

Guide to the Homogenization of Biological Samples

David W. Burden, Ph.D. †

Abstract

Many tools are available for the homogenization of samples. These homogenizers can be generally grouped into those that grind, shear, beat, and shock, though many disruption processes rely on more than one type of force. When developing a disruption scheme, it is critical to define the characteristics of the homogenate and then select the tools that will help to meet the objectives. The examination of methods used to homogenize samples has shown that effectiveness is directly related to the nature of the sample. Samples that start with small particles, such as bacterial cultures, are most effectively disrupted by ultrasonication, but that same method is the poorest for solid muscle. In such cases, samples must be first disaggregated into smaller particles prior to processing. Methods which rely on a single processing step, such as with the high throughput homogenizers, can yield very good sample disruption, but they do not match two-step processes that breakdown samples in a series of steps. The need to process large numbers of samples requires a trade-off with level of effective homogenization.

Table of Contents

Introduction	2
Strategies for Homogenization: Selecting a Method(s)	2
Harvesting Samples	3
Examination of Methods	3
Grinding.....	4
Mortar & Pestle	4
Cryogenic Grinding with Mortar & Pestle.....	4
CryoGrinder for Small Sample Grinding.....	5
Tissues Disruption with Glass Homogenizers	5
Vortexer Bead Beating	7
Shearing.....	7
Blender	7
Rotor-Stator.....	7
Dounce Homogenizer	8
Potter-Elvehjem with PTFE Pestle	8
French Press or French Pressure Cell	8
Beating.....	9
Amalgamators for Tubes	9
High Throughput Homogenizers	9
Shock	11
Sonication.....	11
Combined Methods.....	11
Comparing the Methods	12
Summary	13
Materials and Methods	14
Microorganisms.....	14
Tissue Samples.....	14
Cell Culture	14
Lactate Dehydrogenase Assay.....	14

† OPS Diagnostics, LLC, P.O. Box 348, Lebanon, NJ 08833 TEL (908) 253-3444 Email: david.burden@btc-bti.com

Introduction

The disruption of samples is an early step in the process of isolation and/or quantifying RNA, DNA, proteins, and analytes. Both chemical and mechanical/physical methods are available for disruption, with chemical methods being preferred for many sample types (e.g., *E. coli* and cultured cells). However, many microorganisms, intact tissues, solid specimens, such as seeds, and heavily encased samples are not efficiently disrupted chemically. With such resilient samples mechanical and physical methods that rely on grinding, shearing, beating and shocking are used. Mechanical homogenizers, manual homogenizers, mortar and pestles, sonicators, mixer mills, and vortexers are several of the more common tools used for mechanical and physical disruption.

Sample disruption, or homogenization, is not the most exciting methodology to examine, but none-the-less it does have significant impact on the end results of a process. Though used daily and extensively, methods for sample disruption are not necessarily well understood. Indeed like many well established lab processes, the methods are passed on from researcher to researcher like inheritable family treasures, with little effort expended to decipher the process itself. Being fair, the impact of homogenization on an experiment may be minimal, but at times the choice of tool and its method of use may have a significant impact on the outcome.

Where possible, chemical means may be the preferred method, such as with the lysis of *E. coli* with SDS for plasmid isolation, but it may also introduce unwanted molecules into the lysate. Though useful for nucleic acid isolation, detergents and chaotropes may certainly denature proteins which can make their use undesirable in protein purification schemes. The same is true for the addition of lytic enzymes, which in the case of protein purification, must be subsequently removed. If adding chemicals is impractical or simply does not work, then mechanical and physical disruption of samples is the alternative.

Mechanical/physical methods for disrupting samples include grinding, shearing, beating, and shocking. At times it is difficult to discern between the different forces that are used, but for the sake of simplicity we will segregate the different tools into these

categories. In practice, scientists mix and match disruption methods to meet their needs. Though an ideal disruption method would require only a single step, it is quite common to see two or more methods being used in tandem to obtain the desired result. Indeed the isolation of subcellular fractions could first involve cutting a tissue with scissors, followed by coarse grinding with a handheld homogenizer, and then a final dissociation with a glass Dounce homogenizer. If any one of these steps is omitted, then the results would be less than stellar.

Strategies for Homogenization: Selecting a Method(s)

In choosing the best method to disrupt samples, it is prudent to consider the desired characteristics of the final homogenate and then work backwards as to which method or combination works. For instance, if pieces of intact membrane are required, then the homogenization process must effectively destroy tissue and cells, but prevent complete obliteration of subcellular components. If active proteins are needed, especially those which are heat labile, then processes which generate heat or cause foaming should be avoided. If quantitation of an analyte is the goal, then complete liberation of that analyte is necessary, which implies a thorough dissociation of all cellular structures. Consequently, in the case of homogenization, the ends do justify the means, thus it is valuable to dissect the methods available and to assess both their strengths and limitations.

The tools used for physical and mechanical homogenization produce lysates with different characteristics. Glass homogenizers used for shearing, e.g., Dounce homogenizer, are used to disrupt cultured cells for the preparation of intact nuclei and microsomes, a process dependent upon the clearance between the Dounce pestle and wall of the tube. The shear created by pushing cells past the pestle tear apart whole cells but leave smaller organelles relatively intact. On-the-other-hand, sonication produces microscopic shock waves throughout the sample which destroys cells, organelles, and subcellular structures. Both of these methods are useful as many processes require partial lysis of a sample, as with a presence/absence PCR assay, while other require complete homogenization (as with pharmacokinetic

analysis). As stated previously, it is important to define the type of homogenate needed so that the proper method(s) can be incorporated into the process.

Defining the characteristics of a homogenate is one of two key considerations in selecting tools for a disruption process. The other is throughput. A researcher in a high paced work environment knows the stress of required productivity. Indeed as budgets tighten and staffs are "right sized" the workload of researchers increase. This directly relates to the consideration of which type of homogenizer to use in sample preparation. There are many tools available, but many are designs that have been unchanged in half a century and designed for scientists who might be processing a couple of sample per week. These older homogenizers are often a bottleneck to analysis. Originally mechanical disruption/homogenization of samples relied on slow laborious methods, such as manual homogenization or grinding tissues in liquid nitrogen with mortar and pestle. This type of process may be completely adequate for many labs, but some researchers are under pressure to process hundreds if not thousands of samples daily. Therefore, the level of throughput needs to be considered in designing a scheme.

