# Interference of Sybr®Green in detecting PCR amplicons with the Agilent 2100 bioanalyzer

Application Note

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## Abstract

Sybr® Green I is an intercalating dye commonly used to detect amplification products in real-time PCR. We tested the hypothesis of interference of this dye with the performance of Lab-on-a-Chip technology, which uses a different proprietary intercalating dye. The results demonstrate only minor influences of Sybr® Green I on the performance of the Agilent 2100 bioanalyzer for measurement of a fragment amplified from the PBGD-gene. They prove the general feasibility of using this Lab-on-a-Chip technology for evaluating amplicon quantity, size and purity even in the presence of Sybr® Green I.

## Introduction

Real-time PCR is extensively used for quantifying gene expression in a wide variety of human and animal cells and tissues. One of the most popular dyes for detecting amplification products is Sybr® Green I, which interacts with single and double stranded DNA (ssDNA and dsDNA, respectively) in a non-specific manner. It shows upon binding an increased fluorescence intensity.[1]

Agilent Lab-on-a-Chip technology (Agilent 2100 bioanalyzer) has proved to be an excellent alternative to labor-intensive gel electrophoresis techniques for assessing purity as well as for sizing and quantifying nucleic acid fragments. This popular technique uses an intercalating dye for detection of amplicons. However, there has not been any data published on the effect of using fluorescent dyes, as Sybr® Green I, on the results of DNA sizing and quantification with the Agilent 2100 bioanalyzer.

### Aim of the study

In this study we investigated if detection of PCR products is affected by the presence of an intercalating molecule like Sybr® Green I.

### Design of the study

The design of the experiment is summarized in figure 1. RNA extraction: total RNA was extracted from samples of human adrenal glands (n=5) and from samples of cultured human umbilical vein endothelial cells (HUVEC, n=7) using a Total RNA Isolation Mini kit (Agilent Technologies). Quality and quantity of RNA were assessed on the Agilent 2100 bioanalyzer using Total RNA Nano assay. RT-PCR: cDNA was synthesized using iScript™ cDNA Synthesis Kit (Biorad) on a common thermal cycler. The protocol for reverse trans-cription was: 5 minutes at 25 °C, 30 minutes at 42 °C, 5 minutes at 85 °C. Real time PCR was performed on Light Cycler™ (Roche) using LightCycler Fast-Start DNA Master Hybprobe™ (Roche). For each sample real-time PCR was performed using a mix with equal concentrations of each component (1.25 mM MgCl2, 0.5 µM primer, 1 % DMSO) except for Sybr® Green I 0.5X (Biorad), which was replaced by the same volume of water in the sample without Sybr® Green I. A fragment of Porphobilinogen Deaminase (PBGD) gene was amplified using the following primers: forward CTGAGGAATGCATGTATGCT, reverse ATGCTATCTGAGCCGTCT. Primers were selected based on the published sequence NM000190 from the Genebank database. The protocol for real-time PCR was: denaturation at 95 °C for 10 minutes, 40 cycles of amplification (5 seconds at 95 °C, 10 seconds at 52 °C and 15 seconds at 72 °C) and melting (30 seconds at 95 °C, 10 seconds at 65 °C; temperature increasing until 95 °C with a slope of 0.1 °C/second). The expected amplicon length is 250 bp. DNA assay: PCR fragments were detected on the Agilent 2100 bioanalyzer using a DNA 1000 LabChip Kit, performing a DNA 1000 Assay.

