Supplementary Material

University of Washington Medical Center
Clinical Microbiology Laboratory & CFAR Virology Laboratory Document 1000-100-03

Molecular Diagnosis Manual **Procedure for Extraction and Amplification of *Plasmodium falciparum* and pan-*Plasmodium* 18S rRNA on the Abbott m2000®**		Effective: 01 June 2016
Written by: Sean Murphy, Annette Seilie, Amelia Hanron, Ming Chang, Kurtis Cruz	Reviewed by: Sean Murphy	Approved by: Sean Murphy, M.D., Ph.D.
Revised by: Sean Murphy		

ANNUAL REVIEW

Reviewed by:	Date	Reviewed by:	Date
N/A (first issuance of v3.0 SOP)			

REVISION HISTORY

Version	Date of change	Summary of changes
2.0	22 Feb 2017	Updated to reflect high WBC count interference
3.0	21 July 2017 [redacted]	Added additional rules to Quality Control section regarding discrepancy between Pan *Plasmodium* and *P. falciparum* targets for controls. Details added to Interpretation & Reporting section regarding reporting of *P. falciparum* presence in CHMI and non-CHMI trials. Various grammatical and wording corrections throughout the document for improved clarity and understanding. Updates to following sections with additional detail and corrections to accurately reflect on laboratory standards that have been practiced but not addressed in the present SOP: 1) Quality control measure in RT-PCR reagents section for each reagent 2) Addendum 2 preparation of positive and negative controls to correct numeric values and reflect current practices 3) Reference to *Babesia* assay in the Limitations section as a means to differentiate *Babesia* from *Plasmodium* infections 4) Requirement for a second negative control for all runs with a sample count greater than 48 in the ABBOTT real-time malaria assay procedure. 5) [redacted]. 6) Addition of m2000sp application name for extractions of greater than 48 samples ABBOTT real-time malaria assay procedure updated to include. Listed adequate sample types in Section SPECIMEN. Added the laser cutter and the fume extraction systems in REAGENTS, SUPPLIES AND EQUIPMENT. [redacted] Added PROCEDURE. 5. a. PREPARING SAMPLES AND CONTROLS BEFORE LOADING THEM TO M2000SP, describing preparation steps for the liquid and DBS samples before loading to the m2000sp instrument. Replaced the m2000sp extraction protocol with "1.0ml HIV-1 RNA DBS IUO US TT v11" in Section PROCEDURE.5.b.xii. Added a table in Section PROCEDURE.5.b.vii. listing adequate liquid [redacted] controls for varied tested samples and numbers. Added steps for master mix loading in Sections PROCEDURE. 5.c.xii, xiii and xx, and for setting up RT-PCR plate in Section PROCEDURE. 5. d.iii. [redacted]

		Added and clarified reasons for re-calibration (new mSample prep kit) in DETERMINATION OF PARASITES PER ML WHOLE BLOOD USING STORED STANDARD CURVE.

CLINICAL SIGNIFICANCE

Erythrocyte-stage *Plasmodium* parasites grow in human erythrocytes and cause malarial illness in a density-dependent manner. *Plasmodium* 18S rRNA is a nucleic acid biomarker that can indicate the presence of *Plasmodium* infection. Numerous *Plasmodium* species can infect humans, with *P. falciparum* being the most commonly used species in controlled human malaria infection (CHMI) studies. This quantitative molecular detection assay targets *Plasmodium* 18S rRNA to provide infection detection and estimated parasite density for *Plasmodium falciparum*. In addition, the assay can detect non-*P. falciparum* species including but not limited to *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*.

PRINCIPLE

This single-step real time quantitative reverse transcription polymerase chain reaction (real time qRT-PCR) assay uses specific primer sets and TaqMan fluorescence resonance energy transfer (FRET) probes to target a *P. falciparum*-specific region of the asexual-stage 18S rRNAs as well as a pan-*Plasmodium* genus-specific region of the asexual 18S rRNAs. A multiplexed control primer/probe set also targets the human endogenous TATA-Box binding protein (TBP) mRNA as a low copy number endogenous internal control.

OVERVIEW OF ASSAY VALIDATION

Clinical specimens, contrived parasite-containing specimens (cultured parasites added to whole blood) and contrived synthetic 18S rRNA-containing specimens (whole blood spiked with Armored RNA encoding the full-length P. falciparum 18S rRNA) were used to evaluated performance characteristics including accuracy, correlation, agreement, analytical sensitivity, precision, recovery and carryover. Validation data are contained in a separate report available in the lab. The performance was acceptable and in agreement with those of the preceding 'second generation' assay method.

SPECIMEN

1) Whole blood lysed in Nuclisens Lysis Buffer (50 µL whole blood into 2.0 mL lysis buffer), which preparation is listed in the most current version of SOP# 1000-099-XX.
   a) For fresh whole blood submitted to the lab, prepare the liquid samples per Section Procedure 1. SAMPLE PREPARATION/STORAGE.
   b) If the liquid samples were prepared by the outside facility and stored frozen at ≤-70°C, samples are sent to the Malaria PCR lab on dry ice preferably within 3 months of collection.
   c) Anticoagulants other than EDTA are not acceptable at this time.

2) [redacted]

REAGENTS, SUPPLIES AND EQUIPMENT

Extraction and common consumable materials

- Abbott *m*2000*sp* instrument (Abbott List No. 9K14)
- Abbott *m*2000*rt* instrument (Abbott List No. 9K14)
- Abbott *m*Sample Preparation System (4 x 24 Preps) (Abbott List No. 4J70-24)
- 5 mL Reaction Vessels (Abbott List No. 4J71-20)

- BioMerieux bulk NucliSense Lysis buffer (4 x 1L available)
- 13 mm to 16 mm Sample Tubes filled with 2 mL of bulk lysis buffer
  - i.e., Bioexpress 4 mL tube without cap #T2868-1 and screw caps without rings #T-2868-3
- Alternative tube: Pre-filled BioMerieux 2 mL NucliSense Lysis buffer tubes
- 200 mL Reagent Vessels (Abbott List No. 4J71-60)
- Abbott Master Mix tubes (Abbott List No. 4J71-080)
- 200 µL Disposable Tips (Abbott List No. 4J71-15)
- 1000 µL Disposable Tips (Abbott List No. 4J71-10)
- Abbott Optical Adhesive Covers (Abbott List No. 4J71-75)
- Abbott Adhesive Cover Applicators (Abbott List No. 9K32-01)
- Abbott Splash-Free Support Base (Abbott List No. 9K31-01)
- Abbott 96-Deep-Well Plate (Abbott List No. 4J71-30)
- Abbott 96-Well Optical Reaction Plate (Abbott List No. 4J71-70)
- Calibrated Pipettes: 1-10µL, 10-100µL, 20-200µL, 100-1000µL, 100-2000µL, ranges
- Aerosol resistant pipette tips: 10µL, 100µL, 200µL, 1000µL, 2000µL
- Master Mix Tube (Abbott List No. 4J71-80) Tube racks
- Vortex Mixer
- Microcentrifuge
- Beckman Coulter Allegra 6R Centrifuge (or equivalent)
- Plate shaker, Orbis (or equivalent)
- DNAse-free, RNAse-free Water for Molecular Biology (Sigma-Aldrich # W4502-1L)
- Buffer EB (QIAGEN, #19086)
- 1.5 mL sterile Eppendorf-style tubes, DNase- and RNase-free
- Nuclisens Lysis Buffer, (BioMerieux, #280134)
- Precision water bath model 183 (or equivalent)
- [redacted]
- Abbott m2000
  - The m2000 consists of 'sp' and 'rt' modules for extraction and amplification, respectively.
  - This assay uses an 'offboard' extraction protocol on the 'sp' module to extract 25 µL of whole blood diluted in 1 mL of lysis buffer. The 'sp' module then will combine a mastermix with eluted nucleic acids to prepare an RT-PCR plate for the 'rt' module. The 'rt' module has 5 detection filters (470/530 nm = FAM/SYBR, 520/558 nm = JOE/VIC; 550/586 nm = NED/TAMARA; 581/623 nm = ROX; 648/682 nm = Cy5). A passive ROX reference dye is required for all m2000rt applications.

RT-PCR Reagents

Plasmodium falciparum Primers and Probe

P. *falciparum* specific Probe

- 5'-[6-FAM]-ATTTATTCAGTAATCAAATTAGGAT-3'[Black Hole Quencher 1]
- Synthesized by BioSearch Technologies, Inc. (Dual HPLC purified)
- Reconstitute in Buffer EB to concentrated stock of 100 µM
- Further dilute in DNAse-/RNAse-free water to prepare 10 µM working stocks and store at ≤-70°C.
- **QCing a new lot of probe**: perform parallel test– test two master mixes containing the current and the new lot, respectively. The tested samples must be either Controls or Calibrators. Compare results to current Levey-Jennings chart to ensure that the tested Controls are in range.

P. *falciparum* specific Primers:

- PfDDT1451F21: 5'- GCGAGTACACTATATTCTTAT -3'
- PfDDT1562R21: 5'- ATTATTAGTAGAACAGGGAAA -3'

- Synthesized by BioSearch Technologies, Inc. (HPLC purified).
- Reconstitute each primer in Buffer EB to concentrated stock of 100 µM
- Combine the two primers and further dilute in DNAse-/RNAse-free water to prepare 20 µM working stocks and store at ≤-70°C.
- **QCing a new lot of primers**: perform parallel test– test two master mixes containing the current and the new lot, respectively. The tested samples must be either Controls or Calibrators. Compare results to current Levey-Jennings chart to ensure that the tested Controls are in range.

Pan *Plasmodium* Primers and Probe

Pan-*Plasmodium* Probe

- 5'-[CAL Fluor Orange 560]- ACCGTCGTAATCTTAACCATAAACTA[T(Black Hole Quencher-1)]GCCGACTAG-3'[Spacer C3]
- Synthesized by BioSearch Technologies, Inc. (Dual HPLC purified).
- BHQ chemistry where dC and dU are “propyne” moieties
- Reconstitute in Buffer EB to concentrated stock of 100 µM
- Further dilute in DNAse-/RNAse-free water to prepare 10 µM working stocks and store at ≤-70°C.
- **QCing a new lot of probe**: perform parallel test– test two master mixes containing the current and the new lot, respectively. The tested samples must be either Controls or Calibrators. Compare results to current Levey-Jennings chart to ensure that the tested Controls are in range.

Pan-*Plasmodium* Primers:

- PanDDT1043F19: 5’- AAAGTTAAGGGAGTGAAGA -3’
- PanDDT1197R22: 5’- AAGACTTTGATTTCTCATAAGG -3’
- Synthesized by BioSearch Technologies, Inc. (HPLC purified).
- Reconstitute each primer in Buffer EB to concentrated stock of 100 µM
- Combine the two primers and further dilute in DNAse-/RNAse-free water to prepare 20 µM working stocks and store at ≤-70°C.
- **QCing a new lot of primers**: perform parallel test– test two master mixes containing the current and the new lot, respectively. The tested samples must be either Controls or Calibrators. Compare results to current Levey-Jennings chart to ensure that the tested Controls are in range.

Human TATA-Binding Protein (TBP) Primers and Probe

TBP Probe

- 5'-[Quasar 670]- CACAGGAGCCAAGAGTGAAGAACAGT-3'[Black Hole Quencher-2]
- Synthesized by BioSearch Technologies, Inc. (Dual HPLC purified).
- BHQ chemistry where dC and dU are “propyne” moieties
- Reconstitute in Buffer EB to concentrated stock of 100 µM
- Further dilute in DNAse-/RNAse-free water to prepare 10 µM working stocks and store at ≤-70°C.
- **QCing a new lot of probe**: perform parallel test– test two master mixes containing the current and the new lot, respectively. The tested samples must be either Controls or Calibrators. Compare results to current Levey-Jennings chart to ensure that the tested Controls are in range.

TBP Primers:

- TBP Forward Primer: 5’- GATAAGAGAGCCACGAACCAC -3’
- TBP Reverse Primer: 5’- CAAGAACTTAGCTGGAAAACCC -3’
- Synthesized by BioSearch Technologies, Inc. (HPLC purified).
- Reconstitute each primer in Buffer EB to concentrated stock of 100 µM

- Combine the two primers and further dilute in DNAse-/RNAse-free water to prepare 10 µM working stocks and store at ≤-70°C.
- **QCing a new lot of primers**: perform parallel test– test two master mixes containing the current and the new lot, respectively. The tested samples must be either Controls or Calibrators. Compare results to current Levey-Jennings chart to ensure that the tested Controls are in range.

Bioline SensiFAST™ Probe Lo-ROX One-Step Kit ("SensiFAST") (Bioline catalog #BIO-78005)

- Store at -20°C or colder
- 2x PCR mix
- Ribosafe RNase inhibitor
- Reverse Transcriptase
- Nuclease-free water
- **QC a new lot of SensiFAST kit**: Perform parallel test by testing two master mixes containing current and new enzyme lot numbers with low positive samples of known parasitemia and Calibrators (described in Addendum 1). Estimated parasite concentration of the low positives samples must fall within 0.5 logs of expected values. Calibrators must fall within specified range.

Calibrators and controls

Calibrator

Custom-made, pre-quantified Armored RNA encoding full-length *P. falciparum* 18s rRNA from Asuragen (CoA 512-681-5200)

- Refer to the most current version of Protocol #1000-112-XX for details and handling of the calibrator material.

Liquid Controls

Malaria-positive and malaria-negative lysed whole blood standards

- See ADDENDUM 2: PREPARATION OF MALARIA POSITIVE AND NEGATIVE CONTROLS.
- Controls include NEGATIVE control blood and HIGH and LOW malaria positive control blood.
- All controls contain an equivalent of 50 µL of whole blood in 2 mL lysis buffer.
- Defrost these tubes as described for patient samples.

PROCEDURE

WEAR GLOVES THROUGHOUT THIS PROCEDURE AND CHANGE GLOVES FREQUENTLY OR IF THERE IS POTENTIAL FOR RNASE CONTAMINATION!

1) SAMPLE PREPARATION/STORAGE

a) Blood collection

i) Collect 2–3 mL of whole human blood from clinical trial volunteers by venipuncture into an appropriately labeled 2 mL or 3 mL EDTA Vacutainer tube. The label must contain the correct identifying information approved by the study protocol.

- The preferred blood collection tube is a 2 mL–3 mL K_2–EDTA Vacutainer tube.
- Larger volume tubes are also acceptable but are unnecessary.

ii) Invert the tube gently several times to ensure adequate anticoagulation.

iii) Clotted samples (i.e., due to inadequate mixing) must be rejected.

iv) ***Store the sample at room temperature or 4℃ and transport to the outside Laboratory as soon as possible, ideally within 4-6 hours but no later than 48 hours after collection***.

v) Whole blood must be kept at room temperature or 4°C until aliquoted into lysis buffer tubes and/or cryovials, as described below. Do not store whole blood at ≤0°C prior to lysis in NucliSENS lysis buffer.

NOTE: *If the primary EDTA blood collection tube is to be used for other tests in addition to malaria nucleic acid detection, an aliquot may be removed from the primary EDTA tube ONLY if a single–use sterile needle/syringe barrel assembly is used. The blood collection tube must only be accessed using the needle through the rubber stopper. This modification allows for sampling for blood smears from a single EDTA blood collection tube. Sterile handling and utmost care must be maintained to prevent cross contamination between samples or introduction of contaminating RNases into the primary tube.*

vi) Lysis of whole blood for diagnostic testing

- Whole blood samples must be processed as described below within 48 hours of collection. Record start of processing time.
- Label either a sample tube pre-filled with 2 mL lysis buffer (see Materials above for acceptable tube types) with the correct sample identification, as indicated by the study protocol. LDMS-generated labels are acceptable.
- Uncap the correctly–labeled lysis buffer-containing tube.
- Using a sterile micropipette, transfer 50 µL of EDTA–anticoagulated whole blood directly into the lysis buffer tube. Pipette up and down one time. Ensure that the pipettor does not get contaminated with blood between clinical specimens.
  - o Use a fresh pipette tip with an aerosol–resistant tip for each pipetting action.
  - o Only one tube should be open at any given moment during pipetting steps. All other specimen tubes and vials should be kept closed and physically separated from the one being handled.
- Close the tube immediately after addition of the specimen.
- Vortex the lysis buffer tubes containing specimen.
- Repeat process for all clinical specimens.
- Incubate the lysed samples for 10 minutes at room temperature to release any nucleic acids present in the specimen.
  - o **NOTE**: Lysis buffer uses guanidine thiocyanate to disrupt all cells in the specimen, releasing all nucleic acids that may be present. RNases and DNases in the specimen will be inactivated.
- Transfer and store lysed samples to a ≤-70°C non-cycling freezer. Samples should be shipped in batches to the testing laboratory on dry ice. **Samples in lysis buffer can be stored at ≤-70°C for up to 2 years. Archival samples preserved for longer than this period can also be tested with permission of the Laboratory Director.**

vii) [redacted]

2) SAMPLE RECEIPT AND ELECTRONIC MANIFEST RECEIPT

a) Open the package carefully and note any obvious problems with the shipment. Potential problems observed at this point include missing manifest, incorrect samples or thawed samples. If such problems are identified, enter them in the Non-conforming Event Log and consult the specimen coordinator to resolve the problem.

b) **For EDTA-anticoagulated whole blood samples with requisitions,** record samples on paper requisition and log primary samples onto LDMS as according to the current Malaria Sample Receipt Guideline found in the SOP binder and the Lilith2\Malaria Core folder.
c) **For samples that were received with no requisitions,** initial and time-stamp the paper manifest.
   i) Inventory the samples against the paper manifest.
   ii) Document on the manifest any mismatches or other problem with the samples.
   iii) Respond to the email to acknowledge receipt and condition of shipment.
   iv) Check paper manifest for errors.
   v) Document the shipment condition and any errors in the Malaria Shipment Receipt Log located on the Lilith2 drive.
   vi) Save the electronic manifest to the C:/ drive
   vii) Open @u.washington.edu to obtain the corresponding electronic manifest. In original e-mail, click on "Save" (located to the left of the attached file name).
   viii) Click on "Save" in the File Download Window
   ix) Save manifest on the C:/ drive in a folder named with the batch number

3) IMPORTING ELECTRONIC MANIFEST INTO LDMS

a) In LDMS, open the shipping modules (letter with specimen icon)
b) Shipment type will default to "LDMS Shipping Manifest"
c) Click the "Shipment file location" tab and select C:/ drive
d) Enter the shipment batch number in the "shipment number" window
e) Click the "import" tab
f) Once the manifest data is on the screen, click the "Continue/Cancel" tab
g) Answer the prompted questions:
   i) "No QA/QC has been performed for this shipment. Are you sure you want to complete this import?" → **YES**
   ii) "Would you like to mark these samples as never store?" → **NO**
   iii) "Would you like to adjust the condition of these samples?" → **NO**
   iv) "Import the associated test (assay) setup information?" → **NO**
h) Select the shipment temperature from the drop down menu (typically dry ice).
i) After the batch has imported, record the batch number on the paper manifest.

4) STORING THE IMPORTED ALIQUOTS WITHIN LDMS

a) The aliquot should be stored in Malaria storage box
b) In the LDMS Storage module, click the "Bulk Add" tab
c) Select the appropriate box on the left side of the screen and scan the aliquot barcode to store.
d) Print storage box map and archive appropriately.

5) ABBOTT REALTIME MALARIA ASSAY PROCEDURE

For a detailed description on how to operate the Abbott m2000sp instrument or the Abbott m2000rt instrument, refer to the Abbott m2000sp and m2000rt Operations Manuals, Operating Instructions sections.

a) PREPARING SAMPLES AND CONTROLS BEFORE LOADING THEM TO M2000SP

i) For freshly prepared whole blood lysate samples (e.g., clinical trial samples), proceed to Section Procedure 5.b.iv.
ii) For frozen liquid samples and controls, thaw specimens and controls in a biological safety hood for 20 min. Once thawed, specimens should be processed as quickly as possible. If sample extraction of thawed samples is delayed beyond 6 hours, this should be noted in the computer for the affected sample run(s).
iii) [redacted]

b) M2000sp INSTRUMENT SETUP

i) Turn on the Abbott m2000sp machine and computer.

ii) When the machine is ready, start "Daily Maintenance."

iii) Perform "Extensive Flushes" three times.

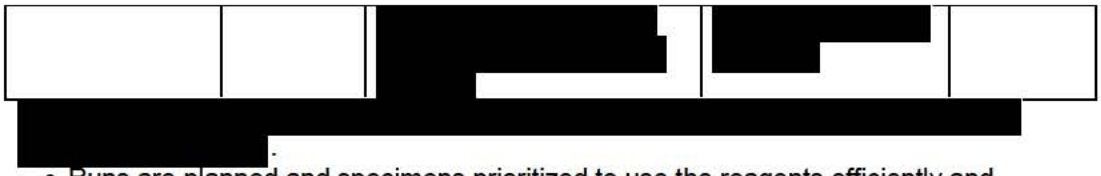
iv) Vortex each assay specimen and each control for 2-3 seconds. Spin at 2000g (3000rpm) for 5 min at 25°C to remove the bubble using Beckman Coulter Allegra 6R Centrifuge with swinging buckets and then remove bubbles with a sterile pipette tip, using a new tip for each vial if necessary.

v) If samples were received in large-size BioMerieux-brand tubes, generate labels for 13- to 16-mm standard tubes (that fit on the m2000 instrument) and transfer ≥1.5 mL of thawed sample to the fresh, labeled tube.

vi) Invert the Abbott *m*Sample Preparation bottles gently to ensure a homogeneous solution without generating any bubbles. If crystals are observed in any of the reagent bottles upon opening, allow the reagent to equilibrate at room temperature until the crystals disappear. Do not use the reagents until the crystals have dissolved. Ensure bubbles or foam are not generated; if present, remove with a sterile pipette tip, using a new tip for each bottle.

vii) The extraction protocol is capable of processing up to 96 tubes. Due to each mSample Prep kit packed for 24 tubes, preferably prepare tubes in increments of 24 tubes. Follow the table below to prepare controls depending on the number and the type of samples processed.

Tested sample type	Total tubes	Liquid sample control	[redacted]	Number of tested samples
Whole blood lysate samples only	Up to 24	One negative control, One low positive control One high positive control		Up to 21
	25 to 48			Up to 45
	49-72	One negative control, One low positive control One high positive control Plus one more negative control placed at the last position.		Up to 68
	73- 96			Up to 92
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]



- Runs are planned and specimens prioritized to use the reagents efficiently and prevent wastage.

viii) Place the liquid high positive, low positive and negative controls, [redacted] followed by the patient samples into the Abbott *m*2000*sp* sample rack.

ix) Check sample volume. The Abbott RealTime Malaria assay minimum sample volume and associated rack requirements on the Abbott *m*2000*sp* are described below.

CAUTION: Do not put a 13 mm tube in a 16 mm rack.
NOTE: Sample tubes identified by the instrument as containing insufficient sample volume (<1.3 mL) will not be processed during the run and will be identified by an insufficient liquid volume error message code in the Process Log and Plate Result screens.

x) Place the 5 mL Reaction Vessels into the *m*2000*sp* 1 mL subsystem carrier.

xi) Load the Abbott *m*Sample Preparation System reagents and the Abbott 96 Deep-Well Plate on the Abbott *m*2000*sp* worktable.

xii) Select the application file from the Run Sample Extraction screen - "1.0ml HIV-1 RNA DBS IUO US TT v11".This extraction protocol specifies sampling of 1 mL of the lysis buffer/blood mix with final elution in 88 µL.

xiii) Start the sample extraction protocol. The extraction takes approximately 2-3.5 hrs for the instrument to complete, depending on sample count.

c) MASTERMIX SETUP

NOTE: The Master Mix Addition protocol must be initiated within 30 minutes after completion of Sample Preparation.
NOTE: Change gloves before handling the Master Mix.

i) Get a bucket of wet ice from R&T room 728.

ii) Place and keep all PCR Mastermix components in wet ice during the Mastermix procedure.

iii) Pull one tube each of one *box of the Bioline SensiFAST LO-ROX One-step RT-PCR kit*:
- 2x SensiFAST Probe LO-ROX mix
- Ribosafe RNase inhibitor mix
- Reverse Transcriptase

iv) Nuclease-free water Clean the PCR set-up workstation. Always wipe down the hoods and workstations with DNA Erase before you start work and again before leaving the station.

v) Prepare reagent master-mix volume according to the number of samples and standards to be run. Arrange the reagent vials in sequence according to the Real-Time RTPCR worksheet. Record all lot numbers for reagents on the setup spreadsheet. The required volumes of each reagent has been calculated by the excel spreadsheet.

vi) Combine the required volumes of each reagent in a Mastermix tube.

vii) Checkmark the "Component added" column on your worksheet as you add each reagent to the Mastermix.

viii) Keep Mastermix tube on wet ice while preparing.

ix) Keep the enzyme and buffer on wet ice at all times.
x) Mix the mastermix solution thoroughly.
- **NOTE**: DO NOT VORTEX MASTERMIX TUBE(S). ADD ALL COMPONENTS, AND THEN INVERT TUBE 15 TIMES TO MIX.

TABLE 1: RT-PCR Setup for 35 µL master mix + 15 µL total nucleic acid template
(NOTE: Total volume shown is for 24 samples plus 4 additional samples as a safety margin.)

PCR Reaction Components	Lot #	Exp Date	Stock conc.		Vol/rxn (µL)	Final conc.		Volume (µL)
2x SensiFAST Probe LO-ROX Mix			2.0	X	25.0	1.0	X	700
Ribosafe RNase inhibitor mix					1.0			28
Reverse Transcriptase					0.5			14
20µM PfDDT primer Mix			20.0	µM	1.0	0.4	µM	28
10µM Pf probe (6FAM)			10.0	µM	0.5	0.1	µM	14
20µM PanDDT primer Mix			20.0	µM	0.5	0.2	µM	14
10µM Pan probe (CAL Fluor Orange 560)			10.0	µM	0.5	0.1	µM	14
10µM TBP Primer Mix			20.0	µM	0.5	0.1	µM	14
10µM TBP Probe (Quasar 670)			10.0	µM	0.5	0.1	µM	14
Nuclease-free water					5.0			140
TOTAL					35.00			

xi) Centrifuge the Master Mix tube for 2 min using Beckman Coulter Allegra 6R centrifuge to ensure the master mix solution is collected at the bottom of the tube
xii) On the m2000sp, print out extraction results of the run by going to deep well results. This will print a barcoded list of samples that were extracted.
xiii) Select a previously-run deep well plate file from the Run Master Mix Addition, which contains an equal number of samples in the present run.
Note: The program, "1.0ml HIV-1 RNA DBS IUO US TT v11", contains the RNA extraction and the master mix loading protocols installed in the Abbott m2000sp instrument and the PCR amplification protocol installed in the Abbott m2000rt instrument. Currently the samples ID file listed in the present extraction run cannot be collected by the generic Master Mix Addition program. Remedies for this issue are listed in Sections xii and xiii.
xiv) You will next be prompted to add reagents/consumables as necessary.
xv) Load the Mastermix tube onto the m2000sp worktable when prompted.
xvi) Select "Bioline" and enter the lot number and expiration date for the RT-PCR kit.
xvii) Select "15(45)/35" for Volume per test.
- This program instructs the instrument to add 35 µL of mastermix plus 15 µL of the template.

xviii) Ensure that there is sufficient mastermix in the tube and check that this corresponds to the volume shown on screen. Click to start Mastermix pipetting. Note that the subsequent *m*2000*rt* protocol must be started within 50 minutes of the initiation of the Master Mix Addition protocol.
xix) Seal the Abbott 96-Well Optical Reaction Plate after the Abbott *m*2000*sp* instrument has completed the addition of samples and master mix.
xx) The m2000sp will not be able to communicate to the m2000rt concerning errors that had occurred during extraction, but it will be able to communicate errors that occurred during master mix addition. Print any errors that had occurred during the extraction process.

d) m2000rt PROTOCOL

i) About 30 minutes before use, switch on and initialize the Abbott *m*2000*rt* instrument in the Amplification Area.

