Longitudinal tracking of *Plasmodium falciparum* clones in complex infections by amplicon deep sequencing

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Supplemental Material

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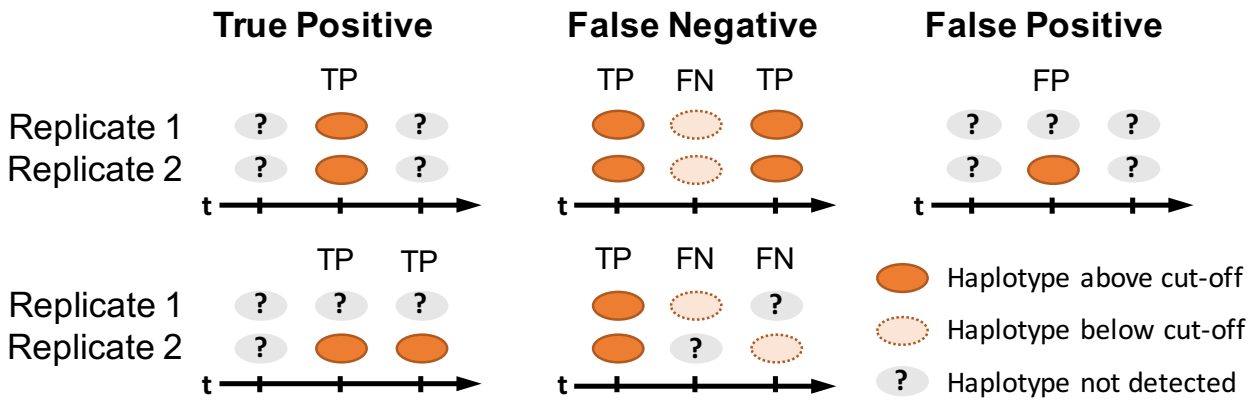
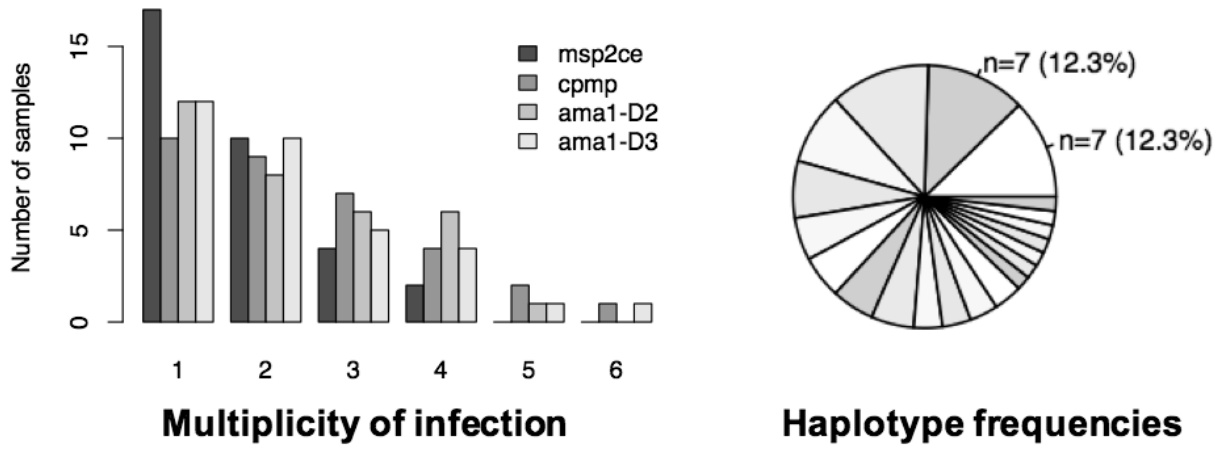
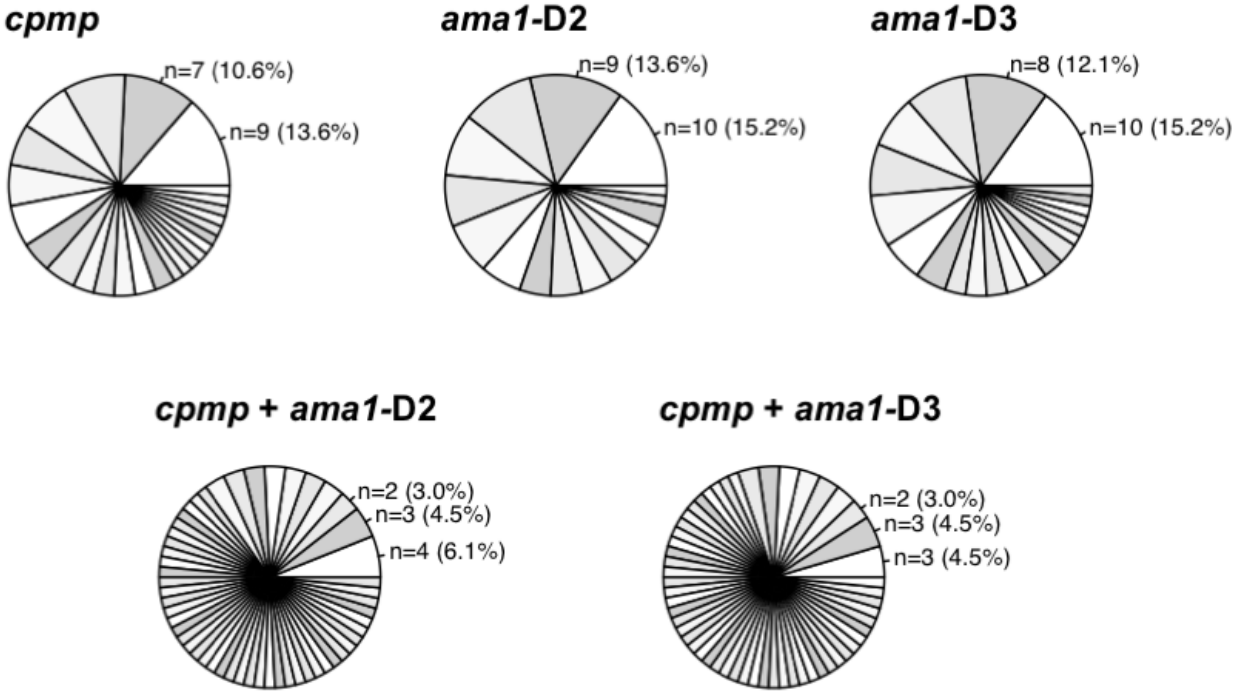


