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

4 See-Lok Ho^a, Ho-Man Chan^a, Amber Wai-Yan Ha^b, Ricky Ngok-Shun Wong^{b,*}, Hung-
5 Wing Li^{a,*}

6 ^aDepartment of Chemistry, Hong Kong Baptist University, Kowloon Tong, Hong Kong,
7 P.R. China

8 ^bDepartment of Biology, Hong Kong Baptist University, Kowloon Tong, Hong Kong,
9 P.R. China

10 *Corresponding author. *Email address:* hwli@hkbu.edu.hk (H.W. Li),
11 rns Wong@hkbu.edu.hk (R.N.S. Wong)

12

13 **Abstract**

14 MicroRNAs (miRNAs) are small non-coding RNAs that regulate human gene expression
15 at the post-transcriptional level. Growing evidences indicated that the expression profile
16 of miRNAs is highly correlated with the occurrence of human diseases including cancers.
17 Playing important roles in complex gene regulation processes, aberrant expression pattern
18 of various miRNAs is implicated in different types and even stages of cancer. Besides
19 localizing in cells, many of these miRNAs are found circulating around the body in a
20 wide variety of fluids such as urine, serum and saliva. Surprisingly, these extracellular
21 circulating miRNAs are highly stable and resistant to degradation, and therefore, are
22 considered as promising biomarkers for early cancer diagnostic via non-invasive
23 extraction from body fluids. Unfortunately, the abundance of these small RNAs is
24 ultralow in the body fluids, making it challenging to quantify them in complex sample
25 matrixes. Establishing a sensitive, specific yet simple assay for an accurate quantification
26 of circulating miRNAs is therefore desirable. Our group previously reported a sensitive
27 and specific detection assay of miRNAs in single molecule level with the aid of total
28 internal reflection fluorescence microscopy. In this work, we advanced the assay to
29 differentiate the expression of a nasopharyngeal carcinoma (NPC) up-regulator hsa-mir-
30 205 (mir-205) in serum collected from patients of different stages of NPC. To overcome
31 the background matrix interference in serum, locked nucleic acid modified molecular
32 beacon (LNA/MB) was applied as the detection probe to hybridize, capture and detect
33 target miR-205 in serum matrix with enhanced sensitivity and specificity. A detection
34 limit of 500 fM was achieved. The as-developed method was capable of differentiating
35 NPC stages by the level of mir-205 quantified in serum with only 10 μ L of serum and the

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

41 **Introduction**

42 Nasopharyngeal Carcinoma (NPC) is a highly metastatic head and neck cancer that
43 originated in the nasopharynx. NPC is highly prevalent in China and Southern Asia. The
44 5-year survival rate for stage III and IV NPC patients is about 50 %. Due to the vague
45 symptoms and its deep location, 70 % of patients were diagnosed as cervical lymph node
46 metastasis and around 30 % of patients were diagnosed as long distant metastasis at the
47 first consultation. In advance NPC, the incidence of local reoccurrence is very high. Due
48 to the distinct metastasis of NPC, the effects of radiotherapy and chemotherapy
49 treatments are only significant at early stages.¹⁻⁴ Therefore, early diagnosis of NPC is
50 extremely necessary.

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

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

71 Northern blotting, reverse transcript polymerase chain reaction and microarray¹³⁻¹⁶ are
72 extensively applied as the routine methods for detection of nucleic acids; unfortunately,
73 the capabilities of detecting miRNAs often hindered by the short length, lack of common
74 features, homology sequence among various miRNA family members and low
75 abundance. Since the content of circulating miRNAs in serum is even lower (~ 20 pM),¹⁷
76 these golden methods often required a large volume of patient serum samples for the
77 detection. In addition, these methods are labor intensive, time consuming, and low
78 sample throughput, which limited the application of these methods for clinical validation
79 and management of disease. Therefore, development of an accurate, precise and sensitive
80 detection method for circulating miRNAs is necessary. Based on our previous published

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

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

104 17-22 °C in melting temperature of the LNA/RNA duplex that contains only a single base
105 mismatch.^{22,23} The LNA modified MB inherits the attractive properties from (i) LNA:
106 enhanced stability and binding affinity, mismatch discrimination capacity of the detection
107 probe; and (ii) MB: lower fluorescence background signal arise from free probe and
108 hence a lower LOD can be achieved.

