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Studying the affinity, kinetic stability, and specificity of RNA/protein interactions: SINE ncRNA/Pol II complexes as a model system

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Summary

The number of documented interactions between proteins and non-coding RNAs (ncRNA) of all types has grown rapidly in the past several years. A current challenge is to experimentally characterize these interactions to ultimately understand their biological roles at a mechanistic level, which will require a combination of multiple experimental techniques. One such category of techniques is biochemical assays that determine the affinity, kinetic stability, and specificity of ncRNA/protein complexes. Here we describe how to experimentally determine these important parameters using electrophoretic mobility shift assays (EMSAs). The interaction between mammalian SINE-encoded ncRNAs and human RNA polymerase II is presented as a model system; however, the experiments could be readily adapted to other ncRNA/protein complexes.

Keywords

protein-RNA interaction; RNA polymerase II; ncRNA; EMSA; affinity; kinetic stability; specificity

Introduction

Recently, an increasing number of reports have used deep sequencing or microarray technologies to identify ncRNAs associated with a variety of proteins (1–3). Moreover, the discovery that much of mammalian genomes are transcribed (4, 5) has fueled the search for novel ncRNA/protein complexes. It is now becoming important to study the parameters governing the interactions between ncRNAs and proteins in these newly found complexes, which will provide important insight into the mechanisms by which the complexes function. Biochemical experiments with purified components are arguably the best means to test whether a given ncRNA binds directly to its putative protein target, and if so, to determine the affinity, kinetic stability, and specificity of the interaction. Such biochemical experiments complement cell-based and molecular genetic techniques, which together, can unravel the mechanisms by which specific ncRNA/protein complexes function.

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Here we describe several biochemical assays we have used to study the interaction between ncRNAs encoded by mammalian SINEs (short interspersed elements) and human RNA polymerase II (Pol II). SINEs are repeat sequences that have amplified in mammalian genomes via retrotransposition. The predominant human SINE is Alu ($\sim 10^6$ copies), whereas in mouse cells the predominant SINEs are B1 and B2 ($\sim 5 \times 10^5$ and $\sim 3 \times 10^5$ copies, respectively) (6). These SINEs can be transcribed by RNA polymerase III to produce ncRNAs. Transcription of SINEs is upregulated in response to a variety of cellular stresses (7, 8). We have shown that in response to heat shock, human Alu RNA and mouse B2 RNA bind to Pol II and repress mRNA transcription (9, 10). Through their interaction with Pol II, Alu and B2 RNAs are brought to the promoters of mRNA genes where they block transcription by preventing Pol II from properly contacting promoter DNA (10–12). Interestingly, B1 RNA also binds tightly to Pol II, however, it does not repress transcription in vitro (10, 13).

Using the interactions between SINE ncRNAs and Pol II as examples, we describe the use of electrophoretic mobility shift assays (EMSAs) to characterize the affinity, specificity, and kinetic stability of ncRNA/protein complexes. These assays rely on monitoring the migration of an ncRNA in a native gel (either polyacrylamide or agarose) in the absence and presence of a protein thought to bind the ncRNA. The position at which an ncRNA or an ncRNA/protein complex migrates in a native gel is mainly dictated by its size and overall charge, but is also influenced by its shape. Because the ncRNA/protein complex is larger than the ncRNA alone, an ncRNA/protein complex will not migrate as far as the free ncRNA; hence the protein is said to “shift” the ncRNA in the gel. An important property of an EMSA is that the gel matrix helps maintain the ncRNA/protein complex while the gel is running, which is referred to as the “caging” effect. If a complex dissociates while the gel is running the gel matrix will keep the ncRNA and protein components from diffusing away from one another such that rebinding occurs. Therefore, the ratio of bound ncRNA to unbound ncRNA after the EMSA should reflect the ratio present in the reaction at the time the gel was loaded. To visualize the data in an EMSA the ncRNA is typically radioactively or fluorescently labeled. Nucleic acid stains, such as ethidium bromide, are less sensitive and often are not adequate to detect the small amounts of ncRNA typically used in EMSAs. Lastly, not all ncRNA/protein complexes behave well in EMSAs; if a complex is not detected by EMSA and a researcher feels strongly that a bona fide interaction exists, other techniques such as nuclease protection assays or filter binding assays should be tried.