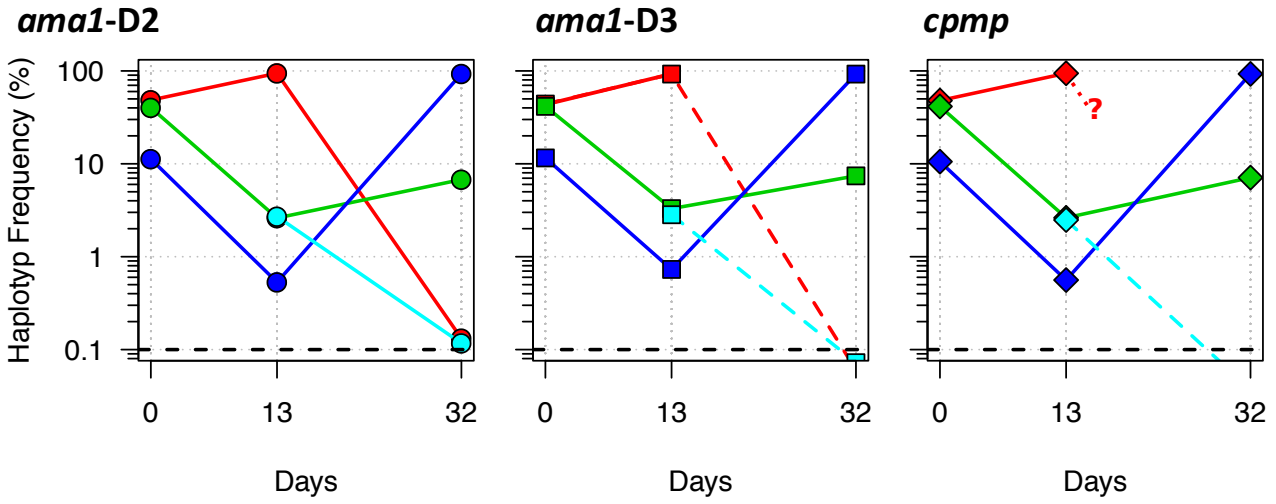
Supplementary Figure S1: Schematic of haplotype classification. Examples show the classification of haplotypes in true-positive (TP), false-negative (FN) and false-positive (FP), based on their detection either in duplicates or in the preceding or succeeding bleeds.



Supplementary Figure S2: Frequency distribution of multiplicity of infection by marker (left) and frequency of *msp2*-CE haplotypes (right) in 33 baseline samples. Marker *msp2*-CE identified 20 different haplotypes. (Frequency distribution of haplotypes of Amp-Seq markers given in Figure 1).

