Data supplement:

Development and Validation of a High-Content Bimolecular Fluorescence Complementation Assay for Small Molecule Inhibitors of HIV-1 Nef Dimerization

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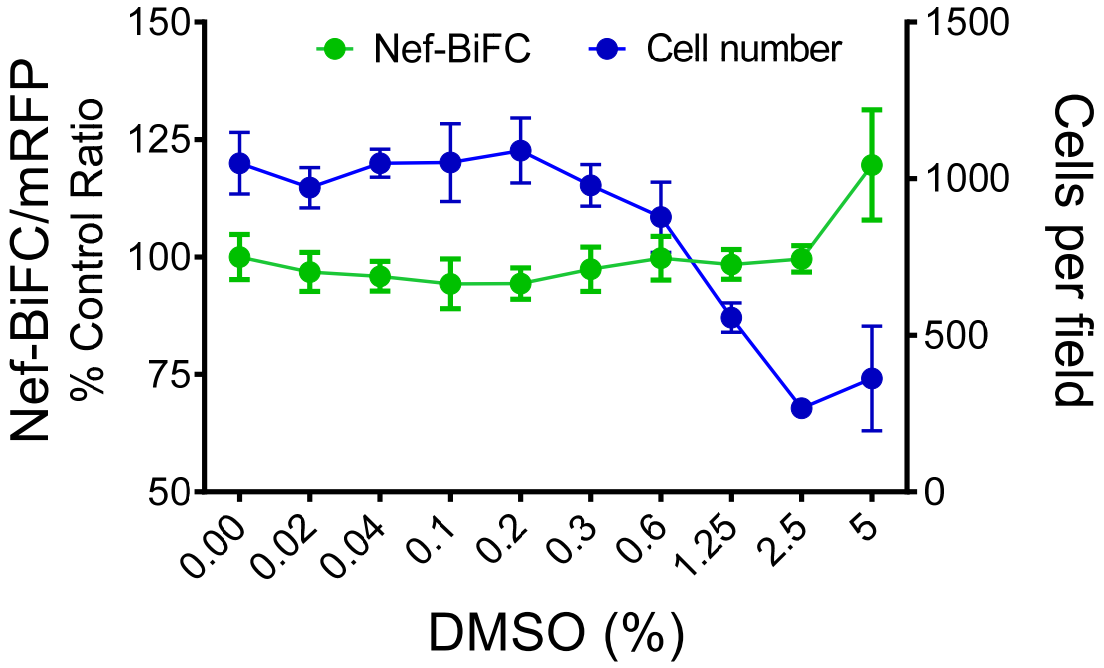
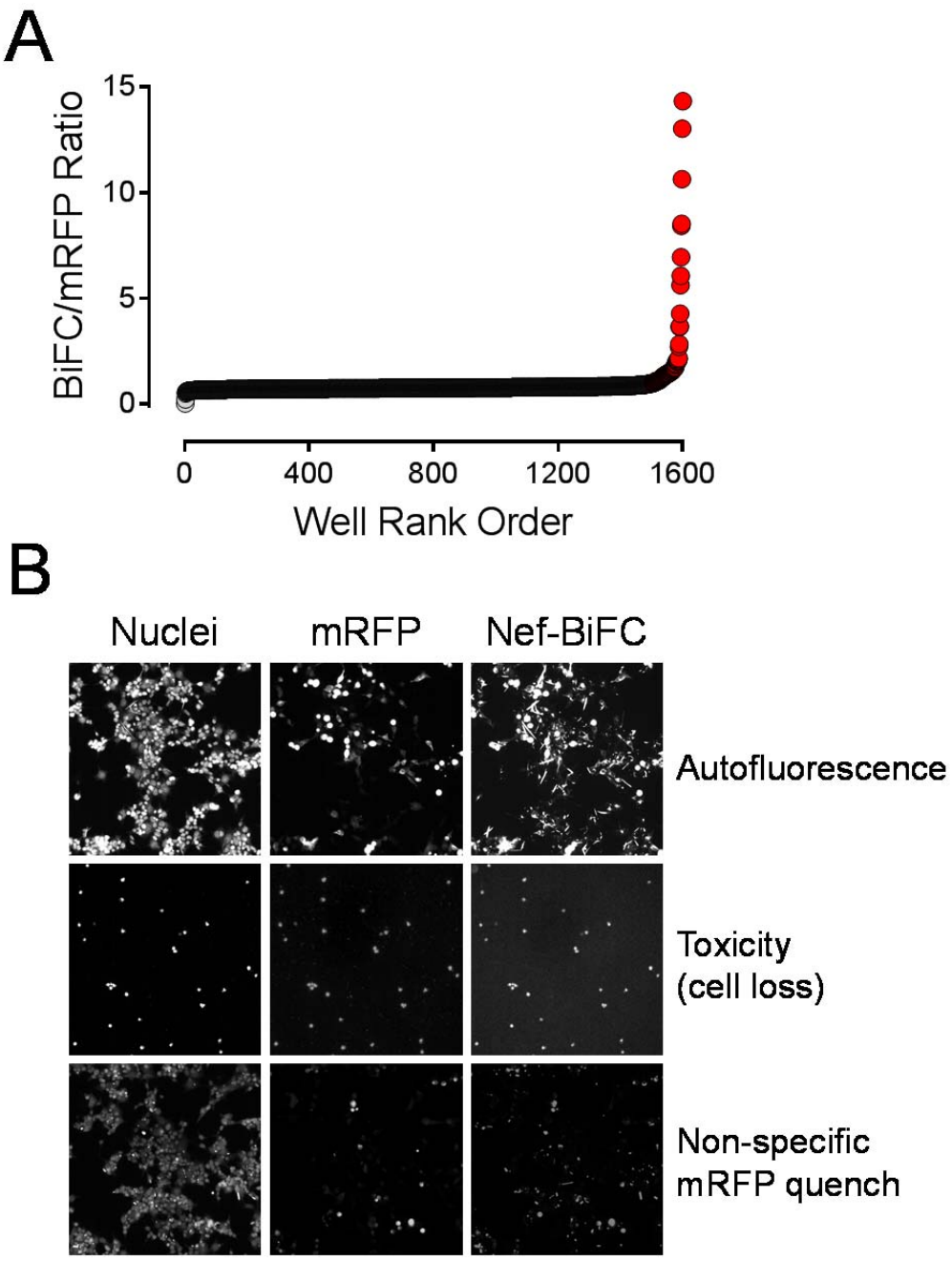
Supplemental Figures S1 – S5