Here we use SINE-encoded ncRNA/Pol II complexes as a model system, however, the experiments described below are readily adaptable to other ncRNA/protein complexes. We first describe an EMSA experiment that provides a measurement of the affinity of an interaction, as reflected by the equilibrium dissociation constant (K_D). We then describe how this basic assay can be modified to perform kinetic experiments and competition experiments that provide insight into the stability and specificity of an ncRNA/protein complex.

2. Materials

All solutions should be prepared using ultrapure water (18 M Ω) and stored as indicated. RNase contamination in any solution or piece of equipment will compromise the experiment; therefore precautions should be taken to keep reactions RNase free. For example, gloves should be worn at all times, reaction and buffer tubes should be kept closed when not in use, and RNase inhibitors can be added to reactions if necessary.

2.1. Solutions for performing binding reactions

Solutions 1–7 are used to make buffer A, buffer B, and folding buffer (described in points 8–10). The solutions should be aliquoted and stored at -20°C , and the buffers should be prepared prior to every experiment.

1. 2 M Tris, pH 7.9: Dissolve 4.84 g Tris base into 10 mL water. Add concentrated HCl until pH is approximately 7.9. Let the solution sit at room temperature for a few hours (since pH is sensitive to temperature for Tris). Check pH and add concentrated HCl until pH is 7.9. Add water to a final volume of 20 mL.
2. 100 mM MgCl₂: Dissolve 1.02 g MgCl₂•6H₂O in water to a final volume of 50 mL.
3. 2 M KCl: Dissolve 7.5 g KCl in water to a final volume of 50 mL.
4. Bovine Serum Albumin (BSA, 20 mg/mL, from Roche).
5. 1 M DTT (1,4 –dithiothreitol): Dissolve 15.4 g in water to a final volume of 50 mL.
6. 80% glycerol: Mix 10 mL 100% glycerol with 2.5 mL water (see Note 1).
7. 1 M HEPES, pH 7.9: Dissolve 4.76 g HEPES into 10 mL water. Check pH and add 4 M NaOH until the pH reaches 7.9. Add water to a final volume of 20 mL.
8. Buffer A: 20 mM HEPES (pH 7.9), 1 mM DTT, 8 mM MgCl₂. If necessary, 20 Units/ μL of the RNase inhibitor RNaseOUT (Life Technologies) can be added to buffer A.
9. Buffer B: 20% glycerol, 20 mM Tris (pH 7.9), 100 mM KCl, 1 mM DTT, 0.05 mg/mL BSA.
10. Folding buffer: 10 mM HEPES (pH 7.9), 1 mM DTT, 4 mM MgCl₂, 10% glycerol, 10 mM Tris (pH 7.9), 50 mM KCl.
11. Pol II: Purified from HeLa cells as previously described (14) and stored in small aliquots at -80°C . Make any necessary dilutions using buffer B.
12. Purified ncRNA: Dilute a portion of purified RNA to a concentration of 1 nM in folding buffer (see Note 2).
13. Competitor ncRNA: Performing the kinetic stability experiments (section 3.3) and the competition experiments to assess specificity (section 3.4) require specific ncRNA competitor molecules, as described in those sections.

2.2. Solutions and equipment for EMSAs

1. 30% acrylamide/Bis solution (37.5:1). Store at 4°C.
2. 1 M magnesium acetate: Dissolve 21.4 g (C₂H₃O₂)₂Mg•4H₂O in water to a final volume of 100 mL.
3. 5X TBE: 0.45 M Tris (pH 8.3), 0.44 M boric acid, 10 mM EDTA. Combine 54 g Tris base, 27.5 g boric acid, 20 mL 0.5 M EDTA (pH 8.0) and water to a final volume of 1 L. The pH should be 8.3. Store at room temperature.
4. 80% glycerol: Mix 80 mL 100% glycerol with 20 mL water (see Note 1). Store at room temperature.
5. Running buffer for native gel: 100 mL 5X TBE, 50 mL 100% glycerol, 5 mL 1 M magnesium acetate, 845 mL water.
6. Ammonium persulfate (APS): 10% solution in water. Store at 4°C.
7. N',N',N',N'-Tetramethylethylenediamine (TEMED). Store at 4°C.
8. 20% ficoll (see Note 3): Purchased as a 20% solution (Sigma-Aldrich)
9. Equipment for running a native gel: Notched glass plate set (20×22 cm, Owl), 1.5 mm thick × 22 cm spacer set (Owl), 1.5 mm thick 20 well comb for 20 cm wide units (Owl), and an Owl electrophoresis system.
10. Gel drying apparatus.
11. Whatman paper (Fisher).
12. Plastic wrap.
13. Phosphorimager. For example, a Typhoon 9400 Scanner (GE Healthcare).

