Electronic Supplementary Material (ESI) for Analytical Methods.
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Supporting Information

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Rapid, instrument-free colorimetric quantification of DNA using Nile Blue

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18 Content of Supporting Information:
19 17 pages (including cover sheet)
20 3 Tables
21 10 Figures
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25 **Determination of a final [DNA] based on each concentration range determined through k-**
26 **means cluster analysis of 5 NB concentrations.** In Table S3 the column "Final determined
27 [DNA] (μg/mL)" represents using the cluster identification/concentration ranges determined for
28 all 5 concentrations of NB to narrow the likely concentration or concentration range of DNA
29 present. For example, Blinded Solution 4 had determined concentration ranges of 15-45,15-45,
30 0-20, 0-40, and 0-60 μg/mL. The concentration range of 0-60 μg/mL becomes narrowed by 0-40
31 μg/mL, and 0-40 μg/mL becomes narrowed by 0-20 μg/mL. From there the remaining
32 concentration ranges were 15-45 μg/mL but would be expected to have an upper-bound of 20
33 μg/mL based on the 0-20 μg/mL range determined. Therefore, the final likely [DNA] would be
34 15-20 μg/mL using this approach.
35 Another example from Table S3 includes Blinded Solution 11 which had determined
36 concentration ranges of 15-45, 55-100,25-30, 70-100, 45-100, and 65-100 μg/mL. The
37 concentration range of 45-100 μg/mL becomes narrowed by 55-100 μg/mL which becomes
38 narrowed by 65-100 μg/mL and by 70-100 μg/mL. The concentration range of 15-45 that was
39 determined does not overlap with any of these narrowed concentration ranges, demonstrating a
40 likely misidentification for that sample. Using the high level of redundancy given by 4/5 of the
41 concentration ranges, this final concentration range can be considered an outlier due to a
42 misidentification, giving a final concentration range of 70-100 μg/mL.
43 Occasionally, multiple misidentifications will affect the final determined concentration,
44 as is the case for Blinded Solutions 1 and 3 in Table S3. Though, for these samples the difference
45 between the true and determined [DNA] was within 5 μg/mL.
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48 **Results of the response of NB to various reagents used in NA preparations.** As shown in
49 Figure S8 all 5 reagents tested (Triton X-100, SDS, ethanol, sodium chloride, and a 1:1
50 phenol:chloroform solution) produced a shift in their mean inverted red colorimetric intensities
51 from that of the true negative (10 mM Tris pH 8.0). Only SDS led to a decrease in the mean
52 inverted red colorimetric intensity and all other reagents led to an increase. A description of the
53 likely interactions causing these shifts for each reagent is given here.
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55 *SDS:* SDS is an anionic surfactant that has a critical micelle concentration of 0.25%
56 (w/v). The concentrations measured here include 0.25, 0.125, 0.06, 0.03, and 0.015%
57 (w/v). At 0.25% SDS the colorimetric response in the red channel is slightly shifted
58 downwards, though a visual colorimetric response from blue to purple is not observed. At
59 all concentrations below 0.25% a significant decrease in the mean inverted colorimetric
60 intensity as well as a visual purple colorimetric response can be seen. Previous work
61 (Mitra, R. K.; Sinha, S. S.; Pal, S. K., Interactions of Nile Blue with Micelles, Reverse
62 Micelles and a Genomic DNA. *Journal of Fluorescence* **2008,** *18* (2), 423-432.) on the
63 interaction of NB and SDS demonstrated that at concentrations of SDS below its critical
64 micelle concentration anionic SDS molecules can interact with cationic NB molecules
65 through electrostatic and hydrophobic interactions. It was also demonstrated that at
66 concentrations at and above the critical micelle concentration NB preferentially resides in
67 the hydrophobic core of the micelle rather than interacting with the anionic, hydrophilic
68 heads of the micelle. When SDS was spiked into a solution of dsDNA a decrease in the
69 inverted red colorimetric is observed, but the decrease is not as significant as the decrease
70 of the true positive (40 μg/mL dsDNA). This illustrates that when SDS (at a

concentration below the critical micelle concentration) and DNA are both present in a sample, NB preferentially interacts with SDS instead of DNA and generates a colorimetric response closer to that of SDS than of just DNA. It is likely that other anionic surfactants would produce similar results as those of SDS, highlighting that care should be taken to minimize the presence of such reagents in DNA samples undergoing analysis with NB.

Triton X-100: Triton X-100 is a nonionic surfactant that has a critical micelle concentration of 0.02% (w/v). The concentrations measured here include 1, 0.1, 0.01, 0.001, and 0.0001% (w/v). At 1% Triton X-100 the colorimetric response in the red channel is unaffected. Though, at concentrations below 1% a significant increase in the inverted red colorimetric is observed with the visual colorimetric response remaining blue. When Triton X-100 was spiked into a solution of dsDNA a slight increase (~10 units) in the inverted red colorimetric intensity as compared to the true positive (40 μg/mL dsDNA) was observed. These results indicate that unlike anionic SDS, nonionic Triton X-100 has significantly less competitive interactions with DNA for NB. It is likely that the increases in the inverted red colorimetric intensity are due to hydrophobic, rather than electrostatic, interactions between Triton X-100 and NB.

