

Production of Recombinant Antibody scFv-Fc Against Notch1 and 2 Using HEK-293T Cell Line**การผลิตรีคอมบิแนนท์แอนติบอดี scFv-Fc ต่อ Notch1 และ 2 โดยใช้เซลล์ไลน์ HEK-293T**

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ABSTRACT

Notch signaling comprises of four different Notch receptors (Notch-1-4) and five ligands (Delta-1, -3, -4 and Jagged1, 2). Notch signaling regulates proliferation, differentiation, and apoptosis of various cell types. Abnormal increases or deficiencies in Notch signaling result in cancer development of various origins. Small molecule γ -secretase inhibitor (GSI) is used for inhibiting Notch signaling but it may have undesirable side effects. ScFv-Fc is a fusion protein of the single-chain variable fragments (scFV) and human immunoglobulin G (IgG) Fc moiety. ScFv-Fc has potential in clinical application over the whole monoclonal antibody for target therapy. The purpose of this research is to produce the recombinant antibody scFv-Fc against Notch1 and 2 in HEK-293T cell line. The results indicated that recombinant anti-Notch1 and 2 scFv-Fc were successfully expressed in HEK-293T cell line as investigated by SDS-PAGE and Western blot.

บทคัดย่อ

วิถีสัญญาณ Notch ประกอบด้วย รีเซปเตอร์ 4 ชนิด (Notch 1-4) และลิแกนด์ 5 ชนิด (Delta-1, 3, 4 และ Jagged 1, 2) วิถีสัญญาณ Notch ทำหน้าที่ควบคุมการเพิ่มจำนวน การเปลี่ยนแปลง ตลอดจนจนถึงการตายของเซลล์หลายประเภท ความผิดปกติของสัญญาณ Notch ส่งผลให้เกิดการพัฒนาเป็นมะเร็งชนิดต่างๆ ได้ มีการใช้ยากดการทำงานของเอนไซม์แกมมาซีเครเตส (GSI) เพื่อการยับยั้งวิถีสัญญาณ Notch แต่มีรายงานว่า GSI ทำให้เกิดผลข้างเคียงที่ไม่พึงประสงค์รีคอมบิแนนท์แอนติบอดีในรูป ScFv-Fc ซึ่งเป็นโปรตีนลูกผสมประกอบไปด้วย scFV และ Fc ของ อิมมูโนโกลบูลินจีของมนุษย์ได้ถูกนำมาประยุกต์ใช้ในการรักษาทางคลินิกเนื่องจากมีคุณสมบัติที่ดีกว่าโมโนคลอนอลแอนติบอดีทั้งโมเลกุลในงานวิจัยนี้มีวัตถุประสงค์ในการผลิตรีคอมบิแนนท์แอนติบอดี scFv-Fc ต่อ Notch1 และ 2 โดยใช้เซลล์ไลน์ HEK-293T จากผลการทดลองพบว่าสามารถผลิตรีคอมบิแนนท์แอนติบอดี scFv-Fc ต่อ Notch1 และ 2 ได้ในเซลล์ไลน์ HEK-293T โดยทดสอบยืนยันการแสดงออกของโปรตีนโดยใช้วิธี SDS-PAGE และ Western blot

Keywords: Notch signaling, Recombinant antibody scFv-Fc, HEK-293T cell line**คำสำคัญ:** วิถีสัญญาณนอติชรีคอมบิแนนท์แอนติบอดีชนิดเอกซีเอพวีเอพีซี เซลล์ไตของตัวอ่อนมนุษย์