3. Methods

It is important to have the native gel prepared before assembling the binding reactions because the reactions are loaded onto a running gel as soon as they are done incubating. Hence the instructions for preparing the native gel are described first, followed by instructions for assembling in vitro binding reactions that will provide a measurement of the affinity of an ncRNA/protein interaction. The ensuing sections describe variations of the experiment to allow the kinetic stability and specificity of the ncRNA/protein interaction to be assessed.

3.1. Prepare the native gel

1. Assemble gel plates and spacers, taping or clamping the plates together as needed for your gel running apparatus.
2. Pour a 4% native polyacrylamide gel. These volumes correspond to gels poured using plates and spacers of the size described in section 2.2. Combine 10.7 mL 30% acrylamide (37.5:1), 8 mL 5X TBE, 400 µL 1 M magnesium acetate, 5 mL 80% glycerol, and 55.9 mL water. Mix well. To polymerize, add 450 µL 10% APS and

110 μ L TEMED. Immediately fill plates with acrylamide mix without getting bubbles in the gel. Insert the comb and let sit until polymerized (~30 min).

3. After the gel is polymerized, place the gel in the gel apparatus and add running buffer to upper and lower reservoirs. Pre-run the gel a minimum of 15 min at 150 V before loading the binding reactions (described below) into the wells.

3.2. EMSAs to determine affinity

To measure the affinity (i.e. K_D) of an ncRNA/protein interaction, set up binding reactions in which the concentration of the ncRNA is well below the K_D for the interaction, and the protein is titrated from below to above the K_D (see Note 4). The K_D is the concentration of protein at which 50% of the ncRNA is in a complex with the protein; the lower the K_D , the higher the affinity. A complete discussion of the theory, experimental considerations, and equations behind measuring a K_D can be found elsewhere (15).

In the example experiment shown in Figure 1, two 32 P-labeled ncRNAs were used: a piece of Alu RNA (Alu-RA RNA) that binds tightly to Pol II and a piece of B2 RNA (B2 RNA(3-73)) that does not detectably bind to Pol II (10). The latter is an important negative control (see Note 5). Lane 1 shows the migration of the unbound Alu-RA RNA and lanes 2–5 show the shift in migration upon titrating Pol II into the binding reactions. Approximately 50% of the Alu-RA RNA was in a complex with Pol II in reactions containing ~2 nM Pol II, which provides an estimate of the K_D for the interaction. Lanes 6–8 show that the migration of the negative control B2 RNA(3-73) does not change upon addition of Pol II. To perform the experiment in Figure 1, follow the protocol below.

1. Fold the ncRNAs (see Note 6). Place the 1 nM dilution of ncRNA at 95°C for 1–2 min, then move to ice.
2. Set up the 20 μ L binding reactions on ice according to Table 1; we use 1.7 mL microcentrifuge tubes. Add the components in the order listed in the table (i.e. add the component in the first row to all tubes, then move to the component in the next row down). Mix the reactions well after each new addition (see Note 7). As the volume of Pol II is increased, the volume of Buffer B is decreased (see Note 8).
3. Incubate the reactions at 30°C for 20 min.
4. Load 18 μ L of each reaction on the native gel while the gel is running; do not add loading dyes to the reactions because they may disrupt the complexes (see Note 9). Add 1 μ L of 20% ficoll prior to loading the reaction if necessary (see Note 3).
5. Run the gel at 150 V for 2–3 hours (see Note 10).
6. After running the gel, how to proceed depends on your method for detecting the ncRNA. To detect 32 P-labeled ncRNA, take apart the gel and transfer it to Whatman paper. Place plastic wrap over the wet gel. Dry the gel using a gel dryer. Place the dried gel on an intensifying screen (typically overnight). Scan the screen using a phosphorimager.
7. Quantitate the signal in each lane using the software program of your choice, drawing a box around each unbound and bound band, as well as a blank region of

the gel near each band to use as a measurement of background. We use ImageJ, which is freely downloadable from NIH (<http://rsbweb.nih.gov/ij/>) to quantitate EMSAs (see Note 11).