Ethanol: Ethanol is a reagent commonly used in the purification of isolated NA. The concentrations measured here include 1, 0.1, 0.01, 0.001, and 0.0001% (w/v). At 1% ethanol the colorimetric response in the red channel is unaffected. Though, at concentrations below 1% a significant increase in the inverted red colorimetric is observed with the visual colorimetric response remaining blue. When ethanol was spiked into a solution of dsDNA no effect on the inverted red colorimetric intensity as compared to the true positive (40 μg/mL dsDNA) was observed. The differences in the inverted red colorimetric intensity observed for NB spiked with ethanol highlight solvation effects on the visual color of NB. Importantly, these effects are not seen when DNA is present demonstrating the interactions between NB and DNA are stronger than those between NB and ethanol.

Sodium chloride: Sodium chloride is a reagent commonly used in the isolation and purification of NA. The concentrations measured here include 0.06, 0.03, 0.015, 0.0075, and 0.004% (w/v). At all concentrations the colorimetric response in the red channel is moderately increased. When sodium chloride was spiked into a solution of dsDNA no effect on the inverted red colorimetric intensity as compared to the true positive (40 μg/mL dsDNA) was observed. It is unclear what causes the differences in the inverted red colorimetric intensity observed for NB spiked with sodium chloride, though importantly, these effects are not seen when DNA is present.

Phenol:Chloroform solution: Phenol:chloroform solutions are commonly used in the isolation of NA. The concentrations measured here include 1, 0.5, 0.25, 0.125, and 0.06% (w/v). At 1% the colorimetric response in the red channel is unaffected. Though, at concentrations below 1% a significant increase in the inverted red colorimetric is observed with the visual colorimetric response remaining blue. When the phenol:chloroform solution was spiked into a solution of dsDNA no effect on the

117 inverted red colorimetric intensity as compared to the true positive (40 µg/mL dsDNA)
118 was observed. It is likely that the hydrophobic nature of these organic solvents creates
119 interactions with NB to cause the differences in the inverted red colorimetric intensity.
120 Importantly, these effects are not seen when DNA is present demonstrating the
121 interactions between NB and DNA are stronger than those between NB and the
122 phenol:chloroform solution.
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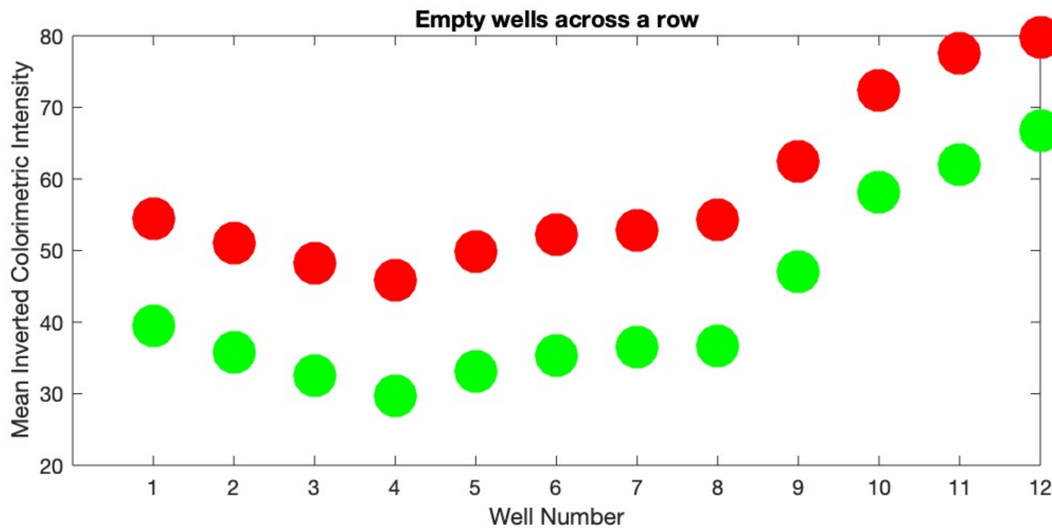
128 **Figure S1.** Plot that visualizes a well plate with the mean inverted red and green colorimetric
129 intensity of the incident light from the art tracing pad (Amazon Product No. B07H7FLJX1)
130 present in each of the wells. Interaction of the well plate and the art tracing pad causes the wells
131 across a row to have variable incident mean inverted colorimetric intensities. The effect can be
132 seen in both the red and green colorimetric channels. To account for this effect, mean inverted
133 red and green colorimetric data from solutions of NB with DNA are subtracted by the mean
134 inverted red and green colorimetric channel from an empty well in the same column as that
135 solution. This normalization process is analogous to a background subtraction performed on
136 spectrometers and is performed for each captured image, making the normalization specific to
137 the image to account for variability between images.



