Hematocrit-independent sampling enables white blood cell counts from patterned dried blood spot cards

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Abstract

The accurate and efficient measurement of white blood cell (WBC) counts is vital for monitoring general patient health and can aid in diagnosing a range of possible infections or diseases. Even with their importance universally acknowledged, access to WBC counts is largely limited to those with access to phlebotomists and centralized clinical laboratories, which house the instrumentation that perform the tests. As a result, large populations of people (e.g., those that are home-bound or live in remote locations) lack facile access to testing. Dried blood spot (DBS) cards are often used to bridge these gaps in access to testing by offering the ability to collect blood at home for ambient shipping to laboratories. However, it is well understood that these cards, which are prepared from cellulose cardstocks without further modification, suffer from variabilities in accuracy and precision due to uncontrolled sample spreading and hematocrit effects, which has hindered their use to determine WBC counts. In this manuscript, we present a method to obtain an accurate WBC count using a patterned dried blood spot (pDBS) card, which comprises collection zones that meter volumes of dried blood. We demonstrate that, unlike the gold standard DBS card (Whatman 903), our pDBS design allows for the collection of a reproducible, average volume of blood volume over the range of hematocrits from 25–55%. We then used qPCR to quantify the *18S rRNA* gene to determine WBC counts from the volumes of blood that are metered in pDBS zones. We observe that WBC counts generated from our method are comparable to those measured by a HemoCue point-of-care WBC analyzer. Our approach to using pDBS cards as a blood collection device has the potential to support at-home sampling and other patient populations that need WBC counts but lack access to clinical facilities.

Introduction

White blood cell (WBC) counts are an integral part of routine health screening and can provide a wealth of information regarding overall patient health.[1] Specifically, counts extending above or below the normal range (4.0–11.0 x 10^3 cells/µL) can indicate bacterial or viral infection, autoimmune disorders, or other diseases (e.g., leukemia).[2–7] The standard clinical method to obtain WBC counts uses a hematology analyzer, which requires vacutainers of liquid whole blood as the specimen.[1,8] While point-of-care platforms have been developed to enable WBC counts (e.g., Hemocue WBC analyzer, Abbott CELL-DYN Emerald analyzer), the costs and relative complexity of these devices make them currently unsuitable for use at home or in limited-resource settings.[9,10] Not only does instrumentation restrict WBCs analysis to a laboratory setting, but also the reliance on a trained phlebotomist to draw blood and a cold chain to transport the blood from the collection site to a centralized facility greatly limits access to this critical determinant of patient health. For example, people who are home-bound or living in a rural or resource-limited settings may have underserved medical needs.[11] The absence of access to clinical care, combined with the disproportionally poor health in these groups, highlight the necessity for improved monitoring and access to health information.

Dried blood spot (DBS) cards—such as the Whatman 903 Protein Saver card—offer a simple method for sample collection, storage, and transportation away to centralized facilities for downstream analyte detection and/or quantification.[12–16] To date, DBS cards have been used to measure markers for a diverse range of medical applications including general health, disease diagnostics and monitoring, newborn screening, drug monitoring, and fertility health.[17–19] While DBS cards are well suited for sampling fingerstick samples of whole blood in limited-resource or at-home settings, extraction of intact WBCs, which would be required for use with a hematology analyzer, is challenging due to the dehydration of the sample (i.e., alter cell size or granularity) and subsequent cellular hemolysis (i.e., loss of cells). Consequently, there have been few successful published reports of quantifying WBCs directly from DBS cards. Efforts to date include extracting

WBCs from blood spotted and subsequently dried onto a variety of substrates (e.g., cellulose paper, polyester membrane, and glass slides). Initial work to extract WBCs from cellulose-based DBS cards provided high total recovery of cells (82%) but required a long extraction protocol with complex steps and analysis via microscopy (ca. 9-10 hours), which greatly limited the utility of this method.[20] A more recent report demonstrated recovery of WBCs from cellulose-based cards and polyester membranes using a much shorter extraction time (< 2 hours) but resulted greatly reduced recovery (1% and 52% for cellulose and polyester, respectively) when WBCs were quantified by flow cytometry.[21] While the analyses showed promise, blood was dispensed directly onto pieces of free-standing material instead of a card or device that was intended be handled by a user, which makes performance difficult to predict. An alternative method to quantify WBCs from a dried blood spot prepared on a glass slide, rather than applied to the porous matrix of a paper or membrane, demonstrated the potential to conduct counts of both red blood cells and white blood cells that retain the shape of their nuclei.[22] While these results highlight a possible route to integrate automated imaging with dried specimens, the approach currently requires several steps (e.g., dilution, timing, spotting) that would benefit from automation when operated by a broad user base.

