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Methodology

Comparison of *Plasmodium falciparum* transfection methods

Tina S Skinner-Adams, Paula M Lawrie, Paula L Hawthorne, Donald L Gardiner and Katharine R Trenholme*

Address: Malaria Biology Laboratory, The Australian Centre for International and Tropical Health and Nutrition, a joint program of the Queensland Institute of Medical Research and the School of Population Health, University of Queensland, Australia

Email: Tina S Skinner-Adams - tinaS@qimr.edu.au; Paula M Lawrie - paulaL@qimr.edu.au; Paula L Hawthorne - paulaHa@qimr.edu.au; Donald L Gardiner - donG@qimr.edu.au; Katharine R Trenholme* - kathT@qimr.edu.au

* Corresponding author

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Abstract

The development of an electroporation based transfection method for *Plasmodium falciparum* has been very successful for the study of some genes but its efficiency remains very low. While alternative approaches have been documented, electroporation of infected red blood cells generally remains the method of choice for introducing DNA into *P. falciparum*. In this paper we compare four published transfection techniques in their ability to achieve stable transfections.

Technology enabling the genetic manipulation of malaria parasites has advanced greatly in recent years. Early success with the transfection of rodent *Plasmodium* species [1] was followed by the achievement of transient [2] and stable transfections in *Plasmodium falciparum* [3–5]. Today transfection technology is instrumental in assessing numerous aspects of *P. falciparum* biology, including gene function [6] and protein targeting [7]. However, progress in this area is still limited by poor transfection efficiencies and by the number and type of genetic modifications that can be achieved with current vectors. New vectors that permit both positive and negative selection are becoming available and will address many of the limitations of the current vectors [8,9]. However, the length of time required to obtain a stable transfection can still be rate-limiting.

Electroporation of infected red blood cells is generally the method of choice for introducing DNA into *P. falciparum*. Alternative approaches including the electroporation of uninfected red blood cells to allow spontaneous uptake of DNA [10] and the use of polyamidoamine dendrimers to transfer DNA across membranes [11] have been investigated in an effort to improve transfection efficiency. Each of these alternative methods have been shown to result in substantially higher levels of reporter signals than is achieved with methods of direct electroporation. However it is not known if these techniques have an improved efficiency in establishing stable transfections. Transient transfection efficiency is believed to be some fifty times higher than stable transfection, but is often of little value to investigators performing transfections experiments with *P. falciparum*.

In order to optimize transfection efficiency and establishment of stable transfections we have compared four published transfection techniques in their ability to achieve stable transfection. These are the electroporation methods of Wu *et al* [2] and Fiddock *et al.* [12], the spontaneous uptake method of Deitsch *et al* [10] and the polyamidoamine dendrimer method of Mamoun *et al.* [11] An additional chemical transfection agent, Effectene (Qiagen) was also tested.

Materials and Methods

Parasites

P. falciparum clone 3D7, obtained from D. Walliker (University of Edinburgh, Edinburgh UK), was cultured as described [13] and cultures containing 10% ring stage parasites were used for all transfection experiment. The experimental drug WR99210 (Jacobus Pharmaceutical Company, New Jersey) was use to select for parasites containing plasmid. The growth and development of each transfection was monitored by Giemsa stained thin smears. Cultures in which drug resistant parasites could be detected before day 31 post transfection were considered to be stably transfected.

Construction of the plasmid vector pHH1-DAG and DNA preparation

The *P. falciparum* expression vector pHH1 [14] was used to construct a pHH1-DAG knockout plasmid for use in all experiments. In this vector, the *T. gondii dhfr-ts* is replaced with the human *dhfr* mutated to encode resistance to WR99210 [12]. The transfection construct pHH1-DAG was made using oligonucleotides 4137D-X5' (GACTCGAGGCCAACTCGAAACTTCCTGC) and 4137E-X5' (GACTCGAGCATCTTTTGCTTGCGGCCGG) to generate an 899 bp PCR product corresponding to part of a gene on the right arm of chromosome 9, (PlasmoDB identifier PFI 1735c) but missing both the 3' and 5' regions. The pHH1 vector was digested with Xho1 and Bgl1 to release the *hsp86* upstream regulatory sequence, the Xho1 and Bgl1 ends were then "filled " and the 899 bp PCR product blunt end cloned into this site to create the vector pHH1-DAG. The plasmid was propagated in PMC103 cells and prepared using Qiagen Mega Prep columns. Sufficient plasmid was prepared for all experiments, pooled and stored at -70°C until required.

Transfection methods

In order to make all transfection experiments as comparable as possible, red blood cells were drawn from a single donor 2–3 hours prior to the transfection being performed. The same donor was used for all experiments. All plasmid used was from a single plasmid pool (see above). While it was not possible to carry out all transfections simultaneously, strict monitoring ensured that all transfections were maintained under the same environmental conditions.

Spontaneous Uptake of DNA from erythrocytes

The method is essentially as described [10]. Briefly, a 240 μl aliquot of erythrocytes was washed 3 times in RPMI 1640 and once in 5 mls cytomix [2]. Fifty μg of DNA was added with cytomix to give a final volume of 400 μl. The cells were transferred to a 0.2 cm cuvette, chilled on ice and electroporated using a Bio-Rad gene pulser and 0.31 kV, 960 μf. After electroporation the erythrocytes were washed once with RPMI 1640 and resuspended to a 5% haematocrit in complete culture media. A 4.5 ml aliquot of this suspension was dispensed into a 150 mm petri dish and inoculated with 0.5 mls of parasite culture. The culture was diluted with DNA loaded RBC's as required until drug pressure was applied on day 6 post transfection and maintained at 5 μm WR99210 thereafter.