138 **Figure S2.** Results of image analysis in the mean inverted red colorimetric intensity for each
139 concentration of NB with concentrations of DNA from 0-100 μg/mL. The data shown are the
140 average of five replicates for each sample and error bars are the standard deviation of those
141 replicates.
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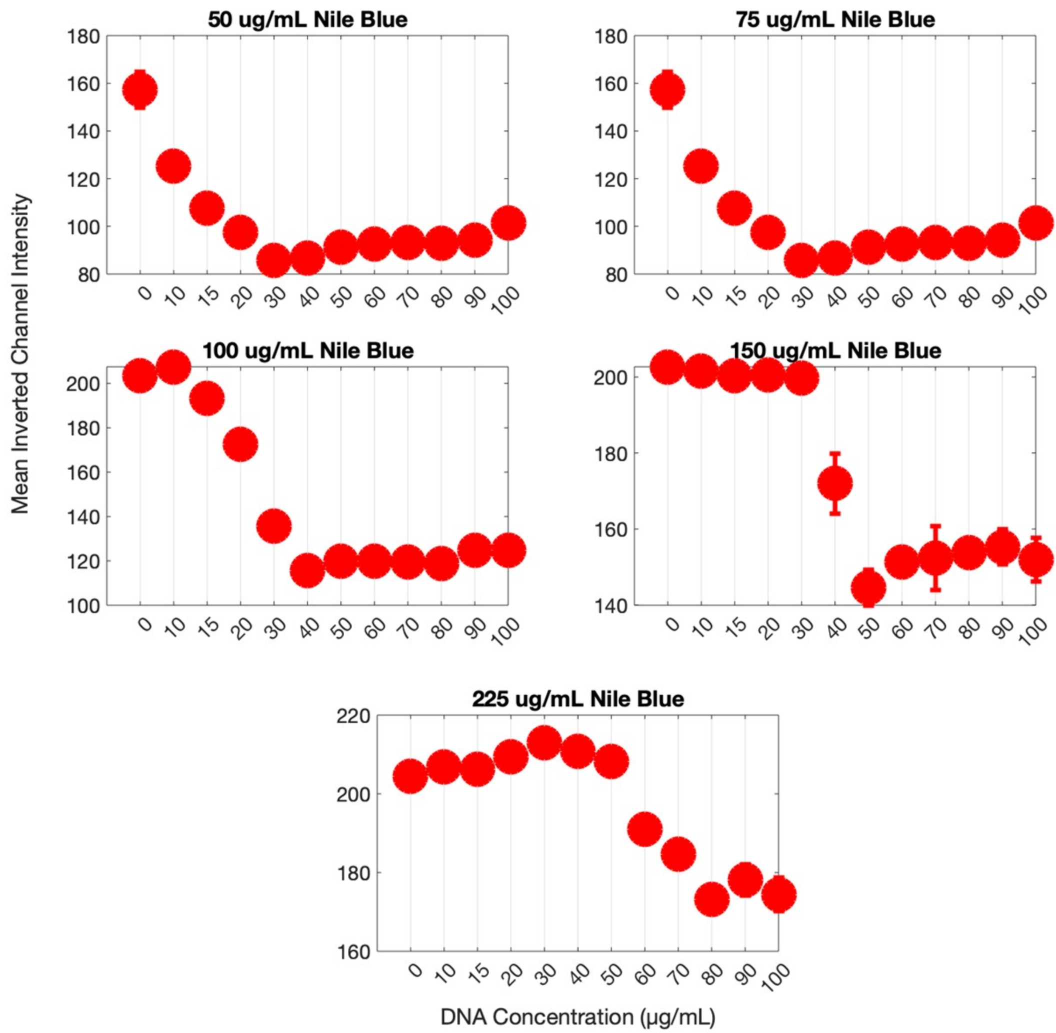

Table S1. Ratio-dependent response of NB to DNA illustrated by the DNA concentrations, both the minimal (low) and maximal (high), that produce a visual purple color when combined with each concentration of NB. The low concentration given is the first concentration of DNA where a visual purple response with NB can be seen. The high concentration is the last concentration where a visual purple response with NB can be seen. Both these high and low concentrations increase as the concentration of NB increases, indicating a positive relationship between NB an DNA. This data was used to determine the range of ratios where the concentration of NB:DNA produces a visual purple response, which is linked to changes in colorimetric intensities via image analysis. The range of ratios was determined to be approximately 1.5-4.0.

