

GLUTATHIONE SENSOR

Sensing Transplant Tissue Degeneration

BY

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I. Abstract

There is a shortage of organs available for donation in the United States today, partly due to the fact that viable organs are often discarded because they fall outside defined organ viability time points. To address this issue, we designed a DNA construct that senses glutathione, which is a marker for kidney degradation. Our construct was designed for and tested in HeLa cells. The PCR to confirm that the construct was true to our design was successful, showing that the plasmid printed was as expected. Transformation of the construct into E. coli, mini-preps in order to amplify the amount of plasmid available, and freezer stock formation in order to store the construct long term were also successful. A transient transfection into HeLa, confirmed against a pComet positive control, was used to test the functionality of the construct. A dose response to glutathione was performed after transfection. At all levels of glutathione, very low levels of fluorescence were observed, showing that the construct may have had a relatively low resolution ability to sense and read out glutathione. High levels of glutathione were observed to be toxic to cells. Due to the low fluorescence observed, an RT-PCR was performed to measure levels of transcribed PAX8 and GFP (the two main elements of our construct). The RT-PCR did show that both proteins were being expressed in our transfected cells. It is unclear whether this is due to our construct sensing glutathione, or whether it is due to other mechanisms and processes within the cell. Our research allows us to conclude that there is the possibility that our construct is working with low levels of readout, but confirmation will require further work in the area. Ideally, this work could provide an easy-to-use, easily transported way to determine the viability of organs, given further research and optimization of the construct. This could potentially address the huge imbalance between organs required and organs available in the United States today.

II. Introduction

There are far more people who require organs than there are organs available for donation. Someone is added to the waitlist almost every ten minutes, and eighteen people die every day because they cannot get the necessary organ transplant (1). People may choose to donate their bodies or their organs to science. In the United States, there are more than 120 million people signed up to be donors (1). With so many organ donors signed up, the shortage may seem a little strange (1). The shortage really exists because organs often cannot be recovered in time from victims. By the time the ambulance gets to scene and victims are found, organs have often degenerated to the point where they cannot be safely transplanted.

The current method of determining whether or not an organ is viable is simply assigning a certain average time of viability - typically a few hours. Because of this, a huge number of potentially viable organs are lost. Not all organs degenerate exactly within that time frame. Some are actually still viable and could save someone's life. That said, some organs may be transplanted when not fully viable, once again due to the inaccuracy of the degeneration time standard. This project seeks to address this problem by developing a system to reliably and easily measure the viability of the organ at the time of its harvesting, thus providing a metric to determine whether it can be successfully transplanted or not.

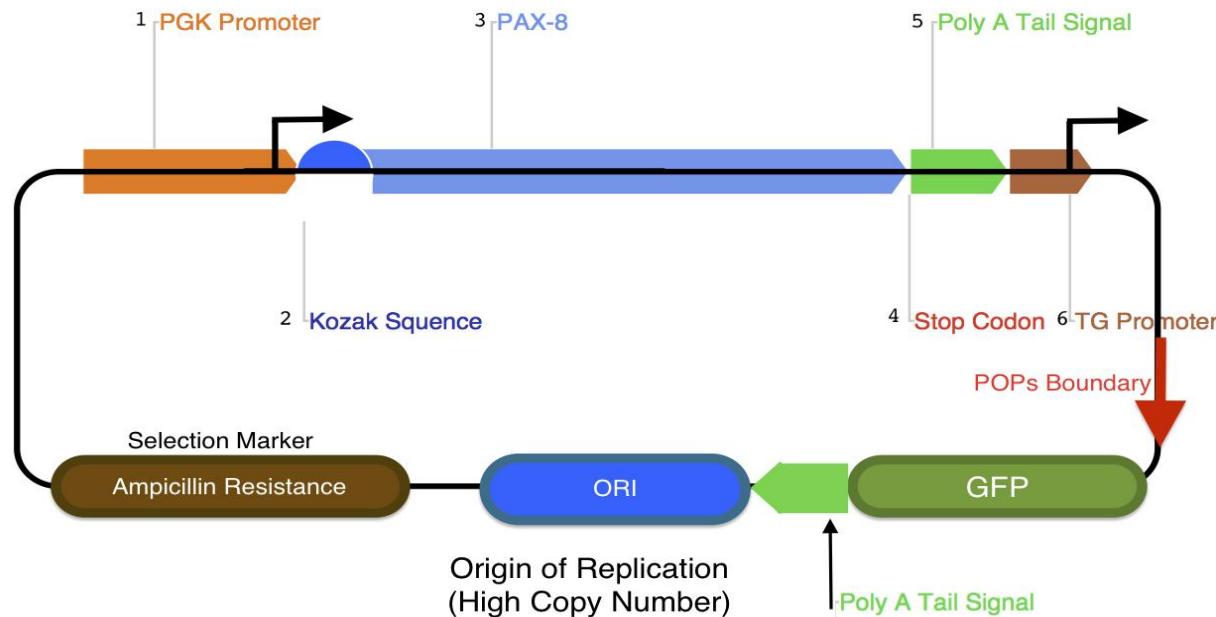
It has been discovered that kidney transplants are among the most successful, with approximately 83.4% of people still living five years after their transplant. It is also one of the most commonly needed transplants (1), making it an ideal target for our project. As kidneys degrade, they exhibit higher levels of alpha-glutathione, a subtype of glutathione, a tripeptide commonly found in mammalian cells (2). Furthermore, lower levels of alpha-glutathione are found in functioning grafts, as compared to non-functioning ones. Sensing total glutathione levels (which is often used as a surrogate marker for alpha-glutathione) would allow us to determine whether or not a kidney is still viable. High levels of glutathione correspond to a kidney that has a high chance of being non-functioning, while low levels correspond to kidneys with a better transplantation prognosis.

