

Full Length Research Paper

Optimization of a protocol for extraction of *Plasmodium falciparum* RNA from infected whole blood samples for use in DNA microarrays

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Accepted 28 March, 2008

This study was carried out to determine the efficiency of two reagents, RNAlater and RNAwiz, for their ability to stabilize *Plasmodium falciparum* RNA in infected whole blood and saponin lysed parasite pellets for use in DNA microarrays. Eight infected blood samples were stored in each of the reagents, and RNA extracted at days 0 and 56 post collection. RNA yields and quality were compared at the different time points between the two test reagents. We show that for both reagents, higher RNA yields and quality is obtained when RNA is isolated immediately after sample collection (day 0), however, results show that RNAwiz storage provides a marginally higher RNA yield compared to RNAlater storage. Our results indicate that whole blood gave slightly higher RNA yields with superior quality as compared to saponin lysed samples when such whole blood samples are stored in RNA wiz, but not in RNAlater. From our results, we recommend RNAwiz as a better reagent for use in storage of whole infected blood intended for extraction of *P. falciparum* RNA for DNA microarrays and other sensitive techniques.

Key words: RNAwiz, RNAlater, RNA extraction, *Plasmodium falciparum*, DNA microarrays.

INTRODUCTION

RNA stabilization is an absolute prerequisite for reliable gene expression analysis. Immediate stabilization of RNA in biological samples is necessary because, directly after harvesting the samples, changes in the gene expression pattern occur due to specific and nonspecific RNA degradation as well as to transcriptional induction (RNAlater Handbook, 2006) Such changes need to be avoided for all reliable quantitative gene expression analyses, such as microarrays analysis, quantitative RT-PCR, such as TaqMan® and LightCycler® technology, and other nucleic acid-based technologies (Lizardi et al., 1995; Dean et al., 2001).

Gene expression studies are increasingly becoming important in malaria research (Schena et al., 1995; Perou et al., 2000; Reymond et al., 2000) where *P. falciparum* infected blood represents the most important sample type for studying the parasite's whole genome (Applied Biosystems information sheet, 2004). Gene expression studies using DNA microarrays require a minimum of 2 µg of intact RNA for hybridization (Hedge et al, 2000). The entire process of isolating parasite RNA from infected red blood cells involves many processes, and steps that are likely to affect the RNA yield and integrity required for down-stream analyses.

Expression studies on infected whole blood samples present many challenges, including small blood sample volumes usually obtained in the field, particularly from malnourished children. The heterogeneity of cell types,

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the different sample handling methods and the duration for handling and processing such blood samples add up to the challenges of parasite RNA extraction and subsequent analysis of parasite gene expression profiles, *ex vivo* (RNAlater Handbook, 2006).

Infected blood samples collected from the field must be transported to the laboratory for processing. The time spent from collection of the blood samples in the field to processing in the laboratory, is usually not less than a few hours, especially so when a large number of samples is to be collected. The extended time between sample collection and processing may probably be one the greatest causes of RNA degradation in the collected samples.

Frequently, many infected blood samples collected in the field have low parasitemia levels. Traditional methods for processing infected blood samples for parasite RNA studies have involved a preliminary lysis of infected red cells using saponin solution (0.1%) to obtain free parasites for analysis. The procedure involves several repeated steps of fractionation and washing before a parasite pellet is obtained. With samples that have a low parasitemia level, this procedure provides the possibility of a significant loss of parasites and loss of RNA stability. Consequently, it is a common observation that many low parasitemia samples subjected to saponin lysis prior to RNA analysis results into little RNA yields, insufficient for gene expression studies (unpublished own laboratory results). The RNA amounts obtained from such samples is usually degraded making it less suited to microarrays and other RNA based analyses. This has been a major draw back of the saponin lysis of infected red cells prior to RNA extraction and analysis.

