Chapter 8

Evaluation of Thermal Stability of RNA Nanoparticles by Temperature Gradient Gel Electrophoresis (TGGE) in Native Condition

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Abstract

Temperature gradient gel electrophoresis (TGGE) is a powerful tool used to analyze the thermal stabilities of nucleic acids. While TGGE is a decades-old technique, it has recently gained favor in the field of RNA nanotechnology, notably in assessing the thermal stabilities of RNA nanoparticles (NPs). With TGGE, an electrical current and a linear temperature gradient are applied simultaneously to NP-loaded polyacrylamide gel, separating the negatively charged NPs based on their thermal behavior (a more stable RNA complex will remain intact through higher temperature ranges). The linear temperature gradient can be set either perpendicular or parallel to the electrical current, as either will make the NPs undergo a transition from native to denatured conformations. Often, the melting transition is influenced by sequence variations, secondary/tertiary structures, concentrations, and external factors such as the presence of a denaturing agent (e.g., urea), the presence of monovalent or divalent metal ions, and the pH of the solvent. In this chapter, we describe the experimental setup and the analysis of the thermal stability of RNA NPs in native conditions using a modified version of a commercially available TGGE system.

Key words Temperature gradient gel electrophoresis, TGGE, Melting temperature, RNA nanoparticle, pRNA, 3-way junction, 3WJ

1 Introduction

Thermodynamic stabilities of RNA NPs are characterized by the ability of an individual sequence to specifically recognize its complementary strand using the nucleotides' ability to engage in canonical (Watson–Crick, i.e., A–U and C–G) and noncanonical interactions, including base-stacking interactions. In general, the stability of nucleic acid molecules formed by the pairing of two strands can be assessed using several biophysical techniques, including spectrophotometric methods (e.g., UV melting) [1]. However, the melting curves acquired by measuring UV absorbance with respect to temperature often have multiple transitions, making it

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impossible to determine the melting point (T_M) specifically for a complex nanoparticle composed of more than two strands. One of the advantages of TGGE is that bands corresponding to the native conformation of a RNA complex can be visualized clearly on a gel matrix. This allows for a direct determination of the T_M value, assuming that there are only two states of RNA folding involved in the melting: the native form (intact RNA complex band detectable on the gel) and the denatured conformation (indeed *any* denatured conformation). Another advantage of TGGE is that it permits the screening of a library of RNA and DNA molecules in order to select the most stable conformation, as has been demonstrated by P. Bevilacqua's group [2, 3]. This feature makes it suitable for SELEX protocols [4]. In addition, TGGE is often used in conjunction with PCR to determine differences in sequence between similar sized DNA or RNA strands [5–7]. This is useful for detecting mutations in DNA or RNA sequences, as a suspected mutant strand can be compared to its original using differences in melting temperatures. The protocol is accurate for up to 50% of the sequence variations of strands containing ~500 base pairs. For example, given a sequence containing 100 mutant variations, the technique could detect upward of 50 [8]. TGGE can also be used to detect structural changes in DNA or RNA due to the addition of intercalator compounds such as ethidium bromide, daunomycin, and doxorubicin [9]. These compounds intercalate between DNA base pairs due to stacking interactions, altering the overall DNA double helix. Like the detection of mutations, one can compare a sample altered with an intercalator to the original strand or polymer and determine whether the intercalator was integrated by analyzing the shift in T_M.

Various TGGE protocols have been documented and the majority of them feature the use of denaturing agents like urea to destabilize the hydrogen forces of attraction in long DNA and RNA molecules [10–12]. These agents, however, decrease the thermal stability of nucleic acids, thereby reducing the melting temperature and rendering direct analysis of the T_M problematic. The protocol described herein utilizes only native conditions, avoiding use of denaturing agents.

