Recommended Genotyping Procedures (RGPs) to identify parasite populations

Developed after an informal consultation organized by the Medicines for Malaria Venture and cosponsored by the World Health Organization, 29-31 May 2007, Amsterdam, The Netherlands
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**Introduction**

The Recommended Genotyping Procedures (RGPs) included in this document are a compilation of protocols from several laboratories (Muséum national d'Histoire naturelle, Paris/France; Swiss Tropical Institute, Basel/Switzerland; Karolinska Institute, Stockholm/Sweden; Ifakara Research Institute, Ifakara/Tanzania). These RGPs differ from SOPs in that they do not include the names of suppliers, product brand names because suppliers and products differ by countries, as do PCR conditions between laboratories. However, these forms can be easily transformed into SOPs by simply filling in the laboratory-specific supplier's names.

These procedures were initiated after experts requested SOPs for genotyping procedures during an informal consultation on "Methods and techniques for clinical trials on antimalarial drug efficacy: Genotyping to identify parasite populations", organized by the Medicines for Malaria and cosponsored by the World Health Organization, 29-31 May 2007, Amsterdam, The Netherlands.

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Notes on optimization of PCR/nested PCR reactions

It must be understood that a unique, universally optimal set of reaction and cycling conditions does not exist for a given pair of oligonucleotide primers that amplify a defined fragment. This is simply because on the one hand thermal cyclers and tubes vary in their characteristics, and, on the other hand, enzymes from different suppliers do not have similar properties (even if purified from the same bacterial stock) nor are the buffers supplied by different companies the same. Therefore, the conditions given in one publication or in a protocol (including SOPs) are not necessarily the ones that will work best in your laboratory.

The only way to optimize the reactions, or to be more precise, to tend towards standardization, is to use a set of control DNA templates with known concentrations of the target sequence: for example a solution where it is known that there are X parasite genomes per µl, or a filter paper with a dried blood drop containing a known number of parasite nuclei.

The optimal conditions (and thus across laboratories, the standard conditions) are those where one gets adequate amplification with the same amount of parasite genomes added in the PCR reaction. For *Plasmodium falciparum* nested PCR genotyping with the usual markers (*msp1*, *msp2* and *glurp*), this is about 10-100 parasite genomes, say an average of 50 parasite genomes. By adequate amplification, it is understood that a single band will be obtained when the DNA template used is purified from a cloned parasite in vitro culture line. In the case of nested PCR: (i) the intensity of the band obtained for the minimum number of parasite genomes (ca. 50) should be similar to that obtained for 5000 or even 50 000 parasite genomes, and (ii) artefact bands due to carry over of the primers added to the primary reaction should not be observed, though this might be difficult to avoid when high numbers of parasite genomes are added in the primary reaction (it would be possible to minimize this by purifying the primary reaction product, but this is costly in time and money). Limitation: Sensitivity of PCR is likely reduced in the presence of human DNA or of whole blood spotted on filter paper that is added directly to the reaction mix.

Annealing temperature and Mg$^{2+}$ concentrations are of course primary factors for specificity and sensitivity of amplification. In this context it should be remembered that using high concentrations of dNTP reduces the effective Mg$^{2+}$ concentration (a dNTP molecule chelates 1 Mg$^{2+}$ ion, thus if one uses 500 µmol/l of each dNTP, and 2 mmol/l Mg$^{2+}$, then effectively there is no Mg$^{2+}$ available for the enzyme at the beginning of the reaction).

Of equal importance is the concentration of the oligonucleotides in the primary reaction. In our experience, as the concentration of oligonucleotides diminishes one obtains cleaner and better amplifications. Apart from the obvious reduction in primer carry-over, this helps diminish non-specific amplification and this means that more of the reagents work to produce the true target. When using filter disks directly in PCR, as it is recommended in the RGPs below, primer concentration during optimization cannot be reduced to a low concentration equal as for using extracted DNA.

In conclusion, the way to optimize PCR is to use a set of defined DNA template standards prepared from cloned parasite lines: start with low primer concentrations followed by increasing primer concentrations at the recommended Mg$^{2+}$ and annealing temperature, and see what the products look like. We strongly advise that one changes one factor at a time if optimization is needed. This might be time consuming, but within a week or two one obtains the best conditions for one’s laboratory.
RGP 001: Sampling and storage of blood samples for genotyping using collection cards

Materials
- Commercially available specialized filter paper cards for blood collection.
  *(complete detailed suppliers and order number to create laboratory-specific SOP).*
  - Most cards are impregnated with a proprietary chemical formula that lyses cell membranes and denatures proteins on contact.
  - Nucleic acids are physically entrapped, immobilized and stabilized for storage at room temperature.
  - Cards protect nucleic acids from nucleases, oxidation, UV damage and microbial and fungal attack.
  - Infectious pathogens in samples applied to some cards are rendered inactive on contact.
  - Samples collected on cards and enclosed in a multi-barrier pouch can be shipped through the post.
  - Short or long term storage according to the supplier’s instructions. Collections cards are placed in a multi-barrier pouch together with desiccant at room temperature (for FTA® Whatman cards at room temperature; for Generation® Capture Cards (Gentra/Qiagen), short term room temperature, but long term –20 °C).

Handling instructions
- Always wear gloves when handling cards to avoid contamination of the cards.
- Store unused cards in a cool, dry place (avoid light and excessive humidity).
- Follow universal precautions when working with biological samples.

Sample application to collection cards
1. Label the filter paper with the appropriate sample identification. Do not add more than one patient onto one card.
2. Clean the finger using a swab with 70% alcohol and prick with a sterile lancet. Squeeze gently to obtain a drop of blood. Wipe this first drop off with a dry cotton wool. Squeeze gently to obtain a second drop of blood.
   As sampling on filter paper is usually done simultaneously with preparing a blood smear, use second drop for preparing a smear, and next drops for spotting on filter paper.
3. Drop blood onto the card in a concentric circular motion within the spotted circle (one 1 inch circle holds about 125 μl whole blood, roughly corresponding to 3 drops of blood). **Important note:** It is necessary that the entire area within the circle is covered with blood.
   Blood may also be drawn into a capillary tube (50-200 μl) to standardize the blood volume before it is added to the filter paper for storage. Whole blood collected with the following anticoagulants EDTA, sodium citrate, ACD can be applied with a pipette. Heparin should be avoided due to the risk of inhibition of PCR.
   Avoid “puddling” of the liquid sample as it will overload the chemicals on the card and do not rub or smear the blood onto the card.
4. Allow the samples to dry for about one hour at room temperature. Do NOT heat assist the sample drying step as this may fix PCR inhibitors onto the matrix.
5. Dried blood spots will appear much darker than freshly spotted ones.
6. Place collection card in a multi-barrier pouch together with desiccant and seal well. Storage at room temperature (optimal in dry and cool environment) until shipment.

Scientific publication

Scientific publications should include information on methods of blood sampling:
- Filter paper type including brand name (i.e. not only "collected on filter paper");
- Amount of blood (volume or number of drops) and/or corresponding blood volume analysed in PCR;
- Storage and transit conditions.

Shipping instructions

<table>
<thead>
<tr>
<th>Transport of shipment to institution where genotyping is performed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Requirements for shipments:</td>
</tr>
<tr>
<td>Frequency of shipment:</td>
</tr>
<tr>
<td>Transport:</td>
</tr>
<tr>
<td>Shipment conditions:</td>
</tr>
</tbody>
</table>
| Documentation included: | commercial invoice  
printed sheet with sample numbers included and to be analysed |

Address for shipment of specimens

<table>
<thead>
<tr>
<th>Key contacts at institution (list below)</th>
</tr>
</thead>
</table>
RGP 002: Sampling and storage of blood samples for genotyping using untreated filter papers

Materials

- Untreated filter paper.

*(complete detailed suppliers and order number to create laboratory-specific SOP).*

- Untreated filter papers, e.g. chromatography papers, may be used for sampling in the field although they are not manufactured for blood collection. The quality of DNA greatly depends on the filter papers and storage conditions.
- Filter papers are practical since they allow shipping and archiving at ambient temperature and are available at low cost but may not give as high quality DNA as collection cards or whole blood.
- Frequently used filter papers such as 3MM® chromatography paper or 903® paper (Whatman) are available as large sheets or small cards.
- Filter papers do not protect the samples from degradation of DNA by nucleases, oxidation, UV damage or microbial and fungal growth especially under humid conditions.

*It is crucial that filter paper samples are kept dry and sealed with desiccant during storage!*

Handling instructions

- Always wear gloves when handling filter paper samples to avoid contamination of the filter papers as well as risk of infectious agents.
- Store unused filter papers in a cool, dry place (avoid light and excessive humidity).
- Follow universal precautions when working with biological samples.
- Infectious pathogens (e.g. HIV, hepatitis) in samples collected on filter papers may be contagious on contact.

Sample application to filter papers

1. Label the filter paper with the appropriate sample identification. Do not add blood from more than one patient per filter paper.

2. Clean the finger using a swab with 70% alcohol and prick with a sterile lancet. Squeeze gently to obtain a drop of blood. Wipe this first drop off with a dry cotton wool. Squeeze gently to obtain a second drop of blood.

   As sampling on filter paper is usually done simultaneously with preparing a blood smear, use second drop for preparing a smear, and next drops for spotting on filter paper.

3. Drop the blood onto the filter paper, preferably three drops per spot. Avoid direct contact between the finger and the paper and do not press the finger onto the paper.

   Blood may also be drawn into a capillary tube (50-200 μl) to standardize the blood volume before it is added to the filter paper for storage. Whole blood collected with the following anticoagulants EDTA, sodium citrate, ACD can be applied with a pipette. Heparin should be avoided due to the risk of inhibition of PCR.

4. Let the blood dissolve in the paper. The size of the spot does not always correlate to the blood volume.

5. Allow the samples to dry for about one hour at room temperature or quickly in direct sunlight (do not leave for long in sunlight since it may damage DNA). Filter paper drying in sunlight is better than in the shade (humidity is the major problem). Do NOT heat-assist the sample drying step.
6. Place the filter papers in individual plastic bags* or envelops together with individual desiccant**. The samples must be fully dried before they are put into plastic bags.

