Fast Detection And Quantification of Plasmodium Species Infected Erythrocytes In A Non-Endemic Region By Using The Sysmex XN-31 Analyzer

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TITLE PAGE

Fast detection and quantification of *Plasmodium* species infected erythrocytes in a non-endemic region by using the Sysmex XN-31 analyzer

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ABSTRACT

BACKGOUND: Due to increased travel from endemic countries, malaria occurs more frequently in non-endemic regions. It is a challenge for diagnostic laboratories in non-endemic countries to provide reliable results, as experience of staff is often limited to only a few cases per year. In this study, we evaluated the diagnostic accuracy of the fully automated Sysmex XN-31 malaria analyzer in a routine diagnostic setting in a non-endemic region.

METHODS: Samples from 112 patients suspected for malaria were examined by the Sysmex XN-31 analyzer to determine the absolute count of malaria-infected red blood cells count (MI-RBC/µl). Microscopic examination of both Quantitative Blood Coat capillary tubes and thick and thin blood films were used as reference methods. Limits of blank (LoB), detection (LoD) and quantification (LoQ) were investigated using an *in vitro Plasmodium falciparum* culture. Nine hundred twenty samples of patients with RBC abnormalities were included to determine which RBC abnormalities trigger indeterminate or false positive results.

RESULTS: No false positive nor false negative results were obtained for the examined patient samples suspected for malaria. For 3% of samples an indeterminate result by the XN-31 was obtained. The Passing-Bablok regression line for diagnostic accuracy of the parasitemia was y = 39.75 + 0.7892x showing a positive bias of about 21% when comparing the MI-RBC results to microscopy. The LoB, LoD and LoQ were calculated to be 4.7, 5.9, and 19.0 infected RBC/µL, respectively. From the 920 abnormal RBC samples collected, 4.6% resulted in a false positive MI-RBC result and almost half of the samples produced indeterminate results. These results were related to increases in nucleated red blood cells, reticulocytes and other abnormal RBC morphologies such as sickle cells.

CONCLUSIONS: Based on the results we conclude that the XN-31 is a fast and reliable screening method in the detection and quantification of *Plasmodium* species in patients However, if an abnormal red blood cell morphology is present, the results of the XN-31 should be interpreted with caution as false positive results can be caused by interfering abnormal erythrocytes.

Keywords: Malaria, diagnosis, flow cytometry, hemocytometry, *Plasmodium*

INTRODUCTION

Malaria is a life-threatening disease caused by the protozoan parasite *Plasmodium,* which is transmitted through the bite of an infected *Anopheles* mosquito. In 2019 there were 229 million infections with 409,000 deaths reported globally.[1] Most of these cases occur in sub-Saharan Africa, which is responsible for 93% of cases and 94% of deaths.[1] Due to the increasing number of global travelers and immigration from endemic countries malaria becomes more relevant in non-endemic countries in moderate climates as well. The five common *Plasmodium* species known to infect humans are *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. *P. falciparum* causes more than 90% of malaria cases and also causes the most fulminant and possibly fatal disease.[2]

Early and accurate diagnosis of malaria is fundamental for successful and timely treatment of the disease, as delay and/or misdiagnosis can result in morbidity and mortality. According to the World Health Organization (WHO), it is recommended to have prompt malaria diagnosis either by microscopy or by a malaria rapid diagnostic test (RDT) in all patients with suspected malaria before treatment is administered.[3] Microscopic examination of thick and thin blood films remains the golden standard according to current CDC guidelines.[4] This method, however, is time consuming and requires the availability of an experienced microscopist to examine the blood films,

which is often a challenge outside office hours and significant variability in applied methods exist among laboratories.[5] Therefore, RDTs for malaria have been developed of which immunochromatographic card tests (ICT) that detect antigens of *Plasmodium* in blood of the host, are most commonly used. These ICTs are easy to perform and provide results within 10-15 minutes.[6] However in case of infections with a low parasitemia, false negative results can be obtained.[7] Furthermore, the sensitivity of RDT testing is decreasing for the detection of *P. falciparum* due to specific mutations in, or complete deletion of, the *HRP-2* gene of *P. falciparum*.[8] Alternative methods like Loop Mediated Isothermal Amplification (LAMP), Real-Time PCR (rt-PCR) and Quantitative Buffy Coat (QBC) fluorescence analysis can be used as reliable alternative screening methods, but are either time consuming, expensive and/or in need of trained staff to be available 24 hours a day, seven days a week.[5,9–11] Hence, no ideal laboratory method is currently available that provides fast and reliable results with information on both the *Plasmodium* species (as these can require different therapy) and the parasitemia value needed to assess the disease severity of patients infected with *P. falciparum* or *P. knowlesi*) without the need of well-trained technicians. This is especially relevant for diagnostic laboratories in non-endemic countries, because these encounter often only a few malaria cases per year and thus lack experience to diagnose malaria by microscopic examination of blood films.[5]

The Sysmex XN-31 hemocytometer is an automated analyzer launched in September 2019 to support malaria diagnosis in whole blood samples in the clinical diagnostic laboratory. Using fluorescence flow cytometry (FFC) technology and a violet semiconductor laser with a 405 nm wavelength, this hemocytometer can detect, specify and quantitate malaria-infected red blood cells (MI-RBC) within a specific area of the scattergram known as the M-gating area. Previous studies

on the XN-31 and its predecessor the XN-30 reported mainly data on *P. falciparum* infected patients in endemic countries [12–16] or on *in vitro* cultures[13,17,18]. The XN-30, was approved for research purposes only, whereas the XN-31 is a CE marked *in vitro* diagnostic device with identical hardware, software, and user interface. The XN-31 has not been evaluated in clinical practice in non-endemic countries nor for *Plasmodium* species other than *P. falciparum* and *P. vivax.*[13,14,16]. In this study we compared the performance of the XN-31 in clinical practice with the current diagnostic workflow in our hospital, which is based on ICT in combination with microscopy by QBC analysis and thin and thick blood film examination. In addition, the limit of blank (LoB), limit of detection (LoD), and the limit of quantification (LoQ) were determined and compared to previous values reported by Sysmex in the Instructions for Use. Since the XN-31 can report indeterminate results that have been suggested to be linked to interference caused by certain RBC abnormalities, such as are commonly observed in peripheral blood smears of patients with sickle cell disease or other hemoglobinopathies.[12] We investigated the specificity of *Plasmodium* infected RBC detection by the XN-31, by examining 920 blood samples derived from patients with a wide array of RBC abnormalities.