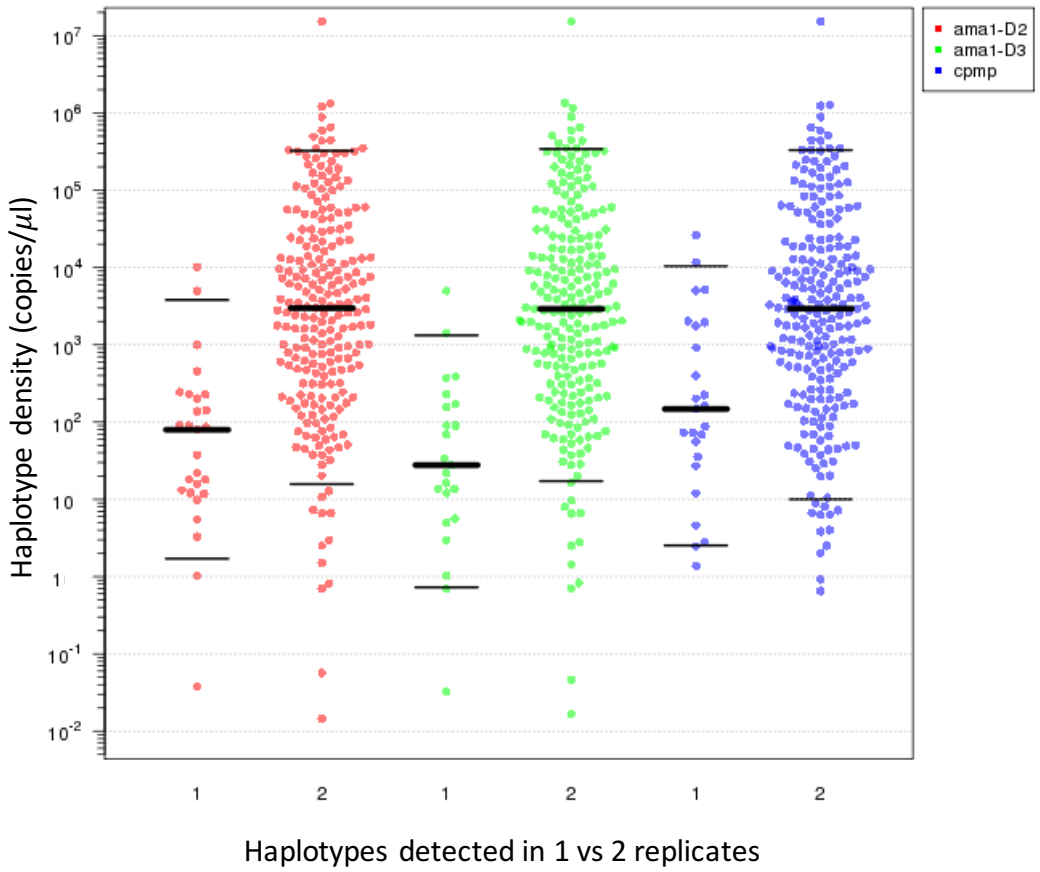


Supplementary Figure S3: Haplotype frequencies by marker in 46 independent samples comprising 66 clones. For marker *cpmp* 25 different alleles were identified, for *ama1*-D2 16 haplotypes and for *ama1*-D3 21 haplotypes. Top panel: haplotypes base on single markers; bottom panel: two-marker haplotypes.

