Quantification of human and *P. falciparum* DNA v1.0

Procedure

Molecular Module

WorldWide Antimalarial Resistance Network (WWARN)

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Version History

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1.0	Creation of procedure	18/11/2010

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Contents

1. Purpose

This procedure is designed to estimate absolute quantification of *Plasmodium falciparum* DNA and human DNA and the ratio of *P. falciparum*: human DNA in DNA extracted from samples of infected blood.

2. Scope

This procedure is intended for use in clinical trials and molecular studies where whole blood samples are collected for next-generation sequencing and genotyping of *P. falciparum* infections. It describes quantification of total DNA and *P. falciparum*- and human-specific quantitative PCR. This procedure is applicable for well-equipped laboratories familiar with real-time PCR.

3. Abbreviations

DNA	Deoxyribonucleic Acid
PCR	Polymerase Chain Reaction
µL	microlitre
mL	millilitre
ng	nanogram
AMA1	*Plasmodium falciparum* Apical Membrane Antigen 1 locus
LRAP	Human Leukocyte-derived arginine aminopeptidase locus
C_T	Cycle threshold

4. Duties and Responsibilities

N/A

5. Materials and Equipment

5.1 Materials

1. Total DNA quantification
    - Qubit Assay Tubes (Invitrogen, catalogue# Q32856)
    - Quant-it dsDNA HS Assay Kit, 500 assays (Invitrogen, catalogue# Q32854)
        - Includes two standards, Quant-iT reagent and Quant-iT buffer

    - HS kit is appropriate for samples with DNA concentration ranging from 0.05 ng/µL - 30 ng/µL. If samples routinely contain > 30 ng/µL DNA, the BR kit for samples ranging from 0.5 ng/µL - 250 ng/µL (Invitrogen, catalogue# Q32853) may be appropriate.
- Micropipets and tips (10 µL, 200 µL and 1000 µL)
- 1.5 mL centrifuge or 15mL conical tubes for master mix (depending on number of samples)

2. Quantitative PCR
    - SYBR green qPCR Master Mix (ABI catalogue# 4309155) or comparable reagent
    - MicroAMP Optical 96-well plates (ABI, catalogue # N801-0560) or comparable optical PCR tubes/plates
    - MicroAMP Optical Adhesive Film Covers (ABI, catalogue # 4311971) or comparable optical PCR film/caps
    - Micropipets and tips (10 µL, 200 µL and 1000 µL)
    - 15 mL conical tubes
    - Parasite-specific primer pair (AMA1 gene):
        - F: 5'-ACGTTGGATGGATTCTCTTTCGATTTCTTTC-'3
        - R: 5'-ACGTTGGATGTGCTACTACTGCTTTGTCCC-'3
    - Human-specific primer pair (LRAP gene):
        - F: 5'-ACGTTGGATGAATTTTCCACTGGATTCCAT-'3
        - R: 5'-ACGTTGGATGTGAACCATGCTCCTTGCATC-'3
    - Parasite DNA standards:
        - 3D7 or another source of pure *P. falciparum* DNA, quantified using Procedure 6.1 below and carefully diluted to the concentrations listed in Table 1.
    - Human DNA standards
        - Human placental DNA (Sigma, catalogue # D4642-5UN) or another source of pure human DNA, quantified using Procedure 6.1 below and carefully diluted to the concentrations listed in Table 1.

Table 1. Human and *P. falciparum* DNA standard concentrations (ng/µL). Samples may need to be diluted to fit within the given human and *P. falciparum* standard range. Alternatively, the range of standards may be extended if samples tend to fall outside of the given ranges.

Human	*P. falciparum*
0.39	0.19
0.78	0.39
1.56	0.78
3.125	1.56
6.25	3.125
12.5	6.25
25	12.5

5.2 Equipment

1. Total DNA quantification
   - Qubit fluorometer (Invitrogen)

2. Quantitative PCR
   - Real-time PCR machine

6. Procedure

6.1 Total DNA quantification

DNA may be quantified using several methods. The procedure for the Qubit Fluorometer has been included here because of its relative low cost, portability and accuracy. Spectramax quantification is a more accurate, but costly and time-consuming alternative. In general, NanoDrop spectrophotometers are NOT sensitive enough to accurately detect the low DNA concentrations (< 10 ng/µL) that are often encountered in leukocyte-depleted samples.

I. Place a 1.5 mL or 15 mL tube on ice. Prepare master mix by adding 1 µL Quant-iT reagent and 199 µL Quant-iT buffer per total number of samples plus two standards.

Table 2. Master mix calculation for 48 samples (50 reactions)

Reagent	**For 1 sample Volume (µL)**	**For 50 samples Volume (µL)**
Quant-iT reagent	1	50
Quant-iT buffer	199	9950
Total volume (µL)	**200**	**10,000**

II. Add master mix, samples and standards to Qubit Assay Tubes as follows:
   a. Add 196 µL master mix and 4 µL sample to each sample tube
   b. Add 190 µL master mix and 10 µL standard to each of two standard tubes.