There are other technologies to measure glutathione, including a variety of small molecule and mechanical sensors. The Yang group in Beijing reported finding a small molecule sensor that fluoresces in the presence of glutathione. Interaction with glutathione changes the chemical structure of the molecule, resulting in fluorescence (3). There are also electrochemical sensors that work in a similar manner, by electrically modifying the molecule in the presence of glutathione (4). However, none of these are genetic sensors of the material, and all are still in an early development stage. A genetic construct could be integrated in a cell culture, and can potentially provide a test of whether the organ is truly viable or not.

The challenge in organ viability testing is to create a technology that can both be easily used and transported on site. This sensor would be easy to use, since all that would be required is a small sample placed in the cell culture, and it would be easy to transport, since cell cultures don't take up a great deal of space, especially if the construct is placed in an organism like E.coli or yeast. Ideally, the sensor should fluoresce relatively soon (within a few hours) if the organ is not viable. The proposed biological device is a genetic construct designed to be integrated into HeLa cells. This seemed the most logical initial testing environment, since the promoters and genes used in our construct are of mammalian origin.

Once the construct has been proven to work in HeLa, an important next step would be to integrate this system into a faster growing organism such as E. coli or yeast, for faster and more convenient analysis. The benefit of yeast would be continuing to use the eukaryotic machinery for transcription and protein folding. Once integrated, the cells will respond to the presence of glutathione by producing GFP. The cells can then be examined and the GFP used as an indicator of glutathione sensing. High glutathione levels will turn on transcription of GFP while low levels will leave the transcription of GFP off. The degree of transcription will provide a metric as to the degree of organ viability.

III. Methods



Device Design

Figure 1: *pGSH* design including our design and standard parts.

Our device consists of six parts ordered below from 5' to 3' as they appear in our plasmid, which is named pGSH (Fig. 1). Each part is described in further detail below. We made no modifications to any of the sequences found in literature. Our device is designed for transfection into HeLa cells. The origin has a high copy number, the resistance marker used is ampicillin. Mechanistic design of the sensor was drawn from previous research on the Thyroglobulin promoter (5). The actuator portion is the GFP gene, which should be activated by our sensor, thus providing a way of reading out the construct's activity. The design was outsourced to DNA 2.0 for printing.

