Quantitative Analysis of MicroRNA in Blood Serum with Protein-Facilitated Affinity Capillary Electrophoresis

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Chapter 20

Quantitative Analysis of MicroRNA in Blood Serum with Protein-Facilitated Affinity Capillary Electrophoresis

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Abstract

MicroRNAs play an important role in gene regulation and disease etiology and are blood-based biomarkers of diseases. Here, we describe a protein-facilitated affinity capillary electrophoresis (ProFACE) method for ultra-sensitive direct miRNA detection as low as 300,000 molecules in 1 mL of blood serum, using single-stranded DNA binding protein (SSB) and double-stranded RNA binding protein (p19) as separation enhancers. This method utilizes either the selective binding of SSB to a fluorescent single-stranded DNA/RNA probe or the binding of p19 to miRNA–RNA probe duplex.

Key words MicroRNA, SSB, p19 protein, Blood serum, Capillary electrophoresis, Laser-induced fluorescence detection

1 Introduction

Many bioanalytical methods have been established to identify and quantify miRNAs include Northern blotting [1], in situ hybridization [2], small RNA library sequencing [3], bead arrays [4], microarray hybridization [5], and reverse transcription (RT)-PCR [6]. While miRNA microarrays allow for massive parallel and accurate relative measurement of all known miRNAs, they lack sensitivity and very expensive to facilitate. PCR-based methods have variation with increasing cycle number [7] and require amplification, making them only semi-quantitative. Here we demonstrated a sensitive and quantitative miRNA detection method by protein-facilitated affinity capillary electrophoresis. Capillary electrophoresis with laser-induced fluorescent detection (CE-LIF) [8] is a promising technique for miRNA detection, where miRNAs are easily separated from most of the other biomolecules because of their strong negative charge. Proteins that were used for this method are single-stranded DNA binding protein (SSB) and RNA binding protein p19 from Carnation Italian ringspot virus. SSB and p19 enhance separation between the excess of the fluorescently labeled

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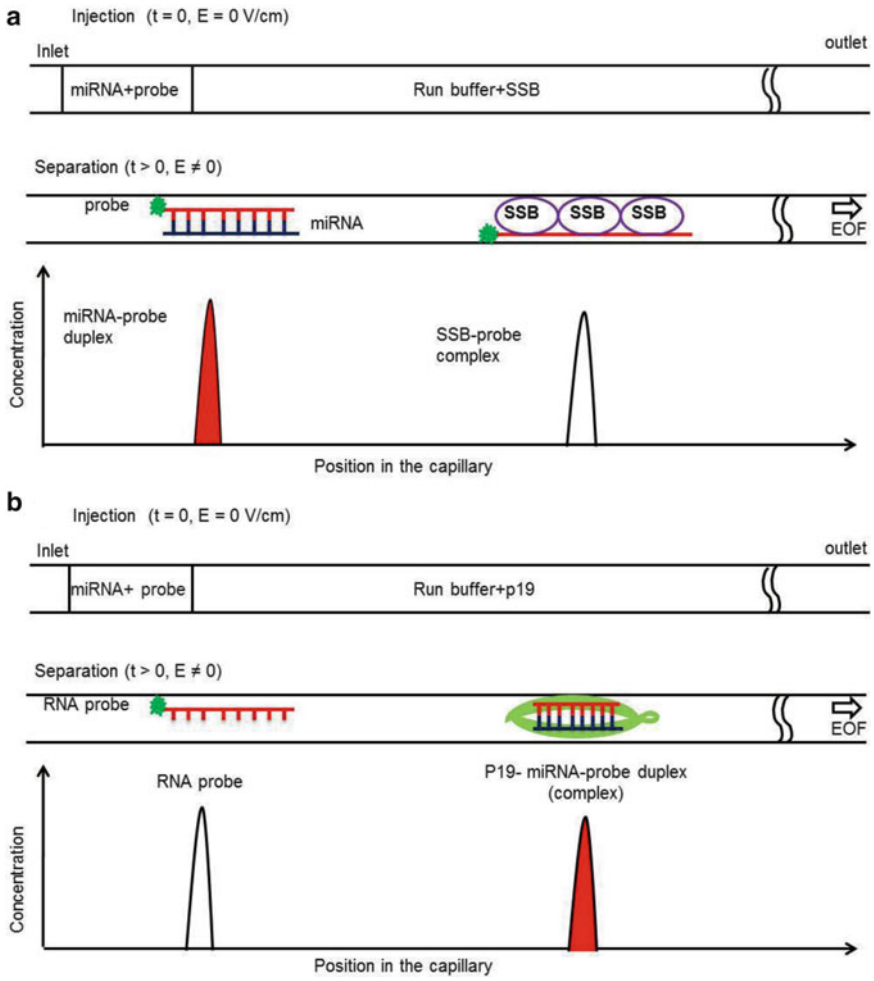