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Introduction

Notch receptors are transmembrane protein found on the surface of many cell types, including cancer cells. Notch signaling controls cell fate in many contexts of development, including proliferation, differentiation and apoptosis (Joutel and Tournier-Lasserre 1998) In mammals, Notch signaling is triggered by binding one of the four Notch receptors (Notch-1-4) and to one of the five ligands (Delta-1, -3, -4 and Jagged1, 2) on the cell surface of between cells, such as cancer cells and a neighbor cell (Fermino *et al.*, 2016). Notch receptors comprises of an extracellular region with 29-36 epidermal growth factor (EGF)-like repeats, negative regulatory region (NRR) and the intra-cellular domain (ICD) that mediates signaling upon activation (Falk *et al.*, 2012). The binding between Notch receptors and ligands triggers proteolytic cleavage of the receptor by γ -secretase within the transmembrane domain of Notch receptor (Falk *et al.*, 2012), resulting in the release of Notch intracellular domain (NICD) into the nucleus, where it binds to the nuclear transcription factor. In the nuclei, Notch transcription complex transcribes the target genes such as *Hes* and *Hey* family, these gene associated with cell differentiation (Van Es *et al.*, 2005). Increases or deficiencies of Notch signaling result in human developmental anomalies and cancer development. Notch signaling acts as tumor suppressor or oncogenic depending on the tumor cell types (Falk *et al.*, 2012). In addition, mutations in different Notch receptors are associated with other diseases such as aortic valve disease (Garg *et al.*, 2005), Allagile syndrome (McDaniell *et al.*, 2006). Previous study has reported that the inhibition of Notch signaling pathways can decrease of tumours (Tammam *et al.*, 2009). Thus, Notch signaling can be a specific and potential therapeutic target for the treatment of cancers and other diseases.

Small molecule γ -secretase inhibitor (GSI) is widely used for inhibiting Notch signaling. GSI equally blocks the processing of all four Notch receptors. Because GSI has multiple substrates besides Notch (Falk *et al.*, 2012), GSI is not ideal to be used for treatment of diseases such as cancer. Therefore, there is a need for specific novel inhibitor of Notch signaling. Whole monoclonal antibodies (mAb) are large in size, that can limit its tumor penetration, and their long serum half-life is inappropriate for some applications such as radioimmunotherapy (Chames *et al.*, 2009). Previous study has generated antibodies in the form of single-chain variable fragments (scFv), but it was reported that they are ineffective for therapeutic applications because of its small size that leads to rapid elimination by renal clearance with short half-life in serum (Chames *et al.*, 2009).

ScFv-Fc is a fusion protein of scFv and human immunoglobulin G (IgG) Fc moiety, and produced as bivalent antibody (Jørgensen, Friis *et al.* 2014). Benefits of the Fc-fusion include increasing a half-life in serum and penetration into tissues (Jørgensen *et al.*, 2014). Moreover, the Fc-region facilitates detection and purification using affinity chromatography such as protein A, protein G (Jørgensen *et al.*, 2014). Therefore, scFv-Fc antibodies have potential for clinical application over the whole mAb, scFv for target therapy.

In 2012, Falk, R. *et al.* generated antibodies in the form of scFv-Fc fusion proteins targeting either the ligand binding domain (LBD) or NRR of Notch receptors. They reported that their antibodies specifically block the activity of Notch1 and 2 via targeting NRR only (Falk *et al.*, 2012). The NRR of Notch receptor contains three of LIN-12-Notch repeats (LNR) and a heterodimerization domain (HD) (Wang, 2011). Receptor/ligand interaction on the cell surface results in a conformational change in the NRR domain, resulting in exposure of a proteolytic cleavage

site on Notch receptor to γ -secretase (Falk *et al.*, 2012). Therefore, NRR can be a target for antibodies that relies on stabilizing NRR and block the cleavage of Notch receptor.

Currently, almost all therapeutic antibodies are produced in mammalian cells because of their superior in protein folding, post-translational modification, secretion and high production yield over prokaryotic cells (Jäger *et al.*, 2013). This type of antibody is indistinguishable from antibodies produced in the body with the lowest immunogenicity (Schirrmann, Büsow, 2010). Therefore, mammalian cells are good alternative host for antibody production. Human embryonic kidney cells 293T (HEK-293T) is an immortalized cell line offer used in transfection study owing to the ease of handling, highly transfectable cell line, strong growth rates and high yield recombinant protein-generation capacity (Dyson, 2016). In addition, HEK-293T cells can be efficiently and easily transfected with plasmid vectors using lipid or polymer based transfection reagents (Schirrmann, Büsow, 2010).

The purpose of this research is to produce the recombinant antibody in the form of scFv-Fc against Notch1 and 2 using HEK-293T cell line.

Objective of the study

To produce the recombinant antibody scFv-Fc against Notch1 and 2 using HEK-293T cell line.

