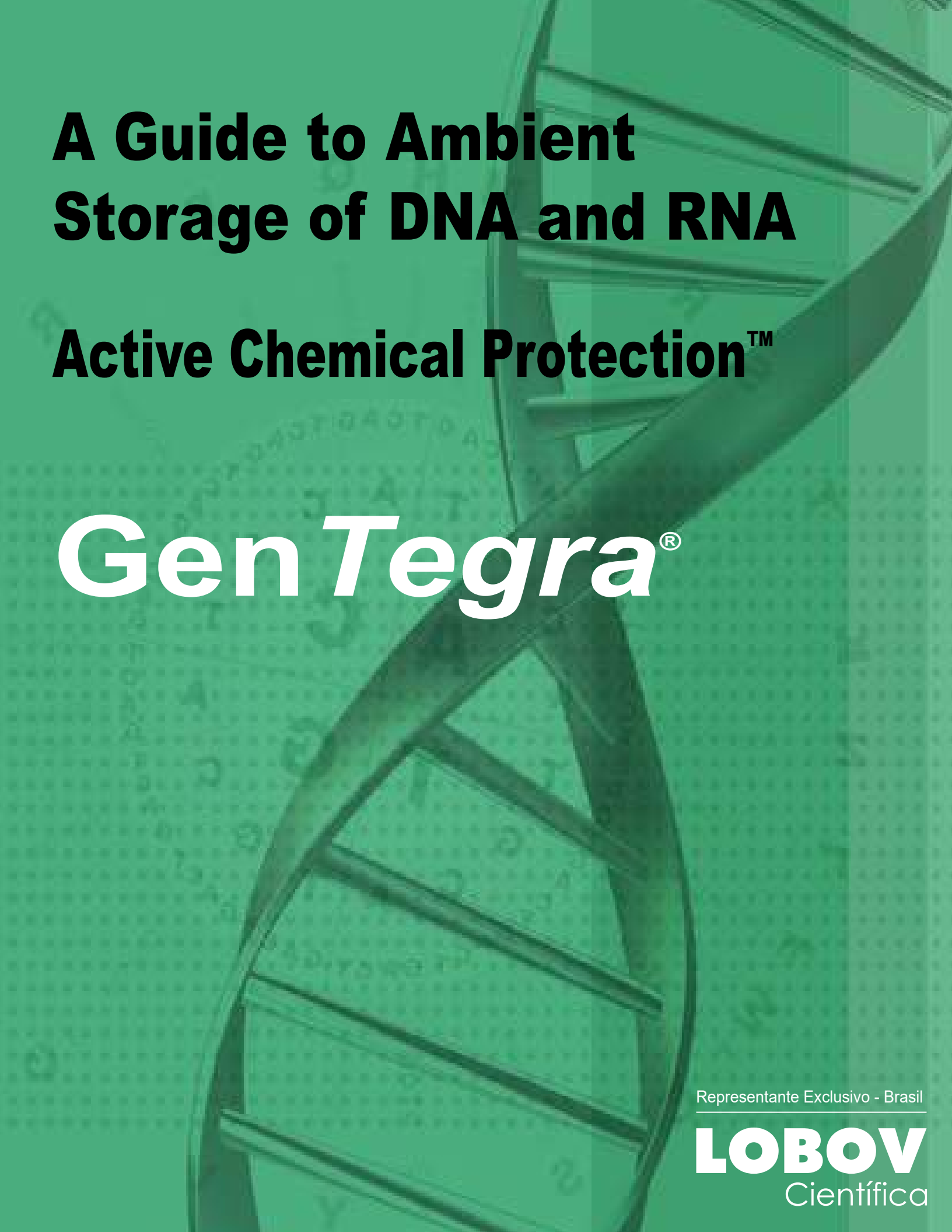
A Guide to Ambient Storage of DNA and RNA

Active Chemical Protection™

GenTegra®



Representante Exclusivo - Brasil

LOBOV Científica

1061 Serpentine Lane, Suite B
Pleasanton, CA 94566

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Nelson, James W.
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Cover design by
Sergio Miller
www.berimbau.com

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PREFACE

My interest in ambient protection and storage began over twenty years ago when I lost all my archived samples due to storm damage. The samples were stored in what was and is a state-of-the-art facility of freezers with electrical generator backup. However, all of these backups was pointless because the storm first took out the electricity and then the flooding that resulted from the rain took out the backup generators. The storm damage was so extensive the power could not be restored for weeks and all my archives of frozen samples and those of all my associates were lost. The final result was a commitment to the thinking, "there must be a better way than relying on freezers to protect valuable irreplaceable samples." From this thinking, Active Chemical Protection™ was developed as the "better way" of protecting valuable irreplaceable samples at ambient temperature!

