CHAPTER 3

RESULTS

3.1 Production of CD4-specific DARPin 23.2

CD4-specific DARPin 23.2 is a bimodular ankyrin with the molecular weight of 14 kDa. The production of CD4-specific DARPin 23.2 was performed in bacteria, *E. coli* strain BL-21 (DE3) carrying pTriEx-CD4-specific DARPin 23.2 under IPTG induction. CD4-specific DARPin 23.2 contained histidine (6xHis) tag at N-terminus. To obtain the purified protein, the purification steps were done using His Trap column. The purified protein was collected as fractions. The purity of the purified protein was determined by SDS-PAGE under reducing condition. The protein was stained with Coomassie Brilliant Blue as shown in Figure 3.1. Purified CD4-specific DARPin 23.2 was found in Fraction 7 to 13. Purified protein in Fraction 9 to 11 showed the highest purity among these fractions, consequently they were pooled together. The Elution buffer was exchanged into PBS through dialysis. Protein concentration of the dialyzed purified DARPin was performed by BCA protein assay kit.

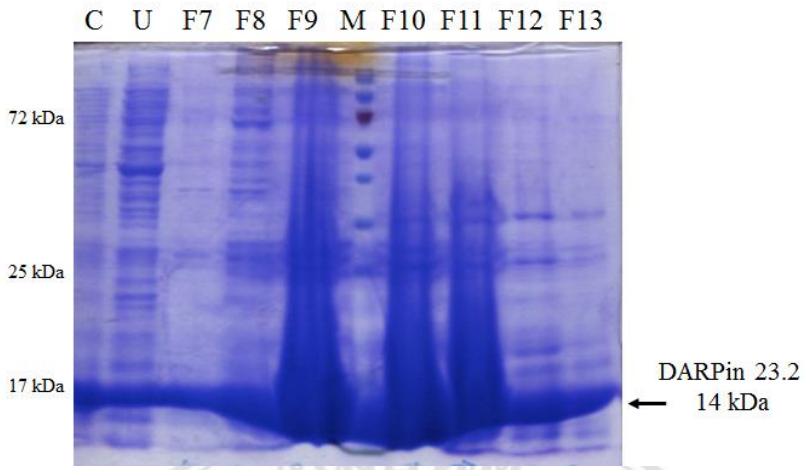

Figure 3.1 Purity of purified CD4-specific DARPin 23.2 considered by SDS-PAGE. (C: bacterial crude extract; U: unbound protein; F: purified protein fraction; M: protein marker)

CD4-specific DARPin 23.2 was further analyzed by Western immunoblotting. The result from chemiluminescent reaction showed the expression of CD4-specific DARPin 23.2 at 14 kDa, whereas lysate from *E. coli* BL-21 (DE3) exhibit no protein when detected with anti-His tag as seen in Figure 3.2.

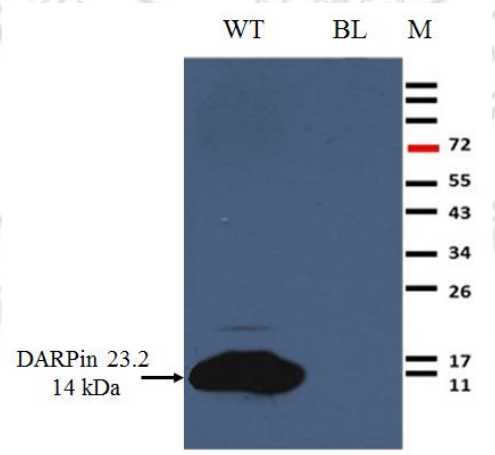

Figure 3.2 Expression of CD4-specific DARPin in purified form detected by Western immunoblotting. (WT: Purified CD4-specific DARPin 23.2 (wild type); BL: crude lysate of BL21 (DE3); M: protein marker)

3.2 CD4-specific DARPin 23.2 Titration in SupT1 cell line

SupT1 is a human T-cell lymphoblastic lymphoma cell line which expresses CD4 molecules on cell surface. The binding of purified CD4-specific DARPin 23.2 to CD4 molecules on SupT1 cell line was determined by flow cytometry in various concentrations. The bound DARPin to CD4 molecule on SupT1 cell surface was detected *via* 6xHis tag at N-terminus. As shown in Figure 3.3A, DARPin bound to CD4 molecules on SupT1 ranging from 10 µg/ml to 10 ng/ml with over 95% positive. The mean fluorescence intensity (MFI) of each concentration was illustrated in Figure 3.3B. The MFI acquired from the binding of 100 ng/ml showed the highest intensity. DARPin at 100 ng/ml was chosen for further experiment.

