Published in final edited form as:

J Parasitol. 2009 February ; 95(1): 253–255. doi:10.1645/GE-1706.1.

Multiple Displacement Amplification for Malaria Parasite DNA

Y. Wang, S. Nair, F. Nosten^{*,†,‡}, and T. J. C. Anderson[§]

Southwest Foundation for Biomedical Research (SFBR), San Antonio, Texas 78245 ^{*}Shoklo Malaria Research Unit (SMRU), Mae Sot, Tak, Thailand [†]Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand [‡]Centre for Tropical Medicine and Vaccinology, Churchill Hospital, Oxford, U.K.

Abstract

Multiple displacement amplification (MDA) using Phi29 has proved to be an efficient, high-fidelity method for whole genome amplification in many organisms. This project was designed to evaluate this approach for use with the malaria parasite *Plasmodium falciparum*. In particular, we were concerned that the AT richness and presence of contaminating human DNA could limit efficiency of MDA in this system. We amplified 60 DNA samples using phi29 and scored 14 microsatellites, 9 single-nucleotide polymorphisms (SNPs), and gene copy number at GTP-cyclohydrolase I both before and after MDA. We observed 100% concordance in 829 microsatellite genotypes and in 499 SNP genotypes. Furthermore, copy number estimates for the GTP-cyclohydrolase I gene were correlated ($r^2 = 0.67$) in pre- and postamplification samples. These data confirm that MDA permits scoring of a range of different types of polymorphisms in *P. falciparum* malaria and can be used to extend the life of valuable DNA stocks.

Modern genetic analyses often involves genotyping of large numbers of genetic markers such as microsatellites, single nucleotide polymorphism (SNPs), or copy number variation for which large amounts of template DNA is required. This is a problem for many parasite species that are small, including blood-borne infections such as malaria in which limited amounts of infected blood may be available from patients. One solution to this problem is to expand DNA stocks in the laboratory using biochemical methods. Polymerase chain reaction (PCR)-based methods that have been used for this purpose, such as primer extension preamplification (Zhang et al., 1992) and degenerate oligonucleotide primed PCR (Telenius et al., 1992), typically resulted in unequal amplification of different genome segments and, therefore, were not ideal (Pinard et al., 2006). In contrast, a more recent method, multiple displacement amplification (MDA), allows enzymatic replication of DNA across the genome with minimal bias and can generate many micrograms of copied DNA from just a few nanograms of starting material (Dean et al., 2002; Hosono et al., 2003). We would like to use this method for expanding depleted stocks of malaria parasite DNA from field-collected parasites. The present experiment was designed to validate that this method faithfully copies the DNA without generating artifacts.

The fidelity and efficiency of MDA has been carefully validated for humans and nonhuman primates (Holbrook, Stabley, and Sol-Church, 2005; Andres et al. 2006; Pinard et al., 2006). However, 2 features of malaria parasites suggest caution when extrapolating from results achieved using other organisms. First, the *P. falciparum* genome is extremely AT rich (Gardner et al., 2002). The performance of MDA using such biased genome composition has not been

evaluated. Second, because parasites infect human blood, DNA prepared from infected blood may also contain contaminating host DNA from white blood cells. Given that the human genome is >100 times larger than the *P. falciparum* genome (23 MB), contaminating human DNA may constitute a significant fraction of the total DNA present in a sample even if buffy coats are removed. We were concerned that the presence of large amounts of contaminating human DNA with a more balanced GC content could limit efficiency of MDA for malaria parasite DNA. Ours is not the first attempt to use of MDA from malaria parasites. This method has been used by Carret et al. (2005) to provide template for comparative genomic hybridization. They reported accurate copying of DNA but provided limited quantitative data allowing assessment of this method. Our interest in MDA was stimulated by the need to genotype additional genetic markers and sequence candidate genes in DNA samples used in a genome screen for drug resistance genes.

The present study was designed to validate MDA for expanding these depleted laboratory stocks of parasite DNA. To do this, we used MDA to expand 60 DNA samples, and genotyped SNPs, microsatellites, and a copy number polymorphism in both original sample and DNA generated after MDA.