Active Chemical Protection™ is designed to protect biological samples from harm better than freezing and without the inherent risks of relying on freezers. Active Chemical Protection is the basis of all of GenTegra's products from DNA, RNA to whole blood, serum and plasma. GenTegra-RNA and GenTegra-DNA are specifically formulated to provide decades of protection, at ambient temperatures, for extracted RNA and DNA samples. For raw samples, such as whole blood, plasma, serum or saliva samples, we offer paper based products using active chemical protection to stabilize these samples on special papers such as GenSaver™, FTA, and GenCollect™.

GenTegra LLC concentrates on protecting samples while they are being transported between collection point and the laboratory and in storage for weeks, months or years. Whether samples are intended for advanced next generation sequencing (NGS), or for pre-analytics our goal is to get the sample to its end use where it can deliver the same results as a freshly collected sample and to do this at ambient temperature conditions without the need for cooling at 4°C, freezing, or shipping on dry ice.

Costs, safety concerns and complex shipping requirements are eliminated when a sample can be shipped in a simple padded envelope and stored at ambient temperatures. GenTegra's products, GenTegra™-DNA, GenTegra-RNA, GenSaver™ papers and GenPlates® allow for ambient transport, as defined by FedEx and UPS (-80°C to +70°C) with complete protection against measurable damage to the nucleic acids being transported. The same high level of protection is provided for years of storage at ambient laboratory conditions.

Michael E, Hogan, PhD
CSO, GenTegra LLC

INTRODUCTION

This publication had its origin in a series of tutorials introducing Active Chemical Protection™ to the life sciences market segment and especially those life science researcher involved in operating and maintaining their own small biorepositories. The tutorial series was met with such acceptance that it was clear a single source that brought all the tutorials into one document and expanded each would be of value.

As the e-book was compiled it also became apparent that there was a need to extend and enhance it content. With that as an introduction you can expect a second edition of this e-Book in the not to distant future which will expand on the topic of ambient sample storage to include collection of samples in remote locations using collection cards that use the same Active Chemical Protection to preserve DNA and RNA in liquid biological samples.

James W Nelson, PhD

GenTegra LLC

A Guide to Ambient Storage of DNA and RNA

Active Chemical Protection™

CHAPTER 1

When freezing is best

Freezing of biological samples is a priori considered the best way to preserve all biological samples but is it really? This belief may stem from the fact that the only practical way to preserve solid tissue samples is by rapid freezing and long term storage at -80°C or in liquid nitrogen. Freezing is the best way to preserve solid tissue samples because chemical preservatives cannot penetrate deeply and quickly enough to stop all biological activity before degradation starts. This may have led to the belief that freezing is also the first and best choice for preserving all other biological samples such as purified DNA or purified RNA. Once a nucleic acid has been carefully removed from the sample and purified it becomes readily accessible to using active chemical preservation to provide a more reliable form of preservation for long term stable storage without the need for refrigeration at -20°C to -80°C.

Freezing is only passive protection

Freezing for long term storage of purified samples provides protection by slowing down all chemically generated attacks. Chemical degradation of DNA and RNA in storage is by oxidation and hydrolysis causing breaks in the nucleic acid strands over time. In general, for each 10°C that the temperature is lowered there is an halving of the rate of all chemical reactions. So dropping the temperature 20°C from room temperature to –20°C would reduce the undesirable effects of oxidation and hydrolysis approximately by a factor of sixteen. The slowing of the reaction rates is the only protection provided by freezing a sample. A recent paper in Biopreservation and Biobanking, Vol 12, #1, 46, 2014, (10.1089/bio.2013.0056) reported "significant degradation

was observed at 25 ng/µl, after only 8 months of storage at –80°C for RNA samples".

