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Effect Of Buffer Composition On PNA-RNA Hybridization Studied In The Microfluidic Microarray Chip

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Abstract: Herin we report that Peptide Nucleic Acid sequences (PNAs) can be used as the probe species for detection of RNA and that a microfluidic microarray (MMA) chip can be used as the platform for detection of hybridizations between immobilized PNA probes and RNA targets. The RNA targets used are derived from influenza A sequences. This paper discusses the optimization of the two probe technologies used for RNA detection and investigates how the composition of the probe buffer or the content of the hybridization solution can influence the overall results. Our data shows that the PNA probe is a better choice over the DNA probe when there is low salt in the probe buffer composition. Furthermore, we have shown that the absence of salt (NaCl) in the hybridization buffer does not hinder the detection of RNA sequences. The results conclude that PNA probes are superior to DNA probes in term of sensitivity and adaptability, asPNA immobilization and PNA-RNA hybridization are less affected by salt content in the reaction buffers unlike DNA probes.

Key words: peptide nucleic acid (PNA), influenza viral RNA, probe buffer, hybridization buffer, salt, formamide, microfluidic microarray (MMA).

Introduction

Peptide nucleic acids (PNAs) are DNA analogs in which the negatively charged deoxyribose phosphate backbone is replaced by an electrically neutral peptide-like backbone.[1-2] Since the PNA probes are not negatively charged, unlike traditional DNA probes, they should be less affected by the salt content of the buffer in which these probes are used.[3] When PNAs are used in the assembly of microarrays, it would be useful to better understand the effects of variation in salt concentration, in both the probe buffer used for printing the PNA probes on solid substrates and the hybridization buffer used for reaction between the probes and the target nucleic acids.

In this paper, PNA probes designed to detect a RNA sequence related to influenza A are employed in the construction of a microfluidic microarray (MMA) chip. The influenza A virus is the causative agent of yearly epidemics and occasional pandemics[4] and numerous microfluidic technologies have been developed to detect the presence of influenza A viral RNAs in clinical samples. Devices employed in these technologies include microfluidic chips,[5-15] integrated Microsystems,[16-20] and lateral flow strips.[8, 21-22] The detection methodologies include RT-qPCR,[15, 17, 20, 23-26] electrophoretic immunoassays,[27] PCR-capillary electrophoresis,[11-14, 18, 29] immunochromatography,[8, 19, 21-22] microarray, [5, 9, 16, 30-32] and electrochemical approaches.[4, 7, 28, 33] Among the microarray detection methods, oligonucleotide probes are most commonly used.[5, 9, 16, 30-32] Whilst PNA probes have been employed to detect various influenza virus strains,[34-36] and one group has employed PNA probes in the microfluidic chip to detect the viral DNA[7]to date, there are no details provided about the buffer compositions in the use of the PNA probes. Therefore, we conducted a study to investigate the effect of buffer composition on PNA probe immobilization and on PNA-RNA hybridization. We believe it is informative to report this study even though, generally, influenza A viral RNAs are detected indirectly after their conversion to cDNA.

In our group, we have previously employed the MMA chip for detection of DNA[37-39] and RNA targets.[40] The microfluidic microarray chip offers several advantages to other designs (e.g. plain microarrays) in that it can be constructed without an expensive robotic spotting system, and used for multiple samples. The use of the chip can also offer fast hybridization rates, and achieve cost-effectiveness.[41-45] The schematic diagrams for the use of the MMA chip are shown in Fig. 1 A-C. The construction of the device follows several steps. Firstly, a PDMS channel plate is sealed with a glass slide that is arrayed with lines consisting the probe molecules (Fig. 1A). Secondly, after removing the first PDMS plate, the glass slide is sealed against a second PDMS plate to admit target samples for

hybridization (Fig. 1B). Thirdly, the PDMS plate is removed and the glass slide is fluorescently scanned for detection (Fig. 1C). An image of the actual experiment used in this study and consisting of 16 probes hybridized with 16 targets is shown in Fig. 1D.

In this paper, we describe the principle of detection of PNA-RNA hybridizations in the MMA chip. This chip has the capability of studying various buffer conditions simultaneously using multiple samples. We report the effect of compositions of reaction buffers (*i.e.* probe buffer and hybridization buffer) on PNA-RNA hybridization results.

Experimental

Nucleic acid sequences

RNA target and DNA probe sequences were synthesized by Integrated DNA Technologies (Coralville IA). PNA probes were purchased from PNA Bio, Inc. (Thousand Oaks, CA). The sequences of the nucleic acid probes as well as RNA targets utilized in this study are shown in Table 1.