METHODS

Sample inclusion

One hundred and twelve EDTA whole blood samples, for which malaria examination was ordered by the physicians, were collected at the Erasmus MC University Medical Center in Rotterdam, the Netherlands, between December 2019 and December 2020, including the follow-up samples of patients after initiation of treatment. In addition, 32 samples from asymptomatic, healthy individuals with no suspicion of malaria were collected for the determination of the Limit of Blank

(LoB). Samples that were older than 24h post collection or had a volume of less than 500µL were excluded. To investigate which RBC abnormalities perturb the MI-RBC examination, blood samples were selected from routine hematology of patients that were not suspected of malaria, comprised a high number of NRBC (>5%) and a relatively normal WBC count of <20 ($x10^3$/ µL). NRBC was used as the primary selection criterion as these frequently co-exist with other RBC abnormalities. With these selection criteria, 920 blood samples were examined with a high amount of NRBCs, reticulocytes and/or morphological RBC abnormalities from patients with thalassemia, sickle cell disease without hemato-oncological diseases, and ICU patients with infections and stress erythropoiesis.

Malaria examination reference methods

In our hospital the standard procedure to diagnose malaria in freshly collected EDTA-blood specimens involves an ICT RDT for malaria antigens and microscopic examination of both a QBC capillary and stained thick and thin blood films. The rapid diagnostic antigen test (Binax NOW® Malaria Test Binax, Inc. Maine, USA) and the QBC analyses were performed according to the manufacturer's instructions. QBC capillaries were examined independently by two technicians by microscopic analysis of two complete rows of the region between the bottom of the capillary and the polynuclear leukocyte layer using an Olympus BX-60 fluorescence microscope equipped with UV-filter, 50× objective and 12.5× oculars (total magnification 625×). Thick blood films were stained with Field's stain (Waldeck GMBH & CO KG, Münster, Germany) and thin blood films were fixed with absolute methanol for three minutes and subsequently stained with Diff Quick (RAL Diagnostics, Martillac, France). Both staining procedures had been optimized for optimal staining of *Plasmodium* parasites as well as Schüffner's dots and Maurer's clefts in infected

erythrocytes. Thick and thin films were examined with regular light microscopes at a total magnification of 1,250x. For all *Plasmodium* positive blood specimens the *Plasmodium* species was confirmed by a real-time PCR method based on the method of Shokoples *et al.*[19]

Flow cytometry analysis

The XN-31 was operated in the Low Malaria (LM) mode, as this mode uses a three times higher sample volume compared to other modes, which lowers the detection limit and thus increases the sensitivity of the method. Using this Low Malaria mode of analysis, the XN-31 provides a complete blood count (CBC), a qualitative result (positive, negative or indeterminate for malaria-infected red blood cells, MI-RBC), a quantitative result (an absolute MI-RBC count and the percentage of *Plasmodium* infected RBC) and a result for the suspected *Plasmodium* species (*P. falciparum* or *P. other*, i.e. non-falciparum, or *P. unknown*). Speciation provided by XN-31 is a suspect flag approved for research use only purposes by the manufacturer. All study samples were also processed on the Sysmex XN-1000 Series analyzer to collect full profile data (complete blood count, CBC), white blood cell differential (WDF), and reticulocytes (RET). The XN-31 results for malaria were compared to those of the combined results of RDT, QBC and rt-PCR analyses in order to determine the negative predictive value (NPV), positive predictive value (PPV), and efficiency. The XN-31 MI-RBC count includes all malaria-infected RBCs, irrespective of life stage. The differentiation into sexual (gametocytes) and non-sexual (rings, mature trophozoites, schizonts) are research use only parameters. The quantification of infected erythrocytes on the XN-31 for both sexual and asexual stages of *Plasmodium* parasites were compared to the counting results obtained by microscopic examination of thin and thick blood films.

The LoB assessment was based on 32 malaria-negative blood samples from healthy individuals with no symptoms and no suspicion for malaria. These samples had CBC values on the Sysmex XN-1000 hemocytometry analyzer within the reference ranges used in our department. The LoB was calculated using the following formula: LoB = mean blank + 1.645(1SD of blank sample).

The LoD was determined using *in vitro* cultured RBC infected with *P. falciparum* NF54 parasites (a generous gift of Dr. M. McCall, Radboudumc, Nijmegen, The Netherlands), that were serially diluted in freshly collected full blood of a healthy donor. The LoD was calculated by the following formula; LoD = LoB + 1.645 (1SD of the sample with the lowest MI-RBC concentration above the LoB with a reproducible qualitative test result).

The LoQ was calculated from the same dilution series and based on the point in the dilution series that exceeded a coefficient of variation (CV) of 20%. Quantification of infected RBC was also determined by microscopic examination of thick and thin blood films. Thin blood films were used to count infected erythrocytes if the parasitemia was above 4783 parasites per µL and thick blood films were used to quantify parasites for all dilutions with a lower parasitemia.

RBC abnormality interferences on XN-31

In addition to a positive or negative result for MI-RBC, the XN-31 can also provide an indeterminate result. Blood films were made for all these XN-31 examined samples to confirm the presence of RBC morphologies. The XN-31 scattergrams of all these samples were compared to true positive *Plasmodium* samples in order to investigate the potential causing interference. The selected blood samples were examined by the XN-31 hemocytometer and thin blood films were

prepared and examined to confirm the presence of RBC morphologies. The XN-31 scattergrams of all these samples were compared to true positive *Plasmodium* samples in order to investigate the potential causing interference.

Statistical analysis

Data analysis was performed by using Analyse-it for Microsoft Excel version 2.30 and Microsoft Excel 2016. Passing-Bablok regression analysis was used to determine accuracy of the MI-RBC produced by the XN-31 compared to microscopy.

RESULTS

Performance of the XN-31 in clinical practice

Due to the COVID-19 pandemic and the drop in international travelers during the time of this study, the number of requests for malaria examination was substantially decreased compared to the pre-COVID-19 period. There were 112 included and 14 of them contained *Plasmodium* parasites based on the results of analysis by RDT, QBC, thin and thick blood film examination and rt-PCR. These 14 positive samples were derived from 8 patients, because 6 samples were follow-up samples after initiation of malaria treatment. The 8 malaria patients were infected with *P. falciparum* (n=6)*, P. malariae* (n=*1)* or *P. vivax* (n=1). Of the 112 samples, 109 were correctly diagnosed by the XN-31, either as negative (n=96) or positive (n=13) and for those the *Plasmodium* species were correctly determined as *P. falciparum* or *P.* other. The XN-31 produced an indeterminate result for the remaining three samples, and therefore, the XN-31 had a positive and negative predictive value of 100% within an efficiency of 96% (**Table 1**). Of the three samples for which an indeterminate result was reported, one sample contained *P. malariae*. The parasitemia

209 in this sample appeared to be very low as only sporadically infected erythrocytes were found in
210 thick and thin blood films. In addition, this sample was also determined to contain microfilaria of
211 *Mansonella perstans*. The other two samples that had an indeterminate XN-31 result did not
212 contain *Plasmodium* infected RBC.