Freezing is, however, anything but passive when it comes to keeping the samples frozen, Energy, usually in the form of electrical power, must be continuously supplied to maintain sample integrity. Because the continuous application of power is so critical these freezer based system are routinely provided a secondary power source in the form of expensive electrical backup systems.

Backup generator
for small biobank.

Backup generator
for large biobank.

From liquid to frozen and back again

Freezing a biological sample is not as simple and error proof as may be assumed. During freezing of a water based sample a wave front is generated as the crystal interface (frozen water molecules) moves through the liquid. This interface can cause significant local shifts in concentration and cause mechanical stresses on large molecules such as DNA, RNA and proteins as it passes through the solution. How this wave front is generated is driven by how fast or slow the freezing takes place. Slowly dropping the temperature will generate larger crystals and large wave fronts while rapid freezing, plunging the sample into liquid nitrogen, for example, will generate many small crystals. And of course the reverse will happen as the sample is thawed. The need to control the potentially harmful effects of freeze and thaw cycles is well documented for protein solutions and may be an issue for the more labile RNA samples.[1]

Active chemical protection™

Active chemical protection is a chemical matrix which provides active protection for purified nucleic acid samples. This specific chemical matrix is designed to scavenge the potentially damaging oxidative species and forms a glass-like physical protective barrier around the nucleic acid. Our GenTegra-DNA matrix is an active chemical protection designed to sacrifice itself to protect the DNA sample.

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[1]Puri M et al. Evaluating Freeze-Thaw Processes Biopharmaceutical Development. BioProcessing International, 13(1) 2015; 34-45.

CHAPTER 2

Active Chemical Protection or freezing?

GenTegra uses a different approach to preserving and protecting DNA, active chemical protection designed to protect against the expected modes of damage. The common modes of chemical degradation of DNA are oxidation and hydrolysis, both of which cause breaks in the DNA strands over time. GenTegra-DNA uses active chemical protection by providing a targeted chemical matrix that is designed to provide active protection against oxidation and hydrolysis. When the DNA is removed from the original tissue or cells it becomes susceptible to external chemical attack such as oxidative attack by free radical and hydrolysis. GenTegra-DNA matrix is made up of specific chemicals that are designed to react sacrificially with reactive chemical species, such as free radicals, preventing damage to the DNA.

Immediate and long term protection

Common mode of delivery for a single sample tube of GenTegra -DNA, active chemical protection, 0.5 mL screw cap tube. Storage is at ambient temperature. Approximately actual size.

Active chemical protection is provided immediately in the initial liquid form when GenTegra-DNA is added to the purified DNA solution or the DNA is added to the sample tube containing the GenTegra-DNA. However, the level of protection increases dramatically when the DNA solution mixed with GenTegra-DNA are dried. As the solution is dried, the GenTegra-DNA concentrates around the DNA and forms a glass-like layer surrounding the DNA. This layer not only provides the protective barrier but also increases the anti-oxidation and anti-hydrolysis matrix concentration compared to the concentration of both the DNA and the active species that might damage the DNA. The presence of this cocoon like barrier around the DNA provides a physical protective barrier in addition to the active chemical protection layer protecting against oxidation and hydrolysis attack.

Near failure proof storage and shipping

Once DNA is dried in the GenTegra matrix it is actively protected against the common modes of degradation and is essentially impervious to many concerns that remain if samples are frozen. Storage of DNA on GenTegra-DNA is at ambient temperatures so power outages, freezer breakdown or forgetting to put the sample back in the freezer are not going to damage the DNA. The term ambient temperature actually includes temperature ranges from –80°C and as high as 70°C for prolonged times providing safe storage almost anywhere in the world without providing heating or cooling. This makes GenTegra matrix ideal for shipping purified samples from remote locations to a central laboratory for analysis, no ice packs or dry ice are required and shipping delays will not adversely affect the samples. About the only things GenTegra-DNA does not protect against is fire and physical damage to the tubes containing the DNA.

