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Direct Quantification of Circulating MiRNAs in Different Stages of Nasopharyngeal Cancerous Serum Samples in Single Molecule Level with Total Internal Reflection Fluorescence Microscopy

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Abstract

MicroRNAs (miRNAs) are small non-coding RNAs that regulate human gene expression at the post-transcriptional level. Growing evidences indicated that the expression profile of miRNAs is highly correlated with the occurrence of human diseases including cancers. Playing important roles in complex gene regulation processes, aberrant expression pattern of various miRNAs is implicated in different types and even stages of cancer. Besides localizing in cells, many of these miRNAs are found circulating around the body in a wide variety of fluids such as urine, serum and saliva. Surprisingly, these extracellular circulating miRNAs are highly stable and resistant to degradation, and therefore, are considered as promising biomarkers for early cancer diagnostic via non-invasive extraction from body fluids. Unfortunately, the abundance of these small RNAs is ultralow in the body fluids, making it challenging to quantify them in complex sample matrixes. Establishing a sensitive, specific yet simple assay for an accurate quantification of circulating miRNAs is therefore desirable. Our group previously reported a sensitive and specific detection assay of miRNAs in single molecule level with the aid of total internal reflection fluorescence microscopy. In this work, we advanced the assay to differentiate the expression of a nasopharyngeal carcinoma (NPC) up-regulator hsa-mir-205 (mir-205) in serum collected from patients of different stages of NPC. To overcome the background matrix interference in serum, locked nucleic acid modified molecular beacon (LNA/MB) was applied as the detection probe to hybridize, capture and detect target miR-205 in serum matrix with enhanced sensitivity and specificity. A detection limit of 500 fM was achieved. The as-developed method was capable of differentiating NPC stages by the level of mir-205 quantified in serum with only 10 μL of serum and the

whole assay can be completed in an hour. The experimental results agreed well with reported and while the quantity of miR-205 determined by our assay was found comparable to that of quantitative reverse transcription polymerase chain reaction (qRT-PCR), supporting that this assay can be served as a promising non-invasive detection tool for early NPC diagnosis, monitoring and staging.

Introduction

Nasopharyngeal Carcinoma (NPC) is a highly metastatic head and neck cancer that originated in the nasopharynx. NPC is highly prevalent in China and Southern Asia. The 5-year survival rate for stage III and IV NPC patients is about 50 %. Due to the vague symptoms and its deep location, 70 % of patients were diagnosed as cervical lymph node metastasis and around 30 % of patients were diagnosed as long distant metastasis at the first consultation. In advance NPC, the incidence of local reoccurrence is very high. Due to the distinct metastasis of NPC, the effects of radiotherapy and chemotherapy treatments are only significant at early stages.[1-4] Therefore, early diagnosis of NPC is extremely necessary.

While the tumorigenesis of NPC is a multistage process relying on spatial and temporal control of the gene expression, studies showed that the expression profile of several mircroRNAs (miRNAs) has appeared to influence the development of NPC.[5] MiRNAs are small non-coding RNA of 19-24 nucleotides. They are important gene regulators that repress over 30 % of the gene expression via translation inhibition or degradation of the target messenger RNAs in the transcriptional level. MiRNAs also involved in multiple biological processes including cell differentiation, proliferation apoptosis and

metabolism. The regulatory function of miRNAs appears to be one of the key factors in cancer pathogenesis.[6-8] Many of the research studies focused on the detection and expression profiling of miRNAs in cancerous cells and tissues, which are difficult and invasive to be extracted. Some of these miRNAs were also discovered circulating in the bloodstream and consequently found in a wide range of body fluids, such as saliva, serum, plasma, and urine. These extracellular miRNAs in the circulatory system are proven to be stable and resistant to RNAse degradation as well as repeated freeze-thaw cycles under the protection of the RNA-induced silencing complex (RISC). Since literature confirmed that expression profiling of these circulating miRNAs also reflects physiological and pathological conditions and in the view that the collection of body fluids are rather non-invasive as compared to the collection of biopsies, tissues and whole cells, the expression profile of serum miRNAs is of high potential to act as a convenient tool for early stage diagnosis, staging and monitoring of NPC.[9-12]

Northern blotting, reverse transcript polymerase chain reaction and microarray[13-16] are extensively applied as the routine methods for detection of nucleic acids; unfortunately, the capabilities of detecting miRNAs often hindered by the short length, lack of common features, homology sequence among various miRNA family members and low abundance. Since the content of circulating miRNAs in serum is even lower (~ 20 pM),[17] these golden methods often required a large volume of patient serum samples for the detection. In addition, these methods are labor intensive, time consuming, and low sample throughput, which limited the application of these methods for clinical validation and management of disease. Therefore, development of an accurate, precise and sensitive detection method for circulating miRNAs is necessary. Based on our previous published

work on direct quantification of miRNAs by single-molecule counting with total internal reflection fluorescence microscopy,[18] we further advanced the single molecule counting assay to quantify the amount of circulating mir-205 in serum samples of NPC patients at different cancer stages.