A limitation to these or any other approaches is that a known volume contained in and extracted from the DBS sample is required to obtain the WBC count, which is expressed volumetrically as "cells per microliter" and not an absolute number. Traditional DBS cards are known to suffer from sampling errors due to uncontrolled sample application and spreading leading to variations in composition (e.g., cellular to plasma ratio) across the blood spot and, consequently, variations in the volume of blood stored within a given area of the blood spot.[23] Ensuring precision metering of a dried sample can enable quantitative analyses comparable to a liquid blood reference. We have previously demonstrated a method to improve the reproducibility of dried blood collection through patterned dried blood spot (pDBS) cards.[16] This volume control—in combination with a quantitative

method that does not require intact cells to measure total WBCs in a punch—provides the potential for obtaining WBC/μL values from DBS cards.

Herein, we describe a method to obtain a patient WBC count using dried blood spots, which relies on the precise metering of the blood collected by our pDBS cards. We demonstrate that metering is independent of the sample hematocrit, over the range of 25–55%, with intra- and inter-card sampling precision superior to Whatman 903 DBS cards. We show that quantitative PCR (qPCR) can be used to measure the total number WBC in a punch from a pDBS card and that accurate WBC counts can then be calculated by applying the standardized, hematocrit-independent volume contained in a punch. This quantitative method, which aligns with standard laboratory practices for how DBS cards are handled, improves upon the capabilities of traditional DBS cards, and will allow, for the first time, patients in remote settings to access a key hematological parameter that is typically restricted to a clinical environment.

Results and Discussion

Determining the volume of blood in a paper punch

We modified the geometry of our previous pDBS card design to create shorter, wider channels (**Figure S1A**) (cite Keith). While we expect that the hematocrit from an average, healthy patient will fall within the normal range for adults (ca. 35–49%), we anticipate that sick patients may have hematocrits that extend beyond those ranges. We therefore chose to demonstrate sample filling in our pDBS cards using hematocrits from 25–55% hematocrit (**Figure S1B**). We designed the channels in pDBS cards to extend beyond defined blood collection zones, patterned into cardstock using wax printing,[24,25] into order to ensure complete filling of zones regardless of the hematocrit. The size of each zone supports their complete removal using a standard 6-mm hole punch.

Based on our previous work, we expected that wax patterning DBS cards can enable reproducible sampling such that each zone can be treated as a replicate.[16,26] We chose to quantify the intracard and intercard reproducibility of volumes collected in punches obtained from 903 and pDBS cards. To measure the amount of whole blood in a dried punch, we generated reference curves at each hematocrit to correlate volumes of whole blood (0–20 µL) with absorbances at 540 nm utilizing Drabkin's reagent (**Figure S2A**). These curves enable us to transform measured absorbances into a volume of collected blood using the linear fit at a certain hematocrit, facilitating a more comprehensive understanding of potential hematocrit effect on sample volume. As expected, we found these liquid reference curves to be volume and hematocrit dependent but patient independent (n = 3 unique donor samples). These results confirm that the relationship between sample volume and hematocrit can be applied to any patient sample to determine the volume of blood stored in a paper punch.

We then used this method to quantify the amount of blood collected in punches from 903 and pDBS cards. We first prepared a sample of whole blood from single donor with a contrived hematocrit of 45% and applied 75 µL of the sample to one 903 card (N = five sample application zones per card) and three pDBS cards. We dried all samples at room temperature for 24 hours, then extracted three replicate punches from the 903 card blood spots (15 total punches) or four punches from pDBS cards (12 total punches). We performed a Drabkin's assay on each punch and converted measured absorbances to volumes of whole blood using the linear fits resulting from calibration curves (**Figure S2B**). The volume stored in a punch from a 903 card is 11.0 ± 0.5 µL and 12.8 ± 0.4 µL from a pDBS card. Results demonstrate there is a statistically significant difference in both the intracard and intercard volumes for the 903 card punches ($p = 0.04$ and 0.01, respectively), while there is no significant difference in these volumes for pDBS cards ($p = 0.22$ and 0.62, respectively; **Figure 1**). These experiments provide support to the perception that 903 cards cannot be used in applications requiring the recovery of precise volumes from sub-punches of the main dried blood spot, providing evidence for why 903 cards are not used clinically

to perform WBC counts even though this would fill a major need in testing access. Conversely, these results are evidence that pDBS cards, through superior sampling, could support such an application from dried blood.

