SUPPORTING INFORMATION

SUPPLEMENTARY METHODS

Western Blot. Protein samples containing 5 μg were separated by SDS PAGE using 12 % acrylamide gels. The protein bands were transferred to nitrocellulose membrane and rabbit serum containing anti-p24 antibody (NIH AIDS Reagents Program) was used at 1 : 100,000. A second anti-rabbit horseradish peroxidase-conjugated antibody (Jackson ImmunoResearch) at 1 : 10,000 and ECL - Western Blot Detection Reagents (GE Healthcare) was used to reveal the presence of cleaved and uncleaved GST-X-mV protein.

SUPPLEMENTARY FIGURE LEGENDS

SUPPLEMENTARY FIGURE 1: Quality assessment of the GST-X-mV fusion protein. (a) Western blot analysis of GST-Gag processing by HIV-1 PR. 5 μg GST-Gag (without mVenus) was incubated with 100 nM protease. Over time samples were removed and analyzed by SDS PAGE and western blot using anti-p24 antibody. Cleavage products appeared overtime, starting with p82, followed by p27, and then p25. (b) MALDI TOF MS spectrum of 10 μM GST-Gag, not containing mVenus, after 2 hrs incubation with 25 nM HIV-1 PR demonstrating the various Gag cleavage products. (c) Time-resolved MALDI mass spectrometry (MS) analysis of protease-mediated GST-MA-CA (without mVenus) processing. Samples were taken over time and analyzed by MALDI MS. (d) The affinity matrix is not cleaved by HIV-1 PR. 5 μg of the monoclonal anti-GST antibody was incubated with 100 nM HIV-1 PR, analyzed by SDS PAGE, and proteins detected by coomassie staining. The light and heavy chain of the antibody were not cleaved by protease.

SUPPLEMENTARY FIGURE 2: Determination of bead binding capacity for GST fusion proteins. (a) The binding of the GST fusion protein, GST-X-mV, to anti-GST antibody coated beads was analyzed to determine the bead binding capacity. For evaluation of bead saturation, differing amounts of GST-p2/NC_{CS}-mV were incubated with 10^6 beads, washed, and then analyzed by flow cytometry. The fluorescence increases with higher concentrations of the fusion protein and follows a sigmoid curve in the semi-log plot. From the saturation plateau we estimate that approximately 0.5 -1.0 x 10^6 molecules of GST-p2/NC_{CS}-mV bind per bead. (b). Emission scans of the fusion protein immobilized on beads. GST-MA/CA_{CS}-mV or GST-4xG_{CS}-mV was immobilized on beads and incubated with protease. The emission spectrum was recorded using a Tecan Saphire fluorescence spectrometer. As can be seen protease does

not alter the spectral features of the mVenus protein. Experimental conditions: t = 60 min; T = 37 ºC; [S: GST-MA/CA_{CS}-mV or GST-4xG_{CS}-mV] = 10 μM; [PR] = 50 nM; excitation wavelength = 488nm. (c) An alternative platform of the GST-X-mV fusion protein. The StrepII-MA-CA-mV fusion protein contains a Strep-tagII instead of the N-terminal GST anchor which binds to strepavidin-coated beads [38] (scheme: upper panel). The lower panel refers to a dilution series of StrepII-MA-CA-mV fusion protein. (d) On bead CE-CBA cleavage analysis of StrepII-MA-CA-mV using protease. StrepII-MA-CA-mV was immobilized on beads, incubated with protease, and then analyzed by flow cytometry. The protease-mediated processing of the MA/CA CS decreases the fluorescence on the beads, which can be quantified. Experimental conditions: t = 60 min; T = 37 ˚C; [S: StrepII-MA-CA-mV] = 1 μM; [PR] = 100 nM.

