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# Improved purification protocol for recombinant human leukocyte antigens using an affinity magnetic agarose

**Thu Zar Ma Ma Moe Min[1,2,3], Sonwit Phanabamrung[3,4], Chanvit Leelayuwat[3,5*]**

[1] Department of Medical Laboratory Technology, University of Medical Technology, Mandalay, Myanmar.
[2] Medical Technology Program, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand.
[3] The Centre for Research and Development of Medical Diagnostic Laboratories (CMDL), Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand.
[4] Biomedical Sciences Program, Graduate School, Khon Kaen University, Khon Kaen, Thailand.
[5] Department of Clinical Immunology and Transfusion Sciences, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand.

**KEYWORDS**
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HEK 293T cell;
Protein expression;
Protein purification;
Anti-DYKDDDDK magnetic agarose method.

**ABSTRACT**

Anti-human leukocyte antigen (HLA) antibodies are a risk factor for graft failure and graft loss in organ transplantation. To develop a new biomolecular technique for anti-HLA antibody detection against HLA antigens, a large quantity of HLA proteins is necessary. The optimization of existing protein purification protocols plays a crucial role in laboratory practice in biomolecular technology. In this work, we presented the efficient expression of recombinant HLA-A and -B proteins in the HEK 293T cell line and an improved protocol of the PierceTM anti-DYKDDDDK magnetic agarose method for HLA protein purification to enhance the quantity of proteins. The percent recoveries of HLA-A and -B proteins were 81.15% and 80.73% at 350–380 µg/mL in the total eluates, respectively. The number of elution times and incubation time in the elution step were the most important to enhance the percent recovery and quantity of purified proteins. In conclusion, the improved purification protocol enables the high-yield production of functional recombinant HLA-A and -B proteins for the establishment of an ion-sensitive field-effect transistor-based immunosensor for anti-HLA antibody detection. Moreover, it could provide a useful guideline for the expression and purification of other DYKDDDDK-tagged recombinant proteins.

***Corresponding author:** Chanvit Leelayuwat, MT, PhD. Department of Clinical Immunology and Transfusion Sciences, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand. Email address: chanvit@kku.ac.th
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## Introduction

The human leukocyte antigen (HLA) system is a cluster of related proteins that are encoded by the major histocompatibility complex (MHC) in humans. The MHC genes are divided into three subgroups: MHC class I, MHC class II, and central MHC or class III according to their locations on the human chromosome[1]. Among them, class I and class II genes are highly polymorphic and directly participated in antigen presentation. The HLA class I molecules: HLA-A, -B, and -C are expressed on the surface of a wide variety of nucleated cells with varying degrees of specificity. They are composed of three extremely polymorphic extracellular domains (α 1-3) and a transmembrane heavy chain associated with a light chain, β2-microglobulin (ß2-m). These cell-surface proteins are immunological barriers to organ and stem cell transplantations[2]. Antibody-mediated rejection (AMR) is strongly associated with graft failure and graft loss in renal transplantation and is caused by pre- and post-transplant HLA antibodies[3-5]. Therefore, detection and analysis of HLA-specific antibodies have become pivotal tasks in every clinical setting.

To set up a new biomolecular technique for anti-HLA antibody detection, a large quantity of HLA proteins is required. Moreover, the cost of commercial HLA proteins remains extremely high with their limited quantity, which is a major issue. Nowadays, many innovative technologies and high-quality protein purification kits manufactured by various suppliers have been introduced[6-8]. Among them, magnetic particle-based methods are frequently used in a wide range of biological applications due to their strong paramagnetic talent, low cost, simplified procedures, and short operating time. Moreover, multiple centrifugation steps are not required in the purification of the affinity-tagged protein utilizing magnetic nanoparticles[9-11].

Herein, the transfection efficiency of the lipofection method was systematically optimized by inserting the green fluorescent protein (GFP) plasmid into the HEK 293T cell line and determined by flow cytometry. In addition, we demonstrated an improved purification protocol that produced high yields of purified recombinant HLA-A*01:01 and -B*07:02 proteins using the commercial Pierce™ anti-DYKDDDDK magnetic agarose kit.

## Materials and methods

***Optimization of transfection efficiency of HLA-A and HLA-B plasmids***

The optimization of transfection conditions was conducted using the plasmid encoding green fluorescence protein (GFP plasmid) according to the optimization protocol provided by the manufacturer of X-treme Gene HP DNA transfection reagent[12]. A constant plasmid DNA concentration was incubated with various amounts of transfection reagent. In this approach, a monolayer of 50,000 cells per well of HEK 293T cells with 70–90% confluency in a 24-well plate is transfected with 1 µg of DNA per well in varying amounts of transfection reagent (1 µL, 2 µL, 3 µL and 4 µL) at the 1:1, 2:1, 3:1, and 4:1 ratios of microliter (µL) transfection reagent to microgram (µg) of plasmid DNA.

The transfection efficiency of the GFP plasmid was evaluated by a flow cytometric method in which, a standard GFP-encoding plasmid was parallelly transfected alongside the HLA-A and -B plasmids. The ratio between the plasmid amount and the transfection reagent was varied to obtain the optimal ratio. After the transfection, the cells were incubated for 48 hours at 37°C in a 5% CO2 incubator allowing the green fluorescent protein expression. Following this incubation period, transfected cells were gently detached from the culture plate by repeated pipetting. A wash step was carried out, followed by centrifugation at 2,000 rpm for 5 mins. The cells were resuspended in the PBS buffer to obtain a single cell suspension.

The HEK 293T cells that had successfully taken up the GFP plasmid DNA and encoded it intracellularly demonstrated vivid green

fluorescence upon exposure to an excitation wavelength of approximately 488 nanometers (nm) measured by flow cytometer. To establish the threshold for distinguishing between GFP-positive and GFP-negative cells, we utilized untransfected HEK 293T cells as a reference.