Demonstrating that stored volume is hematocrit independent

A possible limitation to our proposed approach would be if a patient's hematocrit must be known to determine the volume contained in a pDBS punch. It would be advantageous for the punch volume to be—in practice—entirely independent of the hematocrit, where the accuracy of a WBC count could be preserved without first correcting for a separate measurement. To evaluate the relationship between hematocrit and punch volume in a pDBS card, we determined the sampling precision and intracard and intercard variability using hematocrits ranging from 25–55% (n = 12 punches per hematocrit). Results demonstrate good precision of dried blood volumes collected by pDBS cards, where coefficients of variation are < 6.2% for all hematocrits. We observed no significant intracard differences in punch volume between the four replicates on a single pDBS card and no significant intercard differences in punch volume between replicates across three pDBS cards ($p > 0.05$; **Figure 2A**). Based on these results, we calculated a hematocrit-independent volume (12.1 µL) as the average volume from all punches across the full hematocrit range (**Figure 2B**). While there is no significant intracard or intercard variability, we conducted a two-factor ANOVA for these conditions and confirmed there was a statistically significant, hematocrit-dependent difference in collected volumes ($p < 0.05$). Nevertheless, we hypothesized that a relatively small difference in volume, even if statistically significant using the standard α of 0.05 as a threshold, would not negatively affect the accuracy of a calculated WBC count as any error caused by slightly under- or over-estimating the punch volume was likely negligible compared to experimental errors introduced during our assay workflow (e.g., extraction, dilution). We aimed to demonstrate this assertion by directly comparing measurements of WBC counts from hematocrit-independent and hematocrit-dependent volumes of blood.

Based on the complications with analyzing WBCs recovered from dry specimens using flow cytometry or microscopy, we chose to use quantitative PCR (qPCR) to measure WBC counts as it does not require intact cells, but rather the genetic material within those cells. The basis of our method is to use qPCR to calculate absolute numbers of WBCs in a punch (via a measured Ct) and then calculate WBC counts using the known volume of blood contained in that punch. We chose the *18S rRNA* gene as our gene of interest because it is found in all WBCs and has been used successfully as a reference gene in molecular applications of DBS cards.[27] **Table S1** depicts results from qPCR experiments with volumes of liquid blood that correspond to hematocrit-dependent and hematocrit-independent (i.e., the 12.1 µL average) volumes. We performed measurements in triplicate to calculate the average Ct and standard error of the mean (SEM) for each hematocrit from 25–55%. We next modeled the ideal relationship between sample volume and Ct on the theoretical basis that doubling the volume (and thus doubling target copies of the target gene) results in a ΔCt of -1. We fit the resulting model by non-linear regression (**Table S2**) using Equation 1.

$$Ct = A*\ln(volume) + B \qquad (eq.\ 1)$$

This model allows us to predict a Ct from any input sample volume and any hematocrit. That is, we could compare theoretical differences in Ct that could result from using the average, hematocrit-independent volume instead of the hematocrit-dependent volume. We compared (i) the standard error in Ct measured from technical triplicates (which reflects the workflow error) to (ii) the difference between the theoretical, hematocrit-independent Ct value and the measured, hematocrit-dependent Ct value (which reflects error introduced by a volume discrepancy) (**Table 1**). This analysis confirmed that the error in Ct value, when using hematocrit-dependent volumes compared to the hematocrit-independent average volume, is less than the overall error of our methods (i.e., the SEM of measured Ct). As a result, we concluded that it is acceptable to use

the average, hematocrit-independent volume of 12.1 µL for quantitative applications of this pDBS card assuming patient samples are in this range of hematocrits that includes and extends beyond what is considered healthy.

