Chapter 2

Screening Inhibitory Potential of Anti-HIV RT RNA Aptamers

Margaret J. Lange and Donald H. Burke

Abstract

Aptamers targeted to HIV reverse transcriptase (RT) have been demonstrated to inhibit RT in biochemical assays and as in cell culture. However, methods employed to date to evaluate viral suppression utilize time-consuming serial passage of infectious HIV in aptamer-expressing stable cell lines. We have established a rapid, transfection-based assay system to effectively examine the inhibitory potential of anti-HIV RT aptamers expressed between two catalytically inactive hammerhead ribozymes. Our system can be altered and optimized for a variety of cloning schemes, and addition of sequences of interest to the cassette is simple and straightforward. When paired with methods to analyze aptamer RNA accumulation and localization in cells and as packaging into pseudotyped virions, the method has a very high level of success in predicting good inhibitors.

Key words Aptamer, Human immunodeficiency virus (HIV), Reverse transcriptase, Pseudotyping, RNA accumulation

1 Introduction

The current therapeutic regimen for slowing or preventing replication of human immunodeficiency virus 1 (HIV-1) includes a cocktail of compounds that target several viral proteins such as reverse transcriptase (RT) [1, 2]. Despite the success of these therapies, toxicity, noncompliance, and appearance of drug-resistant viral strains continue to present significant problems. Aptamers are structured nucleic acids generated to bind specific molecular targets through an iterative process termed Systematic Evolution of Ligands by EXponential enrichment, or SELEX [3, 4]. These small nucleic acids bind their targets with high-affinity and -specificity, rivaling antibodies. In addition, aptamers can be produced chemically in a scalable process, making them economical, non-immunogenic, and small enough in size to allow efficient entry into biological compartments [5]. Aptamers can also be chemically modified to increase stability and resistance to nuclease-mediated degradation and subjected to conjugation

Daniel Lafontaine and Audrey Dubé (eds.), *Therapeutic Applications of Ribozymes and Riboswitches: Methods and Protocols*, vol. 1103, DOI 10.1007/978-1-62703-730-3_2, © Springer Science+Business Media New York 2014

chemistries to produce more potent, diverse molecular combinations [5]. Along with these benefits, aptamers may be evolved to bind extracellular, intracellular, and cell-surface targets, giving them broad therapeutic potential [2, 5–7]. Notably, aptamers have been selected to bind critical proteins from several viruses and have been shown to impair the replication of the associated viruses, including HIV [3, 4, 8–15], hepatitis B virus (HBV) [16, 17], human cytomegalovirus (CMV) [18], hepatitis C virus (HCV) [19–23], and influenza virus [24, 25]. Thus, the versatility of potential protein targets for aptamers is quite broad.

Anti-HIV RT aptamers have previously been shown to inhibit HIV replication in cell culture [8, 12, 14, 15, 26]. Most of these studies employed a time-consuming system requiring the development of clonal, stable, aptamer-expressing cell lines and long-term serial passage of HIV in the presence of aptamer to demonstrate inhibition. Recently, we developed a fast, streamlined assay to enable screening of multiple aptamers in a variety of expression contexts using a transient transfection-based single-cycle infectivity assay [26]. Our assay utilizes aptamer-expressing plasmids in concert with an EGFP-expressing HIV proviral plasmid and a VSV-G envelope plasmid. Co-transfection of these plasmids directs the production of VSV-G-pseudotyped HIV from cells that also express the aptamer. The assay system allows for determination of both transfection efficiency and infectivity documented by the presence of EGFP-positive cells. The aptamer accumulates within the cells and is packaged within the EGFP-encoding virus, potentially via interaction with the RT portion of the polyprotein. Through aptamer packaging within the virus, the aptamer is carried to a new cell upon infection, where it remains tightly bound the HIV RT, preventing replication and the appearance of EGFP-positive cells. The system allows rapid evaluation of new aptamers and new designs for their expression context. For example, by assaying several different aptamer expression contexts in which highly active or inactivated ribozymes flank the aptamer, we found that ribozyme catalytic activity was not required for inhibition [26]. Intracellular ribozyme cleavage has been reported to be highly inefficient due to the low magnesium environment of the cell [27]. Thus, our data suggested that the uncleaved or partially cleaved transcripts may be the active forms in all tested expression contexts. The ribozyme structures flanking the aptamer, despite their inactivity, likely provide adequate tertiary structural support to favor proper aptamer folding for binding to the RT. The following method details the use of the single-cycle infectivity assay coupled with the catalytically inactive aptamer expression cassette. The method reliably correlates aptamer biological activity to biochemical studies and provides a fast, efficient screening system. Additionally, the system is designed to be highly versatile in terms of adding or subtracting cassette components.

2 Materials

2.1 Generation of Aptamer-Expressing and Control Plasmids

2.1.1 Plasmids and Oligonucleotides

Parental CMV-driven RNA expression vector (*see* **Note 1**), oligonucleotides for preparation of the parental aptamer expression cassette (Integrated DNA Technologies, Coralville, IA) (Table 1).

2.1.2 Cloning Reagents (See Note 1)

Tris–EDTA (TE) buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA), restriction enzymes with appropriate restriction enzyme buffers (New England Biolabs, Ipswitch, MA), T4 DNA ligase with buffer (New England Biolabs, Ipswitch, MA), Antarctic Phosphatase with buffer (New England Biolabs, Ipswitch, MA), LB-Ampicillin broth (Sigma, St. Louis, MO), LB-Ampicillin agar plates, *Escherichia coli* HB101 competent cells, super optimal broth (SOC) medium (Invitrogen, Carlsbad, CA), thermocycler, 37 °C bacterial culture incubator, 37 °C shaker incubator.

2.2 Determination of Aptamer Bioactivity by Single-Cycle Infectivity Assay

2.2.1 Cell Culture

293FT cells (Invitrogen, Carlsbad, CA) (*see* **Note 2**) maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10 % fetal bovine serum (FBS) (Sigma, St. Louis, MO), 500 μg/mL Geneticin (Sigma, St. Louis, MO), 6 mM L-glutamine (Gibco, Life Technologies, Grand Island, NY), 1 mM sodium pyruvate (Gibco, Life Technologies, Grand Island, NY), 0.1 mM non-essential amino acids (Gibco, Life Technologies, Grand Island, NY), and 1× vitamins (Gibco, Life Technologies, Grand Island, NY). Cells should be maintained at 37 °C in 5 % CO_2. For cell splitting and plating for bioassays: TrypLE Express (Gibco, Life Technologies, Grand Island, NY), sterile 1× Dulbecco's Phosphate Buffered Saline (PBS) (Gibco, Life Technologies, Grand Island, NY), 100 mm tissue culture dishes (ISC Bioexpress, Kaysville, UT), 6-well tissue culture plates (ISC Bioexpress, Kaysville, UT), and 96-well ELISA plates (ISC Bioexpress, Kaysville, UT).

