University of Toronto

Faculty of Applied Science and Engineering

BME440 Biomedical Engineering Technology and Investigation

Lab 5: Cell Culturing and Counting of Serially Diluted HeLa Cell Lines using XTT Assay to Determine Optimal Cell Density through Absorbance Readings for Cell Viability

PRA0101 - November 27, 2023

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1.0 Introduction & Scientific Objective

Mammalian cell culture is a commonly used laboratory technique in biological research as it allows scientists to study cell physiology and biochemistry of cells in-vitro [1]. HeLa cells are a widely used human cervical cancer cell line and are unique due to being the first immortalized cell line [1]. Use of proper aseptic technique ensures the integrity and reproducibility of experimental results in cell culture studies, as they prevent microbial contamination. The XTT Assay, is a standard method for assessing cell viability relying on the reduction of the tetrazolium salt XTT to a colored formazan dye by metabolically active cells [1]. The absorbance values from the formazan dye, measured at 450-475 nm wavelength, directly correlates with the number of viable cells in a sample [1]. The sensitivity of XTT Assay makes it ideal for determining the cell viability and proliferation of cultured cells in response to various experimental conditions [2]. The specific problem that will be addressed in this lab is to accurately determine the relationship between cell number and absorbance values produced from the XTT assay. The scientific objective of this lab is to quantify the cell viability of HeLa cells across varying concentrations using the XTT assay and to analyze the linear range where cell numbers correlate with the absorbance values. We expect to observe a specific linear range in the Absorbance vs. Cell Number graph, indicating the optimal cell density range for a reliable viability assessment in HeLa cells.

2.0 Materials and Methods

Prior to using the biological safety cabinet (BSC) the surface and items were wiped down using 70% ethanol. In BSC, the media was aspirated from the T-25 flask containing HeLa cell line using a glass Pasteur pipette. The cells were washed by adding 5 mL PBS to the flask using 5-mL serological pipette and was aspirated. 2 mL of 0.25% Trypsin-EDTA was added and the flask was incubated at 37°C for 3 minutes. The cells were checked under a microscope to see if they dislodged from the surface. In the BSC, 5 mL of media containing Ca^{2+} and FBS was added using a 10-mL serological pipette. With the same pipette all 7mL of cell suspension was transferred to a 15-mL tube. The cells were centrifuged at 200xg for 5 minutes to form a pellet at the bottom of the tube. In the BSC, all supernatant was aspirated from the 15-mL tube and 3 mL of cell culture media with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin (Pen-Strep) was added to the tube. Using a P-1000 set to 1mL, the pellet in the media was resuspended to mix. 100 µL of cell suspension was set aside into the 1.5mL Eppendorf tube for counting and 100 µL of Trypan Blue dye was added and mixed. Then, 1 mL of culture media was added to 8 1.5-mL Eppendorf tubes and serial dilution was performed by transferring 2 mL of the cell suspension from the 15-mL tube into Tube 1 and 1 mL from Tube 1 to Tube 2 and onward, halving the cell concentration at each step. Using a micropipette, 100 µL from each tube were transferred in triplicates into a 96-well plate. As we introduced bubbles to A1-well during the transfer, we used wells 2-4 for each row instead of 1-3. XTT mixture was prepared by mixing 30 µL of XTT activator into 1.5 mL of XTT reagent. 100 µL of the mixture was added into each sample well. Lastly, the 96-well plate was read at 460 nm after incubating for 1 hour at 37°C.

3.0 Results

Viable cell concentrations were measured using an automated cell counter. As 100µL of cell suspension was mixed with 100µL Trypan blue solution, the initial sample concentrations were halved when taking these measurements. Two separate measurements of concentration and percentages of the live and dead cells in the sample are presented in Table 1 below. Average concentration of live cells from two measurements in Table 1, is doubled and used as the viable cell concentration of the initial sample in Tube 1 (see Table 2). Serial dilutions were performed by transferring 2 mL of liquid from Tube 1 to Tube 2 and onward, until Tube 7, halving the concentration in each step. Cell numbers were calculated by multiplying the cell densities with 100 µL. Calculated viable cell densities and cell numbers in each well are presented in Table 2.