We collected 60 whole blood samples from *P. falciparum*-infected patients Mawker-Thai on the Thailand-Burma border between October 2000 and September 2003. Collection protocols were approved by the Ethical Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand, and by the Institutional Review Board at the University of Texas Health Science Center at San Antonio, Texas. Buffy coats were removed, and DNA was prepared by phenol-chloroform extraction from 1 ml of packed red blood cells. Both *P. falciparum* and human DNA are present in these samples, and loci from both species can be amplified by PCR (data not shown). We employed a 5-10 ng DNA template for all MDA reactions. We used the GenomiPhi DNA Amplification kit (GE Healthcare, Little Chalfont, Buckinghamshire, U.K.). Reactions were conducted in 20- μ l reactions following the manufacturer's instructions. The DNA products generated by MDA were purified on SigmaSpin postreaction purification columns (Sigma-Aldrich, St. Louis, Missouri), and DNA was quantified on a NanoDrop 1000 spectrophotometer (NanoDrop, Wilmington, Delaware).

To evaluate the fidelity of whole genome amplification, we genotyped DNA isolated from blood samples and DNA generated from these samples by MDA using several different polymorphic markers. We genotyped 14 dinucleotide microsatellite markers, 1 marker from each of the 14 chromosomes in *P. falciparum*, using fluorescent end-labeled oligonucleotides following standard procedures. Primer sequences and locus details are shown in Table I, whereas PCR conditions and scoring methods were described previously (Nair et al., 2003). We genotyped 9 SNPs by primer extension (Nair et al., 2002; Anderson et al., 2005) using the ABI SNaPshot kit (Applied Biosystems, Foster City, California). These were known polymorphisms in 6 transporter loci, including 3 in the multidrug resistance locus (*pfmdr1*) on chromosome 5 (Table II). Finally, to evaluate our ability to score copy number variation, we measured gene-copy number at the GTP-cyclohydrolase 1 (*gch*) locus by *TaqMan* PCR. Recent comparative genomic hybridization experiments show abundant variation in copy number at this locus (Kidgell et al., 2006) (M. Ferdig and J. Patel, pers. comm.). We used the $\Delta\Delta C_T$ method to measure copy number relative to a calibrator sample (M363, a field isolate). For analyzing post-MDA samples, MDA amplified M363 was used as the calibrator. Primers and probes for *gch* were F-AATGATAAATGTGAATCTATTAATGAGAATGTT, R-CATTACTTTTATTTCTTCCTTATTTTCTTC, and 6FAM-TTAATAAACAGTC TCTTAAGGATTC (probe). Primers and probes for β -tubulin were F-TGTCTTGGATCACTTGCGCA, R-GTGCCGGAGTTAACACAAC AAA, and VIC-ATCATATTTTTTTCGTCGAAC (probe). Primers were used at a final concentration of 300 nM, whereas probe concentration was 100 nM for both GTP-CH and β -tubulin reactions. All assays were run in quadruplicate in 10- μ l

volumes on 384-well plates on an ABI 7900HT real-time PCR machine, allowing estimation of both relative copy number and 95% confidence intervals. We reject data for which RQ/upper confidence interval >0.4. We validated the assay using a range of template concentrations (1-0.0001 ng). Nair et al. (2007) provide further details of methodology for measuring gene copy number in malaria parasites.

