Challenges for Accurate Quantification of RNA

Takema Hasegawa1, Diana Hapsari1 and Hitoshi Iwahashi2*

1 The United Graduate School of Agricultural Science, Gifu University, 1-1 Yanagido, Gifu, 501-1193, Japan
2 Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

ABSTRACT

Ribonucleic acid (RNA) quantification is an essential technique in biology. There has been remarkable progress in RNA quantification techniques over the recent years; however, the specificity of these techniques to quantify a very small amount of RNA is doubtful because of factors which can inhibit precise quantification. To develop a technique that leads to the most sensitive RNA quantification, these problems must be overcome. In this article, we first review the factors that inhibit precise quantification of RNA: the quality of RNA, secondary structure of RNA, efficiency of the enzyme reaction, annealing conditions, limitations of the experimental protocol and equipment, and detection sensitivity of the equipment. Next, we discuss the possible methods which contribute to these factors: RNA quality control focused on target RNA degradation, isothermal amplification, techniques for avoiding amplification errors, RNase H-dependent PCR, targeting using a fluorescent-labeled probe, targeting using a padlock probe, bridged/locked nucleic acid (BNA/LNA) and peptide nucleic acid (PNA), and the clustered regularly interspaced short palindromic repeat (CRISPR) system. One of the goals for the development of an ultrasensitive RNA quantification technique is the absolute quantification of RNA. Here, we discuss the techniques used for this type of RNA quantification.

Keywords
absolute quantification, accuracy of RNA quantification, isothermal amplification, RNA quality, RNA quantification

1. Introduction

RNA quantification is an essential technique in biology. When the cell responds to an external stimulus, in most situations, a corresponding RNA is transcribed from the DNA; the expressed RNA has many functions, including synthesis of proteins and ribozymes, transcriptional control, and signaling, in the case of mRNA, tRNA, and rRNA, respectively [1, 2, 3, 4, 5]. Measuring the expressed RNA allows us to determine the cell conditions. There are four types of bases that compose an RNA strand (adenine, cytosine, guanine, and uracil), following the Watson-Crick base pairs [6]. Studies on RNA processing enzymes (ligase, reverse transcriptase, and polymerase) have progressed considerably [7, 8, 9, 10]. In general, reverse transcription was performed using DNA primers and reverse transcriptase; the resulting cDNA was then amplified using sequence-specific primers and DNA polymerase to measure the RNA levels. Ligase and RNA polymerase were also used to target and amplify RNA. Nowadays, it is easy to recognize a target sequence, and there are also many methods to detect and quantify the RNA.

There has been remarkable progress in RNA quantification techniques over the recent years. Some reports have described ultrasensitive RNA detection methods, such as isothermal amplification and digital polymerase chain reaction (PCR) [11, 12, 13, 14, 15]. However, these methods are not ideal as in vivo RNA quantification cannot measure all RNA levels, while in vitro RNA quantification cannot measure only a single RNA type. RNA yield, RNA quality, efficiency of the enzyme reactions, and targeting of the probe are considered the main reasons behind these inaccurate results.
Accurate RNA quantification techniques are essential for advances in biology and medical science. A sensitive and accurate RNA quantification enables a deeper understanding of cellular behavior as it relies on absolute quantification. Absolute quantification, a method which evaluates the RNA levels in terms of copy numbers, as opposed to fold changes measured in techniques such as Quantitative PCR, is an essential technique for next-generation biology because the behavior of RNA in cells can be considered in relation to absolute numbers. Since absolute quantification can be expressed by copy/cell or copy/tissue, the results of experiments conducted at different experimental conditions can be shared with accuracy.

Coronavirus Disease 2019 (COVID-19) pandemic by new type coronavirus (SARS-Cov-2) has been occurred in the world [16]. This virus is an RNA virus and infects from human to human. Nowadays, some methods (e.g. PCR and isothermal amplification) targeted to RNA are often used to detect SARS-Cov-2. By the COVID-19 pandemic, virus research is becoming more critical with research on RNA viruses being actively conducted. Establishing a sensitive RNA quantification method enables the early detection of viruses with a low detection limit and enables researchers to observe their behavior. Hence, developing a sensitive and inexpensive RNA quantification method is crucial for keeping ahead of any future infection outbreaks.

To develop an RNA quantification technique, we should know the factors determining the detection sensitivity of each method. This review introduces the factors that cause RNA quantification results unreliable (Table 1). Furthermore, it also discusses a new technique for RNA quantification.

Table 1: Factors that inhibit precise RNA quantification and techniques

| Techniques to solve problems | Factors that inhibit precise quantification |
|-----------------------------|------------------------------------------|
| 3. 1 RNA quality control focused on target RNA degradation | 1. Quality of RNA |
| 3. 2 Isothermal amplification | 2. Secondary structure of RNA |
| 3. 3 Techniques for avoiding amplification error | 3. The efficiency of the enzyme reaction |
| 3. 4 RNase H dependent PCR | 4. Temperature and pH condition |
| 3. 5 Targeting by a fluorescent-labeled probe | 5. Limitation of constitutional change in RNA structure |
| 3. 6 Targeting by padlock probe | 6. The sensitivity of detection equipment |
| 3. 7 RNA (LNA) and PNA | |
| 3. 8 CRISPR system | |
| 4. 1 Digital quantification | |
| 4. 2 RNA detection with an enzyme-free method | |
| 4. 3 Method to dispense the solution more accurately | |

2. Factors that inhibit precise RNA quantification

To develop an RNA quantification technique, we should understand the factors that determine its detection limit. In this section, we focus on six main such factors.
2.1 Quality of RNA

RNA degradation and contamination occur during RNA extraction protocols and its preservation. RNA quality is crucial for RNA quantification [17, 18, 19]. A small difference in RNA quality leads to a large difference in the quantitative results obtained. In general, RNA quality is evaluated based on the yield, contamination, and degradation. Measuring the ultraviolet absorbance of the extracted RNA is a well-known method that can be used to evaluate the yield and contamination. The maximum absorption wavelength of RNA is 260 nm, which is the wavelength for determining RNA concentration. Contamination is evaluated by measuring the absorbance at 280 nm and 230 nm to determine the protein and chemical compound contamination, respectively. Furthermore, the A260/A280 and A260/A230 ratios are used as indices of protein and chemical contamination, respectively [20]. rRNA gel electrophoresis is another well-known method for evaluating RNA degradation. The degree of RNA degradation can be understood from the electrophoretic band shape, and the ratio of fluorescence intensity of two types of rRNA (18S and 28S for eukaryotes, and 16S and 23S for prokaryotes and archaea) [21]. Agilent Technologies has also developed an RNA integrity number (RIN) as an index of RNA degradation [21]. RIN was calculated from the capillary gel electrophoresis data of rRNA. Many researchers use this method because it allows them to determine the degree of RNA degradation as a numerical value.

However, these RNA quality evaluation methods have their own disadvantages. Absorbance measurements cannot be used for small amounts of RNA (i.e., values that are below the detection limit) [22]. Moreover, while an absorbance measurement evaluates contamination, it cannot evaluate the direct effects of contamination on enzymes. rRNA gel electrophoresis also cannot be used for RNA values below the detection limit. Furthermore, this method can be used to evaluate the degradation of rRNA, but not mRNA and non-coding RNA, which is generally the target RNA. There are many types of RNA degrading enzymes (RNases), such as exonucleases, endonucleases, single-strand-specific RNases, double-strand-specific RNases, and sequence-specific enzymes [23]. It is expected that the model of RNA degradation would differ depending on the type of RNA. The evaluation method of RNA degradation using rRNA gel electrophoresis cannot cover subtle levels of degradation of the target RNA. However, remarkable progress has been made in RNA quantification techniques. The amount of target sample and RNA used for research is decreasing. Nevertheless, a more sensitive RNA quality evaluation method is still needed to develop an accurate RNA quantification technique.

