

Cell Health:

Incubation And Ultra-low Preservation

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A roundtable discussion

TOPICS

- > **How to avoid cell culture contamination.**
- > **Methods for remediation, types of cells and incubators.**
- > **The laboratory environment and good practices.**
- > **Ultra-low storage -- cell health and freezer function.**

ROUNDTABLE PARTICIPANTS

BRUNO SAINZ, a research scientist from the University of Illinois at Chicago, Hepatology Section, has 10 years' experience in cell culture and virology.

JEREMIAH KELLY, Associate Professor of Medicine and research faculty member at the Rush Alzheimer's Disease Center, is primarily interested in the neurobiology of Alzheimer's disease.

RICHARD MEAGHER works at Northwestern University's Feinberg School of Medicine as director at the Cell Therapy Processing Facility. His primary research interest is regulation of hematopoiesis, specifically through growth factors and microenvironment.

JOE LAPORTE is National Service Manager for Sanyo Scientific.

DEEPAK MISTRY is Scientific Products Marketing Manager for Sanyo.

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Avoiding cell culture contamination

Moderator: Good morning. We are here to address cell health: how cells are incubated and frozen, and the challenges faced during those operations. Dr. Kelly, can you tell us about your work, the challenges you face?

Dr. Kelly: Because the Rush Alzheimer's Disease Center maintains a biorepository of transformed and untransformed lymphocyte samples from subjects in several NIH-funded studies, we face the challenge of efficiently processing a large number of samples. Our program currently has frozen untransformed lymphocytes from about 2000 subjects, and nearly 1000 transformed lymphocyte samples. We use a method that is well tested for obtaining EBV-transformed cells. We probably have had experience culturing more than 200 cultures at once, in one place. I'm not sure if anybody else has done quite



Dr. Jeremiah Kelly, left, of the Rush Alzheimer's Disease Center, and Deepak Mistry, Marketing Manager for Sanyo Scientific.

as many simultaneously. Our incubators are critical to our efforts. Contamination has never been a problem, I'm happy to say. We worry about mycoplasma, but we've never had any problem with mycoplasma contamination.

Mod: Is that unusual?

Dr. Kelly: I think it's probably because we isolate

the sample in one room. It's a fairly uncomplicated technique. There isn't too much chance of contamination with other cell lines because we usually are not culturing any other type of cell in the space.

We've had some difficulty with fungal contamination previously, but that was related to long-term storage of samples at sub-optimal temperatures. We've gotten past that problem. But I would say that our transformation success has been very good. Contamination has not been an issue and we now have, I think, about 26,000 aliquots of transformed and untransformed lymphocyte samples stored in our cryofreezers.

Proactive vs. reactive contamination control

Mistry: Most researchers and end users I've spoken with have had similar experiences with regards to mycoplasma and contamination. Bacterial contamination occurs on many levels. There is a level that is obvious, where the culture is stopped immediately. Then there is the situation where contamination is not evident. From my experience, any level of bacterial growth affects CO₂ and O₂ levels, and pH as well. Another observation is the difference between proactive and reactive contamination control. Cell lines from a proactive system seem to be more resilient throughout a research project.

Mod: Dr. Kelly, you mentioned having 26,000 samples in cryopreservation. That's from the repository?

Dr. Kelly: This is our repository.

Mod: *I think one thing that Deepak is talking about is that cells often go from incubator to freezer and back again, and that the environment throughout this process is an important part of long-term viability?*

Mistry: Assuming the technique is right and you've done all the right things, I think you could have a better product. Where you have both incubation and preservation, incubation is a little bit more complex in that you're trying to mimic very specific environments. To do that as perfectly as possible, you probably want a decontamination system

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working in real time. Not only to protect your cell cultures, but to maintain that environment.

Mod: Dr. Sainz, would you like to talk about your experiences?

Dr. Sainz: We work with human and mouse hepatocytes and don't have as many cells as Dr. Kelly. There is one primary mouse hepatocyte cell line, and transformed cell lines. Likewise with the human cells, there is one primary line and transformed cell lines. We find that cell culture is extremely important, because hepatocytes removed from the liver do differentiate, thereby losing properties inherent in hepatocytes in vivo. So we take great care in culturing our hepatocytes in an environment that will not promote differentiation, where cells are kept in a state where we can use them for our experiments. We infect our cells with the hepatitis C virus so the conditions under which we perform tests are also important. Slight fluctuations in incubator temperature or CO₂ can dramatically affect incubation and infection. Even a slight increase in

Cell lines from a system with proactive contamination control seem to be more resilient throughout a research project.

CO₂ can result in non-productive infection. So we are constantly looking at both the cell line and how it's growing and behaving over a long period of time. Our infection is a read-out for not only the state of a cell, but of our environment. So many factors are constantly under surveillance.

Mod: Does working with infectious cell lines complicate the process?

Dr. Sainz: It depends on what you mean by complicated. Some viruses are cytopathic. Other viruses, in particular Hepatitis C, are non-cytopathic.

Dr. Kelly: It would be the same with Epstein-Barr virus.