We replaced the single-stranded Locked Nucleic Acid (LNA)-modified complementary sequences by the LNA modified molecular beacon as the detection probe. Molecular beacon (MB) is one of the nucleic acid-based probes that have been widely applied in both *in vitro* and *in situ* imaging and sensitive DNA and RNA detection. MB is a hairpin oligonucleotide of two stems complementary to each other with a fluorophore on one end, acting as a signal reporter, and a quencher on the other end. The loop part contains sequence that is complementary to that of the target. In the absence of target, the hairpin probe stays close and the fluorophore transfer the absorbed energy to the quencher along the irradiative pathway by Förster resonance energy transfer (FRET) due to their close proximity. On the other hand, in the presence of target, hybridization of the loop sequence with the complementary target opens up the hairpin and allows the fluorophores to emit fluorescent signals.[19-21] For a specific detection of the target miRNAs, a couple of LNA analogues were incorporated into the loop part of the MB sequences. LNA is nucleic acid that contains a methylene bridge connecting the 2'-O and 4'-C on the ribose ring. The methylene bridge restricted the ring by locking it in a rigid C3'endo conformation and fixing the nucleotide in three-dimensional space. Upon hybridization, this rigid three-dimensional structure will substantially increase the melting temperature of the duplex. Besides, the LNA analogues significantly destabilize the interaction with oligonucleotide that contains one or more mis-match bases. Study shows a decrease of

17-22 °C in melting temperature of the LNA/RNA duplex that contains only a single base mismatch.[22,23] The LNA modified MB inherits the attractive properties from (i) LNA: enhanced stability and binding affinity, mismatch discrimination capacity of the detection probe; and (ii) MB: lower fluorescence background signal arise from free probe and hence a lower LOD can be achieved.

In brief, the detection strategy of the assay was based on the direct hybridization between the target miRNAs and the LNA modified molecular beacon followed by fluorescence labeling for signal detection by the TIRFM-EMCCD imaging system. Since each fluorescence spot in the image represents a single hybridized miRNA, quantification of miRNAs can be achieved by simply counting the number of spots in the images. The application of the LNA/MB has further lowered the detection limit by an order of magnitude and achieved a LOD of 500 fM. Furthermore, the assay was applied for quantification of mir-205 to differentiate different stages of NPC serum samples. The whole detection assay was straightforward, free of sample enrichment and pre-analysis purification; the quantification can be done in one hour with 10 μL of serum sample. This as-developed assay is of high potential to serve as a useful tool for measuring the changes of miRNAs concentration in biological samples for clinical monitoring of pathological states of cancers.

Experimental

Coverslides pretreatment

All the coverslides were prewashed prior to use. Generally, No.1 22-mm square glass slide (Gold Seal, Electron Microscopy System, USA) were successively sonicated in household detergent for 30 min, acetone for 15 min twice and ethanol for 30 min. Then

the slides were consecutively soaked in Piranha solution (H_2SO_4: H_2O_2 3:1) and sonicated for 30 min, then sonicated again in the solution of HCl: H_2O: H_2O_2 (1: 1: 1) at 60 °C for 30 min, further soaked in Piranha solution for 30 min and sonicated for another 30 min. All the slides were rinsed with distilled H_2O extensively between each step. The slides were finally stored in distilled water and blow-dried with nitrogen gas before use.

Materials and reagents

Tris-NaCl-EDTA hybridization buffer (TNE 250 buffer) was prepared by mixing 20 mM pH 8.0 Tris-HCl (Invitrogen, USA) with 1 mM EDTA (Sigma, USA) and 250 mM sodium chloride (Sigma, USA) in distilled water. The pH of the buffer was adjusted to pH 7.4 with 2 M HCl. The buffer solution was filtered through a 0.22 μm nylon membrane filter and autoclaved prior to use. The commercial available Locked Nucleic Acid (LNA)-modified oligonucleotide probe (miRCURY LNA™ microRNA Detection Probes) complementary to miR-205 was purchased from Exiqon (Denmark) with sequence 5'-C AGA CTC CGG TGG AAT GAA GGA-3'. The LNA-modified molecular beacon (LNA/MB) and synthetic target miRNAs were purchased and HPLC purified from Integrated DNA Technology (USA). The sequences were shown below:

LNA-MB probe for mir-205: 5' TYE563-CGC TCC TCA **G**AC **T**CC **G**GT **G**GA **A**TG **A**AG GAG CG-Iowa Black® RQ-Sp3' (bold: LNA analogue; unbold: DNA analogue); Synthetic mir-149: 5' - UCU GGC UCC GUG UCU UCA CUC CC - 3'; Synthetic mir-200c: 5' - UAA UAC UGC CGG GUA AUG AUG GA - 3'; Synthetic mir-205: 5' - UCC UUC AUU CCA CCG GAG UCU G - 3'. All oligonucleotides were suspended in 500 μL DEPC-water (Ambion, USA) and further diluted to appropriate concentrations

with TNE 250 buffer. The melting temperatures were predicted based on the nearest neighbor method.

