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SHORT PAPER

Comparative Evaluation of Custom TNA Extraction Buffers to Obtain High-quality TNA from Shrimp and Polychaetes

Joanna Gerszon[1,*], Berit Genz[1], Louise Franz[1], Melony Jay Sellars[1], Ralf Joachim Moser[1],

[1]Genics Pty Ltd., Level 5, Gehrman Building, 60 Research Road, St Lucia, Qld 4067, Australia

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Corresponding Author
Tel.: + 40671300895515
E-mail: joanna.gerszon@genics.com.au

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Abstract

Selecting the appropriate lysis buffers is crucial in obtaining high-quality nucleic acids for molecular pathogen detection. In this study, we compared three in-house and one commercial lysis buffer for their effectiveness in extracting high-quality TNA from Tiger shrimp and sandworm samples. Buffer L1 outperformed the commercial counterpart, producing higher TNA concentrations and concordant PCR results for all DNA and RNA targets in the Shrimp MultiPath™. The findings underscore the significance of selecting the right extraction buffers to achieve high-quality nucleic acids for downstream molecular applications and suggest that in-house buffers can be a viable alternative to commercial ones for difficult-to-extract tissues.

Introduction

Extraction of nucleic acids is a critical step for a large number of applications in molecular biology, and always starts with the disruption of tissue and cellular structures of a biological sample.

Cell disruption methods can broadly be categorized into mechanical procedures using shearing forces exerted by blenders, homogenizers, bead mills, presses, and sonication, and non-mechanical procedures that use temperature gradients, osmosis, chemicals, or enzymes to lyse tissues and cells (Geciova et al., 2002; Islam et al., 2017). The choice of disruption methodology is highly dependent on the type of target tissue and target molecule, as well as downstream application. Complex biospecimens might require a combination of cell disruption methods for complete lysis (Balasundaram et al., 2009). In addition, with the view to extract nucleic acid for downstream molecular applications such as polymerase chain reaction (PCR), the chosen extraction procedure must address the presence of potential PCR inhibitors. Marine invertebrates contain complex polysaccharides such as chitin, collagen and calcium carbonate, which have PCR inhibiting properties (Huelsken et al., 2011; Sidstedt et al., 2020; Skujienė & Soroka, n.d.; Zhang et al., 2019).

The use of commercial extraction buffers adds substantial costs to a basic extraction process and does not necessarily guarantee sufficient high-quality DNA and RNA yield (Angthong et al., 2020). In the context of high throughput commercial applications, it is important to balance the costs of the extraction process with the quality and quantity of extracted nucleic acids.

This study aimed to develop an economical buffer for high-throughput, high quality TNA (total nucleic acids) extraction from the complex tissue types Penaeid Giant Tiger shrimp and polychaetes. The formulation of extraction buffer components was based both on rich organic sample types and the potential presence of inhibitors, such as polysaccharides (Lever et al., 2015; Xiao et al., 2015). In-house formulated buffers were compared against a commercially available extraction/lysis buffer with a focus on quantity of extracted nucleic acids and performance in PCR-based assays using real-time qPCR and the MultiPath™ technology, measuring multiple pathogens and β-actin controls simultaneously.

Material and Methods

Extraction Buffers:

(i) Buffer L1: 30 mM Tris, 30 mM EDTA, 800 mM Guanidinium–HCl (GnHCl), 0.5% Triton X-100; pH 10
(ii) Buffer L2: 4M Guanidium Thiocyanate (GITC), 10 mM Tris; pH 7
(iii) Commercially available lysis buffer
(iv) Buffer S3: 66 mM Tris, 3.3% Triton X-100, 1.65 M Guanidinium–HCl (GnHCl), 0.825 M NaCl; pH 7.9

Nucleic Acid Extraction

Penaeid Giant Tiger shrimp *(Penaeus monodon)* and Sandworm (Polychaete *Perinereis helleri)* samples were collected from aquaculture farms. Prior to extraction of total nucleic acids (TNA), pooled shrimp tissue (muscle and organs) and whole sandworm (polychaete) tissue was blended in 5% ethanol in order to standardize tissue input amounts and minimise variations between replicate measurements. Blended shrimp and sandworm tissue was dispensed in equal aliquots of 0.5 g to 12, 2 mL microtubes, and 0.5 mL of each buffer was added (L1, L2 + 1% β-mercaptoethanol (ME), commercial buffer and S3) together with four ceramic and eight glass beads. Tissue samples were homogenized twice for 90 seconds at maximum speed using a Tissue Lyser II (QIAGEN, Hilden, Germany) (Gerszon et al., 2022). A total of 48 shrimp samples and 48 polychaete samples were processed in two runs using the MagMAX CORE Nucleic Acid Purification Kit (Carlsbad CA USA) and KingFisher Flex Purification System in 96 deep-well plate format, with a final elution volume of 50 µL of PCR-grade water.