   NOTE: Do <u>not</u> vortex the mix. Mix by hand-shaking and light centrifugation. Avoid prolonged exposure of the mix to light.

III. Take measurement using Qubit Fluorometer

a. Turn Qubit Fluorometer on by pressing “Home”
b. Select Qubit HS dsDNA Kit (or BR if using Broad Range Kit)
c. When prompted, place Qubit assay tube containing Standard #1 into the tube slot and close the lid
d. Press “Go” to read Standard #1
e. Repeat steps c and d for Standard #2
f. Place samples in the tube slot one at a time, close lid and record concentration as reported on the screen
g. Multiply the reported concentration by the dilution factor (e.g. 4 µL sample in 200 µL total tube volume = 50) to determine the sample DNA concentration in ng/µL.

6.2 Quantitative PCR

Quantitative PCR can be performed using a number of real-time PCR machines and reagents. The procedure given here uses the ABI system and a SYBR green-based detection method. Other machines and reagents may require different reaction mixes, sample volumes and cycling conditions.

I. Calculate the number of samples to be tested. Each standard and sample is quantified in duplicate for both the LRAP and AMA1 primers. A 96-well plate will accommodate 16 samples. A representative plate is given in Figure 1.

	Human DNA standards		Samples 1-16				*P. falciparum* DNA standards		Samples 1-16			
	1	2	3	4	5	6	7	8	9	10	11	12
A	25ng	25ng	1	1	9	9	12.5ng	12.5ng	1	1	9	9
B	12.5ng	12.5ng	2	2	10	10	6.25ng	6.25ng	2	2	10	10
C	6.25ng	6.25ng	3	3	11	11	3.125ng	3.125ng	3	3	11	11
D	3.125ng	3.125ng	4	4	12	12	1.56ng	1.56ng	4	4	12	12
E	1.56ng	1.56ng	5	5	13	13	0.78ng	0.78ng	5	5	13	13
F	0.78ng	0.78ng	6	6	14	14	0.39ng	0.39ng	6	6	14	14
G	0.39ng	0.39ng	7	7	15	15	0.19ng	0.19ng	7	7	15	15
H	water	water	8	8	16	16	water	water	8	8	16	16

LRAP
AMA1

Figure 1. A 96-well plate set up to analyze 16 samples and two standards (human and parasite) in duplicate against each gene target

Image source: Matthew Adams, Center for Vaccine Development, University of Maryland, Baltimore.

II. Place two 15 mL tubes on ice and label them either ‘LRAP’ or ‘AMA1’. Prepare PCR master mix by adding SYBR green qPCR Master Mix reagent,

forward and reverse primers (LRAP primers in the tube labeled 'LRAP'and AMA1 primers in the tube labeled 'AMA1') using the amount per reaction shown in Table 3 multiplied by the number of reactions. It is best to prepare a master mix for a few extra reactions to allow for wastage or pipetting losses.

Table 3. qPCR Master Mixes for LRAP and AMA1 reactions

Reagent	Amount per reaction
SYBR green qPCR Master Mix reagent	12.5 µL
0.24 µM LRAP or AMA1(as appropriate) forward primer	as needed
0.24 µM LRAP or AMA1(as appropriate) reverse primer	as needed
Water to a total mix volume of 23 µL	

NOTE: Do not vortex the mix. Mix by hand-shaking and light centrifugation. Avoid prolonged exposure of mix to light.

III. Add 23 µL of LRAP or AMA1 master mix, as appropriate, to each well. Add 2 µL of sample DNA to each well as shown in columns 3-6 and 9-12 of Figure 1. Finally, add 2 µL of each human DNA standard or water to columns 1-2 and 2 µL of each *P. falciparum* DNA standard to columns 7-8 as shown in Figure 1.

IV. Seal plate thoroughly and cycle under the PCR conditions listed in Table 4 (also shown in Figure 5). Be sure to the label the standards and concentrations, blanks (water) and samples.

Table 4. Quantitative PCR thermal cycling conditions

Stage	Repetitions	Temperature (°C)	Time (minutes:seconds)
1	1	95	10:00
2	5	95	0:45
		56	0:45
		72	0:45
3	29	95	0:45
		65*	**0:45**
		72	0:45
4 (Dissociation)	1	95	0:15
		55	1:00
		95	0:15
		60	0:15

*Data collection occurs in Stage 3, step 3 (shown in **green**)