Mistry: The length of time that your cultures are in the incubator is important. Some researchers incubate for one day or two days, some for a week. What is going on during the incubation? What conditions are fluctuating? Some systems that use HEPA are constantly on. What are the options? Some researchers question HEPA system effectiveness. They have concerns about bacteria collecting within the filters when they become moist.

Dr. Meagher: Our lab handles only human stem cells — from bone marrow, cord blood, human peripheral blood progenitor cells, the whole gamut. At some point we will work with embryonic stem cells. The cells are used as part of our hematopoietic

stem cell transplant program, so contamination is a very serious concern, both environmental and cross-contamination. At this point we don't do a great deal of cell culture per se. Our primary goal is to preserve cells, which we do in standard tissue culture media containing normal nutrients for stem cells. We've moved from fetal bovine products into human serum albumin supplementation.

It's the cell culture environment

LaPorte: Earlier you were talking about how to control cells grown in the incubator. We're seeing a trend towards using pH levels as opposed to the CO₂ levels. We all know that CO₂ usually controls pH, but specifically in the IVF market, many customers will measure the pH every morning, sometimes hourly, and adjust CO₂ levels to obtain the desired pH. Incubators traditionally have controlled CO₂. Every major manufacturer is focused on controlling CO₂ levels.

In reality, without good lab practices, it doesn't matter what incubator you use; you are going to inoculate the incubator, and if it's a resistant organism like mold or yeast, it becomes difficult to control. The best way to deal with contamination is not to introduce it.

Often the problem is not lab practices, but how the tissue culture room is set up. We have sometimes done viable particle counts in tissue culture labs, an effective method to discover the source of contamination. The problem could arise from some other lab sharing the air handling system, or someone who doesn't wear gloves on a particular day.

But usually everyone is using good lab practices, and it's the air that's causing a problem, or people traffic. Biological safety cabinets are assumed to be sterile, but someone walking by quickly can disrupt the air curtain, and you have contamination. Every time a door opens, unless you have a very clean Level 3, Level 4 environment, there's always the potential of introducing contamination.

What happens to airborne contaminants is also quite important. Water pans are a great place for things to grow. Sanyo's approach is to control not only the contaminant landing on the incubator shelves or inside surfaces; organisms that are airborne are controlled by a UV lamp in the back of the incubator.

UV decontamination

Mod: So ultraviolet light is sufficient to kill bacteria and viruses?

Mistry: *Independent testing shows that you can prevent the growth of airborne bacteria, mold and yeast common in incubators, when you have a sterilization process and UV decontamination of circulated, humidified air and humidity pan surface water. While certain species of contaminants can become thermophilic over time, UV disrupts pyrimidine dimers, which does not allow microorganisms to replicate. Remember that while many species are killed after two hours of UV exposure, others may take 10-plus hours.*

Mod: Dr. Meagher?

Dr. Meagher: We hardly ever experience contamination problems because we don't culture cells for the most part. We have seen contamination arising from steps where we feed cells. Whenever you introduce multiple manipulations of cells, the opportunity for contamination goes up exponentially.

Our cells are collected through a medically sterile procedure, and concentrated. We add cryopreservation media and into the freezer they go. We are hyper-alert to the potential for contamination because of the nature of the product, that these cells are going into patients. You have to make sure you do everything exactly the same way every time. We don't use any form of radiation for sterilization due to the fact that these cells are transplanted into humans.

Mod: How vigorous is your sterile technique? How much do you emphasize it?

Dr. Sainz: Sterilization is the foundation of tissue culture. We, fortunately, have never had a contamination problem at UIC. It's a renovated lab in an

old building that had many problems with ducts, air vents, and mold and dust in the environment, so we're conscious of that in our tissue culture rooms. We had specific tissue culture rooms made in our laboratories, so we don't have to walk through. We've had problems in the past, in other facilities where it's very easy for someone walking by, closing a refrigerator door next to the biosafety cabinet to introduce air and cause contamination.

Mod: Do you use both heat and UV sterilization incubators?

Dr. Sainz: We have the Sanyo incubators, so we do have UV sterilization.

Mod: You mentioned some thermophilic species need 24 hours of UV exposure?

Dr. Sainz: Correct.

Mod: So that comes down to how frequently the door is opened, is that right?

Mistry: Our system provides several options. Some incubators only have HEPA, which is not working real time, and you have heat, but heat is really more of a reactive approach.

At any visible indication of contamination you probably want to stop work immediately, autoclave everything in the interior, and run a 24-hour UV protocol. Shutting down costs a lot of time and money. But even more significant is the effort invested to create cell lines.

