Quantifying the Link between Ice Growth and Cryopreservation

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Project Report 1

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1. Introduction

Most breakthroughs in gene therapy and tissue engineering industries are highly dependent on the ability of successfully transporting and storing stem cells and immune cells. This is currently done via cryopreservation using different types of molecules and polymers as cryoprotectants[1]. At present, most used cryoprotectants, such as DMSO (dimethyl sulfoxide) and glycerol, are needed in high concentrations to be effective inducing a certain level of toxicity. This leads to the current challenges in the field, such as reduced and/or different cell function post-thaw and decreased cell viability[2].

Proline has been established to have antifreeze protein activity (AFP) if suitable conditions are met; proline AFP activity is assumed to be based on an ice recrystallisation inhibition activity (IRI)[3]. Polyproline has been shown to be able to increase between 20% - 50% in cell recovery post-thaw in different cell cultures[4]. However, the mechanism behind prolines IRI activity remains unknown. This study investigates proline IRI activity –does proline function in a physical manner by binding to ice crystals and impeding their growth rather than in a biochemical way by changing the cells' protein expression. To test this, this study uses red blood cells (RBC) as model cells; due to RBCs being anucleated cells, one can exclude the proline's IRI activity acting through a biochemical way when analysing the results.

2. Materials and Methods

2.1 RBC freezing and quantification of cell survival

For each experiment 20 ml of sheep blood was used (SB069, Alsever's). The blood was initially centrifuged for 5 minutes at 2000 rpm, then the supernatant was removed and replaced with the same amount of PBS (phosphate-buffered saline).

The blood was incubated for 20 minutes in 1:1 ratio of 10% glycerol in PBS (49767, Sigma Aldrich) or 200 mM L-proline (P6507, Sigma Aldrich), further referred as proline. After incubation the blood was centrifuged for 5 minutes at 2500 rpm, the supernatant removed and PBS was added up to 20 ml of total solution. 0.5 ml of the resulting blood solution was used for each condition tested with 0.5 ml of different glycerol percentages, hence a 1:1 ratio of 0%, 5% and 10% glycerol in PBS or/and proline in blood was established. Two controls were left at room temperature in 1:1 ratio of PBS or lysis buffer (0.32M sucrose, 5mM $MgCl_2$, 1% triton X-100, 10mM tris HCl pH 7.8). Another 0.5 ml of blood was removed and maintained at room temperature without any further modifications as a control for this step. When alanine (A15804, Alfa Aesar) was used as a positive control the same protocol was utilised.

The samples were frozen for 20 minutes at -80°C liquid nitrogen and then warmed up in a water bath at 45°C for 10 minutes. The samples were then centrifuged for 5 minutes at 2000 rpm and 50 µL of supernatant was added to 0.75 ml of ADH assay[5]. For each condition, three repeats of 0.2 ml each per sample were transferred into a 96 well plate and a UV/vis measurement was obtained at 580 nm wavelength (Synergy HT, BioTeK).

2.2 RBC rehydrating time lapse experiment

The same protocol as described above was employed - a 10% glycerol preincubation period was used, then the RBCs was distributed to multiple technical replicates of proline, PBS and alanine. After the 10 minutes warmed up at 45°C, images of the first replicates were taken. In order to slowly rehydrate the cells, 200 µL of PBS was added every 45 minutes until the glycerol concentration reached 3%. After every step of rehydration, one technical replicate was used to take images of the cells.

2.3 Crystal size formation analysis

To test if proline influences the size and shape of water crystals, a cryo-stage set up was employed. Drops of water with PBS, 400 mM proline/proline with 10% glycerol or 400mM alanine/alanine with 10% glycerol were dropped from ~1m into a slide that was frozen on dry ice. The samples were left at -8°C for 30 minutes for the ice crystals to fully grow. As the water would freeze instantly, images at the beginning of the experiment and after the 30 minutes were taken. All the images were captured using an Olympus CX41 microscope attached to a Cannon EOS 500D camera.

To quantify the number of ice crystals formed and their size versus the controls, ImageJ was used to count and to calculate and area versus number of ice crystals per each condition, then to normalize the acquired data.

3. *Results*

3.1 Toxicity testing of glycerol and proline on RBCs

The first RBCs freezing experiments performed with a pre-incubation in 10% glycerol (PBS) could not be quantified as the control cells left in PBS after the preincubation had a significant amount of cell death and hence the survival rate could not be calculated.

