Novel Cell type-specific aptamer-siRNA delivery system for HIV-1 therapy

Jiehua Zhou[1], Haitang Li[1], Shirley Li[2], John Zaia2 & John J Rossi[1*]

[1]Division of Molecular Biology,;[2] Division of Virology, Beckman Research Institute of the City of Hope, Duarte,California 91010

Present address: J. J. Rossi. Division of Molecular Biology, Beckman Research Institute of the City of Hope, Duarte, California 91010, USA. (jrossi@coh.org)

Summary

The successful use of small interfering RNAs (siRNAs) for therapeutic purposes requires safe and efficient delivery to specific cells and tissues. Here we demonstrate cell type-specific delivery of anti-HIV siRNAs via fusion to an anti-gp120 aptamer. The envelope glycoprotein is expressed on the surface of HIV-1 infected cells, allowing binding and interalization of the aptamer-siRNA chimeric molecules. We demonstrate that the anti-gp120 aptamer-siRNA chimera is specifically taken up by cells expressing HIV-1 gp120, and the appended siRNA is processed by Dicer, releasing an anti-*tat/rev* siRNA which in turn inhibits HIV replication. We show for the first time a dual functioning aptamer-siRNA chimera in which both the aptamer and the siRNA portions have potent anti-HIV activities and that gp120 expressed on the surface of HIV infected cells can be used for aptamer mediated delivery of anti-HIV siRNAs.

RNA interference (RNAi) is a process of sequence-specific post-transcriptional gene silencing triggered by small interfering RNAs (siRNA). The silencing is sequence specific and one of the two strands of the siRNA guides the RNA induced silencing complex (RISC) to the complementary target, resulting in cleavage and subsequent destruction of the target RNA[1]. RNAi is rapidly becoming one of the methods of choice for gene function studies, and is also being exploited for therapeutic applications[2, 3]. The

successful therapeutic applications of RNAi are critically dependent upon efficient intracellular delivery of siRNAs[3]. Currently, there are several methods to deliver siRNA *in vivo*. These can be divided into physical and mechanical methods (hydrodynamic tail vein injections in mice[4, 5, 6], electroporation[7, 8, 9], ultrasound[10], and the gene gun[11]); local administration[3] (intravenous injection[12], intraperitoneal injection, subcutaneous injection); and chemical methods (cationic lipids[13, 14], polymers[15, 16, 17, 18, 19, 20], and peptides[21, 22, 23, 24]). However, the delivery efficiency (desired dose), uncontrollable biodistribution and delivery-related toxicitities must be carefully analyzed. Recently, the cell type-specific delivery of siRNAs has been achieved using aptamer-siRNA chimeras[25]. In this system, the aptamer portion mediated binding to the prostate-specific membrane antigen (PSAM), a cell-surface receptor and the siRNAs linked to the aptamer was selectively delivered into PSMA expressing cells resulting in silencing of target transcripts both in cell culture and *in vivo* following intratumoral delivery. In a similar study[26] a modular streptavidin bridge was used to connect lamin A/C or GAPDH siRNAs to the PSMA aptamer. Consequently, this system induced silencing of the targeted genes only in cells expressing the PSMA receptor.

In the present study, we took advantage of the gp120 glycoprotein[27, 28] binding properties of an anti-gp120 RNA aptamer to explore the potential of using this aptamer for delivery of anti-HIV siRNAs into HIV infected cells. Based upon our own previous studies[29, 30], we tested the aptamer as a chimeric transcript with a Dicer substrate RNA duplex (25-30 nt). We utilized a 27 mer siRNA in which one strand is covalently attached to the aptamer, and the second strand is base paired to the upper strand and compared a 27 base pair dsRNA with a 21 base pair dsRNA fused to the aptamer in an analogous fashion. The anti-gp120 aptamer binding to the R5 version of HIV-1 gp120 has been previously demonstrated[31.] This aptamer was shown to neutralize HIV-1 infectivity[31, 32, 33] by direct binding to gp120 in virions. We wanted to determine whether or not the anti-gp120 aptamer could provide selective binding and subsequent

internalization into HIV infected cells which should express gp120 on the cell surface. Although the aptamer alone provided some inhibitory function when tested in this setting, the siRNA chimeras provided more potent inhibition than the aptamer alone, suggesting cooperativity between the siRNA and aptamer portions in inhibiting HIV replication and spread. Our results demonstrate that the gp120 aptamer-siRNA chimeras are internalized in cells expressing gp120 either ectopically or from HIV infection, and moreover the chimeric RNAs provide potent and lasting inhibition of HIV replication in T-cells in culture. These results support the concept of using aptamer-siRNA conjugates for systemic treatment of HIV infection. This approach has the major advantage of not relying upon gene therapy, and the siRNAs can be changed or multiplexed to avert viral resistance.

Design of anti-gp120 aptamer-siRNA chimeras

To enhance the stability of the chimeric RNAs in cell culture and *in vivo*[4, 34, 35, 36, 37], the aptamer and sense strand segment of the siRNAs contained nuclease-resistant 2'-Fluoro UTP and 2'-Fluoro CTP and were synthesized from corresponding dsDNA templates by *in vitro* bacteriophage transcription (**Fig 1**). To prepare the siRNA containing chimeras, in vitro transcribed chimeric aptamer-sense strand polymers were annealed with equimolar concentrations of an unmodified antisense strand RNA. These 2'-Fluoro modified chimeras were stable in cell-culture media for up to 48 hours whereas the unmodified control RNAs were quickly degraded within several minutes (data not presented).

The gp120-binding aptamer which neutralizes R5 strains of HIV-1 has been previously described and characterized[31]. Since the synthetic Dicer substrate duplexes of 25-30 nt have been shown to enhance RNAi potency and efficacy, we chose a 27 mer duplex RNA as the siRNA portion our chimeric molecule. The 27 mer siRNA portion of chimeras (**Ch L-1** and **Ch 1**) targets the HIV-1 *tat/rev* common exon sequence. The

chimeras designed **Ch L-2** and **Ch 2** are identical to **Ch L-1** and **Ch 1** with the exception that the 27 mer duplex is replaced by a 21 base pair duplex. In the **Ch L-1** and **Ch L-2** designs we inserted a four nucleotide linker (CUCU) between the aptamer and siRNA portions to minimize steric interference of the aptamer portion with Dicer. Previous studies on the anti-gp120 aptamer identified the minimal region of the aptamer essential for binding gp120 and showed mutations within this region significantly lower the binding affinity. As controls for aptamer binding we created the chimera designated as **M-1**. As a control for the siRNA mediated silencing we constructed an additional mutant in the siRNA portion which should abrogate RNAi directed cleavage of the target, and this is designated as **M-2**.

Because of competition by the sense (passenger) strand with the anti-sense (guide) strand for RISC entry, the strand selectivity is an important factor for evaluating siRNAs. Therefore, we evaluated these chimeras RNA using the SiCheck reporter system, which readily allows screening of the potencies of candidate sh/siRNAs. The gene silencing of both the sense target (corresponding to the mRNA) and the anti-sense target were tested independently and the selectivity ratios could be calculated as a measure of the relative incorporation of each strand into the RISC. The comparison (**Fig S1**) demonstrated that the **Ch L-1** mediated ~86% knockdown of the sense target; however, knockdown of the anti-sense target is much less (~50 %), indicative of good strand selection (R = 3.2). **Ch 1** also indicated similar knockdown (~ 83%) of the sense target and strand selection (R = 2.9). In contrast, **Ch L-2 and Ch 2** have poorer efficacy (<70%) and strand selectivity (R = 1.9 and 1.6, respectively). These data suggest that the aptamer-27 mer siRNA chimeras indeed enhance the RNAi efficacy and potent, consistent with previous studies in our laboratory[29, 30].

Anti-gp120 aptamer-siRNA chimeras bind and are internalized by cells expressing HIV gp160

Style tag for displayed matter CHO-gp160 cells stably expressing the HIV envelope glycoprotein gp160 were used to test uptake of the chimeric aptamer-siRNAs. These cells do not process gp160 into gp120 and gp41 since they lack the gag encoded proteases required for envelope processing. As a control we used the parental CHO-EE cell line which does not express gp160. The anti-gp120 aptamer and the chimeras were labeled with Cy3 to follow their binding and potential internalization in gp160 expressing cells. Flow cytometric analyses (**Fig 2a**) revealed that the aptamer and chimeras specifically bound to the CHO-gp160 cells but did not bind to the control CHO-EE cells. As anticipated, the **M-1** dramatically reduced binding to the CHO-gp160 expressing cells.

In order to determine if the bound aptamer and chimeras were internalized in the gp160 expressing cells, we carried out Z-axis confocal microscopy and three-dimensional image reconstruction with the CHO-gp160 cells incubated with the Cy3-labeled transcripts. Both the anti-gp120 aptamer (data not presented) and **Ch 1** (**Fig 2b**) were selectively internalized within the CHO-gp160 cells but not the CHO-EE control cells. The **M-1** was also not internalized. Three-dimensional image reconstruction (**Fig 2c**) shows localization of the Cy3-labeled Chimera **Ch 1** in a single cell. To visualize the plasma membranes the cells were stained with the carbocyanine dye DIO.

Anti-gp120 aptamer-siRNA chimeras are processed by Dicer

We next asked whether or not the siRNAs could be processed from the chimeras by Dicer in whole cell extracts that contain good Dicer cleavage activity. The first set of experiments used a 5'-end P^{32} labeled antisense strand to follow Dicer processing (**Fig 3a**). The size of the P^{32} labeled cleavage product(s) indicates from which direction Dicer enters the siRNA and cleaves. When **Ch L-1** was incubated with the cytoplasmic lysate, we observed that the 27 nt antisense strand was processed into a 21-23 nt siRNA. This result suggests Dicer processing preferentially enters from the 5' end of the antisense

strand and cleaves 21 to 23 nt downstream, leaving the 5' end of the antisense strand intact (**Fig 3b**). In contrast, the 21 base siRNAs were not processed further in these extracts.

Anti-gp120 aptamer-siRNA chimeras specifically silence target gene expression

To evaluate whether these anti-gp120 aptamer-siRNA chimeras function in triggering RNAi, we first transfected CHO-gp160 and CHO-EE cells with HIV pNL4-3 Luc. The HIV pNL4-3 has the firefly luciferase under the control of the HIV LTR and is Tat responsive. The anti-*tat/rev* siRNA efficacy is monitored by inhibition of luciferase expression. Subsequent to the transfections the cells were treated with the chimeras in the absence or presence of the transfection reagent Lipofectamine 2000.

Luciferase expression was potently inhibited when **Ch L-1** and **Ch 1** were lipofected into both types of cells (**Fig 4a**). However, in the absence of lipofection, gene silencing from **Ch L-1** and **Ch 1** was specific to CHO-gp160 expressing cells and no inhibition of luciferase was observed in CHO-EE cells. Interestingly, **Ch L-1** and **Ch 1** which are linked to the 27 mer duplex RNA showed somewhat greater efficacy than chimeras **Ch L-2** and **Ch 2**, consistent with our previous observations of Dicer substrates enhancing RNAi[29, 30].

To validate that the siRNAs released from the chimeras were triggering RNAi we transfected CHO-gp160 cells with a Rev-EGFP fusion construct harboring the siRNA targets. The transfected cells were then transfected with **Ch L-1**, **Ch L-2**, 27 mer siRNA or 21 mer siRNA in presence of Lipofectamine 2000. Thirty six hours post transfection total RNA was isolated and subjected to a modified 5'-RACE (Rapid amplification of cDNA ends) technique to identify the specific cleavage products in the Rev portion of the fusion transcript. We assumed that the Ago2 mediated cleavage was between bases 10 and 11 relative to the 5' end of each siRNA. Our Dicer analyses of the 27 mer

revealed that it is cleaved 21-23 nucleotides downstream from the 5' end of the antisense strand (antisense relative to the *tat/rev* target), whereas the 21 mer is not processed further (**Fig 3b**). We expected that the RNAi mediated cleavage site in the target would be shifted by six bases between the 27 mer and the 21 mer derived siRNAs. Fragments of the predicted lengths were obtained from cells treated with the siRNAs or chimeras (**Fig 4b**). Direct sequencing of the excised bands verified the expected PCR product, which demonstrated that cleavage occurred at the predicted position for the siRNA duplex between positions 10 and 11 from the 5' end of the siRNA antisense strand (**Fig S3**). These data provide a formal demonstration that the chimeras produce siRNAs that are incorporated into RISC. As expected, no RACE PCR products were generated from RNA isolated from cells untreated with the chimeras or siRNAs.

