CHAPTER III

RESULTS

3.1 Construction of adenoviral plasmids containing scFv-M61B9 gene

Two platforms of adenoviral plasmid were established for the adenoviral vector production in a packaging cell line (293A). First, the cytoplasmic expression system, the scFv-M61B9cyt gene was amplified from pAdE-scFv-M61B9ER using a set of primer that was designed to have the short restriction sequences for *Sal*I and *Not*I at the upstream and downstream of scFv-M61B9 gene, respectively. The scFv-M61B9cyt gene composed of the V_L and V_H chains tagged with HA sequence at the 3'-end was amplified, subcloned into the expression vector (pAdTrack-CMV) and then named as pAdT-scFv-M61B9cyt. Successful amplification of the scFv-M61B9cyt gene was clearly indicated by the presence of a band at approximately 800 bp (Figure 3.1). The amplified scFv-M61B9cyt fragment was purified and digested with *Sal*I and *Not*I and then ligated into the pAdTrack-CMV plasmid vector which was pre-treated with the same enzyme. The ligated product was transformed into the *E. coli* Novablue and the kanamycin resistant colonies were selected for plasmid purification. The approximately 10 kb plasmids were digested with *Sal*I and *Not*I (Figure 3.2). The result shows the approximately 800 bp band of scFv-M61B9cyt gene and the the band of vector pAdTrack-CMV at approximately 9 kb in lane 2. The scFv-M61B9cyt gene was re-amplified from vector pAdT-scFv-M61B9cyt to prove the pAdTrack vector containing scFv-M61B9cyt (Figure 3.3). These results indicated that the scFv-M61B9cyt gene was successfully inserted into the pAdTrack-CMV vector.

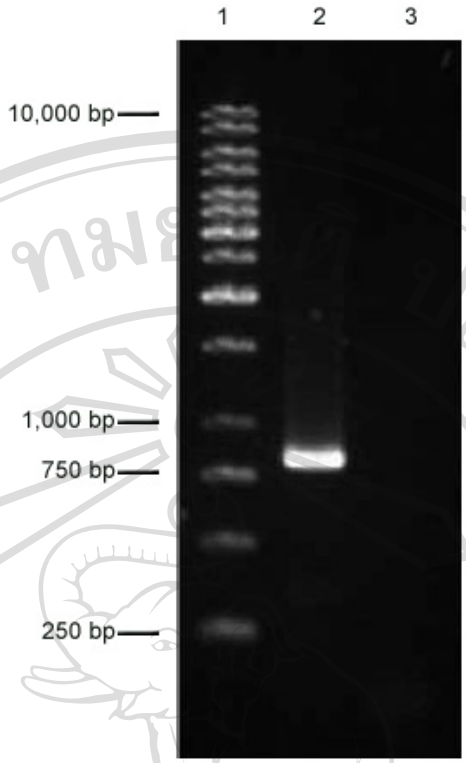


Figure 3.1 PCR product of scFv-M61B9cyt gene. PCR product of scFv-M61B9cyt (800 bp) form pAdE-scFv-M61B9ER and no vector templates were shown in lane 2 and 3, respectively. Lane 1 showed 1 kb DNA marker.

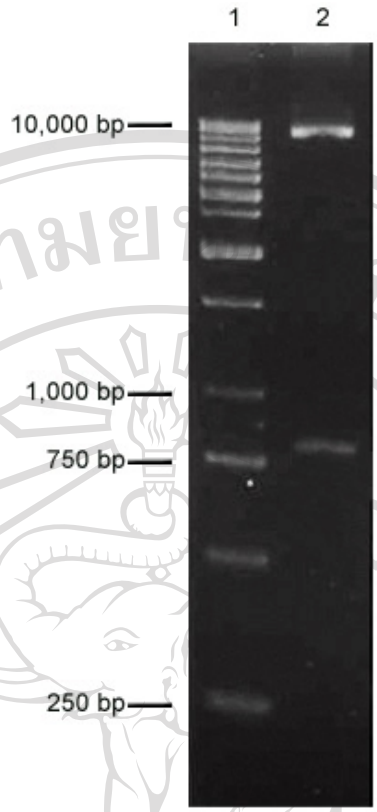


Figure 3.2 Characterization of pAdTrack-CMV containing scFv-M61B9cyt. Lane 2 showed the pAdT-scFv-M61B9cyt digested with *Sal*I and *Not*I restriction enzymes. Lane 1 showed 1 kb DNA marker.