7. Store in a humidity controlled, cool, dry environment (preferably in an air-conditioned room) until shipment. If dry room temperature is not possible, store in refrigerator or freezer but great care must be taken to protect against moisture.

* small zip-locked bags (often used in pharmacies for dispensing pills).
** desiccant pouches (silica gel) can be obtained in bulk.

Scientific publication

Scientific publications should include information on methods of blood sampling:

- Filter paper type including brand name (i.e. not only "collected on filter paper");
- Amount of blood (volume or number of drops) and/or corresponding blood volume analysed in PCR;
- Storage and transit conditions.

Shipping instructions

<table>
<thead>
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<td>Requirements for shipments:</td>
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<td>Frequency of shipment:</td>
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<tr>
<td>Transport:</td>
</tr>
<tr>
<td>Shipment conditions:</td>
</tr>
<tr>
<td>Documentation included:</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

Address for shipment of specimens

Key contacts at institution *(list below)*
RGP 003: Decision tree for genotyping *Plasmodium falciparum* (new infections versus recrudescences)

1. Receipt of samples
2. DNA preparation
   - ok
   - not ok
     - Notify sender
3. Multiplex pPCR
   - *msp1* & *msp2*
4. *msp2* nPCR
   - Worksheet 003
5. Agarose gel
   - ok
   - not ok
     - Repeat twice steps 2 to 5
6. *msp2* Hinf digest & RFLP analysis
   - or
   - Simple comparison of PCR fragments
   - Worksheet 003
7. DNA preparation & glurp pPCR & nPCR
   - Worksheet 005
8. Agarose gel
   - ok
   - not ok
     - Repeat twice steps 7 to 8
9. Analysis RGP 009
   - Worksheet 005
10. *msp1* nPCR
    - Worksheet 007
11. Agarose gel
    - ok
    - not ok
      - Repeat twice steps 10 to 11
12. Analysis RGP 009
    - Worksheet 007
13. RGP 014: Data analysis, outcome classification and reporting
RGP 004: DNA purification from collection cards

Receipt and registration of samples
- Follow procedures in Worksheet 001.

Storage of special filter paper collection cards
- Important: Consult supplier’s instructions, recommendations for different cards may vary.
- For most collection cards, store samples in a multi-barrier pouch together with desiccant at room temperature. Some suppliers recommend long term storage at –20 °C.
- See RGP 001.

DNA purification from 3 mm sample disks

Materials
(complete detailed suppliers and order number below to create laboratory-specific SOP).

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
<th>Product number</th>
<th>Storage temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specialized filter paper collection cards</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Puncher 1/8 inch</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Pipettes (1000 µl, 200 µl, 20 µl, 10 µl)</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>1.5 ml safe-lock tubes</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>500 µl reaction tubes</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>200 µl reaction tubes</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Filter tips (0.5-10 µl, 2-30 µl, 2-200 µl, 200-1000 µl)</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Ethanol absolute</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>DNA purification solution/paper disk wash buffer (specific for filter paper collection card)</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>96-well PCR plates</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
</tbody>
</table>

DNA purification procedure
- Please also consult supplier’s instructions.

Note: The following protocol is recommended for FTA® Whatman treated filter paper collection cards. Deviations from this protocol that are recommended when using Qiagen Generation® Capture Cards are included in brackets at the end of this section.

A 3 mm disk, approximately holding 3-5 µl of dried blood, can be purified in a 1.5 ml microfuge tube or in a 96-well plate.
1. Remove a sample from the treated filter paper card by punching a 3 mm disk with a clean hole-punch (for example, a standard 1/8” pliers type paper punch). To eliminate cross-contamination between donors, punch clean filter paper 3 times between donors.

2. Punch disk directly into a 1.5 ml microfuge tube. Alternatively, for processing large numbers of samples: place the 3 mm disk into a well of a 96-well plate and position plate in robotic workstation (alternatively, perform next steps manually in a 96-well plate using a multichannel pipette).

3. Add 200 µl of the card supplier-specific wash solution and incubate 5 min (for Generation® Capture Cards: 15 min) at room temperature; the DNA will remain bound to the disk while contaminants are released.

4. Mix by pipetting up and down 3 times and then remove as much solution as possible.

5. Repeat steps 3 and 4 twice more for a total of 3 washes with wash solution.

6. Add 200 µl 1x TE and incubate 5 min at room temperature.

7. Remove as much liquid as possible with a pipette.

8. Repeat steps 6 and 7 once.

9. Use humid filter disk straight in PCR or disk may be allowed to dry.

Note: Use washed FTA disk within 3 h at room temperature or store washed disk at +4 °C to –20 °C up to one week according to the supplier. Users have indicated that washed disks were successfully used even after storage of 2 weeks.

For Generation® Capture Cards an alternative protocol is recommended:

6. Add 200 µl 100% ethanol and incubate one minute at room temperature.

7. Remove as much alcohol as possible with a pipette.

8. Repeat steps 6 and 7 once.

9. It is important to dry disk well. Dry the disk containing the DNA at room temperature for at least 1-16 hours to evaporate the alcohol or at least one hour at 50 °C incubator.

After drying, sample disks should be orange to white in color. Purified disks are stable for at least 9 months at room temperature; for long-term storage, store at –20 °C.
RGP 005: DNA extraction from blood collected on untreated filter paper

Receipt and registration of samples
- Follow procedures in Worksheet 001.

Storage of untreated filter paper
- See RGP 002.

DNA extraction from untreated filter paper

Several methods for extracting DNA from blood collected on untreated filter paper are described below. The first is a simple, low cost method which has performed better than the Chelex and methanol methods (Bereczky et al., 2005) but for which long term storage of DNA is not recommended. Others are using commercially available preparation kits that are more costly but have the simplicity of following the manufacturers’ instructions and enables storage of DNA.

Furthermore semi-automated systems may also be used in well equipped laboratories with the need for high throughput of a large number of samples.

To achieve optimal sensitivity of detection from samples with low parasite densities it is important that the DNA template is sufficiently concentrated. Many protocols use small amount of blood and DNA is eluted in relatively large volumes. The template added to the PCR reaction should correspond to at least 0.5 μl of whole blood in each reaction.

TE-buffer method

1. Cut out 1-2 pieces, 3x3 mm or 4 mm diameter punch, from the dried blood spot.
2. Take necessary precautions to avoid cross-contamination between samples and contagious risk (see below).
3. Transfer the cut-out pieces to a 1.5 ml microfuge tube.
4. Add 65 μl of TE buffer [10 mmol/l Tris pH 8.0 (Tris base and Tris-HCl) and 0.1 mmol/l EDTA in distilled water - kept at room temperature]. Make sure that the whole paper-cuts are soaked in the TE buffer.
5. Incubate the tubes at 50 °C for 15 minutes.
6. Press the filter paper piece gently at the bottom of the tube several times using a new pipette tip for each piece. Press rather than smash the “swollen” papers in the TE buffer until the liquid becomes slightly red.
7. Heat at 97 °C in a heating block for 15 min to elute the DNA.
8. Centrifuge the tubes for a few seconds to remove condensation from the lid and the wall of the tube. The paper pieces are left in the tube.
9. Ready to use as template for PCR. Use 3-5 μl in a 20-50 μl total PCR master mix volume.
10. Store the samples at 4 °C until amplification which should be performed within 1-2 days. If samples need to be reanalysed later it is recommended to make a fresh extraction from new filter paper pieces. Long term storage at –20 °C has not been evaluated and should therefore not be recommended.
Commercial extraction methods

- For commercially available extraction kits, follow the manufacturer’s instructions. Update the laboratory-specific SOP to include these instructions and record manufacturer’s name and order number.
- Modification to the protocol may be performed if long DNA fragments are required (Sakihama et al., 2001) but it is usually unnecessary for the genetic sequences in the genotyping protocols.

Avoiding infectious risk

- Handling of filter papers requires the use of gloves to prevent contact with contagious agents (e.g. hepatitis, HIV). Also shipment needs to consider the infectious risk.

Avoiding cross-contamination

- Do not touch the blood with the fingers when manipulating the filter paper.
- Clean the tools used for preparing the filter paper pieces (e.g. scalpel blades, scissors, forceps, punch) in between contact with each sample. Start by putting the blade or scissors in water to dissolve the blood, thereafter soak in 5 mol/l HCl a few seconds, followed by neutralization in 5 mol/l NaOH, rinse again in water and dry properly by wiping with a clean tissue. By using two sets of scissors or scalpels you can alternate with one set being disinfected while using the other.
- If scalpel blades are used, the cuts should be done on a disposable surface, e.g. small yellow stickers (note pads). Remove two or three papers after each time (since the blade usually cuts through at least two papers of the note pad). The cuts can also be done on a glass plate which is cleaned with HCl as described above. The scalpel blade can be used to transfer the cut out filter paper pieces into the Eppendorf tube which minimizes the number of tools to clean.
- If you use the whole blood spot by cutting outside the spot it is also important to clean the tools properly between each sample.

References


Note: The recommended PCR protocols in the following sections are specifically designed for the use of collection cards. If DNA is extracted from untreated filter cards, as described above RGP 005, PCR mixes and cycling conditions will require adjustment.
RGP 006: Multiplex primary PCR for msp1 and msp2

Reagents and materials

*(complete detailed suppliers and order number to create laboratory-specific SOP)*.

