Time Temperature Visual Indicators for Cold Chain Tracking of Biospecimens

by

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A Thesis Presented in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Approved April 2024 by the
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ARIZONA STATE UNIVERSITY

May 2024

ABSTRACT

For cold chain tracking systems, precision and versatility across varying time intervals and temperature ranges remain integral to effective application in clinical, commercial, and academic settings. Therefore, while electronic and chemistry/physics based cold chain tracking mechanisms currently exist, both have limitations that affect their application across various biospecimens and commercial products, providing the initiative to develop a time temperature visual indicator system that resolves challenges with current cold chain tracking approaches. As a result, a permanganate/oxalic acid time temperature visual indicator system for cold chain tracking has been proposed. At thawing temperatures, the designed permanganate/oxalic acid reaction system undergoes a pink to colorless transition as permanganate, Mn(VII), is reduced to auto-catalytic Mn(II), while oxalate is oxidized to CO_2. Therefore, when properly stored and vitrified or frozen, the proposed visual indicator remains pink, whereas exposure to thawing conditions will result in an eventual, time temperature dependent, designed color transition that characterizes compromised biospecimen integrity. To design visual indicator systems for targeted times at specific temperatures, absorbance spectroscopy was utilized to monitor permanganate kinetic curves by absorbance at 525 nm. As a result, throughout the outlined research, the following aims were demonstrated: (i) Design and functionality of 1x (0.5 mM $KMnO_4$) visual indicator systems across various time intervals at temperatures ranging from 25℃ to -20℃, (ii) Design and functionality of high concentration, 5x, visual indicator systems across varying targeted time intervals at temperatures ranging from 25℃ to 0℃, (iii) Pre-activation stability and long-term stability of the proposed visual indicator systems.

ACKNOWLEDGMENTS

I want to extend my gratitude to Dr. Chad Borges for all of his guidance, support, and mentorship throughout my academic and research journey during both my undergraduate and graduate studies. Throughout my research endeavors, Dr. Borges has continuously encouraged me as a researcher and academic, challenging and empowering me to further foster my critical thinking, research aptitude, and scientific knowledge bases. I've learned valuable lessons from his patience, leadership, and flexibility and his support has been invaluable and integral to my scholarly and personal development. Thank you!

I also want to express my gratitude to Dr. Marcia Levitus and Dr. Peter Williams for their encouragement and support throughout my scholarly theses. Their insightful perspectives on analytical methodologies and guidance have allowed me to further expand my scientific foundations and continue development of my research abilities. Thank you!

Additionally, I would also like to express my gratitude to all of the postgraduate, graduate, and undergraduate students within the Borges Lab. A special thanks to Dr. Jorvani Cruz Villarreal, Dr. Nilojan Jehanathan, Schuyler Kremer, Ishmael Quansah, and Aaron Uy!

Furthermore, I would like to acknowledge the National Cancer Institute of the National Institutes of Health, the Innovative Molecular Analysis Technologies (IMAT) Program, and Arizona State University Biodesign. The research throughout this thesis was completed with Grant R21CA250999 and Biodesign facilities.

Finally, I would like to thank my family and friends for their support along my journey.

TABLE OF CONTENTS

Page

CHAPTER Page

LIST OF TABLES

Table Page

Table Page

LIST OF FIGURES

Figure Page

CHAPTER 1

BACKGROUND

1.1 Introduction

Biochemical reactions serve as a template for numerous biological and cellular processes, providing the backbone for interactions between proteins, molecules, and chemical structures. However, these biochemical reactions are governed by the Laws of Thermodynamics, which state: (i) Zeroth Law of Thermodynamics: If two systems are both in thermal equilibrium with a third system, the two systems are also in thermal equilibrium with each other (ii) First Law of Thermodynamics: Energy cannot be created or destroyed, only transferred between systems (iii) Second Law of Thermodynamics: Heat transfer is accompanied with a change in entropy, and therefore work within an entire system must be less than the heat transferred into a system.[1] Within the Laws of Thermodynamics, heat transfer is defined as a change of thermal energy from warmer to cooler systems, resulting in an exchange of atomic/molecular kinetic energy.[1] However, the subsequent change in kinetic energy between systems can significantly impact the reaction rate, as demonstrated by the Arrhenius Equation: $k = A_0 e^{-E_a/k_B T}$

(Equation 1.1.1)[2]

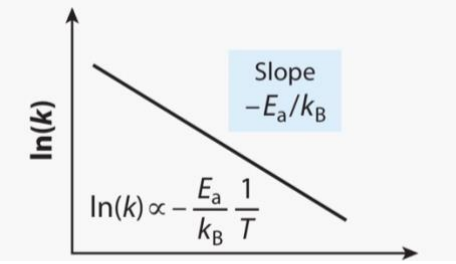


Figure 1.1.1: Arrhenius Plot. Relationship between the natural log of the reaction rate and inverse temperature in Kelvin. Reproduced from Knapp et al. [2]

As shown in *Equation 1.1.1* and *Figure 1.1.1*, the thermodynamic transfer of heat and increase in temperature (T) results in an increased reaction rate vis-à-vis an increase in the reaction rate constant, (k). Therefore, particularly for biochemical samples stored below room temperature, the transfer of

samples and exposure to non-ideal storage conditions can result in unnoticeable thawing that can compromise the molecular integrity of chemical or biological systems. As a result, thawing conditions are not only a threat for biospecimen and pharmaceutical product integrity, but also risk inaccurate research conclusions and non-viable clinical products. Consequently, cold chain tracking devices have been developed to track temperature changes in biospecimens for application in numerous academic, commercial, and clinical fields.

Unfortunately, currently marketed electronic or chemistry/physics-based cold chain tracking devices each have limiting drawbacks that impact their effectiveness for clinical and research aliquots. While electronic cold chain tracking devices have been successful and accurate at tracking temperature changes for containers of biochemical samples at temperatures well below 0°C, they remain expensive and are not applicable for individual vials, limiting their application for smaller or individualized aliquots commonly used in research settings. Adversely, chemistry/physics-based tracking mechanisms constitute an affordable cold chain tracking system that is applicable for individual vials and aliquots. However, many biospecimen samples require storage at temperatures well below 0°C, as such, current chemistry/physics-based tracking mechanisms remain unable to accurately track temperature changes for a variety of research and clinical samples with subzero storage temperatures. As a result, our lab has taken advantage of the permanganate/oxalate reaction mechanism to develop a color-transitioning time temperature visual indicator for cold chain tracking. Within our proposed visual indicators, both antifreeze-free and antifreeze salt-containing eutectic aqueous systems with depressed freezing/melting points will be implemented to increase

application of our proposed indicators to function at subzero storage temperatures. Therefore, the proposed and designed pink to clear color-changing visual indicator will provide a cost-effective cold chain tracking mechanism that is applicable across a large range of temperatures, including subzero, and can be implemented for individual aliquots for use within research settings.

1.2 Reaction Intermediates and Absorbance Spectra

The permanganate/oxalate reaction system to be utilized as our time temperature visual indicator is an auto-catalytic redox reaction characterized by a pink to colorless transition due to the reduction of Mn(VII) to Mn(II), as demonstrated in the following equation:

$$2MnO_4^- + 6H_3O^+ + 5H_2C_2O_4 \rightarrow 2Mn^{2+} + 14H_2O + 10CO_2 \quad \textit{(Equation 1.2.1)}^{3}$$

Therefore, within the mechanism, the previously mentioned pink to colorless transition is governed by the reduction of permanganate [Mn(VII)] to several reaction intermediates that contribute to the absorbance spectra, followed by subsequent reduction to Mn(II).

$$Mn(VII) \rightarrow Intermediates \rightarrow Mn(II) \quad \textit{(Equation 1.2.2)}^{5}$$

However, the intermediates represent a combination of distinct reaction components. Therefore, in their 1994 paper, Pimienta and colleagues utilized UV/Visible spectroscopy to explore and characterize the intermediates present within the reaction mechanism:

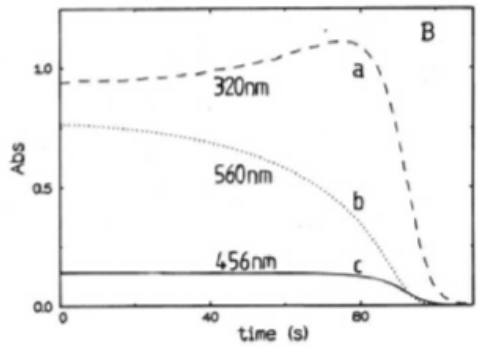


Figure 1.2.1: UV/Visible Spectrum for the $KMnO_4$/Oxalic Acid Reaction. Figure reproduced from Pimienta et al.[5]

Previous research indicated that permanganate absorbs in the region of 490 to 650 nm and therefore in *Figure 1.2.1*, the absorbance kinetics at a wavelength of 560 nm were correlated to the progressive disappearance of MnO_4^-.[4] Additionally, the absorbance spectra at 320 nm showcases progression to a maximum peak before a sharp decline, suggesting that one of the intermediates reaches its maximum value and subsequently decreases until disappearance. However, Pimienta and colleagues identified an isosbestic point at the start of the final kinetic curve, at 456 nm, and subsequently, further experiments were required to determine the identity and total number of reaction intermediates.[5] For isosbestic points, the absorbance remains constant during the course of the reaction and therefore varying concentrations of reactants or products should not impact the absorbance at the isosbestic point.[6] As a result, to further investigate the isosbestic point and characterize the intermediates, Pimienta and colleagues altered the initial concentrations of oxalic acid and sulfuric acid before calculating the wavelength at the isosbestic point across varying reagent concentrations:[5]

Table 1.2.1: Pimienta and Colleagues Isosbestic Experiments. *Varying concentrations of $H_2C_2O_4$ and H_2SO_4 are in molar and their subsequent effect on the isosbestic wavelength and percentage of bio(oxalate)manganate(III) and another intermediate species (x) within the permanganate/oxalic acid reaction is demonstrated. Table data reproduced from Pimienta et al.*[5]

No.	$[H_2C_2O_4]_0$	$[H_2SO_4]_2$	$t_{1/2}$	λ_{isos}	$\%III_2$	%x
1	0.2	0.27	240	450	91	9
2	0.025	0.27	220	452	69	31
3	0.0013	0.27	80	456	40	60
4	0.0013	0.09	180	456	32	68

As seen in *Table 1.2.1*, varying concentrations of oxalic acid result in slightly altered isosbestic wavelengths, suggesting the presence of two or more intermediates within the reaction mechanism. Since varied concentrations of reagents should not impact the wavelength of the isosbestic point, the data in *Table 1.2.1* implies that there are two components that contribute to the intermediate in altered quantities across differing reaction conditions. Otherwise, isosbestic wavelength changes due to varying concentrations of oxalic acid would be attributed to formation of a qualitatively new component of the reaction mechanism at different concentrations of the same reagents, which is unlikely. Therefore, Pimienta and colleagues were able to deduce that there is a second intermediate in the permanganate/oxalic acid reaction, along with bis(oxalate)manganate(III).[5] However, due to the instability of manganese(VI) and manganese(V) in acidic conditions, they were ruled out as potential intermediates, leaving Mn(IV) as the second intermediate in the reaction.[5] Therefore, the following skeleton mechanism outlining the manganese intermediates was constructed:

$$VII + II \rightarrow III + IV \qquad \textit{(Equation 1.2.3)}^5$$

$$IV \rightarrow III \qquad \textit{(Equation 1.2.4)}^5$$

$$III \rightarrow II \qquad \textit{(Equation 1.2.5)}^5$$

Then, the full compounds were substituted into the equations above, resolving the mechanism for the intermediates of the permanganate/oxalic acid reaction system:

$$MnO_4^- + MnC_2O_4 \rightarrow [Mn(C_2O_4)]^- + MnO_2 \qquad \textit{(Equation 1.2.6)}$$

$$MnO_2 \rightarrow [Mn(C_2O_4)]^- \qquad \textit{(Equation 1.2.7)}$$

$$[Mn(C_2O_4)]^- \rightarrow Mn(II) \qquad \textit{(Equation 1.2.8)}$$

Subsequently, identification of the intermediates that are present on the absorption spectrum allowed our lab to utilize extracted rate constants from Pimienta's work[4] to construct MATLAB scripts that simulated the reaction kinetics and concentrations for MnO_4^- and MnO_2. As a result, when designing visual indicators for various time lengths at 25°C (the only temperature at which the rate and equilibrium constants for the complex reaction mechanism are known), varying concentrations of the reactants were simulated to identify the composition that produces the reaction kinetics for specific time intervals, ranging from 5 minutes to multiple hours:

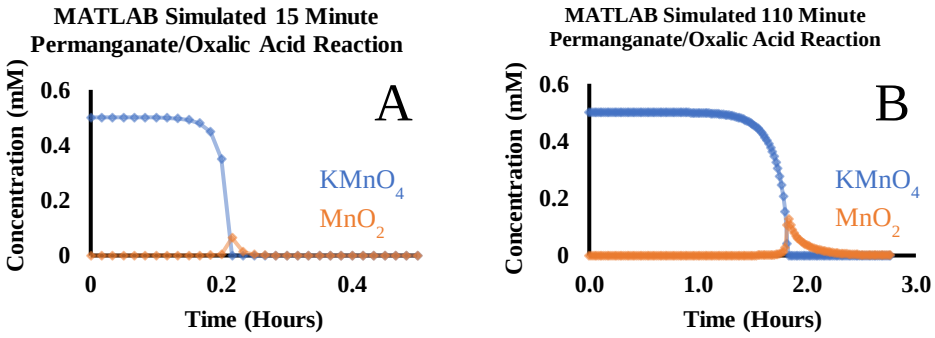


Figure 1.2.2: MATLAB Simulations for 15 and 110 Minute 1x Antifreeze-Free Systems. (A) $[HClO_4]$: 0.07 M, $[Na_2Oxalate]$: 1.38 mM, $[KMnO_4]$: 5.0 x 10^{-1} mM, $[Mn(ClO_4)_2]$: 1 nM (B) $[HClO_4]$: 6 mM, $[Na_2Oxalate]$: 1.38 mM, $[KMnO_4]$: 5.0 x 10^{-1} mM, $[Mn(ClO_4)_2]$: 1 nM.

In *Figure 1.2.2*, the simulated reactions were designed for approximately 15 minutes and 110 minutes, showcasing the concentration changes of MnO_4^- (in blue) and MnO_2 (in orange) as the reaction progresses to completion. Under favorable conditions,

the concentration of MnO_2 relates to an intermediate within the reaction mechanism that is ultimately converted to Mn(II), the final product, after a sharp peak at the final stages of the reaction. However, as further outlined in *Chapter 1.4*, excess MnO_2 precipitates out of solution, impacting the integrity of the visual indicator.

Nevertheless, while the MATLAB script serves as a powerful tool to simulate reaction kinetics for antifreeze-free systems, it is not possible to incorporate other components such as the eutectic antifreeze salts used for subzero temperature indicators (described in *Chapter 1.5*). Therefore, for eutectic systems, designed reaction times will be experimentally tested via systematic trial and error processes and subsequently analyzed via absorbance spectroscopy.

1.3 Reaction Mechanism:

As previously mentioned, the auto-catalytic redox permanganate/oxalate reaction involves a series of simultaneous rate laws and intermediate steps. In the reaction mechanism, the initially pink MnO_4^- is reduced through the previously outlined intermediates (*Equations 1.2.6, 1.2.7, 1.2.8)* to Mn(II), forming a colorless solution. When stored below its freezing point, the solution should remain pink as the reaction is not running while frozen. However, if exposed to non-ideal storage and thawed conditions, the reaction will take place and eventually change from pink to colorless. By keeping the indicator next to the biospecimens or samples through their lifetime, the color change will indicate that the biospecimen or sample was exposed to non-ideal storage or transportation temperatures. For experiments and application, the proposed visual indicators will be designed with varying concentrations of the following reagents: Antifreeze-free or antifreeze-salt-containing aqueous eutectic solvents (Sodium

Perchlorate ($NaClO_4$/-37°C), Magnesium Perchlorate ($Mg(ClO_4)_2$/-67°C) or Lithium Perchlorate ($LiClO_4$/-18°C)), Perchloric acid ($HClO_4$), Disodium oxalate ($Na_2C_2O_4$), Potassium permanganate ($KMnO_4$), and Manganese Perchlorate ($Mn(ClO_4)_2$). While previous research utilized sulfuric acid within the reaction mechanism, our lab substituted perchloric for sulfuric acid to establish the ClO_4^- anion as the common ion in solution with the previously outlined antifreeze salts. Additionally, a strong acid such as perchloric acid will fully dissociate in solution, as shown in *Equation 1.3.1*, while also protonating disodium oxalate to oxalic acid, $H_2C_2O_4$, as shown in Equations *1.3.3 and 1.3.4*:

$$HClO_4 \leftrightharpoons ClO_4^- + H^+ \text{ (K = Strong Acid)} \qquad \textit{(Equation 1.3.1)}^4$$

$$Na_2C_2O_4 \rightarrow C_2O_4^{2-} + 2Na^+ \qquad \textit{(Equation 1.3.2)}$$

$$C_2O_4^{2-} + H^+ \leftrightharpoons HC_2O_4^- \text{ (K = 5 x } 10^{-5} \text{ mol x } L^{-1}\text{)} \qquad \textit{(Equation 1.3.3)}^4$$

$$HC_2O_4^- + H^+ \leftrightharpoons H_2C_2O_4 \text{ (K = 5 x } 10^{-2} \text{ mol x } L^{-1}\text{)} \qquad \textit{(Equation 1.3.4)}^4$$

Consequently, throughout the paper, the reaction will be described as a permanganate/oxalic acid reaction rather than a permanganate/oxalate reaction. However, some of the oxalate anion will not be protonated, and therefore free oxalate anions are able to react in solution with Mn(II), forming a complex that produces Mn(II) oxalate:

$$Mn^{2+} + C_2O_4^{2-} \leftrightharpoons MnC_2O_4 \text{ (K = 1.6 x } 10^{7} \text{ mol}^{-1} \text{ x } L^{-1}\text{)} \qquad \textit{(Equation 1.3.5)}^4$$

Equation 1.3.5 also demonstrates the autocatalytic nature of the reaction as Mn(II) not only serves as the final product of the reaction but is utilized through the reaction to continuously produce more Mn(II) oxalate that will eventually be converted into Mn(II) following a series of intermediate steps. Additionally, Mn(II) oxalate will not contribute to the absorbance spectra and solely serves as an intermediate in the reaction mechanism,

subsequently reacting with permanganate and hydrogen ions in a redox reaction to form Mn(IV) dioxide and Mn(III):

$$4H^+ + MnO_4^- + MnC_2O_4 \rightarrow MnO_2 + Mn^{3+} + 2CO_2 + 2H_2O$$ *(Equation 1.3.6)*[4]

($k = 5 \times 10^3\ mol^{-1} \times L \times s^{-1}$)

Similar to MnO_4^-, Mn(IV) dioxide concentration can be monitored via absorbance spectroscopy (provided its concentrations remains low enough to maintain its solubility) and as one of the intermediates in the conversion of MnO_4^- to Mn(II), Mn(IV) dioxide will react with oxalic acid to form a Mn(IV) oxalic acid complex that will subsequently react with protons in the following reactions:

$$MnO_2 + H_2C_2O_4 \leftrightharpoons [MnO_2, H_2C_2O_4]$$ *($K = 1.77 \times 10^4\ mol^{-1} \times L$)* *(Equation 1.3.7)*[4]

$$2H^+ + [MnO_2, H_2C_2O_4] \rightarrow Mn^{3+} + CO_2 + CO_2^{\cdot -} + 2H_2O$$ *(Equation 1.3.8)*[4]

($k = 8 \times 10^{-2}\ s^{-1}$)

Following the dissociation of the Mn(IV) oxalic acid complex, previously generated Mn(III) reacts with the oxalate anion to form Mn(III) oxalate that undergoes multiple reactions to form the final product of the permanganate/oxalic acid reaction: Mn(II)

$$Mn^{3+} + C_2O_4^{2-} \leftrightharpoons [Mn(C_2O_4)]^+$$ *($K = 2 \times 10^9\ mol^{-1} \times L$)* *(Equation 1.3.9)*[4]

$$[Mn(C_2O_4)]^+ + C_2O_4^{2-} \leftrightharpoons [Mn(C_2O_4)]^-$$ *($K = 1.29 \times 10^8\ mol^{-1} \times L$)* *(Equation 1.3.10)*[4]

$$[Mn(C_2O_4)]^+ \rightarrow Mn^{2+} + CO_2 + CO_2^{\cdot -}$$ *($k = 0.92\ s^{-1}$)* *(Equation 1.3.11)*[4]

$$[Mn(C_2O_4)]^- \rightarrow Mn^{2+} + CO_2 + CO_2^{\cdot -} + C_2O_4^{2-}$$ *($k = 1.3 \times 10^{-3}\ s^{-1}$)* *(Equation 1.3.12)*[4]

$$2CO_2^{\cdot -} \rightarrow C_2O_4^{2-}$$ *($k = 10^9\ mol^{-1} \times L \times s^{-1}$)* *(Equation 1.3.13)*[4]

1.4 – MnO_2 Insolubility

While the previously defined MATLAB script allows our lab to simulate reaction kinetics across a wide range of experimental times, simulated reactions demonstrate that decreased concentrations of oxalic acid result in increased MnO_2 concentrations, which at high enough concentrations become insoluble and derail the reaction kinetics of the designed visual indicators. As previously shown in *Equation 1.3.7*, MnO_2 reacts with oxalic acid to form a Mn(IV) oxalic acid complex that ultimately dissociates into Mn(III) and other byproducts. Therefore, decreased disodium oxalate concentrations that vary from the ideal stochiometric ratios results in increases of MnO_2, which consequently results in incomplete reactions and disrupted reaction kinetics:

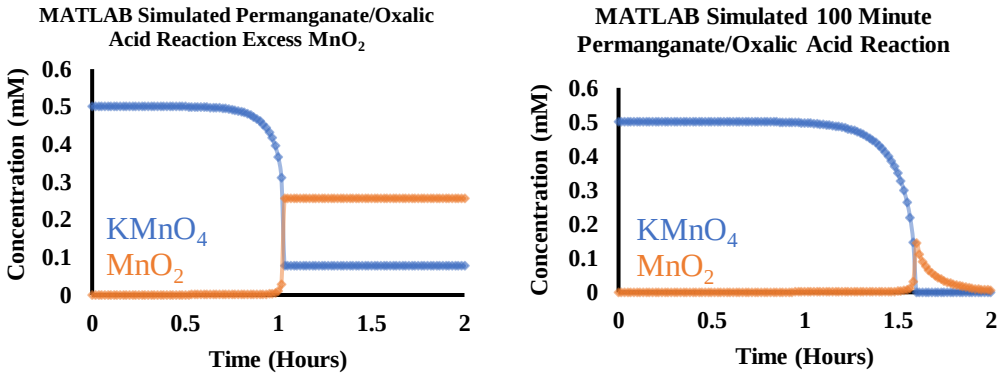


Figure 1.4.1: MATLAB Simulations Demonstrating MnO_2 spike. (A) $[HClO_4]$: 8 mM, $[Na_2Oxalate]$: 700 uM, $[KMnO_4]$: 5.0 x 10^{-1} mM, $[Mn(ClO_4)_2]$: 1 nM (B) $[HClO_4]$: 8 mM, $[Na_2Oxalate]$: 1.38 mM, $[KMnO_4]$: 5.0 x 10^{-1} mM, $[Mn(ClO_4)_2]$: 1 nM.

As shown in *Figure 1.4.1*, a decrease in oxalic acid from nominal concentrations of 1.38 mM to 700 µM results in increased concentrations of MnO_2, as represented by the orange line. Due to the stochiometric ratios within the reaction mechanism, disodium oxalate is a limiting reagent and in conditions with a significant decrease in the concentration of disodium oxalate, there is not enough oxalic acid to react with permanganate in solution, resulting in the outlined MnO_2 precipitation. However, increased concentrations of $MnClO_4$ as a reagent can also result in a disruption of the

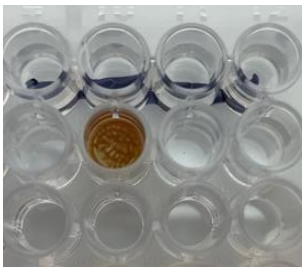


Figure 1.4.2: MnO_2 Precipitate Formation.

desired kinetics curve of MnO_4^-. Subsequently, while MnO_2 serves as an intermediate in the reaction mechanism and therefore smaller concentrations of MnO_2 are produced during the progression of the permanganate/oxalic acid reaction, larger quantities are insoluble, forming a film of brown precipitate that impacts the proposed visual indicator. Therefore, MATLAB simulations and experimental conditions will be adjusted to avoid large quantities and spikes of MnO_2, preserving the color integrity of the visual indicator.

1.5 Implementation of Antifreeze-Salt-Containing Eutectics

As previously mentioned, one of the limitations of current chemistry/physics based cold chain tracking systems is their ability to be active much below 0°C. Therefore, our lab proposes four versions of the permanganate/oxalic acid visual indicator utilizing antifreeze-free or antifreeze-salt-containing aqueous eutectic solvents. While the antifreeze-free visual indicator will remain accurate and reliable for temperatures above 0°C, utilizing antifreeze-salt-containing eutectics will allow for our visual indicators to function (i.e., maintain chemical reactivity) at temperatures down to -67°C, increasing the range for which our visual indicators have application in clinical and research settings. Therefore, while various antifreeze-salt-containing aqueous eutectic solvents such as LiCl and $MgCl_2$ have been previously tested within our lab[13], perchlorate antifreeze salts were chosen due to the inability of permanganate to further oxidize ClO_4^-.

For antifreeze-salt-containing aqueous eutectic solvents, the combination of two components at a specific molar ratio results in a depressed freezing/melting point that is well below the individual freezing points of the two individual components.[7] However, while hydrogen bonding contributes to the decrease in freezing point, the eutectic solutes must remain soluble in aqueous media.[8] Therefore, antifreeze-salt-containing eutectics operate at specific molar ratios and the eutectic or freezing point of the compound exists at a particular chemical composition.[8] As a result, the three antifreeze-salt-containing aqueous eutectic solvents proposed for our visual indicators have specific eutectic temperatures and the subsequent phase diagrams:

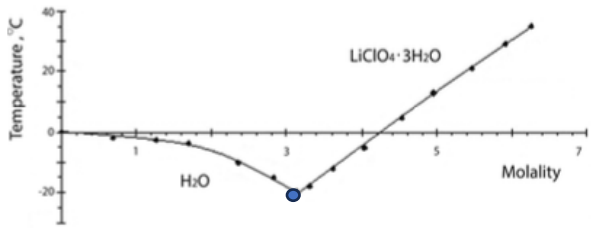


Figure 1.5.1: Phase Diagram for $LiClO_4$. The blue labeled point, the eutectic point, is at 3.11 molal and -18℃. Figure reproduced from Davidian et al.[9]

In *Figure 1.5.1*, the $LiClO_4$ phase diagram showcases that $LiClO_4$ has a eutectic point at -18°C, making the $LiClO_4$ system specifically applicable for biospecimens with acceptable storage temperatures below -18°C. When frozen, the proposed $LiClO_4$ visual indicator systems should remain pink until exposure to thawing or improper storage conditions above -18°C. Therefore, since previous research indicated that $LiClO_4$ has a weight-to-weight ratio of 22% at its eutectic point (equivalent to 3.11 molal), $LiClO_4$ solvents will be created with specific mass ratios to ensure that the solvent is at its eutectic composition in our proposed visual indicator system[9]. On the other hand, $NaClO_4$ has a lower eutectic temperature:

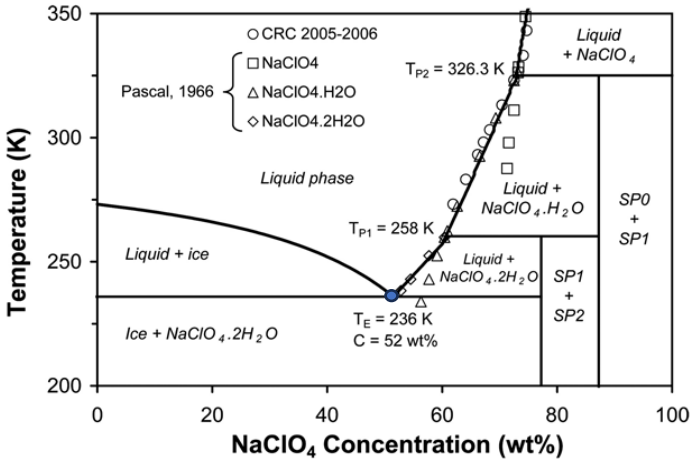


Figure 1.5.2: Phase Diagram for $NaClO_4$. The blue labeled point, the eutectic point, is at 236K or -37℃ and a composition of 52% w/w. Reproduced from Chevrier et al.[10]

As shown in *Figure 1.5.2*, $NaClO_4$ has a freezing point at -37°C and a weight-to-weight ratio of approximately 52% w/w at the eutectic point. Therefore, similar to $LiClO_4$, the $NaClO_4$ solvent will be at its eutectic composition within the proposed visual indicator systems. Additionally, sodium perchlorate systems will be specifically designed and applicable for biospecimen samples with storage temperatures below -37°C, when the visual indicator is frozen. Finally, the phase diagram for $Mg(ClO_4)_2$ is shown below:

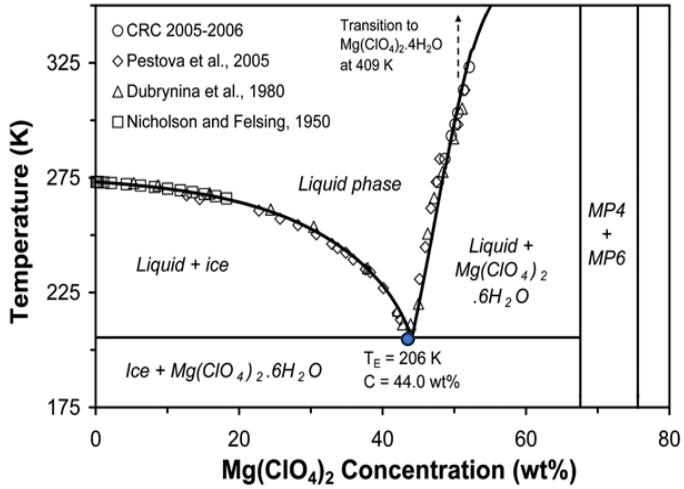


Figure 1.5.3: Phase Diagram for $Mg(ClO_4)_2$. Eutectic point at 206 K or -67℃ at a composition of 44% w/w. The blue point represents the eutectic point. Reproduced from Chevrier et al.[10]

As demonstrated in *Figure 1.5.3,* $Mg(ClO_4)_2$ has a freezing point at -67°C or 206K at its eutectic point while at a weight-to-weight ratio of 44%. Subsequently, visual indicators designed with $Mg(ClO_4)_2$ will be applicable for storage temperatures below -67°C. Overall, implementation of antifreeze-salt-containing eutectics represent a viable option to increase the application of our proposed visual indicators, resolving some of the subzero temperature challenges and limitations present in currently utilized cold chain tracking systems.