Table 1: Cell concentrations and viability percentages from the initial sample, obtained using an automated cell counter and average of the two measurements

	Total Concentration (cells/mL)	Concentration of Live Cells (cells/mL)	Concentration of Dead Cells (cells/mL)	Percentage of Live Cells	Percentage of Dead Cells
Measurement 1	4.93×10^5	4.63×10^5	2.93×10^4	94%	6%
Measurement 2	7.86×10^5	6.45×10^5	1.41×10^4	82%	18%
Average	6.395×10^5	5.54×10^5	2.17×10^4	88%	12%

Table 2: Calculated viable cell densities and number of cells in each well containing 100 µL solution per each serial dilution

Serial Dilution Step	Tube Number	Viable Cell Density (cells/mL)	Cell Number in Each Well
0	Tube 1	1.11×10^6	1.11×10^5
1	Tube 2	5.54×10^5	5.54×10^4
2	Tube 3	2.77×10^5	2.77×10^4
3	Tube 4	1.39×10^5	1.39×10^4
4	Tube 5	6.93×10^4	6.93×10^3
5	Tube 6	3.46×10^4	3.46×10^3
6	Tube 7	1.73×10^4	1.73×10^3
-	Control Tube	0	0

Table 3: Raw absorbance values measured at 460 nm for Wells 2-4 and the average absorbance value of the wells, with the corresponding tube number that the sample was obtained from

		Absorbance at 460 nm (A.U.) (Raw)			
Row	Tube Number	Well 2	Well 3	Well 4	Average
A	Tube 1	1.3105	1.1801	1.1106	1.2004
B	Tube 2	0.7869	0.6633	0.6652	0.7051
C	Tube 3	0.4202	0.4039	0.5645	0.4629
D	Tube 4	0.3601	0.3659	0.3688	0.3649
E	Tube 5	0.3168	0.3101	0.3218	0.3162
F	Tube 6	0.3079	0.2781	0.2798	0.2886
G	Tube 7	0.3043	0.2571	0.3199	0.2938
H	Control Tube	0.1112	0.1118	0.1133	0.1121

Table 4: Absorbance values measured at 460 nm for Wells 2-4 and the average absorbance value of the wells, with the values of the blank control wells subtracted for correction

			Absorbance at 460 nm (A.U.) (Values of the blank control wells subtracted)			
Row	Tube Number	Cell Number	Well 2	Well 3	Well 4	Average
A	Tube 1	1.11×10^5	1.1993	1.0683	0.9973	1.0883
B	Tube 2	5.54×10^4	0.6757	0.5515	0.5519	0.5930
C	Tube 3	2.77×10^4	0.309	0.2921	0.4512	0.3508
D	Tube 4	1.39×10^4	0.2489	0.2541	0.2555	0.2528
E	Tube 5	6.93×10^3	0.2056	0.1983	0.2085	0.2041
F	Tube 6	3.46×10^3	0.1967	0.1663	0.1665	0.1765
G	Tube 7	1.73×10^3	0.1931	0.1453	0.2066	0.1817
H	Control Tube	0	0	0	0	0