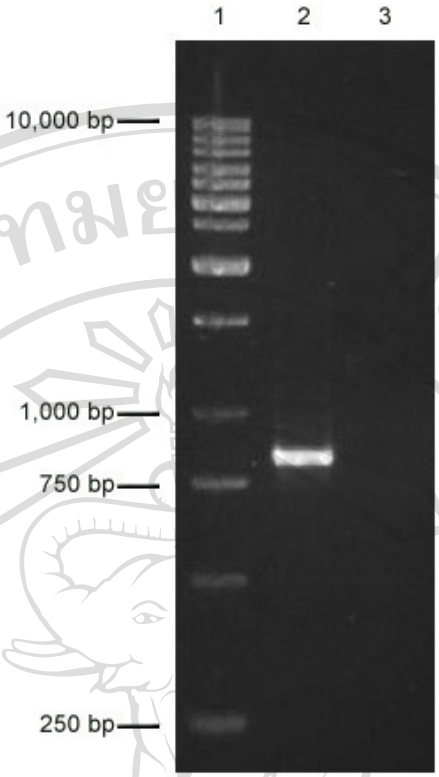


Figure 3.3 PCR product of scFv-M61B9cyt amplified from pAdT-scFv-M61B9cyt. PCR product of scFv-M61B9cyt (800 bp) from pAdT-scFv-M61B9cyt and no vector template were shown in lane 2 and 3, respectively. Lane 1 demonstrated 1 kb DNA marker.

In comparison, the pAdT-scFv-M61B9sec was generated by using the point mutation of pAdT-scFv-M61B9ER. This vector was mutated at the lysine sequence (AAA) of the KDEL region to create a stop codon (TGA) as show in Figure 3.4. The scFv-M61B9sec gene was flanked by a human κ light chain leader sequence at the 5'-end, and a sequence encoding the HA tag at the 3'-end. The point mutation was perform using the mutagenic primers to amplify the mutated pAdTrack vector expressed the scFv-M61B9sec gene. The non-mutated parental DNA template (pAdT-scFv-M61B9ER) was digested with *Dpn*I and the mutated vector (pAdT-scFv-M61B9sec) was subsequently transformed into *E. coli* Novablue. The successfully mutation was shown by using DNA sequencing in Figure 3.4.

The modified pAdTrack containing scFv-M61B9cyt and scFv-M61B9sec gene were digested with *Pme*I and then transformed into *E*. coli BJ5183 which has already transformed with pAdEasy vector to perform homologous recombination. The kanamycin resistant colonies were selected for purification and then digested with *Pac*I to verify proper recombination (Figure 3.5). The DNA fragment band at 4,500 bp can be observed in lane 3 indicated the successful recombination of the adenoviral vector. The pAdEsay vector containing scFv-M61B9cyt and scFv-M61B9sec was proved by PCR (Figure 3.6). The scFv-M61B9cyt and scFv-M61B9sec band at approximately 800 and 880 bp was shown in lane 2, respectively. The newly synthesized recombinant plasmid vector was named pAdE-scFv-M61B9cyt and pAdE-scFv-M61B9sec, respectively (Figure 3.7) and transformed into *E. coli* DH10B for large-scale amplification.

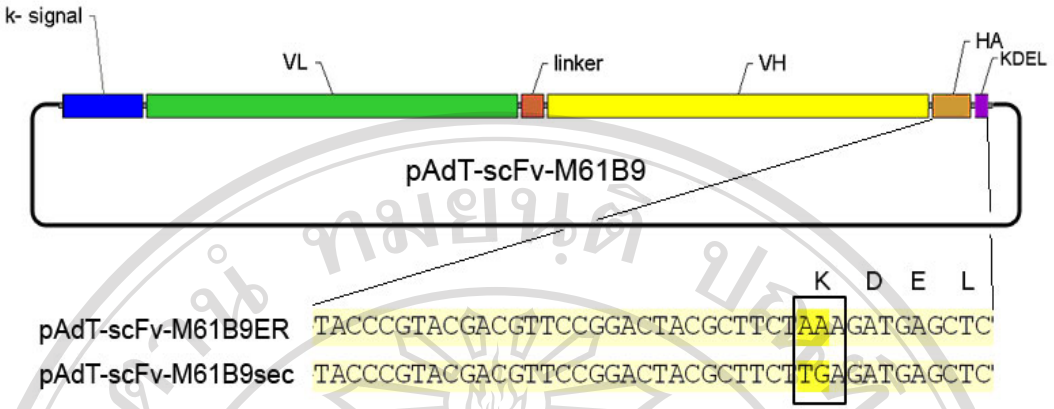


Figure 3.4 The point mutation of pAdT-scFv-M61B9ER was performed to generate pAdT-scFv-M61B9sec. As show in the box, the lysine sequence (AAA) was mutated to the stop codon (TGA).

