Developing a non-isotopic assay for detecting interstrand crosslinks in plasmid-sized duplex DNA

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Interstrand crosslinks (ICLs) consist of covalent links between the two antiparallel strands of double-stranded DNA. These links between two sites on opposing DNA strands prevent their separation and therefore replication and transcription of the DNA molecule (Figure 1). When the cell encounters this malfunction, it initiates cellular processes that lead to apoptosis, otherwise known as programmed cell death. DNA ICL lesions form when a compound known as a crosslinking agent reacts with two DNA nucleotides positioned on opposite DNA strands. As such, DNA ICL agents provide a mode chemotherapeutic treatment for cancer, due to ICL lesion’s ability to trigger cell death in cancer cells.

**Figure 1.** Double-stranded DNA with an interstrand crosslink (Left, Rajski *et al.* 1998). Mitomycin C links two guanine residues with a bisalkylation, otherwise known as an interstrand crosslink (Right, Tomasz *et al.* 1987). Interstrand crosslinks prevent replication and transcription, causing cell death (Noll *et al.* 2014).

Importantly, research has shown the presence of DNA sequence specificity by crosslinking agents (~2-3 base pairs). DNA interstrand crosslinking by Mitomycin C is sequence specific,
only occurring between the two exocyclic amines of a CG sequence (5’-3’). The distance between the two reactive sites of a crosslinking agent (3.4 Å) is compatible with the distance between the two exocyclic amines of neighboring guanine residues (3.1 Å) in a CG sequence (5’-3’) (Figure 2). Crosslinking specificity also discriminates by sequence directionality. Slight differences in distance between exocyclic amines in guanine bases of a CG sequence (5’-3’) compared to a GC sequence (5’-3’) prevent interstrand crosslinks from occurring in the latter, despite the sequence containing the same nucleotides. This sequence-targeted property could be expanded and potentially lead to specific crosslinking within a defined genomic location, potentially allowing for increased specificity with formation of these toxic lesions and prevention unwanted side-effects.

![Figure 2](image.png)

**Figure 2.** Depiction of compatible distance between carbamate and aziridine reactive sites on a general mitosene crosslinking agent and the exocyclic amino groups of antiparallel guanine residues (Bass *et al.* 2013).

To evaluate ICL formation in duplex DNA, a gel shift assay is used to assess the differential mobility of DNA with and without interstrand crosslinks. In an ICL gel shift assay, a double stranded DNA sample is incubated with a crosslinking agent. Subsequently, that entire DNA
sample is denatured using heat and/or a chemical denaturant to overcome the intermolecular forces which hold the two strands of a double stranded DNA molecule and convert it to single strand. Molecules of DNA containing an interstrand crosslink will be unable to denature due to the physical constraints of the ICL, whereas DNA that does not contain an ICL will be denature to single strand. Once the sample is loaded on a gel, the different migration distances of single and double stranded DNA are used to identify ICLs. In a gel-based assay, the single stranded DNA will move quickly and migrate further through the gel, leaving the crosslinked double stranded DNA higher up on the gel. By using gel electrophoresis to separate molecules based on size, we can exploit the different hydrodynamic profiles of double stranded and single stranded DNA to evaluate ICL formation and therefore the ICL activity of cross-linking agents (Figure 3).

**Figure 3.** Depiction of a gel shift assay used to evaluate interstrand crosslinking activity. In the first lane is only single-stranded DNA, which is compared to the second lane which contains a DNA sample that has been incubated with a crosslinking agent and subsequently denatured. The second lane contains interstrand crosslinked DNA, monoalkylations (MA) in single-stranded DNA, and single-stranded DNA.
A subset of interstrand crosslinking agents are known as bisalkylating agents or bifunctional alkylating agents. The ICL-forming compounds contain two independently reactive groups which react with each DNA strand to form persistent carbon-carbon bonds between the two strands. One such agent is mitomycin C (MMC), a aziridine and carbamate containing natural product with antitumor and antibiotic activity derived from the bacteria *S. caespitosus*, which is known to form ICLs after it is activated by a reducing agent (Figure 4).\(^3,4,7\)

**Figure 4.** Mitomycin C (Left), an aziridine and carbamate containing natural product derived from the bacteria *Streptomyces caespitosus*. It has antibiotic and antitumor activity, and is a common chemotherapy treatment due to its bifunctional alkylating (interstrand crosslinking) ability. Leucoaziridinomitose (Right) is a putative intermediate of the MMC ICL mechanism, which occurs after the reductive activation of MMC.

