General Procedure A (GPA) for the Guanidinylation of Aminoglycosides (step a).

To a solution of an aminoglycoside (5 amines, 0.054 mmol) in H_2O (0.5 mL) was added 1,4-dioxane (2.5 mL) and *N*,*N*'-di-Boc-*N*''-triflylguanidine (**6**, 0.82 mmol) in alternating portions so that the solution remained relatively clear. After 5 min, NEt_3 (0.82 mmol) was added at rt. After 3-4 days, the reaction mixture was extracted with $CHCl_3$ (3 x 10 mL), washed with H_2O and brine and dried ($MgSO_4$). The fully guanidinylated, Boc-protected, product can be isolated by flash column chromatography (fcc) on silica gel ($CHCl_3$ / MeOH).

General Procedure B (GPB) for the Deprotection of Guanidinoglycosides (step b).

A solution of TFA / CH_2Cl_2 (1:1, 1 mL) was added to the protected guanidinoglycoside (0.041 mmol) at rt. After approximately 4 h, the solution was diluted with toluene, concentrated in vacuo and dissolved in H_2O. Subsequent lyopholyzation of H_2O provided the deprotected guanidinoglycoside as a fluffy white TFA salt.

Table S1. Summary of guanidinylation reactions.

Aminoglycoside Precursor	No. of amines	Yield % step a	FAB MS $[M]^+$	Yield% step b	ESI MS $[M+H]^+$
Kanamycin A (**1a**)	4	91	1454	100	653
Kanamycin B (**2a**)	5	65	1695	95	694
Tobramycin (**3a**)	5	100	1678	99	678
Paromomycin (**4a**)	5	70	1827	70	826
Neomycin B (**5a**)	6	68	2068	100	867

Boc_{10}-Guanidino Tobramycin.

According to GPA, tobramycin (**3a**, 25.4 mg, 0.054 mmol) was treated with reagent **6** (319 mg, 0.816 mmol) and NEt_3 (0.12 mL, 0.816 mmol). The fully guanidinylated, Boc-protected product (91.2 mg, 100%) was isolated by fcc ($CHCl_3$ / MeOH 98:2). ^1H NMR ($CDCl_3$, 400 MHz) δ = 11.51 (s, 1H), 11.47 (s, 1H), 11.45 (s, 1H), 11.35 (s, 1H), 11.28 (s, 1H), 8.86 (d, J = 3.6 Hz, 1H), 8.48-8.45 (m, 2H), 8.39 (d, J = 8.8 Hz, 1H), 8.15 (d, J = 8.4 Hz, 1H), 5.39 (d, J = 3.6 Hz, 1H), 5.36 (d, J = 3.6 Hz, 1H), 4.96 (d, J = 3.6 Hz, 1H), 4.45-4.25 (m, 3H), 4.05-4.01 (m, 2H), 3.91-3.80 (m, 4H), 3.70-3.62 (m, 2H), 3.56-3.29 (m, 6H), 3.18 (br d, J = 13.2 Hz, 1H), 3.07 (d, J = 11.6 Hz, 1H), 2.43-2.38 (m, 1H), 2.32-2.28 (m, 1H), 2.19-2.14 (m, 1H), 2.01 (br s, 2H), 1.54 (s, 9H), 1.51 (s, 9H), 1.49 (s, 9H), 1.47-1.46 (m, 36H), 1.44 (s, 9H), 1.43 (s, 18H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ = 163.6, 163.2,

163.0, 162.4, 161.8, 158.1, 157.1, 156.4, 155.6, 154.9, 153.08, 153.05, 152.6, 152.4, 99.4, 97.0, 86.7, 83.8 (2C), 83.7, 83.42, 83.41, 79.7, 79.5, 79.41, 79.37, 79.3, 79.2, 75.9, 73.4, 73.3, 71.7, 69.7, 63.4, 62.1, 58.3, 49.5, 48.7, 48.6, 40.9, 35.3, 31.1, 28.44 (3C), 28.39 (3C), 28.3 (3C), 28.2 (12C), 28.1 (3C), 28.1 (3C), 28.0 (3C); MS (FAB) *m/z* (rel intensity) 1811 ([M + Cs]$^+$, 100); HRMS (FAB) *m/z* calcd for $C_{73}H_{127}N_{15}O_{29}$ [M + Cs]$^+$ 1810.7978, found 1810.8131.