2.2.2 Plasmids and Transfection Reagents

Aptamer-expressing plasmids, control plasmids, proviral plasmid pNL4-3-CMV-EGFP (*see* Subheading 3.2) (*see* **Note 3**), vesicular stomatitis virus glycoprotein (VSV-G) plasmid pMD-G (Invitrogen, Carlsbad, CA), polyethylenimine (PEI) transfection reagent [28] (Sigma, St. Louis, MO) (*see* **Note 4**), serum-free DMEM medium, standard DMEM cell culture medium (*see* Subheading 2.2.1), microcentrifuge tubes.

2.2.3 Flow Cytometry Reagents

20 % Paraformaldehyde (*see* **Note 5**), 1× PBS, flow cytometer, microcentrifuge tubes, flow cytometer tubes (if applicable).

2.2.4 Virus Harvesting

0.45 μm syringe filters (Millipore, Billerica, MA), 5 mL syringes, microcentrifuge tubes, −80 °C freezer.

Table 1
Oligonucleotide sequences for assembly of the aptamer expression cassette and primer sequences for amplification of reference genes used in endpoint or qPCR experiments

Name	Sequence
RzModA[a,b]	GCATAATACGACTCACTATAGGGCTAGCGGATTAAACAAG
RzModB[b]	GCGTTTCGTCGCATCCCAGCGACTCATTTCCTTGTTTAATCCGCTAGCCC
RzModC[b]	GCTGGGATGCGACGAAACGCCTTCGGGCGTCCTTGTATCTGAATTCCCTACGAACCC
RzModD[b]	GACAGGGCCCGTTTTCCAGTGTTTTCCCCTTTATCTCCTGGGTTCGTAGGGAATTCAG
RzModE[b]	GGAAAACACTGGAAAACGGGCCCTGTCACGGATTGTGCTTATCCGT
RzModF[b]	GGAGGCGGCCGCTGTTTCGTCCTCACGGACTCATTTCACGGATAAGCACAATCCGTGAC
RzModG[a,b]	TTTTATTAGGAAAGGACAGTGGGAGGCGGCCGCTGTTTCGTCCTC
GAPDH-F	AGAAGGCTGGGGCTCATTTG
GAPDH-R	AGGGGCCATCCACAGTCTTC
GAPDHspliced-F	GGTGAAGGTCGGAGTCAACG
GAPDHspliced-R	GTTGAGGTCAATGAAGGGGTC
U6-F	CGCTTCGGCAGCACATATAC
U6-R	TTCACGAATTTGCGTGTCAT
18S-F	CAGCCACCCGAGATTGAGCA
18S-R	TAGTAGCGACGGGCGGTGTG
HIV Gag-F	TGCTATGTCAGTTCCCCTTGGTTCTCT
HIV Gag-R	AGTTGGAGGACATCAAGCAGCCATGCAAAT

[a]Primers used for amplification of inserts for cloning into aptamer expression cassettes
[b]Restriction enzyme sites are denoted in underlining

2.3 Analysis of Intracellular Aptamer RNA Accumulation and Localization

293FT cells, 6-well tissue culture plates, aptamer-expressing plasmids, control plasmids, PEI transfection reagent (*see* **Note 4**), serum-free DMEM, standard DMEM culture medium (*see* Subheading 2.2.1), 1× PBS, cell scraper, microcentrifuge tubes.

2.3.1 Cells, Plasmids, and Transfection Reagents

2.3.2 RNA Fractionation and Isolation Reagents

TRIzol and TRIzol LS reagent (Invitrogen, Carlsbad, CA) or the Paris RNA Isolation Kit (Ambion, Life Technologies, Grand Island, NY), chloroform (Sigma, St. Louis, MO), TKM buffer (10 mM Tris–HCl pH 7.5, 10 mM KCl, 1 mM $MgCl_2$), Triton X-100, 70 % ethanol, Turbo DNase (Ambion, Life Technologies, Grand Island, NY), nuclease-free water, NanoDrop Spectrophotometer (Thermo-Fisher Scientific, Waltham, MA).

2.3.3 cDNA Synthesis and Real-Time PCR

ImProm II Reverse Transcription System with random hexamer primers (Promega, Madison, WI), primers for amplification of aptamer and aptamer cassette sequences (Integrated DNA Technologies, Coralville, IA), primers for amplification of reference genes for normalization (Table 1) (Integrated DNA Technologies, Coralville, IA), PCR tubes, microcentrifuge tubes, Real Time PCR Plates and Optical Covers (Applied Biosystems, Foster City, CA), Power SybrGreen PCR Master Mix (Applied Biosystems, Foster City, CA), ABI 7500 Real Time RT-PCR Thermocycler (Applied Biosystems, Foster City, CA).

2.4 Analysis of Aptamer Incorporation into Viral Particles

1. Virus-containing supernatant generated from transfection (*see* Subheading 2.2.4), ultracentrifuge, ultracentrifuge-appropriate tubes, TRIzol reagent, chloroform, Turbo DNase, nuclease-free water, 3 M sodium acetate, 100 % ethanol, 70 % ethanol, –80 °C freezer, NanoDrop.

3 Methods

3.1 Generation of Aptamer Expression Cassettes and Controls

The method described in Subheading 3.1 will prepare the constructs to be utilized in the single-cycle infectivity assay (Fig. 1). The cloning process is straightforward and can be easily modified to accommodate a wide range of restriction sites (*see* **Note 1**). The parental aptamer expression cassette generated in this method employs a minimal core (~40 nt) aptamer, flanked by two catalytically inactive ribozymes derived from RzB [29, 30] and sTRSV [31]. The ribozymes on either side of the aptamer allow the aptamer to fold into its active conformation and may decrease the probability of exonuclease degradation [27]. The CMV promoter in these constructs results in high-level expression of aptamer RNA in both the nuclear and cytoplasmic compartments, and this accumulation and localization can be verified using other methods described in this chapter.

