Rapid communication

Reliable transfection of *Plasmodium falciparum* using non-commercial plasmid mini preparations

Tobias Spielmann*, Matthew W.A. Dixon, Maria Hernandez-Valladares, Mandy Hannemann, Katharine R.Trenholme, and Donald L. Gardiner

Infectious Diseases and Immunology Division, Queensland Institute of Medical Research, PO Royal Brisbane Hospital, QLD 4029, Australia.

*Corresponding author. E-mail address: Tobias.Spielmann@gmail.com

Abstract

Transfection of *Plasmodium falciparum* is instrumental in the research of this parasite. However the actual transfection protocol has not changed significantly since the first description and it is generally believed that large amounts of highly pure plasmid DNA are needed for successful transfection. Here we report the transfection of *P. falciparum* using a protocol based on non-commercial minipreparations of plasmid DNA. This method permits the reliable transfection of *P. falciparum* using less resources and costs with a success rate comparable to currently used methods. A moderate throughput may be achieved using this method, providing a first step towards systematic transfection approaches in this parasite.

Keywords: Malaria, *Plasmodium falciparum*, transfection

The development of the transfection technology for *Plasmodium* has greatly increased our understanding of malaria parasites. However, despite the progress in vector development, reflected by a growing number of validated promoters, reporters and selectable markers (Cowman and Crabb, 2005; Gardiner et al., 2003), the actual transfection procedure most commonly used for *P. falciparum* has not changed significantly since its first description (Wu et al., 1995), and transfection of *P. falciparum* is still considered a very difficult technique. The low efficiency of transfection and the long selection times to obtain episomally transfected parasites has hampered further development of the actual transfection protocol, as testing different conditions is very time consuming. This has caused a certain mystification of the procedure and there is anecdotal evidence for small changes in the actual protocol that are believed to increase the success rate of transfections as well as to reduce selection times. However, it is generally believed that large amounts of highly pure plasmid DNA (usually prepared using commercial maxiprep kits) are required which then have to be transfected into often large *P. falciparum* culture volumes. By the time selection is applied (48 hours after transfection) this results in often considerable culture volumes for each single

transfection [10-30ml culture (Skinner-Adams et al., 2003b)] that then have to be maintained for weeks. This incurs significant cost as well as handling time, especially if many transfections have to be performed. However, often several constructs have to be tested, for instance to obtain adequate expression of transgenic GFP fusion proteins, as the transgenic protein might not be tolerated if expressed too highly or during the wrong parasite stage. Gateway technology (Invitrogen) allows the generation of different plasmids with different promoters or tags in a short time (Skinner-Adams et al 2003a; van Dooren et al., 2005), making transfection the actual rate-limiting step for such tests. Also, systematic approaches for gene tagging and gene targeting would currently need vast resources and the presently used methods are not suitable to achieve even a medium throughput.

We have developed a transfection protocol based on non-commercial plasmid mini preparations transfected into 250µl infected red blood cells (corresponding to 5 ml of *P. falciparum* culture) that shows a comparable success rate to methods using commercial maxi preparations and larger culture volumes. Drug pressure was applied 6-12 hours after transfection (Voss *et al.* 2006), thereby reducing the number of gametocytes formed and avoiding expansion of the culture volume (transfections were also successful if the drug was added immediately after transfection, although this was not extensively tested). The mini preparation protocol can also be used to conveniently produce plasmids for DNA sequencing. Once the plasmid has been confirmed to contain no mutations, the remaining plasmid can then be transfected. We successfully used this mini preparation transfection method (MiPreT) for a number of constructs that are based on different transfection vectors and many constructs were tested several times. Resistant parasites usually reached 1% parasitemia between 20 and 35 days after transfection. An estimation of the time after transfection until cultures reached 1% revealed a mean of 27.5 days for the MiPreT method (10 individual transfections). This is comparable to selection times observed in our laboratory using maxi preparation transfection methods (MaxPreT) (Skinner-Adams et al., 2003b). There was no difference between the time constants of MiPreT and MaxPreT transfections with constants of 0.6-

1.0 recorded for both of the methods with no discernable pattern. Plasmids that required very long selection times using MaxPreT (presumably due to adverse effects of the transgene on the parasites), were also successfully transfected using MiPreT. Although transfected parasites were never obtained for certain plasmids, transfections with these plasmids were also always unsuccessful when MaxPreT was used. These constructs were designed to mediate expression of GFP-fusion proteins of early transcribed membrane proteins (ETRAMP) (Spielmann et al., 2003) under the control of the *hsp86* promoter and might have been deleterious to the parasites. Thus the success rate of the method presented here is comparable to using plasmids prepared from commercial maxi preparations.

