Digital PCR genotyping of pepino mosaic virus

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Abstract

Pepino mosaic virus (PepMV) is a plant pathogen causing significant economic losses in tomato production. Sensitive, reliable and robust detection methods are crucial for containing the spread of PepMV and reducing its damaging effects. Digital PCR (dPCR) presents several advantages to conventional real-time quantitative PCR (qPCR), including absolute quantification ability, robust quantitative multiplexing capabilities and straightforward result analysis. Furthermore, dPCR is especially suitable for analysis of complex samples due to its remarkable tolerance to PCR inhibitors, which makes it a promising method for plant virus genotyping. In this chapter, we present two protocols for PepMV genotyping and quantification using one-step reverse transcription digital PCR (RT-dPCR). The first protocol outlines four simplex assays, while the second describes two duplex assays for precise and comprehensive genotyping of PepMV variants.

Keywords Plant virus, detection, quantification, Pepino mosaic virus, PepMV, digital PCR, dPCR, RT-dPCR, duplex

1 Introduction

Pepino mosaic virus (PepMV) is a non-enveloped rod shaped, single-stranded RNA virus belonging to the genus *Potexvirus* ***(1)***. It is a pathogen that affects the genus *Solanum*, and its effects on greenhouse tomato production are economically very damaging ***(2–4)***. PepMV exhibits significant genomic diversity, and five main genotypes are currently recognized: Peruvian (LP); European (Eur); American (US1, US2) and Chilean (Ch2) ***(5, 6)***. Highly virulent and damaging isolates are distinguished from mild isolates by single nucleotide variations ***(7)***. The enforcement of strict hygiene measures required to control the spread of PepMV is challenged by the virus's remarkable resilience ***(8)***. Cross protection strategies have been researched that entail the infection of a plant with a mild strain of a virus to confer resistance to more severe strains ***(9)***. One such strategy, the PMV-1 vaccine has been approved in the European Union ***(10)***. There has also been research into controlling the spread of PepMV by RNA interference using transgenesis ***(11)***. Reliable detection, quantification, and genotyping of PepMV are essential not only for developing, applying, and evaluating approaches to combat it, but also for assessing plant resistance to PepMV, determining PepMV concentrations across various plant tissues and organs, and measuring the prevalence of different PepMV strains in mixed infections. As PepMV can be transmitted through seeds ***(3, 12)***, quantification and genotyping are also important for screening of seed stocks to prevent major economic damage ***(13)***.

Reverse transcription real-time quantitative polymerase chain reaction (RT-qPCR) is a commonly employed method for PepMV quantification and genotyping ***(13, 14)***. This method only allows for relative quantification, as absolute quantification would require standard curves prepared from certified reference materials, which are currently not available for PepMV. The method is also heavily affected by PCR inhibition caused by various substances commonly present in plant material ***(15)***.

Conversely, digital PCR (dPCR) provides absolute quantification of target sequences without requiring standard curves ***(16)***. It has also shown remarkable tolerance to PCR inhibitors, improving the accuracy and repeatability of the results ***(17, 18)***. Further advantages of the method include its robust multiplexing

(detection of more than one target in one reaction) capabilities and straightforward analysis of results ***(19–22)***.

Here we describe two one-step RT-dPCR-based protocols for accurate genotyping and quantification of PepMV. The first protocol was developed for the Bio-Rad QX200 system (suitable also for QX One and QX600) and consists of the following simplex (one target) assays for detection and quantification of PepMV genotypes: PepMV-universal for all genotypes, PepMV-Eur for Peruvian and European genotypes, PepMV-Ch2 for the Ch2 and US2 genotypes, and PepMV-US1 for the US1 genotype. The probes were labeled with 6-carboxyfluorescein FAM. The second protocol was developed for the Stilla Naica platform and describes two duplex assays acquired by combining the simplex assays. The first duplex consists of the PepMV-US1 and PepMV-Universal assays, while the second duplex contains the primers and probes for the PepMV-Eur and PepMV-Ch2 assays. Here the dyes on two probes differ from the ones used in the first protocol, namely, hexachloro-fluorescein (HEX) and cyanine-5 (Cy5) are used instead of FAM for the PepMV-Ch2 and PepMV-US1 assays, respectively. Generally, the level of background fluorescence is higher in multiplexed assays due to larger quantities of fluorescent dyes present. Therefore, double-quenched ZEN probes with non-fluorescent quencher Iowa Black FQ (IBFQ) on 3' end are used in the duplex assays to reduce the background fluorescence.

