Molecular Biology of *Plasmodium falciparum* – a practical course

3rd edition

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Goal of the course

In many cases, students of the Life Sciences (Biology, Biochemistry, Pharmacy, Biomedical Sciences etc.) have few opportunities to do bench work and see the practical side of molecular biology. In this course, we teach a couple of basic techniques almost used by all labs which work in molecular biology trying to show pitfalls and important details. In parallel, we show some aspects of the *Plasmodium falciparum* life cycle and how this parasite is maintained in *in vitro* culture. Afterwards, the attentive student should be able to plan, conduct and critically evaluate experiments done during the course.
Resume of the Activities throughout the course

Day 1: Introduction to *Plasmodium falciparum*. Putting together of experimental groups 1-7. Extraction of genomic parasite DNA. Techniques of preparing solutions and discussion of the gDNA. Seed bacteria *E. coli* strains DH10B, XL1 blue, Sure and BL21 RIL.

Day 2: Introduction in the culture of blood stage *Plasmodium* parasites. Demo experiment: Synchronization of parasites in late trophozoite/schizont stage parasites. Quantification of *P. falciparum* gDNA. Introduction to the polymerase chain reaction (PCR). Setup e start of PCRs using *P. falciparum* gDNA. Inoculation of LB overnight cultures with colonies from plates with *E. coli* DH10B, XL1 blue MRF-, Sure and BL21 RIL.

Day 3: Morning: Demo experiment: Harvesting of ring stage parasites. Analysis of the PCR products and their purification from gels: grupos 1-4 glassmilk, 5-7 glassmilk of fragments run in crystal violet gel. Groups 1-2 chemocompetent DH10B cells, groups 3-4 XL1 blue cells, groups 5-6 SURE cells and group 7 BL21 RIL cells. Setup of the ligation reaction in pGEM T easy and transformation (plus controls). Afternoon: Recovery of trophozoite stage parasites.

Day 4: Morning: harvest of schizont stage parasites. Calculation of the competency of bacteria and analysis of the pGEM transformations. Preparation of RNAs of in 3 groups (ring RNA 1,2 trophozoite RNA 3,4 and schizont RNA groups 5-7). Quantification of RNAs. Inoculation of minipreps form pGEM clones. cDNA synthesis: DNase1, cDNA. Test PCR for quality control with cDNA.

Day 5: Minipreps and quick insert test (digest pGEX2T in parallel), choosing of inserts, purification by glassmilk and ligation in pGEX2T, transformation. PCR with cDNAs and specific oligos. Theoretical background of cloning (making fused constructs).

W E E K E N D

Day 6: Minipreps and analysis of recombinant pGEX2T clones. Setup of the sequencing reaction. Seminars about next generation sequencing (Platform Illumina groups 1-2, Platform 454, groups 3-4, and IonTorrent 5-7), Retransformation of pGEX2T recombinant clones in *E. coli* BL21 RIL.

Day 7: Inoculation of pre-inoculum and main cultures in LB-amp-cam, induce bacteria e harvest. Sequence analysis in the computer, creation of plasmids in silico using APE software. Thematic seminars groups 1 and 2.


Day 9: Western blot, ELISA, evaluation of results. Thematic seminars groups 5-7.

Day 10: Help Desk. Seminars: Production of recombinant protein in Insect cells/Baculovirus (groups 1-3) or in *Pichia pastoris* (4-7). Final exam about contents of the course.
**Introduction**

**Malaria and *Plasmodium***

Human malaria continues to be an important infectious disease and puts a risk almost two thirds of the world's population, mainly in tropical regions of the world, showing a strict connection to poverty. The most virulent malaria causing protozoan species, *Plasmodium falciparum*, is responsible for the main part of the 800,000 malaria caused annual deaths [1,2]. However, recent advances have been achieved resulting in a constant decrease of deaths. This was done by increasing prevention (treated bednets) or quicker access to treatment. Also, a number of vaccine trials are in progress and currently a couple of vaccines look promising. These include antigens RTS/S-AS01 [3], MSP3 [4] but also novel antigens such as PfRh5 [5]. On the other hand, there appears to be a tendency to a higher incidence of severe *P. vivax*-caused malaria cases [6] and the looming loss of treatment efficiency of Artemisinine, the first line drug against *P. falciparum* [7].

In the human host, Malaria infection starts with the intradermal injection of sporozoites with the saliva of infected Anopheles mosquitoes during the blood meal. A part of these sporozoites migrates to the bloodstream and is then entering hepatic tissue where they invade hepatocytes. During the next 6-14 days (depending on the *Plasmodium* species), the parasite transforms into up to 40000 merozoites which then leave the infected cell and start to invade in red blood cells. The process of invasion is a highly coordinated process which takes approximately 2 minutes (revised in [8]), and leads to the formation of a parasitophorous vacuole (Figura 1). The multiplication of the parasites inside the red blood cell - termed schizogony - takes normally 48 h hours (72 h in the case of *P. malariae*) and the sickness is perceived by the infected person when the first blood cells lyse and liberate huge quantities of inflammatory factors ("malaria toxin"). During the intraerythrocytic phase, the parasite greatly modifies the structure of the red blood cell (RBC) and novel antigens appear in the RBC and also on its surface. These antigens have either unknown functions or function as transporters or adhesins. One part of the merozoites transform into sexual forms called gamonts or gametocytes which do not proliferate any more, unless they get transferred into mosquitoes during the next blood meal. When this happens, gametocytes rapidly transform into gametes - either one female or 8 male flagellated gametes. These quickly form a diploid form, the ookinete, which then rapidly egresses from the ingested blood meal, traversing the layer of intestinal epithelial cells and forming a cystic form on the outside of the intestine. Then, meiosis happens and a strong proliferation takes place (sporogony) which results in tens of thousands of sporozoites which - once liberated from the so called oocyst - actively migrate to the salivary gland where they stay in a dormant form. When the next blood meal ensues, the sporozoites again become injected into a new vertebrate host and the life cycle continues. The life cycle is shown in a resumed form in Figure 1. During this course we are going to amplify, express and detect a number of very relevant antigens of the merozoite and the infected red blood cell surface and part of these antigens and their localization are depicted in Figure 1.

**Figura 1: Life cycle and the Plasmodium merozoite.** In the lower part the different steps during the RBC invasion are depicted. A: weak association of the merozoite with the RBC. B: Re-orientation to bring the apical complex and the RBC membrane together. C: Formation of the invasion complex and a tight junction. At this timepoint, invasion can't be reversed. D, E: Injection of factors into the RBC cytosol (rhoptry contents) and active invasion (actin/myosin motor and interaction of proteins secreted onto the RBC membrane and also RBC proteins. F: Resealing of the RBC. G: The parasite starts to evolve inside the parasitophorous membrane which apparently is semi-permeable which means that small molecules up to 1000 Daltons can freely traverse from one side to the other.

**Molecular Biology in this course**

Per definition, molecular biology is understood as the aspects relative to DNA or RNA. Since its discovery by James Watson, Francis Crick, Maurice Wilkins e Rosalind Franklin this area continues in expansion and diversification and the degree of complexity clearly exceeds the quantity of knowledge which can be taught even in undergrad courses which solely focus on this area. However, there are basic concepts and techniques which are used by almost all researchers in this area. In this course, we try to show these basic concepts and techniques. These are basically the same in laboratories which work in the most distant systems and organisms - as a consequence molecular biologists who worked yesterday in the Anas patyrhynchos (“duck”) system, easily adapt to molecular biology laboratories working with Drosophila, Homo sapiens or Plasmodium.

During this course, we follow the steps of our laboratory which focussed on the question which of the many *P. falciparum* antigens may be recognized differently by people with or without immunity to *P. falciparum*. Many of the antigens expressed herein are also vaccine candidates and the procedure is as straight forward as possible.

### Day 1

Goals of the day: Preparation of solutions, media and genomic DNA extraction using blood stage forms of *Plasmodium falciparum* strain 3D7, make LB agar plates, seed bacteria.

The preparation of exact solutions is absolutely important when working with DNA. The secure pipetting and handling of minute quantities of solutions is essential. At this point it is of utmost relevance that the concept of molarity/percentage of solutions is understood. The first solutions that are going to be prepared are necessary for the lysis of *P. falciparum* parasites.

1x PBS from 10x PBS

Concentrated solutions are very frequent in labs because they permit the quick setup of solutions for use, simply by dilution with water. In order to simplify, many times we use 10 times concentrated solutions (10x), but be aware that 1x may be relative and depends on what experiment you're doing.

For the dilution of *n* times concentrated solutions to a 1x dilution, the following formula is used:
where the $\text{Volume}_{1x}$ obviously contains $\text{Volume}_{\text{conc sol.}}$ plus water. Let's take an example: We have 5x solution (five times concentrated) and want to set up 300 ml of a 1x solution. Then, the equation is:

$$\text{Volume}_{\text{conc sol.}} \times 5 = 300 \text{ ml} \times 1 \rightarrow \text{Volume}_{\text{conc sol.}} = \frac{300 \text{ ml} \times 1}{5} \rightarrow \text{Volume}_{\text{conc sol.}} = 60 \text{ ml}.$$ 

By this way, each group should now prepare 20 ml PBS 1x in Falcon tubes 50 ml, using the provided 10x PBS solution.

In the next step, we need to prepare the parasite lysis solution. This solution provokes the solubilization of any membrane (contains strong ionic detergents) and liberates nucleic acids from the parasite. For the efficient separation of nucleic acids from proteins (histones, transcription factors etc) it is also important to degrade these. This is done by the addition of proteinase K from the fungus *Tritirachium album*.

The preparation of the lysis buffer occurs in the same way as the preparation of $n$ times concentrated solutions. In the next example, we prepare 10 ml Tris/HCl, pH 8, 200 mM from a one molar solution of Tris/HCl pH 8.