DNA Quantification

DNA yield was estimated at A_{260} using a Nanodrop spectrophotometer (ClarioSTAR, BMG LABTECH, Germany). DNA purity was determined based on the absorbance measurements at A_{260} and A_{280}. DNA concentration of a subset of samples was also determined using a Qubit 4 fluorometer (Life Technologies, USA) and Qubit™ dsDNA High Sensitivity Assay according to the manufacturer's instructions.

Quantity and integrity of genomic DNA was analysed using a 4200 TapeStation System (Agilent, CA, USA) and genomic DNA Screen Tape Assay according to the manufacturer's protocol.

Real-time qPCR Analysis

qPCR was performed using two assays previously designed and validated at Genics laboratory. All shrimp TNA samples were analysed using a β-actin (β-Actin) qPCR assay with the following primers: 5' - TCC CTC CAC CAT GAA GAT CAA G-3' (forward, Bact_Ex3_31) and 5'-CTG GAA GGT GGA GAG CGA G-3' (reverse, Bact_Ex3_31). All sandworm TNA samples were analysed using the cytochrome *c* oxidase subunit 1 (COX1) qPCR assay with the primers 5' – ACC ATT GTA ACA GCC CATGC-3' (forward COX1_P17) and 5' – TGC TAT GTC TGG GGC ACC TA-3' (reverse COX1_P17). RT-qPCRs were setup using the PowerUp SYBR Green Master Mix according to the manufacturer's specifications and run on the QuantStudio 12K Flex System with the following cycle conditions: 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec. Final melt curve analysis with 95°C for 15 sec and 60°C for 1 min and a terminal heating step to 95°C at a ramp rate of 0.05°C/sec.

Shrimp MultiPath™

In total 48 shrimp and 48 sandworm TNA samples were analysed using the high-throughput PCR-based Shrimp MultiPath™ (SMP) panel (Genics, Brisbane, Australia) to determine the suitability of samples for multiplex PCR analysis. SMP contains 13 shrimp pathogen targets as well as internal control assays for shrimp genomic DNA and mRNA. To run the assay 2 µL of cDNA was used. Each SMP run included a synthetic positive control, an extraction control, and a no-template control. Presence of target genes was determined as copy number per reaction.

Statistical Analysis

Statistical analysis was performed using the MedCalc Statistical Software version 20.111 (MedCalc Software Ltd). Data normality was tested with the Shapiro-Wilk test. Normally distributed data were tested for statistical significance using a one-way ANOVA (unpaired analyses) with Tukey-Kramer post-

hoc test for all pairwise comparisons. Non-normally distributed data were compared using a Kruskal-Wallis test with Dunn post-hoc analysis. P<0.05 was deemed statistically significant. All data was presented as the mean ± standard deviation.

Results and Discussion

The availability of commercial DNA/RNA/TNA extraction kits and associated lysis/extraction buffers greatly simplifies the isolation of nucleic acids from biospecimens and challenging sample matrices. However, an in-depth understanding of the mechanism of action of different buffer components allows the formulation of new compositions of extraction solutions that are dedicated to specific types of specimens. This study focused on Tris, GnHCl, Triton X-100, EDTA, NaCl, and GITC, as key buffer ingredients and formulated the three buffers L1, L2, and S3 with different ionic strength and pH to compare to a commercially available buffer. The study aimed to evaluate the TNA extraction efficacy of the custom formulated buffers L1, L2 and S3 and compared them to the commercially available buffer by assessing the quantity and quality of TNA extracted from shrimp and sandworm samples.

Quantitative analysis using UV spectrophotometric (UV spec) measurements showed that shrimp tissue extraction with L2 buffer resulted in the highest yield of DNA, with mean 367±28 ng/µl; followed by L1 buffer: 332±31 ng/µl; a significantly lower DNA concentration was noted for samples extracted with the commercial buffer, and S3 buffer, with mean 286±43; 157±28 ng/µl, respectively (P<0.05) (Table 1). With sandworm tissue, the quantity of DNA extracted with L2 buffer was almost 18 times lower than DNA yielded with L1 buffer (L1: 995.7±56.7 ; L2: 56.7±24) (P<0.05) (Table 1). Upon investigation, buffer L2 showed a significant drop in pH and associated acidification compared to buffer L1 over the storage period and it is suspected that this pH instability might be the leading cause for lower-than-expected DNA yields in sandworm samples.

