Qualitative Single Base Extension assay for Identification of Single Nucleotide Polymorphism in Mumps Virus Genome

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Abstract

Objective: Objective of the study was to standardize and evaluate suitability of Single Base Extension (SBE) singleplex assay for rapid routine monitoring of genetic homogeneity of virus samples, from productivity improvement experiments in Mumps vaccine. A Single Base Extension assay is a simple, cost effective tool for quick identification of single nucleotide polymorphism (SNP) at the three known sites in Mumps vaccine virus genome. The assay can be developed as a singleplex or multiplex version with potential for detection of up to ten SNP bases in a single reaction.

Methods: SNaPshot kit from Applied Biosystems (ThermoFisher) was used to identify the nucleotide bases at the three SNP sites in Mumps virus genome. Approximate sizing locations of individual primers were determined using the SNaPshot Primer Focus Kit, also from Applied Biosystems. Genetic analyzer 3500 was used for the capillary electrophoresis of the processed samples. Results obtained from the data collection software were analyzed using GeneMapper software.

Findings: Nucleotide G was identified at locations 1073 and 11345 and nucleotide C was identified at location 5261 for all tested mumps vaccine samples, thus demonstrating consistency in genetic homogeneity at the three known SNP hotspots in experimental mumps vaccine virus samples.

Conclusion: The singleplex SBE assay was found to be a reliable and relatively inexpensive tool for routine monitoring of genetic homogeneity of vaccine virus samples, with potential for multiplexing that would reduce the cost and assay time even further.

Keywords: *Mumps virus, Primer Focus Assay, SBE assay, Singleplex, SNP sites*

Introduction

Regulatory organizations are expecting from biological industries, increased usage of molecular methods for ensuring consistency in critical quality attributes of biological products such as live attenuated vaccines. Consistent performance of live attenuated viral vaccines is critical from safety and efficacy point of view. Polymerase chain reaction (PCR) based advanced molecular methods are already being used as batch release criteria for oral polio and influenza vaccines.[1]

Both qualitative and quantitative molecular methods are useful in quality check of vaccines. Qualitative methods supported with adequate validations are simple and readily doable for routine

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genetic monitoring of homogeneity of viruses.

On the other hand, complicated techniques such as Mutant Analysis by PCR and Restriction Enzyme Cleavage (MAPREC) and Matrix-assisted Laser Desorption/Ionization – Time of Flight (MALDI-TOF) have been used for analyzing dynamism in mutant profile of Jeryl Lynn mumps vaccine virus.[2]

Consistency in the quality of experimental vaccines can be effectively monitored, if a simple and quick genetic tool such as the SBE assay can be standardized.

Single base extension, as the name indicates, extends the annealed primer by only one nucleotide before getting terminated because of the presence of dideoxynucleotides (ddNTPs) instead of the usual deoxynucleotides (dNTPs) in the reaction mixture. The ddNTPs present in the reaction mixture are fluorescently labeled, each one having its unique identifiable color.

SNP genotyping can be used for identifying mutations. Single base deletions, additions or replacements can be quickly captured using this technique. Useful features of SBE and its comparison with real time PCR and next generation sequencing have been reported.[3-5]

In this study, we have described an application of the simplest form of this assay for identification of nucleotides at the known SNP locations of mumps virus passaged on different cell substrates.

Mumps virus is an enveloped RNA virus having non-segmented single stranded RNA of negative polarity as a genome. Its genome length is 15384 nucleotides which sequentially represent the seven genes, viz. NP, V/I/P, M, F, SH, HN and L gene.[6]

Kosutic-Gulija et al first reported the genetic heterogeneity of the L-Zagreb mumps vaccine virus.[7] The first site was reported in the Nucleocapsid (NP) gene at position 1073, represented as G1073T. The second site, in F gene was represented as C5261T and the third site being G11345T in the L gene of the virus.

In this study, the virus was passaged multiple times on three different cell substrates viz. HDC, Vero cells and Chicken Embryo Culture (CEC) cells, including a plaque purification passage.

First and the last passage samples were then genetically analyzed for the identification of the nucleotide present at the three known SNP locations, using the SBE assay.

RNA viruses like the mumps virus are known for their high rate of mutations because of non-availability of proof reading mechanism. Therefore it was interesting to find out, whether the changed cell substrate results in mutations at least at the known hotspot SNP sites.

Sequencing of the entire genome may not be feasible always. Hence a technique such as SBE can be a useful firsthand tool for primary analysis in determining genetic homogeneity.

Materials and Methods

Materials

Biological materials such as Mumps vaccine virus, HDC, CEC and Vero cells were available in-house.