Fig. 1 Schematic diagram of the protein-facilitated affinity capillary electrophoresis (ProFACE) assay for miRNA detection. A mixture of a miRNA and fluorescently labeled probe is injected as a small plug into a capillary and subjected to electrophoresis. (**a**) When SSB is added in the run buffer, it binds only to the DNA or RNA probe and enhances the separation of the miRNA–probe duplex from the excess probe. (**b**) When p19 is present in the run buffer, it binds only to the miRNA–RNA probe duplex and shifts it far away from the free probe increasing resolution and sensitivity of the assay (reproduced and modified from ref. 12 with permission from ACS publications)

single-stranded DNA/RNA probe and the miRNA–probe duplex (Fig. 1). Earlier SSB has been used to distinguish single-stranded (ss) from double-stranded (ds) DNA using CE-LIF [9]. It binds to ssDNA (Kd < 50 nM) and ssRNA of eight bases or more in length but does not bind to dsDNA, dsRNA, or dsDNA–RNA hybrids [10]. The affinity of ssDNA to SSB is about ten times higher than that of ssRNA [11]. Due to the binding of the more neutral and bulky SSB, the probe migrates at a faster rate that significantly increases the separation time between the excess probe and

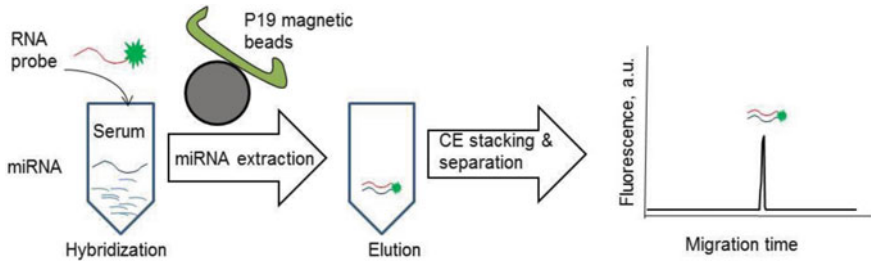

Fig. 2 Pre-concentration of miRNA from serum sample by p19 magnetic beads followed by detection of miRNA by ProFACE-LIF (reproduced and modified from ref. 12 with permission from American Chemical Society)

miRNA–probe duplex (Fig. 1a). On the other hand protein p19 binds only to double-stranded RNA in a size-specific manner. It does not bind to ssRNA, rRNA, mRNA, ssDNA, or dsDNA. p19 binds tightly to 21–23 nt long dsRNA. Binding is enhanced with the 5′ phosphate groups of miRNA due to the interaction between the tryptophan residues on the end capping helices of the p19 dimer which stabilizes the structure. Unlike SSB, p19 binds to the miRNA–probe duplex directly separating it from the excess probe in CE (Fig. 1b). Using these unique binding properties of p19 to miRNA, we developed a method that allowed us to detect ultra-low amount of miRNA from serum sample with p19-coated magnetic beads followed by CE-LIF detection [12] (Fig. 2). Without PCR amplification, as low as 300,000 molecules of miRNA in 1 mL of serum can be measured in a 20 min ProFACE run. The sensitivity of the method is comparable with existing PCR techniques. It can be parallelized to quantitatively detect multiple miRNA-based biomarkers in different biological samples.

Briefly, prior to CE separation miRNAs are incubated with excess of either a fluorescently labeled ssDNA probe or ssRNA probe. Depending on the probe, the annealing temperature is increased to 60 or 55 °C, and then slowly decreased to 20 °C to allow the proper hybridization of the probes with its complementary miRNA. A short plug of hybridized product is then hydrodynamically injected into a capillary and subjected to electrophoresis. Prior to injection, the capillary is prefilled with 25 mM borax buffer. In case of CE separation with SSB or p19, different amount of protein is added to the run buffer.

For serum experiments different concentration of miRNAs and probes are spiked in 1 mL of FBS and hybridized. Obtained hybridized product is then incubated with p19 magnetic beads in p19 binding buffer by shaking for 2 h at room temperature. After extensive washing with wash buffer miRNA duplex is eluted in small amount of elution buffer. Obtained miRNA duplex is then injected into a capillary with sample stacking and subjected to electrophoresis.

2 Materials

Prepare all solution using deionized nuclease-free water and filter all buffer solution through a 0.22 μm filter. Analytical grade reagents should be used for all experiments. Store prepared reagent as specified (*see* **Note 1**).

2.1 Capillary Electrophoresis (CE)

1. Capillary electrophoresis system with a laser source from Beckman Coulter (MDQ, PA800, PA800 plus).
2. Bare fused silica capillary (*see* **Note 2**).
3. Glass vials with caps.
4. Sample vials with strings and caps.

2.2 Other Related Equipments

1. PCR Thermocycler.
2. Magnetic separation rack.
3. Benchtop incubator.
4. Laboratory roller mixer.
5. 0.2 mL PCR tubes and 1.5 mL tubes.