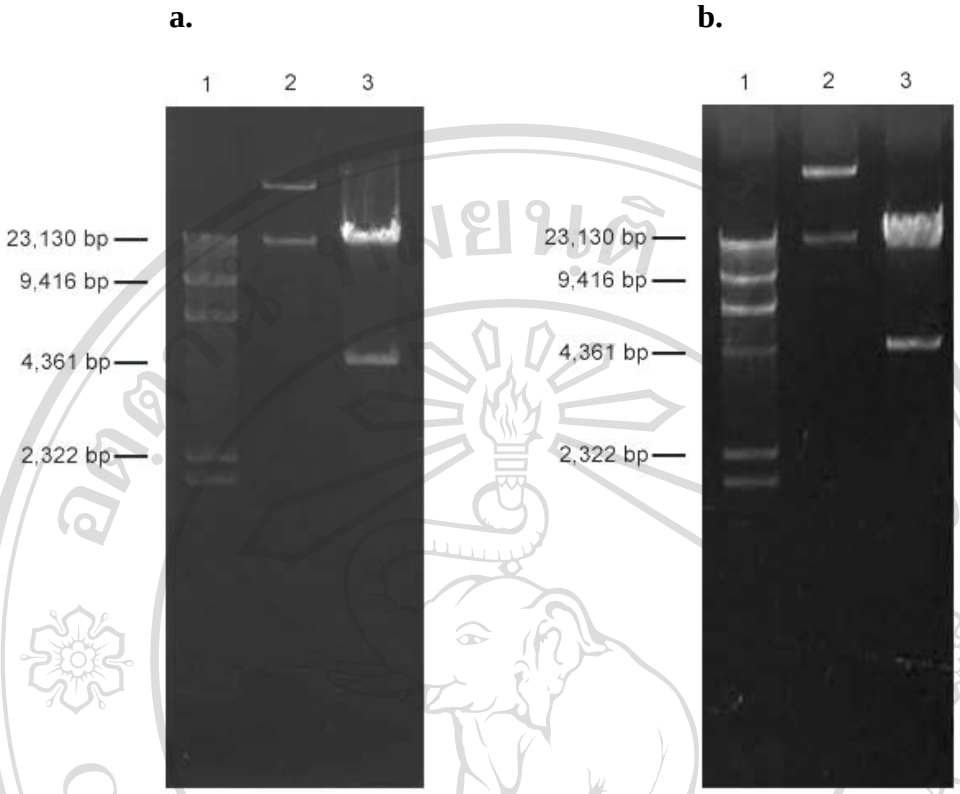


Figure 3.5 Restriction fragment analysis of pAdE-scFv-M61B9cyt (a.) and pAdE-scFv-M61B9sec (b.) using *Pac*I restriction enzyme. The DNA fragment band at 4,500 bp can be observed in lane 3. Lane 2 showed un-digested vector. Lane 1 showed Lamda DNA/*Hind*III marker.

