1.0 Purpose

This protocol provides a method for performing PCRs for neutral microsatellite analysis on genomic DNA extracted from either dried blood spots (DBSs) on Whatman 903 filter paper or whole blood. Microsatellites are tandem repeats of one to six nucleotides in a DNA sequence. Microsatellite loci usually vary in length from between five and forty repeats with dinucleotide, trinucleotide, and tetranucleotide repeats being the most common. The size differences in the lengths of microsatellite loci among various strains of *Plasmodium falciparum* are the basis for multilocus genotyping, a population genetics technique where the sizes of various loci are determined; the combination of these loci lengths is then used to distinguish recrudescence from reinfection in a drug efficacy study. Multilocus genotyping data allows inferences to genetic structure and relatedness as well as allelic variation among strains of a microorganism such as *P. falciparum* [22.1, 22.2].

2.0 Scope

2.1 This SOP applies to microsatellite research and surveillance conducted within the Malaria Branch, DPDM/CGH/CDC.
2.2 Technical personnel should possess a molecular biology background.
2.3 This SOP is only used for research and surveillance purposes.

3.0 Related Documents

Title	Document Control Number
Filter Paper Blood Spot collection and Storage	MBS.DR.C.004
QIAamp DNA mini and blood mini handbook	5th Edition, QIAGEN, May 2016
Decontamination of Work Area and Waste Material and Use of Disinfectant	DPDM.DR.C.003
Applied Biosystems 3130/3130xl Genetic Analyzers user guide	Life Technologies publication # 4477796

4.0 Responsibility

Position	Duties
All laboratory staff	Understand and follow the procedures in the SOP when working on neutral microsatellite analysis in the CDC malaria laboratories.

5.0 Definitions

Term	Definition
SOP	Standard Operating Procedure
DPDM	Division of Parasitic Diseases and Malaria
DNA	Deoxyribonucleic Acid
HiDi	Highly Deionized Formamide

6.0 Equipment/Materials (if applicable)

Item	Definition
Pipettes	Single and Multichannel
Centrifuge	Bench top centrifuge with 96-well microwell plate rotor
Thermocycler	Bench top with 96-well reaction chamber
4°C Refrigerator	
-20°C Freezer	
Applied Biosystems Genetic Analyzer	Sequencer (3130/3130xl) (ThermoFisher Scientific. Inc)
PCR Workstation with UV light	
Vortex	Bench top

7.0 Reagents

Material Name	Other Information	Storage Condition
Promega 2X PCR Buffer	Assay was optimized using this reagent	-20°C
Nuclease Free Water		Ambient
Primers: Forward and Reverse	10 µM working stock solution	-20°C
Hi-Di Formamide		-20°C
GeneScan ROX-350 size standard	Assay was optimized using this reagent	+4°C

8.0 Supplies, Other Materials

8.1 Sterile micropipette tips
8.2 96-well PCR plate, half-skirt.
8.3 Plate Septa, 96 well.
8.4 1.5 or 2.0 ml sterile RNAse and DNAse-free tubes
8.5 Tube racks
8.6 DNAse-free or sterile solution reservoirs or basins
8.7 Benchtop cooler or icebox
8.8 Biohazard bag with a holder or plastic container for tip and tube disposal
8.9 Powder-free latex, vinyl, or nitrile gloves
8.10 Lab wipes
8.11 Permanent markers
8.12 Clean laboratory coat and safety glasses
8.13 70% ethanol (Prepared daily) in a spray bottle, store at ambient temperature.
8.14 10% Bleach (Prepared daily) in a spray bottle, store at ambient temperature.

9.0 Safety Precautions

9.1 Wear personal protective equipment (PPE). At a minimum, don a lab coat, gloves, and safety glasses.
9.2 Follow CDC safety policies and the Biosafety in Microbiological and Biomedical Laboratories manual (BMBL).
9.3 Universal precautions for working with infectious agents must be followed when performing this assay.
9.4 PCR workstations and crosslinker are equipped with UV lights. Caution should be considered during decontamination.
9.5 Use aerosol-resistant pipette tips to minimize PCR contamination.
9.6 Decontaminate all surfaces and pipettes with 70% ethanol, before and after each use according to DPDM.DR.C.003, Decontamination of Work Area and Waste Material and Use of Disinfectant.
9.7 Follow safety data sheets (SDS) when handling all chemicals including those known to be carcinogenic and reproductively toxic (HiDi Formamide).

