Optimizing Transfection- and Transduction-Free siRNA Delivery and Gene Expression Knockdown in Primary Human CD4+ T Cells

Anangi Balasiddaiah

Department of Pharmacy, UiT The Arctic University of Norway

Corresponding authors address: Anangi Balasiddaiah, Department of Pharmacy, UiT The Arctic University of Norway, Phone: 0047 41332735, Email: anangib@gmail.com

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Abstract

T-cell-based therapies have gained momentum in recent decades, revolutionizing treatment options for cancer patients. Currently, CRISPR Cas-9 and RNAi technologies are commonly employed for understanding genetic networks in primary T cells. These technologies involve mainly electroporation or viral vectors to deliver the cargo required for gene editing or silencing and they pose some limitations such as reduced cell viability, poor transfection efficiency, and insertional mutagenesis. Dharmacon™ has developed chemically modified self-delivering siRNAs termed Accell™ siRNAs with wide cell type applicability transcending the need for transfection reagents or viral vectors for intracellular siRNA delivery. In this study, using human primary CD4-T cells as a model, we optimized Accell™ siRNA uptake and gene expression knockdown in unstimulated T cells and optimized a workflow to efficiently knockdown gene expression in primary T cells without compromising cell viability. This method is useful for rapidly investigating gene functions in primary T cells in a resting state.

Introduction

T cells play a crucial role in maintaining normal health and in the outcome of a disease during an infection ([1]). While the immune functions of T cells are protective against viral infections and tumor progression, overactivation of T cells due to genetic abnormalities is a grave harm leading to autoimmune diseases ([1]). In recent years, T cell therapy has garnered immense attention owing to the success of TCR/CAR redirected T cell therapeutics against solid and hematologic cancers ([1]). At this juncture, a revision and enhancement in the gene silencing technologies to manipulate T cells and study the intricacies of genetic networks within T cells are of utmost importance to advance the available therapeutics milieu of T cells.

Gene Knockout by CRISPR-Cas9 and gene expression knockdown by siRNA-enabled RNA interference are the two widely used technologies to manipulate and study gene function in T cells ([2,3]). These technologies utilize viral vectors or electroporation to deliver gene silencing or editing machinery and are limited by imposed genotoxicity and insertional mutagenesis or cytotoxicity due to high voltage ([2,3]). While electroporation has been the most commonly used technique to deliver cargo into T cells, primary T cells are notoriously difficult to transfect without prior activation ([2]), thus limiting the study of gene expression in unstimulated T cells. While a few electroporation methods allow transfection of primary T cells without prior activation, they have demonstrated poor transfection efficiency with a decreased cell viability which further affects gene expression profiles making the electroporation not ideal for T cell gene editing or silencing ([2,3]).

Dharmacon™ has developed novel chemically modified siRNAs called Accell™-siRNAs using a proprietary siRNA sequence design algorithm ([4]). Accell™ siRNAs require no transfection reagents or viral vectors for intracellular delivery and they are known to be effective in hard-to-transfect cell types such as primary neurons and T cells. However, to our knowledge, Accell siRNAs have not been widely explored in primary human T cells although few studies tested Accell™ siRNAs on T cells ([5–13]). In this study, we optimized Accell™-siRNA uptake,

stability, and gene expression knockdown efficiency in unstimulated T cells using freshly isolated human CD4-T cells as a model and propose a reliable workflow to study gene function in unstimulated primary human T cells.

Results

T- cell culture media (X-VIVO-15, TexMACS) show strong Accell siRNA delivery into unstimulated human primary CD4-T cells while Accell siRNA delivery media further improves Accell siRNA delivery

As a starting step, we optimized Accell siRNA delivery into unstimulated primary T cells using freshly isolated human CD4-T cells and Accell-green-non-targeting control siRNA (control siRNA that is not complimentary to any human transcripts). Accell-green-non-targeting control siRNA is 6-FAM labeled that exhibits green fluorescence and allows assessment of Accell siRNA uptake by flow cytometry. Here, we tested conditions covering three different serum-free media (X-VIVO-15 ™, TexMACS ™, Accell siRNA delivery media) supplemented with or without an anti-CD3 antibody. According to Dharmacon™, Accell siRNA delivery media maintains cell health and provides suitable serum-free conditions for the uptake of Accell siRNAs. While Dharmacon recommends this media for use with Accell siRNAs they also encourage customers to test cell-type-specific serum-free media that are regularly used to maintain the particular cells. Therefore, we decided to screen two serum free T-cell culture media (X-VIVO-15 ™, TexMACS) along with Accell siRNA delivery media. Thus, we treated human primary CD4-T cells with Accell-green-non-targeting control siRNA in the presence of different conditions and evaluated the Accell siRNA uptake, stability, and viability at 24 h and 48 h time points by flow cytometry. We found that almost all cells retained high siRNA fluorescent signals while maintaining high cell viability (95%) at all the conditions tested (Fig. 1a and b, Fig. 2a and b). T- cell culture media (X-VIVO-15, TexMACS) displayed strong delivery of Accell siRNAs while Accell siRNA delivery media enhanced siRNA uptake by three-fold compared to X-VIVO ™-15 or Tex MACS™ when we measured the mean fluorescence intensity (MFI) of the siRNA retained by T cells at both the time points (Fig. 1b,