Three probes were used: two were PNA probes (P-PNA, P-PNA-inf) and one was DNA probe (P-DNA). Both PNA probes are 13-mer PNA probe sequences utilizing an 'O monomer' spacer molecule (2-(2-aminoethylethoxy) ethoxy acetic acid) located at the N-terminal position for attachment to the chip surface. The P-PNA probe has the same base sequence as the central region of a 30-mer DNA probe (P-DNA), utilising an amino group with a C6 linker located at the 5' end for surface attachment. This DNA probe was previously used for analyzing RNA samples [40]. The P-PNA-inf probe is a 13-mer PNA probe that was previously reported to probe influenza A viral RNA.[34, 50]

Two target RNA sequences were used. One is complementary to the PNA probe (T-RNA), whilst the other is designed to mimic an influenza A viral RNA target sequence (T-RNA-inf). Both

RNA target sequences were labeled by biotin at the 5' termini. The sequence of T-RNA was so designed that the binding region (section underlined in the T-RNA sequence in Table 1) is complementary to both the PNA probe (P-PNA) and the central region of the oligonucleotide probe (underlined in P-DNA).

The influenza A virus consists of single-stranded RNAs [46-47]. The RNA molecule comprises eight segments that encode for different proteins necessary for transcription, replication and viral assembly [48-49]. The sequence of T-RNA-inf was obtained from the analysis of the 5'untranslated region (UTR) which was conserved among each of the eight RNA segments.[34, 50]

PNA-RNA or DNA-RNA hybridizations were determined by imaging of theCy5 tag (emits at 670 nm when excited at 633 nm using fluorescence spectroscopy) incorporated by conjugation of streptavidin-Cy5 to the 5' biotin moiety on the RNA target sequences.

Materials

Glutaraldehyde (25%), 3-aminopropyltriethoxysilane (APTES), sodium dodecyl sulfate (SDS), sodium borohydride, and bovine serum albumin were obtained from Sigma-Aldrich and were used without further purification. Formamide was purchased from Merck. Ultrapure water (18 MΩ cm) was obtained from a water purification system (Easy pure RF, Dubuque, IA). Streptavidin-Cy5 (1 mg/μL) was purchased from Invitrogen, Life Technologies. Phosphate buffered saline (PBS), saline sodium citrate (SSC), sodium citrate, sodium bicarbonate, concentrated sulfuric acid, hydrogen peroxide (30 %) and ethanol (95 %) were all reagent-grade materials.

The 2% APTES solution was prepared by mixing 2.4 mL of APTES in 117.6 mL of 95% ethanol. The 5% glutaraldehyde solution was prepared by mixing 24 mL of 25% glutaraldehyde, 50 mL of 20X PBS and 46 mL of water.

The plain glass microscope slides (3" x 2") were purchased from Fisher Scientific (Ottawa, ON).

Functionalization of Glass Slides

Four glass slides were washed with detergent (Sparkleen) and rinsed with pure water, as previously described [51-52]. In summary, the glass slides were submerged in piranha solution (3:1 mixture of concentrated H_2SO_4, 30% H_2O_2) for 15 min. at 80 °C. After being rinsed with distilled water, ethanol 95%, then distilled water, the glass slides were dried under N_2. They were then submerged in a 2% APTES solution (under a N_2 gas environment) for 20 min. and then rinsed with 100% ethanol. Dispensing of APTES liquid was performed by a 10"-long needle together with a compensating nitrogen gas balloon. One observation is that the liquid should be yellow or light brown at this point and if the color turns dark and/or particles are found in the liquid it should be discarded.

The glass slides were subsequently baked in the oven for 1 h at 120 °C in air. Thereafter, the glass slides were submerged in a 5% glutaraldehyde solution in the refrigerator for 1 h and then rinsed with pure water. Finally, the glass slides were N_2-dried and stored in the dark at 4 °C before probe printing.

Probe Immobilization

A 2" x 2" PDMS channel plate consisting of microchannels (200-μm wide and 35-μm deep) was fabricated as described elsewhere.[37, 52] The 1.5-mm thick channel plate was sealed against the aldehyde-functionalized glass slide to form the MMA chip. Then, 0.6 μL of nucleic acid probes prepared in the probe buffer (0.15 M sodium bicarbonate with 1 M NaCl, unless noted otherwise) were added into the inlet reservoirs of the MMA chip using a micropipette. The probe solutions were filled through the channels by applying suction at the outlets followed by incubation at room temperature for 1 h. After the microchannels had been rinsed with 1 μL of a wash solution (0.15 M

sodium bicarbonate with 1 M NaCl), the PDMS channel plate was peeled away from the surface. Afterward, the glass slide was treated with the sodium borohydride solution (50mg $NaBH_4$, 15mL PBS 1X, 5mL ethanol 95%, 150 µL SDS 1.5%) for 20 min. The glass chip was then rinsed with pure water for 2 min. and then subsequently dried under N_2. The prepared glass slide was submerged in the blocking solution (10 mL 10X SSC, 150 µL SDS 1.5%, 20mg BSA and made up to 20mL with pure water) to inhibit non-specific binding. The glass chip incorporating the printed probe was then rinsed with deionized water for 2 min. and finally dried as above.

