Chapter 2

Pressure Cycling Technology in Systems Biology

Bradford S. Powell, Alexander V. Lazarev, Greta Carlson, Alexander R. Ivanov, and David A. Rozak

Abstract

Systems biologists frequently seek to integrate complex data sets of diverse analytes into a comprehensive picture of an organism's biological state under defined environmental conditions. Although one would prefer to collect these data from the same sample, technical limitations with traditional sample preparation methods often commit the investigator to extracting one type of analyte at the expense of losing all others. Often, volume further constrains the range of experiments that can be collected from a single sample. The practical solution employed to date has been to rely on information collected from multiple replicate experiments and similar historical or reported data. While this approach has been popular, the integration of information collected from disparate single-analyte sample preparation streams increases uncertainty due to nonalignment during comparative analysis, and such gaps accumulate quickly when combining multiple data sets. Regrettably, discontinuities between separate data streams can confound a whole understanding of the biological system being investigated. This difficulty is further compounded for researchers handling highly pathogenic samples, in which it is often necessary to use harsh chemicals or high-energy sterilization procedures that damage the target analytes. Ultra-high pressure cycling technology (PCT), also known as barocycling, is an emerging sample preparation strategy that has distinct advantages for systems biology studies because it neither commits the researcher to pursuing a specific analyte nor leads to the degradation of target material. In fact, samples prepared under pressure cycling conditions have been shown to yield a more complete set of analytes due to uniform disruption of the sample matrix coupled with an advantageous high pressure solvent environment. Fortunately, PCT safely sterilizes and extracts complex or pathogenic viral, bacterial, and spore samples without adversely affecting the constituent biomolecules valued as informative and meaningful analytes. This chapter provides procedures and findings associated with incorporating PCT into systems biology as a new and enabling approach to preanalytical sample treatment.

Key words: Pressure cycling technology, Barocycling, Sample processing, Biomolecule extraction, Biological threat agent inactivation, Sterilization, Systems biology, Ultra high pressure, Sample preparation

Abbreviations

BSL	Biological safety level
cfu	Colony forming units
FEP	Fluorinated ethylene propylene

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MMIB Muscle Mitochondria Isolation Buffer
PBI Pressure BioSciences, Inc.
PCT Pressure cycling technology, also Barocycling
SPS Sample preparation system
TSB Tryptone Soy Broth

1. Introduction

Sample preparation is often a significant bottleneck to discoveries in systems biology, particularly with regard to conventional means of inactivating infectious organisms or samples associated with complex matrices. Conventional sample preparation techniques frequently consume the entire sample for the isolation of a single class of analyte, such as nucleic acid, protein, or other substance. The presence of background matrix, such as blood, soil, or natural polymer, further limits biomolecule extraction and analysis. Improved methods of sample inactivation, processing, and extraction are needed to safely separate whole biomolecules from various samples containing problematic matrices or contaminated with hazardous biological select agents that do not consequently destroy the variety of analytes being sought for study, whether protein, nucleic acid, and toxin, lipid, carbohydrate, etc. Fortunately, new technologies are now becoming available to achieve nondestructive inactivation and extraction of biomolecules for safer handling, convenient transport, stable storage, and broad target analysis of multiple classes of biomolecular analytes. In this regard, we have found that PCT provides sample processing capabilities that are unmatched by other commercially available techniques.

Conventional inactivation methods, such as pressurized steam (autoclave), irradiation, oxidizers, and cross-linking agents often require facility engineering and can substantially modify or destroy covalent bond structure, preventing high-yield proteomic and metabolomic analyses, as has been reviewed elsewhere (1). While syringe filtration is a popular solution, it is tedious and increases the risk of releasing highly infectious agents. Other physical disruption methods, such as bead milling and sonication, generate excessive heat, which rapidly accelerates the hydrolysis of urea and the formation of isocyanic acid, resulting in the potential carbamylation of primary amines as well as potential health risks from aerosols. These methods are also subject to occasional breach, which again is unsafe when used to extract dangerous biological agents. Popular nucleic acid extraction agents, such as TRIzol®, are hazardous and also modify proteins. A few reports have described methods for the isolation of proteins after a TRIzol® RNA and DNA extraction procedure. Most of the methods are time consuming and require

purification by gel electrophoresis to remove compounds that inhibit subsequent analysis (2–6). Although the purification of proteins either after extraction of DNA and RNA or by a conventional method appeared to be comparable, it cannot be determined if the conventional method against which the comparison was made was optimal.

These impediments to complete systems biology research and development can be circumvented using a new approach called ultra-high pressure cycling technology (PCT) (see Subheading 1.2) that employ unique physical conditions to inactivate microorganisms and extract biomolecules while concurrently preserving their covalent structure and function for sensitive analysis. The reported applications for PCT are diverse and have been expanding, particularly its use as a successful means of isolating biomolecules (7–11), see Table 1. The central advantage of PCT is its capability to rapidly alternate in programmed cycles of high and low pressure to induce dissolution of matrix from sample and biomolecules from each other. During PCT, samples are placed within disposable sample containers called "pressure utilized to lyse samples for extraction" (PULSE) tubes and are subjected to up to 60,000 psi (414 MPa) in less than 3 s and returned to ambient pressure in less than 1 s. Maximum and minimum pressures, timing of cycles, and the number of cycles are controlled using a computer or programmable logic controller interface of the Barocycler instrument (Pressure BioSciences, South Easton, MA). PULSE Tube temperature is controlled using a peripheral circulating water bath. Risk of exposure to the researcher is mitigated by safety features of the PCT sample preparation system (SPS). Furthermore, the PULSE Tubes can be used to transport and store the disrupted samples after pressure treatment, significantly minimizing the risk of contamination associated with handling. Improved approaches to inactivation of pathogens will enhance the safety and security of all personnel involved in molecular study of infectious pathogens, from sample handling, through transport, archival, and analysis. This technology has the potential to lower costs and time associated with inactivation treatment, transport, storage, and documentation due to effective and rapid biological inactivation and resultant room temperature stability of extracted bioanalytes.

Although PCT separates proteins from noncovalently associated components through disruption of natural intermolecular forces, an early study demonstrated that some proteins may be recovered with full activity. Bradley et al. (7) identified the optimal temperature, applied pressure, cycle frequency, and number of cycles for PCT inactivation of lambda phage in spiked human plasma samples, and found that varying these parameters did not affect the activities of alkaline phosphatase and total amylase in human plasma samples subjected to the same treatments. Increased yields of *Escherichia coli* proteins were observed on 2D gels after PCT compared to

Table 1
Pressure cycling applications

Application	Sample type	Instrument	Comments	References
Native protein isolation, immunoaffinity enrichment	Cells, tissues, whole organisms	Barocycler NEP3229 and NEP2320; Pressure Cycling Technology (PCT) Shredder, Shredder SG3	Extraction of poorly soluble membrane-associated and integral membrane proteins in their native conformation	(71)
Systems biology	Biological fluids, cells, tissues, whole organisms	Barocycler NEP3229 or NEP2320; PCT Shredder, Shredder SG3	Detergent-free concurrent extraction of proteins, nucleic acids, and lipids	(8, 67)
Proteomics: protein extraction	Cells, tissues, whole organisms	Barocycler NEP3229 or NEP2320; PCT Shredder, Shredder SG3	Pressure-enhanced extraction of hydrophobic membrane proteins	(9–11, 41, 72)
Genomics, transcriptomics, DNA sequencing: extraction of nucleic acids	Cells, tissues, whole organisms	Barocycler NEP3229 or NEP2320, PCT Shredder, Shredder SG3	Better recovery of nucleic acids from difficult to lyse samples. Intact or minimally sheared genomic DNA	(73, 74)
Concurrent decontamination and extraction of multiple analytes from highly pathogenic organisms	Samples containing BSL-3 and BSL-4-rated material	Barocycler NEP3229 and NEP2320	Synergy of high temperature, pressure, and chemistry inactivates pathogens	(1)
Pressure-enhanced enzymatic reactions	Samples for proteomics and glycoproteomics	Barocycler NEP3229 and NEP2320	Pressure helps to speed up digestion and decrease the amount of denaturants	(40, 41, 44, 75)
Structural biology, protein conformation studies	Samples of proteins, protein complexes, lyposomes, cells, or microorganisms	Barocycler HUB440	Real-time pressure-perturbation spectroscopy (UV/Vis, fluorescence, EPR, NMR)	(76)

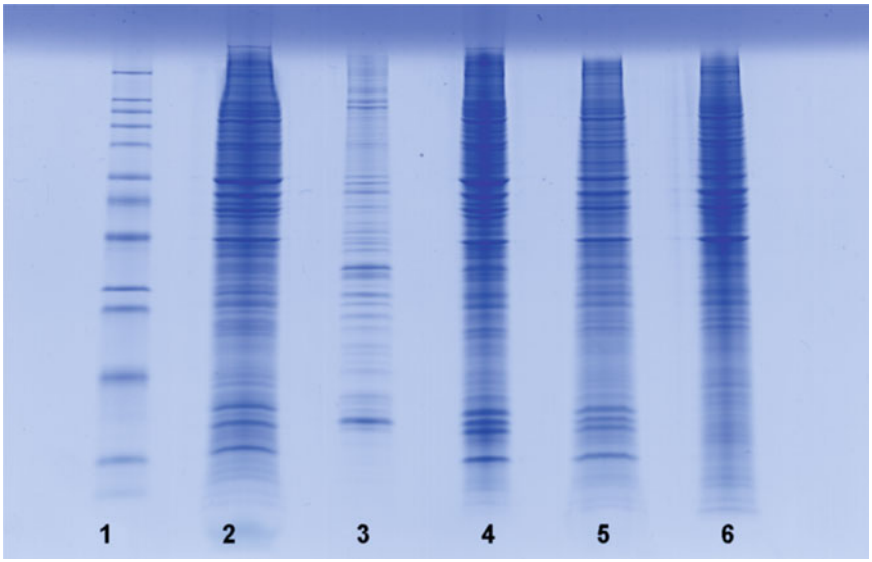

Fig. 1. Comparison of different protein extraction techniques from human fibroblasts cells. (*1*) Molecular weight standard, (*2*) ProteoSolve SB, (*3*) residual protein from insoluble fraction—ProteoSolve SB, (*4*) Trizol, (*5*) Ambion PARIS, (*6*) Quagen Allprep.

samples prepared on a bead mill (10), sonicated (11), or ground in glass homogenizers with or without sonication (9). PCT technology can also inactivate problematic microorganisms and has been shown effective for a wide range of molecular diagnostic analytes including blood, swabs, bone, hair, mummified and formalin-fixed tissue (12–14), fossilized amber (15), soil, and other problematic matrices (16, 17). Finally, when comparisons were made between samples prepared by PCT using the ProteoSolve SB chemistry or prepared by different kits (TRIzol, AllPrep kit, PARIS kit), several protein species were preferentially extracted by PCT-optimized SPS (Fig. 1).

1.1. A Need for New Tools and Methods

The value of systems biology rests on its ability to uncover meaningful interrelation of information concerning different classes of biomolecules with other knowledge of the biological entity being investigated. Until now, systems biology investigations have generally tested one or a few classes of biomolecule with reliance on prior information to construct an understanding or test a hypothesis. A clearer picture evolves as each data stream is integrated into the growing model of the system. For example, total soluble protein may be recovered, trypsinized, and then identified by mass spectrometry to list gene products associated with a given disease state. The new findings of protein levels or posttranslational modification may then be compared to lists from microarray heat maps, promoter activity profiles, or secondary metabolic activities, with each data set

having been collected independently on a "separate but equivalent" test system. There are understandable limitations to the number of concurrent tests that can be performed on a single sample, commonly including sample amount, yield of extracted target, and assay sensitivity. Other features can further reduce sample testing, such as interference by background matrix (consider fossilized tissue set in amber (15)) or dangerously infectious bacteria or viruses that require safe inactivation. Most current inactivation methods stem from standard means of decontamination that largely destroy the biomolecule of interest. Fortunately, a technology exists that can inactivate dangerous pathogenic organisms and extract whole biomolecules from various samples matrices in a simple user-friendly platform, as described throughout this chapter.

1.2. The Promise of Pressure Cycling Technologies

PCT is a recently developed sample preparation technology that is rapidly growing in application across many fields of biology and is uniquely suited for systems biology (8). PCT can extract multiple concurrent biomolecular targets from complex and problematic sample matrix and has demonstrated the capability to inactivate some of the most dangerous pathogenic microbes while preserving molecule integrity for sophisticated analysis. A survey of PCT applications is given in Table 1. Unlike common physical-based cell disruption technologies (homogenization, sonication, microfluidization, etc.), PCT does not impart shear force and heating is controllable. This has facilitated the development of simple methods that isolate intact and functional organelles (18) (see Subheading 3.5). Furthermore, PCT treatment in combination with an amphipathic solvent system inactivates enzymes and maintains the inactive state, which makes the method suitable for recovery of almost any biological molecule: peptide, protein, RNA, DNA, carbohydrate, lipid, metabolite, and their combinations. Moreover, PCT-treated sample held in this solvent is stable at room temperature, which extends the shelf life and simplifies the handling of special biological samples. Importantly, PCT enables safe and simple inactivation of dangerous pathogens without imparting covalent damage onto target molecules as occurs during standard methods of decontamination. Furthermore, because PCT allows the extraction of multiple molecule types from limited or complex sample, it effectively provides target amplification. Thus, PCT provides great flexibility and a common sample preparation platform for experimental design and the integration of multiple findings. These PCT capabilities far exceed those of conventional sample preparation methods and therefore can uniquely benefit research programs in systems biology.

