Guide to the Disruption of Biological Samples – 2012

Version 1.1

David W. Burden, Ph.D.[†]

Abstract

When developing a disruption scheme, it is prudent to define the characteristics of the desired homogenate and then select the methods, reagents, and tools that will help to meet the objectives. There is a vast selection of chemistries and tools that have been endlessly combined to disrupt samples. Detergents, chaotropes, and lytic enzymes are often effective for lysing cells and tissues, many times alone, but also when combined with mechanical methods. Mechanically disrupting samples using homogenizers, which can be grouped into those that grind, shear, beat, and shock, is commonplace when chemical methods alone are insufficient. 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 first be 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 may require a trade-off with the effectiveness of homogenization.

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

(P) This symbol denotes that there is a link to a related protocol.

Introduction

Disruption is an early and fundamental step in any research which involves analyzing, separating, or isolating some component from an intact sample. This includes the isolation/harvesting of cellular components or quantification of 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 (e.g., seeds), and heavily encased samples are not effectively disrupted chemically. With chemically resistant samples, mechanical and physical methods that rely on grinding, shearing, beating and shocking can be 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 often scantly detailed in protocols even though it does have significant impact on the end results of a process. Many research articles will simply state that a sample was "homogenized" in a defined buffer, without specifying what type of homogenizer was employed. When specific homogenizers are mentioned, such as the Dounce or bead beater, little additional information is provided to detail its use. Consequently, methods used for sample disruption are also not necessarily well understood. Indeed like many well established lab processes, homogenization methods are passed on from researcher to researcher like inheritable family treasures, with little effort expended to decipher the process itself. This leads to significant variation in methodology between laboratories. Being fair, the impact of homogenization on an experiment may be minimal, but at times the choice of tools, chemistries, and their method of use may have a significant impact on the outcome.

Where possible, chemical disruption may be the preferred method, such as lysing *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 making their application to protein purification impractical. The same is true for the addition of lytic enzymes, which in the case of protein purification, must be subsequently removed. If chemical disruption 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. Grinding, which is done with such tools as a mortar and pestle, involves applying force downward on a sample in conjunction with a separate tangential (i.e., rotating) force. Shearing is like that of a blender where a force is tangentially applied to a sample. Directly impacting a sample with a ball or hammer is beating. Shock is similar to beating, but there is no physical implement contacting the sample, just shockwaves. At times it is difficult to discern between the different forces that relate to each method. For instance, grinding is a combination of shearing and beating, 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. For instance, the isolation of subcellular fractions could first involve cutting a tissue with scissors, followed by course shearing with a handheld homogenizer, and then a final dissociation with a glass Dounce homogenizer. If any one of these steps is omitted, then the degree of homogenization would be reduced.

There are several methods we employ to evaluate homogenization methods which will be highlighted in this guide. First, homogenization releases cytosolic enzymes and several of these can be assayed and used as a relatively measurement of sample disruption. Lactate dehydrogenase is routinely used in our laboratories for this assessment. Similarly, measuring soluble protein can be used as an indicator of homogenization efficiency. Microscopic observation of samples also provides useful information on the extent by which samples are homogenized.

Strategies for Disruption: Selecting a Method(s)

The approach used to disrupt tissues, cells and/or matrices is dependent upon the objectives of the researcher. This might appear as an obvious statement to the experienced scientists, but to the individual just getting started in a particular line of investigation it might not be as apparent. Deciding on a disruption method should be approached in a reverse direction, i.e., the final product should dictate the tools and methods used to produce it. For instance,

producing a lysate which includes active protein requires a method that avoids denaturing conditions and harsh treatment of the sample, while isolating RNA may involve the exact opposite.

For discussion purposes, the methods used for disrupting samples has been divided into chemical and physical means. This dichotomy is relatively artificial as most homogenization/disruption processes make use of a combination of mechanical and chemical methods. However, in its simplest form, disruption can be broken down as illustrated in Figure 1.

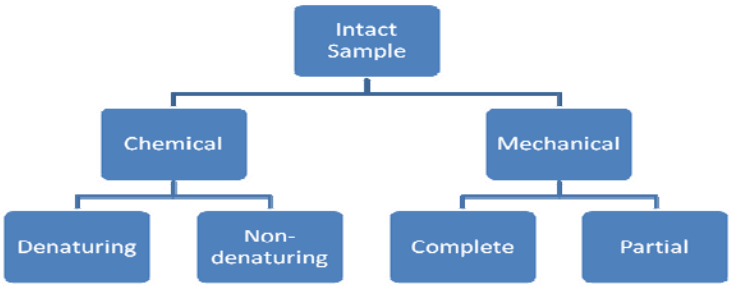


Figure 1. Simple schematic of sample disruption options. This simplified scheme does not consider that methods are normally combined during sample disruption. Mechanical homogenization normally makes use of buffers or lysis solutions just as chemical lysis normally requires that the sample contains small particles, which is normally created using a homogenizer.

In developing a good method to disrupt samples, it is necessary to consider the desired characteristics of the final homogenate/lysate and then work backwards as to which chemistries and tools will work alone, or in combination, to yield that product. To do this, knowledge of the limitations of the target molecules is necessary. 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. In isolating RNA, great care needs to be taken to prevent omnipresent RNases from degrading the target by lysing samples under highly denaturing conditions. 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. Table 1 provides a summary of major targets and factors which should be considered during sample disruption.

Table 1. Major biochemical targets and sample processing considerations.

Target	Application	Considerations
Protein	Activity Assays/ Characterization	Liberation of active protein is normally done under non-denaturing conditions (i.e., no use of strong detergents or chaotropes). Samples should be kept cold to avoid inactivating heat labile proteins. Proteases may be liberated during homogenization, thus protease inhibitors may be added to the sample. For further information on retaining protein activity, see this link.
Protein	Immunoassays	Antibodies can bind to denatured proteins readily, thus lysates prepared for immunoassays are often done using surfactants. Protein samples for Western blots are often boiled in SDS.
RNA	Quantitation/ Cloning	RNA in intact cells and tissues can be stable. Once it is released by homogenization, RNases can rapidly degrade the target. Consequently, isolating RNA usually involves minimizing RNase activity by preparing samples under highly denaturing conditions using chaotropes and by maintaining very low temperatures during sample disruption.
DNA	Detection/ Quantitation/ Cloning	DNA is also threatened by nucleases upon liberation from an intact sample. However, DNase is much easier to control by the addition of EDTA which chelates Mg^{+2}, an ion necessary for DNase activity. Many DNA isolation protocols still make use of detergents and chaotropes.
Drugs	Quantitation	Pharmacokinetic analysis of residual drugs often uses organic based extractions with acetonitrile and methanol. The major considerations when disrupting samples with such solvents is vapor pressure created from processing samples in closed containers and flammability.

(P) This symbol denotes that there is a link to a related protocol.

Although defining the characteristics of a homogenate is a major consideration in selecting methods for a disruption process, it is not the only one. A second major consideration is sample throughput. A researcher in a high paced work environment knows the stress of generating mass data. Indeed as budgets tighten and staffs are "right sized", the workload of researchers increase. In these environments, sample preparation is often the bottleneck. There are many chemistries and tools available, but it is the homogenizer type which can clog a process pipeline. Many homogenizer designs have been unchanged in half a century and were designed for scientists who might process a couple of sample per week. These older homogenizers were the centerpiece of slow laborious protocols, such as grinding tissues in liquid nitrogen with mortar and pestle. This type of process may be completely adequate for many labs, but it can be very inadequate when hundreds, if not thousands of samples must be processed daily. Therefore, the level of throughput needs to be considered in designing a scheme.

