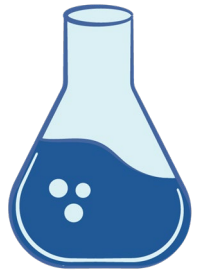
ULT Set Point Effects on RNA Quality: A Comparative Analysis

presented by Christine Alencar MS, LEED Green Associate

Sustainable Labs
UVA Sustainability

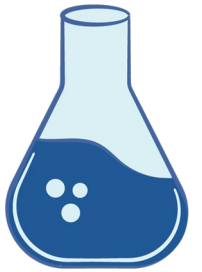
Learning Objectives

1. Learn about the research study performed to answer the question of **whether -70°C is equal to -80°C** in its ability to preserve highly sensitive biological samples for up to one year under standard lab conditions;
2. **Understand a researcher's hesitation** in changing any lab operation that has worked for them in the past;
3. Explore creative strategies for **sustainability professionals to partner with researchers** to develop high-level research projects to answer the call for more peer-reviewed evidence supporting sustainable lab practices; and
4. Examine how cold storage demands fit into **big-picture lab sustainability** challenges as well as solutions.



Roadmap

1. Sustainability at UVA
2. Cold Storage: a brief history
3. Inspiration for the study
4. Methods
5. Results
6. Conclusions & Lessons Learned



Sustainability at the University of Virginia

University of Virginia

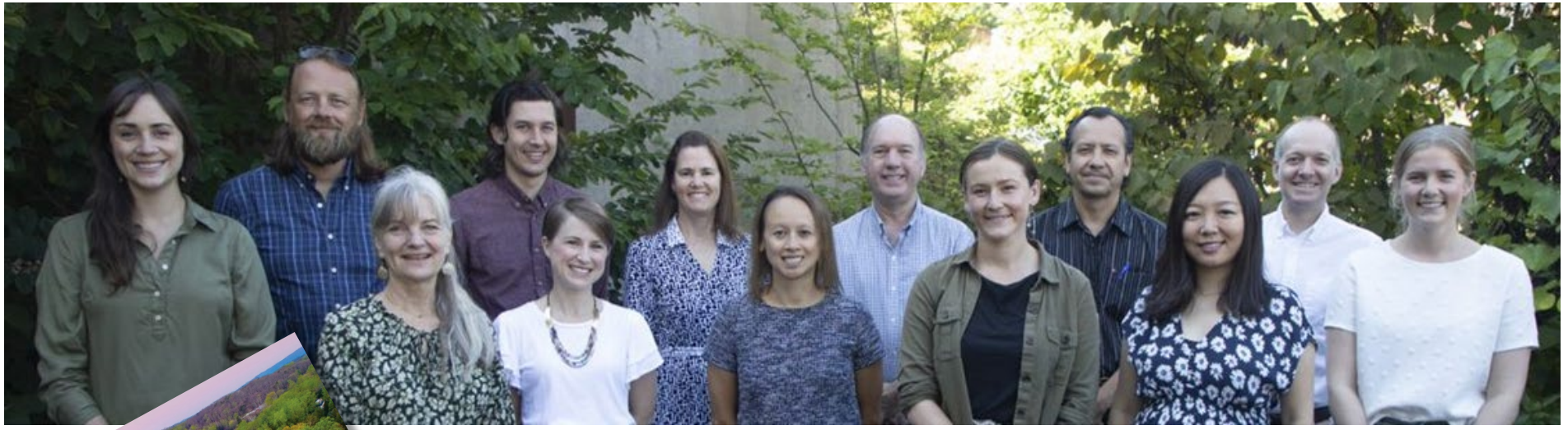
- 500 buildings
- 18M square feet
- R-1 research University
- Health System, L1 Trauma Center
- UNESCO World Heritage Site