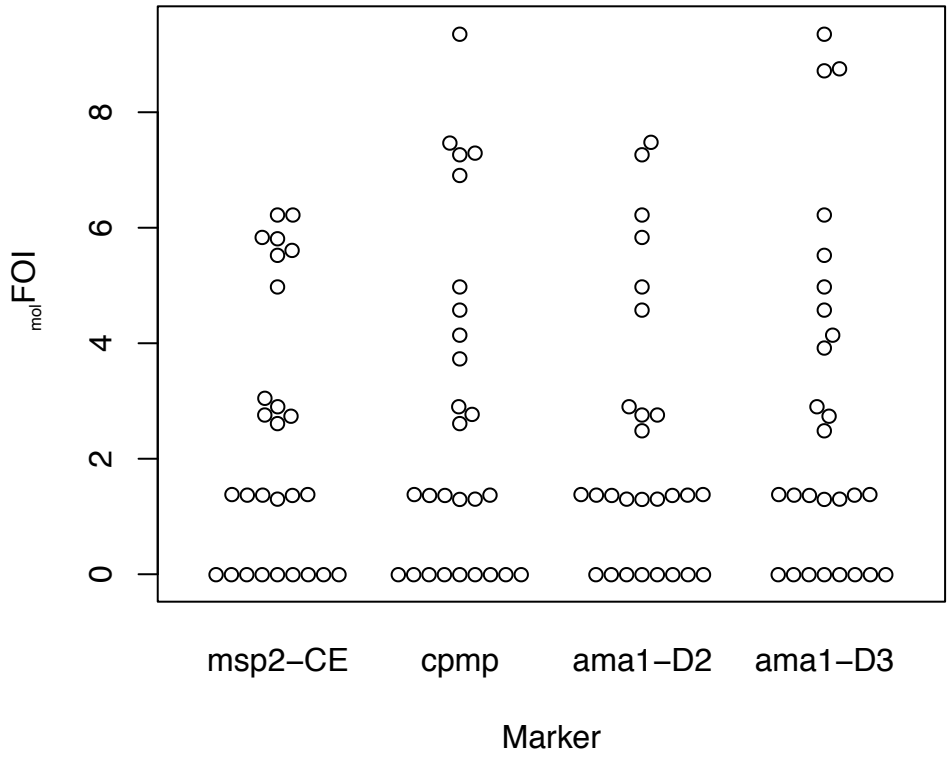


Days	Clone 1			Clone 2			Clone 3			Clone 4		
	●	■	◆	●	■	◆	●	■	◆	○	□	◇
0	48.5	44.0	48.0	40.2	41.5	41.4	11.3	11.6	10.6	-	-	-
13	94.4	92.6	94.3	2.61	3.29	2.62	0.53	0.73	0.56	2.71	2.83	2.49
32	0.13	0.06	n.d.	6.77	7.42	7.10	93.1	92.5	92.9	0.12	0.07	0.04

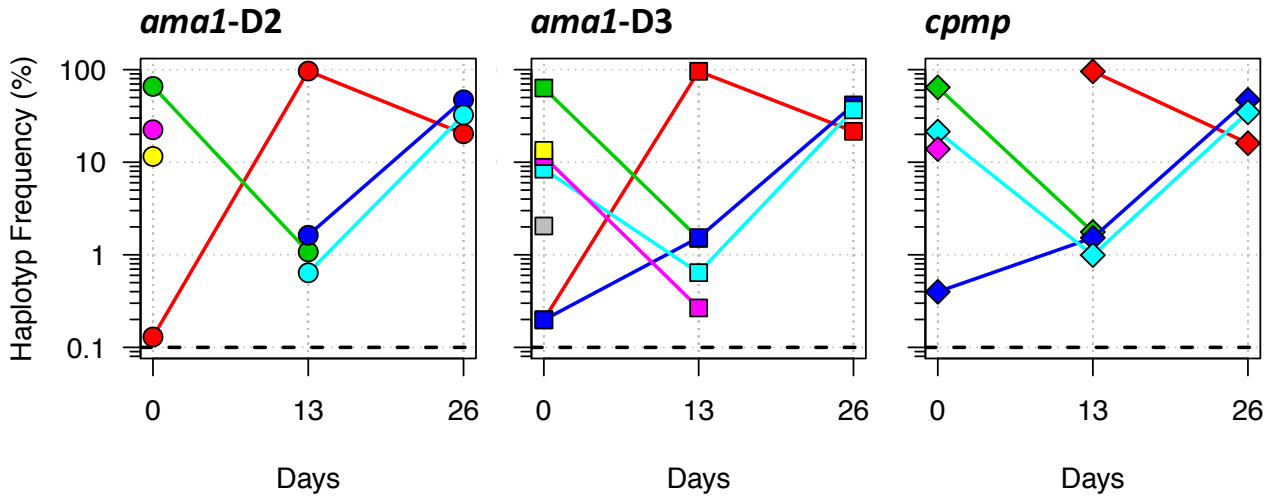
Supplementary Figure S4: Within-host haplotype frequencies of Amp-Seq markers in longitudinal samples from one child. Inserted table lists within-host multi-locus haplotype frequencies in percent. Multi-locus haplotypes have the same colour-code in figures and table. Solid line represents persisting haplotypes above cut-off criteria (true-positive haplotypes). Dashed line represents persisting haplotypes falling below cut-off criteria (false-negative haplotypes detected below cut-off criteria). Dotted line and question mark indicate a false-negative haplotype that was not detected (n.d.) but could be imputed based on the established multi-locus haplotypes from the preceding sample. Black dashed line represents cut-off criteria of the Amp-Seq genotyping method.