NB Concentration (μg/mL)	DNA Concentration (μg/mL)		Ratio of NB:DNA	
	Low	High	Low	High
50	15	30	3.3	1.7
75	20	40	3.8	1.9
100	30	50	3.3	2
150	50	70	3.0	2.1
225	60	80	3.8	2.9

188 **Figure S3.** Elbow plot showing the percentage of variance in the red and green colorimetric
189 intensity data that can be explained by an increasing cluster number for each of the
190 concentrations (50, 75, 100, 150, and 225 µg/mL) of NB.
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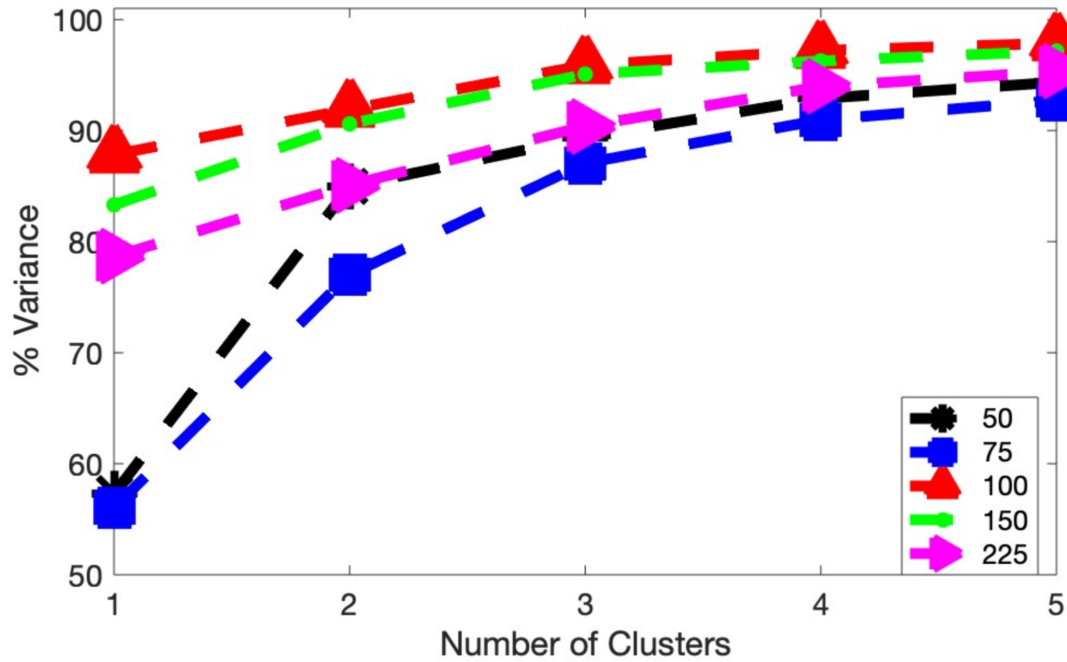

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195 **Figure S4.** Cluster plots formed from k-means cluster analysis of the colorimetric response (red
196 and green channel) for each of the remaining concentrations of NB with DNA concentrations
197 from 0-100 µg/mL. Each concentration had 5 replicates (data points) and the formed clusters are
198 enclosed in error ellipses representing the 95% confidence interval. The concentration range (in
199 µg/mL) within each cluster is given next to the cluster.
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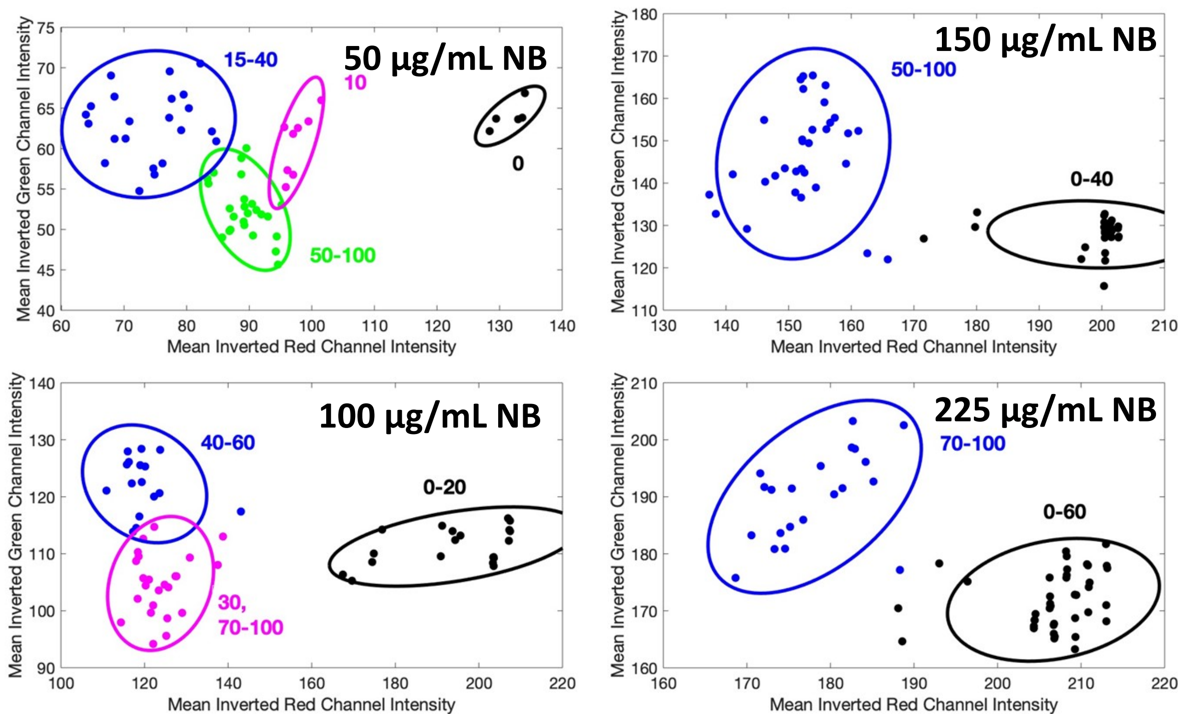

