Studies on a Method to Measure MicroRNA as a Diagnostic Marker

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Abbreviations

AUROC: Area under the receiver operating characteristic curve

BCL2: B cell lymphoma 2

Bst DNA Polymerase: *Bacillus stearothermophilus* DNA Polymerase

CA19-9: Carbohydrate antigen 19-9

cDNA: complementary DNA

CEA: Carcinoembryonic antigen

CI: Chemiluminescence intensity

CLL: Chronic lymphocytic leukemia

CS: Cover sequence

CV: Coefficient of variation

CYLD: Cylindromatosis

dNTP: deoxyribonucleotide triphosphate

EMT: Epithelial to mesenchymal transition

HBV: Hepatitis B virus

HDA: Helicase-dependent amplification

HDL: High density lipoprotein

LAMP: Loop-mediated isothermal amplification

L-TEAM: Low-temperature amplification

miRNA: microRNA

mRNA: messenger RNA

NASBA: Nucleic acid sequence-based amplification

NEB: New England Biolabs

NERS: Nicking endonuclease recognition sequence

NF-kB: Nuclear factor-kappa B

NGS: next generation sequencing

PTEN: Phosphatase and tensin homologue

RAKE: RNA-primed, array based Klenow enzyme

RCA: Rolling circle amplification

RLU: Relative light unit

RPA: Recombinase polymerase amplification

qRT-PCR: Quantitative reverse transcription - polymerase chain reaction

SD: Standard deviation

SDA: Strand displacement amplification

SMART: Signal mediated amplification of RNA technology

ssDNAs: single-stranded DNAs

TBS: Target binding sequence

TGF: Transforming growth factor

ZEB: Zinc-finger E-box-binding homeobox

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Preface

MicroRNAs (miRNAs) are functional small non-coding RNAs of 19−24 nucleotides. The miRNAs have the function of post-transcriptional gene silencing by regulating the translation of mRNA into proteins [1]. Although only 26 years have passed since the discovery of the first miRNA, the findings that miRNAs play important roles in many intracellular regulatory processes have accumulated. In addition, miRNAs are highly expected to bring a breakthrough in the medical field of diagnosis and treatment of diseases. Before going into the main topic of this thesis, I would like to give an overview of the history of research on miRNAs so far.

History of research on miRNAs in the regulatory processes

In 1993, the first miRNA, *lin-4* was discovered as a negative regulator in developmental timing in the nematode *Caenorhabditis elegans* [2, 3]. It was suggested that *lin-4* down-regulated *lin-14* mRNA translation via an antisense RNA-RNA interaction with their 3′ untranslated regions. In 2000, *let-7* was discovered as a heterochronic switch gene in *C. elegans* as a second miRNA. The *let-7* regulated developmental timing by repressing the translation of heterochronic gene, especially *lin-41* [4]. The successive regulation of heterochronic gene activities by the *lin-4 and let-7* in *C. elegans* is summarized in Fig. 1. The expression levels of LIN-14 and LIN-28

proteins are decreased by the *lin-4* expression at the end of first larval stage of *C. elegans*, which is caused by the hybridization of the *lin-4* to mRNA of *lin-14* and *lin-28*. This change of the expression progresses the larval stages. The expression level of LIN-41 protein is decreased by the *let-7* expression at the late larval stage, which is caused by the hybridization of the *let-7* to mRNA of *lin-41*. The expression level of LIN-29 protein is increased by the decrease of LIN-41 protein. This leads to progression to the adult stage of *C. elegans* [4]. The *let-7* was highly conserved in a wide range of species, including human, mouse, chicken, frog, zebrafish, flies, abalone and sea urchin [5]. Since then, hundreds of miRNAs were found in worms, flies, fish, frogs, mammals and flowering plants [6]. Although most miRNAs in zebrafish were expressed in a highly tissue-specific manner during segmentation and later stages, they were not expressed during early development. This finding indicated that the function of miRNAs was differentiation or maintenance of tissue identity, not establishment of tissue fate [7]. The role of miRNAs is to regulate many processes such as proliferation, differentiation, apoptosis and development [1, 7–8].

The first evidence of the relationship between miRNA and cancer was reported in 2002. The expression levels of miR-15 and miR-16 in human tissues of chronic lymphocytic leukemia (CLL) decreased due to the deletion of chromosome 13q14 which had the cluster of miR-15 and miR-16 [9]. The miR-15 and miR-16 induced apoptosis by interacting with the mRNA of B cell lymphoma 2 (BCL2) at a posttranscriptional level. The decrease of expression levels of miR-15 and miR-16 led to carcinogenesis [10]. These miRNAs are designated as tumor suppressive miRNAs

because the targets of miRNAs are oncogenes. After that, many kinds of miRNAs closely related to various types of cancers were identified from the miRNA expression profile analysis in the cancer tissues [11−16].

I would like to introduce good examples of miRNAs that are deeply involved in the process of cancer metastasis. The invasion and metastasis are triggered by the transition from epithelial phenotype to mesenchymal phenotype of cells [17]. The EMT (Epithelial to Mesenchymal Transition) is controlled by the interaction between Zinc-finger E-box-binding homeobox 1 (ZEB1), ZEB2 which are E-cadherin transcriptional repressors and miR-200 family (miR-200a, -200b, -200c, -141 and -429) shown in Fig. 2. The miR-200 family inhibits the translation of mRNA of ZEB1 and ZEB2. Reciprocally, ZEB1 and ZEB2 proteins repress the transcription of miR-200 family [18]. This double negative feedback loop controls EMT in tumorigenesis and MET in development [17]. The miRNAs and mRNAs form a complicated gene expression regulatory network to maintain homeostasis of the organisms.

The relationships between aberrant expression of miRNAs and diseases are not confined to cancers. The different expression patterns of miRNAs were found in the tissues of cardiovascular diseases and neurological disorders [19−20].

History of research on miRNAs in blood

In 2007, Valadi et al. [21] reported that exosomes which were membrane vesicles of endocytic origin released by many cells into blood contained both mRNAs and miRNAs. In 2008, Mitchell et al. [22] reported that miRNAs were present in plasma in

a stable form that was protected from ribonuclease activity. They also found that the concentration of miR-141 in serum distinguished prostate cancer patients from healthy people [22]. Their findings opened the field of miRNA studies in blood. As a mechanism of stable existence of miRNA in blood, the following two reasons have been identified. One reason is that most of miRNAs are bound to protective proteins such as Argonaute complexes, high density lipoprotein (HDL) and other RNA-binding proteins [22−25]. The other reason is that some miRNAs are inside or outside membrane vesicles such as exosomes (diameter 50−150 nm), microvesicles (diameter 100−1000 nm) and apoptotic bodies [22−26]. The circulating miRNAs in serum and plasma became new potential biomarkers for cancer diagnosis, prognosis and detection of recurrence [27−29]. Furthermore, in 2016, circulating miRNAs have been considered as a new type of signaling molecules [26]. The miRNAs secreted as form of extracellular vesicles by cells are transported by blood and received by distant cells. The transported miRNAs change gene expression of the recipient cells such as hormones or cytokines. The miR-210 containing extracellular vesicles which were secreted from cancer cells promoted angiogenesis in endothelial cells [30]. These findings show a possibility that cancer metastasis can be diagnosed by measuring miRNAs in blood [31].

In summary, there is a possibility that miRNAs become useful cancer diagnostic markers because miRNAs have the following characteristics: First, aberrant expression of miRNAs in tissues and bodily fluids is cancer specific. Second, aberrant expression of miRNAs occurs in the early stage of the cancer. Third, miRNAs were stable in blood, urine and saliva even ribonucleases are present. As of August in 2019, 2654 human

miRNAs are registered in the miRNA database, miRBase ver.22.1 [32, 33].

My research theme on miRNAs

From the above findings, I have come to think that miRNAs may show good diagnostic performances beyond existing cancer markers. Therefore, my research theme was set for the studies on a method to measure miRNA as a diagnostic marker.

Current miRNA studies fall into three categories. The first category is fundamental research which elucidates the function of miRNAs in the regulatory processes of proliferation, differentiation, apoptosis and development. The second category of the research is to identify the relationships between miRNAs and diseases and to conduct clinical research with miRNAs as a diagnostic marker or a therapeutic drug in medical laboratories. The third category of the research is to establish an analytical method of miRNAs in a sample, which is the basis of other research. My research falls into the third category. The ultimate goal of my research is that my proposed measurement method of miRNAs becomes one of standard measurement methods of miRNAs in clinical laboratories.

Review of diagnostic performances of miRNAs

I would like to review previous research results on the diagnostic performance of miRNA in blood for the detection of colorectal cancer and pancreatic cancer.

As a serum diagnostic marker for a colorectal cancer, miR-92, miR-23a, miR-1246, miR-200c, miR-21, let-7 g, miR-181b, miR-92a, miR-203 were identified [16].

The miR-21 is one of the most important oncogenic miRNAs which is found in many types of cancer. The one of targets of miR-21 is mRNA of tumor suppressors (Phosphatase and tensin homologue (PTEN), Cylindromatosis (CYLD)) and leads to increase of Nuclear Factor-kappa B (NF-kB) activity which results in an inflammatory disease and cancer [16]. The accuracy and clinical value of miR-21 in serum and plasma as a diagnostic marker for a colorectal cancer was assessed by conducting meta-analysis [34]. In seven studies, the pooled clinical sensitivity of miR-21 for the detection of colorectal cancer was 75% (each study was from 52% to 90%). The pooled clinical specificity was 84% (each study was from 74% to 91%). The pooled area under the receiver operating characteristic curve (AUROC) was 0.86 (each study was from 0.653 to 0.927). The results suggest that miR-21 in serum and plasma is a diagnostic marker for colorectal cancer with moderate clinical sensitivity and clinical specificity [34]. As an existing colorectal cancer marker, Carcinoembryonic antigen (CEA) is used with clinical sensitivity of ~ 80%, a clinical specificity of ~ 70% for detecting recurrent colorectal cancer [35].

Pancreatic cancer is a 4th leading cancer in death in Japan and the number of deceased people by pancreatic cancer was 31,809 in 2015 [36]. The best validated and most clinically useful diagnostic marker of pancreatic cancer is Carbohydrate antigen 19-9 (CA19-9) [37]. The clinical sensitivity of CA19-9 was 79–81% and the clinical specificity of CA19-9 was 80–90% in symptomatic patients [37]. However, the clinical sensitivity of CA19-9 in stage I and II patients were 40–47% and 58–78% respectively, which showed that CA19-9 was not an effective marker for early detection of pancreatic

cancer [38, 39]. At the current situation, the poor detection of early stage of pancreatic cancer resulted in the 5-year survival rate of only 12% which was the worst in all organ-specific cancers [40]. When pancreatic cancer was diagnosed at stage 0, Ia and Ib, the 5-year survival rate improved 86%, 69% and 60%, respectively [40]. Therefore, a serological diagnostic marker exceeding CA19-9 for the early detection of pancreatic cancer is required [38].

The review paper showed that miR-16, miR-155, miR-196a, miR-200a, miR-200b, miR-210 and miR-18a were identified as pancreatic cancer markers in blood [41]. Other validation studies showed that the clinical sensitivity of pancreatic cancer ranged from 83% to 95% by using 7 miRNAs (miR-20a, miR-21, miR-24, miR-25, miR-99a, miR-185, miR-191) [42], 10 miRNAs (miR-26b, miR-34a, miR-122, miR-126, miR-145, miR-150, miR-223, miR-505, miR-636, miR-885.5p) [43], 3 miRNAs (miR-486-5p, miR-126-3p, miR-106b-3p) [44] and 9 miRNAs (miR-16, miR-24, miR-27a, miR-30a.5p, miR-323.3p, miR-20a, miR-25, miR-29c, miR-483.5p) [45]. The validation studies showed that the clinical specificity of pancreatic cancer ranged from 42% to 90% [42−45]. In addition, Kojima et al. [46] studied the diagnostic value of miRNAs in serum for the detection of pancreatic cancer. The results showed that a combination of eight miRNAs (miR-6075, miR-4294, miR-6880-5p, miR-6799-5p, miR-125a-3p, miR-4530, miR-6836-3p, and miR-4476) achieved clinical sensitivity, clinical specificity and AUROC for pancreatic cancer as 80%, 98% and 0.953 respectively [46]. The results of CA19-9 in the same test cohort showed clinical sensitivity of 66%, specificity of 93%, and AUROC of 0.682, respectively. This study

showed that the combination of eight miRNAs were clinically more valuable rather than CA19-9 to identify patients with pancreatic cancers [46].

Existing measurement methods of miRNAs

A lot of researches on a measurement method of miRNAs have been conducted worldwide. However, the measurement of miRNAs is still challenging because miRNAs have unique characteristics of short length (19−24 nucleotides), low concentration and sequence homology among the miRNA family [47]. Research activities have been conducted to develop a miRNA quantification method for practical use [48−51]. Current three major measurement methods of miRNAs are quantitative reverse-transcription PCR (qRT-PCR), microarray and next generation sequencing (NGS) [32, 52]. These three major methods and three novel methods with new emerging technologies for measuring miRNA are described briefly in the below.

To measure miRNA in a sample by qRT-PCR assay, the extraction and purification of miRNA is conducted. In TaqMan miRNA qRT-PCR assay, stem-loop primer is used for capturing miRNA. When the stem-loop primer is hybridized with miRNA, the 3′ end of the stem-loop primer is extended by reverse transcription reaction and the complementary DNA (cDNA) is generated. The generated cDNA is amplified by PCR with a miRNA-specific forward primer and a reverse primer in a thermal cycler. The amplified DNA is detected by hybridization of TaqMan fluorescent probe. In other SYBR-green-based qRT-PCR assay, miRNA is polyadenylated at the 3′ end and oligo-d(T) is used as a reverse transcription primer. Reverse transcription reaction is

performed, and the cDNA is generated. The generated cDNA is amplified by PCR and detected by intercalation of SYBR green dye to the double stranded DNA [32].

To measure miRNAs in a sample by microarray assay, the extraction and purification of miRNA is conducted. The miRNAs purified from a sample are labeled with fluorescent dye. The fluorescent dye-labeled miRNAs hybridize with the complementary cDNA sequences which are attached on a slide. After washing, the fluorescent intensity by the hybridized miRNAs with the attached cDNA sequences is measured. The advantage of microarray assay is that many kinds of miRNAs can be detected at one time with low cost. The disadvantage of microarray assay is that the specificity of miRNAs which have similar sequences is imperfect and absolute quantification is not achieved [32].

To measure miRNAs in a sample by RNA sequencing assay, the extraction and purification of miRNA is conducted. The miRNAs are reverse transcribed to cDNA library. Adaptor ligation is conducted to cDNA library. The cDNA with adaptor is immobilized on surface and solid-phase PCR is conducted. The amplified DNA is measured by next-generation sequencing platforms. RNA sequencing assay has several advantages. First, high accuracy is achieved in distinguishing miRNAs that are very similar in sequence. Second, novel miRNAs can be identified. On the other hand, the disadvantages of RNA sequencing assay are the computational support for data analysis is required, and the measurement cost is high. Due to these disadvantages, it is not suitable for clinical laboratory tests for daily use [32].

These three major measurement methods require time-consuming pretreatment,

manual operation and a standalone instrument. Among the three methods, qRT-PCR is most frequently used for diagnostic purposes due to a highly sensitive and quantitative assay. However, the measurement cost of qRT-PCR is high and the throughput is low. The details are described in Chapter II.

New measurement methods of miRNAs

The alternative measurement methods of miRNAs to overcome the above disadvantages have been expected and are being developed [32]. The two novel methods which don't require the amplification and labeling of miRNAs are described briefly in the below.

In a splinted ligation assay for miRNAs, both 3′ end of a miRNA and 5′ end of an oligonucleotide hybridizes with 'bridge' oligonucleotide. The schematic illustrations of the assay are shown in Fig. 3. The gap between miRNA and the oligonucleotide is ligated by T4 DNA ligase. The ligated oligonucleotide hybridizes with a complementary biotinylated capture probe and a fluorescence probe. The set of the biotinylated capture probe with the ligated oligonucleotide and the fluorescence probe binds to a streptavidin coated surface. After washing, fluorescence intensity is measured [32].

In RNA-primed, array based Klenow enzyme (RAKE) assay for miRNA, a miRNA hybridizes with a complementary DNA which is attached to a microarray slide. The schematic illustrations of the assay are shown in Fig. 4. Exonuclease I degrades unhybridized DNA probes. After washing, Klenow DNA polymerase I extends DNA strand from 3′ end of a miRNA with incorporation of the biotinylated dATPs.

Fluorescent dye-labeled streptavidin is added. After washing, fluorescence intensity is measured [54].

Existing isothermal amplification of a nucleic acid

In my research, I adapted a new isothermal DNA amplification to a fully automated immunoassay analyzer for miRNA measurement. Therefore, I would like to overview existing isothermal amplification methods developed so far. An isothermal amplification of a nucleic acid is a method to amplify a nucleic acid at a constant temperature without raising or lowering the temperature of reaction solutions. The following isothermal amplification methods have been developed since the early 1990s to overcome the disadvantages of qRT-PCR. Loop-mediated isothermal amplification (LAMP), strand displacement amplification (SDA), rolling circle amplification (RCA), helicase-dependent amplification (HDA), nucleic acid sequence-based amplification (NASBA), recombinase polymerase amplification (RPA) and signal mediated amplification of RNA technology (SMART). The advantages of the isothermal amplification methods are that simplified protocols can be applied and minimal sample preparation is acceptable [55−57].

The common feature of the isothermal amplification methods is to use polymerase which has strand displacement activity with lack of 5′ → 3′ exonuclease activity. The strand displacement activity synthesizes a new DNA strand while dissociating the hydrogen bond of the double stranded template DNA by itself. The former DNA strand is displaced without degradation. DNA can be synthesized at a constant temperature and

the synthesis is not inhibited by the secondary structure of DNA [58]. As representative isothermal amplification methods using polymerase with the strand displacement activity, one emerging assay for the detection of miRNAs and two clinically approved assays for the detection of nucleic acids of infectious diseases are described in the below.

Rolling circle amplification (RCA) assay for miRNA is an isothermal amplification method by which many research reports have been published [55−57]. The schematic illustrations of the principle of RCA assay are shown in Fig. 5. A miRNA hybridizes with a padlock probe by cooling down from around 65 ºC to room temperature gradually. After annealing, T4 DNA ligase is added and circular DNA is formed by the ligation. Then, phi29 DNA polymerase with the strand displacement activity is added. As a miRNA serves as a primer, a long single-stranded DNA with multiple copies of the complementary sequence of circular DNA is generated. Fluorescence probes hybridize with the amplified sequences and after washing, the fluorescence intensity is measured [32, 53].

Clinically approved nucleic acid assay with an isothermal amplification

Among isothermal amplification methods, there are only two methods which are used in a clinically approved assay. The two methods are explained briefly in the below.

Loop-mediated isothermal amplification (LAMP) method is used in a clinically approved assay. The in-vitro diagnostic products with LAMP method by Eiken Chemical Co., Ltd. are used in clinical laboratories for detecting SARS corona virus,

Influenza virus, mycoplasma *pneumoniae*, legionella, Mycobacterium tuberculosis and other infectious diseases [59]. LAMP assay achieves highly efficient amplification and sensitive detection of nucleic acids of infectious diseases, which are measured in one pot with 60 ^{o}C incubation in less than one hour. The schematic illustrations of the principle of an amplification method of LAMP are shown in Fig. 6. The LAMP method needs four different primers in a target gene. The inner primer F1c-F2 hybridizes with the 3′ end of the target gene to initiate an extension reaction. The extended strand is displaced from the target gene by DNA polymerization from the outer primer F3 with DNA polymerase. A stem loop structure is formed at the 5′ end of the extended strand. A third primer (B1c-B2) hybridizes with the 3′ end of the extended strand to initiate an extension reaction. The newly extended strand is displaced from the previous extended strand by DNA polymerization from a fourth primer B3. The double stem loop structures at both 3′ end and 5′ end are formed in single-stranded DNA. This DNA with dumbbell-like structure has self-amplification activity by the following two extension reactions. The one extension reaction is polymerization from the 3′ end of stem loop structure. The second extension reaction occurs after two inner primers (F1c-F2, B1c-B2) hybridize with the two loop portions. These two extension reactions occur in succession at a constant temperature and sequences complementary to each other are alternately and repeatedly generated in large quantities [55–57, 59]. However, LAMP method cannot be applied for the detection of miRNAs because it needs four different primers in a target gene to start amplification. The four different primers cannot be set in the short length miRNA of only 19–24 nucleotides.