Optimization of the hybridization conditions

Selection of detection probe. To select the detection probe that possess the highest binding affinity towards the miRNA target, single stranded LNA (ssLNA) probe and a LNA-modified molecular beacon (LNA/MB) probe were prepared and hybridized with the target mir-205. Briefly, 0 and 10 pM of the target was incubated with 10 pM of ssLNA probe and LNA/MB probe respectively at 54 ˚C for 60 min. The optimal detection probe was applied for the rest of the experiment. *Hybridization temperature.* To determine the optimal hybridization temperature, 10 pM of synthetic mir-205 was incubated with 10 pM of LNA/MB probe at 49 °C, 54 °C and 59 °C respectively for 60 min. *Hybridization time.* To determine the optimal hybridization time, 10 pM of synthetic mir-205 were incubated with 10 pM of LNA/MB for 30 min, 45 min, 60 min, and 120 min respectively.

Single molecule detection of target miRNA.

The calibration curve was established by correlating the averaged number of single fluorescent molecules counted in captured images at each concentration of spiked miRNAs. Synthetic mir-205 with the final concentration of 0, 0.25, 0.5, 1, 10, 50, 100 pM was hybridized with 100 pM LNA-MB probe at the optimal condition. The hybridization and labeling of the hybrids were performed according to the following procedures. All the oligonucleotides were diluted to 1 nM with TNE 250 buffer (pH 7.4). The hybridization cocktail contained appropriate amount of target miRNA strand, 100 pM LNA-MB probe and top up to a final volume of 50 µL with TNE 250 buffer (pH 7.4).

Then the cocktail was incubated in a dry heating block (*AccuBlock* Digital Dry Bath D 1100, USA) at the optimal temperature and time as determined. To demonstrate the role of MB in LNA/MB probe, synthetic mir-205 with a final concentration of 0, 1, 10, 20, 50, 100, 200 pM was hybridized with 200 pM ssLNA probe at optimal conditions. After incubation, 1 μL of 100 nM YOYO-1 Iodide (YOYO) was then added to the hybridization cocktail to label the hybrids with a ratio of the YOYO and the hybridized base pair at 1:3 (dye/bp) at room temperature. The YOYO-labeled hybrids were kept equilibrium for 5 min and 10 μL of the solution was pipetted onto the prewashed coverslides for TIRFM imaging.

Selectivity of the LNA/MB probe

To study the selectivity of the assay, four synthetic miRNAs samples (mir-149, mir-200c, mir-205, and mixture of mir-149, mir-200c, mir-205) with a final concentration of 10 pM each were prepared and hybridized with 40 pM LNA-MB probe at optimal conditions. The hybrids were then labeled with YOYO (1:3 dye/bp) and visualized under the TIRFM imaging system.

Quantification of the target miRNAs in serum

Serum samples were obtained from the Nasopharyngeal Carcinoma Area of Excellence Research Tissue Bank under the Center for Nasopharyngeal Carcinoma Research. The serum samples were stored at - 80 °C prior to use without further modification. The serum sample was diluted 5-fold with TNE 250 buffer prior to quantification of target miRNAs by standard addition approach. Consequently, synthetic mir-205 of a final concentration of 0, 1, 2, 5, 10 and 15 pM was spiked respectively into hybridization cocktails that each contained 7 μL of diluted serum and LNA-MB probe with a final

concentration of 100 pM. The solutions were finally topped up with TNE 250 buffer to 40 μL and incubated at the optimal condition.

Imaging system and data analysis. A prism type total internal reflection fluorescence microscopy was setup as mentioned before. Briefly, the sample coverslip was placed between a fused-silica isosceles prism (CVI, laser USA) and a 60 × oil type objective that equipped on an Olympus IX71 inverted microscope with a HQ 535/50 (Chroma) band-pass filter. A 488-nm diode laser (Newport, USA) was used as the excitation source to excite the YOYO-1 dye. The fluorescence image of the hybrid was captured by an Electron Multiplying Charge Coupled Device (EMCCD) (PhotonMax 512, Princeton Instrument, USA) incorporated with a Uniphase mechanical shutter (Model LS2Z2, Vincent Associates, USA) and a driver (Model VMM-T1, Vincent Associates, USA) in external synchronization and frame-transfer mode. The exposure time of both camera and shutter were set at 100 ms. The multiplication gain and the delay time of the shutter drive were set at 4000 and 10 ms respectively. In general, fluorescent images of 10 sequential frames each were acquired on 10 different coordinates from a single slide using the WinSpec/32 software (version 2.5.22.0, USA) provided by Princeton Instruments. All the images were analyzed by a public-domain image processing software *Image J*. Generally, five frames (frame 3 to 7) of the image series with a region of interest (ROI) of 200 pixels square at the center of the image were chosen for analysis due to better signal homogeneity. The single molecule counting was performed by using the *Analyze Particles* function in the *Image J* software. Threshold was set as three times the standard deviation of the intensity of the image and size of the particles was set as 2-10 pixels to reduce the false positive signal generated by the background noise. Each bright spot in

the fluorescent image represents a single-molecule; the number of bright spots in the five frames was summed up (accumulation of the number of diffusing molecules detected in 250 ms), while the summed spots from 10 different image series from a single slide were averaged to eliminate localization effect. All the experiments were done in triplicates and the error bars refer to the standard error of mean of the experiments.