Cell Medium, Minimum Essential Medium with Hank's Salts & L-Glutamine was from

Invitrogen, USA. It was supplemented with 10% Fetal Bovine Serum from Morgate, Australia.

Tissue Culture Flasks were from Becton Dickinson and 96 well plates were from Corning Inc, USA.

Shrimp Alkaline Phosphatase was from USB

Corporation, USA.

DNase, RNase and pyrogen free microcentrifuge tubes and micropipette tips were procured from Eppendorf, Germany and PCR Tubes were obtained from Axygen Inc, USA.

RNA extraction kit, QIAMP Mini RNA kit catalog no. 52906 and PCR purification kit, catalog no. 28106 were from QIAgen, Germany.

AccessQuick RT-PCR system, catalog A1702 was from Promega.

SNaPshot multiplex kit, catalog no. 4323161, Primer Focus Kit, Catalog no. 4329538 and GeneScan120 Liz Size Standard, Catalog no. 4324287 and Hi-Di Formamide were from Thermo Fisher USA.

Designed Primers were synthesized from Sigma Genosys.

Virus growth and extraction of RNA

Four consecutive passages of the L-Zagreb mumps virus, including one plaque purification passage were performed on each of the three cell substrates, viz; HDC, Vero cells and CEC.

Briefly, monolayers of the respective cells in TCFs were infected with preceding passage virus and infected cultures were incubated at 33 ± 1°C temperature. Multiple harvests were collected at predefined intervals. Homogenized virus pool was prepared from all the harvests which was titrated and stored at -60°C.

RNA was extracted from all the homogenized virus pool samples using QIAgen Mini RNA extraction kit as per the instructions provided in the kit. Briefly, heat inactivated virus samples were first lysed with the lysis buffer, loaded onto the spin columns, washed twice using the wash buffer and finally eluted with the elution buffer from the kit. All the purified RNA samples were appropriately labeled and stored at -60°C till further use.

Preparation of DNA amplicons and SBE assay

Promega's single tube RT-PCR AccessQuick system was used to prepare cDNA from the RNA templates using random hexameric primers. For the preparation of amplicons, gene-specific primers for the NP gene, F gene and L gene covering the SNP sites were designed and used. Refer Table 1 for the list of primers designed.

Table 1: Details of primers designed and their properties.

Primer Name	5'à 3' Sequence	Nucleotides	GC %
N-F	GCCTTCTTTCTCACCCTCAA	20	50
N-R	GTACCGACTCCCATAGCATAAC	22	50
*1073-F	ATCGAGGTCTCGGAGAACAA	20	50
*1073-R	CAACAAAGCAAGGTATCTGG	20	45
F-F	GGATAACCAGCTTGCAACTTTC	22	45.5
F-R	CCATTAGACCGGCACTTAGTATC	23	48
*5261-F	TGACGCCTGCAGTGGTTCAAGCAA	24	54.2
*5261-R	GCAGCAGAAATTGAAGTAGATAAT	24	33.3
L-F	TCATGTAGTCGCCTGTTCAATA	22	40.9
L-R	CTCCACGAAGCATGGGATTA	20	50
*11345-F	AGACCCATTAGTGTCTGCAATTGCTGAT	28	42.9
*11345-R	AGACAGCCAGCCTTAATTAATCGTTTCA	28	39.3

*Nucleotide position in Mumps genome, -F: Forward primer, -R: Reverse primer

The above primer pairs were designed using online primer design tools. The primers were aligned with the consensus sequence of the L-Zagreb mumps virus downloaded from the NCBI GenBank Accession No. AY685920.

Individual amplicons were prepared in separate reactions. Briefly, reverse transcription of RNA was done using AMV reverse transcriptase, followed by PCR amplification with AccessQuick Master Mix and corresponding primer pairs, as shown in Table 2.

Table 2 : Details of reaction set up and processing conditions used for single tube RT-PCR.

Sr. No.	Reagent	Quantity (µL)	RT-PCR Conditions	Temperature (oC)	Time (minutes)
01	Master Mix	30.0	Reverse Transcription	45	45
02	Purified Water	17.1	Initial Denaturation	95	2
03	Gene-Specific Forward Primer	2.7	Cyclic* denaturation	95	0.3
04	Gene-Specific Reverse Primer	2.7	Cyclic* annealing	60	0.5
05	Reverse Transcriptase	1.5	Cyclic* extension	72	0.5
06	RNA template	6.0	Final Extension	72	5.0
	Total	60.0	Storage	4 or -20	∞

*Cyclic: 50 cycles.

