Supplementary information

Title: Development Of The Epitope-Derived Peptide As Alternative Detection Element For Rapid Fluorescent Diagnostic Assay To Detect Avian Influenza Virus

Duong Tuan Bao[‡1], Do Thi Hoang Kim[‡1], Hyun Park[‡1], Bui Thi Cuc[1], Nguyen Minh Ngoc[1], Nguyen Thi Phuong Linh[1], Nguyen Chien Huu[1], Trinh Thi Thuy Tien[1], Nguyen Thi Viet Anh[1], Tung Dao Duy[1], Chom-Kyu Chong[2], Seung-Taek Yu[3], Do-Young Choi[3], Seon-Ju Yeo[§1]

Supplementary Method and Result

I. Expression of recombinant antigens in *E.coli*

II. Optimization of moue IgG (mIgG) for co-conjugation with peptide on Eu NP

III. Experimentally measured values of peptide Kd (the equilibrium dissociation constant)

IV. Sandwich FLISA assay as peptide-linked immunoassay using 96-well plate for reliability of FICT

Supplementary Figures

I. Expression of recombinant antigens in *E. coli*

Polymerase chain reaction (PCR) amplification was performed as follows: 5 min at 94 °C, 30 cycles of 30 sec at 95 °C, 60 sec at 63 °C, 90 sec at 72°C, and 10 min at 72 °C, for H5 HA1; 5 min at 95 °C, 30 cycles of 30 sec at 94 °C, 30 sec at 64 °C, 45 sec at 72 °C, and 10 min at 72 °C, for H5 HA2 and H7 HA1. *E. coli* BL21 (DE3) bacteria were transformed with the plasmids and selected on ampicillin plates for antigen expression. Transformed bacteria were seeded in 10 mL Luria-Bertani (LB) broth overnight and transferred to 250 mL fresh LB medium. The bacteria were continuously grown at 37 °C under 250 rpm agitation until the optical density at 600 nm (OD_{600}) reached 0.58. Then, induction was performed with the addition of 0.5 M isopropyl β-D-1-thiogalactopyranoside (IPTG). After a 3 h induction, the bacterial pellet from a 10 mL sample was resuspended in 100 μL binding buffer (10 mM imidazole, 300 mM NaCl, 20mM Tris-HCl, pH 7.9), sonicated, and treated with a buffer (10mM imidazole, 300 mM NaCl, 20 mM Tris-HCl, 8M urea, pH 7.9) to dissolve the inclusion bodies. After centrifugation at 12,000 rpm for 30 min the presence of the dissolved antigen in the supernatant was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Refolding was performed in freshly prepared refolding buffer (100 mM Tris-HCl, pH 7.5, 0.5 M L-arginine, 0.2 mM EDTA) at 10 ^{o}C for 24 h and a second buffer (20 mM PBS pH 7.5, 300 mM NaCl, 10 mM Imidazole, 100 mM Urea) for another 24 h. The protein solution was concentrated with Centricon filter unit (**Figure S1**).

II. Optimization of moue IgG (mIgG) for co-conjugation with peptide on Eu NP

For co-conjugation of mIgG, 10 µL Eu NP (0.2 µm, 1% w/t) was added to 500 µL 0.1 M Tris-HCl (pH 7.0) and incubated for 1 h at 25 ºC in the presence of 0.13 mM EDC and 10 mM Sulfo-NHS. The EDC and Sulfo-NHS surplus was removed by centrifugation at 27,237 × g for 5 min. The activated Eu NP was then mixed with 30 µL of 10 µM peptide in 500 µL 0.1 M sodium phosphate (pH 8.0) and allowed to react for 1 h at 30 ºC and differential mIgG concentration was added for 1 hr at 30 ºC. After centrifugation at 27,237 × g for 5 min, the conjugate was collected, washed with 2 mM phosphate-buffered saline (PBS) (pH 8.0), resuspended in 100 µL of storage buffer (1% bovine serum albumin (BSA) in PBS) and stored at 4ºC.

Figure S2A showed the 2 pmole of mIgG did not induce the non-specific reaction at test line but it showed CL values. Therefore, effect of 2 pmole of mIgG on P2 conjugate was evaluated. P2 conjugate without mIgG showed 10 HAU/mL as the detection limit (**Figure S2B**) and co-conjugation with mIgG had 20 HAU/mL as detection limit (**Figure S2C**).

III. Experimentally measured values of peptide K_d (the equilibrium dissociation constant)

The antibody (1C5) (10 μg/mL) was coated on 96-well plate at 4 ºC for 12 h and 5% BSA was used as a blocking agent at 37 ºC for 2 h to prevent non-specific reaction. After washing the plate with PBST (0.1%), 1280 HAU/mL of H5N3 virus and negative antigen (the protein concentrated from egg allantoic cavity) were allowed to bind to the antibody at 37 ºC for 2 h. After washing the plate, the serially diluted different conjugates (Eu NP-conjugated peptides) as ligands were reacted with virus at 37 ºC for 1 h. After washing, fluorescence was measured using an Infinite F200 microplate reader with filter set of 355 nm/612nm (excitation/emission wavelength) (TECAN, Männedorf, Switzerland). Data (n = 3) are shown as mean ± SD. K_d was determined with GraphPad Prism 5.0 software. **Figure S3** indicates the saturation binding curves indicating the binding affinity for each peptide. The K_ds of P0, P1, P2, and P3 were 180.5 ± 20.66, 206.2 ± 21.52, 178.1 ± 41.66, and 446.7 ± 51.01 nM, respectively.

IV. Sandwich FLISA assay as peptide-linked immunoassay using 96-well plate for reliability of FICT assay

Antibody (1C5) (10 μg/mL) was coated on 96-well plate at 4 ºC for 12 h and 5% BSA blocked the plate at 37 ºC for 2 h to prevent non-specific reaction. After washing the plate with PBST (0.1%), the serially diluted viruses were allowed to bind to antibody at 37 ºC for 2 h. After washing the plate, 36 nM of two different conjugates (Eu NP-conjugated 3F11 or P2) were reacted with antigen at 37 ºC for 1 h. After washing, fluorescence was measured using an Infinite F200 microplate reader with filter set of 355 nm/612nm (excitation/emission wavelength) (TECAN, Männedorf, Switzerland). **Figure S8A** displays the overview of step-by-step of sandwich peptide-linked immunoassay. The lowest detectable virus titer was 20 HAU/mL by 3F11 conjugate (**Figure S8B**) and 80 HAU/mL by P2 peptide conjugate (**Figure S8C**), indicating that the performance of P2 peptide was at least 4-fold lower than 3F11 antibody in this assay.

Table S1. Primers for the generation of recombinant antigen

Target	Primers
rHA1 of H5N3	FW: 5'- GGATCCGGAAATGGCACACAATGGAAAGCT-3'
	REV: 5'-GCGGCCGCAGAGCCTCCACCCCCGTTTATTGCACCCATTGGAGTTT-3'
rHA2 of H5N3	FW: 5'- GGATCCGGAAATGGCACACAATGGAAAGCT-3'
	REV: 5'-GCGGCCGCAGAGCCTCCACCCCCGTTTATTGCACCCATTGGAGTTT-3'
rHA1 of H7N1	FW: 5'-AGGGATCCAGGAGARGTTTGTTATCCT-3'
	REV: 5'-GCGGCCGCAACCTGTACTCCACTCTGAAT-3'

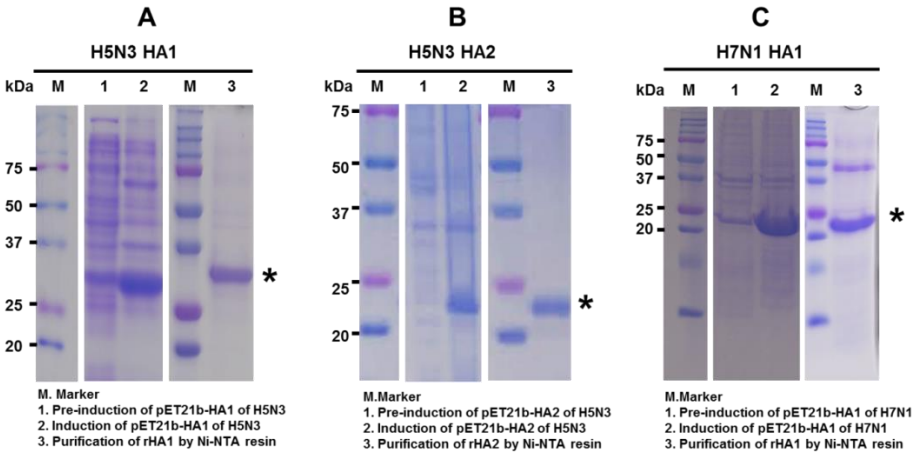


Figure S1. SDS-PAGE analysis of recombinant antigens. After induction with IPTG, antigens were expressed in *E. coli*. H5N3 rHA1 (30 kDa) (A), H5N3 rHA2 (22 kDa) (B), and H7N1 (22 kD) were purified under denaturing conditions, refolded, and purified using a Ni-NTA resin. Asterisks indicate the induced bands corresponding to the antigens.