Strand displacement amplification (SDA) method is other isothermal amplification method which is used in a clinically approved assay. The in-vitro diagnostic products with SDA method by Becton Dickinson and company Ltd. are used in clinical laboratories for detecting Chlamydia trachomatis and Neisseria Gonorrhoeae [55–57, 60]. The SDA assay achieves highly efficient amplification and sensitive detection of nucleic acids of infectious diseases, which are measured with 54 ^{o}C incubation. The schematic illustrations of the principle of an amplification method of SDA are shown in Fig. 7. The SDA method needs four different primers in a target gene. The inner primers (S1, S2) hybridize with the 3′ end of the target gene to start an extension reaction. The extended strands are displaced from the target gene by DNA polymerization from the outer primers (B1, B2) with DNA polymerase. The opposite synthesized DNAs hybridize and double-stranded DNA which has S1 and S2 is formed. The outer primers (B1, B2) and the inner primers (S1, S2) hybridize with the double-stranded DNA and primer extensions are started. After hybridization of the opposite synthesized DNAs, short double-stranded DNA which has S1 and S2 is formed. Restriction endonuclease cleaves only the original S1 and S2 sequence because the restriction endonuclease can not cleave synthesized DNA sequence in which thiol-modified deoxycytidine triphosphate (dCsTP) is incorporated. The deoxyribonucleotide triphosphate (dNTP) used in the SDA method consist of dATP, dGTP, dTTP and dCsTP. Nicking reaction occurs in the original inner primer sequence on one strand and from this portion, the extension of the strand is started. The inner primers (S1, S2) hybridize with the synthesized DNAs and the primer extension is started. Nicking reaction and

polymerization occur alternately and repeatedly. These reactions occur in succession at a constant temperature and the sequences are generated in large quantities [55–57, 60]. However, SDA method cannot be applied for the detection of miRNAs because it needs four different primers in a target gene to start amplification. The four different primers cannot be set in the short length miRNA of only 19–24 nucleotides.

New isothermal amplification method used in my studies

A novel isothermal DNA amplification method, low-temperature amplification (L-TEAM) was proposed by Komiya [61]. The isothermal amplification method was filed as a patent (PCT/JP2011/078717, WO/2012/077819, filing date: 2011-12-12) under the name “Method and kit for detecting target nucleic acid”. The L-TEAM method was granted Japanese patent (No. 6126381, grant date: 2017-4-14) and US patent (No. 09845495, grant date: 2017-12-19). The L-TEAM method needs 2 DNA templates (Sequence conversion DNA and Signal amplification DNA), DNA polymerase, nicking endonuclease and dNTP (dATP, dGTP, dCTP and dTTP). The schematic illustrations of the principle of L-TEAM method are shown in Fig. 8.

The first template (Sequence conversion DNA) is composed of a first arbitrary sequence, an endonuclease recognition sequence which is used for a nicking reaction and a sequence complementary to the target nucleic acid in the direction from 5′ to 3′. The second template (Signal amplification DNA) is composed of the same first arbitrary sequence, an endonuclease recognition sequence which is used for a nicking reaction and the same first arbitrary sequence. A target nucleic acid hybridizes with the

complementary portion of the Sequence conversion DNA, the target nucleic acid serves as a primer to start an extension reaction by DNA polymerase. Next, an endonuclease cleaves the sequence and from this position, the oligonucleotide is synthesized by DNA polymerase. The previous synthesized sequence is displaced. The repeat of DNA polymerization and nicking reaction generate a sequence complementary to the first arbitrary sequence, which is called a Signal DNA with linear amplification. Subsequently, the Signal DNA from the Sequence conversion DNA hybridizes with the sequence of the Signal amplification DNA. DNA polymerization occurs, and a nicking endonuclease cleaves the sequence. From this position, the oligonucleotide is synthesized by DNA polymerase. The previous synthesized Signal DNA is displaced. The repeat of DNA polymerization and nicking reaction generate Signal DNAs with exponential amplification [61]. This exponential amplification method may cause false positive results because the amplified Signal DNAs may contaminate other reaction vessels due to the open system of immunoassay analyzers. I modified L-TEAM to a cascade reaction that consists only of linear amplification and adapted it to an immunoassay analyzer, ARCHITECT i system (Abbott Japan, Tokyo, Japan) to measure miRNAs. The details are described in Chapter II.

Unique features of enzymes used in my studies

The DNA polymerase with strand displacement activity and nicking endonuclease used in my research have the following unique features: I used *Bacillus stearothermophilus (Bst)* DNA Polymerase, Large Fragment which lacks the N-terminal

5′ → 3′ exonuclease domain of *Bst* DNA Polymerase, Full Length. The *Bst* DNA Polymerase, Full Length originally lacks 3′ → 5′ proofreading exonuclease activity. So, *Bst* Polymerase, Large Fragment has neither 5′ → 3′ exonuclease activity nor 3′ → 5′ proofreading exonuclease activity [62]. Taq DNA Polymerase has 5′ → 3′ exonuclease activity, which means Taq DNA Polymerase does not have strand displacement activity. Bst DNA Polymerase, Large Fragment has other unique ability of extending DNA sequence to RNA primer. Taq DNA Polymerase does not have the ability of extending DNA sequence to RNA primer [63].

I used Nb.BbvCl as nicking endonuclease, which was a mutant of R.BbvCl from *Bacillus brevis*. R.BbvCl is an unusual restriction endonuclease in that it recognizes the asymmetric seven base-pair sequence in double-stranded DNA and cleaves as follows; CCTCAGC / GCTGAGG → CC^TCAGC / GC^TGAGG. The cleaved site is shown in ^. R.BbvCl is a heterodimer and possesses two different catalytic sites for strand hydrolysis. Nb.BbvCl is a genetically engineered mutant in which R2 subunit of R.BbvCl is catalytically inactivated by mutagenesis and R1subunit remains functional. Nb.BbvCl recognizes the asymmetric seven base-pair sequence in double-stranded DNA and cleaves only one strand of the sequence as follows; CCTCAGC / GCTGAGG → CCTCAGC / GC^TGAGG [64, 65].

Evaluation items in my studies

I developed a fully automated miRNA assay by adapting an isothermal DNA

amplification method to standard immunoassay analyzers. To develop the assay, I investigated the following evaluation items. The first evaluation item was an improvement of detection sensitivity of Signal DNA by hybridization reaction. The factors which were investigated for the improvement of detection sensitivity were 1) sequences and length of Signal DNA, 2) length of the capture DNA probes and chemiluminescent detection DNA probes, 3) concentration of the capture DNA probes and chemiluminescent detection DNA probes, 4) ingredients of reaction solution. The second evaluation item was an improvement of specific amplification reaction of Signal DNA from miRNA and reduction of non-specific amplification reaction. The factors which were investigated for the improvement of specific amplification and the reduction of non-specific amplification were 1) sequences and length of Signal DNA, 2) length of Converter DNA and Cascade DNA, 3) concentration of Converter DNA and Cascade DNA, 4) types of polymerase and nicking endonuclease, 5) concentration of polymerase and nicking endonuclease. 6) dNTP concentration, 7) amplification method, 8) assay protocol.

Patents

During the research period, the following four PCT patent applications were filed. The 1, 2 and 3 were granted US patent. The 4 is being examined by US patent office. All applications are being examined by Japan and European patent office.

1. “Covered sequence conversion DNA and detection methods” - Inventor: Ken Komiya, Makoto Komori, Toru Yoshimura, PCT application No.: WO/2015/114469,

PCT filing date: 2015-1-15, US patent No.: 10036077, US patent grant date: 2018-7-31.

2. “Sequence conversion and signal amplifier DNA having locked nucleic acids and detection” - Inventor: Ken Komiya, Makoto Komori, Toru Yoshimura, PCT application No.: WO/2016/059473, PCT filing date: 2015-10-13, US patent No.: 10208333,

US patent grant date: 2019-2-19.

3. “Sequence conversion and signal amplifier DNA having poly DNA spacer sequences and detection methods using same” - Inventor: Ken Komiya, Makoto Komori, Toru Yoshimura, PCT application No.: WO/2016/059474, PCT filing date: 2015-10-13, US patent No.: 10316353, US Patent grant date: 2019-6-11.

4. “Sequence conversion and signal amplifier DNA cascade reactions and detection methods using same” - Inventor: Makoto Komori, Toru Yoshimura, PCT application No.: WO/2016/103234, PCT filing date: 2015-12-24.

Chapter II:

Measurement of microRNA with isothermal DNA amplification on fully automated immunoassay analyzers

Summary

MicroRNAs (miRNAs) in a blood sample are usually measured by quantitative reverse transcription PCR (qRT-PCR), microarray and next generation sequencing (NGS) which requires time-consuming pretreatment, manual operation and a standalone instrument. To overcome these disadvantages, miRNA testing has been developed using the automated analyzers routinely used in clinical laboratories. An isothermal DNA amplification reaction was adapted to a fully-automated immunoassay analyzer that conducts extraction, amplification and detection processes at 37°C in 44 minutes. In a reaction vessel, a pre-designed single-stranded signal DNA was amplified in the presence of miRNA, using DNA templates, DNA polymerase, and nicking endonuclease. Then, the amplified signal DNA was hybridized by one DNA probe attached to a magnetic particle and another DNA probe labeled with acridinium ester. After the chemiluminescence reaction, luminescence intensity was automatically measured. The automated assays of cancer-related miRNAs were implemented on the analyzer with throughput of 66 tests per hour. In the assays with one-step amplification, three miRNAs (miR-21-5p, miR-18a-5p and miR-500a-3p) at concentrations lower than 100 fM were automatically detected and the cross reactivity for miR-21-5p with fifteen similar miRNAs was not higher than 0.02%. In the assay with two-step amplification,

detection sensitivity and amplification rate for miR-21-5p were 3 fM and 103-fold, respectively. The coefficient of variations (CVs) in the measurement at the target concentrations from 5 fM to 1000 pM were less than 8%. Furthermore, I also achieved automated nucleic acid detection in human serum. The proposed fully-automated miRNA assays showed high sensitivity, low cross reactivity and reproducibility suitable for clinical use.

Introduction

MicroRNAs (miRNAs) are functional small non-coding RNAs of 19−24 nucleotides that are involved in regulating many biological processes, including proliferation, differentiation, apoptosis and development [1, 32]. The aberrant expression of miRNAs is closely related to cancer [10, 12−14]. The miRNAs are stable in blood because most of miRNAs are bound to protective proteins such as Argonaute complexes and some miRNAs are inside or outside membrane vesicles such as exosomes [22−25]. Recently, circulating miRNAs have attracted attention as new potential biomarkers for cancer diagnosis, prognosis and detection of recurrence [27, 42, 66−70]. However, the measurement of miRNAs is still challenging because miRNAs have cumbersome characteristics; short length (19−24 nucleotides), low concentration and sequence homology among the miRNA family [47]. Research activities have been conducted to develop a miRNA quantification method for practical use [48−51].

The current major measurement methods of miRNAs are quantitative reverse-transcription PCR (qRT-PCR), microarray and next generation sequencing

(NGS) [32, 52]. While both microarray and NGS are suitable for screening and discovery purposes, qRT-PCR is still the first choice for validation and clinical tests with a high number of samples [70]. The qRT-PCR has been preferably used due to its high sensitivity and high specificity [32]. But qRT-PCR in clinical laboratories has several disadvantages. First, a large qRT-PCR instrument used only for detecting nucleic acids is needed [55, 71]. Second, measurement cost is high due to the expensive instrument and the manual operation required for extraction and purification processes [72, 73]. Third, throughput is decreased by the long purification process due to blood coagulation under high-temperature conditions [73]. These disadvantages of qRT-PCR make it difficult to introduce miRNAs into clinical laboratories as cancer diagnostic markers [74, 75]. In addition, the manual operation has risk of human error.

Fully-automated immunoassay analyzers are widely used in clinical laboratories to measure various proteins and chemical compounds as diagnostic markers in biological samples including serum, plasma and urine [76–78]. In most fully-automated immunoassay analyzers, luminescence intensity of chemiluminescent substrates is detected, such as acridinium ester, ruthenium-complex and luminol, after the labeled antibodies or antigens bind to the analytes [79]. The existing fully-automated immunoassay analyzers can be used to process and measure miRNAs in serum under physiological temperature conditions. This avoids blood coagulation and the long purification process, eliminates the need to perform manual extraction processes and eliminates the need to transfer the sample to the standalone qRT-PCR instrument. Implementation of miRNA testing on the automated immunoassay analyzers will lead to

shorter test time and cost reduction, and thus, is significantly advantageous both for medical facilities and patients [74, 75].

According to the meta-analysis of clinical studies about miR-21-5p as a gastric cancer marker in serum and plasma, the cut off concentrations of miR-21-5p were 37.3 fM, 50 fM and 59.5 fM at three sites [67]. Therefore, a miR-21-5p assay needs a detection sensitivity around 50 fM. A previous study reported a detection sensitivity of miRNA at 1 pM using an acridinium labeled antibody to a heterohybrid of miRNA and DNA on a fully-automated immunoassay analyzer without DNA amplification [74]. To establish a more sensitive miRNA measurement, development of a method for DNA amplification is needed. However, the clinically approved methods of DNA amplification, such as loop-mediated isothermal amplification (LAMP) and strand displacement amplification (SDA), require the high reaction temperature at 60°C and 54°C, respectively [59, 80]. Although rolling circle amplification (RCA) achieves DNA amplification at 30−37°C, it requires several hours for ligation reaction of padlock probe before DNA amplification [81, 82].

Dr. Komiya recently proposed a novel isothermal DNA amplification method, low-temperature amplification (L-TEAM) and demonstrated leak-free amplification [83]. In this method, pre-designed signal DNA is amplified exponentially in the presence of a target nucleic acid at constant 37°C with the use of two DNA templates. In the present study, I adapted the miRNA measurement via L-TEAM for use with a fully-automated immunoassay analyzer.

The exponential DNA amplification using immunoassay analyzers could lead to

false positive results because the automated immunoassay analyzers are open systems. There is a possibility that the amplified signal sequence may contaminate other reaction vessels via a pipette or aerosol in the analyzer. In the reaction design of exponential amplification including PCR and L-TEAM, in which the end product as an amplified signal triggers amplification of the end product itself, contamination severely interferes amplification results. To minimize potential false positive results due to contamination, I modified L-TEAM to a cascade reaction that consists only of linear amplification. In the cascade reaction, successive sequence conversion of the generated single-stranded DNAs (ssDNAs) reduces the risk of false positive results by avoiding self-triggered exponential amplification and achieves higher amplification rates in comparison to one-step linear amplification.

In this paper, I first describe the automated assays for three miRNAs, i.e., miR-21-5p, miR-18a-5p and miR-500a-3p, that were reported as cancer diagnostic markers [27, 66, 68], implemented on a fully-automated immunoassay analyzer with one-step amplification reaction. Then, I report the assay performances upon the detection of miR-21-5p and miR-200 families that were reported as colorectal cancer markers [84, 85] on the analyzer with two-step amplification reaction.

Materials and methods

Materials for amplification and detection

All oligodeoxyribonucleotides and oligoribonucleotides were synthesized by Gene Design (Ibaraki, Japan). The Converter DNA and Cascade DNA were purified via ion exchange HPLC. The sequences of oligodeoxyribonucleotides and oligoribonucleotides and the chemical modification are described in Table 1. Bst DNA Polymerase, Large Fragment, Nb.BbvCI, NEB Buffer 2 (final concentration of 10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1% Tween 20, pH 7.9) and dNTPs were purchased from New England Biolabs Japan (Tokyo, Japan). Streptavidin-coated magnetic particle, Dynabeads® M-270 Streptavidin was purchased from Thermo Fisher Scientific K. K. (Kanagawa, Japan). The magnetic particles are uniform in size, having a diameter of 2.8 μm [86].

Scheme of Signal DNA amplification

The isothermal one-step signal DNA amplification (one-step amplification) reaction for miRNA detection requires one DNA template called Converter DNA, DNA polymerase having strand displacement activity, nicking endonuclease, dNTPs and a target miRNA. Converter DNA comprises Signal DNA1 generation sequence, nicking endonuclease recognition sequence (NERS), cover sequence 1 (CS1) which is complementary to Signal DNA1 generation sequence, and target binding sequence (TBS) which is complementary to a target miRNA in the 5′ to 3′ direction (Fig. 9a). At

the reaction temperature, Converter DNA forms a hairpin structure comprising an 18-bp stem and avoids binding of Signal DNA1 released from a Converter DNA to the Signal DNA1 generation sequence of another Converter DNA.

When miRNA hybridizes with the TBS of Converter DNA, DNA extension occurs from the 3′ end of miRNA by DNA polymerase (step i, ii in Fig. 9b). During DNA extension, the hairpin structure is opened due to the strand displacement activity of DNA Polymerase, and the DNA strand complementary to Converter DNA is generated (step iii). Then, nicking endonuclease recognizes its recognition sequence in the double-stranded form. Upon nicking reaction, Signal DNA1 is generated (step iv). After that, DNA extension occurs again from the 3′ end of nicked position. As a consequence, the formerly-generated Signal DNA1 is released because of the strand displacement activity of DNA Polymerase (step v). These polymerization and nicking reactions repetitively occur and linearly amplify Signal DNA1, only in the presence of miRNA without the needs of reverse transcription reaction and tag-adding reaction for generating primer-binding sequence that are required in qRT-PCR.

The isothermal two-step cascade signal DNA amplification (two-step amplification) reaction is also achieved by layering two consecutive linear signal DNA amplification reactions (Fig. 9b, c). In the second step reaction, hybridization of Signal DNA1 with the Signal DNA1 binding sequence of Cascade DNA further triggers the next linear amplification of Signal DNA2, whose sequence is different from that of Signal DNA1, via the repetitive polymerization and nicking reaction similarly to the first step reaction.

Investigation on the effect of cover sequence introduction to Converter DNA and comparison of nicking endonucleases

I investigated the amplification behavior in the one-step amplification assay for miR-24-3p using Converter DNAs with and without cover sequence (Table 1a). Amplification of Signal DNA was monitored by detecting the fluorescence emitted upon hybridization with the molecular beacon on a real-time PCR system, CFX 96 (BioRad Japan, Tokyo, Japan). The amplification reaction was performed at constant 37.0^{o}C in 25-μL reaction mixtures of NEB Buffer 2 (final concentration of 10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1% Tween 20, pH 7.9), with a target single-stranded DNA having the sequence same as miR-24-3p termed miD-24-3p, Converter DNAs with or without cover sequence, Bst DNA Polymerase, Large Fragment, Nt.AlwI or Nb.BbvCI, and dNTPs. Fluorescence detection was implemented at intervals of about 69 seconds for 120 times. The final concentrations of Bst DNA Polymerase, Large Fragment, Nt.AlwI or Nb.BbvCI, and dNTPs were 0.08 units/μL,0.1 units/μL, and 200 μM each, respectively. The final concentrations of the target DNA, Converter DNAs, and the molecular beacon were 1, 100, and 100 nM, respectively.

Preparation of Capture DNA probe attached to magnetic particles

Capture DNA probe (13 nucleotides (nt) in length) labeled with biotin-triethylene glycol spacer at its 3′ end was incubated with streptavidin-coated magnetic particles for 30 minutes at a room temperature. After washing the magnetic particles under magnetic attraction, a 0.05% (w/v) particle solution was prepared.

Preparation of Chemiluminescence DNA probe

Chemiluminescence DNA probe (10 nt) modified with amino group-six carbon spacer at its 5′ end was reacted with NHS-conjugated acridinium ester provided by Abbott Laboratories (IL, USA) for overnight at a room temperature. The resulting Chemiluminescence DNA probe labelled with acridinium ester was separated by reverse phase HPLC (Nihon Waters K.K., Tokyo, Japan), and diluted to 100 nM.

