The carba-LNA modified siRNAs targeting HIV-1 TAR region downregulates HIV-1 replication successfully with Enhanced Potency

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Figure S1

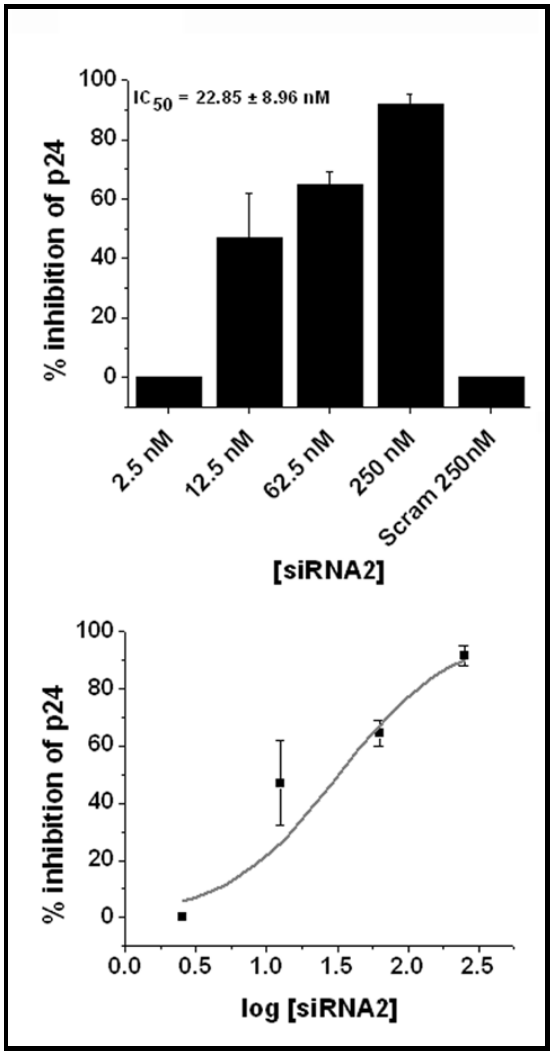


Figure S1: Dose response studies using p24 ELISA for native siRNA2 showing % inhibition over virus control at various doses. The dose titration sigmoidal plot is placed below bar plot. Results are a cumulative of at least three independent experiments. Error bars represent ±SD from mean. p24 ELISA was carried out with culture supernatants, 48 h post-co-transfection of pNL4-3 and varying doses of siRNA.

Figure S2

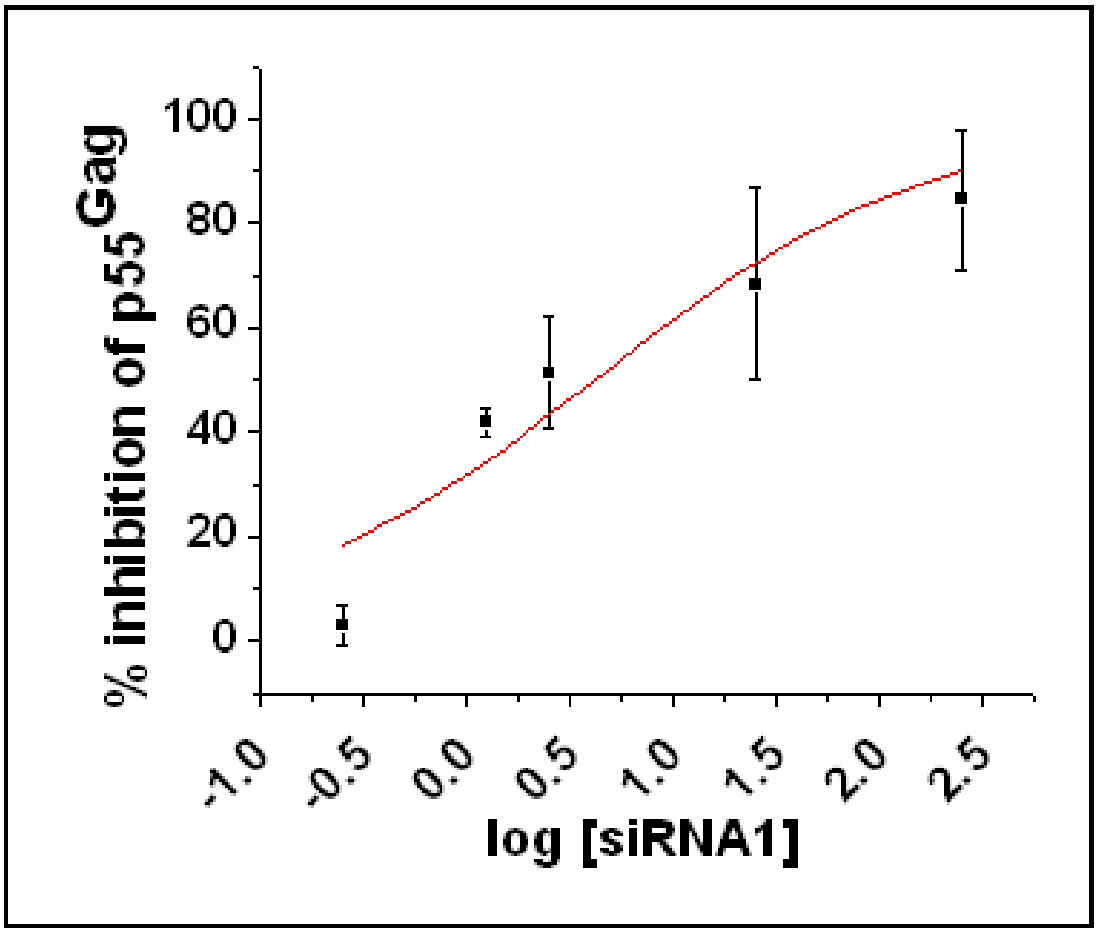


Figure S2: Dose titration plot obtained from Western Blot of native siRNA1 showing % inhibition of $p55^{Gag}$ over virus control for various doses of siRNA1. Results are cumulative of at least two independent experiments. Error bars represent ±SD from mean. Figures were generated by sigmoidal fit of log [siRNA] vs % inhibition of $p55^{Gag}$ plot using Origin software.