Sample disruption throughput is dependent upon whether samples come into direct contact with the homogenizer during processing. With glass homogenizers, such as the Dounce, Potter-Elvehjem, and conical ground glass, and rotor-stators time is required to clean and decontaminate the homogenizer itself following sample processing. With the case of rotor-stators, this may involve disassembling the shaft. Glass homogenizers may need to be washed with detergents and then decontaminated by baking. With manual cryogenic grinding, the time required is even greater as the liquid nitrogen chilled mortar and pestle must be allowed to warm before cleaning. If manual methods are used for processing many samples, then it is necessary to have many of these homogenizers available.

Ultrasonication probes are the exception to this issue as they can be readily cleaned. As the probes are metal and cleanable, they can be washed and decontaminated with alcohol.

Homogenizers that isolate the sample from the homogenizer are usually more accommodating for high throughput sample processing. Bead beaters are effective this way as samples are in tubes and the tubes are agitated. The homogenizer itself never touches the sample. This is also true for bath sonicators where the sample tube is immersed in a bath.

Harvesting Samples

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

RNA and protein profiles are not uniform between cells in a heterogeneous sample. Therefore, it is important to consider whether the position of analytes within a sample is significant or whether the concentration or presence of the analyte in relative concentration to the whole sample is important. If location is important, then care needs to be exercised in not only maintaining the position of materials within the sample, but the impact of stress imparted by handling and storage should also be considered. Alternatively, homogeneous samples, such as a shaking bacterial culture, can be viewed as a uniform collection of cells.

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. There is usually little concern about losing cells from a culture as it is generally assumed that all the cells are genetically identical and physiologically the same. However consideration should be given to the target molecule and its stability once the manipulation of the sample begins. For instance, *E. coli* cultured at 37˚C in a shake flask is in a very different environment than *E. coli* embedded in a pellet following centrifugation. Berstein et al. (2002) reported that the half-life of approximately 80% of *E. coli* mRNAs are between 3 and 8 minutes, well within the time it can take to simply harvest cells from culture. Consequently, the RNA profile of a microorganism may represent the conditions of sample collection rather than culturing.

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 survive based on the environmental parameters surrounding that mass and disaggregating the biofilm will certainly change the behavior of the bacteria. The physiology of *Pseudomonas aeruginosa* cells differ upon its position within biofilms (Pérez-Osorio et al., 2010), thus disruption

during harvesting and storage may affect gene expression based on changes to microenvironments. 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. Handling of oat seedlings, soybean hypocotyls, and potato plants all have shown changes in transcript levels based on the handling (Green, 1993). For 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 as the collagen is sufficiently stable. For other proteins, this may not be the case (see link for more on the stability of proteins).

Original strategies for the isolation of RNA from animal tissues were believed to be highly dependent upon the harvesting method. Rapid harvest and freezing was believed to be critical to retaining RNA within the sample. This was followed by cryogenic grinding or homogenization under highly denaturing conditions (Kirby, 1965). It was widely taught that RNA degraded very rapidly once an organism was sacrificed. Currently, many of the original notions of the methods needed to isolate RNA impressed upon "experienced" scientists appear to be in question.

RNA is apparently not as fragile a molecule as once believed. Sharova et al. (2009) examined a wide spectrum of mouse embryonic stem cell mRNAs for their half-life and found all but a few to have a half-life of over 7 hours. This type of stability is in stark contrast to the notion that RNA turnover in cells should be measured in minutes. RNA levels in porcine retinal pigment cells (Malik et al., 2003) were relatively stable up to five hours after extracting intact eyes from sacrificed animals. Another eye opener (no pun intended) is that RNA in harvested tissues and deceased animals, including cadavers, may be relatively stable post-mortem for several hours (Lee et al., 2005), though there is significant variability between tissues and individuals. This is based upon the tissue remaining intact. Once tissues are dissected, the rate of RNA degradation increases (Fajardy et al., 2009). Generalizing, it appears that RNA can be stable for several hours in post-mortem samples as long as tissues have not been dissected or homogenized. Once homogenized, RNA is at risk of degrading.

Harvesting most biological samples involves some type of refrigeration. All molecules, including DNA, RNA, and protein, will remain intact as long as they are stored below -130°C, either in cryogenic freezers, vapor phase freezers, or submersed in liquid nitrogen (see link for more information on cryogenic storage). Many researchers routinely archive tissues in -80˚C ultralow freezers, though some biological activity can exist even at this temperature.

Chemical Disruption Methods

It is very possible to disrupt a biological sample using nothing more than water and a blender. This would be considered a mechanical approach to sample disruption. However, most methods use lysis buffers/solutions instead of water as they provide a degree of stability when isolating specific biomolecules. Virtually all lysis solutions address pH (which is why they are usually called lysis buffers) but they may also control ionic strength, osmotic strength, and the activity of nucleases and proteases. When isolating membrane proteins, surfactants are normally used to partition the membrane proteins from the membrane to surfactant particles, called micelles. Other common lysis buffer additives include lytic enzymes, which can liberate cellular contents from cell wall envelopes, and chaotropes that disrupt the ordered structure of biological systems which protects biomolecules from enzymatic degradation.

Lysis buffers in many instances can be used to lyse cells and tissues without the assistance of mechanical homogenizers. Indeed one of the most common disruption methods relies on lysing *Escherichia coli* with an alkaline solution of SDS (the detergent sodium dodecylsulfate) for plasmid isolation. Similarly, adherent tissue

culture cells can be lysed with high concentrations of chaotropic guanidine salts (e.g., chloride or isothiocyanate). For solid and resilient samples, lysis buffers are commonly used in combination with a mechanical disruption method. This is particularly true for tissues which are very dense, like organs and seeds. Some microorganisms which are resistant to chemical and enzymatic lysis, such as members of the genus *Mycobacterium*, must also be disrupted mechanically.

Other additives protect liberated biomolecules from denaturation, oxidation and enzymatic degradation. Reducing agents protect free thiol groups from oxidation, especially cyteine located in the active site. Protease inhibitors are regularly added to prevent protein degradation from proteases released from cells during homogenization.

Surfactants/Detergents[1]

Surfactants, which are commonly called detergents, have the characteristic of disrupting the distinct interface between hydrophobic and hydrophilic systems. Biological membranes, the most obvious hydrophobic/hydrophilic interfaces, are the primary target of detergents. Indeed with the example of *E. coli* and SDS, the detergent completely (and effectively) obliterates the distinct interface separating the cell from its environment, i.e., the membrane. However, SDS also has the ability to unfold (denature) cytosolic proteins and partition membrane proteins into small detergent droplets (micelles). Depending upon the detergent used and its concentration, the impact these surfactants have on biological systems will vary greatly.

Detergents have at least two fundamental properties, namely a water soluble hydrophilic head and a hydrophobic (oil soluble) tail. These properties allow detergents to insert into and then disperse membranes, in addition to unfolding proteins. Depending upon the chemical makeup of the hydrophilic and hydrophobic ends its action on proteins and membranes will vary. Not all surfactants are chemically equal as some are capable of completely solubilizing membranes and denaturing proteins while others, like mild surfactants, will disassociate loosely bound proteins.

A major characteristic of surfactants is whether the hydrophilic group is ionic or non-ionic. Ionic surfactants tend to be better at solubilizing membranes and denaturing proteins. With ionic surfactants, the hydrophilic moiety is typically a sulfate or carboxylic group for anionic surfactants or ammonium group for cationic surfactants. SDS (sodium dodecyl sulfate), is an anionic detergent with a sulfate hydrophilic head and 12 carbon tail (dodecyl or lauryl) which is important not only in the lab but also in many household detergents. Sodium deoxycholate is a carboxylic based anionic detergent derived from bile salts which is commonly used in many lysis buffers. CTAB (cetyltrimethylammonium bromide) is a cationic detergent widely used in the isolation of DNA from plants.