MMC is commonly used for stomach or pancreas adenocarcinoma but has also been used as a chemotherapy treatment for bladder, breast, colorectal, head and neck, and non-small cell lung cancer.\(^7\) After reductive activation, MMC forms a putative aziridinomitose species as an intermediate, called a leucoaziridinomitose (Figure 4). The leucoaziridinomitose serves as a bifunctional electrophile, which is then attacked twice by the nucleophilic DNA, first to create a monoalkylation and then a second time to form an interstrand crosslink (Appendix 1).\(^4,9\) This
creates persistent covalent bonds between the two DNA strands. Understanding the mechanistic aspects of DNA interstrand crosslinking by these compounds is an active area of research, because these DNA lesions are known cytotoxic DNA lesions, believed to cause cell death by preventing DNA replication.5

![Chemical Structure](image)

**Figure 5.** Chemical structure of (1S, 2S)-6-desmethyl-(methyaziridino)mitosene, also known as UMMS (Rink *et al.* 2005).

A collection of modified aziridinomitosene compounds similar to the putative leucoaziridinomitosene intermediate of the MMC ICL mechanism have been synthesized for assessment of ICL activity and to probe the mechanistic aspects of the crosslinking activity.6 UMMS is one of these novel aziridinomitosene compounds and has been shown to form ICLs in synthetic duplex oligonucleotides at rates similar to MMC (~1-2%) (Figure 5).4 Additionally, this particular aziridinomitosene was shown to exhibit the same sequence specificity as MMC, forming ICLs only between the exocyclic amines of guanines in a CG sequence (5’-3’) (Figure 6).

The ultimate goal of this research is to test the ICL activity of UMMS with ~100x-larger duplex DNA molecules through the development of a non-isotopic gel-based assay. If ICL concentration in DNA reacted with UMMS measured by gel-shift assay is high enough, it lends this class of molecules the potential for use as treatment of certain cancers including non-small
cell lung cancer. Additional analogues of UMMS will also be tested to elucidate the mechanism of interstrand crosslinking.

**Figure 6.** D-PAGE Gel demonstrating that the sequence specificity of UMMS as a bisalkylating agent is analogous to MMC in the formation of interstrand crosslinks in small synthetic oligonucleotides. As shown in the gel shift assay above, crosslinks formed by UMMS only occur in a CG sequence (5’-3’) regardless of the surrounding sequence of nucleotides (Rink et al. 2005).

Many methods exist for DNA quantification, including UV/vis spectrophotometry, intercalating dyes (SYBR, ethidium bromide), radiolabeling, emulsion PCR (ddPCR), and 5’ hydrolysis probes paired with real-time quantitative PCR (rt-qPCR). All of these methods can reliably quantify DNA with variable levels of sensitivity. However, radiolabeling and intercalating dyes can quantify a mixed sample containing single and double stranded DNA. These two methods involve different modes of interaction of the signaling species with each DNA molecule. A radiolabel is covalently tethered whereas a molecular dye is non-covalently associated. There are
two methods for radiolabeling, enzymatic and chemical. For the enzymatic method, DNA precursors tagged with an isotope, typically a $^{32}$P-dNTP, are incorporated during the production of stock DNA samples. The $^{32}$P-labeled DNA is run on a gel, and the dried gel is then imaged using phosphorimagery to obtain a digital gel image and quantitative measures of DNA. This method is specific, sensitive, and can reliably quantify single and double strand, but requires the use of radioactive materials and specialized equipment.

An extensive literature search did not reveal evidence for the use of a gel-based assay for quantification of both ss and ds DNA species, which does not require the use of radioactive materials. Research by UPS undergraduates Leah Stromberg and Sabrina Close has shown that SYBR Gold stain can detect both single- and double-strand DNA with a sensitivity ~1000 times greater than the current standard, ethidium bromide. The use of SYBR Gold to quantify the ratio of single- and double-stranded DNA as needed for a viable ICL assay remains to be established. The first goal of this project was to establish a quantifiable relationship between single- and double-stranded signal intensities of DNA stained with SYBR Gold, that could be used to determine a reliable concentration for the two DNA forms.