The formula is:

$$\text{Volume}_{\text{conc sol.}} \times \text{Concentration}_{\text{conc sol.}} = \text{Volume}_{\text{use}} \times \text{Concentration}_{\text{use}}$$

which transforms to:

$$\text{Volume}_{\text{conc sol.}} \times 1 \text{ M} = 10 \text{ ml} \times 0.2 \text{ M}, \text{ or } \text{Volume}_{\text{conc sol.}} = 10 \text{ ml} \times 0.2, \text{ or } \text{Volume}_{\text{conc sol.}} = 2 \text{ ml}$$

Note that the units for molar (M) are eliminated. Please set up now 1 ml of the 4x *Plasmodium* lysis buffer:

<table>
<thead>
<tr>
<th>4X Plasmodium lysis buffer</th>
<th>Stock solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 mM Tris/HCl pH 8</td>
<td>1 M Tris/ Tris/HCl pH 8</td>
</tr>
<tr>
<td>4 mM EDTA pH 8</td>
<td>0,5 M EDTA pH 8</td>
</tr>
<tr>
<td>0,8 M NaCl</td>
<td>5 M NaCl</td>
</tr>
<tr>
<td>4% SDS</td>
<td>20% SDS</td>
</tr>
</tbody>
</table>

Prepare also 1 ml TE:

<table>
<thead>
<tr>
<th>TE</th>
<th>Stock solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM Tris/HCl pH 8</td>
<td>1 M Tris/ Tris/HCl pH 8</td>
</tr>
<tr>
<td>1 mM EDTA pH 8</td>
<td>0,5 M EDTA pH 8</td>
</tr>
</tbody>
</table>
Each group should now get a styropor box with ice.

The groups 1-7 receive one tube with 100 µl compacted blood infected with *P. falciparum*. **Attention:** This is alive parasite which is still able to infect you if you inject it in your blood stream or rub it onto mucosas! First, we separate the parasite from the RBC which is done by incubating with an isotonic saponin solution. Saponin interacts with cholesterol which is contained only in the RBC membrane but not in parasite membranes.

- Add 1,25 ml 1xPBS to the RBC pellet and mix by inverting the closed tube.
- add 150 µl 1% Saponin (provided).
- incubate 5 min on ice, inverting the tube from time to time. The solution must get transparent, such as currant juice.
- centrifuge for 5 min at 12000 rpm in an Eppendorf fuge.
- Localize the blackish pellet and discard cautiously all supernatant without mixing the pellet, but you may delete also the whitish membrane fraction on top of the pellet
- Resuspend the pellet in 1 ml 1x PBS.
- centrifuge for 5 min at 12000 rpm.
- Discard the supernatant and dissolve the pellet (~50 µl) in 250 µl TE.
- Add 100 µl Plasmodium 4x lysis buffer, as prepared before.
- Add 20 µl solução Proteinase K (10 mg/ml, provided)
- incubate 3 h or o/n a 37°C

During this step the membranes are solubilized by SDS and the proteins are degraded by Proteínas K which works at a pH8 optimum of 8 and in the presence of 1% SDS. In the next step, we get rid of lipids and protein remains by sequential Phenol, Phenol-Chloroform and Chloroform extraction (**Attention: Phenol is toxic, causes cancer e burns. Use gloves and don't contaminate the tubes on the outside!**).

- add 400 µl Phenol and incubate rocking for 10 min
- centrifuge for 5 min at 8000 rpm in the Eppendorf fuge.
- transfer the aqueous supernatant (don't mess with the whitish interphase) to a new tube and add 400 µl Phenol-Chloroform
- incubate rocking for 10 min
- centrifuge for 5 min at 8000 rpm in the Eppendorf fuge.
- transfer the supernatant to a new tube and add 400 µl Chloroform
- incubate rocking for 10 min
- centrifuge for 5 min at 8000 rpm in the Eppendorf fuge.
- add 800 µl de Etanol 100%, invert the tube and monitor the solution: a transparent viscous material should transform in a whitish cotton-like hair-thick stuff. Leave then for 15 min on ice.
- centrifuge for 10 min at 8000 rpm and 4°C in an Eppendorf fuge. Check the position of the tube's lid in order to know where to expect the sometimes very small pellet.
- Localize the pellet and retrieve the supernatant
- Add 1 ml Ethanol 70% and invert the tube 5 times, incubate 5 min a RT on the bench

At this point, the pellet is depleted of salt and contaminants, while genomic DNA and higher molecular weight RNAs remain precipitated in the pellet.
• centrifuge for 5 min at 8000 rpm in the Eppendorf fuge.
• discard with caution all liquid and spin down the empty tube and collect with a P20 all remaining liquid.
• open the tube and let the pellet air-dry at briefly on a Kleenex wipe on the bench
• add 50 µl TE, dissolve by pipetting up and down. Don’t let the DNA stick to the pipet tip. Then store at 4°C.

**Seed E. coli DH10B, BL21 DE3 pLys RIL, SURE and XL1 blue MRF**

One of the most important tools in the steps that follow are competent bacterial cells. To obtain them it is totally important that the bacteria don’t get contaminated with strains that don’t get competent. One step towards this goal is to seed the bacteria in a correct way in order to contain separated colonies which also permits to define if a stock is contaminated or not. The best way to properly seed bacteria is the “3-streak” method using a bacterial inoculating loop (Figure 2).

Before the actual plating we need LB agar plates with or without antibiotics. We prepare 1 litre of LB-Agar in two Erlenmeyers of 1 l.

**LB - agar medium premixed**: dissolve 35 g in 1 Liter of deionized water, autoclave for 20 min a 121°C.

• after cooling to ~45°C, add 500 µl ampicillin 100 mg/ml (1000X) in one of the Erlenmeyers with 500 ml liquid LB-agar, mix without introducing air bubbles.
• Make 8 plates without antibiotics (write always on the bottom what kind of plate it is), then add 300 µl de ampicillin 100 mg/ml.
• let the agar jellify in the dark
• store at 4°C wrapped in saran wrap
**Figura 2: The three streak method.** Note that every after 3 parallel lines the inoculating device is sterilized in the flame. Doing so, you guarantee that there is a serial dilution occurring which then results in single colonies.

Another criterion is the adequate use of antibiotics which already select our bacterial strains. In the case of *E. coli* DH10B (or DH5α), streptomycin at 50 µg/ml is applied on the plate. The strain BL21 DE3 pLys RIL is supplemented with Chloramphenicol (34 µg/ml). XL1 blue MRF e *E. coli* SURE are grown in the presence of 12.5 µg/ml Tetracyclin.

**Procedure:**

Initially, we apply 10 µl of 100 mg/ml Streptomycin, 20 µl of 34 mg/ml Chloramphenicol, and 20 µl Tetracyclin (12.5 mg/ml) on four LB-agar plates (one plate for each pair of groups, group 7 does BL21 RIL) and spread the liquid using the Drigalski spreader, sterilized by burning 70% Ethanol. This ensures that the spreader itself does not heat up - so don’t hold the spreader into the Bunsen-flame while it burns. Let the plates dry for 15 min at 37°C to evaporate excess liquid (or alcohol).

From a suspension of bacteria done in 0.5 ml PBS, seed the DH10B strain on the streptomycin plate, the BL21 RIL on the chloramphenicol plate and Sure and XL1 on the tetracyclin plates as indicated in Figure 2.

1. Write **on the bottom** of the plate the name of the bacterial strain and the date.
2. First, sterilize the inoculating loop in the flame. Afterwards, cool it down holding it in the middle of the agar plate.
3. Dive the loop in the solution that contains either *E. coli* DH10B/BL21/XL1blue/Sure. Verify that there is a liquid film in the loop.
4. Paint this liquid on the agar **without** hurting the agar surface, do 3 lines.
5. Sterilize the loop, rotate the plate 90°, and repeat the process in step 3. The first of the new lines should cross all three previous lines, the second only two and the last line should only cross one of the former lines.
6. Repeat step 5.
7. Close the plate and incubate it at 37°C for 12-16 h (until next day).

For the next day, we are going to need culture media in order to grow competent bacteria. Groups 1 and 2 will grow *E. coli* DH10B, 3 and 4 will grow XL1 blue cells, groups 5 and 6 will grow Sure cells and group 7 will grow BL21 RIL cells. Each group should then prepare 100 ml SOB in 1 litre Erlenmeyers (you can set up 400 ml in one go and split it in 4 Erlenmeyers). We will use a lot of Agar plates, too. One group should prepare one more litre of LB-agar in two Erlenmeyers or Schott bottles of 1 l.

Recipe for **1 litre SOB**: 5 g yeast extract, 20 g tryptone, 10 mM NaCl, 2,5 mM KCl, dissolve in 900 ml deionized water and adjust pH to 7.5 (pH paper) with approx. 2,5 ml 10 M NaOH (provided **Attention: caustic!**), autoclave for 20 min at 121°C, **afterwards** add MgSO₄ to 20 mM final concentration (1 M solution provided).

Recipe for **1 litre LB-Agar** (Originally *Lysogeny broth*, aka Luria broth, Luria Bertani broth ou Lennox broth): 10 g Tryptone, 5 g Yeast extract, 10 g NaCl, 15 g Agar. Cool to <50°C, add concentrated
antibiotics and spread approx. 20 ml per Petri plate. Leave drying at RT in the dark, then store at 4°C in saran wrap.

Check your bench and clean it up before you leave. Store the products and solutions in the fridge or freezer and discard your ice. See you tomorrow!

Day 2

Goals of the day: Quantify gDNA, organize and pipet PCR with your oligos and gDNAs, analysis of the result, see in vitro culture of *P. falciparum* separating trophozoites/schizonts, inoculate bacteria in 5 ml pre-cultures. Get an ice bucket.