Harvesting Samples

Though the focus of this article is on homogenization methods, it is important to note that methods used for sample collection and harvesting can significantly impact results as well. Like selecting the best homogenization method, a suitable harvesting method is also required. Depending upon the analyte or component being sought, the method of collecting, harvesting, and subsequently storing should be carefully considered.

Cultured microbes tend to be robust, unless they are highly sensitive to environmental stress (e.g., oxygen intolerance). Typically microorganisms can be harvested and handled while chilled and subsequently processed, but this is for laboratory cultures. Samples collected from the environment are completely different as populations of mixed cultures can rapidly change in numbers, adhere to surfaces, and alter their metabolic profiles based on the means by which they are collected and stored. Bacteria embedded in biofilms are surviving based on the environmental parameters surrounding that mass, and disaggregating the biofilm will

certainly change the behavior of the bacteria. Whether or not such changes impact the ultimate result of the collection and homogenization process needs to be considered.

What is true for bacteria is also true for other organisms. Plants certainly have periods of active metabolism where harvesting and storage conditions will impact the levels and conditions of analytes. For instance, during the germination of seeds, cells not only experience rapid growth, but they are also adjusting to environmental stimuli. Certainly cotyledons harvested and left on a lab bench over lunch will have a very different RNA profile than freshly collected and homogenized samples. However, with seeds that are dried and relatively dormant, the collection and homogenization process probably has a much smaller impact on the levels and condition of analytes.

The haste by which animal tissues need to be harvested and processed is directly related to the stability of the components being sought after. DNA, RNA, proteins, and the myriad of other solutes available from biological samples are all different regarding their stability once harvested from the source. It is important that this variable is considered when designing a homogenization scheme.

For instance, human skin is a major source of collagen for biomedical devices. This skin is typically collected from cadavers well after the donor has expired. Obviously collagen is sufficiently stable and can be collected days after death. For other proteins this may not be the case (see link for more on the stability of proteins). This is quite the opposite for RNA, where it is best to sacrifice animals, immediately harvest tissues, and then transfer samples to a beaker of liquid nitrogen. Rapid harvest and freezing is critical to retaining RNA within the sample. Once frozen, RNA will remain intact as long as it is kept below -130°C, either in cryogenic freezers, vapor phase freezers, or submersed in liquid nitrogen (see link for more information on cryogenic storage). RNA stored at ultralow temperatures (e.g., -80°C) will degrade over time, thus it is not recommended for long term storage.

Examination of Methods

For simplicity, the methods used for sample disruption have been divided into four

groups: grinding, shearing, beating, and shocking. Many engineers may cringe by this delineation, but we are approaching the topic practically and as biologists. Foremost, it must be highlighted that many methods make use of more than one force, as with conical homogenizers which grind and shear. We attempt to note this where it happens. Additionally, there are many tools and methods which are not discussed simply because there are not time and resources to examine all options.

Grinding

Mortar and pestle is the best known tool for grinding, but many tools are used for grinding, including grain mills, coffee grinders, and some more refined laboratory apparatuses including vortexers and glass homogenizers. Grinding relies on creating friction by sandwiching the sample between two hard surfaces that slide against each other. Grinding causes tearing and ripping of samples, much like shearing, but differs in that there is direct contact between sample and homogenizer.

In its various forms grinding can be used on wet, dry, and frozen samples. A relatively soft sample placed between two hard, scraping surfaces will wear down. With adequate patience, solids can be reduced to very fine particles by grinding, part of which is dependent upon the topology of the grinding surfaces.

The key characteristic of grinding is that friction generates heat and at times it can be significant. Consequently, the heat tolerance of the analyte should be a factor when considering grinding methods for disruption. Frequently samples are frozen prior to grinding, especially with liquid nitrogen, which can be used for chilling both samples and homogenizer. This cryogenic grinding makes samples brittle and fracture easily, but it also preserves analytes that are heat labile or which may rapidly degrade upon liberation, such as RNA.

Certainly the oldest tool used for grinding is the mortar and pestle, making its debut long before the dawn of civilization. It is still a popular grinding tool in the lab, being used for some of the most advanced analytical processes. Many researchers use grinding beads and vortexers for lysing microbes and larger grinding balls for tissue samples. Various configurations of bench top vortex mixers can be used with micron sized grinding spheres to rupture bacteria, yeast,

and molds. Larger tissues samples can be disrupted in plastic tubes (e.g., polycarbonate) with larger stainless steel grinding balls. Vortexers, though readily available, tend to be poor as compared to other methods discussed below. Several of the glass tissue homogenizers use grinding forces along with shearing to effectively disrupt cultured cells and tissues.

Mortar & Pestle: Mortar and pestles are still widely used for sample homogenization. In life science labs, their widest use is for the grinding tissue frozen with liquid nitrogen (see below) however they are indispensable for grinding solids at room temperature. For the single occasional sample it is fine, but when throughput is necessary, alternative tools like mixer mills are more practical.

Strengths – Mortar and pestles are easy to use and relatively inexpensive to purchase. With dry grinding, it is possible to generate very small particles.

Limitations – Throughput with mortar and pestles is low. Contamination issues may also be a concern as the grinding will generate dust. Though sturdy, many sets are made of glass or porcelain which can chip or crack if dropped.

Cryogenic Grinding with Mortar & Pestle: Grinding samples frozen with liquid nitrogen using a mortar and pestle is a widely used method. The mortar and pestle are cleaned and placed in a Styrofoam tub or cooler where liquid nitrogen is poured or dispensed onto the mortar and pestle. Care is needed to avoid splattering liquid nitrogen when the mortar and pestle first start chilling. After several minutes the set will be cooled and a fog will usually settle over the apparatus. The sample may already be frozen or it can be snap frozen by dropping it into a beaker of liquid nitrogen (use plastic beakers). If the sample is taken from a -80°C freezer, let it sit on the surface of the mortar to chill further. To grind, hold the pestle with a gloved hand and firmly press on the sample while twisting. The sample will typically shatter into small pieces, some of which may fly from the mortar so use added caution with biohazardous materials. The fragmented pieces of the sample will continue to get smaller as the sample is ground using a circular motion with downward pressure. Once the grinding is completed, residual sample must be tapped or scraped from the pestle. The sample must

then be transferred into a receiving vessel using a pre-chilled spatula. If the sample is to be subsequently stored as frozen, pre-chill the tube of vial that will hold the ground sample.

Strengths – The mortar and pestle, whether it is used for grinding at room temperature or with liquid nitrogen, is a good standard method for reducing samples into small particles. The apparatus is relatively inexpensive and is available in ceramics to metals.