1.6 Absorbance Spectroscopy

Absorbance spectroscopy will be utilized to monitor experimental reaction kinetic curves for the permanganate/oxalic acid reaction systems. While our MATLAB script demonstrated various intermediate species that absorb to lesser extents at differing wavelengths in the visible spectrum, our spectrophotometers will be focused on the absorbance of permanganate. By utilizing a specific wavelength, we are able to ensure that a majority of the absorbance recorded is due to MnO_4^-, with minimal absorbance from other analyte species within the reaction mechanism. Previous literature on the absorbance of permanganate generated numerous absorbance values at different wavelengths, illustrating that MnO_4^- can absorb at a range of wavelengths, with a λ_{max} of 525 nm. As a result, our spectroscopy experiments will be performed at a wavelength of 525 nm, the wavelength with the greatest sensitivity to MnO_4^-, to maximize its absorbance.[11] Consequently, our lab will acquire kinetic curves at 525 nm to "monitor" reaction length, utilizing absorbance spectroscopy to achieve proposed targeted runs times across replicates and different reaction systems.

In absorbance spectroscopy, photons of a particular wavelength are passed through a solution, exciting electrons of various analytes and chemicals. For the permanganate/oxalic acid reaction, there are various absorbing species such as MnO_4^-, MnO_2, and bis(oxalate)manganate(III), as indicated in *Chapter 1.2*. When these analytes are exposed to a light source, their electrons temporarily become excited and "jump" to a higher energy state before eventually falling back down to their ground state energy level without emitting photons. However, because photons are interacting with absorbing species, the power of the light beam after passing through a solution full of analytes is less than the incident power. Therefore, the transmittance or fraction of the light beam that passes through the solution can be determined by the following equation:

$$T = \frac{P}{P_0} \qquad \textit{(Equation 1.6.1)}^{12}$$

In *Equation 1.6.1*, T represents the transmittance or the fraction of light that passes through the analytes in solution, P is equal to the final power of the light beam, and P_0 illustrates the initial power of the light beam. Utilizing the final value of transmittance, the absorbance of the absorbing species can be calculated via the following equation:

$$A = -\log(T) = -\log\left(\frac{P}{P_0}\right) \qquad \textit{(Equation 1.6.2)}^{12}$$

However, while the absorbance of a particular analyte can be calculated via *Equations 1.6.1 and 1.6.2*, absorbance spectroscopy is governed by the Beer-Lambert Law. In Beer's Law, the absorbance of a chemical is directly related to the product of the molar absorptivity, path length, and concentration of the analyte or absorbing species, as shown below:

$$A = \varepsilon bc$$ *(Equation 1.6.3)*[12]

In *Equation 1.6.3*, A represents the absorbance, ε serves as the molar absorptivity in L x mol^{-1} x cm^{-1}, b is equal to the pathlength in centimeters, and c represents the absorbing species' concentration, in molarity. Therefore, the absorbance or concentration of a particular species, such as MnO_4^-, can either be calculated or analyzed via absorbance spectroscopy.

Our experimental methods will include collecting absorbance data on different spectrophotometers, a Cary 60 UV/Visible Spectrophotometer, a ThermoFisher UV/Visible MultiSkan Go Spectrophotometer or a SpectraMax iD5 Standard Multi-Mode Microplate Detection Platform. While the ThermoFisher UV/Visible Spectrophotometer and SpectraMax iD5 Standard Multi-Mode Microplate Detection Platform can perform spectroscopy analysis at temperatures ranging from room temperature to 65°C, the reaction kinetics for visual indicators systems at cooler temperatures, particularly subzero, cannot be accurately acquired using these instruments. Therefore, the Cary 60 UV/Visible Spectrophotometer will be used for reaction systems within the temperature range of -20°C to 4°C. To achieve low temperatures, the Cary Spectrophotometer will be connected to a circulating cooling bath, qChanger controller and nitrogen gas line as shown below:

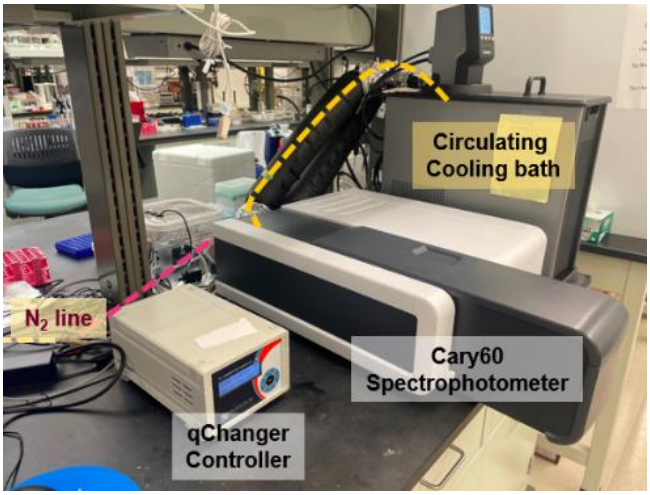


Figure 1.6.1: Cary 60 UV/Visible Spectrophotometer Setup in the Borges Lab.

In *Figure 1.6.1*, the qChanger Controller controls the temperature within the Cary 60 Spectrophotometer while the circulating cooling bath circulates a cooled 47% v/v polyethylene glycol and water mixture that is generally set for a couple of degrees under the target temperature. Additionally, a N_2 line is connected to the spectrophotometer at a pressure of approximately 10-20 psi to prevent condensation on the quartz cuvettes within the spectrophotometer at cold temperatures. The qChanger controller additionally allows the use of up to six cuvettes simultaneously by rotating the cuvettes during the absorbance readings.

1.7 Objectives

Currently, there are two main cold chain tracking mechanisms for clinical and academic settings: chemistry/physics aqueous based cold chain tracking and electronic cold chain tracking. However, both currently utilized methods have various limitations and therefore, research settings continue to lack cold chain tracking systems that are affordable, effective, and consistently accurate at subzero temperatures. As a result, we propose a permanganate/oxalic acid visual indicator system that resolves some of the

previously outlined challenges for cold chain tracking of archived biospecimens and clinical samples. Therefore, the objectives for this thesis are:

I. Demonstrate the functionality of 1x (0.5 mM $KMnO_4$) visual indicator systems and subsequent design of 14 outlined targeted indicators.

II. Explore the functionality of higher concentration indicator systems (5x or 10x) and design of 11 proposed high concentration targeted indicators.

III. Investigate the pre-activation stability and long-term stability of the visual indicator systems.

The thesis will be divided into 5 distinct chapters. The first chapter provides background on the proposed permanganate/oxalic acid system, including explanations of reaction mechanisms, absorbance spectroscopy, and implementation of antifreeze-salt-containing eutectics. The second chapter outlines the functionality of the 1x visual indicator systems, including the design of the proposed targeted indicators and application of MATLAB simulations. Moreover, the third chapter explores high concentration visual indicators (5x or 10x) and outlines their subsequent design to achieve additional targeted indicators. Additionally, the fourth chapter explores the pre-activation stability and long-term stability of the proposed permanganate/oxalic acid indicators. Finally, the fifth chapter provides conclusions and future outlook for the visual indicator systems.

CHAPTER 2

1X TIME TEMPERATURE VISUAL INDICATOR SYSTEMS

2.1 Introduction

Previous research within our lab explored the functionality of the visual indicator systems, demonstrating that targeted run times could be achieved and reproduced across controlled temperatures with various antifreeze-free and antifreeze-salt-containing aqueous eutectic solvents.[12] Therefore, to evaluate the use of the visual indicator system across various solvents and reaction conditions, our lab proposed to design specific time-temperature indicators based on the permanganate/oxalic acid reaction, aiming for the following reaction times and temperatures:

Table 2.1.1 Proposed 1x Targeted Visual Indicators

Antifreeze-Free	Indicator #1 – 1x Antifreeze-Free System Running 60 Minutes at 25℃
	Indicator #2 – 1x Antifreeze-Free System Running 90 Minutes at 25℃
	Indicator #3 – 1x Antifreeze-Free System Running 120 Minutes at 25℃
	Indicator #4 – 1x Antifreeze-Free System Running 18 Hours at 4℃
	Indicator #5 – 1x Antifreeze-Free System Running 24 Hours at 4℃
	Indicator #6 – 1x Antifreeze-Free System Running 60 Minutes at 0℃
$NaClO_4$	Indicator #7 – 1x $NaClO_4$ System Running 5 Minutes at 25℃
	Indicator #8 – 1x $NaClO_4$ System Running 60 Minutes at 0℃
	Indicator #9 – 1x $NaClO_4$ System Running 7 Days at -20℃
$Mg(ClO_4)_2$	Indicator #10 – 1x $Mg(ClO_4)_2$ System Running 5 Minutes at 25℃
	Indicator #11 – 1x $Mg(ClO_4)_2$ System Running 60 Minutes at 0℃
	Indicator #12 – 1x $Mg(ClO_4)_2$ System Running 7 Days at -20℃
$LiClO_4$	Indicator #13 – 1x $LiClO_4$ System Running 5 Minutes at 25℃
	Indicator #14 – 1x $LiClO_4$ System Running 60 Minutes at 0℃

As shown in *Table 2.1.1*, there are 14 proposed indicators designed for the 1x permanganate/oxalic reaction systems, ranging from 5 minutes to 7 days. Within a research setting, the impact of thawing conditions on a research or clinical sample depends on the contents and storage temperature of the particular sample or biospecimen. Therefore, designed visual indicators for cold chain tracking need to be applicable across

various temperature ranges and time scales, prompting our experimental planning to design 14 indicators for the 1x system.

However, the functionality of each of our proposed indicators will be further explored across various temperatures. For example, Indicator #1 is designed to run for 60 minutes at 25°C, but the same reaction system (same composition) is also experimentally tested at 4°C. In a research setting, if a particular aliquot or clinical sample cannot be exposed to 25°C for more than 60 minutes, our proposed Indicator #1 can be applicable to monitor biospecimen integrity. Therefore, if Indicator #1 is exposed to thawing conditions for 60 minutes at 25°C, the visual indicator will appear clear, indicating a non-viable sample. However, cooler, or warmer temperatures will also affect the kinetics of the reaction system and subsequent reaction times. As a result, if Indicator #1 is exposed to thawing conditions at 4°C, the reaction will slowly progress but will not turn clear after only 60 minutes, indicating exposure to thawing conditions but not complete loss of biospecimen integrity.

Nevertheless, before designing our proposed visual indicators, we utilized our previously outlined MATLAB simulations to investigate the impact of varying concentrations of the following reagents: perchloric acid, sodium oxalate, and manganese perchlorate. As previously mentioned, the permanganate/oxalic acid reaction is autocatalytic, resulting in the sharp decrease in MnO_4^- at the end of the reaction, but hydronium ions are also reactants. Therefore, either manganese perchlorate or perchloric acid can be added in small quantities to increase the reaction kinetics:

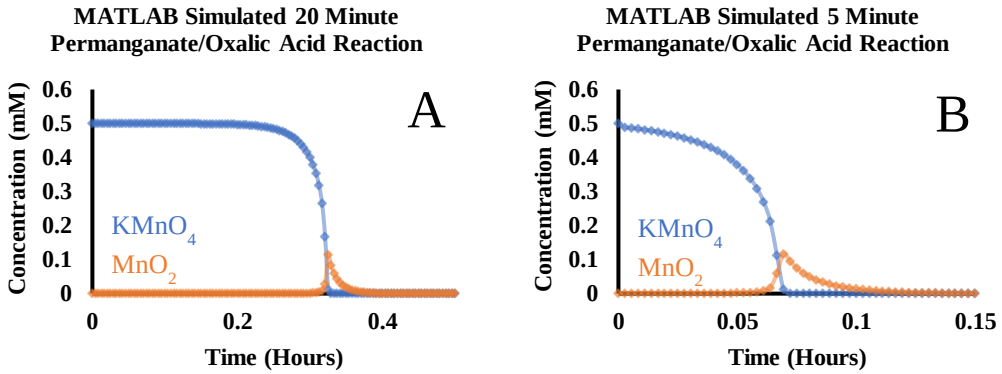


Figure 2.1.1: MATLAB Simulations Demonstrating the Effect of Increasing $Mn(ClO_4)_2$. (A) $[HClO_4]$: 50 mM, $[Na_2Oxalate]$: 1.38 mM, $[KMnO_4]$: 5.0 x 10^{-1} mM, $[Mn(ClO_4)_2]$: 1.0 x 10^{-6} mM. (B) $[HClO_4]$: 50 mM, $[Na_2Oxalate]$: 1.38 mM, $[KMnO_4]$: 5.0 x 10^{-1} mM, $[Mn(ClO_4)_2]$: 1.0 x 10^{-2} mM.

As seen in *Figure 2.1.1*, addition of the manganese perchlorate catalyst decreased the reaction time of the simulated reaction from approximately 20 minutes to 5 minutes, as expected. As a catalyst within the reaction, additional Mn(II) ions react with oxalic anions as shown in *Equation 1.3.5*, forming Mn(II) oxalate that subsequently reacts with MnO_4^- in *Equation 1.3.6*, decreasing the reaction time of the visual indicator system. Moreover, despite nitric acid washing procedures (described below) and use of a laminar flow hood, trace quantities of Mn(II) and MnO_2 are likely present among the reactants and lab materials. Therefore, when applicable, reaction compositions were constructed with small concentrations of manganese perchlorate to minimize the effect of extraneous manganese ions that could be present from the environment. Additionally, increasing the concentration of acid within solution resulted in increased reaction rate kinetics and subsequently decreased reaction time:

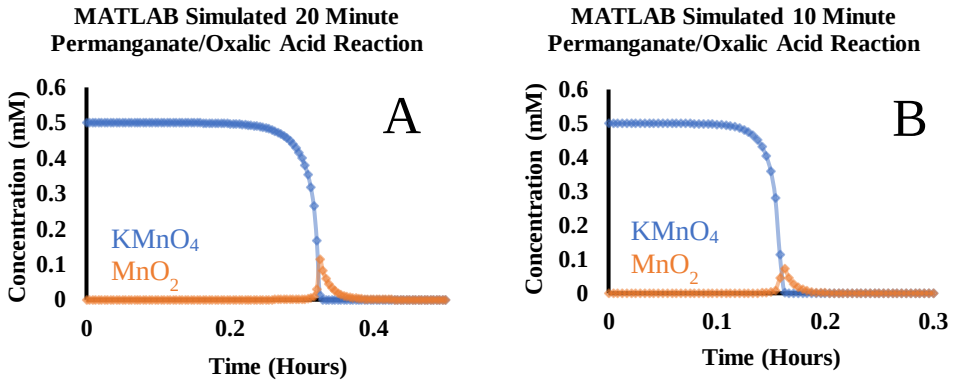


Figure 2.1.2: MATLAB Simulations Demonstrating Effect of Increasing $HClO_4$. (A) [$HClO_4$]: 50 mM, [$Na_2Oxalate$]: 1.38 mM, [$KMnO_4$]: 5.0 x 10^{-1} m, [$Mn(ClO_4)_2$]: 1.0 x 10^{-6} mM. (B)[$HClO_4$]: 90 mM, [$Na_2Oxalate$]: 1.38 mM, [$KMnO_4$]: 5.0 x 10^{-1} mM, [$Mn(ClO_4)_2$]: 1.0 x 10^{-6} mM.

As seen in *Figure 2.1.2*, increasing the final concentration of $HClO_4$ from 50 mM to 90 mM decreased the simulated reaction time of the 1x antifreeze-free reaction by approximately half, illustrating that varying $HClO_4$ concentrations could be an effective tool in adjusting reaction systems to achieve targeted indicators. Subsequently, decreases in the concentration of $HClO_4$ will be utilized to design and produce longer visual indicator systems, particularly for indicators such as #9 and #12 in *Table 2.1.1*. Nevertheless, decreasing the concentration of $HClO_4$ consequently decreases proton concentrations within solution, resulting in the progressive accumulation of MnO_2 and potential disruption of our visual indicator, as previously outlined. However, since MnO_2 reacts with oxalic acid within the permanganate/oxalic acid mechanism, varying concentrations of disodium oxalate will be tested to explore increasing the reaction time of the visual indicator systems without the formation of MnO_2. Subsequently, MATLAB simulations of increasing $Na_2Oxalate$ concentrations generated the following results:

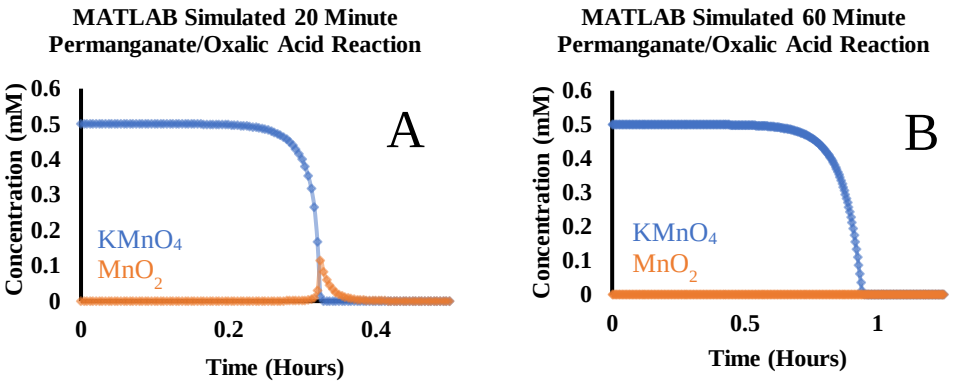


Figure 2.1.3: MATLAB Simulations Demonstrating Effect of Increasing $Na_2Oxalate$. (A) $[HClO_4]$: 50 mM, $[Na_2Oxalate]$: 1.38 mM, $[KMnO_4]$: 5.0 x 10^{-1} mM, $[Mn(ClO_4)_2]$: 1.0 x 10^{-6} mM. (B) $[HClO_4]$: 50 mM, $[Na_2Oxalate]$: 5 mM, $[KMnO_4]$: 5.0 x 10^{-1} mM, $[Mn(ClO_4)_2]$: 1.0 x 10^{-6} mM.

As shown in *Figure 2.1.3*, increasing the final concentration of $Na_2Oxalate$ from 1.38 mM to 5 mM resulted in an increase of simulated reaction time from approximately 20 minutes to 60 minutes without an MnO_2 spike. Therefore, varying $Na_2Oxalate$ concentration provides another valuable tool to adjusting reaction length time, particularly when $HClO_4$ concentrations cannot be further decreased due to MnO_2 spikes. Finally, the last component of the permanganate/oxalic visual indicator systems is potassium permanganate, which provides the pink coloration that characterizes the visual indicator. However, to ensure similar permanganate coloration across various indicator systems, the concentration of MnO_4^- will remain consistent across the 1x indicator systems. Therefore, 1x time temperature indicators will be designed with varying quantities of perchloric acid, disodium oxalate, and manganese permanganate.

2.2 Methods and Materials

2.2.1 Materials

The spectrophotometers utilized throughout experimental procedures were the Cary 60 UV/Visible Spectrophotometer (Agilent Technologies), ThermoFisher

UV/Visible MultiSkan Go Spectrophotometer (ThermoFisher), and the SpectraMax iD5 Standard Multi-Mode Microplate Detection Platform (Molecular Devices). To complement the Cary 60, the circulating cooling bath was purchased from Fisher Scientific whereas the nitrogen gas tanks were purchased and refilled at the ASU Stores - Gas Services. Additionally, for the Cary 60 Spectrophotometer, 1 mm path length Quartz Cuvettes (Agilent Technologies) were used while skirted, clear polystyrene 96 well plates (Thomas Scientific) were utilized in the (MultiSkan Go) ThermoFisher and (iD5 Standard Multi-Mode Microplate Detection Platform) SpectraMax spectrophotometers.

Moreover, a laminar flow hood (Terra Universal) and a Purelab Flex 3 Lab Water Purification System (ELGA Labwater) were purchased and utilized throughout experiments. Additionally, the following solvents and acids were used throughout experiments: Nitric acid (HNO_3, Cat. No. 87003-261, 67-70% ARISTAR PLUS for trace metal analysis, VWR), Sodium perchlorate hydrate ($NaClO_4$, Cat. No. 381225, 99.99% pure on trace metal basis, Sigma-Aldrich), Magnesium perchlorate ($Mg(ClO_4)_2$, Cat. No. 63095, Sigma-Aldrich), Perchloric acid ($HClO_4$, Cat. No. 311421, Sigma-Aldrich), Lithium perchlorate anhydrous ($LiClO_4$, Cat. No. 431567, 99.99% pure on trace metals basis, Sigma-Aldrich), Manganese perchlorate ($Mn(ClO_4)_2$, Cat. No. 44318, 99.995% pure on metals basis, Fisher Scientific), Sodium oxalate ($Na_2C_2O_4$, Cat. No. 379735, Sigma-Aldrich) and Potassium permanganate ($KMnO_4$, Cat. No. 223468, 99.0%, Sigma-Aldrich).

2.2.2 Safety Considerations

As outlined, experimental reagents and chemicals include flammable, volatile, and corrosive reagents. Therefore, appropriate personal protective equipment and a fume hood were utilized during experimental procedures to protect from physical or personal injury due to direct contact with solvents or chemical compounds. Appropriate personal protective equipment included solvent resistant gloves, lab coats, protective eyewear, and protective Cryo-Gloves when handling samples in the liquid nitrogen or -80°C freezer storage systems. Safety instructions and considerations were followed to avoid instrument and equipment damage.

2.2.3 Nitric Acid Decontamination Procedure

Particularly in the early stages of experimental testing, environmental and labware contamination impacted the replicability and consistency of the permanganate/oxalic acid indicators. As previously alluded to, exogenous Mn(II) was likely present in some of the labware and reagents, and therefore a nitric acid decontamination procedure was developed to improve reaction precision and consistency among replicates. Consequently, prior to experimental runs, all glassware, 96 well plates or quartz cuvettes, pipette tips, and Eppendorf tubes were thoroughly washed and soaked overnight with 2% nitric acid.

A 2% nitric acid solution was prepared using high purity 18.2 MΩ*cm deionized water in a previously nitric acid washed Teflon bottle. The inside of the Eppendorf tubes or labware was rinsed with nitric acid, for a total of three times, before the labware was subsequently filled with the nitric acid for overnight soaking in the fume hood.

For pipette tips, racks of 10 µL and 200 µL pipette tips were suspended overnight in containers full of 2% nitric acid whereas 1 mL pipette tips were collected and stored

overnight in a previously washed glass beaker. For 10 µL and 200 µL pipette tips, racks of each respective pipette tip were placed into a container that allowed the nitric acid level to rise above the pipette rack. Washed labware was used as a weight over the pipette rack to ensure it stayed under the nitric acid level. For 1 mL pipette tips, batches of pipette tips were collected in a previously washed glass beaker filled with 2% nitric acid covering all tips. A washed glass watch was placed over the beaker to ensure that all the 1 mL pipettes were fully suspended for overnight washing.

After overnight washing, pipette tips were drained and shaken to release any residual nitric acid droplets before being placed in the laminar flow hood for air-drying. Similarly, containers with overnight nitric acid were drained and transferred to the laminar flow hood for air-drying. After the air-drying process was completed, all the previously washed labware and materials were stored and covered in rinsed food saver vacutainers until use.

2.2.4 Experimental Procedure for 1x Visual Indicators at 25℃

Experimental reactions were designed for each of the 14 indicators for subsequent kinetic analysis in the spectrophotometer at 525 nm. Firstly, the antifreeze-salt-containing eutectics were quantified via a CETAB precipitation titration method that was potentiometrically monitored with a perchlorate selective electrode before being transferred from their glass containers into smaller aliquots to minimize contamination due to solution transfer. Additionally, stock solutions of manganese perchlorate, perchloric acid, and sodium oxalate were prepared with 18.2 MΩ*cm deionized water and stored in Eppendorf tubes at room temperature until use for experiments. Due to the

oxidizing capacity of permanganate, a fresh stock of permanganate was prepared daily. Moreover, between experiments within a day, the permanganate stock was stored at 4°C.

Following stock solution preparation, solutions of the designed reactions were prepared and mixed within wells in a washed 96 well plate. The stock solutions in Eppendorf tubes were vortexed three times and then pipetted in the plate individually. The solvent was added first, followed by perchloric acid, manganese perchlorate, and sodium oxalate to generate consistency for interday and intraday replicates. Before the addition of permanganate, the 190 µL mixture was mixed. A blank was prepared using 10 µL of water instead of permanganate solution. Following mixing, a washed plate cover was placed over the 96 well plate and the solutions were incubated at room temperature for 5 minutes. During the 5-minute incubation of the reagents, the settings for the spectrophotometer were configured and prepared. Spectrophotometers were set to measure permanganate absorbance at a wavelength of 525 nm and a temperature of 25°C. For 5-minute reactions, time intervals between readings were set to 10 seconds, but for reactions greater than 5 minutes, intervals between readings were set to 15 seconds.

After the 5-minute incubation, the plate was transferred to the workplace in front of the spectrophotometer. Then, 10 µL of 10 mM permanganate solution was quickly transferred into each of the wells, generally prepared in triplicate. A 200 µL pipette was used to quickly mix each final solution three times. Then, the plate was transferred to the plate reader and absorbance readings were acquired. After kinetics runs were completed, the collected absorbance data were saved and processed using Excel as the time between adding the permanganate and the beginning of absorbance readings was added to the

reaction time while the blank was subtracted from all of the solutions. After data analysis, cleanup procedures were performed for lab materials and equipment.

2.2.5 Experimental Procedure for 1x Visual Indicators at <25℃

Experiments designed and tested at temperatures below 25°C utilized the Cary 60 UV/Visible Spectrophotometer. As previously mentioned, the Cary 60 Spectrophotometer was connected to a qChanger controller, circulating cooling bath, and nitrogen gas system, allowing the temperature of the spectrophotometer to be adjusted to the specific temperature at which the kinetic analysis was to be performed. Clean quartz cuvettes were placed into the spectrophotometer before the connected qChanger controller was set to the experimental temperature. While the qChanger controller, which itself has a limited peltier cooling system, was set to the designed reaction temperature, the circulating cooling bath was set to a lower temperature, generally a couple of degrees lower than the qChanger. For example, if the qChanger was set for 0°C, the circulating cooling bath was set for -2°C to ensure that the spectrophotometer reaches and maintains the target temperature. Additionally, the nitrogen gas system was set to approximately 10-20 psi to prevent cuvette condensation throughout the reaction. Moreover, while the spectrophotometer has a light that blinks green when the cuvettes are at the target temperature, generally occurring after a couple of minutes, we gave the system approximately 30 minutes to achieve the target temperature before running any experimental analysis.

