Supporting Material.

Material and Methods

Plasmid Construction. The pRSF-Duet1 was obtained from Novagen (EMD Biosciences, Darmstadt, Germany), the TetA(B) gene (*Tn10*) from laboratory stocks of a pBAD vector, the HIV-1 protease gene was a gift from Kelli L. Kuhen (Genomics Institute of the Novartis Research Foundation), and the HCV NS3-NS4a fusion gene lacking the helicase domain was synthesized by Genscript (Piscataway, NJ). DNA primers were obtained from IDT DNA (see Supplementary Table 1). Restriction enzymes were obtained from New England Biolabs (Bethesda, MD), and sequencing performed by Genewiz (San Diego).

The pRSF-Duet1 vector was first modified to replace the kan resistance marker with β-lactamase (*bla*) from a pET101D (Invitrogen) vector. The *bla* gene was PCR amplified with primers AMPtoRSF F and AMPtoRSF R, digested with *DrdI* and *SphI*, and ligated to the similarly digested pRSF-Duet1 vector to create pRSF(Amp).

The pRSF-Duet1 vector harbors two T7 promoters; the second was replaced by an *rrnB* terminator to stop transcription of the first gene cassette and a *GlnS* promoter to drive *Tn10* in the second gene cassette. To place *Tn10* under the *GlnS* promoter, it was PCR amplified with TetF NdeI and TetR PstI, digested with *NdeI* and *PstI*, and ligated to a similarly digested pEVOL vector.[1] Conveniently this vector harbors an *rrnB* terminator upstream of the GlnS promoter; therefore the *rrnB-PGlnS-Tn10* cassette was amplified with primers PtetT SalI F and PtetT XhoI R, digested with *SalI* and *XhoI* and ligated into the similarly digested pRSF(Amp) vector to create pRSF-Tet. This replaced the second T7 promoter with the *rrnB-GlnS* terminator-promoter region and maintained the T7 terminator at the end of the cassette.

Overlap PCR was used to insert protease sequences into the *Tn10* gene. The HIV protease sequence (SQNYPIV) was designed with primers OVRtetHIV F and OVRtetHIV R; the HCV protease sequence (GEAGDDIVPCSMSYTWT) with primers lTetHCV F and lTetHCV R, and the HCV protease sequence (DDIVPCSMS) with primers sTetHCV F and sTETHCV R. PCR was performed in 2 steps. Forward primers (F) were combined with primer PtetT XhoI R in set 1 PCRs and Reverse primers (R) were combined with primer PtetT SalI F in set 2 PCRs. PCRs from set 1 and set 2 were gel extracted, mixed at a 1:1 ratio, and overlap PCR amplified with primers PtetT SalI F and PtetT XhoI R. The overlap PCR products were digested with *SalI* and *XhoI* and ligated to the similarly digested pRSF-Tet.

Mutant (catalytically inactive) proteases genes were created with a modified Quikchange mutagenesis (Stratagene, La Jolla, CA) protocol. The catalytic aspartate (D25) of HIV protease was mutated to an alanine using primers HIV D256A F and HIV D25 R. The mutant NS3 protease S139A was synthesized by Genscript. These mutations render the protease inactive allowing for a positive control as the cells will be viable in the presence of tetracycline.

Mutant or wild type HIV and NS3 were cloned into their respective pRSF-Tet vectors by PCR amplification with primers HIV NcoI F and HIV EcoRI R; or HCV NcoI F and HCV EcoRI R, to create pRSF-HIV and pRSF-HCV, respectively. PCR products were digested with *EcoRI* and *NcoI* and ligated to the similarly digested respective pRSF-

Tet vector. pRSF-protease vectors sequences and maps are listed below (see Supplementary Figs. 1 and 2).