RNA extraction procedures should be performed carefully. When extracting RNA from cells or viruses, most methods involve filtration or separation operations to remove DNA and proteins. RNA loss always occurs during surgery. This loss is not always the same for each sample [24, 25]. When performing an absolute RNA quantification, it is necessary to consider the RNA loss which can take place through extraction and handling protocols.

2.2 Secondary structure of RNA

RNA quantification results also depend on the RNA structure. When targeting highly structured RNA, annealing of the ssDNA and inhibition of reverse transcriptase activity occur; thus, an accurate reverse transcription cannot be performed [26, 27, 28]. In addition, when targeting short RNAs, such as siRNA, it is difficult to perform an accurate RNA quantification because it is easily affected by degradation, and the reverse transcription efficiency is also reduced [29, 30]. The more sensitive the RNA quantification method, the more dependent it is to the RNA structure. Even in the comprehensive analysis of RNA-seq, which targets a small amount of sample, it is necessary to consider the influence of the structure of each RNA on the reverse transcription efficiency and ultimately the quantification of the target RNA.
2.3 Efficiency of the enzyme reaction

We use enzymes such as reverse transcriptase and DNA polymerase to measure RNA content in most of the cases. Because enzyme reactions do not always happen at the maximum efficiency, much attention must be paid to enzyme usage when developing more sensitive RNA quantification methods [31, 32, 33]. DNA polymerase is often used for RNA quantification but causes amplification errors, even if the DNA polymerase has proofreading activity [34, 35]. Furthermore, DNA polymerase also amplifies non-target sequences and primer dimers in some cases [36]. This shows that DNA polymerase can start synthesizing DNA from a point that is not properly annealed. If the 3′ end of the primer anneals even with a few bases, there is a risk that DNA synthesis will be initiated. Reverse transcriptase is also often used for RNA quantification, but the reverse transcriptase reaction is not ideal because its efficiency is influenced by the RNA structure [37].

PCR, such as real-time PCR, digital PCR, and RNA sequencing, is another technique often used for RNA quantification. However, PCR is not suitable for sensitive RNA quantification. PCR can amplify a target DNA logarithmically twice. It has the advantages associated with the use of PCR, but has a disadvantage in terms of the sensitivity of RNA quantification. As mentioned above, the accuracy of DNA polymerase is not 100 %, and it has some associated errors [31]. RNA quality also affects the accuracy and amplification efficiency of DNA polymerase [36]. PCR logarithmically amplifies not only the target DNA but also these errors. If there are many target sequences in a sample, we may obtain results before errors occur and are amplified. However, if the number of target sequences in a sample is low, the results are unreliable because the errors are amplified before the target sequences. Risk factors, such as targeting errors and primer dimers, are also increased. Therefore, when developing a new sensitive RNA quantification method, the use of PCR should be avoided, and a way to solve this problem should be identified.

2.4 Annealing conditions

When targeting a certain sequence, two sequences that bind to each other, such as a primer and probe, which are complementary to each other, are used in most cases. Adenine (A), uracil (U), thymine (T), cytosine (C), and guanine (G) bind to each other via a hydrogen bond because the primer and probe anneal to the target sequence via hydrogen bonds. However, the annealing ratio changes with temperature and salt concentration [38, 39]. The lower the temperature and salt concentration, the stronger the hydrogen bonds. The weaker the hydrogen bond, the more specific sequences can be annealed, thus increasing the specificity [40, 41]. If the hydrogen bond is too weak, annealing cannot be performed. Even though the annealing process can be controlled by temperature and salt concentration, it is difficult to control it entirely [42]. The primer and template RNA or DNA amplicon repeats the divergence and annealing in an equilibrium state [43, 44]. The equilibrium cannot be biased toward a 100 % accurate annealing. The equilibrium state of annealing is affected by the sequence pool in the sample [45]. It is unlikely that primers or probes will anneal to 100 % of the target DNA accurately. It is not an exaggeration to say that the limit of RNA quantification is determined by this targeting error.

2.5 Limitations of experimental protocol and equipment

In general, many researchers use a micropipette to prepare solutions. When properly calibrated and controlled, a micropipette can achieve an accuracy of over 99.5 % [46]. This value is sufficient for carrying out ordinary experiments. On the other hand, when aiming at a sensitive absolute RNA quantification, pipetting may not be possible. For example, when performing quantitative PCR, a standard curve is prepared by serially diluting a standard nucleic acid. For more sensitive quantification, the standard must be prepared using a standard curve of 1 to 100 copies of standard nucleic acids. However, this is difficult to achieve. When a low-concentration standard
nucleic acid is diluted using a simple liquid dilution procedure, such as pipetting, the diluted standard nucleic acid varies [47]. According to the Poisson distribution, when a low copy number of DNA is dispensed, the probability that a given number of molecules will be x in number or Px, is expressed by the following formula:

\[ P_x = \frac{\mu^x \times e^{-\mu}}{x!} \]

Px: Probability of a given number of molecules to be x; \( \mu \): Average copy number; x: Actual DNA copy number per fixed area.

Table 2 shows the establishment of the copy number in the solution that is obtained when a solution with an average concentration of 1 or 2 copies/well is dispensed. As shown in the table, a copy of one RNA per well cannot be dispensed in a standard laboratory. Further, this method is applied to liquid dilution using a micropipette and liquid separation via diffusion into a microcell. Even using digital PCR methods, one copy per well cannot be controlled accurately; thus, correction is performed using a Poisson distribution [48]. When studying sensitive absolute RNA quantitation, equipment to control one copy of RNA is needed.

| Actual DNA copy number per fixed area (x) | 0 | 1 | 2 | 3 | 4 | 5 |
|------------------------------------------|---|---|---|---|---|---|
| Average copy number (\( \mu \))          | 1 | 36.8 | 36.8 | 18.4 | 6.1 | 1.5 | 0.3 |
| 2                                         | 13.5 | 27.1 | 27.1 | 18.0 | 9.0 | 3.6 |

2.6 Sensitivity of the detection equipment

Another factor that determines the limit of RNA detection is the sensitivity of the detection equipment. Most RNA detection methods, including RT-PCR, one of the most commonly used RNA detection methods, use DNA amplification because it is challenging to measure the target RNA directly using the detection equipment that are currently in use in standard laboratories. However, DNA amplification for RNA detection increases the risk of amplification errors. Ideally, the sample RNA taken for the quantification should project the same concentration value as the target RNA. Technologies that detect single-molecule RNA, such as fluorescence microscopy and single-molecule fluorescence in situ hybridization (smFISH), have been developed [49, 50, 51, 52]. However, they are not widely used because they are too expensive for researchers to use in ordinary experiments.

3. Developing RNA quantification

Six main factors that determine the RNA detection limit were introduced in the previous section. In this section, we introduce RNA quantification methods and techniques that can solve these problems. In addition, we discuss the requirements for a more sensitive RNA quantification.