10.0 Sample Information/Processing

10.1 Enter sample ID, gene, and sequencing primer names into sample sheet of sequence analyzer before sequencing run. Simplified numbers, such as 1, 2, 3, etc., should be avoided: the identification labels should be specific.

11.0 Quality Control

Use Positive and Negative Controls

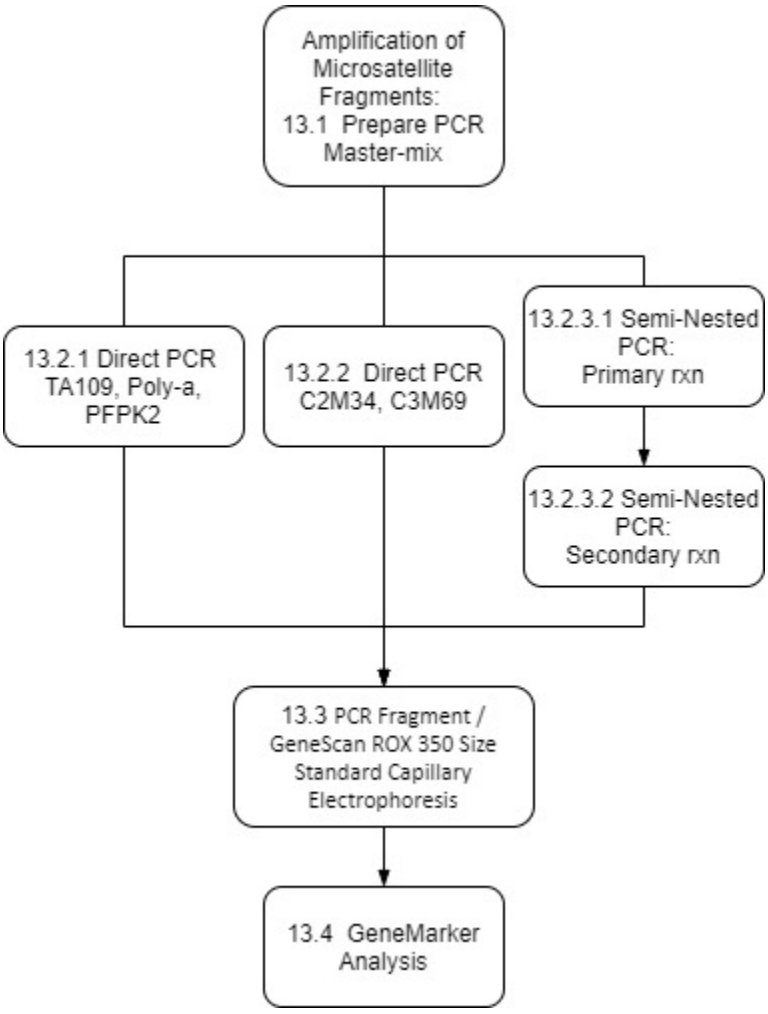
11.1 The positive controls are obtained from cultured *P. falciparum* strains, such as 3D7 or 7G8 stored at -20°C.
11.2 Negative control is non-infected human DNA, stored at -20°C.

11.3 Positive, negative, and blank control wells must be assayed simultaneously with test samples on the assay test plate.

11.4 If negative and blank controls show histogram peaks upon analysis, the control results are rendered invalid and the assay must be repeated.

12.0 Flowchart/Workflow

The workflow consists of three different PCR reactions, performed in any order – a Direct PCR using primers TA109, Poly-α, and PFPK2, a Direct PCR using primers C2M34 and C3M69, and a Semi-Nested PCR for primers TA1 and 2490 (semi-nested: primary PCR product is used in a secondary PCR with labeled primers). The products from the PCR reactions will be used in the Capillary Electrophoresis step (13.3). See Section 23.1 "Neutral Microsatellite Primer Information" for details on the correct primers to use.