2.3 CE Solutions

1. CE running buffer stock solution: 200 mM Sodium tetraborate (borax), pH 9.2. Add about 250 mL of water to a 1 L glass beaker (*see* **Note 3**). Weigh 76.274 g of sodium tetraborate and transfer to the beaker. Add water to bring the volume to 900 mL. Mix and bring final volume to 1 L by adding water.
2. CE running buffer working solution: 25 mM sodium tetraborate (borax), pH 9.2. Add 25 mL of 200 mM borax (from previous step) to 175 mL of water to obtain 200 mL of working solution.
3. CE wash solution: 100 mM NaOH. Weigh 4 g NaOH pallets and add slowly to 500 mL of water, stirring continuously. When pallets dissolve completely bring volume to 1 L by adding water.
4. CE wash solution: 100 mM HCl. Measure 8.33 mL of concentrated HCl (12 M) with graduated pipette. Put HCl into 1 L volumetric flask then add water until it reaches 1 L.

2.4 Storage and Incubation Solutions

1. Stock solution of 200 mM Tris-Acetate, pH 8.2: Add about 100 mL of water to a 500 mL glass beaker. Weigh 12.114 g of Tris base and transfer to the beaker (*see* **Note 4**). Add water to bring the volume to 400 mL. Mix and adjust pH with glacial acetic acid (*see* **Note 5**). Bring final volume to 500 mL by adding water.
2. Stock solution of 200 mM NaCl: Add about 250 mL of water to 1 L glass beaker. Weigh 11.68 g of NaCl and transfer to the beaker. Mix and add water to bring the final volume to 1 L.
3. Stock solution of 50 mM EDTA: Add about 25 mL of water to 100 mL glass beaker. Weigh 0.93 g of disodium salt of

EDTA and transfer to the beaker (*see* **Note 6**). Mix and add water to bring the final volume to 50 mL. Adjust pH to 8 with 10 M NaOH.

4. Incubation buffer: 50 mM Tris-acetate, pH 7.9, 50 mM NaCl, 1 mM EDTA. Add 12.5 mL of 200 mM Tris-acetate, 5 mL of 200 mM NaCl and 1 mL of 50 mM EDTA to 31.5 mL of water to have the final volume of 50 mL.
5. Storage buffer: 10 mM Tris, 0.1 mM EDTA. Add 2.5 mL of 200 mM Tris (*see* **Note 7**) and 0.1 mL of 50 mM EDTA to 47.4 mL of water to have the final volume of 50 mL.
6. 25× Wash buffer: 500 mM Tris–HCl, 2,500 mM NaCl, 25 mM EDTA.
7. 1× BSA-wash buffer: dilute the 25× wash buffer and 100× BSA stocks (both comes with the kit) with sterile water to a final concentration of 1× wash buffer and 1× BSA.

2.5 MicroRNA (miRNA) and Hybridization Probes

1. Resuspend synthetic miRNA (*see* **Note 8**) in storage buffer to obtain final concentration of 100 μM and store it at –20 °C (*see* **Note 9**).
2. Resuspend synthetic DNA and RNA probes complementary to miRNA (*see* **Note 10**) as described above (*see* **Note 9**).

2.6 Proteins for Affinity CE Separation

1. SSB—Single-stranded DNA binding protein (Sigma): Prepare the stock solution of SSB by resuspending in incubation buffer. Prepare 100 nM SSB from stock solution by diluting with incubation buffer.
2. p19 siRNA binding protein (BioLabs, New England, cat. # M0310L).

2.7 Blood Serum Components

1. p19 miRNA detection kit (BioLabs, New England, cat. # E3312S) (*see* **Note 11**).
2. 100 % fetal bovine serum.
3. tRNA from yeast.
4. 1× p19 elution buffer: 20 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA, 0.5 % SDS.

3 Methods

Keep all miRNAs, probes, and proteins on ice during the procedures.

3.1 Hybridization of miRNA with DNA and RNA Probes

1. Prepare three 1 mL samples of 1 μM stock solution by adding 10 μL of 100 μM of each of the following: miRNA and probes (DNA and RNA) to 990 μL of incubation buffer to obtain three samples of 1 mL stock solutions.

2. Prepare three 1 mL samples of 200 nM working solution by adding 200 μL of 1 μM of each of the following: miRNA and probes (DNA and RNA) to 800 μL of incubation buffer to obtain three samples of 1 mL working solutions.
3. Mix together 25 μL of 200 nM miRNA and 50 μL of 200 nM DNA probe. Add 25 μL of incubation buffer to final volume of 100 μL. Hybridize the mixture in PCR thermocycler at 60 °C (*see* **Note 12**).
4. Mix together 25 μL of 200 nM miRNA and 50 μL of 200 nM RNA probe. Add 25 μL of incubation buffer to final volume of 100 μL. Hybridize the mixture in PCR thermocycler at 60 °C (*see* **Note 13**).

3.2 CE Separation Procedure for DNA Probe Without SSB

1. Prepare the capillary and the cartridge following the manufacturer instruction (*see* **Note 14**).
2. Take six glass CE vials and add the following into each vial separately: 1.5 mL of HCl, 1.5 mL of NaOH, 1.5 mL of H_2O and add 1.5 mL of 25 mM borax buffer (to three separate vials each). Arrange them on inlet buffer tray in the same order as they are prepared (*see* **Note 15**).
3. Take two more glass vials and fill one of them with 25 mM borax buffer. Add 0.3 mL of water to the other vial where the waste will be collected. Arrange them on outlet buffer tray (*see* **Note 16**).
4. Prepare a sample vial with spring on. Add 20 μL of hybridized miRNA–DNA sample (from Subheading 3.1, **step 3**) into a PCR tube. Place the tube into the sample vial. Place the sample vial on sample tray (*see* **Note 17**).