2 Materials

2.1 Samples

1. Extracted sample DNA (*see* **Note 1**).
2. Positive and negative controls (*see* **Note 2**).

2.2 Simplex assays on the Bio-Rad QX200 platform

2.2.1 Reaction preparation and thermal cycling

1. 1-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad, USA; includes the master mix, reverse transcriptase and dithiothreitol (DTT))

2. Rainin pipettes with corresponding presterilized BioClean LTS tips with filter (*see* **Note 3**).
3. Two UV chambers (*see* **Note 4**).
4. Pierceable Foil Heat Seal (Bio-Rad, USA) or equivalent.
5. Heat Sealer (Eppendorf, Germany) or equivalent.
6. T100™ Thermal Cycler (Bio-Rad, USA) or equivalent.
7. Pipettes.
8. Nuclease-free microcentrifuge tubes.
9. twin.tec semi-skirted 96-well plates (Eppendorf, Germany).
10. Vortex and microcentrifuge.
11. Centrifuge with rotor for 96-well plate centrifugation.

2.2.2 Droplet generation and readout

1. QX200™ Droplet Generator or Automated droplet generator (AutoDG) (Bio-Rad, USA).
2. DG8 Cartridge Holders, Cartridges and Gaskets (for QX200 droplet generator) (Bio-Rad, USA).
3. Droplet Generation Oil for Probes (for QX200 droplet generator) (Bio-Rad, USA).
4. DG32 Automated Droplet Generator Cartridges (for AutoDG) (Bio-Rad, USA).
5. Automated droplet Generation Oil for Probes (for AutoDG) (Bio-Rad, USA).
6. Pipette Tips for the AutoDG™ System (Bio-Rad, USA).
7. Pipette Tip Waste Bins for the AutoDG™ System (Bio-Rad, USA).
8. Nuclease-free water.
9. Droplet Reader Oil (Bio-Rad, USA).
10. QX200™ Droplet Reader (Bio-Rad, USA).
11. QuantaSoft analysis software or QXManager Software (Bio-Rad, USA).
12. Cooling blocks.

2.3 Duplex assays on the Stilla Naica platform

2.3.1 Reaction preparation

1. qScript XLT One-Step RT-qPCR ToughMix (no ROX) (Quanta BioSciences, USA) or equivalent (*see* **Note 5**).
2. Fluorescein (FITC; 1 µM).
3. Pipettes.
4. Nuclease-free microcentrifuge tubes.
5. Vortex and microcentrifuge.

2.3.2 Digital PCR and data analysis

1. Geode partitioning and thermocycling instrument (Stilla Technologies, France).
2. Prism3 or Prism6 detection and imaging instrument (Stilla Technologies, France).
3. Sapphire chips (Stilla Technologies, France).
4. Antistatic wipes.
5. Low-lint, high absorbency, and chemically inert wipes.
6. Crystal Reader and Crystal Miner software (Stilla Technologies, France).

3 Methods

3.1 Simplex assays on the Bio-rad QX200 platform

3.1.1 Preparation of reaction mixtures

1. Prepare the reaction mixtures by mixing in nuclease-free tubes 5 µL master mix with 1 µL DTT (300 mM), 2 µL reverse transcriptase, nuclease free water, primers, and probes, according to **Table 1**, up to a final volume of 16 µL (*see* **Note 5**). Vortex the mixtures and centrifuge them briefly (*see* **Note 6**).
2. Distribute the mixtures into 96-well polypropylene plates (*see* **Note 7**).

3. Add 4 µL of each RNA sample or non-template control (NTC) into the wells containing the reaction mixtures (*see* **Note 5**). Seal the 96-well plates with pierceable foil. Vortex and centrifuge briefly.

