Enabling whole genome amplification from picogram quantities of genomic DNA with the phi29-XT WGA Kit

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INTRODUCTION

Recent advancements in Next-Generation Sequencing (NGS) technologies have revolutionized molecular biology. However, the challenge of obtaining sufficiently high quantity and quality genomic DNA (gDNA) for downstream NGS analysis remains a major limitation in the fields of cancer research, prenatal testing, preimplantation genetic testing, single cell analysis, forensic science, and metagenomics. To address this issue, Whole Genome Amplification (WGA) emerged as a prominent tool that can amplify gDNA from very low, and even single cell amounts[1]. WGA technology can be broadly classified into two categories: (i) PCR-based WGA and (ii) isothermal WGA. Among the isothermal WGA technologies, the most widely used method is Multiple Displacement Amplification (MDA). Here, we demonstrate the utility of the phi29-XT WGA Kit (NEB #E1604) for the amplification of gDNA by MDA, including the human genome and single cell inputs. The phi29-XT WGA Kit is a fast and broadly applicable solution for generating high yields of quality DNA from various input sources.

MDA WITH THE PHI29-XT WGA KIT

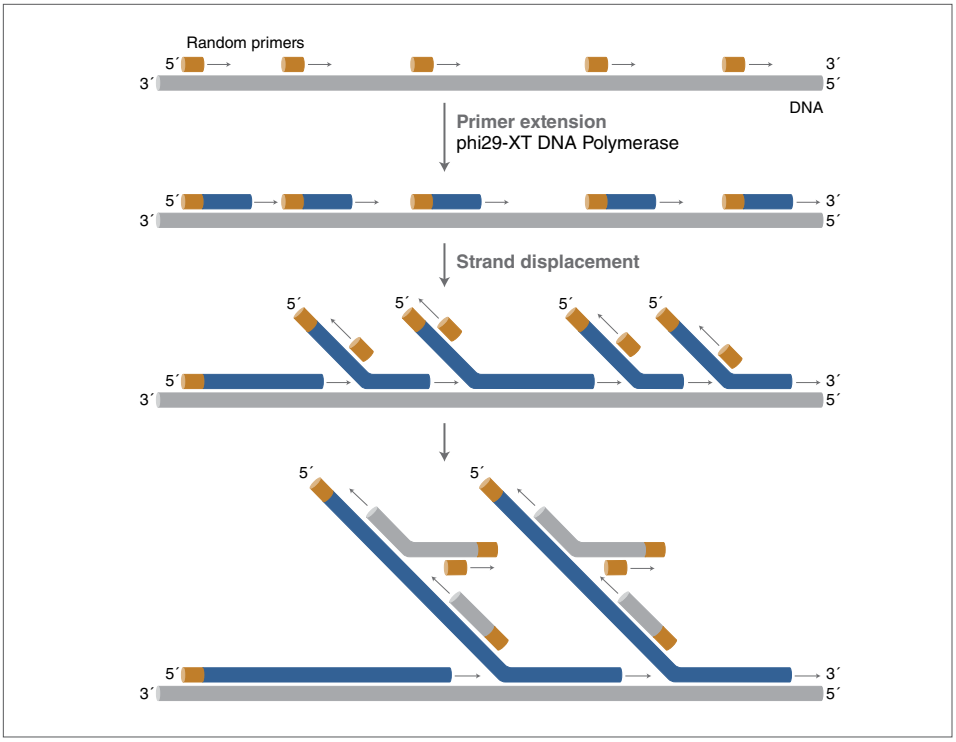
WGA by MDA is a powerful technique to amplify very small amounts of gDNA using random primers. Random primer utilization obviates the need for primer design and enables the amplification of unknown DNA sequences. For the reaction to proceed, gDNA must be denatured by heat or alkaline treatment, followed by annealing of random primers to the DNA and amplification by a strand-displacing DNA polymerase (Figure 1). As the DNA polymerase displaces the non-template strand of the DNA, random primers anneal to the displaced DNA, which triggers additional displacement amplification. This exponential process results in long, branched networks of DNA structures.

