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Evaluation of Differential Evolution Algorithms for Optimization of Jurkat Cell Cryopreservation Using Multicomponent Osmolyte Solutions

Abstract:
The current industry standard for cell preservation involves the use of dimethyl sulfoxide (DMSO) as a cryoprotective agent, despite the biotoxicity that results after long periods of exposure. Multicomponent osmolyte solutions are a promising alternative but require time intensive experiments for optimization. This study examined the ability of a differential evolution (DE) algorithm to optimize the preservation of Jurkat cells, an immortalized T lymphocyte line, in a three component osmolyte solution. The algorithm compared the post-thaw recovery of the cells suspended in solutions of various concentrations of trehalose, glycerol, and isoleucine (TGI) to the 10% DMSO control. The algorithm identified nine formulations of osmolyte solutions for each generation which were compared to the best solutions from the previous generation. The plates were frozen with a cooling rate of -1 degree Celsius per minute and fluorescence readings were taken to determine cell viability post-thaw. The objective of the algorithm was to mutate the previously successful formulations to guess new formulations that will have greater success. The improved formulations were created based on comparing the post-thaw viability of previous generations and the new mutant formulations. The new generation created as a result of these comparisons ideally would have a higher average viability than the previous. After various iterations were performed, the algorithm would converge on one final solution that was the most optimal for Jurkat cryopreservation. Two variations of the algorithm were used to compare speed of convergence and optimal conditions. The experimental procedure resulted in the mutual convergence on an optimal combination of $0\,\mu L$ trehalose solution, $60\,\mu L$ glycerol solution, $80\,\mu L$ isoleucine solution, and $160\,\mu L$ Normosol-R with the sorting algorithm converging a generation earlier than the non-sorting counterpart. However, the results of this experiment are subject to fault due to the high viability of Generation 0 and inconsistent control variables. Further extensions of this experiment should be carried out to prove the validity of the results and improve upon the methodologies used. This study aids in validating the use of a DE algorithm to improve modern methods of optimizing the preservation of cell types in non-DMSO solutions and should be applied to various cell types and osmolyte combinations in the future.

Introduction:

Regenerative medicine is undergoing many developmental and implementation phases in hopes to expand conventional medicine to successfully address unsolved medical disorders. However, the possibilities of biomanufacturing and personalized medicine are subject to the limitations of existing methods of preservation for these systems [1]. Current conventional use of DMSO as a cryoprotective agent for these cells results in poor outcomes for cell survival, making it unsuitable to reliably preserve aggregates and tissues [2-6]. When DMSO treated cells are infused in patients, adverse side effects can result ranging from nausea and vomiting,

cardiovascular effects, or even death [7]. In response to these challenges, this experiment aims to broaden the applications of multicomponent osmolyte solutions to the preservation of cells and tissues. Research has identified that extending the use of multicomponent osmolyte solutions to preserve cell types is a promising alternative. Improving this process would result in more reliable preservation of this important cell type and alleviate further obstacles preventing the widespread adoption and implementation of biomanufacturing technologies. The difficulty in implementing the use of these solutions is identifying the optimal concentrations of each component that will result in the best viability post-thaw. In order to reduce the time intensive labor of determining these optimal conditions, a differential evolution algorithm, similar to the algorithm developed by Storn and Price [8], can be utilized to dramatically reduce the number of experiments in which it takes to determine an ideal three component osmolyte solution. Although not technically necessary, the use of an algorithm or alternative optimization aid is critical in the timely assessment of an experiment with numerous variables that require assessment. Analyzing all combinations of a three component solution as well as studying the effects of cooling rate, incubation, nucleation temperature, or additional osmolyte components, exponentially increases the number of required experiments. The benefits of such an algorithm are quickly apparent.

Research Questions:

1. Will the sorting and non-sorting versions of the differential sorting algorithm converge on the same solution?
2. Will both versions converge at the same iteration?
3. How will viability post-thaw of the optimal solution compare to the 10% DMSO control?

