Supplemental Data for:

A highly sensitive aptamer-based HIV reverse transcriptase detection assay

By

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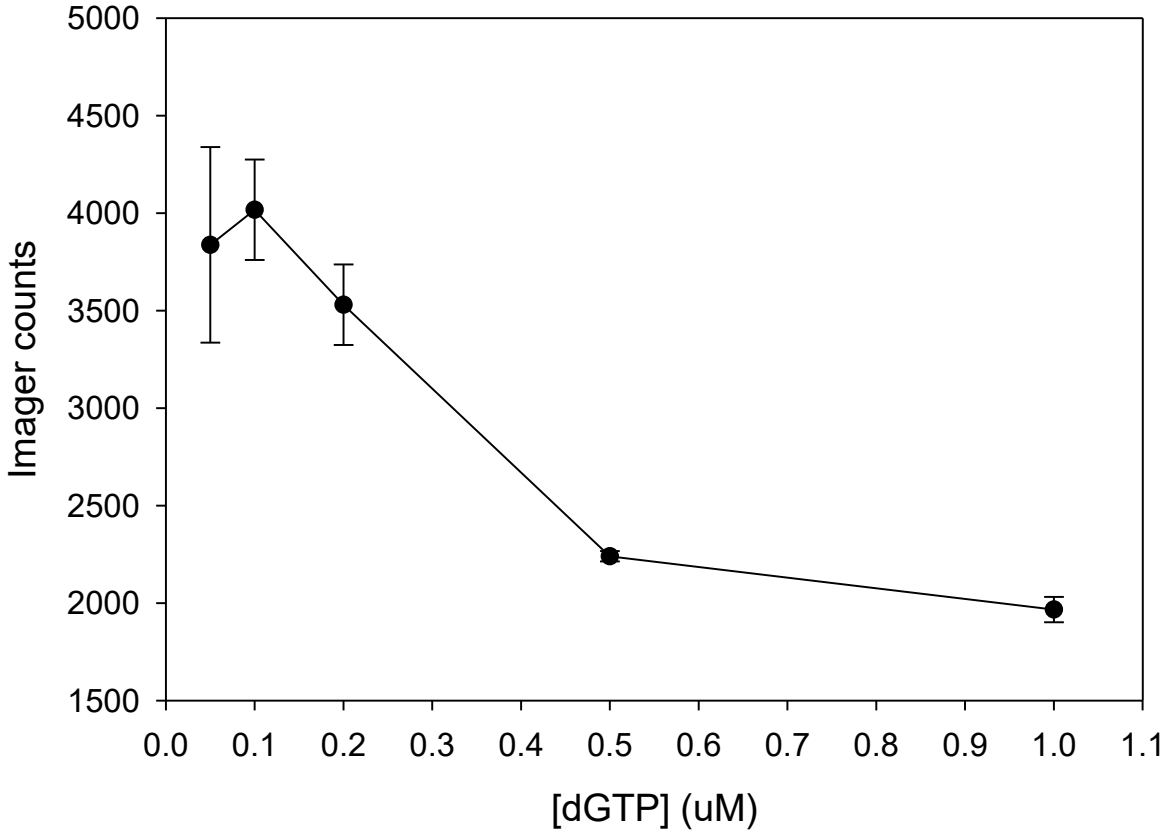

Figure S1. Imager signal level for gel assay vs. concentration of non-radioactive dGTP. A standard Aptamer-based RT Detection Assay was performed for gel analysis with 1 x 10^7 molecules of RT per well and 2.5 µCi (3000 Ci/mmol, 10 µCi/µl) of radiolableled dGTP which was equivalent to ~ 0.033 µM in the 25 µl assay volume. Unlabeled dGTP was added to the assay to bring the final dGTP concentration to 0.05, 0.1, 0.2, 0.5, or 1 µM dGTP. The Labeling step was then carried out for 4 hours before processing and running the samples on a gel. Results shown are an average from triplicate wells with the error bars representing standard deviations.