Then, the indicator solution and blank were prepared in 2 mL Eppendorf tubes. Indicator solutions were prepared as outlined in the previous section. Since the quartz cuvettes used hold a volume of 100 μL, enough indicator solution was prepared for

triplicates. The volume of each reagent in the designed reaction composition was doubled and mixed into an Eppendorf tube. Subsequently, the Eppendorf tube was vortexed three times, followed by incubation. For antifreeze-free and lithium perchlorate systems to be tested at 4°C or 0°C, the antifreeze-free solutions were incubated in an ice bath for approximately 10 minutes while lithium perchlorate systems were incubated in an ice bath for approximately 30 minutes before experimental analysis to ensure depressed temperatures for indicator solutions. However, the magnesium and sodium perchlorate systems required longer and colder incubation temperatures, particularly for indicators at -20°C. Therefore, all magnesium and sodium perchlorate systems were incubated overnight in a 20.59% w/w sodium chloride bath in the -20°C freezer for the 0°C, 4°C, and -20°C target temperatures. Similarly, the freshly prepared permanganate solution was cooled in the 4°C refrigerator before experiments.

While the indicator solutions were incubating, a 200 μL blank was prepared in a separate 2 mL Eppendorf tube and 100 μL was transferred directly into one cuvette within the spectrophotometer. Then, the settings of the spectrophotometer were inputted to record permanganate absorbance (525 nm) for a designated time and specific temperature. Indicator solutions were activated by adding cooled permanganate to the cooled Eppendorf tube and vortexing three times. After mixing, 100 μL of the solution was quickly transferred into three separate cuvettes within the spectrophotometer and the reaction kinetics program was started. The time between the addition of permanganate and first absorbance point (time delay) was applied to the data points. The blank was

subtracted from each of the triplicates during data processing. After experiments, cuvettes were emptied, and nitric acid washed.

2.2.6 Determining the Absorbance for Reaction Completion

To determine the absorbance value for the end of the reaction, solutions were prepared in Eppendorf tubes with various absorbance values ranging from 0-0.1 in increments of 0.01. Once the solutions were prepared, they were visually analyzed by multiple lab members to determine the final absorbance at which the pink MnO_4^- could no longer be observed and the solution was visually determined to be clear. However, while the absorbance cutoff was observed to be 0.03 for reaction systems at room temperature, Beer's law was utilized to adjust the absorbance cutoff for reaction systems at cooler temperatures:

$$A = \varepsilon bc \qquad \textit{(Equation 2.2.6.1)}^{13}$$

For spectrophotometers at 25°C, a 96 well round bottom plate was utilized during absorbance spectroscopy and measurements demonstrated a path length of 0.555 cm. Therefore, utilizing the measured path length, an absorbance of 0.03, and a molar absorptivity[13] of 2.34 x 10^3 L/(mol x cm), the concentration of MnO_4^- at the absorbance cutoff was calculated:

$$0.03 = \left(2.34\ x\ 10^3\ \frac{L}{mol\ x\ cm}\right)\ x\ (5.5\ x\ 10^{-1} cm)\ x\ c$$

$$c = \frac{0.03}{\left(2.34\ x\ 10^3\ \frac{L}{mol\ x\ cm}\right)\ x\ (5.5\ x\ 10^{-1} cm)}$$

$$c = 23\ uM$$

Then, the absorbance cutoff for the Cary 60 spectrophotometer was calculated by using the molar absorptivity[12] of 2.34 x 10^3L/(mol x cm), a new path length of 1 mm, and the previously calculated MnO_4^- concentration of 23 μM:

$$A = \left(2.34\ x\ 10^3\ \frac{L}{mol\ x\ cm}\right)\ x\ (1.0\ x\ 10^{-1} cm)\ x\ (2.3\ x\ 10^{-5}\ M)$$

$$A = 0.005$$

Therefore, the absorbance cutoffs for reaction kinetics were determined to be 0.03 for the ThermoFisher UV/Visible MultiSkan Go Spectrophotometer and SpectraMax iD5 Standard Multi-Mode Microplate Detection Platform, while an absorbance cutoff of 0.005 was calculated for the Cary 60 UV/Visible Spectrophotometer.

2.3 Results and Discussion

Data collection was completed in cooperation with Dr. Jorvani Cruz Villarreal under the guidance of Dr. Chad Borges.

2.3.1 Experimental Validation of MATLAB Simulations

As previously mentioned, MATLAB simulations demonstrated that increases in the concentration of $Mn(ClO_4)_2$ and $HClO_4$ result in increased reaction rate kinetics. Therefore, to evaluate the utility of the previously outlined MATLAB simulations, experiments were performed to empirically test the effect of varying $Mn(ClO_4)_2$, $HClO_4$, and Na_2Oxalate concentrations.

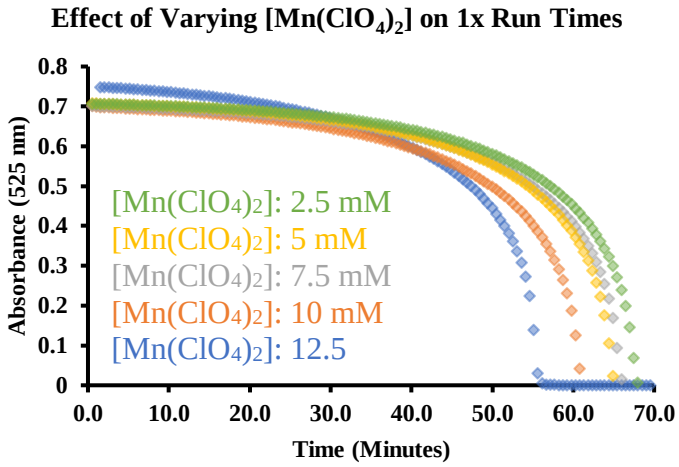


Figure 2.3.1.1: Experiments Demonstrating Effect of Increasing $Mn(ClO_4)_2$. Constant Concentrations Across All Reactions: $[HClO_4]$: 10 mM, $[Na_2Oxalate]$: 1.38 mM, $[KMnO_4]$: 5.0 x 10^{-1} mM.

As illustrated in *Figure 2.3.1.1*, empirical trends matched the previously outlined MATLAB simulations and theoretical background as increases in the concentration of $Mn(ClO_4)_2$ from 2.5 to 12.5 mM resulted in reaction times decreasing from approximately 70 minutes to 55 minutes. Additionally, while the reactions with $Mn(ClO_4)_2$ concentrations of 5 mM and 7.5 mM ended at approximately the same time, potential contamination or pipetting errors could have contributed to similar runs times for the two permanganate/oxalic acid systems with different $Mn(ClO_4)_2$ concentrations. Nevertheless, empirical data for adjusting $Mn(ClO_4)_2$ concentrations in the proposed 1x visual indicator system demonstrated that increases in the concentration of $Mn(ClO_4)_2$ were positively correlated with quicker reaction kinetics and therefore decreased run times. Similarly, the effect of adjusting $HClO_4$ concentration was experimentally tested in different reaction systems. Similar to the previously simulated MATLAB reaction

kinetics, experimental reaction kinetics validated that increases in $HClO_4$ concentrations resulted in quicker reaction rate kinetics and shortened reaction times.

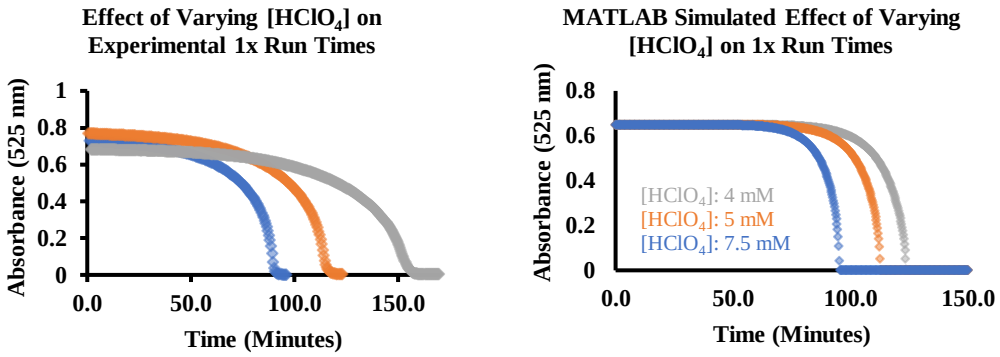


Figure 2.3.1.2: Experiments Demonstrating Effect of Increasing $HClO_4$. ***Blue Line*** *- [$HClO_4$]: 7.5 mM, [$Na_2Oxalate$]: 1.38 mM, [$KMnO_4$]: 5.0 x 10^{-1} mM,* ***Orange Line*** *– [$HClO_4$]: 5 mM, [$Na_2Oxalate$]: 1.38 mM, [$KMnO_4$]: 5.0 x 10^{-1} mM,* ***Grey Line*** *– [$HClO_4$]: 4 mM, [$Na_2Oxalate$]: 1.38 mM, [$KMnO_4$]: 5.0 x 10^{-1} mM.*

In *Figure 2.3.1.2*, increasing the concentration of $HClO_4$ from 4 to 7.5 mM decreased the reaction time from approximately 160 to 90 minutes, confirming MATLAB simulations and theoretical conclusions that explored the relationship between increased $HClO_4$ concentrations and permanganate/oxalic acid reaction run times. Therefore, experimental data demonstrated that when designing visual indicators for specific targeted run times, increasing concentrations of $Mn(ClO_4)_2$ and $HClO_4$ resulted in decreased run times, allowing our lab to achieve shortened run times for indicators #7, #10, and #13. Additionally, experiments were subsequently designed to investigate the empirical trends present for varying concentrations of $Na_2Oxalate$ across the 1x indicator systems with antifreeze-free and antifreeze-salt-containing aqueous eutectic solvents:

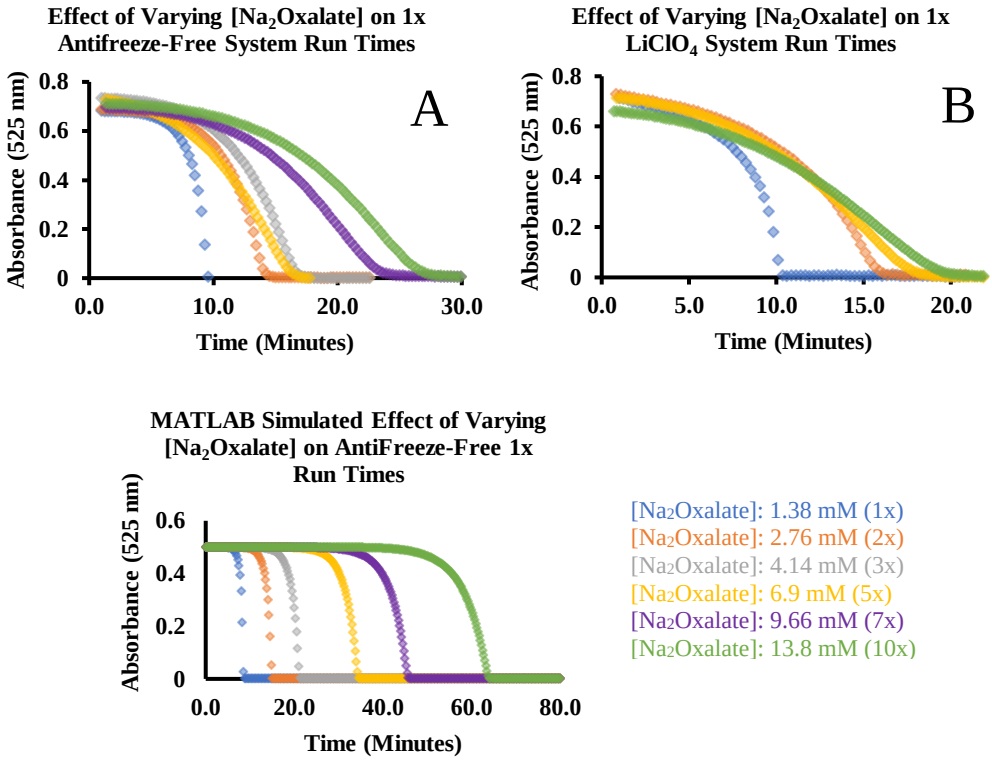


Figure 2.3.1.3: Experiments and MATLAB Simulations Demonstrating the Effect of Increasing $Na_2Oxalate$ for Antifreeze-free and $LiClO_4$ systems. Constant Concentrations: $[HClO_4]$: 100 mM, $[KMnO_4]$: 5.0 x 10^{-1} mM. ***Blue Curve*** *- [$Na_2Oxalate$]: 1.38 mM (1x),* ***Orange Curve*** *- [$Na_2Oxalate$]: 2.76 mM (2x),* ***Grey Curve*** *- [$Na_2Oxalate$]: 4.14 mM (3x),* ***Yellow Curve*** *- [$Na_2Oxalate$]: 6.9 mM (5x),* ***Purple Curve*** *- [$Na_2Oxalate$]: 9.66 mM (7x),* ***Green Curve*** *- [$Na_2Oxalate$]: 13.8 mM (10x).*

As shown in *Figure 2.3.1.3*, increasing concentrations of sodium oxalate resulted in an increase in the reaction time of the visual indicator systems, as previously simulated in the MATLAB script. However, the addition of excess sodium oxalate altered the curve of the reaction system, as seen in the antifreeze-free system in *Figure 2.3.1.3*. While the blue curve, 1x sodium oxalate, has the steep and pronounced absorbance falloff which characterizes the autocatalytic nature of the permanganate/oxalic acid reaction, the green curve, 10x sodium oxalate, showcased a more progressive decrease in MnO_4^-. A similar trend was observed in the $LiClO_4$ system as the 1x sodium oxalate reaction produced the ideal kinetic curve while the 10x sodium oxalate reaction resulted in a flattened curve.

Nevertheless, the empirical trend for varying sodium oxalate concentration was also explored in the sodium perchlorate and magnesium perchlorate systems:

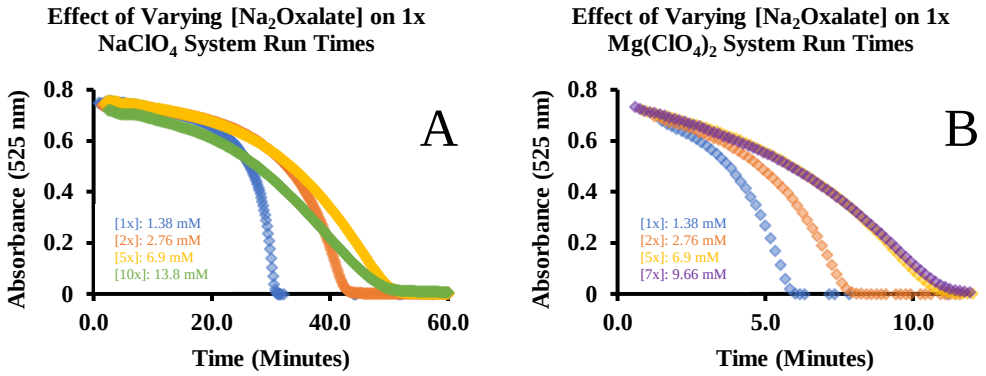


Figure 2.3.1.4: Experiments Demonstrating the Effect of the Increasing $Na_2Oxalate$ for $NaClO_4$ and $Mg(ClO_4)_2$ Systems. Constant Concentrations: $[HClO_4]$: 100 mM, $[KMnO_4]$: 5.0 x 10^{-1} mM. ***Blue Curve*** *- [$Na_2Oxalate$]: 1.38 mM (1x),* ***Orange Curve*** *- [$Na_2Oxalate$]: 2.76 mM (2x),* ***Yellow Curve*** *- [$Na_2Oxalate$]: 6.9 mM (5x),* ***Purple Curve*** *- [$Na_2Oxalate$]: 9.66 mM (7x),* ***Green Curve*** *- [$Na_2Oxalate$]: 13.8 mM (10x).*

As shown in *Figure 2.3.1.4*, the 1x $Mg(ClO_4)_2$ and $NaClO_4$ systems demonstrated similar trends for increasing sodium oxalate concentrations as the 1x antifreeze-free and $LiClO_4$ systems. While increased sodium oxalate concentration resulted in longer run times, there was a similar change in curve shape, shifting away from the ideal absorbance curve for the proposed visual indicators. For the purposes of the visual indicator, we envisioned a rapid transition from a clearly distinguishable pink color to a colorless solution, allowing users to identify the integrity of their sample quickly and easily. Therefore, while higher concentrations of sodium oxalate were required in specific systems to reach targeted run times, reactions were designed to best preserve the steep absorbance trajectory seen in the 1x oxalate reactions. Additionally, when the final "endpoints" at each sodium oxalate concentration for the four visual indicator systems were compared, the following results were obtained:

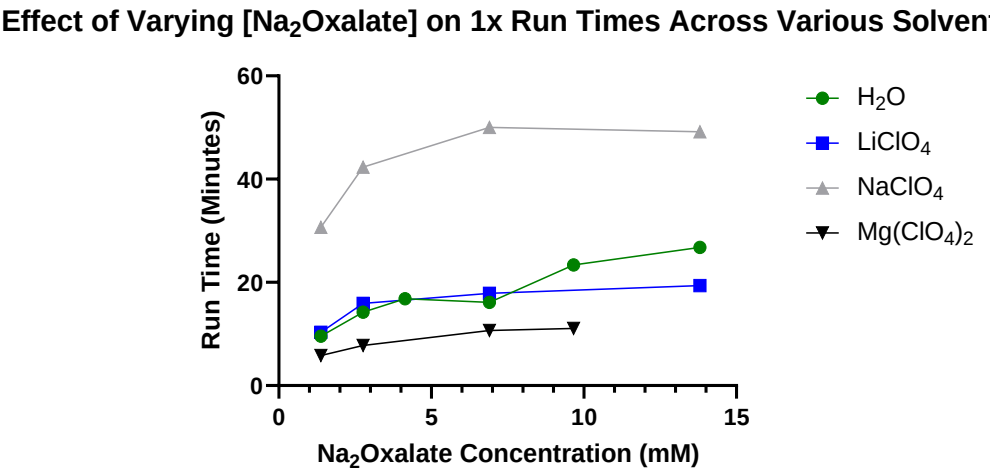


Figure 2.3.1.5: End Points of Experiments Demonstrating Effect of Increasing $Na_2Oxalate$ in 1x Indicator Systems. 1x [$Na_2Oxalate$]; 1.38 mM, 2x [$Na_2Oxalate$]; 2.76 mM, 3x [$Na_2Oxalate$]; 4.14 mM, 5x [$Na_2Oxalate$]; 6.9 mM, 7x [$Na_2Oxalate$]; 9.66 mM, 10x [$Na_2Oxalate$]; 13.8 mM.

As shown in *Figure 2.3.1.5*, increasing sodium oxalate concentration was positively correlated with increased final run times for the 1x visual indicator systems. Additionally, the absorbance spectra for each solvent and their respective final reaction endpoints demonstrated a plateau of run times at increasing sodium oxalate concentrations. For example, the 5x and 10x sodium oxalate concentrations in the $NaClO_4$ system completed their respective reactions at similar times but with varying absorbance curves as shown in *Figure 2.3.1.4*. Therefore, instead of utilizing maximum concentrations of sodium oxalate, reactions were subsequently designed with concentrations of sodium oxalate that still achieved the maximum run time for the system but preserved the integrity and steepness of the permanganate/oxalic acid absorbance curve. Overall, across varying reagents, experimental data validated previously outlined theoretical conclusions and MATLAB simulations, demonstrating that reaction systems can be designed with varying concentrations of manganese perchlorate, perchloric acid,

and sodium oxalate to achieve specific run times and absorbance spectra for the previously outlined indicators.

2.3.2 Comparison of MATLAB Simulations and Experimental Data

While the MATLAB simulations have been utilized as an effective tool to understand various aspects of reaction kinetics and the impact of various reagent changes on the overall reaction system, further investigation was required to compare MATLAB simulated and experimentally collected data sets. Therefore, experimental data was gathered to compare the general progression of the kinetic curves before further testing was performed to determine the accuracy of MATLAB designed simulations. Firstly, experimentally acquired kinetic curves at a wavelength of 525 nm were recorded for the 15-minute 1x antifreeze-free reaction under the same conditions and concentrations simulated in the MATLAB script:

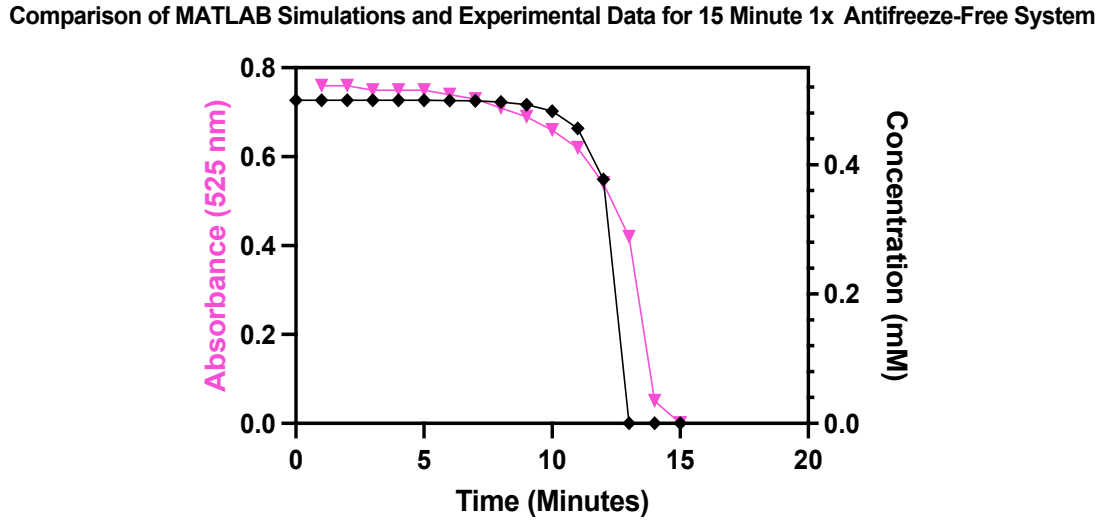


Figure 2.3.2.1: Comparison of Experimental Data and MATLAB Simulation for 15 Minute 1x Antifreeze-Free System. The pink curve corresponds to the experimental data, with the y-axis representing the MnO_4^- absorbance across the length of the reaction. On the other hand, the black curve represents the MATLAB simulated reaction kinetics with the y-axis corresponding to the concentration of MnO_4^-. [$HClO_4$]: 0.07 M, [$Na_2Oxalate$]: 1.38 mM, [$KMnO_4$]: 5.0 x 10^{-1} mM, [$Mn(ClO_4)_2$]: 1 nM.

As seen in the two curves in *Figure 2.3.2.1*, the MATLAB simulation provides an accurate simulation and representation of MnO_4^- kinetic curves for our visual indicators. While the MATLAB script predicted a run time of approximately 13 minutes, the experimental data produced an average run time of approximately 14.25 minutes, emphasizing the value of the MATLAB scripts for 1x antifreeze-free visual indicator design. Additionally, the comparison between MATLAB simulations and experimental data was further extended and analyzed with longer running systems, producing the following results:

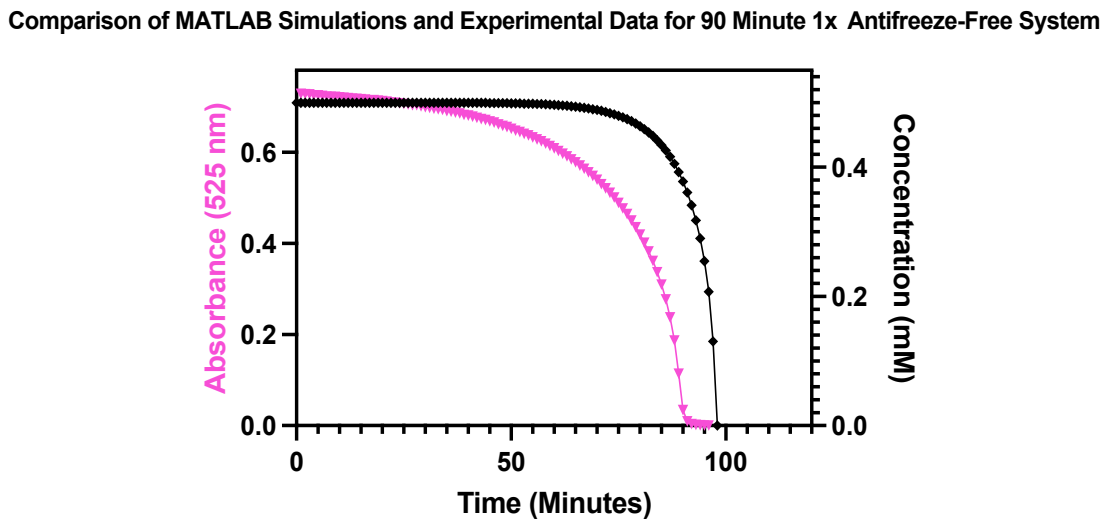


Figure 2.3.2.2: Comparison of Experimental Data and MATLAB Simulation for 90 Minute 1x Antifreeze-Free System. The pink curve corresponds to the experimental data, with the y-axis representing the MnO_4^- absorbance across the length of the reaction. On the other hand, the black curve represents the MATLAB simulated reaction kinetics with the y-axis corresponding to the concentration of MnO_4^-. [$HClO_4$]: 7.5 mM, [$Na_2Oxalate$]: 1.38 mM, [$KMnO_4$]: 5.0 x 10^{-1} mM, [$Mn(ClO_4)_2$]: 1 nM.

Similarly, the comparison between the MATLAB simulated reactions and the experimental data for the 90-minute antifreeze-free system continued to demonstrate that the MATLAB script can accurately predict approximate run times for the 1x antifreeze-free reaction systems. While there is a greater dissimilarity between the general curvature of the two reaction sets, particularly in comparison to shorter reactions,

the approximated end time remains accurate within 10% as the experimental run time was approximately 90 minutes whereas the simulated run time was precisely 98 minutes. Additionally, discrepancies between the simulated and experimental reaction kinetics could have arisen from a variety of challenges with experimental data collection, including but not limited to contamination with exogenous Mn(II), MnO_2, or other environmental particles. Nevertheless, MATLAB simulations have been a valuable tool for design of our targeted indicators, providing accurate run times for 1x antifreeze-free systems and a more comprehensive understanding of various reaction components and their subsequent effects on the permanganate/oxalic acid reaction.