3.1.2 Manual droplet generation using Bio-Rad QX200^{TM} droplet generator

1. Turn on the droplet generator by connecting it to a power source.
2. Insert the DG8 cartridge into the cartridge holder.
3. Transfer 20 µL of each reaction sample into the sample wells on the cartridge.
4. Transfer 70 µL of droplet generation oil into each oil well on the cartridge.
5. Secure the DG8 cartridge gasket over the cartridge.
6. Press the button on the droplet generator to open it.
7. Place the holder with the cartridge into the droplet generator.
8. Close the lid on the droplet generator to begin droplet generation. After completion (indicated by all lights shining green), remove the holder with the cartridge from the instrument and remove the gasket from the cartridge.
9. Transfer the droplet suspensions (40 µL) onto a 96-well plate placed on a cooling block.
10. Repeat droplet generation and plate transfer steps for all the samples.
11. Seal the plate harboring the droplets with pierceable foil (*see* **Note 8)**. Do not vortex or centrifuge the plate.

3.1.3 Automatic droplet generation using Bio-Rad AutoDG

1. Turn on the automatic droplet generator and configure the sample plate in the instrument software.
2. Place the DG32 cartridges, pipette tip boxes without box lids (*see* **Note 9**) and the tip waste bin into their dedicated holders in the instrument (as indicated on the instrument screen).
3. Place the sealed 96-well plate containing the prepared reactions into its dedicated holder (named "sample plate") in the instrument.
4. Place the cooling block (*see* **Note 10**) into the holder named "droplet plate" and place an empty 96-well plate into the cooling block for droplet collection.

5. Check if there is enough automated droplet generation oil present and change the oil if needed (*see* **Note 11**).
6. Tap “START Droplet Generation” and “Start Run” to initiate droplet generation.
7. After the run is finished and the instrument door unlocked, remove the plate with droplets from the instrument and discard the used consumables.
8. Seal the plate harboring the droplets with pierceable foil (*see* **Note 8**). Do not vortex or centrifuge the plate.

3.1.4 Thermal cycling

1. Transfer the plate into a thermocycler and run the PCR under the conditions shown in **Table 2**.

3.1.5 Droplet reading and data analysis

1. Transfer the 96-well plate into the QX200 Droplet Reader (*see* **Note 12**).
2. Run the QuantaSoft software on the connected computer.
3. Flush the instrument by clicking “Setup” -> “Instrument routines” -> “Flush System” in the QuantaSoft software.
4. Click “Setup” -> “Template” -> “New” to configure the plate layout and enter the necessary information. Save the created template.
5. Click “Run” to initiate droplet reading.
6. After reading is finished, save the experiment data, and remove the plate from the instrument.
7. Open the experiment in the QuantaSoft software and click “Analyze” to start data analysis.
8. Imaging results are visualized as a scatterplot of fluorescence amplitudes for each droplet (**Fig. 1**). Set the fluorescence thresholds for distinguishing between positive and negative droplets. The thresholds can be set simultaneously for all samples in a given assay. Use NTCs as a guide for determining the negative population and threshold setting (*see* **Note 13**).
9. The software calculates the concentration of target copies in each sample based on the set thresholds. The results can be viewed under the “Concentration” tab and can be exported in .csv format for further analysis in spreadsheet software such as Excel (Microsoft).

3.2 Duplex assays on the Stilla Naica platform

3.2.1 Preparation of reaction mixtures

1. Prepare the reaction mixtures by mixing 13.75 µL ToughMix (2x) (no ROX) with 2.75 µL FITC (final concentration, 100 nM), nuclease free water, primers, and probes, according to **Table 3** (*see* **Notes 6** and **14**). Vortex and centrifuge briefly.