DNA microarrays, a high throughput technology used to study whole genomes of organisms among its many other applications, is increasingly adopted in many African laboratories. This technique requires intact, undegraded RNA (Ambion technical notes, 2006). Obtaining high quality RNA from *P. falciparum* becomes a necessary requirement prior to microarrays analysis. Optimal methods for blood sample storage before analysis and optimal sample storage durations are needed in order to obtain high quality RNA for DNA microarrays. This is particularly important under a typical African setting where the infrastructure and tropical climate are limiting.

The need for high quality RNA for DNA microarrays has called for a study to optimize methods for use in *P. falciparum* RNA extraction from infected whole blood samples. This study was therefore designed to optimize a protocol for sample storage and cell preparation that can use whole blood samples, without lysing, to isolate parasite RNA for use in DNA microarrays using the laboratory *P. falciparum* strain 3D7 as a surrogate of field isolates. The time for sample storage (0 versus 56 days), storage reagents (RNAwiz and RNAlater) and parasite preparation (saponin lysis versus whole blood) were thus compared in terms of RNA yield and quality.

MATERIALS AND METHODS

Samples

This study used 3D7 laboratory strain parasites kindly provided by Research Laboratory in Muheza Designated District Hospital (DDH), Tanzania. Estimation of the level of parasitemia was based on microscopy of thin and thick smears on glass microscopic slides. Parasitemia was calculated as the average percentage of infected red blood cells on duplicate slides at 10 different microscopic fields in each slide, and cross examined by 3 independent microscopists. On average 400ul all of samples adjusted to a parasitemia level of 4% were used.

Saponin lysis procedure

Eight 3D7 blood sample pairs (16 samples), 300 µL each, were used for this experiment. Four sample pairs (8 samples) were subjected to saponin lysis as described below. The 8 samples were briefly centrifuged to obtain a red blood cell pellet at the bottom of an RNase free micro centrifuge tube. The supernatant was aspirated using an RNase free pipette and tip. Red cell pellets from each tube were transferred to respectively labeled 15 mL falcon tubes followed by the addition of 2 volumes of the 0.2% saponin solution to each falcon tube. The mixture was pipetted up and down 5 - 10 times for thorough mixing. The tubes were left to stand for 2 min. Samples were resuspended by addition of 10 mL of RNase free phosphate buffered saline (PBS, Dulbecco, USA) to each tube. The resuspended samples were transferred to 50 mL falcon tubes containing 10 mL of RNase free PBS and centrifuged at 10, 000 rpm for 10 min. The tiny dark pellet was transferred to an RNase free 1.5 mL micro centrifuge tube and stored at minus 80°C.

Whole blood processing and storage

Eight of the blood samples (not subjected to saponin lysis) were processed as follows: 300 µL of blood was placed in 1.5 mL microcentrifuge tubes. The tubes were centrifuged at 2800 rpm for 3 min, and the supernatant containing plasma was sucked off with subsequent washing of samples in 500 µL of 1x PBS 3 times. Then to each tube containing infected red blood cells, 500 µL of either RNAwiz or RNAlater were added. The mixture was homogenized by vortexing for 20 – 30 s and left to stand at room temperature for 10 min, and then stored at -80°C overnight.

Sample storage reagent and duration

Two reagents for storage of blood samples intended for parasite RNA extraction, RNAwiz® and RNAlater™ (both from Ambion, Inc., Austin, Texas, USA), were compared for their suitability as storage reagents for blood samples intended for RNA extraction in terms of yield and quality RNA obtained from such samples. Therefore, four of the saponin-lysed cells were mixed with 500 µL of RNAlater and the other 4 samples mixed with the same volume of RNAwiz. All samples were gently mixed by vortexing to obtain a homogenous sample-reagent mixture and kept at -80°C. RNA in samples stored in RNAlater was extracted on the same day (day 0) in two of the samples and after 56 days (8 weeks) from the other 2 RNAlater stored samples. The remaining 4 saponin lysed samples were directly mixed with 500 µL RNAwiz, and processed in the same procedure as described above for samples stored in RNA later. The 8 infected, un-lysed blood samples were either stored in 500 µL RNAwiz or RNA later as described for the saponin lysed samples above. The time points for RNA extraction were the same as described above for the lysed samples.