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
<th>Product number</th>
<th>Storage temperature</th>
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</thead>
<tbody>
<tr>
<td>H₂O distilled (or Milli-Q)</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>10x PCR Buffer</td>
<td></td>
<td></td>
<td>−20 °C</td>
</tr>
<tr>
<td>dNTPs (nucleotides) 2 mmol/l</td>
<td></td>
<td></td>
<td>−20 °C</td>
</tr>
<tr>
<td>MgCl₂ 25 mmol/l</td>
<td></td>
<td></td>
<td>−20 °C</td>
</tr>
<tr>
<td>Taq polymerase I (5 U/µl)¹</td>
<td></td>
<td></td>
<td>−20 °C</td>
</tr>
<tr>
<td>Specific oligos (primer)</td>
<td></td>
<td></td>
<td>−20 °C</td>
</tr>
<tr>
<td>Tris EDTA (TE) buffer concentrate</td>
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<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Mineral oil²</td>
<td></td>
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</tr>
<tr>
<td>Pipettes (1000 µl, 200 µl, 20 µl, 10 µl)</td>
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<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>500 µl reaction tubes</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Filter tips (0.5-10 µl, 2-30 µl, 2-200 µl, 200-1000 µl)</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>96-well PCR plates³</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
</tbody>
</table>

¹ Several laboratories that have tested this protocol reported variation in sensitivity of PCR depending on the Taq enzyme used. We recommend that each user should test different enzymes to identify the optimal polymerase.

² Only if a thermal cycler is used that does not have a heated lid.

³ Depends on choice of single tubes or PCR plates. The latter allow using a multi-channel pipette.

Equipment required

*(complete detailed suppliers and order number to create laboratory-specific SOP)*.

- Thermocycler;
- Centrifuges for pre-PCR quick spin to collect mixture of master mix plus template on filter disk at bottom of tube/plate:
  - minifuge for tubes
  - centrifuge with adaptor for plates.
Preparation of dNTPs

Deoxynucleotides are degraded by repeated freeze-thawing. Therefore, aliquots of mixtures of all 4 dNTPs are prepared in a quantity specific for the needs and throughput of a particular lab. An aliquot should not be re-frozen more than twice. dNTP stock solution is made up in 1xTE with each nucleotide (dATP, dTTP, dGTP and dCTP) at a concentration of 2 mmol/l.

PCR primers for multiplex primary PCR for \textit{msp1} and \textit{msp2}

- PCR primers are dissolved in 1x TE (stock and working solution).
- Prepare stock solution with a concentration of 100 μmol/l and store at -20°C.
- For working solution, dilute stock solution to a concentration of 10 μmol/l. Keep aliquots at -20°C.
- Primers for primary PCR:

(\textit{complete supplier information to create laboratory-specific SOP}).

For \textit{msp1}

\begin{align*}
\text{M1-OF} & \quad 5'\text{-CTAGAAGCTTAAAAAGATGCAGTATTG-3'} \\
\text{M1-OR} & \quad 5'\text{-CTTAAATAGTATTCTAATTCAAGTGATCA-3'}
\end{align*}

For \textit{msp2}

\begin{align*}
\text{M2-OF} & \quad 5'\text{-ATGAAGGTAAATTAAAACATTGTCTATTATA-3'} \\
\text{M2-OR} & \quad 5'\text{-CTTTGTTACCATCGGTACATTCT-3'}
\end{align*}

Internal quality controls

**Control 1:** \textit{P. falciparum} 3D7, FC27 and RO33 in vitro culture strains spotted together on FTA collections cards. These three strains correspond to the three allelic families of \textit{msp1} as follows: 3D7 strain harbors a K1-type \textit{msp1} allele, FC27 strain harbors a Mad20 type \textit{msp1} allele, and Ro33 strain harbors the Ro33-type \textit{msp1} allele. There are two allelic families for \textit{msp2}: the 3D7 strain harbors a 3D7-type allele, the FC27 strain harbors a FC27-type allele. A mixture of these three positive controls will be provided from MMV or WHO on demand at two concentrations: (i) in such a concentration that a 3 mm filter disk approximately contains 200 parasites of each strain; and (ii), in a concentration that a 3 mm filter disk approximately contains 2000 parasites of each strain.

An alternative option to obtain control DNA is via Malaria Research and Reference Reagent Resource Center (www.mr4.org).

**Control 2:** No template control (NTC) = empty filter paper (without blood spot) that has gone through the washing procedure; this serves as negative control for all steps (punching, washing and master mix).

**Control 3:** \textit{P. falciparum} negative human DNA (whole blood; any source) = negative control

Scheme for applying these controls:

- **Control 1** (positive control) to be included in each PCR.
- **Control 2** (NTC) to be included in each PCR; if PCR is performed in 96-well plates, 4 NTCs randomly distributed in the plate are to be used.
- **Control 3** (human DNA) to be included only upon change of primer batch (to confirm specificity of new batch) or at least bi-annually (to check for potential mix ups). In endemic areas care must be taken for identifying a confirmed negative blood sample. Slide negative by microscopy is not sufficient. Negative by \textit{Plasmodium} species PCR is acceptable.
Change of lot numbers, batches and quality assurance

- A test PCR has to be performed when introducing a new batch of primers. nPCR results of the 2 positive controls (2 different concentrations) are recorded in Worksheet 004, or Worksheet 008, respectively, and compared to previous results for trend control (see RGP 013 quality assurance).

Master mix multiplex pPCR msp1&2 and pPCR conditions

**Note 1:** This protocol is designed for parasite DNA attached to a 3 mm disk that remains in the pPCR reaction; a minimum of 75 µl reactions is required to cover the entire disk in a 0.5 ml tube.

**Note 2:** For purified parasite DNA in solution (obtained by the Chelex extraction method or with a purification kit) the total volume should be reduced to save costs. Primer concentrations can be reduced to as low as 30-50 nmol/l final concentration for each primer. Such low primer concentrations are not suitable for PCR directly on filter disks. Low primary primer concentrations help to reduce artefact bands in nested PCR.

<table>
<thead>
<tr>
<th>Primary mix</th>
<th></th>
<th>Thermoprofile primary PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O distilled (or Milli-Q)</td>
<td>44 µl</td>
<td>94 °C - 2 min</td>
</tr>
<tr>
<td>10x PCR Buffer</td>
<td>7.5 µl</td>
<td>94 °C - 30 sec</td>
</tr>
<tr>
<td>dNTPs (2 mmol/l)</td>
<td>7.5 µl (200 µmol/l final conc.)</td>
<td>54 °C - 1 min</td>
</tr>
<tr>
<td>MgCl₂ (25 mmol/l)</td>
<td>6 µl (2 mmol/l final conc.)</td>
<td>30 cycles¹</td>
</tr>
<tr>
<td>fw primer M1-OF</td>
<td>2.25 µl (300 nmol/l final conc.)</td>
<td>72 °C - 1 min</td>
</tr>
<tr>
<td>fw primer M2-OF</td>
<td>2.25 µl (300 nmol/l final conc.)</td>
<td>72 °C - 5 min</td>
</tr>
<tr>
<td>rev primer M1-OR</td>
<td>2.25 µl (300 nmol/l final conc.)</td>
<td>Then go to room temperature</td>
</tr>
<tr>
<td>rev primer M2-OR</td>
<td>2.25 µl (300 nmol/l final conc.)</td>
<td></td>
</tr>
<tr>
<td>Taq Polymerase (5 U/µl)</td>
<td>1 µl</td>
<td></td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>75 µl</strong></td>
<td></td>
</tr>
</tbody>
</table>

¹As the performance of different thermal cyclers can differ substantially, the suggested PCR profile and cycle number requires optimization to the conditions in each laboratory.

- Fill out Worksheet 002.
- Prepare master mix for all samples to be amplified (plus positive and no-template controls) according to 2.3. in a template-free room dedicated to PCR with dedicated no-template pipettes; use aerosol protected pipette tips.
- For adding the master mix to the template (on disks), change workspace (bench permitted for working with template). Add 75 µl to the reaction tubes or to a 96-well plate that already contains the washed and dried blood collection disks.
- If thermocycler lacks a heated lid, overlay reaction with 2 drops of mineral oil.
- To decrease risk of contamination, quick spin all tubes and plates containing filter disk before opening tubes or removing caps or PCR film from plates.

After this multiplex pPCR is completed, continue with RGP 007 for msp2 nPCR. Only in case of a recrudescence, msp1 nPCR will be performed at a later stage (after genotyping of glurp) according to RGP 011.
Reference

RGP 007: *msp2* family-specific nested PCR

Pre-PCR procedures and multiplex pPCR are described in RGP 004 and RGP 006.

Reagents and materials
*(complete detailed suppliers and order number to create laboratory-specific SOP).*

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
<th>Product number</th>
<th>Storage temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O distilled (or Milli-Q)</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>10x PCR Buffer</td>
<td></td>
<td></td>
<td>–20 ºC</td>
</tr>
<tr>
<td>dNTPs (nucleotides) each 2 mmol/l</td>
<td></td>
<td></td>
<td>–20 ºC</td>
</tr>
<tr>
<td>MgCl₂ 25 mmol/l</td>
<td></td>
<td></td>
<td>–20 ºC</td>
</tr>
<tr>
<td>Taq Polymerase I (5 U/µl)</td>
<td></td>
<td></td>
<td>–20 ºC</td>
</tr>
<tr>
<td>Specific oligos (primer)</td>
<td></td>
<td></td>
<td>–20 ºC</td>
</tr>
<tr>
<td>Tris EDTA (TE) buffer concentrate</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Mineral oil¹</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Pipettes (1000 µl, 200 µl, 20 µl, 10 µl)</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>500 µl reaction tubes</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Filter tips (0.5-10 µl, 2-30 µl, 2-200 µl,</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>200-1000 µl)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>96-well PCR plate</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
</tbody>
</table>

¹Only if a thermal cycler is used that does not have a heated lid.

Equipment required
*(complete detailed suppliers and order number to create laboratory-specific SOP).*

- Thermocycler;
- Centrifuges for pre-PCR quick spin to collect mixture of master mix plus template at bottom of tube/plate:
  - minifuge for tubes
  - centrifuge with adaptor for plates.

nPCR primers

- PCR primers are dissolved in 1x TE (stock and working solution).
- Prepare stock solution with a concentration of 100 µmol/l.
- For working solution, dilute stock solution to a concentration of 10 µmol/l. Keep aliquots at –20 ºC.
- Primers for semi-nested family-specific PCR (Falk et al., 2006):
  - S1fw: 5’- GCT TAT AAT ATG AGT ATA AGG AGA A -3’
  - M5rev: 5’- GCA TTG CCA GAA CTT GAA-3’
  - N5rev: 5’ - CTG AAG AGG TAC TGG TAG A-3’

Internal quality controls
- See RGP 006.