SUPPLEMENTARY FIGURE 3: (a) Time resolved cleavage kinetics of the protease thrombin and the substrates GST-p2/NC_{CS}-mV or GST-Thr_{CS}-mV using on bead CE-CBA. GST-p2/NC_{CS}-mV or GST-Thr_{CS}-mV was immobilized on beads and incubated with thrombin. Over time samples were taken and analyzed by flow cytometry. The normalized fluorescence intensity vs. time plot showed a decrease of fluorescence intensity for the correct enzyme - substrate pair. The resulting values could be fitted using a single exponential equation. The incorrect enzyme-substrate pair showed no fluorescence decay. Experimental conditions: t = 60 min; T = 37 ˚C; [GST-Thr_{CS}-mV] = 1 μM; [thrombin] = 8 U, buffer = 20 mM Tris-HCL pH 8.4, 150 mM NaCl, 2.5 mM CaCl2. (b) Time resolved cleavage kinetics of HIV protease and the substrates GST-p2/NC_{CS}-mV or GST-Thr_{CS}-mV using on bead CE-CBA. The experiment was carried out as described previously in a. Only the HIV-1 substrate showed a fluorescence decay indicating cleavage of the appropriate substrate. Experimental conditions: t = 60 min; T = 37 ºC; [GST-p2/NC_{CS}-mV] = 1 μM; [PR] = 50 nM; buffer = 20 mM MES pH 6.0, 200 mM NaCl, 2.5 % glycerol at 37 ºC.

SUPPLEMENTARY FIGURE 4: Comparison of IC_{50} determination utilizing CE-CBA and fluorogenic substrate assay for the low affinity aspartyl peptidase inhibitor, pepstatin A and the high affinity HIV-1 inhibitor, amprenavir (a) Shown are the normalized rFU values vs. the log[inhibitor] concentration plotted values of the in solution CE-CBA cleavage analysis. Selected concentrations of pepstatin A were incubated with 0.025 μM PR and 5 μM GST-MA/CA_{CS}-mV at 37 ºC and then captured on beads and analyzed by flow cytometry as described previously. The IC_{50} was determined using the 4 parameter logistic model from Origin 7.0. (b) Selected concentrations of pepstatin A were incubated with 0.025 μM PR and 30 μM of the fluorescently labeled anthranilyl protease substrate Abz-Thr-Ile-Nle-p-nitro-Phe-Gln-Arg-NH2, H2992. The initial velocity was calculated and the IC_{50} was determined using the 4 parameter logistic model by Origin 7.0. (c) CE-CBA-based IC_{50} analysis of amprenavir. Selected concentrations of amprenavir were incubated with 0.0025 μM PR and 5 μM GST-MA/CA_{CS}-mV at 37 ºC and then captured on beads and analyzed by flow cytometry as described previously. The IC_{50} for high affinity inhibitor was determined using the Morrison quadratic equation [21].

(d) FSA-based IC_{50} determination of amprenavir. A concentration series of the PI amprenavir was incubated with 0.0025 μM PR and 30 μM of the fluorescently labeled anthranilyl protease substrate, H2992. The initial velocity was calculated and the IC_{50} was determined using the Morrison quadratic equation [21]. Mean and standard deviation values are shown. Assessment of protease inhibitor potency, protease inhibitor resistance, and screening for new inhibitors has been aided through the use of FSA [22]. Since FSA is an accept standard methodology for obtaining IC_{50} values for protease inhibitors, we evaluated whether in solution CE-CBA produced similar or distinct IC_{50} values as compared to FSA for the moderate affinity μM protease inhibitor pepstatin A, and the high affinity, nM clinical HIV-1 protease inhibitor amprenavir. The IC_{50} values determined for pepstatin A, 1.6 ± 0.1 μM, and amprenavir, 0.19 ±

0.05 nM, were obtained from in solution CE-CBA analysis of GST-MA/CA_{cs}-Vm. Utilizing FSA, with the fluorescently labeled anthranilyl substrate Abz (aminobenzoyl)-Thr-Ile-Nle-p-nitro-Phe-Gln-Arg-NH2, the pepstatin A and amprenavir IC_{50}s were found to be 1.1 ± 0.2 μM and 0.18 ± 0.03 nM, respectively.

SUPPLEMENTARY FIGURE 5: Validation of the multiplexed HTS CE-CBA robustness. The Z'-factor is a statistical means to pre-quantify the suitability of a particular assay for use in a full-scale, high-throughput screen (*36*). Z'-factors in the range 0.5 - 1 indicate, with closer to 1 a better score, that the assay tested is highly suitable for use as a high-throughput screen. Shown are the rFU values from the positive control (without protease, indicating 100 % inhibition of protease processing) and negative control (with protease, indicating no inhibition of protease processing) of on bead CE-CBA using GST-CA-p2-mV (n = 3, each run 100 samples). The Z'-factor was calculated according to the equation by Zhang and co-workers: Z'-factor = $1 - (3\sigma_{(+)} + 3\sigma_{(-)})/|\mu_{(+)} - \mu_{(-)}|$. $\sigma_{(+)}$ and $\sigma_{(-)}$ represents the standard deviation and $\mu_{(+)} - \mu_{(-)}$ represents the positive and negative control mean values (*36*). The Z'-factor determined was 0.837 ± 0.003 (mean ± s.e.m.), indicating that the on bead GST-CA-p2-mV CE-CBA is suitable for high-throughput screening of inhibitors targeting Gag MA-CA or CA-p2.

