DISRUPTING AND LYSING CELLS WITH THE VIBRA-CELL ULTRASONIC PROCESSOR

The disruption of cells is an important method in the field of proteomics and in the isolation and preparation of intracellular products. Isolation of subcellular fractions and concentration of proteins allow for more efficient identification and study of proteins of interest. From research levels through to production, many areas of biotechnology, particularly recombinant technology, necessitate the use of ultrasonics for cell disruption. Cell disruption focuses on obtaining the desired product from within the cell, and it is the cell wall that must be disrupted to allow access to the contents of the cell.

All cells have a plasma membrane, a protein-lipid bilayer that forms a barrier separating cell contents from the extracellular environment. Lipids constituting the plasma membrane are amphipathic, having hydrophilic and hydrophobic moieties that associates spontaneously to form a closed bimolecular sheet. Membrane proteins are embedded in the lipid bilayer, held in place by one or more domains spanning the hydrophobic core. In addition, peripheral proteins bind the inner or outer surface of the bilayer through interactions with integral membrane protein or with polar lipid head groups. The nature of the lipid and protein content varies with cell type.

In animal cells, the plasma membrane is the only barrier separating cell contents from the environment, but in plants the plasma membrane is also surrounded by a rigid cell wall. Plant cell walls consist of multiple layers of cellulose. These types of extracellular barrier confer shape and rigidity to the cells. Plant cell walls are particularly tough, making them very difficult to disrupt mechanically or chemically. The lack of an extracellular wall in animal cells make them relatively easy to lyse.

Soft, fresh plant tissue can often be disrupted by sonicating in a lysis buffer. Other plant tissues, like pine needles, need to be ground dry, without liquid nitrogen. Some hard, woody plant materials require freezing and grinding in liquid nitrogen prior to being ultrasonically processed. Plant cell suspension cultures and calluses can be lysed by sonication in a lysis buffer for 30 seconds to 2 minutes. The diversity of plants and plant tissue make it impossible to give a single recommendation for all samples. However, one should be aware that most plant tissues typically contain polysaccharides and polyphenols that can coprecipitate with RNA and inhibit downstream assays. Treating a plant tissue lysate with polyvinylpyrrolidone (PVP) will precipitate such problematic components from the lysate before the actual RNA isolation is carried out.

Single-cell organisms (micro-organisms) consist of a semipermeable, tough, rigid outer cell wall surrounding the protoplasmic membrane and cytoplasm. The cytoplasm is made up of nucleic acid, protein, carbohydrates, lipids, enzymes, inorganic ions, vitamins, pigments, inclusion bodies, and about 80% water. In order to isolate and extract any of these substances from inside the cell, it is necessary to break the cell wall and protoplasmic membrane. In some cases the cell may excrete the desired substance without assistance, but in most cases, the cells must be lysed in order for these intracellular substances to be released. Breaking cell membranes and releasing the contents present significant challenges. The process must be fast and thorough to

maximize the protein yield. Because the energy applied must be great enough to break the cell membranes or walls, yet gentle enough to avoid physically or chemically damaging cell content, the Sonics' Vibra-Cell with its variable intensity capability is ideally suited for this application.

Microorganisms differ greatly in their sensitivity to ultrasonic disintegration. For example, the most readily disintegrated are the rod-like forms (bacilli), while the spherical organisms (cocci) are much more resistant. The group Mycobacteria, to which the tuberculosis organism belongs, is particularly difficult to disrupt.

Yeast, gram-positive bacteria, and to a lesser extent, gram-negative bacteria have considerably harder cell walls in comparison to animal cells, and require relatively high power for cell disruption.

Bacteria are extremely diverse; therefore, it is difficult to make one recommendation for all bacteria. Ultrasonic processing will lyse most Gram positive and Gram negative bacteria, including mycobacteria. Typically, glass beads and lysis solutions are added to a bacterial cell pellet and the sample is sonicated for a few minutes. It is possible to lyse some Gram negative bacteria by sonication in lysis solution alone. Bacteria cell walls can be digested with lysozyme to form spheroplasts. Gram positive bacteria usually require more rigorous digestion (increased incubation time, increased incubation temperature, etc.) than Gram negative organisms. The spheroplasts are then easily lysed in a GITC lysis buffer with ultrasonics.

Gram negative bacteria typically require 10 to 15 minutes of processing, while staphylococcus requires 20 to 30 minutes.

With the Vibra-Cell, the level of intensity that should be used is application dependent. For example high intensity might be recommended for the break up of cells, but should never be used when the release of intracellular components might be objectionable e.g. Organelle isolation.