Limitations – A significant problem in cryogenic grinding with mortar and pestle is that small samples can be essentially lost when ground into the surface of the mortar. This makes sample recovery difficult and leads to poor yields. Another major disadvantage of mortars and pestles is that the number of samples that can be processed is low. This limitation is even more pronounced with cryogenic grinding as the mortar and pestle must be warmed between uses and cleaned. Labs that process significant numbers of samples cryogenically must also dedicate significant shelf space to the mortar and pestle sets.

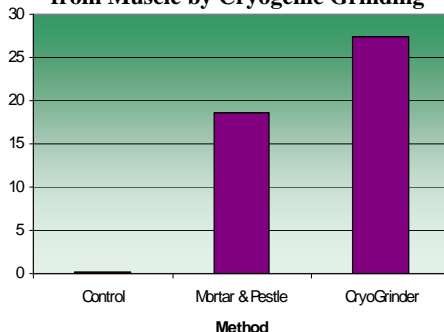
CryoGrinder™ for Small Sample

Grinding: As small samples are difficult to recover from standard mortar and pestles, the [CryoGrinder™](#) serves as an alternative tool for cryogenic grinding. Samples of 100 mg or less which are pulverized using a mortar and pestle chilled with liquid nitrogen are spread as a fine powder over the mortar surface. This is difficult to collect. The CryoGrinder™, which is essentially a miniature mortar and pestle, possesses a small well and associated pestle designed for samples less than 100 mg. Following grinding, collecting pulverized sample is more efficient. The CryoGrinder™ is used similarly to a standard mortar and pestle, in that the CryoGrinder™ is chilled and then samples are added to the well. The CryoGrinder is also powered by a handheld cordless screwdriver.

Cryogenic grinding is useful as a first step in preparing samples for chemical lysis or subsequent mechanical processing. Its true value is that samples can be reduced from large solid items to small particles without tremendous input of heat. With smaller particle size, the sample can be rapidly dissolved, as is done for RNA isolation.

As compared to the mortar and pestle, the CryoGrinder™ does generate smaller particles as was determined by comparative enzyme liberation studies.

Relative LDH Enzyme Activity Liberated from Muscle by Cryogenic Grinding

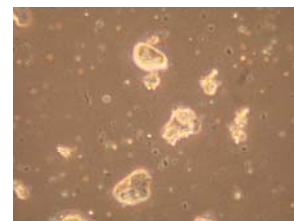


Strengths – The CryoGrinder™ is effective at grinding small samples while frozen. It is more effective than the mortar and pestle as measured by the release of LDH from muscle tissue homogenized by both methods. The CryoGrinder™ generates smaller particles than the mortar and pestle. With the addition of an extraction buffer, small particles will more readily dissolve into solution than larger particles. Another advantage is that the CryoGrinder™ is motorized which allows for a greater number of samples to be processed without added fatigue.

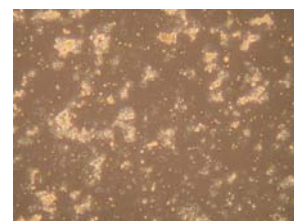
Limitations – Sample size for the CryoGrinder™ must be small (100 mg or less) for the pestle to be pressed effectively against the mortar. The mortars are also small and must be kept within a reservoir (e.g., CryoCooler) so that they remain cold.

Tissues Disruption with Glass

Homogenizers: Original methods for homogenizing tissues made use of glass homogenizers. The tools available for this include ground glass homogenizers such as the Potter-Elvehjem, conical, and Tenbroeck. These tissue grinders are closely related to the Dounce and Potter-Elvehjem (when used with a PTFE pestle), but the latter rely on shearing forces and will be discussed below. Glass tissue grinders have tight fitting mortars and pestles with ground glass surfaces. The surfaces are coarse like a very fine emery paper so that the pestles can dig into tissues being gripped by the mortar. Tissues processed in glass tissue grinders are often chilled on ice, and



Cryogrinding muscle reduced the sample size, but yielded relatively low homogenization efficiency of 30%.



Conical glass tissue grinder produced a good homogenate though total efficiency was 42%.

the Tenbroeck pestle, which is hollow, can be filled with cold liquid to cool from the inside.

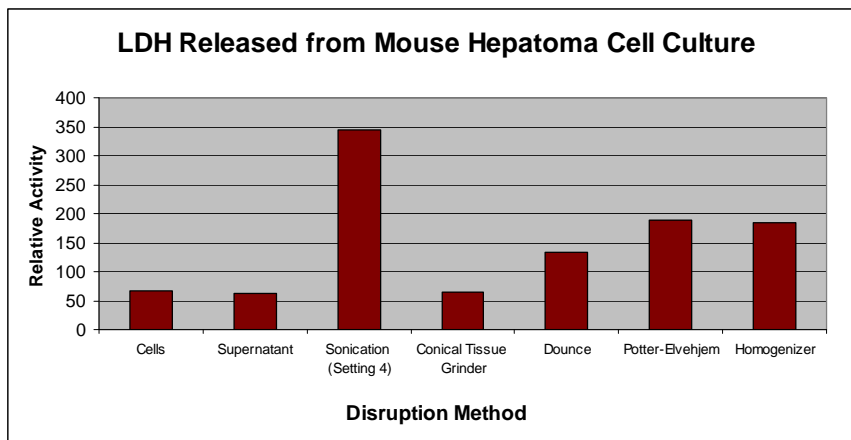
The actual process of grinding is relatively simple and involves adding an extraction buffer and the tissue to the mortar, and then slowly pressing the pestle on to the sample with a twisting motion. The piston is raised and lowered while twisting to help turn the sample to expose all sides to grinding. After the pestle is turned 360° it is slowly removed from the tube (a strong vacuum helps to create shearing forces). This action is repeated nine more times.

Since they are glass, the homogenizers can be washed and sterilized before use. If residual detergents on the glass is a concern, then cleaning can be done with a 1% solution of sodium carbonate (which serves as a very nice wetting agent) followed by rinses with 3% acetic acid. The homogenizers can also be baked to further

decontaminate the glass if the application is for RNA or DNA isolation.

Strengths – Glass tissue grinders are inexpensive and easy to use. They work relatively well and generate very fine homogenate. In single-step disruption experiments, conical glass homogenizers liberate about half as much enzyme as compared to larger more expensive high throughput homogenizers. They generate about 40% of the best two-step process. They are very easy to clean and decontaminate.

