties	
Effects of inhalation: Not established, avoid inhalation	
Effects of ingestion: Not established, avoid ingestion	
Effects of skin absorption: Not established, avoid contact with skin	
Suggested First Aid	
Inhalation: Seek medical advice	
Ingestion: Seek medical advice	
Contact with eyes: Wash with copious amounts of water. Seek medical advice	
Contact with skin: Wash thoroughly with water.	
Action on Spillage and Method of Disposal	
Spillage of vial contents should be taken up with absorbent material wetted with an appropriate disinfectant, such as hypochlorite. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.	

15. LIABILITY AND LOSS

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16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 0.8 g
Toxicity Statement: Toxicity not assessed
Veterinary certificate or other statement if applicable. **Attached: Not Applicable**

17. CERTIFICATE OF ANALYSIS

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http://www.who.int/bloodproducts/publications/TRS932Annex2_Inter_biolefstandardsrev2004.pdf (revised 2004). They are officially endorsed by the WHO Expert Committee on Biological Standardization (ECBS) based on the report of the international collaborative study which established their suitability for the intended use.

WHO Reference Reagent
NIBSC code: 23/174
Instructions for use

1. INTENDED USE

23/174 is proposed to be establish as a WHO Reference Reagent (RR) for High-threshold-Type 3-MAPREC assay control for HTS platform, with a mean estimate for mutation U472C of 1.04% (95% CI: 1.01%–1.08%). Additionally, 23/174 can be employed for validation and implementation of WG-HTS for whole-genome SNP profiling (variant frequencies spanning approximately 1% to 75% across genomic positions).

2. CAUTION

This preparation is not for administration to humans or animals in the human food chain.

Biological Agent- Identification: The material contains liquid frozen, poliovirus type 3 (Sabin) material. Material is infectious in nature and must be handled inside a Microbiological safety cabinet.

Biological Agent- Controls: This preparation should be regarded as potentially hazardous to health. This material may be handled at Containment Level 2 (CL2) following approval from the local biosafety committee.

3. UNITAGE

Based on a WHO collaborative study, the single nucleotide polymorphism (SNP) estimates for 23/172 are as follows: U472C (1.04%), A769G (1.50%), A1003G (2.22%), U1867C (1.30%), C2493U (75.56%), C2950U (6.38%), C3640U (1.88%), A3766G (1.52%), A4171G (1.45%), A4872G (12.93%), A4872C (2.90%), A4884G (2.78%), G4925A (5.08%), A4935G (6.36%), U5832C (34.00%) where; Sabin 3 sequence (GenBank ID AY184221) is used as the reference for contig assembly.

4. CONTENTS

Country of origin of biological material: United Kingdom.
Each vial contains approximately 0.8 ml of poliovirus type 3 (Sabin) grown in serum free medium.

5. STORAGE

The material should arrive frozen.
Unopened vials should be stored at -70℃ or below.

21. DIRECTIONS FOR OPENING

Vials have a screw cap. The cap should be removed by turning anticlockwise. Care should be taken on removal of cap to prevent loss of the content.

22. USE OF MATERIAL

23/174 is proposed to be establish as a WHO Reference Reagent (RR) for High-threshold-Type 3-MAPREC assay control for HTS platform. Additionally, 23/174 can be employed for validation and implementation of WG-HTS for whole-genome SNP profiling. Appendix 1 of this report provides an example of intended use of this material using Illumina systems.

23. STABILITY

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label.

24. REFERENCES

1. Report on the WHO collaborative study to investigate the utility of next generation sequencing (NGS) as a molecular test of virus stocks used in the manufacture of Poliovirus vaccine (Oral). WHO/BS/2019.2359, October 2019.
2. Report on the WHO collaborative study to investigate the utility of next generation sequencing (NGS) as a molecular test of virus stocks used in the manufacture of Type 1 and 2 Poliovirus vaccine (Oral). WHO/BS/2022.2438, October 2022.
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14. MATERIAL SAFETY SHEET

Classification in accordance with Directive 2000/54/EC, Regulation (EC) No 1272/2008: Not applicable or not classified

Physical and Chemical properties	
Physical appearance: Liquid	Corrosive: No
Stable: Yes	Oxidising: No
Hygroscopic: No	Irritant: No
Flammable: No	Handling: See caution, Section 2
Other (specify):	
Toxicological properties	
Effects of inhalation: Not established, avoid inhalation	
Effects of ingestion: Not established, avoid ingestion	
Effects of skin absorption: Not established, avoid contact with skin	
Suggested First Aid	
Inhalation: Seek medical advice	
Ingestion: Seek medical advice	
Contact with eyes: Wash with copious amounts of water. Seek medical advice	
Contact with skin: Wash thoroughly with water.	
Action on Spillage and Method of Disposal	
Spillage of vial contents should be taken up with absorbent material wetted with an appropriate disinfectant, such as hypochlorite. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.	

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Net weight: 0.8 g
Toxicity Statement: Toxicity not assessed
Veterinary certificate or other statement if applicable. **Attached: Not Applicable**

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