Materials and Methods:

Cell culture:

Jurkat cells (ATCC TIB-152), a human T-lymphocyte line, whose identity was confirmed by Short Tandem Repeat (STR) profiling was used in this investigation. The cells were cultured in high-glucose RPMI 1640 (Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; Qualified, Life Technologies). Cultures were kept at densities ranging from 1×10^5 to 3×10^6 cells/mL.

Control Curves:

Control curves for Jurkat cells suspended in Normosol-R and 10% DMSO were created to calculate the absolute viability of cells post-thaw. For the Normosol-R control curve, cells were resuspended in Normosol-R to achieve a concentration of 12 million/mL. Six replicates of $50\ \mu L$ were seeded and the solution was diluted to half and the new concentration was seeded. This process was continued until 10 different concentrations were seeded. For the 10% DMSO control curve, cells were resuspended in Normosol-R to achieve a concentration of 8 million/mL. In each testing well, $25\ \mu L$ of 20% DMSO was added. Six replicates of $25\ \mu L$ were seeded and the solution was diluted to half and the new concentration was seeded. This process was continued until 10 different concentrations were complete. For both control curves, after 30 minutes of incubation time with Calcein-AM/PI the fluorescence data was taken.

Solution Formulation:
Solutions were made at twice their final concentration in Normosol-R. Solution formulations were determined by the DE algorithm and consisted of various formulations of trehalose, glycerol, and isoleucine. The highest possible solution concentration was selected to be ⅛ of the solubility limit for each particular solute. The parameter space was divided into equal increments and assigned a level between 0 and 5. Level 0 represented a zero volume of the solution in question, where level 5 represented the maximum possible volume. Each level increment corresponded to $20\mu L$ of the solution, making $100\mu L$ the volume associated with level 5.

Cells were centrifuged at 1000 RPM for 10 minutes and resuspended in Normosol-R. The cells were resuspended to achieve 6 million cells/mL. The cell suspension and 2x solutions were combined 1:1 for a total volume of $50\mu L$ in each well. Clear-bottom black 96 well plates (Corning, NY, USA) were used. The final cell concentration of each well was 300,000 cells/well.

Freezing experiments:
Cells were frozen in 96 well plates with 6 replicates for each solution formulation. Nine formulations were tested simultaneously and compared to a 10% DMSO control. Cells were incubated for an hour at room temperature before being sealed with silicon well covers. The covers were used to prevent desiccation throughout the experimental process.

Samples were cryopreserved using a control rate freezer (Series III Kryo 10; Planer, Middlesex, USA). A plastic rack was used to hold samples during freezing. The freezing profile was as follows: (1) start at 20°C, (2) -10°C/min to 0°C, (3) hold at 0°C for 15 minutes, (4) -1°C/min to -8°C, (5) -50°C/min to -45°C, (6) +15°C/min to -12°C, (7) -1°C/min to -60°C, and (8) -10°C/min to -100°C. The freezing profile contains periods of rapid cooling and rewarming in order to help nucleate ice in the solution external to the cell.

After completion of the freezing profile, the samples were transferred to a liquid nitrogen freezer where they were stored for at least 24 hours until thaw.

Thawing and post-thaw assessment:
Cells were thawed in a 37°C water bath (ThermoFisher Scientific, Waltham, MA) for no longer than 3 minutes.

Viability:
Cell viability was determined with calcein acetoxymethyl (Calcein-AM, Life Technologies) and propidium iodine (PI, Life Technologies). Calcein-AM/PI dye was added to each well at a 1:1 ratio to the solution volume, for a total of $50\mu L$ introduced to each well. The 96 well plates were covered with tin foil to avoid light exposure. The plates were incubated for 30 minutes at 37°C and CO_2 at 5% vol%. A Synergy HT microplate reader (Biotek, Winooski, VT) was used to

measure the fluorescence of each plate at 530/590 nm and 485/528 nm. Viability was determined using existing control curves with known numbers of live and dead cells in each well which allowed the absolute viability to be determined using the line of best fit. Fluorescence readings for the experimental plates were correlated to the corresponding viability. The post-thaw recovery of each formulation was the ratio of the number of live cells post-thaw to the number of seeded live cells.

