Quantification of single-strand DNA by sequence-specific counting in capillary flow cytometry

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Abstract
In this study, we report an approach to achieve sequence-specific counting of single DNA molecules, which is required for more versatile applications of the previously reported absolute DNA quantification technique based on flow cytometric DNA single molecule counting. While using the same capillary-based flow cytometric setup, fluorescence activation of a target DNA was made with a number of fluorescent oligonucleotide probes of complementary sequences to that of a target DNA. The feasibility of the proposed approach was tested with 7 kb single-strand M13 DNA as the target DNA for sequence specific counting for quantification. Sample preparation, the number of fluorescent oligonucleotide probes, and hybridization conditions mainly matter for the performance of the proposed method. Using a set of 30 sequence-specific fluorescent probes with a selected hybridization buffer, acceptable performance was confirmed through comparison with other conventional methods such as digital polymerase chain reaction (dPCR), UV spectrophotometry, and deoxyribonucleoside monophosphate analysis by mass spectrometry. Proven comparability to the dPCR method confirmed the feasibility of the proposed approach. With further improvement in instrumentation, the proposed method is expected to become established as a reference measurement procedure for sequence-specific quantification of nucleic acids working under a uniquely straightforward measurement principle.

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(Some figures may appear in colour only in the online journal)

1. Introduction

Many different methods have been developed for the quantification of DNA. Each one utilizes a different measurement principle and accordingly exhibits particular intrinsic strengths and weaknesses in applications to specific biological samples. Therefore, researchers are required to select the most appropriate method for DNA quantification depending on the purpose of analysis and the properties of the samples to be analyzed. For example, UV spectrophotometry, the most common method, provides the simplest and fastest measurement format. Absorbance at 260 nm UV is measured for a DNA sample in a cuvette with a defined path length. Traditionally, cuvettes with a 1 cm path length were used for UV...
spectrophotometry, but more recently measurement instruments requiring extremely low amounts (0.5–2 µl) of samples have been widely adopted [1, 2]. One limitation of such microvolume measurement systems is that sensitivity is greatly reduced due to a shortened path length, down to 0.2–1 mm. For the quantification of low-concentration DNA samples, fluorometry could be an alternative; it has been reported that 10 pg µl\(^{-1}\) to 100 ng µl\(^{-1}\) DNA can be quantified by fluorometry [3, 4]. One limitation of fluorometry is that it requires the parallel analysis of reference material to assign quantity values to the samples, because fluorescent intensity is not an intrinsic property of DNA. Other approaches include chemical analysis of the building blocks or elements of DNA, with measurements of the total phosphorous (P) content in DNA being reported as approaches for absolute quantification [5–8]. Such methods are unique because they could provide a metrological traceability of DNA quantity to SI units. Here, the traceability is linked to the quantity of P as expressed in the SI units of mol kg\(^{-1}\) [5]. Other chemical approaches have utilized the chromatographic separation and quantification of nucleotides, nucleosides, or nucleobases [9–12]. Various analytical methods including liquid chromatography-UV, liquid chromatography-mass spectrometry (LC-MS), and capillary electrophoresis-UV have been employed for DNA quantification via building block analysis. DNA quantity from measurements of building blocks could also be traceable eventually to the P quantity expressed in mol kg\(^{-1}\) units when those measurement are calibrated with reference materials of which values are eventually linked to the certified value on P [12].