3.2.2 Droplet generation and thermal cycling

1. Clean the bottom of each chip by sliding it on an antistatic wipe and a low-lint, high absorbency and chemically inert wipe.
2. Remove the white caps off the Naica sapphire chip inlets and load 25 µL of each reaction mixture into the inlets. Do not insert the pipette tip into the oil phase. Close the inlets with tall caps included with the chips.
3. Turn on the power switch at the back panel of the Geode.
4. Connect the pressure source to the pressure inlet at the back of the instrument and switch on the power source (*see* **Note 15**).
5. Place the chips into their dedicated spaces in the instrument.
6. Set the partitioning and thermal cycling conditions as indicated in **Table 4** (see **Note 16**).
7. Press the “Run” icon on the instrument screen, press the “Open” icon and select the correct cycling program in the “local/templates” subfolder
8. Press the “Open” icon and then the “Play” icon to start the program.
9. Once the run is over, touch the screen, open the lid of the instrument, remove the Sapphire chips, and close the lid.

3.2.3 Droplet imaging and data analysis

1. Turn on the power switch at the back of the Prism instrument and press the wake-up button on the front panel.
2. Log in with your username and password.

3. Click on the “Crystal Reader” icon on the instrument or a computer to launch the reader software.
4. Click on the “OPEN TRAY” button in the software or press the “Eject” button on the instrument to eject the tray.
5. Insert the Sapphire chips in the Sapphire tray holder.
6. Place the tray holder on the tray and gently push it towards the back of the tray.
7. Click on the “CLOSE TRAY” button in the software or press the “Eject” button on the instrument to retract the tray.
8. Click on the “NEW EXPERIMENT” button in the software, press “BROWSE” and select the “ScanningTemplate_Prism6_SapphireChip_QuantaBio-qScript-XLT-1-step-RT-qPCR-Thoughmix_Taqman_v1.1.ncx” template.
9. Enter the name of the experiment in the “New Experiment” field and add potential additional information into the “Comments” field.
10. Under the “Experiment Details” tab, check the detection channels you want to utilize and enter the target and fluorophore name for each one (see **Note 17**).
11. Select the chambers to scan and enter the sample name and type for each chamber.
12. For multiplex assays, a color compensation matrix needs to be prepared according to manufacturer instructions.
13. Click on the “SCAN” button, define the location to save scanned data, and confirm that the correct chips are loaded to initiate the scanning of droplets.
14. After the scanning is completed, click “OK” to display the results.
15. Open the experiment file in the Crystal Miner software.
16. Imaging results are visualized as a scatterplot of fluorescence amplitudes for each droplet (**Fig. 2**). Set the fluorescence thresholds for determining positive and negative droplets. It is advised to set the thresholds in the 2D view to account for possible crosstalk between the fluorescence channels (**Fig. 3**).
17. Quantification results are presented by the software as the number of target copies per µL reaction. The results can be exported in .csv format for further analysis in spreadsheet software.

4 Notes

1. Different methods can be used to extract RNA from plant material. For the development of the assays described here, RNeasy Plant mini kit (Qiagen, Germany) was used with some modifications to the manufacturer's instructions. Briefly, the protocol involved the homogenization of 200 mg of plant material in 900 µL of lysis buffer, followed by loading 600 µL of the homogenate onto RNeasy Mini Spin columns. RNA was eluted from the columns by applying two consecutive aliquots of 50 µL each (i.e., 100 µL total) of RNase-free warm water (65°C).
2. At least non-template controls (NTC) and positive controls containing DNA of tested target(s) should be included. It is recommended to also include a negative extraction control to test for potential contamination during RNA extraction and positive extraction controls, which can be either internal or external. Internal positive controls are prepared for each sample and consist of RNA extracted from samples spiked with exogenous nucleic acid that has no relation with the target virus or endogenous nucleic acid and is used to confirm successful extractions. Naturally infected host tissue or spiked healthy host tissue can serve as an external positive control. During initial assay testing and during the validation process an additional control should be incorporated consisting of healthy material of the host plant to assess the background signal produced by the matrix.
3. Use of Rainin tips is recommended for all steps of the protocol, as well as upstream sample preparation. Other tips can have rough tip openings which can lead to shedding of plastic debris in the reaction mixture that can cause shearing of droplets and detection of artifacts during droplet imaging.
4. This is recommended as the preparation of reaction mixture and addition of samples should be performed at separate locations using dedicated laboratory equipment to prevent cross-contamination. Both steps should be performed at locations different from the one used for RNA extraction.
5. It is recommended to prepare the mixtures with 10 % excess volume to offset pipetting losses.