Fig. 2b). In addition, T cells cultured in Accell siRNA media displayed similar levels of siRNA fluorescent signals whereas T cells in T cell culture media (X-VIVO-15, TexMACS) retained markedly different levels of siRNA fluorescent signals, indicating heterogeneity in uptake among the T cells cultured in T cell culture media (Fig 1b, Fig 2b). The addition of anti-CD3 antibody neither affected cell viability nor siRNA uptake (Fig. 1a and b, Fig. 2a and b). Of note, the addition of siRNA to the cells did not affect cell viability compared to the cells without siRNA indicating that Accell siRNAs are not toxic to CD4-T cells. Regardless of the conditions tested, strong siRNA delivery was pronounced at 24 h after siRNA treatment. The siRNA levels were sustained until 48 h (Fig 1b, Fig 2b) indicating that siRNA uptake is probably complete by 24 h. In conclusion, these data indicate that Accell siRNA delivery into human primary CD4-T cells is highly effective at all three serum-free media tested while Accell siRNA delivery media yields the highest siRNA uptake.

Accell siRNA causes efficient gene expression knockdown in unstimulated primary human CD4-T cells

To optimize gene expression knockdown in human T cells using Accell siRNAs, we further tested the Accell siRNA smart pool targeting GAPDH gene transcripts in unstimulated primary human CD4-T cells. As a control, we treated T cells with Accell non-targeting siRNA (control siRNA that is not complimentary to any human transcripts) to assess non-sequence-specific effects on the cells (Fig.3a). Here, T cells were cultured only in Accell siRNA delivery media as it improved siRNA uptake in siRNA delivery experiments as shown in Fig.1b and Fig. 2b. In addition, we tested anti-CD3 antibody to check its effects on Accell siRNA mediated gene expression knockdown (Fig. 3 a). Treatment of CD4-T cells with the Accell siRNA smart pool targeting GAPDH resulted in a clear knockdown of GAPDH at the transcript level (Fig. 3 b). In the absence of anti-CD3 antibody, GAPDH gene expression knockdown efficiency reached 48% at 48 h and 62% at 72 h. In the presence of an anti-CD3 antibody, GAPDH gene expression knockdown efficiency reached 68% at 48 h (20% increase compared to without anti-CD3 antibody condition at 48 h) and 72% at 72 h (10%

increase compared to without anti-CD3 antibody condition at 72 h) as shown in Fig.3 b. These data indicate that Accell siRNA causes efficient knockdown at the transcript level in unstimulated primary human CD4-T cells and the addition of an anti-CD3 antibody further enhances the knockdown by 10-20%.

Discussion

In the present study, we optimized Accell siRNA delivery and gene knockdown in human primary CD4-T cells without pre-activation. We optimized a method that allows efficient gene expression knockdown and rapid gene function analysis in freshly isolated primary human CD4-T cells (Fig. 4). Accell siRNAs by Dharmacon are two decades old and have been successfully used to knock down gene expression in difficult-to-transfect cell types such as primary neurons, NK cells, and T cells. While the idea of exploitation of Accell siRNAs for the gene expression knockdown in T cells is not new, to our knowledge, a few studies tested Accell siRNAs on T cells ([5–13]) and the majority of these studies worked with T cells expanded or preactivated. In agreement with our results, these studies demonstrated efficient gene knockdown of primary T cells.