1.3. Current Applications of High Pressure in the Life Sciences and Biotechnology

Most people are very familiar and comfortable with temperature control equipment—from household to the laboratory and industrial settings temperature control play a significant role in our lives. Pressure is a thermodynamic parameter orthogonal to temperature and is equally important in defining the state of matter and molecular interactions. Pressure, unlike temperature, travels through liquid media at the speed of sound, independent of diffusion or convection kinetics. Therefore, an attempt to use high hydrostatic pressure as a synergistic thermodynamic parameter, or as an alternative to high temperature, should theoretically allow more precise control of chemical reactions in shorter intervals of time. However, laboratory adoption of pressure technologies was historically limited by material science and engineering constraints required to generate and maintain extremely high levels of pressure in a confined space.

Hydrostatic pressure is already being used quite successfully in a range of research and industrial applications. For example, high hydrostatic pressure is currently used in the chemical and food industries to modulate synthetic reactions and enzyme catalysis, and as a nonthermal sterilization technology. The use of high hydrostatic pressure is also gaining acceptance in the life sciences and new applications are emerging. Among these applications are the development of vaccines, food allergy inhibition, infectious agent inactivation in transfusion products and transplant organs, and protein crystallization and X-ray crystallography (19, 20). Since the 1960s, high-pressure bioscience and biotechnology research has been developed mainly in Europe and Japan, where funding for high-pressure chemical and biological research has exceeded $100 million/year. Most of these applications to date employ static pressure.

The specific applications of PCTs to systems biology are turning out to be just as varied. These include the inactivation of pathogenic microbes; the preparation of diverse sample sets from a number of complex matrices such as tissue, insect, and spore samples; isolation of eukaryotic organelles; and acceleration of enzymatic reactions. Some of these methods employ PCT procedures sequentially to enable unique sample processing and rapid workup for molecular analysis. These and other applications are briefly addressed below.

1.3.1. Agent Inactivation

A valuable capability of the PCT platform is its unique capacity to inactivate dangerous microbial pathogens while preserving analyte for subsequent analysis by sensitive methods such as mass spectrometry. While this utility can be applied to inactivation and extraction of more widely studied blood-borne infectious agents, such as the hepatitis viruses or human immunodeficiency virus (HIV), we have focused on pretreatment and processing of two select biological threat agents as examples of dangerous and problematic samples:

Bacillus anthracis spores and the mosquito-borne Venezuelan eastern encephalomyelitis virus (VEE). These agents were chosen to demonstrate PCT treatment as each presents an especially intractable difficulty with regard to inactivation and subsequent analysis. In both cases, standard methods of inactivation greatly limited subsequent systems biology. As a positive-strand RNA virus, a treated VEE sample must either be free of infectious RNA or demonstrate full biological inactivity before passage outside of a biological safety level 3 (BSL-3) containment environment. Alternatively, the *B. anthracis* endospore is one of the most durable microbes and we must show full sterility before passage outside of BSL-3 containment. Unfortunately, standard decontamination procedures, such as pressurized steam, formaldehyde gas, or ionizing radiation, damage the covalent integrity of the very biomolecules intended for recovery and analysis. Limitations of standard methods for inactivation of biological select agents have been reviewed elsewhere (1). In contrast, we have found that *B. anthracis* spore samples inactivated and extracted by PCT are suitable for subsequent biomolecular analysis (see Subheading 3.4).

1.3.2. Sample Preparation

Studies pioneered by Pressure BioSciences, Inc. (PBI) (formerly known as Boston Biomedica, Inc.) have shown that the application of alternating hydrostatic pressure to biological specimens can result in more rapid, reproducible, and safe sample preparation, which can be finely controlled by modulating the pressure, temperature, and number of pressure cycles (17). The PCT SPS was the first PCT-based product developed and commercialized by PBI in 2002. Since then, the company has explored the use of PCT to lyse various cell types, extract nucleic acids, control of enzymatic activity, inactivate pathogens, and control molecular binding and elution (21–31).

PCT offers unique advantages compared to other high-pressure processes. First and most significantly, the PCT SPS employs alternating pressure rather than static pressure. Pressure between ambient and high levels, such as 15–60,000 psi (0.104–414 MPa) depending on instrument specifications, can be generated in a few seconds and depressurized on a millisecond time scale. Incubation periods at each pressure level may also be varied, which can be as short as a few seconds or as long as minutes per cycle. Manipulations of these parameters allow for greater control of pressure effects on a sample. This rapid cycling feature enables the extraction of bacterial spores for the construction of spore proteome lists whose contents are not contaminated by alterations associated with germination, which is triggered by high static pressure (1).

PCT processes are fundamentally different from conventional sample preparation methods, including other physical disruption methods, such as homogenization, sonication, bead beating, and French press. These generate high shearing forces, causing uneven energy distribution in a sample, leading to possible rupture of

covalent bonds and heating of the specimens. Resulting heat dissipation relies on heat diffusion rates, even when the sample is surrounded by a thermally controlled medium. Conversely, hydrostatic pressure travels uniformly throughout the entire depth of the sample, supplying an equal amount of energy to every cell in a tissue block simultaneously, devoid of rapid mass transfer, cavitation, or mechanical collisions. Therefore, the PCT process reduces the likelihood of irreversible damage to many labile molecules. This consideration is also supported by the data generated in our laboratory. Hydrostatic pressure is synergistic with thermal treatment predominantly due to the phenomenon of compression heating. Note that various materials are subject to different compression heat generation under identical pressure. Such compression heating is proportional to the compressibility (bulk modulus) of the material, and therefore, lower for the least compressible materials such as water, and much higher for lipids, oils, and other highly compressible substances, such as aliphatic hydrocarbons (paraffin) (32, 33). This phenomenon may be successfully used in cell disruption by exerting higher energies onto the lipid bilayer than to the aqueous matrix of the cells. Consequently, lipid membranes transiently exhibit higher fluidity during the application of high pressure and are destabilized during subsequent rapid depressurization of the system. We expect a similar effect to occur during the deparaffinization of the paraffin-embedded tissue specimens.

1.3.3. Protein Extraction and Fractionation

PCT provides unique value for the design of proteomics investigations. Many methods currently used for tissue lysis and protein extraction rely on aggressive chemicals or vigorous physical shearing forces to disrupt and solubilize samples. For example, bead milling, sonication, and rotor-stator homogenization are commonly used for tissue homogenization. These procedures may result in damage to covalent bonds due to heating, shearing, and/or to protein denaturation. PCT-based extraction methods exhibit unique orthogonal features compared to conventional methods. For example, as pressure is applied to a sample in solution, the disruption of protein structures, such as cellular membranes, breaks open the cells, allowing for the release of their contents. Consequently, pressure cycling-based methods release at least as many, if not more, proteins as other current extraction methods (9–11, 17). Further, in several cases (34–36), distinct protein species were found in the pressure cycling extracts; in particular, high molecular weight species, as well as hydrophobic proteins and molecular complexes.

1.3.4. Subcellular Fractionation

Proteomes of whole cells or tissues can be highly complicated and difficult to dissect and analyze. However, the proteome may be greatly simplified by focusing on isolated molecular complexes or organelles such as mitochondria. Conventional methods for the extraction of biomolecules or organelles from cells and tissues can

lack reproducibility or be too damaging for the extraction of delicate structures. High hydrostatic pressure acts preferentially on the more compressible components of the sample, such as the lipid constituents of the plasma membrane. Under high pressure, the lipids in the plasma membrane transiently form an unstable, interdigitated structure. When the pressure is rapidly released, the destabilized membrane ruptures leading to the release of intracellular contents. The large size of the plasma membrane lipid bilayer makes it particularly vulnerable to damage during pressure cycling, while smaller protein complexes or organelles, e.g., nuclei and mitochondria, can be more tolerant and may require higher pressures for complete disruption.

PCT destabilizes intermolecular interactions by rapidly and repeatedly raising and lowering pressure in the reaction vessel from ambient to high levels (up to 60,000 psi [414 MPa]). The Barocycler instrument allows the user to adjust the pressure that is applied to the sample, permitting precise control of the extent of sample disruption depending on the composition and structure of subcellular membrane components. Therefore, by using PCT, it becomes possible to selectively disrupt plasma membranes and release intracellular complexes in a relatively mild and highly reproducible manner liberating the user from the tedious task of manual tissue homogenization (18, 35).

1.3.5. Acceleration of Enzymatic Hydrolysis

Hydrostatic pressure has been previously shown to enhance enzymatic hydrolysis by chymotrypsin and pepsin (37, 38), as well as by the enzymes alcalase, neutrase, corolase 7089, corolase PN-L, papain (39), and trypsin (40). In our experiments, we have confirmed the positive effects of pressure and additional benefits of alternating hydrostatic pressure (pressure cycling) for several enzymatic reactions including proteinase K, PNGase F, Lys-C, and lysozyme.

Current evidence demonstrates that tissue digestion by proteinase K is accelerated under pressure, resulting in faster isolation of intact genomic DNA both at 55°C and at ambient temperature. Other recent work has focused on enzymatic digestion for peptide analysis. PCT has been shown to significantly accelerate digestion, which frequently requires up to 24 h under ordinary atmospheric pressure. Enzymatic digestion combined with pressure cycling has been shown to improve efficiency of digestion without sacrificing specificity (41–44).

1.3.6. Tick Mitochondrial DNA, Borrelia burgdorferi DNA, and Total Bacterial DNA Detection

PCT has also proven to be extremely effective at freeing bioanalytes from complex matrices such as arthropod vectors. For example, PCT has allowed us to efficiently extract *B. burgdorferi* (the causative agent of Lyme disease) DNA from ticks for real-time PCR analysis and detection. In an illustrative experiment, 21 tick samples from various sources were extracted using the PCT Shredder (further described in Subheading 2.1.2). Of the 14 ticks obtained from the Maryland Department of Agriculture, five were identified

Table 2
Detection of *B. burgdorferi* and total bacterial load in the ticks by real-time PCR

Species	Number of ticks	*B. burgdorferi* detection		Total bacterial detection	
		Positive	Ct values	Positive	Ct values
I. scapularis	5	3	20.28; 23.69; 22.15	5	15.94; 14.11; 17.68; 17.07; 22.41
A. americanum	6	1	29.67	6	17.87; 20.35; 20.15; 21.04; 16.83; 19.91
D. variabilis	3	0		3	17.75; 21.03; 17.86
Unidentified ticks	7	1	23.20	7	19.24; 17.08; 20.11; 17.22; 16.08; 23.20; 24.25

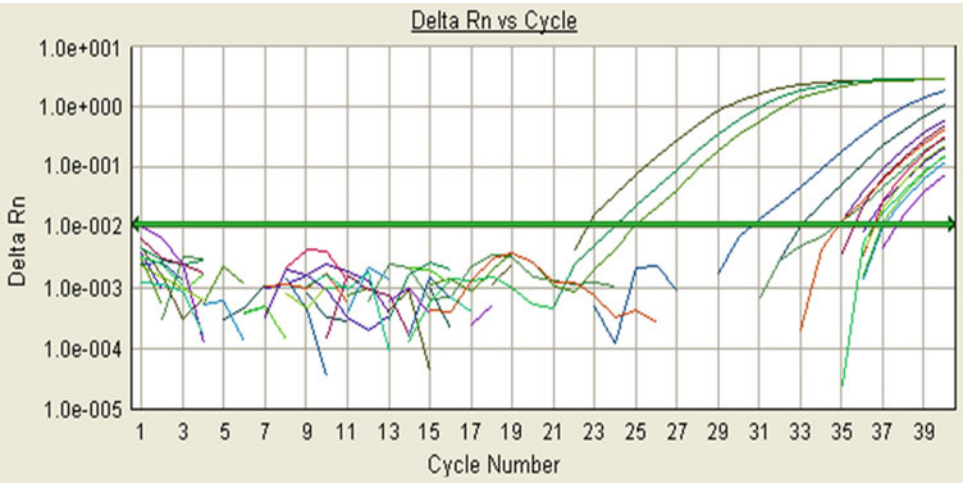

Fig. 2. Real-time PCR detection of *Borrelia burgdorferi* in tick DNA isolated using the PCT Shredder and Pressure Cycling Technology. Real-time PCR detection of *B. burgdorferi* 23S rRNA gene, generating a 75 bp fragment with a Taqman probe. Three extractions out of five on *Ixodes scapularis* were significantly positively amplified (about 10^8–10^9 copies). Two are less strongly amplified. One out of nine *Amblyomma americanum* and *Dermacentor variabilis* are less strongly amplified.