WGA by MDA is typically performed with the bacteriophage phi29 DNA Polymerase (phi29), due to its inherently high fidelity, high processivity, and strong strand-displacement activity[2]. An engineered phi29, phi29-XT DNA Polymerase, shares these properties and has improved thermostability, sensitivity, and amplification rates than the wild-type phi29. These features make phi29-XT an ideal enzyme choice for WGA and therefore was chosen to power our WGA kit.

MATERIALS

- phi29-XT WGA Kit (NEB #E1604)
- NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina (NEB #E7805)
- LAMP Fluorescent Dye (NEB #B1700)
- Quant-iT® PicoGreen®

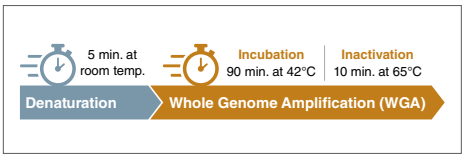
FIGURE 1: **Schematic representation of WGA by MDA**



During WGA by MDA, random primers (orange) anneal to the DNA template and are extended by the strand-displacing phi29-XT DNA Polymerase (arrows). As the polymerase synthesizes new DNA, it displaces downstream strands, generating single-stranded regions that serve as new templates for additional primer binding and extension. This results in a continuous, exponential amplification of the genomic DNA.

The phi29-XT WGA Kit generates high quantities of DNA products in as little as 1.5 hours with low bias and high fidelity. This kit includes dNTPs and exonuclease-resistant random primers for WGA from trace amounts of gDNA and single cells. The phi29-XT WGA Kit protocol is user-friendly, consisting of just two steps– denaturation and amplification (Figure 2).

FIGURE 2: **phi29-XT WGA Kit workflow**



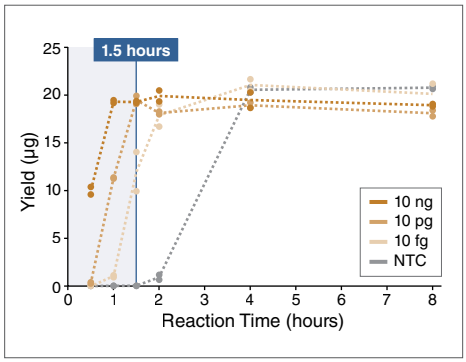
The phi29-XT WGA Kit offers a streamlined workflow with just a few minutes of hands-on time, enabling efficient whole genome amplification in under two hours.

To evaluate the performance of the phi29-XT WGA kit, we used two key metrics: DNA yield and GC bias. The GC bias is a measure of the relationship between GC content and read coverage across a genome. Biases can occur during WGA, library prep, sequencing, and mapping. For these experiments, we used the same NEB library prep kit, sequencing platform, and analysis pipeline to assess only the biases introduced during amplification. The experiments reported hereafter, unless otherwise stated, were carried out with the standard phi29-XT WGA Kit protocol, which includes 1X phi29-XT DNA Polymerase for WGA, 1X phi29-XT Reaction Buffer for WGA, 1 mM dNTPs, and 50 µM Exonuclease-Resistant Random Primers. Standard reactions were carried out at 42°C for 1.5 hours, then heat-inactivated at 65°C for 10 minutes. Libraries for NGS were prepared using the NEBNext® Ultra II FS DNA Library Prep Kit for Illumina (NEB #E7805) with 50 ng DNA input and 4 PCR cycles. Reaction yields were quantified using Quant-iT PicoGreen dsDNA Reagent.