Automated miRNA detection on the analyzer

A fully-automated immunoassay analyzer, ARCHITECT i system (Abbott Japan, Tokyo, Japan) is used for measuring proteins and chemical compounds in serum, plasma and urine as diagnostic markers in about 30 minutes at clinical laboratories [76]. In the present study, automated miRNA assays were implemented on the immunoassay analyzer as follows. One sample solution and six operational solutions were placed on the analyzer, and the miRNA assay was started. Initially, a 40-μL sample solution containing synthetic miRNA in 10 mM Tris-HCl and 0.01% BSA (pH 8.0) was dispensed into a reaction vessel. Then, a 50-μL solution containing detergent was mixed for 7 minutes at 37°C on the fully-automated analyzer. The role of detergent is to liberate miRNAs when the sample is serum. A 30-μL solution of this mixture was transferred to another reaction vessel. Next, a 40-μL solution of Converter DNA, a 40-μL pre-mixed solution of Bst DNA Polymerase, Large Fragment and Nb.BbvCl, and a 20-μL dNTPs solution were dispensed into the reaction vessel and incubated for 7

minutes at 37°C. In this amplification reaction mixture containing Bst DNA Polymerase, Large Fragment and Nb.BbvCI at 0.07 and 0.09 units/μL, respectively, hybridization of target miRNA to Converter DNA primes DNA extension and Signal DNA1 is linearly amplified by the repetitive polymerization and nicking reaction. After 7 minutes, 70-μL of this reaction mixture was transferred to the other reaction vessel. A 50-μL solution of Capture DNA probe attached to the magnetic particle was dispensed in the reaction vessel and incubated for 18 minutes at 37°C. In this amplification and capturing reaction mixture containing Bst DNA Polymerase, Large Fragment and Nb.BbvCI at 0.04 and 0.05 units/μL, respectively, amplification of Signal DNA1 continues and hybridization of Signal DNA1 to a capture DNA probe concurrently occurs (Fig. 10). After washing the magnetic particles under magnetic attraction, a 50-μL solution of acridinium-labeled Chemiluminescence DNA probe was dispensed and incubated for 4 minutes at 37°C. In this hybridization reaction mixture, Chemiluminescence DNA probe hybridizes to Signal DNA1 captured by Capture DNA probe on the magnetic particle. After washing the magnetic particles again under magnetic attraction, Pre-trigger solution containing hydrogen peroxide and Trigger solution containing sodium hydroxide of ARCHITECT i system (Abbott Japan) were dispensed and incubated for 18 seconds at 37°C. The chemiluminescence of acridinium at the wavelengths in the range of 400 to 500 nm is measured by the optical system of the analyzer. In principle, the intensity of chemiluminescence is proportional to the amount of amplified Signal DNA1. In the one-step amplification assays, Converter DNA-21, Converter DNA-18 and Converter DNA-500, which were designed for the

corresponding target miRNAs (miR-21-5p, miR-18a-5p and miR-500a-3p), were used. The final concentrations of Converter DNA and each dNTP were 1.4 nM and 100 μM, respectively. In the two-step amplification assays for miR-21-5p and miR-200 family, the reaction was performed similarly as the above described with the Converter DNAs, each having the TBS for the corresponding target miRNA (miR-21-5p, miR-200a, miR-200b and miR-200c), except for the final concentrations of Cascade DNA and each dNTP were 4.2 nM and 10 μM, respectively.

Generation of the calibration curve for estimating the amplification rate

The serially diluted Signal DNA solutions were spiked in 10 mM Tris-HCl, 0.01% BSA (pH 8.0) and automatically measured similarly to the corresponding miRNA assays with Converter DNA, DNA polymerase, nicking endonuclease, dNTPs, Capture DNA probe, and Chemiluminescence DNA probe.

Automated nucleic acid detection in human serum on the analyzer

The ssDNA having the sequence the same as miR-21-5p, termed miD-21-5p, was spiked directly into normal human serum. The sample was measured via the two-step amplification assay for miR-21-5p.

Results and discussion

The effect of cover sequence introduction to Converter DNA and comparison of nicking endonucleases in efficiency of amplification reaction

Fluorescence intensity representing the Signal DNA amplification in the isothermal one-step amplification assay of the target miD-24-3p increased in the order of Converter DNA-24-1 without the cover sequence and with the recognition sequence of Nt.AlwI, Converter DNA-24-2 with the cover sequence and the recognition sequence of Nt.AlwI, and Converter DNA-24-3 with the cover sequence and the recognition sequence of Nb.BbvCI. (Fig. 11). I concluded that the introduction of the cover sequence to Converter DNA promoted the Signal DNA amplification and the amplification efficiency with Nb.BbvCI is higher than that with Nt.AlwI in the reaction at constant 37 ^{o}C.

Automated miRNA detection with one-step amplification on the analyzer

I measured different concentrations of synthetic miR-21-5p from 0.01 to 1000 pM in triplicate, and 0 pM in replicates of 5 via the one-step amplification assay for miR-21-5p using Converter DNA-21 (Table 1b). The Converter DNA was designed to form a hairpin structure by the introduction of Cover sequence as shown in Fig. 9. The hairpin structure accelerates DNA amplification by preventing Signal DNA released into the solution from hybridizing to Signal generation sequence of intact Converter DNA (Fig. 11). The assay results were obtained in 44 minutes after initial sample dispense

with throughput of 66 tests per hour. The resulting dose response curve of the miR-21-5p assay is shown in Fig. 12a. The coefficient of variations (CVs) of chemiluminescence intensity (CI) for the target at concentrations of 1 pM and higher were less than 6%. The CVs of CI even at low target concentrations both of 10 and 100 fM were 10% and that of the background CI at 0 pM was 13%. The detection sensitivity was in the range from 10 to 100 fM. Similarly, I also measured miR-18a-5p and miR-500a-3p on the analyzer with Converter DNA-18 and Converter DNA-500, respectively (Fig. 12a).

The one-step amplification assays for miR-18a-5p and miR-500a-3p, which were developed by changing the TBS of Converter DNA-21, exhibited comparable sensitivity as shown in the dose response curves (Fig. 12a). In the present assay system, the target miRNA is easily altered only by changing the TBS of Converter DNA since miRNA is converted to the common Signal DNA to be detected with the common Capture DNA probe and Chemiluminescence DNA probe. More than a hundred circulating miRNAs in blood have been so far identified as diagnostic, prognostic, or predictive biomarkers for different types of cancers [69]. The proposed assay allows the use of a common detection system and promotes the development of multiple miRNA assays.

Next, I investigated the cross reactivity of the one-step amplification assay for miR-21-5p to evaluate the sequence specificity. I selected fifteen human miRNAs which have sequences similar to that of miR-21-5p according to the miRNA database, miRBase [32, 33]. The numbers of bases identical to those in miR-21-5p sequence ranged from 8 to 14 (Table 2). I measured the samples of each synthetic miRNA at the

concentrations from 0 to 1000 pM. The RLU obtained with the synthetic miRNA was read off of the dose response curve of miR-21-5p and represents the interfering miR-21-5p concentration (Fig. 12b). The cross reactivity was evaluated with the ratio of the interfering miR-21-5p concentration to each synthetic miRNA concentration. No cross reactivity was observed for fourteen miRNAs (miR-15a-5p, miR-30a-3p, miR-195-5p, miR-491-3p, miR-16-1-3p, miR-34a-5p, miR-200a-3p, miR-337-5p, miR-340-5p, miR-425-5p, miR-9-1-5p, miR-338-3p, miR-505-5p, miR-660-5p) (Fig. 12b). At the highest, 0.02% of cross reactivity was found in miR-590-5p at the concentration of 1000 pM, which has 13 bases identical to those in miR-21-5p sequence. These results indicated high sequence specificity of the proposed miR-21-5p assay.

Automated miR-21-5p detection with two-step amplification on the analyzer

The detection sensitivity of the one-step amplification assay for miR-21-5p in the range from 10 to 100 fM was not sufficient because the cut off concentration of miR-21-5p in serum and plasma for cancer diagnosis is around 50 fM [67]. I extended the one-step amplification assay to the two-step amplification assay by layering linear amplification reactions [87]. I measured different concentrations of synthetic miR-21-5p from 0 to 1,000 pM in replicates of 5 on the analyzer (Table 1c). The obtained dose response curve is shown in Fig. 13a. The curve fitting of the dose response curve with fitted equation and correlation coefficient is shown in Fig. 13b. For determining the precise detection limit, I further measured different concentrations of synthetic miR-21-5p from 0 to 50 fM in the samples in replicates of 20 (Fig. 13c). The sample

with 0-fM (blank) and 3-fM miR-21-5p showed the mean CI of 79 relative light unit (RLU) (standard deviation (SD) 15) and 322 RLU (SD 30), respectively. The 3 fM of miR-21-5p was distinguished from the blank sample (P (two-tailed unpaired *t*-test) < 10^{-21}). The two-step amplification assay achieved the detection sensitivity value of 3 fM for miR-21-5p, significantly lower than the cut off concentrations in serum around 50 fM [67]. This highly sensitive detection is attributed not only to the introduction of Cover sequence and layering linear amplification reactions, but also to the application of chemiluminescence reaction with Capture DNA probe and Chemiluminescence DNA probe emulating the common sandwich assay in immunoassay. This reaction design is suitable for the fully-automated immunoassay analyzer.

In Fig. 13a, the CVs of CI for the target at concentrations from 0.01 pM to 1000 pM were less than 8%. The CV of CI even at low target concentration of 0.001 pM and that of the background CI at 0 pM were both 18%. In Fig. 13b, the CVs of CI for the target at concentrations of 3 fM were 9%, and those at five concentrations from 5 to 50 fM were less than 8%. These results indicated the high sensitivity and reproducibility of the assay.

Amplification rates as the ratios of amplified Signal DNA2 concentration to the target miR-21-5p concentration were estimated by using the calibration curve of Signal DNA2. The calibration curve of Signal DNA2 was generated in the measurement with serially diluted Signal DNA2 in 10 mM Tris-HCl, 0.01% BSA (pH 8.0) (Fig. 14a). The mean amplification rate for the target concentrations from 0.1 to 10 pM was 103-folds (Fig. 14b). For comparison, I also estimated the amplification rate in the one-step

amplification assay based on the results shown in Fig. 12a. The obtained mean amplification rate for the target miR-21-5p at concentrations from 0.1 to 10 pM was 26-folds (Fig. 15a, 15b). The cascading of Signal DNA generation achieved the higher amplification rate in the two-step amplification assay for miR-21-5p than that in the one-step amplification assay.

I also investigated whether precursor miR-21-5p (60 nt), which contains mature miR-21-5p sequence and forms a secondary structure, and mature miR-21-5p (22 nt) were distinguished in the two-step amplification assay. The samples of synthetic precursor miR-21-5p and mature miR-21-5p at the concentrations from 0 to 1000 pM were measured. The cross reactivity evaluated as the ratio of interfering concentration of precursor miR-21-5p to the concentration of mature miR-21-5p was, at the highest, 0.01% at the concentration of 1000 pM (Fig. 16). The result indicated that the miR-21-5p assay with Converter DNA-21 preferably detected mature miR-21-5p than precursor miR-21-5p.

Automated miR-200 family detection with two-step amplification on the analyzer

The miR-200 family consists of miR-200a, miR-200b, miR-200c, miR-141 and miR-429 which have highly homologous sequences (Table 1c) [85]. I investigated the cross reactivity of the two-step amplification assays for miR-200a, miR-200b and miR-200c by evaluating the ratio of interfering concentrations of the other members of miR-200 family to the concentration of the target miRNA. The samples of synthetic miRNAs, each containing miR-200a, miR-200b, miR-200c, miR-141 or miR-429 at the

concentrations from 0 to 1000 pM, were measured in three respective assays with Converter DNAs having TBS for miR-200a, miR-200b and miR-200c. In the assay for miR-200a, the cross reactivity values were not higher than 0.20, 0.01, 0.30 and 0.19% for miR-200b, miR-200c, miR-141 and miR-429, respectively (Fig. 17a). In the assay for miR-200b, the cross reactivity values were not higher than 0.06, 0.64, 0.02 and 0.01% for miR-200a, miR-200c, miR-141 and miR-429, respectively (Fig. 17b). In the assay for miR-200c, the cross reactivity values were 0.003, 0.44, 0.07 and 0.002% for miR-200a, miR-200b, miR-141 and miR-429, respectively (Fig. 17c). The similar miRNA sequences did not cause the false positive signal higher than 0.64%, showing high sequence specificity of the two-step amplification assays for miR-200 family detection.

These results indicated that my miRNA assays with two-step amplification on the analyzer have high reproducibility and sequence specificity and could be clinically useful in cancer detection.

Automated nucleic acid detection in human serum on the analyzer

I performed the two-step amplification assay in normal human serum containing the ssDNA on the analyzer. MiD-21-5p spiked in serum samples at different concentrations from 0.001 to 1000 pM and a blank at 0 pM were measured in replicates of 10 and 20, respectively on the automated analyzer. The obtained dose response curve is shown in Fig. 18a. The curve fitting of the dose response curve with fitted equation and correlation coefficient is shown in Fig. 18b.

To determine the sensitivity of the assay, I further measured different concentrations of miD-21-5p in serum samples from 1 to 100 fM in replicates of 10 and a blank at 0 fM in replicates of 20 (Fig. 18c). The mean CI calculated for the target miD-21-5p at the concentrations of 0 and 10 fM were 840 RLU (SD 60) and 1101 RLU (SD 100), respectively. The 10 fM of miD-21-5p in serum was distinguished from the blank serum sample (P (two-tailed unpaired *t*-test) < 10^{-8}). The CVs of CI in the measurement with the target miD-21-5p in serum at concentrations from 0 to 1000 pM were less than 8% except for 9% at 0.01 pM, which again represented the high reproducibility of the assay. The mean amplification rate for the target concentrations from 0.1 to 10 pM was 126-fold (Fig. 19a, 19b). These results indicated that the two-step amplification assay for ssDNA in normal human serum had high detection sensitivity and reproducibility.

The physiological temperature condition of constant 37^{o}C eliminated the need to purify the miRNA due to blood clotting caused by high-temperature conditions. Also, detergent addition, which is required for detaching miRNAs from the protein or breaking down exosomes in human serum [88], was implemented in the fully-automated process.

Conclusions

The fully-automated miRNA measurement was developed by adapting an isothermal DNA amplification reaction to the existing automated immunoassay analyzer, ARCHITECT i system. The reaction temperature at constant 37^{o}C eliminates the

purification process and results in a testing time of 44 minutes and a throughput of 66 tests per hour. The proposed assay for a cancer-related miRNA showed high detection sensitivity, high reproducibility and low cross reactivity. Implementation of the measurement of miRNAs as potential cancer markers together with immunoassays for the conventional protein markers on the single automated analyzer would allow the precise diagnosis and reduce the cost of nucleic acid test. I believe my work will enable early cancer diagnosis based on high throughput measurement of multiple biomarkers in full automation, which is useful both for patients and clinical laboratories in terms of testing time, cost, and accurate diagnosis.

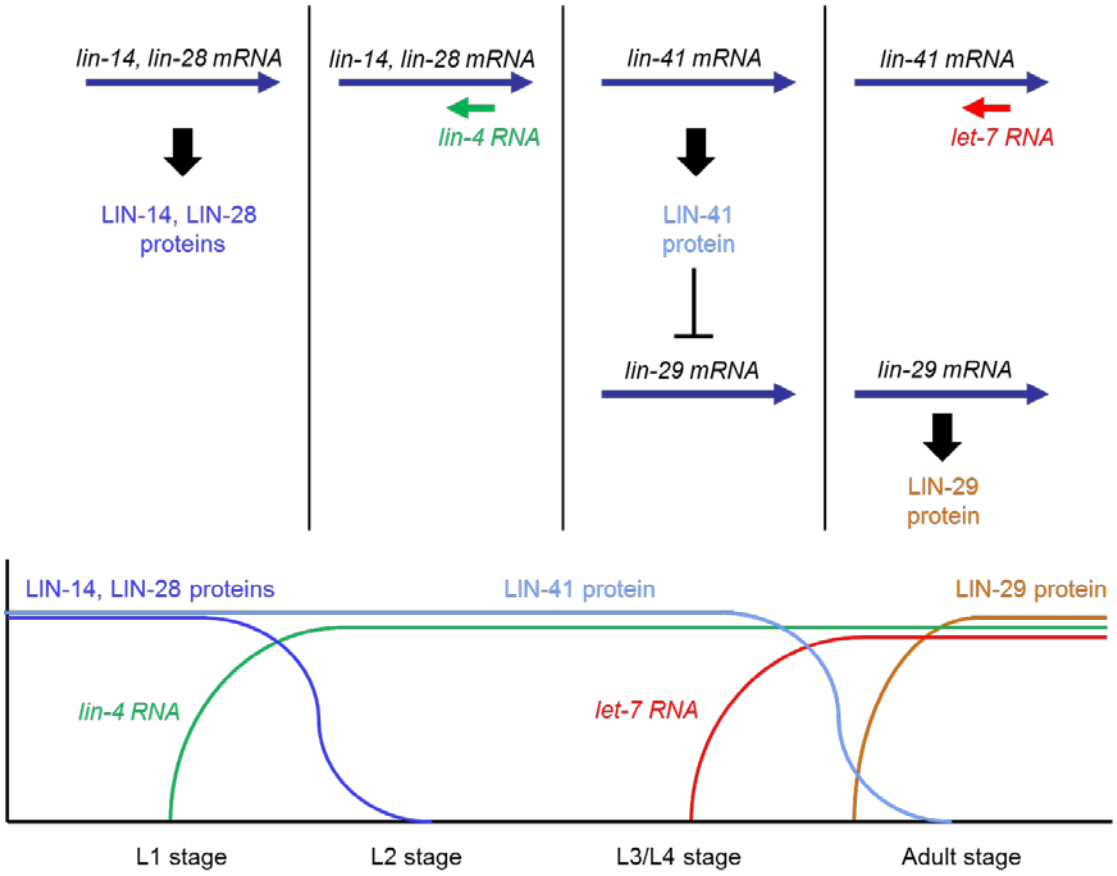


Fig. 1 The schematic illustrations of successive regulation of heterochronic gene activities by the *lin-4 and let-7* in *C. elegans*. The expression levels of LIN-14 and LIN-28 proteins are decreased by the *lin-4* expression at the end of first larval stage. The expression level of LIN-41 protein is decreased by the *let-7* expression at the late larval stage. The expression level of LIN-29 protein is increased by the decrease of LIN-41 protein. Fig. 1 was drawn by modifying the Figure 5 in [4].

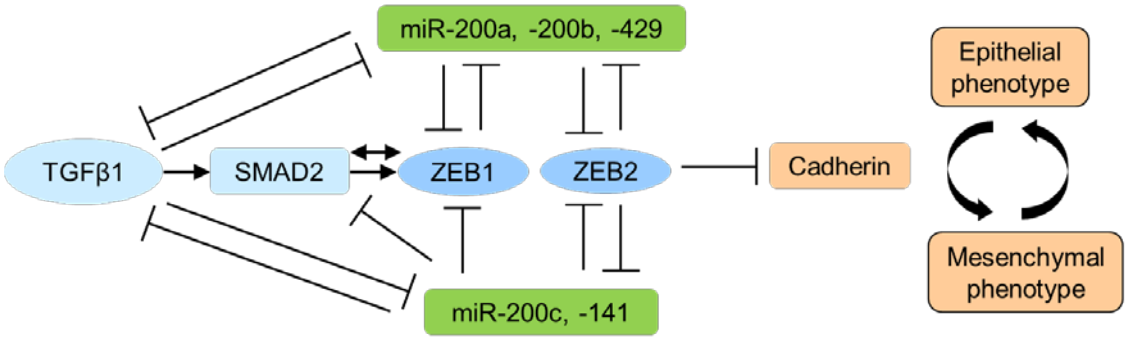


Fig. 2 The schematic illustrations of double negative Zinc-finger E-box-binding homeobox 1 (ZEB1) and ZEB2 which are E-cadherin transcriptional repressors and miR-200 family (miR-200a, -200b, -200c, -141 and -429) feedback loop of TGF β pathway. EMT (epithelial to mesenchymal transition) is induced by the repression of Cadherin. The Fig. 2 was drawn by modifying the Figure 1 in [17].