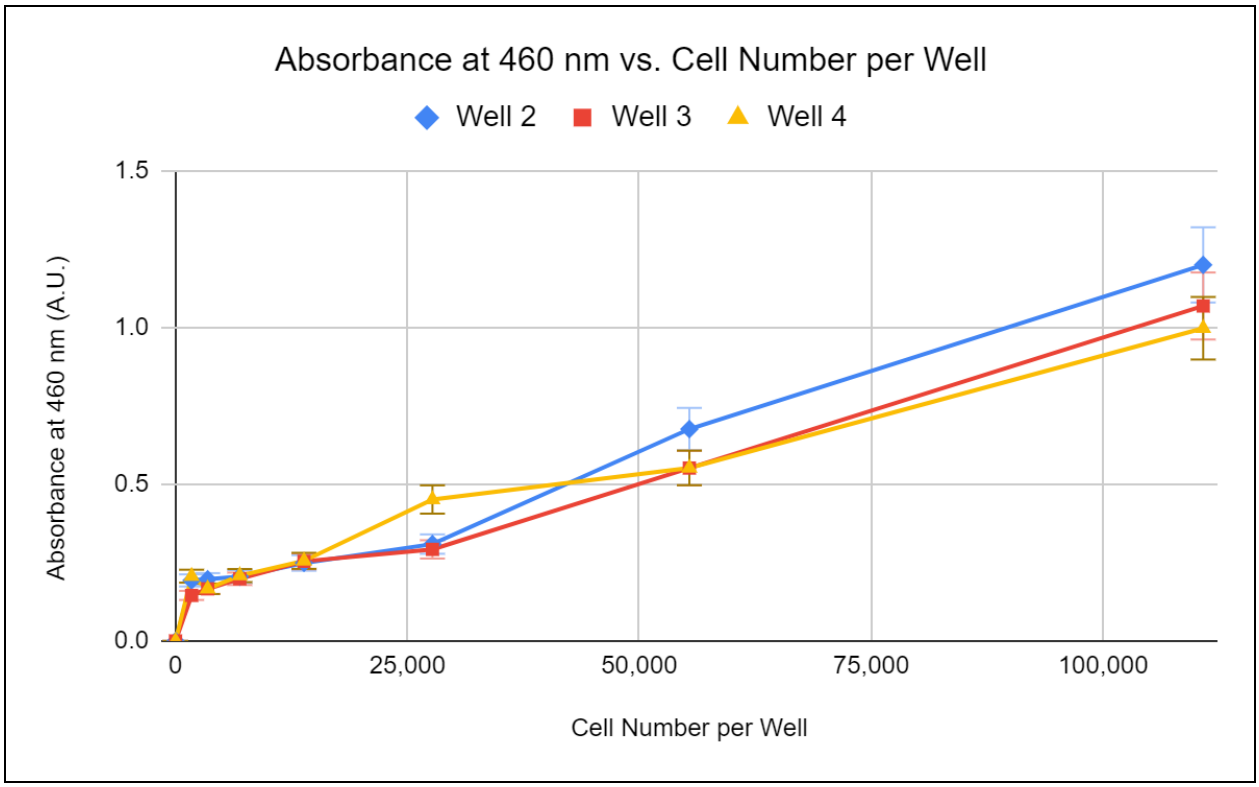

Figure 1: Absorbance at 460 nm (A.U.) vs. number of viable HeLa cells for wells 2-4
The samples were diluted in series and the cell concentrations were halved in each dilution by adding equal parts of culture media. 100 µL of each dilution was transferred into the rows A-G for wells 2-4. Row H in wells 2-4 contained the control samples with only culture media. The absorbance values were measured at 460 nm and are presented in Table 3. This graph was plotted with the background corrected values in Table 4. The cell numbers in this graph are plotted from row H to A (from left to right). Linear portions of the curves approximately range from 3.463×10^3 to 1.108×10^5 cells per well.