THE PHI29-XT WGA KIT GENERATES HIGH YIELDS IN SHORT REACTION TIMES

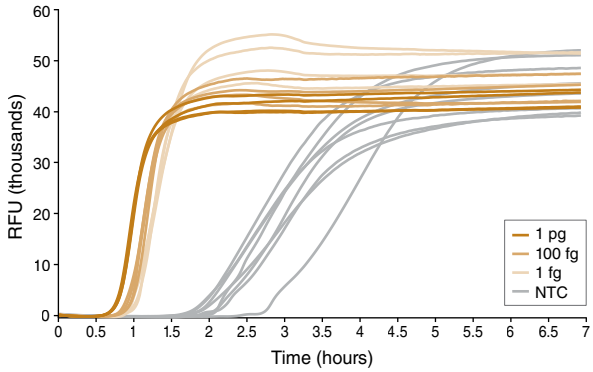
The phi29-XT DNA Polymerase included in this kit can generate over 10 µg of DNA from 10 fg of human gDNA when incubated at 42°C for 1.5 hours. For larger template quantities (>100 pg), the reaction time can be reduced, with maximum yield typically achieved in about 1 hour. Extended incubation time beyond 1.5 hours for WGA reactions without a template (NTC) may result in the production of nonspecific DNA products (Figure 3), which is a previously reported occurrence for phi29 DNA polymerase when using random primers[2]. These nonspecific products exhibit delayed amplification kinetics compared to reactions involving even femtogram quantities of gDNA template, as illustrated in Figure 4. Consequently, nonspecific amplification products do not significantly contribute to the overall pool of WGA products when a gDNA template is included in the reaction.

FIGURE 3: **phi29-XT DNA Polymerase generates high yields of product in a short period of time, even from 10 fg genomic DNA input**



phi29-XT DNA Polymerase generates high yields of product in a brief period of time, even from 10 fg of genomic DNA input. Duplicate WGA reactions were carried out using various amounts of human genomic DNA as input and were incubated for 0.5 to 8 hours at 42°C, followed by heat inactivation at 65°C for 10 minutes. Reaction yields (dots) were quantified using Quant-iT® PicoGreen® dsDNA Reagent and were averaged (line) to determine the yield at each reaction temperature. NTC = no template control. The 10 fg input does not include the complete human genome (~6-7 pg in diploid cell) but is included to demonstrate the sensitivity of the assay.

FIGURE 4: **Tracking WGA in real time: rapid product formation from ultra-low DNA inputs**



Real-time monitoring of WGA reaction kinetics reveals rapid generation of product with as little as 1 fg of human gDNA input. Comparison of amplification kinetics of different template concentrations (1 fg to 1 pg) of human gDNA with a no template control (NTC). Four replicates were used for human gDNA, and 8 replicates for NTCs. Real-time monitoring of WGA reactions was conducted by adding an intercalating fluorescent dye (NEB #B1700S) at a final concentration of 0.5X.

HIGH YIELD FROM VARIOUS gDNA INPUT MATERIALS

Achieving sufficient DNA yields for downstream applications such as high-quality sequencing can be challenging, especially from distinct types of input material such as single genomes, mixed genomes, and single-cell samples. The phi29-XT WGA Kit overcomes these challenges, and users can now obtain substantial amounts of high-quality DNA from various low-input sources such as human gDNA, bacterial gDNA, microbiome samples, and more complex starting materials including gDNA extracted from blood, blood spots or buccal swabs. With all the samples we tested, the phi29-XT WGA Kit was able to produce more than 10 µg of DNA from as little as 10 pg of extracted gDNA or from a single cell. (Table 1).

REACTION SCALING

The phi29-XT WGA Kit is flexible, allowing reaction volumes to be scaled down to 10 µL or up to 50 µL while maintaining a high yield (>5 µg of DNA) and with minimal impact on GC bias (Figure 5). GC Bias is a measure of the relationship between GC content and read coverage across a genome. The ideal normalized coverage is 1.0 if all the GC content windows are represented proportionally to how they occur in the reference genome.