Alternatively, to check the impact of gene knockdown, functional evaluation of the targeted gene can be performed if the targeted gene is involved in cell proliferation or apoptosis or a specific signaling pathway.. Most of these studies reported clear protein knockdown in T cells treated with Accell siRNAs. Three of these studies used human T cells ([8–10]) while the rest worked with mouse T cells ([5–7,11]) for their siRNA experiments. One of these studies used an elegant approach where they treated T cells with siRNA for 6 days before they used them for functional assays (3 days after siRNA treatment, they changed the medium with fresh medium containing siRNA, and then siRNA treatment was continued for three more days) ([8]). This could be a better strategy when protein knockdown is not observed despite transcript knockdown.

Accell siRNAs can enter the cells without any reagent or vehicle and are stable but a downside is that most of the Accell siRNAs designed by Horizon discovery are not tested on cells and therefore unexpected results will be expected. For example, we tested 4 Accell siRNAs targeting four different genes but only 2 of them worked in our hands (only 2 siRNAs were able to cause gene knockdown). Another limitation with Accell siRNA is that they may not be useful to target inducible genes like inducible phosphatases as they are expressed shortly depending on the stimulus, and the experiments need to be conducted within that short window of time which is not enough for Accell siRNA to knockdown the target gene.

Given the importance of T cells in TCR/CAR therapy and autoimmune diseases, our work has clinical implications as our method optimization for siRNA delivery and gene knockdown in human CD4-T cells can aid in rapidly understanding the function of genes in T cells that play an important role in anti-tumor immunity or autoimmune diseases.

Acknowledgements:

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Materials and Methods

PBMC isolation, T cell sorting, T cell culture with Accell siRNAs, and Flow cytometry

Buffy coats from healthy donors were purchased from Universitetssykehuset Nord-Norge HF (UNN) with permission. Peripheral blood mononuclear cell (PBMCs) isolation was performed with density gradient centrifugation over Lymphoprep (Stem cell technology #07811). CD4 -T cell sorting was performed using CD4-T cell isolation kit (Miltenyi Biotec, 130-096-533) by manual cell separation with LS columns and QuadroMACS separator.

For T cell experiments with Accell green-non-targeting siRNA, 5 M purified CD4-T cells were resuspended in 500 ul serum free media (X-VIVO-15, Lonza, 02-060Q; TexMACS, Myltenyi Biotec, 130-097- 196; Accell siRNA delivery media, Horizon Discovery, B-005000-500) and then were seeded in 48 well plate and then treated with 1 uM siRNA (Horizon discovery, D-001950-01-20) in the presence or absence of aCD3 antibody (Tonbo Biosciences, 40-0038-u500). After 24 h and 48 h of treatment, the cells were stained with Near IR Live/dead dye and then the cells were acquired on BD Fortessa. The data was analyzed on Cytobank.

For knockdown experiments with GAPDH targeting siRNA, 5 M T-cells were resuspended in 500 ul Accell siRNA delivery media and then were treated with GAPDH targeting siRNA smart pool (Horizon discovery, D-001930-10-20) or control non-targeting siRNA (Horizon discovery, D-001910-10-20) at 1uM in the presence or absence of aCD3 antibody. After 48 h and 72 h after treatment, T-cells were pelleted for RNA extraction, c-DNA synthesis, and qPCR.

For siRNA knockdown experiments with CD5 targeting siRNA, 5 M T-cells were resuspended in 500 ul Accell siRNA delivery media and then were treated with CD5 targeting siRNA smart pool (Horizon Discovery, E-007848-00-0020) or control non-targeting siRNA at 1uM. Cells were collected at 48 h and 96 h and then stained with Near IR Live/dead dye and anti-CD5 antibody and then the cells were acquired on BD Fortessa to measure the surface levels of CD5 protein on T cells. The data was analyzed on Cytobank.

RNA extraction, cDNA synthesis, qPCR

Quick RNA miniprep kit (Zymo Research, R1055) was used to extract RNA from T cells by following the protocol provided with the kit. During extraction, RNA was treated with DNAase-1 to remove the presence of the residual amount of genomic DNA. High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Thermoscientific, 4374966) was used to synthesize cDNA for qPCR. Briefly, 200 ng of total RNA was used for cDNA synthesis in 20 ul volume reaction. After cDNA synthesis, cDNA was diluted four times by adding 60 ul of sterile water. PCR reaction contained 5 ul of 2 X PowerUp™ SYBR™ Green Master Mix (Thermoscientific A25918), 3 ul of CDNA template, and 2 ul of water in a final volume of 10 ul. qPCR was performed on Light cycler 96 (Roche). Light cycler 96 stware was used æællto analyze the quality of the data and extract CT values. Actin was used as a reference or internal control gene to normalize the mRNA levels between different samples and the data was analyzed using formula 2^ (CT of reference gene- CT of gene of gene of interest) as described earlier ([14,15]). The sequences of the primers used are listed in the table below. The primers listed were validated using positive and negative controls before using them in qPCR and all the primer sets yielded the expected amplicon size, amplification was not found when RNA alone was included as a template.

