Chapter 9

Label-Free Determination of the Dissociation Constant of Small Molecule-Aptamer Interaction by Isothermal Titration Calorimetry

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Abstract

Isothermal titration calorimetry (ITC) is a powerful label-free technique to determine the binding constant as well as thermodynamic parameters of a binding reaction and is therefore well suited for the analysis of small molecule—RNA aptamer interaction. We will introduce you to the method and present a protocol for sample preparation and the calorimetric measurement. A detailed note section will point out useful tips and pitfalls.

Key words RNA, Aptamer, Small molecule, ITC, K_d, In vitro transcription

1 Introduction

In the last couple of years, aptamers have been introduced as powerful tools in Medicine, Molecular Biology and Synthetic Biology (reviewed in Ref. [1]). The high binding affinity and specificity of aptamers is the prerequisite for many applications; therefore the detailed characterization of their binding behavior is an important step after selection.

A plethora of methods exists to determine the dissociation constant (K_d) of aptamers, including: filter binding assays, fluorescence anisotropy, equilibrium dialysis, surface plasmon resonance (SPR), and differential scanning calorimetry [2]. For most of these methods either the ligand or the aptamer has to be labeled, radioactively or by a fluorescent dye, which may influence the interaction between the aptamer and the ligand and is not always easy to perform. A powerful and label-free method to determine the K_d of aptamers is isothermal titration calorimetry (ITC) [3]. The concentration of aptamer which is needed for one reaction is about 10 μM per ITC experiment, an amount easily obtained by in vitro transcription in any wet lab.

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ITC directly measures the change in the enthalpy (ΔH) caused upon ligand binding. The instrument contains two identical cells, a sample cell and a reference cell. The reference cell is filled with water or buffer and the sample cell contains either the ligand or the aptamer. Defined injections into the sample cell are carried out by a syringe containing the second component. Each injection causes heat effects in the sample cell which is due to dilution effects, stirring and heat changes through the interaction of the different molecules. The amount of power which is needed to compensate these heat changes is directly measured and plotted against time (shown in Fig. 1a).

The energy which is needed until the steady state level is reached again is directly proportional to the energy of the reaction. Since ΔH is directly measured and K_a can be calculated by fitting the ITC binding isotherm, ΔG can be determined using the following equation:

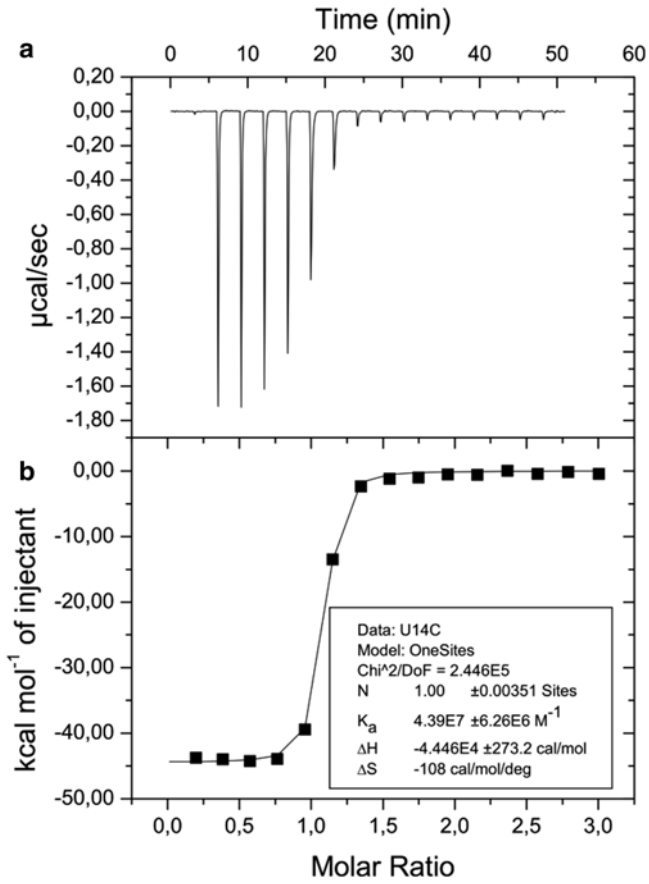

Fig. 1 Example of results obtained by an ITC experiment. The raw data of the experiment are shown in (**a**). Each spike resembles an injection. (**b**) shows the calculated integrals of each spike (*black squares*) and the fit to calculate K_a (*black line*). For fitting the one site binding model was used

$$\Delta G = -RTlnK_a$$

in which ΔG is the Gibbs free energy, R is the gas constant and T is the temperature in Kelvin. The entropy (ΔS) can also be calculated:

$$\Delta G = \Delta H - T\Delta S$$

Hence, ITC is a powerful technique which determines the K_d as well as other thermodynamic parameters and the stoichiometry of the reaction.