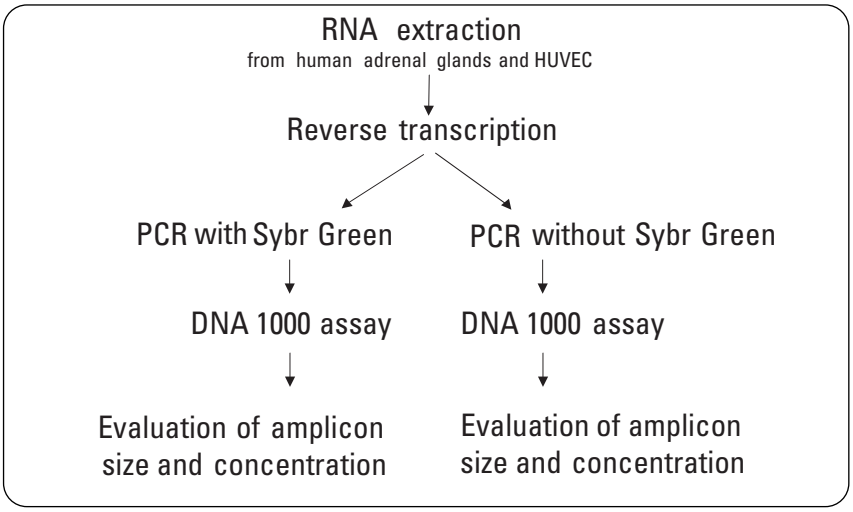


**Figure 1**
**Sample generation for comparative experiments.**

### Statistical analysis

Data are expressed as mean ±SD, and statistical evaluation was performed using the student's test for paired data with SPSS (vers 13.0, SPSS Italy, Bologna). Values of $P<0.05$ were considered to be statistically significant.

## Results

Each sample showed only one peak in the electropherogram in accord with the result of the real-time PCR melting curve (data not shown). As compared to the amplicons without Sybr® Green I, those with the dye showed a decrease of the migration time relative to the marker used as internal standard (-0.46 ± 0.28 seconds) and therefore a decrease of the estimated size (-3.92 ± 2.15 bp). See examples of electropherograms in figure 2 for identical samples amplified with or without Sybr® Green I. The mean size of the amplicon was 229 ± 2 bp with Sybr® Green and 232 ± 2 bp without Sybr® Green I (mean ± SD, $P<0.0001$) (figure 3), however, this corresponded to a size difference of only 1.3 %. The amplicon is sized as 230 bp instead of the expected 250 bp. Considering that absolute sizing accuracy is specified with ± 10 % CV, the result is within these expectations. There was an increase of the amplicon concentration for samples with Sybr® Green I (5.75 ± 4.33 ng/µL) as compared to samples without Sybr® Green I. The mean concentration of the amplicon was 32.9 ± 6.2 ng/µL with Sybr® Green I and 27.1 ± 5.70 ng/µL without Sybr® Green I (difference in quantity is 17.6 %; mean ± SD, $P=0.001$; figure 4). Sybr® Green I within the amplification did not induce the formation of additional signal within sizing region, which is the area between the lower (15 bp) and upper (1500 bp) internal marker of the kit reagents. No additional signal was found in front of the lower marker or above the upper marker in any sample either (figure 5).

## Discussion

An accurate sizing and quantification of PCR products (amplicons) is crucial for many molecular, biological and diagnostic applications. Since the Lab-on-a-Chip technology is increasingly used to this end, we investigated if the incorporation of Sybr® Green I in the amplicons affected the accuracy of these measurements. This study showed two important results. First, the incorporation of Sybr® Green I in the amplicons does not preclude the amplicons' detection with the Lab-on-a-Chip technology and therefore, Sybr® Green I can be used for RT-PCR whenever characterization of the amplicons by Lab-on-a-Chip is wanted. Second, Sybr® Green I caused a slight, albeit significant, decrease of the apparent amplicon size along with an increase in the concentration of PCR products, as detected by DNA 1000 assay on the Agilent 2100 bioanalyzer.

The structure of the internal dye used with the Agilent 2100 bioanalyzer is proprietary and therefore unknown to the authors. However, Sybr® Green I has an 497/520 nm excitation/emission spectrum, the Agilent 2100 bioanalyzer has red excitation laser (around 635 nm) and detection window at 674-696 nm. Thus, it is altogether implausible that Sybr® Green I fluorescence is probably directly detected by the Agilent 2100 bioanalyzer. In addition, it is unlikely that a conflict between Sybr® Green I and the Agilent internal dye happens. However, some influence of Sybr® Green I occurs, thus increasing fluorescence of the PCR product with a subsequent increase of its concentration computation.