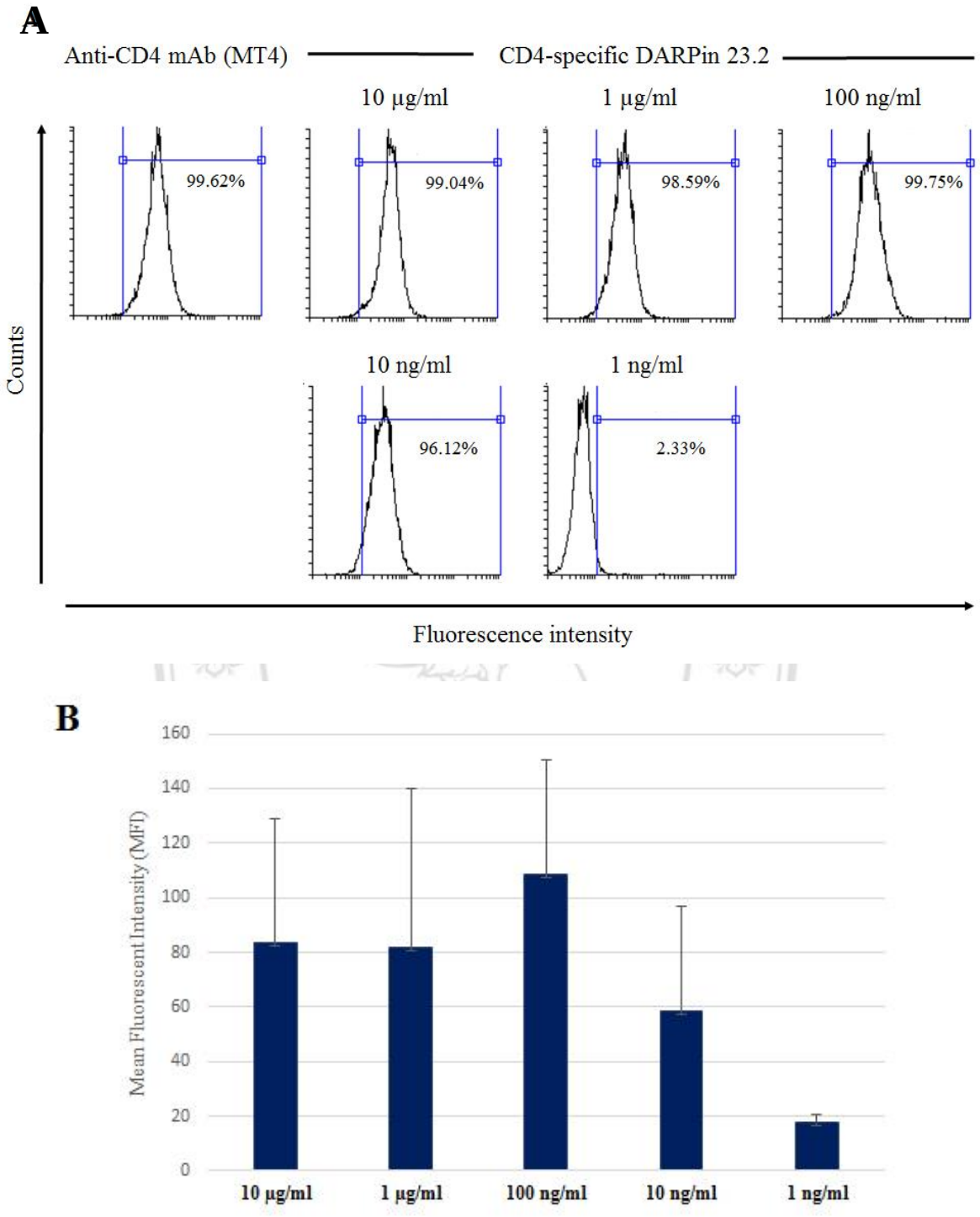

Figure 3.3 Binding of CD4-specific DARPin 23.2 in various concentrations to CD4 molecules expressed on SupT1 cell surface shown in histogram (A) and mean fluorescent intensity (MFI) (B).

3.3 CD4-specific DARPin 23.2 binding on PBMC

To determine the specificity of CD4-specific DARPin 23.2 on primary cells, the binding was investigated using PBMC. The PBMC was isolated from a donor using ficoll-hypaque centrifugation. Purified CD4-specific DARPin 23.2 at 100 ng/ml was used to determine the binding to CD4 molecules on PBMC. DARPin was traced by mosuse anti-His and RPE conjugated rabbit anti-mouse immnunoglobulins and CD3-FITC. The reactions were analyzed by flow cytometry.

The lymphocytes were gated according to FSC and SSC dot plot profile. T-lymphocytes were characterized by expression of CD3 and CD4. There was 21.32% of T-lymphocytes among the gated lymphocytes, detected by monoclonal antibody against CD3 and CD4. The purified CD4-specific DARPin 23.2 bound specifically to CD4 molecules on primary cells since it exhibited comparable proportion of 18.63% comparing to anti-CD4 mAb, as shown in Figure 3.4.

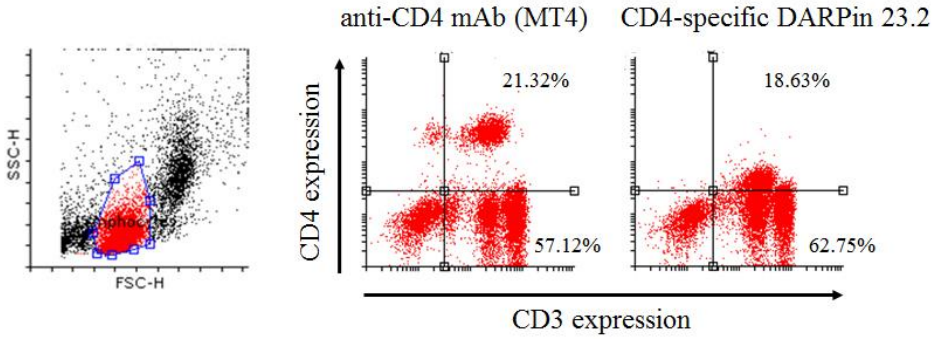

Figure 3.4 CD4-specific DARPin 23.2 binding on PBMC

3.4 Generation of mutated CD4-specific DARPin 23.2

According to Nimmanpipug *et al.* [33], phenylalanine at position 100 (F100) displayed the highest binding free energy (GBTOT) among the members of region 3. To improve the binding affinity of CD4-specific DARPin 23.2, F100 was a candidate for mutational analysis. Further computational mutation into other amino acids was performed. The mutated DARPin, along with CD4 molecule, were minimized and calculated the interaction energy. The result suggested the mutation of F100 to arginine (F100R) would slightly improve the binding affinity between CD4-specific DARPin and CD4 molecule. Nonetheless, mutation of F100 into glycine (F100G) would abolish the binding interaction.

3.4.1 Generation of CD4-specific DARPin (F100R) and CD4-specific DARPin (F100G)

The original vector, pTriEx-CD4-specific DARPin 23.2 went through site-directed mutagenesis process to generate CD4-specific DARPin (F100R) and CD4-specific DARPin (F100G). Specific primers designed to induce the mutation were used for each mutation, as indicated in Table 2.1. *Dpn*I treatment eliminated the original vector and left only the mutated one. The mutated vectors were transformed into *E. coli* strain XL1-Blue and plated on LB agar containing ampicillin and tetracyclin. The isolate colonies were randomly picked. The vector pTriEx-CD4-specific DARPin (F100R) or pTriEx-CD4-specific DARPin (F100G) were prepared by plasmid minipreparation. The vector was analyzed by restriction enzyme, *Xba*I and *Hind*III. As shown in Figure 3.5, all vectors exhibited the right fragment at 4,707 and 701 bp. The clones were confirmed by sequencing, as seen in Figure 3.6. F100R clone 3 and 8 and F100G clone 2 showed correct mutated sequence.

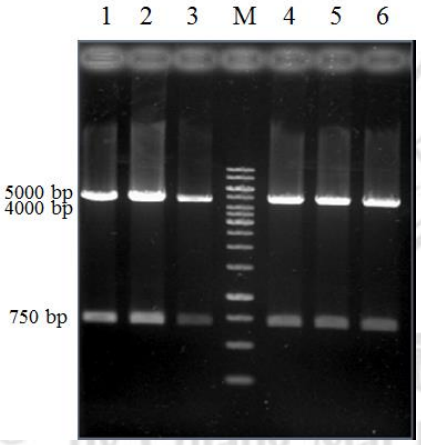

Figure 3.5 Restriction enzyme analysis of pTriEx-CD4-specific DARPin mutants, F100R and F100G with *Xba*I and *Hin*dIII. Lane 1 illustrated DNA fragments from pTriEx-CD4-specific DARPin 23.2 (WT). Lane 2 and 3 represented pTriEx-CD4-specific DARPin 23.2 (F100G) clone 2 and 8 respectively. Lane 4 – 6 represented pTriEx-CD4-specific DARPin 23.2 (F100R) clone 3, 5 and 8 respectively. M was 1 kb DNA marker.