The above quantification methods focus on the optical or chemical characteristics of DNA regardless of sequence contexts. However, not only quantitative information but also the sequence context of DNA is important in many biological and medical analyses. For example, discriminative measurements of specifically targeted sequences of DNA are critical for the quantification of genetically modified organisms, pathogenic viruses, and mutation frequencies [13–15]. As follows, polymerase chain reaction (PCR) is a distinctive technology for the quantification of DNA, since it measures even a tiny amount of DNA in a sequence-specific manner. Taking this advantage, real-time PCR and digital PCR (dPCR) have become well established and widespread applied PCR formats for DNA quantification. In real-time PCR, DNA quantities are calculated by comparing the amplification profiles of a target DNA with those of calibrators of the same sequences [16]. Digital PCR works by PCR amplification of single DNA molecules sparsely partitioned among thousands of small reaction units [17]. In this format, a small portion of the partitions contain one or more DNA molecules with the correct sequence, which will result in positive fluorescent signals, while the rest of the reaction units without correct targets will result in negative signals [18, 19]. The fraction of positive reaction units over the total units leads to the copy concentration of the target DNA in the sample. dPCR provides noteworthy advantages for DNA quantification based on the following features: (i) it can detect and quantify a tiny amount of DNA in a sequence-specific manner; (ii) it is minimally influenced by competitors or matrices [20]; and (iii) principally, it does not require calibrators for quantification, representing the counting of individual molecules. Despite all its merits, dPCR should not be regarded as error-free, because perfect success in PCR amplification of single-copy DNAs is not guaranteed. Any failure in PCR amplification leads to underestimation of the DNA quantity. A number of reports have indicated that amplification efficiencies and subsequent absolute quantification efficiencies by dPCR are affected by various experimental parameters, such as primer and probe positions, annealing temperatures, and cycle numbers [21–23]. This weakness is even more crucial when dPCR is applied to the absolute quantification of RNA, in which reverse transcription is generally not expected to be perfect or effectively calibrated [24]. We have previously reported that individual DNA molecules activated by fluorescent dyes could be directly counted in capillary flow cytometry (FCM) [25–27]; similar flow cytometric DNA quantification has also been recently reported by other teams [28–30]. We were interested in the direct counting of individual molecules because of its potential for substantial reduction of measurement uncertainty from its simple and straightforward measurement principle. In our results, quantities of DNA measured by the counting method were equivalent to the quantities from other methods such as P analysis and nucleotide analysis. Subsequently, an international comparison study on the absolute quantification of plasmid DNA was performed, which found an equivalence between dPCR, isotope dilution-mass spectrometry (IDMS), and counting technology [31]. However, the counting approaches were only demonstrated to detect and quantify large and discrete DNA molecules in a sequence-non-specific manner. In such quantification, performance could be critically harmed by the inclusion of non-target DNA molecules, e.g. impurities or matrix DNA. In addition, considering that many molecular biological analyses are concerned with the genetic elements of specific sequences, sequence-non-specific counting methods are limited in applications. In order to overcome this limitation, we aimed to develop a sequence-specific counting and quantification method for DNA and RNA. The non-specific fluorescent tagging of the previous works needed to be replaced with sequence-specific fluorescence activation.

We proposed the use of a set of a multiple number of sequence specific probes, FAM-labeled oligonucleotides of sequences complementary to the sequence of a target DNA. Although relatively more expensive than unlabeled oligonucleotides, 5′-FAM-labeled oligonucleotides of high sequence accuracy are readily attainable by custom gene synthesis. A number of these probes were to be attached to the target single-strand DNA through sequence-specific hybridization as depicted in figure 1(a). We might be able to fluorescently activate the target DNA to a level sufficient for flow cytometric detection if the number of FAMS attached surpasses the minimum required to generate genuine event signals. The more probes attached, the more distinctive signals from the DNA-probe hybrids. One concern here is that the excessive free FAM probes would result in high background noise. Unlike non-specific staining dyes, e.g. SYBR™ Gold, free FAM probes are fluorescent since their fluorescence is not quenched by surrounding water molecules. The target DNA-probe hybrids
localize FAMs within them, whereas FAMs of free probes are homogeneously distributed in the solution. Therefore, the FCM detector was expected to detect the passage of a DNA-probe hybrid as a genuine event, as it would create a peak shape change instantaneously in the level of total fluorescence detected. Ideally, fluorescence produced by free probes should be constant to result in a flat level. Unfortunately, however, the local concentration of free probes in the detection zone could be instantaneously fluctuated by various reasons, which might result in misleading signals for false events. This possibility would rise as the concentration of free probes increases. Maximizing the signals for true events while minimizing the signals causing false events as depicted in figure 1(b) would be one of the key requirements for the accuracy of the proposed method, which was to be systematically investigated in this work. In addition, quantitative hybridization of target DNAs in a very diluted concentration would not be readily achieved considering the kinetics and thermodynamics involved. In this regard, optimization of DNA-probe hybridization would require a vast amount of time and effort to reach a satisfactory condition. The feasibility of the proposed method was to be assessed by comparing with other DNA quantification methods of different measurement principles such as dPCR, UV spectrophotometry, and quantification of hydrolyzed nucleoside by isotope dilution–mass spectrometry (ID-MS). It is necessary as this approach was to be first tried. Furthermore, appropriate certified reference materials to firmly judge the performance of the proposed method were not available. Comparability of the result of the proposed method to the results of other methods would help in assessing its feasibility. Comparability especially with dPCR, a method quantifying DNA sequence specifically, would strongly support the feasibility of the proposed approach, as it was aimed at quantifying DNA in a sequence-specific manner.

