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GoEnrich: creating high quality genomic DNA resources from limited voucher specimen tissues or museum specimens of at-risk species for conservation-friendly use in the validation of environmental DNA assays

Mark Louie D. Lopez[1], Matthew T. Bonderud[1], Isabel G. Ma[1], Vanessa C. Thompson[1] and Caren C. Helbing[1*]

Abstract

Objective Environmental DNA (eDNA) methods are crucial for monitoring populations, particularly rare and cryptic species. For confident eDNA application, rigorous assay validation is required including specificity testing with genomic DNA (gDNA). However, this critical step is often difficult to achieve as obtaining fresh tissue samples from at-risk species can be difficult, highly limited, or impossible. Natural history museum collections could serve as a valuable and ethical voucher specimen resource for eDNA assay validation. The present study demonstrates the effectiveness of whole genome amplification (WGA) in providing enough gDNA to assemble high quality mitogenomes from which robust targeted eDNA assays can be designed.

Results Using fresh and historical museum tissue samples from six species spanning fish, birds, and mammals, we successfully developed a WGA method with an average yield of 380 to 1,268 ng gDNA per 20 µL reaction. This gDNA was used for whole genome shotgun sequencing and subsequent assembly of high quality mitogenomes using *mtGrasp*. These mitogenomes were then used to develop six new robust, targeted quantitative real time polymerase chain reaction-based eDNA assays and 200 ng WGA-enriched yielded satisfactory C_q values and near 100% detection frequencies for all assays tested. This approach offers a cost-effective and non-invasive alternative, streamlining eDNA research processes and aiding in conservation efforts.

Keywords Whole genome amplification, eDNA assay validation, Museomics, qPCR, At-risk species

*Correspondence:
Caren C. Helbing
chelbing@uvic.ca
[1]Department of Biochemistry and Microbiology, University of Victoria, Victoria, BC V8P 5C2, Canada

Introduction

Environmental DNA (eDNA), genetic material obtained from environmental samples, has enhanced biodiversity monitoring offering a sensitive and cost-effective means of detecting cryptic species compared to traditional methods [1, 2]. However, data quality and reliability issues have undermined trust in eDNA applications [3, 4]. Validating targeted qPCR-based eDNA assays requires ensuring assay specificity using genomic DNA (gDNA) from target and non-target species; however, obtaining tissue samples for gDNA isolation is challenging for elusive or endangered species due to permit processes [3–5]. Researchers have explored natural history museums as potential sources of DNA [6], extracting gDNA from voucher specimens, though optimal methods for gDNA isolation and processing remain underexplored due to variations in specimen preparation and archival conditions [7].

Museum specimens are commonly stored under ambient conditions, either desiccated or in liquid-preserved collections, with some housed in refrigerated cold rooms. Over time, prolonged exposure to these conditions causes long strands of high molecular weight DNA to degrade into shorter fragments, diminishing both the size and quantity of DNA [8]. A substantial portion of dried specimens, often taxidermized hides, pose challenges in preserving intact DNA sequences suitable for downstream molecular analysis [9]. Furthermore, DNA degradation can occur when specimens are preserved in alcohol, typically 70–75% ethanol, where decreasing alcohol concentrations due to evaporation may lead to DNA hydrolysis [10]. Fragmented DNA and DNA that is chemically crosslinked by formaldehyde-based preservatives complicate downstream analysis such as qPCR due to the limited availability of intact gDNA [11].

Recent advances in museum sample genomic studies (museomics) have employed whole genome amplification (WGA) to amplify genomic DNA in an unbiased way [12]. However, these techniques were not tested for enhancing genomic resources for validating qPCR-based eDNA assays. The present study presents a workflow utilizing WGA-enriched gDNA from fresh and historical museum samples for genome skimming and mitochondrial genome assembly. Additionally, we demonstrate the use of enriched gDNA to validate six targeted qPCR-based assays.