Change of lot numbers, batches and quality assurance
- A test PCR has to be performed when introducing a new batch of primers. nPCR results of the positive controls (different concentrations) are recorded in Worksheet 004 msp2 and compared to previous results for trend control (see RGP 013 quality assurance).

Master mix family-specific nested msp2 PCR and PCR conditions

<table>
<thead>
<tr>
<th>Nested Mix</th>
<th>Thermoprofile nested PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O distilled (or Milli-Q)</td>
<td>94 ºC - 2 min</td>
</tr>
<tr>
<td>10x buffer</td>
<td>32.5 µl</td>
</tr>
<tr>
<td>5.0 µl</td>
<td>94 ºC - 30 s</td>
</tr>
<tr>
<td>dNTPs (2 mmol/l)</td>
<td>5.0 µl (200 µmol/l)</td>
</tr>
<tr>
<td>5.0 µl</td>
<td>50 ºC - 45 s</td>
</tr>
<tr>
<td>MgCl2 (25 mmol/l)</td>
<td>3.0 µl (1.5 mmol/l)</td>
</tr>
<tr>
<td>3.0 µl</td>
<td>70 ºC - 1 min 30 s</td>
</tr>
<tr>
<td>S1fw Primer (10 µmol/l)</td>
<td>1.5 µl (300 nmol/l)</td>
</tr>
<tr>
<td>1.5 µl</td>
<td>70 ºC - 5 min</td>
</tr>
<tr>
<td>Family-specific reverse primer</td>
<td>1.5 µl (300 nmol/l)</td>
</tr>
<tr>
<td>N5 or M5 (10 µmol/l)</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>Taq polymerase (5 U/µl)</td>
<td>49 µl²</td>
</tr>
<tr>
<td>Total volume</td>
<td>49 µl²</td>
</tr>
<tr>
<td>primary PCR product is added to nPCR</td>
<td>1 µl</td>
</tr>
</tbody>
</table>

1As the performance of different thermal cyclers can differ substantially, the suggested PCR profile and cycle number requires optimization to the conditions in each laboratory.
2 50 µl volume is required for subsequent RFLP analysis.

- Fill out Worksheet 003.
- Prepare two master mixes for the amplification of 3D7-type and FC27-type alleles in a separate tube for all samples to be amplified, plus for one positive control for each family-specific PCR. The positive control is a mixture of the following three in vitro culture strains: 3D7, which carries a 3D7 msp2 allele; the FC27, which carries the FC27 msp2 allele; and RO33 which also carries a 3D7-type allele. Master mixes should be prepared in a template-free room dedicated to PCR with dedicated no-template pipettes: use aerosol protected pipette tips.
- Aliquot master mix to reaction tubes or 96-well plate in a template-free room. Change workspace for adding template (pPCR product).
- If thermocycler lacks a heated lid, overlay reaction with 2 drops of mineral oil. The primary PCR product is added through the oil layer to the reaction mixture. Use for each DNA sample a separate tip.

*Note: To decrease risk of contamination, quick spin all tubes and plates containing extracted DNA or PCR product before opening tubes or removing caps or PCR film from plates. Condensation of liquid from hot PCR reactions can cause contamination problems upon opening of tubes or plates. Opening must be done without any delay after the centrifugation step.*

**Post-PCR procedures**

**Reagents and material**

*(complete detailed suppliers and order number to create laboratory-specific SOP).*

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
<th>Product number</th>
<th>Storage temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O distilled (or Milli-Q)</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Restriction enzyme Hinfl</td>
<td></td>
<td></td>
<td>−20 ºC</td>
</tr>
<tr>
<td>10x Buffer for restriction enzyme</td>
<td></td>
<td></td>
<td>−20 ºC</td>
</tr>
<tr>
<td>Glycerol</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>HCl 37%</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>EDTA</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Trizma base</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Boric acid</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Xylene cyanol FF</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Acrylamide/bisacrylamide (37.5:1)</td>
<td></td>
<td></td>
<td>+4 ºC</td>
</tr>
<tr>
<td>Ammonium persulfate</td>
<td></td>
<td></td>
<td>+4 ºC</td>
</tr>
<tr>
<td>TEMED</td>
<td></td>
<td></td>
<td>+4 ºC</td>
</tr>
<tr>
<td>1 kb ladder</td>
<td></td>
<td></td>
<td>+4 ºC</td>
</tr>
<tr>
<td>Agarose</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
</tbody>
</table>

**Equipment required**

- Gel documentation system Microwave
- Mini gel apparatus.

**Preparation of 5x TBE buffer stock**

- 54 g Tris
- 27.5 g boric acid
- 20 ml 0.5 mol/l EDTA pH 8.0
- H₂O distilled (or Milli-Q) add up to 1l solution.

**Preparation of blue juice**
- 30% glycerol
- 10 mmol/l Tris-HCl pH 7.5
- 10 mmol/l EDTA pH 8.0
- 0.25% of both bromophenol blue and xylene cyanol.

**Preparation of the 1kb ladder (0. 1 µg/µl)**
- 600 µl H₂O distilled (or Milli-Q)
- 300 µl blue juice
- 100 µl 1 kb ladder stock (stock: 1 µg/µl).

**Preparation of the ethidium bromide staining solution**
(for staining of gel after electrophoresis)
- 10 µl of a 10 mg/ml ethidium bromide stock solution (stable for 1 year at +4 °C in a dark container)
- 100 ml 0.5x TBE.

(staining solution can be reused and kept for about 2 weeks in a dark container at room temperature)

**Electrophoresis**

**Preparation of an agarose gel 2%**
- Take 0.5x TBE buffer and dissolve agarose (min gel: 50 ml 0.5x TBE + 1g agarose) by heating up the mixture in microwave until boiling and all agarose particles are dissolved. Cool to 50 °C before pouring in gel casting mold. Allow the cast gel to set for 30 min at room temperature before removing the well-forming combs and using.

**Gel electrophoresis of msp2 PCR products**
- After the nested PCR run the amplified product is checked on a 2% agarose gel. Take 4 µl of the nested PCR product and mix with 1 µl blue juice.
- In case no RFLP analysis is performed, the PCR products amplified from the paired samples obtained from a patient must be run side by side on the gel in order to obtain the optimum comparison.
- There should be a minimum of two sets of markers per gel.
- Gels are stained for 30 min by total immersion in the ethidium bromide staining solution and then destained in water for 15 minutes (this improves visibility of bands), then visualized in a 312 nm UV transilluminator and documented using an electronic photographic documentation system.

**Alternative gel protocol (recommended by G. Snounou)**
- For preparation of a 2% high resolution agarose gel follow RGP 012.

**Analysis**
- For PCR-RFLP analysis of msp2 continue with RGP 008.
- For simple sizing and binning PCR products proceed according to RGP 009.
- Printouts of all documented gels are added to Worksheet 003.
- Results are to be listed in Worksheet 003.
- Follow RGP 014 (data analysis) for definition of recrudescence versus new infection and procedures for reporting genotyping data and extra information.
**Further data analysis & reporting**

- Continue genotyping procedure according to **RGP 003** (decision tree).
- Follow up samples showing a new infection will not be processed further.
- Follow up samples showing a recrudescence will be subject to *glurp* genotyping **RGP 010**.

Reference

RGP 008: *msp2* genotyping by PCR-RFLP on family-specific PCR products

Follow RGP 006 and RGP 007 for primary and nested PCR of *msp2*. To increase the discrimination power of *msp2* genotyping, all positive samples are subject to restriction digest followed by fragment analysis on high percentage agarose gels or preferably on polyacrylamide (PAA) gels.

Reagents and materials

*(complete detailed suppliers and order number to create laboratory-specific SOP)*.

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
<th>Product nr</th>
<th>Storage temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipettes (1000 µl, 200 µl, 20 µl)</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Pipette tips 2-200µl, 200-1000µl</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>500 µl reaction tubes</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>H₂O distilled (or Milli-Q)</td>
<td></td>
<td></td>
<td>No storage</td>
</tr>
<tr>
<td>Restriction enzyme Hinf I</td>
<td></td>
<td></td>
<td>–20 ºC</td>
</tr>
<tr>
<td>10x Buffer Hinf I</td>
<td></td>
<td></td>
<td>–20 ºC</td>
</tr>
<tr>
<td>Restriction enzyme Dde I</td>
<td></td>
<td></td>
<td>–20 ºC</td>
</tr>
<tr>
<td>10x buffer Dde I</td>
<td></td>
<td></td>
<td>–20 ºC</td>
</tr>
<tr>
<td>Restriction enzyme ScrF I</td>
<td></td>
<td></td>
<td>–20 ºC</td>
</tr>
<tr>
<td>10x Buffer ScrF I</td>
<td></td>
<td></td>
<td>–20 ºC</td>
</tr>
<tr>
<td>EDTA</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Trizma base</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Boric acid</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Acrylamide/bisacrylamide (37.5:1)</td>
<td></td>
<td></td>
<td>+4 ºC</td>
</tr>
<tr>
<td>Ammonium persulfate</td>
<td></td>
<td></td>
<td>+4 ºC</td>
</tr>
<tr>
<td>TEMED</td>
<td></td>
<td></td>
<td>+4 ºC</td>
</tr>
<tr>
<td>1 kb ladder</td>
<td></td>
<td></td>
<td>+4 ºC</td>
</tr>
</tbody>
</table>

Equipment required

- Vertical gel electrophoresis system
- Digital gel documentation system.
Restriction digest with Hinf I for PCR-RFLP

Note: Only samples with amplified fragments are subject to digestion. It is quite possible with samples from study areas of low multiplicity of infection (MOI), that only one of the two family-specific nPCRs yields one or more nPCR products.

- Digest 7 µl of nested PCR product with the restriction enzyme Hinf I.