Nucleic acids contain pentoses (ribose) linked to pyrimidins or purins (Figure 3). The pyrimidin and purin groups possess a specific spectrum of absorbance which permits to measure their quantity in aqueous solutions. However, in our gDNA prep there is also a lot of RNA which also absorbs light. Therefore, it is impossible to adequately measure the DNA content of our samples. As a semiquantitative method, we will analyze an aliquot of our DNA by electrophoresis in agarose gels, stained with the intercalating reagent ethidium bromide.
form RNA and DNA. Note that the bases (purines and pyrimidins) are connected via a nitrogen to the sugar molecule in position 1. The sugars are linked together by phosphodiester bonds (stars, an hydroxyl group reacted with one acid group from the phosphate) forming polymers. Note that DNA consists of deoxy-ribose which means that carbon 2 in the sugar is reduced and contains two hydrogens. Origin of the pictures: in clockwise sense: uic.edu, Absoluteastronomy.com, knowledgeclass.blogspot.com.

In order to prepare an agarose gel, one group prepares a solution of 40 ml 1x TAE (provided) containing 0.4 g agarose in a 250 ml Erlenmeyer. Melt the agarose in the microwave for 1 min and let the solution (without any visible undissolved resin) cool down to under 50°C. **Use a thermo-glove to hold and mix the solution.**

While the agarose is cooling down prepare the gel tray as shown in figure 4. Insert the comb.
**Figure 4: Mounting the gel tray for agarose gel electrophoresis.** Take care that the tape closes tightly at the edges. Don’t pour gel solutions hotter than 50°C since they spoil the acryl matrix of the tray and tend to disconnect the tape and the ethidium bromide solution will leak on the support/table.

When cooled down to <50°C, add 1 µl ethidium bromide solution (10 mg/ml) in the gel solution and mix. **Attention! Ethidium bromide is causing cancer and passes the skin barrier. It is also volatile and evaporates from too hot solutions. Don’t breathe over the gel when you pour it into the tray. Use gloves.** After pouring the gel, take off your gloves and leave them in place, you will need them afterwards.

Right now, each group should prepare one Eppendorf tube with 5 µl gDNA and add enough 6x loading buffer to get a final concentration of 1x loading buffer (provided). How much would that be in order to reach 1x?

<table>
<thead>
<tr>
<th>Stock solution 50x TAE: 242 g Tris Base (MW=121.1), 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA pH 8.</th>
<th>6X gel loading buffer: 6X TAE, 50% (v/v) glycerol, 0.01% bromophenolblue.</th>
</tr>
</thead>
</table>

After the gel has jellified, retrieve the comb and put the gel in the electrophoresis chamber and fill with 1xTAE solution so that the gel gets submerse. Load your samples and don’t forget the molecular weight marker. Connect the power cords and run the gel at 120 Volts for approx 20 min. Always consider that the DNA is negatively loaded and will migrate to the positive pole. After the run, the differently sized DNA fragments will be separated and can be visualized under UV light in a photodocumenter (“eagle eye”).

**PCR (polymerase chain reaction)**

After confirming that you have genomic DNA we proceed to the specific amplification of DNA fragments which represent you gene using the DNA polymerase chain reaction. For this, prepare first you 10x PCR buffer using the solutions which are provided. Everyone who is in doubt if his/her gDNA is good enough can receive a backup of pre-tested 3D7 or NF54 gDNA.

<table>
<thead>
<tr>
<th>10x PCR buffer</th>
<th>Stock solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM TrisCl pH 8.8</td>
<td>1 M TrisCl pH 8.8.</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>1 M MgCl₂</td>
</tr>
<tr>
<td>500 mM KCl</td>
<td>3 M KCl</td>
</tr>
</tbody>
</table>

Add milliQ water to a final volume of **0.5 ml**.

Localize your oligonucleotides for the gene you're amplifying. The oligos come in a mixed solution of 5 pmol/µl. Mix them briefly and spin them down and put them back on ice. Do the same with the 2.5 mM dNTP solution. Prepare two 0.2ml tubes writing at the lateral part of the tube (**writing on the lid will go away in the next step!**) what’s in there: “gDNA” or "+" and “control” or "-" plus your group number.
Pipet the reaction in the following way, adding each component in the indicated sequence (use **exclusively** the P20 pipet) in the tube marked "control" or "gDNA/group XY":

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>water milliQ</td>
<td>26.4 µl</td>
</tr>
<tr>
<td>10x PCR buffer</td>
<td>4 µl</td>
</tr>
<tr>
<td>dNTPs (2.5 mM each)</td>
<td>4 µl</td>
</tr>
<tr>
<td>Oligo Mix (5pmol/µl)</td>
<td>4 µl</td>
</tr>
<tr>
<td>Taq Polimerase (5U/µl)</td>
<td>0.2 µl</td>
</tr>
</tbody>
</table>

Adjust the pipet to 19.5 µl, mix the solution by pipetting up and down *slowly*, without leaving liquid at the tube wall and transfer 19.5 µl to the “gDNA/group No. XY” tube. Add 0.5 µl of your gDNA and close the tubes. If you use another gDNA (e.g., DNA backup), you have to recalculate the reaction above for three reactions. Store your reaction on ice until each group is ready.

When every group is ready, the reaction is put in the thermocycler. The following program is run: 94°C, 40 s, 53°C, 40 s, 65°C, 1.5 min). In the next step, the amplified fragments will be visualized again in ethidium bromide stained gels. An additional test is inserted at this point and groups 5-7 will run their fragments in crystal violet (CV) stained gels which permit the visualization without exposing the DNA to harmful UV radiation. Normally, fragments purified in CV gels are ligated easier in the following cloning step.

Groups 1-4 prepare a common EtBr stained 1% TAE-agarose gel, while groups 5-7 prepare a gel with 1% CV-TAE. Add to you completed PCR reaction samples 6x TAE loading buffer (groups 1-4) or 6x CV TAE buffer (groups 5-7). How much 6x buffer you have to add to the 20 µl reaction volume to give you 1x loading buffer concentration?

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**Gel de Crystal violet**

- **CV stock solution**: 2 mg/ml Crystal violet in pure methanol (store at 4°C).
- **6x CV-TAE loading buffer** (0.1 mg/ml *Crystal Violet* in 6xTAE): 100 µl 6x TAE loading buffer without bromophenolblue (provided) + 5 µl *Crystal Violet* stock ≈ 6x CV-TAE.
- **1% CV-TAE gel**: 40 ml TAE + 0.4 g agarose "cloning grade", heat, cool down to 50°C, add 50 µl *Crystal Violet* stock.

Run both gels for 20 min at 120 V. Groups 5-7 must monitor the gel during the run because weak bands are easy lost and get invisible. Unfortunately, the sensibility of detection of CV gels is 10 times lower than for EtBr stained gels. After the run, the gels are quickly photographed and the bands are excised with a scalpel. Try to cut as small but as much as possible of the band. Try to not exceed 1-2 mm thick cut pieces. The gel slice is put in a new Eppendorf tube labelled with the group number and the name of the amplified gene.
For the next step, one group should now prepare 50 ml of NEW wash buffer: 20 mM Tris/Cl pH7.5, 0.01 M NaCl, 2 mM EDTA, ≥55% (v/v) EtOH in milliQ H₂O. Leave on ice (Stocks: 1 M Tris pH 7.5, 0.5 M EDTA, pH8, 5 M NaCl, 100% EtOH).

**Purification of DNA fragments with Glassmilk**
- cut band in Eppendorf 1.5 ml
- add 3 volumes of NaI (6 M), typically 300-400 µl and incubate at 37°C until no more gel residue is visible
- add 5 µl Glassmilk (100 mg/ml em 3 M NaI, mix before use)
- incubate 5 min at RT, mix from time to time
- centrifuge 1 min at “full speed”, discard supernatant using a P1000 pipet
- wash the pellet by adding 1 ml NEW wash buffer (**washing is: vortex, centrifuge, discard supernatant with pipet P1000**), re-centrifuge 10 s and remove residual volume using a P20 or P200, let dry for 5 min at RT on the bench (open tube, to evaporate alcohol)
- Add 15-20 µl TE, LoTE ou H₂O, mix well and incubate 5-10 min at RT (the right pH to release DNA from the silica matrix is between 6 and 8)
- centrifuge for 1 min at full speed
- Pipet the supernatant into a new tube labelled with "PCR fragment gene XY group XY". Don’t transfer silica. Protect the writing on the tube with transparent 3M tape ("tesafilm", "durex")
- The isolated fragment is ready to use. Maintain it on ice for the next step or store it at -20°C.

**Purification of trophozoite/schizont stage parasites**

This experiment should be conducted by a volunteer under supervision of an experienced person. The use of gloves and the conscience that the manipulation has to be done under sterile conditions is essential. Localize the following items before you start: 1 fresh 15 ml tube - steril, complete RPMI medium, incomplete RPMI medium, Voluven 6%, 2 cell culture bottles 75cm², Ethanol 70%, glass slides and blood smear slider, pipet boy, sterile pasteur pipets, steril plastic or glass pipets and a bucket for discarding, one water proof text marker, one pencil and a lighter. Parasites in mixed stage culture (1 bottle 75cm²) have their medium removed. The parasitemia and parasite forms are checked by a thin blood smear. The parasites are then removed into a 15 ml Falcon tube using 10 ml incomplete RPMI medium. The parasite suspension is centrifuged at 1500 rpm in a Sorvall benchtop centrifuge for 5 min at RT.