The cyclic peptide library was prepared as previously described by Tavassoli and Benkovic. To confirm library preparation and diversity, the pBK-In plasmid was sequenced both as a pool and as isolated library plasmids (see Supplementary Fig. 3). Sequencing results yielded the expected codon distribution and presence of the TAG codon. The pEVOL plasmids employed were previously described by Young *et. al.*

Assay Optimization. The most effective conditions for the selection were determined by transforming BL21(DE3) or DH10B(DE3) with wild type or a catalytically inactive HIV protease mutant (D86A) and growing bacteria at a range of IPTG and tetracycline concentrations. BL21(DE3) or DH10B(DE3) *E. coli* harboring the pEVOL-*p*BzF plasmid and pRSF-HIV-Tet(HIV) or pRSF-Mutant HIV-Tet(HIV) were grown overnight in 2xYT media at 37 ºC to saturation with chloramphenicol (40 μg/mL) and ampicillin (100 μg/mL) and diluted in 2xYT to an OD_{600} of 0.1. The cultures were then spotted (2 μL/drop) on LB Agar plates containing a range of tetracycline concentrations (0-25 μg/mL) and IPTG concentrations (0, 0.001, 0.01, and 0.1 mM) and incubated for 48 hours at 37 ºC. Cell viability was then assessed under the varied conditions to ascertain the optimal concentrations of Tet and IPTG for the selections to be 7 μg/mL and 0.001 mM respectively (see Supplementary Fig. 4) in the BL21(DE3) strain. Interestingly, differential tetracycline sensitivity was not observed in the DH10B(DE3) strain.

Cyclic Peptide Growth Curves. A cyclic peptide plasmid containing the sequence SQNYPIV was prepared according to the previous protocol. [2] To assess the effects of an unnatural amino acid on the SQNYPIV competitive inhibitor the TAG codon used to replace each residue of the peptide. Thus, 7 mutants were prepared with a modified Quikchange mutagenesis (Stratagene, La Jolla, CA) protocol to contain the TAG codon at each amino acid position of the sequence. Cyclic peptide plasmids were then transformed into electrocompetent *E. coli* BL21(DE3) cells possessing the pEVOL-*p*AcF and pRSF-HIV-Tet(HIV) plasmids and plated on LB agar containing chloramphenicol (20 μg/mL), ampicillin (50 μg/mL) and kanamycin (50 μg/mL). Isolated colonies were picked and grown to saturation in 2xYT media with chloramphenicol, ampicillin, and kanamycin, then diluted to OD_{600} 0.1 and transferred to a 96 well plate (100 μL/well) in triplicate and in the presence or absence of tetracycline (5 μg/mL), arabinose (0.02%) and *p*-acetylphenylalanine (1 mM). A negative control was also employed utilizing the pRSF-Mutant HIV-Tet(HIV) plasmid. The plate was then incubated in a Molecular Biosystems Spectromax 250 plate reader at 37 ºC with constant shaking. OD_{600} measurements were taken every 10 minutes over the span of 10 hours (see Supplementary Fig. 5). In the absence of arabinose no growth was observed, but when grown in the presence of p-acetylphenylalanine and arabinose all mutants were capable, to some extent, of inhibiting HIV protease, albeit no mutant demonstrated a significantly improved inhibition of the natural competitive inhibitor. However, these experiments demonstrate the feasibility of utilizing cyclic peptides to inhibit HIV protease.