Guanidino-Tobramycin·5TFA (3b).

Boc-protected, guanidino-tobramycin (69.1 mg, 0.041 mmol) was deprotected according to GPB, (TFA / CH_2Cl_2 (1:1, 1 mL)) and provided **3b** (50.9 mg, 99%). ^{1}H NMR (DMSO, 400 MHz) δ = 8.33 (br s, 1H), 8.07 (br s, 1H), 7.85 (br s, 1H), 7.65-7.13 (m, 17H), 5.40 (d, J = 3.2 Hz, 1H), 5.29 (br s, 2H), 5.12 (br s, 1H), 4.95 (d, J = 2.8 Hz, 1H), 4.52 (br s, 3H), 4.20 (dd, J = 9.6, 2.4 Hz, 1H), 3.81-3.71 (m, 2H), 3.58-3.29 (m, 11H), 2.02-1.93 (m, 2H), 1.58-1.47 (m, 2H), 1.24-1.22 (m, 1H); ^{13}C NMR (DMSO, 100 MHz) δ = 158.1, 157.6, 156.5, 156.4, 155.9, 97.1, 95.2, 81.2, 78.5, 74.5, 72.8, 71.4, 69.7, 67.8, 63.1, 59.9, 57.1, 50.3, 49.7, 48.9, 41.1, 33.9, 33.2; MS (ESI-positve) *m/z* (rel intensity) 678 ([M + H]$^+$, 100), 714 ([M + H +HCl]$^+$, 30), 750 ([M + H + 2HCl]$^+$, 19); HRMS (MALDI) *m/z* calcd for $C_{23}H_{47}N_{15}O_9$ (M + H)$^+$ 678.3754, found 678.3738.

Conditions for Binding Assays

Fluorescence anisotropy and solid-phase experiments were conducted at 22°C in a buffer containing 30 mM HEPES (pH 7.5), KCl (100 mM), sodium phosphate (10 mM), NH_4OAc (20 mM), guanidinium HCl (20 mM), $MgCl_2$ (2 mM), NaCl (20 mM), EDTA (0.5 mM), and Nonidet P-40 (0.001%). This complex mixture of cations and anions is found to minimize the non-specific binding of ligands to the Rev-RRE complex and to maximize the reversibility of the Rev-RRE interaction (as evident in both anisotropy and solid-phase assays).

Design of Rev Peptides

Both N-terminus and C-terminus modified $Rev_{34\text{-}50}$ peptides were synthesized and studied. Succinylation of the N-terminus, amidation of the C-terminus, and addition of four alanines has been shown to increase the α helicity of the Rev peptide, and thereby increase the affinity and specificity to the RRE [R. Tan, L. Chen, J.A. Buettner, D. Hudson, A.D. Frankel, *Cell* **1993,** *73*, 1031-1040]. In this case, these four alanines also serve as a spacer for subsequent fluorescein modification. The N-terminus free amine of the "N-terminus modified" peptide is used for conjugation to fluorescein (resulting in "Fl-Rev"). The Cys of the "C-terminus modified" peptide is conjugated to fluorescein (resulting in "Rev-Fl"). For the non-labeled C-terminus modified Rev peptide ("Rev-IA"), the C-terminal Cys is capped as a thioether acetamide derivative (SCH_2CONH_2) to prevent dimerization.