213 **Table 1**: Performance of XN-31 compared to the combination of parasitological examinations used in routine
214 patient care
215

Overall Result of Parasitological Examinations			
		Positive	**Negative**
XN-31 Result	**Positive**	13	0
	Indeterminate	1	2
	Negative	0	96
	Total	14	98
Predictive Value and Efficiency			
PPV		**NPV**	**Efficiency**
100%		100%	96%

216 PPV, positive predictive value, defines the probability of having *Plasmodium* in a sample with a
217 positive result. NPV, negative predictive value, describes the probability of not having
218 *Plasmodium* in a sample with a negative test result. Efficiency is the proportion of correctly
219 classified samples as negative or positive among all samples.
220

221 To investigate the correlation between the parasitemia determination by the XN-31 and
222 microscopic examination, Passing-Bablok analysis was performed on 12 of the 14 positive
223 samples. For one positive sample the parasitemia could not reliably be determined by microscopy
224 because the patient was treated for malaria long enough that the parasite morphology in infected
225 erythrocytes was too aberrant to be reliably determined. In addition, in the *P. malariae* sample too
226 few infected erythrocytes were present that the parasitemia could not be accurately be determined
227 by microscopy. On the 12 remaining positive samples, Passing-Bablok analysis was performed
228 with the parasitemia results from the XN-31 and the parasitemia determined in thin and thick blood
229 films to determine the accuracy of the XN-31 across a range of distinct concentrations of

Plasmodium infected RBC. The parasitemia of the XN-31 correlated well with the parasitemia determined by microscopy (**Figure 1)** with a proportional bias of 21% (y-intercept of 39.75 and slope of 0.79). In addition, quantification of *Plasmodium* infected RBC in a dilution series prepared from *in vitro* cultured RBC infected with *P. falciparum* in freshly collected blood of a healthy donor, demonstrated a good agreement between the parasitemia determined by the XN-31 and microscopic methods as well as a clear linear response with a best fit line of y = 440.7 + 1.12x (**Figure 2**).

Determination of XN-31 detection limits for *Plasmodium* infected erythrocytes

As shown in **Table 2** the LoB was determined to be 4.7 infected RBC/µL and the LoD was determined to be 5.9 infected RBC/µL. In addition, a dilution series of *in vitro* cultured *P. falciparum* was used to determine the LoQ. As shown in **Figure 3** the lowest concentration at which the %CV was still below 20% was 19/µL, which had a CV of 19%.

Table 2: Determination of LoB and LoD for the XN-31

Determination of LoB for MI-RBC# (/µL)	
Mean #MI-RBC of blanks (32 samples)	2.4
1SD	1.4
LoB = mean blank + 1.645(1SD of blank sample)	4.7
Determination of LoD for MI-RBC# (/µL)	
Mean #MI-RBC of Target LoD Sample (10 replicates)	4.9
1SD	0.7
LoD = LoB + 1.645 (1SD of low conc. sample)	5.9

LoB was calculated using samples of patients not infected with *P. falciparum*. The LoD was calculated based on the LoB and 1SD of the dilution sample mean with a reproducible positive test result with the lowest number of MI-RBCs above the LoB. Abbreviations: LoB, limit of blank; LoD, limit of detection; SD, standard deviation.

RBC abnormalities interfering with XN-31 analysis

In order to examine which RBC abnormalities could trigger indeterminate results, 920 samples from 254 unique patients with RBC abnormalities (ranging from 1 to 16 samples per patient) were selected from regular patient care hemocytometry. Included samples contained no *Plasmodium* parasites, had greater than 5% NRBC's and less than $20x10^9$ WBC/L, or came from the hematology clinic. Processing of these 920 blood samples on the XN-31 generated 449 negative, 429 indeterminate and 42 false positive results for the detection of *Plasmodium* infected RBC. The MI-RBC values of these false positive samples varied substantially as a wide range of 20 to 44,310 infected RBC/μL was observed. This result demonstrates that the false positive results did not only occur with low MI-RBC values being incorrectly detected. **Table 3** shows the frequency of occurrence of specific RBC abnormalities (as determined by the XN-1000) in samples classified by XN-31 as positive, negative or indeterminate for MI-RBC qualitative judgment. Upon reviewing the results for these samples on the XN-1000, more than 67% of the samples with an MI-RBC false positive result and 62% of the samples with indeterminate result had greater than 10% NRBCs. It is clear in **Table 3** that there are lower percentages of samples with increased NRBCs and reticulocytes that are triggering the false positive MI_RBC results. There were also samples with lower numbers of NRBC and reticulocytes triggered an indeterminate or false positive MI-RBC result. Therefore, thin blood films of all false positive MI-RBC samples, were manually re-evaluated by two trained microscopists to examine the abnormal RBC morphology present in these samples. In total 42 samples from 31 patients gave false positive MI-RBC results. Of these, 15/42 samples (36%) were from 8 sickle cell disease patients with sickle cells present in their blood films. In the examined group of abnormal RBC samples especially the sickle cell patients triggered many a false positive or an indeterminate MI-RBC result and never a negative result apart from the few samples from sickle cell patients for which no sickle cells in their blood

films could be found. Furthermore false positive or indeterminate MI-RBC results were also generated with the samples from premature newborns (6/42), having high numbers of reticulocytes and NRBC counts. Other patient groups and RBC anomalies occurring in the false positive MI-RBC result group were hemochromatosis (8/42), beta thalassemia (12/42), leukemia/lymphoma (6/42), and sepsis (1/42). Many patients in the false positive MI-RBC group had multiple diagnoses and had multiple samples taken at different time points. Many patients in the false positive MI-RBC group had multiple diagnoses, notable all hemochromatosis samples came from a single patient with beta thalassaemia.

