A Study on Enhanced Fluorescence Signal-to-noise by using the Stray Light Shutter for Quantitative PCR Chip

Liang-Chieh Chao[1], Chun-Han Chou[1], Hsin-Yi Tsai[1], Kuo-Cheng Huang[1] and Dar-Bin Shieh[2]
[1]*Taiwan Instrument Research Institute, National Applied Research Laboratories, Hsinchu, Taiwan*
[2]*Institute of Oral Medicine, School of Dentistry, National Cheng Kung University, Tainan, Taiwan*

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Abstract: Quantitative polymerase chain reaction (qPCR) is the most important inspection technique for virus, especially for coronavirus disease, in this year. The qPCR chip and device were planned to develop because of the characteristics of fast inspection time, high accuracy and small system volume. Therein, the fluorescence intensity was the important signal in qPCR device, which represented the positive or negative reaction after the DNA was amplified and bound on the fluorescence dye, but the fluorescence signal was easily to be affected by the excitation and scattered light. The mini spectrometer was employed to receive the fluorescence intensity in PCR chip in this study, and the optical simulation was progressed, and the stray light shutter (SLS) was added to improve the signal-to-noise ratio (SNR) of fluorescence. The analysis results showed that the SNR of fluorescence can be enhanced from 3.14 to 16.78 by using the SLS with shape of extend component aperture, which the protruding structure was at the direction away from center. The results from this manuscript can provide the important reference information to the developer of qPCR chip, whom can obtain the high SNR fluorescence signal in qPCR inspection process for disease.

1 INTRODUCTION

Quantitative polymerase chain reaction (qPCR also called real time PCR, RT PCR) is an important laboratory technique in molecular biology application based on the PCR technique, which was one of the most common method in a large amount of disease detection, such as virus and bacterial inspection (Espy et al., 2006). The qPCR technique was widely applied in clinical disease inspection (Kralik et al., 2017), especially for the coronavirus disease 2019 in this year. In qPCR process, the standard double strand DNA would be separated into two single strand DNA by increasing the reagent to specific temperature, which the temperature was about 92-95 ℃ depends on the reagent. In addition, the specific primer DNA would bind on the single standard DNA with the annealing of reagent's temperature to 58-62 ℃, and the specific primer DNA will extend while the temperature raised to about 72 ℃. With the repeat cycle of increased and decreased temperature of biological sample included DNA, the amount of DNA can be amplified with 2 to the 30th power in the PCR process. It looks on the amplification of a targeted DNA in the regular PCR process, and it collected the result within the fluorescent signal instead of post process such as Gel electrophoresis. There are two type of methods for products detection in real-time PCR process: (1) non-specific fluorescent dyes directly intercalated with any double-stranded DNA and (2) specific DNA probes connected with oligonucleotides being labelled with fluorescent reporter (Bustin et al., 2009). When the DNA of sample included the specific fragment of disease, qPCR could provide the quantitative fluorescence information after each thermal cycle. Therefore, the fluorescence intensity could present positive or negative reaction to the tested target DNA. Therein, in consideration of the inspection cost, except the usage in clinical diagnosis, most of the experiment would choose SYBR Green for the fluorescence sample.

In addition, the previous study had proved that the SYBR Green can also get the precise result as TaqMan probes can made. The applicability was demonstrated by measuring the copy number in three different genetic contexts, which included the quantification of gene rearrangement, the detection and quantification in cell lines and cancer biopsies,

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and the detection of deletions in dominant optic atrophy (Ponchel et al., 2003). The results showed that the presented assay had important clinical application and can providing accuracy diagnostic results in short time. Four gerne expression profiles (A1, A2A, A2B, A3) of adenosine receptors in breast cancer tissue were analysed by optimized TaqMan and SYBR Green quantitative RT PCR (Tajadini et al., 2014). The result showed that the efficiency for TaqMan and SYBR Green methods in all genes were calculated mor than 95 %, and the correlations of mean normalized data of each gene in two methods were positive and significant. SYBR Green I-based duplex qPCR was developed for simultaneous detection of virus (Zheng et al., 2020). Therein, the classical swine fever virus (CSFV) and porcine circovirus 3 (PCV3) were simultaneously inspected in one sample that amplified by their distinct melting temperatures. The experimental results showed that the qPCR with fluorescence dye of SYBE Green was a reliable diagnostic tool to monitor the disease in clinical field.

