varATS real-time PCR for P. falciparum quantification

Aim and application

- This procedure describes a (quantitative) real-time PCR (qPCR) assay for the detection and/or quantification of P. falciparum in blood samples, using var gene acidic terminal sequence (varATS, ˜59 copies/genome) as a target. This protocol is adapted from Hofmann et al, PLoS Med, 2015 [1]
- The protocol is applicable to studies aiming to detect and/or quantify Plasmodium falciparum in blood samples. Sensitivity of this PCR is
    - For DNA extracted from 200 ul whole blood: 0,05 parasites/µl
    - For DNA extracted from filter paper: 0,4 parasites/µl

Principle

- Quantitative PCR for the diagnosis and quantification(based on a standard curve) of *Plasmodium falciparum* using a fluorescent TaqMan® probe.

Reagents

- Primers/probe:
    - var fw: 5’ – CCCATACACAACCAAYTGGA – 3’
    - var rev: 5’ – TTCGCACATATCTCTATGTCTATCT – 3’
    - var probe: 6 – FAM-TRTTCCATAAATGGT-NFQ-MGB
- TaqMan Universal Master mix, no UNG (ThermoFisher)
- Nuclease free water.
- Samples:
    - gDNA from Positive controls.
    - gDNA from Standard curves.

Apparatus, equipment and materials

- Equipment:
    - StepOne Plus thermocycler (Applied Biosystems)
    - StepOne software v2.3
- Materials
    - Bioplastics qPCR plates – ALG/0001260
    - Sealing foil – LAB/006558

Sample for analysis

- gDNA extracted from:
    - Whole blood
    - Blood on filter paper

Responsibilities

- Procedures must be carried out by a trained researcher or lab technician.

Definitions and abbreviations

- DNA: deoxyribonucleic acid

- gDNA: genomic DNA
- µl: microliter
- ml: milliliter
- min: minutes
- nM: nanomolar
- NTC: negative template control
- qPCR: quantitative/real-time polymerase chain reaction
- SC: standard curve
- sec: seconds
- conc: concentration

Method

1. Sample preparation – Standard curve

- Synchronize a P. falciparum culture using sorbitol as prescribed in the SOP to obtain a culture containing only rings.
- Make a thin film and count the parasitaemia (against =3000 RBC's) - A
- Count the amount of RBC/µl using the Neubauer counting chamber. - B
- Use A and B to calculate the amount of P. falciparum per µl
- Dilute your Pf culture to 100.000 par/µl in fresh whole blood(or any other known parasite density)
- Dilute 1/10 until 0,001par/µl in fresh whole blood(or similar density)

2. qPCR - Work on ice the entire procedure!!

- Create a new Experiment file using Excel Template varATS PCR:
- Design your PCR plate taking into account it should include:
  - Standard curve, in triplicates (reference range 100.000 to 0,01 parasites/µl)
  - Positive control
  - NTC and/or Negative control
  - 2 Reference samples, in case of multi-plate experiments (reference sample can be the same as the positive control)
    - 100 par/ml as sensitivity test of the assay
    - Any other sample of the standard curve

NOTE: For multi-plate experiments/studies standard curve only needs to be added in the first study plate. In subsequent plates, always include a reference sample that was present in your standard curve plate. This sample will be used for normalization before applying standard curve equation for quantification.

In pre-PCR room (155/01/10):

- Prepare Mastermix for the desired number of samples.
  For one reaction mix the following volumes:
  - Universal Master mix - 10,0µl
  - Var fw (10µM) - 1,6 0µl
  - Var rev (10µM) -

1,6 0µl
- Var Probe (10µM) - 0,8 0µl
- H_2O - 1,0 µl

(total volume 15 µl)

In laboratory 155/01/01

- Distribute the 15,0 µl/well into a white qPCR-plate.
- Load 5 µl of DNA and cover with sealing foil.
- Protect the wells with sealing foil.
- Spin the plate again for 2min at 2000rpm in centrifuge 02880(155/01/01) or centrifuge 03741(155/04/41)