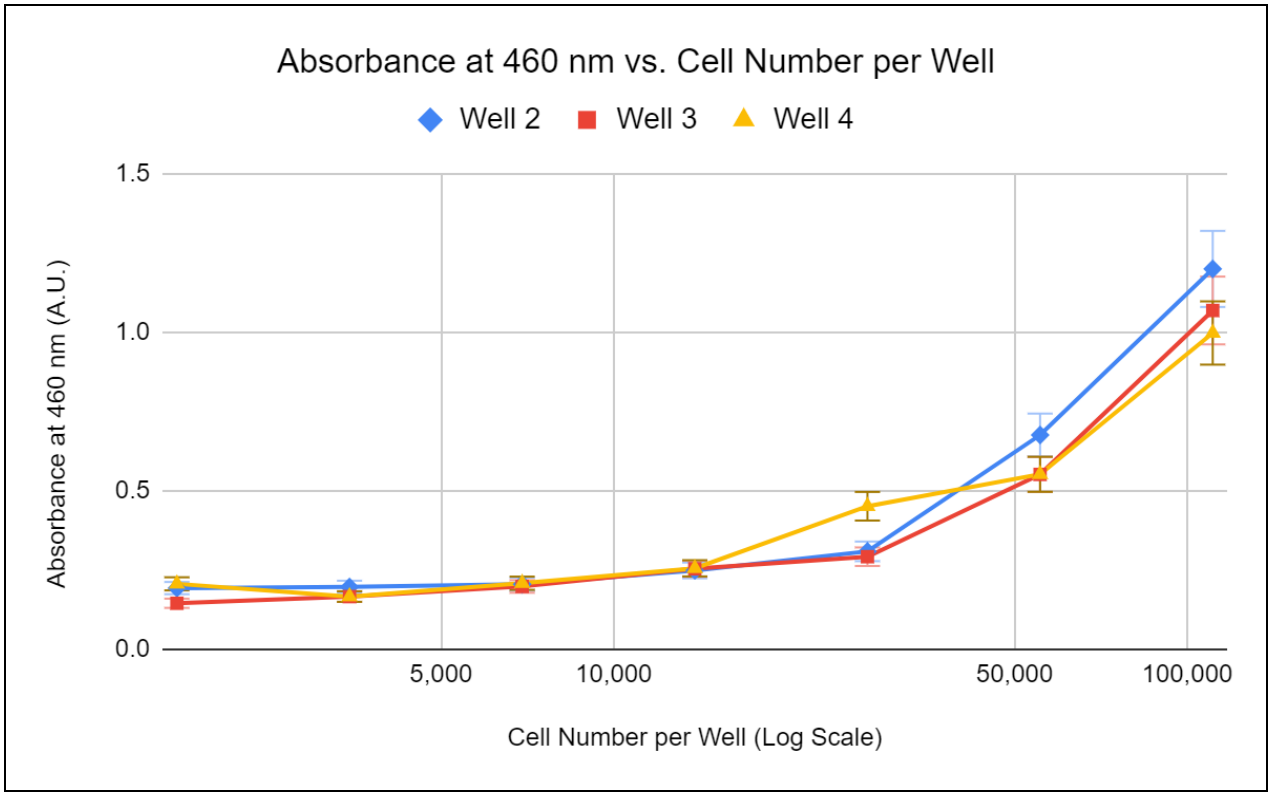

Figure 2: Absorbance at 460 nm (A.U.) vs. number of viable HeLa cells for wells 2-4
The samples were diluted in series and the cell concentrations were halved in each dilution by adding equal parts of culture media. 100 µL of each dilution was transferred into the rows A-G for wells 2-4. Row H in wells 2-4 contained the control samples with only culture media. The absorbance values were measured at 460 nm and are presented in Table 3. This graph was plotted with the background corrected values in Table 4. The cell numbers in this graph are plotted from row H to A (from left to right) and scaled logarithmically.