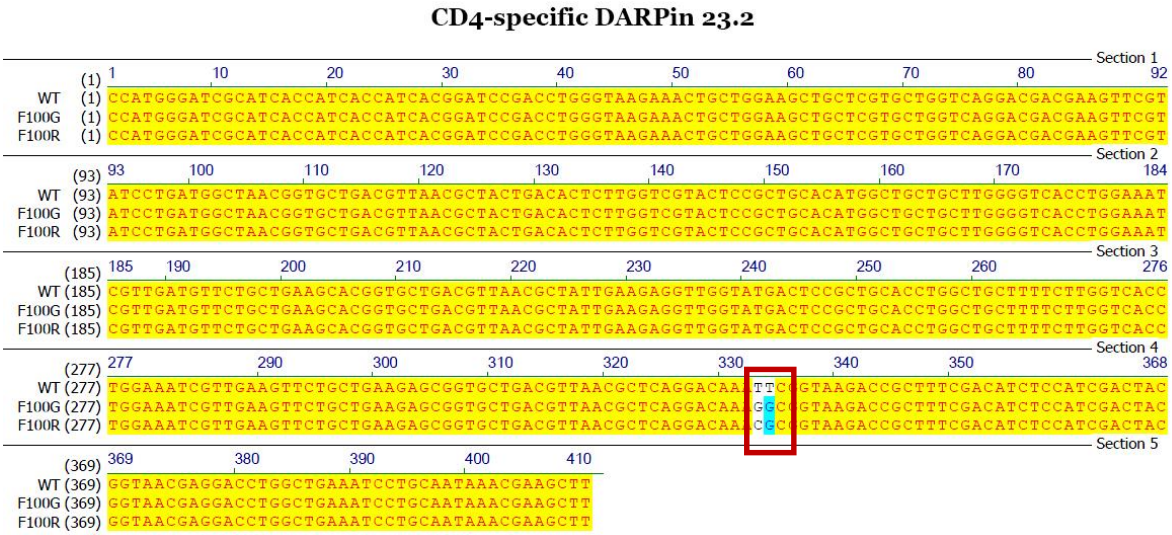

Figure 3.6 Aligned sequencing results of CD4-specific DARPins, at position 100 (red box), phenylalanine of WT displayed TTC, whereas F100R displayed CGC and F100G showed GGC.

3.4.2 Production and purification of mutated CD4-specific DARPin

Large scale production of mutated CD4 specific DARPin was conducted in bacterial system. Vector carrying CD-specific DARPin mutants, F100R clone 3 and F100G clone 2, were transformed into expression bacteria, BL21 (DE3). Both DARPin mutants were expressed under IPTG induction. Purification was performed using HiTrap HP affinity column. The purity of the purified protein was determined by SDS-PAGE under reducing condition.

As seen in Figure 3.7, CD4-specific DARPin (F100R) was found at 14 kDa from fraction 15 – 26. Fraction 19 to 21 produced the highest purity and eventually pooled together. Elution buffer was exchanged into PBS by dialysis. Protein concentration was verified by BCA protein assay kit. To determine the expression of purified DARPin (F100R), 100 µg/ml of purified protein was electrophoresed and blotted on PVDF membrane. DARPin (F100R) had 6×His tag at N-terminus likewise wild type. When probed with anti-His tag mAb, purified CD4-specific DARPin (F100R) was found at correct molecular weight, as seen in Figure 3.8.

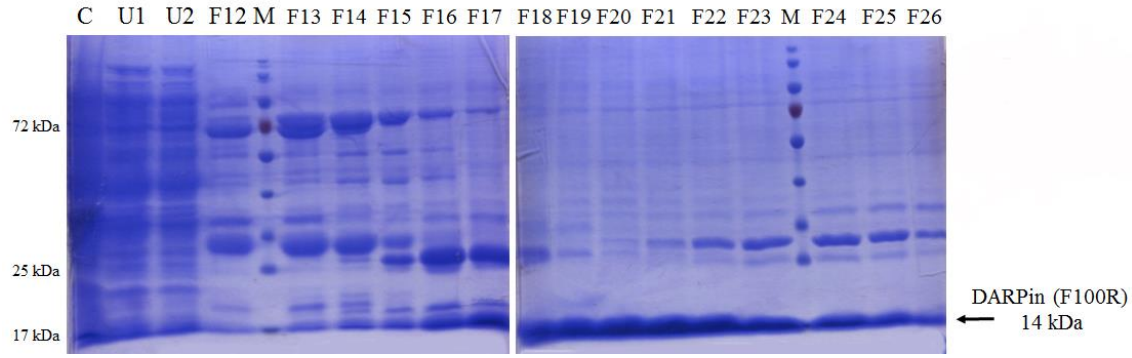

Figure 3.7 Purity of CD4-specific DARPin (F100R) eluted fractions determined by SDS-PAGE. DARPin was found at 14 kDa. (C: bacterial crude extract; U: unbound protein; F: purified protein fraction; M: protein marker)

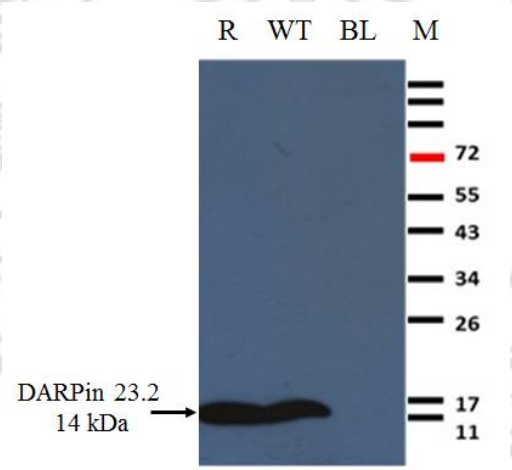

Figure 3.8 Expression of purified CD4-specific DARPin 23.2 (F100R) detected by Western immunoblotting. (R: CD4-specific DARPin 23.2 (F100R); WT: CD4-specific DARPin 23.2 (wild type); BL: crude lysate of BL21 (DE3); M: protein marker)

The same process of determining eluted protein purity was performed for CD4-specific DARPin (F100G). Stained protein fractions from SDS-PAGE presented DARPin as a major band from Fraction 14 to 26, with the highest purity at Fraction 19 – 24. The pooled protein fraction was electrophoresed and blotted onto PVDF membrane. After probing with anti-His tag following with HRP-conjugated anti-mouse immunoglobulins, the chemiluminescent reaction was visualized by X-ray film. In Figure 3.9, CD4-specific DARPin (F100G) was found at correct size of 14 kDa.

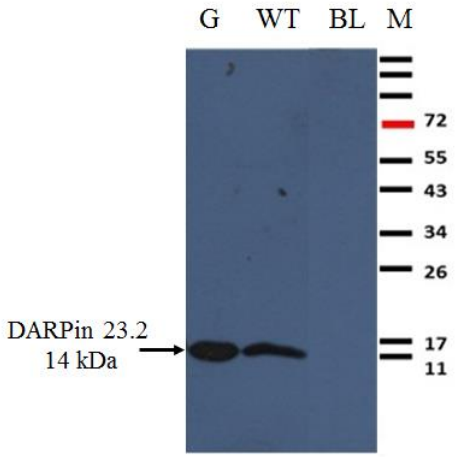

Figure 3.9 Expression of purified CD4-specific DARPin (F100G) detected by Western immunoblotting. (G: CD4-specific DARPin 23.2 (F100G), WT: CD4-specific DARPin 23.2 (wild type); BL: crude lysate of BL21 (DE3); M: protein marker)

3.5 Binding of mutated CD4-specific DARPin to CD4 molecules

To investigate the binding affinity to bind to CD4 molecules of CD4-specific DARPin mutants, F100R and F100G comparing to wild type, the binding was discovered by flow cytometry. As SupT1 expressed CD4 molecule **o**n cell surface, it was included in this experiment. The earlier binding titration of CD4-specific DARPin (WT) had verified the final concentration to be further used at 100 ng/ml. Both DARPin mutants along with wild type at final concentration of 100 ng/ml, were incubated with SupT1 on ice. The unbound DARPin was washed out. DARPins were tracked with anti-His tag following by RPE conjugated rabbit anti-mouse immunoglobulins. The data revealed no binding of both DARPin F100R and F100G, as illustrated in Figure 3.10. The experiment was repeated with additional concentration of DARPin mutants at 1 µg/ml and 10 ng/ml. Both DARPin mutants certified negative binding activity to CD4 molecules. This suggested that mutation to either arginine or glycine abolished the binding affinity of CD4-specific DARPin 23.2.