- After returning to the clean bench, the medium is removed and the volume of the compacted erythrocytes is measured = 1 Vol.
- Add **1.4 volumes** complete RPMI and gently mix by pipetting.
- Add **2.4 volumes** Voluven 6% and mix without introducing air bubbles.
• Close the tube and incubate for 30 min in an upright position at 37°C. Afterwards, be sure that you see a defined line of deposited RBC and a turbid, sometimes greyish supernatant. This supernatant contains the late trophozoite sand schizonts. Very late schizonts and ring stage parasites are not recovered from the supernatant.
• Transfer the supernatant into the initial 75cm² culture flask.
• Label two more empty 75cm² flasks and pipet 10 ml of complete medium inside each.
• Add **2.1 ml fresh blood** to the purified trophozoites
• Add complete medium to this bottle to reach **15 ml**.
• After mixing, take out 10 ml and pipet **5 ml** in the other two bottles which already have 10 ml clean medium
• Into the initial flask, add **10 ml** complete medium
• Close the taps of all flasks without really closing them air-tight: It is essential that there is exchange of atmosphere
• Put the bottles back into the air-tight box and light the candles. Close the lid of the box and watch the candles go out without production excess smoke. If the candles have a smoky burnout, open the lid and repeat the candle-burning step.
• Put the box in the incubator at 37°C.
• **Arrange the sterile hood so that everything is clean and nice. Take the trash to the washing device or the hospital trash. Leave no traces.**

After all, the pre-cultures for the competent bacteria are inoculated. For this 5 ml SOB, are retrieved from the autoclaved 100 ml and put into a 50 ml Erlenmeyer. Add the necessary amount of antibiotic for selection (2.5 µl Streptomycin for the DH10B culture, 5 µl tetracyclin for Sure and XL1 blue and 5 µl Chloramphenicol for BL21 RIL). Using the inoculation loop sterilized in the flame, inoculate one single colony of each plate into the 5 ml SOB medium. Put the cultures on the orbital shaker at RT and 100 rpm.

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**Check your bench and clean it up before you go. Store your stuff in the fridge or freezer and discard your ice bath. See you tomorrow!**

**Day 3**

Goals of the day: Preparation of competent cells: DH10B, BL21 RIL, XL1blue and Sure. Harvest ring stage parasites (early in the day), trophozoites (latest timepoint possible). Ligate amplified fragments in pGEM T easy, transform in DH10B and XL1blue. Get you ice bucket with a lot of ice.

First we need to inoculate 2-5 ml of the preculture in the remaining SOB (95 ml) at RT with the necessary antibiotics already inside. Put the culture on the orbital shaker at 100 rpm and RT. Be sure that the CCMB80 is on ice.

**Harvest of ring stage parasites**

A volunteer should now prepare the flow as described before, however, without new culture flasks. Instead, place a bottle with 1xPBS and a new Falcon tube 15 ml. Trizol should also be at reach. The sterile hood should have been switched on with circulation and UV light for 20 min. After this, the volunteer should retrieve the culture box from the incubator and open it in the hood retrieving one of the bottles **without wetting** the tap. The other bottles remain in the box, which can be closed
burning the candle as before. Watch out for smoke production. Return the box to incubator. Afterwards:

- Aspirate the culture medium.
- Prepare a thin blood smear, stain it and verify that it contains mostly ring stage parasites. Estimate the percentages of each form.
- Retrieve the parasites resuspending them with 12 ml 1x PBS and put them into a 15 ml tube.
- Add 1,5 ml Saponin 1%.
- Incubate 10 min on ice, inverting the tube from time to time
- When the solution is completely transparent ("currant juice"), centrifuge the tube at high speed (2800 rpm Sorvall) or 4000 rpm (Eppendorf) in a swing out rotor.
- Aspirate the supernatant but localize the pellet before, it may be quite small and whitish. Leave approx. 500 µl in the tube. The following steps can be done outside the flow.
- Wash the pellet once with 1x PBS and transfer to a new Eppendorf tube labelled “3D7 ANEL, day XY”. Centrifuge it at 12000 rpm, 4°C or RT for 5 min.
- Discard the supernatant and add up to max. 100 µl 1x PBS, resuspend the pellet until its homogenous
- Add 1ml Trizol. Mix on the vortex for 15-30 seconds, there should be no particles inside.
- Store the tube in the freezer -20°C.

Please clean the flow as before, leave no traces.

**Ligation setup: A/T cloning in pGEM T easy**

In the next step, we are going to ligate the purified PCR fragments in a entry plasmid vector for this kind of fragment ("A/T cloning vector"). The chosen vector is pGEM T easy (Promega). First set your purified fragment on ice. In figure 5 you will find a map of the plasmid showing its most important restriction sites.
Figure 5: Plasmid map of pGEM T easy. In the bottom, the exact sequence of the multiple cloning site is shown. Note that the polylinker lies inside the coding sequence of the lacZ alpha domain (copied from the Promega site).

Now localize the components of the cloning kit in the central ice bucket: vector pGEM T easy (yellow cap), 2x ligation buffer (white cap) and the T4 ligase enzyme (green cap). The kit is expensive and normally it is sufficient to use a 2.5 µl reaction volume - if you are able to pipet such as small volume! Prepare a tube with the identification: “pGEM - Number of the group or name of amplified gene” Using the provided P10 and adequate tips for this purpose and put 0.75 µl of your fragment in this tube. Store it on ice.

Next a volunteer pipets the mastermix for all ligation reaction which contains all the other components. Use also the P10 for this.

Ligation:

1 reaction 7 reactions

0.25 µl 1.75 µl Vector pGEM T easy

1.25 µl 8.75 µl 2x Ligation buffer
Each group now should pipet immediately 1.75 µl of this master solution on their fragment and mark this tube with an "L" for ligation on top of the lid. Mix the reaction by flicking the tube and apply a short spin to collect the volume at the bottom of the tube. Incubate the reaction in a cold water bath (~10 °C). Theoretically, 90% of the fragments which can be ligated, will do so in a very short time such as 15 min. We leave the reaction running while we take care of the competent cells we are growing in parallel.

**Preparation of competent E. coli**

For the preparation of competent bacteria it is absolutely mandatory to work with very clean and residue free glassware, specifically no soap or detergent of any kind should be present at any step. More important perhaps, don’t use glassware that has only been autoclaved but is otherwise used for maxipreparations of plasmids which could give you pretransformed bacteria! This means that all glassware that you will want to use should be thoroughly rinsed in deionized water and then baked at 180°C for 2 h. The second critical point is to not let overgrow bacteria, better go for less yield and higher efficiency than for more but not so competent cells. The third point is to work always with ice-cold material, like prechilled tubes, solutions and centrifuges. You can even prechill a box of P200 tips which are used in the end of the process to aliquot your bacteria. The fourth point is that you bacteria should grow exponentially. If you fell that the main culture isn’t growing so vigorously as in other occasions, go for a new inoculum. If after the first centrifugation you already see a blackish pellet, you should discard this lot. Try to prepare competent bacteria always form a freshly grown plate (less than 1 week in the fridge). **Don’t even think to contaminate you bacterial strain and if your lab seems unaware of this point, store yourself a glycerol backup of the grown bacteria in liquid nitrogen.** Our experience shows that someday there will be people asking for your stock!

Prepare frozen Eppendorf tubes labelled with the type of bacteria you will produce and maintain the tubes closed in the -20°C until use. Likewise, you should label your tubes with a "10" if you prepare DH10B, or a "RIL" if you prepare BL21 RIL cells, and so on with Sure ("S") or XL1 blue cells ("XL1"). When finally your main culture has grown to a OD\textsubscript{600} of 0.4, transfer the culture in to two prechilled new 50 ml Falcon tubes.

- Centrifuge the suspension for 10 min a 3500 rpm in the cold Eppendorf centrifuge at 0°C.
- Discard the supernatant as much as you can without losing the pellet, the supernatant goes into the biological waste.
- Return the tubes to the ice bath. Now resuspend the pellet smoothly in 1 ml CCMB80 buffer.
- Add another 15 ml CCMB80 to each tube and join the liquids in one tube and store it on ice.
- Leave on ice for 20 min.
- Centrifuge for 10 min at 3000 rpm and 0 °C, afterwards discard the supernatant in the biological waste.
- resuspend the cells smoothly in 1 ml CCMB80 without heating them, the cells are very fragile now.
- Take out 50 µl and add 200 µl LB in a new eppendorf tube and measure the OD\textsubscript{600nm}. The solution should have an OD of 1 a 1,5. If the value is higher than that, add more CCMB80 until the OD is right.
- Incubate another 20 min on ice.
- Always on ice, aliquot 125 µl with the frozen P200 tips in the prechilled tubes.
Finally, the tubes ate transferred to -80°C freezer where they should stay for ≥ 30 min until use. Stored like this, they increase the competency during the next 24 h and can be used for month or years.

Buffer CCMB80 is: 10 mM KOAc pH 7.0 (10 ml of a 1M stock), 80 mM CaCl₂·2H₂O (11.8 g/L) 20 mM MnCl₂·4H₂O (4.0 g/L), 10 mM MgCl₂·6H₂O (2.0 g/L), 10% glycerol (100 ml/L) adjust the pH down to 6.4 with 0.1 M HCl if necessary. Sterilize by filtering through a 0.22 micron filter and store it in the dark at 4°C (black precipitate may form - MnO₂ - but this does not seem to interfere).

Calculating the competency of competent bacteria

The unit that describes the competency of bacteria is colony forming units per µg of plasmid (cfu/µg).

Figure 6 illustrates in a ludicrous form which competency is desirable and which is insufficient.

![Figure 6: Classifying the quality of competent bacterial cells](image)

**Figure 6: Classifying the quality of competent bacterial cells.** Bacteria with 10⁵ cfu/µg may be used in retransformations of ready plasmids. Bacteria with 10⁶ cfu/µg may be used for simple clonings (sticky end cloning of small and middle sized DNA fragments). Bacteria with 10⁷ cfu/µg are useful in A/T cloning and blunt end clonings. Bacteria with 10⁸ cfu/µg or more are appropriate for library cloning and difficult DNAs and everywhere where you look for a huge number of colonies.

In order to measure the competency is it necessary to have a pure and quantified plasmid. This plasmid is then diluted to minute concentrations where the expected number of colonies becomes countable on a normal 10 cm agar plate. So, first thing to consider is what value of competency you expect to see. A good value to start with is 10⁶ cfu.