Further, relative DNA purity compared to protein contamination was measured via A_{260} and A_{280} ratio and determined to be suitable (a ratio of ~1.8) and comparable across all samples and treatments (Table 1). The measured DNA integrity numbers (DIN) for shrimp samples ranged from 1.5 to 1.8, and sandworm DNA ranged from 1.5 to 6.1 which is sufficient to run the SMP workflow (Table 1). To characterize the quality of extracted DNA, the quantitative abundance of β-Actin

Table 1. DNA concentration, purity, and DNA integrity number (DIN) comparison of different extraction buffers. The purity parameter was calculated based on the $A_{260/280}$ absorbance ratio. Data is presented as means ± SD of n = 12 independent measurements.

BUFFER	Concentration [ng/µL] (Mean±SD)		Purity[a] A_{260}/A_{280} (Mean±SD)		DIN[b] (Mean±SD)	
	Shrimp	Worms	Shrimp	Worms	Shrimp	Worms
L1	332±31	995.7±193	1.99±0.02	1.86±0.1	1.8±0.2	5.7±0.1
L2	367±28	56.73±24	2.03±0.01	1.81±0.03	1.5	4.4±2.0
CB	286±43	240.5±243	2.01±0.01	1.86±0.02	1.8±0.4	6.1±0.3
S3	157±28	759±181	1.90±0.02	1.87±0.02	1.7±0.07	1.5±0.1

[a] – A ratio of 1.8 – 2.0 is an acceptable range of good quality DNA

[b] – DIN values range from 1 to 10, where 1 indicates highly degraded gDNA and 10 represents highly intact gDNA

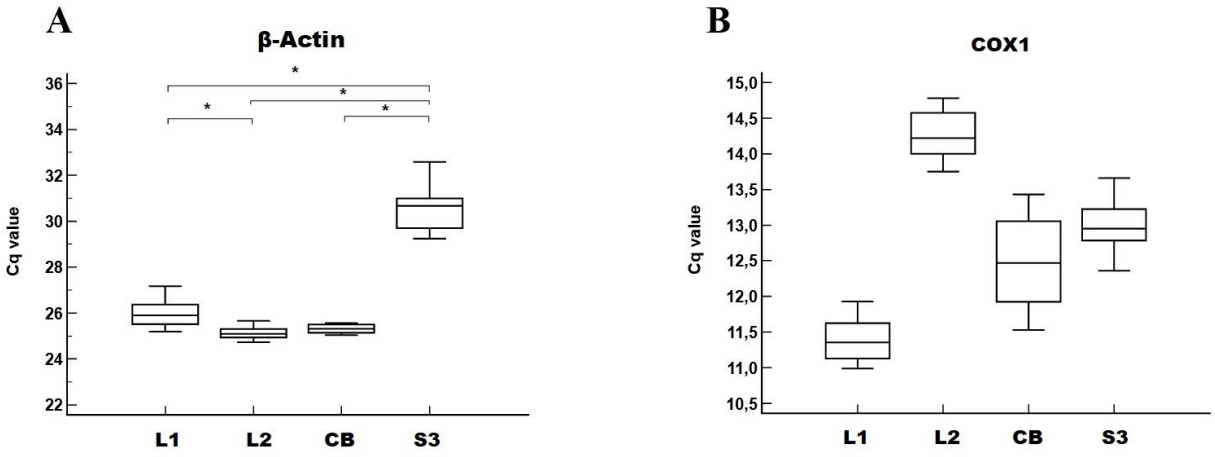

Figure 1. Comparison of qPCR Cq values targeting (A) the β-actin gene in shrimp samples and (B) the COX1 gene in sandworm samples. Data is presented as box and whisker plots indicating the median, the 25th and 75th percentiles as box, and the 10th and 90th percentiles as whiskers. * P<0.05.

(shrimp) and COX1 (sandworm) genes was assessed via qPCR. Sample DNA extracted with buffer L2 and commercial buffer showed the lowest Cq values (the highest copy number) in β-actin SYBR qPCR, with mean 25.12±0.2 and 25.37±0.3, respectively, while S3 sample DNA showed the highest Cq values (low copy number) and was substantially deviated from other treatment groups (P<0.05) (Figure 1A). The amplification of a 114 bp fragment of the sandworm COX1 gene showed that amongst four examined buffers, L1 resulted in the highest copy number (Figure 1B).