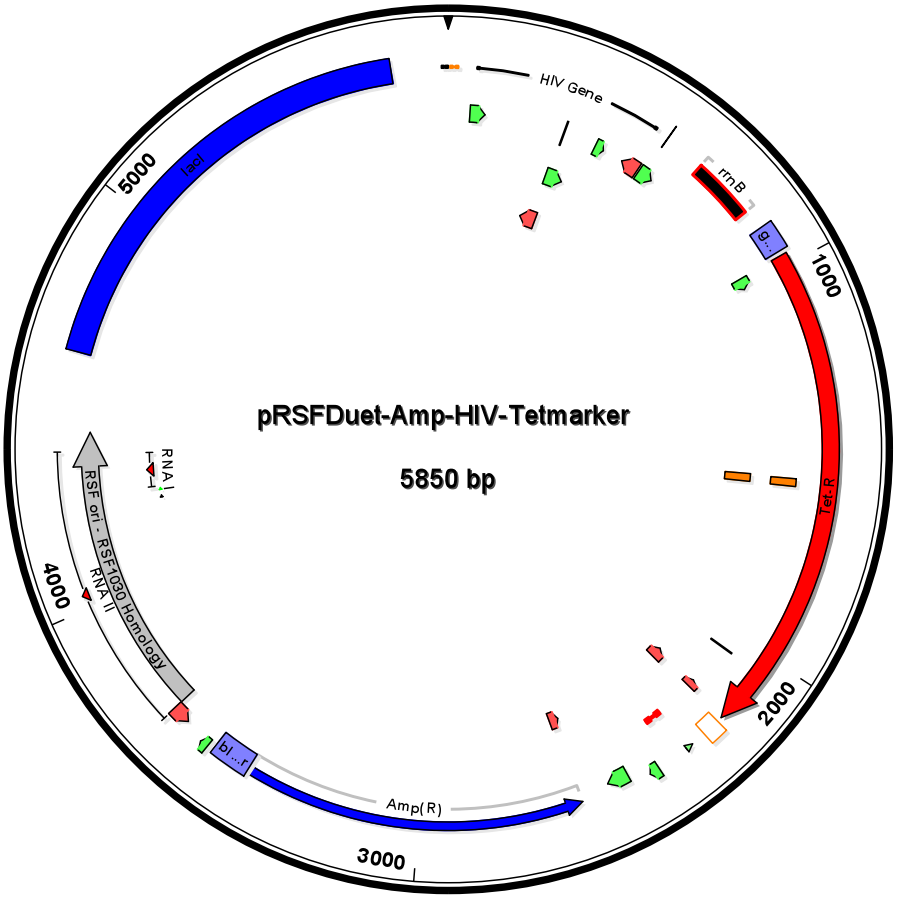
Cyclic Peptide Inhibition Asssay. Independently synthesized cyclic peptides (CPC Scientific) were obtained and 2 mM stocks were prepared in DMSO and serial dilutions were prepared in distilled water. HIV protease was purchased from EMD Biosciences (Darmstadt, Germany) and used in conjunction with a SensoLyte 520 HIV-1 Protease Assay Kit (AnaSpec, Fremont, CA). HIV protease was diluted to a concentration of 0.5 μg/mL using 1X Assay buffer and incubated for 30 min at 37 ^{o}C with peptides (either linear or cyclic) at final concentrations of 0.1, 0.5, 1, and 2 μM in 1X Assay buffer in triplicate. The diluted fluorogenic HIV substrate was then added and the rate of cleavage was measured using a Spectramax Gemini EM (Ex/Em= 490/520 nm; Molecular Probes) over a 1 hour period. The initial slope of the curves was then utilized to calculate IC_{50} values.