Supplementary Table 1

CS	PR	k_{cat}/K_M ($mM^{-1}s^{-1}$)	fold increase
NC-p1(wt)*	wt	37.5 ± 3.9	1.0
NC-p1(K436E,I437V)*	wt	121.4 ± 7.7	3.2
NC-p1(wt)	wt	14.2 ± 0.2	1.0
NC-p1(A431V)	wt	30.9 ± 0.5	2.2
NC-p1(wt)	I84V	4.1 ± 0.01	0.3
NC-p1(A431V)	I84V	11.5 ± 0.9	0.8
p1-p6(wt)	wt	6.7 ± 0.2	1.0
p1-p6(L449F)	wt	29.7 ± 0.2	4.4
p1-p6(wt)	I84V	1.6 ± 0.3	0.2
p1-p6(L449F)	I84V	7.0 ± 0.3	1.0
p1-p6(T456S)	wt	13.8 ± 0.5	2.0

* = experiment was carried out at 37 °C

k_{cat}/K_M mean values and s.e.m. of wild type and mutant Gags and PRs were calculated from CE-CBA experiments (n = 3) as previously described.

Supplementary Table 2

CS	PR	Rate (rFU s^{-1})	fold increase
NC-p1(wt)	wt	77	1.0
NC-p1(A431V)	wt	185	2.4
NC-p1(K436E,I437V)	wt	439	5.7
p1-p6(wt)	wt	946	1.0
p1-p6(L449F)	wt	6167	6.5

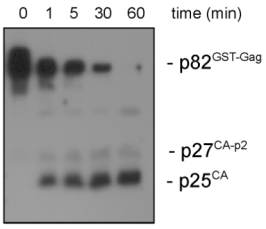
Representative initial rate values of wild type and mutant Gags and PRs were calculated from CE-CBA experiments (n = 2).

Supplementary Table 3

CS	PR	IC_{50} (nM)	fold increase
NC-p1(wt)	wt	0.19 ± 0.05	1.0
NC-p1(A431V)	wt	0.17 ± 0.05	0.9
NC-p1(K436E,I437V)	wt	0.18 ± 0.05	0.9
p1-p6(wt)	wt	0.17 ± 0.04	1.0
p1-p6(L449F)	wt	0.21 ± 0.04	1.2

IC_{50} mean values and s.e.m. of wild type and mutant Gag and PR were calculated from CE-CBA experiments (n = 3) as previously described

a



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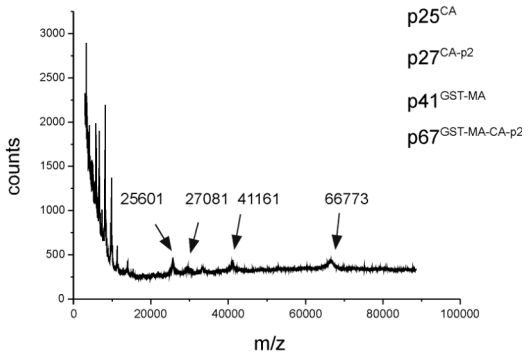

