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Pressure Cycling Technology (PCT) Applications in Extraction of Biomolecules from Challenging Biological Samples

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Abstract
The Barocycler ™ NEP3229, a commercially available pressure cycling instrument from Pressure BioSciences, can be used to process up to 0.5 g solid or 1.5 mL liquid samples in a single-use PULSE™ Tube. The Barocycler can rapidly generate alternating hydrostatic pressure between ambient and 235 MPa within a 3 second rise time and millisecond pressure drop time. It was found that, during an exposure to multiple cycles of pressure, biomolecules, such as nucleic acids, proteins, lipids and small molecules can be extracted into a lysis buffer from cells and tissues. The composition of the lysis buffer, pressure cycling parameters, and process temperature (4-50ºC) conditions can be adjusted for specific applications. Here, we describe our recent developments in the extraction of biomolecules from important yet recalcitrant biological samples. In an effort of extracting proteins for proteomic studies, samples of microorganisms (*Escherichia coli, Saccharomyces cerevisiae, Vibrio parahaemolyticus, Rhodopseudomonas palustris*), nematodes (*Caenorhabditis elegans*), plant (*Strelitzia reginae*) and animal tissues such as whole *Danio rerio* (zebra fish), rat liver, brain and adipose tissue, were processed using PCT. 2D gel electrophoresis, liquid chromatography and mass spectrometry were employed in the analysis of the PCT-extracted protein species. It was shown that extracts obtained by PCT frequently yield more protein species, particularly among low abundance, high molecular weight, hydrophobic and basic proteins, than many conventional extraction techniques. PCT can also be beneficial for the extraction and enrichment of nucleic acids from small or difficult to lyse samples. For example, PCT protocols have been developed for DNA extraction from hard-to-break materials, such as bone fragments and teeth, hair and skin. These samples can be in minute quantity, such as single hair or small bloodstain spot on a single cotton thread. In the absence of pulverization and substantial de-calcification, DNA molecules were successfully extracted from exhumed ancient bone fragments for sequence analysis. These studies illustrate the capabilities and potential of PCT applications for extraction of various analytes from challenging biological samples leading to new opportunities in drug discovery, diagnostics and biotechnology.

Keywords: high hydrostatic pressure, protein extraction, nucleic acid extraction, sample preparation, proteomics, genomics, Pressure Cycling Technology.

1. Introduction

Cells and tissues in many biological samples contain difficult to solubilize structural elements, such as, complex cell walls, cuticles, shells, elements of cytoskeleton and components of high mineral or collagen content. However, the vast majority of modern analytical methods require an input of fully dissolved analytes. Conventional solubilization methods often depend on total disruption or homogenization of the samples. These

extraction processes are often laborious and difficult to automate. For certain analyses, it is preferred that biomolecules retain their structure and/or function. However, many popular homogenization techniques may be disruptive to biomolecular complexes and individual molecules of interest. Since sample preparation is a critical prerequisite for a successful molecular analysis, efficient and reliable extraction procedures are essential, particularly those addressing the extraction of various analytes from recalcitrant samples.

A novel approach for sample preparation has been developed using alternating levels of hydrostatic pressure, which is called, Pressure Cycling Technology (PCT) [1-2]. PCT sample preparation can be carried out in a cyclic pressurization device, called a Barocycler NEP3229, which is equipped with disposable sample containers called PULSE (Pressure Utilized to Lyse Samples for Extraction) Tubes. PCT was found effective in processing a wide variety of biological samples. This article provides several illustrations of this technique. Common sample preparation challenges are summarized in Table 1.

Table 1. Common Challenges in Biological Sample Preparation

Nature of Challenges	Examples	Current Methods
Rigid cell walls (polysaccharides or proteoglycans)	• microorganims • spores • fungi • plant tissues • algae • connective tissue • vascular tissues	Bead milling, freezer mill, sonication, enzymatic digestion
High protein or lipid content	• adipose tissue • brain • seeds	Grinding in liquid nitrogen
Mineralization or highly crosslinked polypeptides	• teeth • bone • hair • fingernails	Pulverization in liquid nitrogen
Digestive enzymes	• liver • pancreas	Homogenization at low temperatures
Complex matrices	• soil • food • stool	Soaking in buffer, homogenization