Materials and methods

1. To generate HEK-293T cell line that stably expresses scFv-Fc against Notch1 and 2

1.1 Plasmids preparation

The plasmids anti-Notch1_E6-pBIOCAM5 and anti-Notch2_B9-pBIOCAM5 (Addgene, USA) encoded scFv-Fc against Notch1 and 2, respectively. These plasmids were used to transformed *E.coli* DHT α . The transformed bacteria were streaked on a LB agar containing ampicillin (50 μ g/ml) and incubated for 16-18 hrs at 37°C. The single colony was picked and cultured in LB broth containing ampicillin (50 μ g/ml). Cell pellets were collected and the plasmid was extracted using QIAprep Spin Miniprep Kit (Qiagen, Germany). The sizes of the plasmids were confirmed by *Bam*HI digest. The concentration and purity of nucleic acid was measured at 260/280 nm using NanoDrop™2000/2000c Spectrophotometer (Thermo Scientific, USA).

1.2 Transfection of HEK-293T cells

1.2.1 Cell culture

HEK-293T cell line (ATCC® CRL-3216™) were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 1% HEPES free acid, 1% sodium pyruvate and 1% penicillin-streptomycin. Cells were maintained in 5% CO₂ at 37 °C.

1.2.2 Transfection

HEK-293T cells were seeded at 5x10⁴ cells/ well in 24-well plates for 24 hrs. At the time of transfection, cells were washed with PBS. Plasmids (anti-Notch1_E6-pBIOCAM5 and anti-Notch2_B9-pBIOCAM5) (1 μ g) were mixed with FuGene6™ transfection reagent (Promega, USA) according to the manufacturer's instruction and the complex were added to cells. Cells transfected with only transfection reagent and empty vector were used as negative controls.

1.2.3 Selection for stable transfectant

Geneticin (G418) (Gibco, USA) was used for selection of stable transfectants. After 48 hrs of transfection, transfected HEK-293T cells were cultured in fresh media containing G418 (1 mg/ml). Fresh media containing G418 were added every 3-4 days, until stable cells were obtained. The culture supernatant and protein lysate were harvested to detect the recombinant anti-Notch1 and 2 scFv-Fc.

1.2.4 SDS-PAGE and Western blot

The samples (culture supernatant and protein lysate) were subjected to 8 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis visualized by using Coomassie staining and the samples were subjected to 8% SDS-PAGE and then transferred onto PVDF membrane (Millipore) using semi-dry electroblotter (Bio-Rad). The membrane with transferred protein was blocked by using 3 % (w/v) skim milk in PBS-T solution (0.1 % v/v Tween-20 in PBS) for 5 min, 2 times at room temperature and probed overnight with the anti-His tagged rabbit antibody at 1: 2000 (Cell Signaling Technology, USA). After washing with PBS-T for several times, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG at 1:4000 (GE Healthcare, UK). The band was visualized with high performance chemiluminescence X-ray film.

2. To purify the recombinant anti-Notch1 and 2 scFv-Fc

2.1 Sample preparation

HEK-293T cell line with stable transfection was cultured in DMEM medium and incubated in 5% CO₂ and humidified atmosphere at 37 °C for 5-7 days. The supernatant was harvested and centrifuged to recover culture supernatant at 3,000xg for 30 min. Saturated ammonium sulfate solution to supernatant was added and bring the final concentration to 50% saturation of ammonium sulfate and transferred to 4°C overnight. The precipitate was recovered and resuspended in 0.1 volume of the starting volume in PBS. The samples were dialyzed against PBS for 72 h with PBS changes every 12 h at 4°C.

2.2 Purification by protein G affinity column

The sample was adjusted to pH 8.0 by adding 1/10 volume 1 M Tris (pH 8.0). The Protein G Sepharose™ 4 Fast Flow (GE Healthcare, UK), an affinity resin with protein G immobilized was packed onto the column and equilibrated with 20 mM sodium phosphate (pH 7.0), then the antibody solution was passed through the column. The column was washed with 20 mM sodium phosphate (pH 7.0). The protein was eluted with 100 mM glycine buffer (pH 3.0). The antibody fraction was collected in microcentrifuge tube. The antibody in each fraction was detected using 8% SDS-PAGE and Western blot as described above.

Results

1. Plasmids used in this study

To confirm the sizes of the plasmids, the plasmids were digested by restriction enzymes (*Bam*HI). As shown in Figure1, the results indicated that the size of plasmid anti-Notch1_E6-pBIOCAM5 was 7010 bp and the size of plasmid Notch2_B9-pBIOCAM5 was 6995 bp.