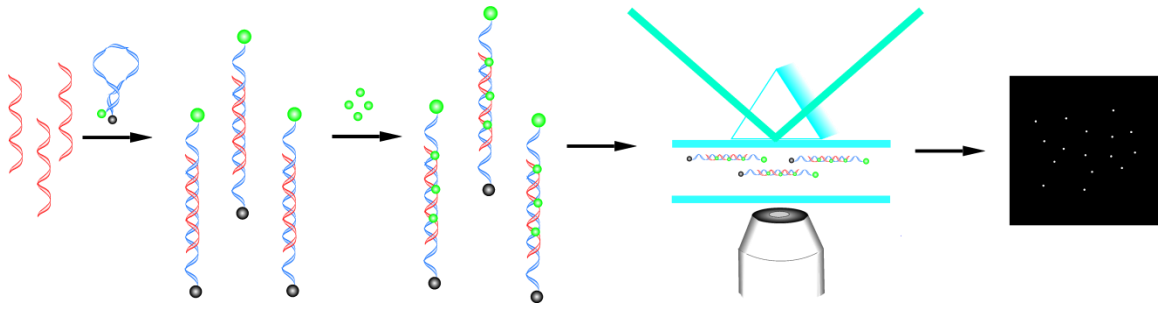


Figure 1. Schematic illustration of the solution-based hybridization assay for the direct quantification of miRNAs with TIRFM.

Detection strategy

Single-molecule detection (SMD) is a highly valuable technique that is widely applied in the field of bioscience and biotechnology for monitoring the enzymatic kinetics, DNA mapping, and nucleic acid adsorption and desorption dynamics, and detection of biomolecules like nucleic acids and proteins for early-stage disease diagnostics. Optical microscopy particularly total internal reflection fluorescence is commonly employed in SMD due to its high sensitivity.[24-28] Schematic illustration of the assay for the direct detection of miRNAs by single molecule counting is shown in figure 1. We previously reported that solution based hybridization is more efficient than surface based, solution based hybridization strategy was applied in the assay.[29] In order to increase the signal

intensity, the hybridized duplexes were labeled with an intercalating fluorescent dye YOYO-1. Due to binding mechanism of YOYO-1, it exhibits a much higher binding affinity towards double-stranded oligonucleotides. It shows about 400-fold intensity enhancement upon binding to double stranded nucleic acid. Afterward, the labeled hybrids were visualized under the TIRFM-EMCCD imaging system. A 488 nm cyan iodide laser was used as the excited source with an angle of incidence of ~ 70°, the penetration depth of the evanescence field generated at the glass/ water interfaces was calculated to be approximately 115 nm by $d= \lambda/4\pi\ (n_1^2 sin^2\theta - n_2^2)^{-1/2}$ (where d corresponds to the penetration depth, λ corresponds to the wavelength of the light source, n_1 and n_2 are the reflective index of glass and water, and θ *is the angle of incidence* respectively). A central region of 200 square pixels, equivalent to 53 × 53 μm^2 in actual dimension, in the EMCCD image was chosen to be the sampling region for homogeneous excitation intensity from the laser. In the TIRFM image, the fluorescent signal from a single molecule was visualized as an individual bright spot. By counting the bright spots in the sample region, quantification of the target miRNAs was achieved.

Optimization

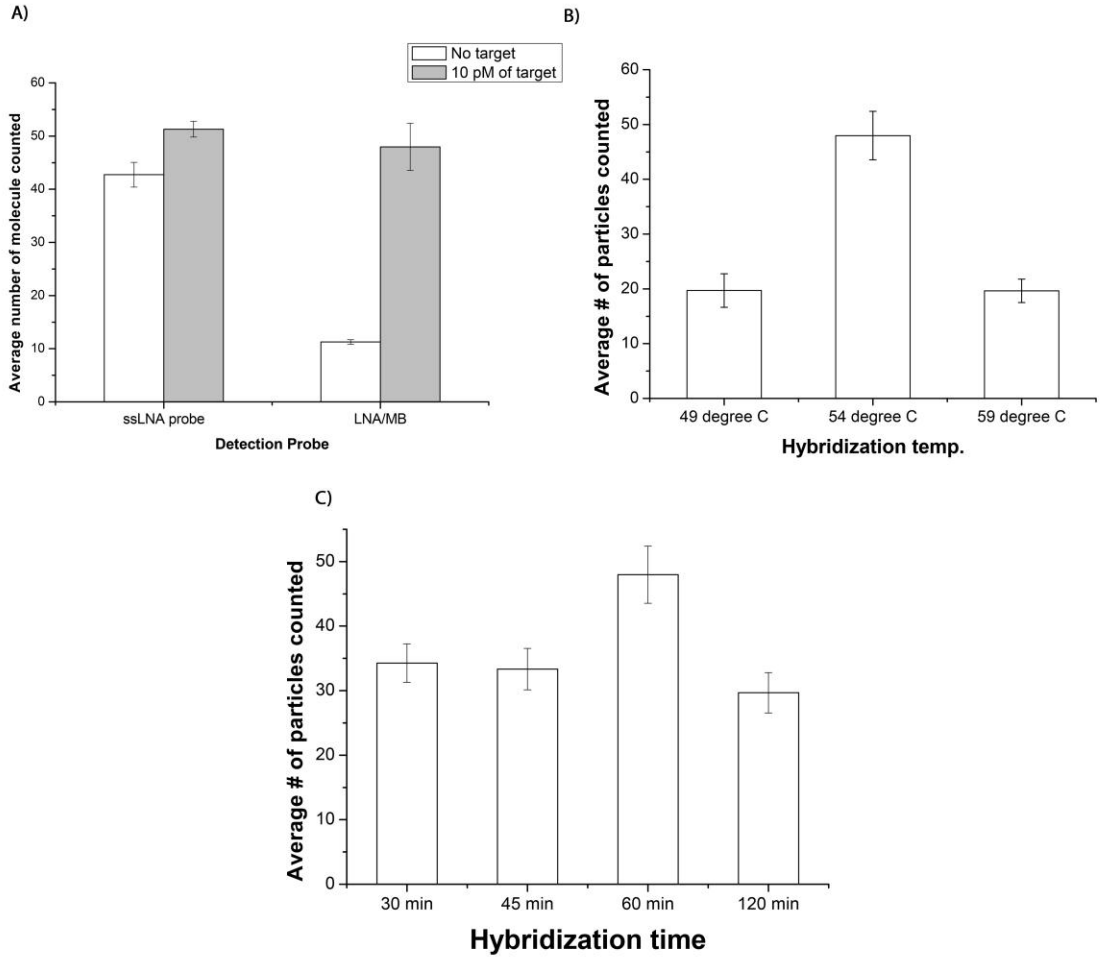
As the detection was based on the hybridization of the probe and the target, an optimal hybridization condition is very crucial. Here, we studied the effects of probe design, hybridization temperature and time on the hybridization efficiency. In recent years, locked nucleic acid (LNA) probe has been widely applied in biosensing. In the LNA analogue, methylene bridge locked the ribose ring into a rigid conformation and hence increased the melting temperature of the hybrid. By introducing a number of LNA analogues into a sequence of DNA or RNA not only can enhance the binding affinity of a