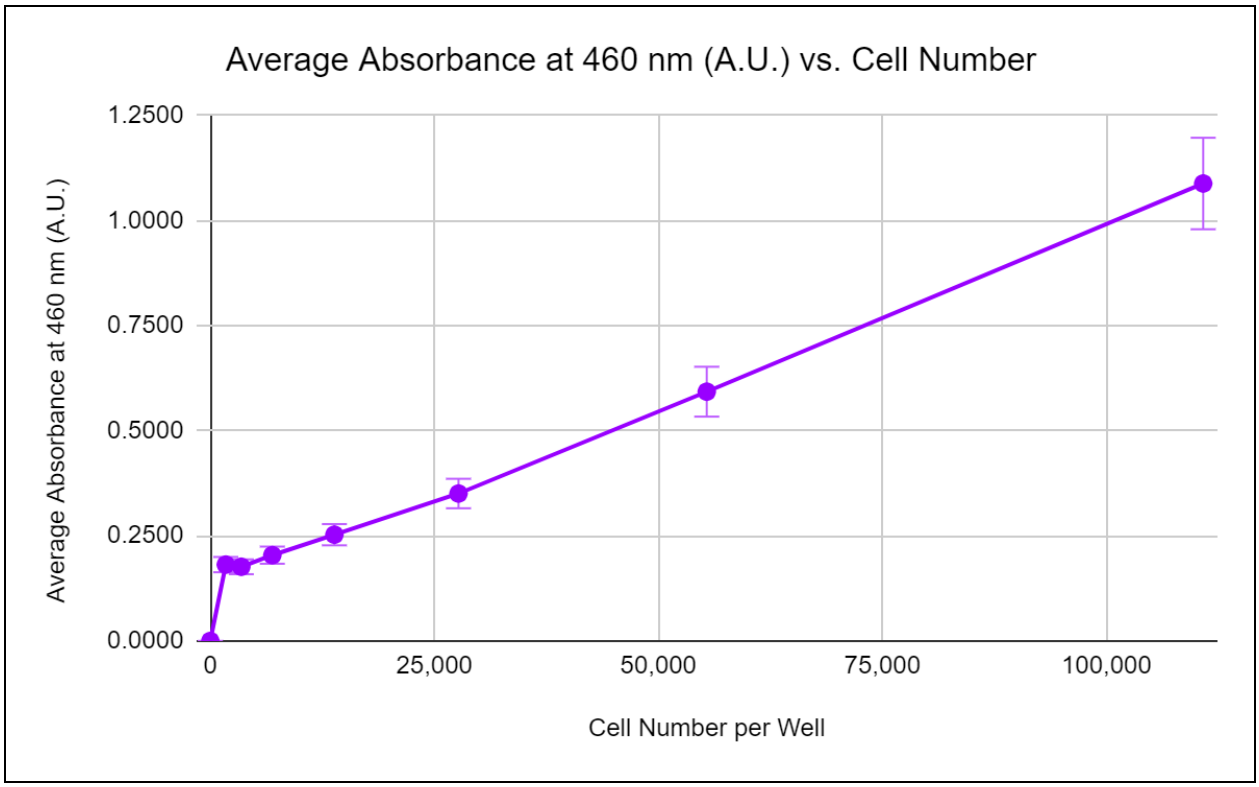

Figure 3: Average absorbance at 460 nm (A.U.) of wells 2-4 vs. number of viable HeLa cells The samples were diluted in series and the cell concentrations were halved in each dilution by adding equal parts of culture media. 100 µL of each dilution was transferred into the rows A-G for wells 2-4. Row H in wells 2-4 contained the control samples with only culture media. The absorbance values were measured at 460 nm and are presented in Table 3. This graph was plotted with the background corrected values in Table 4. The cell numbers in this graph are plotted from row H to A (from left to right). Linear portions of the curves approximately range from 3.463 x 10^3 to 1.108 x 10^5 cells per well.