3.1 RNA quality control focused on target RNA degradation

We discussed the possible methods that contribute to the quality of RNA, in section 2.1. RIN is used to evaluate RNA degradation; in general, it evaluates the quality of rRNA and not that of mRNA and target RNA. Moreover, RIN cannot be used for formalin-fixed paraffin-embedded (FFPE) samples or for a minimal number of RNA samples [53, 54]. A more sensitive RNA quality evaluation method than RIN is needed for absolute RNA quantification. A
method that can improve RNA quantification is RNA degradation evaluation, which focuses on the degree of degradation in each mRNA region. RNA degradation by cellular RNases depends on the RNA sequence, region, and folding structure [23]. Therefore, we can use the difference in the degree of degradation of each RNA region for RNA degradation evaluation. The transcript integrity number (TIN) value is calculated by evaluating the survival rate of each mRNA region using RNA-Seq data, and by using this method, it is possible to perform a more sensitive evaluation than by using RIN [55]. On the other hand, it is considered that RNA degradation can be quickly evaluated by designing a standard RNA having an easily degraded region and a region that is not likely to be degraded and evaluating the survival rate of each region of this standard RNA [25]. The standard RNA can also be used to measure the loss of the final yield during RNA extraction protocols [25].

3.2 Isothermal amplification

We discussed the possible methods that could contribute to the efficiency of the enzymatic reaction in section 2.3. Some isothermal amplification methods are faster than PCR because they do not require temperature changes [56]. Furthermore, since that are resistant to enzyme inhibitors, a more stable gene amplification can be obtained than when using PCR [56]. Additionally, since that are performed at the optimum temperature of the enzyme, the enzyme inhibition reaction is unlikely to occur in the latter stage of amplification [56]. Moreover, some isothermal amplification methods do not amplify DNA logarithmically but linearly. Therefore, isothermal amplification can be used to measure a small number of RNAs. Isothermal amplification has been used for virus detection, SNP detection, sex detection, and RNA amplification [57, 58, 59]. Examples of isothermal amplification are loop-mediated isothermal amplification (LAMP) [60], rolling circle amplification (RCA) [61], transcription-mediated amplification (TMA) [62], nucleic acid sequence-based amplification (NASBA) [62], strand displacement amplification (SDA) [63], isothermal multiple displacement amplification (IMDA) [64], helicase-dependent amplification (HAD) [65], single primer isothermal amplification (SPIA) [66], and SmartAmp [58, 67]. Some isothermal amplification methods can detect 0 – 10 copies of a sequence [68, 69, 70]. Even in real-time quantification, where the amplification rate is likely to be affected, quantification up to the zeptomole order is possible [14]. However, isothermal amplification has the disadvantage of having an amplification power that is too strong. This creates a risk of amplifying a non-target amplicon [14]. In addition, detection using a probe and treatment with a restriction enzyme can be used to confirm the amplification accuracy, but in such instances, it is not as easy to measure the melting temperature (Tm) value for real-time qPCR.

3.3 Techniques for avoiding amplification errors

We discussed the possible methods contributing to the enzyme reaction efficiency and annealing conditions in sections 2.3 and 2.4, respectively. Even if a method with an amplification power capable of detecting one copy of RNA is developed, it cannot be used for RNA quantification if there is a risk that the background noise becomes high due to amplification errors. At the same time, it is challenging to eliminate the amplification errors using gene amplification. Therefore, methods for correcting or stopping amplification errors have been developed. Among these, two methods are effective in achieving a highly accurate gene amplification. The first method is using a sequence-specific probe. Because the probe can recognize the target sequence, we know whether this amplicon is correct or not [13, 71]. The second method is to improve the accuracy of DNA amplification. However, it is difficult to dramatically improve the accuracy of primer hybridization and enzyme amplification reactions. Therefore, the technique of stopping erroneous amplification can contribute to further the improvement in the accuracy of DNA amplification. For example, the Taq MutS enzyme recognizes a DNA mismatch and binds to it [58, 72] (Fig.1).
MutS binding inhibits DNA polymerase reactions. This technique was used for SmartAmp, which is an isothermal amplification, and significantly improved the accuracy of DNA amplification.

![Diagram of Taq Must stop a mis-amplification](image)

Figure 1: Taq Must stop a mis-amplification (redrawn according to Mitani et al. [58])

### 3.4 RNase H-dependent PCR

We discussed a possible method that contributes to the annealing conditions in section 2.4. One of the causes of non-specific amplification in PCR and isothermal amplification is primer annealing errors. Several bases at the 3’ end of the primer bind to the non-target region and further amplification occurs producing an erroneous amplicon from the annealing error. Once a non-specific amplicon is produced, it serves as a template to enhance non-specific amplification. RNase H-dependent PCR (rhPCR) is one of the methods used to prevent non-specific amplification [73, 74] (Fig.2). In the rhPCR, in which RNase H2 enzyme and rh primer with a blocking modification at the 3’ end and 6th DNA base from the 3’ end is replaced with RNA. The rh primer prevents the erroneous amplification by blocking the 3’ end. RNase H2 cleaves the rh primer RNA only when the rh primer and the DNA sample form an entirely complementary strand. The cleaved rh primer removes the blocking modification and initiates DNA amplification. Although many current studies are attempting to determine SNPs, DNA amplification accuracy can be expected to improve; thus, the rh PCR technique can be used when more sensitive DNA amplification is required.
3.5 Targeting using a fluorescent-labeled probe

We discussed the possible methods that contribute to the enzyme reaction efficiency, annealing conditions, and sensitivity of the detection equipment outlined in sections 2.3, 2.4, and 2.6, respectively. Reverse transcription is often used to amplify the targeted RNA. However, reverse transcription involves two steps: primer annealing and the reverse transcription reaction, and these reactions do not always progress optimally. The efficiency of reverse transcription decreases depending on the RNA structure and length. One way to solve these problems is to target the probe; and because the probe can anneal with short sequences (10–20 nt), it can be used for short RNAs, such as miRNAs [75, 76]. For a highly structured RNA, efficiency can be maintained by targeting a site that is not as highly structured. Moreover, because the probe can skip the reverse transcription step, the loss of RNA until the quantification process can be reduced.

Several types of probes have been developed. Fluorescent-labeled DNA probes are often used to detect target RNAs, both in vivo and in vitro [77]. Different types of fluorescently labeled probes can be used. The simplest one, is a probe with one fluorescent labeling compound modified at the end of the ssDNA. However, this probe requires amplification of the target sequence or washing to remove all the non-specifically boundrobe sequences after targeting. This probe is not suitable because it involves an additional procedure to detect a small number of RNAs. Simultaneously, a probe that fluoresces when it forms a double strand with the target sequence is also developed. The exciton-controlled hybridization-sensitive fluorescent oligonucleotide (ECHO) probe is a probe with a fluorescent compound (typically thiazole orange) modified in the middle position of ssDNA [78] (Fig.3). When the ECHO probe forms a double strand with the target sequence, the fluorescent compound is inserted into the double-stranded DNA and emits fluorescence. Thiazole orange emits some fluorescence, even when it is not inserted in the double-stranded DNA, but its fluorescence is weakened when the distance between them is extremely short. Since this can reduce the background noise, an ECHO probe has been developed by modifying two thiazole orange molecules at the same position in the ssDNA. The ECHO probe has been applied not only to PCR but also to isothermal amplification [71].
A probe utilizing fluorescence resonance energy transfer (FRET) and quenching of fluorescence by a quencher has also been developed [79]. By modifying a fluorescent substance and a quencher at both ends of ssDNA with a hairpin structure, it is possible to suppress fluorescence in a single step and emit fluorescence at the time the double-stranded DNA binds to the target sequence. By using two fluorescent probes and by targeting adjacent sequences of the target RNA, FRET can occur by bringing two types of fluorescent compounds close to each other, and this specific fluorescence can be measured. However, these methods have some points that need to be improved for sensitive RNA quantification, such as the competition between the target sequence and the probe sequence itself, and the difference in the annealing efficiency between the target sequences.