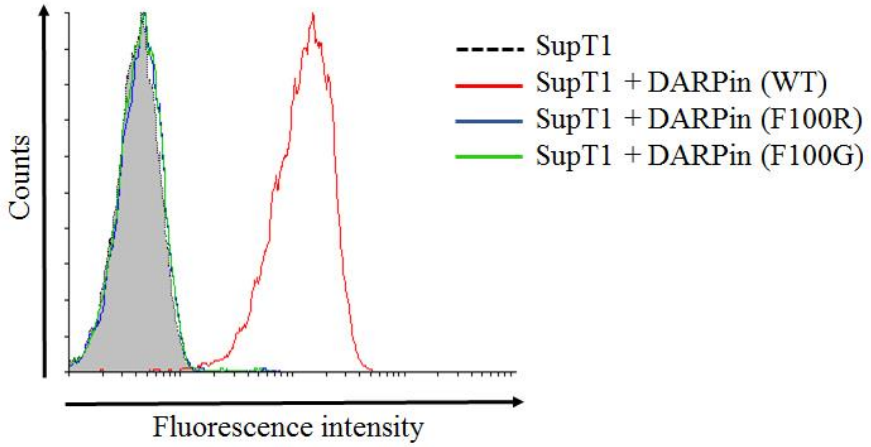

Figure 3.10 Overlay flow cytometry data on the binding of CD4-specific DARPin mutants, F100R and F100G to CD4 molecules on SupT1 cell line. Comparing to wild type DARPin, both DARPin mutants demonstrated negative binding ability to CD4.

3.6 Adenovirus receptors and CD4 expression profile of HeLa cell line

Adenovirus serotype 5 (Ad5) utilizes CAR and αv-integrins as receptors to enter the target cells. In this experiment, adenovirus was used as a vector to deliver CD4 gene and resulted in CD4 expression on cell surface. To evaluate the efficacy of adenovirus transduction, the expression of CAR and αv-integrins were detected by flow cytometry. Histogram displayed the positive expression for both CAR (44.61%) and αv-integrins (98.24%) as seen in Figure 3.11. This results ascertained the transduction efficiency of Ad5 in HeLa cell line.

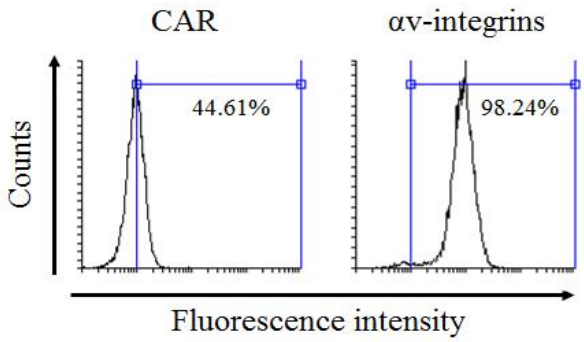

Figure 3.11 Adenovirus receptors for Ad5, CAR and αv-integrins profile on HeLa cell surface

3.7 CD4 expression profile on HeLa cell line

To appraise the expression of CD4 molecule on HeLa cell surface, the study was done by flow cytometry. HeLa cells was stained for CD4 using anti-CD4 mAb (MT4) and CD4-specific DARPin 23.2 while using isotype control and irrelevant DARPin as negative control. The result certified that HeLa cells didn't express CD4 on cell surface, as demonstrated in Figure 3.12.

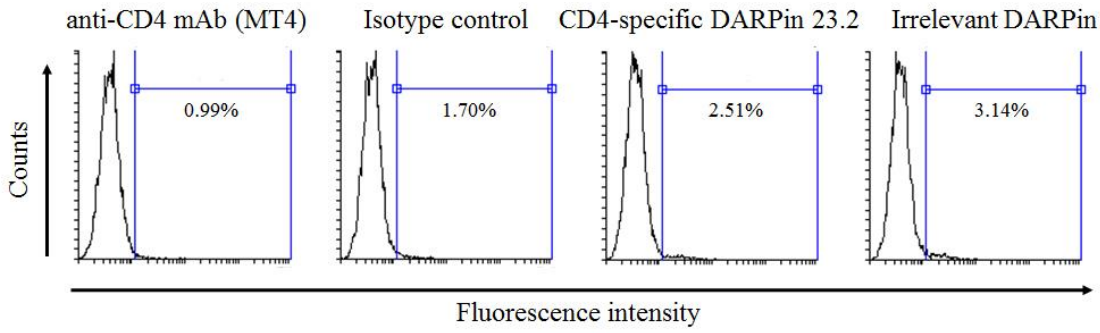

Figure 3.12 Negative CD4 expression profile on HeLa cell surface

3.8 Production of Ad5-CD4 (K35A) and Ad5-CD4 (K35AQ40A)

In the study of Wisitponchai *et al*. [34], the computational interaction between CD4 and CD4-specific DARPin 23.2 revealed the key amino acids in this binding on CD4 molecule, K35, Q40 and Q33. To evaluate these hotspots, CD4 gene was mutated at position 35 into alanine (K35A). The double mutation version of CD4 had an additional mutation at position 40 (K35AQ40A).

3.8.1 Generation of CD4 (K35A) and CD4 (K35AQ40A)

To generate CD4 (K35A) and CD4 (K35AQ40A) gene from the original vector, pCDM8-CD4 (WT), site-directed mutagenesis was conducted by using specific primers as listed in Table 2.3. From the process, the wild type vector was depleted by *Dpn*I treatment, leaving only mutated vectors. The mutated pCDM8-CD4 (K35A) was transformed into E. coli MC1061/P3. The transformed clone were selected by the resistance to ampicillin, kanamycin and tetracycline. Isolated colonies were randomly picked and screened for CD4 gene in transformed mutated vector by PCR. As presented in Figure 3.13, the positive amplified CD4 gene at 1,320 bp was seen from clone1, 2, 4 and 5.

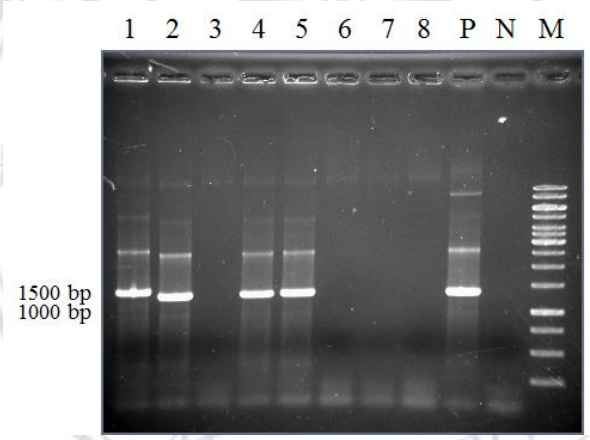

Figure 3.13 Screening of CD4 (K35A) gene by PCR in MC1061/P3 carrying pCDM8-CD4 (K35A) where lane 1 – 8 represented clone 1 to 8 respectively. (P: positive control; N: negative control; M: 1 kb DNA marker)

Positive clones from PCR were further analyzed by restriction enzyme analysis. In order to do this, pCDM8-CD4 (K35A) was extracted from the bacteria by plasmid minipreparation. Digesting with *Nhe*I for overnight, every selected clones resulted in the presence of 8,000 and 2,000 bp fragments, as seen in Figure 3.14. Sequencing verified the mutation sequence. Among the three selected clones, only clone 5 was successfully mutated from lysine to alanine at position 35, as seen in aligned sequences in Figure 3.15.