2. The PCT Sample Preparation System for Biological Sample Extraction

The Barocycler NEP3229 instrument was developed for generating the alternating levels of hydrostatic pressure. The instrument can generate cycles of pressure between ambient and 280 MPa, within a 3 second rise time and nearly instantaneous depressurization time. Dwell times of 5-60 seconds at either ambient or high pressure can be set by the user. Pressure chamber temperature can be regulated between 4 and 50ºC by an external circulating water bath. The single-use PULSE Tube is designed for processing up to 1.5 mL liquid samples (cell suspensions) or up to 500 mg of solid tissue immersed into the adequate amount of lysis

buffer resulting in the equivalent total sample volume of ~ 1.5 mL. Together, the Barocycler and PULSE Tube form the basis of the PCT Sample Preparation System (PCT SPS). The PCT SPS allows simultaneous processing of up to 3 samples within a few minutes. For typical tissues or cultured cells, satisfactory extraction results are usually obtained using a protocol consisting of 5 one minute cycles at 235MPa, at ambient temperature without further optimization in the pressure cycling conditions. Extraction buffers are usually chosen based on the requirements of subsequent steps. For example, nucleic acids are extracted with common chaotropic agents for subsequent affinity purification [1,3]. Total protein extraction may be performed in a buffer containing high concentration of denaturants and detergents or even distilled water for SDS-PAGE, 2D gel electrophoresis or multidimensional liquid chromatography [2,4]. In some cases, buffers containing lower concentration of chaotropic salts or detergents can be used directly for PCR amplification of DNA or immunoassays, thus bypassing tedious purification steps [1]. Because samples processed in PULSE Tubes are fully contained in a vessel manufactured from solvent-resistant polypropylene, extraction can also be conducted in the presence of certain organic solvents. The extraction parameters can be optimized according to the specific sample type and downstream application requirements.

3. Processing Challenging Biological Sample Using PCT SPS

When some 'difficult' samples are processed, such as the ones listed in Table 1, special processing parameters often need to be evaluated and optimized based on the nature of the physical and chemical structure of the specimen. Several examples of these specimens are described below.

3.1 Microorganisms: Spores of Gram-positive bacteria

Bacillus sp. are rod-shaped, aerobic or facultative, and endospore-forming bacteria. The spores of *Bacillus sp.* are particularly hard to lyse by either physical or chemical means due to its structure and composition. The surface of *Bacillus* spore is complex [5]. *Bacillus* spore core is surrounded by the core wall, the cortex and the spore coat. The core wall is composed of peptidoglycan as the vegetative cell wall. The cortex is composed of a unique peptidoglycan with three repeat subunits, it also contains meso-diaminopimelic acid, and has very little cross-linking between tetrapeptide chains. The outer spore coat represents 30-60 percent of the dry weight of the spore. The spore coat proteins have a relatively high content of cysteine and hydrophobic amino acids. They are highly resistant to treatments that solubilize most other proteins. In addition, the cytoplasmic water activity is very low in these spores. These make *Bacillus* spores highly resistant to stringent environmental conditions, such as heat, pressure, chemicals, dyes, and lytic enzymes.

Extraction of biomolecules, such as DNA, from *Bacillus sp.* spores typically includes homogenization steps, such as bead beating or sonication. It was reported that only 10 and 30% of total DNA presented in the extracts. In our experiments, *Bacillus subtilis* spores were prepared based on the method described in [6]. The spores were then pelleted and suspended in ATL buffer (QIAGEN, Valencia, CA) with 1% Triton 100 or saturated with Guanidinium HCl supplemented with 1% CHAPS 3-[(3-cholamidopropyl) dimethylammonio]-1 propanesulfonate, 1% Triton and 1% SDS. Samples were then PCT-treated at 35 kpsi for five 1 minute cycles at 22°C. Positive control samples were prepared by bead beating for five 30 sec treatments with 30 sec cooling breaks between treatments. Negative controls

were prepared using identical samples and buffers without any mechanical treatment. After extraction, DNA was purified using QIAGEN DNeasy Mini kit from 250 µL of clarified supernatants. PCT extracts were then split into two aliquots each. One aliquot was heated at 70°C for 10 min prior to adding ethanol according to the DNeasy minikit procedure. The other aliquot was processed at ambient temperature. Resulting DNA products were dissolved in 100 µL of AE buffer and visualized on an agarose gel. Figure 4 shows that PCT in combination with the appropriate buffer selection is capable of releasing DNA from *Bacillus* spores. DNA yields appear to be comparable in samples extracted using either the PCT or a bead beating method.