Anti-gp120 aptamer-siRNA chimeras inhibit HIV gp120 mediated cell fusion and HIV-1 infection CEM T-cells

Essential to the use of the aptamer-siRNA chimeras in treating HIV infection is that the aptamer allows internalization of the chimeras in HIV infected cells. We first demonstrated by Northern blot analyses that chimeric delivered siRNAs were detectable in HIV infected CEM cells directly which were treated with the chimeras. The Northern blotting data of **Figure 5a** demonstrate that the siRNAs from chimera are internalized in HIV infected CEM cells since the 27 mer was processed to 21-23 base siRNAs in these cells, but not in the gp120-negative uninfected CEM cells, suggesting that the chimeras specifically delivered siRNA into the infected CEM cells through anti-gp120 aptamer.As expected, the 21 or 27 mer duplex siRNAs in absence of the aptamers were not detectable in the CEM cells owing to the lack of internalization (**Fig 5a**). Since a little of non-specific bindings existed on the cells surface, tiny 21 or 27 RNA from chimeras (**Ch L-1, Ch 1** and **Ch L-2**) also were hybridized in uninfected CEM.

To further confirm siRNA function after internalization to infecterd CEM cells, qRT-PCR was preformed to evaluate the *tat/rev* gene expression. Aptamer or chimeras were added directly to media containing infected CEM cells. After 7 days, treated cells were harvested, the total RNA was extracted and the extent of *tat/rev* gene inhibition was determined by quantitative RT-PCR expression assays. We find that the treatment of infected CEM cells with the chimeras is able to induce silencing of the *tat/rev* gene, while the aptamer alone did not affect *tat/rev* gene expression (**Fig 5b**). These results provide further support that the aptamer delivered siRNA triggers RNAi.

In HIV-1 infection, gp120 expressed at the cell surface will induce syncytia formation between infected and uninfected cells due to interactions between gp120 and CD4[27, 28]. We therefore sought to determine if the aptamer and chimeras would have an impact on syncitia formation in cell culture. In this assay, the HIV-1 infected-CEM cells were incubated with siRNA or chimeras RNAs. Subsequently, the uninfected MT2 cells expressing CD4 were added into infected-CEM cells treated with RNA. After 48 h of co-incubation at 37°C, cells syncytia were analyzed microscopically. The treatment of the HIV infected cultures with the aptamer and chimeras resulted in a clear reduction in syncytia formation (Data not presented). We also asked if the aptamer and chimeras prevent HIV replication in an acute infection assay by monitoring HIV-1 Gag p17 via an immunofluorescence assay (IF). These assays revealed a marked reduction in p17 expression in cells cells treated with the anti-gp120 aptamer and even more pronounced reduction with the Ch L1 chimera (**Fig S4**).

To further verify the activity of the anti-HIV activity of the chimeras in inhibition of HIV-1 replication, we carried out the following assays. In the first assay, HIV-1 was first mixed with the chimeras or aptamer and subsequently the viruses were used to infect CEM cells. In this assay the infectivities of the aptamer or chimera treated virus were significantly reduced and viral replication was suppressed out to two weeks

(**Fig 5c**). **Ch L-1** was the most effective inhibitory agent. In the second experiment, the aptamer or chimeras were incubated with HIV infected-CEM cells. At different days post treatment with the aptamer and chimeras, aliquots of the media were assayed for viral p24 antigen levels. The results of these analyses (**Fig 5d**) showed that all of the aptamer containing RNAs inhibited p24 production, but the strongest inhibition was observed with **Ch L-1** treatment, again consistent with our results from the other assays. These data, together with the inhibition of cell fusion and p17 expression, demonstrate that the anti-gp120 aptamer-siRNA chimera system can strongly inhibit HIV-1 replication and infection. Moreover, the suppression is attributed to the combined affect of the aptamer binding gp120 and RNAi.

Anti-gp120 aptamer-siRNA chimeras do not trigger an interferon response

It has been reported previously that siRNAs delivered by liposomes or polyplex reagents can non-specifically activate inflammatory cytokine production (TNFα, IL-6 and IL-12) as well as IFN responsive genes, which in turn can trigger cellular toxicity[38, 39, 40]. We therefore assessed the induction of type I interferon regulated gene expression by our anti-gp120 aptamer-siRNA chimeras using quantitative RT-PCR expression assays. As a positive control, we incubated the target cells with poly(IC). We find that the treatment of HEK293 cells with the chimeras did not significantly induce expression of the interferon-β and p56 genes (**Fig 6a**). Since CEM cells are difficult to be transfect with control molecules such as poly (IC), we used IFN-α as a positive control to confirm upregulation of p56 and OAS1 gene expression. As we observed in the HEK293 transfection assays, treatment of CEM cells with the chimeras did not induce type I IFN responses (**Fig 6b**). Similar results were obtained using HIV infected CEM cells treated with the chimeras, suggesting that the gp120 mediated internalization of the chimeras does not trigger toxic IFN responses.

Discussion

Aptamers are nucleic acid species that have been engineered through repeated rounds of *in vitro* selection to bind to various molecular targets such as small organic molecules,

proteins, nucleic acids, and even cells[41, 42, 43]. Because aptamers are capable of binding with high specificity to their ligands at low nano- to picomolar dissociation constants they can be used as molecular drugs for both basic research and clinical purposes[44, 45, 46, 47, 48].

The success of RNAi-based clinical applications is dependent upon the efficiency of siRNA delivery to target cells. In this report, we have capitalized upon the exquisite specificity of a gp120 aptamer to deliver anti-HIV siRNAs into HIV infected cells with the net result that replication and spread of HIV is strongly inhibited by the combined action of the aptamer and siRNA targeting the *tat/rev* common exon of HIV-1.

We utilized the HIV-1 envelop protein gp120 as a model receptor for targeted intracellular delivery of anti-HIV siRNAs. Cell type-specific binding and uptake of chimeric aptamer-siRNA conjugates were achieved through the interaction of the aptamer portion with gp120 on the cell surface of infected cells. To insure the stability of our RNA chimeric molecules in sera, we utilized the RNA stabilizing 2'-Fluoro backbone modifications of pyrimidines on the aptamer and siRNA sense strand. The antisense strand was no chemically modified, but was in fact stabilized by virtue of its base pairing to the modified sense strand.

Notably, the cell type-specific gene silencing revealed that the siRNAs were successfully delivered into cells and entered into the RNAi pathway by interaction of the anti-gp120 aptamer with gp120 expressed on the cell surface. Interestingly, the chimeras containing a 27 mer duplex RNA gave better efficacy in gene silencing than the corresponding 21 mer duplex containing chimeras. The 27 mer duplex alone was also more potent than the 21 mer duplex when these RNAs were delivered by lipofection. We attribute this increased potency to Dicer processing of the 27 mer wherein the processed 21-23 mer siRNAs are more readily handed off to RISC. It is of interest that we never observed complete processing of the 27 mer into 21-23 mers in

our Northern Blot analyses of cells treated with the chimeras. This may in part be a consequence of the high intracellular concentrations achieved by aptamer delivery, but may also reflect that the design of our blunt ended duplexes is sub-optimal for Dicer processing. We observed that rather than enter the duplex from the 2 base 3' overhang, Dicer cleavage initiated following entry onto the duplex from the blunt end of the duplex. To this end we are testing other structures of the siRNA portion of the aptamers to achieve more complete Dicing.

An interesting observation is that analyses of the target cleavage products by a 5'-RACE technique further demonstrated that neither the 27 mer or 21 mer siRNAs underwent processing to trigger duplexes with two base 3' overhangs on both ends of the siRNAs. In fact for both the 21 mer and 27 mer derived siRNAs, the target mRNA was cleaved between positions 10 and 11 relative to the blunt 5' end of the siRNA antisense strand. These results suggest that unprocessed 27 mer as well as Dicer processed 27 mer antisense strands may be incorporated into RISC. Given the results from the cell extract Dicing reaction, which revealed that the 27 mer is not processed at the 5' end of the antisense but only at the 3' end, it is not possible to determine whether all, some or none of the activated RISC was derived from intact 27 mer antisense getting incorporated directly into RISC.

Aptamers that bind to viral or cellular proteins with high affinity and specificity are useful for therapeutic applications. In this study, both aptamer and chimeras can dramatically suppress the replication and production of HIV-1 in a variety of assays. These results demonstrate important attributes of the anti-gp120 aptamer as both inhibitors of HIV via direct binding to virion or intracellular gp120 and as a cell type specific delivery vector for therapeutic siRNAs.

Because the anti-gp120 aptamer is responsible for the targeted delivery of siRNAs, gp120 expression is necessary for cell type-specific transport. This is in

essence a safety feature which could be capitalized upon to deliver siRNAs that target HIV or even cellular messages essential for viral replication. Since only HIV infected cells would bear the inhibitory action of the siRNA, this approach greatly minimizes potential off-target effects by the siRNAs.

The dual inhibitory potential of the aptamer-siRNA fusion is an important point of discussion. Both the aptamer and chimeras showed strong inhibition of syncitial cell formation, expression of HIV-1 Gag p17 and HIV replication and spreading in HIV-1 infected-CEM T-lymphocytes. The anti-gp120 aptamer neutralizes HIV-1 infectivity via blocking the interaction of gp120 and CD4, and the siRNA silences *tat/rev* expression. Thus, the anti-gp120/HIV chimeras serve a double-function and therefore provide greater efficacy than either the aptamer or siRNA applied alone. Finally, we show that the aptamer mediated delivery of siRNAs via binding to gp120 and subsequent internalization does not trigger type I interferon gene responses in different cell lines.

In summary, this strategy provides a new paradigm for delivery of anti-HIV siRNAs by allowing selective delivery to HIV infected cells and dual function inhibition of HIV replication and spread. Moreover, the aptamer and siRNAs can be readily changed to accommodate genetic changes in the virus, making, making this an attractive approach for systemic anti-HIV therapy.

Methods

Materials. Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich, all restriction enzymes were obtained from New England BioLabs (NEB) and all cell culture products were purchased from GIBOC (Gibco BRL/Life Technologies, a division of Invitrogen.); DuraScribe T7 transcription Kit (EPICENTRE Biotechnologies); Silencer siRNA Labeling Kit (Ambion); Vybrant Cell-Labeling Solution (Molecular Probes, Invitrogen); Galacto-Star System (Applied Biosystems);

CHO-Env Transfectants (CHO-WT and CHO-EE) (NIH); Magi cell line (NIH); HEK 293 cell line (ATCC); Random primers (Invitrogen); Bio-Spin 30 Columns (Bio-Rad); Recombinant Human Dicer Enzyme Kit (Genlantis); Lipofectamine 2000 (Invitrogen)

siRNAs. siRNA and antisense strand RNA were purchased from Integrated DNA Technologies (IDT). Site I (*tat/rev*) 27 mer: Sense sequence: 5'-GCGGAGACAGCGACGAAGAGCUCAUCA-3'; Antisense: 5'-UGAUGAGCUCUUCGUCGCUGUCUCCGCdTdT-3'; Site I (*tat/rev*) 21 mer: Sense sequence: 5'- GCGGAGACAGCGACGAAGAGC-3'; Antisense: 5'-GCUCUUCGUCGCUGUCUCCGCdTdT-3';

Aptamer-siRNA chimeras. (The 27 or 21 mer sense strand is marked in bold and mutated nucleotides are underlined).