Fire destroys laboratory at research facility

To use, add water and go

After storage or shipping of a sample protected by GenTegra-DNA the sample is reconstituted by adding molecular biology grade water to the sample. The matrix of DNA and GenTegra dissolves quickly and is ready for immediate use in all downstream

analysis without the need for any intermediate purification step, just dissolve and go.

To summarize, GenTegra-DNA uses active chemical protection to protect DNA from hydrolysis and oxidation at a wide range of ambient temperature. This dry stabilization technique protects while shipping at ambient temperatures without dry ice or cold packs. To use stored samples, simply add water, mix and aliquot a sample, then dry the remaining sample and return to storage.

CHAPTER 3

Active Chemical Protection of RNA

GenTegra-RNA is designed to provide the same level of ambient temperature protection for RNA as GenTegra-DNA provides to DNA. The two products start much the same but GenTegra-RNA is then enhanced to provide the additional protection that is required to stabilize RNA. RNA is much more labile and subject to forms of degradation that are just not much of a worry when dealing with DNA, particularly from RNases that are pervasive in the environment. DNases do not represent the same level of threat when working with purified DNA. A recent publication reports that using GenTegra-RNA for shipping RNA samples delivered better RNA than did shipping frozen on dry ice and yielded **17% more scaffolds*** than the frozen samples.

Immediate and long term protection

GenTegra-RNA starts with the same chemicals that provide the same ambient temperature protection as GenTegra-DNA against hydrolysis and oxidation, but adds a potent RNase inhibitor. This powerful RNase inhibitor provides protection immediately in

Common mode of delivery for a single sample tube of GenTegra-RNA, active chemical protection, 0.5 mL screw cap tube. Storage is at ambient temperature. Approximately actual size.

the liquid state. When a sample is dried in GenTegra-RNA the RNase inhibitor remains present but the RNase enzymes are inactive in the dry state. Later, when the RNA sample is rehydrated for analysis, after shipping or for retesting after a period of storage, the RNase inhibition becomes active again protecting the RNA against any trace of RNase activity that may still be present.

Similar where it counts

Although the formulations are significantly different between GenTegra-DNA and GenTegra-RNA they both share the common advantage that neither cause any interference in the downstream analysis. No further purification is required for any of the common genomic analyses: PCR, qPCR, Sanger sequencing or NGS. With most current analytical techniques requiring far less than the isolated sample, both GenTegra-DNA and GenTegra-RNA also share the ability to be dried and rehydrated 5 times without loss of protection, allowing aliquots to be easily taken and the residual dried down for further ambient temperature storage.

To summarize, GenTegra-DNA and GenTegra-RNA use active chemical protection to protect against hydrolysis and oxidation at a wide range of ambient temperatures and GenTegra-RNA adds additional protection against any RNase activity that is present. Active chemical protection while shipping at ambient temperatures eliminates the need for dry ice, wet ice or cold packs and the expense of expedited overnight shipping. To use RNA samples dried on GenTegra-RNA, simply add back water, mix and remove an aliquot, then dry the remaining sample and return it to storage.

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* http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0050226

CHAPTER 4

Working with small sample volumes , <20 µL

"My sample is gone! What happened to it?", is all too often an issued when working with small sample volumes. When tubes of GenTegra®-DNA and GenTegra®-RNA are manufactured each tube contains 21 µl of GenTegra matrix. This 21 µl is carefully added to the tube so that the volume of GenTegra matrix is placed at the bottom of the tube. Immediately after adding the 21 µl of GenTegra solution the tubes are dried. As the solution dries it leaves behind a residual layer of GenTegra matrix on the walls of the tube at a height equal to the original 21 µl. This is why we recommend a minimum sample volume of 20 µl be added to the tubes in normal use. A 20 µl sample volume ensures the sample dissolves all the GenTegra matrix on the sides of the tube. For samples smaller than 20 µl special care and consideration must be used.

