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CHAPTER ONE

INTRODUCTION

DNA Quantification Methods

Quantification of DNA is a required process in forensic casework laboratories.[3] DNA typing amplification kits require an optimal range of template DNA concentration. When the optimal concentration of template DNA is added to the amplification reactions, interpretation of the results is usually straightforward; however, not quantifying DNA before amplification can cause problems with the profiles obtained. If less than optimal DNA is put into the assay, the assay can generate incomplete or partial results. If more than optimal DNA is added, the signal can saturate the detector and cause interpretation issues. Accuracy in DNA quantification is important so that these problems do not occur. Inhibition in samples can cause these problems by appearing to lower the concentration of DNA in the sample, possibly even causing the sample to be falsely negative. A false negative is a sample that contains DNA but quantifies as though it did not.

While quantification assays were originally created and performed by the laboratory performing the test, contemporary kits are commercially available from the manufacturer and commonly used by forensic labs. Of these quantification methods, ultraviolet spectroscopy (UV-Vis) absorbance was the first method commonly used. This method determined quantity and quality of DNA by taking the relative absorbance at 260 nm (for DNA) and 280 nm (for proteins) and used the ratio to determine purity of DNA. Pure DNA produces a ratio of 1.7. Additionally, pure, single-stranded DNA at an absorbance of 1 absorption unit (AU) has a quantity of 33 µg/mL. Problems occurred due to UV-Vis having the inability to distinguish between DNA sources. Purity estimates of DNA are limited to the ratio of DNA to proteins, and numerous contaminating compounds with absorption in the same spectral range (e.g phenol, a compound used in DNA extraction, fluoresces at 270 nm). By contrast, yield gels, agarose gels stained with ethidium bromide, separate DNA by electrophoresis and allowed the user to determine the amount of

DNA by the size of the band. This method allowed for more accurate quantification of total DNA, but still could not differentiate between DNA sources. Inhibition also could not be detected by yield gels[3,4].

The slot blot was the next method used; this was a DNA-hybridization colormetric based assay. It was performed by placing the DNA samples, 30 or more, onto a single nylon membrane. This membrane allowed biotin-labeled DNA probe to be hybridized to target DNA and cross-linked to the membrane. Standard quantities of DNA were run in tandem with the samples so that the DNA could be more accurately quantified. The slot blot allowed more accurate quantification of DNA, and was specific to human and primate DNA using D17Z1, a 40 base pair region on chromosome 17. Although the slot blot was capable of quantification and specie determination, it was still unable to determine if inhibition was present.[5]

The Picogreen assay was the first "automated" DNA quantification method and used a 96 well microtiter plate with a DNA interchelating dye. An interchelating dye's fluorescence increases when bound to double stranded DNA. This method requires a standard curve using known DNA standards run with the samples for better quantification results. Picogreen was not human specific, but could determine quantities of DNA down to 250 pg. Another method known as End-point PCR uses one STR locus or *Alu* repeat region to determine quantity of human DNA in the sample. This method also uses interchelating dyes and standard curves to quantify DNA. End-point PCR was human specific and could quantify down to 100 pg of DNA. Inhibition could be determined, but it required using multiple serial dilutions of DNA to determine if inhibition was present. This dilution series required additional valuable sample and expensive consumables making it sub-optimal.[4,5,6]

Real-time (RT-PCR) or quantitative PCR (qPCR) is the current standard technique used to quantify DNA. qPCR tests fluorescent signal every cycle of PCR to determine the amount of DNA in the reaction. The tube or tray in qPCR is sealed and therefore the only changes in the tube are caused by the PCR reaction. This allows the relative fluorescence, the amount of change from one sample to a reference

standard dilution series, to be used to quantify the sample. A forensic scientist can then analyze the data using a computer that calculates the quantity of DNA based on the known standard dilution series. There have been several approaches used for qPCR, the most common of which is the Taqman assay (a flourogenic assay), which incorporates a fluorogenic 5' probe that hybridizes to a complementary single stranded DNA target and then is hydrolyzed as the PCR process takes place. Hydrolysis of the probe separates the 5' flourophore from the 3' quencher covalently linked to the probe, resulting in an increase in fluorescence each amplification cycle. Typical qPCR reactions may extend up to 40 cycles allowing unknowns to be quantified when compared to standards. The standards in the Quantifilier® kit range from 50 ng/µL to 0.023 ng/µL providing accurate quantification over a large range of DNA template concentrations.[4,5]

A fluorogenic assay is based on a probe that has 2 different dyes, a fluorophore and a quencher, fused at opposite ends of the probe. This probe binds specifically to a region internal to the target amplicon and is designed to not anneal until after the primers are bound and extension has started. The Taqman probe is covalently modified to contain a fluorophore at the 5' end and a fluorescence quenching molecule (the quencher) the 3' end, which quenches the signal from the dye on the 3' end when these dyes are in close proximity. The Quantifiler® Kit uses the TaqMan assay which contains a FAM (blue) dye on the 5' end as the reporter and a non-fluorescent quencher (NFQ) on the 3' end that does not interfere with the signal when bound allowing quenching of the FAM dye. Also, a minor groove binder (MGB) is used on the 3' end to raise the melting temperature to allow shorter probes, 62 bp in the Human kit, while allowing the probe to anneal at the correct temperature. In the Quantifiler® kit the probe is bound to the NFQ. While extension is occurring, the Taq polymerase used to extend the DNA then hydrolyzes the probe displacing it from the DNA strand and releasing the fluorophore from the quencher thereby allowing the fluorescence to increase during each cycle of DNA amplification. This process is shown in Figure 1.[5,7]

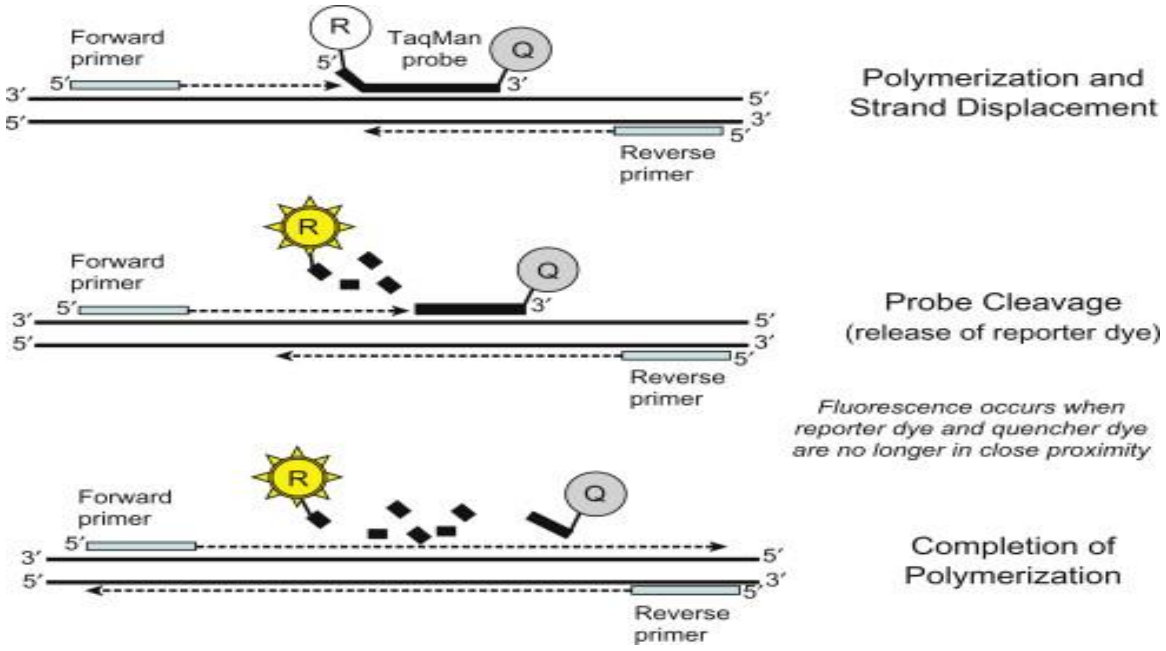

Figure 1: Basic process of qPCR after the Probe is annealed through extension.[5]

If the probe is unable to bind to the DNA sequence then qPCR will not give any result. In the Quantifiler® kit there is also an internal PCR control (IPC) that helps determine if the polymerase, assay, and the instrument are working correctly. The IPC has a VIC (green) reporter dye and a synthetic DNA sequence so that it cannot be confused with the Human DNA in either sequence or dye reporting.[5,7]

After the qPCR run is completed, the data generated is analyzed by a computer. The major components of this analysis are involve the curves of fluorescent signal growth per cycle, and the standard curve which will be used to quantify the unknown DNA. The signal graph can also show if inhibition is present during the PCR reaction, based on deviation of the slope of the growing fluorescence curve relative to the expected rate of increase. The plot of the cycle number versus a log scale of the DNA concentration should be linear during the exponential phase. This range is where the threshold of the process occurs, which, depending on which cycle of the PCR crossing the threshold occurs, is used to determine the quantity of the DNA in the unknowns. The linear range is where the process loses efficiency and deoxynulceotide triphosphates (dNTPs) or the primers are used at different rates. Finally, the PCR reaction plateaus when PCR products accumulate and the reactants become depleted (Figure 2).

The important measure comes in what is called the cycle threshold (C_T), also known as the quantification cycle (C_q): where the fluorescence reaches a threshold, an arbitrary number but, normally about 0.2, that is set by the software to be above the noise in the beginning of the PCR process. The more quickly the fluorescence crosses the threshold the higher the quantity of DNA in the sample. The log of the DNA concentrations is then compared to the C_q to determine the standard curve, resulting in a negatively sloped line, Figure 2.

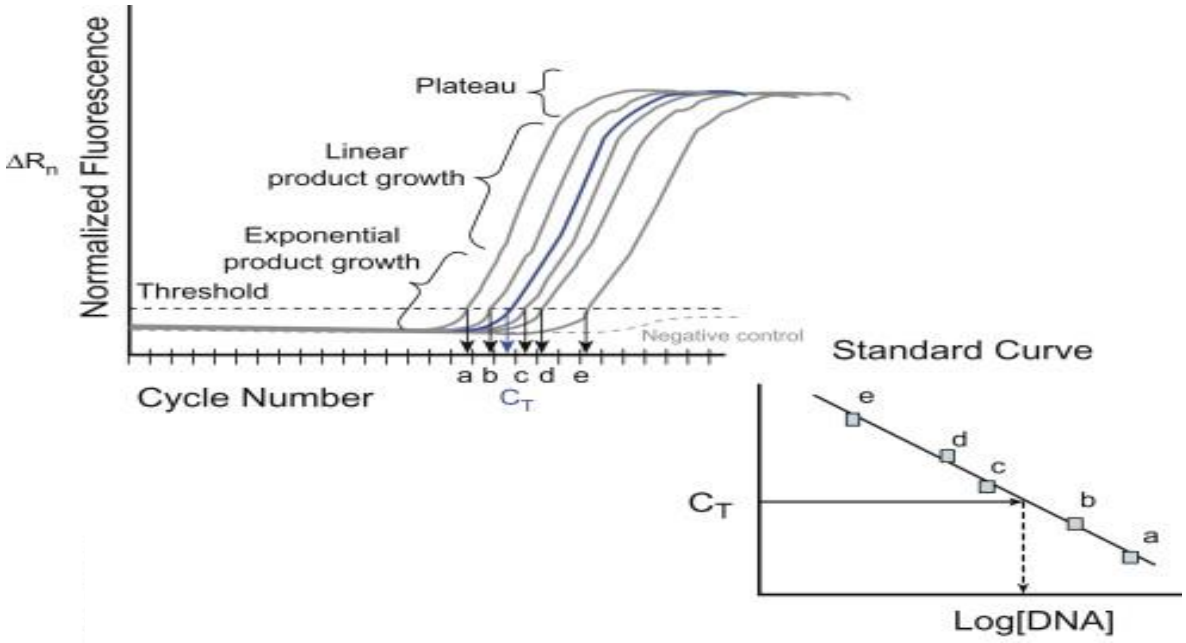

Figure 2: Graphs of the qPCR fluorescent growth by cycle number and the standard curve generated by the standards.[5]

As long as PCR amplification rate occurs the same within each sample, the standards can then be used to determine the amount of input DNA in the unknowns, in ng/µL. This is based on the C_q values of the standards and then comparing the C_q value of each of the unknowns. Forensic qPCR normally uses 8 standards and 3 different controls to determine how well the standards were made. The two positive controls are quantified at 1.0 ng/µL and 0.1 ng/µL, and a non-template control (NTC), i.e. the negative control, has a quantification value of 0 ng/µL to determine if contamination is present. After quantification is complete, the sample is then diluted so that the optimal amount of DNA enters the STR amplification.[5]

According to Guesini et al, the problem with qPCR methods is that the most common method of interpretation, the C_q method, has problems because it is user dependent and requires efficiency to be the same in all reactions. If the efficiency is not the same, the estimates generated are "strongly impaired". They stipulate that the efficiency of the reactions is the most important assumption in the C_q method. Also stated, is that inhibitors generate inaccurate results with qPCR by changing the efficiency of the reaction and with strong inhibition can cause false negatives.[8]

Droplet Digital PCR

A new process recently developed for the quantification of DNA is droplet digital PCR (ddPCR). This process works similarly to qPCR in the amplification step, but has a major change before PCR occurs. Instead of having all of the PCR components into one well of a plate, ddPCR instead separates the PCR components into 20,000 nanoliter-sized droplets. This partitioning of the sample into water-oil emulsion droplets lets each droplet act as a separate PCR reaction, Figure 3. During the quantification step, ddPCR uses absolute quantification, which counts each droplet as a positive or negative, instead of the relative quantification used by qPCR. When correctly diluted, absolute quantification uses positive and negative fluorescent data from up to 20,000 droplets to calculate the starting number of targets in each reaction assuming a Poisson distribution.[9,10]

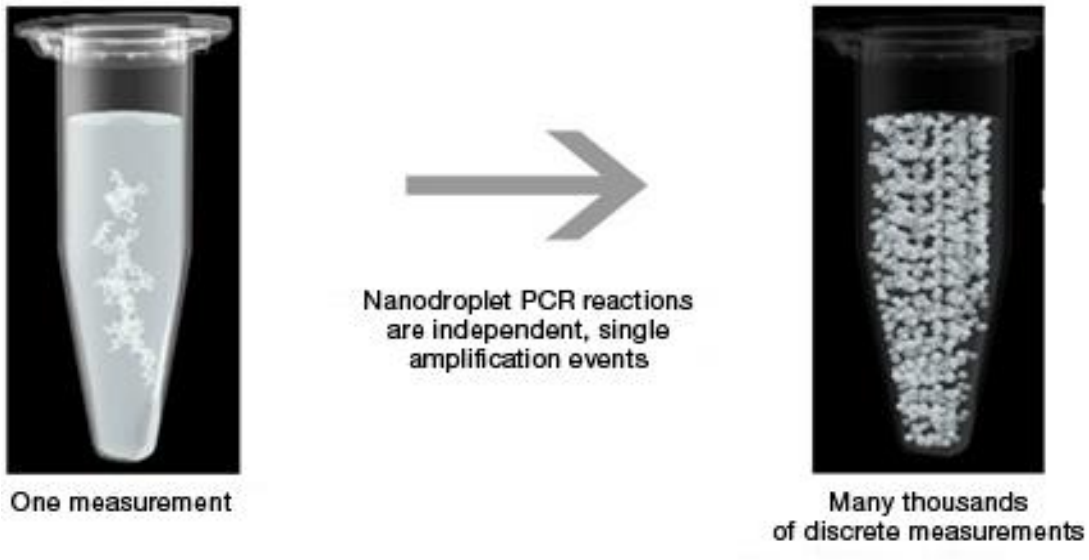

Figure 3: Difference in samples between standard qPCR and ddPCR.[9]

During this process, ddPCR uses microfluidics to divide the PCR samples into emulsion droplets. These each represent one independent reaction in the whole of the 20,000 droplets. Each droplet allows PCR amplification of template to occur and is read after the PCR process to be called positive or negative. The data are fit into a Poisson distribution and the actual number of template molecules are calculated. The 20,000 sample droplets allow more complex statistical analyses to occur due to the absolute quantification instead of using relative fluorescence. Absolute quantification saves time because creation of standards is not necessary and also saves reaction volume by not having to use reactions on the standard dilution series or the other reference samples, such as the non-template control. The counting of positive and negative reactions allows small differences in DNA quantity to be distinguished between samples with a high signal-to-noise ratio thanks to small background noise compared to a strong signal. Lower signal, as might be expected from PCR with reduced efficiency in the presence of inhibition, is still detected by absolute positive and negative droplets. By contrast in standard qPCR, inhibition can be misinterpreted as lower abundance of target amplicon.[9]

Droplets for ddPCR are created using a reaction master mix composed of dNTPs, Taq DNA polymerase, fluorescently labeled oligonucleotide probes, reaction buffer, and template DNA. The droplets are made from an oil-water mixture and partitioned into the 20,000 nanodroplets of equal size and volume in a disposable microfluidic chambers using 20 µL of starting sample, Figure 4 (adapted from reference 9).

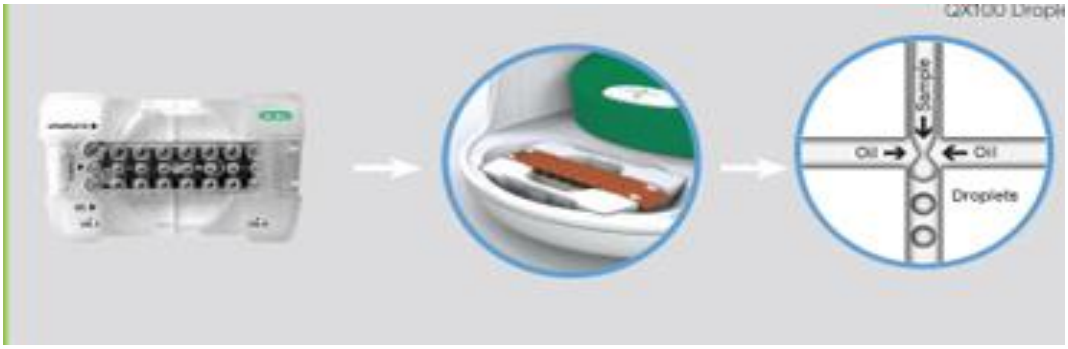

Figure 4: How droplets are generated by the droplet generating instrument.[9]

Next, the droplets are transferred to a 96-well plate for standard PCR amplification to occur in each droplet to generate the fluorescent signal. Finally, the droplets are read one at a time through the droplet detector, Figure 5.