Supplementary Figure S5: Density of true-positive haplotypes detected in only one or both replicates. X-axis, haplotypes detected in 1 versus 2 replicates by Amp-Seq marker. Y-axis, haplotype density by qPCR measured as 18S rRNA gene copies per μl whole blood. Points represent individual haplotypes; colours represent individual markers. Black horizontal bar represents 5, 50 and 95-percentile. Wilcoxon rank sum test with continuity correction: W=1000 and p-value=$2x10^{-9}$ for *ama1*-D2, W=700 and p-value=$5x10^{-9}$ for *ama1*-D3, W=1000 and p-value=$5x10^{-5}$ for *cpmp*.



Supplementary Figure S6: Frequency distribution of molecular force of infection ($_{mol}$FOI) by marker. A total of 117 samples from 27 individuals (on average 4.3 samples per individual [min: 2, max: 7]) were used to estimate force of infection (FOI).



Multi-locus haplotype names	Day 0			Day 13			Day 32		
	ama1-D2 %	*ama1*-D3 %	*cpmp* %	*ama1*-D2 %	*ama1*-D3 %	*cpmp* %	*ama1*-D2 %	*ama1*-D3 %	*cpmp* %
Haplotype 1	65.9	63.3	64.4	1.1	1.5	1.8			
Haplotype 2	0.1	0.2		96.6	96	95.6	20.3	21.6	16.1
Haplotype 3		0.2	0.4	1.6	1.5	1.5	47.3	41.6	47.2
Haplotype 4		8.4	21.4	0.6	0.6	1	32.4	36.8	34.6
Haplotype 5	22.4								
Haplotype 6	11.5								
Haplotype 7		11.5			0.3				
Haplotype 8		13.4							
Haplotype 9		2.0							
Haplotype 10			13.9						

Supplementary Figure S7: Within-host haplotype frequency of Amp-Seq markers in longitudinal samples from 1 child representing an unresolvable multi-locus haplotype.

Inserted table lists within-host haplotype frequencies for all markers with a possible solution of partly established multi-locus haplotypes for the major haplotypes. Multi-locus haplotypes 1-3 match well in frequencies of individual haplotypes at day 0, 13 and 32. In contrast, multi-locus haplotype 4 does not match in frequencies of individual haplotypes at day 0. This could be explained by a complex shared haplotype situation with one or several clones detected only at day 0 and 13, e.g. haplotypes 5-10. Solid lines represent persisting haplotypes.

Supplemental Tables

Supplementary Table S1: PCR Primer sequence for Amp-Seq and *msp2*-CE genotyping and sequence library preparation.

Primer for primary PCR	
cpmp_prim_F	CGATACAGGACATATAGA
cpmp_prim_R	TTCAATAACATTTACTAGG
Pfama1_F5	TGCGTATTATTATTGAGC
Pfama1_R613	GTGTTGTATGTGATGCTC
Primer for nested PCR	
ama1_D2_F_Linker	GTGACCTATGAACTCAGGAGTC**GGTCCTAGATATTGTAATAAAG**
ama1_D2_R_Linker	CTGAGACTTGCACATCGCAGC**CATGTTGGTTTGACATTAAA**
ama1_D3_F_Linker	GTGACCTATGAACTCAGGAGTC**TACTACTGCTTTGTCCCATC**
ama1_D3_R_Linker	CTGAGACTTGCACATCGCAGC**TCAGGATCTAACATTTCATC**
cpmp_F_Linker	GTGACCTATGAACTCAGGAGTC**CATAAGTCATTAAAATTTATGGAT**
cpmp_R_Linker	CTGAGACTTGCACATCGCAGC**CGTTACTATCAAGATCGTTAATATC**
Primer for msp2 CE genotyping	
msp2_S2_fw	GAAGGTAATTAAAACATTGTC
msp2_S3_rev	GAGGGATGTTGCTGCTCCACAG
msp2_S1-fw	GCTTATAATATGAGTATAAGGAGAA
msp2_FC27-rev	GCATTGCCAGAACTTGAA
msp2_3D7-rev	CTGAAGAGGTACTGGTAGA
Primer for sequence library PCR (XXXXXX=barcode)	
Forward	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGC
	TCTTCCGATCTXXXXXXXXGTGACCTATGAACTCAGGAGTC
Reverse	CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAAC
	CGCTCTTCCGATCTXXXXXXXXCTGAGACTTGCACATCGCAGC

