Fast & efficient isolation of phage genomic DNA using the Monarch® HMW DNA Extraction Kits

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INTRODUCTION

Viruses that infect bacteria, also known as bacteriophages (or phages), are found everywhere. Approximately 20% of the total deoxyribonucleotides in the biomass of any microbiome are viral in origin (1). However, only a very small percentage of nucleotides derived from bacteriophages has been sequenced; therefore much about the genomes of bacteriophages is still unknown. A significant diversity of DNA base modifications has been observed in bacteriophages, including chemically simple modifications, such as methyl groups, as well as more complex substituents derived from sugars, amino acids, or polyamines (2,3). Understanding these DNA modifications and how they are made not only holds scientific clues for understanding phage-host interactions/conflicts, but may also lead to new ways to manipulate DNA. The ongoing discovery of new DNA modifications in phages suggests that many more are waiting to be found. Thus, efficient methods for isolating bacteriophage DNA for genome analysis and characterization of noncanonical nucleosides are needed and are of great interest to the field.

Historically, phage DNA has been isolated using genomic DNA purification methods employing silica, for which numerous kits are commercially available. However, these methods often present challenges with low DNA yields, low purity, and DNA shearing – all of which can negatively impact downstream studies. In the past, we utilized a phenol/chloroform-based DNA purification protocol, which employed a glass rod for spooling the DNA upstream of genomic and chemical analyses (4). While the DNA purified with this protocol is satisfactory, the procedure is cumbersome, uses hazardous chemicals, and requires significant hands-on time. Therefore, we continued looking for more convenient and robust solutions to isolate bacteriophage DNA.

Here, we describe an alternative approach for fast and efficient isolation of phage genomic DNA that offers benefits over both silica-based methods and modified approaches using glass rod-based methods. The Monarch High Molecular Weight (HMW) DNA Extraction Kit (NEB #T3050, T3060) employs an innovative approach to DNA capture using large glass beads. It offers a convenient, fast, user-friendly method, while still providing higher DNA yields with high purity. This workflow has been developed for use with different sample types including cultured cells, blood, bacteria and yeast, and supports several downstream applications including long-read sequencing and genome mapping. In this technical note, we examine and demonstrate the use of the recently developed protocol to isolate genomic DNA from bacteriophages and compare its performance to existing methods. The phages processed include those from the Gram-negative bacteria *Xanthomonas* (phage Xp12), from *Escherichia* (phages Cba120 and T4), and from Gram-positive bacteria *Bacillus* (phage SP8 and the jumbo phage G). The sizes of these phage genomes range from ~65 kb to ~168 kb and 497 kb for the jumbo phage (Table 1). DNA quality, quantity and size were assessed by UV-Vis spectrophotometry and pulsed-field gel analysis; DNA modifications were detected using liquid chromatography/mass spectrometry (LC/MS).

MATERIALS

- Monarch HMW DNA Extraction Kit (NEB #T3060)
- Lambda DNA PFG Ladder (NEB #N0341)
- Nucleoside Digestion Mix (NEB #0649)
- RNase A (NEB #T3018)
- DNase I (NEB #M0303)
- Isopropanol
- Ethanol

METHODS

Phage preparation

Bacteriophage plaques were obtained by the double agar overlay method (5) and as described in the phage DNA purification protocol provided with the Monarch kit. For scaled-up liquid growth of bacteriophages, growth media was inoculated with the bacterial host strain at a 1:100 dilution and incubated with shaking for 1 hour in flasks. After this initial lag phase, the cultures were infected with phage plaques picked from plates added directly into the medium. Cultures were shaken until lysed or a drop in absorbance at 600 nm was observed. The culture mixtures were then pelleted by centrifugation at 12,000 x *g* for 20 minutes to remove cellular debris and unlysed host bacteria. Next, bacteriophages were precipitated by addition of

TABLE 1: **Obtained yield and purity of bacteriophage genomic DNA samples purified with the Monarch HMW DNA Extraction Kit**