2 Materials

2.1 ITC Instrumentation

A variety of ITC instruments is available on the market. All of them work the same way; they measure the heat changes in the sample cell after the injection of one binding partner. The various instruments are able to measure binding constants (K_a) from 10^3 to 10^9 M^{-1} [2]. A common instrument is the MicroCal iTC200, which is convenient for most applications. In the last couple of years the instruments have become more and more sensitive with the advantage that up to 80 % less sample is needed. The TA Instruments Low Volume Nano ITC, for example, is able to detect as little as 0.05 μJ. Consequently, the sample cell volume can be reduced from 1 ml to 180 μl. Another advantage of low sample instruments is the faster equilibration time, so the injection interval can be shortened. The number of injections also differs between a low volume instrument and a normal volume instrument. With a normal cell instrument about 20–40 injections per experiment are carried out compared to 15 injections with a low volume instrument. As a result, more data points are collected with a normal cell instrument resulting in a better resolution.

All things considered, low volume instruments produce accurate data sets for high enthalpy reactions with binding constants between 10^3 and 10^8 M^{-1}. For low enthalpy reactions instruments with a normal cell volume produce more accurate data.

2.2 Plasmid

1. Recommended plasmid for in vitro transcription: pSP64 Promega. The 3 kbp long plasmid contains a β-lactamase gene as a resistance marker for the selection in *E. coli*, a multiple cloning site and an origin of replication resulting in multiple copies of plasmid per cell (*see* **Note 1**).

2.3 Enzymes and Buffer

1. T7 RNA polymerase for in vitro transcription: The polymerase can be purchased from many suppliers (e.g., New England Biolabs). It can also be purified in the lab following the protocol described by Ref. [4]. In vitro transcription buffer (*see* **Note 2**):

250 mM $Mg(Ac)_2$, 1 M Tris–HCl pH 8.0, 1 M DTT, 200 mM spermidine.

2. 10× measurement buffer (*see* **Note 3**): 200 mM Na-cacodylate pH 6.8, 100 mM $MgCl_2$, 1 M NaCl.

3 Methods

3.1 General Aspects for In Vitro Transcription

About 5 nmol of RNA is needed for an ITC experiment. The RNA can be chemically or enzymatically synthesized. The enzymatic synthesis by in vitro transcription is a well-established method and can be performed in any lab. For in vitro transcription the sequence of choice needs to be placed on a plasmid downstream of a T7 promoter. At the end of the sequence a restriction site is needed so the plasmid can be linearized by the respective restriction enzyme. During transcription, the T7 RNA polymerase binds to its promoter, transcribes the DNA template into RNA and falls off at the end of the template at the restriction site. This type of transcription is called run-off transcription. The T7 RNA polymerase binds to the template multiple times, this way it produces a high amount of RNA. Thereby, up to several milligrams of RNA can be produced.

3.2 Template Construction for In Vitro Transcription

1. We recommend using the plasmid pSP64 from Promega, a small high copy plasmid. The sequence of choice can easily be inserted into the vector using the restriction sites *Hin*dIII and *Eco* RI of the plasmids multiple cloning site (*see* **Note 4**).
2. The insert should contain the following features: a T7 promoter, the sequence which should be transcribed and a restriction site for run-off transcription (Fig. 2).
3. The T7 promoter sequence is shown in Fig. 2c. The first one of the two Gs at the end will be the first nucleotide of the transcript (*see* **Note 5**).

3.3 3′-End Heterogeneity

1. The T7 RNA polymerase (as well as other polymerases) is known to add non-coded nucleotides to the 3′-end. To avoid this heterogeneity we recommend using a ribozyme for defined 3′ ends. This ribozyme is placed at the 3′ end of the template sequence (Fig. 2a). After the RNA is transcribed the ribozyme cuts the RNA leaving a defined 3′ end.
2. Two ribozymes can be used, the hepatitis delta virus (HDV) ribozyme and the hammerhead ribozyme [5, 6]. For 3′-end heterogeneity the HDV ribozyme is the ribozyme of choice as it has the advantage that it is sequence independently (*see* **Note 6**). The hammerhead ribozyme can also be used, but the sequence needs to be modified depending on the template sequence since the template is part of the hammerhead sequence.