***Expression of HLA-A and HLA-B plasmids***

HLA-A and -B plasmid DNA clones carry the cDNA sequences of HLA-A*01:01 (GenBank accession no. NM001242758) and HLA-B*07:01 (GenBank accession no. NM005514) in the pcDNA3.1+/C-(K)-DYK vector (GenEZ™ ORF clones) (Supplementary Figures S1A and S1B), which were expressed in HEK 293T cells as a tagged protein with a C-terminal DYKDDDDK tag. The transfection reagent, X-treme Gene HP DNA transfection reagent, and the optimized transfection protocol were used to transfect HLA-A and HLA-B-inserted plasmids into HEK 293T cells. The pcDNA3.1+/C-(K)-DYK without target HLA-A and -B cDNA was transfected into the cells as the MOCK control. The final volume of 100 µL of transfection mixture, containing 2 µL of the transfection reagent and 1 µg of each plasmid DNA and Dulbecco's Modified Eagle Medium (DMEM) media, was incubated for 15 min at room temperature and then added to HEK 293T cells ($3.5 \times 10^5$ cells per well in a 6-well plate). For the large-scale production, five petri dishes were used for each experiment, and the final volume of 1000 µL of transfection mixture containing 18 µL of the transfection reagent, 12 µg of each plasmid DNA, and DMEM media was incubated for 15 min at room temperature. After that, transfection mixtures were plated onto HEK 293T cells ($2.5 \times 10^6$ cells per petri dish), and cell culture plates were incubated at 37°C with 5% $CO_2$ for 72 h.

***Optimization of DYKDDDDK-tagged HLA-A and -B proteins purification***

The transfected HEK 293T cells were collected and lysed by ice-cold Pierce IP lysis buffer with protease enzyme buffer. Then, the lysate was centrifuged at 13,000 g for 10 min at 4°C. The supernatant was transferred to a new tube as crude proteins and Pierce™ Anti-DYKDDDDK Magnetic Agarose Kit was used to purify the DYKDDDDK-tagged HLA-A and -B proteins in the crude lysate. All purification steps, including binding the DYKDDDDK-tagged HLA proteins onto the magnetic beads, 3 times washing the beads with DYKDDDDK-tagged target proteins, and 3 times of elution steps for eluting the target protein from the beads were done to begin with the manufacturer's protocol (SOP1)[13]. Then, SOP1 was systemically optimized in each step of purification process because the C-terminal DYKDDDDK-tagged HLA-A and -B proteins were not successfully purified using SOP1.

Firstly, in SOP2 and 3, 100 µL of magnetic agarose slurry and 500 µL of binding buffer, pH 7.2 were added into a Eppendorf Protein LoBind®, 1.5 mL tubes and mixed well. The tube was placed into a magnetic stand to collect the beads against the side of the tube, and then the supernatant was discarded. This wash was repeated a total of 3 times. After that, 700 µL of lysate containing DYKDDDDK-tagged protein was added to the pre-washed magnetic agarose and inverted to mix, and the samples were incubated with mixing for 45 min at room temperature. The beads were collected using a magnetic stand, and the supernatant was discarded. The flow-through fraction was saved for subsequent downstream analysis. The beads with DYKDDDDK-tagged target proteins were washed twice with 500 µL of 10 mM PBS (pH 7.2) containing protease inhibitor and once with 500 µL of deionized water. A magnetic stand was used to collect the beads, and the supernatant was removed. Finally, the DYKDDDDK-tagged recombinant proteins were eluted from the beads by the acid elution method. 100 µL of IgG elution buffer, pH 2.8, with protease inhibitor was added to the tube, mixed well, and incubated for 5 min in SOP2 and for 12–15 min in SOP3 at room temperature with frequent vortexing. After incubation, the magnetic agarose was collected with a magnetic stand, and then

the supernatant that contained the eluted target protein was removed and kept in 1.5 mL microtubes. To neutralize the low pH, 15 µL of neutralization buffer (1 M Tris, pH 8.5) was added to the 100 µL of eluate immediately. The elution step was done three times for complete recovery of the abundant target. The process of optimizing the protocols, SOP1, 2 and 3 is illustrated in figure 1 and shown in supplementary table S1. The optimized protocol of SOP3 is described in supplementary.

The purified protein samples were aliquoted into small portions in Eppendorf Protein LoBind®, 1.5 mL tubes for further study. The concentration of all fractions including purified target proteins was analyzed by the Pierce™ BCA Assay. In addition, the percent recoveries for purified target proteins in total eluates were calculated by dividing the amount of purified target protein recovered (µg) by the amount of total crude proteins added and multiplying the results by 100.

***Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and western blot***

The purified protein abilities were detected using DYKDDDDK-tagged antibody (HRP) (Genscript), HLA-A and HLA-B purified MaxPab mouse polyclonal antibodies (Abnova) with the western blotting method. The working concentrations of crude and purified proteins were 10 µg/mL and 1 µg/mL, respectively, according to the manufacturer's protocol[14]. The proteins separated on a 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis at 150 volts for 90 mins were electronically transferred onto polyvinylidene fluoride membranes (PVDF). The PVDF membranes were blocked with 5% skim milk in 0.1% TBS-T at RT for 1 h and then incubated with 0.5 µg/mL of anti-DYKDDDDK tag antibody (HRP) or anti-HLA-A and -B at 4 °C for 18 h. After incubation, the membranes were treated with 0.1% TBS-T for 3 times and probed with anti-HLA-A and -B were incubated with HRP-conjugated goat anti-mouse secondary antibodies (Biolegend) at RT for 1 h. The bands of protein were detected with the enhanced luminol-based chemiluminescent (ECL) western blotting substrate kit (Amersham™, GE Healthcare UK) according to the manufacturer's instructions and finally imaged with the chemiluminescent imaging system (Amersham Imager 600, Cytiva, USA). The outline of the designed experiment for three different protocols of protein purification is summarized in figure 2.