Subsequently, Shrimp MultiPath™ was used to further evaluate the efficacy of the tested extraction buffers. The SMP panel contains several DNA and RNA pathogen targets as well as two internal control assays, targeting DNA and mRNA fragments of the housekeeping gene, β-actin, which are designed to validate the quality of extracted DNA (β-Actin_gDNA) and RNA (β-Actin_mRNA). Assay β-Actin_gDNA detected the highest copy number in commercial buffer group samples with mean 671±445, followed by L1 buffer: 564±153, and L2: 522±59 copy number / reaction (Figure 2A). As in the qPCR analysis, a significantly lower copy number was detected in S3 group samples (P<0.05). A similar trend was observed for the β-Actin_mRNA assay. All samples passed the RNA control assay, L1, commercial buffer, and L2 group samples with a copy number higher than the limit of quantitation (HIGH), whereas S3 group samples occurred as an outlier, with significantly lower, deviating 150±60 copy number / reaction (Table 2). Subsequently, sandworm TNA samples were tested for the presence of

Table 2. Shrimp β-actin genomic DNA (β-Actin_gDNA) and messenger RNA (β-Actin_mRNA), *Enterocytozoon hepatopenaei pathogen* (EHP), *Infectious hypodermal and hematopoietic necrosis virus* (IHHNV), *Gill associated virus* (GAV-YHV2), *White spot syndrome virus* (WSSV) assay performance as reported on Shrimp MultiPath™. HIGH score represents samples with copy number higher than the limit of quantitation.

BUFFER	β-Actin_gNDA	β_Actin_mRNA	EHP	IHHNV	GAV	WSSV
L1	564±153	HIGH	142±52	808±141	1377±204	HIGH
L2	522±59	HIGH	121±48	na	na	na
CB	671±445	HIGH	82±51	1066±314	1408±195	HIGH
S3	359±32	428±131	150±60	na	na	na

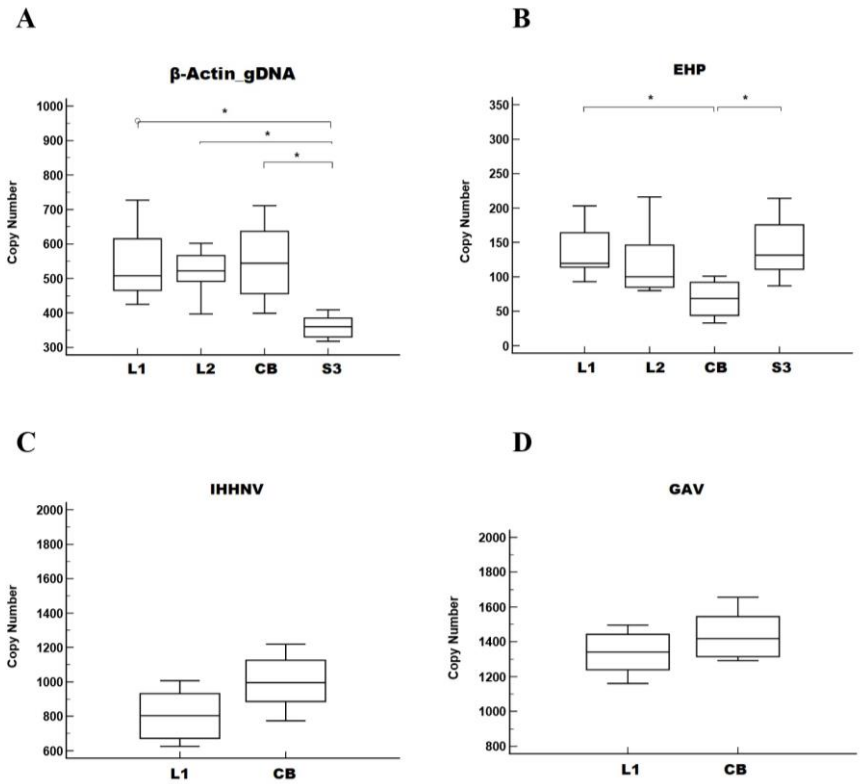

Figure 2. Molecular assessment of TNA quality extracted from shrimp and sandworm samples using buffers L1, L2, commercial buffer (CB), and S3. DNA/RNA quality was assessed by quantifying the copy number / reaction of (A) shrimp β-actin genomic DNA (β-Actin_gDNA), (B) *Enterocytozoon hepatopenaei pathogen* (EHP) (sandworm samples), (C) *Infectious hypodermal and hematopoietic necrosis virus* (IHHNV), (D) *Gill associated virus* (GAV-YHV2) using Shrimp MultiPath™ panel. Data is presented as box and whisker plots indicating the median, the 25th and 75th percentiles as box, and the 10th and 90th percentiles as whiskers. * P<0.05

Enterocytozoon hepatopenaei pathogen (EHP), which was previously reported in this species. In contrary to shrimp samples, comparative analysis of EHP copy number in sandworm samples indicated that S3 buffer performed the best, together with L1. The detection rate was significantly higher in both groups in comparison to the commercial buffer samples ($P<0.05$) (Figure 2B).