A reliable gel-based quantification assay that does not require radioisotopes and also allows detection of low yield DNA species would be the first of its kind reported. In addition, such a protocol would be invaluable to labs using undergraduates or without access to radioactive materials. Eventually this assay will be used to establish the relative bisalkylation reactivity of the novel aziridinomitosene, UMMS, toward duplex DNA by measuring the quantity of interstrand crosslinks (ICLs) in UMMS-treated plasmid DNA samples.

In this work, SYBR Gold stained DNA will be purified on a 1% agarose gel via gel electrophoresis, then visualized and quantified using Bio-Rad’s Image Lab Software ($\lambda = 325$ nm),
followed by retrieval with glass-wool elution and orthogonal quantification via UV/vis spectrophotometry. A response curve relating the ds DNA signal intensity to ss DNA signal intensity will be generated from the gel images of SYBR Gold labeled DNA. This regressions can then be used to elucidate the ICL activities of novel aziridinomitosenes.

**Goals.**

DNA ICL activity has been previously reported for aziridinomitosene derivatives in synthetic double-stranded oligonucleotides (14 bp). The goal of this project is to ICL formation in plasmid-sized DNA. The larger nucleic acid substrate will afford increased assay sensitivity of the assay. The plasmid pUC19 (~2600 bp) represents a ~100x increase in DNA size and is commercially available. The key accomplishment of this project was the development of a purification and isolation protocol which produces a double-stranded, linear, uniform DNA substrate free from contamination that can be reproducibly transformed from its ds state to 100% fully denatured single strand.

**Results and Discussion.**

Plasmid DNA exists in multiple isoforms of DNA. In a gel shift assay identifying ICLs, two bands are expected, a lower band for single-stranded DNA and an upper band for double stranded crosslinked DNA. However, circular plasmid DNA samples can contain nicked open circle (1) and supercoil (4) forms besides the desired open circle plasmid DNA (2) (Figure 7). The
extra bands present due to these forms could confound results of the gel shift assay, especially the supercoil band which has a similar migration distance to single stranded linear DNA. Linearizing the circular plasmid reduces isoform complexity.

**Figure 7.** A plasmid DNA sample can contain many different structural forms of DNA, each with a different migration pattern. Shown above are nicked (1), open circle (2), and supercoil (4). To avoid potentially confounding results, a plasmid digest is performed using HindIII as the enzyme to create a sample of linear double-stranded DNA (3).

The double-stranded circular plasmid was digested with enzyme HindIII to yield a double-stranded, linear DNA substrate (3). Gel electrophoresis was performed to confirm the presence of and isolate the double-stranded linear DNA (Figure 8). The digest procedure has a % yield of double-stranded linear DNA, but a portion of the plasmid DNA remains uncut in the supercoil form. Only the bands containing double-stranded linearized DNA are excised from the gel.
Figure 8. Gel purification of the DNA from the HindIII enzymatic digest. Lane 1 is a size ladder, and lanes 2-5 show the digested DNA. Boxed in red is the linear double-stranded DNA. The faint band below is remnant supercoil plasmid DNA.

The dissolved DNA was removed from the excised gel bands using solid phase extraction column chromatography. Glasswool columns were manufactured in-house for the purpose of eluting the dissolved DNA. This column method involves puncturing a PCR tube in which a filter is created using compressed glasswool (Figure 9). The band slice containing the double-stranded linearized DNA was placed on the glasswool filter, and the entire PCR tube is placed inside an eppendorf microcentrifuge tube (1.5 mL). Centrifugation was then performed to elute the DNA dissolved in TAE running buffer, trapping the solid agarose behind on the glass wool. Not only is this glasswool filter technique cost-effective, additional difficulties were encountered when commercial sephadex columns were utilized for the purpose of solid phase extraction of dissolved DNA, as many commercial columns contain fungicides. The fungicides in questions have chemical properties that confound DNA quantification results (i.e. lambda max 260).
Figure 9. A scheme of the glass wool elution procedure. The gel slice on the left is the crude DNA from the enzymatic HindIII digest, and the band in red is excised and used for the SPE. The final eluted DNA is shown as pure on the gel slice to the right.