2. Experimental

2.1. Preparation of study materials

M13mp18 single-strand DNA was purchased from New England Biolabs (#N4040S, USA). To make homogeneous samples, three independent M13 DNA samples were pooled and then divided into five aliquots. Forty sequence-specific fluorescent probes were designed using Primer3 V0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0) and then synthesized by Macrogen (Korea). Each probe was 20 nt long and labeled with fluorescein amidite (FAM) at the 5′ end with 5 nt intervals between the neighboring probes. Sequence information of the target sequence and corresponding probes are presented in figure S-1 (stacks.iop.org/MET/V/A/mmedia).

2.2. Hybridization conditions

Two different buffers, PCR buffer (Qiagen) and PerfectHyb™ Plus Buffer (Sigma), were tested for the hybridization of the probes to the M13 DNA. As a final hybridization condition, PerfectHyb™ Plus Buffer was used for M13 DNA (17.3 nM) and a probe set consisting of 30 different probes (266 nM each). The hybridization reactions were performed in a Veriti 96-well thermal cycler (Applied Biosystems) under the following conditions: initial denaturation at 80 °C for 5 min, 30 touchdown cycles from 80 to 50 °C (−1 °C/cycle, each cycle for 1 min), and final hybridization at 40 °C for 60 min.

2.3. FCM counting

After hybridization, DNA samples were 1000-fold diluted in 1X TE buffer to an approximate level of 500 000 copies µl−1. For counting, 5 µl of the diluted sample was further diluted in 1.5 ml of FCM running buffer (5 mM Tris/HCl at pH 9.5) resulting in levels of 1500–2000 copies µl−1. All dilution processes were performed by gravimetry. A 1 µl long and 50 µm × 50 µm square capillary tube (Polymeric Technolo-
gies) was used as the fluidic channel of the capillary FCM, in which a DNA sample was loaded by pressure. The sample volume was estimated based on comparison of results by various volume measurement methods in a previous study [26]. The density of the test sample (1.01 mg µl−1) was measured to be slightly higher than pure water. Applying its exact density, the sample mass was determined to be 1.31 mg. In order to better focus the DNA molecules to the center of the capillary, two independent driving forces of pressure (0.04 MPa) and reverse electric field (−10 kV) were applied. DNA molecules passing through the detection window were counted until the loaded sample was completely passed through the detection window, producing no more positive counts. For this, the sample solution was pushed with a pure buffer solution immediately following behind. Photons detected by an avalanche photodiode were collected for every 80 µs, and the cumulative photon counts were converted to analogue electric signals for initial data processing such as threshold setting and judgement of true events. Then the converted analogue signals were saved as a numerical data file for further analysis to calculate true DNA counts. Details of the flow cytometric setup are available in the previous report [26].

2.4. Digital PCR

Droplet digital-PCR (ddPCR) experiments were performed on a Q200 platform (Bio-Rad). Four different primer-probe combinations targeting M13 DNA were initially prepared and compared. The two primer-probe combinations finally selected are as follow:

Combination 1
Forward primer: CGGTATTTTTATAATGGCGATG
Reverse primer: CCTTCTGACCTGAAAGCGTA
Probe: FAM-TGATTTGGCGGATAAAGAG-BHQ-1
Combination 2
Forward primer: AATACGAGCAAGGCGATAG
Reverse primer: GAGGCCACCGAGTAAAAGAG
Probe: HEX-AAGGTTATTTGGCGGATAG-BHQ-1
ddPCR reactions were performed using ‘ddPCR™ supermix for probes’ (Bio-Rad). The Veriti 96-well thermal cycling conditions were 95 °C for 10 min, and 80 cycles of 94 °C for 30 s and 57 °C for 60 s. The ddPCR data were analyzed using the QuantaSoft software version 1.7.4 (Bio-Rad). The droplet
volume was calculated based on the value suggested by the manufacturer (Bio-Rad, 0.85nL), and the uncertainty arising from the droplet volume was reflected in the Type B uncertainty [32].