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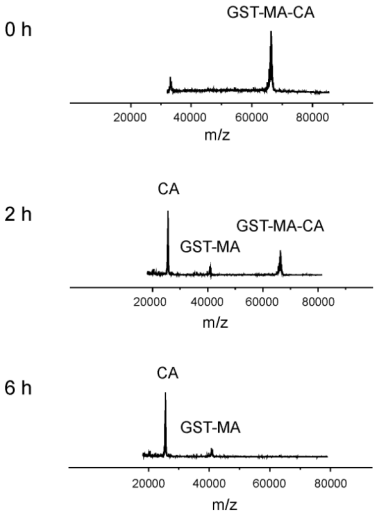
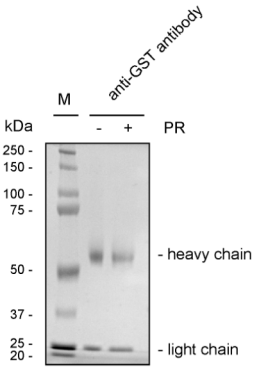
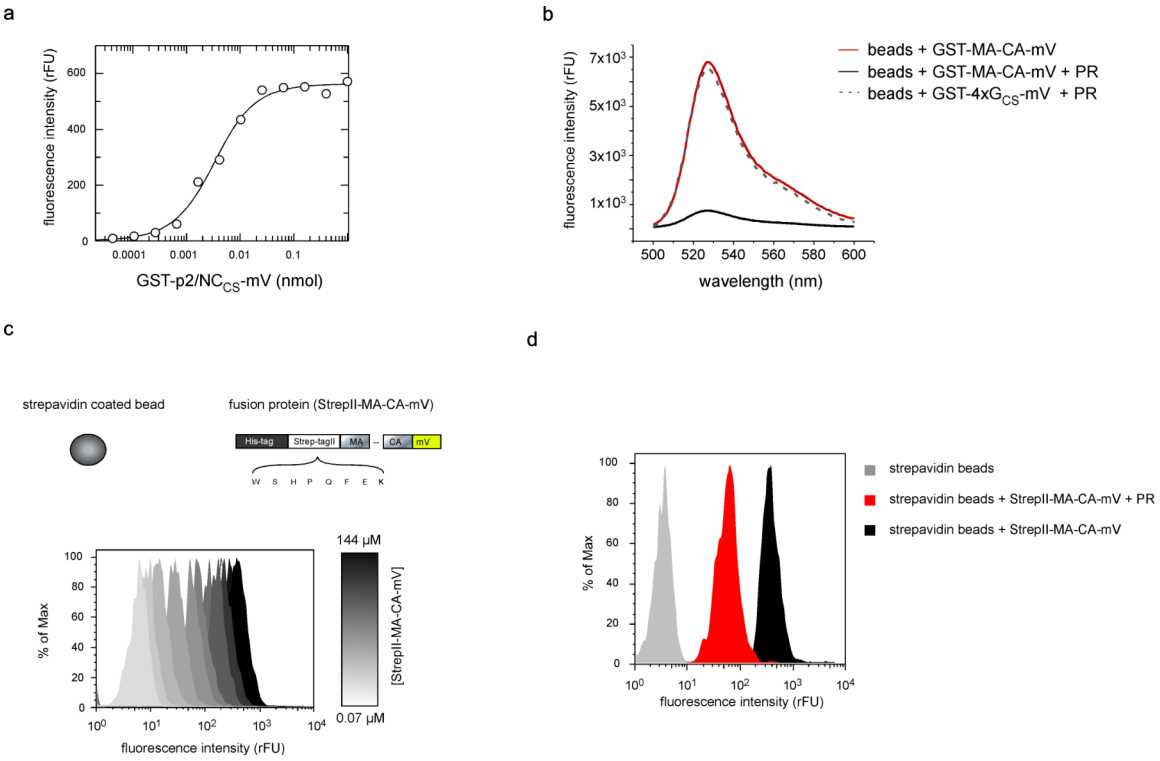


	MALDI MS determined MW	theoretical MW
GST-MA-CA	= 66,320 Da	66,352 Da
GST-MA	= 40,725 Da	40,767 Da
CA	= 25,594 Da	25,603 Da