NOTE: The Abbott *m*2000*rt* requires 15 minutes to initialize.
NOTE: Remove gloves before returning to the sample preparation area.

ii) Place the sealed optical reaction plate into the Splash Free Support Base for transfer to the Abbott *m*2000*rt* instrument.

iii) Use the "Import and Setup" tab to import the *m*2000*sp* test orders via the Network Drive.
- Select the appropriate run (master mix addition transferred from the m2000sp).
- Select the mSample prep lot number and expiration date used for the RNA extraction.
- Select the current/updated calibrators and control lot numbers.
- On the plate map screen, type and confirm all samples ID following the printout list for the extracted samples.
- Utilize the deep well plate results barcode list to enter the relevant sample names into the PCR. Take extra care to be sure wells with samples are not left unmarked as the machine may not collect results for those properly. If any of the samples had failed extraction, note that error in the sample name or comment at this point.
- Designate the wells for each liquid control.

iv) Place the Optical Reaction Plate in the *m*2000*rt*

v) Select the protocol "Mal_BiolineMultiEXT LDA 0.1".
- This program includes the following RT-PCR cycling parameters:
  - Reverse transcription: 10 minutes at 48°C
  - Denaturation of RT: 2 minutes at 95°C
  - PCR amplification x 45 cycles
  - Denaturation: 95°C for 5 seconds
  - Annealing/Extension: 50°C for 35 seconds
    NOTE: Sample volume is 50 µL per well.

vi) Click START. The *m*2000*rt* program will run for approximately 1.5 hours.

vii) While the RT-PCR is running, seal and label the Deep Well plate(s) on the m2000sp (these plates contain ~40-50 µL of remaining total nucleic acids). Store the plates at 4°C temporarily during the run and if the run completes successfully, store the plate at -70°C or colder. Some or all of these materials may be returned to the clinical trial site or the original investigators. These plates are not barcoded, so they need to be adequately labeled with the date of the assay, the clinical trial details that they are associated with, and tech initials.

NOTE: The m2000rt protocol must be started within 50 minutes of the initiation of the Master Mix Addition protocol.
NOTE: If unable to transfer the *m*2000*sp* test order, enter sample IDs manually in the *m*2000*rt* in the correct PCR tray locations according to the "Wells for Selected Plate" grid, found on a detail screen under "PCR Plate Results" on the *m*2000*sp*.

e) POST-PROCESSING PROCEDURES:

i) When RT-PCR cycling is completed, remove the Optical plate. Place the Abbott 96-Well Optical Reaction Plate in a sealable plastic bag
- NEVER open or access the post-PCR plate in the CFAR laboratory! If further analysis of a particular batch is needed, place the PCR plate in a sealed plastic

bag and perform further studies in an off-site laboratory.

ii) Clean the Splash Free Support Base before next use.

iii) Click on 'Results' and select the run of interest.

iv) Click "Analyze" and ensure that the parameters are set as follows:
- *P. falciparum*:
  - Fixed threshold: 0.015
  - Initial sensitivity: 0.0001 / Final sensitivity: 2.50
- Pan-*Plasmodium*:
  - Fixed threshold: 0.01
  - Initial sensitivity: 0.0001 / Final sensitivity: 2.50
- TBP:
  - Fixed threshold: 0.05
  - Initial sensitivity: 0.0001 / Final sensitivity: 2.50

v) Examine the control reactions for each batch and ensure that the CN for *P. falciparum* and Pan-*Plasmodium* of the high and low positive controls fall within +/- 3SD of the mean CN (cycle number specific to the Abbott LDA software and equivalent to the conventional threshold cycle) based on Levey-Jennings plots. For the negative control, no malaria amplicon should be detectable. If new controls are made, the acceptable ranges will be re-set over time.

vi) Examine the high, low and negative controls and ensure that the TBP RNA all amplify within their individual, acceptable ranges as determined by Levey-Jennings plot monitoring.

vii) Enter the CN of all targets into the Levey-Jennings QC file for all controls.

viii) The program will use the stored standard curve to calculate the RNA copies per reaction (although this is displayed as "copies/mL in the Abbott m2000rt platform). *It is important to realize that this calculation is copies per mL of lysis buffer, which contained RNA/DNA from 25 µL of whole blood (using lab-filled sample tubes with 2 mL lysis buffer).*

ix) Export raw data file using a writable CD. Sort by sample location and copy and paste the contents of the file into the final report template. The template will automatically calculate the actual RNA copies/mL of processed lysis buffer (and corresponding log value) and convert this to RNA copies/mL of whole blood and subsequently to estimated parasites/mL whole blood using an assay-specific conversion factor.

x) If problems arose during processing at extraction, RT-PCR or post-run steps, enter the problems in the Non-conforming Event Log.

xi) Archive the m2000sp/rt data to a CD-ROM. Take the CD to a PC-based computer and transfer the data to the appropriate sub-folder within the "Malaria Core" directory on the Lilith2 drive.

xii) Ensure that printed and electronic records are archived appropriately. The information for archive includes 1) printed LDMS shipping/receipt manifest (if received via LDMS – otherwise hardcopy receipt of another style manifest), 2) printed m2000sp extraction map, 3) printed m2000rt RT-PCR results, 4) final Excel spreadsheet report.

xiii) If same/next day reporting is requested, send the final Excel spreadsheet to Drs. Murphy ([redacted]) and Chang ([redacted]) and send an accompanying text message to alert Dr. Murphy to the new data. Indicate to Drs. Murphy/Chang the following: 1) standards in or out of range, 2) internal controls in or out of range, 3) any atypical amplification curves, 4) any technical or sample-related issues. Reports can be signed out in-person in the main laboratory or remotely using an FDA 21CFR part 11 compliant electronic signature program.

f) Storage of remaining sample materials

i) Depending on the size of the EDTA Vacutainer tubes used for blood collection, some residual whole blood will remain in the original EDTA tube. This material is referred

to as “remaining whole blood”. In addition, one mL of the original 2 mL lysis buffer will remain in the processed sample tubes since the Abbott m2000sp aspirates 1 mL of the lysate. This material is referred to as “remaining lysed blood”. In addition, at least one archival sample of 50 µL blood in lysis buffer will also remain and is referred to as “archival lysis buffer tube”. Finally, there is also a deep well plate containing the eluates from the m2000sp extraction that is referred to as “remaining nucleic acid eluate”.

ii) Store the remaining whole blood, remaining lysed blood and the remaining nucleic acid eluate (deep well plate) for at least one work day in a ≤-70°C non-cycling freezer. In some clinical trials, additional whole blood aliquots may be made and stored as archival material.
iii) Store the archival lysis buffer tube and any additional archival materials in a ≤-70°C non-cycling freezer.
iv) Once results have been analyzed, confirmed and accepted with no errors, any unused remaining whole blood, remaining lysed blood and remaining nucleic acid eluate (deep well plate) may be disposed of appropriately.

QUALITY CONTROL

One high positive control, one low positive control and one malaria-negative control are extracted and tested by RT-PCR in parallel with every run. Up to 92 clinical specimens and 4 controls can be tested at once. Refer to the Levey-Jennings plots for expected CN for the controls.

Concern for RT-PCR inhibitors or extraction problems: If the TBP RNA target is detected with a later than expected CN in any of the control samples (as determined by Levey-Jennings monitoring), this should raise concern for poor sample extraction, degradation of the control lot number, and the possibility of inhibitors in the extraction reagents. Runs that result in problematic control CNs should be entirely re-tested at the RT-PCR step. If the repeat RT-PCR fails, a new aliquot of controls must be extracted/amplified to examine the quality of the controls. If the problem does not arise again, the entire run should be repeated from the beginning with fresh samples. However, the technician must consult a Research Scientist or the Laboratory Director if problems with the TBP mRNA of the controls persist.

Quantitative results of the *P. falciparum* and Pan-Plasmodium channels for the positive controls must be within 0.5 $\log_{10}$ copies/mL whole blood difference for each control level. Should the discrepancy between channels be greater than 0.5 $\log_{10}$ copies/mL whole blood for the positive controls, the technician must repeat the RT-PCR. If the problem arises again, the technician must consult a Research Scientist or the Laboratory Director prior to taking further actions.

If TBP detection is late or not detected for individual clinical samples within a run, and the TBP of the controls are within acceptable range, the sample’s documentation should be assessed for possible deviations from the accepted sample preparation and storage protocol. At a minimum, clinical samples with undetectable or TBP CNs >33 cycles should be re-tested at the RT-PCR step. If the repeat RT-PCR fails, new samples should be obtained and the extraction/amplification process repeated. Consult a Research Scientist or the Laboratory Director if delays in TBP CNs are observed in clinical samples.

Concern for amplicon contamination: If the negative control is positive for either *Plasmodium* target with a CN <40 cycles, this raises concern for amplicon contamination. Any samples that resulted as Malaria-positive would need to be re-tested using remaining extracted nucleic acids at the RT-PCR step. If the NEG sample is contaminated on a repeat RT-PCR run, the entire run should be repeated using fresh samples starting from extraction. Consult a Research Scientist or the Laboratory Director if contamination is ever suspected.

Unsatisfactory samples: If samples are received in an unsatisfactory manner (thawed while in transit, tubes defrosted, tube breakage, missing/altered labels, a significant delay from collection to lysis buffer addition, etc.), consult a Research Scientist or the Laboratory Director.

INTERPRETATION & REPORTING

For *P. falciparum* CHMI trials, the Excel reporting file will report the Pan-Plasmodium 18S rRNA RT-PCR CN (called C_T for 'crossing threshold' in the report) and the estimated number of parasites per mL of whole blood. A separate column will qualitatively indicate whether or not the *P. falciparum* channel was also positive only in cases where the Pan-Plasmodium channel was positive. [redacted] Note that the *P. falciparum*-specific conversion factor (7400 copies of P. falciparum 18S rRNA per ring-stage P. falciparum parasite) may not be applicable to non-*P. falciparum* infections [redacted]

Estimated parasite densities will be reported for samples containing ≥ 20 estimated parasites per mL of whole blood. Results are reported as "Not Detected" for samples that do not produce a CN for the malaria target (i.e., target not detected). For samples that produce late CNs resulting in parasite calculations between 10-20 parasites per mL of whole blood, these samples will be qualitatively reported as "Low positive <20 parasites/mL of whole blood, below the limit of quantification", whereas samples with CNs resulting in estimates of <10 parasites per mL of whole blood will be reported as "Not Detected". The low positive category allows for slight variation in RNA copy number per parasite for samples estimated to contain a single parasite; this range of acceptability allows for variation of +/- 0.3 $\log_{10}$ copies in a single parasite.

[redacted]

The negative control should not generate a CN in the FAM or CAL Fluor Orange 560 (VIC) channel, and the positive malaria controls must be within their respective CN ranges as specified in historical Levey-Jennings plots before accepting results. Runs with control results not corresponding to expected values must be further investigated and/or repeated; consult a Research Scientist before taking action.

Evaluate the amplification curves of all samples for P. *falciparum,* Pan *Plasmodium* and TBP RNA using m2000rt LDA program. Document and print out any atypical amplification curves. The fluorescent signal graphs should be evaluated for all samples, especially when the RNA copy number is near the limit of quantification or detection.

Record QC results and sample data in appropriate worksheets. Print the worksheet-generated Report in hard copy and/or PDF format for review by Research Scientist and Medical Director prior to delivery to client.

LIMITATIONS

Poor sample preservation (repeated thawing, significant delays in processing, etc.) can reduce RNA concentrations.

High leukocyte concentrations may modestly decrease the quantitative values reported by this assay. If high leukocytes are present (>25x10^9/L) generating TBP CN <26, consult a Research Scientist so that a sample can be diluted and retested.

Plasmodium gametocytes may lead to positive results in this assay since these forms of the parasite express both S- and A-type 18S rRNAs and also possess genomic DNA genes encoding all 18S rRNA isoforms. In the event that gametocytes were suspected, a thick smear could be evaluated and/or a gametocyte-specific RT-PCR could be performed. Sporozoites may also cross-react although it would be extremely unlikely to detect sporozoites in peripheral blood in all but immediate post-challenge periods.

Babesia species may cause false positive results due to weak cross-reactivity with the pan-*Plasmodium* qRT-PCR (~1000-fold weaker binding than to *Plasmodium*) but do not cross-react with the *P. falciparum* qRT-PCR. In the event that *Babesia* was suspected, a thick blood smear could be evaluated and/or sequencing of the pan-*Plasmodium* amplicon could be performed. Alternatively, a *Babesia* specific RT-PCR could be performed.

DETERMINATION OF PARASITES PER ML WHOLE BLOOD USING STORED STANDARD CURVE

A standard curve (see below) of Armored RNA encoding the *P. falciparum* 18S rRNA is required are the following occurrences:

- A new lot of Bioline mastermix is used.
- A new lot of Abbott mSample Preparation System is used.
- A new version of the m2000 application specification file is installed.
- An optical calibration of the Abbott m2000rt is performed.
- Six months has elapsed since the last calibration.

This curve is compared to previous curves generated in the initial method validation. If the repeated standard curve falls within the expected ranges established during method validation, the stored standard curve can be used to calculate parasitemia.

The following is an example of the calculation based on the mean standard curve from the validation (the actual regression calculation is performed in the m2000rt software using a stored standard curve:

***EXAMPLE: P. falciparum* 18S rRNA copies/mL blood Std. Curve (example only)**

= (10^((Sample CN – Y-intercept)/(slope)))(88 µL eluate/15 µL template)(1000 µL/25 µL)
= (10^((Sample CN – 43.82)/(-3.155)))(88µL/15µL)(1000µL/25µL)
= (10^((Sample CN – 43.82)/(-3.155)))(234.66)

Ring-stage *P. falciparum* parasites per mL blood (uses conversion factor from Abbott m2000 Method Validation for this specific assay 1000-100-01)

= (18S rRNA copies/mL blood) / (7400 copies per ring-stage parasite)

When the standard curve is re-run at periodic intervals, the slope and Y-intercept must be assessed and compared to previous runs. The repeated standard curve should have a +/- 2SD slope of -3.0 to -3.5 on the *P. falciparum* FAM channel and +/-2SD slope of -3.2 to -3.6 on the Pan *Plasmodium* CAL Fluor Orange 560 channel. If standard curve values are outside this range, consult a Research Scientist.

ADDENDUM 1: RE-CALIBRATION WITH AN ARMORED RNA STANDARD CURVE

See latest version of Protocol 1000-112-XX for all recalibration methods.

ADDENDUM 2: PREPARATION OF MALARIA POSITIVE AND NEGATIVE CONTROLS

1. Liquid sample controls

a. To prepare HIGH positive controls:

i. Obtain 5 mL of EDTA-anticoagulated whole human blood by venipuncture or as fresh excess from the main laboratory (drawn within the past 12 hours).
ii. To make approximately 38 tubes of controls, aliquot 47.5 mL of lysis buffer into a 50 mL conical tube. Or scale up the preparation in a 200-mL plastic ware (Milipore Stericup Filter unit).
iii. Add 2.5 mL of whole blood to this tube and vortex 15 seconds.
iv. Thaw one "1% parasitemia" tube (in 1 mL lysis buffer) on ice until thawed. These high concentration tubes are located in $\leq$-70^{o}C.
v. Add 0.5 mL of the 1% parasitemia tube to the conical tube and vortex for 15 seconds.
vi. Aliquot 1.3 mL of lysed parasite-containing whole blood that was just created into 4 mL simport tubes or suitable plastic tubes made of made of polypropylene with similar diameter that can withstand $\leq$-70^{o}C freezing. Ensure that tubes are labeled "HIGH MAL POS" with the date of preparation.
vii. The resulting parasitemia of the HIGH MAL POS control is 0.01% parasitemia (4x107 parasites/mL).
viii. Store tubes upright in a $\leq$-70^{o}C freezer until use.

NOTE: If additional 1% parasitemia tubes are unavailable, additional material will need to be cultured according to Protocol #1000-102-01.

b. To prepare LOW positive controls:

ix. Obtain 5 mL of EDTA-anticoagulated whole human blood by venipuncture or as fresh excess from the main laboratory (drawn within the past 12 hours).
x. To make approximately 38 tubes of controls, aliquot 47.5 mL of lysis buffer into a 50 mL conical tube. Or scale up the preparation in a 200-mL plastic ware (Millipore Stericup Filter unit).
xi. Add 2.5 mL of whole blood to this tube and vortex 15 seconds.
xii. Collect a tube of 0.001% parasitemia stock from the freezer (1mL)
xiii. Add 1 mL of the 0.001% parasitemia stock to the 50 mL conical tube to achieve a sample approximating 0.00002% parasitemia or 800 parasites/mL.
xiv. Aliquot 1.3 mL of the 0.00002% solution into 5mL simport tubes or suitable plastic tubes made of polypropylene with similar diameter that can withstand $\leq$-70°C freezing. Ensure that tubes are labeled "LOW MAL POS" with the date of preparation.
xv. The resulting parasitemia of the LOW MAL POS control is 0.00002% parasitemia (800 parasites/mL).
xvi. Store tubes upright in a $\leq$-70^{o}C freezer until use.
NOTE: If additional 1% or 0.01% parasitemia tubes are unavailable, additional material will need to be cultured according to Protocol #1000-102-01.

c. To prepare NEGATIVE controls:

i. Obtain 5 mL of EDTA-anticoagulated whole human blood by venipuncture or as fresh excess from the main laboratory (drawn within the past 12 hours).
ii. The blood donors are historically free of malaria.
iii. To make approximately 38 tubes of controls, aliquot 47.5 mL of lysis buffer into a 50mL conical tube. Or scale up the preparation in a 200-mL plastic ware (Millipore Stericup Filter unit).
iv. Add 2.5 mL negative EDTA whole blood into the conical tube for a final volume of 50 mL

lysed negative blood. Vortex well.

v. Aliquot 1.3 mL of lysed negative blood into 4mL simport tubes. Ensure that tubes are labeled "NEGATIVE" with the date of preparation, tech., initial and the temperature storage.

vi. Transfer all tubes upright in a ≤-70°C freezer until use.

NOTE: Ensure that a plug is used if the 1-5 mL pipette is used. The 1-5 mL disposable tips are not filter plugged and there is a risk of contamination of a plug is not used.

2. [redacted]

b. [redacted]

ADDENDUM 3: EXAMPLE SCREENSHOTS FROM STANDARD ASSAY WORKSHEETS

Example of LDMS import worksheet

Import Manifest

QA/QC Not performed

Batch Number: 9654

Setup Date: **Sorted by:** Protocol/ID2 **Shipped:** No **Import Date** 16/Sep/2011

Shipped From:

Lab ID: [redacted]
Lab Name: [redacted]
Country: [redacted]
Contact: [redacted]
Phone #: [redacted]
Fax #: [redacted]
E-mail: [redacted]

Shipped To:

Lab ID: [redacted]
Lab Name: RETROVIRUS LABORATORY - CAP# 2483716-08
RESEARCH & TRAINING BUILDING
300 NINTH AVE
SEATTLE WA 98104-2499
Country: UNITED STATES
Contact:
Phone #: (206) 897-5210
Fax #: (206) 897-5237
E-mail: [redacted]

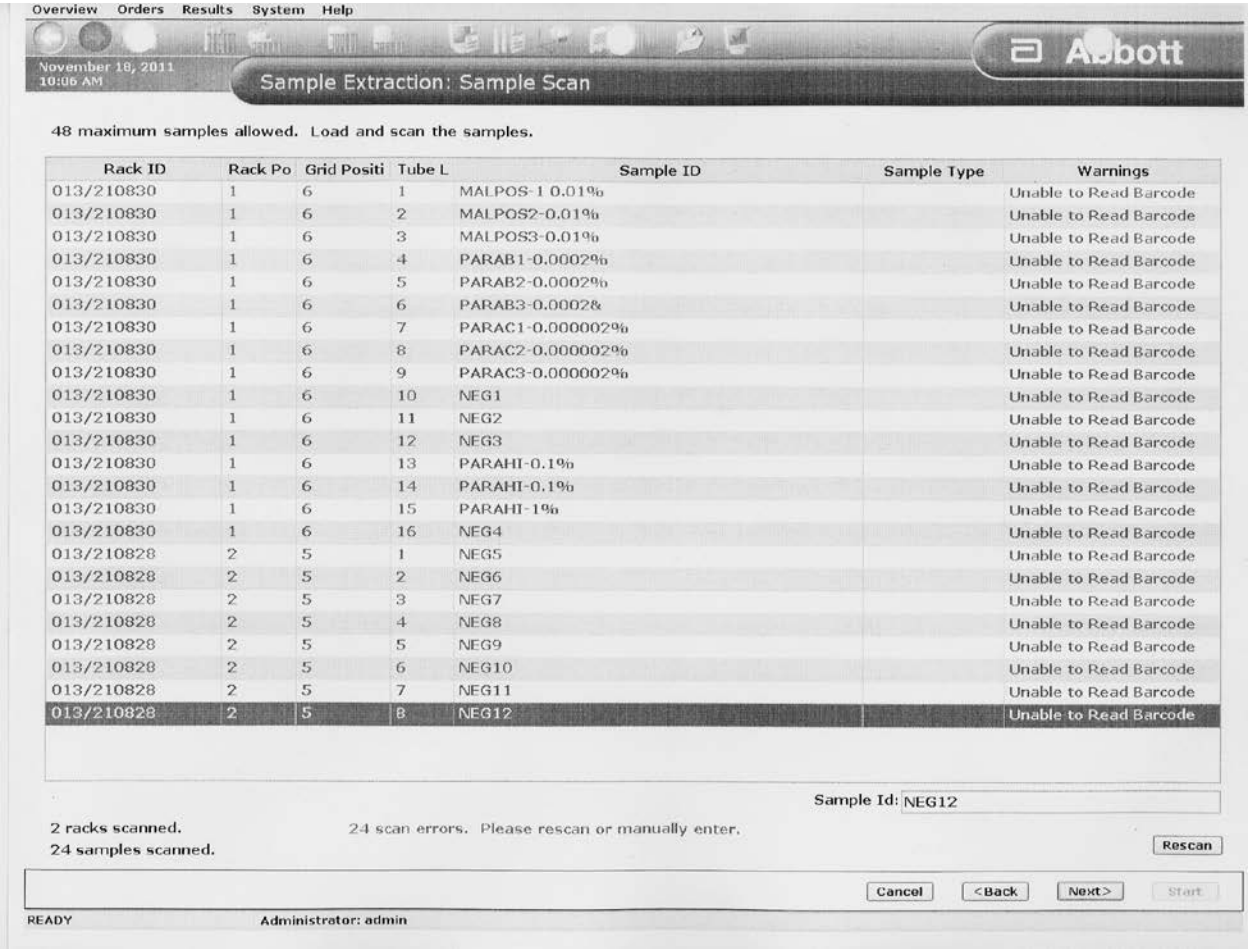
Number of Specimens: 2
Sending Lab Batch Number: 2,000
Comments:

Import Manifest **Box Name:** [redacted]

Batch Number: 9654 **Setup Date:** **Shipped:** No **Import Date** 16/Sep/2011

Spec ID	Global Spec ID	Group/Prot	PID/ID1	VID	Site	Spec Date or Harvest Date	Spec Time	Prim	Add	Der	Sub A/D	Volume	Pos
[redacted]				9.00 Inf	[redacted]		13:20	BLD	EDT	PL1	N/A	1.10 ML	1, A
				9.00 Inf			13:20	BLD	EDT	PL1	N/A	1.10 ML	2, A

Example of Extraction worksheet



Overview Orders Results System Help

November 18, 2011
10:06 AM

Sample Extraction: Sample Scan

Abbott

48 maximum samples allowed. Load and scan the samples.

Rack ID	Rack Po	Grid Positi	Tube L	Sample ID	Sample Type	Warnings
013/210830	1	6	1	MALPOS-1 0.01%		Unable to Read Barcode
013/210830	1	6	2	MALPOS2-0.01%		Unable to Read Barcode
013/210830	1	6	3	MALPOS3-0.01%		Unable to Read Barcode
013/210830	1	6	4	PARAB1-0.0002%		Unable to Read Barcode
013/210830	1	6	5	PARAB2-0.0002%		Unable to Read Barcode
013/210830	1	6	6	PARAB3-0.0002&		Unable to Read Barcode
013/210830	1	6	7	PARAC1-0.000002%		Unable to Read Barcode
013/210830	1	6	8	PARAC2-0.000002%		Unable to Read Barcode
013/210830	1	6	9	PARAC3-0.000002%		Unable to Read Barcode
013/210830	1	6	10	NEG1		Unable to Read Barcode
013/210830	1	6	11	NEG2		Unable to Read Barcode
013/210830	1	6	12	NEG3		Unable to Read Barcode
013/210830	1	6	13	PARAHI-0.1%		Unable to Read Barcode
013/210830	1	6	14	PARAHI-0.1%		Unable to Read Barcode
013/210830	1	6	15	PARAHI-1%		Unable to Read Barcode
013/210830	1	6	16	NEG4		Unable to Read Barcode
013/210828	2	5	1	NEG5		Unable to Read Barcode
013/210828	2	5	2	NEG6		Unable to Read Barcode
013/210828	2	5	3	NEG7		Unable to Read Barcode
013/210828	2	5	4	NEG8		Unable to Read Barcode
013/210828	2	5	5	NEG9		Unable to Read Barcode
013/210828	2	5	6	NEG10		Unable to Read Barcode
013/210828	2	5	7	NEG11		Unable to Read Barcode
013/210828	2	5	8	NEG12		Unable to Read Barcode

Sample Id: NEG12

2 racks scanned.
24 samples scanned.

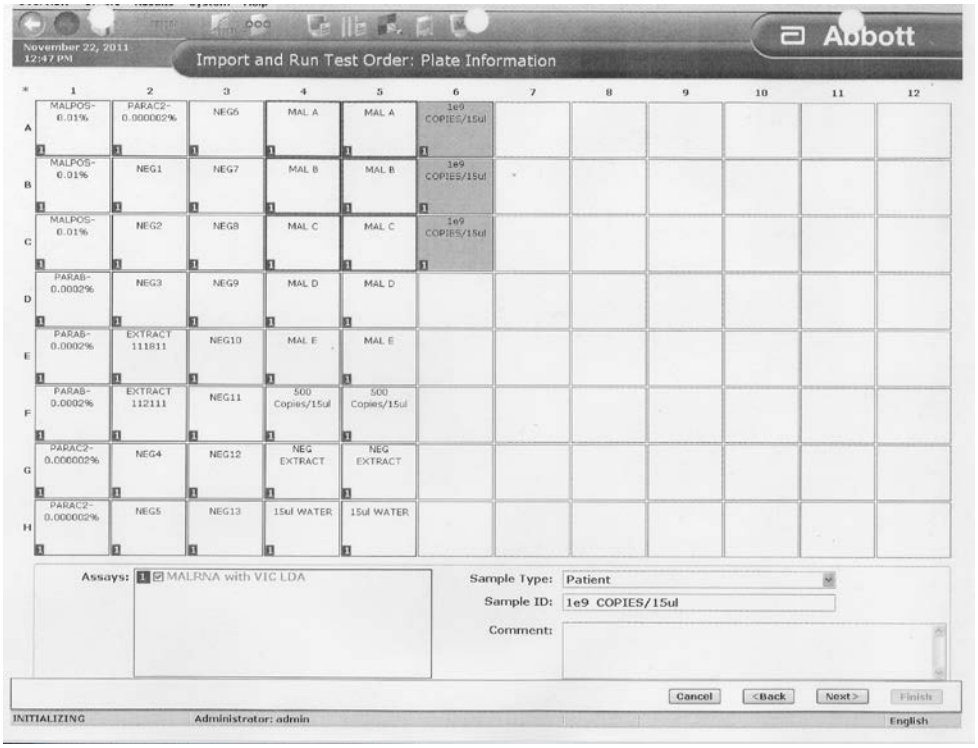
24 scan errors. Please rescan or manually enter.

Rescan

Cancel <Back Next> Start

READY Administrator: admin

Example of RT-PCR worksheet



REFERENCES

- Murphy SC, Prentice JL, Williamson K, Wallis CK, Fang FC, Fried M, Pinzon C, Wang R, Talley AK, Kappe SHI, Duffy PE, Cookson BT. 2012. Real-time quantitative reverse transcription PCR for monitoring of blood-stage *Plasmodium falciparum* infections in malaria human challenge trials *Am J Trop Med Hyg*. 86(3):383–94.
- Murphy SC, Daza G, Chang M, Coombs RW. 2012. Laser cutting eliminates nucleic acid cross-contamination in dried blood spot processing. *J Clin Microbiol*. 50(12): doi: 10.1128/JCM.02549-12
- Mangold KA, Manson RU, Koay ESC, Stephens L, Regner M, Thomson Jr. RB, Peterson LR, Kaul KL. 2005. Real-Time PCR for Detection and Identification of *Plasmodium* spp. *J Clin Microbiol,* 43(5):2435–40.

ASSOCIATED PROTOCOLS & GUIDANCES (Note that 'XX' indicates the most current version)

- *In vitro* culturing of *P. falciparum* 3D7 (#1000-102-XX)
- Handling and use of Armored RNA calibrators (#1000-112-XX)
- Second-generation *P.falciparum*-only qRT-PCR (#1000-74-XX)
- Preparation of liquid blood samples and dried blood spots intended for later testing by UW Plasmodium spp. 18S rRNA qRT-PCR (#1000-099-XX)
- Non-conforming Event Procedure and Report (# 1000-103-XX)
- Guidelines for Sample Receipt, Accessioning and Processing for Malaria Whole Blood Samples with Requisition (# 1000-104-XX)
- Training Log (# 1000-105-XX)
- CFAR/UW protocol on records retention, training, etc.
- Abbott m2000sp/rt operations manuals

METHOD DEVELOPMENT & VALIDATION

Third generation assay for the molecular detection and quantification of blood-stage *Plasmodium* from human whole blood

Protocol #1000-100-01

Sean C. Murphy, MD, PhD

Amelia Hanron, MLS (ASCP)

Annette M. Seilie

Zachary Billman

Ming Chang, PhD

Robert Coombs, MD, PhD

This project was supported by the Divisions of Microbiology & Virology (Laboratory Medicine, UWMC), the Bill and Melinda Gates Foundation, and the Center for AIDS Research (UW)

August 1, 2016

NOTE: Minor modifications made to this document on February 22, 2017 to add 1) calculations for confidence intervals to precision estimates, 2) replace two-sided t-tests for stability with one-sided t-tests more appropriate for degradation measurements, 3) add three specimens in the Accuracy comparison for 600 para/mL level to bring total to 18 samples, 4) revise analytical sensitivity table to include calculations using two different cutoffs. These changes were made to align with the Biomarker Qualification package being submitted to FDA CDER.