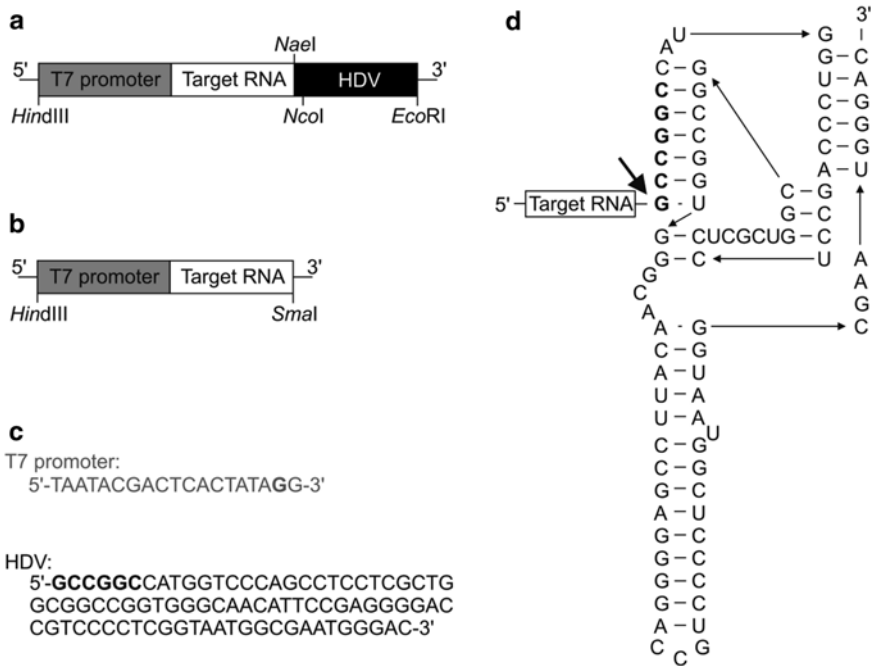

Fig. 2 The constructs for run-off transcription are shown. The T7 promotor is shown in *grey*, the target sequence in white and the HDV ribozyme in black. In (**a**) the construct with HDV ribozyme is shown. The sequence can be cloned into the pSP64 vector by using the restriction sites *Hin*dIII and E*co*RI. For run-off transcription the *Eco*RI restriction site can be used. (**b**) shows the construct without the HDV ribozyme. In this case the restriction sites *Hin*dIII and *Sma*I are used. For run-off transcription the restriction site *Sma*I is used. (**c**) shows the sequence of the T7 promotor with the G in *bold* being the first nucleotide of the transcript. The sequence of the HDV is shown in *black* with the *Nae*I cleavage site indicated in *bold letters*. The secondary structure of the HDV ribozyme is shown in (**d**). The *bold letters* indicate the *Nae*I cleavage site and the *arrow* between the target sequence and the HDV indicates the cleavage site of the ribozyme

3.4 Plasmid Preparation

1. For a large scale in vitro transcription 1–2 mg plasmid is needed (*see* **Notes 7** and **8**).
2. The plasmid pSP64 has to be digested either with the restriction enzyme *Eco*RI, which cleaves the plasmid straight after the HDV ribozyme, or an enzyme which cuts straight after the sequence (*see* **Note 9**).
3. After digestion the plasmid DNA is purified by phenol–chloroform extraction (*see* **Note 10**).

3.5 In Vitro Transcription

1. For the in vitro transcription all components are mixed together as listed in Table 1. It is very important to follow the order given in Table 1; otherwise magnesium phosphate might precipitate.
2. The reaction is mixed by carefully pipetting up and down and is afterwards incubated at 37 °C over night (*see* **Note 11**).