Limitations – Homogenizing with glass tissues grinders inevitably will leave fibrous and membranous components relatively intact. Certain tissues even with prolonged grinding are difficult to disaggregate. Throughput with these homogenizers is also low unless multiple units are available. Glass homogenizers are also prone to breakage.



The above graph displays relative levels of lactate dehydrogenase liberated from tissue culture cells by shock, grinding, and shearing. The effectiveness of sonication, as will be discussed below, is evident as it released nearly twice as much enzyme activity as the best glass homogenizer. However, glass tissue grinders are a fraction of the cost of an ultrasonication system (\$70 vs. \$5000), thus for a limited number of samples, glass homogenizer may be effective. The degree of disruption (as measured by activity) demonstrates the value of each homogenizer system.

The Potter-Elvehjem with PTFE pestle effectively shears cells with its mechanically driven pestle, while the Dounce homogenizer proves less effective, but traditionally retains many cellular organelles intact. The conical tissue grinder which relies on friction to disrupt tissues is less effective as the cells are small and are not effectively gripped between the pestle and tube. Interestingly, the rotor-stator homogenizer (last column) is about equal in effectiveness to the much less expensive glass homogenizers.

Vortexer Bead Beating: Though not their intended use, vortexers are routinely used to disrupt samples. This method relies on the addition of grinding beads to tube and then repeatedly vortexing the sample. Typically used for the lysis of microorganisms, vortexers can disrupt tissues by using large grinding beads (>2 mm) made of zirconium or stainless steel.

Homogenizing samples by vortexing generates significant amounts of heat due to the friction created by the grinding balls. Many protocols call for bursts on the vortexer interspersed with cooling on ice. Several vortexer models are available that hold multiple microfuge tubes and that pulse (alternating on and off) in order to help dissipate heat.

Strengths – Vortexers are available to most researchers and thus can be applied to homogenization at no cost. New vortex mixers are relatively inexpensive. Standard single tube vortex mixers can be used for all size tubes. Multitube vortexers can homogenize full racks of tubes.

Limitations – Vortex mixers are designed for mixing and lack the same power as true homogenizers, thus they are usually less effective at sample disruption. With microbes, homogenization rates are around 50% as compared to true bead beaters. Though partially effective, vortexers may be adequate for many applications.

Shearing

Homogenizers such as blenders work by shearing which is created by a tangential force being applied to the sample. There are several tools that disrupt by shearing, including blenders, rotor-stators, and some of the glass homogenizers, all of which made their entrance into the lab in the middle of last century.

In the pre-WWII era samples were prepared by chopping and dicing, and then in the 1940s, blenders arrived. These were truly the first in a series of innovations in sample homogenization. Through the 1940s and 50s, the number of tools commercialized for sample disruption increased dramatically (including the Dounce homogenizer, Potter-Elvehjem homogenizer, and French press - named for their inventors), all of which disrupt by shearing. It was also in the 1950s that the first rotor-stator homogenizers started seeing use in the labs.

Blender: One of the early innovations applied to sample homogenization was the Waring blender which made its appearance early during World War II. This simple device was instrumental to early work in protein purification and analyte isolation. Samples are placed in the blender with extraction buffer and then blended. The blades shear and cut tissues reducing tissues in size significantly.

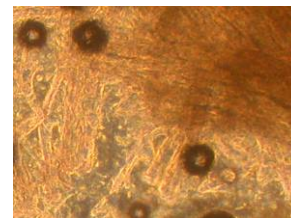
Strengths – Blenders are readily available with even household devices being suitable for many lab applications. They can process large samples quickly and are easy to use. Lab blenders are available in stainless steel which allows for decontamination and sterilization.

Limitations - Blending can create vortexes which cause foaming and result in significant protein denaturation. Blenders also provide a coarse homogenate which is not always suitable for efficient extractions.

Rotor-Stator: The rotor-stator, or what is commonly known as the handheld homogenizer, was first commercialized for the laboratory in the 1950s. This homogenizer is one of the most widely used tools for homogenizing plant and animal tissues. Rotor-stators are designed with an outer stationary tube (stator) and a inner turning shaft (rotor) which is connected to a motor. At the bottom of the rotor-stator are slots on both the tube and shaft. This design is essentially the same as an electric razor. When running at 10,000-20,000 rpm, samples pressed into the slots of the rotor-stator are efficiently sheared.

Rotor-stators come in many different widths and bottom slot configurations. It would be speculative to identify the intended application for each rotor-stator type, but in practice these larger shaft assemblies are used to macerate animal and plant tissues of increasing mass. The shearing action of the homogenizers produces a very uniform homogenate in relatively little time. Like other homogenization techniques, the rotor-stators can generate heat, thus some of the more advanced models come with temperature probes that shut down the units if the temperature rise is extreme.

Rotor-stators are available as handheld units and larger stand supported models. Some are modified workshop routers, but they can also be very complex programmable models with numerous features. Most are the size of handheld kitchen mixers, though with



Blenders are an effective first step in reducing the size of samples. However as depicted in this image of blended heart, many microfibrils remain intact.



Rotor-stator homogenizer had a relative efficiency of 27.6% as compared to other methods.

much greater power. Sample sizes which can be processed on handheld homogenizers range from less than 1 ml up to 40 L or more.

Strengths – Rotor-stators can be very effective at homogenizing a wide array of samples. The shafts come in a variety of sizes capable of a wide range of volumes. Indeed process scale rotor-stators are commonly used in various industries. Sample disrupted with rotor-stators can be very homogeneous.

Limitations – The initial cost for rotor-stators varies, but at minimum it will be several thousand dollars. Furthermore, different motor units will be required for very small and large samples. Each shaft is \$1000 or more. Shafts are difficult to clean which requires the unscrewing of the rotor shaft from the stationary outer stator housing. When homogenizing fibrous samples such as muscle, threads of connective tissue can be caught within the shaft assembly, making rapid cleaning and decontamination a problem. High speed homogenization also generates heat and possible vortices which can denature proteins.