Figure S3

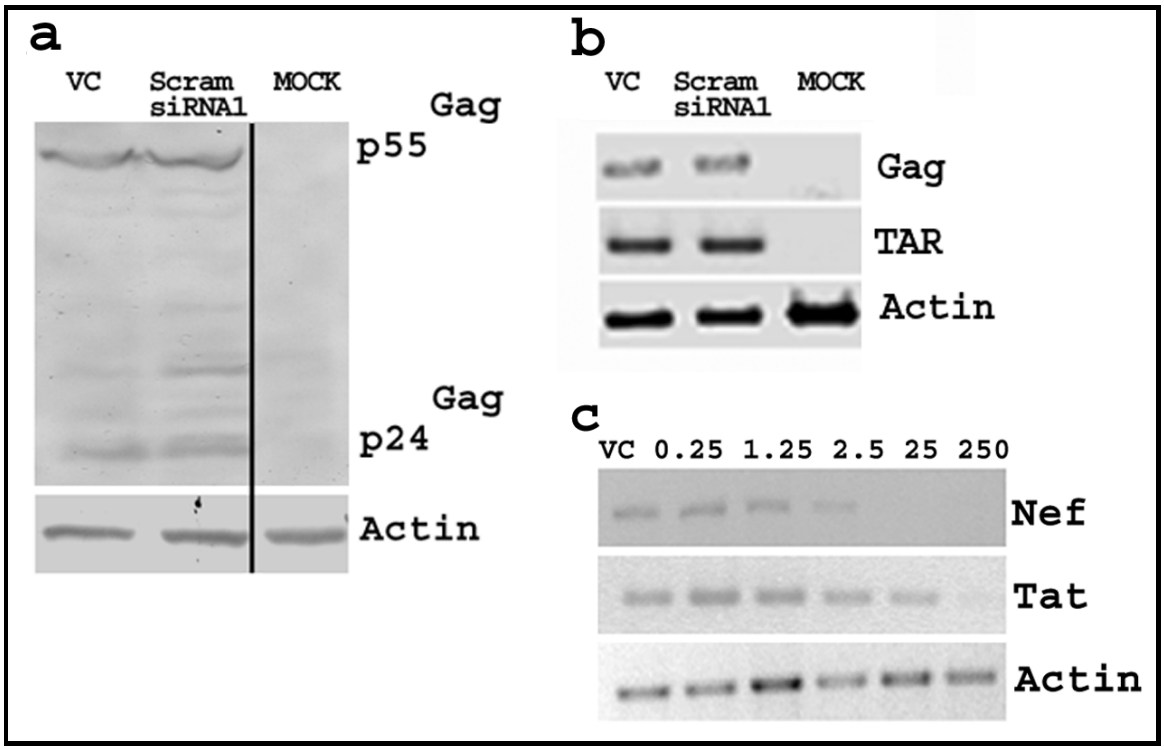
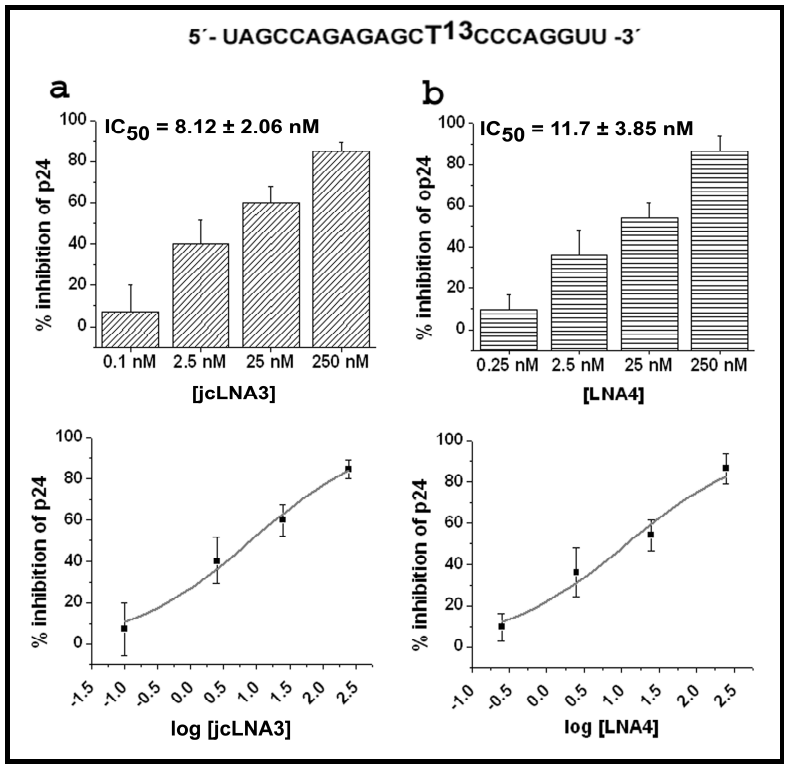
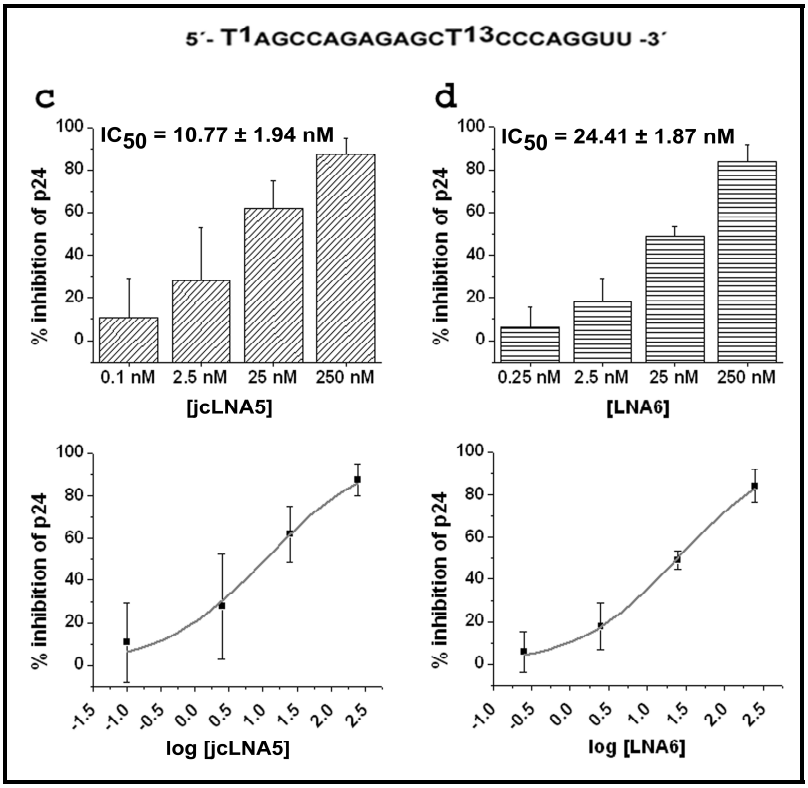