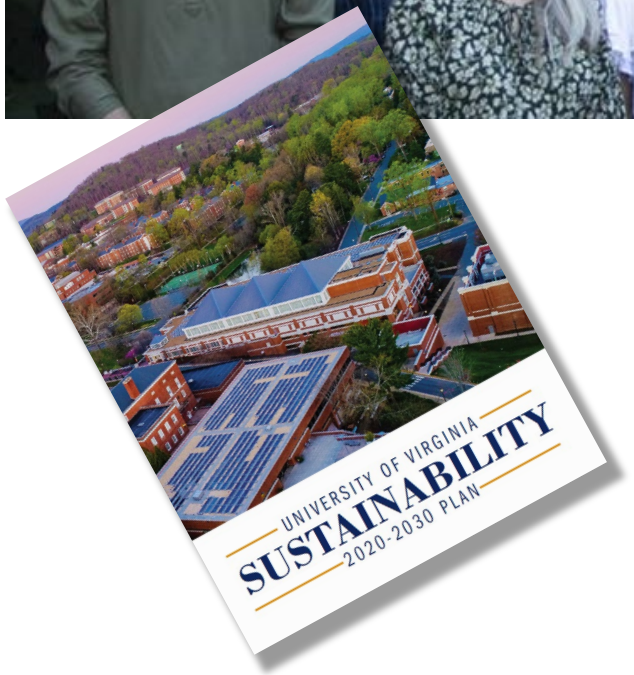
LEED required for all capital projects

74 LEED certified projects representing over 4 million square feet + ~15 projects in progress

6 USGBC Virginia Leadership Awards in the past 2 years

Sustainability at the University of Virginia





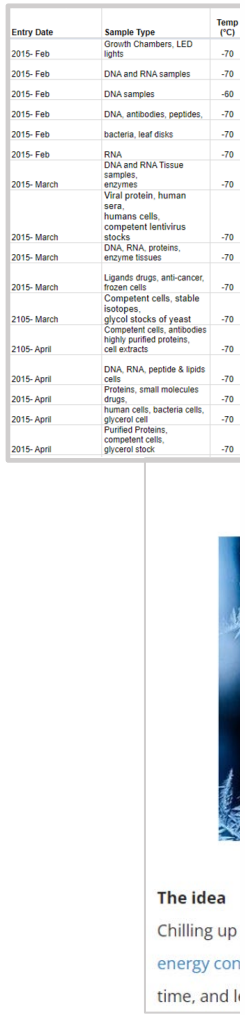
Meet the Office for Sustainability

- Manages UVA's energy portfolio, among other initiatives
- Recently published UVA's 2020-2030 Sustainability Strategic Plan, including goals such as being **carbon neutral by 2030** and **fossil fuel free by 2050**
- Lab sustainability **essential** for achieving goals

Lab Cold Storage

A brief history…

- Ultra low freezers are a top contributor to lab energy use (plug loads, BTUs)
- -70°C once the standard
- 1980's, new refrigerants capable of reaching -80°C
- Green Labs initiatives worked to re-introduce -70°C
- Units saw 30 – 40 % energy reduction for old and new alike
- My Green Lab, CU Boulder, many more encouraged labs to "warm up", and some did!
- UVA confirmed similar savings during 2018 Pilot Lab Program



Entry Date	Sample Type	Temp (°C)
2015- Feb	Growth Chambers, LED lights	-70
2015- Feb	DNA and RNA samples	-70
2015- Feb	DNA samples	-60
2015- Feb	DNA, antibodies, peptides,	-70
2015- Feb	bacteria, leaf disks	-70
2015- Feb	RNA	-70
2015- March	DNA and RNA Tissue samples, enzymes	-70
2015- March	Viral protein, human sera, humans cells, competent lentivirus stocks	-70
2015- March	DNA, RNA, proteins, enzyme tissues	-70
2015- March	Ligands drugs, anti-cancer, frozen cells	-70
2105- March	Competent cells, stable isotopes, glycol stocks of yeast	-70
2105- April	Competent cells, antibodies highly purified proteins, cell extracts	-70
2015- April	DNA, RNA, peptide & lipids cells	-70
2015- April	Proteins, small molecules drugs,	-70
2015- April	human cells, bacteria cells, glycerol cell	-70
2015- April	Purified Proteins, competent cells, glycerol stock	-70

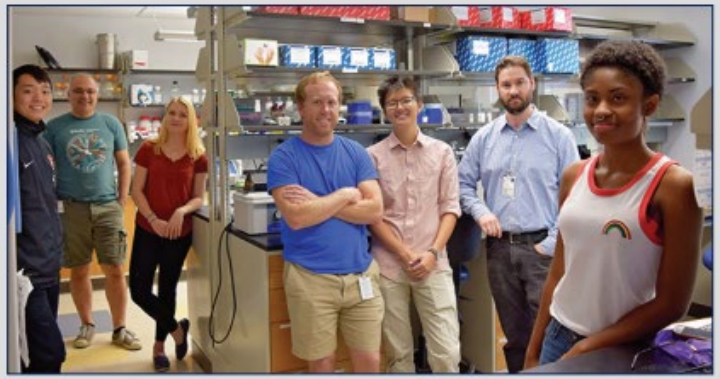
The idea

Chilling up

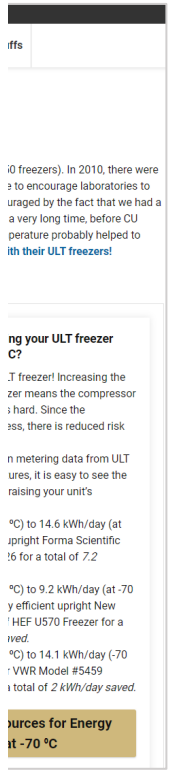
energy con

time, and l

BIG WINS FOR THE CIVELEK LAB: SWITCHING FROM -80 TO -70°C ON ULTRA-LOW TEMP FREEZERS



Despite occupying the oldest building out of all the Pilot Labs, Dr. Civelek's team had the most recently renovated space, smallest physical footprint, and lowest lab population. Odds were stacked against them in achieving significant savings through this program, but their willingness to adopt cutting edge sustainability practices helped them realize an overall lab energy reduction of 15.6%, the largest reduction of all three Pilot Labs. These savings were achieved after switching to -70°C ULT freezer setpoints from -80°C.