Fluorescent probes can be used to achieve sensitive RNA quantitation, but there is some need for improvement. It is necessary to consider that the annealing efficiency of the target sequence changes depending on the RNA pool sequence and that the background noise is suppressed. In addition, because there are restrictions on the equipment and conditions for directly detecting a single RNA, DNA amplification is often performed. The risks of using DNA amplification for RNA quantification have been extended.

3.6 Targeting using padlock probe

We discussed the possible methods that contribute to the secondary structure of RNA in section 2.2. The padlock probe is an ssDNA designed with complementary sequences of the target sequence at both ends [80] (Fig.4). Circular ssDNA is synthesized by binding to the target sequence and is connected by a ligase. Circular ssDNA serves as a template for RCA DNA amplification. The target RNA can be detected by detecting long ssDNAs synthesized using RCA. Because long ssDNA is a repetitive sequence, the detection method can be used not only for the staining of DNA with SYBR Green, but also for detection using a probe [81]. It can detect sequences up to the attomole order [14]. Padlock probes and RCA have been used to detect very small amounts of RNA and miRNAs. It has also been applied to the visualization of RNA in cells in situ [81].
On the other hand, the Padlock probe has disadvantages. Since padlock probe targeting also uses DNA-RNA hybridization, the annealing efficiency must be considered. Ligase circulates the Padlock probe, but its reaction efficiency is not 100%. The utilization of various ligases, such as T4 DNA ligase, RNA ligase, and SplinR ligase, has been studied [82, 83, 84]. It is also necessary to pay attention to padlock probe dimer formation. When the ends of each of the two Padlock probes are annealed and bound to the target sequence, a linear Padlock probe dimer is formed. This dimer does not become as a strong starting point for amplification as a circular Padlock probe, and it also causes background noise.

3.7 BNA (LNA) and PNA

We discussed the possible methods that contribute to the enzyme reaction efficiency and the annealing conditions in sections 2.3 and 2.4, respectively. Bridged/locked nucleic acid (2,4-BNA/LNA) and peptide nucleic acid (PNA) are used to increase the Tm value of the modified nucleic acid and increase the efficiency of hybridization with the target sequence [85, 86] (Fig.5). Ribose, one of the constituents of DNA, can change its structure into several conformations. As the degree of freedom in the structure of nucleic acids is high, the Tm value decreases. Therefore, LNA (BNA) is designed as a crosslinking structure on the ribose of DNA. This crosslinked structure locks the structural change of ribose and reduces the degree of freedom of the DNA structure. Therefore, the Tm value is increased. On the other hand, PNA is a compound in which the sugar-phosphate skeleton of nucleic acids is replaced with a skeleton, with N-(2-aminoethyl) glycine as a unit, and a base is bound with a methylene carbonyl bond. PNA also increases the Tm value by lowering the degree of freedom of the skeleton.
The modified nucleic acid with an increased Tm value makes hybridization with the target sequence more robust. Therefore, the specificity can be improved compared to that of the usual primer. In addition, because it can be designed to be shorter than the usual primers, the research design options are increased. The modified nucleic acid can stop the DNA amplification reaction by blocking modification on the terminal, in addition to the start of amplification and targeting. In SNP detection studies, the detection of SNPs by stopping the SNP amplification region with a modified nucleic acid has been studied [87].

An excellent advantage of using LNA and PNA is the improvement in the Signal/Noise (S/N) ratio. As the modified nucleic acid has a strong affinity for the target sequence, it is possible to design a study under reaction conditions (e.g., high temperature and low pH) that do not generate noise due to nonspecific amplification, thereby improving the S/N ratio [88]. It can be expected that a high S/N ratio must be achieved for absolute RNA quantification, and that the use of modified nucleic acids will help achieve this goal.

### 3.8 CRISPR system

We discussed the possible methods that contributes to the secondary structure of RNA and the sensitivity of the detection equipment, as explained in sections 2.2 and 2.6, respectively. The CRISPR system is an immune system found in bacteria [89, 90]. It is possible to cleave any sequence by designing a guide RNA. CRISPR is mainly used as a genome-editing tool [91]. On the other hand, it is also being studied for RNA quantification. CRISPR Cas13a is characterized by the formation of a complex with a guide RNA, and when it recognizes a sequence complementary to the guide RNA, it cleaves the surrounding RNA randomly [92]. A Specific High-sensitivity Enzymatic Reporter Unlocking (SHERLOCK) RNA detection system utilizing this characteristic was studied [93] (Fig.6). SHERLOCK uses the guide RNA-Cas13a to target a sequence and short-chain RNA modified with a fluorescent compound and a quencher at both ends. When the target RNA is present, guide RNA-Cas13a acquires RNase activity and cleaves the modified RNA. When the modified RNA is cleaved, the quencher leaves and the fluorescent compound exhibit fluorescence. As a result, target RNA was detected. The application of SHERLOCK for virus detection has been studied previously [92].

Figure 5: The chemical formulas of DNA, LNA, and PNA monomer
The CRISPR Cas system has the potential for RNA quantification because it can target a sequence and cleave RNA or DNA. It is important to note that the reaction efficiency of the enzyme is almost 100%. To apply the CRISPR system for RNA quantification, it is necessary to optimize the targeting efficiency and cleavage efficiency of the target sequence.

4. Absolute quantification of RNA

Absolute quantification of RNA quantifies RNA by copy number. Evaluating the number of RNAs with a specific copy number is an essential technique for next-generation biology because the behavior of RNA in cells can be considered with a specific image. With the recent development of RNA quantification technology, absolute quantification of RNA has become possible. In this section, we introduce technologies that can be used for absolute quantification of RNA.

4.1 Digital quantification

We discussed the possible methods that contribute to the efficiency of the enzyme reaction (section 2.3), annealing conditions (section 2.4), and digital quantification methods that have the potential to be used for absolute RNA quantification (section 2.6). A typical example is digital PCR. In digital PCR, the target DNA is divided into many microsections, and gene amplification is performed using PCR [11, 94]. The target DNA is quantified by counting the positive sections used for DNA amplification in the entire microsection. Digital PCR can ignore the differences in DNA amplification rates between the samples. This method can be used for more sensitive quantification than real-time PCR, which quantifies RNA based on the DNA amplification rate.

On the other hand, it cannot be said that a complete absolute quantification has been performed yet [95]. When two or more target genes are in the same section, they are difficult to determine. Although a correction method has been established and accurate quantification can be performed, it still cannot be claimed as a complete absolute quantification.

Digitizing RNA quantification is an effective method to obtain an RNA absolute quantification. Any method used to detect a single RNA can be digitized to perform absolute RNA quantification. Unless a machine that can directly detect a single RNA is widely used, the target RNA needs to be amplified. The development of an absolute RNA quantification method will be based on digital DNA amplification, as DNA amplification carries the risk of differences in amplification rate between samples, thus affecting the results.
4.2 RNA detection using an enzyme-free method

We discussed the possible methods that contribute to the efficiency of the enzyme reaction in section 2.3. Most methods used for RNA detection utilize DNA amplification by polymerase. However, polymerase is an enzyme, and the amplification reaction is error-prone. Further, the more complicated the method, the more necessary it is to ensure accuracy in each process, as the results tend to be unstable. There are limits to biological reactions. Therefore, enzyme-free nucleic acid detection methods have also been investigated.