probe towards the target sequence, but also increase the stability of the hybrid as well as capability of mis-match discrimination of the detection probe. By incorporating a fluorophore-quencher pair into probe (LNA/MB), a significantly greater difference in molecules counts, with and without target molecules, was achieved as shown in figure 2A. The molecule counts of the detection probe-alone (background) were eliminated by 60 % with the application of the quencher molecule in MB as compared with that of linear LNA probe. Given the fact that LNA/MB gives relatively higher signal-to-background, LNA/MB was used as the detection probe for the rest of the experiment.

Studies show that the optimal hybridization temperature for nucleic acid is 20-25 °C below the melting temperature of the detection probe.[30] Since the difference in hybridization temperature for LNA and miRNAs from the same family with a single base mismatch could be small (depends on the mutation position and length of target), the hybridization temperature plays a key role on the specificity and efficiency of the assay. To study the effect of hybridization temperature, the temperature was set at 15-25 °C below the melting temperature of the LNA/MB probe (74 °C, nearest neighbor method), which was 49, 54, and 59 °C. As elucidated by the counts of hybrid attained at different temperatures in figure 2B, the hybridization efficiency at 54 °C, i.e. 20 °C below the melting temperature, was the highest. It agreed with the literature that hybridization affinity of LNA probe is highly temperature dependent.[31] Herein, the hybridization temperature was set as 54 °C for the entire study.

Incubation time also plays an important role on hybridization efficiency. As shown in figure 2C, hybridization time of 30, 45, 60 and 120 min was studied. It shows that the number of hybrids increased while increasing the incubation time and reached the

maximum at 60 min. The number of counts dropped after 120 min of incubation. This phenomenon agreed with our previous reported result that the prolonged incubation might cause the change of structure of both the target and the probe.[18] Hence, the incubation time was set at 60 min for the entire experiment.



287

288 **Figure 2.** Optimization of hybridization conditions A) performance of ssLNA and
289 LNA/MB probe with complementary target mir-205; B) effect of hybridization
290 temperature and C) effect of incubation time on the hybridization efficiency. Error bars,
291 standard error of mean, n = 3.

292

Quantification of synthetic mir-205

To demonstrate the performance of the developed detection assay, a calibration plot (figure 3A) of the molecules counts as a function of the concentration of the target mir-205 was constructed under the optimal hybridization mentioned above. Briefly, synthetic mir-205 of different concentrations 0-100 pM was hybridized with 100 pM LNA/MB in solution and labeled with YOYO dye (1:3 dye/bp) respectively. By single-molecule counting on the series of images, the calibration of the molecules counts as a function of the target concentration was constructed. A good linear correlation coefficient of 0.998 was obtained with a limit of detection at 500 fM (*LOD = blank + 3× standard error of mean of blank*). The limit of quantification was calculated to be 2 pM (*LOQ = blank + 10× standard error of mean of blank*). As study showed that the level of miRNAs in the cancer patient's serum sample is around 20 pM,[17] this as-developed assay is capable of quantifying the trace amount of circulating miRNAs in serum samples.

To emphasize the important role of the fluorophore-quencher pair in MB on the assay performance, a calibration plot for quantification of mir-205 with ssLNA probe was constructed under the optimal hybridization conditions (figure 3B) for comparison with that of LNA/MB. A good linear correlation coefficient of 0.998 and a limit of detection of 8 pM were achieved. The limit of detection fell into the same magnitude range as the reported.[18] It indicated that the replacement of the ssLNA probe by LNA/MB was capable to improve the sensitivity of the assay by more than 10 times.