201

202 **Table S2.** Concentration ranges of DNA that can be inferred based on the k-means cluster
203 analysis of the colorimetric response (red and green channel) for each of the concentrations of
204 NB.
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[Nile Blue]	50 μg/mL	75 μg/mL	100 μg/mL	150 μg/mL	225 μg/mL
[DNA] (μg/mL)	0	0	0-20	0-40	0-60
	10	10-15	25-30, 70-100	45-100	65-100
	15-45	20-50	35-65		
	50-100	55-100			

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218 **Table S3.** Concentration ranges of DNA in blinded samples inferred from the k-means cluster
219 analysis of the colorimetric response (red and green channel) for each of the blinded samples of
220 DNA ran against all concentrations of NB. Cells highlighted orange are those where the
221 concentrations were incorrectly identified. The column "Final determined [DNA] (µg/mL)"
222 represents using the cluster identification/concentration ranges determined for all 5
223 concentrations of NB to narrow the likely concentration or concentration range of DNA present.
224 For example, Blinded Solution 4 had determined concentration ranges of 15-45,15-45, 0-20, 0-
225 40, and 0-60 µg/mL. The concentration range of 0-60 µg/mL becomes narrowed by 0-40 µg/mL,
226 and 0-40 µg/mL becomes narrowed by 0-20 µg/mL. From there the remaining concentration
227 ranges were 15-45 µg/mL but would be expected to have an upper-bound of 20 µg/mL based on
228 the 0-20 µg/mL range determined. Therefore, the final likely [DNA] would be 15-20 µg/mL
229 using this approach. A more detailed description of this process is given in the text at the start of
230 the SI.

Blinded Solution Number	Cluster identifications or concentration ranges for each blinded solution (µg/mL)					Final determined [DNA] (µg/mL)	True [DNA] (µg/mL)
	50	75	100	150	225		
1	15-45	15-45	35-65	45-100	0-60	45	50
2	10	10-15	0-20	0-40	0-60	10	10
3	15-45	15-45	25-30, 70-100	0-40	0-60	25-30	35
4	15-45	15-45	0-20	0-40	0-60	15-20	20
5	15-45	15-45	35-65	45-100	0-60	45	45
6	50-100	55-100	25-30, 70-100	45-100	65-100	70-100	85
7	50-100	55-100	35-65	45-100	65-100	65	65
8	50-100	55-100	25-30, 70-100	45-100	65-100	70-100	100
9	15-45	10-15	0-20	0-40	0-60	15	15
10	50-100	55-100	25-30, 70-100	45-100	65-100	70-100	90
11	15-45	55-100	25-30, 70-100	45-100	65-100	70-100	75
12	15-45	15-45	25-30, 70-100	0-40	0-60	25-30	25
13	0	0	0-20	0-40	0-60	0	0
14	50-100	15-45	35-65	45-100	0-60	50-60	60
15	50-100	15-45	35-65	45-100	0-60	50-60	55
16	15-45	15-45	25-30, 70-100	0-40	0-60	25-30	30
17	0	10-15	0-20	0-40	0-60	0-10	5
18	50-100	55-100	25-30, 70-100	45-100	65-100	70-100	80
19	15-45	15-45	35-65	0-40	0-60	35-40	40
20	15-45	10-15	25-30, 70-100	45-100	65-100	70-100	95
21	10	15-45	25-30, 70-100	45-100	65-100	70-100	70