Most methods which do not use enzymes have been devised to detect hybridized DNA by using only the RNA-DNA hybrid. In nucleic acid circuits, the target RNA triggers a reaction to change the DNA structure and hybridization [96]. By combining catalytic hairpin assembly (CHA) and a hybridization linkage reaction (HCR), methods to manipulate the distance of the fluorescently modified DNA to cause FRET and to detect the RNA have been developed. Studies in which DNA has the activity of a DNAzyme only when a target sequence exists, by utilizing structural DNA changes, and in which detection is performed using the reaction, have also been conducted [97]. For example, designing a DNA probe encoding a DNAzyme with peroxidase activity and H2O2-mediated colorless 2,2'-azino-bis (3-ethylbenzothiazolin-6-sulfonic acid) when targeting the target RNA, it can catalyze the oxidation of ABTS^2- to green ABTS^-.

Nanoparticles are also used for enzyme-free RNA detection. For example, DNA is modified on the surface of gold nanoparticles and gold film, and a DNA super-sandwich targets the target RNA. A method for electrically quantifying RNA using an amplified surface plasmon resonance (SPR) biosensor has also been developed [98].

The enzyme-free methods have the advantage that the accuracy and efficiency of the enzyme reaction can be ignored during the quantification. By evaluating the hybridization efficiency of DNA and RNA and the detection sensitivity of the device, it is possible to develop a better RNA quantification method.

4.3 Method to dispense the solution more accurately

We discussed the possible methods that contribute to the topics discussed in section 2.5 about the limitations of the experimental protocol and equipment. When performing a sensitive absolute quantification of RNA, it is desirable to evaluate one copy of RNA as a standard substance. However, in ordinary research facilities and micropipette operations, the accuracy of solution dispensing is limited. Therefore, methods for dispensing a smaller amount of solution and a standard nucleic acid into one copy/well have been developed.

In emulsion PCR, a water-in-oil (W/O) emulsion is utilized to generate numerous droplets of the reaction mixture in a bulk oil phase, capable of performing many independent reactions in parallel [99]. The liquid volume of this droplet is controlled to the femtolitre (10^-15 L) order. T-junction and flow-focusing generate droplets by separating the liquid phase from a thin capillary with oil from the outside. The droplet liquid volume and accuracy are highly dependent on the flow rate, liquid properties, and device shape. A theoretical model that controls the droplet size, such as by the shape of a fixed flow junction has been developed [100, 101, 102, 103]. These technologies are applied to the droplet digital PCR.

It is difficult to dispense RNA as one copy/well. In addition, there are few standard products in which RNA is dispensed as one copy/well. In order to develop an RNA quantification method that can measure up to one copy of RNA, a standard product in which RNA is dispensed as one copy per well is required. In recent years, although not regarding RNA, standard products have been developed in which DNA is dispensed as one copy/well [104]. In this method, cells are discharged into the well while controlling the number of cells with an inkjet head, instead of dispensing the DNA itself into the well. Using this method, they succeeded in creating a highly accurate copy/well reference material. On the other hand, this method cannot be applied because RNA is transcribed many times in cells.
and is unstable. However, the method of creating a standard product by controlling a considerable substance containing one nucleic acid, instead of dispensing the nucleic acid itself, could be applied to the production of a single copy/well RNA reference substance.

5. Discussion

Table 3 lists the advantages of each technique. These techniques contribute to the accuracy of RNA quantification. However, it is necessary to pay attention while considering isothermal amplification and targeting by the padlock probe because these have a relatively high risk of errors. Most techniques are costly because they require new equipment, especially modified DNA and special enzymes. RNA quality control focused on target RNA degradation can reduce the cost because it can be evaluated at the same time as the quantitative experiment. Part of the isothermal amplification for avoiding amplification error can use unmodified DNA, and the cost can be reduced. We evaluated whether the same experimental design could be used for many types of RNA. We evaluated an underlying method to determine whether there were many experimental reports and history.

| Technique                                      | Accuracy | Cost  | Versatility | Underlying method |
|------------------------------------------------|----------|-------|-------------|-------------------|
| 3. 1 RNA quality control focused on target RNA degradation | ++       | ++    | ++          | -                 |
| 3. 2 Isothermal amplification                   | -        | +     | ++          | +                 |
| 3. 3 Techniques for avoiding amplification error | +        | +     | ++          | -                 |
| 3. 4 RNase H dependent PCR                      | +        | -     | -           | -                 |
| 3. 5 Targeting by a fluorescent-labeled probe  | -        | -     | -           | ++                |
| 3. 6 Targeting by padlock probe                | -        | -     | +           | +                 |
| 3. 7 BNA (LNA) and PNA                         | +        | -     | -           | +                 |
| 3. 8 CRISPR system                             | +        | -     | +           | -                 |
| 4. 1 Digital quantification                     | ++       | -     | ++          | +                 |
| 4. 2 RNA detection with an enzyme-free method  | +        | -     | -           | -                 |
| 4. 3 Method to dispense the solution more accurately | +   | -     | +           | +                 |

++ means that the technique has a very high contribution. + means that the contribution is high. While, - means that the technique does not give any contribution to the factor.

To develop a more sensitive RNA quantification method, we need to solve the problems elaborated in this review. RNA structure mainly affects reverse transcription efficiency. For this, it is useful to use a probe that can target short sequences. The efficiency of the enzyme reaction mainly affects the rate and accuracy of DNA amplification. For this, it is better to ignore the amplification rate using digital amplification instead of quantifying the target RNA at the amplification rate, as done using real-time quantification.

It is considered that an RNA absolute quantification is developed by combining digital DNA amplification and isothermal amplification because the amplification power of isothermal amplification is stronger than that of traditional PCR. However, we need to be careful about the amplification errors as isothermal amplification has a very strong amplification power, it has an amplification error risk associated. Amplification errors increase the background signal of digital quantification and obscure an accurate signal. To combine digital DNA amplification and isothermal amplification, we need to improve the accuracy while maintaining a high amplification power. The Taq MutS enzyme, fluorescence probes, and rhPCR are expected to contribute to improving the accuracy of DNA amplification.
Moreover, the enzyme-free method is significant because the enzyme reaction efficiency can be ignored. Annealing mainly affects the accuracy of DNA amplification. Therefore, it is adequate to control the Tm value using BNA or PNA. Furthermore, non-specific amplification is initiated by the complementation of only a few bases at the 3’ end. To prevent non-specific amplification, it is also useful to modify the 3’ end, as in rhPCR. Overall, we believe that a sensitive absolute RNA quantification technology will be developed by evaluating and managing RNA quality at the mRNA level and applying RNA detection methods with a high S/N ratio based on digital quantification methods. In the current scenario, the advantage of using DNA amplification is that it is inexpensive because existing detection devices can be used. On the other hand, the advantage of using the enzyme-free method is that it is more accurate, although it is costly.

An absolute quantification of 1–10 copies of RNA require standard RNA, which is also controlled by 1–10 copy numbers. However, commonly used current research facilities cannot be used to control RNA at such low concentration levels. Currently used micropipettes are limited to dispensing a minimum of 0.5 µL, but it is necessary to dispense less than that amount for more sensitive research experiments. The emulsions can be controlled to the order of fL. It is expected that liquid separation/mixing/detection technologies using emulsions will be developed and that their costs will be reduced.