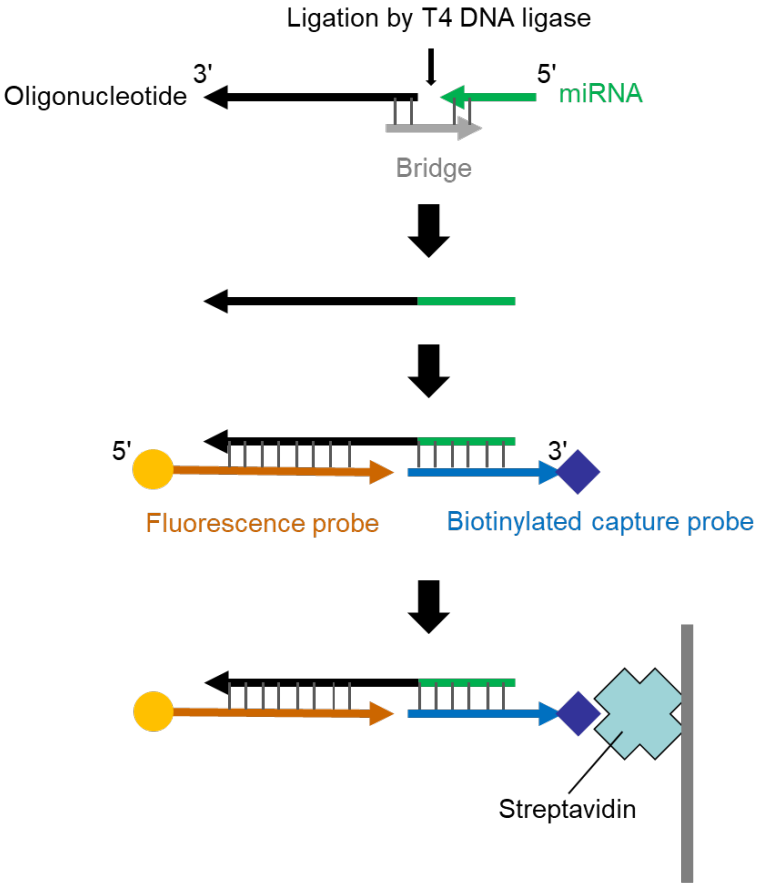


Fig. 3 The schematic illustrations of the principle of a splinted ligation assay for miRNAs. The splinted ligation assay is a new measurement method of miRNAs which doesn't require the amplification and labeling of miRNAs.

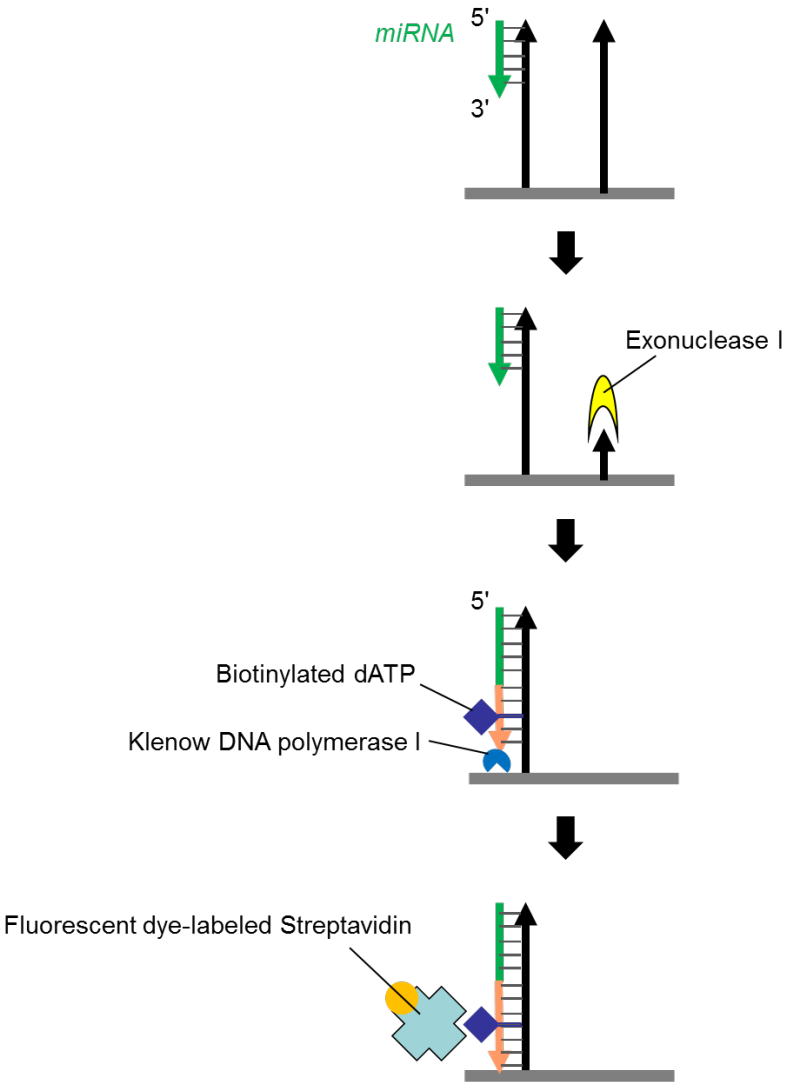


Fig. 4 The schematic illustrations of the principle of a RNA-primed, array based Klenow enzyme (RAKE) assay for miRNAs. The RAKE assay is a new measurement method of miRNAs which does not require the amplification and labeling of miRNAs.

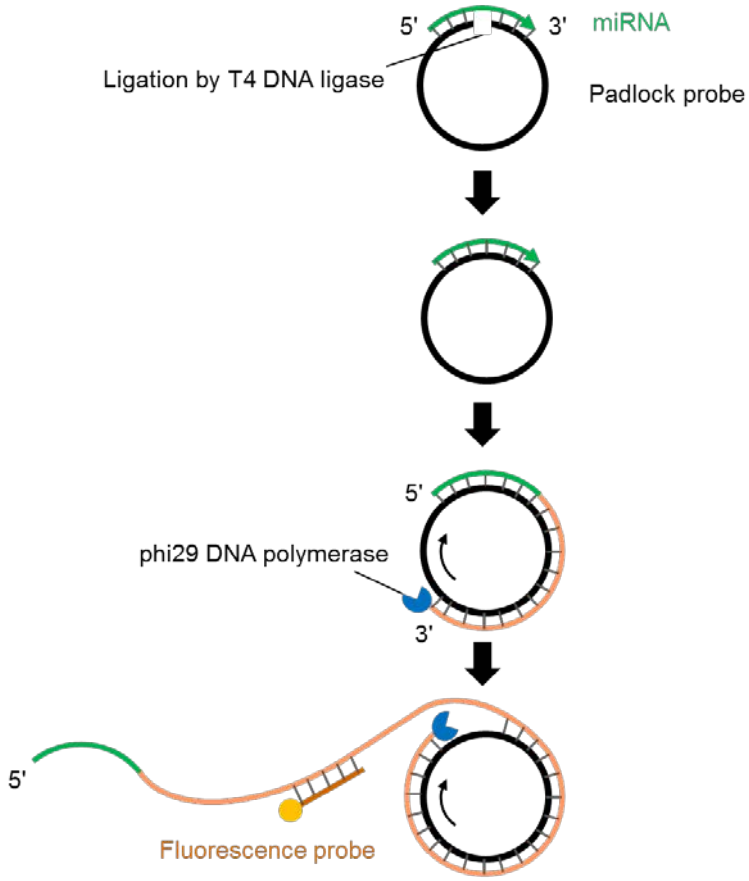
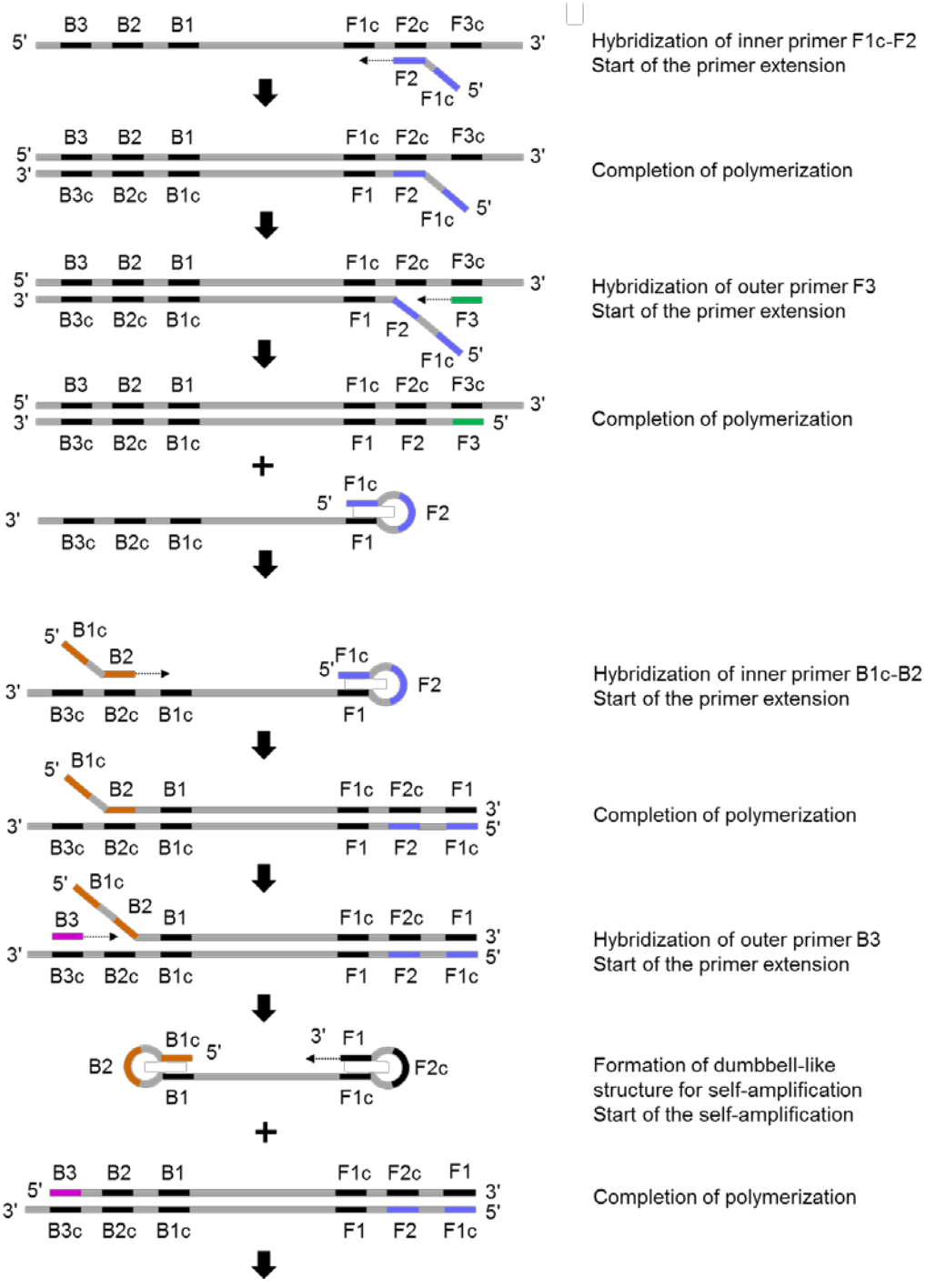


Fig. 5 The schematic illustrations of the principle of an isothermal DNA amplification method, rolling circle amplification (RCA) assay for miRNAs. The RCA assay is an emerging miRNA method which uses polymerase with the strand displacement activity.


B3 B2 B1 F1c F2c F3c
5' 3'
F2
F1c 5'
B3c B2c B1c F1 F2 F3
Hybridization of inner primer F1c-F2
Start of the primer extension
Completion of polymerization
Hybridization of outer primer F3
Start of the primer extension
Completion of polymerization
Hybridization of inner primer B1c-B2
Start of the primer extension
Completion of polymerization
Hybridization of outer primer B3
Start of the primer extension
Formation of dumbbell-like
structure for self-amplification
Start of the self-amplification
Completion of polymerization

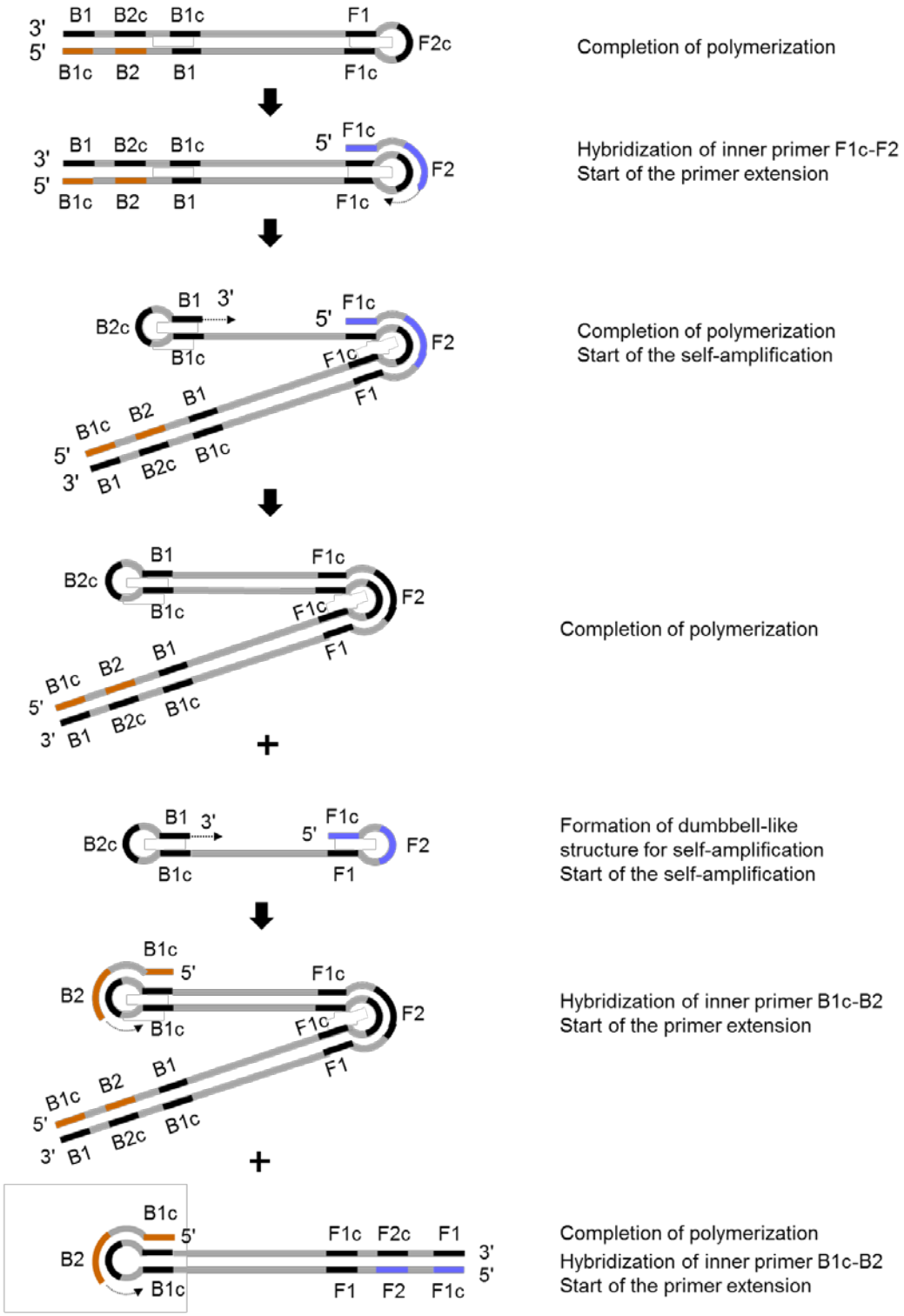
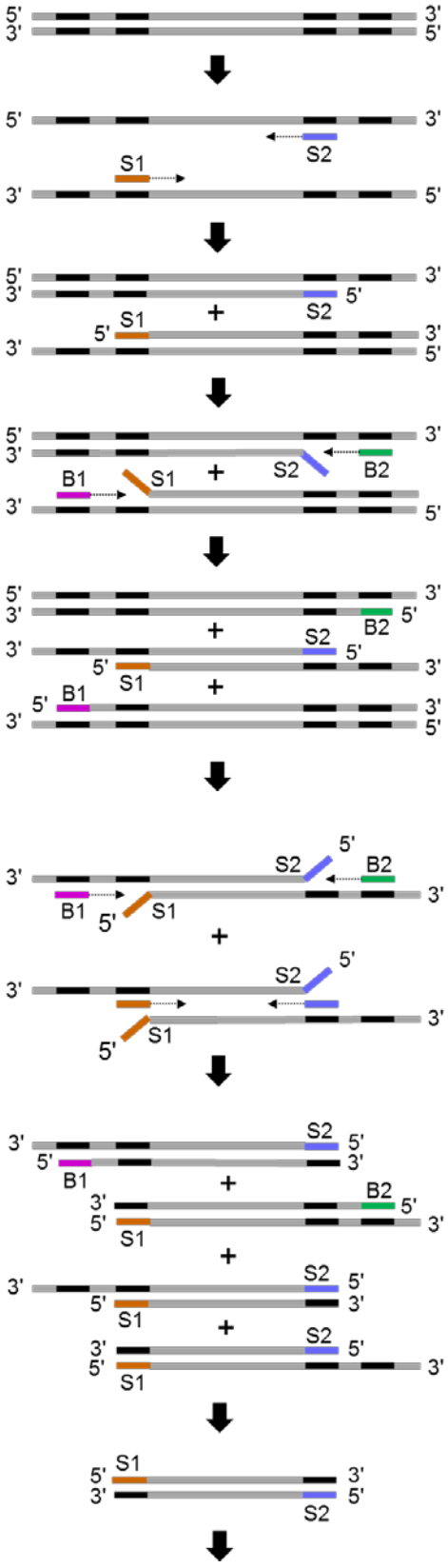


Fig. 6 The schematic illustrations of the principle of an isothermal DNA amplification method, loop-mediated isothermal amplification (LAMP). The LAMP

method is used in a clinically approved assay. The LAMP assay achieves highly efficient amplification and sensitive detection of nucleic acids of infectious diseases, which are measured in one pot with 60 °C incubation in less than one hour.


5'
3'
3'
5'
Denature
S1
S2
Hybridization of inner primers
Start of the primer extension
Completion of polymerization
B1
B2
Hybridization of outer primers
Start of the primer extension
Completion of polymerization
Hybridization of the opposite
synthesized DNAs
Hybridization of outer primers
Start of the primer extension
Hybridization of inner primers
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Completion of polymerization
Hybridization of the opposite
synthesized DNAs

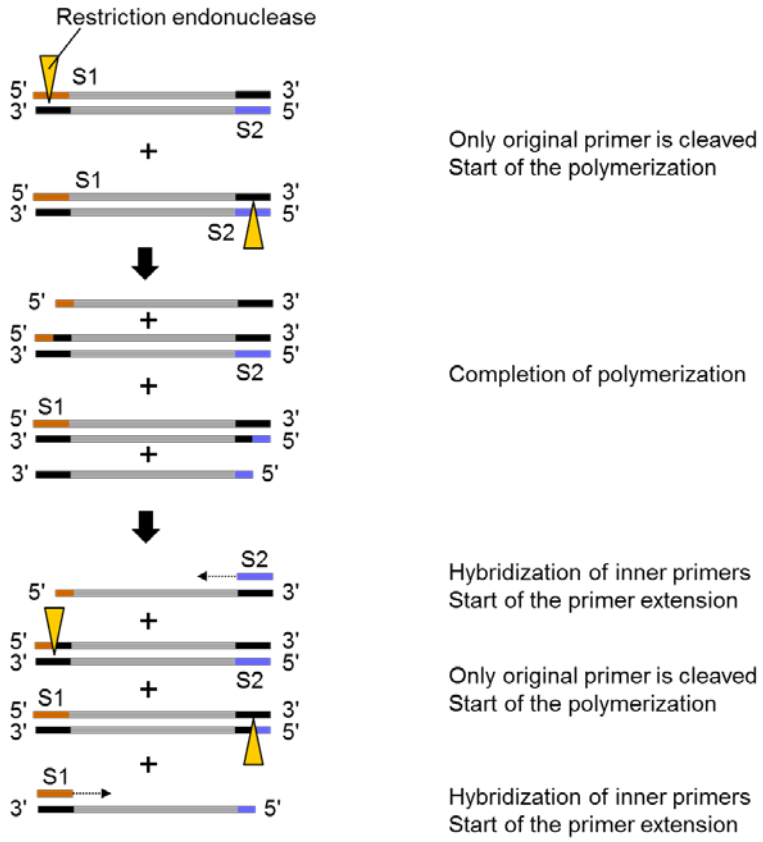


Fig. 7 The schematic illustrations of the principle of an isothermal DNA amplification method, strand displacement amplification (SDA). The SDA method is used in a clinically approved assay. The SDA assay achieves highly efficient amplification and sensitive detection of nucleic acids of infectious diseases, which are measured with 54 °C incubation.