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

122 **Experimental**

123 **Coverslides pretreatment**

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

127 the slides were consecutively soaked in Piranha solution (H₂SO₄: H₂O₂ 3:1) and sonicated
128 for 30 min, then sonicated again in the solution of HCl: H₂O: H₂O₂(1: 1: 1) at 60 °C for
129 30 min, further soaked in Piranha solution for 30 min and sonicated for another 30 min.
130 All the slides were rinsed with distilled H₂O extensively between each step. The slides
131 were finally stored in distilled water and blow-dried with nitrogen gas before use.

132 **Materials and reagents**

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

143 LNA-MB probe for mir-205: 5' TYE563-CGC TCC TCA **GAC TCC GGT GGA** ATG
144 AAG GAG CG-Iowa Black® RQ-Sp3' (bold: LNA analogue; unbold: DNA analogue);
145 Synthetic mir-149: 5' - UCU GGC UCC GUG UCU UCA CUC CC - 3'; Synthetic mir-
146 200c: 5' - UAA UAC UGC CGG GUA AUG AUG GA - 3'; Synthetic mir-205: 5' -
147 UCC UUC AUU CCA CCG GAG UCU G - 3'. All oligonucleotides were suspended in
148 500 µL DEPC-water (Ambion, USA) and further diluted to appropriate concentrations

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

151 **Optimization of the hybridization conditions**

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

163 **Single molecule detection of target miRNA.**

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

172 Then the cocktail was incubated in a dry heating block (*AccuBlock* Digital Dry Bath D
173 1100, USA) at the optimal temperature and time as determined. To demonstrate the role
174 of MB in LNA/MB probe, synthetic mir-205 with a final concentration of 0, 1, 10, 20, 50,
175 100, 200 pM was hybridized with 200 pM ssLNA probe at optimal conditions. After
176 incubation, 1 μ L of 100 nM YOYO-1 Iodide (YOYO) was then added to the
177 hybridization cocktail to label the hybrids with a ratio of the YOYO and the hybridized
178 base pair at 1:3 (dye/bp) at room temperature. The YOYO-labeled hybrids were kept
179 equilibrium for 5 min and 10 μ L of the solution was pipetted onto the prewashed
180 coverslides for TIRFM imaging.

181 **Selectivity of the LNA/MB probe**

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

187 **Quantification of the target miRNAs in serum**

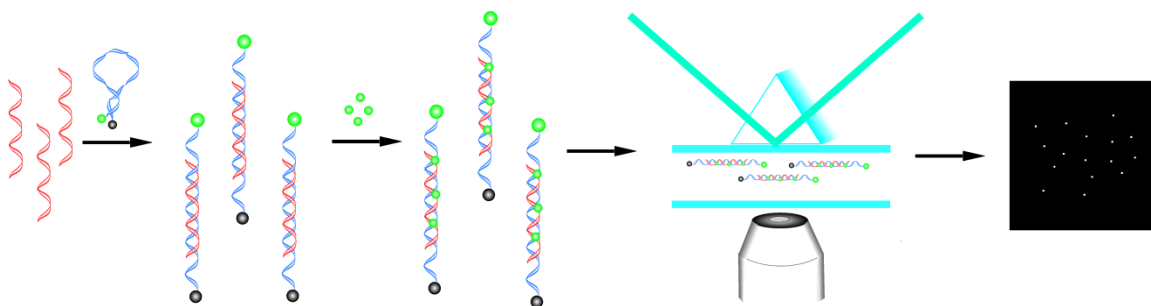
188 Serum samples were obtained from the Nasopharyngeal Carcinoma Area of Excellence
189 Research Tissue Bank under the Center for Nasopharyngeal Carcinoma Research. The
190 serum samples were stored at - 80 °C prior to use without further modification. The
191 serum sample was diluted 5-fold with TNE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

195 concentration of 100 pM. The solutions were finally topped up with TNE 250 buffer to
196 40 μ L and incubated at the optimal condition.

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

218 the fluorescent image represents a single-molecule; the number of bright spots in the five
219 frames was summed up (accumulation of the number of diffusing molecules detected in
220 250 ms), while the summed spots from 10 different image series from a single slide were
221 averaged to eliminate localization effect. All the experiments were done in triplicates and
222 the error bars refer to the standard error of mean of the experiments.