2.5. Nucleoside analysis by ID-MS

Quantification of M13 DNA by stable isotope-labeled DNA mass spectrometry was performed following previously reported procedures [12]. In brief, the same amount of stable isotope-labeled E. coli genomic DNA was spiked in the M13 DNA and deoxyribonucleoside monophosphate (dNMP) standards, which were then enzymatically hydrolyzed into dNMP monomers by DNase I (Takara) and Phosphodiesterase I (Thermo-Fisher), and further hydrolyzed into deoxyribonucleosides (dNs) by Shrimp Alkaline Phosphatase (Takara). The resulted hydrolysates were analyzed via LC-MS using an Agilent 1200 series LC system (Agilent) and API 4000 MS system (SCIEX).

3. Results and discussion

3.1. Sample preparation

To perform sequence-specific counting, we selected M13 single-strand DNA as a model target DNA. For hybridization of a target DNA with oligonucleotide probes, the target DNA needs to become single-stranded to provide hybridization sites to the probes. Initially, we tested an approach of melting double-stranded DNAs to single-stranded, then hybridizing the single-stranded with an excessive amount of FAM-labeled oligonucleotide probes. In our preliminary work, this process turned out to be of an unsatisfactorily low yield. Under the tired condition, the melt complementary strand probably overwhelmed the probes in competing for the hybridization sites. Comprehensive understanding of this aspect needed a great deal of investigation of the thermodynamics and kinetics involved in melting, transporting, and competition in hybridization. Although this was an important matter to be eventually resolved, we were to go forward to test the overall scheme of the proposed approach. We decided to use single-stranded DNAs as the target DNA for the moment, since their hybridization with oligonucleotide probes could be efficiently achieved because of freely available hybridization sites to probe. Single-strand DNA could be prepared using lambda exonuclease on 5’ phosphorylated amplicons [33], which was quite successful in our own experiment. However, the resulting samples after such treatments were not pure enough to ultimately demonstrate comparability among different DNA quantification methods, since some of them were prone to overestimation with the elevated impurities. Alternatively, M13 DNA from a commercial source has been known to be of high purity and integrity, and was selected as an excellent single-strand target DNA. Furthermore, its length is 7249 bp long to provide a sufficient number of hybridization sites for fluorescence activation. We favored bigger DNA, since this would disperse less along the
center axis of the flow channel, which would improve the uniformity of the sensitivity of detection. Straying away from the focused detection zone accordingly reduces sensitivity in detection. Design and preparation of FAM-labeled oligonucleotide probes were performed according to the sequence of the designated hybridization site on M13 DNA (figures 1(a) and S-2). Quantitative hybridization was not readily achieved as expected. For a sparse amount of the target DNA, the first condition for a probe to hit the right segment of the target DNA for hybridization would not happen frequently, which would be even more serious for extremely diluted M13 DNA. In our previous work, the upper limit of the concentration of the target DNA turned out to be 8000 copies µL⁻¹ for the flow cytometric setup [26]. Otherwise, non-linearity of quantification occurred due to the events of simultaneous detection of multiple entities caused by insufficient spacing among DNAs. Even the signal-processing algorithm for identifying such events was not effective if it surpassed the upper limit concentration. An alternative approach to overcome this limitation was to hybridize the target M13 DNA in a higher concentration (17.3 nM compared to initially tried 2.2 pM) then quantitatively dilute it to within the upper limit (figure 1(c)), which dramatically improved the efficiency of hybridization. The relative amount of each FAM-oligonucleotide probe to a hybridization mixture was optimized to 15X of the concentration of M13 DNA, 266 nM for each probe. For this optimization, the number of FAM-oligonucleotide probes fixed to 30 according to Arjun Raj’s report [34], of which validity was to be assured later (see the following section). Although the larger amount of a probe accelerated its hybridization to M13 DNA, the excessive free probes were to increase the background noise, and hence interfered with true events. We attempted selective removal of free probes using ultra-centrifugal filter after hybridization, but it failed due to almost loss of DNA-probes (figure S-3). The use of a larger amount of probes sharply increased background noise, which was not confidently excluded from true events. On the other hand, the use of a smaller amount of probes led to a weaker signal for true events indicating incomplete hybridization. Under the given condition, we observed these at 20X and 10X concentration of probes, respectively. Therefore, 15X was chosen as the optimum concentration of probes. This optimum could vary by various other conditions for hybridization and detection. Major influencing conditions for hybridization, such as the number of probes and buffer matrix, are described in the following sections in detail. One notable aspect in detection is relative concentrations of DNA-probe hybrids and free probes. The magnitude of fluorescence produced by a DNA-probe hybrid is presumably independent of its concentration, unless it surpasses the upper limit. In contrast, background noise caused by free probes is dependent upon their concentrations. Interfering noise comes from the fluctuation of their local concentrations, which is proportional to their concentrations. Therefore, dilution of a sample as much as possible would be beneficial to improve the signal to noise ratio of FCM detection (figure 1(c)). It must be noted, however, that excessive dilution would increase measurement uncertainty as events become too rare to obtain statistically sound results. Under the given condition (1500–2000 count per sample), the number of FAMs on free probes constantly exposed to the detector as the background level was 30–40 whereas FAMs on a DNA-probes hybrid was 30. Therefore, signal to noise was interpretable as fluorescence from 30 FAMs interfered by the momentary change in fluorescence produced by 30–40 FAMs. Under this condition, confident discriminating of background noise from true events was possible, as described in Data Processing section.