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235 **Figure S5.** Changes in the mean inverted red colorimetric intensity of the response of NB with
236 DNA solutions ranging from 0-100 µg/mL at various starting pH values (2-12) of NB. The
237 maximal difference in the colorimetric response from that of the normal pH of NB (pH=4) was
238 12%, demonstrating that the effect of the starting pH of NB on the colorimetric response is
239 relatively low. Importantly, the differences in the measured colorimetric intensity do not affect
240 the observed trend (i.e. a decrease in the mean inverted red colorimetric intensity). This effect
241 was observed when combining the solutions of NB in equal volumes with the solutions of DNA
242 which are prepared in 10 mM Tris at pH 8.0. It is likely that upon addition of the DNA solution
243 to NB, the pH of the entire solution (NB + DNA) is closer to that of the DNA solution (pH=8.0)
244 than of the initial NB solution (pH=2-12)
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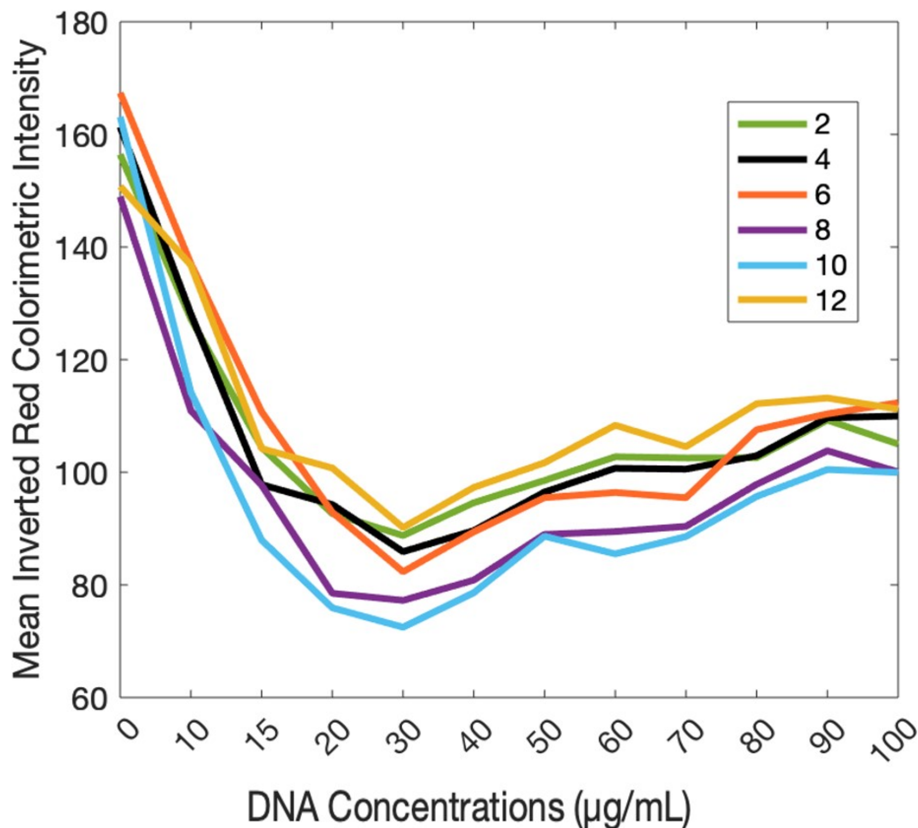
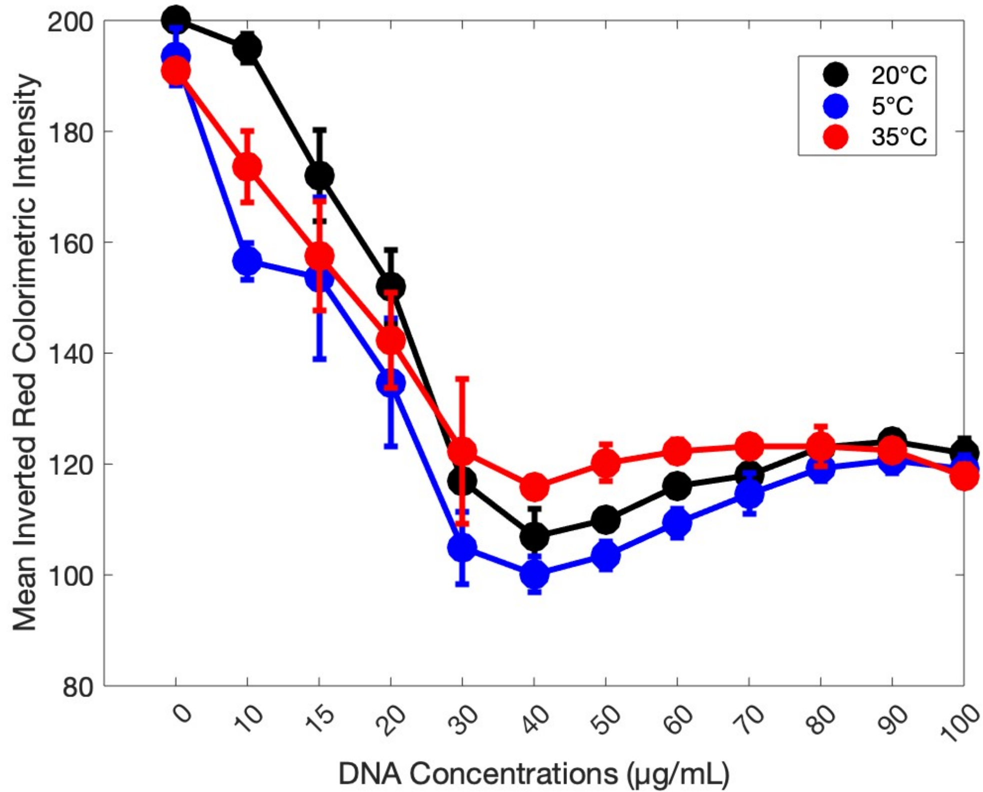


248 **Figure S6.** Changes in the mean inverted red colorimetric intensity of the response of NB with
249 DNA solutions ranging from 0-100 µg/mL at incubation temperatures of 5 °C (fridge), 20 °C
250 (room temperature), and 35 °C (oven). At 5 °C and 35 °C the measured colorimetric intensities
251 of the concentrations of DNA tested were on average 8.5% and 4.5% less than the response at 20
252 °C. This demonstrates that when this method using NB is performed at temperatures within the
253 range of 5-35 °C the effect of varying temperatures is negligible and observed trend in the
254 response of NB to DNA is preserved.