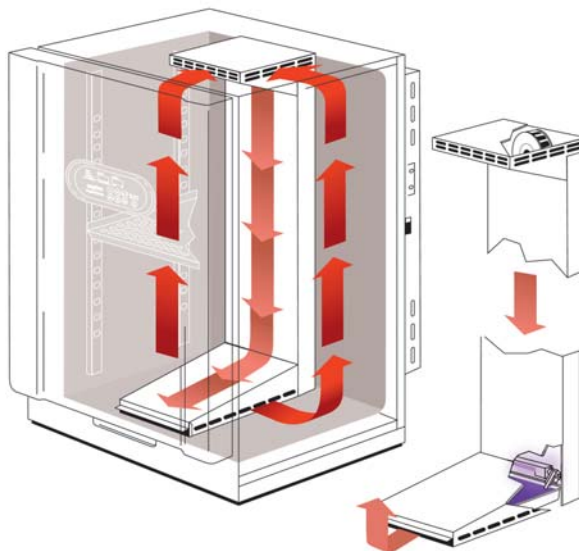
Mod: Dr. Kelly, sterility is obviously very important in your facility. Can you address how you do things?

Dr. Kelly: I have encountered contamination problems with facilities previously, with traffic running through cell culture rooms. I've also seen incubators that have had contamination that simply cannot be remediated. We're lucky at Rush to have a separate tissue culture room that's completely isolated, with a separate air system. We have minimized the steps taken for the isolation of our cells, the movement of the cells into the tissue culture dishes, movement from the incubator to the microscope for counting and assessment of viability, movement to the tissue culture hood for feeding. The major thing is minimizing steps taken outside of the incubator. And then, we do the usual things done in any sort of tissue culture operation. You have decontamination, using fresh coats, and frequent glove changes, use 70% ethanol wipe downs before and after feeding cells. We have a QA program that's modest by some standards, but we clean our incubators about every three months with 70% ethanol. We change our water pan about every eight weeks. And we UV-disinfect every night.

Mod: The entire room?

Dr. Kelly: The entire room, the biosafety cabinets.

LaPorte: Many people have misconceptions



The airflow process within the SANYO MCO-20AIC chamber reveals the relative position of the blower and UV lamp in relationship to the humidity pan and out-flow ducts at the base of the chamber.

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regarding UV lamps. We have a timer on our incubator that indicates when the UV light isn't functioning properly. UV lights will still glow in the visible purple range that everyone sees, even when the effectiveness of the lights has tapered off. So often, even in common applications like biosafety cabinets, one assumes that if a light is glowing, the disinfection capability is still effective. So it's important to monitor the number of hours the bulb is used.

Mod: What is a bulb's useful lifetime?

LaPorte: It depends.

Mistry: On how often you do 24 hour cycles, and other factors.

LaPorte: We know that a typical bulb operates effectively for up to 3000 hours. Typically it lasts three years.

Mod: You mentioned minimizing steps that you take in the various processes. Can you offer any insights as to how to organize culture processes?

Dr. Kelly: Key is having a separate tissue culture room, if you can afford that, and having a separate air source for room ventilation. The third factor would be having a small area in which your equipment is very closely spaced and everything else is placed next to it. We have two benches that are very close to each other, and our incubator sits right next to our biosafety cabinets, within a couple steps of the incubators and microscope. The tech only needs to turn and take a couple of steps to go from one instrument to another. And glove changes are easy. Only one technician works at one time in the room.

Mod: By design?

Dr. Kelly: Yes.

Many people have misconceptions regarding UV lamps. UV lights will still glow in the visible purple range that everyone sees, even when the effectiveness of the lights has tapered off. So often, even in common applications like biosafety cabinets, it's important to monitor the number of hours the bulb is used.

LaPorte: We've seen contamination in older facilities that are retrofitted for tissue culture. To control contamination, be as clean as possible. Researchers will often have two biosafety cabinets, and run them constantly to attempt to collect contaminants on the HEPA filters. But often those facil-



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Active Background Contamination Control™ delivers automatic UV cycling to destroy contaminants *in situ* all day, all night, all the time, while inCu safe® copper-enriched interior components stop surface contamination before it starts.

ities generate so much heat, from those biosafety cabinets running, that they employ an auxiliary cooling system. And often, facilities will put in a water-cooled system. *Probably 70-80% of the times I see contamination, air handlers are in the room and by the very nature of how they operate, the refrigeration systems in them will produce condensation that drips off into a pan. It's almost impossible to keep those pans clean. Doing viable particle counts can help you keep an eye on this.*

Mod: Is that something people should do on a regular basis?

LaPorte: Some of the larger pharmaceutical companies, as well as operating rooms or post-op rooms in hospitals, will do this. But I am surprised by how many people ask what we mean. We've had to do a particle count just to show them the problem isn't with the incubators, or even from lab practices. Often you can take the particle count with the fans and all the control systems off, and when you turn them back on the room floods with all sorts of contaminants. Whenever we run into contamination issues, the first thing we do when we walk in the room is look up. And when you do you often see duct work and airflow coming right straight into the front of the incubator. Every time you open the door, whatever's up in the building system, or even if it's isolated to your particular room, can cause contamination issues.

Mod: Were architectural designers used during your retrofit?

Dr. Sainz: I don't think architects were aware of this. It's difficult at a state university to involve all the players, to properly look at the designs and plan out

how the room should be designed. Oftentimes you're limited by space to the existing ductwork. Obviously in a new building you have the luxury of all these different steps. We have people in our lab who have had experiences with incubators in areas that weren't ideal, and they bring that knowledge with them. Having someone in the lab who's experienced, that has been tremendous.