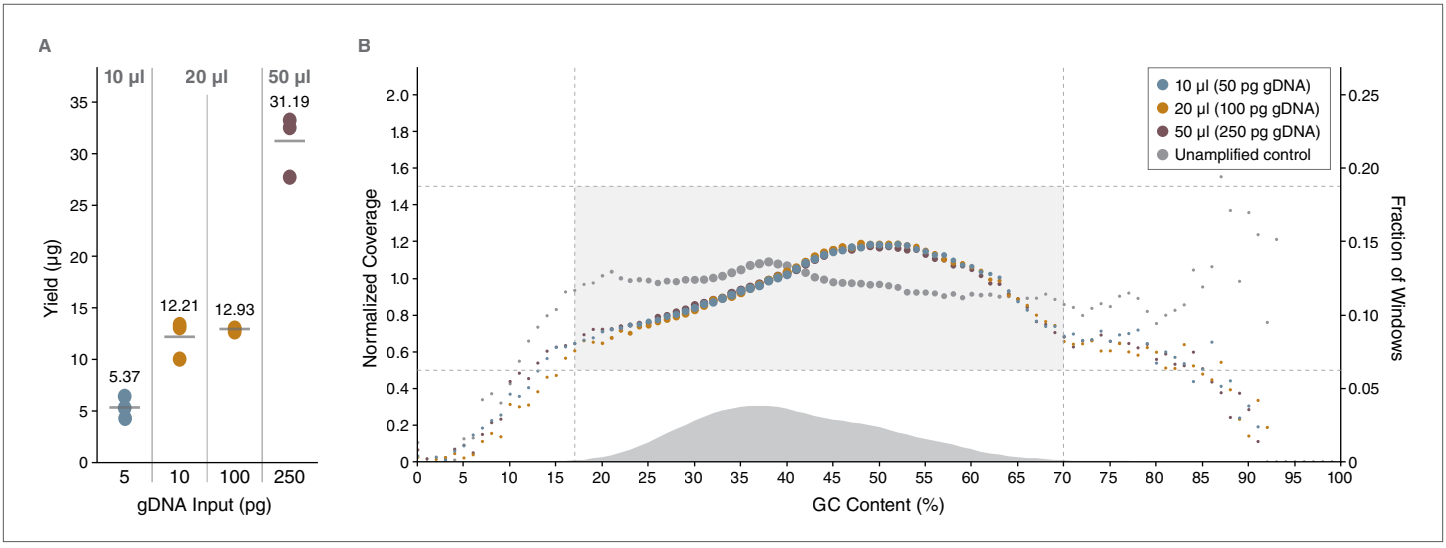
GC biases are introduced during amplification and genomic library preparation. To assess the GC bias generated during WGA, the GC bias profiles for WGA-amplified samples were compared to the unamplified control sample (Figure 5, gray dotted line). The phi29-XT WGA Kit provided uniform GC coverage, even with the expected polymerase-induced variation, regardless of the GC content of the gDNA or reaction volume tested. Indeed, the GC coverage remained close to the theoretical normalized coverage of 1.0, and close to the unamplified control, especially in the region of interest, encompassing 99% of the GC content windows for the human genome.

TABLE 1: **phi29-XT WGA Kit is broadly applicable to a variety of input sources, consistently yielding more than 10 µg of DNA**

GENOME	SAMPLE TYPE	INPUT MATERIAL	INPUT AMOUNT	YIELD (µg)
Single genomes	Human	Human DNA	10 pg	18.5
		Human Single Cell	1 cell	15.8
		Blood	10 pg	18.3
	Bacterial	*H. influenzae*	10 pg	20.5
		E. coli	10 pg	22.9
		B. pertussis	10 pg	13.2
Mixed genomes	Bacterial	*H. influenzae* + *E. coli* + *B. pertussis*	10 pg	23.7
	Microbiome	Oral microbiome	10 pg	22.3
		Gut microbiome	10 pg	21.8
		Mycobiome	10 pg	19.7
	Environmental	Environmental microbial community	10 pg	12.3
	Forensics	Blood spot	10 pg	15.6
		Buccal swab	10 pg	18.6

Average reaction yields from triplicate WGA reactions from various input materials. DNA yield is quantified using Quant-iT® PicoGreen® dsDNA Reagent (Invitrogen, #P11496). Since on average, a human somatic cell contains approximately 6–7 pg of DNA (diploid DNA content), we used 10 pg of gDNA as minimum amount of input material for all samples tested to ensure uniform genome coverage for downstream NGS analysis. Applications involving difficult to lyse cells, such as fungal, plant and Gram-positve bacterial cells, may require an additional lysis step to extract the gDNA prior to WGA.