8. Subtract the background intensity value from each respective signal intensity value. Use these background-subtracted intensities for all subsequent calculations of the fraction of ncRNA bound in each lane (bound/(bound+unbound)).

3.3. Measuring kinetic stability

To measure the rate at which a complex dissociates, complexes are formed, and then a large excess of unlabeled ncRNA is added to reactions. As complexes dissociate, the excess unlabeled ncRNA will sequester the free protein and keep it from rebinding the less abundant labeled ncRNA, thereby allowing dissociation of the labeled ncRNA/protein complexes to be measured in the absence of detectable re-association (see Note 12). The dissociation of B1 RNA/Pol II complexes is provided as an example experiment (Figure 2) (13). In panel A, lane 1 shows the ratio of bound ³²P-labeled B1 RNA to free B1 RNA at the beginning of the time course (time zero). As the time course proceeds (lanes 2–10) the amount of bound B1 RNA decreases and the amount of unbound B1 RNA increases, indicative of dissociation. As shown in panel B, quantitating the fraction of B1 RNA bound at each time point allows the rate constant for dissociation to be determined.

1. To perform the experiment shown in Figure 2, set up one large 240 μ L binding reaction according to Table 2. 240 μ L easily allows ten 20 μ L aliquots to be removed over the time course of dissociation (see Note 13). Follow the directions in section 3.2 for folding the ncRNA and adding the reaction components.
2. After incubating the large binding reaction, remove a 20 μ L aliquot and load it on a running native gel (see Note 14).
3. Add 10 μ L of a 120 nM stock of unlabeled B1 RNA that was diluted into folding buffer (see Note 15). Mix well. Start a timer.
4. Remove 20 μ L aliquots at the following time points (in min) and load on the running gel: 1, 5, 10, 20, 30, 45, 60, 75, 90 (different time courses may be needed for different complexes).
5. Quantitate the data (see section 3.2, points 7 and 8) and plot the fraction bound (bound/(bound+unbound)) versus time. If a rate constant is desired, fit the data with a single exponential (see Note 12).

3.4. Determining binding specificity by performing competition experiments

The site on a protein that binds an ncRNA will often associate with other RNAs, either specifically or non-specifically. Information on the specificity of an interaction can be obtained from competition experiments that determine the relative affinities with which a protein binds one ncRNA versus another. For example, a competition binding experiment might reveal that a protein has a 100-fold greater affinity for its biologically relevant target RNA compared to a competitor RNA. This means that when the target RNA and the competitor RNA are present at equal concentrations in excess of the protein, the protein will

bind the target RNA over the other RNA 99 times out of 100. A range of relative affinities obtained from competition experiments performed with a variety of RNAs will give an overall impression of a protein's binding specificity. Competition experiments are also useful for screening mutant or deletion constructs of an ncRNA against the wild-type construct to determine whether the mutation or truncation altered binding affinity.

Competition binding reactions are assembled such that the protein chooses whether to bind a labeled target ncRNA or an unlabeled competitor ncRNA at different molar ratios of competitor to target. In the example experiment shown in Figure 3, we used labeled B2 RNA and unlabeled B1 RNA as the competitor to determine the relative affinity of Pol II for binding these two ncRNAs (13). The data show that when the molar ratio of B1 RNA to B2 RNA was 1:1 the amount of the B2 RNA/Pol II complex decreased by 50%. Since the ncRNAs were both in excess of Pol II in this reaction, the result indicates that B1 RNA and B2 RNA bind Pol II with similar affinities, and Pol II does not have specificity for binding one of these ncRNAs over the other.

1. To perform the experiment in Figure 3 set up 20 μ L binding reactions on ice according to Table 3, following the directions in section 3.2 (see Note 16).
2. Incubate the binding reactions and run on a native gel, as described in section 3.2.
3. Quantitate the data (see section 3.2, points 7 and 8) and calculate the fraction bound (bound/(bound+unbound)). Compare the change in fraction bound to the change in the ratio of unlabeled competitor ncRNA:labeled target ncRNA. Determine the ratio that causes the fraction bound to decrease by 50% from the starting point. This ratio provides an estimate of the relative affinities with which the two ncRNAs bind the protein.