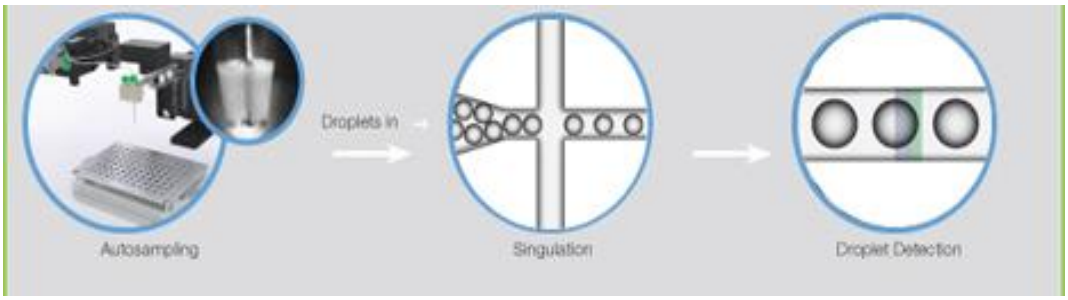

Figure 5: How the droplets are read by the instrument. [9]

Software bundled with the instrumentation allows for visual graphs of the positive and negative reactions. This is shown as a graph where the positive reactions are above and the negative below a threshold

fluorescence. These graphs indicate the precise number of droplets counted as compared to the bulk fluoresce of dyes determined by qPCR, Figure 6.

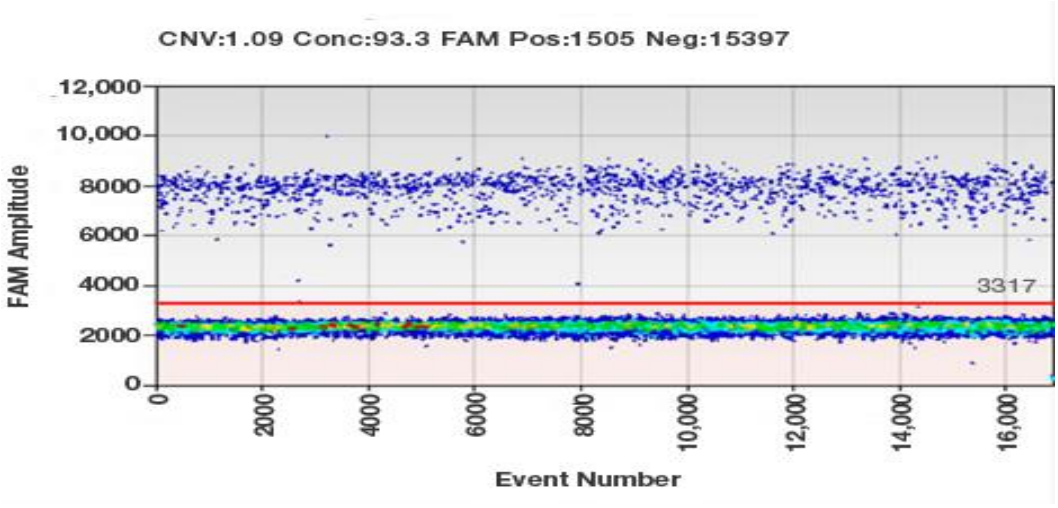

Figure 6: Results from a single ddPCR run. The number of events, i.e. droplets, is compared to the FAM Amplitude, ie amount of total fluorescence, in each individual droplet. The Red line is a manually controlled threshold line which distinguishes between positive and negative droplets. [9]

The samples with lower fluorescence might have begun with less template or could have had reduced efficiency of amplification due to inhibitors in the PCR reactions. With proper dilution, the number of starting target molecules would fit the Poisson distribution with the majority of droplets beginning with a single target copy. This distribution facilitates highly accurate quantification of beginning numbers of target molecules in the sample. [9]

Since the droplets are randomly distributed some will contain no template, some will contain one template molecule, etc., Figure 7. The random distribution follows a Poisson distribution and facilitates analysis. Poisson's law of small numbers states that random quantifiable effects allow predictions to be made. For ddPCR, these predictions can determine the amount of template molecules in a droplet after analyzing the fluorescence data.

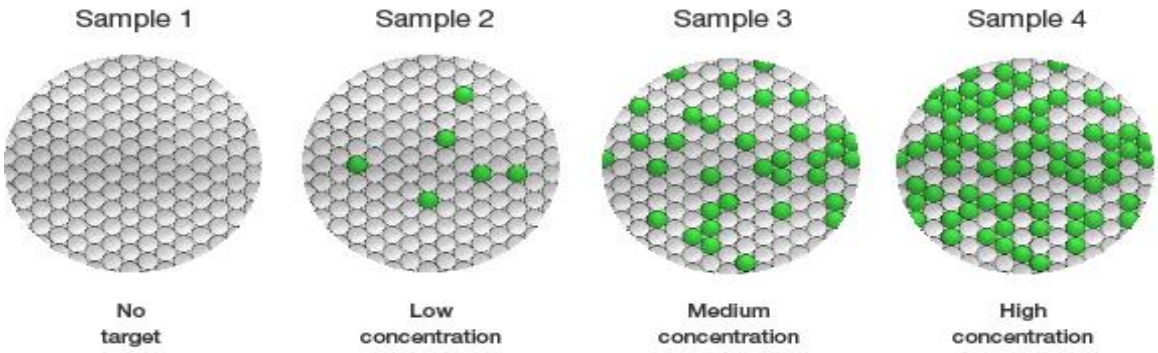

Figure 7: Random distribution of template molecules due to droplet creation. [10]

The output for the Poisson calculations ends up in copies of target molecules per sample based on the number of positive droplets, *p*. This can be used instead of standard curves to determine concentration, even between instruments and laboratories, due to the absolute nature of the quantification. The plots shown correspond to the 95% confidence level of the distribution, Figure 8.

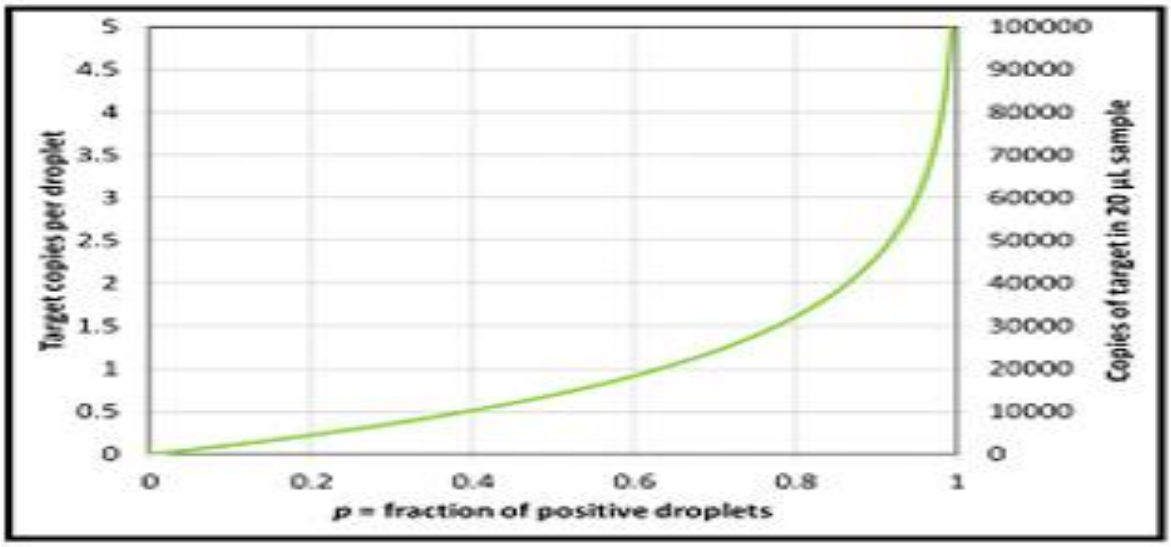

Figure 8: Positive droplets correlate to number of target copies in a 20 µL sample. Independent variable is the fraction of positive droplets, dependant is number of target copies in a 20 µL sample. [10]

As this graph shows, the detection limit for ddPCR is anywhere in the range from 1-100,000 target copies, or one copy in a single droplet to an average of five copies per droplet across all droplets. Based on the statistics, ddPCR is most effective at lower concentrations of DNA. ddPCR when compared

to qPCR is able to determine rare events seen in low quantities over the microreactions. For example, a commonly used concentration in forensics is 1 ng of input DNA into the assay, which corresponds to only 303 copies of the target genome in the 20 µL reaction volume.[10]

Quantification of DNA samples using the ddPCR platform is potentially advantageous to the forensic biology community because it offers a lower chance of false negative results, increased precision and fewer control samples than the standard qPCR platform, thereby increasing laboratory throughput. Samples that are negative for quantification, including false negatives, results do not undergo subsequent DNA testing.

A DNA quantification standard series is required for quantifying DNA using qPCR; a standard curve is created from the standard series and unknown samples are compared to it. The counting method used with ddPCR does not need a DNA quantification standard series. This saves 16 wells out of the 96 in a 96-well plate used by both qPCR and ddPCR; this means 17% of the plate can be used for samples instead of the standard series. Eliminating the need for these standards can increase throughput in a laboratory and decrease reagent and handling costs. This would also help decrease the evidence backlog- the collection of cases that have not been tested within 30 days of submission to a forensic laboratory.[11] As of 2009, there were more than 100,000 backlogged cases retained by 168 laboratories nation-wide.[11] Implementing ddPCR technology into forensic laboratories may improve detection of inhibited samples, decrease measurement errors, increase plate utilization, and ultimately, serve to reduce the number of backlogged cases.

PCR inhibitors

According to Opel et al (2010)[1], not much was known of the underlying causes of PCR inhibition. That paper proposed three different potential mechanisms: the inhibitor binds directly to the polymerase, the inhibitor interacting with the DNA, and finally, the inhibitor interacting with the polymerase during the PCR extension step. Opel suggests that humic acid binds directly to DNA, limiting the amount of

template in the PCR reaction. Also, Sutlovic et al[12] noted that humic acid increases the melting temperature (T_m) of template DNA. Heavy metal inhibitors interact with the DNA molecule by degrading and binding to the DNA molecule.[13,14] Therefore, even though magnesium is required for PCR processes, heavy metals do not affect the polymerase by binding to it where the magnesium would.

Inhibition of PCR has been a concern for forensic samples because of inhibitors causing false negatives and creating problems for STR amplification, but previous methods were limited in their ability to detect inhibition. Quantification using qPCR instrumentation was the first method to reliably detect and quantify inhibition. The C_q of a sample increases with inhibition, and this can be detected by an increase in the internal PCR control and human DNA detector, Figure 9.

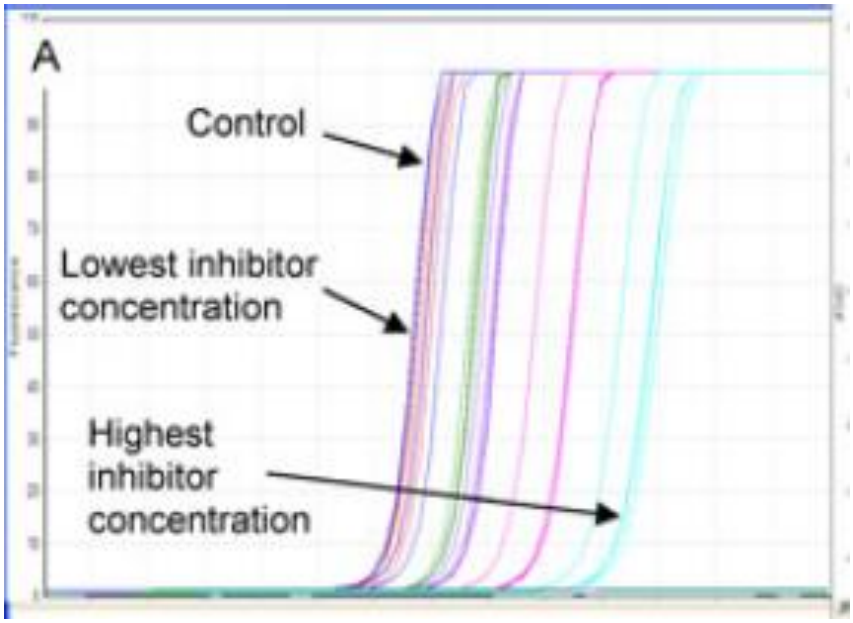

Figure 9: Humic Acid study performed by Opel et al. The cycle number is the independent variable, detected fluorescence is the dependent variable. In the presence of increasing humic acid, inhibition results in higher cycle number to reach maximum fluorescence, mimicking lower starting target concentration and indicating reduced PCR efficiency.[1]

While this defined case illustrates the effect of inhibition, in casework situations it is more difficult to ascertain if the increase in C_q is due to inhibition or concentration of DNA in the sample. It is important to note that the effects of inhibition can be lessened by dilution of the sample or further purification of the sample, but these also decrease the concentration or the total amount of DNA in the sample. Because of

this inhibition problem, multiple qPCR kits have been tested previously, Quantifiler®[13] and Quantiplex[15], and the metal-treated samples have caused C_q shift for both the DNA input and the IPC.

Several studies involving ddPCR have compared it to qPCR, and some have mentioned that ddPCR handles inhibition better[16,17], but none have done as extensive of a study on how specific inhibitors affect the ddPCR process for forensic samples. Dingle et al states that ddPCR may offer an advantage compared to qPCR because of ddPCR partitioning the sample into droplets. Partitioning of the sample into droplets allows for the DNA to be counted by the Poisson distribution, and this same principle applies to the inhibitors in the sample as well. Whereas in qPCR, amplification is dependent on the concentration of inhibitor in the total sample and can cause an inaccurate quantification.[17] These specific inhibitors, Al, Cu, Fe, and humic acid, can be found in soil or in the body. Droplet digital PCR could be important for cases where there is a small quantity of DNA present to be able to continue analysis to get a DNA profile for a victim where qPCR results might be interpreted as a failure.

These experiments used *Tert*[18] and *Sox2*[19] as the probes for quantification. *Tert* is a human probe for DNA that targets telomerase, which is a protein that maintains the ends of telomeres thus helping keep DNA from degrading. In this system, *Tert* was bound to HEX, which is a green fluorophore. *Sox2* is a human probe that targets a transcription factor involved in regulation of embryonic development and determines cell fate. This is required for stem cell maintenance. In this system, *Sox2* was bound to FAM, which is a blue fluorophore. The probes emit different wavelengths of light, HEX (556 nm) and FAM (518 nm)[20], so that they can be run in the same reaction, or multiplexed.

The metals used in this experiment were Al, Cu, and Fe (High Purity Standards, Charleston, SC). These metals were then made into a stock solution and pH adjusted, between 3 and 5, using 3 M ammonium hydroxide (NH_4OH) and 1 M hydrochloric acid (HCl).[13] The buffering of the metals with the pH adjustment made it to where the pH of the metal input would not interfere with the pH needed to perform PCR of above 8, and the ddPCR supermix (BIO-RAD) has a buffering system that has the pH set

at 8.3.[21] The humic acid was a stock solution prepared with technical grade humic acid (Sigma-Aldrich, St. Louis, MO) and TE-4 buffer (10 mM Ultrapure™ Tris-HCl, pH 8.0, Invitrogen Corporation, Carlsbad, CA, and Ultrapure™ EDTA, pH 8.0 GIBCO Products, Grand Island, NY).[21] The final pH values and concentrations for the solutions are below in Table 1.

Table 1: Inhibitor concentrations and pH values.

Inhibitor	Concentration	pH
Al	570 ppm	4.0
Cu	1400 ppm	4.9
Fe	1200 ppm	3.8
Humic Acid (HA)	500 ng/µL	<2

It is important to make sure the levels of inhibition used could be seen in actual casework and these concentrations and the concentrations used in the experiment are close to biologically relevant levels according to studies performed before this one.[13,22]

CHAPTER TWO

MATERIALS AND METHODS

Preparation of DNA Samples

DNA samples were prepared using a 50 ng/µL stock solution of DNA from Serological Research Institute (SERI, Richmond, CA) and diluting the DNA with Ultrapure™ DNase/RNase-Free Distilled Water (Invitrogen™) until the desired concentration of DNA was reached.

In the sensitivity study, the DNA was diluted to 33 ng/µL and underwent serial dilution until the final concentration was 0.045 ng/µL. For all other experiments, the DNA solution was diluted to 5 ng/µL.

Table 2: Serial Dilution for Sensitivity Study.