FIGURE 5: **phi29-XT WGA Kit ensures robust amplification performance from 10 to 50 µl reaction volumes**



phi29-XT WGA Kit reactions scale well between 10-50 µL reaction volumes. Yield (µg) (A) and GC-bias (B) obtained from quadruplicate WGA reactions carried out using 3 reaction volumes with human genomic DNA (NA19240) as input. The input template amount was scaled to maintain the proportion with the other reaction components. Incubation was performed for 1.5 hours at 42°C, followed by heat inactivation at 65°C for 10 minutes. Dot sizes are proportional to the frequency in which the GC content window is present in the human genome (filled dark grey area). The light grey box indicates the region of the plot that encompasses 99% of the GC content values (17% to 70%) for the human genome (NCBI, GRCh38) and ± 50% normalized coverage. For each reaction volume tested, the coverage remained within 50% of the expected frequency for 99% of the GC content windows, which is consistent with thorough, unbiased WGA amplification.

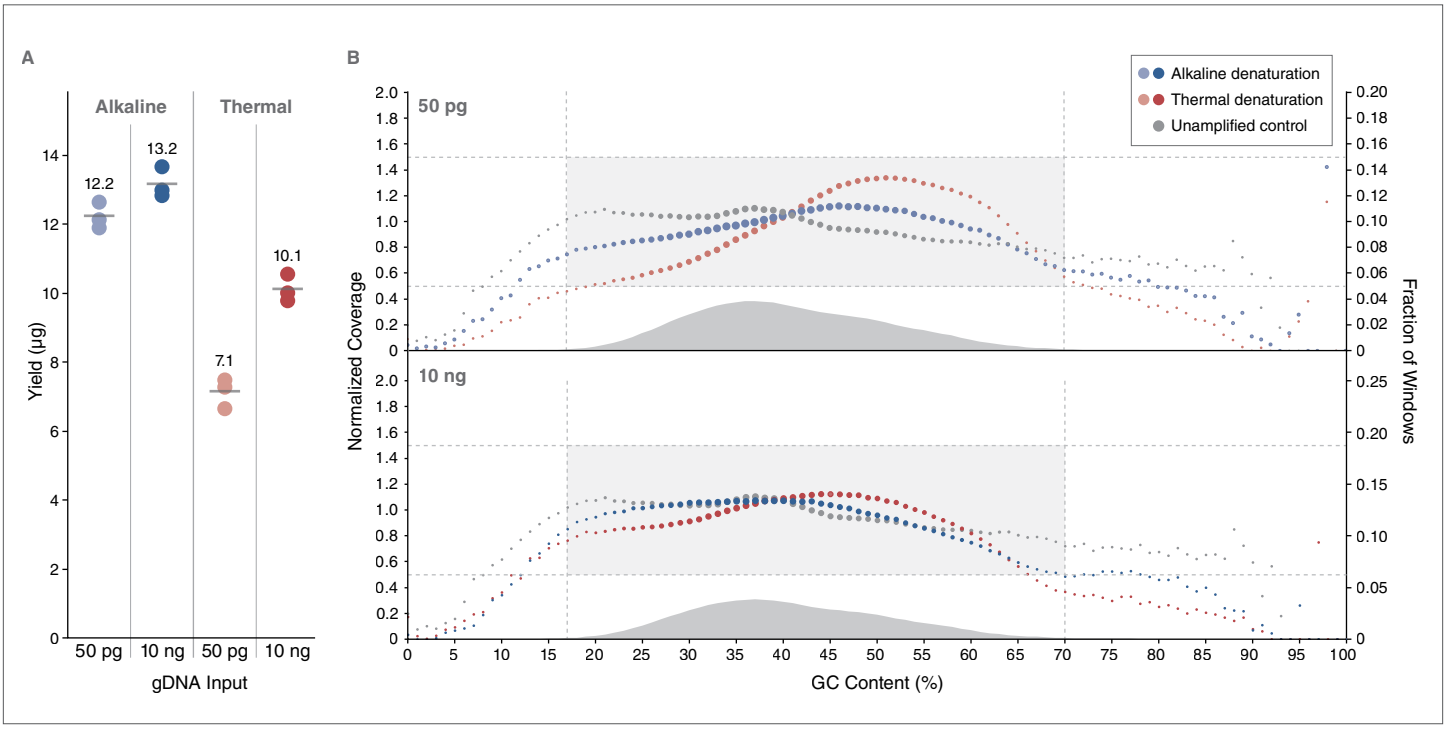
ALKALINE VS THERMAL DENATURATION

The first step in MDA is binding exonuclease-resistant random primers to denatured gDNA, followed by a strand displacement activity. The initial denaturation method used in this process contributes to bias in the reaction. Typically, MDA reactions that were initially denatured with alkali showed an improved yield and sequencing data quality as compared to those initiated with thermal denaturation[3,4]. The phi29-XT WGA Kit protocol uses alkaline denaturation, which ensures the best gDNA integrity for downstream NGS analysis. The quality of whole genome amplified DNA is also dependent on the amount of template used in the reaction. Figure 6 shows