Calculate how you should dilute a plasmid with de 1.5 µg/µl in order to obtain 30 colonies, assuming that you expect an efficiency of 10⁶ cfu/µg. We transform 1 µl of a plasmid.

**Transformation**

- Prepare two Eppendorf tubes writing on them the name of your construct such as pGEM-EBA175 and put 1 µl of your ligation in each (we use XL1 blue or Sure and DH10B), store on ice.
- For all groups: Thaw two tubes of competent Sure, XL1 blue, 3 tubes of DH10B and one tube BL21 on ice.
- Add 25 µl of DH10B in all different ligations and store the rest of the cells. Groups 1-4 use XL1 blue for the other tube and groups 5-7 use Sure for the second tube. Each group has now their ligation transforming in DH10B and XL1 or Sure cells.
• One group should now prepare a test plasmid (provided) and dilute it to an appropriate concentration and transform 1 µl of this solution in 25 ul of the remaining bacteria Sure, XL1 blue, DH10B and also the BL21 cells.
• Leave the tubes for 30 min on ice.
• Meanwhile, each group prepares 2 LB amp plates with 4 µl 1 M IPTG e 20mg/ml X-gal (you can prepare a mastermix for all groups and spread 24 ul with the Drigalski spreader). Sure and XL1 blue cells should be plated also with 20 ul tetracyclin, while the BL21 receiving plate should get 20 ul Chloramphenicol. Put the plates in the 37°C incubator to heat.
• Write on each plate (bottom!) which antibiotic is used (ex. LB-amp, LB-amp-tet, LB-amp-cam) and which bacteria is plated containing which construct, such as "pGEM-MSP9".
• After 30 min on ice, transfer the tubes to a 42°C water bath for exact 60 s. Return the tubes to the ice bath.
• After 1-2 min, add 125 µl LB without anything, close the tube and put it in the 37°C incubator.
• Incubate por 20min.
• Then, using the Drigalski, spread the whole volume of transformed bacteria on the plates and store at 37°C.

Harvest of trophozoites
A volunteer should then repeat exactly the steps done this morning when harvesting the ring stage parasites. There should now be a bigger pellet than seen in the morning. Resuspend it finally in 1 ml Trizol, label it "3D7 trophozoites" and freeze it at -20°C.

Check your bench and clean it up before you go. Store your stuff in the fridge or freezer and discard your ice bath. See you tomorrow!

Day 4
Goals of the day: Harvest of schizonts, calculation of the competency using the plates with the control plasmids. Preparation of total RNA of the three blood stage forms. RNA gel, cDNA synthesis. Setup of the PCR checking the transcription of your genes in the three blood stage forms. Inoculation of minipreps from the pGEM clones. Get yourself an ice bucket with ice.

Harvesting schizonts
A volunteer should now recover the schizont stage parasites as was done before for ring stage and trophozoite stage parasites. In the end, there should be a huge black parasite pellet and this is due to the high amount of hemozoin - the malaria pigment. Resuspend the pellet in 1 ml Trizol.

Calculating competency
Count the colonies on the plates. Consider the quantity of DNA that you transformed in µg. Divide the number of colonies by the amount of plasmid transformed (in µg) and compare the result with the picture in Figure 6. In your transformations using ligated pGEM, take a look at the colonies: They are uniform in size and appearance? What’s their color?

Afterwards, store the plates in the fridge or leave them on the bench until the colony size reaches a diameter of 1 mm.
Purification of parasite total RNA
When working with RNA it is necessary that you are totally aware that from the time point of the first precipitation RNases may attack your RNA. This means that you must not touch - even with gloves - the inside of the Eppendorf tube containing your material, including the lid! Who is not totally sure of his/her handling qualifications should use gloves.

- Thaw RNA at 37 °C
- Vortex for 15 seconds
- leave 15 min on the bench at RT
- add 200 µl RNAse free chlorofom, close tube
- mix manually for 15 seconds
- centrifuge at 4°C, at 12000 rpm, 15 min
- transfer the supernatant to a new tube labelled "RNA 3D7 troph/ring/schizont", add 500 µl Isopropanol 100 % RNAse free
- leave 15 min on the bench at RT (~20-25°C).
- centrifuge for 60 min ("over lunch") at 12000 rpm, 4°C, take care to direct the tube lid to a position that lets you know afterwards where the pellet is expected.
- Afterwards discard the supernatant
- Add 500 µl EtOH 75%-80% RNAse free, you don't need to mix now
- Centrifuge again for 5 min 12000 rpm, 4 °C, afterwards discard the supernatant completely (short spin and take out the rest with a P200)
- let the pellet air dry at RT.
- Resuspend the pellet in 20 µl MilliQ water RNAse free.
- At this point, you can freeze your RNA at -80°C. As we proceed directly, please stick the tube with duct tape to the vortex and let it vortex for 10 min at low speed - the drop with the RNA should stay in one at the bottom of the tube. Afterwards, put the tube on ice.

Testing the quality of the RNA in a TBE gel
In order to check if no degradation of RNA occurred, a small aliquot of the RNA is run on a TBE gel stained with EtBr. For this, 2 µl of each RNA are mixed with 3 µl milliQ water RNAse free in a new tube containing 1 µl loading buffer for TBE gels (6x loading buffer Fermentas tube). A volunteer should prepare an 0.8% TBE gel using the gel tray specific for RNA which is handled only with gloves. O running buffer is 1x or 0.5x TBE. Load the gel and run it for 30 min and take a photo ("eagle eye").

cDNA synthesis
In the next step, a complementary DNA strand is synthesized using total RNA as a substrate and reverse transcriptase from the Moloney Murine Leucemia virus (MMuLV). This enzyme was optimized for the use in molecular biology. Reverse Transcriptases normally possess two activities: A RNA-dependent DNA polymerase 5'-3’ and a RNase H activity, which is an DNA-dependent RNase 5’-3’ (Figura 7).
Figure 7: Activities of Reverse Transcriptase (and RNase H+). Note that the enzyme needs a small DNA primer in order to synthesize DNA. In this case the primer is a oligo dT which hybridizes in all RNAs which contain a long stretch of “A”s such as messenger RNAs.

There are enzymes available which exert a reverse transcriptase activity whithout RNase H ("Superscript"). Herein, we use the common RNase H+ RT. The RNase H activity becomes important when you want to amplify genes bigger than 1000 nt from your cDNA since RNA hybridizes stronger to DNA than another complementary DNA strand. There are other ways than oligo dT to prime cDNA production. Commonly, researchers use random hexamers (dN₆) which hybridize to any sequence in a random way. In cases, where very few RNA is available or there is many background/host RNA (intracellular organism) one can also use directly a gene specific antisense oligo for cDNA synthesis which then limits further analysis to the targeted gene. Herein, we use random hexamers.

Since every RNA produced in batch using the Trizol method may contain small amounts of genomic DNA, a previous DNA elimination is mandatory before cDNA synthesis. Small amounts of gDNA would later work as a template in PCR analysis and spoil the test to evaluate the timepoint/phase of transcription of the genes looked at in our experiment. Therefore, we include a DNAse 1 treatment of our RNA before cDNA synthesis.

**DNAse1 treatment and reverse transcription**

- Localize DNAse1 buffer and EDTA 50 mM, vortex, spin and put on ice.
- Prepare a water bath at 20°C and put in a swimming device for tubes
- Switch on the heating block, 65°C.
- Pipet using the P20 into new tube m(note that you have three tubes, ring troph and schizont RNAs!):  
  - X µl RNA (judge from the TBE gel result)
  - Y µl água milliQ
  - 1 µl DNAse buffer
  - 1 µl DNAse 1 (directly out of the freezer, don’t leave it on ice), *short spin*.
  - incubate for 20 min at 20°C.
  - add 1 µl EDTA 50 mM, flick the tube and apply a short spin.
While DNase 1 is denatured, prepare two new tubes per RNA, labelled “ring +RT, ring –RT, troph +RT, troph –RT, schiz +RT, schiz –RT”. Thaw/localize the tubes 5X RevertAid buffer and 10 mM dNTPs. Pipet in the tubes marked with “… RT-“:

- 11 µl DNase1 RNA
- 2 µl random oligos (10 pmol/µl)
- 11,5 µl H₂O
- heat for 5 min at 65°C
- return to the ice bucket, then apply a short spin.
- add 4µl dNTPs 10 mM (don't confound with the PCR dNTP's 2.5 mM)
- add 8 µl reaction buffer 5X
- add 2µl RNAse inhibitor
- transfer 28.5 µl to the tube labelled “.....RT+”.
- add to this tube 1.5 µl RevertAid, mix by flicking, short spin, and incubate for 5 min at RT.
- transfer the tubes to a dry heat block at 42°C or to a water bath at 42°C and incubate for 50 min.

Afterwards, the reaction can be heat-inactivated at 70°C for 10 min or frozen directly. Normally, one would then check the viability of the cDNA versus the RT- sample doing a test PCR with a transcript known to be present in all three stages. To facilitate, our lab has a test kit where there has to be added solely Taq Pol and template DNA. The target gene in this case is fructose bisphosphat aldolase. Localize the tube “1x K2 Mix”. A volunteer should now pipet this reaction. There are three tubes which are tested and three controls (3 tubes cDNA RT+, and 3 controls RT-) plus one positive control (gDNA de P. falciparum 3D7) and one negative control (water).

- Prepare a strip tube with 8 tubes and write on the side what you are going to pipet: r(ing RT)+, r(ing RT)- etc.
- pipet 8x 15 µl 1x K2 mix = 120 µl in the negative control tube (the one with water as template)
- add to this tube 8x 0.08 µl = 0.64 µl (P20! not P10) Taq Pol, leave the tip on the pipet.
- modify the volume to 15 µl and aliquot 15 µl in each tube.
- Then, add 1 µl of each template (cDNAs or RT- control, gDNA) in the individual tubes.
- close tubes
- insert in the thermocycler and run the following program:
  - 94°C 40 s
  - 54°C 40 s
  - 72°C 40 s
  29 repetitions (30 cycles).