Notes

1. 100% glycerol is very viscous and difficult to pipet; therefore, carefully pour 100% glycerol into a conical and then add water to the appropriate volume.
2. Purified RNA for in vitro binding reactions is most often made by in vitro transcription from an engineered DNA template using T7 RNA polymerase. Creating the DNA requires cloning the sequence encoding the ncRNA of interest into a plasmid containing a T7 promoter or using PCR to generate a DNA template containing a T7 promoter and the sequence encoding the ncRNA. In vitro transcription reactions (often with an [α - 32 P]NTP) are performed and the ncRNA is gel-purified. Detailed descriptions and protocols for generating ncRNAs by in vitro transcription are contained on several company and research lab websites, as well as in the literature (9, 16, 17).
3. For some interactions, adding ficoll to binding reactions just prior to loading them on a native gel can help complexes resolve better in the gel and cause bands to be sharper. The effect of adding ficoll varies for different ncRNA/protein complexes and hence should be tested on a case-by-case basis.

4. Meeting these experimental criteria require having an estimate of the K_D , which can be obtained from a preliminary experiment in which the ncRNA concentration is set as low as possible and the protein is titrated over a broad range. For high affinity complexes, setting the concentration of the ncRNA below the K_D can be challenging because the ncRNA will be difficult to detect. If this is the case, use the lowest detectable amount of ncRNA, and present the K_D as an upper estimate (e.g. $K_D < 1$ nM). To obtain an accurate measurement of a K_D a protein titration with many points is required, and typically the data are fit with a binding equation using non-linear regression (15). Alternatively, a minimal titration of the type shown in Figure 1 can be used to estimate the K_D .
5. It is important to include an RNA that does not bind the protein of interest to control for the specificity of the assay. Non-specific interactions between nucleic-acid binding proteins and RNA can be detected by EMSAs. If it is suspected that non-specific interactions are interfering with the assay, a small amount of competitor nucleic acid (e.g. 10–50 ng of poly(dGdC)•poly(dGdC) can be added to the binding reactions to disrupt such interactions.
6. It is important that ncRNAs are folded prior to the experiment. We have found that SINE-encoded ncRNAs bind tightly to Pol II most reproducibly when they are folded by diluting the ncRNA into buffer, heating to 90°C to denature any structure, and then rapidly cooling on ice to re-fold the ncRNA.
7. In the case of Pol II, we keep it on dry ice prior to use. After all other components are added to reactions and mixed well, thaw the Pol II, dilute as needed, add it to reaction tubes, and then snap-freeze any remaining Pol II in liquid nitrogen. Our experience is that many proteins handled in this manner can often survive 2–4 freeze-thaw cycles without losing activity, although this needs to be determined for each protein.
8. The 20 μ L binding reactions are assembled such that half of the reaction volume consists of buffer A and half of the reaction volume consists of buffer B. Hence, the components in buffer A and buffer B are twice as concentrated as needed in the final binding reaction. The ncRNAs are diluted in folding buffer, which is comprised of half buffer A and half buffer B. The Pol II is stored and diluted in buffer B. Therefore, the volume of buffer A plus 50% of the volume of ncRNA should equal 10 μ L, and the volume of buffer B plus Pol II plus 50% of the volume of ncRNA should equal 10 μ L. Maintaining these ratios when titrating components or adding additional components will maintain the appropriate final concentration of each buffer component: 10 mM HEPES (pH 7.9), 1 mM DTT, 4 mM $MgCl_2$, 10% glycerol, 10 mM Tris (pH 7.9), 50 mM KCl.
9. For some protein/ncRNA complexes it is important to load the gel while it is running so that complexes do not dissociate while samples are sitting in the wells of the gel in the absence of a current. In this case, it is very important to not touch the running buffer while loading the gel. The absence of loading dyes makes it difficult to watch the sample sink into the wells, however the greater concentration of glycerol in the samples compared to the gel running buffer ensures the samples

are dense enough to settle to the bottom of the wells. If desired, dyes can be loaded in lanes that do not contain sample so that their migration can be monitored as the gel runs.