In addition to detergents with a net charge, zwitterionic detergents are a class of surfactants that possess both anionic and cationic groups and have a net charge of zero. The zwitterionic detergent CHAPS, a derivative of cholic acid, is effectively used for isolating membrane proteins.

Non-ionizing detergents have a head which is polar, but uncharged, such as a glycoside (sugar) or polyethylene chain, tend to be milder and less likely to denature proteins, but still capable of dispersing some membranes. They often act to dissociate loosely interacting molecules. These surfactants, such as Triton X-100, Brij-35, NP-40 and Nonidet P-40, are widely used in immunoassay wash solutions at low concentrations, but also in lysis buffers at higher concentrations.

The value of detergents when applied to isolating membrane proteins is related to their ability to form micelles. In aqueous environments and at the correct concentrations, detergents will spontaneously form small particles called micelles where the hydrophilic heads orient outward and the hydrophobic tails congregate inwards. The concentration at which this occurs is called “critical micelle concentration” or CMC. Depending upon the detergent, the molecular weight of the micelles can range from 1200 to 80,000 daltons.

[1] The terms surfactant and detergent are used very loosely throughout scientific literature (including here). As surfactants are defined as chemicals that interact with hydrophobic/hydrophilic interfaces, detergent is chemically defined as an alkylbenzylsulfonate, a specific subgroup of surfactant.

(P) This symbol denotes that there is a link to a related protocol.

Micelles dissolve into and disrupt cell membranes which then disperse into membrane/micelle hybrids particles. Proteins embedded in cellular membranes are picked up by these micelles. A constant equilibrium between monomer detergent molecules and micelles ideally lead to a dispersion of membrane proteins so that each micelle contains one protein. For protein purification, the protein/micelle acquires characteristics which allow it to be separated from other membrane proteins/micelles.

Detergents and their use are application specific and not always predictable.

Table 2. Detergents used for sample preparation and their properties.

Detergent	Type	Characteristics	Use Level
SDS (sodium dodecylsulfate)	Anionic	Strong detergent used to disrupt membranes and denature proteins	Commonly used between 1-10%
Sodium Deoxycholate	Anionic	Derived from bile salts. Effective at solubilizing proteins and disrupting protein-protein interactions.	Common use level is 0.5%.
CTAB (cetyltrimethylammonium bromide)	Cationic	Popular cationic detergent used for the isolation of DNA from plants. Polysaccharides associated with plants are insoluble in CTAB and high concentrations of NaCl. This can be used to effectively separate DNA from plant carbohydrates.	For DNA isolation buffers, typical use level is 2%.
NP-40 (nonyl phenoxypolyethoxyl ethanol)	Non-ionic	Generally mild surfactant which can dissolve cytoplasmic membranes but not nuclear membranes. Useful for isolating nuclei.	Use at 0.1 to 1%.
Nonidet P-40 (octylphenoxypolyethoxyethanol)[2]	Non-ionic	This mild surfactant is useful for disrupting cytoplasmic membranes of cultures cells, but lacks the strength to emulsify nuclear membranes. Consequently it can be used to harvest cytoplasmic proteins and analytes.	Use at 0.1 to 1%. (P)
Triton X-100	Non-ionic	This is a mild surfactant/surfactant that has polyethylene oxide as a hydrophilic group and a tetramethylbutyl phenyl group as the hydrophobic portion.	For lysis solutions, up to 5%. In wash solutions, 0.1-0.5%.
Polysorbate 20 (Tween 20, Polyoxyethylene (20) sorbitan monolaurate)	Non-ionic	This surfactant is a heavily modified sorbitol in which polyoxyethylenes serve as the hydrophilic group and a 12 carbon lauric acid as the hydrophobic end. It is a very biomolecule friendly surfactant, being used in foods, pharmaceuticals, and in wash solutions for assays.	Typically used at very low concentrations of 0.1%.

Chaotropes

Where surfactants are used to disrupt the interface between hydrophobic and hydrophilic systems, chaotropes are used to disrupt the weak interactions between molecules, like hydrogen bonding in water and hydrophobic interactions between proteins. Chaotropes are effective at denaturing proteins that can cause havoc on freshly homogenized samples, which is the rationale for adding chaotropes to RNA lysis buffers. Common chaotropes used in lysis buffers include sodium iodide, guanidine HCl, guanidine isothiocyanate, and urea.

Unlike surfactants which are used at relatively low concentrations, chaotropes are used at high molarities. Guanidine salts, used extensively for RNA isolation, is used at 6M concentrations. Sodium iodide, which at times is used like guanidine, is also used at 6M. Urea is often used at 9.5M. Very often chaotropes are used in combination with detergents so that biological systems can not only be denatured, but emulsified as well.

Chaotropes are widely used and applicable to nucleic acid isolation procedures which use silica based resins/gels for purification. Nucleic acids liberated from tissues lysed in chaotropic agents, such as 6M guanidine, supplemented with Proteinase K (an unusually hearty protease that is active in both denaturing conditions and elevated

[2] Nonidet P-40 is often designated NP-40 which is a chemically similar but different surfactant.

(P) This symbol denotes that there is a link to a related protocol.

temperatures) will adsorb to silica gel upon the addition of ethanol. The very clean nucleic acids can be eluted with water or TE buffer.

Enzymes

Enzymatic treatment of tissues and cells can be a very effective first step in processes where cell walls and extracellular matrices may provide unwanted contaminants in a cell/tissue extract. On-the-one-hand, primary hepatocytes can be generated from sacrificed rats where the liver has been perfused with a combination of trypsin and collagenase. This allows for the harvesting of viable, intact cells from a tissue that releases vast amounts of proteolytic enzymes when homogenized mechanically. Similarly, yeast cells can be treated with cell wall degrading enzymes to yield protoplasts (naked cells) and cell wall shells, or what is commonly referred to as ghosts. This is a common step in traditional transformation procedures used with yeast, but it can also be used to selectively harvest periplasmic enzymes, cell wall mannans or similar component. Likewise, plants can be treated with cellulases to yield protoplast while filamentous fungi can be treated with chitinase.

Proteases are used in sample disruption to disaggregate tissues and release individual cells, or in the case of genomic DNA isolation, attack other proteins that may either bind up the DNA (histones) or threaten the final product (nucleases). Proteases such as trypsin, dispase, and collagenase are used to release cells from tissues and culture plates. However outside of this application, they are undesirable in disrupting samples as they also degrade receptors and other surface proteins. Proteinase K is extensively used in DNA isolation as it is resistant to SDS and heat, both which are used in the typical genomic DNA isolation procedure.

Highly specific lytic enzymes are very useful in sample preparation protocols. With plant, yeast, and molds, cell wall degrading enzymes can be used to either rupture cells in hypertonic buffers or generate protoplasts in isotonic lysis buffers. Most cell wall degrading enzyme preparations are a combination of enzymes as cells walls are typically composed of a mixture of polymers. Yeast cell walls contain glucans and mannans, molds contain chitin, glucan, and galctomannans, and plants have a combination of cellulose and xylans.

Lysis solutions using enzymes at a minimum require a buffer. When generating protoplasts, cells are typically treated with buffered enzyme in the presence of an osmotic stabilizer, such as 1 M sorbitol. The enzymes tend to degrade holes in the cell wall which then allow the protoplast to escape. Gentle centrifugation of protoplasts allows for the separation of empty shells from the cell membrane and its contents. This can be an effective method of separating periplasmic enzymes from other cell associated proteins.