Well	DNA conc.
A	33 ng/µL
B	11 ng/µL
C	3.67 ng/µL
D	1.22 ng/µL
E	0.41 ng/µL
F	0.14 ng/µL
G	0.045 ng/µL

Preparation of Inhibitor Solutions

The metals used in this experiment were Aluminum, Copper, and Iron which were analytical standards in solution (High-Purity Standards, Charleston, SC). The solutions were ~21 mM. The solutions were then pH adjusted (Al, 4.0 pH; Cu, 4.9 pH; Fe, 3.8 pH), and buffered with 3 M ammonium hydroxide (NH_4OH) and 1 M hydrochloric acid (HCl). Dilutions were prepared with Ultrapure™ DNase/RNase-Free Distilled Water (Invitrogen™). Humic acid stock solution, 506.758 ng/µL, was prepared by using technical grade humic acid (Sigma-Aldrich) and TE-4 buffer (10 mM UltraPure™ Tris-HCL, pH 8.0, Invitrogen Corporation and 0.1 mM UltraPure™ EDTA, pH 8.0, GIBCO Products, Grand

Island, NY). Dilutions were prepared with Ultrapure™ DNase/RNase-Free Distilled Water (Invitrogen™).[13,23]

Preparation of ddPCR Mixture

Into 0.1 mL 8- tube strips, 10 µL of 2x ddPCR Supermix for Probes (no dUTP) (BIO-RAD) was added. After, the probe, 20x PrimePCR™ ddPCR™ Copy Number Assay: *Tert* and *Sox2* (BIO-RAD), was added to each well. Then DNA and inhibitor were added, using water to fill to the 20-µL reaction volume.

Sample Preparation for the Sensitivity Study

To determine the optimal DNA input for further experiments, a sensitivity study was performed. DNA stock at 33 ng/µL was serially diluted 1:2 with ultrapure water until the final concentration was 0.045 ng/µL. 1 µL of DNA was added to each sample well with different columns receiving different amounts of probe.

Table 3: Input DNA Concentrations for Sensitivity Study.

Well	DNA conc.
A	33 ng/µL
B	11 ng/µL
C	3.67 ng/µL
D	1.22 ng/µL
E	0.41 ng/µL
F	0.14 ng/µL
G	0.045 ng/µL
H	Negative Control

The amount of probe was different in different columns: columns 1 and 2 had 1 µL of *Tert* only, 3 and 4 had 1 µL of *Sox2* only, and 5 and 6 had 0.5 µL of both *Tert* and *Sox2* to see if the probes could be multiplexed.

Sample Preparation for the Probe Study

After determining the probes could be multiplexed, the optimal amount of probe to input into each reaction was determined. 5 ng/µL of DNA was input into reactions with either 0.5 ng/µL each or 1 ng/µL each of both *Tert* and *Sox2*. Then ultrapure water was added until the final volume was 20 µL.

Metal Concentration Preparation

After the optimal amount of probe was determined, 5 ng/µL of DNA was added to wells with 1 µL of both *Tert* and *Sox2*. Then metals were diluted from stock solutions to reach 7 mM for metals and 166.6 mM for humic acid which was the starting concentration for the inhibition study.

Sample Preparation for Inhibition Study (7 mM)

The diluted inhibitor solutions were added 1 µL starting with well F and increasing by 1 µL until 6 µL were input in well A. 5 ng/µL of DNA was added to each well along with 1 µL of both *Tert* and *Sox2*. Then water was added until the final volume was 20 µL. Duplicate lanes of each sample were used.

Table 4: Amount of Inhibitor added to the study.

Well		Al	Cu	Fe	HA
A	6 µL of Inhibitor Solution	42.25 mM	42.92 mM	42.97 mM	1000 ng/µL
B	5 µL of Inhibitor Solution	35.21 mM	35.60 mM	35.81 mM	833.33 ng/µL
C	4 µL of Inhibitor Solution	28.17 mM	28.48 mM	28.65 mM	666.67 ng/µL
D	3 µL of Inhibitor Solution	21.13 mM	21.36 mM	21.49 mM	500 ng/µL
E	2 µL of Inhibitor Solution	14.08 mM	14.24 mM	14.32 mM	333.33 ng/µL
F	1 µL of Inhibitor Solution	7.04 mM	7.12 mM	7.16 mM	166.67 ng/µL
G	Positive Control				
H	Negative Control				

Sample Preparation for Aluminum Study (7 mM)

After the inhibition study, the diluted aluminum solution was then used to determine the effects Al has on ddPCR. Other than the positive and negative control, all wells contained *Tert* and *Sox2*, but not

all wells had DNA input, Table 5. 2 lanes were prepared, one lane was amplified and one lane was not amplified.

Table 5: Aluminum Study Wells.

Well	Well Contents
A	*Tert*+ *Sox2*+ Al
B	*Tert*+ *Sox2*+ Al
C	*Tert*+ *Sox2*+ Al
D	*Tert*+ *Sox2*+ DNA+ Al
E	*Tert*+ *Sox2*+ DNA+ Al
F	*Tert*+ *Sox2*+ DNA+ Al
G	Positive Control
H	Negative Control

This was to determine what effect aluminum had on the sample and if the effect was strengthened by amplification than just with the aluminum.

Droplet Creation, DNA Amplification, and Droplet Reading

The samples were loaded on the droplet generation (DG) chip, DG8 (BIO-RAD). 70 µL of DG oil (BIO-RAD) were then added to the chip. The chip was placed into the Droplet Generator machine (BIO-RAD). Following droplet creation, the droplets were then pipetted into a 96 well plate and placed into a thermocycler (C1000 Touch, BIO-RAD), with conditions that are in Table 6.

Table 6: Thermocycler Conditions.

Cycle Conditions	Temperature	Time	Settings	
Hold 1	95 °C	10 min	50% ramp	(~2-3 °C/sec)
40 cycles of:	94 °C	30 sec	Heated lid	105 °C
	60 °C	60 sec		
Hold 2	98 °C	10 min		
Hold 3	4 °C	∞		

Following PCR, the plate was transferred into the QX100 Droplet Reader (BIO-RAD) and the droplets are read and analyzed using Quantasoft software (BIO-RAD).

Further Analysis

Means and standard deviations for positive droplets, concentrations, average amplitude, and Chi-square "Goodness of Fit" results were performed on Microsoft Excel. Outliers determined by average ± 3 standard deviations.

CHAPTER THREE

RESULTS

Sensitivity Study

The results of the sensitivity study indicated a linear correlation of the positive number of droplets with increasing DNA concentration, Figure 10.

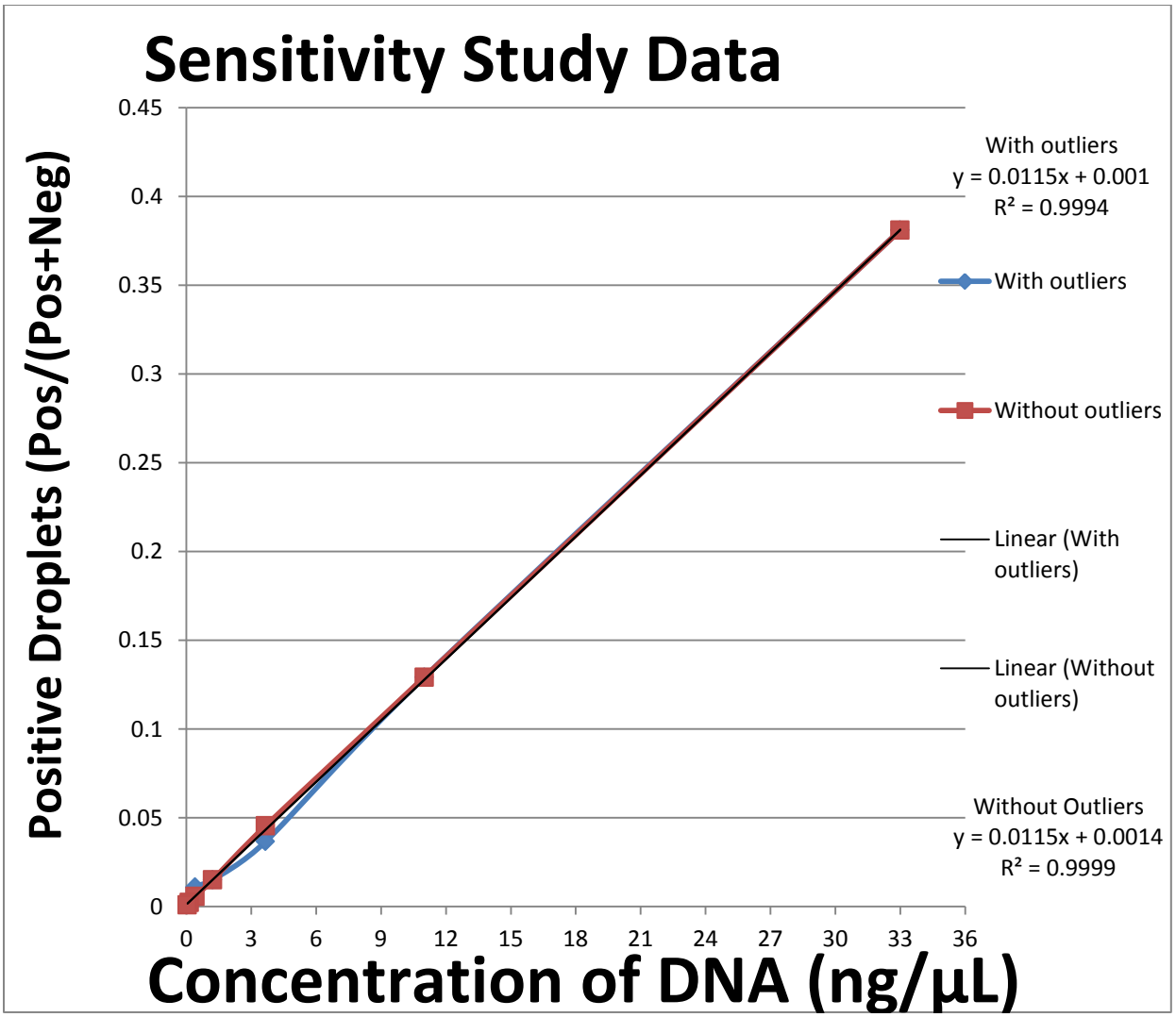

Figure 10: Graphical Representation of Sensitivity Study Data. Concentration of DNA vs Positive Droplet Ratio. Linear equation above line descriptions is with outliers, below is without outliers.

This line was created by averaging all replicates from all six lanes, including the multiplexed lanes. The trend line for the data with outliers removed had a correlation coefficient of R^2=0.9999, outliers have no

effect on the correlation coefficient based on this experiment. The trend line shows that the efficiency of the reaction is constant no matter the quantity of DNA input or number of probes. From this data, 5 ng/µL was chosen for continued study. This value was chosen because 5 ng/µL is close to forensically relevant levels (approximately 1 ng/µL), but still provides sufficient positive droplets to determine if droplets were lost due to inhibition rather than of random sampling error. 5 ng/µL in theory should result in approximately 1500 positive droplets where-as 1 ng/µL would result in only 300 possible positive droplets.

Another aspect of this study was not only to determine optimal DNA template concentration, but was also used to determine if this amount of DNA would work for either singleplex or multiplex reactions. For the singleplex 1 µL of probe was used. Therefore for the multiplex reactions, only 0.5 µL of each probe was used total to keep the total amount of probe 1 µL. The manuals for ddPCR use 1 µL of target probes and 1 µL of wild type probes. In this case both probes are targets for forensic uses, and therefore determining if 1 µL of probe total, or 1 µL of each probe (2 µL) would be better for continuing study.

Probe Study

For the probe study, it was important to determine if 1 µL of probe, 125 nM of each probe[24], total or 1 µL, 250 nM, of each probe was better for detection. If the change in fluorescent amplitude was not significant, the savings of using less probe would outweigh the higher fluorescent amplitude of more probe. The average amplitudes for each probe were determined and the results are listed in Table 7 below.

Table 7: Amplitudes of Probe Study.

Amount of probe		Average Amplitude (RFU)	Average of Both Probes (RFU)		Probe	Ratio of 1.0 µL of each probe/ 0.5 µL of each probe
A	0.5 µL *Sox2*	3161.64	E (A+B)/2	2878.12	*Sox2* (C/A)	2.83
B	0.5 µL *Tert*	2594.59			*Tert* (D/B)	2.23
C	1.0 µL *Sox2*	8959.73	F (C+D)/2	7516.76	(*Sox2*+*Tert*)/ 2	2.53
D	1.0 µL *Tert*	5789.19			Both (F/E)	2.61

Using 1 µL of each probe showed an amplitude of over 2.5 times more than the amplitude of 0.5 µL, therefore further experiments used 1.0 µL of each probe. This shows the probes still limit the speed of the reaction.

Inhibition by Copper

Copper was diluted and the starting concentration of copper was 7.16 mM and reached 42.92 mM. Figures 11-12 are the duplicate copper lanes tested to determine the inhibitor input for further studies.

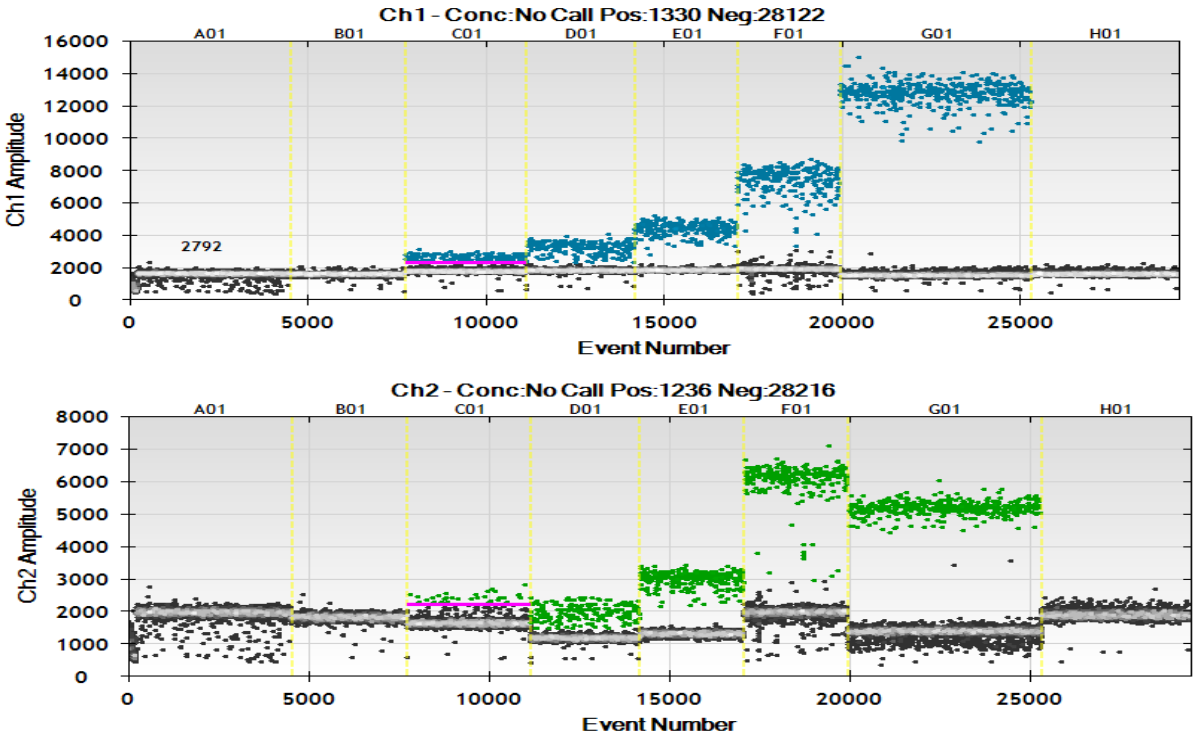

Figure 11: Copper Study Lane A. Event number is number of droplets read in total for the lane. Single lane multiplex with *Sox2*, blue positives, top, and *Tert*, green positives on bottom. Wells were: A01- 42.92 mM, B01- 35.60 mM, C01- 28.48 mM, D01- 21.36 mM, E01- 14.24 mM, F01- 7.12 mM, G01- Positive Control, H01- Negative Control.

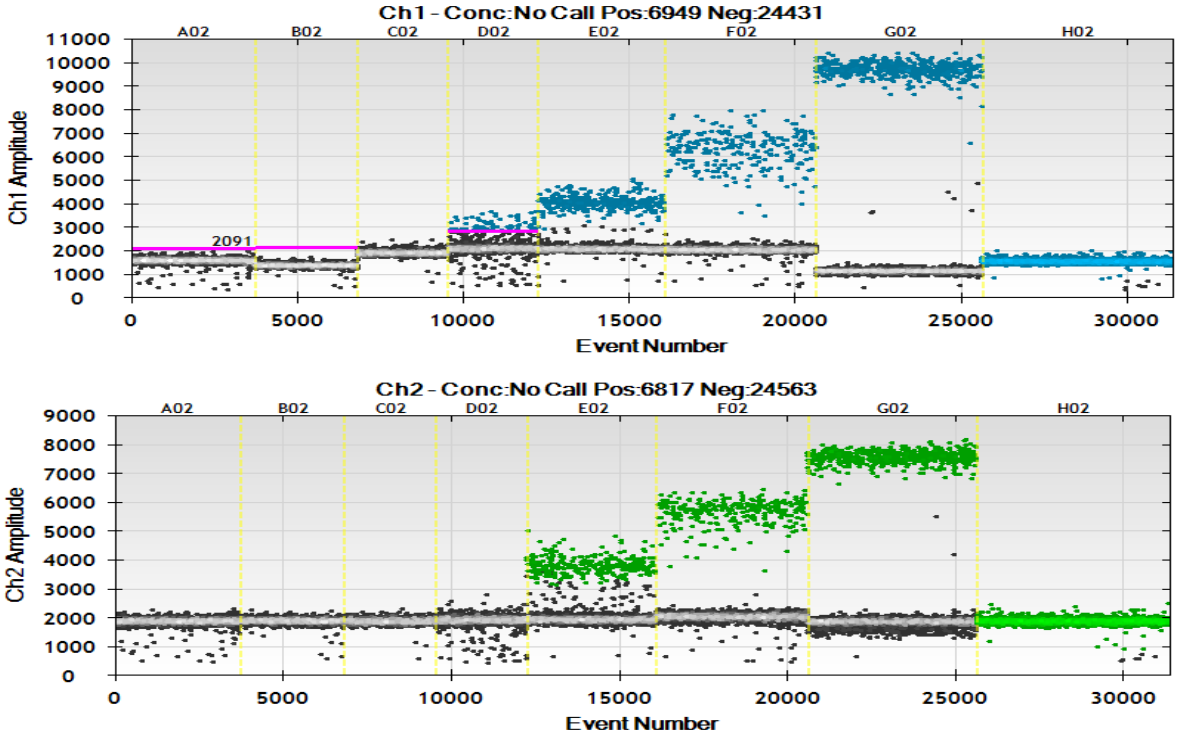

Figure 12: Copper Study Lane B. Event number is number of droplets read in total for the lane. Single lane multiplex with *Sox2*, blue positives, top, and *Tert*, green positives on bottom. Wells were: A02- 42.92 mM, B02- 35.60 mM, C02- 28.48 mM, D02- 21.36 mM, E02- 14.24 mM, F02- 7.12 mM, G02- Positive Control, H02- Negative Control.