Aptmer:
5'- GGGAGACAAGACUAGACGCUCAAUGUGGGCCACGCCCGAUUUU
ACGCUUUUACCCGCACGCGAUUGGUUUGUUUCCC - 3'
Chimera L-1 sense strand:
5'- GGGAGACAAGACUAGACGCUCAAUGUGGGCCACGCCCGAUUUU
ACGCUUUUACCCGCACGCGAUUGGUUUGUUUCCC*CUCU***GCGGAGACAGC**
GACGAAGAGCUCAUCA -3'
Chimera 1 sense strand:
5'- GGGAGACAAGACUAGACGCUCAAUGUGGGCCACGCCCGAUUUU
ACGCUUUUACCCGCACGCGAUUGGUUUGUUUCCC**GCGGAGACAGCGACG**
AAGAGCUCAUCA -3'
Chimera L-2 sense strand:
5'- GGGAGACAAGACUAGACGCUCAAUGUGGGCCACGCCCGAUUUU
ACGCUUUUACCCGCACGCGAUUGGUUUGUUUCCC*CUCU***GCGGAGACAGC**
GACGAAGAGC -3'
Chimera 2 sense strand:
5'- GGGAGACAAGACUAGACGCUCAAUGUGGGCCACGCCCGAUUUU

ACGCUUUUACCCGCACGCGAUUGGUUUGUUUCCC**GCGGAGACAGCGACG**
AAGAGC -3’

Mutant-1 sense strand:

5’- GGGAGACAAGACUAGACGCUCAAUGUGGGCGGGGCCCGAUUUU
ACCGUUUUCAAAGCACGCGAUUGGUUUGUUUCCC*CUCU***GCGGAGACAGC**
GACGAAGAGC -3’

Mutant-2 sense strand:

5’- GGGAGACAAGACUAGACGCUCAAUGUGGGCCACGCCCGAUUUU
ACGCUUUUACCCGCACGCGAUUGGUUUGUUUCCC*CUCU*
GCGGAGACAGCG
UGUAAGAGCUCAUCA -3’

Chimera L-1, chimera 1 and Mutant-1 antisense strand:

5’- UGAUGAGCUCUUCGUCGCUGUCUCCGCdTdT-3’

Chimera L-2, chimera 2 antisense strand:

5’- GCUCUUCGUCGCUGUCUCCGCdTdT-3’

Mutant-2 antisense strand:

5’- UGAUGAGCUCUUACACGCUGUCUCCGCdTdT-3’

Generation of aptamer and chimera RNAs by *in vitro* transcription. Double-stranded DNA templates were directly generated by PCR and the resulting PCR products were recovered using a QIAquick Gel purification Kit. Chimera sense strands were transcribed from its PCR generated DNA templates using the DuraScription Kit (Epicentre, Madison, WI) according to the manufacturer’s instruction. In the transcription reaction mixture, the canonical CTP and UTP were replaced with 2’-F-CTP and 2’-F-UTP to produce RNA that is resistant to RNase A degradation. The reactions were incubated at 37 °C for 16 h, and subsequently purified with Bio-Spin 30 Columns (Bio-Rad) following ethanol precipitation. RNA was treated by CIP to remove the initiating 5’-triphosphate. To prepare the chimeras, the chimeras harboring only the sense strand RNA was combined with the appropriate antisense RNA in HBS buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2.7 mM KCl), heated to 95 °C for 3 min and then cooled to 37°C slowly. Incubation continued at 37°C for 10

min. Fluorescent aptamer and chimeras were generated using the Silencer siRNA Labeling Kit (Ambio) according to the manufacturer's instructions.

Cell culture. HEK 293 cells and CEM cells were purchased from ATCC and cultured in DMEM and RPMI 1640 supplemented with 10% FBS respectively, according to their respective data sheets. CHO-WT and CHO-EE cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. They are grown in GMEM-S. Cells were cultured in a humidified 5% CO_2 incubator at 37 °C.

Cell-surface binding of aptamer-siRNA chimeras (Flow cytometry analysis). CHO-WT gp160 or CHO-EE cells were washed with PBS, trypsinized and detached from the plates. After washing cells twice times with 500 μL 1×HBS buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2.7 mM KCl, 0.01% BSA). Cell pellets were resuspended in 1*HBS buffer and incubated at 37°C for 30 min. Cells were then pelleted and resuspended in 50 μL of 1*HBS (prewarmed to 37°C) containing either 400 nM Cy3-labeled aptamer or Chimera RNAs. After incubation at 37°C for 40 min, cells were washed three times with 500 μL of 1×HBS prewarmed to 37°C, and finally resuspended in 350 μL of 1×HBS buffer prewarmed to 37°C and analyzed by flow cytometry.

Cellular binding and uptake studies. (Confocal Microscopy analysis). The CHO-WT gp160 and CHO-EE cells lines were grown in 8-well chambered-slide with seeding at 1×10^5 in GMEM-S medium to allow 50%-70% confluence in 24 h. On the day of experiments, cells were washed with 250 μL prewarmed PBS. And incubated with 250 μL prewarmed completely growth medium for 30 min at 37°C. Cy3-labeled RNAs at 20 nM of final concentration were added into the media and incubated at 37°C for 1.5 hrs. Subsequently, cells were washed three times with 250 μL of prewarmed PBS, fixed with 4% formaldhydes for 10 min. The cells were stained by treatment with 100 μL of Vybrant Cell-Labeling Solution (DIO membrane dye, Molecular Probes, Invitrogen)

according to the manufacturer's instructions. The images were collected using a Zeiss LSM 510 upright 2 photon confocal microscopy system under water immersion at 40 magnifications. Images were combined and deconvoluted to reconstruct a three-dimensional image.

Analysis of chimera processing. Sense RNAs were annealed with equal moles of 5'-end-labeled antisense strands in HBS buffer in order to form chimeric dsRNA. The chimeras or dsRNAs were incubated at 10 nM final concentration in the absence of target mRNA in HCT116 cell lysates for varying times (20 min, 60 min and 120 min). Reactions were stopped by phenol/chloroform extraction and the RNAs were collected for electrophoresis in a denaturing 20% polyacrylamide gel. The gels were subsequently dried and exposed to X-ray film.

Dual luciferase assays. (Day 1) CHO-gp160 and CHO-EE cells were transfected with pNL4-3. Luc.R-.E- (NIH AIDS Research and Reagent Program, Germantown, MD) and pRSV-Renilla using Lipofectamine 2000. pNL4-3.Luc.R-.E- is an Env- Vpr- non-infectious clone containing the firefly luciferase (F-Luc) gene inserted into the *nef* gene. (Day 2) Cells which transiently expressed pNL4-3.Luc were seeded in 24-well plates at 50-70% confluency. For siRNA, (Day 3) cells were transfected with 200 nM RNA using Lipofectamine 2000. For aptamer-mediated siRNA delivery, (Day 3) cells were incubated in 400 μL refresh complete growth media for 30 min at 37°C. The chimeras RNAs were added directly to the media (400 μL) at a final concentration of 200 nM chimeras. Cells were harvested for analysis on day 4. The expression of the pNL4-3.Luc and normalizing control Renilla luciferase were detected by the Dual-luciferase Reporter Assay System (Promega, USA) according to the manufacturer's instructions. All samples were transfected in triplicate and the experiment was performed a minimum of three times.

5'-RACE PCR assay. Total RNA (5 μg) from CHO-gp160 cells treated with different siRNAs and chimeras was ligated to a GeneRacer adaptor (Invitrogen) without prior treatment. Ligated RNA was reversed transcribed using a gene specific primer 1 (GSP-Rev 1: 5'-TCACCCTCTCCACTGACAGAGAACTT -3'). To detect cleavage products, PCR was preformed using primers complementary to the RNA adaptor (5'-cDNA primer: 5'- GGACACTGACATGGACTGAAGGAGTA -3') and gene-specific primer 2 (GSP-Rev 2: 5'- TAACCTCTCAAGCGGTGGTAGCTGAA -3'). Amplification products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. The identity of the specific PCR products was confirmed by sequencing of the excised bands.

Northern Blot analysis of CEM cells. CEM cells were infected by HIV NL4-3 for 10 days. Prior to adding the various RNAs, the infected-CEM cells were gently washed 3 times to clear out free virus. $5x10^4$ cells were incubated with refolded RNAs at 400 nM final concentrations in 96-well plates at 37°C. The total RNAs were harvested on the 7^{th} day post application for analysis with STAT-60 (TEL-TEST "B", Friendswood, TX) according to the manufacturer's instructions. Two micrograms of total RNAs were electrophoresed in a15% polyacrylamide-8 M urea gel and then transferred to a Hybond N+ membrane (Amersham pharmacia Biotech, USA). Prehybridization and hybridization were carried out using PerfectHyb Plus Hybridization buffer (Sigma, USA) at 37°C with 3 pmol of a 21-mer DNA oligonucleotide probe end-labeled with T4 polynucleotide kinase and γ-^{32}P-ATP. Filters were washed three times at 37°C for 15 min, prior to autoradiography. We also probed for human U6 snRNA as an internal RNA loading standard.

qRT-PCR analysis. CEM cells were infected with HIV NL4-3 for 10 days. Prior to analyses,the infected-CEM cells were gently washed 3 times to eliminate free virus. The infected CEM cells were treated directly with the aptamer or Ch L-1 (400 nM). After 7

d, total RNAs were isolated with STAT-60 (TEL-TEST "B", Friendswood, TX). Expression of the tat/rev coding RNAs was analyzed by quantitative RT-PCR using 2X iQ SyberGreen Mastermix (BIO-RAD) and specific primer sets at a final concentration of 400 nM. Primers were as follows: *tat/rev* forward primer: 5'-GGCGTTACTCGACAGAGGAG -3'; *tat/rev* reverse primer: 5'-TGCTTTGATAGAGAAGCTTGATG -3'; GAPDH forward primer 1: 5'- CAT TGA CCT CAA CTA CAT G-3'; GAPDH reverse primer 2: 5'- TCT CCA TGG TGG TGA AGA C-3'.

RNA-Stat60 was used to extract total RNA according to the manufacturer's instruction (Tel-Test). Residual DNA was digested using the DNA-free kit per the manufacturer's instructions (Ambion). cDNA was produced using 2 μg of total RNA Moloney murine leukemia virus reverse transcriptase and random primers in a 15 μL reaction according to the manufacturer's instructions (Invitrogen). GAPDH expression was used for normalization of the qPCR data.

HIV-1 challenges and p24 antigen assay. Method 1: NL4-3 virus was incubated with refolded RNAs at 37°C for 1 h. Subsequently, viruses were gently washed with PBS and used to infect CEM cells. The culture supernatants were collected at different times post infection (7 d, 11 d, 15 d and 18 d) for p24 antigen analyses. Method 2: CEM cells were infected with HIV NL4-3 for 10 days. Prior to RNA treatments the infected-CEM cells were gently washed with PBS three times to remove free virus. 1.5×10^4 infected CEM cells and 3.5×10^4 uninfected CEM cells were incubated with refolded RNAs at 400 nM final concentration in 96-well plates at 37°C. The culture supernatants were collected at different times (3 d, 5 d, 7 d and 9 d). The p24 antigen analyses were performed using a Coulter HIV-1 p24 Antigen Assay (Beckman Coulter) according to the manufacturer's instructions.

Interferon assay (qRT-PCR analysis). For HEK293 cells, the cells were transfected with siRNA and chimeras RNAs (50 nM) or 200 ng poly(IC) using lipofectamine 2000 (Invitrogene). For infected CEM cells, cells were directly treated with chimera RNAs (400 nM) or IFN-alpha (100 U/mL). After 24 h, total RNAs were isolated with STAT-60 (TEL-TEST "B", Friendswood, TX). Expression of human mRNAs encoding IFN-β, p56(CDKL2) and OAS1 were analyzed by quantitative RT-PCR using 2X iQ SyberGreen Mastermix (BIO-RAD) as described above and specific primer sets for these genes at final concentrations of 400 nM. Primers were as follows: GAPDH primer 1: 5'- CAT TGA CCT CAA CTA CAT G-3'; GAPDH primer 2: 5'- TCT CCA TGG TGG TGA AGA C-3'; IFN-β forward, 5'-AGACTTACAGGTTACCTCCGAA-3'; IFN-β reverse, 5'-CAGTACATTCGCCATCAGTCA-3'; P56 (CDKL2) forward, 5'-GCCTCCTTGGGTTCGTCTATAA-3'; P56 (CDKL2) reverse, 5'-CTCAGGGCCCGCTCATAGTA-3'; OAS 1 forward, 5' -GGAGGTTGCAGTGCCAACGAAG-3'; OAS 1 reverse, 5'-TGGAAGGGAGGCAGGGCATAAC-3';

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Figure Legends.

Figure 1: Predicted secondary structure for anti-gp120 aptamer-siRNA chimeras. The region of anti-gp120 aptamer responsible for binding to gp120 is outlined in green. The siRNA part of the chimera consists of 27 bps as an example here, targeting Site-I of HIV-1 *tat/rev*. Two mutated chimeras M-1 (mutant aptamer) and M-2 (mutant siRNA) were constructed as experimental controls. Mutated regions are shown in magenta.

Figure 2a: Binding affinity assay. Cy3-labeled RNAs were tested for binding to CHO-gp160 cells and CHO-EE control cells. Cell surface binding of Cy3-labeled aptamer-siRNA chimeras were assessed by flow cytometry.

Figure 2b: Binding and uptake of **Ch 1** to CHO-gp160 cells. CHO-gp160 cells and CHO-EE control cells were grown on chamber slides and incubated with 20 nM of **Ch 1** in culture

medium for 2 hours. Cells were washed in PBS three times, fixed and stained with DIO (a plasma membrane dye), washed and analyzed by confocal microscopy.

Figure 3: Analysis of chimera processing. 21-23 nt RNA fragments are produced following incubation of chimera RNAs in HCT116 cell extracts. (**a**) Chimera sense strands were annealed with equal molar equivalents of 5'-end P^{32}-labeled antisense oligos. (**b**) The cleavage products or denatured strands were visualized following denaturing polyacrylamide-gel electrophoresis. Note that the major Dicer product (marked by a white arrow) of the 27 mer aptamers is processed from the 5' end of the antisense strand since the 21 base product harbors the 5' ^{32}P label.