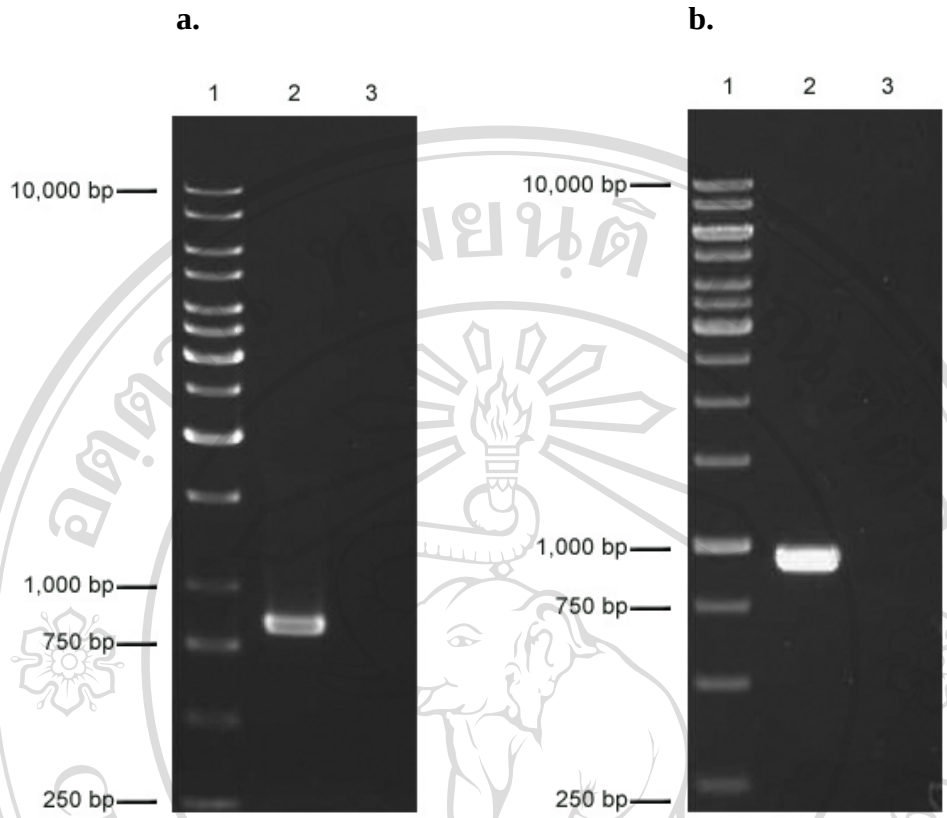
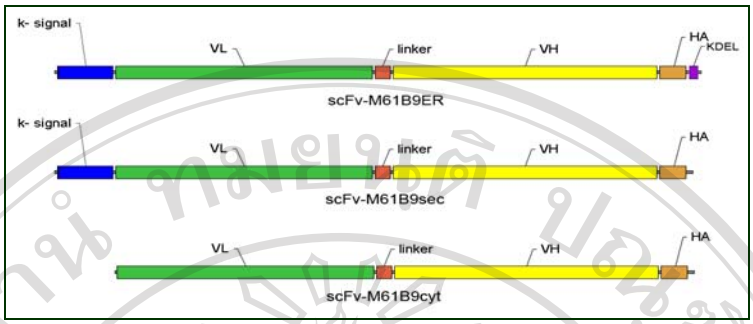


Figure 3.6 ScFv-M61B9 gene amplification of pAdE-scFv-M61B9cyt (a.) and pAdE-scFv-M61B9sec (b.). ScFv-M61B9cyt gene (800 bp) and scFv-M61B9sec gene (880 bp) were shown in lane a.2 and b.2, respectively. Non-specific amplification from no vector template was shown in lane 3. Lane 1 showed 1 kb DNA marker.

a.



b.

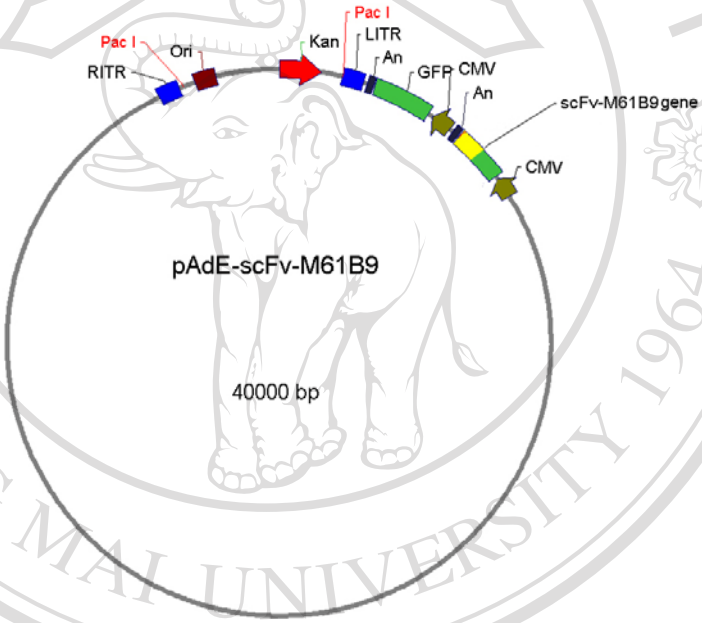


Figure 3.7 Schematic illustration of recombinant adenoviral plasmid vector. a.) The scFv-M61B9ER, scFv-M61B9sec and scFv-M61B9cyt gene was shown in the box. VL, light chain variable region; VH, heavy chain variable region; HA, HA-tag. b.) The pAdE-scFv-M61B9 containing the scFv-M61B9ER, scFv-M61B9sec and scFv-M61B9cyt gene was generated by homologous recombination. An, polyadenylation site; LITR, left-hand ITR and packaging signal; RITR, right-hand ITR; Ori, origin of replication; Kan, kanamycin resistant gene; CMV, cytomegalovirus promoter.