Dr. Kelly: We were fortunate in being able to participate in the design of our lab. Rush was converting a floor of the Cohn Building, the main research building, and we were able to design a tissue culture room to minimize contamination.

Mod: As much as you can afford to put in up-front would probably be cost effective in the long run, would it not?

Mistry: Yes, because you don't have much control over what's already in place.

Mod: Does acting as a biorepository impose a higher standard?

Dr. Kelly: Yes, it's very important. We do have a responsibility to maintain our cells for other investigators and we're supported by NIH for this.

Mod: Is transporting cells an issue?

Dr. Kelly: To other investigators?

Mod: Yes, in terms of contamination. I mean is it something that's easily managed?

Dr. Kelly: It isn't a problem.

Mod: You use UV sterilization?

Dr. Kelly: That's the primary method we use in our tissue culture room. We have two Sanyo and two Thermo incubators. It's important to have the best incubator. But if you are in contaminated surroundings due to bad duct work, that shoots everything.

Mistry: We understand that it's a combination of many different things, but it seems to be the environment, the exterior environment that's an issue. And as we all know, in many labs the environment changes day to day and it could be something that could be at equilibrium for a certain point of time but then change due to something that is unseen.

Efficacy of UV decontamination



Dr. Kelly: Have you compared your product to other company's products? Under different conditions of contamination?

Mistry: We just commissioned a study by a very well-known rapid microbiological detection company that works with some of the top pharmaceutical companies. We wanted to understand the differences between the two methods. What we found was that they were statistically equivalent. But we did see effectiveness of UV decontamination with the more thermophilic species.

Cell preservation

Mod: Let's move on to preservation. We've cultured a lot of cells, now what?

Mistry: How are you going to archive them? And in Dr. Kelly's case, how are you going to be able to share them? With repositories, long- and short-term archiving is critical.

Mod: Dr. Kelly, perhaps you could discuss the procedures you use, what you find to be the best for cell health?

Dr. Kelly: We collect blood samples from subjects in retirement communities around the Chicago area for one study, and from other locations around the country for our other major study. Samples are returned to us in cell separation tubes that separate mononuclear cells from red cells and platelets. So all we have to do is spin down the sample, take off the mononuclear cells, and wash, count and aliquot them. We save some cells for transformation and the rest we cryopreserve or pellet for DNA extraction.

Those we save for transformation immediately go into culture. They're EBV-transformed and they are then saved in our repository. We track all samples using Freezerworks (Dataworks Development, Inc.,

METHOD	UV	HIGH HEAT	
	SANYO	Brand F(+140°C)	Brand H(+90°C)
TEST RESULTS, MAXIMUM LOG REDUCTIONS			
Bacteria	> 4.5	> 4.5	> 4.5
Yeast	> 2.9	> 2.9	> 2.9
Mold	> 2.7	> 2.7	> 2.7
DECONTAMINATION OPTIONS			
Overnight	✓	✓	✓
Active Background Contamination Control™	✓	⊘	⊘

Independent testing confirms that exposure to ultraviolet light at 253.7nm and heat sterilization at +90°C and +140°C are equally effective in decontaminating an incubator interior chamber against organisms selected for testing. Additionally, unlike incubators that depend solely on heat for decontamination, the patented SANYO SafeCell™ UV system continues to protect against contamination during normal operation by combining the passive resistance of copper-enriched stainless steel with UV decontamination of circulated, humidified air.

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Mountlake Terrace, WA), which we find very satisfactory.

Freezerworks, which operates with our data entry system for the Alzheimer's Disease Center projects at Rush, integrates, in real time, clinical and biological data. Each of the aliquots of transformed and untransformed lymphocyte samples is assigned a bar code and initially placed into a gradient freezing system for a week, then into a cryofreezer for long-term storage. We currently have 17 -86°C freezers that we operate continuously, and three cryo-freezers. We use a system from Rees Scientific Corp. (Trenton, NJ) for monitoring. We have a 48-port operating system, one of the largest available, to monitor freezer temperatures 24 hours a day. In addition to transformed and untransformed lymphocyte specimens, our program stores samples of DNA, serum, plasma and urine specimens collected from subjects enrolled in several ongoing studies. The largest component of the frozen biospecimen repository at the Rush Alzheimer's Disease Center is brain tissue obtained from nearly 600 individuals who generously donated their brain at the time of death as enrollees in either the Rush Religious Orders Study or the Rush Memory and Aging Project.

Dr. Meagher: At this point we keep track of all our samples using manual tracking. We're taking a look at a system, InfoDot (Key Surgical Inc., Eden Prairie, MN), which is a two-dimensional bar code you can stick onto the bag before freezing. The same system is used to track surgical instruments in operating rooms. You can then follow a sample bag, map it, and locate it easily. It's similar to, but different from, a normal bar code. Supposedly the scanner used for this product will find a bag through cryogenic nitrogen vapor.

Ultra-low and cryogenic preservation

Mod: How do you preserve your cells at ultra-low temperatures?