Looking at the death percentage of RBCs before incubation and after showed that after the preincubation there was a significant cell death in comparison to before preincubation. Hence, toxicity testing of cells while left in 10% glycerol and 200 mM proline was performed (Figure 1).

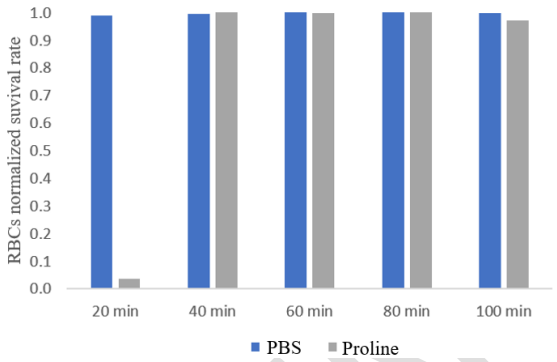

Figure 1| Testing of toxicity of proline and glycerol in blood cells. Blood was added in a 1:1 ratio to either 10% glycerol or proline (200mM) and tested for cell survival every 20 min.

As leaving the cells in glycerol or proline for a long period of time does not show to increase their death rate, another freezing experiment was performed where a 0.5 mL blood sample was taken and analysed after each step on the freezing process. This showed that after the pre-incubation of blood in 10% glycerol, if cells are resuspended in PBS, the shock of exchanging saline medium results in high cell death. Hence, all control cells were left in the original 10% glycerol at room temperature for all future experiments.

3.2 Preincubation with glycerol shows most RBC recovery post-thaw

In order to test if proline preincubation increases the survival rate of RBCs, data was compared from experiments with 10% glycerol pre-incubation, 200mM proline preincubation and no preincubation (Figure 2).

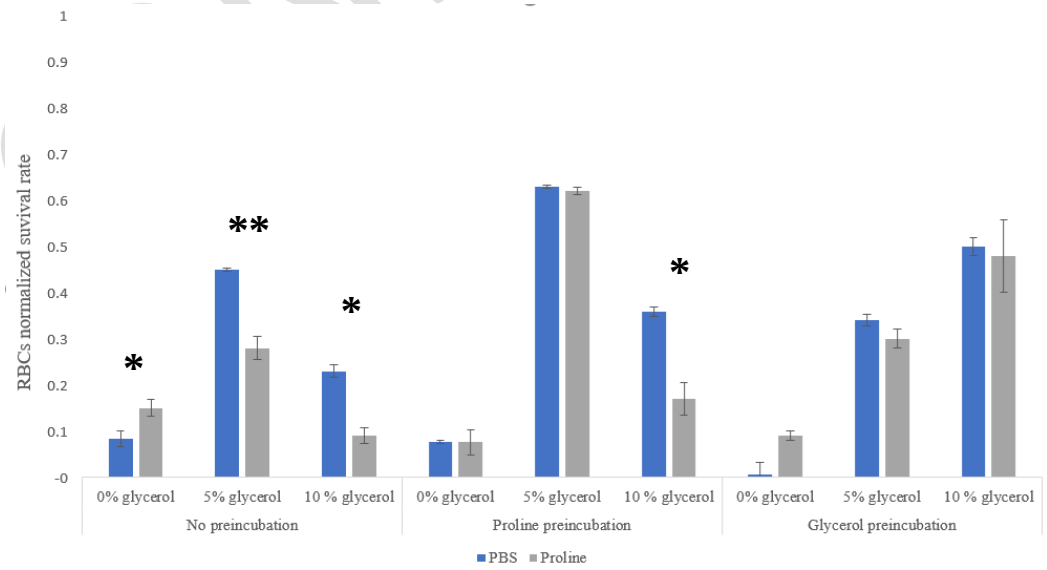

Figure 2| Effect of different preincubation methods on RBCs survival post-thaw. Error bars are calculated based on standard derivation of all 3 technical replicates for each condition. *- p values <0.05; **-p value <0.005

Although proline preincubation shows an increase in survival at 5% glycerol, all preincubation conditions present a certain degree of increase at 5% glycerol. The glycerol preincubation method presents the highest increase in overall survival of RBCs, hence it was utilized in all further experiments.