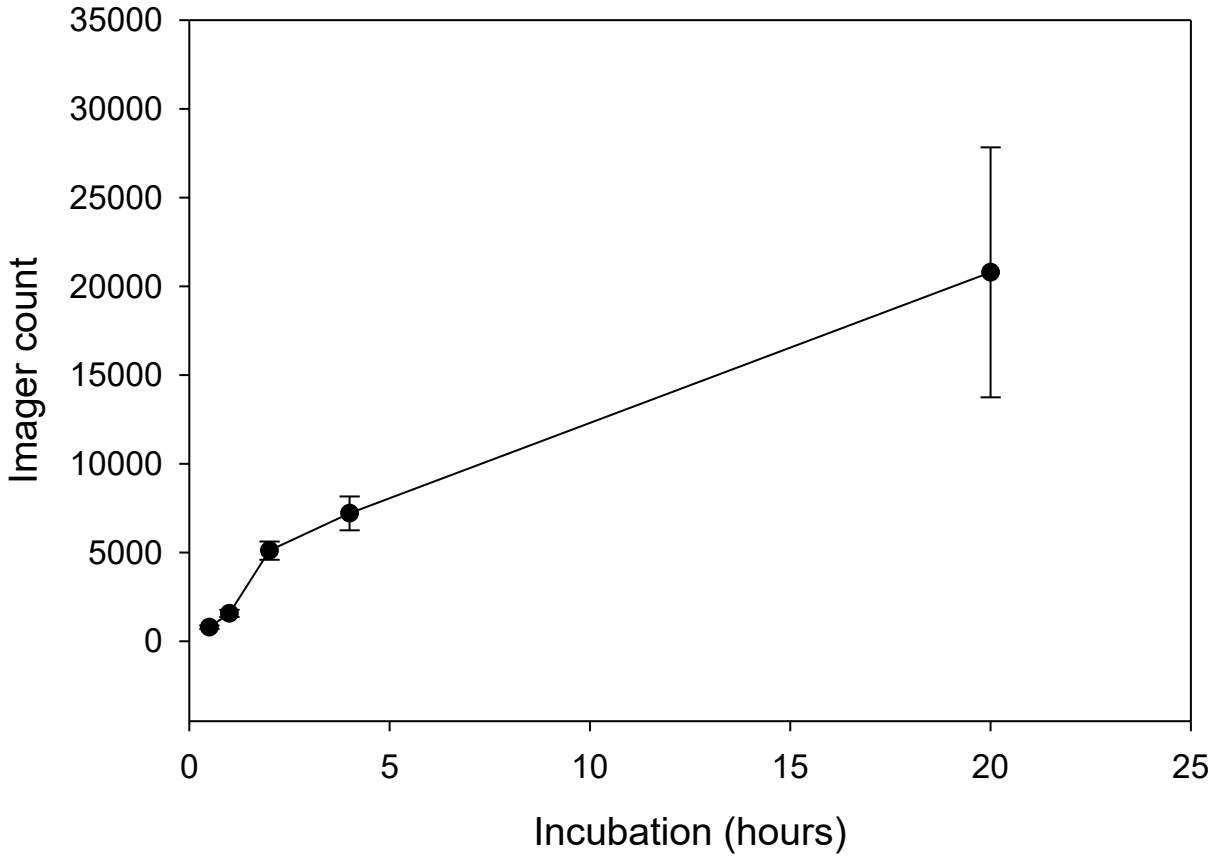

Figure S2. Imager signal level for gel assay vs. time of Labeling step. A standard Aptamer-based RT Detection Assay was performed for gel analysis with 1 x 10^7 molecules of RT per well and 2.5 µCi (3000 Ci/mmol, 10 µCi/µl) of radiolabeled dGTP (0.2 µM final concentration) in a 25 µl assay volume. The Labeling step was carried out for 0.5, 1, 2, 4, or 20 hours before processing and running the samples on a gel. Results shown are an average from triplicate wells with the error bars representing standard deviations.