Figure 4a: Aptamer-siRNA chimeras-mediate silencing of pNL4-3 luciferase. CHO-gp160 cells or CHO-EE cells transfected with pNL4-3 luc were incubated with 200 nM of the experimental RNAs in the presence or absence of the transfection reagent lipofectamine 2000. In the absence of the transfection reagent inhibition of pNL4-3 luc expression was only observed for CHO-gp160 cells. These results are consistent with the aptamer mediated binding to gp160 and internalization of the chimera followed by processing into siRNAs. The data were normalized with Renilla luciferase expression and represent the average of three replicate assays.

Figure 4b: Cleaved mRNA from CHO-gp160 cells previously transfected with either saline (untreated), Tat-Rev site I 27-mer siRNA, 21-mer siRNA, **Ch L-1** and **Ch L-2** RNAs, was ligated to an RNA adaptor and reverse transcribed using a gene-specific primer. Depicted is an agarose gel electorphoresis of the 5'-RACE-PCR amplification products using a primer specific to the RNA adaptor and a reverse primer (GSP-Rev-2) to Rev-EGFP, indicated specific siRNA-mediated cleavage products of Rev-EGFP mRNA.

Figure 5a: Northern Blots of infected CEM cells. Infected CEM cells were directly treated with siRNA and Chimeras. The 27 Chimera RNA is partially processed to a 21 mer siRNA following uptake into the CEM cells. Total RNAs were hybridized with a 21-mer P^{32}-labeled oligonucleotide probe. U6 RNA was used as an internal loading control.

Figure 5b: Aptamer-mediated inhibition of expression of *tat/rev* in infected CEM cells. Cells were incubated with the wild type aptamer or **Ch L-1** for 7 days prior to RNA extraction. Gene expression for *Tat/rev* and GAPDH was assayed by qRT-PCR. Data represent the average of three replicates.

Figure 5c: Chimera RNAs inhibit HIV infection. HIV-1 NL4-3 was incubated with the various RNAs at 37°C for 1 h. Subsequently,the treated virions were used to infect CEM cells. The culture supernatant was collected at different time (7 d, 11 d, 15 d and 18 d) for p24 antigen analyses. Data represent the average of duplicate assays.

Figure 5d: The siRNAs delivered by the chimera RNAs inhibit HIV-1 replication in previously infected CEM cells. 1.5×10^4 infected CEM cells and 3.5×10^4 uninfected CEM cells were incubated at 37C with the various RNAs at a final concentration of 400 nM. The culture supernatant was collected at different time points (3 d, 5 d, 7 d and 9 d) for p24 antigen analyses. Data represent the average of triplicate measurements of p24.

Figure 6: IFN assays. IFN-β, the interferon response gene encoding P56 (CDKL2) and OAS1,mRNAs were measured by quantitative RT-PCR. The expression of these interferon response genes was, not significantly induced by the siRNAs or chimeric RNAs, whereas expression of these genes was induced by poly(IC) in HEK 293 cells or by IFN-alpha in infected CEM cells. Gene expression levels are normalized to GAPDH mRNA expression levels. The data represent the average of triplicate measurements.

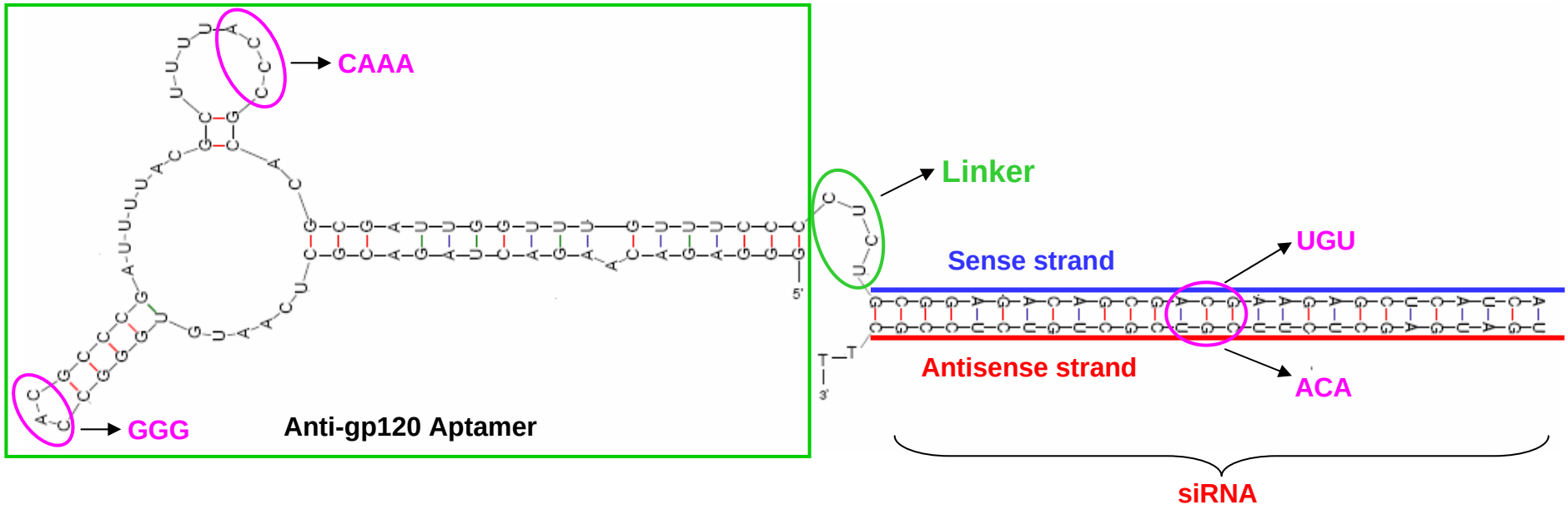
Supplementary materials:

Fig S1: Gene silencing activity and strand selectivity of chimera RNAs and siRNAs. Dual luciferase assays of Renilla luciferase harboring the siRNA targets in the 3' UTR were carried out using the dual luciferase psiCHECK vector. The siRNA mediated knockdown of sense (white bars) and anti-sense (gray bars) targets are shown. All RNAs are normalized to the value of the corresponding buffer control. The strand selectivity was calculated: $R_{buffer} = 1.0$; $R_{27\ mer\ siRNA} = 2.2$; $R_{21\ mer\ siRNA} = 4.9$; $R_{Ch\ L\text{-}1} = 3.2$; $R_{Ch\ L\text{-}2} = 1.9$; $R_{Ch\ 1} = 2.9$; $R_{Ch\ 2} = 1.6$; $R_{M\text{-}2} = 1.2$, respectively.

Fig S2: Confocal microscope images were combined and deconvoluted to reconstruct a three-dimensional image. Three-dimensional image reconstruction shows localization of the Cy3-labeled **Ch 1** interior to the plasma membrane of a single cell.

Fig S3: RACE PCR sequence.

Figure S4: Immunofluorescence assay of HIV-1 p17. HIV-1 infected CEM cells were incubated with 400 nM of aptamer or chimeras (**Ch L-1** and **Ch L-2**) in culture media for **a**) 24 hours and **b**) 72 hours. Cells were washed with PBS, fixed, permeabilized and blocked with NGtS. After incubation with the primary antibody (anti-p17), a FITC-conjugated secondary antibody (Ho-α-Mu-FITC) was added to detect the p17 antibody. Cells were washed, resuspended in 15 μL hard mounting medium and spotted on a microscope slide for confocal microscopy imaging.



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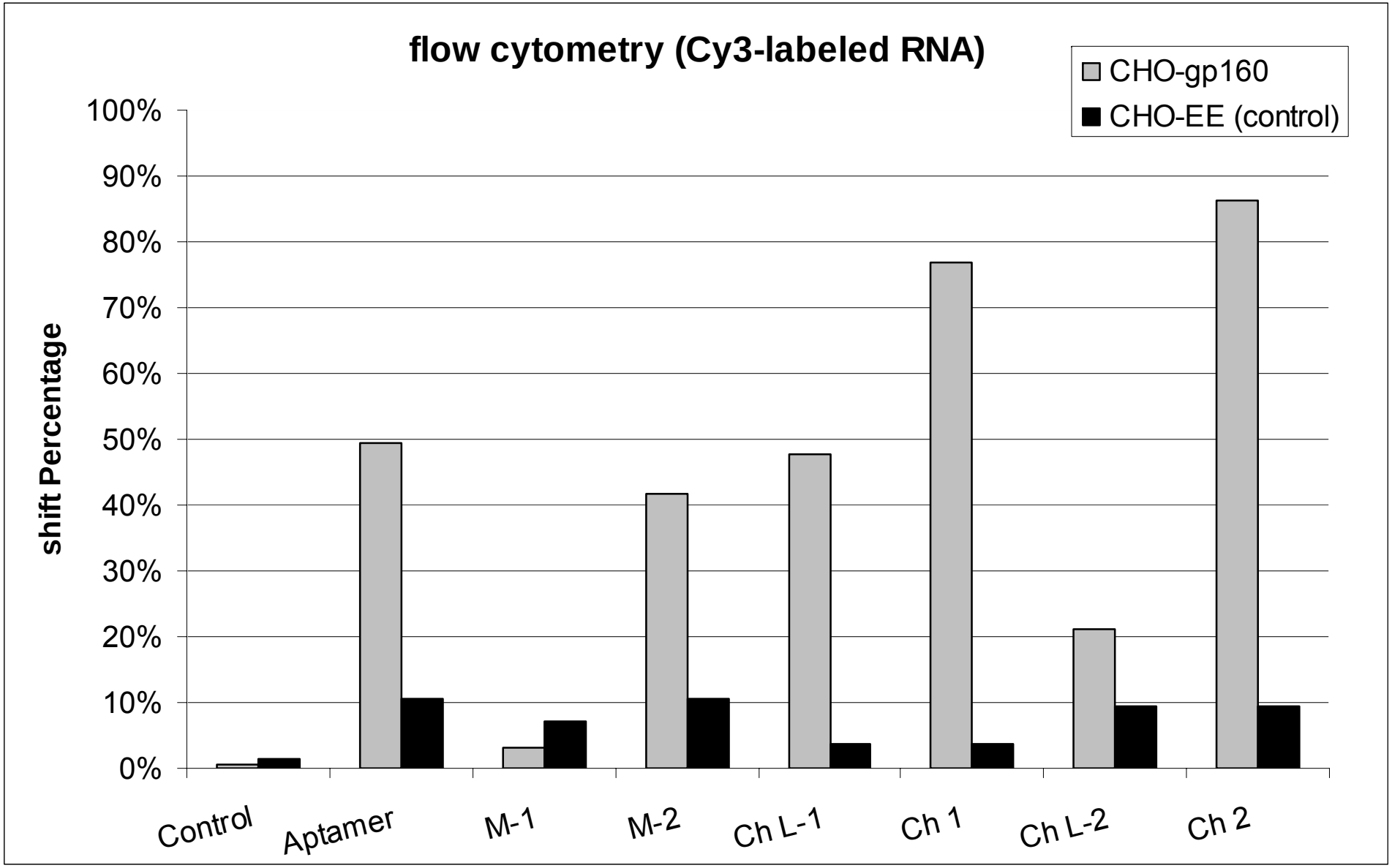