In qPCR laboratory

- Open StepOne software and log in with your username(MALARIA).
- Open an existing Template file for varATS qPCR or, alternatively, create a new file selecting Set-up>Advanced set-up. In case you open an Existing template file, check the following conditions have not been modified, and update your plate design.
- To create a new file first go to Set-up>Experiment properties and select the following settings: StepOnePlus instrument / Quantitation Standard Curve / TaqMan Reagents / Standard (2h)
- Move to Set-up>Plate setup and Define targets and samples tab. In the left panel enter a new target name for varATS with FAM as reporter and NFQ-MGB as quencher.
- In the right panel enter a new sample name for the Positive control/Reference well
- Click on Assign targets tab and assign the varATS target to each position according to your plate design. Assign the position of Reference sample.
- For standard curve dilutions, select S in the Task cell, and click on the Define and then set-up Standards button. In the dialogue box enter the parameters of your standard curve including Quantity and dilution factor. Close the dialogue box
- In the lower panel select ROX as reference dye
- Move to Set-up>Run Method and enter reaction volume and cycling conditions as follows (activate data collection in the 55ºC step)

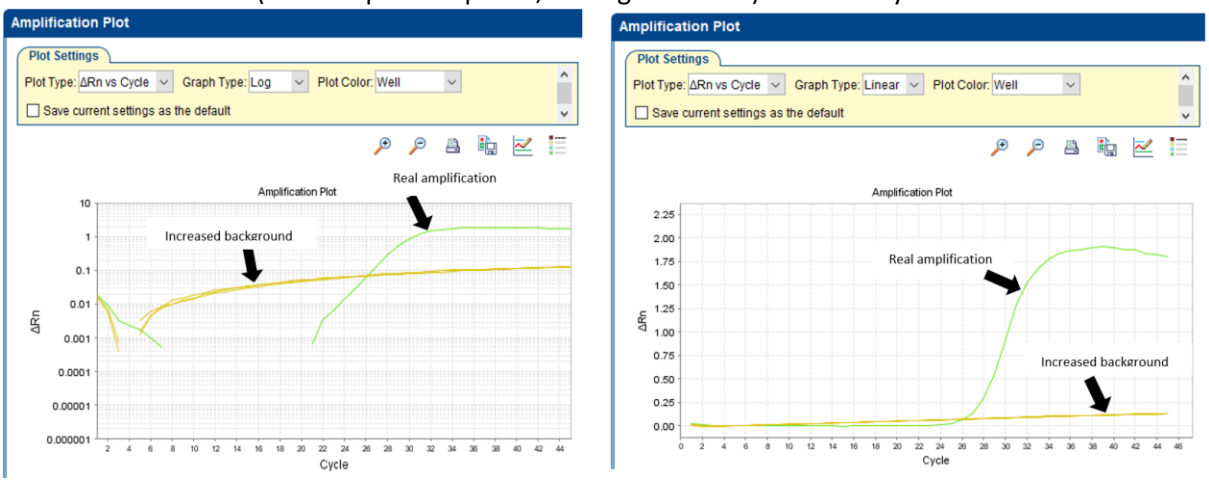
Step	Conditions	# cycles
Initial Denaturation	10’ – 95°C	1x
Denaturation	15’’ – 95°C	45x
Annealing& Elongation	60’’ – 55°C	
Holding	15°C	1x

- Save as new Experiment (in addition, you can save as Template and use it for further experiments)

3. Analysis using StepOne Software v2.3

3.1. Analysis of the standard curve plate

- In StepOne software, go to Analysis >Amplification Plot and check results of positive and negative controls.
- Check amplification curves for true amplification or increased background curves.
- True amplification can be recognized by the standard amplification phases (ground, exponential, linear and plateau phase).
- Increased background can be recognized by early arising curve with a continuously increasing fluorescence (without plateau-phase; see Figure below). After analysis a low Ct-value is noticed.



By changing Graph type from log to linear, these differences will be more clear.