In the qPCR system, the accuracy of the result strongly depends on the correctness, sensitivity and resolution of the fluorescent signal. In the commercial PCR System. The most common fluorescent detector is modulated CMOS Camera or the photodiode. In modulated CMOS camera, every pixel has 1 amplifier with it. Therefore, the detecting signal of the tiny fluorescence variation can be easier observed than CCD type camera, and also the driving voltage can be lower at the same time. Based on the advantages above, CMOS component has been more and more common use in the Fluorescence lifetime imaging microscopy (FLIM) techniques. The researcher (Chen et al., 2015) used the frequency-domain fluorescence lifetime imaging microscopy (FD-FLIM) composed with Olympus TIRFM microscope and CMOS camera capturing the scattered laser and analysed the intensity and modulation. The result proved that the calibration in the CMOS camera observation would be necessary in the imaging process, which can minimize the effect of bleaching or background interference. Anitoa's ultra-low light CMOS biosensor was used in a handheld, real-time qPCR system (BioOptics World, 2015). The several types of pathogen DNA and RNA, including hepatitis B/C and E coli. The detection limit of four copies per sample was achieved and over nine orders of magnitude in dynamic range. The CMOS biosensor had the enough sensitivity to replace the photomultiplier tubes and cooled CCDs in applications medical and scientific instruments.

However, the colour filter should be used on the photodiode or CMOS camera when multi colour of fluorescence signal needed to de detect in one sample for various diseases. Hence, the optical component which has the characteristics of spectrum separation becomes an important component in future application for detection of compound disease in clinical inspection field. The spectrometer has the characteristics of wavelength separation, and the information of detected light intensity at each specific wavelength can be recorded independently. In the study, the spectrometer was employed to receive the fluorescence intensity in qPCR device. In addition, the package shape of the spectrometer and the effect the stray light was analyzed, and the stray light shutter was investigated to prevent the interference of fluorescence by excitation light and enhance the fluorescence signal-to-noise ratio. In the future, we will consider the design and the results that investigated in this manuscript, and we can get the more precise results in different concentration of DNA than previous device.

2 EXPERIMENTAL PRINCIPLE AND SETUP

The total power of an incident light is equal to the outer light, and the power can be divided into the reflected, transmitted, scattered, and absorbed. There is fluorescent particle in the liquid in the qPCR sample, and fluorescence is emitted when the substance or particle is excited and then absorbing the light or electromagnetic radiation. Generally, the fluorescence occurs when an electron of molecule jumps to ground state by emitting the light from the excited state, and the emission light ceases nearly immediately when the excitation light stops. Therefore, the distribution of emission light intensity of fluorescence particles can be simulated by the scattering model.

2.1 Scattering Model Principle

If the substance is a particle, the particle would absorb the light energy and re-emit the light with different intensity in different directions. Therein, the Rayleigh scattering theory was employed when the dimension of particle was smaller than the light wavelength, and the Mie scattering theory was used when the particle size was larger than the wavelength of light. However, there is lots of calculation time in the light intensity of each light beam. Total integrated scattering (TIS)