Other Additives

Aside from buffers, there are several other important components of homogenization buffers that warrant discussing. When the objective is to purify an active protein, homogenization buffers may contain many additional components. These can generally be viewed as additives that will help to retain the active form of the protein and those that prevent the degradation of the protein.

The cytosol contains high concentrations of solutes in a reduced environment. Protein concentrations have been estimated to be as high as 30 mg/ml. Liberating the contents of cytosols causes the solutes to become rapidly diluted which not only can cause non-associated solutes to diffuse, but also protein subunits and co-factors. Osmotic stabilizers, such as sucrose or sorbitol, can be added to help bind up water and prevent dissociation of related solutes.

With many homogenization methods, high volumes of air are also introduced into the system, where the oxygen can shift the environment from reduced to oxidized. Within cells, oxygen tension is extremely low, essentially anaerobic, thus the introduction of oxygen and their related radicals can lead to deleterious effects. Reducing agents such as glutathione, dithiothreitol, and β-mercaptoethanol can react with oxidized species and prevent their negative consequences.

For protein isolation procedures, it is very common to add protease inhibitors to the homogenization buffer and/or homogenate. In animal cells, proteases are contained in lysosomes where their function is to recycle the amino acids and breakdown foreign material. Dependent upon the cell and tissue type, the concentration lyosomes and associated enzymes can be high. The proteases contained within are heterogeneous and capable of degrading

proteins at many different locations. Generally there are exoproteases which cleave both the amino and carboxyl terminal residues, as well as endoproteases which can attack specific peptide bonds.

Plants are generally believed to lack lysosomes, but rather use vacuoles in much the same manner. Microorganisms also have proteases, but these are usually located in the periplasmic space. Similar to animal cells, disruption of plant and microbial cells releases the proteases which then can degrade proteins in the homogenate.

The deleterious action of proteases can be reduced by keeping samples cold while processing and by adding protease inhibitors. Though it is impractical to inhibit every type of proteases, several inhibitors can drastically reduce proteolytic acitivity. These inhibitors are summarized in Table 3.

Table 3. Commonly used protease inhibitors used during sample processing.

Inhibitor	Specificity	Use Level	Comment
PMSF	Cysteine and serine proteases	0.1 – 1 mM (24-240 µg/ml)	PMSF has a short half life in water. Dissolve in ethanol and add just before processing.
EDTA	Metalloproteases	1 mM (0.37 mg/ml)	EDTA chelates divalent cations which are required by metalloproteases.
Pepstatin A	Acid Proteases (aspartyl peptidase)	1-2 µM (0.5-1.0 µg/ml	Prepare 1 mg/ml in ethanol (stable at -20˚C). Use by diluting 1 µl/ml.
Leupeptin	Serine and thiol proteases	10-100 µM (5-50 µg/ml)	Prepare 25 mg/ml stock and use 0.2-2 µl/ml.
Aprotinin	Serine proteases	0.1-0.8 µM (0.65-5.2 µg/ml)	Use 1 µl/ml of 5 mg/ml stock in water (0.76 mM).

When homogenizing plants, the disruption of the vacuole is much like disrupting a lysosome. Proteases are released in addition to phenolic oxidases. Plants contain substantial concentrations of phenolic compounds which when oxidized can react with proteins. Plant homogenate turns black as a result of these reactions. The addition of a phenolic scavenging reagent, such as polyvinylpyrrolidone used at a concentration of 0.5-2%, can bind the phenolics and prevent their oxidation and subsequent reaction with proteins.

Mechanical Disruption Methods

Disrupting cells and tissues by applying a force not inherent to the sample is considered a mechanical disruption method. Mechanical homogenization procedures generate lysates with characteristics different than those produced by chemical lysis. By avoiding detergents and chaotropes, many cytosolic proteins may remain intact following liberation from the cell. This is useful for protein isolation and enzyme assays. However, mechanical homogenization may simply be the tool used to rapidly disrupt cells and tissues with the use of denaturing reagents, especially during RNA isolation procedures. Regardless of the mechanical approach, whether it is to beat, grind, shear, or explode cells, they are tools that can be applied in many different ways to sample preparation.

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 due to a lack of time and resources to examine all options. This section focuses on the more widely used methods.

In our effort to better understand mechanical disruption, many of the homogenizers and methods have been compared by analyzing samples following homogenization. In certain instances, such as with yeast and muscle tissue, microscopic observation can be very useful for determining the extent of disruption. In other instances, a measurement of the DNA, RNA, or protein released into the supernatant has been used. In many processes, especially where more than one homogenizer was used to process a sample, relative efficiencies of homogenization

was performed by measuring lactate dehydrogenase (LDH) released from the cells/tissues. Theses comparisons will be noted throughout the following sections.

Grinding

Grinding relies on creating friction by sandwiching the sample between two hard surfaces that slide against each other. Forces on the sample are two-fold, namely downward pressure accompanied by a tangential shearing force. Grinding causes tearing and ripping of samples, much like shearing, but differs in that there is direct contact between sample and homogenizer. Mortar and pestle is the best known tool for grinding, but others are grain mills and certain types of glass homogenizers. 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.

In its various forms grinding can be used on wet, dry, and frozen samples, however it is most efficient on solid samples. One key characteristic of grinding is that friction generates heat and at times can be significant. Consequently, the heat tolerance of the analyte should be considered when selecting a grinding method. Frequently samples are frozen prior to grinding, commonly with liquid nitrogen, which can be used for chilling both sample and homogenizer. This cryogenic grinding makes the sample brittle and fracture easily, but it also preserves analytes that are heat labile or which may rapidly degrade upon liberation, such as RNA. Large traditional mortar and pestle are useful for cryogenic grinding as the mass of the homogenizer acts as a cold reservoir.

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. A miniaturized version of the mortar and pestle, the CryoGrinder™, is used for scaled down cryogenic grinding of small samples in the milligram range. Several of the glass tissue homogenizers, such as conical glass and Tenbroeck, use grinding forces to effectively disrupt cultured cells and tissues.

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

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

Limitations – Throughput with mortar and pestle 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 frozen samples 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 (use a protective glove) 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, the ground 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 frozen, pre-chill the tube or 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. The relatively large mass of the mortar and pestle serve as a cold reservoir which helps to prevent sample thawing.

Limitations – A significant problem in cryogenic grinding with mortar and pestle is that small samples (e.g., 10-20 mg) 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. As the mortar and pestle may be in the -150˚C range following grinding, they must be warmed to room temperature (slowly) between uses and cleaned. Consequently, if many samples are processed daily, many sets are needed. Labs that process significant numbers of samples cryogenically must 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. Small samples, which are pulverized cryogenically using a mortar and pestle, 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 (P). 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 wrench.

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 (see below).

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. As the CryoGrinder™ generates smaller particles than the mortar and pestle the small particles will more readily dissolve into extraction buffers. 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 liquid nitrogen reservoir (e.g., CryoCooler™) so that they remain cold.

Figure 2. CryoGrinder™ is useful for the cryogenic grinding of very small samples, less than 100 mg.