- Omit increased background curves for the analysis.
- Adjust threshold if necessary.
- For standard curve triplicates, omit outliers.
- Go to Analysis panel>Standard curve and check Ct differences and efficiency. Consider removing standard curve points close to sensitivity limit if amplification was not optimal.
- Export Standard curve amplification plot image and equation and save it as .jpg files. Export Ct results table into Excel and transfer data into a new “Std Curve” sheet of the varATS_Analysis Template.xlsx
- Copy Equation parameters, CtREF and Ct limits of standard curve in the upper box of the template. Efficiency of the standard curve needs to be =85%

3.2. Analysis of samples in Study Plates (not containing a standard curve):

- In StepOne software, check results of positive and negative controls, the amplification profiles of each sample, and position of automated thresholds.
- Export results into a new Excel file and transfer Ct values for the Reference and each sample into “study plate” sheet of the varATS_Analysis Template.xlsx
- Normalization of Ct values using the CtREF in present plate compared to Std curve plate is automatically generated using formula: Ctsample * (CtREFstdcruve/CtREFstudyplate)
- It is recommended to analyze each new plate in a different excel sheet.
- Samples with Ct values above the Ctmax of the standard curve will be considered negative. Samples with Ct values below the Ctmin of the standard curve will be considered “higher than first point in standard curve” (alternatively, these samples can be diluted until their Ct fits within standard curve range)

3.3. Validity of the test

- See validation reports:
  - L:\Biomedical Sciences\Parasitology\Malariology\LABORATORY GENERAL\Validations\QPCR\varATS qPCR
- Validation Report MMV P218-24012019-first draft
- Results are valid when the positive controls(all replicates) are positive and the negative controls (all replicates) give no amplification.
  - If the positive control from DNA extraction is negative (=2 replicates)
    ✍ Repeat from DNA extraction
  - If the negative control from DNA extraction is positive (=2 replicates)
    ✍ Repeat from DNA extraction
  - If the NTC is positive (=2 replicates)
    ✍ Repeat PCR
- The results can be accurately quantified until 50 par/ml = 0,05 par/µl.
- The test is able to detect parasites until 10 par/ml, however the quantification in the range between 50 par/ml and 10 par/ml is not accurate and therefore these samples (2 or 3 replicates positive) are reported as 'Low positive' for clinical purposes. Samples with only one positive replicate of the three tested with parasite densities <50 P/ml are considered "non-detected" or negative samples.
- Therefore, the PCR decision tree summarizes as follows:
  - 2-3 replicates positive with □50 par/ml
    - Positive with x (normalized density, calculated from standard curve) par/ml.
  - 1 replicate positive with □50 par/ml
    - Repeat PCR
    - After repetition if =1 replicate positive with =50 par/ml, report POSITIVE with x(normalized density, calculated from standard curve) par/ml.
  - 2-3 replicates positive with <50 par/ml
    - Low Positive
  - 1 replicate positive with <50 par/ml
    - Negative

      !! *Remark:* if the TBS is positive in this case, repeat from PCR to confirm negative PCR.
      - If =1 replicate is positive and the quantification is **<50 p/ml**
        ✍ Report as *<50 p/ml*
      - If all 3 replicates are negative
        ✍ Report as *negative*
      - If any other result, follow the "decision tree".
  - If the parasite density of 1 replicate is =50 p/ml
    - *REPEAT THE PCR!!!! If the same result is obtained report as positive with normalization of parasite density. If any other result, follow the "decision tree".*

Quality control

1. First line control

- Positive controls
  - Positive extraction control
  - Positive PCR control(this can be the SC reference)
- Negative control
  - Negative extraction control
  - NTC
- See 3.3 Validity of the test

2. Second line control

3. Third Line control

Technical validation

- See 3.3 Validity of the test

Recording and interpretation of results

- See 3. Analysis using StepOne Software v2.3

Clinical validation/release of results

- N/A

Reporting

- If the PCR is used for clinical studies, the reporting will be discussed in the study-specific lab manual

Storage of samples

- Samples are stored 2-8°C during the PCR
- Samples are stored -20°C after finalization of the test.

Training

- Follow the unit's training plan

Method validation and literature

- *Hofmann N, et al. (2015) Ultra-Sensitive Detection of Plasmodium falciparum by Amplification of Multi-Copy Subtelomeric Targets. PLoS Med 12(3): e1001788. doi:10.1371/journal. pmed.1001788*
- *L:\BiomedicalSciences\ Parasitology\ Malariology\ LABORATORYGENERAL\ Validations\ QPCR\ varATS qPCR*
- *Validation Report MMV P218-24012019-first draft*

Comments

N/A.

Safety and environment

- Refer to the general safety documents that are relevant to this document. If necessary indicate any specific points of interest and/or risks to personnel or the surroundings.

Attachments and forms for completion

- varATS_experiment template.xlsx
- varATS_Analysis_template.xlsx