Table 1. Distribution of experimental treatments.

Reagent	Duration (days)	Saponin (n = 8)	Whole (n = 8)
RNAlater	0	2	2
RNAlater	56	2	2
RNAwiz	0	2	2
RNAwiz	56	2	2

Table 2. RNA yields from 3D7 samples stored for different durations, storage reagents and cell preparation methods.

Storage reagent	Duration (days)	Cell preparation method (n = 2)	UV spec quality OD (260/280)	ug/ul*
RNAlater	0	Saponin	1.62	1.02
		Whole	1.40	0.77
RNAlater	56	Saponin	1.54	0.87
		Whole	1.02	0.70
RNAwiz	0	Saponin	1.7	1.55
		Whole	1.85	1.69
RNAwiz	56	Saponin	1.5	1.25
		Whole	1.73	1.35

*= (A260/0.025) x 0.1.

Parasite RNA extraction from whole blood samples

For both cell preparation methods, a standard single step RNA extraction protocol using RNAwiz followed by ethanol precipitation was used as per manufacturer's instructions. Samples were thawed at room temperature for 8 - 10 min. To each sample 200 μ l of chloroform were added. Tubes were inverted up and down for 15 - 20 s at room temperature for 8 min to obtain a homogenous mixture. The tubes were centrifuged at 81.5 X g for 15 min at 4°C. The upper most aqueous layer was carefully transferred into new, individually wrapped and respectively labeled RNAse free 1.5 ml microcentrifuge tubes. To each tube, 2 volumes of 100% ethanol were added, followed by 0.2 volumes of 10.5 M NH₄OAc and 2 μ L of 5 mg/mL glycogen. RNA was precipitated by placing the tubes at -20°C for 2 h followed by centrifuging the tubes at 81.5 X g for 10 min at 4°C. RNA was seen as a whitish gelly material at the bottom at the tube. The pellet was washed 2 times using 1 mL of 100% ethanol, each time centrifuging the tubes at 7000 rpm for 5 min at 4°C and dumping supernatant. Pellets were air dried for 5 min and resuspended in 20 - 30 μ L of RNAase free water.

RNA quality and quantity check

The quality of RNA extracted from the experimental samples, stored and prepared differently for RNA extraction, was checked using the ultra fine Agilent bioanalyser 2000® and UV spectrometry. The quantity of the RNA was checked using UV spectrometry. Optical densities (OD) readings of extracted RNA were taken at 260 and 280 nm wavelengths. The reading at 260 nm allowed for estimate calculations of RNA concentration in the samples using the following formula: RNA conc. (μ g/mL) = OD₂₆₀ x 40 x dilution factor. Where OD₂₆₀ = Optical density at 260 nm, whereas the factor '40' in the formula is based on that an OD of 1 corresponds to ~ 40 μ g/mL for single stranded RNA. The ratios OD₂₆₀/OD₂₈₀ gave an estimate of the RNA purity (Table 1).

RESULTS AND DISCUSSION

Duration of storage of samples

The RNA yields among samples stored for different durations (day 0 versus 56 days) prior to RNA extraction was compared among samples stored in the same storage reagent and cell preparation. Results in Table 2 indicate that, for each storage reagent, higher RNA yields were obtained from samples processed immediately (day 0) as compared to RNA yields at 56 days. Results in Table 2 show that 1.2 versus 0.7 μ g/ μ L of RNA were obtained in saponin-lysed, RNAlater stored samples at days 0 and 56 respectively, whereas 0.77 vs 0.7 μ g/ μ L of RNA were obtained from whole blood, RNAlater stored samples at days 0 and 56, respectively.