1. TABLE OF CONTENTS

2. ACKNOWLEDGEMENTS

Since 2008, the Murphy Laboratory in the University of Washington Medical Center (UWMC)'s Department of Laboratory Medicine developed a series of molecular malaria biomarker detection assays to support Seattle-based malaria clinical trials. The first-generation assay relied on LightCycler dual hybridization probe technology and was supported through the UWMC Microbiology Laboratory. A second generation assay using TaqMan hydrolysis probes was subsequently developed at the Harborview Medical Center site in space shared with the Center for AIDS Research (CFAR). The second-generation assay utilized the Abbott m2000 platform in order to accommodate larger clinical trials. This third generation assay was developed to further improve the second generation assay by providing P. falciparum and Pan-Plasmodium-specific channels that accommodate the known sequence diversity present in field strains. The third generation assay relied on the invaluable contributions of Research Scientists Annette M Seilie and Ming Chang, as well as Medical Laboratory Scientist Amelia Hanron. The malaria laboratory also continues to rely on the generous support and space provided by CFAR Retrovirology Lab Director Robert Coombs. We also thank the other technologists and support staff in the Divisions of Retrovirology and Microbiology.

Many groups have contributed to the first- , second- and/or third-generation assays. Advice and technical support were received from the Divisions of Molecular Virology (1616 Eastlake), Retrovirology (Harborview Research & Training Building), Microbiology (UWMC and Harborview Medical Center) and others within the Department. Linda Cook, Jane Kuypers and technologist Nancy Wright were instrumental in the production of synthetic control reagents used in both generations of the assay. Caroline Wallis, Ferric Fang and staff of Harborview Microbiology Laboratory also provided clinical samples used to evaluate the specificity of assay reagents. Gametocyte- and sporozoite-containing samples were kindly provided by Matt Fishbaugher, Will Betz and Stefan Kappe of the Center for Infectious Disease Research (CIDR). Other CIDR collaborators involved in various phases of method development have included Patrick Duffy (now National Institutes of Health, Rockville, MD), Ruobing Wang, Angela Talley and Sara Healy. James Kublin (Fred Hutchinson Cancer Research Center, Seattle, WA) has also provided instrumental support and advice since he first pointed out the need for such a test to Dr. Murphy in 2008. We also thank Abbott Molecular, Inc., especially Drs. Gavin Cloherty and Clifford Chan for providing a research m2000 platform for development and validation of our assay.

Finally, we thank the Bill and Melinda Gates Foundation and in particular Sophie Allauzen and the late Alan Magill for providing ongoing and generous financial support and guidance for the development and validation of the third generation assay and for supporting its use in the ongoing Drug Development Tool Biomarker Qualification pathway at the FDA.

3. SUMMARY

Malaria infection with *Plasmodium* parasites has been diagnosed by microscopy for over 100 years. The analytical sensitivity of detection by thick blood smear (TBS) microscopy is ~5-50 asexual parasites per µl (2000-20,000 parasites per mL) for expert-level microscopists. Molecular detection methods permit more sensitive detection of Plasmodium DNA or RNA biomarkers. Detection at lower parasite densities allows for diagnosis earlier. In the setting of 'controlled human malaria infection' trials, earlier detection may reduce participant discomfort and accelerate the time to rescue treatment. The first [1] and second [2] generation assays have detected parasite biomarkers in whole blood 2-4 days earlier than TBS because of the improved analytical sensitivity of molecular approaches. Quantification by molecular methods also make it possible to determine the kinetics of asexual parasite growth in challenge trials [1, 2], which can help prioritize candidate drugs and vaccines based on relative efficacy.

Most molecular approaches are based on detection of conserved regions of parasite DNA encoding the 18S ribosomal RNA (rRNA) and/ or the 18S rRNA itself [3]. This document concerns the development and validation of a third-generation assay intended to replace the second-generation assay is described herein.

Here, we validated a real time quantitative RT-PCR assay to detect asexual-type *Plasmodium falciparum* and pan-*Plasmodium* genus-specific 18S rRNAs using specific primers and TaqMan probes. Briefly, 50 µL of EDTA anticoagulated whole blood is added to 2 mL of guanidinium-based lysis buffer and samples are freshly tested or stored at ≤-70°C. Total nucleic acids are extracted from half of this lysed sample (1 mL containing the equivalent material from 25 µL of whole blood), and multiplexed RT-PCR is performed. The assay provides absolute RNA copy number quantification using a three-$\log_{10}$ standard curve consisting of synthetic Armored RNA standard curve in negative whole blood. The analytical sensitivity is 20 parasites per mL of whole blood. The method is highly reproducible and is monitored with Levey-Jennings plots of similarly-extracted high, low and negative controls in each assay. The method passed all criteria set forth in a validation plan with respect to accuracy, correlation, precision, analytical sensitivity, analytical specificity, reportable range and carryover.

4. ABBREVIATIONS

- %AT, percent adenine and thymine
- %CV, coefficient of variation
- %GC, percent guanine and cytosine
- BLAST, Basic Local Alignment Search Tool
- CFAR, Center for AIDS Research
- CFO560, CAL Fluor Orange 560
- CI, confidence interval
- CIDR, Center for Infectious Disease Research (formerly Seattle Biomed)
- CT, crossing threshold
- FN, false negative
- FP, false positive
- FRET, fluorescence (or Förster) resonance energy transfer
- HMC, Harborview Medical Center
- Lysate: 25 uL human EDTA whole blood lysed in 1 mL bioMerieux NucliSENS lysis buffer.
- MCTC, Malaria Clinical Trials Center
- mRNA, messenger RNA
- NASBA, nucleic acid-based sequence amplification
- NCBI, National Center for Biotechnology Information
- NIH, National Institutes of Health
- PCR, polymerase chain reaction
- qPCR, quantitative PCR
- qRT-PCR, quantitative RT-PCR
- rDNA, ribosomal DNA gene
- rRNA, ribosomal RNA
- RT-PCR, reverse transcription PCR
- SD, standard deviation
- SSU, small subunit
- TBP, the gene encoding human TATA-Box Binding Protein
- Tm, melting temperature
- TN, true negative
- TP, true positive
- UWMC, University of Washington Medical Center

5. METHOD DEVELOPMENT

a. Sequence specificity of Plasmodium-specific reagents

The 18S rRNA genes of *P. falciparum* are the most common molecular target in molecular *Plasmodium* assays. In *P. falciparum*, there are four 18S (or small subunit, SSU) rRNA genes that are differentially expressed during the parasite lifecycle [4, 5]. Asexual or 'A'-type rRNAs encoded by genes on chromosomes 5 (MAL5_18S) and 7 (MAL7_18S) are predominantly expressed in the blood-stage of infection and persist into the mosquito blood meal, whereas 'S' type rRNAs encoded on chromosomes 1 (MAL1_18S) and 11 (MAL11_rRNA) are predominantly expressed during oocyst maturation through sporozoite formation [4]. However, the control of expression is not absolute. In some non-falciparum species such as *P. vivax*, another sexual type 18S rRNA (type 'O') is present as well.

i. Primers for *P. falciparum* nucleic acid amplification

For the current third generation assay, the sequence region encompassing the previously used PL1473/PL1679 primer pair [6] were evaluated for the development of *P. falciparum* specific primers. The PL1473/PL1679 pair can be used to detect all *Plasmodium spp*. known to infect humans [6] and generates a 214-bp amplicon. This primer pair is perfectly matched to asexual-stage 18S rRNA genes MAL5_18S and MAL7_18S; the reverse primer P1679 lacks the two base pair insertion present in both sexual-type 18S rRNA transcripts. When combined with *P. falciparum*-specific probes, this approach formed the basis for the UWMC first- and second-generation assays and this approach is suitable for clinical trials using defined strains such as *P. falciparum* NF54 or 3D7. However, in epidemiology studies conducted in our laboratory, we encountered samples from the Shokolo Malaria Research Unit (SMRU) in Thailand that were positive using a Thai qPCR method [7] but negative by the UWMC PL1473/PL1679 second-generation assay (unpublished BIOME study in collaboration with PATH, UW, SMRU, UCSF and the BMGF. Since this suggested that field strain variation could result in false negative results for the PL1473/PL1679 primer pair developed by Mangold et al., we sought to identify even more conserved sequences for the third generation assay.

We analyzed potential primers against available *Plasmodium* sequences for *in silico* mismatches and identified sequences with 100% conservation amongst asexual-stage *P. falciparum* sequences that also had the greatest number of mismatches against sexual-stage *P. falciparum* and other *Plasmodium* spp. Potential primer pairs were synthesized and tested by SYBR-Green qRTPCR using RNA extracted from pure blood-stage culture of *P. falciparum* strain 3D7, then further examined for amplicon purity by melting curve analysis. Of the potential primer designs, the PfDDT1451F21 (GCGAGTACACTATATTCTTAT) and PfDDT1562R21 (ATTATTAGTAGAACAGGGAAA) primer pair produced the best separation in melt curve analyses. PfDDT1451F21 / PfDDT1562R21 primer pair can be used to specifically detect *P. falciparum*. The primer is 100% matched to the A-type -stage 18S rRNA consensus sequence for *P. falciparum* 3D7 (MAL5_18S/PF3D7_0531600 and MAL7_18S/PF3D7_0725600), whereas there are mismatches in the sexual-type transcripts from chromosomes 1 and 11 (MAL1_18S/PF3D7_0112300 and MAL11_rRNA/PF3D7_1148600, respectively) (**Figure 1**). The *P. falciparum*-specific primer sequences are conserved in 10 full or partial length *P. falciparum*-derived A-type 18S rRNA sequences available in GenBank (**Appendix 1**) but are not in non-*P. falciparum* species (**Figure 2 and Table 1**).

ii. Hydrolysis (Taqman) probe for *P. falciparum* nucleic acid detection

The TaqMan probe for *P. falciparum* detection used in this assay was originally designed for the second generation [2] and targeted the same region targeted by the original FRET dual hybridization probe in the first-generation assay [2]. The probe is currently manufactured by LCG BioSearch Technologies (5'-[6-FAM]-ATTTATTCAGTAATCAAATTAGGAT-3'[Black Hole Quencher 1]). The probe is 100% matched to the A-type -stage 18S rRNA consensus sequence for *P. falciparum* 3D7 (MAL5_18S/PF3D7_0531600 and MAL7_18S/PF3D7_0725600), whereas there are mismatches in the sexual-type transcripts from chromosomes 1 and 11 (MAL1_18S/PF3D7_0112300 and MAL11_rRNA/PF3D7_1148600, respectively) (**Figure 1**). In addition, the *P. falciparum*-specific probe is conserved in 10 full or partial length *P.*

falciparum-derived A-type 18S rRNA sequences available in GenBank (**Appendix 1**) but are not in non-*P. falciparum* species (**Figure 2 and Table 1**).

FIGURE 1. Alignment of *P. falciparum* sexual- and asexual-type 18S rRNAs to RT-PCR reagents. Reference sequences for the two A-type (PF3D7_0513600 and PF3D7_0725600) and two S-type (PF3D7_0112300 and PF3D7_1148600) 18S rRNA genes of *P. falciparum* were aligned and the pan-*Plasmodium* and *P. falciparum*-specific primers and probes were overlaid. Dots represent 100% conserved sequences. Solid green fill indicates the position of forward and reverse pan-*Plasmodium* primers and green text indicates the position of the corresponding probe. Solid red fill indicates the position of forward and reverse *P. falciparum*-specific primers and red text indicates the position of the corresponding probe. Gene names refer to Plasmodb.org identifiers.

TABLE 1. Primer/probe conservation compared to available GenBank sequences

Species[a]	Sequences evaluated	Pf 100% conservation	Pan-*Plasmodium* 100% conservation
P. falciparum	10	10/10 PfDDT1451F21 10/10 probe 10/10 PfDDT1562R21	10/10 PanDDT1043F19 10/10 probe 10/10 PanDDT1197R22
P. vivax	19	N/A	19/19 PanDDT1043F19 18/19 probe[a] (1/19 1bpΔ) 18/19 PanDDT1197R22
P. ovale wallikeri	13[b]	N/A	13/13 PanDDT1043F19 11/13 probe 11/13 PanDDT1197R22
P. ovale curtisi	7	N/A	7/7 PanDDT1043F19 7/7 probe 7/7 PanDDT1197R22
P. malariae	14	N/A	14/14 PanDDT1043F19 14/14 probe 14/14 PanDDT1197R22
P. knowlesi	132[c]	N/A	131/132 PanDDT1043F19 129/132 probe 127/132 PanDDT1197R22
P. brasilianum	14	N/A	14/14 PanDDT1043F19 13/14 probe (1/14 1bpΔ) 14/14 PanDDT1197R22

[a] There were 1 bp substitutions in each of the probe and reverse primer sequence that occurred in 1/19 *P. vivax* sequences. The substitutions occurred in the same sequence (DQ660817.1) and this sequence was A-type but divergent from the other 18 available P. vivax sequences.
[b] Two *P. ovale wallikeri* sequences that align most closely to A-type genes but are divergent from the other *P. ovale wallikeri* and *P. ovale custisi* A-type sequences may represent erroneous sequence deposits in GenBank.
[c] Of 132 available A-type *P. knowlesi* genes, only 1-5 sequences showed 1-2 bp changes in the primer or probe binding regions.

Figure 2. Alignment of consensus asexual-type 18S rRNAs of the four major species of human-infecting *Plasmodium sp*. against RT-PCR assay reagents. Reference sequences for A-type 18S rRNA genes of *P. falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi* were aligned across the pan-*Plasmodium* and *P. falciparum*-specific amplicon region. Sequences of primers and probes were overlaid. Dots represent 100% conserved sequences. Solid green fill indicates the position of forward and reverse pan-*Plasmodium* primers and green text indicates the position of the corresponding probe. Solid red fill indicates the position of forward and reverse *P. falciparum*-specific primers and red text indicates the position of the corresponding probe. Additional sequences are listed in Appendix 1.

iii. Primers for Pan-*Plasmodium* nucleic acid amplification

We identified five published primer/probe sets reported to target pan-*Plasmodium* genus 18S rRNAs by qPCR [6, 8-11] and tested primer-probe combinations *in silico* and *in vitro*. We had previously used the Mangold primers [6] and since we previously identified false negative field samples missed by these primers, we did not evaluate these primers further. Of the remaining primers, we analyzed regions of *Plasmodium* 18S rRNAs encompassed by these primer sets using sequences available in Genbank/PlasmodDB to assess sequence conservation across A-type 18S rRNAs of human-infecting malaria species. One primer set was eliminated prior to testing due to amplicon proximity to the 3' end of the 18S rRNA, and another was eliminated due to amplicon overlap with the PfDDT1451F21 / PfDDT1562R21 region. Several pairs [8, 10, 11] were synthesized and tested by qRT-PCR for multiplex performance.

Of the tested pair, Plasmo1 / Plasmo2 [8], matched 100% of all aligned asexual-stage full-length *Plasmodium spp.* sequences and performed the best but was ultimately deemed to be unsuitable under qRT-PCR thermocycling conditions developed for the above-described *P. falciparum* specific PfDDT1451F21 / PfDDT1562R21 pair (48°C for 10 min, 95°C for 2 min, and 45 cycles of 95°C for 5 s and 50°C for 35 s on the Abbott m2000*rt* platform) due to non-specific amplicon production. These amplicons were generated following RT-PCR but not PCR (data not shown) indicating that the primers generated spurious products during the low temperature RT step. The Plasmo 1/Plasmo 2 region was re-evaluated. A new pair PanDDT1043F19 (AAAGTTAAGGGAGTGAAGA) and PanDDT1197R22 (AAGACTTTGATTTCTCATAAGG) performed well in initial qRT-PCR assays and demonstrated 100% sequence conversation with human-infecting Plasmodia (**Figure 2** and **Table 1**). The amplicon does not overlap the *P. falciparum* PfDDT1451F21 / PfDDT1562R21 region (**Figure 2**). The PanDDT1043F19 / PanDDT1197R22 amplicon contains conserved and variant nucleotides that permit both pan-*Plasmodium* primer construction and yet simultaneously allows for species identification by amplicon sequencing if needed. Beyond human-infecting Plasmodia, the pan-*Plasmodium* primers also display 100% homology to rodent-infecting species *P. yoelii, P. chabaudi* and *P. berghei* and 100% homology to primate-infecting *P. cynomolgi* and *P. reichenowi*.

iv. Hydrolysis (Taqman) probe for Pan-*Plasmodium* nucleic acid detection

In parallel with pan *Plasmodium* primer pair design, we evaluated the probes corresponding to the published primer/probe sets mentioned above [8-11]. The Plasmo1 / Plasmo2 primers were originally reported with a hydrolysis (TaqMan) probe [8], which was subsequently evaluated against the qRT-PCR-compatible PfDDT1451F21 / PfDDT1562R21 primer pair. This probe showed 100% conservation with human-infecting Plasmodia (**Figure 2** and **Table 1**). Beyond human-infecting Plasmodia, the pan-*Plasmodium* probe also displays 100% homology to rodent-infecting species *P. yoelii, P. chabaudi* and *P. berghei*, 100% homology to primate-infecting *P. cynomolgi* and near 100% homology to *P. reichenowi* (35/36 bp probe homology). The pan-*Plasmodium* probe is currently manufactured by LCG BioSearch Technologies (5'-[CAL Fluor Orange 560]- ACCGTCGTAATCTTAACCATAAACTA[T(Black Hole Quencher-1)]GCCGACTAG-3'[Spacer C3]).

b. Total nucleic acid extraction

i. Abbott m2000 sp

The Abbott m2000sp platform is the front-end extraction apparatus for the integrated, semi-automated, m2000 system. The m2000sp uses silica-based extraction following off-board cell lysis and DNase and RNase inactivation by guanidinium thiocyanate-based lysis buffer. m2000sp extraction leads to recovery of high quality RNA and DNA across a range of sample types, including whole blood.

ii. Whole blood extraction protocol for malaria

The Abbott m2000 protocol for whole blood extraction on the sp instrument aspirates 1 mL of bioMerieux NucliSENS lysis buffer containing 25 μL of EDTA whole blood (Lysate) from a sample tube containing >1.3 mL of total sample. For this reason, samples are prepared to contain 50 μL of whole blood in 2 mL lysis buffer. The extracted nucleic acid is eluted in 53 μL buffer. We previously observed qRT-PCR inhibition if volumes of ≥100 μL of whole blood in 2 mL lysis buffer were extracted.

Recovery of total nucleic acids by the m2000sp system varies by target length with shorter targets less efficiently recovered. Plasmodial 18S rRNAs, like other eukaryotic 18S rRNAs are 1.9-2.1 kb long. The full-length MAL5_18S rRNA from chromosome 5 is 2,092 bp and the MAL7_18S sequence is 2,087 bp. The second-generation method validation determined that the Abbott m2000sp provides ~100% recovery for *Plasmodium* 18S rRNA (107% [95%CI 60-154%] – see second-generation report), indicating nearly complete target recovery. The extraction method is unchanged from second to third generation assay and, as such, this type of recovery study were not performed for the third generation assay.

c. Endogenous internal control mRNA target

A human TATA-box binding protein (TBP) mRNA (GenBank: NM_001172085) serves as an endogenous internal control mRNA to monitor overall extraction and RT-PCR efficiency and sample integrity. This gene is abundant in humans and shows low variability across multiple samples and tissue types. In addition, in method development studies, co-amplification of this target multiplexed with the P. falciparum and pan-Plasmodium targets had little to no effect on the performance characteristics of the individual Plasmodium-targeted reactions. TBP mRNA generates positive reactions in a C_T range approaching that of the *Plasmodium* limit of detection. TBP C_Ts are monitored for the control samples and are expected to be relatively stable, but results from individual patients could vary more significantly due to variations in white blood cell content between patients. This target is multiplexed with the *P. falciparum* and Pan *Plasmodium* 18S rRNA targets in the validated assay.

d. RT-PCR

i. Multiplexed qRT-PCR design

To provide absolute quantification of parasite RNA by RT-PCR, whole blood is extracted, and 18S rRNA is co-amplified with the endogenous internal control mRNA in a multiplexed format. The final RT-PCR method uses the Bioline SensiFAST™ Probe Lo-ROX One-Step kit, the previously described *Plasmodium* genus-specific primers and TaqMan CAL Fluor Orange 560 (CFO560) Black Hole Quencher Probe, *P. falciparum*-specific primers and TaqMan 6-FAM Black-Hole Quencher probe, as well as primers and a Quasar 670-labeled probe for the internal control under conditions specified in the standard protocol (Protocol #1000-100-01).

ii. Armored RNA standard curve

Absolute quantification is achieved by using a standard curve of Armored RNA encoding the full-length *P. falciparum* 18S rRNA target (Pf-Armored RNA) diluted in negative whole blood. This standard curve is compared to parasite-containing whole blood samples of known parasite density. The Pf-Armored RNA was purchased from Asuragen at $5x10^{11}$ copies per mL of stock solution. Ten-fold serial dilutions of Pf-Armored RNA – from $5.3x10^{7}$ to $5.3x10^{2}$ copies per mL lysate– were diluted in whole blood and amplified by *P. falciparum* (**Figure 3A**) and pan-*Plasmodium* qRT-PCR (**Figure 3B**). The three highest concentration dilutions were subsequently retested in triplicate to generate standard curves for both Pf (**Figure 3D**) and Pan qRT-PCR (**Figure 3B**). We also tested the previously described serial dilutions of cultured blood-stage parasites serving as an External Quality Assurance panel, in which parasite counts were evaluated by qPCR and qRT-PCR assays in five other laboratories, including by the UWMC's second-generation assay [12]. In this way, we were able to acquire the conversion factor necessary to calculate the parasites per mL of blood from the copies of the Armored RNA.

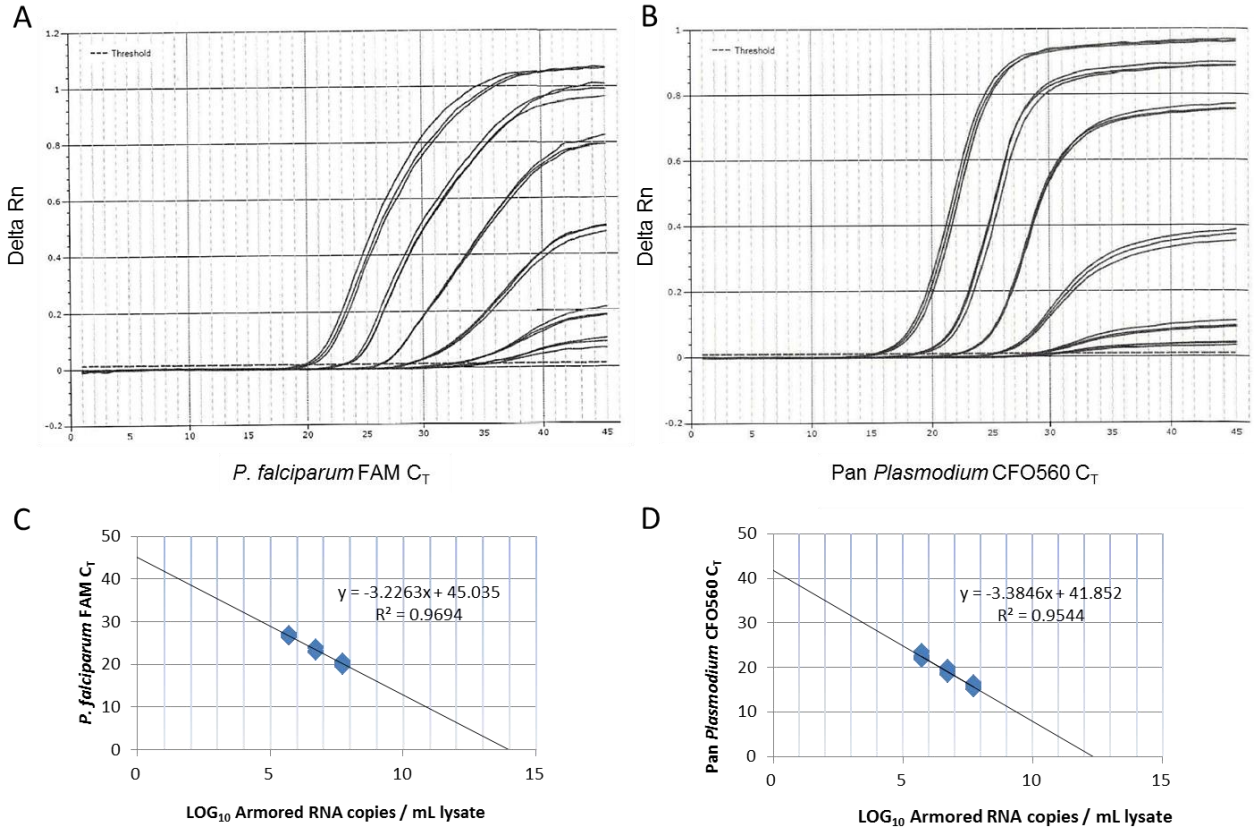


Figure 3. Multiplex *Plasmodium* RT-PCR fluorescence output and standard curves. Pf-Armored RNA diluted into whole blood as 10-fold dilutions from $5.3x10^7$ to $5.3x10^3$ and final 2-fold dilution to $2.15x10^3$ copies per mL lysate were tested using the *P. falciparum*-specific reagents (**A**) and the pan-*Plasmodium* reagents (**B**). Preliminary standard curves were derived by linear regression for *P. falciparum* (**C**) and pan-*Plasmodium* (**D**) targets.

iii. Assay format

Up to 92 clinical samples plus controls (*Plasmodium* high and low controls and 1-2 negative controls) can be extracted and amplified tested in each batch. Quantitative data is provided for all samples with an estimated parasite density of ≥20 parasites per mL of whole blood. The Armored RNA standard curve is regenerated after the m2000*rt* instrument is optically calibrated every six months or when a new lot of enzyme kit is used.

6. METHOD VALIDATION

a. Validation plan and available specimens

A plan for the *Plasmodium* qRT-PCR validation was provided to the lab to guide testing and analysis of the data. The validation followed the plan from 1st and 2nd generation validations with minor modifications. Data generated from Pf-Armored RNA standards per mL lysate were used to evaluate the standard curve, reportable range and carryover and limit of detection. Data generated from *parasite-containing whole blood samples* (10 to $4x10^7$ parasites per mL blood) were used to evaluate accuracy, precision, analytical sensitivity, analytical specificity, reportable range and carryover. The following summarizes the findings of this third-generation assay validation.

b. Standard curve assessment

Pf- Armored RNA standards in whole blood at $5.3x10^7$, $5.3x10^6$ and $5.3x10^5$ copies per mL lysate were tested in triplicate to develop a standard curve. A total of four runs were conducted for this validation study. The graphs of the standard curves are shown in **Figure 4** and the mean curve derived from all of the results is in **Table 2**.

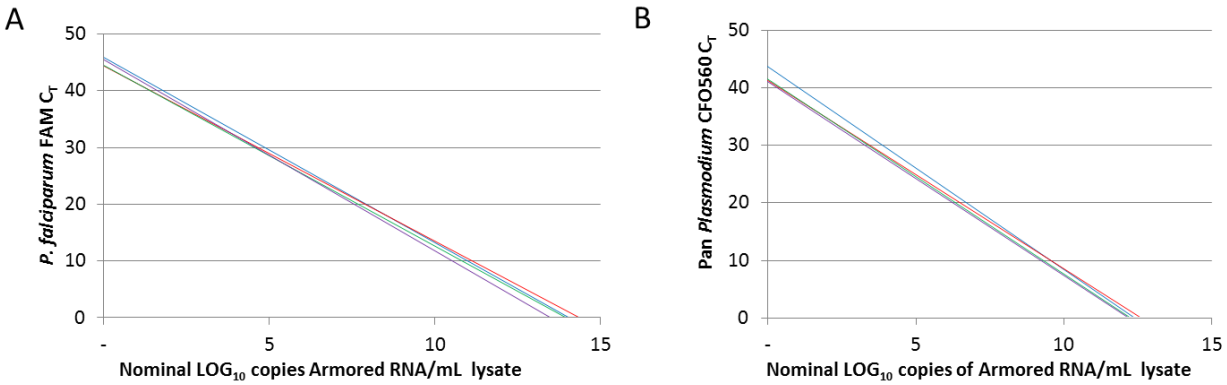


Figure 4. Three-point standard curve analyses. Repeated testing of the Pf-Armored RNA standard curve by P. *falciparum* (**A**) and Pan *Plasmodium* (**B**) qRT-PCR. n=4 runs with $5.3x10^7$, $5.3x10^6$ and $5.3x10^5$ copies per mL lysate.

