

Evaluation of the analytical performance of the novel NS-Prime system and examination of temperature stability of fecal transferrin compared with fecal hemoglobin as biomarkers in a colon cancer screening program



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ABSTRACT

Objective: To examine the analytical aspects of fecal transferrin (Tf) and hemoglobin (Hb) measured on the NS-Prime analyzer for use in a colon cancer screening program.

Designs and methods: Method evaluation and temperature stability studies for fecal Tf and Hb were completed. A method comparison was carried out against the NS-Plus system using samples collected from 254 screening program participants. A further 200 samples were analyzed to help determine suitable reference limits for fecal Tf using these systems.

Results: The assay for fecal Tf showed acceptable linearity, precision, and recovery, and showed minimal carryover with low potential for impact by the prozone effect. The 95th percentile for fecal Tf obtained for the reference population was 4.9 µg/g feces. The collection device sufficiently maintained fecal Tf and Hb stability for at least 7 days at room temperature, 4 °C, and –20 °C. Fecal Tf and Hb were most stable at 4 °C and –20 °C, but showed considerable loss (20–40%) of both proteins at 37 °C within the first 7 days. Mixing small amounts of blood into diluted fecal samples maintained at 37 °C for various time periods showed > 50% loss of both proteins within 1 h of incubation.

Conclusions: The NS-Prime analyzer showed acceptable performance for fecal Tf and Hb. These studies suggest that use of both Tf and Hb together as biomarkers will result in higher positivity rates, but this may not be attributed to greater stability of Tf over Hb in human feces.

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1. Introduction

Colorectal cancer (CRC) is the second leading cause of cancer death in Canadian men, and the fourth leading cause of cancer death in the world [1,2]. Most cases of CRC arise from premalignant adenomatous polyps which release small

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amounts of blood into the stool [3,4]. Colorectal cancer screening by fecal occult blood tests (FOBT) and detection of treatable cancer at early stages can secure favorable outcomes and reduce the mortality rate by as much as 60% [5]. According to the guidelines established by the Canadian Cancer Society (CCS), individuals between 50 and 75 years of age should have an FOBT performed every two years [6–8]. Currently, many FOBT-based CRC screening methods are available, such as the guaiac fecal occult blood test (g-FOBT), and fecal immunochemical test (FIT) for hemoglobin (Hb), making screening more convenient [7–13]. Previous studies have criticized the g-FOBT due to high false positivity results and lack of sensitivity to clinically significant lesions [9,10]. Accordingly, FIT was reported to have superior sensitivity [11–13]. However, the complexity of the fecal matrix due to the degree of hydration, the absorption of proteins into fecal materials, and the ongoing proteolytic degradation and loss of protein antigenicity due to bacteria action, all pose challenges to biomarker measurement in the stool.

Transferrin (Tf), which is mainly responsible for the transport of iron in the blood, has also been identified as a potential biomarker for CRC [14,15]. Bleeding into the gastrointestinal tract releases both Tf and Hb into feces where it can be measured by immunoassay-based tests [15–18]. Furthermore, it has been reported from *in vitro* studies [15], and implied from studies done on CRC screening populations [17], that Hb is more sensitive to degradation by enterobacteria and digestive enzymes than Tf. While the original source of data substantiating the latter claim is not clear, it has been hypothesized that increased time in the bowel due to constipation or bleeding from lesions located further up the bowel in the ascending colon may yield false negative results when Hb is used alone as a screening biomarker. Use of Tf with Hb may improve sensitivity for CRC through the presumed resistance of Tf against degradation [14,16]. Few investigations appear in the literature assessing the relative stability of blood proteins in the fecal matrix, but there are some that show superior stability of Tf over Hb against loss of antigenicity as determined by immunoassay [15]. Furthermore, the fact that the relative recovery of Hb from feces is only about 5 times higher than that of Tf, while blood concentrations of Hb is approximately 100 fold higher than Tf [16], also suggests greater stability of Tf in the fecal matrix. Recently, combining Tf with Hb as fecal CRC biomarkers had shown to produce higher positivity rates in patients with cancer or premalignant lesions [14,16–18].

This study is an investigation into the suitability of fecal Tf measurements done by an automated immunoassay system for potential use as an alternative or adjunct diagnostic marker to FIT using fecal Hb in a CRC screening program. Other previous studies demonstrated that Hemo Techt NS-Plus (Alfresa Pharma) automated system showed acceptable performance characteristics [19,20]. Consequently, the NS-Plus system was used in this study for fecal Tf and Hb evaluation and for comparison purposes. Hemo Techt NS-Prime (Alfresa Pharma) automated system is a new system with several enhancements over its predecessor, the NS-Plus system. These enhancements improve convenience for workflow, including increased patient throughput and extended on-board reagent storage, compared to its predecessor. The purpose of this study is to examine the analytical performance of this system using laboratory manipulated and community-collected fecal samples in order to confirm suitability of this system for CRC screening using Hb as a primary biomarker, and Tf as an investigational biomarker.