the impact of alkaline and thermal denaturation with two human gDNA template concentrations (50 pg and 10 ng). The effects of thermal denaturation are more pronounced with lower template inputs, whereas for the highest concentration of template examined, both the yield and GC bias demonstrate less susceptibility to the denaturation process.

SINGLE-CELL WGA

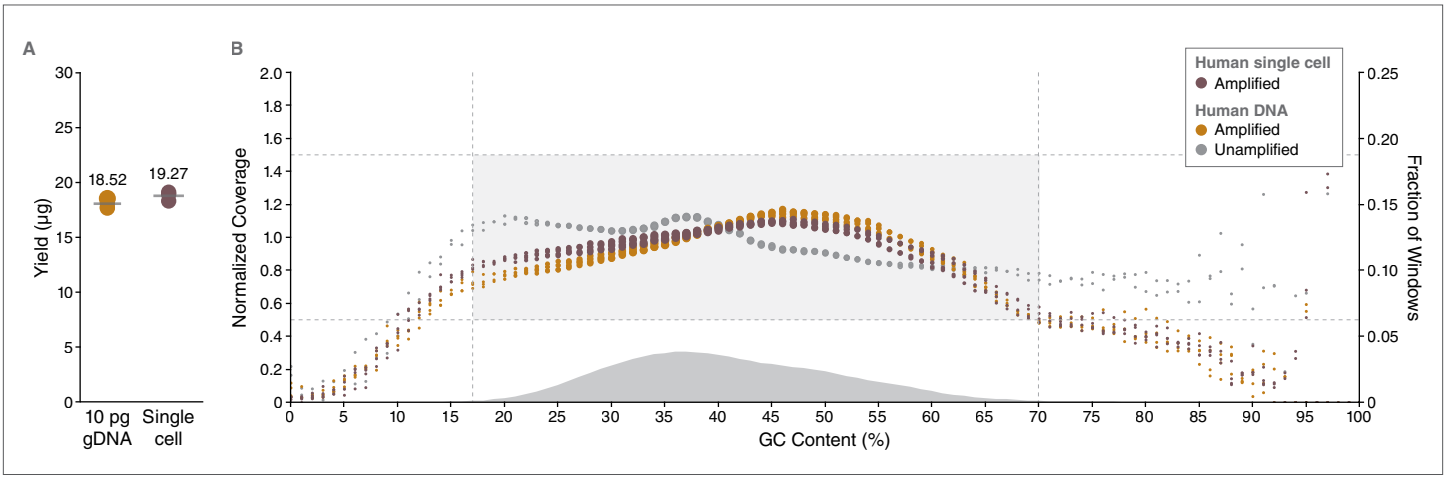
The challenge of collecting and sequencing all DNA fragments from a single cell remains a significant barrier in genomics. While sequencing individual DNA molecules with lengths of thousands of bases has become feasible, there are several obstacles and limitations when it comes to comprehensive single-cell sequencing. For instance, a diploid human cell contains 6-7 pg of genomic DNA. This small amount necessitates amplification before proceeding with microarray or NGS analyses[5]. Therefore, to achieve full genome sequencing from a single cell, whole-genome amplification (WGA) is an essential tool. We tested the phi29-XT WGA Kit with a single human cell and obtained low GC bias as well as a similar yield when compared with WGA products obtained from gDNA purified from bulk human cells (Figure 7). One common bias resulting from single cell WGA is the stochastic loss of template information that is most prevalent at low input concentrations, where only a few early amplification events determine the composition of the final WGA product[6]. To address this, a typical strategy is to pool multiple individual amplified reactions from single cells[7,8]. The phi29-XT WGA Kit demonstrates a high degree of reproducibility from cell to cell (Figure 7), ensuring reliability when multiple individual WGA products from single cells need to be combined.