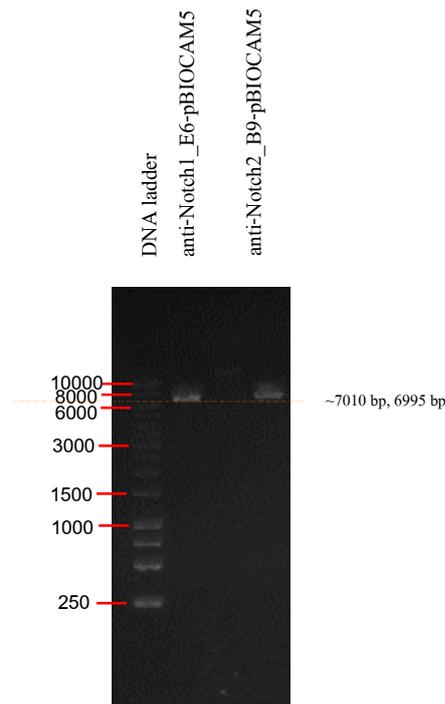


Figure1 Agarose gel electrophoresis (1%) of the plasmids used in this study anti-Notch1_E6- pBIOCAM5 and anti-Notch2_B9-pBIOCAM5 recombinant plasmids were extracted using QIAprep Spin Miniprep Kit, digested with *Bam*HI and separated in 1% agarose gel electrophoresis as labelled

2. Recombinant anti-Notch1 and 2 scFV-Fc

To confirm the expression of recombinant anti-Notch1 and 2 scFV-Fc in cell lysate and culture supernatant, HEK-293T cells were transfected with plasmids anti-Notch1_E6-pBIOCAM5, anti-Notch2_B9-pBIOCAM5. Empty vector without an insert was used as negative control. The expression of anti-Notch1 and 2 scFv-Fc was analyzed by Western blot (Fig. 2A, B). The results showed bands detected with anti-His tagged antibody (Cell Signaling Technology, USA) against His tagged protein of anti-Notch1 and 2 scFv-Fc to have a molecular weight of approximately. 58 kDa (Fig. 2A, B) that were detected in both supernatant and cell lysate. The control negative was shown as empty vector (Fig. 2A) and culture supernatant (Fig. 2B). β -actin was used as a loading control. Based on these preliminary results, HEK-293T cell line was used to generate stable transfectants in the next experiment.

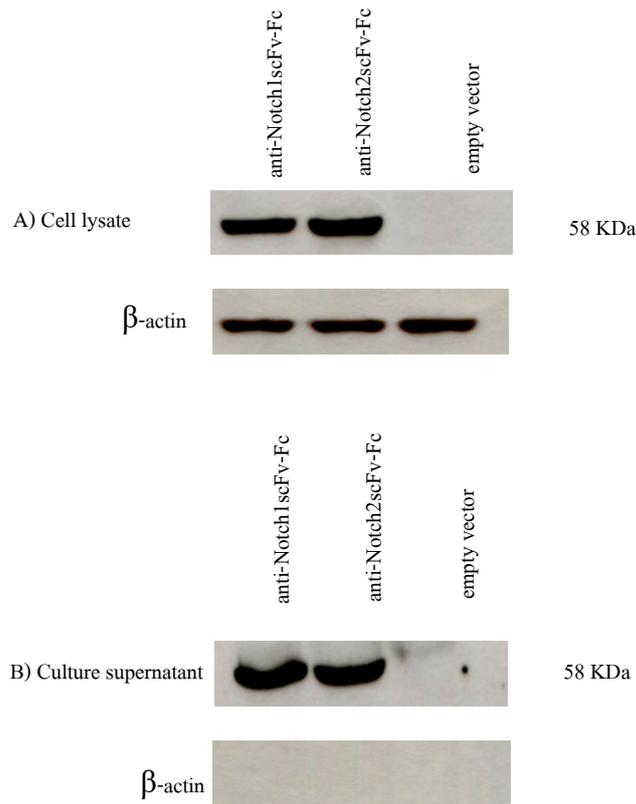


Figure2 Detection of scFv-Fc by Western blot. A) Cell lysate B) Culture supernatant were subjected to 8% SDS-PAGE and then transferred on to PVDF membrane using semi-dry electroblotter. The primary antibody was probed with the anti-His rabbit antibody at 1:2000 and the secondary antibody was horseradish peroxidase (HRP)-conjugated anti-rabbit IgG at 1:4000. The band was visualized with chemiluminescence X-ray film. β -actin was used as a loading control.