N-terminus modified: $_{NH2}$AAAATRQARRNRRRRWRERQR$_{am}$

C-terminus modified: $_{suc}$ TRQARRNRRRRWRERQRAAAAC$_{am}$

Synthesis of Rev Peptides

Synthesis was carried out using standard Fmoc/HBTU chemistry on ABI Applied Biosystems 431A peptide synthesizer using protected amino acids purchased from Calbiochem. Protected amino acids used in the synthesis were: Fmoc-Arg-(Pbf)-OH, Fmoc-Asn-(Trt)-OH, Fmoc-Gln-(Trt)-OH, Fmoc-Trp-(Boc)-OH, Fmoc-Thr-(tBu)-OH, Fmoc-Glu-(OtBu), and Fmoc-Cys-(Trt)-OH. For the C-terminus modified peptide, the first peptide coupling was done manually to avoid isomerization problems associated with Cys. Fmoc-Cys-(Trt)-OH was converted into its symmetric anhydride by reaction with 0.45 equivalents of DCC in dry methylene chloride for 10 min. The anhydride was separated from the DCU precipitate by vacuum filtration and methylene chloride was subsequently removed in vacuo. The solid anhydride was then dissolved in 30% N-methylpyrrolidone (NMP) in DMF and added to deprotected Rink Amide MBHA resin. The coupling lasted 2.5 hours at 25°C and was monitored by ninhydrin (Kaiser Test). Upon completion, the resin was washed with methylene chloride followed by NMP, and loaded onto the ABI synthesizer. The remainder of the peptide synthesis was carried out using standard HOBt/HBTU chemistry and monitored with a built-in conductivity meter. For both peptides, the first round of automated coupling reactions was done as a "double coupling" to ensure efficiency. Upon completion of the automated synthesis, the N-terminus modified resin was washed (with methylene chloride) and coupled to fluorescein by reaction with ~100 equivalents of 5-carboxyfluorescein succimidyl ester (Molecular Probes) in DMSO for 1 h at room temperature. The C-terminus modified resin was washed with DMF and reacted with 0.3 M succinic anhydride, 0.3 M 1-hydroxybenztrizole hydrate, and 0.03 M DMAP in DMF, for 1 h at room temperature. Both reactions were monitored using the Kaiser Test. Upon completion of each reaction, the resin was washed (in methylene chloride) and cleaved from the resin at room temperature for 2.5 h in a "cleavage cocktail" containing 88% TFA, 5% water, 5% phenol, and 2% triisopropylsilane (v/v). The filtrate was then mixed with 15 volumes of 2% acetic acid, and extracted 4 times with diethyl ether. Crude peptides were purified on a C-18 semiprep HPLC column with an isocratic mixture of either 19% or 14% acetonitrile (0.1% TFA) in water (0.1% TFA) (for the fluorescein and non-fluorescein containing peptides respectively). The purified peptides were lyophilized and the C-terminus modified peptide was reacted with 100 equivalents of 5-iodoacetamidofluorescein in 100 mM sodium phosphate (pH 8.0), 2 mM EDTA, and 30% (v/v) DMSO at room temperature in the dark for 2 h. Or, separately, the purified C-terminus modified peptide was reacted with iodoacetamide (85 mM in 75% DMF/water (v/v) and 20 mM HEPES, pH 8.0) at 25°C in the dark for 2 h. The resulting peptides ("Rev-Fl" and "Rev-IA", respectively) were then purified on a C-18 semiprep HPLC column with an mixture of acetonitrile (0.1% TFA) and water (0.1% TFA); isocratic conditions were 19% acetonitrile for Rev-Fl and 14% acetonitrile for Rev-IA. Electrospray mass spectrometry confirmed masses for Fl-Rev (3,079 amu), $_{NH2}$-Rev (2,721), Rev-Fl (3,312 amu), and Rev-IA (2,982 amu). Analytical HPLC confirmed a greater than 98% purity for each peptide. Molecular extinction coefficients were taken as 73,500 cm^{-1} M^{-1} for Fl-Rev (498 nm), 77,000 cm^{-1} M^{-1} for Rev-Fl, (498 nm), and 5,600 cm^{-1} M^{-1} for both $_{NH2}$-Rev and Rev-IA (280 nm).