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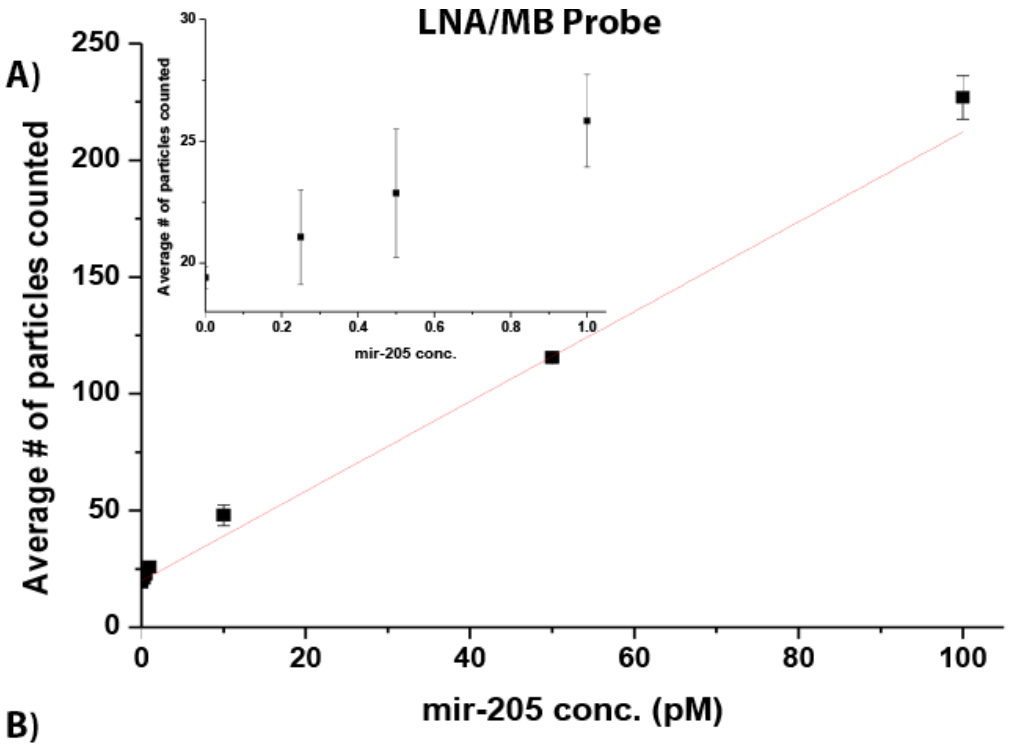
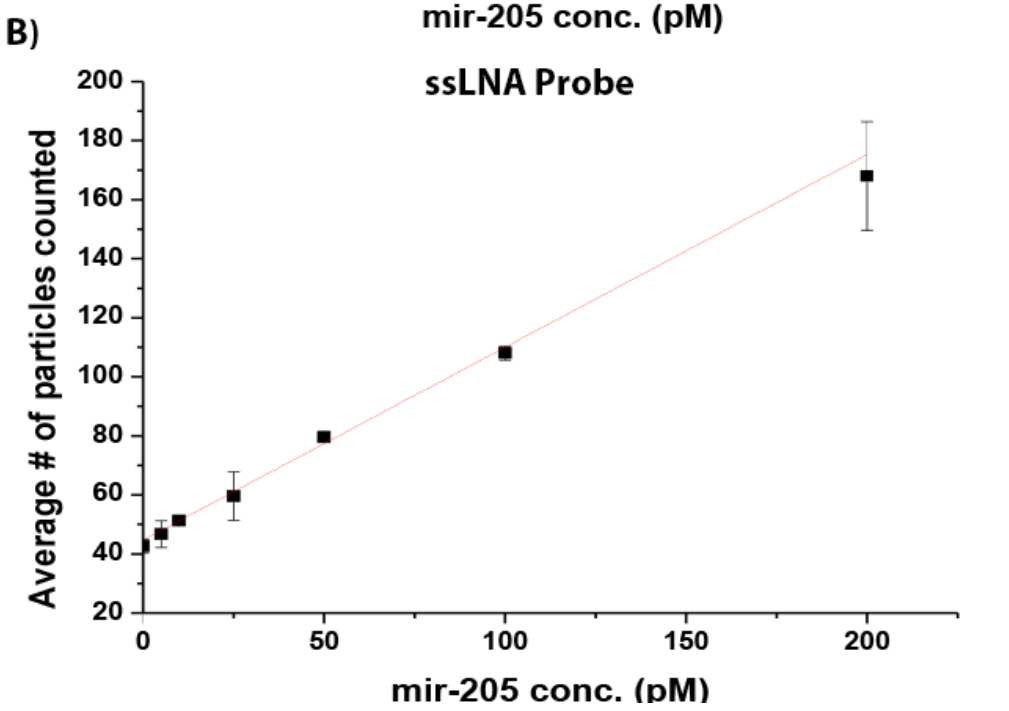

A)
LNA/MB Probe
Average # of particles counted
mir-205 conc. (pM)
0
50
100
150
200
250
0
20
40
60
80
100
Average # of particles counted
mir-205 conc.
20
25
30
0.0
0.2
0.4
0.6
0.8
1.0


B)
ssLNA Probe
Average # of particles counted
mir-205 conc. (pM)
20
40
60
80
100
120
140
160
180
200
0
50
100
150
200

316

Figure 3. Calibration plot for the quantification of mir-205, different concentration of mir-205 were hybridized with a) LNA/MB probe and b) single-stranded LNA probe at 54 °C for 1 hr. Error bars, standard error of mean, n = 3.