Seen above in Figure 9, solid phase extraction (SPE) of DNA from the post-digest purification gel using the glasswool elution procedure successfully recovers only the double stranded linear DNA. After SPE of the dissolved DNA, an additional centrifugation was added to remove any potential agarose contaminants that flowed through the makeshift column. As expected, recovering of the dissolved DNA without the use of a commercial column requires the addition of a desalting procedure to the overall purification scheme. UV/vis spectrophotometry confirmed the high salt content of DNA isolated via SPE (Figure 9). A standard ethanol precipitation was optimized to suit our substrate. Shown in Figure 10, after the glasswool elution, isolated double-stranded linear DNA has high levels of salt contamination, which are eliminated after the ethanol precipitation.
Figure 10. Quantification of DNA samples is performed with UV/vis spectrophotometry. On the left is the UV/vis spectrum of DNA isolated from the glass wool elution procedure. The high signal intensity at 230 nm indicates salt contamination. The spectrum on the right is the same DNA sample after a desalting procedure, ethanol precipitation.

UV/vis provides qualitative and quantitative data regarding the DNA substrate. It confirms that the purification protocol developed for the pUC19 commercial plasmid (digest, gel purification, glasswool elution, agarose spin, and ethanol precipitation) yields double-stranded linear DNA with minimal salt content and protein content, that can be reliably quantified.

After development of this purification protocol to create a stock of reliably quantified, linear double-stranded DNA, gels were run to begin to establish a relationship between SYBR Gold signal intensity in double- versus single-stranded DNA. For these test gels, size ladder and uncut pUC19 plasmid were used as standards for running distances, and the other samples either had or had not been denatured. Denaturation of the DNA samples was performed using a combined chemical (DMSO) and heat (100 °C) denaturation. In order to establish a relationship between concentration and signal intensity, every single-stranded DNA concentration represented was
replicated with a double-stranded DNA sample. The signal volume would be extrapolated for the double and single stranded bands of the gel and compared to establish a relationship.

**Figure 11.** Gel shift assay of the double-stranded linear DNA versus single-stranded linear DNA. In gel 1 (Left), lane 1 is double-stranded linear DNA, and lane 2 shows the single-stranded DNA that was treated with 60% DMSO and an incubation at 100 ℃. In gel 2 (Right), the initial results of the denaturation procedure are shown. Lane 1 is a size ladder. Lane 2 shows the double-stranded linear DNA. Lane 3 shows DNA treated with chemical and heat denaturation procedure, but incomplete denaturation and limited migration. Direct addition of SYBR Gold to all lanes.

However, unnatural band characteristics were observed almost immediately in samples treated with the denaturation protocol. Rather than seeing a clear difference in migration between single- and double-stranded DNA (Figure 11, Gel 1). Thick, wavy bands in only “single-stranded” lanes with the same migration distance as the double-stranded DNA indicated interference with the denaturation protocol (Figure 11, Gel 2). After examining samples for degradation, DNase contaminants, suboptimal pH conditions, salt contamination, and correcting for remaining salt contamination and suboptimal pH, the wavy bands persisted. The remaining culprits were dye and stain contamination. In the purification protocol devised above, the circular commercial pUC19 is first digested with HindIII enzyme to produce a linear, double-stranded DNA product. This sample is run on a gel to isolate cut DNA, which is then removed and recollected via glass-wool elution.
and further spun to remove remaining agarose. The DNA isolated after this process has been exposed to salt contaminants from the gel and staining contaminants from the gel visualization process. An ethanol precipitation is performed to remove salt contaminants. However, DNA samples are stained with SYBR Gold via direct addition of the stain to each DNA sample, meaning SYBR Gold can potentially contaminate the DNA sample. The ethanol precipitation does not appear to remove all contaminants from the dye, and SYBR Gold stain appears to inhibit the denaturation of DNA by both chemical and heat denaturation. This was confirmed by the gel in Figure 12 shown below. The denaturation procedure and preparation of DNA samples remained identical to previous gels, except rather than perform direct addition of SYBR Gold to DNA samples, the gel was post-stained, so SYBR Gold could not affect migration of the band.

**Figure 12.** Gel shift assay of the double-stranded linear DNA versus single-stranded linear DNA in a post-stained gel (SYBR Gold). Lane 1 is DNA treated with the chemical and heat denaturation procedure and is the only lane with direct addition of SYBR Gold. As shown above, the band in lane 1 has no denaturation and abnormal migration, which confirms interference from SYBR Gold. Lane 2 is double-stranded DNA with no direct addition of SYBR Gold. Lane 3 shows the single-stranded DNA without direct addition of
SYBR Gold that was treated with 60% DMSO and an incubation at 100 °C. Although denaturation is incomplete, single-stranded DNA is present and the band migration is as expected.