as *Ixodes scapularis*, six were *Amblyomma americanum*, and three were *Dermacentor variabilis*. Real-time PCR detection of *B. burgdorferi* and total bacterial load was performed on all the tick DNA preps using primers published by Courtney et al. (45) and by Black and Piesman (46). As shown in Table 2, DNA specimens extracted from three out of five *I. scapularis* ticks, one out of six *A. americanum* ticks, and one out of seven unidentified ticks randomly collected from domestic dogs in Massachusetts were found to be positive for *B. burgdorferi* by real-time PCR amplification of *B. burgdorferi*-specific 23S rRNA gene (see Fig. 2). All of the 21 tick DNA preps were found

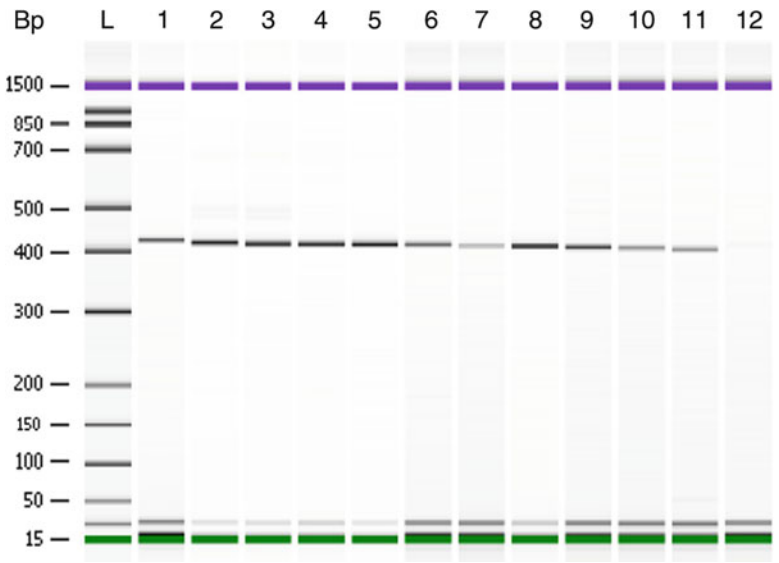

Fig. 3. Mitochondrial DNA isolated from ticks using the PCT Shredder and Pressure Cycling. *Lanes 1–11*: Positive amplification of tick mitochondrial 16S rDNA gene from tick DNA preps isolated using PCT and PCT shredder. *Lane 12* is a PCR negative control. The amplicon produced is approximately 400 bp.

to be positive for general bacterial-specific 16S rDNA, indicating a relatively constant level of total bacterial load. As shown in Fig. 3, positive control PCR assays on all the tick DNA preps demonstrated positive amplification of tick mitochondrial 16S rDNA gene (see Table 2).

2. Materials

2.1. Instruments

2.1.1. Ultra High Pressure Cycler Systems

The PCT SPS has been used to extract protein, DNA, RNA, and small molecules from many types of samples (8, 17, 47, 48). The mechanisms of action of the PCT SPS differ significantly from other sample preparation methods. Specifically, lysis occurs when samples are subjected to alternating cycles of ambient and high pressure, up to 60,000 psi (414 MPa), in the hydrostatic reaction chamber of the Barocycler. The Barocyclers NEP3229, NEP2320, and HUB440 (when equipped with an optional sample chamber) have external chiller hookups and automatic fill and dispense valves. The Barocyclers NEP3229 and NEP2320, with an optional maximum operating pressure of 45,000 psi (310 MPa), have an integrated microprocessor with an easy-to-use keypad, while the HUB440, with a maximum operating pressure of 60,000 psi (414 MPa), has manual or analog external signal control and optional data acquisition and control system with PBI software.

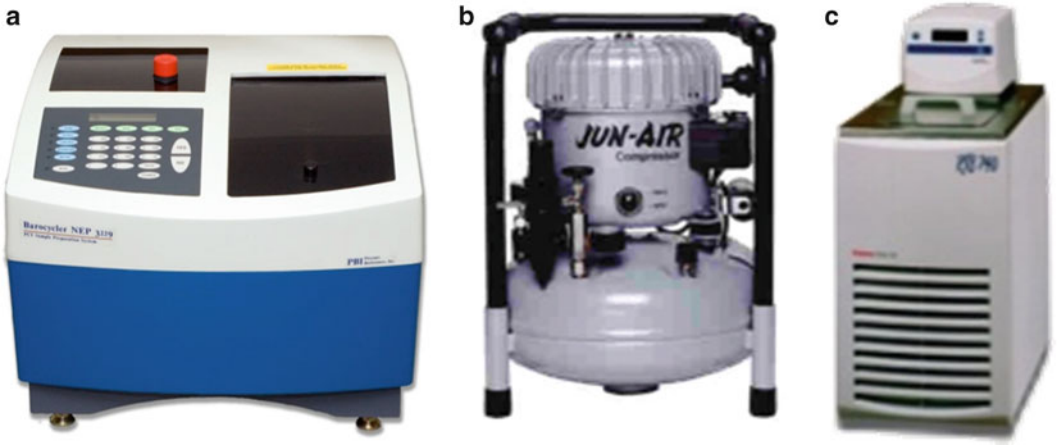

Fig. 4. Typical pressure cycling equipment. Barocycler NEP2320 (**a**) with air compressor (**b**) and optional circulating water bath (**c**).

The larger NEP3229 has a 30 mL capacity chamber, which can accommodate up to 3 PULSE Tubes or 48 MicroTubes and comes with an external hydraulic pump. The smaller NEP2320 has a 12 mL capacity chamber, which holds a single PULSE Tube or 12 MicroTubes, uses pneumatic pressure, and can take advantage of available laboratory compressed air, bottled compressed air, or a stand-alone compressor (Fig. 4). The HUB440 also requires an external air pressure source. Pressure is generated by the hydraulic pump or air compressor to create high- and ultra-high hydrostatic pressure inside the Barocycler reaction chamber. Exquisite control of the PCT process is accomplished by specialized components, a programmable controller, and software. The Barocycler instrument reaction chambers are temperature controlled using a peripheral circulating water bath. Safety features in the PCT system design significantly reduce risk of exposure to the researcher to pathogens (1).

The key advantages of the PCT SPS in biomolecular extraction, sample fractionation, and enzymatic digestion include reproducibility, lack of shearing forces, and flexible sample handling. In addition, because pressure is evenly distributed throughout the sample almost instantaneously, common variations due to gradient effects observed in other extraction methods, e.g., freeze-thawing, boiling, sonication, bead beating, mechanical homogenization, and so on are avoided. Depending on the nature of the sample, PCT parameters may be adjusted to optimize reaction efficiency by changing the temperature, increasing or decreasing the pressure and/or number and duration of pressure cycles, and by modifying extraction chemistry (e.g., by adding denaturants and/or detergents).

2.1.2. Tissue Shredders

The recent introduction of a small tool called the "PCT Shredder" has expanded the utility of Barocycling systems for processing of

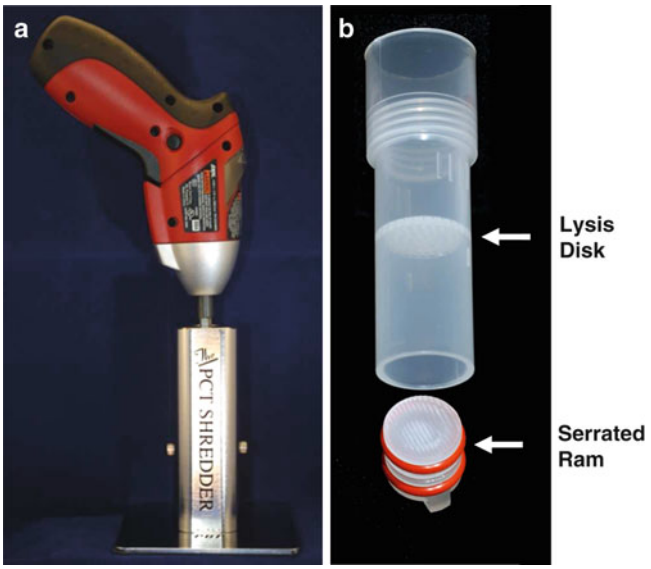

Fig. 5. The PCT Shredder and PULSE Tube. (**a**) The PCT Shredder driver and stand. (**b**) FT500-S Shredder PULSE Tube with serrated ram. Tissue is placed between the lysis disk and the ram. Extraction reagent is placed into the upper chamber.

fibrous, solid, or otherwise resilient samples such as nematodes, cartilage and muscle as well as tough plant material (e.g., cotton fibers, pine needles, bark, roots, etc.). The system uses FT500-S PULSE Tubes with a serrated ram that grinds the sample against the perforated lysis disk (Fig. 5b). Shredding time is typically 10–20 s for tough samples such as skeletal muscle. The metal shredder stand (Fig. 5a) can be chilled ahead of time to reduce sample heating during grinding. These sample containers may then be processed in the Barocycler.

To increase the reproducibility of homogenization in the PCT Shredder, sample processing must be performed using a controlled amount of force exerted on to the rotating tool. While the PCT Shredder relies on operator control, the new generation of this product, the Shredder SG3, has been outfitted with a latch mechanism (Fig. 6). This modification ensures that each sample is processed under identical conditions, independent of operator effort. As in the original model, the spring-loaded shredder base holds the sample, while the latch mechanism keeps the rotational tool in a fixed position relative to the spring. In its current design, this prototype exerts approximately 25 lb of force onto the PULSE Tube in the vertical direction, when latched.

2.1.3. Bead Beaters

While efficiency of the improved PCT Shredder has been successfully demonstrated for many resilient sample types, including *Dermacentor* and *Ixodes* ticks, this single-sample tool does not readily lend itself to parallel high-throughput processing. Bead beating provides an

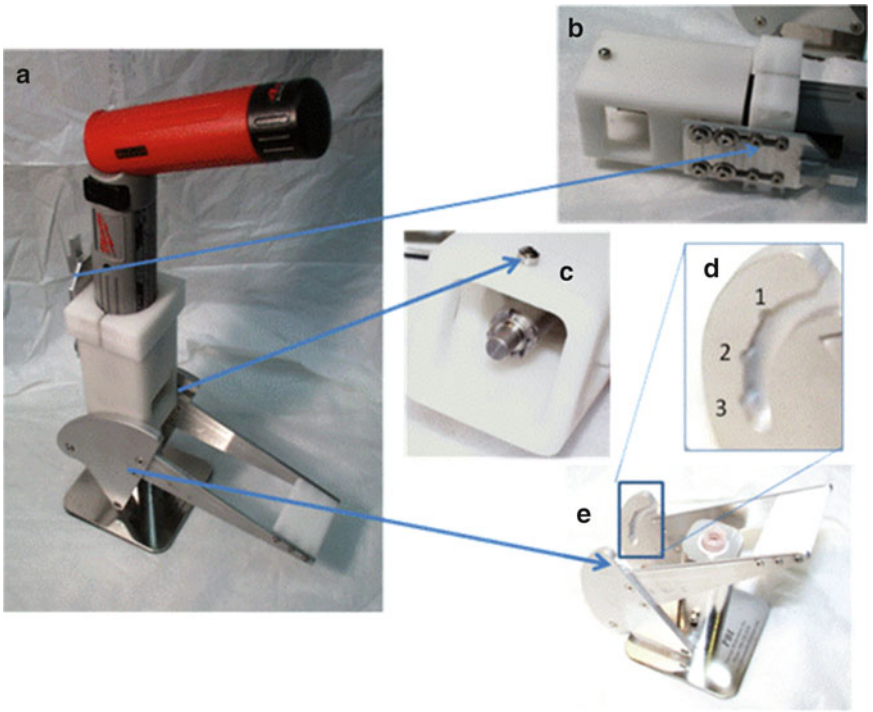

Fig. 6. Shredder Generation 3 (SG3). (**a**) Assembled unit. (**b**) Driver mount hardware. (**c**) Bearings provide smooth operation of the latch against the rails (**d**, **e**). Three preset locking positions (**d**) are provided. The cordless driver is equipped with a user replaceable rechargeable battery and is capable of running up to 100 samples on a single battery charge.

alternative method for achieving mechanical sample disruption of multiple samples in a microtiter plate format. Bead beating is considered as an established method of sample homogenization: a large body of literature is available to prove the performance of this method in biological sample preparation, including applications for disruption of arthropods (49, 50). However, to determine optimal specifications, such as frequency, amplitude, and the choice and number of beads per tube required to provide reproducible disruption of tough arthropod exoskeleton, we performed a series of experiments using the MiniBeadbeater-1 (BioSpec Products) and 0.2-mm zirconia beads in a microcentrifuge tube of appropriate shape and dimensions to model the in-well Barocycler sample containers. Successful tick disintegration was demonstrated in a 30 s run (see Fig. 7).