Forward barcode		Reverse barcode	
Fwd_1	TAGATCGC	Rev_1	TAAGGCGA
Fwd_2	CTCTCTAT	Rev_2	CGTACTAG
Fwd_3	TATCCTCT	Rev_3	AGGCAGAA
Fwd_4	AGAGTAGA	Rev_4	TCCTGAGC
Fwd_5	GTAAGGAG	Rev_5	GGACTCCT
Fwd_6	ACTGCATA	Rev_6	TAGGCATG
Fwd_7	AAGGAGTA	Rev_7	CTCTCTAC
Fwd_8	CTAAGCCT	Rev_8	CAGAGAGG
Fwd_13	TGGTGGTA	Rev_9	GCTACGCT
Fwd_14	TTCACGCA	Rev_10	CGAGGCTG
Fwd_15	AGCACCTC	Rev_11	AAGAGGCA
Fwd_16	CAAGGAGC	Rev_12	GTAGAGGA
Fwd_17	ATTGGCTC	Rev_13	ATGCCTAA
Fwd_18	CACCTTAC	Rev_14	ACGCTCGA
Fwd_19	CTAAGGTC	Rev_15	AGTCACTA
Fwd_20	GAACAGGC	Rev_16	ATCCTGTA
		Rev_17	CGCATACA
		Rev_18	CTGGCATA
		Rev_19	GATAGACA
		Rev_20	GCTAACGA
		Rev_21	GTGTTCTA
		Rev_22	TCCGTCTA
		Rev_23	CCTAATCC
		Rev_24	GACAGTGC

Supplementary Table S2: Location and size of the amplicons.

	cpmp	*ama1*-D2	*ama1*-D3
From	1895	775	1281
To	2324	1253	1796
Size	430	479	516

Supplementary Table S3: Summery of sequence coverage (total read numbers) by Amp-Seq marker.

	cpmp	*ama1*-D2	*ama1*-D3
1st Qu.	247	2292	2997
Median	794	3386	4716
Mean	1117	3682	5189
3rd Qu.	1632	5143	6906
Max	6376	11570	34240

Supplementary Table S4: Summary of multi-locus haplotype (MLH) inference based on longitudinal samples from 33 children.

Status of MLH inference	Samples	Multi-locus haplotypes	Single-locus haplotypes		
			cpmp	*ama1*-D2	*ama1*-D3
	n	n	n	n	n
Full established MLH	78 [1]	116 [1]	116	103	103
Partly established MLH [2]	49	64	135	130	126
Unresolvable MLH [3]	8	0	20	18	18
Incomplete datasets [4]	13	0	7	11	11
Total	140	180	258 [5]	244 [5]	240 [5]

n number of samples or haplotypes.

[1] 45 out of 78 samples with fully established multi-locus haplotypes were single clone infections.

[2] Samples were multi-locus haplotypes could be established for some but not for all clones of a sample.

[3] Samples were no multi-locus haplotype could be established.

[4] Samples with missing genotyping results for any of the markers.

[5] Total number of parasite clones detected in 140 samples was 277.

Supplementary Table S5: Overview of sample selection criteria applied for different types of analyses.

Analysis Type	Samples n	Children n	Selection Criteria
Baseline H_e and MOI	33	33	Baseline (or first bleed available) sample.
Multi-locus H_e	46	33	Samples with a resolvable multi-locus haplotype that were separated by a treatment plus ≥2 consecutive *P. falciparum* negative samples from the same child.
$_{mol}$FOI	117	27	Children with a complete set of replicates.
Sensitivity and false discovery rate	48	12	Children that did not received antimalarial treatment during the timespan analysed and harboured at least one haplotype that was detected at 3 consecutive bleeds.
Reproducibility	139	33	True-positive haplotypes.

Supplementary Table S6: Numbers of missed haplotypes due to imperfect detection either at baseline, in any intermediate sample, or prior to haplotype clearance. Haplotypes from 48 longitudinal samples from 12 children were classified into true-positive (TP) and false-negative haplotypes. Two types of false-negative haplotypes (missed clones) can be differentiated: (FN_i) False-negative haplotypes detected but below cut-off criteria and (FN_{ii}) false-negative haplotypes not detected but imputed.