<table>
<thead>
<tr>
<th>Reaction mix (total volume 20 µl)</th>
<th>7 µl nested PCR product</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10.5 µl H₂O distilled (or Milli-Q)</td>
</tr>
<tr>
<td></td>
<td>2 µl 10x Hinf I restriction enzyme buffer</td>
</tr>
<tr>
<td></td>
<td>0.5 µl (5 units) Hinf I</td>
</tr>
</tbody>
</table>

- A master mix is prepared for all digests and aliquots are added to the nested PCR products. After 2 h incubation at 37 °C, add 5 µl blue juice to stop the reaction and load 20 µl of the total volume on a 10% PAA gel (see below).

Electrophoresis

The preferable method to separate small restriction fragments is by polyacrylamide gel electrophoresis.

**Preparation of a PAA gel 10%**

For pouring 1 vertical PAA gel

- 17 ml H₂O distilled (or Milli-Q)
- 10 ml 5x TBE
- 15 ml acrylamide/bisacrylamide
- 20 µl TEMED
- 500 µl of 10% ammonium persulfate.

**Electrophoresis of 10% PAA gel**

- As length standard 1.5 µg of 1 kb ladder is used. PAA Gels are run in 1x TBE buffer (1:5 diluted stock solution) in a vertical gel electrophoresis apparatus for 2.5 h at 200 V.
- Staining of the gel for 15 min in ethidium bromide staining solution.
- Visualize on a 312 nm UV plate (UVP transilluminator).
- Save under unequivocal identifier. Printouts of all documented gels are added to Worksheet 003.

**Alternative gel protocols**

- If no setup is available for PAA gel electrophoresis, either a standard 3.5% agarose gel can be used. Alternatively, for preparation of high resolution agarose gels follow RGP 012.

Analysis

- Follow RGP 014 (data analysis) for definition of recrudescence versus new infection and procedures for reporting genotyping data and extra information.

**FC27-type alleles**

- PCR amplified with S1fw plus M5 rev (FC27-type alleles) are digested with Hinf I. There are two or three resulting restriction fragments per allele. See Figure 1 for a graphical representation of the repeat units and Hinf I restriction sites.
- 96 bp fragment: Three fragments only occur, if at least 2 copies of the 96 bp repeat are present. This is indicated by a 96 bp fragment. More than two copies of the 96 bp repeat result in a fragment of double intensity relative to the other fragments of this allele.

- The fragment at the 5’ end is 148 bp in most of the FC27-type alleles. However, in Africa several new alleles have been observed that varied in this fragment due to duplication of a 9 bp repeat. But most of these variants are very rare.

- The fragments so far observed at the 3’ end of the PCR product differ from each other only by the number of 36 bp repeats. In multiple infections, this results in a ladder of these fragments.

**PCR-RFLP of FC27 allelic family**

![Diagram of PCR-RFLP of FC27 allelic family](image)

**Figure 1.** Organization of the 96 bp and 36 bp repeats and Hinf I restriction sites of PCR products of the most frequent FC27-type msp2 alleles. The variable Hinf I fragment at the 3’ end of FC27-type alleles varies according to the copy number of the 36 bp repeat. Note that if one 96 bp fragment appears in a Hinf I digest, in actual fact two 96 bp repeats are present in the allele. H: Hinf I restriction site; S1 fw and M5 rev: location of nested PCR primers, whereby only M5 targets a family-specific region.

**3D7-type alleles**

- PCR amplified with S1fw plus N5 rev (3D7-type alleles) is digested with Dde I, and for optimal resolution a further separate digest is performed with ScrF I (no double digest). See Figure 2 for a graphical representation of the repeat units and Dde I and ScrF I restriction sites. Field surveys showed that about 72% of all 3D7-type alleles are cut by both enzymes. About 20% of 3D7 type alleles were not cut by ScrF I, and 8% were not cut by Dde I (Felger et al. 1999; Flück et al., 2007).
**Figure 2:** Structural organization of PCR products of 3D7-type *msp2* alleles. The repeats, indicated as boxes, vary in size (4 to 10 amino acids), sequence and copy number (1 to 15 copies), and are often in a scrambled array. S1 fw and N5 rev: nested primers, depicted as arrows, whereas only the reverse primer N5 targets a family-specific sequence. S1 fw primer is located in the 5’ conserved region. Poly T: polythreonine stretch responsible for further size variation; S = ScrF1 restriction site; D = Ddel. The few size-variants of semi-conserved ScrF1 and Ddel restriction fragments at the 3’ end that have been observed so far are shown in base pairs (bp).

- Results of the PCR-RFLP analysis are to be listed in **Worksheet 003**.

Further data analysis & reporting

- Continue genotyping procedure with other marker genes according to **RGP 003** (decision tree).
- Follow up samples showing a new infection will not be processed further.
- Follow up samples showing a recrudescence will be subject to *glurp* genotyping **RGP 010**.

References


RGP 009: Sizing of individual nPCR fragments

Material
- Gel documentation system.

Procedure
- Agarose gels (standard electrophoresis grade or high resolution agarose) are made and stained according to **RGP 007** or **RGP 012**, respectively.
- The MMV/WHO consensus meeting made the following recommendation: Interpretation of bands in agarose gels should be performed by software on digitized images or, if this is not available, by two independent experienced readers.

Size determination by gel documentation software
- For documenting gels it is recommended to use a digital camera in combination with a gel documentation system. In many programs, it is possible to determine the size of the bands automatically. This automated procedure has the advantage of being unbiased by the experimenter. (Care must be taken to correcting gel smiles or distortions, some software are not capable of doing this.)

(Insert here the procedures for size determination of the analysis system used in your laboratory).
- An example for procedures applied together with a commercial system could be the following: Using the xxx gel documentation system with the xyz software, select the Tool Box “Analysis Tools” and then “Mol. Weight”. A function box appears where the used molecular weight marker can be selected from a standard library, or the size of the bands of the marker in use can be recorded manually. After selecting “Add Band” in the “Query” menu, the cursor can be pointed on the band of interest, and by clicking the size of the band is displayed in the function box.

Visual determination of fragment size
- If a gel documentation system with corresponding software is not available for sizing individual fragments, bins need to be defined on an enlarged gel picture, and a ruler to be used to define areas for individual bins of about 20 bp. The smaller the bins the higher is the resolution of the genotyping technique. The gel pictures are enlarged on an A4 paper to increase the accuracy of migration distance measurements.
- Greatest care is required for determining fragment sizes. Poor resolution of fragments results in apparent little diversity of the marker gene and thus increases the number of false recrudescences.

Interpretation of the genotype patterns
- PCR products amplified from the paired samples obtained from a patient must be run side by side on the gel in order to obtain the optimal comparison. The genotype pattern for each sample consists of the different DNA bands seen in each lane. If the genotype pattern for the two paired samples of a study participant are the same or any bands are shared between the two samples, then the interpretation is recrudescence. A new infection is indicated when the patterns are completely different. This definition is explained in great detail in Appendix 2 of the WHO/MMV document “Methods and techniques for clinical trials on antimalarial drug efficacy: Genotyping to identify parasite populations”.

Analysis and reporting
- Follow **RGP 014**.
RGP 010: *glurp* primary and nested PCR

*Note: According to RGP 003, msp1 genotyping is only to be performed if preceding msp2 and glurp typing have indicated a recrudescence.*

Pre-PCR procedures are described in RGP 004.

**Reagents and materials**

*(complete detailed suppliers and order number to create laboratory-specific SOP)*

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
<th>Product number</th>
<th>Storage temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O distilled (or Milli-Q)</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>10x PCR buffer</td>
<td></td>
<td></td>
<td>−20 °C</td>
</tr>
<tr>
<td>dNTPs (nucleotides) 2 mmol/l</td>
<td></td>
<td></td>
<td>−20 °C</td>
</tr>
<tr>
<td>MgCl₂ 25 mmol/l</td>
<td></td>
<td></td>
<td>−20 °C</td>
</tr>
<tr>
<td>Taq polymerase I (5 U/µl)</td>
<td></td>
<td></td>
<td>−20 °C</td>
</tr>
<tr>
<td>Specific oligos (primer)</td>
<td></td>
<td></td>
<td>−20 °C</td>
</tr>
<tr>
<td>Tris EDTA (TE buffer)</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Mineral oil¹</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Pipettes (1000 µl, 200 µl, 20 µl, 10 µl)</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>500 µl reaction tubes</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Filter tips (0.5-10 µl, 2-30 µl, 2-200 µl, 200-1000 µl)</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>96-well PCR plate</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
</tbody>
</table>

¹ Only if a thermal cycler is used that does not have a heated lid.

**Equipment required**

*(complete detailed suppliers and order number to create laboratory-specific SOP)*

- Thermocycler;
- Centrifuges for pre-PCR quick spin to collect mixture of master mix plus template on filter disk at bottom of tube/plate: minifuge for tubes; if plates are used, a centrifuge with adaptor for plates is needed.
Recommended Genotyping Procedures (RGPs) to identify parasite populations version 1 - October 2008

Preparation of dNTPs

Deoxynucleotides are degraded by repeated freeze-thawing. Therefore, aliquots of mixtures of all 4 dNTPs are prepared in a quantity specific for the needs and throughput of a particular lab. An aliquot should not be re-frozen more than twice. dNTP stock solution is made up in 1xTE with each nucleotide (dATP, dTTP, dGTP and dCTP) at a concentration of 2 mmol/l.