Selectivity

To demonstrate the selectivity of LNA/MB probe, four samples of NPC associated miRNAs, (i) mir-149; (ii) mir-200c; (iii) mir-205 and; (iv) a mixture of all these three miRNAs with a final concentration of 10 pM each; were hybridized with 40 pM LNA/MB at optimal conditions. As illustrated in figure 4, the signal detected in the samples that contained mir-149-only and mir-200c-only was 6 % and 1.4 %, respectively, ((signal from samples – blank)/ signal from mir-205 × 100%) of those contained mir-205-only and the mixture of all three miRNAs. Moreover, the signal detected in mixture was comparable to the one with mir-205 only. It elucidated that LNA/MB probe can discriminate mir-205 from the rest of the miRNAs. This LNA/MB probe shows a high selectivity towards the complementary target.

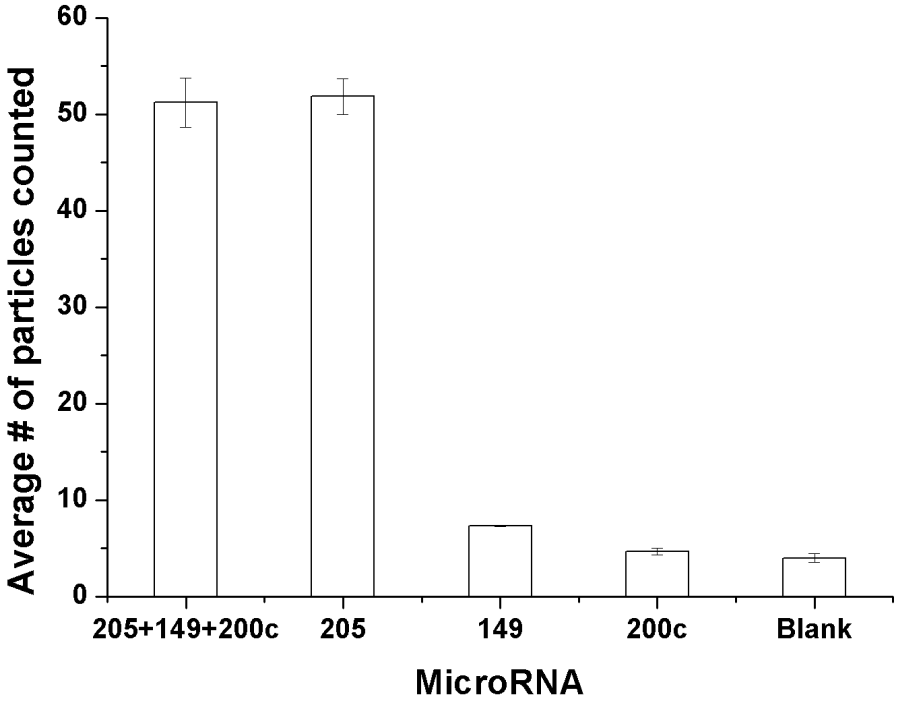
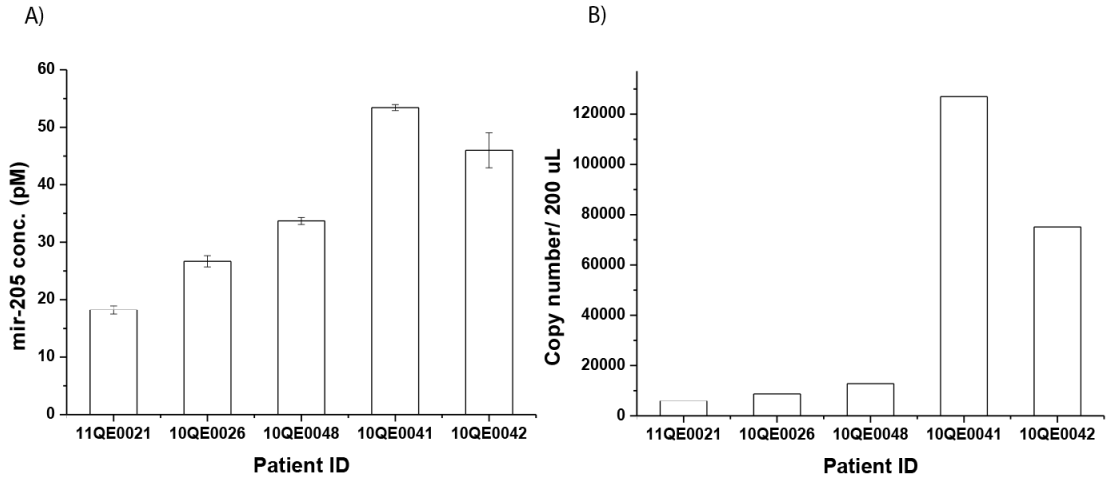


Figure 4. Study on the selectivity of the assay. The LNA/MB is capable of differentiating the target miRNAs from others miRNAs. Error bar, standard error of mean, n=3.