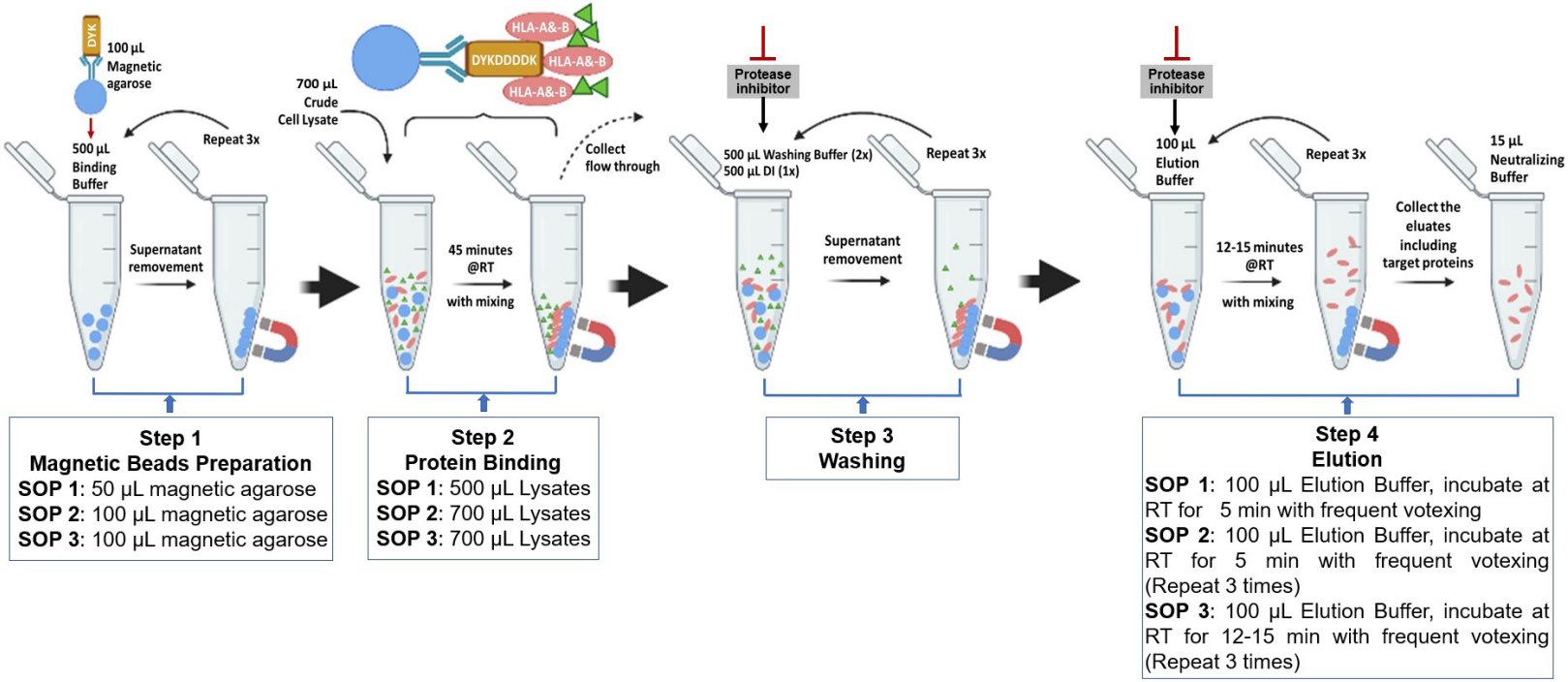


**Figure 1** Schematic diagram of DYKDDDDK-tagged HLA-A and -B proteins purification protocol including the optimization steps. Three standard operating protocols were implemented as SOP1, SOP2, and SOP3. In the boxes for each step, the optimized factors for optimized protocols were shown.

***Statistical Analysis***

All experiments were carried out in triplicate if not stated otherwise in the figures. The purified protein concentrations in eluates were shown as the mean ± SD of three independent experiments. The statistical analysis was performed by using SPSS Statistics for Windows, version 19.0 (SPSS, Inc., Chicago, IL, USA). The distribution of the collected data in this study was tested by the Shapiro-Wilk test. Statistical significance was conventionally accepted when $p$-value < 0.05. One-way ANOVA with Tukey's test or independent student t-test (with normal distribution) was analyzed for eluate 1 (E1) and 2 (E2), respectively.

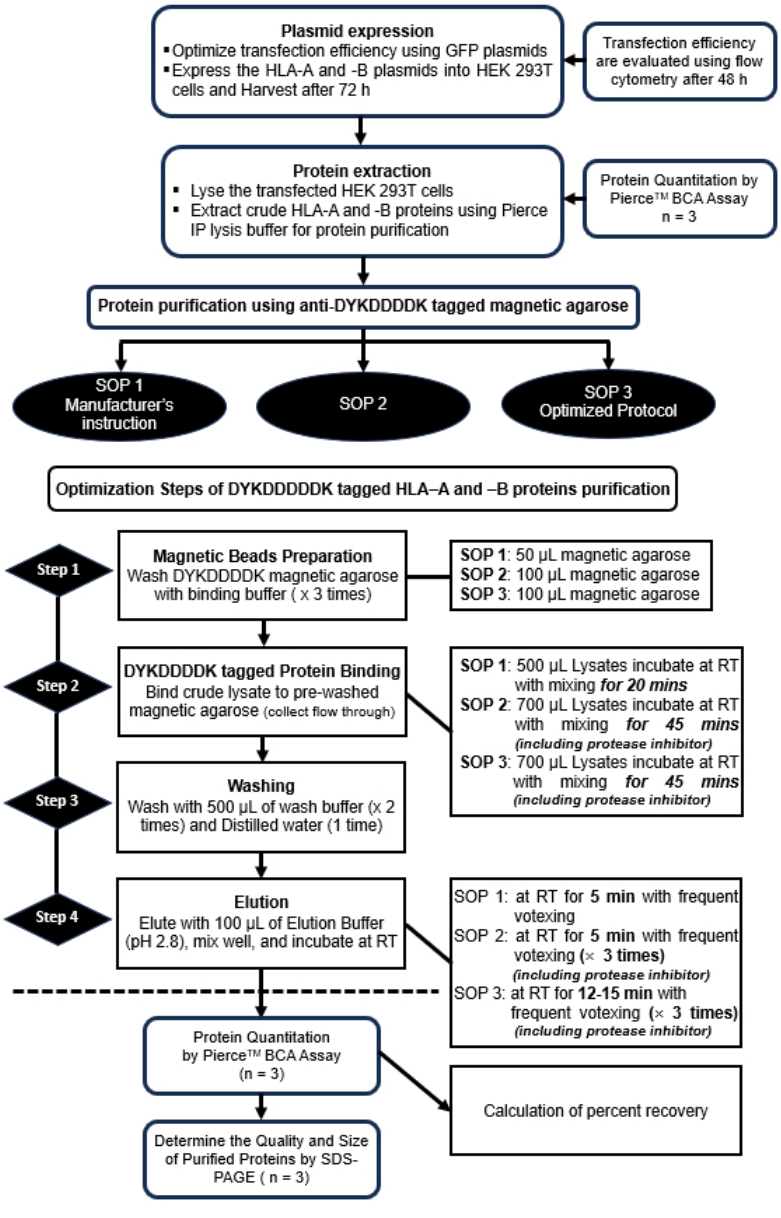


**Figure 2** A workflow overview of three DYKDDDDK-tagged HLA-A and -B proteins purification protocols by Pierce™ anti-DYKDDDDK magnetic agarose kit.

**Note:** The optimized factors in each step for optimized protocols were shown; n indicates the number of technical triplicates used in this study.