Supplementary Table 1. List of primers used for cloning

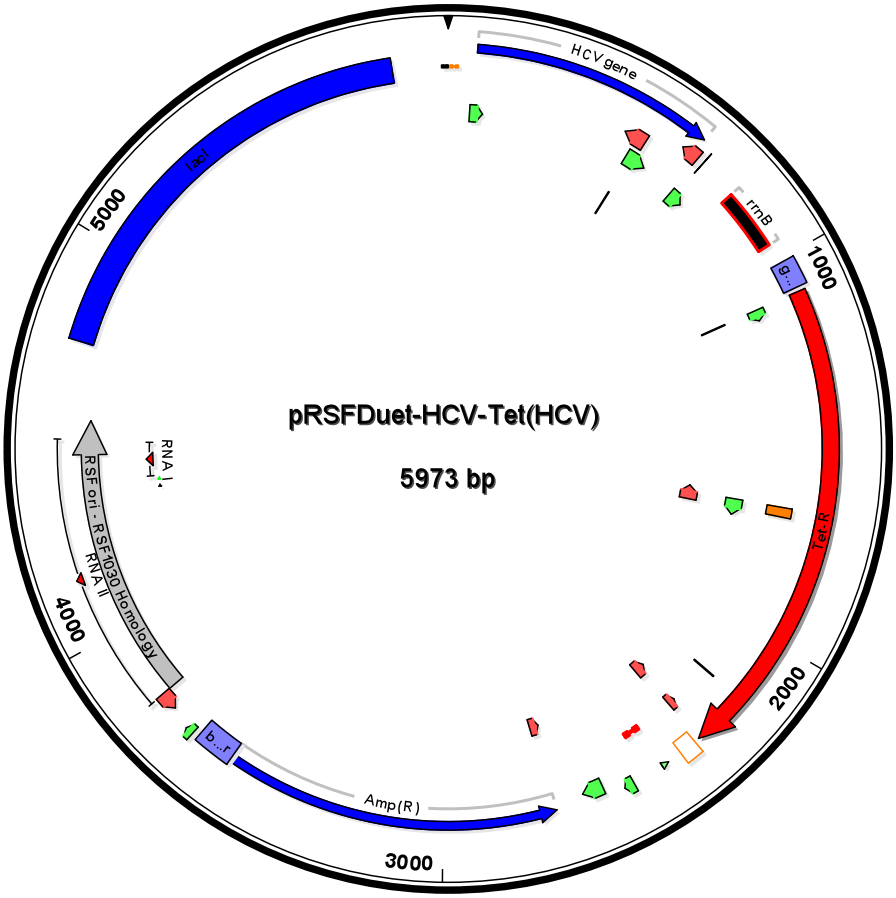
Primer	Sequence
rpsL-pET F	CAC CAT GGC AAC AGT TAA CCA GCT GGT ACG CAA A
rpsL-pET R	ATT AAG CCT TAG GAC GCT TCA CGC CAT ACT TGG A
AMPtoRSF F	ACAAGCTGACGACCGGGTCGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTC
AMPtoRSF R	GAGCGCTGCATGCCAAACATGAGAATTAATTCTTGAAGACGAAAGGGC
TetF NdeI	GTTCAGGTCATATGATGAAATCTAACAATGCGCTCATCGTCATCC
TetR PstI	GTTCAGGTCTGCAGTCAGGTCGAGGTGGCCCGGCT
PtetT SalI F	CCTGCAGGTCGACTGAGTTTAAACGGTCTCCAGCTTGGCTGTT
PtetT XhoI R	TACCAGACTCGAGTCAGGTCGAGGTGGCCCGGCTC
OVRtetHIV F	CGT CGA AGT CAG AAC TAC CCG ATC GTT CGA CCG ATG CCC TTG AGA GCC TTC
OVRtetHIV R	AAC GAT CGG GTA GTT CTG ACT TCG ACG CTC TCC CTT ATG CGA CTC CTG CAT TAG G
lTetHCV F	TGACGACATCGTTCCGTGCTCTATGTCTTACACCTGGACCCGACCGATGCCCTTGAGAGC
lTetHCV R	GACATAGAGCACGGAACGATGTCGTCACCAGCTTCACCACGCTCTCCCTTATGCGACTCC
sTetHCV F	TGACGACATCGTTCCGTGCTCTATGTCTCGACCGATGCCCTTGAGAGCCTTC
sTETHCV R	CGAGACATAGAGCACGGAACGATGTCGTCACGCTCTCCCTTATGCGACTCCTGC
HIV NcoI F	GAGATATACCATGGTTTTTAGGGAAGATCTGGCCTTCCTACA
HIV EcoRI R	CGAGCTCGAATTCTCAAAAATTTAAAGTGCGACCAATCTGAGTCAACAGA
HCV NcoI F	GAGATATACCATGGGCAGCGTGGTTATTGTGGGTCGC
HCV EcoRI R	CCGAGCTCGAATTCTCAAGAGCGCATCGTGGTTTCCAGATTTTCCAC

.