Dr. Meagher: We like to collect twice as many cells as we need, in case we have to repeat a transplant procedure, so we treat all our stored cells as if we were going to use them in patients. We freeze all cells indicated for autologous transplants, and a good number for allogeneic transplant as well. Sometimes we don't get sufficient cells if the patient is sick, but that isn't an issue from healthy donors.

We use standard freezing protocols, and don't do much with the cells beforehand because we don't expand the cells. Most of what we do is

protocol-driven because we work in a medical environment. For freezing, basically we use controlled-rate freezing and DMSO, both of which are standard, routine freezing protocols.

Dr. Sainz: Our system is not as elaborate as the one at Rush. We culture cells for very long periods. When we are ready to cryopreserve we do the basic protocol of re-suspending the cells in 90% FBS and 10% DMSO. We also perform gradient freezing in isopropanol at -86°C . We currently have two -86°C freezers in our laboratory and we will keep the cells at -86°C for about a week, and then transfer them to long-term storage in liquid nitrogen.

We have had success in keeping cells long term at -86°C . These are adherent cells, not as susceptible to cell death, so we can get some very good long-term storage.

Mod: How long are your cells in freezer storage before you take them out, start working with them?

Dr. Sainz: It depends. We make many clones of our cells and we also have repositories of our major lab stocks of cells. We'll keep the major lab stocks at liquid nitrogen temperatures. We maintain clones and cells we work with constant-



Joe LaPorte, National Service Manager, Sanyo Scientific, discusses cryopreservation with Bruno Sainz, a research scientist with the University of Illinois, Chicago, Hepatology Section.

ly at -86°C for long-term storage. We get very good viability out of the cells.

Mod: Do you freeze quickly and thaw slowly? Or is it vice versa?

Dr. Sainz: For cryopreservation, you want to freeze slowly and then thaw quickly. We perform thawing at 37°C in a water bath, then transport cells into the tissue culture hood. Adherent cells do very well under this protocol. Hepatocytes are very hearty cells, so we tend not to have problems with tak-

ing them out of long-term cryopreservation.

Mod: Dr. Kelly? Are your cells durable?

Dr. Kelly: Reasonably durable.

Dr. Meagher: We store all our cells at liquid nitrogen temperatures, -195°C , but we use vapor-phase freezers. We previously used liquid nitrogen, but moved to the new vapor phase freezers because we had concerns about bags breaking and cross-contamination when the cells are stored in liquid. With the vapor-phase freezers you can maintain essentially the same storage conditions as with liquid nitrogen, without the liquid.

We use polypropylene bags, which are very strong and durable, but they can become brittle at cryogenic temperatures. No material is 100% reliable. We found it was difficult to work with bags when moving samples into and out of a racking system that's under liquid nitrogen.

Mistry: It seems to me that what's probably most important on a daily basis is reliability. What factors are important with respect to reliability in -86°C storage?

Dr. Kelly: It's tremendously important to us, with the number of freezers we maintain, and the preciousness of our materials, to have products from a company with a good service plan. On average, we have a breakdown about every 10 days, and by that I mean a breakdown in temperature maintenance, where something triggers an alarm. I'm

Dr. Kelly: Yes. Any time the alarm is triggered we must remove all samples to a spare freezer. We oftentimes don't have much in terms of residual freezer space and so that can be a huge problem for us. And we're filling our spaces continuously with brain tissue from the new autopsies.

Mod: What about a continuous service?

Dr. Kelly: We have a very nice arrangement now with the company that's providing our freezers. We're pretty happy with them.

Mod: Might the system trigger alarms even though a cell-threatening event did not occur?

Dr. Kelly: My techs tell me problems sometimes arise with probe position due to samples moving around the freezer, so you have to be very careful about how you position your samples. There could also be conflicts between the freezer's own sensor and the Rees sensor.

Dr. Meagher: We have few if any problems with our isothermal vapor phase nitrogen freezers. They break down rarely, but we expect them to have a life span of 15-20 years, depending on maintenance. In all the years I've been doing this, I've only seen one freezer fail catastrophically. It was a 25-year old liquid-phase nitrogen freezer, not a vapor phase freezer, and it imploded. That was back at Walter Reed.

Our freezers use an automated filling system, with a high- and low-temperature sensor that determines the liquid nitrogen fill. We keep the sensors set in such a way that we have three to five days, if the freezer is not opened, to take care of things if the freezer stops working properly. When that happens we off-load samples into a backup freezer, fix the broken one, and use that as the backup for next time.

PERFORMANCE	SANYO	Brand N	Brand R	Brand F
Temp. Uniformity Range (setpoint@ -80.0°C)	4.2° C	9.0° C	12.5° C	7.7° C
Temp. at Top of Chamber (setpoint@ -86.0°C)	-86.0° C	-77.5° C	-81.4° C	-81.4° C
Max Warming Point, 10 sec. Door Opening	-75.0° C	-34.3° C	-57.7° C	-76.8° C
Noise Level, 1 meter from unit	43.8 dB(A)	51.7 dB(A)	52.0 dB(A)	72.0 dB(A)

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not talking about total breakdown, where equipment needs replacement, but we very frequently have had to bring in a temporary freezer while another freezer is being fixed. Because of the increasing number of samples we will have to store in the next few years, we expect we will need to add an average of 2-3 freezers a year.