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260 **Figure S7.** Changes in the mean inverted red colorimetric intensity of the response of NB with
261 DNA solutions ranging from 0-100 µg/mL when imaged under different incident lighting
262 conditions. These conditions were measured using the art tracing pad previously described, using
263 an iPAD with a white background produced by the free app "ColorLumina", using ambient light
264 against a white background, and on the art tracing pad using a clear-sided well plate in place of
265 the black-sided well plate. Compared to the art tracing pad, the measured colorimetric intensities
266 for all three alternatives were significantly lower, demonstrating the decrease in the amount of
267 incident light from all three alternatives. However, the observed NB response to DNA is
268 relatively preserved in each system and only the absolute colorimetric intensity values are
269 affected.
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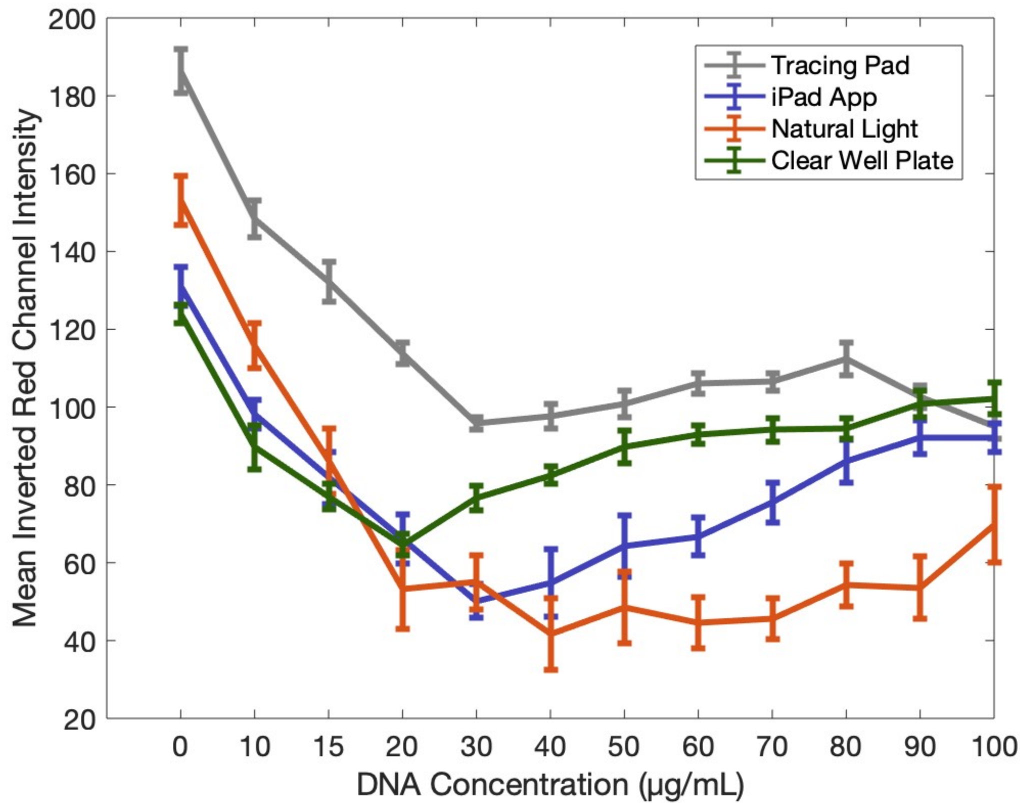