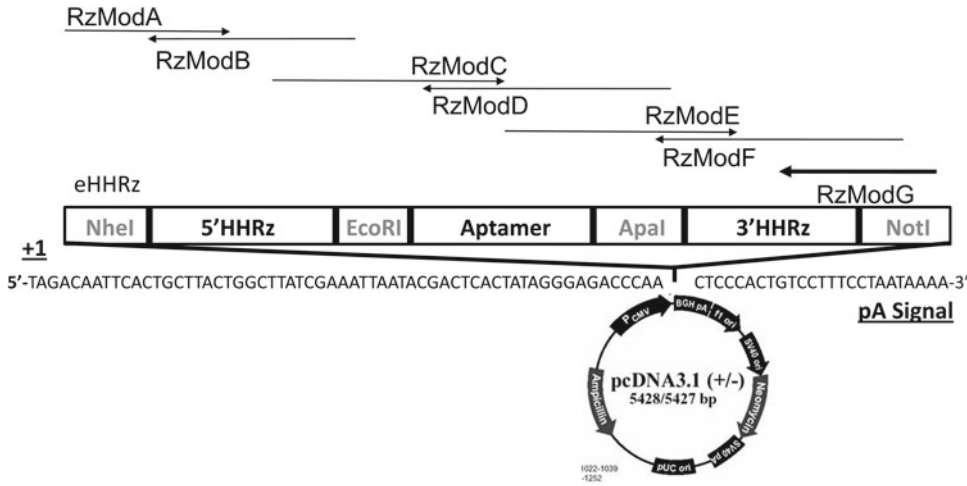

Fig. 1 Cloning schematic for insertion of the catalytically inactive ribozyme cassette into the parental CMV-driven vector

1. Reconstitute the oligonucleotides found in Table 1 at a concentration of 100 μM using TE buffer prepared in nuclease-free water (*see* **Note 1**). Dilute each of the oligonucleotides to 10 μM using nuclease-free water, except for the two outermost oligonucleotides (RzModA and RzModG, Table 1), which should be diluted to 25 μM. In a PCR tube, combine 1 μL of each oligonucleotide with 5 μL 10× Taq Buffer, 1.25 μL 25 mM $MgCl_2$, 1 μL 10 mM dNTPs, 1 U Taq Polymerase, and enough water to bring the final volume to 50 μL. Amplify the reaction using a thermocycler (*see* **Note 6**).

   Initial denaturation (5 min at 95 °C).

   30 cycles of denaturation (30 s at 95 °C), annealing (30 s at 60 °C), and extension (45 s at 72 °C).

   Final extension (10 min at 72 °C).

   Final hold (4 °C).

2. Ethanol precipitate the PCR product from Subheading 3.1, **step 2**, by adding 1/10 volume of 3 M sodium acetate and 3 volumes of 100 % ethanol in a microcentrifuge tube. Precipitate the PCR product for 1 h at −80 °C or overnight at −20 °C. Centrifuge the solution at full speed (16,100×*g* for Eppendorf 5415D) using a microcentrifuge for 30 min at 4 °C. Remove the supernatant, and gently wash the pellet once using cold 75 % ethanol. Repeat the centrifugation. Remove the supernatant, and air-dry the pellet for approximately 10 min (*see* **Note 7**). Resuspend the pellet in 25–50 μL nuclease-free water, depending on band intensity of the PCR product by ethidium bromide staining in an agarose gel. Further dilution of the product prior to ligation into the cassette may be required to achieve a 3:1 molar ratio of insert to vector.

3. Prepare the parental CMV-driven vector (*see* **Note 1**) and parental aptamer expression cassette for cloning by digesting each component with restriction enzymes NheI-HF and NotI-HF (½ μL each enzyme (10 U), 3 μg DNA, 1× NEB buffer 4, 1× BSA, water). After 1 h at 37 °C, inactivate the enzymes by incubation for 20 min at 65 °C. Subsequent phosphatase treatment of the vector is optional but may help reduce vector religation.
4. Purify the digested vector using a PCR Clean-Up or Gel Extraction Kit. The parental aptamer expression cassette may be purified or ethanol precipitated as in Subheading 3.1, **step 3**. Quantify the purified vector and insert using a NanoDrop spectrophotometer.
5. Ligate the cassette and vector using a molar ratio of 3:1 (insert:vector). Perform ligation in a 20 μL reaction volume using T4 DNA ligase (1× NEB ligase buffer with ATP, ½ μL T4 DNA ligase (200 U), 3:1 insert:vector, water).
6. Transform 10 μL of a 20 μL ligation reaction to 50 μL HB101 competent cells. Incubate on ice for 30 min. Heat shock the cells for 1 min at 42 °C and place back on ice for 5 min. Add 200 μL SOC medium and incubate at 37 °C for 1 h with shaking. Plate 100 μL on LB plates containing ampicillin. Incubate overnight at 37 °C. Pick colonies and screen for the presence of the correct insert (*see* **Note 8**). Confirm by DNA sequencing.
7. After verification by sequencing, proceed to clone in any aptamer or control RNA of choice by amplifying the aptamer or control sequence with primers to append EcoRI and ApaI restriction sites to the 5′ and 3′ ends, respectively (*see* **Note 1**). Proceed as for the cassette cloning (*see* **Note 9**).

3.2 Determination of Aptamer Bioactivity by Single-Cycle Infectivity Assay

The method described in Subheading 3.2 allows for the determination of aptamer bioactivity in the context of the aptamer expression construct made in Subheading 3.1. The method utilizes a single-cycle infectivity assay where aptamer-expressing plasmid is first transfected into the cell, followed by the proviral plasmid pNL4-3-CMV-EGFP and VSV-G envelope plasmid, pMD-G [26]. The HIV-$1_{NL3\text{-}4\text{-}derived}$ CMV-EGFP plasmid (pNL4-3-CMV-EGFP) used in single-cycle infectivity assays was kindly provided by Vineet KewalRamani (National Cancer Institute [NCI]—Frederick). This proviral vector lacks the genes encoding *vif*, *vpr*, *vpu*, *nef*, and *env* and has a CMV immediate early promoter-driven EGFP in place of *nef*. As such, the virus can undergo only one round of replication. Thus, the virus is produced in the presence of aptamer, allowing the aptamer to be effectively packaged within the pseudotyped virus, presumably via interaction with the RT [14, 26]. Upon infection of fresh cells, aptamer interaction with the RT prevents DNA synthesis and, thus, the appearance of EGFP-positive cells.

To validate the results obtained from the bioactivity assay, it is strongly suggested to proceed with Subheadings 3.3 and 3.4, which verify that the aptamer accumulates to sufficient levels in total, cytoplasmic, and nuclear compartments of the cell, as well as to verify that the aptamer packages efficiently within the pseudotyped viral particles.