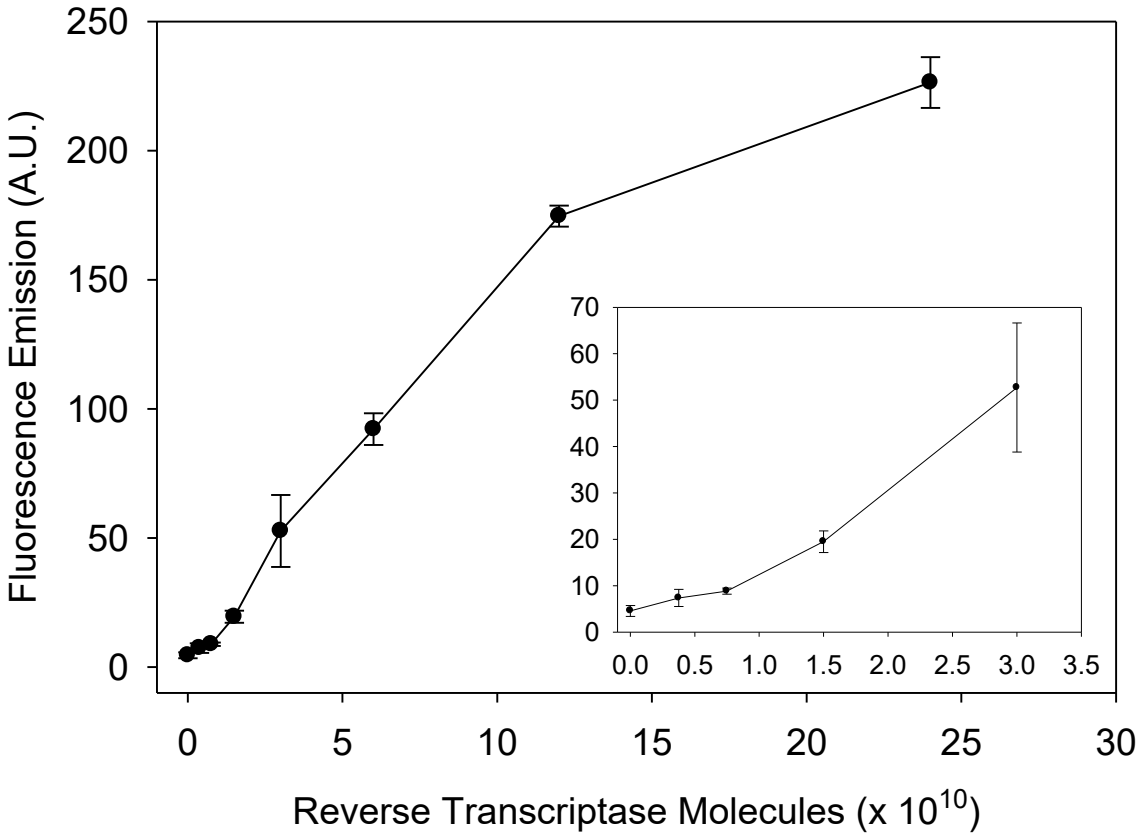

Figure S3. Determination of HIV-1 RT detection limit using the EnzChek Reverse Transcriptase Assay Kit. Different amounts of RT enzyme preparation (RT molecule (by weight) (x 10^{10}): 0, 0.38, 0.75, 1.5, 3, 6, 12, 24) were used in the EnzChek assay using the manufacturer's recommended protocol. Results shown are an average from triplicate wells with the error bars representing standard deviations. Based on ~1.7 x 10^{11} RT molecules per unit of enzyme (see main text), the detection limit for our enzyme in the EnzChek assay was ~0.75 x 10^{10} molecules of RT of ~0.04 units. The manufacturer's stated detection limit is 0.02 units. Therefore, our enzyme appears to be a little less active than the company's reference enzyme, but the difference is quite small (only about 2-fold). Based on these results the HIV RT used in these experiments would be comparable to other wild type HIV-1 RTs. "A.U." - Arbitrary Units.

Table S1. Detection of various RTs in Aptamer-based RT assay

[a]Enzyme	[b]Imager counts	[c]Relative detection	[d]K_d (pM)
HIV-1 (HXB2 wild type)	32,223 ± 10,364	1	27 ± 5
HIV-1 K65R	32,771 ± 3,346	1	204 ± 47
[e]HIV-1 AZTr	10,685 ± 1,796	0.33	131 ± 15
HIV-1 M184V*	16,111 ± 2,638	0.50	15 ± 4
HIV-1 K103N*	19,339 ± 4,090	0.60	8.5 ± 3.5
HIV-1 type A/E	24,265 ± 718	0.75	45 ± 20
HIV-2*	6,508 ± 503	0.20	8.8 ± 3.1
MuLV RT	1,235 ± 472	0.04	65 ± 12
AMV RT	---	ND	>10,000
PFV RT	693 ± 250	0.02	250 ± 50
Klenow (exo-)	---	ND	>10,000
Human DNA Pol α	---	ND	>10,000
Taq	---	ND	>10,000

[a]All drug resistant mutants were derived from the HXB2 sequence with the indicated mutations. 2×10^7 RT molecules (based on weight) were used in each assay. A plasmid clone for HIV-1 wild type and HIV-1 K65R and AZTr proteins were from Dr. Michael Parniak (University of Pittsburgh). HIV-1 type A/E was from Stefan Sarafianos (Emory University). Plasmid clones for HIV-2, HIV-1 M184V, and HIV-1 K103N were from Dr. Stephen Hughes (NIH HIV Dynamics and Replication Program) *Taq*, MuLV, AMV, and Klenow were from New England Biolabs. Human DNA Pol α was from Chimerx. PFV was provided by Dr. Edward Arnold (Rutgers). Abbreviations: MuLV- Maloney murine leukemia virus; AMV- avian myeloblastosis virus; PFV- prototype human foamy virus.