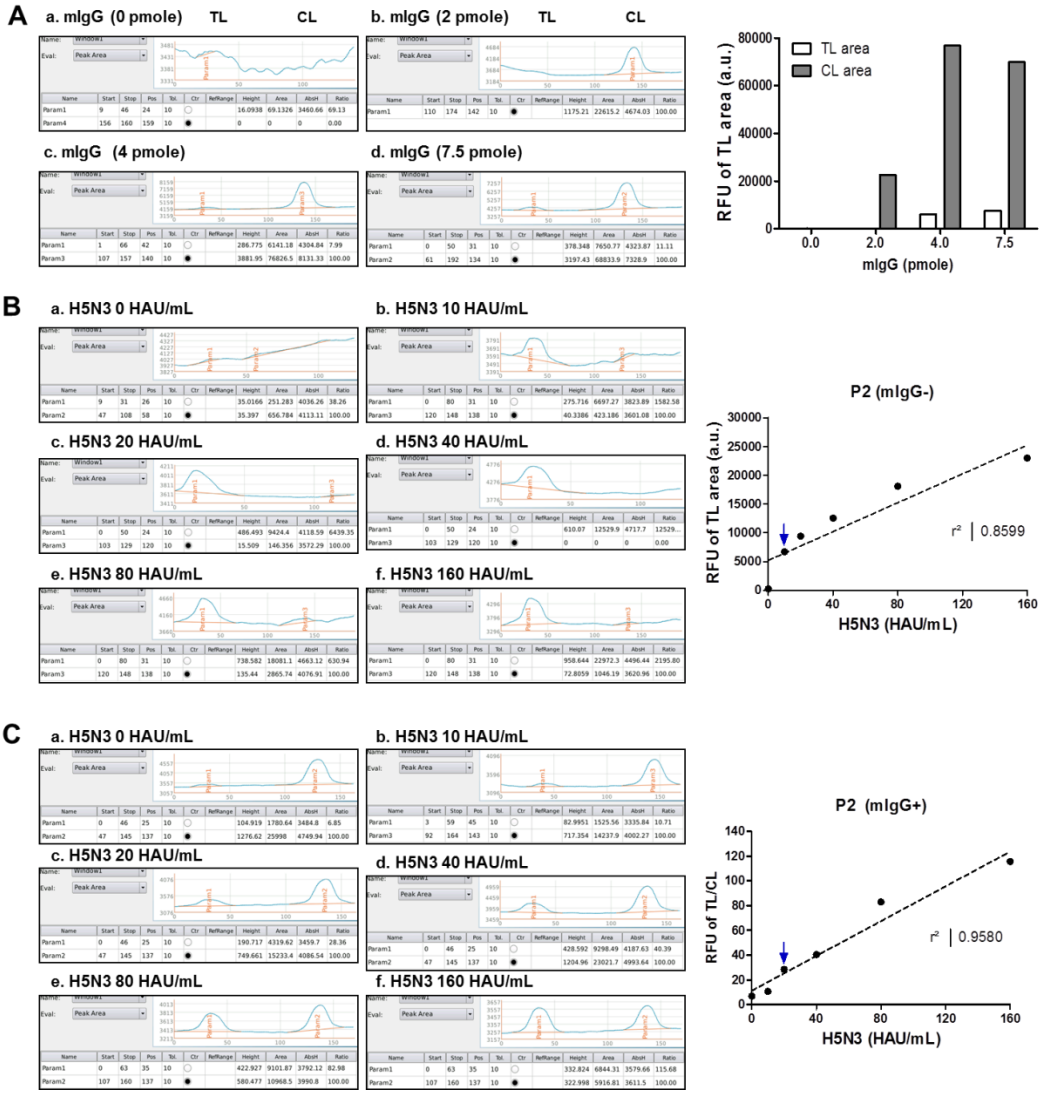


Figure S2. Optimization of conjugate with mouse IgG. Various amounts (2–7.5 pmol) of mouse IgG were tested for peptide conjugate to determine the suitable CL values without non-specific binding of the peptide at TL values in DW (A). TL and CL values were determined by serially diluted virus from 10-160 HAU/mL with P2-conjugate (B) and P2-mouse IgG-conjugate (C). H5N3 0 HAU/mL, negative antigen protein concentrated from egg allantoic cavity

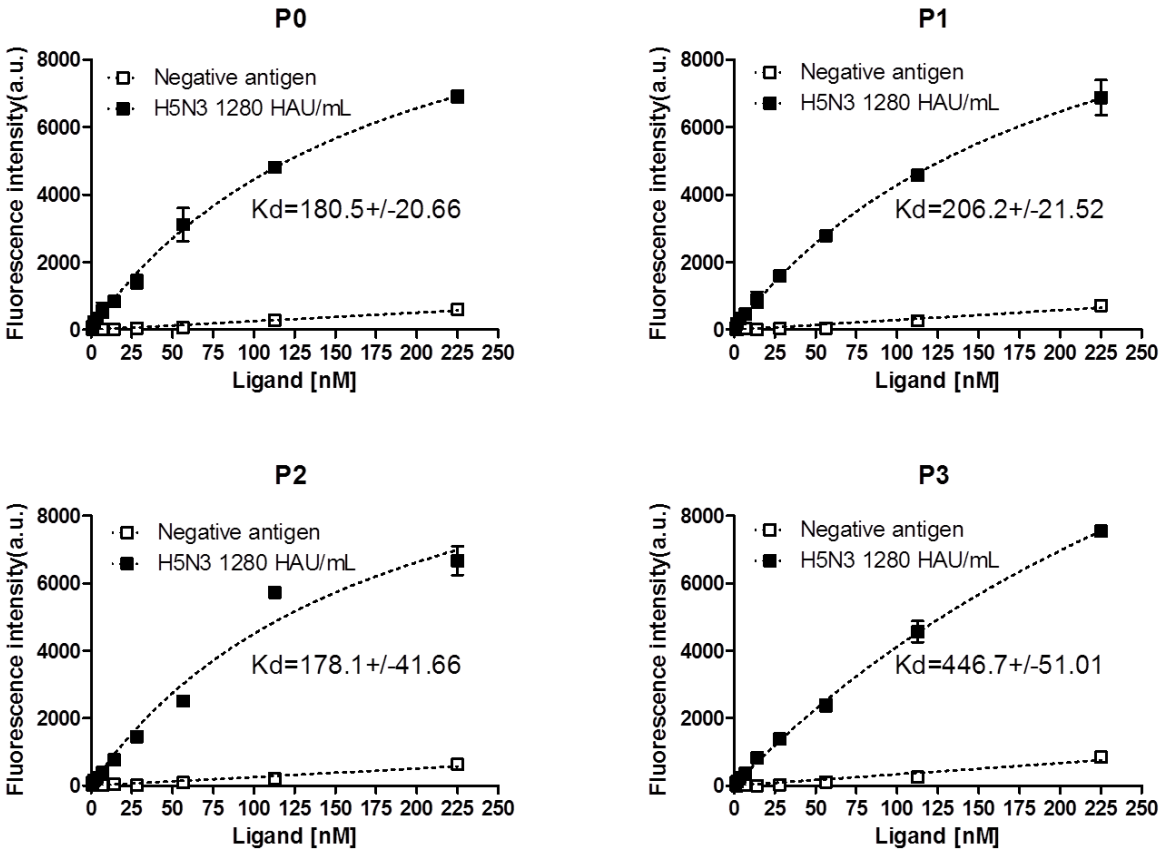
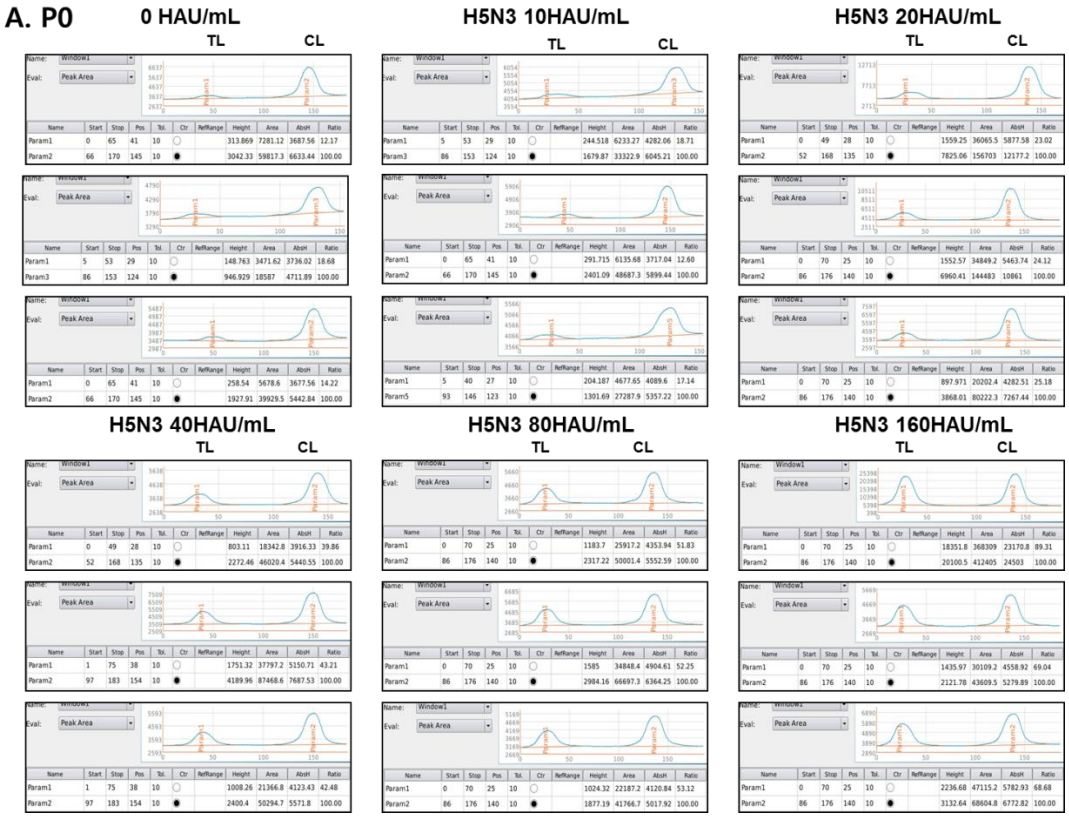