In the design of this experiment the assumption of equally efficient amplification with and without

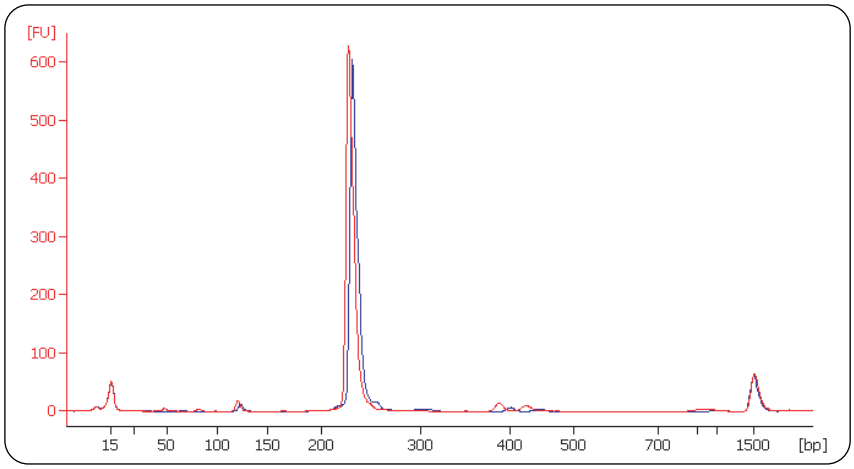


**Figure 2**
**Amplification products after PCR with (red) or without (blue) Sybr®Green addition analyzed with the DNA 1000 assay.**

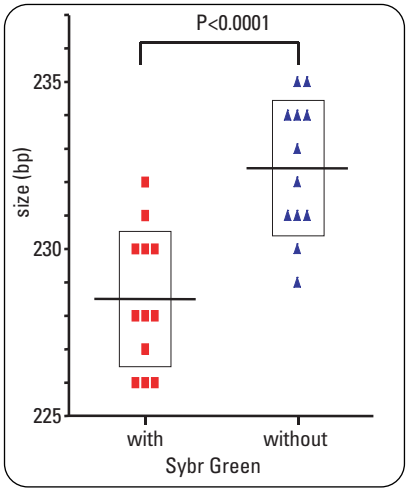


**Figure 3**
**Influence of Sybr®Green I on DNA1000 sizing results. The scatter plot shows a significant decrease (mean ± SD; P<0.0001) in bp size for samples with Sybr®Green I (red squares) compared to samples without (blue triangles).**

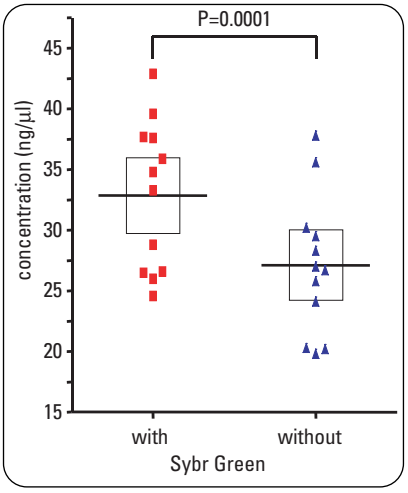


**Figure 4**
**Influence of Sybr®Green I on DNA1000 quantitation results. The scatter plot shows a significant increase (mean ± SD; P=0.0001) in concentration for samples with Sybr®Green I (red squares) versus sample without (blue triangles).**