Table 2. Three-point standard curve analyses

***P. falciparum* (n=4 runs)**	
Slope	-3.23 cycles/$\log_{10}$(+/- 2 SD = -3.47 to -2.98)
Y-intercept	45.04 cycles (+/- 2 SD = 43.55 to 46.52)
r^2	0.996 (acceptable >0.975)
Pan *Plasmodium* (n=4 runs)	
Slope	-3.38 cycles/$\log_{10}$ (+/- 2 SD = -3.59 to -3.17)
Y-intercept	41.85 cycles (+/- 2 SD = 39.44 to 44.26)
r^2	0.998 (acceptable >0.975)

The standard curve allows direct calculation of the $\log_{10}$ RNA copy number per RT-PCR reaction (using 15 μL of template from 53 μL of eluate). The standard curve is re-evaluated at least every six months or when reagents lot numbers are changed or the Abbott m2000rt instrument is optically calibrated. The repeat curve must fall within the range above in order to continue to use the standard curve values defined in this validation study. The copy number can be converted to estimated parasites per mL of whole blood using a conversion factor derived below. The standard protocol provides copy numbers and estimated parasites per mL based on the pan-Plasmodium qRT-PCR standard curve.

To derive a conversion factor relating 18S rRNA copies/mL to estimated parasites per mL, we utilized the same approaches as previously reported [1, 2]. Archival EQA samples [12] were tested with the third-generation assay and copy numbers were generated using the Pf-Armored RNA standard curves. Compared to the nominal TBS-based parasite densities for the EQA samples [12], the third generation assay estimated that there were $7.4x10^3$ 18sRNA copies per parasite (3.87 $\log_{10}$ 18S rRNA copies per parasite; median 3.87 $\log_{10}$; 95% CI 3.79 – 3.94 $\log_{10}$; n=22 EQA-validated samples). This conversion factor is consistent with assay-specific conversion factors determined for the first- ($1x10^4$ copies/parasite [1]) and second-generation assays ($3.5x10^3$ copies/parasite [2]). Thus, to calculate the estimated parasite density per mL of whole blood in this assay, the 18S rRNA copy number per mL of lysate (equivalent to 50% of the nucleic acids from 50 μL of whole blood) is divided by 7400 (18S rRNA-to-parasite conversion) and multiplied by 40 (conversion to 1 mL of whole blood).

c. Correlation

Parasite-containing specimens (high, medium and low concentration) and negative control specimens were tested in duplicate over 20 total runs by different operators. The nominal and observed positive and negative results were compared. Log_{10} transformed data for observed and nominal parasites per mL showed excellent correlation across a range of parasite densities (**Figures 5A:** *P. falciparum*, r^2 = 0.99, slope = 0.94 and **Figure 5B** Pan-*Plasmodium*, r^2 = 0.99, slope = 0.98). The target correlation was r^2 > 0.95.

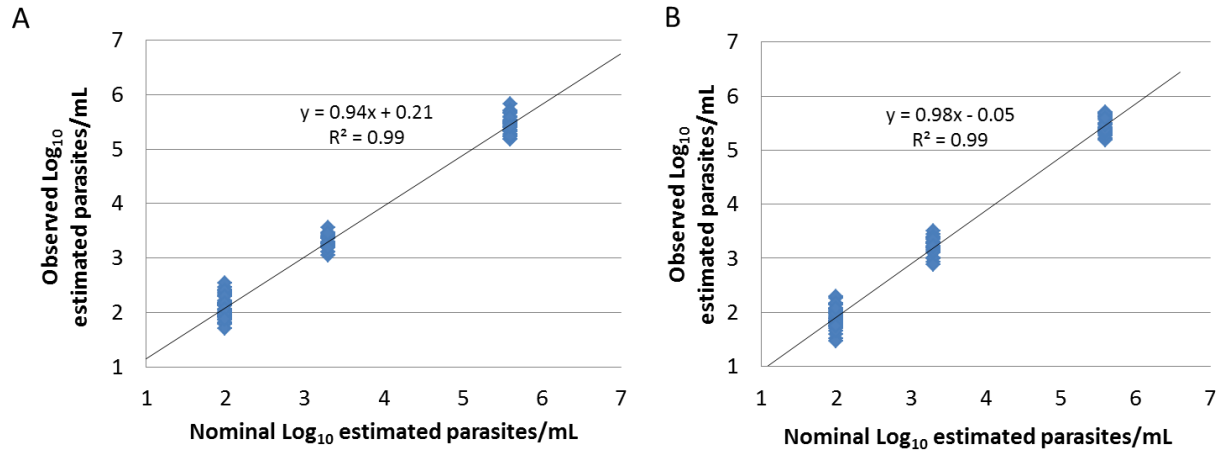


Figure 5. Correlation evaluation for (A) *P. falciparum* and (**B**) pan-*Plasmodium* *q*RT-PCR.

d. Accuracy – Trueness studies

The same data evaluated for correlation was also evaluated for trueness (closeness) to the nominal value. All parasite-containing specimens were positive by our assay across the wide range of parasite densities, and all negative controls were negative (100% sensitive, 100% specific). All high, medium and low parasitemia samples were correctly categorized by the assay. Quantitative qRT-PCR data and nominal values were compared using a Bland-Altman plot as log_{10} parasites per mL of whole blood. Differences between nominal and observed estimates (recovery) were plotted (**Figures 6A** *P. falciparum* and **Figure 6B** Pan-*Plasmodium*). Of 106 samples in this dataset, the average log_{10} difference (bias) across all samples was 0.03 log_{10} parasites per mL (95%CI -0.36 to 0.41 log_{10} parasites per mL) in the *P. falciparum* RT-PCR and 0.11 log_{10} parasites per mL (95%CI -0.23 to 0.44 log_{10} parasites per mL) in the pan *Plasmodium* RT-PCR. The maximum absolute difference in log_{10} recovery from the nominal value was 0.53 log_{10} parasites per mL in the P. *falciparum* RT-PCR and 0.53 log_{10} parasites per mL in the pan-*Plasmodium* RT-PCR. There was no evidence of concentration-dependent differences in recovery. There were no established validation targets for this criterion.

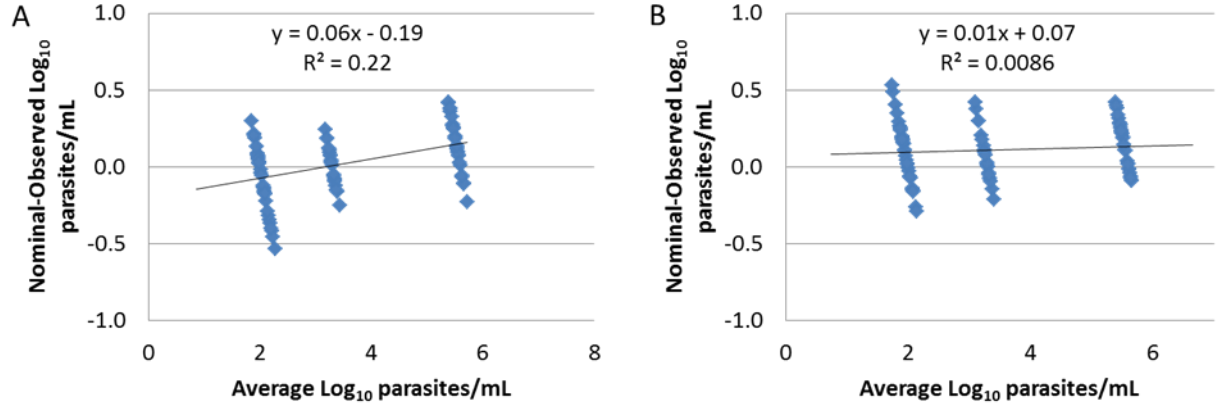


Figure 6. Bland-Altman difference plots. (A) *P. falciparum* and (**B**) pan-*Plasmodium* qRT-PCR.

e. Precision

Within-run (repeatability) and between-run (within lab reproducibility) precision was determined by testing duplicate high, medium, low and negative samples over 20 runs (only 6 runs with duplicates for medium samples) judged against the nominal standard values as determined by blood smear. Intra- and inter-assay components of variation were calculated [13]. The percent coefficient of variation (%CV = Standard deviation / mean) are reported in **Tables 3-4**. Confidence intervals were calculated as suggested by CLSI. The assay performed within the precision criteria set forth in the validation plan.

Table 3. Precision - *P. falciparum* qRT-PCR

Control	Samples per run	Total runs	Nominal parasites per mL whole blood	*P. falciparum* channel	
				Intra-assay %CV within run (95% CI)	Inter-assay %CV within lab (95% CI)
High	2	20	$4x10^5$	1.95% (1.49-2.82%)	2.90% (2.38-3.73%)
Mid	2	6	$2x10^3$	1.35% (0.87-2.96%)	3.98% (3.12-5.49%)
Low	2	20	$1x10^2$	6.45% (4.93-9.31%)	9.88% (8.09-12.69%)
Acceptance criteria				*<10%*	*<15%*

Table 4. Precision – Pan-*Plasmodium* qRT-PCR

Control	Samples per run	Total runs	Nominal parasites per mL whole blood	Pan-*Plasmodium* channel	
				Intra-assay %CV within run (95% CI)	Inter-assay %CV within lab (95% CI)
High	2	20	$4x10^5$	1.34% (1.03-1.94%)	2.74% (2.24-3.51%)
Mid	2	6	$2x10^3$	2.62% (1.69-5.78%)	5.52% (4.33-7.61%)
Low	2	20	$1x10^2$	6.63% (5.08-9.58%)	10.17% (8.33-13.06%)
Acceptance criteria				*<10%*	*<15%*

f. Reference Interval

The reference interval of the healthy, normal population for this assay is “not detected”.

g. Analytical sensitivity

Analytical sensitivity of this assay is mainly defined by blood sampling volume. The limit of detection of the assay is best described by the Poisson distribution for samples containing 1 or 2 parasites, on average, per 50 µL extraction volume since stochastic fluctuations in blood collection and sampling affect assay sensitivity more than RT-PCR sensitivity. The Poisson distribution predicts that the frequency distribution of the number of parasites present in any single 50 uL whole blood volume prior to extraction and will indicate with what frequency a sample containing 1 parasite per tube (on average), will actually contain zero parasites and be detected as negative. For a 50 µL blood volume containing *on average* a

single parasite per tube, the Poisson distribution predicts probabilities of 36.8%, 36.8%, 18.4%, 6.1%, 1.9% for 0, 1, 2, 3 or ≥4 parasites, respectively, to be actually present in any one sample.

A range of dilutions (10-250 parasites per mL) from previously quantified clinical samples were tested. At and above 20 parasites per mL, ≥95% of samples (20 per level) were positive. Only 70% (14/20) of samples at 10 parasites per mL were positive, suggesting that the dilution series reached the nominal one parasite per sample density in the 10-20 parasite/mL range and that the dilution series was within two-fold of the intended concentration (**Table 5**). To ascertain whether the assay could continue to detect RNA at concentrations below what would be expected from a single parasite in the sample, whole blood samples were made containing 1325 copies of the Pf-Armored RNA per mL lysate, equivalent to 0.36 parasites per 50 μL of whole blood – all were positive (20/20 samples, **Table 5**) with clear, unambiguous C_T curves for both *P. falciparum* and pan-*Plasmodium* targets.

To further corroborate the ability of the test to detect a single parasite in a tube and to differentiate this from a true negative sample, we considered whether the C_Ts of low positive samples would ever overlap the C_Ts of true negative blood. None of 105 known negative samples generated any C_T value for the *P. falciparum* or pan-*Plasmodium* assays (hence C_T = 45 cycles). In contrast, the C_Ts for low positive samples (nominal value 20 parasites per mL) was 32.6 cycles (range 28.8-35.8 cycles) for *P. falciparum* and 29.3 (range 26.1-32.3 cycles) for pan-Plasmodium.

Collectively, these data support the conclusion that the third generation assay can detect the 18S rRNA content of a single parasite in a 50 uL aliquot of whole blood. The assay will therefore report quantitative results to a parasite density of 20 estimated parasites per mL of whole blood and will denote low positive results for samples with 10-19 estimated parasites per mL to allow for single parasites with a less-than-average 18S rRNA load. All other samples with lower or absent parasite densities will be reported as '<20 parasites per mL'. This approach is consistent with reporting for the first- and second-generation assays. For this assay, the LoD and LoQ are 20 parasites per mL.

Table 5. Analytical sensitivity analyses

Nominal Parasites / mL	Number of Replicates	3rd generation assay		2nd generation assay
		P. *falciparum* # detected (%)	Pan-*Plasmodium* # detected (%)	P. *falciparum* # detected (%)
Clinical Samples diluted in whole blood, individually processed				
250	10	10 (100%)	10 (100%)	10 (100%)
100	10	10 (100%)	10 (100%)	10 (100%)
50	20	19 (95%)	19 (95%)	18 (90%)
50*	20	19 (95%)	19 (95%)	17 (85%)
20	20	19 (95%)	19 (95%)	18 (90%)
20*	20	16 (80%)	14 (70%)	12 (60%)
10	20	14 (70%)	14 (70%)	12 (60%)
10*	20	8 (40%)	8 (40%)	4 (20%)
Pf-*Armored-RNA in whole blood, processed in bulk*				
7**	20	18 (90%)	20 (100%)	ND

*Number and % detection using a cutoff of <10 estimated parasites/mL as "Not Detected". In other rows, positive/negative are based on detection of any copies/mL lysate.

**Based on 1325 copies of Pf-Armored-RNA corresponding to the full-length 18S rRNA; ND, not done

Mixed *Plasmodium* species infections can also occur, and it is possible to detect both *P. falciparum* plus another more abundant non-*P. falciparum* infection with this assay. Since clinical specimens with mixed species were unavailable, we made contrived specimens that mimicked a mixed specimen by diluting a clinical *P. falciparum* specimen to densities of $2x10^2$ (10X LOD) to $2x10^5$ parasites per mL into a clinical sample containing *P. vivax* at a constant density of $2x10^5$ parasites per mL. *P. falciparum* was detected at all concentrations (n=2 per condition), even when present at 1:10,000 compared to high density *P. vivax* parasites (**Figure** 7).

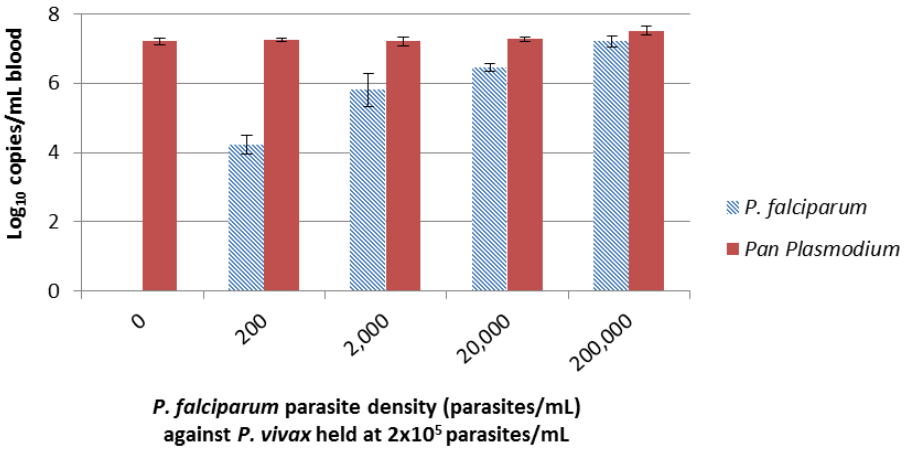


Figure 7. Detection of *P. falciparum* in the setting of mixed *P. vivax* co-infection. Error bars show 95%CI (n=2/level).

h. Analytical specificity

i. Inclusivity

Inclusivity of the *P. falciparum* and Pan *Plasmodium* reagents were judged by testing of pre-extracted DNA from *P. falciparum, P. vivax, P. malariae, P. knowlesi,* and *P. ovale* (8.33E-4 ng/uL) spiked into extracted malaria-negative eluates. Genomic DNA was a kind gift of the CDC. Such measurements could not be performed using clinical whole blood RNA samples because *in vitro* culture of non-*P. falciparum* species is not feasible and clinical specimens are seldom available locally for any species other than *P. falciparum* and *P. vivax*. The *P. falciparum* RT-PCR reagents *only* detected the *P. falciparum* DNA, while the pan-*Plasmodium* target was detected in all five of the known human-infecting *Plasmodium* species. DNA from *P. brasilianum* was unavailable.

Clinical specimens with intact malaria RNA were available for *P. falciparum* and *P. vivax*. In all such samples (*P. falciparum* n=12; *P. vivax* n=2), the *P. falciparum* channel was positive only for samples diagnosed as *P. falciparum* by TBS whereas the Pan-*Plasmodium* channel was positive in all *P. falciparum* and *P. vivax* samples positive by TBS. All TBS diagnoses for these clinical samples were made by the UWMC and HMC Clinical Microbiology laboratories, which are both enrolled in malaria TBS EQA through CAP. To extend inclusivity studies beyond human-infected *Plasmodium*, samples were also tested for *P. yoelii* 17XNL and *P. berghei* ANKA, two *Plasmodium* rodent strains. For these strains, the pan-*Plasmodium* qRT-PCR was positive and the *P. falciparum* qRT-PCR was negative.

ii. Exclusivity

The third-generation assay was tests against whole blood containing CMV (Cytomegalovirus, 1x10 IU/mL), EBV (Epstein-Barr Virus, $1x10^5$ IU/mL), HIV-1 (average viral load 4.76 $\log_{10}$ RNA copies/mL), HIV-2 (average viral load 3.24 $\log_{10}$ RNA copies/mL), *Trypanosoma brucei rhodesiense* ($1x10^6$ parasites per mL*), T.b. gambiense* ($1x10^6$ parasites per mL*), T. b. brucei* ($1x10^6$ parasites per mL*), T. cruzi* ($1x10^6$ parasites per mL) and *Babesia microti* (>5% parasitemia). With the exception of *B. microti*, none of the exclusivity samples were detected by the third generation assay.

Despite a number of nucleotide mismatches, the related Apicomplexan parasite *Babesia microti* can be detected with the third generation Pan-*Plasmodium* test, albeit with poor detection compared to Plasmodia. *Babesia* was undetectable by *P. falciparum* qRT-PCR. Despite the nearest neighbor cross-reactivity of the third-generation assay, the C_T for the *Babesia microti* sample occurred approximately 10 cycles later than when a *Babesia*-specific RT-PCR is performed suggesting that the *Plasmodium*-specific reagents bind only weakly to the *Babesia* sequences. Nonetheless, *Babesia* is listed as one potential source of false-positives due to cross reactivity. Such specimens could be further investigated by blood smear and/or amplicon sequencing to differentiate between *Babesia* and *Plasmodium*.

In addition to non-*Plasmodium* pathogens, we also tested sexual-stage gametocytes and sporozoites for 18S rRNA production. Cultured *P. falciparum* gametocytes and dissected sporozoites were obtained from CIDR. Both gametocytes and sporozoites were detectable by the *P. falciparum* and Pan *Plasmodium* reagents. These phases of the life cycle will be noted as possible causes of positive results.

iii. Analytical interferences

The third generation assay was tested for analytical interferences due to leukocytosis (range $19.3x10^9$ cells/L to $64.7x10^9$ cells/L, normal range $4\text{-}11x10^9$ cells/L), hemolysis (up to >500 mg hemoglobin/dL, normal range <50 mg/dL), lipemia (up to 299 mg/dL, normal <40 mg/dL), bilirubinemia (up to 19.9 mg/dL, normal <2.5 mg/dL) and heparinized plasma (40 USP heparin/mL). For the pan-Plasmodium channel where quantification is being reported, none of the hemolyzed, lipemic, bilirubinemic or heparin-containing samples gave statistically different results compared to controls ($p>0.05$ by Student's t-tests). Leukocytosis samples were statistically different than controls ($<25x10^9$ WBC/L: $p=0.02$; $>25x10^9$ WBC/L: $p=0.002$) although the magnitude of the absolute difference was relatively modest ($<25x10^9$ WBC/L: 0.12 $\log_{10}$ parasites/mL less than controls; $>25x10^9$ WBC/L: 0.34 $\log_{10}$ parasites/mL less than controls). The shift is likely due to competition for RT-PCR reagents since the concentration of the control TBP mRNA would be higher in high leukocyte-containing samples; in the highest leukocytosis samples (64.7x109 WBC/L), the TBP C_T was 25.9 cycles compared to the usual range of 28-29 cycles. The third-generation biomarker assay SOP contains a disclaimer about samples with very high leukocyte counts.

i. Reportable range

The reportable range of parasite densities by RT-PCR was determined by assaying a wide range of high through low density specimens. High density specimens ($1x10^7$ parasites per mL) were added to serial dilution samples to ascertain the quantitative linearity of the RT-PCR curve. The $\log_{10}$ transformed data appeared to be linear (**Figure 8**) based on linear regression and analysis by runs tests ($p = 0.29$ for pan-Plasmodium and $p = 0.07$ for *P. falciparum*; Prism, GraphPad). A second- or third-degree polynomial line fitting curve did not improve upon the fit (data not shown).

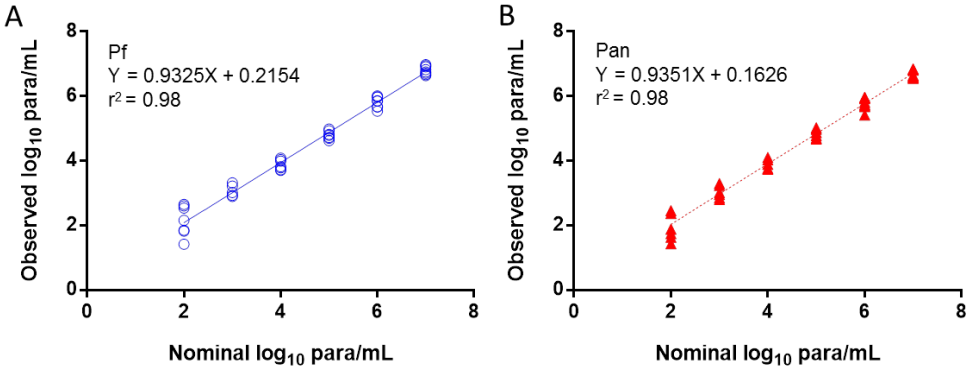


Figure 8. Correlation for reportable range samples tested by (A) *P. falciparum* or (B) pan-*Plasmodium* qRT-PCR channels in multiplex qRT-PCR.

The differences between the nominal and observed values ($\log_{10}$ parasites/mL) were plotted against the nominal value (**Figure 9A-B**). For the pan-*Plasmodium* channel, the bias was +0.133 $\log_{10}$ parasites per

mL (95% Limits of Agreement -0.328 to 0.593) and for *P. falciparum*, the bias was +0.092 log_{10} parasites per mL (95% Limits of Agreement -0.396 to 0.581). A criterion of acceptability within our laboratory is the ability to reliably report 3.5-fold changes in concentration (~+/-0.55 log_{10} units). Nearly all samples were within this range, with a few at the lowest template concentration ($1x10^2$ parasites per mL) slightly exceeding this differences. This dilution series was derived from cultured material in whole blood. Cultured parasites contain multiply-infected erythrocytes that can affect the Poisson distribution and such cells could account for the increased variability at the lowest density tested. Samples in this series showed minimal deviation from the nominal values. Thus, for positive samples from the LoD to $1x10^7$ parasites per mL, the result can be reported directly. For samples with parasite densities $>1x10^7$/mL, extracted eluate can be diluted 1:100 and RT-PCR repeated if indicated. In practice, this should be a rare occurrence and may be deemed to be clinically unnecessary since such parasites should be easily observed by TBS.

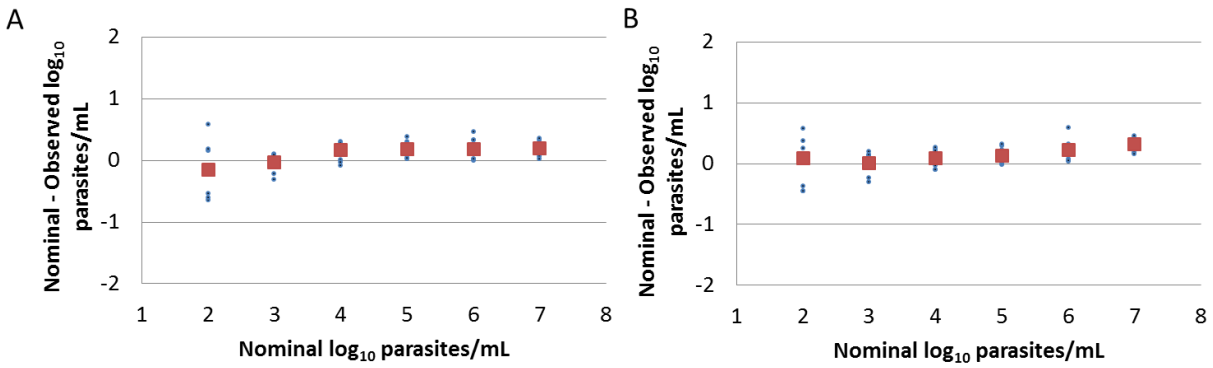


Figure 9. Reportable range of (A) *P. falciparum* and (B) pan-*Plasmodium* qRT-PCR. Per protocol, quantitative results are reported from the pan-*Plasmodium* value.

j. Analyte stability

To determine a range of acceptable pre-processing times, a low parasitemia clinical specimen ($2x10^2$ estimated parasites /mL) that was close to proposed clinical treatment thresholds was aliquoted and stored for 24-96 hours at 4°C or room temperature (24°C) before being processed into lysis buffer and frozen. One-sided unpaired Student's t-tests were performed to determine if a significant difference existed. Storage for 96 hr at 4°C resulted in a statistically significant difference compared to starting concentrations (p=0.02), but other conditions did not show statistically significant differences when log_{10} data was evaluated (**Table 6**). The pan-*Plasmodium* data showed the same pattern when evaluated on a linear scale, whereas the linear scale *P. falciparum*-specific data showed statistically significant differences at 48 and 72 hr (but not 96 hr) of room temperature storage (data not shown). For individual aliquots, all aliquots stored at 4°C and processed in ≤48 hours and all aliquots stored at room temperature and processed in ≤24 hours (n=4/condition) produced results within 0.55 log_{10} parasites/mL of those processed immediately (0 hours) for both *P. falciparum* and Pan *Plasmodium* (**Figure 10A-B).** Several aliquots stored for 72 and 96 hrs had results that deviated from nominal values by >±0.55 log_{10} parasites/mL.

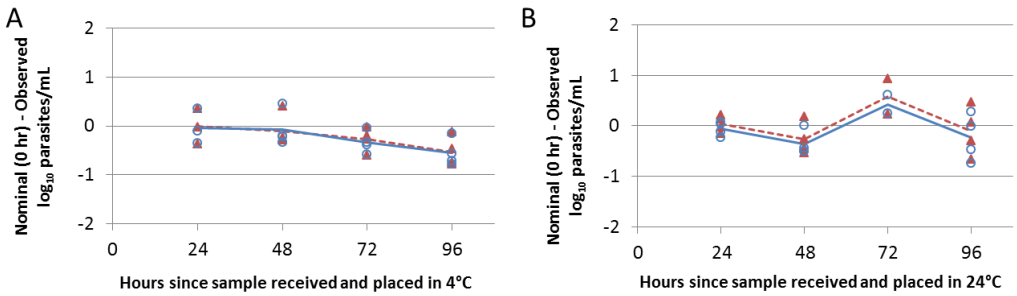


Figure 10. Analyses of biomarker in whole blood stored at different temperatures and for different times. Stability of unprocessed whole blood collected and immediately placed at 4°C (**A**) or 24°C (**B**) for *P. falciparum* (open blue circles, solid line) and Pan *Plasmodium* (filled red triangles, dashed line).

Table 6. One-sided paired *t*-tests of stability samples

Sample type	Temp.	24 hr	48 hr	72 hr	96 hr
Pan-*Plasmodium* 2x10^2 p/mL	4°C	0.485	0.337	0.089	*0.020*
	24°C	0.399	0.123	0.117	0.358
***P. falciparum* 2x10^2 p/mL**	4°C	0.449	0.383	0.068	*0.020*
	24°C	0.364	0.059	0.057	0.211

n=4 samples/condition/timepoint vs. 0 hr samples. Calculated using $\log_{10}$ estimated parasites per mL data. Italicized data were statistically significant at $p<0.05$.

For this reason, the protocol states that samples should be processed into lysis buffer within 48 hours of collection. If storage is required for more than 12 hr, we recommend storage at 4°C. This recommendation and the above data are similar to findings from validation of the first- and second-generation assays. Irreplaceable samples received and/or handled outside of these time limits and/or conditions can be reported with a comment.