PCR primers (according to Snounou 2002 and G. Sounou personal communication)

- PCR primers are dissolved in 1x TE (stock and working solution).
- Prepare primer stock solution with a concentration of 100 µM.
- For working solution, dilute stock solution to a concentration of 10 µmol/l. Keep aliquots at -20 ºC.
- Primers for primary PCR glurp:
  - G-F3: 5’- ACATGCAAGTGGTATCCTGAAG -3’
  - G-F4: 5’-TGTAGGTACCACGGGTTCTTGTGG-3’ (same as nested primer)
- Primer for nested PCR glurp:
  - G-NF: 5’-TGTTCACTGACAAATTAGTTTAGATCA -3’
  - G-F4: 5’-TGTAGGTACCACGGGTTCTTGTGG-3’ (same as primary primer)

Internal quality controls

**Control 1**: *P. falciparum* 3D7, FC27 and RO33 in vitro culture strains spotted together on FTA collections cards. A mixture of these three positive controls will be provided from MMV or WHO on demand at two concentrations: (i) in such a concentration that a 3 mm filter disk approximately contains 600 parasites; and (ii), in a concentration that a 3 mm filter disk approximately contains 6000 parasites.

*Note: The single pair of nested glurp primers derive from conserved regions of the gene and amplify all in vitro culture strains.*

An alternative option to obtain control DNA is via Malaria Research and Reference Reagent Resource Center (www.mr4.org).

**Control 2**: No template control (NTC) = empty filter paper (without blood spot) that has gone through the washing procedure; this serves as negative control for all steps (punching, washing and master mix).

**Control 3**: *P. falciparum* negative human DNA (whole blood; any source) = negative control

Scheme for applying these controls:

- **Control 1** (positive control) to be included in each PCR
- **Control 2** (NTC) to be included in each PCR; if PCR is performed in 96-well plates, 4 NTCs randomly distributed in the plate are to be used.
- **Control 3** (human DNA) to be included only upon change of primer batch (to confirm specificity of new batch) or at least bi-annually (to check for potential mix ups). In endemic areas care must be taken for identifying a confirmed negative blood sample. Slide negative by microscopy is not sufficient. Negative by *Plasmodium* species PCR is acceptable.

Change of lot numbers, batches and quality assurance

- A test PCR has to be performed when introducing a new batch of primers. nPCR results of positive controls (mixed in vitro culture strains in different concentrations) are recorded in **Worksheet 006** and compared to previous results for trend control and compared to previous results for trend control (see **RGP 013** quality assurance).
Master mix *glurp* pPCR and pPCR conditions

*Note: This protocol is designed for parasite DNA attached to a 3 mm disk that remains in the pPCR reaction; a minimum of 75 µl reactions are required to cover the entire disk in a 0.5 ml tube.*

<table>
<thead>
<tr>
<th>Primary mix</th>
<th>Thermoprofile primary PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O distilled (or Milli-Q)</td>
<td>94 °C - 2 min</td>
</tr>
<tr>
<td>10x PCR buffer</td>
<td>94 °C - 30 sec</td>
</tr>
<tr>
<td>dNTPs 2 mmol/l</td>
<td>54 °C - 1 min</td>
</tr>
<tr>
<td>MgCl₂ 25 mmol/l</td>
<td>72 °C - 1 min</td>
</tr>
<tr>
<td>G-F3 Primer (10 µmol/l)</td>
<td></td>
</tr>
<tr>
<td>G-F4 Primer (10 µmol/l)</td>
<td></td>
</tr>
<tr>
<td>Taq polymerase (5 U/µl)</td>
<td></td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>’75 µl</strong></td>
</tr>
</tbody>
</table>

1 As the performance of different thermal cyclers can differ substantially, the suggested PCR profile might require optimization to the conditions in each laboratory.

- Fill out Worksheet 005.
- Prepare master mix for all samples to be amplified (plus a positive and several no template controls; per plate 4 negative no-template controls recommended) in a template-free room dedicated to PCR with dedicated no template pipettes; use aerosol protected pipette tips.
- For adding the master mix to the template (on disks), change workspace (bench permitted for working with template). Add 75 µl to the reaction tubes or to a 96 well plate that already contains the washed and dried blood collection disks.
- If thermo cycler lacks a heated lid, overlay reaction with 2 drops of mineral oil.
- To decrease risk of contamination, quick spin all tubes and plates containing extracted DNA or PCR product before opening tubes or removing caps or PCR film from plates.

Master mix *glurp* nested PCR and PCR conditions

<table>
<thead>
<tr>
<th>Nested mix</th>
<th>Thermoprofile nested PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O distilled (or Milli-Q)</td>
<td>94 °C - 2 min</td>
</tr>
<tr>
<td>10x PCR buffer</td>
<td>94 °C - 30 sec</td>
</tr>
<tr>
<td>dNTPs (2 mmol/l)</td>
<td>59 °C - 1 min</td>
</tr>
<tr>
<td>MgCl₂ (25 mmol/l)</td>
<td>72 °C - 1 min</td>
</tr>
<tr>
<td>G-NF Primer (10 µmol/l)</td>
<td></td>
</tr>
<tr>
<td>G-F4 Primer (10 µmol/l)</td>
<td></td>
</tr>
<tr>
<td>Taq polymerase (5 U/µl)</td>
<td></td>
</tr>
<tr>
<td><strong>Total volume master mix</strong></td>
<td><strong>49 µl</strong></td>
</tr>
</tbody>
</table>

1 As the performance of different thermal cyclers can differ substantially, the suggested PCR profile might require optimization to the conditions in each laboratory.
Recommended Genotyping Procedures (RGPs) to identify parasite populations  version 1 - October 2008

| primary PCR product is added to nPCR | 1 µl |

1 As the performance of different thermal cyclers can differ substantially, the suggested PCR profile might require optimization to the conditions in each laboratory.
2 Reaction works well in a volume of 20 µl. But this requires precision pipettes and 96 well plates or 0.2 ml or 0.1 ml reaction tubes.

- Fill out Worksheet 005.
- Prepare master mix for all samples to be amplified (plus positive and no template controls) in a template-free room dedicated to PCR with no template pipettes; use aerosol protected pipette tips.
- Aliquot master mix to reaction tubes or 96 well plate in a template-free room. Change workspace for adding template (pPCR product).
- If thermo cycler lacks a heated lid, overlay reaction with 2 drops of mineral oil. The primary PCR product is added through the oil layer to the reaction mixture. Use for each DNA sample a separate tip.
- To decrease risk of contamination, quick spin all tubes and plates containing primary PCR product before opening tubes or removing caps or PCR film from plates.

Post-PCR procedures

**Reagents and material**

*(complete detailed suppliers and order number to create laboratory-specific SOP)*.

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
<th>Product number</th>
<th>Storage temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O distilled (or Milli-Q)</td>
<td>Room temperature</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>Room temperature</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCl 37%</td>
<td>Room temperature</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>Room temperature</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trizma base</td>
<td>Room temperature</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boric acid</td>
<td>Room temperature</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>Room temperature</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xylene cyanol FF</td>
<td>Room temperature</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 kb ladder</td>
<td>+4 ºC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agarose</td>
<td>Room temperature</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>+4 ºC/dark</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Equipment required**

- Gel documentation system
- Microwave
• Mini gel apparatus.

Preparation of 5x TBE buffer stock
• 54 g Tris
• 27.5 g boric acid
• 20 ml 0.5 mol/l EDTA pH 8.0
• H₂O distilled (or Milli-Q) add up to 1 l solution.

Preparation of blue juice
• 30% glycerol
• 10 mmol/l Tris-HCl pH 7.5
• 10 mmol/l EDTA pH 8.0
• 0.25% of both bromophenol blue and xylene cyanol.

Preparation of the 1kb ladder (0.1 µg/µl)
• 600 µl H₂O distilled (or Milli-Q)
• 300 µl blue juice
• 100 µl 1 kb ladder stock (stock: 1 µg/µl).

Preparation of the ethidium bromide staining solution
(for staining of gel after electrophoresis)
• 10 µl of a 10 mg/ml ethidium bromide stock solution
• 100 ml 1x TBE.

(The staining solution can be kept for maximum 2 weeks in a dark container)

Electrophoresis
• The glurp allelic variants range between approximately 1200 bp and 600 bp. Resolution of these variants can be achieved using 2% agarose.

Preparation of an agarose gel 2%
• Take 0.5x TBE buffer and dissolve agarose (mini gel: 50 ml 0.5x TBE + 1 g agarose) by heating up the mixture in microwave until boiling and all agarose particles are dissolved. Cool to +50 °C before pouring in gel casting mold. Allow the cast gel to set for 30 minutes at room temperature before removing the well-forming combs and using.

Gel electrophoresis of nPCR products
• After the nested PCR run the amplified product is run on a 2% agarose gel. Take 4 µl of the nested PCR product and mix with 1 µl blue juice.
• PCR product amplified from the paired samples obtained from a patient must be run side by side on the gels in order to obtain the optimum comparison.
• Gels are stained for 30 min by total immersion in the ethidium bromide staining solution and then destained in water for 15 minutes, then visualised in a 312 nm UV transilluminator, and documented using an electronic photographic documentation system.

Analysis of gels
• Printouts of all documented gels are added to Worksheet 005.
• glurp consists of a constant region within which two repeat regions are found. The repeat region RII harbours a repeat unit of approximately 60 bp, which is present in a variable copy number in different parasite lines.
• For sizing and binning PCR products and for interpretation of gels proceed according to RGP 009.
• Follow RGP 014 on procedures for reporting genotyping data and provision of extra information.
• Results are to be listed in Worksheet 005.

Further data analysis & reporting
• Continue genotyping procedure according to RGP 003 (decision tree).
• Follow up samples showing a new infection compared to the corresponding baseline sample will not be processed further.
• Follow up samples showing a recrudescence will be subject to msp1 family-specific nPCR genotyping, RGP 011.

Reference
RGP 011: *msp1* family-specific nested PCR

*Note: According to RGP 003, msp1 genotyping is only to be performed if preceding msp2 and glurp typing have indicated a recrudescence.*

Pre-PCR procedures and multiplex pPCR are described in RGP 004 and RGP 006.