Quantification of WBCs from pDBS cards

We first analyzed liquid samples of whole blood to correlate measured Ct values with known WBC counts obtained by a Hemocue WBC analyzer as our gold standard point-of-care method.[9] We then created contrived samples with 20,000–320,000 total WBCs, which corresponded to WBC counts of 1,600–26,000 WBC/µL, falling far above and below the normal WBC count range. We then extracted DNA from each sample and performed qPCR for the *18S rRNA* gene (n = 3 replicates). **Figure S3** shows the resulting calibration curve relating total numbers or counts of WBCs to a Ct value. Results show good linearity (R^2 = 0.960) between the logarithm of WBC number and *18S rRNA* Ct values, providing confidence to our method to obtain a WBC count from a known punch volume of dried blood.

We acquired a whole blood sample from a single donor (WBC count: 4,600 WBC/µL) and made three contrived samples with target counts of ~4,000, 8,000, and 12,000 WBC/µL by adding or removing donor plasma. Using a Hemocue WBC analyzer, we then confirmed these counts to be 4,200, 7,600, and 11,900 WBC µL. We spotted 75 µL of each contrived sample on pDBS cards (n = 3 cards per WBC count), allowed the pDBS cards to dry for 24 hours, extracted genomic DNA from each punch, and obtained a Ct value by qPCR (**Table S3**). Due to how we prepared the contrived samples at targeted WBC counts, they also each had a different final hematocrit. Although we suggested previously that small differences in sampling volumes caused by the hematocrit should not affect the final WBC count, we sought to confirm this approximation experimentally. Using both the average punch volume (12.1 µL) and the hematocrit-dependent volumes, in combination with the obtained corresponding *18S rRNA* Ct value, we quantified WBC counts from pDBS cards (**Figure 3A**). We determined there was no significant difference in the

counts obtained using the average punch volume compared to the hematocrit-dependent punch volumes, thereby validating our approach.

The Clinical Laboratory Improvement Amendments of 1988 (CLIA) regulations suggest WBC counts from standard methods to be ≤10% error.[28] We calculated the error in WBC count from pDBS cards using the average, hematocrit-independent sample volume to be between 3–20%, depending on the WBC count (**Figure 3B**). In two out of three contrived clinical blood samples (4,200 and 7,600 WBC/μL), the accuracies of counts from pDBS cards fell outside of CLIA guidelines (20% and 18%, respectively). The disagreement between measured and actual counts is not caused by volume estimation, as the error for hematocrit-dependent volumes are higher than those using the average punch volume. For the sample with the highest WBC count (11,900 WBC/μL), the accuracy from pDBS cards (3%) was well within the suggested limits. We attribute the higher variability for the lower WBC count samples to the current high touch required for this assay (i.e., extraction, purification, analysis). As our CVs are consistent at each WBC count, subsequent investigations would benefit the WBC count versus Ct calibration curve having a larger donor sample size and automation of key assay steps. This approach would enhance the ability to account for potential variations in donor blood sample and provide a more accurate WBC count. Additionally, each of these cards are manufactured by hand, adding to the potential for error. However, as these cards are not diagnostic, slight variability in WBC counts, especially underestimation at low counts, still provides valuable insight to a patient that follow-up testing may be necessary.

Conclusion

In this manuscript, we present a method to obtain a quantitative WBC count from a dried blood spot card. These measurements are made possible by patterning the cardstock with features that result in the distribution, collection, and storage of a reproducible metered volume of blood in replicate zones. While we show these volumes are dependent on the hematocrit of the

sample of blood, we also demonstrate that using the average volume obtained from a range of hematocrits (25–55%) introduces less experimental error in measurements of Ct obtained by qPCR than the full experimental workflow itself (e.g., elution, extraction, aliquoting, mixing). As a result, we show that using an average, hematocrit-independent volume of blood stored in a pDBS zone can enable the calculation of WBC counts with accuracies to the gold standard that near CLIA recommendations across the range of counts for healthy donors. These results from pDBS cards are in contrast with applications of the standard Whatman 903 DBS card, which has not been shown to support measurements of WBC counts. We propose one possible rationale for the omission of this highly desirable, clinical measurement, which is that 903 cards do not support the collection of reproducible volumes of blood within or across sample application zones. Our observations are consistent with commonly accepted limitations of DBS cards discussed in the literature.