The ability to control the amplitude at the probe tip is a prerequisite for process optimization. And because each application requires its own set of processing parameters, due to variation in volume and composition, the optimum amplitude can only be determined empirically. When processing a new sample, it is recommended that the amplitude be set first at 50% (30% with a microtip) and then increased of decreased as required.

Prior to sonication, cells can be treated with various agents to aid the disruption process. Lysis can be promoted by suspending cells in a hypotonic buffer, which causes them to swell and burst more readily by physical shearing. Lysozyme can be used to digest the polysaccharide component of yeast and bacterial cell walls. Alternatively, processing can be expedited by treating tough cells with glass beads to facilitate the crushing of cell walls. This treatment is commonly used with yeast cells. Viscosity of a sample typically increases during lysis due to the release of nucleic acid material. DNase can be added to samples (25-50 µg/ml) to reduce this problem. Nuclease treatment is not required for sonicated material because sonication shears chromosomes. Because proteolysis can be a

problem whenever cells are manipulated; it is advisable to add protease inhibitors to samples undergoing lysis.

NOTE:

All living organisms contain proteolytic enzymes (proteases and peptidases). Proteases are required for a variety of cellular functions, such as cellular repair or the digestion of extracellular material. In whole cells, protease activity is tightly regulated by compartmentalization or inhibitors to prevent damage to cellular proteins. Cell lysis disturbs this regulation and proteolytic degradation of the sample becomes a concern. Therefore, addition of protease inhibitors to cell lysis buffers is often required. Protease inhibitors are biological or chemical compounds that functions by reversibly or irreversibly binding to the protease. Proteases generally belong to one of four evolutionarily distinct enzyme families based on the functional groups involved in cleavage of the peptide bond. Therefore, several different types of inhibitors are generally required to protect proteins from proteolysis during extraction and purification.

Cellular disruption is the first step in RNA isolation and one of the most critical steps affecting yield and quality of the isolated RNA. Typically, cell disruption needs to be fast and thorough. Slow disruption, for example placing cells or tissue in guanidinium isothiocyanate (GITC) lysis solution without any additional physical shearing, may result in RNA degradation by endogenous RNase released internally, yet still inaccessible to the protein denaturant, GITC. This is especially a concern when working with tissues high in endogenous RNase such as spleen and pancreas. Incomplete disruption may also result in decreased yield because some of the RNA in the sample remains trapped in intact cells and, therefore, is unavailable for subsequent purification. For most samples, thorough disruption can be monitored by close inspection of lysate after disruption. There should be no visible particulates, except when disrupting materials containing hard, non-cellular components, such as connective tissue or bone.

When processing difficult cells with the Vibra-Cell, pretreatment with an enzyme to “weaken” the cell walls is beneficial. Lysozyme, byaluronidase, glycosidase, glucalase, lyticase, zymolase and lysostaphin digestion are among the enzymatic methods frequently used with yeast. Lysozyme, zymolase and lysostaphin digestion are among the enzymatic methods frequently used with bacteria and yeast to dissolve a coat, capsule, capsid or other structure not easily sheared by ultrasonics. Enzymatic treatment is usually followed by sonication in a GITC lysis buffer. Collogenase may be used with collogen, lysostaphin with staphylococcus, and trypsin hyaluronidase with liver and kidney.

Yeast can be extremely difficult to disrupt. To process yeast sonicate in a tube containing the sample, guanidinium-based lysis buffer and small glass beads (0.5 – 1 mm). Enzymatic pretreatment as outlined above is strongly recommended.

To disrupt filamentous fungi, scrape the mycelial mat into a cold mortar, add liquid nitrogen, grind to a fine powder with a pestle, then sonicate in lysis buffer to solubilize completely. As fungi may also be rich in polysaccharides, pretreatment with polyvinylpyrrolidone (PVP) may be beneficial.

Disruption of cells found in soil and sediments is accomplished by 1) isolating the bacterial cells from the material prior to the RNA isolation procedure. This is accomplished by homogenization of wet soil in a mechanical blender followed by a slow speed to pellet the bacteria cells. From this point, cells can be lysed as described above for bacteria, or 2) isolating RNA from the soil or sediment directly. For example, adding soil to a diatomaceous earth and lysis buffer, and then sonicating. The sample is then centrifuged to remove solid debris.

Cultured cells are relatively easy to disrupt with the Vibra-Cell. Cells grown in suspension are collected by centrifugation, rinsed to remove culture medium, and then lysed by sonicating in GITC lysis buffer. Placing the flask or plate on ice while washing and lysing the cells will further protect the RNA from endogenous Rnases released during the disruption process.