276 **Figure S8.** Colorimetric response of NB with various reagents to determine the specificity of NB
277 towards DNA. **A)** All reagents were prepared as solutions at the 5 concentrations shown in the
278 figure (w/v %) and combined with a 75 µg/mL solution of NB. The response given across each
279 reagent and concentration range was compared to that of the response of the true negative (10
280 mM Tris pH 8.0). See the text above for a description of each reagent's response. **B)** For each
281 reagent the concentration that produced the greatest change in the mean inverted red colorimetric
282 intensity was spiked into solutions of 40 µg/mL dsDNA and combined with a 75 µg/mL solution
283 of NB. The response given across each reagent was compared to that of the response of the true
284 positive (40 µg/mL dsDNA). See the text above for a description of each reagent's response.
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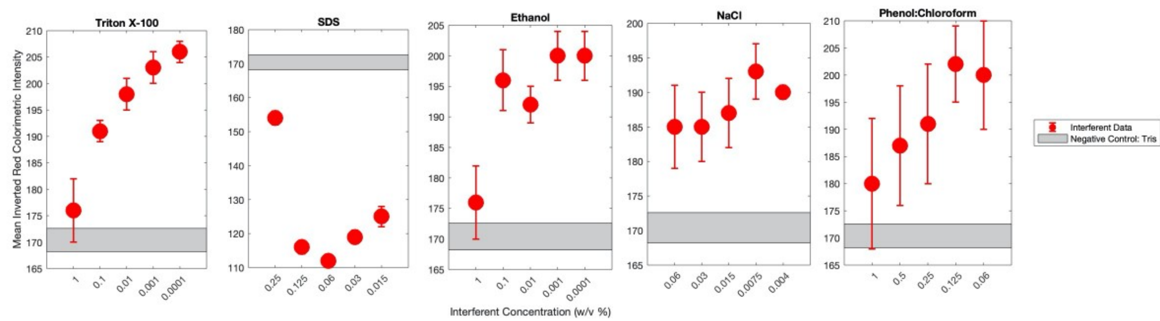
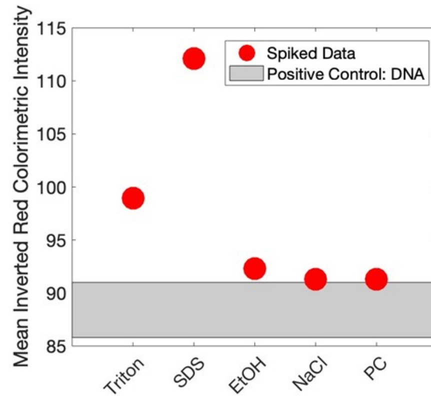


B



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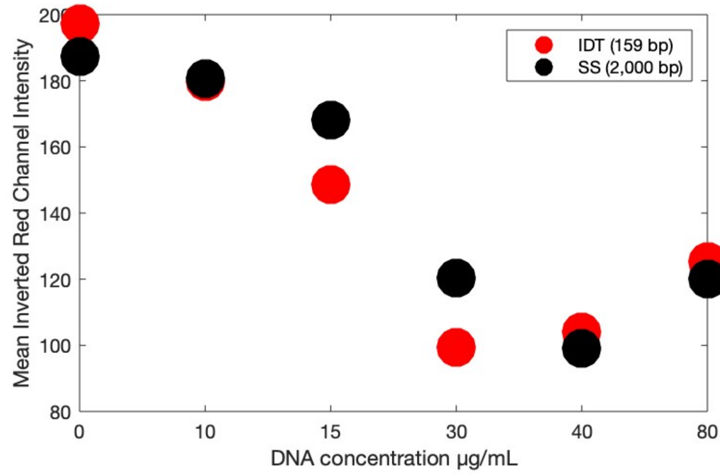
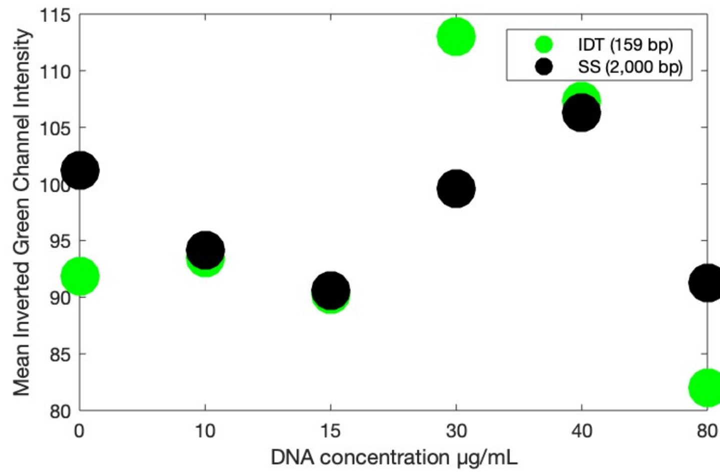
290 **Figure S9.** Colorimetric response of a 75 µg/mL solution of NB titration against various
291 concentrations of dsDNA of either 159 base pairs (IDT) or 2,000 base pairs (salmon sperm, SS)
292 in length.





293 **Figure S10.** Migration distance of NB spots on paper strips when exposed to concentrations of
294 DNA at 0, 50, and 100 µg/mL. Solutions of NB were added to Ahlstrom 319 filter paper strips
295 prepared at a width of 3 mm and a length of 60 mm using wax to create hydrophobic barriers. 4
296 µL spots of 225 µg/mL NB were added to the start of the strip and allowed to dry. The strips
297 were placed in solutions of 0, 50, and 100 µg/mL of DNA (see inset image). Through capillary
298 action the DNA solutions move up the paper strip, coming into contact with the NB spot. Once
299 the solutions had moved up ¾ the length of the paper strip they were removed from solution and
300 placed on a paper towel for three minutes before imaging in natural light. These results
301 demonstrate that as the concentration of DNA increases, the distance NB migrates down the
302 paper strip increases. At 0 µg/mL the NB spot does not migrate beyond the initial spotted
303 location. At 50 and-100 µg/mL of DNA the spot of NB appears to migrate down the paper lane,
304 visualized by the blue color of NB. Additionally, the distance traveled by the 100 µg/mL sample
305 is greater than that of the 50 µg/mL sample.
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