Table 3: Detection of *Plasmodium* infected RBCs in routine blood samples with RBC abnormalities using the XN-31 analyzer

	XN-31 Result for the detection of *Plasmodium* infected RBCs		
Sysmex XN-1000 Parameter (# out of 920)	NEGATIVE (% out of 920)	INDETERMINATE (% out of 920)	POSITIVE (% out of 920)
NRBC >10% (404)	111 (12%)	265 (29%)	28 (3%)
RET >1.5% (699)	251 (27%)	411 (45%)	37 (4%)
RBC ABN Flag (448)	105 (11%)	313 (34%)	30 (3%)
RET ABN SCAT (251)	22 (2%)	205 (34%)	24 (3%)
Total	489	1194	119

Most samples included comprise more than a single abnormality.
Abbreviations: NRBC, nucleated red blood cell; RET, reticulocytes; WBC ABN, white blood cell abnormal; RBC ABN, red blood cell abnormal; RET ABN SCAT, reticulocyte abnormal scattergram.

To determine whether a true positive MI-RBC sample can be distinguished from a false positive MI-RBC sample, we compared the MI-RBC scattergrams of the XN-31 analyses. In **Figure 4**, a representative example of a true positive MI-RBC sample and a false positive sample are shown. The events in the forward scatter light and side-fluorescent light (FSC and SFL) scattergram of a true positive MI-RBC sample demonstrate a compact and perpendicular pattern with the defined

clusters of RBC infected by one or multiple ring forms as explained by Pillay et al.[12] (shown in the green circles in **Figure 4A**). On the other-hand the scattergrams of false positive MI-RBC samples demonstrate a dispersed pattern at a 45°C angle (located within the orange circle) (**Figure 4B**).

DISCUSSION

Our study shows that the XN-31 can be used in clinical practice as a fast and easy screening assay for malaria that provides reliable qualitative and quantitative results. Therefore, XN-31 can easily be integrated in regular 24/7 patient care diagnostics settings in non-endemic counties and can provide all required information to clinicians to timely start proper treatment. Compared to other screening assays, the XN-31 is the only test that can provide rapid results (< 1 minute) to determine the presence of *Plasmodium* infected RBC with a detection limit equivalent to thick blood film examination[5] in combination with a *Plasmodium* species differentiation and an accurate parasitemia quantification. The XN-31 can accurately determine the parasitemia in samples with low numbers of infected erythrocytes, as the limit of detection and quantification was determined to be 5.9 and 19 infected RBC per µL, respectively. Thereby the detection limit of the XN-31 is equivalent to thick and thin blood examination which has on average a detection limit of ~ 10 parasites per µL.[5]

Although we did not get any false positive or false negative results for samples of patient suspected for malaria, it is known that submicroscopic malaria exists.[20] In these cases the number of infected erythrocytes is below the detection limit of thick blood film examination, and thus also below the detection limit of the XN-31. Therefore, false negative results can occur, but most submicroscopic

malaria cases are asymptomatic and occur in patients from endemic areas with extensive immunity against malaria or in patients infected with a benign, non-falciparum, *Plasmodium* species that will present with a typical and characteristic fever pattern returning every 48 or 72 hours.[21–23] Hence, for patients whom malaria is clinically suspected, but a negative result is obtained, regardless of which test is used, repeat testing should be undertaken periodically. In specific cases further examinations by more sensitive methods can be indicated for patients for which a negative result by the XN-31 has been generated. In our analytical performance evaluation we achieved an LoD of ~6 MI-RBC/µL, which is significantly lower than the 20 MI-RBC/µL cut-off set for qualitative judgment of MI-RBC present or absent. The threshold for defining a sample as positive could therefore possibly be adjusted by the manufacturer.

Next to the hypothetical possibility of false negative results due to patients with very low parasitemia, our study demonstrated that indeterminate and false positive MI-RBC results can occur as well. Examination of a large panel of blood samples of patients with a variety of RBC abnormalities demonstrated not only a high frequency of indeterminate results (~ 50%), but also false positive results (~ 5%). These false positive MI-RBC results were predominantly encountered for blood samples from sickle cell patients and patients with increased numbers of nucleated erythrocytes and/or reticulocytes. Conditions of stressed erythropoiesis such as may occur in thalassemia or other hemoglobinopathies are mentioned in the instructions for use as potentially giving an erroneous MI-RBC positive result. Analysis of the scattergrams demonstrated substantial differences between the true positive and false positive MI-RBC samples, and therefore, future refinement of the automatic interpretation script of the XN-31 for the obtained scattergrams should result in improved performance of the XN-31. Thus, caution is required for patients with abnormal

blood cell morphology and review of the scattergram is advised when authorizing results. It should however be noted that in our study we deliberately enriched the number of samples measured with those expected to cause interferences and that the actual occurrence of such issues may be substantially lower in the routine setting where only samples from patients suspected to have malaria will be measured on XN-31.

In order to better determine what could produce indeterminate and false positive MI-RBC results by the XN-31, abnormal RBC samples were examined. For detection of MI-RBC, the software determines the number of events in the M-gating area as shown in **Figure 4.** If the number of detected events exceeds a certain threshold an indeterminate or positive result will be generated according to clustering patterns defined by algorithms. When interfering cell types are present that produce a scattergram with a distinct cluster of particles in the M-gating area, the algorithm will override the presence of generalized background scatter, producing a false positive MI-RBC result. This also means that when 20 or more parasites/μL are present, but no distinct cluster can be detected due to interference, an indeterminate result will be produced that is marked with an abnormal scattergram flag. Examination of the panel of abnormal RBC samples showed that a number of diseases and conditions frequently occurred in both the false positive and indeterminate MI-RBC results group; beta thalassemia, leukemia, lymphoma, premature newborns, and sickle cell disease. The observation occurring most frequently for the false positive samples was sickle cell disease in crisis. Although there were no sickle cell patients included in the study population for suspected *Plasmodium* infection, it can be hypothesized that a patient with sickle cell disease and malaria should get a valid MI-RBC present result (cluster detected) although the actual parasitemia value would be overestimated. Premature newborns, beta thalassemia and