Dounce Homogenizer: Not long after the arrival of the Waring blender the Dounce homogenizer was designed. Though this device looks like a ground glass homogenizer, it relies on pushing the sample between the sides of the tube and the pestle. Shearing forces are created as the sample and liquid squeeze up and past the pestle. The Dounce homogenizer is most effective at lysing tissue culture cells and finely diced tissue in order to generate lysates where there are still intact subcellular particles. If there is a need for membrane fragments and organelles, then the Dounce homogenizer is a good tool to use. Once the sample is placed in the tube, the pestle is inserted, pressed down, and then lifted. This up and down motion is repeated which causes the sample to be sheared repeatedly. The shearing force can be controlled to an extent by using different pestles with different diameters. The larger diameter pestle is tighter fitting and creates greater shear, while the opposite is true for the smaller pestle.

Strengths – An inexpensive device that is very effective for mildly lysing cells. Dounce homogenizers are easy to use, clean, and decontaminate.

Limitations – Solid tissue is not effectively homogenized using a Dounce homogenizer. If individual cells are processed, they must first be disaggregated from solid tissue or dissociated from tissue culture plates which is time consuming. Throughput is low. The devices are fragile and can break easily.

Potter-Elvehjem with PTFE Pestle: By changing the type of pestle used in a Potter-Elvehjem homogenizer, it effectively changes from a grinder to a shearing homogenizer. For shearing, the pestle is connected to a variable speed lab motor. For sample processing, tissue is placed in the sample and the pestle is rotated at 600-750 rpm. The tube is repeatedly pressed up on the pestle where shearing forces disrupt the sample.

Strengths – The Potter-Elvehjem homogenizer is relatively inexpensive, though the motor can cost several hundred dollars. They are easy to use and clean, and samples can be kept cold on ice during processing. The homogenizer is effective for disrupting animal cells for the generation of subcellular components.

Limitations – Using a Potter-Elvehjem homogenizer with a motorized PTFE pestle is good for disrupting cells, but not every efficient at homogenizing solid tissue. Muscle homogenized by this method was incomplete and significant sample left as solid. Overall the process was poor in a one-step method. When used in conjunction with other homogenizers, it was effective.

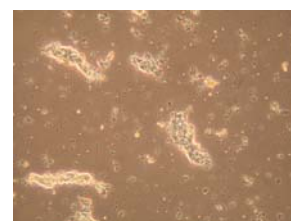
French Press or French Pressure Cell: The French press came into use in the early 1960s. It was developed in the late 1940s for the disruption of microorganisms. It works by forcing cells through a tiny orifice under extremely high pressure, e.g., 20,000 psi. As the cells move from high pressure to low pressure, they expand and shatter.

The French press is very useful for periodic cracking of microorganisms, but it is much less useful for routine cell disruption. The samples that are used in French pressure cells must be fluid, such as previously homogenized tissue, blood, microbes, or other fine particulate fluids. When disrupting microbes, cells are often harvested by centrifugation and the cell paste is processed.

French pressure cells cost upward of \$3500, with the smaller cells being used for samples



Dounce homogenizer produces 32% relative homogenization of solid muscle as compared to other processes.



Potter-Elvehjem homogenizer with motorized PTFE pestle yields 36% relative disruption of muscle as compared to other systems.

of 1-2 ml. Larger presses process upwards of 30-40 ml.

Strengths – The French press is a very effective and efficient tool. Homogenates generated by French press rival ultrasonication in degree of thoroughness of disruption. Sample homogenates are very uniform.

Limitations – Sample sizes are relatively small and throughput is very low. For any samples other than single cells or microbial cultures, a pre-homogenization step is first necessary. French pressure cells can be expensive relative to the number of samples that can be processed. Because of the small orifice, the French press can clog.

Beating

Beating is in many ways similar to grinding, but the act of beating a sample using a projectile makes it distinguishable. Most bead beating methods rely on placing a sample and beads in a tube and rapidly shaking them back and forth. Bead beating has been used for years for the disruption of microorganisms, originally using small glass beads and dental amalgamators (i.e., the shakers that dentists use to mix up the components of metal fillings). Bead beating is simply quite effective, though traditionally it was a bottleneck due to the limited number of samples that could be processed. Some labs took it upon themselves to increase the throughput by adapting paint shakers to process samples. At times these were effective, but not totally satisfactory. To remedy this limitation, several companies developed bead beaters (also called mixer mills) that could handle racks of tubes or even microwell plates.

Amalgamators for Tubes: Dental mixers, or amalgamators, have been used to bead beat microorganisms for years. This simple instrument allows a tube to be locked into a little shaking arm which then oscillates rapidly. When bacteria, yeast, or molds are added to the tube with grinding beads, the amalgamator effectively grinds the cells within several minutes.

These beads beaters are moderately effective and relatively low cost. Several vortexers have been modified to provide similar actions, with the added value of being able to process multiple tubes.

Strengths – These are moderately effective homogenizers at a very reasonable price.

Vortexer units are available with a pulsing feature which helps to reduce the effect of heat generated during homogenization.

Limitations – The individual tube bead beaters are rather effective, though throughput is low. For labs running limited samples, this unit might be adequate. Vortexer units are less effective, but hold greater numbers of samples. Depending upon the application, lower lysis efficiency may not matter.

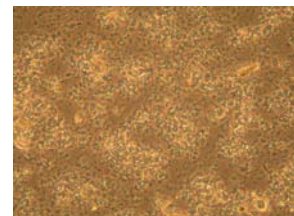
High Throughput Homogenizers: With the rise of high throughput screening strategies, sample homogenization became a bottleneck. To circumvent the logjam, various approaches were taken to homogenize samples *en masse*. The simplicity and effectiveness of bead beating was applied to a larger format, the result being high throughput homogenizers. These devices, formally known as mixer mills, in the simplest form, shake tubes or microwell plates at speeds up to 1600 rpm with grinding balls.

Mixer mills are not new, but their adaptation to the SBS formats (i.e., Society of Biomolecular Screening standardized plate dimensions) is relatively new as compared to other homogenization technologies. Essentially a microwell plate, set of vials, or rack of tubes is locked into a moving platform. Normally each well/tube has a sample and one or more grinding balls. For processing, the homogenizer violently shakes the samples causing the grinding ball(s) to impact the samples against the tube walls. Processing for 1-2 minutes usually thoroughly homogenizes most samples.

High throughput homogenizers are versatile as they can be used for a wide array of sample types and sizes. The most important aspect of getting good disruption from a high throughput homogenizer is to properly match the sample size (mass and volume) with a suitable well size and grinding ball. Generally, all samples and buffers should take up no more than a third of the volume of the vessel. Thus with a leaf punch of 10 mg with 200 μ l of extraction buffer can easily be processed in a 1.2 ml round well, 96 deep well plate using a standard 5/32" grinding ball. Note that a round well was specified as square deep well plates afford samples to hide in the corners avoiding homogenization. Deep well plates should be used for small samples of 50 mg or less. As



High throughput homogenizer with deep well plate and 5/32" grinding ball produced very fragmented muscle and a relative disruption efficiency of 52%.