PHAGE	BACTERIAL HOST	GENOME SIZE	BASE MODIFICATION	PHAGE INPUT	YIELD (µg)	PURITY READING A_{260}/A_{280}	PURITY READING A_{260}/A_{230}
XP12	*Xanthomonas oryzae*	63,783 bp	5-mdC	1,011 pfu	45	1.72	2.05
SP8	*Bacillus subtilis*	138,741 bp	5-hmdU	1,010 pfu	32	1.99	2.09
CBA120	*Escherichia coli* (strain ATCC 700728)	157,304 bp	5-NeOmdU	1,012 pfu	90	2.30	1.90
T4	*Escherichia coli*	168,903 bp	5-a-gmdC, 5-b-gmdC	1,011 pfu	81	1.77	1.97
G	*Bacillus megaterium*	49,7531 bp	Uncharacterized modified 5-hmdC	107 pfu	24	1.80	1.88

polyethylene glycol (average molecular weight 8,000 Da – PEG8000), 1 M NaCl and incubation at 4°C, for hours to overnight. Precipitated phages were collected by centrifugation at 12,000 x *g* for 10 minutes and resuspended in a phage resuspension buffer composed of 50 mM Tris-HCl pH 7.5 buffer, 10 mM $MgCl_2$, and 100 mM NaCl. Resuspended phages were treated with 5 µg/ml RNase A (NEB #T3018) and 10 µg/ml DNase I (NEB #M0303) for 1 hour at room temperature to degrade host nucleic acids before the following DNA extraction.

DNA extraction using Monarch HMW DNA Extraction Kits

The Monarch HMW DNA Extraction Kit for Tissue was used to isolate bacteriophage genomic DNA following the provided protocol for bacteriophage DNA purification. Briefly, the bacteriophage lysate was concentrated as previously described using polyethylene glycol 8000 (PEG-8000) and sodium chloride solution (10% PEG8000 and 1 M NaCl final concentration) followed by centrifugation and resuspension in 300 µl of a buffer composed of 5 mM $MgSO_4$ in water. The concentrated phage solution was then lysed using 300 µl of HMW gDNA Tissue Lysis Buffer and 20 µl of Proteinase K for 45 minutes at 56°C in a thermal mixer with agitation set at 300 rpm. After lysis, 300 µl Protein Separation Solution was added, gently mixed, and centrifuged at 16,000 x *g* for 10 minutes to partition proteins from phage genomic DNA, resulting in an aqueous top layer and hydrophobic bottom layer containing residual protein. Two DNA capture beads were added to the transferred aqueous layer and the DNA precipitation was initiated by the addition of 550 µl of isopropanol. After 25 slow and gentle manual inversions, the DNA was bound to the beads. The supernatant was removed by careful pipetting and the bead-bound DNA was washed twice with 500 µl gDNA Wash Buffer. After pouring the beads into the provided retainer, residual alcohol from the wash buffer was efficiently removed by a pulse spin in a benchtop minifuge. To elute the DNA, the beads with the DNA were immediately transferred to 100 µl of gDNA Elution Buffer II and samples were incubated at 56°C for 5 minutes to allow for elution of the DNA from the beads. The bead/eluate mixture was then transferred to the bead retainer sitting in a 1.5 low bind microcentrifuge tube and the beads were separated from the extracted phage gDNA by centrifugation for 1 minute at 12,000 x *g*. The purified DNA was prepared for use by warming to 50°C for 2–3 intervals of 10 minutes interspersed with occasional pipetting up and down with a P200 wide bore tip. DNA samples were stored at 4°C for short-term storage before chemical and spectroscopic analysis.

DNA quantification and spectroscopic characterization

DNA quantification and purity analysis was carried out on a NanoDrop® One microvolume spectrophotometer from Thermo Fisher Scientific® Inc and a DropSense® 16 spectrophotometer from Trinean®, now Unchained Labs®. DNA elution/resuspension buffer composed of 10 mM Tris-HCl pH 9.0 and 0.1 mM EDTA served as reference blank.

Nucleoside analysis of isolated bacteriophage genomic DNA using LC/MS

Approximately 1 µg of isolated bacteriophage DNA was treated with 1 µl of Nucleoside Digestion Mix (NEB #0649) in a 20 µl reaction volume using the supplied buffer and incubated at 37°C for at least two hours to overnight. The produced nucleosides were then subjected to liquid chromatography (LC) analysis. LC analyses were performed on an Agilent® 1290 UHPLC system equipped with a diode array detector and a single quadrupole MS detector using a reverse phase Waters® XSelect® HSS T3 XP column (100 Å pore size, 2.5 µm resin size, and bed dimensions of 2.1 × 100 mm) at a flow rate of 0.6 ml/min with a binary gradient from 2% solvent A (10 mM ammonium acetate, pH 4.5) to 100% B (methanol) and the effluent monitored by UV absorbance at 260 nm.