## Results

### *Optimization of transfection efficiency*

To optimize the transfection efficiency, HEK 293T cells were transfected with 1 µg of GFP plasmid DNA per well with varying amounts of transfection reagent. The cells were collected 24 h after transfection, and the transfection efficiencies were evaluated by the flow cytometric method. The transfection efficiencies were 95.9%, 97.5%, 98.1%, and 97.2% at the different ratios of transfection reagent and plasmid, 1:1, 2:1, 3:1, and 4:1, respectively (Figure 3). The highest transfection efficiency was determined to be 98.1% at a 3:1 ratio of transfection reagent to GFP plasmid. This ratio was consistent with the recommended ratio by Roche Diagnostics GmbH, Germany[12]. It also had a similar efficiency rate of 97.5% at a 2:1 ratio of transfection reagent to GFP plasmid.

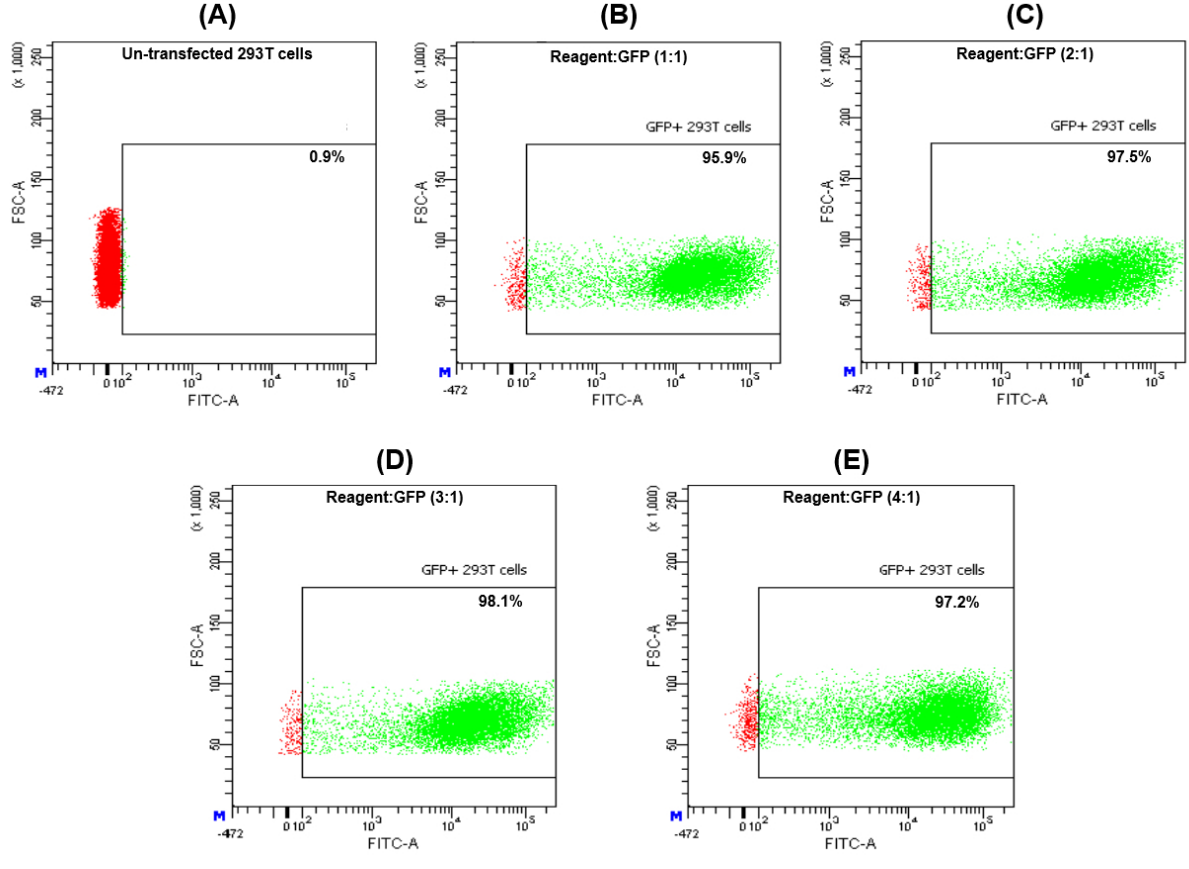


**Figure 3** The optimization of transfection efficiency by using GFP plasmid DNA by flow cytometry (Scatter plots represented the flow cytometry analysis of transfectants) and HLA-A plasmid DNA by SDS-PAGE. (A) un-transfected HEK 293T as a negative control; the transfection regent and GFP plasmid ratios of 1:1, 2:1, 3:1, and 4:1 in (B), (C), (D), and (E), respectively.

### *Purified HLA-A and -B proteins concentration and percent recovery*

The optimal conditions for protein expression and purification were used to produce large amounts of protein. The concentrations of HLA-A and -B proteins were very low in the eluates, 18 ± 11.96 µg/mL and 12 ± 8.32 µg/mL, respectively using SOP1 (Supplementary Table S2).

Then, both HLA-A and HLA-B proteins were produced, and purification was carried out using

an optimized protocol (SOP2). As shown in figure 4A and 4B, SOP2 considerably enhanced the quantities of isolated HLA-A and HLA-B proteins in E1 when compared to SOP1 ($p$-value = 0.017 for HLA-A and $p$-value = 0.008 for HLA-B).

Finally, the number of elution times and incubation times in the elution step condition were increased, and SOP3 was established to increase target protein yield. We found an average increase in purified protein yield in E1 and E2 of between 2- and 3-fold using SOP3 when compared with SOP2 (Supplementary Table S2). The statistical analysis indicated no significant difference in purified protein concentration in E1 between SOP2 and 3 of HLA-A ($p$-value = 0.335) and HLA-B ($p$-value = 0.244) (Figure 4A and 4B). Despite this, the protein concentration in E1 as determined by SOP1 and SOP3 showed highly significant differences, with $p$-value = 0.004 for HLA-A and $p$-value = 0.002 for HLA-B. Similarly, for purified HLA-A ($p$-value < 0.001) and HLA-B ($p$-value = 0.001), very significant differences in E2 were observed between SOP1 and SOP3 (Figure 4A and 4B).