Name of the gene	Primer sequence	Product size
GAPDH (Forward primer)	CTTTTGCGTCGCCAG	140 bp
GAPDH (Reverse primer)	TTGATGGCAACAATATCCAC	
Actin (Forward primer)	CTTCGCGGGCGACGAT	103 bp
Actin (Reverse primer)	CACATAGGAATCCTTCTGACCC	

Western blotting

5-10 M CD4-T cells are lysed in 60 ul MKK lysis buffer and stored for 5 min at RT for completion of lysis. Then the samples were spun at high speed (12000 rpm for 10 min) and 52 ul of cell lysate was collected without touching the pellet. To 52 ul of cell lysate, 20 ul of 4 X LDS buffer (Novex Life

technologies, NP0008) and 8 ul of 10 X Bolt samples reducing agent (Novex Life technologies, B0009) were added and incubated at 70°C for 10 min to reduce the proteins in the samples. 2 or 10 ul of reduced samples were loaded and separated on Bolt™ 8-12%, Bis-Tris, 1.0 mm, Mini Protein Gels (Thermo scientific, NW04120BOX). XCell SureLock™ Mini-Cell and XCell II™ Blot Module (Invitrogen, EI0002) were used for protein transfer to odyssey nitrocellulose membranes (LI-COR, 926-31092). After transfer, the membranes were blocked with Intercept (TBS) blocking buffer (LI-COR, 927-60001) for 1 hour and then the membranes were transferred to 50 ml falcon tube containing 5 ml of Intercept (TBS) blocking buffer containing the primary antibody and 0.1% Tween and then the tube was kept for rotation overnight on a rotator in a cold room. After overnight rotation, the membranes were washed with 1x TBS+0.1% Tween three times on a shaker at RT. Then membranes were transferred to TBS buffer containing 0.1% tween and IRDye secondary antibody (LI-COR) and then rocked for 1 h on a shaker followed by three washes with 1x TBS+0.1% Tween. The membranes were probed on Odyssey Sa imager and raw data files were analyzed using Image studio version 5.2. All the primary and secondary antibodies used for probing are listed in table below.

Name of the antibody	**company**	**Catalog number**	**dilution**
GAPDH antibody	Cell signaling Technology	#2118	1:1000
GAPDH antibody	Santacruz biotechnology	Sc-47724	1:200
Actin antibody	Cell signaling Technology	#4968	1:1000
Donkey anti-mouse 680 LT	LI-COR	926-68022	1:10000
Donkey anti-rabbit 680 RD	LI-COR	926-68073	1:10000
Goat anti-mouse 800 CW	LI-COR	925-32210	1:10000
Donkey anti-rabbit 800 CW	LI-COR	926-32213	1:10000

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Step by step by protocol

Reagents needed

5X siRNA buffer

CD4 T cell isolation kit

siRNA smart pools and Non-targeting siRNA as a control

Accell siRNA delivery medium

RNAase free water

Lymphoprep

Method

Reconstitution of siRNA

Prepare 1X siRNA buffer (Add required RNAase-free water to 5X siRNA buffer to make it 1X)

Reconstitute siRNA with the required amount of 1X siRNA buffer to make a siRNA stock solution of 50 uM (Add 1X siRNA buffer to the vial containing lyophilized siRNA and resuspend siRNA with a pipette multiple times and shake the vial on an orbital shaker for 60 min, give a short spin, and then store it at -20°C until the usage).

siRNA treatment of T cells

Isolate pBMCs from a buffy coat by density gradient centrifugation (ideally get a fresh buffy coat and isolate T cells on the same day)

Isolate T cells from PBMCs by a method of choice (we used CD4-T cell isolation kit and QuadroMACS separator for manual isolation of CD4-T cells from PBMCs)

Count and seed CD4-T cells at 5M/0.5 ml in Accell siRNA delivery medium supplemented with penicillin and streptomycin in a 48-well plate and then add aCD3 antibody at 1ug/ml and rest the cells for 30 min in the CO2 incubator.

Add siRNA to the cells at a final concentration of 1uM (10 ul of 50 uM stock siRNA to 500 ul of cell suspension) and incubate the cells in the incubator (5% CO_2) for 48 h to 96 h depending on the downstream assays.