Reagents and materials

*(complete detailed suppliers and order number to create laboratory-specific SOP).*

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
<th>Product number</th>
<th>Storage temperature</th>
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</thead>
<tbody>
<tr>
<td>H₂O distilled (or Milli-Q)</td>
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<td>Room temperature</td>
</tr>
<tr>
<td>10x buffer B</td>
<td></td>
<td></td>
<td>–20 ºC</td>
</tr>
<tr>
<td>dNTPs (nucleotides) 2 mmol/l</td>
<td></td>
<td></td>
<td>–20 ºC</td>
</tr>
<tr>
<td>MgCl₂ 25 mmol/l</td>
<td></td>
<td></td>
<td>–20 ºC</td>
</tr>
<tr>
<td>Taq Polymerase I (5U/µl)</td>
<td></td>
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<td>–20 ºC</td>
</tr>
<tr>
<td>Specific oligos (primer)</td>
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</tr>
<tr>
<td>Tris EDTA (TE) buffer concentrate</td>
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<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Mineral oil¹</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Pipettes (1000 µl, 200 µl, 20 µl, 10 µl)</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>500 µl reaction tubes</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Filter tips (0.5-10 µl, 2-30 µl, 2-200 µl, 200-1000 µl)</td>
<td></td>
<td>Room temperature</td>
<td></td>
</tr>
<tr>
<td>96-well PCR plate</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
</tbody>
</table>

¹ Only if a thermal cycler is used that does not have a heated lid.

Equipment required

*(complete detailed suppliers and order number to create laboratory-specific SOP).*

- Thermocycler;
- Centrifuges for pre-PCR quick spin to collect mixture of master mix plus template at bottom of tube/plate:
  - minifuge for tubes
  - centrifuge with adaptor for plates.

PCR primers for msp1

- PCR primers are dissolved in 1x TE.
• Prepare stock solution with a concentration of 100 µmol/l.
• For working solution, dilute stock solution to a concentration of 10 µmol/l. Keep aliquots at –20 °C.

Primers for the family-specific nested msp1 PCRs

K1 allelic family: M1-KF and M1-KR
• M1-KF: 5’-AAA TGA AGA AGA AAT TAC TAC AAA AGG TGC-3’
• M1-KR: 5’-GCT TGC ATC AGC TGG AGG GCT TGC ACC AGA -3’

MAD20 allelic family: M1-MF and M1-MR
• M1-MF: 5’-AAA TGA AGG AAC AAG TGG AAC AGC TGT TAC-3’
• M1-MR: 5’-ATC TGA AGG ATT TGT ACG TCT TGA ATT ACC -3’

RO33 allelic family: M1-RF and M1-R2
• M1-RF: 5’-TAA AGG ATG GAG CAA ATA CTC AAG TTG TTG-3’
• Ro33-R2: 5’-CAA GTA ATT TTG AAC TCT ATG TTT TAA ATC AGC GTA-3’

Internal quality controls
• Same as described for pPCR in RGP 006.

Change of lot numbers, batches and quality assurance

A test PCR has to be performed when introducing a new batch of primers. nPCR results of the 2 positive controls (2 different concentrations) are recorded in Worksheet 008 and compared to previous results for trend control.

Master mix msp1 nPCR and nPCR conditions

<table>
<thead>
<tr>
<th>Nested mix</th>
<th>Thermoprofile nested PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O distilled (or Milli-Q)</td>
<td>94 °C - 2 min</td>
</tr>
<tr>
<td>10x Buffer</td>
<td>94 °C-30 sec</td>
</tr>
<tr>
<td>dNTPs 2mM each</td>
<td>59 °C-1 min</td>
</tr>
<tr>
<td>MgCl₂ 25mM</td>
<td>30 cycles¹</td>
</tr>
<tr>
<td>M1-(K/M/R)F primer 10µmol/l</td>
<td>72 °C-1 min</td>
</tr>
<tr>
<td>M1-(K/M/R)R primer 10µmol/l</td>
<td>Final extension : 72 °C-5 min</td>
</tr>
<tr>
<td>Taq Polymerase (5 U/µl)</td>
<td></td>
</tr>
<tr>
<td>Total volume master mix per sample</td>
<td>48 µl²</td>
</tr>
<tr>
<td>primary PCR product is added to nPCR</td>
<td>2 µl</td>
</tr>
</tbody>
</table>

¹As the performance of different thermal cyclers can differ substantially, the suggested PCR profile and cycle number requires optimization to the conditions in each laboratory.
²Reaction works well in a volume of 20 µl. But this requires precision pipettes and 96 well plates or 0.2 ml or 0.1 ml reaction tubes.
• Fill out Worksheet 007.
• Prepare master mix for all samples to be amplified plus for one positive control plus for several no-template controls in a template-free room dedicated to PCR with dedicated no-template pipettes; use aerosol protected pipette tips. The positive control is a mixture of the 3 culture strains 3D7, FC27, and RO33; 3D7 carries a K1-type \( msp1 \) allele, FC27 carries a Mad20-type \( msp1 \) allele, and RO33 carries a RO33-type \( msp1 \) allele.
• Aliquot master mix to reaction tubes or 96-well plate in a template-free room. Change workspace for adding template (pPCR product).
• If thermocycler lacks a heated lid, overlay reaction with 2 drops of mineral oil. The primary PCR product is added through the oil layer to the reaction mixture. Use for each DNA sample a separate tip.

Note: To decrease risk of contamination, quick spin all tubes and plates containing extracted DNA or PCR product before opening tubes or removing caps or PCR film from plates. Condensation of liquid from hot PCR reactions can cause contamination problems upon opening of tubes or plates. Opening must be done without any delay after the centrifugation step.

Post-PCR procedures

Reagents and material

(For detailed suppliers and order number to create laboratory-specific SOP).

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
<th>Product number</th>
<th>Storage temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>H(_2)O distilled (or Milli-Q)</td>
<td></td>
<td></td>
<td>No storage</td>
</tr>
<tr>
<td>Glycerol</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>HCl 37v%</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>EDTA</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Trizma base</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Boric acid</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Xylene cyanol FF</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>Agarose</td>
<td></td>
<td></td>
<td>Room temperature</td>
</tr>
<tr>
<td>1 kb ladder</td>
<td></td>
<td></td>
<td>+4 °C</td>
</tr>
</tbody>
</table>

Equipment required
• Digital gel documentation system
• Microwave
• Gel electrophoresis system (minigel).

Preparation of 5x TBE buffer stock
• 54 g Tris
• 27.5 g boric acid
• 20 ml 0.5 mol/l EDTA pH 8.0
• H₂O distilled (or Milli-Q) add up to 1 l solution.

*Preparation of blue juice*
• 30% glycerol
• 10 mmol/l Tris-HCl pH 7.5
• 10 mmol/l EDTA pH 8.0
• 0.25% of both bromophenol blue and xylene cyanol.

*Preparation of the 1kb ladder (0.1 µg/µl)*
• 600 µl H₂O distilled (or Milli-Q)
• 300 µl blue juice
• 100 µl 1 kb ladder stock (stock: 1µg/µl).

*Preparation of the ethidium bromide staining solution*  
(for staining of gel after electrophoresis)
• 10 µl of a 10 mg/ml ethidium bromide stock solution (stable for 1 year at +4 ºC in a dark container)
• 100 ml 0.5x TBE.
(staining solution can be reused and kept for about 2 weeks in a dark container at room temperature)

*Electrophoresis*

*Preparation of an Agarose gel 3%*
• Take 0.5x TBE buffer and dissolve agarose (3 g agarose and 100 ml 0.5x TBE) by heating up the mixture in microwave until boiling and all agarose particles are dissolved. Cool to 50 ºC before pouring in gel casting mold. Allow the cast gel to set for at least 30 min at room temperature before removing the well-forming combs and using.

*Gel electrophoresis of msp1 PCR products*
• After the nested PCR run the amplified product is checked on a 3% agarose gel. Take 4 µl of nested PCR product and mix with 1 µl blue juice. Usage of combs with broader teeth is of advantage.
• PCR product amplified from the paired samples obtained from a patient must be run side by side on the gels in order to obtain the optimum comparison.
• There should be a minimum of two sets of markers per gel.
• Gels are stained for 30 minutes by total immersion in the ethidium bromide staining solution and then destained in water for 15 minutes (this improves visibility of bands), then visualized in a 312 nm UV transilluminator, and documented using an electronic photographic documentation system.

*Alternative gel protocol (recommended by G. Snounou)*
For preparation of a 3% high resolution agarose gel follow RGP 012.

*Analysis*
• For sizing and binning PCR products proceed according to RGP 009.
• Results are to be listed in Worksheet 007.
• Printouts of all documented gels are added to Worksheet 007.
• Follow **RGP 014** (data analysis) for definition of recrudescence versus new infection and procedures for reporting genotyping data and extra information.

**Further data analysis and reporting**

• Continue genotyping procedure according to **RGP 003** (decision tree).

Reference

RGP 012: Alternative high resolution gel electrophoresis

Material
- High resolution agarose (such as NuSieve® or MetaPhor® agarose).

Advantage
- Gels (2-4%) made with high resolution agarose provide higher resolution for resolving DNA fragments in the range of 200 to 800 bp and almost reach the resolution of acrylamide gels. Gels look nicer and the bands are clearer, at least for the msp1 and msp2 markers. The issue of cost is of course an important one, and re-use of the gels has been very helpful.