Mistry: Do you have upright freezers?

Dr. Kelly: Yes.

Mistry: Is there one specific problem that's been reoccurring?

Dr. Kelly: Maintaining temperature control is always a problem.

Mod: You mean constant temperatures?

Ultra-low freezer operation and reliability

Dr. Kelly: I want to stress how crucial it is for groups like ours who do human research to have the very best systems in place to maintain and protect our biospecimens. They are incredibly precious and cannot be replaced. They are from individuals who have given us sometimes more than 15 years of clinical data and have donated their brain and in some cases their spinal chords, muscle, nerve samples, and lymphocytes. We just can't lose these samples.

Mistry: I think this is a good point for Joe to provide some perspective as he's had experience with several manufacturers.

LaPorte: As freezers have evolved over the years, many technical issues have arisen due to the

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Montreal Protocol, which controls the production of ozone depleting substances. Almost all ultra-low freezers on the market before the Montreal Protocol contained refrigerants that harm the ozone layer.

The refrigerants these freezers can use are limited and the temperatures are extreme. Manufacturers focus on high-volume refrigerants for building systems, home air conditioners, etc. Unfortunately the ultra-low temperature market represents just 1/10th of 1% of the entire refrigeration market.

Often breakdowns are related to the discharge temperatures or the heat the compressors are running under. Ultra-low systems use a cascade system, meaning one compressor cools another and that's what cools the box to remove the heat.

There's also a misunderstanding with many researchers that they can treat an ultra-low freezer almost like they do a home freezer. I'm going to buy 400 lbs. of steaks and throw it in my freezer, and it'll eventually get cold. You can't do that with an ultra-low freezer. They're storage freezers, they're not blast freezers.

Ultra-low freezers operate differently than regular domestic freezers. So putting many products into them creates problems in heat removal, because that's basically the nature of refrigeration — we're taking heat energy from one place and transferring it to another place. So, many failures are related to too much room temperature product being placed in the freezer at once.

We see many failures related to simple maintenance items such as cleaning the filters. "I'm going to wait until that little light comes on the freezer that says 'clean filter' before I do it." You can't rely on those, because they often won't come on until the freezer is under stress.

One very important thing is to keep ice off the gaskets and to clean the filters. It will prolong the life of the freezer.

Dr. Kelly: We actually have a local representative of the company that makes the freezer who comes in and makes the call, checks the freezer and actually will replace the freezer if need be with a loaner unit, if he needs to take the one that's having problems.

LaPorte: Are most of your problems occurring after you put product into the freezer?

Dr. Kelly: I would say our problems often result from packing many samples of different shapes and sizes. Because with frozen brain tissue, packages are often irregularly shaped.

LaPorte: Actually ultra-low freezers run easier when they're fuller, as long as there's some room for air, because you have a large mass of prod-

uct that helps maintain the temperature.

Dr. Kelly: I would wonder if you have irregularly shaped samples, like brain tissue, whether there might be a circulation problem.

LaPorte: It depends on the age of the freezer. One thing we deal with daily that we never even used to hear of a few years ago is validation. Biotech and pharmaceutical companies, particularly, must show the FDA that the product in freezers is kept within temperature boundaries. Some of the older designs cannot maintain narrow temperature ranges throughout the freezer. In some cases the temperature might go as high as -55°C in some parts of the freezer, depending on the loading, how much ice there is, how good the gaskets are, and the quality of service.

So an installed monitoring system will read one temperature and the system probe will read another, and in many cases the unit sensor is going to be located in the coldest spot in the freezer. And the monitoring sensor is in the center. So often one responds faster than the other.

In many cases, it's product distribution and where the sensors are located that will cause issues. It's an education process; people need to understand what these freezers are capable of doing. We recommend chest freezers wherever possible, but most labs don't have the room for them.

Dr. Kelly: We don't currently have the room to use multiple chest freezers.

Dr. Meagher: For a while, we used a chest freezer from one vendor that got down to -135°C . It worked fine, but -135°C is not -195°C . If you need very long-term storage, you want liquid nitrogen temperatures. Stem cell work, especially cord blood stem cells, are a long-term proposition. You may not use the cells for five, ten, twenty years or longer. You want a freezer that's very cold, provides very uniform temperature, and will keep going and going. Liquid nitrogen does that for us.

Mod: People are working with smaller and smaller samples, more sensitive samples, storing the samples for long periods of time. How does temperature uniformity, or lack of it, affect viability?

LaPorte: Drug manufacturers must keep their clinical trial samples for upwards of 25 years, in case there's an issue with the drug or they have to go back and look at it again, which means you may have to keep viability for 25 years. Now with Hep C you'll have viability at -86°C for long periods of time, but not every cell line is going to be like that. You might have viability at -85°C for the products at the bottom, but the products at -60°C and -65°C at the top might not have that kind of

viability. *Imagine if you have to go back and test your samples 20 years after the fact and you find that everything at the top of every one of your ultra-low freezers has very low viability. That can be disastrous. You can't always go by the display. If it says -85°C , it's not necessarily true that everything in that freezer is at -85°C .*

In Sanyo's case, freezers are designed in a very specific manner, as far as the flow of the refrigerant in the walls of the freezer, so that we keep uniformity very tight inside the freezer. Every manufacturer has addressed that in one way or another. But we're finding, especially with the pharmaceutical companies and the biotechs, that many freezers just aren't meeting the standard for product storage. They're either having to relax the standard or they're having to look at, okay, what kind of mapping am I getting on these freezers?