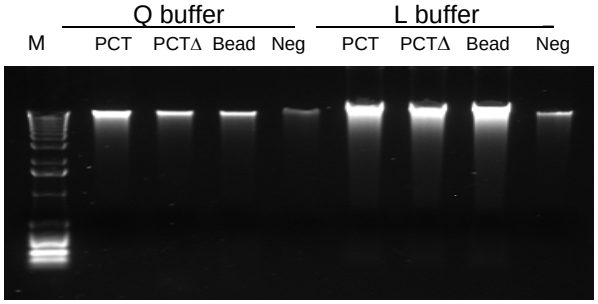


Fig. 1 Agarose gel comparing genomic DNA extracted by PCT or bead beating from *B. subtilis* spores and comparing two extraction buffers (Q and L).

One of the challenges in the detection of bacterial spores is that very low copy numbers of spores (ideally as few as 1-10 copies) must be detected. This requires maximum efficiency in extraction and amplification of the DNA. As shown above, DNA extractions from serial diluted spore samples were compared using the PCT and the bead beating methods, and 'negative' controls without physical treatment. The crude lysates was further purified using the QIAGEN DNeasy kit and copy numbers of spore DNA was quantitatively analyzed using a real-time PCR method. However, it was found that it is difficult to accurately determine the extraction efficiency of PCT or bead beating due to the presence of relatively large signals in the negative controls, presumably due to exogenous bacterial DNA retained on the surface of the spores. In other experiments, spores were incubated directly in PULSE Tubes containing cultural medium. After the incubation for a few hours, the spores converted to the vegetative form of the bacterium and were allowed to replicate. This method has several advantages. First, more cells containing target DNA are formed during replication. This results in an increase in sensitivity. Not only is vegetative form safer than the spores, the vegetative form is easier to lyse. This allowed for DNA to be extracted directly by PCT SPS, without the requirement of changing buffer or opening the PULSE Tube. This approach not only increases the sensitivity but potentially offers the researcher a safer method of processing pathogenic microbes.

3.2 Nematodes: organisms with cuticles

Nematodes, such as *Caenorhabditis elegans,* are covered by a protective outer skin called cuticle that is naturally colorless and partly translucent. The cuticle is resistant to host digestive enzymes and resilient to mechanical disruption in most nematodes. It is also relatively resistant to many chemicals, but allows the passage of water molecules and certain small water-soluble ions. The cuticles also appear to function as described as the "hydrostatic skeleton of nematodes". Since the body cavities of nematodes contain pressurized fluids, the cuticle apparently serves to maintain the body at a constant diameter by resisting the internal pressure of these fluids. A variety of bioorganic compounds have

been identified in the cuticles of many nematodes. These include amino acids, proteins, carbohydrates, lipids, RNA, ascorbic acid, ATP and hemoglobin. The conventional method in extracting biomolecules from *C. elegans* is based on sonication.

It was found that DNA, RNA and proteins could be extracted from *C. elegans* using the five one-minute cycles between ambient and 235 MPa pressure in various extraction buffers (Figure 2). However, elevated concentration of chaotrope salts and detergents are necessary in the extraction buffer. For example, DNA was extracted with saturated guanidinium chloride (room temperature) with 1% CHAPS, followed by DNeasy purification (QIAGEN). RNA of *C. elegans* was extracted in 4M guanidine isothiocyanate (GITC) and 1% NP40. RNeasy kit (also from QIAGEN) was employed to purify the RNA from the crude lysates produced by PCT and sonication. For total proteins, a lysis buffer containing 35 mM Tris, 8M urea, 4% CHAPS, and 65 mM DTT (pH 8.0) was used. The control samples were prepared by sonication at 4°C for five rounds of 30 sec ultrasonic treatment interrupted with 30 sec cooling intervals on ice. PCT extractions yielded more DNA, RNA and higher yield of proteins. It was shown that high concentrations of denaturants, detergents and reducing agents are necessary for the extractions of biomolecules from nematodes surrounded with resilient cuticle.