MetaPhor® gel preparation and re-use
- Gels are prepared by boiling. Ideally a microwave oven should be used to boil the gel solution, it is particularly suited when a previously used gel is re-boiled.
- When high resolution agarose gels are prepared for the first time it is imperative to add the correct amount of powder slowly to the appropriate volume of electrophoretic buffer, in order to avoid "lumps". The boiling should be done with frequent mixing of the solution during the boiling time. It is important to make sure that all the powder has dissolved, in order to obtain a homogeneous gel solution. This is achieved by boiling and mixing the solution until no undissolved agarose powder, which is transparent at this stage, can be seen.
- Following boiling, allow the gel to cool down to 50-60 °C, pour in the sealed gel plate, and then place the well-former combs, and allow at least thirty minutes for the gel to set. For high resolution agarose gels it is important to place the gel, after it has fully set, for at least another 30 min at 4° C before removing the combs and running the samples. Preferably the buffer in which the gel is run should also be cooled to 4 °C. When run cold, high resolution agarose gels give better results.
- The electrophoretic buffer of choice is TBE buffer, which has good buffering capacity and results in less heating, during electrophoresis, than other types of buffer. Furthermore it does not require pH adjustment when a stock solution is prepared. It is preferable to store the TBE Buffer stock in a plastic container, as the borate leaves an insoluble deposit on glass.
- Following use, gels can be stored indefinitely at room temperature, provided they are submerged in TBE Buffer. This buffer contains EDTA which will prevent any organisms from growing and subsequently degrading the gel. It is advisable to change the TBE Buffer once or twice in the first few days of storage, as this will decrease the amount of ethidium bromide and tracking dye in the gel by diffusion.
- A previously used gel is broken-up and placed in a bottle, and simply boiled as above. Care must be taken that the final volume is made up to the original gel volume (with water) after each boiling. This can be done if boiling is made in the same bottle, which can be marked at the appropriate level with a piece of autoclave tape.
- With frequent re-use, up to 10 to 15 times in our experience, the resolving power and integrity of the gel is retained. The only problems likely to be encountered are reduction in the gel volume by loss of gel material during frequent re-boiling (this might alter the agarose concentration), and in particular the accumulation of dust and other dirt particles in the gel! The amount of money saved when a large number of gels are used adds up to very large sums with time.
- It is important point to bear in mind that re-used gels will always contain small amounts of ethidium bromide and should therefore, be handled with care. In addition, because of the potential of altered mobility of DNA in the presence of ethidium bromide, re-used gels might not be deemed suitable for accurate molecular weight determination and size comparison of amplification product between different gels.
• Using family-specific primers (Snounou et al., 1999) the size range of \textit{msp1} allelic variants is ca. 100 bp to 250 bp, and for \textit{msp2} allelic variants is ca. 250 bp to 550 bp. For the \textit{msp1} marker use 3-3.5\% gels and for the \textit{msp2} marker 2.5-3\% gels. It should be noted that at these high resolution agarose concentrations, the gels are quite fragile and should be handled with care if one wishes to avoid tearing and breakage.

\textit{Preparation of 5x TBE buffer stock}  
• 54 g Tris  
• 27.5 g boric acid  
• 20 ml 0.5 mol/l EDTA pH 8.0  
• H\textsubscript{2}O distilled (or Milli-Q) add up to 1 l solution.

\textit{Preparation of blue juice}  
• 30\% glycerol  
• 10 mmol/l Tris-HCl pH 7.5  
• 10 mmol/l EDTA pH 8.0  
• 0.25\% of both bromophenol blue and xylene cyanol.

\textit{Preparation of the 1kb ladder (0.1 \mu g/\mu l)}  
• 600 \mu l H\textsubscript{2}O distilled (or Milli-Q)  
• 300 \mu l blue juice  
• 100 \mu l 1 kb ladder stock (stock: 1 \mu g/\mu l).

\textit{Preparation of the ethidium bromide staining solution}  
(for staining of gel after electrophoresis)  
• 10 \mu l of a 10 mg/ml ethidium bromide stock solution (stable for 1 year at +4 °C in a dark container)  
• 100 ml 0.5x TBE.  
(staining solution can be reused and kept for about 2 weeks in a dark container at room temperature)

Reference  
RGP 013: Evaluation of assay sensitivity and trend controls (quality assurance)

Positive control

- Parasites from three *P. falciparum* in vitro culture strains (see Table 1) with known parasite densities will be prepared by Swiss Tropical Institute on FTA© Whatman cards and distributed via MMV or WHO. Positive controls will be provided at such a concentration that a 3 mm filter disk approximately contains 200 parasites of each of the three strains (in total: 600 parasites). In addition, a higher density positive control will be provided, that will introduce with each filter disk about 2000 parasite per strain into the pPCR. The high density positive controls can be used to optimize suboptimal procedures.

- Keep collection card in a multi-barrier pouch together with desiccant (for humidity control) at room temperature.

Table 1: *P. falciparum* in vitro culture strains provided as positive controls

<table>
<thead>
<tr>
<th>Culture strain</th>
<th>Allelic family</th>
<th>Allelic family</th>
<th>Allelic family</th>
</tr>
</thead>
<tbody>
<tr>
<td>FC27</td>
<td>Mad20</td>
<td>FC27</td>
<td>genotyping does not involve family-specific nPCR</td>
</tr>
<tr>
<td>3D7</td>
<td>K1</td>
<td>3D7</td>
<td></td>
</tr>
<tr>
<td>Ro33</td>
<td>Ro33</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Monitoring of assay sensitivity

- Trend analyses in PCR tests are standard procedures in accredited laboratories. To analyse trends in sensitivity, all PCR results of positive controls are monitored. Positivity of the controls (2 different concentrations) is continuously listed in Worksheet 004 for *msp2*, Worksheet 006 for *glurp* and Worksheet 008 for *msp1*. These worksheets documenting the trend analysis must be kept in each laboratory and provided upon request by the commissioning party.

Determination of end point sensitivity

- Sensitivity of all procedures including DNA purification and PCR should be assessed during initial set up and validation of genotyping experiments plus at regular intervals. An in vitro culture strain can be obtained from Malaria Research and Reference Reagent Resource Center ([www.mr4.org](http://www.mr4.org)). Using diluted cultured parasites, both the efficiency of DNA purification and of pPCR plus nPCR can be assessed.

- Starting from *P. falciparum* in vitro cultures (strains 3D7, K1, Po33), serial dilutions should be performed according to Table 2. The diluted cultures should be spotted on specialized filter paper blood collection cards according to RGP 001. The cards should be kept and re-used for later sensitivity testing.
Table 2: Dilutions of in vitro culture strain and loading of PCR products on agarose gel

<table>
<thead>
<tr>
<th>lane</th>
<th>PCR product</th>
<th>lane</th>
<th>PCR product</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PBS</td>
<td>7</td>
<td>25 parasites/µl</td>
</tr>
<tr>
<td>2</td>
<td>0.01 parasite/µl</td>
<td>8</td>
<td>50 parasites/µl</td>
</tr>
<tr>
<td>3</td>
<td>0.1 parasite/µl</td>
<td>9</td>
<td>75 parasites/µl</td>
</tr>
<tr>
<td>4</td>
<td>1 parasite/µl</td>
<td>10</td>
<td>750 parasites/µl</td>
</tr>
<tr>
<td>5</td>
<td>5 parasites/µl</td>
<td>11</td>
<td>7500 parasites/µl</td>
</tr>
<tr>
<td>6</td>
<td>10 parasites/µl</td>
<td>12</td>
<td>75000 parasites/µl</td>
</tr>
</tbody>
</table>

- For sensitivity testing DNA purification and PCR should be performed according to RGP 006 and RGP 010 (glurp primary and nested PCR) together with the nPCR protocols (RGP 007 for msp2 nested PCR; RGP 011 for msp1 nPCR).
- Nested PCR products should be detected on an agarose gel according the respective protocol. The endpoint is determined as the last positive result in the dilution series. Lane 6 (10 parasites/µl) should give a positive result. Reproducibly positive must be the dilution 25 parasites/µl.
- To assess the reproducibility of the end point determined, the tests performed should be repeated.
- To complete the documentation on sensitivity testing, photographs of gels should be pasted in below.

photographs of gels
RGP 014: Data analysis, outcome classification and reporting

Data analysis

- The classification of treatment outcomes is based on an assessment of the parasitological and clinical outcome of antimalarial treatment according to the latest guidelines of WHO.
- Genotyping analysis should be performed as indicated in the flow chart shown in Appendix 3 of “Methods and techniques for clinical trials on antimalarial drug efficacy: genotyping to identify parasite populations”. By adopting the most stringent definition of new infection versus recrudescence as shown in Appendix 2 of “Methods and techniques for clinical trials on antimalarial drug efficacy: genotyping to identify parasite populations”, only mutually exclusive results with respect to trial outcomes can be obtained.
- For sizing of individual nPCR fragments RGP 009 should be followed.

Reporting

The genotyping report should provide the following information:

- Description of the method including the type of collection cards used and the protocol actually followed (with reference to the recommended RGPs). Deviations from RGPs need to be stated (e.g. using of Whatman® 3MM filter paper instead of treated collection cards).
- Information on number and IDs of samples received, samples evaluable, samples not evaluable.
- Excel sheet (preferable as .pdf so it cannot be changed afterwards) comprising all genotyping results for each marker gene analyzed whereby “R” stands for Recrudescence and “N” stands for New infection. One column should indicate the final genotyping result.
- If none of the 3 marker genes was PCR-positive, proceed to *Plasmodium falciparum* species PCR.

For regulatory purposes, a full documentation has to go to the study sponsor who is preparing the regulatory submission. In addition, one copy needs to be kept at the analyzing centre in case of an FDA/EMEA inspection. The documentation must include the Excel sheet for sample registration (Worksheet 001), all PCR worksheets plus labeled gel images for all markers.

Reporting of additional data in case of > 10% failure rate

- The commissioning party conducting the trial will analyse the data. As primary outcome of treatment efficacy trials, two estimates of treatment failure rates or risk are calculated:
  - Failure unadjusted by genotyping
  - Failure adjusted by genotyping.
- If the PCR-corrected failure rate > 10%, extra information should be given.
- The following information should be reported to allow the assessment of the probability to misclassify a new infection as a recrudescence due to chance.
  1. Mean MOI calculated from at least 50 random samples from baseline for the respective site using the highest discriminatory marker.
  2. For all genotyped samples, the presence of gametocytes at day of failure.
  3. Allelic frequencies of all genotypes determined in at least 50 baseline samples (the same samples as used for determination of MOI). Capillary electrophoresis provides the most exact fragment sizing and thus the most adequate determination of the frequency of specific alleles. If only electrophoresis gel analysis is available, the frequency of the dominant genotypes need to be reported, and bin sizes should be limited to < 20 bp in order to increase the resolution of genotyping).
- For regulatory clinical trials reporting of all additional data (i, ii, and iii) is mandatory. For surveillance purposes, only (i) and (ii) are required, but (iii) is optional.