The reaction is going to be analyzed the next day and can stay in the thermocycler without problems. Inoculate now the recombinant pGEM minipreps for tomorrow, do 6 clones per plate (every group has then six minipreps). Use 1.5 ml TB amp to grow the colonies in 15 ml tubes (the reused ones), please label your tubes at least with your group number/gene name! Use the inoculation loop to inoculate your colonies. Don't close the tubes too tightly and put them in the shaker at 37°C.
Check your bench and clean it up before you go. Store your stuff in the fridge or freezer and discard your ice bath. See you tomorrow!

Day 5
Goals of the day: make minipreps, run analytic gel with products from RT-PCR. Quantitative preparation of pGEX2T BamH1/EcoR1 cut vector, BamH1/EcoR1 restriction of pGEM minipreps, purification and ligation in pGEX2T, transformation in DH10B/XL1 blue.

Miniprep of plasmid DNA (Sambrook et al. 1991, [9])
The miniprep procedure is an essential procedure in almost every lab that works on molecular biology aspects. It should be conducted carefully in order to facilitate downstream steps which require a certain quality of plasmid DNA that is being prepared (subcloning, sequencing etc.). The procedure consists in the change of medium against an isotonic buffer, then the lysis of bacteria with denaturation of both plasmid an genomic DNA using NaOH and ionic detergents (SDS). Afterwards the DNA is renatured and protein-SDS-gDNA complexes are precipitated and the remaining plasmid is precipitated with isopropanol. then, plasmid DNA is desalinized using Ethanol/water and dissolved in low ionic strength buffer and treated with RNase A.

- Pellet the 1.5 ml TB cultures you inoculated the day before (12000 rpm, 1 min), discard supernatants
- Dissolve the bacterial pellet in 200 µl Buffer 1 (vortex)
- Lyse bacteria adding 400 µl Buffer 2 (10 min RT, mex gently inverting the tube)
- Precipitate proteins adding 300 µl Buffer 3, mix vigorously, then store on ice for 5 min
- Pellet proteins for 5 min 12000 rpm at 4°C or RT
- Retrieve supernatant cautiously to a new tube and add 600 µl Isopropanol, invert the tube about 5 times to mix (optionally leave 10 min a RT)
- Pellet 5 min, 12000 rpm, 4°C or RT
- Wash pellet with 1 ml EtOH 70%, centrifuge for 2 min 12000 rpm, RT.
- Air-dry pellet at RT, resuspend in 50 µl TE/RNase 10 µg/ml
- Analyze 2-4 µl by restriction analysis when you have fragments bigger than 1000 nt, and 4-6 ul when your expected fragments are smaller than 1000 nt.

Tampão 1 miniprep: 10 mM Tris/HCl pH 7,5 ou 8, 1% Glicose (w/v), 25 mM EDTA pH 8,
Tampão 2: 0,2 M NaOH, 1% SDS.
Tampão 3: 3 M Acetato de potássio, 5 M Acido acético
Restriction analysis

Next, we check if the fragment was indeed inserted into the plasmid during the ligation. Take a look at the plasmid map again and design how your plasmid would be if there was a your fragment inside, not forgetting that you introduced a BamH1 site (GGATCC) at one end of your insert.

We are going to digest the clones in a way that the fragment of correct clones can already be recovered from the analytic gel and transferred to the expression vector pGEX2T, predigested with BamH1 and EcoR1. Please setup the following reactions as follows:

<table>
<thead>
<tr>
<th>6 µl Miniprep DNA</th>
<th>Mastermix for 6 reactions?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 µl tampão &quot;3&quot;</td>
<td>µl</td>
</tr>
<tr>
<td>0.3 µl EcoR1</td>
<td>µl</td>
</tr>
<tr>
<td>0.3 µl BamH1</td>
<td>µl</td>
</tr>
<tr>
<td>6.9 µl H₂O</td>
<td>µl</td>
</tr>
</tbody>
</table>

incubate for 2 h at 37°C (incubator).

Whenever possible use the principle "mastermix" to pipet your reactions. Afterwards run a 1% TAE agarose gel and excise the insert of one of the digestions (400-1000 bp) and purify with Glassmilk as described before (chapter PCR). Group 4 should digest in parallel the empty pGEX2T vector (Maxiprep or grown from competency test plates) with the same enzymes and should see one huge ~5 kB band.

Ligation of fragments with sticky (cohesive) ends

After purification of vector and inserts, both are ligated. In Figure 8, the map and polylinker region of this plasmid is shown.
Figure 8: Map of the plasmid pGEX2T. LacI encodes the lac repressor which represses transcription from the lac/tac promoter.

A ligation of fragments with cohesive ends occurs in a slightly different buffer compared to blunt end or A/T ligations. The buffer consists of Tris/Cl pH8, ATP, MgCl₂, and Dithiotreitol, but does not contain polyethylene glycol (PEG) which condenses DNA molecules (PEG is contained in the pGEM T easy buffer). Before the ligation, the fragments maintained on ice are exposed to 42°C in order to destroy any hydrogen bridges between cohesive ends which may be dimers/oligomers of the same molecules that would give non-sense ligation products. Pipet:

<table>
<thead>
<tr>
<th>Tube</th>
<th>&quot;Lig +&quot;</th>
<th>&quot;Lig control&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector pGEX2T digested with BamH1/EcoR1</td>
<td>0.5 µl</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>Insert digested with BamH1/EcoR1</td>
<td>1.5 µl</td>
<td>--</td>
</tr>
</tbody>
</table>

__________________________ heat to 42°C for 3 min e return the tubes to the ice bath

Add:

<table>
<thead>
<tr>
<th>Component</th>
<th>&quot;Lig +&quot;</th>
<th>&quot;Lig control&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>MilliQ water</td>
<td>2.3 µl</td>
<td>3.8 µl</td>
</tr>
<tr>
<td>Ligation buffer 10x</td>
<td>0.5 µl</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>Ligase (0.2 Weiss U/µl)</td>
<td>0.2 µl</td>
<td>0.2 µl</td>
</tr>
</tbody>
</table>
Apply a short spin and put the tube in a cold water bath (10°C) in a styropor box. Incubate for 30 min. Afterwards, transform 1 µl of each reaction in the most competent bacteria DH10B or XL1 blue MRF-as described above. Label the plates properly as before! The agar plates will be taken out of the incubator on saturday and minipreps will be inoculated for you on sunday night.

**Analysis of the cDNA quality**

Now we take a look at the cDNA quality - if there is cDNA and if there is no PCR product in the RT-PCR sample. Prepare a 1.5% TAE Agarose gel. Load the gel with the PCR products done with the K2 oligomix. Evaluate the size and intensity of the formed products. Check that there is no product in the RT-samples.

*Check your bench and clean it up before you go. Store your stuff in the fridge or freezer and discard your ice bath. See you on monday!*

**Day 6**

Goals of the day: Minipreps and analysis of pGEX2T recombinants. Eventually, setup of sequencing reactions. Seminars about next generation DNA sequencing (Plataforma MySeq, groups 1-2, Plataforma 454, groups 3-4, and Ion Torrent 5-7), Retransformation of recombinant pGEX2T clones in E. coli BL21.

**Minipreps of recombinant pGEX2T plasmids**

Analyze the performance of the ligation reaction counting colonies on the vector only and the vector plus insert plate. Harvest and process the minipreps which were grown and purify the pGEX plasmids as done with the pGEM derivates. Verify the presence of recombinant clones by restriction analysis (with BamH1 e EcoR1) as was done before with the pGEM clones. When ready, chose two clones for retransformation in BL21 for expression and sequencing.

**Sanger sequencing of plasmids (dideoxy sequencing)**

In order to check if there was no introduction of mutations in our PCR fragments (Taq Polymerase introduces ≥1 error per 1000 bp synthesized), we monitor the integrity of our fragment by semi-automatic sequencing using the ABI 3550 platform. Sanger sequencing, developed by Frederick Sanger in 1976, is the synthesis of a DNA starting from a small DNA primer a and a single stranded DNA substrate using a DNA polymerase in the presence of dideoxy dNTPs labelled with fluorescent molecules. In the case of the ABI3550 platform, the dideoxy nucleotides are marked with fluorescent markers which emit different light wavelengths. For example, dideoxy A emits "red" light, dideoxy "C" blue light and so on. When the dideoxy base is incorporated, the polymerization necessarily stops at this point (dideoxy base!) and the created fragment has the colour of the incorporated dideoxy base and a specific weight which corresponds to all nucleotides incorporated in this fragment. Then, in a capillary connected to a laser and a prism that differentiates light wavelengths the signal is captured. In figure 9, the principle of Sanger sequencing is resumed.
**Dideoxy-Terminator Sequencing**

A. The sequencing primer anneals at its target site

B. DNA Polymerase binds at 3' end of primer

C. Primer is extended with random termination of the growing chain by labelled dideoxy nucleotides

*Figures of all lengths from 1 to several hundred bases are made.
All oligos of a specific length are labelled with the same colored dye.
Origins are separated on a polyacrylamide gel capable of resolving 500 from 1000.*

**Figure 9: Principle of dideoxy sequencing.**

Note that the random incorporation of terminators (labelled dideoxy nucleotides) leads to the production of various fragment sizes and all of them marked with one of the four dyes used for each dideoxy nucleotide. The fragments are then separated in an adequate matrix (polyacrylamide matrix, specific resins etc.) and analyzed using laser light and specific detectors.