To accommodate situations where samples can be processed into lysis buffer within 48 hrs but where immediate testing or freezing is unavailable for lysis buffer-treated samples, we lysed *P. falciparum* infected whole blood (4x10^5 parasites per mL) in NucliSENS lysis buffer and stored the lysis buffer for 24-96 hours prior to freezing at -80°C. Although some loss of RNA integrity was seen over the 96 hours, both *P. falciparum* and Pan *Plasmodium* targets remained within 0.55 $\log_{10}$ copies per mL lysate of the original value (0.16 $\log_{10}$ copies / mL lysate average loss for *P. falciparum* RT-PCR and 0.22 $\log_{10}$ copies / mL lysate loss for Pan *Plasmodium* RT-PCR (n=4/condition). TBP mRNA integrity was also maintained. Thus, lysed samples can be maintained at 4C or room temperature for up to 96 hrs post-lysis buffer treatment. In practice, the lab retains leftover extracted samples at 4°C until the RT-PCR run is completed and reviewed in the event that a RT-PCR run must be repeated from extracted materials.

mRNAs are known to degrade rapidly with sample freeze-thaw. Based on this information, frozen whole blood was not approved as an acceptable sample in first- and second- generation assays. To better assess this for the third-generation assay, whole blood clinical samples were frozen and thawed at -70°C once or up to five times prior to processing. Samples frozen and thawed one time with immediate processing into lysis buffer showed no difference in $\log_{10}$ recovery for *P. falciparum*, pan-*Plasmodium* or TBP mRNA (n=9). However, after five freeze-thaw rounds, both 18S rRNA and TBP mRNA were compromised. Therefore, in consultation with the laboratory director, the laboratory *may* accept archival frozen whole blood samples provided that the samples were promptly frozen at ≤-70°C and that they were temperature monitored to ensure that freezing was consistent prior to thawing and testing. All clients outside our locale are requested to collect samples into NucliSENS lysis buffer for *optimal* sample preservation.

k. Carryover

Carryover was tested by running high target samples and negative samples interspersed in the same run. The data from the negative samples and the "known" high positive samples were evaluated to see if there was any carryover between samples that could affect an unknown result. All results for high positive samples (n=207) were found to be positive, and all results for known negative samples (n=105) were negative in this validation. In this dataset, 44 of these negative samples followed a high positive sample that exceeds the range of parasite densities observed in CHMI trials. In practice, the negative control is included to monitor for accumulated contamination throughout the assay.

l. Accuracy: comparison of second and third generation assay

The second-generation *P. falciparum* assay has been used in a number of clinical trials [1, 2, 14] with strong correlation to values determined by blood smears and to results from a number of outside laboratories [12]. The present third-generation assay is a successor to the second-generation assay, designed to increase its applicability to clinical trials occurring in malaria endemic fields. To establish compatibility between the well-characterized second- generation assay with the third, we tested archival samples that were previously tested in the published malaria EQA exercise [12]. These samples were at the following concentrations: $3x10^5$, $6x10^3$, $6x10^2$, $6x10^1$ and 6 parasites per mL of whole blood. Observed $\log_{10}$ 18S rRNA copies/mL of the second generation assay compared closely with those of the third-generation assay (**Figure 11**).

Rate of detection by the third-generation assay was compared to the reported detection rate by the second-generation assay (**Table 7**). Both the *P. falciparum* and Pan *Plasmodium* targets of the third-generation assay closely matched the percent of samples detected as reported by the second-generation assay, suggesting that assay sensitivity of the third-generation assay closely resembles that of the second-generation, if not slightly improved based on improved detection rates at low densities. This assay is therefore qualitatively and quantitatively aligned with the second-generation assay.

Table 7. Comparison of second- and third-generation assay detection of low parasite densities

Nominal Para/mL	Gen. 3 assay			Gen. 2 assay	
	# of replicates	*P. falciparum* # detected (%)	Pan *Plasmodium* # detected (%)	# of replicates	*P. falciparum* # detected (%)
600	18	18 (100%)	18 (100%)	20	20 (100%)
60	21	17 (81%)	17 (81%)	15	11 (73%)
6	21	3 (14%)	3 (14%)	15	1 (6%)

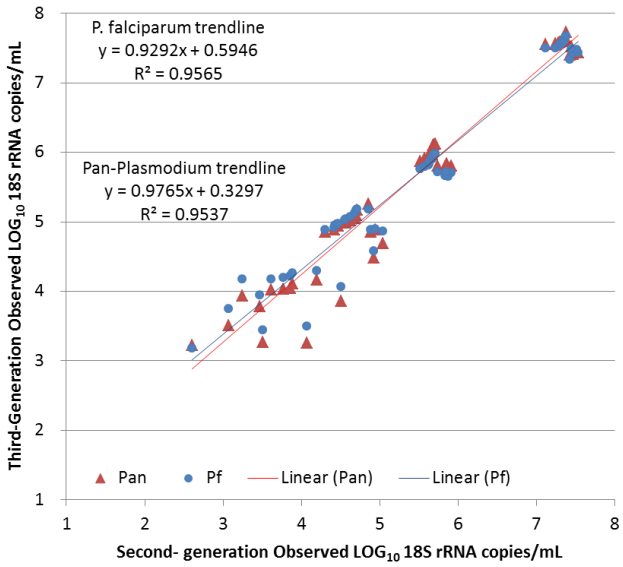


Figure 11. Comparison of third- and second-generation RT-PCR results. *P. falciparum* qRT-PCR (blue) and Pan-*Plasmodium* (red).

m. Reporting scheme for *P. falciparum* and pan-*Plasmodium* results

The pan-*Plasmodium* qRT-PCR demonstrated earlier and more linear RT-PCR characteristics compared to the *P. falciparum* qRT-PCR. To simplify clinical trial reporting, data for CHMI studies with *P. falciparum* will be reported as 'estimated parasites per mL' based on the linear regression of the pan-*Plasmodium* 18S rRNA copy number. The pan-Plasmodium C_T will also be reported. *P. falciparum* qRT-PCR will be reported as a qualitative result and the *P. falciparum* C_T will not be reported. For epidemiology or other studies, custom reports can be generated to meet client-specific needs. Both the pan-*Plasmodium* and *P. falciparum*-specific RT-PCRs will be maintained as qRT-PCR assays using Levey-Jennings plots and other QC steps in the event that quantification from both pan and *P. falciparum* RT-PCR channels is needed for samples with possible mixed infections.

7. COMPARISION TO FIRST AND SECOND GENERATION ASSAYS

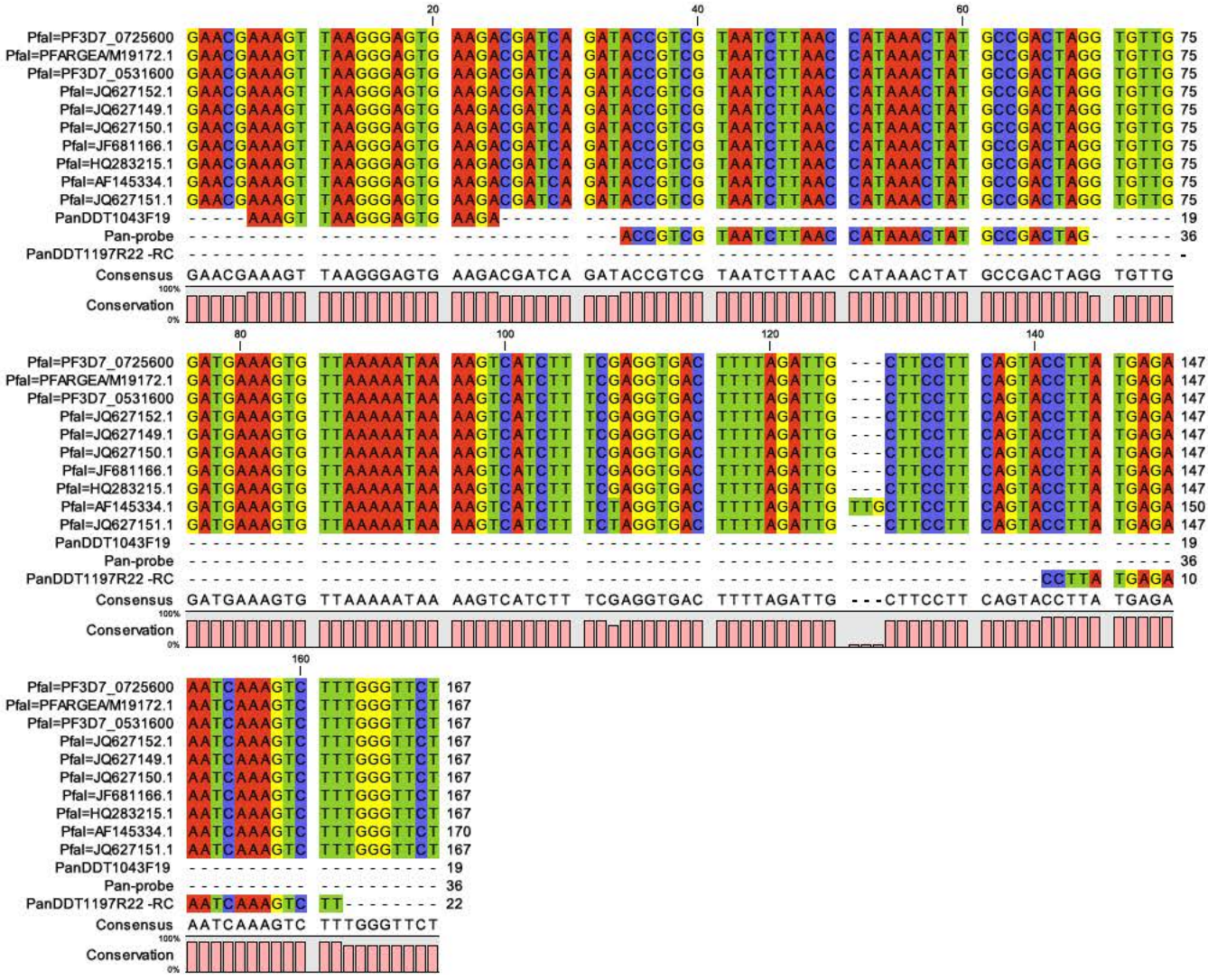
Characteristic	1st generation assay	2nd-generation assay	3rd generation assay
Extraction platform	BioMerieux easyMAG	Abbott m2000sp	Abbott m2000sp
RT-PCR platform	Roche LightCycler 2.0	Abbott m2000rt	Abbott m2000rt
RT-PCR kit	Qiagen Multiplex	AgPath	Bioline
Primers	PL1473F/PL1679R18	PL1473F/PL1679R18	PfDDT1451F21/ PfDDT1562R21 PanDDT1043F19/ PanDDT1197R22
Probe	Dual-hybridization probes	TaqMan probe	TaqMan probes
Internal control	Competitive	Non-competitive exogenous, in-house *in vitro* transcript RNA	Non-competitive Endogenous, Human House-keeping gene (TBP)
Analytical sensitivity	40 parasites per mL (report to 20)	40 parasites per mL (report to 20/mL)	20 parasites per mL (report to 20/mL)
Species specificity	*P. falciparum**	*P. falciparum* only	*P. falciparum &* pan *Plasmodium*
Precision – within-run **– between run**	0.60 / 1.11 / 1.79% (hi/med/low) 1.63 / 1.88 / 4.05% (hi/med/low)	0.81 / 1.78 / 3.52% (hi/med/low) 2.25 / 3.24 / 6.30% (hi/med/low)	*P.fal:* 1.33/ 0.46/ 3.01% (hi/med/low) 2.40/ 2.36/ 5.66% (hi/med/low) *Pan:* 0.91/ 0.82/ 3.00% (hi/med/low) 2.18/ 3.21/ 6.43 (hi/med/low)
Carryover	None detected	None detected	None detected
Clinical samples per run	22	92	92

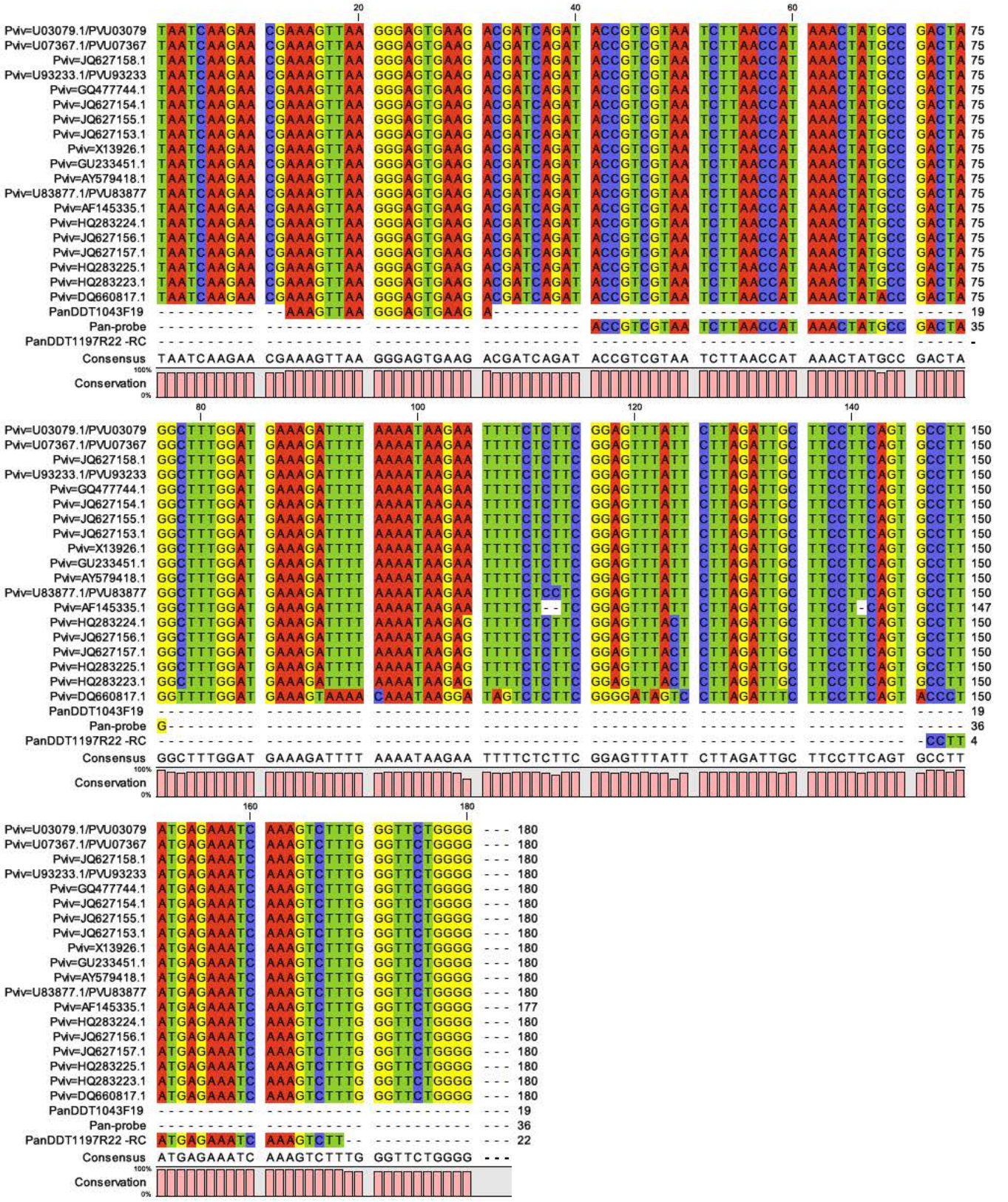
8. SUMMARY

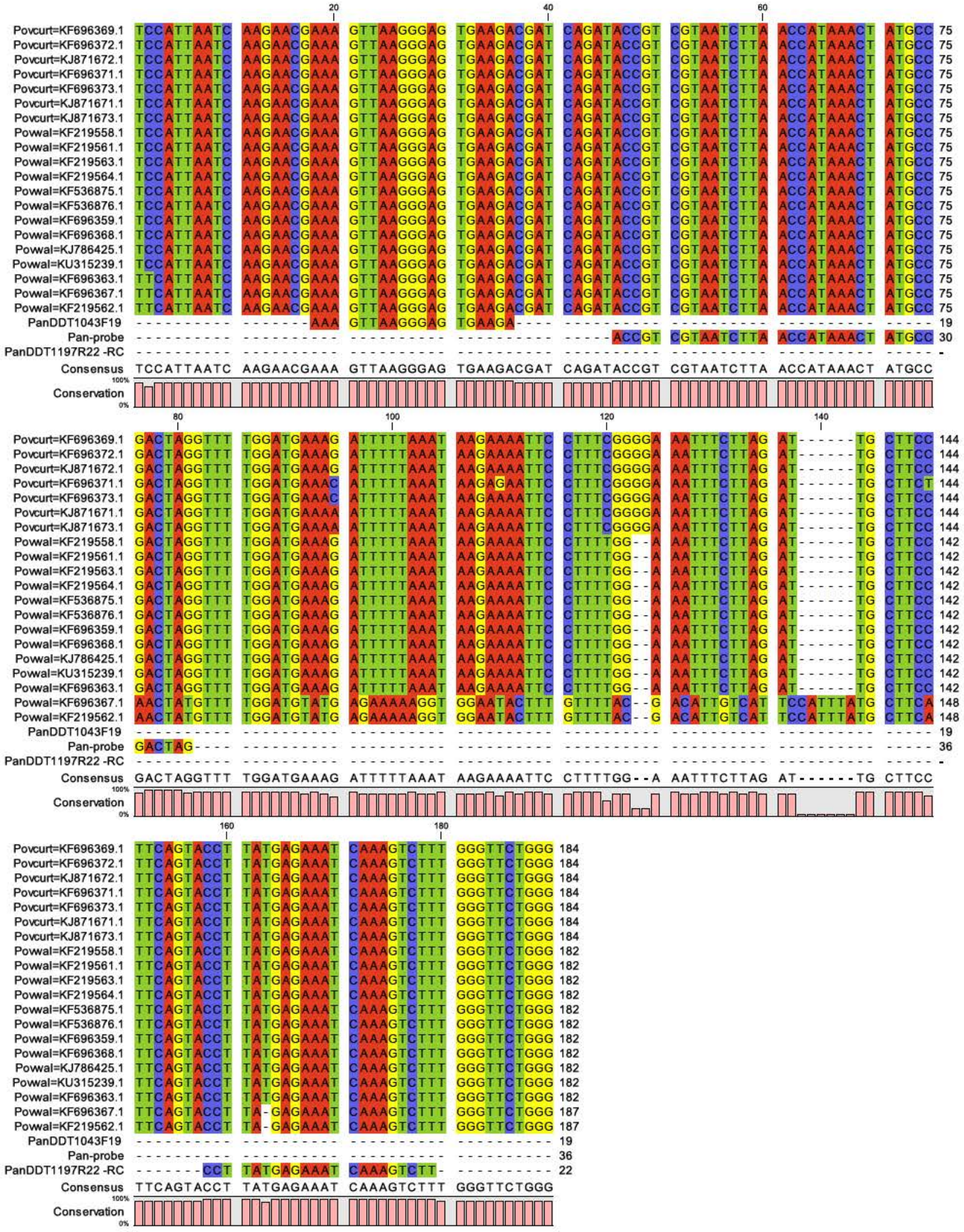
The validation data demonstrates that the third-generation TaqMan probe-based assay on the Abbott m2000 platform is highly sensitive and specific across a wide range of parasite densities. The assay fulfilled all of the criteria set forth in the validation plan with respect to accuracy, correlation, precision, analytical sensitivity, analytical specificity and carryover. The data also determined a parasite conversion factor of 7400 copies of the 18S rRNA per parasite. The protocols and validation are complete for all assay components, and the assay can be used on clinical trial specimens.

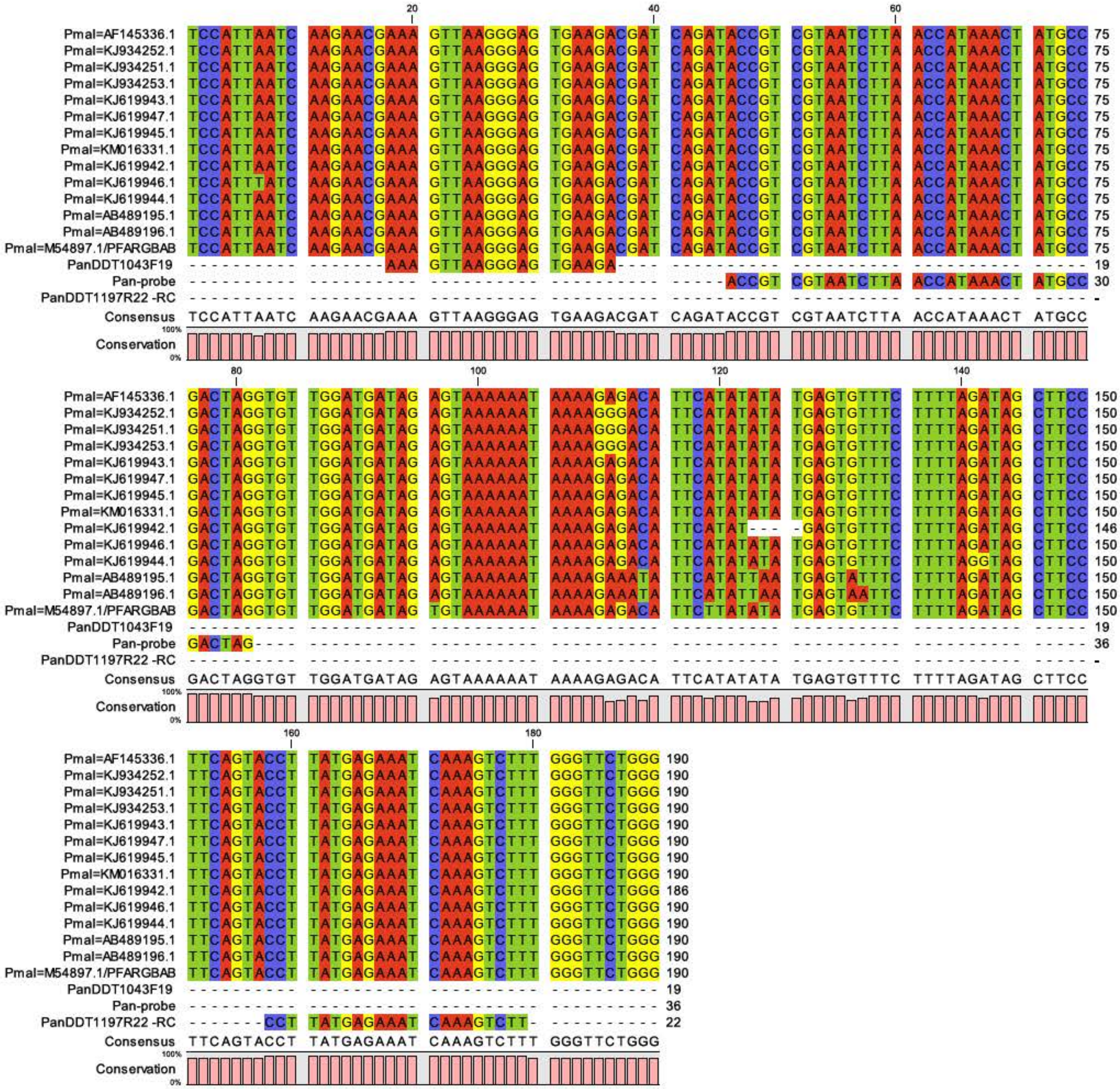
9. REFERENCES

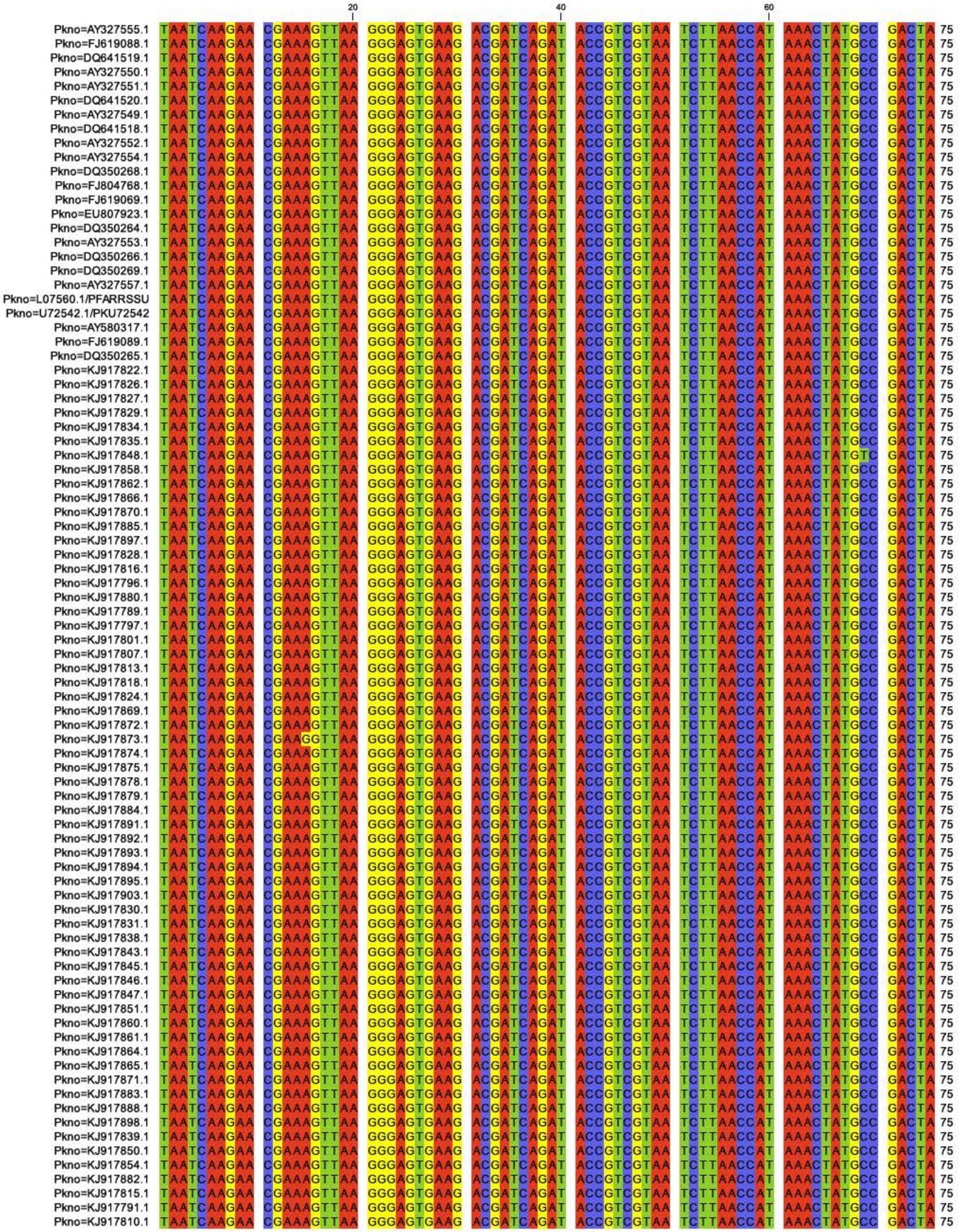
1. Murphy SC, Prentice JL, Williamson K, Wallis CK, Fang FC, Fried M, Pinzon C, Wang R, Talley AK, Kappe SH, et al: Real-time quantitative reverse transcription PCR for monitoring of blood-stage Plasmodium falciparum infections in malaria human challenge trials. *Am J Trop Med Hyg* 2012, 86:383-394.
2. Murphy SC, Daza G, Chang M, Coombs R: Laser cutting eliminates nucleic acid cross-contamination in dried-blood-spot processing. *J Clin Microbiol* 2012, 50:4128-4130.
3. Murphy SC, Shott JP, Parikh S, Etter P, Prescott WR, Stewart VA: Malaria diagnostics in clinical trials. *Am J Trop Med Hyg* 2013, 89:824-839.
4. McCutchan TF, de la Cruz VF, Lal AA, Gunderson JH, Elwood HJ, Sogin ML: Primary sequences of two small subunit ribosomal RNA genes from Plasmodium falciparum. *Mol Biochem Parasitol* 1988, 28:63-68.
5. Gunderson JH, Sogin ML, Wollett G, Hollingdale M, de la Cruz VF, Waters AP, McCutchan TF: Structurally distinct, stage-specific ribosomes occur in Plasmodium. *Science* 1987, 238:933-937.
6. Mangold KA, Manson RU, Koay ES, Stephens L, Regner M, Thomson RB, Jr., Peterson LR, Kaul KL: Real-time PCR for detection and identification of Plasmodium spp. *J Clin Microbiol* 2005, 43:2435-2440.
7. Imwong M, Hanchana S, Malleret B, Renia L, Day NP, Dondorp A, Nosten F, Snounou G, White NJ: High-throughput ultrasensitive molecular techniques for quantifying low-density malaria parasitemias. *J Clin Microbiol* 2014, 52:3303-3309.
8. Rougemont M, Van Saanen M, Sahli R, Hinrikson HP, Bille J, Jaton K: Detection of four Plasmodium species in blood from humans by 18S rRNA gene subunit-based and species-specific real-time PCR assays. *J Clin Microbiol* 2004, 42:5636-5643.
9. Singh B, Bobogare A, Cox-Singh J, Snounou G, Abdullah MS, Rahman HA: A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. *Am J Trop Med Hyg* 1999, 60:687-692.
10. Kamau E, Tolbert LS, Kortepeter L, Pratt M, Nyakoe N, Muringo L, Ogutu B, Waitumbi JN, Ockenhouse CF: Development of a highly sensitive genus-specific quantitative reverse transcriptase real-time PCR assay for detection and quantitation of plasmodium by amplifying RNA and DNA of the 18S rRNA genes. *J Clin Microbiol* 2011, 49:2946-2953.
11. Mahajan B, Zheng H, Pham PT, Sedegah MY, Majam VF, Akolkar N, Rios M, Ankrah I, Madjitey P, Amoah G, et al: Polymerase chain reaction-based tests for pan-species and species-specific detection of human Plasmodium parasites. *Transfusion* 2012, 52:1949-1956.
12. Murphy SC, Hermsen CC, Douglas AD, Edwards N, Petersen I, Fahle G, Adams M, Berry AA, Billman Z, Daza G, et al: External quality assurance of malaria nucleic acid testing for clinical trials and eradication surveillance. *PLoS One* 2014, 9:e97398.
13. Chesher D: Evaluating assay precision. *Clin Biochem Rev* 2008, 29 Suppl 1:S23-26.
14. Hodgson SH, Douglas AD, Edwards NJ, Kimani D, Elias SC, Chang M, Daza G, Seilie AM, Magiri C, Muia A, et al: Increased sample volume and use of quantitative reverse-transcription PCR can improve prediction of liver-to-blood inoculum size in controlled human malaria infection studies. *Malar J* 2015, 14:33.