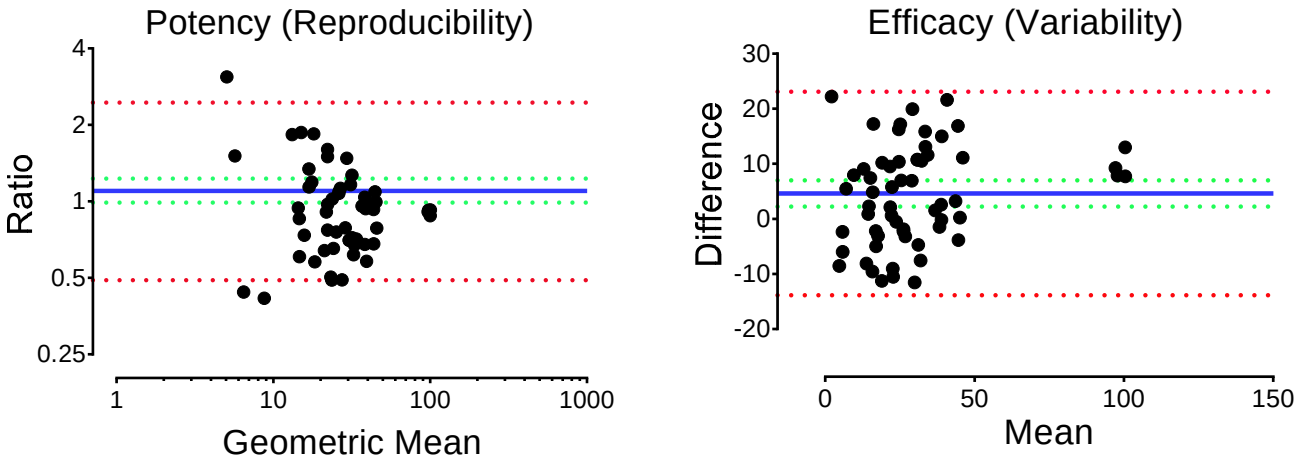
Supplemental Tables S1 and S2



Supplemental Figure S1. Comparison of the Nef-BiFC/mRFP fluorescence ratio at increasing concentrations of DMSO in 293T cells expressing the Nef-BiFC biosensor. Transfected cells were cultured in the absence or presence of the DMSO concentrations shown. Forty-eight hours later, nuclei were stained with Hoechst 33342, and images were acquired in the DAPI (nuclei), Texas red (mRFP) and FITC (Nef-BiFC) channels. Nef-BiFC/mRFP ratios were calculated and are plotted as the mean percent of untreated controls ± S.D. for sixteen wells (*green*). The number of cells present per well was estimated from the nuclear stain and is plotted as the mean ± S.D. for sixteen wells (*blue*). DMSO enhances cell loss above 0.6%; all subsequent screening assays were performed at 0.1% DMSO to minimize this effect.

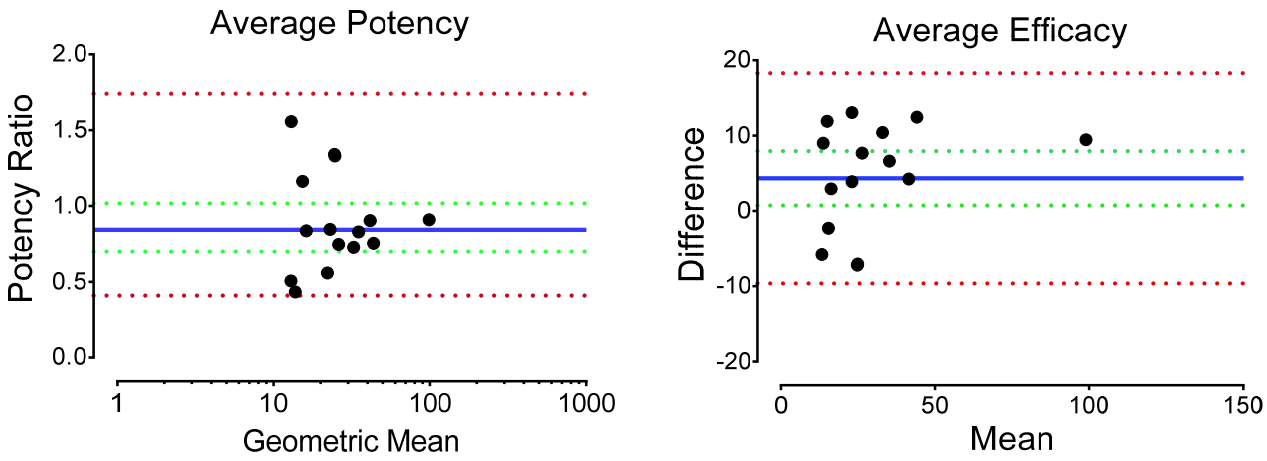


Supplemental Figure S2. Pre-processing of pilot screen data from the NCI Diversity Set III. The NCI Diversity Set III of ~ 1,600 compounds was screened using the automated Nef-BiFC assay in transfected 293T cells as described in the text. Nef-BiFC/mRFP ratios were calculated for each well, and outliers were defined as having a Nef-BiFC/mRFP ratio greater than three standard deviations above the mean of the untreated control wells for each plate. *Top:* The graph shows the rank order of the BiFC/mRFP ratios for all 1,600 compounds, with the outliers highlighted in red. *Bottom:* Representative ArrayScan images of treated cells producing artificially high BiFC/mRFP ratios. Such compounds typically were autofluorescent (top row), caused substantial cell loss as a surrogate measure of toxicity (middle row) or were non-specific quenchers of mRFP fluorescence (bottom row).