Figure S3. Comparison of Kd values. The antibody (1C5) captured H5N3 virus and the serially diluted different conjugates (Eu NP-conjugated peptides) were reacted with virus. After washing, the saturation of fluorescence was measured to determine K_d value of each peptide.


A. P0
0 HAU/mL
H5N3 10HAU/mL
H5N3 20HAU/mL
H5N3 40HAU/mL
H5N3 80HAU/mL
H5N3 160HAU/mL
TL
CL


B. P1
0 HAU/mL
H5N3 10HAU/mL
H5N3 20HAU/mL
H5N3 40HAU/mL
H5N3 80HAU/mL
H5N3 160HAU/mL
TL
CL

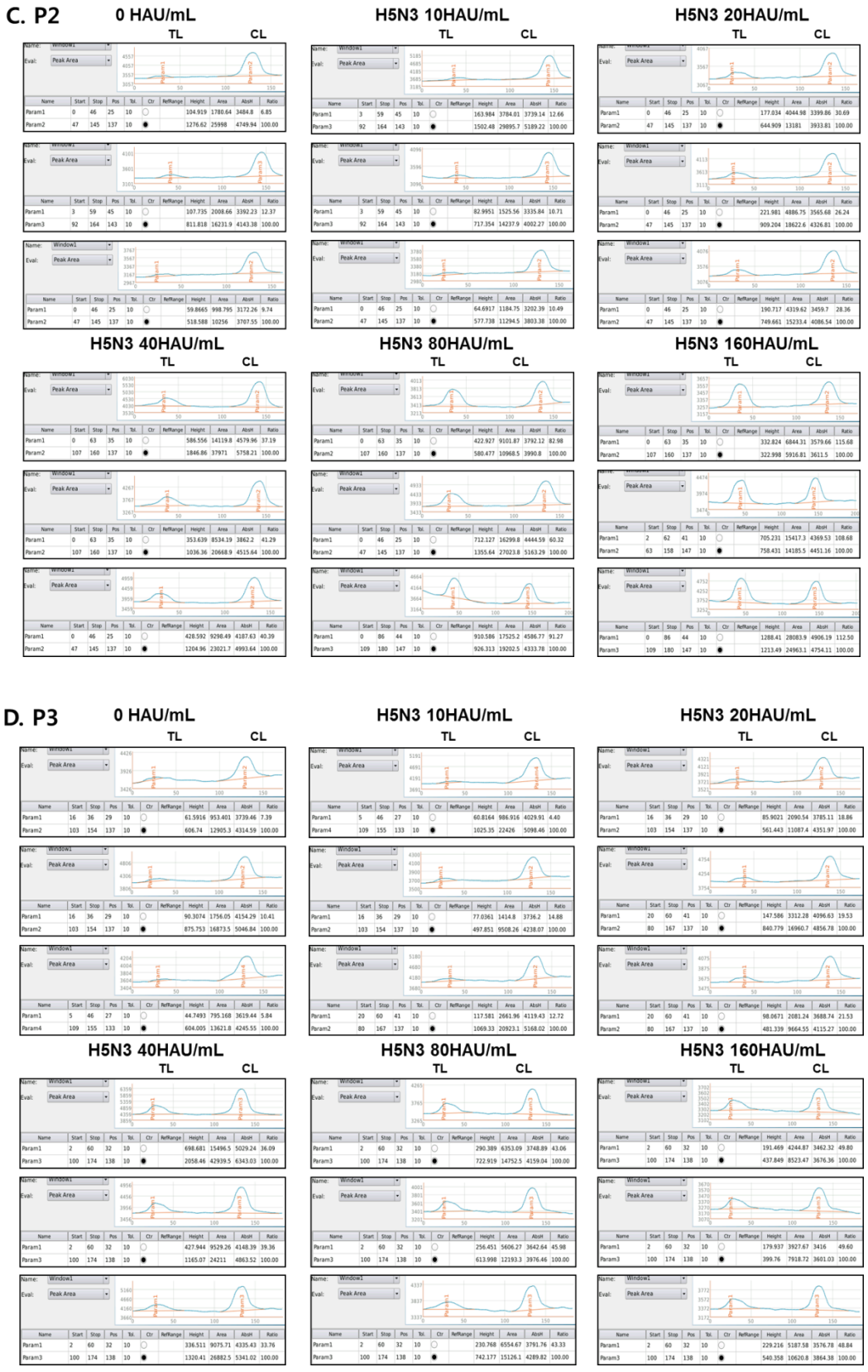
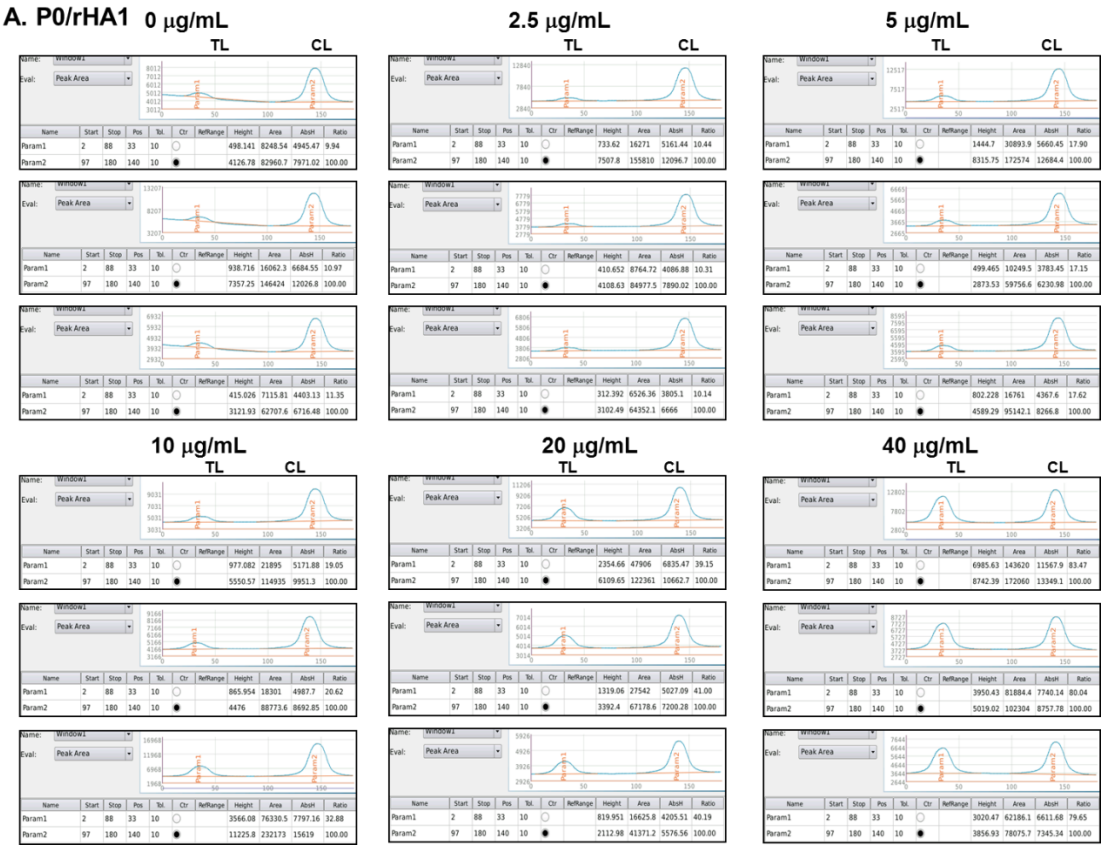
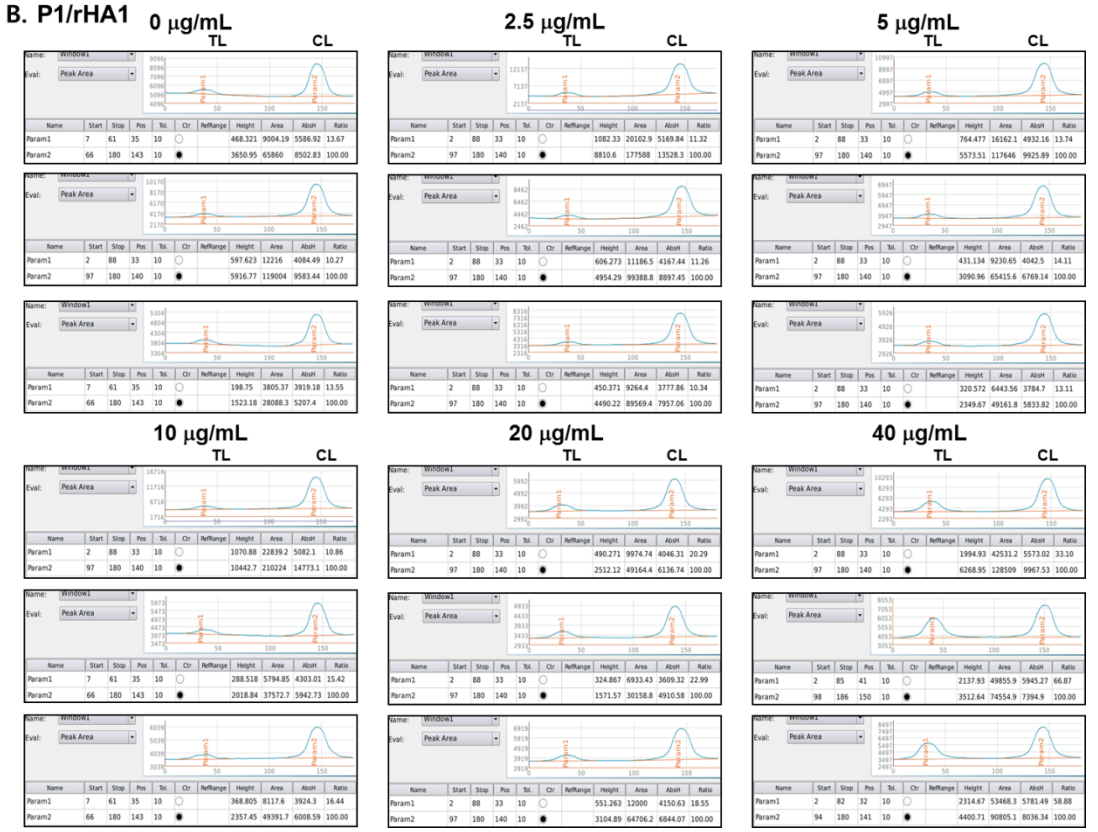
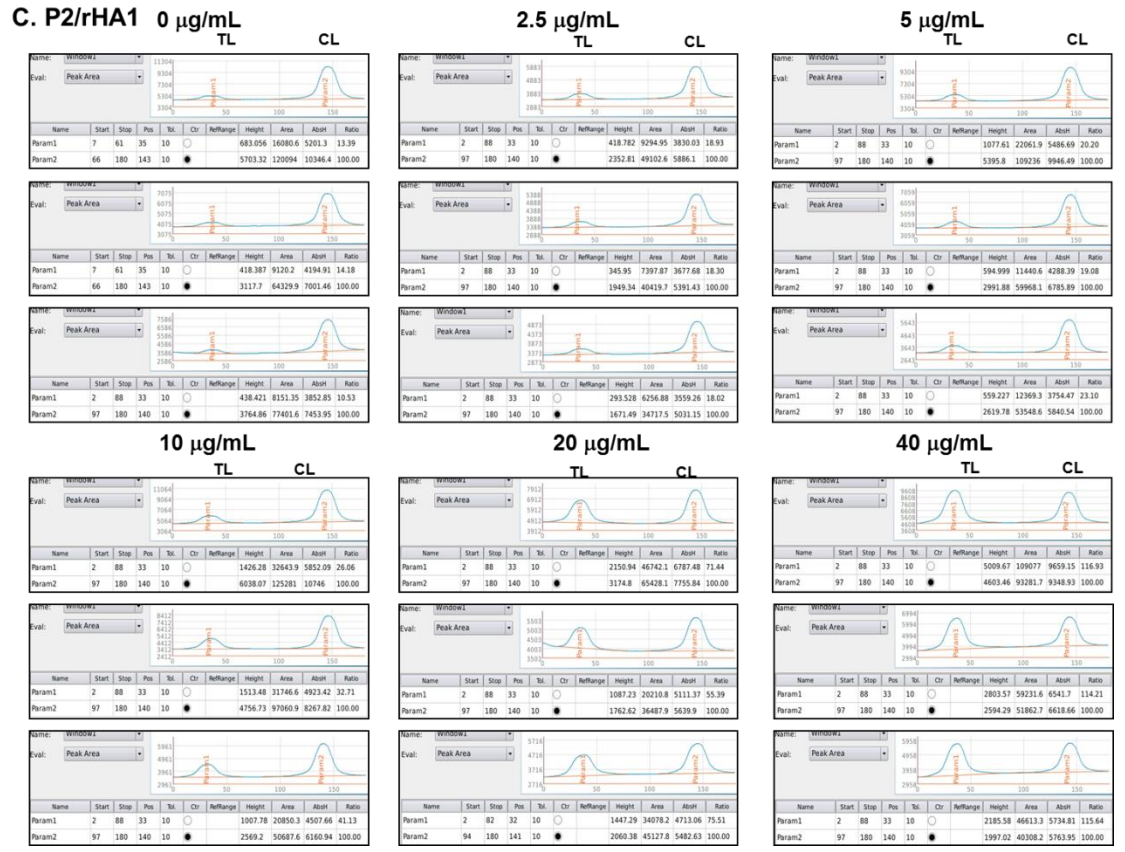
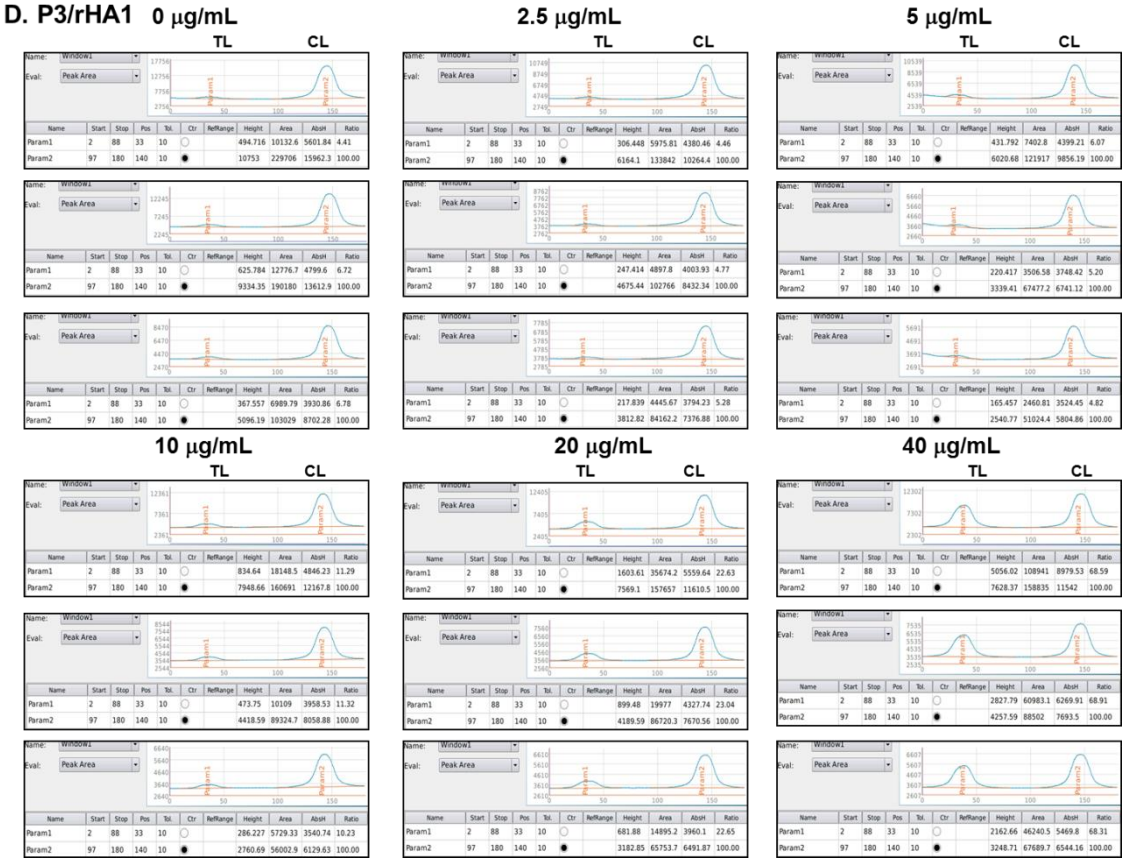