TGGE can be implemented in a fashion that is either perpendicular or parallel to the electrical current (Fig. 1). In a perpendicular analysis, multiple aliquots of a single purified RNA NP are placed into each well corresponding to known temperatures gradually increasing from left to right (as shown in Fig. 1a). After electrophoresis is complete, the band corresponding to the RNA complex sample eventually undergoes thermal denaturation at a certain temperature. This allows for the assessment of the apparent T_M value of the NPs. As such, there are various RNA NPs whose T_M values have been evaluated: pRNA 3WJ [13] and RNA triangle, [14] RNA squares and pentagons, [15] RNA tecto-squares [16]

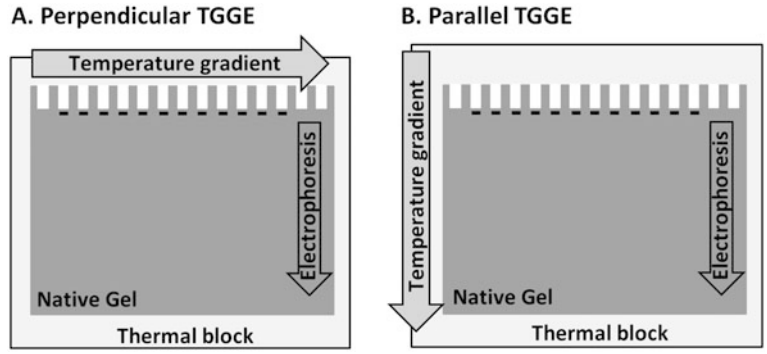

Fig. 1 Demonstration of (**a**) perpendicular and (**b**) parallel settings of temperature gradient gel electrophoresis

and nano-rings [17], RNA nano-cubes [18], and polyhedrons [19]. Moreover, perpendicular TGGE can be used to extract thermodynamic parameters of RNA nanoparticles. This has been demonstrated by P. Guo's group, where the T_M parameters of pRNA 3WJ NP have been analyzed as a function of concentration [20]. In contrast to perpendicular TGGE, parallel TGGE allows for the screening of multiple nanoparticles in high-throughput fashion. This is especially useful when comparing the thermal stabilities of a library of RNA nanoparticles at once. One example of such an experiment was the comparison the stabilities of 3WJ NPs composed of RNA, DNA, 2′-fluoro uridine and cytidine modified RNA strands (2′F-U/C RNA), respectively [13]. Multiple samples are run side-by-side through a temperature range that is gradually increased from top to bottom as the nucleic acid complexes continue to migrate from their wells (Fig. 1b). The heat-resistant RNA constructs migrate more slowly as the hydrogen and stacking interactions between participating strands form stable complexes. As a tendency, perpendicular analysis is more useful for examining the thermodynamic properties of a single NP, whereas parallel analysis is more useful for comparing the properties of several NPs.

The stability of RNA NPs is important for many aspects of application in nanomedicine, RNA synthetic biology, biomedical diagnostics, etc. This chapter focuses on the TGGE method to study the melting behavior of an RNA NP in native conditions using total RNA staining to visualize the bands. The method utilizes a commercially available TGGE system with modifications that are detailed in the methods section.

2 Materials

All solutions are prepared using double deionized water (dd H_2O) that has been passed through a filter (0.2 μm pore size). All reagents are of analytical grade, either purchased from a certified company or prepared in the laboratory. The reagents are typically stored at room temperature unless noted otherwise.

2.1 Native Polyacrylamide Gel Electrophoresis (PAGE).

1. Tris–borate–magnesium chloride (TBM, pH = 8.0) buffer; 1× buffer: 89 mM Tris base, 86 mM boric acid, and 10 mM $MgCl_2$.
2. Tris–magnesium saline (TMS) buffer, pH = 8.0; 1× buffer: 50 mM Tris–HCl, 100 mM NaCl, 10 mM $MgCl_2$.
3. Acrylamide–bis-acrylamide, 40% solution (mix ratio 29:1). Store at 4 °C. This reagent is available from several vendors, e.g., Thermo Fisher Scientific, Sigma-Aldrich. Alternatively, weigh 38.7 g of acrylamide and 1.3 g bis-acrylamide and transfer to a 150-mL graduated beaker containing about 50 mL of water. Mix for about 30 min using on a magnet stirrer and add dd H_2O up to 100 mL mark on the beaker. It is suggested to filter the resulting mixture through a 0.2 μm. The solution can be stored at 4 °C for at least 1 month if properly wrapped with aluminum foil (*see* **Note 1**).
4. Native gel loading buffer (6× concentrated): weigh 4 g sucrose, 25 mg bromophenol blue, and 25 mg of xylene cyanol and transfer to a graduate 15-mL falcon test tube. Bring the total volume up to 10 mL with dd H_2O.
5. Ammonium persulfate (APS): 10% solution in water (*see* **Note 2**).
6. TEMED: *N*, *N*, *N′*, *N′*-tetramethyl-ethylenediamine. Store at 4 °C (*see* **Note 3**).