A similar trend in RNA yields was observed with samples stored in RNAwiz at days 0 and 56. Thus, RNA yields from saponin-lysed, RNAwiz stored samples at days 0 and 56 were 1.55 and 1.25 μ g/ μ L respectively. When RNA was extracted from whole blood samples stored in RNAwiz, it was observed that 1.69 and 1.35 μ g/ μ L were obtained at days 0 and 56 respectively. This indicates that the yields of parasite RNA obtained from samples at day 0 were consistently higher than those obtained at day 56. In addition, the results also indicate higher yields from samples stored in RNAwiz as compared to RNAlater.

When the yields from samples stored in RNAlater were compared to those stored in RNAwiz, it was generally observed that, for extraction of *Plasmodium falciparum*

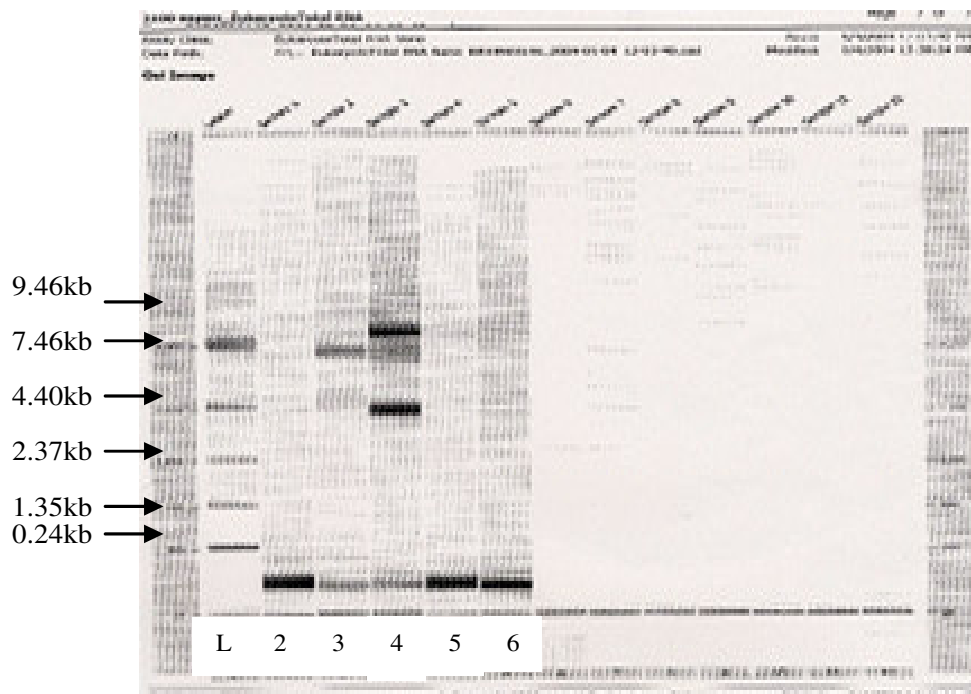


Figure 1. An Agilent Bioanalyzer® picture showing RNA fragments from saponin lysed and whole blood. L = RNA ladder with 6 synthetic poly (A) tailed RNAs ranging from 0.24-9.5Kb (in size). In a top-bottom order, fragments are 9.49, 7.46, 4.40, 2.37, 1.35, and 0.24. Lane 2 = RNA from whole blood in RNAlater, at day 0; Lanes 3 and 4 = RNA from whole blood, stored in RNAwiz at day 0 and 56 respectively; Lanes 5 and 6 = RNA from whole and Saponin lysed blood in RNAlater, at day 56.