This was the step down effect that was desired, but the baseline was not lowered for many of the inhibited samples. The negative sample in lane B was left as positive to show that manual input of negative and positives is needed at times; meaning the computer will call all the negatives positive because there is not two clouds of droplets. The amplitudes between the Positive control and the first two inhibited samples are 37.6% and 60.7% for E and F wells, respectively, for *Sox2*, and 53.5% and 91.7% for *Tert*. The *Tert* percentages are higher because of the F01 well amplitude being greater than the amplitude for the Positive control.

Copper was repeated in the inhibition study, with similar results. Copper showed the same results as it did in the previous experiment, slowly inhibiting signal amplitude until the baseline was reached. Figures 13-14 are the duplicate lanes from this study.

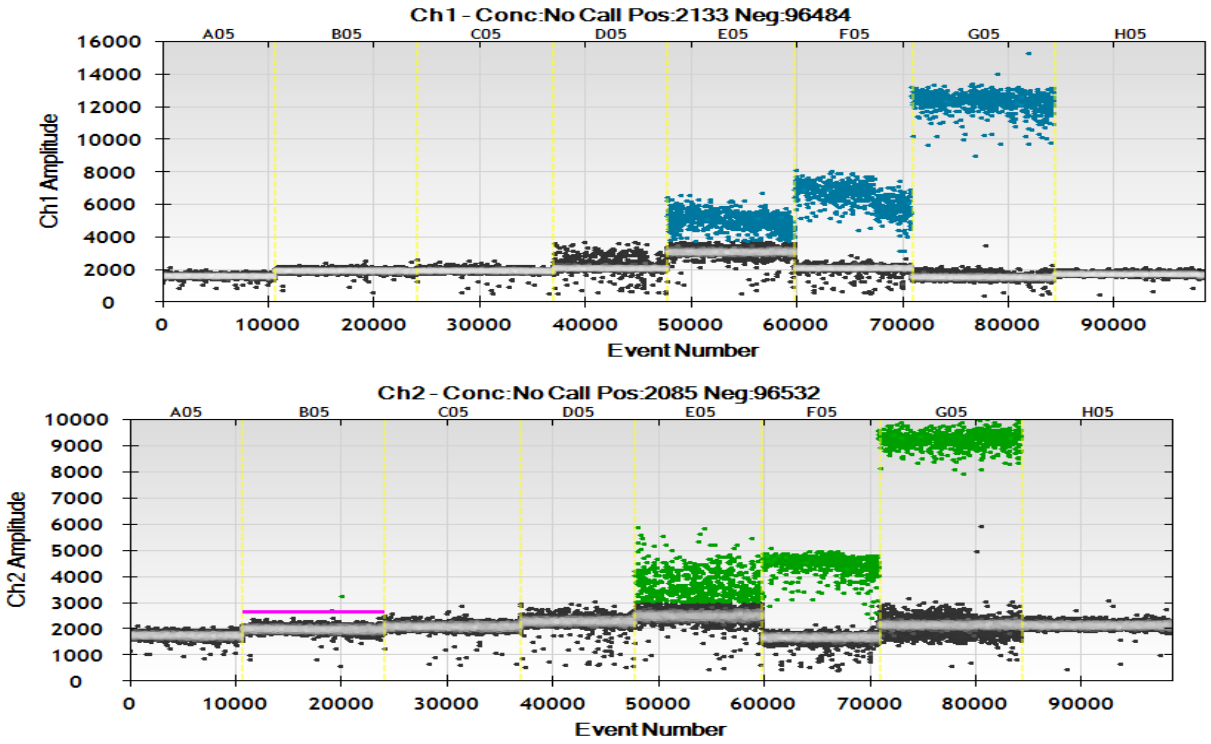

Figure 13: Inhibition Study: Copper Lane A. Event number is number of droplets read in total for the lane. Single lane multiplex with *Sox2*, blue positives, top, and *Tert*, green positives on bottom. Wells were: A05- 42.92 mM, B05- 35.60 mM, C05- 28.48 mM, D05- 21.36 mM, E05- 14.24 mM, F05- 7.12 mM, G05- Positive Control, H05- Negative Control.

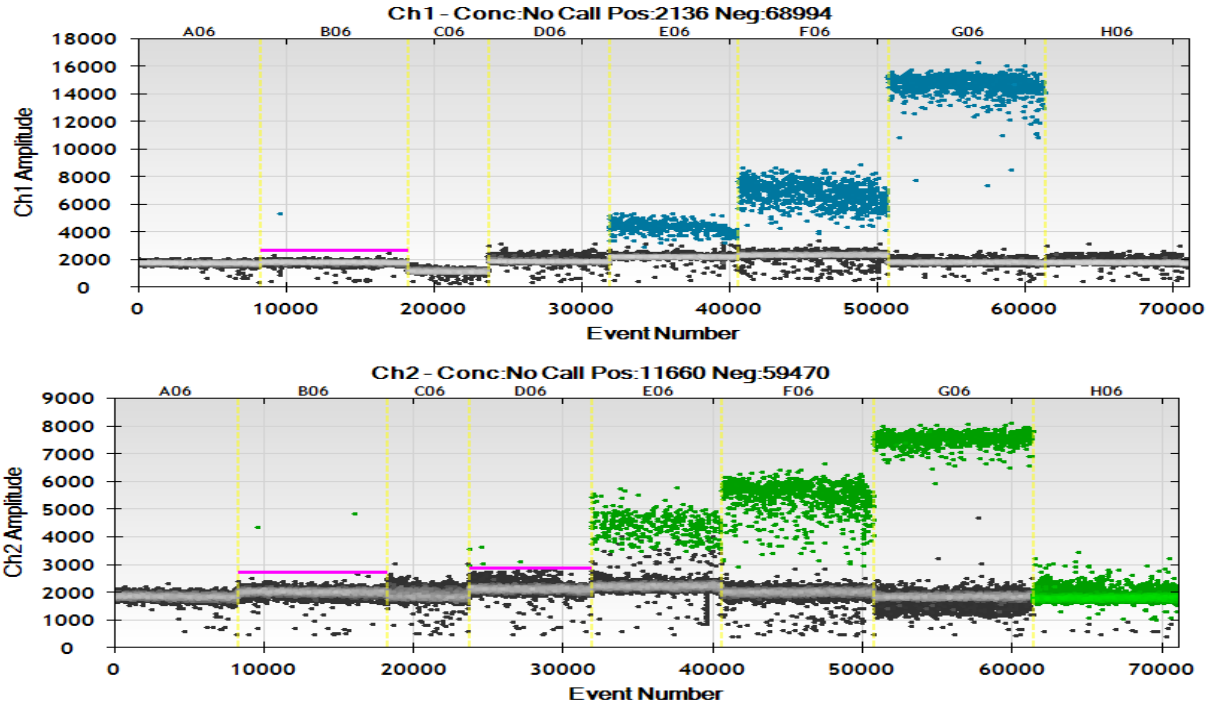

Figure 14: Inhibition study: Copper Lane B. Event number is number of droplets read in total for the lane. Single lane multiplex with *Sox2*, blue positives, top, and *Tert*, green positives on bottom. Wells were: A06- 42.92 mM, B06- 35.60 mM, C06- 28.48 mM, D06- 21.36 mM, E06- 14.24 mM, F06- 7.12 mM, G06- Positive Control, H06- Negative Control.

The amplitudes between the Positive control and the first two inhibited samples are 34.6% and 48.8% for E and F wells, respectively, for *Sox2*, and 35.0% and 58.8% for *Tert*. The *Sox2* percentages are a bit lower than before because the second lane has a higher amplitude for the Positive control. Average of the wells for F and G for positive droplet ratio was 0.135 for the Positive control, G, and 0.138, F, for the first inhibited well. This means that 13.5% and 13.8 % of all droplets read were positive. Therefore, even though amplitude is lost, the amount of positive droplets is consistent with small amounts of inhibition, Figure 15.

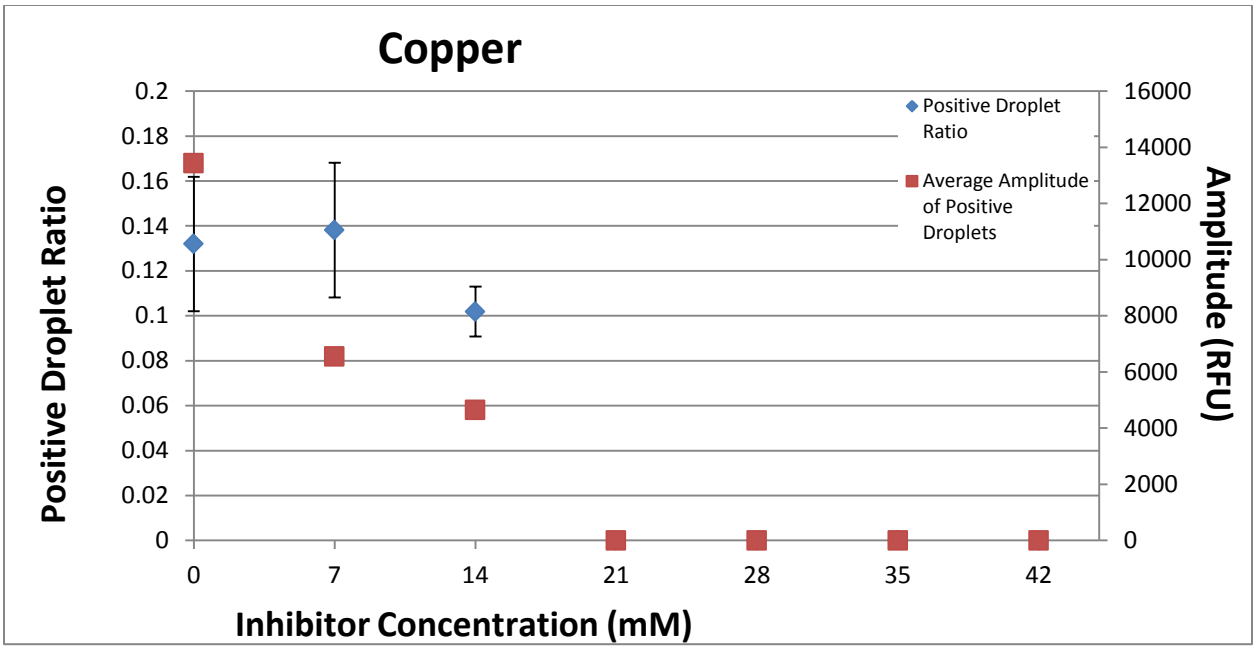

Figure 15: Copper: Positive Droplet Ratio and Average Amplitude of Positive Droplets (RFU, Sox2 and Tert Combined) compared to the concentration of inhibitor input (mM). The blue diamonds are the positive droplet ratio and the red squares are the red squares.

This is a very strong conclusion for ddPCR, because the lowered amplitude shows that the quantification of DNA is correct and that there is some problem with the sample.

Inhibition by Iron

Only one lane of iron out of the duplicate lanes was successful, Figure 16.

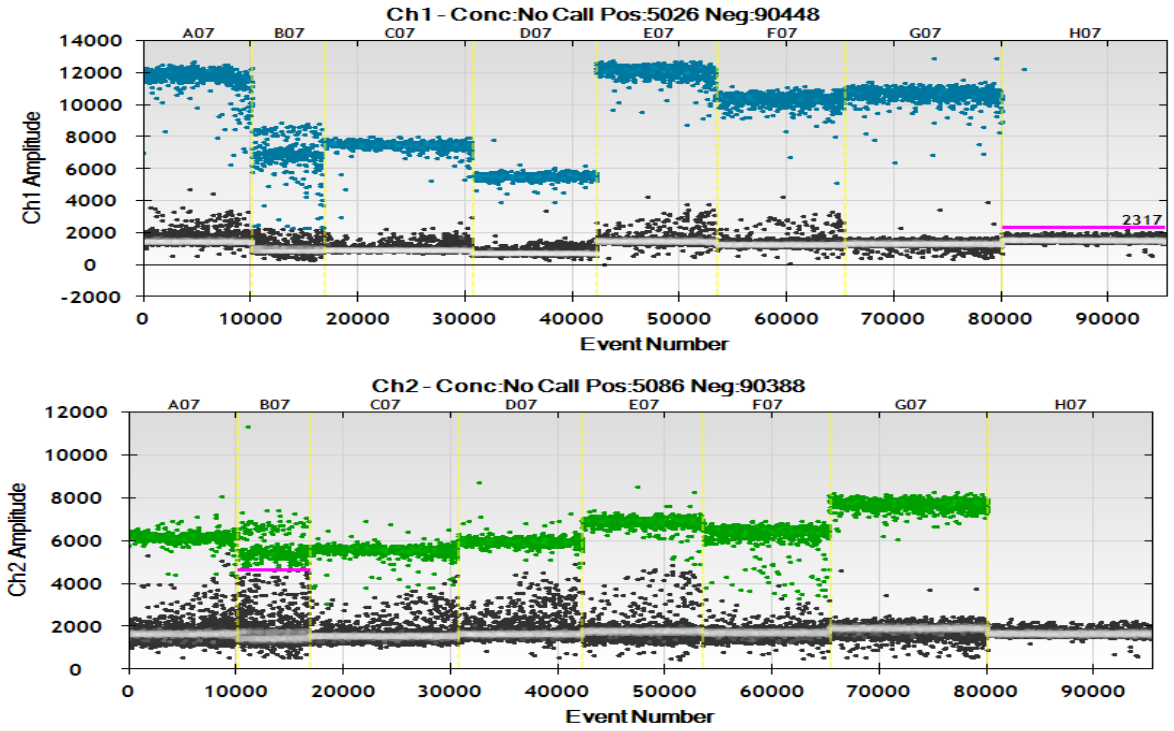

Figure 16: Inhibition Study: Iron Lane. Event number is number of droplets read in total for the lane. Single lane multiplex with *Sox2*, blue positives, top, and *Tert*, green positives on bottom. Wells were: A07- 42.97 mM, B07- 35.81 mM, C07- 28.65 mM, D07- 21.49 mM, E07- 14.32 mM, F07- 7.16 mM, G07- Positive Control, H07- Negative Control.

The duplicate iron lane was considered a failure with multiple failed wells, including the positive control. Iron had a small effect on the droplets, but overall did not cause any major issues with the positive droplets until extreme inhibition was present. Some droplets had the droplet rain effect, where there are non-negative positive droplets between the positive cloud and negative cloud. However, iron did not cause catastrophic failures as was seen for high concentrations of copper in previous tests. The percentage of positive droplets was an average of 12.0 ± 1.2% for all of the wells for Fe. The positive droplet amplitude, and therefore the efficiency of the reaction, was lowered for the 21 mM to 35 mM wells, showing some inhibition occurred, but the positive droplet ratio was not greatly affected until 35 mM, Figure 17.

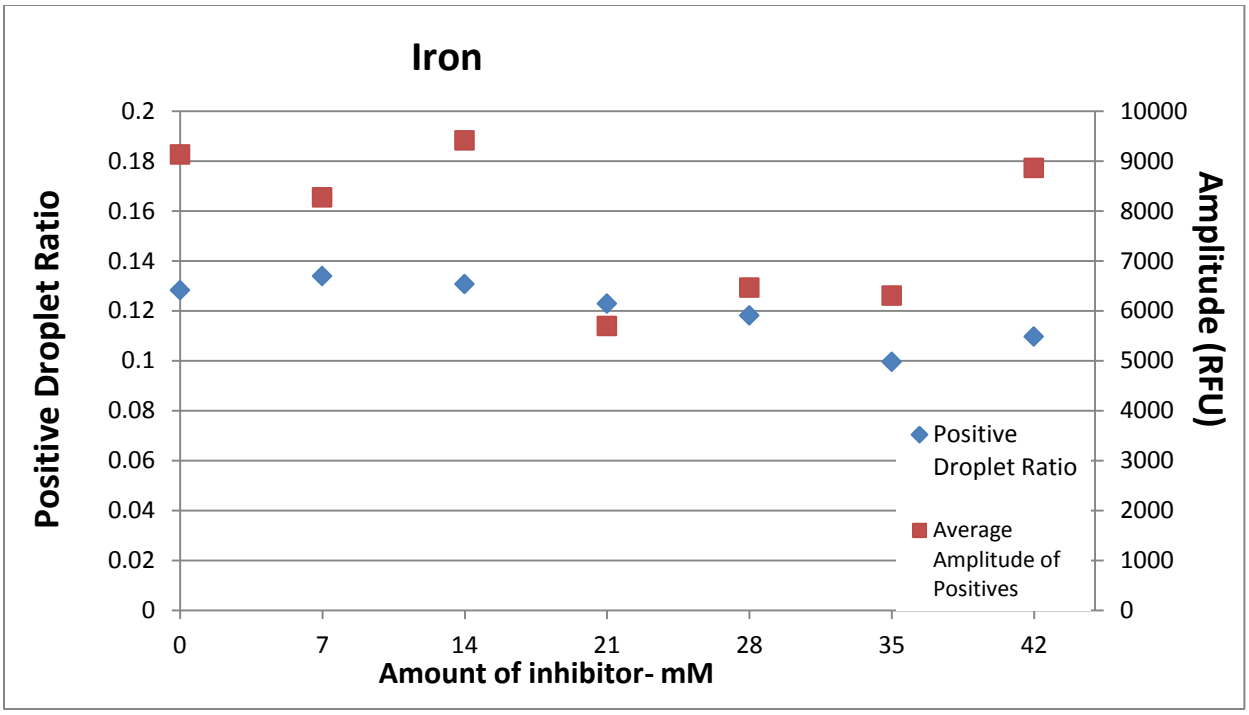

Figure 17: Iron: Positive Droplet Ratio and Average Amplitude of Positive Droplets (RFU, Sox2 and Tert Combined) compared to the concentration of inhibitor input (mM). The blue diamonds are the positive droplet ratio and the red squares are the red squares.