2.2. Sample Tubes

Aside from the standard PCT laboratory equipment, a short list of expendables is required to support most pressure cycling sample preparation protocols. Chief among these are disposable sample tubes, which have been specifically designed for use in pressure cyclers. The tubes allow for safe and sterile handling of the sample from preprocessing the sample with a tissue shredder to sterile room-temperature storage of the sample after the pressure

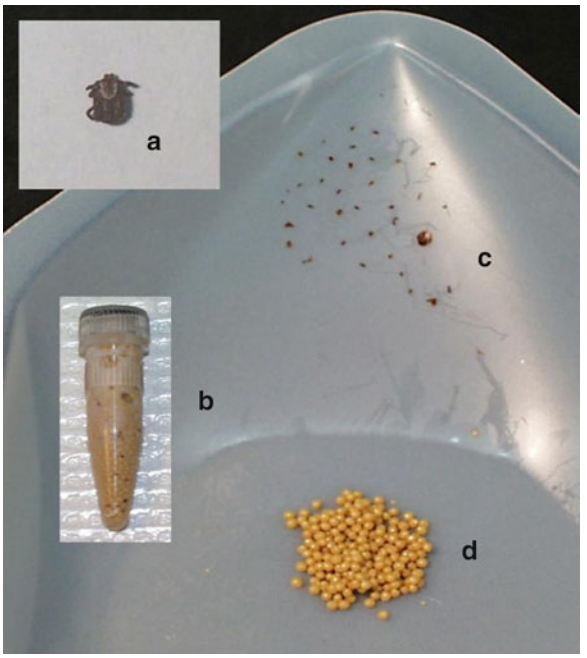

Fig. 7. Processing ticks with a bead beater. (**a**) Wild tick, mass approximately 2 mg. (**b**) Tick lysate after 30 s of bead beating. (**c**, **d**) The zirconia beads (**d**) thoroughly broke apart the exoskeleton of the tick (**c**).

cycling process. PCT sample tubes will vary based on your application and the type of Barocycler system being used. Several pressure cycling tubes designed to handle different sample volumes and matrices are briefly described below.

2.2.1. PULSE Tubes

The first generation of consumable sample containers (FT500 PULSE Tubes) for the PCT SPS was developed to accommodate relatively large 1.4 mL samples and enable several applications described below. In this original PULSE Tube design, pressure is transmitted to the sample by a movable ram (Fig. 8). When the Barocycler chamber is pressurized, the ram pushes the specimen from the sample chamber through the lysis disk and into the fluid retention chamber. When pressure is released, the sample (now partially homogenized) is pulled back through the lysis disk by the receding ram. The sample is lysed by the combination of physical passage through the lysis disk and the rapid pressure changes.

FT500-S PULSE Tubes can also be used with the tissue shredders, described earlier, to support pre-PCT processing of difficult sample matrices such as plant and insect samples. For complete disintegration of the smallest insects, PULSE Tubes are available with photochemically etched stainless steel disks secured against the lysis disk (Fig. 9).

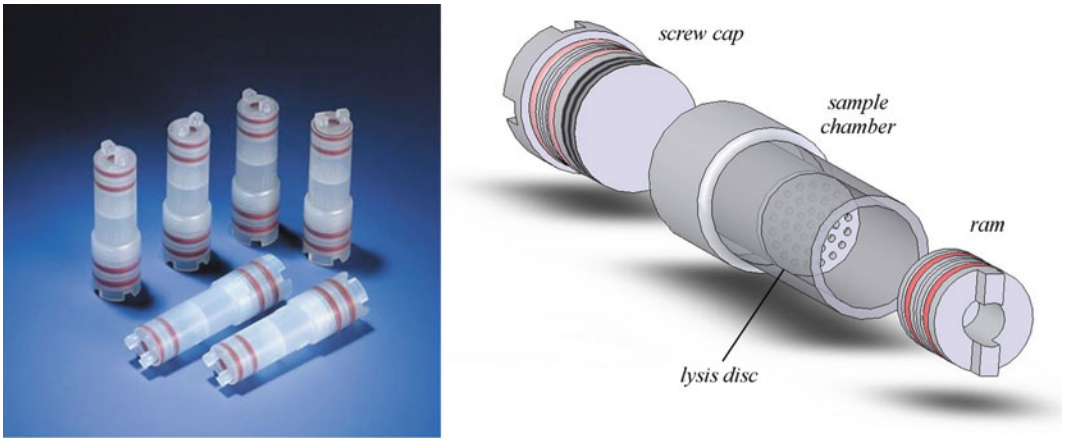

Fig. 8. Specially designed PULSE Tubes for disrupting cells and tissues by PCT. At high pressure, the movable ram compresses the sample. The perforated lysis disk macerates solid tissues increasing the surface area which is exposed to the extraction buffer.

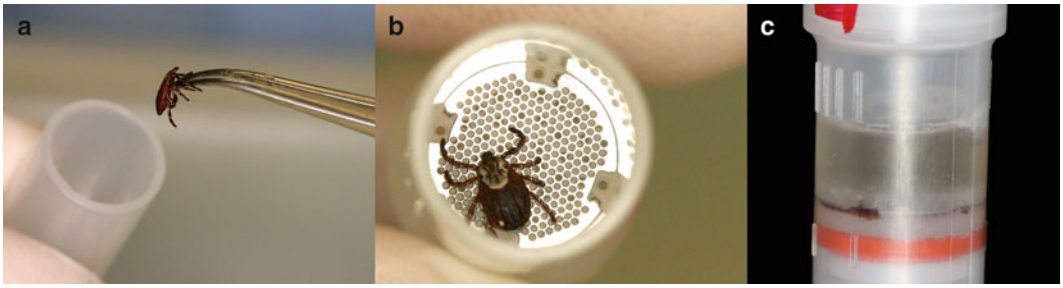

Fig. 9. Homogenization of a small tick using the FT-500MS PULSE Tube outfitted with a metal lysis disk. (**a**) Placement of the tick in the Shredder PULSE Tube-MS; (**b**) tick in the Shredder PULSE Tube prior to homogenization; (**c**) tick in the Shredder PULSE Tube after homogenization.

For liquid samples, FT500-ND PULSE Tubes without a lysis disk are available. These tubes can accommodate volumes from 200 µL to 1.4 mL.

2.2.2. PCT MicroTubes

To address the strong demand for smaller sample volumes and to enable higher sample throughput, new disposable processing containers named "PCT MicroTubes" have been developed (Fig. 10). These containers have no moving parts and efficiently transmit hydrostatic pressure to the sample by flexible deformation of the polymer walls. They are suitable for cell/tissue lysis, PCT-fractionation, as well as in-solution and in-gel protein digestion applications. The PCT MicroTube was designed to meet the critical requirements of modern proteomic analysis by mass spectrometry. It is made from fluorinated ethylene propylene (FEP). This fluoropolymer was selected because of its unique features. FEP is highly inert and retains its integrity within an extremely wide temperature

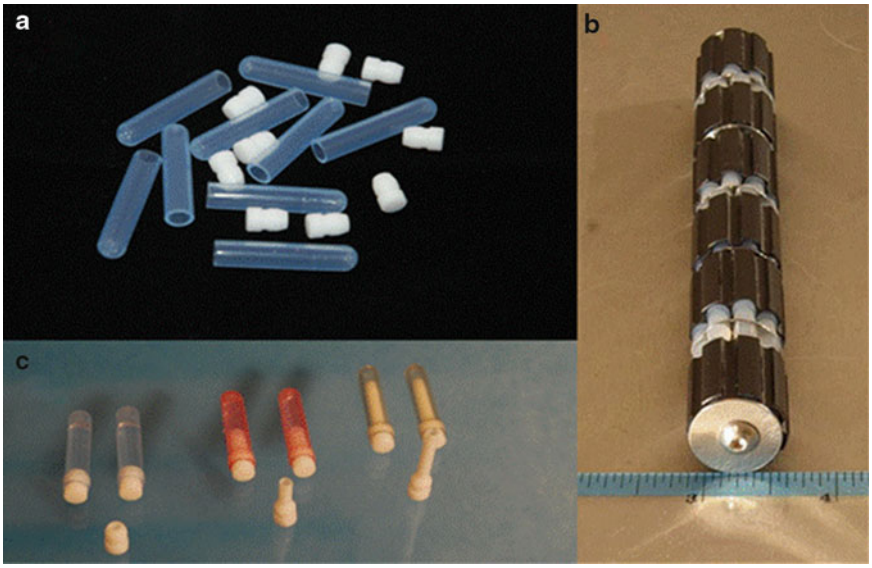

Fig. 10. PCT MicroTubes. (**a**) Standard PCT MicroTubes. (**b**) PCT MicroTube cartridge system. (**c**) PCT MicroTubes with gel-picking caps available in 50, 100, and 150 μL sizes.

range (−200 to +150°C). The PCT MicroTube's outstanding chemical resistance, nonwetting surface, and negligible protein and nucleic acid binding, help to ensure nearly complete sample recovery—features that are essential for high-pressure-enhanced enzymatic proteolysis.

It is essential to avoid the presence of air pockets in sealed PCT tubes as this adds unnecessary compressibility and risks oxidation of susceptible sample components by dissolved oxygen and very high partial pressure. Void volume is eliminated in PULSE Tubes by use of a movable plunger. However, with PCT MicroTube this is accomplished by choosing the properly sized displacement cap. These PCT MicroTube closures, termed MicroCaps, also meet the criteria of chemical resistance and low binding, as they are manufactured from polytetrafluoroethylene (PTFE), a polymer selected for sealing sample containers for high pressure applications. The dimensions of the MicroTubes are fixed to provide maximum ergonomic convenience in handling small containers while wearing laboratory gloves, whereas the PCT MicroCaps are available in three versions, displacing air from the MicroTubes and resulting in effective sample volume at 50, 100, or 150 μL, respectively (Fig. 10c). In addition, all PCT MicroCaps have the unique feature of being used as gel spot pickers to excise small protein spots from a polyacrylamide gel. The PCT MicroCap holds the gel protein spot safely within the cap, while it is transferred to the MicroTube, ready for processing by PCT. This approach reduces the likelihood of cross-contamination between gel spots and substantially simplifies the gel spot picking and transfer process.

A specialized cartridge system has been designed to hold multiple PCT MicroTubes (up to 12 in a Barocycler NEP2320 and up to 48 in the Barocycler NEP3229) in the pressure chamber of the Barocycler instrument (Fig. 10b). The PCT MicroTube cartridge system keeps these containers sealed during rapid cycles of hydrostatic pressure even at temperatures exceeding the boiling point of the sample components. PCT MicroTubes withstand centrifugation at centrifugal forces up to 14,000 $\times g$ thus enabling stepwise fractionation of cell lysates by the reagents of increasing stringency directly in a single container.

2.3. Reagents and Kits

While pressure-cycling technologies are extremely flexible with respect to the use of standard pre- and postprocessing buffers and reagents, several PCT-specific reagent kits are available for use according to the classes of biomolecules sought for isolation.

2.3.1. ProteoSolve LRS and SB Kits

Recently, PBI developed a detergent-free sample preparation technique, which allows for the concurrent isolation and fractionation of proteins, nucleic acids, and lipids from cells and tissues. This method uses a synergistic combination of cell disruption by PCT and a reagent system using perfluorinated alcohols and other reagents contained in the ProteoSolve-SB kit that dissolves and partitions distinct classes of molecules into separate fractions. Applications of perfluorinated alcohols as solvents are widespread in polymer chemistry, chromatography, and NMR spectroscopy. However, many groups also have adopted these solvents for solubilization and extraction of hydrophobic membrane proteins in proteomics workflow (36, 51–57), as well as an important mobile phase component for HPLC (58), CZE (59) and LC–MS analysis of nucleic acids (60). Perfluorinated alcohols possess unique physicochemical properties. Polar, amphipathic, and volatile in nature, these compounds act together as strong chaotropic agents and detergents, and are conveniently removed after use. As solvents, fluorinated alcohols exhibit strong hydrogen bonding, mix freely with water, and will associate and dissolve a wide variety of compounds with receptive sites such as oxygen, double bonds, or amine groups, while remaining practically immiscible with aliphatic hydrocarbons (61). However, upon application of high hydrostatic pressure, fluorinated alcohols such as trifluoroethanol and hexafluoroisopropanol (HFIP) form metastable mixtures with aliphatic compounds that are possible only under pressure (62). This phenomenon has been used in development of several pressure-dependent tissue lysis and sample extraction kits recently commercialized by PBI. Several authors previously reported the effectiveness of fluorinated alcohols in solubilizing hydrophobic proteins (63) and in causing rapid conformational changes of proteins (34, 64–66). Our preliminary experiments suggest that the combination of high hydrostatic pressure and optimized chemical reagents

rapidly and reversibly denatures sample proteins, and thus minimizes undesired enzymatic activity, which could cause sample degradation during storage at temperatures above −80°C.