is defined as the ratio of the total power generated by contributions of scattered radiation included the forward and backward to the incident radiation, shown as Fig. 1. In the generally TIS situation (Harvey et al., 2012), the incident beam on the sample was nearly normal, and the integration was carried from the small values to almost 90 degrees. When the light scattered from the specular reflection is small and if the substance is surface material, the TIS will be affected by the surface roughness. In addition, the bidirectional scattering distribution function (BSDF) was used to describe the scattered light (Pfisterer et al.). The phenomenon of BSDF is usually split into the reflected and transmitted light, which is separated to the BRDF (bidirectional reflectance distribution function) and BTDF (bidirectional transmittance distribution function). In general, the vector I is the incident light, vector R is the specular light, and the vector S is the scattered light, shown as Fig. 2. Therein, the specular light presented the mirror reflected or scattering light. The projection light of specular light and scattered light on the plane was defined as β and β_0, and the variation was x. When the scattering phenomenon was caused by an isotropic random rough surface, and the dimension of rough structure was relatively smaller than the wavelength of scattered light. The ABg scattering model can usually described the required scattering probability distribution well and was suitable to the polished optical surfaces. Therefore, the ABg scattering model was used to fit to the BSDF which is plotted as a function of $|\beta - \beta_0|$(Won, 2014). The ABg scattering model was widely used to evaluate scatter results through ABg parameters, and written as Eqn. (1).

$$BSDF(\vec{x}) = \frac{A}{B+|\vec{x}|^g} \quad (1)$$

where A must be equal to 0 or larger than 0. B must be larger than 1E-12, unless g is equal to 0. If g is equal to 0, then B can be equal to 0. In addition, no scattering will occur if A is equal to 0. In the manuscript, the intensity distribution was analyzed by ABg model, and the fitting curve was similar to the Lambertian scattering type. The Lambertian scattering model was usually used to present the scattering intensity of uniform rough surface. The probability of the scattered light on the projection vector β is the same everywhere in the unit circle, and the BSDF is 1/π.

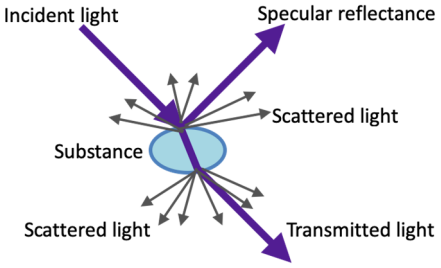

Figure 1: The schematic of the total integrated scattering.

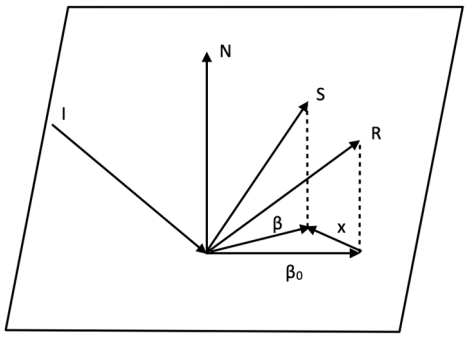

Figure 2: The schematic of the total integrated scattering.

2.2 Experimental Setup

The FRED optical engineering software was employed to simulate the fluorescence distribution that received by the spectrometer in qPCR chip. Therein, the excitation light was the blue LED with wavelength of 465 nm and half angle of ±9°, and the spectrometer was used to receive the fluorescence. In addition, the spectrometer can detect the light intensity of 11 wavelengths because of the nano interference filter deposited on standard CMOS silicon sensor, which the detection wavelength was 350-1000 nm. The dimension of sensing area was 780 μm × 520 μm, and the diameter of the detection aperture was 0.9 mm with height of 1 mm. The gap between the surface of spectrometer and the qPCR chip was 1 mm. Due to the bottom of the PCR chip was sealed by the plastic thin film to prevent outflowing of liquid reagent, which the reflection ratio of plastic thin film and chamber of qPCR chip was set at 30% and 80% in the simulation process, respectively. The schematic of the experimental setup was shown in Fig. 3. In addition, the reagent was composed of water and some biological component, so the setting parameter of refractive index was 1.336 in the simulation process. The setting parameter of scattering ratio was 0.6, which means the 100% of incident light and 60% of light will be scattered.