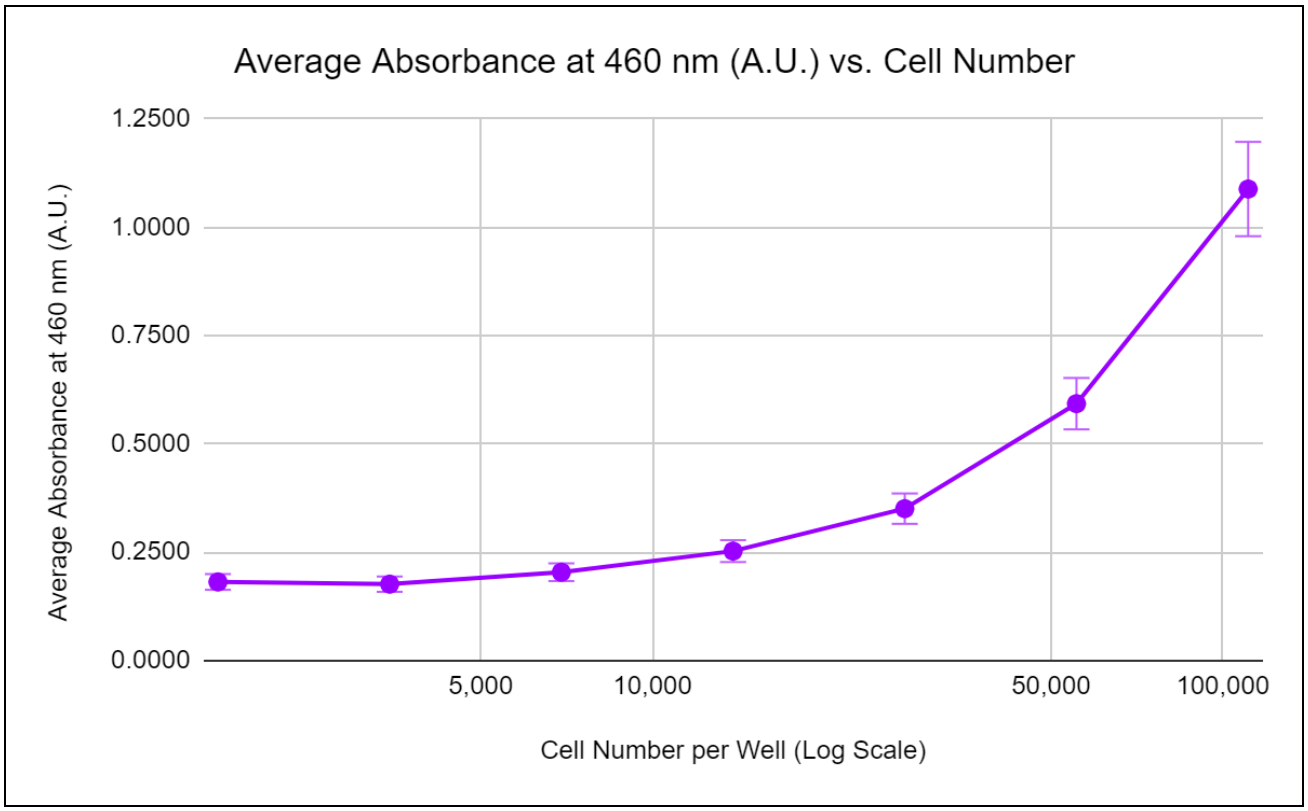

Figure 4: Average absorbance at 460 nm (A.U.) of wells 2-4 vs. number of viable HeLa cells The samples were diluted in series and the cell concentrations were halved in each dilution by adding equal parts of culture media. 100 µL of each dilution was transferred into the rows A-G for wells 2-4. Row H in wells 2-4 contained the control samples with only culture media. The absorbance values were measured at 460 nm and are presented in Table 3. This graph was plotted with the background corrected values in Table 4. The cell numbers in this graph are plotted from row H to A (from left to right) and scaled logarithmically.

3.1 Summary of Results

The results of the XTT assay using HeLa cells demonstrate a clear correlation between cell viability and absorbance values measured at 460 nm. The absorbance values, corrected by subtracting the values from the control wells, were plotted against the number of viable cells. In *Figure 1* and *Figure 3*, a linear relationship is observed within a specific range of cell concentrations. This linear portion, ranging from approximately 3.463×10^3 to 1.108×10^5 cells per well, indicates the range within which the assay provides reliable and consistent results for cell viability. Outside of this range, the relationship deviates from linearity, suggesting reduced accuracy in either very low or high cell concentrations.

4.0 Discussion

Based on the results represented in *Figures 1 and 3,* the optimal range of cell densities to proceed with for the XTT assay for HeLa cells would be approximately from 3.463×10^3 to 1.108×10^5 cells per well (3.463×10^4 to 1.108×10^6 cells/mL). This range is determined by where the absorbance values present a linear relationship with the cell number, indicating that changes in absorbance are directly proportional to the changes in the cell number. By choosing the range to be in the linear region, we ensure that the quantification of the cell viability is accurate, reliable and reproducible. Thus, cell densities outside of this range would not be recommended. Below 3.463×10^4 cells/mL, the XTT assay might not be sensitive enough to detect small changes in cell viability as the cell numbers might be below the assay's limit of detection. The lack of sensitivity may result in inaccuracy in the data as the assay cannot reliably measure the cell viability at these low concentrations. At high cell densities, above 1.108×10^6 cells/mL, we would expect the assay to reach a saturation point where absorbance no longer increases proportionally with the number of cells (see Appendix A). This nonlinearity would make it challenging to interpret the results accurately at higher cell densities. As higher cell densities were not measured, the aforementioned saturation point is not visible in *Figures 2 and 4.*