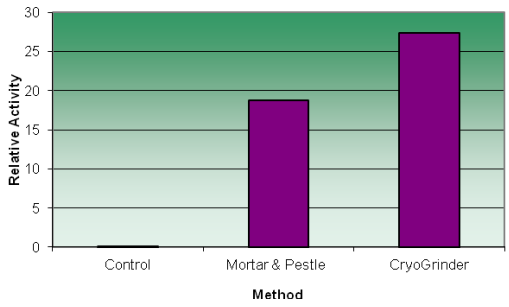


Figure 3. Comparison of LDH liberated by cryogenic Methods. Mouse muscle was cryogenic ground using mortar and pestle and CryoGrinder™. Greater LDH activity was harvested from CryoGrinder™ homogenized muscle.

(P) This symbol denotes that there is a link to a related protocol.

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 course like a very fine emery paper so that the pestles can dig into tissues being gripped by the mortar and shear the sample as it is turned. Tissues processed in glass tissue grinders are often chilled on ice. The Tenbroeck pestle, which is hollow, can be filled with cold liquid to cool from the inside.

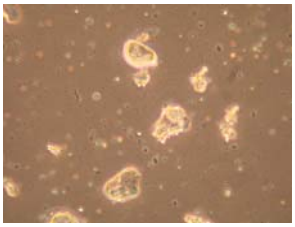


Figure 4. Mouse muscle homogenized with CryoGrinder™. Though particles are slightly larger than other methods, homogenization was relatively complete.

The actual process of grinding is relatively simple and involves adding an extraction buffer and the tissue to the homogenizer tube, 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. This action is repeated.

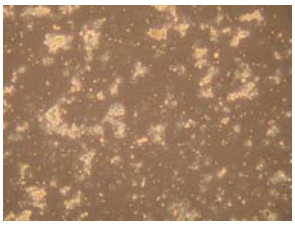


Figure 5. Mouse muscle homogenized with a conical glass homogenizer. Though the homogenate appears fine, significant connective tissue did not homogenize. In comparison to other methods, the conical glass homogenizer was 42% as efficient (see Fig 20).

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 at 280˚C 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. Glass tissue grinders are approximately 30-40% as efficient as the best methods (see Fig. 20), but the relative cost is fractional ($70 vs. $15,000). They are very easy to clean and decontaminate.

Figure 6. Tenbroek (left) and conical (right) glass homogenizers.

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.

(P) This symbol denotes that there is a link to a related protocol.

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, biological samples were homogenized 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.

Though mistaken for bead beating, many researchers shear microorganisms and small sections of tissue using small grinding beads and vortexers. Various configurations of bench top vortex mixers can be used with micron sized grinding spheres to rupture bacteria, yeast, and molds. Larger spheres, like 7/64" stainless steel grinding balls, are usually used with tissues. Vortexers, though readily available, tend to be poor as compared to other methods discussed below.

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. Coffee grinders have essentially the same mechanism as a blender and can be used with seeds and kernels. However, liquid samples cannot be processed with coffee grinders.

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 and clean. Lab blenders are available in stainless steel which allows for decontamination and sterilization.

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

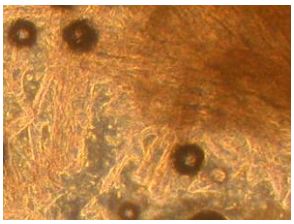


Figure 7. Mouse heart muscle homogenized with a blender. Blenders are an effective first step in disaggregating samples. However as depicted in this image, many microfibrils remain intact. Blenders can also introduce air bubbles (dark spots) which can be detrimental to some analytes.

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

Homogenizing samples by vortexing can generate significant amounts of heat due to the friction created by the grinding beads. 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 used for homogenization at no cost. New vortex mixers are relatively inexpensive. Standard single tube vortex mixers can be used for all size tubes while pulsing vortexers can handle up to 12 microfuge tubes. Multitube vortexers can homogenize full racks of tubes.

(P) This symbol denotes that there is a link to a related protocol.

Limitations – Vortex mixers are designed for mixing and lack the same power as true homogenizers; thus, they are usually much 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.

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 an inner turning shaft (rotor) which is connected to a motor. At the bottom of the rotor-stator are slots on both the stator and rotor. 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.

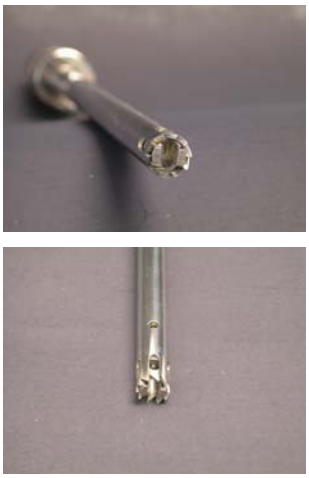


Figure 8. Top (upper) and side (bottom) views of a rotor/stator. The outer shaft (stator) remains stationary while the inner shaft (rotor) turns at speeds up to 20,000 rpm. Rotor/stators can be very effective on homogenizing powders and pills, but can generate only course homogenates when used on tougher tissues.

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 (P). 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 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 are designed for different sample types and sizes. Indeed, process scale rotor-stators are commonly used in various industries. Samples disrupted with rotor-stators can be very homogeneous.

Limitations – The initial cost for rotor-stators varies, but at minimum it will be an investment of several thousand dollars. Furthermore, different motor units will be required for very small and large samples. Each rotor-stator 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 become caught within the shaft assembly, making rapid cleaning and decontamination a problem. High speed homogenization also generates heat and possible vortexes that can denature proteins.

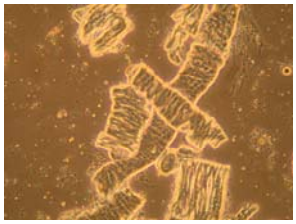


Figure 9. Mouse muscle disrupted using a rotor-stator homogenizer. Rotor-stator homogenizer had a relative efficiency of 27.6% as compared to other methods (see Fig. 20).

(P) This symbol denotes that there is a link to a related protocol.

Dounce Homogenizer: Not long after the arrival of the Waring blender, the Dounce homogenizer was introduced. Though this device looks like a ground glass homogenizer, it relies on pushing a 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, causing 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 a greater shear, while the opposite is true for the smaller pestle.

Strengths – The Dounce homogenizer is an inexpensive device that is very effective for mildly lysing cells. They typically are purchased with two different diameter pestle heads (type A and B) which provides some control over the extent of shearing. 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.

Figure 11. Though the homogenate appears very fine, significant tissue was left intact. The Dounce homogenizer produces 32% relative homogenization of solid muscle as compared to other processes (see Fig. 20).

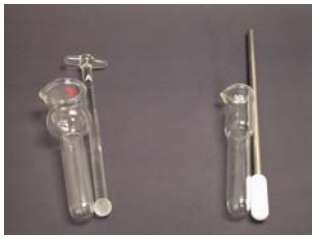


Figure 10. Two glass homogenizers used for shearing cells. The Dounce homogenizer (left) is manually operated and disrupts cells by forcing them between the pestle and tube wall. The Potter-Elvehjem homogenizer can be hand operated or attached to a motor. The pestle can be PTFE or glass.

Potter-Elvehjem with PTFE Pestle: Potter-Elvehjem homogenizers can be used for both grinding tissues and shearing cells. By changing from a ground glass pestle to a PTFE one, it effectively changes from a grinder to a shearing homogenizer. For shearing, the pestle can be 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 very efficient at homogenizing solid tissue. Muscle homogenized by this method was incomplete. For solid tissue, homogenizing with a Potter-Elvehjem is not overly effective. Overall the process was poor in a one-step method. When used in conjunction with other homogenizers, it was effective.

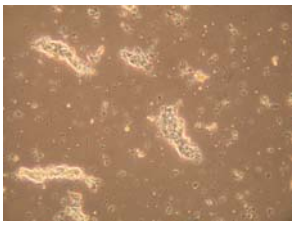


Figure 12. Potter-Elvehjem homogenizer with motorized PTFE pestle yields 36% relative disruption of muscle as compared to other systems. Significant solid tissue remained intact (see Fig. 20).