3.3 Proline IRI activity shows to be concentration dependent

Many known cryoprotectants show to have concentration dependent IRI activity – a specific concentration mixed with another cryoprotectant such as glycerol will significantly increase the overall survival rate of cells[8]. Hence, different concentrations of proline were tested with 10% glycerol (Figure 3).

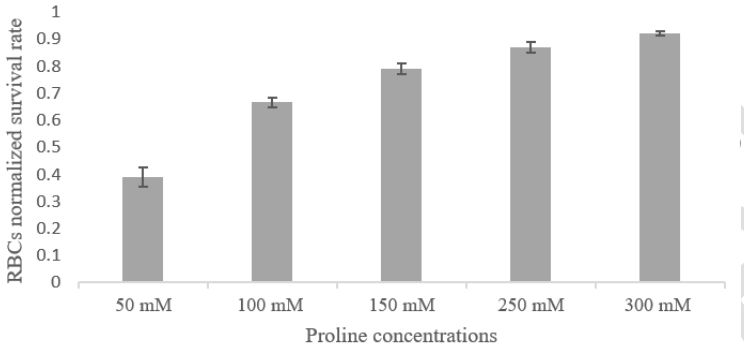

Figure 3| Proline shows concentration dependent IRI effect in RBCs.

As proline does show to have a higher IRI activity if concentration is increased, further experiments were performed using varied concentrations and compared overall results.

3.4 Alanine as a positive control in assessing IRI activity

As previous experiments have shown proline have some IRI activity but not very conclusive, an experiment using alanine as a positive control has been performed. Alanine has a very similar structure to proline (Figure 4A-B) and although is a primary amino acid found in many of the cryo-protective proteins in nature, it has been shown that alone it does not have any IRI activity[6,7]. Hence, using alanine as a positive control versus proline would indicate if the activity seen so far with proline is itself significant.

For this experiment 3 different biological replicates have been done (Supplementary Table 1). Quantifying the results (Figure 4C) shows that proline does not present any significant increase in IRI activity than alanine – both alanine and proline present the same RBCs survival rate at both 200mM and 400mM concentrations in 10% glycerol.

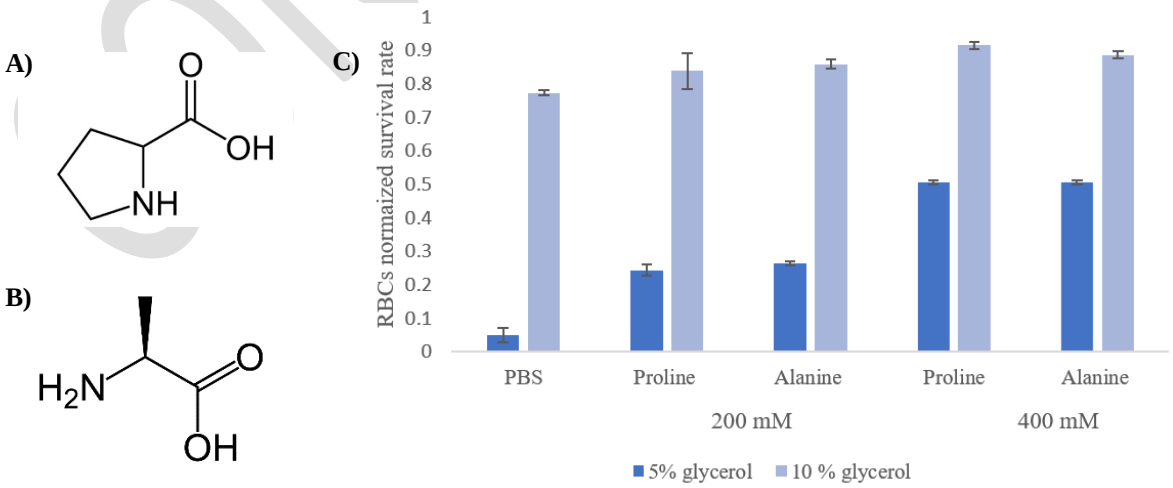

Figure 4| A) Proline structure **B)** Alanine structure **C)** Proline IRI activity at different concentrations quantified using alanine as positive control. Error bars are calculated based on standard derivation of all 3 technical replicates for each condition.

