Optimization of polycistronic antiCCR5 artificial microRNA leads to improved accuracy of its lentiviral vector transfer and more potent inhibition of HIV-1 in $CD4^+$ T-cells

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Supplementary text and figures:

Supplementary Methods

Plasmid construction. The mic20 flank plasmid, which contained miR20 flanking sequences, was obtained by inserting the miR20 Fla2 rev, miR20 Fla3 for, miR20 Fla1 for, and miR20 Fla4 rev annealed oligos (Table S1) into the pcDNA6.2-GW/miR-neg control plasmid linearized by BamHI and XhoI (Invitrogen). To obtain the miR1001, miR1002, and miR1001lg amiRNA coding sequences, four oligos for each amiRNA (Table S1) were annealed and ligated with the BsmBI-digested mic20 flank plasmid. The BglII/SalI fragment from miR13lg [17] encoding the amiRNA was ligated into the BamHI/SalI sites of miR1002 to generate the tandem miR1002+miR13lg construct, while the BglII/SalI fragment of miR1002 was ligated into the BamHI/SalI of miR13lg to generate the miR13lg+miR1002 tandem construct. Generation of miR13lg+miR13lg and the H1sh13lg rev plasmid with sh13lg was previously described [17]. Four oligos (Table S1) were annealed and inserted into the BglII/HindIII sites of the pRNA-H1/Neo plasmid (Genscreen) to generate the pH1-sh1005 plasmid. Single microRNAs, sh1005 or tandem amiRNAs were transferred to the LTR VECT lentivector [22], the EGFP lentivector (LTR VECT wherein the puromycin resistance gene was replaced with the EGFP reporter), or the EF1α-EGFP lentivector (wherein in addition to the EGFP reporter being replaced, the CMV promoter was replaced by the EF1α promoter).To transfer the constructs into lentiviral vectors, the amiRNA plasmids were digested with BglII/SalI, and the fragments that contained amiRNAs

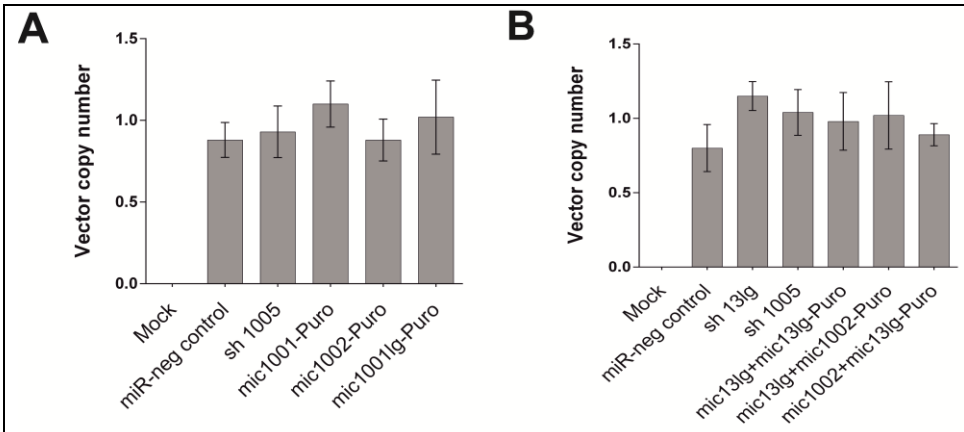
were cloned into the corresponding BamHI/XhoI sites of the different lentivectors. The resulting vectors were designated mic1001-Puro, mic1001lg-Puro, mic1002-Puro, mic1002+mic13lg-Puro, mic13lg+mic1002-Puro, and mic13lg+mic13lg-Puro for vectors with the CMV promoter and the puromycin marker; mic1002-EGFP, mic13lg+mic13lg-EGFP, mic1002+mic13lg-EGFP and mic13lg+mic1002-EGFP for vectors with the CMV promoter and the EGFP marker; and EF-mic13lg+mic13lg-EGFP and EF-mic13lg+mic1002-EGFP for vectors with the EF1α promoter and the EGFP marker. To transfer sh1005 and sh13lg with the H1 promoter into LTR VECT, the XbaI(blunted)/BglII fragments from plasmids pH1-sh1005 and pH1-CCR5 shRNA [17] were cloned into the BamHI/ClaI (blunted) sites of LTR VECT, generating sh1005 and sh13lg vectors containing the corresponding short hairpins in reverse orientation. The empty vectors LTR VECT, EGFP and EF1α-EGFP were used as corresponding controls As a nonsilensing negative micro RNA control we used lentivector contained miR-neg micro RNA, which is predicted not to target any known vertebrate gene. The sequence of this amiRNA was derived from pcDNA™6.2-GW/miR-neg control plasmid (Invitrogen).