The graph above also shows the discrepancy in amplitude in the 21-35 mM wells. Also determined was the number of positive droplets between the positive and negative clouds and the percent of the total amount of droplets those droplets are, Table 8.

Table 8: Iron: Number of positives between positive and negative clouds and the percentage of those droplets of the whole.

Iron	Number of Positives between positive and negative clouds	Percent of droplets between positive and negative clouds
42 mM	295	2.852
35 mM	248	3.73
28 mm	256	1.841
21 mM	329	2.84
14 mM	156	1.394
7 mM	110	0.926
Positive control	30	0.204

As the table shows, the amount of these droplets increases greatly between the positive control and the inhibited wells going up to nearly 4% of all the droplets read in the wells to be these in between droplets.

Inhibition by Humic Acid

Humic acid had almost no demonstrable effect on droplets in this experiment, Figures 18-19.

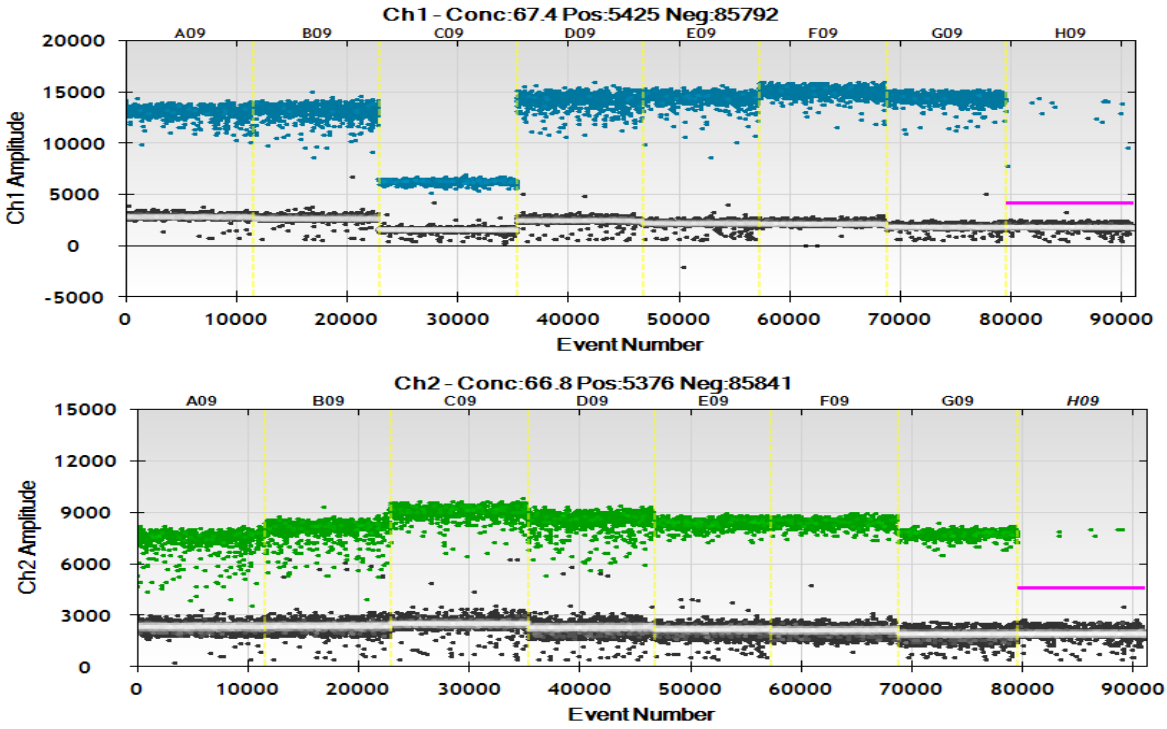

Figure 18: Inhibition Study: Humic Acid Lane A. Event number is number of droplets read in total for the lane. Single lane multiplex with *Sox2*, blue positives, top, and *Tert*, green positives on bottom. Wells were: A09- 1000 ng/µL, B09- 833.33 ng/µL, C09- 666.67 ng/µL, D09- 500 ng/µL, E09- 333.33 ng/µL, F09- 166.67 ng/µL, G09- Positive Control, H09- Negative Control.

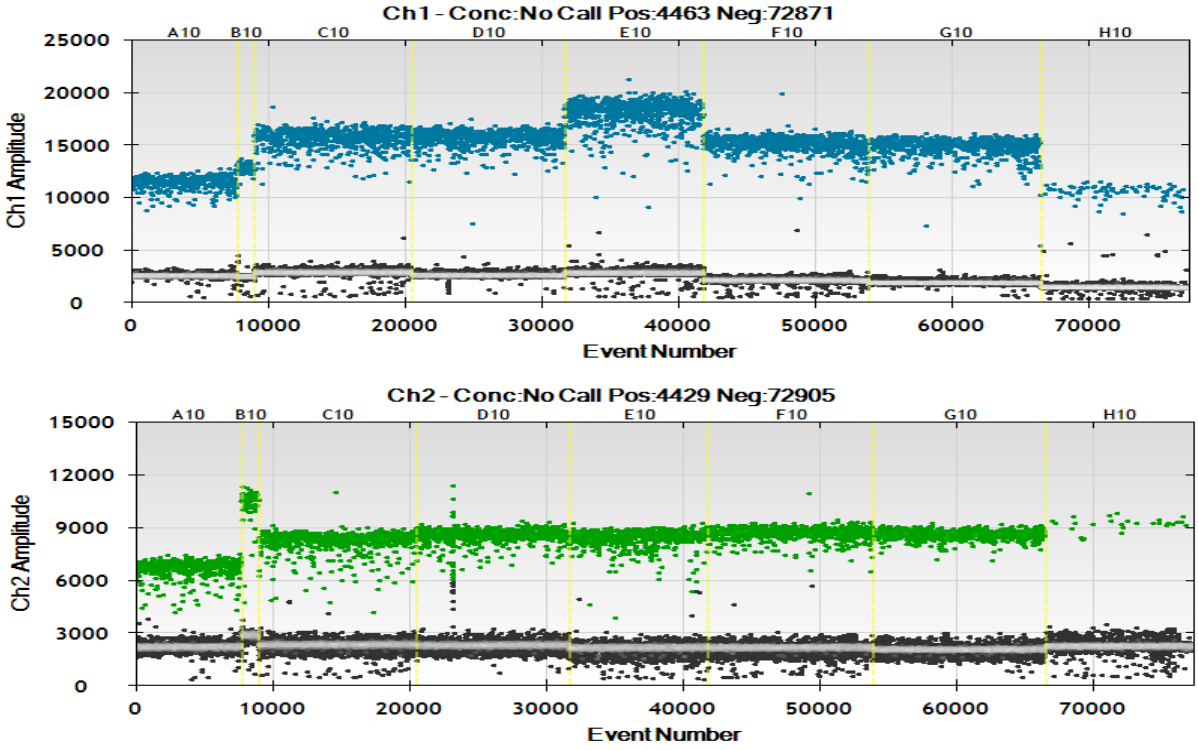

Figure 19: Inhibition Study: Humic Acid Lane B. Event number is number of droplets read in total for the lane. Single lane multiplex with *Sox2*, blue positives, top, and *Tert*, green positives on bottom. Wells were: A10- 1000 ng/µL, B10- 833.33 ng/µL, C10- 666.67 ng/µL, D10- 500 ng/µL, E10- 333.33 ng/µL, F10- 166.67 ng/µL, G10- Positive Control, H10- Negative Control.

The wells were in line with the Positive Control except for Lane A, Well C09 which showed a lower amplitude, which meant that the efficiency of the reaction was lowered in that well showing some possible inhibition. Overall, the wells showed the same amplitude and quantification of DNA, this shows that the efficiency is not changed by this inhibitor. These samples showed some false positives in the H wells, this is likely due to operator error. The average percentage of positive droplets was 12.9 ± 0.9% for both lanes combined. Figure 20 shows the percent of positive droplets compared to the concentration of inhibitor for humic acid.

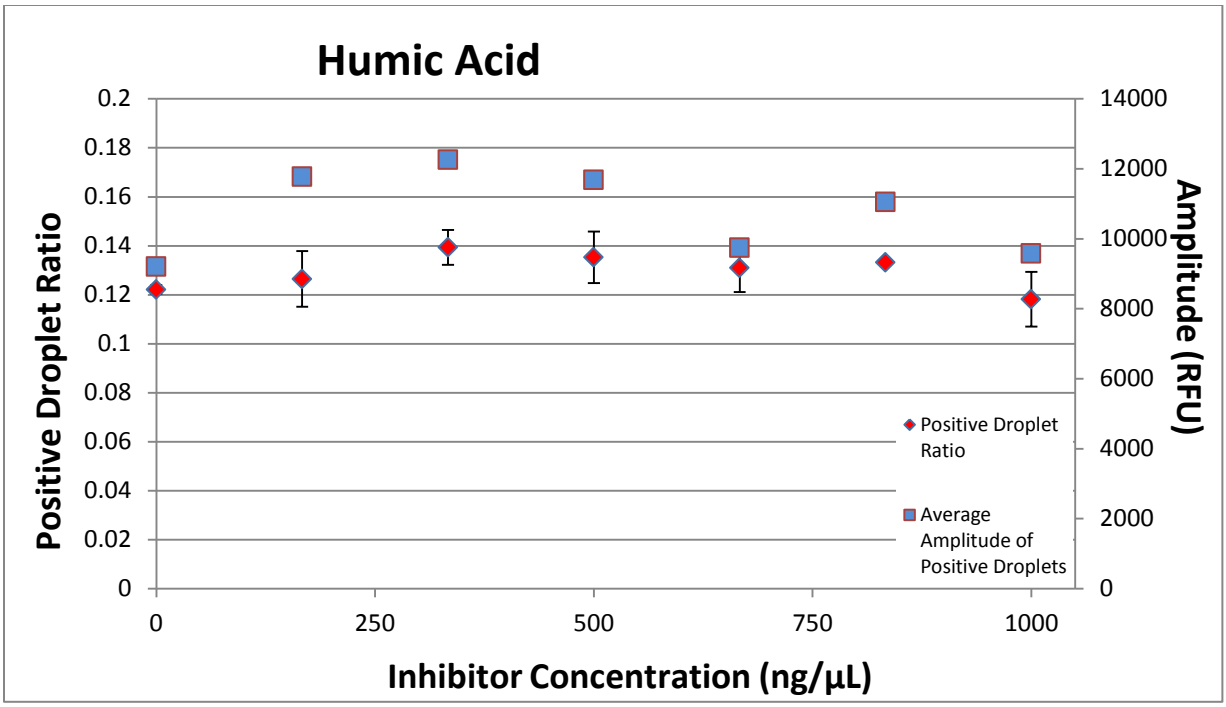

Figure 20: Humic Acid: Positive Droplet Ratio and Average Amplitude of Positive Droplets (RFU, Sox2 and Tert Combined) compared to the concentration of inhibitor input (mM). The red diamonds are the positive droplet ratio and the blue squares are the red squares.

The graph shows that the percentage of positive droplets for humic acid is consistent with any concentration of inhibitor. This is confirmed by Chi-square "Goodness of Fit" at α=0.05, Table 9.

Table 9: Chi-square "Goodness of fit" values by inhibitor concentration for humic acid, α=0.05. H_0- positive droplet ratio of inhibited sample= positive droplet ratio of positive control; H_A- positive droplet ratio of inhibited sample≠ positive droplet ratio of positive control, k=1. Lower values, closer to 0, show more independence.

Inhibitor concentration	Chi-square p-value
1000 ng/µL	0.975
833.33 ng/µL	0.964
666.67 ng/µL	0.966
500 ng/µL	0.953
333.33 ng/µL	0.970
166.67 ng/µL	0.967

Also determined was the number of positive droplets between the negative and positive clouds, this data is in Table 10.

Table 10: Humic acid: Average number of positives and percent positives between clouds.

Humic acid	Average number of positives between clouds	Percent positives between clouds
1000 ng/µL	74	0.757886
837 ng/µL	55	0.872116
666 ng/µL	79.5	0.665049
500 ng/µL	93.5	0.828350
337 ng/µL	93.5	0.906271
166 ng/µL	56	0.474777
Positive Control	41	0.350412

The positive control had the lowest number of positives between the clouds of all of the samples, but none of the samples deviated as far as some did in the iron wells. The highest well has only 0.91% of droplets being this droplets between the wells, compared to 0.35% in the positive control.

Inhibition by Aluminum

Aluminum caused problems with some mechanism in the PCR reaction, Figures 21-22.

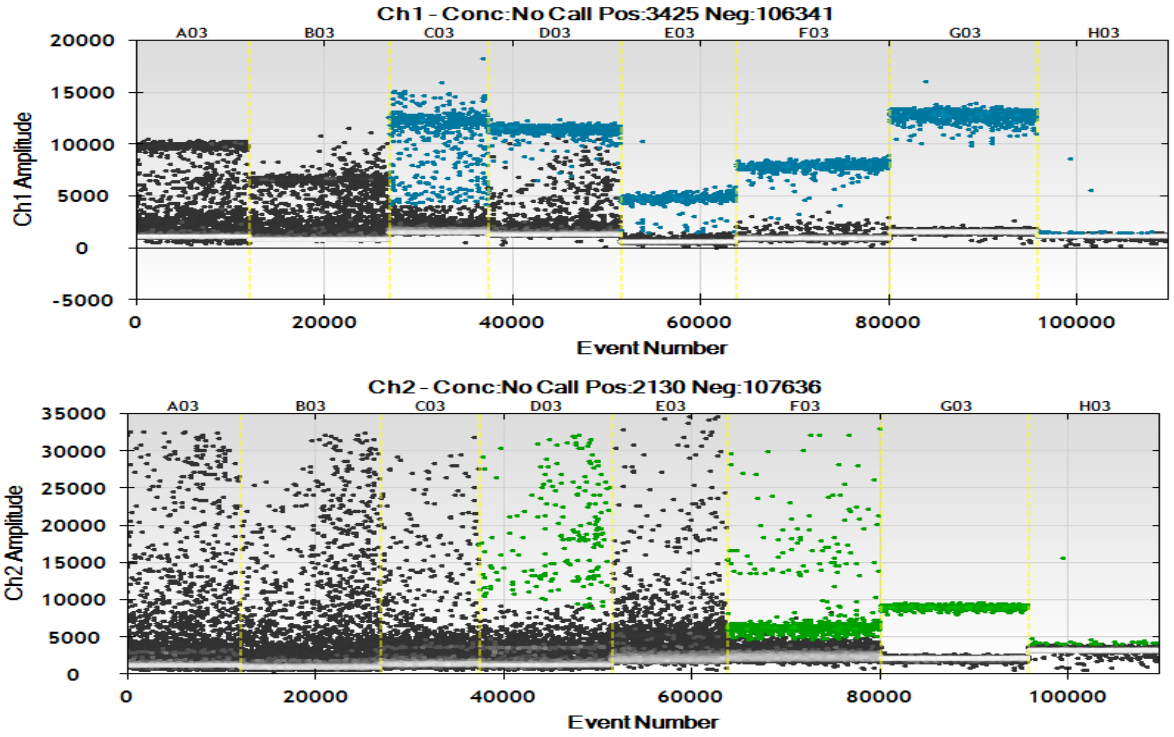

Figure 21: Inhibition Study: Aluminum Lane A. Event number is number of droplets read in total for the lane. Single lane multiplex with *Sox2*, blue positives, top, and *Tert*, green positives on bottom. Wells were: A03- 42.25 mM, B03- 35.21 mM, C03- 28.17 mM, D03- 21.13 mM, E03- 14.08 mM, F03- 7.04 mM, G03- Positive Control, H03- Negative Control.

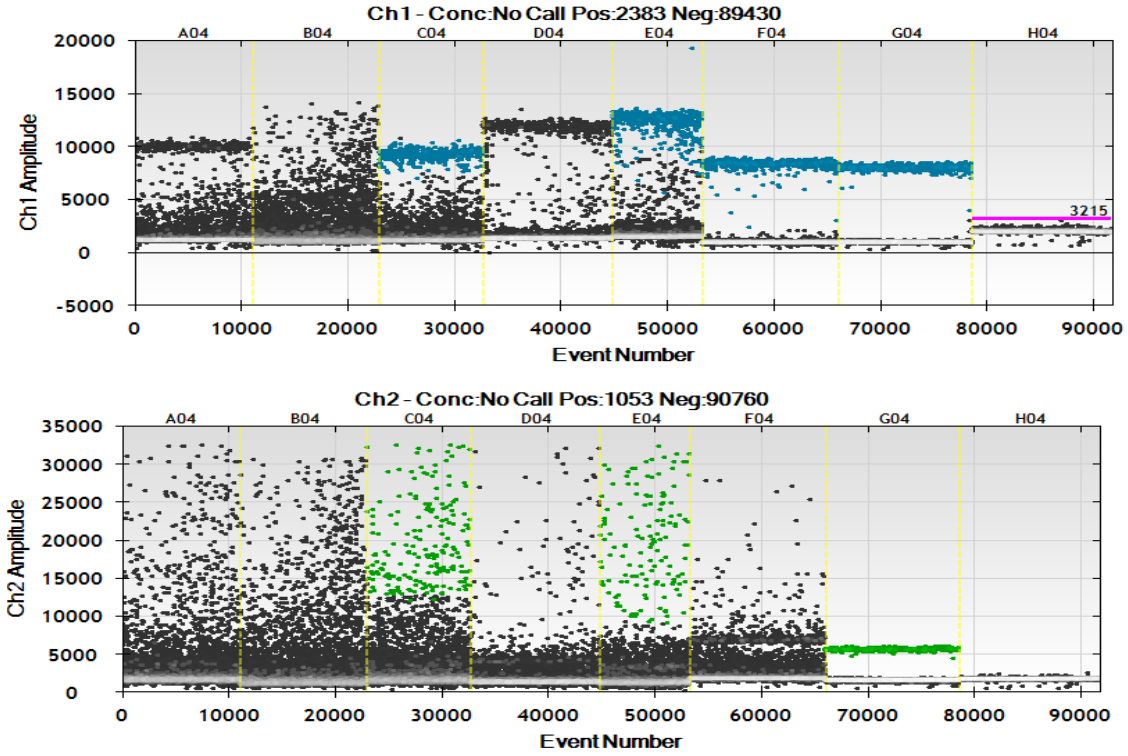

Figure 22: Inhibition Study: Aluminum Lane B. Event number is number of droplets read in total for the lane. Single lane multiplex with *Sox2*, blue positives, top, and *Tert*, green positives on bottom. Wells were: A04- 42.25 mM, B04- 35.21 mM, C04- 28.17 mM, D04- 21.13 mM, E04- 14.08 mM, F04- 7.04 mM, G04- Positive Control, H04- Negative Control.