2.2 Gel Electrophoresis System

In principle, any commercially available or custom-made TGGE can be used to unravel thermostability of RNA nanoparticles. As an example, a TGGE apparatus from Biometra GmbH with added modification was utilized in the current protocol. The gel casting using the original glass plates was inconvenient in our trials; therefore, we searched for alternative casting plates and electrophoresis systems that can compensate for the commercial recommended counterparts. Below is the suggested list of materials that could fit into the 9 × 9 cm area temperature block of the TGGE System.

1. Electrophoresis unit that fits into the temperature block of the utilized TGGE system. In this work, a SE250 Mighty Small II Mini Vertical Electrophoresis Unit has been used (**Hoefer, Inc.) has been used, including the following:**

(a) Lower **b**uffer chamber.
(b) Upper **b**uffer chamber/cooling core.
(c) Safety lid with high voltage leads.
(d) Casting cradle w/sealing gasket set.
(e) Casting clamp assemblies.
(f) Cams—4 pcs.
(g) Glass plates, rectangular, 10 × 8 cm.
(h) Alumina plates, notched, 10 × 8 cm.
(i) Spring clamps.
(j) Combs, 15 well (0.75 mm).
(k) T-spacers (0.75 mm).
(l) Gel seal.

2.3 Temperature Gradient Gel Electrophoresis

The protocol is based on the use of a 115 V TGGE System (e.g., Biometra GmbH) complete with:

(a) TGGE controller with integrated power supply, and control function of temperature and electrophoresis conditions.
(b) TGGE-electrophoresis unit with two removable buffer chambers, Peltier-element powered gradient block, and control cable.
(c) Buffer wicks.
(d) Cover films.
(e) Plastic clamps.

2.4 RNA Nanoparticles.

1. Gel-purified RNA nanoparticles. The concentration can be diluted to 50 ng/μL using 1× TMS buffer (or a buffer of choice). Alternatively, if the yield of RNA self-assembly is greater than 90%, the preassembled NPs can be prepared without purification.

2.5 Analysis

1. A gel imaging system.
2. Gel quantification software (e.g., Image J, Image Quant or equivalent).
3. Data analysis and graphing software (e.g., Origin, Excel or equivalent).

3 Methods

The customized TGGE procedure implies two general steps: (1) insertion of the RNA NP into the gel matrix using a standard vertical electrophoresis system and (2) transfer of the glass plate

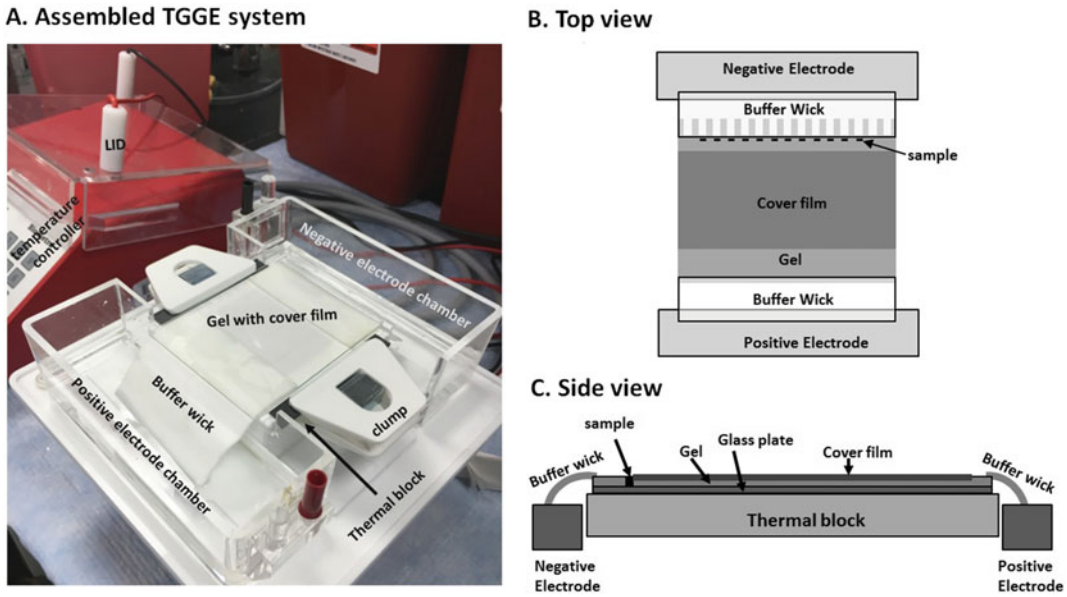

Fig. 2 A detailed view of customized TGGE system. (**a**) Photograph of complete assembly unit. Schematic top and side views with descriptions of components are shown in panels **b** and **c**, respectively

containing polyaclylamide (PAA) gel into the TGGE apparatus for evaluation of thermal stability. Figure 2 depicts the custom-assembled TGGE. The method primarily describes perpendicular TGGE but this protocol also applies for the parallel version as well.