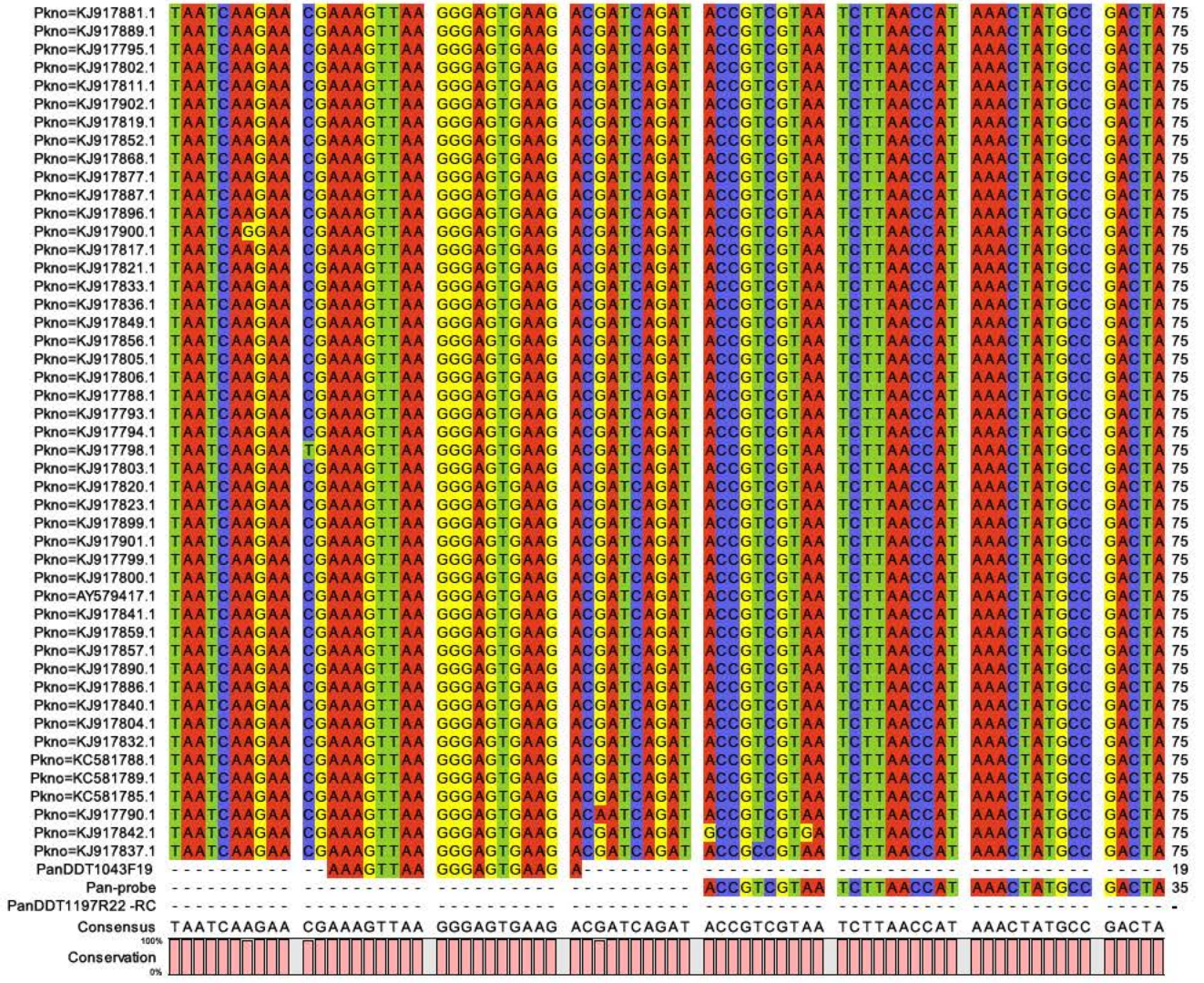

Pfal=PF3D7_0725600 GAACGAAAGT TAAGGGAGTG AAGACGATCA GATACCGTCG TAATCTTAAC CATAAACTAT GCCGACTAGG TGTTG 75
Pfal=PFARGEA/M19172.1 GAACGAAAGT TAAGGGAGTG AAGACGATCA GATACCGTCG TAATCTTAAC CATAAACTAT GCCGACTAGG TGTTG 75
Pfal=PF3D7_0531600 GAACGAAAGT TAAGGGAGTG AAGACGATCA GATACCGTCG TAATCTTAAC CATAAACTAT GCCGACTAGG TGTTG 75
Pfal=JQ627152.1 GAACGAAAGT TAAGGGAGTG AAGACGATCA GATACCGTCG TAATCTTAAC CATAAACTAT GCCGACTAGG TGTTG 75
Pfal=JQ627149.1 GAACGAAAGT TAAGGGAGTG AAGACGATCA GATACCGTCG TAATCTTAAC CATAAACTAT GCCGACTAGG TGTTG 75
Pfal=JQ627150.1 GAACGAAAGT TAAGGGAGTG AAGACGATCA GATACCGTCG TAATCTTAAC CATAAACTAT GCCGACTAGG TGTTG 75
Pfal=JF681166.1 GAACGAAAGT TAAGGGAGTG AAGACGATCA GATACCGTCG TAATCTTAAC CATAAACTAT GCCGACTAGG TGTTG 75
Pfal=HQ283215.1 GAACGAAAGT TAAGGGAGTG AAGACGATCA GATACCGTCG TAATCTTAAC CATAAACTAT GCCGACTAGG TGTTG 75
Pfal=AF145334.1 GAACGAAAGT TAAGGGAGTG AAGACGATCA GATACCGTCG TAATCTTAAC CATAAACTAT GCCGACTAGG TGTTG 75
Pfal=JQ627151.1 GAACGAAAGT TAAGGGAGTG AAGACGATCA GATACCGTCG TAATCTTAAC CATAAACTAT GCCGACTAGG TGTTG 75
PanDDT1043F19 ----- AAAGT TAAGGGAGTG AAGA---------- ---------- ---------- ---------- ---------- ----- 19
Pan-probe ---------- ---------- ---------- ---ACCGTCG TAATCTTAAC CATAAACTAT GCCGACTAG- ----- 36
PanDDT1197R22 -RC ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----- -
Consensus GAACGAAAGT TAAGGGAGTG AAGACGATCA GATACCGTCG TAATCTTAAC CATAAACTAT GCCGACTAGG TGTTG
100%
Conservation
0%
Pfal=PF3D7_0725600 GATGAAAGTG TTAAAAATAA AAGTCATCTT TCGAGGTGAC TTTTAGATTG ---CTTCCTT CAGTACCTTA TGAGA 147
Pfal=PFARGEA/M19172.1 GATGAAAGTG TTAAAAATAA AAGTCATCTT TCGAGGTGAC TTTTAGATTG ---CTTCCTT CAGTACCTTA TGAGA 147
Pfal=PF3D7_0531600 GATGAAAGTG TTAAAAATAA AAGTCATCTT TCGAGGTGAC TTTTAGATTG ---CTTCCTT CAGTACCTTA TGAGA 147
Pfal=JQ627152.1 GATGAAAGTG TTAAAAATAA AAGTCATCTT TCGAGGTGAC TTTTAGATTG ---CTTCCTT CAGTACCTTA TGAGA 147
Pfal=JQ627149.1 GATGAAAGTG TTAAAAATAA AAGTCATCTT TCGAGGTGAC TTTTAGATTG ---CTTCCTT CAGTACCTTA TGAGA 147
Pfal=JQ627150.1 GATGAAAGTG TTAAAAATAA AAGTCATCTT TCGAGGTGAC TTTTAGATTG ---CTTCCTT CAGTACCTTA TGAGA 147
Pfal=JF681166.1 GATGAAAGTG TTAAAAATAA AAGTCATCTT TCGAGGTGAC TTTTAGATTG ---CTTCCTT CAGTACCTTA TGAGA 147
Pfal=HQ283215.1 GATGAAAGTG TTAAAAATAA AAGTCATCTT TCGAGGTGAC TTTTAGATTG ---CTTCCTT CAGTACCTTA TGAGA 147
Pfal=AF145334.1 GATGAAAGTG TTAAAAATAA AAGTCATCTT TCTAGGTGAC TTTTAGATTG TTGCTTCCTT CAGTACCTTA TGAGA 150
Pfal=JQ627151.1 GATGAAAGTG TTAAAAATAA AAGTCATCTT TCTAGGTGAC TTTTAGATTG ---CTTCCTT CAGTACCTTA TGAGA 147
PanDDT1043F19 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----- 19
Pan-probe ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----- 36
PanDDT1197R22 -RC ---------- ---------- ---------- ---------- ---------- ---------- -----CCTTA TGAGA 10
Consensus GATGAAAGTG TTAAAAATAA AAGTCATCTT TCGAGGTGAC TTTTAGATTG ---CTTCCTT CAGTACCTTA TGAGA
100%
Conservation
0%
Pfal=PF3D7_0725600 AATCAAAGTC TTTGGGTTCT 167
Pfal=PFARGEA/M19172.1 AATCAAAGTC TTTGGGTTCT 167
Pfal=PF3D7_0531600 AATCAAAGTC TTTGGGTTCT 167
Pfal=JQ627152.1 AATCAAAGTC TTTGGGTTCT 167
Pfal=JQ627149.1 AATCAAAGTC TTTGGGTTCT 167
Pfal=JQ627150.1 AATCAAAGTC TTTGGGTTCT 167
Pfal=JF681166.1 AATCAAAGTC TTTGGGTTCT 167
Pfal=HQ283215.1 AATCAAAGTC TTTGGGTTCT 167
Pfal=AF145334.1 AATCAAAGTC TTTGGGTTCT 170
Pfal=JQ627151.1 AATCAAAGTC TTTGGGTTCT 167
PanDDT1043F19 ---------- ---------- 19
Pan-probe ---------- ---------- 36
PanDDT1197R22 -RC AATCAAAGTC TT-------- 22
Consensus AATCAAAGTC TTTGGGTTCT
100%
Conservation
0%

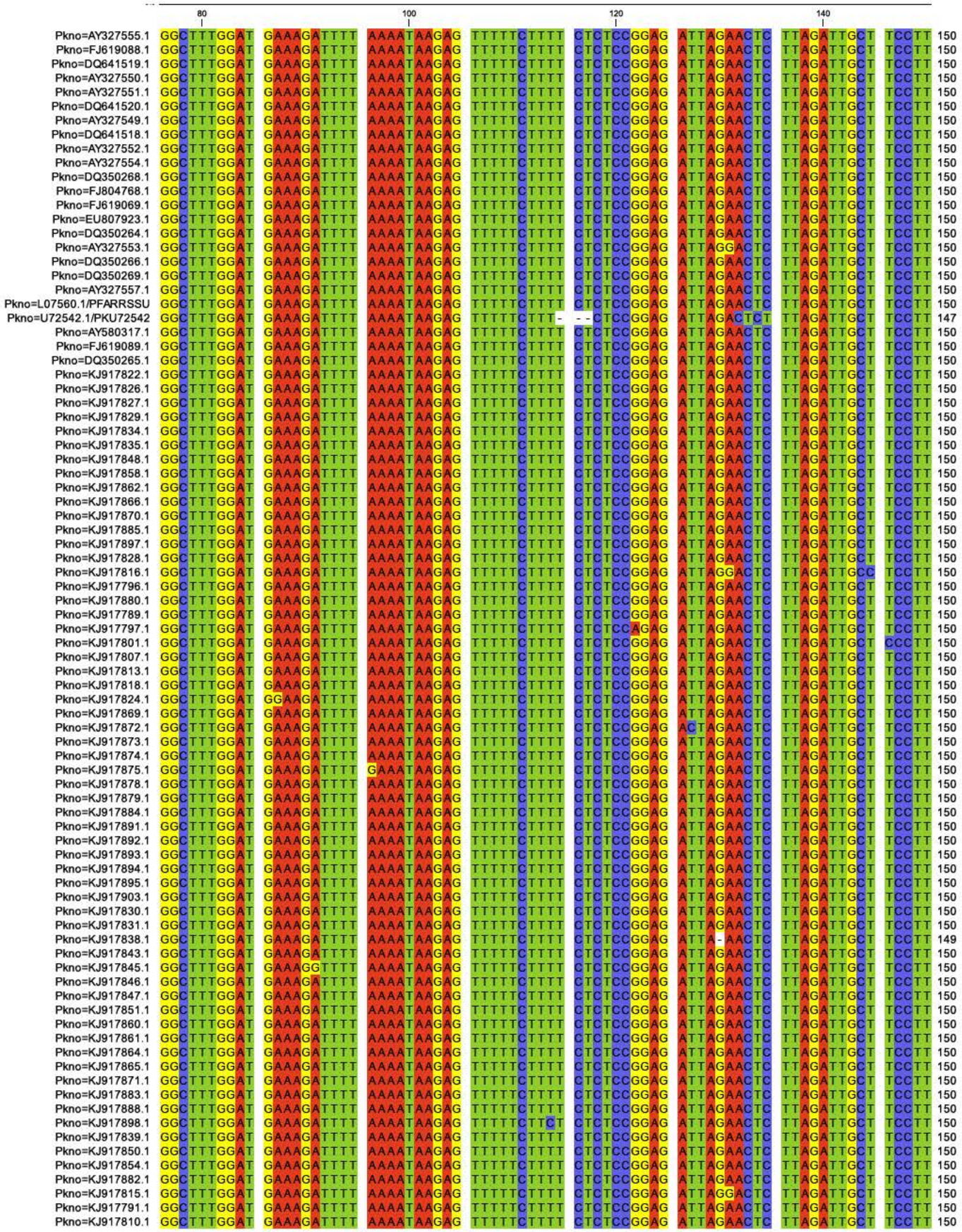

Pviv=U03079.1/PVU03079
Pviv=U07367.1/PVU07367
Pviv=JQ627158.1
Pviv=U93233.1/PVU93233
Pviv=GQ477744.1
Pviv=JQ627154.1
Pviv=JQ627155.1
Pviv=JQ627153.1
Pviv=X13926.1
Pviv=GU233451.1
Pviv=AY579418.1
Pviv=U83877.1/PVU83877
Pviv=AF145335.1
Pviv=HQ283224.1
Pviv=JQ627156.1
Pviv=JQ627157.1
Pviv=HQ283225.1
Pviv=HQ283223.1
Pviv=DQ660817.1
PanDDT1043F19
Pan-probe
PanDDT1197R22 -RC
Consensus
Conservation
100%
0%

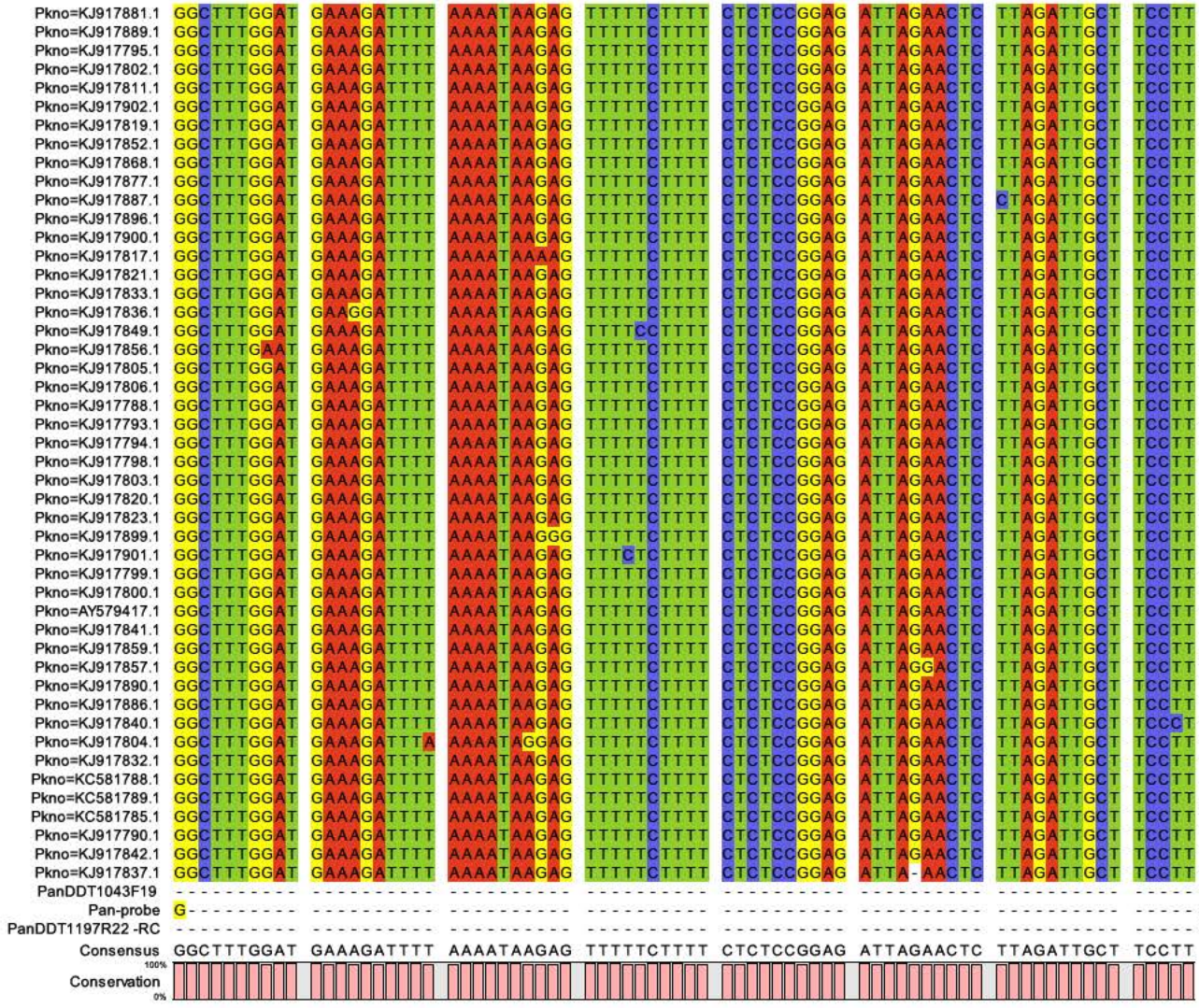

Povcurt=KF696369.1
Povcurt=KF696372.1
Povcurt=KJ871672.1
Povcurt=KF696371.1
Povcurt=KF696373.1
Povcurt=KJ871671.1
Povcurt=KJ871673.1
Powal=KF219558.1
Powal=KF219561.1
Powal=KF219563.1
Powal=KF219564.1
Powal=KF536875.1
Powal=KF536876.1
Powal=KF696359.1
Powal=KF696368.1
Powal=KJ786425.1
Powal=KU315239.1
Powal=KF696363.1
Powal=KF696367.1
Powal=KF219562.1
PanDDT1043F19
Pan-probe
PanDDT1197R22 -RC
Consensus
Conservation
100%
0%

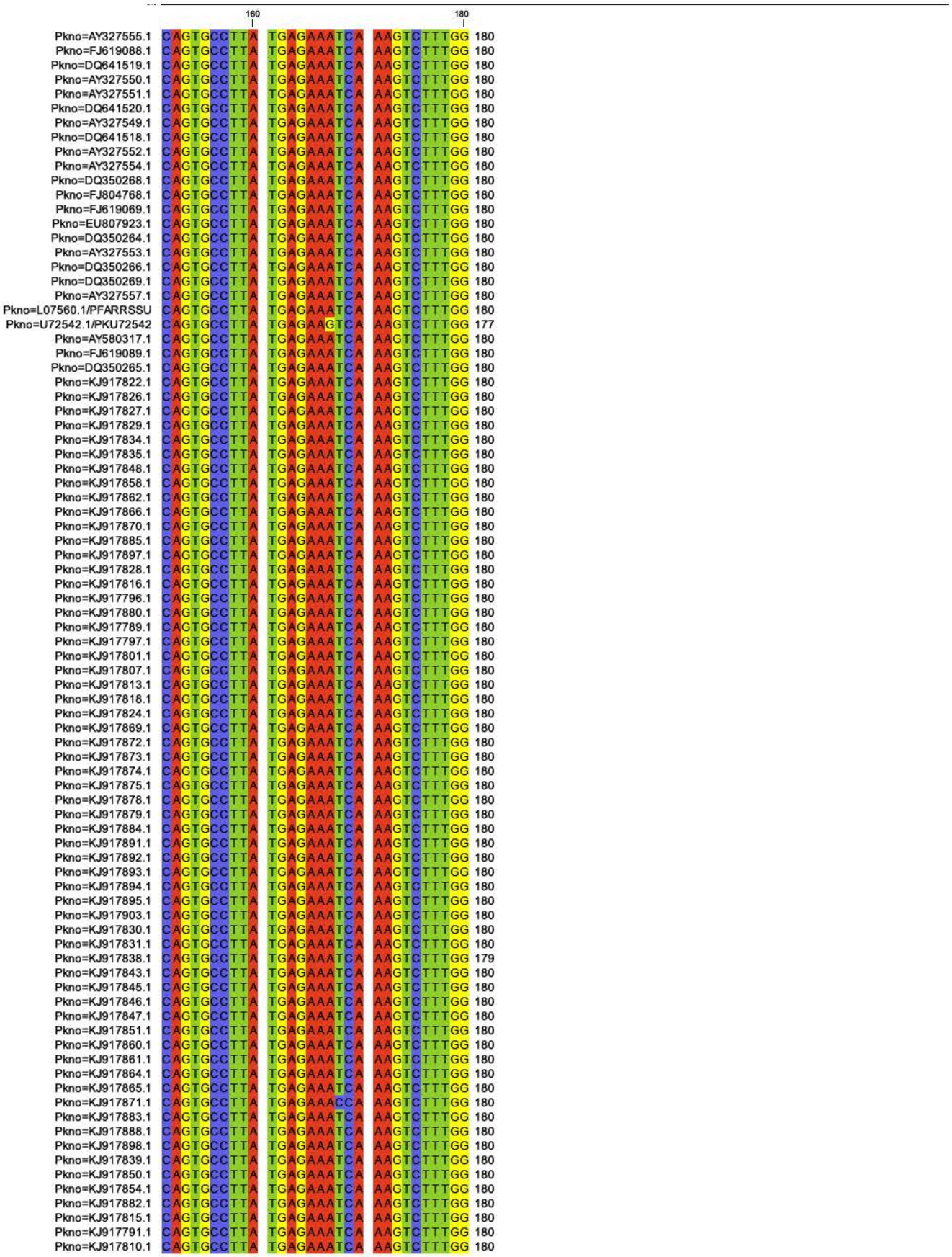

20
40
60
Pmal=AF145336.1 TCCATTAATC AAGAACGAAA GTTAAGGGAG TGAAGACGAT CAGATACCGT CGTAATCTTA ACCATAAACT ATGCC 75
Pmal=KJ934252.1 TCCATTAATC AAGAACGAAA GTTAAGGGAG TGAAGACGAT CAGATACCGT CGTAATCTTA ACCATAAACT ATGCC 75
Pmal=KJ934251.1 TCCATTAATC AAGAACGAAA GTTAAGGGAG TGAAGACGAT CAGATACCGT CGTAATCTTA ACCATAAACT ATGCC 75
Pmal=KJ934253.1 TCCATTAATC AAGAACGAAA GTTAAGGGAG TGAAGACGAT CAGATACCGT CGTAATCTTA ACCATAAACT ATGCC 75
Pmal=KJ619943.1 TCCATTAATC AAGAACGAAA GTTAAGGGAG TGAAGACGAT CAGATACCGT CGTAATCTTA ACCATAAACT ATGCC 75
Pmal=KJ619947.1 TCCATTAATC AAGAACGAAA GTTAAGGGAG TGAAGACGAT CAGATACCGT CGTAATCTTA ACCATAAACT ATGCC 75
Pmal=KJ619945.1 TCCATTAATC AAGAACGAAA GTTAAGGGAG TGAAGACGAT CAGATACCGT CGTAATCTTA ACCATAAACT ATGCC 75
Pmal=KM016331.1 TCCATTAATC AAGAACGAAA GTTAAGGGAG TGAAGACGAT CAGATACCGT CGTAATCTTA ACCATAAACT ATGCC 75
Pmal=KJ619942.1 TCCATTAATC AAGAACGAAA GTTAAGGGAG TGAAGACGAT CAGATACCGT CGTAATCTTA ACCATAAACT ATGCC 75
Pmal=KJ619946.1 TCCATTTATC AAGAACGAAA GTTAAGGGAG TGAAGACGAT CAGATACCGT CGTAATCTTA ACCATAAACT ATGCC 75
Pmal=KJ619944.1 TCCATTAATC AAGAACGAAA GTTAAGGGAG TGAAGACGAT CAGATACCGT CGTAATCTTA ACCATAAACT ATGCC 75
Pmal=AB489195.1 TCCATTAATC AAGAACGAAA GTTAAGGGAG TGAAGACGAT CAGATACCGT CGTAATCTTA ACCATAAACT ATGCC 75
Pmal=AB489196.1 TCCATTAATC AAGAACGAAA GTTAAGGGAG TGAAGACGAT CAGATACCGT CGTAATCTTA ACCATAAACT ATGCC 75
Pmal=M54897.1/PFARGBAB TCCATTAATC AAGAACGAAA GTTAAGGGAG TGAAGACGAT CAGATACCGT CGTAATCTTA ACCATAAACT ATGCC 75
PanDDT1043F19 ---------- -------AAA GTTAAGGGAG TGAAGA---- ---------- ---------- ---------- ----- 19
Pan-probe ---------- ---------- ---------- ---------- -----ACCGT CGTAATCTTA ACCATAAACT ATGCC 30
PanDDT1197R22 -RC ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----- -
Consensus TCCATTAATC AAGAACGAAA GTTAAGGGAG TGAAGACGAT CAGATACCGT CGTAATCTTA ACCATAAACT ATGCC
100%
Conservation
0%
80
100
120
140
Pmal=AF145336.1 GACTAGGTGT TGGATGATAG AGTAAAAAAT AAAAGAGACA TTCATATATA TGAGTGTTTC TTTTAGATAG CTTCC 150
Pmal=KJ934252.1 GACTAGGTGT TGGATGATAG AGTAAAAAAT AAAAGGGACA TTCATATATA TGAGTGTTTC TTTTAGATAG CTTCC 150
Pmal=KJ934251.1 GACTAGGTGT TGGATGATAG AGTAAAAAAT AAAAGGGACA TTCATATATA TGAGTGTTTC TTTTAGATAG CTTCC 150
Pmal=KJ934253.1 GACTAGGTGT TGGATGATAG AGTAAAAAAT AAAAGGGACA TTCATATATA TGAGTGTTTC TTTTAGATAG CTTCC 150
Pmal=KJ619943.1 GACTAGGTGT TGGATGATAG AGTAAAAAAT AAAAGAGACA TTCATATATA TGAGTGTTTC TTTTAGATAG CTTCC 150
Pmal=KJ619947.1 GACTAGGTGT TGGATGATAG AGTAAAAAAT AAAAGAGACA TTCATATATA TGAGTGTTTC TTTTAGATAG CTTCC 150
Pmal=KJ619945.1 GACTAGGTGT TGGATGATAG AGTAAAAAAT AAAAGAGACA TTCATATATA TGAGTGTTTC TTTTAGATAG CTTCC 150
Pmal=KM016331.1 GACTAGGTGT TGGATGATAG AGTAAAAAAT AAAAGAGACA TTCATATATA TGAGTGTTTC TTTTAGATAG CTTCC 150
Pmal=KJ619942.1 GACTAGGTGT TGGATGATAG AGTAAAAAAT AAAAGAGACA TTCATAT--- -GAGTGTTTC TTTTAGATAG CTTCC 146
Pmal=KJ619946.1 GACTAGGTGT TGGATGATAG AGTAAAAAAT AAAAGAGACA TTCATATATA TGAGTGTTTC TTTTAGATAG CTTCC 150
Pmal=KJ619944.1 GACTAGGTGT TGGATGATAG AGTAAAAAAT AAAAGAGACA TTCATATATA TGAGTGTTTC TTTTAGGTAG CTTCC 150
Pmal=AB489195.1 GACTAGGTGT TGGATGATAG AGTAAAAAAT AAAAGAAATA TTCATATTAA TGAGTATTTC TTTTAGATAG CTTCC 150
Pmal=AB489196.1 GACTAGGTGT TGGATGATAG AGTAAAAAAT AAAAGAAATA TTCATATTAA TGAGTAATTC TTTTAGATAG CTTCC 150
Pmal=M54897.1/PFARGBAB GACTAGGTGT TGGATGATAG TGTAAAAAAT AAAAGAGACA TTCTTATATA TGAGTGTTTC TTTTAGATAG CTTCC 150
PanDDT1043F19 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----- 19
Pan-probe GACTAG---- ---------- ---------- ---------- ---------- ---------- ---------- ----- 36
PanDDT1197R22 -RC ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----- -
Consensus GACTAGGTGT TGGATGATAG AGTAAAAAAT AAAAGAGACA TTCATATATA TGAGTGTTTC TTTTAGATAG CTTCC
100%
Conservation
0%
160
180
Pmal=AF145336.1 TTCAGTACCT TATGAGAAAT CAAAGTCTTT GGGTTCTGGG 190
Pmal=KJ934252.1 TTCAGTACCT TATGAGAAAT CAAAGTCTTT GGGTTCTGGG 190
Pmal=KJ934251.1 TTCAGTACCT TATGAGAAAT CAAAGTCTTT GGGTTCTGGG 190
Pmal=KJ934253.1 TTCAGTACCT TATGAGAAAT CAAAGTCTTT GGGTTCTGGG 190
Pmal=KJ619943.1 TTCAGTACCT TATGAGAAAT CAAAGTCTTT GGGTTCTGGG 190
Pmal=KJ619947.1 TTCAGTACCT TATGAGAAAT CAAAGTCTTT GGGTTCTGGG 190
Pmal=KJ619945.1 TTCAGTACCT TATGAGAAAT CAAAGTCTTT GGGTTCTGGG 190
Pmal=KM016331.1 TTCAGTACCT TATGAGAAAT CAAAGTCTTT GGGTTCTGGG 190
Pmal=KJ619942.1 TTCAGTACCT TATGAGAAAT CAAAGTCTTT GGGTTCTGGG 186
Pmal=KJ619946.1 TTCAGTACCT TATGAGAAAT CAAAGTCTTT GGGTTCTGGG 190
Pmal=KJ619944.1 TTCAGTACCT TATGAGAAAT CAAAGTCTTT GGGTTCTGGG 190
Pmal=AB489195.1 TTCAGTACCT TATGAGAAAT CAAAGTCTTT GGGTTCTGGG 190
Pmal=AB489196.1 TTCAGTACCT TATGAGAAAT CAAAGTCTTT GGGTTCTGGG 190
Pmal=M54897.1/PFARGBAB TTCAGTACCT TATGAGAAAT CAAAGTCTTT GGGTTCTGGG 190
PanDDT1043F19 ---------- ---------- ---------- ---------- 19
Pan-probe ---------- ---------- ---------- ---------- 36
PanDDT1197R22 -RC -------CCT TATGAGAAAT CAAAGTCTT- ---------- 22
Consensus TTCAGTACCT TATGAGAAAT CAAAGTCTTT GGGTTCTGGG
100%
Conservation
0%