Supplementary Table S1. Oligonucleotides sequences

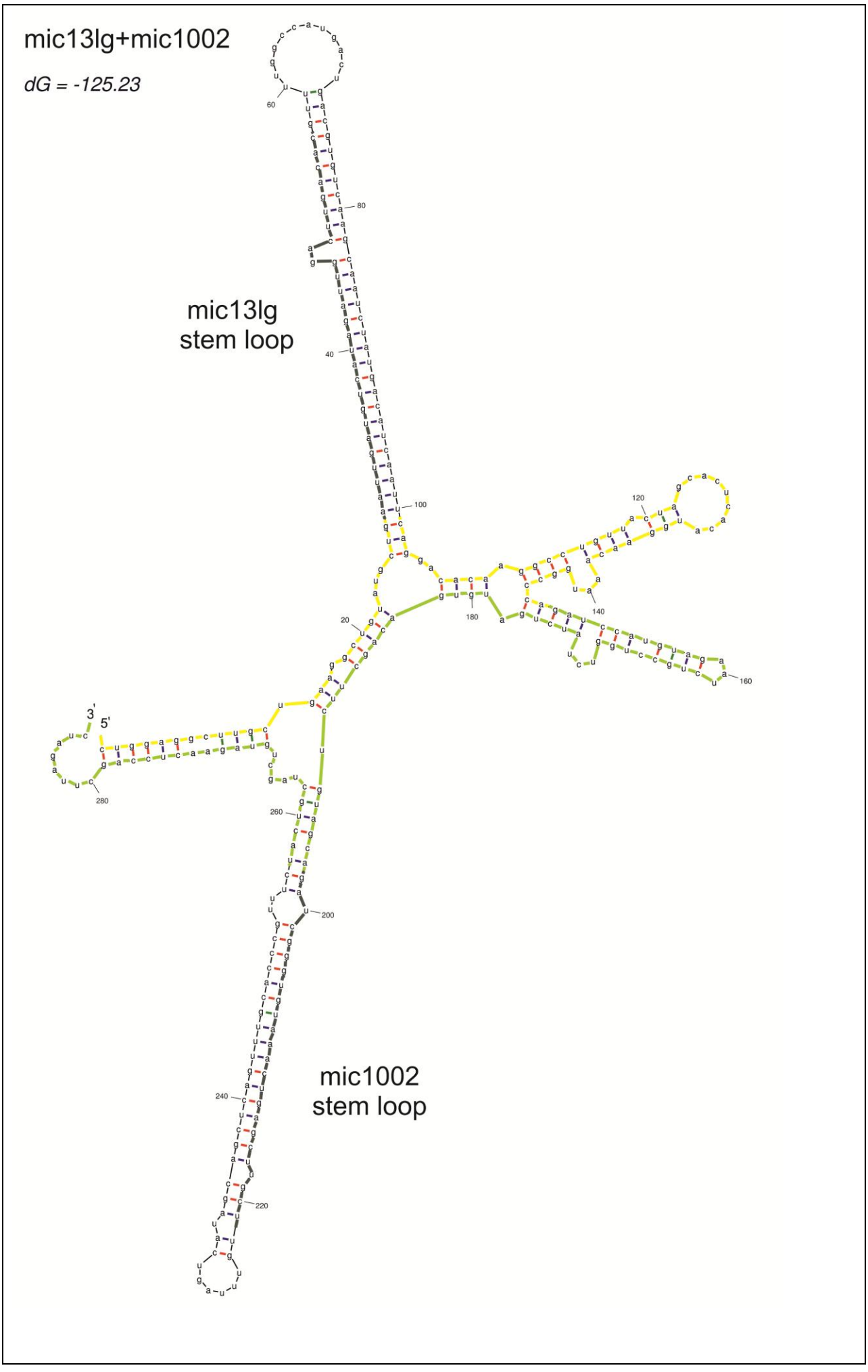
№	Name	5’ - 3’ sequence	
1	miR20 Fla2 rev	Tagcacgtctctcagaagctgtcacatcagatagaccaggcagattctacatg	For cloning mic20 flank plasmid
2	miR20 Fla3 for	Gctacgatccgtctcggctagctgtagaactccagcttagatctggccgcac	
3	miR20 Fla1 for	Gatccatgtagaatctgcctggtctatctgatgtgacagcttctgagagacgt	
4	miR20 Fla4 rev	Tcgagtgcggccagatctaagctggagttctacagctagccgagacggatcg	
5	sh1005 O1 for	Gatctccgagcaagctcagtttacaccttgtc	For cloning sh1005
6	sh1005 O2 rev	Gtcggacaaggtgtaaactgagcttgctcgga	
7	sh1005 O3 for	Cgacggtgtaaactgagcttgctcttttt	
8	sh1005 O4 rev	Tcgaaaaaaagagcaagctcagtttacacc	
9	Mi1002 -20 O1 for	Tctgtagcagatcgggtgtaaactgagcttgcttgttta	For cloning miR1002