Although there was not complete conversion of double-stranded DNA to single-stranded DNA in the gel subjected to the denaturation protocol, a portion of the treated DNA was able to convert to single-strand and migrate the appropriate distance on the gel without the appearance of a thick, wavy band.

Issues arose with denaturation, and less than 100% conversion of double-stranded to single-stranded DNA was observed in DNA samples that were treated with the denaturation protocol. In order to gain complete control of the substrate and convert 100% of double-stranded DNA to single-stranded DNA in samples subjected to the denaturation procedure, a protocol which avoids all potential contaminants was devised. Ghost protocol follows the procedure outlined above.

Figure 13. A scheme of the Ghost protocol.
However, all gels are post-stained and instead of staining all cut DNA samples for isolation via gel electrophoresis after HindIII digest, a section of the gel containing the size ladder and one aliquot of the DNA digest is excised and post-stained with SYBR Gold. After the 40 minute post-staining period with agitation is complete, the gel is reassembled and visualized using a trans-illuminator. Using the stained DNA aliquot as a guide, the position of the unstained aliquots is approximated, and they are isolated using the procedure outlined above (Figure 13). Additionally, all samples are sunk using 50% glycerol instead of 6x Dye to avoid further dye contamination. Therefore, the DNA substrate isolated at the end of ghost protocol is free from dye and salt contamination, excluding potential contaminants obtained during industrial processing of the pUC19 plasmid.

**Figure 14.** A gel confirming the presence of DNA after DNA purification via ghost protocol, which demonstrates the destabilization of the desalting equilibrium. In lane 1 is crude, unpurified digested DNA. Lanes 2 and 3 show what is supposed to be linear double-stranded DNA isolated via ghost protocol. However, only single-stranded DNA is visible, despite the lack of a denaturation treatment.

One unexpected result of the addition of Ghost protocol to the purification procedure is the destabilization of the double-stranded to single-stranded equilibrium in the isolated DNA stock. The desalting procedure combined with the removal of any salt contact from dyes or stains, means that the DNA isolated is completely desalted. The DNA isolated with Ghost protocol and the
purification procedure isolated above was observed to convert to single-strand without chemical or heat denaturation (Figure 14). This is easily remedied with the addition of 1X TAE buffer to each sample before loading to ensure an optimal salt concentration to stabilize double-stranded DNA.

After success with the purification and denaturation procedures due to this implementation of Ghost protocol, the next step was to acquire SYBR Gold signal intensity data for double- and single-stranded DNA. Concentration gradient gels were run to establish the signal volume intensity over a range of DNA amounts (Figure 15). Although the SYBR Gold manufacturer advertises that SYBR Gold can be used to visualize and quantify picogram amounts of DNA, so far the smallest amount experimentally observed with SYBR Gold is 1 nanogram of double-stranded DNA.

![Figure 15](image)

**Figure 15.** A concentration gradient gel to establish the range of signal volume intensity for both double- and single-stranded DNA.

Using gels with concentration gradients, linear regressions of signal volume intensity were created. For the range of concentrations evaluated, the signal intensities of SYBR Gold for single- and double-stranded DNA appear to be linear. The regressions for single- and double-stranded DNA
signal intensity with SYBR Gold both have high confidence, with $R^2 > 0.95$ (0.9635, 0.9997) (Figure 16).

**Figure 16.** Plot demonstrating the linear relationship between SYBR Gold signal volume intensity and DNA concentration in both double-stranded (blue) and single-stranded (orange) DNA.

Future work on this assay will focus on defining limits of detection and quantification of SYBR Gold for single- and double-stranded forms of DNA. Ultimately, this dye-based assay will be used to evaluate plasmid DNA crosslinking by the aziridinomitosene-based alkylating agents. If these alkylating agents have high levels of ICL forming activity, they could potentially have the same uses as other bisalkylating agents, such as mitomycin C, as chemotherapeutic compounds.