FIGURE 6: **Alkaline denaturation enhances yield and reduces GC bias**



Alkaline denaturation of input gDNA improves yield and minimizes GC bias during WGA reactions compared to using thermal denaturation. Yield (µg) (A) and GC-bias (B) obtained from triplicate WGA reactions with alkaline (blue) and heat (red) human gDNA (NA19240) denaturation at two template concentrations (50 pg and 10 ng). Alkaline denaturation outperforms thermal denaturation at both gDNA template concentrations. Thermal denaturation was accomplished by incubating gDNA at 95°C for 5 min.

FIGURE 7: **The phi29-XT WGA Kit enables single-cell WGA with yields and GC bias comparable to purified gDNA, and consistent cell-to-cell reproducibility**

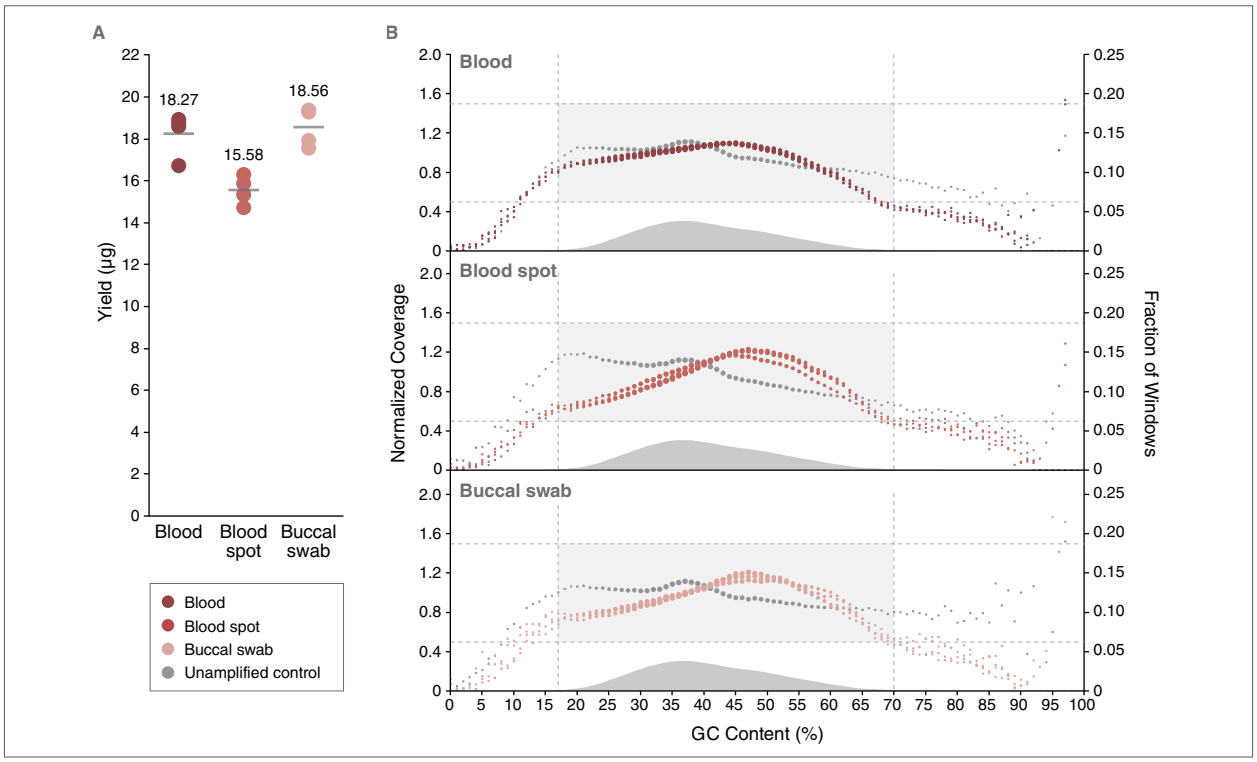


WGA carried out with a single human cell generates comparable yield and GC bias to WGA with 10 pg extracted human gDNA purified from bulk human cells as input material. Yield (µg) (A) and GC-bias (B) obtained from triplicate WGA reactions from single human cells (purple) and human purified gDNA (gold).

WGA ON MOCK FORENSIC SAMPLES

Forensic investigations require the amplification of trace amounts of DNA, typically accomplished by MDA. The phi29-XT WGA Kit is suitable for such applications due to potential drawbacks in PCR-based WGA methods, which could result in incomplete coverage of genetic loci. To test this kit for forensic applications, we used mock forensic samples, such as gDNA extracted from blood, blood spots and buccal swabs. Our tests revealed that the phi29-XT WGA Kit can produce a high concentration of DNA, irrespective of the type of template tested. Additionally, the amplification was highly uniform across the entire GC profile of the human genome, suggesting suitability for use in forensic applications (Figure 8).