3. Purification of recombinant anti-Notch1 and 2 scFv-Fc by protein G affinity column

To obtain purified recombinant antibodies by protein G affinity column. HEK-293T cell line stably transfection was cultured in DMEM media and 100 ml of culture supernatants were harvested at 5-7days. Because the human Fc domain is used in the fusion protein, it could be readily purified by using protein G affinity column. After protein precipitation by ammonium sulfate, samples were mixed with the binding buffer containing 20 mM sodium phosphate before loading onto the column to increase the binding efficiency. Non-specific binding was collected in the flow-through fraction. After washing the column with binding buffer containing 20 mM sodium phosphate to remove non-specific binding proteins, the sample was eluted with 100 mM glycine (Fig. 3A-C). The results indicated that the fractions of anti-Notch1 and 2 were appeared many bands, when detected by SDS-PAGE analysis (Fig. 3A, B). In the other hand, the samples detected by Western blot using anti-His antibody for specific against His tagged protein of anti-Notch1 and 2 display a single band with a molecular weight approx. 58 kDa (Fig. 3A, B). However, this band (a molecular weight of approx. 58 kDa) was appeared on SDS-PAGE of anti-Notch2 scFv-Fc (Fig. 3B), but does not appeared in anti-Notch1 scFv-Fc (Fig. 3A) because anti-Notch1 scFv-Fc have low concentration of protein. Moreover, the other bands besides anti-Notch1 and 2 scFv-Fc on SDS-PAGE were appeared as well in controls (Fig. 3C).