SBE assay was performed using SNaPshot multiplex kit to identify the bases at the 3 known SNPs sites, the details of which are listed in Table 3 below.

GeneMapper version 5 software was used for the analysis of the electropherograms. Internal sizing standard GeneScan 120 LIZ was included in each sample. The samples to be tested were loaded into 96 well plates. The 8 capillary array of the instrument took a run time of less than 20 minutes for analyzing 8 samples in a column simultaneously.

Table 3 Details of reaction set up and processing conditions used for SBE Assay.

Sr. No.	Reagent	Quantity (µL)	Temperature (oC)	Time (second)
01	*RRM	5.0	---	---
02	SNP-specific Primer	1.0	96	10
03	Gene-specific Amplicon	3.0	50	5
04	Purified Water	1.0	60	30
Twenty Five cycles of the above 3 steps are performed and then the PCR product is held at 4oC until the next step of post-reaction clean up.				
05	Reaction Mixture	10.0	37	60
06	Shrimp Alkaline Phosphatase	1.0	72	15
Store at 4oC until loading for capillary electrophoresis.				
07	HiDi Formamide	9.0	---	---
08	GeneScan 120 LIZ Size Standard	0.5	---	---
09	Cleaned up PCR Sample	0.5	95	5
Load for Capillary Electrophoresis.				

*Ready Reaction Mix: It contains fluorescently labeled ddNTPs, reaction buffer and AmpliTaq DNA polymerase.

Validation of SBE assay

Performance consistency of the SBE assay was checked through resolution of primers and its sensitivity, linearity and reproducibility. Resolution of individual SNP-specific primer was first checked using the Primer Focus Kit as per the kit instructions.

Reproducibility was checked by running a primer focus assay on duplex primers, i.e. the shortest and the longest primers were combined in one reaction.

Linearity of the assay was checked by running 3 serial double dilutions of the original sample. Sensitivity was evident from the results obtained for the low titer mumps virus grown on Human Diploid Cells (HDC).

Results

Results of Virus growth and RNA extraction

Virus content for HDC-mumps ranged from 2.1 to 2.7 log $CCID_{50}$ per 0.5 ml over the 3 consecutive passages. That for Vero-mumps ranged from 4.3 to 5.4 log $CCID_{50}$ per 0.5 ml and for CEC-mumps, it was from 5.7 to 6.5 log $CCID_{50}$ per 0.5 ml.

Thus, Comparison of last passage virus content revealed almost 2.5 log more virus in Vero-mumps than HDC-mumps and 1.1 log more virus in CEC-mumps than in Vero-mumps reflecting different degrees of adaptation on different cell substrates.

Results of preparation of DNA-amplicons & SBE assay

Gel electrophoresis of gene-specific amplicons revealed sizes of around 200 to 300 basepairs, which were purified and sequenced. More than 99% identity with L-Zagreb mumps sequence (GenBank Accession No. AY685920) was obtained for Vero-mumps and CEC-mumps partial sequences blasted at NCBI, whereas for HDC-mumps, it was more than 84 % identity.

SBE results indicated presence of only variant A population for all the samples because nucleotide G was detected at SNP-1 and SNP-3 locations whereas nucleotide C was detected at SNP-2 location.

Representative results of SBE assay for the last passage of CEC grown mumps virus for all the three SNP hotspots are shown in Fig.1. Black peaks were observed for SNP-1 and SNP-3 as seen in fig. 1 (a) and (c), whereas blue peak was observed for SNP-2 as seen in fig. 1(b).

Fig.1. Results of SBE assay

Fig. 1(a) Peak detected at SNP-1.