363 hemochromatosis are associated with stressed or disturbed erythropoiesis as well and we speculate
364 that triggering of indeterminate and false positive results is highly correlated with diseases and
365 conditions associated with the presence of immature cells in the erythrocyte lineage or with
366 severely abnormal RBC morphology.
367
368 Although in our study population of patients suspected for a *Plasmodium* infection, none of the
369 mentioned diseases were present, it is very well possible as RBC abnormalities occur relatively
370 frequently in the population in malaria endemic areas.[24] Some of these abnormalities can even
371 cause mortality in malaria patients making it even more important to understand exactly which
372 RBC abnormalities cause indeterminate or positive result on the XN-31. Mortality in sickle cell
373 patients with malaria is a problem in endemic countries that have a high prevalence of sickle cell
374 disease. More than 80% of people that have sickle cell disease live in sub-Saharan Africa where
375 most *Plasmodium* deaths occur.[25] In one study from Cameroon, it was found that 2 out of every
376 10 sickle cell patients who died had malaria.[26] To date, there have been preliminary studies done
377 how interferences affect the results of the XN-31, but there is some discrepancy in the results and
378 what conditions can trigger an abnormal scattergram on the XN-31.[14,15] Further need to be done in
379 order to determine whether the XN-31 can properly detect *Plasmodium* infected RBC in patients
380 with sickle cell disease and other diseases that significantly affect red blood cell morphology
381 and/or erythropoiesis.
382
383 When an indeterminate result occurs, it is clear that additional examinations by other methods are
384 required to confirm whether *Plasmodium* infected RBC are present or not to prevent the reporting
385 of a false positive MI-RBC result, which could lead to misdiagnosis. To mitigate the possibility of

386 having a false positive MI-RBC result with potentially serious consequences, we recommend to
387 evaluate the hemocytometric scattergrams to determine whether or not there is distinct cluster
388 formation of particles within the M-gating area where parasites are detected, as seen in a true
389 positive *Plasmodium* sample. In case of abnormal RBC morphology and a positive malaria result,
390 *Plasmodium* infected RBC should be confirmed by microscopic examination of thick and/or thin
391 blood films. However the scattergrams of true positive and false positive MI-RBC samples are
392 different and therefore further refinement of XN-31 gating and interpretation algorithms should be
393 able to increase the specificity.
394
395 Finally, there are some limitations of this study. Firstly, the number of included patients for
396 diagnostic accuracy was smaller than anticipated due to travel restrictions during the COVID-19
397 outbreak. Secondly, the LoD and LoQ studies were performed using *P. falciparum* parasites
398 cultured *in vitro* in RBC as there were no patient samples with a parasitemia high enough to dilute
399 serially across the linear range. Although the use of *in vitro* cultures on the XN-31 is not approved
400 in the specifications, our results showed that no interferences were present if the dilution series is
401 prepared by dilution of *P. falciparum* parasites cultured in RBC *in vitro* in freshly collected blood
402 of a healthy donor. Using this method accurate results were obtained for the LoB, LoD, and LoQ.
403
404 **CONCLUSION**
405 The XN-31 is a promising alternative for rapid diagnostic antigen tests, as mutations in the *HRP-*
406 *2* gene will not interfere with the accuracy of results on the XN-31. However, our study
407 demonstrated that false positive results can occur in sickle cell patients or other RBC abnormalities
408 such as elevated NRBCs and/or reticulocytes and confirmation by the reference method is

necessary. However, the scattergrams of true positive and false positive MI-RBC samples are different and therefore further refinement of XN-31 gating and interpretation algorithms should be able to increase the specificity. And until that has been achieved, caution is required for patients with abnormal blood cell morphology. Ultimately, we showed that the XN-31 can be a fast and accurate screening method for the detection and quantification of *Plasmodium* infected RBC in blood samples of patients suspected for malaria.

DECLARATIONS

Ethics approval

Ethical clearance for the use of residual blood samples for scientific purposes was granted by the Medical Ethics Review Board of the Erasmus MC, University Medical Center Rotterdam, the Netherlands (MEC 2012-047).

Consent for publication

Not applicable.

Availability of data and materials

The data that support the findings of this study are available from Erasmus MC, Department of Clinical Chemistry but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of Erasmus MC, Department of Clinical Chemistry.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

TK, YB, RK, JH, and HR all contributed to the study design, study execution, interpretation of the data, and substantially revised the drafts. TK performed the statistical analysis and drafted the work.

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REFERENCES

1. World Health Organization. *World Malaria Report: 20 Years of Global Progress and Challenges*. Geneva: WHO; 2020. https://www.who.int/publications/i/item/9789240015791. Accessed November 9, 2021.
2. Snow RW. Global malaria eradication and the importance of *Plasmodium falciparum* epidemiology in Africa. *BMC Med*. 2015;13(1):14-16. doi:10.1186/s12916-014-0254-7
3. World Health Organization. Malaria: diagnostic testing. 2016:1-3. http://www.who.int/csr/resources/publications/drugresist/malaria.pdf.

4. CDC. Malaria Diagnostic Tests. Malaria Diagnostic Tests. https://www.cdc.gov/malaria/diagnosis_treatment/diagnostic_tools.html#tabs-1-1. Published 2020. Accessed April 26, 2021.

5. Boonstra MB, Koelewijn R, Brienen EAT, et al. Malaria diagnosis in a malaria non-endemic high-resource country: high variation of diagnostic strategy in clinical laboratories in the Netherlands. *Malar J*. 2021;20(1):411. doi:10.1186/s12936-021-03889-7

6. Mishra M, Misra R. Immunochromatographic Methods in Malaria Diagnosis. *Med Journal, Armed Forces India*. 2007;63(2):127. doi:10.1016/S0377-1237(07)80054-2

7. Portero J-L, Rubio-Yuste M, Descalzo MA, et al. Accuracy of an Immunochromatographic Diagnostic Test (ICT Malaria Combo Cassette Test) Compared to Microscopy among under Five-Year-Old Children when Diagnosing Malaria in Equatorial Guinea. *Malar Res Treat*. 2010;2010:1-6. doi:10.4061/2010/858427

8. Cheng Q, Gatton ML, Barnwell J, et al. *Plasmodium falciparum* parasites lacking histidine-rich protein 2 and 3: A review and recommendations for accurate reporting. *Malar J*. 2014;13(1):1-8. doi:10.1186/1475-2875-13-283

9. Charpentier E, Benichou E, Pagès A, et al. Performance evaluation of different strategies based on microscopy techniques, rapid diagnostic test and molecular loop-mediated isothermal amplification assay for the diagnosis of imported malaria. *Clin Microbiol Infect*. 2020;26(1):115-121. doi:10.1016/j.cmi.2019.05.010

10. Ponce C, Kaczorowski F, Perpoint T, et al. Diagnostic accuracy of loop-mediated isothermal amplification (LAMP) for screening patients with imported malaria in a non-endemic setting. *Parasite*. 2017;24. doi:10.1051/parasite/2017054