d

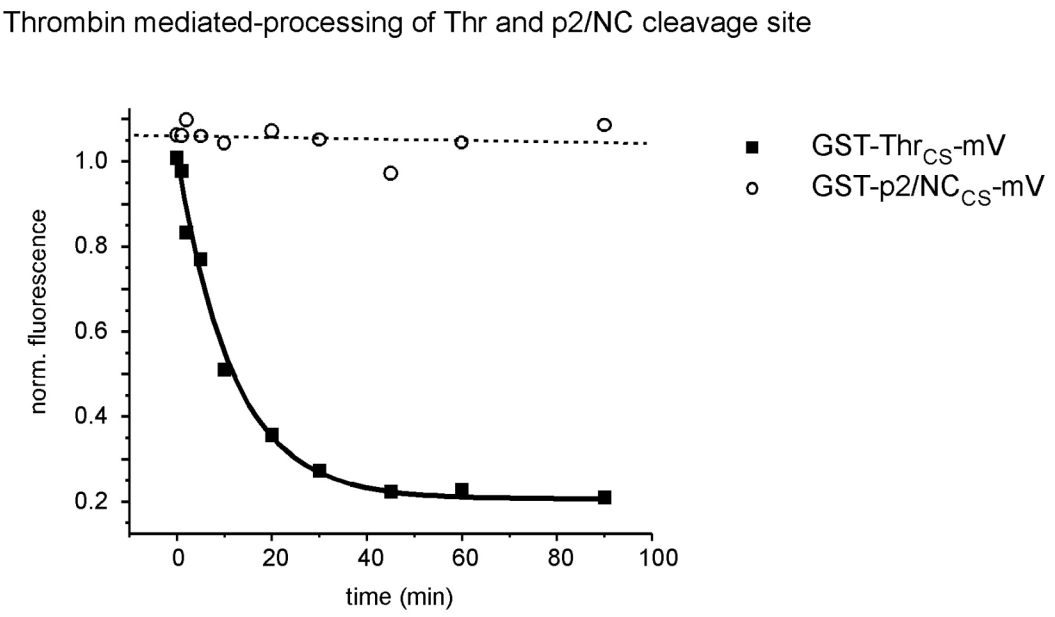


Supplementary Figure 1



Supplementary Figure 2

a



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