1. Generate high-quality plasmid DNA preparations using standard methods (*see* **Note 10**). Dilute each of the plasmids to a concentration of 100 ng/μL using nuclease-free water.
2. The day prior to transfection, plate 300,000–500,000 293FT cells in 6-well plates, aiming for ~60–70 % confluence the next day (*see* **Note 11**). Media volume should be 2 mL per well. Plate enough wells to accommodate all of the aptamer-expressing plasmids and control plasmids, in addition to "no-plasmid" and "mock" wells.
3. Prepare transfection mixtures for the aptamer-expressing plasmids. Combine 97 μL serum-free medium (DMEM) with 3 μL PEI transfection reagent (3 μL PEI/μg DNA) (*see* **Note 4**). Incubate the mixture at room temperature for 5 min. Add 10 μL (for a total of 1 μg DNA using 100 ng/μL stock) of aptamer-expressing plasmid to the transfection mixture. Filler DNA (1 μg) should be used for "no-plasmid" and "mock" wells (*see* **Note 12**). Flick the tube to mix, and briefly spin the liquid out of the cap. Allow the mixture to incubate at room temperature for 30 min (*see* **Note 13**). Add the mixture dropwise to the 293FT cells in 6-well plates so as to not disrupt transfection complexes or the cells. Gently swirl the plates to mix and place in the cell culture incubator.
4. After 4 h, remove the medium from the cells and gently replace it with 2 mL of fresh medium (*see* **Notes 2** and **4**).
5. Prepare a second transfection mixture for each well, containing the proviral plasmid and envelope plasmid for production of VSV-G-pseudotyped HIV-1 in the presence of aptamer. Amounts may be multiplied depending on sample number to allow for a single mastermix. Combine 48.5 μL serum-free medium with 3 μL PEI transfection reagent for each well. Incubate the mixture at room temperature for 5 min. In a separate tube, combine 48.5 μL serum-free medium with 2.5 μL pNL4-3-CMV-EGFP (250 ng) and 1.5 μL pMD-G (150 ng) for each well (*see* **Notes 14** and **15**). Incubate the mixture at room temperature for 5 min. After the incubation, add the DNA-containing mixture to the PEI-containing mixture. Flick the tube to mix, and briefly spin the liquid out of the cap. Allow the mixture to incubate at room temperature for 30 min (*see* **Note 13**). Add the mixture dropwise to the 293FT cells in 6-well plates, taking care not to disrupt the

cells. Gently swirl the plates to mix and place in the cell culture incubator.

6. After 4 h, remove the medium from the cells and gently replace it with 2 mL of fresh medium (*see* **Note 4**).
7. 48 h after the second media change, harvest the VSV-G-pseudotyped virus by syringe filtering the supernatant using a 0.45 μm syringe filter. To minimize freeze–thaw cycles, aliquot the virus into sterile 1.7 mL tubes, keeping the following applications in mind: infectivity assays (50–150 μL), p24 ELISA (100 μL), and harvesting of viral RNA (1 mL) (*see* **Note 16**). Store the cell-free virus at −80 °C until future use.
8. After harvesting the virus, collect the cells by scraping or trypsinization and transfer them to a fresh 1.7 mL microcentrifuge tube. These cells will be used in Subheading 3.4 for analysis of aptamer accumulation and localization (use scraping method). Perform Subheading 3.4 immediately upon cell harvest. Alternatively, transfection efficiency may be determined by flow cytometry using the cells after fixation for 30 min in 4 % paraformaldehyde (use trypsinization method; *see* Subheading 3.2, **step 12**) (*see* **Note 5**).
9. To determine infectivity of the harvested virus, plate 293FT cells in 6-well plates (as for the transfection in Subheading 3.1, **step 2**) the day prior to infection (*see* **Note 17**).
10. Thaw one aliquot of harvested virus for measuring infectivity and mix well by gently inverting the tube. Use 50–150 μL of the harvested virus (*see* **Note 18**) to infect the 293FT cells plated for infection (Fig. 2). For an individual assay, each sample should be infected with the same volume of viral supernatant.
11. Thaw another aliquot of virus to perform a p24 ELISA for determination of the amount of p24 present in the viral aliquots. Dilutions of 1:10 and 1:100 are recommended for the p24 ELISA to ensure that obtained values are within the range of the standard curve. A protocol for generating a homemade p24 ELISA is available [32]. Commercial kits may also be utilized. These p24 values will be used to normalize the infectivity data to be collected in Subheading 3.2, **step 12**.
12. After 24–48 h, remove the cell culture medium from each well (*see* **Note 19**). Harvest the cells by trypsinization with 200 μL TrypLE Express, wash the well once with 800 μL 1× PBS, and transfer the 1 mL cell suspension to a 1.7 mL microcentrifuge tube. Centrifuge the cells at 300 × *g* for 10 min at 4 °C. Remove the supernatant, and resuspend the cells in 500 μL 4 % paraformaldehyde (*see* **Note 5**). Incubate the cells for 30 min at room temperature or for 1 h at 4 °C. Wash the cells once with 1× PBS, centrifuge at 300 × *g* for 10 min, remove the

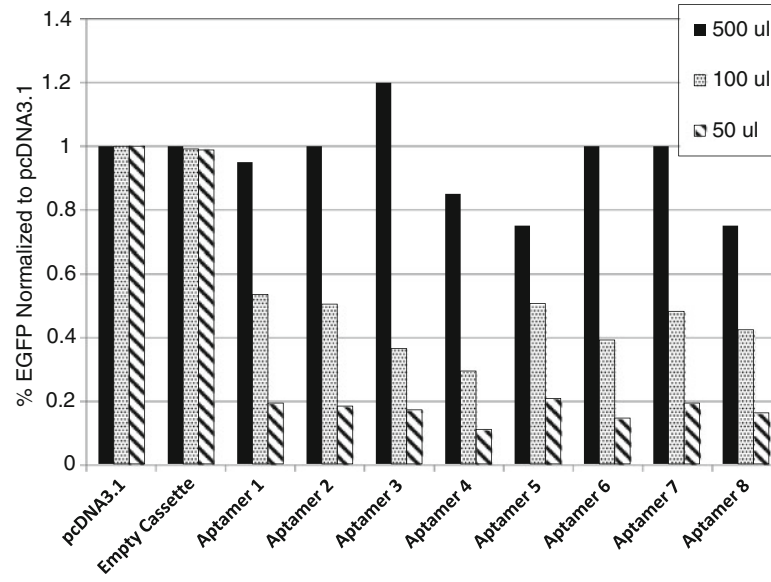

Fig. 2 Single-cycle infectivity assay performed with several different amounts of viral supernatant demonstrating the informative range of the infectivity assay. 293FT cells were transfected with 1 mg aptamer-expressing plasmid or control plasmid (pcDNA3.1 and empty cassette). After 4 h, medium was changed and cells were transfected with proviral and envelope plasmids to produce VSV-G-pseudotyped HIV-1 virions in the presence of aptamer. After 4 h, the medium was changed. Viral supernatant was collected after 48 h by syringe filtration, and fresh 293FT cells were infected with 50, 100, or 500 mL viral supernatant. Infected cells were collected after 24 h, fixed with 4 % paraformaldehyde, and the percentage of EGFP-positive cells was determined by flow cytometry. Data were normalized to pcDNA3.1

supernatant, and resuspend the cells in 500 μL 1× PBS. The cells can now be analyzed using a flow cytometer for the percentage of GFP-positive (infected) cells. Following analysis, infectivity can be normalized to the amount of p24 present per sample.