Figure S4. Quantitative analysis by rapid FICT (Figure 5B)


A. P0/rHA1
0 μg/mL
2.5 μg/mL
5 μg/mL
10 μg/mL
20 μg/mL
40 μg/mL
TL
CL


B. P1/rHA1
0 μg/mL
2.5 μg/mL
5 μg/mL
10 μg/mL
20 μg/mL
40 μg/mL
TL
CL


C. P2/rHA1 0 μg/mL
TL
CL
2.5 μg/mL
TL
CL
5 μg/mL
TL
CL
10 μg/mL
TL
CL
20 μg/mL
TL
CL
40 μg/mL
TL
CL


D. P3/rHA1 0 μg/mL
TL
CL
2.5 μg/mL
TL
CL
5 μg/mL
TL
CL
10 μg/mL
TL
CL
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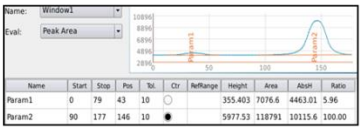
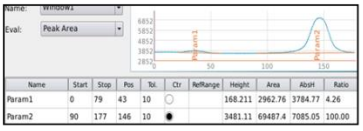
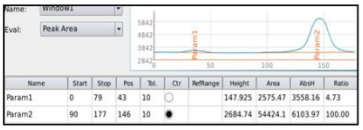
TL
CL
40 μg/mL
TL
CL