Evaluation of Rev Peptides

The C-terminus modified peptide is found to be superior to the N-terminus peptide. Gel shift mobility assays indicate that perturbation of RRE affinity is significantly worse when the N-terminus modified peptide is conjugated to fluorescein compared to the addition of fluorescein to the C-terminus modified peptide. Furthermore, upon binding the RRE, fluorescence quenching of "Fl-Rev" is much more significant (~30%) than quenching of "Rev-Fl" (~10%) (this makes Rev-Fl better suited for fluorescence anisotropy experiments). These differences can be rationalized by examining the solution structure of the Rev-RRE complex which shows the N-terminus of the Rev peptide buried in the major groove of the RRE and the C-terminus free in solution [J.L Battiste, H. Mao, N.S. Rao, R. Tan, D.R. Muhandiram, L.E. Kay, A.D. Frankel, and J.R. Williamson, *Science* **1996,** *273*, 1547-1551].

Synthesis of 67-nt RRE

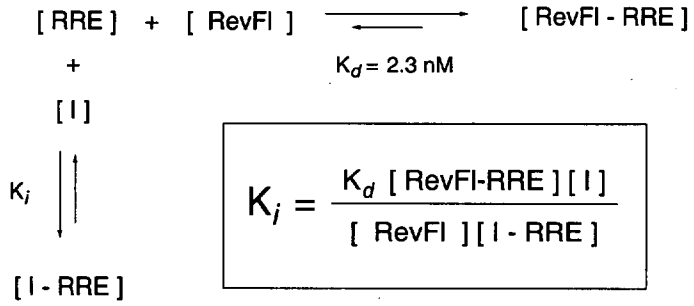
The synthesis of the 5' biotinylated RRE transcripts is a modification of the procedure described [M. Hendrix, E.S. Priestley, G.F. Joyce,and C. Wong *J. Am. Chem. Soc.* **1997,** *119*, 3641-3648]. To generate RNA transcripts with a phosphorothioate 5'-end, 8 mM guanosine monophosphorothioate (purchased from USB) was included in the transcription reaction (along with 2 mM of each NTP), this approach was found to be more efficient than the previously described method. For the transcription of the tri-phosphate 5' end, no GMPS was included in the reaction. T7 RNA polymerase was utilized for "run-off" in vitro transcription with a complementary, 83-nt DNA template as described by Uhlenbeck [Uhlenbeck, O.C.; Milligan, J.F. *Methods in Enzymology* **1989,** *180*, 51-62]. Synthesis of the 83-nt DNA template was carried out using standard phosphoramidite chemistry. The oligonucleotide was purified using denaturing polyacrylamide gel electrophoresis (PAGE), followed by extraction and multiple rounds of ethanol precipitation. The sequence and homogeneity of the template was confirmed using di-deoxy sequencing techniques. Transcription products were also purified using denaturing PAGE, extraction and multiple rounds of ethanol precipitation. Transcripts with a phosphorothioate 5' end were reacted with Iodoacetyl-LC-Biotin (purchased from Pierce) as described [M. Hendrix, E.S. Priestley, G.F. Joyce,and C. Wong *J. Am. Chem. Soc.* **1997,** *119*, 3641-3648] and re-purified as above. The expected sequence of the 67-nt RNA transcript was verified by 5'-end labeling a RNA transcript (having a 5' hydoxyl) with ^{32}P and subsequent enzymatic digestion (RNase T1, A, and U2). The molecular extinction coefficient for the biotinylated and non-biotinylated 67-nt RNA transcript was taken as 741,400 cm^{-1} M^{-1} (at 260 nm). Before use, RNA transcripts were folded in a buffer containing 30 mM HEPES (pH 7.5), KCl (100 mM), $MgCl_2$ (2 mM), and EDTA (0.5 mM) by heating to 90°C and cooling to room temperature over 30 min.