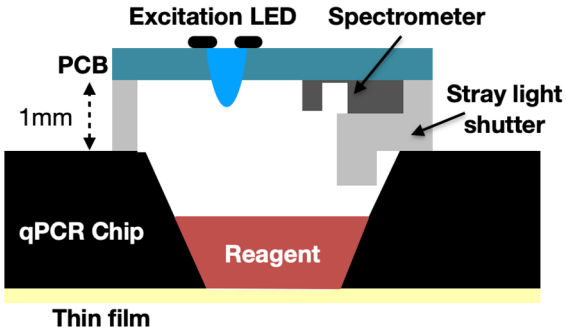

Figure 3: The schematic of simulation and experimental setup.

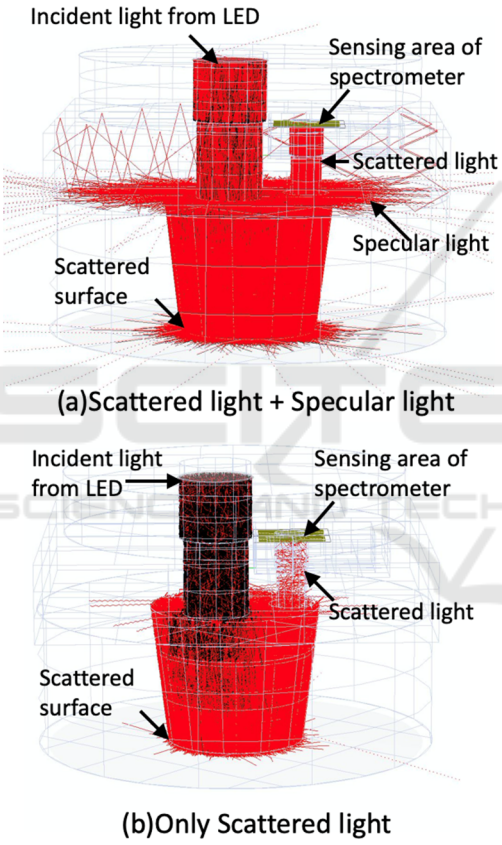

Figure 4: The Ray trace of (a) specular light and scattered light and (b) only scattered light.

3 EXPERIMENTAL RESULT AND DISCUSSION

Initially, the stray light has an evidently effect in the actual experiment, and the signal variation of the fluorescence intensity that received by spectrometer was small. Therefore, the analysis of the scattered light in the experimental setup and the solution was presented to obtain a high signal-to-noise ratio results for the following actual qPCR experiments. Therein, the specular light in the simulation software was determined as the noise because it does not excite the fluorescence substance and directly reflect, shown as Fig. 4(a). In addition, the specular light was set as absorption light, and the real signal received by the spectrometer was the scattered light that generated by the fluorescence light, which was defined as the signal and shown as Fig. 4(b). Therefore, the choose and the shape of the stray light shutter was designed, and the light intensity of signal and noise was analyzed in the following sections.

3.1 Effect of Stray Light

In order to investigate whether the stray light shutter can enhance the SNR or not, the same gap between the surface of spectrometer and chamber of qPCR chip was fixed at 1mm. Initially, there was no stray light shutter at the light path region, shown as Fig. 5 (a). In the analysis progress, the light distribution of all rays included specular light (defined as noise) and scattered light was shown in Fig.6 (a). The light distribution at the corner can be removed when the specular light was set as be absorbed, which the light distribution can present that the only scattering light from the fluorescence substance and shown as Fig. 6(b). The results showed that the specular light was generated by the incident light on the chamber of qPCR chip, so the protruding structure was designed to occlude the specular light from the side wall of qPCR chip's chamber. Therein, the protruding structure was defined as the stray light shutter (SLS) and the protruding position was designed at the semicircle away from the center (Fig. 5 (b)) and near the center (Fig. 5(c)).