1. Murine Phosphoglycerate Kinase-1 promoter (PGKp) : 500 base pairs
This promoter is constitutively activated in mammalian cells as, in its biologic setting, it promotes expression of a kinase that catalyzes the formation of ATP from ADP and 1,3-BPG. In our device, this promoter is used to constitutively express PAX-8. Sourced from snapgene.com (6).
2. Kozak Sequence : 6 base pairs
This Kozak Sequence initializes the translation of PAX-8. This sequence was sourced from the BIOE 44 lecture on eukaryotic gene design.
3. Bovine PAX-8 : 1371 base pairs, 457 amino acids
PAX-8 is one of many *cis*-acting transcription factors that activate the Thyroglobulin promoter. PAX-8 activity is downregulated in the absence of glutathione (7). Sourced from bovinegenome.org (8).
4. TAG Stop Codon : 3 base pairs
The TAG stop codon here arrests translation of PAX-8.
5. Poly-A Tail Sequence : 225 base pairs
This sequence recruits proteins which catalyze the addition of the poly-A tail characteristic of mature mRNA in eukaryotes. The poly-A tail forms a loop with a protein complex and the 5' cap of the mRNA, aiding in the initiation of translation. This sequence was sourced from algosome.com (9).
6. Rat Thyroglobulin Promoter: 194 base pairs

In rats the transcription of Thyroglobulin is upregulated by the interaction between this promoter sequence and PAX-8. This sequence was sourced from Sinclair *et al.* (10). A Rat TG Promoter was used due to constraints on size and the limited selection of TG promoter sequences in the literature. The 3' end of this promoter delineates the POPs boundary of our part.

Experimental Design

1. Perform mini-preps and make freezer stocks.
2. PCR on pGSH to confirm construction
3. pGSH transfection success and construct functionality assay
4. Cell viability in glutathione assessment
5. RT-PCR to measure PAX8 and GFP expression in pGSH transfected cells

E. coli cells containing pGSH (provided by DNA 2.0) were streaked on LB + ampicillin agar plates (12). Single colonies were picked and grown up overnight in liquid LB + ampicillin media. These stocks were added 1:1 to 50% glycerol in barcode tubes (013302652, 0133023846, 0133026153; plasmid "CJMSBJPD") to be frozen (12). They were also used to perform DNA extraction using the QIAprep Spin Miniprep Kit (13).

Once a reasonable amount of plasmid had been generated from the stab, a PCR reaction and gel electrophoresis were performed with the mini-prepped plasmid in order to ensure that the plasmid provided was the plasmid designed and requested. The PCR reaction and gel were run according to protocols also provided earlier in this class (14). The negative control for the PCR was a reaction with the primers but without plasmid DNA, to ensure that there was no contamination or unexpected amplification.

Next, in order to test both the success of a transient transfection in HeLa, and the functionality of the construct, a twenty-four well experiment was designed, based on the transfection protocol provided earlier in the class (15). Two wells were transfected with pComet. This was the positive control for transfection, since it was shown that this transfection worked earlier in the class, showing strong fluorescence signal. Two wells were left untransfected. This was the negative control for transfection. No fluorescence should be observed in these wells. The other 20 wells were dosed with varying levels of glutathione in duplicate. That is, ten different doses were administered - 0nM, 16.5nM, 66.6nM, 264nM, 1.05uM, 4.22uM, 16.9uM, 67.5M, 250uM, 1mM. The 0 nM dosage was included to account for the fact that HeLa already has a basal level of glutathione. The goal was observe whether fluorescence could be seen that that basal level or not. To further ensure that the glutathione did not interfere with transfection, one of the pComet wells was also dosed with 1mM glutathione. The success of the transfection and of the construct's functionality were measured using fluorescence microscopy. Brightfield images were taken, along with GFP images, and they were superimposed to ensure that any GFP signal that was reported was from live, viable cells.

In addition, the above microscopy images and experimental design were also used to assess cell viability in glutathione, since this would allow a definition of the upper limits of the system. After taking images, the number of dead cells in each brightfield images were compared across all images. Qualitative observations were made based on what was observed. Here, the pComet functioned as a control since it is representative of a common transfection, while the non-transfected cells functioned as a negative control. The relative concentration of dead cells can be compared to the proportion observed in pComet to assess how high or low they are.

Our final step was to perform an RT PCR for transcription levels of the genes in our plasmid, namely PAX8 and GFP. DNA samples were isolated from the transfected cells described above using Qiagen's RNEasy Extraction Kit (16) and Invitrogen's SuperScript III cDNA Kit (17). The positive pComet wells and the negative wells were used. Since we observed the most fluorescence at low concentrations, our 0 nM dosage and 16.5 nM dosage wells were also used for this experiment.