Hybridization and Detection

The glass slide with probe line arrays was covered with a second 2" x 2" PDMS channel plate to form the MMA chip assembly. The straight channels were aligned orthogonal to the printed probe lines on the slide. Hybridization reactions occur at the intersections between the channels and the probe lines. The target RNA samples were diluted in the hybridization buffer (1X SSC, unless noted otherwise, with 0.15% SDS). After the target solutions were denatured at 95 °C for 5 min, they were quickly cooled in ice just prior to their hybridization to immobilized probes. The target solutions (0.6 µL) were added to the inlet reservoirs of the MMA chip using a micropipette. Target solutions in different reservoirs were then drawn into the channels simultaneously by suction. Probe-target hybridization was carried out in an oven at 40 °C for incubation for 30 min. The microchannels were rinsed with 1µL of hybridization buffer immediately after incubation. SA-Cy5 solutions (0.6 µL, 1 mg/mL) were added to the inlet reservoirs using a micropipette, and the solutions were then drawn into the channels by suction simultaneously. After a further incubation step (30 min. at room temperature), the microchannels were rinsed immediately with 1 µL of wash buffer (1X PBS and 0.2% Tween 20)

For analysis, the glass slide was imaged on a confocal laser fluorescent scanner (Typhoon Trio+ variable mode imager) purchased from GE Healthcare (Baie d'Urfé, QC). The scan conditions were spatial resolution: 25 µm, excitation wavelength: 633 nm, emission wavelength: 670 nm, and photomultiplier tube voltage: 600V. The scanned image was analyzed by the ImageQuant 5.2 software. In the data quantification procedure, lines were drawn manually to cover the rectangular hybridization patches on the glass slide, and the average fluorescent intensity of the patch was recorded in the relative fluorescent unit (RFU).

Results and Discussion

Effect of probe buffer composition

RNA targets were hybridized to DNA and PNA probes in the presence of different amounts of salt in the probe buffers. The hybridization results are shown in Figure 2A. Here, the RNA targets were applied along the vertical direction, intersecting with the probe lines created in the horizontal orientation. Experiments were conducted using two concentrations of RNA targets (T-RNA). Stronger fluorescent signals were observed for the DNA-based probes (P-DNA) when prepared in the probe buffer containing the high salt concentration (1 M NaCl) rather than the low salt one (0.1 M NaCl). It is assumed this observation arises as the DNA probe possesses a negatively charged backbone and accordingly it requires the high ionic strength conditions provided by the 1M NaCl solution to reduce the Columbic repulsion between the negatively charged backbone and the glass slide during the probe immobilization step as previously reported. [55-56] Another observation is that much stronger signals are observed for the PNA-based probes (P-PNA) rather than the DNA-based probes (P-DNA). As shown in Fig. 2B, enhancement in signal intensity of 521% was found for the 10-nM RNA target, and 317%

for the 20-nM target, when P-PNA rather than P-DNA was the probe using 0.1 M NaCl as the probe buffer.

Furthermore, there was considerably less influence of salt concentration on RNA hybridization efficiencies when using the PNA-based probes (P-PNA). For instance, the hybridization signals (depicted in Fig. 2B) were similar for the 10-nM RNA target when the PNA probes were immobilized using buffers of different salt contents (i.e. the signal was decreased by just 28.8% when 0.1 M instead of 1 M NaCl was used). On the other hand, the DNA-based probes were less robust in which the signal decreases were higher when the probe buffer contained 1 M instead of 0.1 M NaCl (i.e. the signals were decreased by 83.9% and 66.4% for 20-nM and 10-nM RNA, respectively). These data supports the notion that PNA probes are more robust than DNA probes in terms of sensitivity and adaptability.[34-36]

Effect of hybridization buffer composition

Hybridizations involving RNA targets generally require a high temperature that functions to remove secondary structures of the single-stranded nucleic acid molecules. Nevertheless, formamide can also be used to achieve the same function allowing hybridization to be conducted at more reasonable temperatures.[53-54]

The results of RNA hybridizations conducted in formamide-containing hybridization buffer are shown in Fig.3A. Stronger signal intensities are observed for T-RNA targets when prepared in the hybridization buffer containing 30% formamide (Fig. 3B) indicating the utility of formamide to minimize any secondary structural motifs inherent in the target RNA sequences. Such a signal enhancement was observed even when the PNA probes (P-PNA) were printed at a low-salt probe

buffer containing 0.1 M NaCl. Similar observations were obtained in Fig. 3A for the RNA target (T-RNA-inf) which is related to the influenza A viral RNA sequence.