3.2. Optimization of the number of fluorescent oligonucleotide probes

Dependence of the counting results upon the number of FAM-labeled oligonucleotide probes to be hybridized to M13 DNA is shown in figure 2. Applications of 10–40 FAM probes confirmed the expectation of signal intensity proportional to the number of applied FAM probes. Weak signals from M13 DNA hybridized with a smaller number of FAM probes possibly caused underestimation of its quantity. In this experiment, we set the threshold level for rejecting irrelevant noise counting. Under this level, background noise overwhelms so that many false positive events will be produced if counted. Detailed discussion on this matter is presented in the following section, Data Processing. With a smaller number of FAM probes, chances are high for some M13 DNA-FAM probe hybrids to produce a weaker signal than the threshold level, which leave them uncounted. Under the given condition, the counting results with 10 and 20 probes were 1577 ± 126 and 3102 ± 346 (n = 3), respectively, whereas those with 30 and 40 probes were 4195 ± 523 and 4210 ± 687, respectively. On the other hand, a larger number of FAM probes would strengthen the signal intensity greater than the threshold level to avoid underestimation. The result with 30 probes was comparable to that with 40 probes, implying that significant underestimation would not happen with 30 probes. However, the number of FAM probes is desired to be minimized, considering the high expense associated with their synthesis. Therefore, the set of 30 probes was chosen as the optimum probe set, which is within the range of the number of fluorescent probes reported in previous work [34] where the set of 12–48 fluorescent probes each with 20 nt length was acceptable in the detection of RNA in cells based on confocal microscopy. This optimum number of probes could be reduced with further improvement of instrumentation as well as the optimization of experimental conditions. Nevertheless, the optimum probe number found was reasonable to go ahead to mature the proposed method.

3.3. Optimization of hybridization conditions

In this work, we initially used 1X PCR buffer for hybridization of M13 DNA with FAM-labeled oligonucleotide probes, of which a number were fixed to 30 after optimization described in the previous section. With this buffer, however, even the largest quantity produced was overly smaller than the result by UV spectrophotometry (up to 60%), which suggested possible underestimation by the proposed method. One outstanding
possibility for the underestimation was incomplete hybridization between the target DNA and probes. Other parameters involved in hybridization such as temperature and hybridization time were varied to observe no dramatic increase in count numbers. Alternatively, we replaced PCR buffer with a well-known commercial buffer (PerfectHyb™ Plus) widely used in Northern blot and Southern blot experiments [35, 36]. As observed in other work [37], the hybridization efficiency and accordingly DNA counts dramatically increased with the use of the commercial hybridization buffer (figure 3(a)). The aid of buffer composition to enhance hybridization would be more critical to the proposed method compared to general PCR. Ordinary PCR exploits iterated hybridization and polymerization reactions in which a small degree of incomplete hybridization in each cycle could be compensated for in later cycles. Contrastingly, only one cycle of hybridization reaction is employed in our approach, then the achievement of complete hybridization is critical to secure accurate quantification. Also observed was the trend that the increase in the concentration of the hybridization buffer led to the increase in hybridization efficiency, which reached a plateau at 40%, implying that the maximum hybridization aid from the buffer composition has been attained. Completeness of hybridization would be demanding to achieve with lower-concentrated target DNA considering both the kinetics and thermodynamics of hybridization. As shown in figure 3(b), the use of PCR buffer as the hybridization buffer showed a dramatic decrease in hybridization efficiency along the dilution of the target DNA. In contrast, however, the special purpose hybridization buffer, PerfectHyb™ Plus buffer, maintained its efficiency down to 1 ng µl⁻¹ of M13 DNA. For assuring complete hybridization, 10 ng µl⁻¹ M13 DNA was mixed with probes in 40% PerfectHyb™ Plus buffer for assessment of quantification performance of the proposed method. The optimized hybridization condition for complete hybridization should not lead to enhanced non-specific hybridization to avoid overestimation with samples of complex DNA matrices. Various negative controls were subject to the optimized hybridization condition. As shown in figure 3(c), negative controls of ‘Buffer only’, ‘M13 target only’, ‘M13 with incorrect fluorescent probes’, and ‘Probes only’ did not give significant counts as desired. Even more important, ten times more the amount (100 ng µl⁻¹) of yeast RNA mixed with the probes did not produce significantly increased counts compared to probes only, indicating virtually no interference by mismatched tagging. Unlike the condition for sequence-specific counting, non-specific tagging with SYBR™ Gold resulted in meaningful counts for both single-strand M13 DNA and M13 DNA hybridized with non-fluorescent probes (figure 3(d)).