Differential Evolution Algorithm:

The optimization aspect of this experiment was computed using a differential sorting algorithm that had been previously developed by Chia-Hsing Pi, a graduate student in the Biopreservation Core Resource Lab, under the guidance of Professor Allison Hubel derived from the findings of Storn and Price [8]. Two versions of this computer algorithm were tested. One version was a sorting algorithm, which would sort the viability results of the previous generation high to low and mutant results low to high and compare the results and select the best recovery. The other non-sorting version would choose the best overall viability and create the next formulations using these values. The differences between these methods are depicted in Figure 1 with artificial results. These experiments were repeated until the convergence upon one ideal formulation, which would be the optimal cryoprotective agent for the given cell type.

Sorting

G0	M1	G1
0.37	0.88	0.88
0.45	0.85	0.85
0.52	0.61	0.61
0.76	0.23	0.76
0.97	0.21	0.97

G1 Average Viability: 0.814

Non-Sorting

G0	M1	G1
0.45	0.23	0.45
0.37	0.85	0.85
0.52	0.61	0.61
0.97	0.88	0.97
0.76	0.21	0.76

G1 Average Viability: 0.728

Figure 1. Sorting vs Non-Sorting Algorithm Methods

Results

The control curve consisted of 10 groups of varying concentrations with 6 replicates each. The fluorescence readings for each of the groups were consistent relative standard deviations between 4 and 19.5% for both the Normosol-R and 10% DMSO control curves, the results of which are shown in Figures 2 and 3 respectively.