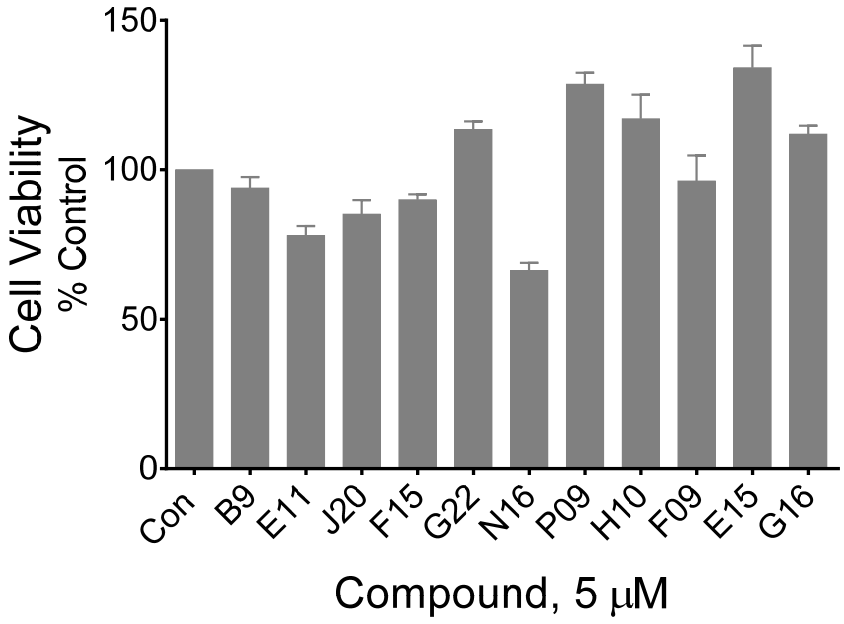
Supplemental Figure S3. Assessment of compound reproducibility and variability. Automated Nef-BiFC assays were conducted in transfected 293T cells using the top 1% of compounds from the pilot screen of the NCI Diversity Set, which span a range of potencies. Two independent trials were conducted on separate days with each compound tested in quadruplicate wells. *Left panel:* Comparison of the potency values between the two independent runs showed the assay meets established NIH criteria[1] for reproducibility with a MR = 1.10 (*blue line*), RLs in the range 0.99-1.23 (*green lines*), MSR = 2.21 and LsA in the range of 0.49-2.45 (*red lines*). *Right panel:* Compound efficacy also passed established criteria with an MD = 4.61 (*blue line*), DLs in the range 2.23-6.99 (*green lines*), MSD = 18.46 and LsAd in the range -13.85 to 23.08 (*red lines*).

[1] http://www.ncbi.nlm.nih.gov/books/NBK83783



Supplemental Figure S4. Average compound reproducibility and variability. The replicate values obtained in Supplemental Figure S3 were averaged for a global comparison of compound potency and efficacy. The Ratio-GM plot (*left panel*) meets the NIH criteria[1] for reproducibility with a MR =0.84 (*blue line*), RLs in the range 0.69-1.02 (*green lines*), MSR=2.06 and LsA in the range 0.41-1.74 (*red line*). The Difference-Mean plot (*right panel*) compared compound efficacy and passed all established criteria with an MD = 4.32 (*blue*), DLs in the range 0.72-7.93 (*green lines*), MSD=13.96 and LsAd in the range -9.63 to 18.28 (*red lines*).