Main text

Methods

Tissue collection and extraction

The present study includes freshly collected and historical museum tissue samples. The goldeye (Actinopterygii: *Hiodon alosoides*, te-HIAL) and cisco (Actinopterygii: *Coregonus artedi*, te-COAR) fresh tissues were obtained from the Alberta Ministry of Environment and Protected Areas. Fresh tissue samples of Northern goshawk (Aves: *Accipiter gentilis*, av-ACGE) were sourced from the Beatty Biodiversity Museum. Fresh tissue samples were preserved in 70% ethanol and stored at -20 °C until DNA extraction. Museum voucher specimens, comprising dried tissue samples of snowshoe hare (Mammalia: *Lepus americanus*, ma-LEAM), bison (Mammalia: *Bison bison*, ma-BIBI), and bald eagle (Aves: *Haliaeetus leucocephalus*, av-HALE), were obtained from the Royal British Columbia Museum in Victoria, Canada. Possession and disposal of these wildlife tissue samples for genetic analysis are covered under British Columbia Wildlife Act permit #NA22-782623. DNA extraction was performed using the DNeasy Blood and Tissue kit (Qiagen, Ontario, Canada), and quantification was conducted using the Qubit™ 1X dsDNA High Sensitivity Assay Kit (Invitrogen, Massachusetts, USA). Further details on the samples used in the present study are provided in Table 1.

Whole genome amplification (WGA) of genomic DNA

A modified method by Hutchison et al. [13] was adapted in the present study. A master mix was created by combining 20 ng gDNA, 1 µL 100 µM random hexamer primers (Cat. R016 and R106, Fidelity Systems, Maryland, USA), and 0.5 µL Phi29 10X buffer (Cat. M0269S, New England Biolabs, Ontario, Canada), adjusted to 5 µL

Table 1 Details of the samples used in the validation of the targeted qPCR-based eDNA assays in the present study. The WGA-gDNA was used as source material for whole genome shotgun sequencing and mitogenome assembly using *mtGrasp*

Type	Class	Scientific name	Species code	Collection date	Museum sample accession number	Sequence length (bp)	Number of contigs	GenBank Accession
Fresh tissue	Aves	*Accipiter gentilis*	av-ACGE	2019	UBCTB002332	16,197	1	PQ049665
	Actinopterygii	*Coregonus artedi*	te-COAR	2022	UVICCOAR-1	16,820	1	PQ040454
	Actinopterygii	*Hiodon alosoides*	te-HIAL	2022	UVICHIAL-1	16,619	1	PQ040453
Museum voucher specimen	Mammalia	*Bison bison*	ma-BIBI	1989	RBC17040	16,320	1	PQ049661
	Aves	*Haliaeetus leucocephalus*	av-HALE	1980	RBC19978	18,645	1	PQ049664
	Mammalia	*Lepus americanus*	ma-LEAM	1980	RBC16127	17,042	1	PQ049662

RBC: Royal British Columbia Museum; UBCCTB: University of British Columbia - Beatty Biodiversity Museum Cowan Tetrapod Collection; UVIC: University of Victoria

with UltraPure-dH_2O (Invitrogen, Massachusetts, USA). The mixture underwent heating at 95 °C for 5 min, then quick cooling by plunging on ice to 4 °C. This mix was added to a mixture of 1.5 µL Phi29 10X buffer, 1 µL Phi29 DNA Polymerase (10,000 U/mL, New England Biolabs), 8 µL 2.5 mM dNTPs (FroggaBio, Ontario, Canada), and 4.5 µL of UltraPure-dH_2O, resulting in a 20 µL final reaction volume. Incubation at 30 °C for 18 h followed, with subsequent deactivation of Phi29 polymerase at 65 °C for 10 min in a BioRad thermocycler. WGA products were purified using the QIAquick PCR purification kit (Cat. 28104, Qiagen, Ontario, Canada), and a portion was subjected to electrophoresis on a 0.8% agarose gel and visualized with Gel Red (Gold Biotechnology, Missouri, USA) for DNA size and integrity confirmation. DNA sizes were compared with a 1Kb DNA Ladder RTU (GeneDireX, Taipei, Taiwan).