Figure S3: Western blot and RTPCR. a: Western blot of scrambled siRNA1 and mock transfected sample; b: RTPCR of scrambled siRNA1 (scram siRNA1 was used at 250 nM) and mock transfected sample for Gag, TAR and actin primers and c: RTPCR using Nef, Tat and actin primers and concentrations in nM is indicated above the gel. Blots and gels are representative of at least two independent experiments Western blot of scrambled samples was performed with 40 μg of total protein isolated from cells 48 h post co-transfection of pNL4-3 and 250 nM each of scram siRNA1. For mock only transfection reagents were used with no plasmid or siRNA and all other conditions remaining same. The blot was probed with HIV-1 α-p24 and α-Actin antibodies. The thin line in Figure a denotes that a lane was edited out since the sample was not in the context of this manuscript. RTPCR was performed with 1 μg of RNA isolated from cells 48 h post co-transfection of pNL4-3 and varying doses of siRNA. The PCR was performed with HIV-1 Gag, TAR, Nef and Tat along with human actin specific primers.

Figure S4


5´- UAGCCAGAGAGCT13CCCAGGUU -3´
a
IC50 = 8.12 ± 2.06 nM
% inhibition of p24
0.1 nM 2.5 nM 25 nM 250 nM
[jcLNA3]
b
IC50 = 11.7 ± 3.85 nM
% inhibition of op24
0.25 nM 2.5 nM 25 nM 250 nM
[LNA4]
% inhibition of p24
log [jcLNA3]
% inhibition of p24
log [LNA4]


5´- T1AGCCAGAGAGCT13CCCAGGUU -3´
c
IC50 = 10.77 ± 1.94 nM
% inhibition of p24
0.1 nM 2.5 nM 25 nM 250 nM
[jcLNA5]
d
IC50 = 24.41 ± 1.87 nM
% inhibition of p24
0.25 nM 2.5 nM 25 nM 250 nM
[LNA6]
% inhibition of p24
log [jcLNA5]
% inhibition of p24
log [LNA6]

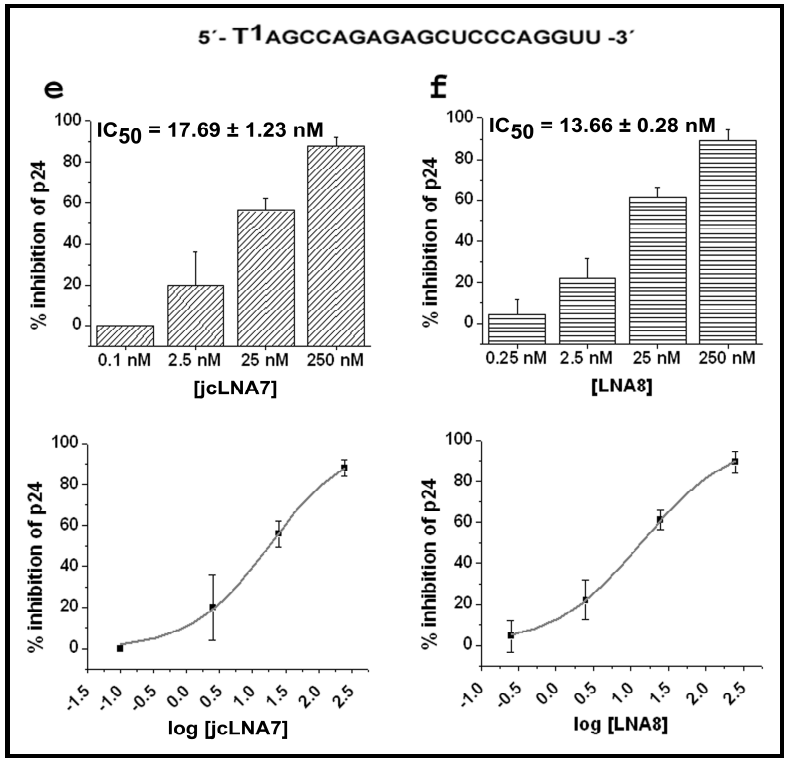
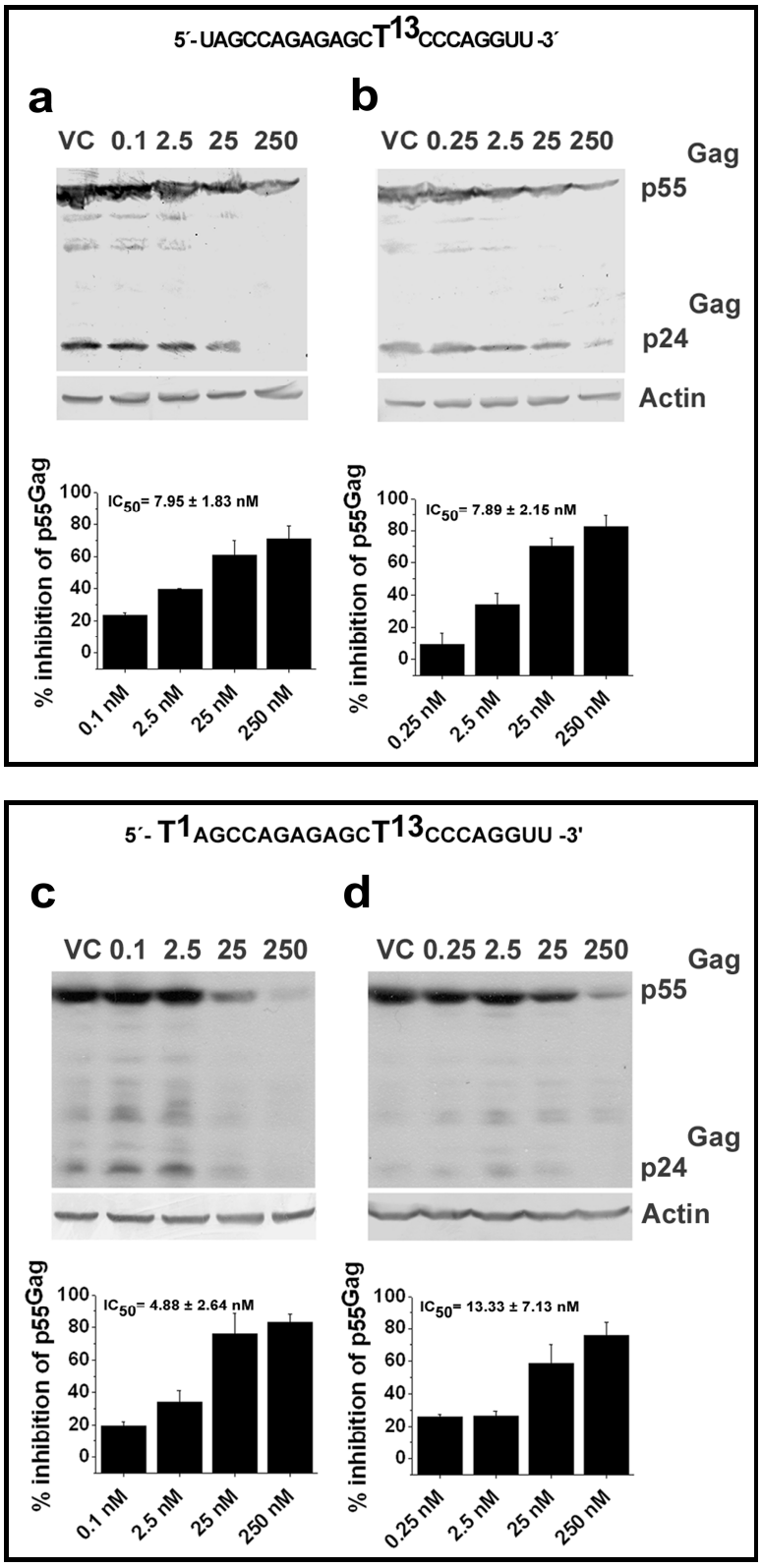