Sample volumes >10 µl to <20 µl

Working with sample volumes between 10 µl and 20 µl is relatively straight forward. Once the sample has been added carefully to the bottom of the tube it is mixed with the pipette by several in and out actions. For these small volumes **do not vortex the sample,** it is too easy to lose sample on the sides of the tube and the sample volume is too small to be unaffected by the wetting it will leave behind on the side walls. If the sample is high on the side walls after mixing it is almost impossible to ensure you can recover it at a later date even if a larger recovery volume is used. A brief centrifugation can help ensure the sample and matrix are in the bottom of the tube before drying. For volumes of 10-20 µl, we recommend rehydration in 20 µl to ensure recovery of all the sample. For volumes smaller than 20 µl, withdraw the sample more slowly to allow maximum flow of sample into the pipette tip and ensure maximum recovery.

Most tubes used in the laboratory today are low binding but if the sample is placed in the tube above the GenTegra coated portion at the bottom some binding may occur. The GenTegra matrix generally prevents binding of DNA or RNA to the walls of the tube but

may not counteract binding of the sample to the tube wall if sample is already dried and as a consequence binding has already occurred. To prevent the possibility of binding to the sides of the tube, extra caution needs to be taken to carefully place the sample at the bottom of the tube where the GenTegra matrix coats the sides of the tube.

GenTegra Protection and sample volumes <10 µl

Sample volumes of 10 µl or less pose a special challenge, not in protecting the sample but in recovering the sample later. Samples of less than 10 µl are easily protected because of the presence of an excess of the protective matrix. Even when small volumes are carefully placed into the bottom of a tube it is all too easy to deposit that small volume on the side of the tube rather than on the bottom. When the same small volume is used to recover the sample it may not be placed where the original sample was placed and therefore the sample will appear to be lost or reduced when in reality it is still in the tube. The recovery volume of water must come in contact with the dried DNA/RNA sample in order to recover the sample. Do not vortex, to mix volumes <10 µl, gentle re-pipetting is advised.

To summarize, for sample volumes less than 20 µl we recommend recovering with a volume at least twice the original sample volume and preferably using 20 µl. For sample volumes <5 µl we recommend a recovery volume of at least 10 µl. Small samples will be well protected by GenTegra but if the sample is lost due to handling difficulties it is still lost, protected or not. If you can accept the dilution, using a recovery volume of 20 µl for all samples less than 20ul will reduce the risk of inadvertent sample loss due to simple pipetting errors.

CHAPTER 5

Active Chemical Protection in the Biobank

There are three key issues that drive every biobank today: sample protection, controlling cost, and limited space. Whether the biobank operates a repository or an archive the efficient use of space is important. And eventually as the storage needs grow and more and more freezers are added, a lack of available space becomes a looming concern.

Saving space: Sample containers & freezer inefficiency

A quick measurement of any freezer will show that approximately 30% of the space it occupies is used for the refrigeration equipment and the insulation to maintain the temperature. This is 30% lost storage space when compared to an ambient storage method that does not require any power equipment or insulation. Simply treating samples with GenTegra-DNA and moving them out of the freezer allows you to store 30% more samples in the same space. And as no power is needed to protect the samples, power outages are no longer a concern for the samples stored dry on GenTegra-DNA.

When you look into a freezer used for storing biological samples it is often filled with cardboard boxes that hold either 100 small sample tubes or 81 slightly larger sample tubes. This format is often the de-facto format when sample preparation and purification are done manually. The tubes, though small, allow for labeling that identifies the samples. It is also a convenient format when it is necessary to withdraw a sample that has been requested for further study. This sample box format is, however, one of the most space-inefficient formats. Improved space efficiency is found by using the 96-tube rack format or by using

standard 96-well microplates. When compared to the standard cardboard sample tube boxes these 96-tube racks are capable of storing nearly 3 times as many samples in the same space. And a standard 96-well microplate is even more efficient, storing >5 times as many samples as a cabinet or freezer full of 100 tube cardboard boxes.

If you need more capacity without increasing your biobank's space requirements, consider adopting a more space efficient form of storage labware, and at the same time "freezer proof" your samples with GenTegra-DNA to keep them safe, secure and immune to power failures or natural disasters. And these gains in space, economics and security can be made with minimum cost in time and budget, and maximum benefit to your biobank.