2.3.3 Design and Experimental Testing of 1x Visual Indicators

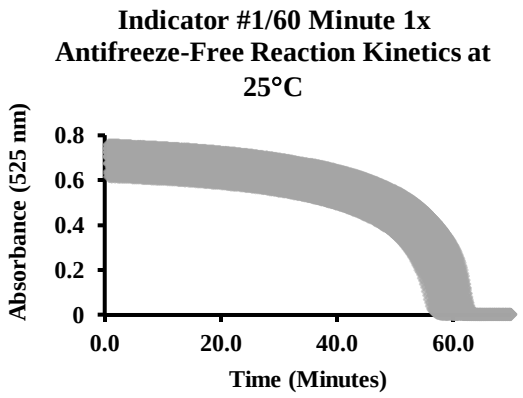
As previously outlined, 14 indicators were proposed to demonstrate the functionality of the 1x permanganate/oxalic acid visual indicator system for specific applications using a variety of solvents, including antifreeze-free and eutectic solutions. Within the 14 proposed indicators for the 1x reaction system, six of the indicators are antifreeze-free, three implemented sodium perchlorate, three incorporated magnesium perchlorate, and two utilized lithium perchlorate. Additionally, to ensure ideal stochiometric ratios, the 1x indicator systems were designed with final sodium oxalate and potassium permanganate concentrations of 1.38 mM and 0.5 mM, respectively. Varying concentrations of perchloric acid and manganese perchlorate were implemented to achieve different targeted run times. Within our reaction systems, an average run time within 10% of the targeted run time represented successful reaction construction for each indicator system. The first three targeted indicators designed were the 60-minute, 90-minute, and 120-minute 1x antifreeze-free systems at 25°C:

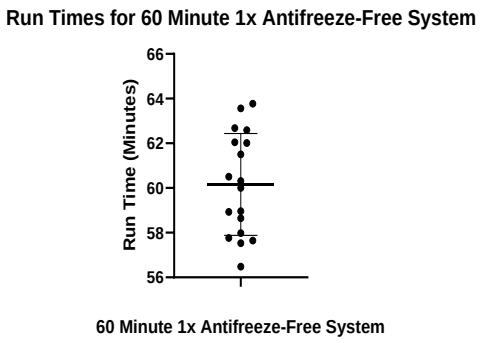
Table 2.3.3.1 Performance Summary of Targeted Indicators #1, #2, #3

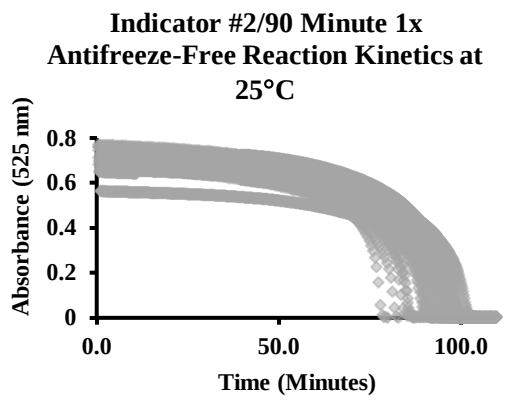
Indicator	Average (Minutes)	SD (Minutes)	Interday CV	Intraday CV
#1/60 Minute 1x Antifreeze-Free	60.2 (n=18)	2.3	3.8%	1.5%
#2/90 Minute 1x Antifreeze-Free	92.0 (n=21)	6.3	6.8%	3.2%
#3/120 Minute 1x Antifreeze-Free	123.5 (n=18)	6.1	4.9%	2.5%

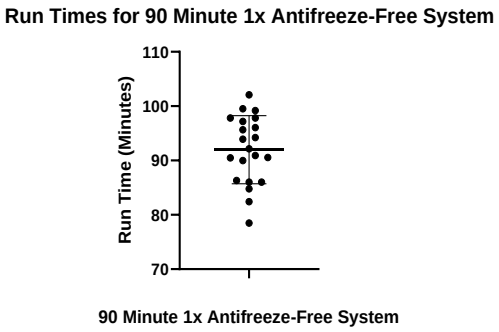
As shown in *Table 2.3.3.1*, each of the first three indicators were successfully designed and replicated, producing the following kinetic curves:

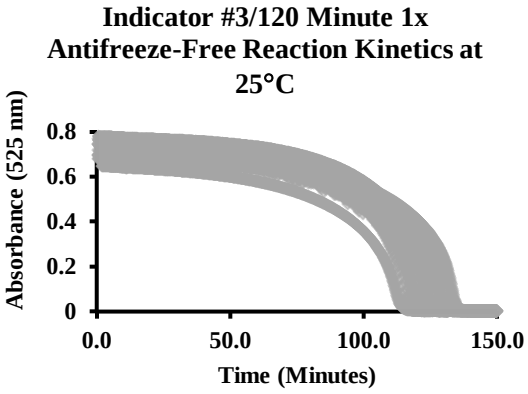
Table 2.3.3.2 Summary of Absorbance Spectra for Targeted Indicators #1, #2, #3. *Targeted Indicator #1 (n=18) - [$HClO_4$]: 10 mM, [$Na_2Oxalate$]: 1.38 mM, [$KMnO_4$]: 5.0 x 10^{-1} mM, [$Mn(ClO_4)_2$]: 100 µM, Targeted Indicator #2 (n=21) - [$HClO_4$]: 7.5 mM, [$Na_2Oxalate$]: 1.38 mM, [$KMnO_4$]: 5.0 x 10^{-1} mM, Targeted Indicator #3 (n=18) - [$HClO_4$]: 5 mM, [$Na_2Oxalate$]: 1.38 mM, [$KMnO_4$]: 5.0 x 10^{-1} mM. In the run time plots, data analysis of the plots showcases the mean and standard deviation across all the replicates within an indicator system.*

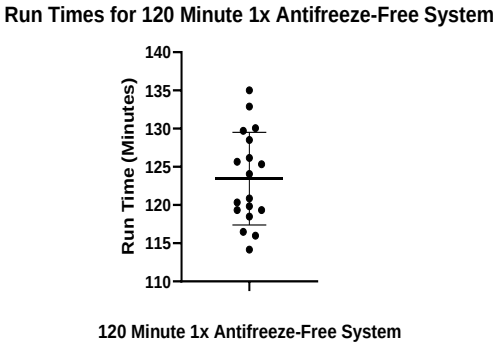












As demonstrated across the three absorbance spectra in *Table 2.3.3.2*, the antifreeze-free indicators at 25°C were designed with accurate replicates across multi-day sets of data. The 60-minute 1x antifreeze-free system at 25°C had an average run time of 60.2 minutes (SD±2.3). Additionally, the reaction system demonstrated good interday and intraday precision with CV values of approximately 3.8% and 1.5%, showcasing our ability to continuously replicate the indicator system across various days with fresh stock solutions of permanganate. Similarly, the 90-minute 1x antifreeze-free system at 25°C totaled an average run time of 92.0 minutes (SD±6.3). However, as the reaction time increased for our proposed indicator, we expected the standard deviation between run times to subsequently increase, as shown. Nevertheless, the second indicator system also

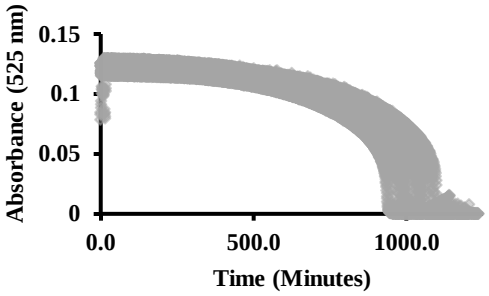
demonstrated interday and intraday precision with CV values of approximately 6.3% and 3.2%. Finally, the 120-minute 1x antifreeze-free system at 25°C had an average run time across 18 replicates of 123.5 minutes (SD±6.1). Additionally, the system demonstrated similar interday and intraday precision, with CV values of 4.9% and 2.5%. For indicators #1-3, we envision applications in both clinical and research settings, including use for transportation of biological or biospecimen samples such as blood after withdrawal from patients. Set at 25°C, these reaction systems would be ideal for biological samples that cannot be exposed to room temperature conditions for more than 60, 90, or 120 minutes. However, for samples requiring storage on ice, the following indicators were proposed:

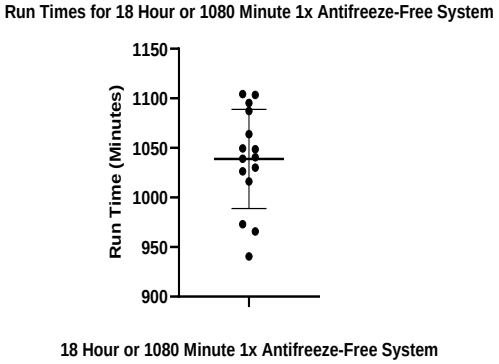
Table 2.3.3.3 Performance Summary of Targeted Indicators #4, #5, #6

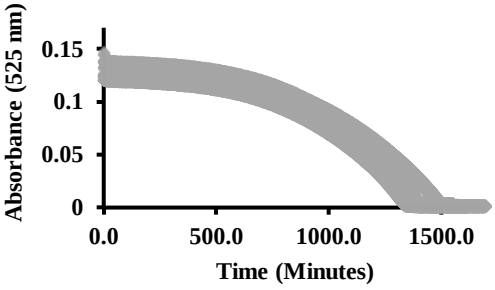
Indicator	Average	SD	Interday CV	Intraday CV
#4/18 Hour 1x Antifreeze-Free at 4°C	17.3 Hours (n=15)	0.8 Hours	4.8%	4.5%
#5/24 Hour 1x Antifreeze-Free at 4°C	23.6 Hours (n=9)	1.1 Hours	4.9%	1.8%
#6/1 Hour 1x Antifreeze-Free at 0°C	58.0 Minutes (n=12)	2.2 Minutes	3.7%	1.7%

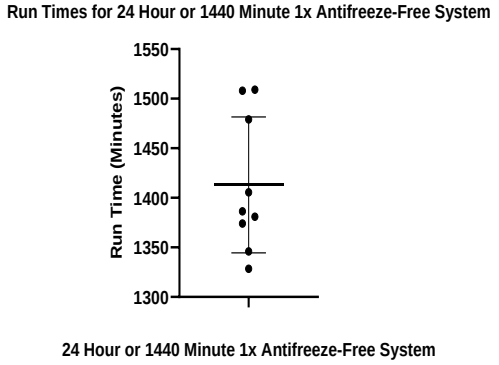
Similarly, the following kinetic curves for indicators #4, #5, and #6 were produced:

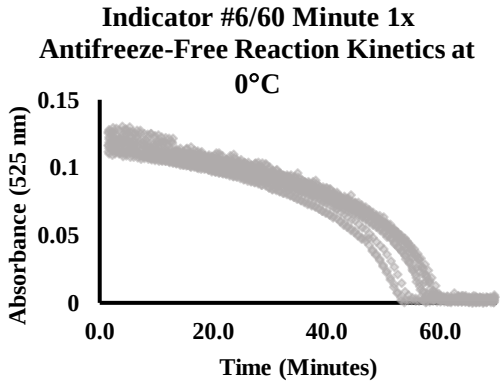
Table 2.3.3.4 Summary of Absorbance Spectra for Targeted Indicators #4, #5, #6. *Targeted Indicator #4 (n=15) - [$HClO_4$]: 7 mM, [$Na_2Oxalate$]: 1.38 mM, [$KMnO_4$]: 5.0 x 10^{-1} mM, Targeted Indicator #5 (n=9) - [$HClO_4$]: 6.25 mM, [$Na_2Oxalate$]: 2.5 mM, [$KMnO_4$]: 5.0 x 10^{-1} mM, Targeted Indicator #6 (n=12) - [$HClO_4$]: 100 mM, [$Na_2Oxalate$]: 1.38 mM, [$Mn(ClO_4)_2$]: 37.5 µM, [$KMnO_4$]: 5.0 x 10^{-1} mM. In the run time plots, data analysis of the plots showcases the mean and standard deviation across all the replicates within an indicator system.*

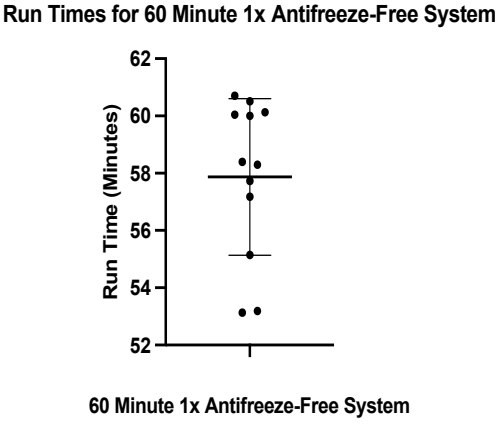












As shown in *Table 2.3.3.4*, the permanganate/oxalic reaction was successfully designed and adjusted to achieve proposed indicators #4, #5, and #6. For indicator #4 (18-hour 1x antifreeze-free system at 4°C), the average run time was 17.3 hours (SD±0.8)

over 15 replicates, within the accepted plus or minus 10% deviation, equivalent to 1.8 hours for this system. Additionally, the designed reaction showcased accurate interday and intraday precision, with CV values of 4.8% and 4.5%, respectively. Moreover, indicator #5 (24-hour 1x antifreeze-free system at 4°C) had an average run time of approximately 23.6 hours (SD±1.1) across 9 replicates. While the standard deviation demonstrated a greater range of completed run times, the final concentration of sodium oxalate was increased from the ideal concentration of 1.38 mM to 2.5 mM to achieve the targeted reaction length. Therefore, increasing the disodium oxalate concentration beyond "ideal" conditions and subsequently altering the kinetic curve could have contributed to the increased range of final run times. Nevertheless, the indicator system demonstrated similar interday and intraday precision to previous indicators, with CV values of 4.9% and 1.8%, respectively. Finally, the last 1x antifreeze-free indicator designed was indicator #6 (60-minute 1x antifreeze-free system at 0°C), which resulted in an average run time of 58.0 minutes (SD±2.2) across 12 replicates. For indicators #4-6, we envision similar applications for monitoring the integrity of biological or biospecimen samples as the previously defined indicators #1-3, with the possibility of monitoring biospecimen integrity over longer periods of time, such as overnight storage. However, these indicators would be applicable specifically for samples that need to either remain on ice or in conditions just above the freezing point, complementing the previously outlined indicators that were designed for room temperature conditions.

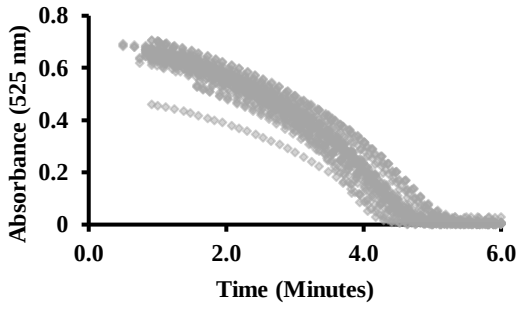
Following successful design of the antifreeze-free 1x visual indicators, we subsequently explored the visual indicators utilizing the aforementioned antifreeze-salt-containing aqueous eutectic solvents, beginning with sodium perchlorate:

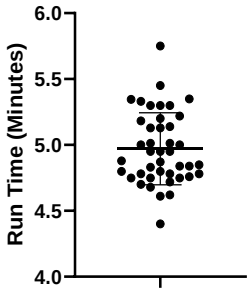
Table 2.3.3.5 Performance Summary of Targeted Indicators #7, #8, #9

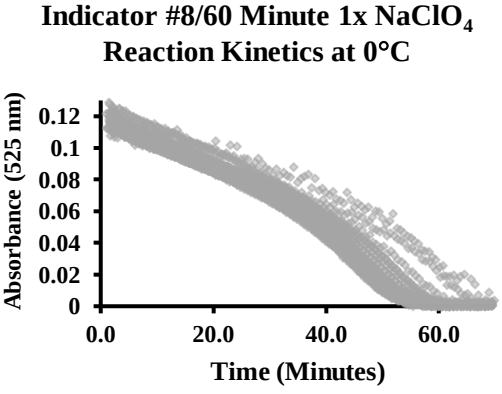
Indicator	Average	SD	Interday CV	Intraday CV
#7/5 Minute 1x $NaClO_4$ at 25°C	5.0 Minutes (n=42)	0.3 Minutes	5.0 %	3.7%
#8/60 Minute 1x $NaClO_4$ at 0°C	57.2 Minutes (n=27)	3.7 Minutes	6.2%	2.4%
#9/7 Day 1x $NaClO_4$ at -20°C	6.9 Days (n=3)	0.1 Day	-	1.9%

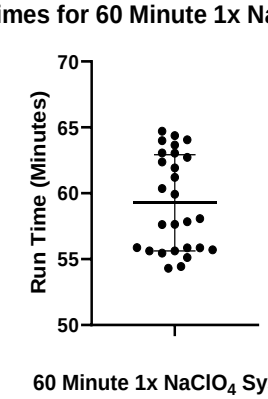
The following kinetic curves for the sodium perchlorate systems were produced:

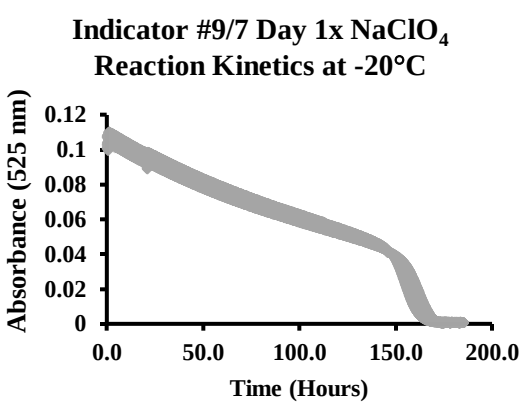
Table 2.3.3.6 Summary of Absorbance Spectra for Targeted Indicators #7, #8, #9. *Targeted Indicator #7 (n=42) - [$HClO_4$]: 100 mM, [$Na_2Oxalate$]: 1.38 mM, [$Mn(ClO_4)_2$]: 2.3 x 10^{-1} mM, [$KMnO_4$]: 5.0 x 10^{-1} mM, Targeted Indicator #8 (n=27) - [$HClO_4$]: 100 mM, [$Na_2Oxalate$]: 1.38 mM, [$Mn(ClO_4)_2$]: 2.7 x 10^{-1} mM, [$KMnO_4$]: 5.0 x 10^{-1} mM, Targeted Indicator #9 (n=3) - [$HClO_4$]: 10 mM, [$Na_2Oxalate$]: 1.38 mM, [$Mn(ClO_4)_2$]: 5.0 x 10^{-1} mM, [$KMnO_4$]: 5.0 x 10^{-1} mM. In the run time plots, data analysis of the plots showcases the mean and standard deviation across all the replicates within an indicator system.*

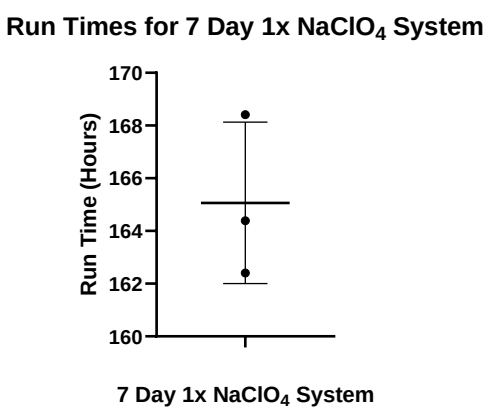












As shown in *Tables 2.3.3.5 and 2.3.3.6*, the 1x sodium perchlorate visual indicator systems were successfully designed and consistently replicated across various days. While the previously outlined antifreeze-free visual indicator systems are applicable for temperatures at or above the freezing point (0°C), implementation of antifreeze-salt-containing eutectics in the visual indicator systems allowed for reaction design at temperatures below 0°C, such as indicator #9. For indicator #7 (5-minute 1x $NaClO_4$ system at 25°C), replicates of the reaction system generated an average run time of 5.0 minutes (SD±0.3). Additionally, the reaction demonstrated strong precision and consistency with interday and intraday CV values of approximately 5.0% and 3.7%. Similarly, indicator #8 (the 60-minute 1x $NaClO_4$ system at 0°C) was successfully designed and replicated with an average run time of 57.2 minutes (SD±3.7) across 27 replicates. Moreover, replicates across various days demonstrated strong precision with interday and intraday CV values of approximately 6.2% and 2.4%. Additionally, for indicator #9 (7-day 1x $NaClO_4$ at -20°C), the average reaction time was 165.1 hours (SD±0.1) across three replicates, equivalent to 6.9 days. However, while the reaction set demonstrated strong intraday precision with a CV value of 1.9%, additional reaction sets

are required to investigate the interday precision of the reaction system. Nevertheless, the proposed 1x sodium perchlorate visual indicator systems were designed for long-term storage of biospecimen or clinical samples. More specifically, the 5-minute 1x $NaClO_4$ system was designed for reactions that cannot be exposed to thawing conditions without a loss of biospecimen integrity while the 60-minute reaction system was proposed for samples that can be exposed to a few cycles of thawing during transportation or sample analysis without loss of sample integrity. However, the 7-day reactions system was designed specifically for long-term storage of aliquots or samples at subzero temperatures, achieving cold chain tracking at storage temperatures well below those previously defined for antifreeze-free systems.

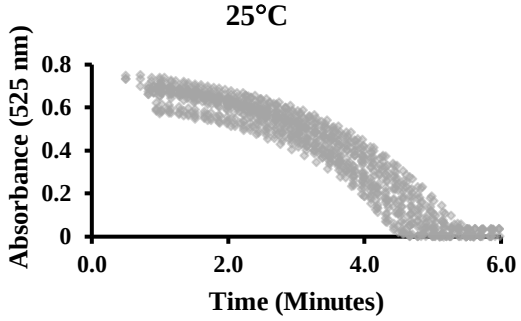
Three 1x proposed magnesium perchlorate visual indicator systems were explored:

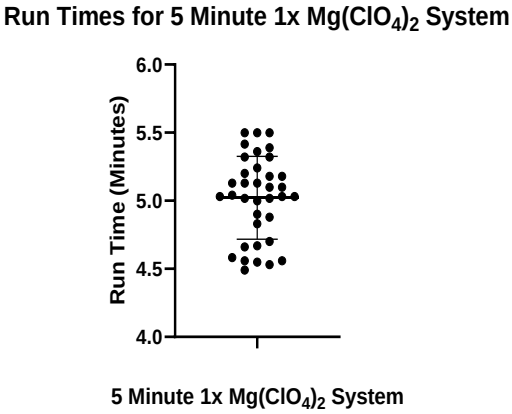
Table 2.3.3.7 Performance Summary of Targeted Indicators #10, #11, #12

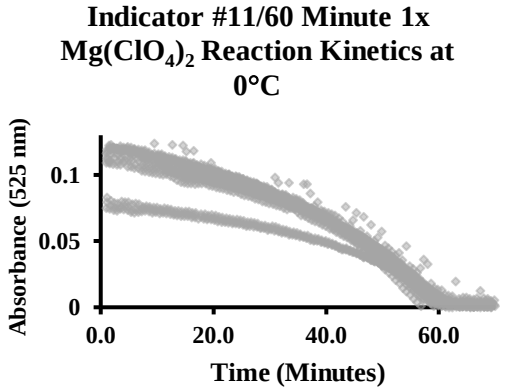
Indicator	Average	SD	Interday CV	Intraday CV
#10/5 Minute 1x $Mg(ClO_4)_2$ at 25°C	5.0 Minutes (n=36)	0.3 Minutes	6.1%	3.4%
#11/60 Minute 1x $Mg(ClO_4)_2$ at 0°C	62.8 Minutes (n=15)	2.4 Minutes	3.8%	1.7%
#12/7 Day 1x $Mg(ClO_4)_2$ at -20°C	5.1 Days (n=3)	0.1 Days	-	1.2%

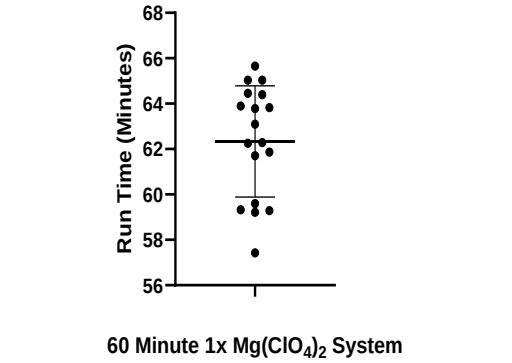
Similarly, the kinetic curves of the indicators are as shown:

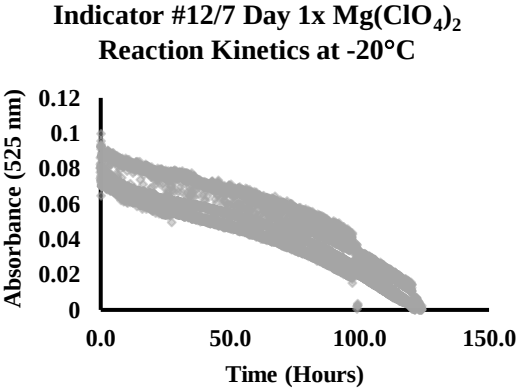
Table 2.3.3.8 Summary of Absorbance Spectra for Targeted Indicators #10, #11, #12. *Targeted Indicator #10 (n=36) - [$HClO_4$]: 100 mM, [$Na_2Oxalate$]: 1.38 mM, [$Mn(ClO_4)_2$]: 2.75 x 10^{-2} mM, [$KMnO_4$]: 5.0 x 10^{-1} mM, Targeted Indicator #11 (n=15) - [$HClO_4$]: 100 mM, [$Na_2Oxalate$]: 1.38 mM, [$Mn(ClO_4)_2$]: 4.5 x 10^{-2} mM, [$KMnO_4$]: 5.0 x 10^{-1} mM. Indicator #12 (n=3) – [$HClO_4$]:8 mM, [$Na_2Oxalate$]: 11.04 mM, [$KMnO_4$]: 5.0 x 10^{-1} mM. In the run time plots, data analysis of the plots showcases the mean and standard deviation across all the replicates within an indicator system.*

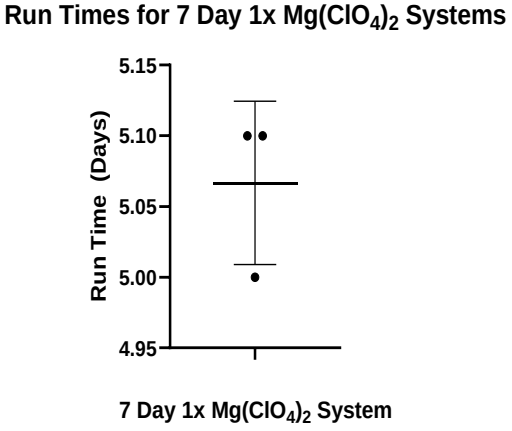












As demonstrated in *Tables 2.3.3.7 and 2.3.3.8,* the 1x magnesium perchlorate visual indicator systems were successfully constructed and replicated across various days. For indicator #10 (5-minute 1x $Mg(ClO_4)_2$ system at 25°C), 36 replicates generated an

average run time of approximately 5.0 minutes (SD±0.3), well within the 10% CV required for the visual indicators. Additionally, the reaction system demonstrated strong intraday precision with a CV value of 3.4% while the interday precision also highlighted the consistency of the reaction across various reagent stocks with a CV of 6.1%. Moreover, indicator #11 (60-minute 1x $Mg(ClO_4)_2$ system at 0°C), showcased similar consistency with an average run time of 62.8 minutes (SD±2.4) across 15 replicates. Nevertheless, while the box plot demonstrated increased variation in the end times of the reaction system, the proposed visual indicator showed strong consistency across replicates with interday and intraday CV values of 3.8% and 1.7%. Finally, indicator #12 (7-day 1x $Mg(ClO_4)_2$ system at -20°C) was only able to be extended to a run time of 5.1 days (SD±0.3). Therefore, further experimental testing is required to explore potentially increasing the reaction length of the system. Nevertheless, similar to the previously defined sodium perchlorate indicator systems, the 5-minute magnesium perchlorate system was designed for the long-term storage of aliquots that cannot be exposed to thawing cycles while the 60-minute magnesium perchlorate system was designed for samples that can be exposed to a limited number of thawing cycles during sample preparation or analysis.

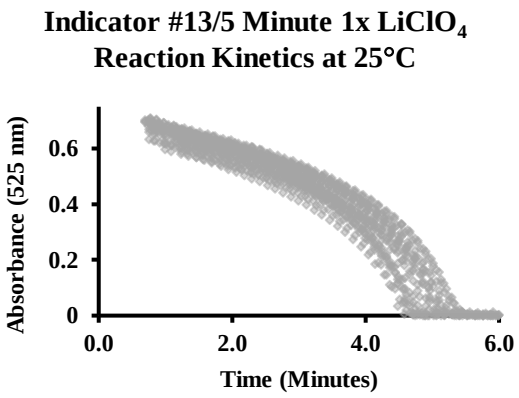
The last 1x eutectic visual indicator system explored was lithium perchlorate:

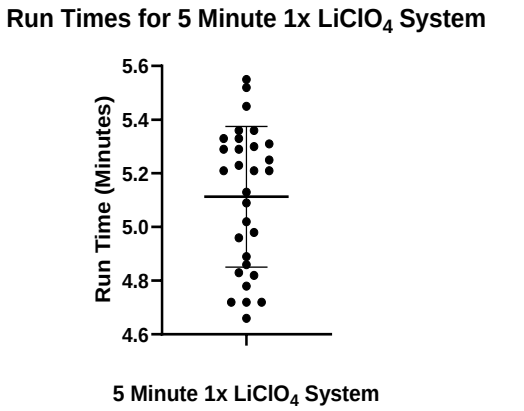
Table 2.3.3.9 Performance Summary of Targeted Indicators #13, #14

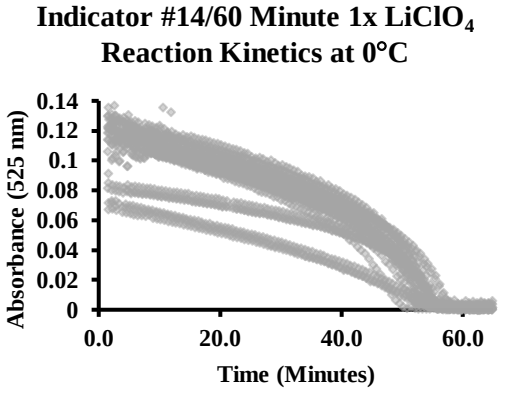
Indicator	Average (Minutes)	SD (Minutes)	Interday CV	Intraday CV
#13/5 Minute 1x $LiClO_4$ at 25°C	5.1 (n=30)	0.3	5.1%	3.0%
#14/60 Minute 1x $LiClO_4$ at 0°C	56.2 (n=24)	1.5	2.7%	1.8%

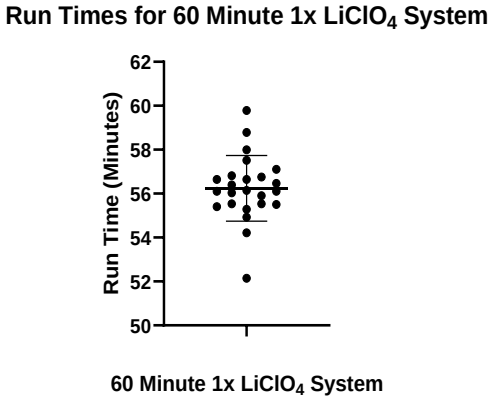
Additionally, the kinetic curves for the lithium perchlorate systems are shown:

Table 2.3.3.10 Summary of Absorbance Spectra for Targeted Indicators #13, #14. *Targeted Indicator #13 (n=30) - [$HClO_4$]: 100 mM, [$Na_2Oxalate$]: 1.38 mM, [$Mn(ClO_4)_2$]: 6 x 10^{-2} mM, [$KMnO_4$]: 5.0 x 10^{-1} mM, Targeted Indicator #14 (n=24) - [$HClO_4$]: 100 mM, [$Na_2Oxalate$]: 1.38 mM, [$Mn(ClO_4)_2$]: 1.0 x 10^{-1} mM, [$KMnO_4$]: 5.0 x 10^{-1} mM. In the run time plots, data analysis of the plots showcases the mean and standard deviation across all the replicates within an indicator system.*









Similarly, *Tables 2.3.3.9 and 2.3.3.10* demonstrated the successful construction of the lithium perchlorate visual indicator systems. For indicator #13 (5-minute 1x $LiClO_4$ visual indicator), 30 replicates across various days generated an average run time of approximately 5.1 minutes (SD±0.3). Moreover, interday and intraday calculations highlighted the consistency and precision of the reaction system with CV values of 5.1% and 3.1%. Additionally, indicator #14 (the 60-minute 1x $LiClO_4$ system) demonstrated an

average run time of 56.2 minutes (SD±1.5) across 24 replicates. Similarly, the reaction system was tested across various days with different permanganate stock solutions and the interday and intraday precision was analyzed, generating CV values of 2.7% and 1.8%, respectively. Overall, replicates across all fourteen 1x indicators demonstrated strong precision and accuracy. However, there were kinetic curve inconsistencies and varying starting absorbance values among some of the indicators, particularly for indicators run at lower temperatures. While we initially hypothesized that challenges with condensation or partially blocked light paths contributed to inconsistent kinetic curves, these hypotheses would have demonstrated an increase in absorbance. Therefore, further experiments are required to explore variation in kinetic curves at lower temperatures. In regard to varying starting absorbance values, we hypothesize that due to permanganate samples being freshly prepared daily, the starting absorbance values will inevitably vary slightly among reaction replicates.