Notes

Do not add serum or BSA to the Accell siRNA delivery medium as they are known to inhibit uptake of Accell siRNAs by the cells.

Cell culture medium (Accell siRNA delivery medium) can be replaced with serum-supplemented medium after 48 h of siRNA treatment if needed.

siRNA can be quality controlled on nanodrop if needed to make sure that the vial contains an estimated concentration of siRNA.

Ideally, collect the cells at 48 h and 72 h time points for checking mRNA knockdown, and 72 h and 96 h time points for protein knockdown. However, we suggest that different time points need to be tested for different genes. In our hands, we observed efficient knockdown in transcript levels at both 48 h and 72 h time points.

We recommend using siRNA smart pools (4 siRNAs targeting transcripts of the same gene in mixed form) rather than individual siRNA.

Figure.1

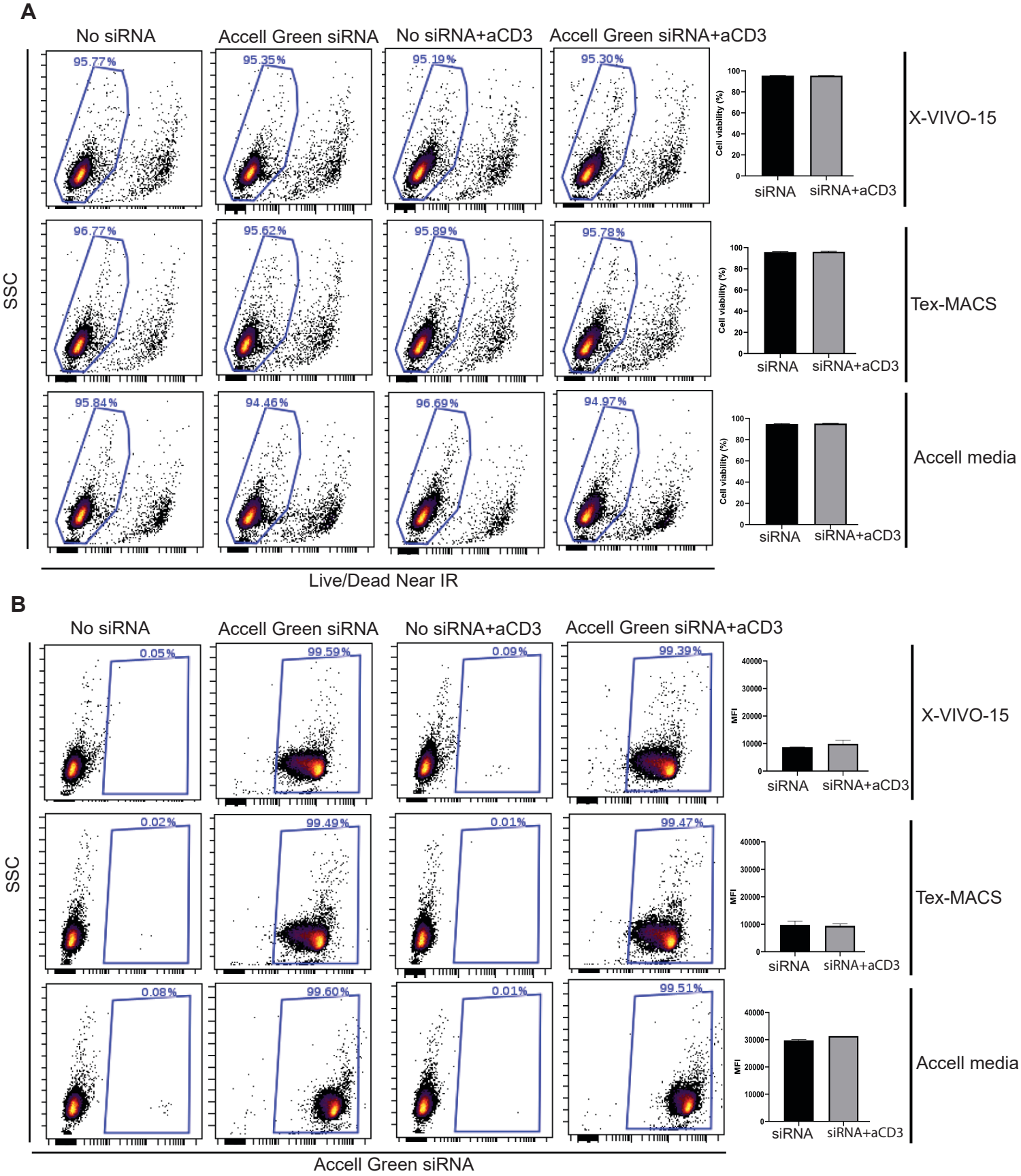


Figure 1: Flowcytometry analysis of purified human CD4-T cells after 24 h of treatment with Accell green-non-targeting siRNA. The conditions tested were three different serum-free cell culture media (X-VIVO 15, Tex MACS, Accell media) supplemented with or without aCD3 