With the SLS, the irradiance of signal (scattering light) and the noise (specular light) were analyzed under different scattering ratios. The results showed that signal was linear relationship to the scattering ratio by using away from center SLS and without using SLS, but most signal was occluded by using near center SLS (Fig.7(a)). In addition, the noise intensity can be evidently decreased by using the away from center SLS (Fig.7(b)), and the SNR can be enhanced about 4 -5 times compared to the SNR without using SLS (Fig.7(c)). On the contrary, the SNR of fluorescence by using near center SLS was lower than that without using SLS. Therefore, the SLS that with protruding structure away from center was the important component to enhance the SNR of fluorescence in qPCR process.

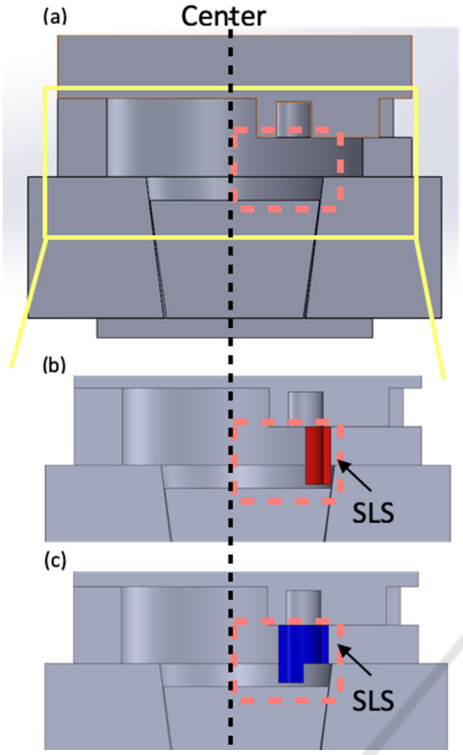

Figure 5: The simulation setup of (a) without stray light shutter (SLS), (b) with SLS away from centre, and (c) with SLS near the centre.

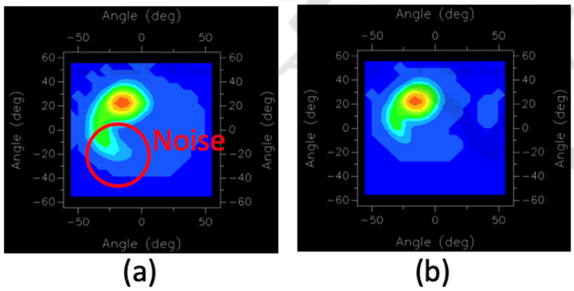

Figure 6: The light distribution of (a) all rays and (b) only scattering rays received by sensor.

3.2 Shape Effect of Stray Light Shutter

The signal decreased obviously when the light was isolated by the designed structure. Hence, four different shapes of SLS were designed and employed to analyze the signal, noise and SNR (Fig. 8(a)). Therein, there was almost no signal when the shape of SLS was the same to the aperture of spectrometer and excitation light (Fig. 8(b)). The signal was slightly higher with protruding structure of component half aperture shape than component aperture. There was the highest signal can be obtained when the structure between the excitation light and spectrometer was removed and designed only the protruding structure away from the center. In addition, there was 60 % of the highest signal by using the SLS with shape of extend component aperture. In this shape, the noise also decreased to the half value compared to the full aperture. Therefore, the highest SNR can be evaluated and obtained by using the SLS with shape of the extend component aperture, which the SNR was 16.78(Fig. 8(c)).

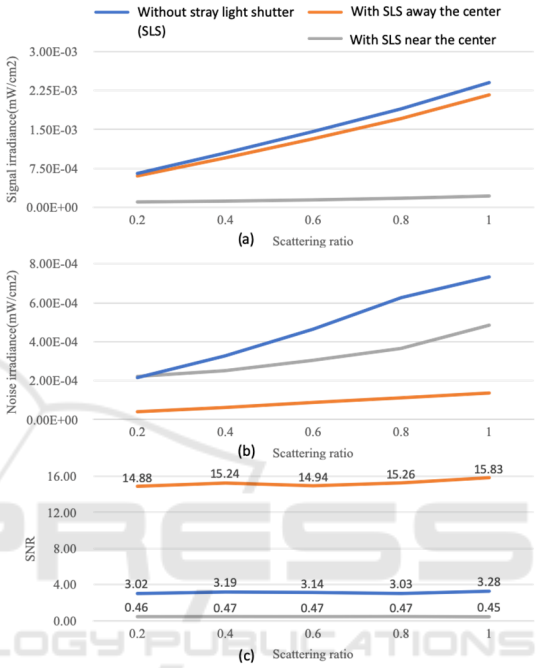

Figure 7: The (a) signal, (b) noise and (c) SNR of fluorescent under different scattering ratios by without using SLS and with using away from centre and near centre SLS.