3.3 Analysis of Intracellular Aptamer RNA Accumulation and Localization

The method described in Subheading 3.3 assesses required components (accumulation and localization) of the single-cycle aptamer bioactivity assay performed in Subheading 3.2. For inhibition to occur, the aptamer must accumulate to significant levels within the cell [26]. If the levels of aptamer are not sufficient to bind the available virus-associated RTs, inhibition is not expected to be observed. This method validates the bioactivity assay by providing additional confirmation of both positive and negative outcomes. If the accumulated RNA levels of aptamer are high, yet inhibition does not occur, it is likely that the specific aptamer utilized may not efficiently bind to and/or inhibit the RT or that it may localize to a nonproductive biological compartment. Binding may occur at many surfaces on the RT [33] and as such may bind in a manner

that does not yield inhibition of RT activity. If the levels of aptamer expression are high and inhibition does occur, the assay confirms the positive result. Finally, if the levels of aptamer expression are low and a negative result is obtained, the reasons for low-level aptamer expression should be explored and the expression system should be optimized (*see* **Note 15**). This validation is best used in concert with the determination of aptamer packaging with the virus (Subheading 3.4), which is an additional important component in terms of aptamer-mediated inhibition of viral replication.

1. This protocol will utilize the transfected cells collected by scraping into 1× PBS from Subheading 3.2 (*see* Subheading 3.2, **step 8**). Alternatively, a separate transfection may be performed (*see* **Note 20**).
2. Separate the cells collected in Subheading 3.2, **step 8**, into two 1.7 mL microcentrifuge tubes. One tube will be used for total RNA isolation, while the other will be used for cytoplasmic and nuclear fractionation prior to RNA isolation. Pellet the cells by centrifugation for 10 min at 4 °C and 300 × *g*. Remove the supernatant.
3. For total RNA isolation, resuspend the cell pellet in TRIzol reagent according to the manufacturer's instructions. Alternatively, the column-based Paris RNA Isolation kit may be used. After completing the TRIzol or Paris RNA Isolation kit protocol, DNase-treat the sample using Turbo DNase (5 μL 10× buffer, 2 μL or 4 U Turbo DNase in an ~50 μL sample) for 1 h at 37 °C (*see* **Note 21**). Inactivate the reaction by adding 11 μL of the provided inactivation resin, mixing well, incubating for 2 min at room temperature, centrifuging at 16,100 × *g* for 3 min, and transferring the supernatant to a fresh tube. The DNase-free RNA may now be quantified using a Nanodrop spectrophotometer. Make sure that the quality of the isolated RNA is high according to the spectrophotometer (*see* **Note 22**). RNA may be subjected to subsequent precipitations to enhance quality using Subheading 3.1, **step 3**.
4. The Paris RNA Isolation kit may also be used for fractionation and RNA isolation. Alternatively, the following method may be used: For cytoplasmic and nuclear fractionation, resuspend the cell pellet in 500 μL ice-cold TKM buffer and incubate on ice for 5 min. Add 15 μL of 10 % Triton X-100 to lyse the cells while keeping the nuclei intact. Incubate the lysis mixture for 10 min on ice. Centrifuge the samples at 500 × *g* for 5 min at 4 °C. Transfer the supernatant containing the cytoplasmic material to a fresh tube with care not to disrupt the nuclear pellet. Repeat the lysis process an additional time for the nuclear pellet to ensure that cytoplasmic contaminants are eliminated. After the second lysis process, wash the nuclear pellet once in 500 μL TKM buffer prior to RNA extraction. Isolate cytoplasmic and nuclear RNA

using TRIzol reagent as per the manufacturer's instructions. Due to the liquid component of the cytoplasmic fraction, TRIzol LS reagent should be used (*see* **Note 23**). Complete the remainder of the protocol as instructed in Subheading 3.3, **step 3**.

5. Isolated total, cytoplasmic, and nuclear RNA may now be used for synthesis of cDNA using the ImProm II Reverse Transcription System as per the manufacturer's instructions. Briefly, at least 200 ng of RNA is suggested for synthesis of cDNA using this protocol. Reaction conditions are as follows: 4 mL 5× reaction buffer, 2 μL 25 mM $MgCl_2$, 1 μL 10 mM dNTPs, 1 μL random hexamer primers (0.5 μg), 0.5 μL RNasin (20 U), 1 μL ImProm II reverse transcriptase, and water to 20 μL. Random hexamer primers, oligoDT primers, or gene-specific primers may be used. Always include "no-RT" controls to ensure that any amplification is not due to genomic or plasmid DNA contamination (*see* **Note 21**). Reactions should be performed at 42 °C for 1 h, with inactivation at 99 °C for 5 min. Samples should be kept at −20 °C.
6. The cDNA generated in Subheading 3.3, **step 5**, may be used for subsequent endpoint PCR or qPCR analysis. This protocol describes the quantitative determination of aptamer expression levels in each RNA fraction using the relative quantity qPCR method. Primers for several reference genes to enable normalization can be found in Table 1 (*see* **Note 24**). Additionally, obtain primers specific to the aptamer constant regions or the entire aptamer expression cassette to enable amplification of aptamer sequences within the cDNA (*see* **Note 25**). Determine amplification efficiency for each set of primers using the Pfaffl method prior to performing qPCR [34]. Perform real-time RT-PCR (qPCR) using PowerSybr Green PCR Master Mix with the appropriate primers according to the manufacturer's instructions. The formula for calculating the relative quantity is as follows: CT values for the reference genes (e.g., GAPDH) are subtracted from the CT values of the sample (e.g., $\Delta CT = CT_{aptamer} - CT_{GAPDH}$) to determine the ΔCT. Then, the ΔCT values for the negative control (e.g., pcDNA3.1 vector) are subtracted from the ΔCT values for the samples ($\Delta\Delta CT = \Delta CT_{sample} - \Delta CT_{control}$). The ΔΔCT values are then used to calculate relative quantity (RQ), using the equation $RQ = 2^{-}\Delta\Delta^{CT}$. Thus, graphs are represented as quantity relative to the specified reference gene, with subsequent normalization to sample controls (set to 1).

3.4 Analysis of Aptamer Incorporation into Viral Particles

The method described in Subheading 3.4 provides an additional experimental layer of validation to the single-cycle infectivity assay, verifying positive and negative results. When paired with the method in Subheading 3.3 to determine aptamer accumulation levels and the bioassay in Subheading 3.2, the method has been

highly successful in predicting aptamers that will be successful viral inhibitors.