Figure S4: Dose response studies using p24 ELISA for jcLNA and corresponding LNA modified siRNA1 showing % inhibition over virus control at various doses. a: jcLNA3, b: LNA4, c: jcLNA5, d: LNA6, e: jcLNA7, f: LNA8. The sequence for a particular jcLNA/LNA pair is represented above the bar plots with the position(s) of modification highlighted. Respective dose titration sigmoidal plots are placed below each bar plot. Results are cumulative of at least three independent experiments. Error bars represent ±SD from mean. p24 ELISA was carried out with culture supernatants 48 h post-co-transfection of pNL4-3 and varying doses of siRNA

Figure S5


5´-UAGCCAGAGAGCT13CCCAGGUU-3´
a
b
VC 0.1 2.5 25 250
VC 0.25 2.5 25 250
Gag p55
Gag p24
Actin
% inhibition of p55Gag
IC50= 7.95 ± 1.83 nM
IC50= 7.89 ± 2.15 nM
0.1 nM
2.5 nM
25 nM
250 nM
0.25 nM
5´-T1AGCCAGAGAGCT13CCCAGGUU-3'
c
d
VC 0.1 2.5 25 250
VC 0.25 2.5 25 250
IC50= 4.88 ± 2.64 nM
IC50= 13.33 ± 7.13 nM

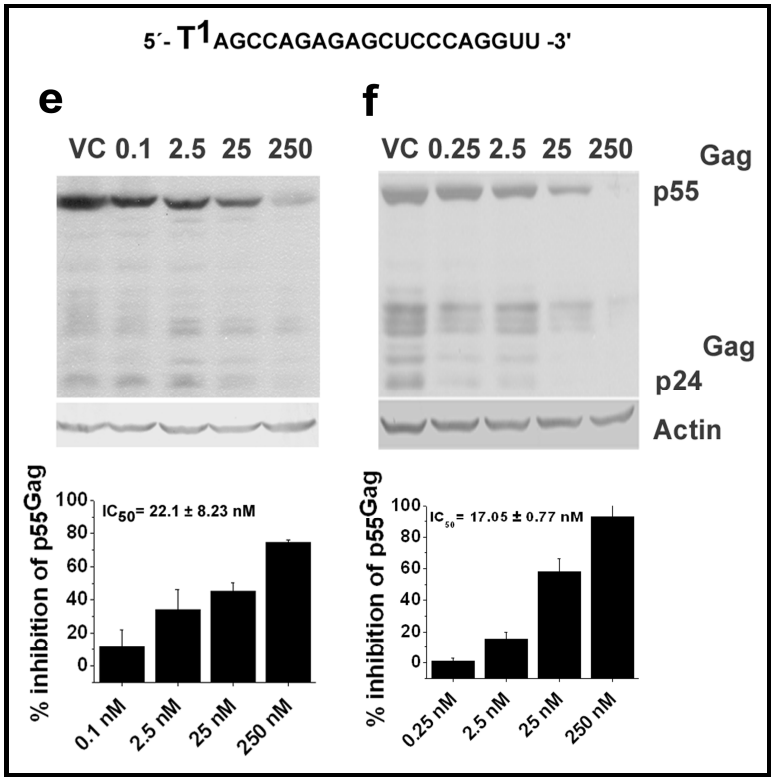


Figure S5: Dose response studies using Western blot for jcLNA and LNA modified siRNA1. a: jcLNA3, b: LNA4, c: jcLNA5, d: LNA6, e: jcLNA7, f: LNA8. The sequence for a particular jcLNA/LNA pair is represented above the blot with the position(s) of modification highlighted. Concentrations in nM are indicated above the gels. For dose titration sigmoidal plots refer to Figure S6. Results are cumulative of at least two independent experiments. Error bars represent ±SD from mean. Western blot was performed with 40 ug of total protein isolated from cells 48 h post co-transfection of pNL4-3 and varying doses of siRNA. The blots were probed with HIV-1 α-p24 and α-Actin antibodies. Band intensities of $p55^{Gag}$ were calculated and normalized with that of actin intensity for each sample using ImageJ software. % inhibition was calculated from the normalized intensity over virus control (VC).