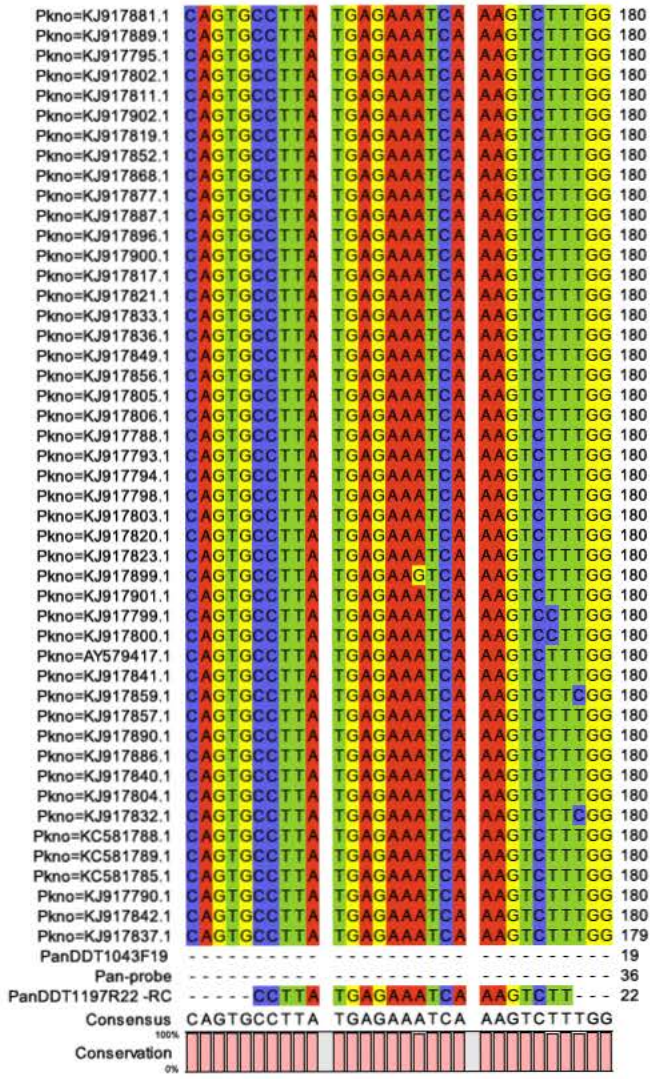

20
40
60
Pkno=AY327555.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=FJ619088.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=DQ641519.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=AY327550.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=AY327551.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=DQ641520.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=AY327549.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=DQ641518.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=AY327552.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=AY327554.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=DQ350268.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=FJ804768.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=FJ619069.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=EU807923.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=DQ350264.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=AY327553.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=DQ350266.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=DQ350269.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=AY327557.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=L07560.1/PFARRSSU TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=U72542.1/PKU72542 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=AY580317.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=FJ619089.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=DQ350265.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917822.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917826.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917827.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917829.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917834.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917835.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917848.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGTC GACTA 75
Pkno=KJ917858.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917862.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917866.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917870.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917885.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917897.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917828.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917816.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917796.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917880.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917789.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917797.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917801.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917807.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917813.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917818.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917824.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917869.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917872.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917873.1 TAATCAAGAA CGAAGGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917874.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917875.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917878.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917879.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917884.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917891.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917892.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917893.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917894.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917895.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917903.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917830.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917831.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917838.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917843.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917845.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917846.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917847.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917851.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917860.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917861.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917864.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917865.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917871.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917883.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917888.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917898.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917839.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917850.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917854.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917882.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917815.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917791.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917810.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75

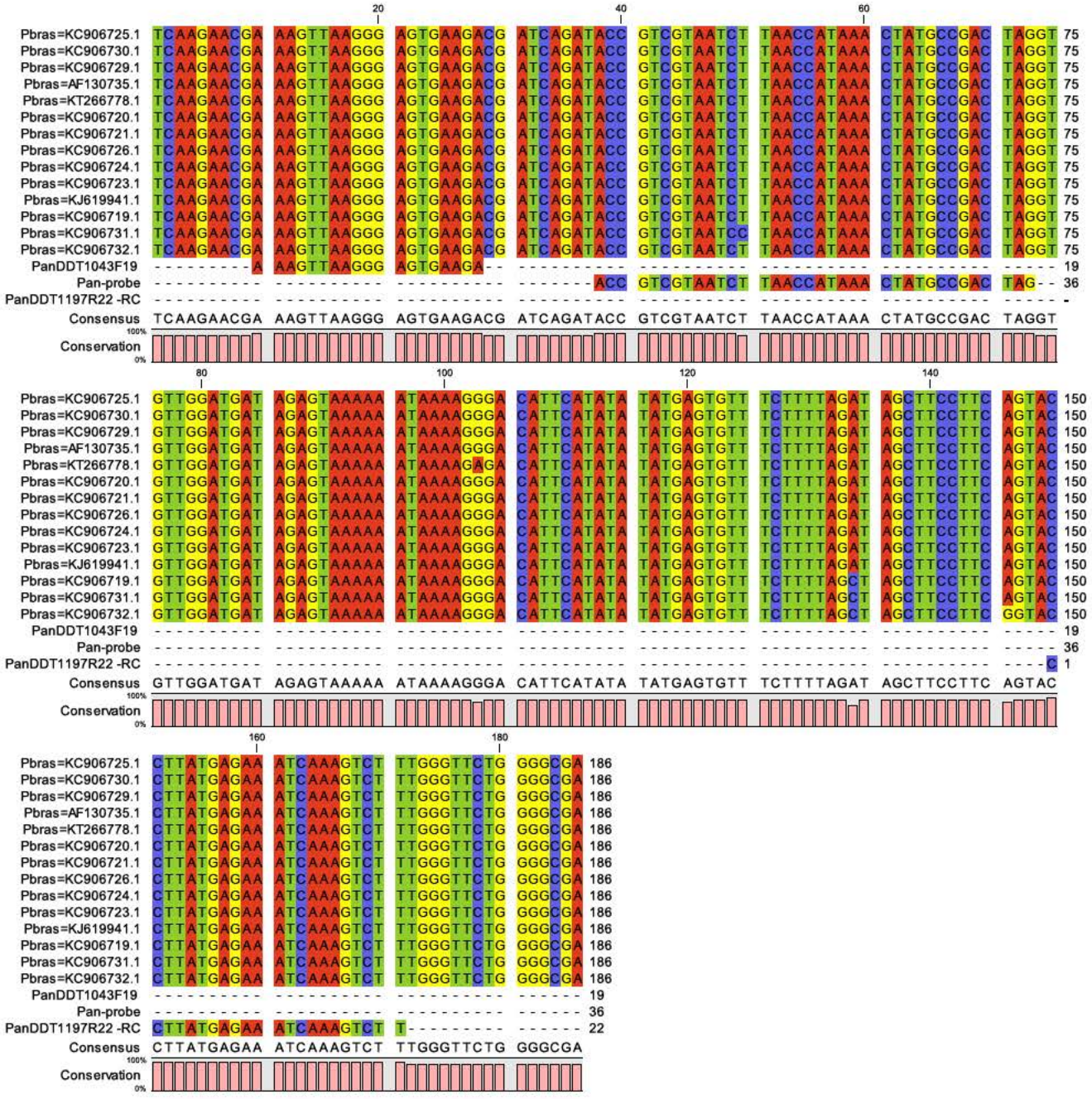

Pkno=KJ917881.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917889.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917795.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917802.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917811.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917902.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917819.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917852.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917868.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917877.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917887.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917896.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917900.1 TAATCAGGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917817.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917821.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917833.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917836.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917849.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917856.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917805.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917806.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917788.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917793.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917794.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917798.1 TAATCAAGAA TGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917803.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917820.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917823.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917899.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917901.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917799.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917800.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=AY579417.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917841.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917859.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917857.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917890.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917886.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917840.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917804.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917832.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KC581788.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KC581789.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KC581785.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917790.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACAATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917842.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT GCCGTCGTGA TCTTAACCAT AAACTATGCC GACTA 75
Pkno=KJ917837.1 TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGCCGTAA TCTTAACCAT AAACTATGCC GACTA 75
PanDDT1043F19 ---------- --AAAGTTAA GGGAGTGAAG A--------- ---------- ---------- ---------- ----- 19
Pan-probe ---------- ---------- ---------- ---------- ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA 35
PanDDT1197R22 -RC ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----- -
Consensus TAATCAAGAA CGAAAGTTAA GGGAGTGAAG ACGATCAGAT ACCGTCGTAA TCTTAACCAT AAACTATGCC GACTA
100%
Conservation
0%

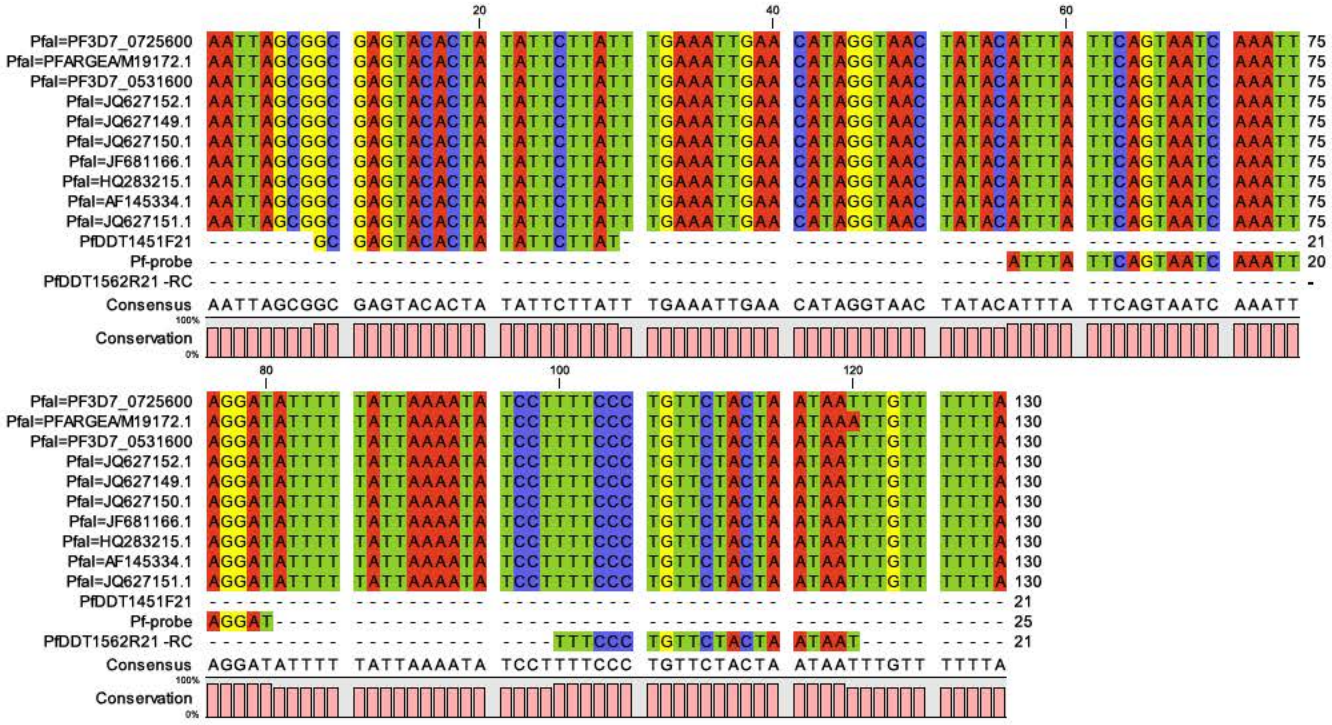

80
100
120
140
Pkno=AY327555.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=FJ619088.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=DQ641519.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=AY327550.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=AY327551.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=DQ641520.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=AY327549.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=DQ641518.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=AY327552.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=AY327554.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=DQ350268.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=FJ804768.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=FJ619069.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=EU807923.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=DQ350264.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=AY327553.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGGACTC TTAGATTGCT TCCTT 150
Pkno=DQ350266.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=DQ350269.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=AY327557.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=L07560.1/PFARRSSU GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=U72542.1/PKU72542 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTT- ---CTCCGGAG ATTAGACTCT TTAGATTGCT TCCTT 147
Pkno=AY580317.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=FJ619089.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=DQ350265.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917822.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917826.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917827.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917829.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917834.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917835.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917848.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917858.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917862.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917866.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917870.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917885.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917897.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917828.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917816.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGGACTC TTAGATTGCC TCCTT 150
Pkno=KJ917796.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917880.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917789.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917797.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCAGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917801.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT CCCTT 150
Pkno=KJ917807.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917813.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917818.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917824.1 GGCTTTGGAT GGAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917869.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917872.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ACTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917873.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917874.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917875.1 GGCTTTGGAT GAAAGATTTT GAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917878.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917879.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917884.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917891.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917892.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917893.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917894.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917895.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917903.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917830.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917831.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917838.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTA-AACTC TTAGATTGCT TCCTT 149
Pkno=KJ917843.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917845.1 GGCTTTGGAT GAAAGGTTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917846.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917847.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917851.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917860.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917861.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917864.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917865.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917871.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917883.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917888.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917898.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTCT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917839.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917850.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917854.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917882.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917815.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGGACTC TTAGATTGCT TCCTT 150
Pkno=KJ917791.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917810.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150


Pkno=KJ917881.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917889.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917795.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917802.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917811.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917902.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917819.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917852.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917868.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917877.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917887.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC CTAGATTGCT TCCTT 150
Pkno=KJ917896.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917900.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917817.1 GGCTTTGGAT GAAAGATTTT AAAATAAAAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917821.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917833.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917836.1 GGCTTTGGAT GAAGGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917849.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTCCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917856.1 GGCTTTGAAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917805.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917806.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917788.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917793.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917794.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917798.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917803.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917820.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917823.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917899.1 GGCTTTGGAT GAAAGATTTT AAAATAAGGG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917901.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTCTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917799.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917800.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=AY579417.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917841.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917859.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917857.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGGACTC TTAGATTGCT TCCTT 150
Pkno=KJ917890.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917886.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917840.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCCT 150
Pkno=KJ917804.1 GGCTTTGGAT GAAAGATTTA AAAATAGGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917832.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KC581788.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KC581789.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KC581785.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917790.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917842.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT 150
Pkno=KJ917837.1 GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTA-AACTC TTAGATTGCT TCCTT 149
PanDDT1043F19 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----- 19
Pan-probe G--------- ---------- ---------- ---------- ---------- ---------- ---------- ----- 36
PanDDT1197R22 -RC ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----- -
Consensus GGCTTTGGAT GAAAGATTTT AAAATAAGAG TTTTTCTTTT CTCTCCGGAG ATTAGAACTC TTAGATTGCT TCCTT
100%
Conservation
0%


160
180
Pkno=AY327555.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=FJ619088.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=DQ641519.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=AY327550.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=AY327551.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=DQ641520.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=AY327549.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=DQ641518.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=AY327552.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=AY327554.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=DQ350268.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=FJ804768.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=FJ619069.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=EU807923.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=DQ350264.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=AY327553.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=DQ350266.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=DQ350269.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=AY327557.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=L07560.1/PFARRSSU CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=U72542.1/PKU72542 CAGTGCCTTA TGAGAAGTCA AAGTCTTTGG 177
Pkno=AY580317.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=FJ619089.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=DQ350265.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917822.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917826.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917827.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917829.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917834.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917835.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917848.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917858.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917862.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917866.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917870.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917885.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917897.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917828.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917816.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917796.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917880.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917789.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917797.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917801.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917807.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917813.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917818.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917824.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917869.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917872.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917873.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917874.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917875.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917878.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917879.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917884.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917891.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917892.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917893.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917894.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917895.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917903.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917830.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917831.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917838.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 179
Pkno=KJ917843.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917845.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917846.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917847.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917851.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917860.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917861.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917864.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917865.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917871.1 CAGTGCCTTA TGAGAAACCA AAGTCTTTGG 180
Pkno=KJ917883.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917888.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917898.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917839.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917850.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917854.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917882.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917815.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917791.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917810.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180


Pkno=KJ917881.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917889.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917795.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917802.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917811.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917902.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917819.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917852.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917868.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917877.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917887.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917896.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917900.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917817.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917821.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917833.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917836.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917849.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917856.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917805.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917806.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917788.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917793.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917794.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917798.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917803.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917820.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917823.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917899.1 CAGTGCCTTA TGAGAAGTCA AAGTCTTTGG 180
Pkno=KJ917901.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917799.1 CAGTGCCTTA TGAGAAATCA AAGTCCTTGG 180
Pkno=KJ917800.1 CAGTGCCTTA TGAGAAATCA AAGTCCTTGG 180
Pkno=AY579417.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917841.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917859.1 CAGTGCCTTA TGAGAAATCA AAGTCTTCGG 180
Pkno=KJ917857.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917890.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917886.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917840.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917804.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917832.1 CAGTGCCTTA TGAGAAATCA AAGTCTTCGG 180
Pkno=KC581788.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KC581789.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KC581785.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917790.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917842.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 180
Pkno=KJ917837.1 CAGTGCCTTA TGAGAAATCA AAGTCTTTGG 179
PanDDT1043F19 ---------- ---------- ---------- 19
Pan-probe ---------- ---------- ---------- 36
PanDDT1197R22 -RC -----CCTTA TGAGAAATCA AAGTCTT--- 22
Consensus CAGTGCCTTA TGAGAAATCA AAGTCTTTGG
100%
Conservation
0%


20
40
60
Pbras=KC906725.1 TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCAGATACC GTCGTAATCT TAACCATAAA CTATGCCGAC TAGGT 75
Pbras=KC906730.1 TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCAGATACC GTCGTAATCT TAACCATAAA CTATGCCGAC TAGGT 75
Pbras=KC906729.1 TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCAGATACC GTCGTAATCT TAACCATAAA CTATGCCGAC TAGGT 75
Pbras=AF130735.1 TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCAGATACC GTCGTAATCT TAACCATAAA CTATGCCGAC TAGGT 75
Pbras=KT266778.1 TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCAGATACC GTCGTAATCT TAACCATAAA CTATGCCGAC TAGGT 75
Pbras=KC906720.1 TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCAGATACC GTCGTAATCT TAACCATAAA CTATGCCGAC TAGGT 75
Pbras=KC906721.1 TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCAGATACC GTCGTAATCT TAACCATAAA CTATGCCGAC TAGGT 75
Pbras=KC906726.1 TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCAGATACC GTCGTAATCT TAACCATAAA CTATGCCGAC TAGGT 75
Pbras=KC906724.1 TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCAGATACC GTCGTAATCT TAACCATAAA CTATGCCGAC TAGGT 75
Pbras=KC906723.1 TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCAGATACC GTCGTAATCT TAACCATAAA CTATGCCGAC TAGGT 75
Pbras=KJ619941.1 TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCAGATACC GTCGTAATCT TAACCATAAA CTATGCCGAC TAGGT 75
Pbras=KC906719.1 TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCAGATACC GTCGTAATCT TAACCATAAA CTATGCCGAC TAGGT 75
Pbras=KC906731.1 TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCAGATACC GTCGTAATCC TAACCATAAA CTATGCCGAC TAGGT 75
Pbras=KC906732.1 TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCAGATACC GTCGTAATCT TAACCATAAA CTATGCCGAC TAGGT 75
PanDDT1043F19 ---------A AAGTTAAGGG AGTGAAGA-- ---------- ---------- ---------- ---------- ----- 19
Pan-probe ---------- ---------- ---------- -------ACC GTCGTAATCT TAACCATAAA CTATGCCGAC TAG-- 36
PanDDT1197R22 -RC ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----- -
Consensus TCAAGAACGA AAGTTAAGGG AGTGAAGACG ATCAGATACC GTCGTAATCT TAACCATAAA CTATGCCGAC TAGGT
100%
Conservation
0%
80
100
120
140
Pbras=KC906725.1 GTTGGATGAT AGAGTAAAAA ATAAAAGGGA CATTCATATA TATGAGTGTT TCTTTTAGAT AGCTTCCTTC AGTAC 150
Pbras=KC906730.1 GTTGGATGAT AGAGTAAAAA ATAAAAGGGA CATTCATATA TATGAGTGTT TCTTTTAGAT AGCTTCCTTC AGTAC 150
Pbras=KC906729.1 GTTGGATGAT AGAGTAAAAA ATAAAAGGGA CATTCATATA TATGAGTGTT TCTTTTAGAT AGCTTCCTTC AGTAC 150
Pbras=AF130735.1 GTTGGATGAT AGAGTAAAAA ATAAAAGGGA CATTCATATA TATGAGTGTT TCTTTTAGAT AGCTTCCTTC AGTAC 150
Pbras=KT266778.1 GTTGGATGAT AGAGTAAAAA ATAAAAGAGA CATTCATATA TATGAGTGTT TCTTTTAGAT AGCTTCCTTC AGTAC 150
Pbras=KC906720.1 GTTGGATGAT AGAGTAAAAA ATAAAAGGGA CATTCATATA TATGAGTGTT TCTTTTAGAT AGCTTCCTTC AGTAC 150
Pbras=KC906721.1 GTTGGATGAT AGAGTAAAAA ATAAAAGGGA CATTCATATA TATGAGTGTT TCTTTTAGAT AGCTTCCTTC AGTAC 150
Pbras=KC906726.1 GTTGGATGAT AGAGTAAAAA ATAAAAGGGA CATTCATATA TATGAGTGTT TCTTTTAGAT AGCTTCCTTC AGTAC 150
Pbras=KC906724.1 GTTGGATGAT AGAGTAAAAA ATAAAAGGGA CATTCATATA TATGAGTGTT TCTTTTAGAT AGCTTCCTTC AGTAC 150
Pbras=KC906723.1 GTTGGATGAT AGAGTAAAAA ATAAAAGGGA CATTCATATA TATGAGTGTT TCTTTTAGAT AGCTTCCTTC AGTAC 150
Pbras=KJ619941.1 GTTGGATGAT AGAGTAAAAA ATAAAAGGGA CATTCATATA TATGAGTGTT TCTTTTAGAT AGCTTCCTTC AGTAC 150
Pbras=KC906719.1 GTTGGATGAT AGAGTAAAAA ATAAAAGGGA CATTCATATA TATGAGTGTT TCTTTTAGCT AGCTTCCTTC AGTAC 150
Pbras=KC906731.1 GTTGGATGAT AGAGTAAAAA ATAAAAGGGA CATTCATATA TATGAGTGTT TCTTTTAGCT AGCTTCCTTC AGTAC 150
Pbras=KC906732.1 GTTGGATGAT AGAGTAAAAA ATAAAAGGGA CATTCATATA TATGAGTGTT TCTTTTAGCT AGCTTCCTTC GGTAC 150
PanDDT1043F19 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----- 19
Pan-probe ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----- 36
PanDDT1197R22 -RC ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----C 1
Consensus GTTGGATGAT AGAGTAAAAA ATAAAAGGGA CATTCATATA TATGAGTGTT TCTTTTAGAT AGCTTCCTTC AGTAC
100%
Conservation
0%
160
180
Pbras=KC906725.1 CTTATGAGAA ATCAAAGTCT TTGGGTTCTG GGGCGA 186
Pbras=KC906730.1 CTTATGAGAA ATCAAAGTCT TTGGGTTCTG GGGCGA 186
Pbras=KC906729.1 CTTATGAGAA ATCAAAGTCT TTGGGTTCTG GGGCGA 186
Pbras=AF130735.1 CTTATGAGAA ATCAAAGTCT TTGGGTTCTG GGGCGA 186
Pbras=KT266778.1 CTTATGAGAA ATCAAAGTCT TTGGGTTCTG GGGCGA 186
Pbras=KC906720.1 CTTATGAGAA ATCAAAGTCT TTGGGTTCTG GGGCGA 186
Pbras=KC906721.1 CTTATGAGAA ATCAAAGTCT TTGGGTTCTG GGGCGA 186
Pbras=KC906726.1 CTTATGAGAA ATCAAAGTCT TTGGGTTCTG GGGCGA 186
Pbras=KC906724.1 CTTATGAGAA ATCAAAGTCT TTGGGTTCTG GGGCGA 186
Pbras=KC906723.1 CTTATGAGAA ATCAAAGTCT TTGGGTTCTG GGGCGA 186
Pbras=KJ619941.1 CTTATGAGAA ATCAAAGTCT TTGGGTTCTG GGGCGA 186
Pbras=KC906719.1 CTTATGAGAA ATCAAAGTCT TTGGGTTCTG GGGCGA 186
Pbras=KC906731.1 CTTATGAGAA ATCAAAGTCT TTGGGTTCTG GGGCGA 186
Pbras=KC906732.1 CTTATGAGAA ATCAAAGTCT TTGGGTTCTG GGGCGA 186
PanDDT1043F19 ---------- ---------- ---------- ------ 19
Pan-probe ---------- ---------- ---------- ------ 36
PanDDT1197R22 -RC CTTATGAGAA ATCAAAGTCT T--------- ------ 22
Consensus CTTATGAGAA ATCAAAGTCT TTGGGTTCTG GGGCGA
100%
Conservation
0%