3.2 Generation of recombinant adenovirus expressing scFv-M61B9

The packaging cell line (293A) which constitutively express the E1 gene products required for propagation of the recombinant adenovirus were transfected by lipid-DNA complexes compost of *Pac*I digested adenoviral plasmid vector (pAdE-scFv-M61B9cyt or pAdE-scFv-M61B9sec) and lipofection reagent. To assess how soon the packaged viral particles could be observed, transfected cells were monitored by GFP expression using inverted fluorescence microscope. GFP expression was visible at 7 day post transfection, representing the fraction of the population that was transfected (Figure 3.8). The culture supernatant containing newly recombinant adenovirus vector was named Ad5-scFv-M61B9cyt or Ad5-scFv-M61B9sec and collected to transduce into HeLa cells.

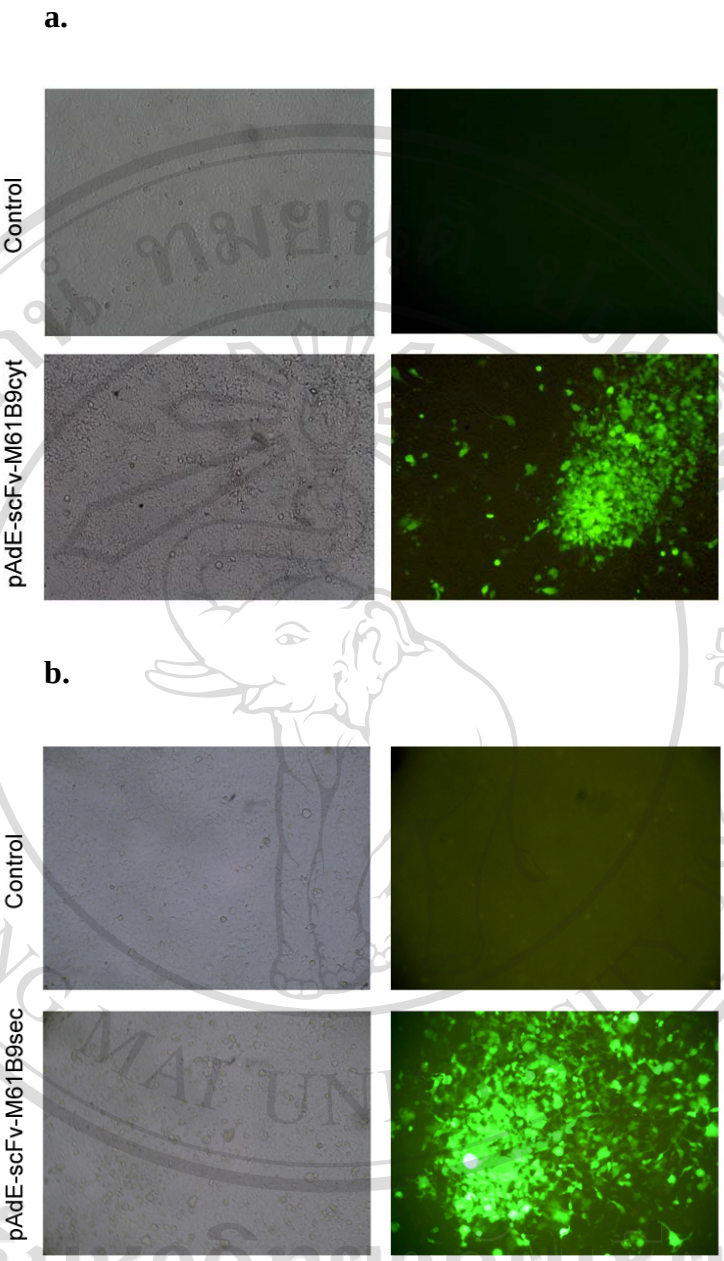


Figure 3.8 Adenovirus-producing foci after transfection of 293A cells. *Pac*I-treated pAdE-scFv-M61B9cyt (a.) or pAdE-scFv-M61B9sec (b.) was transfected into 293A cells and GFP expression was visualized by fluorescence microscopy. Comet-like adenovirus-producing foci become apparent in day 7. No such foci were observed in the cells transfected with no vector (control).

3.3 Production of scFv in each compartment of mammalian cells

To produce the scFv-M61B9 in cytoplasm, Ad5-scFv-M61B9cyt was transduced into HeLa cells. After 48 hr, the GFP positive cells were observed and collected to harvest scFv-M61B9. Accordingly, the Ad5-scFv-M61B9sec and Ad5-scFv-M61B9ER was transduced into HeLa cells to produce scFv-M61B9 in a secretory system and ER system, respectively (Figure 3.9). The scFv-M61B9 in each compartment was further analyzed by using indirect ELISA and western immunobloting.