2. Material and methods

2.1. Preparation of materials

Stock solutions of 100 g/L Hb as hemolysate were prepared by exhaustive washing of red blood cells with normal saline solution, followed by lysis of the cells with water and freezing at -70°C . Stock solutions of 3 g/L Tf were prepared in a serum pool. The concentration of Tf in the serum pool was measured by immunoturbidometry on an Architect c16000 (Abbott Diagnostics) clinical chemistry system. Complete stool samples were provided by healthy adult volunteers (from 24 to 50 years of age), and were manually mixed and stored frozen in weighed aliquots of approximately 20 g each for further study.

For studies requiring Tf and Hb in the fecal samples, 1,500 μL of normal saline solution containing known amounts of dilute Tf or Hb solution was added to aliquot samples of approximately 20 g of stool to give the desired target concentrations. Typically, the 20 g fecal samples were spiked with sufficient hemolysate to produce Hb concentrations of between 20 and 60 $\mu\text{g/g}$ feces, and sufficient serum to produce Tf concentration of between 10 and 30 $\mu\text{g/g}$ feces. These samples were maintained at 4°C during preparation in order to limit protein degradation during experiment set up. Following this, the fecal samples were manually mixed for 30 sec on ice prior to further sampling into the FIT collection devices, which were provided by the manufacturer and required by the testing systems. Samples measuring approximately 10 μg were aliquoted into separate FIT collection vials, using the manufacturer provided sampling probe, and allowed to equilibrate for at least 48 h at 4°C . This insured complete dispersal into the collection vial stabilizing solution. The collection vials were then sent to a central laboratory and tested for Tf and Hb by the Hemo Techt NS-Plus and NS-Prime analyzer systems.

Assay imprecision was determined using quality control materials (IFOBT NS-Prime controls) and human feces samples spiked with hemolysate and serum. Precision data was collected on 21 different days over a 4-week period and covered 3 different calibrations. Recovery studies were conducted by spiking fecal samples with small volumes of hemolysate and human serum with known amounts of Hb and Tf, respectively. The recovery of Tf and Hb from each individual sample was measured and compared to predicted concentrations. Carryover was determined by using a sample with sufficient

hemolysate and human serum added to bring Hb concentrations to approximately 200,000 $\mu\text{g/g}$ feces and Tf concentrations to 6,000 $\mu\text{g/g}$ feces. This sample was then analyzed in a sequence proceeded by a sample with low Hb and Tf (~ 2 μg Hb/g feces; ~ 1 μg Tf/g feces) in the instrument sample rack. Linearity was examined by quintuplicate measurements of samples with added hemolysate and serum to give the equivalent of about 2–200 μg Hb/g feces (10–1000 $\mu\text{g/L}$ Hb) and about 1–100 μg Tf/g feces (5–500 $\mu\text{g/L}$ Tf). The limit of blank was determined as the mean value plus 2 standard deviations of 10 measurements from unused FIT collection vials containing sample stabilizing solutions. Analytical specificity for Tf and Hb was determined by mixing bovine and porcine Hb (at 10 mg/mL; or equivalent of up to 20,000 μg Hb/g feces) or plasma from these species (100 $\mu\text{L/mL}$ saline; or equivalent of up to 200 μL plasma/g feces) with feces samples. The spiked amount of both Hb and plasma added to the fecal sample were varied from 0 to 20 μL . The impact of the prozone effect was determined by adding different amounts of hemolysate or serum giving final concentrations of up to 6000 $\mu\text{g/g}$ feces for Tf and 200,000 $\mu\text{g/g}$ feces for Hb. All samples were analyzed on the NS-Prime analyzer system.

The temperature stability of samples collected in the FIT collection vials was examined using samples from 3 different volunteers for each time interval (0, 2, 4, 7, 14, and 30 days) and temperature (~ 23 $^{\circ}\text{C}$, 37 $^{\circ}\text{C}$, 4 $^{\circ}\text{C}$, and frozen at -20 $^{\circ}\text{C}$) and through up to 3 freeze/thaw cycles using two different concentrations of Tf and Hb, as previously described [19]. The stability of Tf and Hb in the fecal samples at 37 $^{\circ}\text{C}$ was confirmed by three different experimental designs. In the first, Tf and Hb were minimally diluted ($\sim 10\%$) in human feces samples by mixing saline containing hemolysate and serum with approximately 20 g of feces to produce approximately 50 μg Tf/g feces and 100 μg Hb/g feces. These samples were then incubated for up to 48 h at 37 $^{\circ}\text{C}$ with removal of aliquots into FIT collection vials for measurement at select intervals. The stability of Tf and Hb was also determined based on experimental designs previously reported [15]. A second experimental design involved mixing 5 μL of whole blood diluted in 500 μL of saline with a 4.5 g fecal sample, followed by incubation at 37 $^{\circ}\text{C}$, and sampling into FIT collection vials over a 4-h period. In other experimental designs, saline solution was substituted with Tris-buffered saline solution (pH 8) and processed as above, or 5 μL of blood with 4 mL of Tris-buffered saline solution (pH 8) was added to a 1 g fecal sample followed by incubation at 37 $^{\circ}\text{C}$ with sampling into FIT collection vials over a 4-h period. All samples were analyzed on the NS-Prime analyzer system.