This problem occurred in all cases for the *Tert* probe, and at higher concentrations in the *Sox2* probe. This effect was unexpected and it was not clearly obvious what caused it so further experiments were needed to determine the cause of this effect.

Mechanism of Inhibition by Aluminum Study

The lane that was amplified showed the same high intensity droplets and showed the same readings in lanes that had no DNA input in them, Figures 23-25.

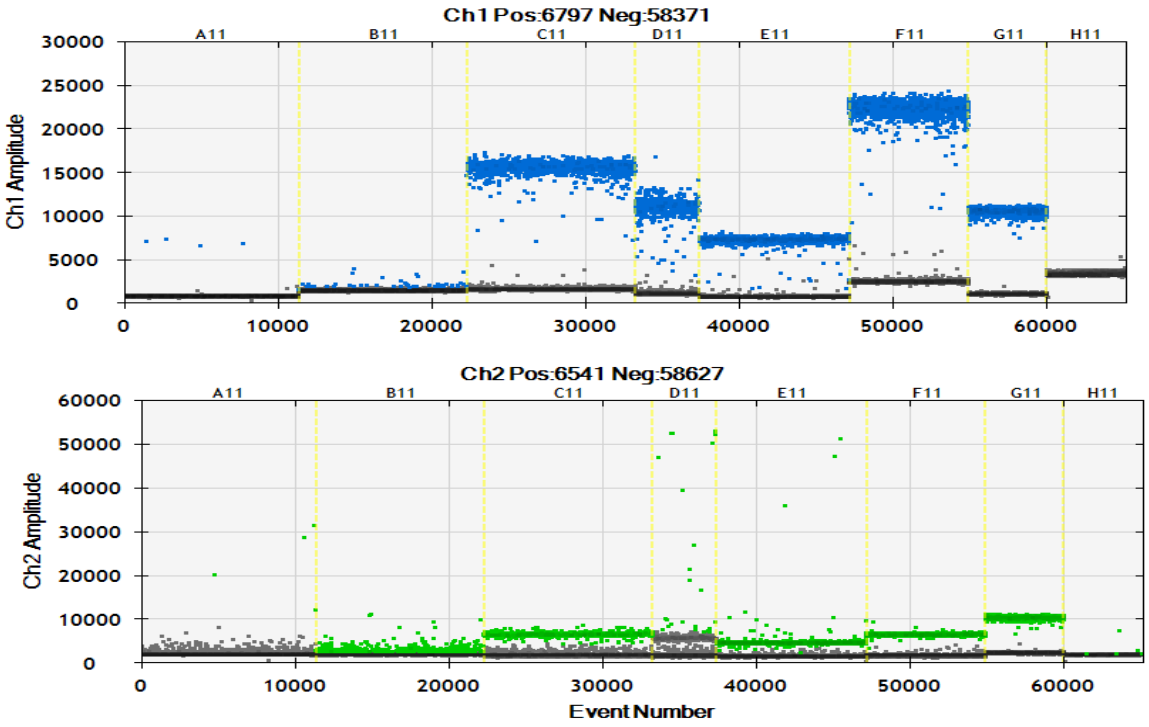

Figure 23: Aluminum Amplified lane at 7.04 mM unscaled. Event number is number of droplets read in total for the lane. Single lane multiplex with *Sox2*, blue positives, top, and *Tert*, green positives on bottom. Wells were: A11- *Tert*+*Sox2*+Al(7.04 mM), B11- *Tert*+*Sox2*+Al(7.04 mM), C11- *Tert*+*Sox2*+Al(7.04 mM)+DNA(5ng/µL), D11- *Tert*+*Sox2*+Al(7.04 mM)+DNA(5ng/µL), E11- *Tert*+*Sox2*+Al(7.04 mM)+DNA(5ng/µL), F11- *Tert*+*Sox2*+Al(7.04 mM)+DNA(5ng/µL), G11- Positive Control, H11- Negative Control.

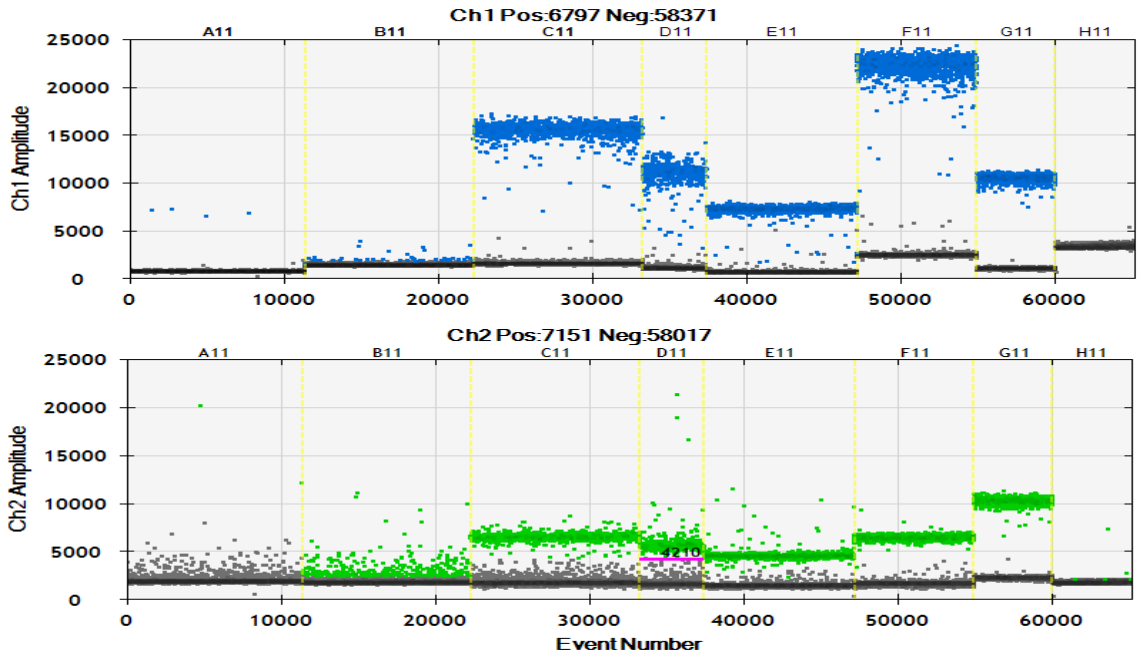

Figure 24: Aluminum Amplified Lane Scaled to 25,000 RFU. Event number is number of droplets read in total for the lane. Single lane multiplex with *Sox2*, blue positives, top, and *Tert*, green positives on bottom. Wells were: A11- *Tert*+*Sox2*+Al(7.04 mM), B11- *Tert*+*Sox2*+Al(7.04 mM), C11- *Tert*+*Sox2*+Al(7.04 mM)+DNA(5ng/µL), D11- *Tert*+*Sox2*+Al(7.04 mM)+DNA(5ng/µL), E11- *Tert*+*Sox2*+Al(7.04 mM)+DNA(5ng/µL), F11- *Tert*+*Sox2*+Al(7.04 mM)+DNA(5ng/µL), G11- Positive Control, H11- Negative Control.

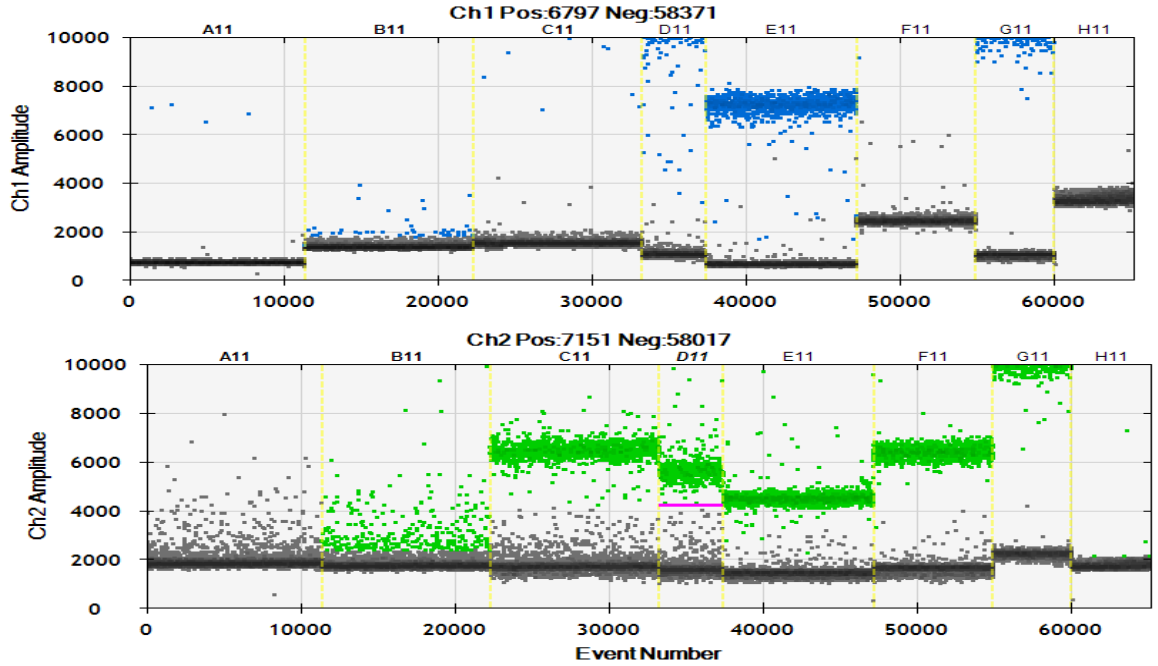

Figure 25: Amplified lane scaled to 10,000 RFU. Event number is number of droplets read in total for the lane. Single lane multiplex with *Sox2*, blue positives, top, and *Tert*, green positives on bottom. Wells were: A11- *Tert*+*Sox2*+Al(7.04 mM), B11- *Tert*+*Sox2*+Al(7.04 mM), C11- *Tert*+*Sox2*+Al(7.04 mM)+DNA(5ng/µL), D11- *Tert*+*Sox2*+Al(7.04 mM)+DNA(5ng/µL), E11- *Tert*+*Sox2*+Al(7.04 mM)+DNA(5ng/µL), F11- *Tert*+*Sox2*+Al(7.04 mM)+DNA(5ng/µL), G11- Positive Control, H11- Negative Control.

This shows that this inhibitory effect could be caused by Al either emitting in the same spectrum as the FAM and HEX, or it could cause displacement of the flourophores causing false positives. The amount and percent of positives between the clouds to determine the full effect that was caused by the inhibition, Table 11.

Table 11: Number and Percent of Droplets between Positive and Negative clouds in the amplified lane.

Aluminum Study- Amplified Lane		
Positives in wells with no DNA		Percent positives in wells without DNA
A	236	2.061
B	330	3.028
Number of positives between positive and negative cloud		Percentage of droplets between positive and negative clouds compared to total (%)
C	249	2.266
D	82	1.996
E	64	0.652
F	35	0.453
Positive control (G)	7	0.137

The percentage of droplets was much greater in the wells that had the inhibitor in them, up to 3%, showing that this may be the best way to determine inhibition. However, this process does take an extra amount of time, but would only need to be done when inhibition is suspected.

The same effect, Figures 26-28, occurred even when the droplets were not amplified, but to a lesser extent.

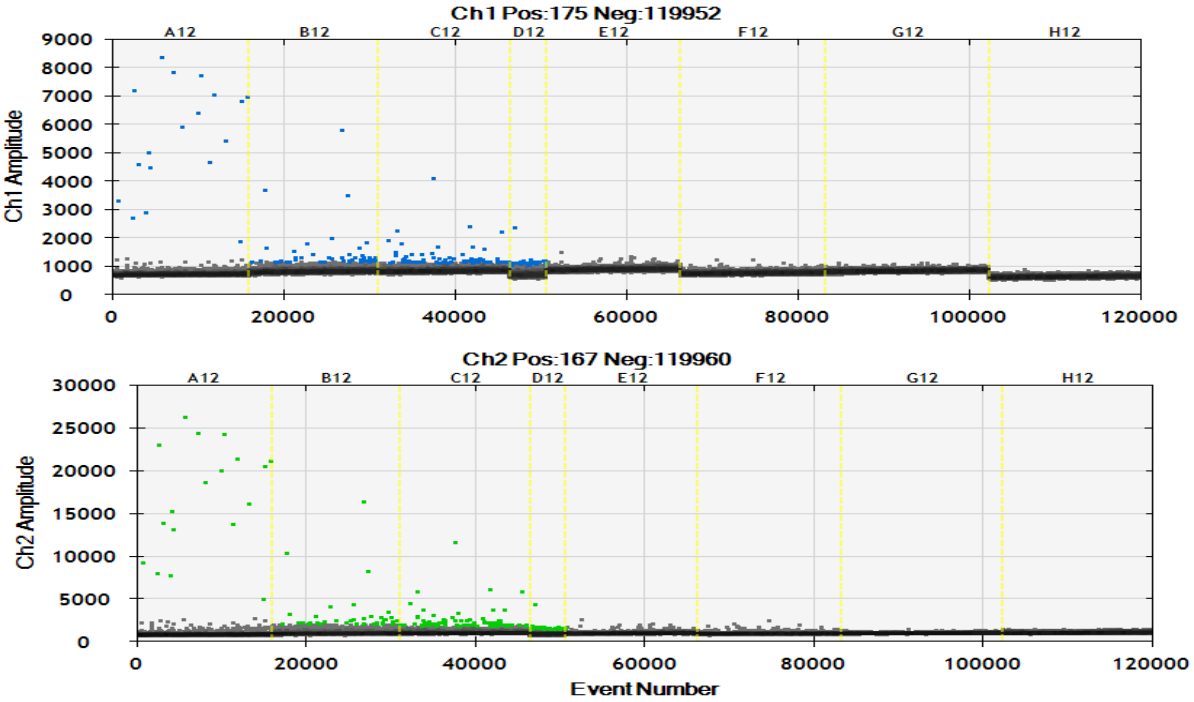

Figure 26: Aluminum Non-amplified Lane Unscaled. Event number is number of droplets read in total for the lane. Single lane multiplex with *Sox2*, blue positives, top, and *Tert*, green positives on bottom. Well number is above the droplet readings. Wells were: A12- *Tert*+*Sox2*+Al(7.04 mM), B12- *Tert*+*Sox2*+Al(7.04 mM), C12- *Tert*+*Sox2*+Al(7.04 mM), D12- *Tert*+*Sox2*+Al(7.04 mM)+DNA(5ng/µL), E12- *Tert*+*Sox2*+Al(7.04 mM)+DNA(5ng/µL), F12- *Tert*+*Sox2*+Al(7.04 mM)+DNA(5ng/µL), G12- Positive Control, H12- Negative Control.

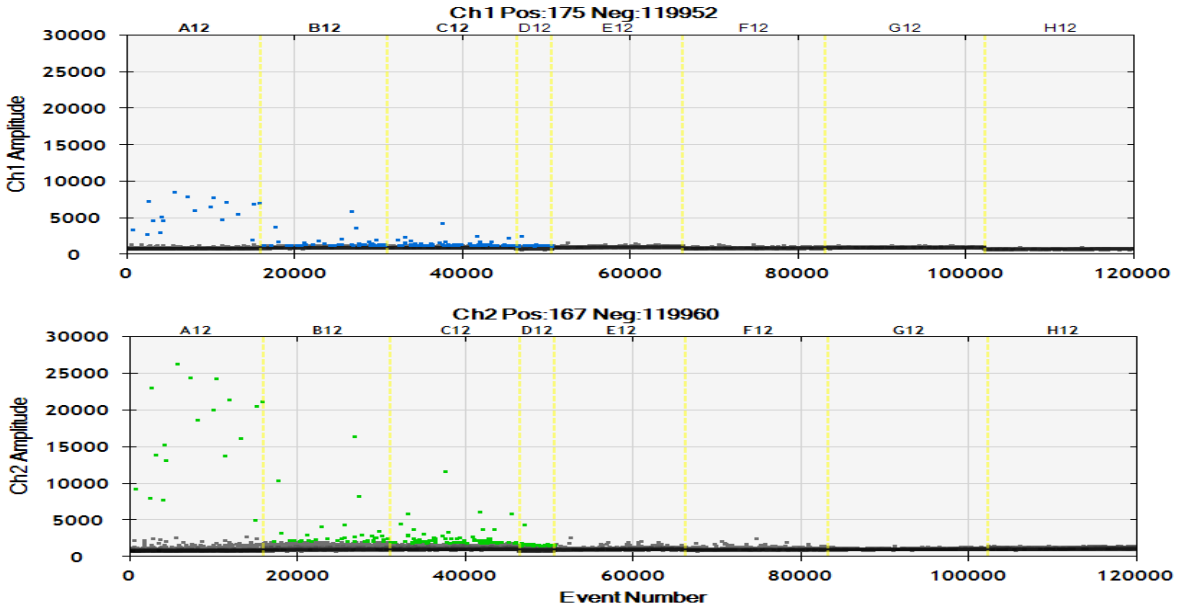

Figure 27: Aluminum Non-amplified Lane Scaled to 30,000. Event number is number of droplets read in total for the lane. Single lane multiplex with *Sox2*, blue positives, top, and *Tert*, green positives on bottom. Well number is above the droplet readings. Wells were: A12- *Tert*+*Sox2*+Al(7.04 mM), B12- *Tert*+*Sox2*+Al(7.04 mM), C12- *Tert*+*Sox2*+Al(7.04 mM), D12- *Tert*+*Sox2*+Al(7.04 mM)+DNA(5ng/µL), E12- *Tert*+*Sox2*+Al(7.04 mM)+DNA(5ng/µL), F12- *Tert*+*Sox2*+Al(7.04 mM)+DNA(5ng/µL), G12- Positive Control, H12- Negative Control.