Pulse-field gel electrophoresis analysis of the isolated bacteriophage genomic DNA

Pulsed field gel electrophoresis was performed on the Bio-Rad® CHEF-DR®III System. Approximately 150 ng of each isolated bacteriophage DNA was separated on a 1% agarose gel in 0.5X TBE buffer with power supply setting of switch times 0.5–94 seconds, 6 V/cm, 120° angle at 14°C for 20 hours. The gel was then stained with 1 µg/ml ethidium bromide in TBE buffer at dark for 1 hour. Followed by destaining in water for 0.5 hour, the gel was documented using an AlphaImager® EC HP camera system irradiated at 254 nm UV light. Lambda DNA PFG Ladder (NEB #N0341) served as the sizing reference.

RESULTS

Effective lysis of bacteriophage capsid

Several bacteriophages previously domesticated in the lab were chosen for testing the phage DNA purification protocol provided with the Monarch HMW DNA Extraction Kit for Tissue. Isolation and purification of the phage DNA using the provided phage protocol in the kit performed well for all phages tested. The lysis reagent effectively broke up the bacteriophage capsid resulting in the release of genomic DNA.

Efficient phage DNA extraction and isolation

For all five bacteriophages tested, the DNA capturing on the beads was straightforward. The DNA precipitated on the beads was visible for most phages but less clear for jumbo phage G due to lower phage input. Compared to the DNA extraction protocol previously established in our lab using phenol/chloroform extraction and a glass rod for DNA spooling, the hands-on time for the new protocol using the Monarch DNA Extraction kit was noticeably less (Table 2). Moreover, the overall time from the beginning of lysis to the endpoint of obtaining good quality and alcohol-free DNA for downstream analysis or sequencing is significantly shorter. The main difference in time between the preparation approaches is due to the removal of carryover ethanol. In the case of phenol/chloroform extraction, the sample is incubated overnight,

TABLE 2: **Hands-on time for bacteriophage genomic DNA purification between the phenol/chloroform-based protocol or the Monarch HMW DNA Extraction Kit**

	TIME SPENT PER STEP				
METHOD	PHAGE LYSIS	PROTEIN SEPARATION	DNA BINDING AND WASHING	REMOVAL OF CARRYOVER ETHANOL	DNA ELUTION
Original method – Phenol/Chloroform	0.5–1 hr.	20–30 min.	20 min.	Overnight	24 hr.
Monarch HMW DNA Extraction Kit	0.5–1 hr.	11 min.	10 min.	None	1–24 hr.

while the residual alcohol is evaporated gradually from the spooled DNA. In contrast, using the Monarch kit, the residual ethanol from the wash buffer is efficiently removed via a quick pulse spin in a benchtop minifuge.

Highly-pure DNA isolated from bacteriophages with minimal RNA and other contaminants

Purified bacteriophage DNA was assayed via UV-Vis spectrophotometric analysis. The collected spectra (Figure 1B) all appeared very clean – a typical nucleic acid UV absorbance profile with the characteristic peak at 260 nm was observed, and no other absorbance signal was observed. The spectrophotometric readings were used to determine the DNA concentration and the purity ratios (A_{260}/A_{280} and A_{260}/A_{230}). The purity level of the extracted DNA is generally good and falls in the higher range or close to the quality threshold. For A_{260}/A_{280} ratios, the readings are within the range between 1.7 – 2.3, indicating either minimal protein carryover (values <1.8) or RNA contamination (values >1.9) (6). However, it is possible that the unusual nucleoside content of the phage DNA results in minimal changes in the UV-vis spectrum and hence could explain the atypical A_{260}/A_{280} values.