13.0 Examination (Test) Procedure

13.1 Amplification of Microsatellite Fragments: Preparation of PCR Master-mix

13.1.1. To prevent contamination with DNA and amplicons, preparation of primers and master-mix buffer must be performed in different and/or physically separated work areas from DNA and PCR working areas.

13.1.2. Turn on the UV light in the PCR workstation (designated for DNA work) for 20 minutes (or as recommended by the manufacturer) before use.

13.1.3. PCR master-mix preparation can be performed on assigned clean lab space or clean workstation.

13.1.4. Label 200µl thin-walled PCR tubes or 96-well plate according to testing format.

13.1.5. Thaw PCR reagents (2X Promega buffer and primers) at room temperature, then vortex and microcentrifuge briefly and place in bench-top cooler.

13.1.6. Use a sterile 2 ml tube or solution basin to prepare enough PCR master-mix to accommodate the number of sample and control reactions, and extra reactions (1-4) needed to ensure sufficient volume.

13.1.7. Master-mix preparations are listed in Section 13.2 below. Prepare a **Direct PCR** or **Primary PCR**, using a master-mix for each locus primer set according to the volumes listed in Section 13.2.

13.1.8. Mix the reagents, centrifuge it briefly and place the master mix tube or basin on ice. Aliquot appropriate volume of master-mix to reaction tubes.

13.1.9. Relocate master-mix PCR tubes/plate to the DNA workstation and add DNA template into the corresponding tube or wells, ensuring that pipette tips are changed between samples.

13.1.10. Cap the tube or seal the PCR plate tightly.

13.1.11. Briefly centrifuge (5-10 seconds) to collect all reagents into the bottom of the tube/well. Relocate tubes/plate to a designated thermocycler room and place tubes/plate into the thermocycler machine.

13.1.12. Select a pre-programmed PCR protocol or set up a new protocol based on the cycling profiles in the table below in Section 13.2. Check the accuracy of the program cycling parameters before initiating the run.

13.1.13. Upon completion of the cycling program:

a) If performing Direct PCR, place the PCR product at 4°C overnight.

b) If performing Semi-Nested Primary PCR, proceed immediately to the secondary PCR step. **Secondary PCR** must be performed in a separate PCR workstation to prevent contamination with amplicons. The appropriate master-mix volume is listed in Section 13.2.3.2. Add the primary PCR product into the secondary PCR master-mix. Proceed with steps 13.1.8 through 13.1.12. Upon completion of the cycling program, place the secondary PCR product at 4°C overnight.

13.2 Preparation of Master-mix and PCR Reactions

13.2.1. Direct PCR Reaction: TA109, Poly-α, and PFPK2[22.4]

a) PCR reaction:

Item	**Direct PCR (1x)**
2 X Promega Master-mix	7.5 µl
Water	4.3 µl
Forward Primer (10uM)	0.6 µl
Reverse Primer(10uM)	0.6 µl
Total Master-mix	13 µl
DNA	**2.0 µl**
Total volume	15 µl

b) PCR Cycling Parameters

94°C, 2 min

94°C, 20 sec |
45°C, 20 sec |40 cycles
65°C, 30 sec |

65°C, 2 min
4°C, ∞

13.2.2 Direct PCR reaction: C2M34 and C3M69[22.5]

a) PCR reaction

Item	**Direct PCR (1x)**
2 X Promega Master-mix	7.5 µl
Water	4.3 µl
Forward Primer (10uM)	0.6 µl
Reverse Primer(10uM)	0.6 µl
Total Master-mix	13 µl
DNA	**2.0 µl**
Total volume	15 µl

b) PCR Cycling Parameters

94°C, 2 min

94°C, 30 sec |
50°C, 30 sec |5 cycles
60°C, 30 sec |

94°C, 30 sec |
45°C, 30 sec |40 cycles
60°C, 30 sec |

4°C, ∞

13.2.3. Semi-nested PCR for loci TA1 and 2490[22.4]

13.2.3.1 Primary PCR: TA1 (TA1-FOR + TA1-REV) and 2490 (2490-FOR + 2490-REV)

a) Primary PCR Reaction:

Item	**Primary PCR (1x)**
2 X Promega Master-mix	7.5 µl
Water	4.3 µl
Forward Primer (10uM)	0.6 µl
Reverse Primer(10uM)	0.6 µl
Total Master-mix	13µl
DNA	**2.0 µl**
Total volume	15 µl

b) Primary PCR Cycling Parameters

94°C, 2 min

94°C, 30 sec |
42°C, 30 sec |25 cycles
40°C, 30 sec |
65°C, 40 sec |

65°C, 2 min
4°C, ∞

13.2.3.2 Secondary PCR: TA1 (TA1-FOR HEX + TA1-REV) and 2490 (2490-FOR + 2490-REV FAM)

a) Secondary PCR reaction:

Item	**Secondary PCR (1x)**
2 X Promega Master-mix	7.5 µl
Water	5.3 µl
Forward Primer (10uM)	0.6 µl
Reverse Primer(10uM)	0.6 µl
Total Master-mix	14µl
Primary PCR product	**1.0 µl**
Total volume	15 µl

b) Secondary PCR Cycling Parameters

94°C, 2 min

94°C, 20 sec |
45°C, 20 sec |25 cycles
65°C, 30 sec |

65°C, 2 min
4°C, ∞

13.3 **PCR Fragment Capillary Electrophoresis on ABI 3130 xl**

13.3.1. Prepare a master-mix by combining 480 ul of HiDi formamide with 20 ul ROX-350 DNA ladder for a total of 500 ul. Additional HiDi-ROX master- mix can be prepared depending on the sample number.

13.3.2 Add 10.5 µl of HiDi-ROX master-mix into designated wells of a 96-well, half-skirt plate.

13.3.3 Add 1.5 µl of PCR fragment (from 13.1.13) to the appropriate well and mix with the HiDi-ROX master-mix. Briefly spin the plate with a centrifuge.

13.3.4. Relocate 96-well, half-skirt plate to ABI 3130 xl Genetic Analyzer and run

fragment analysis as per manufacturer's instructions.

13.4 **Analysis of Microsatellites Data using GeneMarker® Software**

13.4.1. Open GeneMarker® software.

13.4.2. Panels should be utilized for all markers. To import panel: Go to Tools- Panel Editor- File- Import Panels. After importation, Go to:

13.4.3. "File" →"Open Data" → Click "Add" →Search for a folder, then add samples to project→ Click "Open", then click "Ok", then click "Run"

13.4.4. A window will open within "Template Selection", then select:

13.4.5. "Panel" → select the panel based on the size marker assayed with samples

13.4.6. "Size Standard" → GS350

13.4.7. "Standard Color" → Red

13.4.8. "Analysis Type" → Fragment

13.4.9. After selecting all options in the "Template Selection" box, click "Next" to proceed to the "Data Process- Fragment Analysis" box.

13.4.10. "Data Process- Fragment Analysis" → Maintain the manufacturer's standard settings. Only change: ""Min Intensity:200"; "Max Intensity: 60,000"; "Percentage for > 5" Global Max; and the "Local Region for % > 30".

13.4.11. Click "Next" to proceed to the "Additional Setting- Fragment Analysis" box→ Maintain the manufacturer's standard settings.

13.4.12. "Allelic Ladder": None

13.4.13. "Positive Control Template": None

13.4.14. "Allele Elevation": option(s)

13.4.15. "Peak Score": "Reject: < 1.00" and "Check:7.00" < "Pass"

13.4.16. After confirmation, Click "Ok".

13.4.17. Click "Ok", a second time to confirm the data process.

13.4.18. Click the "Marker" selection, located next to the toolbar, and select the same size marker assayed with samples. Select "Allele List" located next to the "Report" option.

13.4.19. The minimum relative fluorescent units to be considered as a positive peak is above 200 rfu.

13.4.20. The standard cutoff range for minor peaks is 30% of the primary peak for multiple alleles. If more than 1 peak appears, use the highest peak as the standard peak and select other peaks that have a minimum of 30% height using the standard peak as a reference.

13.4.21. For calling multiple alleles, we refer to the peak pattern in positive controls in the same plate in order to exclude the non-specific amplification.

13.4.22. Peak pattern references are shown in Section 23.2.

13.4.23. Right-click within the observed peak and confirm allele size, based on the original allele size and panel. Use references listed in Anderson's paper [3].