3.5 Time lapse rehydration of RBCs

Employing the glycerol preincubation protocol leads to a dehydration of the RBCs post-thaw. To test whether rehydrating the cells back to health without leading to further cell death is possible, a slow rehydration protocol was used (Figure 5). Images were taken of the RBCs in proline after every rehydration step to see any phenotypical differences (Figure 6). Alanine was only used as a control to measure average cell survival.

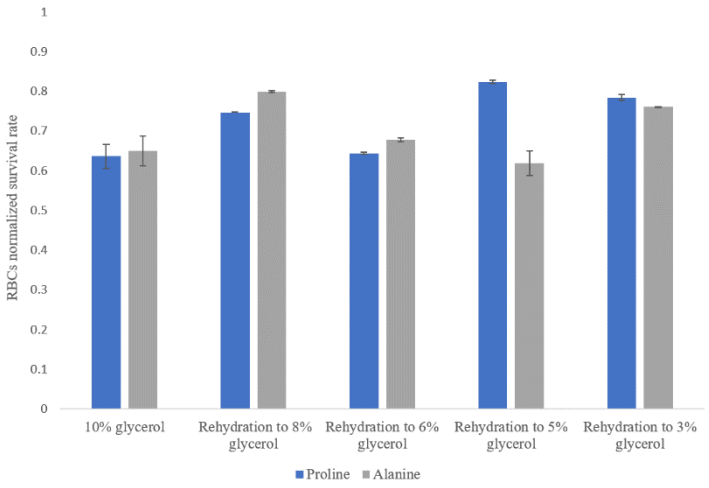

Figure 5| Slow rehydration of RBCs post-thaw. The cells were slowly rehydrated by adding 0.2 mL PBS every 45 minutes. The concentration of proline and alanine used was 200mM.

By comparing phenotype versus cell survival at each step, one cannot imply any correlation. The cells are at an average of 0.7 normalized survival rate however small variations in survival rate could be due to differences in blood variability.

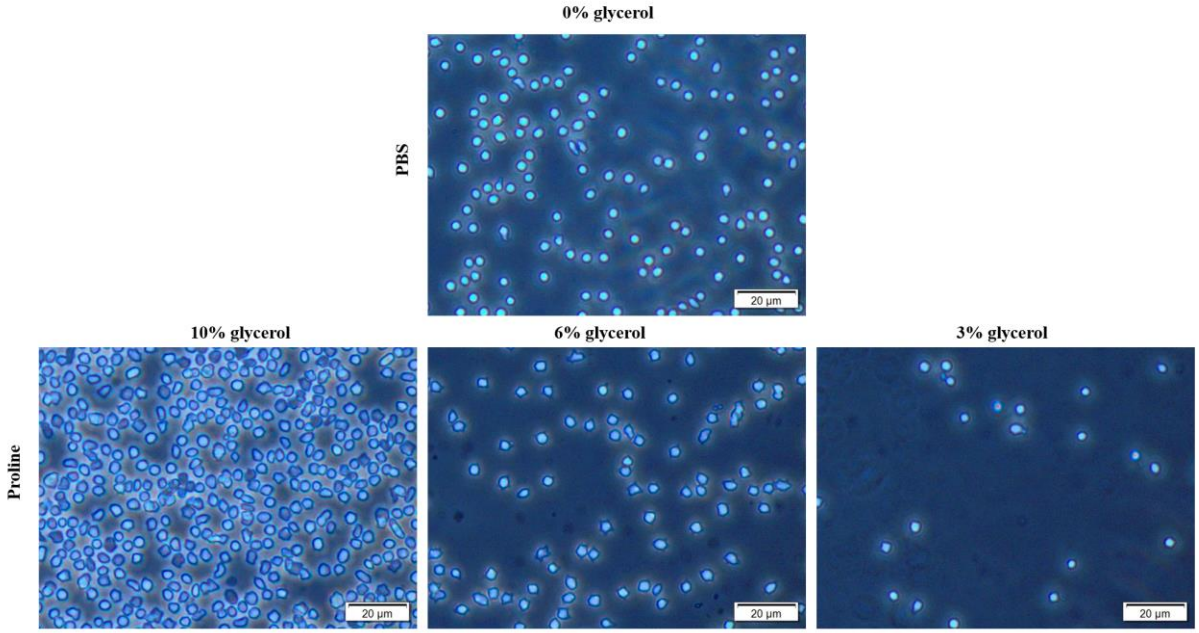

Figure 6| RBCs post-thaw slow rehydration with PBS. As concentration of glycerol decreases, the cells slowly return to their normal phenotype - the cells appear to have a healthier phenotype (rounder and bigger) at 3% glycerol than the ones from 10% glycerol.