While some point-of-care platforms exist to obtain WBC counts, these instruments are not realistic for at home or in limited-resource settings due to cost and complexity of their operation. Conversely, our pDBS card design allows for at-home or remote patient sampling and simple transfer to a clinical setting by mail. This approach allows for more direct communication between a patient and clinical lab (e.g., through laboratory-developed tests), thereby improving access to routine testing and increased agency for patients over personal health information. While the focus of these efforts resulted in a new method to quantify WBC counts in blood, we anticipate that the ability to produce metered volumes of dried specimens will have additional applications in healthcare where a volumetric determinant is required for accuracy of a measurement (e.g., parasitemia in blood and viral load in plasma).

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Figure 1. Intracard and intercard sampling variability for Whatman 903 and pDBS cards. 75 µL of whole blood was applied to five sample collection circles on one 903 card and sample inlets on three pDBS cards. Zones were punched from replicate blood spots (n = 15 total punches for 903 cards, n = 12 total punches for pDBS cards). Replicates from each card are indicated by markers with the same color. Blood volumes stored in each punch were determined using the method detailed in Figure S2. A two-factor ANOVA test (no replication, α=0.05) yielded a significant difference in the intracard (within the same blood spot) and intercard (across blood spots) punch volumes for the 903 cards, while no significant differences were observed for pDBS cards.

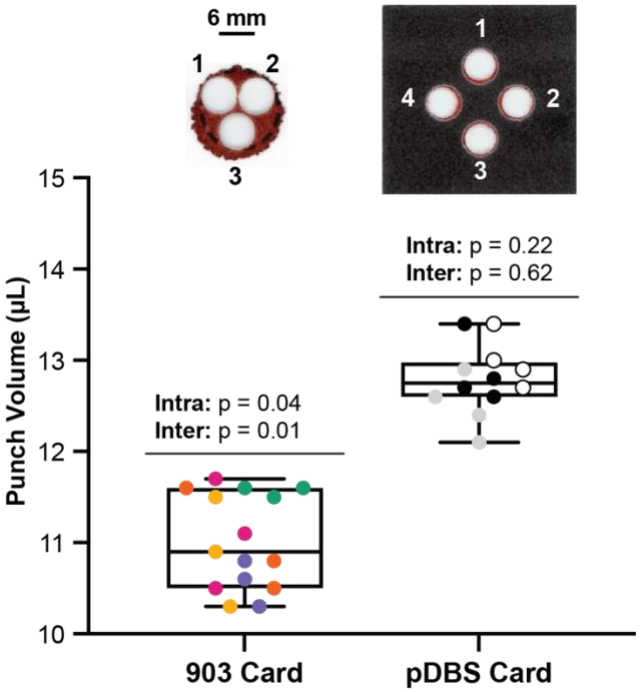


Figure 2. Determining pDBS punch volume and card variability across the full hematocrit range. **(A)** Table of hematocrit-dependent, punch volumes (average and standard deviation, coefficient of variation; n = 12) and associated card variabilities. Both intracard and intercard variabilities was obtained using two-factor ANOVA (no replication, α = 0.05). **(B)** Average punch volume from pDBS cards as a function of hematocrit. Three cards (n = 12 punches) were analyzed per hematocrit. The average punch volume (solid green line) across all hematocrits is 12.1 ± 1.0 µL. The shaded green area represents the 95% confidence interval.

A

Hct (%)	Punch volume ± SD (µL)	CV (%)	Intracard variability, p-value	Intercard variability, p-value
25	11.3 ± 0.7	5.7	0.49	0.26
30	11.0 ± 0.7	6.1	0.74	0.91
35	11.3 ± 0.6	6.2	0.09	0.51
40	12.2 ± 0.5	4.1	0.71	0.89
45	12.8 ± 0.4	2.9	0.22	0.62
50	12.9 ± 0.4	3.4	0.09	0.19
55	13.4 ± 0.4	3.0	0.86	0.80