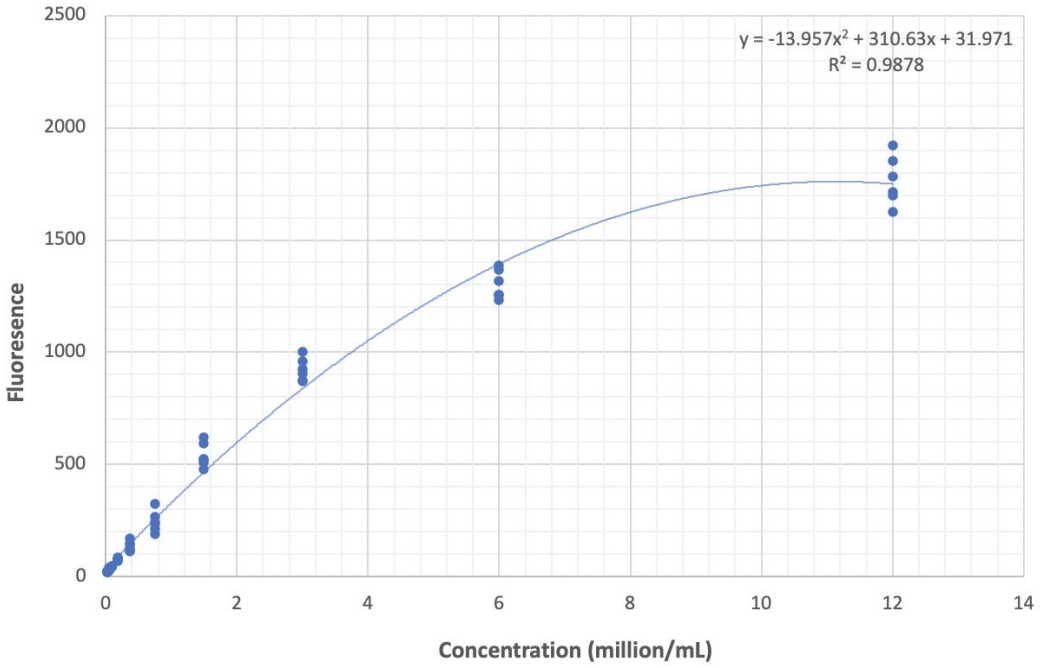

Figure 2. Jurkat Cell Control Curve in Normosol-R

The three highest relative standard deviations were present for a concentration of 0.375 million/mL with a relative standard deviation of 19.08%, a concentration of 0.1875 million/mL with a relative standard deviation of 16.34%, and a concentration of 0.046875 million/mL with a relative standard deviation of 14.09%. All other experimental groups demonstrated relative standard deviations below 10%.

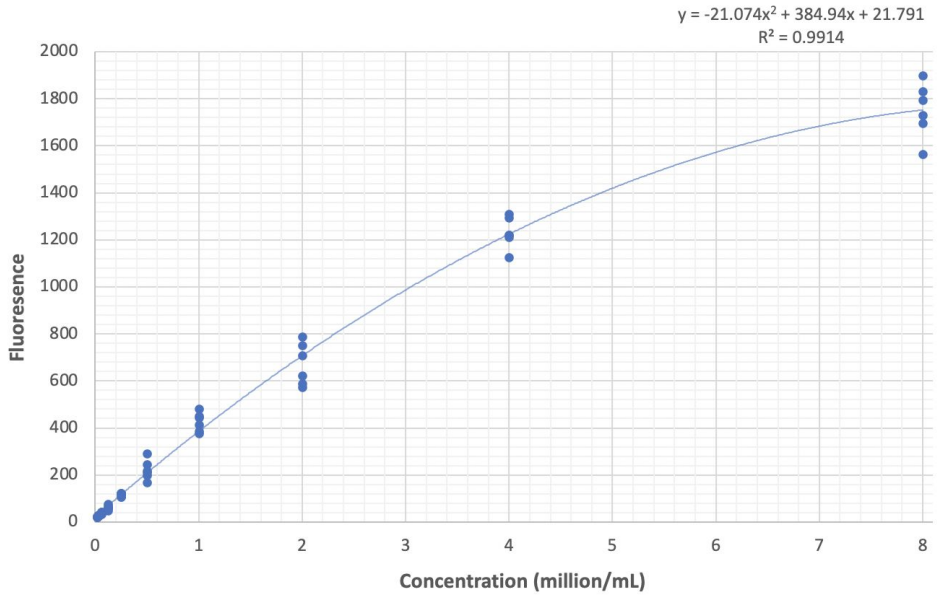

Figure 3. Jurkat Cell Control Curve in 10% DMSO

The three highest relative standard deviations were present for a concentration of 0.5 million/mL with a relative standard deviation of 18.64%, a concentration of 0.125 million/mL with a relative standard deviation of 15.03%, and a concentration of 1 million/mL with a relative standard deviation of 13.24%. All other experimental groups demonstrated relative standard deviations below 10%.

Viability for the sorted algorithm results are shown in Table 1. The bolded viability values correspond to the highest post-thaw recovery demonstrated between the current mutant and the previous generation. Collectively these bolded values provide the viability for the next generation.

Table 1. Results for Sort Algorithm by Trial Group

	G0 (Sorted)	M1 (Sorted)	G1	G1 (Sorted)	M2 (Sorted)
1	0.191	**0.608**	0.608	**0.535**	0.233
2	0.419	**0.551**	0.551	**0.551**	0.239
3	**0.535**	0.467	0.535	**0.586**	0.324
4	**0.586**	0.419	0.586	**0.608**	0.423
5	**0.614**	0.416	0.614	**0.614**	0.453
6	**0.673**	0.389	0.673	**0.673**	0.453
7	**0.712**	0.373	0.712	**0.712**	0.496
8	**1.066**	0.329	1.066	**1.066**	0.496
9	**1.449**	0.300	1.449	**1.449**	0.526
Control	1.320	0.638			0.571

From the results presented in Table 1 it is apparent that Generation 0 (G0) had a high viability. All but two formulations resulted in the highest viability post-thaw and carried on to Generation 1 (G1). These results provided Generation 0 with an overall unusually high viability. Which was not expected at this phase of the experiment.