Both cell lysate and culture supernatant of transduced cells was used to detect for scFv-M61B9 by using ELISA. The CD147-BCCP protein was captured by coated avidin protein for detecting the scFv-M61B9 in the sample. The survivin-BCCP and avidin were used as controls. Cell lysate and culture supernatant were added and followed by HRP-conjugated anti-HA antibody. Anti-CD147 mAb, anti-SVV mAb and anti-BCCP mAb were added and traced by HRP-conjugated goat anti-mouse immunoglobulins antibody as controls. From cells transduced with Ad5-scFv-M61B9ER, scFv-M61B9 was detected in the cell lysate only. Whiles, the scFv-M61B9 expressed by the Ad5-scFv-M61B9sec-transduced cells was found not only in the cell lysate but also in the culture supernatant. On the other hand, the expression of scFv-M61B9 generated by the pAdE-scFv-M61B9cyt-transduced cells can not be observed (Figure 3.10).

In addition, the localization of scFv-M61B9 produced in HeLa cells transduced with Ad5-scFv-M61B9 containing the signal sequence (Ad5-scFv-M61B9ER and Ad5-scFv-M61B9sec) was investigated. Ad5-scFv-M61B9ER, the representation of the signal sequence containing vector, was transduced into HeLa

cells. The extracted scFv from each compartment including cytoplasmic, ER and nuclear compartment of the transduced cells were separated by reducing SDS-PAGE and then blotted onto PVDF membrane. The scFv-M61B9 was detected by using HRP-conjugated anti-HA Ab. The band at approximately 35 kDa was observed in lane 4 (Figure 3.11) exhibited the scFv-M61B9 was generated and retained in the ER system.