13.4.24. Peak sizes for the positive and negative controls should be validated first.

13.4.25. When the analysis is complete, go to File → Save project. The data can also be exported as an Excel file.

13.4.26. Samples with a green box to the left of the allele location (number), indicate successful amplification.

13.4.27. Samples with a red box to the left of the allele location (number) indicate an unsuccessful amplification and the microsatellite fragment amplification will need to be repeated.

13.4.28. Samples with a yellow box to the left of the allele location (number) may indicate that the sample (s) exhibit a low concentration of the ROX or a low PCR reaction quality. In such cases, the sample(s) allele location should be reviewed and confirmed.

13.4.29. HEX-labeled samples – peaks in green; FAM-labeled – peaks in blue.

14.0 Method Performance Specifications (if applicable)

14.1 This SOP is not to be used as a screening test for malaria diagnosis or as a test to confirm the presence of malaria infection.

14.2 Interpretation and application of the results should be done by qualified persons with expertise in microsatellites and malaria infections.

15.0 Calculations: N/A

16.0 Reference Values/Alert Values: N/A

17.0 Interpretation of Results

17.1 The PCR results involve the amplification of the microsatellite loci of interest followed by fragment electrophoresis on a capillary sequencer and fragment analysis using **GeneMarker®** software (SoftGenetics State College, USA). **Note:** If amplification is not successful on the first attempt, the amplification and analysis will be repeated a second time. If, after a second attempt, the sample fails to amplify, the sample will be categorized as NA (not amplified) at the locus being tested.

18.0 Results Review and Approval

18.1 Controls and analysis results are reviewed by a second individual before the results are reported.

18.2 The results will not be reported when controls are invalid.

18.3 The allele size for all samples can be exported as an Excel file. For an additional exporting option, allele size and allele height, both, can be exported into one file.

18.4 Once the analysis is complete and there is a consensus with regard to the data, the data will be reported. The project SGF data files can be reported in the standard Excel template (provided within GeneMarker®) and sent to review and approval by the supervisor and then stored in an access-controlled multi-user share drive. Raw data cells should be locked to prevent unintentional deletions or changes.

19.0 Reporting Results; Guidelines for Notification

19.1 A supervisor or principal investigator will report a summary of results to the corresponding program epidemiologists or project officers in the USA or other countries, depending on the project.

20.0 Sample Retention and Storage

20.1 Relocate unused PCR products and store them at -20°C until the experiment is completed.

20.2 Relocate unused isolated DNA samples and controls and store them at -20°C once the experiment is completed.

21.0 Records Management

21.1 Records are maintained in the Laboratory Multi-User SharePoint. Individual Controlled Laboratory notebooks contain daily information of assay details.

22.0 References

22.1 Anderson TJ, Su XZ, Bockarie M, Lagog M, Day KP: Twelve microsatellite markers for characterization of Plasmodium falciparum from finger-prick blood samples. *Parasitology* 1999, 119.

22.2 Anderson TJ, Su XZ, Roddam A, Day KP: Complex mutations in a high proportion of microsatellite loci from the protozoan parasite Plasmodium falciparum. *Mol Ecol* 2000, 9:1599-1608.

22.3 Anderson TJ, Haubold B, Williams JT, Estrada-Franco JG, Richardson L, Mollinedo R, Bockarie M, Mokili J, Mharakurwa S, French N, et al: Microsatellite markers reveal a spectrum of population structures in the malaria parasite Plasmodium falciparum. *Mol Biol Evol* 2000, 17:1467-1482.

22.4 Roper C, Pearce R, Bredenkamp B, Gumede J, Drakeley C, Mosha F, Chandramohan D, Sharp B. Antifolate antimalarial resistance in southeast Africa: a population-based analysis. *Lancet* 2003, 361(9364):1174-81.

22.5 Nair S, Williams JT, Brockman A, Paiphun L, Mayxay M, Newton PN, Guthmann JP, Smithuis FM, Hien TT, White NJ, Nosten F, Anderson TJ. A selective sweep driven by pyrimethamine treatment in southeast asian malaria parasites. *Mol Biol Evol* 2003, 20(9):1526-36.