FIT samples collected by participants in a CRC screening program were de-identified and used in the method comparison study and for the Tf reference range study. Based on the CRC screening program eligibility criteria, all subjects were from 50 to 74 years of age and were without personal history of CRC, Crohn's disease, colitis, bleeding hemorrhoids, recent history of rectal bleeding, or of a first-degree relative with CRC. The analytical performance of the NS-Prime system was assessed by comparing results from 254 samples covering the analytical range and measured using the in-house method (Hemo Tech NS-Plus). These samples were archived after their original analysis and stored frozen at -20 $^{\circ}\text{C}$. Samples were then thawed and re-measured on the same day by both systems. Fecal Tf and Hb levels were also determined in a separate set of 200 adult CRC screening program participants (all 50–74 years of age) to determine levels of Tf in the screening population. Only one sample per participant was analyzed for both studies. All components of these studies were reviewed and approved by the local ethics review board.

3. Results

3.1. Precision

Precision of the NS-Prime analyzer and assays for fecal Tf and Hb were determined using vendor-provided quality control materials and minimally diluted mixed fecal samples. The within-run imprecision for Hb and Tf was higher when spiked human feces samples (6.1% and 14.1% at 36.8 and 75.4 μg Hb/g feces, respectively; 6.4% and 8.3% at 29.4 and 64.8 μg Tf/g feces, respectively) were used compared to when manufacturer-prepared quality control materials ($< 1\%$ in all cases for 193 and 90 $\mu\text{g/L}$ Hb, and 145 and 47 $\mu\text{g/L}$ Tf) were used, but in all cases was less than 15% (Supplementary Table 1). The between-run imprecision for Tf and Hb was between 5% and 10% over 21 days using the manufacturer provided quality control materials and, for Hb, similar to that previously reported by us using the NS-Plus analyzer system [19].

3.2. Linearity and limit of detection studies

Linearity for fecal Tf and Hb measurements on the NS-Prime analyzer was examined by quintuplicate measurements of diluted calibrator solutions to give solutions with approximately 5–50 $\mu\text{g/g}$ feces (or 25–250 $\mu\text{g/L}$) for Tf, and 25–250 $\mu\text{g/g}$ feces (or 125–1250 $\mu\text{g/L}$) for Hb. The recovery ranged from 92.1 to 117.4% for Hb using hemolysate, and 99.1–119.8% for Tf using human serum (Supplementary Table 2). The limit of blank was 0.2 μg Tf/g feces and 0.1 μg Hb/g feces. A sample containing 1 μg Hb/g feces showed a %CV of 13%, and a sample containing 0.6 μg Tf/g feces showed a %CV of 12%. Specificity of the assay for human Hb and Tf was determined by challenging the method with very high concentrations of bovine and porcine Hb, and using bovine and porcine plasma as a source of Tf. Bovine Hb and plasma added to the collection vial gave results ranging from 1 to 19 $\mu\text{g/g}$ feces for Hb, and 0.4–0.8 $\mu\text{g/g}$ feces for Tf. Porcine Hb and plasma gave results ranging from 1 to 6 $\mu\text{g/g}$ feces for Hb, and 0.4 to 2 $\mu\text{g/g}$ feces for Tf.

3.3. Prozone effect

Assessment for the prozone effect was carried out by adding increasing volumes of hemolysate or plasma directly to the FIT collection vials. On analysis of Hb, all samples showed decreasing results that were still above the upper limit of the analytical measurement range of the assay. Similar manipulation with Tf gave results above the analytical measurement range for most concentrations, but use of 20 μL of serum with 3 g/L Tf gave a result of 281 ng/mL (which is equivalent to 56 $\mu\text{g/g}$ feces). These studies using unrealistically high levels of blood proteins suggest that the prozone effect is not a likely cause for false negative results using typical diagnostic cutoffs for Tf or Hb. There was no evidence of significant carryover ($\leq 0.01\%$) based on measurement of blank samples following very high concentration samples with the highest concentrations in the blank samples amounting to less than 2 μg Hb/g feces or less than 1 μg Tf/g feces.

Comparison of performance of the NS-Prime system to the NS-Plus system for Tf and Hb was determined using a variety of samples collected from CRC screening eligible subjects (Supplementary Fig. 1). The correlation coefficient was 0.9986 and 0.8874 for Tf and Hb, respectively. Disagreement between Hb values showed greatest variability at high concentrations,