antibody. A) T cell viablity based on Live/dead Near IR staining. Cells shown in the gates are viable cells. The cells shown in the plots are gated on Total T cell gate. T cell viabilty is aslo presented in graphs on the right side. B) siRNA uptake in percentage measured based on the siRNA flourescent signal retained in T cells. Mean flourescent intensity (MFI) of the signal is shown in the graphs on the right side. Cells shown in the plots are gated on live T cell population shown in Fig 1a.

Figure.2

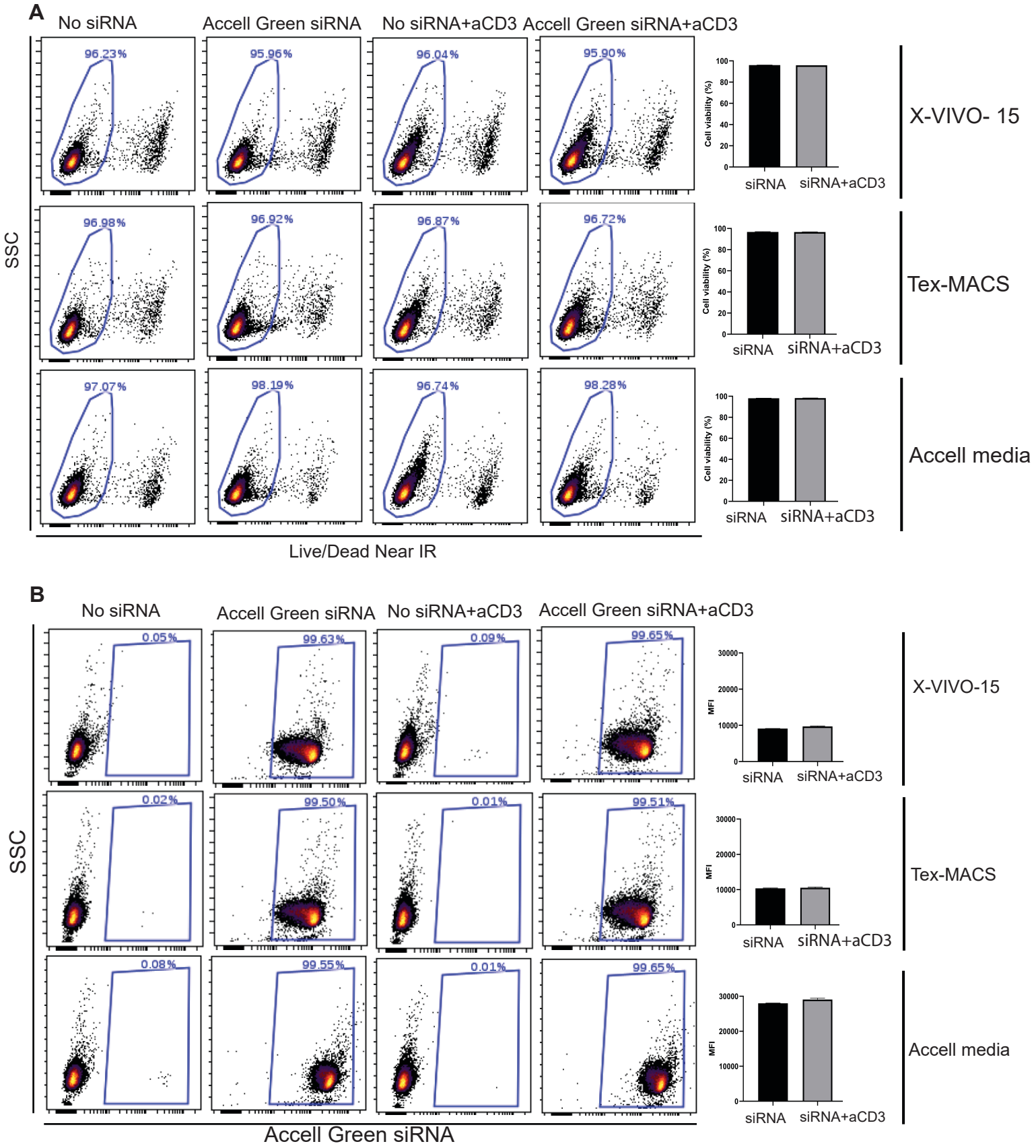
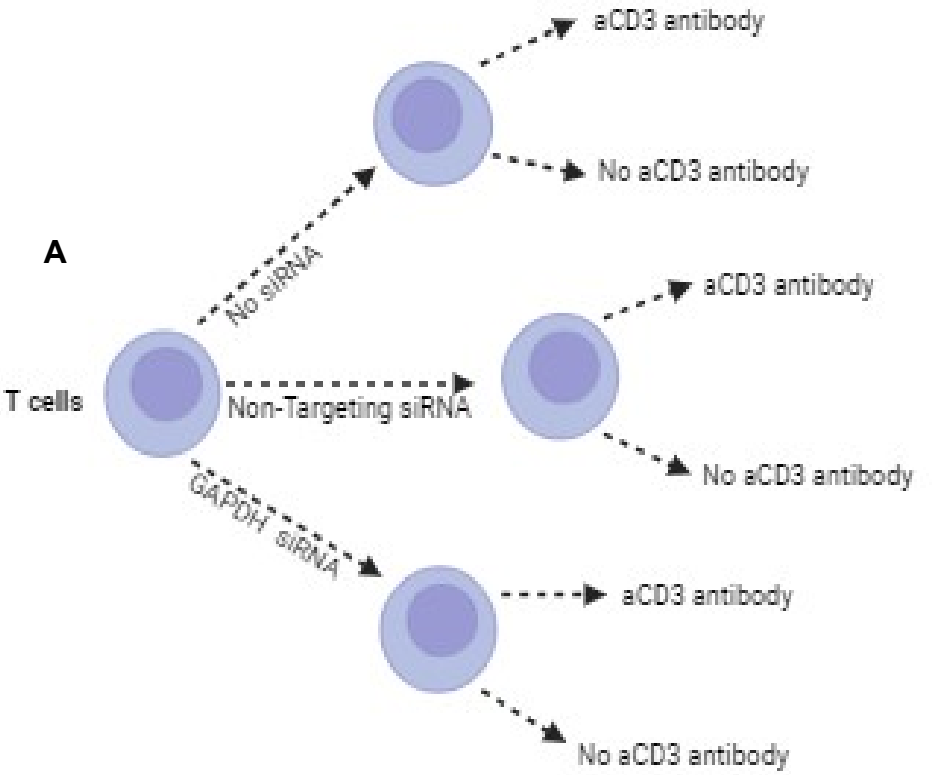