Library preparation, whole genome shotgun sequencing, and mitogenome assembly

The WGA-enriched gDNA samples were sent to the Michael Smith Genome Sciences Centre, BC Cancer, Vancouver, Canada for library preparation and whole genome sequencing. The TruSeq DNA PCR-free library prep kit (Cat. 20015963, Illumina Inc., California, US) was used for library preparation using the Microlab NIMBUS liquid handling robot (Hamilton Robotics, Nevada, USA). A total of 500 ng gDNA was fragmented by sonication with Covaris LE220 (Covaris, Massachusetts, USA) and underwent end repair and size selection of 300–400 bp fragments using PCRClean DX paramagnetic beads (Cat. C-1003-50, Aline Biosciences, Massachusetts, USA). After 3' A-tailing, TruSeq adapters (Cat. 20015963, Illumina Inc., California, USA) were added and purified with PCRClean DX paramagnetic beads. Quantification of PCR-free genome libraries was done using a qPCR Library Quantification kit (Cat. 07960140001, KAPA, Massachusetts, USA) before sequencing on an Illumina NovaSeq 6000 instrument with S4 reagents in a single run, generating 150 bp paired end reads (Illumina Inc., California, USA). Mitogenomes were assembled, circularized, and standardized using mtGrasp (Mitochondrial Reference-Grade Genome Assembly and Standardization Pipeline, v. 1.1.0), aligning sequences from 5' to 3' end with tRNA-Phe as the initial sequence [14, 15].

eDNA assay design and validation

The eDNA assays for detecting *L. americanus* (eLEAM2), *H. leucocephalus* (eHALE), and *C. artedii* (eCOAR7) were fully validated through prior studies [15, 19], while the rest of the assays were validated as part of the current work (Table S2). All eDNA assays were designed and validated based on our established workflow [5], with performance characteristics meeting or exceeding Canadian standards [3, 4]. Mitogenomes of target and confounding species (from the National Center for Biotechnology Information (NCBI); Table S1) were aligned using MAFFT v7.490 [16], and phylogenetic trees were generated with RAXml-NG v. 1.2.2 [17]. *Unikseq* was used to identify unique regions in the target species' whole mitogenome sequences to increase the likelihood of finding suitable regions for robust qPCR assay design [18]. Primers and probes were selected using Beacon Designer 8.21 (PREMIER Biosoft, California, USA) based on identified regions. Successful primer and probe sequences for each eDNA assay are listed in Tables S2 and S3.

In vitro specificity validation involved testing 10 ng WGA-enriched target species' gDNA (2 µL of 5 ng/µL gDNA per 20 µL reaction) and non-target species' gDNA following methodologies outlined in previous studies [5, 18, 19]. qPCR validation of multiple primer pairs tested on gDNA from both target and sympatric species was done using QIAcuity EG PCR Kit (Cat. 250111, Qiagen). The thermocycler profile included an initial denaturation at 95 °C for 2 min, followed by 50 cycles of 15 s at 95 °C, 30 s at 64 °C, and 45 s at 72 °C. High-end specificity validation of primer-probe assay was carried out using QIAcuity Probe PCR Kit (Cat. 250101, Qiagen) with 25 technical replicates per sample. Amplification was observed in only a few replicates with the 10 ng WGA-enriched gDNA from older museum samples as input. To enhance amplification frequency, subsequent tests utilized higher input amounts of 100, 200, and 1,000 ng per reaction. Lastly, serial dilutions of synthetic double-stranded DNA (gBlocks®, IDT, Iowa, USA) were prepared to construct standard curves (Figures S1 – S6) to assess assay sensitivity [20].

Statistical analysis

To minimize the impact of outliers, the data is presented as median C_q values. Friedman repeated measures test was employed to identify significant differences ($p \leq 0.05$) among the test groups. Within each group, pairwise comparisons between treatments were conducted using the Wilcoxon Signed Rank test to determine statistical significance ($p \leq 0.05$).

Results

WGA enrichment of fresh and museum gDNA samples

After WGA-enrichment, the sample concentrations ranged from 19 to 63.4 ng/µL with overall yields between 380 and 1,268 ng per 20 µL reaction. Changes in the fragment size of gDNA samples before and after WGA enrichment were observed (Fig. 1). The gDNA samples extracted from fresh tissues showed less smearing, whereas museum voucher specimens exhibited significant fragmentation, ranging from 100 to 1,000 bp. The