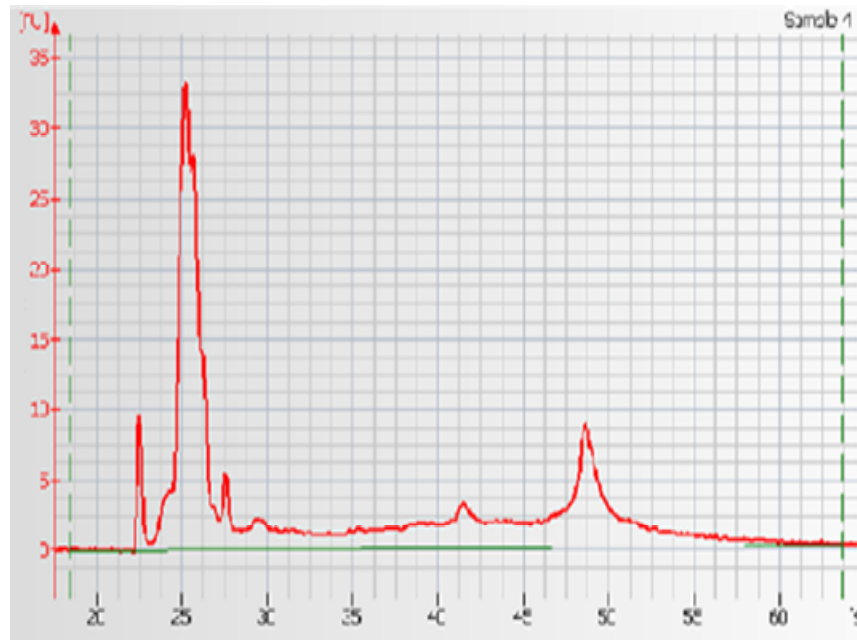
RNA in infected blood samples, RNAwiz storage provides a marginally higher RNA yield compared to RNAlater storage. As regards to whether infected blood samples should be saponin lysed before RNA extraction or not, results from this study indicate that slightly higher RNA yields are obtained from whole blood when such whole blood samples are stored in RNA wiz, but not in RNA later.

Our study shows that RNAlater stabilizes RNA in pure parasite pellets better than washed red blood cells. RNAlater is an aqueous tissue storage reagent that rapidly permeates tissues to stabilize and protect cellular RNA. RNAlater has been extensively tested on tissues such as brain, heart, kidney, spleen, liver, testis, skeletal muscle, fat, lung and thymus (RNAlater Handbook, 2006). RNAlater is also effective for *E. coli*, *Drosophila*, tissue culture cells, white blood cells, and some plants. RNAlater has not been widely used for preserving RNA in whole blood, plasma, or sera. Because of their high protein content, these fluids are likely to form an insoluble the Bioanalyzer 2000®, results show an overall trend that in any case, samples processed immediately on collection gave RNA of superior quality. Results for RNA quality from some of the samples are presented in Figure 1. In this figure, it is shown that RNA from washed red cells, stored in RNAwiz at day 0 (lane 3) and at day 56 (lane 4) were superior in quality than the other tested

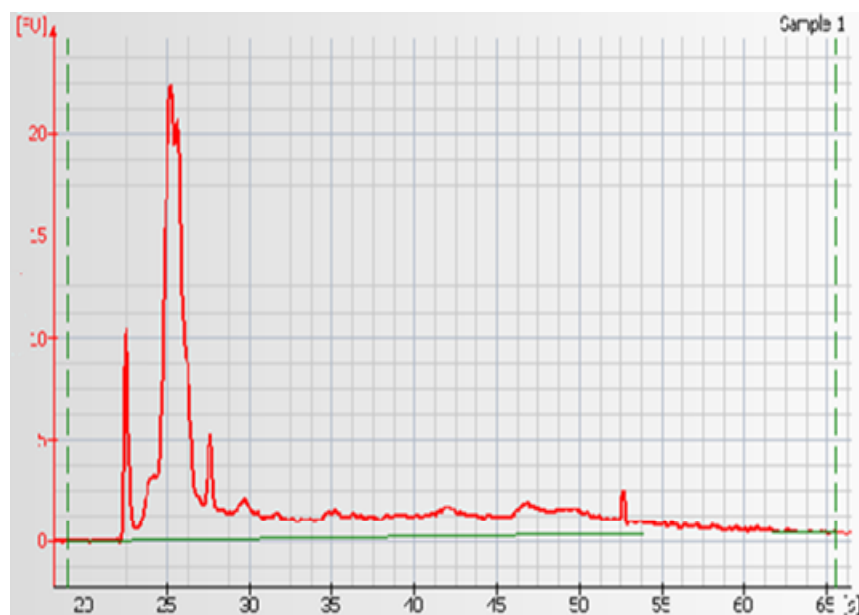
precipitate if they are mixed with RNAlater (RNAlater Handbook, 2006). However, the effectiveness of this reagent in RNA stabilization in washed red blood cells and pure parasite pellets from saponin lysis has not been tested. The results from this study provide the evidence that RNAlater can effectively be used for storage and stabilization of RNA in washed red blood cells as well as in pure parasite pellets. The slight reduction in the yield of RNA in washed red blood cells may be a result of proteins in the red cells themselves and also plasma proteins that may not be completely removed during red cell washing with PBS.