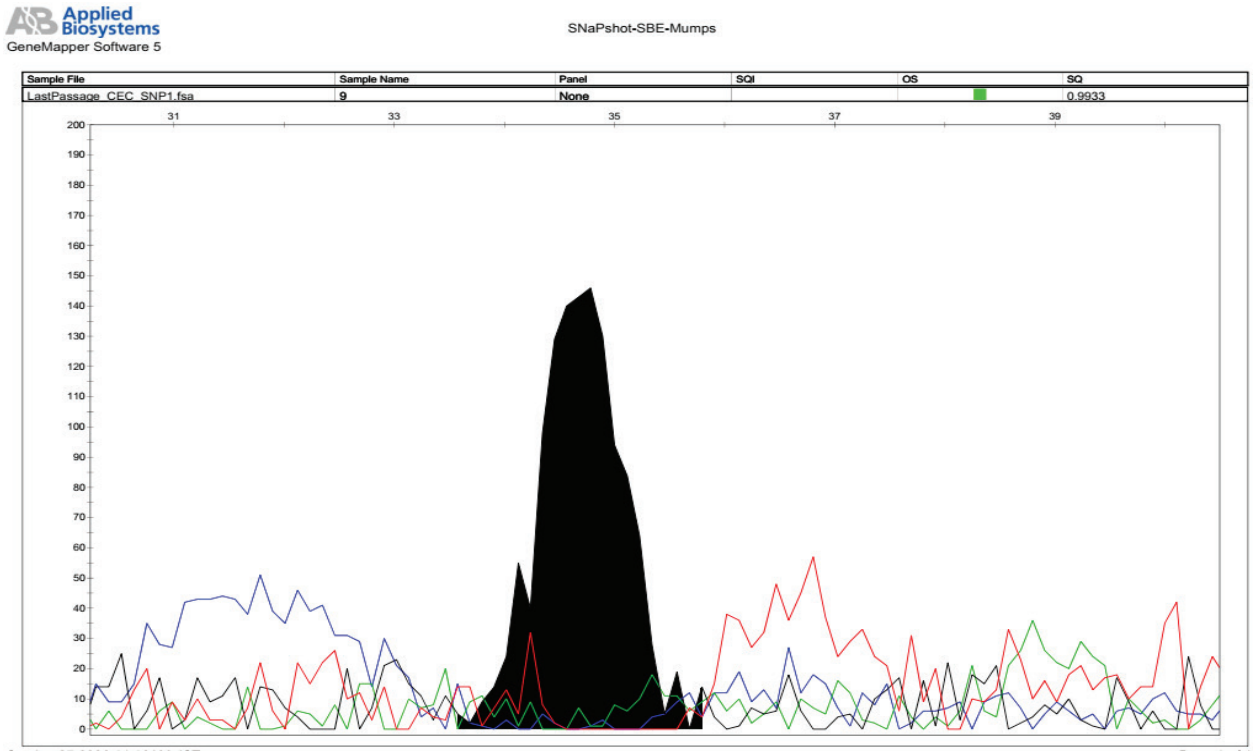


Fig. 1(b) Peak detected at SNP-2.