**Methods.**

**DNA Preparation.**
Experimental DNA comes from the ~2,600 bp plasmid pUC19. Circular pUC19 plasmids were cut and linearized with the enzyme HindIII, previously established to cut pUC19 around 1,100 bp. Digested DNA was purified via 1% 1X TAE agarose gel electrophoresis to visualize linearized segments which were excised and isolated using glass-wool elution. Decanting the retrieved DNA solution after 15-minute incubation on ice followed by a 15-minute spin at 14,000 rpm removed further agarose contaminants. Further desalting was performed using ethanol precipitation. The final product was confirmed using UV/vis spectrophotometry to determine its concentration and its 260/230 ratio to determine its purity and gel electrophoresis to assess the DNA structure.

**Ghost Protocol.**

To gain complete substrate control and avoid other possible contaminants, ghost protocol was used during DNA purification and isolation. Ghost protocol follows the procedure outlined above. However, instead of staining the cut DNA samples for isolation via gel electrophoresis after HindIII digest, a section of the gel containing the size ladder and one aliquot of the DNA digest was excised and stained with SYBR Gold. After the 40-minute post-staining period with agitation was complete, the gel was reassembled and visualized using a trans-illuminator. Using the stained DNA aliquot as a guide, the position of the unstained aliquots was approximated, and they were isolated using the procedure outlined above. Additionally, all samples were sunk using 50% glycerol instead of 6x Dye to avoid further dye contamination. Therefore, the DNA substrate isolated at the end of ghost protocol was free from dye and salt contamination, excluding potential contaminants obtained during industrial processing of the pUC19 plasmid.
Denaturation.

The method of denaturation was optimized to include a mix of chemical and heat denaturation. Successful denaturation was observed in samples with 60% DMSO after a 2-2.5 minute incubation in boiling water (100 °C).

Methods Specifications.

Gel Conditions.

Analytical, Purification, Digest.

A 1% 1X TAE mini gel was run at 80V for between 45 minutes and an hour depending on the level of separation desired. All gels were post-stained to avoid dye interference during the run using a 10,000 fold dilution of SYBR Gold stain in 1X TAE buffer for 40 minutes with light agitation. Gels were imaged with Bio-Rad’s Image Lab Software (325 nm) and visualized using a transilluminator as a guide for excision.

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**Denaturation.**

A 1% 1X TAE mini gel was run at 80V for between 1.5 and 2 hours depending on the level of separation desired. All gels were post-stained to avoid dye interference during the run using a 10,000 fold dilution of SYBR Gold stain in 1X TAE buffer for 40 minutes with light agitation. Gels were imaged with Bio-Rad’s Image Lab Software (325 nm) and visualized using a transilluminator as a guide for excision.
References.

1 Stromberg, L. 2016. *Investigation of Non-Isotopic Methods for Quantification of DNA in Agarose Gels*. University of Puget Sound Department of Chemistry.


Scheme 1. Proposed mechanism for the formation of a bisalkylation crosslink by Mitomycin C between two antiparallel guanine residues (Warner et al. 2015)
Appendix 2.

Additional Findings.

SYBR Gold appears to affect the ability of linearized double-stranded DNA to denature when introduced to the sample matrix before loading either to stain the sample or as trace contamination. As a result, all gels were post-stained, and samples were purified and isolated according to ghost protocol.

DMSO appears to affect the signal intensity of SYBR Gold. During the optimization of the denaturation method, different amounts of neat DMSO were added to DNA samples which were stained identically. However, samples with 70% DMSO had higher signal intensity than samples with 60% DMSO, despite identical amounts of DNA and identical staining.

DNA duplex appears to be destabilized by ghost protocol. The isolation of linear, duplex DNA from pUC19 plasmid appears to produce a product that reverts to a single-stranded form without the application of chemical or heat denaturants. To avoid complications, samples isolated via ghost protocol were isolated in pH 8 1X TAE buffer.

The method of denaturation was optimized to include a mix of chemical and heat denaturation. Successful denaturation was observed in samples with 60% DMSO after a 2-2.5 minute incubation in boiling water.
A sample concentration gradient of single- and double-stranded DNA samples ranging from approximately 10-40 ng of DNA per samples showed a linear relationship between signal intensity and DNA concentration. The function relating double-stranded DNA amount (ng) to signal intensity (volume) had an $R^2=0.947$ and the function relating double-stranded DNA amount (ng) to signal intensity (volume) had an $R^2=0.866$. 