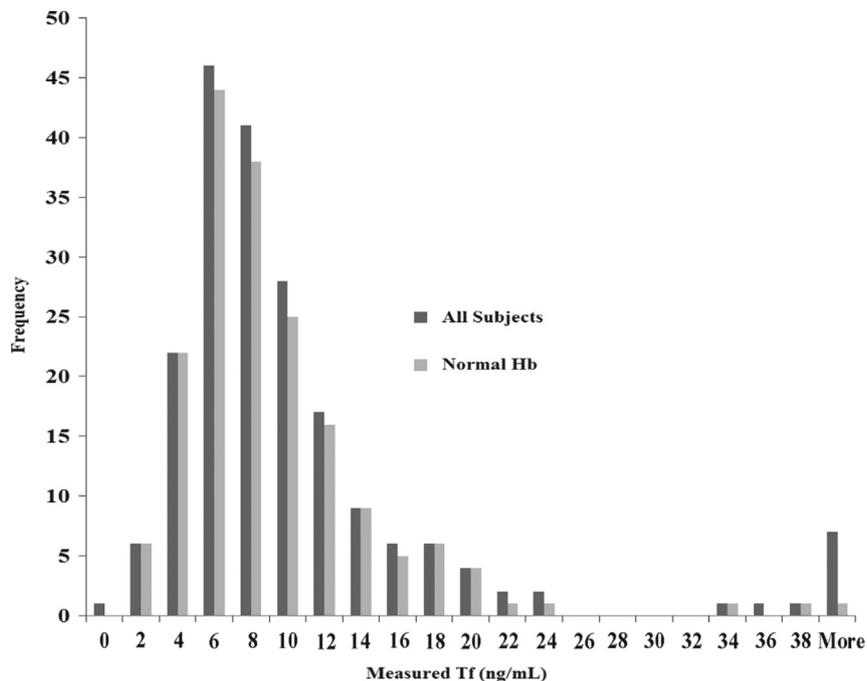


Fig. 1. Distribution of Tf levels among an adult population eligible for CRC screening using the NS-Prime analyzer system. Open bars represent values for all subjects screened ($n=200$). Closed bars are for data after excluding cases with Hb greater than 20 $\mu\text{g/g}$ feces ($n=180$).

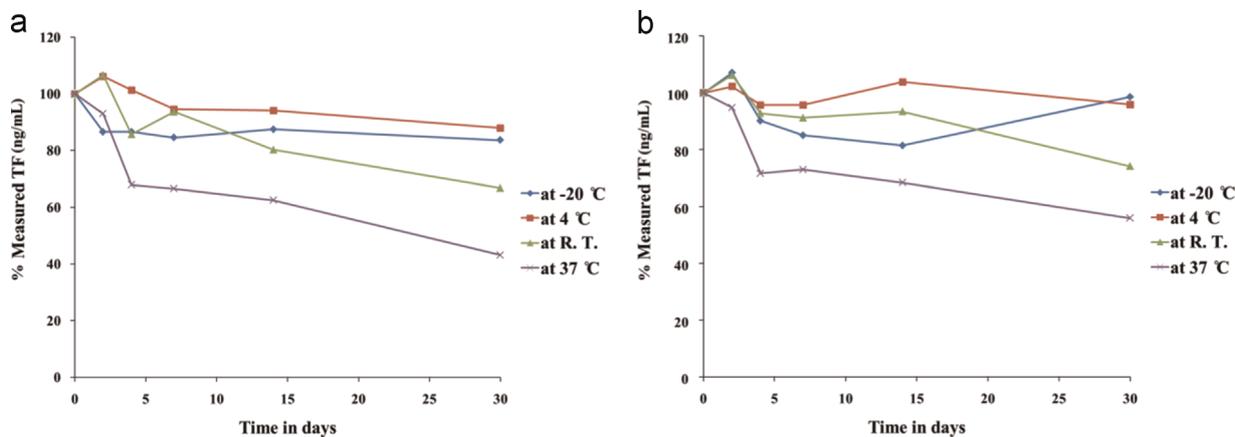


Fig. 2. Temperature stability of Tf maintained in FIT collection vials at different timed intervals. Two different concentrations of Tf were examined: (a) 12.5 μg Tf/g feces, and (b) 25 μg Tf/g feces.

beyond the analytical measurement range of the assay, and in samples requiring dilution.

3.4. Reference limits

Samples from 200 consecutive subjects from a CRC screening eligible population were used to determine Hb and Tf on the NS-Plus and NS-Prime analyzer systems. Subjects ranged in age from 50 to 74 years (62.0 ± 6.3 years) and consisted of 106 females and 94 males. The 99th, 95th, and 90th percentile estimates for Tf results for the complete population were 29.3 $\mu\text{g/g}$ feces, 4.9 $\mu\text{g/g}$ feces, and 3.4 $\mu\text{g/g}$ feces, respectively, on the NS-Prime system and similar to that obtained using the NS-Plus system. About 180 subjects showed normal Hb (less than 20 $\mu\text{g/g}$ feces) on the NS-Prime system. The 99th, 95th, and 90th percentile estimates for Tf in patients with normal fecal Hb were 6.8, 3.6, and 3.0 $\mu\text{g/g}$ feces, respectively. Histograms showing Tf data for the NS-Prime system, before and after excluding samples with high Hb, is shown in Fig. 1. The 99th, 95th, and 90th percentile estimates for fecal Hb in this same population were 533.2, 51.0, and 16.1 $\mu\text{g/g}$ feces, respectively, on the NS-Prime system.