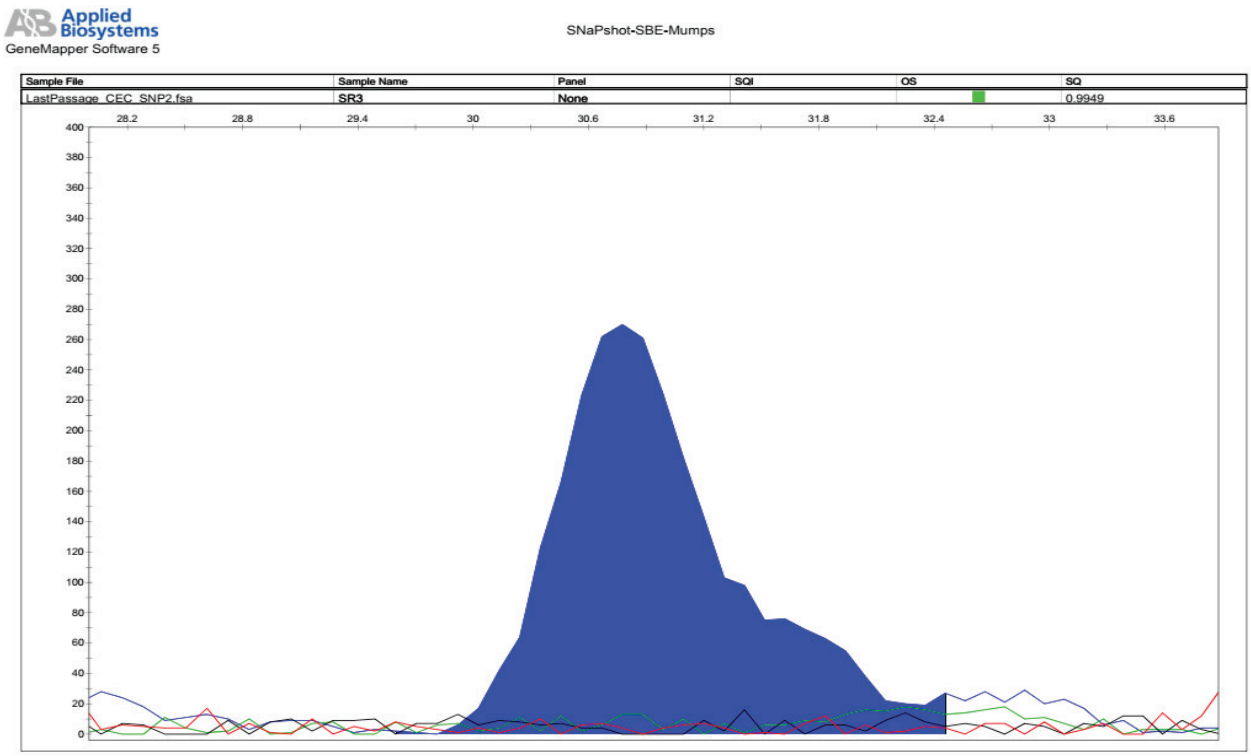


Fig. 1(c) Peak detected at SNP-3.

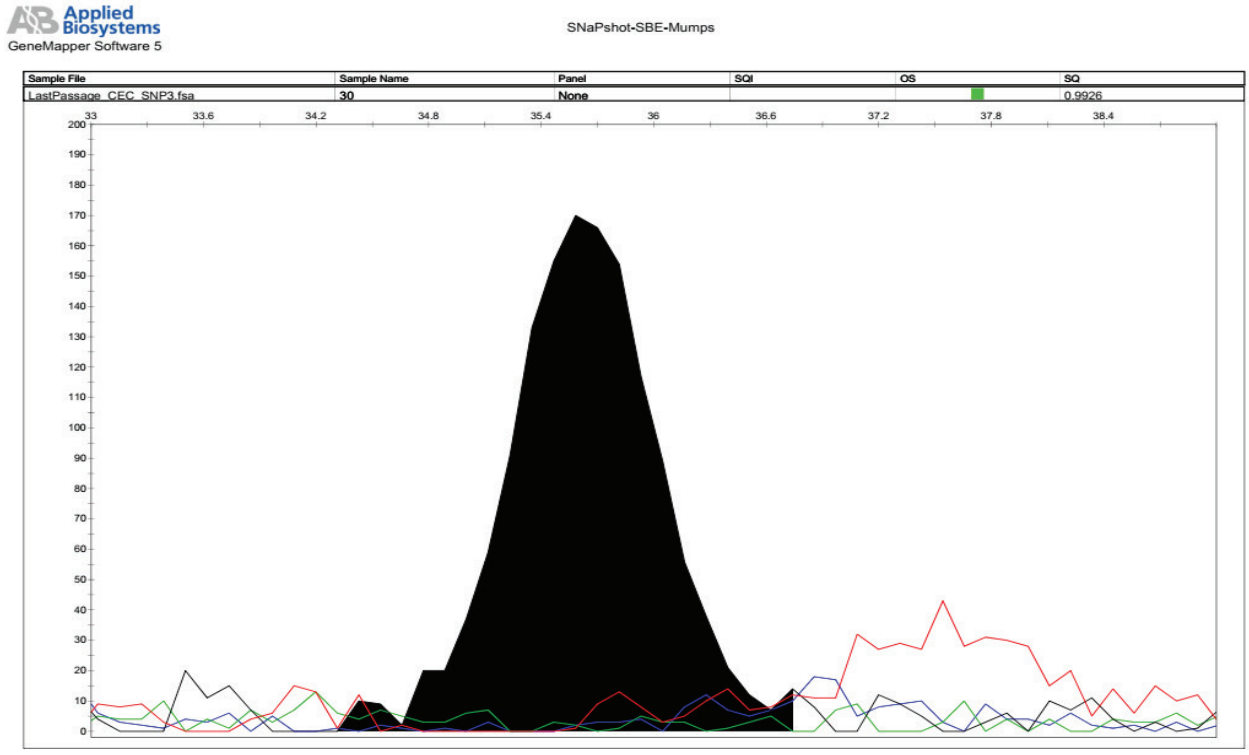


Fig. 2 depicts the results of validation of SBE assay.

Primer focus assay revealed that all the three SNP-specific primers could be resolved between 30 to 40 base pair size range and all the four fluorescent dyes were distinguishable as seen in Fig. 2(a).

Differences in the heights and sizes of the peaks are because of the differences in the molecular weights of the fluorochromes and their degree of affinities.

Sensitivity of the assay was evident from the fact that peaks were detected for HDC-mumps samples with as low as 2.1 log $CCID_{50}$ per 0.5 ml titer.

As is evident from the Fig. 2(b), the peak height and area of the original sample were found to be decreasing linearly over the three dilutions thus confirming linear response.