Mistry: We are going to publish data in a white paper format that focuses on SANYO's application-specific compressor technology, developed for use specifically in an ultra-low temperature freezer. It further demonstrates how SANYO ultra-low freezers deliver better chamber temperature uniformity necessary for stability of stored product, and better viability of frozen biological materials.

Model MDF-U73VC with optional sliding drawer racks holds 57,600 1.8 ml vials in a compact 9.5 sq. ft. footprint. From short-term storage to long-term archiving as low as -86°C , SANYO ultra-low freezers maintain stable temperatures at all levels for post-thaw reproducibility.

LaPorte: It gets back to the issue of reliability, and that many people just don't trust ultra-low freezers any more. One could purchase an old chest freezer, plug it in and forget it. Technology was different. The old -70°C or -75°C freezers used a refrigerant that hasn't been available for years. It's just

not available in the industrialized world anymore. You can get it in some of the less-developed countries, but you can't use it here.

And we've seen many times where customers get one failure after another and they wonder, "Are there any alternatives for that?" Dr. Kelly, you mentioned you're storing at -120°C for

very long-term storage, for infinite viability and everyone has read the papers on, once you get below -130°C , then you can almost consider it to be infinite storage.

The decision has to be made: "Am I going to work with liquid nitrogen or with mechanical refrigeration?" Liquid nitrogen will store at -196°C , but presents issues of cross-contamination. "My alternative is to store in the vapor phase, but I'm not going to get that really low temperature that liquid nitrogen can give me. So what else do I do? Use mechanical refrigeration? How long can I expect it to run to get my return on investment plus ongoing energy costs?"

Biopharma companies will ask, "If my ultra-low is not reliable, how am I going to trust a -150°C that's mechanical?"

We see many failures related to simple maintenance items such as cleaning the filters. "I'm going to wait until that little light comes on the freezer that says 'clean filter' before I do it. They often won't come on until the freezer is under stress.

Dr. Kelly: And I think also, price. But I'm not sure what the price differential would be.

Mistry: It's almost a long-term investment with LN_2 . When you take that initial hit, if you've got a mechanical freezer, if you look at it over time, there are some benefits and it could equalize at some point.

Mod: It sounds like you're unsure about long-term cost benefits.

Mistry: It's hard to just make a firm average on every system. An end user has to weigh what they want, what they need.

LaPorte: We're going through this now. We have a major pharmaceutical company looking at keeping their core cell lines in our -150°C freezers. And we're going through a stratification study, a whole factor acceptance testing study, which is showing that our freezer does what it's expected to do.

In their case, they made a decision to go with mechanical refrigeration because their requirement for the storage of their product meant the product could never get above -135°C . The future of their entire organization is tied up in these -150°C freezers. So they made a decision.



Cell Health:

Incubation And Ultra-low Preservation

We went through an exhaustive study — you'd be surprised how many sensors aren't really reading the temperatures the units are running at.

In this case, we're able to show that we had temperature uniformity well within the range of their requirements. As I understand it, -130°C is the magic number for the glass transition phase. And if we can get that 5°C safety margin and keep everything below that temperature, then we should be fine. And in this case, compared to liquid nitrogen in the vapor phase, it has many advantages as far as being able to use the entire volume of the freezer as opposed to just the bottom or just certain parts.

There's also a misunderstanding with many researchers that they can treat an ultra-low freezer almost like a home freezer. I'm going to buy 400 lbs. of steaks and throw it in and it'll eventually get cold. You can't do that with ultra-low freezers; they're storage freezers, not blast freezers.

Dr. Kelly: And there are space requirements with the tanks.

LaPorte: When you're doing repositories then OSHA gets involved. And then you have issues like, okay what happens if we tip a tank over or displace the oxygen in the room with LN_2 vapor? Mechanical refrigeration is still recommended and all of our units have LN_2 backups. So if anything happens, then it kicks in liquid nitrogen to control the temperature. Power failures do happen, and there's always the unexpected scenario.

Repair or replace?

Mistry: Dr. Kelly, you talked about service being a major factor for you. I think you know that the compressor is at the heart of the freezer. Some manufacturers make their own compressors, and some source them. This factor can be important on the long haul, on a service side, and also on a reliability side.

LaPorte: *One of the major failures of ultra-low freezers is the discharge temperature. It doesn't matter who makes the freezer, the compressors run a lot hotter than a typical compressor does in a home freezer.* And the choice of the refrigerants and the oils in the compressors has a big effect on this as well.