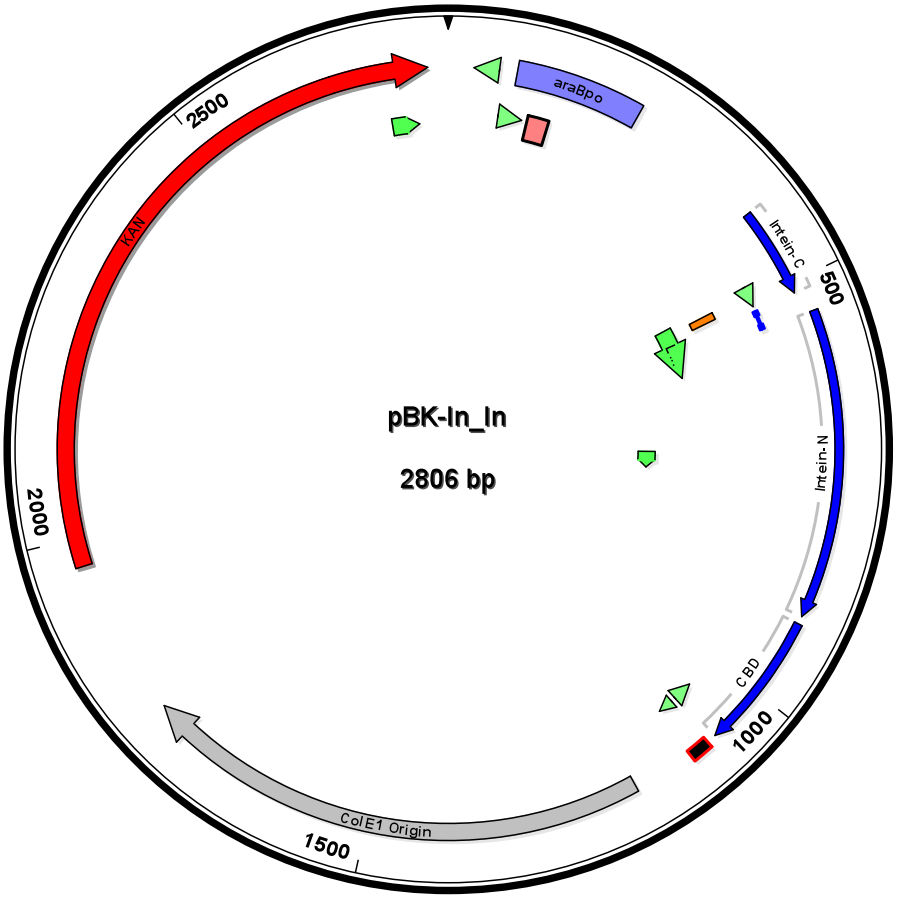
Supplementary Figure 1. Map of pRSF-HIV-Tet(HIV)



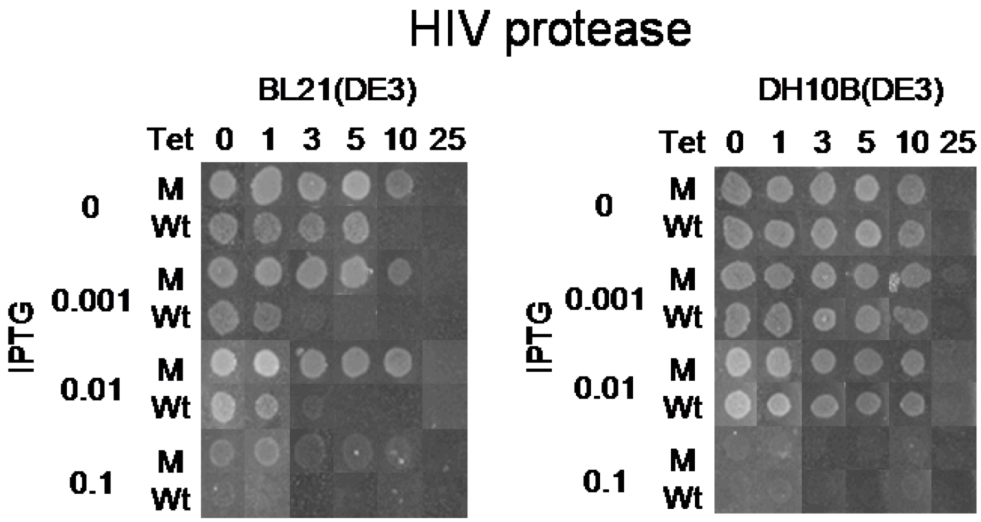
Supplementary Figure 2. Map of pRSF-HCV-Tet(HCV)