Although 1X SSC (saline-sodium citrate) is the buffer commonly used for hybridization experiments, we tested the capacity of our system to deliver reasonable results when using low-salt hybridization buffers such as C (15 mM sodium citrate) in the presence of 30% formamide. A comparison of hybridizations obtained in SSC and C buffers is illustrated in Fig. 4A. It is found that intensities with signal-to-noise ratio greater than 2 are still observed using hybridization buffers prepared in the low-salt conditions such as C buffer without NaCl added, suggesting that the need of high salt for reducing Columbic repulsion is not as critical when PNA probes, rather than DNA probes, are used. In fact, using PNA probes signals are still observable against the T-RNA-inf target sequence even at 5nM using low-salt hybridization buffers (i.e. C buffer) are used (Fig. 4B).

Conclusion

The microfluidic microarray (MMA) chip has been employed to study the effects of buffer compositions on PNA-RNA hybridization experiments. The presence of salt in the probe buffer and hybridization buffer does result in better signals for the detection of RNA targets, although hybridization still occurred to a reasonable extent in the absence of NaCl. Moreover, the presence of formamide in the hybridization buffer offers better RNA hybridization when conducted at a relatively low temperature of 40 °C. The MMA chip results support the notion that the PNA probe is more useful than the DNA probe in low ionic strength conditions with respect to assay sensitivity and experimental adaptability for RNA detection.

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339 **Table 1.** Sequences of probes (P) and targets (T) used to study PNA-RNA hybridizations

Identifier	Sequence
	Probes
P-DNA	H_2N-C6-CTGTATTG**AGTTGTATCGTGT**GGTGTATTT
P-PNA	H_2N-O-O-AGTTGTATCGTGT
P-PNA-inf	H_2N-O-O-CCTTGTTTCTACT
	Targets
T-RNA	biotin-ACGGAGCGCAAAAUACACC**ACACGAUACAACU**CAAUACAGUCGACGCCUA
T-RNA-inf	biotin-A**GUAGAAACAAGG**CCUGCUUUUG

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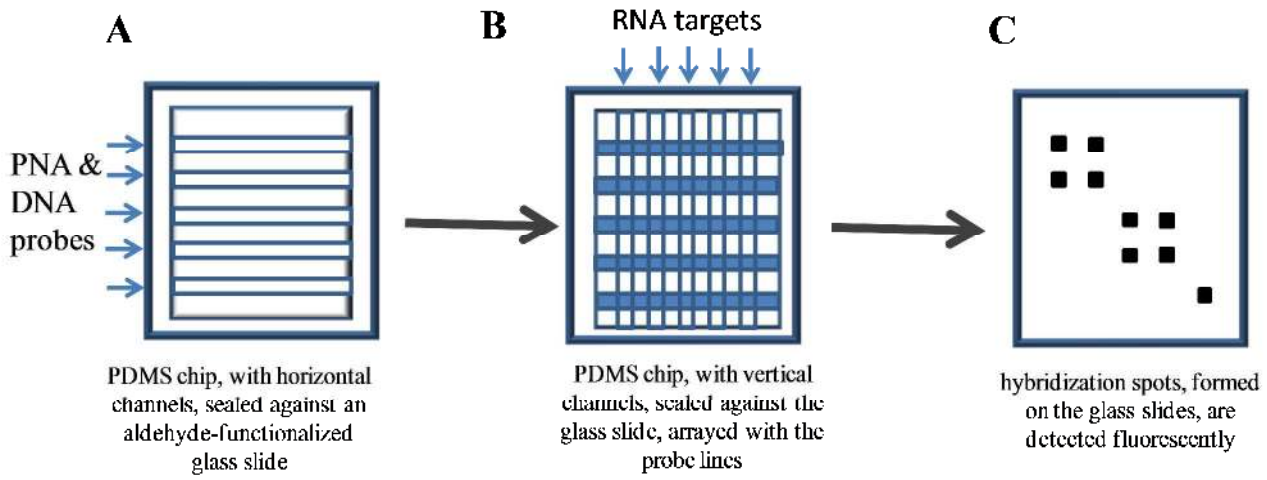
Fig. 1. Detection procedure using the microfluidic microarray (MMA) chip. The schematic diagrams of printing the probe array (A), hybridization with the RNA targets (B), detection of fluorescent results on the glass slide surface (C); an image of the experimental results (D).