1. Thaw 1 mL aliquots of virus-containing supernatant harvested in Subheading 3.2, **step 7**. Transfer the supernatant to an appropriate high-speed centrifuge tube, and adjust the sample volume with PBS to ensure that each sample contains an equal volume of supernatant prior to centrifugation. Centrifuge the samples at 125,000 × *g* for 1 h at 4 °C using a Beckman Coulter TL-100 ultracentrifuge with the TLA 45 rotor. Remove the supernatant, and resuspend the viral pellet in TRIzol Reagent (Invitrogen) as per the manufacturer's instructions. TRIzol LS reagent may be used if a portion of liquid remains over the viral pellet as per the manufacturer's instructions. Addition of 5–10 μg glycogen as a carrier to the aqueous phase during RNA isolation is recommended to improve RNA yields. Alternatively, a column-based viral RNA isolation kit (e.g., Qiagen QIAmp Viral RNA Mini kit) may be used. After completing the TRIzol protocol, proceed as in Subheading 3.3, **step 3**.
2. Isolated viral RNA may now be used for synthesis of cDNA using the ImProm II reverse transcription system as described in Subheading 3.3, **step 5**. For this protocol, ≥200 ng of RNA is suggested for synthesis of cDNA. Always include "no-RT" controls to ensure that any amplification is not due to genomic or plasmid DNA contamination (*see* **Note 21**).
3. The cDNA generated in Subheading 3.4, **step 2**, may be used for subsequent endpoint PCR or qPCR analysis as in Subheading 3.3, **step 6**. Primers for HIV GAG as a reference gene to enable normalization to the viral genome are located in Table 1. Primers specific to the aptamer constant regions or aptamer expression cassette obtained in Subheading 3.3, **step 6**, may be used for amplification of aptamers in viral cDNA.

4 Notes

1. Use of a parental CMV-driven vector: The parental vector utilized in our original study was obtained from the laboratory of Vinayaka R. Prasad [14]. This vector was a modified pcDNA3.1 vector, and the final sequence differs from the original pcDNA3.1 in that several cloning sites have been removed and/or moved. We modified this vector by changing the ribozymes used in the original cassette. While the cloning in this method can utilize pcDNA3.1, slight modifications to the restriction enzymes utilized would be required. For example, cloning of the NheI–NotI aptamer expression cassette into pcDNA3.1 would result in the presence of two ApaI restriction sites (one within the aptamer expression cassette and the

other downstream of the NotI restriction site). Other CMV-driven vectors would be equally acceptable, provided that the restriction sites were modified appropriately. Although we do not anticipate issues with use of the aptamer expression cassette in other CMV-driven vectors, our results are based solely on use of the modified pcDNA3.1 vector.

2. The transfection and infection efficiencies of the 293FT cells decrease with age or passage. We recommend using low-passage 293FT cells (<30 passages) for all transfections and infections. Additionally, 293FT cells are very sensitive to forces generated during removing medium, adding medium, adding transfection mixtures, etc. Extreme care should be taken due to the sensitivity, extreme care should be taken not to disrupt the cells from the plate during such procedures.
3. The pNL4-3-CMV-EGFP proviral plasmid was kindly provided by Vineet KewalRamani (NCI—Frederick). This proviral vector lacks the genes encoding *vif*, *vpr*, *vpu*, *nef*, and *env* and has a CMV immediate early promoter-driven EGFP in place of *nef*.
4. Use of the PEI transfection reagent. Other transfection reagents may also be used. PEI provides highly efficient and consistent transfection of 293FT cells. PEI utilized in this study was made according to the literature [28]. The PEI-DNA transfection mixture should be added dropwise to the cells. Refrain from vortexing or vigorously pipeting the PEI-DNA transfection complex, as these may produce sheer forces that disrupt the PEI-DNA complexes and reduce transfection efficiency. Vortexing after formation of the complex is not recommended. Additionally, dropwise addition of the mixture is crucial to maintaining the complex formation upon addition to the cells. As with many transfection reagents, PEI can be toxic to cells. We have found that using over 6 μL of PEI in a single transfection in a 6-well plate can result in cellular toxicity. Notably, different preparations of PEI will have different toxicity levels, and toxicity should be evaluated prior to the use of the reagent. Due to the associated toxicity, we recommend two separate transfections: one for the aptamer-expressing plasmids and the other for the proviral plasmids. Changing the cell culture medium in between the transfections will prevent accumulation of PEI toxicity within the well.
5. Making of paraformaldehyde solutions for cell fixation: The 4 % paraformaldehyde solution should be diluted from a 20 % paraformaldehyde solution. For 20 % paraformaldehyde stock solution preparation, use the following recipe:

   200 g paraformaldehyde in 800 mL MilliQ water.

   1 mL 10 N NaOH, added dropwise until solubilization occurs.

   Bring up to 1 L with MilliQ water, and heat to 65 °C with stirring to dissolve.

Aliquot in desired volume, and store at −20 °C.

To make 4 % paraformaldehyde working stock.

Mix 100 mL 20 % paraformaldehyde with 50 mL 10× PBS and bring up to 500 mL with MilliQ water.

20 % Paraformaldehyde may be stored at 4 °C for up to 2 weeks or at −20 °C or 20 °C for long-term storage.

The 4 % paraformaldehyde solution should be diluted fresh from the 20 % solution for use.

6. Additional parent aptamer expression cassette for cloning into the parental vector (*see* **Note 1**) may be generated using the outer oligonucleotides (RzModA and RzModG, Table 1) as primers. Dilute the outermost oligonucleotides to 10 μM prior to PCR. Amplify the reaction as in Subheading 3.1, **step 1**.
7. Do not allow the pellet to dry completely. If the pellet is allowed to dry completely, there will be difficulty solubilizing the pellet.
8. To expedite the screening process, PCR may be performed after resuspending the bacterial colony in 10 μL LB-AMP broth and incubating the resuspension for 2–3 h at 37 °C. PCR may be performed with 2 μL of the 10 μL culture. Alternatively, one-half of the bacterial colony may be directly utilized in the PCR reaction.
9. Full-length aptamers (>40 nt) simplify screening of the PCR amplicon by size. Additionally, use of aptamer sequences larger than 100 nt enables easier quantification of aptamer accumulation by real-time RT-PCR. Including segments that are present within all aptamer transcripts allows one set of RT/PCR primers to be used in all RNA quantification assays.
10. Use of maxi preparations of DNA is highly recommended. These preparations typically yield high-quality DNA. Assay variability using lower quality mini preparations of DNA has been encountered. Optimally, DNA should have a 260/280 ratio of ~1.6–1.8 and a 260/230 ratio ≥2.
11. Age or passage number of the 293FT cells affects cellular proliferation speed. Older cells may take longer to reach sufficient levels of confluence than newer cells. This should be taken into consideration when plating prior to experimental procedures.
12. Filler DNA should not affect the experimental outcome. Generally, we utilize a plasmid that expresses mCherry via the CMV promoter as filler (pCMV-mCherry). Salmon sperm DNA may also be used. Experiments should be performed to evaluate the filler DNA prior to introduction into single-cycle infectivity assays using aptamer-expressing plasmids.
13. The mixture may be incubated for 20 min to 2 h without any detrimental effect on transfection efficiency.