RNAwiz is a reagent used for extraction of nucleic acids from different homogenized tissues. Its ability to stabilize RNA in samples has not been previously determined. Our results show RNAwiz to be a better RNA stabilizer in both washed red cells and in parasite pellets with marginally higher RNA yields in whole washed red cells.

When the samples were analyzed for their quality using samples with RNA extracted at day 0 being of a slightly better quality than that extracted at day 56. When the samples were subjected to further quality analysis it was clearly shown that samples stored in RNAlater had a variable degree of degradation as shown in Figure 3. Figure 2 show Agilent Bioanalyzer 2000 ® runs of RNA from whole blood, stored in RNAwiz at day 0 (Figure 2A)



A



B

Figure 2. Agilent Bioanalyzer 2000® plots showing RNA quality from the same parasite sample extracted from whole blood at day 0 (A) and day 56 (B), both stored in RNAwiz.

and at day 56 (Figure 2B). Results in Figure 2 consistently agree to that shown in Figure 1 in that storage of washed red cells in RNAwiz give intact RNA with insignificant differences in their integrity over different time points of extraction. Hybridization in a DNA microarrays experiment of RNA from washed red blood cells in RNAwiz extracted at day 56 provided scatter graphs

shown in Figures 4A (un-normalized) and B (normalized) indicating the suitability of the RNA obtained from the samples for DNA microarrays and other techniques that require high quality RNA samples.

From this study, we conclude and recommend RNAwiz to be a suitable reagent that can be used not only for extraction of RNA from washed red cell samples but also

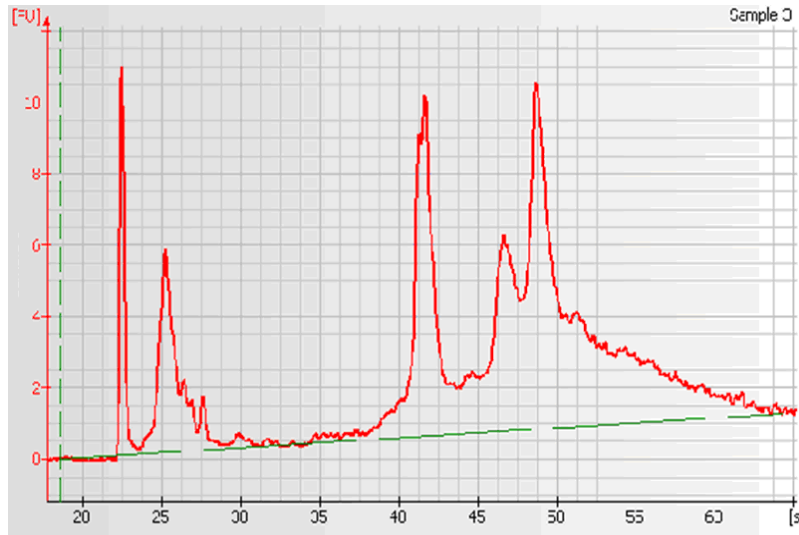


Figure 3. An Agilent Bioanalyzer 2000® plot showing the quality of RNA extracted from whole infected blood, stored in RNAlater at day 0.