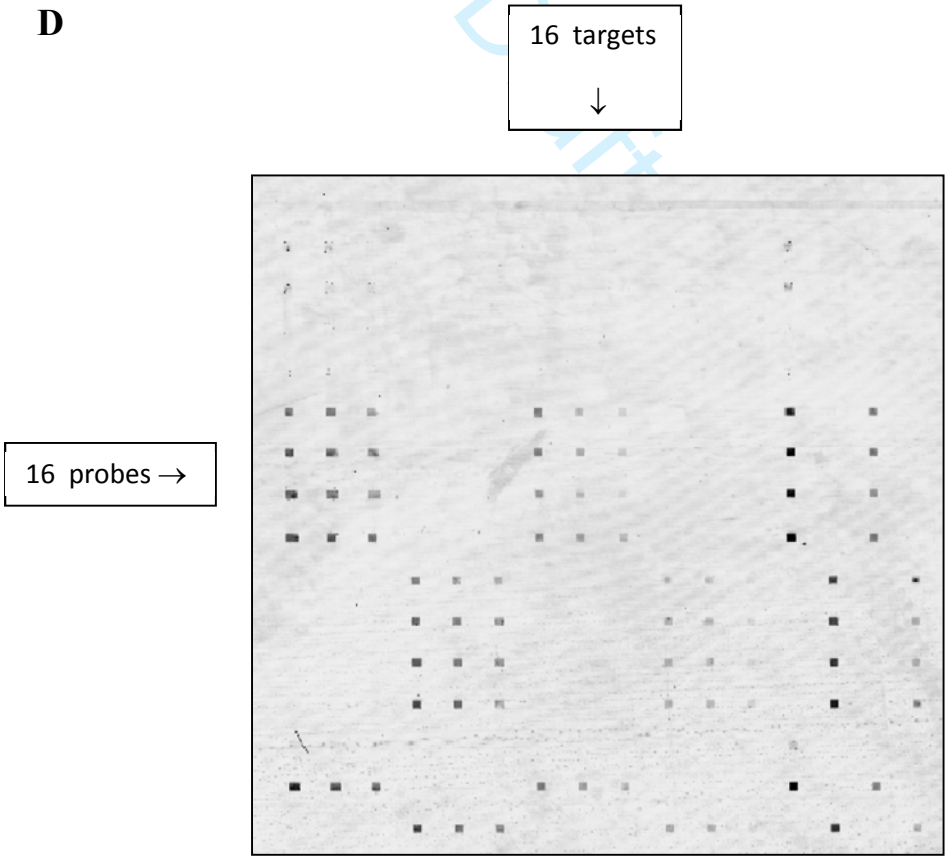


Fig. 2. (A) Image and (B) background-subtracted intensity of hybridization of T-RNA with the complementary DNA probe (P-DNA) and PNA probe (P-PNA) in different low-salt buffer (0.1 M NaCl) and high-salt spotting buffer (1 M NaCl). The probe solutions contain P-DNA (20 μM), 0.15 M sodium bicarbonate, and low/high salt content. The target solutions contain T-RNA (20 nM or 10 nM), 1X SSC (saline-sodium citrate) buffer, and 0.15% SDS.