High throughput homogenizer with 15 ml vials and two large 7/16" grinding balls produced a fine homogeneous lysate with a relative efficiency of 81%.

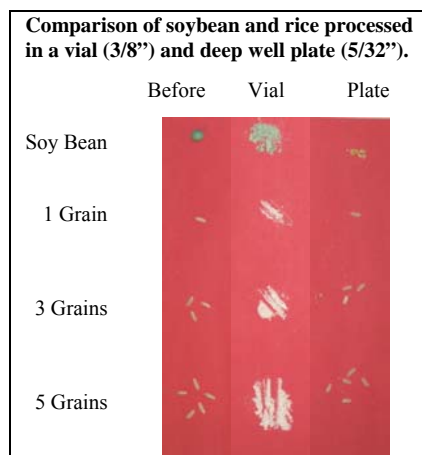
sample size increases, polycarbonate vials are the next best choice for grinding.

Any tube or container that can be locked into a high throughput homogenizer can be used to disrupt samples. Indeed squat 60 ml polycarbonate jars have been used to homogenize complete animal organ systems. Most samples however are under 1 gram. When sample size is larger than 100 mg, a 24 well format is an effective format to use. Polycarbonate vials (4 ml) are available in the SBS 24 well format which allows for liquid handling of homogenate. In this format, larger grinding balls (3/8") are used. Polycarbonate is the choice material to use for homogenization vials as it is hard and pushes back on grinding balls. Though polypropylene vials will work, as with the deep well plates, it tends to soften as the tube heats with processing. With harder samples, like seeds, the ball can wedge in the tube with the sample. For samples that require organic solvents that melt polycarbonate, such as phenol and chloroform (including Trizol™), polyethylene vials are available that are solvent compatible.

High throughput homogenizers can be used for wet or dry grinding. Wet grinding as primarily described above, is just that, homogenizing with solvent. Dry grinding is popular in the analysis of seeds and plant materials. In this approach, the seed is processed with the ball without solvent. The key factor in dry grinding, especially seeds, is that the samples tend to be very hard and as such require a disproportionately large grinding ball. For instance, a grain of rice is only about 20 mg, and easily fits in a well of a deep well plate, but the 5/32" grinding balls used in that format have insufficient mass to crack the rice. To smash the rice requires a 4 ml polycarbonate vial with a 3/8" grinding ball.

Many agricultural biotechnology labs analyze seeds from field trials. This involves pooling seeds prior to homogenizing. To accomplish this, larger polycarbonate vials are available that can hold up to 15 corn kernels and numerous smaller seeds (several grams). Using a larger vial allows for larger grinding balls, and in the case of corn, two 7/16" balls are used. This process generates corn meal that can be used for a range of tests from starch composition to genetic analysis. The larger vials reduce throughput using platform homogenizers, but it still is preferable to using coffee grinders which required

cleaning after each sample. In one laboratory using coffee grinders to process corn, throughput was increased from 5 samples per hour to 100 samples.



These larger vials have found new application in biopharmaceutical labs and are now being used more extensively for high throughput analysis of animal tissues for pharmacokinetic analysis. Residual drug levels are often assessed from dosed animals, a labor intensive process that requires homogenization of tissues and organs, usually with rotor-stator homogenizers. Organs and tissues can be homogenized in 15 ml vials very effectively in a one-step process that produces very fine homogenate. The larger 7/16" balls used with 15 ml vials provide additional force that differentiates the degree of homogenization, even when compared to proportionally smaller samples in deep well plates.

Strengths - High throughput homogenizers are designed for processing hundreds of samples daily, but their overall effectiveness makes them useful for lower throughput operations as well. The fact that the grinding balls and vessels (i.e., plates vials, tubes) are separate from the mechanical mechanisms makes minimizing cleaning and cross-contamination issues as most lab treat the plates, vials, and grinding balls as disposables (though balls and some vials can be reused).

Limitations – High throughput homogenizers require an initial investment of \$7000 to \$15,000. The use of consumables may increase sample process costs, but that must be measured against reduced labor and overhead charges.

Not all high throughput homogenizers shake plates the same way, as some use a figure “8” paint shaker motion while two designs have a linear motion. The paint shaker type homogenizers tend to give different lysis efficiencies between wells as not all samples follow the same shaking path. Linear motion homogenizers yield comparable processing for each sample.

Shock

Shock waves are also used for disrupting samples, with ultrasonication being the best example. Shock is pressure. Several tools are available that disrupt samples by using pressure differential.

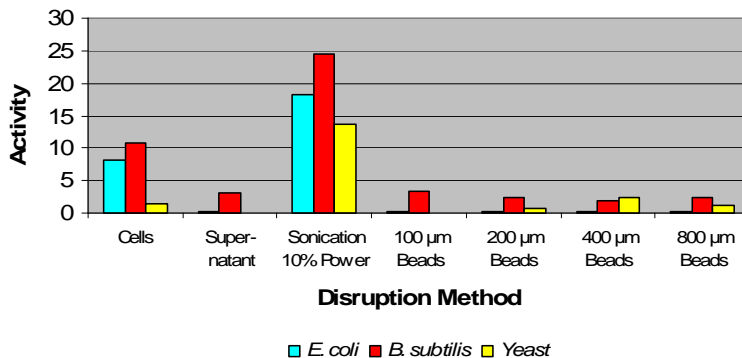
Sonication: Sonication is one process by which samples can be disrupted by pressure. In this case, the pressure is created by a probe that rapidly expands and contracts at high frequencies. The probe undergoes this high frequency oscillation due to the piezoelectric effect, a phenomena that occurs when oscillating current is applied to certain crystals, such as quartz and sodium potassium tartrate. (See [link](#) for further explanation on piezoelectric effect.) When a current is applied to these crystals, they contract, while reversing the current causes them to expand. Rapid oscillation of the current causes tiny shock waves. Sonicators are designed with the crystals being attached to a metal probe so that the energy of the shock waves is focused to a small area.