3.3 Creating a Method for CE Separation in Karat Software and Running the Sample

1. From the instrument setup window, select Initial Condition tab. Select "current" from auxiliary data channels. Insert 15 °C for cartridge and 4 °C for sample storage temperature. Analog output scaling factor keeps as 1.
2. From the instrument setup window, select the LIF Detector initial Conditions tab. Under electropherogram channel 1 select "acquisition enabled" and insert 1,000 RFU for dynamic range. Select normal for filter settings with peak width 16–25. Select direct for signal. Excitation and emission wavelengths are 488 nm and 520 nm, respectively. Keep data rate at 4 Hz for both channels.
3. From the instrument setup window, select the Time Program tab. Click in the "Event" column to display the down arrow. Click on the down arrow to display the popup menu. Scroll down and select Rinse… command. Set the parameters by selecting "pressure" for pressure type, with value of 20.0 psi for 2 min. Select the tray position from inlet and outlet box as

Table 1
Instrument setup for CE separation

	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary
1		Rinse—pressure	20.0 psi	2.00 min	BI:A1	BO:A2	Forward
2		Rinse—pressure	20.0 psi	2.00 min	BI:B1	BO:A2	Forward
3		Rinse—pressure	20.0 psi	2.00 min	BI:C1	BO:A2	Forward
4		Rinse—pressure	20.0 psi	2.00 min	BI:D1	BO:A2	Forward
5		Inject—pressure	0.3 psi	2 s	SI:A1	BO:A1	Override, forward
6		Wait		0.10 min	BI:E1	BO:A1	
7	0.00	Separate—voltage	12.5 kV	15 min	BI:F1	BO:A1	0.17 min ramp, normal polarity
8	1.00	Autozero					

shown in Table 1. Keep the pressure direction to "forward". Press ok (*see* **Note 18**).

4. Following Table 1, create three more rows of rinsing event.
5. Then from the popup menu of Event column select Inject… command to create the fifth row. Select "pressure" for injection type with the values of 0.3 psi for 2 s. Keep pressure direction to forward and select the tray position as in Table 1.
6. For sixth row of Event select Wait… command. For duration insert 0.10 min and select the tray position as in Table 1.
7. Now select Separate… command from popup menu. Select "voltage" as a separation type with the value of 20.0 kV for 15.00 min at 0.17 ramp time. Select "At time" with 0.00 min. Select the tray position as in Table 1, and keep the polarity "Normal".
8. For the last row of Event select Autozero… command from popup menu and select at time inserting 1.0 min (*see* **Note 19**).
9. Save the method. To make a single run, click the blue arrow (single run button) located on the toolbar. The single run acquisition window will be displayed. Enter a sample ID for the run. Enter a path name where the data acquired for this run will be stored. Enter a file name to be used to save the data and then click "start" to start the run (Fig. 3).

3.4 CE Separation Procedure for DNA Probe with SSB

1. Use the same capillary if it is still ok or prepare the capillary following Subheading 3.2 [1].
2. Mix together, 75 μL of 200 mM borax and 300 μL of 100 nM SSB. Add 225 μL of water to final volume of 600 μL (*see* **Note 20**).

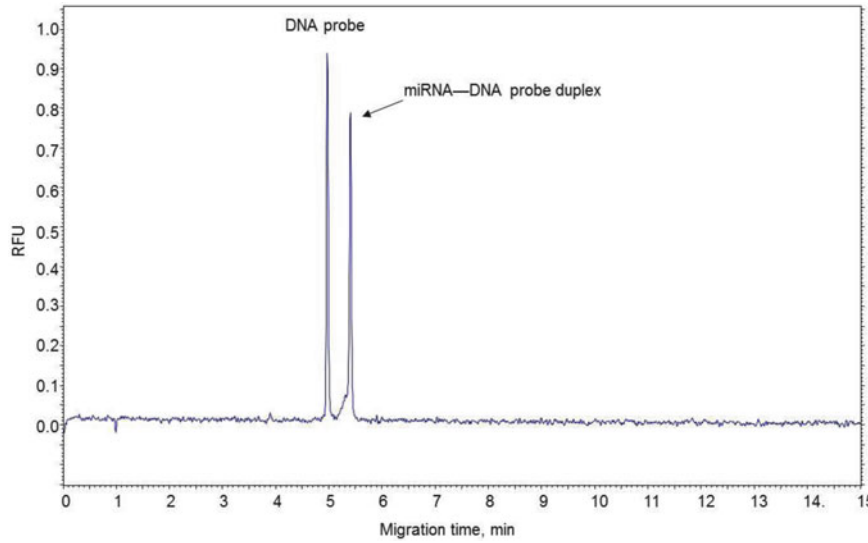

Fig. 3 Separation of miRNA–DNA probe duplex from the excess DNA probe without SSB. The sample contains 40 nM miRNA and 90 nM DNA probe