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total of *2 kWh/day saved.*

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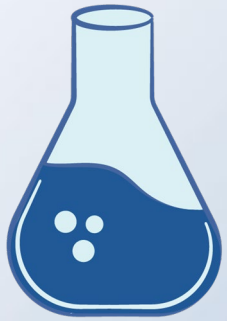
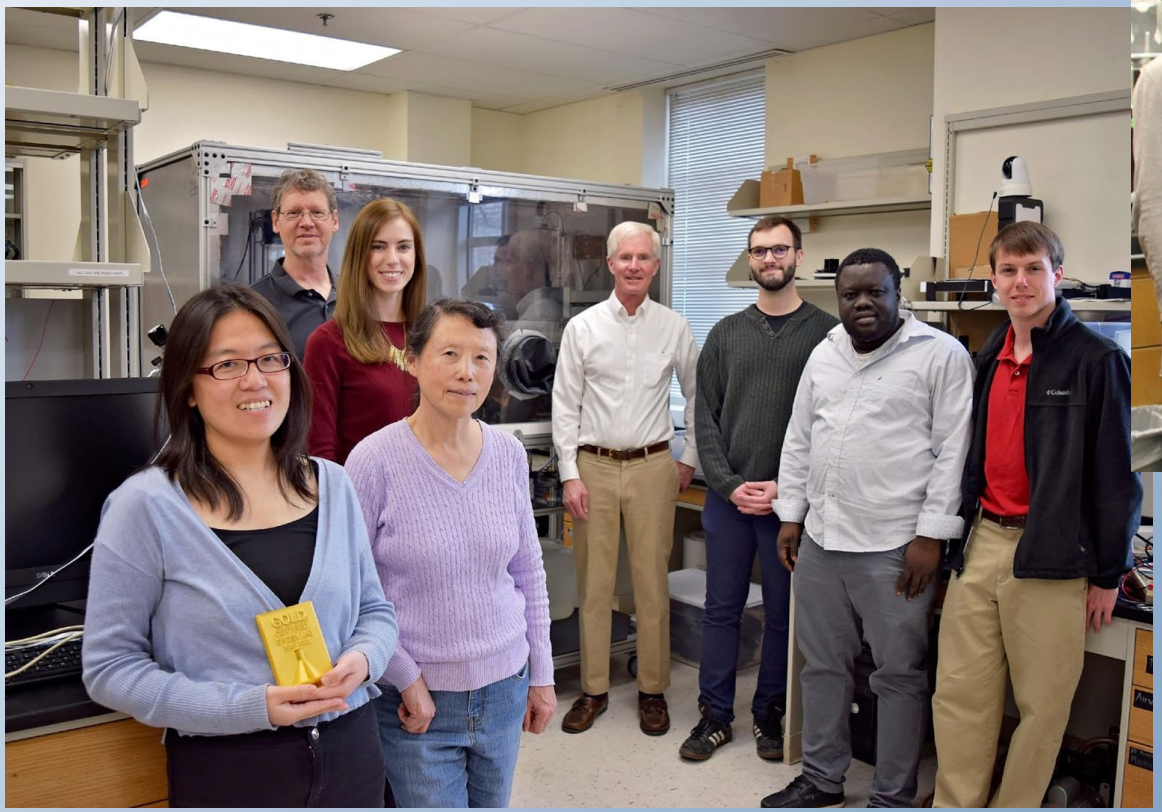


But many (or most) labs at UVA remained skeptical, citing lack of peer reviewed evidence.



Literature

Farkas, D.H., et al. 1996. Specimen collection and storage for diagnostic molecular pathology investigation. Archives of Pathology and Laboratory Medicine 120: 591-596. *Solid tissue can be stored at -70°C for at least two years for future RNA-based testing. Extracted RNA can be stored as a precipitate in ethanol at -70°C and is “stable indefinitely”. Tissue should be snap frozen in liquid nitrogen as soon as possible after excision and stored at -70°C. “The most common source of RNases is the skin,” and researchers should take great care to avoid even the slightest skin contact with any equipment, tubes, or materials associated with extracting RNA. However, these **points are not cited or corroborated in the paper but written as common knowledge.**

Faugeroux, D., 2016. Ultra-low temperature freezer performance and energy use tests. UC Riverside Office of Sustainability. *Most modern freezers take about nine hours to warm from -80°C to -50°C. At -70°C, a researcher may lose 1.5 to 3 hours of sample rescue time, still leaving **at least six hours for intervention in units set to -70°C.**

Molnar, A., et al. 2021. Lyophilization and homogenization of biological samples improves reproducibility and reduces standard deviation in molecular biology techniques. Amino Acids 53: 971-928. *”We demonstrated that **lyophilized samples stored at 4°C for 20 months can yield protein and RNA of similar quantity and quality to -80°C storage…”**

Paradise, A., et al. 2019. In search of energy efficiency opportunities for laboratory-grade freezers. Prepared by the Center for Energy Efficient Laboratories (CEEL) Emerging Technologies Program. *Studied **relationship between heat output from freezers and subsequent strain on HVAC equipment. Emphasis on developing more room temperature sample storage technologies**. ”On average, a reduction of 1kWh in freezer consumption results in an additional 0.11 kWh in HVAC electricity savings and a penalty of 0.023 therms.”