Once the samples were isolated, the PCR reaction and gel electrophoresis were performed as before, using protocols provided for this class (14). Twelve separate PCR reactions were run on two separate 10 well gels, as described in Table 2. The results were read out using a gel imager, and all results were compared against a 1kb+ ladder to ensure that the bands were in the expected locations.

Rxn.	Primers	DNA Sample
1	GFP Primers	No DNA (Negative Control #1 - For Primer Validity)
2	GFP Primers	No Transfection Well Extract (Negative Control #2 - For Construct)
3	GFP Primers	0 nM Construct Transfection Well Extraction

4	GFP Primers	16.5 nM Construct Transfection Well Extraction
5	PAX8 Primers	No DNA (Negative Control #1 - For Primer Validity)
6	PAX8 Primers	No Transfection Well Extract (Negative Control #2 - For Construct)
7	PAX8 Primers	0 nM Construct Transfection Well Extraction
8	PAX8 Primers	16.5 nM Construct Transfection Well Extraction
9	Construct Primers	No DNA (Negative Control - Primer Validity)
10	Construct Primers	Mini-Prepped Plasmid DNA (Positive Control)
11	pComet GFP Primers	No DNA (Negative Control - Primer Validity)
12	pComet GFP Primers	Transfected pComet Well Extraction (Positive Control)

Table 2: Experimental Design of RT PCR and PCR Samples

IV. Results

Experiment 1: Transform construct into *E. coli* and make freezer stocks.

Our streaking was successful, as shown by the colony growth in the image of the plate below. This plate was used to pick colonies for Miniprep DNA extraction and also for glycerol stock creation.

Figure 2: LB+amp plate streaked with pGSH *E.coli*, used for DNA extraction and stock creation.



Experiment 2: PCR to confirm DNA construction

PCR and Gel electrophoresis, using pGSH and primers designed around the PAX8 sequence and the TG promoter, revealed an amplicon of length ~1300 base pairs. The PAX8 gene is 1371 base pairs long. This result suggests that no major construction errors occurred by confirming that the PAX8 gene of correct length is present in pGSH. Our negative control shows no band, as expected, telling us that there is no contamination or unexpected amplification occurring. . (Note: pComet results are explained later on, under the RT-PCR experiments.)

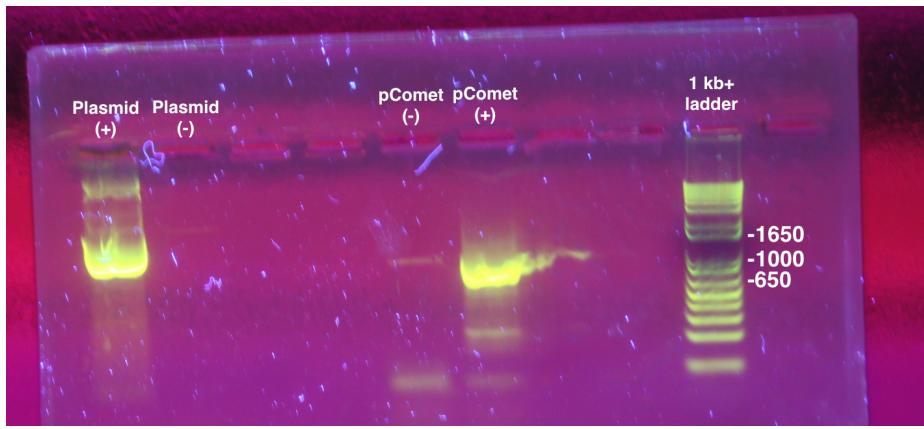
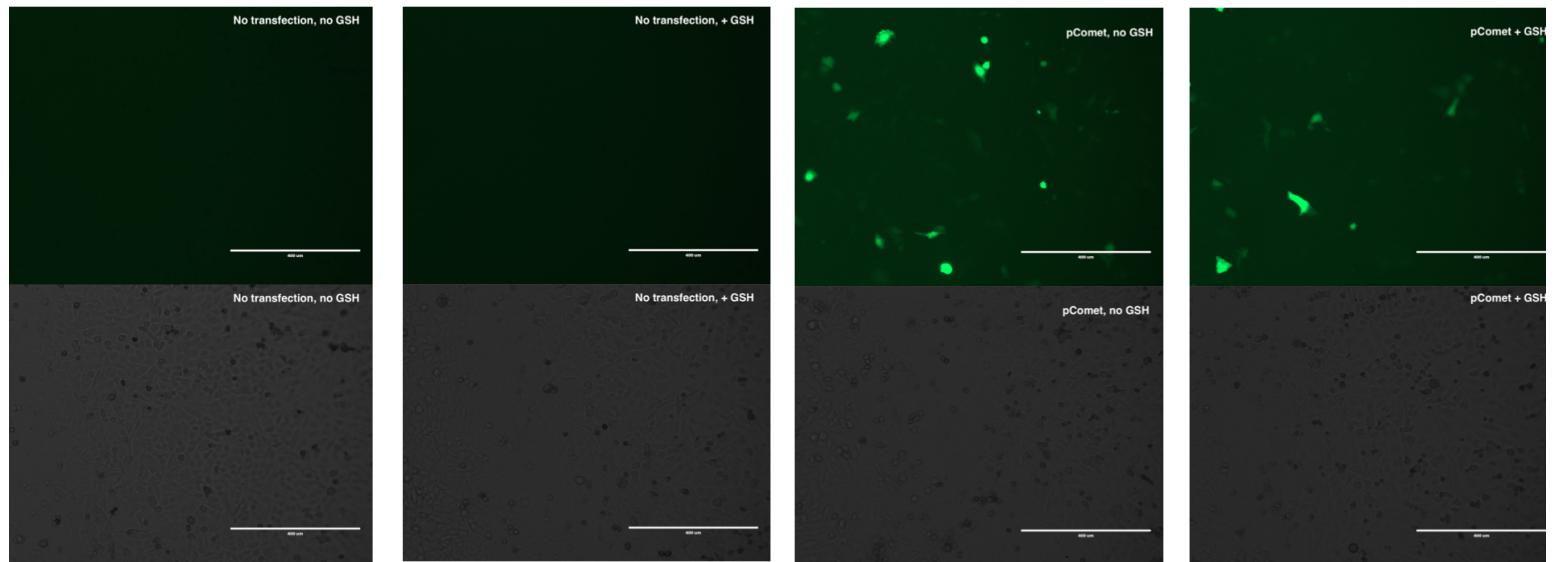