In order to run a sequencing reaction we use the components from the BigDye 3.1 kit (Applied Biosystems). The minuscule reaction is pipetted in 0.2 ml PCR tubes or in 96 well plate:

- Miniprep recombinant pGEX2T 2 µl
- primer Mix “pGEX seq” (1.25 pmol/µl) 4 µl
- BigDye Mix (provided) 4 µl

The assay is conducted in a PCR machine in a room of the Parasitology Department of the ICB2. After the reaction, 90 µl Isopropanol 66% are added and the assay is centrifuged at 12000 rpm for 20 min at RT. Use 0.5 ml support tubes in 1.5 ml tubes with cut lids. After centrifugation, discard the supernatant (turning upside down the tube on paper towels) and add 100 µl Isopropanol 75%. Centrifuge again 10 min at 12000 rpm and RT. Afterwards the supernatant is discarded again and the pellet is air-dried in the dark and delivered to the technician who operates the sequencer.

Retransform now the two chosen plasmids (0.5 µl) in *E. coli* BL21 as done before. Label the bottom of the LB amp plate and divide the plate in 2. Don’t forget to add chloramphenicol on your LB amp plate before you spread the transformed bacteria. Spread each transformation on one half. Put your plates in the incubator at 37°C.
Check your bench and clean it up before you go. Store your stuff in the fridge or freezer and discard your ice bath. See you tomorrow!

Day 7
Goals of the day: Inoculate pre-inoculum and main culture in LB-amp-cam, induce bacteria and harvest. Analysis of the sequences in the computer, mount in silico the created plasmids, using the program APE. Specific group seminars.

Production of recombinant GST proteins – 1. Step: growth
Using colonies from the Agar plates plated out yesterday, inoculate one colony in prewarmed 5 ml LB-amp-cam supplemented with 100 µl Glucose 40% in a 50 ml Erlenmeyer. Incubate on the shaker at 37°C. Grow for 2 hours and check if the medium got slightly cloudy. Then transfer to 100 ml prewarmed LB-amp-cam in a 500 ml Erlenmeyer and continue to grow shaking at 37°C. When the culture shows strong growth and a OD$_{600}$ nm > 0.6 add 20 µl IPTG 1 M and continue to grow at room temperature for 3 h. Afterwards, harvest the bacteria in 50 ml Falcon tubes for 15 min at 2800 rpm. Add 2 ml PBS-1% Triton supplemented with lysozyme (100 µg/ml), incubate for 5 min mixing from time to time and then store the tube at -20°C in the freezer.

Computational sequence analysis
The plasmids sequences are delivered in *.ab1 format which can be opened in software which read this format such as Chromas lite. In order to simplify, the supervisor analyzes the sequences using DNAstar software which includes an estimation of the sequence quality. Afterwards the sequences are delivered to each group and analyzed individually using PlasmDB and NCBI’s blast site. Groups with bad sequences should copy the correct sequence from the plasmodb sequence using the oligos and the plasmdb sequence ID. Starting with the amplified sequences, we proceed to mount the plasmids in APE (free download from the internet, windows).

Check you bench and clean it before leaving. Store your stuff in the freezer or fridge and empty your ice bath. See you tomorrow!

Day 8

Recombinant GST protein production – 2. purification
Starting with the lysed pellets from protein-producing bacteria, we are going to purify in a one step protocol the GST fusion proteins. This is possible due the fact that GST has a strong affinity to reduced glutathion. Specifically, soluble GST containing proteins are exposed to a resin that contains immobilized glutathione and stick to it. After exhaustive washings, only GST fused proteins still bind to the resin and these are retrieved by soluble glutathione by competition. The procedure is as follows:

- Thaw the frozen bacteria in a water bath.
• Make sure that the solution is very viscous (free genomic DNA) and transparent.
• Using a 5 ml syringe with a G21 needle, pass the solution 10 times through the needle without creating too much foam. This will shear the DNA and decrease the viscosity.
• Add 3 ml more PBS-Triton 1% and centrifuge for 15 min at 4°C and 2800 rpm (Sorvall).
• Transfer the supernatant with the soluble proteins to a 15 ml tube
• Add 200 µl glutation sepharose slurry (50% em PBS, provided).
• incubate rocking for 1 h at RT. Close the tube firmly.
• Centrifuge at 1500 rpm for 1 min in the Sorvall centrifuge and discard the supernatant with a P1000. Your proteins are supposed to have adhered to the resin.
• Add 15 ml PBS–Triton X100 and mix the resin by inverting the tube a couple of times
• Centrifuge at 2000 rpm for 1 min and discard the supernatant directly from the tube but take care to not lose the resin.
• Repeat the last two steps 3 times using 15 ml PBS
• Try to have only ≤ 200 µl compacted resin.
• Add 500 µl elution buffer (50 mM Tris pH 7.5, 10 mM reduced Glutathion) and mix with a P1000, transferring all resin to an Eppendorf tube.
• Incubate 30 min at RT, mixing from time to time (very slow vortex)
• Then precipitate the resin at 12000 rpm, 1 min at RT. Transfer the supernatant into a new Eppendorf and store on ice. Collect the resin with PBS to 15 ml tube to recycle the resin.

Preparing an SDS-Polyacrylamide gel (for SDS-PAGE)
A very important step in protein purification is the control of purity and integrity of the cleaned protein. Recombinant *P. falciparum* proteins are very often truncated due to the uncommon codon usage. We will prepare 3 gels with the same content (7 produced recombinant proteins, pure GST and a molecular weight standard): One is stained to visualize the proteins, and two are transferred to nitrocellulose membranes to verify if the proteins are recognized by a pool of malaria exposed patient sera and the sera of never exposed persons. The gel system used herein will be the one introduced by Laemmli [10] and consists of a gel with a low percentage and a low pH (6.8, stacking gel) that concentrates the proteins and another gel with a high pH (8.8) and a relatively high acrylamid concentration (6-15%, running gel) to neatly separate the proteins. As the proteins purified herein have molecular weights between 30 and 60 kDa, a 10% SDS-PAGE is adequate to visualize and resolve the proteins.

Before starting, familiarize yourself with the apparatus which will hold the gels (Mini Protean II system BioRad) and mount the glass plates between which the gel will be made. Mark until which point you will fill with running gel. Afterwards prepare the gel solutions for the running and the stacking gel.

(One volunteer for all groups) Use gloves. Pipet in a 50 ml tube:

<table>
<thead>
<tr>
<th>4x Running gel buffer</th>
<th>3.75 ml</th>
<th>(1.5 M TrisCl pH 8.8, 0.4% SDS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide 40%</td>
<td>3.75 ml</td>
<td></td>
</tr>
<tr>
<td>MilliQ water</td>
<td>7.5 ml</td>
<td></td>
</tr>
<tr>
<td>TEMED</td>
<td>90 µl</td>
<td></td>
</tr>
</tbody>
</table>
Add 120 µl of a fresh 10% ammonium persulphate solution and immediately fill the gel solution between the plates until the mark you did. Then add slowly 1 ml 20% methanol/bromophenolblue on top of the gel. Let the gel polymerize and monitor the process looking at the remaining running gel solution. Once it is polymerized, discard the methanolic blue solution on top of the gel by turning it over the sink. Don’t drop it.

(One volunteer for all groups) **Use gloves.** Pipet in a 15 ml tube:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>4x Stacking gel buffer</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>Acrylamide 40%</td>
<td>0.7 ml</td>
</tr>
<tr>
<td>milliQ water</td>
<td>3.7 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>60 µl</td>
</tr>
</tbody>
</table>

Add 60 µl of the 10% ammonium persulphate solution and add on top of the running gel until the gel occupies almost all space until 1 mm under top border of the smaller plate. Insert the comb. Let the gel polymerize and monitor the process looking at the rest of the stacking gel solution.

Prepare 2 litres of 1x Running buffer (0.192 M glycine, 0.025 M Tris base, 0,1% SDS) from the 10x Running buffer stock which **does not contain SDS.** Aliquot 60 µl of your recombinant protein in an Eppendorf tube and add 20 µl 4x Loading buffer (0,25 M TrisCl pH6.8, 6% SDS, 20% glycerine). Heat this mixture at 95°C for 5 min. Then return to RT and spin down the liquids.

Put the gels into the gel chamber and add 1x running buffer: The electrodes must be covered and the buffer should have contact with the top of the gel. Check the buffer reservoir in the middle: there should be no leakage. Now take out the combs and wash the wells with a 2 ml seringe with a fine needle. Then load your samples and record the sequence how you put the samples in relation to the molecular weight marker. Run the electrophoresis with 120 Volts and switch the current off when the blue bromophenol line reaches starts to bleed out of the gel into the lower reservoir (1-1.5 h).

Afterwards use gloves to retrieve one gel from between the plates and stain it with Coomasie brilliant blue solution (1% Coomasie brilliant blue, 50% methanol, 7.5% glacial acetic acid - in water), optionally put it for 15 seconds in a microwave which accelerates staining. Leave it for ~15 min in the staining solution. Then, throw away the coomassie solution and rinse once with clean water. Then add destaining solution (20% methanol, 10% glacial acetic acid, in water). While this is running, take out the other two gels and prepare them for wet transfer onto Hybond C membranes. The setup for this procedure is shown in Figure 10.
Figure 10: Mounting na SDS gel for transfer to a membrane. Note that the gel should be between the black side of the gel cassette and the membrane. Inverting this will transfer the proteins to the buffer and you lose the proteins.

It is important that everybody watches what the person does who is mounting the gel-membrane sandwich. It is imperative that i) the membrane is wetted in transfer buffer (1x running buffer without SDS + 10% methanol) and that ii) no air bubbles are trapped between gel and membrane and that iii) the orientation is right and the proteins are not transferred to the whatman paper instead of the membrane. When the sandwich is mounted, it is transferred to the transfer tank which is filled with the buffer in which the sandwich was mounted. As a general rule, the electric field should be 1 V per cm² gel for one hour. Normally, transfers at 80 Volts for 1 h are sufficient.

After the transfer, the apparatus is unmounted and and rinsed with deionized water. The membrane is bathed briefly in Ponceau stain and photographed (0.2 g Ponceau, 1 % glacial acetic acid in water). The transferred proteins may appear if there is more than ~1 microgram in one band on the membrane. Afterwards the membranes are stored humid in Saran wrap (“vitafilme”).