3. Take three glass CE vials and add the following into each vial separately: 1.5 mL of HCl, 1.5 mL of NaOH, and 1.5 mL of H_2O. Prepare three plastic vials with strings in. Add 200 μL of borax–SSB mixture (from **step 2**) to two PCR tubes. Place them into two of the prepared plastic vials (rinsing buffer and running buffer, respectively). Add 200 μL of 25 mM borax into another PCR tubes and place it into the third plastic vial (mocking buffer). Arrange them in inlet buffer tray as described earlier (*see* **Note 15**).
4. Take another plastic vial with string in. Add 200 μL of borax–SSB mixture (from **step 2**) to a PCR tube and place it into the prepared plastic vial. Add 0.3 mL of water another glass vial where the waste will be collected. Arrange them on outlet buffer tray (*see* **Note 16**).
5. Prepare a plastic sample vial with spring in. Add 20 μL of hybridized miRNA–DNA probe sample (from Subheading 3.1, **step 3**) into a PCR tube. Place the tube into the sample vial. Place the sample vial on sample tray (*see* **Note 17**).
6. Use the same method that was created previously (Subheading 3.3) to run the sample. Only chose a different file name to save the data and then click "start" to start the run (Fig. 4).

3.5 CE Separation Procedure for RNA Probe Without and with SSB

1. Follow the procedure as described in Subheadings 3.2, 3.3 and 3.4. Use miRNA–RNA probe hybridized sample (from Subheading 3.1, **step 4**) instead (Figs. 5 and 6, respectively).

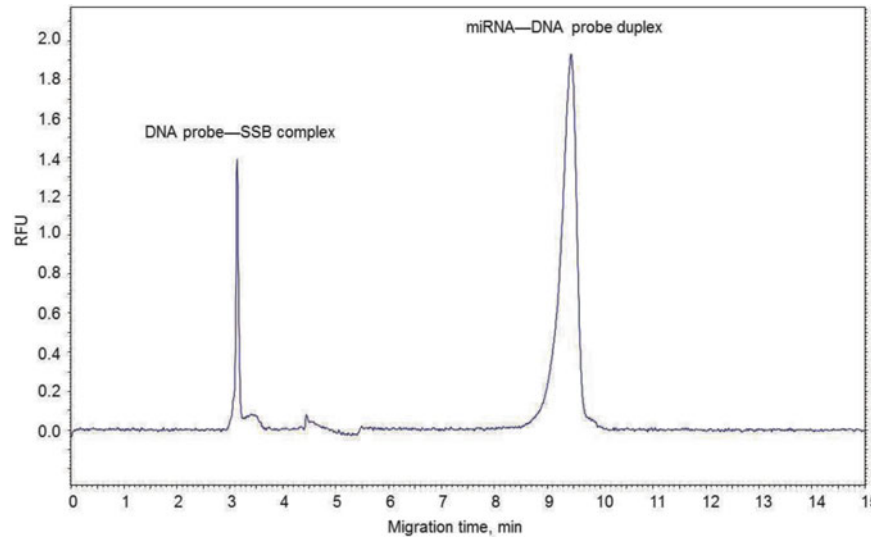

Fig. 4 Separation of miRNA–DNA probe duplex from the excess DNA probe with 50 nM SSB. The sample contains 40 nM miRNA and 90 nM DNA probe

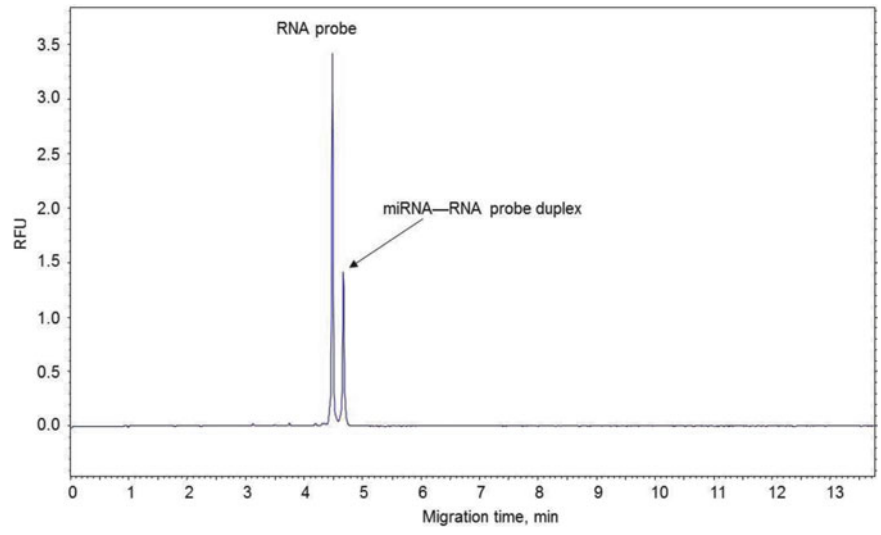

Fig. 5 Separation of miRNA–RNA probe duplex from the excess RNA probe without SSB. The sample contains 40 nM miRNA and 100 nM RNA probe

3.6 CE Separation Procedure for RNA Probe with p19

1. Mix together 75 μL of 200 mM borax and 5 μL of p19 protein. Add 520 μL of water to final volume of 600 μL (*see* **Note 21**).
2. Follow the procedure as described in Subheading 3.3 and 3.4 using miRNA–RNA probe hybridized sample instead (Fig. 7).