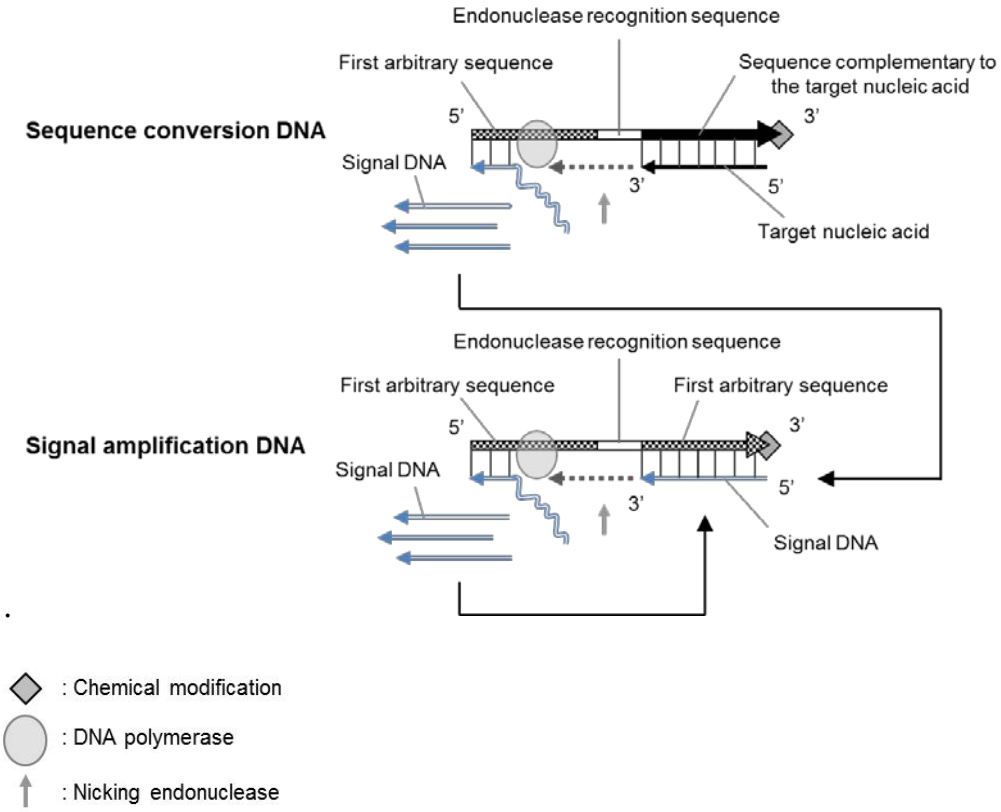


Fig. 8 The schematic illustrations of the principle of an isothermal DNA amplification method, low-temperature amplification (L-TEAM) proposed by Komiya. The method generates exponential amplification of Signal DNAs with 37 ºC incubation.

a

one-step amplification assay on the real-time PCR system

miD-24-3p	5' TGGCTCAGTTCAGCAGGAACAG 3'
Converter-24-1	5' TGATAGCCCTGTACAATGCTGCTCAGAGATCCCTGTTCCTGCTGAACTGAGCCA-(I) 3'
Converter-24-2	5' TGATAGCCCTGTACAATGCTGCTCAGAGATCCATTGTACAGGGCTATCACTGTTCCTGCTGAACTGAGCCA-(I) 3'
Converter-24-3	5' TGATAGCCCTGTACAATGCCTCAGCCATTGTACAGGGCTATCACTGTTCCTGCTGAACTGAGCCA-(I) 3'
Molecular beacon	5' (F)-CGCGATGATAGCCCTGTACAATGCTGCTTCGCG-(D) 3'

b

one-step amplification assay on the immunoassay analyzer

miR-21-5p	5' UAGCUUAUCAGACUGAUGUUGA 3'
miR-18a-5p	5' UAAGGUGCAUCUAGUGCAGAUAG 3'
miR-500a-3p	5' AUGCACCUGGGCAAGGAUUCUG 3'
Converter-21	5' TCTTGCGTATCTTCTCCACCTCAGCTGGAGAAGATACGCAAGATCAACATCAGTCTGATAAGCTA-(I)-(I) 3'
Converter-18	5' TCTTGCGTATCTTCTCCACCTCAGCTGGAGAAGATACGCAAGACTATCTGCACTAGATGCACCTTA-(I)-(I) 3'
Converter-500	5' TCTTGCGTATCTTCTCCACCTCAGCTGGAGAAGATACGCAAGACAGAATCCTTGCCCAGGTGCAT-(I)-(I) 3'
Signal DNA1	5' TGAGGTGGAGAAGATACGCAAGA 3'
Capture DNA probe 1	5' CTTCTCCACCTCA-(B-TEG) 3'
Chemiluminescence DNA probe 1	5' (NH_2-C6)-T^{L}C^{L}TTGC^{L}GTAT 3'

c

two-step amplification assay on the immunoassay analyzer

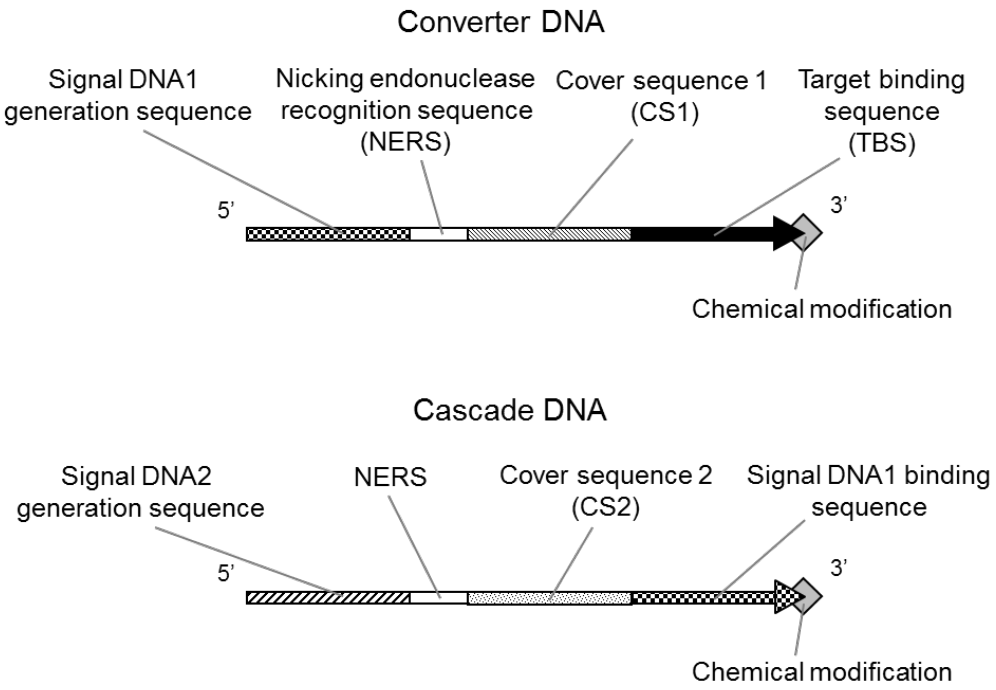
miR-21-5p	5' UAGCUUAUCAGACUGAUGUUGA 3'
miD-21-5p	5' TAGCTTATCAGACTGATGTTGA 3'
Precursor miR-21-5p	5' UAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAGUCGAUGGGCUGUC 3'
miR-200a	5' UAACACUGUCUGGUAACGAUGUU 3'
miR-200b	5' UAAUACUGCCUGGUAAUGAUGAC 3'
miR-200c	5' UAAUACUGCCGGGUAAUGAUGGA 3'
miR-141	5' UAACACUGUCUGGUAAAGAUGGC 3'
miR-429	5' UAAUACUGUCUGGUAAAACCGU 3'
Converter-21S	5' TCTTGCGTATCTTCTCCACCTCAGCGCAAGATCAACATCAGTCTGATAAGCTA-(I)-(I) 3'
Converter-200a	5' TCTTGCGTATCTTCTCCACCTCAGCGCAAGAAACATCGTTACCAGACAGTGTTA-(I)-(I) 3'
Converter-200b	5' TCTTGCGTATCTTCTCCACCTCAGCGCAAGAGTCATCATTACCAGGCAGTATTA-(I)-(I) 3'
Converter-200c	5' TCTTGCGTATCTTCTCCACCTCAGCGCAAGATCCATCATTACCCGGCAGTATTA-(I)-(I) 3'
Cascade DNA	5' AGCAGCAACATAGCAGAACCTCAGCGCTGCTTCTTGCGTATCTTCTCCA-(I)-(I) 3'
Signal DNA2	5' TGAGGTTCTGCTATGTTGCTGCT 3'
Capture DNA probe 2	5' TAGCAGAACCTCA-(B-TEG) 3'
Chemiluminescence DNA probe 2	5' (NH_2-C6)-AGCAGCAACA 3'

Table 1 (**a**) Oligodeoxyribonucleotide sequences used for investigating the effect of cover sequence introduction to Converter DNA and comparison of nicking endonucleases. The underlined and double-underlined letters of Converter-24-1, 24-2 and Converter-24-3 represent the recognition sequences of Nt.AlwI and Nb.BbvCl, respectively. Converter-24-1 has no cover sequence. Converter-24-2 and Converter-24-3 were designed to form the hairpin structure with 18-bp stems. (F) and (D) represent 6-carboxyfluorescein (6-FAM), and dabcyl, respectively. The molecular beacon, which was designed to form the hairpin structure with a 5-bp stem, has the sequence

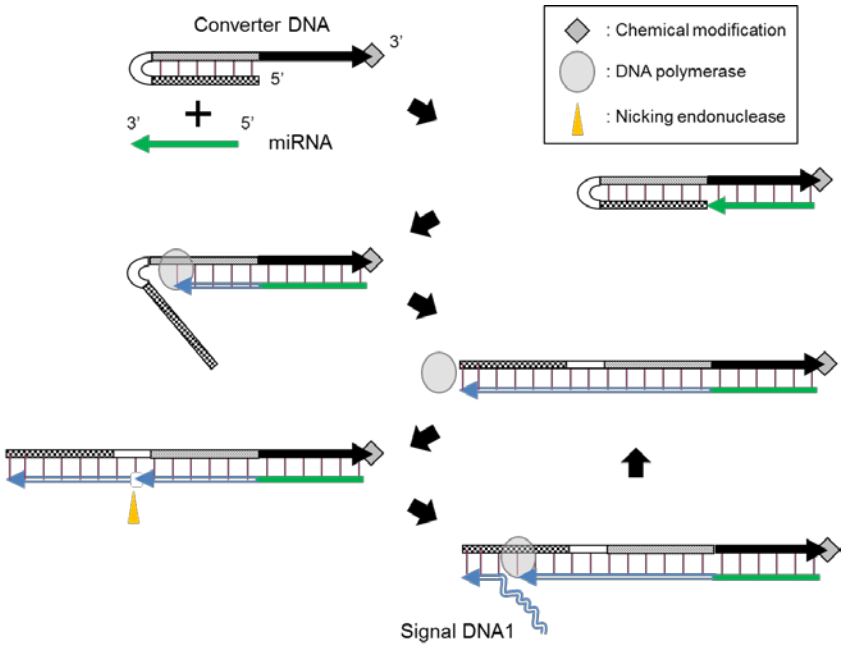
complementary to that of Signal DNA. Upon hybridization to the Signal DNA, the fluorophore, 6-FAM and the quencher, dabcyl attached to the 5′ and 3′ end ends of the molecular beacon becomes apart, resulting in fluorescence emission.

Oligodeoxyribonucleotide and oligoribonucleotide sequences used in the (**b**) one-step and (**c**) two-step amplification assays for miRNA measurement on the analyzer. The 3′ end of Converter DNA and Cascade DNA were chemically modified with two consecutive inverted deoxyribothymidines, represented by (I)-(I), to avoid unexpected extension reactions. Since the GC-content of Chemiluminescence DNA probe 1 was low, Locked Nucleic Acid, which is an artificial nucleic acid having a bridged structure in the ribose portion, was introduced into it as indicated by the superscript L symbol for assuring high detection sensitivity. (B-TEG) and (NH_2-C6) represent biotin with triethylene glycol spacer and primary amine with six carbon spacer, respectively. The underlined letters represent the recognition sequences of Nb.BbvCl.

a


Converter DNA
Signal DNA1 generation sequence
Nicking endonuclease recognition sequence (NERS)
Cover sequence 1 (CS1)
Target binding sequence (TBS)
5'
3'
Chemical modification
Cascade DNA
Signal DNA2 generation sequence
NERS
Cover sequence 2 (CS2)
Signal DNA1 binding sequence
5'
3'
Chemical modification

b


Converter DNA
3'
5'
3'
5'
miRNA
: Chemical modification
: DNA polymerase
: Nicking endonuclease
Signal DNA1

c

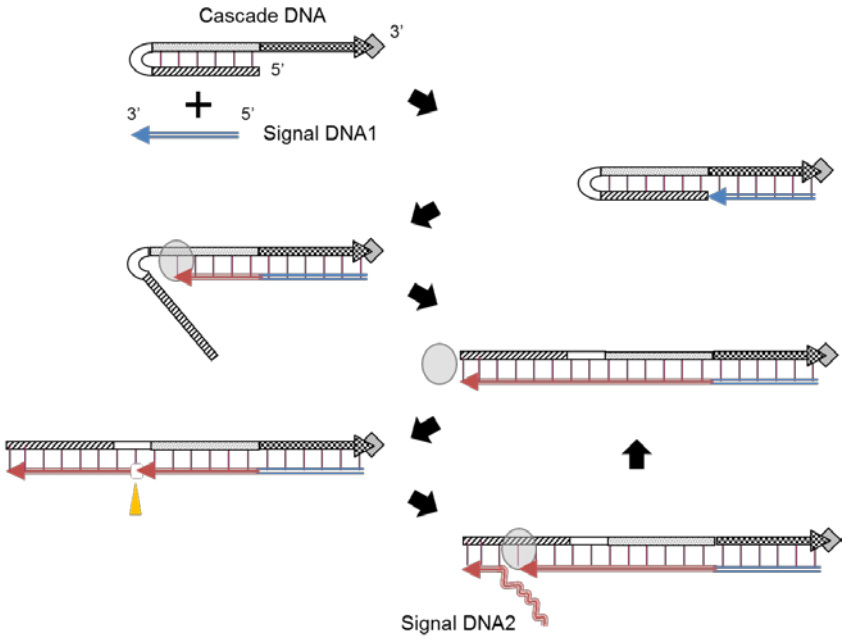


Fig. 9 The schematic illustrations of (**a**) the sequence domains of Converter DNA and Cascade DNA, (**b**) linear signal DNA amplification reaction in the one-step amplification assay or the first step of the two-step amplification assay, (**c**) linear signal DNA amplification reaction in the second step of the two-step amplification assay. Red vertical lines represent base pairings.

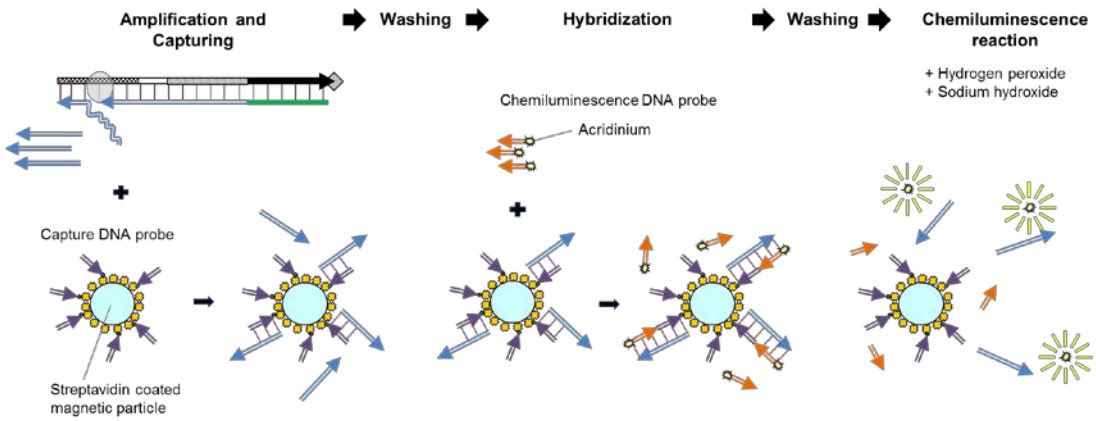


Fig. 10 The schematic illustrations of capturing of Signal DNA followed by chemiluminescence reaction on the fully-automated immunoassay analyzer. The analyzer is used for measuring proteins and chemical compounds in serum, plasma and urine as diagnostic markers in about 30 minutes at clinical laboratories.

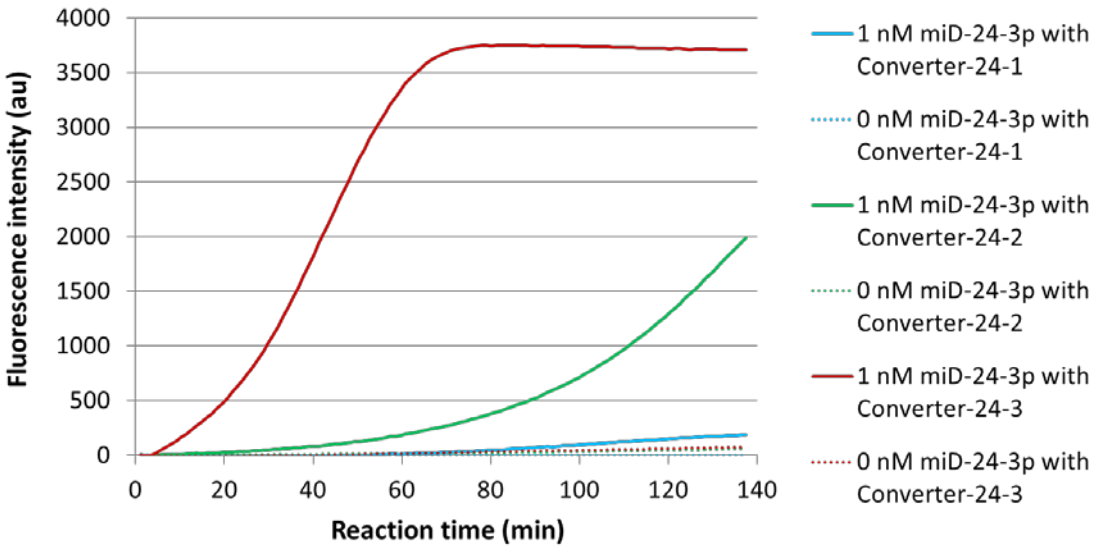
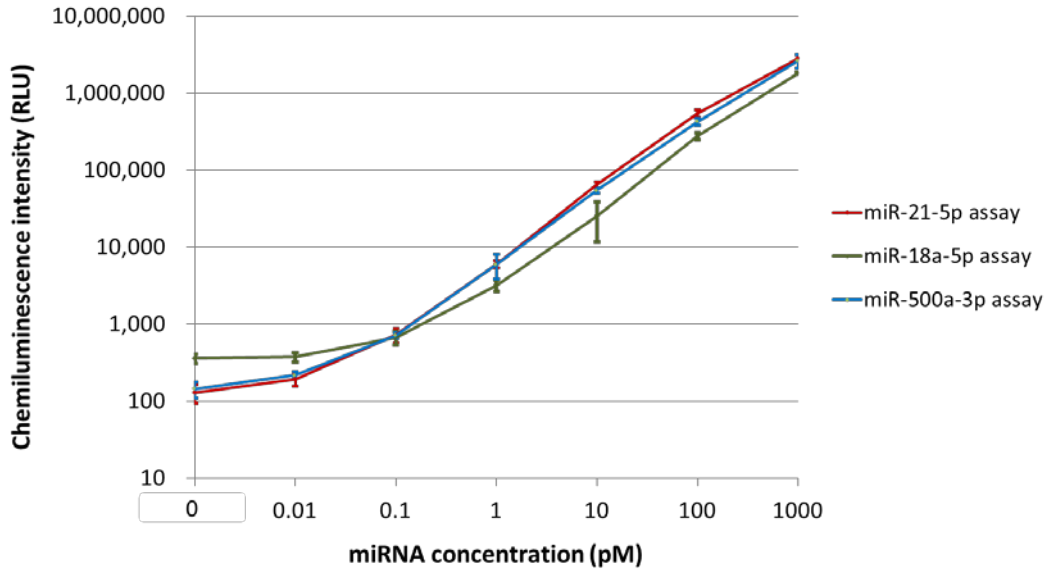