Figure S6:

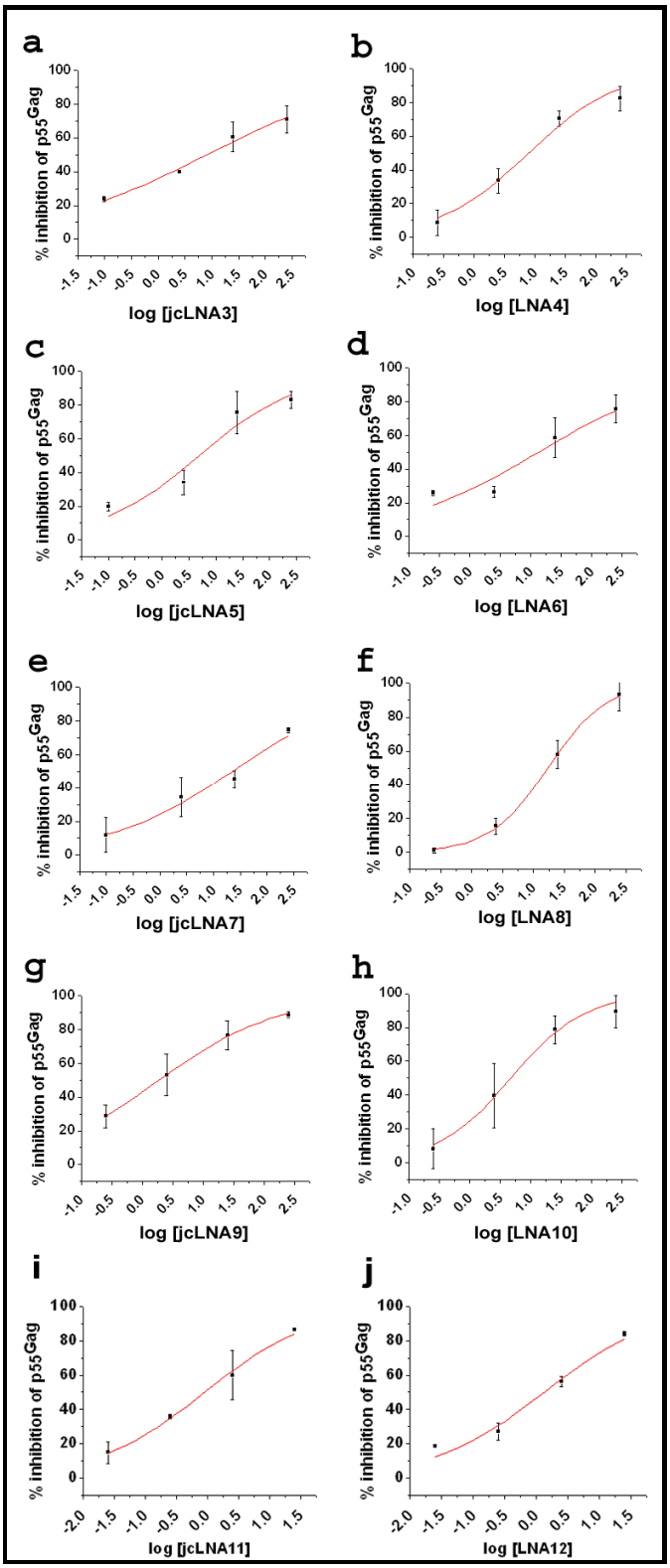

a
% inhibition of p55Gag
log [jcLNA3]
b
% inhibition of p55Gag
log [LNA4]
c
% inhibition of p55Gag
log [jcLNA5]
d
% inhibition of p55Gag
log [LNA6]
e
% inhibition of p55Gag
log [jcLNA7]
f
% inhibition of p55Gag
log [LNA8]
g
% inhibition of p55Gag
log [jcLNA9]
h
% inhibition of p55Gag
log [LNA10]
i
% inhibition of p55Gag
log [jcLNA11]
j
% inhibition of p55Gag
log [LNA12]

Figure S6: Dose titration plot obtained from Western Blot of jcLNA-T and corresponding LNA-T modified siRNA1 showing % inhibition of $p55^{Gag}$ over virus control for various doses of siRNAs. a: jcLNA3, b: LNA4, c: jcLNA5, d: LNA6, e: jcLNA7, f: LNA8, g: jcLNA9 and h: LNA10, i: jcLNA11 and j: LNA12. Results are cumulative of at least two independent experiments. Error bars represent ±SD from mean. Figures were generated by sigmoidal fit of log [siRNA] vs % inhibition of $p55^{Gag}$ plot using Origin software.