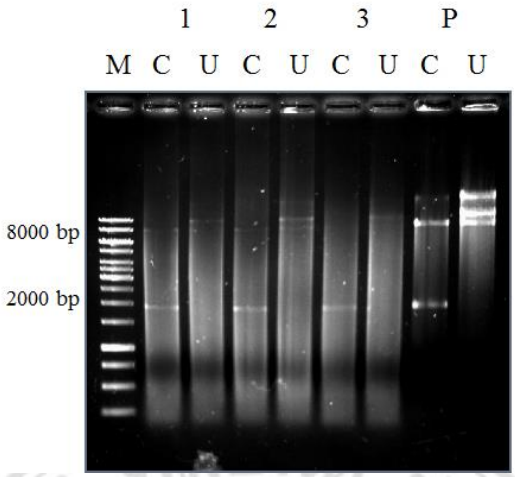

Figure 3.14 Clone screening of pCDM8-CD4 (K35A) using *Nhe*I digestion. M represented 1 kb DNA marker. Lane 1 -3 represented pCDM8-CD4 (K35A) clone 2, 4, and 5 respectively. Wild type CD4 in pCDM8 plasmid was used as positive control (P). All three selected clones generated complete digestion and resulted in 8,000 and 2,000 bp fragments in *Nhe*I cut condition (C) when comparing to uncut condition (U).

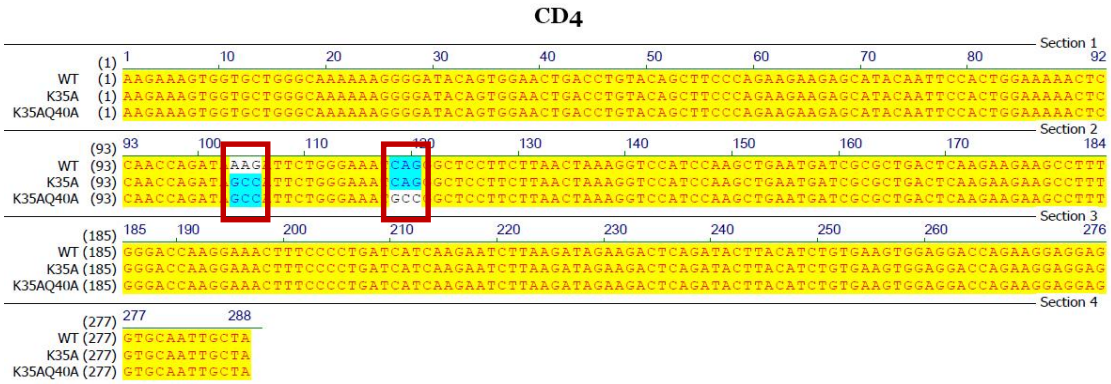

Figure 3.15 Aligned sequencing results for D1 of human CD4. For CD4 (K35A), lysine at position 35 was mutated into alanine. Additional mutation of glutamine at position 40 to alanine was performed for CD4 (K35AQ40A).

For double mutated version of CD4 (K35AQ40A), sequencing-proved pCDM8-CD4 (K35A) went through another site-directed mutagenesis using a pair of specific primers for CD4 (K35AQ40A) listed in Table 2.3. The same procedures of screening were applied for selection of MC1061/P3 carrying pCM8-CD4 (K35AQ40A). Ten clones were randomly picked and screened for CD4 (K35AQ40A) by colony PCR. In Figure 3.16, all selected clones illustrated the mutated CD4 gene at 1,320 bp. Only clone 1, 2, 4, 5, 6 and 7 were further cultured to extract plasmid using plasmid minipreparation kit. Digestion with *Nhe*I for overnight resulted in two DNA fragments at 8,000 and 2,000 bp. All six clones were seen with the correct DNA fragment size in Figure 3.16. Sequencing confirmed the alanine sequence for both position 35 and 40 as aligned in Figure 3.15 for clone 6.

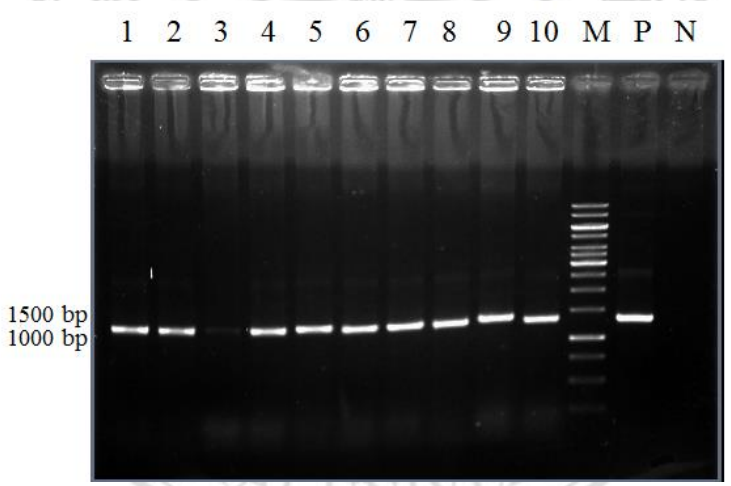

Figure 3.16 Screening of CD4 (K35AQ40A) by PCR in MC1061/P3 carrying pCDM8-CD4 (K35AQ40A) clone 1 to 10 loaded in lane 1 through 10 respectively. (P: positive control; N: negative control; M: 1 kb DNA marker)

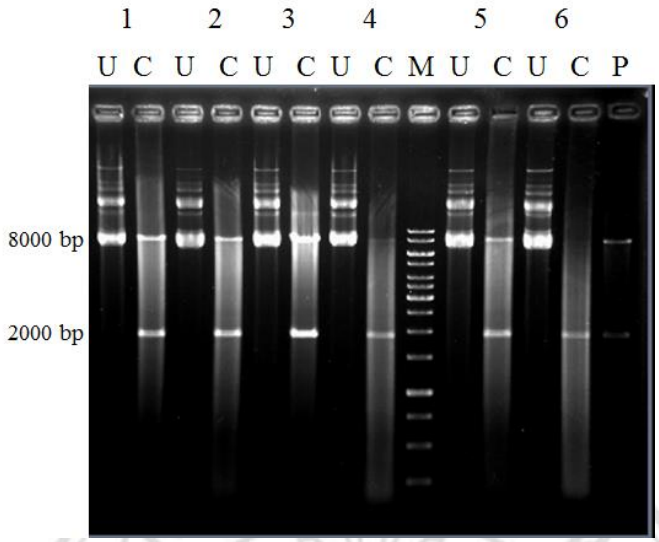

Figure 3.17 Clone screening of pCDM8-CD4 (K35AQ40A) using *Nhe*I digestion. All six selected clones generated complete digestion and resulted in 8,000 and 2,000 bp fragments *Nhe*I cut condition (C) when comparing to uncut condition (U). Wild type CD4 in pCDM8 plasmid was used as positive control (P).