11. Pöschl B, Waneesorn J, Thekisoe O, Chutipongvivate S, Panagiotis K. Comparative diagnosis of malaria infections by microscopy, nested PCR, and LAMP in Northern Thailand. *Am J Trop Med Hyg*. 2010;83(1):56-60. doi:10.4269/ajtmh.2010.09-0630

12. Pillay E, Khodaiji S, Bezuidenhout BC, Litshie M, Coetzer TL. Evaluation of automated malaria diagnosis using the Sysmex XN-30 analyser in a clinical setting. *Malar J*. 2019;18(1):15. doi:10.1186/s12936-019-2655-8

13. Tougan T, Suzuki Y, Itagaki S, et al. An automated haematology analyzer XN-30 distinguishes developmental stages of falciparum malaria parasite cultured in vitro. *Malar J*. 2018;17(1):1-8. doi:10.1186/s12936-018-2208-6

14. Zuluaga-Idárraga L, Rios A, Sierra-Cifuentes V, et al. Performance of the hematology analyzer XN-31 prototype in the detection of *Plasmodium* infections in an endemic region of Colombia. *Sci Rep*. 2021;11(1):5268. doi:10.1038/s41598-021-84594-y

15. M'baya B, Mfune T, Samon A, Hwandih T, Münster M. Evaluation of the Sysmex XN-31 automated analyser for blood donor malaria screening at Malawi Blood Transfusion Services. *Vox Sang*. 2021;(June):1-8. doi:10.1111/vox.13208

16. Post A, Kaboré B, Reuling IJ, et al. The XN-30 hematology analyzer for rapid sensitive detection of malaria: A diagnostic accuracy study. *BMC Med*. 2019;17(1):1-12. doi:10.1186/s12916-019-1334-5

17. Toya Y, Tougan T, Horii T, Uchihashi K. Lysercell M enhances the detection of stage-specific *Plasmodium*-infected red blood cells in the automated hematology analyzer XN-31 prototype. *Parasitol Int*. 2021;80(October 2020):102206. doi:10.1016/j.parint.2020.102206

18. Tougan T, Toya Y, Uchihashi K, Horii T. Application of the automated haematology

analyzer XN-30 for discovery and development of anti-malarial drugs. *Malar J*. 2019;18(1):1-12. doi:10.1186/s12936-019-2642-0

19. Shokoples SE, Ndao M, Kowalewska-Grochowska K, Yanow SK. Multiplexed real-time PCR assay for discrimination of *Plasmodium species* with improved sensitivity for mixed infections. *J Clin Microbiol*. 2009;47(4):975-980. doi:10.1128/JCM.01858-08

20. Pousibet-Puerto J, Cabezas-Fernández MT, Lozano-Serrano AB, et al. Submicroscopic Malaria in Migrants from Sub-Saharan Africa, Spain. *Emerg Infect Dis*. 2019;25(2):349. doi:10.3201/EID2502.180717

21. T B, L O, I F, C D. Asymptomatic malaria infections: detectability, transmissibility and public health relevance. *Nat Rev Microbiol*. 2014;12(12):833-840. doi:10.1038/NRMICRO3364

22. EE B, JJ van H, PJ van G, et al. A case report of transfusion-transmitted *Plasmodium malariae* from an asymptomatic non-immune traveller. *Malar J*. 2013;12(1). doi:10.1186/1475-2875-12-439

23. A W, AW D, I H, et al. High Rates of Asymptomatic, Sub-microscopic *Plasmodium vivax* Infection and Disappearing *Plasmodium falciparum* Malaria in an Area of Low Transmission in Solomon Islands. *PLoS Negl Trop Dis*. 2015;9(5). doi:10.1371/JOURNAL.PNTD.0003758

24. Min-Oo G, Gros P. Erythrocyte variants and the nature of their malaria protective effect. *Cell Microbiol*. 2005;7(6):753-763. doi:10.1111/J.1462-5822.2005.00524.X

25. Archer NM, Petersen N, Clark MA, Buckee CO, Childs LM, Duraisingh MT. Resistance to *Plasmodium falciparum* in sickle cell trait erythrocytes is driven by oxygen-dependent growth inhibition. *Proc Natl Acad Sci U S A*. 2018;115(28):7350-7355.

doi:10.1073/pnas.1804388115

26. Eleonore NLE, Cumber SN, Charlotte EE, et al. Malaria in patients with sickle cell anaemia: Burden, risk factors and outcome at the Laquintinie hospital, Cameroon. *BMC Infect Dis*. 2020;20(1):1-8. doi:10.1186/s12879-019-4757-x

FIGURES

Figure 1: Accuracy of XN-31 parasitemia determination in clinical patient samples compared to parasitemia determined by microscopy

Figure 2: Accuracy of XN-31 for dilution series of *in vitro* cultured *P. falciparum* compared to microscopy
A linear dilution series was prepared of *in vitro* cultured *P. falciparum* infected RBC in freshly obtained blood of a healthy donor, after which the parasitemia was determined by XN-31 and microscopic examination of blood films. Line of best fit is in red comparing the results of the XN-31 MI-RBC# (/µL) to the microscopy results.

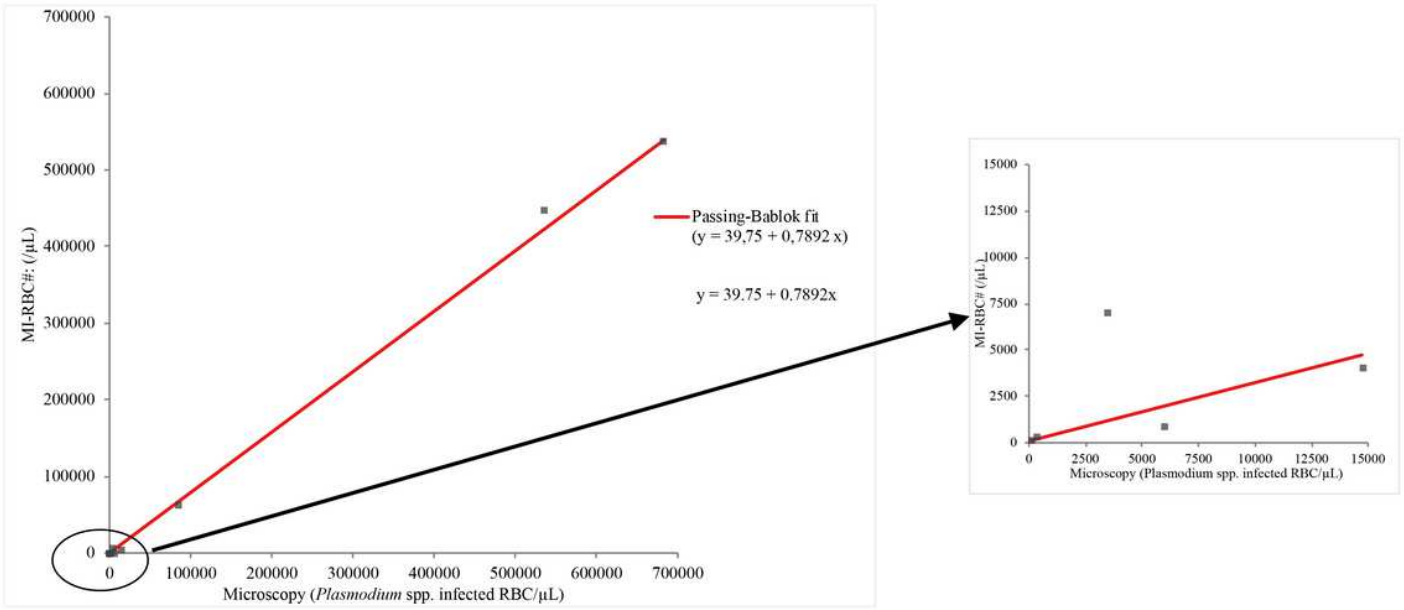
Figure 3: Determination of Limit of Quantification (LoQ) of the XN-31