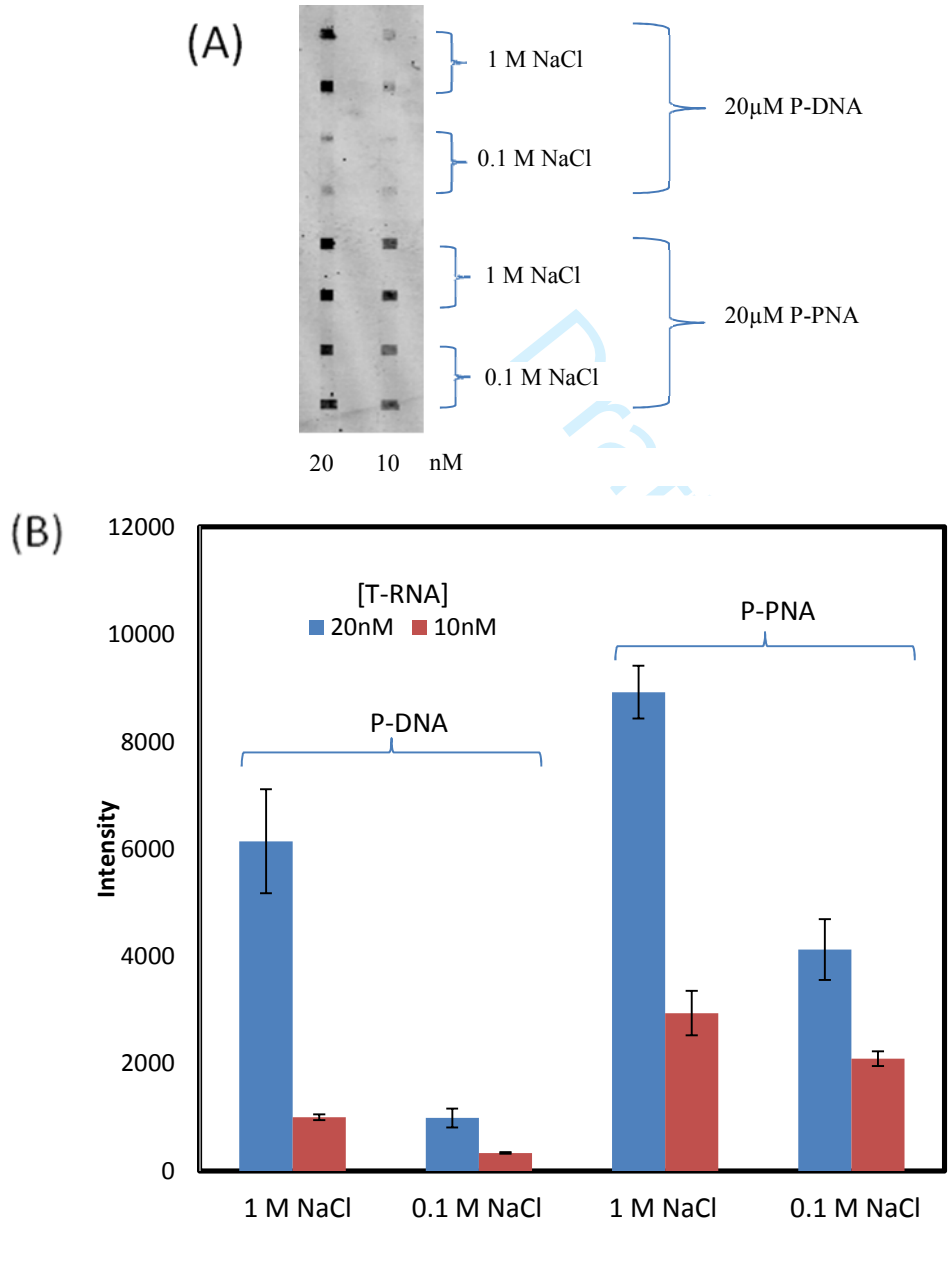
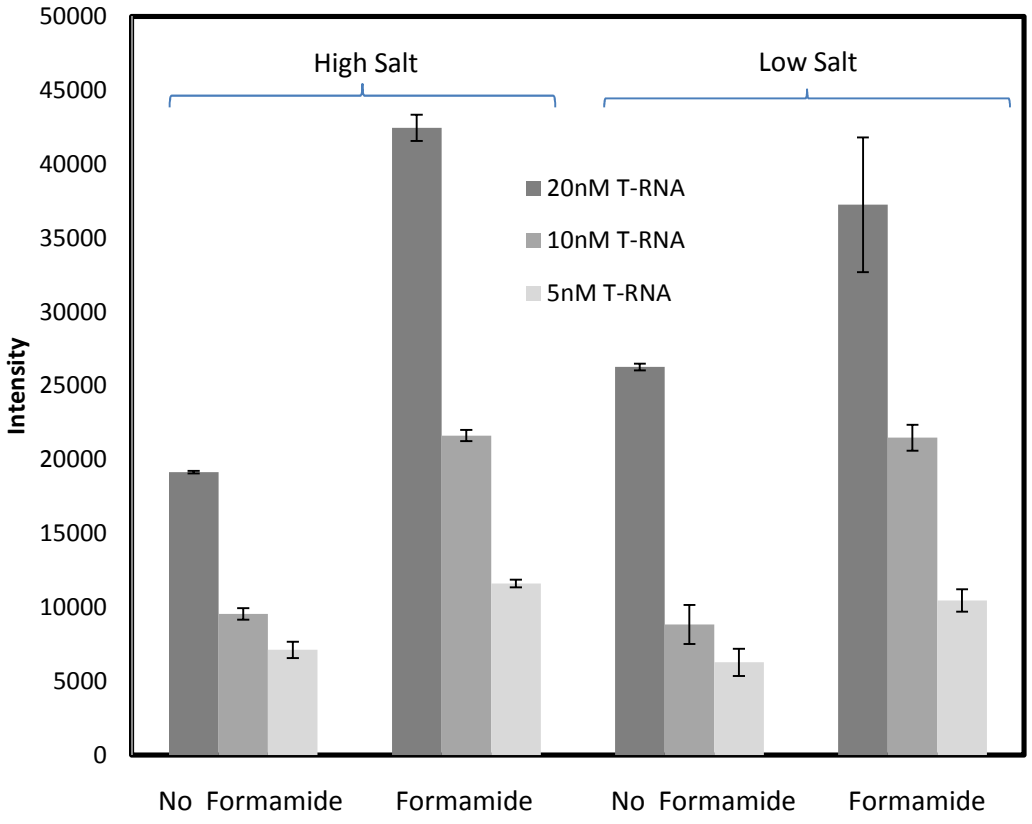