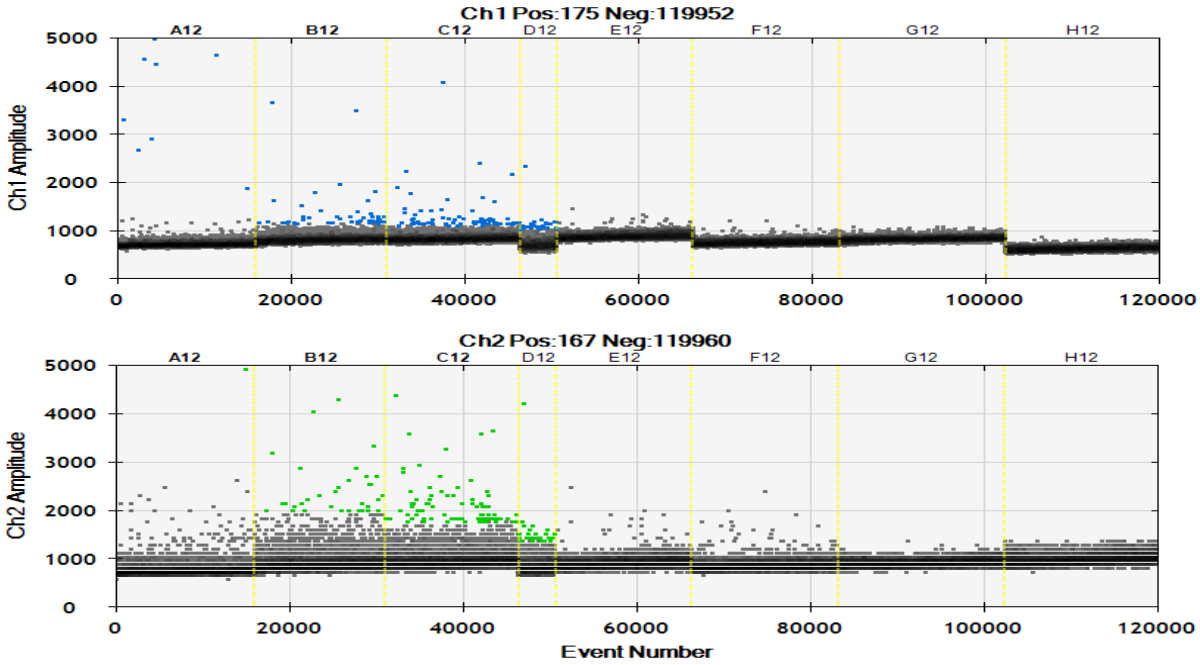

Figure 28: Aluminum Non-amplified lane scaled to 5,000 RFU. Event number is number of droplets read in total for the lane. Single lane multiplex with *Sox2*, blue positives, top, and *Tert*, green positives on bottom. Well number is above the droplet readings. Wells were: A12- *Tert*+*Sox2*+Al(7.04 mM), B12- *Tert*+*Sox2*+Al(7.04 mM), C12- *Tert*+*Sox2*+Al(7.04 mM), D12- *Tert*+*Sox2*+Al(7.04 mM)+DNA(5ng/µL), E12- *Tert*+*Sox2*+Al(7.04 mM)+DNA(5ng/µL), F12- *Tert*+*Sox2*+Al(7.04 mM)+DNA(5ng/µL), G12- Positive Control, H12- Negative Control.

The positive and negative control were blank and had no positive droplets. However, there were positive droplets in the other wells. Though the amount of positives is not large, 100 droplets or less in all cases, the fact that there are any in the wells show that the positive droplet calls were caused by aluminum.

CHAPTER FOUR

DISCUSSION AND CONCLUSIONS

The input amount of DNA, 5 ng/µL, was used because it was close to forensically relevant levels and the efficiency of the reaction was not changed based on DNA input. Also, there was concern because the mechanism for how ddPCR dealt with inhibition was not known. It was not certain if inhibition in ddPCR would make some droplets negative, lower the amplitude, or something completely different. 5 ng/µL has roughly 1500 droplets appear positive out of a possible 20,000, enough to allow inhibition to show if we lost a percentage of droplets; whereas, 1 ng/µL only has roughly 300 droplets. However, there was concern if 30 out of 300 droplets were lost due to inhibition or random error; if there is a significant loss from 1500 droplets, it was very likely to be due to inhibition.

Humic acid did not show a significant change in results for ddPCR: not causing a difference in positive droplet percentage with increased inhibition, unlike qPCR where it has been shown to raise C_q values. Humic acid did cause a small increase in droplets between the positive and negative clouds, but not as high of a percentage as the metals did. When higher concentrations of humic acid are added, enhancements are needed to improve qPCR performance in older qPCR kits.[1]

Iron showed some inhibition of ddPCR, but did not greatly affect results until 35 mM input, causing a small change in percentage of positive droplets with increased inhibition. Iron caused more droplets to be between the positive and negative clouds, up to 3.7% of droplets. For qPCR, iron shows increases in IPC C_q, even at the lowest levels of inhibition, and lowers the DNA concentration shown the PCR reaction by binding to the DNA molecules. In Figures 29-30, Iron is represented by red.

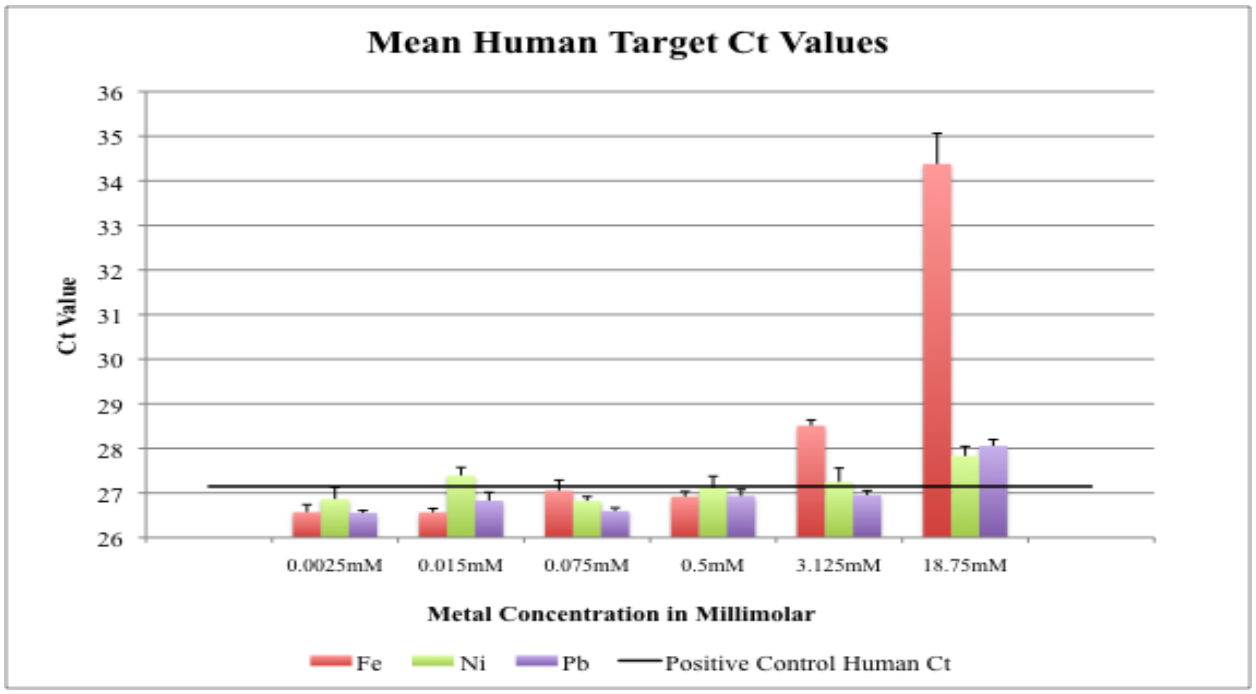

Figure 29: Mean Human Target C_q Values for Quantiplex. Concentration, mM, of metal is compared to the C_q. Red is Iron, the metal of interest for this paper. The solid line is the mean C_q for the controls.[15]

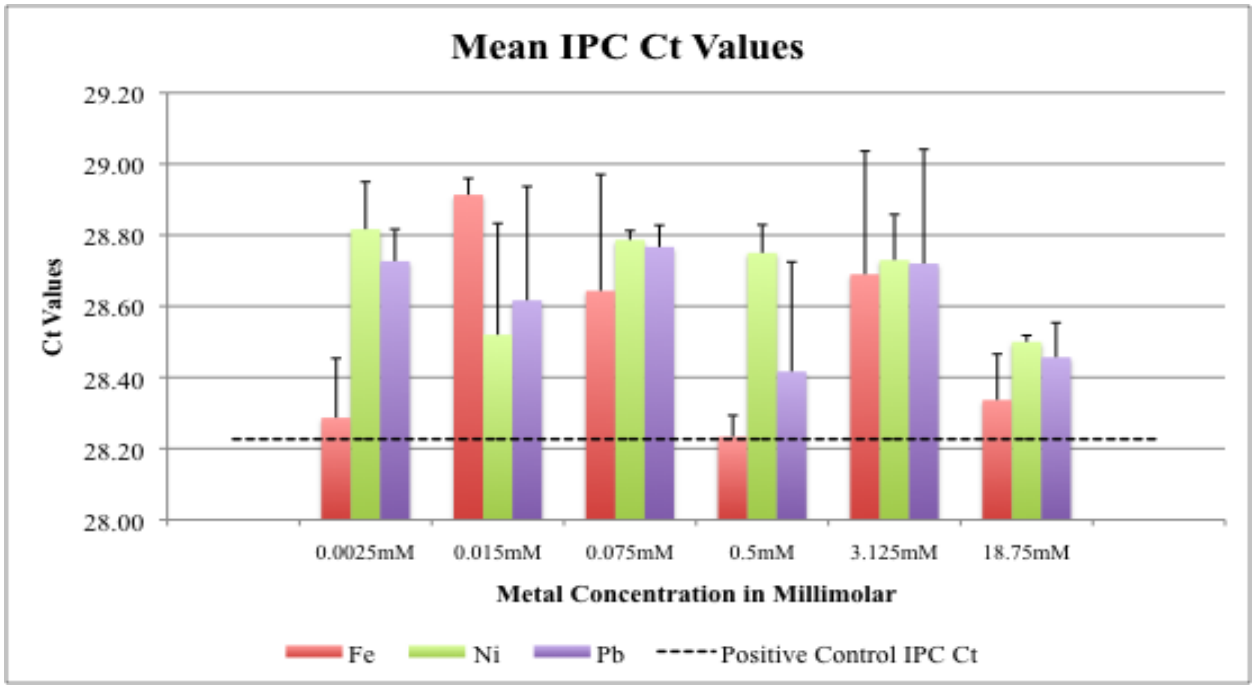

Figure 30: Mean IPC C_q Values for Quantiplex. Concentration, mM, of metal is compared to the C_q. Red, Fe, is the metal of interest for this paper. The dashed line is the mean C_q for the controls.[15]

Iron does not change the Human probe as quickly as the IPC, but the drastic change in IPC C_q at the smallest amount of inhibition can show that there are inhibitors in the well. Iron can also lower the quantification of DNA in the sample for qPCR, Figure 31.

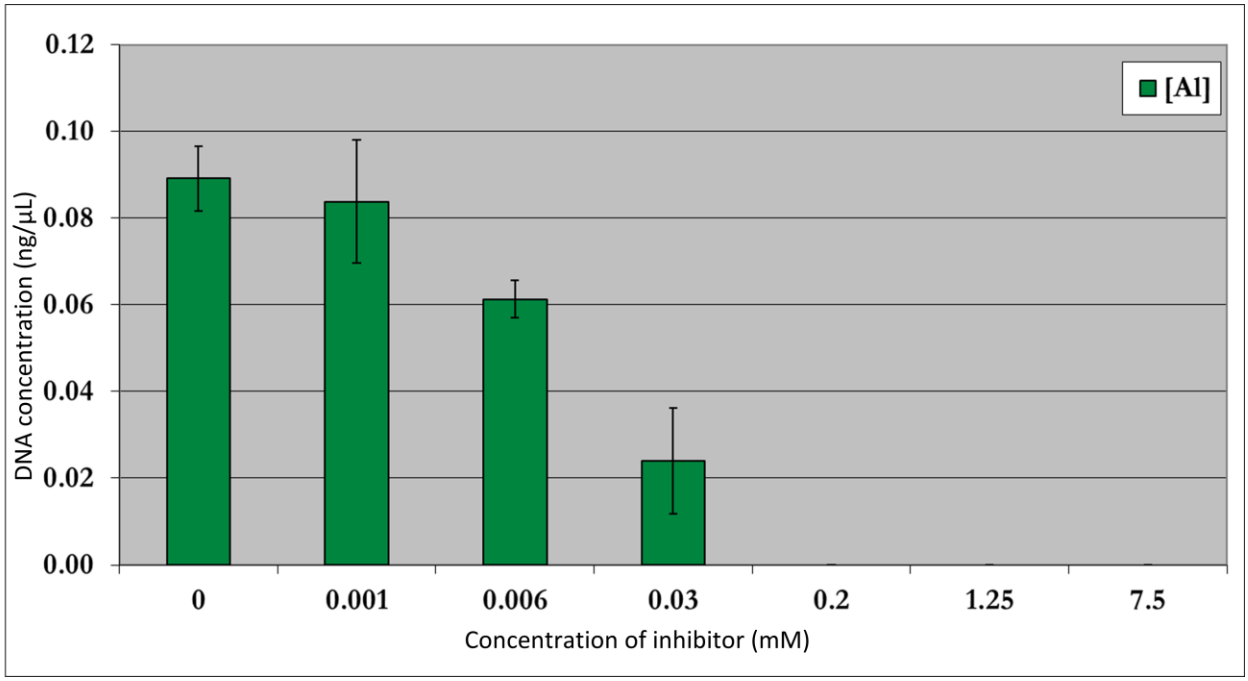

Figure 31: Iron: DNA concentration, ng/µL, based on the concentration of inhibitor, mM, added to the PCR reaction.[13]

Iron can lower the DNA concentration to 0 in qPCR, it never had as strong of an effect on ddPCR. Overall, iron affects qPCR more than ddPCR. Copper showed a decrease in amplitude correlating with the amount of copper added for ddPCR. qPCR is affected more strongly when looking at the IPC. Copper raises the IPC with very little inhibitor added and increases as inhibitor concentration increases. With low concentration of inhibitor using ddPCR, copper just lowers amplitude and can get a close positive droplet ratio to the control. In previous studies using older kits, copper was reported to result in random estimated DNA concentration for Quantifiler, including causing a large problem in the well with the most inhibition causing the DNA concentration to go off scale, Figure 32-33.