(P) This symbol denotes that there is a link to a related protocol.

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 rupture.

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 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. Due to the small orifice, the French press can clog.

Beating

Beating involves crushing a sample with a projectile. Most beating methods rely on placing a sample and projectile in a tube and rapidly shaking them back and forth. The most common method is bead beating which uses grinding balls or small beads, though cylinders and irregular shapes have also been used. Bead beating has been in practice 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 can be quite effective if done correctly, 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 (formerly called mixer mills) that could handle racks of tubes or even microwell plates.

Though simple in theory, bead beating can be quite complex as several factors must work together to effectively homogenize samples. The tube, or microwell plate, used in bead beating is not just a sample holder, but actively participates in the homogenization. The projectiles, henceforth referred to as balls or beads, must also be carefully matched to the sample. The stroke length of the amalgamator or mixer mill is also critical, as is the physical motion it imparts on the sample tube. Finally, sample mass, volume, and extraction buffer all impact the effectiveness of the homogenization.

Tubes/Plates: The objective of bead beating is to sandwich a sample between a grinding bead, or ball, and a hard surface. That hard surface can be either another bead/ball, or in many cases, the tube or deep well plate being used for the processing. With microorganisms and micron sized beads, the colliding surfaces are more likely two different beads. When processing tissues or seeds, the collision is between the tube and ball. Balls and beads, as will be discussed below, are typically hard and dense materials like glass, ceramics, or steel. Consequently, to get a good homogenate the tube must also be hard to push back on the grinding ball. A metal tube would be ideal, but in reality, metal tubes (though they have been used) are impractical because of their high cost. The alternative to metal is plastic tubes.

Not any plastic is suitable for bead beating as some are very brittle and will not hold up to the repeated impact of the ball (which is why glass tubes are not used). For example, polystyrene tubes, though widely used for labware, crack very readily. Polypropylene is much more durable, being the material of choice for microfuge tubes and deep well plates. It holds up well to bead beating especially when small beads are used for lysing bacteria. However, when used with large grinding balls, polypropylene tends to soften as the sample warms. This dampens the impact

between the grinding ball and tube. High density polyethylene works relatively well with grinding balls. It also has the added feature of being compatible with many organic solvents used for the extraction of small molecules from tissues during pharmacokinetic analysis.

Polycarbonate is the best plastic for tubes and vials used for bead beating. It is extremely durable, clear, and impact resistant even at cryogenic temperatures. The major limitation with polycarbonate is that it is incompatible with several solvents commonly used for extraction including: phenol, chloroform, acetone, and acetonitrile. Consequently, for dry grinding or extraction using aqueous buffers, polycarbonate vials yield the best homogenate.

The last point about tubes and plates regards the closures used to seal them. If samples are processed with liquids, then the cap must have some type of gasket (an O-ring or seal) to prevent liquid from leaking out. Special care needs to be observed if the extraction buffer is an organic solvent, such as methanol or acetonitrile, as the vapor pressure of the solvent can push the solvent (and the analytes) out of the tube if the seal is insufficient. Polypropylene microfuge tubes are often used for organic extractions, and experience has shown that all brands of tubes do not seal equally. This is the same for deep well plates, as push-on sealing mats vary greatly on their tightness of seal. We have observed that tight fitting polypropylene sealing mats, whether round or square, tend to seal much better than mats made of PTFE. Furthermore, self-adhesive sheets do not work well, and heat sealed sheets are only suitable for very small beads (e.g., <600 micron) when used with deep well plates.

Balls/Beads: Not discussing the beads used for bead beating would be similar to teaching cooking without referring to pots and pans. The beads (and grinding balls) used during the homogenization process are the most important component of the process, thus discussing what products are available and how to use them is important. First, bead beating not only uses beads, but grinding balls, ceramic satellites, and grinding resins. Our lab usually refers to any spherical media of 3 mm or less as a bead. Above that size it miraculously becomes a grinding ball. The larger media are normally made of stainless steel while the smaller beads are either silica (glass) or ceramic (zirconium silicate or zirconium oxide).

Non-spherical media is very effective at disrupting tough fibrous samples, such as muscles, skin, and sclera. On the small size, garnet, cut wire, silica gel, and similar sharp grinding media can effectively slice resilient samples, particularly when used in conjunction with a larger grinding ball. Satellites (shaped like Saturn) and cylinders can also be effective breaking up tough samples due to the sharpness of their edges. We routinely use a combination of garnet (a hard, sand-like mineral) and zirconium oxide satellites for tougher animal tissues.

It is important to mix and match grinding beads/balls and sample types in order to get effective disruption. Table 4 summarizes some of the more common configurations for balls, beads, and tubes/vials.

Table 4. Grinding media and their applications.

Media	Sample	Configuration	Comment[3]
100 µm Beads	Bacteria	Microfuge Tubes Deep Well Plates	Tubes or wells should 1/6 volume of beads and 1/3 volume of cell suspension.
200 µm Beads	Bacteria/Yeast	Microfuge Tubes Deep Well Plates	Amounts as above. Good for small yeasts (*Pichia*) and larger bacteria.
400 µm Beads	Yeast	Microfuge Tubes Deep Well Plates	Best size for *Saccharomyces*. Beads and culture broth should not exceed half volume.
500 µm Garnet	Skin/Sclera/Muscle	Polycarbonate Vials (4 ml)	Abrasive for tearing. For tough samples use with a 6 mm satellite. Use amounts as above.
800 µm Beads	Fungi	Microfuge Tubes Deep Well Plates	Good for pollen, mycelia, and algae. Volume should be the same as 100 µm beads.
1.0-1.7 mm Beads	Leaf Tissue/Soil	Microfuge Tubes	Process up to several leaf punches. Do not overfill tubes with buffer.
2.8-3.0 mm Beads	Plant Materials	Microfuge Tubes	Mass of sample should be kept to under 50 mg and buffer less than 500 µl.
5/32" Grinding Balls	Plant/Animal Tissues/Insects	Deep Well Plates	Animal tissues should be <100 mg while plants should be <50 mg. Use 1 ball per well.
6 mm ZO Satellites	Plant/Animal Tissues	Deep Well Plates Polycarbonate Vials (4 ml)	Use as above. When stainless steel may interfere with assays, zirconium oxide can be used.
3/8" Grinding Balls	Seeds/Animal Tissues	Polycarbonate Vials (4 ml) Polyethylene Vials (4 ml)	Use for rice grains, kernels, other seeds, on tissues (<150 mg). Do not overfill vials.
7/16" Grinding Balls	Pooled Seeds/ Organs/Tissues	Polycarbonate Vials (15 ml) Custom Container	Good for large samples and pooled seeds for field trials. Container must be relatively large. Use 2 balls for 15 ml vials or more for custom containers (up to 125 ml).

Grinding media, whether beads, balls or satellites, can be purchased in bulk or pre-dispensed in grinding tubes. Beads can be purchased in a raw form, but these need to be cleaned prior to use. Raw beads contain numerous contaminants from dirt, to skin cells, to hair, to insect parts. Fine micro-particles in silica beads can bind analytes and lower yields upon homogenization.

Processed beads can be purchased which are acid washed to remove contaminants. Molecular biology grade beads are also available which have been washed, baked, and packaged aseptically. These beads are also quality control tested. For homogenizing samples with very few cells, as with environmental samples with low cell counts, low binding beads can be used. These beads are chemically treated so that less analyte bind to the beads upon liberation during bead beating. These have been shown effective for detecting low levels of bacteria by quantitative PCR, increasing both linearity and sensitivity of assays. Consequently, low binding beads have found use in tools used for biological weapons detection.