3.1 RNA NP Insertion into 7% Polyacrylamide Gel Matrix at Native Conditions

1. Prepare and set up a vertical electrophoresis apparatus (see instructions for the electrophoresis unit, Subheading 2.2).
2. Prepare PAA mixture by mixing 0.875 mL of 40% PAA, 0.5 mL of 10 × TBM, and 3. 625 mL of dd H_2O. Add 60 μL of ammonium persulfate and 6 μL of TEMED.
3. Cast gel with a 8 cm × 10 cm × 0.75 cm gel cassette. Use 15-well combs.
4. Wait until the gel polymerizes for ~30 min, fill the inner and outer chambers with 1× TBM buffer, carefully remove 15-well comb and using a syringe wash formed wells (*see* **Note 4**).
5. Mix purified RNA nanoparticles with 6× native loading buffer. To increase the density of the RNA samples it is recommended to use 20 μL of RNA (50 ng/μL) and 10 μL of the 6× gel loading buffer.
6. Add 2 μL of the resulting mixture to the central 11 out of 15 wells and electrophorese at 70 V at room temperature until the sample has entered the gel (*see* **Note 5**).
7. After the electrophoresis is complete (~5–10 min depending on the NP size), disassemble glass plates with a spatula leaving

PAA gel on the small plate (white alumina plate, notched, available from GE Lifesciences http://www.gelifesciences.com/) together with T spacers (*see* **Note 6**).

3.2 Application of the PAA Gel with Prerun RNA NP to the TGGE Thermal Block

At this point the RNA NPs have entered the gel matrix and it can be safely transferred to the TGGE system. Prior to transferring, the back side of the aluminum plate should be thoroughly cleaned. Overall, the protocol is similar with respect to the description in the TGGE manual from Biometra (please refer to technical report. http://www.biometra.de/files/themes/biometra_standard/pdfs/Manual_TGGE_Mini_Oct_2009.pdf). However, since the original plates are not in use, it is important to match the gels' wells with the temperature line # 1 (L1, closest to temperature T1) to line #6 (L6, closest to temperature T2) (as shown in Fig. 3) on the temperature block to obtain accurate temperature reading.

1. Fill buffer chambers with ~250 mL of 1× TBM buffer and place buffer wicks inside the chambers. Let buffer wicks soak for ~10 min.