The energy coming from the tip of a sonicator is extreme. Anyone misguided enough to touch the tip of a sonicator knows it is like touching red hot metal. Ultrasonic probes and baths oscillate up and down at 20,000 cycles/second though the amplitude of the oscillation is very short. A typical oscillation involves a contraction when the electrical current is applied and an expansion when the current is reversed. When the probe contracts, negative pressure causes the liquid to flow up with the probe while the expansion of the crystals pushes the liquid. At a rate of 20 kHz, the liquid turns into a zone of microscopic shockwaves.

As liquids cannot flow as fast as crystals oscillate, during the contraction small vacuum cavities are formed. When the crystals expand, the cavities rapidly implode and create microscopic shock waves. This process, known as cavitation, is extremely powerful when the collective energy of all the imploding cavities is combined. The

cavities are formed and collapse in microseconds.

Relative Disruption Efficiency of Microorganisms

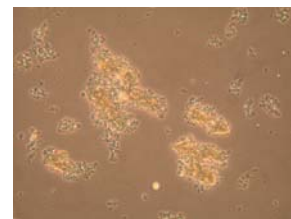


Strengths – For single cells, cell suspensions, and microorganisms, sonication is probably the most effective homogenization method. The extremely powerful forces generated by cavitation are capable of disrupting most cell samples in seconds.

Limitations – As effective as sonication can be, it also has the greatest limitations. It is a powerful method if the samples contain small particles, i.e., either cells or c homogenized tissue. For solid tissue, sonication is a very poor method. Sonication also generates a tremendous amount of heat which can denature many proteins. This can be offset by short bursts coupled with incubations on ice, but that becomes laborious and time consuming. Throughput is also an issue for sonication. If cross-contamination is not an issue, then a single probe sonicator can be used on multiple samples one at a time. There are multi-probe sonicator heads, but the sample to sample variability with this system is unknown.

Combined Methods

The scientific literature reveals an interesting pattern in the way researchers have historically homogenized samples. Foremost, scientists in their traditionally independent manner rely on no specific tool in any one field to homogenize samples. The methods and procedures that are employed support the notion of innovative and independent thought upon which science is built. However, there is one characteristic that occurs frequently, which is very effective sample disruption typically



Sonicator with micro probe tip yielded the lowest efficiency of all methods on muscle at 2.6%.

uses two or more of the methods discussed above. Most methods are done with two steps using different tools.

Using two steps to homogenize samples is done out of practicality. The first step in the process is used to reduce the size of the sample to coarse particles while the second step further reduces or obliterates those particles. The second step of the process is unsuccessful without the initial processing step. Perhaps the most apparent tool that does not work on solid samples is ultrasonication.

On microorganisms and cell suspensions, sonication is very effective at lysing cells as compared to bead beating. However, on solid tissue, sonication is highly ineffective. With solid mouse muscle used as a comparative substrate, sonication was only useful to cook the muscle. On the solid, no disruption occurred, and in this particular case, no active enzyme was liberated. However, if sonication is used as a second step, as with following cryogrinding, then the effectiveness proved better than all others. Indeed the combination of CryoGrinder and sonication liberated greatest amount of enzyme and became the standard by which the other methods were measured.

Other combinations also provided to be effective. Cryogrinder in conjunction with the Dounce, Potter-Elvehjem, and conical glass homogenizers all generated homogenates better than those methods alone. The rotor-stator and sonicator also were an effective combination.

The trade off with the two-step homogenization processes is that it slows throughput. Certainly the liberation of analytes is greater, but processing time per sample is increased. As compared to the high throughput homogenizers, two-step homogenization process is more thorough, but it is off-set by lower productivity by the researcher. Consequently, it is necessary to establish a goal of the homogenization process within the larger objective of the researcher process. If the objective is to process a vast number of samples, then high throughput methods are warranted. If pure analytical data is required, then two-step processing of samples should be pursued.

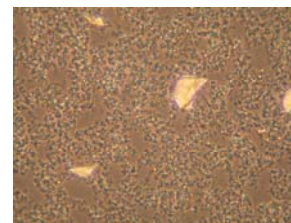
Comparing the Methods

The release of analytes from biological tissue is one of the first processing steps post-harvest. To do this, a myriad of tools are available for disrupting samples, however it is not always clear as to the strengths and limitations of these tools.

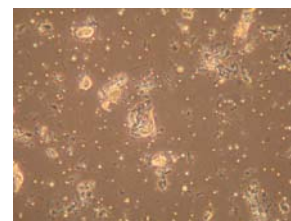
In high throughput environments, where each sample cannot be readily scrutinized, it is fundamentally important that the disruption step is uniform and does not drastically increase the variability of sample analysis. In order to assess the effectiveness of sample disruption, our team examined the tools and handling options on a variety of biological samples, including cultured cells, bacteria, yeast, liver, muscle, heart, lung, and plant tissue (seeds and leaf punches). Disruption methods employed included liquid shearing, sonication, cryogrinding, bead beating and mechanical homogenization. Disrupted samples were measured for lactate dehydrogenase release as determined with an INT assay, yield of nucleic acids, resulting particle size and homogenate consistency, and ease of handling of homogenate with a Biomek FX Assay Workstation.

Generally the efficiency of sample disruption was inversely proportional to its throughput, the exception being with high throughput bead beating. Manual glass homogenizers can process large samples to generate suspensions that have good LDH activity, extremely small particle sizes, and are readily liquid handled. However, throughput was laboriously slow and residual fibrous tissues often remained adhered to the homogenizer. Mechanical homogenization yielded very fluid samples; even of tough tissues such as muscle however resulting particle sizes (observed microscopically) were relatively large. Sample debris cleared by centrifugation retained considerable enzyme activity. Sonication was effective for disrupting small particles, but yield poor results on solid samples. Sonication, though relatively fast, requires a prior disruption step for any solid samples.

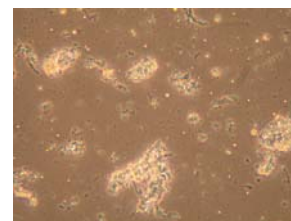
High throughput bead beating (mixer mill) was very effective at disrupting samples, yielding the highest LDH activity. However, sample size was limited to 100 mg/well using deep well plates. Cryogenic grinding was also examined as a method for preparing samples for repeated handling. Frozen tissue powder could be measured and



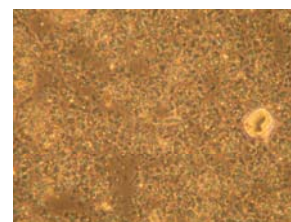
Rotor-stator combined with sonication homogenized at a relative efficiency of 37.2%.