We found high fidelity of whole genome amplification using the MDA method. There were 6 microsatellite genotyping failures for DNA templates both before and after MDA, and paired comparisons were available for 829 (98.7%) of a possible of 840 genotypes. In these 829 microsatellite genotypes, we observed complete concordance in allele size (Table I). For the SNP data, there were 25 and 22 genotyping failures pre- and post-MDA, and paired comparisons were possible for 499 (92.4%) of a possible 540 genotypes. We observed just 3 discrepancies (99.4% concordance) for these 499 SNP genotypes. Re-genotyping of these discrepant samples revealed errors in our original genotype data. Therefore, the concordance was in fact 100% (Table II) as observed with the microsatellites. Gene copy number was also scored. We observed a reasonable correlation ($r^2 = 0.67$) when we plotted relative copy number estimates (log transformed) derived from the original DNA stocks and MDA-derived DNA (Fig. 1). This correlation is not perfect, and suggests some caution when using MDA-derived DNA for *TaqMan* PCR copy number estimation. We found that MDA was efficient, resulting in an average of ~1,000-fold expansion in the amount of DNA. The efficiency of this method was higher, with more input template, as observed by others (Bergen et al., 2005). We used a 10-ng input DNA for 56/60 samples, which generated a mean of 10.3 μ g (SD = 2.2 μ g; range = 3.3-19.8 μ g) of product. For the remaining 4 samples, we used a 5-ng input due to the lack of original DNA, generating considerably less (mean = 5.4 μ g) product (Mann-Whitney *U*-test, $P = 0.025$).

These results confirm those of Carret et al. (2005) who showed good correspondence between DNA samples before and after MDA in comparative genomic hybridization experiments, suggesting that SNPs can be reliably scored. Furthermore, these data demonstrate that microsatellite loci, which are widely used in malaria research, can also be reliably scored after MDA. We conclude that MDA is an efficient process for expanding laboratory stocks of *P. falciparum* DNA. The product generated is suitable for a range of downstream genotyping applications, including SNPs, microsatellites, and, to a lesser extent, copy number amplifications. Although there was a significant amount of residual human DNA present in the original DNA sample, this does not seem to pose a problem. We emphasize that these experiments were designed to evaluate the performance of MDA using high-quality DNA template prepared from packed red blood cells containing single clone malaria infections. In many projects, finger prick blood samples are absorbed onto filter papers and dried. The DNA extracted from these blood spots may be of lower quality and frequently contains multiple malaria genotypes. Work at the Broad Institute (Boston, Massachusetts) provides encouraging results using MDA with such samples. They used MDA to generate large quantities of DNA genome sequence from finger prick blood spots and report excellent concordance (Volkman et al., 2007). More generally, MDA has multiple applications in parasitology, allowing multiple loci to be genotyped from parasite species of small size or larval stages, and simplifying construction of genetic libraries and genome sequencing from species that are difficult to culture.

Acknowledgments

We thank the patients at the Mawker-Thai clinic for donating blood and parasites and the field staff at SMRU for assistance. This investigation was funded by NIH R01 AI48071 (to T.J.C.A.). This investigation was conducted in facilities constructed with support from Research Facilities Improvement Program Grant C06 RR013556 from the National Center for research Resources, National Institutes of Health. The SMRU is part of the Wellcome-Trust

Mahidol University-Oxford Tropical Medicine Research Program supported by the Wellcome Trust of Great Britain. F.N. is a Wellcome Trust Senior Clinical Fellow.