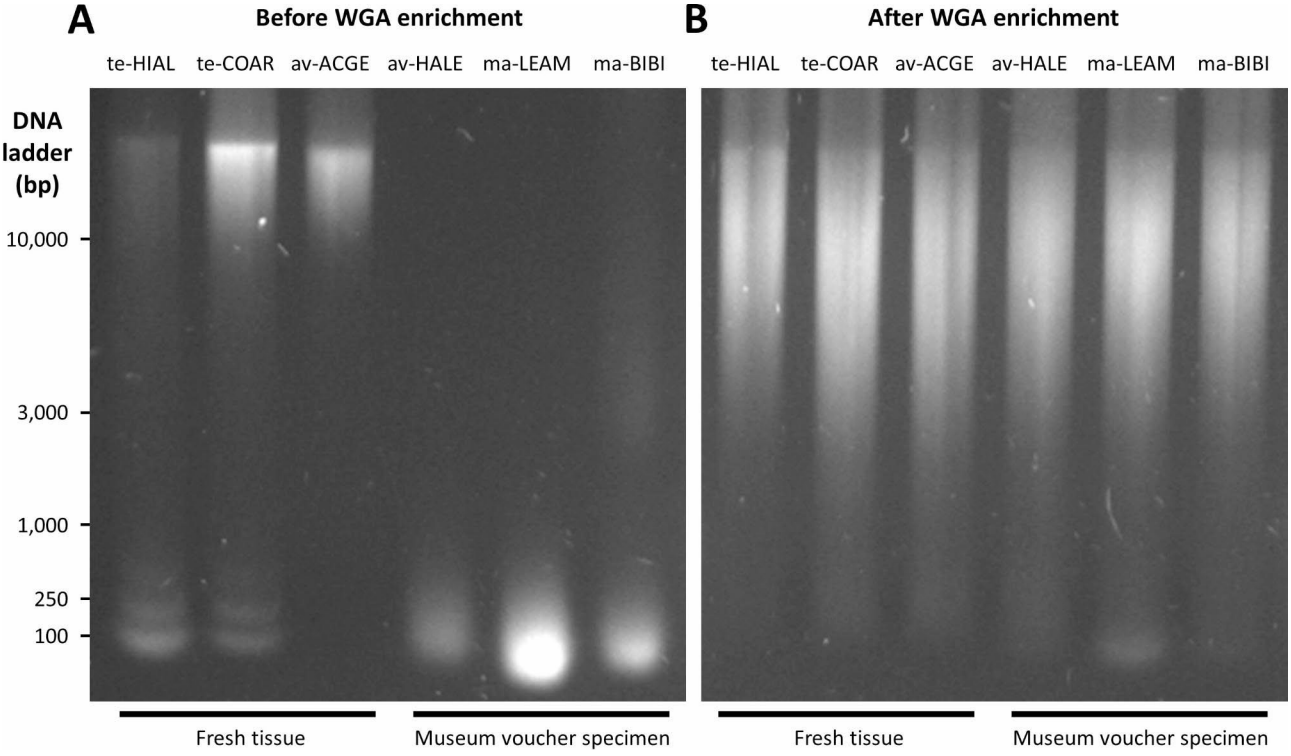

Fig. 1 Gel images of gDNA (**A**) before and (**B**) after whole genome amplification (WGA) enrichment. Each gDNA sample (100 ng) was run in a 0.8% agarose gel stained with Gel Red. gDNA samples: av-ACGE, *Accipiter gentilis*; ma-BIBI, *Bison bison*; te-COAR, *Coregonus artedi*; av-HALE, *Haliaeetus leucocephalus*; te-HIAL, *Hiodon alosoides*; ma-LEAM, Lepus *americanus*

WGA-enriched gDNA displayed enhanced fragment size, with a smear observed at 3,000 bp and larger.

Assembled mitogenomes

Libraries were constructed from WGA-enriched gDNA and resulted in 1.92 to 2.3 million reads (1.9 ± 0.4 million) per library. We successfully constructed mitogenomes for all six species examined in the present study (Table 1). With *mtGrasp*, we successfully assembled a single contig for each sample, suggesting a high sequencing depth of the mitogenome. The assembled mitogenomes ranges from 16,197 to 18,645 base pairs.

WGA-enriched gDNA samples for eDNA assay specificity validation

The assays developed in the present study were thoroughly validated for their specificity by testing them against the gDNA of the target species, sympatric species in the same environment, and common contaminating DNA such as human, cat, and dog. There was no cross-amplification of non-target species for any of the six eDNA assays (Table S4).