223



224

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

227 **Detection strategy**

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

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

252 **Optimization**

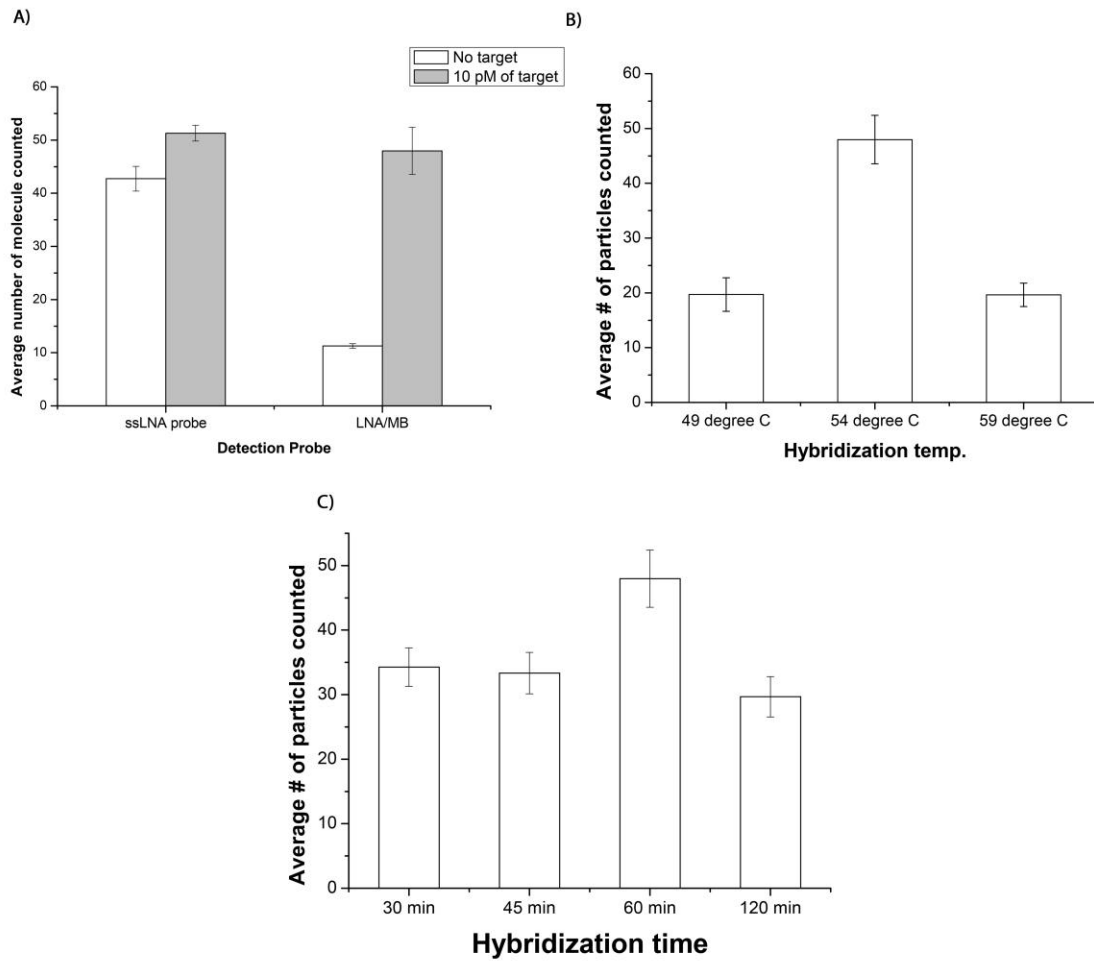
253 As the detection was based on the hybridization of the probe and the target, an optimal
254 hybridization condition is very crucial. Here, we studied the effects of probe design,
255 hybridization temperature and time on the hybridization efficiency. In recent years,
256 locked nucleic acid (LNA)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

260 probe towards the target sequence, but also increase the stability of the hybrid as well as
261 capability of mis-match discrimination of the detection probe. By incorporating a
262 fluorophore-quencher pair into probe (LNA/MB), a significantly greater difference in
263 molecules counts, with and without target molecules, was achieved as shown in figure
264 2A. The molecule counts of the detection probe-alone (background) were eliminated by
265 60 % with the application of the quencher molecule in MB as compared with that of
266 linear LNA probe. Given the fact that LNA/MB gives relatively higher signal-to-
267 background, LNA/MB was used as the detection probe for the rest of the experiment.