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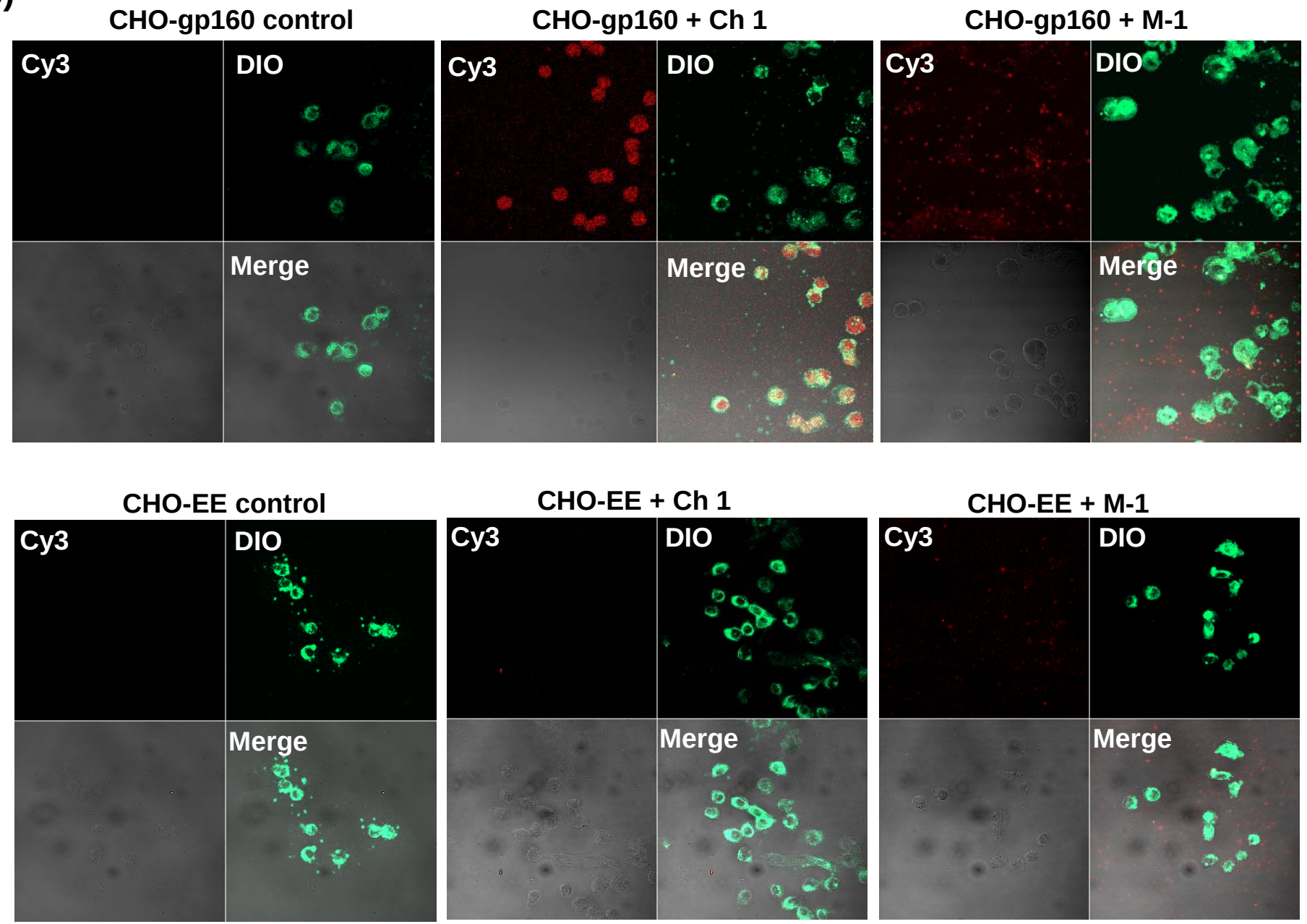

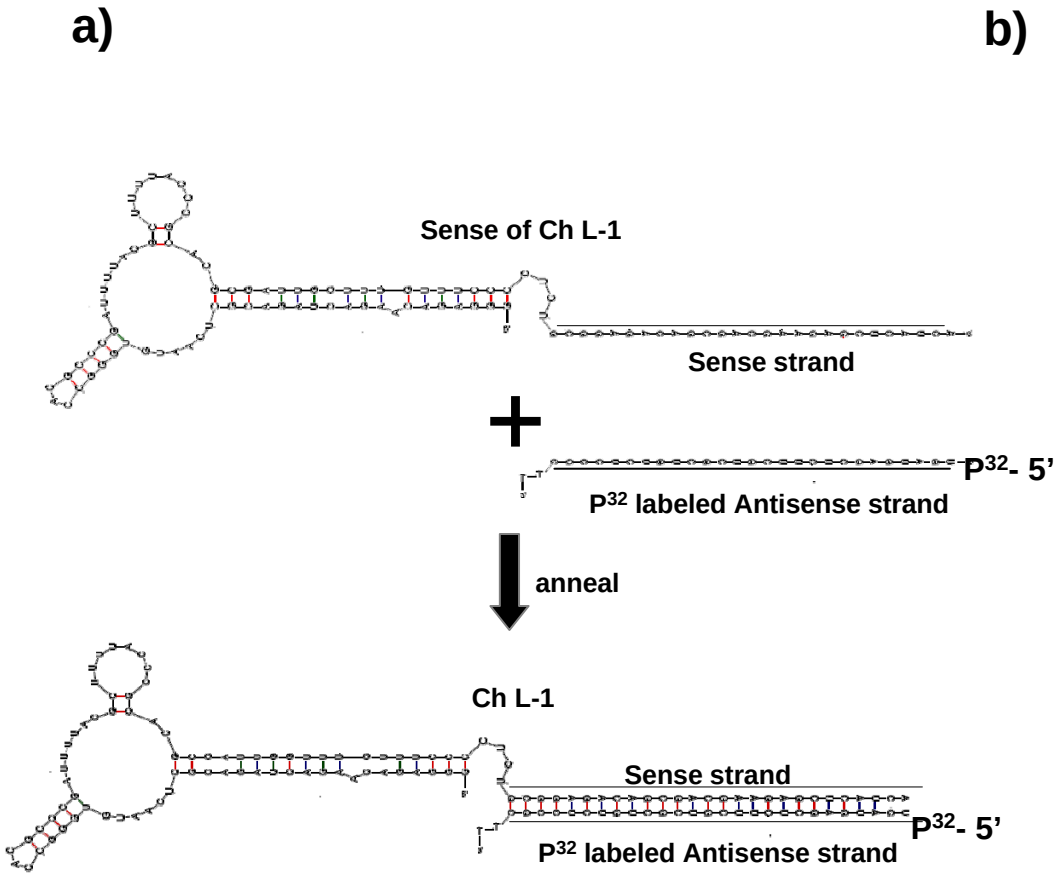

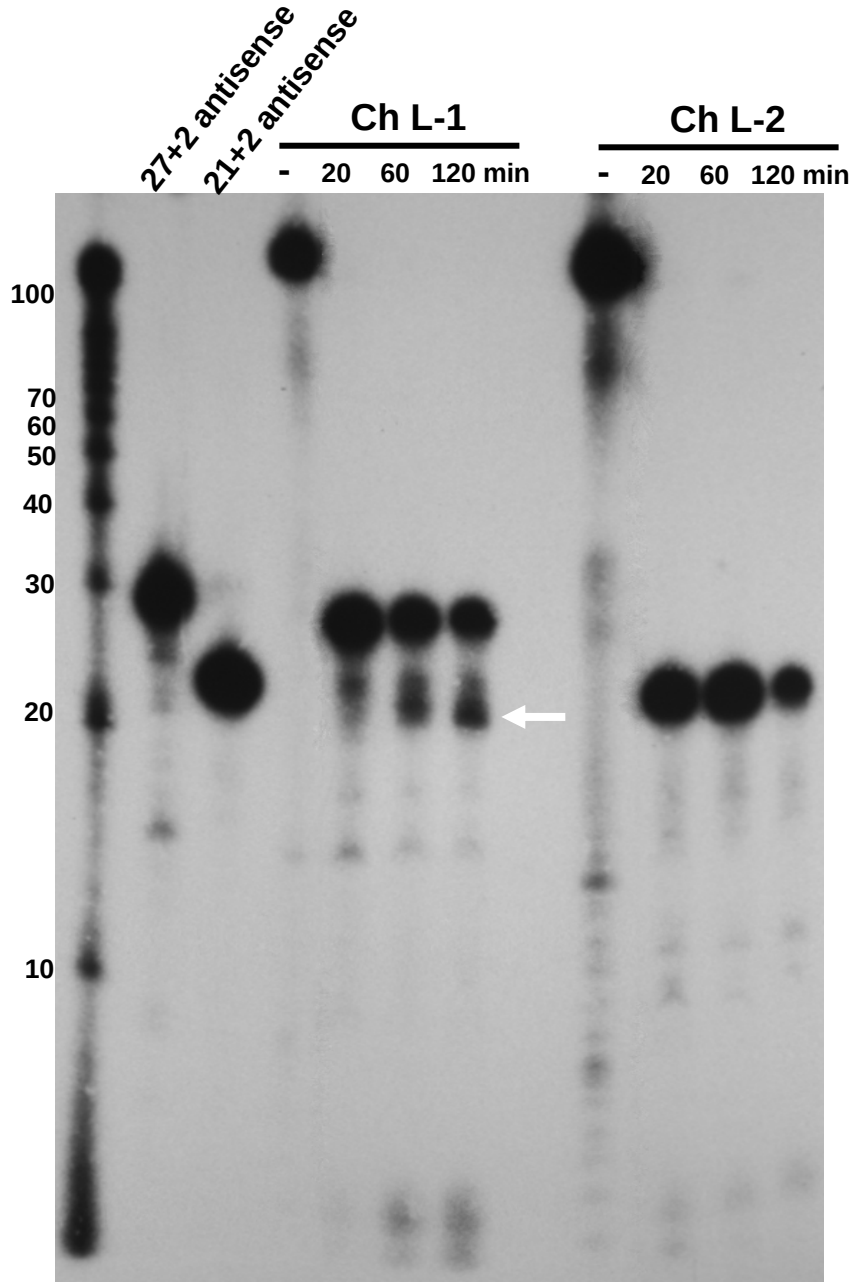

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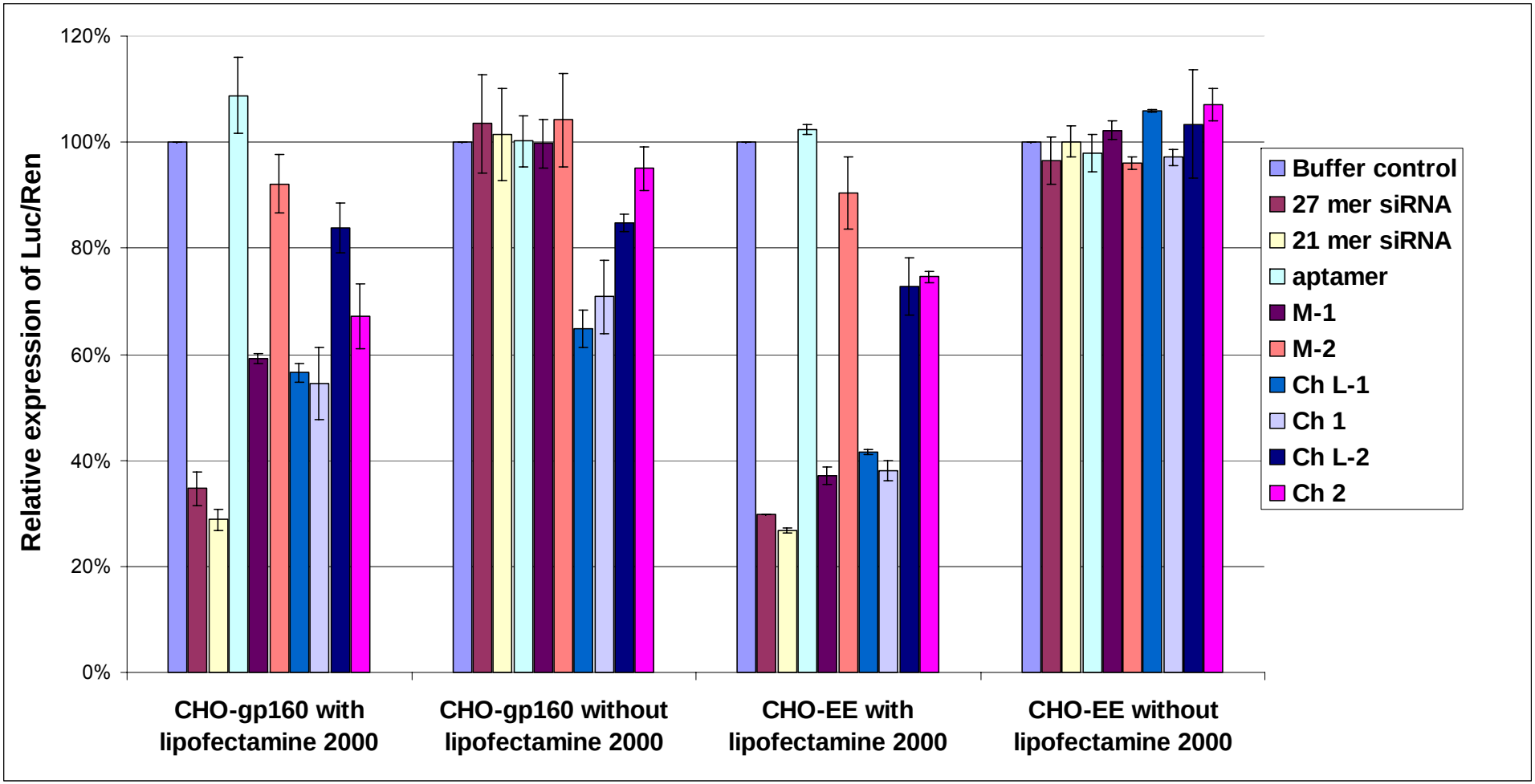