Quantification of mir-205 in different stages of nasopharyngeal carcinoma patients' serum sample. Over the past few years, mir-205 was reported to be abnormally up regulated in head and neck squamous cell carcinoma (HNSCC) and esophageal squamous cell carcinoma (ESCC). Study showed that mir-205 might play an important regulatory role in the stepwise development of NPC.[5] Here, as a proof-of-concept, we applied single molecule detection assay to quantify the amount of mir-205 in five patient serum samples. Since the fluorescent dye YOYO-1 has no selectivity towards oligonucleotides, like DNA, mRNA, and other small RNAs, in order to eliminate the unwanted signal

generated from the complex sample matrix and to avoid sophisticated pretreatment steps, standard addition strategy was adopted here. Briefly, synthetic mir-205 was spiked into the sample matrix with the MB probes. The original concentration of mir-205 in the samples was obtained by extrapolating the calibration curve. Five independent calibrations were prepared for the five serum samples, stage one: 11QE0021, stage two: 10QE0026 and 10QE0048, and stage three: 10QE0041 and 10QE0042, which were collected from different NPC patients. The original concentrations of mir-205 in these samples were determined. All five calibration plots (S2) showed a good correlation between the concentration of mir-205 and the number of counted molecules with coefficients of determination above 0.992. Since the serum samples were diluted five times throughout the analysis, the amount of circulating mir-205 in each samples estimated has to be multiplied by the conversion factor of five in order to obtain the actual mir-205 contents. As depicted in figure 5A, the content of the circulating mir-205 were found to be 18.2 ± 0.7, 26.7 ± 1.0, 33.6 ± 0.6, 53 ± 0.5 and 46.0 ± 3.0 pM in stage one: 11QE0021, stage two: 10QE0026 and 10QE0048, and stage three: 10QE0041 and 10QE0042 NPC patients' serum samples respectively. The results agreed with literature reported that mir-205 is an up-regulator in NPC. qRT-PCR analysis was also performed for the five patient serum samples for comparison (Figure 5B). qRT-PCR result also described an increase in mir-205 expression as cancer progress. However, there is variation between the results obtained from these two different approaches particularly in serum sample at early cancer stage where the content of mir205 is relatively low. We anticipated that the differences might be caused by the variation arising from the isolation, purification, and the amplification steps in qRT-PCR while single molecule

TIRFM approach is free of sample-pretreatment and amplification. Moreover, the LNA/MB probe used in this assay is more specific than the cDNA primer in qRT-PCR, and may therefore provides higher target probing efficiency particularly at low abundance of target miR205 which is crucial for early cancer diagnosis. In general, both the single molecule detection and the accredited qRT-PCR methods demonstrated the upregulation of mir-205 expression in NPC patients. The detection assay developed here is of high potential as a non-invasive tool for the application for expression profiling on circulating miRNAs in early cancer diagnosis.



375

376 **Figure 5.** Quantification of circulating mir-205 contents in NPC patients' serum samples
377 of stage one NPC patient (patient ID: 11QE0021), stage two NPC patients (patient ID:
378 10QE0026 & 10QE0048), and stage three NPC patients (patient ID: 10QE0041 &
379 10QE0042) by A) single molecule TIRFM assay and B) qRT-PCR assay respectively.

380 **Conclusion**

381 In the work, we have developed a rapid, pre-analysis purification and sample enrichment
382 free, yet sensitive detection assay for the quantification of miRNAs using a LNA
383 modified MB detection probe and solution-based hybridization approach with TIRFM.
384 This as-developed assay is capable of quantifying the content of NPC associated mir-205
385 in patients' serum samples at different NPC stages. The content of mir-205 in the serum
386 samples from different stages was found elevated and agreed with the qRT-PCR results
387 and other studies that mir-205 is an up-regulator for NPC. The developed detection assay
388 is direct, simple, and of high potential to serve as a promising tool for clinical application.

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394

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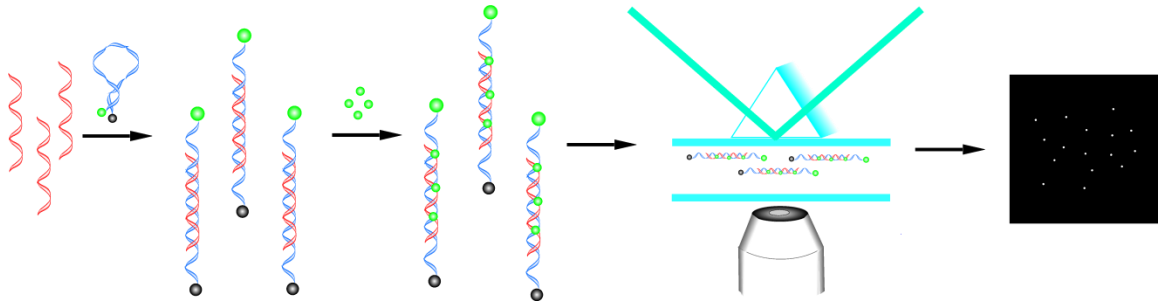

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