3.8.2 Construction of pAdTrack-CD4 (K35A) and pAdTrack-CD4 (K35AQ40A)

To construct transfer vector carrying transgene in adenovirus system, both CD4 mutant genes were cloned and ligated into modified pAdTrack-CMVGFP. CD4 (K35A) was amplified from pCDM8-CD4 (K35A) clone 5 using Phusion® High-Fidelity DNA Polymerase. The PCR product and backbone pAdTrack-CMVGFP were purified and treated with *Sfi*I for overnight at 50°C. Both *Sfi*I-treated CD4 (K35A) and pAdTrack-CMVGFP were purified again. The gel electrophoresis shown in Figure 3.18, illustrated purified *Sfi*I-treated CD4 (K35A) at 1,320 bp and *Sfi*I-treated pAdTrack-CMVGFP at 10,000 bp. The same procedure was performed to obtain CD4 (K35AQ40A) insert.

The purified *Sfi*I-treated CD4 (K35A) or CD4 (K35AQ40A) insert and modified pAdTrack-CMVGFP backbone were ligated using T4 DNA ligase at 4°C for overnight. The ligated products were transformed into *E. coli* XL1-Blue and plated on LB agar containing kanamycin and tetracycline. The selected clones were initially screened by PCR then the PCR-positive clones were tested again with restriction enzyme analysis. Figure 3.19 exhibited the correct DNA bands of 10,000 and 1,320 bp when treated with

*Sfi*I in both clones. Both clones contained the right sequence for CD4 (K35A) confirmed by sequencing. The same results were obtained from the construction of pAdTrack-CMVGFP-CD4 (K35AQ40A).

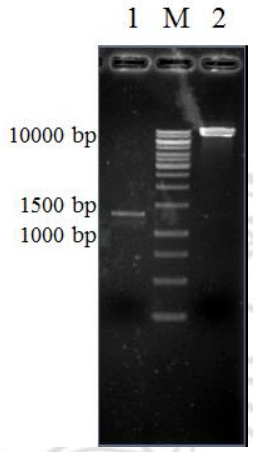

Figure 3.18 Purified *Sfi*I-treated CD4 (K35A) and *Sfi*I-treated pAdTrack-CMVGFP. Lane 1 represented purified SfiI-treated CD4 (K35A). Lane 2 represented purified SfiI-treated pAdTrack-CMVGFP backbone. (M: 1 kb DNA marker)

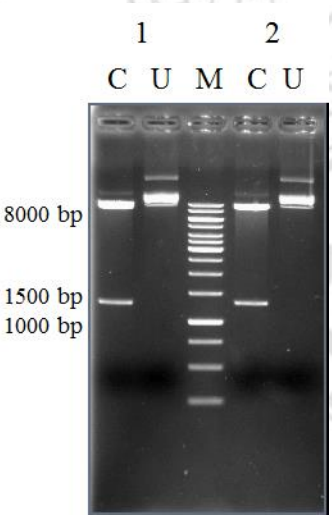

Figure 3.19 Screening of pAdTrack-CMVGFP-CD4 (K35A) using restriction enzyme analysis of *Sfi*I. Correct digestion products of 10,000 and 1,320 bp were detected in both clones. (C: *Sfi*I cut condition; U: uncut condition; M: 1 kb DNA marker)

3.8.3 Recombined adenovirus vectors

The pAdEasy system requires the homologous recombination of transfer vector and pAdEasy to complete the recombinant adenoviral genome. Recombined plasmids were extract and screened with *Pac*I and *Sfi*I. As illustrated in Figure 3.20, digestion with *Pac*I resulted in DNA fragment of 30,000 and 3,000 bp whereas treatment with *Sfi*I gave fragments of 23000, 7000 and 1,320 bp.

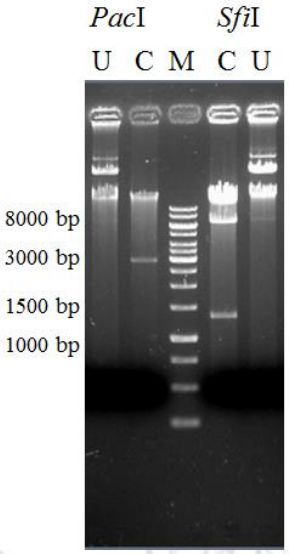

Figure 3.20 Screening of pAdET-CD4 (K35A) using restriction enzyme analysis. (C: cut; U: uncut; M: 1 kb DNA marker)

The recombined vector was transformed into XL1-Blue. Large scale extraction of pAdET-CD4 (K35A) and pAdET-CD4 (K35AQ40A) was done using HiSpeed® Plasmid Midi Kit. The vectors were tested with *Pac*I and *Sfi*I digestion as described above.

3.8.4 Generation and amplification of Ad5-CD4 (K35A) and Ad5-CD4 (K35AQ40A)

In transfected 293A cells, GFP expression could be observed under inverted fluorescent microscope 24 h post transfection. The comet formation gradually formed. Culture media was added to the cells in day 3 post transfection. At day 7 post transfection, 293A cells were harvested. To release adenovirus, the transfected cells went through 4 times of freezing/thawing cycle. Cell lysate was stored at -70°C as the first adenoviral

stock and further amplification. The adenoviral production was performed as described in method section until it reached three 15-cm tissue culture dishes. The transduced cells were harvested and collected cell pellet in total medium volume of 1 ml. After four cycle of freezing and thawing, only clarified cell lysate containing Ads was kept at -70°C for further experiments.

3.8.5 Adenovirus titration in HeLa

To test the transductivity of concentrated Ads, the investigation was done by flow cytometry. Twenty four hours prior to transduction, HeLa cells were plated in 24-well plate at 1×10^5 cells/well. The cells reached 70 – 80% confluence at the time of transduction. Three versions of Ad5-CD4 were transduced into the cells in the final volume of 100 µl. After 36 hours of incubation, the transduced cells were harvest and tested the viability using supravital dye, 0.2% trypan blue. Transductivity of adenovirus were observed via GFP expression by flow cytometry.

HeLa cells transduced with Ad5-CD4 (WT) of 2.5, 5, 10, 15, 20 µl showed viability of 93.65%, 96.48%, 94.74%, 92.38%, and 77.25% respectively. As illustrated in Figure 3.21, GFP expression was seen at 31.78%, 66.15%, 85.20%, 72.28% and 64.02% respectively. In conclusion, transduction of Ad5-CD4 (WT) of 10 µl was chosen for further experiment as it retained the highest cell viability and GFP expression.

Ad5-CD (K35A) was transduced into HeLa cells with the same set of volume as performed with Ad5-CD4 (WT). Cell viability as determined by 0.2% trypan blue were 9335%, 90.22%, 74.87%, 58.79% and 64.00% respectively. Analyzed by flow cytometry, GFP expression was seen at 50.86%, 91.46%, 85.97%, 76.4% and 67.16% respectively. For further experiment, 5 µl-reaction was chosen since it illustrated the highest viability and GFP expression comparable to the Ad5-CD4 (WT) transduction condition.

HeLa cells transduced with Ad5-CD4 (K35AQ40A) using the same set of volume as performed with Ad5-CD4 (WT). Their cell viability are 94.14%, 95.00%, 85.71%, 72.10% and 45%. Transductivity of this virus as demonstrated in GFP expression were in order of 64.14%, 88.99%, 73.71%, 63.75% and 60.39%. Since the 5 µl-reaction

exhibited the highest viability and GFP expression, this volume was chosen for further experiment.