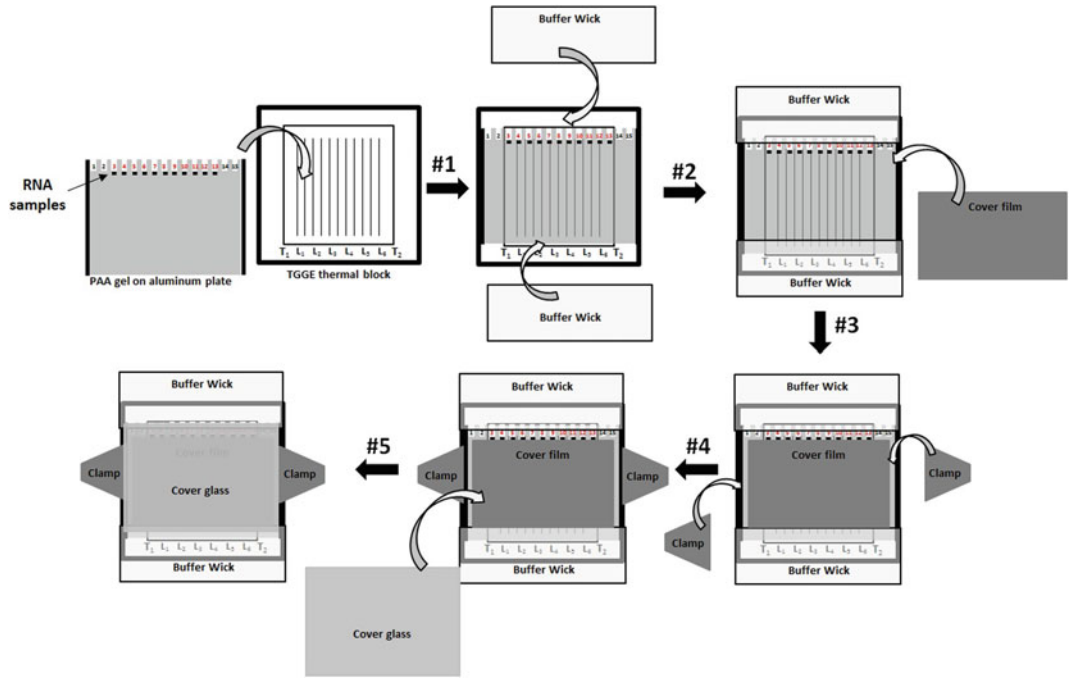

Fig. 3 A step-by-step diagram of TGGE assembly procedure. *Step 1*: transfer gel containing RNA nanoparticles into TGGE thermal block so wells 3–13 will be positioned between lower and higher temperatures T1 and T2, respectively. *Step 2*: attach presoaked buffer wicks to allow current conduction between two electrodes. *Step 3*: gently cover the gel with flexible cover film and avoid formation of any empty space or bubbles between gel and cover film. This is a critical point as bobbles will cause gel drying effect ruining experiment. *Step 4*: fix the position of the glass plate with clamps placed on the gel spacers and thermal block and finally place a cover glass on top of the overall assembly as shown in **step 5**

2. Gently wipe the surface of the thermal block with clean water. Place the glass plate containing gel on the thermal block (refer to schematic diagram of Fig. 3) (*see* **Note 7**).
3. Appropriate adjustments are required to ensure temperature ranges match the gel wells and lines L1 and L6 in the thermal block. Figure 3 **step 1** demonstrates the appropriate alignment of the gel's wells and the lines on thermal block. From the 15 wells only 11 wells undergo precise temperature changes (wells numbered 3–13, highlighted in red in Fig. 3). Wells 1 and 2 can be additionally used for a molecular weight ladder or monomer controls as needed. Noteworthy, wells 14 and 15 can be omitted since the maximum temperature is reached at T2 (corresponding to well 13).
4. Carefully attach the presoaked buffer wicks to the top and the bottom of the gel and cover the gel with a cover film available from the manufacturer (Fig. 3, **steps 2** and **3**) (*see* **Note 8**).
5. Fix the gel position on the thermal block with clamps provided by manufacturer as shown in Fig. 3 **step 4** (*see* **Note 9**).
6. Place the cover glass on the resulting assembly in such a way that it attaches to the top of the buffer wicks (*see* **Note 10**).
7. Close the safety lid which will connect the two electrodes. The TGGE system is now ready to run.
8. Program the TGGE temperature controller to the desired temperature range according to manufacturer's manual.
9. Electrophorese at constant 100 V for about 1 h (*see* **Note 11**).
10. When run is complete, carefully disassemble the whole system and place the gel into solution containing ethidium bromide for 20 min.
11. Rinse the gel twice with sufficient amount of dd H_2O to remove excess of Eth.Br. and destain the gel for another 10 min.
12. Visualize RNA bands on the gel using a gel imaging system and paper-print the actual size of the gel.

3.3 Calculate the Temperature Within Each Well and Determine Melting Temperature

1. Align the printed gel with the thermal block as it was before the experiment. Lane 3 and 13 should correspond to the starting and final temperatures T1 and T2 respectively. Since there was a linear gradient from L1 to L6, the analysis of the temperature on each well will be similar to the corresponding lines on the thermal block.
2. Calculate the estimated temperatures corresponding to each well using the following example:

As an example, let the temperature range be set to a linear gradient starting from L1 = 30 °C to L6 = 60 °C, which