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

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

283 maximum at 60 min. The number of counts dropped after 120 min of incubation. This
284 phenomenon agreed with our previous reported result that the prolonged incubation
285 might cause the change of structure of both the target and the probe.¹⁸ Hence, the
286 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

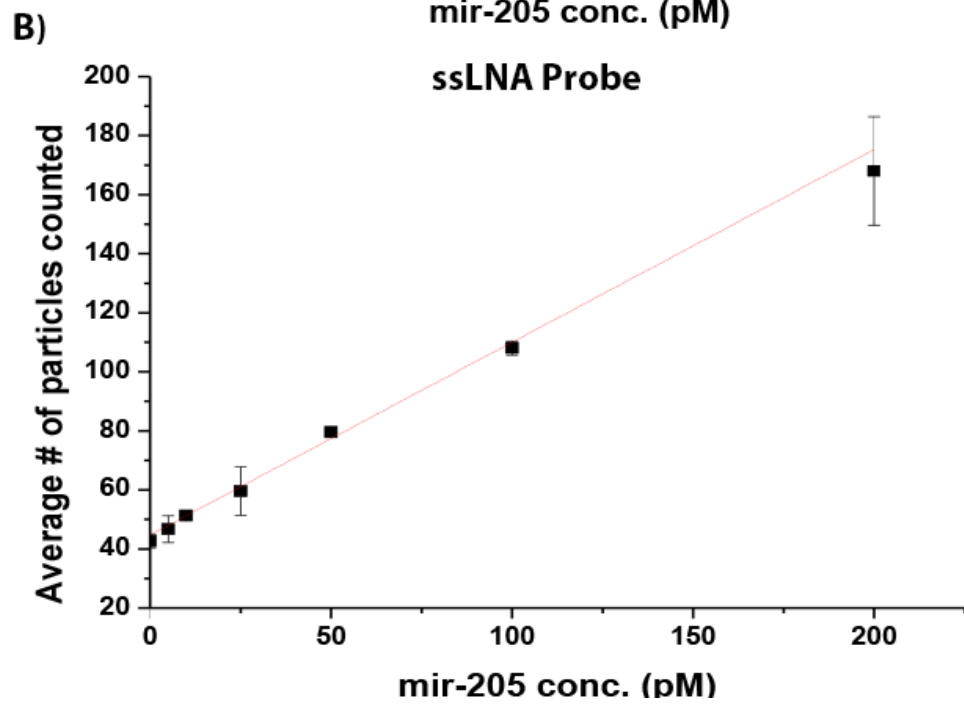
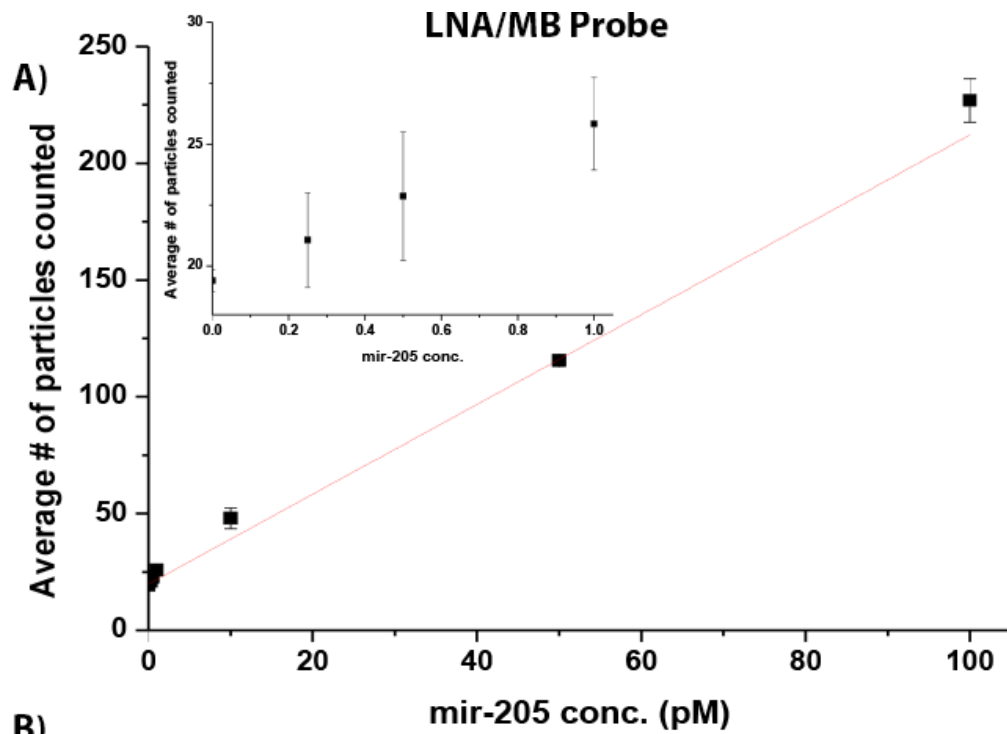
293 **Quantification of synthetic mir-205**