This PCT-assisted liquid–liquid extraction method has been used for the simultaneous extraction and efficient recovery of proteins, DNA, RNA, and lipids from biological samples such as cell cultures and tissues (8, 67). After extraction, the sample is separated by centrifugation into three fractions: a lipid-containing upper phase, a protein-containing lower phase, and an insoluble fraction (pellet and interface), which contains the DNA and RNA, as well as a small amount of protein. The DNA and/or RNA can be isolated from the residual solid fraction by one of a number of standard methods or kits. The dissolved sample proteins can be isolated from the lower (polar) phase by the removal of the volatile solvent. This can be accomplished by either evaporation of the solvent under vacuum or by precipitating the protein using a reagent included in the kit. The resulting protein pellet can then be reconstituted in a buffer provided for 2D electrophoresis or in another suitable reagent for downstream protein analysis by SDS-PAGE or other applications. Due to the strong chaotropic properties of perfluorinated alcohols, most proteins isolated in the solvent are irreversibly denatured and do not retain any enzymatic activity. In addition, the denatured proteins may no longer be recognized by antibodies generated against native protein structure. The lipids, in the upper apolar phase can be subjected to further separation or can be analyzed directly. The lipid carrier reagent is compatible with most popular separation methods employed for lipid extracts, such as normal phase HPLC, TLC, and direct analysis using MALDI-TOF mass spectrometry with popular matrices (e.g., dihydroxybenzoic acid (DHB) for positive ionization mode). While the lipid carrier reagent is present in excess, its aliphatic hydrocarbon constituents are easily separated from and tend not to interfere with ionization of sample-derived lipid molecules. If desired, analysis of the lipid composition can be performed using GC–MS or LC–MS/MS. In this case, especially when the sample contains sufficient amount of lipids to form a visible liquid phase, the addition of the lipid carrier reagent may be omitted, as the excess of its components may interfere with the chosen separation method.

2.3.2. PBI Mitochondria Isolation Kits

Mitochondria isolation from solid tissue is usually carried out using labor-intensive homogenizer-based methods (68) that require extensive operator experience and are prone to high variability between researchers. To facilitate efficient and reproducible mitochondria preparation, kits for isolating mitochondria from rat kidney, skeletal muscle, and lung were developed as semiautomated methods to generate mitochondria-enriched preparations using the PCT Shredder or the Shredder SG3 for initial tissue homogenization, and PCT for additional tissue lysis and extraction of

mitochondria. The protein profiles of control mitochondria samples isolated by homogenizer are essentially the same as the test samples prepared using the PCT Shredder with or without PCT (see Fig. 11). Coomassie blue-stained SDS-PAGE gels and Western blots have confirmed that lung mitochondrial samples isolated by homogenizer are essentially the same as the samples prepared using the PCT Shredder with or without pressure cycling, indicating that pressure cycling can be used to easily and reproducibly generate high-quality mitochondrial preparations (Fig. 12). Analysis of mass spectra from rat skeletal muscle mitochondria isolated using PCT confirms that the samples are highly enriched in mitochondrial proteins (Fig. 13). In addition, the similarity of protein distribution between samples prepared by both methods supports the conclusion that PCT can be used for effective and reproducible preparation of mitochondria-enriched fractions for proteomic analyses (18).

3. Methods

Most of the methods described below employ the NEP3229 Barocyler SPS and specific reagent kits available from PBI (South Easton, MA, USA), except as noted. For experiments requiring pressure above 35,000 psi (241 MPa), a commercial version of the Barocycler NEP3229 instrument, which has been modified to expand the pressure range to 45,000 psi (310 MPa), was used. Barocycler NEP2320 is capable of reaching 45,000 psi (310 MPa) with an optional upgrade package available from PBI. Subheading 3.3 for inactivation and extraction of purified *B. anthracis* spores employs the modified NEP3229 Barocyler SPS (see Note 1). Both Barocylcer SPS employ an external water circulator bath to control temperature of the sample chamber throughout the treatment and an associated source of pressure to drive cycling hydrostatic pressure as programmed.

3.1. Preparing the NEP3229 Barocycler for Operation

1. Power on, adjust water levels, prime the system, and preheat at least 30 min before use. First, toggle on power switches for the NEP3229 Barocyler, and the water circulator bath. Set the water circulator temperature control to 55°C and top off the circulator bath with deionized water as this fluid contacts the Barocycler chamber (see Note 1).
2. While the SPS is preheating, unscrew the Barocyler chamber lid and inspect the appearance of the lubricant on its threading. Wipe off and reapply fresh lubricant if warranted (see Note 1).
3. View the water inside the Barocycler chamber and adjust level using deionized water and a large disposable pipette (e.g., 50 mL) until level meets the top of the narrowing in the neck of the chamber, which would be situated immediately below the height of the lid when applied and properly tightened.

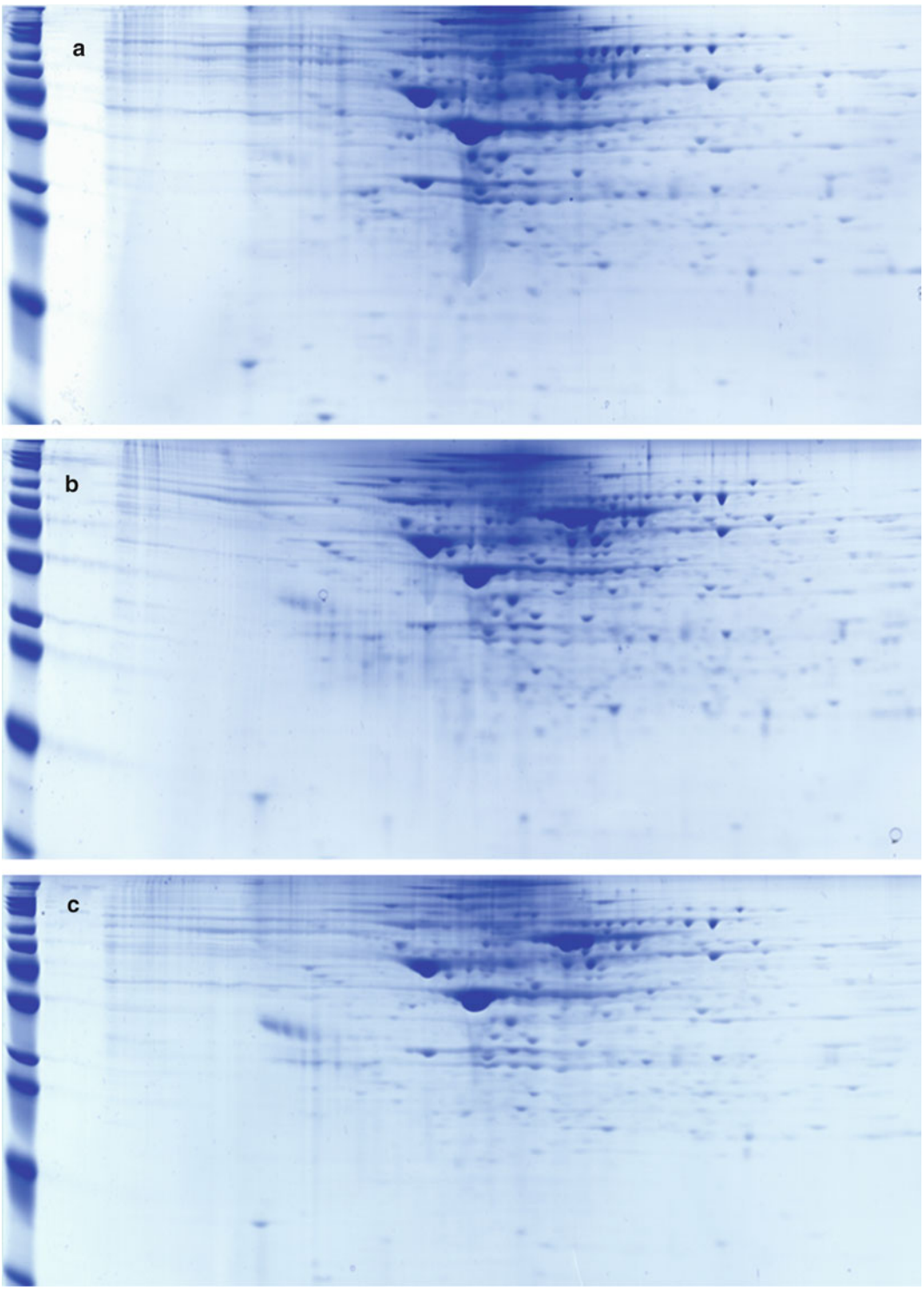

Fig. 11. The total protein profile of rat lung mitochondria. 2D PAGE of mitochondria prepared using the control homogenizer method (**a**); The *PCT Shredder* alone (**b**); or *The PCT Shredder* followed by PCT at 10,000 psi (**c**) (125 μg/gel). Result: No significant differences in protein pattern were observed on 2D gels in any of the samples, further supporting the hypothesis that the Shredder, with or without PCT, can be used to prepare a mitochondria-enriched fraction from tissues.

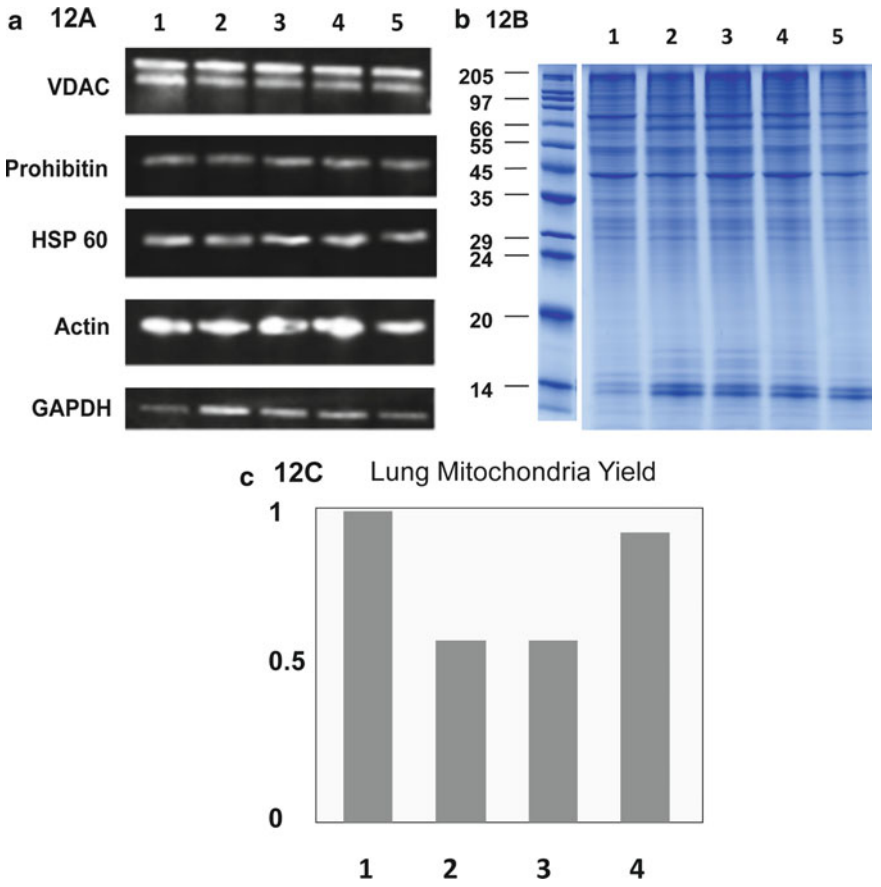

Fig. 12. (**a**) Western blots were probed with antibodies to VDAC (outer mitochondria membrane), Prohibitin (inner mitochondria membrane), HSP60 (mitochondrial matrix), actin and GAPDH (cytosolic protein). The presence of GAPDH in all the lung mitochondria preparations is likely due to carryover of cytoplasmic material and could be further reduced by additional washing of the mitochondrial pellet. The presence of actin in the mitochondrial pellet is expected, as it has been reported to be associated with mitochondria (4). Samples as follows: *1* Homogenizer control; *2* Shredder alone; *3* Shredder with PCT at 10,000 psi for 5 cycles; *4* Shredder with PCT at 20,000 psi for 5 cycles; *5* Shredder with PCT at 20,000 psi for 15 cycles. (**b**) SDS-PAGE protein load was adjusted to 8 μg/lane. Samples as follows: *1* Homogenizer control; *2* Shredder alone; *3* Shredder with PCT at 10,000 psi for 5 cycles; *4* Shredder with PCT at 20,000 psi for 5 cycles; *5* Shredder with PCT at 20,000 psi for 15 cycles. (**c**) Mitochondria were prepared as above. Protein yield, expressed as milligrams per gram tissue, was measured by Bradford assay and normalized to mass of starting material. Samples as follows: *1* Homogenizer control; *2* Shredder alone; *3* Shredder with PCT at 10,000 psi for 5 cycles; *4* Shredder with PCT at 20,000 psi for 5 cycles.

4. Place the empty sample holder into the chamber. Screw the chamber lid back on using a chamber closure tool, removable height indicator, and arrow marker to ensure proper tightness. Close the Plexiglas outer chamber door.
5. Program the Barocycler as described in Subheadings 3.2–3.5.
6. Prime the system fluid by running through 2–3 cycles of the program (see Note 2), then abort the run and adjust water level if needed. Reclose the chamber and allow the system to achieve full temperature while preparing your samples.