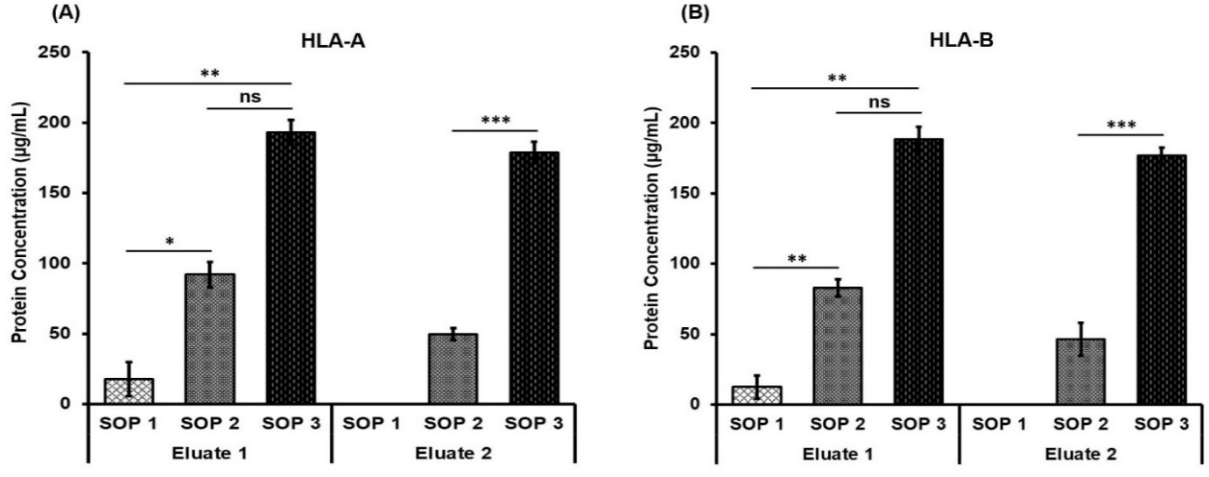


**Figure 4** Comparison of purified protein concentrations amongst different SOPs.
**Note:** (A) Comparison of SOP1, SOP2, and SOP3 for purified HLA-A protein concentration in eluates 1 and 2. (B) Comparison of SOP1, SOP2, and SOP3 for purified HLA-B protein concentration in eluates 1 and 2. Each dataset illustrated the mean and standard deviation obtained from three independent experimental triplicates (ns = no significant, * $p$-value < 0.05, ** $p$-value < 0.01, *** $p$-value < 0.001).

In addition, the percent recoveries for purified target proteins in total eluates were calculated by dividing the amount of purified target protein recovered (µg) by the amount of total crude proteins added and multiplying the results by 100[15]. The percent recovery of HLA-A and -B proteins in E1 purified by SOP3 is remarkably higher than those obtained by SOP1 and SOP2 (Table 1). There were no statistically significant differences in the percent recovery of E1 between SOP1 and SOP2, as well as between SOP2 and SOP3, for both purified proteins ($p$-value > 0.05). Although the percent recovery of E1 for HLA-A between SOP1 and 3 was statistically significant ($p$-value = 0.018), there was no significant difference for HLA-B ($p$-value = 0.093) (Supplementary Figure S2A). It was interesting to note that the purified HLA-A and HLA-B proteins in total eluates had percent recoveries of 81.15% and 80.73%, respectively, using SOP3 (Table 1). Moreover, the statistical analysis revealed highly significant differences in the total eluates of HLA-A ($p$-value = 0.005) and HLA-B ($p$-value = 0.001) by SOP3 compared to that of SOP2 (Supplementary Figure S2B).

**Table 1** Percent recoveries of purified HLA-A and -B proteins using different SOPs

| Proteins amount in Total Eluates | HLA-A (% Recovery) | | | | HLA-B (% Recovery) | | | |
|---|---|---|---|---|---|---|---|---|
| | SOP1 | SOP2 | SOP3 | *p*-value | SOP1 | SOP2 | SOP3 | *p*-value |
| E1 [C (µg)] | 13.48 ± 9.14 | 21.88 ± 5.98 | 42.18 ± 2.99 | 0.018 | 15.11 ± 12.3 | 24.13 ± 3.40 | 41.72 ± 4.57 | 0.093 |
| E1+E2 [C (µg)] | ND* | 33.71 ± 9.52 | 81.15 ± 3.62 | 0.005 | ND* | 37.5 ± 3.95 | 80.73 ± 7.33 | 0.001 |

**Note:** ND*, not done.

***Evaluation of the quality of purified HLA-A and -B proteins by SDS-PAGE and western blot analysis***

The quality and size of purified HLA-A and -B proteins were assessed using SDS-PAGE and western blot analysis. By using SOP1, the bands were not seen in elutes even though the thick bands were observed at 52 kDa in accordance with the predicted size of the Genscript company in crude lysates and flowthrough fractions of both proteins against anti-HLA-A and -B antibodies (Figure 5A and 5B). Moreover, the thick bands were detected at 52 kDa of HLA-A and -B proteins when probed with DYKDDDDK tag antibody (HRP) (Figure 5C).

When protein purification was carried out using the optimized protocol (SOP2) (Supplementary Table S1), the more-intense single bands in E1 for both HLA proteins were observed compared to SOP1. The weak single bands were found in E2, while no band was seen in eluates 3 (E3) of SOP2 for isolated HLA-A and -B (Figure 5D and 5E).

The 2x sample buffer containing DTT was directly added to the beads to test the target proteins on the beads. The mixture was incubated for 5 min at 100 °C before being spun down. Next, the magnetic beads were collected in a magnetic stand, and the supernatants were used for the SDS-PAGE and western blotting. The target proteins were found to be thick bands in the supernatants of both proteins suggesting that they were not fully eluted from the beads (Figure 5F).

After purification using a modified elution step condition (SOP3) (Supplementary Table S1), the single thick bands were detected in E1 and E2 of both purified DYKDDDDK-tagged HLA-A and -B proteins (Figure 5G and 5H). Nonetheless, no bands were detected in E3 in all experiments using SOP2 and 3. It could be due to denaturation of the target purified proteins. These findings revealed that the first repeated elution steps were covered only for complete recovery of highly abundant targets.