On the other hand, if there is a reliable RNA standard, sensitive RNA absolute quantification is possible, even at a micropipette-level research facility. If there is a standard RNA in which 1–10 copies of RNA are accurately controlled, a new RNA quantification method can be developed and evaluated based on that standard RNA. It is necessary to develop better standards for low-molecular-weight RNA and standard RNA products based on these standards.

RNAseq is widely used because of the development of next-generation sequencing techniques. RNAseq can comprehensively quantify transcription; however, when focusing on individual RNAs, its accuracy is insufficient from the viewpoint of absolute quantification. Strengthening techniques for accurate, fast, and inexpensive absolute quantification of some target sequences allows for a more detailed observation of organisms. Only then the behavior of the RNA in a cell can be considered in a more concrete image. In addition, the demand for technological development related to virus detection has increased in recent years. Developing more accurate RNA quantification methods will contribute to developments in biology and will help in staying ahead of future outbreaks.

REFERENCES

[1] Dinger ME, Mercer TR and Mattick JS (2008) RNAs as extracellular signaling molecules. J. Mol. Endocrinol., 40(4): 151–159.
[2] Hannon GJ (2002) RNA interference. Nature, 418 (6894): 244–251.
[3] Ha M and Kim VN (2014) Regulation of microRNA biogenesis. Nat. Rev. Mol. Cell Biol., 15(8): 509–524.
[4] Horning DP and Joyce GF (2016) Amplification of RNA by an RNA polymerase ribozyme. Proc. Natl. Acad. Sci. USA., 113(35): 9786–9791.
[5] Samanta B and Joyce GF (2017) A reverse transcriptase ribozyme. Elife., 6:e31153.
[6] Xia T, SantaLucia J Jr, Burkard ME, Kierzek R, Schroeder SJ, Jiao X, Cox C and Turner DH (1998) Thermodynamic parameters for an expanded nearest-neighbor model for formation of RNA duplexes with Watson-Crick base pairs. Biochemistry, 37(42): 14719–14735.
[7] Ho CK and Shuman S (2002) Bacteriophage T4 RNA ligase 2 (gp24.1) exemplifies a family of RNA ligases found in all phylogenetic domains. Proc. Natl. Acad. Sci. USA., 99(20): 12709–12714.
[8] Sauter KB and Marx A (2006) Evolving thermostable reverse transcriptase activity in a DNA polymerase scaffold. Angew. Chem. Int. Ed. Engl., 45(45): 7633–7635.
[9] Rahman M, Uddin M, Sultana R, Moue A and Setu M (2013) Polymerase chain reaction (PCR): A short review. Anwer Khan Modern Medical College Journal, 4(1): 30–36.
[10] Lohman GJ, Zhang Y, Zhelkovsky AM, Cantor EJ and Evans TC Jr (2014) Efficient DNA ligation in DNA-RNA hybrid helices by Chlorella virus DNA ligase. Nucleic Acids Res., 42(3): 1831–1844.
[11] Vogelstein B and Kinzler KW (1999) Digital PCR. Proc. Natl. Acad. Sci. USA., 96(16): 9236–9241.
[12] Hindson CM, Chevillet JR, Briggs HA, Gallicchio ET, Ruf IK, Hindson BJ, Vessella RL and Tewari M (2013) Absolute quantification by droplet digital PCR versus analog real-time PCR. Nat. Methods, 10(10): 1003–1005.
[13] Deng R, Zhang K, Sun Y, Ren X and Li J (2017) Highly specific imaging of mRNA in single cells by target RNA-initiated rolling circle amplification. Chem. Sci., 8(5): 3668–3675.
[14] Tian W, Li P, He W, Liu C and Li Z (2019) Rolling circle extension-actuated loop-mediated isothermal amplification (RCA-LAMP) for ultrasensitive detection of microRNAs. Biosens. Bioelectron., 128: 17–22.
[15] Xue T, Liang W, Li Y, Sun Y, Xiang Y, Zhang Y, Dai Z, Duo Y, Wu L, Qi K et al. (2019) Ultrasensitive detection of miRNA with an antimonene-based surface plasmon resonance sensor. Nat. Commun., 10(1): 28.
[16] Chakraborty I and Maitry P (2020) COVID-19 outbreak: Migration, effects on society, global environment and prevention. Sci. Total Environ., 728: 138882.
[17] Fleige S and Pfaffl MW (2006) RNA integrity and the effect on the real-time qRT-PCR performance. Mol. Aspects Med., 27(2-3): 126–139.
[18] Kashofer K, Vietler C, Pichler M and Zatloukal K (2013) Quality control of RNA preservation and extraction from paraffin-embedded tissue: implications for RT-PCR and microarray analysis. PLoS One, 8(7): e70714.
[19] Li X, Nair A, Wang S and Wang L (2015) Quality control of RNA-seq experiments. Methods Mol. Biol., 1269: 137–146.
[20] Manchester KL (1996) Use of UV methods for measurement of protein and nucleic acid concentrations. Biotechniques, 20(6): 968–970.
[21] Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M, Lightfoot S, Menzel W, Granzow M and Ragg T (2006) The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Mol. Biol., 7: 3.
[22] Aranda R 4th, Dineen SM, Craig RL, Guerrieri RA and Robertson JM (2009) Comparison and evaluation of RNA quantification methods using viral, prokaryotic, and eukaryotic RNA over a 10(4) concentration range. Anal. Biochem., 387(1): 122–127.
[23] Houseley J and Tollervey D (2009) The many pathways of RNA degradation. Cell, 136(4): 763–776.
[24] Kresse SH, Namløs HM, Lorenz S, Berner JM, Myklebost O, Bjerkhagen B and Meza-Zepeda LA (2018) Evaluation of commercial DNA and RNA extraction methods for high-throughput sequencing of FFPE samples. PLoS One, 13(5): e0197456.
[25] Hasegawa T, Takahashi J and Iwahashi H (2018) RNA quality control using external standard RNA. Pol. J. Microbiol., 67(3): 347–353.
[26] Amann RI, Ludwig W and Schleifer KH (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev., 59(1): 143–169.
[27] Malboeuf CM, Isaacs SJ, Tran NH and Kim B (2001) Thermal effects on reverse transcription: improvement of accuracy and processivity in cDNA synthesis. Biotechniques., 30: 5.
[28] Mayer G, Müller J and Lüsse CE (2011) RNA diagnostics: real-time RT-PCR strategies and promising novel target RNAs. Wiley Interdiscip Rev. RNA., 2(1): 32–41.
[29] Dabney J and Meyer M (2012) Length and GC-biases during sequencing library amplification: a comparison of various polymerase-buffer systems with ancient and modern DNA sequencing libraries. Biotechniques., 52(2): 87–94.
[30] Fishman A, Light D and Lamm AT (2018) QsRNA-seq: a method for high-throughput profiling and quantifying small RNAs. Genome Biol., 19(11): 133.
[31] McNerney P, Adams P and Hadi MZ (2014) Error rate comparison during polymerase chain reaction by DNA polymerase. Mol. Biol. Int., 2014: 287430.
[32] Bauer RJ, Zhelkovsky A, Bilotti K, Crowell LE, Evans TC Jr, McReynolds LA and Lohman GJS (2017) Comparative analysis of the end-joining activity of several DNA ligases. PLoS One, 12(12): e0190062.
[33] Schwaber J, Andersen S and Nielsen L (2019) Shedding light: The importance of reverse transcription efficiency standards in data interpretation. Biomol. Detect. Quantif., 17: 100077.
[34] Potapov V and Ong JL (2017) Examining sources of error in PCR by single-molecule sequencing [published correction appears in PLoS One, 12(7): e0181128.
[35] Bębenek A and Ziuzia-Graczyk I (2018) Fidelity of DNA replication—a matter of proofreading. Curr. Genet., 64(5): 985–996.
[36] Schrader C, Schielke A, Ellerbroek L and Johne R (2012) PCR inhibitors - occurrence, properties and removal. J. Appl. Microbiol., 113(5): 1014–1026.