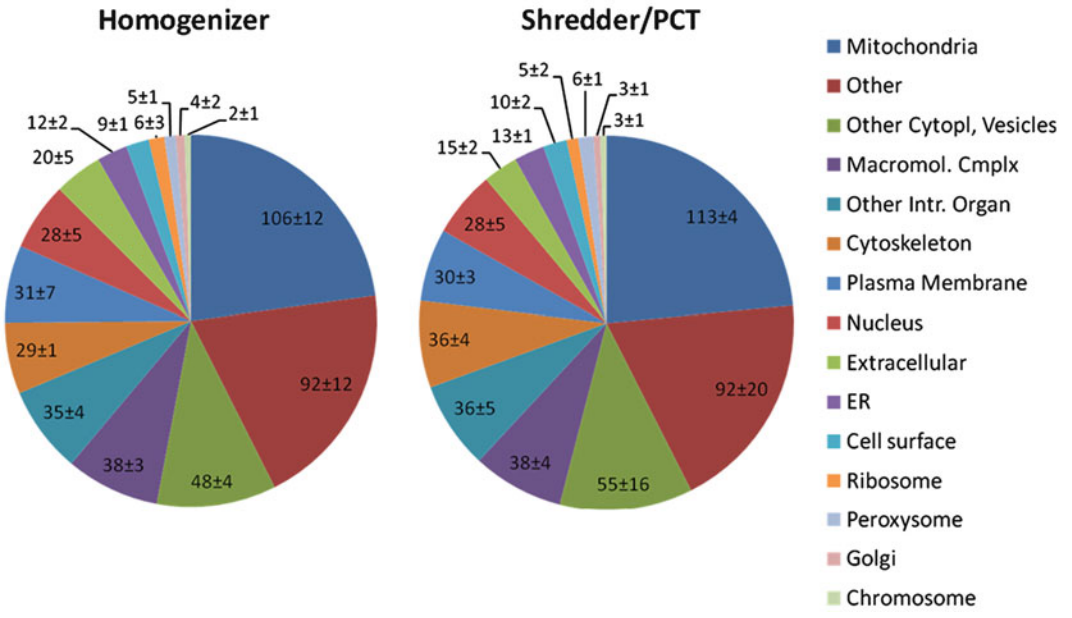

Fig. 13. Mass spectrometry analysis of mitochondria from rat skeletal muscle. Proteomic analysis of mitochondria prepared by conventional homogenizer and Shredder/PCT at 10,000 psi ($n = 3$ individual preps per method, 1–3 technical replicates per prep). Trypsin digests of mitochondria-enriched samples were subjected to nanoLC–MS/MS analysis on an LTQ-Orbitrap. Average of seven runs for each method (*error bars* represent standard deviation). Only proteins with an identification confidence level of $\geq$90% were included. Using GO terms protein localization data analysis (STRAP 1.0 (5)), these data confirm that the samples are highly enriched in mitochondrial proteins. In addition, the similarity of protein distribution between samples prepared by both methods supports the conclusion that PCT can be used for effective and reproducible preparation of mitochondria-enriched fractions for proteomic analyses. Further analysis is under way to determine if there are subtle differences in protein composition or quantity between methods.

3.2. Processing Vegetative Bacterial Cells

Based on our preliminary experiments and previously published materials (69), we have designed an initial protocol for extraction of *B. burgdorferi* DNA using a combination of PCT Shredder tool and hydrostatic pressure cycling. This protocol with its listed modifications (described in Subheading 3.4) has been used for all tick and culture extractions:

1. Prepare the Barocycler as described in Subheading 3.1 and heat to 54°C, then program it using one of the following cycling parameters: (a) 30 cycles consisting of 55 s at 20,000 psi and 5 s at atmospheric pressure, then 120 cycles consisting of 5 s at 45,000 psi and 5 s at atmospheric pressure. (b) 30 cycles consisting of 55 s at 20,000 psi and 5 s at atmospheric pressure, then 60 cycles consisting of 10 s at 45,000 psi and 10 s at atmospheric pressure. (c) 30 cycles consisting of 55 s at 20,000 psi and 5 s at atmospheric pressure, then 30 cycles consisting of 20 s at 45,000 psi and 10 s at atmospheric pressure.

2. Pellet the bacterial cell culture by centrifuging at 8,000 × *g* for 10 min at 4°C. For each milliliter of cell culture, resuspend the pelleted cells in 100 μL lysis buffer containing 50 mM Tris–HCl, pH 8.0; 25 mM EDTA; 500 mM NaCl and 1% NP-40.
3. Add 100 μL 20 mg/mL proteinase K (Qiagen) for each milliliter of lysis buffer used.
4. Load the sample into the PULSE Tubes (see Note 3).
5. Immediately before processing sample, prime system again as described in Subheading 3.1, step 6. Without delay, remove chamber lid, lower preloaded sample holder into the chamber, reaffix lid, and start program (see Subheading 3.1 regarding the need to prime, load, and start the processing without unnecessary delay).
6. After completing the run, remove the chamber lid, remove the sample holder, and carry it to a biosafety cabinet for recovery of sample from the PULSE Tubes.
7. Transfer the lysate into a clean microcentrifuge tube and perform standard DNA extraction protocols.

3.3. Processing Bacterial Spores

1. Program Barocycler with "99kill" method described in Note 2.
2. Load 0.7 mL each of spore samples and the amphipathic solvent (Reagent A from ProteoSolve LRS or ProteoSolve SB kits) in PULSE Tubes as described in Notes 3 and 4.
3. Insert loaded PULSE Tubes into the sample holder, affixing each with a Lawrence Clip (see Fig. 14).
4. Immediately before processing the sample, prime the system again as described in Subheading 3.1, step 6. Without delay, remove the chamber lid, lower the preloaded sample holder into the chamber, reaffix the lid, and start the program (see Subheading 3.1 regarding the need to prime, load, and start the processing without unnecessary delay).
5. After completing the run, remove the chamber lid, remove the sample holder and carry it to a biosafety cabinet for recovery of sample from PULSE Tubes.
6. Test a portion of the PCT-treated sample to confirm killing as described in Note 5.
7. Treated sample may be stored at 4°C indefinitely or processed for subsequent molecular analysis as needed.

3.4. Arthropod-Borne Bacteria: Extracting *B. burgdorferi* from Ticks

1. Preheat the Barocycler to 54°C.
2. Place one tick in the ram end of a FT500 PULSE Tube, insert the ram, and add 100–200 μL lysis buffer (50 mM Tris–HCl, 25 mM EDTA, 500 mM NaCl, 1% NP_4O, pH 8.0) to the cap

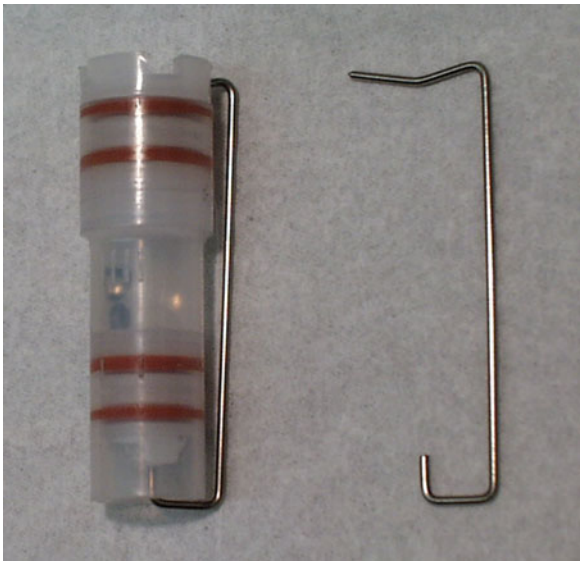

Fig. 14. The "Lawrence" clip is a stainless steel retainer clip designed to keep the ram in the PULSE Tube at temperatures slightly above boiling point of the solvent.

end depending on the size of the tick. Add RNase at this step if desired. Close the PULSE Tube.

3. Shred the sample forcefully at 10 s intervals, briefly chilling on ice in between, for a total of 20–30 s or until the tick is observed to be disintegrated. If using the Shredder SG3, begin shredding on setting 2 for 5 s and then move to setting 3 for 5 s (see Note 6).
4. After shredding, add 1.0–1.1 mL lysis buffer and 100 µL 20 mg/mL proteinase K (Qiagen, Cat# 19131) to each of the sample containers. The final volume of reagents in each tube should equal 1.4 mL. Close the tubes, vortex thoroughly, and process in the Barocycler for 20 cycles consisting of 55 s at 20,000 psi and 5 s at atmospheric pressure. This pressure cycling program has been optimized to enhance proteinase K activity.
5. Reprogram the Barocycler, still at 54°C, for 120 cycles consisting of 5 s at 45,000 psi and 5 s at atmospheric pressure. This pressure cycling program is optimized for *Borrelia* lysis.
6. Transfer the samples to microcentrifuge tubes. Add 210 µL 10% hexadecylammonium bromide (CTAB) and incubate at 65°C for 20 min.
7. Add an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) to each sample, mix well by vortexing, and incubate at room temperature for 3–5 min.
8. Centrifuge at 10,000 × *g* for 10 min at 4°C.
9. Carefully collect the upper, aqueous phase of each sample and reextract with an equal volume of chloroform:isoamyl alcohol

(24:1) to remove any trace of phenol carryover. Discard the lower phase.

10. Centrifuge at 10,000 × *g* for 10 min at 4°C and transfer the aqueous phase to a new microcentrifuge tube. Discard the lower phase.
11. To precipitate DNA, add a 2× volume of precooled 2-propanol and a one tenth volume of 3 M sodium acetate. Incubate at −70°C for 30 min or −20°C for 2 h.
12. Pellet the sample by centrifugation for 15 min at 4°C at 13,000 × *g*.
13. Discard the supernatant, being careful not to disturb the pellet. Wash the pellet with 500 µL of 75% ethanol and centrifuge again at 13,000 × *g* for 8 min.
14. Clean up the DNA over a column such as the Qiagen DNeasy column. Elute about 30 µL for a small tick.
15. PCR detection can be performed using an Applied Biosystems 7500 Real-Time PCR thermocycler. A 75 bp *B. burgdorferi*-specific 23S rRNA gene fragment is amplified. The primers are described in Courtney et al. (45) and by Black and Piesman (46).

3.5. Preparation of Functional Skeletal Muscle Mitochondria from Rat Tissue Using the PBI Mitochondria Isolation Kit: Rat Muscle

1. Chill the PCT Shredder stand by placing it into a refrigerator or −20°C freezer at least 30 min before sample processing. If using a Barocycler for the PCT-enhanced protocol, be sure that the unit has reached 4°C before start of experiment.
2. Dilute PCT MMIB (Muscle Mitochondria Isolation Buffer) stock to 1×. Adjust pH to 7.4 with 0.1 N KOH, if required.
3. Prepare a slurry of partly frozen PBI MMIB by placing 8–10 mL of PBI MMIB into a small beaker or large test tube. Chill the buffer in a −80°C freezer for about 20 min; alternately, partly immerse in liquid nitrogen for about 1 min. When the buffer begins to freeze, break up the ice and mix well, until the solution is the consistency of slush.
4. Place 0.5–0.7 g of freshly excised muscle tissue into ice-cold PBI MMIB slurry to rapidly cool the tissue. For best results, the samples must be kept on ice, or at 4°C, for all subsequent steps. Transfer cooled tissue to a small dish and mince with scissors into small pieces. This step increases surface area and helps to wash away blood.
5. Prepare a working enzyme solution by adding 100 µL of Protease Type XXIV (Sigma, St. Louis, MO), 25 mg/mL in 10 mM HEPES, to 10 mL cold 1× PBI MMIB.
6. Transfer the minced tissue into the diluted enzyme solution. Continue to mince the tissue while incubating in the enzyme solution at 4°C or on ice for 5 min. After 5 min, aspirate the

enzyme solution and wash tissue pieces twice with 5 mL of fresh 1× PBI MMIB. Alternatively, minced tissue may be washed by pouring the entire sample (minced tissue and enzyme solution) into a large round-bottom test tube. Allow the minced tissue to settle to the bottom and gently pour off enzyme solution. Repeat twice with fresh 1× PBI MMIB to wash away enzyme.

7. Split the minced tissue into three approximately equal portions, and transfer to three FT500-S PULSE Tubes, using up to ~0.2 g of tissue/tube. This protocol can be easily scaled up to accommodate more tissue. However, to ensure efficient tissue homogenization, do NOT overload the PULSE Tubes. Use more PULSE Tubes if necessary. Using the PULSE Tube Tool, insert a serrated ram and *gently* compress the tissue between the ram and the lysis disk. If a large volume of wash buffer was carried over with the minced tissue, aspirate and discard it before proceeding. Add 0.5 mL of fresh PBI MMIB to the cap side of the PULSE Tube. Close the tube with the PULSE Tube cap.
8. Shred samples. If using the PCT Shredder, insert the PULSE Tube into precooled shredder stand, cap side up. Seat the shredder driver tip into the PULSE Tube cap. Shred for 10 s while pushing down on the driver. If using the Shredder SG3, insert PULSE Tube into the precooled Shredder SG3 base, cap side up. Seat the shredder driver tip into the PULSE Tube cap; adjust the lever to setting 2 and shred for 10 s.
9. After shredding, withdraw the PULSE Tube from the stand, and check to confirm that the shredder ram is now flush with the lysis disk and that all of the tissue has been forced through the holes of the lysis disk. If a significant amount of intact tissue is still present between the ram and lysis disk, cool the sample on ice and repeat shredding for an additional 10 s.
10. Before PCT processing, adjust the volume of each PULSE Tube to 1.4 mL with 0.7–0.8 mL additional PBI MMIB. Set a pre-chilled Barocycler for 10,000 psi, 20 s ("Time 1") and 5 s at atmospheric pressure ("Time 2"), for 5 cycles. Process samples one at a time in the NEP2320 or three at a time in the NEP3229. As each tube comes out of the Barocycler, place it on ice.
11. Remove the PULSE Tube caps and transfer homogenates to a large, round-bottom centrifuge tube (such as Beckman 355642 or equivalent) using a plastic transfer pipette. To recover the full sample volume, use the PULSE Tube tool to push up on the ram until the ram presses on the lysis disk. Rinse each PULSE Tube with additional PBI MMIB (1–2 mL), and pool the washes with the homogenate. Adjust total homogenate volume to ~15 mL (or less, if using smaller centrifuge tubes).