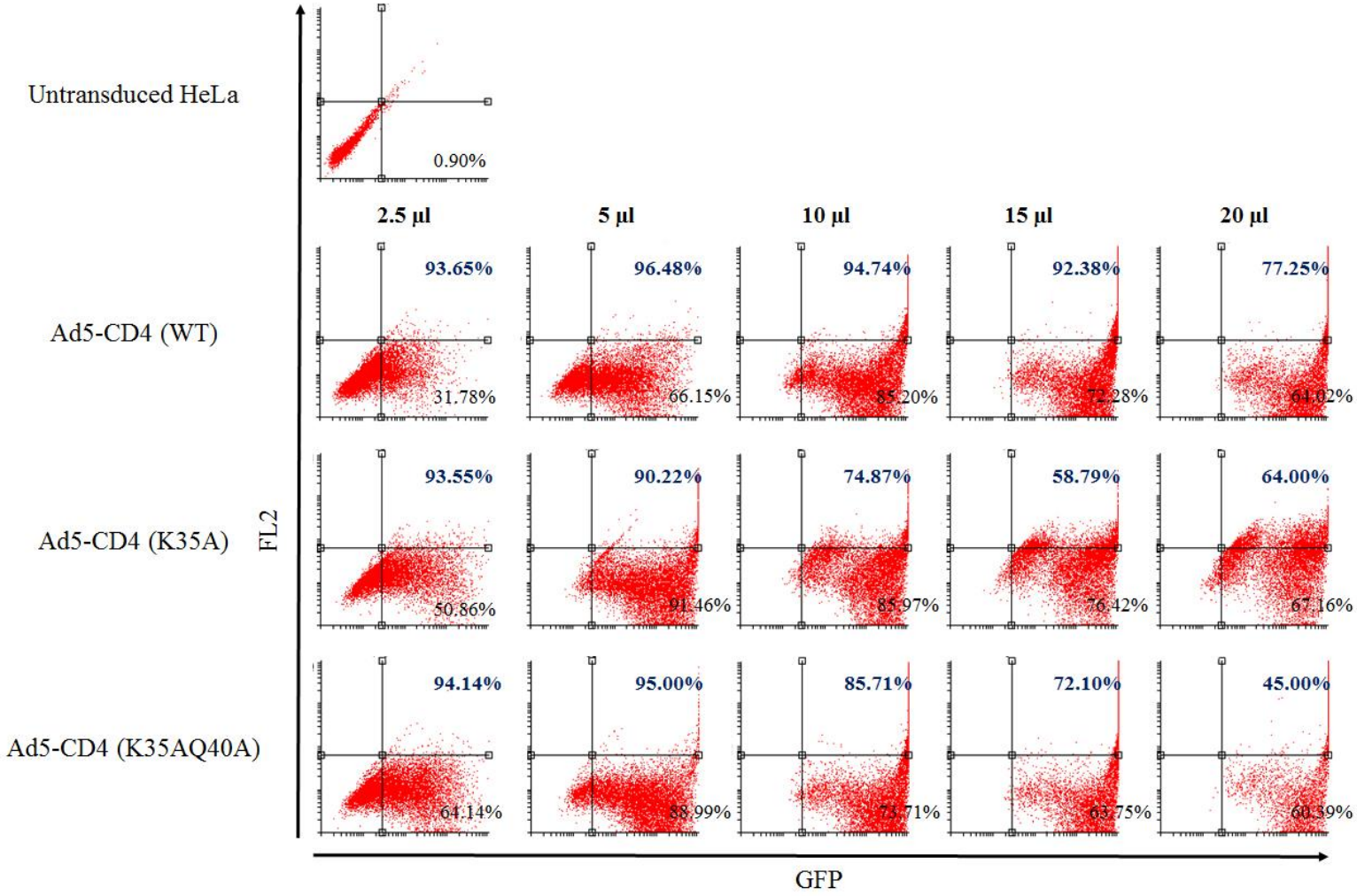

Figure 3.21 Transduction efficiency of adenovirus carrying CD4 genes (WT, K35A, K35AQ40A) in HeLa cells. Cell viability determined by 0.2% trypan blue were shown in blue letters.

3.8.6 CD4 expression in HeLa cells transduced with Ad5-CD4

To determine the ability to express CD4 molecules on cell surface when transduced by Ad, the transduced cells were stained by mAb against CD4 and analyzed by flow cytometry. HeLa cells were transduced with all three versions of Ad5-CD4 according to the volume concluded in the previous experiment. After 36 hours of transduction, the cells were harvested and stained with anti-CD4 mAb (MT4) following by RPE conjugated rabbit anti-mouse immunoglobulins.

From the data shown in Figure 3.22A, anti-CD4 mAb (MT4) recognized CD4 in HeLa cells transduced with Ad5-CD (WT) (65.1%) as well as both CD4 mutants, 60.14% positive for CD4 (K35A) and 69.50% positive for CD4 (K35AQ40A). The comparable positive percentage were observed among the three versions of CD4. The experiments were performed in triplicate. The summarized expression of CD4 by transduction of Ad was illustrated in Figure 3.22B in bar graph of mean ± SEM. Isotype control mAb was included in this experiment. It showed no binding to untransduced HeLa cells.