10. Running the gel at too high a voltage will heat the gel plates and potentially disrupt ncRNA/protein complexes or unfold the ncRNA.
11. If using ImageJ on data collected with a Typhoon scanner each signal intensity (for samples and background) should be corrected using the following equation:

$$\text{corrected signal} = \text{signal}^2$$

This correction is required because software running the Typhoon scanner saves the square root of signal intensity, and ImageJ does not automatically square the signal. In addition, if signal intensities were not determined using rectangles of the same size, then each corrected signal must be multiplied by the area of the rectangle used for quantitation prior to subtracting background and further analyzing the data.

12. Rates of dissociation are typically measured when the association reaction is either blocked from occurring or not detectable. A more complete discussion of the theory and experimental considerations behind measuring dissociation rate constants, and the single exponential equations used to fit data can be found elsewhere (15).
13. Multiply the volume of each reaction component by the number of desired time points (plus one or two extra to account for volume loss during pipetting) to obtain the final volumes of each component to add to the large binding reaction. Importantly, the rate of dissociation is independent of the fraction of ncRNA that is bound at the beginning of the reaction; however, it is best to choose a condition under which most of the ncRNA is bound to maximize the dynamic range of the assay.
14. This aliquot will provide a measurement of the amounts of protein/ncRNA complex and free ncRNA present prior to adding the excess unlabeled ncRNA. It can be considered the zero time point.
15. These reactions used a 100-fold molar excess of unlabeled B1 RNA. To ensure that the amount of unlabeled ncRNA is sufficient to block the association reaction a control can be performed in which the excess unlabeled ncRNA and the labeled ncRNA are mixed together prior to adding protein. No complex formation should be observed.
16. When performing competition experiments it is important that the concentration of labeled ncRNA is above that of the protein and ideally at least 10-fold above the K_D for the interaction. Under this condition, most of the labeled ncRNA will not be bound to protein.

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Figure 1.

Alu-RA RNA binds to Pol II with an apparent K_D of approximately 2 nM. B2 RNA(3-73) does not bind to Pol II over the concentration range tested. The EMSA shows the migration of the ^{32}P -labeled ncRNAs before and after the addition of purified Pol II. The figure is reproduced with permission from Mariner, et. al. 2008.

Figure 2.

Complexes between B1 RNA and Pol II dissociate slowly. **(A)** The EMSA shows Pol II-bound and unbound ^{32}P -labeled B1 RNA over a time course after the addition of unlabeled B1 RNA to block the association reaction. **(B)** The data from panel A were quantitated and plotted as the fraction of B1 RNA bound to Pol II over time. The data were fit with a first-order exponential decay equation to determine a rate constant for dissociation of $1.5 \times 10^{-4} \text{ s}^{-1}$. The figure is reproduced with permission from Wagner, et. al. 2010.

Figure 3.

The competition experiment reveals that B1 RNA and B2 RNA have similar relative affinities for binding to Pol II. The EMSA shows the fraction of ^{32}P -labeled B2 RNA that remains bound to Pol II as the concentration of unlabeled B1 RNA increases. The ncRNAs were incubated at the molar ratios indicated. The figure is reproduced with permission from Wagner, et. al. 2010.

Table 1

Reaction component (μL)	Reaction number							
	1	2	3	4	5	6	7	8
Buffer A	9.5	9.5	9.5	9.5	9.5	9.5	9.5	9.5
Buffer B	9.5	7.2	8.8	8.1	7.5	9.5	8.5	7.5
1 nM Alu-RA RNA	1	1	1	1	1	–	–	–
1 nM B2 RNA(3-73)	–	–	–	–	–	1	1	1
3.5 nM Pol II	–	2.3	–	–	–	–	–	–
35 nM Pol II	–	–	0.7	1.4	2	–	1	2

Table 2

Reaction component (μL)	
Buffer A	114
Buffer B	97.2
1 nM ³² P-B2 RNA	12
35 nM Pol II	16.8

Table 3

Reaction component (μL)	Reaction number					
	1	2	3	4	5	6
Buffer A	8	8	8	8	8	8
Buffer B	6.9	6.9	6.9	6.9	6.9	6.9
Folding buffer	3	2.7	2	–	2	–
100 nM ³² P-B2 RNA	1	1	1	1	1	1
100 nM B1 RNA	–	0.3	1	3	–	–
1000 nM B1 RNA	–	–	–	–	1	3
35 nM Pol II	1.1	1.1	1.1	1.1	1.1	1.1