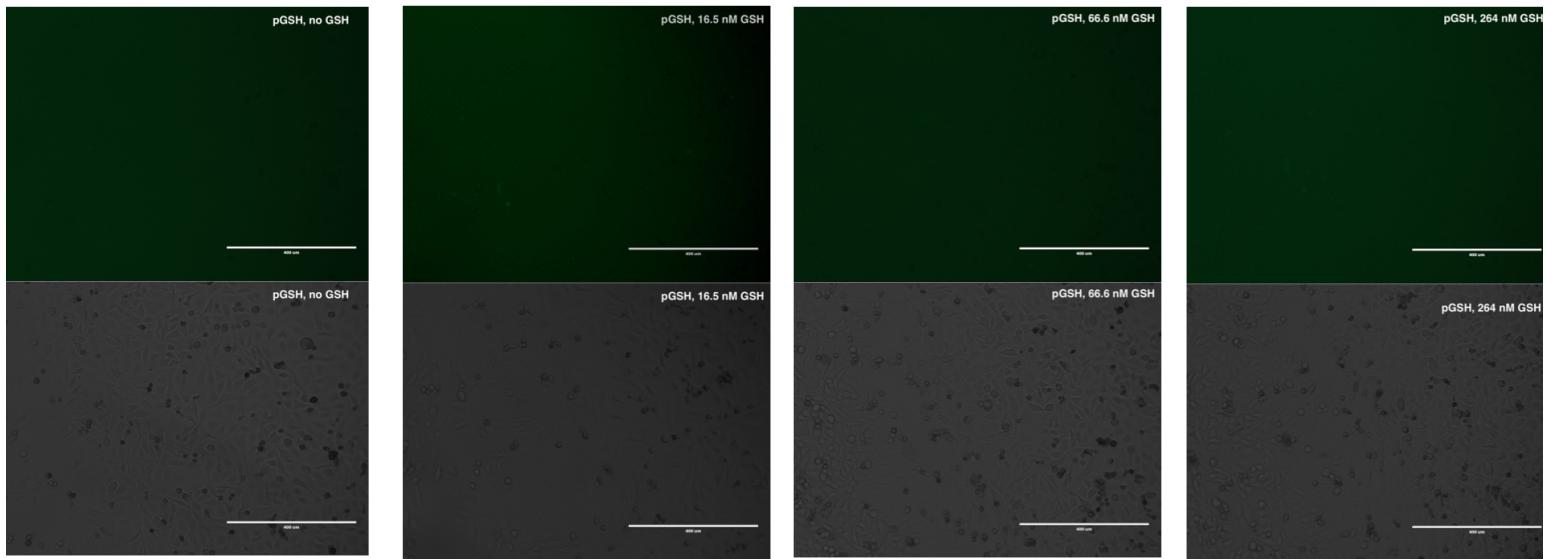
Figure 3: PCR Results for PCR performed on mini-prepped plasmid DNA. RT-PCR results for RT-PCR performed on extracted DNA from HeLa cells transfected with pComet.