When the next mutant generation, Mutant 2 (M2), was compared to Generation 1's recovery, the viability was lower so the differential evolution algorithm declared that the solution had converged. This resulted in the optimal combination of $0\ \mu L$ trehalose solution (level 0), $60\ \mu L$ glycerol solution (level 3), $80\ \mu L$ isoleucine solution (level 4), and $160\ \mu L$ Normosol-R with convergence that occurred at Generation 1. This convergence occurred too quickly to be considered reliable and is the fault of Generation 0 having such a high recovery. The optimal solution cannot be considered final due to this premature convergence and unlikely recovery of the first generation.

Figure 4. Jurkat Sorting Optimization in TGI Solution

Another indication that this result is invalid is due to the recovery rates above 100%, which can be easily seen in Figure 4. This experiment was performed using control curves and viability is expressed in absolute terms. For the results to have a recovery of over 100% means that cells would have had to split and grow while the cells were frozen in the solution in the nitrogen freezer. This degree of growth and proliferation while frozen is unexpected and unlikely to occur.

The viability of the non sorting algorithm trials are provided in Table 2 with the same format as presented in Table 1. However, this data lacks the sorting component that was previously used due to the nature of the algorithm.

Table 2. Results for Non Sort Algorithm by Trial Group

	G0	M1	G1	M2	G2	M3
1	**0.535**	0.069	**0.535**	0.160	**0.535**	0.041
2	**0.419**	0.111	**0.419**	0.178	**0.419**	0.251
3	**0.614**	0.312	**0.614**	0.080	**0.614**	0.335
4	**0.586**	0.528	**0.586**	0.344	**0.586**	0.133
5	0.673	**0.695**	**0.695**	0.337	**0.695**	0.092
6	**1.066**	0.370	**1.066**	0.370	**1.066**	0.112
7	0.191	**1.026**	**1.026**	0.145	**1.026**	0.316
8	**0.712**	0.251	0.712	**0.835**	**0.835**	0.053
9	**1.449**	0.469	**1.449**	0.291	**1.449**	0.154
Control	1.320	0.918		0.899		0.349

Since Generation 0 for the experiment is shared between the sorting and non sorting versions of the algorithm, the same trend of an unusually high recovery of Generation 0 can also be seen

here. Only one formulation was taken from Mutant 1 (M1) and Mutant 2 to complete their respective following generations.

The non sorting version of the algorithm converged at Generation 2 due to the following mutant, Mutant 3 (M3), having lower viability than Generation 2 (G2). The non sorting version of the algorithm also converged on the optimal solution combination of $0\ \mu L$ trehalose solution (level 0), $60\ \mu L$ glycerol solution (level 3), $80\ \mu L$ isoleucine solution (level 4), and $160\ \mu L$ Normosol-R. This is because these results were carried on from Generation 0 and prevailed at convergence, which can be seen in the representations of Table 1 and Table 2.