Figure S7

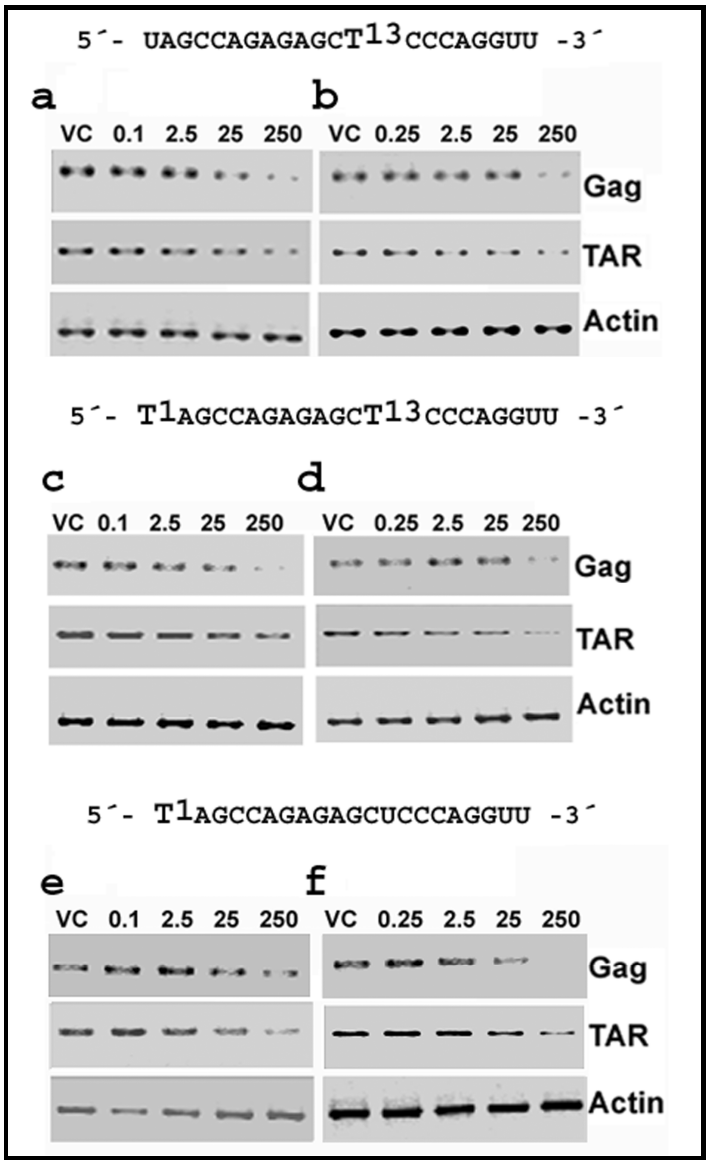
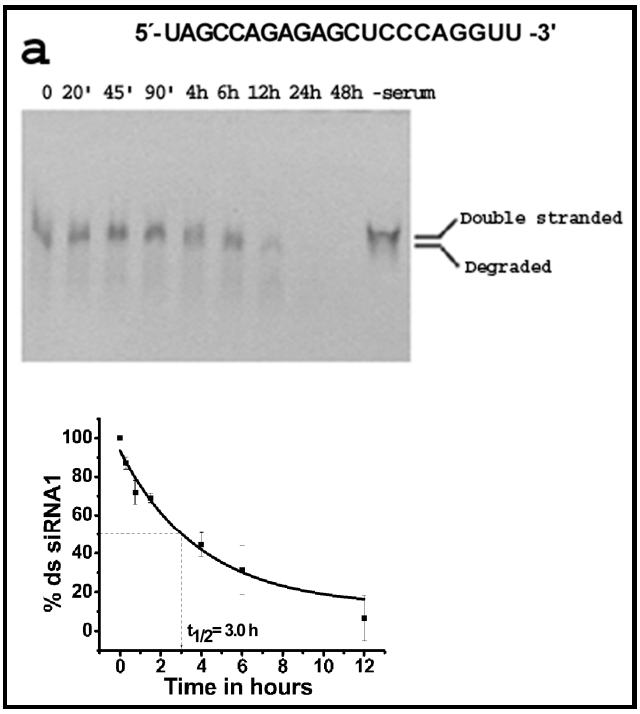
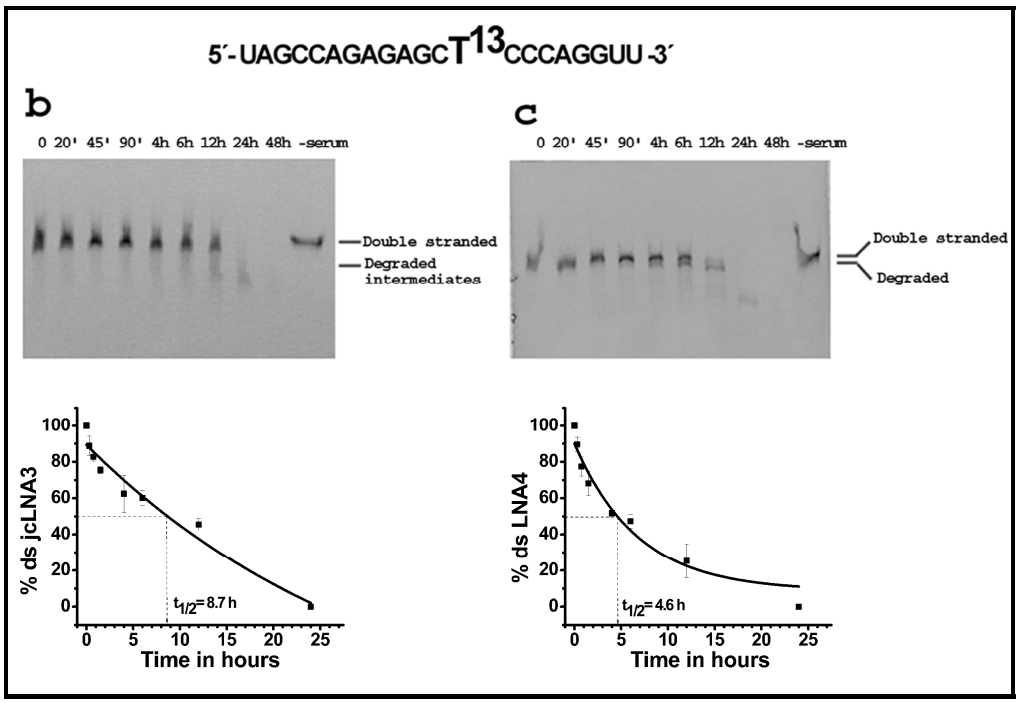


Figure S7: Dose response studies using RTPCR for jcLNA and LNA modified siRNA1. a: jcLNA3, b: LNA4, c: jcLNA5, d: LNA6, e: jcLNA7, f: LNA8. The sequence for a particular jcLNA/LNA pair is represented above the gels with the position(s) of modification highlighted. Concentrations in nM are indicated above the gels. Gels are representative of at least two independent experiments. RTPCR was performed with 1 μg of RNA isolated from cells 48 h

post co-transfection of pNL4-3 and varying doses of siRNA. The PCR was performed with HIV-1 Gag and TAR along with human actin specific primers.