[b]Imager counts were an average of 3 repeats ± standard deviations using the filter-based aptamer assay.

[c]Values were set relative to the HXB2 (type B clone) wild type enzyme.

[d]Values are an average of 2-4 experiments ± standard deviations. Binding was tested to the parent 38NT2,4methyl aptamer shown in Fig. 1B. Filter binding assays were performed as described previously (Miller et al. 2016, Protein Science 25(1):46-55).

[e]AZTr is an AZT resistant RT with the following mutations: D67N, K70R, T215F, and K219Q.

ND- Not Detected.

*Enzyme contains N-terminal 6 His Tag

Aptamer-based HIV reverse transcriptase detection assay

Protocol for: "A highly sensitive aptamer-based HIV reverse transcriptase detection assay"

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Materials

1. Tween-20 (Thermo Fisher Scientific Surfact Amps 20, product # 28320),
2. dGTP (Roche Applied Sciences)
3. α-P^{32} dGTP (PerkinElmer, (3000 Ci/mm, 10 μCi/ul)
4. DEAE Filtermats (90 x 120 mm, part # 1450-522) (PerkinElmer)
5. Streptavidin coated plates (8 well strips on 96 well plates) (Pierce™, ~10 pm/well binding capacity, Catalog number: 15125).
6. Microplate adhesive film (VWR)
7. DNA Aptamers (Integrated DNA Technologies): 5'-Biotin or PC (Photo-Cleavable) Biotin-CCCCCCCCCCCCCCCCCCCCCmCCmCCCCTTCGGTGCTTTGCACCGAAGGGGGGG-3', mC denotes 2'-O-methyl C. (Note: Aptamers were ordered purified by HPLC or PAGE). 5'-Biotin for the PAGE Gel detection assay and 5'-PC-Biotin for the Filter or liquid scintillation detection assays)
8. 1 M Tris-HCl (pH=7.5)
9. 1 M DTT
10. 0.5 M EDTA (pH=8)
11. 10% BSA solution
12. 2 M KCl solution
13. 5 M NaCl solution
14. 1 M $MgCl_2$ solution
15. Formamide (molecular biology grade)
16. Xylene cyanol
17. Bromophenol blue
18. 0.5 M $NaP0_4$ (pH=7)
19. Urea
20. Boric Acid
21. Tris base
22. HIV reverse transcriptase (RT) (Note: An HXB2 wild type clone was used to obtain purified RT in our experiments. Any source of purified wild type HIV RT should be adequate as a standard although the aptamer used in these assays has been tested extensively only with subtype B RT.)

Equipment

1. 96-well plate orbital shaker
2. 37ºC incubator, or 37ºC room
3. Protective screens for radioactivity

4. Phosphorimager and imager screens
5. Shaker (either rocker or orbital, Filter detection assay only)
6. PAGE gel electrophoresis equipment (PAGE detection assay only)
7. Handheld UV light (UVP Model UVM-57, 302 nm, Filter detection assay only)
8. Speedvac vacuum drier (optional)
9. Scintillation counter (optional)

Aptamer assay protocol

The assay is broken down into 6 steps. Both the Gel detection and Filter detection assays are described.

Step 1, Aptamer binding

NOTE: This step can also be carried out overnight in preparation for the next day. Alternatively, aptamers can be attached to plates as below, followed by washing of the wells with water, patting dry, wrapping in air tight plastic, and storing the plates for several days at 4ºC in the dark.

1. Wash plate 3X with Binding buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM dithiothreitol (DTT), 0.1 mg/ml Bovine Serum Albumin (BSA), 1 mM EDTA (pH=8), and 0.1% Tween-20). All washes were conducted using an 8 channel multichannel pipette set at 200 µl. Solution was added to all wells, then poured off or pipetted as noted.