Fluorescence Anisotropy

Fluorescence anisotropy experiments were conducted essentially as described [supporting information of Kirk, S.R.; Luedtke, N.W.; Tor, Y. *J. Am. Chem. Soc.* **2000**, *122*, 980-981] except that, here, 100 nM of the RRE is used for all displacement experiments. IC_{50} values are dependent on the assay conditions (especially on RNA concentrations). In the supporting information of Kirk, et al. *JACS* **2000**, *122*, 980, 8.5 nM of the RRE is used, and an IC_{50} value of 0.8 μM is measured for neomycin's disruption of the Rev-RRE complex. As theory predicts, neomycin's IC_{50} value increases by approximately a factor of 10 when 100 nM of the RRE is used.

See Figure **S1(a)** for fluorescence anisotropy data showing RevFl-RRE association. For displacement experiments (**Figure S2(b)**), a Rev-RRE complex was formed by mixing 10 nM Rev-Fl with 100 nM of the pre-folded 67-nt RRE (having a triphosphate on 5' end) in a thermocontrolled (22°C) cuvette. Inhibitors were then titrated and both total fluorescence and anisotropy were monitored (Perkin Elmer LS-50B fluorimeter, maximum slit widths, Excitation 490 nm, Emission 530 nm, 2 second integration time, 6 readings averaged per concentration). The K_d of the RevFl-RRE interaction is calculated (Figure S1(a)) by titration of the prefolded 67-nt RRE fragment into a solution of 10 nM Rev-Fl (See: T. Heyduk, J.C. Lee, *Proc. Natl. Acad. Sci. USA* 1990, *87*, 1744-1748. F.W. Sevenich, J. Langowski, V. Weiss, K. Rippe, *Nucleic Acids Res.* 1998, *26*, 1373-1381).

The K_d obtained for Rev-Fl binding to the RRE was used to calculate a K_i value for Rev-IA. Upon binding the RRE, only minor changes in the emission spectrum of fluorescein were seen (about 10% quenching of Rev-Fl). We have taken the change in anisotropy as being directly proportional to the fraction of Rev-Fl bound by the RRE. Sufficient mixing time was always provided to allow for equilibrium to be reached. From a simple, three component, competitive binding equilibrium, the following equation can be derived:



$$K_i = \frac{K_d\,[\text{RevFl-RRE}]\,[\text{I}]}{[\text{RevFl}]\,[\text{I - RRE}]}$$

By this analysis, a K_i of 1.2±0.8 nM is calculated for Rev-IA, which compares to a K_d of 2.3±0.5 nM for Rev-Fl. This shows that addition of fluorescein has a only minor effect on RRE affinity. Since binding stoichiometries of the other inhibitors are unknown, IC_{50} values (inhibitor concentrations which give 50% displacement of RevFl) are reported.

a.

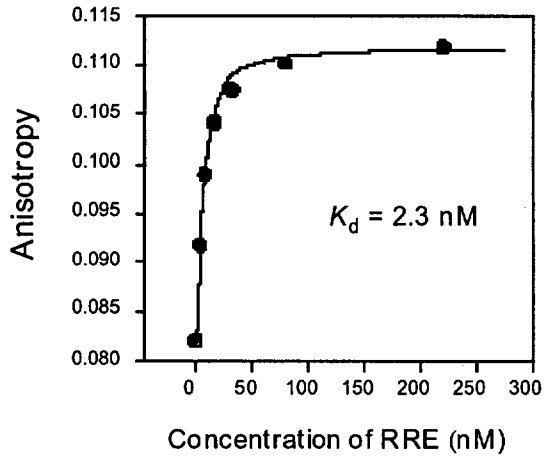


Figure S1a. Both line shape and gel shift analysis indicate a 1:1 complex of the RRE to Rev-Fl. Given this, a K_d of 2.3 nM was calculated by nonlinear regression using the equation:

$$A = A_0 + \Delta A \frac{([RNA]_{total} + [Rev\text{-}Fl]_{total} + K_d) - \sqrt{([RNA]_{total} + [Rev\text{-}Fl]_{total} + Kd)^2 - 4[RNA]_{total}[Rev\text{-}Fl]_{total}}}{2[Rev\text{-}Fl]_{total}}$$

A= anisotropy of the Rev-Fl
A_0= anisotropy of the Rev-Fl in the absence of RNA
ΔA=the total change in anisotropy at saturation of the Rev-Fl

b.