Figure S8


a
5´-UAGCCAGAGAGCUCCCAGGUU -3'
0 20' 45' 90' 4h 6h 12h 24h 48h -serum
Double stranded
Degraded
% ds siRNA1
t1/2= 3.0 h
Time in hours


5´-UAGCCAGAGAGCT13CCCAGGUU-3´
b
0 20' 45' 90' 4h 6h 12h 24h 48h -serum
Double stranded
Degraded intermediates
% ds jcLNA3
t1/2= 8.7 h
Time in hours
c
0 20' 45' 90' 4h 6h 12h 24h 48h -serum
Double stranded
Degraded
% ds LNA4
t1/2= 4.6 h
Time in hours

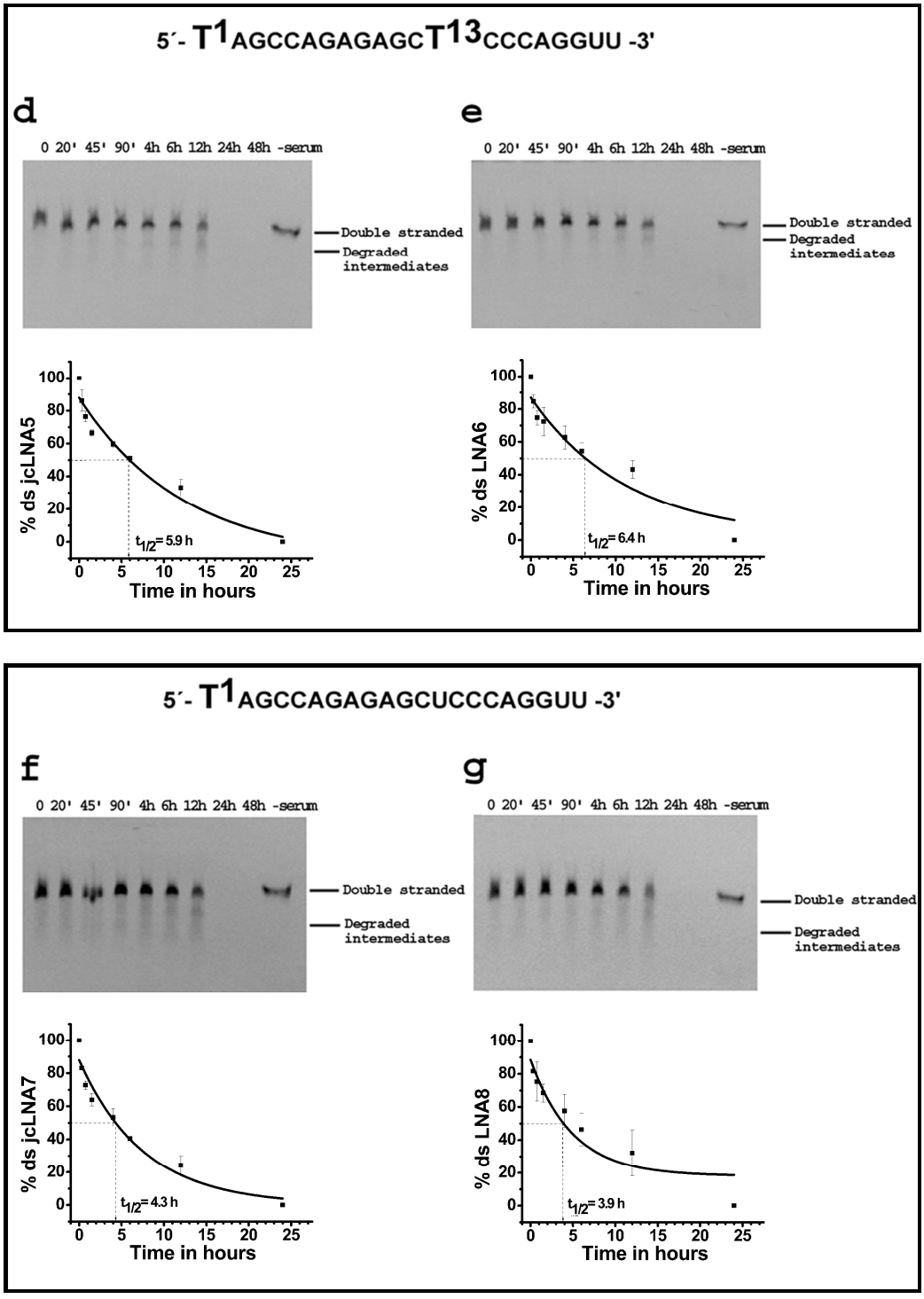


Figure S8: Serum stability of chemically modified double stranded siRNAs targeting TAR1 at different time points. a: native siRNA1, b: jcLNA3, c: LNA4, d: jcLNA5, e: LNA6, f: jcLNA7, g: LNA8. The sequence for a particular jcLNA/LNA pair is represented above the gels

with the position(s) of modification highlighted. Respective best fit exponential decay curve of the mean % double stranded form showing the mean $t_{1/2}$ value is represented below the gels. Gels and curves are representative of at least three experimental repeats. Error bars represent ±SD from mean.

Table S1: Sequence details of native siRNA 1 and 2 along with the respective scrambled sequences

Region	**Name of siRNA**	**Sequence**
TAR1	siRNA1 (Antisense)	5'- UAG CCA GAG AGC UCC CAG GUU -3′
	siRNA1 (sense)	5'- CCU GGG AGC UCU CUG GCU AUU -3'
	Scram (Antisense)	5′ AUAUACCAGGGGGACGCCCUU 3′
	Scram (sense)	5' GGGCGUCCCCCUGGUAUAUUU 3'
TAR2	siRNA2 (Antisense)	5'- CCA GGC UCA GAU CUG GUC UUU -3‘
	siRNA2 (sense)	5'- AGA CCA GAU CUG AGC CUG GUU -3'
	Scram (Antisense)	5′ UCACAUCGACGUCUGGGUCUU 3′
	Scram (sense)	5' GACCCAGACGUCGAUGUGAUU 3'

Table S2: Sequence details of jcLNA and LNA modified siRNA1 targeting TAR1 region. The underlined and bold residues indicate the site of modification. The sense strand sequence is same as that for siRNA1