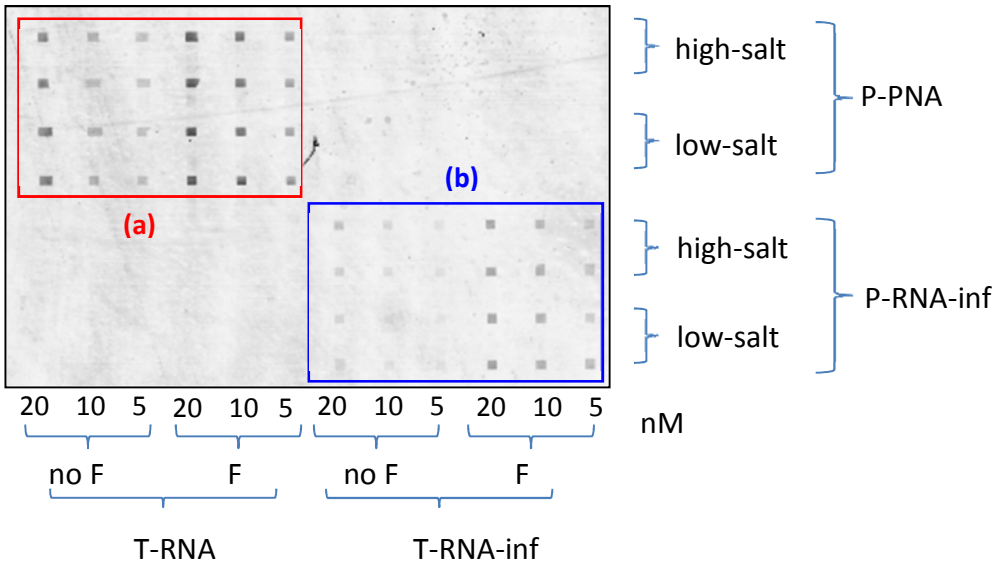