3.5. Temperature stability

In this study, the effect of temperature on Tf and Hb in FIT collection vials was further investigated by analyzing samples on the NS-Prime system at time intervals of 0, 2, 4, 7, 14 and 30 days at two different concentrations (Figs. 2 and 3). Non-homogeneity of samples, in spite of manual mixing, is a significant factor contributing to variability in results in studies like

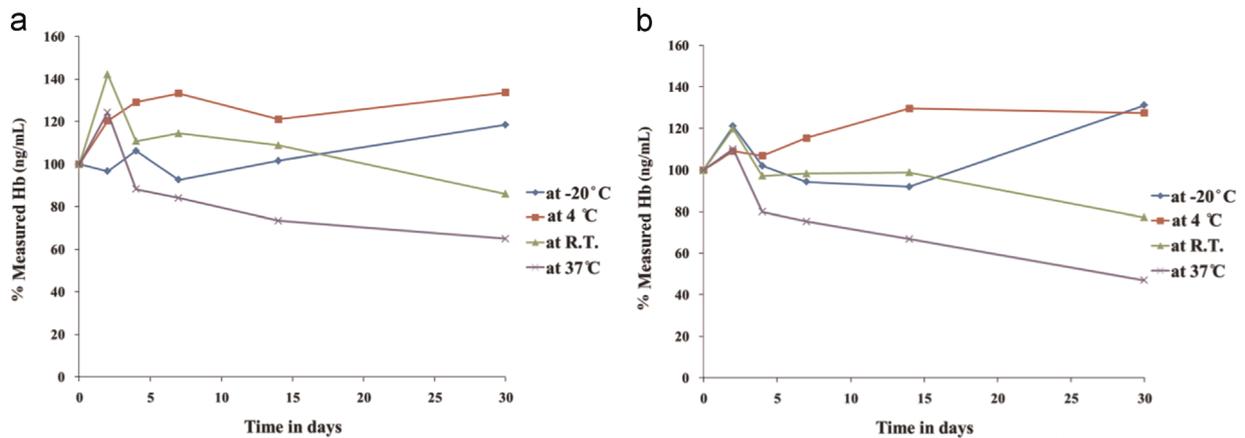


Fig. 3. Temperature stability in Hb maintained in FIT collection vials at different timed intervals. Two different concentrations of Hb were examined: (a) 25 μg Hb/g feces, and (b) 50 μg Hb/g feces.

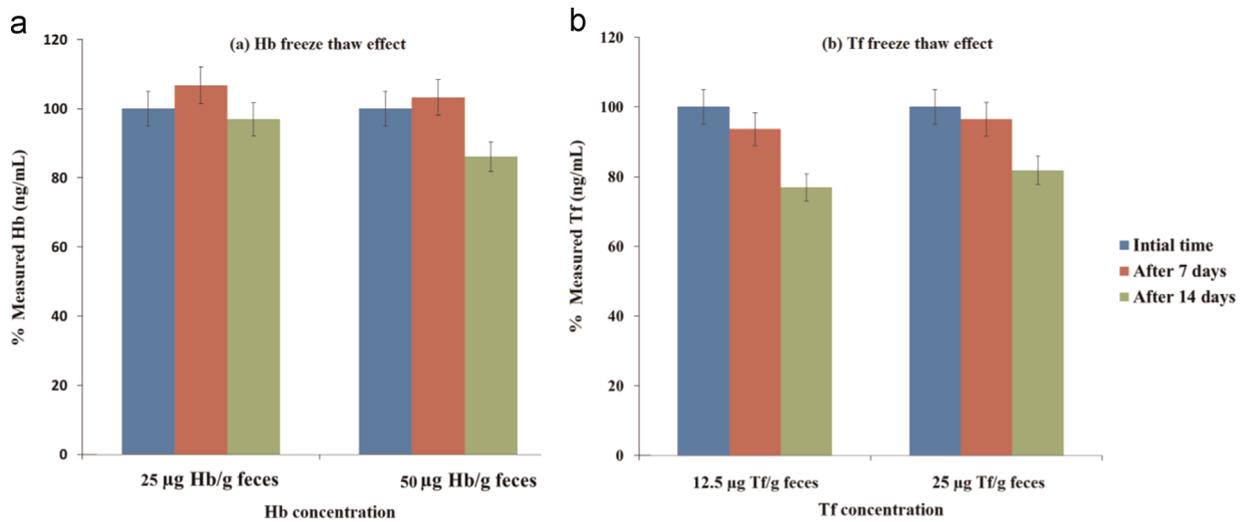


Fig. 4. Effect of freezing and thawing on the levels of Hb and Tf maintained in FIT collection vials. Graphs show (a) freeze–thaw effect on Hb level and (b) freeze–thaw effect on Tf level.

this one. However, considering this, the collection device maintained acceptable fecal Tf and Hb stability for at least 7 days at 37 °C, room temperature (~23 °C), 4 °C, and –20 °C. After 7 days of incubation, the effect of temperature became more significant. Both Tf and Hb were maintained within acceptable stability limits when stored at –20 °C and 4 °C over the entire 30-day time course, however, increasing the temperature to 23 °C resulted in more degradation in Tf and Hb and loss of antigenicity. At 37 °C, the Tf and Hb values were significantly lower after 7 days (Figs. 2 and 3). Fig. 4 shows the effect of freeze/thaw cycles on Tf and Hb. While Tf was minimally affected by a single freeze/thaw cycle, substantial loss of antigenicity occurs for Tf during subsequent freeze/thaw cycles (Fig. 4b). However, consistent with previous findings using the NS-Plus system [19,20], Hb was minimally affected by 2 freeze/thaw cycles, but these present results suggest more substantive loss of antigenicity through repeated freezing and thawing beyond this (Fig. 4a).