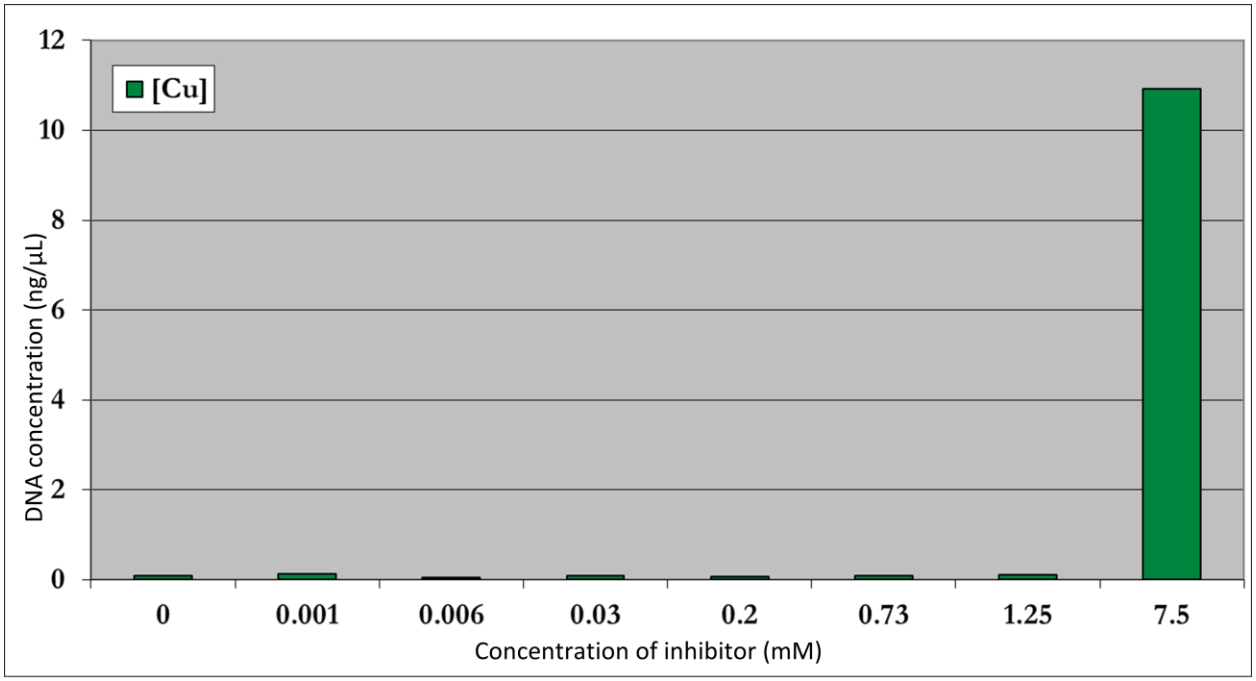

Figure 32: Copper: DNA concentration, ng/µL, based on the concentration of inhibitor, mM, added to the PCR reaction. Unzoomed to show problems with the highest concentration well.[13]

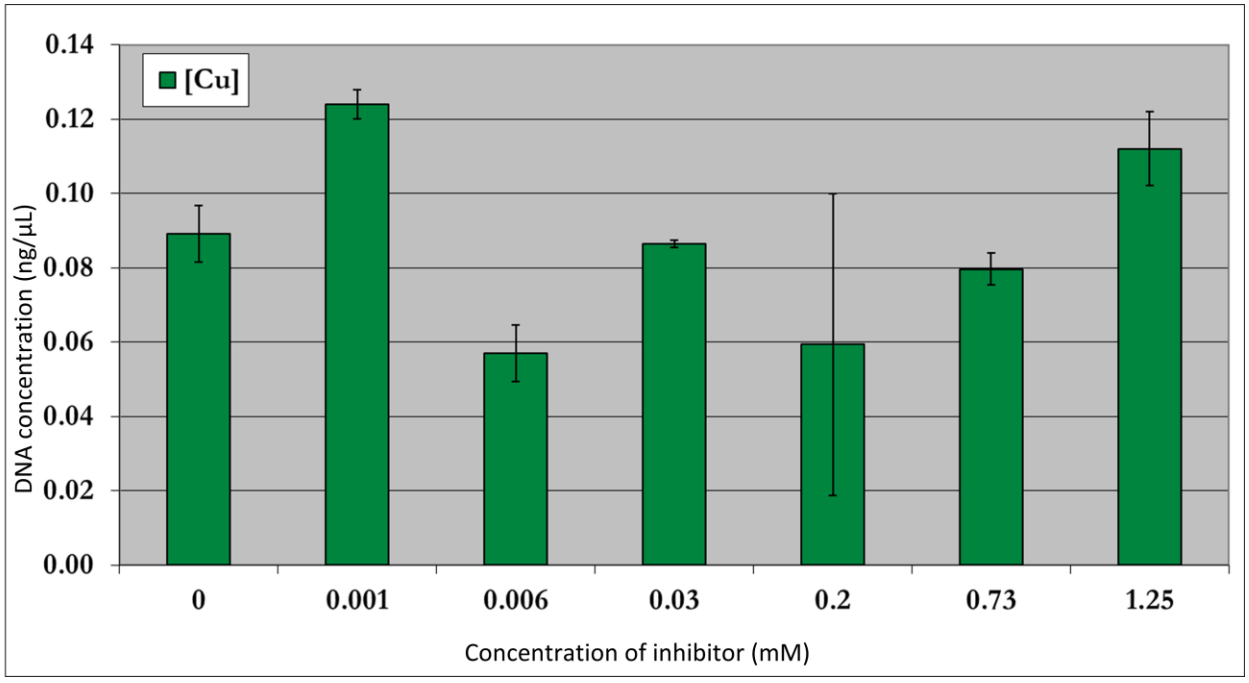

Figure 33: Copper: DNA concentration, ng/µL, based on the concentration of inhibitor, mM, added to the PCR reaction. Zoomed in to show the lower inhibitor concentrations.[13]

Copper causes a large issue with the Quantifiler kit, causing random DNA concentrations, but still giving results that were mostly near the positive control other than the highest concentration of inhibitor which called a large DNA concentration. With Quantiplex, Cu, purple, generally raised the IPC and Human Target C_q values, but the C_q lowered for the highest inhibition for the IPC, Figure 34-35.

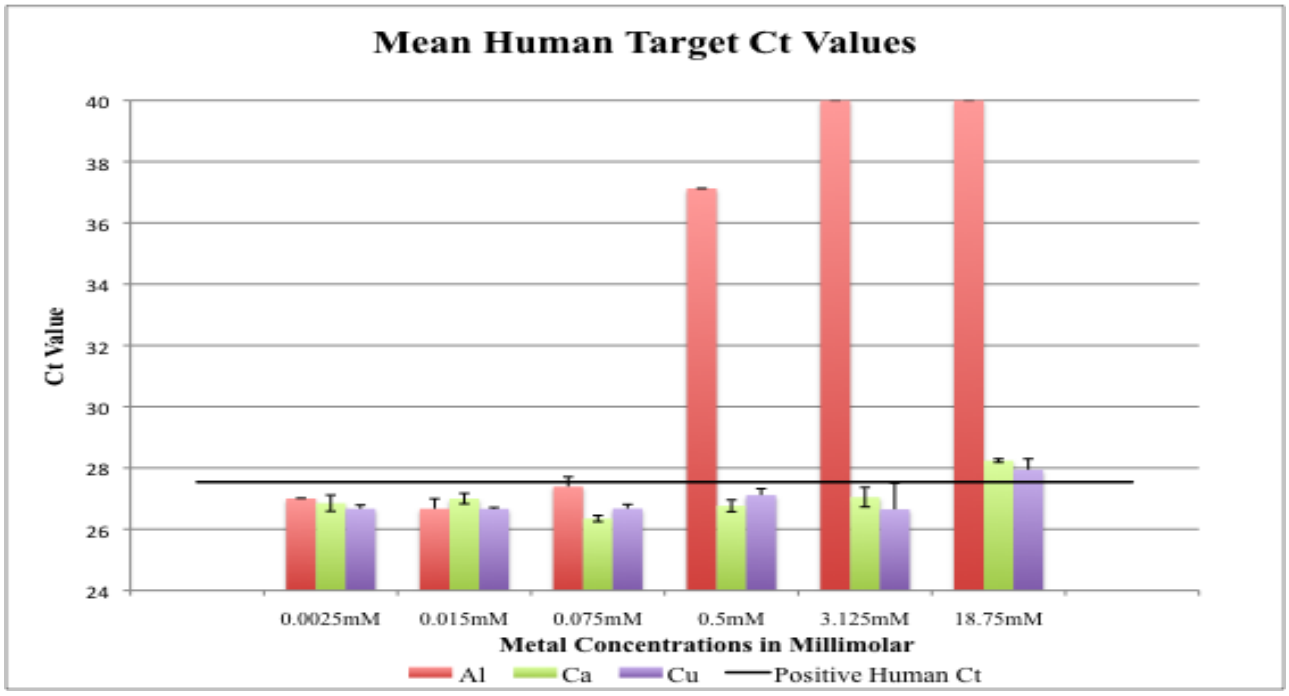

Figure 34: Mean Human Target C_q Values for Quantiplex. Concentration, mM, of metal is compared to the C_q. Red, Al, and Purple, Cu, are of interest for this paper. The solid line is the mean C_q for the controls.[15]

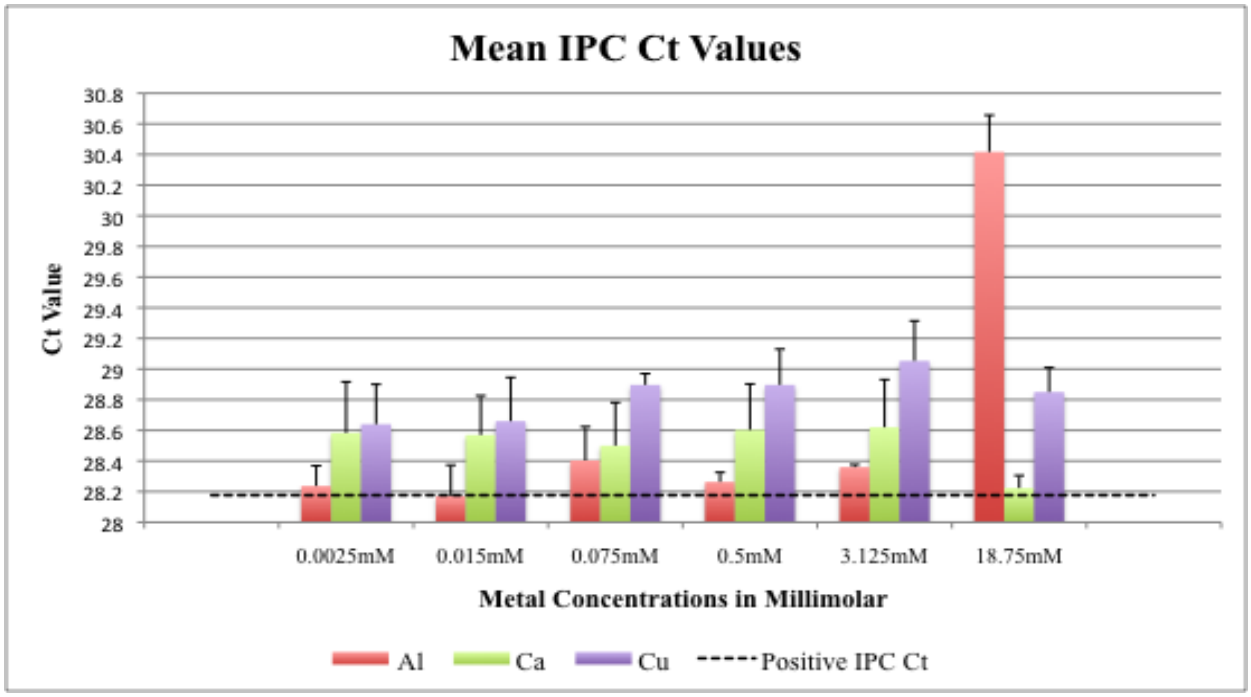

Figure 35: Mean IPC C_q Values for Quantiplex. Concentration, mM, of metal is compared to the C_q. Red, Al, and Purple, Cu, are of interest for this paper. The dashed line is the mean C_q for the controls.[15]

Copper showed the strongest overall effect on the IPC C_q raising it well above the positive control in each well. Copper did not increase the Human C_q as strongly as the IPC. Aluminum has a strong effect on ddPCR, making results unusable. Even at the lowest concentrations of inhibitor used, aluminum causes droplet rain and lowered amplitude. With any inhibition, Al can cause droplets that have high amplitudes, 20,000 RFUs plus, that are not seen otherwise. Aluminum can also cause positive droplets in wells that were not amplified, up to 100 positive droplets. In wells that were amplified, aluminum caused up to 2% of droplets to be between the positive and negative wells and causing up to 3% of droplets to be positive in wells with no DNA in them. Aluminum was also found to be the strongest inhibitor of qPCR, red in Figures 34-35, increasing both human and IPC C_q also increasing the Human target to 40 cycles causing a null quantification. With strong inhibition in qPCR, aluminum will even have an estimated DNA concentration of 0, Figure 36.

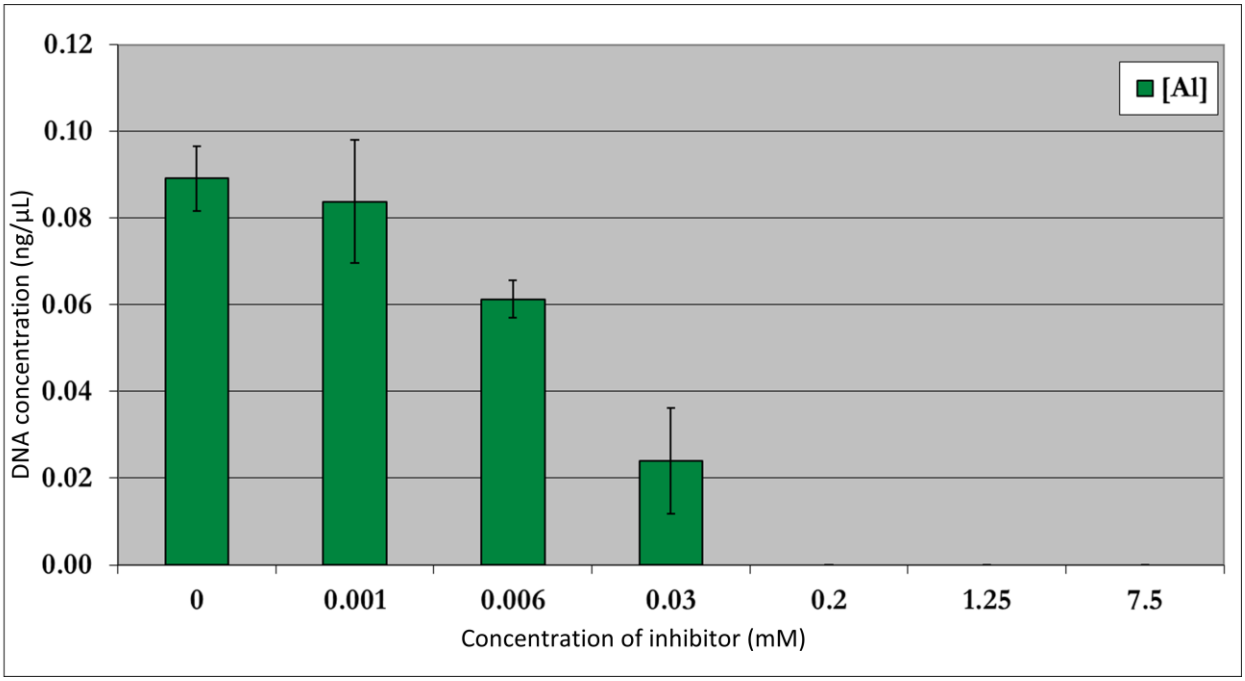

Figure 36: Aluminum: DNA concentration, ng/µL, based on the concentration of inhibitor, mM, added to the PCR reaction.[13]

In both ddPCR and qPCR, this is problematic, because aluminum is one of the most abundant metals in bone samples, and has been reported to have the strongest inhibitory effect.[13,15]

The most interesting result from copper is that it lowered the amplitude of the droplets, but, at low inhibitor concentrations, did not change the efficiency of the reaction as the quantification of DNA was the roughly equivalent to the positive control. This shows a good strength for ddPCR, in some cases it can accurately report the quantity of DNA through inhibition.

Copper and iron, although both metals, showed different effects and binding affinities in the ddPCR reaction mixture. Copper lowered both probes equally showing that it binds equally strongly to both probes. However, iron showed a stronger binding affinity to *Tert* causing droplet rain at much lower concentrations than with *Sox2* meaning that it could bind to the different DNA sequences in the probes with different affinity. Also, iron caused a drop in amplitude in *Sox2* in multiple wells that did not occur with *Tert*. This shows iron can lower the amplitude in some cases with extreme inhibition, but not all

cases. Overall, iron does not cause a change efficiency as most wells showed close to the same quantification of DNA.

In ddPCR reactions, aluminum creates an effect that causes varying amplitudes for droplets, and completely changes how the *Tert* probe interacts with the droplet reader. At high concentrations the data generated cannot be used, and even at low concentrations the data is suspect. Without DNA in the well and no amplification, aluminum still caused many positive droplets of random amplitudes and, when amplified, the wells with DNA showed the same effect. Aluminum even caused positive droplets to be read when the well was not amplified, causing amplitudes in *Tert* that were well above normal for positive droplets. This droplet effect was lowered in the non-amplified lane with wells with DNA. This means aluminum was causing issues with either the probes and primers or the reading instrument itself. Aluminum in ddPCR reactions could interact with the primers, allowing them to release the flourophores without needing the DNA strand to replicate. Aluminum could also simply emit at the same wavelength as the flourophores which is why it can be detected. Either of these answers could be likely, and would need more testing to determine the root cause of the problem.

From what was determined by this study, ddPCR can be a good platform for quantification of DNA for forensic purposes. It does not change efficiency based on the concentration of DNA added, which is very important when an unknown amount of DNA is in a sample. Multiple probes can be multiplexed which is important for determining if there is male DNA in the sample. ddPCR has ways to not have inhibition show the full effect. Inhibition primarily shows by a lowering of amplitude of positive droplets in the reaction even to the baseline negative, but also creating a higher percentage of droplets that are between the positive and negative clouds. However, ddPCR currently has a major problem with aluminum, which would cause any aluminum not cleaned from the DNA purification step to cause a large problem with DNA quantification. Overall, ddPCR is a solid platform that could become very strong for forensic uses after the problem with aluminum is resolved.

In summary, compared with previous studies performed with qPCR, at comparable concentrations, both humic acid and iron showed little affect on ddPCR function. Iron affects qPCR results more strongly than humic acid, especially with strong inhibition with iron. Although, humic acid treated samples do not affect PCR processes greatly with newer systems and kits that have protection against inhibition. Copper is a strong inhibitor for PCR, but affects ddPCR more strongly than qPCR with the data found in this study. Copper in previous studies never caused a null DNA concentration estimation where it causes the amplitude for droplets to lower to the baseline in ddPCR. Aluminum is the strongest inhibitor of PCR tested, and harms qPCR performance greatly. However, in any concentration Al can make ddPCR readings unusable, even without amplification. This is important because aluminum is one of the most common metals in bone samples and could affect forensic casework.[13,15]

Copper lowers the amplitude of inhibited samples and this could be a good way to determine inhibition before continuing with further processes. Aluminum needs to be studied more in conjunction with ddPCR to determine the best course of action with an aluminum inhibited sample. Lastly, these experiments need to be tested at lower DNA concentrations to determine how lower concentrations of DNA are affected by inhibitors with ddPCR.

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