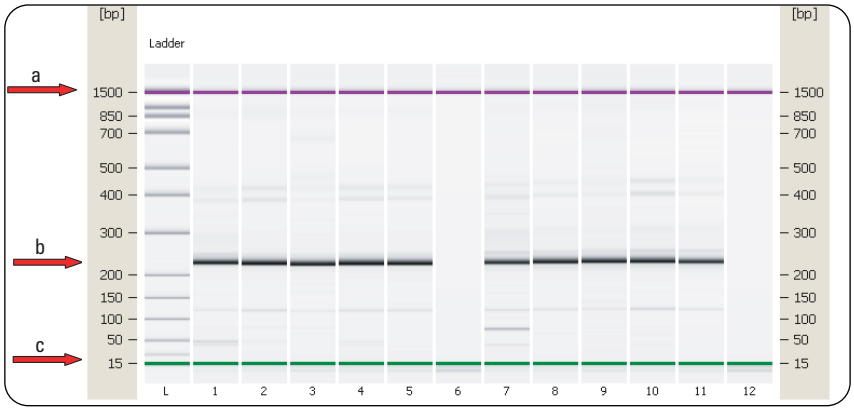


**Figure 5**
**Purity of PCR fragments generated with or without Sybr®Green I. Example of typical the analysis results in the 2100 bioanalyzer gel view. No dominant additional bands appear besides those expected fragments identified by arrows corresponding to the: a) upper marker, b) amplicon, c) lower marker. Samples 1-5: PCR with Sybr®Green I; Samples 6: no template control PCR with Sybr®Green I, water; Samples 7-11: PCR without Sybr®Green I; Samples 12: no template control PCR without Sybr®Green I, water.**

Sybr® Green I is underlying. Both PCR reactions, with and without Sybr® Green I were carried to 40 cycles of amplification to achieve the plateau phase and therefore should represent the maximal amount of product possible. Accordingly, the concentration of product determined by the Agilent 2100 bioanalyzer might also simply represent the different amplification efficiencies. Notwithstanding this possibility, it is quite important to underline that the amount of amplicons measured with and without Sybr® Green I were found to be quite close to each other in terms of analytical specification for the quantification accuracy (which is ± 30 %). Size determination for this fragment was at 230 bp whereas the expectation from sequence data was at 250 bp. This deviation is an individual and characteristic feature of fragment in this experimental set-up and might be sequence dependent. Interestingly, with Sybr® Green I we did observe, along with the change of concentration, a slight decrease in the retention time of the amplicons, resulting in a decrease of their estimated size by approximately 1 %.

Sybr® Green I structure was determined to be [2-[N-(3-dimethylaminoprpyl)-N-propylamino]-4-[2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methyldiene]-1-phenyl-quinolinium]$^{+}$ [2], and carries two positive charges in standard conditions. One positive charge is due to electron deficiency of the conjugated, mesomeric heteroaromatic systems linked by a methine bridge, and the other one is due to the protonation of the amino group of the 3-dimethylaminopropyl residue. These positive charges are thought to contribute to the binding of Sybr® Green I to DNA [2]; it was also suggested that Sybr® Green I can bind to dsDNA by intercalation and surface binding [3,4]. The delay of dsDNA in electrophoresis is likely caused by charge neutralization (diminished coulombic repulsion between negative charges of phosphate groups) and conformational rigidification of the dsDNA helical structure [5,6], due to intercalation in the double helix. Theoretically, Sybr® Green I and the internal dye used for detection of amplicons in the Agilent 2100 bioanalyzer might or might not compete for binding to DNA in an intercalating mode. In the first case one would expect a decrease in fluorescence intensity, along with negligible changes of the electrophoretic mobility. In the latter eventually one would predict no change of fluorescence intensity along with minimal or unchanged electrophoretic mobility. As we did observe an increase of both, fluorescence intensity and electrophoretic mobility, our results are difficult to explain at the molecular level. Therefore, further research on the interactions of DNA with Sybr® Green I and the internal dye is necessary.

## Conclusion

In summary, this study shows the following:

1. Amplicons containing Sybr® Green I can be detected without disturbances using the DNA 1000 assay in the Agilent 2100 bioanalyzer.
2. The incorporation of Sybr® Green I in the amplicons results in a small but significant increase of fluorescence intensity and a small but significant increase in the electrophoretic mobility. These changes must be taken into account when using Sybr® Green I in the Agilent 2100 bioanalyzer.

Since the impact of Sybr® Green I is low, the Agilent 2100 bioanalyzer proved to be a precise tool for amplicon verification after real-time PCR in our hands. It is a complementary technique to the melting point determination. It allows a useful crosscheck of fragment sizes, product contamination and amplicon concentration.

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