6. It is important to mix the reaction mixtures well, as failure to do so can result in reduced reproducibility of results due to the viscosity of the master mix. Vortex with lower speeds to prevent bubble formation.
7. Use of polypropylene plates is recommended to minimize DNA binding to the plate and thus improve repeatability. Plates must be semi-skirted to fit into the instrument. Eppendorf twin.tec semi-skirted plates and Bio-Rad ddPCR 96-well plates have been proven to be suitable for this application.
8. It is recommended to begin thermal cycling within 30 minutes of sealing the plate. Alternatively, sealed plates can be stored at 4 °C for up to 4 hours before thermal cycling.
9. Only Bio-Rad's AutoDG Pipette tips should be used, as other tips can damage the instrument.
10. The cooling blocks should be cooled in a freezer for at least 2 hours before use to prevent droplet/oil emulsion evaporation. Dark purple color indicates that the block is at the correct working temperature. If the block is pink in color, it should not be used.
11. It is recommended to load the oil bottle ahead of time to save time during experiments. To do so, open the instrument, remove the cap from the bottle of Auto DG oil and insert it into the tower of the oil delivery system in the instrument. Touch the Oil Type button and touch the "Probes" droplet. If loaded correctly, the instrument will perform the flush and prime procedure. Afterwards, touch the "OK" button to return to the home screen. Auto DG Probes oil should be used for hybridization probe-based dPCR experiments.
12. The droplet reader can be switched on in advance to warm up the instrument.
13. Generally, thresholds should be set just above the negative cluster found in NTCs. Sometimes the negative cluster in a given reaction can have a higher fluorescence than the negative cluster in NTCs. The thresholds should be set separately for such reactions.
14. Simplex assays described in the previous subsection can also be adapted for the Stilla platform.
15. The pressure delivered to the instrument should be within 1,250 ± 50 mbar and should never exceed 1,300 mbar.
16. A new reaction program can be created by clicking "Programs" -> "New Program" in the main panel of the instrument software. Modify the program conditions in the created program and

then click on the “Save” icon. The programs are saved in the local/scripts folder. Programs can also be imported to or exported from a USB stick.

17. For the 6-color Naica system at least three channels need to be switched on, we recommend the third channel to be the furthest from the detection channels.