To further compare MiPreT and MaxPreT, we transfected 3D7 parasites from the same culture side by side using two previously described plasmids. Plasmid 1 was based on vector pHHC*/DR0.28 (O'Donnell et al., 2002) and mediates expression of a myc-tagged ETRAMP4 (ETRAMP4myc) under the control of the *hsp86* promoter (Spielmann et al., 2006). The second plasmid was vector pARL, which mediates expression of GFP under the *crt* promoter (Crabb et al., 2004). All transfections were successful and 1% parasitemia was reached 22-29 days after transfection (table 1). The transgene was expressed in parasites obtained by the MiPreT method as evident from immunofluorescence assays or fluorescence of live parasites (Figure 1). To reproduce these results we chose a different commonly used parasite strain (D10) and repeated the side by side test of MiPreT and MaxPreT. Again none of the transfections failed and 1% parasitemia was reached between 24-34 days after transfection. These side by side tests further confirmed our previous results that the performance of MiPreT and MaxPreT are comparable.

To assess the viability of transfections utilizing MiPreT, vector integrity was confirmed. Stability of the vector from both transfection methods was verified using both vector rescues and Southern blotting (Figure 2). Genomic DNA (gDNA) was extracted from the following transfections

3D7pARL MaxPreT, D10pARL MiPreT, 3D7 ETRAMP4myc MiPreT and D10 ETRAMP4myc MaxPreT. The gDNA was digested with *EcoR*I and *EcoR*V and probed with hDHFR. The Probe specifically hybridised to a 1900kb band in all pARL and ETRAMP4myc transfected lines, this size is consistent with the expected restriction pattern for episomally maintained vector (Figure 2 A). For vector rescue, precipitated gDNA from the transfected parasites was transformed into *E. coli* and plasmid DNA was extracted. The plasmids were then digested using *Bgl*II and *EcoR*V and compared to un-transfected vector. All rescued plasmids tested showed an identical digestion pattern to un-transfected vector (Figure 2 B).

The MiPreT method was then used to assess the localization of two members of the ETRAMP family that have not previously been localized (Spielmann et al., 2003). The full length coding sequences of *etramp5* (PFE1590w) and *etramp11.1* (PF11_0039) were cloned into the pHHC*/DR0.28 vector containing a myc tag as previously described for *etramp4* (Spielmann et al., 2006). This resulted in vectors pHHC*/DR0.28-ETRAMP5myc and pHHC*/DR0.28-ETRAMP11myc which were transfected into 3D7 parasites using the MiPreT method. The resultant parasite lines ETRAMP5myc and ETRAMP11.1myc were analysed in immunofluorescence assays using anti-myc antibodies (Figure 3). Both myc-tagged proteins were detected in a peripheral stain around the parasite similar to previously analysed ETRAMP members (Spielmann et al., 2003; Spielmann et al., 2006). This suggests that these ETRAMP members also reside in the prasitophorous vacuole membrane and further demonstrates the usefulness of the MiPreT method.

Presently it is generally accepted that large amounts of highly pure DNA is required for successful *P. falciparum* transfection. However, our results show that a cheap and simple plasmid preparation is sufficient to achieve reliable transfection of this parasite. The comparatively small parasite culture volumes and the method to prepare plasmid DNA might even be suitable for automation. We hope that this will encourage further efforts to simplify this important technique. Moreover this

method provides a significant step towards high throughput approaches to enable systematic targeting of *P. falciparum* gene function, which will be the logical next step in the post-genomic era of this parasite.

Acknowledgements

We thank Drs. Alan Cowman, Brendan Crabb and Akin Adisa for vectors. T. S. is supported by a fellowship from the Swiss National Science Foundation. M.D. is supported by an ANZ Trustees PHD scholarship. K. T and D. G are supported by NHMRC program grant number 290208 and by a generous donation from Mark Nicholson, Alice Hill and the Tudor Foundation. M.H.-V. was supported by a Human Frontiers Science Program short-term fellowship.

References

Cowman, A.F., and Crabb, B.S. 2005. Genetic manipulation of *Plasmodium falciparum*. In: Sherman, I.W., (Ed.), Molecular Approaches to Malaria, ASM Press, Washington DC pp. 50-67.

Crabb, B.S., Rug, M., Gilberger, T.W., Thompson, J.K., Triglia, T., Maier, A.G., Cowman, A.F., 2004. Transfection of the human malaria parasite *Plasmodium falciparum*. Methods Mol Biol 270, 263-276.

Gardiner, D.L., Skinner-Adams, T.S., Spielmann, T., Trenholme, K.R., 2003. Malaria transfection and transfection vectors. Trends Parasitol 19, 381-383.

Hawthorne P.L, Trenholme K.R, Skinner-Adams T.S, Spielmann T, Fischer K, Dixon M.W.A, Ortega M.R, Anderson K.L, Kemp D.J, Gardiner D.L. 2004. A novel *Plasmodium falciparum* Ring Stage Protein, Rex, is located in Maurer's Clefts. Mol Biochem Parasitol 136, 181–189.