This study attempted to closely simulate fecal consistency and constituents to investigate the stability of Hb and Tf in feces at 37 °C in an effort to simulate potential for degradation of proteins and loss of antigenicity in the fecal matrix prior to delivery of the sample and collection for screening purposes (Fig. 5). The results of these investigations show significant loss of antigenicity of both Hb and Tf within hours after mixing and maintaining these minimally diluted samples at 37 °C.

4. Discussion

The NS-Prime analyzer system represents a new system which provides automated analysis of fecal proteins in samples preserved in special collection vials. Measurement of fecal Hb represents the standard biomarker for CRC screening in individuals with population risk for the disease. A number of other potential feces-derived biomarkers for CRC have also been explored with fecal Tf receiving the most attention as a potential alternative or adjunct to Hb. This study first evaluated the analytical performance of the NS-Prime system for measurement of fecal Hb and Tf. This study showed higher imprecision for spiked human feces samples, possibly reflecting the lack of homogeneity of the manually mixed and minimally diluted samples, and variability due to sampling with the kit sampling probe. In connection with this, preliminary studies using manual mixing of undiluted samples showed even greater variability (results not shown), hence, dilution of samples by about 10% was required to bring within-run sampling imprecision to less than 15% at concentrations of Tf and Hb used in these studies. Nevertheless, both within-run and between-run imprecision was determined to be acceptable. Furthermore, linearity and limit of detection results suggest analytical sensitivity and performance at lower concentrations was sufficient for both proteins at levels found in healthy individuals. The levels of Tf and Hb from bovine and porcine sources used in the specificity studies were in great excess of that which would be expected to contaminate stool *in vivo*, so it has also been concluded that interference by undigested dietary protein from these animal species is unlikely to pose a significant problem for these assays. Studies for carryover and prozone effect were also completed, and the results suggested that the NS-Prime system is robust enough to prevent misclassification of patient sample results. Additionally, comparison of performance of the NS-Prime system to the NS-Plus system (an analyzer already in use by many colon cancer screening programs for measurement of fecal Hb) showed that the two systems give similar estimates of both Tf and Hb. The temperature stability of Hb in the FIT collection device using the NS-Plus system had been previously examined and those results suggested acceptability for use in a population-based screening program involving samples sent in through the postal system [19,20]. This study shows that the temperature stability of fecal Tf is similar to that of fecal Hb once collected into the

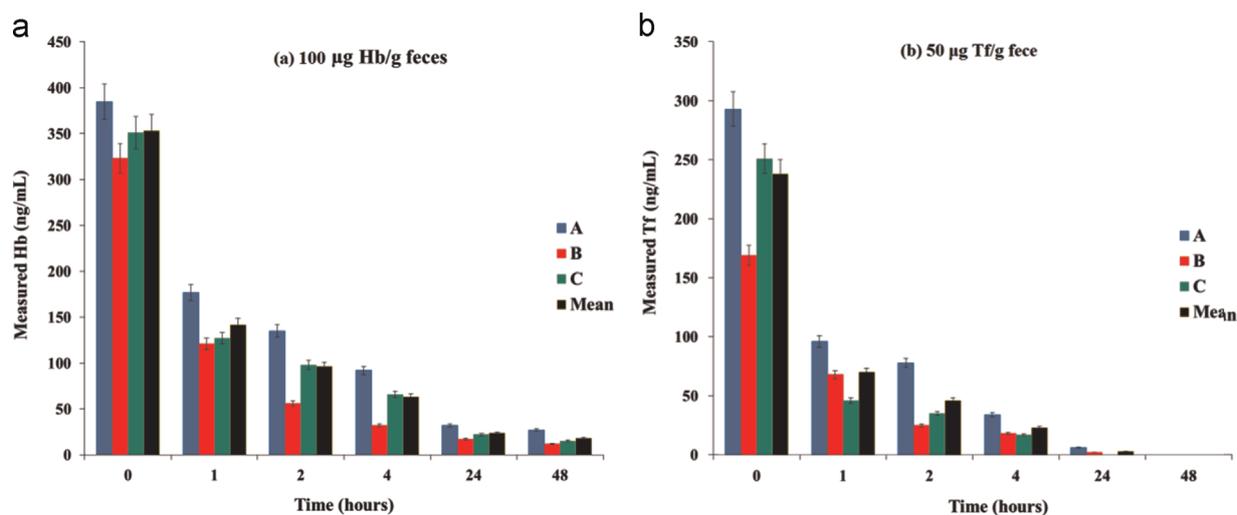


Fig. 5. Stability of Hb and Tf in minimally diluted fresh fecal samples maintained at 37 °C. Approximately 20 g fecal samples were diluted by adding Hb and Tf in 1.5 mL of saline on ice. Samples were then incubated at 37 °C for up to 48 h and sampled into FIT collection vials at indicated intervals. A, B and C represent different fecal samples collected from three volunteers.

sampling containers and stored. Taken together, these results suggest acceptable analytical performance of the NS-Prime system for use for CRC screening using Hb, and investigational measurement of Tf.