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Figure captions

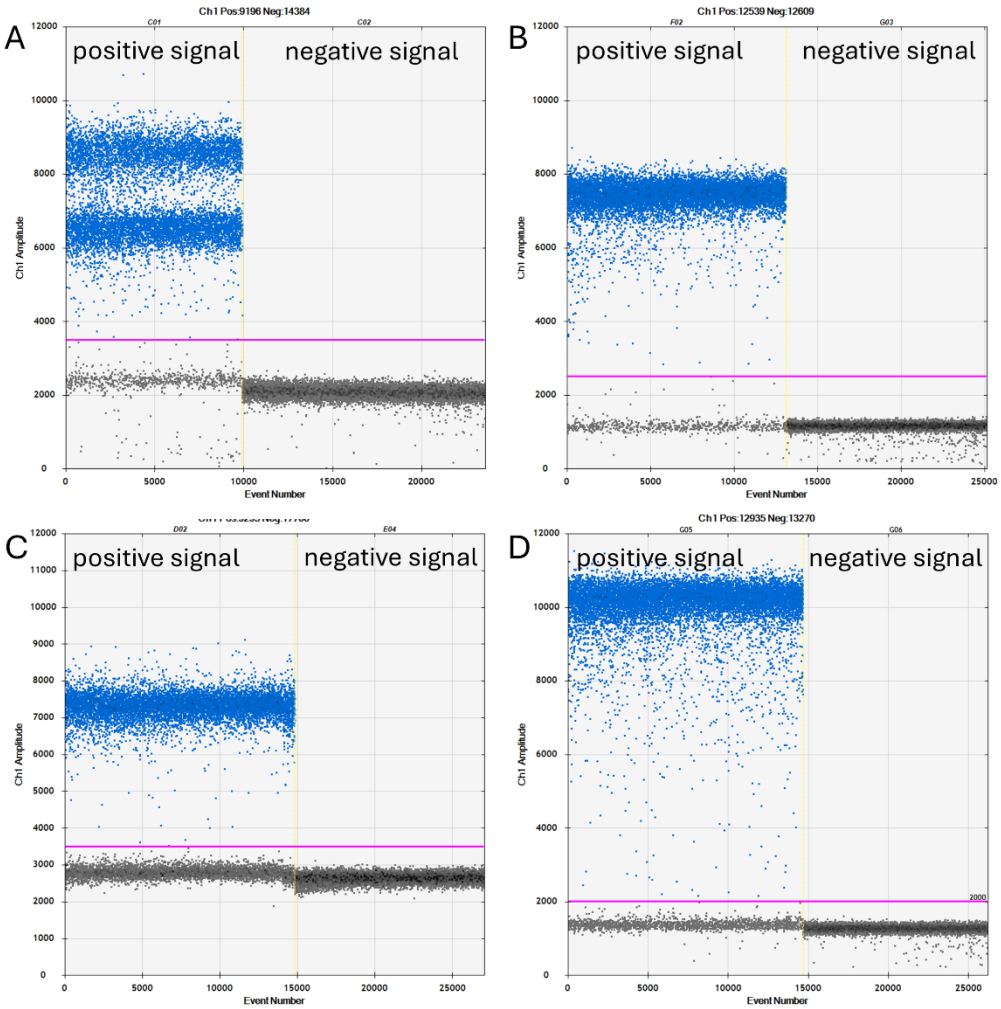

Fig. 1: Amplification plots for simplex assays on QX200. The left part of each panel represents a positive reaction (positive signal) while the right sides of the panel represent non-template controls (negative signal). The pink line represents the set threshold. A – universal assay, B – EUR assay, C-CH2 assay, D – US2 assay