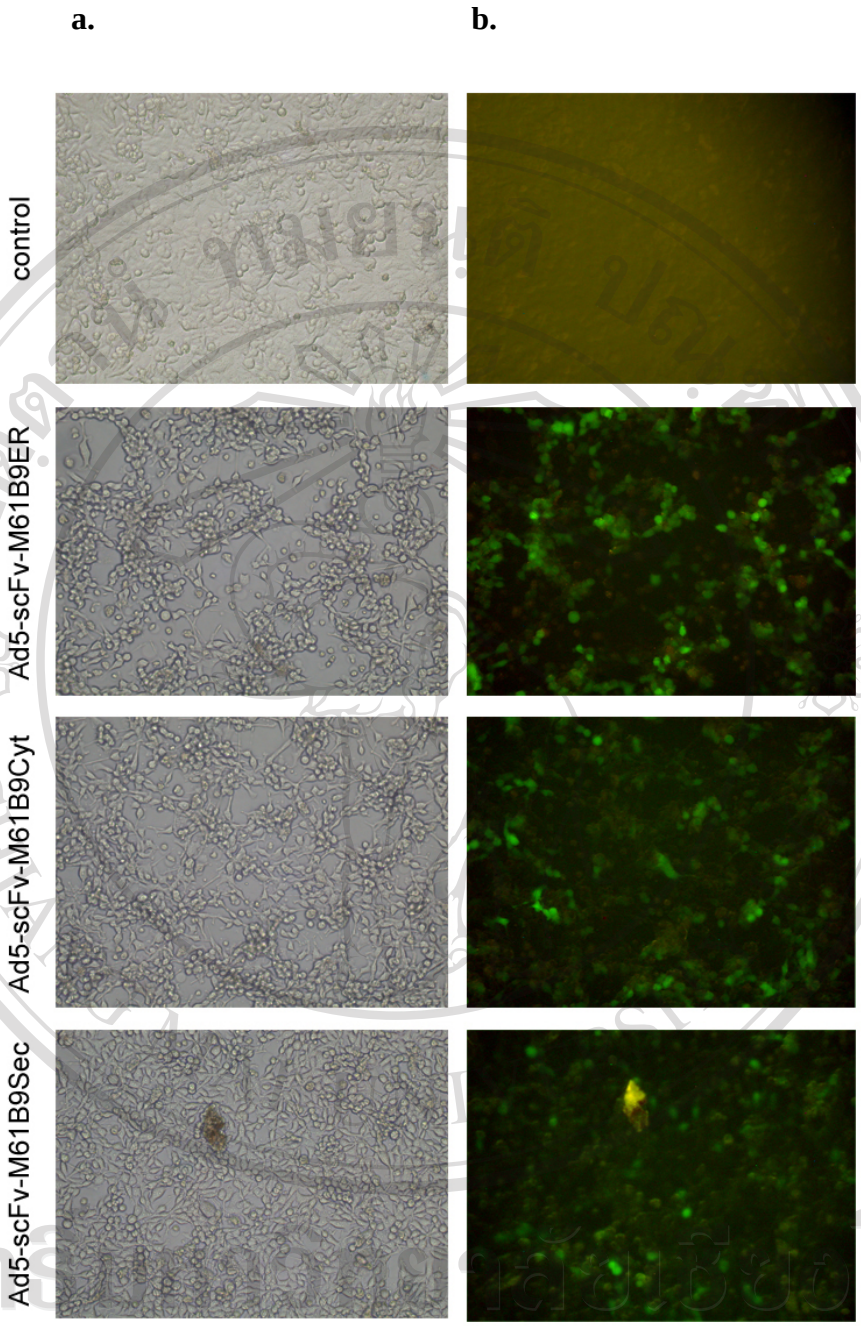


Figure 3.9 Transduction of the adenovirus vector containing scFv-M61B9 gene in HeLa cells. The transduced cells were shown by light microscopy (a.). The GFP positive cells can be observed at 2 day post transduction by using fluorescence microscopy (b.).

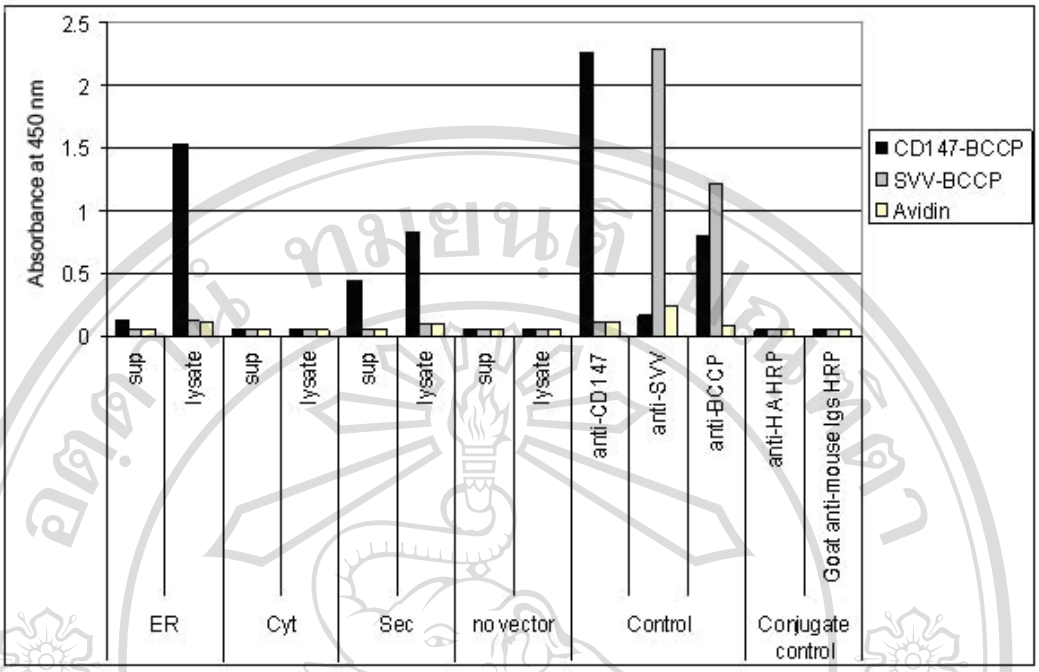


Figure 3.10 The detection of scFv-M61B9 in culture supernatant (sup) and HeLa cell lysate (lysate). The solid phase was coated with Avidin (white bar) and Avidin-captured proteins; CD147-BCCP (black bar) and survivin-BCCP (gray bar). Both culture supernatant and cell lysate from the HeLa cells transduced with Ad5-scFv-M61B9ER (ER), Ad5-scFv-M61B9cyt (Cyt), Ad5-scFv-M61B9sec (Sec) and no transduced cells (no vector) were added and detected the scFv-M61B9 by adding HRP-conjugated anti-HA Ab. The antibody control; anti-CD147 mAb, anti-survivin mAb (anti-SVV) and anti-BCCP mAb were followed by HRP-conjugated goat anti-mouse immunoglobulins antibody.