[37] Fungtammasan A, Tomaszkiewicz M, Campos-Sánchez R, Eckert KA, DeGiorgio M and Makova KD (2016) Reverse transcription errors and RNA-DNA differences at short tandem repeats. Mol. Biol. Evol., 33(10): 2744–2758.

[38] Fuchs J, Fiche JB, Buhot A, Calemzuk R and Livache T (2010) Salt concentration effects on equilibrium melting curves from DNA microarrays. Biophys. J., 99(6): 1886–1895.

[39] Markegard CB, Gallivan CP, Cheng DD and Nguyen HD (2016) Effects of concentration and temperature on DNA hybridization by two closely related sequences via large-scale coarse-grained simulations. J. Phys. Chem. B., 120(32): 7795–7806.

[40] Rychlik W, Spencer WJ and Rhoads RE (1990) Optimization of the annealing temperature for DNA amplification in vitro. Nucleic Acids Res., 18(21): 6409–6412.

[41] Roux KH (2009) Optimization and troubleshooting in PCR. Cold Spring Harb Protoc., 2009(4): pdb.ip66.

[42] Ruiz-Villalba A, van Pelt-Verkuij E, Gunst QD, Ruijter JM and van den Hoff MJ (2017) Amplification of nonspecific products in quantitative polymerase chain reactions (qPCR). Biomol. Detect. Quantif., 14: 7–18.

[43] Wilkins Stevens P, Henry MR and Kelso DM (2019) DNA hybridization on microparticles: determining capture-probe density and equilibrium dissociation constants. Nucleic Acids Res., 27(7): 1719–1727.

[44] Bielec K, Sozanski K, Seynen M, Dziekan Z, Ten Wolde PR and Holyst R (2019) Kinetics and equilibrium constants of oligonucleotides at low concentrations. Hybridization and melting study. Phys. Chem. Chem. Phys., 21(20): 10798-10807.

[45] Binder H and Preibisch S (2005) Specific and nonspecific hybridization of oligonucleotide probes on microarrays. Biophys. J., 89(1): 337–352.

[46] Furukawa S, Kohguchi K, Okazawa K, Morinaga M, Oohubo M, Tsujioka T and Tohyama K (2018) Effects of handling micropipettettes on dispensing accuracy and results of a questionnaire survey of each institution. Japanese Journal of Medical Technology, 67(1): 44–51 (Japanese).

[47] Faddy MJ and Smith DM (2008) Extended poisson process modelling of dilution series data. Appl. Statist., 57(4): 461–471.

[48] Majumdar N, Banerjee S, Pallas M, Wessel T and Hegerich P (2017) Poisson plus quantification for digital PCR systems. Sci. Rep., 7(1): 9617.

[49] Shah S, Lubeck E, Schwarzkopf M, He TF, Greenbaum A, Soin CH, Lignell A, Choi HM, Gradinaru V, Pierce NA et al. (2016) Single-molecule RNA detection at depth by hybridization chain reaction and tissue hydrogel embedding and clearing. Development, 143(15): 2862–2867.

[50] Xie F, Timme KA and Wood JR (2018) Using single molecule mRNA fluorescent in situ hybridization (RNA-FISH) to quantify mRNAs in individual murine oocytes and embryos. Sci. Rep., 8(1): 7930.

[51] Kaur G, Lewis JS and van Oijen AM (2019) Shining a spotlight on DNA: Single-molecule methods to visualise DNA. Molecules., 24(3): 491.

[52] Li G and Neuret G (2019) Multiplex RNA single molecule FISH of inducible mRNAs in single yeast cells. Sci. Data., 6(1): 94.

[53] Chung JY, Cho H and Hewitt SM (2016) The paraffin-embedded RNA metric (PERM) for RNA isolated from formalin-fixed, paraffin-embedded tissue. Biotechniques., 60(5): 239–244.

[54] Wehmas LC, Wood CE, Chorley BN, Yauk CL, Nelson GM and Hester SD (2019) Enhanced quality metrics for assessing RNA derived from archival formalin-fixed paraffin-embedded tissue samples. Toxicol. Sci., 170(2): 357–373.

[55] Tsai CC, Shih HC, Ko YZ, Wang RH, Li SJ, and Chiang YC (2016) Direct LAMP assay without prior DNA purification for sex determination of papaya. Int. J. Mol. Sci., 17(10): 1630.
[60] Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N and Hase T (2000) Loop-mediated isothermal amplification of DNA. Nucleic Acids Res., 28(12): E63.

[61] Lizaridi PM, Huang X, Zhu Z, Bray-Ward P, Thomas DC and Ward DC (1998) Mutation detection and single-molecule counting using isothermal rolling-circle amplification. Nat. Genet., 19(3): 225–232.

[62] Compton J (1991) Nucleic acid sequence-based amplification. Nature, 350(6313): 91–92.

[63] Walker GT, Fraiser MS, Schram JL, Little MC, Nadeau JG and Malinowski DP (1992) Strand displacement amplification-an isothermal, in vitro DNA amplification technique. Nucleic Acids Res., 20(7): 1691–1696.

[64] Dean FB, Hosono S, Fang L, Wu X, Faruqi AF, Bray-Ward P, Sun Z, Zong Q, Du Y, Du J et al. (2002) Comprehensive human genome amplification using multiple displacement amplification. Proc. Natl. Acad. Sci. USA., 99(8): 5261–5266.

[65] Vincent M, Xu Y and Kong H (2004) Helicase-dependent isothermal DNA amplification. EMBO Rep., 5(8): 795–800.

[66] Kurn N, Chen P, Heath JD, Kopf-Sill A, Stephens KM and Wang S (2005) Novel isothermal, linear nucleic acid amplification systems for highly multiplexed applications. Clin. Chem., 51(10): 1973–1981.

[67] Inoue Y, Morii T, Toyoda Y, Sakurai A, Ishikawa T, Mitani Y, Hayashizaki Y, Yoshimura Y, Kurahashi H and Sakai Y (2010) Correlation of axillary odour to a SNP in the ABCC11 gene determined by the Smart Amplification Process (SmartAmp) method. J. Plast. Reconstr. Aesthet. Surg., 63(8): 1369–1374.

[68] Iwamoto T, Sonobe T and Hayashi K (2003) Loop-mediated isothermal amplification for direct detection of Mycobacterium tuberculosis complex, M. avium, and M. intracellulare in sputum samples. J. Clin. Microbiol., 41(6): 2616–2622.

[69] Hill J, Beriwal S, Chandra I, Paul VK, Kapil A, Singh T, Wadowsky RM, Singh V, Goyal A, Jahnukainen T et al. (2008) Loop-mediated isothermal amplification assay for rapid detection of common strains of Escherichia coli. J. Clin. Microbiol., 46(8): 2800–2804.

[70] Kasahara K, Ishikawa H, Sato S, Shimakawa Y and Watanabe K (2014) Development of multiplex loop-mediated isothermal amplification assays to detect medically important yeasts in dairy products. FEMS Microbiol. Lett., 357(2): 208–216.