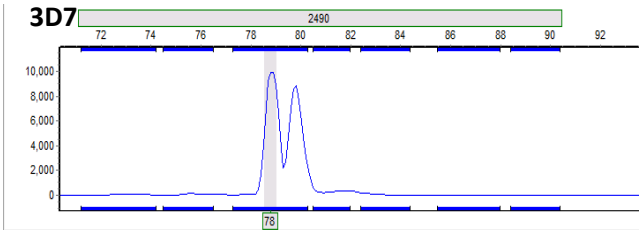
23.0 Attachments/Appendix

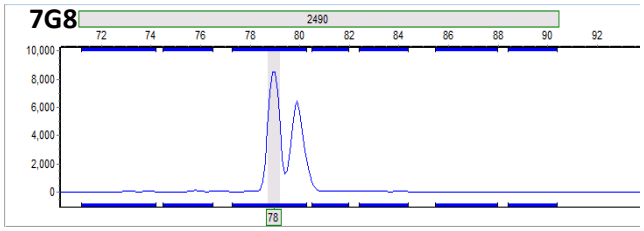
23.1 Seven Neutral Microsatellite Loci Primer sets for *Plasmodium falciparum* genotyping

Primer	Sequence	Tag	Size of 3D7 Allele	Size of 7G8 Allele	Chromosome	Tandem Repeats
Neu1_TA1_FOR	CTA CAT GCC TAA TGA GCA	None				
Neu1_TA1_REV	TTT TAT CTT CAT CCC CAC	None				
Neu1_TA1_FOR_HEX	CC GTC ATA AGT GCA GAG C	5' HEX	183	171	6	3
NEU3_POLYA_FOR	AAA ATA TAG ACG AAC AGA	None				
NEU3_POLYA_REV_FAM	GA AAT TAT AAC TCT ACC A	5' FAM	150	183	4	3
NEU7_PFPKA_FOR	CTT TCA TCG ATA CTA CGA	None				
NEU7_PFPK2_REV_HEX	AAA GAA GGA ACA AGC AGA	5' HEX	168	171	12	3
NEU9_TA109_FOR_FAM	GG TTA AAT CAG GAC AAC AT	5' FAM				
NEU9_TA109_REV	CCT ATA CCA AAC ATG CTA AA	None	172	163	6	3
NEU12_2490_FOR	TTC TAA ATA GAT CCA AAG	None				
NEU12_2490_REV	ATG ATG TGC AGA TGA CGA	None				
NEU12_2490_REV_FAM	AGA ATT ATT GAA TGC AC	5' FAM	78	78	10	3
313_C2_FOR_FAM	TC CCT TTT AAA ATA GAA GAA A	5' FAM				
313_C2_REV	GAT TAT ATG AAA GGA TAC ATG	None	262	232	2	2
383_C3_FOR_HEX	AA TAG GAA CAA ATC ATA TTG	5' HEX				
383_C3_REV	AGA TAT CCA GGT AAT AAA AAG	None	173	151	3	2

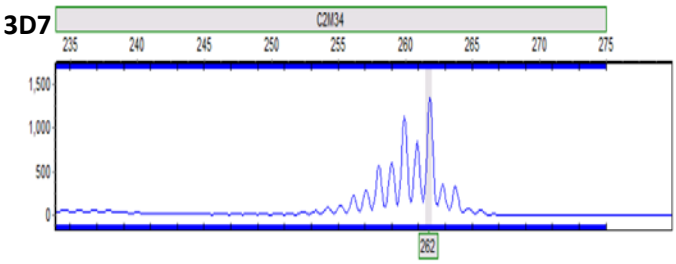
23.2 Complementary table: peak pattern references in control strains for microsatellite loci