Finally, while reactions were designed for the 14 proposed visual indicators, each indicator was subsequently also tested at varying temperatures, producing the following results:

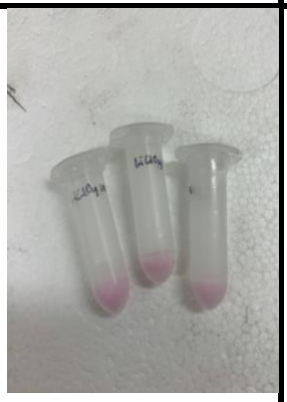
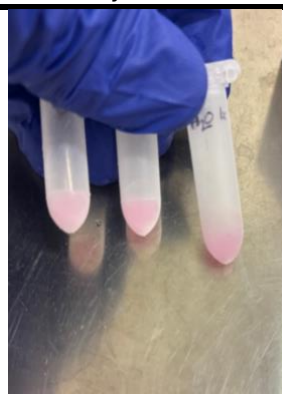
Table 2.3.3.11 Summary Run Time and Precision Characteristics of 1x Targeted Visual Indicators. *Bolded Indicators represent the original 14 proposed indicator systems.*

Solution Composition (Freezing/Melting Point)	25℃	4℃	On ice (0℃)	-20℃	-80℃/-160 ℃
Antifreeze-Free (0℃) 1x System (Indicator #1)	**Avg: 60.2 Minutes (SD±2.3) n=18**	Avg: 11.0 Hours (SD±0.5) n=12			Stays Pink
Antifreeze-Free (0℃) 1x System (Indicator #2)	**Avg: 92.0 Minutes (SD±6.3) n=21**	Avg: 17.0 Hours (SD±0.3) n=3			Stays Pink

Antifreeze-Free (0℃) 1x System (Indicator #3)	**Avg: 123.5 Minutes (SD±6.1) n=18**	Avg: 22.3 Hours (SD±0.7) n=6			Stays Pink
Antifreeze-Free (0℃) 1x System (Indicator #4)	Avg: 97.1 Minutes (SD±5.9) n=21	**Avg: 17.3 Hours (SD±0.8) n=15**			Stays Pink
Antifreeze-Free (0℃) 1x System (Indicator #5)	Avg: 118.4 Minutes (SD±4.0) n=15	**Avg: 23.6 Hours (SD±1.1) n=9**			Stays Pink
Antifreeze-Free (0℃) 1x System (Indicator #6)	Avg: 3.8 Minutes (SD±0.1) n=18		**Avg: 58.0 Minutes (SD±2.2) n=12**		Stays Pink
52% (w/w) $NaClO_4$ (-37℃) 1x System (Indicator #7)	**Avg: 5.0 Minutes (SD±0.3) n=42**		Avg: 69.5 Minutes (SD±3.0) n=6	Avg: 20.4 Hours (SD±2.7) n=3	Stays Pink
52% (w/w) $NaClO_4$ (-37℃) 1x System (Indicator #8)	Avg: 4.5 Minutes (SD±0.2) n=27		**Avg: 59.3 Minutes (SD±3.7) n=27**	Avg: 15.9 Hours (SD±1.2) n=3	Stays Pink
52% (w/w) $NaClO_4$ (-37℃) 1x System (Indicator #9)	Avg: 44.2 Minutes (SD±1.4) n=12			**Avg: 6.9 Days (SD±0.1) n=3**	Stays Pink
44% (w/w) $Mg(ClO_4)_2$ (-67℃) 1x System (Indicator #10)	**Avg: 5.0 Minutes (SD±0.3) n=36**		Avg: 71.4 Minutes (SD±2.6) n=15	Avg: 20.6 Hours (SD±1.1) n=6	Stays Pink
44% (w/w) $Mg(ClO_4)_2$ (-67℃) 1x System (Indicator #11)	Avg: 4.6 Minutes (SD±0.3) n=12		**Avg: 62.3 Minutes (SD±1.6) n=18**	Avg: 15.9 Hours (SD±0.5) n=3	Stays Pink
44% (w/w) $Mg(ClO_4)_2$ (-67℃) 1x System (Indicator #12)	Avg: 34.5 Minutes (SD±1.4) n=12	-		Avg: 5.1 Days (SD±0.1) n=3	Stays Pink
22% (w/w) $LiClO_4$ (-18℃) 1x System (Indicator #13)	**Avg: 5.1 Minutes (SD±0.3) n=30**		Avg: 79.5 Minutes (SD±2.9) n=6	Avg: 24.5 Hours (SD±1.4) n=9 (Visual Inspection)	Stays Pink
22% (w/w) $LiClO_4$ (-18℃) 1x System (Indicator #14)	Avg: 4.0 Minutes (SD±0.4) n=27		**Avg: 56.2 Minutes (SD±1.5) n=24**		Stays Pink

As expected, decreasing temperatures resulted in longer reaction times due to slower reaction kinetics, allowing for our proposed indicators to be applied to a range of temperatures that track the stability of actual biomolecules at different temperatures, depending on the ideal storage temperature of biospecimens and aliquots. Nevertheless, at -80°C and -140°C, the proposed 1x visual indicators are frozen or vitrified, remaining light pink:

Table 2.3.3.12: 1x Visual Indicators at -80℃

1x $NaClO_4$ System	1x $Mg(ClO_4)_2$ System	1x $LiClO_4$ System	1x Antifreeze-Free System
			

Overall, *Table 2.3.3.11* demonstrates the functionality and replicability of the proposed visual indicator systems, allowing for further applicational testing to determine the effectivity of the proposed visual indicator systems for cold chain tracking.

2.4 Conclusions

In total, 13 of the 14 proposed visual indicators were successfully designed and experimentally tested across varying temperatures, ranging from 25℃ to -80/-140℃. Within the design process of our proposed visual indicators, MATLAB simulations of the permanganate/oxalic reaction were successfully implemented after experimental data validated the precision and accuracy of MATLAB simulations and simulated trends of

varying reagent concentrations. As a result, among the targeted indicators bolded in *Table 2.3.3.11*, only the -20℃ $Mg(ClO_4)_2$ system (Indicator #12) has yet to be fully developed due to challenges with extending the reaction to the 7-day target. Nevertheless, the remaining 13 targeted visual indicators were subsequently replicated with accurate reproducibility across varying days, highlighting the consistency of the proposed visual indicators for application in clinical and academic fields. Additionally, the implementation of antifreeze-salt-containing aqueous eutectic solvents within the permanganate/oxalic acid reaction increased the applicability of our proposed cold chain tracking mechanism for samples or aliquots at lower temperatures, particularly -20℃ and -80/-160℃. Overall, initial design of the proposed permanganate/oxalic acid visual indicator system has shown promising results to be a cost-friendly cold chain tracking system that resolves some of the previously defined challenges with currently utilized cold chain tracking mechanisms in clinical and academic settings.

CHAPTER 3

HIGH CONCENTRATION (5X) TIME TEMPERATURE VISUAL INDICATOR SYSTEMS

3.1 Introduction

While the functionality of our proposed visual indicator systems at 1x concentrations of permanganate (0.5 mM) was previously explored across 14 different 1x indicator systems, challenges with visualization based on the color of the frozen indicator systems prompted our lab to explore the functionality of high concentration systems. As shown in *Table 2.3.3.12,* the outlined 1x systems stored in the -80℃ freezer had a light pink color, potentially impacting the ability of users to qualitatively assess the integrity of their samples. Additionally, previous research on the long-term stability of 1x indicator systems identified similar challenges in visualization of frozen indicator systems that can be easily masked by frost on sample vials, impacting the ability for users to easily determine whether their sample has been exposed to thawing conditions.[12] Therefore, the 11 indicators stated in *Table 3.1.1* were proposed for high concentration systems that generate a more intense pink coloration compared to the previously developed 1x systems.

Table 3.1.1 Proposed 5x Targeted Visual Indicators

Antifreeze-Free	Indicator #15 – 60 Minute 5x Antifreeze-Free System at 25℃ Indicator #16 – Maximum Length 5x Antifreeze-Free System at 4℃
$NaClO_4$	Indicator #17 – 5 Minute 5x $NaClO_4$ System at 25℃ Indicator #18 – 15 Minute 5x $NaClO_4$ System at 25℃ Indicator #19 – 60 Minute 5x $NaClO_4$ System at 0℃
$Mg(ClO_4)_2$	Indicator #20 – 5 Minute 5x $Mg(ClO_4)_2$ System at 25℃ Indicator #21 – 15 Minute 5x $Mg(ClO_4)_2$ System at 25℃ Indicator #22 – 60 Minute 5x $Mg(ClO_4)_2$ System at 0℃
$LiClO_4$	Indicator #23 – 5 Minute 5x $LiClO_4$ System at 25℃ Indicator #24 – 15 Minute 5x $LiClO_4$ System at 25℃ Indicator #25 – 60 Minute 5x $LiClO_4$ System at 0℃

As shown in *Table 3.1.1*, eleven high concentration (5x) visual indicator systems were proposed, with run times defined based on temperatures from 0℃ to 25℃. Out of the proposed 5x visual indicator systems, two indicators are antifreeze-free, three are formulated with sodium perchlorate (-37°C), three utilize magnesium perchlorate (-67°C), and three implement lithium perchlorate (-18°C). Additionally, unlike the previously outlined 1x visual indicators whose ideal final concentrations of sodium oxalate and potassium permanganate were 1.38 mM and 0.5 mM, the ideal final concentrations of sodium oxalate and potassium permanganate for the 5x visual indicators are 6.9 mM and 2.5 mM. However, while the ideal concentration of potassium permanganate will remain constant across all the proposed visual indicator systems to ensure a consistent starting pink color and MnO_4^- absorbance across indicators systems, varying concentrations of sodium oxalate are used when increased concentrations are required to achieved proposed targeted run times. Unfortunately, the MATLAB simulations are unable to simulate the permanganate/oxalic acid reaction for systems involving antifreeze-salt-containing aqueous eutectic solvents, and therefore design of the proposed indicators is performed experimentally via trial-and-error methods.

3.2 Methods and Materials

Similar materials and methods from Chapter 2 were also utilized for design of high concentration visual indicator systems.

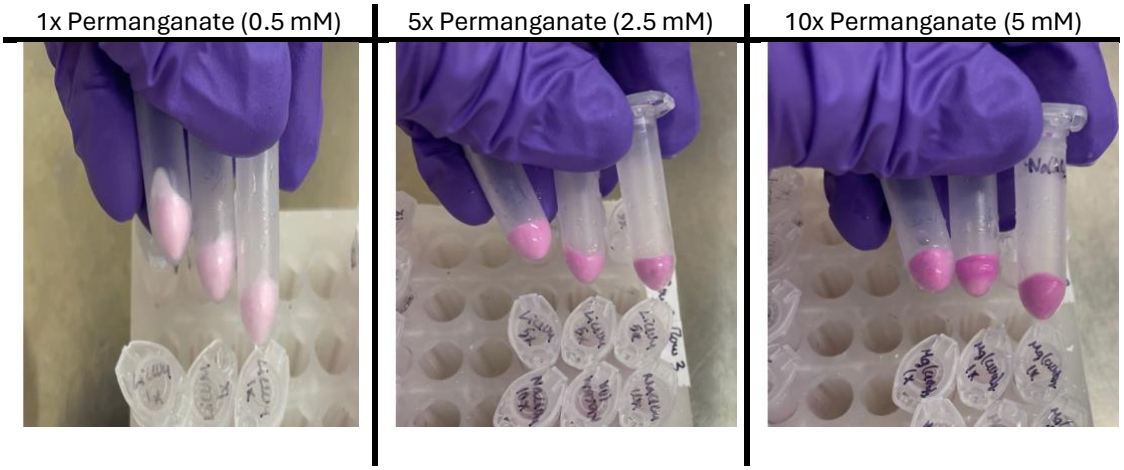
3.3 Results and Discussion

Data collection was completed in conjugation with Dr. Jorvani Cruz Villarreal under the guidance of Dr. Chad Borges.

3.3.1 Color Intensity for 1x, 5x, and 10x Permanganate

To demonstrate the change in pink color intensity following freezing, various permanganate concentrations were tested in a sodium perchlorate reaction system:

Table 3.3.1.1 Comparison of Color Intensity for 1x, 5x, and 10x Permanganate. *Left: [$HClO_4$]: 100 mM, [$Na_2Oxalate$]: 1.38 mM, [$Mn(ClO_4)_2$]: 5.0 x 10^{-2} mM, Middle: [$HClO_4$]: 250 mM, [$Na_2Oxalate$]: 6.9 mM, [$Mn(ClO_4)_2$]: 5.0 x 10^{-2} mM, Right: [$HClO_4$]: 375 mM, [$Na_2Oxalate$]: 13.8 mM, [$Mn(ClO_4)_2$]: 5.0 x 10^{-2} mM.*



As expected, *Table 3.3.1.1* demonstrates that increasing the concentration of permanganate results in a more intense pink coloration, allowing users to visually inspect our proposed visual indicators with greater ease and certainty. Therefore, both 5x and 10x permanganate concentrations were explored for the proposed visual indicators to provide an alternative for the previously defined 1x indicator systems.

3.3.2 Functionality of High Concentration Visual Indicators

When initially exploring the functionality of high concentration (5x or 10x) indicator systems, experimental results quickly demonstrated that increasing the concentration of both permanganate and sodium oxalate resulted in faster run times, challenging our ability to meet the specific run times outlined in *Table 3.1.1.* For example, Indicator #15 (60-minute 5x/10x antifreeze-free system at 25°C) was unable to be achieved utilizing 10x concentrations of sodium oxalate (13.8 mM) and permanganate

(5 mM), as shown in *Figure 3.3.2.1*, comparing the maximum length 1x and 10x antifreeze-free systems.

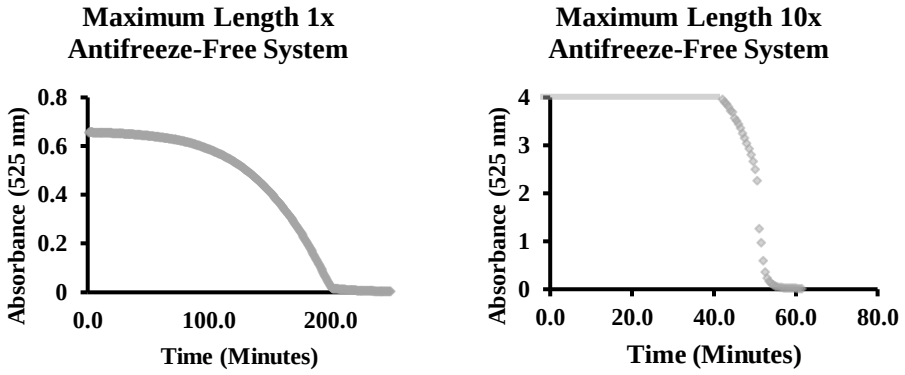


Figure 3.3.2.1 Comparison of 1x and 10x Max Length Antifreeze-Free Reactions at 25℃. 1x Antifreeze-Free Reaction Composition: [$HClO_4$]: 4 mM, [$Na_2Oxalate$]: 2.5 mM, [$KMnO_4$]: 0.5 mM. 10x Antifreeze-Free Reaction Composition: [$HClO_4$]: 37.5 mM, [$Na_2Oxalate$]: 13.8 mM, [$KMnO_4$]: 5 mM. Note: For the 10x visual indicator systems, the spectrophotometers had a maximum absorbance of 4.0 and therefore the grey line in the figure indicates that recorded absorbances at those time points were over the threshold for the ability of the spectrophotometer.

As seen in *Figure 3.3.2.1,* the maximum length 10x antifreeze-free system resulted in a run time of approximately 55 minutes whereas the maximum length 1x antifreeze-free system had a final run time of approximately 200 minutes. Therefore, while the previously designed 1x indicator systems achieved the outlined 1x targeted run times with the possibility to further extend reaction times, the initially designed 10x visual indicator systems were unable to achieve the targeted run times present in *Table 3.1.1.* Additionally, 10x indicator demonstrated the following progression:

Table 3.3.2.1 Progression of 10x $LiClO_4$ Reaction System. *[$HClO_4$]: 50 mM, [$Mn(ClO_4)_2$]: 250 μM, [$Na_2Oxalate$]: 13.8 mM, [$KMnO_4$]$_2$: 5 mM.*

60% Reaction Completion	80% Reaction Completion	Reaction Completion

As seen in *Table 3.3.2.1*, progression of the 10x $LiClO_4$ reaction system demonstrated a color transition from pink to brown to colorless, disrupting the proposed color transition. Within the proposed visual indicators, visual inspection of a pink color was designed to represent a viable sample whereas the transition to a clear color demonstrated non-viable samples whose biochemical integrity have potentially been comprised. Therefore, to avoid various color transitions in different visual indicators and potential challenges such as excess CO_2 formation, the 10x systems were no longer pursued and the focus was shifted to 5x higher concentration visual indicator systems. Similarly, visual inspection of a proposed 5x $LiClO_4$ visual indicator was performed, demonstrating the following results:

Table 3.3.2.2 Progression of 5x $LiClO_4$ Reaction System. *[$HClO_4$]: 100 mM, [$Mn(ClO_4)_2$]: 50 µM, [$Na_2Oxalate$]: 6.9 mM, [$KMnO_4$]$_2$: 2.5 mM.*

Beginning of the Reaction	80% Reaction Completion	Reaction Completion

As demonstrated in *Table 3.2.2.2,* the 5x $LiClO_4$ visual indicator system showcased the pink to colorless transition that characterizes the proposed visual indictor. The initially dark pink color turns to lighter pink towards the end of the reaction before rapidly changing to colorless, indicating aliquot or biospecimen exposure to thawing or improper storage conditions. Therefore, for higher concentration visual indicators, the 5x system were subsequently explored and designed for proposed indicators #15-25. While the ideal sodium oxalate and permanganate concentrations for the 5x indicator systems were 6.9 mM and 2.5 mM, each of the systems were tested with varying concentrations of sodium oxalate to investigate the effect of increased oxalate concentrations on 5x reaction run times and kinetics curves.

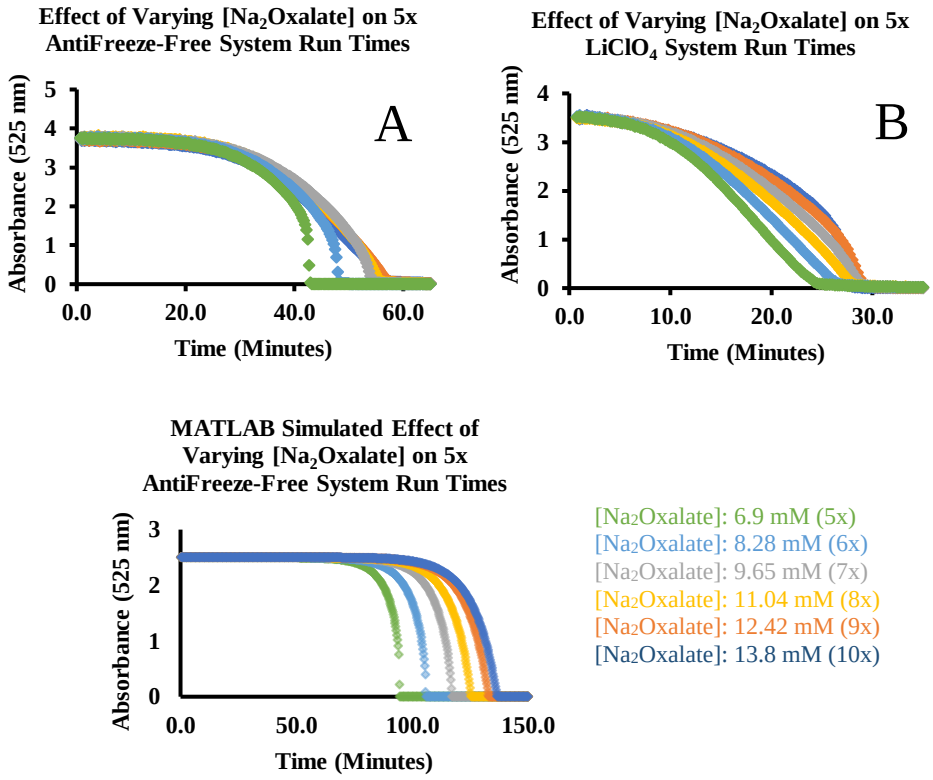


Figure 3.3.2.2: Experiments Demonstrating Effect of Increasing $Na_2Oxalate$ for 5x Antifreeze-Free and $LiClO_4$ Systems. (A) Constant Concentrations: $[HClO_4]$: 40 mM, $[KMnO_4]$: 2.5 mM. (B) Constant Concentrations: $[HClO_4]$: 50 mM, $[Mn(ClO_4)_2]$: 5 µM, $[KMnO_4]$: 2.5 mM. ***Green Curve*** *- $[Na_2Oxalate]$: 6.9 mM (5x),* ***Light Blue Curve*** *- $[Na_2Oxalate]$: 8.28 mM (6x),* ***Grey Curve*** *- $[Na_2Oxalate]$: 9.65 mM (7x),* ***Yellow Curve*** *- $[Na_2Oxalate]$: 11.04 mM (8x),* ***Orange Curve*** *- $[Na_2Oxalate]$: 12.42 mM (9x)* ***Dark Blue Curve*** *- $[Na_2Oxalate]$: 13.8 mM (10x).*

As shown in *Figure 3.3.2.2*, similar to the 1x visual indicators, increasing the concentration of oxalate from 6.9 mM to 13.8 mM in the 5x permanganate systems resulted in increased reaction times for the antifreeze-free indicator, from approximately 45 to 60 minutes. However, a similar modification in the kinetic curves was identified in higher concentration oxalate systems, shifting away from the sharp and rapid transition in the green (6.9 mM Oxalate) and blue (8.28 mM Oxalate) kinetic curves that characterize the permanganate/oxalic acid visual indicator towards a more progressive decrease in the permanganate absorbance, as seen in the yellow (11.04 mM Oxalate) or dark blue (13.8

mM Oxalate) curves. Nevertheless, experimental data indicated that small increases of oxalate, such as from 6.9 mM to 9.66 mM, increased the reaction time of the system while minimizing the modification of the kinetic curve and subsequent pink-to-clear transition of the visual indicator. Therefore, within the context of reaction design, while the ideal concentrations of sodium oxalate and permanganate for the 5x indicators were 6.9 mM and 2.5 mM, increased reaction times can be achieved by increasing the concentration of sodium oxalate. On the other hand, for the $LiClO_4$ system, increasing oxalate concentration resulted in faster run times but a similar disruption in the kinetic curve was seen as the concentrations of oxalate approached 13.8 mM. Therefore, 5x $LiClO_4$ systems were designed with the ideal concentration of oxalate, 6.9 mM, preserving the integrity of the kinetic curve while maximizing the reaction time of the indicator systems. Additionally, the effect of increasing oxalate concentrations was tested in the higher concentration $Mg(ClO_4)_2$ and $NaClO_4$ systems:

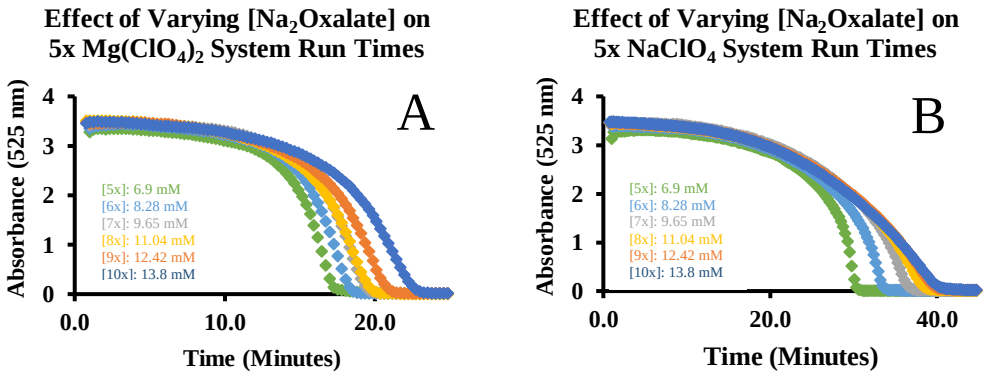


Figure 3.3.2.3: Experiments Demonstrating Effect of Increasing $Na_2Oxalate$ for 5x $Mg(ClO_4)_2$ and $NaClO_4$ Systems. (A) Constant Concentrations: $[HClO_4]$: 40 mM, $[KMnO_4]$: 2.5 mM, Solvent: 165 µL $Mg(ClO_4)_2$ (B) Constant Concentrations: $[HClO_4]$: 125 mM, $[Mn(ClO_4)_2]$: 10 µM, $[KMnO_4]$: 2.5 mM, Solvent: 149 µL $NaClO_4$. ***Green Curve*** *- $[Na_2Oxalate]$: 6.9 mM (5x),* ***Light Blue Curve*** *- $[Na_2Oxalate]$: 8.28 mM (6x),* ***Grey Curve*** *- $[Na_2Oxalate]$: 9.65 mM (7x),* ***Yellow Curve*** *- $[Na_2Oxalate]$: 11.04 mM (8x),* ***Orange Curve*** *- $[Na_2Oxalate]$: 12.42 mM (9x)* ***Dark Blue Curve*** *- $[Na_2Oxalate]$: 13.8 mM (10x).*

As demonstrated in *Figure 3.3.2.3*, the 5x $NaClO_4$ and $Mg(ClO_4)_2$ systems showcased similar trends to the 5x antifreeze-free system for increasing oxalate concentration. As the final oxalate concentration was increased, reaction time was subsequently increased but at the expense of a modified kinetic curve. Therefore, in reaction design for the $Mg(ClO_4)_2$ and $NaClO_4$ systems, oxalate concentrations were kept to concentrations of 6.9 mM (5x) or 8.28 (6x) to achieve target times without disruption of the proposed visual indicator kinetics. Moreover, the effect of increasing oxalate concentrations was quantitatively analyzed in *Figure 3.2.3.3*, demonstrating similar results as the previously outlined kinetic curves:

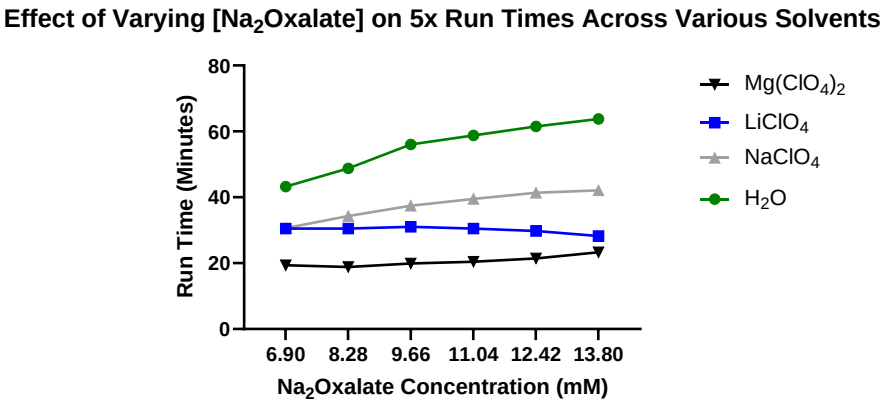


Figure 3.3.2.4: End Points of Experiments Demonstrating Effect of Increasing $Na_2Oxalate$ in 5x Indicator Systems. 1x [$Na_2Oxalate$]: 1.38 mM, 2x [$Na_2Oxalate$]: 2.76 mM, 3x [$Na_2Oxalate$]: 4.14 mM, 5x [$Na_2Oxalate$]: 6.9 mM, 7x [$Na_2Oxalate$]: 9.66 mM, 10x [$Na_2Oxalate$]: 13.8 mM.