[71] Lenzhava A, Ishida T, Ishizu Y, Naito K, Hanami T, Katayama A, Kogo Y, Soma T, Ikeda S, Murakami K et al. (2010) Exciton Primer-mediated SNP detection in SmartAmp2 reactions. Hum. Mutat., 31(2): 208–207.

[72] Biswas I and Hsieh P (1996) Identification and characterization of a thermostable MutS homolog from Thermus aquaticus. J. Biol. Chem., 271(9): 5040–5048.

[73] Dobosy JR, Rose SD, Beltz KR, Rupp SM, Powers KM, Behlke MA and Walder JA (2011) RNase H-dependent PCR (rhPCR): improved specificity and single nucleotide polymorphism detection using blocked cleavable primers. BMC Biotechnol., 11: 80.

[74] Liu J, Huang S, Sun M, Liu S, Liu Y, Wang W, Zhang X, Wang H and Hua W (2012) An improved allele-specific PCR primer design method for SNP marker analysis and its application. Plant Methods, 8(1): 34.

[75] Abe T, Goda K, Futami K and Furuichi Y (2009) Detection of siRNA administered to cells and animals by using a fluorescence intensity distribution analysis polarization system. Nucleic Acids Res., 37(7): e56.

[76] Zhang P, Zhang H, Welchko RM, Thompson RC, Xu S and Tumer DL (2020) Combined microRNA and mRNA detection in mammalian retinas by in situ hybridization chain reaction. Sci. Rep., 10(1): 351.

[77] Bao G, Rhee WJ and Tsourkas A (2009) Fluorescent probes for live-cell RNA detection. Annu. Rev. Biomed. Eng., 11: 25–47.

[78] Ikeda S, Kubota T, Yuki M and Okamoto A (2009) Exciton-controlled hybridization-sensitive fluorescent probes: multicolor detection of nucleic acids. Angew. Chem. Int. Ed. Engl., 48(35): 6480–6484.

[79] Didenko VV (2001) DNA probes using fluorescence resonance energy transfer (FRET): designs and applications. Biotechniques, 31(5): 1106–1121.

[80] Banér J, Nilsson M, Mendel-Hartvig M and Landegren U (1998) Signal amplification of padlock probes by rolling circle replication. Nucleic Acids Res., 26(22): 5073–5078.

[81] Larsson C, Koch J, Nygren A, Janssen G, Raap AK, Landegren U and Nilsson M (2004) In situ genotyping individual DNA molecules by target-primed rolling-circle amplification of padlock probes. Nat. Methods, 1(3): 227–232.

[82] Zhang J, Li Z, Wang H, Wang Y, Jia H and Yan J (2011) Ultrasensitive quantification of mature microRNAs by real-time PCR based on ligation of a ribonucleotide-modified DNA probe. Chem. Commun. (Camb.), 47(33): 9465–9467.

[83] Zhang P, Liu Y, Zhang Y, Liu C, Wang Z and Li Z (2013) Multiplex ligation-dependent probe amplification (MLPA) for ultrasensitive multiplexed microRNA detection using ribonucleotide-modified DNA probes. Chem. Commun. (Camb.), 49(85): 10013–10015.
[84] Schneider N and Meier M (2017) Efficient in situ detection of mRNAs using the Chlorella DNA ligase for padlock probe ligation. RNA, 23(2): 250–256.

[85] Braasch DA and Corey DR (2001) Locked nucleic acid (LNA): fine-tuning the recognition of DNA and RNA. Chem. Biol., 8(1): 1–7.

[86] Gaylord BS, Heeger AJ and Bazan GC (2002) DNA detection using water-soluble conjugated polymers and peptide nucleic acid probes. Proc. Natl. Acad. Sci. USA., 99(17): 10954–10957.

[87] Fouz MF and Appella DH (2020) PNA clamping in nucleic acid amplification protocols to detect single nucleotide mutations related to cancer. Molecules., 25(4): 786.

[88] Kiviniemi M, Nurmi J, Lövgren T and Ilonen J (2005) Locked nucleic acid (LNA) probes in high-throughput genetic analysis: application to an assay for type 1 diabetes-related HLA-DQB1 alleles. Clin. Biochem., 38(11): 1015–1022.

[89] Horvath P and Barrangou R (2010) CRISPR/Cas, the immune system of bacteria and archaea. Science, 327(5962): 167–170.

[90] Deveau H, Garneau JE and Moineau S (2010) CRISPR/Cas system and its role in phage-bacteria interactions. Annu. Rev. Microbiol., 64: 475–493.

[91] Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA et al. (2013) Multiplex genome engineering using CRISPR/Cas systems. Science, 339(6121): 819–823.

[92] Gootenberg JS, Abudayyeh OO, Lee JW, Esseletzbichler P, Dy AJ, Joung J, Verdine V, Donghia N, Daringer NM, Freije CA et al. (2017) Nucleic acid detection with CRISPR-Cas13a/C2c2. Science, 356(6336): 438–442.

[93] Kellner MJ, Koob JG, Gootenberg JS, Abudayyeh OO and Zhang F (2019) SHERLOCK: nucleic acid detection with CRISPR nucleases., Nat. Protoc., 14(10): 2986–3012.

[94] Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, Bright IJ, Lucero MY, Hiddessen AL, Legler TC et al. (2011) High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Anal. Chem., 83(22): 8604–8610.

[95] Quan PL, Sauzade M and Brouzes E (2018) dPCR: A technology review. Sensors (Basel), 18(4): 1271.

[96] Wang H, Li C, Liu X, Zhou X and Wang F (2018) Construction of an enzyme-free concatenated DNA circuit for signal amplification and intracellular imaging. Chem. Sci., 9(26): 5842–5849.

[97] Wu H, Liu Y, Wang H, Wu J, Zhu F and Zou P (2016) Label-free and enzyme-free colorimetric detection of microRNA by catalyzed hairpin assembly coupled with hybridization chain reaction. Biosens. Bioelectron., 81: 303–308.

[98] Wang Q, Liu R, Yang X, Wang K, Zhu J, He L and Li Q (2016) Surface plasmon resonance biosensor for enzyme-free amplified microRNA detection based on gold nanoparticles and DNA supersandwich. Sensors and Actuators B: Chemical., 223: 613–620.

[99] Zhu Z, Jenkins G, Zhang W, Zhang M, Guan Z and Yang CJ (2012) Single-molecule emulsion PCR in microfluidic droplets. Anal. Bioanal. Chem., 403(8): 2127–2143.

[100] Steijin VV, Kleijn CR and Kreutzer MT (2010) Predictive model for the size of bubbles and droplets created in microfluidic T-junctions. Lab. Chip., 10(19): 2513–2518.

[101] Gupta A and Kumar R (2010) Effect of geometry on droplet formation in the squeezing regime in a microfluidic T-junction. Microfluid. Nanofluid., 8: 799–812.

[102] Sivasamy J, Wang TN, Nguyen NT and Kao LT (2011) An investigation on the mechanism of droplet formation in a microfluidic T-junction. Microfluid. Nanofluid., 11: 1–10.

[103] Schneider T, Burnham DR, VanOrden J and Chiu DT (2011) Systematic investigation of droplet generation at T-junctions. Lab. Chip., 11(12): 2055–2059.

[104] Seo M, Takabatake R, Izumi S, Unno H, Kawashima Y, Ki U, Hatada S, Katoh I, Nakazawa S, Matsumoto T et al. (2019) Novel bioprinting application for the production of reference material containing a defined copy number of target DNA. Anal. Chem., 91(20): 12733–12740.