Three technical replicates of a sample containing primer duplex showed excellent reproducibility as is evident in the electropherograms in Fig. 2(c). Not only size, i.e. mobility but also the peak height, i.e. fluorescence intensity are seen matching in all the three replicates for all the peaks.

Fig.2. Results of Validation of SBE Assay

Fig. 2(a) Exemplary Primer Resolution with respect to Size and Fluorochromes

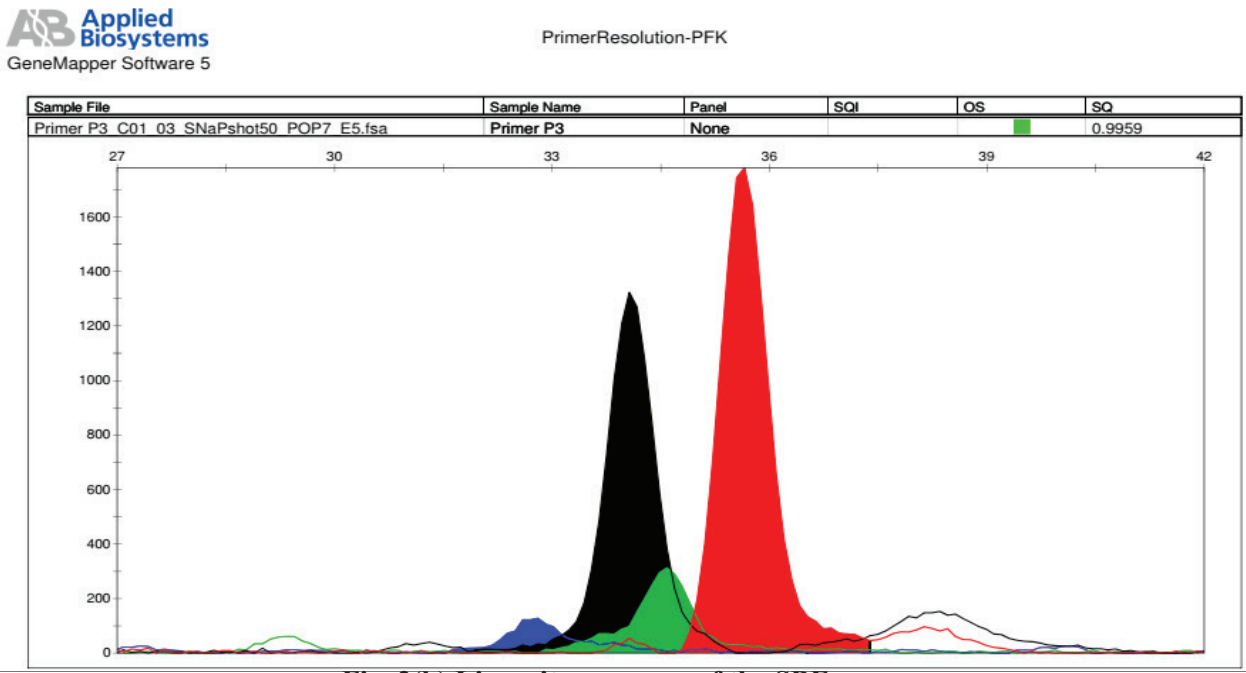


Fig. 2(b) Linearity response of the SBE assay

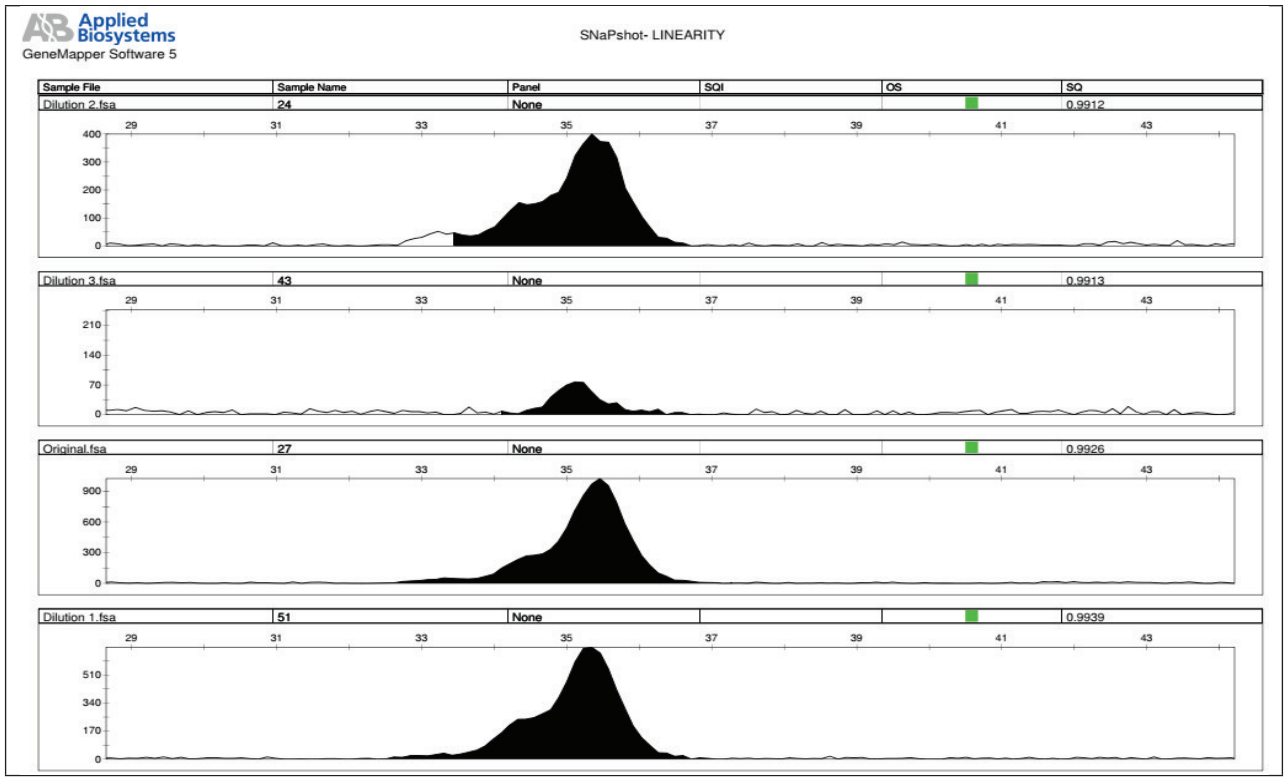
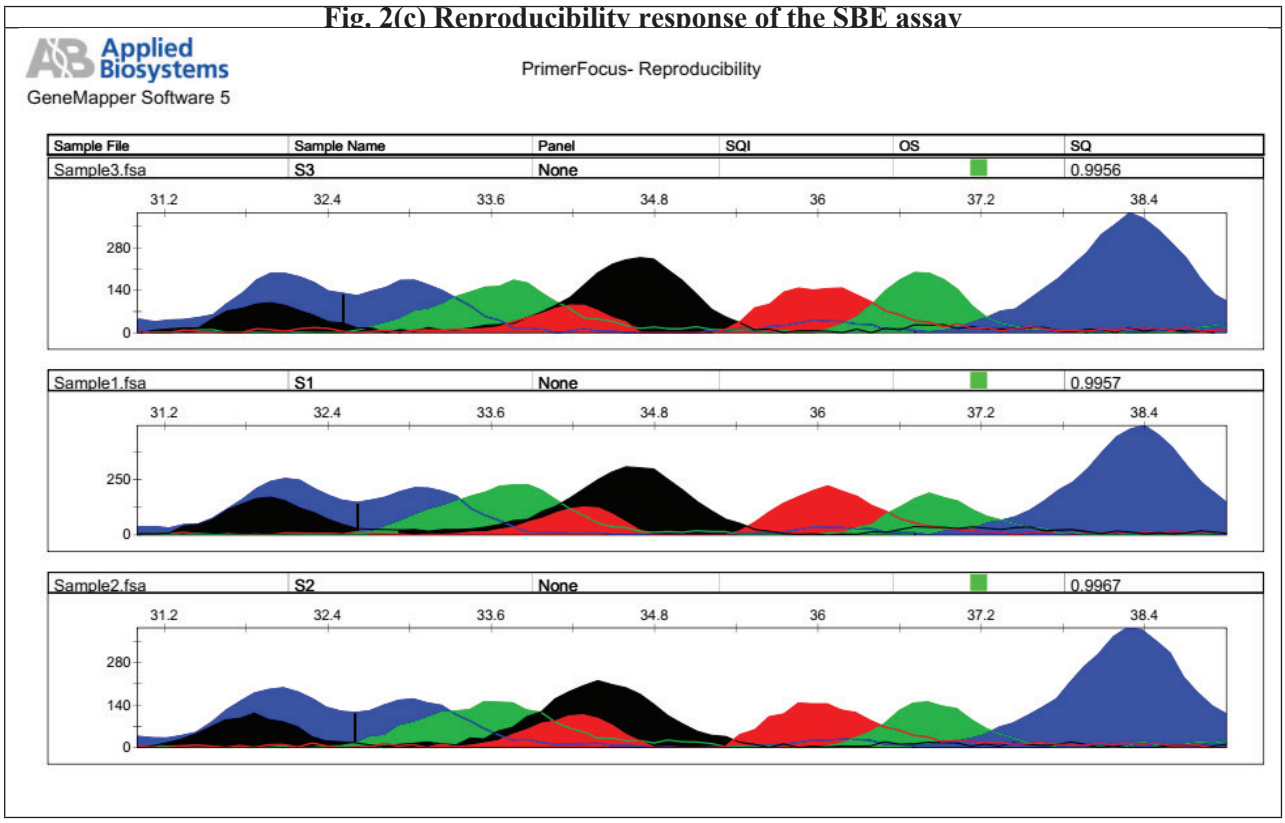