In *Figures 2 and 4*, which are plotted with a logarithmic scale for the cell numbers, the general trend of the curves follow a gradual increase in the absorbance with an increase in cell number, indicating a linear relationship. This linear relationship is clearly visible, when cell numbers are plotted using a linear-scale in *Figures 1 and 3.* At lower cell densities, the amount of formazan dye produced (indicated by the absorbance at 460 nm) is directly proportional to the number of viable cells as each cell contributes to the reduction of the XTT tetrazolium salt to the coloured formazan dye. As the cell number increases further, we would expect the curve to plateau and present a non-linear relationship, as it hits the saturation point (see Appendix A). However, this point is not visible in *Figures 2 and 4* as the curve continues to increase. The extended linear range could be attributed to the specific metabolic activities of HeLa cells. The XTT assay may have a broader linear range with HeLa cells than initially anticipated, which indicates that the saturation point could occur at a higher cell number than used in the experiment. The concentration of XTT reagent in the assay could have been sufficient to avoid early saturation, suggesting for future experiments higher cell densities may be needed to determine the point of saturation.

As a follow-up experiment to this lab, a drug sensitivity assay can be conducted to evaluate the effect of anticancer drugs on HeLa cell viability [3]. As the recommended range of cell densities are known, the cells can be exposed to a range of drug concentrations to assess the dose-response relationship. As a result the IC50 values (the concentration of drug that is required for 50% inhibition in vitro) can be determined for different drugs for comparison [3].

5.0 Conclusion

In conclusion, the experiment established a linear relationship between the absorbance at 460 nm and the number of viable HeLa cells using the XTT assay, within a specific cell density range. The optimal range was determined to be approximately 3.463×10^3 to 1.108×10^5 cells per well, where the results of the XTT assays are a reliable and proportional measure of cell viability, supporting our initial hypothesis. Cell densities below and above of this range are not recommended due to decreased sensitivity and potential saturation of the assay, respectively, although the expected saturation point was not observed within the tested densities. In order to determine the actual saturation point, the cell numbers could be increased for future experiments.

The findings and techniques from this lab are relevant for applications beyond measuring cell viability, and may be applied to pharmacological research to assess cytotoxicity of drugs [4], in genetic studies to understand the impact of gene manipulation on cell metabolism [5], and in cancer research to study the effects of different treatments on cancer cell proliferation [6].

6.0 References

(1) *BME 440 – Lab 5: Intro to Mammalian Cell Culture, Cell Counting and XTT Assay For Cell Viability,* 2021; pp. 1-11.

(2) *Development and Applications of the XTT Assay for Cell Viability, Journal of Cellular Biochemistry,* 1988.

(3) Cai, L.; Qin, X.; Xu, Z.; Song, Y.; Jiang, H.; Wu, Y.; Ruan, H.; Chen, J. *Comparison of Cytotoxicity Evaluation of Anticancer Drugs between Real-Time Cell Analysis and CCK-8 Method*. ACS Omega 2019, 4 (7), 12036-12042. DOI: 10.1021/acsomega.9b01142.

(4) Xenometrix AG. *XTT Cytotoxicity Test, Cell Viability Test*. Available at: https://www.xenometrix.ch/shop/XTT-Cytotoxicity-Test-Cell-Viability-Test. [Accessed: December 7, 2023].

(5) Kunik, T.; Tzfira, T.; Kapulnik, Y.; Gafni, Y.; Dingwall, C.; Citovsky, V. Genetic *Transformation of HeLa Cells by Agrobacterium*. Proc. Natl. Acad. Sci. U.S.A. 2001, 98 (4), 1871-1876. DOI: 10.1073/pnas.98.4.1871. Epub 2001 Jan 30. PMID: 11172043; PMCID: PMC29349.