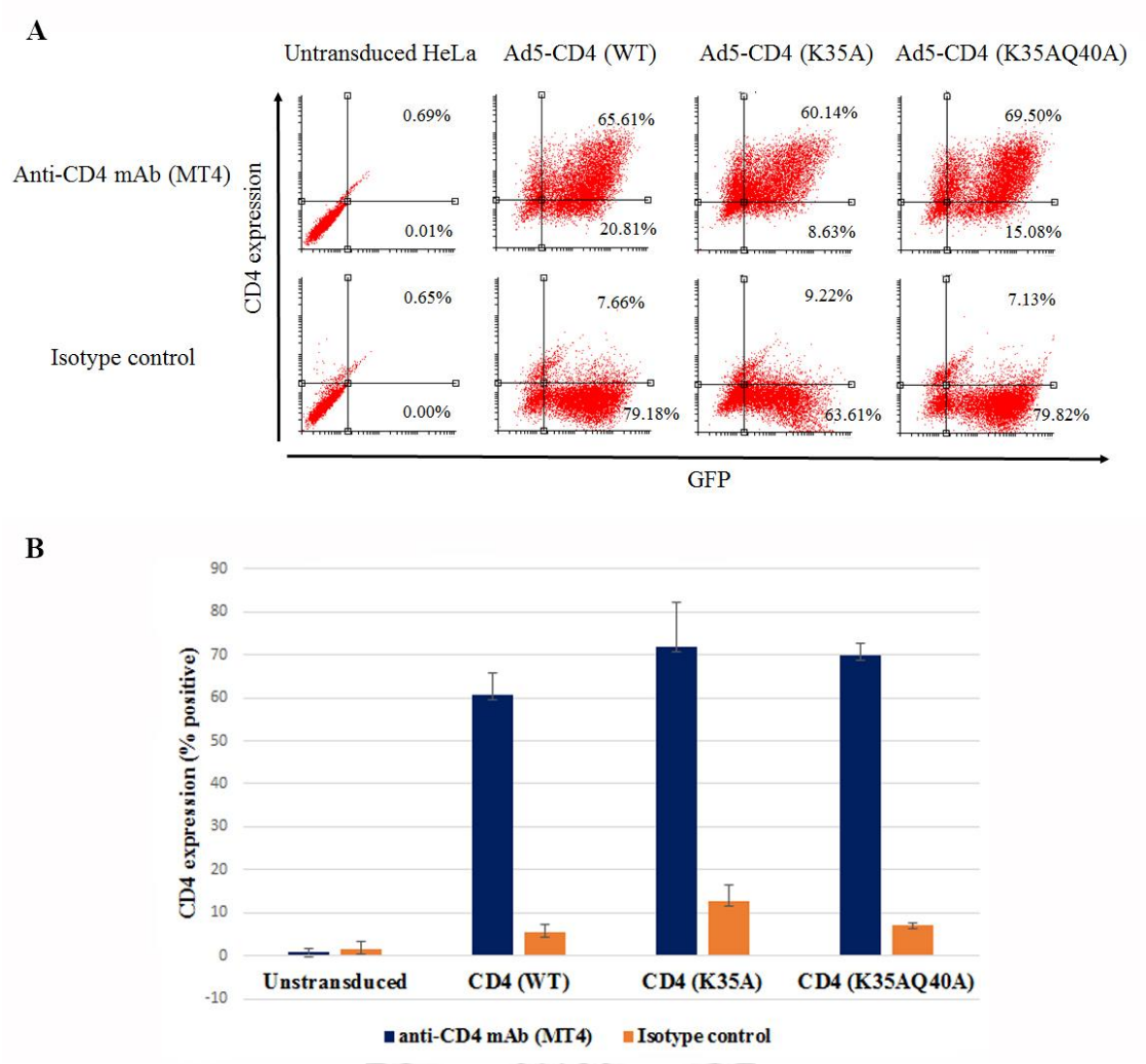

Figure 3.22 Expression of CD4 molecules in HeLa cells transduced with Ad5-CD4 tested by anti-CD4 mAb (MT4). MT4 recognized both CD4 (K35A) and CD4 (K35AQ40A) comparing to CD4 (WT) as seen in dot plot analysis of flow cytometry data (A). The summarized data of the triplicate repeat experiments was presented in bar graph of mean ± SEM (B).

3.9 Binding of CD4-specific DARPin 23.2 to CD4 mutants

To investigate the ability of CD4-specific DARPin 23.2 to bind to mutated CD4 molecules, the binding was performed in HeLa cells transduced with Ad5-CD4 (K35A) and Ad5-CD4 (K35AQ40A). Briefly, after 36 hours of virus incubation, transduced HeLa cells were harvested. One hundred ng/ml of CD4-specific DARPin was added to the transduced cells following by anti-His tag and RPE conjugated rabbit anti-mouse immunoglobulins. The data was analyzed by flow cytometry. Irrelevant DARPin was used as negative DARPin.

From the analyzed data shown in Figure 3.23A, CD4-specific DARPin 23.2 bounds specifically to CD4 (WT) at 75.58%. DARPin 23.2 still bound specifically to both forms of CD4 mutants, 79.97% positive for CD4 (K35A) and 82.57% positive for CD4 (K35AQ40A). These binding reflected the unimportance of either K35 or Q40 on CD4 molecule as they illustrated positive binding to CD4-specific DARPin similar to the binding of CD4 (WT). The summarized data of the triplicate repeat experiment was presented in bar graph of mean ± SEM in Figure 3.23B.

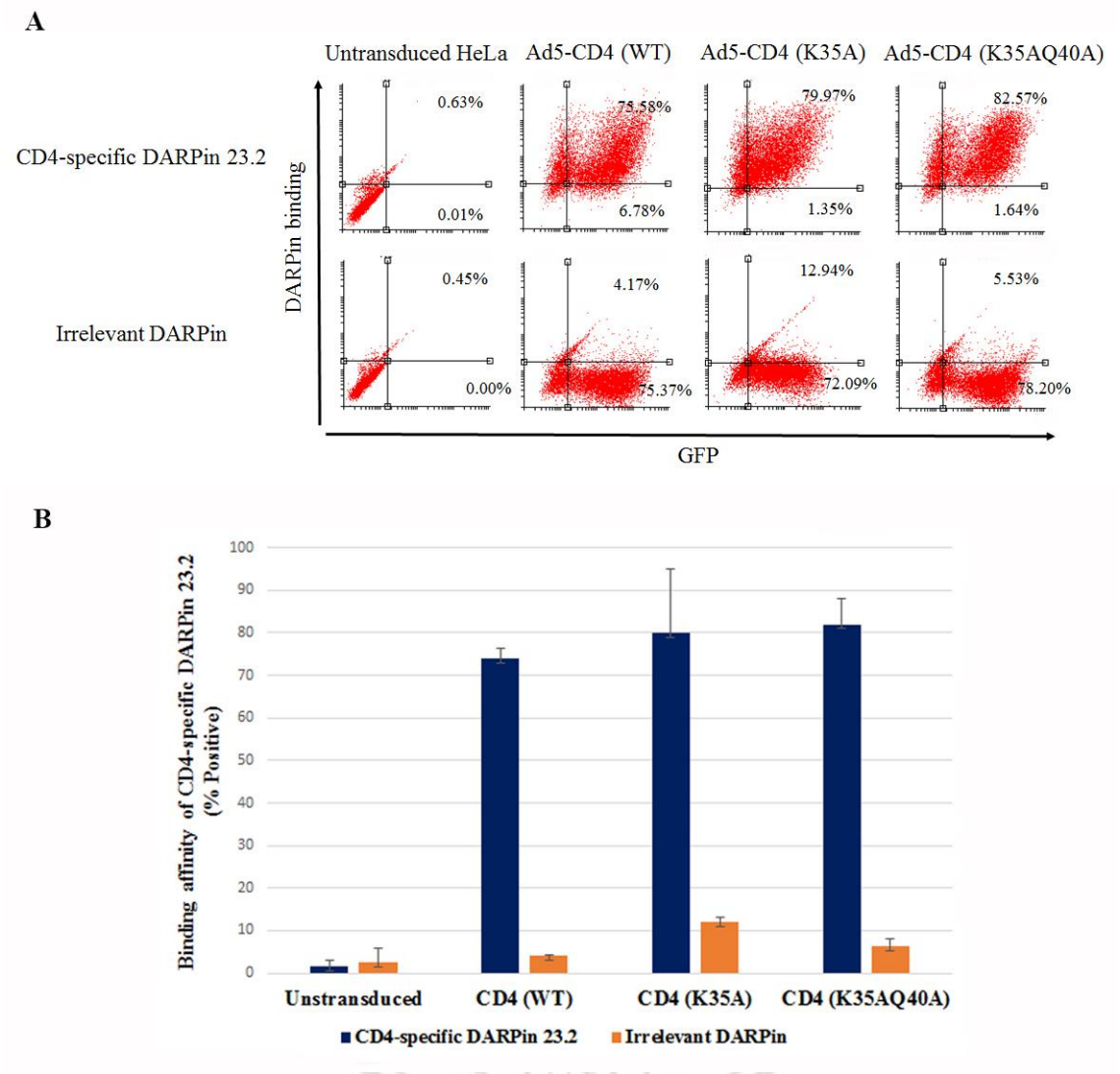

Figure 3.23 Binding affinity of CD-specific DARPin 23.2 to CD4 molecules in HeLa cells transduced with Ad5-CD4. Dot plot analysis of flow cytometry data illustrated all similar positive binding pattern in CD4 (K35A) and CD4 (K35AQ40A) comparing to CD4 (WT) (A). The summarized data of the triplicate repeat experiments was presented in bar graph of mean ± SEM (B).