10	Mi1002-20 O4 rev	Tagcagtagaaacgggtgcaaactgagctgcta	
11	Mi1002-20 O3 for	Gtcatagcagctcagtttgcacccgtttctact	
12	Mi1002-20 O2 rev	Tgactaaacaagcaagctcagtttacacccgatctgcta	
13	Mi1001 -20 O1 for	Tctgtagcacggtgtaaactgagcttgctcgtagtgtt	For cloning miR1001
14	Mi1001-20 O4 rev	Tagcagtacggaagtaaactgaacttgctctagata	
15	Mi1001-20 O3 for	Tagttatctagagcaagttcagtttacttccgtact	
16	mi1001-20 O2 rev	Actaaacactacgagcaagctcagtttacaccgtgcta	
17	Mi1001lg-20 O1 for	Tctgtagcacgtggatcgggtgtaaactgagcttgctcgtagtgtt	For cloning miR1001lg
18	Mi1001lg-20 O4 rev	Tagcagtacgaaggatcaggtgcaaactgaacttgctctagata	
19	Mi1001lg-20 O3 for	Tagttatctagagcaagttcagtttgcacctgatccttcgtact	
20	Mi1001lg-20 O2 rev	Actaaacactacgagcaagctcagtttacacccgatccacgtgcta	