With limited access to voucher specimens, being able to confidently use WGA-enriched gDNA for assay validation enables individual labs to confidently demonstrate that an eDNA assay is performing as expected. We compared the abilities of the non-enriched and WGA-enriched gDNA to effectively act as templates to amplify amplicons. Utilizing 10 ng/reaction non-enriched and WGA-enriched gDNA from fresh tissue samples (Fig. 2A) resulted in comparable C_q values and detection frequencies for *C. artedi* (te-COAR) and *H. alosoides* (te-HIAL), but a 14 C_q increase for *A. gentilis* (av-ACGE) WGA-enriched DNA compared to non-enriched gDNA was observed.

In the case of museum samples (Fig. 2B), increasing the input amounts of WGA-enriched gDNA improved C_q values and detection frequencies. Specifically, 10 ng/reaction WGA-enriched gDNA had very high C_q values (43 to 49) and reduced detection frequencies (12 to 64%) compared to 10 ng/reaction non-enriched gDNA. Increasing the concentration of WGA-enriched gDNA to 200–2000 ng/reaction lowered the C_q values while greatly improving detection frequency to near-100% among replicates for all assays tested (Fig. 2B). All eDNA assays in the present study meet the sensitivity criteria set by the Canadian Standard Association (Table S5).

Discussion

The present study shows the efficacy of WGA enrichment in generating complete mitogenomes from gDNA samples extracted from limited fresh or museum voucher