3.3.3 Absorbance Endpoint of the 5x Visual Indicators

As outlined in *Chapter 2*, for permanganate/oxalic acid reactions at 25°C, the absorbance cutoff was determined to be 0.03 whereas for permanganate/oxalic acid reactions at temperatures <25°C, the absorbance cutoff was calculated from Beer's law to be 0.005. However, higher concentration systems, particularly systems with increased

sodium oxalate concentrations produced long "tails" at which the absorbance would decrease in small intervals, demonstrated below:

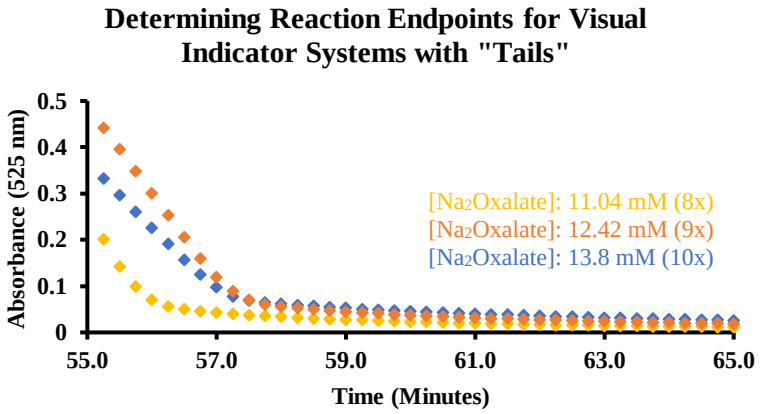


Figure 3.3.3.1: Experiments Demonstrating Visual Indicator Systems with "Tails". Constant Concentrations: [$HClO_4$]: 40 mM, [$KMnO_4$]: 2.5 mM, Solvent: 175 µL H_2O. ***Yellow Curve*** *- [$Na_2Oxalate$]: 11.04 mM (8x),* ***Orange Curve*** *- [$Na_2Oxalate$]: 12.42 mM (9x)* ***Dark Blue Curve*** *- [$Na_2Oxalate$]: 13.8 mM (10x).*

As showcased in *Figure 3.3.3.1,* higher concentrations of sodium oxalate in the 5x system resulted in extended run times but produced a "tail" for the kinetic curves. However, at the beginning of the "tail," the absorbance at 525 nm did not reach the previously defined absorbance cutoff of 0.03 for 25°C or 0.005 for <25°C, but subsequently reached the cutoff value after approximately 10-15 additional minutes. Nevertheless, visual inspection of the reaction solution at the beginning of the "tail" in follow-up experiments demonstrated that at the beginning of the "tail," the reaction solution was clear, indicating completion of the visual indicator transition from pink to colorless. As a result, for reactions which produced an absorbance "tail," both at 25°C and at temperatures <25°C, the endpoint of the reaction was selected at the beginning of the "tail" while reactions without a tail had their endpoint defined by the stated absorbance cutoffs determined in Chapter 2.

3.3.4 Design and Experimental Testing of 5x Visual Indicators

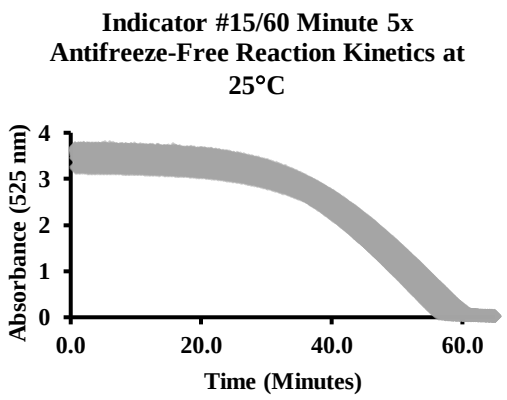
The outlined 11 high concentration (5x) systems were designed and replicated across various days. Within the 11 proposed 5x visual indicators, two are antifreeze-free systems, three are $NaClO_4$ systems, three utilize $Mg(ClO_4)_2$, and three implement $LiClO_4$. After experimental design and testing, the following results were produced:

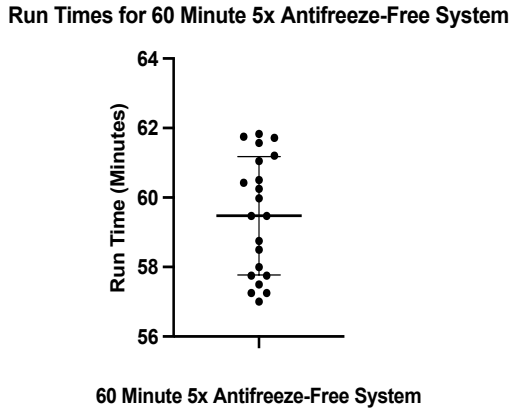
Table 3.3.4.1 Performance Summary of Targeted Indicators #15, #16

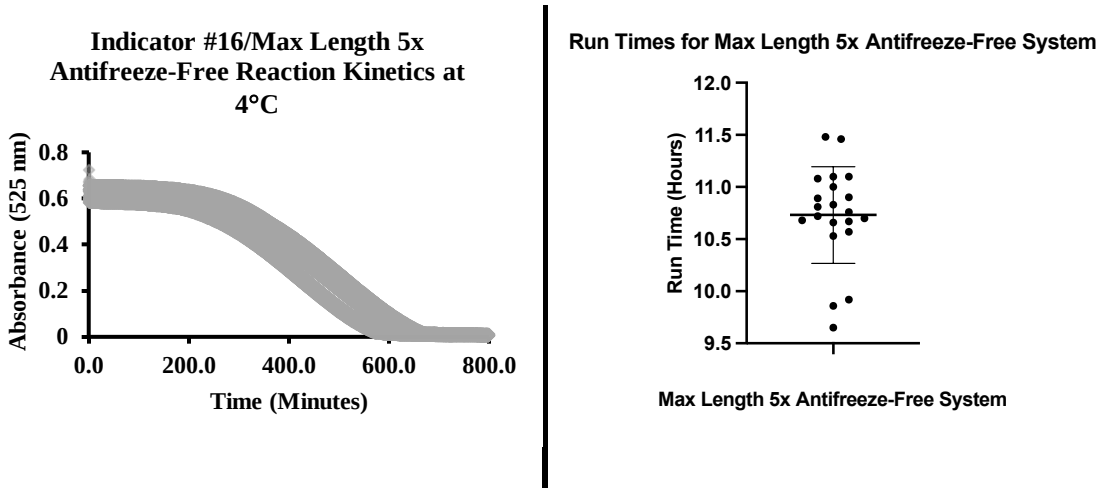
Indicator	Average	SD	Interday CV	Intraday CV
#15/1 Hour 5x Antifreeze-Free at 25℃	59.3 Minutes (n=21)	1.7 Minutes	2.9%	0.6%
#16/Max Length 5x Antifreeze-Free at 4℃	10.7 Hours (n=21)	0.5 Hours	4.3%	1.5%

Additionally, the 5x antifreeze-free systems generated the subsequent kinetic curves:

Table 3.3.4.2 Summary of Absorbance Spectra for Targeted Indicators #15, #16. *Targeted Indicator #15 (n=21) - [$HClO_4$]: 40 mM, [$Na_2Oxalate$]: 13.8 mM, [$KMnO_4$]: 2.5 mM, Targeted Indicator #16 (n=21) - [$HClO_4$]: 27.5 mM, [$Na_2Oxalate$]: 13.8 mM, [$KMnO_4$]: 2.5 mM. In the run time plots, data analysis of the plots showcases the mean and standard deviation across all the replicates within an indicator system.*







As shown in both *Tables 3.3.4.1 and 3.3.4.2,* the high concentration antifreeze-free systems were successfully designed and replicated across various days. For indicator #15 (60-minute 5x antifreeze-free system at 25℃), the average run time is 59.3 minutes (SD±1.7) across 21 replicates. Additionally, the system demonstrated strong interday and intraday precision with CV values of 2.9% and 0.6%. However, for data analysis and endpoint determination, the reaction produced a tail due to a final sodium oxalate concentration of 13.8 mM (10x) and a final permanganate concentration of 2.5 mM (5x). Therefore, as previously alluded to, the endpoint of the reactions was determined by the beginning of the tail rather than the utilized absorbance cutoff for 1x systems. Similarly, indicator #16 (maximum length reaction 5x antifreeze-free system at 4℃) utilized the beginning of the tail as the endpoint of the reaction, producing an average run time of 10.7 hours (SD±0.5) across 21 replicates. While the originally proposed 5x antifreeze-free indicator at 4℃ was targeted for 18 hours, the 5x antifreeze-free system at 4℃ only reached a maximum length of approximately 10.7 hours. Subsequent experiments to decrease the acid concentration or increase the oxalate concentration resulted in incomplete reactions or MnO_2 spikes. Nevertheless, the

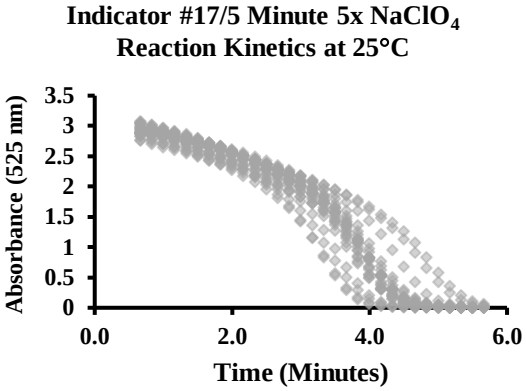
maximum length reaction was successfully replicated both within same day replicates and across various days, with interday and intraday CV values of 4.3% and 1.5%. Similar to the 1x antifreeze-free indicators, the designed 5x indicators were proposed to provide a visual indicator change for biospecimens and clinical samples that were either to be transported or temporarily stored. Therefore, samples could be exposed to room temperature conditions during transportation for up to 60 minutes while aliquots and clinical products could be temporarily stored in the refrigerator for up to 10.7 hours. Following successful design and replication of the antifreeze-free indicators, higher concentration indicators with sodium perchlorate were explored and designed:

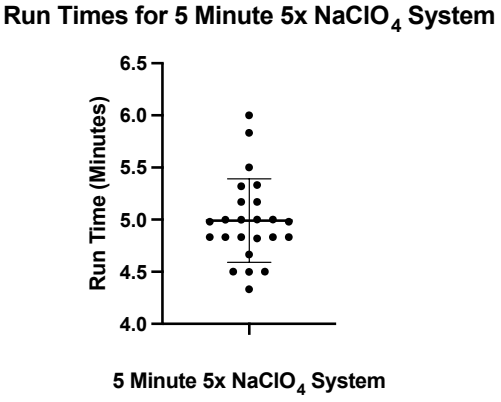
Table 3.3.4.3 Performance Summary of Targeted Indicators #17, #18, #19

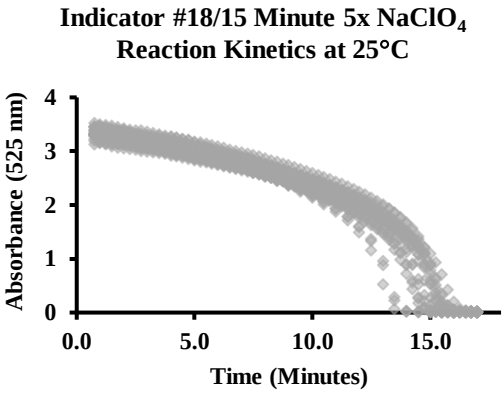
Indicator	Average (Minutes)	SD (Minutes)	Interday CV	Intraday CV
#17/5 Minute 5x $NaClO_4$ at 25℃	5.0 (n=24)	0.4	8.0%	3.1%
#18/15 Minute 5x $NaClO_4$ at 25℃	15.6 (n=18)	1.0	6.4%	2.1%
#19/60 Minute 5x $NaClO_4$ at 0℃	55.2 (n=18)	2.3	4.2%	3.4%

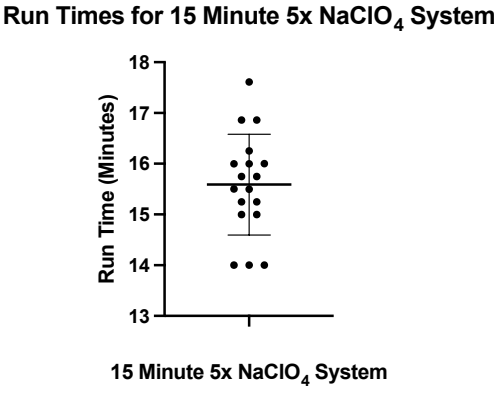
Similarly, the higher concentration sodium perchlorate indicators produced the following kinetic curves:

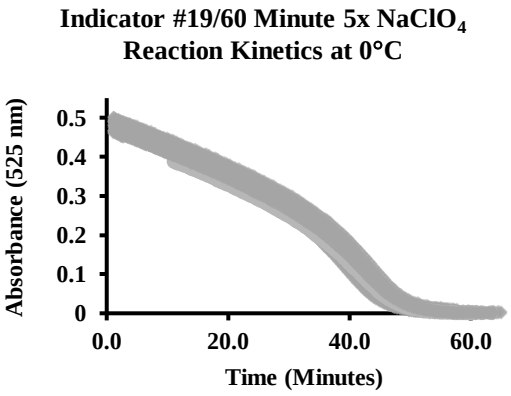
Table 3.3.4.4 Summary of Absorbance Spectra for Targeted Indicators #17, #18, #19. *Targeted Indicator #17 (n=24) - [$HClO_4$]: 100 mM, [$Na_2Oxalate$]: 6.9 mM, [$Mn(ClO_4)_2$]: 1.52 mM, [$KMnO_4$]: 2.5 mM, Targeted Indicator #18 (n=18) - [$HClO_4$]: 100 mM, [$Na_2Oxalate$]: 6.9 mM, [$Mn(ClO_4)_2$]: 5.2 x 10^{-1} mM, [$KMnO_4$]: 2.5 mM, Targeted Indicator #19 (n=18) - [$HClO_4$]: 200 mM, [$Na_2Oxalate$]: 8.28 mM, [$Mn(ClO_4)_2$]: 1.7 mM, [$KMnO_4$]: 2.5 mM. In the run time plots, data analysis of the plots showcases the mean and standard deviation across all the replicates within an indicator system.*

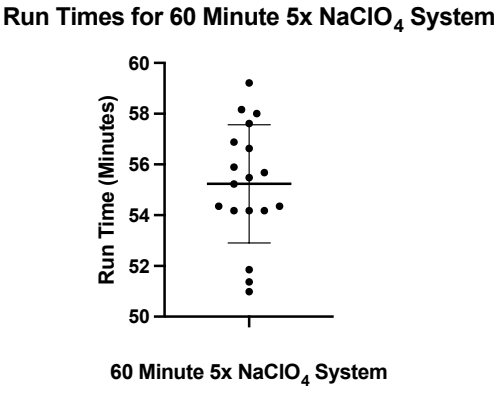












As demonstrated in *Tables 3.3.4.3 and 3.3.4.4,* high concentration sodium perchlorate systems were successfully constructed and reproduced across various intraday and interday replicates. For indictor #17 (5-minute 5x $NaClO_4$ system at 25℃),

the average run time across 24 replicates was 5.0 minutes (SD±0.4). Additionally, unlike the previous higher concentration antifreeze-free indicators, the sodium perchlorate indicators utilized the previously defined absorbance cutoffs of 0.03 for reactions at 25℃ and 0.005 for reactions at temperatures less than 25℃. For indicator #17, the reaction system demonstrated consistent precision across interday and intraday replicates, producing CV values of 8.0% and 3.1%, respectively. Similarly, indicator #18 (15-minute 5x $NaClO_4$ reaction system at 25℃) was successfully designed with an average run time of 15.6 minutes (SD±1.0) across 18 replicates, within the 10% deviation range for targeted indicator run times. Additionally, the reaction system showcased consistent interday and intraday precision, with CV values of 6.4% and 2.1%, demonstrating the reproducibility of the reaction system. Finally, indicator #19 (60-minute 5x $NaClO_4$ system at 0℃) produced an average run time of 55.2 minutes (SD±2.3) across 18 replicates over 6 different days. Therefore, the reaction system was reproducible and consistent across newly prepared stock solutions with interday and intraday CV precision values of 4.2% and 3.4%. Moreover, similar to the 1x sodium perchlorate systems, the higher concentration sodium perchlorate systems were designed to monitor biospecimens and aliquots during long-term storage. However, due to challenges with frost, the high concentration of permanganate provides a more intense coloration that allows users to quickly identify the integrity of their sample. Therefore, the 5-minute and 15-minute systems were designed for samples that either cannot be exposed to thawed conditions or can be exposed to one thawing cycle whereas the 60-minute system was designed for aliquots that can be exposed to a few thawing cycles without a significant impact to

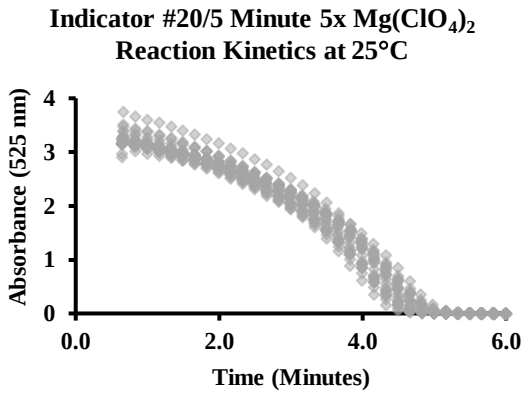
biospecimen integrity. After design of the 5x sodium perchlorate systems, similar experiments were performed for higher concentration magnesium perchlorate systems:

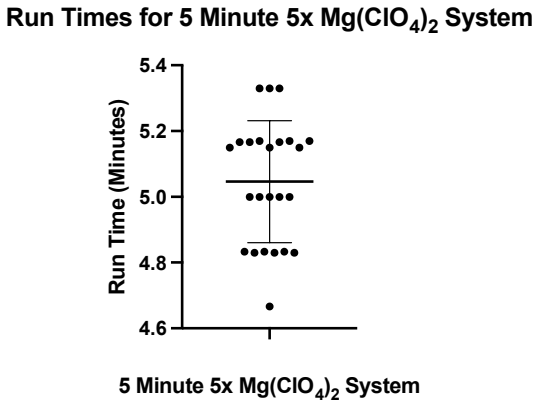
Table 3.3.4.5 Performance Summary of Targeted Indicators #20, #21, #22

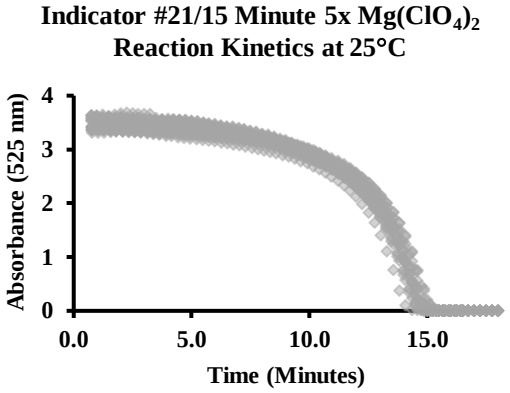
Indicator	Average (Minutes)	SD (Minutes)	Interday CV	Intraday CV
#20/5 Minute 5x $Mg(ClO_4)_2$ at 25℃	5.1 (n=24)	0.2	3.7%	1.0%
#21/15 Minute 5x $Mg(ClO_4)_2$ at 25℃	15.0 (n=18)	0.3	1.9%	1.0%
#22/60 Minute 5x $Mg(ClO_4)_2$ at 0℃	61.3 (n=21)	1.4	2.2%	1.0%

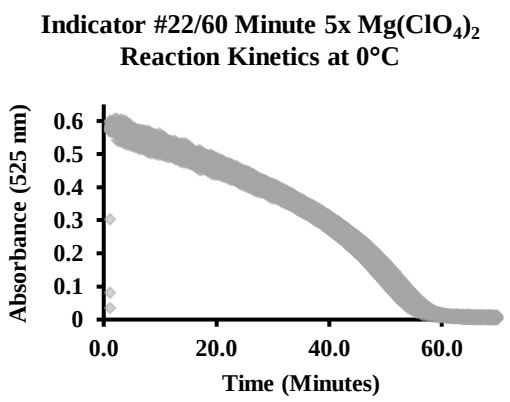
Moreover, the following kinetics curves were obtained:

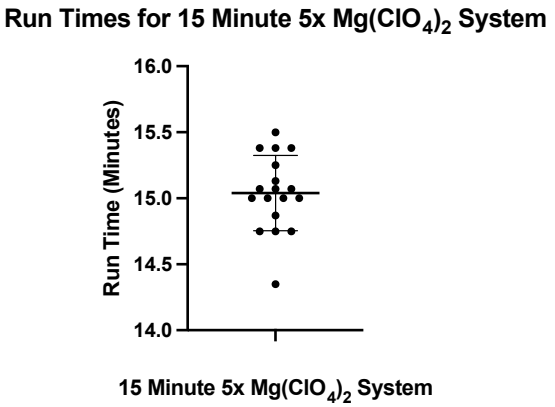
Table 3.3.4.6 Summary of Absorbance Spectra for Targeted Indicators #20, #21, #22. *Targeted Indicator #20 (n=24) - [$HClO_4$]: 120 mM, [$Na_2Oxalate$]: 8.28 mM, [$Mn(ClO_4)_2$]: 5.0 x 10^{-1} mM, [$KMnO_4$]: 2.5 mM, Targeted Indicator #21 (n=18) - [$HClO_4$]: 70 mM, [$Na_2Oxalate$]: 6.9 mM, [$KMnO_4$]: 2.5 mM, Targeted Indicator #22 (n=21) - [$HClO_4$]: 160 mM, [$Na_2Oxalate$]: 8.28 mM, [$Mn(ClO_4)_2$]: 8.0 x 10^{-1} mM, [$KMnO_4$]: 2.5 mM. In the run time plots, data analysis of the plots showcases the mean and standard deviation across all the replicates within an indicator system.*

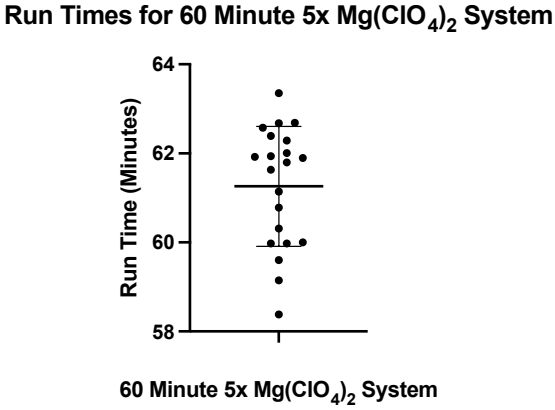












As demonstrated in *Tables 3.3.4.5 and 3.3.4.6,* the 5x magnesium perchlorate systems were designed and successfully replicated across multiple days with varying stocks. For indicator #20 (5-minute 5x $Mg(ClO_4)_2$ system at 25℃), 24 replicates across 8 different days produced an average run time of 5.1 minutes (SD±0.2). Additionally, the magnesium perchlorate system demonstrated consistent precision and replicability, with interday and intraday CV values of 3.7% and 1.0%. Similar to the previously defined 5-minute 5x sodium perchlorate reaction, the 5-minute 5x magnesium perchlorate indicator system was designed with application to aliquots that are unable to be exposed to thawing conditions without a loss of biospecimen integrity. On the other hand, indicator #21 (15-minute 5x $Mg(ClO_4)_2$ system at 25℃) was similarly designed to

monitor temperature changes during aliquot storage. However, the 15-minute 5x indicator system is applicable to samples or aliquots that can undergo one or two thawing cycles. After design of indicator #21, 18 replicates across 6 days demonstrated an average run time of 15.0 minutes (SD±0.3). Moreover, while the 5-minute 5x $Mg(ClO_4)_2$ reaction system was designed with ideal 5x concentrations of sodium oxalate and permanganate, the 15-minute reaction was designed with a 6x concentration of sodium oxalate and 5x concentration of permanganate to ensure accurate reaction kinetics that achieve the targeted run time. Additionally, across the interday and intraday replicates, the reaction system demonstrated strong precision and reproducibility with CV values of approximately 1.9% and 1.0%. Finally, indicator #22 (60-minute 5x $Mg(ClO_4)_2$ system at 0℃) was also successfully designed with a 6x concentration of sodium oxalate, producing an average run time of 61.3 minutes (SD±1.4) across 21 replicates. Moreover, the reaction system similarly demonstrated accurate reproducibility both within intraday replicates and interday replicates, generating CV values of 1.0% and 2.2%.