294 To demonstrate the performance of the developed detection assay, a calibration plot
295 (figure 3A) of the molecules counts as a function of the concentration of the target mir-
296 205 was constructed under the optimal hybridization mentioned above. Briefly, synthetic
297 mir-205 of different concentrations 0-100 pM was hybridized with 100 pM LNA/MB in
298 solution and labeled with YOYO dye (1:3 dye/bp) respectively. By single-molecule
299 counting on the series of images, the calibration of the molecules counts as a function of
300 the target concentration was constructed. A good linear correlation coefficient of 0.998
301 was obtained with a limit of detection at 500 fM ($LOD = blank + 3 \times standard\ error\ of$
302 $mean\ of\ blank$). The limit of quantification was calculated to be 2 pM ($LOQ = blank +$
303 $10 \times standard\ error\ of\ mean\ of\ blank$). As study showed that the level of miRNAs in the
304 cancer patient's serum sample is around 20 pM,¹⁷ this as-developed assay is capable of
305 quantifying the trace amount of circulating miRNAs in serum samples.

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

313

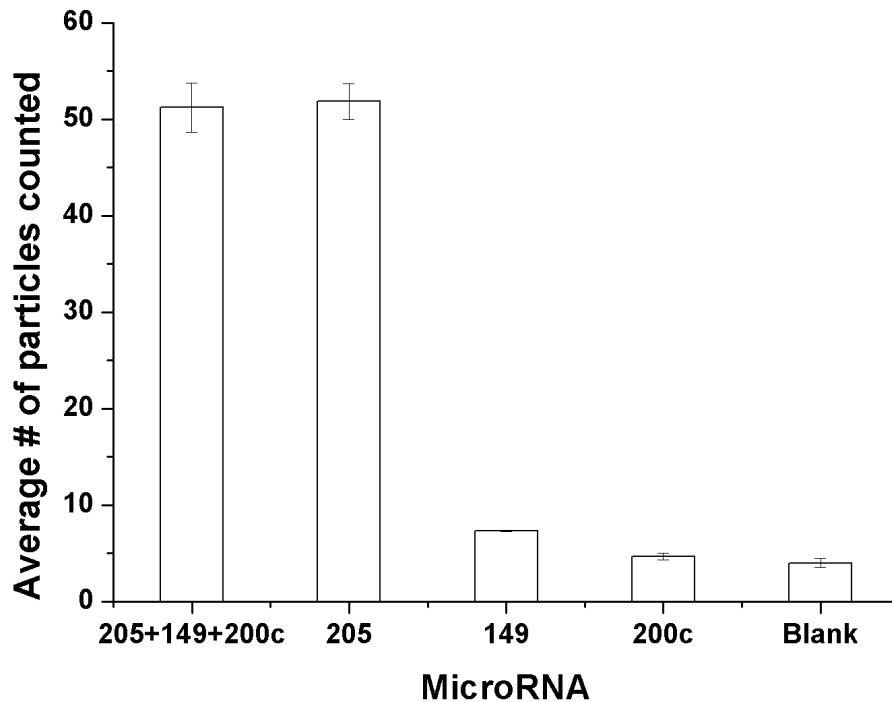
314



317 **Figure 3.** Calibration plot for the quantification of mir-205, different concentration of
318 mir-205 were hybridized with a) LNA/MB probe and b) single-stranded LNA probe at 54
319 °C for 1 hr. Error bars, standard error of mean, n = 3.