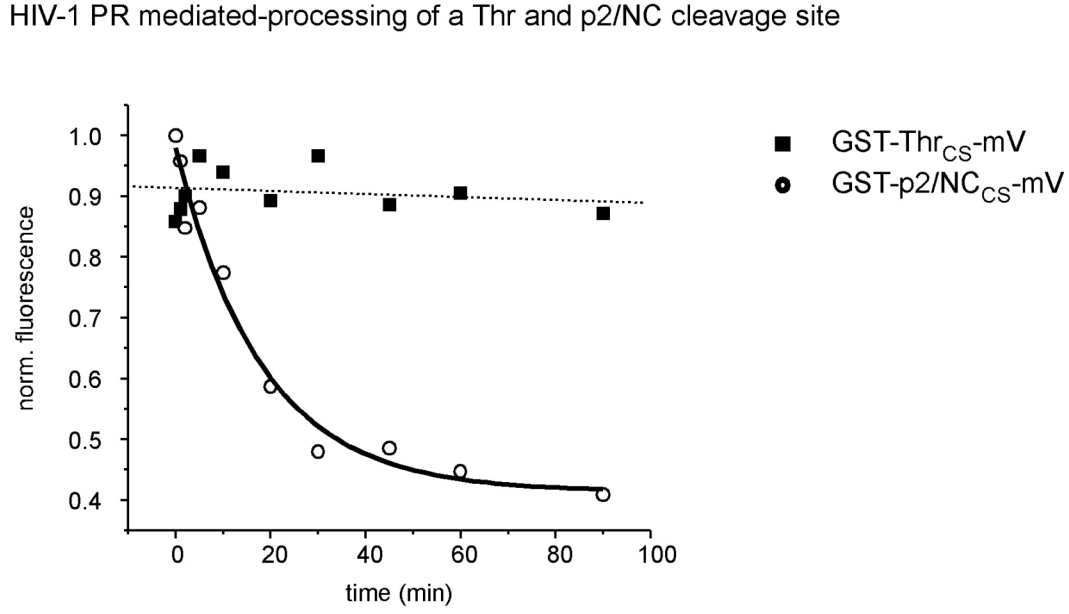
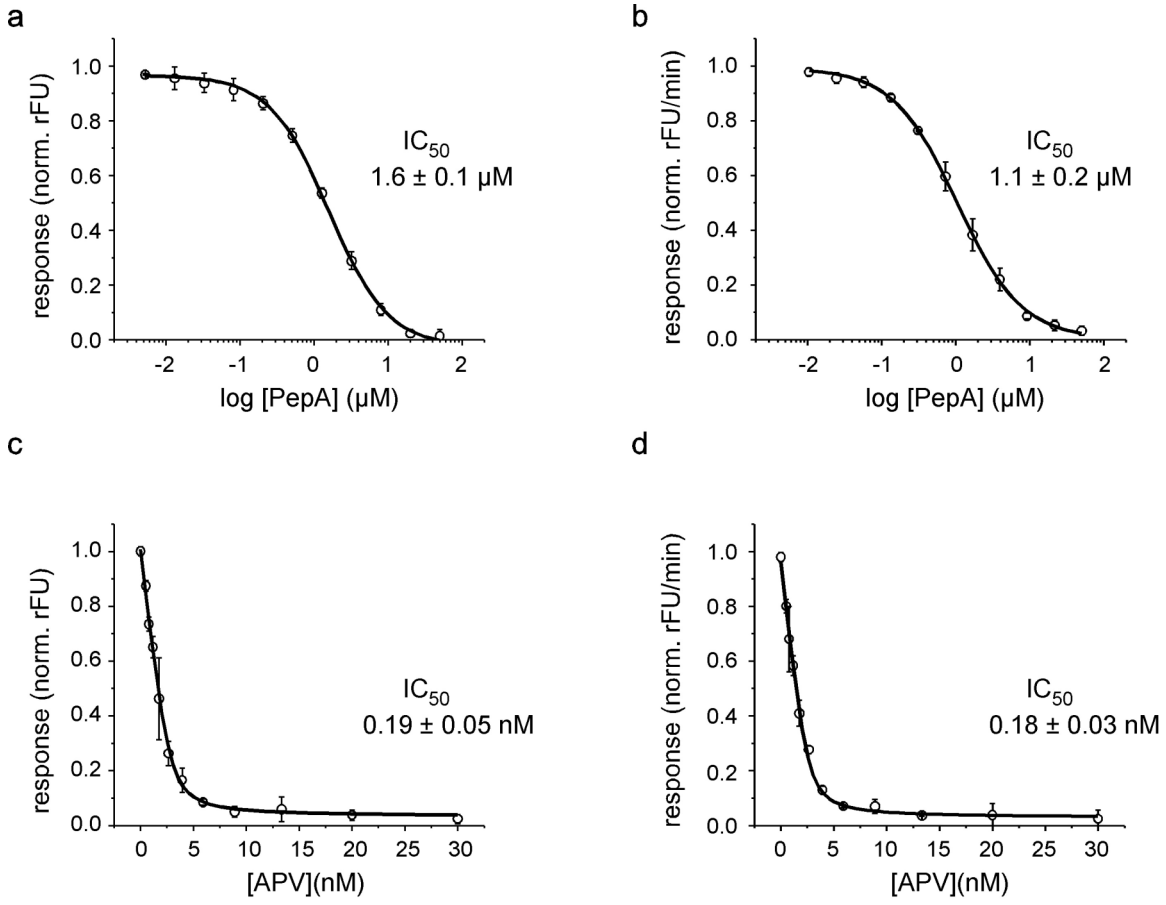
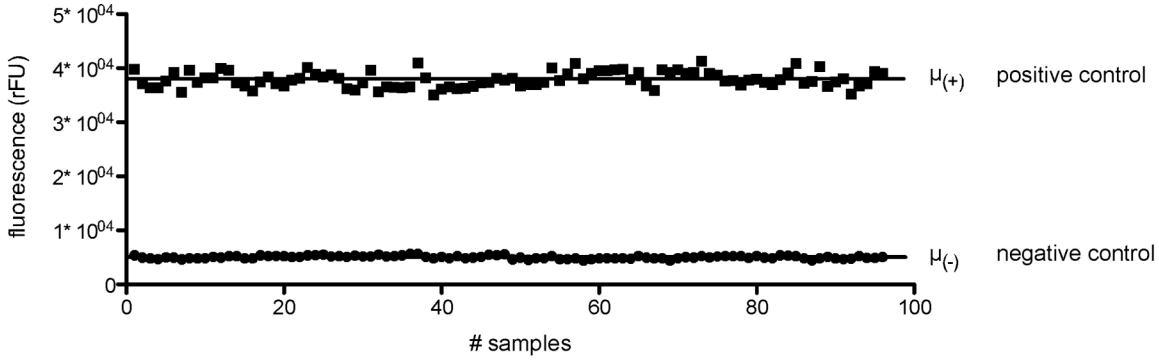


Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5