Table 1
In vitro transcription for RNA preparation

Add H_2O 10 ml	RNase-free water
800 µl	$Mg(Ac)_2$ 250 mM (*see* **Note 12**)
2000 µl	Tris–HCl 1 M pH 8.0
200 µl	Dithiothreitol (DTT) 1 M
100 µl	Spermidine 200 mM
2 mg	Digested plasmid
400 µl	ATP 100 mM
400 µl	UTP 100 mM
400 µl	GTP 100 mM
400 µl	CTP 100 mM
15 µg/ml	T7 RNA polymerase

3.6 HDV Cleavage and RNA Purification

1. The RNA cleaves itself straight after transcription if a ribozyme such as the HDV ribozyme is used. The ribozyme folds into its catalytic active conformation during the incubation at 37 °C. It results in two RNA species, your RNA of interest and the free HDV ribozyme.
2. To eliminate the HDV RNA as well as the plasmid DNA, the in vitro transcription is separated by polyacrylamide gel electrophoresis (*see* **Note 13**). Therefore, the RNA dissolved in deionized formamide containing 25 mM EDTA before it is loaded onto four polyacrylamide gels (20 cm × 20 cm) containing 8 M of urea. For the gels TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA) is used.
3. The RNA is detected using UV shadowing, cut out and sliced into small pieces (about 0.5 cm × 0.5 cm).
4. To elute the RNA the gel pieces are placed into 40 ml of 300 mM NaAc (pH 6.5) over night at 4 °C. The RNA is filtered (pore size 0.45 µm) to remove remaining gel residues and separated into four different 50 ml reaction tubes.
5. The RNA is precipitated by adding ethanol to a final concentration of 35 ml into each reaction tube (about 27 ml).
6. The samples are incubated at −20 °C for 1 h and afterwards the RNA is pelleted by centrifugation (1 h, 10,000 × *g*, 4 °C).
7. The supernatant is removed, each pellet is washed with 10 ml of 70 % (v/v) ethanol and the samples are again centrifuged (15 min, 10,000 × *g*, 4 °C).

8. The ethanol is removed completely and the samples are dried at room temperature for 5 min (*see* **Note 14**).
9. It is recommended to dissolve the RNA in RNase-free water but if necessary the RNA can also be dissolved in buffer.
10. After the purification the RNA has to be quality checked by gel electrophoresis.

3.7 Sample Preparation

1. Before using an aptamer, it has to be refolded properly to its folding protocol, which is individual for each aptamer.
2. To adjust the buffer to its final concentration a higher concentrated buffer is prepared. In most cases a 10× measurement buffer is convenient (*see* Subheading 2.3). Finally, the buffer is diluted by adding the RNA in the desired concentration and RNase-free water to the final volume. A good buffer to start with is 20 mM Na-cacodylate pH 6.8, 10 mM $MgCl_2$, and 100 mM NaCl [7, 8]. As the salt concentrations significantly influence the binding constant, the buffer conditions might have to be adjusted (*see* **Note 3**).
3. If no studies on the binding behavior of the aptamer exist, a good start is to titrate 100 μM of the ligand into 10 μM of the RNA. For the iTC200 instrument, 350 μl of sample volume with 10 μM of RNA are needed. The titrant is needed in a volume of 80 μl.
4. It is very important that the RNA and the ligand have the same salt concentration because otherwise heat changes, which are due to dilution effects and not due to ligand binding, can be observed. Some ligands need to be dissolved in dimethylsulfoxid (DMSO) due to higher stability. In this case the DMSO concentration needs to be considered and should not exceed 2 % (v/v).

3.8 ITC Measurement

1. In general the ITC instrument should be placed in a room with constant temperature. The instrument should be prevented from vibrations during a run. Both temperature changes and vibrations affect the instrument, which leads to inaccurate measurements.
2. Before the ITC instrument can be used for RNA samples, it should be cleaned thoroughly. Some instruments, like the iTC200 instrument, have a special cleaning program with a detergent (provided from the manufacturer) which should be used. Afterwards it is important to rinse the sample cell a couple of times with RNase-free water.
3. Before the measurement can be started, the sample has to be loaded into the sample cell. This is a very critical step because air bubbles must not be placed insight the sample cell. Remaining air bubbles completely prevent data analysis.

Table 2
Experimental parameters for the MicroCal iTC200

Total number of injections	16
Cell temperature	25 °C
Reference power	6 µcal/s
Initial delay	180 s
Syringe concentration	Concentration of the ligand in the syringe
Cell concentration	Concentration of RNA in the sample cell
Stirring speed	1000 rpm

Table 3
Injection parameters for the MicroCal iTC200

Injection volume	2.49 µl[a]
Spacing	180 s
Filter period	5 s

[a]The first injection differs from all other injections because of a volumetric error. The error occurs because of a backlash in the motorized screw used to drive the syringe [9]. As a consequence only 0.2 µl of titrant is injected in the first injection