Selecting appropriate screening cutoffs remains a challenge for CRC screening programs using quantitative FIT methodologies, often showing screen-positive rates higher than anticipated using percentile-based cutoffs determined from healthy (and often younger) subjects. For example, our previous work estimated a cutoff of 10 μg Hb/g feces based on 95th percentile estimates in a young healthy population [19], whereas a screening cutoff of 20 μg Hb/g feces applied to an older screening-program-eligible population showed a positivity rate of about 10%. Hence, the screening cutoffs have a significant impact on program positivity rates, down-stream effects on the need for confirmatory diagnostic procedures and the costs associated with these procedures. This study addressed reference intervals making use of a CRC eligible population to give a 5% positivity rate. The results suggest a screening cutoff of 4 or 5 $\mu\text{g}/\text{g}$ feces for Tf would be appropriate based on 95th percentile estimates for an adult population undergoing CRC screening. This cutoff is remarkably close to that recommended by the manufacturer. Accordingly, using a 5 μg Tf/g feces cutoff and a 20 μg Hb/g feces cutoff, Tf identified 3 positive samples (out of 180) not identified by Hb, but the overall positivity rate was higher for Hb (20/200) when compared to Tf (10/200). This is consistent with results from a recent study examining screening populations using quantitative assays for both proteins [16]. Addressing the diagnostic yield with the 5% positivity rate should be addressed as further validation of the legitimacy of 5 μg Tf/g feces as an effective one for identification of clinically significant lesions.

Fecal Tf is claimed to have an advantage over fecal Hb as a biomarker for CRC screening due to its increased stability in feces under physiological conditions. This claim largely draws on the earlier work of Uchida, et al., who used highly diluted feces in a Tris-buffered saline solution with added blood to examine protein stability [15]. Other studies have examined the temperature stability of hemoglobin in feces by measurement of heme-related pseudoperoxidase activity [21] and by FIT methodology [22], but at temperatures of around 20 °C, and showed significant loss of heme or hemoglobin antigenicity within 4 days in the fecal matrix. The temperature stability of both Hb and Tf in human feces was determined at 37 °C in this study in an effort to provide data to confirm the reported greater fecal stability of Tf over Hb, and to support the rationale for using Tf as a complimentary or alternative biomarker to Hb. The results from this study using minimally diluted human fecal samples were qualitatively different from previous results of others [15]. We further used lysed whole blood added to buffered stool samples that were minimally diluted or diluted 5 fold with 100 mM Tris-buffered saline solution (pH 8.0) in an effort to more closely simulate the experimental design of others to examine fecal stability of Hb and Tf (result not shown) [15], all yielding qualitatively similar results to those found using minimally diluted fecal samples. Hence, these studies failed to demonstrate superior antigen epitope stability of Tf over Hb using this assay system, nor did it support an advantage of Tf over Hb as a marker for colonic bleeding. It is acknowledged that fresh fecal samples without *in vitro* manipulation are not homogenous samples and may differ significantly across different individuals based on diet and intestinal flora. Procedures carried out here to make the samples homogenous to examine stability in presence of fecal bacteria were likely to accelerate breakdown of these proteins, compared to the situation *in vivo* where blood is not well mixed with the fecal matrix, and depending on location of a bleeding lesion within the bowel, may be concentrated along the surface of the stool. Nevertheless, there is no evidence based on these studies that Tf has any antigen stability advantage over Hb.

5. Conclusion

This study shows comparable analytical performance of NS-Prime automated analyzer system to that of the NS-Plus system for measurement of Hb and Tf. Furthermore, the reference limits for Tf were determined using a CRC screening-eligible population using both NS-Prime and NS-Plus systems. Our studies examining the stability of Hb and Tf in fecal samples maintained at 37 °C failed to demonstrate superior temperature stability for Tf. Nevertheless, further study examining the diagnostic performance of fecal Hb and Tf against patients followed up with colonoscopy is warranted to determine if Tf offers a diagnostic advantage in CRC screening when used with or instead of Hb.

Conflict of interest

There is no conflict of interest with the company who provided reagents and instruments.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.plabm.2015.07.002>.