Detergent cell lysis is sometimes used in conjunction with ultrasonic processing to facilitate disruption. Detergents break the lipid barrier surrounding cells by solubilizing proteins and disrupting lipid:lipid, protein:protein and protein:lipid interactions. Detergents, like lipids, self-associate and bind to hydrophobic surfaces. They are composed of a polar hydrophilic head group and a nonpolar hydrophobic tail and are categorized by the nature of the head group as either ionic (cationic or anionic), nonionic or zwitterionic. Their behavior depends on the properties of the head group and tail.

NOTE:

When working with detergents, do not use a Vibra-Cell equipped with a probe with a replaceable tip. Use a solid probe instead.

Unfortunately, there is no standard protocol available for selecting a detergent to use for membrane lysis, the ideal detergent will depend on the intended application. In general, nonionic and zwitterionic detergents are milder and less denaturing than ionic detergents and are used to solubilize membrane proteins when it is critical to maintain protein function and/or retain native protein:protein interactions for enzyme assays or immunoassays. Zwitterionic detergents and nonionic detergents are commonly used for these purposes. In contrast, ionic detergents are strong solubilizing agents and tend to denature proteins, thereby destroying protein activity and function. There are a few commonly used ionic detergents that are only mildly denaturing, including sodium cholate and sodium deoxycholate.

The choice of detergent for cell lysis also depends on sample type. Animal cells, bacteria and yeast all have differing requirements for optimal lysis due to the presence of absence of a cell wall. Because of the dense and complex nature of animal tissues, they require both detergent and sonication. In addition to the choice of detergent, other important considerations for optimal cell lysis include the buffer, pH, salt concentration and temperature. Consideration should be given to the compatibility of the chosen detergent with downstream applications. For example, if the detergent used for lysis must be removed, then a dialyzable detergent should be selected.

If pretreatment with enzymes or detergents cannot be used, the freeze / thaw method should be considered. The freeze / thaw method is commonly used to lyse bacterial and

mammalian cells. The technique involves freezing a cell suspension for 10 minutes in a dry ice and isopropanol bath and then thawing the material at room temperature immediately prior to ultrasonic processing. The freeze / thaw cycle should be repeated three times prior to being subjected to the ultrasonics. This method of lysis causes cells to swell and ultimately break as ice crystals form during the freezing process and then contract during thawing. This pretreatment has been shown to effectively release recombinant proteins located in the cytoplasm of bacteria and is recommended when lysing mammalian cells. However, this method for lysis is not recommended when working with nitrilases as it has been noticed that purified nitrilases suffer structural damages upon freezing.

Most animal tissues can be processed fresh (unfrozen). However, it is important to keep them cold and to process them quickly (within 30 minutes) after dissection. When disrupting fresh tissue, the cells need to be sheared immediately at the time the GITC lysis solution is added. This can be done by dispensing the lysing solution in the tube, adding the tissue and immediately sonicating. Samples should never be left sitting in lysis solution, undisrupted. Hard tissues should be first treated in a blender or a mechanical homogenizer.

Animal tissues that have been frozen after collection should be disrupted by grinding in liquid nitrogen with a mortar and pestle. During this process, it is important that the equipment and tissue remain at cryogenic temperatures. The tissue should be dry and powdery after grinding. Grinding should be followed by sonication in a GITC lysis buffer. Processing frozen tissue in this way is cumbersome and time consuming, but effective.

Processing a sample with the Vibra-Cell will typically cause the temperature of the sample to increase especially with small volumes. Since high temperatures inhibit cavitation, the sample temperature should be kept as low as possible - preferably just above its freezing point. This can be accomplished by pulsing the ultrasonics on and off while keeping the sample vessel immersed in an ice bath. While processing the sample occasionally touch the vessel to ensure that the sample is relatively cool.

Increasing hydrostatic pressure (typically 15-60 psi) and viscosity can enhance cell disruption. For microorganisms, the addition of glass beads in the 0.5 to 1mm size range promotes cell disruption. Beads are almost a prerequisite when working with spores and yeast. A good ratio is one volume of beads to two volumes of liquid. Glass beads are available from:

Cole-Parmer Instruments
625 East Bunker Court
Vernon Hills, Illinois 60061
USA
Phone: 1-800-323-4340
Fax: 847-247-2929
Email: info@coleparmer.com

Fisher Bioblock Scientific
Parc d'innovation – BP 50111
F-67403 illkirch cedex
France
Phone: 03 88 67 14 14
Fax: 03 88 67 11 68
Email: infos@bioblock.fr

When processing a sample with ultrasonics, always immerse the probe deep enough below the surface of the sample to inhibit aerosoling or foaming, foaming substantially

reduces cavitation. Processing at a lower power setting without foam is much more effective than processing at a higher power setting with foam. Decreasing the power, increasing processing time and lowering the temperature of the sample will usually prevent aerosoling and foaming. Do not use any antifoaming agents or surfactants.