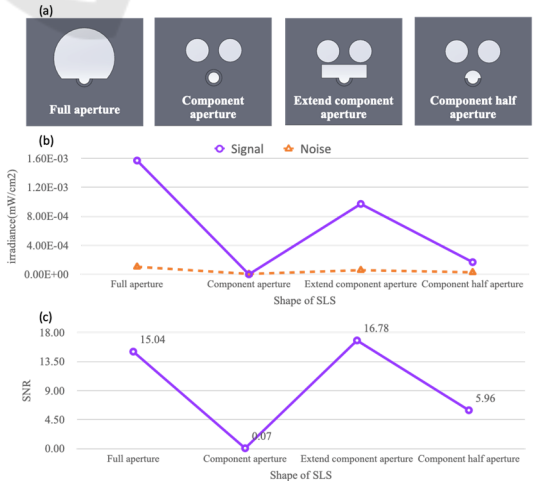

Figure 8: The (a) the design shape of the stray light shutter (SLS), (b) the irradiance of signal and noise and (c) the SNR of fluorescence using different shape of SLSs.

3.3 Effect of Detection Angle

From the above results, the suitable shape of SLS was determined. However, several signal would be blocked by the package of spectrometer because of the vertical structure with height of 1 mm. Therefore, the expand angle of the package of spectrometer was simulated to detect more rays and obtain the better package shape of spectrometer, shown as Fig. 9(a). The irradiance of signal and noise that received by spectrometer with SLS of extend component aperture shape and without SLS were analyzed, and the results showed than the higher signal can be obtained with the increase of expand angle, shown as Fig. 9(b). The signal was gradually saturated when the expand angle was set at 30 and 40 degree. In addition, the SNR with SLS was about 5-6 times compared to the SNR without SLS, and the SNR with expand angle of 40 degrees can be enhanced 7.3% relative to without expand angle (Fig. 9(c)). The comparison of the SNR under different parameters were summarized in Table 1. Therefore, the spectrometer package can be modified to expand the detection angle and the SLS can be added in the actual experimental setup to enhance the SNR of fluorescence.

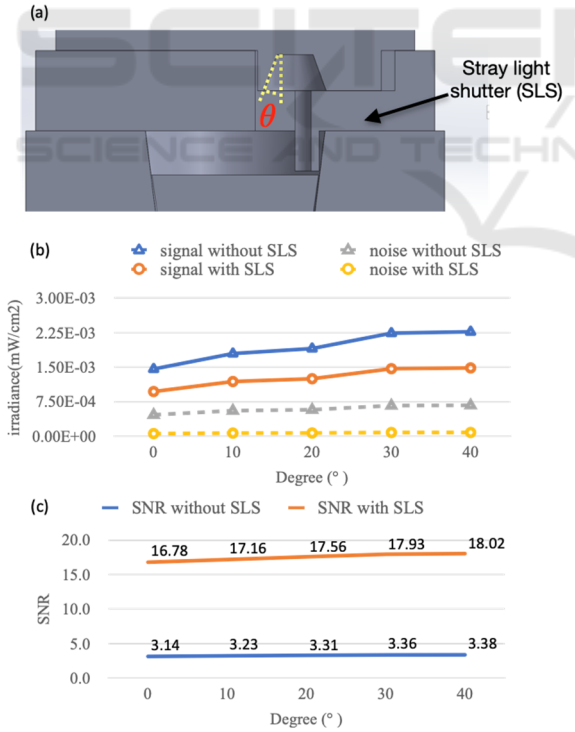

Figure 9: The (a) chematic of expand angle of spectrometer package, (b) the irradiance of signal and noise and (c) SNR of fluorescence without and with SLS under different expand angles.