Experiment 3: Plasmid Transfection Success and Construct Functionality Assessment

In the construct functionality experiment, high levels of GFP fluorescence in both pComet transfection control wells (glutathione at 0nM and 1mM), observed with fluorescence microscopy, suggest that plasmid transfection was successful and not inhibited by experimentally relevant glutathione concentrations. (Figure 3C,D). The lack of signal in the negative control confirms that there is no unexpected signal observed (Figure 3A,B). Experimental wells show

extremely low levels of fluorescence (Figure 3E-3N). This does not provide conclusive proof that our construct worked, because the signal is too low to be sure. Further testing is required.





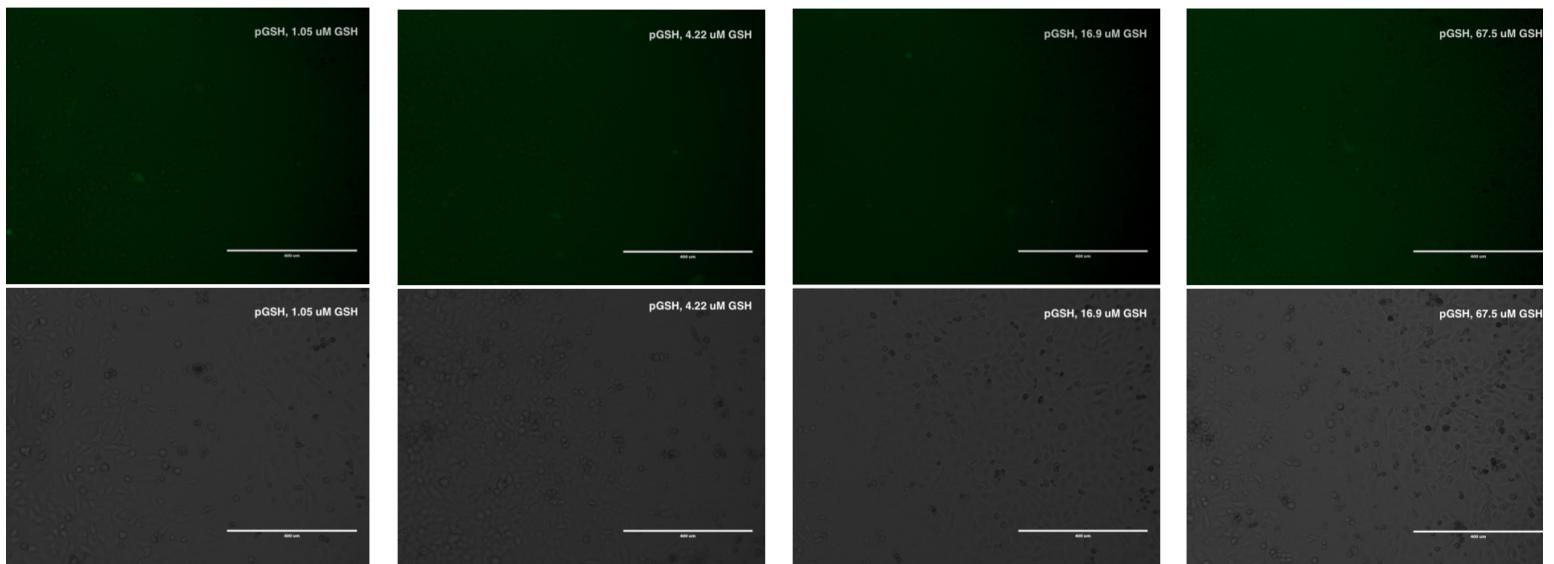


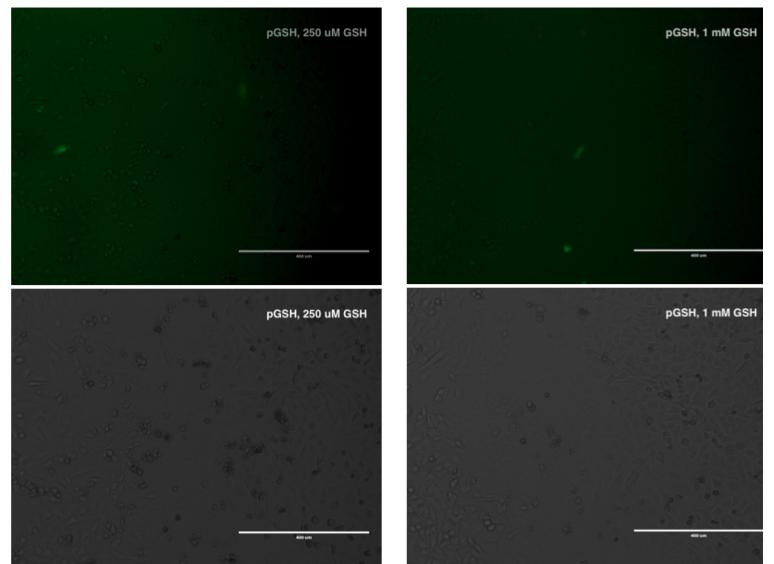
Figure 4: Fluorescent images of HeLa cells post-transfection. HeLa cells were seeded overnight in two 12-well plates at a cell density of 10^5 cells per well, followed by transfection in media containing varying concentrations of glutathione. Cells were incubated for 24h, and fluorescent images were taken of each well. The plasmid pComet, which results in green fluorescence when successfully transfected, was used as a control. Image Gain was adjusted evenly across images using ImageJ to normalize contrast levels.

Experiment 4: Cell viability in Glutathione

It was qualitatively observed, based on the light microscopy images shown above, that increasing concentrations of glutathione decreased cell viability. pComet wells and no transfection wells functioned as controls here also. They provided baselines against which to assess whether cell death appeared to be increasing compared to normal or decreasing compared to normal.

Experiment 5: RT-PCR to assess PAX8 and GFP transcription