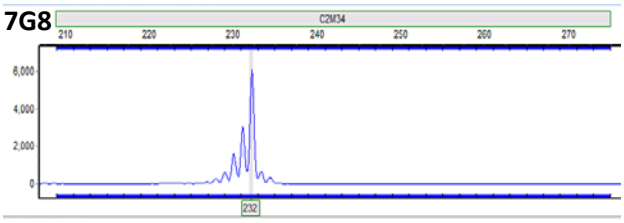
2490



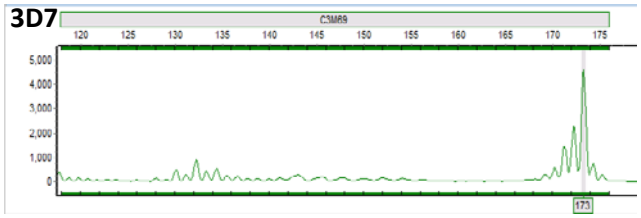


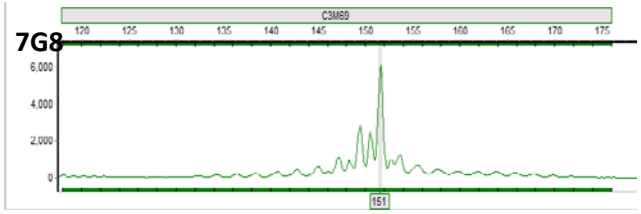
C2





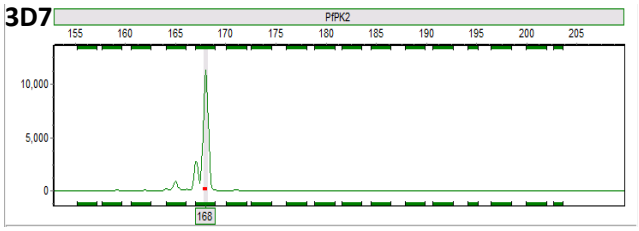
C3



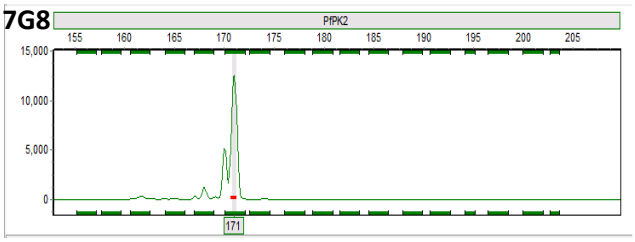


PFPK2

3D7

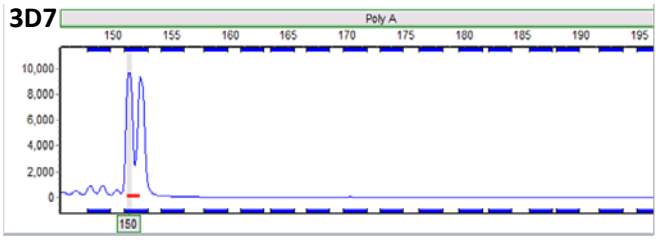


7G8

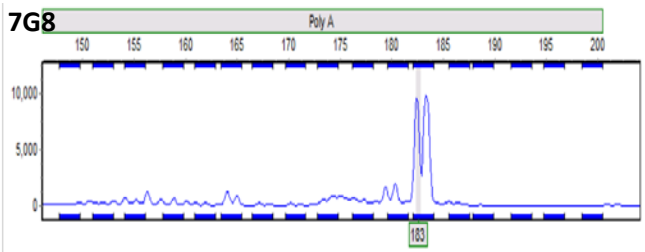


PolyA

3D7

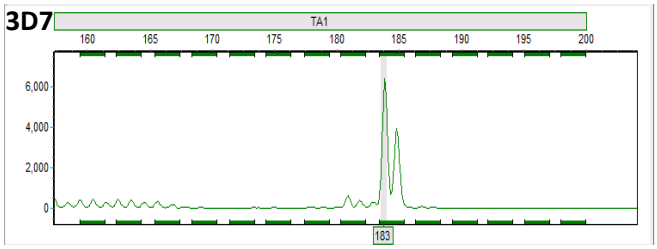


7G8



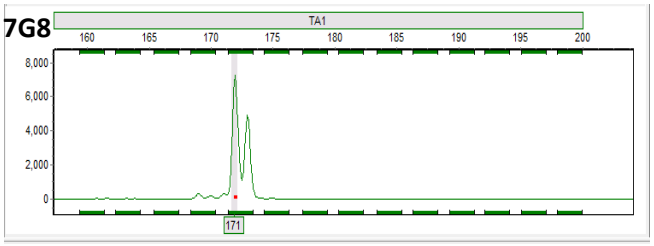
TA1:

3D7

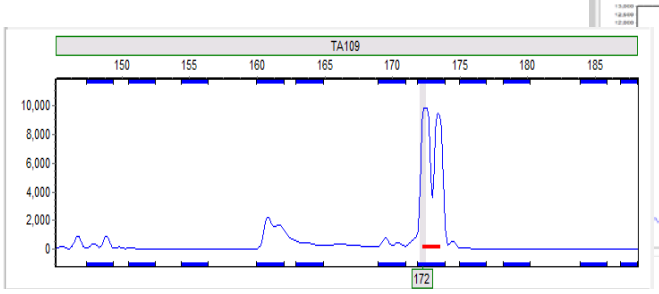


TA1:

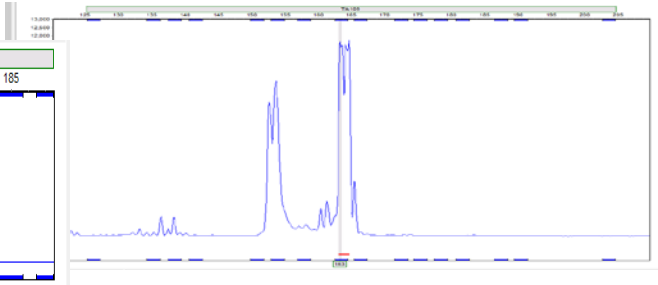
7G8



TA109: 3D7



TA109: 7G8



23.3 Example for plate design: Preparation of Master-mix, for Un-Nested PCR: TA109, PolyA & PFPK2 (Full Plate)

Plate 1												
	1	2	3	4	5	6	7	8	9	10	11	12
A	3001744727	3001744754	3001744763	3001744791	3001744799	3001744664	3001744672	3001744680	3001744805	3001744864	3001744730	3D7
B	3001744728	3001744755	3001744764	3001744792	3001744800	3001744665	3001744673	3001744681	3001744806	3001744811	3001744731	3D7
C	3001744734	3001744756	3001744781	3001744793	3001744801	3001744666	3001744674	3001744682	3001744808	3001744809	3001744732	7G8
D	3001744735	3001744758	3001744782	3001744794	3001744802	3001744667	3001744675	3001744683	3001744807	3001744810	3001744733	7G8
E	3001744750	3001744759	3001744785	3001744795	3001744803	3001744668	3001744676	3001744684	3001744815	3001744817	3001744736	Neg
F	3001744751	3001744760	3001744786	3001744796	3001744804	3001744669	3001744677	3001744685	3001744816	3001744818	3001744737	Neg
G	3001744752	3001744761	3001744789	3001744797	3001744662	3001744670	3001744678	3001744686	3001744813	3001744819	3001744738	Neg
H	3001744753	3001744762	3001744790	3001744798	3001744663	3001744671	3001744679	3001744687	3001744814	3001744729	3001744739	Neg

23.4 Example for plate calculation:

	Un-Nested PCR (1x)	**Un-Nested PCR (110x)**
2 X Promega Master-mix	7.5 µl	825 µl
Water	4.3 µl	473 µl
Forward Primer (10uM)	0.6 µl	66 µl
Reverse Primer(10uM)	0.6 µl	66 µl
Total Master Mix	13 µl	1430 µl
DNA	**2.0 µl**	**2.0 µl**
Total volume	15 µl	

Notes:

23.4.1 Design plate using an Excel spreadsheet, Word table format, or any other resource of preference.

23.4.2 Utilize last column of the table for assay controls. Use at least 1 positive and 1 negative control.

23.4.3 Prepare an extra volume to avoid pipetting error. Example above: Full plate (96 wells), prepare a volume for 110 samples.

24.0 Revision History

Ver #	DCR #	Changes Made to Document	Date
01	MBK.2017.020	New Document	03/31/2017

25.0 Approval Signatures

Approved By: Ira Goldman 3/26/2021
Author Date

Print Name and Title

Approved By: Samaly S. Svigel 3/26/2021
Author Date

Print Name and Title

Approved By: Zhiyong (Jane) Zhou 3/26/2021
Reviewer Date

Print Name and Title

Approved By: Venkatachalam (Kumar) Udhayakumar 03/27/2021
Approver Date

Print Name and Title