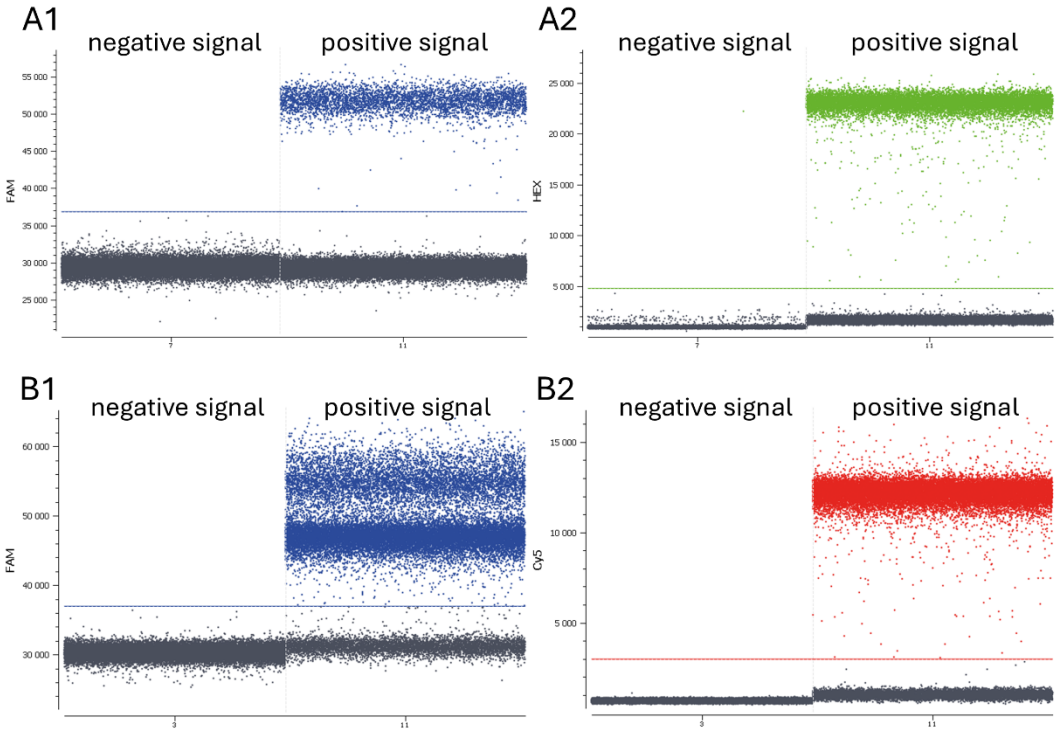

Fig. 2: Amplification plots for duplex assays on Naica. The left part of each panel represents the negative template control, while the right sides of the panel represent positive reaction (positive signal) template controls (negative signal). The blue, green and red lines represent the set threshold. Panels A1 and A2 present the EUR-Ch2+US2 duplex (A1 – EURL target A2Ch2+US2 target), panels B1 and B2 present the universa-US1 duplex (B1 – universal, B2 – US1).

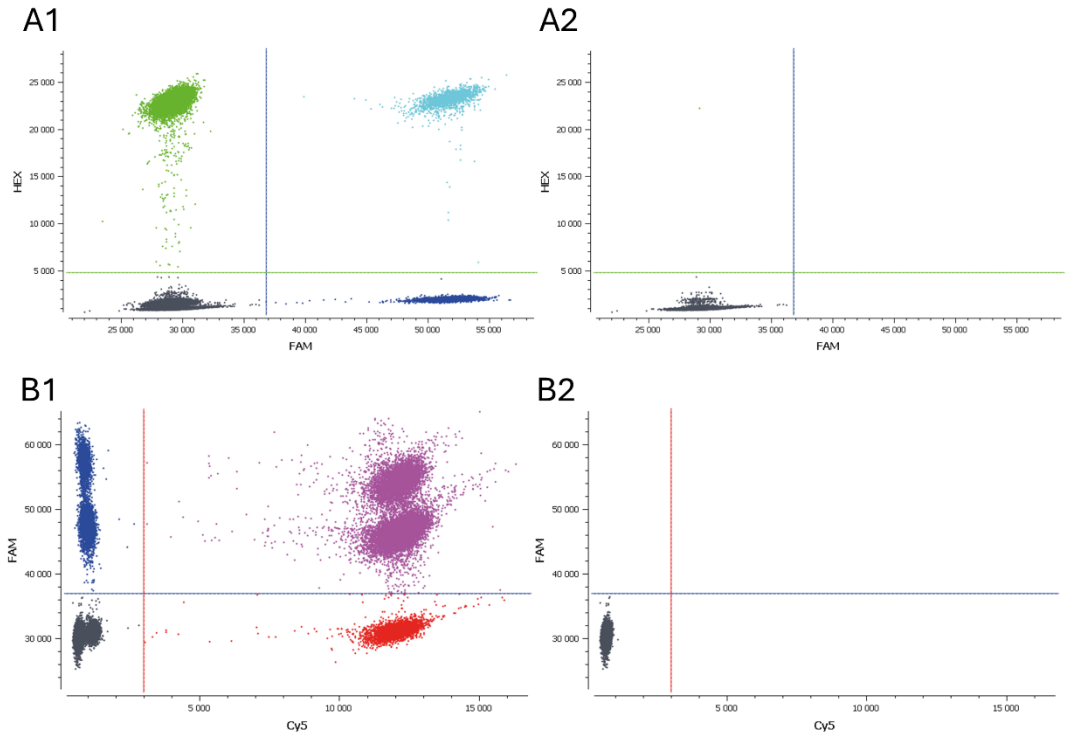

Fig. 3: Amplification plots for duplex assays on Naica in 2D view. The blue, green and red lines represent the set thresholds. Panels A1 and A2 present the EUR-Ch2+US2 duplex (A1 – positive signal, A2 - NTC), panels B1 and B2 present the universa-US1 duplex (B1 – positive signal, B2 – NTC).