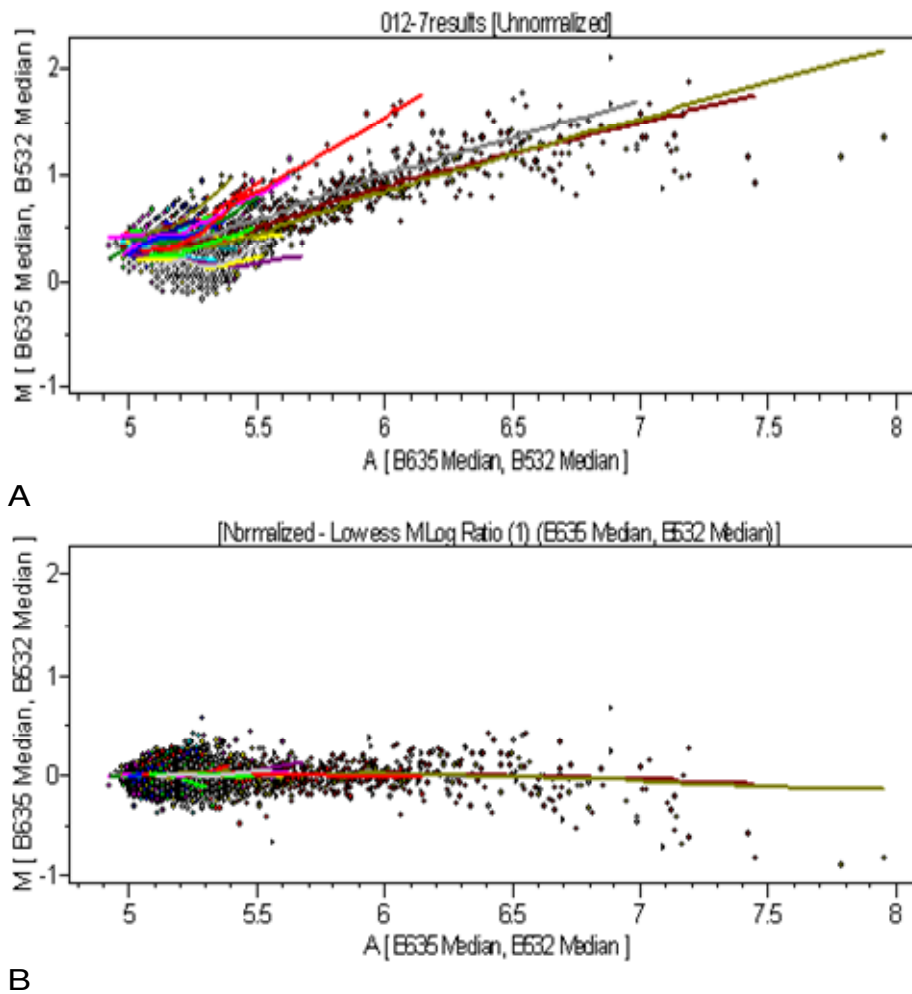


Figure 4. Un-normalized (A) and Lowess-normalized (B) scatter graphs for DNA microarrays hybridized using RNA samples obtained from washed red blood cells stores in RNAwiz and extracted at day 56 of storage.

for storage of such samples prior to RNA extraction. The RNA obtained from RNAwiz stored red blood cells is of high quality and is suitable for sensitive downstream techniques such as DNA microarrays. This provides the opportunity for working with small amounts of blood samples usually collected in the field to obtain RNA for high throughput techniques.

ACKNOWLEDGEMENTS

The authors acknowledge the support of the MOMS Clinical Research Laboratories at Muheza Designated District Hospital, Muheza Tanzania for providing space for this work. The Seattle Biomedical Research Institute (Seattle Washington) is acknowledged for providing financial and technical assistance.

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