Fig. 3. (A) Background-subtracted intensity and (B) images of hybridization of (a) T-RNA with the complementary P-PNA probe and (b) T-RNA-inf with the complementary P-PNA-inf probe; both probes were prepared in different low-salt (0.1M NaCl) and high-salt (1 M NaCl) probe buffers containing 0.15 M $NaHCO_3$. The targets were prepared in the 1X SSC buffer with formamide (F) or without formamide (no F), and 0.15% SDS. The concentration of formamide is 30%. The concentrations of T-RNA and T-RNA-inf are 5, 10, and 20 nM.

(A)

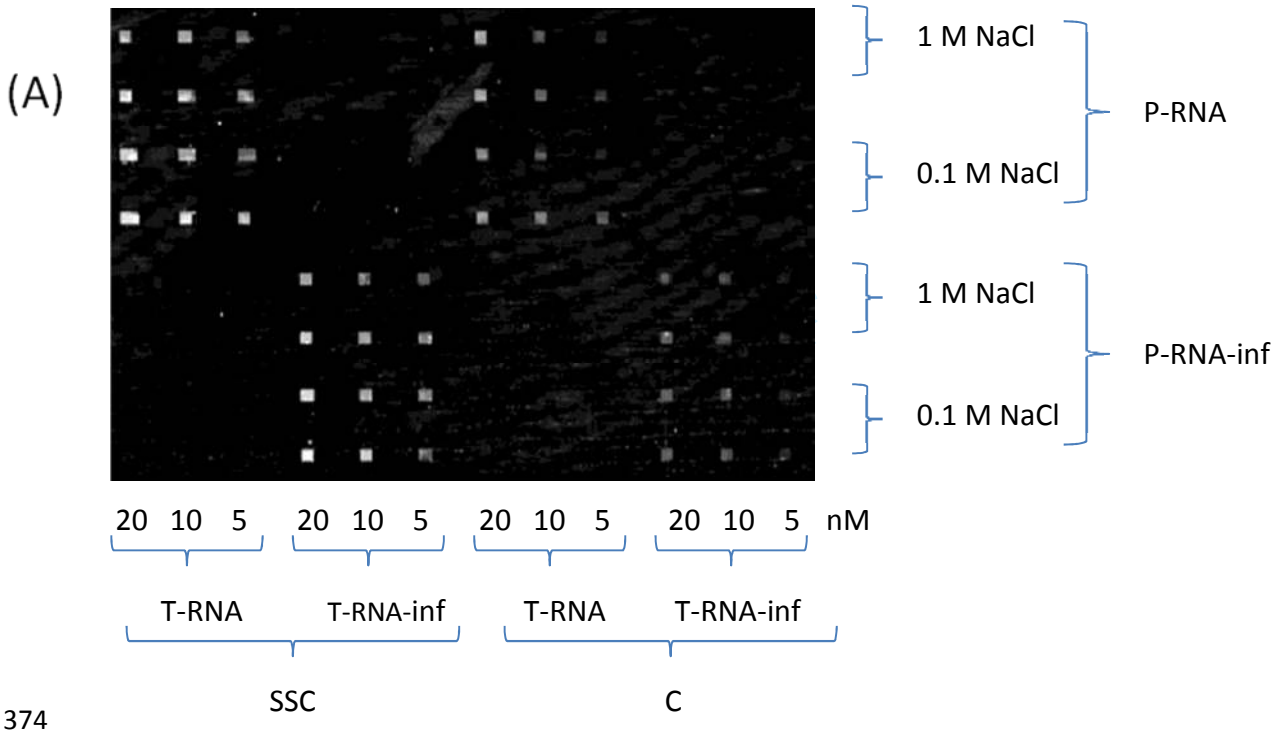


(B)

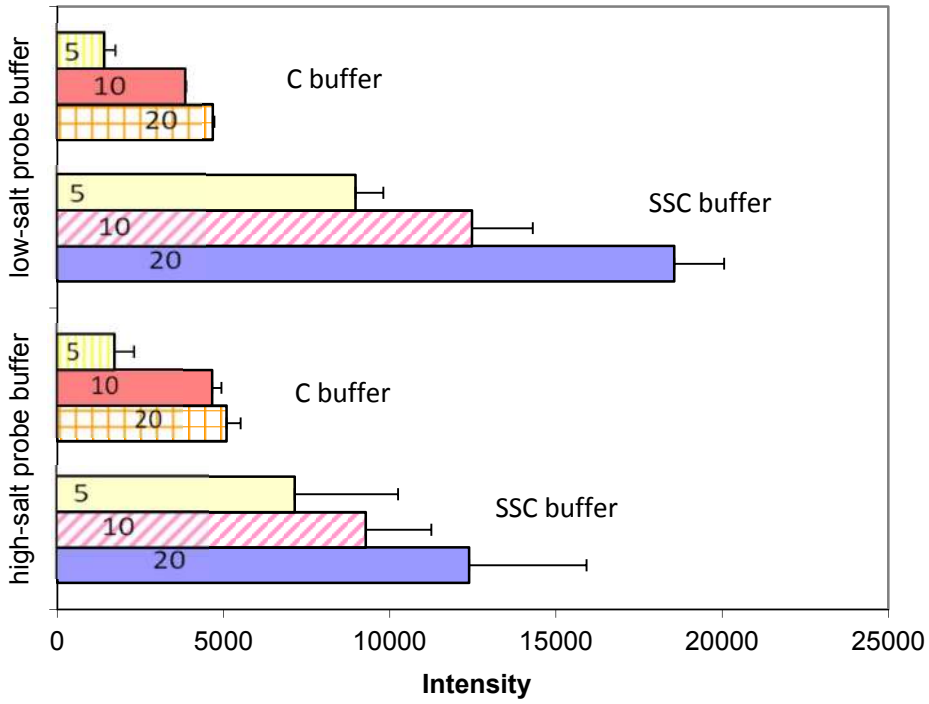

(a)
(b)
high-salt
low-salt
P-PNA
high-salt
low-salt
P-RNA-inf
20 10 5 20 10 5 20 10 5 20 10 5
nM
no F
F
no F
F
T-RNA
T-RNA-inf

365

Fig. 4. (A) Images of hybridizations of T-RNA with the complementary PNA probe (P-RNA), and of T-RNA-inf with the complementary PNA probe (P-RNA-inf). The images are shown with white patches and black background for clarity. The probes were prepared in low-salt (0.1 M NaCl) and high-salt (1 M NaCl) probe buffers containing 0.15 M $NaHCO_3$. The target solutions were prepared in 1X SSC solution or 15 mM C solutions, which all contained 30% formamide. (B) Background-subtracted intensity of hybridization of T-RNA-inf with the complementary P-PNA-inf probe when two probe buffers and two hybridization buffers are used. The numbers represent the T-RNA-inf concentration in nM.



(B)


low-salt probe buffer
high-salt probe buffer
C buffer
SSC buffer
C buffer
SSC buffer
5
10
20
5
10
20
5
10
20
5
10
20
0
5000
10000
15000
20000
25000
Intensity

376