Fig. 2(c) Reproducibility response of the SBE assay



Discussion

As a part of a doctoral programme, studies on productivity improvements in L-Zagreb mumps

vaccine were undertaken. In this context, a mini-review describing evolution of viral vaccine production technology with respect to change in cell substrate and culture methodology has already been published.[8] So also a research article describing adaptation of L-Zagreb strain-based mumps vaccine on different cell substrates has been reported.[9] Further, the effect of changes in culture methods and cell substrates on key quality attributes of mumps vaccine have been evaluated.[10] In this research, the qualitative SBE method was found to be a useful tool for quick genetic analysis of experimental mumps vaccine virus samples.

Mumps vaccine, an RNA virus vaccine, is known for its changing in-vitro and in-vivo

properties.[11] It also exhibits quasispecies nature with defective interfering particles.[12]

Hence, it is very important to analyze and ensure the consistency in quality of the vaccine at every stage of the experiment, which can be achieved by the availability of a quick molecular method capable of judging the impact of changes.

Genetic consistency was observed at all the three SNPs, irrespective of changes in cell substrate and number of passages. Based on this evidence, it might be useful to undertake whole genome sequence analysis by advanced methods such as next generation sequencing.

The results of primer focus assay confirmed the suitability of the designed primers for the work. Red fluorescent dye was having maximum affinity and the blue dye showed minimum affinity with black and green dyes showing intermediate affinities. All the SBE assays were performed in singleplex configuration.

Multiplexing was deliberately avoided in first runs owing to a lot of optimizations necessary to achieve perfection. System variables such as pH, temperature, primer size, buffer type, its concentration, applied voltage and the type of polymer used can affect the electrophoretic mobility of the sample.

Sensitivity of the SBE assay was evident from the results obtained for the low virus titer, i.e. HDC grown mumps virus, for which the titers were around 2.0 log $CCID_{50}$ as against 5.0 to 6.0 log $CCID_{50}$ titers of Vero grown and CEC grown viruses.

The assay was found to be accurate based on the appropriate negative results obtained for all the negative controls. Negative controls in the form of 'No Template Control' and 'No Primer Control' were included in every SBE assay, besides 'No RT Control' in case of RT-PCR. Positive controls were available with the assay kits.

Given the high infidelity of RNA viruses, it could be possible that more and more mutations will become evident, especially under circumstances such as change in cell substrates. However, today not enough data is available for the biomarkers in mumps genome responsible for critical functionalities. When bioinformatics in this field gets enriched, SBE assays could become an indispensable tool, owing to its shorter lead time and relatively inexpensive nature.

This study has demonstrated that a simple, qualitative molecular method of identifying nucleotide bases at the known locations can be a useful preliminary tool for comparison of the genetic homogeneity of the virus populations adapted to grow on different cell substrates.

This study has reported for the first time, application of SBE for detection of bases at the known SNP hotspots of the L-Zagreb mumps virus grown on

HDC, CEC and Vero cells.

Conclusion

Overall the SBE method was found to be a practical first hand alternative to verify genetic homogeneity. Although, it could not be a substitute for the whole genome sequencing, it could be useful in initial characterization of biological samples as well as to quickly understand the impacts of critical changes done in the production process of biologics.

The multiplexing potential of this tool can save a lot of time and resources, especially when high throughput screening is essential for large number of samples.

This study was performed with singleplex configuration for each sample and for each of the three SNP sites, but in future, carefully optimized multiplexing assays can be developed, especially if more number of SNP locations are to be evaluated.

Conflict of Interest: Authors, who are employees of Serum Institute, declare that there is no competing / conflicting interest for the work carried out as described in the manuscript.

Source of Funding: There was no external funding received for this work.

Ethical Clearance: The study is exempt from ethics committee review because no animals or humans were involved in it.

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