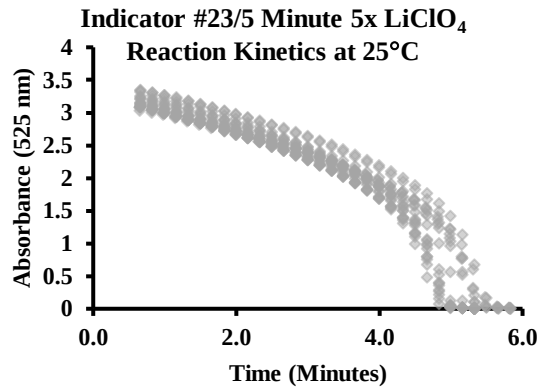
The final three proposed high concentration visual indicators systems were constructed with $LiClO_4$:

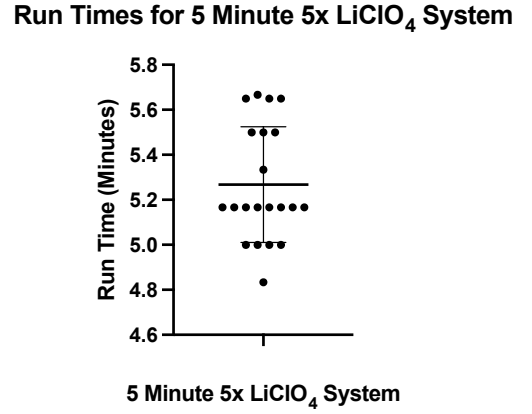
Table 3.3.4.7 Performance Summary of Targeted Indicators #23, #24, #25

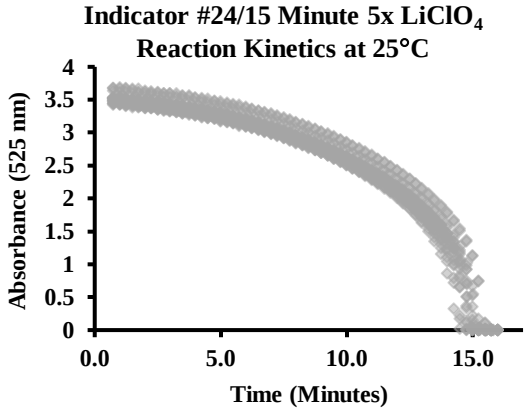
Indicator	Average (Minutes)	SD (Minutes)	Interday CV	Intraday CV
#23/5 Minute 5x $LiClO_4$ at 25℃	5.3 (n=21)	0.3	4.9%	1.8%
#24/15 Minute 5x $LiClO_4$ at 25℃	15.2 (n=18)	0.3	2.2%	1.2%
#25/60 Minute 5x $LiClO_4$ at 0℃	61.0 (n=18)	2.8	4.6%	1.4%

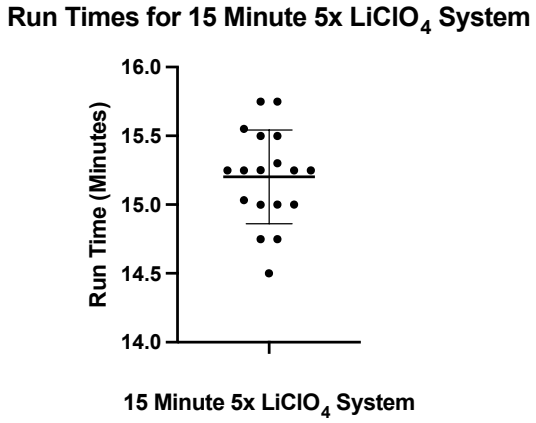
Additionally, the collected data produced the following kinetic curves:

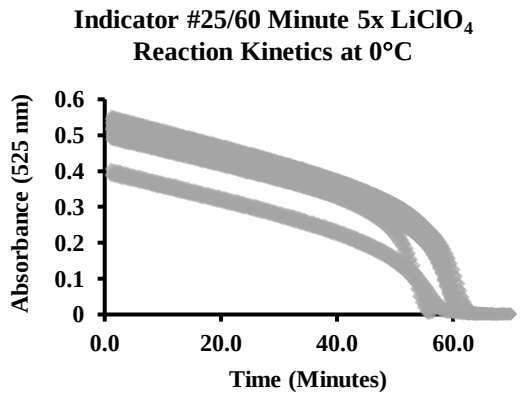
Table 3.3.4.8 Summary of Absorbance Spectra for Targeted Indicators #23, #24, #25. *Targeted Indicator #23 (n=21) - [$HClO_4$]: 100 mM, [$Na_2Oxalate$]: 6.9 mM, [$Mn(ClO_4)_2$]: 7.5 x 10^{-1} mM, [$KMnO_4$]: 2.5 mM, Targeted Indicator #24 (n=18) - [$HClO_4$]: 100 mM, [$Na_2Oxalate$]: 6.9 mM, [$Mn(ClO_4)_2$]: 5.0 x 10^{-2} mM, [$KMnO_4$]: 2.5 mM, Targeted Indicator #25 (n=18) - [$HClO_4$]: 100 mM, [$Na_2Oxalate$]: 8.28 mM, [$Mn(ClO_4)_2$]: 1.075 mM, [$KMnO_4$]: 2.5 mM. In the run time plots, data analysis of the plots showcases the mean and standard deviation across all the replicates within an indicator system.*

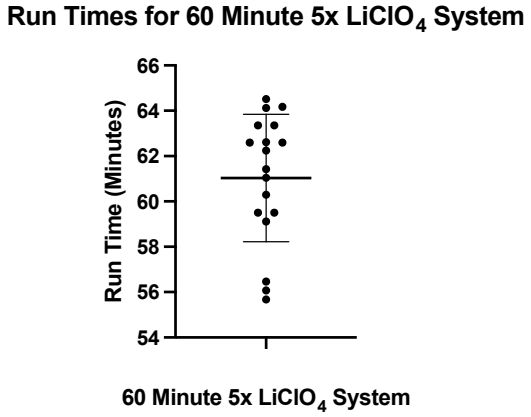












As demonstrated in *Tables 3.3.4.7 and 3.3.4.8,* the 5x $LiClO_4$ visual indictor systems were successfully constructed and replicated utilizing absorbance spectroscopy.

Similar to the other 5x eutectic systems, the 5-minute and 15-minute systems were designed for storage of samples with minimal exposure to thawing conditions whereas the 60-minute system was proposed for aliquots that required repetitive use and exposure to thawing conditions for transportation or analysis. Firstly, indicator #23 (5-minute 5x $LiClO_4$ reaction at 25℃) had an average run time of 5.3 minutes (SD±0.3) across 7 days of reaction replicates. Within those 7 days, the reaction system showcased strong replicability and precision, with interday and intraday CV values of 4.9% and 1.8%. On the other hand, indicator #24 (15-minute 5x $LiClO_4$ system at 25℃) had 18 replicates across six different days, producing an average run time of 15.2 minutes (SD±0.3). Additionally, the 15-minute reaction demonstrated increased precision in comparison to the 5-minute reaction, with interday and intraday CV values of 2.2% and 1.2%. Moreover, the final 5x $LiClO_4$ system, indicator #25 or the 60-minute 5x $LiClO_4$ reaction system at 0℃ showcased an average run time of 61.0 minutes (SD±2.8) across 18 replicates. Nevertheless, the reaction system demonstrated similar precision to the previous higher concentrations systems with interday and intraday CV values of 4.6% and 1.4%. Overall, the high concentration systems were successfully designed and tested in experimental environments, producing the following table of designed higher concentration visual indicators:

Table 3.3.4.9: Summary Run Time and Precision Characteristics of 5x Targeted Visual Indicators

Solution Composition (Freezing Pt)	25℃	4℃	On ice (0℃)
Antifreeze-Free (0℃) 5x System (Indicator #15)	**Avg: 59.3 Minutes (SD±1.7) n=21**		
Antifreeze-Free (0℃) 5x System		**Avg: 10.7 Hours (SD±0.5)**	

(Indicator #16)		**n=21**	
52% (w/w) $NaClO_4$ (-37℃) 5x System (Indicator #17)	**Avg: 5.0 Minutes (SD±0.4) n=24**		
52% (w/w) $NaClO_4$ (-37℃) 5x System (Indicator #18)	**Avg: 15.6 Minutes (SD±1.0) n=18**		
52% (w/w) $NaClO_4$ (-37℃) 5x System (Indicator #19)			**Avg: 55.2 Minutes (SD±2.2) n=18**
44% (w/w) $Mg(ClO_4)_2$ (-67℃) 5x System (Indicator #20)	**Avg: 5.1 Minutes (SD±0.2) n=24**		
44% (w/w) $Mg(ClO_4)_2$ (-67℃) 5x System (Indicator #21)	**Avg: 15.0 Minutes (SD±0.3) n=18**		
44% (w/w) $Mg(ClO_4)_2$ (-67℃) 5x System (Indicator #22)			**Avg: 61.3 Minutes (SD±1.3) n=21**
22% (w/w) $LiClO_4$ (-18℃) 5x System (Indicator #23)	**Avg: 5.3 Minutes (SD±0.3) n=21**		
22% (w/w) $LiClO_4$ (-18℃) 5x System (Indicator #24)	**Avg: 15.2 Minutes (SD±0.3) n=18**		
22% (w/w) $LiClO_4$ (-18℃) 5x System (Indicator #25)			**Avg: 61.0 Minutes (SD±2.8) n=18**

3.4 Conclusions

Similar to the 1x visual indicator systems described in *Chapter 2*, 11 high concentration indicator systems were designed and replicated across numerous days to construct specialized reaction systems for our proposed visual indicator research. While the 1x reaction systems function across varying time intervals and temperatures, previous studies highlighted challenges with visualization of 1x visual indicator systems, particularly at subzero temperatures, prompting investigation and development of higher concentration systems with a more intense pink coloration. As a result, higher concentration systems that utilized increased concentrations of MnO_4^- were compared

with the previously defined 1x reaction systems, successfully demonstrating the contrast in coloration that allows for more seamless and user-friendly implementation of our proposed visual indicators at subzero storage temperatures. After comparison of various visual indicator systems, the progression and functionality of the higher concentration systems, 5x and 10x, was explored. However, 10x indicators showcased a pink to brown to clear transition or excess CO_2 formation and were subsequently deemed unapplicable for the purposes of our proposed indicators. Nevertheless, 5x visual indicator systems demonstrated the outlined pink to clear transition and therefore, the 11 proposed indicators were designed and replicated with 5x or 2.5 mM permanganate. Overall, encouraging process has been made in the development of higher concentration visual indicator systems that provide users with more concentrated reaction systems specifically designed for use and application at subzero storage temperatures that have previously affected the ability of users to effectively utilize the proposed cold chain tracking mechanism.

CHAPTER 4

PRE-ACTIVATION STABILITY AND LONG-TERM STABILITY STUDIES FOR VISUAL INDICATORS

4.1 Introduction

While previous chapters outlined the design and functionality of various proposed targeted indicators, experimental designs have included preparing solutions within Eppendorf tubes or a 96-well plate, incubating the reagents without permanganate for 5 minutes and subsequently collecting absorbance data and reaction kinetics upon "activation" with permanganate. However, initial prototypic devices for our proposed visual indicators implement a mechanism where all the reagents are mixed together in a capsule that separates dry permanganate within a glass ampoule.[13] Therefore, users will be able to "release" permanganate from the glass ampoule into solution, subsequently "activating" the reaction upon mixing of permanganate and the pre-mixed reaction solution. In this case, perchloric acid, sodium oxalate, manganese perchlorate, and either water or an antifreeze-salt-containing aqueous eutectic solvent will be pre-mixed and incubated together until activation by the user. As a result, we explored the pre-activation stability of the reagents components, except for the permanganate, within polypropylene Eppendorf tubes.

Additionally, previous research investigated the long-term stability of the permanganate/oxalic acid reaction at -80℃, below the freezing point of the eutectic and antifreeze-free solvents[12]. Theoretically, upon freezing, the permanganate/oxalic acid reaction should either be halted or significantly slowed, preventing the pink to colorless transition that characterizes compromised biospecimens or aliquots. Therefore, if samples

or aliquots are stored at the proper temperature, the permanganate/oxalic acid reaction should not progress, preventing the pink to colorless transition. As a result, we continued previous studies and explored the long-term stability of the permanganate/oxalic acid reaction across various indicator systems to determine the stability of the reaction mechanism and proposed indicators in frozen conditions.

4.2 Methods

4.2.1 Experimental Procedure for Pre-Activation Studies

All materials were nitric acid washed as described in *Chapter 2*. Reactions were prepared for pre-activation studies of the reagents, without permanganate. First, washed 2.0 mL Eppendorf tubes were labeled with their corresponding incubation time and reaction: Indicators #15, #16, #18, #21, or #24. Each indicator was prepared with 6 replicates for each of the following incubation times: 5 minutes, 3 days, 1 week, 2 weeks, 1 month, 2 months, 3 months, 4 months, 5 months, and 6 months. Stock concentrations for each of the reagents were prepared with 18.2 MΩ*cm deionized water and stored in 15 mL Falcon Tubes. To prevent variation due to stock concentrations, enough stock solutions were prepared to be utilized for all of the pre-activation studies. Varying quantities of the antifreeze-free or antifreeze-salt-containing aqueous eutectic solvents, perchloric acid, manganese perchlorate, and sodium oxalate were mixed together depending on the indicator composition for all of the incubation times except 5 minutes. Following mixing, the Eppendorf tubes were vortexed three times and subsequently stored at room temperature. Any remaining stock solutions were stored within a separate container and utilized for either the 5-minute incubation reaction or blanks. A fresh 100 mM potassium permanganate solution was prepared daily throughout all experiments due

to the oxidizing capacity of permanganate. When the permanganate was not used in a particular experiment, it was stored in the refrigerator at 4°C.

For the 5-minute control reactions, solutions of the designed reactions were prepared and mixed within wells in a washed 96 well plate. When mixing solutions in the laminar flow hood, the stock solutions were vortexed three times and then pipetted in their respective wells. The solvent was added first, followed by perchloric acid, manganese perchlorate, and sodium oxalate to generate consistency. Before the addition of permanganate, the volume in each well totaled 195 µL with the exception of the blank which totaled 200 µL. Additionally, before the diluted solution of permanganate was added, the remaining reagents within solution were mixed three times with a 200 µL pipette. Following mixing, a washed plate cover was placed over the 96 well plate and the solutions incubated at room temperature for 5 minutes. During the 5-minute incubation of the reagents, the settings for the spectrophotometer were configured and prepared. The spectrophotometer was set to be measured at a wavelength of 525 nm and the temperature of 25°C. After 5 minutes incubation of all the reagents without the permanganate, the plate was transferred to the workplace in front of the spectrophotometer.

However, solutions that were incubated for various time periods greater than the control or 5-minute incubation time had a slightly different protocol. After the specified incubation time, a 200 µL pipette was used to transfer the solutions from the Eppendorf tubes (n=6) for a particular indicator set to the 96-well plate. No additional incubation time was required, and the solutions were mixed three times with the 200 µL pipette before transportation to the workplace in front of the spectrophotometer.

After 96-well plate preparation, 5 µL of the 100 mM permanganate stock solution was quickly transferred into each of the wells, before a 200 µL pipette was used to quickly mix each final solution three times. Following mixing of each indicator solution, the plate was transferred to the plate reader and absorbance readings were acquired. After kinetics runs were completed, the data was subsequently saved and the time between adding the permanganate and the beginning of absorbance readings was added to the reaction time. Additionally, during data analysis, the blank was subtracted from samples and reactions were determined to be complete at an absorbance of 0.03 following blank subtraction. Finally, cleanup procedures were performed.

4.2.2 Experimental Procedure for Long-Term Stability Studies

All the labware, including Eppendorf tubes, pipette tips, 96-well plates, and glassware underwent the nitic acid washing procedure stated in *Chapter 2*. Three 2.0 mL Eppendorf tubes were labeled with their respective reaction and start date. Then, stock concentrations of each of the reagents were prepared in Eppendorf tubes with 18.2 MΩ*cm deionized water and stored until use. The indicators were prepared in Eppendorf tubes and vortexed three times and subsequently incubated at room temperature for 5 minutes. After completion of incubation, potassium permanganate was pipetted into each of the three Eppendorf tubes before the Eppendorf tubes were vortexed three times and immediately transferred into a dry ice/ethanol bath for flash freezing. The tubes remained in the dry ice/ethanol bath for 10 minutes before they were directly transported into a cardboard box and stored in the -80°C freezer. If multiple long-term stability indicators were prepared consecutively, the procedure was repeated for another set of Eppendorf tubes. After experiments were performed, cleanup procedures were performed.

4.3 Results and Discussion

4.3.1 Pre-Activation Studies for Visual Indicators

For pre-activation studies of the permanganate/oxalic acid reaction implementing various antifreeze free and antifreeze-salt-containing aqueous eutectic solvents, absorbance spectroscopy was utilized to determine the average run time the following incubation times: 5 Minutes (Control), 3 Days, 1 Week, 2 Weeks, 1 Month, 2 Months, 3 Months, 4 Months, 5 Months, and 6 Months. The first visual indicator system explored was the 5x $Mg(ClO_4)_2$ system. Across all the incubation times except for the 5-minute control, a cloudy precipitate formed within solution and addition of permanganate to the solution resulted in the brown/red precipitate shown below, compromising the permanganate/oxalic acid reaction.

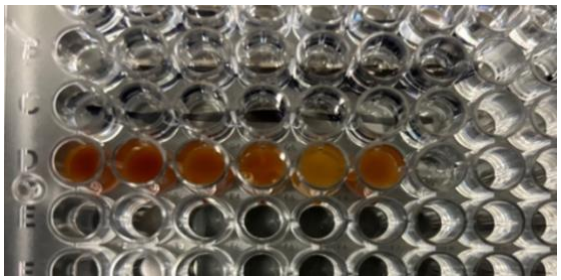


Figure 4.3.1.1: Precipitate Formation $Mg(ClO_4)_2$ Pre-Stability Studies.

Therefore, further experiments are currently being explored to investigate the formation of a cloudy precipitate in solution. Our current hypotheses are either magnesium oxalate precipitating out of solution or bacterial growth present within the solution mixture. Subsequently, a sample of cloudy $Mg(ClO_4)_2$ was tested for potential bacterial growth, and we are currently awaiting the results before further exploration of

the $Mg(ClO_4)_2$ systems. Nevertheless, the $LiClO_4$ and $NaClO_4$ reaction systems were subsequently explored, generating the following results:

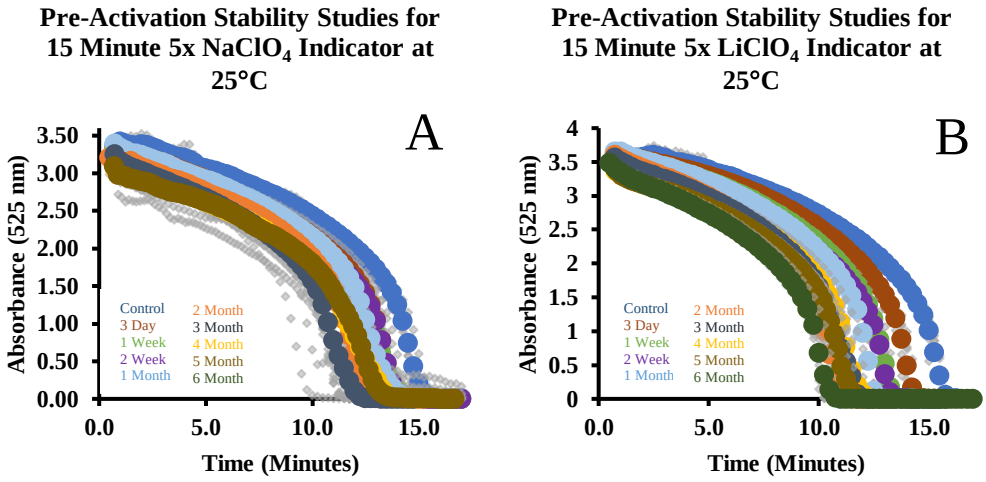


Figure 4.3.1.2: Pre-Stability Studies for 15-Minute 5x $NaClO_4$ and $LiClO_4$ Indicators. (A) $[HClO_4]$: 100 mM, $[Na_2Oxalate]$: 6.9 mM, $[Mn(ClO_4)_2]$: 5.2 x 10^{-1} mM, $[KMnO_4]$: 2.5 mM. (B) Targeted Indicator #24 - $[HClO_4]$: 100 mM, $[Na_2Oxalate]$: 6.9 mM, $[Mn(ClO_4)_2]$: 5.0 x 10^{-2} mM, $[KMnO_4]$: 2.5 mM. Colored curves represent run time averages for varying incubation time whereas light grey data points in the background represents all the experimental data and replicates. Incubation Times: Dark Blue – "Control or 5 Minutes", Maroon – 3 Day, Green – 1 Week, Purple – 2 Weeks, Light Blue – 1 Month, Orange – 2 Months, Dark Grey – 3 Months, Yellow – 4 Months, Brown – 5 Month, Dark Green – 6 Month.

As shown in *Figure 4.3.1.2*, initial experiments investigating the impact of varying incubation times demonstrated that increasing incubation time of the reagents without permanganate in Polypropylene Eppendorf tubes shortens the reaction time of the 5x indicator systems. Unfortunately, some of the $NaClO_4$ replicates with a 6-month incubation time did reach reaction completion when activated with permanganate and therefore an average run time for $NaClO_4$ 6-month incubation was not achieved. Nevertheless, the final run times of the $LiClO_4$ and $NaClO_4$ systems were compared across varying incubation times, as shown below:

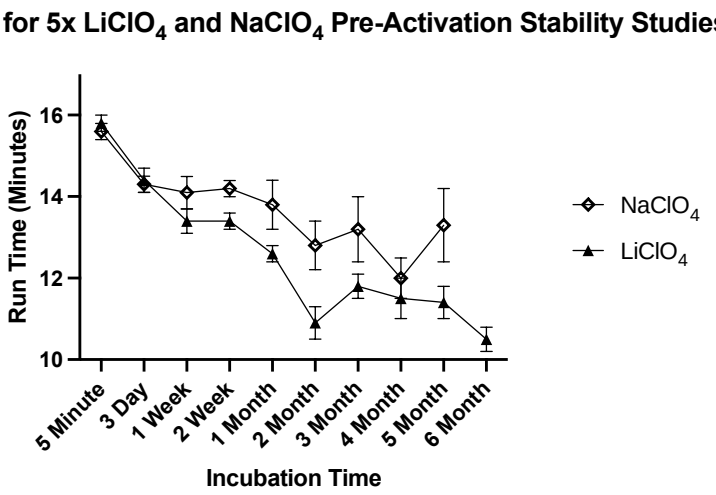


Figure 4.3.1.3: Pre-Stability Run Times for 15-Minute 5x $NaClO_4$ and $LiClO_4$ Indicators.

Similarly, *Figure 4.3.1.3* further illustrated that increasing the incubation time of the reaction reagents resulted in a progressive decrease in the average run time of the permanganate/oxalic acid systems, potentially impacting the accuracy and application of our proposed visual indicators in academic and clinical contexts. In both the pre-activation studies for $NaClO_4$ and $LiClO_4$, the control reactions with 5-minute incubation resulted in run times of 16 minutes while absorbance kinetics after multiple months of incubation of the reagents resulted in an average run times of approximately 12 minutes. However, further investigation is required to determine the explanation for decreased reaction time across increased incubation times. Interactions between the chemical reagents within solution, particularly after the introduction of antifreeze-salt-containing aqueous eutectic solvents, could influence the designed reaction times whereas other potential explanations could include reagent reactions with the Polypropylene plastic or other environmental impacts on the chemical reaction system. Therefore, further pre-activation studies will test the proposed visual indicators in various storage materials and investigate potential chemical interactions between reagents

to ensure accurate application of the designed visual indicators. However, while the eutectic systems demonstrated decreased run times, pre-activation studies of the 5x antifreeze-free systems produced the following results:

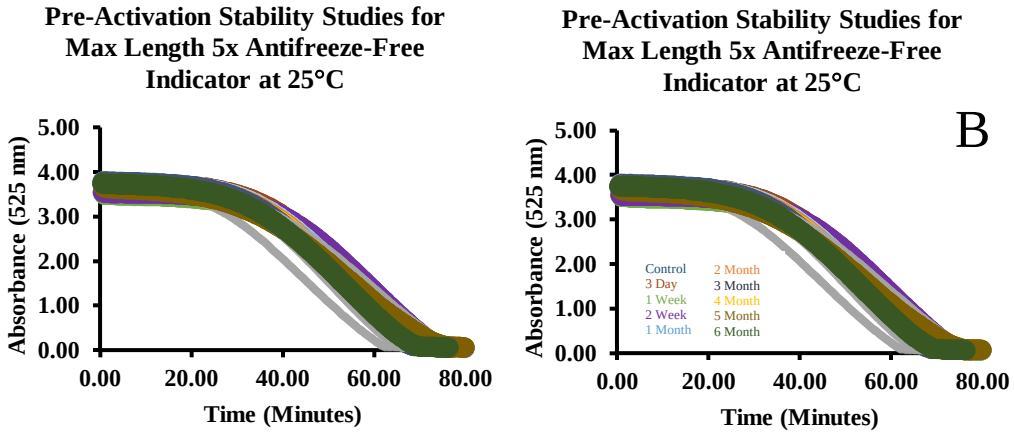


Figure 4.3.1.4: Pre-Stability Studies for 5x Antifreeze-Free Indicators. (A)[$HClO_4$]: 40 mM, [$Na_2Oxalate$]: 13.8 mM, [$KMnO_4$]: 2.5 mM, (B) [$HClO_4$]: 27.5 mM, [$Na_2Oxalate$]: 13.8 mM, [$KMnO_4$]: 2.5 mM. Colored curves represent run time averages for varying incubation time whereas light grey data points in the background represents all the experimental data and replicates. Incubation Times: Dark Blue – "Control or 5 Minutes", Maroon – 3 Day, Green – 1 Week, Purple - 2 Weeks, Light Blue – 1 Month, Orange – 2 Months, Dark Grey - 3 Months, Yellow - 4 Months, Brown – 5 Month, Dark Green – 6 Month.

Additionally, the average run times at each incubation time were calculated and compared across both the 5x antifreeze-free systems:

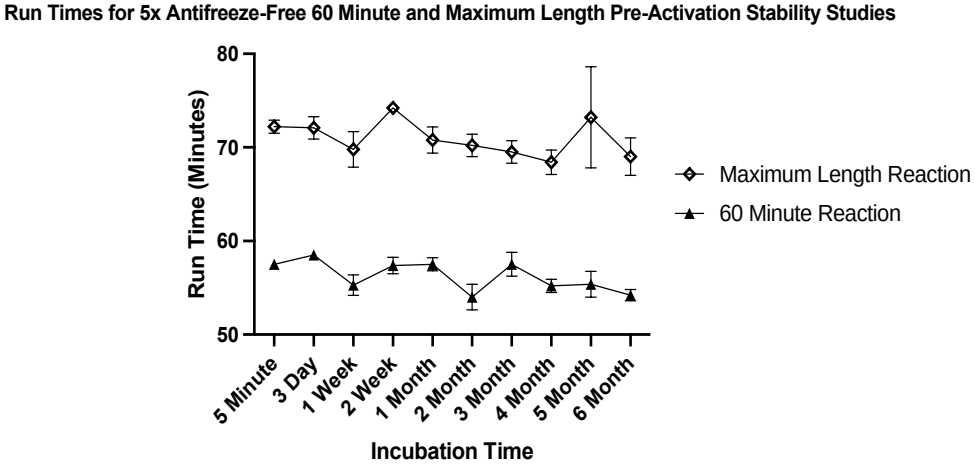


Figure 4.3.1.5: Pre-Activation Run Times for 5x Antifreeze-Free Indicators.

As demonstrated in *Figures 4.3.1.4 and 4.3.1.5,* unlike the previously explored indicators with antifreeze-salt-containing aqueous eutectic solvents, antifreeze-free indicators demonstrated minimal variation in average run time as the incubation time of the reagents without permanganate was increased. While slight variations in run time exist across various incubation durations, freshly prepared permanganate stocks were prepared daily and therefore account for interday variation. Additionally, consistent run times for the 5x antifreeze-free systems across varying incubation times suggests that eutectic solvents might be responsible for the previously outlined changes demonstrated in the 5x $LiClO_4$ and $NaClO_4$ systems. Potentially, the introduction of either additional metal ions or perchlorate ions results in increased metal-ion complexing or additional reactions that impact the permanganate/oxalic acid reaction over time, resulting in the decreased run times demonstrated in *Figures 4.3.1.2* and *4.3.1.3.* Therefore, future experiments will include monitoring the stability of reactions between eutectic solvents and other chemical reagents, such as perchloric acid or sodium oxalate, to further investigate reaction time changes that occurred with increased incubation times.