4. Prior to each run, the sample cell should be cleaned with water and a second time with buffer to remove any residue from the previous experiment. To load the RNA sample into the cell it is very convenient to use a Hamilton pipet. For the above mentioned reasons, check the pipet for air bubbles before loading.
5. Furthermore, ensure that the sample cell is filled to the top. If the instrument has not been used for a while you might also check if the reference cell is fully filled.
6. The titrant needs to be loaded into the syringe. In most cases, the instrument is programmed to do that automatically.
7. The experimental parameters as well as the injection parameters depend on the instrument and on the aptamer. For the instrument MicroCal iTC200, one set of parameters is listed in Tables 2 and 3. The run itself is carried out by the instrument automatically (*see* **Note 15**).

3.9 Experimental Considerations

1. Only the ΔH values can be measured directly by ITC. By fitting a suitable model to the ITC isotherm, the association constant (K_a) can be calculated. As a consequence, it is important to receive maximum data points for the fitting process, as the shape of the binding isotherm dictates the accuracy of K_a.

2. The shape of the isotherm depends on K_a and the concentration of the interacting components in the sample cell.
3. The product of the stoichiometry of the reaction (n), the binding constant K_a and the concentration of aptamer in $[M]$ provides a value which is known as the Brandt's "c" value [3].

$$c = n \times K_a \times aptamer conc.[M]$$

This value should be between 10 and 500 and is adjusted by varying the concentration of the aptamer in the sample cell.

4. As a control experiment, it is important to titrate the titrant into buffer without the aptamer. This experiment shows the dilution effects caused by each injection. This heat effects caused by dilution of the titrant are subtracted from the experimental data subsequently.
5. As a second control experiment, buffer has to be titrated into the aptamer in order to see heat effects, which are due to dilution of the aptamer. Normally, these effects are very small and therefore negligible.

3.10 Data Analysis

1. The thermodynamic parameters can be calculated using the software provided from the manufacturer, which is either Origin or the software provided by TA Instruments.
2. The software calculates the area below each peak. A baseline is adjusted automatically. It is important to check whether the baseline was adjusted correctly (if not it must be adjusted manually).
3. The calculated heat effects from the control experiment are subtracted from the data to get only the heat effects from the interaction between the ligand and the aptamer. Normally, this is also done by the provided software.
4. The first injection is never reliable [9]; it should be removed from the data so that it is not included into the fit. This results in a more accurate calculation of K_a.
5. A binding model is fitted to the integrals. These integrals result from the calculated area of each peak and as the concentrations of ligand after each injection are known, the data can be plotted as molar changes in enthalpy against molar ratio (Fig. 1b).
6. The fit itself is managed automatically by the software but the right binding model has to be chosen. In most cases, one ligand binds one aptamer; therefore, the one site binding model should be used. Nevertheless, there are also other binding models available which should be taken into account.

3.11 Analyzing Aptamer Mutants with ITC

In order to understand the interaction between aptamer and ligand in detail, the binding behavior of aptamer mutants can be analyzed. ITC has the advantage that both the binding enthalpy and the

entropy of the binding partners can be determined; thus, the effect of the mutation on binding behavior can be identified. Nevertheless, a change in ΔG can either be due to a change in ΔH (e.g., different amount of hydrogen bonds) or due to a change in the entropy (ΔS). Such mutant analysis was done for a tetracycline binding aptamer [10]. Here, the mutants A13U and A9G both have the same ΔG values but differ in ΔH and ΔS. For A13U this is due to a reduced binding enthalpy. A9G on the other hand has a more unfavorable entropic term than the wild type aptamer. The crystal structure of the aptamer–ligand complex, which was later solved, verified these observations [11]: Whereas for A13U a direct contact between the RNA and the ligand is missing, A9 is important for the formation of the binding pocket; hence, A9U destabilizes the ground state of the aptamer.

4 Notes

1. It is important to use high copy plasmids as high amounts of plasmid are needed for large scale in vitro transcription.
2. For the complete experiment, it is important to use RNase-free water. Buffers should be autoclaved for 30 min. Gloves should be used at any time.
3. In principle, different buffers can be used. So if any further experiments are planned with the RNA, e.g., NMR spectroscopy or the analysis of UV melting profiles, it is convenient to use the same buffer for all experiments. This allows an improved comparability of the experiments. If possible, Tris buffers should be avoided as they have a strongly temperature dependent pK_a and a large heat of ionization [2]. Spermidine can be added to a final concentration of 1 mM to shield unspecific binding sites [8].
4. For ITC experiments, defined 3′-ends are not required. Nevertheless, there are many applications such as NMR spectroscopy or X-ray crystallography where it is required to have a defined RNA sequences. To be able to compare all experiments, it is necessary to use the same RNA; in this case, we recommend using a ribozyme for defined 3′-ends.