O'Donnell, R.A., Freitas-Junior, L.H., Preiser, P.R., Williamson, D.H., Duraisingh, M., McElwain, T.F., Scherf, A., Cowman, A.F., Crabb, B.S., 2002. A genetic screen for improved plasmid segregation reveals a role for Rep20 in the interaction of *Plasmodium falciparum* chromosomes. EMBO J 21, 1231-1239.

Skinner-Adams, T.S., Hawthorne, P.L., Trenholme, K.R., Gardiner, D.L., 2003a. GATEWAY vectors for *Plasmodium falciparum* transfection. Trends Parasitol 19, 17-18.

Skinner-Adams, T.S., Lawrie, P.M., Hawthorne, P.L., Gardiner, D.L., Trenholme, K.R., 2003b. Comparison of Plasmodium falciparum transfection methods. Malar J 2, 19.

Spielmann, T., Fergusen, D.J., Beck, H.P., 2003. *etramps*, a new *Plasmodium falciparum* gene family coding for developmentally regulated and highly charged membrane proteins located at the parasite-host cell interface. Mol Biol Cell 14, 1529-1544.

Spielmann, T., Gardiner, D.L., Beck, H.P., Trenholme, K.R., Kemp, D.J., 2006. Organization of ETRAMPs and EXP-1 at the parasite-host cell interface of malaria parasites. Mol Microbiol 59, 779-794.

van Dooren, G.G., Marti, M., Tonkin, C.J., Stimmler, L.M., Cowman, A.F., McFadden, G.I., 2005. Development of the endoplasmic reticulum, mitochondrion and apicoplast during the asexual life cycle of *Plasmodium falciparum*. Mol Microbiol 57, 405-419.

Voss TS, Healer J, Marty AJ, Duffy MF, Thompson JK, Beeson JG, Reeder JC, Crabb BS, Cowman AF. 2006. A var gene promoter controls allelic exclusion of virulence genes in *Plasmodium falciparum* malaria. Nature. Feb 23;439(7079):1004-8.

Wu, Y., Sifri, C.D., Lei, H.H., Su, X.Z., Wellems, T.E., 1995. Transfection of Plasmodium falciparum within human red blood cells. Proc Natl Acad Sci U S A 92, 973-977.

Figure Legend

Fig. 1. Transgene expression in 3D7 parasites transfected using MiPreT. **A.** Detection of ETRAMP4myc in acetone fixed cells using an anti-myc antiserum. The green anti-myc stain (myc)

overlayed with Hoechst stained nuclei (n) shows the typical staining pattern for ETRAMP4. **B.** GFP fluorescence in the parasite cytoplasm in live parasites. GFP fluorescence in the parasite cytoplasm is seen in a single and a triple infected red blood cell. bf, bright field; n, nuclei. Size bars, 5µm.

MaxPreT transfections were done using Maxiprep DNA (Qiagen) as described (Skinner-Adams et al., 2003b) using previously described electroporation conditions (Wu et al., 1995). Minipreps for MiPreT were prepared by inoculating 5ml LB medium with *E. coli* colonies containing the appropriate plasmid (rich media such as 2xYT are not suitable to reach good yields) in a 50ml conical tube. *E. coli* were grown for 16-20 hours at 37ºC, cells were pelleted and transferred into a 1.5ml tube, briefly spun and the remaining medium was removed. The pellet was loosened by vigorous vortexing and bacteria were lysed by adding 400µl alkaline lysis buffer (0.1 M TrisHCl pH 8.0, 0.1 M NaOH, 0.5 % SDS, 1 mM EDTA, with RNaseA added to 50µg/ml prior to use). Tubes were inverted several times and vortexed briefly. Then 200µl 3M NaAcetate pH 5.3 was added, the sample mixed well and centrifuged at maximum speed in a table top centrifuge for 5-10 minutes. The supernatant was transferred to a fresh tube and precipitated by adding 550µl isopropanol, stored for 10 minutes and centrifuged for 20 minutes. The pellet was briefly dried, resuspended in 100µl TE buffer and phenol/chloroform extracted followed by precipitation using 1/10 volume 3M NaAcetate pH 5.3 and 3 volumes 100% ethanol. DNA was pelleted by centrifugation for 30 minutes and the pellet washed and sterilised using 75% ethanol. All the following steps were performed in a laminar air flow cabinet. The 75% ethanol was removed and the DNA pellet dried in the laminar air flow cabinet. The mini prep DNA pellet represents approximately 40-60ug DNA. The pellet was then resuspended in 30µl TE, stored for 5 minutes and 700µl cytomix (Wu et al., 1995) were added. The parasite culture to be transfected was pelleted, 250µl of the packed cells were added to the DNA solution, mixed and transferred into a 0.4 cm electroporation cuvette. Parasites were transfected using standard bacterial transformation conditions as described (Wu et al., 1995). The transfected parasites were added to a fresh 60 mm dish containing 6ml fresh media and one drop of uninfected blood to compensate for the lysis of red

blood cells. Transfected cultures were put on drug (WR99210 at 5nM concentration) 6-12 hours (or immediately) after transfection and fed every day until parasites had died off. Then the cultures were fed every second day until 1% parasitemia was reached.