Each point in the graph represents the mean of 10 replicates in that concentration and the %CV associated with those replicates. The LoQ is the lowest concentration of infected erythrocytes with a %CV <20 (indicated with the red line), which is in this case an MI-RBC of 19 parasites/µL. Abbreviations: LoQ, limit of quantification; CV, coefficient of variation.

Figure 4: Comparison of XN-31 scattergrams of a true positive *Plasmodium falciparum* sample with a false-positive MI-RBC sample

Figure A is a scattergram of a true positive MI-RBC samples containing erythrocytes infected with *Plasmodium falciparum*. **Figure B** is a scattergram of a sample of a patient in sickle crisis that produced a false positive MI-RBC result by the XN-31. The red particles are what the XN-31 suspects to be a *Plasmodium* infected red blood cell*,* the teal particles are the leukocytes, and the dark blue particles are the non-infected red blood cells or debris. In **Figure A,** a true positive sample, the cluster of events is vertical (green circles), whereas the false-positive events identified in the MI-RBC channel in the sickle cell crises samples cluster at a 45 ° angle (orange circle in panel B).
Abbreviations: FSC, forward scatter light; SFL, side-fluorescent

Figures



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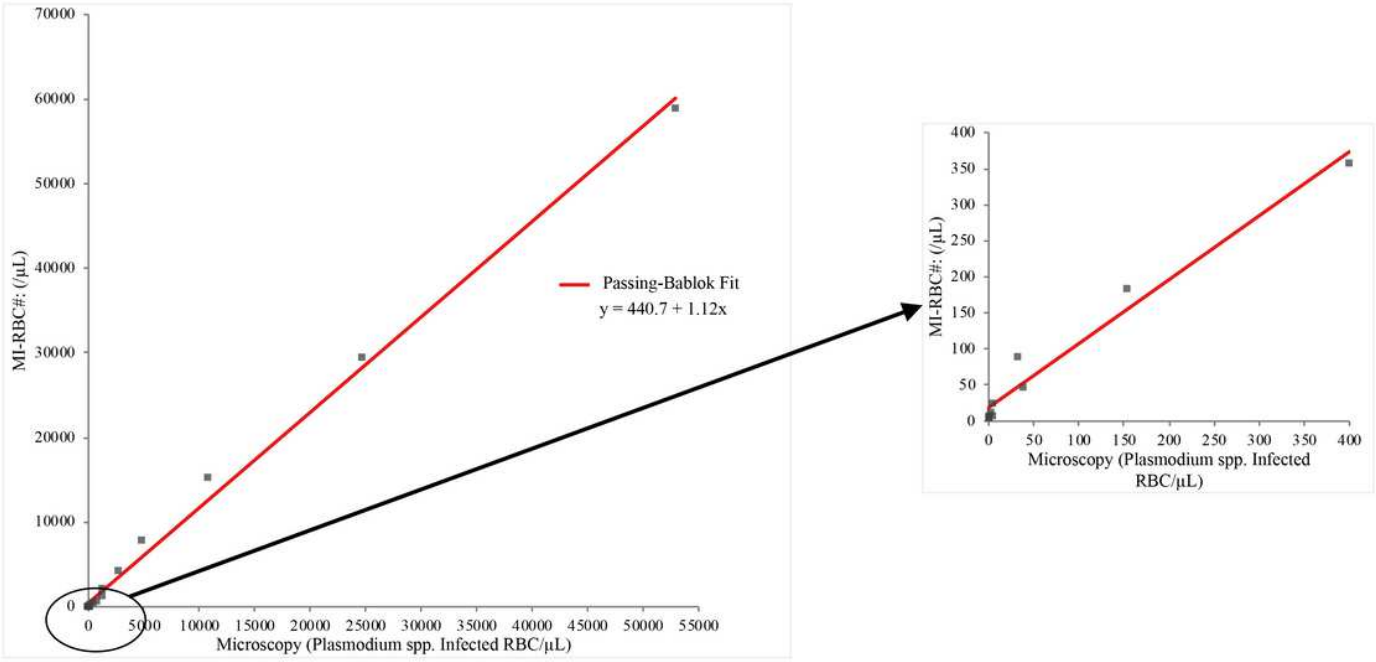
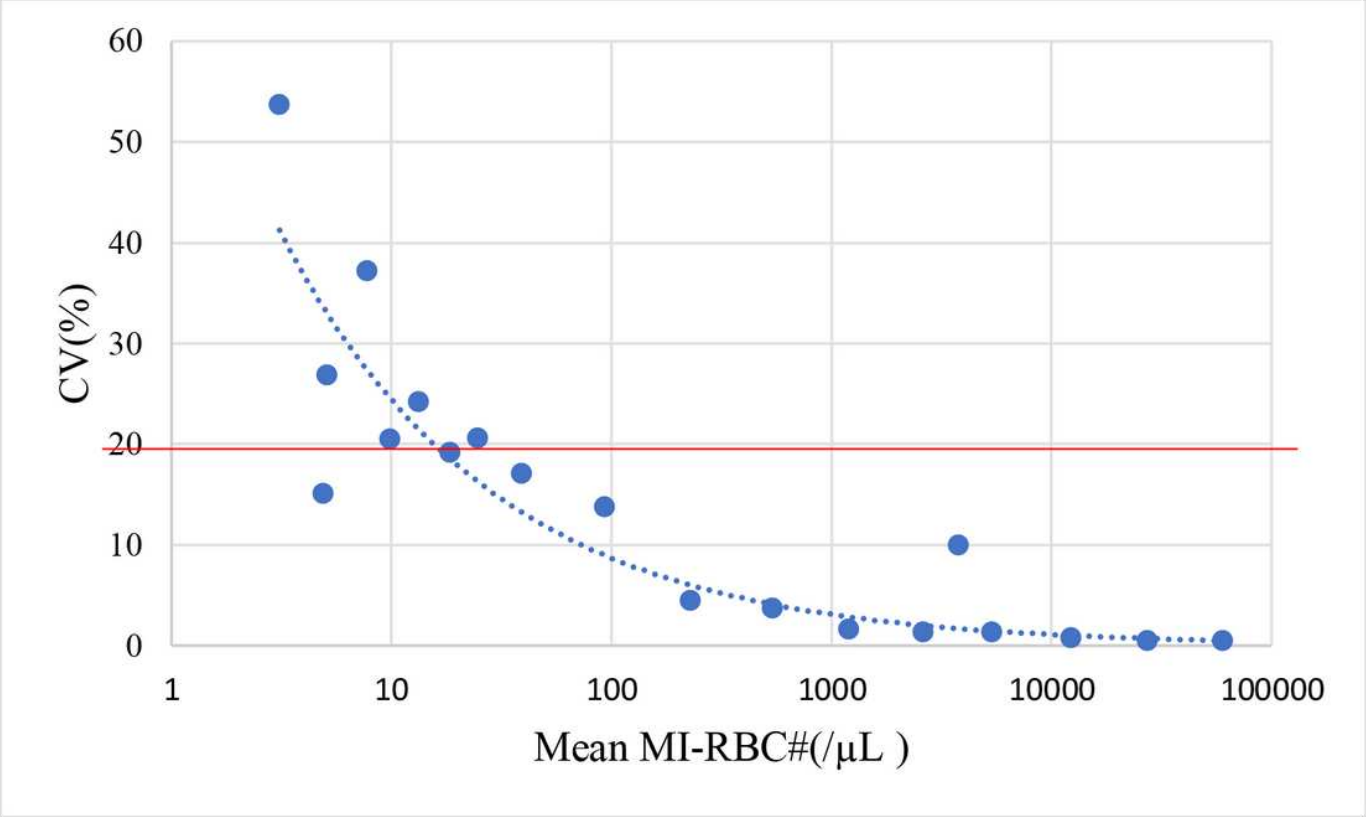