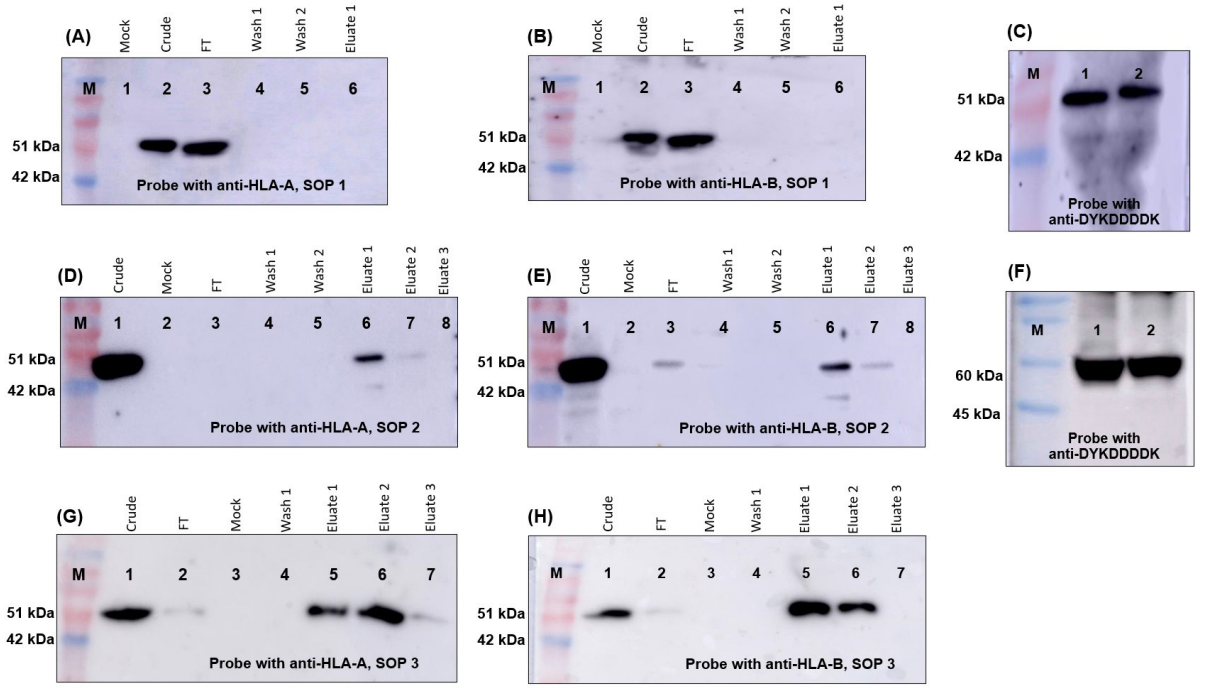


**Figure 5** The quality evaluation of purified proteins by SDS-PAGE and western blot analysis.
**Note:** (A) and (B) Purified HLA-A and -B proteins by probing with specific HLA-A and -B antibodies, respectively. (C) Crude lysates by probing with DYKDDDDK tag antibody (HRP). Lane M, Protein Marker; Lane 1, Crude lysate of HLA-A; Lane 2, Crude lysate of HLA-B. (D) and (E) Crude lysates and purified HLA-A and -B proteins by probing with specific HLA-A and -B antibodies, respectively. (F) Checking the elution steps using the sample directly removed from saved magnetic beads used for purification by probing with DYKDDDDK tag antibody (HRP). Lane M, Protein marker; Lane 1 and 2: Sample from the beads that was used for HLA-A and -B proteins purification, respectively. (G) and (H) Crude lysates and purified HLA-A and -B proteins by probing with specific HLA-A and -B antibodies, respectively.

## Discussion

Protein structural and functional information is frequently required in large quantities and at high purity for the purpose of developing a new biomolecular technique. The low efficiency levels of exogenous protein expression and purification are frequently encountered in order to obtain sufficient amounts of native protein[16]. The human embryonic kidney 293 (HEK 293) cell is one of the most commonly used mammalian cell lines in research platforms for protein production[17]. The HEK 293T cells are originated from HEK 293 cells along with the SV40 large T antigen, which can bind to SV40 enhancers of expression vectors and stably express to improve protein production[18].

In this study, GFP plasmid was transfected into the HEK 293T cell line by using the X-treme GENE HP DNA transfection reagent. The transfection efficiency was systematically optimized using GFP and evaluated by flow cytometry. Our results on GFP plasmid showed that the transfection efficiencies achieved under these optimized conditions had a high expression level. Although the highest percentage of transfection efficiency was 98.1% at a 3:1 ratio of transfection reagent to plasmid, the transfection percentage (97.5%) was similar at a 2:1 ratio of transfection reagent to plasmid. Therefore, the optimal transfection condition was determined as a 2:1 ratio of transfection reagent and plasmid in our study. Lipofection

systems utilizing the X-treme Gene HP DNA transfection reagent and the HEK 293T cell line were effective in achieving high expression of HLA-A and HLA-B genes in the pcDNA3.1+/C-(K)-DYK vector.

Another important facet and the main objective of this study is to optimize the purification method using the anti-DYKDDDDK magnetic agarose system for isolating the recombinant HLA-A and -B proteins from crude cell lysates. The development of universal purification and capture strategies for native proteins has been challenging due to the diversity of proteins and their biochemical properties[19]. The small epitope tags, such as the DYKDDDDK (FLAG), c-Myc, and HA tags, which have a short amino acid sequence attached to the N- or C-terminus of a recombinant protein, are able to strongly and precisely bind their respective immunoaffinity resins[20,21]. Magnetic resins can purify the affinity-tagged protein without multiple centrifugation steps. Moreover, magnetic nanoparticles (MNPs) can be reused due to their superparamagnetic capability; consequently, it can reduce the high cost of conventional protein purification methods such as chromatography, centrifugation, and membrane separation and their turn-around times[10].

In this work, the Pierce™ Anti-DYKDDDDK Magnetic Agarose system was used to purify the DYKDDDDK-tagged recombinant HLA-A and -B proteins. A high-affinity rat monoclonal antibody (clone L5) covalently attaches to a magnetite-embedded agarose core particle by recognizing the small amino acid sequence, DYKDDDDK. The Pierce™ Anti-DYKDDDDK Magnetic Agarose recognizes the DYKDDDDK-tagged proteins with the tag on either the N- or C-terminus[13]. At the beginning, to purify crude lysates, we applied the manufacturer's protocol, represented as SOP1. Unfortunately, the C-terminally DYKDDDDK-tagged HLA-A and -B proteins were not purified successfully. The purified protein concentrations were very low in the eluates of HLA-A and -B, and no bands were identified in the eluates in the SDS-PAGE profile. The possible cause is that all target proteins cannot bind to the magnetic beads adequately. It depends on the ratio of magnetic agarose slurry to crude lysate volume for sufficient binding of antigen and antibody. In addition, the target proteins did not elute from the beads. Therefore, SOP2 was established, which significantly boosted the concentrations of purified proteins in total eluates for HLA-A and -B. These concentrations were not enough for use in further studies. In addition, SDS-PAGE and western blot analysis revealed an indistinct band in E2 and a blank in E3. In this condition, the DYKDDDDK-tagged proteins could not be eluted from the beads, although most of the target proteins were bound to the magnetic beads. In this case, the target proteins were found to be thick bands in the supernatants of both proteins. According to this finding, most of the target proteins could bind to the magnetic beads. We identified two potential causes for these issues. In this work, the acid elution protocol was applied using a commercial IgG elution buffer at pH 2.8. The first reason was that the elution conditions were too mild, and the second reason was that purified proteins in those eluates were degraded because a strong acid buffer was used in the elution step even though we added the neutralizing buffer, 1 M Tris, at pH 8.5 immediately. Therefore, the elution condition was optimized to a more stringent method in which the incubation time of the elution step was increased to 12–15 min, ensuring that 15 min was not exceeded to avoid the captured antibodies leaching from the beads. Moreover, the protease inhibitor was also added to the washing buffer and elution buffer to prevent the degradation of target proteins. The repeated elution step was performed three times as SOP2 to obtain the highly purified protein yield. The pH of a protein solution influences the success of protein crystallization[22]. In addition, in order to maximize stability, the pH of the storage buffer should be at least one unit away from the pI values of the target protein[23]. In this study, the pI values of the produced DYKDDDDK-tagged HLA-A