Cryogrinding followed by Dounce homogenizer were 44.6% efficient as compared to other methods.



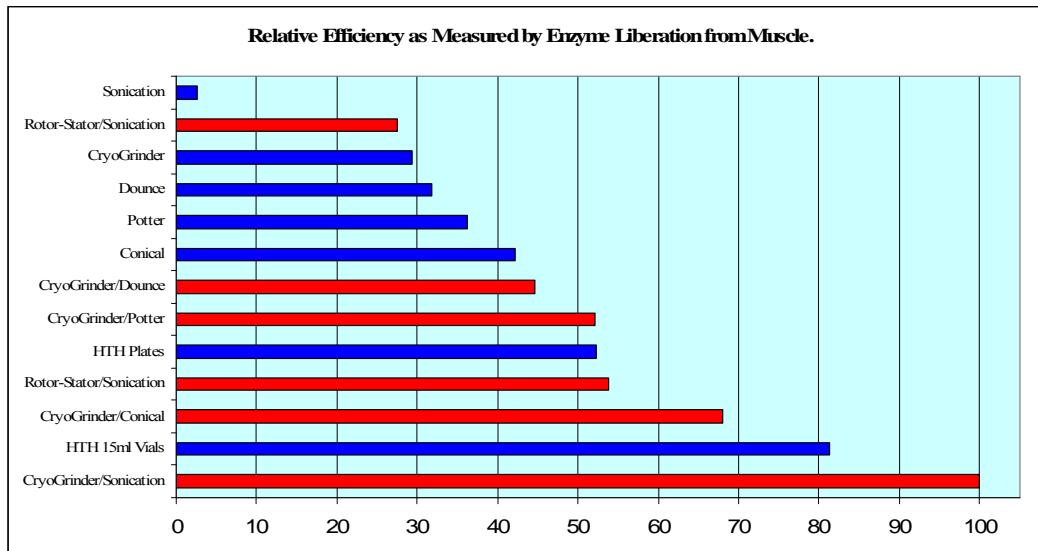
Combining the CryoGrinder and Potter-Elvehjem homogenizer yielded an improved efficiency of 52.1%.



Cryogenic grinding followed by sonication proved to be the most efficient combination for homogenization with a relative efficiency of 100%.

subsequently homogenized to yield high levels of LDH activity and nucleic acids. A

matrix of biological tissue versus sample disruption method is discussed.



This relative efficiency chart is very revealing as regards the effectiveness of any single method on sample homogenization. The blue bars represent a single processing step while red bars are two-step processes. Most single step processes are less than 50% effective as the most effective two-step combination. The force of grinding balls with the high throughput homogenizers provide the best single-step results, and with that, the large vials are grinding balls suggest that greater force produces better disruption. Sonication, interestingly, brackets both ends of the scale showing its ineffectiveness on solid samples but efficiency at finishing a two-step process. Although many one-step processes are only partially effective as compared to alternatives, it is important to realize that if chemical lysing methods are used subsequently, the resulting homogenization may be completely adequate. This is certainly the case for cryogenic grinding where tissues are effectively dissolved with detergents and chaotropes.

Summary

Effective disruption of biological samples is a process that starts with sample collection and proceeds through the homogenization process. To generate a homogenate that is suitable, it is prudent to define the characteristics required in the fine product and then choose the best method or combination of methods that will produce that product.

All methods used to disrupt samples have strengths and weaknesses. Lower throughput methods can be used to process larger samples with good results, especially as many are low in cost. High throughput methods are also effective, but initial costs may be a limitation.

Many tools used to homogenize samples, by themselves, are not that effective at disruption samples, but when combined with other tools can be very useful. Ultrasonication is the classic example as it is a poor method when used on solid samples, such as muscle, but yields the best results when combined with an early processing step. Many of the shearing methods produce fine homogenates but fail to disrupt all tissues. They can be valuable in producing lysates with intact subcellular components. Bead beating with high throughput homogenizers can be a very effective one-step homogenization method however effectiveness decreases with sample and grinding ball size.

Materials and Methods

Microorganisms: Stock cultures of *E. coli*, *B. subtilis*, and *S. cerevisiae* were used in this study. Bacteria were cultured on trypticase soy broth in baffled flasks, overnight at 30°C. Yeast was cultured on YPD broth (1% yeast extract, 2% peptone, 2% dextrose) in baffled flasks, overnight at 28°C. For enzyme assays, the microorganisms were diluted in PBS to a concentration that yielded optical densities between 0.1 and 1.0.

Tissue Samples: Female CD1 mice, 42 days (Charles River Laboratories) were sacrificed and spleen, heart, lungs, kidneys, leg muscles, and liver were extracted, frozen, and stored at -140°C.

Cell Culture: Mouse hepatoma cells were cultured on DMEM with 10% fetal calf serum with penicillin, streptomycin, and glutamine. Cells were cultured in flasks at 37°C in 5% CO₂. For testing, cells were trypsinized, harvested by centrifugation, and diluted in PBS to a density to yield a reading of 0.1 to 1.0 for LDH assays.

Lactate Dehydrogenase Assay: Mouse muscle was homogenized and lysates were assayed for the release of lactate dehydrogenase from cells and tissues. Several one-step methods and two-step homogenization methods were employed. For one-step methods, a known mass of muscle was placed in a tube and 10 volumes of Complete Homogenization Buffer with protease inhibitors was added (OPS Diagnostics), followed by homogenization as described in Table 1. For two-step homogenization methods, homogenization protocols were run in tandem (e.g., mechanical homogenization followed by sonication). For all approaches, total muscle lysates were cleared by centrifugation prior to analysis to remove LDH bound to debris and tissue particles. A colorimetric NAD linked assay was used to measure released lactate dehydrogenase. The LDH substrate solution is prepared by mixing 2 ml of 1 M TRIS, pH 8, with 8 ml water, followed by the addition of 49 mg lithium lactate (Sigma L-1500), 100 µl of INT stock (33 mg/ml iodinitrotetrazolium chloride (Sigma I-8377) in DMSO), 0.9 mg phenazine methosulfate (Sigma P-9625), and 8.6 mg β-nicotinamide adenine dinucleotide (Sigma N-0632). Briefly, 50 µl of lysate (or lysate diluted in PBS for concentrated samples) is added to a well of a

microtiter plate followed by 125 µl of substrate solution. The plate is placed in a kinetic plate reader and optical densities at 490 nm are measured every 30 sec. for 5 min. LDH activity is calculated as the maximum slope of the reaction.