320 **Selectivity**

321 To demonstrate the selectivity of LNA/MB probe, four samples of NPC associated
322 miRNAs, (i) mir-149; (ii) mir-200c; (iii) mir-205 and; (iv) a mixture of all these three
323 miRNAs with a final concentration of 10 pM each; were hybridized with 40 pM
324 LNA/MB at optimal conditions. As illustrated in figure 4, the signal detected in the
325 samples that contained mir-149-only and mir-200c-only was 6 % and 1.4 %, respectively,
326 $((\text{signal from samples} - \text{blank}) / \text{signal from mir-205} \times 100\%)$ of those contained mir-205-
327 only and the mixture of all three miRNAs. Moreover, the signal detected in mixture was
328 comparable to the one with mir-205 only. It elucidated that LNA/MB probe can
329 discriminate mir-205 from the rest of the miRNAs. This LNA/MB probe shows a high
330 selectivity towards the complementary target.



331

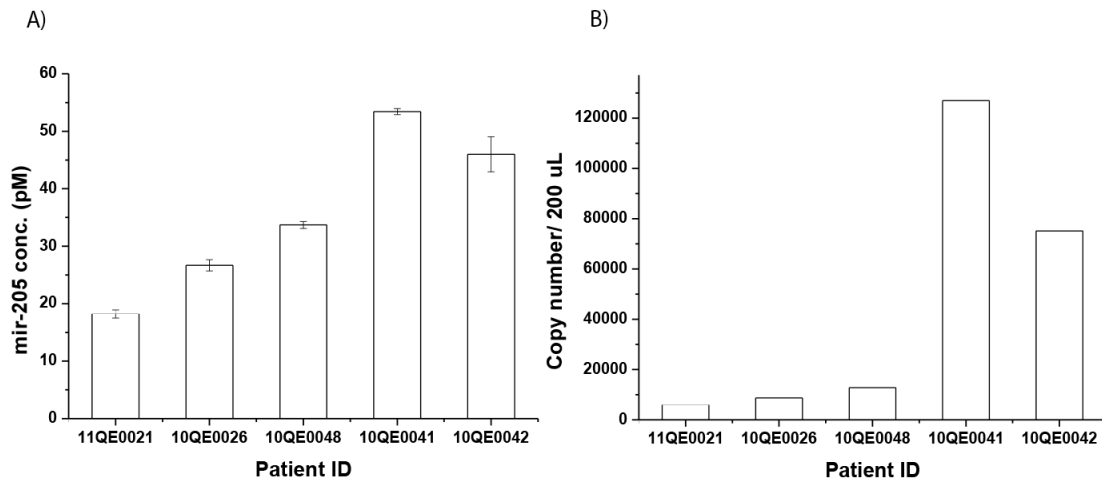
332 **Figure 4.** Study on the selectivity of the assay. The LNA/MB is capable of
 333 differentiating the target miRNAs from others miRNAs. Error bar, standard error of
 334 mean, n=3.

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

343 generated from the complex sample matrix and to avoid sophisticated pretreatment steps,
344 standard addition strategy was adopted here. Briefly, synthetic mir-205 was spiked into
345 the sample matrix with the MB probes. The original concentration of mir-205 in the
346 samples was obtained by extrapolating the calibration curve. Five independent
347 calibrations were prepared for the five serum samples, stage one: 11QE0021, stage two:
348 10QE0026 and 10QE0048, and stage three: 10QE0041 and 10QE0042, which were
349 collected from different NPC patients. The original concentrations of mir-205 in these
350 samples were determined. All five calibration plots (S2) showed a good correlation
351 between the concentration of mir-205 and the number of counted molecules with
352 coefficients of determination above 0.992. Since the serum samples were diluted five
353 times throughout the analysis, the amount of circulating mir-205 in each samples
354 estimated has to be multiplied by the conversion factor of five in order to obtain the
355 actual mir-205 contents. As depicted in figure 5A, the content of the circulating mir-205
356 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
357 one: 11QE0021, stage two: 10QE0026 and 10QE0048, and stage three: 10QE0041 and
358 10QE0042 NPC patients' serum samples respectively. The results agreed with literature
359 reported that mir-205 is an up-regulator in NPC. qRT-PCR analysis was also performed
360 for the five patient serum samples for comparison (Figure 5B). qRT-PCR result also
361 described an increase in mir-205 expression as cancer progress. However, there is
362 variation between the results obtained from these two different approaches particularly in
363 serum sample at early cancer stage where the content of mir205 is relatively low. We
364 anticipated that the differences might be caused by the variation arising from the
365 isolation, purification, and the amplification steps in qRT-PCR while single molecule