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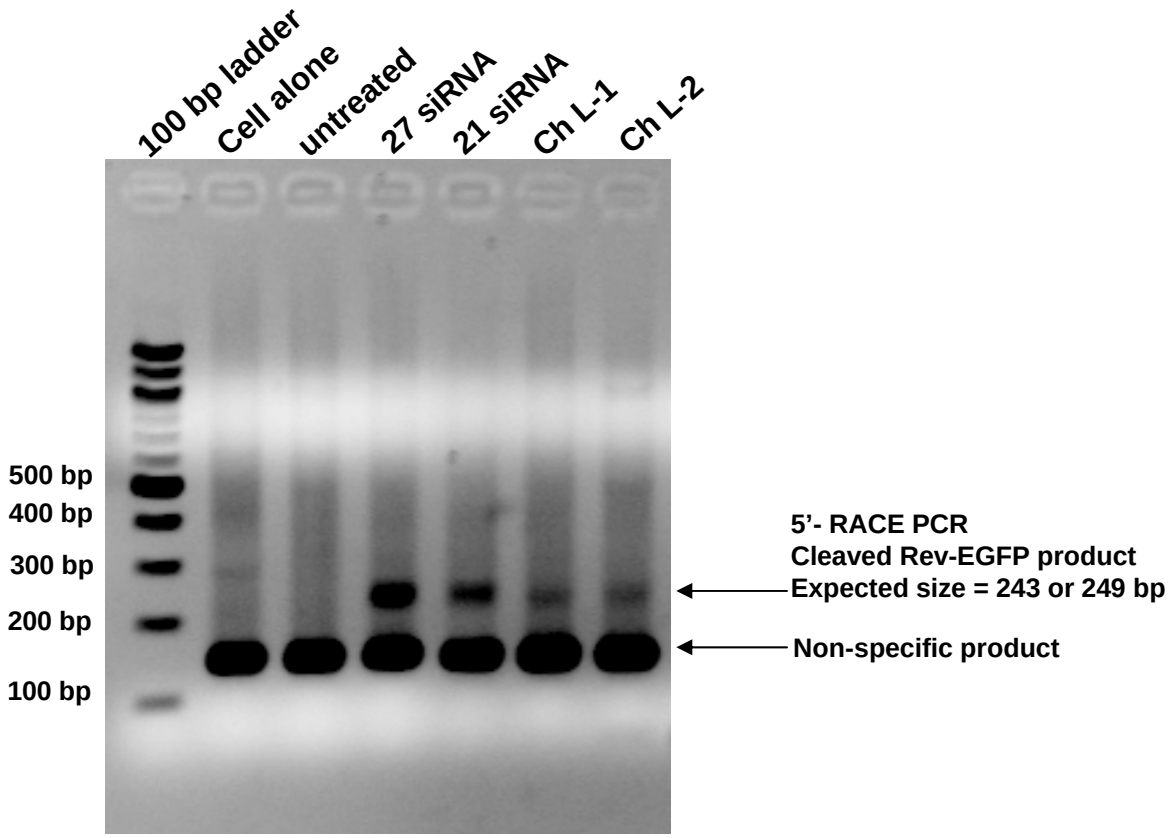


RACE PCR

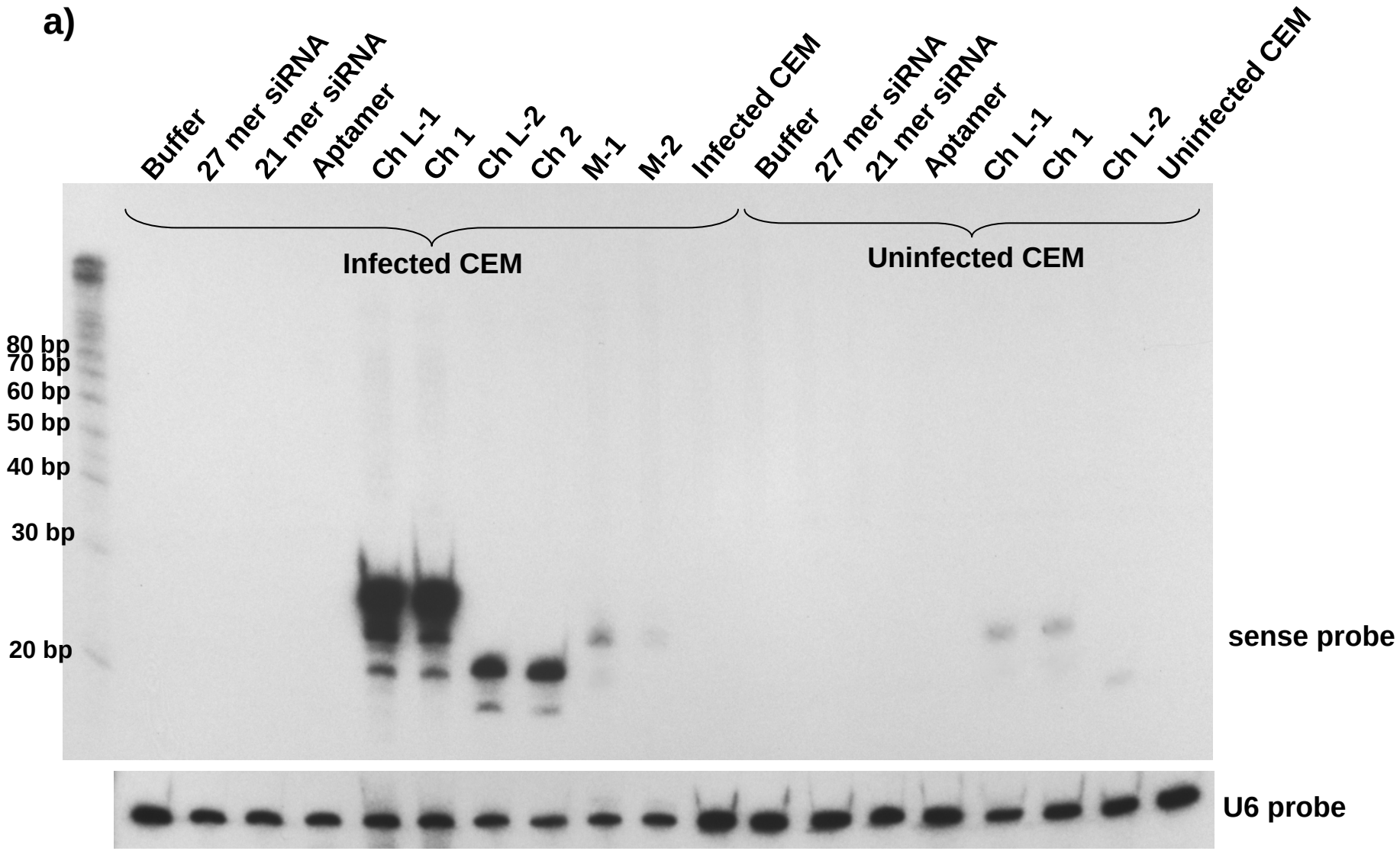
21+2 mer antisense strand 3'- dTdT CGCCUCUGUCGCUGCUUCUCG -5'

10 nt

Target sequence of Tat/Rev GCGGAGACAGCGACGAAGAGCTCATCA

10 nt

27+2 mer antisense strand 3'- dTdT CGCCUCUGUCGCUGCUUCUCGAGUAGU -5'



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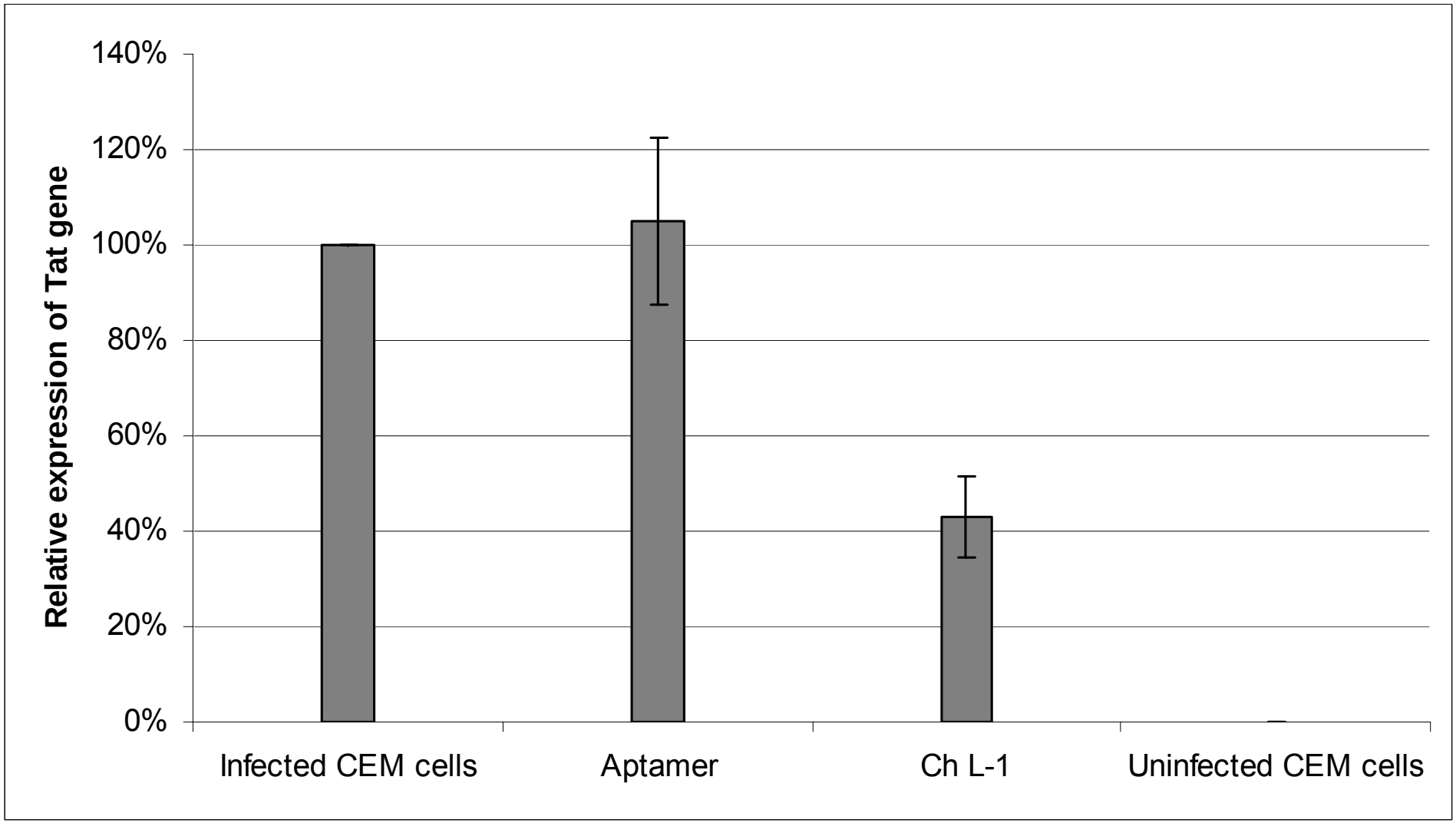