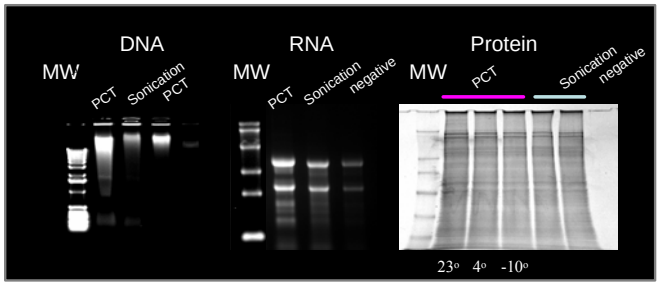
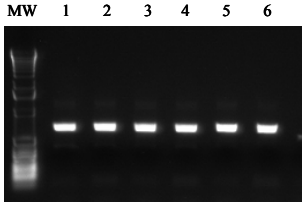


Fig. 2 *C. elegans* extraction comparing PCT with sonication. Panel A: Genomic DNA bands are shown with PCT treatment at -20 and 4°C, left and right respectively. Panel B: Ribosomal RNA bands are shown. Panel C shows the extracted total protein, which PCT was done at 23, 4 and -10°C as labeled.

In a parallel study [7], liquid cultures of *C. elegans* are grown to maturity then collected and washed. Worms were suspended in a lysis buffer (35 mM Tris, 8M urea, 4% CHAPS, 65mM DTT, pH 8.0) that is suitable for membrane protein extraction and treated using 5 cycles of 20 seconds each at 207 MPa, room temperature. Up to 37% higher protein yields ($t=4.71$, $p<.01$) were obtained using PCT relative to sonication. In the 2D gels, it was found that more high molecular weight proteins (possibly due to higher extraction efficiency and decreased proteolysis) as well as more basic proteins were released by PCT compared to sonication. The combination use of the lysis buffer and PCT effectively rupture the tough cuticle allowing for extraction of soluble proteins as well as membrane proteins. Analysis of gel spots on a 2D gel revealed membrane proteins including ATP synthase subunits and several transmembrane channel proteins. By electronic microscopic examination, it was determined that sonication produced largely disintegrated lysate. In contrast, there are many 'empty' cuticles remaining in the PCT lysates. These examples illustrated that, by a combining strong lytic buffers with PCT, high efficiency extraction of biomolecules from cuticle-protected nematodes can be achieved. The ability of reducing the amount of debris and particulate matter offered by PCT extraction is another distinctive advantage of this novel method, since it facilitates easier 'clean up' procedures than those following the conventional total homogenizations, such as sonication.

3.3 Plant tissue samples: wheat seeds

Plant tissue can be resistant to disruption due to high content of water-insoluble polysaccharides, and lipids. Extraction of biomolecules from some plant tissue presents both physical and chemical challenges. Here, extracted genomic DNA from wheat seeds is shown. The released and then purified DNA was amplified by PCR and visualized on an agarose gel (Fig. 3). One or five grains of wheat seeds were processed with 0.5 mL extraction buffer (room temperature saturated guanidinium HCl and 1% CHAPS, pH not adjusted). Inert polypropylene inserts were placed into the PULSE Tube to adjust the total sample volume to the required minimum of 1.3 ml. Pressure cycles between ambient and 235 MPa were applied five times in 1 min duration each. The process temperature was held at 4ºC. After PCT, samples were transferred into microcentrifuge tubes and clarified by centrifugation at 13,000 ×g, 4°C for 2 min. Supernatants (400 µL) were purified using QIAGEN DNeasy Plant Mini kits according to the manufacturer's instructions. DNA was amplified by PCR using ABI 9700 thermocycler with universal primers probing for a non-coding region of its chloroplast DNA [8]. These data demonstrate that the PCT SPS can efficiently extract DNA from hard-to-lyse plant tissue and that the DNA is suitable for further analysis. In addition to seeds, nucleic acids and proteins were successfully extracted from plant flower, leaf, root, stem, pine needles, and corn kernel.



Extraction Method	PCT				Mortar and Pestle	
Lane Number	1	2	3	4	5	6
No. of Seeds	1	5	1	5	1	5

Fig. 3 PCR amplified DNA products from DNA extracted by PCT SPS or mortar/pestle grinding controls. Excellent results were obtained from the PCT extracted samples.