FIGURE 8: **Robust WGA performance across common forensic DNA sources with phi29-XT WGA Kit**



WGA reactions carried out with gDNA extracted from mock forensic samples (blood, blood spot, or buccal swap) generate high yields of low GC bias WGA product. Yield (µg) (A) and GC-bias (B) obtained from quadruplicate WGA reactions from gDNA extracted from blood, blood spot and buccal swab. After extraction, 10 pg of gDNA was used as a template, and WGA reactions were carried out with the standard phi29-XT WGA protocol.

CONCLUSIONS

Examining DNA from diverse matrices is a growing practice in modern molecular biology laboratories. However, in numerous clinical or biological applications, starting material can be limited, for example in single cells (meta) genomics, forensic investigations, preimplantation genetic testing, the study of minimal residual cancer, and liquid biopsies approaches. Here, we demonstrated how the phi29-XT WGA Kit offers a solution to faithfully amplify template gDNA when initial quantities are low, so that the trace gDNA can be interrogated in downstream analysis. The phi29-XT DNA Polymerase for WGA combines high processivity with strand displacement and proofreading activities, producing DNA suitable for sequencing applications. Also, the optimal reaction temperature of 42°C improves amplification of high GC sequences and has been found to produce less bias and chimeras than wild-type phi29 DNA Polymerase that is typically active at 30°C[9,10]. The phi29-XT WGA Kit represents a significant advance in routine and specialized WGA applications from ultra-low gDNA concentrations. The high-efficiency and easy-to-use workflow enables more robust and reliable sequencing of these precious samples, minimize sequencing bias, and accommodates a variety of input materials.

References

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