Fig. 11 Mean fluorescence curves in the isothermal one-step amplification assay of the target miD-24-3p with Converter DNA-24-1 without the cover sequence and with the recognition sequence of Nt.AlwI, Converter DNA-24-2 with the cover sequence and the recognition sequence of Nt.AlwI, and Converter DNA-24-3 with the cover sequence and the recognition sequence of Nb.BbvCl. Each sample was measured in triplicate and averaged.

a



b

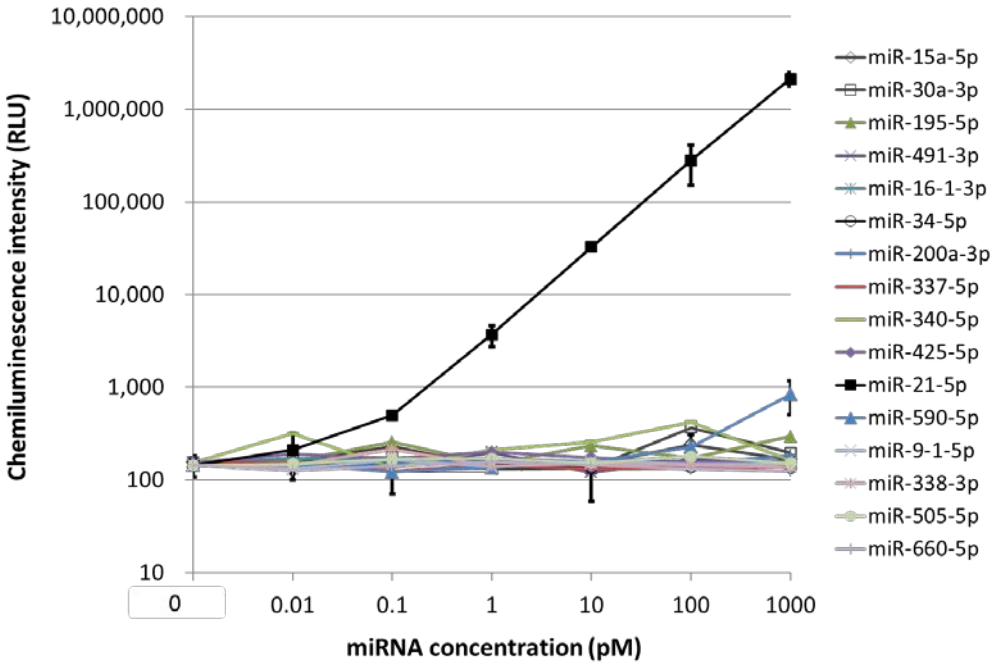


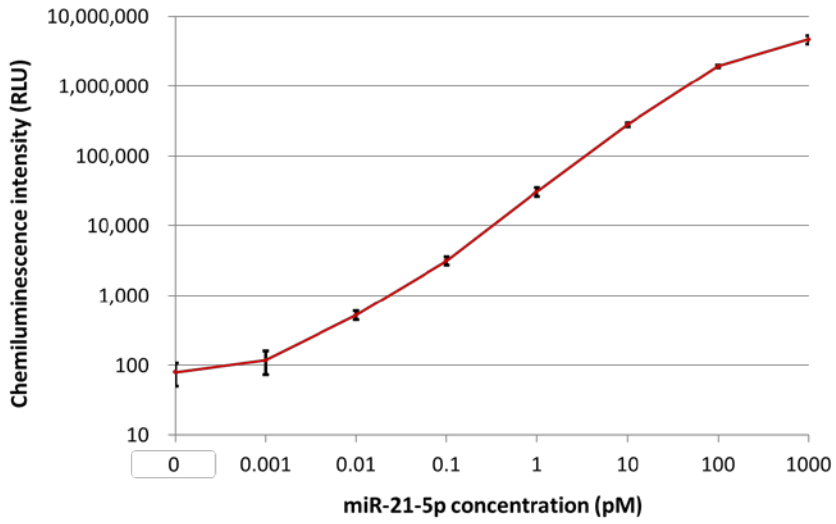
Fig. 12 (**a**) Dose responses in the respective one-step amplification assays for miR-21-5p, miR-18a-5p, and miR-500a-3p on the analyzer. The bar shows ± 2 standard deviations. Each sample was measured in triplicate except for the blank sample

(replicate (rep.) = 5). (**b**) Cross reactivity test of fifteen human miRNAs which have sequences similar to that of miR-21-5p in the one-step amplification assay for miR-21-5p on the analyzer. The bar shows ± 2 standard deviations. Each miRNA sample was measured in triplicate except for the blank sample (rep. = 5).

Human miRNAs	Number of identical bases	Sequences
miR-21-5p		**5' U A G C U U A U C A G A C U G A U G U U G A 3'**
miR-15a-5p	11	5' U A G C A G C A C A U A A U G G U U U G U G 3'
miR-30a-3p	14	5' C U U - U C A G U C G G A U G U U U G C A G C 3'
miR-195-5p	13	5' U A G C A G C A C A G A A - - A U A U U G G C 3'
miR-16-1-3p	12	5' C C A G - U A U U A A C U G U G C U G C U G A 3'
miR-200a-3p	9	5' U A A C A C U G U C U G G U A A C G A U G U 3'
miR-340-5p	11	5' U U A U A A A G C A A U G A G A C U G A U U 3'
miR-425-5p	8	5' A A U G A C A C G A U C A C U C C C G U U G A 3'
miR-9-1-5p	13	5' U C U U U G G U U A U C U A G C U G U - - A U G A 3'
miR-505-5p	8	5' G G G A G C C A G G A A G U A U U G A U G U 3'
miR-491-3p	8	5' C U U A U G C A A G A U U C C C U U C U A C 3'
miR-34a-5p	14	5' U G G C A G U G U C U U A C U G - - G U U G U 3'
miR-337-5p	11	5' G A A C G G C U U C A U A C A G G A G U U 3'
miR-590-5p	13	5' G A G C U U A U C A U A A A A G U G C A G 3'
miR-338-3p	14	5' U C C A G C A U C A G - - U G A U G U U G 3'
miR-660-5p	12	5' U A C C C A U U G C A U A U C G G A G U U G 3'

Table 2 miRNA sequences used in the cross reactivity test by one-step amplification assay on the analyzer. The symbol * indicates bases identical to those of miR-21-5p. The numbers of identical bases range from 8 to 14. The highest number of consecutive identical bases is 8.

a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1,000,000
100,000
10,000
1,000
100
10
Chemiluminescence intensity (RLU)
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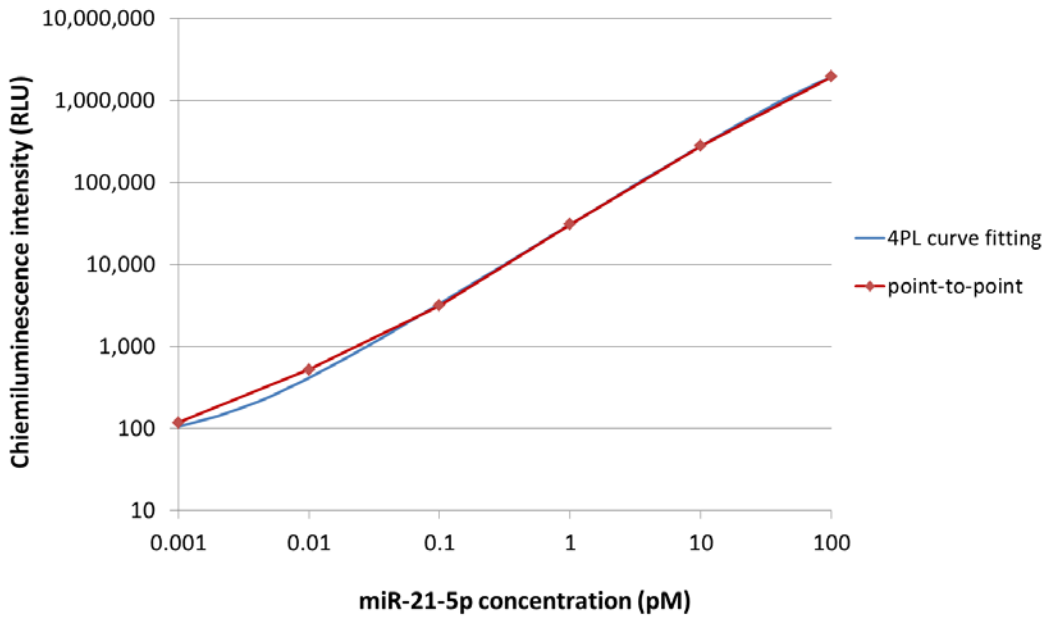
0.001
0.01
0.1
1
10
100
1000
miR-21-5p concentration (pM)

b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1,000,000
100,000
10,000
1,000
100
10
Chiemiluminescence intensity (RLU)
0.001
0.01
0.1
1
10
100
miR-21-5p concentration (pM)
4PL curve fitting
point-to-point

c

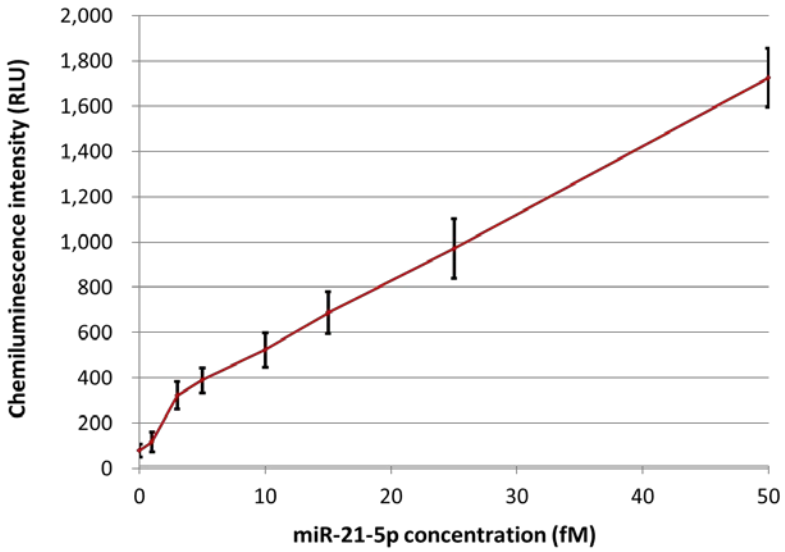
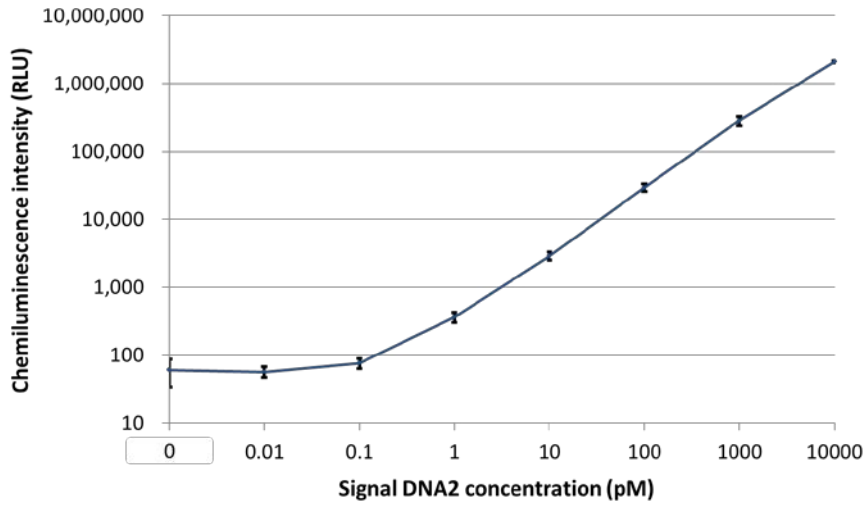


Fig. 13 (**a**) Dose response in the two-step amplification assay for miR-21-5p at the concentrations from 0 to 1,000 pM on the analyzer. The bar shows ± 2 standard deviations. Each sample was measured in replicates of 5. (**b**) The four parameter logistic (4PL) curve fitting of the dose response curve in the two-step amplification assay for miR-21-5p at the concentrations from 0 pM to 100 pM on the analyzer. The fitted equation was $Y = 6521131 + (69.73642 – 6521131) / (1 + (X/241.1466)^{0.9764099})$. The correlation coefficient was 1.0. Each sample was measured in replicates of 5. (**c**) Dose response for the detection sensitivity test in the two-step amplification assay for miR-21-5p at the concentrations from 0 to 50 pM on the analyzer. The bar shows ± 2 standard deviations. Each sample was measured in replicates of 20.

a



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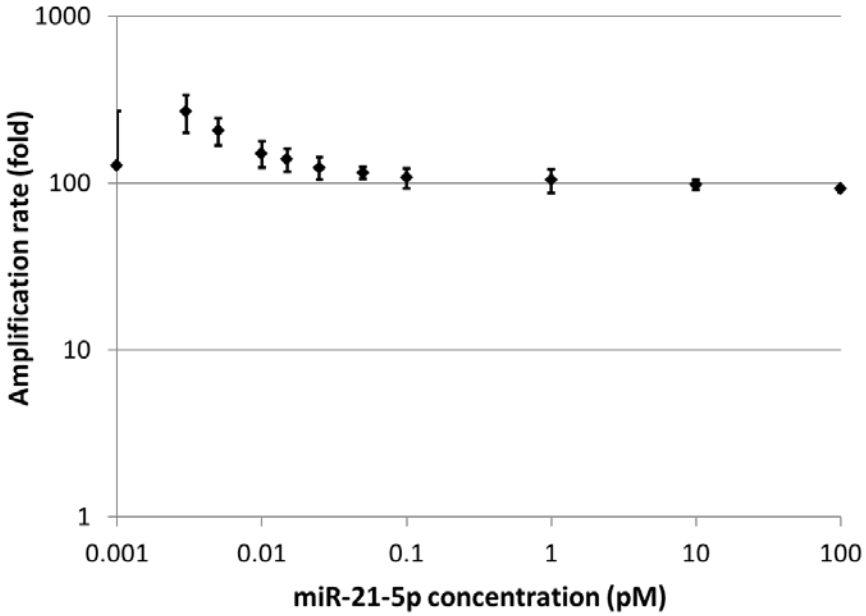
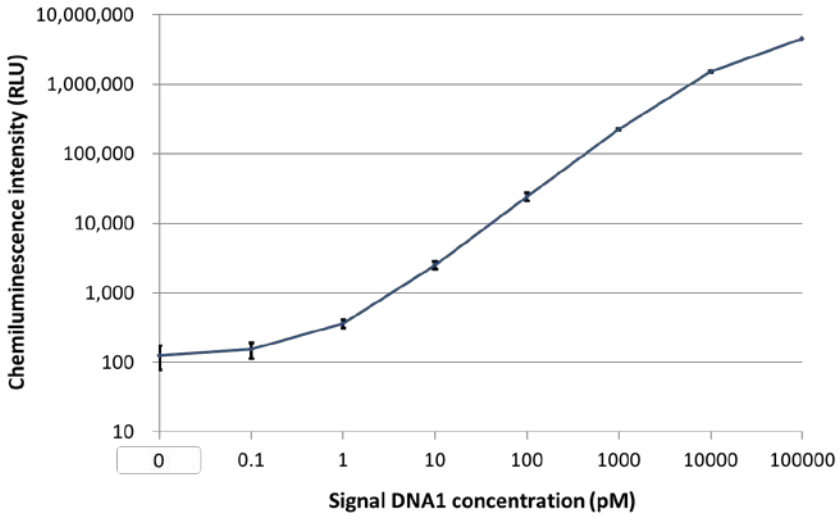


Fig. 14 (**a**) Calibration curve of Signal DNA2 spiked in the unreacted samples that were prepared same as the two-step amplification assay for miR-21-5p on the analyzer for calculating the amplification rates shown in Fig. 14 (b). The bar shows ± 2 standard deviations. Each sample was measured in triplicate. (**b**) Plot of amplification rates of

Signal DNA2 concentrations to those of the target miR-21-5p in the two-step amplification assay for miR-21-5p on the analyzer. The bar shows ± 2 standard deviation of the amplification rate. The samples containing the target miR-21-5p at concentrations from 0.001 to 0.1 pM were measured in replicates of 20. Those at concentrations from 1 to 100 pM were measured in replicates of 5.

a



b

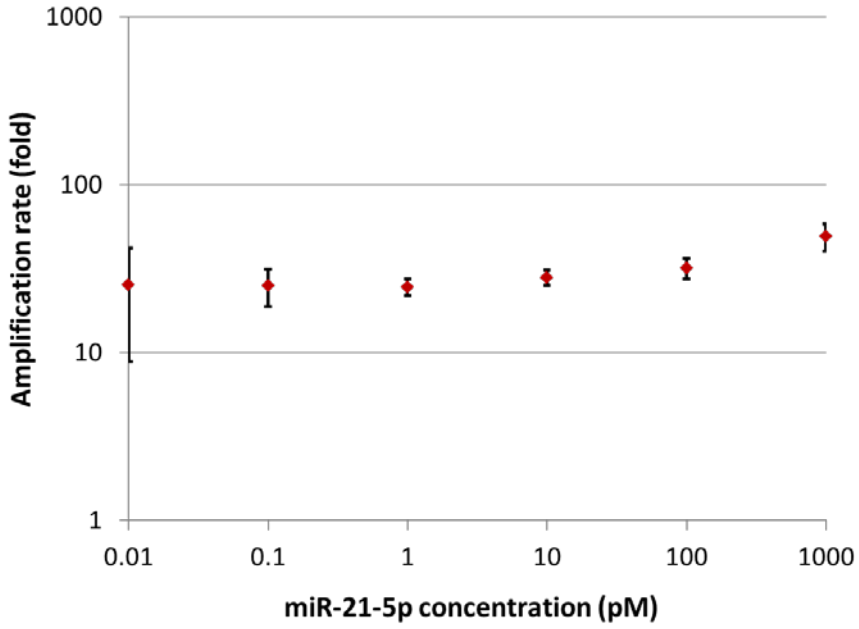


Fig. 15 (**a**) Calibration curve of Signal DNA1 spiked in the unreacted samples that were prepared same as the one-step amplification assay for miR-21-5p on the analyzer for calculating the amplification rates shown in Fig. 15 (b). The bar shows ± 2 standard deviations. Each sample was measured in triplicate. (**b**) Plot of amplification rates of

Signal DNA1 concentrations to those of the target miR-21-5p in the one-step amplification assay for miR-21-5p on the analyzer. The bar shows ± 2 standard deviation of the amplification rate. Each sample was measured in triplicate.