Table 1: This comparison of the SNR under different parameters.

Expand angle(°) / SLS	0	40
Without SLS	3.14	3.38
With SLS	16.78	18.02

4 CONCLUSIONS

In the results of qPCR technology, the intensity of fluorescence signal plays an important role because it indicated the positive or negative reaction of tested target disease. Hence, the optical simulation results can provide the referential information for designing the experimental setup to obtain the higher SNR fluorescence signal in qPCR chip. From the simulation results, the 5 times of SNR of fluorescence can be enhanced by adding a stray light shutter (SLS) with protruding structure away from centre. In relative to the original package and without adding SLS, the SNR of fluorescence can be enhanced from 3.14 to 18.02 by adding SLS and expanding the 40 degrees of spectrometer package. From the above simulation results, the better fluorescence signal can be obtained by referring simulation setup and set up in the actual experiments in the future, which the higher fluorescence SNR can present the better performance of qPCR technology.

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REFERENCES

Espy, M. J., Uhl, J. R., Sloan, L. M., Buckwalter, S. P., Jones, M. F., Vetter, E. A., Yao, J. D. C., Wengenack, N. L., Rosenblatt, J. E., Cockerill, F. R., Smith, T. F., 2006. Real-time PCR in Clinical Microbiology: Applications for routine Laboratory testing, *Clinical Microbiology Reviews*, Vol. 19, 165-256.

Kralik, P., and Ricchi, M., 2017. A Basic Guide to Real Time PCR in Microbial Diagnostics: Definitions, Parameters, and Everything, Frontiers in Microbiology, Vol. 8, 108.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, M., Nolan, T., Pfaffl,

M. W., Shipley, G. L., Vandesompele, J., Wittwer, C. T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments, *Clinical Chemistry*, Vol. 55, 611-22.

Ponchel, F., Toomes, C., Bransfield, K., Leong, F. T, Douglas, S. H., Field, S. L, Bell, S. M., Combaret, V., Puisieux, A., Mighell, A. J., Robinson, P. A., Inglehearn, C. F., Issacs, J. D., Markham, A. F., 2003. Real-time PCR based on SYBR-Green I fluorescence: An alternative to the TaqMan assay for a relative quantification of gene rearrangements, gene amplifications and micro gene deletions, *BMC Biotechnology*, Vol. 18, 1-13.

Tajadini, M., Panjehpour, M., Javanmard, S. H., 2014. Comparison of SYBR Green and TaqMan method in quantitative real-time polymerase chain reaction analysis of four adenosine receptor subtypes, *Advanced Biomedical Research*, Vol. 3, 85.

Zheng, H. H., Zhang, S. J., Cui, J. T., Zhang, J., Wang, L., Liu, F., Chen, H. Y., 2020. Simultaneous detection of classical swine fever virus and porcine circovirus 3 by SYBR green I-based duplex real-time fluorescence quantitative, *Molecular and Cellular Probes*, Vol. 50, 101524.

Chen, H., Holst, G., Gratton, E., 2015. Modulated CMOS camera for fluorescence lifetime microscopy, *Microscopy Research and Technology*, Vol. 78, 1075-81.

BioOptics World Editors, 2015. Low-light CMOS biosensor enables detection of four copies of pathogen DNA per sample, *BioOptics World*.

Harvey, J. E., Choi, N., Schroeder, S., Duparré, A., 2012. Total integrated scatter from surfaces with arbitrary roughness, correlation widths and incident angles, *Optical Engineering*, Vol. 51, 013402.

Pfisterer, R., Scatter and BSDF Measurements: Theory and Practice, *Photonics Marketplace*.

Won, Y. 2014. A Study of Scattering Characteristics for Micro-scale Rough Surface.