Riesgo, A., et al. 2012. Optimization of preservation and storage time of sponge tissues to obtain quality mRNA for next-generation sequencing. Molecular Ecology Resources 12: 312-322. *Use of RNAlater® or flash freezing in liquid nitrogen are **key steps in the RNA preservation process.**

Sandusky, G.E., et al. 2009. Lessons learned in human tissue banking for acquiring high quality biospecimens for translational genomic research: A perspective of the I U Simon Cancer Center Tissue / Fluid BioBank. Human Genome: Features, Variations, and Genetic Disorders, 277-293. ***No loss of RNA yield or quality for samples stored less than two years at -80°C. Some degradation beyond two years.**

Wan, E., et al. 2010. Green technologies for room temperature nucleic acid storage. Current Issues in Molecular Biology 12:135-142. ***RNAstable®, a room temperature storage solution for RNA, maintained sample integrity for 11 days. Study was limited and unable to continue testing beyond 3-4 weeks. Interestingly, RNAstable® has been discontinued by MilliporeSigma.**

Wu, J., et al. 2011. Stability of extracted RNA at various storage temperatures and through multiple freeze-thaw cycles. International Society for Biological and Environmental Repositories, Annual Meeting, poster # BSS06. ***Extracted RNA stable for up to ten freeze-thaw cycles.**

Literature, *summarized…*

- To our knowledge, no longitudinal study using bioinfomatic analysis to compare RNA sample quality after storage at different temperature settings
- Evidence that RNA can withstand up to 10 freeze-thaw cycles without significant degradation
- RNA sensitive to contamination (especially via RNAse enzymes). How we collect samples may be more important than how we store them
- Scientists hesitate to switch to -70°C, wanting more time to rescue samples in emergency situations, but -80°C provides only 1.5 to 3 extra hours. With samples able to withstand several freeze-thaw cycles (above), some see value in reducing load on ULTs by switching to -70°C
- Cutting edge techniques can help science circumvent the need for ultra cold storage (but these techniques are not yet widely used, accepted, or made available

What would bioinformatic analysis reveal about RNA sample storage at -70°C and -80°C?

- RNA widely used
- Known to be sensitive to collection, processing, and storage
- Submitted proposal to UVA Environmental Stewardship Subcommittee
- Awarded $15k for comparative study utilizing bioinformatic analysis of RNA stored for 1 year at -70°C and -80°C
- Crafted MOU with Eppendorf® and UVA Procurement to obtain two identical ULTs on loan (740h CryoCube®)
- UVA Sustainable Labs team connected with campus researchers and technical staff to coordinate and assign project tasks

UNIVERSITY of VIRGINIA

SUSTAINABILITY · UVA
From the Ground Up

Freezer Temperature Effects on RNA Quality
Memorandum of Understanding

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January 7, 2021

Project Description

The average ultra-low temperature (ULT) lab freezer set to -80°C (current standard practice) consumes as much energy as a single American household. However, we know that a ten-degree increase can reduce individual unit consumption up to 40% and strong anecdotal evidence in both the Green Labs and Scientific communities suggests that most lab materials can be safely stored at -70°C. With nearly 500 ULTs accounted for on our campus, shifting the scientific community's stance on cold storage standards towards -70°C has the potential to save approximately 2,755 kWh/day.

The RNA-seq study will provide robust, peer reviewed evidence to show that sensitive biological samples can be stored safely at -70°C degrees, allowing us to reduce energy consumption and extend the life of ULT units, a significant contribution to UVA's 2030-2050 Sustainability Goals. Additionally, monitoring energy consumption via Hampshire devices will allow for a robust energy-use analysis of the two ULTs used throughout the study. Results will be published in a peer reviewed science journal and presented at the International Institute for Sustainable Laboratories annual conference in 2022 or 2023, therefore providing evidence to scientists around the world that this is an effective approach to cold storage. The Green Labs Working Group has secured project funding from UVA's Committee on Sustainability to cover the costs of the following:

1. Sample preparation and RNA extraction
2. Library preparation and quality control
3. Sequencing services by selected vendor
4. Computer hours for bioinformatic analysis
5. Installation of Hampshire Devices and subsequent freezer monitoring by UVA Facilities Management for 2 ULT units used in study

The Smart Labs Project Associate will provide project management, oversight, and review. Gabriel Alencar PhD will be performing lab operations as well as computational analysis of results. Kristin Floyd is providing guidance on behalf of UVA Procurement. Green Labs Intern and Working Group co-chair, Pumoli Malapati, will perform study write-up, facilitate stakeholder communications, and provide periodic updates to the Green Labs Working Group. Eppendorf has agreed to provide, at no cost to the University, delivery of two ultra-low temperature lab freezers (two 740h or two 740hi models) to the School of Medicine Campus. One year following delivery, both units will be available to UVA research labs for purchase at a discount of at least 50% off the current list price of $18,200 (740h). Freight costs for both units shipping to and, if returning at the end of the study, from the University back to Eppendorf will be the financial responsibility of Eppendorf. As a sponsor, Eppendorf will receive acknowledgement in all publications resulting from this study, including but not limited to peer reviewed journal articles, blog article writeups, and a feature on the UVA Green Labs web page.