3.6 Ice crystal growth and reshaping

To analyse if proline does actively reshape and control ice crystal growth, a cryo-stage like model was utilized. The number of ice crystals and their size was quantified by normalization of the data for all conditions (Figure 7).

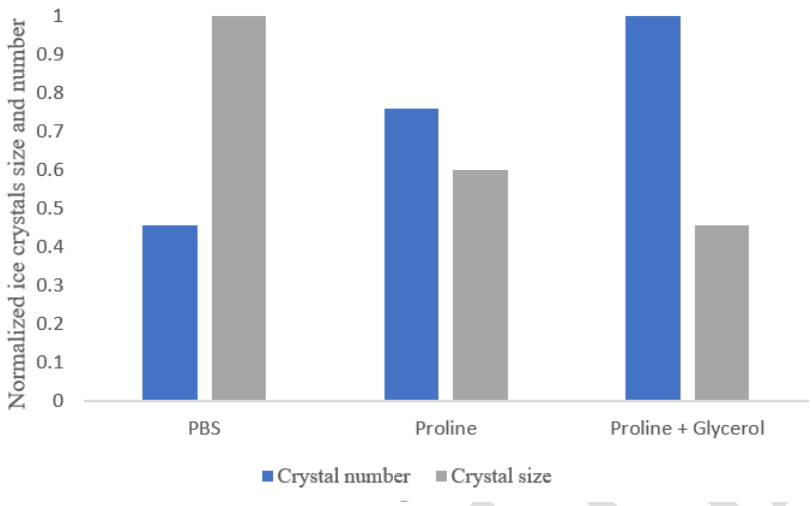

Figure 7| Quantifying proline's ability to model ice crystal growth alone and in combination with glycerol. A concentration of 400mM proline and 10% glycerol was employed.

Alanine was unable to be quantified due to its solubility – it is not as soluble in water as proline, hence creates a more viscous liquid that does not freeze instantly and reshapes the ice crystals growth (Figure 8). Proline presents a clear trend – alone it increases ice crystal number but decreases their size, however when glycerol is added the size of the crystal are decreasing further while their number increases.