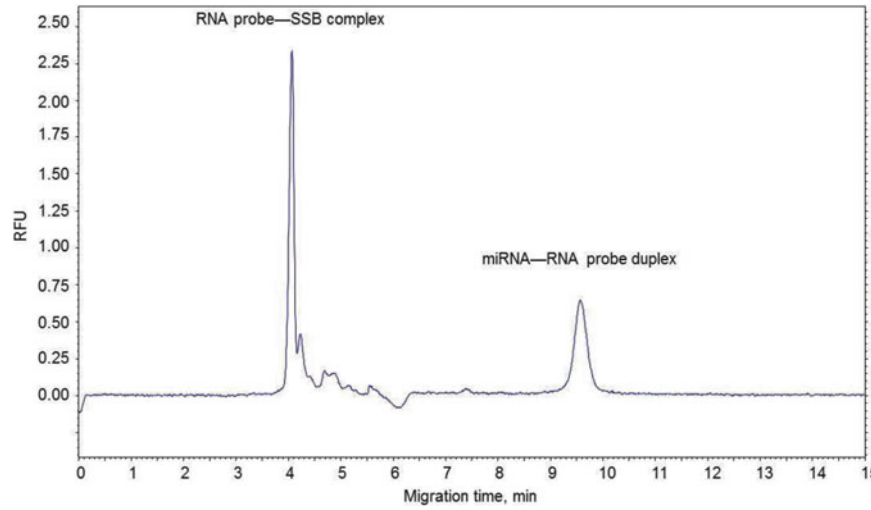

Fig. 6 Separation of miRNA–RNA probe duplex from the excess RNA probe with 50 nM SSB. The sample contains 40 nM miRNA and 100 nM RNA probe

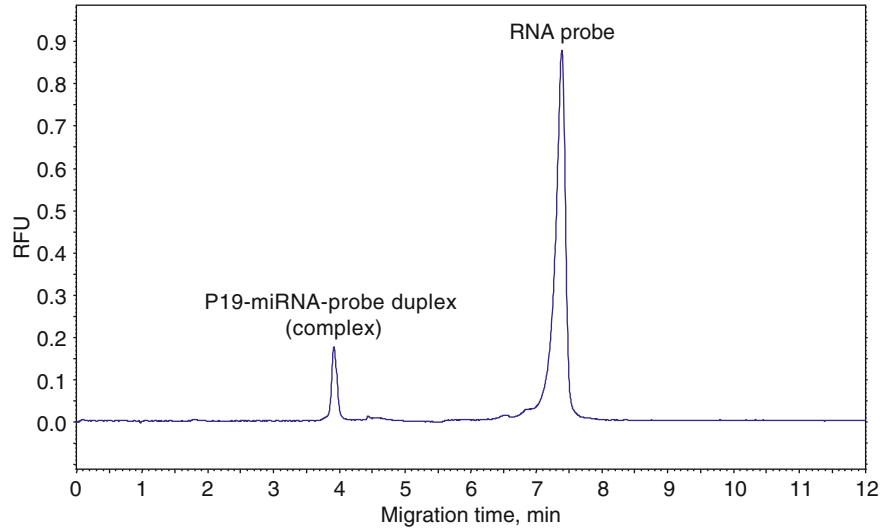

Fig. 7 Separation of miRNA–RNA probe duplex from the excess RNA probe with 250 nM p19 protein. The sample contains 40 nM miRNA and 100 nM RNA probe

3.7 MicroRNA Pre-concentration from Serum Using p19 Beads

1. For hybridization of miRNA with RNA probes in serum, mix together 1 mL of 100 % FBS, 0.55 μL of 200 nM miRNA (from Subheading 3.1, **step 2**), 5.5 μL of 200 nM miRNA probe (from Subheading 3.1, **step 2**), 1 μL of 100 μM tRNA (diluted from stock), 1 μL of murine RNase inhibitor (40 U, miRNA kit), and 91.95 μL of p19 binding buffer (*see* **Note 11**). Transfer the mixture into 11 PCR tubes, 100 μL each. Hybridize the mixture in PCR thermocycler at 75 °C (*see* **Note 22**). The final concentration would be 100 pM RNA and 1 nM RNA probe.