Sequence of antisense strand	**Codes for jcLNA**	**Codes for LNA**
5′- $U^1A^2G^3$ $C^4C^5A^6$ $G^7A^8G^9$ $A^{10}G^{11}C^{12}$ $\underline{T}^{13}C^{14}C^{15}$ $C^{16}A^{17}G^{18}$ $G^{19}U^{20}U^{21}$ -3′	jcLNA3	LNA4
5′- $\underline{T}^1A^2G^3$ $C^4C^5A^6$ $G^7A^8G^9$ $A^{10}G^{11}C^{12}$ $\underline{T}^{13}C^{14}C^{15}$ $C^{16}A^{17}G^{18}$ $G^{19}U^{20}U^{21}$ -3′	jcLNA5	LNA6
5′- $\underline{T}^1A^2G^3$ $C^4C^5A^6$ $G^7A^8G^9$ $A^{10}G^{11}C^{12}$ $U^{13}C^{14}C^{15}$ $C^{16}A^{17}G^{18}$ $G^{19}U^{20}U^{21}$ -3′	jcLNA7	LNA8
5′- $U^1A^2G^3$ $C^4C^5A^6$ $G^7A^8G^9$ $A^{10}G^{11}C^{12}$ $U^{13}C^{14}C^{15}$ $C^{16}A^{17}G^{18}$ $G^{19}\underline{T}^{20}U^{21}$ -3′	jcLNA9	LNA10
5′- $T^1A^2G^3$ $C^4C^5A^6$ $G^7A^8G^9$ $A^{10}G^{11}C^{12}$ $U^{13}C^{14}C^{15}$ $C^{16}A^{17}G^{18}$ $G^{19}\underline{T}^{20}U^{21}$ -3′	jcLNA11	LNA12

Experimental Section

Western blot. 48 h post transfection cells were trypsinised and harvested by centrifugation at 5000 g for 5 min at 4^{o}C followed by washing once with PBS. Harvested cells were lysed in RIPA buffer (containing 50 mM Tris pH 7.5, 150 mM NaCl, 1mM EDTA, 1% NP-40 (v/v), 1% Na-deoxycholate (w/v), 0.1% SDS (w/v) and centrifuged at 10000g for 10 min at 4^{o}C. The supernatant was collected and protein content was measured by Bradford assay; subsequently 1X Laemelli buffer was added and the samples boiled in a water bath. 40 μg of the protein samples were subjected to 12% SDS-PAGE (in Biorad Mini PROTEAN Tetra Cell electrophoresis apparatus). The proteins were then transferred on a nitrocellulose membrane in a Biorad western blot unit. Subsequently the membrane was blocked with 5% BSA (Albumin bovine serum, Sigma) in 1X TBS-T for 2 h at room temperature. Primary antibodies namely mouse anti-HIV-1 p24 (Santa Cruz Biotechnology) was added at 1:200 dilution or mouse antiactin (Santa Cruz Biotechnology) was added at 1:200 dilution and incubated overnight at 4^{o}C. The following day the blots were washed thrice with 1X TBS-T and incubated with horse-radish peroxidase (HRP)(Santa Cruz Biotechnology)-conjugated secondary antibody or alkaline phosphatase (ALP)(Sigma)-conjugated secondary antibody at room temperature for 2 h. Following three washes as earlier the blots were subjected to chemiluminescence and exposed to X-ray films (in case of HRP conjugated secondary antibodies) or were treated with 5 Bromo 4 Chloro 3 Indolyl Phosphatase Disodium Salt (BCIP) (SRL) -Nitro Blue Tertazolium chloride (NBT) (SRL) solution for colorimetric assay (in case of ALP conjugated secondary antibodies).

The developed and fixed X-ray films or the blots after BCIP-NBT treatment were scanned. Densitometric analyses of the bands were done with ImageJ software[66, 67] [Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/,

1997-2009]. The $p55^{Gag}$ intensities were normalized with the actin intensities and percentage inhibition was calculated over the normalized intensity of virus control (VC). IC_{50} value was calculated using Origin® 6.1 software as for ELISA.

RNA isolation and RTPCR. Total cellular RNA was isolated from transfected cells using TRI (Sigma) according to the manufacturer's protocol. 1 μg of total RNA was subjected to DNAse1 treatment as per the manufacturers instruction and then subjected to reverse transcription in a 25 μl mix containing 300 ng of random hexamer (Invitrogen), dNTP (0.2 mM) (Sigma), DTT (5 mM) (Invitrogen), Porcine RNAse Inhibitor (20 U) (New England Biolabs) and reverse transcriptase (100 U) (Invitrogen). Following reverse transcription 2.5 μl of cDNA was subjected to PCR in a 25 μl mix containing of each forward and reverse primers (0.4 μM) (Sigma), $MgCl_2$ (1.5 mM), dNTP (200 μM) and Taq (1.5U) (Genei). The following primers were used:

RT-PCR primer position in pNL4.3 complete sequence (AF324493)

Gag Forward: 5'–ATA ATC CAC CTA TCC CAG TAG GAG AAA T–3'

Gag Reverse: 5'–TTT GGT CCT TGT CTT ATG TCC AGA ATG C – 3'

From position 1544 to 1658: 114 bp PCR product

TAR Forward: 5' -CAA AGA CTG CTG ACA TCG A- 3'

TAR Reverse: 5' -CAC ACA ACA GAC GGG CAC A- 3'

From 320 to 577 bp: 257 bp PCR product

Beta actin Forward: 5'-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3'

Beta actin Reverse: 5'- TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3'

In our experiments actin primers were added in the same mix containing Gag primers since the cycle parameters of both were same (multiplex). The cycle parameters are described in Table S3. The PCR products were subjected to 1.5% Agarose gel electrophoresis stained with ethidium bromide and visualized on a GelDoc™ (BioRad).

Table S3: PCR cycle parameters

	Initial	Denaturation	Annealing	Extension	No.of

	Denaturation				**Cycles**
Gag/Actin multiplex	94°C – 5 min	94°C – 1 min	55°C - 45s	72°C -1 min	31
Nef	95°C – 2 min	94°C – 1 min	60°C – 1 min	72°C -1 min	33
TAR	95°C – 4 min	95°C – 1 min	54°C - 40s	72°C - 30s	30
Tat	95°C – 2 min	94°C – 1 min	55°C - 30s	72°C - 45s	33