The RT-PCR experimental control was the pComet RT-PCR. We had clear results from microscopy in that well, so if the RT-PCR was working, we would expect to see a clear band in the pComet lane, and no band in the negative control (indicating no contamination). Looking back at Figure 3, this is exactly what was observed. This means that our experiment is working as expected, and also that the pComet is showing high levels of GFP and minimal primer contamination, which corresponds with the results from the microscopy. This means that the rest of our results can be interpreted knowing that the experimental setup is correct, and it provides a confirmation for the control pComet images shown in Figure 4.



The results of our RT-PCR experiment for the negative controls functioned as expected. Though we see low levels of contamination, those lanes appear essentially clear, as expected. Furthermore, our GFP cell negative control (which is a lane with no construct transfection, but with DNA) shows what is probably a contaminant, but does not show GFP since the band is too high. That is as expected. We should not see GFP expression if there has been no transfection.

Both PAX8 and GFP mRNA transcripts are being produced nearly identically in HeLa cells transfected with PGSH. This result was found regardless of the addition of additional glutathione. For PAX8, this shows that the constitutive promoter works as expected. Expression should occur, regardless of dosing. The expression of GFP in wells with and without additional glutathione, may be explained by two possibilities. The first possibility is that, the promoter sequence paired with GFP has a baseline level of expression that is independent of the activation of PAX8 by glutathione. The second possibility is that the wild type intracellular concentration of glutathione in HeLa is sufficient to activate PAX8 and induce expression of GFP. Since RT-PCR was only performed on wells with the lowest levels of glutathione, it cannot be concluded whether or not PAX8 transcriptional regulation of TG is being activated by glutathione.