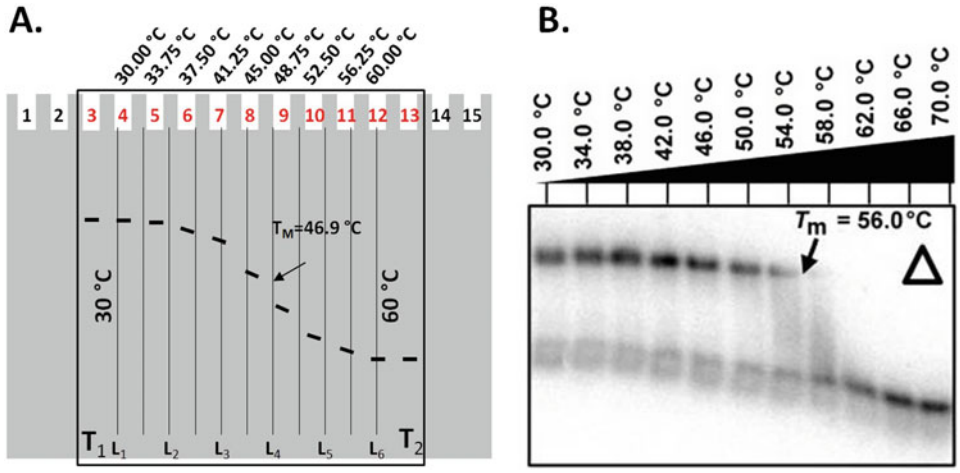

Fig. 4 Example of RNA complex melting point determination. (**a**) Calculation of temperature on each PAA well based on known the temperatures of L1–L6. (**b**) Melting temperature of P-32 labeled RNA triangle NP determined using TGGE system. (Image obtained from ref. [15] with permission from NAR, Oxford University Press)

corresponds to temperatures in the gel's wells #4 and #12, respectively (Fig. 4).

(a) The operating temperature range can be found by subtracting 60 °C (well #12)—30 °C (well #4) = 30 °C.

(b) To estimate the temperature increment corresponding to adjacent wells, divide the temperature range over the total numbers of wells excluding the first well (9–1 = 8), resulting in 30 °C /8 = 3.75 °C.

(c) Next, estimate the temperature corresponding to each well by adding the appropriate number of temperature increments. For this example, one obtains for well 4 a temperature of 30 °C (equal to T1), for well 5: 30 °C + 3.75 °C = 33.75 °C, for well 6: 33.75 °C + 3.75 °C, etc.

3. In general, the inflection point of the location of the gel bands as a function of temperature corresponds to the melting point of the analyzed nanoparticle (*see* **Note 12**). The T_M point can be determined accurately using a gel quantification software mentioned in the materials section. For these purposes, integrate the intensity of the RNA NP bands on each well and divide this value by the total intensity of the well. This will determine the relative fraction of formed nanoparticle in percent.

4. Plot the data (with temperature as x-axis and remaining fraction of NP as y-axis) using a graphing software, e.g., Origin. Fit the data points to a nonlinear fitting curve using sigmoid function to determine 50% nanoparticle formation, this corresponds to the melting temperature.

4 Notes

1. It is important to wear a mask when weighing acrylamide. Prepare aqueous solution of acrylamide powder in a fume hood only.
2. It is convenient to have 1.6 mL Eppendorf test tubes containing 1 g of APS. When needed, add 1 mL of dd H_2O and mix well to have fresh APS solution.
3. Storage at 4 °C reduces the characteristic TEMED smell.
4. It is advisable to insert the combs on its 1/5 part into the gel, this will decrease the depth of the resulting wells allowing better washing.
5. The final RNA amount should be at least 20 ng/well to detect RNA NP using total staining with ethidium bromide (Et.Br.).
6. To ensure that the PAA gel sticks to the small alumina plate but not to the large plate it is recommended to wipe the large glass plate with a siliconized reagents, e.g., Sigmacoat or a Gel Slick.
7. We found that the manufacturer's recommended thermal coupling solution can be omitted. The alumina glass plate is a very good heat conductor and it makes full contact with the surface of the thermal block.
8. Avoid dislocation of the gel at this step.
9. Small paper clips can be used as alternatives. Attach clippers on plastic T-spacers; do not clip on the aluminum plate.
10. The cover plate should gently lie on the wicks, do not squeeze them on the gel.
11. The timing depends of the size of RNA NP and the percentage of the native gel. Based on our studies, it takes 60 min to run an RNA triangle NP on 7% native TGGE [14] and 50 min for a pRNA 3WJ analyzed on 15% TGGE [13].
12. This is a coarse way of calculating TM for RNA NP, and generally useful when comparing thermal stabilities between nanoparticles where the exact melting point is not critical. The more precise way of calculating TM requires the use of a radiolabeled strand allowing the quantification of the remaining fraction of NP.

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