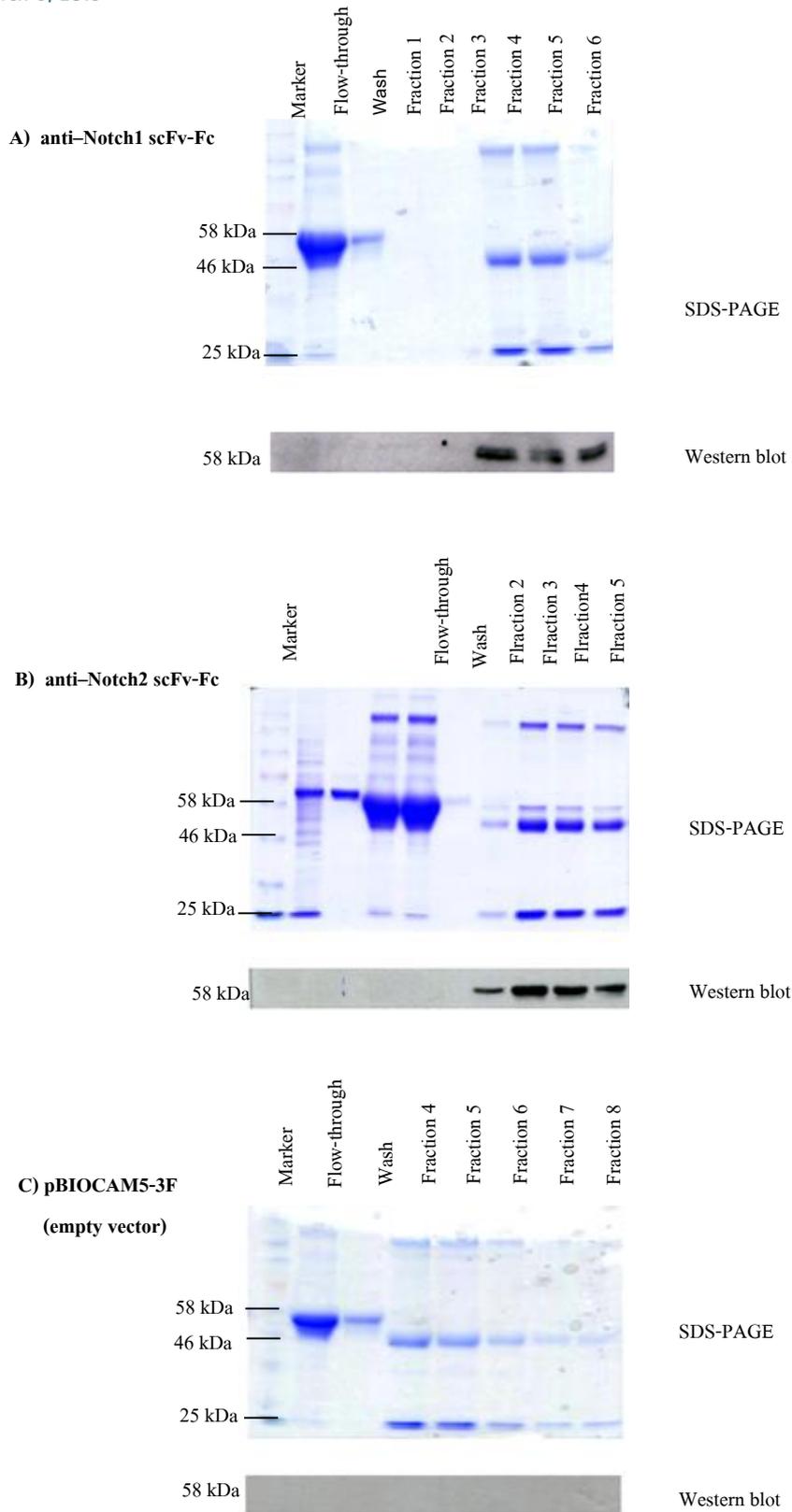


Figure3 Purification of recombinant scFv-Fc by protein G affinity column. A) anti-Notch1 scFv-Fc B) anti-Notch2 scFv-Fc C) pBIOCAM5-3F (empty vector) were purified by protein G affinity column, subjected to 8%SDS-PAGE analysis visualized by using Coomassie staining and were subjected to 8% SDS-PAGE and then transferred onto PVDF membrane using semi-dry electroblotter. The primary antibody was probed with the anti-His rabbit antibody at 1:2000 and the secondary antibody was horseradish peroxidase (HRP)-conjugated anti-rabbit IgG at 1:4000. The band was visualized with chemiluminescence X-ray film.

Conclusions and Discussion

This study reported the production of recombinant scFv-Fc against Notch1 and 2 in HEK-293T cell line. The recombinant antibody is increasingly used as therapeutic proteins for target therapy in cancer and chronic inflammation as having superior, properties than using conventional drugs. ScFv-Fc is a fusion protein of scFV and human immunoglobulin G (IgG) Fc moiety (Jørgensen *et al.*, 2014). Benefits of the Fc-fusion include increased half-life in serum and penetration into tissues (Jørgensen *et al.*, 2014). Moreover, Fc-region facilitates detection and purification such as protein A or protein G (Jørgensen *et al.*, 2014). In 2012, Falk, R. *et al.* generated antibodies in the form of scFv-Fc fusion proteins targeting NRR of the Notch receptors (Falk *et al.*, 2012). They reported that these antibodies can specifically block the activations of Notch1 and 2. Therefore, in this study we are interested in producing scFv-Fc fusion proteins according to a study by Falk, R. *et al.* (2012) but using HEK-293T cell line. There are two plasmids (anti-Notch1_E6-pBIOCAM5 and anti-Notch2_B9-pIPCAM5) that were used in this study. We found that the fractions of samples purified by protein G affinity column appeared as more than one band, when detected by SDS-PAGE. The samples were subjected to Western blot using anti-His tagged antibody for detection of specific anti-Notch1 and 2 scFv-Fc. The results indicated that a single protein band of anti-Notch1 and 2 were shown at a molecular weight of approx. 58 kDa (Fig. 3A, B). Although, the calculated molecular weight of anti-Notch1 and 2 were 55 kDa and 53 kDa, respectively. The difference might be attributed to the post-translational modification of the protein. Other protein bands appearing on SDS-PAGE may result from contaminant or bovine IgG in 10% FBS added to media for culture HEK-293T cell. Therefore, in the future we will decrease the percentage of FBS in the culture media or use serum-free media to decrease the effect protein of bovine IgG. In the future, we will characterize biological functions of obtained antibodies in cancer cells and macrophages, include binding activity and blocking activity for obtained efficiency the recombinant antibody scFv-Fc against Notch1 and 2 used as therapeutic proteins for target therapy in cancer.

In conclusion, this study demonstrated the production of recombinant anti-Notch1 and 2 scFv-Fc in HEK-293T cell line.

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