LITERATURE CITED

- ANDERSON TJ, NAIR S, QIU WG, SINGLAM S, BROCKMAN A, PAIPHUN L, NOSTEN F. Are transporter genes other than the chloroquine resistance locus (*pfcr*) and multi drug resistance gene (*pfmdr*) associated with antimalarial drug resistance? *Antimicrobial Agents for Chemotherapy* 2005;272:1153–1161.
- ANDRES O, RONN AC, FERRANDO A, BOSCH M, DOMINGO-ROURA X. Sequence quality is maintained after multiple displacement amplification of non-invasively obtained macaque semen DNA. *Biotechnology Journal* 2006;1:466–469. [PubMed: 16892275]
- BERGEN AW, QI Y, HAQUE KA, WELCH RA, CHANOCK SJ. Effects of DNA mass on multiple displacement whole genome amplification and genotyping performance. *BMC Biotechnology* 2005;5:24. [PubMed: 16168060]
- CARRET CK, HORROCKS P, KONFORTOV B, WINZELER E, QURESHI M, NEWBOLD C, IVENS A. Microarray-based comparative genomic analyses of the human malaria parasite *Plasmodium falciparum* using Affymetrix arrays. *Molecular and Biochemical Parasitology* 2005;144:177–186. [PubMed: 16174539]
- DEAN FB, HOSONO S, FANG L, WU X, FARUQI AF, BRAY-WARD P, SUN Z, ZONG Q, DU Y, DU J, DRISCOLL M, SONG W, KINGS-MORE SF, EGHOLM M, LASKEN RS. Comprehensive human genome amplification using multiple displacement amplification. *Proceedings of the National Academy of Sciences USA* 2002;99:5261–5266.
- GARDNER MJ, HALL N, FUNG E, WHITE O, BERRIMAN M, HYMAN RW, CARLTON JM, PAIN A, NELSON KE, BOWMAN S, et al. Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* 2002;419:498–511. [PubMed: 12368864]
- HOLBROOK JF, STABLEY D, SOL-CHURCH K. Exploring whole genome amplification as a DNA recovery tool for molecular genetic studies. *Journal of Biomolecular Technology* 2005;16:125–133.
- HOSONO S, FARUQI AF, DEAN FB, DU Y, SUN Z, WU X, DU J, KINGSMORE SF, EGHOLM M, LASKEN RS. Unbiased whole-genome amplification directly from clinical samples. *Genome Research* 2003;13:954–964. [PubMed: 12695328]
- KIDGELL C, VOLKMAN SK, DAILY J, BOREVITZ JO, PLOUFFE D, ZHOU Y, JOHNSON JR, LE ROCH K, SARR O, NDIR O, et al. A systematic map of genetic variation in *Plasmodium falciparum*. *PLoS Pathology* 2006;2:e57.
- MU J, FERDIG MT, FENG X, JOY DA, DUAN J, FURUYA T, SUBRAMANIAN G, ARAVIND L, COOPER RA, WOOTTON JC, et al. Multiple transporters associated with malaria parasite responses to chloroquine and quinine. *Molecular Microbiology* 2003;49:977–989. [PubMed: 12890022]
- NAIR S, BROCKMAN A, PAIPHUN L, NOSTEN F, ANDERSON TJ. Rapid genotyping of loci involved in antifolate drug resistance in *Plasmodium falciparum* by primer extension. *International Journal for Parasitology* 2002;32:852–858. [PubMed: 12062556]
- NAIR S, NASH D, SUDIMACK D, JAIDEE A, BARENDT M, UHLE-MANN AC, KRISHNA S, NOSTEN F, ANDERSON TJ. Recurrent gene amplification and soft selective sweeps during evolution of multidrug resistance in malaria parasites. *Molecular and Biochemical Evolution* 2007;24:562–573.
- NAIR S, WILLIAMS JT, BROCKMAN A, PAIPHUN L, MAYXAY M, NEWTON PN, GUTHMANN JP, SMITHUIS FM, HIEN TT, WHITE NJ, NOSTEN F, et al. A selective sweep driven by pyrimethamine treatment in southeast Asian malaria parasites. *Molecular and Biochemical Evolution* 2003;20:1526–1536.
- PINARD R, DE WINTER A, SARKIS GJ, GERSTEIN MB, TARTARO KR, PLANT RN, EGHOLM M, ROTHBERG JM, LEAMON JH. Assessment of whole genome amplification-induced bias through high-throughput, massively parallel whole genome sequencing. *BMC Genomics* 2006;7:216. [PubMed: 16928277]
- TELENIUS H, CARTER NP, BEBB CE, NORDENSKJOLD M, PONDER BA, TUNNACLIFFE A. Degenerate oligonucleotide-primed PCR: General amplification of target DNA by a single degenerate primer. *Genomics* 1992;13:718–725. [PubMed: 1639399]

VOLKMAN SK, SABETI PC, DECAPRIO D, NEAFSEY DE, SCHAFFNER SF, MILNER DA JR.