Assembled Our Team…

Gabriel Alencar PhD,
Class of 2019

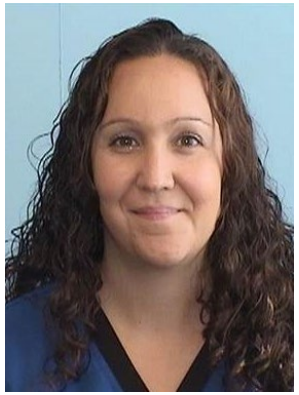
Dakota Delong-Maxey,
Class of 2021

Pumoli Malapati,
Class of 2021

Olivia Regehr,
Class of 2023

Sohel Shamsuzzaman PhD,
UVA Cardiovascular
Research Center

UVA Genome Analysis
& Technology Core,
Katia Sol-Church PhD

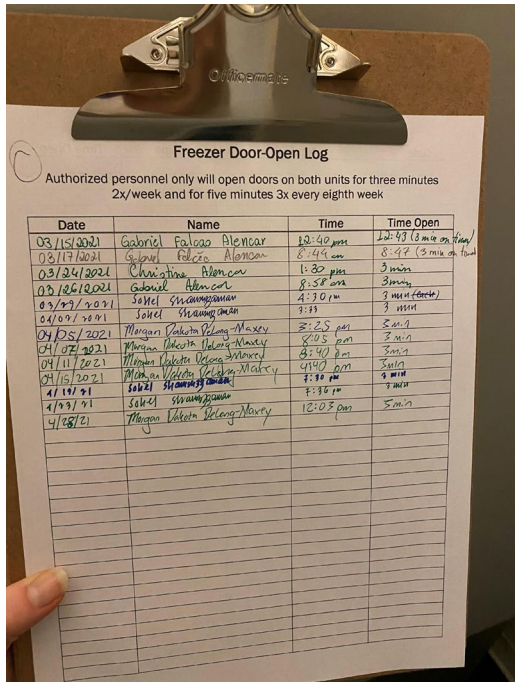


Jennifer Williams,
UVA Health Pathologist

Methods

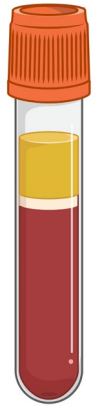
Methods

- Owens lab (UVA Cardiovascular Research Center) provided remnant animals based on existing animal research protocols approved by Institutional Animal Care and Use Committee
- UVA Sustainable Labs allocated Student Employee hours to literature research, scheduled door openings, and preventative maintenance on both units to mimic standard lab practices.
- Designed three experiments…



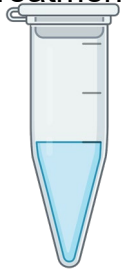
Methods, Experiment 1

n = 3

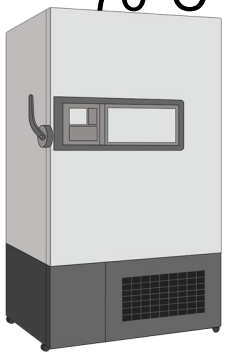


Trizol RNA isolation

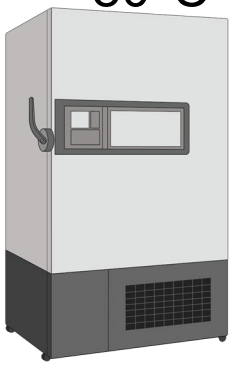
Samples divided for different temperature treatments



*-70°C

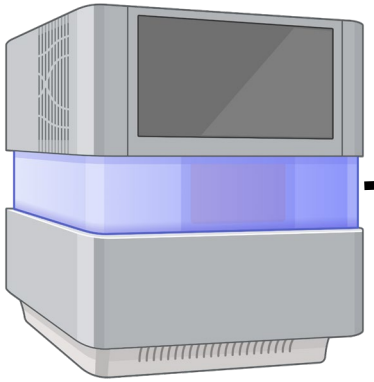


*-80°C



*Stored for 1 year with identical door-opening schedule

Illumina NextSeq 2000, UVA Genome Analysis & Technology Core Facility

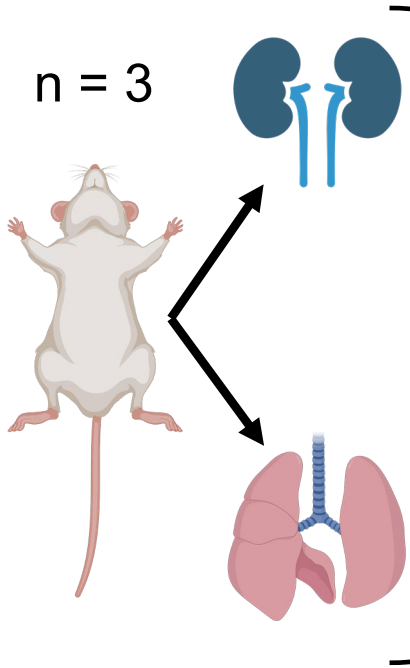


Performed RNA concentration and quality control followed by library prep for sequencing