E. P0/rHA2 0 μg/mL

TL CL

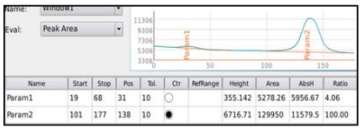


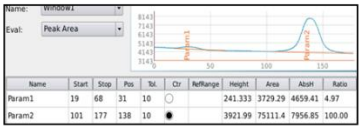


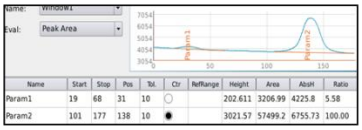


2.5 μg/mL

TL CL





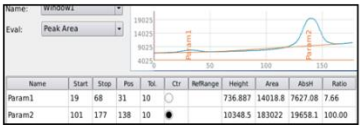


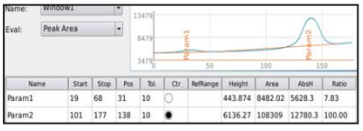
5 μg/mL

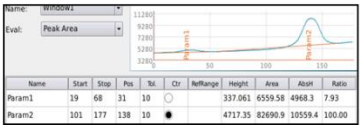
TL CL

10 μg/mL

TL CL

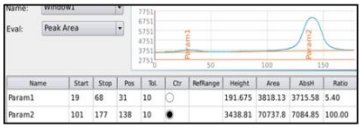


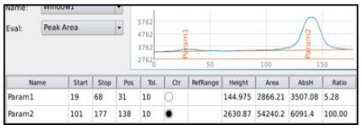




20 μg/mL

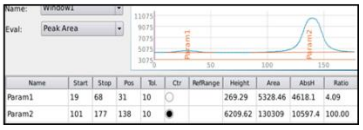
TL CL

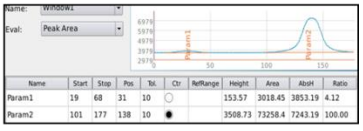


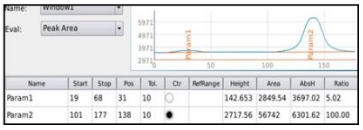


40 μg/mL

TL CL

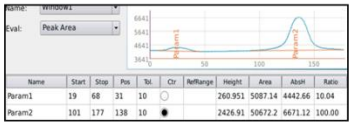


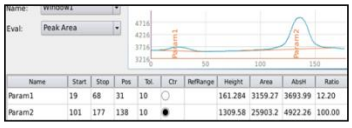


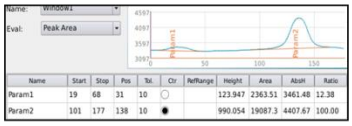


F. P1/rHA2 0 μg/mL

TL CL

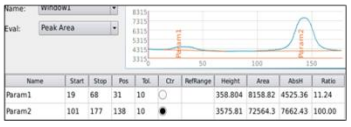


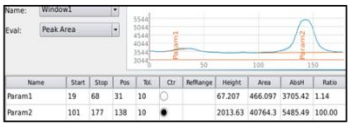


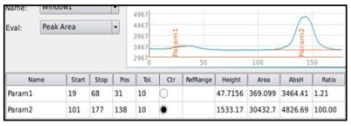


2.5 μg/mL

TL CL

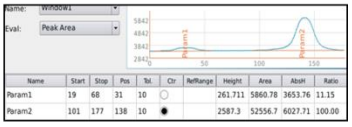


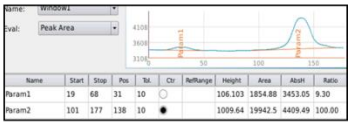




5 μg/mL

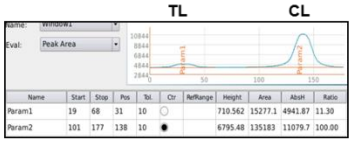
TL CL





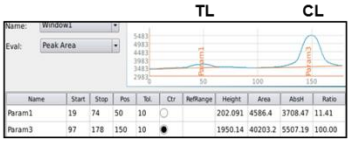
10 μg/mL

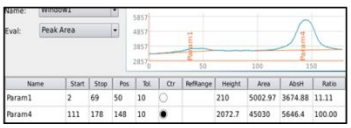
TL CL



20 μg/mL

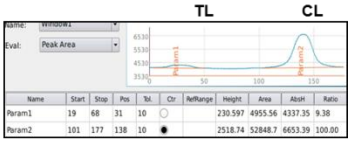
TL CL





40 μg/mL

TL CL



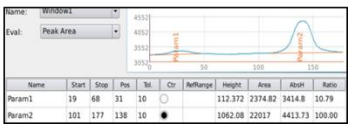
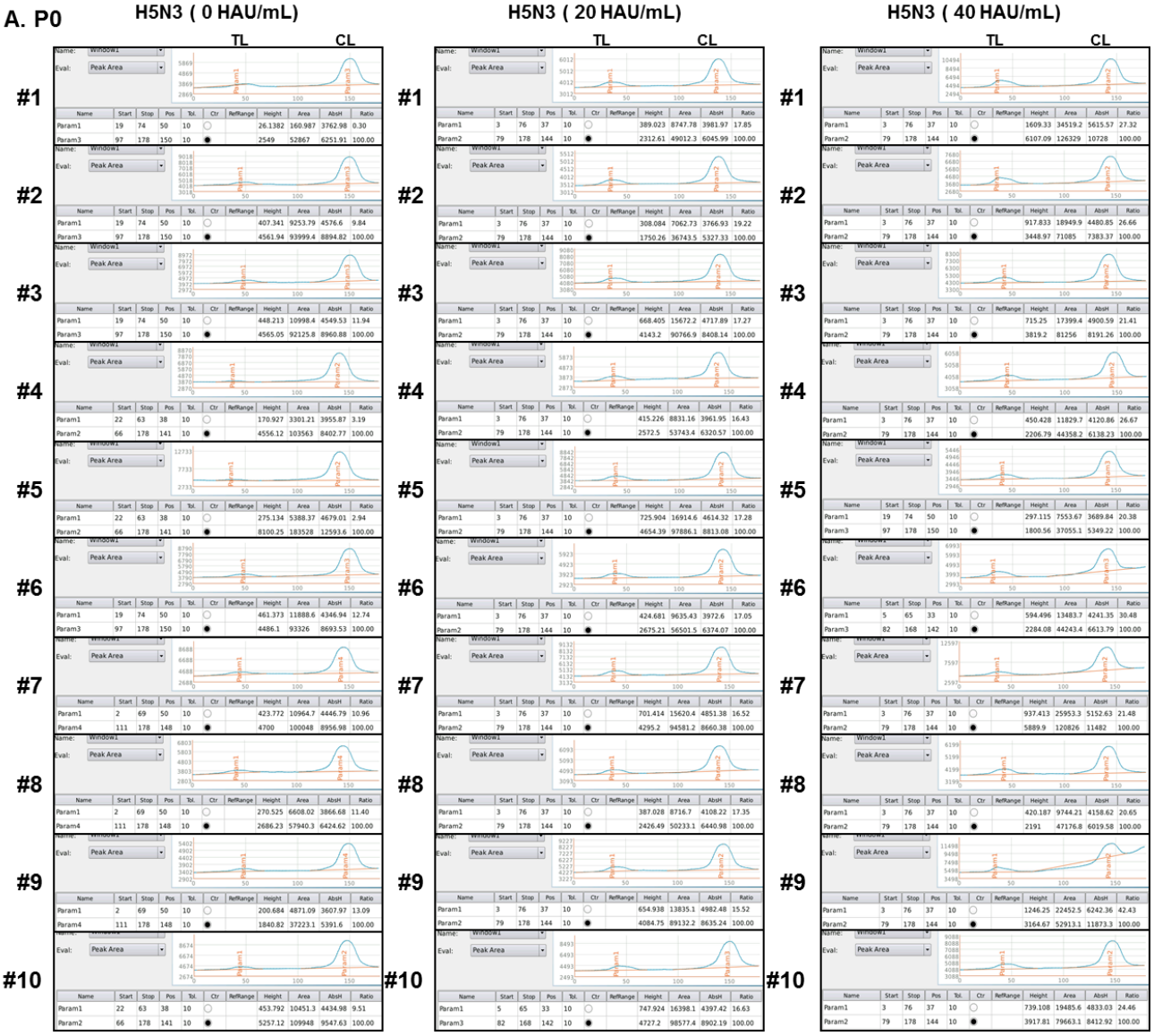


Figure S5. Quantitative analysis by rapid FICT (Figure 5C)