2. Control: Mix together 1 mL of 100 % FBS, 55 μL of 200 nM miRNA probe (from Subheading 3.1, **step 2**), 1 μL of 100 μM tRNA (diluted from stock), 1 μL of murine RNase inhibitor (40 U, miRNA kit), and 43 μL of p19 binding buffer (*see* **Note 11**). Transfer the mixture into 11 PCR tubes, 100 μL each. Hybridize the mixture in PCR thermocycler at 75 °C (*see* **Note 22**). The final concentration would be 10 nM RNA probe (*see* **Note 23**).
3. Add 20 μL of p19 beads into a 1.5 mL tube. Resuspend p19 beads with a brief vortex.
4. Place the tube with p19 beads in a magnetic separation rack. Remove the supernatant from beads.
5. Transfer all 1,100 μL of hybridized serum mixture of miRNA sample from PCR tubes to 1.5 mL tube with p19 magnetic beads. Incubate the mixture at room temperature by shaking on a benchtop roller mixer for 2 h.
6. Follow the same procedure as described above for control as well.
7. To remove the unbound RNA heat up the 1× BSA-wash buffer to 37 °C (*see* **Note 24**). Remove supernatant from dsRNA-p19 beads binding reaction using the Magnetic Separation Rack.
8. Wash the beads pellet in 500 μl of 1× BSA-wash buffer (*see* **Note 25**).
9. Repeat the wash seven times following the procedure as described above (*see* **Note 26**).
10. Follow the **steps 1–5** simultaneously for control as well.
11. Heat up 40 μl of 1× p19 elution buffer from p19 miRNA detection kit to 37 °C (*see* **Note 27**).
12. After the last wash, remove as much of the supernatant as possible without touching the beads pellet.
13. To elute isolated ds miRNA add 40 μl of pre-warmed 1× p19 elution buffer to the beads pellet and incubate for 10 min at room temperature with shaking followed by 10 more minutes incubation at 37 °C.
14. Carefully remove the supernatant containing miRNA-RNA probe hybrid from the tube using the magnetic rack and place into a clean PCR tube. Keep it frozen at −20 °C until ready for analysis.
15. Follow **steps 1–4** simultaneously for the control sample as well.

3.8 Detection of miRNA by Capillary Electrophoresis

1. Prepare the instrument and accessories following the procedures described as in Subheading 3.2. Follow through **step 1** (*see* **Note 28**) to **step 3**.
2. Add 20 μL of miRNA sample obtained from previous section to a PCR tube and place it into a plastic vial as described in Subheading 3.2, **step 4**.

Table 2
Instrument setup for CE separation with sample stacking

	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary
1		Rinse—pressure	20.0 psi	2.00 min	BI:A1	BO:A2	Forward
2		Rinse—pressure	20.0 psi	2.00 min	BI:B1	BO:A2	Forward
3		Rinse—pressure	20.0 psi	2.00 min	BI:C1	BO:A2	Forward
4		Rinse—pressure	20.0 psi	2.00 min	BI:D1	BO:A2	Forward
5		Inject—pressure	1.5 psi	10 s	SI:A1	BO:A1	Override, forward
6		Inject—pressure	0.3 psi	2 s	BI:C1	BO:A1	No override, forward
7		Inject—pressure	0.3 psi	2 s	BI:E1	BO:A1	No override, forward
8		Wait		0.10 min	BI:E1	BO:A1	
9	0.00	Separate—voltage	12.5 kV	15 min	BI:F1	BO:A1	0.17 min ramp, normal polarity
10	1.00	Autozero					

3. Prepare the control following the same procedure as **step 2** (*see* **Note 29**).
4. For creating the method follow the steps as described in Subheading 3.3. For Time program, follow Table 2 instead of Table 1. Save the method. To make a single run, click the blue arrow (single run button) located on the toolbar. The single run acquisition window will be displayed. Enter a sample ID for the run. Enter a path name where the data acquired for this run will be stored. Enter a file name to be used to save the data and then click "start" to start the run (Fig. 8).

4 Notes

1. All CE wash solutions should be stored at room temperature. Stock buffer solution should be kept at 4 °C.
2. Capillary length should be 30 cm or 50 cm (20 or 40 cm from an injection to a detection point, respectively) with an o.d. of 365 μm and an i.d. of 75 μm.
3. In order to dissolve sodium tetraborate faster put the stirrer into the beaker with water first. Then slowly add in the sodium tetraborate crystal, warm up the solution to about 30 °C. Once the crystals are in, turn on the heat at a low temperature about 45–50 °C. Leave it stirring for 5–6 h until dissolved. But be sure to bring the solution to room temperature before adjusting pH.