Bioinformatic Analysis, Gabe Alencar PhD

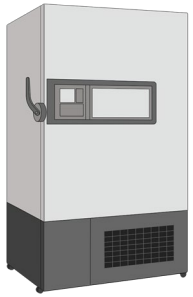
Methods, Experiment 2

n = 3

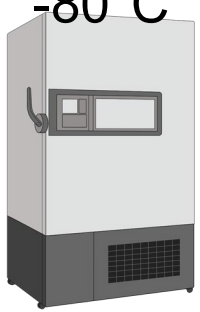


Samples flash frozen in LN

*-70°C



*-80°C



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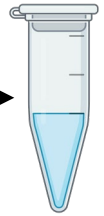
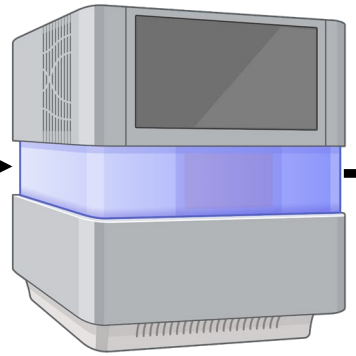


RNA extracted (Qiagen RNA kit), UVA Biorepository & Tissue Research Facility

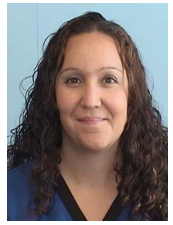


Illumina NextSeq 2000, UVA Genome Analysis & Technology Core Facility

Bioinformatic Analysis, Gabe Alencar PhD

Methods, Experiment 3

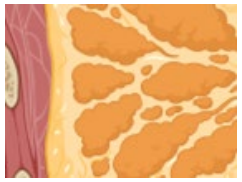
Jennifer Williams,
UVA Health Pathologist

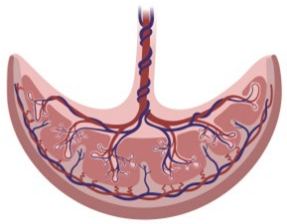


*Stored for 1 year with identical door-opening schedule

Sohel Shamsuzzaman
PhD, UVA
Cardiovascular Research Center

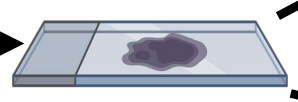
n = 2





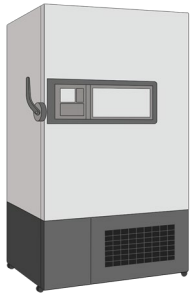
n = 2

Sample preparation,
Cryostat

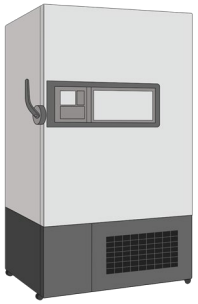


n = 15 / sample / temperature

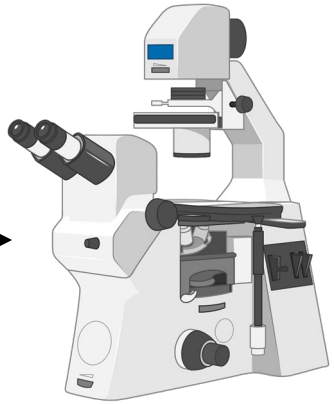
*-70°C



*-80°C



H&E staining



Experiment 1

Tissue

↓

Extracted RNA

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Samples divided evenly between -70°C and -80°C freezers stored under standard lab conditions for one year

↓

Samples removed

↓

Core performed RNA concentration and quality control followed by library prep for sequencing

↓

Bioinformatic Analysis

Experiment 2

Tissue

↓

Samples divided evenly between -70°C and -80°C freezers stored under standard lab conditions for one year

↓

Samples removed

↓

Biorepository & Tissue Research Facility Extracted RNA

↓

Core performed RNA concentration and quality control followed by library prep for sequencing

↓

Bioinformatic Analysis

Experiment 3

Tissue

↓

Prepared slides

↓

Slides divided evenly between -70°C and -80°C freezers stored under standard lab conditions for one year

↓

Samples removed, stained with H&E

↓

Captured cell images

↓

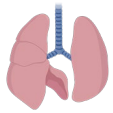
Analyzed cell images for differences using ImageJ / Fiji image analysis software

Results

Results Exp. 1 & 2: RNA Integrity

Table 1. Quality control of samples by **R**NA **I**ntegrity **N**umbers and millions of reads (in parentheses)