3.4. Data processing

No meaningful counts observed for various negative controls mentioned above was the result of appropriate data processing. In the range of weak signal level, there are noises of various origins. At extremely low level, electronic noise of the system is rejected so as not to interfere with fluorescence signals. Above this, Raman scattering of water results in the noisy baseline when the activation laser is on. There were also optical signals generated by water-borne particulates of unknown origins. These noise sources often produce a significant number of event-like peaks even for a pure buffer solution (figure 4(a)). In addition, the excess amount of free FAM
probes causes the fluctuation of background fluorescence resulting in a large number of counts. These noise counts, unrelated to our DNA-probe hybrids, are of large fluctuation, and compromise the repeatability of measurement if included in event counting. The adverse effect of the noise counting was reduced by background subtraction, but not to the satisfactory level. We set the threshold level (at 0.44 V, indicated with an arrow in figure 4(a)) to count only likely-genuine events of DNA-probe hybrids, with which signals lower than the threshold level were excluded from further signal processing as seen in figure 4(b). The threshold level was modestly set that some number of counts are usually observed for the negative control of FAM probes only (the middle panel of figure 4(b)). This amount of counts was then subtracted from the count for a sample (the top panel of figure 4(b)), which gave the final result. The final result was usually of an undistorted Gaussian distribution on the signal intensity axis (the bottom panel of figure 4(b)). The resulted distribution is consistent with what we commonly observed from samples of no noise issues with high signal intensities. For example, $R^2$ values for Gaussian fitting of two cases, pBR322 (0.993) and M13 DNA (0.992), were high and close to each other (data not shown). This suggests a successful and valid noise rejection by the applied data processing. Furthermore, some variation in the threshold level did not lead to a significant change in the final result. For example, three different threshold levels of 0.4, 0.44, and 0.5 V showed variability of 0.7% in final DNA counts, which was taken into account as a Type B uncertainty. Overall, the applied data processing fit the proposed measurement principle, and was robust enough to work with an acceptable level of measurement uncertainty.

3.5. Comparison with other methods

Validation of a newly proposed measurement procedure is usually made using a certified reference material (CRM) or an approved reference measurement procedure. Deviation of the result of the proposed method from the certified reference value of the CRM or from the result by the reference procedure indicates the possible measurement bias of the proposed method. Unfortunately, however, neither of them is available to date. Instead, we assessed the comparability of the proposed method with other conventional methods such as dPCR, UV spectrophotometry, and ID-MS as shown in figures 5(a), S-4, and table S-1. Result values are also tabulated in table S-2. The result for the proposed method agreed with the results by dPCR, whereas results by ID-MS and UV were significantly higher (~18%). Although the results of all deployed methods were not comparable, the agreement found between two sequence-specific methods (FCM and dPCR) was promising. ID-MS and UV spectrometry measure all DNAs regardless of their sequences, whereas the FCM method and dPCR quantify only the DNA containing the specified sequences. Therefore, ID-MS and UV spectrophotometry are prone to overestimation...
Figure 4. Threshold setting and blank correction. Data processing of raw data of sequence-specific counting (a) Raw data from samples and controls. Buffer blank (square), probe-only control (circle) and hybridized M13 sample (triangle) were analyzed under the same experimental conditions. The arrow indicates the final threshold value, 0.44 V. (b) Signals after application of the threshold. The signals from the hybridized M13 sample before deconvolution (top) were subtracted by the signals from probe-only control (middle) to yield deconvoluted signals directly representing M13 DNA counts (bottom).