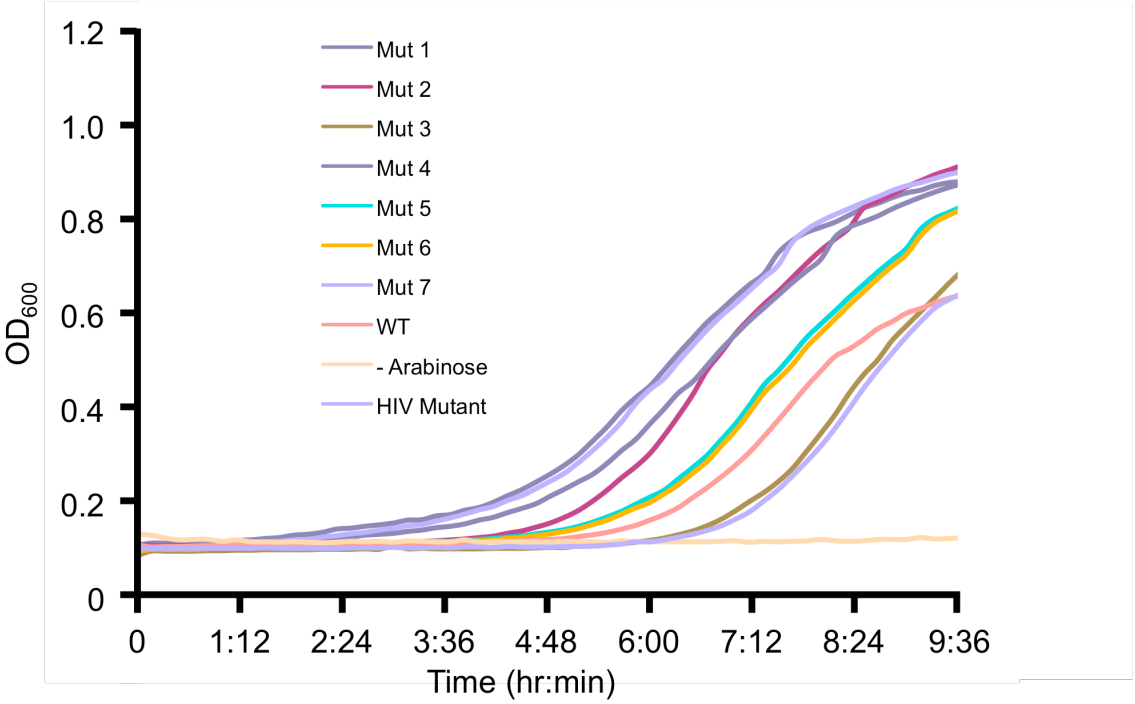
Supplementary Figure 3. Map of pBK-In



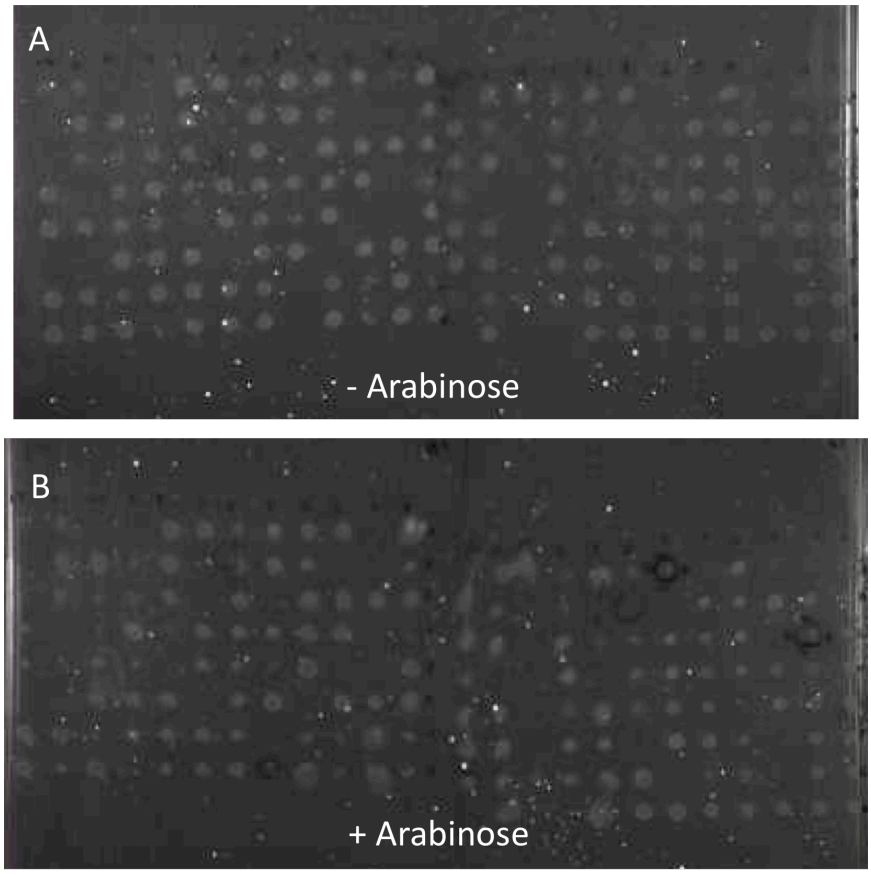
Supplementary Figure 4. Optimization of Selection Conditions.