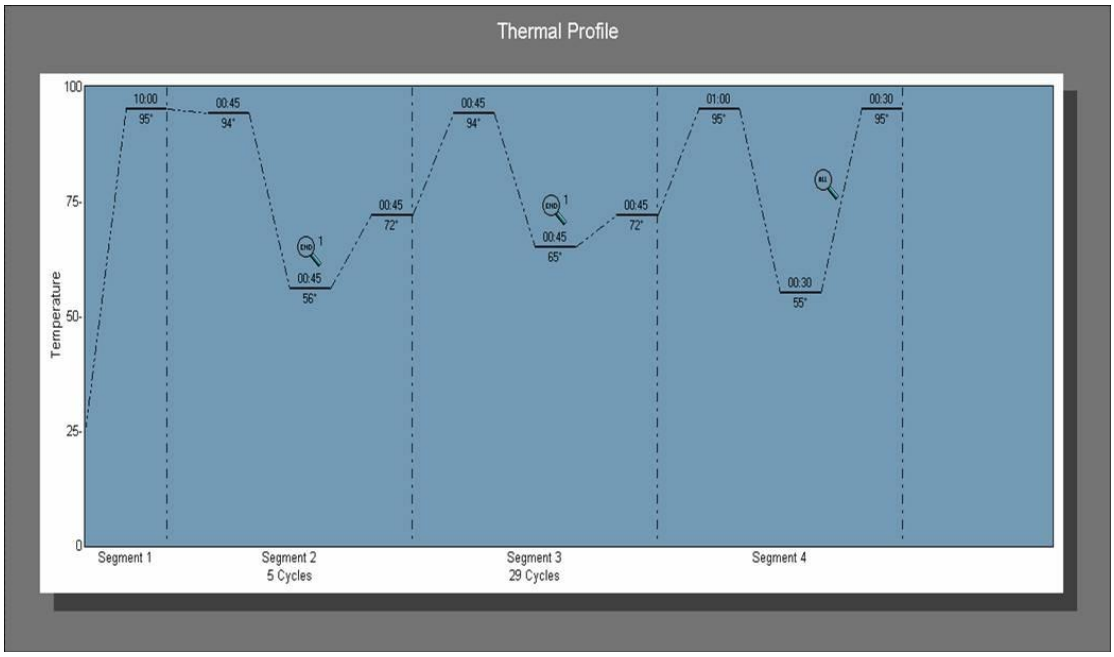

Figure 2. Quantitative PCR thermal cycling conditions

Image source: Susana Campino and Sarah Auburn, Wellcome Trust Centre for Human Genetics, Oxford and Wellcome Trust Sanger Institute, Hinxton

6.3 Quality check and calculation of total *P. falciparum* DNA and *P. falciparum* to human DNA ratio

I. Check the quality of your quantification reaction.
   a. Standards: visually examine the fit of the standard curve to your DNA standards. If necessary, remove outliers and reanalyze. If > 2 data points do not fall on the standard curve, it may be necessary to repeat the qPCR reaction.
   b. Samples: check that C_T estimates among duplicate samples are close in value. As a general rule, samples with a standard deviation of C_T value > 0.4 should be repeated.

II. Using the qPCR worksheet template tab, fill in the values from DNA quantification and qPCR. See the qPCR worksheet example tab or Appendix A for an example with data from a mock experiment.
   a. qPCR estimated [DNA] (ng/µL) is the mean concentration of LRAP and AMA1 derived from each pair of duplicate samples run in quantitative PCR. These values are used to determine the proportion of human and *P. falciparum* DNA in each sample.
   b. Estimation of total DNA concentration by the Qubit method is described in step 6.1.III.g. The concentration of *P. falciparum* DNA in the sample (ng/µL) is calculated by the equation (total DNA concentration multiplied by proportion of *P. falciparum* DNA calculated in step 6.3.II.a).

c. The elution volume of the DNA extraction is estimated at 275 µL for a Qiagen Midi Kit extraction and 575 µL for a Qiagen Maxi Kit extraction, although this may vary depending on extraction technique and/or elution volume. The Qiagen kit 'high concentration' (rather than maximum yield) elution method is preferred for DNA extraction. The concentration of *P. falciparum* DNA calculated in step 6.3.IIb above is multiplied by the elution volume to determine the total amount (ng) of *P. falciparum* DNA in the sample.

7. References

N/A

Appendix A: example qPCR worksheet

Sample name	qPCR estimated [AMA1] ng/µL	qPCR estimated [LRAP] ng/µL	Proportion human DNA	Proportion *P. falciparum* DNA	Qubit or Spectramax estimate [total DNA] ng/µL	*P. falciparum* [DNA] ng/µL	Elution volume (µL)	Total *P. falciparum* DNA (ng)
1	0.538	0.036	**0.06**	0.94	1.617	1.516	575	**871**
2	0.838	0.149	**0.15**	0.85	4.419	3.752	275	**1032**
3	2.37	1.7	**0.42**	0.58	9.03	5.26	275	**1446**
4	2.14	0.09	**0.04**	0.96	5.23	5.02	275	**1380**
5	0.502	0.073	**0.13**	0.87	1.873	1.635	575	**940**
6	0.476	0.27	**0.36**	0.64	1.939	1.237	575	**711**
7	1.63	0.23	**0.12**	0.88	5.37	4.71	275	**1294**
8	1.62	0.26	**0.14**	0.86	4.52	3.89	275	**1071**