3.4 Animal tissue samples: genomic DNA from pig bone

To demonstrate that PCT is suitable for difficult animal samples, an extraction of DNA from porcine femoral bone is shown here. To eliminate the possibility of DNA contamination from soft connective and muscle tissue, the bone was prepared for extraction by first removing tissue with a razor; the bone was subsequently broken apart with a hammer for exposure and removal of soft tissue contained within, and then washed with 10% bleach for 5 min followed by extensive water wash. Bone fragments were then produced by shattering the bone with a hammer.

To optimize the release of DNA from bone fragments, a series of time course experiments were conducted by incubating bone fragments of approximately 250 mg in various acids for 0 min to 12 hours at ambient pressure prior to PCT treatment. Optimum DNA release was obtained by incubating the bone fragments in 1% acetic acid (pH 4.8) for 60 minutes, followed by PCT treatment (see Figure 4). Negative controls were incubated under identical conditions, but not exposed to pressure cycles. DNA released from PCT-processed bone was compared to DNA released from bone that had been first chilled in liquid nitrogen and subsequently pulverized using a tissue pulverizer (BioSpect Products, Bartlesville, OK). Each extract was purified using a QIAGEN DNeasy kit according to the manufacturer's instructions. The resulting DNA was amplified by PCR using primers for pig β-actin DNA. PCR products were analyzed using a 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA). Yield of DNA was determined semi-quantitatively by integrating the resulting DNA PCR product bands.

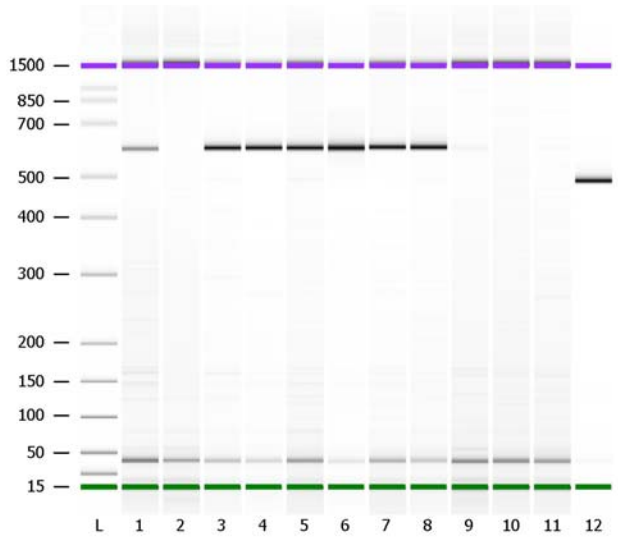


Fig. 4 PCR products examined using the Agilent BioAnalyzer. Lanes 1-8 were from samples incubated with acetic acid (pH 4.9) and saturated EDTA for 60 min and followed by 10 cycles of PCT at 4 °C. Lanes 9-11 were incubated with acetic acid for 1 hr but no PCT. Lanes 12 was a PCR positive control.

4. Conclusions

The PCT Sample Preparation System (PCT SPS) is a significant advancement in modern sample preparation. The System is suitable for the initial steps of sample preparation for genomics, proteomics and purification of biomolecules from cells and tissues. It offers several advantages over conventional cell and tissue homogenization methods, including reproducibility, safety, convenience, speed and precise control over the process. These features are inherent in the design of the system and in the biophysical properties of PCT. Pressure travels through the sample rapidly and delivers precisely identical amount of energy to every cell in the biological sample. Pressure cycling does not create high shearing forces, which could deliver sufficient energy to rupture covalent bonds. The PULSE Tube is compatible with many solvents, including organic solvents, use of which could be problematic with open extraction systems, such as mortal-and-pestle grinding, and Dounce homogenization. Moreover, pressure cycling is an orthogonal sample preparation technique which may be used to reveal analytes that may not be released by other methods. The examples shown in this publication demonstrate that PCT provides a platform technology that is the basis for improved extraction of biomolecules from several types of challenging biological samples. The biophysical process of PCT may also be incorporated into various designs of fully automated sample preparation systems, thus significantly reducing the bottleneck of sample preparation in biological research.

4. References

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