Marker	Baseline sample	Any intermediate sample	Sample prior to clearance
	n	n	n
TP haplotypes			
msp2-CE	29	34	23
cpmp	39	45	31
ama1-D2	36	44	29
ama1-D3	36	43	29
FN_i haplotypes			
msp2-CE	2	6	2
cpmp	1	0	3
ama1-D2	0	1	2
ama1-D3	1	0	3
FN_{ii} haplotypes			
msp2-CE	n/a [1]	5	n/a [1]
cpmp	0	2	1
ama1-D2	1	0	0
ama1-D3	1	2	0

[1] FN_{ii} haplotypes cannot be imputed at the beginning of infection or prior to clearance for marker *msp2*-CE.

Supplementary Table S7: Reproducibility of true-positive haplotypes in technical replicates. Reproducibility only decreased when clone densities fell below 1000 copies 18S rRNA gene per μl whole blood and/or within-host frequency below 1% (FIG S5).

	cpmp			***ama1*-D2**			***ama1*-D3**		
	n_1	**n_2**	**q**	**n_1**	**n_2**	**q**	**n_1**	**n_2**	**q**
	25	235	0.949	28	228	0.942	23	226	0.952
Haplotype density (copies/μl)									
>1000	7	148	0.977	2	146	0.993	2	142	0.993
100-1000	6	52	0.945	8	50	0.926	5	51	0.953
10-100	8	23	0.852	13	22	0.772	10	22	0.815
<10	4	12	0.857	5	10	0.800	6	11	0.786
Haplotype proportion within a sample (%)									
>10	13	172	0.964	16	165	0.954	11	167	0.968
1-10	4	55	0.965	4	47	0.959	5	46	0.948
<1	8	8	0.667	8	16	0.800	7	13	0.788

n_1 number of clones detected only with one of the replicates.

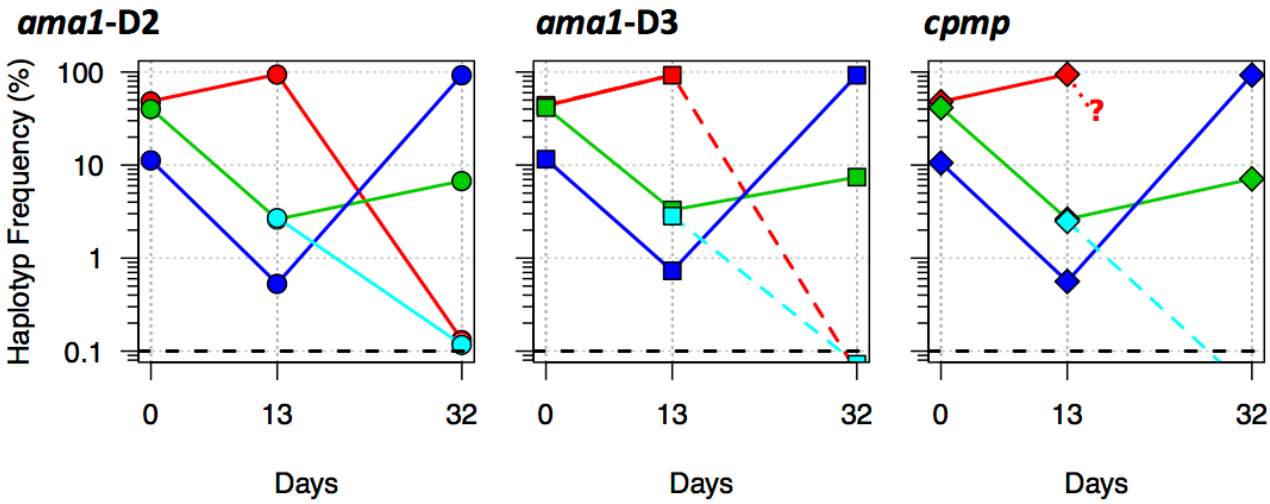
n_2 number of clones detected with both replicates.

q detectability as descripted in Bretscher et al. 2010.

Supplemental Text S1

Example of multi-locus haplotype inference

Below an example of *P. falciparum* infection dynamics is shown for one child in great detail to illustrate our strategy for inferring a multi-locus haplotype that combines SNP data from three molecular markers *ama1*-D2, *ama1*-D3, and *cpmp.* Within-host haplotype frequency data of the example is shown in Table S8 and corresponding graphic illustration in FIG S7.



Supplementary Figure S8: Within-host haplotype frequencies of Amp-Seq markers in longitudinal samples from one child. Multi-locus haplotypes have the same colour-code in figures. Solid line represents persisting haplotypes above cut-off criteria (true-positive haplotypes). Dashed line represents persisting haplotypes falling below cut-off criteria (false-negative haplotypes detected below cut-off criteria). Dotted line and question mark indicate a false-negative haplotype that was not detected but could be imputed based on the established multi-locus haplotypes from the preceding sample. Black dashed line represents cut-off criteria of the Amp-Seq genotyping method.