and -B proteins were 6.09 and 5.57, respectively. Therefore, the pH of the elution buffer was adjusted by adding neutralizing buffer at 7.2. Finally, we could successfully set up a reliable and functionally optimized protocol, represented as SOP3, for the purification of the DYKDDDDK-tagged HLA-A and -B proteins with a high yield concentration (170–200 µg/mL).

Furthermore, the overall percentage recovery of purified HLA-A (81.15%) and -B proteins (80.73%) in total eluates obtained by SOP3 was significantly higher than those obtained by SOP1 and 2. Therefore, this study improved the effectiveness of the protocol for recombinant HLA protein purification by using an anti-DYKDDDDK magnetic agarose system.

There is no study of the purification method of DYKDDDDK-tagged recombinant HLA-A and -B proteins using the commercial kit, the Pierce™ anti-DYKDDDDK magnetic agarose system in the laboratory practice. Although the selected purification kit was used according to its manual, DYKDDDDK-tagged recombinant HLA-A and -B proteins were not successfully purified. In practice, we encountered many factors that influenced the purification of DYKDDDDK-tagged recombinant HLA-A and -B proteins using the Pierce™ anti-DYKDDDDK magnetic agarose method. We verified the crucial factors in each step of protein purification, including the magnetic agarose slurry volume (100 µL), crude lysate volume (700 µL), the incubation time for DYKDDDDK-tagged protein binding to pre-washed magnetic agarose beads (45 min), the elution step (3 times), increased the incubation time in the elution step (12–15 min) and added the protease inhibitor to the washing buffer and elution buffer.

In this study, the potential of the Pierce™ anti-DYKDDDDK magnetic agarose kit with an improved and reliable protocol was evaluated to purify the DYKDDDDK-tagged recombinant HLA-A and -B proteins. The purified HLA-A and -B proteins obtained using this improved, reliable protocol have been successfully applied in the establishment of an ion-sensitive field-effect transistor-based immunosensor for the detection of relevant anti-HLA antibodies, especially in the field of kidney transplantation[24]. Furthermore, this improved protocol would become an important tool that could provide a valuable guideline for the production and purification of other DYKDDDDK-tagged recombinant proteins.

## Conclusion

For protein production, the X-treme GENE HP DNA transfection reagent was used to transfect HEK 293T cells with HLA-A and HLA-B inserted pcDNA3.1+/C-(K)-DYK plasmids. The transfection conditions were optimized using the plasmid encoding green fluorescent protein, reporting on conditions that would ensure optimal expression. In this study, we explored and evaluated the critical factors at each step of the purification process. In summary, an improved and functional protocol of the Pierce™ Anti-DYKDDDDK Magnetic Agarose system was introduced in the DYKDDDDK-tagged recombinant HLA-A and -B protein purification, increasing the percent recovery of the purified high yield proteins that can be used *in vitro* studies.

## Take home messages

An improved and functional protocol of the Pierce™ Anti-DYKDDDDK Magnetic Agarose system was introduced in the DYKDDDDK-tagged recombinant HLA-A and -B protein purification, increasing the percent recovery of the purified high yield proteins that could provide a useful guideline for the purification of other DYKDDDK-tagged recombinant proteins.

## Conflicts of interest

The authors declare no conflict of interest.

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## Supplementary

**Table S** Comparison of concentrations of HLA-A and -B proteins between SOP 1, 2 and 3

| Steps of purification | HLA-A (µg/mL) | | | HLA-B (µg/mL) | | |
|---|---|---|---|---|---|---|
| | SOP 1 | SOP 2 | SOP 3 | SOP 1 | SOP 2 | SOP 3 |
| Crude lysate | 2014 ± 46.30 | 2058 ± 76.38 | 2051 ± 37.61 | 1931 ± 43.45 | 1949 ± 112.11 | 1965 ± 34.30 |
| Flow through | 1676 ± 52.50 | 1481 ± 17.32 | 1436 ± 36.31 | 1720 ± 40.65 | 1459 ± 42.72 | 1351 ± 40.23 |
| Wash fraction | 103 ± 13.92 | 104 ± 20.05 | 112 ± 12.99 | 95 ± 13.92 | 113 ± 20.46 | 104 ± 12.33 |
| Wash fraction | 30 ± 10.25 | 36 ± 26.73 | 45 ± 13.23 | 24 ± 10.41 | 30 ± 15.21 | 57 ± 14.08 |
| Eluate 1 | 18 ± 11.96 | 92 ± 9.01 | 193 ± 8.45 | 12 ± 8.32 | 83 ± 6.29 | 189 ± 8.73 |
| Eluate 2 | ND* | 50 ± 4.33 | 179 ± 7.52 | ND* | 46 ± 11.81 | 177 ± 5.94 |
| Eluate 3 | ND* | 35 ± 2.89 | 49 ± 10.90 | ND* | 31 ± 6.29 | 49 ± 4.33 |