Bradford test
In order to measure the quantity of proteins on the membrane, a Bradford test is conducted in 96well plate using a BSA serial dilution as standard. For this, three groups (triplicate values) should work out a serial dilution of 0.1% BSA in PBS as follows: The first well receives 20 µl BSA 0.1%, the next 5 wells receive 10 µl PBS. Then, 10 µl are pipetted form the BSA well to the next well with PBS and mixed by pipetting. Then 10 µl are retrieved and taken to the next well and mixed equally. This is repeated until the forelast well is diluted and then 10 µl of 4 times diluted BSA are discarded with the tip. The sixth well contains only 10 µl PBS. Now, put 10 ul of your proteins in lateral wells and add 90 µl Bradford reagent (BCA reagent diluted 1:4 in PBS (1 part BCA + 3 parts PBS). Measure the result in a photometer at 595 or 605 nm. Analyze the result producing a graph in Excel or equal, making a
standard curve with BSA and reading out the values of your proteins. We need these values for the next step.

**ELISA, 1: Coating**

With the quantified proteins on ice, go ahead and fill 12 horizontal wells of a microplate with 0.2 µg of your protein in 50 µl coating buffer (NaCO₃ 50 mM at pH 9.6). Similarly, put 0.2 µg GST in the last line (12 wells). When all groups pipetted their proteins, the 96well plate is covered stored in the refrigerator at 4°C.

*Clean your bench and store your things/solutions in the freezer or fridge. Throw away your ice. See you tomorrow!*

**Day 9**

Goals of the day: Perform the immunoblot, measure patient plasma antibodies against your proteins in ELISA.

**Western blot**

In this experiment, we check if a frequently exposed (asymptomatic) individual recognizes recombinant proteins differently compared to an acutely infected person. The principle is as follows: After blocking potential protein binding sites with an excess of non-related protein (4% skimmed milk proteins in PBS plus 0.1% Tween20 – a non-ionic mild detergent) for 1 h at RT (or overnight at 4°C), the antisera are pipetted over the membrane and specific IgG – if present - will then recognize the blotted proteins. After exhaustive washings, an antibody anti-IgGhuman conjugated to an enzyme, capable of performing a colorimetric or chemoluminescent reaction is added. In Figure 11, the schematic outline of a western/immunoblot is given.

![Western blot diagram](image)

**Figure 11: Principle of the Western blot.** The conjugated enzymes are usually alcaline phosphatase, peroxydase from horse radish or even chromophors such as Cy5, Quantum dots or Alexa colorants.
Manipulate the membranes which came off the transfer with forceps or using gloves. First we block the membranes with 4% “Molico” skimmed Milk in PBS-Tween20 (PBS-T). The membranes should be covered with sufficient liquid (≥20 ml per membrane, in two boxes).

- Incubate the membranes for 1 h at RT on the seesaw (or overnight at 4°C)
- Wash the membranes with 30 ml PBS-T, 5 min on the seesaw.
- Apply 10 ml PBS-T 1% leite on each membrane (Mark one Box “as” and the other “acute mal”). Add 4 µl of the "AS" plasma to the Box AS e 4 µl of the plasma “acute mal” to the other box. Incubate 1 h at RT on the seesaw.
- Discard the liquid with antibody.
- Wash the membranes with an excess of PBS-T for three times, discarding the PBS-T each time. It’s better to wash too much then too less.
- Apply 10 ml of PBS-T 1% milk and add 4 µl de antiHuman IgG peroxydase.
- Incubate 1 h on the seesaw, then discard the solution.
- Wash the membrane 3 times as before. Someone should now switch on the very sensitive camera in the dark room and reserve it for the next hour. The camera works best when cooled to -15°C.
- Take the membranes out of the Box and put them on saran wrap four times the size of the membrane and protect it from drying.
- Prepare the developing solution (Western pico, Pierce/KPL): 1 ml Luminol + 1 ml oxygenized water in a 2 ml Eppendorf (this is solution A and B of the kit). NEVER EVER MIX THE STOCK SOLUTIONS IN THEIR BOTTLES!
- Put the membranes, a P1000 pipet plus tips, gloves and paper towels in a tray and go to the dark room with the fotodocumenter.
- Take a photo of the white membrane and then pour the revelation liquid over the membrane (take off the upper layer of saran wrap) and take serial photos of the chemoluminescent signals and store them on your pendrive.
- Clean the photodocumenter. Back to the lab, clean the membrane boxes with deionized water.

**ELISA 2. developing**

The proteins in the microplate now adhered firmly to the surface of the plate wells. Now the remaining protein binding sites are blocked with non-reactive proteins and then we apply plasma samples with specific antibodies against the coated proteins. Localize if there are: Plasmas on ice, PBS-T 4% skimmed milk (same as for the western blot), PBS-T 1% skimmed milk. PBS-T in the sprayerbottle, Eppendorfs, paper towels.

- Discard the coating solution in the sink and wash the wells with PBS-T once.
- Beat the plate uside down on the paper towels to get rid of remaining liquid.
- Add 50 µl PBS-T 4% Milk per well and incubate covered for at least 30 min.

We should have 8 proteins on the plate (7 from each group plus pure GST). Each well is filled with 50 ul antibody solution in PBS/1% milk, so calculate before how much antibody you need to have enough (without wasting) antibody to have a 1:500 dilution. Include in your calculation a couple of wells more in order to have excess volume in case the pipetting is not 100% exact.

- Wash the wells once with PBS-T
• Apply the antibody 1:500 in PBS-T milk in columns (remember that in the horizontal lines there are the different proteins, this means that every protein is tested against each plasma)
• Incubate closed for 1 h at RT.
• Prepare the anti-human IgG-peroxidase conjugate – the same as in western blots – in a 1:1500 dilution and store it on ice, prepare 6 ml per plate.
• Discard the liquid, by inverting the plate and wash the wells 4 times with PBS-T as before.
• After the last washing, dry the plate by hitting it on paper towels, upside down.
• Add 50 µl of the antiHumanIgG peroxidase solution and and incubate for 1 h at RT.
• Discard the liquid by inverting and wash the wells 4-5 times with PBS-T.
• Beat the plate to dryness on a paper towel as before.
• Prepare the developing mix: TMB solution and hydrogen peroxyd solution mixed 1:1, 5.5 ml total volume in a multichannel pipettor bench.
• Prepare another multichannel pipettor bench with 1 M HCl Take care, this has pH 0 and harms you if you touch it.
• Using the multichannel pipet, apply 50 µl of the TMB substrate and switch on the timer to 3 to 5 min when you start pipetting the first column. There is no need to change the tips.
• After applying to all wells, discard the tips and stick new ones to the multichannel pipet.
• If wells get too blue before 5 min pass, then stop the reaction at that time point by pipetting 50 µl 1 M HCl.
• With the stopped plate, proceed to measuring the extinction of the wells in a plate reader at 450/595 nm wavelengths

Show the results in a graph in Excel, decrease the values for each plasma and protein by the values obtained for each plasma with GST.

Check and clean your bench before you go. Store your stuff in the fridge or freezer and get rid of your ice bath. See you tomorrow!

Day 10

Help desk – Revision of all that was done then lunch.

At 14.00: written exam

Evaluate the course: what could be better?

Annex: Oligos used to amplify genes from *P. falciparum*.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Proteins</th>
<th>Expressed parts</th>
<th>Peptide characteristics</th>
<th>Immune epitopes</th>
<th>Primers pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF80310c</td>
<td>MSP4</td>
<td>Exon 1</td>
<td>C</td>
<td>B and T</td>
<td>F_GCAGTCGGGGAAAGAAAAACTAAATGTGG</td>
</tr>
<tr>
<td>PF10_0345</td>
<td>MSP3</td>
<td>C-terminal</td>
<td>C</td>
<td>B</td>
<td>F_GCAGTCACCTTATTTAGTTGGTG</td>
</tr>
<tr>
<td>PFF0995c</td>
<td>MSP5</td>
<td>N-terminal</td>
<td>P</td>
<td>?</td>
<td>F_GCAGTCGAAAAMGGGAAGGTTGGTTTAC</td>
</tr>
<tr>
<td>MAL7P1.176</td>
<td>EBA175</td>
<td>N-terminal</td>
<td>Duffy-like</td>
<td>B (NKND)</td>
<td>F_GCAGTCTGTGAGAAAGGATATTGATCC</td>
</tr>
</tbody>
</table>
C, conserved; P, polymorphic. Restriction site are indicated in bold letters. F, primer forward e R, primer reverse.

### Bibliografia


### Annex 2
Guidelines for the outfit of figures in the report (which are valid for master and doctoral theses and scientific publications).

The figure and its primary result has to be shown as complete as possible. Large gel pics should be cropped and only the interesting parts are shown. The legend must be complete and relate to all data that are visible in the picture in order to turn the picture auto-explicative. See the example how it could be done:

Raw image with marks which part of the gel is to be used:
And here the edited image:

![Image of gel electrophoresis](image_url)

**Figura 1**: Digestão de 4 clones pGEX4T1-Pfmsp10 (1-4) com as enzimas BamHI e EcoRI analisado em gel de TAE agarose 1%. A flecha vermelha indica a ocorrência do fragmento referente a Pfmsp10 e no lado esquerdo os tamanhos dos fragmentos do peso molecular estão informados. O asterisk indica vetor plasmídial não cortado e o quadrado RNA remanescente da bactéria. O sinal nos pocos deplasms de plasmídeo indica que houve presença de gDNA da bactéria de alto peso molecular. Foram digeridos 5 ul de um miniprep de 50 ul total.

**References**

The references should be inserted with a reference manager. We recommend Mendeley which is freeware and creates a plugin for MS word and Open/LibreOffice writer. Whenever you cite work from others, put the reference.