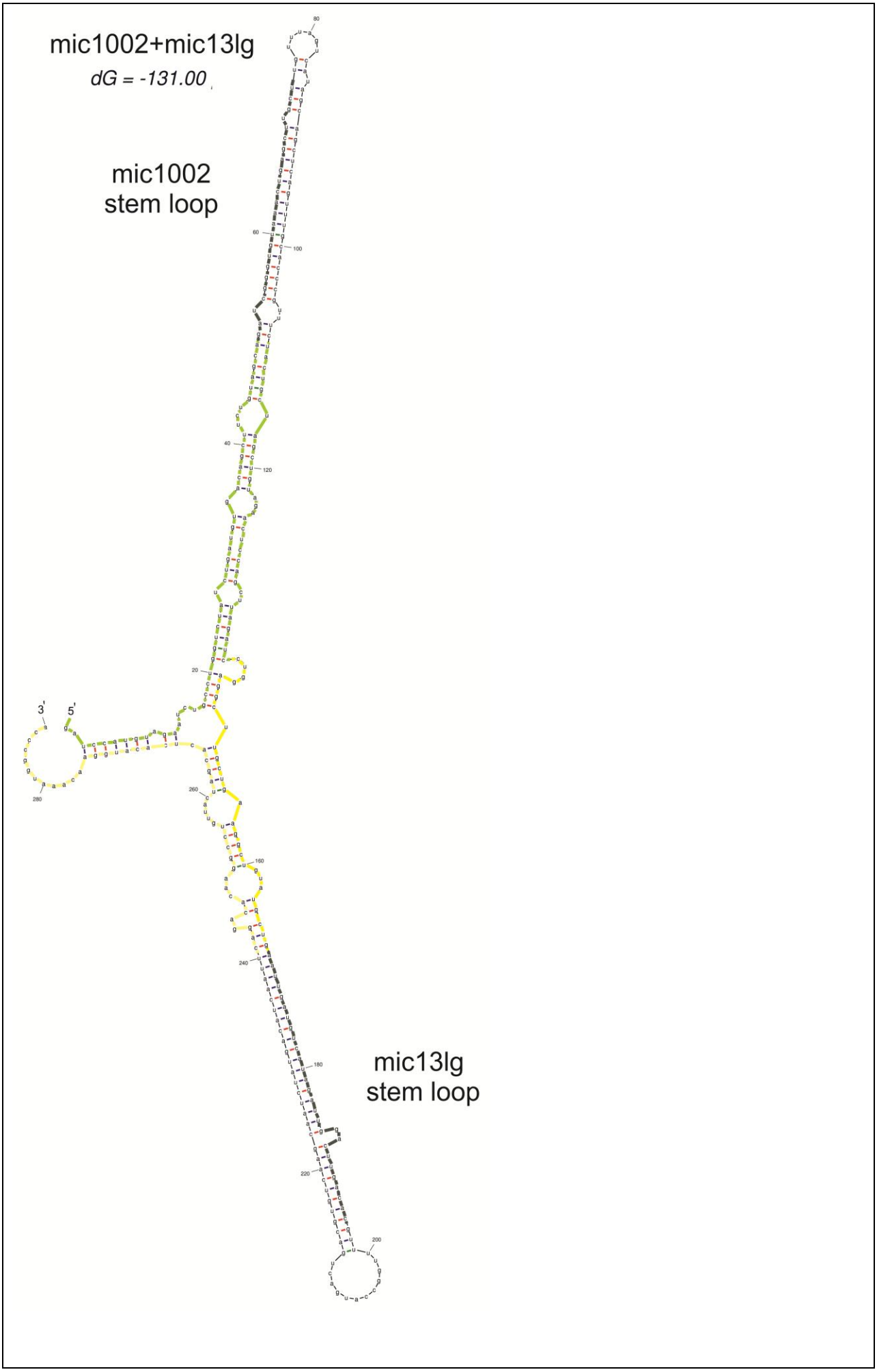
Supplementary Table S2. Characteristics of mic13lg and mic1002 amiRNAs

Name	AntiCCR5 sequence	miRNA flanking sequence
mic13lg (28 nt)	aattgatgtcatagattggacttgacac	miR-155
mic1002 (24 nt)	gatcgggtgtaaactgagcttgct	miR-20