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

374



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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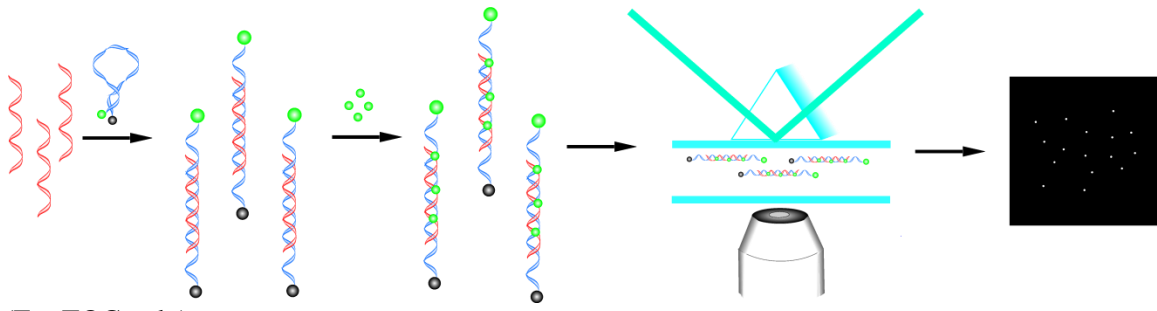
394