MDF-C2156VANC is ideally suited for ultra-low and cryogenic storage in laboratories, long-term preservation and storage of blood, specimens and components, and in testing of various types. Mechanical Freezer

Preservation (-150°C) provides users with numerous advantages: uniform cryogenic storage temperatures; no worries about sample contamination; no liquid supply problems; no danger of sudden liquid eruptions; and low operational costs.



One particular manufacturer decided they no longer wanted to make a compressor for the ultra-low industry. So what happened is the manufacturers of these freezers had to go to all the compressor manufacturers and say, "Okay, which one of these is going to be best for our application?"

But on the service end of it, the compressors are more expensive than the standard air conditioning compressor. And the service people are often in a quandary with what they offer their customers. Eventually any freezer is going to fail. Whether it's five or twenty years down the road, you have to make the decision, "Am I going to fix it or buy a new one?"

Dr. Kelly: Let me make sure that I understand how you work with the service end. This is a contract arrangement that Sanyo, lets say, has with a local service that delivers this as needed to people who buy your units?

LaPorte: Yes. We do direct service here in Chicago. In most of the rest of the nation and Canada we use third-party service personnel that are trained on our product.

Dr. Kelly: So you would say that's a norm for most manufacturers' freezers?

LaPorte: Not necessarily. Many manufacturers use their own direct people in the major centers. In smaller markets they might subcontract it. If they get overflow service, they'll subcontract to a service organization that's used to working on their product. That happens quite often because of the amount and volume of service.

Dr. Kelly: So you're only likely to encounter the situation where the contractor would offer somebody else's compressor in the smaller cities, rather than the major cities.

LaPorte: We run into it everywhere. And we make it very specific, at service training, that we know contract service organizations have a business to run, but don't presume to represent this as a direct replacement.

Dr. Kelly: I suspect that probably happens more often than you think, to many laboratories. And most people don't understand that that actually is occurring.

LaPorte: Companies wonder if they should get a part from the original manufacturer when a third-party replacement is cheaper and seems to work.

Dr. Kelly: So you do think that it does make a significant difference?

LaPorte: Yeah. That's often the case.

Dr. Kelly: So in other words, you're buying the box with the compressor, and the type of refrigerant that's used and the sensors and so on and if your compressor fails, and you buy a compressor from another manufacturer that's installed by your local service representative, you could very well be operating with a different product in a sense.

Mistry: Yes.

Mod: And so the solution is to, for the customer, to specify that they want OEM parts?

LaPorte: A major burnout repair on an ultra-low freezer can cost upwards of \$3,000.

Dr. Kelly: I can tell you that the low price they're quoting for -80°C freezers is about \$9,000. It's gone down from \$11,000.

LaPorte: So then you have to decide whether to repair it or buy a new one.

Mod: So service providers are paid for the actual service provided, not a flat fee?

LaPorte: Often, unless you have a service contract.

Mistry: I want to ask the researchers which is more important, footprint or volume?

Dr. Sainz: We chose uprights because we had no space. We would have preferred a chest.

Dr. Kelly: We could never go with chests. Just don't have the room.

Mistry: So I would say pretty much every inch that you can save for the volume capacity is a strong selling point.

Mod: And a chest freezer is preferred because temperature is more uniform?

Dr. Sainz: Yes, you basically keep the cold inside the unit. Every time a researcher opens a -86°C door, they know that they're losing temperature.

LaPorte: That's why an inventory system is great to have. People have a map — they know exactly where to go and exactly how to get it. I responded to calls when I worked at the service company

where customers would say, "This freezer is warming up on me all the time." And I'll be in the lab working on that freezer and further down the lab someone else has an ultra-low door wide open, and they're taking samples to their desk and sorting through them and working on the bench and they go on to the computer for a minute and meanwhile the freezer door has been sitting open and everyone ignores the alarm. Lab practices for ultra-low freezers are just as important, maybe more important, than they are for contamination control.

Dr. Kelly: I think that for a biorepository, the major issue is providing secure, long-term storage of large numbers of samples in freezers that require a limited amount of maintenance. I think excellent freezer temperature control systems and high-quality and easily available on-site freezer maintenance and repair services are the factors that concern me the most.

Dr. Sainz: I think with contamination, the equipment is important but environment and practices are probably just as important. As for the products we use, researchers should educate themselves, because these decisions can have long-term effects on the research.

LaPorte: Depending on a researcher's environment and technique, there is only so much you can do with respect to contamination control. If you get too aggressive, you're affecting the cell lines. If you're having contamination problems, look up. If you see vents too close to the incubators then there's a good chance you're going to have a problem and may wish to consider viable particle counting.

Mod: How much time is involved in a viable particle count?

LaPorte: At larger institutions, biosafety officers will do it. We offer those services as well.

I cannot stress enough, for cryopreservation, to clean filters, and keep ice off the door gaskets. It's boring, but it's a lot better than having a freezer warm up and losing your samples. Cleaning filters can reduce the amount of energy a freezer uses by 10 to 25 percent.

If you decide to have a freezer serviced as opposed to purchasing a new one, take a good look at what you're being offered. Make sure you know what you're getting with a service provider.

Mod: It's been a great discussion. Thank you all for participating.

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