Amalgamators: Dental mixers, or amalgamators, have been used or adapted to bead beat microorganisms for years. This simple instrument allows a tube to be locked into a small 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 in as little as 30 seconds.

These bead beaters are effective and relatively low cost. Several vortexers have been modified to provide similar actions (but yield poorer results), with the added value of being able to process multiple samples.

Strengths – These are 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. However, it must be emphasized that vortexers used for bead beating are not as effective as true oscillating amalgamators.

[3] A general comment for all samples is that extraction buffers should not contain detergents as foaming with prevent beads and balls from moving freely during processing.

(P) This symbol denotes that there is a link to a related protocol.

Limitations – The individual tube bead beaters are rather effective, though throughput is low. Some models can hold up to three tubes. For labs running a few samples, such units might be adequate. Vortexer units are less effective, but hold greater numbers of samples. Depending upon the application, lower lysis efficiency may not matter.

Multitube Homogenizers: The second generation bead beaters moved from single tube formats to homogenizers that can handle up to 24 microfuge tubes. The first instrument in the category was the FastPrep®, which in many ways looks like a microtube centrifuge (a.k.a., microfuge). Microfuge tubes placed in the round rack are oscillated at high speed (up to 8000 rpm) in very short vibratory-like motion. A similar model is the Precellys®, which operates in much the same manner. Variations on both machines have been made that allow for oversized microtubes (e.g., 7 ml) and even 15 and 50 ml conical tubes.

Strengths – The ability to process multiple samples make these machines very useful to the point where some of been used for clinical work. For microorganisms these machines are effective. For tissues, using course media such as garnet allows for many tissues to be shredded.

Limitations – The stroke or oscillating pattern of these machines are very small and thus they tend to work less effectively at homogenizing very resilient tissues, such as skin and sclera. Samples also get very warm by the heat generated by the 8000 rpm motion. Some machines have shutdown features due to overheating which frustrates many researchers. Additionally, these units tend to be very expensive for their capacity, costing around $10,000.

High Throughput Homogenizers/Mixer Mills: With the rise of high throughput screening strategies, sample homogenization became the 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, resulting in high throughput homogenizers (also known as mixer mills). These devices in the simplest configuration shake racks of tubes or microwell plates at speeds up to 1800 rpm.

Mixer mills are not new, but their adaptation to the SBS formats (i.e., Society of Biomolecular Screening standardized plate dimensions) is relatively recent 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 with 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 thoroughly homogenizes most samples. Similarly, microorganisms can be disrupted using beads.

Several brands of high throughput homogenizers are available, such as the Geno/Grinder®, HT Homogenizer®, and Retsch® Mixer Mill. Though these can all hold plates and racks of tubes, they differ significantly in the method of operation. The path or motion which the plates follow during processing is different for these homogenizers, with the Geno/Grinder® and HT Homogenizer® having a linear motion while the Retsch® Mixer Mill follows a figure "8" motion. With linear motion homogenizers, all wells or tubes follow the same path, thus processing between samples is the same. With figure "8" motion machines, like the originally modified paint shakers, wells on the outside of the plate (or tubes on the outside of a rack) follow a different path than samples towards the middle. This leads to differential processing of samples and increased variation in analyte yield. For analytical work, this could skew results based on the positioning of the sample during homogenization. Indeed one user manual for a figure "8" homogenizer recommends flipping the plate halfway through processing to generate more uniform results.

High throughput homogenizers are versatile as they can be used for a wide array of sample types and sizes. The most important aspect of resulting in good disruption from a high throughput homogenizer is to properly match the sample size (mass and volume) with a suitable well size and grinding media. Generally, samples and buffers should take up no more than a third of the volume of the vessel. Thus a leaf punch of 10 mg with 200 µl of extraction buffer can easily be processed in a 96 deep well plate (1.2 ml round well) 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 sample size increases, polycarbonate vials are the next best choice for grinding.

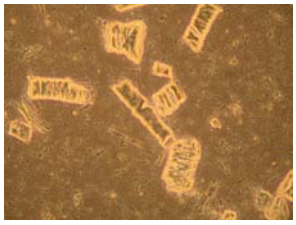
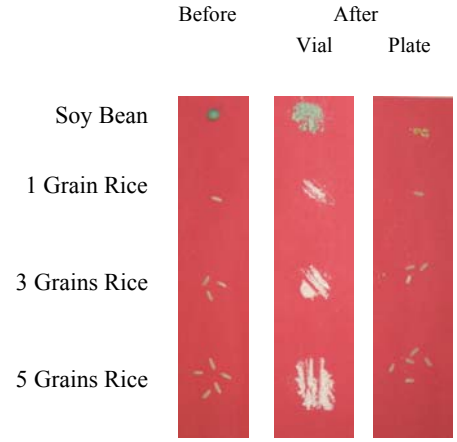


Figure 13. High throughput homogenizer with deep well plate and 5/32" grinding ball produced very fragmented muscle and a relative disruption efficiency of 52% (see Fig. 20). (P)

Any impact resistant tube or container that can be locked into a high throughput homogenizer can be used for disrupting samples. Indeed short 60 ml polycarbonate jars have been used to homogenize complete animal organ systems. Most samples however are less than 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 pulverize the rice requires a 4 ml polycarbonate vial with a 3/8" grinding ball. A comparison of soybean and rice processed in deep well plates and 4 ml vials can be seen in Table 5.

Table 5. Comparison of Rice and Soy Processed in Deep Well Plates and Polycarbonate Vials.



Samples in plates were ground with 5/32" stainless steel grinding balls while larger 3/8" balls were used with 4 ml vials.

Many agricultural biotechnology labs analyze seeds from field trials (P) by pooling seeds prior to homogenizing. To accomplish this, larger polycarbonate vials (15 ml) 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 (P). 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. For instance, in a laboratory using coffee grinders to process corn, throughput was increased from five samples per hour to 100 samples using high throughput methods.

(P) This symbol denotes that there is a link to a related protocol.

These larger polycarbonate 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 (Figure 14).

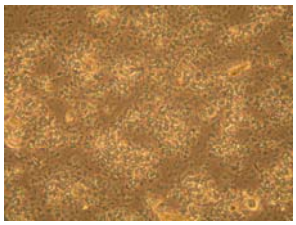


Figure 14. High throughput homogenizer with 15 ml vials and two large 7/16" grinding balls produced a fine muscle homogeneous lysate with a relative efficiency of 81% (see Fig. 20).

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, and tubes) are separate from the mechanical action used to power the disruption minimizes cleaning and cross-contamination issues. Most researchers treat the plates, vials, and grinding balls as disposable items (though balls and some vials can be reused) which also helps to minimize contamination/clean-up issues.

Limitations – High throughput homogenizers require an initial investment of $7000 to more than $15,000. The use of consumables may increase sample processing 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 yield 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 caused by a rapid change in pressure. Several tools are available that disrupt samples by using pressure differential.

Sonication: Sonication (also referred to as ultrasonication) 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 a 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. As liquids cannot flow as fast as crystals oscillate, small vacuum cavities are formed during the contraction. 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.

Strengths – For 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 is also has the greatest limitations. It is a powerful method if the sample contains small particles, i.e., either cells or homogenized tissue. For solid tissue, sonication is a very poor method (Fig. 15). 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.