One rather surprising result that the PAX8 cell(-) lane also shows similar expression patterns. This lane represents a lane where DNA was loaded, but the DNA did not contain any construct DNA since those wells were never transfected. Therefore, we would have expected to see no PAX8 expression. The expression of PAX8 can be explained by the fact that HeLa cells have been shown to have low levels of PAX8 expression (18). However, there is some debate about the actual level of PAX8 expression in HeLa, so further work would be required to ascertain what is causing this result.

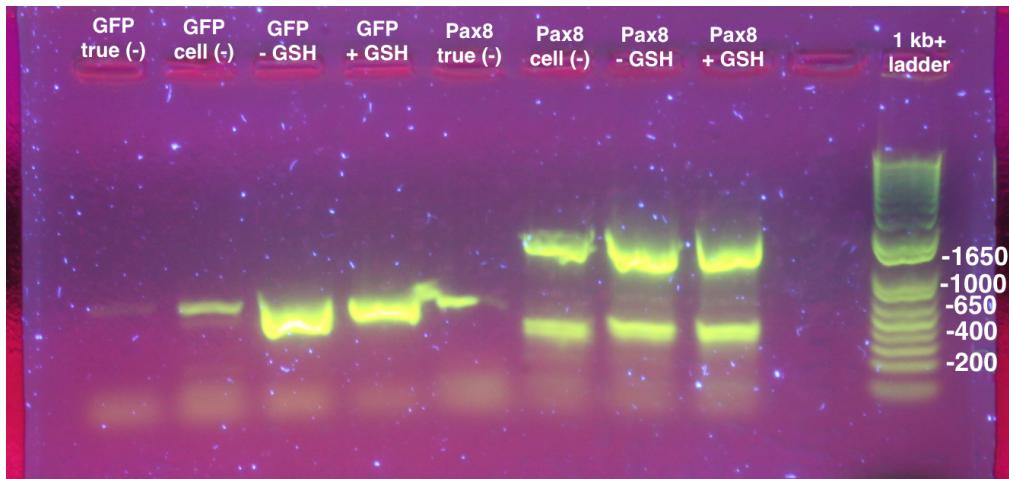


Figure 5: RT-PCR Results for RT-PCR performed on cDNA library. RNA extraction was performed on transfected HeLa cells, followed by incubation with reverse transcriptase to form cDNA library.

V. Discussion

Given our experimental results, it is possible that our DNA construct exhibits at least partial functionality, but does not function well enough to distinguish varying concentrations of glutathione in the extracellular space. However, our data cannot conclusively prove that statement quite yet. It is also possible that the expression we observed in our RT-PCR is simply due to baseline expression of PAX8 by the HeLa cells themselves. That would tell us that while the readout of the constructor is working, the PAX8 is not functioning quite as desired. However, even in that case, we would expect to see increasing GFP readout with increasing glutathione concentration, which is unfortunately not what was observed.

One potential cause of the failure of consistent transcription of the TG promoter in the presence of glutathione is the absence of other transcription factors such as TTF1 and TTF2 which together with PAX8 activate the TG promoter (10). These could not be included in our construct due to size constraints, but it is highly probable that the construct would function much better with these co-transcription factors included.

A good next step would be to quantify protein levels in the cells. The quantification would allow a better readout of whether GFP concentration is actually a baseline value, or whether it increases with increasing glutathione. The appropriate controls (i.e. non-transfected HeLa cell samples) would also allow us to determine whether or not PAX8 is natively present in HeLa cells as the above RT-PCR seems to suggest.

With some further work, this construct could be evaluated to see what is actually occurring in the system. Given those results, and perhaps given the ability to add in the co-transcriptional factors, it is possible that it could be optimized to work in HeLa. From there, it could be further optimized for a faster growing organism like E. coli or yeast, and then, potentially be used as a viable, relatively easy way to check organs for degradation exactly the time of collection. This

could be incredibly useful in organ preservation applications, and could potentially allow for a few more people to receive organs that may otherwise have been discarded.

VI. Author Contributions

The idea of assessing organ viability was initially conceptualized by Chris Mathy, but developed into a project by all three group members (Jon Deaton, Shivani Baisiwala, Chris Mathy). The DNA construct was designed collectively by all group members using Gene Designer 2.0. The construct functionality experiment was designed by Jon Deaton and Chris Mathy, while the PCR and rtPCR experiments were designed by Shivani Baisiwala. All experiments and maintenance of cell cultures were carried out by all three group members. The project proposal and final paper were written collectively by all group members

VII. References

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