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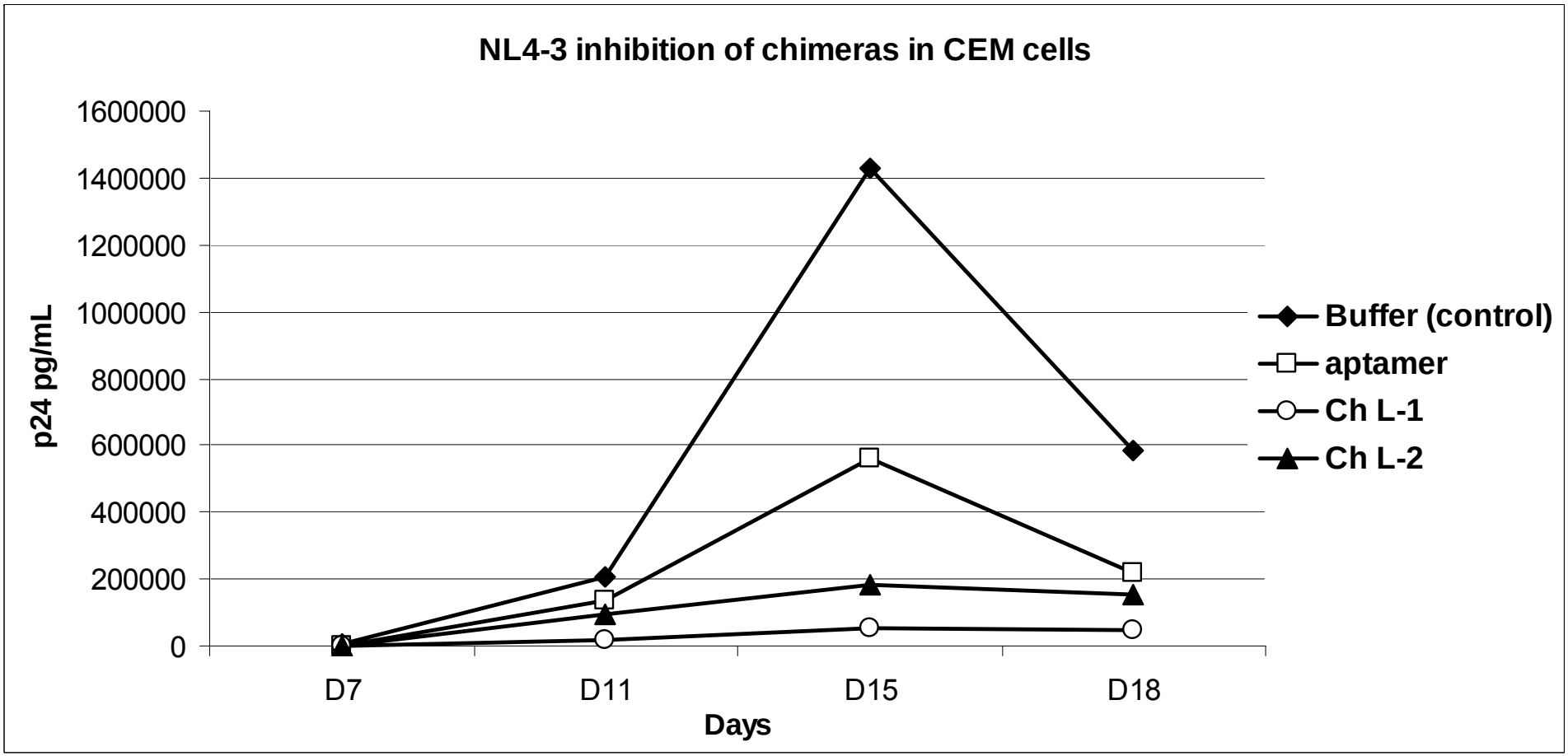

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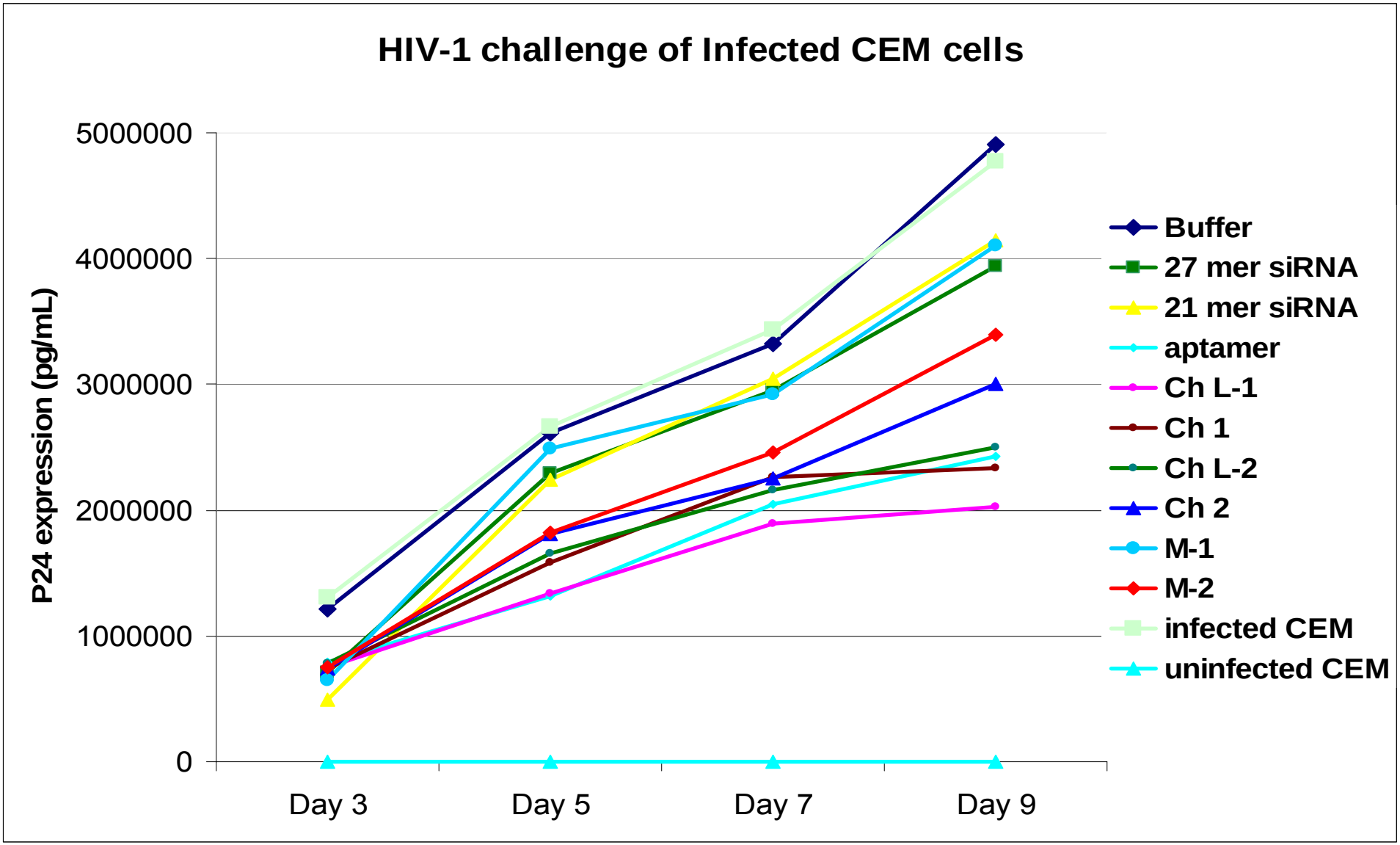

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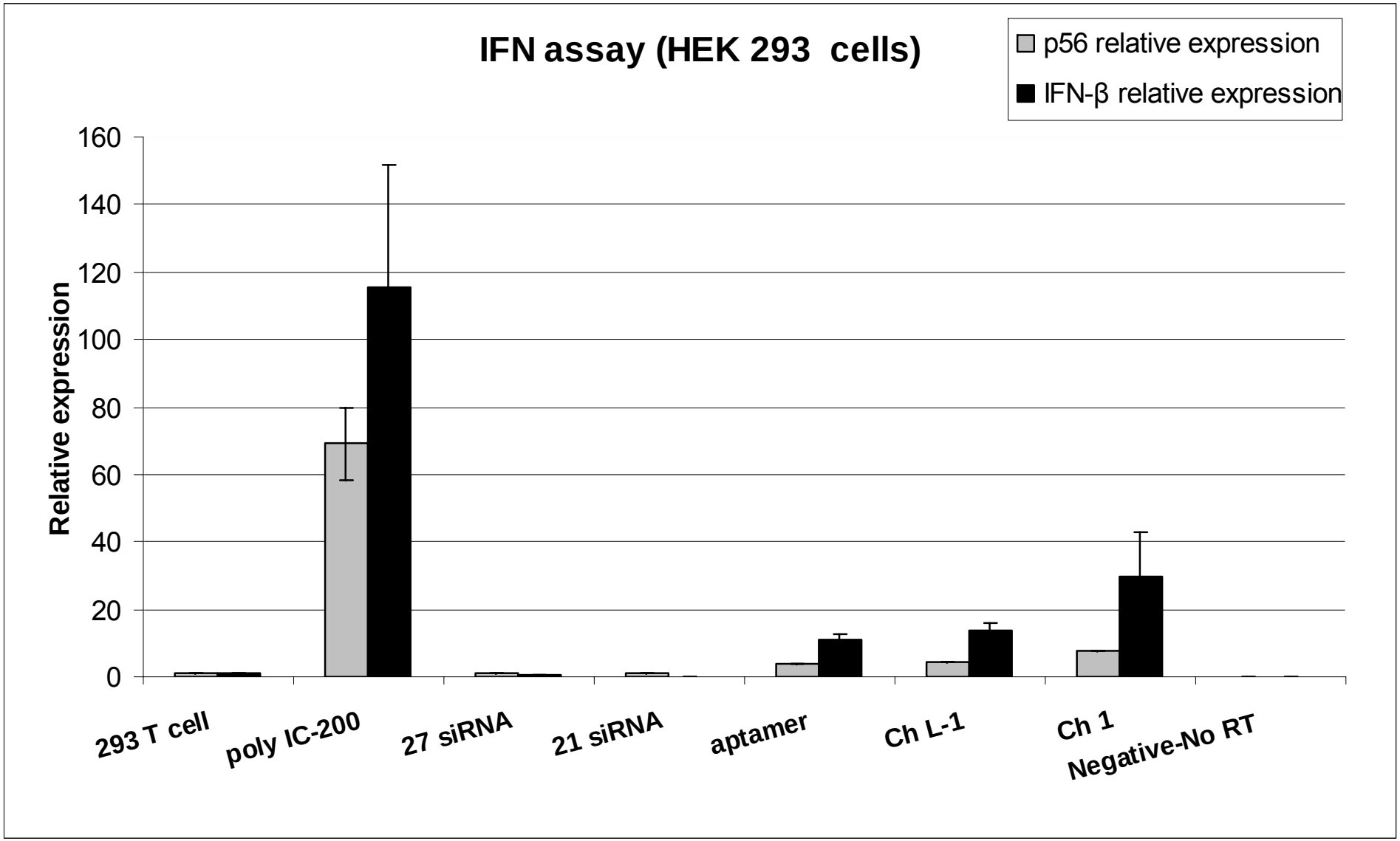

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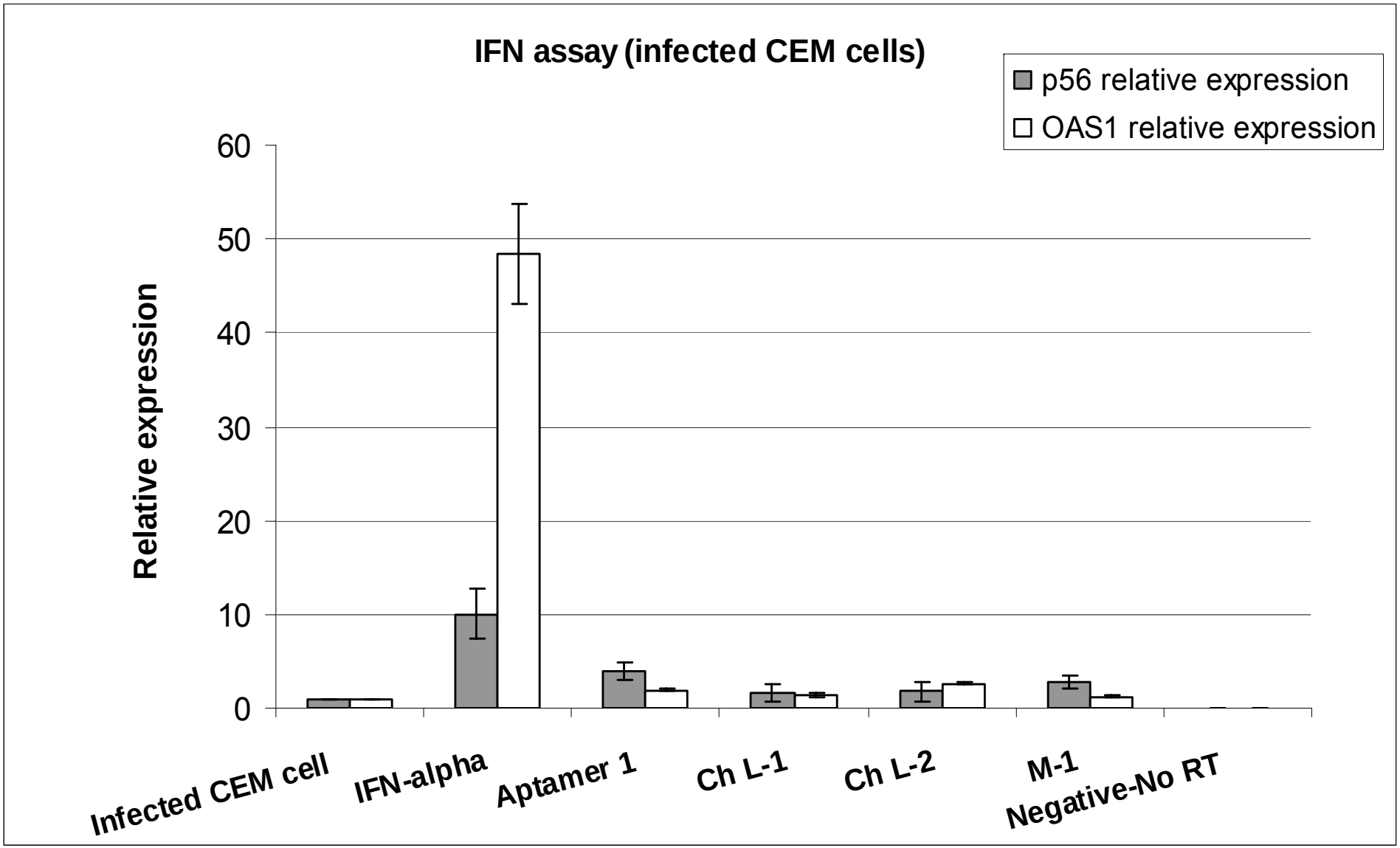