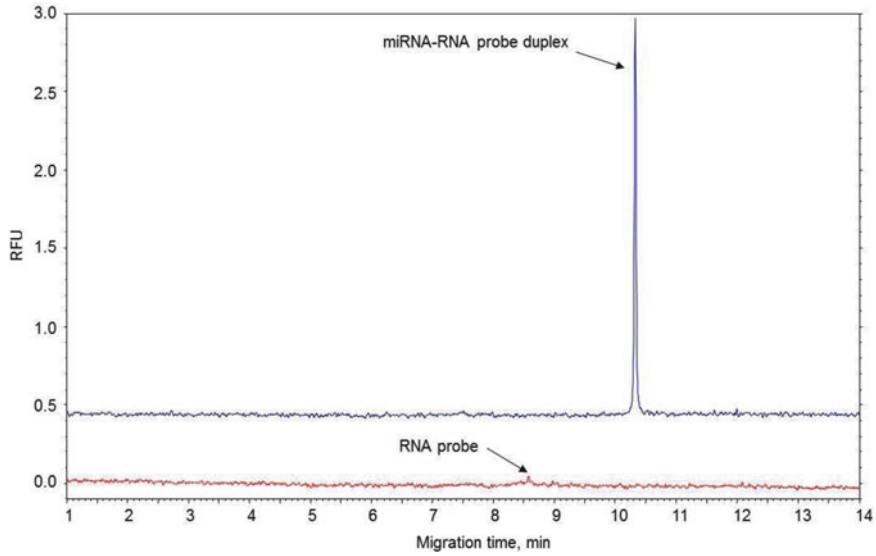

Fig. 8 Electropherogram of serum sample containing 50 fM miRNA with 1.25 nM RNA probe and 10 nM RNA probe alone as a control (*red*)

4. Follow the same procedure as above to dissolve Tris base.
5. Add acetic acid slowly to reach the desired pH, it might take about 2.5–3.0 mL.
6. Warming the water up to 40 °C along with stirring will make EDTA dissolve faster.
7. Prepare 200 mM Tris as in Subheading 2.4, **item 1** without adding acetic acid.
8. Synthetic miRNAs should be 21–23 nt long. All miRNAs and complementary probes should be ordered with 5′ phosphorylation modification.
9. For instance if you receive your oligo as lyophilized (freeze-dried) solids at a concentration of 25.2 nmol from manufacturer, resuspending it in 252 μL storage buffer will give a concentration of 100 μM. To prevent degradation of oligo by repeated freezing and thawing, oligo should be aliquoted into several tubes and freeze at −20 °C. The ones that are not required for immediate use freeze them at −80 °C for long-term use.
10. All DNA and RNA probes should be 3′ fluorescently labeled.
11. This kit includes: 1× p19 Beads, 1× p19 binding buffer, 1× p19 wash buffer, 1× p19 elution buffer, 10× BSA, RNase inhibitor. Store all the components at 4 °C.
12. Set up the following annealing program: Heat up at 60 °C for 2:00 min. Set the header temperature to 60 °C. Add 40 cycles

of decrement steps of 1 °C every 3 s and hold at 20 °C. Press enter when the cycle is done then wait until header temperature decreases to 20 °C before taking it out. Freeze at –20 °C until ready to use.

13. Set up the following annealing program: Heat up at 55 °C for 2:00 min. Set the header temperature to 55 °C. Add 35 cycles of decrement steps of 1 °C every 3 s and hold at 20 °C. Press enter when the cycle is done then wait until header temperature decreases to 20 °C before taking it out. Freeze at –20 °C until ready to use.
14. Capillary is 30 cm in total length, 20 cm from the injection to the detection point. Make detection "window" using window maker (MicroSolv Technology corp., USA). Follow the instruction provided. Try to make the "window" as small as possible to avoid the breakage, etc. Be careful not to bend the "window" as it is fragile. When installing the capillary into the cartridge, be very slow and gentle not to break. Once installed a cartridge with a new capillary into the machine, condition it by rinsing the capillary with 1 M NaOH solution for 15 min. Same capillary can be reused many times as long as it is not broken or clogged.
15. Place vials with 1.5 mL of HCl, 1.5 mL of NaOH, 1.5 mL of H_2O, and 1.5 mL of 25 mM borax buffer (three vials) into inlet tray from BI-A1 to BI-F1, respectively. First vial with borax buffer is a rinsing buffer (BI:D1), second vial with borax buffer is a mocking buffer (BI:E1), and the third one is a running buffer (BI:F1). Make sure caps are dry before placing them on.
16. Place the buffer vials on BO-A1 and waste vial in BO-A2. Make sure that caps are dry before placing them on.
17. Place the sample vial on SI-A1. Make sure that cap is dry before placing it on.
18. A row with inserted parameter will appear as shown in Table 1.
19. At the end, Time Program will look like Table 1.
20. In this experiment rinsing buffer and running buffer (inlet and outlet) are supplemented with 50 nM SSB.
21. In this experiment rinsing buffer and running buffer (inlet and outlet) are supplemented with p19 siRNA binding protein.
22. Set up the following annealing program: Heat up at 75 °C for 5 min and then decreased the temperature to 45 °C in decrement step of 1 °C every 3 s. Then hold at 45 °C for 7 h to allow the hybridization before decreasing the temperature to 20 °C.
23. In this experiment excess amount of single-stranded RNA probe is used as a control. Since single-stranded RNA does not bind to p19.

24. Keep the wash buffer in a benchtop incubator all the time to maintain the temperature at 37 °C during the wash process.
25. For each wash, before removing the supernatant shake the beads on a benchtop shaker for 5 min at room temperature.
26. To minimize any loss of the beads during the wash steps, allow the beads to settle to the bottom of the tube before using the magnetic rack. Beads are drawn to the side of the tube using the magnetic rack and the supernatant is carefully removed with a micropipette.
27. Put the elution buffer in the same benchtop incubator as previous step to warm up to 37 °C.
28. For this separation use the longer capillary (50 cm long) instead to achieve better resolution.
29. Run the control first then run the sample using the same CE method.

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