B

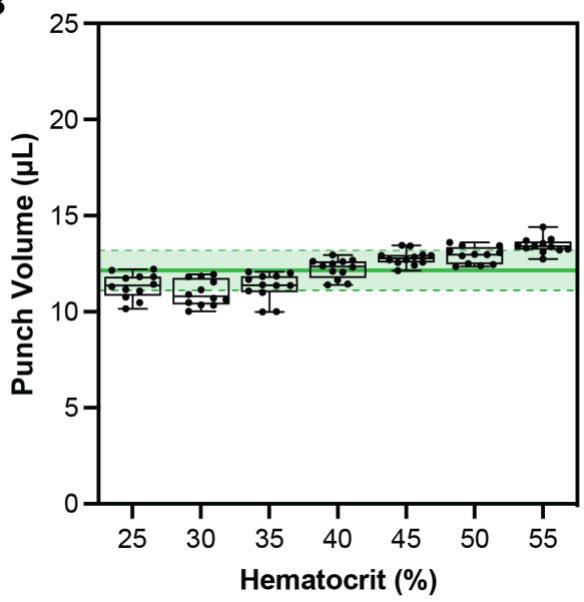


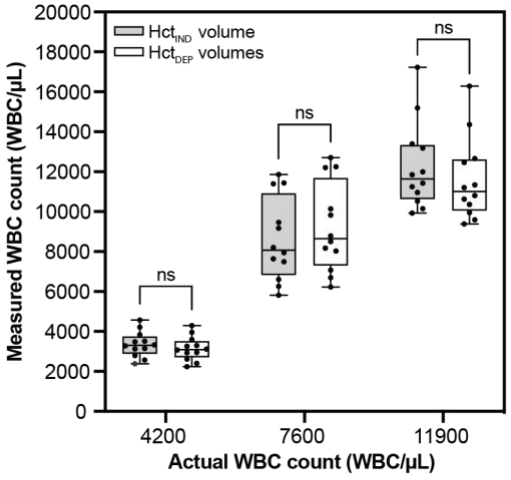
Table 1. Measured and theoretical *18S rRNA* Ct values for hematocrit-dependent (Hct_{DEP}) and hematocrit-dependent (Hct_{IND}) volumes

	Hct_{DEP}			Hct_{IND}			
Hct (%)	Volume (µL)	Avg Ct	SEM	Volume (µL)	Model Ct*	δCt†	|δCt| ≤ Hct_{DEP} SEM?
25	11.3	24.4	0.2	12.0	24.3	-0.1	yes
30	11.0	24.8	0.1	12.0	24.7	-0.1	yes
35	11.3	24.4	0.1	12.0	24.3	-0.1	yes
40	12.1	23.3	0.1	12.0	23.3	0.1	yes
45	12.8	23.0	0.2	12.0	23.1	0.1	yes
50	12.9	22.6	0.3	12.0	22.7	0.1	yes
55	13.4	22.4	0.3	12.0	22.5	0.2	yes

* Hct_{IND} Theoretical Ct determined using best fit lines (Table S2)
† δCt = Ct(Hct_{IND},theoretical – Hct_{DEP},measured)

Figure 3. WBC counts determined using hematocrit-independent (Hct_{IND}) or hematocrit-dependent (Hct_{DEP}) volume methods. **(A)** WBC counts (WBC/µL) were determined using total WBC number per punch determined by qPCR and dividing by either the (i) Hct_{IND} punch volume or (ii) Hct_{DEP} punch volumes (Figure 2B) for each sample. Three pDBS cards (n = 12 punches) were compared. A two-tailed Student's t-test (α = 0.05) yielded p-values of 0.44, 0.53, and 0.47, providing no significant difference between WBC counts calculated using the Hct_{IND} versus Hct_{DEP} volume methods. (B) Table of measured WBC counts using Hct_{IND} volume and Hct_{DEP} volumes methods with associated errors compared to WBC counts obtained using a Hemocue WBC analyzer (actual count).

A



B

Actual WBC count ± SD (WBC/µL)	Hct (%)	Method	Punch volume (µL)	Measured WBC count ± SD (WBC/µL)	CV (%)	Error (%)
4200 ± 58	50	Ind	12.1	3354 ± 638	19	20
		Dep	12.9	3146 ± 598	19	25
7600 ± 100	35	Ind	12.1	8609 ± 2079	24	18
		Dep	11.3	9218 ± 2226	24	21
11900 ± 120	45	Ind	12.1	12257 ± 2175	18	3
		Dep	12.8	11586 ± 2056	18	3

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