NOTE: EDTA was included in the binding buffer to chelate Mg^{2+} that may be present in cell media or lysates. This helps prevent nuclease activation or extension of the aptamer by polymerase or RT in the presence of non-radioactive nucleotides from cell or media sources. The concentration of EDTA can be increased if solutions with high Mg^{2+} concentrations are used.

IMPORTANT: The major source of contamination in this assay is RT. Extra care should be taken to make sure no solutions or other contaminating sources (e.g. pipet, gloves) transfer RT to the plates or buffers at any time. If possible use filtered pipet tips for any step with RT and discard after each use. If filter tips are not used in wash steps, be very careful with the multichannel pipette that solutions do not get into the pipette nozzle where they can access contaminating materials.

2. Mix aptamer in Binding buffer at a concentration of 20 pm of 5'-biotinylated (Gel assay) or 5' PC-Biotin biotinylated (filter assay) 33NT2,4methyl+20C aptamer per 25 µl of Binding buffer. For all steps using the PC-Biotin aptamer, the plate should be covered with foil to avoid light exposure.

NOTE: Each well was capable of binding ~10 pm of aptamer in a 100 µl volume with both the floor and walls of the well coated with streptavidin. Therefore, 20 pm was in excess to achieve maximal binding in 25 µl. The choice to use 25 µl was mainly to decrease the amount of radioactivity and aptamer used in the assays. Assays using 100 µl of material and the same concentration of aptamer and radioactivity yielded similar results. It is possible that even less aptamer would be adequate although this was not extensively tested. Using 96-well plates with ~ 5-fold greater streptavidin binding capacity than those used did not affect the results.

3. Add 25 µl of aptamer Binding buffer solution to each well and incubate 2 hours at room temperature with mild shaking at 500 rpm (orbital 96-well shaker was used).

Step 2, RT binding

1. Mix RT standards in 25 µl of Binding buffer. Standards should span the range of expected RT amounts. In our assays we used 5-fold dilutions starting at 1 x 10^8 RT molecules in the highest standard and 6400 in the lowest (7 total standards and a no RT control). The assay, as described

below, is essentially linear over this range. However, linearity for higher amount of RT can be obtained by increasing the dGTP concentration and/or shortening the incubation time. Further accuracy can be obtained by doing duplicate wells or multiple dilutions, especially for the samples being measured.
2. Mix samples to be tested by adding sample and appropriate dilutions to Binding buffer for a total volume of 25 µl. If the amount of sample being added is greater than 5 µl we recommend diluting in 2X binding buffer.
3. Wash wells from the Aptamer binding step above 4X with 200 µl of Binding buffer.
4. Make sure wells are dry for the RT binding step by patting several times onto a large Kimwipe.
5. Add 25 µl of RT in Binding buffer to the appropriate wells.
6. Incubate 2 hours at room temperature with mild shaking at 500 rpm.
NOTE: Incubating for only 1 hour modestly reduced the signal while incubations for longer than 2 hours did not improve results.

Step 3, RT washing
1. Prepare the labeling mix by adding 2.5 µCi of α-P^{32} dGTP (3000 Ci/mm, 10 µCi/ul) per 25 µl and unlabeled dGTP (final dGTP concentration in reactions was 0.2 µM) in Labeling buffer (50 mM Tris-HCl, pH=8, 80 mM KCl, 6 mM $MgCl_2$, 1 mM DTT).
NOTE: The amount of radiolabeled nucleotide used can be decreased for detection of higher amounts of RT. Total dGTP can also be increased to improve assay linearity for higher amounts of RT at the expense of sensitivity.
2. Carefully remove the RT-binding mix using a multichannel pipette by placing the pipette tip into the well without scratching against the floor or walls.
3. Wash the wells 5X with 200 µl of Binding buffer. Take care to not cross-contaminate wells.
4. Wash each well 1 time with 200 µl Labeling buffer containing no dGTP.
5. Make sure wells are dry for the Labeling step by patting several times onto a large Kimwipe.

Step 4, Aptamer labeling
1. Add 25 µl of the labeling mix from above to each well.
2. Cover the wells with microplate adhesive film.
3. Place the plate in a 96-well orbital shaker inside a 37ºC incubator or in a 37ºC room.
4. Incubate at 350 rpm for the desired time.
NOTE: Assay detection improved about 4-fold between 2-20 hours. For very small amounts of RT a 16 hour incubation would be ideal while larger amounts can be incubated for less than 2 hours with good results. Upon long incubations, condensation onto the film and well walls typically occurs. Shaking at 350 rpms in the Labeling step can be omitted if desired.
IMPORTANT: Discard all radioactive materials according to regulations.