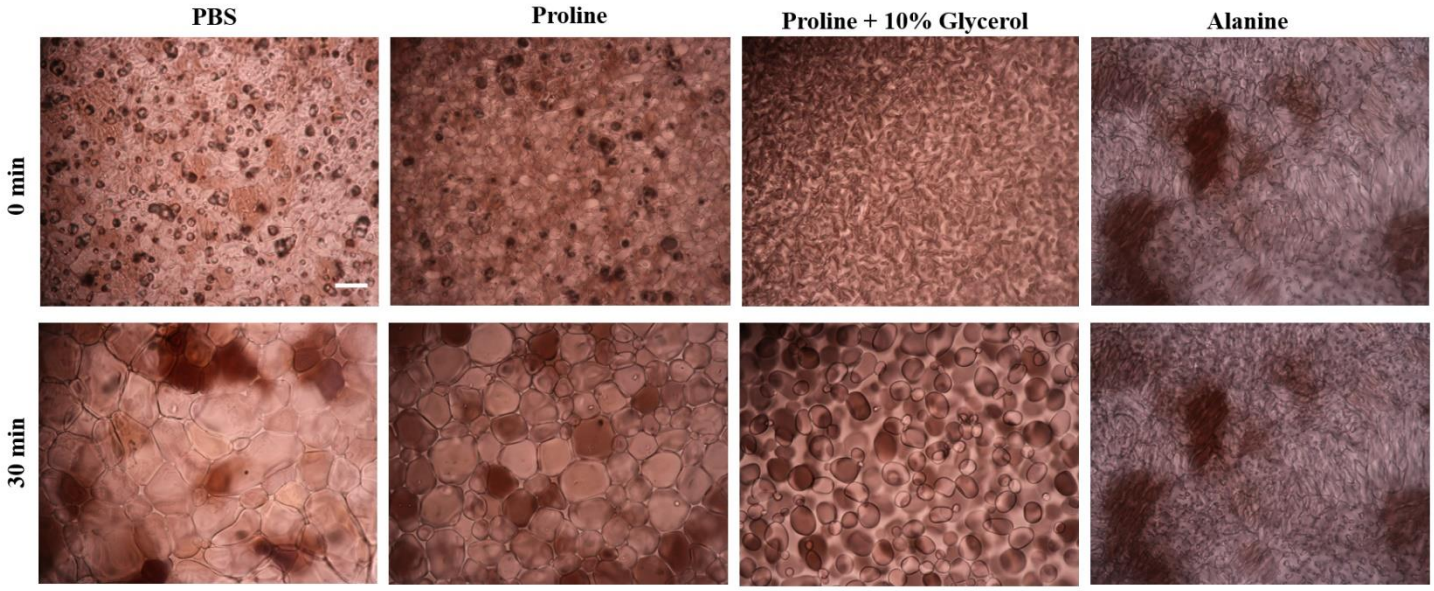

Figure 8| Ice crystal growth after 30 minutes in different conditions. Scale set at 20 µm. Proline and alanine concentration used at 400 mM.

4. Discussion

Prolines has been shown to have AFP activity, however determining whether this activity is based on metabolic pathways due to proline being an important amino acid, or on a physical manner by binding and reshaping ice growth is still to be discover. Performing different experiments on RBCs using PBS and alanine as controls has been generating controversial results.

Method wise, glycerol preincubation does present the highest cell survival rate. These results are as expected as preincubation with glycerol allows the cells to dehydrate eliminating water from inside the cells, hence decreasing the cell death due to crystals forming inside the cells (Figure 2). By showing that slow rehydration of the cells is possible post-thaw without inducing further cell death, one can assume that the survival rate of cells post-thaw presented in Figure 2 will be similar to the number of cells ready to be used after rehydration.

To what degree does proline presents an IRI activity remains inconclusive due to several factors. Firstly, although proline does show to have concentration dependence activity, when compared to alanine it does not present any significant differences (Figure 4). Thus, it is hard to quantify if differences in cell survival are due to proline IRI activity or differences between biological replicates (i.e. blood variability).

Secondly, when analysing ice crystal growth and size, proline shows to affect growth and size of ice crystals. The crystals formed with proline and proline with glycerol are smaller (Figure 8) than the PBS control, which related to the results from RBCs survival – 10% glycerol with proline do present the highest survival rate. However, this can be due to changes in viscosity which can influence ice crystal formation. This concept is proven by alanine, which is less water soluble than proline and by changing the viscosity of the solution, resulting in the reshaping of the ice crystal formation and making it unquantifiable.

Future work is required to acquire a higher understanding of prolines IRI activity. Using cell cultures of platelets might help reduce the errors due to high RBCs variability, which is responsible for part of the variation present between different biological replicates. Furthermore, performing a flow cytometry experiment on cells post-thaw would generate a more accurate reading of cell survival than the AHD assay. As currently the volume of RBCs used for each experiment is quite low, any errors appearing throughout the freezing steps will be increased. Hence, using flow cytometry we can quantify survival per cell rather than per volume.

5. Conclusion

Proline presents inconclusive results in relation to its IRI activity. It does show to control ice crystal growth however this could be due matrix variability and viscosity, however when used in blood it does present a concentration dependent activity but overall cell survival is equal to controls. Therefore, the addition of proline itself could be changing the cell survival rate to a certain degree. As the viscosity changes after proline addition, which affects ice crystal growth formation, there will be a change in cell survival rates. In conclusion, using RBCs as a model might not be representative enough to establish prolines IRI activity mechanism.

6. Acknowledgements

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7. References

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8. Supplementary information

Supplementary Table 1| Normalized data of 3 distinct biological replicates of RBCs survival rate post-thaw

		5% glycerol	10% glycerol
Biological replicate 1	PBS	0.049	0.773
	Proline 200mM	0.242	0.838
	Alanine 200mM	0.263	0.859
	Proline 400mM	0.505	0.914
	Alanine 400mM	0.50	0.886
Biological replicate 2	PBS	0.235	0.791
	Proline 200 mM	0.373	0.825
	Alanine 200 mM	0.357	0.826
Biological replicate 3	PBS	0.576	0.728
	Proline 200 mM	0.717	0.880
	Alanine 200 mM	0.282	0.677