   If you do not want to use a ribozyme because defined 3′-ends are not required, you might want to use the restriction sites *Hin*dIII and *Sma*I to clone your fragment, containing the T7 promoter, into the pSP64 vector. *Sma*I has the advantage that it leaves blunt ends after digestion, which reduces the possibility that unspecific nucleotides are added.
5. Two Gs are standard and show good transcription efficiency. One G is also working but the transcription efficiency decreases

significantly. Three Gs on the other hand increase the transcription efficiency even further.

6. To generate defined 3′-ends, the HDV ribozyme is used. Before starting, it is important to check that the HDV and your RNA of choice do not have the same size. In this case it is not possible to use the HDV as the RNA and the HDV ribozyme cannot be separated by electrophoresis. In this case, the hammerhead ribozyme can be used as it is 20 bp shorter than the HDV ribozyme.
7. It is also possible to use PCR products or annealed oligonucleotides as a template for the in vitro transcription but the transcription is less efficient. If a PCR product or annealed oligonucleotides are used, the sequence 5′-CAAG-3′ should be placed downstream of the T7 promotor to increase the transcription efficiency.

   For the large scale transcription described in this article, about 1–2 mg of template are needed. The easiest way to receive such high amounts of template is to use plasmid DNA, so we recommend using plasmids.
8. For the preparation of high amounts of plasmid, the Qiagen Maxi- or Giga-preparation kit is recommended
9. We recommend digesting 4–6 mg of plasmid overnight, with 5 times less enzyme than would be needed to digest the amount of DNA in 1 h. The amount of restriction enzyme should not exceed 10 % (v/v). This is due to the glycerol in which restriction enzymes are stored. If the amount of glycerol is too high the restriction enzyme is inactive.
10. 1 volume of phenol–chloroform–isoamyl alcohol (25:24:1) is added to the digested plasmid, the sample is transferred into a MaXtract tube (Qiagen) and centrifuged. The aqueous phase is transferred into a new MaXtract (Qiagen) tube, 1 volume of chloroform–isoamyl alcohol (24:1) is added and the sample is centrifuged again. The supernatant is transferred into a new reaction tube, the plasmid DNA is precipitated by adding isopropyl alcohol and 0.1 volume of NaAc (3 M, pH 6.5). Afterwards it is incubated at –20 °C for 30 min and pelleted by centrifugation. The pellet is washed with 70 % (v/v) of ethanol, centrifuged, and after drying the pellet at 37 °C it is resolved in a suited amount of water (about 2 ml). 200 ng of plasmid DNA is quality checked on a 1 % (w/v) agarose gel.
11. A successful transcription is very often accompanied by white clouds of precipitated pyrophosphate. If there is no precipitation visible, *t* this does not imply that the transcription did not work. Regardless, you can still carry on with the purification.

    The success of a transcription has to be analyzed by polyacrylamide gel electrophoresis.

12. The magnesium concentration can be varied from 15 to 30 mM to optimize the transcription.
13. Before the transcription is loaded onto the gel, its salt concentration must be reduced by ethanol precipitation.
14. If the RNA is dried for too long, it is impossible to resolve it. Drying the RNA for 5 minutes at room temperature is sufficient, if the supernatant is carefully removed.
15. Total number of injections: the accuracy of the syringe dictates the number of injections because by doing too many injections the minimal injection volume is reached. Cell temperature: The K_a strongly depends on the temperature, so the temperature must be adjusted to the experimental needs. Reference Power: The reference power depends on the reaction. For strongly exothermic reactions, it should be adjusted to a value around 5 μcal/s. For very endothermic reactions, it should be adjusted to a value around 0.5 μcal/s. Initial Delay: This parameter is necessary to establish a baseline before the first injection and is specific of each experiment. Syringe and Cell concentration: These parameters depend on the K_a of the aptamer (*see* also Subheading 3.5). Stirring Speed: If the stirring speed is too low, mixing takes too long and as a consequence the spacing time has to be adjusted. However, the solution in the sample cell might contain suspended particles; then, very fast stirring is required.

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