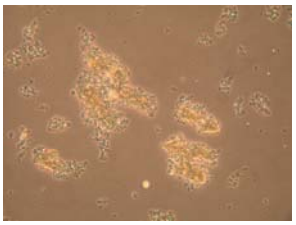


Figure 15. Sonicator with micro probe tip yielded the lowest efficiency of all methods on muscle at 2.6% (see Fig. 20).

Combined Mechanical Methods

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 disrupt samples. The methods and procedures that are employed support the notion of innovative and independent thought upon which science is built. However, there is a pattern that occurs frequently which is very effective, that being the use of two or more of the methods discussed above. Most sample processing is done with two steps using different homogenizers.

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 less or unsuccessful without the initial processing step. In order to compare single-step and two-step homogenization methods, equal sized samples of mouse muscle were homogenized with the methods sited above and with two-step combinations of those methods. The lysates generated from those processes were then assayed for lactate dehydrogenase (P) which provided data on the relative efficiencies of the method (Fig. 20).

On microorganisms and cell suspensions, sonication is very effective at lysing cells as compared to bead beating. Perhaps the most apparent tool that does not work on solid samples is ultrasonication, but is very effective when applied to samples first reduced in size. With solid mouse muscle used as a comparative substrate, sonication essentially cooked 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 then the effectiveness was highly increased. Ultrasonication following cryogrinding (Fig. 16) was far superior to either of those methods alone, more than doubling their combined LDH activity (Fig. 20). Indeed the combination of CryoGrinder™ and sonication liberated greatest amount of enzyme and became the standard by which the other methods were measured.

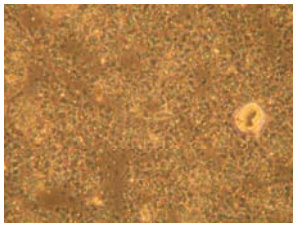


Figure 16. Cryogenic grinding followed by sonication proved to be the most efficient combination for homogenization with a relative efficiency of 100%. The density of small size of particles exceeded all other methods (see Fig. 20).

(P) This symbol denotes that there is a link to a related protocol.

Other combinations also proved to be effective. CryoGrinder™ in conjunction with the Dounce (Fig. 17), Potter-Elvehjem (Fig. 18), and conical glass homogenizers all generated homogenates better than those methods alone. The rotor-stator and sonicator also were an effective combination (Fig 19).

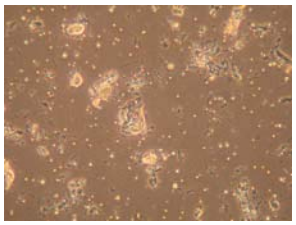


Figure 17. Cryogrinding mouse muscle followed by Dounce homogenizer was 44.6% as efficient as compared to other methods (see Fig. 20).

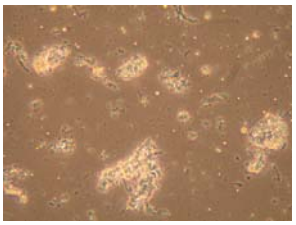


Figure 18. Combining the CryoGrinder and Potter-Elvehjem homogenizer on mouse muscle yielded an improved efficiency of 52.1% (see Fig. 20).

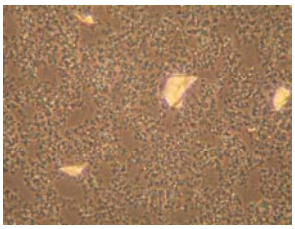


Figure 19. Mouse muscle processed first with a rotor-stator and then sonicator has a relative efficiency of 54%. This is greater than the sum of the individual efficiencies (see Fig. 20).

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 and weigh it against productivity demands. 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.

Generally the efficiency of sample disruption is inversely proportional to its throughput, the exception being with high throughput bead beating (Fig. 20). High throughput homogenizers (mixer mills) are very effective at disrupting samples, yielding the highest LDH activities for a single step homogenization processes. 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 though resulting particle sizes (observed microscopically) were relatively large. However, samples with removed debris cleared by centrifugation retained considerable enzyme activity.

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 provides the best single-step results, and with that, the large vials and grinding balls suggest that greater force produces better disruption. Sonication, interestingly, borders both sides of the effectiveness scale showing its ineffectiveness on solid samples but is highly efficient 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.

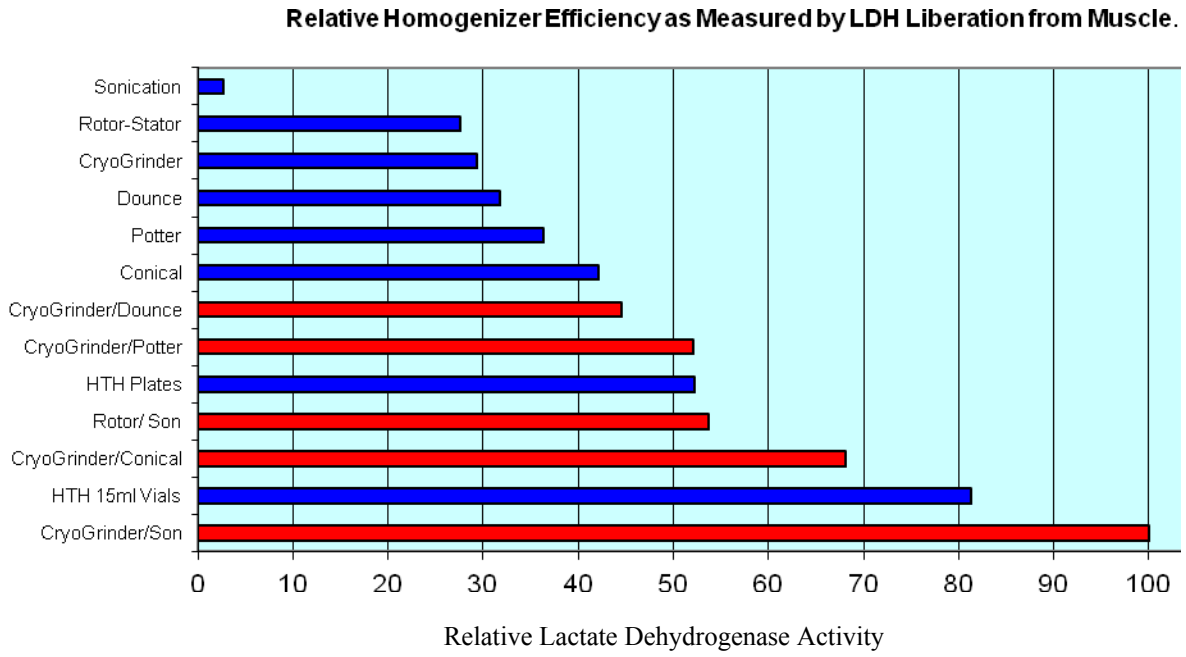


Figure 20. This relative efficiency chart is very revealing as regards the effectiveness of any single method on sample homogenization. The blue bars represent a single processing step while red bars are two-step processes.

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 final product and then choose the best method or combination of methods that will produce that product.

Chemical and mechanical methods, by themselves, can be used to disrupt samples, but normally are used in some combination to achieve a desired homogenate. At a minimum, buffers, chaotropes, and surfactants are common additives that help to solubilize and maintain biomolecules released during mechanical processing. For the isolation of biomolecules which are labile or sensitive to degradation, protective chemistries partnered with a mechanical method are necessary to obtain quality homogenates.

All methods used to disrupt samples have strengths and weaknesses. Lower throughput methods can be used to process larger samples with good results, and many are economical. High throughput methods are also effective, but the initial investment will be greater.

Many tools when used alone to homogenize samples are not as effective at disrupting samples but when combined with each other 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.

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