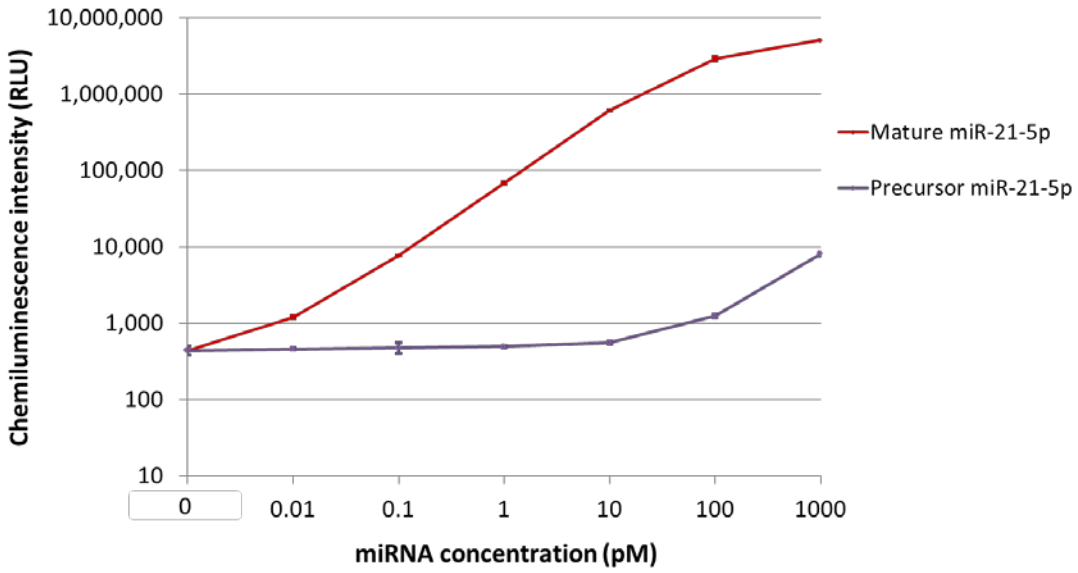
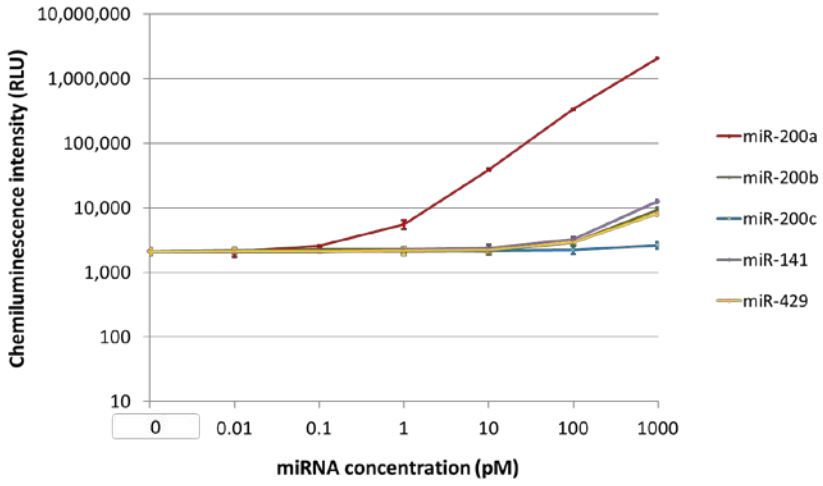


Fig. 16 Cross reactivity test of precursor miR-21-5p in the two-step amplification assay for miR-21-5p on the analyzer. The bar shows ± 2 standard deviations. Each sample was measured in triplicate except for the blank sample (rep. = 5).

a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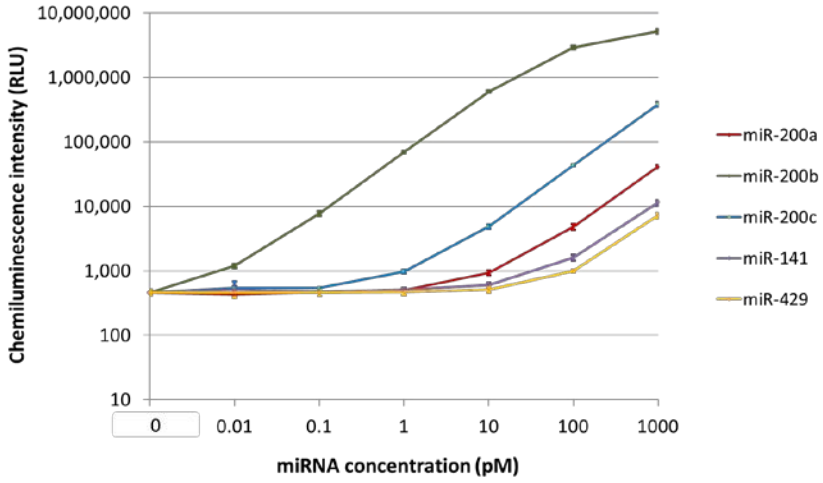
1,000,000
100,000
10,000
1,000
100
10
Chemiluminescence intensity (RLU)
0
0.01
0.1
1
10
100
1000
miRNA concentration (pM)
miR-200a
miR-200b
miR-200c
miR-141
miR-429

b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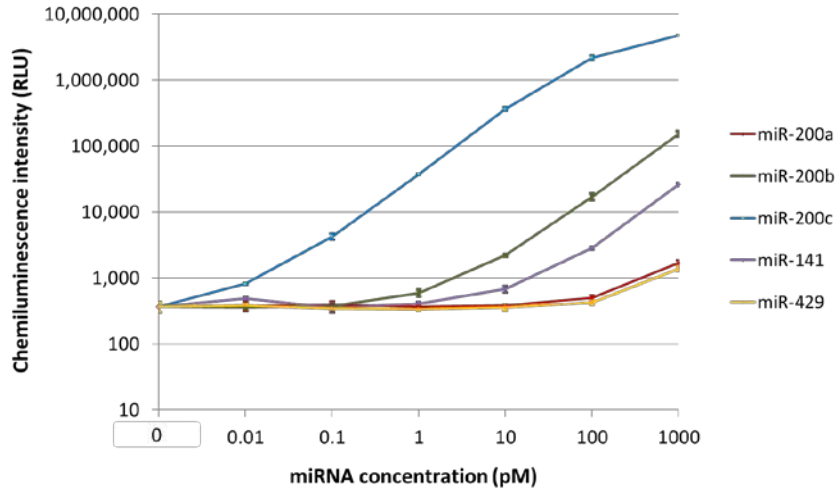
1,000,000
100,000
10,000
1,000
100
10
Chemiluminescence intensity (RLU)
0
0.01
0.1
1
10
100
1000
miRNA concentration (pM)
miR-200a
miR-200b
miR-200c
miR-141
miR-429

c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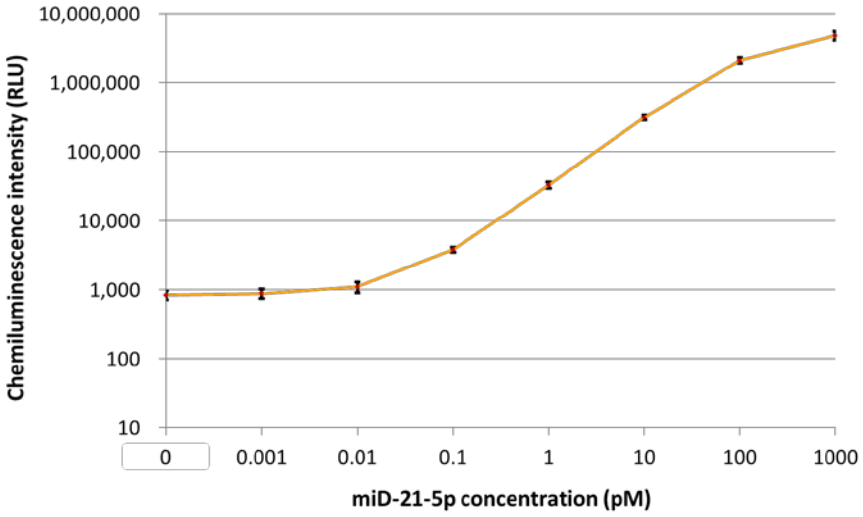
1,000,000
100,000
10,000
1,000
100
10
Chemiluminescence intensity (RLU)
0
0.01
0.1
1
10
100
1000
miRNA concentration (pM)
miR-200a
miR-200b
miR-200c
miR-141
miR-429

Fig. 17 (**a**) Cross reactivity test of four miRNAs (miR-200b, miR-200c, miR-141, miR-429) in miR-200 family in the two-step amplification assay for miR-200a on the analyzer. The bar shows ± 2 standard deviations. Each sample was measured in triplicate except for the blank sample (rep. = 5). (**b**) Cross reactivity test of four miRNAs (miR-200a, miR-200c, miR-141, miR-429) in the two-step amplification assay for miR-200b on the analyzer. The bar shows ± 2 standard deviations. Each sample was measured in triplicate except for the blank sample (rep. = 5). (**c**) Cross reactivity test of four miRNAs (miR-200a, miR-200b, miR-141, miR-429) in the two-step amplification assay for miR-200c on the analyzer. The bar shows ± 2 standard deviations. Each sample was measured in triplicate except for the blank sample (rep. = 5).

a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1,000
100
10
Chemiluminescence intensity (RLU)
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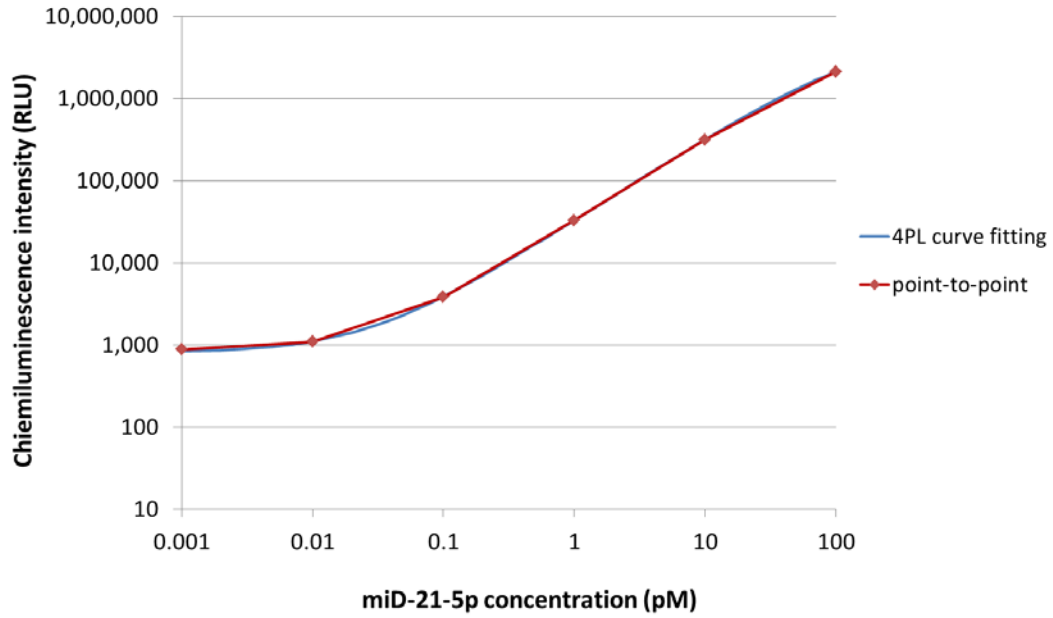
0.01
0.1
1
10
100
1000
miD-21-5p concentration (pM)

b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1,000
100
10
Chiemiluminescence intensity (RLU)
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miD-21-5p concentration (pM)
4PL curve fitting
point-to-point

c

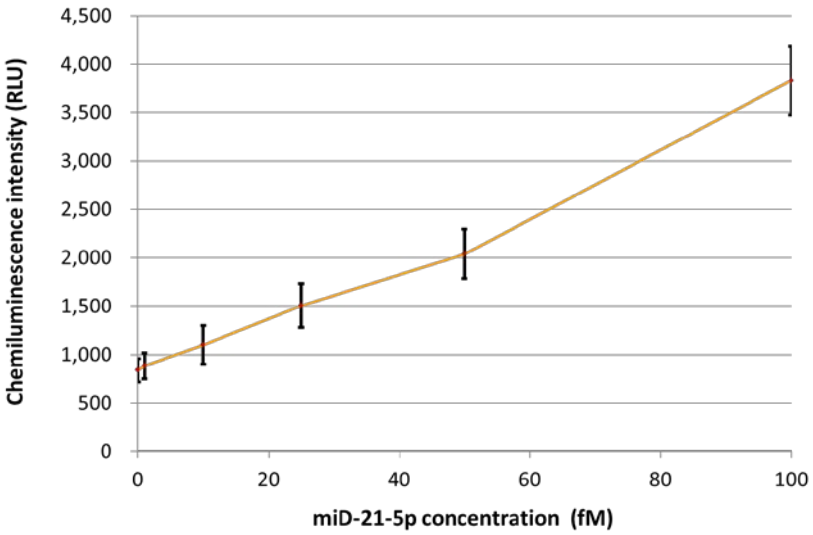
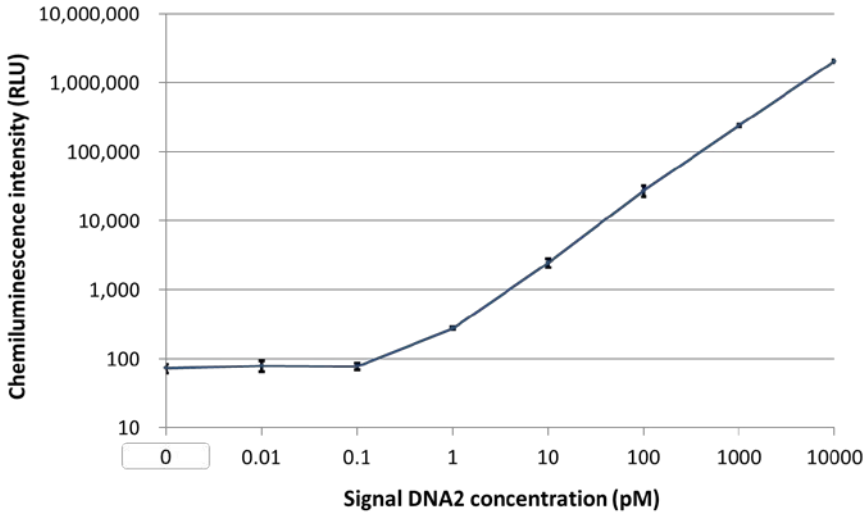


Fig. 18 (**a**) Dose response on the spiked miD-21-5p, which was the single-stranded DNA with the same sequence as miR-21-5p, in human serum at the concentrations from 0 to 1,000 pM in the two-step amplification assay for miR-21-5p on the analyzer. The bar shows ± 2 standard deviations. Each sample was measured in replicates of 10 except for the blank serum sample (rep. = 20). (**b**) The four parameter logistic curve fitting of the dose response curve in the two-step amplification assay for miD-21-5p at the concentrations from 0 pM to 100 pM on the analyzer. The fitted equation was $Y = 5437186 + (810.5592 - 5437186) / (1 + (X/154.5906)^{1.016908})$. The correlation coefficient was 1.0. Each sample was measured in replicates of 5. (**c**) Dose response for the detection sensitivity test on the spiked miD-21-5p in human serum at the concentrations from 0 to 100 fM in the two-step amplification assay for miR-21-5p on the analyzer. The bar shows ± 2 standard deviations. Each sample was measured in replicates of 10 except for the blank serum sample (rep. = 20).

a



b

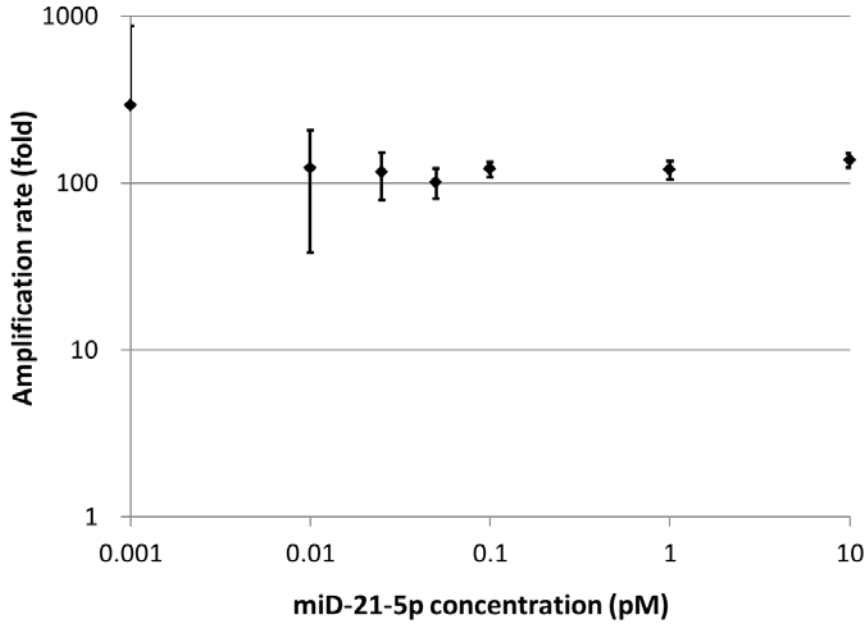


Fig. 19 (**a**) Calibration curve of Signal DNA2 spiked in the unreacted samples that were prepared same as the two-step amplification assay for miR-21-5p on the analyzer for calculating the amplification rates shown in Fig. 19 (b). The bar shows ± 2 standard deviations. Each sample was measured in triplicate. (**b**) Plot of amplification rates of

Signal DNA2 concentrations to those of the target miD-21-5p in human serum in the two-step amplification assay for miR-21-5p on the analyzer. The bar shows ± 2 standard deviation of the amplification rate. Each sample was measured in replicates of 10.

Chapter III:

Concluding Remarks

The circulating miRNAs in blood have attracted attention as new potential biomarkers for cancer diagnosis, prognosis and detection of recurrence. However, the current measurement methods of miRNAs including qRT-PCR, microarray and next generation sequencing have several disadvantages such as time-consuming pretreatment, manual operation, a standalone instrument, low throughput and expensive test cost, which make it difficult to introduce a miRNA test as a cancer biomarker into clinical laboratories. In this research shown in the Chapter II, I adapted an isothermal DNA amplification reaction to a fully automated immunoassay analyzer, ARCHITECT i system (Abbott Japan, Tokyo, Japan) which was widely used in clinical laboratories to measure proteins and chemical compounds as diagnostic markers. In a reaction vessel, miRNA was converted to a pre-designed single-stranded DNA as a signal by using DNA templates, DNA polymerase with strand displacement activity and nicking endonuclease. The Signal DNA was amplified by the repetitive enzymatic reaction. Then, the amplified Signal DNA was hybridized by one DNA probe attached to a magnetic particle and another DNA probe labeled with acridinium ester. After the chemiluminescence reaction, luminescence intensity was automatically measured.

I developed three assays of cancer related miRNAs (miR-21-5p, miR-18a-5p and miR-500a-3p) by only changing the target binding sequence of Converter DNA with

one-step amplification on the fully automated immunoassay analyzers. The testing time was 44 minutes. The throughput was 66 tests per hour. The cross reactivity for miR-21-5p with fifteen similar human miRNAs which had identical bases from 8 to 14 was not higher than 0.02%.

To improve detection sensitivity, I extended the one-step amplification assay to the two-step amplification assay by layering linear amplification reactions. The two-step amplification assay achieved the detection sensitivity value of 3 fM for miR-21-5p, significantly lower than the cut off concentrations in serum around 50 fM. This highly sensitive detection is attributed not only to the introduction of Cover sequence and layering linear amplification reactions, but also to the application of chemiluminescence reaction with Capture DNA probe and Chemiluminescence DNA probe emulating the common sandwich assay in immunoassay. This reaction design is suitable for the fully-automated immunoassay analyzer. The CVs in the measurement of miR-21-5p at the target concentrations from 5 fM to 1000 pM were less than 8%.

I investigated the discrimination of the precursor and mature microRNA. The assay detected the mature miR-21-5p than the precursor miR-21-5p.

I investigated the cross reactivity for miR-200 family which have highly homologous sequences with the two-step amplification. The similar miRNA sequences did not cause the false positive signal higher than 0.64%.

These results indicated that the proposed fully-automated miRNA assays showed high sensitivity, low cross reactivity and reproducibility suitable for clinical use.

Furthermore, I achieved the automated nucleic acid detection in human serum. As a

next step, the automated native miRNA detection in human serum on the analyzer is planned.

This method has huge potential to resolve the current disadvantages of miRNA measurement method, such as time-consuming pretreatment, manual operation, a standalone instrument, high measurement cost and low throughput. The characteristics of this method are that 1) purification is not required, 2) manual operation is not required, 3) existing fully automated immunoassay analyzers in clinical laboratories can be used, 4) short testing time (44 minutes) and high throughput (66 tests per hour).

This method would make it possible to measure potential diagnostic nucleic acid markers like miRNAs together with conventional protein markers on existing fully automated immunoassay analyzers in clinical laboratories. It is useful for patients and clinical laboratories in terms of testing time, cost, and accurate diagnosis.

Perspectives

Factors to cause differences of miRNA test results

A lot of research papers on miRNAs have been published as potential diagnostic markers for many diseases. However, very few miRNAs have been introduced into clinical laboratories as diagnostic markers. One of the reasons is that the test results about concentrations of same miRNAs in human blood samples were different between various clinical studies. The reasons for the difference in miRNA concentration are that the following factors were not considered sufficiently in the clinical studies. First factor is the characteristics of patient population in terms of age, sex, ethnic group, disease stage and disease category. Second factor is the measurement method such as qRT-PCR, microarray and NGS, and the product of reagents and instruments for the measurement methods. Third factor is the pre-analytical processing method of a blood sample such as blood collection tube type, standing time and temperature after blood drawing, centrifugation speed / time for separation of blood cells and sample storage conditions before miRNA testing [89, 90]. The reason why the difference of pre-analytical processing conditions affect miRNA concentration is that majority of solid tumor-associated circulating miRNAs are highly expressed in blood cells such as red blood cells, platelets, myeloid cells and lymphoid cells [89, 91]. The cell contamination and cell disruption like hemolysis in the pre-analytical processing of a blood sample impact the test results of miRNA concentration [91].