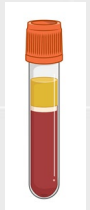


	Exp. 1, Whole Blood		Exp. 2, Kidney		Exp. 2, Lung	
	-70°C	-80°C	-70°C	-80°C	-70°C	-80°C
Mouse 1	1.5 (9M)	1.5 (8M)	5.9 (27M)	~~7 (rm)~~	5.6 (27M)	2.3 (27M)
Mouse 2	1.6 (9M)	1.6 (8M)	6.8 (21M)	7.1 (31M)	5.4 (20M)	5.8 (26M)
Mouse 3	1.7 (7M)	1.6 (7M)	5.8 (23M)	7.3 (34M)	~~5.6 (rm)~~	6 (25M)

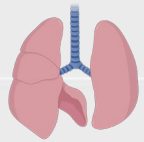
- Analyzed whole genome for kidney and lung, only analyzed most expressed genes for blood due to poor RIN numbers
- RIN is a quality control measure, helps researchers decide whether a sample is worthy of sequencing

Results, Exp. 1 & 2: Principal Component Analysis

Results: Experiment 1



Results: Experiment 2



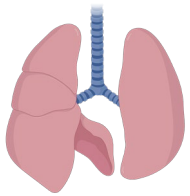
- Each datapoint represents thousands of genes
- Tissue type is greatest source of variance
- -70°C and -80°C samples show strong similarities
- This analysis does not identify differences!

Results: Experiment 2

Results, Exp. 1 & 2: Genes showing differences between -70°C and -80°C

Table 2. Differential Expression Analysis, Experiment 2

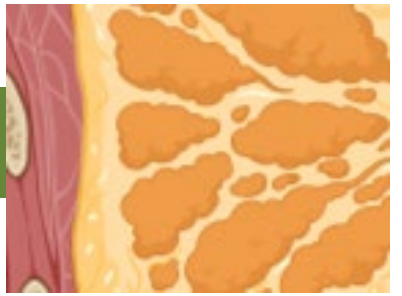
Significant altered genes in kidney stored at -70°C and -80°C		
Gene Name	**Fold Change (log2)**	**Adjusted p value**
Pcyt2	0.47	0.046
Hexb	-0.27	0.024
Rn45s	0.42	0.040
Hao2	-0.37	0.040
Lars2	0.49	0.024
Significant altered genes in lung stored at -70°C and -80°C		
Gene Name	**Fold Change (log2)**	**Adjusted p value**
Cryaa	-21.49	3.86E-04
Ooep	-21.45	3.86E-04

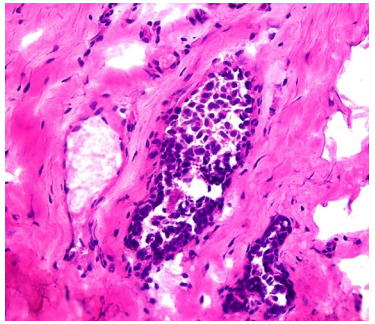
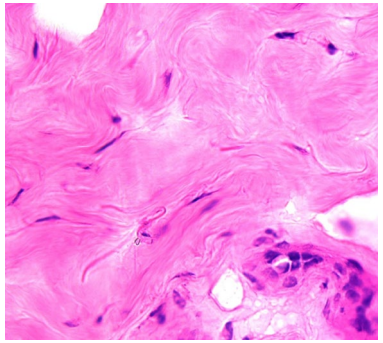
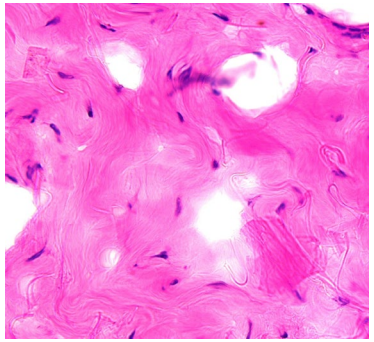
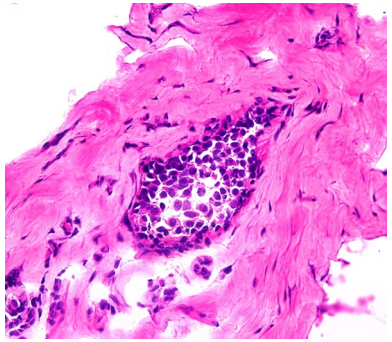
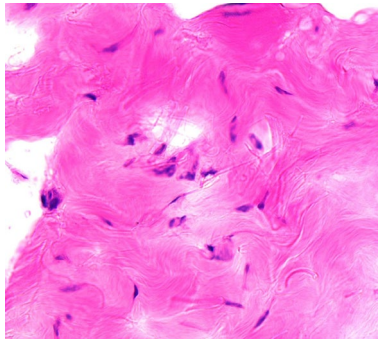
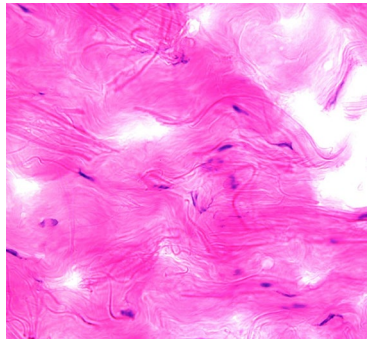


Low level significance: Regionality, ubiquity throughout organism, induced expression. Differences unlikely due to storage temperature.