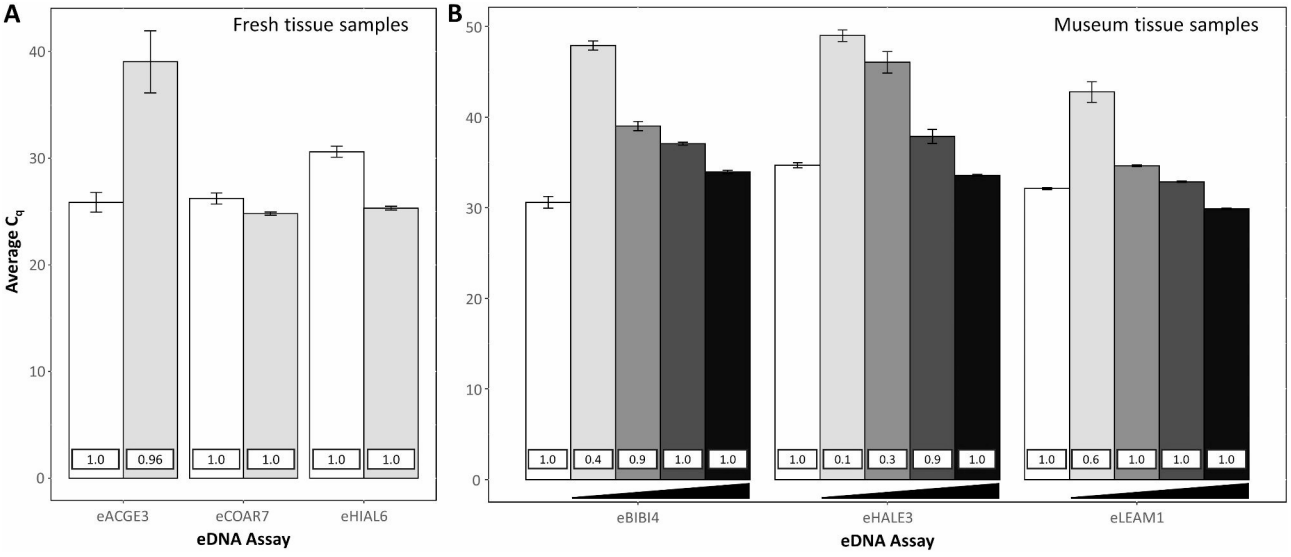

Fig. 2 Comparison of non-enriched and WGA-enriched gDNA performance as a template source for eDNA assay amplicon generation. Average C_q ± SD (bars and whiskers, respectively) and detection frequency (white inset boxes) ($N = 25$ technical replicates) are shown. **A**) Amplification performance of 10 ng non- and WGA-enriched gDNA input (white and light gray bars, respectively) from fresh tissue samples using eACGE3, eCOAR7, and eHIAL6 assays. **B**) Amplification performance of non-enriched (10 ng/reaction) and different concentrations of WGA-enriched gDNA input (10, 100, 200, or 1,000 ng/reaction, indicated by the black bevel and progressively darker shaded bars) from museum tissue samples using eBIBI4, eHALE3, and eLEAM1 assays ($N = 25$ technical replicates). Higher C_q values correspond to lower concentrations of amplified DNA. eDNA assays and respective target species: eACGE3, *Accipiter gentilis*; eBIBI4, *Bison bison*; eCOAR7, *Coregonus artedi*; eHALE3, *Haliaeetus leucocephalus*; eHIAL6, *Hiodon alosoides*; eLEAM1, *Lepus americanus*

specimens. Moreover, the enriched gDNA can serve as a replenishable source of genetic material for eDNA assay validation. Providing an accessible way to grow the mitogenome sequence repositories will enhance the development of more reliable qPCR-based eDNA tools [5]. Unbiased selection of unique sequences of the target taxon rather than relying on short, barcoded regions of the mitogenome increases the likelihood of designing a taxon-specific assay while satisfying the rules of good primer and probe design for qPCR [15].

In vitro specificity assessments necessitate the collection of gDNA from target and sympatric taxa, sourced from voucher specimens [5, 21]. Acquiring tissue samples for gDNA isolation, especially from elusive, endangered, and regulated species, often entails cumbersome permit procedures and can negatively impact animal health. Leveraging natural history museums as repositories of gDNA resources can circumvent these challenges. WGA-enriched gDNA facilitates the preservation of requisite samples, bolstering their concentration and volume through unbiased amplification [7].

Limitations

We used six separate targeted qPCR assays and evaluated the specificity of the primers and probes using WGA-enriched gDNA. More assays on additional specimens, including even older museum specimens, is desired.

Conclusion

WGA is an innovative approach that effectively amplifies the original template DNA, enabling the production of a substantial quantity of genetic information from small quantities of gDNA. The present study demonstrates the successful application of WGA enrichment to generate complete mitogenomes using small amounts of gDNA from endangered species and museum voucher specimens. WGA-enriched gDNA samples were also useful in the comprehensive validation of eDNA assay specificity by increasing the concentration and volume of the original gDNA samples needed for the validation. This is particularly advantageous in minimizing the need for continuous destructive sampling of museum samples, arduous permit applications, and expensive and intrusive collection of rare and endangered species.

Abbreviations

av-ACGE	Northern goshawk (Aves: Accipiter gentilis)
av-HALE	Bald eagle (Aves: Haliaeetus leucocephalus)
eDNA	Environmental deoxyribonucleic acid
gDNA	Genomic deoxyribonucleic acid
ma-BIBI	Bison (Mammalia: Bison bison)
ma-LEAM	Snowshoe hare (Mammalia: Lepus americanus)
qPCR	Quantitative real-time polymerase chain reaction
te-HIAL	Goldeye (Actinopterygii: Hiodon alosoides)
te-COAR	Cisco (Actinopterygii: Coregonus artedi)
WGA	Whole genome amplification

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13104-024-06936-z.

Supplementary Material 1

Acknowledgements
Not applicable.

Author contributions
MLDL, MTB, CCH designed the research; MLDL, MTB, IGM performed the experiments; MLDL, VCT, CCH analyzed the data; MLDL, MTB, IGM, VCT, CCH prepared the manuscript. MLDL, MTB, IGM, VCT, CCH read and approved the final manuscript.

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Data availability
The datasets supporting the conclusions of this article are included within the article and its additional files. Mitochondrial genomes assembled in the present study were uploaded to National Center for Biotechnology Information's (NCBI) nucleotide database (see Table 1 for accession numbers).

Declarations

Ethics approval and consent to participate
Ethics approval from the University of Victoria's Animal Care Committee was not required for the present study according to the Canadian Council on Animal Care as the obtained samples were opportunistically subsampled from existing provincial government and museum collections. Possession and disposal of these wildlife tissue samples for genetic analysis are covered under British Columbia Wildlife Act permit #NA22-782623.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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