Step 5, Aptamer removal
1. Carefully remove the radioactive material from the wells with a multichannel pipette. Place material into a glass dish or other container that is behind a protective shield. We added paper towels to the glass dish to absorb liquids.
2. Rinse the wells 5X with 200 µl of 0.5 M $NaPO_4$ (pH=7) and discard the wash material in the glass dish above, then incubate in 200 µl of 0.5 M $NaPO_4$ (pH=7) for 30 min at room temperature, shaking at 500 rpm. Discard material, wash with of 0.5 M $NaPO_4$ 2X, and then with water 3X, placing all washes in the glass dish.

NOTE: The streptavidin-biotin linkage is very stable allowing several washes to remove unincorporated dGTP. Take care to minimize light exposure for PC-Biotin aptamers.

3. Make sure wells are dry for the Aptamer removal step by patting several times onto a large Kimwipe.

4. Release aptamers from well by one of two methods:

(A) For the 5'-biotinylated 33NT2,4methyl+20C aptamer for PAGE gel detection:

1. Add 25 µl of 90ºC formamide buffer (90% formamide, 10 mM EDTA (pH=8), 0.025% bromophenol blue and xylene cyanol) to each well and heat in a microwave for 4 min (carefully monitored to make sure plate does not melt. May vary with different microwaves).
2. Incubate at room temperature for 30 min, shaking at 500 rpm.
3. Remove formamide buffer from each well and use directly for loading onto PAGE gels (see below).

(NOTE): This typically releases over ½ of the total radioactivity on the plate. Some material is not released and may include material sequestered within the plate coating/matrix or some aptamers that are not release from the streptavidin.

(B) For PC-Biotin 5'-biotinylated 33NT2,4methyl+20C aptamer for filter detection:

1. Add 25 µl of water to each well.
2. Exposed to UV light for 10 min using a handheld UV light mounted directly on top of the open wells. We typically mounted the light on a stand so that the exposure area was resting on the top of the wells.

NOTE: The appropriate strength and wavelength (~300 nm) light source must be used for optimal release. This may require testing. We used a new UVP Model UVM-57, 302 nm light in our assays and ~10 min was required for optimal release. Too long an exposure can degrade the DNA.

3. Dry the water in the wells with a speedvac.

NOTE: Remove rotor from speedvac during drying.

4. Add 10 µl of water to resuspend material in each well. Alternatively, 10 µl from the 25 µl of water in the well can be used directly for the next step with a small loss in sensitivity.

NOTE: The level of radioactivity release is similar to the gel assay described above.

Step 6, Aptamer detection

(A) For the 5ˈ-biotinylated 33NT2,4methyl+20C aptamer for PAGE gel detection:

1. Prepare a standard 15% denaturing 7M urea PAGE gel for electrophoresis in Tris-Boric Acid-EDTA (TBE) running buffer.

NOTE: A thicker gel (0.8 mm) with a 32 well comb (BRL Gibco S2 or similar) can be used for loading up to ~18 µl of sample per well. We recommend skipping spaces between samples to prevent run-over of sample and also prevent spreading of the radioactive signal between wells during exposure to the imager screen. The samples need only be run a short distance (~15 cm or less) as there is no need to resolve bands. Beware of thicker gels cracking during the drying procedure. Alternatively, a thinner gel (0.4 mm) can be used and less sample can be loaded. For samples with high signal, gels can be exposed without drying.

2. Expose wet or dried gels to the imager screen for several minutes to several hours depending on level of signal.

3. Develop and quantify with a phosphorimager.

(B) For PC-Biotin 5'-biotinylated 33NT2,4methyl+20C aptamer for filter detection:

1. Pipette 10 µl of material to each square of a 96 square DEAE Filtermat. Cut the Filtermat as needed to accommodate all samples.

NOTE: As above, make sure samples are separated enough to prevent the radioactive signals from one well from interfering with adjacent wells.

2. Air dry, then place Filtermats in a container with 0.5 M $NaPO_4$ (pH=7) and incubated with shaking (orbital or rotary shaker) for 30 min at room temperature.
3. Decant the liquid into radioactive liquid waste.
4. Repeat the filter wash step for 5 min.
5. Rinse filters with distilled water.
6. Dry filter using a heat lamp.
7. Wrap in plastic wrap and expose to the imager screen as above.