A. P0



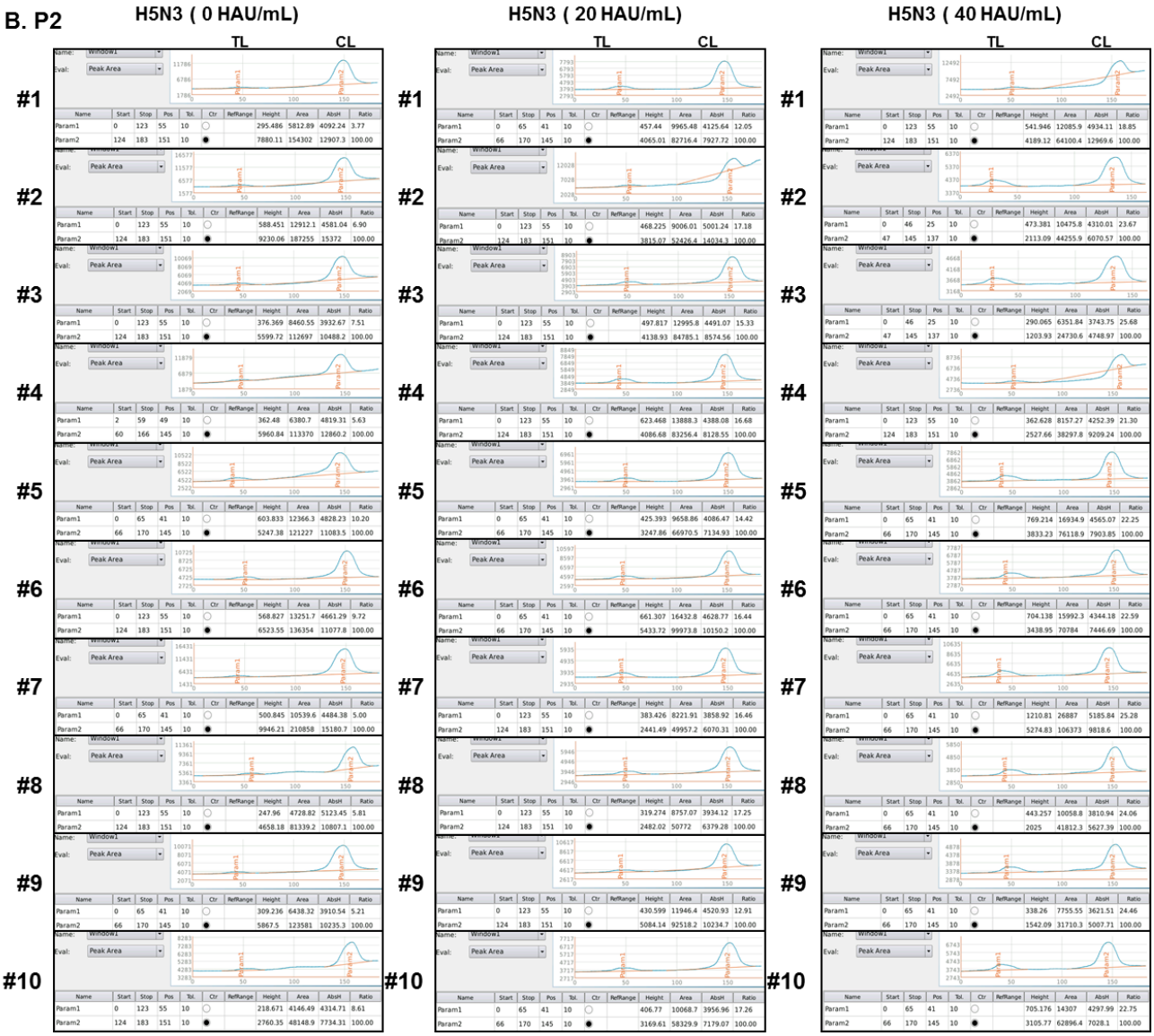
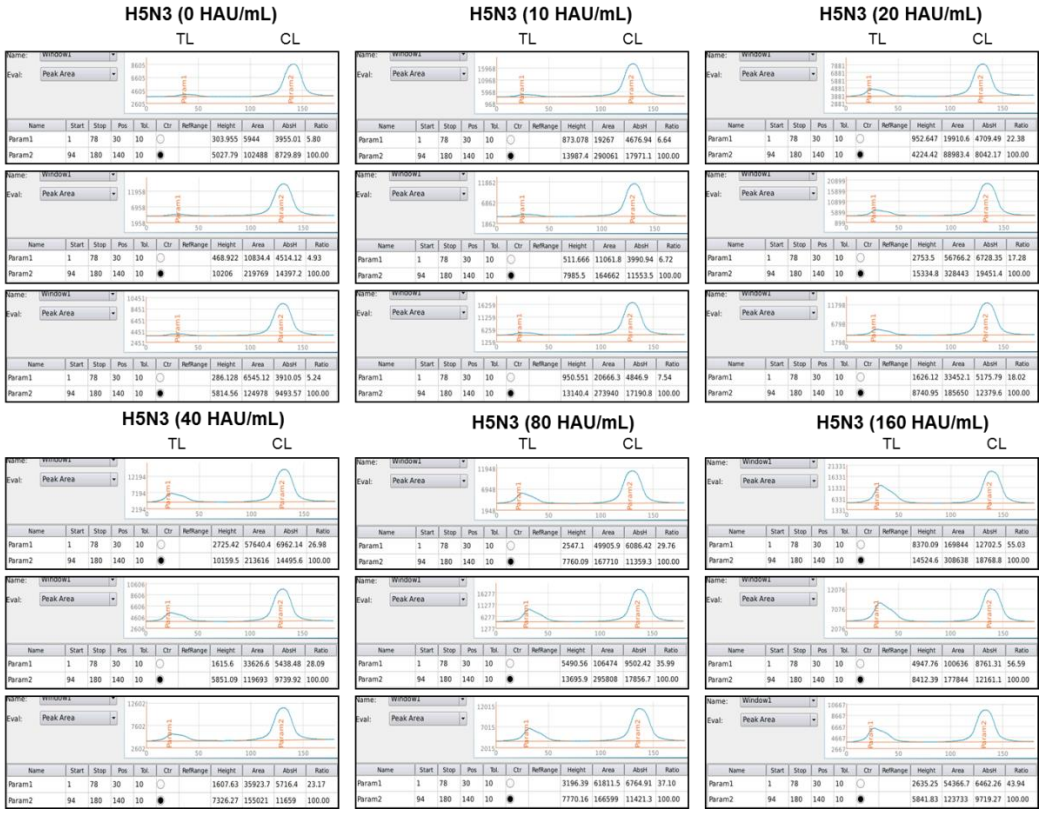
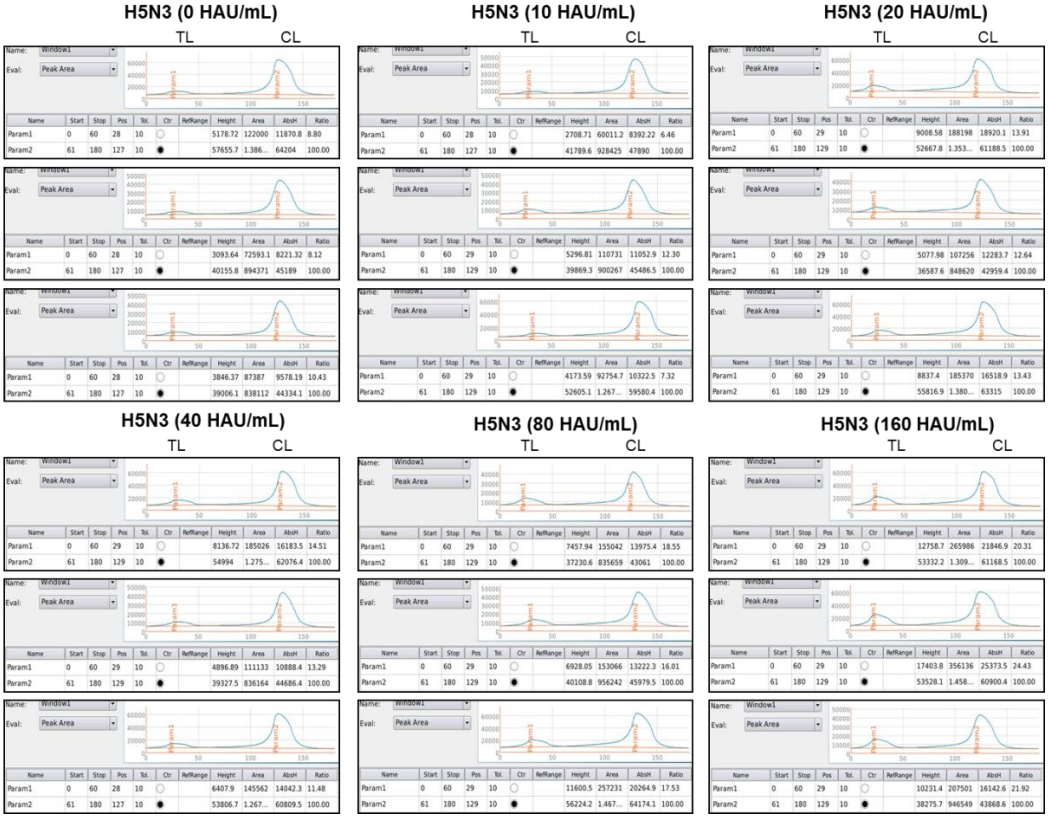


Figure S6. Efficiency of P0 and P2 as detection elements in FICT for human nasopharyngeal specimens. (Figure 6)