**Note:** ND* = Not Done

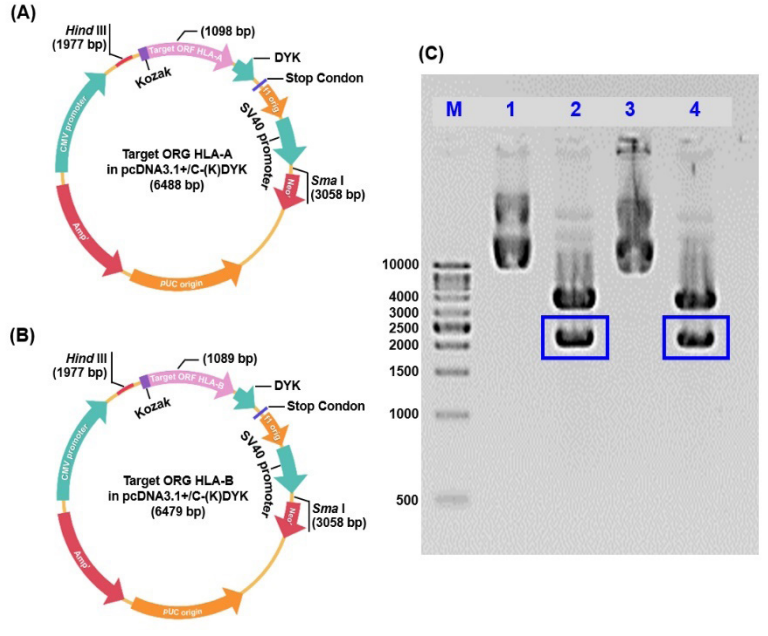


**Figure S1** The illustration of HLA-A and -B plasmids and the restriction enzyme analysis.
**Note:** (A and B) Schematic representation of the pcDNA3.1$^+$/C-(K)-DYK vector map with target ORF HLA-A with (6488 bp) and -B gene (6479 bp). (C) Restriction digestion of HLA-A and -B/pcDNA3.1$^+$/C-(K)-DYK with *Hind*III and *Sma*I. Lane M: DNA Ladder. Lane 1 and 3: The undigested clones, HLA-A and -B DNA clones showing the bands of 6488 bp and 6479 bp represent the full size of plasmid, respectively. Lane 2 and 4: After digestion with *Hind*III and *Sma*I, the bands at 4400 and 2000 bp in the blue boxes indicate the inserted target HLA-A and -B genes.

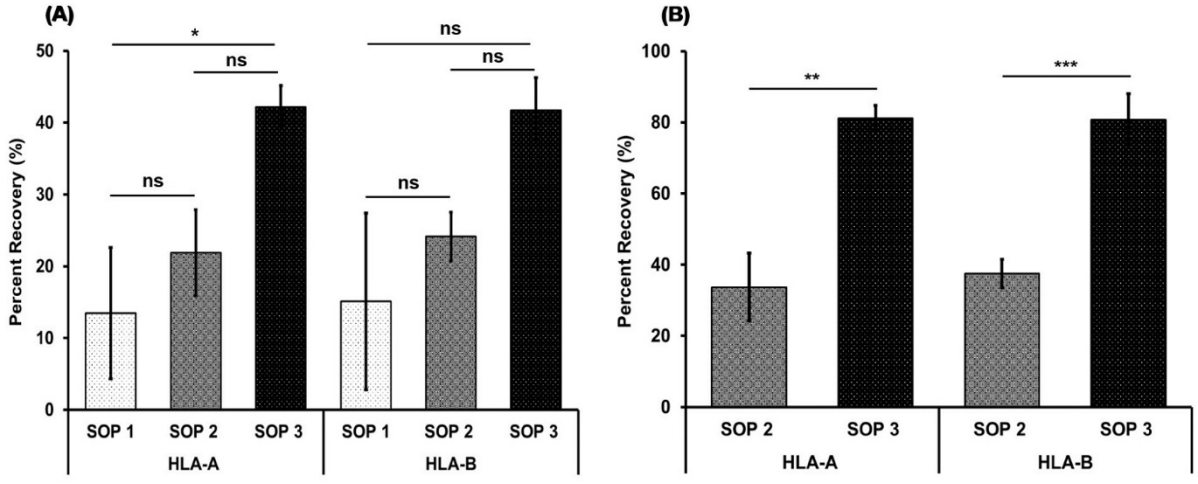


**Figure S2** The percent recovery of each SOPs for purified HLA-A and -B proteins.

**Note**: (A) The percent recovery of purified HLA-A and -B proteins in eluate 1 by SOP 1, 2 and 3. (B) The percent recovery of purified HLA-A and -B proteins in total eluates (Eluates 1 and 2) by SOP 2 and 3. Each dataset illustrated the mean and standard deviation obtained from three independent experimental triplicates. (ns = no significant, * $p$-value < 0.05, ** $p$-value < 0.01, *** $p$-value < 0.001).

## 1. HLA-A and HLA-B cDNA ORF clones

**HLA-A cDNA ORF clones (Product ID-OHu29153)**
**(NCBI Ref.Seq: NP_001229687.1) _ HLA-A*01:01**

MAVMAPRTLLLLLSGALALTQTWAGSHSMRYFFTSVSRPGRGEPRFIAVGYVDDTQFVRFDSDAASQKMEPRAPWIEQEG
PEYWDQETRNMKAHSQTDRANLGTLRGYYNQSEDGSHTIQIMYGCDVGPDGRFLRGYRQDAYDGKDYIALNEDLR
SWTAADMAAQITKRKWEAVHAAEQRRVYLEGRCVDGLRRYLENGKETLQRTDPPKTHMTHHPISDHEATLRCWALG
FYPAEITLTWQRDGEDQTQDTELVETRPAGDGTFQKWAAVVVPSGEEQRYTCHVQHEGLPKPLTLRWELSSQPTIPIVGI
IAGLVLLGAVITGAVVAAVMWRRKSSDRKGGSYTQAASSDSAQGSDVSLTACKV

**HLA-B cDNA ORF clones (Product ID-OHu26926D)**
**(NCBI Ref.Seq: NM_005514.6) _ HLA-B*07:02**

MLVMAPRTVLLLLSAALALTETWAGSHSMRYFYTSVSRPGRGEPRFISVGYVDDTQFVRFDSDAASPREEPRAPWIEQEG
PEYWDRNTQIYKAQAQTDRESLRNLRGYYNQSEAGSHTLQSMYGCDVGPDGRLLRGHDQYAYDGKDYIALNEDLR
SWTAADTAAQITQRKWEAAREAEQRRAYLEGECVEWLRRYLENGKDKLERADPPKTHVTHHPISDHEATLRCWALG
FYPAEITLTWQRDGEDQTQDTELVETRPAGDRTFQKWAAVVVPSGEEQRYTCHVQHEGLPKPLTLRWEPSSQSTVPIV
GIVAGLAVLAVVVIGAVVAAVMCRRKSSGGKGGSYSQAACSDSAQGSDVSLTA