Electroporation method 1

The method was essentially as described by Wu *et al*. [2]. Briefly, parasitized red blood cells were pelleted and resuspended in cytomix containing 150 μg DNA to give a final volume of 800 μl. Electroporations were carried out in 0.4 cm cuvettes using settings of 2.5 kV, 25 μf. Electroporated samples were immediately mixed with 30 mls complete culture media and fresh RBC's added to give a 5% haematocrit in a 150 mm petri dish. Cultures were maintained without drug pressure for 48 hours and then maintained on 5 μm WR99210 thereafter

Electroporation method 2

The method was essentially as described by Fidock and Wellems [12]. Briefly, parasitized red blood cells were pelleted and resuspended in cytomix containing 150 μg DNA to give a final volume of 800 μl. Electroporations were carried out in 0.4 cm cuvettes using settings of 0.31 kV and 960 μf. Electroporated samples were immediately mixed with 30 mls complete culture media and fresh RBCs added to give a 5% haematocrit in a 150 mm petri dish. Cultures were maintained without drug pressure for 48 hours and then maintained on 5 μm WR99210 thereafter

Chemical Transfection method 1 (Superfect)

The method is essentially as described by Mamoun *et al*. [11] and the manufacturer (Qiagen). 10 μ DNA was incubated with 40 μl superfect reagent for 10 mins and then mixed with 1 ml of complete culture media. This mix was then added drop wise to 240 μl packed parasitized red blood cells. Cultures were maintained for 48 hours without drug pressure and were diluted with fresh red blood cells when required.

Chemical Transfection method 2 (Effectene)

This method was carried out according to the manufacturers instructions (Qiagen), 2 μg DNA was mixed with buffer EC to a volume of 300 μl, 16 μl of enhancer was added and the mix incubated for 5 mins at room temperature. 60 μl of Effectene reagent was added to the mix and incubated for a further 10 mins before the addition of 1 ml complete culture medium. This DNA mix was then added drop wise to 240 μl packed parasitized red blood cells. After DNA addition 4.5 ml of complete media was added to the cells. Cultures were maintained for 48 hours without drug pressure and were diluted with fresh red blood cells when required.

Table 1: In vitro efficacy of *P. falciparum* transfection techniques.

Method of Transfection	Parasite Positive (Day)	1% Parasitaemia (Day)	Success Rate (%)
Spontaneous Uptake (Deitsch *et al.* 2001)			100%
Transfection 1	19	24	
Transfection 2	16	C	
Transfection 3	19	24	
Transfection 4	17	24	
Average	**18**	**24**	
Electroporation A (Wu *et al.* 1995)			100%
Transfection 1	30	36	
Transfection 2	30	37	
Transfection 3	17	20	
Transfection 4	16	19	
Average	**23**	**28**	
Electroporation B (Fiddock *et al.* 1997)			25%
Transfection 1	N/A	N/A	
Transfection 2	N/A	N/A	
Transfection 3	16	19	
Transfection 4	N/A	N/A	
SuperFect (Mamoun *et al.* 1999)			0%
Transfection 1	N/A	N/A	
Transfection 2	N/A	N/A	
Effectene			0%
Transfection 1	N/A	N/A	
Transfection 2	N/A	N/A	

N/A Not achieved **C** culture lost due to contamination

Results and Discussion

All transfections performed using the high voltage/low capacitance method of Wu *et al.* [2] resulted in stable transfection (Table 1). However, only one of four transfections performed using the low voltage/high capacitance method of Fiddock *et al.* [12] was successful. This contrasts with previous findings [12] and may be because in previous studies the success of transfection was measured by expression of a reporter gene, whereas we only considered a transfection to be successful if continuous growth of parasites after drug selection was achieved. Differences in viability post-electroporation are not believed to be responsible for the different transfection efficiencies of these 2 protocols as no difference in parasite growth was observed. We acknowledge that other factors relating to red cells, parasites, vectors or equipment may have contributed to the observed differences, investigating these factors was beyond the scope of this study.

The spontaneous uptake method of Deitsch *et al.* [10] was the most successful of all the methods assessed. All four cultures transfected using this technique grew out after drug selection. This technique was labour intensive, requiring the addition of DNA loaded red blood cells for several days before drug selection. However, as fewer parasites were required, less media and other consumables were needed. The electroporation of uninfected red cells rather than the parasites themselves is likely to be an important factor in the success of this method as it has no impact on parasite viability. This technique has also been shown to be successful in achieving stable transfections with smaller amounts of DNA than the 200 μg used here making this method of transfection extremely favourable.

Success with the SuperFect method has been previously repored [11] and the protocol used during this study was essentially as described, however we were unable to use either the SuperFect or Effectene reagent to generate stable transfectants. Optimization experiments with both the SuperFect and the Effectene protocols may be the key to improving transfection efficiencies with these chemical reagents, however, the very nature of these products and their method of DNA delivery suggests that they may be of limited value in this area of research.

Conclusions

Genetic manipulation of *P. falciparum* relies on transfection techniques which have a low level of efficiency and multiple transfections are often required before a stable transfection can be established. We have examined established and novel transfection techniques in order to determine the method most likely to result in stable transfections. Our data suggest that electroporation is the best method to deliver DNA into these parasites and that the spontaneous uptake protocol of Deitsch *et al.* [10] may be the most efficient of those currently in use.

Authors' contributions

TSA participated in the design of the study, performed the transfections and drafted the manuscript. KT conceived of the study and participated in its design and coordination. PL and PH, carried out plasmid preparations and tissue culture. DG designed and built the vector. All authors read and approved the final manuscript.

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