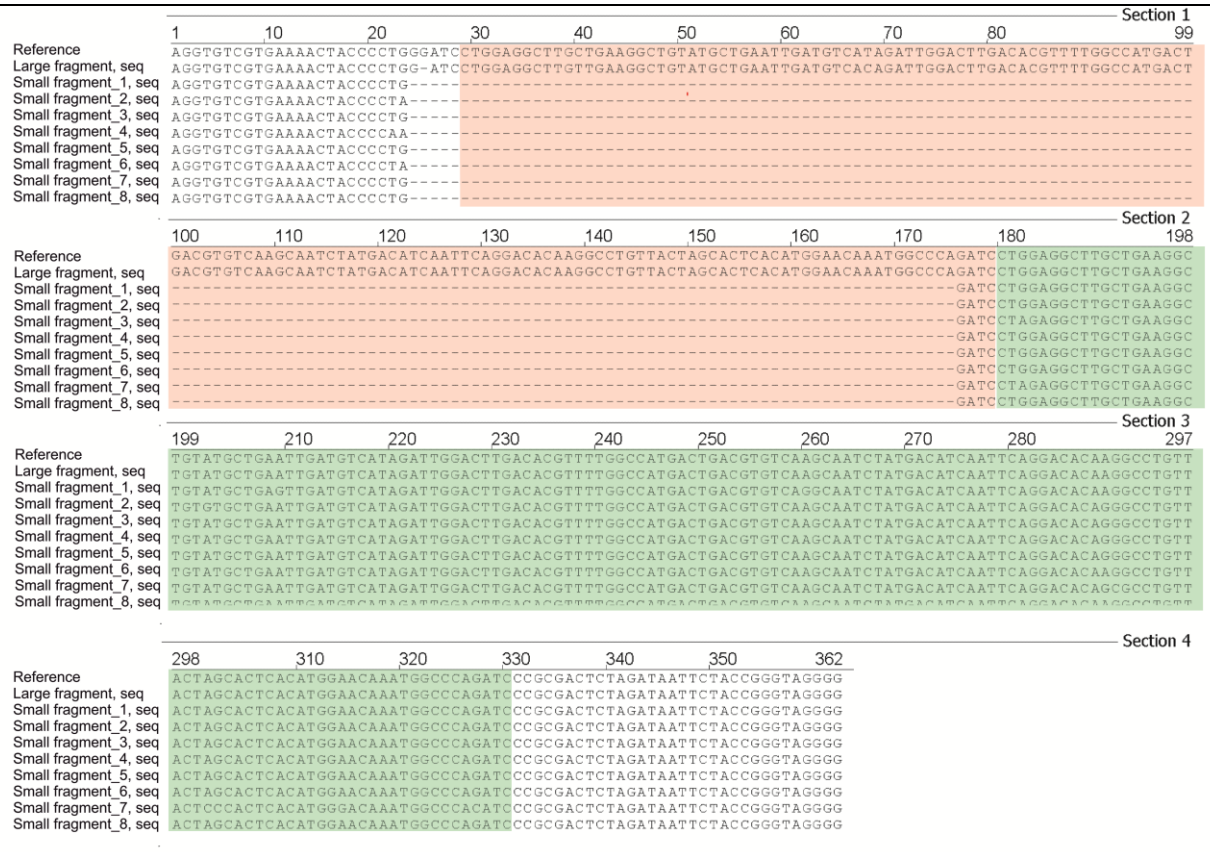


Supplementary Figure S1. Average transgene copy number per cell in transduced HT1080 CCR5-EGFP cells were determined by qPCR using previously described primers and probes [17]. The β-globin gene was used for normalization of the quantitative results. A - copy number for experiments described in Fig.1c. B - copy number for experiments described in Fig.2a.