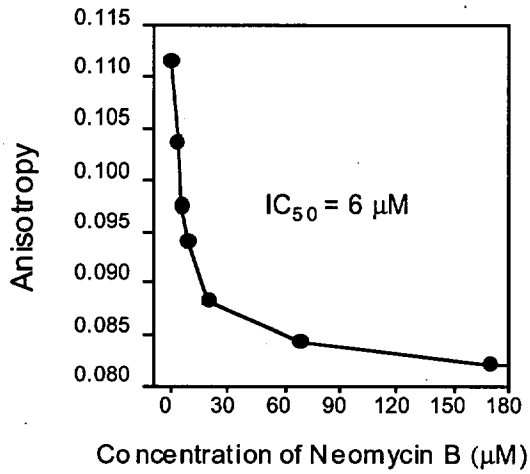


Figure S1b. Example of a fluorescence anisotropy displacement experiment, where the Rev-RRE complex is disrupted with increasing concentration of inhibitor.

Assembly of the Solid-Phase Immobilized Rev-RRE Complex

Solid-phase based experiments were done essentially as described [Luedtke, N.W.; Tor, Y. *Angew Chem. Intl. Ed.* **2000,** *39*, 1788-1790]. "ImmunoPure" beaded agarose covalently modified with stretavidin was purchased from Pierce. Immediately before use, the resin is washed with 3 volumes of buffer (under "Experimental Conditions")with gentle mixing (by inversion of the tube). After each wash (4 total) the slurry of solid-support and buffer is gently centrifuged (1,000 rpm 30 s) to settle the agarose beads, and the buffer removed by pipette. Following the last wash, 500 µl of dilute biotinylated RRE (40 nM in buffer) is added to 1 mL of a 40% slurry (400 µl of beads and 600 µl of buffer) and immediately inverted to mix. The slurry is incubated at room temperature for 1.5 h with constant inversion for mixing. By monitoring absorbance of the supernatant at 260 nm, the efficiency of RRE immobilization is calculated. The loading efficiency is >95% for biotinylated RRE and is <1% for non-biotinylated RRE. Following RRE immobilization, 1 equivalent of Rev-Fl is added, incubated for 1 h, and the supernatant quantified for fluorescence intensity. Under these conditions, non-specific binding of Rev-Fl to the solid-support is not observed (as determined by addition of RevFl to streptavidin-agarose which lacks immobilized RRE).

Since a linear relationship between fluorescence intensity and concentration of Rev-Fl is established by a calibration of UV absorbance and fluorescence emission, the concentration of Rev-Fl in the supernatant (following incubation with the immobilized RRE) can be calculated by its fluorescence intensity. It is found that under the above loading conditions, ~85% of Rev-Fl is bound by the immobilized RRE. The K_d of the RevFl-RRE interaction on solid support is then calculated by assuming that 85% of the RRE is bound by Rev-Fl (which is confirmed by mixing a known volume of gel with 8M guanidinum HCl and quantifying the amount of Rev-Fl present on the gel itself). The effective "surface concentration" of the RRE is irrelevant in this special circumstance, since the surface volume of free RRE cancels with the surface volume of Rev-RRE complex:

K_d = [RRE][Rev-Fl]/[complex] = (moles of free RRE)[Rev-Fl] / (moles of complex)

A K_d of 2.5 +/- 2 nM is measured (the same affinity as measured by fluorescence anisotropy) and is found to be independent of the "effective loading" of the RRE on the agarose (from 0.1 pmoles RRE/ml gel through 2.0 pmoles RRE/ml gel). This suggests that the immobilized RRE complexes are non-interacting over this loading range.