14. A mastermix may be prepared for proviral and envelope plasmid transfections. However, prior to addition of DNA to the PEI mixture, the two separate plasmids should be mixed in their appropriate amounts to ensure that each plasmid has equivalent access to the PEI once introduced to the PEI mixture.
15. It is important that aptamer RNA expressed in the cell does not get overwhelmed by the amount of virus produced. If too much virus is produced, the results will be skewed toward a lack of inhibition. We have optimized the amount of proviral and envelope plasmids in our system. We recommend performing proviral titrations in the presence of aptamer to determine the appropriate amount of proviral and envelope plasmid to include based on the amount of aptamer RNA generated from the aptamer-expressing plasmids.
16. Freeze–thaw cycles should be minimized. Care should be taken to ensure that all sample measurements are taken using samples with equal freeze–thaw cycles. Variation in sample concentrations and experimental measurements may be observed with multiple freeze–thaw cycles.
17. For infectivity determination, cells may be plated the day prior to or on the day of infection. Care should be taken to ensure that the confluency will be between 60 and 70 % regardless of the day of plating (*see* **Note 11**).
18. Use of too much or too little virus-containing supernatant will skew the results (Fig. 2). The amount of medium used for infection depends on the amount of virus produced and the confluency of the cells to be infected. Too much virus will saturate the EGFP signal, while too little virus will not provide sufficient positive cells for proper analysis and normalization. The optimal final percentage of EGFP-positive cells for the controls should be 5–10 %.
19. Cells may be harvested for infectivity determination upon the appearance of EGFP-positive cells by fluorescence microscopy. Fluorescence may appear between 24 and 48 h. We recommend at least 24-h infection time.
20. Differences in aptamer accumulation and localization may be observed depending on the presence or the absence of the proviral transfection within the experimental sample. Additionally, if a separate transfection is performed, it should be done side by side with the transfection containing proviral and envelope plasmids to ensure that the cells are in the same state (*see* **Notes 2** and **11**).
21. DNase treatment of the RNA samples is essential to ensure that the observed signal is not because amplification of contaminating genomic or plasmid DNA did not occur.

Amplification of contaminating DNA will give false-positive results and will also result in amplification in the "no-RT" cDNA samples.

22. Care should be taken to ensure the highest quality of RNA. The optimal 260/280 ratio for pure RNA should be ~2.0. Additionally, the 260/230 ratio should be ≥2. Certain RNA isolation protocols may yield low-quality RNA and benefit substantially from subsequent RNA precipitations.
23. TRIzol LS reagent is a more highly concentrated form of TRIzol reagent that accounts for additional "liquid sample" remaining in the tube, assuring the final concentration to be appropriate for nucleic acid or protein isolation.
24. Fractionated RNA samples require specific housekeeping genes. For cytoplasmic samples, spliced GAPDH is recommended (Table 1). For nuclear samples, U6 RNA is recommended (Table 1). Unspliced GAPDH or 18S RNA may be used for total RNA preparations (Table 1). Endpoint PCR to determine cytoplasmic contamination in nuclear samples (and vice versa) is recommended prior to performing qPCR.
25. Aptamer-specific primers may vary with your choice of aptamer due to differences in the content of the constant regions.

References

1. Joshi PJ, Fisher TS, Prasad VR (2003) Anti-HIV inhibitors based on nucleic acids: emergence of aptamers as potent antivirals. Curr Drug Targets Infect Disord 3(4):383–400
2. Gopinath SC (2007) Antiviral aptamers. Arch Virol 152(12):2137–2157. doi:10.1007/s00705-007-1014-1
3. Burke DH, Scates L, Andrews K, Gold L (1996) Bent pseudoknots and novel RNA inhibitors of type 1 human immunodeficiency virus (HIV-1) reverse transcriptase. J Mol Biol 264(4):650–666. doi:10.1006/jmbi.1996.0667
4. Tuerk C, MacDougal S, Gold L (1992) RNA pseudoknots that inhibit human immunodeficiency virus type 1 reverse transcriptase. Proc Natl Acad Sci U S A 89(15):6988–6992
5. Burnett John C, Rossi John J (2012) RNA-based therapeutics: current progress and future prospects. Chem Biol 19(1):60–71. doi:10.1016/j.chembiol.2011.12.008
6. Zhou J, Rossi JJ (2011) Aptamer-targeted RNAi for HIV-1 therapy. Methods Mol Biol 721:355–371. doi:10.1007/978-1-61779-037-9_22
7. Syed MA, Pervaiz S (2010) Advances in aptamers. Oligonucleotides 20(5):215–224. doi:10.1089/oli.2010.0234
8. Cohen C, Forzan M, Sproat B, Pantophlet R, McGowan I, Burton D, James W (2008) An aptamer that neutralizes R5 strains of HIV-1 binds to core residues of gp120 in the CCR5 binding site. Virology 381(1):46–54. doi:10.1016/j.virol.2008.08.025
9. Li N, Wang Y, Pothukuchy A, Syrett A, Husain N, Gopalakrisha S, Kosaraju P, Ellington AD (2008) Aptamers that recognize drug-resistant HIV-1 reverse transcriptase. Nucleic Acids Res 36(21):6739–6751. doi:10.1093/nar/gkn775
10. Neff CP, Zhou J, Remling L, Kuruvilla J, Zhang J, Li H, Smith DD, Swiderski P, Rossi JJ, Akkina R (2011) An aptamer-siRNA chimera suppresses HIV-1 viral loads and protects from helper CD4(+) T cell decline in humanized mice. Sci Transl Med 3(66):66ra66. doi:10.1126/scitranslmed.3001581
11. Held DM, Kissel JD, Thacker SJ, Michalowski D, Saran D, Ji J, Hardy RW, Rossi JJ, Burke DH (2007) Cross-clade inhibition of recombinant human immunodeficiency virus type 1 (HIV-1), HIV-2, and simian immunodeficiency virus SIVcpz reverse transcriptases by RNA pseudoknot aptamers. J Virol 81(10):5375–5384. doi:10.1128/JVI.01923-06
12. Chaloin L, Lehmann MJ, Sczakiel G, Restle T (2002) Endogenous expression of a high-affinity pseudoknot RNA aptamer suppresses