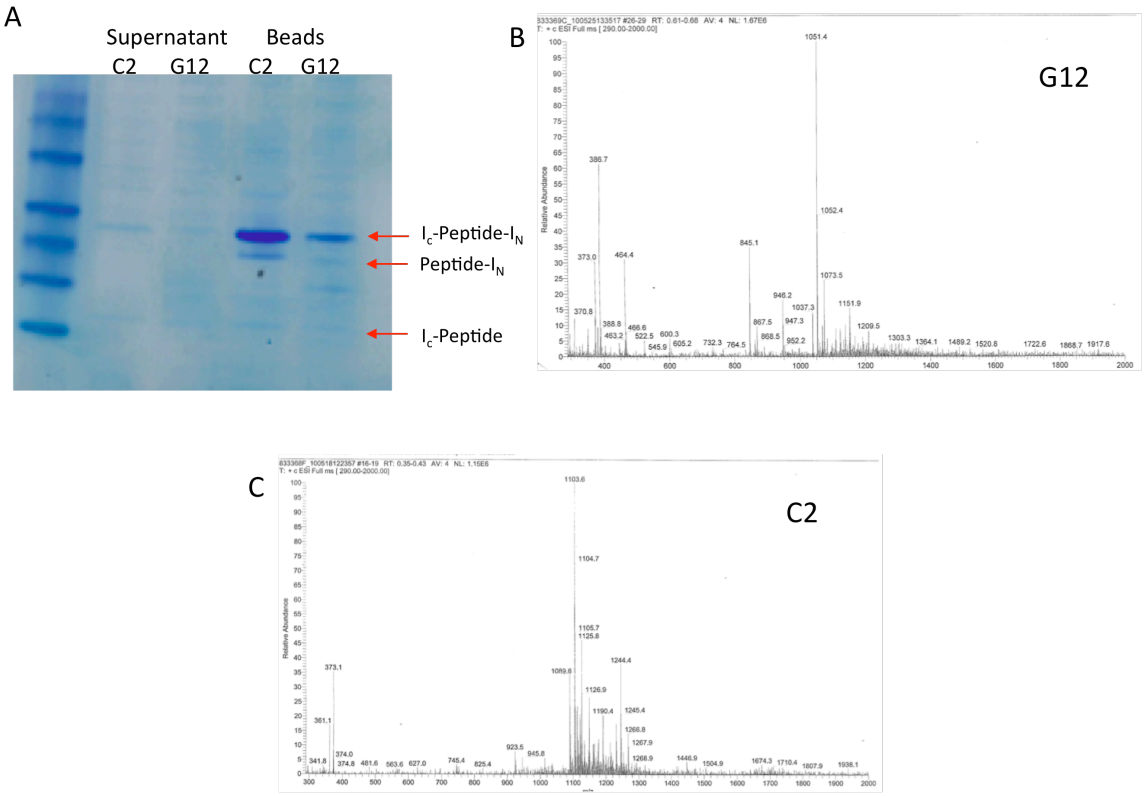
Supplementary Figure 5. Growth Curves in the Presence of an SQNYPIV Cyclic Peptide Inhibitor (WT), and its Corresponding Unnatural Amino Acid Containing Analogs (Mut 1-Mut7).