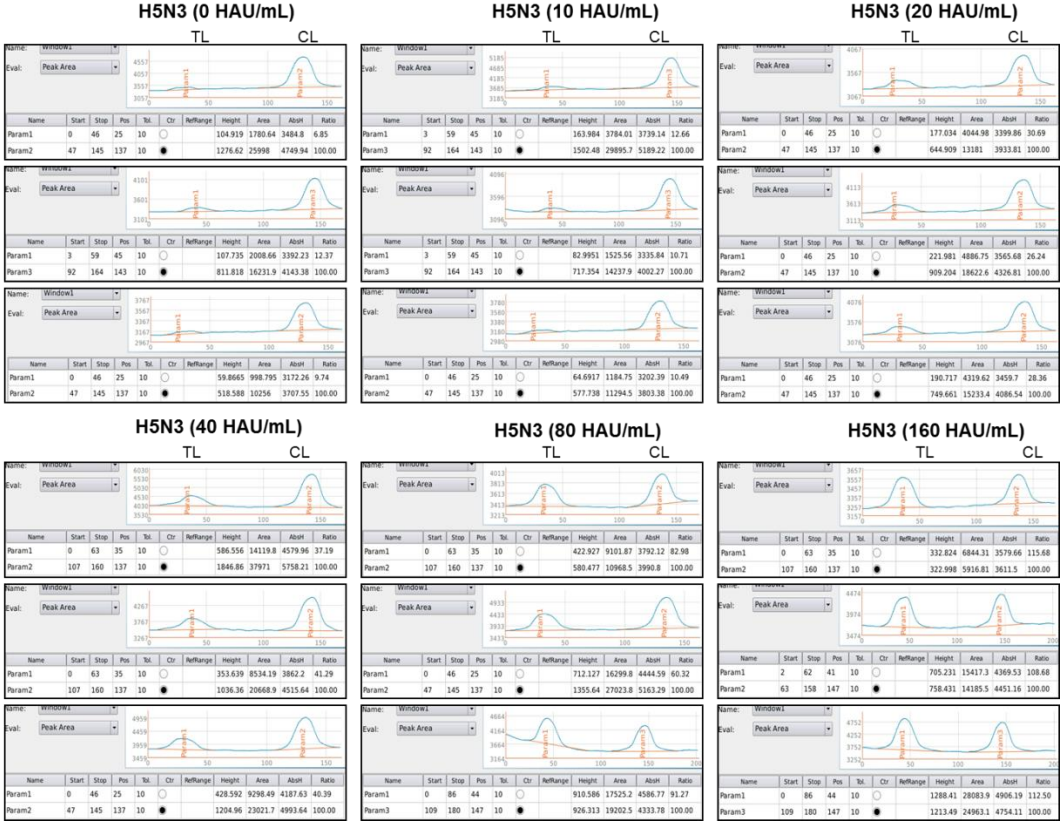
A. 3F11/1C5-DW



B. 3F11/1C5-Specimen



C. P2/1C5-DW



D. P2/1C5-Specimen

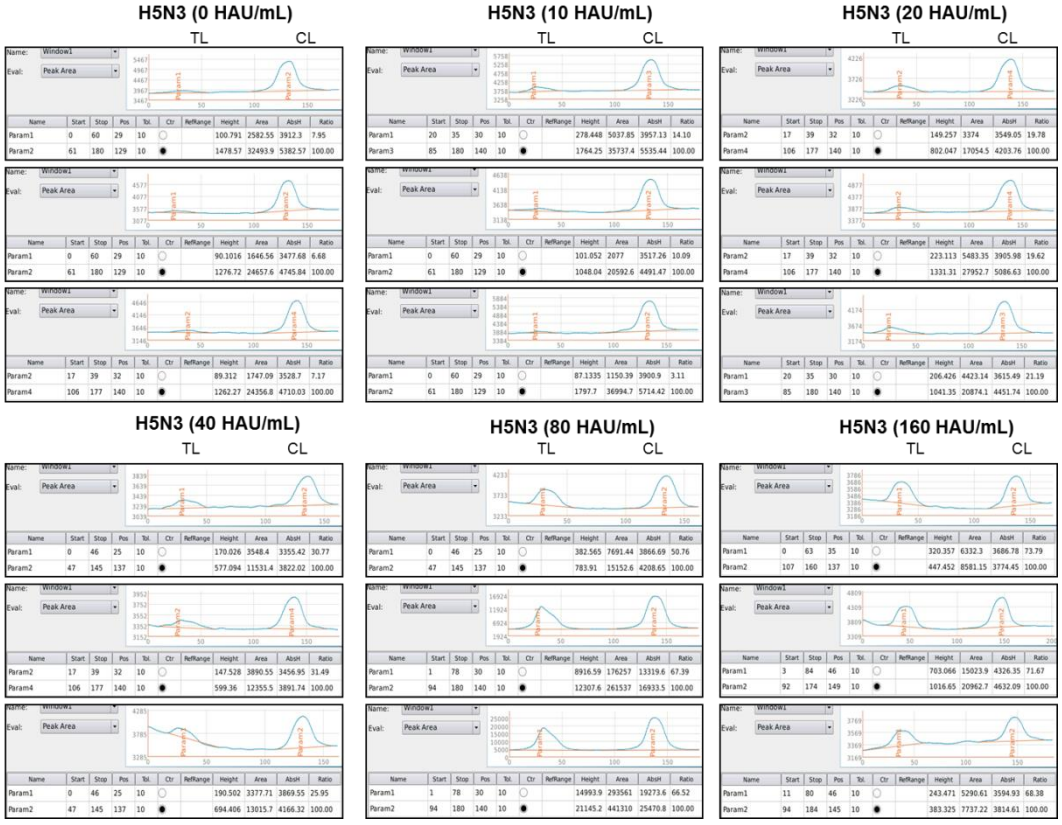


Figure S7. Comparison between P2 peptide-FICT and regular FICT employing the 3F11 antibody (Figure 7)

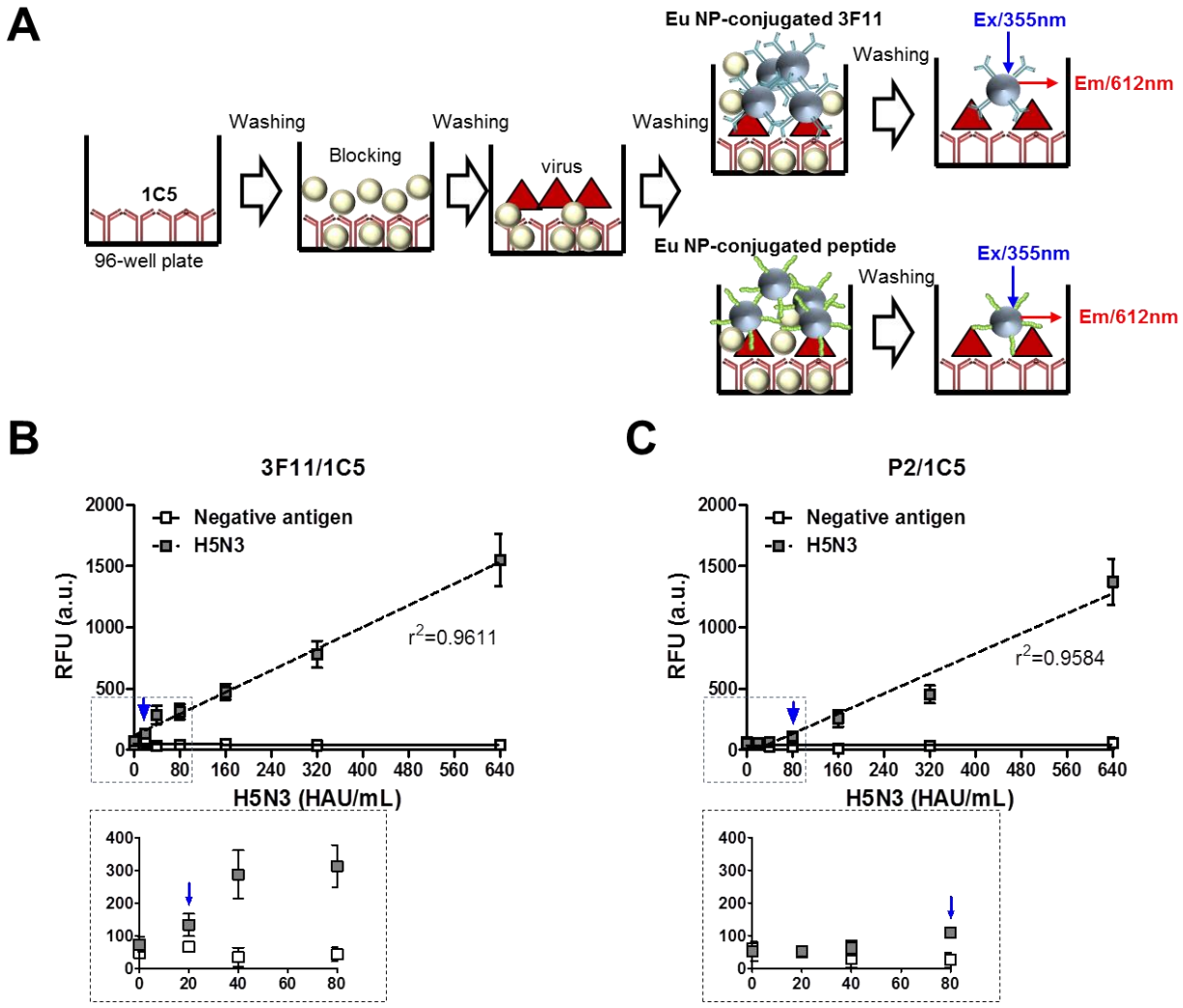


Figure S8. Sandwich FLISA assay as peptide-linked immunoassay using 96-well plate. The procedure of step-by step of 96-well plate-mediated immunoassay is shown (**A**). Antibody (1C5) was coated on plated microplate and virus was added detected by Eu NP-conjugated 3F11 (**B**) or Eu NP-conjugated P2 peptide (**C**). Blue arrow indicates the LOD of this assay.