12. Centrifuge the homogenate at 4°C for 8 min at 1,000 × *g* to pellet tissue debris and red blood cells, as well as any unlysed cells and nuclei. Mitochondria will remain in the supernatant.
13. Gently pour the supernatant into a second round-bottom centrifuge tube. Avoid transferring the milky material that may be floating just above the debris pellet. Centrifuge the supernatant for 8–10 min at 14,000 × *g* to pellet the mitochondria-enriched fraction. After centrifugation, gently pour off and discard the supernatant. *The mitochondria-enriched pellet may consist of two layers: a darker bottom layer composed of intact mitochondria and a pale top layer that can be discarded.*
14. To separate the layers, gently tap the bottom of the tube on the bench several times. This will cause the top layer to slide down the side of the tube while the lower layer remains attached. The pale material can then be easily aspirated and discarded.
15. Add ~0.5 mL fresh PBI MMIB and gently break up the pellet. Transfer the suspension to a microcentrifuge tube (provided with the kit). Using the plastic pestle provided with the kit, gently homogenize to break up any clumps until a homogenous suspension is formed. Rinse the large tube with an additional ~0.5 mL of PBI MMIB and pool with the suspension. Centrifuge the sample at 14,000 × *g* at 4°C for 8–10 min. Rinse the plastic pestle with dH_2O for use in step 16.
16. Aspirate and discard the wash supernatant. Add 20–30 μL of fresh PBI MMIB to the mitochondria-enriched pellet. Using the plastic pestle, gently homogenize the pellet to form a uniform suspension. *The isolated mitochondria are more stable in suspension and will remain functional longer if kept concentrated. Therefore, use the smallest possible volume when suspending the final pellet.*
17. The mitochondria enriched sample is now ready for use. For assays that require functional mitochondria, store the final sample on ice. If additional purification is desired, the final suspension can be subjected to density gradient centrifugation.

4. Notes

1. *Barocyler instrument systems and use requirements*: While the Barocycler NEP3229 is a self-contained instrument, the Barocycler NEP2320 SPS requires an air compressor. The compressed air source must be able to supply at least 1.2 cfm of air flow at 105 psi. For experiments at elevated temperatures, both Barocycler models also require a circulator water bath. The following equipment has been used and is recommended: Neslab RTE-140 circulator bath for the NEP3229 Barocycler,

Fig. 15. Barocycler 2320 front panel user interface.

and Thermocube circulator bath for the NEP2320 Barocyler. Because the fluid in these baths contact the Barocycler sample chamber, which contains stainless steel components exposed to variable temperature (4–95°C), it is important to avoid the use of any corrosive chemicals and change the water periodically according to instructions of the manufacturer. The NEP3229 SPS has a larger chamber and lid than the NEP2320. This allows the NEP3229 SPS to simultaneously process up to three of the 1-mL PULSE Tubes. However, the commercially available version of the NEP3229 is currently capable of reaching the maximum pressure of 35,000 psi, while the smaller Barocycler NEP2320 is available in a configuration capable of reaching 45,000 psi. To achieve desired throughput and performance, modifications were made by the manufacturer to the NEP3229 instrument to reach the 45,000 psi pressure level. The threads of a larger chamber closure of the NEP3229 require periodic application of the thin coat of the Tri-Flow TF23015 industrial grade fluoropolymer-based lubricant available from PBI. Before each use, the closure should be inspected, cleaned, and fresh lubricant reapplied, if needed. Wipe off existing lubricant if it appears as dark gray instead of white or if more than 2 weeks have passed since the last application. Reapply a fresh thin coat of the lubricant only to the threadwork. Remove all grease except on the threads, and particularly on the bottom surface of the chamber lid which contacts water.

Fig. 16. The PULSE Tube Key has two ends for opening and closing the PULSE Tube cap and setting the ram at the appropriate position.

2. *Programming the modified NEP3229*: Programming the Barocycler entails entry of numerical values for pressure, time, and temperature then alphanumeric name (see Fig. 15) as follows: (a) Set the first pressure at 45,000 psi for a hold of 5 s; (b) set the second pressure at ambient for a hold of 5 s; (c) set number of cycles to 99; (d) choose *Save* and *Name* the program (e.g., "99kill"). Test the program and prime the fluid system before processing the sample as follows: (a) Press *Run* and allow passage through at least 2 or 3 cycles; (b) observe the LED readout on barocycler instrument panel to confirm each stage achieves desired pressure setting; (c) while the system is at ambient pressure phase, abort the test run by depressing the large red knob. The lid is not removable if aborted with pressure in the chamber. (d) Unscrew the chamber lid, view the water level, and readjust if needed.
3. *Loading PULSE Tubes and MicroTubes*: For the FT500 series tubes, load solid samples into the ram end of the pulse tube, then insert the ram using the bottom side of the PULSE Key (see Fig. 16). Add buffer to the cap end, being sure that the final volume of buffer and sample equals 1.4 mL. When loading FT500-ND tubes, insert the ram into the PULSE tube and push to correct height (air space inside the capped tube should be minimized) using the bottom side of the PULSE Wrench (see Fig. 16). Add sample and liquid reagents to the cap end. PCT MicroTubes are loaded by placing the desired number of MicroTubes in the provided rack and filling them by pipetting from the bottom up. Add small volumes to larger volumes, as depositing small volumes of liquid onto the nonwetting surface of the tube may be challenging. 150, 100, and 50 µL caps are provided in racks and may be picked up using the MicroTube Capper, then inserted into the tube. Release the cap by depressing the end of the MicroTube Capper. Under loading the MicroTube may cause it to become dented during pressure cycling; in this case, use a gel loading tip to withdraw the sample. If working with infectious agents inside a biosafety

cabinet, wipe the exterior of the sealed tubes with fresh peroxide (not bleach), before removing from the cabinet.

4. *Priming the NEP3229 SPS for anthrax spores*: Reproducible, complete killing of anthrax spores was achieved using rapid cycling, medium heat, and extraction solvent as described in Subheading 3.3 and Note 5. These conditions are attained and controlled over the optimized method of 99 cycles using the modified NEP3229 Barocyler SPS, which attains a rapid ramp 5 s between ambient pressure and the maximum pressure of 45,000 psi. The method was designed to minimize pressure-induced germination of spores to preserve biomolecule content for comparison of proteomes between different production lots of spore reagent material. While this NEP3229 method was developed for complete killing of anthrax spores, it is a useful starting point from which methods specific for other types of spores may be inactivated and extracted for systems biology using the Barocyler systems. The biology and molecular content of spores including development of new sample extraction methods for mass spectrometry has been reviewed in detail elsewhere (1).
5. *Viability testing of PCT-treated B. anthracis spores*: Full loss of viability (i.e., complete "killing") must be demonstrated before *B. anthracis* spore material is analyzed using sophisticated instrumentation as required in a systems biology study. This is true whether instrumentation is located inside the containment suite or located outside containment, at BSL-2, as is more common. To measure spore killing, plate dilutions of the PCT-treated sample and untreated control sample on tryptone soy broth (TSB) or other nutrient agar and incubate at 37°C 5% CO_2. Inspect plates periodically and count any cfu for up to 2 weeks. Viability is calculated from cfu using (1).

$$\text{Viability} = \frac{\left(\frac{\text{cfu}}{\text{mL}} \text{ for PCT sample}\right) * (\text{volume of PCT sample in mL})}{\left(\frac{\text{cfu}}{\text{mL}} \text{ for control sample}\right) * (\text{volume of control sample in mL})} \quad (1)$$

We achieve inactivation of anthrax spores by PCT at a level that is equivalent to autoclave decontamination (70): 10^8-fold decrease in spore viability.

6. *Challenges of working with small ticks and other arthropods*: Sometimes the bodies of very small ticks, such as those of the *Ixodes* genus, may get stuck in the holes of the lysis disk or in the serrations of the ram. If this happens, place 1 mL of lysis buffer in the tube and close the tube. This forces the ram away from the lysis disk. Vortex, then remove the 1 mL buffer and save for the PCT step. Push up the ram, close the cap, and continue shredding the sample.

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References

1. Powell BS, Cybulski R (2011) Inactivation and extraction of bacterial spores for systems biological analysis. In: Ivanov AR, Lazarev A (eds) Sample preparation in biological mass spectrometry. Springer, New York
2. Banerjee S, Smallwood A, Chambers AE, Nicolaides K (2003) Quantitative recovery of immunoreactive proteins from clinical samples following RNA and DNA isolation. Biotechniques 35:450–452, 454, 456
3. Hummon AB, Lim SR, Difilippantonio MJ, Ried T (2007) Isolation and solubilization of proteins after TRIzol extraction of RNA and DNA from patient material following prolonged storage. Biotechniques 42:467–470, 472
4. Nolan RL, Teller JK (2006) Diethylamine extraction of proteins and peptides isolated with a mono-phasic solution of phenol and guanidine isothiocyanate. J Biochem Biophys Methods 68:127–131
5. Riol H, Jeune B, Moskovic A, Bathum L, Wang E (1999) Optimized lymphocyte protein extraction performed simultaneously with DNA and RNA isolation: application to the study of factors affecting DNA, RNA, and protein recovery from lymphocytes of the oldest individuals. Anal Biochem 275:192–201
6. Tolosa JM, Schjenken JE, Civiti TD, Clifton VL, Smith R (2007) Column-based method to simultaneously extract DNA, RNA, and proteins from the same sample. Biotechniques 43:799–804
7. Bradley DW, Hess RA, Tao F, Sciaba-Lentz L, Remaley AT, Laugharn JA Jr, Manak M (2000) Pressure cycling technology: a novel approach to virus inactivation in plasma. Transfusion 40:193–200
8. Gross V, Carlson G, Kwan AT, Smejkal G, Freeman E, Ivanov AR, Lazarev A (2008) Tissue fractionation by hydrostatic pressure cycling technology: the unified sample preparation technique for systems biology studies. J Biomol Tech 19:189–199
9. Ringham H, Bell RL, Smejkal GB, Behnke J, Witzmann FA (2007) Application of pressure cycling technology to tissue sample preparation for 2-DE. Electrophoresis 28:1022–1024
10. Smejkal GB, Robinson MH, Lawrence NP, Tao F, Saravis CA, Schumacher RT (2006) Increased protein yields from *Escherichia coli* using pressure-cycling technology. J Biomol Tech 17:173–175
11. Smejkal GB, Witzmann FA, Ringham H, Small D, Chase SF, Behnke J, Ting E (2007) Sample preparation for two-dimensional gel electrophoresis using pressure cycling technology. Anal Biochem 363:309–311
12. Chesnick IE, Mason JT, O'Leary TJ, Fowler CB (2010) Elevated pressure improves the rate of formalin penetration while preserving tissue morphology. J Cancer 1:178–183
13. Fowler CB, Chesnick IE, Moore CD, O'Leary TJ, Mason JT (2010) Elevated pressure improves the extraction and identification of proteins recovered from formalin-fixed, paraffin-embedded tissue surrogates. PLoS One 5: e14253
14. Fowler CB, Cunningham RE, Waybright TJ, Blonder J, Veenstra TD, O'Leary TJ, Mason JT (2008) Elevated hydrostatic pressure promotes protein recovery from formalin-fixed, paraffin-embedded tissue surrogates. Lab Invest 88:185–195
15. Smejkal GB, Poinar GO, Righetti PG (2009) Will amber inclusions provide the first glimpse of a Mesozoic proteome? Expert Rev Proteomics 6:1–4
16. Okubara PA, Li C, Schroeder KL, Schumacher RT, Lawrence NP (2007) Improved extraction of Rhizoctonia and Pythium DNA from wheat

roots and soil samples using pressure cycling technology. Can J Plant Pathol 29:304–310