20
40
60
Pfal=PF3D7_0725600 AATTAGCGGC GAGTACACTA TATTCTTATT TGAAATTGAA CATAGGTAAC TATACATTTA TTCAGTAATC AAATT 75
Pfal=PFARGEA/M19172.1 AATTAGCGGC GAGTACACTA TATTCTTATT TGAAATTGAA CATAGGTAAC TATACATTTA TTCAGTAATC AAATT 75
Pfal=PF3D7_0531600 AATTAGCGGC GAGTACACTA TATTCTTATT TGAAATTGAA CATAGGTAAC TATACATTTA TTCAGTAATC AAATT 75
Pfal=JQ627152.1 AATTAGCGGC GAGTACACTA TATTCTTATT TGAAATTGAA CATAGGTAAC TATACATTTA TTCAGTAATC AAATT 75
Pfal=JQ627149.1 AATTAGCGGC GAGTACACTA TATTCTTATT TGAAATTGAA CATAGGTAAC TATACATTTA TTCAGTAATC AAATT 75
Pfal=JQ627150.1 AATTAGCGGC GAGTACACTA TATTCTTATT TGAAATTGAA CATAGGTAAC TATACATTTA TTCAGTAATC AAATT 75
Pfal=JF681166.1 AATTAGCGGC GAGTACACTA TATTCTTATT TGAAATTGAA CATAGGTAAC TATACATTTA TTCAGTAATC AAATT 75
Pfal=HQ283215.1 AATTAGCGGC GAGTACACTA TATTCTTATT TGAAATTGAA CATAGGTAAC TATACATTTA TTCAGTAATC AAATT 75
Pfal=AF145334.1 AATTAGCGGC GAGTACACTA TATTCTTATT TGAAATTGAA CATAGGTAAC TATACATTTA TTCAGTAATC AAATT 75
Pfal=JQ627151.1 AATTAGCGGC GAGTACACTA TATTCTTATT TGAAATTGAA CATAGGTAAC TATACATTTA TTCAGTAATC AAATT 75
PfDDT1451F21 --------GC GAGTACACTA TATTCTTAT- ---------- ---------- ---------- ---------- ----- 21
Pf-probe ---------- ---------- ---------- ---------- ---------- -----ATTTA TTCAGTAATC AAATT 20
PfDDT1562R21 -RC ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----- -
Consensus AATTAGCGGC GAGTACACTA TATTCTTATT TGAAATTGAA CATAGGTAAC TATACATTTA TTCAGTAATC AAATT
100%
Conservation
0%
80
100
120
Pfal=PF3D7_0725600 AGGATATTTT TATTAAAATA TCCTTTTCCC TGTTCTACTA ATAATTTGTT TTTTA 130
Pfal=PFARGEA/M19172.1 AGGATATTTT TATTAAAATA TCCTTTTCCC TGTTCTACTA ATAAATTGTT TTTTA 130
Pfal=PF3D7_0531600 AGGATATTTT TATTAAAATA TCCTTTTCCC TGTTCTACTA ATAATTTGTT TTTTA 130
Pfal=JQ627152.1 AGGATATTTT TATTAAAATA TCCTTTTCCC TGTTCTACTA ATAATTTGTT TTTTA 130
Pfal=JQ627149.1 AGGATATTTT TATTAAAATA TCCTTTTCCC TGTTCTACTA ATAATTTGTT TTTTA 130
Pfal=JQ627150.1 AGGATATTTT TATTAAAATA TCCTTTTCCC TGTTCTACTA ATAATTTGTT TTTTA 130
Pfal=JF681166.1 AGGATATTTT TATTAAAATA TCCTTTTCCC TGTTCTACTA ATAATTTGTT TTTTA 130
Pfal=HQ283215.1 AGGATATTTT TATTAAAATA TCCTTTTCCC TGTTCTACTA ATAATTTGTT TTTTA 130
Pfal=AF145334.1 AGGATATTTT TATTAAAATA TCCTTTTCCC TGTTCTACTA ATAATTTGTT TTTTA 130
Pfal=JQ627151.1 AGGATATTTT TATTAAAATA TCCTTTTCCC TGTTCTACTA ATAATTTGTT TTTTA 130
PfDDT1451F21 ---------- ---------- ---------- ---------- ---------- ----- 21
Pf-probe AGGAT----- ---------- ---------- ---------- ---------- ----- 25
PfDDT1562R21 -RC ---------- ---------- ----TTTCCC TGTTCTACTA ATAAT----- ----- 21
Consensus AGGATATTTT TATTAAAATA TCCTTTTCCC TGTTCTACTA ATAATTTGTT TTTTA
100%
Conservation
0%

University of Washington Medical Center
Clinical Microbiology Laboratory Document # 1000-112-01

Molecular Diagnosis Manual **Procedure for receipt, aliquoting and use of Armored RNA encoding full-length *P. falciparum* 18S rRNA**		Effective: 1 July 2016
Written by: Sean Murphy	Reviewed by: Ming Chang	Approved by: [redacted] Sean Murphy, M.D., Ph.D.
Revises or supersedes:		Revised by:

ANNUAL REVIEW			
Reviewed by:	Date	Reviewed by:	Date
N/A (first issuance as SOP)			

REVISION HISTORY	
Date of change	Summary of changes

CLINICAL SIGNIFICANCE

This protocol describes the ordering, receipt, handling and use of an Armored RNA encoding the full-length 18S rRNA of *Plasmodium falciparum* for use in RT-PCR assays.

PRINCIPLE

A custom-ordered Armored RNA from Asuragen® encodes the full-length 18S rRNA of *P. falciparum* and serves as a calibrator in the laboratory's qRT-PCR assay. The standard curve produced by the Armored RNA should be tested at least every 6 months or when reagent lot numbers are changed. If the standard curve produced falls within expected ranges established during method validation of the third generation malaria qRT-PCR assay, then the standard curve can be used to calculate estimated parasitemia.

SPECIMEN

Clinical specimens are *not* required for this procedure.

CLINICAL INDICATION AND CLINICAL UTILITY

This protocol supports the *P. falciparum* and Pan *Plasmodium* quantitative RT-PCR procedure used to provide sensitive detection and quantification of blood-stage parasites from malaria vaccine clinical trial specimens.

REAGENTS AND SUPPLIES

1) Custom-Made Armored RNA encoding the 18s RNA (Asuragen, CoA 512-681-5200)

Pf 18S (WAS-1) Target Sequence
5'AACCTGGTTGATCTTGCCAGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCAAGTGAAAGTATA
TATATATTTTATATGTAGAAACTGCGAACGGCTCATTAAAACAGTTATAGTCTACTTGACATTTTTATTA
TAAGGATAACTACGGAAAAGCTGTAGCTAATACTTGCTTTATTATCCTTTGATTTTTATCTTTGGATAA
GTATTTGTTAGGCCTTATAAGAAAAAAGTTATTAACTTAAGGAATTATAACAAAGAAGTAACACGTAAT
AAATTTATTTTATTTAGTGTGTATCAATCGAGTTTCTGACCTATCAGCTTTTGATGTTAGGGTATTGGC
CTAACATGGCTATGACGGGTAACGGGGAATTAGAGTTCGATTCCGGAGAGGGAGCCTGAGAAATAG
CTACCACATCTAAGGAAGGCAGCAGGCGCGTAAATTACCCAATTCTAAAGAAGAGAGGTAGTGACAA

GAAATAACAATGCAAGGCCAATTTTTGGTTTTGTAATTGGAATGGTGGGAATTTAAAACCTTCCCAGA
GTAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAA
AATTGTTGCAGTTAAAACGCTCGTAGTTGAATTTCAAAGAATCGATATTTTATTGTAACTATTCTAGGG
GAACTATTTTAGCTTTTCGCTTTAATACGCTTCCTCTATTATTATGTTCTTTAAATAACAAAGATTCTTTT
TAAAATCCCCACTTTTGCTTTTGCTTTTTTTGGGGATTTTGTTACTTTGAGTAAATTAGAGTGTTCAAA
GCAAACAGTTAAAGCATTTACTGTGTTTGAATACTATAGCATGGAATAACAAAATTGAACAAGCTAAAA
TTTTTTGTTCTTTTTTCTTATTTTGGCTTAGTTACGATTAATAGGAGTAGCTTGGGGACATTCGTATTCA
GATGTCAGAGGTGAAATTCTTAGATTTTCTGGAGACGAACAACTGCGAAAGCATTTGTCTAAAATACT
TCCATTAATCAAGAACGAAAGTTAAGGGAGTGAAGACGATCAGATACCGTCGTAATCTTAACCATAAA
CTATGCCGACTAGGTGTTGGATGAAAGTGTTAAAAATAAAAGTCATCTTTCGAGGTGACTTTTAGATT
GCTTCCTTCAGTACCTTATGAGAAATCAAAGTCTTTGGGTTCTGGGGCGAGTATTCGCGCAAGCGAG
AAAGTTAAAAGAATTGACGGAAGGGCACCACCAGGCGTGGAGCTTGCGGCTTAATTTGACTCAACAC
GGGGAAACTCACTAGTTTAAGACAAGAGTAGGATTGACAGATTAATAGCTCTTTCTTGATTTCTTGGA
TGGTGATGCATGGCCGTTTTTAGTTCGTGAATATGATTTGTCTGGTTAATTCCGATAACGAACGAGAT
CTTAACCTGCTAATTAGCGGCGAGTACACTATATTCTTATTTGAAATTGAACATAGGTAACTATACATT
TATTCAGTAATCAAATTAGGATATTTTTATTAAAATATCCTTTTCCCTGTTCTACTAATAATTTGTTTTTT
ACTCTATTTCTCTCTTCTTTTAAGAATGTACTTGCTTGATTGAAAAGCTTCTTAGAGGAACATTGTGTG
TCTAACACAAGGAAGTTTAAGGCAACAACAGGTCTGTGATGTCCTTAGATGAACTAGGCTGCACGCG
TGCTACACTGATATATATAACGAGTTTTTAAAAATATGCTTATATTTGTATCTTTGATGCTTATATTTTG
CATACTTTTCCTCCGCCGAAAGGCGTAGGTAATCTTTATCAATATATATCGTGATGGGGATAGATTAT
TGCAATTATTAATCTTGAACGAGGAATGCCTAGTAAGCATGATTCATCAGATTGTGCTGACTACGTCC
CTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATTGAAAGATATGATGAATTGTTTGGACAAGA
AAAATTGAATTATATTCTTTTTTTTCTGGAAAAACCGTAAATCCTATCTTTTAAAGGAAGGAGAAGTCG
TAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTA-3’

Details from CoA from Asuragen

Catalog Number: 49594
Lot Number: DL16-109
Total Volume: 10 mL
Concentration: 5 x 1011 copies/mL
Number of Vials: 100
Volume/Vial: 100 µL
Storage buffer: TSMIII (10 mM Tris, 100 mM NaCl, 1 mM MgCl2, 0.1 % gelatin, 0.3 % Microcide III; pH 7.0)

2) RNA Storage Solution (Invitrogen, AM7000)
3) BioMérieux NucliSens EasyMag Lysis Buffer (4x1mL bottles, Category No. 280134)
4) Bioexpress 4 mL tube without cap #T2868-1 and screw caps without rings #T-2868-3
5) Calibrated Pipettes: 1-10µL, 10-100µL, 20-200µL, 100-1000µL, 100-2000µL, ranges
6) RNase-/DNase-free, aerosol resistant pipette tips: 10µL, 100µL, 200µL, 1000µL, 2000µL
7) Eppendorf-style 1.5 mL microcentrifuge tubes, DNase- and RNase- free
8) Vortex Mixer
9) -80°C freezer
10) RNase-/DNase-free work space

PROCEDURE

1) RECEIPT AND PREPARATION OF ARMORED RNA FOR USE
    a. Immediately store all stock custom-made Armored RNA of 5e11 copies/mL (or 5e8 copies/µL) at -70°C or lower.
    b. Preparation of 1e7 copies/µL aliquots for standard curves and general use.
        i. The Armored RNA is received from Asuragen at 5e11 copies/mL (or 5e8 copies/µL) in 100µL aliquots.
        ii. Thaw at least 2mL of RNA Storage Solution
        iii. Thaw exactly one tube of the Armored RNA.
        iv. Dilute the Armored RNA by 1:5 to obtain and initial dilution of 1e8 copies/µL

v. Further dilute the solution by 1:10 to obtain a final concentration of 1e7 copies/µL.
vi. Aliquot the 1e7 Armored RNA copies/µL solution in 30µL tubes. Store in -70°C or lower overnight before use.
vii. These small aliquots are for single-use and should be discarded after each use.

2) QUALITY ANALYSIS AND STANDARD CURVE CALIBRATION
   a. Preparation of standard curve samples for extraction
      i. The standard curve for the third-generation assay will be derived from Armored RNA diluted from $5.3x10^7$ to $5.3x10^5$ copies per mL of lysed negative whole blood
      ii. To prepare samples needed for the standard curve, obtain approximately 15mL of lysed malaria negative sample. This can be 15mL worth of freshly prepared lysed material (375µL of negative whole blood to 15mL of bioMérieux Nuclisens EasyMag lysis buffer) or 15mL of thawed, pre-lysed malaria negative controls.
      iii. Prepare one aliquot of 5mL lysed negative in 15mL falcon tubes, and three aliquots of 4.5mL lysed negative. Keep the aliquots on ice or at 2-8°C and used as quickly as possible. Label the 5mL aliquot as Calibrator "A" and the other aliquots as Calibrators "B", and "C".
      iv. Remove a single frozen 30uL aliquot of Armored RNA of 1e7 copies/ µL from -80°C and allow to defrost for ~5 minutes. Do not expose remaining aliquots to room temperature conditions.
      v. Spike Calibrator "A" or "CAL A" with 26.5µL of the single-use 1e7 Armored RNA copies/µL solution cap, vortex and quickspin. This will is the first of 3 calibrator levels. Proceed down TABLE 2, taking care to thoroughly mix, spin and change pipette tips between dilution steps.

TABLE 1. Plasmodium 18s rRNA Encoding Armored RNA Standard Curve Set Up

Stock Calibrator Level	Stock volume (mL)	NEG in lysis buffer (mL)	Final copies/mL lysis buffer	Nominal Log10 copies / mL of lysis buffer	Calibrator Label
			5.3E+07	7.72	"CAL A"
"CAL A"	0.5	4.5	5.30E+06	6.72	"CAL B"
"CAL B"	0.5	4.5	5.30E+05	5.72	"CAL C"

      vi. Prepare 1.3mL aliquots of each Calibrators "MAL A" through "MAL C". There should be sufficient sample to prepare 3 aliquots each.
      vii. Follow the Third Generation Assay SOP (Document #: 1000-100-01) to extract and run the samples as calibrators on the Abbott m2000sp and rt machines.
      viii. The resulting standard curve should observe conditions in Table 2a, b. If values are outside of these ranges, consult a Research Scientist or the Laboratory Director.

Table 2a. Acceptable Range for *P. falciparum* Standard Curve

Slope	-3.23 cycles/$\log_{10}$(+/- 2 StDev = -3.47 to -2.98)
Y-intercept	45.04 cycles (+/- 2 StDev = 43.55 to 46.52)
r^2	0.996 (acceptable >0.975)

Table 2b. Acceptable Range for Pan *Plasmodium* Standard Curve

Slope	-3.38 cycles/$\log_{10}$ (+/- 2 StDev = -3.59 to -3.17)
Y-intercept	41.85 cycles (+/- 2 StDev = 39.44 to 44.26)
r^2	0.998 (acceptable >0.975)

EXAMPLE OF ACTUAL USE

The following is an example of the calculation based on the mean standard curve from the validation (the actual regression calculation is performed in the m2000rt software using a stored standard curve:

EXAMPLE: P. falciparum 18S rRNA copies/mL blood
= (10^((Sample CN – Y-intercept)/(slope)))(53 µL eluate/15 µL template)(1000 µL/25 µL)
= (10^((Sample CN – 43.82)/(-3.155)))(53µL/15µL)(1000µL/25µL)
= (10^((Sample CN – 43.82)/(-3.155)))(141.33)

Ring-stage *P. falciparum* parasites per mL blood (uses conversion factor from Abbott m2000 Method Validation)

Estimated parasites/mL = (18S rRNA copies/mL blood) / (7400 copies per ring-stage parasite)

REFERENCES:

Product manuals for the commercial products listed above.

Asuragen®

Armored RNA Quant®
Pf 18S (WAS-1)

Armored RNA Quant® Pf 18S (WAS-1)

- **Catalog Number:** 49594
- **Lot Number:** DL16-109
- **Total Volume:** 10 mL
- **Concentration:** 5 x 10^{11} copies/mL
- **Number of Vials:** 100
- **Volume/Vial:** 100 µL
- **Storage buffer:** TSMIII (10 mM Tris, 100 mM NaCl, 1 mM $MgCl_2$, 0.1 % gelatin, 0.3 % Microcide III; pH 7.0)
- **Storage:** ≤ -15 °C

Pf 18S (WAS-1) Target Sequence

AACCTGGTTGATCTTGCCAGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCAAGTGAAAGTATATATATATTTTATATGTAGA
AACTGCGAACGGCTCATTAAAACAGTTATAGTCTACTTGACATTTTTATTATAAGGATAACTACGGAAAAGCTGTAGCTAATACT
TGCTTTATTATCCTTTGATTTTTATCTTTGGATAAGTATTTGTTAGGCCTTATAAGAAAAAAGTTATTAACTTAAGGAATTATAACA
AAGAAGTAACACGTAATAAATTTATTTTATTTAGTGTGTATCAATCGAGTTTCTGACCTATCAGCTTTTGATGTTAGGGTATTGGC
CTAACATGGCTATGACGGGTAACGGGGAATTAGAGTTCGATTCCGGAGAGGGAGCCTGAGAAATAGCTACCACATCTAAGGAA
GGCAGCAGGCGCGTAAATTACCCAATTCTAAAGAAGAGAGGTAGTGACAAGAAATAACAATGCAAGGCCAATTTTTGGTTTTGT
AATTGGAATGGTGGGAATTTAAAACCTTCCCAGAGTAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGC
TCCAATAGCGTATATTAAAATTGTTGCAGTTAAAACGCTCGTAGTTGAATTTCAAAGAATCGATATTTTATTGTAACTATTCTAGG
GGAACTATTTTAGCTTTTCGCTTTAATACGCTTCCTCTATTATTATGTTCTTTAAATAACAAAGATTCTTTTTAAAATCCCCACTTTT
GCTTTTGCTTTTTTTGGGGATTTTGTTACTTTGAGTAAATTAGAGTGTTCAAAGCAAACAGTTAAAGCATTTACTGTGTTTGAATA
CTATAGCATGGAATAACAAAATTGAACAAGCTAAAATTTTTTGTTCTTTTTTCTTATTTTGGCTTAGTTACGATTAATAGGAGTAG
CTTGGGGACATTCGTATTCAGATGTCAGAGGTGAAATTCTTAGATTTTCTGGAGACGAACAACTGCGAAAGCATTTGTCTAAAAT
ACTTCCATTAATCAAGAACGAAAGTTAAGGGAGTGAAGACGATCAGATACCGTCGTAATCTTAACCATAAACTATGCCGACTAG
GTGTTGGATGAAAGTGTTAAAAATAAAAGTCATCTTTCGAGGTGACTTTTAGATTGCTTCCTTCAGTACCTTATGAGAAATCAAA
GTCTTTGGGTTCTGGGGCGAGTATTCGCGCAAGCGAGAAAGTTAAAAGAATTGACGGAAGGGCACCACCAGGCGTGGAGCTT
GCGGCTTAATTTGACTCAACACGGGGAAACTCACTAGTTTAAGACAAGAGTAGGATTGACAGATTAATAGCTCTTTCTTGATTTC
TTGGATGGTGATGCATGGCCGTTTTTAGTTCGTGAATATGATTTGTCTGGTTAATTCCGATAACGAACGAGATCTTAACCTGCTA
ATTAGCGGCGAGTACACTATATTCTTATTTGAAATTGAACATAGGTAACTATACATTTATTCAGTAATCAAATTAGGATATTTTTA
TTAAAATATCCTTTTCCCTGTTCTACTAATAATTTGTTTTTTACTCTATTTCTCTCTTCTTTTAAGAATGTACTTGCTTGATTGAAAAG
CTTCTTAGAGGAACATTGTGTGTCTAACACAAGGAAGTTTAAGGCAACAACAGGTCTGTGATGTCCTTAGATGAACTAGGCTGC
ACGCGTGCTACACTGATATATATAACGAGTTTTTAAAAATATGCTTATATTTGTATCTTTGATGCTTATATTTTGCATACTTTTCCTC
CGCCGAAAGGCGTAGGTAATCTTTATCAATATATATCGTGATGGGGATAGATTATTGCAATTATTAATCTTGAACGAGGAATGCC
TAGTAAGCATGATTCATCAGATTGTGCTGACTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATTGAAAGATATG
ATGAATTGTTTGGACAAGAAAAATTGAATTATATTCTTTTTTTTCTGGAAAAACCGTAAATCCTATCTTTTAAAGGAAGGAGAAG
TCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTA

Asuragen®

Armored RNA Quant®
Pf 18S (WAS-1)

Quality Control Test	Specifications	Test results
Dideoxynucleotide Sequencing of Plasmid DNA Target	Actual sequence matches target	Actual sequence matches target
ARQ Batch Stock Quantification	$R^2 \geq 0.95$ for standard curve	0.98
	Sample replicate variability ≤ 20 %	3.2 %
ARQ Identity	PCR band seen at appropriate size when compared to DNA size standard(s)	PCR band seen at appropriate size when compared to DNA size standard(s)
ARQ Integrity	Test and report	Extracted Armored RNA Quant migrates as an intact band similar in size to that of the RNA Reference Control and RNA Extraction Control.

Manager: [redacted] **Date:** 24 Jun 16

Note: For research use only, not for diagnostic purposes. Inclusion of the Armored RNA Quant® Pf 18S (WAS-1) preparation into a commercial product, testing service or process requires a license from Asuragen, Inc.

Eligibility criteria for trials using the biomarker for the proposed COU

In CHMI studies, the study population is enrolled from a population of healthy, malaria-naïve volunteers aged 18 through 45-50 years old. Subjects are expected to be negative for the biomarker at baseline. Inclusion and exclusion criteria are intended to mitigate risks associated with *Plasmodium* infection and known and unknown risks potentially associated with study products. The following inclusion/exclusion criteria are representative of criteria used in most CHMI studies of drug and vaccine trials. Criteria for first-in-human trials may be more restrictive. Standardized CHMI inclusion/exclusion criteria were developed [52] with the involvement of USMMVP, Sanaria, University of Maryland, Oxford University, Radboud University, Seattle Biomedical Research Institute, KEMRI-Wellcome Kilifi Research Programme, USAID, US FDA, NIAID, PATH Malaria Vaccines Initiative and the European Vaccine Initiative. Since the development of these criteria, screening has expanded to include assessment for cardiovascular disease risks.

Volunteers participating in CHMI trials should meet the following criteria. Some variations in these criteria are permitted from trial to trial depending on the nature of the drug or vaccine product under investigation.

i. General CHMI inclusion criteria

- Aged 18-50 years old. In specific situations, if it is determined that it is appropriate for individuals >50 years old to be included, additional screening should be included as necessary to protect the safety of volunteers.
- Volunteer must understand and sign written informed consent form.
- Volunteer should be free of clinically significant health problems as established by medical history, clinical examination and general laboratory evaluation.
- Volunteer should be available for the duration of the study, reachable by mobile phone or pager and should provide information on two emergency contacts to assist with making contact.
- Volunteer should reside nearby the trial center during days 0-42 following CHMI.
- Female volunteers should take adequate contraceptive precautions or be of non-childbearing potential. (Note that in some studies, women of childbearing potential may be excluded.)
- Volunteer must agree not to travel to a malaria endemic region during the in-person visit portion of the trial.
- Volunteer should be in good health, as determined by vital signs (heart rate, blood pressure, oral temperature), medical history, screening 12-lead ECG and laboratory tests*, and a physical examination.
    - *Typical screening laboratory tests include hemoglobin, white blood cell count, platelet count, glucose (random), serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), serum creatinine, HIV/hepatitis screening, urine protein and urine blood.

ii. General CHMI exclusion criteria

- History of malaria infection or vaccination, continuous residence in malaria-endemic area for ≥5 years, travel to malaria-endemic area in previous 6 months, or prior participation in malaria research study.
- Use of malaria chemoprophylaxis or chemotherapy within 90 days prior to study.
- Use of systemic antibiotics with known antimalarial activity within 30 days prior to the study (such as trimethoprim-sulfamethoxazole, doxycycline, tetracycline, clindamycin, erythromycin, fluoroquinolones and azithromycin)
- Plans to travel to malaria endemic areas during the study period
- A history of a chronic systemic immune modulating disorder, such as lupus, rheumatoid arthritis, vasculitis, scleroderma, cancer and diabetes mellitus. In addition all significant contraindications to the antimalarial to be used should be exclusion criteria. For example psoriasis and porphyria are contraindications to chloroquine use.

- Symptoms, physical signs and laboratory values suggestive of systemic disorders, including renal, hepatic, cardiovascular, pulmonary, neurological, skin, immunodeficiency, psychiatric (including suicide risk within 3 years), active neoplastic disease or other conditions, which could interfere with the interpretation of the study results or compromise the health of the volunteer.
- Any confirmed or suspected immunosuppressive of immunodeficient condition including HIV and asplenia
- A history of allergic disease or reactions likely to be exacerbated by mosquito bites, malaria infection or antimalarial drugs
- The confirmed or suspected presence of Hepatitis B, C or HIV
- The confirmed or suspected presence of hemoglobin S, hemoglobin C, hemoglobin E, thalassemia or Glucose-6-phosphate dehydrogenase deficiency
- Pregnant or lactating volunteers (or planning to start during study period)
- The chronic use (defined as more than 14 days) of immune modulating drugs within 6 months prior to malaria challenge or the use of immunoglobulins or blood products within 3 months prior to malaria challenge. Inhaled and topical steroids are generally allowed.
- Suspected or known alcohol or illicit drug abuse that, in the opinion of the investigator, may interfere with the subject's ability to comply with the protocol. Some protocols include partial or complete restrictions on alcohol, tobacco and/or other recreational drug use and some protocols delineate a specific timeframe to which the abuse applies.
- Volunteers unable to be closely followed, for social, geographical or psychological reasons
- The participation in another clinical trial which requires the use of an investigational or non-registered drug, vaccine or medical device within 30 days before the malaria challenge or any time during the study period for that participant
- Plans to undergo surgery (elective or otherwise) between enrollment and the end of the study.
- Vital sign abnormalities including systolic blood pressure >140 mm Hg or diastolic blood pressure >90 mm Hg; resting heart rate <40 or >100 beats per minute or oral temperature ≥ 38°C (100.4°F).
- Body mass index (BMI) ≥ 35 (exact cutoff varies by study)
- Any Grade 1 or higher clinically significant laboratory abnormality on screening
    - Screening laboratory tests include hemoglobin, white blood cell count, platelet count, glucose (random), serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), serum creatinine, HIV/hepatitis screening, urine protein and urine blood. Some studies also assess for sickle trait. Any clinically significant Grade 1 or higher value for any screening test is exclusionary. Testing can be repeated in persons with non-clinically significant abnormalities to ascertain eligibility.
- Algorithmic evidence of moderate to high coronary risk, using locally appropriate coronary risk evaluation scoring systems. At our center, such risk consists of moderate risk or higher categories for fatal or non-fatal cardiovascular event within 5 years (>10%) determined by non-invasive criteria for cardiac risk according to the National Health and Nutrition Examination Survey (NHANES I) and/or an abnormal ECG.
    - Risk factors include sex, age (years), systolic blood pressure (mm Hg), smoking status (current vs. past or never), body mass index (BMI; kg/mm_2), reported diabetes status (yes/no), current treatment for raised blood pressure (yes/no).
    - Abnormal screening ECG findings include evidence of pathologic Q waves and significant ST-T wave changes; left ventricular hypertrophy; any non-sinus rhythm excluding isolated premature atrial or ventricular contractions; right or left bundle branch block; QT/QTc interval >450 ms; or advanced (secondary or tertiary) A-V heart block.
- Acute febrile illness (oral temperature ≥ 38°C [100.4°F]) or other acute illness on or up to 3 days before product administration (drug or vaccine) and/or CHMI.
- Any condition that would, in the opinion of the site investigator, place the subject at an unacceptable risk of injury or render the subject unable to meet the requirements of the protocol or compromise the interpretation of data or the scientific integrity of the protocol.