replication of HIV-1. Nucleic Acids Res 30(18):4001–4008
13. Kolb G, Reigadas S, Castanotto D, Faure A, Ventura M, Rossi JJ, Toulme JJ (2006) Endogenous expression of an anti-TAR aptamer reduces HIV-1 replication. RNA Biol 3(4):150–156
14. Joshi P, Prasad VR (2002) Potent inhibition of human immunodeficiency virus type 1 replication by template analog reverse transcriptase inhibitors derived by SELEX (systematic evolution of ligands by exponential enrichment). J Virol 76(13):6545–6557
15. Ramalingam D, Duclair S, Datta SA, Ellington A, Rein A, Prasad VR (2011) RNA aptamers directed to human immunodeficiency virus type 1 Gag polyprotein bind to the matrix and nucleocapsid domains and inhibit virus production. J Virol 85(1):305–314. doi:10.1128/JVI.02626-09
16. Zhang W, Ke W, Wu SS, Gan L, Zhou R, Sun CY, Long QS, Jiang W, Xin HB (2009) An adenovirus-delivered peptide aptamer C1-1 targeting the core protein of hepatitis B virus inhibits viral DNA replication and production in vitro and in vivo. Peptides 30(10):1816–1821. doi:10.1016/j.peptides.2009.07.006
17. Feng H, Beck J, Nassal M, Hu KH (2011) A SELEX-screened aptamer of human hepatitis B virus RNA encapsidation signal suppresses viral replication. PLoS ONE 6(11):e27862. doi:10.1371/journal.pone.0027862
18. Kaiser N, Lischka P, Wagenknecht N, Stamminger T (2009) Inhibition of human cytomegalovirus replication via peptide aptamers directed against the nonconventional nuclear localization signal of the essential viral replication factor pUL84. J Virol 83(22):11902–11913. doi:10.1128/JVI.01378-09
19. Umehara T, Fukuda K, Nishikawa F, Sekiya S, Kohara M, Hasegawa T, Nishikawa S (2004) Designing and analysis of a potent bifunctional aptamers that inhibit protease and helicase activities of HCV NS3. Nucleic Acids Symp Ser 48:195–196. doi:10.1093/nass/48.1.195
20. Kikuchi K, Umehara T, Fukuda K, Kuno A, Hasegawa T, Nishikawa S (2005) A hepatitis C virus (HCV) internal ribosome entry site (IRES) domain III-IV-targeted aptamer inhibits translation by binding to an apical loop of domain IIId. Nucleic Acids Res 33(2):683–692. doi:10.1093/nar/gki215
21. Jones LA, Clancy LE, Rawlinson WD, White PA (2006) High-affinity aptamers to subtype 3a hepatitis C virus polymerase display genotypic specificity. Antimicrob Agents Chemother 50(9):3019–3027. doi:10.1128/AAC.01603-05
22. Kikuchi K, Umehara T, Nishikawa F, Fukuda K, Hasegawa T, Nishikawa S (2009) Increased inhibitory ability of conjugated RNA aptamers against the HCV IRES. Biochem Biophys Res Commun 386(1):118–123. doi:10.1016/j.bbrc.2009.05.135
23. Romero-Lopez C, Diaz-Gonzalez R, Barroso-delJesus A, Berzal-Herranz A (2009) Inhibition of hepatitis C virus replication and internal ribosome entry site-dependent translation by an RNA molecule. J Gen Virol 90(Pt 7):1659–1669. doi:10.1099/vir.0.008821-0
24. Cheng C, Dong J, Yao L, Chen A, Jia R, Huan L, Guo J, Shu Y, Zhang Z (2008) Potent inhibition of human influenza H5N1 virus by oligonucleotides derived by SELEX. Biochem Biophys Res Commun 366(3):670–674. doi:10.1016/j.bbrc.2007.11.183
25. Park SY, Kim S, Yoon H, Kim KB, Kalme SS, Oh S, Song CS, Kim DE (2011) Selection of an antiviral RNA aptamer against hemagglutinin of the subtype H5 avian influenza virus. Nucleic Acid Ther 21(6):395–402. doi:10.1089/nat.2011.0321
26. Lange MJ, Sharma TK, Whatley AS, Landon LA, Tempesta MA, Burke DH, Johnson MC (2012) Robust suppression of HIV replication by intracellularly expressed reverse transcriptase aptamers is independent of ribozyme processing. Mol Ther 20:2304–2314. doi:10.1038/mt.2012.158
27. Khvorova A, Lescoute A, Westhof E, Jayasena SD (2003) Sequence elements outside the hammerhead ribozyme catalytic core enable intracellular activity. Nat Struct Biol 10(9):708–712. doi:10.1038/nsb959
28. Reed SE, Staley EM, Mayginnes JP, Pintel DJ, Tullis GE (2006) Transfection of mammalian cells using linear polyethylenimine is a simple and effective means of producing recombinant adeno-associated virus vectors. J Virol Methods 138(1–2):85–98. doi:10.1016/j.jviromet.2006.07.024
29. Saksmerprome V, Roychowdhury-Saha M, Jayasena S, Khvorova A, Burke DH (2004) Artificial tertiary motifs stabilize trans-cleaving hammerhead ribozymes under conditions of submillimolar divalent ions and high temperatures. RNA 10(12):1916–1924. doi:10.1261/rna.7159504
30. Roychowdhury-Saha M, Burke DH (2007) Distinct reaction pathway promoted by non-divalent-metal cations in a tertiary stabilized hammerhead ribozyme. RNA 13(6):841–848. doi:10.1261/rna.339207
31. Burke DH, Greathouse ST (2005) Low-magnesium, trans-cleavage activity by type III, tertiary stabilized hammerhead ribozymes with stem 1 discontinuities. BMC Biochem 6:14. doi:10.1186/1471-2091-6-14

32. Aiken C (2009) Cell-Free Assays for HIV-1 Uncoating. Methods Mol Biol 485:41–53. doi:10.1007/978-1-59745-170-3_4
33. Ditzler MA, Bose D, Shkriabai N, Marchand B, Sarafianos SG, Kvaratskhelia M, Burke DH (2011) Broad-spectrum aptamer inhibitors of HIV reverse transcriptase closely mimic natural substrates. Nucleic Acids Res 39(18):8237–8247. doi:10.1093/nar/gkr381
34. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29(9):e45

http://www.springer.com/978-1-62703-729-7

Therapeutic Applications of Ribozymes and Riboswitches
Methods and Protocols
Lafontaine, D.A.; Dubé, A. (Eds.)
2014, XI, 278 p. 53 illus., 19 illus. in color., Hardcover
ISBN: 978-1-62703-729-7
A product of Humana Press