Figure 2

Accuracy of XN-31 for dilution series of in vitro cultured P. falciparum compared to microscopy A linear dilution series was prepared of in vitro cultured P. falciparum infected RBC in freshly obtained blood of a healthy donor, after which the parasitemia was determined by XN-31 and microscopic examination of blood films. Line of best fit is in red comparing the results of the XN-31 MI-RBC# (/µL) to the microscopy results.



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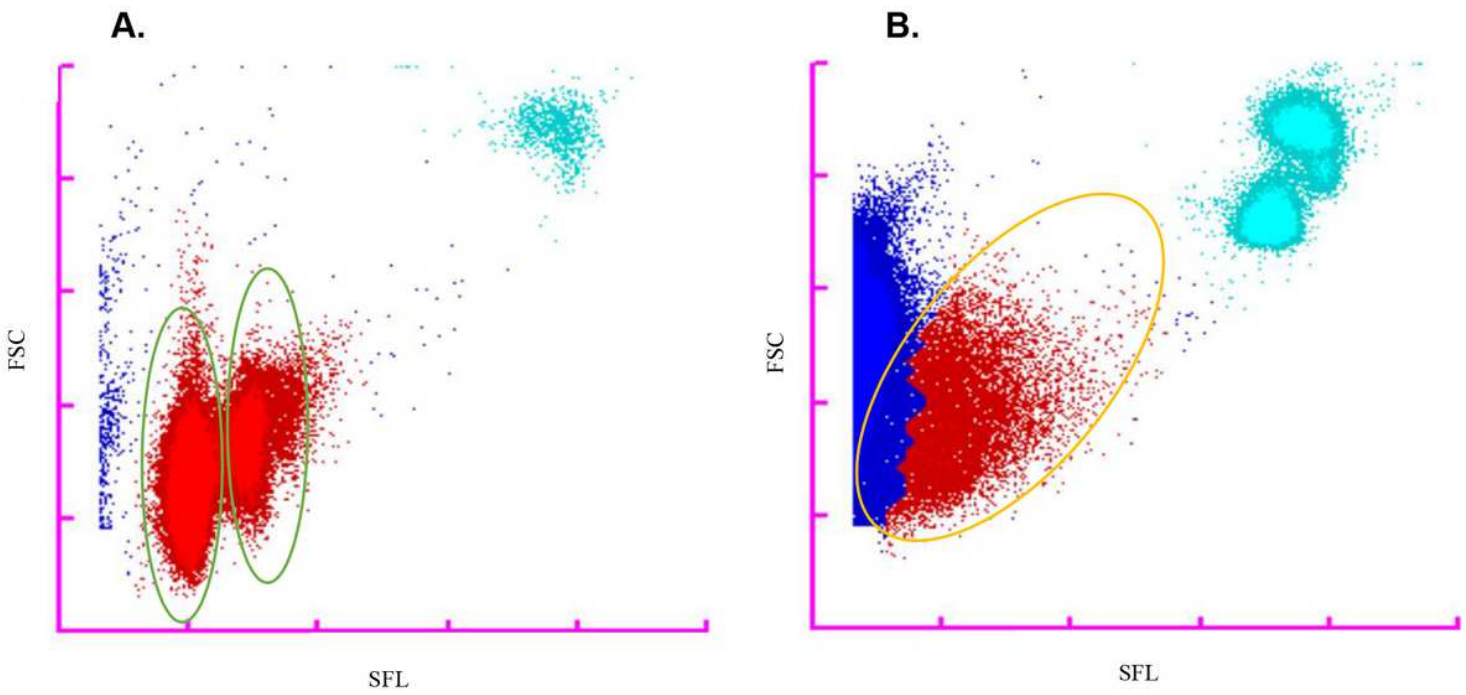

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Comparison of XN-31 scattergrams of a true positive Plasmodium falciparum sample with a false-positive MI-RBC sample

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