Reduced operating costs with ambient temperature storage

Efficient use of space saves money in obvious ways but how does one save money by moving samples from freezers to ambient storage when the preservation chemistry costs money? A recent publication[1] by an ISBER study group looking at the costs of freezer storage found a reasonable low end cost for freezer storage to be $2.40 per sample over a 10-year period. A major US pharmaceutical company estimates their biobank costs at $0.50 per sample per year or $5.00 per sample for a 10-year period.

GenTegra offers a product especially designed for biobanks using automated DNA purification systems, GenTegra-DNA BULK. This product is designed to easily fit into the automated purification process and to allow automated addition of GenTegra-DNA immediately following the purification step. The samples are then dried and are ready for long term storage. This product provides GenTegra-DNA at a much lower cost per sample. Biobanks with yearly sample rates of 5,000 or more will see per sample costs

even lower. Besides a lower 10-year cost for using ambient dry storage, ambient technology also offers safe and secure storage with freedom from concerns about power outages or power backup systems. Ambient dry storage on GenTegra-DNA does not require air conditioning or humidity control for safe storage. The acceptable temperature range for storage is -80°C to +72°C. When air-conditioning fails for any reason it is of no concern when samples are protected by GenTegra-DNA.

Space efficient, highly secure and costly

One biobank requirement where additional cost is justifiable is secure sample tracking. Many samples in the freezers are individually labeled by hand either with a Sharpie® or a freezer label. Both of these, to a lesser or greater degree, are subject to failure of the labeling system. This issue has been addressed with the 2D 96-tube rack labeling system that provides a permanent 2D barcode on each tube. This format is space efficient and incredibly secure in its labeling as the barcodes are an integral part of each tube. However, this format is among the most expensive formats as the 2D barcode adds a cost of about $0.30 per tube and the use of a screw cap adds another $0.28[2]. Cost drops with volume usage but it is still an expensive per sample cost for storage. These extra costs may be justifiable because of the security provided and the ease of interfacing to an electronic sample tracking systems that are also required for tracking samples in a large biobank.

Individual 2D bar-code shown above.

Comparative size of tubes shown at right.

An interesting side point is that manual labeling systems, hand written with a Sharpie or using an adhesive label are much more secure and less subject to failure by peeling or falling off due to the adhesive failing when stored at ambient temperature.

If you need more capacity without increasing your biobank's space requirements, consider adopting a more space efficient form of storage labware, and at the same time "freezer proof" your samples with GenTegra-DNA to keep them safe, secure and immune to power failures or natural disasters. These gains in space, economics and security can be made with minimum cost in time and budget, and maximum benefit to your biobank's security.

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[1] Preservation of Biospecimens at Ambient Temperature: Special Focus on Nucleic Acids and Opportunities for the Biobanking Community; Volume 14, Number 2, 2016 BIOPRESERVATION AND BIOBANKING; A Review from the International Society for Biological and Environmental Repositories (ISBER) Biospecimen Science Working Group/p>

[2]Prices are for minimum quantity of one box or one bag, somewhat lower prices may be obtained with larger quantities.