Based on the summary of results including DNA yield, qPCR performance and Shrimp MultiPath™ performance, buffer L1 was selected for further evaluation and compared to the commercial buffer as a reference/control.

The next stage of the experiment aimed to test the concordance of SMP results of selected DNA- and RNA-based pathogens comparing L1 and commercial buffer extracted samples. For this purpose, shrimp samples which previously tested positive for DNA viruses, *Infectious hypodermal and hematopoietic necrosis virus* (IHHNV), *White spot syndrome virus* (WSSV) as well as RNA virus, *Yellow-head virus type 2* or *Gill associated virus* (GAV-YHV2) were selected. Buffer L1 and commercial buffer extracted samples performed at a similar level with respect to pathogen detection and copy number determination. The pathogen profile for L1 buffer extracted samples was as follows: IHHNV 808±141 copies / reaction; GAV 1377±204 copies / reaction; WSSV: HIGH (above limit of quantitation); and for commercial buffer: IHHNV 1066±314 copies / reaction; GAV 1408±195 copies / reaction; WSSV: HIGH, resulting in 100% concordance measured for detection of all analyzed DNA and RNA targets (Table 2, Figure 2C-D).

The presented results show that the composition of extraction buffers and the concentration of individual compounds have a considerable effect on the extraction of high-quality nucleic acids. Guanidinium hydrochloride (GnHCl) is known as a protein denaturing agent and is used in many buffers at a very high concentration, up to 6 M (Weidner et al., 2022). Even though the chaotropic salt alters the secondary and tertiary structures of many proteins and inhibits RNases, it has also been shown that 4 M GnHCl is not effective with respect to inhibiting all DNA nuclease activity at room temperature (Pramanick et al., 1976). Hence, a more effective buffer proved to be buffer L1 which is supplemented with EDTA, a chelating agent for ions such as Ca^{2+}, Zn^{2+}, Mg^{2+}, and Mn^{2+}, which are the most common cofactors for nucleases. Another reason for the notable difference between L1 and S3 buffer efficacy could be the addition of 0.825 M NaCl to buffer S3. Although NaCl plays a key role in disrupting molecular interactions by keeping proteins soluble and increasing the ionic strength of the buffer, it is essential to optimize its concentration, as it displays either dispersive or binding effects on DNA, causing its precipitation along with proteins (Besbes et al., 2011). Another reason for the performance of buffer L1 might be attributed to the high pH level. The use of alkaline extraction buffers results in higher DNA yields (Lever et al., 2015), whereas S3 buffer, as with most of the conventional phosphate- or TE-based solutions had pH 7.9.

Maintaining a constant pH is critical for nucleic acid isolation, as fluctuations in pH levels can cause damage to nucleic acids, affect the activity of enzymes involved in the isolation process, or interfere with downstream applications. Buffer L2 and a commercial buffer used in this study have a neutral pH 7.0 and overall, both performed well in experiments with shrimp tissue initially. However, over time, the pH of buffer L2 decreased significantly, resulting in lower performance.

The formulation of the in-house buffers can have several benefits, including customization; buffers can be customized to meet the specific needs of the particular experiment. This allows for a greater flexibility in the experimental design and can improve the quality and reproducibility of data generated. Furthermore, custom formulation can lead to unique and adapted extraction solutions for challenging, complex sample matrices. Moreover, it is possible that in-house buffers can improve the integrity and efficiency of the yield of the extracted nucleic acid.

All utilized approaches to quantitatively assess the yield and purity of nucleic acids, together with detection of pathogen targets with Shrimp MultiPath™ showed that among three in-house prepared buffers, L1 performs equally well compared to the commercially available buffer and can be used as a suitable alternative for these complex tissue types and the PCR-based Shrimp MultiPath™ downstream workflow.

Ethical Statement

All samples were collected on commercial shrimp farms during routine diagnostic sampling after permission from the farm owner. No specific permits from an animal ethics committee were required.

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Author Contribution

JG: Conceptualization, Data Curation, Investigation, Writing - original draft; BG: Investigation, Methodology; LF: Methodology, Sample acquisition; MS: Funding Acquisition, Writing - review and editing; RM: Conceptualization, Data Curation, Supervision, Writing -review and editing.

Conflict of Interest

JG, BG, LF, RM and MS are affiliated with Genics Pty Ltd, which provides one of the methods used in the present paper (Shrimp MultiPath™) as a commercial service.

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