Tables

Table 1: Primers and probes used in simplex PepMV genotyping assays

Target and Method	**Name**	**DNA Sequence of the Oligonucleotide (5'-sequence-3')**	**Final Concentration in the PCR [nmol/l]**
PepMV-Universal	KL05-48 forward 1	ACTCCTAGAGCTGACCTCAC	200
	KL05-51 reverse 1	TCTCCAGCAACAGGTTGGTA	200
	KL05-49 forward 2	ACTCCTAGAGCTGATCTTAC	200
	KL05-52 reverse 2	TCACCTGCAACTGGTTGATA	200
	KL05-50 probe	FAM-TGTCAGCTTGCATTTACTTCCAAAA-TAMRA	400
PepMV-Eur	Eur-rep forward	TGGGTCAAGAAAGTGGAAAAATTAG	900
	Eur-rep reverse	TGCATGAAAGCTGCAATGGT	900
	Eur-rep probe	FAM-TGCTGTCAAGTCAAAGCCTGGCCA-TAMRA	200
PepMV-Ch2	Ch2-cp forward	TGGGTTTAGCAGCCAATGAGA	900
	Ch2-cp reverse	AACTTTGCACATCAGCATAAGCA	900
	Ch2-cp probe	FAM-CGGACCTGCCATGTGGGACCTC-TAMRA	200
PepMV-US1	US1-cp forward	AGCGCGTGCTTATGCTGAT	900
	US1-cp reverse	CGTGAGAGTGCTGGATTTGAAG	900
	US1-cp probe	FAM-CCGCACAACTCATAGGTGCCACCC-TAMRA	200

Table 2: Thermal cycling conditions for the QX200 platform

Cycling step	**T (°C)[a]**	**Time**	**Number of cycles**
Reverse transcription	50	60 min	1
Enzyme activation	95	10 min	1
Denaturation	95	30 s	40
Annealing and elongation	56	1 min	
Enzyme deactivation	98	10 min	1

Table 3:Primers and probes used in PepMV genotyping assays on the Stila naica platform

Target and Method	**Name**	**DNA Sequence of the Oligonucleotide (5'-sequence-3')**	**Final Concentration in the PCR [nmol/l]**
Duplex 1 (Eur + Ch2)	Eur-rep forward	TGGGTCAAGAAAGTGGAAAAATTAG	900
	Eur-rep reverse	TGCATGAAAGCTGCAATGGT	900
	Eur-rep probe	FAM-TGCTGTCAAGTCAAAGCCTGGCCA-ZEN/IBFQ	200
	Ch2-cp forward	TGGGTTTAGCAGCCAATGAGA	900
	Ch2-cp reverse	AACTTTGCACATCAGCATAAGCA	900
	Ch2-cp probe	HEX-CGGACCTGCCATGTGGGACCTC-ZEN/IBFQ	200
Duplex 2 (US1 + universal)	US1-cp forward	AGCGCGTGCTTATGCTGAT	900
	US1-cp reverse	CGTGAGAGTGCTGGATTTGAAG	900
	US1-cp probe	Cy5-CCGCACAACTCATAGGTGCCACCC-ZEN/IBFQ	200
	KL05-48 forward 1	ACTCCTAGAGCTGACCTCAC	200
	KL05-51 reverse 1	TCTCCAGCAACAGGTTGGTA	200
	KL05-49 forward 2	ACTCCTAGAGCTGATCTTAC	200
	KL05-52 reverse 2	TCACCTGCAACTGGTTGATA	200
	KL05-50 probe	FAM-TGTCAGCTTGCATTTACTTCCAAAA-ZEN/IBFQ	400

Table 4: Cycling conditions for the Naica Geode

Cycling step	**T (°C)**	**Pressure (mbar)**	**Time**	**Number of cycles**
Partition	40	Atmospheric pressure to +950	12 min	1
cDNA synthesis	50	+950	10 min	1
Enzyme activation	95	+950	1 min	1
Denaturation	95	+950	30 s	45
Annealing and elongation	56	+950	15 s	
Release	Down to 25	Down to atmospheric pressure	33 min	1

[a]Ramp rate should be set to 2.5 °C/s.