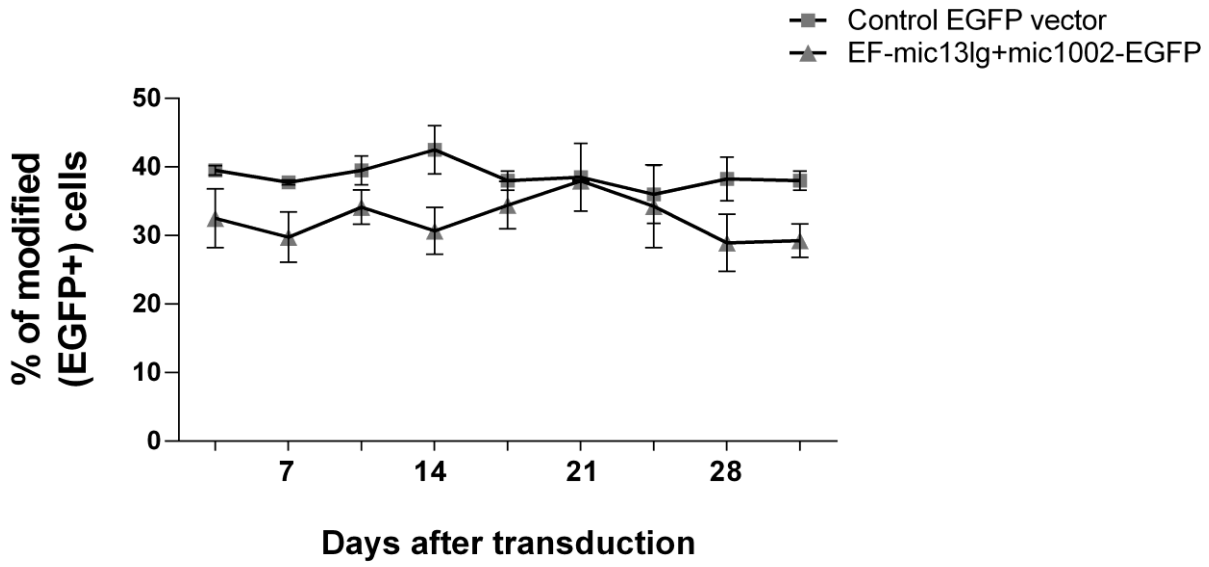

mic13lg+mic1002
dG = -125.23
mic13lg
stem loop
mic1002
stem loop
3'
5'
20
40
60
80
100
120
140
160
180
200
220
240
260
280


mic1002+mic13lg
dG = -131.00
mic1002
stem loop
mic13lg
stem loop
3'
5'

Supplementary Figure S2. Secondary structures of mic13lg+mic1002 and mic1002+mic13lg polycistrons obtained by folding of RNA sequences using the M-fold web server (http://unafold.rna.albany.edu/?q=mfold). mic13lg and mic1002 flanking sequences are depicted by yellow and green lines, respectively. Antisense chain in both amiRNA is shown as a black bold line. Stem-loop strutures of mic13lg and mic1002 including their antisense guide chains are identical for both polycistrons. Shuffling of two amiRNA sequences in polycistron caused structural changes in the amiRNA flanking region. These structural differences may affect the processing of amiRNA precursor, and therefore, the level of mature amiRNA in the cells.



Supplementary Figure S3. Sequence analysis of PCR fragments (depicted in Figure 3F) which were generated by amplification of genome DNA of amiRNAs-transduced $CD4^+$ lymphocytes. All products of the PCR reaction were cloned into T-vector and single clones were analyzed by Sanger sequencing. Sequencing confirmed the presence of tandem amiRNA in long fragments and the presence of only one copy of single amiRNA in short fragments. First mic13lg amiRNA repeat is highlighted in orange, second repeat depicted in green color.



Supplementary Figure S4. Percentage of EF-mic13lg+mic1002-EGFP vector-modified $CD4^+$ cells ($EGFP^+$) remains stable during one month of *in vitro* culture. The fractions (%) of GFP-positive cells was measured over time in EF-mic13lg+mic1002-EGFP vector transduced cells and in the cells transduced with control EGFP vector.