Fig. 2. Genomic analysis of transfections. **A.** Southern blot illustrating the presence of pARL and ETRAMP4myc vectors in 3D7 and D10 transfected lines from MiPreT and MaxPreT transfections. **B.** Representative vector rescues from 3D7 pARL(MaxPreT) and D10 pARL(MiPreT) parasite lines. Both untransfected and rescued vector show the same restriction pattern (4687 and 2722bp).

Fig. 3. ETRAMP5myc and ETRAMP11.1myc transgene analysis of MiPreT transfected parasites using immunofluorescence assays. Cells were fixed with acetone and probed with an anti-myc serum (cy 2, green), co-stained with a serum detecting REX1 (TexasRed, red) localising to the Maurer's clefts (Hawthorne et al, 2004) and Hoechst nuclei staining (blue). Each panel shows 3 representative cells from different sections. **A.** ETRAMP5myc parasites showing localization to the periphery of late ring and early trophozoite stage parasites. **B.** ETRAMP11.1myc parasite line with localization of the transgene to the periphery of ring stage parasites. Size bars, 5μm.

Table 1. Side by side comparison of the mini preparation transfection protocol with the standard method using commercial maxi preparations.

Plasmid	Maxi preparation protocol	Mini preparation protocol
ETRAMP4myc	29*(3D7), 26*(D10)	28*(3D7), 24*(D10)
pARL	22*(3D7), 31*(D10)	24*(3D7), 34*(D10)

* Days after transfection until 1% parasitemia was reached; parasite line used indicated in brackets

Figure 1

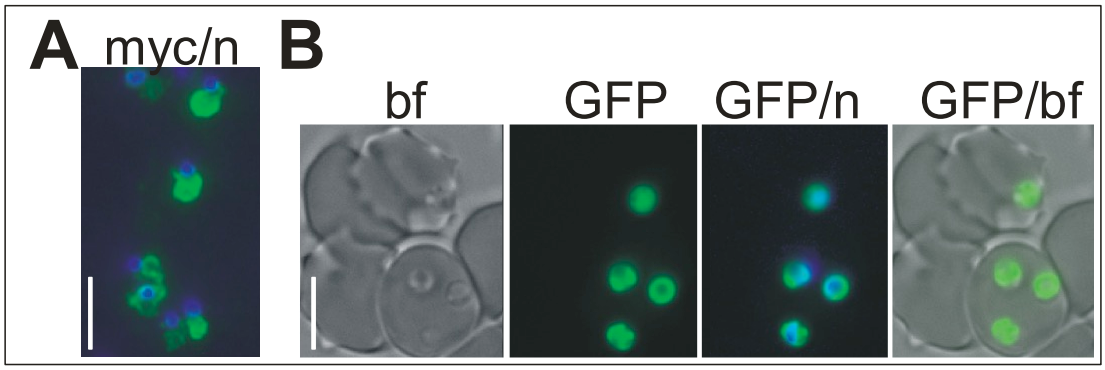

Figure 2.

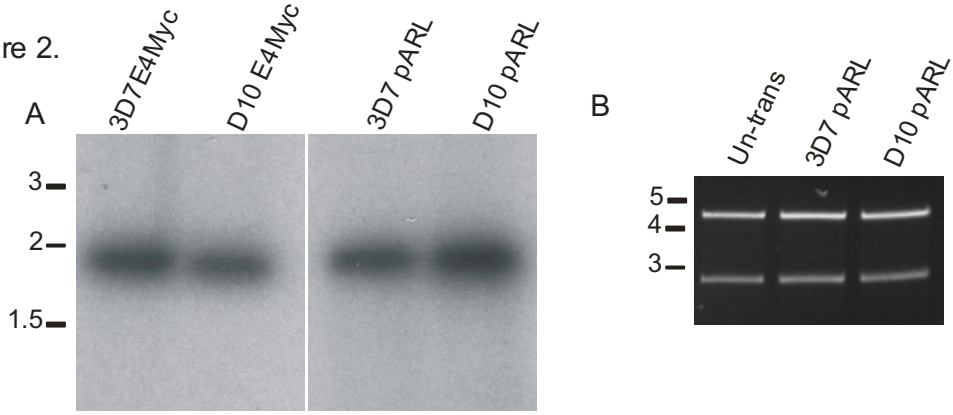

Figure 3.