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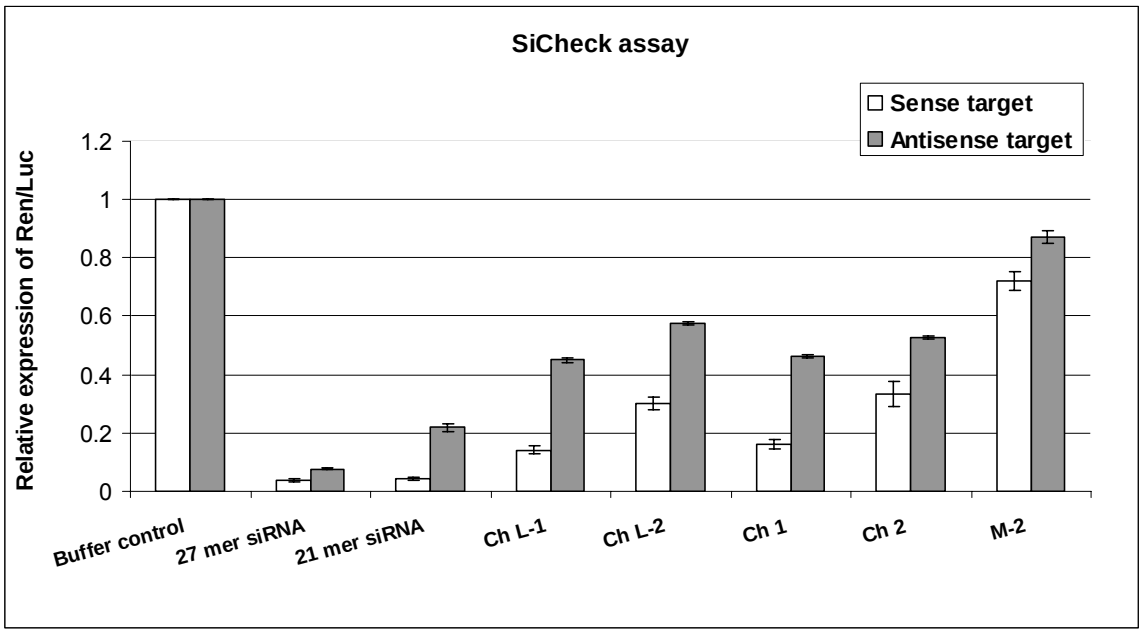


Figure S2:

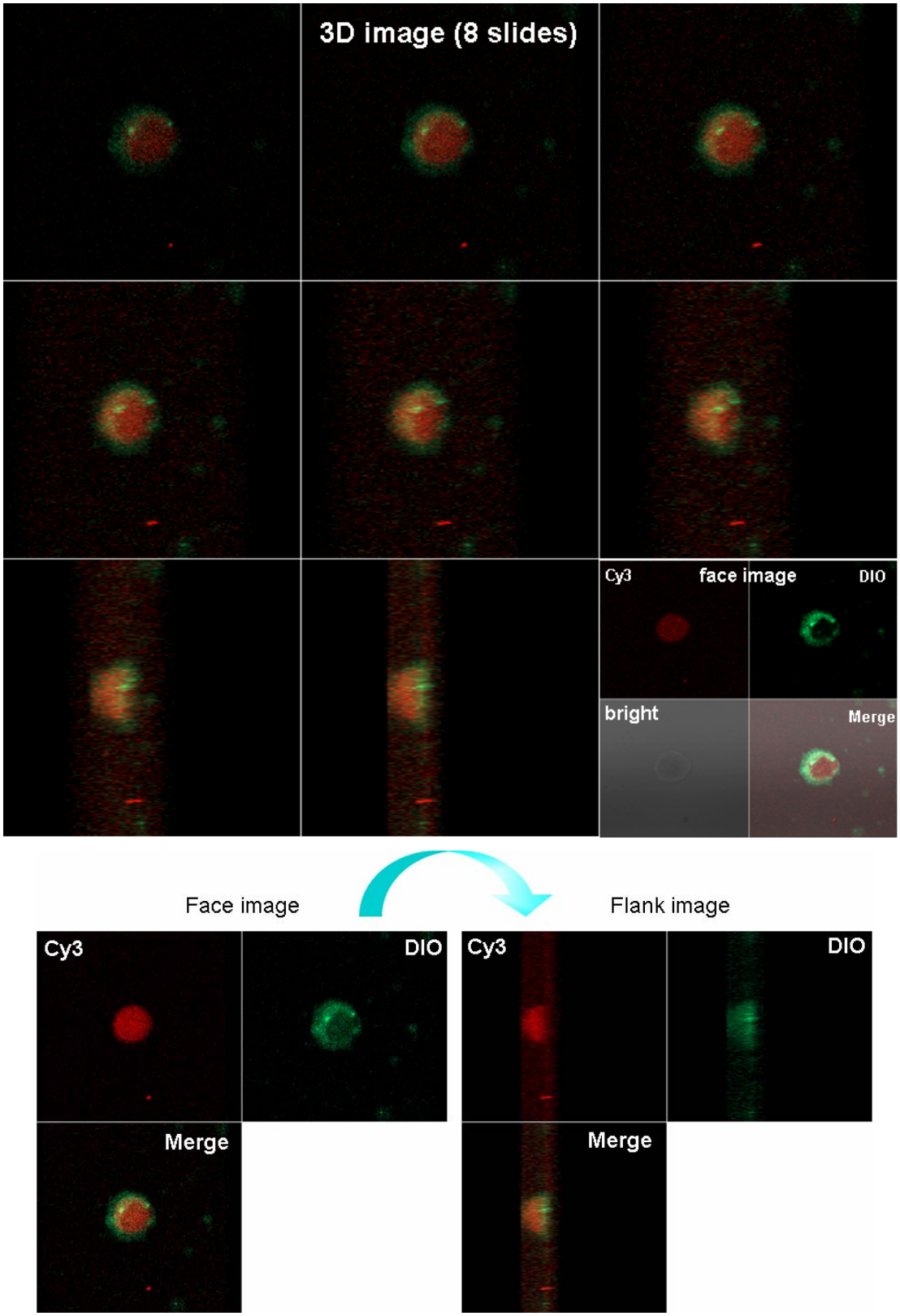


Fig S3:

For 27 mer duplex RNA, RACE PCR product was cloned into TA vector and sequenced.

RACE PCR Product exact sequence (243 bp): 5'- GGA CAC TGA CAT GGA CTG AAG GAG TAG AAA GAG CTC ATC AGA ACA GTC AGA CTG ATC AAG CTT CTC TAT CAA AGC AAC CCA CCT CCC AAT CCC GAG GGG ACG CGT CAG GCG CGC AGG AAT AGA AGG CGC CGG TGG AGA GAG AGA CAG AGA CAG ATC CAT TCG ATA TCT GAA CGG ATC CTT GGC ACT TAT CTG GGA CGA TCT GCA GAG CCT GTG CCT CTT CAG CTA CCA CCG CTT GAG AGG TTA -3'

For 21 mer duplex RNA, RACE PCR product was gel purified and directly sequenced using relative forward primer (5'-cDNA primer 1) and reverse primer (GSP primer 2).

RACE PCR Product exact sequence (249 bp):

5'- GGA CAC TGA CAT GGA CTG AAG GAG TAG AAA GAC GAA GAG CTC ATC AGA ACA GTC AGA CTG ATC AAG CTT CTC TAT CAA AGC AAC CCA CCT CCC AAT CCC GAG GGG ACG CGT CAG GCG CGC AGG AAT AGA AGG CGC CGG TGG AGA GAG AGA CAG AGA CAG ATC CAT TCG ATA TCT GAA CGG ATC CTT GGC ACT TAT CTG GGA CGA TCT GCA GAG CCT GTG CCT CTT CAG CTA CCA CCG CTT GAG AGG TTA -3'

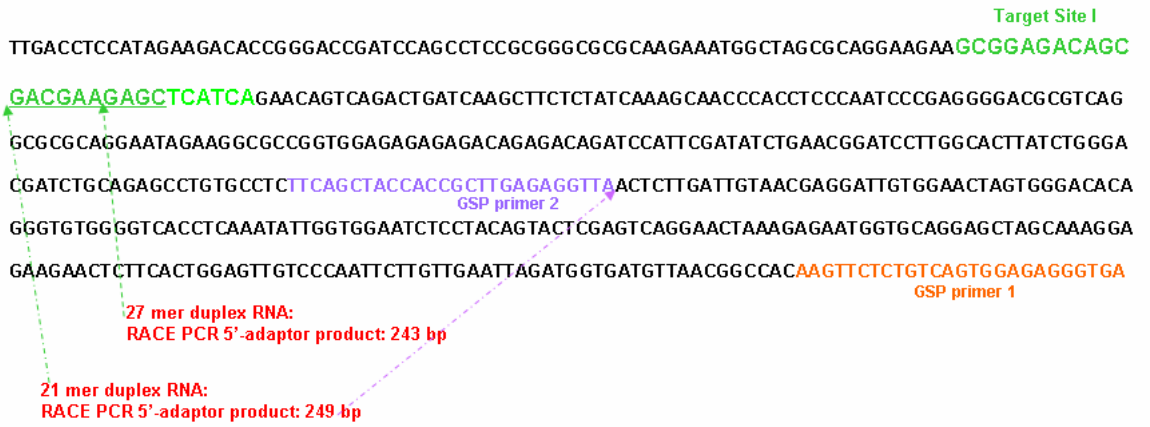
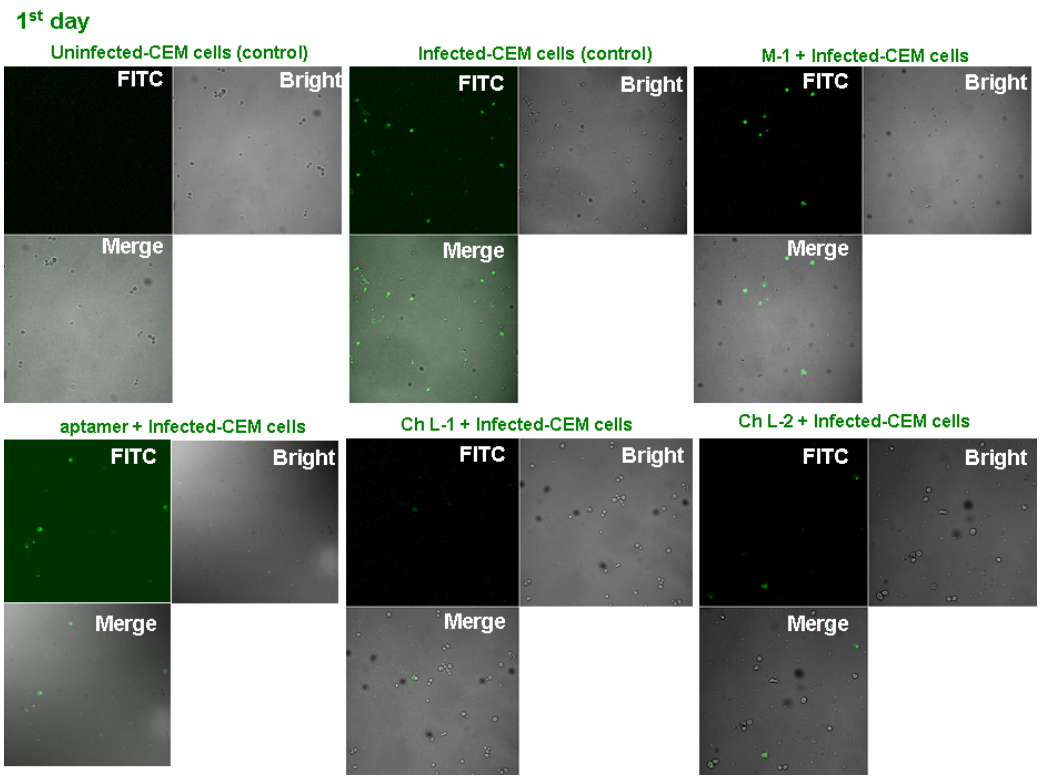


Figure S4: a)



b)