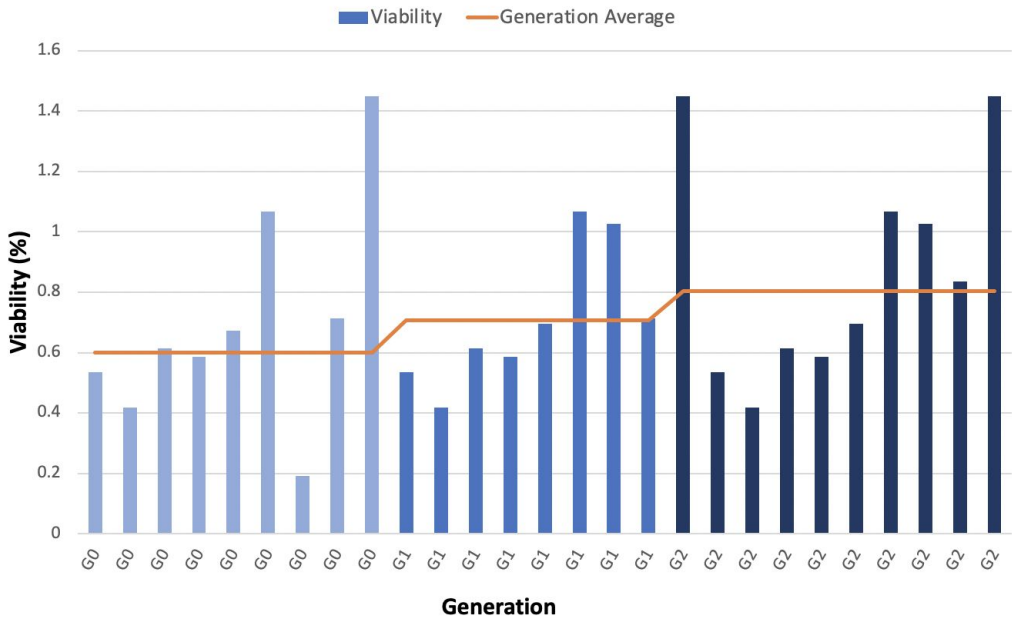

Figure 5. Jurkat Non Sorting Optimization in TGI Solution

When evaluating the results of the experiment and its shortcomings, it is important to take note of the viability of the control wells. Table 1 and Table 2 both demonstrate a digressing trend of the post-thaw recovery of the control well. The results between all mutant and G0 results should be comparable, but instead a downward trend is apparent. This serves as another primary explanation as to why the following results did not exceed the viability of previous generations.

It can be noted that in this experiment, the sorting version of the algorithm converged faster than the non sorting version. This result is expected, as demonstrated in Figure 1, but it is unknown if this experiment provided sufficient data and evidence to prove this hypothesis. This experiment also resulted in the two versions of the algorithm converging on the same solution but this was an artificial by-product of the high viability of Generation 0. Although a mutual convergence on the same generation is ideal and an indication of the functionality and

effectiveness of the two algorithms, this experiment alone does not sufficiently prove that both versions of the algorithm will result in the same optimal combination of components.

Discussion

Primary findings from this experiment conclude that the differential sorting algorithm is sensitive to experimental shortcomings such as high starting viability and susceptible to errors in cell count, seeding density, fluorescence reading consistency, freezing methods, and Calcein-AM/PI handling.

As mentioned above, the viability of Generation 0 is an important factor for asserting the reliability of the results obtained by the experimental process discussed and for conducting an accurate and complete run of the DE algorithm. A more appropriate and expected recovery of the first generation would be closer to 20-40%. This occurrence outlined a drawback of the DE algorithm that can only be identified by experimental reasoning. The additional flaws of the experiment are not related to the DE algorithm itself, but instead the steps leading up to its use.

The resulting viabilities of over 100% indicates an additional error in the experimental practice. Cells do not experience an optimal environment during freezing and often experience damage that leads to cell death, thus representing the importance of improving freezing practice. The most reasonable explanation for this phenomenon is that a higher number of cells than expected were seeded into the corresponding wells of the experiment and thus resulting in higher viability readings. This source of error outlines the importance of reliable cell counting prior to seeding. Seeding the cells into the wells for each of the replicates posed as a step prone to discrepancies. The seeding concentration was calculated based on the cell counts obtained from the hemocytometer. The average of three counts was used to calculate the volume of the re-suspension solution. Thus the seeding density was dependent on the concentration of the representative samples that were counted for the experiment. In the future, taking and counting additional samples would improve the accuracy of the average concentration that is used to calculate the seeding density. If the sample taken for the hemocytometer was not an accurate representation of the actual density of the parent flask, then the seeded density would not be the desired value.