17. Tao F, Behnke J, Li C, Schumacher R, Lawrence NP (2006) Applications of pressure cycling technology (PCT) in proteomics. In: Smejkal GB, Lazarev A (eds) Separation methods in proteomics. CRC Taylor & Francis, Boca Raton, pp 3–18
18. Gross VS, Greenberg HK, Baranov SV, Carlson G, Stavrovskaya IG, Lazarev AV, Kristal BS (2011) Isolation of functional mitochondria from rat kidney and skeletal muscle without manual homogenization. Anal Biochem 418 (2):213–223
19. Fourme R, Kahn R, Mezouar M, Girard E, Hoerentrup C, Prange T, Ascone I (2001) High-pressure protein crystallography (HPPX): instrumentation, methodology and results on lysozyme crystals. J Synchrotron Radiat 8:1149–1156
20. Visuri K, Kaipainen E, Kivimaki J, Niemi H, Leisola M, Palosaari S (1990) A new method for protein crystallization using high pressure. Biotechnology (N Y) 8:547–549
21. Hess RA, Reinhard LA (1999) Unusual properties of highly charged buffers: large ionization volumes and low barrier hydrogen bonds. JACS 121:9867–9870
22. Laugharn JA Jr, Tao F, Sciaba-Lentz L, Bradley DW, Hess RA (1998) Sample preparation for molecular diagnostics by pressure cycling. In: Ludwig H (ed) High pressure bioscience & biotechnology. Springer, New York
23. Dreier GH, Tao F, Hess RA, Cheung CY, Sciaba LE, Green DJ, Laugharn JA Jr (1999) A bioseparation apparatus with high-pressure fluid injection and fluid sampling. Anal Biochem 269:223–229
24. Cheung CY, Green DJ, Litt GJ, Laugharn JA Jr (1998) High-pressure-mediated dissociation of immune complexes demonstrated in model systems. Clin Chem 44:299–303
25. Dusing S, Li C, Behnke J, Manak M, Schumacher R (2001) Inactivation of viruses in plasma by cycled pulses of high pressure. In: Hayashi R (ed) Trends in high pressure bioscience and biotechnology. Elsevier Science B.V., Amsterdam, pp 355–359
26. Green DJ, Litt GJ, Laugharn JA Jr (1998) Use of high pressure to accelerate antibody: antigen binding kinetics demonstrated in an HIV-1 p24: anti-HIV-1 p24 assay. Clin Chem 44:341–342
27. Laugharn J, Dreier G, Rudd E, Green D (2000) Pressure cycling reactor and methods of controlling reactions using pressure. BBI BioSeq, Inc., US Patent 6,036,923
28. Laugharn JA Jr, Hess RA, Tao F (2000) Nucleic acid isolation and purification. BBI BioSeq, Inc., US Patent 6,111,096
29. Laugharn JA Jr, Hess RA, Tao F (2000) Pressure-enhanced extraction and purification. BBI BioSeq, Inc., US Patent 6,120,985 and 6,274,726
30. Remaley A, Hess R, Fischer S, Sampson M, Manak M (2000) Pre-analytical sterilization of serum by pressure cycling treatment: a novel procedure to prevent laboratory acquired infections. Clin Chem 46:A39
31. Rudd EA (1997) Reversible inhibition of lambda exonuclease with high pressure. Biochem Biophys Res Commun 230:140–142
32. Balasubramanian S, Balasubramaniam VM (2003) Compression heating influence of pressure transmitting fluids on bacteria inactivating during high pressure processing. Food Res Int 36:661–668
33. Rasanayagam V et al (2003) Compression heating of selected fatty food materials during high-pressure processing. J Food Sci 68:254
34. Fatima S, Mishra A, Sen P, Khan RH (2008) Characterization of fluoroalcohols-induced intermediates of *Mucor miehei* lipase at low pH. Protein Pept Lett 15:346–352
35. Gross V, Lazarev A, Lawrence N, Schumacher R (2008) Isolation of mitochondria from cell cultures by PCT for proteomic analysis. Biotechniques 45:99–100
36. Yao H, Stuart RA, Cai S, Sem DS (2008) Structural characterization of the transmembrane domain from subunit e of yeast F1Fo-ATP synthase: a helical GXXXG motif located just under the micelle surface. Biochemistry 47:1910–1917
37. Chicon R, Belloque J, Recio I, Lopez-Fandino R (2006) Influence of high hydrostatic pressure on the proteolysis of beta-lactoglobulin A by trypsin. J Dairy Res 73:121–128
38. Chicon R, Lopez-Fandino R, Quiros A, Belloque J (2006) Changes in chymotrypsin hydrolysis of beta-lactoglobulin A induced by high hydrostatic pressure. J Agric Food Chem 54:2333–2341
39. Penas E, Restani P, Ballabio C, Prestamo G, Fiocchi A, Gomez R (2006) Evaluation of the residual antigenicity of dairy whey hydrolysates obtained by combination of enzymatic hydrolysis and high-pressure treatment. J Food Prot 69:1707–1712
40. Lopez-Ferrer D, Petritis K, Hixson KK, Heibeck TH, Moore RJ, Belov ME, Camp DG II, Smith RD (2008) Application of pressurized solvents for ultrafast trypsin hydrolysis in

proteomics: proteomics on the fly. J Proteome Res 7:3276–3281

41. Freeman E, Margolin E, Ivanov AR (2011) Pressure-assisted lysis of mammalian cell cultures prior to proteomic analysis. In: Ivanov AR, Lazarev A (eds) Sample preparation in biological mass spectrometry. Springer, New York
42. Getie-Kebtie M, Lazarev A, Eichelberger M, Alterman M (2010) Label-free mass spectrometry-based relative quantification of proteins separated by one-dimensional gel electrophoresis. Anal Biochem 409:202–212
43. Lee B, Lopez-Ferrer D, Kim BC, Na HB, Park YI, Weitz KK, Warner MG, Hyeon T, Lee SW, Smith RD, Kim J (2011) Rapid and efficient protein digestion using trypsin-coated magnetic nanoparticles under pressure cycles. Proteomics 11:309–318
44. Lopez-Ferrer D, Petritis K, Robinson EW, Hixson KK, Tian Z, Lee JH, Lee SW, Tolic N, Weitz KK, Belov ME, Smith RD, Pasa-Tolic L (2011) Pressurized pepsin digestion in proteomics: an automatable alternative to trypsin for integrated top-down bottom-up proteomics. Mol Cell Proteomics 10:M110.001479
45. Courtney JW, Kostelnik LM, Zeidner NS, Massung RF (2004) Multiplex real-time PCR for detection of *Anaplasma phagocytophilum* and *Borrelia burgdorferi*. J Clin Microbiol 42:3164–3168
46. Black WC IV, Piesman J (1994) Phylogeny of hard- and soft-tick taxa (Acari: *Ixodida*) based on mitochondrial 16S rDNA sequences. Proc Natl Acad Sci U S A 91:10034–10038
47. Clements RT, Smejkal G, Sodha NR, Ivanov AR, Asara JM, Feng J, Lazarev A, Gautam S, Senthilnathan V, Khabbaz KR, Bianchi C, Sellke FW (2008) Pilot proteomic profile of differentially regulated proteins in right atrial appendage before and after cardiac surgery using cardioplegia and cardiopulmonary bypass. Circulation 118:S24–S31
48. Tao F, Li C, Smejkal G, Lazarev A, Lawrence NP, Schumacher RT (2007) Pressure cycling technology (PCT) applications in extraction of biomolecules from challenging biological samples. In: Abe F, Suzuki A (eds) Fourth international conference on high-pressure biosciences and biotechnology, J-STAGE, Tsukuba, pp 166–173
49. Crowder CD, Matthews HE, Schutzer S, Rounds MA, Luft BJ, Nolte O, Campbell SR, Phillipson CA, Li F, Sampath R, Ecker DJ, Eshoo MW (2010) Genotypic variation and mixtures of Lyme *Borrelia* in *Ixodes* ticks from North America and Europe. PLoS One 5: e10650
50. Rijpkema SG, Herbes RG, Verbeek-De Kruif N, Schellekens JF (1996) Detection of four species of *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks collected from roe deer (*Capreolus capreolus*) in The Netherlands. Epidemiol Infect 117:563–566
51. Canelle L, Bousquet J, Pionneau C, Hardouin J, Choquet-Kastylevsky G, Joubert-Caron R, Caron M (2006) A proteomic approach to investigate potential biomarkers directed against membrane-associated breast cancer proteins. Electrophoresis 27:1609–1616
52. Deshusses JM, Burgess JA, Scherl A, Wenger Y, Walter N, Converset V, Paesano S, Corthals GL, Hochstrasser DF, Sanchez JC (2003) Exploitation of specific properties of trifluoroethanol for extraction and separation of membrane proteins. Proteomics 3:1418–1424
53. Redeby T, Emmer A (2005) Membrane protein and peptide sample handling for MS analysis using a structured MALDI target. Anal Bioanal Chem 381:225–232
54. Redeby T, Roeraade J, Emmer A (2004) Simple fabrication of a structured matrix-assisted laser desorption/ionization target coating for increased sensitivity in mass spectrometric analysis of membrane proteins. Rapid Commun Mass Spectrom 18:1161–1166
55. Wang H, Qian WJ, Mottaz HM, Clauss TR, Anderson DJ, Moore RJ, Camp DG II, Khan AH, Sforza DM, Pallavicini M, Smith DJ, Smith RD (2005) Development and evaluation of a micro- and nanoscale proteomic sample preparation method. J Proteome Res 4:2397–2403
56. Zhang H, Lin Q, Ponnusamy S, Kothandaraman N, Lim TK, Zhao C, Kit HS, Arijit B, Rauff M, Hew CL, Chung MC, Joshi SB, Choolani M (2007) Differential recovery of membrane proteins after extraction by aqueous methanol and trifluoroethanol. Proteomics 7:1654–1663
57. Zuobi-Hasona K, Crowley PJ, Hasona A, Bleiweis AS, Brady LJ (2005) Solubilization of cellular membrane proteins from *Streptococcus mutans* for two-dimensional gel electrophoresis. Electrophoresis 26:1200–1205
58. Muetzelburg MV, Hoffmann R (2008) Separation of multiphosphorylated peptide isomers by CZE. Electrophoresis 29:4381–4385
59. Mitulovic G, Stingl C, Steinmacher I, Hudecz O, Hutchins JR, Peters JM, Mechtler K (2009) Preventing carryover of peptides and proteins in nano LC-MS separations. Anal Chem 81:5955–5960
60. Apffel A, Chakel JA, Fischer S, Lichtenwalter K, Hancock WS (1997) Analysis of

oligonucleotides by HPLC–electrospray ionization mass spectrometry. Anal Chem 69:1320–1325

61. HFIP technical product information. DuPont Chemical Solutions http://www2.dupont.com/Polymer_Specialties/en_US/assets/downloads/HFIP_Tech_Sheet_jan2012.pdf
62. Lazarev AV, Gross VS (2008) Extraction and partitioning of molecules. US Patent Application 20080300386, filed on 2 June 2008
63. Chatterjee C, Gerig JT (2007) Interactions of trifluroethanol with the Trp-cage peptide. Biopolymers 87:115–123
64. Cort JR, Liu Z, Lee GM, Huggins KN, Janes S, Prickett K, Andersen NH (2009) Solution state structures of human pancreatic amylin and pramlintide. Protein Eng Des Sel 22:497–513
65. Forbes JG, Jin AJ, Ma K, Gutierrez-Cruz G, Tsai WL, Wang K (2005) Titin PEVK segment: charge-driven elasticity of the open and flexible polyampholyte. J Muscle Res Cell Motil 26:291–301
66. Naseem F, Khan RH (2008) Pea lectin in alkaline conditions: formation of molten globule-like intermediate and its structural and thermal studies under the influence of hexafluoroisopropanol. Protein Pept Lett 15:606–611
67. Freeman E, Gross V, Romanovsky I, Ivanov AR (2011) Adipose tissue lysis and protein extraction followed by ms-based proteomic profiling reveals constituents of oxidative stress in obesity. In: Ivanov AR, Lazarev A (eds) Sample preparation in biological mass spectrometry. Springer, New York
68. Rasmussen HN, Andersen AJ, Rasmussen UF (1997) Optimization of preparation of mitochondria from 25-100 mg skeletal muscle. Anal Biochem 252:153–159
69. Carroll JA, Stewart PE, Rosa P, Elias AF, Garon CF (2003) An enhanced GFP reporter system to monitor gene expression in *Borrelia burgdorferi*. Microbiology 149:1819–1828
70. Dang JL, Heroux K, Kearney J, Arasteh A, Gostomski M, Emanuel PA (2001) *Bacillus* spore inactivation methods affect detection assays. Appl Environ Microbiol 67:3665–3670
71. Schneider LV, Likhte V, Wright A, Chu F, Cambron E, Baldwin-Burnett A, Krakow J (2012) Recovery and immunoaffinity enrichment of intact membrane proteins from metastatic ovarian cancer tissue. International Journal of Proteomics, August 2012 (in press)
72. Patel N, Solanki E, Picciani R, Cavett V, Caldwell-Busby JA, Bhattacharya SK (2008) Strategies to recover proteins from ocular tissues for proteomics. Proteomics 8:1055–1070
73. Tao F (2008) Challenges and current solutions in proteomic sample preparations. In: Mine Y, Miyashita K, Shahidi F (eds) Nutrigenomics and proteomics in health and disease: food factors and gene interactions. Wiley, New York
74. Yu C, Cohen LH (2004) Tissue sample preparation—not the same old grind. LC GC N Am. 2003;21:1038–1048
75. Szabo Z, Guttman A, Karger BL (2009) Rapid release of N-linked glycans from glycoproteins by pressure-cycling technology. Anal Chem 82:2588–2593
76. McCoy J, Hubbell WL (2011) High-pressure EPR reveals conformational equilibria and volumetric properties of spin-labeled proteins. Proc Natl Acad Sci U S A 108:1331–1336

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