(6) Altun, A.; Şahin İnan, Z. D.; Yulak, F. *Death Receptor-Dependent Apoptosis and Cell Cycle Delay Induced by Bioymifi in Human Cervical Cancer Cells*. Pak. J. Pharm. Sci. 2022, 35 (4), 1031-1036. PMID: 36008899.

(7) American Type Culture Collection. *XTT Cell Proliferation Assay Kit Instruction Manual, Catalog Number 30-1011K (1000 Assays)*. American Type Culture Collection, 2011. Available at: https://www.atcc.org/. [Accessed: December 7, 2023].

Appendix A

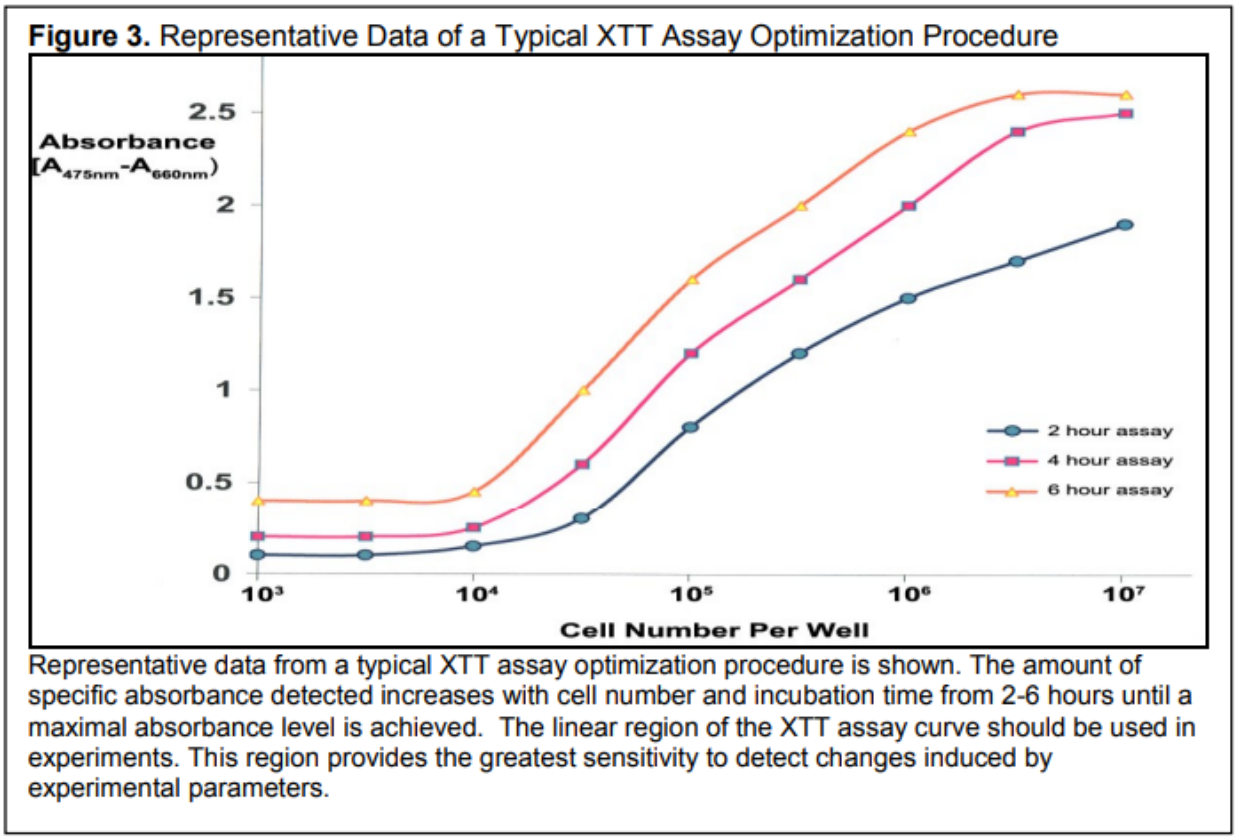

Figure A1: Reference absorbance vs. cell number per well graph for comparison and to show expected saturation behavior of the curve for higher cell densities above 10^6 cells per well