with impurity DNAs in the sample when aiming to quantify DNAs of specific sequences. Even for those two sequence-specific quantification methods, what was measured could be different. The proposed method detects DNAs that can accommodate 30 probes. Shorter DNAs bear weaker fluorescence signals to be buried in the background noise. We described the decrease in detected DNA with a smaller number of probes in the previous section (figure 2). In the case of dPCR, however, any DNA containing the sequence fragment amplified by PCR will be detected. In this case, the applied PCR amplified the region of 110 bp in length. Differences in possible contribution to overall quantified results among different methods are presented in figure 5(b). Accordingly, comparison among the results of these methods is to be in the following order: UV spectrophotometry $\geq$ ID-MS $\geq$ dPCR $\geq$ FCM. In the case of disagreement, differences among the results are governed by the purity of the test sample. The observed results in this work imply that there could be a substantial amount of DNA impurities with shorter than 110 bp or originated from other than M13 DNA. Due to this kind of imperfection in the sample preparation, the utilization of chemical measurement methods is not always successful in quantification of specific DNAs. Nevertheless, the close agreement between two sequence-specific quantification methods was encouraging to go forward for further development of the proposed approach.

3.6. Measurement uncertainty and future improvement

Various uncertainty sources of the proposed measurement procedure are summarized in table 1. These sources are further categorized to either statistical sources or systematic sources in their natures, which are usually labeled Type A or Type B [38], respectively. Sampling errors, instrumental drifts, and day-to-day or tube-to-tube variations of hybridization efficiencies were regarded as Type A uncertainty sources. Homogeneity and stability of samples, adsorption of DNA molecules, overlapping and out-of-focus detection of DNA, possibly causing underestimation, were regarded as Type B uncertainty sources. Although most of those uncertainty components were confidently budgeted, two important uncertainty components were not the case. Uncertainty arising from insufficient tagging of target DNA was still uncertain, even though their existence was not considered high as discussed in previous sections. The other uncertainty of difficulty in assessment was the one arising from possible straying away of probe–DNA hybrids from the detection zone. Although we do not expect a substantial portion of DNA-probe hybrids to escape from detection and quantification, current instrumentation is not capable of providing a necessary spatial resolution to track individual DNA-probe hybrids. In this situation, the use of a CRM or a reference method solves the problem with which the measurement bias of the proposed method can be assessed. It is unfortunate, however, that neither is available to date. Putting this unknown bias aside, we calculated the combined uncertainty of the method reflecting all identified uncertainty sources, which turned out to be 3.85% (Table S-3). Utilizing the results from other methods, the theoretically possible maximal value of the unknown bias could be found. For this, we assume the proposed method produced highly negatively biased value due to incompleteness of hybridization
Figure 5. Interpretation of the results from different DNA quantification methods. (a) Grouping of results depending on measurand definitions. Counting (FCM) and dPCR are sequence-specific methods, while ID-MS and UV spectrophotometry are sequence-non-specific. Error bars indicate expanded uncertainty. (b) Inclusion and exclusion of different nucleic acid targets depending on different measurand definitions.

Table 1. Uncertainty sources in the sequence-specific counting.

| Procedures                  | Causes of uncertainty                           | Treatment of uncertainty |
|-----------------------------|------------------------------------------------|--------------------------|
| Sampling                    | Homogeneity of samples                          | Type A($u_s$)            |
|                             | Day-to-day variations                           | Type A($u_s$)            |
|                             | Adsorption of samples in tubes and capillary wall| Type B($u_b$)            |
| Hybridization               | Hybridization efficiency                        | Type A($u_s$) and Type B($u_b$) |
| Detection                   | Repeatability of multiple runs                  | Type A($u_s$)            |
|                             | Overlapping and out-of-focus positioning in the capillary | Type B($u_b$) |
| Data acquisition and processing | Instrumental drifts                            | Type A($u_s$)            |
| Systematic                  | Threshold setting                               | Type B($u_b$)            |
|                             | Estimation of capillary volume                  | Type B($u_b$)            |
|                             | Weighing                                       | Type B($u_b$)            |