DAILY JP, SARR O, NDIAYE D, et al. A genome-wide map of diversity in *Plasmodium falciparum*. Nat. Genetics 2007;39:113–119. [PubMed: 17159979]

ZHANG L, CUI X, SCHMITT K, HUBERT R, NAVIDI W, ARNHEIM N. Whole genome amplification from a single cell: Implications for genetic analysis. Proceedings of the National Academy of Sciences USA 1992;89:5847–5851.

FIG. 1.

Concordance between measures of copy number before and after MDA. Graph shows the correlations between relative copy number estimates (log transformed) of *gch* measured before and after MDA. The slope of the relationship between log template concentration and ΔC_T was 0.01, within the acceptable range. The slope is close to 1, whereas the correlation coefficient (r^2) is 0.67.

Table 1

Dinucleotide microsatellite markers amplified in this study. The base pair position of the microsatellite repeats amplified in the genome sequence is shown. Amplification conditions were the same for all loci and were as described previously (Nair et al., 2003). The number of alleles (A), expected heterozygosity (H_e) and percentage of match between microsatellite genotypes measured before and after MDA are shown.

Chr*	Position	Forward	Reverse	A	H_e	% Match
1	271320	TGTAGTTTTTATTATGTACGC	GAACGTTCAATTTGACATAAT	12	0.86	100
2	628374	AATCTAGGAGAAATAAATGG	TCAAAATGGGAAACTTATATT	12	0.88	100
3	993684	TTTTCCCATGTGTATTTAGT	ACCATTTCTAACAAACAAAAA	15	0.9	100
4	549788	GGATTGGATTACAAGAAAAAT	CTACTCACAAAATTTGAAACA	9	0.71	100
5	750347	ATGGAAATAAGATAGCATCA	TATTTACATAATCAGCAAA	4	0.44	100
6	1053065	TGAATGTATGGAGGAGTAAC	CATATAAAATAAAAAGAACATCTCA	4	0.35	100
7	1247164	GTACATTCTGCTTTTGTCTGT	ATAGCATACTCTGTGAAAA	6	0.79	100
8	999712	GGTCATTTAAGTCTTTTGCTA	TTTGTCTAAATGTGTTTGA	7	0.75	100
9	446874	GATAITTTATCGAAATGGTCA	TGAGCTTTTATTTACATTTTATAGT	13	0.87	100
10	299641	AGGAAGATAATAACATGAACA	TTGGGTATATTTTCATACAAAC	10	0.83	100
11	1393700	TTTCTTCACITTTTACCTTG	ACATATAATGGATAAACCTTTT	10	0.85	100
12	100517	AGAACATAAGAAACGATTTG	ATTTTGTACTGTTTGGTG	10	0.78	100
13	751761	CAAAATTTTGTGAGGATTCA	ATTGAATGTAAAGGAAATG	11	0.69	100
14	897528	GTCAGATGAAAGGGTACATA	CTTTTCTTTGTGTGATTTT	6	0.8	100

* Chr = chromosome.

Single-nucleotide polymorphisms (SNPs) genotyped in this study. Gene names and IDs are shown. The SNPs are labeled following Mu et al. (2003) and Anderson et al. (2005).

Table II

Locus-SNP	Chr*	PlasmoDB ID	Mutations	% Common allele	n	% Match
G25-intron	14	PF14-0679	G-A [†]	0.85	60	100
G47-241	5	PFE0775c	L-V	0.93	60	100
G49-146	8	PF08-0078	Q-E	0.74	57	100
G49-1046	8	PF08-0078	L-I	0.79	56	100
G54-141	14	PF14-0260	Y-Y [†]	0.62	60	100
G70-105	12	PFL0620c	E-K	0.51	59	100
pfindr1-1042	5	PFE1150w	N-D	0.93	60	100
pfindr1-86	5	PFE1150w	N-Y	0.95	57	100
Pfindr1-184	5	PFE1150w	Y-F	0.63	51	100

* Chr = chromosome.

[†] G54 SNP is silent, whereas G-A nucleotide change in G25 is in noncoding region.