References

- [1] Canadian Cancer Society's Advisory Committee on Cancer Statistics. Canadian Cancer Statistics, Toronto, ON, Canadian Cancer Society 2014, from <http://www.cancer.ca/en/cancer-information/cancer-101/canadian-cancer-statistics-publication/?region=on>.
- [2] Cancer Research UK, Worldwide Cancer Mortality Statistics, Retrieved February 02, 2014, from <http://www.cancerresearchuk.org/cancer-info/cancerstats/world/mortality/>.
- [3] G. Lal, S. Gallinger, Familial adenomatous polyposis, *Semin. Surg. Oncol.* 18 (2000) 314–323.
- [4] D. Cunningham, W. Atkin, H.J. Lenz, H.T. Lynch, B. Minsky, B. Nordlinger, et al., Colorectal cancer, *Lancet* 375 (2010) 1030–1047.
- [5] J. He, J.E. Efron, Screening for colorectal cancer, *Adv. Surg.* 45 (2011) 31–44.
- [6] D.J. Leddin, R. Enns, R. Hilsden, V. Plourde, L. Rabeneck, D.C. Sadowski, H. Singh, Canadian Association of Gastroenterology position statement on screening individuals at average risk for developing colorectal cancer, *Can. J. Gastroenterol.* 24 (2010) 705–714.
- [7] J.S. Mandel, T.R. Church, J.H. Bond, F. Ederer, M.S. Geisser, S.J. Mongin, et al., The effect of fecal occult-blood screening on the incidence of colorectal cancer, *N. Engl. J. Med.* 343 (2000) 1603–1607.
- [8] R. Labianca, G.D. Beretta, B. Kildani, L. Milesi, F. Merlin, S. Mosconi, et al., Colon cancer, *Crit. Rev. Oncol. Hematol.* 74 (2010) 106–133.
- [9] B. Levin, D. Brooks, R.A. Smith, A. Stone, Emerging technologies in screening for colorectal cancer: CT colonography, immunochemical fecal occult blood tests, and stool screening using molecular markers, *CA Cancer J. Clin.* 53 (2003) 44–55.
- [10] J.F. Collins, D.A. Lieberman, T.E. Durbin, D.G. Weiss, Accuracy of screening for fecal occult blood on a single stool sample obtained by digital rectal examination: a comparison with recommended sampling practice, *Ann. Intern. Med.* 142 (2005) 81–85.
- [11] J.E. Allison, L.C. Sakoda, T.R. Levin, J.P. Tucker, I.S. Tekawa, T. Cuff, et al., Screening for colorectal neoplasms with new fecal occult blood tests: update on performance characteristics, *J. Natl. Cancer Inst.* 99 (2007) 1462–1470.
- [12] F.A. Oort, J.S. Terhaar Sive Droste, R.W. Van Der Hulst, H.A. Van Heukelem, R.J. Loffeld, I.C. Wesdorp, et al., Colonoscopy-controlled intra-individual comparisons to screen relevant neoplasia: faecal immunochemical test vs. guaiac-based faecal occult blood test, *Aliment. Pharmacol. Ther.* 31 (2010) 432–439.
- [13] A. Parra-Blanco, A.Z. Gimeno-García, E. Quintero, D. Nicolás, S.G. Moreno, A. Jiménez, et al., Diagnostic accuracy of immunochemical versus guaiac faecal occult blood tests for colorectal cancer screening, *J. Gastroenterol.* 45 (2010) 703–712.
- [14] P. Jin, Z.T. Wu, M.M. Meng, X. Wang, X.W. Wang, L.J. Gong, et al., Combined fecal transferrin test and immuno fecal occult blood test for detecting colorectal cancer and advanced adenoma in asymptomatic and symptomatic populations, *J. Cancer Sci. Ther.* 4 (2012) 243–248.
- [15] K. Uchida, R. Matsuse, N. Miyachi, S. Okuda, S. Tomita, H. Miyoshi, et al., Immunochemical detection of human blood in feces, *Clin. Chim. Acta* 189 (1990) 267–274.
- [16] Y. Takashima, T. Shimada, T. Yokozawa, Clinical benefit of measuring both haemoglobin and transferrin concentrations in faeces: demonstration during a large-scale colorectal cancer screening trial in Japan, *Diagnosis* 2 (2015) 53–59.
- [17] J.G. Chen, J. Cai, H.L. Wu, H. Xu, Y.X. Zhang, C. Chen, et al., Colorectal cancer screening: comparison of transferrin and immuno fecal occult blood test, *World J. Gastroenterol.* 18 (2012) 2682–2688.
- [18] J.Q. Sheng, S.R. Li, Z.T. Wu, C.H. Xia, X. Wu, J. Chen, et al., Transferrin dipstick as a potential novel test for colon cancer screening: a comparative study with immuno fecal occult blood test, *Cancer Epidemiol. Biomark.* 18 (2009) 2182–2185.
- [19] E. Randell, M. Kennell, A. Taher, S. Antle, F. Bursey, T. Tavenor, et al., Evaluation of Hemo Tech NS-Plus system for use in a province-wide colorectal cancer screening program, *Clin. Biochem.* 46 (2013) 365–368.
- [20] P. Catomeris, N.N. Baxter, S. Boss, L. Paszat, L. Rabeneck, E. Randell, et al., Su1216 effect of temperature, time and freeze/thaw cycles on the stability of fecal hemoglobin using five commercial fecal immunochemical test (FIT) methods and one Guaiac method. AGA abstracts, *Gastroenterology* 146 (2014) S-404.
- [21] G.P. Young, M.A. Sinatra St, D.J. John, Influence of delay in stool sampling on fecal occult blood test sensitivity, *Clin. Chem.* 42 (7) (1996) 1107–1108.
- [22] L.F. Brown, C.G. Fraser, Effect of delay in sampling on haemoglobin determined by faecal immunochemical tests, *Ann. Clin. Biochem.* 45 (6) (2008) 604–605.