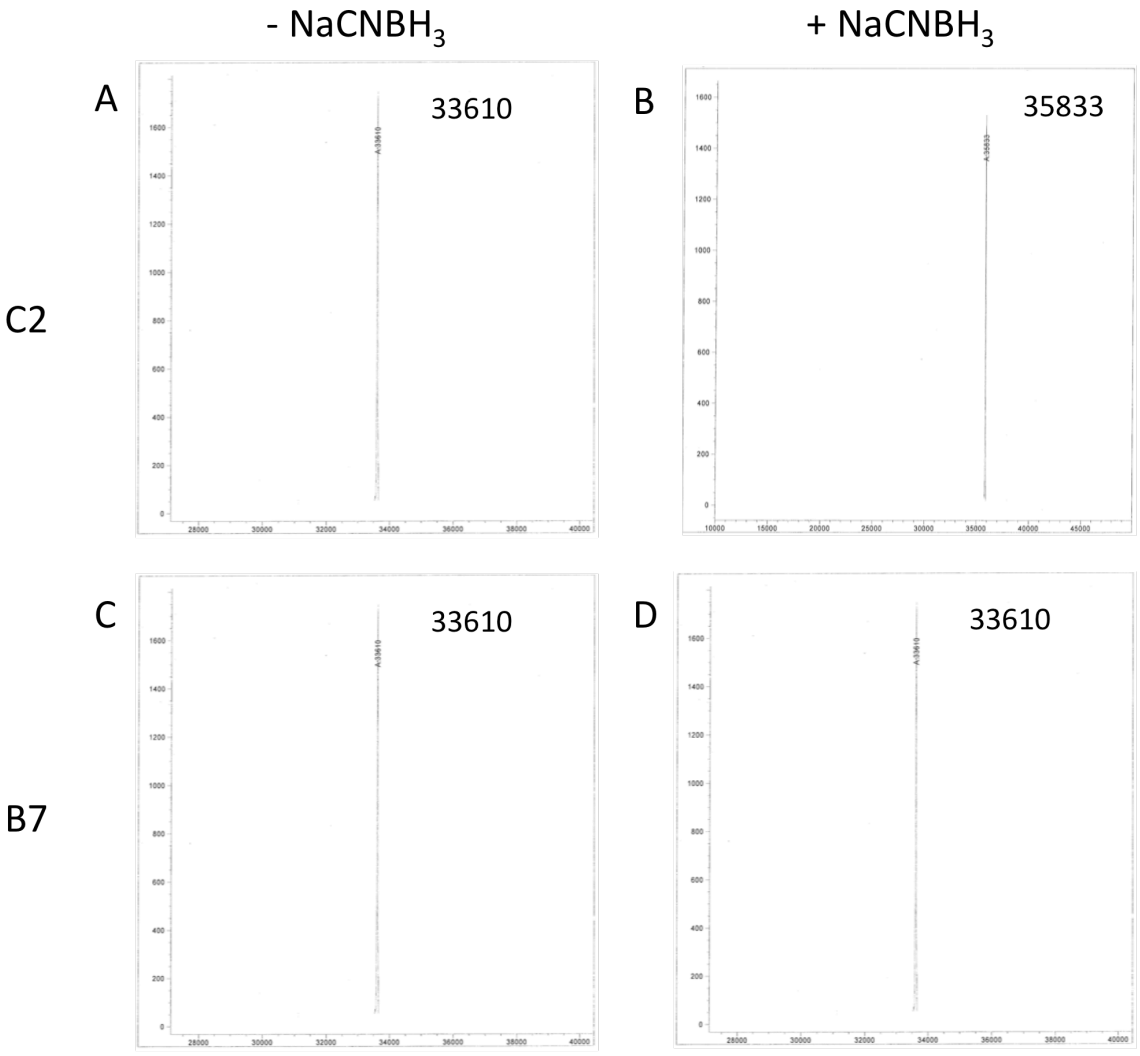
Supplementary Figure 6. Initial identification of cyclic peptide hits. Positive colonies were grown in liquid culture and stamped on plates containing appropriate inductants and *p*BzF in the absence (A) or presence (B) of arabinose.



Supplementary Figure 7. Validation of the expression of cyclic peptide hits. A) SDS-PAGE analysis of both boiled chitin beads and supernatant. B) Mass spectrum of G12 hit from a large scale expression (1L) followed by MeOH extraction. C) Mass spectrum of C2 hit from a large-scale expression (1L) followed by MeOH extraction.



Supplementary Figure 8. Mass spectra of cyclic peptides C2 and B7 incubated with HIV protease (0.5 μg) in the presence or absence of $NaCNBH_3$ (5 mM) for 16 h at 37 ^{o}C. The C2 analog increases in mass by 2223 Da only in the presence of reductant, corresponding to 2 cyclic peptides linked to the HIV protease dimer (1 per subunit). Similar results were obtained for cyclic peptide G12, also possessing an aryl ketone (data not shown).



References.

1 Young, T. S., Ahmad, I., Yin, J. A. & Schultz, P. G. An Enhanced System for Unnatural Amino Acid Mutagenesis in E. coli. *J Mol Biol*, (2009).

2 Tavassoli, A. & Benkovic, S. J. Split-intein mediated circular ligation used in the synthesis of cyclic peptide libraries in E. coli. *Nat Protoc* **2**, 1126-1133, (2007).