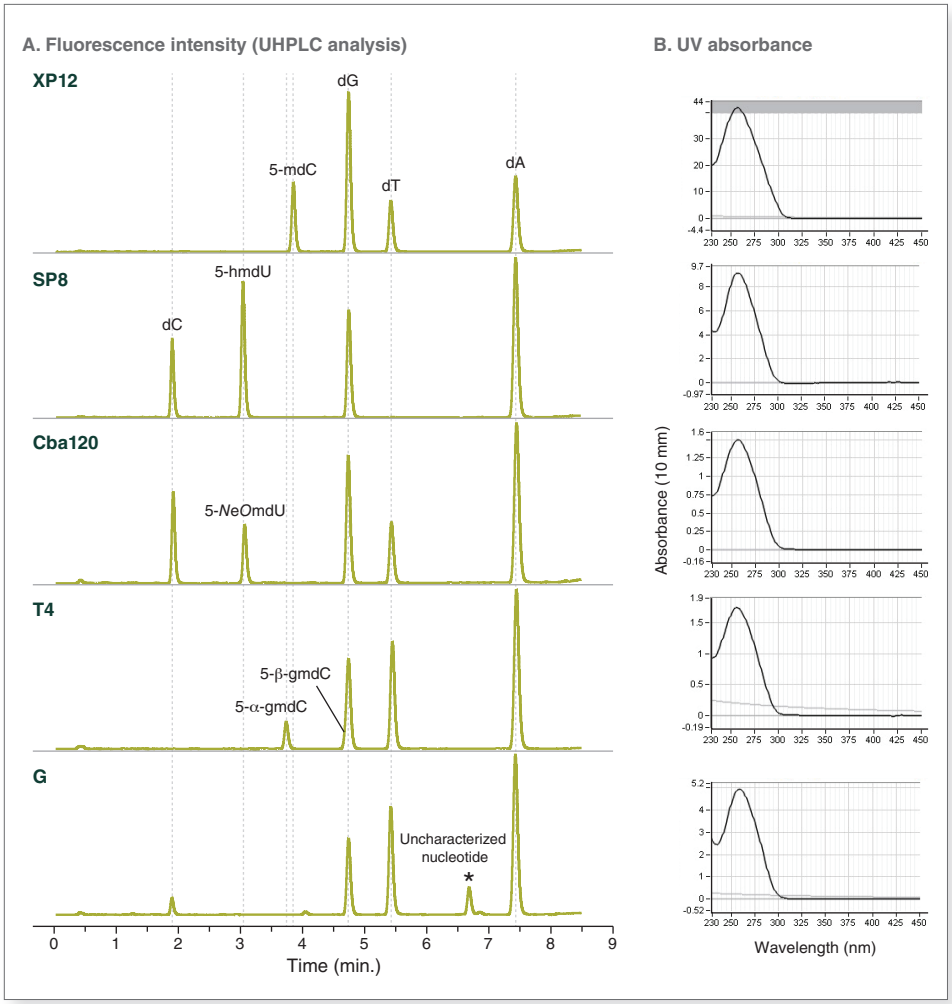
For A_{260}/A_{230} ratios, which is generally considered to be the more sensitive purity indicator, the readings fall between 1.88–2.05 range, in which DNA is generally considered to be pure (6).

Ultra-high-performance liquid chromatography (UHPLC)-based analysis was used to investigate the nucleotide chemical composition of the purified bacteriophage DNA. The isolated phage genomic DNA was digested to nucleosides using the blend of nucleases and phosphatases and then the mixture of nucleosides was subjected to the UHPLC separation and detection. LC traces of each bacteriophage DNA purified are shown in Figure 1A. The results indicate that all phage genomic DNA purified was of high purity. The baseline of each LC trace is almost flat and no undesirable non-2´-deoxynucleoside species, such as ribonucleosides from RNA carryover or small molecule carryover from the extraction kit, were observed. This also confirms that the higher than usual A_{260}/A_{280} purity ratios observed in some samples were likely to be caused by specific absorbance properties of the individual nucleosides, rather than by RNA contamination, as discussed before.

FIGURE 1: **Bacteriophage DNA purified with the Monarch HMW DNA Extraction Kit is of high purity and performs well in downstream applications**

A. 1 µg of purified bacteriophage DNA was digested to nucleosides using the Nucleoside Digestion Mix (NEB #M0649) and the mixture was subjected to UHPLC-MS analysis. LC traces are shown for each phage. Peaks of all nucleotides are marked in the figure. Traces show no RNA peaks or any other contamination signals