Standardization of pre-analytical processing of a blood sample and a normalization method

The standardization of the pre-analytical processing of a blood sample is important for reducing the differences of miRNA measurement concentrations between the clinical studies. The degree to which each parameter of the pre-analytical processing affects miRNA measurement concentrations should be investigated. From the results, acceptable conditions of each parameter are clarified, and standardized method of the pre-analytical processing is established.

The standardization of a normalization method is also important for reducing the differences of miRNA measurement concentrations between the clinical studies. The normalization methods with endogenous controls and spike-in controls are used for minimizing the technical variability and removing systemic bias among different measurements. The small nuclear RNA such as RNU6B, RNU44 and RNU48 or miR-16, let-7a, miR-142-3p, 18S rRNA are often used as endogenous controls. The *C. elegans* miRNAs such as cel-miR-39, -43, -54 and -238 are used for spike-in controls. The differences of normalization controls cause the differences of miRNA concentration [89]. The common normalization control should be established to compare miRNA concentrations in blood samples accurately in the different clinical studies.

The investigation for standardization of the pre-analytical processing and normalization method of a blood sample is necessary to measure miRNA concentrations accurately in clinical laboratories. When a miRNA diagnostic product using my measurement method on the fully automated immunoassay analyzers come to market

and available for customers, the product would be expected to contribute to investigating the standardization method of the pre-analytical processing and the normalization method.

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References

1. Esteller M: Non-coding RNAs in human disease. Nat Rev Genet 2011;12:861−874
2. Lee RC, Feinbaum RL, Ambros V.: The C. elegans heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to lin-14. Cell 1993;75:843−854
3. Wightman B, Ha I, Ruvkun G: Posttranscriptional regulation of the heterochronic gene *lin-14* by *lin-4* mediates temporal pattern formation in C. elegans. Cell 1993;75:855−862
4. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G: The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature 2000;403:901−906
5. Pasquinelli AE, Reinhart BJ, Slack F, Martindale MQ, Kuroda MI, Maller B, Hayward DC, Ball EE, Degnan B, Muller P, Spring J, Srinivasan A, Fishman M, Finnerty J, Corbo J, Levine M, Leahy P, Davidson E, Ruvkun G: Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature 2000;408:86−89
6. Alvarez-Garcia I, Miska EA: MicroRNA functions in animal development and human disease. Development 2005;132:4653−46626
7. Wienholds E, Kloosterman WP, Miska E, Alvarez-Saavedra E, Berezikov E, Bruijn E, Horvitz HR, Kauppinen S, Plasterk RHA: MicroRNA expression in zebrafish embryonic development. Science 2005;309:310−311
8. Ambros V: The functions of animal microRNAs. Nature 2004;431:350−355

9. Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, Aldler H, Ratten S, Keating M, Rai K, Rassenti L, Kipps T, Negrini M, Bullrich F, Crose CM: Frequent deletions and down-regulation of microRNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A 2002;99:15524−15529
10. Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, Wojcik SE, Aqeilan RI, Zupo S, Dono M, Rassenti L, Alder H, Volinia S, Liu C, Kipps TJ, Negrini M, Crose CM: MiR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci U S A 2005;102:13944−13949
11. Kloosterman WP, Plasterk RHA: The diverse functions of microRNAs in animal development and disease. Development Cell 2006;11:441−450
12. Lu, J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR: MicroRNA expression profiles classify human cancers. Nature 2005;435:834−838
13. Calin GA, Croce CM.: MicroRNA signatures in human cancers. Nat Rev Cancer 2006;6: 857–866
14. Rosenfeld N, Aharonov R, Meiri E, Rosenwald S, Spector1 Y, Zepeniuk M, Benjamin H, Shabes N, Tabak S, Levy A, Lebanony D, Goren Y, Silberschein E, Targan N, Ben-Ari A, Gilad S, Sion-Vardy N, Tobar A, Feinmesser M, Kharenko O, Nativ O, Nass D, Perelman M, Yosepovich A, ShalmonB, Polak-Charcon S, Fridman E, Avniel A, Bentwich I, Bentwich Z, Cohen D, Chajut A, Barshack I: MicroRNAs accurately identify cancer tissue origin. Nature Biotech 2008;26:462−469

15. Sarver AE, Li L, Kartha RV, Subramanian S: MicroRNAs in the malignant transformation process (Chapter 1). Advances in Experimental Medicine and Biology 2015;889:1−21
16. Ress AL, Perakis S, Pichler M: MicroRNAs and colorectal cancer (Chapter 6). Advances in Experimental Medicine and Biology 2015;889:89−103
17. Hill L, Browne G, Tulchinsky E: ZEB/miR-200 feedback loop: at the crossroads of signal transduction in cancer. Int J Cancer 2013;132:745−754
18. Sundararajan V, Gengenbacher N, Stemmler MP, Kleemann JA, Brabletz T, Brabletz S: The ZEB1 / miR-200c feedback loop regulates invasion via actin interacting proteins MYLK and TKS5 Oncotarget 2015;6:27083−27096
19. Ono K: MicroRNAs and cardiovascular remodeling (Chapter 10). Advances in Experimental Medicine and Biology 2015;888:197−213
20. Grasso M, Piscopo P, Crestini A, Confaloni A, Denti MA: Circulating microRNAs in neurodegerative diseases (Chapter 7). Experientia Supplementum 2015;106:151−169
21. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO: Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of generic exchange between cells. Nat Cell Biol 2007; 9:654−659
22. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, Lin DW, Urban N, Drescher CW, Knudsen BS, Stirewalt DL, Gentleman R, Vessella RL, Nelson PS, Martin DB, Tewari M: Circulating microRNAs as stable blood-based markers for cancer

detection. Proc Natl Acad Sci U S A 2008;105:10513−10518

23. Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF, Mitchell PS, Bennett CF, Pogosova-Agadjanyan EL, Stirewalt DL, Tait JF, Tewari M: Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proc Natl Acad Sci U S A 2011;108:5003−5008
24. Turchinovich A, Weiz L, Langheinz, Burwinkel B. Characterization of extracellular circulating microRNA. Nucleic Acids Res 2011;39:7223−7233
25. Zhu H, Fan GC: Extracellular/circulating microRNAs and their potential role in cardiovascular disease. Am J Cardiovasc Dis 2011;1:138−149
26. Fujita Y, Yoshioka Y, Ochiya T: Extracellular vesicle transfer of cancer pathogenic components. Cancer Science 2016;107:385−390
27. Kosaka N, Iguchi H, Ochiya T: Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. Cancer Science 2010;101:2087−2092
28. Boeri M, Verri C, Conte D, Roz L, Modena P, Facchinetti F, Calabrò E, Croce CM, Pastorino U, Sozzi G: MicroRNA signatures in tissues and plasma predict development and prognosis of computed tomography detected lung cancer. Proc Natl Acad Sci. U S A 2011;108:3713−3718
29. Silva J, Garcia V, Zaballos A, Provencio M, Lombardia L, Almonacid L, Garcia JM, Dominguez G, Pena C, Diaz R, Herrera M, Varela A, Bonilla F: Vesicle-related microRNAs in plasma of non small cell lung cancer patients and correlation with survival. Eur Respir J 2010;37:617−623

30. Kosaka N, Yoshioka Y, Fujita Y, Ochiya T: Versatile roles of extracellular vesicles in cancer. J Clin Invest 2016;126:1163−1172
31. Bouyssou JMC, Manier S, Huynh D, Issa S, Roccaro AM, Ghobrial IM: Regulation of microRNAs in cancer metastasis. Biochim Biophys Acta 2014;1845:255−265
32. Pritchard CC, Cheng HH, Tewari M. MicroRNA profiling: approaches and considerations. Nat Rev Genet 2012;13:358−369
33. Kozomara A, Birgaoanu M, Griffiths-Jones S. miRBase: from microRNA sequences to function. Nucleic Acids Res 2019;47:D155−D162
34. Xu F, Xu L, Wang M, An G, Feng G: The accuracy of circulating microRNA-21 in the diagnosis of colorectal cancer: systematic review and meta-analysis. Colorectal Dis 2015;5:O100−O107
35. Duffy MJ: Carcinoembryonic Antigen as a marker for colorectal cancer: Is it clinically useful? Clin Chem 2001;47:624−630
36. Population dynamics statistics 2015 by Ministry of Health, Labor and Welfare http://www.mhlw.go.jp/toukei/saikin/hw/jinkou/suikei15/
37. National Comprehensive Cancer Network: Pancreatic Adenocarcinoma Guidelines Version 2. 2016
38. Mitsunaga S, Kojima M, Ikeda M, Ochiai A: Serum microRNAs as tumor markers for diagnosis of pancreatic cancer. Pancreas 2017;32:56−61
39. Japan Pancreas Society: Pancreatic Cancer Clinical Practice Guideline 2013
40. Kanno A, Masamune A, Hanada K, Maguchi H, Shimizu Y, Ueki T, Hasebe O, Ohtsuka T, Nakamura M, Takenaka M, Kitano M, Kikuyama M, Gabata T, Yoshida

K, Sasaki T, Serikawa M, Furukawa T, Yanagisawa A, Shimosegawa T: The present situation of early diagnosis for pancreatic cancer. Pancreas 2017;32:16−22

41. Subramani R, Gangwani L, Nandy SB, Arumugam A, Chattopadhyay M, Lakshamanaswamy R: Emerging roles microRNAs in pancreatic cancer diagnosis, therapy and prognosis (Review). Intl J Onco 2015;47:1203−1210
42. Liu R, Chen X, Du Y, Yao W, Shen L, Wang C, Hu Z, Zhuang R, Ning G, Zhang C, Yuan Y, Li Z, Zen K, Ba Y, Zhang CY: Serum microRNA expression profile as a biomarker in the diagnosis and prognosis of pancreatic cancer. Clin Chem 2012;58:610−618
43. Schultz NA, Dehlendorff C, Jensen BV, Bjerregaard JK, Nielsen KR, Bojesen SE, Calatayud D, Nielsen SE, Yilmaz M, Hollander NH, Andersen KK, Johansen JS: MicroRNA biomarkers in whole blood for detection of pancreatic cancer. JAMA 2014;311:392−404
44. Cao Z, Liu C, Xu J, You L, Wang C, Lou W, Sun B, Miao Y, Liu X, Wang X, Zhang T, Zhao Y: Plasma microRNA panels to diagnose pancreatic cancer: Results from a multicenter study. Oncotarget 2016;7:41575−41583
45. Johansen JS, Calatayud D, Albieri V, Schultz NA, Dehlendorff C, Werner J, Jensen BV, Pfeiffer P, Bojesen SE, Giese N, Nielsen KR, Nielsen SE: The potential diagnostic value of serum microRNA signature in patients with pancreatic cancer. Int J Cancer 2016;139:2312−2324
46. Kojima M, Sudo H, Kawauchi J, Takizawa S, Kondou S, Nobumasa H, Ochiai A: MicroRNA markers for the diagnosis of pancreatic and biliary-tract cancers. PLoS

One 2015;10:2469−2490

47. Hong C, Baek A, Hah SS, Jung W, Kim DE: Fluorometric detection of microRNA using isothermal gene amplification and graphene oxide. Anal Chem 2016;88:2999−3003
48. Kalogianni DP, Kalligosfyri PM, Kyriakou IK, Christopoulos TK. Advances in microRNA analysis. Anal Bioanal Chem 2018;410:695−713
49. D'Agata R, Spoto G. Advanced methods for microRNA biosensing: a problem-solving perspective. Anal Bioanal Chem 2019;411:4425-4444
50. Liu Y, Zhang J, Tian J, Fan X, Geng H. Multiplex detection of microRNAs by combining molecular beacon probes with T7 exonuclease-assisted cyclic amplification reaction. Anal Bioanal Chem 2017;409:107−114
51. Clancy E, Burke M, Arabkari V, Barry T, Kelly H, Dwyer RM, et al. Amplification-free detection of microRNAs via a rapid microarray-based sandwich assay. Anal Bioanal Chem 2017;409:3497−3505
52. Ach RA, Wang H, Curry B: Measuring microRNAs: comparisons of microarray and quantitative PCR measurements, and of different total RNA prep methods. BMC Biotechnology 2008;8:69
53. Wu X, Zhu S, Huang P, Chen Y: Highly specific quantification of microRNA by coupling probe-rolling circle amplification and Forster resonance energy transfer. Anal Biochem 2016;502:16−23
54. Nelson PT, Baldwin DA, Scearce LM, Oberholtzer JC, Tobias JW, Mourelatos Z: Microarray-based, high-throughput gene expression profiling of microRNAs

2004;1:155−161

55. Zhao Y, Chen F, Li Q, Wang L, Fan C: Isothermal amplification of nucleic acids. Chem Rev 2015;115:12491−12545
56. Deng H, Gao Z: Bioanalytical applications of isothermal nucleic acid amplification. Anal Chim Acta 2015;853:30−45
57. Yan L, Zhou J, Zheng Y, Gamson AS, Roembke BT, Nakayama S, Sintim HO: Isothermal amplified detection of DNA and RNA. Molec Biosyst 2014;10:970−1003
58. Nippon gene Inc. website: http://www.nippongene.com/english/product/modifying-enzymes/bst-dna-polymerase.html
59. Notomi T, Mori Y, Tomita N, Kanda H: Loop-mediated isothermal amplification (LAMP): principle, features, and future prospects. J Microbiol 2015;53:1−5
60. Becton Dickinson Inc.: Package insert of BD Probe Tec ET Chlamydia trachomatis amplified DNA Assay.
61. Komiya K: Method and kit for detecting target nucleic acid 2011; the patent application No. PCT/JP2011/078717
62. Aliotta JM, Pelletier JJ, Ware JL, Moran LS, Benner JS, Kong H: Thermostable *Bst* DNA polymerase I lacks a 3′ → 5′ proofreading exonuclease activity. Genetic Analysis 1996;12:185−195
63. New England Biolabs Inc.: Polymerase & Amplification technology:82−85
64. Heiter DF, Lunnen KD, Wilson GG: Site-specific DNA-nicking mutants of the

heterodimeric restriction endonuclease R.BbvCl. J. Mol. Biol. 2005;348:631−640

65. New England Biolabs Inc. website: https://www.neb.com/products/r0631-nbbbvci

66. Toiyama Y, Takahashi M, Hur K, Nagasaka T, Tanaka K, Inoue Y, Kusunoki M, Boland RC, Goel A. Serum miR-21 as a diagnostic and prognostic biomarker in colorectal cancer. J Natl Cancer Inst 2013;105:849−859

67. Zeng Z, Wang J, Zhao L, Hu P, Zhang H, Tang X, He D, Tang S, Zeng Z. Potential role of microRNA-21 in the diagnosis of gastric cancer: a meta-analysis. PloS One 2013;8:e73278

68. Morimura R, Komatsu S, Ichikawa D, Takeshita H, Tsujiura M, Nagata H, Konishi H, Shiozaki A, Ikoma H, Okamoto K, Ochiai T, Taniguchi H, Otsuji E. Novel diagnostic value of circulating miR-18a in plasma of patients with pancreatic cancer. Br J cancer 2011;105:1733−1740

69. Faltejskova PV, Slaby O. Circulating blood-borne microRNAs as biomarkers in solid tumors. EXS 2015;106:75−122

70. He Y, Lin J, Kong D, Huang M, Xu C, Kim TK, Etheridge A, Luo Y, Ding Y, Wang K. Current state of circulating microRNAs as cancer biomarkers. Clin Chem 2015;61:1138−1155

71. Navarro E, Serrano-Heras G, Castano MJ, Solera J. Real-time PCR detection chemistry. Clin Chim Acta 2015;439:231−250

72. Nolan T, Bustin SA. PCR Technology: Current Innovations. 3rd Ed. CRC Press 2013;357

73. Amendola A, Coen S, Belladonna S, Pulvirenti FR, Clemens JM, Capobianchi MR.

Improving clinical laboratory efficiency: a time-motion evaluation of the Abbott m2000 RealTime and Roche COBAS AmpliPrep/COBAS TaqMan PCR systems for the simultaneous quantitation of HIV-1 RNA and HCV RNA. Clin Chem Lab Med 2011;49:1283−1288

74. Kappel A, Backes C, Huang Y, Zafari S, Leidinger P, Meder B, Schwarz H, Gumbrecht W, Meese E, Staehler CF, Keller A. MicroRNA in vitro diagnostics using immunoassay analyzers. Clin Chem 2015;61:600−607
75. Kricka L, Wilson R: RNA testing now automated. Clin Chem 2015;61:571−572
76. Yoshimura T, Fujita K, Kinukawa H, Matsuoka Y, Patil RD, Beligere GS, Chan SS, Dowell BL, Sokoll L, Elliott D, Chan DW, Scheuer C, Hofmann K, Stieber P, Sakurai Y, Iizuka M, Saegusa H, Yamaguchi K. Development and analytical performance evaluation of an automated chemiluminescent immunoassay for pro-gastrin releasing peptide (ProGRP). Clin Chem Lab Med 2009;47:1557−1563
77. Ingen HE, Chan DW, Hubl W, Miyachi H, Molina R, Pitzel L, Ruibal A, Rymer JC, Domke I. Analytical and clinical evaluation of an electrochemiluminescence immunoassay for the determination of CA 125. Clin Chem 1998;44:2530−2536
78. Slev PR, La'ulu SL, Roberts WL. Intermethod differences in results for total PSA, free PSA, and percentage of free PSA. Am J Clin Pathol 2008;129:952−958
79. Serdarevic N. The comparison between different immunoassays for serum carbohydrate antigen (CA19-9) concentration measurement. Acta Inform Med 2018;26:235-239
80. Taylor SN, Van Der Pol B, Lillis R, Hook EW 3rd, Lebar W, Davis T, Fuller D,

Mena L, Fine P, Gaydos CA, Martin DH. Clinical evaluation of the BD ProbeTec™ Chlamydia trachomatis Qx amplified DNA assay on the BD Viper™ system with XTR™ technology. Sex Transm Dis 2011;38:603−609

81. Wu X, Zhu S, Huang P, Chen Y. Highly specific quantification of microRNA by coupling probe-rolling circle amplification and Forster resonance energy transfer. Anal Biochem. 2016;502:16−23
82. Le BH, Seo YJ. Highly sensitive MicroRNA 146a detection using a gold nanoparticle-based CTG repeat probing system and isothermal amplification. Anal Chim Acta. 2018;999:155−160
83. Komiya K, Komori M, Noda C, Kobayashi S, Yoshimura T, Yamamura M. Leak-free million-fold DNA amplification with locked nucleic acid and targeted hybridization in one pot. Org Biomol Chem 2019;17:5708-5713
84. Toiyama Y, Hur K, Tanaka K, Inoue Y, Kusunoki M, Boland R, Goel A. Serum miR-200c is a novel prognostic and metastasis-predictive biomarker in patients with colorectal cancer. Ann Surg 2014;259:735−743
85. O'Brien SJ, Carter JV, Burton JF, Oxford BG, Schmidt MN, Hallion JC, Galandiuk S. The role of the miR-200 family in epithelial-mesenchymal transition in colorectal cancer: a systematic review. Int J Cancer 2018;142:2501−2511
86. https://www.thermofisher.com/jp/ja/home/brands/product-brand/dynal/streptavidin-coupled-dynabeads.html
87. Komiya K, Yamamura M. Cascading DNA generation reaction for controlling DNA nanomachines at a physiological temperature. N Gener Comput 2015;33:213−229

88. Yoo CE, Kim G, Kim M, Park D, Kang HJ, Lee M, Huh N. A direct extraction method for microRNAs from exosomes captured by immunoaffinity beads. Anal Biochem 2012;431:96−98

89. Butz H, Patocs A: Technical aspects related to the analysis of circulating microRNAs. Experientia Supplementum Chapter 3 2015;106:55-71

90. Page K, Guttery DS, Zahra N, Primrose L, Elshaw SR, Pringle JH, Blighe K, Marchese SD, Hills A, Woodley L, Stebbing J, Coombes RC, Shaw JA: Influence of plasma processing on recovery and analysis of circulating nucleic acids. PLos ONE 2013;8:e77963

91. Pritchard CC, Kroh E, Wood B, Arroyo JD, Dougherty KJ, Miyaji MM, Tait JF, Tewari M: Blood cell origin of circulating microRNAs: a cautionary note for cancer biomarker studies. Cancer Prev Res 2012;5:492−497