An additional source of seeding error would occur if the cell suspension used for seeding was not a single cell suspension. Clumps of cells could appear in some wells while others are deprived of those cells. This would cause the viability for certain wells to experience higher or lower values that deviated from the expected viability. Precursors to this experiment also demonstrated the importance of ensuring consistency in the fluorescence readings between replicates. Discrepancies can be caused by errors in the initial cell count when seeding or functionality of Calcein-AM, which will be discussed further later. If results are inconsistent between the replicates the results are not reliable due to the impact that outliers can have on the average fluorescence reading. This error could cause inaccurate viability results post-thaw. Thus, the consistency of the concentration of the cell suspension throughout was vulnerable to variability based on the thoroughness of mixing.

An additional variable that could impact the reliability of the resulting viability is the freezing methods used. During freezing, the silicon lids occasionally became dislodged from the well of the plate. This primarily occurred around the edges of the plate. Since Normosol-R was seeded in the surrounding wells of the 96-well plate, the wells most impacted were not critical in the experiment. However, if the silicon lifted from the experimental wells, the solution was at risk of evaporation. This could cause the osmolarity and concentration of the solution to change and deviate from the desired formulation. In the future, identifying a feasible method to consistently control and reduce the risk of evaporation would aid in the reliability of the experiment.

Lastly, another feature of the experiment that could cause variability in the viability is the Calcein-AM. The use of this compound is sensitive to the incubation time, the presence of bubbles in wells, and the concentration of Calcein-AM used [9]. These variables must be rigidly controlled in order to avoid inconsistencies and erroneous readings associated with the dye. Low fluorescence values and poor replicates are results of inconsistencies in Calcein-AM practices.

Conclusion

The interest of identifying a DMSO-free method to effectively cryopreserve cells and tissues is of paramount importance to the field of biotechnology and preservation. The use of osmolyte solutions in naturally occurring biological systems such as plants and insects has allowed these life forms to survive adverse living conditions [10]. Various sugars, sugar alcohols, proteins, and amino acids have demonstrated potential to maintain cell viability post-thaw.

For example, glycerol's interactions with water is able to inhibit ice crystallization [11] and stabilize proteins during freezing [12]. It has also been hypothesized that trehalose has the potential to lower the transition temperature of cell membranes [13]. After the cell reaches this temperature, the membrane changes from a fluid state to a more rigid and crystalline condition, thus reducing the permeability. It has been demonstrated that specific combinations of these osmolyte solutions, such as glycerol and trehalose, can have a powerful impact on the crystallization of water and form natural deep eutectic systems (NADES) [14]. However, the concentration and complex interactions between these compounds is important to understand to achieve an optimal balance for cell preservation. The utilization of the DE algorithm discussed through the experimental process presented has immense benefits under proper implementation. The objective of this investigation was to understand the relationship between trehalose, glycerol, and isoleucine for optimal preservation of Jurkat cells. This study presents the results of the investigation and identifies the shortcomings associated with the algorithm itself as well as the experimental procedure. Additional improvements on cell counting and seeding, freezing methods, and component handling can be made from the shortcomings of this investigation.

Future Directions:

A more reliable extension of this experiment should capitalize on the errors deduced from this process to conduct a more controlled experiment to identify the optimal TGI formulation for

preserving Jurkat cells. The experiment should compare the two methods of the DE algorithm to determine which version identifies the result the fastest. Additionally, the versions should be compared to ensure that the end result, the optimal solution, is found to be the same by both.

In the future, this experiment could be extended to optimize the preservation of various cell types in osmolyte solutions of various components. The DE algorithm could also be extended to take into account other factors that impact the viability of cells post-thaw. Such variables could include the freezing profile, nucleation temperature, solution concentration and scope, and incubation time.

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