CHAPTER 6

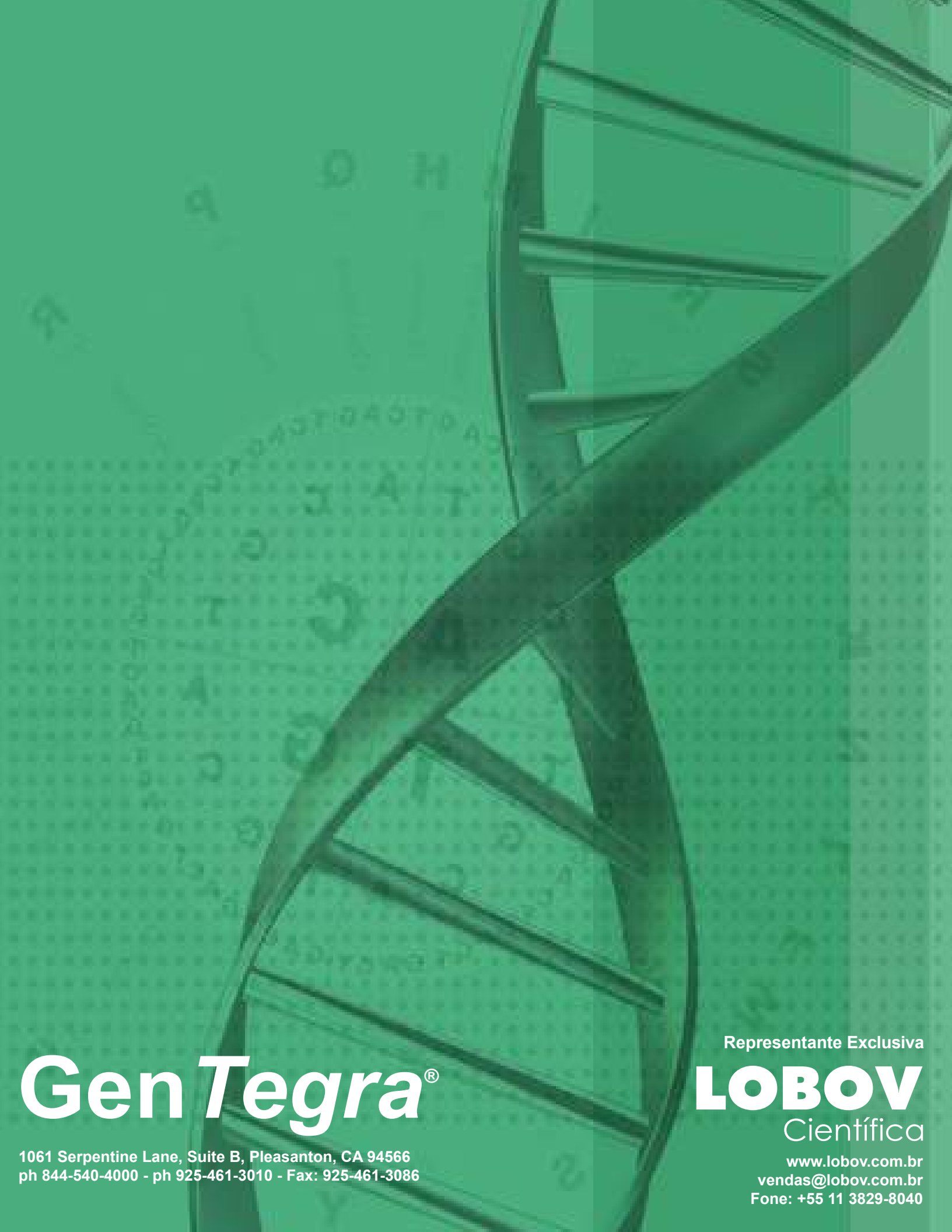
Automation and Cost Reduction

When sample numbers reach significant numbers it is often time to consider automation as a way to both keep up with the sample load and to help reduce the overall costs of sample processing. Automation reduces costs by allowing more samples to be processed faster with less human labor and increases the reliability of the sample processing. Automation also opens the door to significantly reducing the costs involved in using Active Chemical Protection. A significant component of the cost of ACP is the direct costs of delivering ACP in individual sample tubes. Automation allows the use of what is called BULK reagent format for adding GTD protection to the purified DNA.

GenTegra-DNA Dry BULK is designed to work most conveniently with automation robotics. BULK is delivered dry and rehydrated just before use. This ensures that GenTegra-DNA maintains its 3-year shelf life. When used the BULK is made-up by simply adding water and then transferred into the appropriate solvent reservoir on the robotic platform. A simple change to the control software allows the robot to dispense the required 5-µL of GenTegra-DNA solution to the purified DNA. After this additional the samples are ready for drying and storage.

BULK GenTegra-DNA bottle

BULK formulations of GenTegra-DNA are routinely available in sample sizes of 1,000, 2,000 and 4,000 depending upon the number of samples being processed daily or weekly. Custom size bottles are also available to meet the needs of the individual automation requirements.



GenTegra®

1061 Serpentine Lane, Suite B, Pleasanton, CA 94566
ph 844-540-4000 - ph 925-461-3010 - Fax: 925-461-3086

Representante Exclusiva

LOBOV Científica

www.lobov.com.br
vendas@lobov.com.br
Fone: +55 11 3829-8040