During cavitation, free radicals are formed which, if they are allowed to accumulate, can greatly affect the biological integrity of the sample by reacting with proteins, polysaccharides, or nucleic acids. Although during short periods of processing their formation is not normally considered a problem; for longer durations, the addition of free radical scavengers such as, carbon dioxide, N_2O, cysteine, reduced glutahione, dithiothreitol or other SH compounds, might be beneficial. Saturating the sample with a protective atmosphere of helium or nitrogen gas, or dropping a small pellet of dry ice in the sample, will also inhibit free radical formation. Whereas it is true that gas is required for effective cellular disruption, it is not necessary that the vapor phase be oxygen or air since any gas except carbon dioxide will work just as well.

Following ultrasonic processing, the cell debris can be centrifuged at 15,000 rpm for 10 minutes.

Since the greatest concentration of energy is beneath the probe, it is imperative that the sample be kept as close to the tip as possible, liquids are easily processed because the free moving cells circulate repeatedly below the probe. Solid materials however have a tendency to be repelled by the ultrasonic, and should be processed in a vessel large enough to accommodate the probe, yet small enough to restrict sample movement. For small samples, conical shaped test tubes are recommended. Although plastic tubes work well, glass and stainless steel tubes usually work better than plastic ones.

Make sure that the probe is not touching the bottom of the vessel. Allowing the probe to contact the vessel will decrease the power output, and cause minute grey glass particles to migrate into the sample. Although these glass particles will not adversely affect the chemical composition of the sample, they will form a thin grey layer on centrifuging. If the probe has to come in contact with a solid sample, use a standard 20mm (3/4”) diameter stainless steel centrifuge tube cut to 70mm (3”) length. Do not use a glass tube. Microtips must never come in contact with anything but the liquid, because the stress resulting at the point of contact with a hard surface will cause the microtip to fracture. Although larger probes will not fracture if they come in contact with a glass vessel, they may cause the vessel to fracture.

Before using the Vibra-Cell, place the tip in 100% ethanol and energize the power supply for a few seconds to remove any residual substances. If still concerned about contamination from previous use, clean the probe with a disinfectant such as 20% Virkon solution and rinse with distilled water. Probes are autoclavable.

To inhibit sample loss in test tube due to sticking, siliconize the test tube as follows: Wash and dry the test tube thoroughly, coat with silicone, then air dry. “Sigmacote” manufactured by Sigma Chemical Co., 3050 Spruce Street, St. Louis, Missouri 63103, USA, phone (314) 771-5765, is ideally suited for that purpose.

High viscosity and concentration are problematic. 2,000 cps and 15% concentration by weight are maximum limits. Ultrasonic processing propagates sound waves through the sample. If the sample is so dense that it will not pour or circulate easily it will absorb the sound waves, and be too thick for ultrasonic processing.

Use the Cup Horn for processing pathogenic, radioactive, and biohazardous materials in complete isolation without probe intrusion. Because plastic tubes have a tendency to absorb vibrations, it is preferable to contain the sample in a stainless steel tubes or glass tubes when working with a cup horn. To expedite processing, add glass beads to the sample. If desired, crushed ice can also be added to the water inside the cup horn, in order to optimize cooling. Processing samples in a Cup Horn will usually take 4 times longer than processing with direct probe intrusion.

Various methods can be implemented to measure the efficiency of ultrasonic disruption. Typically, counting the cells using a microscope is a satisfactory method. However, for greater accuracy, a protein assay should be used. This procedure is widely recognized as a good method for measuring cell disruption by taking into account the amount of protein released after disruption. The disrupted cells are then tested and checked against this number for percentage breakage.
There are several types of protein assays. The most common is the Folin Reaction (Lowry Assay) method, as it is comparatively simple and provides consistent results. This colorimetric method has a sensitivity to protein of around 8 μg / mL in the assay solution.
The assay turns blue in the presence of proteins due to the reaction of copper ions in the alkaline solution with protein and the reduction of phosphomolybdate- phosphotungstic acid in the Folin reagent by aromatic amino acids in the treated protein.
Fractional protein release, Rp, is calculated using the following equation and multiplying the result by 100:

$$\mathbf{Rp} = \frac{\mathbf{Cf - Cb}}{\mathbf{Ct - Cb}}$$

Cf = Free protein
Ct = total protein
Cb = Background protein

This gives the actual disruption percentage, taking into account the background levels of protein before disruption.