$u_s$: standard uncertainty, $u_b$: maximal uncertainty from uncorrection of bias, $u_c$: combined uncertainty including calculable Type B uncertainties

and undetected DNA-probe hybrids. It is also assumed that the highest result by UV spectrophotometry was bias-free. Then, the difference of two values is assigned as the negative bias of the proposed method, which is 18.4% (Table S-2). Maximal uncertainty from the uncorrected maximal bias was calculated as suggested in previous reports [39, 40] using the following equation:

$$U_e(95\%) = U + E|b| = ku_c + E|b|.$$  

In this equation, bias($b$) is 18.4% as described above, and $u_c$ is the combined uncertainty without consideration of the bias, 3.85% (Table S-3). The coverage factor for ($k$) for 95% is 2.36. The enlarging coefficient corresponding to $b/\nu_c$ was approximated to 0.842 according to the references [40]. Then, the most conservatively assessed expanded uncertainty of the proposed method was calculated to be 24.6%, whereas the least was 9.09% assuming zero bias. Therefore, the true expanded uncertainty of the proposed measurement procedure is to be between 9.09% and 24.6%. Although not certain, it is likely that the correct uncertainty is rather close to the lower limit. The high limit might come from overestimation of UV spectrophotometry due to non-specific detection of all sorts of DNA in the sample (Figure 5(b)). This interpretation was supported by the tight comparability of the value of the proposed method to those of dPCR, another sequence-specific quantification method. Deviation was as small as 2.8% (Table S-2). Along the progresses in the improvement of instrumentation and further optimized hybridization conditions, the uncertainty of the proposed method would be firmly reduced. The detection system of the flow cytometric setup is to be redesigned to get its sensitivity immune to the dispersion of the trajectories of individual DNA-probe hybrids. One direction is to obtain spatial resolution of detection using an arrayed detector. The other direction is to narrow down the flow channel to have equivalent sensitivity across the whole width of the flow channel. Success in this improvement would enable us to distinguish the causes of weak signal; incomplete hybridization or stray- ing way from sensitive detection zone, which will facilitate directional optimization of the measurement procedure. Continuing improvement of the proposed method hopefully will lead to establishment of a highly reliable reference measurement procedure for sequence-specific quantification of various target DNAs. This method is unique as it works under a straightforward measurement principle. In particular, this method obviates the need of reverse transcription in dPCR for quantification of RNA. Considering the uncertainty in the yield of reverse transcription, the proposed method could be developed to be a welcomed resource for bias correction of dPCR procedures for RNA quantification.

4. Conclusions

In this study, we first demonstrated the feasibility of sequence-specific DNA quantification by flow cytometric counting of target DNAs tagged with a number of FAM-labeled sequence-specific oligonucleotide probes. After successful optimization
for the model M13 DNA, the proposed method resulted in a comparable value to the results of other conventional DNA quantitation methods such as dPCR, UV spectrophotometry, and ID-MS. For sufficient fluorescence activation, about 30 FAM-labeled oligonucleotide probes were needed. In addition, the selection of a buffer solution played a critical role in successful hybridization of target DNA with probes. Against the fluorescent background noise from excess free probes, DNA-probe hybrids were successfully detected as genuine events in the optimized condition. The maximum measurement uncertainty could be up to 24.6%, referencing the result of UV spectrometry that highly likely overestimated due to unknown DNA impurities in the sample. However, the uncertainty decreased to 10.7% if compared to the result of dPCR that quantifies DNA in a sequence-specific manner. Along the future improvement of instrumentation to make detection sensitivity immune to the dispersion of the trajectories of individual DNA-probes hybrids, we will be able to firmly reduce the measurement uncertainty of the proposed method.

We value this measurement method because it would work for quantification of specific RNAs without reverse transcription. The reverse transcription step required for quantifying the quantification of specific RNAs without reverse transcription sensitivity immune to the dispersion of the trajectories of individual DNA-probes hybrids, we will be able to firmly reduce the measurement uncertainty of the proposed method.

The reverse transcription step required for quantifying RNA by dPCR could be a substantial uncertainty source in that a reliable reference measurement procedure working without reverse transcription is highly desired.

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Author contributions

S-R P and I C Y conceived and directed the project. H-B Y carried out most experiments with the help of C E L K-S H led the improvement of fluorescence detection of the flow cytometric setup. All authors approved the final manuscript.

Conflict of interests

The authors confirm no financial or non-financial competing interests.

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