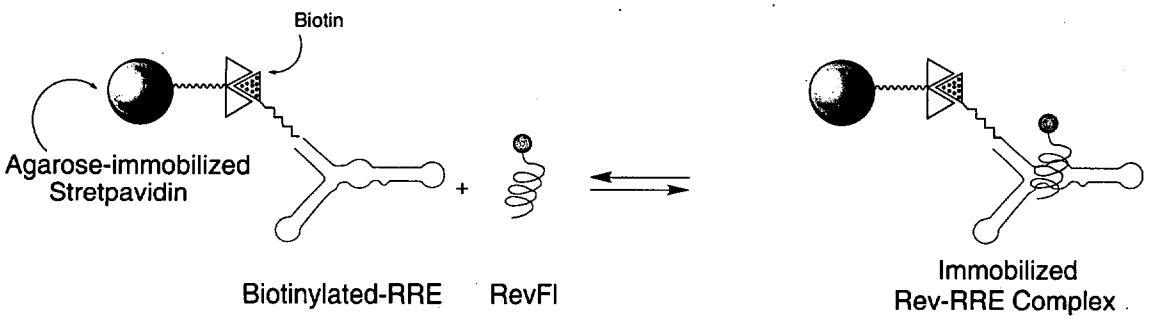


Figure S3a. Assembly of solid-phase immobilized Rev-RRE complex.

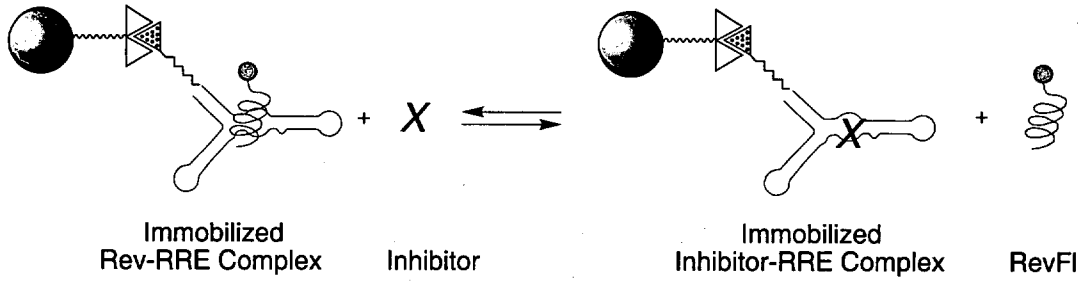


Figure S3b. Displacement of the Rev-Fl peptide into solution by inhibitor "X".

Conducting the Solid-Phase Assay

In 0.75 mL siliconized tubes, 200 µL of buffer (see "Experimental Conditions") is added, followed by 30 µL of a 30% slurry (gel volume / total volume) of the immobilized RevFL-RRE complex (at 0.5 pmole RRE/µL of gel). A small volume of concentrated inhibitor and/or competitor is added, mixed gently, and incubated at room temperature for 60 minutes with constant inversion for mixing. The tube is then gently centrifuged (1,000 rpm for 30 s) to settle the beads. 220 µL of supernatant is then added to 780 µL of "quantification buffer" (4M guanidinium HCl, 125 mM Tris HCl (pH 9.0), 125 mM KCl, 25 mM $MgCl_2$, 0.01% Nonidet P-40), and quantified for fluorescence intensity (Perkin Elmer LS-50B fluorimeter, maximum slit widths, Excitation 490 nm). Controls which establish the minimum (no inhibitor added) and maximum signals (200 µL of 8M guanidinium HCl added instead of buffer) are done with each set of experiments. IC_{50} values can then be established by measuring the concentration of inhibitor needed to displace half of RevFl into solution (see Luedtke, N.W.; Tor, Y. *Angew Chem. Intl. Ed.* **2000,** *39*, 1788-1790 for further details and an example titration).