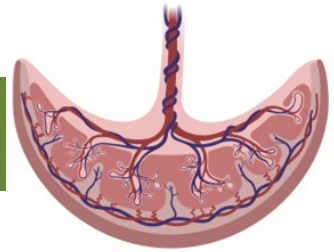
High fold change + low p-value; More expression in -80°C, what does this mean? Probably nothing. Very few reads on one sample (out of five) from -80°C cohort. One read per gene for a single sample.

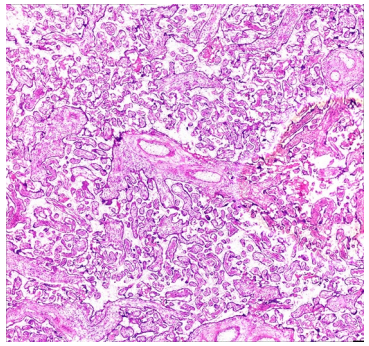
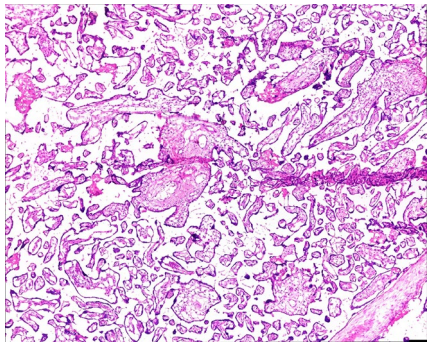
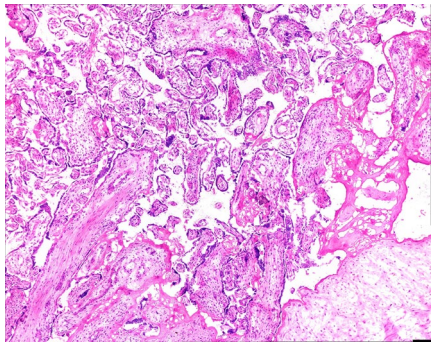
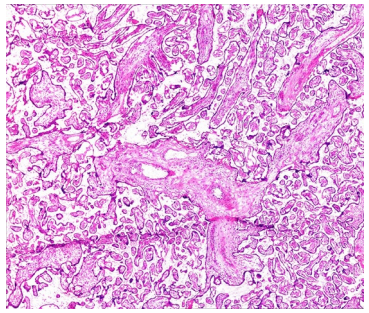
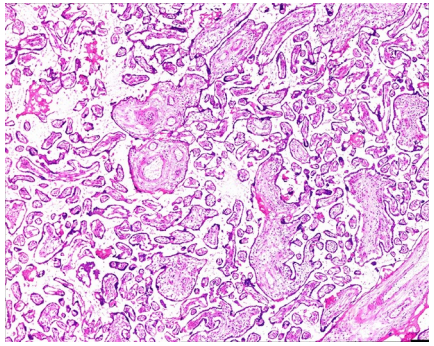
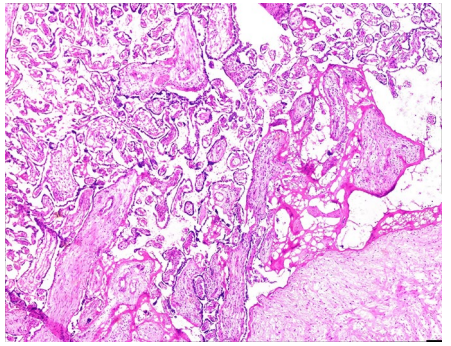
Results, Exp. 3: ImageJ, Fiji analysis of breast tissue



	B1, Slide 1	B2, Slide 5	B2, Slide 15
-80°C			
-70°C			

Results, Exp. 3: ImageJ, Fiji analysis of placenta



	P1, Slide 1	P1, Slide 5	P2, Slide 5
-80°C			
-70°C			

Conclusions & Lessons Learned

Conclusions & Lessons Learned

1. **No significant bioinformatic (RNA) or histological (slides) differences** in RNA or tissue sample quality when stored at -70°C or -80°C for up to one year.
2. **How you gather and process** RNA (and tissue) samples is likely more important than what temperature the sample is stored at. Proper collection and processing prevent degradation.
3. Resources to tap in to:
    - **Core facilities**
    - Sample **repositories** (like the UVA Biorepository and Tissue Research Facility)
    - Journals like **Biopreservation and Biobanking**, and **Archives of Pathology and Laboratory Medicine**
    - Organizations like the **International Institute for Biological and Environmental Repositories**

What's Next?

1. Write and submit **paper for peer review**
2. Explore, research, and demand **more options** for warmer / ambient storage technologies (like RNAstable®)
3. Work with vendors to change **warranty policies**
4. **Engage campus researchers**:
    - How valuable is the extra 1.5 - 3 hours intervention time? Does your unit have monitoring? Will a single thaw make a difference?
    - Emphasize cutting edge techniques like room temperature sample storage, lyophilization, and use of repositories and core facilities to strengthen research
        - Lower costs & less space use
        - Better outcomes & more reliable results
        - Increased accessibility & equity in research

Questions?

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Sustainable Labs
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