4.3.2 Long-Term Stability Studies for Visual Indicators

As previously mentioned, visual indicators stored at subzero temperatures will be frozen and therefore the proposed permanganate/oxalic reaction should not be progressing to a significant degree. However, to test the long-term stability of the permanganate/oxalic reactions at temperatures below the eutectic freezing point, designed reactions were flash-frozen in a dry ice/ethanol bath and stored in the -80°C freezer. Over time, the progress of these frozen indicators was monitored and recorded to explore the long-term stability of the visual indicator systems:

Table 4.3.2.1 Monitoring a Flash-Frozen 1x $NaClO_4$ Indicator Stored at -80℃. *[$HClO_4$]: 10 mM, [$Na_2Oxalate$]: 1.38 mM, [$KMnO_4$]: 5 x 10^{-1} mM*

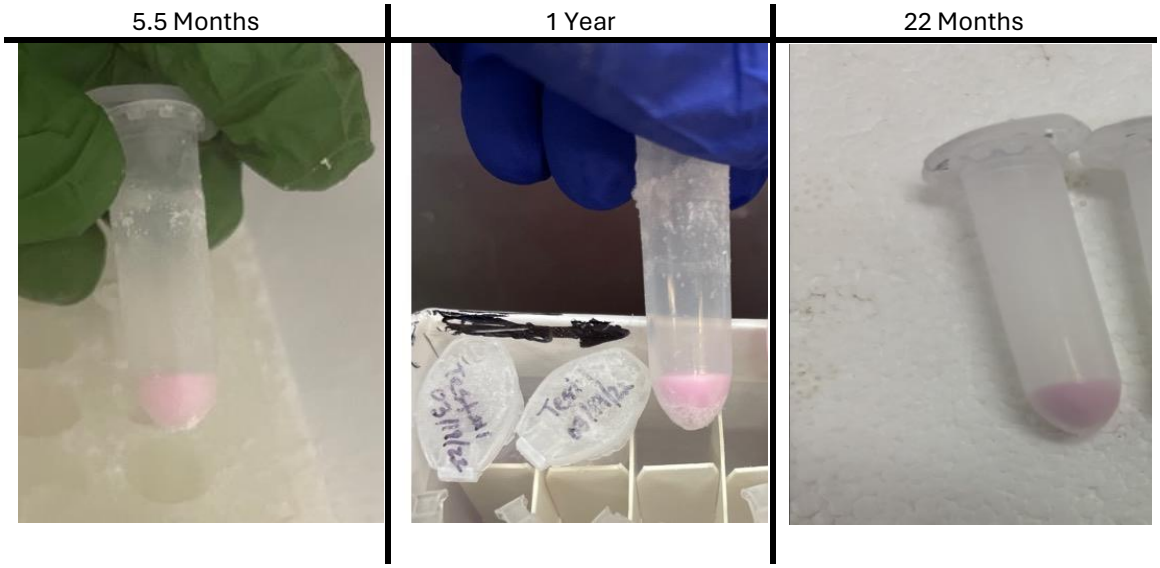
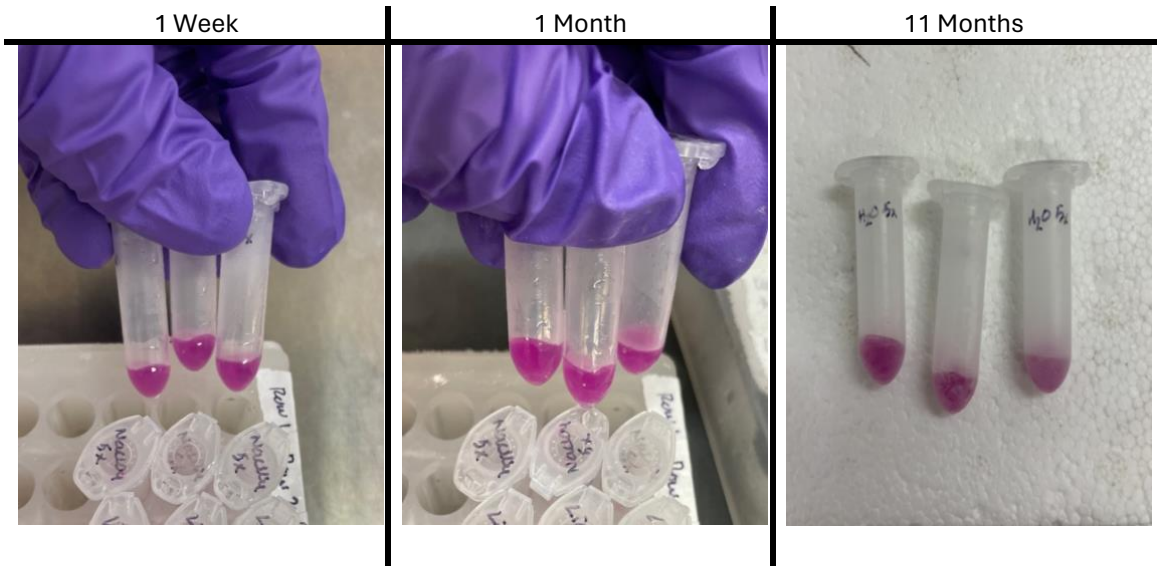


Table 4.3.2.2 Monitoring a Flash-Frozen 5x $LiClO_4$ Indicator Stored at -80℃. *[$HClO_4$]: 250 mM, [$Na_2Oxalate$]: 6.9 mM, [$Mn(ClO_4)_2$]: 50 µM, [$KMnO_4$]: 2.5 mM*



As demonstrated in *Figures 4.3.2.1 and 4.3.2.2*, the long-term stability of the 1x $NaClO_4$ and 5x $LiClO_4$ reactions were confirmed as the triplicates maintained the pink coloration shown across all three images. Additionally, the long-term stability of the proposed indicators was tested across a variety of reaction times and solvents:

Table 4.3.2.3 Summary of Long-Term Stability Indicators at -80 ℃. *Boxes highlighted in green represent indicators that demonstrate long-term stability and continued pink coloration across all three replicates. All systems demonstrated long-term stability and no significant color changes.*

Duration Since Flash-Freezing	Antifreeze-Free	$LiClO_4$	$Mg(ClO_4)_2$	$NaClO_4$
23.5 Months			Length at 25°C: 30 Minutes 1x Reaction System	Length at 25°C: 120 Minutes 1x Reaction System
13 Months	Length at 25°C: 3-5 Minutes 1x Reaction System	Length at 25°C: 1-3 Minutes 1x Reaction System		Length at 25°C: 6-8 Minutes 1x Reaction System
13 Months	Length at 25°C: 3-5 Minutes 5x Reaction System	Length at 25°C: 4-6 Minutes 5x Reaction System		Length at 25°C: 9-12 Minutes 5x Reaction System
12.5 Months			Length at 25°C: 20-40 Seconds 1x Reaction System	
12.5 Months			Length at 25°C: 3-5 Minutes 5x Reaction System	
11.5 Months		Length at 25°C: 5 Minutes 1x Reaction System	Length at 25°C: 5 Minutes 1x Reaction System	Length at 25°C: 5 Minutes 1x Reaction System
4 Months			Length at 25°C: 5 Minutes 5x Reaction System	
4 Months			Length at 25°C: 15 Minutes 5x Reaction System	
1.5 Months			Length at 25°C: 5 Minutes 5x Reaction System	
1.5 Months			Length at 25°C: 15 Minutes 5x Reaction System	

As demonstrated in *Figure 4.3.2.3*, all the previously prepared long-term stability samples remained in a frozen state with consistent pink coloration. Overall, *Figure 4.3.2.3* showcases the long-term stability of the permanganate/oxalic acid visual indicator systems in their frozen states across various antifreeze-free and antifreeze-salt-containing aqueous eutectic solvents. Therefore, for applicational purposes of the proposed visual indicators, our indicators will maintain the pink coloration that characterizes proper biospecimen storage in their frozen state until thawing or improper storage conditions results in the progression of the chemical reaction and the previously outlined pink to colorless transition.

4.4 Conclusions

While previous experiments have outlined reaction design and reproducibility for both the 1x and 5x visual indicator systems, this chapter focused on the pre-activation stability and long-term stability of the proposed indicator systems. As previously outlined, the proposed prototype for our visual indicator system includes pre-mixed reagents in liquid form and dry permanganate that can be activated by the user. Therefore, the pre-stability of the pre-mixed solution including solvent, perchloric acid, magnesium perchlorate and sodium oxalate was explored to determine if long-term incubation of the reagents affected the reaction system. Unfortunately, pre-stability experiments for antifreeze-salt-containing aqueous eutectic solvents produced surprising results as the $Mg(ClO_4)_2$ system produced a cloudy precipitate, potentially from bacterial growth, while the $LiClO_4$ and $NaClO_4$ systems demonstrated decreased reaction time as the incubation time of the reagents increased. Therefore, further experiments will explore the stability and interactions between eutectic solvents and other chemical reagents

within the permanganate/oxalic acid mechanism while additional pre-stability experiments will be performed in other lab materials such as polyethylene terephthalate. However, pre-stability experiments with antifreeze-free systems showcased little variation in average run time as the incubation time of the system increased and therefore our proposed visual indicator antifreeze-free systems are available and prepared for application in cold chain tracking. Additionally, the long-term stability of various proposed indicators was tested to explore reaction progression when the visual indicator systems are frozen. Therefore, various long-term stability systems composed of different solvents were prepared and flash-frozen in a dry ice/ethanol bath before being transferred to the -80°C freezer. Across different time intervals, ranging from a few weeks to years, the visual indicators showcased long-term stability with consistent coloration. Overall, further progress was achieved in developing the proposed visual indicators for application in research or clinical settings. While further experiments are required to investigate the pre-stability of eutectic systems, research findings confirmed the pre-activation stability for antifreeze-free systems and the long-term stability of the visual indicators for both eutectic and antifreeze-free systems.

CHAPTER 5

CONCLUSIONS AND OUTLOOK

5.1 Conclusions and Future Initiatives

In the past couple of years, our lab has made sustained and progressive efforts to develop time temperature indicators for cold chain tracking in clinical and research settings. Currently, research labs and clinical environments utilize two cold chain tracking mechanisms: physics/chemistry based tracking and electronic tracking. However, while electronic cold chain tracking is effective and accurate across a variety of temperatures, including subzero temperatures, increased costs and inapplicability to individual aliquots limit its application for research settings. On the other hand, physics/chemistry based cold chain tracking allows for individual aliquot tracking but currently lacks accuracy and application at subzero temperatures due to the freezing point (0°C) of antifreeze-free systems. Therefore, our lab has developed a permanganate/oxalic acid time temperature visual indicator for cold chain tracking, resolving cost and subzero challenges with currently utilized cold chain tracking methods.

The first aim was to demonstrate the functionality of the 1x time temperature visual indicators across various antifreeze-free and antifreeze-salt-containing aqueous eutectic solvents, as outlined in *Chapter 2*. Often, aliquots or clinical samples are unknowingly exposed to thawing conditions that influence biospecimens with potentially detrimental effects to research and clinical outcomes. Therefore, we have proposed a permanganate/oxalic acid visual indicator system that resolves some of the challenges with current cold chain tracking mechanisms and allows researchers and clinicians to monitor the integrity of their biospecimens and aliquots accurately and effectively.

Within our proposed visual indicator systems, a designed pink to colorless transition is implemented to indicate biospecimen or aliquot exposure to thawing or improper storage conditions. Therefore, when users visually analyze the proposed visual indicator, a pink coloration demonstrates proper storage and intact biospecimen integrity whereas a colorless indicator showcases exposure to thawing conditions that impact the aliquot's integrity for research findings and clinical outcomes. Within the first aim, we developed a more comprehensive understanding of the permanganate/oxalic reaction system and its reaction components before constructing reaction systems with both antifreeze-free and antifreeze-salt-containing aqueous eutectic solvents. As a result, 13 of the 14 proposed 1x time temperature indicators were successfully designed and replicated with varying stock solutions, demonstrating the accuracy and reproducibility of our proposed visual indicator systems. Therefore, the functionality and application of the proposed 1x visual indicator systems was explored and successfully achieved as thirteen 1x visual indicator systems were constructed for cold chain tracking across room temperature, freezing, and subzero temperatures.

Following completion of the first aim, the second aim explored the functionality of visual indicator systems with higher permanganate concentrations, as outlined in *Chapter 3*. Particularly for long-term storage of samples below the freezing point of antifreeze-free or antifreeze-salt-containing aqueous eutectic solvents, previous research demonstrated that the pink coloration of the 1x visual indicators was partially masked by frost.[12] Therefore, higher concentration systems were proposed to include an increased concentration of permanganate and more intense pink coloration that allows users to easily determine the integrity of their samples at storage temperatures such

as -80/-160°C. Within the second aim, both 5x and 10x indicator systems were explored, but 10x indicator systems demonstrated CO_2 accumulation or a pink to brown to colorless transition due to the accumulation of MnO_2, and therefore efforts were focused to design higher concentration visual indicator systems with 5x permanganate concentrations. As a result, eleven 5x higher concentration visual indicator systems were successfully designed and replicated across various solvents, reaction lengths, and temperatures, providing an alternative to the previously outlined and designed 1x visual indicators.

Additionally, following successful design of 1x and 5x visual indicator systems, the third aim investigated the pre-activation stability and long-term stability of the visual indicator systems, as demonstrated in *Chapter 4*. As previously outlined, current prototypes of our proposed visual indicators include a capsule that separates dry permanganate from the rest of the mixed reaction components. Therefore, when users want to activate the visual indicator, the permanganate mixes with the reaction solution and reaction progression begins. However, since the remaining reaction components are incubated together, potentially for extended periods of time until reaction activation, the pre-stability of the reaction components without permanganate in Polypropylene Eppendorf tubes was explored. Following experimentation, pre-stability studies demonstrated that in eutectic systems, increased incubation times resulted in decreased run times, affecting the accuracy and precision of our proposed visual indicators. Therefore, additional experiments are required to investigate interactions between different reagents. However, pre-stability studies for antifreeze-free systems demonstrated limited variation between reaction times for samples with varying incubation times. Therefore, perchlorate-containing eutectic solvents could be potentially

reacting with other components in solution or the storage vessel itself, affecting the designed reaction times for our proposed visual indicators. As a result, prototypic designs might have to include separated ampoules for both eutectic solvents and permanganate, and therefore only upon activation would the eutectic solvent and permanganate be mixed with the remaining reagents. Nevertheless, additional experimentation and testing are required to resolve challenges with pre-activation stability of the reagents.

Moreover, the second component of the third aim investigated the long-term stability of the reaction system at frozen conditions. When samples are stored at the proper temperatures and conditions, our designed visual indicators should be frozen and therefore not progressing at significant rates. As a result, the long-term stability of various visual indicator systems was explored at -80°C and demonstrated that across varying solvents and systems, the reactions showcase minimal progression when frozen, preserving the integrity of the visual indicators. Consequently, visual indicator reaction progression will remain halted or slowed in frozen conditions until samples and visual indicators are exposed to thawing conditions which result in the pink to colorless transition of the visual indicator reactions.

Overall, the functionality of proposed 1x and 5x visual indicator systems was successfully demonstrated and 25 visual indicators were designed for application in cold chain tracking. Nevertheless, future initiatives include applicational testing of our visual indicators for cold chain tracking, and additional pre-activation stability testing of antifreeze-salt-containing aqueous eutectic solvents. As a result, our lab is currently implementing our developed visual time-temperature indicator with aliquots used within the lab. Individualized aliquots are equipped with a proposed visual indicator prepared in

a 1.5 mL Eppendorf tube to monitor biospecimen integrity across the biospecimen lifetime including all manipulation steps such as transportation, freeze-thaw cycles, and long-term storage. Additionally, as discussed in *Chapter 4*, further experiments are required to explore the pre-activation stability of the eutectic systems, ensuring that our proposed visual indicators remain accurate in both clinical and research settings. Nonetheless, we have achieved accurate and successful design of 25 proposed visual indicators systems and will continue development of the proposed permanganate/oxalic acid reaction system to increase the accuracy and efficiency of the system for cold chain tracking.

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APPENDIX A

MATLAB CODE FOR REACTION KINETICS SIMULATIONS

MATLAB CODE FOR REACTION KINETICS SIMULATIONS:

```
function xprime = KMnO4_Pimienta_CatOnly_H2O_Annotated_HClO4(t,x)
%Explanation of variables: x(1) is MnO4, x(2) is MnO2, x(3) is MnO2,H2C2O4, x(4) is
Mn3+, x(5) is
%MnC2O4+, x(6) is Mn(C2O4)2-, x(7) is Mn2+, x(8) is MnC2O4, x(9) is H2C2O4,
%x(10) is HC2O4-, x(11) is C2O42-, x(12) is HClO4, x(13) is ClO4-, x(14) is H+, x(15)
is CO2dot-, x(16) is -OH
%To execute this script, type the following commands, hitting enter after
%each one, into the Command Window:
%clear
%t_int = 0:60:7200
%Classic 1-hr run (2% HNO3 pre-rinsed labware):
%[t,x] =
ode23s(@KMnO4_Pimienta_CatOnly_H2O_Annotated_HClO4,[t_int],[0.5e-3;0;0;0;0;0;
0.6e-7;0;0;0;1.38e-3;10e-3;0;1e-7;0;1e-7])
%Should be 5-min run: [t,x] =
ode23s(@KMnO4_Pimienta_CatOnly_H2O_Annotated_HClO4,[t_int],[0.5e-3;0;0;0;0;0;
0.6e-7;0;0;0;1.38e-3;100e-3;0;1e-7;0;1e-7])
%hr = t/3600
%plot(hr,x(:,1),'-o',hr,x(:,2),'-x')
% or  plot(hr,x(:,1),'-o',hr,x(:,2),'-x',hr,x(:,15),'-')
% or plot(t,x(:,1),'-o',t,x(:,2),'-x')

%Notes:
%-The t_int = ... command defines the time points (in seconds) at which the
%concentration of each reaction component will be calculated.
%-The numbers with semicolons between them in the [t,x] =... command
%are the initial starting concentrations of the x(1), x(2), x(3) variables
%in units of molar (M).
%-Times are calculated in seconds but are converted to hrs with the hr = ...
%command
%-The concentration of any variable can be plotted over time by modifying
%the plot(hr,x(:,1),'-o',hr,x(:,2),'-x',hr,x(:,8),'-') command. This exact
%command plots MnO4, MnO2, and MnC2O4

%The following are rate constants:
kAf = 5e3;
kAr = 1e5;
kBf = 5e3;
kBr = 1e8;
kCf = 1e7;
kCr = 0.0001;
```

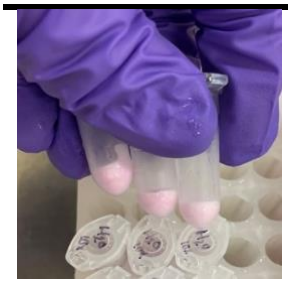
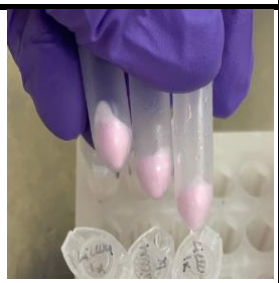
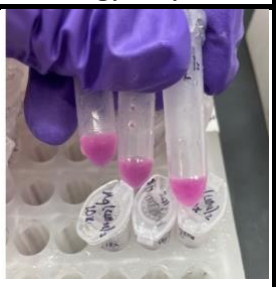
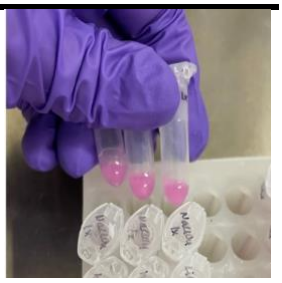
```
kDf = 1.27e3;
kDr = 1e5;
kEf = 1.6e9;
kEr = 1e2;
kHf = 1.77e6;
kHr = 1e2;
kJf = 2e11;
kJr = 1e2;
kKf = 1.29e10;
kKr = 1e2;
kM = 1.3e-3;
kN = 1e9;
kF = 5e3;
kI = 8e-2;
kL = 0.92;
kwf = 1e2; %Needed in order to take the autoprotolysis of water into account.
kwr = 1e16; %Needed in order to take the autoprotolysis of water into account.
%The following sets up a one-dimensional array
xprime = zeros(16,1); %The output must be a column vector
%The following are the simultaneous differential equations that comprise
%the model of the chemical system
xprime(9) = -1*kAf*x(9) + kAr*x(10)*x(14) - kHf*x(2)*x(9) + kHr*x(3);
xprime(10) = kAf*x(9) - kAr*x(10)*x(14) - kBf*x(10) + kBr*x(11)*x(14);
xprime(11) = kBf*x(10) - kBr*x(11)*x(14) - kEf*x(7)*x(11) +
kEr*x(8) - kJf*x(4)*x(11) + kJr*x(5) - kKf*x(5)*x(11) + kKr*x(6) + kM*x(6) +
kN*x(15)^2;
xprime(12) = -1*kCf*x(12) + kCr*x(13)*x(14);
xprime(13) = kCf*x(12) - kCr*x(13)*x(14);
%xprime(14) = kDf*x(13) - kDr*x(14)*x(15);
xprime(14) = kAf*x(9) - kAr*x(10)*x(14) + kBf*x(10) - kBr*x(11)*x(14) +
kCf*x(12) - 4*kF*x(1)*x(8) - 2*kI*x(3) + kwf - kwr*x(14)*x(16);
xprime(16) = kwf - kwr*x(14)*x(16);
xprime(1) = -1*kF*x(1)*x(8);
xprime(7) = -1*kEf*x(7)*x(11) + kEr*x(8) + kL*x(5) + kM*x(6);
xprime(8) = kEf*x(7)*x(11) - kEr*x(8) - kF*x(1)*x(8);
xprime(2) = kF*x(1)*x(8) - kHf*x(2)*x(9) + kHr*x(3);
xprime(3) = kHf*x(2)*x(9) - kHr*x(3) - kI*x(3);
xprime(4) = kF*x(1)*x(8) + kI*x(3) - kJf*x(4)*x(11) + kJr*x(5);
xprime(5) = kJf*x(4)*x(11) - kJr*x(5) - kKf*x(5)*x(11) + kKr*x(6) - kL*x(5);
xprime(6) = kKf*x(5)*x(11) - kKr*x(6) - kM*x(6);
xprime(15) = kL*x(5) + kM*x(6) - 2*(kN*x(15)^2) + kI*x(3);
```

APPENDIX B

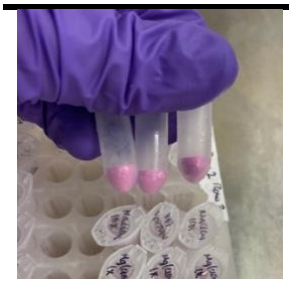
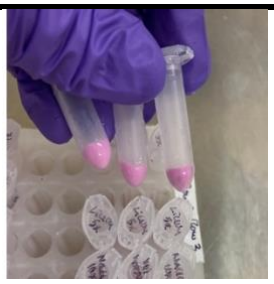
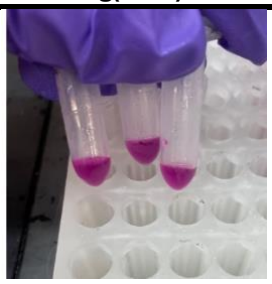
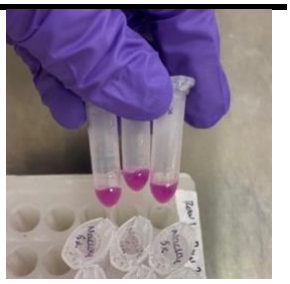
LONG-TERM STABILITY IMAGES

LONG-TERM STABILITY IMAGES:

Initial 1x Long-Term Stability Images

$LiClO_4$	$NaClO_4$	$Mg(ClO_4)_2$	Antifreeze-Free
			
1x $LiClO_4$ System Length at 25°C: 1-3 Minutes After 1 Week	1x $NaClO_4$ System Length at 25°C: 6-8 Minutes After 1 Week	1x $Mg(ClO_4)_2$ System Length at 25°C: 20-40 Seconds After 1 Month	1x Antifreeze-Free System Length at 25°C: 3-5 Minutes After 1 Week

Initial 5x Long-Term Stability Images

$LiClO_4$	$NaClO_4$	$Mg(ClO_4)_2$	Antifreeze-Free
			
5x $LiClO_4$ System Length at 25°C: 4-6 Minutes After 1 Week	5x $NaClO_4$ System Length at 25°C: 8-12 Minutes After 1 Week	5x $Mg(ClO_4)_2$ System Length at 25°C: 3-5 Minutes After 1 Month	5x Antifreeze-Free System Length at 25°C: 3-5 Minutes After 1 Week

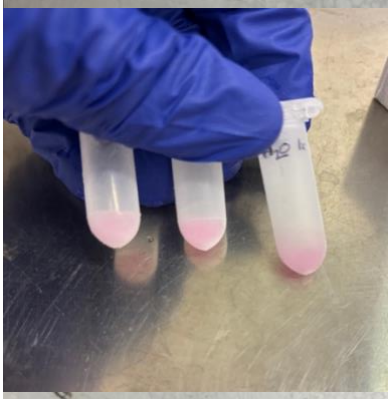
Updated 1x and 5x Long-Term Stability Images

1x $LiClO_4$ System Length at 25°C: 5 Minutes Duration in -80°C: 11.5 Months	

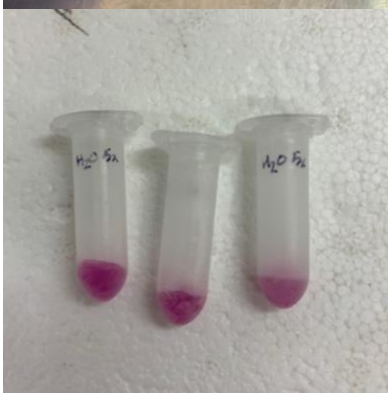
1x $Mg(ClO_4)_2$ System
Length at 25°C: 5 Minutes
Duration in -80°C: 11.5 Months

1x $NaClO_4$ System
Length at 25°C: 5 Minutes
Duration in -80°C: 11.5 Months

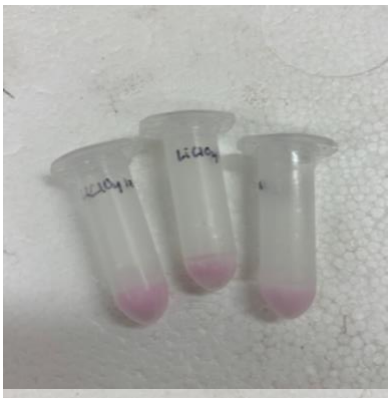
1x Antifreeze-Free System
Length at 25°C: 3-5 Minutes
Duration in -80°C: 13 Months



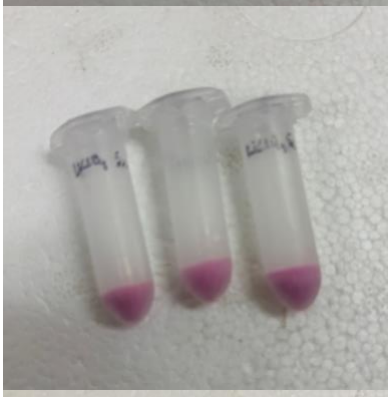
5x Antifreeze-Free System
Length at 25°C: 3-5 Minutes
Duration in -80°C: 13 Months



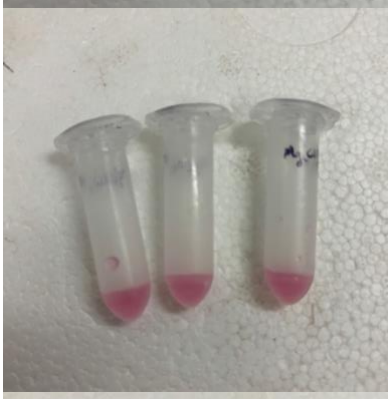
1x $LiClO_4$ System
Length at 25°C: 1-3 Minutes
Duration in -80°C: 13 Months



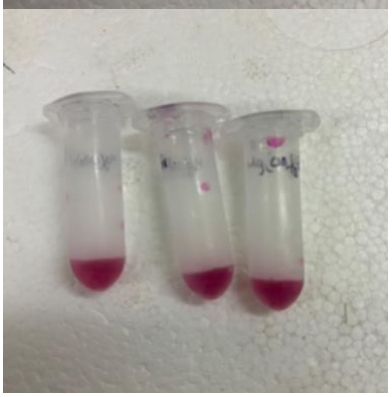
5x $LiClO_4$ System
Length at 25°C: 4-6 Minutes
Duration in -80°C: 13 Months



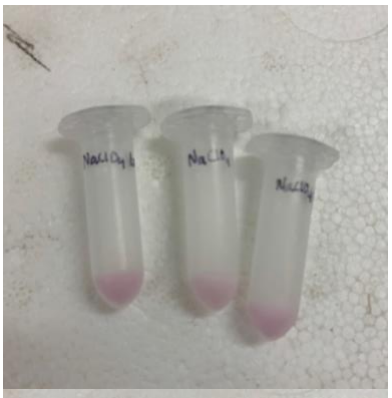
1x $Mg(ClO_4)_2$ System
Length at 25°C: 3-5 Minutes
Duration in -80°C: 12.5 Months



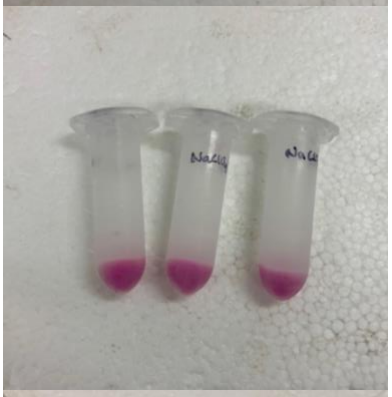
5x $Mg(ClO_4)_2$ System
Length at 25°C: 20-40 Seconds
Duration in -80°C: 12.5 Months



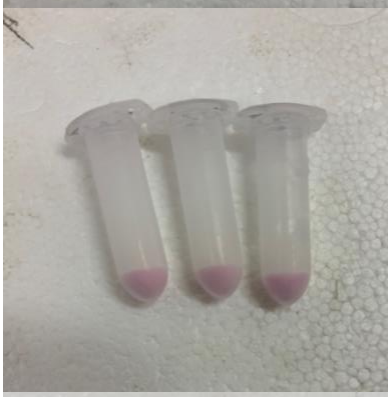
1x $NaClO_4$ System
Length at 25°C: 6-8 Minutes
Duration in -80°C: 13 Months



5x $NaClO_4$ System
Length at 25°C: 9-12 Minutes
Duration in -80°C: 13 Months

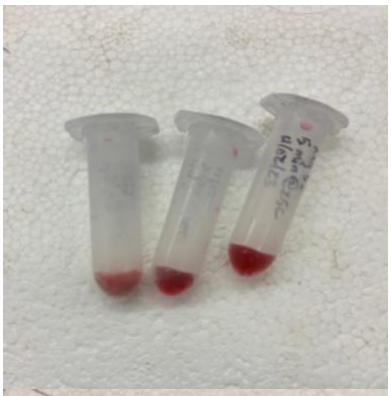


1x $NaClO_4$ System
Length at 25°C: 120 Minutes
Duration in -80°C: 23.5 Months



1x $Mg(ClO_4)_2$ System
Length at 25°C: 30 Minutes
Duration in -80°C: 23.5 Months

5x $Mg(ClO_4)_2$ System
Length at 25°C: 5 Minutes
Duration in -80°C: 4 Months



5x $Mg(ClO_4)_2$ System
Length at 25°C: 15 Minutes
Duration in -80°C: 4 Months