Figure 2: Flowcytometry analysis of purified human CD4-T cells after 48 h of treatment with Accell green-non-targeting siRNA. The conditions tested were three different serum-free cell culture media (X-VIVO 15, Tex MACS, Accell media) supplemented with or without aCD3 antibody. A) T cell viablity based on Live/dead Near IR staining. Cells shown in the gates are viable cells. The cells shown in the plots are gated on Total T cell gate. T cell viabilty is aslo presented in graphs on the right side. B) siRNA uptake in percentage measured based on the siRNA flourescent signal retained in T cells. Mean flourescent intensity (MFI) of the signal is shown in the graphs on the right side. Cells shown in the plots are gated on live T cell population shown in Fig 2a.

Figure.3



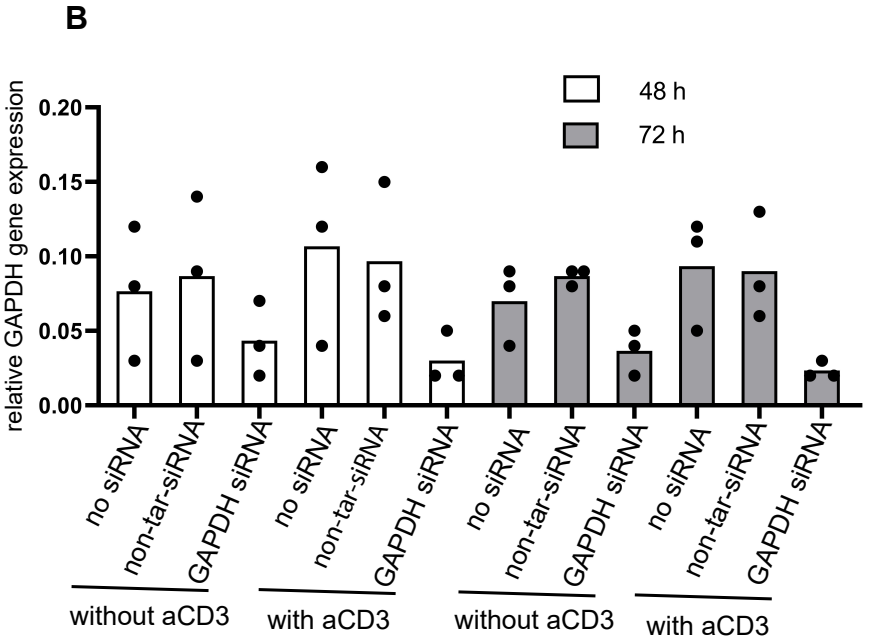


Figure 3. Knockdown of GAPDH in CD4-T cells using Accell siRNA. A) Experimental setup describing the conditions used in the experiment. B) qPCR measurement of GAPDH mRNA levels in purified CD4-T cells treated with Accell siRNA targeting GAPDH in the presence or absence of aCD3 antibody. T cells were cultured in Accell siRNA delivery media. As a control non-targeting siRNA was used. Each data point in the graph represents a donor, n=3 donors. T cells were purfied from three blood donors and then treated with the siRNA followed by qPCR. Relative expression of GAPDH is shown and actin was used a reference gene.

Figure. 4

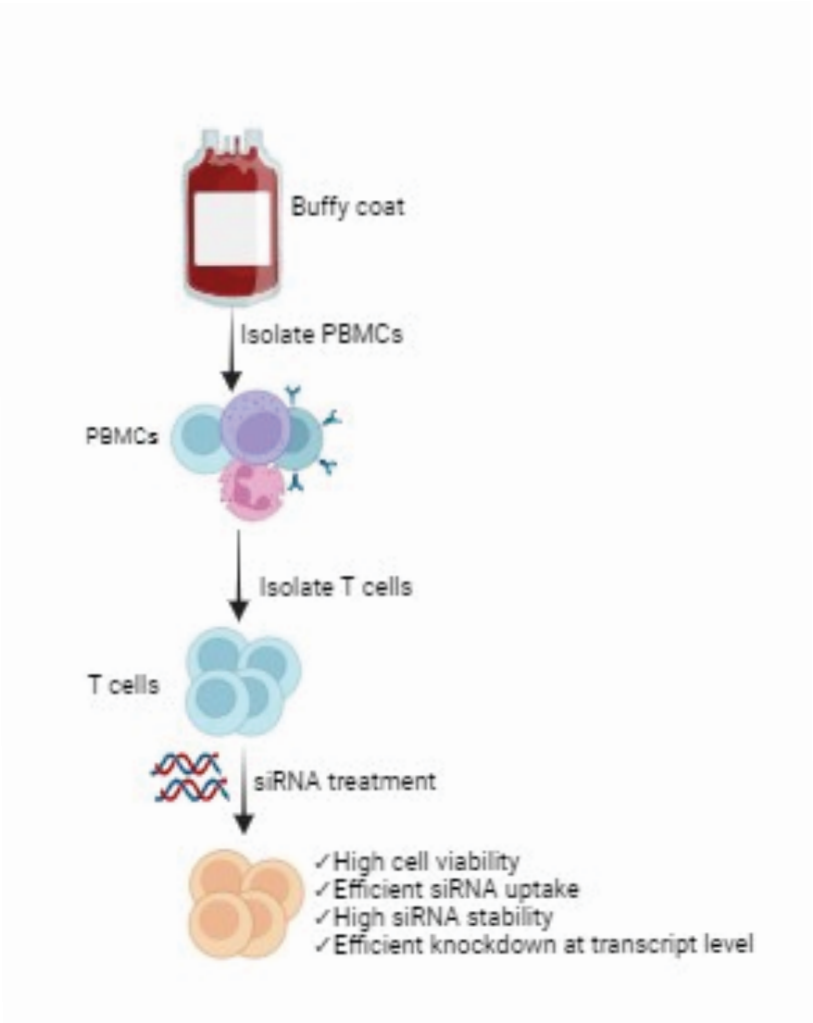


Figure. 4 Graphical abstract for the optimized method for Accell siRNA delivery and gene knockdown in primary human CD4-T cells. The graphical abstract was generated using Biorender.