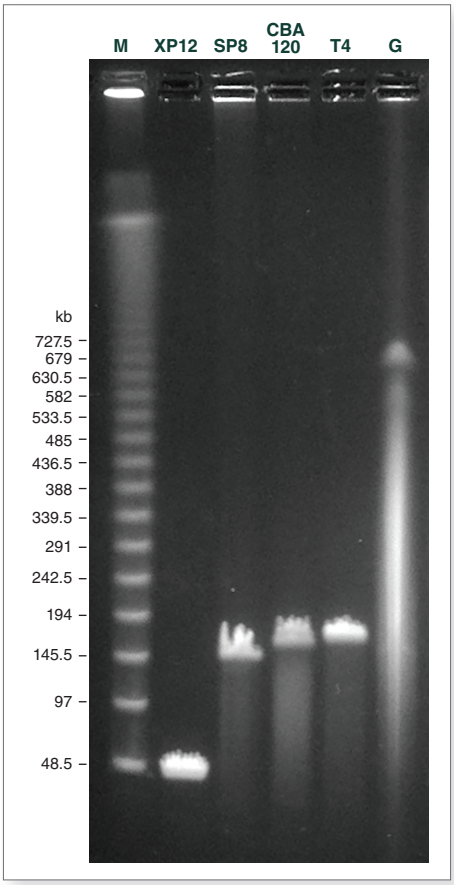
B. All purified phage DNAs were examined spectrophotometrically for quality and quantity. UV absorbance spectra are shown for each phage DNA next to the LC traces.



The five selected bacteriophages are expected to have modified nucleotides present in their DNA, i.e., 5-methyl-2´-deoxycitide (5-mdC) for phage XP12, 5-hydroxymethyl-2´-deoxyuridine (5-hmdU) for phage SP8, 5-(2-aminoethoxy) methyl-2´-deoxyuridine (5-*Ne*OmdU) for phage CBA120, 5-(α-d-glucosyl)oxymethylcytidine (5-α-gmdC) and 5-(β-d-glucosyl)oxymethylcytidine (5-β-gmdC) for phage T4, and an uncharacterized modification on cytidine for phage G. These modified nucleosides were resolved adequately as observed in the LC traces of each phage.

FIGURE 2: **Bacteriophage DNA purified with the Monarch HMW DNA Extraction Kit demonstrates high integrity**

Pulsed field gel analysis illustrates high integrity of the purified bacteriophage DNA and confirms expected size for most DNA samples. 150 ng DNA was loaded per lane. Lane 1: phage XP12 DNA, Lane 2: phage SP8 DNA, Lane 3: phage CBA120 DNA, Lane 4: phage T4 DNA, Lane 5: phage G DNA. M: Lambda DNA PFG Ladder (NEB #N0341).



Integrity of DNA preserved during extraction resulting in intact phage genomic DNA

The long phage DNA molecules can easily get sheared or fragmented during manipulation when performing DNA extraction procedures. Therefore, the integrity of the extracted DNA was examined on an agarose gel using pulse-field gel electrophoresis (PFGE) (Figure 2). The result showed the DNA from phage XP12, SP8, CBA120, and T4 appeared at the expected size and was highly intact with only minimal shearing observed. The DNA of phage G was observed on a position slightly lower in the gel than expected as compared to the size reference ladder. Given that phage G is apparently highly modified with uncharacterized modifications as shown in the UHPLC result, the modification may affect the mobility of DNA when traveling on the agarose gel during PFGE. The characterization of the unknown modification as well as the modification's effect on potentially changing the DNA's biophysical property remain to be elucidated.

CONCLUSION

The new Phage Isolation Protocol for use with the Monarch HMW DNA Extraction Kit for Tissue was used to successfully isolate DNA from several bacteriophages with a simpler and significantly faster workflow. Phage DNA ranging from 63 kb to almost 500 kb was purified with good yields of up to 90 µg, enabling successful use in several downstream applications. The purified phage DNA was of high purity and DNA integrity based on UV-VIS and PFGE analysis and performed well in LC-MS nucleotide analysis; LC-MS analysis also confirmed that the isolated phage DNA was free of RNA and other contaminants.

The discovery of phage DNA modifications has great potential for developing new technologies for handling DNA, for DNA enrichment and for new DNA sequencing methods. Finding new DNA modifications and identifying the biological mechanism for their installation is an area of high interest in current scientific research efforts. By having access to new rapid and high-performing methods such as those described here for extraction of phage DNA will reduce workflow time and facilitate faster and more efficient discovery processes.

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