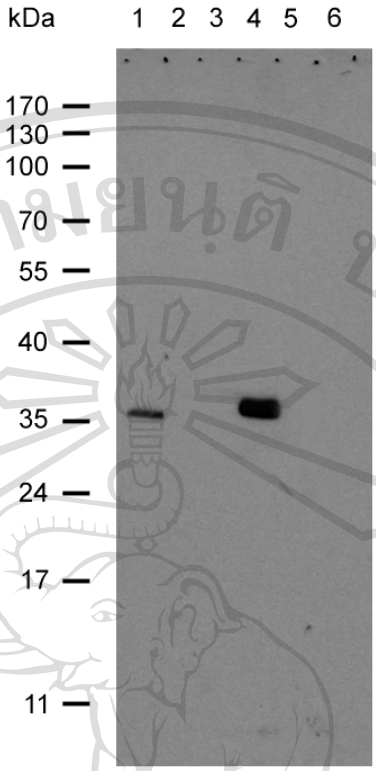


Figure 3.11 The localization of scFv-M61B9 in HeLa cells transduced by Ad5-scFv-M61B9ER. The cytoplasmic fraction proteins (lane 3), ER fraction proteins (lane 4), nuclear fraction proteins (lane 5) and culture supernatant proteins (lane 6) from Ad5-scFv-M61B9ER transduced cells were separated by reducing SDS-PAGE and then blotted to the PVDF membrane. The whole cell lysate from Ad5-scFv-M61B9ER transduced cell lysate (lane 1) and untransduced cell lysate (lane 2) were used as control. The blotted proteins were probed with HRP-conjugated anti-HA Ab. The immuno-reactive bands were visualized by chemiluminescent substrate detection system.

3.4 Functional scFv produced in mammalian cells

The binding activity and specificity of scFv-M61B9 produced in the secretory system was shown by indirect ELISA (Figure 3.10). The scFv-M61B9 which was produced by the HeLa cell transduced with Ad5-scFv-M61B9ER or Ad5-scFv-M61B9sec can specifically bind to the CD147-BCCP fusion protein. The specific binding was confirmed by using the western immunobloting. The scFv-M61B9 reacted only with the CD147 molecule separated on SDS-PAGE under reducing condition at approximately 37 kDa in lane 1 (Figure 3.12). This indicated the specific binding of scFv-M61B9. The bands at 17 kDa detected by anti-BCCP mAb demonstrated the degradation of fusion proteins.

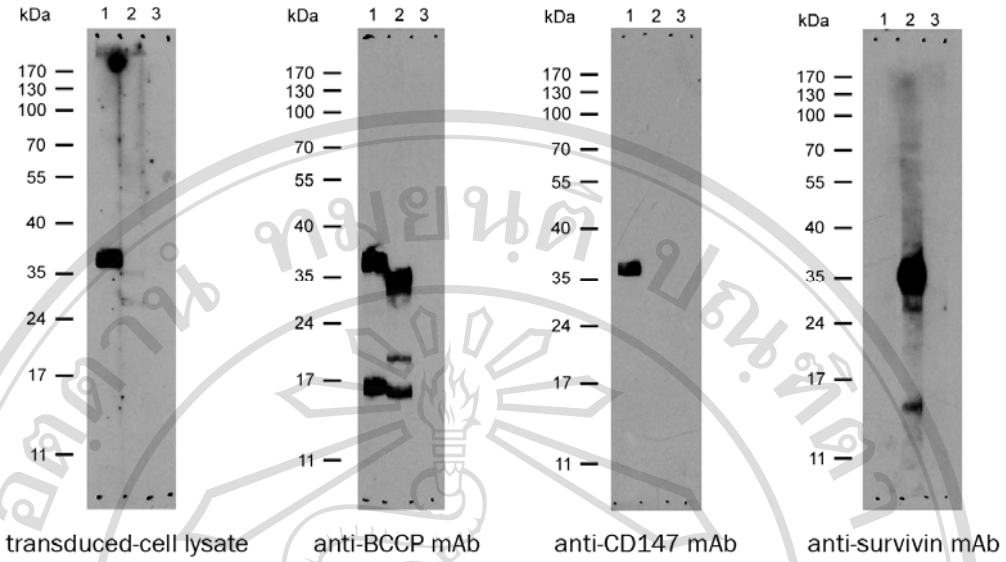


Figure 3.12 The binding activity and specificity of scFv-M61B9 produced in HeLa cells. The bacterial proteins containing CD147-BCCP (lane 1), survivin-BCCP fusion protein (lane 2) and no fusion proteins (lane 3) were separated and blotted onto PVDF membrane. The blotted proteins were probed with trasduced-cell lysate which obtained from HeLa cell trasduced with Ad5-scFv-M61B9ER. The mouse anti-BCCP mAb, mouse anti-CD147 mAb M61B9 and mouse anti-survivin mAb were used as controls. The immuno-reactive bands were visualized by chemiluminescent substrate detection system.