[1] http://www.ncbi.nlm.nih.gov/books/NBK83783



Supplemental Figure S5. Cytotoxicity assessment of hit compounds. 293T cells were cultured in 96-well plates and incubated with the compounds that reproducibly inhibited Nef dimerization in the BiFC assay (Figure 6) at a final concentration of 5.0 μM. After 48 h, cytotoxicity was assessed using the Cell Titer Blue assay (Promega) and the manufacturer's protocol. Compounds were assayed in triplicate, and data are presented as mean percent viability relative to the DMSO-only control (Con) ± S.D.

Class	Day	Plates	Max/Min	Pass/Fail	Mean	SD	CV	Z-factor	S:B
Intra-plate	1	1	max	Pass	0.86	0.06	7.32	0.56	2.69
		2	max	Pass	0.9	0.07	7.37	0.56	2.77
		3	min	Pass	0.32	0.02	5.6	0.53	2.81
		4	min	Pass	0.31	0.02	6.11	0.54	2.9
		All		**Pass**				**0.53**	**2.77**
	2	1	max	Pass	0.95	0.07	7.22	0.64	3.28
		2	max	Pass	0.94	0.06	6.6	0.63	3.17
		3	min	Pass	0.29	0.01	4.67	0.68	3.24
		4	min	Pass	0.3	0.01	5.04	0.67	3.13
		All		**Pass**				**0.63**	**3.23**
	3	1	max	Pass	0.98	0.07	6.72	0.52	2.72
		2	max	Pass	0.92	0.06	6.32	0.52	2.8
		3	min	Pass	0.36	0.03	6.99	0.52	2.56
		4	min	Pass	0.35	0.03	7.95	0.53	2.63
		All		**Pass**				**0.51**	**2.63**
Inter-plate	1	1 and 2	max	Pass	0.88	0.07	7.82		
		3 and 4	min	Pass	0.32	0.02	5.94	0.52	2.75
	2	1 and 2	max	Pass	0.95	0.07	6.95		
		3 and 4	min	Pass	0.29	0.01	5.02	0.64	3.28
	3	1 and 2	max	Pass	0.95	0.07	7.26		
		3 and 4	min	Pass	0.36	0.03	7.62	0.49	2.64
Day-to-day	1 & 2	All	max	Pass	0.91	0.08	8.23		
			min	Pass	0.31	0.02	6.8	0.50	2.94
	2 & 3	All	max	Pass	0.95	0.07	7.11		
			min	Pass	0.33	0.04	12.19	0.47	2.88

Supplemental Table S1. Three-day variability assays. Nef-BiFC assays were independently tested in four sets of 384-well plates on three separate days. Assays on each day consisted of two plates each of the wild-type Nef-BiFC biosensor (max) and dimerization-defective Nef-4D mutant as negative control (min). All plates passed quality control with a Z-factor > 0.5.

Well ID (alias)	PubChem Substance	BiFC/mRFP Ratio	Control % Inhibition	Z-score	Pseudo % Inhibition
E11	NSC31762	x	x	x	x
J20	NSC50648	x	x	x	x
F15	NSC66020	x	x	x	x
G22	NSC168221	x	x	x	x
N16	NSC140873	x	x	x	
P09	NSC156565	x	x	x	x
H10	NSC108753	x	x	x	x
F09	NSC326375	x	x	x	x
E09	NSC146771	x	x	x	x
E15	NSC227186	x	x	x	x
E12	NSC119805	x	x	x	x
G16	NSC298197	x	x	x	x
F06	NSC67546	x	x	x	x
F13	NSC51351	x	x	x	x
C03	NSC121868	x	x	x	x
C04	NSC73053	x	x	x	x

Supplemental Table S2. Top 1% of compounds identified as potential inhibitors of HIV-1 Nef dimerization from a pilot screen of the NCI Diversity Set III using the Nef-BiFC assay. The corresponding PubChem NSC number for each compound is hyperlinked to the PubChem record which includes the chemical structure. For details of data analysis, please see main text.