Supplementary Table S8: Within-host haplotype frequencies (WHHF) in percent of individual Amp-Seq markers observed in longitudinal samples from one child. Haplotypes of individual markers (termed alleles) are sorted by WHHF of day 0. **Haplotypes 1-4** represent multi-loci haplotypes composed of one allele of each of the 3 markers.

Multi-locus haplotype names	Day 0			Day 13			Day 32		
	ama1-D2 %	*ama1*-D3 %	*cpmp* %	*ama1*-D2 %	*ama1*-D3 %	*cpmp* %	*ama1*-D2 %	*ama1*-D3 %	*cpmp* %
Haplotype 1	48.5	44.0	48.0	94.4	92.6	94.3	0.13	0.06	
Haplotype 2	40.2	41.5	41.4	2.61	3.29	2.62	6.77	7.42	7.10
Haplotype 3	11.3	11.6	10.6	0.53	0.73	0.56	93.1	92.5	92.9
Haplotype 4	-	-	-	2.71	2.83	2.49	0.12	0.07	0.04

The inference of multi-marker haplotypes started with identification of alleles that belong to the dominant parasite clone. A dominant Haplotype was defined by a within-host haplotype frequencies (WHHF) >54%.

Inference of multi-marker Haplotypes at Day 0

At Day 0 of this example, 2 different alleles per marker occurred at similar WHHF (listed by marker in Supplemental Table S8. At Day 0 no dominant Haplotype was evident, therefore any increase or decrease of in WHHF of these alleles at Day13 was interrogated: one allele of each of the 3 markers showed an increase of approx. +46%, while the remaining 3 alleles of similar frequency revealed a decrease by approx. -38%. Based on these recoded frequency changes we combined those alleles from each marker, which all increased by approx. +46%, into multi-locus **Haplotype 1** (Supplementary Figure S8, Day 0 in red).

Alleles that constituted **Haplotype 1** were not considered in next steps of inference. Additional multi-locus haplotypes of Day 0 were inferred by combining the alleles of similar frequency which showed a decrease in WHHF for all 3 markers of approx. -38%, thus defining multi-locus **Haplotype 2** (Supplementary Figure S8, Day 0 in green). For the next steps of inference, all alleles associated with multi-locus **Haplotypes** 1 and **2** were no more considered. The remaining alleles

constituted multi-locus **Haplotype 3** with ~11% WHHF for all markers (Supplementary Figure S8, Day 0 in blue).

Multi-marker Haplotypes at Day 13

The dominant alleles in all 3 markers of the Day 13 sample were consistent with multi-locus **Haplotype 1** characterized by ~93% WHHF for all 3 markers (Supplementary Figure S8, Day 13 in red). Again this multi-locus haplotype was no more considered in the next steps of Day 13 haplotype inference. Next two multi-locus haplotypes with similar WHHF were observed. In agreement with allele combinations found at Day 0, multi-locus **Haplotype 2** was identified by an increase of these alleles at Day 32 of approx. +4% (Supplementary Figure S8, Day 13 in green). After excluding alleles constituent multi-locus **Haplotypes 1** and **2** an additional new multi-locus **Haplotype 4** with similar WHHF as **Haplotype 2** was found (Supplementary Figure S8 Day 13 in light blue). The remaining alleles, all with frequencies below 1%, corresponded to multi-locus **Haplotype 3** (Supplementary Figure S8, Day 13 in blue).

Multi-marker Haplotypes at Day 32

The dominant clone in the Day 32 sample corresponds to multi-locus **Haplotype 3**, characterized in this sample by a steep increase of, ~93% WHHF for all markers (Supplementary Figure S8, Day 32 in blue). Alleles of this dominant clones are no more considered in the next step of inference. The dominant clone in this step corresponds to **Haplotype 2** with ~7% WHHF for all markers (Supplementary Figure S8, Day 32 in green). In the next step all alleles of **Haplotypes 3** and **2** were no more considered. But no further multi-locus haplotypes could be established, as WHHF of the remaining alleles were below the 0.1% WHHF cut-off criteria for some of the markers. However, as the inferred multi-locus haplotypes of Day 0, 13 and 32 match for all samples and marker ama1-D2 showed a WHHF above the cut-off criteria, the multi-locus Haplotypes **1** and **4** could be imputed (Supplementary Figure S8, Day 32 in light blue and red).