395 **References**

- 396 (1) Lee, A. W. M.; Lin, J. C.; Ng, W. T. *Semin. Radiat. Oncol.* **2012**, *22*, 233-244.
397 (2) Qin, A. Y.; Zhang, X. W.; Liu, L.; Yu, J. P.; Li, H.; Wang, S. Z. E.; Ren, X. B.; Cao, S. *Eur. J. Cell*
398 *Biol.* **2013**, *92*, 54-60.
399 (3) Tao, Y. G.; Bidault, F.; Bosq, J.; Bourhis, J. *Onkologie* **2008**, *31*, 574-575.
400 (4) Geara, F. B.; Sanguineti, G.; Tucker, S. L.; Garden, A. S.; Ang, K. K.; Morrison, W. H.; Peters, L.
401 *J. Radiother. Oncol.* **1997**, *43*, 53-61.
402 (5) Luo, Z. H.; Zhang, L. Y.; Li, Z.; Li, X. Y.; Li, G.; Yu, H. B.; Jiang, C.; Dai, Y. F.; Guo, X. F.; Xiang, J. J.;
403 Li, G. Y. *BMC Med. Genomics* **2012**, *5*:
404 (6) Esquela-Kerscher, A.; Slack, F. J. *Nat. Rev. Cancer* **2006**, *6*, 259-269.
405 (7) Esteller, M. *Nat. Rev. Genet.* **2011**, *12*, 861-874.
406 (8) Zhang, B. H.; Pan, X. P.; Cobb, G. P.; Anderson, T. A. *Dev. Biol.* **2007**, *302*, 1-12.
407 (9) Cookson, V. J.; Bentley, M. A.; Hogan, B. V.; Horgan, K.; Hayward, B. E.; Hazelwood, L. D.;
408 Hughes, T. A. *Cell. Oncol.* **2012**, *35*, 301-308.
409 (10) De Guire, V.; Robitaille, R.; Tetreault, N.; Guerin, R.; Menard, C.; Bambace, N.; Sapieha, P.
410 *Clin. Biochem.* **2013**, *46*, 846-860.
411 (11) Koberle, V.; Pleli, T.; Schmithals, C.; Alonso, E. A.; Haupenthal, J.; Bonig, H.; Peveling-
412 Oberhag, J.; Biondi, R. M.; Zeuzem, S.; Kronenberger, B.; Waidmann, O.; Piiper, A. *PLoS One*
413 **2013**, *8*, e75184.
414 (12) Si, H. Y.; Sun, X. M.; Chen, Y. J.; Cao, Y.; Chen, S. M.; Wang, H. C.; Hu, C. J. *J. Cancer Res. Clin.*
415 *Oncol.* **2013**, *139*, 223-229.
416 (13) Levesque-Sergerie, J. P.; Duquette, M.; Thibault, C.; Delbecchi, L.; Bissonnette, N. *BMC Mol.*
417 *Biol.* **2007**, *8*, 93.
418 (14) Luo, X.; Zhang, J.; Wang, H. J.; Du, Y. Y.; Yang, L.; Zheng, F. Y.; Ma, D. *Biotechnol. Lett.* **2012**,
419 *34*, 627-633.
420 (15) Nelson, P. T.; Baldwin, D. A.; Searce, L. M.; Oberholtzer, J. C.; Tobias, J. W.; Mourelatos, Z.
421 *Nat. Methods* **2004**, *1*, 155-161.
422 (16) Varallyay, E.; Burgyan, J.; Havelda, Z. *Nat. Protoc.* **2008**, *3*, 190-196.
423 (17) Tian, T.; Xiao, H.; Zhang, X. L.; Peng, S.; Zhang, X. E.; Guo, S.; Wang, S. R.; Liu, S. M.; Zhou, X.;
424 Meyers, C.; Zhou, X. *Chem. Commun.* **2013**, *49*, 75-77.
425 (18) Chan, H. M.; Chan, L. S.; Wong, R. N. S.; Li, H. W. *Anal. Chem.* **2010**, *82*, 6911-6918.
426 (19) Catrina, I. E.; Marras, S. A. E.; Bratu, D. P. *ACS Chem. Biol.* **2012**, *7*, 1586-1595.
427 (20) Peng, Z. Y.; Young, B.; Baird, A. E.; Soper, S. A. *Anal. Chem.* **2013**, *85*, 7851-7858.
428 (21) Tsourkas, A.; Behlke, M. A.; Bao, G. *Nucleic Acids Res.* **2002**, *30*, 4208-4215.
429 (22) Jorgensen, S.; Baker, A.; Moller, S.; Nielsen, B. S. *Methods* **2010**, *52*, 375-381.
430 (23) Valoczi, A.; Hornyik, C.; Varga, N.; Burgyan, J.; Kauppinen, S.; Havelda, Z. *Nucleic Acids Res.*
431 **2004**, *32*, e175.
432 (24) Forkey, J. N.; Quinlan, M. E.; Goldman, Y. E. *Prog. Biophys. Mol. Biol.* **2000**, *74*, 1-35.
433 (25) Ishii, Y.; Yoshida, T.; Funatsu, T.; Wazawa, T.; Yanagida, T. *Chem. Phys.* **1999**, *247*, 163-173.
434 (26) Mashanov, G. I.; Tacon, D.; Knight, A. E.; Peckham, M.; Molloy, J. E. *Methods* **2003**, *29*, 142-
435 152.
436 (27) Taguchi, H.; Ueno, T.; Tadakuma, H.; Yoshida, M.; Funatsu, T. *Nat. Biotechnol.* **2001**, *19*,
437 861-865.
438 (28) Wazawa, T.; Ueda, M. In *Microscopy Techniques*, Rietdorf, J., Ed., **2005**, pp 77-106.
439 (29) Ho, S. L.; Chan, H. M.; Wong, R. N. S.; Li, H. W. *Anal. Chim. Acta.* **2014**, *823*, 61-68.
440 (30) You, Y.; Moreira, B. G.; Behlke, M. A.; Owczarzy, R. *Nucleic Acids Res.* **2006**, *34*, e60

441 (31) Wang, L.; Yang, C. Y. J.; Medley, C. D.; Benner, S. A.; Tan, W. H. *J. Am. Chem. Soc.* **2005**, *127*,
442 15664-15665.

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